Facultad de Farmacia

Departamento de Farmacia y Tecnología Farmacéutica

Programa oficial de doctorado en Medicina Clínica y Salud Pública

DESARROLLO, CARACTERIZACIÓN Y ESTUDIO DE ESTABILIDAD DE UN MEDICAMENTO CELULAR PARA EL TRATAMIENTO DE LA ISQUEMIA CRÍTICA DE MIEMBROS INFERIORES EN PACIENTES DIABÉTICOS TIPO II

TESIS DOCTORAL

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Licenciada en Farmacia

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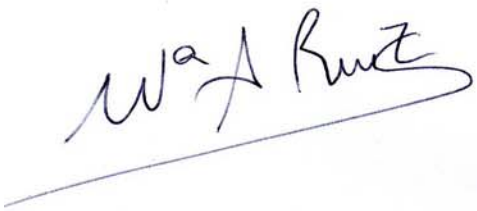

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En Granada, 09 de febrero de 2014

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por su apoyo incondicional.

“Sólo hay felicidad donde hay virtud y esfuerzo serio,
pues la vida no es un juego”

Aristóteles

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ÍNDICE GENERAL

ABREVIATURAS

AEMPS	Agencia Española de Medicamentos y Productos Sanitarios
ASCs	Células Madre Adultas
ATMP	Medicamentos de Terapias Avanzadas
ATMSCs	Células Madre Mesenquimales de Tejido Adiposo
BMMSCs	Células Madre Mesenquimales de Medula Ósea
CAT	Comité de Terapias Avanzadas
CFU-F	Colonias Formadores de Unidades Fibroblastoides
CHM-I	Complejo Mayor de Histocompatibilidad I
CHM-II	Complejo Mayor de Histocompatibilidad II
CHMP	Comité de Medicamentos de Uso Humano
CLI	Isquemia Crítica de Miembros Inferiores
CTMP	Medicamentos de Terapia Celular Somática
DM	Diabetes Mellitus
EMA	Agencia Europea del Medicamento
EPCs	Células Progenitoras del Endotelio
ESCs	Células Madre Embrionarias
EU	Unión Europea
FDA	Food and Drug Administration
FTIR	Espectrofotometría de infrarrojos
GCP	Buenas Prácticas Clínicas
GLP	Buenas Prácticas de Laboratorio
GMP	Normas de Correcta Fabricación
GTMP	Medicamentos de Terapia Génica
HGF	Factor de Crecimiento Hepático
hMSCs	Células Madre Mesenquimales Humanas
HSCs	Células Madre Hematopoyéticas
ICH	International Conference de Armonización
IDO	Indolamina 2,3-dioxigenasa
IL	Interleucina
INF	Interferón
iPS	Células Madre Pluripotentes Inducidas
ISCT	Sociedad Internacional de Terapia Celular
LAL	Lisado de Amebocitos Limulus

LD	Límite de Detección
MCB	Banco Celular Maestro
MCI	Masa Celular Interna
MHLW	Ministerio de Salud, Trabajo y Bienestar
MSCs	Células Madre Mesenquimales
NK	Células asesinas
NO	Óxido Nítrico
OMS	Organización Mundial de la Salud
PC	Medicamentos de Terapias Avanzadas Combinados
PD	Pie Diabético
PGE	Prostaglandinas
Ph. Eur.	Farmacopea Europea
QCP	Programa de Control de Calidad
SDC	Agar Sabouraud Dextrosa Cloranfenicol
T reg	Células T reguladoras
TASC	Trans Atlantic Inter-Society Consensus
TEP	Medicamentos de Ingeniería Tisular
TGF- β1	Factor de Crecimiento Transformante-β1
TPB	Caldo de Tioglicolato y Penasa
TSA	Triptona Soja Agar
TSPB	Caldo de Penasa Soja y Triptona
UFC	Unidad Formadora de Colonia
VEGF	Factor de Crecimiento Endotelial Vascular
VIH	Virus Inmunodeficiencia Humana
WCB	Banco Celular de Trabajo

CAPÍTULO I.
INTRODUCCIÓN

I.1. NANOMEDICINA Y TERAPIAS AVANZADAS

Los nuevos avances en Medicina Regenerativa y Medicina Personalizada han proporcionado nuevas terapias basadas en la Nanomedicina y las Terapias Avanzadas. Estas áreas emergentes del campo biomédico ofrecen nuevas oportunidades para tratar patologías o disfunciones en las cuales aún no existen tratamientos farmacológicos eficaces como son el cáncer, la esclerosis múltiple, la infección por del virus de la inmunodeficiencia humana (VIH) y el Parkinson entre otras (Chauchan y cols., 2013; Connick y cols., 2013; Nair y cols., 2013; Ramana y cols., 2013; Seo y cols., 2013: Hayashi y Onoe, 2013).

La Nanomedicina conlleva un nuevo planteamiento centrado en el diseño, investigación, caracterización y desarrollo de las propiedades de la materia a escala nanométrica (≤ 100 nm) (Chen y cols., 2013), para el diagnóstico, tratamiento y/o prevención de enfermedades (Duncan y cols., 2011; Glenn y Boyce, 2012). A este respecto, la Nanomedicina ofrece numerosas ventajas en comparación con los tratamientos clásicos aplicados hasta el momento, incluyendo la liberación sostenida de agentes terapéuticos, administración dirigida de fármacos a células o tejidos específicos, mejora de la formulación de fármacos insolubles en agua, vehiculización de grandes biomoléculas y reducción de efectos secundarios (Wang, 2012; Kettiger y cols., 2013).

Por su parte el término "Terapias Avanzadas" incluye varias estrategias terapéuticas, basadas en los avances más vanguardistas de la investigación biomédica (Belardelli y cols., 2011) como son la Terapia Celular, Terapia Génica e Ingeniería Tisular.

El término "Medicamento de Terapias Avanzadas" (ATMP del inglés Advanced Therapy Medicinal Product) cubre los siguientes medicamentos para uso humano (Galvez y cols., 2013):

- Medicamento de Terapia Celular Somática (CTMP del inglés Somatic Cell Therapy Medicine Product).
- Medicamentos de Terapia Génica (GTMP del inglés Gene Therapy Medicine Product).
- Producto de Ingeniería Tisular (TEP del inglés Tissue-Engineered Products).
- Medicamentos de Terapias Avanzadas Combinados (PC).

Todos ellos son medicamentos biológicos con propiedades para tratar y/o prevenir enfermedades humanas, o para restaurar, corregir o modificar funciones fisiológicas ejerciendo principalmente una acción farmacológica, inmunológica o metabólica.

Los ATMP pueden ser de origen:

- **Autólogo:** donante y receptor tienen el mismo origen (mismo individuo).
- **Alogénico:** donante y receptor son individuos distintos; el donante es el individuo sano y el receptor es el individuo enfermo.
- **Xenogénico:** el donante es de origen animal y el receptor es la persona con la patología a tratar.

El uso de medicamentos autólogos evita problemas de rechazo del tratamiento y las reacciones de tipo inmunológico en el receptor, además de reducir los problemas éticos y regulatorios que implicaría el uso de productos alogénicos y/o xenogénicos (Prasongchean y Ferretti, 2012). Por otra parte, el trasplante autólogo también tiene limitaciones como la escasez de muestras para la obtención de la célula, gen o tejido sano.

I.1.1. CÉLULAS MADRE COMO MEDICAMENTO

En el ámbito de la biotecnología y las nuevas terapias, la Terapia Celular, tiene por objetivo reparar, reemplazar o recuperar la función biológica de un tejido u órgano dañado, utilizando para ello como estrategia terapéutica el uso de células madre (Mimeault y cols., 2007; Totey y cols., 2009; Zuba-Surma y cols., 2011; Hoggatt y Scadden, 2012; Chun y Liang, 2012).

I.1.1.1. CÉLULAS MADRE: DEFINICIÓN

Las células madre son conocidas como *células troncales* o *células stem,* aunque el nombre más adecuado es el de células progenitoras; sin embargo el término de células madre es el que se ha posicionado en la bibliografía.

Las células madre son células no especializadas, con capacidad de dividirse de forma asimétrica (autorrenovarse) durante períodos de tiempo indefinidos en la vida de un

individuo y que bajo condiciones apropiadas o señales correctas del microambiente en el que viven, pueden dar origen (diferenciarse), a través de divisiones asimétricas, a diferentes linajes con características y funciones especializadas, como por ejemplo glóbulos rojos, miocitos, neuronas, hepatocitos, etc. (Donovan y cols., 2001; Prosper y cols., 2004) (Figura 1).

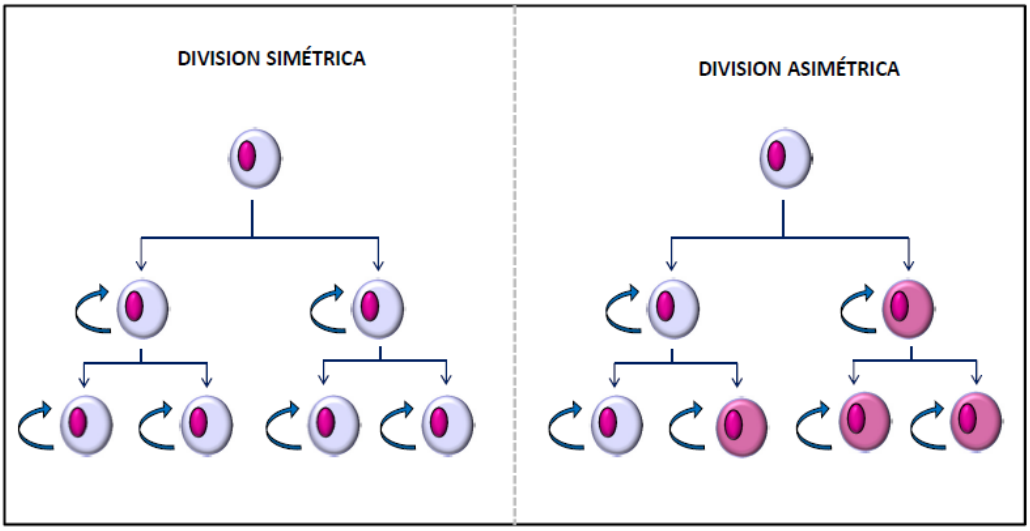

Figura 1. División simétrica y asimétrica de una célula madre.

Las células madre se caracterizan por ser células no diferenciadas, con capacidad de proliferar *ex vivo* e *in vivo* diferenciándose en células especializadas maduras (Herberts y cols., 2011).

I.1.1.2.CÉLULAS MADRE: CLASIFICACIÓN

Las células madre pueden clasificarse según dos criterios: por su potencialidad y por su origen.

De acuerdo al tipo de tejido que originan, las células madre se clasifican en cuatro tipos: totipotentes, pluripotentes, multipotentes y unipotentes (Weissman y cols., 2001; Lakshmipathy y Verfaillie, 2005) (Figura 2).

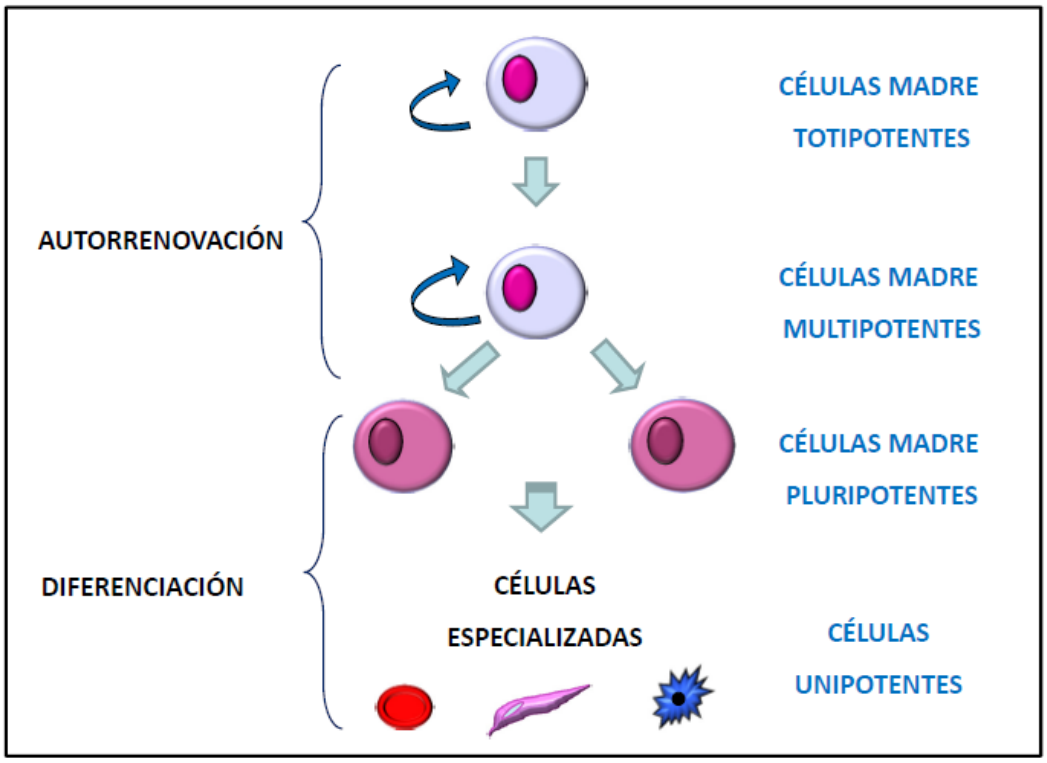

Figura 2. Clasificación de las células madre según su potencialidad.

1. Totipotentes

Células madre capaces de diferenciarse hacia cualquier tipo celular, formando un organismo completo. La célula madre totipotente por excelencia es el cigoto, formado tras la fecundación. Este tipo de células solo están presentes en el embrión en los primeros estadíos de división (hasta la etapa de 8-16 células).

2. Pluripotentes

Son aquellas células que no pueden formar un organismo completo pero pueden dar lugar a los distintos tipos celulares del embrión, es decir, todos los tipos celulares que proceden de las tres capas embrionarias, de la línea germinal y del saco vitelino. Pertenecen a este grupo las células madre embrionarias aisladas de la masa celular interna (MCI) del blastocito.

3. Multipotentes

Células más comprometidas, que pueden derivar a los distintos tipos celulares de su propia capa o linaje embrionario de origen. La mayoría de las células madre adultas pertenecen a este grupo.

4. Unipotentes

Células muy comprometidas y con un grado de diferenciación muy limitado, capaces de dar lugar únicamente a un tipo celular.

Según su origen se las clasifica en células madre embrionarias (ESCs del inglés Embryonic Stem Cells), células madre adultas (ASCs del inglés Adult Stem Cells) y células madre pluripotentes inducidas (iPS del inglés Induced Pluripotent Stem) (Sykova y cols., 2013) (Figura 3).

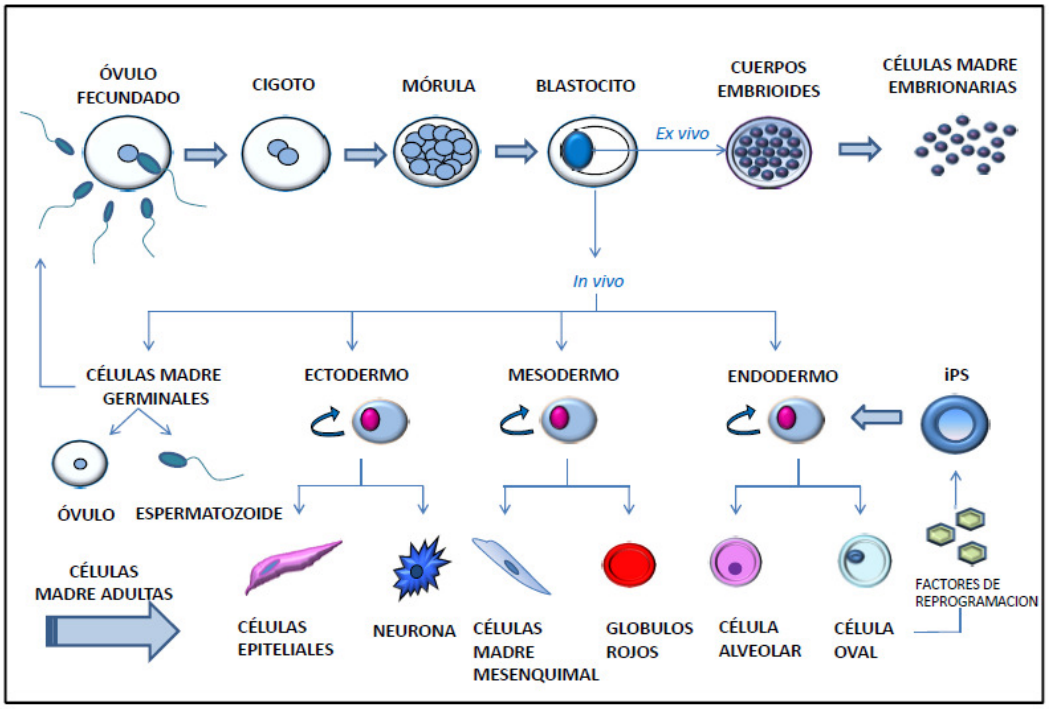

Figura 3. Esquema jerárquico de las células madre.

1. Células Madre Embrionarias (ESCs)

Las ESCs son células madre pluripotentes, se obtienen del interior de la masa celular del embrión en fase de blastocito. Se trata de pre-embriones de cinco o seis días con aproximadamente entre 100 y 200 células. El blastocito está formado por dos tipos de células y una gran cavidad interior (Figura 4):

- **Trofoblasto**: capa externa del blastocito que dará lugar a la placenta y a las envolturas embrionarias.
- **Masa celular interna (MCI):** formará todos los tejidos del cuerpo humano.

Las primeras ESCs se obtuvieron de médula ósea de ratón en 1980 (Evans y Kaufman, 1981), más tarde en 1998, Thomson aisló las primeras ESCs de origen humano de embriones de clínicas de fertilización *in vitro* (Thomson y cols., 1998).

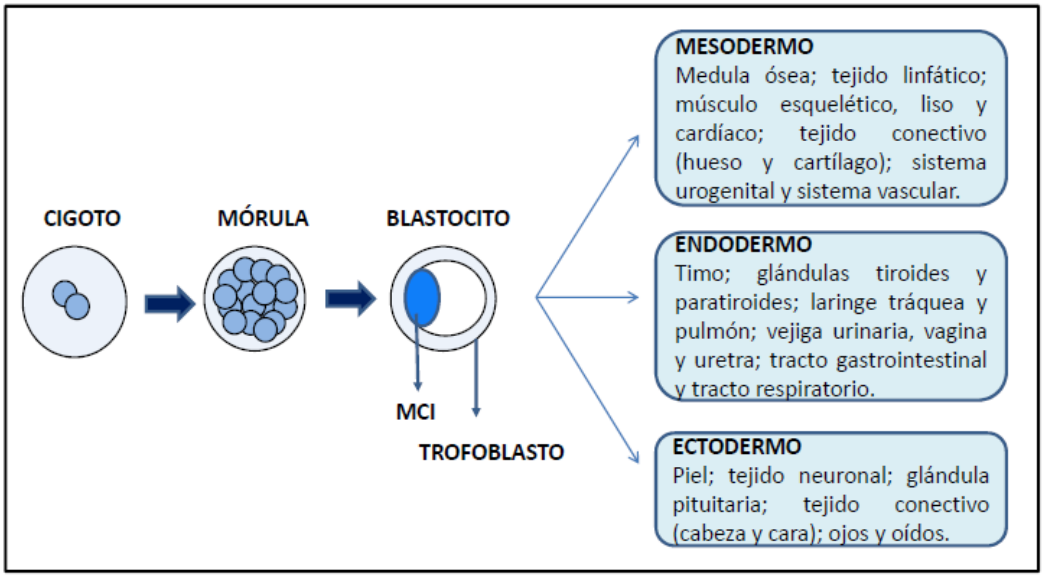

Figura 4. Esquema de diferenciación de los tejidos humanos a partir del blastocito.

Se caracterizan por su capacidad para permanecer en un estado proliferativo no diferenciado durante un período prolongado de tiempo, pudiendo además diferenciarse a diferentes linajes celulares (Bajada y cols., 2008). Son las células madre más versátiles y tienen la capacidad de dar origen a todos los tipos celulares de las tres láminas germinales del individuo: endodermo, mesodermo y ectodermo (Arkell y Tam, 2012).

Las ESCs se han utilizado experimentalmente en diversos modelos animales, demostrando con éxito su capacidad de generar células hepáticas, células madre hematopoyéticas, tejido neural y cardíaco entre otros (Odorico y cols., 2001). No obstante, las consideraciones éticas y preocupaciones de seguridad por el riesgo de formación de teratomas o rechazo inmune en el trasplante y su aplicación clínica en seres humanos es aún limitada (Riess y cols., 2007; Hmadcha y cols., 2009).

2. Células Madre Adultas (ASCs)

Las ASCs son células madre multipotentes y unipotentes, abarcan una variedad de células indiferenciadas localizadas en tejidos adultos, independientemente de la edad del organismo. Se caracterizan por su alto potencial proliferativo (siendo menor que el que presentan las ESCs), por su capacidad sustancial de autorrenovarse y por la capacidad de diferenciarse en al menos un tipo de célula madura funcional (Hmadcha y cols., 2009; Caplan, 2013).

Las ASCs se dividen de forma equilibrada tanto asimétrica como simétricamente (Zhong y Chia, 2008; Wu y cols., 2008). Su capacidad de sufrir divisiones mitóticas asimétricas da lugar a dos células hijas diferenciadas para generar progenitores que posteriormente se diferencian en tipos celulares maduros con funciones especializadas. Alternativamente, se someten a divisiones simétricas de una manera estocástica para producir más células madre, manteniendo las propiedades de células madre.

Las primeras ASCs descritas hace 50 años fueron las células madre hematopoyéticas (HSCs del inglés Hematopoietic Stem Cells) de médula ósea, capaces de autorrenovarse y diferenciarse a células hematopoyéticas multipotentes, que dan lugar a todos los linajes de las células sanguíneas (Siminovitch y cols., 1963). Las HSCs están presentes en la médula ósea y en el cordón umbilical. Debido a su amplia utilización en trasplantes han sido ampliamente estudiadas.

Más tarde, Friendenstein y cols., (Friedenstein y cols., 1976) encontraron otra población de células madre con características similares a las células de la médula ósea y la llamó unidad formadora de colonias de fibroblastos, actualmente conocidas como células madre estromales o mesenquimales (MSCs del inglés Mesenchymal Stem Cells). En los últimos años se han hallado ASCs en casi todos los órganos del individuo adulto:

piel, hígado, páncreas, sangre, médula ósea, riñón, intestino, vasos sanguíneos, sistema nervioso central, pulpa dental, tejido adiposo, músculo esquelético y corazón, entre otros (Young y cols., 2001; Austin y Lagasse, 2003; Peng y cols., 2004; Guettier, 2005; Levy y cols., 2005; Torella y cols., 2006; Baglioni y cols., 2009; Nikravesh y cols., 2011; Nagasawa y cols., 2011; Abe y cols., 2012; Morad y cols., 2013; Silberstein y Lin, 2013).

Las ASCs son las más utilizadas en la Terapia Celular actual, siendo por ello ampliamente estudiadas como potenciales agentes terapéuticos para una gran variedad de enfermedades (González y Bernad, 2012) (Tabla 1).

Tabla 1. Principales células madre adultas estudiadas para el tratamiento de diversas patologías en el área de la Terapia Celular.

Células Madre Adultas	Patologías
Mioblastos	Infarto de miocardio
Células madre mesenquimales	Artrosis Esclerosis múltiple Fistula perianal Isquemia crítica de miembros inferiores Isquemia crítica de miembros inferiores en pacientes diabéticos Regeneración Muscular Regeneración Ósea Tratamiento de la enfermedad de injerto contra el huésped
Células madre hematopoyéticas	Infarto de miocardio Isquemia crítica de miembros inferiores Isquemia crítica de miembros inferiores en pacientes diabéticos
Células limbo-corneales	Patología corneal
Condrocitos	Artrosis Regeneración del cartílago
Melanocitos	Vitíligo
Fibroblastos	Piel artificial
Queratinocitos	Piel artificial
Células dendríticas	Cáncer
Hepatocitos	Cirrosis hepática Trasplantes

3. Células Madre Pluripotentes Inducidas (iPS)

Las células iPS son células madre pluripotentes derivadas artificialmente de una célula madre somática no pluripotente, por inserción de factores de transcripción en la célula (Zhou y Ding, 2010; Sipp, 2010; Ho y cols., 2012). Los primeros ensayos de transferencia de material nuclear a una célula somática fueron realizados por Gurdon,

transfiriéndolo a un ovocito e induciendo su pluripotencia por estímulos electro-químicos (Gurdon y cols., 1958). Años más tarde, esta técnica fue mejorada por Takahashi y Yamanaka (Takahashi y Yamanaka, 2006), demostrando que las células madre pluripotentes podrían ser generadas a partir de fibroblastos de ratón por la expresión de cuatro genes exógenos: OCT3/4, Sox2, CMYC y KLF44 (OKSM) (Takahashi y cols., 2007).

Las células iPS son células madre adultas reprogramadas a comportarse como ESCs, considerándose similares pero no idénticas (Kim y cols., 2012). Existen diferencias a nivel de la expresión génica, en la metilación del ADN, en la estabilidad del fenotipo pluripotente así como en la memoria epigenética de la célula (Philonenko y cols., 2011; Kim y cols., 2012; Yi y cols., 2012; Spinelli y cols., 2013).

La Tabla 2 muestra las diferencias existentes entre los tres tipos celulares: ESCs, ASCs e iPS. Desde el punto de vista clínico, ASCs son las más utilizadas por seguridad y eficacia demostrada, además de por su fácil obtención (Keller y cols., 2012; Lalu y cols., 2012; Sng y Lufkin, 2012). No obstante presentan múltiples limitaciones aún por resolver tales como: la identificación de las señales moleculares que inician su activación, protocolos de aislamiento y cultivos más sencillos, obtención de protocolos de diferenciación *in vitro* que consigan aumentar su plasticidad, identificación de más tipos celulares, aumento de la viabilidad celular en el producto final, estabilidad genética de los cultivos y obtención del número de células en el menor tiempo posible manteniendo sus características intrínsecas (Galvez-Martín y cols., 2013).

Tabla 2. Atributos, limitaciones y problemas éticos de los diferentes tipos de células en Terapia Celular.

	Células Madre Embrionarias	Células Madre Adultas	Células Madre Inducidas
Atributos	Pluripotentes Genéticamente compatible con el paciente Alta capacidad de expansión y cultivo	Multipotentes-Unipotentes Genéticamente compatible con el paciente Bajo riesgo tumorigénico Aplicación clínica demostrada	Pluripotentes Genéticamente compatible con el paciente
Limitaciones	Número limitado de líneas celulares disponibles Rechazo inmune Riesgo de crear teratomas Uso clínico restringido	Tipos celulares limitados Difíciles de identificar Limitada capacidad de expansión	Riesgo de mutagénesis por el uso vectores virales Riesgo de cáncer y senescencia Riesgo de crear teratomas Uso clínico restringido
Problemas Éticos	Destrucción de blastocitos humanos Consentimiento informado para donación de óvulos y blastocitos Mal uso de la clonación reproductiva	Sin consideraciones éticas mayores	Sin consideraciones éticas mayores

I.1.1.3. MEDICAMENTOS DE TERAPIA CELULAR SOMÁTICA (CTMP)

La Terapia Celular comenzó con los primeros trasplantes de médula ósea con éxito en 1968 (Noort y cols., 2002), evolucionando hacia la búsqueda de nuevos tratamientos. Las terapias basadas en células madres abarcan la recogida de la muestra biológica (biopsia), depuración, manipulación, transporte y caracterización de las células con fines terapéuticos.

Se considera CTMP la utilización en seres humanos de células somáticas vivas y/o tejidos, que presenten propiedades profilácticas, de diagnóstico o terapéuticas distintas de sus propiedades fisiológicas y biológicas originales. Sin embrago no todos los productos celulares se consideran medicamentos. Un CTMP debe cumplir con al menos una de las siguientes condiciones (Martín y cols., 2012):

- Las células son sometidas a "manipulación sustancial", alterándose sus características biológicas, funciones o propiedades relevantes para obtener el efecto terapéutico. Teniendo en cuenta la complejidad metodológica de los productos de Terapia Celular, la Agencia Europea del Medicamento (EMA del inglés European Medicines Agency) ha definido las manipulaciones "NO" sustanciales (Tabla 3), por lo que todas aquellas manipulaciones que no estén incluidas son consideradas sustanciales. Una de las principales manipulaciones llevadas a cabo en el desarrollo de un CTMP será por tanto la expansión mediante cultivo celular *ex vivo* ya que altera sustancialmente las características de las células aisladas de un tejido.

- Cambio en la función esencial de la célula, es decir la célula con uso terapéutico no está destinada a realizar su función biológica inicial.

Por tanto un CTMP se define como aquel medicamento de Terapia Celular somática para uso en seres humanos de células somáticas vivas, tanto autólogas, como alogénicas o xenogénicas, cuyas características biológicas han sido alteradas sustancialmente como resultado de su manipulación, para obtener un efecto terapéutico, diagnóstico o preventivo por medios metabólicos, farmacológicos e inmunológicos.

Tabla 3. Modificaciones No sustanciales. Anexo 1, Reglamento (EC) No 1394/2007.

Modificaciones No Sustanciales
Corte
Trituración
Moldeo
Centrifugación
Imbibición en disoluciones antibióticas o antimicrobianas
Esterilización
Irradiación
Separación
Concentración
Purificación celular
Filtración
Liofilización
Congelación
Criopreservación
Vitrificación

I.1.2. MEDICAMENTOS DE TERAPIA GÉNICA (GTMP)

La Terapia Génica implica la expresión de genes terapéuticos en una célula, proporcionando en ella nuevas funciones, con la finalidad de aliviar o curar enfermedades.

La Terapia Génica consta de tres partes fundamentales: las células diana, el transgen terapéutico y el vector de transferencia génica. La célula diana es aquella que se pretende modificar genéticamente pudiendo o no coincidir con la célula afectada por la patología. El vector, que puede ser de origen viral o no viral, tiene que ser seguro y capaz de expresar el gen deseado durante un período prolongado de tiempo en una población suficientemente grande de células para producir un efecto biológico (Muñoz-Ruiz y Regueiro, 2012). Los vectores no-virales serían preferibles por razones de seguridad biológica; sin embargo, la baja eficiencia de los mismos en células primarias y la inestabilidad del transgen limitan su utilización, siendo los vectores virales los más utilizados (Tabla 4) (Lechardeur y Lukacs, 2002; Mancheño-Corvo y Martín-Duque, 2006).

Tabla 4. Vectores virales usados en Terapia Génica.

Vector	Diana Celular
Adenovirus	Pulmón, tracto respiratorio
Retrovirus	Células T y fibroblastos.
Lentivirus	Células madre en proliferación
Virus adenoasociado	Células madre en proliferación

Se considera GTMP, al producto obtenido mediante un conjunto de procesos de fabricación destinados a transferir, *in vivo* o *ex vivo*, un gen profiláctico, de diagnóstico o terapéutico, tal como un fragmento de ácido nucleico, a células humanas/animales para su posterior expresión *in vivo*. Los medicamentos de terapia génica no incluyen las vacunas contra enfermedades infecciosas (Galvez y cols., 2011).

I.1.3. PRODUCTOS DE INGENIERÍA TISULAR (TEP)

La Ingeniería Tisular se define como la aplicación de los principios y métodos de la ingeniería hacia el desarrollo de sustitutos biológicos, utilizando células viables o no, combinadas con biomateriales y otros factores para reparar, mejorar y/o regenerar la función tisular (Pomahac y cols., 1998).

Actualmente la Ingeniería Tisular combina el aporte de células indiferenciadas o no y sus componentes extracelulares, sobre una matriz, reproduciendo una estructura tridimensional tisular, que sea funcional y semejante al tejido, añadiendo factores que aceleren la proliferación celular para ser trasplantadas a la estructura dañada y conseguir su regeneración (Langer, 2000; Song y cols; 2004) (Figura 5).

Un medicamento de Ingeniería Tisular (TEP) es aquel que contiene o está formado por células o tejidos manipulados por ingeniería, y del que se alega que tiene propiedades para regenerar, restaurar o reemplazar un tejido humano (Galvez y cols., 2013). El TEP podrá contener células o tejidos de origen humano, animal o ambos. Las células o tejidos podrán ser viables o no. Podrá también contener otras sustancias, como productos celulares, biomoléculas, biomateriales, sustancias químicas, soportes o matrices (Raghunath y cols., 2005; Stoddart y cols., 2009).

Las células o tejidos se considerarán manipulados por ingeniería si han sido sometidos a manipulación sustancial y/o no están destinados a emplearse para la misma función o funciones esenciales en el receptor y en el donante.

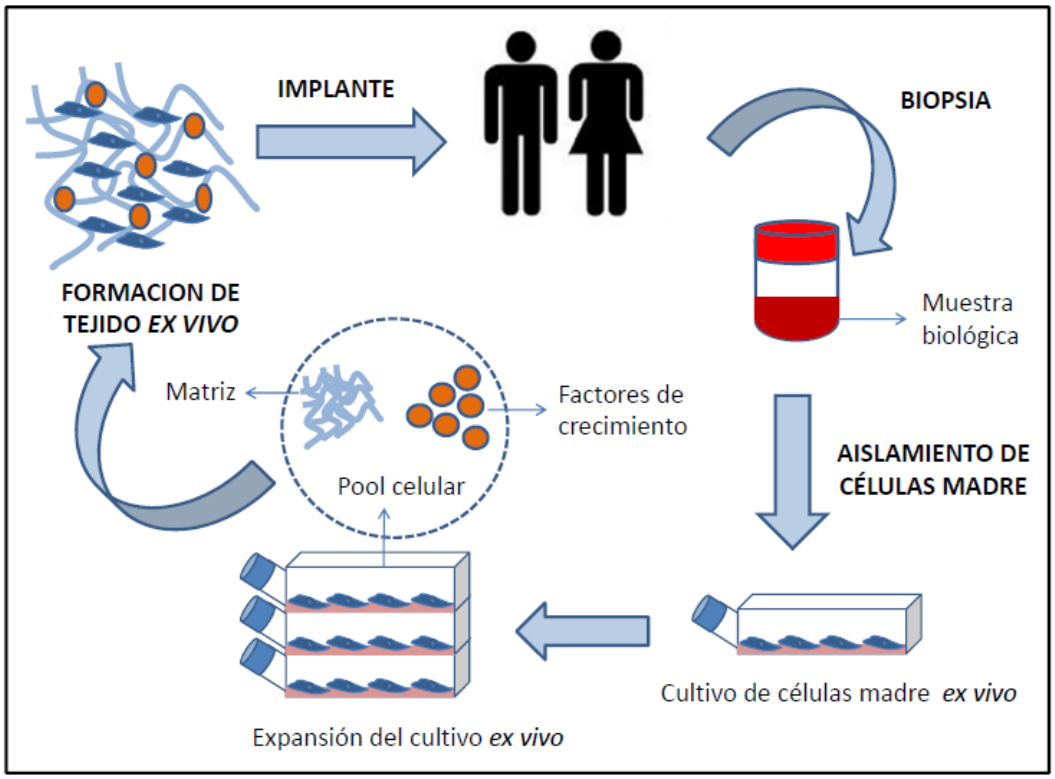

Figura 5. Etapas y componentes de la Ingeniería Tisular en la formación de un tejido.

I.1.4. MEDICAMENTOS DE TERAPIAS AVANZADAS COMBINADOS (PC)

Un PC es aquel que cumple las siguientes condiciones (Gálvez y cols., 2013):

- Incorporar, como parte integrante del mismo, uno o más productos sanitarios.
- La parte celular o tisular debe contener células o tejidos viables, o su parte celular o tisular que contenga células o tejidos no viables tiene que poder ejercer en el organismo humano una acción que pueda considerarse fundamental respecto de la de los productos sanitarios mencionados.

I.1.5. MEDICAMENTOS DE TERAPIAS AVANZADAS COMERCIALIZADOS

El uso de células madre, genes y tejidos como activos farmacológicos en la terapéutica actual es ya una realidad. Actualmente ya existen ATMP comercializados en todo el mundo. La Tabla 5 muestra un resumen de todos los ATMP comercializados actualmente, siendo los CTMP los más estudiados y desarrollados.

Tabla 5. Medicamentos de Terapias Avanzadas comercializados internacionalmente.

	Medicamento	**Activo Biológico**	**Mercado**
CTMP	**Chondorcelet®**	Condrocitos autólogos	Europa
	Epicel®	Queratinocitos alogénicos	Estados Unidos
	Carticel®	Condrocitos autólogos	
	Prochymal ®	Mesenquimales alogénicas	Canadá
	Chondron™	Condrocitos autólogos	India
	Laviv®	Fibroblastos autólogos	Japón
	Cartistem®	Mesenquimales alogénicas	Corea
	Cupistem®	Mesenquimales autólogas	
	HeratiCellgram®	Mesenquimales autólogas	
GTMP	**Provengene®**	Células autólogas CD54+ activadas con PAPGM-CSF	Estados Unidos
TEP	**Glybera®**	Alipogén tiparvovec	Europa
	Apligraf®	Matriz de colágeno con queratinocitos y fibroblastos autólogos	Estados Unidos

En concreto en España, tan solo se comercializa el Chodrocelet® (TiGenix). En el año 2012 obtuvo la autorización de comercialización en la EMA, un año más tarde en el 2013 se acordó el precio de venta con la Agencia Española de Medicamentos y Productos Sanitarios (AEMPS). Debido a su alto coste y a los problemas logísticos que conlleva el trasporte de las muestras biológicas y del medicamento, solo se oferta en 13 hospitales.

Por otro lado, el Sistema Nacional de Salud incluye tres tratamientos con células madre en la cartera de servicios públicos:

- Trasplante autólogo de condrocitos.
- Auto-implante de queratinocitos (piel artificial) para el tratamiento de quemados.
- Tratamiento de lesiones corneales con células madre limbocorneales.

I.2. CÉLULAS MADRE MESENQUIMALES (MSCs)

I.2.1. CONCEPTO Y DEFINICIÓN

Las MSCs son células madre multipontentes, derivadas del estroma medular de varios tejidos humanos. El tejido mesenquimal, generalmente denominado mesénquima, es el tejido del organismo embrionario y se divide en dos tipos de tejido mesenquimal:

- **Tejido mesenquimal no especializado**: formado íntegramente por tejido conjuntivo laxo (sostiene órganos y epitelios en su lugar y posee una gran variedad de fibras proteicas, colágeno y elastina); y tejido conjuntivo denso (posee abundantes fibras colágenas, es menos flexible pero más resistente, está presente en tendones, ligamentos, en la dermis profunda, en el periostio y en el pericondrio).

- **Tejido mesenquimal especializado:** formado por el tejido adiposo, tejido cartilaginoso, tejido óseo, tejido hematopoyético, sangre y tejido muscular, estriado y liso.

El mesénquima hace referencia también a los tejidos de sostén o de relleno que conforman los órganos, en contraposición al parénquima o tejido principal de un órgano.

Las MSCs son capaces de autorrenovarse y diferenciarse en múltiples linajes celulares de origen mesodérmico, tales como tejido adiposo, hueso, tendón, cartílago y músculo (Jiang y cols., 2002). Bajo las condiciones apropiadas también pueden diferenciarse a linaje endodérmico y ectodérmico (Kim y Cho, 2013).

Estas células fueron descritas por primera vez en 1968 por Friedenstein y cols. Utilizando ratones y cobayas, identificaron unas células fusiformes adherentes al plástico que eran capaces de regenerar tejido óseo *ex vivo* (Friedenstein y cols., 1968). En 1976 Friedenstein y colaboradores identificaron una población de células adherentes de la médula ósea que formaban parte del estroma medular y que daban origen al microambiente hematopoyético, con morfología fibroblastoide fusiforme (Friedenstein

y cols., 1976), denominándolas Unidades Formadoras de Colonias Fibroblastoides (CFU-F, del inglés Colony Forming Unit-Fibroblasts).

En el año 2006, la Sociedad Internacional de Terapia Celular (ISCT del inglés Internacional Society Cellular Therapy) propuso tres criterios para definir las MSCs (Dominici y cols., 2006):

- Deben ser adherentes en cultivo.
- Expresar los antígenos de superficie específica CD73, CD90 y CD105 en ausencia de antígenos hematopoyéticos como CD34, CD45, marcadores de monocitos, macrófagos y linfocitos B.
- Deben ser capaces de diferenciarse *in vitro* en osteoblastos, adipocitos y condrocitos bajo condiciones estándar de cultivo.

Sin embargo, incluso estos criterios mínimos definidos para armonizar los criterios de identificación de MSCs no son definitivos, ya que pueden existir diferencias en función de la fuente del tejido de obtención, de las condiciones de cultivo y de la especies (humano y diferentes animales). Su fenotipo está determinado por su microambiente específico (Watt y cols., 2013), por lo que a día de hoy no existe uno o varios marcadores que permitan caracterizar las MSCs *in vivo* de forma inequívoca, y por tanto la caracterización de estas células se lleva a cabo por la combinación de marcadores de superficie y por su capacidad de diferenciación (Lin y cols., 2010; Du Rocher y cols., 2012; Zimmerlin y cols, 2013).

Las MSCs fueron descritas como células fibroblastoides, pero se ha observado que según la fuente de obtención, la morfología de las MSCs puede variar (Kern y cols., 2006). Las células aisladas del cordón umbilical presentan una morfología ovoide, frente a la morfología fibroblastoide de las células obtenidas de médula ósea y tejido adiposo (Figura 6).

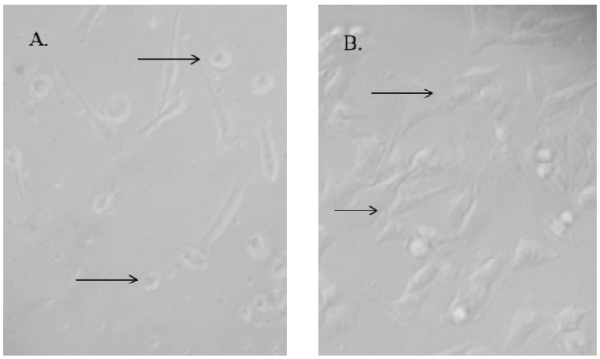

Figura 6. Morfología de Células Madre Mesenquimales humanas. A. Células obtenidas del cordón umbilical, con morfología ovoide. B. Células obtenidas de médula ósea, más prominentes y fibroblastoides (Modificada de Páez, 2007).

I.2.2. FUENTES BIOLÓGICAS DE OBTENCIÓN

Las MSCs se han aislado a partir de aspirados de médula ósea, tejido adiposo, músculo liso y cardíaco, líquido amniótico, placenta, sangre del cordón umbilical, sangre periférica y pulpa dental (Zvaifler y cols., 2000; Zuk y cols., 2001; Rodriguez-Lozano y cols., 2011; Bitsika y cols., 2012; Pojda y cols., 2013).

En la actualidad, la médula ósea, el tejido adiposo y la sangre del cordón umbilical son consideradas como las principales fuentes de obtención de MSCs (Tomar y cols., 2010; Kuhbier y cols., 2010; Menard y cols., 2013), siendo las fuentes más fiables para su posterior aplicación clínica. La médula ósea y el tejido adiposo son consideradas las fuentes más idóneas por su alta disponibilidad y por su fácil procesamiento bajo procedimientos estandarizados, pudiendo obtener un número elevado de células en cultivo *ex vivo*. De ambas, el tejido adiposo es una fuente mucho más rica y accesible que la médula ósea, pudiendo obtener biopsias biológicas de tejido adiposo por procedimientos menos invasivos (Kuhbier y cols., 2010). En particular, las MSCs derivadas de tejido adiposo (ATMSCs) se diferencian respecto a las MSCs derivadas de médula ósea (BMMSC) en su inmunofenotipo ya que expresan diferentes marcadores. Las ATMSCs expresan niveles más altos de CD34, CD49d, y CD54, mientras que las BMMSC expresan niveles más elevados de CD106 (De Ugarte y cols., 2003; Menard y cols., 2013). La capacidad de diferenciación de las MSCs también varía. Las ATMSCs se diferencian más fácilmente a cardiomiocitos y las BMMSCs tienen mayor potencial

condrogénico. Además existen diferencias en cuanto a su expresión génica, perfil proteómico y propiedades inmunológicas (Noël y cols., 2008; Mosna y cols., 2010). Sobre la obtención de MSCs de sangre de cordón umbilical son varios los factores limitantes, siendo el principal la obtención y procesamiento de la muestra, además de las connotaciones éticas y regulatorias que conlleva su origen alogénico (Bieback y cols., 2004).

I.2.3. AISLAMIENTO Y CULTIVO DE CÉLULAS MADRE MESENQUIMALES DE TEJIDO ADIPOSO

El tejido adiposo deriva del mesodermo embrionario y contiene una población heterogénea de células estromales tales como las MSCs, células endoteliales, células de músculo liso, fibroblastos, y otros tipos celulares circulantes como leucocitos o HSCs (Tholpady y cols., 2006; Mizuno y cols., 2012).

La biopsia de tejido adiposo puede ser de origen autólogo o alogénico. La extracción del tejido (30-50 g) se lleva a cabo a través de métodos quirúrgicos, ya sea por lipoaspirado o por exéresis quirúrgica (Figura 7).

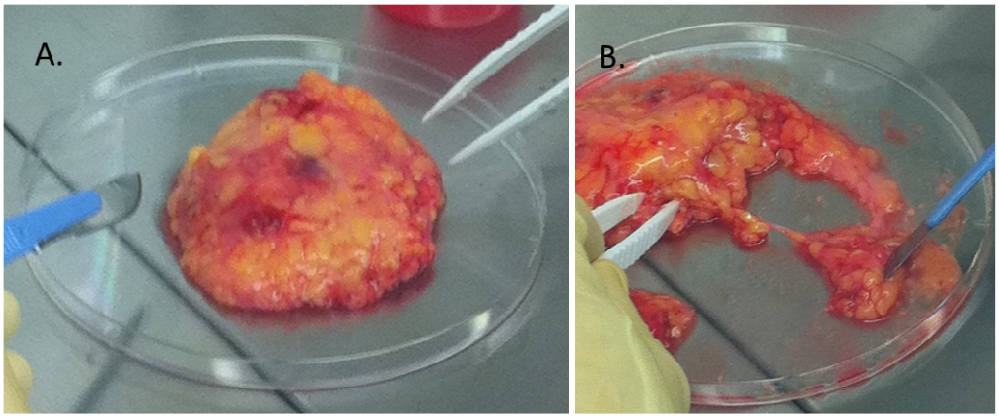

Figura 7. Tejido adiposo obtenido por exéresis. A. Recepción del tejido adiposo en cabina de flujo laminar y pesada. B. Limpieza del tejido adiposo y eliminación del tejido conjuntivo.

Una vez obtenida la muestra, esta es transportada hasta el laboratorio de procesamiento en un recipiente estéril y a una temperatura entre 4-8 ºC.

Ya en el interior de la cabina de flujo laminar, el tejido es lavado, disgregado, digerido enzimáticamente, centrifugado y filtrado (Figura 8). El pool celular obtenido es sembrado en placas (flask, factorías o hiperflask) suplementado con un medio de expansión. El aislamiento de las MSCs se basa en su adherencia a las superficies plásticas, por tanto en la primera siembra las células no adherentes (estromales) son eliminadas en los cambios de medio, obteniendo una única población de células adheridas al plástico.

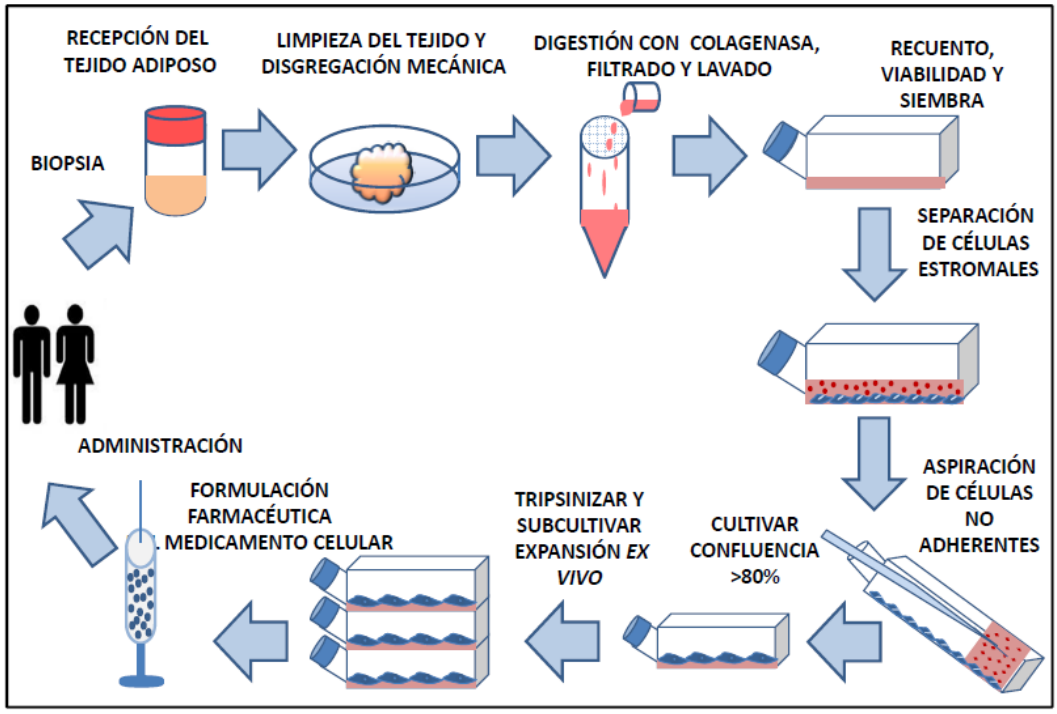

Figura 8. Esquema del proceso de aislamiento y expansión *ex vivo* de ATMSCs hasta la obtención del número de células requeridas para el tratamiento indicado.

Para la obtención de la dosis celular requerida en Terapia Celular, será necesario subcultivar días, incluso semanas, realizando un pase celular una vez obtenida la confluencia del 80% del cultivo (Figura 9) (Brinchmann, 2008).

El potencial de expansión *ex vivo* de las MSCs es limitado, ya que el mantenimiento de un cultivo durante largos periodos de tiempo, provoca el envejecimiento del cultivo ocasionando perdida de la multipontecialidad de las células, senescencia replicativa (Stolzing y cols., 2006; Côté y cols., 2007; Koch y cols., 2012) y disminución de su capacidad de diferenciación (Digirolamo y cols., 1999). Se cree que la senescencia

celular actúa como un mecanismo supresor de tumores capaces de detener el crecimiento celular para reducir el riesgo de malignidad (Beauséjour y cols., 2007; Choudhary y cols., 2012; Pan y cols., 2013).

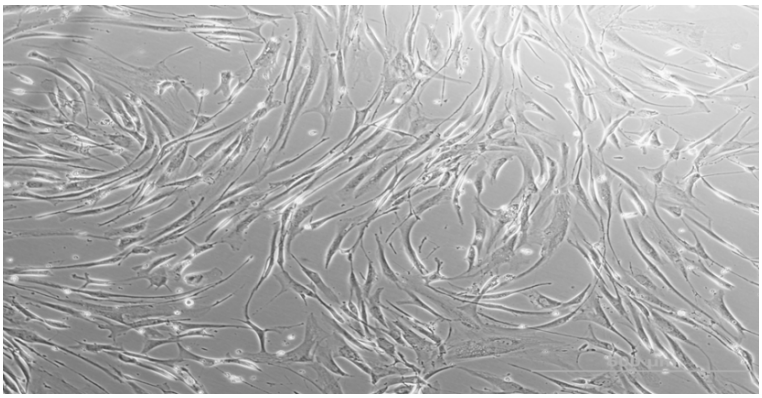

Figura 9. MSCs adheridas en cultivo con una confluencia del 80%**.**

Otro efecto que se ha observado durante la expansión *ex vivo* prolongada de las MSCs es la acumulación de alteraciones genéticas y epigenéticas (Wagner, 2012), dando lugar a transformaciones espontáneas que originan células inmortales con gran similitud a las cancerosas (Ueyama y cols., 2011; Bochkov y cols., 2011; Binato y cols., 2012). Rubio y colaboradores describieron que un cultivo de MSCs mantenido durante más de 20 pases tiene un mayor riesgo de aparición de alteraciones cromosómicas (Rubio y cols., 2005), sin embargo, más recientemente, Binato y colaboradores han descrito que dicha inestabilidad puede aparecer en pases más tempranos (pase 4) (Binato y cols., 2012). Estos resultados son contradictorios, pero todos ellos están de acuerdo en que las MSCs poseen inestabilidad genética y pueden causar transformaciones espontáneas que resulten en tumores (Wang y cols., 2005; Tolar y cols., 2007; Zhang y cols., 2009).

I.2.4. PROPIEDADES FARMACOLÓGICAS

Las MSCs, al igual que las ASCs en general, tienen menor potencial proliferativo y plasticidad que las ESCs, sin embargo ofrecen numerosas ventajas: facilidad de obtención, gran potencial de expansión y crecimiento *ex vivo*, carencia de problemas éticos para su obtención y escasa tendencia a formar tumores.

Su uso terapéutico se basa en su capacidad de secretar citoquinas y factores de crecimiento, migrando al lugar donde existe el daño tisular y adquiriendo propiedades antiinflamatorias, inmunomoduladoras, angiogénicas y antitrombogénicas (Phinney, 2012; Bernardo y cols., 2012; Le Blanc y cols., 2012).

I.2.4.1. CAPACIDAD INMUNOREGULADORA Y ANTIINFLAMATORIA

Las MSCs ejercen efectos inmunomoduladores en todas las células implicadas en la respuesta inmune, tanto *in vivo* como *ex vivo*, a través de mecanismos que todavía no están completamente definidos (Nauta y Fibbe, 2002). Las MSCs se caracterizan funcionalmente por ser capaces de inducir la supresión de la proliferación de linfocitos T (English y cols., 2009; Burr y cols., 2013) y de células asesinas (NK del inglés Natural Killer) (Spaggiari y cols., 2008) además de suprimir la funcionalidad de los linfocitos B (Corcione y cols., 2006; Uccelli y cols., 2006) e inhibir la maduración de las células dendríticas (Nauta y cols., 2006; Ramasamy y cols., 2007; Lisianyĭ, 2013).

Por otro lado el efecto inmunosupresor de las MSCs se debe al contacto célula-célula y a su capacidad de producir factores solubles, incluyendo factor de crecimiento transformante-β (TGF- β del inglés Transforming Growth Factor Beta), factor de crecimiento hepático (HGF del inglés Hepatocyte Growth Factor), óxido nítrico (NO del inglés nitric oxido), interleucinas (IL-4. IL-6, IL-10), prostaglandina E2 (PGE-2), interferón-γ (IFN-γ) e indolamina 2,3-dioxigenasa (IDO) (De Miguel y cols., 2012; Kim y Cho, 2013; Lisianyĭ, 2013). Además las MSCs inducen un entorno inmunosupresor mediante la activación de generación de células T reguladoras (Tregs), directamente o de forma indirecta a través de células dendríticas (Figura 10) (Zappia y cols., 2005; Di Ianni y cols., 2008; Ye y cols., 2008; Madec y cols., 2009; Patel y cols., 2010; Choi y cols., 2012; Engela y cols., 2012; Lee y cols., 2013; Plock y cols., 2013).

Las MSCs presentan una baja inmunogenicidad entre la célula donante y receptora debido a la expresión intermedia del complejo mayor de histocompatibilidad clase I (CMH-I), y no expresan CMH clase II (CMH-II) en su superficie, lo que reduce su antigenicidad (Plock y cols., 2013). Además no presentan expresión para moléculas coestimuladoras como CD80, CD86 y CD40 (Stagg y cols., 2006; De Miguel y cols.,

2012), lo cual las hace idóneas para su aplicación en trasplantes alogénicos (Wood y cols., 2012; Machado y cols., 2013).

A pesar de que los mecanismos de acción exactos por los que las MSCs ejercen sus funciones siguen siendo estudiados, es ampliamente aceptado que pueden constituir una herramienta de alto potencial en Medicina Regenerativa y Terapia Celular.

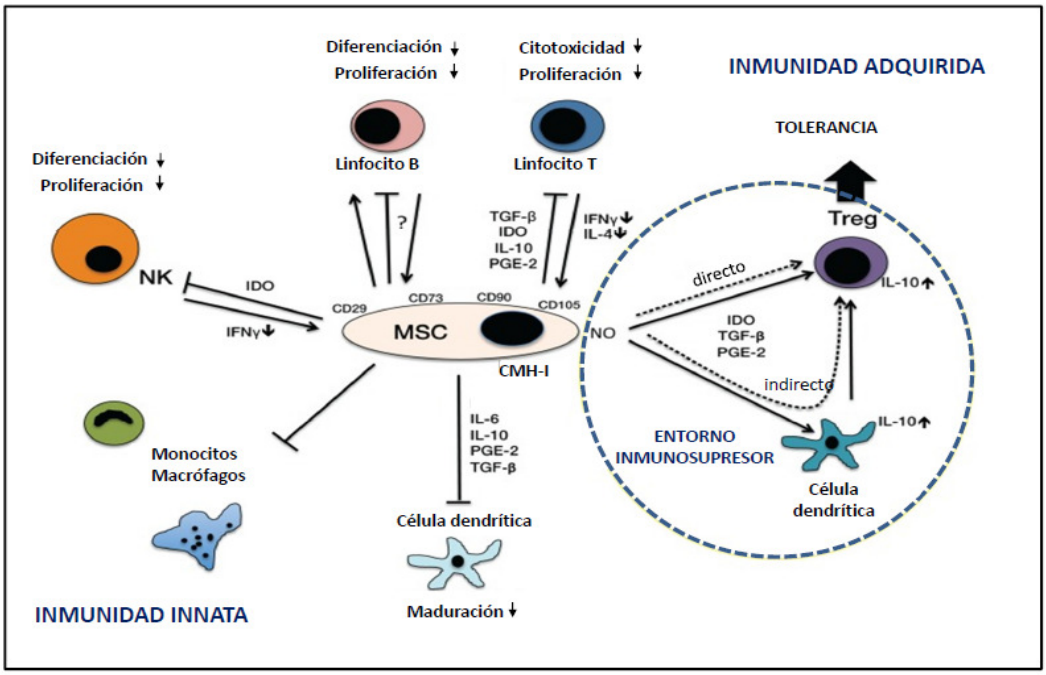

Figura 10. Función inmunológica de las MSCs. Las flechas indican la activación o la inducción, las barras en T indican el bloqueo de la función, en particular la inhibición de la proliferación, la diferenciación, la citotoxicidad y la maduración. (Modificada de Plock y cols., 2013).

I.2.4.2. CAPACIDAD ANGIOGÉNICA Y ANTITROMBÓTICA

Las MSCs presentan una capacidad angiogénica intrínseca debido a su potencial de diferenciación *in vivo* a células vasculares, promoviendo la formación de vasos (Kachgal y Putnam, 2011). Las MSCs secretan múltiples factores de crecimiento angiogénicos, siendo el más importante el factor de crecimiento endotelial vascular (VEGF del inglés Vascular Endothelial Growth Factor) a niveles bioactivos (Bhang y cols., 2009), adquiriendo un papel significativo en la regeneración vascular, cicatrización e isquemias.

I.2.5. INTERÉS EN TECNOLOGÍA FAMACEÚTICA

El desarrollo y la fabricación de MSCs como CTMP debe llevarse a cabo en las mismas condiciones que un medicamento convencional, cumpliendo las normas de correcta fabricación (GMP, del inglés Good Manufacturing Practice), recogidas en la Directiva 2003/94/EC de la Comisión del 8 de octubre de 2003 por la que se establecen los principios y directrices de las prácticas correctas de fabricación de los medicamentos de uso humano y de los medicamentos en investigación de uso humano (Sensebé y cols., 2013; Galvez y cols., 2013).

La producción de MSCs como medicamentos requiere una amplia caracterización del componente celular para garantizar la identidad, pureza, potencia, viabilidad, tumorigenicidad, control de calidad y estabilidad genómica de las células, para su aplicación clínica (Ra y cols., 2011; Martín, y cols., 2012). Toda la caracterización de las MSCs se debe realizar con protocolos normalizados, con el fin de no interferir en la calidad, seguridad y eficacia del producto celular final (Tonti y Mannello, 2008; Phinney, 2012; Greco y Rameshwar, 2012).

Actualmente las vías de administración de las MSCs son diversas: intramuscular, intra-arterial, intravenosa y transdérmica (Horie y cols., 2009; Hosoyama y cols., 2012; Han y cols., 2012; Kim y cols., 2013).

El diseño de la formulación galénica del producto final, dependerá de la concentración celular predeterminada, posología y del órgano o tejido diana:

- **Concentración celular** se define en millones de células por kg de paciente a tratar o por cm^2 cuando la formulación requiere un andamio o membrana.
- **Posología,** el tratamiento puede requerir una única dosis o varias, e incluso tratamientos de larga duración que requieran una administración pautada en el tiempo.
- **Diana**, en el caso de dianas locales, la concentración celular podrá ser mayor. Sin embargo en la administración sistémica se deberá definir la capacidad de agregación celular, para evitar la formación de trombos.

En cuanto a la dosificación, no existen aún datos farmacodinámicos cualitativos ni cuantitativos de estas células, siendo actualmente objeto de estudio en muchos grupos de investigación.

I.2.6. APLICACIONES CLÍNICAS

Las células madre mesenquimales humanas (hMSCs del inglés Human Mesenchymal Stem Cells) son las más estudiadas en el área de desarrollo clínico. Sus aplicaciones terapéuticas se centran principalmente en su administración local y trasplante sistémico (Abdallah y Kassem, 2008), tanto como CTMP, GTMP, TEP y PC (Chen y cols., 2006; Kurtzberg y cols., 2013; Gálvez y cols., 2013).

Actualmente se están llevando a cabo 1.929 ensayos clínicos, en fase de reclutamiento, con células madre en todo el mundo, de los cuales 249 ensayos clínicos se realizan con hMSCs; 30 de ellos en España (clinicaltrial.gov, consultado Noviembre 2013) (Figura 11).

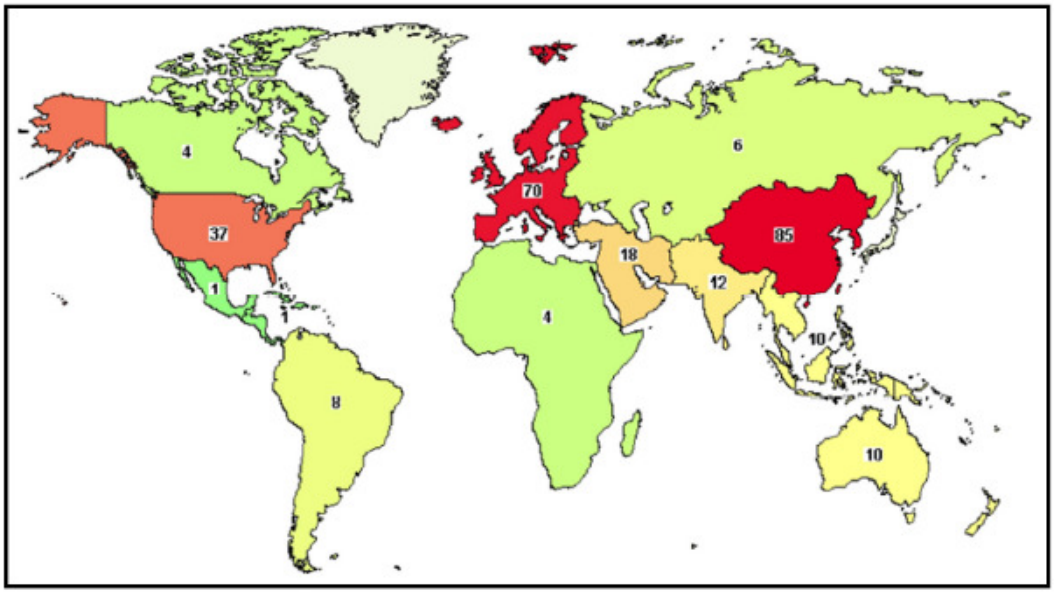

Figura 11. Representación de los ensayos clínicos activos con hMSCs.

Sus efectos terapéuticos están siendo ampliamente explorados en ensayos clínicos como tratamiento potencial en distintas patologías: diabetes (Abdi y cols., 2008), enfermedades neurológicas (Uccelli y cols., 2011; Baek y cols., 2012), patologías cardíacas (Gnecchi y cols., 2012; Mathiasen y cols., 2012) o enfermedades reumáticas (Djouad y cols., 2009) (Tabla 6).

Los resultados de los ensayo clínicos llevados a cabos hasta el momento con este tipo de células han concluido determinando que son seguras y eficaces (Le Blanc y cols., 2008; Prasad y cols., 2011; Wang y cols., 2012; Murphy y cols., 2013).

Tabla 6. Principales patologías estudias en fase clínica con hMSCs.

Patología	**Nº de Ensayos Clínicos**
Injerto contra huésped	26
Leucemia	9
Esclerosis Lateral Amiotrófica	9
Esclerosis Múltiple	25
Artritis	18
Enfermedades inmunológicas	27
Diabetes	40
Alteraciones del sistema digestivo	38
Enfermedades del sistema nervioso central	32
Carcinoma	5
Enfermedades del sistema endocrino	17
Fibrosis	16
Enfermedades gastrointestinales	38
Enfermedad cardiaca	36
Cirrosis	14
Enfermedad hepática	51
Enfermedades metabólicas	22
Enfermedades musculo esqueléticas	29
Necrosis	14
Osteoartritis	12
Enfermedades del tracto respiratorio	10
Enfermedades reumáticas	16
Enfermedades urológicas	11
Enfermedades vasculares	32

*clinicaltrial.gov (Noviembre 2013)

I.3. DIABETES E ISQUEMIA CRÍTICA

I.3.1. DIABETES

La Diabetes Mellitus (DM) es una enfermedad metabólica crónica y compleja que se caracteriza por la deficiencia absoluta o relativa de insulina. El efecto de la diabetes no controlada es la hiperglucemia (aumento del azúcar en sangre), que con el tiempo daña gravemente órganos y sistemas, originando múltiples complicaciones como neuropatías periféricas y lesiones vasculares. La DM está estrechamente asociada con enfermedades cardiovasculares: enfermedad de las arterias coronarias, enfermedad cerebro-vascular y con enfermedad arterial periférica (Setacci y cols., 2013).

La mayoría de los casos de DM se encuentran agrupados en dos categorías:

- **Diabetes de tipo I,** también llamada insulinodependiente o juvenil. Se caracteriza por una producción deficiente de insulina y requiere la administración diaria de esta hormona. Actualmente se desconoce la causa de este tipo de DM, no pudiéndose prevenir con el conocimiento actual.

- **Diabetes de tipo II,** se debe a una utilización ineficaz de la insulina. Este tipo representa el 90% de los casos mundiales y se debe en gran medida a un peso corporal excesivo y a la inactividad física (Gerich, 2007).

La DM es una de las enfermedades crónicas con mayor impacto socio-sanitario no solo por su alta prevalencia, sino también por su elevada morbimortalidad (Soriguer y cols., 2012). Actualmente supone la sexta causa de muerte en países desarrollados. Su prevalencia varía entre varias poblaciones, siendo las más afectadas aquellas que han adquirido un estilo de vida occidental. Los aspectos genéticos son importantes; tener un familiar de primer grado afectado confiere un 40% de riesgo de padecer DM tipo II (International Diabetes Federation, 2009; Tripathi y Srivastava, 2006). Las estimaciones de la Organización Mundial de la Salud (OMS) en referencia a la prevalencia de la DM la sitúan en el 6,5% de la población mundial con una tendencia creciente al 7,8% para el año 2030 (OMS, 2012). En España, se estima que la prevalencia de diabetes tipo II es

del orden del 62% en grupos con edades de 30 a 65 años, y del 10% para grupos de edades entre 30 y 89 años (Martínez-Castelao, 2005; Ruiz-Ramos y cols., 2006).

La DM puede dar lugar a la aparición de lesiones cutáneas y úlceras en el 30% de los pacientes (Vuolo, 2007) debido a la afectación de la enfermedad diabética sobre vasos, nervios y tejido epitelial. La DM conlleva a una disminución de la funcionalidad celular, reduciendo la capacidad de migración y proliferación celular además de la formación vasos (Hill y cols., 2003).

I.3.1.1. PIE DIABÉTICO

El Grupo de Consenso sobre Pie Diabético de la Sociedad Española de Angiología y Cirugía Vascular define el Pie Diabético (PD) como una alteración clínica de base etiopatogénica y neuropática, inducida por una hiperglucemia mantenida, en la que con o sin coexistencia de isquemia, y previo desencadenante traumático, produce lesión y/o ulceración del pie (Lavery y cols., 1996). El PD conlleva la infección, la ulceración y la destrucción de los tejidos del pie asociado con anormalidades neurológicas y vasculopatías periféricas de diversa gravedad en las extremidades inferiores (Kim y Steinberg, 2013). Sin embargo, desde el punto de vista práctico, se denomina PD a todas las lesiones que los diabéticos presentan en su miembro inferior, sean de causa isquémica o no (Isner y Rosenfield, 1993; Black y cols., 2005).

El PD es una de las principales complicaciones de la DM, afectando aproximadamente al 15% de los pacientes diabéticos (Boulton, 1994) y representa la principal causa de amputación de los miembros inferiores en países desarrollados (Murray y Boulton, 1995).

En la fisiopatología del PD existen tres factores fundamentales: la neuropatía, la isquemia y la infección (Bennett, 2000; Shiling, 2003):

1. Neuropatía diabética

Engloba tanto la neuropatía autómica como sensitiva o motora. Se origina por inhibición sensorial (no existe conciencia de un daño repetitivo sobre algún punto de su pie), como por la "simpatectomía" del diabético (que condiciona vasodilatación

mantenida, aumento de la reabsorción ósea y colapso articular). Predispone a los microtraumatismos inadvertidos (Martínez-Castelao, 2005).

2. Isquemia

Es secundaria a las lesiones arterioescleróticas. Desde el punto de vista fisiopatológico, la ateromatosis arterial en el enfermo diabético no presenta elementos diferenciables con respecto al no diabético, pero sí una determinada mayor prevalencia en su morfología y topografía. Frecuentemente las lesiones son multisegmentarias y afectan al sector fémoro-poplíteo y tibio-peroneo de forma bilateral. Se produce alteración de la vasculopatía periférica originando isquemia crítica del miembro inferior.

3. Infección

Se origina por una alteración microvascular, impidiendo la migración leucocitaria y originando por tanto una disfunción inmunitaria que dará lugar a una menor capacidad de respuesta a las infecciones. Es secundaria a las alteraciones inmunológicas y a la situación de isquemia descrita.

Existen varias clasificaciones para el PD, aunque la más utilizada es la clasificación de Wagner (Wagner, 1981), la cual muestra una excelente correlación tanto con el porcentaje de amputaciones como con la morbimortalidad (Tabla 7).

Conforme las lesiones adquieren un grado superior, aumenta la posibilidad de sufrir una amputación más extensa y aumenta asimismo la mortalidad asociada. En cuanto a la etiopatogenia del PD, los Grados 0, I, II y III se correlacionan con lesiones neuropáticas y los IV y V con lesiones isquémicas.

Tabla 7. Clasificación del Pie Diabético, según Wagner.

Grado	Lesión	Características
0	Ninguna, pie de riesgo	Callos gruesos, cabezas de metatarsianos prominentes, dedos en garra, deformidades óseas (pie de Charcot).
I	Úlceras superficiales	Destrucción del espesor total de la piel.
II	Úlcera profunda	Penetra la piel, grasa, ligamentos pero sin afectar a hueso. Infectada.
III	Úlcera profunda más absceso (poliomielitis)	Extensa y profunda, secreción, mal olor.
IV	Gangrena limitada	Necrosis de una parte del pie o de los dedos, talón o planta.
V	Gangrena extensa	Todo el pie afectado; efectos sistémicos.

I.3.2. ISQUEMIA CRÍTICA DEL MIEMBRO INFERIOR

La isquemia crítica del miembro inferior (CLI, del inglés Critical Limb Ischemia) es una de las principales manifestaciones de aterosclerosis sistémica que afecta a las extremidades inferiores, siendo la fase final de la enfermedad arterial periférica.

CLI se ha definido por la Trans Atlantic Inter-Society Consensus (TASC) como el cuadro caracterizado por dolor crónico en reposo, úlceras o gangrena atribuible a la enfermedad arterial oclusiva (Dormand y Rutherford, 2000; Sacks, 2003; Nogren y cols., 2007). Así pues, la CLI es la última fase del proceso de aterosclerosis que puede conllevar la amputación del miembro e incluso la muerte si no se consigue una rápida y eficaz revascularización. CLI se caracteriza por un riesgo de amputación mayor del 50% (Yang y cols., 2010) y bajo pronóstico de supervivencia.

La incidencia de CLI es de 500-1.000 casos por millón y año (Raval y Losordo, 2013). Entre el 15-30% de los pacientes con claudicación intermitente desarrollarán CLI a lo largo del curso de su enfermedad (Nogren y cols., 2007). Aproximadamente el 1% de los adultos mayores de 50 años padecen esta enfermedad, lo que supone que en Europa

y Estados Unidos entre 1.5 y 2 millones de personas sufren CLI (Weitz y cols., 1996). Aunque su patogénesis no es del todo conocida, sí existen una serie de factores de riesgo bien definidos (Raval y Losordo, 2013) (Tabla 8).

Tabla 8. Factores de riesgo ateroescleróticos

Factores de Riesgo	
Modificables	Hiperlipemias
	Factores genéticos
	Tabaquismo
	Diabetes
	Obesidad
	Sedentarismo
	Factores psíquicos
	Hipertensión
	Hipercolesterolemia
No modificables	Sexo
	Edad

I.3.2.1. DIAGNÓSTICO CLÍNICO

El diagnóstico clínico de la CLI está basado en la sintomatología provocada por la disminución del aporte arterial a las extremidades inferiores ocasionando distintos grados de afectación que varían desde una claudicación leve hasta el dolor en reposo y la ulceración, siendo más significativas cuanto más importante sea el déficit de perfusión tisular.

En 1920 se estableció la clasificación de Fontaine para graduar el grado de afectación de aterosclerosis sistémica (Fontaine y cols., 1954). Su carácter práctico hace que se siga utilizando en la actualidad en Europa (Tabla 9):

1. Grado I

A pesar de existir lesiones ateromatosas el paciente se encuentra asintomático, ya sea porque la obstrucción del vaso no es completa o porque se han desarrollado mecanismos de compensación a expensas de arterias colaterales.

2. Grado II

Se caracteriza por la presentación de claudicación intermitente. Al fracasar los mecanismos de compensación, el paciente presenta dolor muscular a la deambulación por hipoxia tisular. La gran mayoría de las veces se localiza a nivel de la masa gemelar y el dolor obliga al paciente a detenerse tras recorrer algunos metros; con el reposo desaparece el dolor. A efectos prácticos este grupo se divide en dos subgrupos:

- IIa: el paciente claudica a distancias superiores a 150 m.
- IIb: el paciente claudica a menos de 150 m.

3. Grado III

Se caracteriza porque el paciente presenta dolor en reposo. Se suele localizar en dedos y pies, es continuo, progresivamente intolerable, empeora con la elevación de la extremidad y mejora con el declive de la misma. Se acompaña de alteraciones de la sensibilidad y la piel aparece fría y pálida, aunque a veces, con el pie en declive, éste puede verse eritematoso.

4. Grado IV

Se caracteriza por la aparición de úlceras y necrosis en la extremidad, y por la incapacidad del paciente para caminar. Pueden ser lesiones más o menos extensas y aparecen entre el 1% y el 3% de todos los pacientes que desarrollan síntomas.

Tabla 9. Clasificación de Fontaine.

Estadio	Clínica
I	Asintomático
II	Claudicación leve
IIa	Claudicación moderada-grave
IIb	Dolor isquémico en reposo
IV	Ulceración o gangrena

El cuadro clínico de la CLI se caracteriza por (Slovut y Sullivan, 2008):

- Dolor en reposo, de origen isquémico persistente que requiere analgesia durante más de 2 semanas.
- Y/o ulceración o gangrena del pie o de los dedos del pie.
- Presión sistólica en el tobillo ≤ 50 mmHg.
- Presión sistólica en el dedo del pie ≤ 30 mmHg.

El pronóstico de los pacientes con CLI es sombrío. Este cuadro ocasiona aproximadamente 150.000 amputaciones en Europa y Estados Unidos cada año. Tras la aparición del cuadro de CLI, en un año solamente el 50% sobrevive libre de amputación (el 25% habrá muerto y el otro 25% habrá requerido una amputación mayor). En concreto, las cifras de mortalidad de este cuadro son dramáticas: 25% en el primer año, 31.6 % a los 2 años y más del 60% a los 3 años (Hirsch y cols., 2006).

I.3.2.2. ISQUEMIA CRÍTICA DEL MIEMBRO INFERIOR EN PACIENTES DIABÉTICOS

La DM es uno de los factores de riesgo ateroscleróticos más importante en pacientes con enfermedad arterial periférica, aumentando el riesgo de desarrollar un proceso isquémico. La CLI es 20 veces más frecuente en pacientes diabéticos, y está presente, por término medio en el 7% de todos ellos (Procházka y cols., 2010), siendo más prevalente en pacientes diabéticos tipo II (Lanzer, 2001). En el momento del diagnóstico el 8% de los pacientes diabéticos muestran evidencias clínicas de padecer CLI, 10 años después del diagnóstico, el porcentaje se incrementa hasta el 15% y a los 20 años de seguimiento llegan al 45% (Flagia y cols., 2009; Kamalesh y cols., 2009; Ruitir y cols., 2010). La DM es responsable del 50-70% de todas las amputaciones no traumáticas (Criqui, 2001).

En el paciente diabético además de existir una disminución de aporte sanguíneo en el miembro inferior, presenta otros desordenes que influyen en la CLI (Dolan y cols., 2002; Prompers y cols., 2008; Claesson y cols., 2011):

- Oclusión de las arterias distales.
- Defecto en la migración y función de progenitores endoteliales.

- Aumento en el tono vasomotor y atenuación de la respuesta a estímulos vasodilatadores.
- Atenuación de los mecanismos de señalización de los monolitos.
- Atenuación de la señalización mediante factores de crecimiento.
- Reducción de los niveles de factores tisulares del crecimiento. Estos a su vez regulan la cicatrización, mediante la estimulación o inhibición de la proliferación, el movimiento y la actividad de biosíntesis celulares.
- Disminución de la respuesta angiogénica, como consecuencia de la disfunción endotelial.
- Desequilibrio entre las enzimas proteolíticas y sus inhibidores.
- Alteración de la función de los macrófagos.
- Alteraciones en la migración y proliferación de los fibroblastos.
- Alteraciones del balance entre la acumulación de los componentes de la matriz extracelular y su remodelación por las metaloproteinasas de la matriz.

Todos estos aspectos favorecen la isquemia y neuropatía en pacientes diabéticos con CLI, derivando en problemas circulatorios que pueden complicarse hasta la amputación.

I.3.2.3. TRATAMIENTO

Los pacientes diagnosticados con CLI tienen un alto riesgo de amputación y mayor morbilidad y mortalidad cardiovascular. Los objetivos del tratamiento para la CLI se basan en el alivio del dolor isquémico, curación de las úlceras, prevención de la pérdida del miembro, mejora de la calidad de vida del paciente y prolongación de la supervivencia (Mangiafico y Mangiafico, 2011). La revascularización, bien quirúrgica o endovascular es el principal tratamiento recomendado para la CLI (Black y cols., 2005), asociado con terapias farmacológicas concomitantes que promuevan una reducción del riesgo cardiovascular además de actuar sobre los factores de riesgo modificables (Abou-Zamzam y cols., 2007). En el caso de los pacientes que no pueden someterse a revascularización ni amputación existen alternativas tales como la compresión neumática intermitente o la estimulación de la médula ósea, que pueden mejorar la sintomatología y promover la cicatrización de la herida (Slovut y Sullivan, 2008).

1. Tratamiento quirúrgico

Técnicas hiperemieantes de revascularización directa y endoluminales son los tratamientos quirúrgicos de elección en CLI:

- Entre las técnicas hiperemiantes, la más utilizada es la simpatectomía lumbar, que en sí misma no debe plantearse como tratamiento primario de una claudicación en miembros inferiores, pero que en casos seleccionados puede mejorar los síntomas al aumentar el flujo cutáneo.
- La técnica de revascularización directa se basa en la actuación directa sobre la arteria lesionada para eliminar directamente la placa de ateroma responsable de la obstrucción, mejorando el flujo colateral.
- Las técnicas endoluminales consisten en la eliminación de estenosis u obstrucciones mediante la utilización de catéteres percutáneos que disponen de balones expansibles o mallas metálicas capaces de dilatar o repermeabilizar estos vasos.

2. Tratamiento farmacológico

El tratamiento farmacológico implica la administración de analgésicos, cuidado local de la herida, tratamiento de la infección y fármacos que actúen sobre el riesgo cardiovascular tales como antiagregantes, hemorreológicos, anticoagulantes y antitrombóticos (Lumsden y cols., 2009):

- Antiagregantes, interfirieren en la agregación plaquetaria, inhibiendo la formación de trombos. Ej.: ácido acetilsalicílico, dipiridamol, trifusal, ticlopidina, prostaglandinas y eicosanoides.
- Hemorreológicos, actúan aumentando el flujo sanguíneo tisular mediante la reducción de la viscosidad sanguínea y aumento de la deformidad del hematíe, además de un cierto efecto antiagregante. Ej.: pentoxifilina y dextranos.
- Anticoagulante, las heparinas en sus diversas formas (sódica, cálcica, bajo peso molecular), están prácticamente limitadas a las formas agudas o críticas de la isquemia.
- Antitrombóticos, actúan reduciendo las complicaciones cardiovasculares en pacientes sometidos a reconstrucción vascular quirúrgica, mejorando la

revascularización arterial y la permeabilidad del injerto Ej.: estatinas y bloqueante β.

Como se ha señalado anteriormente, el objetivo principal del tratamiento de la CLI es la revascularización quirúrgica o endovascular (Black y cols., 2005). Sin embargo un 30% de los pacientes con CLI no son candidatos para la revascularización debido a un alto riesgo quirúrgico o a la anatomía vascular desfavorable (Mamidi y cols., 2012), al igual que sucede con la mayoría de los pacientes diabéticos que presentan alterada su capacidad de revascularización, por lo que la enfermedad culminará con la amputación y/o la muerte del paciente. El tratamiento farmacológico, como única alternativa para estos pacientes sin posibilidades de revascularización, no ha demostrado que afecte favorablemente al curso de CLI (Isner y Rosenfield, 1993). De hecho, en la actualidad, la mayor parte de las sociedades científicas recomiendan habitualmente para estos pacientes la amputación como única opción, a pesar de su evidente implicación disfuncional, junto con su conocida morbimortalidad asociada (Campbell y cols., 1994). Por ello, es evidente que se necesitan nuevas estrategias terapéuticas viables y eficaces para el tratamiento de la CLI.

I.3.3. ISQUEMIA CRÍTICA DEL MIEMBRO INFERIOR Y TERAPIA CELULAR

La Terapia Celular presenta el uso de células madre como tratamiento alternativo en los pacientes diabéticos con CLI que no pueden ser tratados por revascularización quirúrgica. Esta estrategia se basa en la activación de la angiogénesis, lo cual conlleva la proliferación de los vasos colaterales, mejorando por tanto el proceso isquémico del miembro (Freedman e Isner, 2002; Morishita y cols., 2004; Ylä-Herttuala, 2006; Nikol, 2007) y el pronóstico de la CLI.

La angiogénesis terapéutica induce la formación de una red de capilares en el tejido isquémico mediante la alteración del medio local proporcionando un entorno proangiogénico (Davies, 2012). La angiogénesis terapéutica puede ocurrir a través de dos mecanismos: la vasculogénesis y la angiogénesis (Risau, 1997):

- **Vasculogénesis**: fenómeno que tiene lugar en la etapa embrionaria y que consiste en el desarrollo de vasos sanguíneos. En este proceso intervienen células progenitoras del endotelio (EPCs del inglés Endotelial Progenitor Cells) y las HSCs, ambas de origen mesenquimal, compartiendo antígenos de superficie como el CD34 y CD133. La formación de los vasos sanguíneos se inicia con la formación de islotes vasculogénicos, compuestos en su interior por HSCs y rodeadas por EPCs.

- **Angiogénesis:** consiste en la formación de nuevos capilares a partir de vasos preexistentes. Se inicia por la vasodilatación y aumento de la permeabilidad. La isquemia por sí misma, o bien la liberación de citoquinas, o la activación de moléculas de adhesión provocan la liberación de EPCs desde la médula ósea. Estas EPCs acuden a las porciones tisulares isquémicas en un proceso denominado "homing", en donde se diferencian en células endoteliales que generan nuevos vasos sanguíneos. La principal citoquina liberada por la isquemia para promover el homing de las EPCs es el VEGF (Wojakowski y Tendera, 2005), además de granulocitos y macrófagos (GM-CSF del inglés Granulocyte-Macrophage Colony Stimulating Factor), angiopoyetina, etc. (Losordo y Dimmeler, 2004) (Figura 12). El tratamiento con estatinas también ha mostrado la capacidad de estimular la migración de EPCs desde la médula ósea (Vasa y cols., 2001).

El uso de las MSCs, está tomando gran importancia para la regeneración vascular en patologías isquémicas por su actividad angiogénica (Kim y cols., 2013; Watt y cols., 2013).

Las ATMSCs secretan múltiples factores de crecimiento angiogénicos, siendo el más importante el VEGF a niveles bioactivos (Rehman y cols., 2004). Por otra parte, el efecto antiinflamatorio, inmunomodulador y el carácter multipotencial de las MSCs ha demostrado ser capaz de resolver casos puntuales para los que hasta ahora no se tenía una oferta terapéutica mejor. Por este motivo, estas células pueden ser una alternativa factible a la hora de revascularizar una CLI.

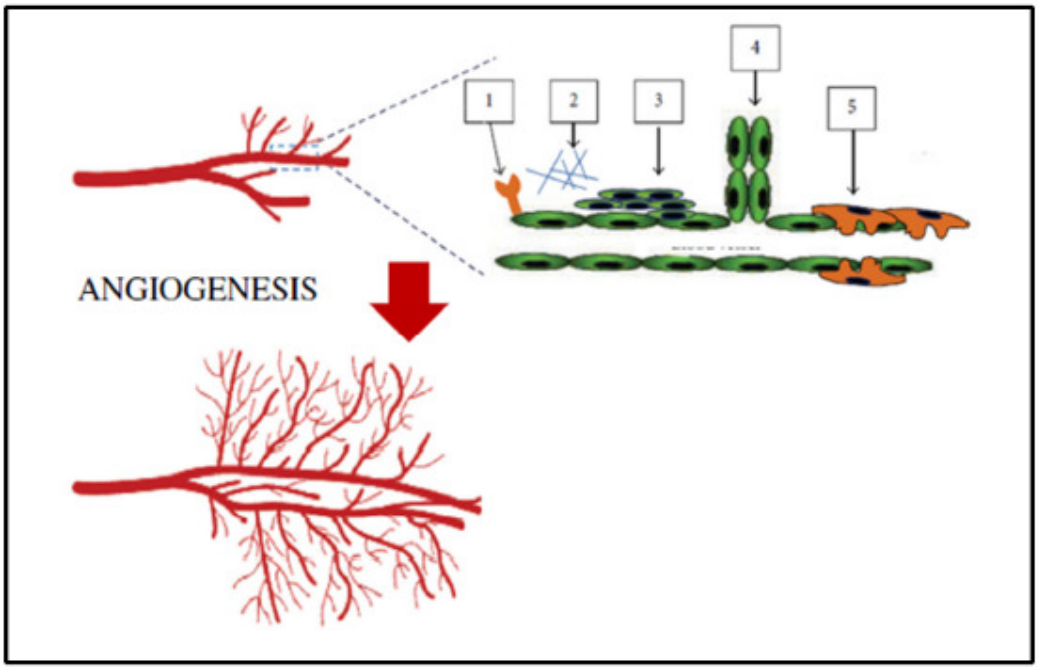

Figura 12. Proceso fisiológico de angiogénesis. 1- Factores angiogénicos (VEGF) unidos a sus receptores en las EPCs, activando la señal de transducción. 2- Formación de una matriz de metaloproteinasas. 3- Proliferación y migración de EPCs. 4- Expresión de las integrinas por EPCs, facilitando su adhesión a la matriz extracelular y su migración para formar el vaso. 5- Estabilización por TGF-β, (Modificada de Yoo y Kwon, 2013).

Los primeros ensayos clínicos llevados a cabo concluyen en que la administración de MSCs es segura y va asociada a significativos beneficios terapéuticos en pacientes diabéticos con CLI (Das y cols., 2013; Gremmels y cols., 2013; Yang y cols., 2013).

Como se ha comentado anteriormente, en los casos más complejos de CLI, la solución de revascularización quirúrgica o endovascular es limitada. Además, en concreto los pacientes diabéticos tienen disminuidos los mecanismos fisiológicos de angiogénesis y reendotelización, por lo que el curso de la enfermedad es más severo y acelerado. En este contexto, el tratamiento con hMSCs, con sus demostradas propiedades de angiogénesis y cicatrización de úlceras cutáneas, puede tener un máximo interés.

La aplicación en Terapia Celular de ATMSCs podría ser una alternativa terapéutica en el tratamiento de la CLI. Aunque las EPCs y HSCs han demostrado

su eficacia (Iba y cols., 2002), estas fuentes potenciales de células angiogénicas tienen limitaciones para la angiogénesis terapéutica debido a la gran cantidad de muestra necesaria para obtener una dosis celular (Bhang y cols., 2009). Por el contrario las ATMSCs pueden aislarse fácilmente a partir de una pequeña muestra de tejido adiposo la cual es obtenida por métodos simples y mínimamente invasivos, además de poder ser expandidas *ex vivo* hasta conseguir la dosis celular requerida. Pero su principal beneficio es su capacidad de diferenciación vascular junto con sus efectos paracrinos promoviendo la revascularización y reparando los tejidos isquémicos (Nakagami y cols., 2005; Cai y cols., 2007) (Tabla 10).

Tabla 10. Efectos terapéuticos de las principales células madre en el tratamiento de la CLI.

Tipo Celular	**Efectos Terapéuticos**
MSCs	Promover la proliferación celular, síntesis de colágeno, liberación de factores de crecimiento, cicatrización, regeneración tisular y neovascularización.
EPCs	Promover la vascularización, secreción de factores de crecimiento pro-angiogénicos y diferenciación en células endoteliales.
HSCs	Secreción de factores de crecimiento angiogénicos, antiinflamación local, promover la vascularización y diferenciación en células endoteliales.

Por otra parte, el efecto antiinflamatorio, inmunomodulador y el carácter multipotencial de las MSCs ha demostrado ser capaz de resolver casos puntuales para los que hasta ahora no se tenía una oferta terapéutica mejor. Por este motivo, estas células pueden ser una alternativa factible a la hora de favorecer la regeneración vascular y propiciar una mejoría en la revascularización que acontece en una CLI.

CAPÍTULO II.

OBJETIVO Y PLAN DE TRABAJO

Las hMSCs son células pluripotentes con actividad angiogénica e inmunomoduladora con efectos demostrados en la revascularización del miembro inferior, presentándose como una alternativa al tratamiento quirúrgico y farmacológico existentes en aquellos pacientes cuya tasa de recuperación es muy baja.

El producto a fabricar deberá ser tratado como medicamento ya que para su obtención sufre una manipulación sustancial (expansión *ex vivo*), por lo que se trata de un CTMP cuyas células deberán ser viables en el momento de la administración.

En base a las experiencias positivas que hasta el momento existen y la prospectiva actual de los medicamentos basados en Terapia Celular somática, el objetivo principal de este proyecto será el diseño, desarrollo, caracterización y estudio de estabilidad de un medicamento celular con hMSCs de origen autólogo derivadas de tejido adiposo, destinado al tratamiento de la CLI en pacientes diabéticos tipo II, para su estudio en un ensayo clínico fase I-II.

Con estas premisas y conocidas las propiedades revascularizantes de las hMSCs en pacientes diabéticos con CLI, se planteó el desarrollo de una suspensión celular de administración intra-arterial. Para ello se estudió la elección de sus excipientes, concentración celular, control de calidad, caracterización celular y estabilidad.

En base a todo lo anterior y para la consecución del objetivo planteado para este estudio, el plan de trabajo propuesto fue:

1. Actualizar el conocimiento sobre los avances en Terapias Avanzadas y Nanomedicina, en concreto en Terapia Celular.
2. Definir un CTMP y revisar los requerimientos legales para su investigación y desarrollo.
3. Diseñar el protocolo necesario para la fabricación del CTMP objeto de estudio. Normalización de la técnica de obtención de hMSCs. Estudio de la viabilidad y confluencia de siembra.
4. Puesta a punto de la fabricación aséptica de hMSCs en una sala blanca bajo condiciones GMP.
5. Diseño de un programa de control de calidad microbiológica para los materiales de partida y de acondicionamiento, productos intermedios y producto final implicados en la fabricación de un CTMP.

6. Formulación galénica de la suspensión celular con hMSCs. Estudio y selección de los excipientes más idóneos así como de la concentración celular.
7. Desarrollo, caracterización y estudio de la influencia que microesferas de glucosa pueden tener sobre la viabilidad celular.
8. Caracterización físico-química del CTMP centrándonos en el análisis de pH, estudio reológico de la forma de dosificación y morfología de las células.
9. Estudio microbiológico del medicamento celular tal y como define la Farmacopea Europea para el análisis de su esterilidad, endotoxinas y micoplasma.
10. Caracterización inmunofenotípica y genética de las hMSCs.
11. Estudio de la capacidad de diferenciación de las células contenidas en el medicamento diseñado.
12. Evaluación de la estabilidad del medicamento celular objeto de estudio en función de la temperatura y tiempo de conservación. Adicionalmente se estudian los posibles fenómenos de desestabilización del medicamento celular mediante análisis óptico de estabilidad acelerada.
13. Definición de las condiciones de transporte y almacenamiento del medicamento final.

CAPÍTULO III.
RESULTADOS

RELACIÓN DE TRABAJOS CIENTÍFICOS PUBLICADOS

- **Gálvez P**, Ruiz A, Clares B. The future of clinical medicine in new therapies: Cell, gene and nanomedicine. Medicina Clínica. 2011; 137(14):645-649.
- **Gálvez P**, Bermejo M, Ruiz MA, Gallardo V, Clares B. Isolation and characterization of the environmental bacterial and fungi contamination in a pharmaceutical unit of mesenchymal stem cell for clinical use. Biologicals. 2012; 40:330-337.
- **Gálvez P,** Ruiz MA, Gallardo V, Clares B. Regulatory considerations in production of a cell therapy medicinal product in Europe to clinical research. Clinical and Experimental Medicine. 2014; 1:25-33.
- **Gálvez P,** Clares B, Hmadcha A, Ruiz MA, Soria B. Development of a cell-based medicinal product: regulatory structures in the European Union British Medical Bulletin. 2013; 105:85-105.
- **Gálvez P,** Clares B, Bermejo M, Hmadcha A, Soria B. Standard requirement of a microbiological quality control program for the manufacture of human mesenchymal stem cells for clinical use. Stem Cells and Development. 2014; in press.
- **P. Gálvez-Martín,** A. Hmadcha, B. Soria, A.C. Calpena-Campmany, B. Clares-Naveros. Study of the stability of packaging and storage conditions of human mesenchymal stem cell for intra-arterial clinical application in patient with critical limb ischemia. European Journal of Pharmaceutics and Biopharmaceutics. 2013; in press.
- **P. Gálvez**, M.J. Martin, J.A. Tamayo, A.C. Calpena, B. Clares. Development and characterization of glucose microspheres for enhancing viability of a human stem cell suspension with clinical application. Sometido.

III.1. THE FUTURE OF CLINICAL MEDICINE IN NEW THERAPIES: CELL, GENE AND NANOMEDICINE

Los avances científicos de las últimas décadas dirigen el campo de la Medicina Regenerativa hacia una nueva era, donde las terapias convencionales se apoyarán en los avances biotecnológicos cuyo máximo exponente son la Terapia Celular, Génica y la Nanomedicina. Todo ello ha provocado un gran impacto en las expectativas de los pacientes que sufren patologías sin tratamientos eficaces. Numerosos grupos de investigación centran sus esfuerzos en el desarrollo de nuevas terapias que además de tratar puedan mejorar la calidad de vida del paciente.

Las células madre, genes y nanopartículas son términos ya conocidos, pero su implicación en las nuevas terapias y sus propiedades en Medicina Regenerativa requieren de una revisión y actualización de los conocimientos en esta área.

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Revisión

El futuro de la medicina clínica hacia nuevas terapias: terapia celular, génica y nanomedicina

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RESUMEN

Las terapias avanzadas aportan un nuevo concepto de medicamento personalizado de origen autólogo, alogénico o xenogénico, basado en células (terapia celular), genes (terapia génica) o tejidos (ingeniería tisular), que, junto a los nanosistemas, ofrecen avances en el diagnóstico, la prevención y el tratamiento de enfermedades. En esta revisión se describirán los fundamentos y campos de actuación de la terapia celular, génica y nanomedicina.

The future of new therapies in clinical medicine

Keywords:
Cell therapy
Gene therapy
Nanomedicine
Nanotechnology

ABSTRACT

Advanced therapies provide a new concept of personalized medicament of autologous, allogeneic or xenogeneic origin, based on cells (cell therapy), genes (gene therapy) or tissues (tissue engineering), which, together with nanosystems, provide new advances in the diagnosis, prevention and treatment of diseases. The basis and different fields of action of cell therapy, gene therapy and nanomedicine are described in this review.

El desarrollo tecnológico y los avances aportados por la industria biotecnológica[1] en la última década han dado lugar a nuevos fármacos y empleo de métodos de transferencia génica[2], técnicas de imagen molecular[3], reprogramación celular y/o transdiferenciación[4], abriendo un camino de nuevas terapias para una medicina más racional y personalizada. Actualmente, existen terapias incipientes con gran potencial de desarrollo.

Terapia celular

Tiene por objetivo reparar, reemplazar o recuperar la función biológica de un tejido u órgano dañado[5], utilizando para ello células vivas. Se basa en la utilización en clínica de células madre, cuyas principales características son su capacidad de autorrenovación y la potencialidad de diferenciarse a células especializadas[6]. Según su potencial de diferenciación se clasifican en[7]: *totipotenciales*, *pluripotenciales y multipotenciales*.

Se han encontrado células madre adultas en médula ósea, cordón umbilical, sangre, córnea, retina, cerebro, músculo esquelético, tejido adiposo, pulpa dental, hígado, piel y epitelio gastrointestinal. En este sentido, cabe destacar los avances científicos llevados a cabo en los siguientes tipos celulares:

- Hematopoyéticas, que son las más estudiadas y mejor caracterizadas; el trasplante alogénico de progenitores hematopoyéticos ha demostrado que existen células madre multipotenciales hematopoyéticas en la médula ósea y en la sangre periférica.
- Mesenquimales (MSC), que son las células del estroma no hematopoyéticas, capaces de diferenciarse y contribuir a la regeneración de tejidos (hueso, cartílago, músculo, ligamento, tendón y tejido adiposo[8]).
- Embrionarias, que se obtienen de la masa celular interna de un blastocisto[9]; se diferencian *in vitro*, sin pérdida de pluripotencialidad, generando la mayoría de los linajes somáticos, incluyendo

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Tabla 1
Resumen de los trastornos patológicos en los que se centran los ensayos clínicos en terapia celular

Trastorno	Tratamiento celular
Infarto de miocardio	Células musculares o mioblastos
Infarto de miocardio	Células madre hematopoyéticas
Patología corneal	Células limbocorneales
Patología de suelo pelviano	Mioblastos esqueléticos
Isquemia crítica crónica de miembros inferiores	Células mesenquimales
Isquemia crítica crónica de miembros inferiores en pacientes diabéticos	Células CD133+
Vitíligo	Melanocitos
Tratamiento de la enfermedad del injerto contra el huésped	Células mesenquimales
Fístula perianal	Células mesenquimales
Esclerosis múltiple	Células mesenquimales
Glioblastoma multiforme	Células dendríticas
Angiogénesis	Células CD133+
Cirrosis hepática	Hepatocitos

los cardiovasculares. Su uso conlleva gran controversia científica[10] y bioética, y por tanto su traslación a la clínica está impedida.
- Células IP (pluripotentes inducidas), que son células adultas diferenciadas y especializadas a las que se ha reprogramado y convertido en células madre pluripotentes, comportándose como células madre embrionarias con nuevas perspectivas para la terapia celular y génica[11].

Tanto regeneración como diferenciación están reguladas por señales inter e intracelulares[12], haciendo de ellas una herramienta crucial para el desarrollo de las terapias avanzadas en enfermedades, para las cuales los tratamientos convencionales no son eficaces: disfunciones hormonales[13] (diabetes mellitus tipo 1[14] o déficit en la hormona del crecimiento), lesiones cardiovasculares[15] (insuficiencia cardiaca congestiva[16], enfermedad arterial periférica[17], infarto de miocardio[18], pacientes trasplantados[19]), enfermedades neurodegenerativas[20] (Parkinson, Alzheimer, corea de Huntington), lesiones osteoarticulares[21,22], distrofias musculares[23] o lesiones en las que hay que regenerar el epitelio, como la fístula perianal (tabla 1).

Esta aparente variedad informa de cómo los principios subyacentes de la terapia celular son comunes, de ahí las ventajas de abordar su investigación, desarrollo e implementación clínica de forma conjunta.

Terapia génica

La "terapia génica" (somática o germinal), definida como el tratamiento de una enfermedad a través de la manipulación genética, consiste en transferir al organismo el material genético corregido con la finalidad de prevenir o tratar enfermedades[24]. El gen terapéutico se puede aplicar en el órgano afectado o administrar el complejo gen/vehículo por vía sistémica *(in vivo)* o, alternativamente, se aíslan las células del paciente, se corrige la mutación y se le vuelven a administrar *(ex vivo)* (fig. 1).

Los tipos de terapia génica que se realizan en estos momentos son: suplementación génica positiva o negativa, supresión génica y destrucción autolítica[25]. Sólo una pequeña fracción de los ensayos de terapia génica va dirigida a corregir defectos monogénicos. Asimismo, la mayoría de los ensayos se centran en reparar mutaciones adquiridas a lo largo de la vida del individuo y que conducen a trastornos tan dispares como el cáncer y las enfermedades cardiovasculares. Hasta el momento los ensayos clínicos en terapia génica se han realizado en pacientes con diabetes mellitus, fibrosis quística, deficiencia de alfa-1-antitripsina, leucemia o infarto de miocardio[26–28], entre otros.

Uno de los principales problemas de la terapia génica es encontrar un vector adecuado (vírico o no vírico) que sea seguro y eficaz. Sin embargo, los vectores actualmente disponibles (basados en el virus de la leucemia murina de Moloney, o el virus de la inmunodeficiencia humana[29]) confieren una expresión débil y transitoria de los genes terapéuticos y, además, se asocian con trastornos oncogénicos e inmunológicos que suscitan la aparición de anticuerpos neutralizantes que pueden inhibir la reinoculación del vector, o respuestas celulares que pueden eliminar las células que expresan el transgen. No obstante, las funciones del sistema inmunitario pueden ser aprovechadas en aproximaciones de terapia génica para vacunar contra microorganismos patógenos o para eliminar células tumorales. A este respecto, cada vez es más evidente que el éxito de este tipo de tratamientos depende en gran medida de interacciones con el sistema inmune, de forma que tanto la formación de anticuerpos como la inducción de respuestas celulares pueden actuar a favor o en contra de la terapia génica. Por ello, se podrían definir dos áreas principales de investigación: estrategias que eliminen respuestas inmunes no deseadas contra vectores y transgenes (inmunosupresión e inducción de tolerancia), y estrategias para inducir respuestas inmunes más potentes y duraderas contra patógenos y tumores.

Cabe destacar los logros conseguidos en esta disciplina mediante el uso de células madre. El estudio de células madre hematopoyéticas (HSC) dirigido a la transferencia de genes se trata de una estrategia terapéutica para el tratamiento de una serie de trastornos causados por defectos hematopoyéticos de un único gen, especialmente para algunas inmunodeficiencias primarias[30].

Los estudios actuales se basan en la transferencia *ex vivo* de los genes terapéuticos en HSC autólogo. Las HSC implantadas fueron genéticamente modificadas; estos ensayos tuvieron éxito debido al

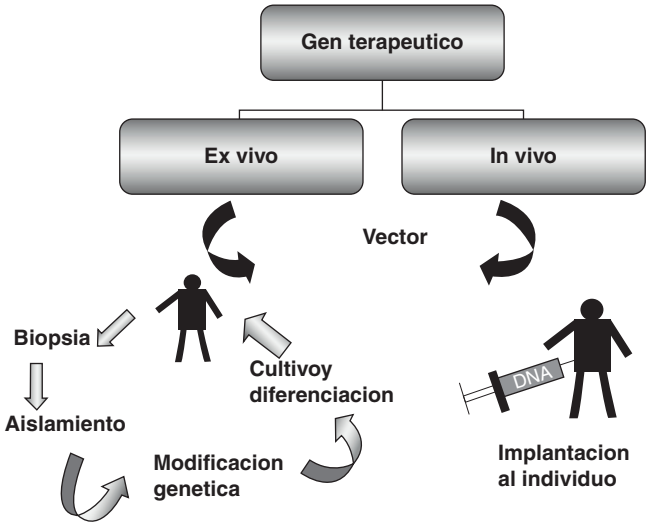

Figura 1. Esquema de las diferentes estrategias terapéuticas en terapia génica.

marcado selectivo del gen linfoide precursor corregido que permite la reconstitución del sistema inmune[31]. En la última década, se han logrado importantes avances en el tratamiento de inmunodeficiencia combinada severa (SCID)-X1, la SCID deficiente en adenosina deaminasa (ADA), y la enfermedad granulomatosa crónica (EGC). Resultados de los ensayos de terapia genética en SCID han demostrado a largo plazo el restablecimiento de la competencia inmunológica y el beneficio clínico en más de 30 pacientes[32].

Por último, es preciso resaltar las evidencias conseguidas en cuanto a que el genoma humano es sensible al entorno nutricional en un doble sentido: los nutrientes pueden regular los genes y, además, los genes influyen en el efecto de la dieta[33], lo que plantea nuevamente posibilidades sobre el metabolismo lipídico y, por tanto, sobre las enfermedades cardiovasculares.

Nanomedicina

Organismos como el *U.S. National Institute of Health*, la *U.K. Royal Society and Royal Academy of Engineering* y la *European Science Foundation* acuñaron el término de nanomedicina, cuyo objetivo es "el control, la reparación y la mejora integral de todos los sistemas biológicos humanos, trabajando desde el nivel molecular con dispositivos de ingeniería y nanoestructuras para lograr beneficios médicos". Precisamente, la Iniciativa Nacional Nanotecnológica (NNI) fue creada en el año 2001, con objeto de acelerar y explotar el progreso en la ciencia y la ingeniería de nanoestructuras.

La nanomedicina y nanotecnología están desarrollando investigaciones significativas, en la sensibilidad diagnóstica y pronóstica[34], protección de tejidos trasplantados[35,36] y farmacocinética del principio activo, aumentando su especificidad y eficacia[37].

Varias nanoplataformas como fullerenos, nanotubos, puntos cuánticos, nanoporos, dendrímeros, liposomas, nanoburbujas y nanopartículas están siendo estudiadas y/o ya comercializadas (fig. 2)[38].

Los liposomas[39] fueron los primeros nanosistemas, aunque su rápida degradación en el torrente circulatorio y su especificidad por el sistema retículo endotelial dio lugar al desarrollo de los llamados liposomas superficialmente modificados.

El uso clínico de liposomas abarca diferentes líneas de investigación, como el transporte de antiinfecciosos, ADN, oligonucleótidos, ribosomas y adyuvantes inmunológicos[40]. Particularmente importante es pues la aplicación de los virosomas en vacunas ya comercializadas como la de la hepatitis B, fiebre amarilla, *Pseudomonas aeruginosa*, Difteria-Tétanos-*Pertussis*-Hepatitis B y Difteria-Tétanos-*Pertussis*-*Haemofilus*.

Como alternativa a la inestabilidad de los liposomas surgen las nanopartículas, bien nanoesferas constituídas por una matriz polimérica, bien nanocápsulas formadas por núcleo con cubierta polimérica. Los polímeros poliláctico, poliglucólico o chitosan han ganando importancia gracias a su atoxicidad, carácter promotor y versatilidad.

Por su parte, los dendrímeros se caracterizan por la existencia de un núcleo, que determina el tamaño, forma, dirección y multiplicidad, una zona intermedia de capas concéntricas o capas de amplificación y una superficie con un número previsto de grupos funcionales. Encontramos estudios con antivíricos, citostáticos, agentes de contraste, vacunas, terapia génica[41–43],

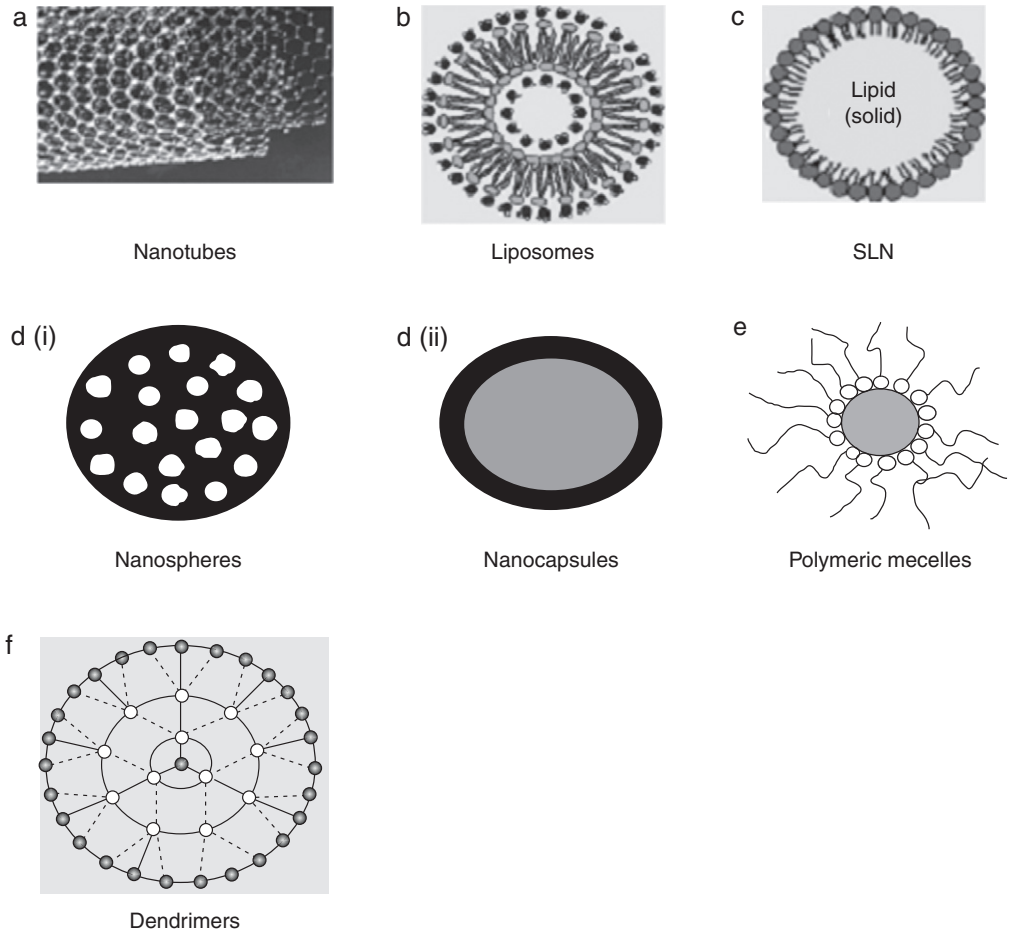

Figura 2. Nanovectores. a. Nanotubos. b. Liposomas. c. Nanopartículas lipídicas. d. (i) Nanoesferas. (ii) Nanocápsulas. e. Micelas poliméricas. f. Dendrímeros.

así como los compuestos de plomo para el desarrollo de fármacos frente al virus de la inmunodeficiencia humana tipo 1 (VIH-1)[44].

Interesantes también son las nanoestructuras de péptidos bioactivos por su potencial capacidad para combinar la bioactividad de objetivos múltiples de biocompatibilidad[45] en medicina regenerativa. Con este fin existen estudios de nanofibras con péptidos bioactivos para la reparación tisular en la enfermedad cardiovascular isquémica[46,47].

Diversas enfermedades son objetivo de nanovehículos[48], aunque es el cáncer la que destaca en el campo de la nanotecnología. En este sentido se están desarrollando nuevos métodos que eliminan resistencias[49–51], para detección y diagnóstico[52–54], con encapsulación de distintos anticancerosos[55–57] y con empleo de nanopartículas de oro[58] o liposomas magnéticos[59] como sistemas transportadores inteligentes.

Conclusiones

La caracterización preclínica de las formas de dosificación revisadas en este trabajo es compleja por la variedad de materiales, propiedades superficiales, reactividad, y la tarea de rastrear los componentes individuales o compuestos en estudios *in vivo*.

Aunque aún persisten grandes problemas para alcanzar el potencial terapéutico que ofrecen todos los sistemas que componen las nuevas terapias avanzadas, existe una esperanza generalizada de que todos ellos presentarán un gran beneficio en la terapéutica actual y futura.

Conflicto de intereses

Los autores declaran no tener ningún conflicto de intereses.

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III.2. REGULATORY CONSIDERATIONS IN PRODUCTION OF A CELL THERAPY MEDICINAL PRODUCT IN EUROPE TO CLINICAL RESEARCH

El uso de células como agentes terapéuticos constituye el campo de la llamada Terapia Celular. Este concepto es relativamente complejo en comparación con el uso de fármacos convencionales, y no sólo implica el trasplante de células a un paciente, sino también toda una serie de tecnologías relativas al aislamiento, caracterización, cultivo, diferenciación y modificación genética de las células trasplantadas que debe realizarse en base a la regulación vigente.

Debido a la novedad y complejidad que ha supuesto el uso de células madre como medicamento en los últimos años, la EMA requirió la redacción de normas armonizadas que garantizasen el desarrollo de estos productos en base a unos estándares de calidad y seguridad.

El objetivo de este artículo ha sido estudiar el concepto de ATMP, y en concreto aquellos requerimientos implicados en el desarrollo de un CTMP. Se han definido los requisitos necesarios para su diseño, las normas de fabricación en condiciones asépticas, las instalaciones requeridas para dicha fabricación, la cualificación mínima del personal implicado y todos los procesos de validación necesarios para la puesta punto del protocolo de fabricación del CTMP, objeto de estudio en esta tesis.

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REVIEW ARTICLE

Regulatory considerations in production of a cell therapy medicinal product in Europe to clinical research

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Abstract The development of new drugs using stem cells has become a clinic alternative for the treatment of different diseases such as Alzheimer's, diabetes and myocardial infarction. Similar to conventional medicines, stem cells as new medicinal products for cell therapy are subjected to current legislation concerning their manufacture process. Besides, their legality is determined by the Regulatory Agencies belonging to the Member State of the European Union in which they are being registered. With the evolution of therapy that uses cells as medicines, there is a need to develop the appropriate legislative and regulatory framework capable of ensuring their safety and effectiveness. However, few works have been published regarding the regulations that these products must comply through production and commercialization processes. The present work is focused on the description of key events during clinical development and cell production of stem cells as drugs. Such as the regulations, requirements and directives involved in the production of cell therapy medicinal products, from the clinical design stage to its commercialization in Europe.

Keywords Stem cells · Advanced therapy · Cell therapy · Clinical trial

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Introduction

The translational process of using new advances therapies in clinical and medical practices has contributed to recent regulatory policies in the United States and Europe, creating new regulatory regimes for advanced and complex treatments such as advanced therapy medicinal products (ATMP). These products are new concepts of medical treatment and include three major types of products such as gene therapy, somatic cell therapy and tissue engineered products that are of particular relevance for regenerative medicine [1].

The regulatory issues to be addressed for the clinical development of these new therapies are much more complex as compared with traditional drugs [2]. The regulatory framework for ATMPs was established with the Regulation on Advanced Therapies (Regulation No 1394/2007), which came into force in December 2008. This lays down specific rules for the evaluation, authorization and commercialization of ATMPs [3, 4].

To ensure the administration of cells and tissues safely and reliable for clinical use, national and international guidelines and regulations have been issued to regulate the collection, process, evaluation, preservation, storage and distribution of all ATMP for application in human [5].

Cell therapy is mainly based on the transplantation of live human cells in order to achieve new treatments for diseases, some of which are as yet incurable.

Although numerous clinical trials based on cell therapy are already being carried out in Europe [6], little works have been published concerning the regulatory guidelines on development of cell therapy medicinal products (CTMP).

Scientific advances have opened up an enormous potential opportunity for all types of cell-based therapeutics, in this

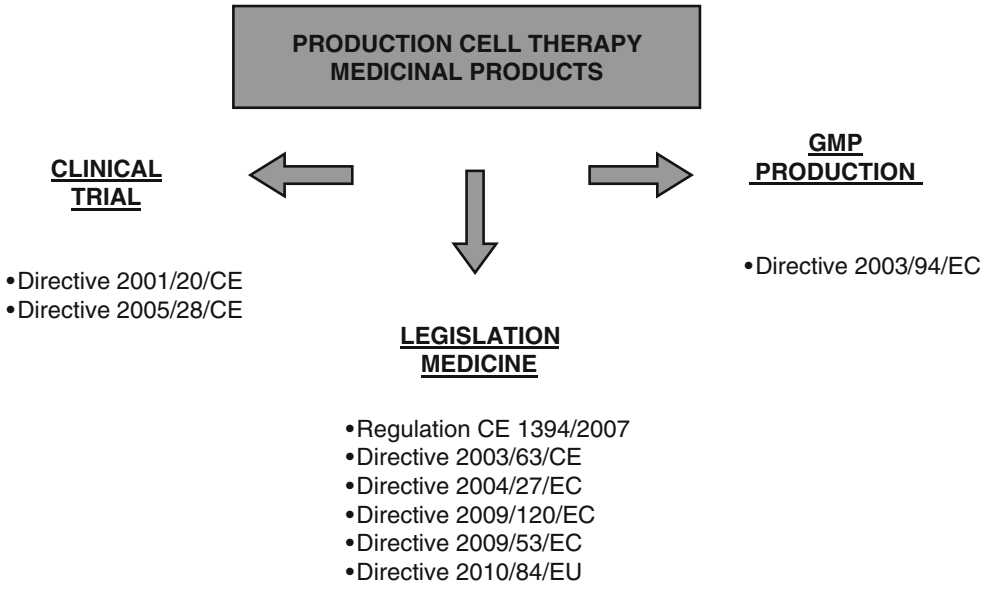

Fig. 1 Summary of current legislation applicable in cellular medicine. Good manufacturing practice (GMP)

sense, an appropriate legislative and regulatory framework must be established in order to complete the assessment of safety, quality and efficacy.

The experience gained from blood banks in the areas of quality control, production and medicine monitoring will undoubtedly be of great service to the field of pharmaceutical cell manufacturing process. In this work, we consider the need for developing pre-clinical studies, clinical trials as well as legal, ethical and scientific requirements to use stem cells products as medicines.

The clinical development of CTMP requires several stages: preclinical phase including animal testing, definition of the optimal cell dose, dosage form, route of administration, mechanism of action, etc.

In this article, we will describe the minimum requirements for clinical development of a CTMP, which currently represent one of the most complex categories of novel pharmaceutical products. Regulatory and practical aspects will be discussed in accordance with European rules; equally, the essential elements involved are also attempted to identify (Fig. 1).

The definition of a human cell therapy medicinal product

The objective of cell therapy is to repair, replace or recover the biological function of damaged tissue or organ [7], because of their ability to differentiate into specific cells, using CTMP as a therapeutic strategy.

Cell therapy uses mainly stem cells; they act as potential active principles in the development of new drugs for the treatment of diseases. Stem cells are broadly classified into two categories: embryonic stem cells (ESC) and adult stem cells (ASC).

ESC may be obtained from the first stages of embryonic development, when the fertilized ovule is a compact sphere morula [8]. ASC may be found in most tissues from a totally developed individual in bone marrow, brain, skin, skeletal muscle, liver, pancreas or placenta, among others (Table 1). The methods for obtaining stem cells are very different; there is not a uniform standard protocol for obtaining these cells, not even in its title. It would be advisable that there was a sole criterion for appointment of a CTMP and for the unification of the obtaining protocols.

Several clinical trials are conducting with CTMC in Europe. The most used stem cells for the development of these trials are ASC, particularly mesenchymal stem cells (MSCs). Currently, the public clinical trials database http://clinicaltrials.gov shows 279 clinical trials with MSCs in the world for a very wide range of therapeutic applications, from which 41 are being conducted in Europe. The number of clinical trials with ESC is much lower than ASC; currently, only 15 clinical trials are being performed in the world and particularly 2 in Europe.

Major clinical fields of application of CTMP are chronic diseases as cancer [9], heart diseases as cardiac regeneration [10], diabetes [11] and genetic diseases [12].

Cells may be derived from autologous, allogeneic or xenogeneic source. Autologous products are those in which donor and recipient cells come from the same person. Allogeneic products are derived from cells or tissues removed from a donor and applied to another person (receiver). Xenogeneic cellular products are those in which viable animal somatic cell preparations are adapted for application into a human recipient.

Table 1 Classification of the most used tissue sources of adult stem cell for a cell therapy medicinal product

Tissue sources	Stem cells	Main field of clinical application
Bone marrow	Adult mesenchymal stem cells (MSCs)	Leukaemia
	Endothelial progenitor cells (haematopoietic cells)	Myocardial infarction
		Diabetes mellitus (type 1 and 2)
	T lymphocytes	Stroke patients
		Critical limb ischaemia
		Amyotrophic lateral sclerosis
Blood	Adult mesenchymal stem cells (MSCs)	Haematopoietic transplantation
	Endothelial progenitor cells (haematopoietic cells)	
Adipose tissue	Adult mesenchymal stem cells (MSCs)	Autoimmune diseases
	Endothelial progenitor cells (haematopoietic cells)	Diabetes mellitus (type 1 and 2)
		Critical limb ischaemia
		Crohn's disease
Umbilical cord	Adult mesenchymal stem cells (MSCs)	Liver cirrhosis
	Endothelial progenitor cells (haematopoietic cells)	Aplastic anaemia
		Diabetes mellitus (type 1 and 2)
Liver	Hepatocytes	Liver cirrhosis
		Carcinoma
		Alcoholic hepatitis
		Liver failure
Maxillofacial tissues	Adult mesenchymal stem cells (MSCs)	Maxillary cyst
		Maxillary bone loss
Iliac crest	Adult mesenchymal stem cells (MSCs)	Heart failure
	Endothelial progenitor cells (haematopoietic cells)	Myocardial infarction
Orofacial bones	Adult mesenchymal stem cells (MSCs)	Dental implants
	Endothelial progenitor cells (haematopoietic cells)	Craniofacial injuries
Chondral tissue	Chondrocytes	Articular cartilage defects of ankle
		Osteochondritis dissecans
Skin	Keratinocytes	Skin graft
		Burn wound
		Leg ulcer
Pancreas	Pancreatic β islet cells	Diabetes mellitus (type 1 and 2)
		Carcinoma
Brain	Neuronal Cells	Alzheimer's disease
		Parkinson's disease
Placenta	Adult mesenchymal stem cells (MSCs)	Aplastic anaemia
	Multipotent progenitor cells (haematopoietic cells)	
Corneal epithelial	Limbal stem cells	Limbal stem cell deficiency
Skeletal muscle	Myoblasts	Myocardial infarction

A CTMP is made up of manipulated cells, cellular components, lysate cells, proliferating cells, genetically modified cells and cells used with matrix and other inert sanitary products, for use in humans. These cells as active principle, together with a selected excipient, constitute the composition of the final medicinal product.

The definition of a stem cell–based medicine as a medicine was established by regulation (EC) No 1394/2007 of the European Parliament and of the council on 13th November, 2007 on ATMP (chapter 1, article 2) and amending directive 2001/83/EC of the European Parliament and council on 6 November 2001, establishing a

community code on medicines for use in humans (annex I, part IV, in accordance with its latest modification) and regulation (EC) No 726/2004.

A stem cell is considered a drug when:

- Unmodified stem cells are used for a biological different function in donor and recipient. The regulation does not consider the following as substantial manipulations: cutting, crushing, moulding, centrifuging, imbibition in antibiotic or antimicrobial solutions, sterilizations, irradiation, separation, concentration or cell purification, filtering, lyophilization, freezing, cryopreservation and vitrification.
- Modified stem cells that have been extensively manipulated/modified or subjected to an engineering process. This manipulation includes the expansion or activation of autologous cell populations ex vivo (e.g. adoptive immuno-therapy), the use of allogeneic and xenogeneic cells associated with medical devices used ex vivo or in vivo.

In order to better explain above ideas, the term "cell factories" was adopted, which clearly indicates the manipulations cells undergo.

On the other hand, the following processes provide examples of somatic cell therapy medicines [13]:

- Cells manipulated to modify their immunological, metabolic or other types of functional properties in qualitative or quantitative aspects.
- Classified, selected and manipulated cells that are subsequently subjected to a manufacture process with the aim of obtaining a finished medicinal product.
- Manipulated cells combined with non-cellular components that carry out the initially intended action in the finished medicinal product.
- Derivatives from autologous cells expressed in vitro in specific culture conditions.
- Genetically modified cells or cells that have been subjected to some other type of manipulation to express functional homologous or non-homologous properties that were not previously expressed.

The concept of using stem cells in medicinal products has been generally associated with several types of adult stem cells. Stem cells are cells with the ability to grow and differentiate into more than 200 cell types of specialized cells found in the body [14]. The main examples that have been studied are hematopoietic stem cells, mesenchymal cells, hepatocytes, neurons, fibroblasts and chondrocytes among other lineages [15, 16].

The development of a CTMP constitutes an alternative therapeutic strategy to conventional clinical therapy, and its use has contributed to the successful treatment of some diseases, for which no effective cure was previously available [17, 18].

Clinical development of a cellular therapy

Clinical application of CTMP should be subjected to the same regulatory principles as any other medicinal product for human use. For the development of a clinical trial, sufficient data of efficacy and safety should be provided to support its realization. Technical requirements of a CTMP for quality, safety and efficacy must be more specific than other medicine. These products are in the early stages of its development, there being still a lot to be done and more scientific and clinical evidences are required.

Phases of development of CTMP

Prior to the approval of a new medicine for prescription and use in human, its effectiveness, safety and comparison with other available therapies are required [19]. These requirements involve multidisciplinary and collaborative work by specialists in fields such as pharmacology, immunology, genomics and transplant medicine [20]. The translational process of cellular therapy to clinical trial must be conducted in accordance with regulation, establishing the safety, efficacy and quality of medicinal products for marketing approval in the EU. A CTMP should be well characterized and must meet certain criteria of identity, purity, stability, potency and safety.

The development of CTMP for clinical use is divided into three main phases: preclinical phase, clinical phase and post-marketing surveillance trial (commercialization; Fig. 2).

This first step is conformed by preclinical research on toxicity and assessment of the biological activity of the cells. The preclinical development of a cellular therapy should provide enough information in order to select the cells dosage to be used during the clinical stage. The dosage level must be safe, and the information about the administration method, the target organs and any possible adverse reactions must also be provided. Preclinical studies should be carried out in conformity with the provisions relating to the application of the principles of good laboratory practice (GLP) laid down in Directive 2004/10/EC [21] and Directive 2004/09/EC [22].

The clinical phase will depend on the test design (phase I, II or III). The performance of clinical trials with CTMP must comply with the principles and requirements stipulated in Directive 2001/20/EC [23], in consideration of the basic principles for their use in human beings [24]. In the setup of each clinical trial, investigators incorporate ethical,

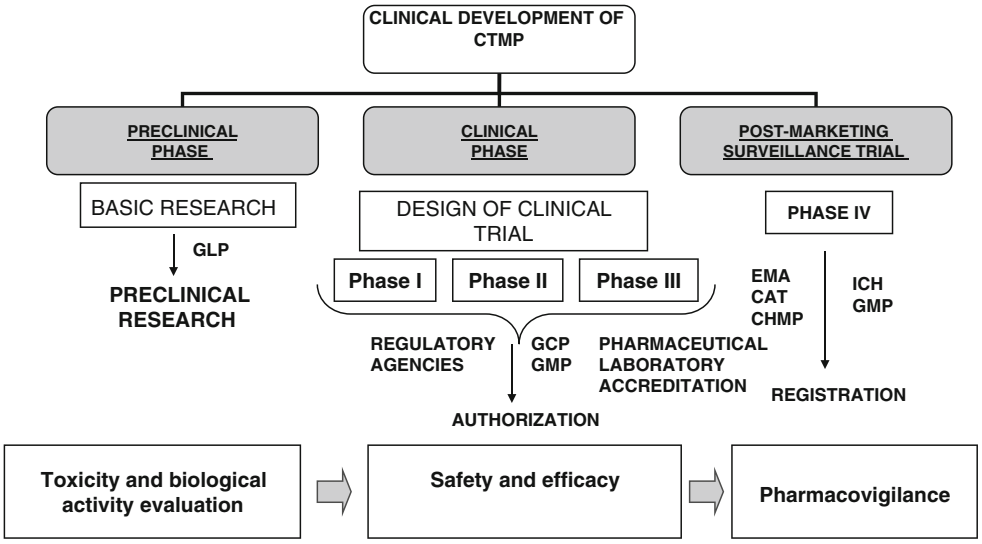

Fig. 2 Clinical development of cellular medicine. Good laboratory practice (GLP), good clinical practice (GCP), good manufacturing practice (GMP), European medicines agency (EMA), committee for advanced therapies (CAT), committee for medicinal products for human use (CHMP) and international conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH)

political, legal, financial and regulatory considerations as protocol is established [25].

Clinical trials of CTMP have to be designed and performed according to the overarching principles and ethical requirements laid down in good clinical practice (GCP) as laid down in Directive 2005/28/EC [26].

CTMP in clinical phase is considered an investigational medicine and should be in compliance with the principles of GMP, as set out in Commission Directive 2003/94/EC [27] related to medicinal products for human use and investigational medicinal products for human use.

Pharmaceutical requirements, installation and personnel supplies for a correct production of a CTMP

Several factors are involved in the transferral of basic research to the clinical environment: production regulations and requirements concerning installations, personnel and processes, representing important circumstances in the production process for the pharmaceutical development of a CTMP.

Production

The manufacture of CTMP as medicine requires ex vivo expansion of stem cell populations. This ex vivo expansion is considered to be a substantial manipulation by Regulation (EC) No 1394/2007. The legislation requires that the research in medicinal products used in clinical trials must be produced according to GMP guidelines. Stem cell–based products intended for clinical use should be produced via a robust manufacturing process governed by a quality control programme sufficient to ensure consistent and reproducible final product (Fig. 3).

A production process is divided into several stages: procurement of tissue or cells and processing at various phases to yield a well-predefined/well-characterized cell suspension; expansion under conditions supporting growth of undifferentiated cells; purification of the intended biologically active cell population.

Installations

The first step in the development of a CTMP process is the design, construction and validation of the physical infrastructure and equipment required [28].

A cellular production laboratory must comply with minimum requirements to ensure the aseptic properties of the product being manufactured. These types of installations are commonly known as white rooms, GMP rooms or clean rooms, and their design must include measures to safeguard aspects relating to product, staff and environmental protection.

Environmental parameters should be strictly controlled: air particles, temperature, humidity, air flow, air pressure and lighting. All air-conditioning systems must be equipped with filters capable of retaining particles from the exterior.

Clean rooms are classified in accordance with the degree of purity of air or air flow required: at an international level, the committee of the international standards organization (ISO) decided to set out an international standard for clean rooms, with the aim of establishing general criteria to govern their conditions, without specific reference to any particular product manufacturing process (Table 2).

In the pharmaceutical industry, clean rooms for the manufacture of medicines adopt the classifications described

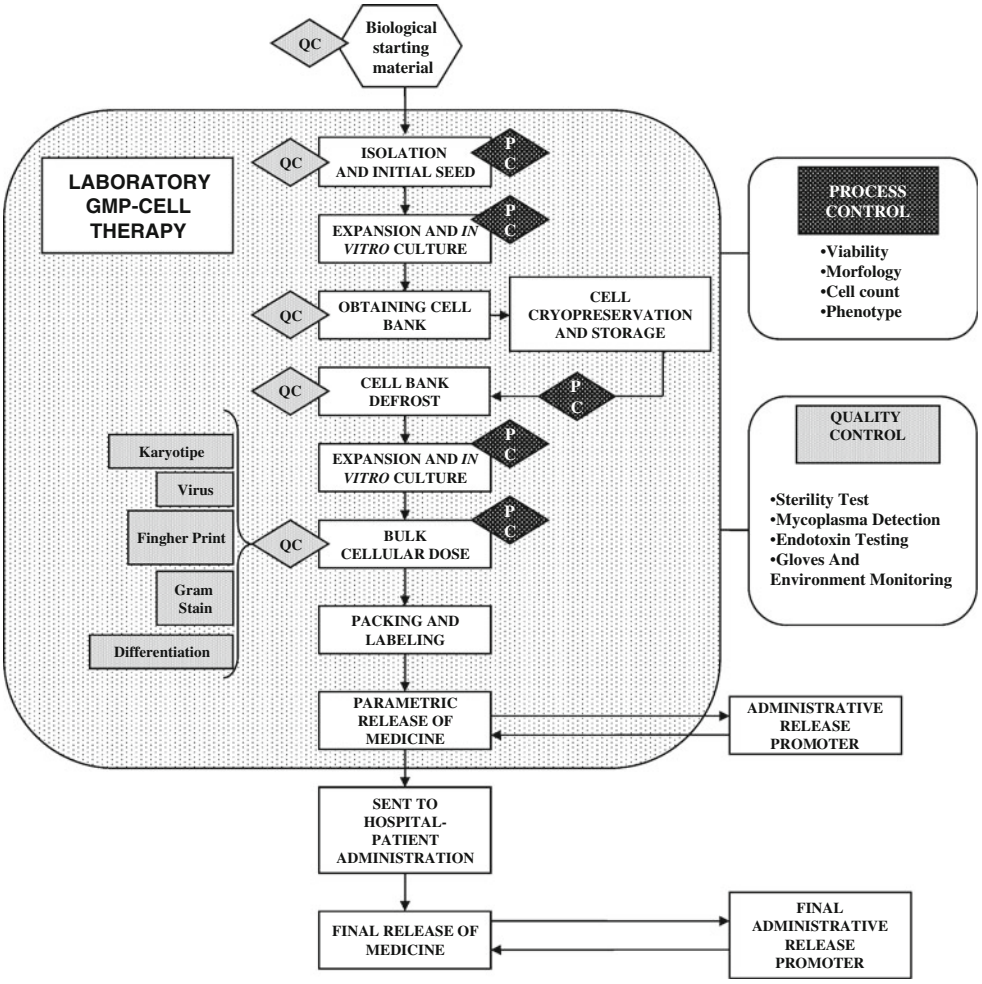

Fig. 3 Scheme of manufacturing process of autologous cell therapy medicinal products. Good manufacturing practice (GMP), process control (PC) and quality control (QC)

Table 2 Regulation ISO-14.664: Clean rooms and associated controlled environments

ISO	Specifications
ISO-14644-1: 1999	Classification of air cleanliness
ISO-14644-2: 2000	Specifications for testing and monitoring to prove continued compliance
ISO-14644-3: 2005	Test methods
ISO-14644-4: 2001	Design, construction and start-up
ISO-14644-5: 2004	Operations
ISO-14644-6: 2007	Vocabulary
ISO-14644-7: 2004	Separative devices (clean air hoods, glove boxes, isolators and mini-environments)
ISO-14644-8: 2006	Classification of airborne molecular contamination

ISO International Standard Organization

in the GMP and are classified into different environments, in accordance with the degree of air quality required. Every manufacturing operation requires an appropriate functional level of environmental cleanliness to minimize the risk of microbial or foreign particle contamination of the product itself or the materials used in its manipulation (Table 3).

Access to these areas must be made through isolated access portals reserved for authorized personnel, equipment and materials with effective filtering ventilation systems. An appropriate level of cleanliness must be maintained, and production installations must be equipped with exhaustively controlled air quality systems.

Table 3 Classification of air to the manufacture of sterile medicinal products

Grade	Specifications
A	The local zone for high risk operations. Normally such conditions are provided by a laminar air flow work station. Laminar air flow systems should provide a homogeneous air speed in a range of 0.36–0.54 m/s (guidance value) at the working position in open clean room applications
B	For aseptic preparation and filling, this is the background environment for the grade A zone
C; D	Clean areas for carrying out less critical stages in the manufacture of sterile products

In addition, a minimum number of compartments for the production of a medicine are required: entrance area, store, dirty and clean clothing changing rooms, sterile distributor, production room, packaging room, conditioning room and a quality control laboratory.

Another important parameter affecting the degree of air purity and consequently, the quality of CTMP, is the control of static pressure inside the room. Pressure gradients should be defined on an ascending or descending scale according to the process to be carried out. In the case of sterile product manufacture, the interval of pressure between one compartment and the next should fall between 10 and 15 positive or negative Pascals [28], to prevent any possible contamination of the product.

Personnel

The establishment and maintenance of a satisfactory system of quality guarantee and the appropriate manufacture of medicines depends on people. Consequently, a CTMP laboratory requires sufficient and technically well-qualified staff to be able to guarantee the quality of specialities and the performance of the necessary controls. The organizational chart of the laboratory must include information on persons who occupy the posts of technical manager and his stand in replacement, as well as the quality control and production managers. Those in charge of production and quality control must be independent and sufficiently qualified to perform all manufacturing tasks.

The manufacturer must ensure that all staff working in production areas or in the control laboratory (including technical, maintenance and cleaning personnel) are appropriately trained, together with any other staff whose work may affect the quality of the product.

In addition to the sterility of changing rooms and the materials used to finish the interior of the clean room itself, careful attention should also be given to the movement of staff.

The necessary clothing required for clean room staff are as follows: overalls, head covers, masks, sterile area footwear, breeches (for visiting personnel), gloves, goggles, hoods and leggings.

The physical ambient working conditions should be controlled with ideal working temperatures established between 20 and 25 °C. When special garments are required, including head coverings (hoods) and foot coverings, temperatures are often reduced to between 18 and 22 °C.

Ideal ambient humidity levels have been established at between 30 and 55 %. Humidity levels that are set too low may cause staff to dehydrate. In addition, it is necessary to set appropriate thermo hydrometric conditions required for the manufacturing process, in which the control of ambient humidity is especially important.

Validation of the productive process

Permission for developer or manufacturer of the CTMP laboratory issued by the competent national authorities is necessary to validate all processes involved in production. The manufacturing process of a CTMP, including collection and transport of biological samples, handling and culturing of cells, and packaging and transport should be carried out and validated in accordance with the harmonized tripartite guideline (ICH) [29], to demonstrate that the whole manufacturing process has been carried out under controlled conditions. All validation activities should be planned and described in a validation master plan (VMP) or equivalent documents. The VMP should be a summary document which is brief, concise and clear [30].

The validation process requires proof that any procedure, process, equipment, material, activity or system complies with GMP guidelines and materially produces a foreseeable result, so as to be able to guarantee the repetition of products in accordance with company quality standards [31]. Since the year 1992, the validation of systems, equipment and procedures has been obligatory by law in the European Union and is considered as a continual on-going process.

Three types of protocols have been established for each of the activities involved in the validation process:

- Installation validation protocol
- Operation validation protocol.
- Process and design validation protocol.

Commercialization

Given the rapid advance of scientific progress in cellular and molecular biotechnology, the Council of the European Union approved regulation (EC) No 1394/2007 for advanced therapies proposed by the European parliament, with the aim

of creating a single legal framework that encompasses gene therapy, somatic cell therapy and tissue engineering. In consequence, any application for an authorization to commercialize advanced therapy biotechnological medicines is evaluated following a centralized procedure coordinated by the European medicines agency (EMA) [32]. The committee for advanced therapies (CAT), created for such a purpose, has representatives from all EU member states (Iceland and Norway are not member states but are included as they are part of the European economic area), as well as medical and hospital staff and patient association representatives [33]. This multidisciplinary committee of experts has the task of assessing the quality, safety and efficacy of advanced therapy medicines and to monitor the main scientific advances made in the industry.

On the basis of this description, the main mission of CAT is to prepare a report on every application made to the EMA, before the committee for medicinal products for human use adopts a definitive resolution on the concession, modification, suspension or revocation of an authorization for the commercialization of a particular medicine. CAT is responsible for the evaluation of the marketing authorization applications for this novel class of products. A favourable resolution for any given medicinal product will grant the commercialization of the product within any member state of the EU [34].

The owner, an authorization, to commercialize CTMP must guarantee a system that ensures that every product and its components may be examined during the process of obtaining, manufacture, cryo-preservation, packaging, storage, transport and delivery [35].

Conclusions

Over recent years, cell therapy has gained considerable importance as an alternative treatment of multiple illnesses. The realization of the full potential of CTMP in a clinical setting demands robust scientific evidence, supported by legitimate regulatory requirements to ensure their safety and efficacy. In consequence, the need has arisen to become aware of the regulations governing its manufacture and use in Europe. In both clinical trials and clinical use, the use of CTMP with respect to conventional medical products leads to changes in procedures, manufacture and regulations. As shown in this work, the production of a cell therapy medicinal product must be carried out in accordance with a consolidated legislative framework for the whole of Europe, based on clear scientific evidence, for the development of appropriate pre-clinical models that guarantee maximum safety.

The development of a CTMP is carried out not only by the pharmaceutical industry, but also hospitals, universities and research centres, both publics and privates. In any case, its development includes laboratory phase, preclinical phase of animal testing and clinical phase in human, prior to an application for registration and marketing. There is, therefore, a need to adopt a multidisciplinary approach to addressing key issues pertaining to the design and functional assessment of such products. All this development involves a high economic cost, which must bear the entity promoting the development of the CTMP. It is a serious problem for public entities.

In order for this process to be carried out in a harmonized way, this work seeks to describe the basic concepts involved in design and authorization of clinical trials, as well as the accreditation of the pharmaceutical laboratory that considers a cell as a medicine. Thus, harmonization initiatives in the area of CTMP to develop common guidelines on safety, efficacy, quality and multidisciplinary issues in pharmaceutical regulation would be very helpful. Several harmonization initiatives already exist, such as ICH composed of industry and regulatory agency representatives from the United States, Europe and Japan (along with several non-voting observers). In such a complex and fast moving field, it seems unlikely that an ideal regulatory framework which anticipates any potential technological development would be developed in the short term. In recent years, there has been a boom in the development of cell therapies that are in clinical phase. Only a limited number of CTMP are translated into products for clinical development and marketing authorization. In fact, only a single cell therapy medicinal product has been authorized in Europe, ChondroCelect.

The manufacture of a CTMP involves high production costs, and extreme logistical complexity, due to specific characteristics, such as a generally short shelf live, and a relatively long-term return on investment. Under such circumstances, continual sources of information and staff training will become necessary in order to ensure that hospitals, clinics and the industry will be capable of producing CTMP in the future.

Conflict of interest The authors declare that they have not any conflict of interest concerning this article.

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III.3. DEVELOPMENT OF A CELL-BASED MEDICINAL PRODUCT: REGULATORY STRUCTURES IN THE EUROPEAN UNION

Las Terapias Avanzadas, como la Terapia Génica, la Terapia Celular Somática y la Ingeniería Tisular, presentan nuevas posibilidades de tratamiento para las enfermedades humanas. El diseño de un ATMP es complejo y debe ser regulado por la normativa vigente, con el fin de preservar la salud pública. En el año 2003 se publicó la Directiva 2003/63/CE describiendo los medicamentos de Terapia Celular y Génica y en qué casos se consideran medicamento. Años más tarde en el 2007 se publica el Reglamento 1394/2007 en el que se amplía el concepto de ATMP, incluyendo los productos de Ingeniería Tisular y Medicamentos de Terapias Avanzadas Combinados, en los que se incorpora un producto sanitario. Hasta el año 2003 los productos derivados de estas terapias se regían por la normativa de trasplantes Ley 30/1979, de 27 de octubre, sobre extracción y trasplante de órganos.

Las células madre tienen un enorme potencial en el campo de la Medicina Regenerativa debido a su capacidad de autorrenovación y de diferenciación en múltiples linajes *in vivo* y *ex vivo*. Este trabajo ha permitido definir las consideraciones regulatorias del producto celular a diseñar, definiéndolo como un CTMP debido a que las células objeto de estudio (hMSCs) deben ser expandidas *ex vivo*, lo cual es considerado como una manipulación sustancial de la célula y por tanto el producto celular final debe considerarse un medicamento.

Además se han definido las fases de desarrollo que requiere un CTMP en Europa, incluyendo las primeras etapas de hipótesis e investigación básica, estudios preclínicos, ensayos clínicos, fabricación, registro, comercialización y estudios de post comercialización.

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Development of a cell-based medicinal product: regulatory structures in the European Union

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Introduction: New therapies with genes, tissues and cells have taken the emerging field for the treatment of many diseases. Advances on stem cell therapy research have led to international regulatory agencies to harmonize and regulate the development of new medicines with stem cells.

Sources of data: European Medicines Agency on September 15, 2012.

Areas of agreement: Cell therapy medicinal products should be subjected to the same regulatory principles than any other medicine.

Areas of controversy: Their technical requirements for quality, safety and efficacy must be more specific and stringent than other biologic products and medicines.

Growing points: Cell therapy medicinal products are at the cutting edge of innovation and offer a major hope for various diseases for which there are limited or no therapeutic options.

Areas timely for developing research: The development of cell therapy medicinal products constitutes an alternative therapeutic strategy to conventional clinical therapy, for which no effective cure was previously available.

Keywords: regulatory agencies/advanced therapies/cell therapy

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Introduction

Advanced therapy medicinal products (ATMP) are a new medicinal product category, including somatic cell therapy medical products (sCTMP), gene therapy medical products (GTMP) and tissue-engineered products (TEP). Cells, genes and engineered tissues are regarded as new active substances in the development of medicines. Aside from the well-established bone marrow transplantation, advances in cell therapy in the last decade have promoted the development of multiple lines of research for the development of a sCTMP.

The application of cells as pharmacologic active substance has the purpose to repair, replace or recover the biologic function of damaged tissue or organs. Cell therapy is an alternative for the treatment of both high prevalent chronic and rare diseases, including immune[1] and cardiovascular diseases,[2] diabetes and their complications,[3–6] neurodegenerative disorders,[7] inflammatory diseases such as Crohn's disease,[8] musculoskeletal diseases,[9] cancer,[10] etc.

Development of new medicines for the treatment of untreated disease aims to improve the patient's quality of life with new and specific therapies either addressed to the right target, thus minimizing side effects or using new strategies and mechanisms of action that open new therapeutic avenues. In this context, Europe has pioneered the development of advanced therapies; however, most of the preclinical and clinical research is investigator driven and less frequently involving spin-offs and small biotech companies. Academic institutions and small and medium enterprises are not so familiar with regulatory issues as conventional pharmaceutical industry. In this work, we aim to describe the regulatory issues to consider during the different steps in the process of innovation for new advanced therapies as sCTMP.

The design, development and authorization of a medicine are a long and complex process. Regulation needs to be applied from the early stages of development of a new medicine to ensure that it meets the requirements of quality, efficacy and safety for administration in humans. This applies to all medicinal products whether of chemical or biologic origin.[11] In Europe, sCTMP meet the definition of a medicinal product as described in Directive 2001/83/EC[12] amended by two subsequent directives (2003/63/EC and 2009/120/EC)[13,14] and completed by Regulation (EC) No. 1394/2207.[15] sCTMP have a specific regulation to harmonize legal framework and to promote research in this field.

This review describes and discusses the different stages in the development of a sCTMP, and both regulatory and practical requirements, from a European perspective.

Regulatory agencies

The three most important regulatory agencies that regulate the development of a medicine for human use are European Medicines Agency (EMA) in the European Union (EU), Food and Drug Administration (FDA) in the USA and Ministry of Health, Labour and Welfare (MHLW) in Japan.

Coordination of technical requirements at the international level is achieved through the International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Its main mission is to achieve management in the technical guidelines on the quality, safety and efficacy of new medicines[16] (Table 1). This organization brings together regulatory authorities, pharmaceutical industry and scientific experts from the USA, Japan and Europe.

Regulatory agencies also review the indications for use, content of the package leaflet, dosage, side effects, warnings and contraindications. Finally, they also ensure post regulatory pharmacovigilance.

European Medicines Agency

EMA is a decentralized agency of the EU located in London (www.ema.eu). Its main responsibility is the protection and promotion of human and animal health, through the evaluation and supervision of medicines for human and veterinary use.[17] This evaluation is done by experts from the national medicines agencies from the EU countries, who are members of different committees within the EMA: Committee for Medicinal Products for Human Use (CHMP), Committee for Medicinal Products for

Table 1 Categories and topic codes to the ICH of Technical Requirements for Registration of Pharmaceuticals for Human Use

Category	Code	Applications
Quality	Q	Concerning the stability studies, the definition of relevant limits for impurities testing and a focus on product quality
Security	S	Guidelines for potential hazards such as carcinogenicity, genotoxicity and reproductive toxicity
Efficacy	E	For the design, development, safety and reporting of clinical trials. There are also guidelines for medicinal products derived from biotechnology processes, use of pharmacogenetics and genomic techniques for the production of more specific drugs
Multidisciplinary	M	For crosscutting themes that do not conform to only one of the above categories (quality, safety and efficacy).

Veterinary Use, Pediatric Committee, Committee for Orphan Medicinal Products, Committee on Herbal Medicinal Products and Committee for Advanced Therapies (CAT). In July 2012, a new committee was created at the EMA, the Pharmacovigilance and Risk Assessment Committee. Scientific committees also receive input from different working parties (experts in different areas). Through these committees, EMA provides scientific advice for the development of new medicines.

The CAT started working in 2009 to promote the development of advanced therapies and to the pursuit of innovation in new treatments with ATMP. The CAT consists of experts in the field of ATMP nominated by the EU Member States, the CHMP and the European Commission. Its main responsibility is to prepare a draft opinion on the quality, safety and efficacy of a product for the final approval on each ATMP marketing authorization application submitted to the EMA, before the CHMP adopts the final opinion on the medicine. CAT offers a query system for the classification of ATMP. This procedure is optional, free of charge and may take place at any stage of the development of a sCTMP or any ATMP in advance of applying for a marketing authorization.[18,19] CAT is involved in the certification of quality and/or nonclinical data, in the development of scientific guidelines and reflection papers and in regulatory procedures and scientific articles. Regulatory aspects for cell-based therapy medicinal products will be described in this paper in more detail.

Food and Drug Administration

FDA is the agency responsible for authorizing the marketing of new medicines in the USA[20,21] The FDA's Center for Biologics Evaluation and Research is responsible for ensuring the safety, purity, potency and effectiveness of many biologic products (cells, genes, tissues, blood components and derivatives, vaccines, etc.) for the prevention, diagnosis and treatment of human diseases. Because of their biologic origin, cell therapy medicinal products are regulated in the Code of Federal Regulations under Title 21 PART 1271, Human Cells, Tissues and Cellular And Tissue-Based Products.[22,23] To promote the development of cell therapy medicinal products, the FDA has formed a working group with the pharmaceutical industry called Critical Path Initiative to act in a proactive fashion regarding the development of new medicinal products.

Ministry of Health, Labour and Welfare

MHLW is the Japanese consumer protection agency. The Pharmaceutical and Medical Devices Agency (PMDA) is responsible for the scientific

evaluation of applications for marketing authorization for new medicines in Japan. The development of a cell therapy medicinal product for human use in Japan is governed by the Guideline on Clinical Research Using Human Stem Cells (July 3, 2006 amended in full, November 1, 2010). The PMDA has a committee of experts in medicine and bioethics to review all human stem cell clinical research. This committee is based in this guideline and the latest scientific findings.[24] On the other hand, there is a specific regulation for clinical trials (Phases I–III, and post regulatory), regulated by the Pharmaceutical Affairs Law (1960 Law 145).

Advanced therapies in Europe

To harmonize the technical aspects of new medicines, in terms of quality, safety and efficacy, the EU proposed a plan of action for the development of new biotech medicines. This plan included genes and cells products as a biologic medicinal product. ATMP have to fulfill the same scientific and regulatory standards as all other medicinal products. Directive 2001/83/EC[12] (Annex I, part IV) consolidated all the regulation of biotechnological products for human use and defined for the first time the terms: GTMP and sCTMP. In 2003, Directive 2003/63/EC[13] amended Directive 2001/83/EC on medicinal products for human use. Later, in 2008, the regulation of biologic medicines was updated and included tissue engineering to the area of advanced therapies, Regulation (EC) No. 1394/2007.[15] This regulation lays down specific rules concerning the authorization, supervision and pharmacovigilance of ATMP. A transitional period was defined to comply with these requirements. In the case of cell and gene therapy, the period ended on December 30, 2011. However, for tissue engineering products, a 4-year period was defined that will end on December 30, 2012.

In 2009, Commission Directive 2009/120/EC[14] was indeed the legal document that was established after the Regulation (EC) No. 1394/2007 to implement the changes into 2001/83/EC. Any organization (pharmaceutical industry, hospitals, public or private research) should comply with the requirements of this regulation from January 1, 2013, for the development of any ATMP of use in humans. According to the European regulation,[12–15] ATMPs include sCTMP, GTMP, TEP and combined advanced therapy products (CATP).

In addition, Directive 2004/23/EC,[25] Directive 2006/17/EC[26] and Directive 2006/86/EC[27] describe the quality and safety standards for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. These directives apply to products classified as no ATMP (no medicine). ATMP is not considered a medicine, when the cells that compose it, have the same essential

function in the donor as in the recipient and when the cells do not subject to any substantial manipulation.[15] For ATMP, these directives only apply in relation to donation, procurement and testing of biologic samples that will be obtained from the cells, genes and/or tissues. With regard to ATMP with blood components or blood cells, Directive 2002/98/EC[28] will also apply.

Somatic cell therapy medicinal products

sCTMP are defined as biologic medicinal products that contain or consist of cells or tissues that have been subjected to substantial manipulation so that biologic characteristics, physiologic functions or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function in the recipient and the donor or/and they are presented as having properties, or are used in or administered to human beings, with a view to treat, prevent or diagnose a disease through the pharmacologic, immunologic or metabolic action of their cells or tissues.[12,14]

On term of regulation, the following manipulations are considered 'no substantial': cutting, crushing, shaping, centrifuging, soaking in antibiotic or antimicrobial solutions, sterilizations, irradiation, cell separation, cell concentration, etc.... all of them are described in the Annex 1, Regulation No. 1394/2007.[15] In contrast, processes that modify biologic characteristics, physiologic functions or structural properties of the cells or tissues are considered 'substantial'.[29]

Active substances for a sCTMP may be manipulated cells, cellular components, lysate cells, proliferating cells and genetically modified cells. The materials in combination with cells should be considered as starting materials and, thus, form part of the active substance.[14] Cells may be of autologous, allogeneic or xenogeneic origin. Autologous products are those in which donor and recipient of cells is the same person. Allogeneic products are derived from cells or tissues removed from a donor and applied to another person. A xenogeneic cellular product includes animal-viable somatic cell adapted for application into a human recipient.

Gene therapy medicinal products

GTMP is defined as a biologic medicinal product containing an active substance that contains or consists of a recombinant nucleic acid used in, or administered to, human beings with a view to regulate, repair,

replace, add or delete a genetic sequence.[12,14] Its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains or to the product of genetic expression of this sequence. GTMP shall not include vaccines against infectious diseases.

Tissue-engineered products

TEP are defined as a product that contains or consists of engineered cells or tissues used for regenerating, repairing or replacing a human tissue (Chapter 1—Article 2, Regulation No. 1394/2007).[15] The biologic origin of the cells and tissues may be human or animal. These products contain viable or nonviable cells or tissues and additional substances such as cellular products, biomolecules, biomaterials, chemical substances, scaffolds or matrices.

As mentioned above, as for sCTMP, cells or tissues are considered engineered, if they fulfill at least one of the following conditions: cells or tissues have been subjected to substantial manipulation, so that biologic characteristics, physiologic functions or structural properties relevant for the intended regeneration, repair or replacement are achieved or the cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor.[15]

Combined advanced therapy products

CATP are products that incorporate as an integral part of the product, one or more medical devices or one or more active implantable medical devices. These products must contain viable cells or tissues, or their cellular or tissue part containing nonviable cells or tissues must be liable to act on the human body with action that can be considered as primary to that of the devices referred to.[15]

A medical device or an active implantable medical device should meet the essential requirements described in Directive 93/42/EEC[30] concerning medical devices and Directive 90/385/EEC[31] relating to active implantable medical devices, to ensure an appropriate level of quality and safety. Both directives were amended by Directive 2007/47/EC.[32] EN/ISO 10993-18[33] and EN/ISO 10993-19[34] describe some guidelines about additional substance that should be identified and characterized in chemical and physical terms (porosity, density, microscopic structure and particular size). Safety, suitability and biocompatibility of all structural components and additional substances are a must in developing these products. CATP may also incorporate structural components that are not identical or used in the same way as in a

medical device. All structural components should be fully characterized and evaluated for their suitability for the intended use. Any medical device used in addition or combined to the cells should be described and its function underpinned by means of chemical, biologic, physical and mechanical properties.[35]

Stages of research on the development of a sCTMP in Europe

In contrast to the conventional innovation model in the pharmaceutical industry, advanced therapies are usually developed by small and medium enterprises and academia (hospitals, universities, etc.). In any case, their development includes several phases: experimental observations, preclinical phase [animal testing under Good Laboratory Practice (GLP)[36] conditions] and clinical trials [Phases I–IV under Good Clinical Practice[37] (GCP), Good Manufacturing Practice[38] (GMP) and Good Pharmacovigilance Practice[39] (GVP), prior to an application for registration and marketing]. All these phases must address the critical points in the development of cellular therapy. Clinical development should be approved by national medicines agencies, marketing authorization applications should go to the EMA with the exception of national hospital exemption, also in compliance with national requirements. The Regulation (EC) 1394/2007 describes that it is possible to prepare a sCTMP in a hospital under the exclusive professional responsibility of a medical practitioner for an individual patient. sCTMP will be manufactured on a nonroutine basis according to specific quality standards. The competent authority of the member state of Union Europe should authorize the manufacturing of these products, and each member state should authorize a hospital exemption clause, ensuring the compliance of traceability and pharmacovigilance with European requirements.[15] National agencies are currently developing rules for hospital exemption with quality requirements similar to those applied by EMA.

The main critical points in the development of a new sCTMP are selection of cellular biologic sample, cell type and its production process, including biopsy, active substance formulation (qualitative and quantitative composition), selection of pharmaceutical form: cell concentration of the medicinal product, route of administration, dose (single dose, multidose), detailed instructions for use, application, implantation or administration, dose–response relationship, shelf-life and stability, pharmacologic properties, quality properties of a product, assessment of adverse reactions and evaluation of the risk–benefit balance that must always be positive.[12–15,35,40] Both national agencies and CAT can provide regulatory advice during the development of a sCTMP.[41]

On the other hand, the EMA has published several guides that describe regulatory aspects for the development of a sCTMP, CHMP/410896/06,[35] CHMP/CPWP/708420/09[40] and CHMP/CPWP/83508/09[42] among others.

Experimental observations

At this stage of development, experimental observations are carried to study the active ingredients (e.g. differentiated progenitor or stem cells) and establish their mechanism of action in order to develop a sCTMP.

The active ingredients (cells) are classified in consonance with the tissue localization from which they are obtained and their function (embryonic stem cells may be obtained from the first stages of embryo development; adult stem cells are found in the bone marrow, adipose tissue, etc.; mononuclear cells from the bone marrow, etc.) and by their potentiality (pluripotential, etc.). Currently, there is much diversity in the name of these cell-derived products. There is no unified naming convention that would facilitate research in this area, giving common information sources in the development of a product. Nomenclature for these active substances should be standardized at the European level, with a consensus for all sCTMP.

Preclinical research

Preclinical development of a sCTMP refers to the set of studies on efficacy and safety of the active substance (cells) to be performed in biologic systems distinct to the human, which may give substantial information to address a Phase I-IIa pilot study. These may include *in vitro* work with cellular models, preclinical experiments using animal cells in animal models and preclinical work using human cells in immunocompromised mice.[43] Although there are no perfect models that mimic the human disease, these approaches may provide enough information for ethical committees and regulatory agencies to decide for further development based on the proper balance between risk and benefit for a specific situation.

The selection of animal models and species should be scientifically justified. The phase of preclinical research is focused on toxicity and assessment of the biologic activity of the cells,[44] through pharmacodynamic, pharmacologic, pharmacokinetic, biodistribution, tumorigenicity and interactions with other cellular or not components studies.

Nonclinical studies performed in animals and physiologic models in the laboratory aim to analyze the physicochemical properties and

behavior of the compound both *in vivo* and *in vitro*. When relevant animal models cannot be developed, *ex vivo* and/or *in vitro* studies may replace animal studies. Data from preclinical studies must be interpreted in terms of the animal species used because human stem cells are very different from an animal model. Usually, two or more species (a rodent and nonrodent) are used in these experiments because the medicine application could affect differently.

Nonclinical studies should be carried out in conformity with the provisions related to GLP laid down in Directive 2004/10/EC[36] and Directive 2004/9/EC[45] on the inspection and verification of GLP. Animal tests are designed not only to show the safety and proof of concept for efficacy of the new medicinal product, but also to assess its biodistribution, pharmacodynamics and toxicity. In contrast to small and biologic molecules, the control of the amount of drug absorbed into the blood, its chemical breakdown in the body, the short-, mid- and long-term toxicity, its breakdown products (metabolites) and the rate of excretion usually measured in preclinical studies[46] are difficult to evaluate with cellular medicines. Nonclinical aspects are described in guideline 35 of the ICH S6 and should be considered. The number of animals, their genders, the frequency and duration of monitoring should be appropriate to detect possible adverse effects.[35] An effort should be made to introduce new tests that respond to these questions when using cells as medicines.

GLPs are a set of principles that provide a framework within which laboratory studies are planned, performed, monitored, recorded, reported and archived to ensure the reliability of data generated within a compliant laboratory.[47] A key feature of GLP is the generation of quality control methods and data management within the cell culture laboratory.[48] GLP principles were set by the Organization for Economic Co-operation and Development (OECD). This organization has established the Mutual Acceptance of Data system in OECD member countries for the mutual acceptance of nonclinical safety study data.[49] For purposes of assessment and other uses related to the protection of human being and his environment, other member countries should accept the GLP principles.

The OECD has issued a series of recommendations for compliance with the GLP. These guidelines focus on the assessment of chemicals for very different applications and also apply to medicines.

Clinical research (and follow-up)

Clinical trials can be classified according to their purpose as Phase I, Phase II, Phase III and Phase IV.

Clinical trials in Phase I are the first step in investigating a substance or new drug in humans. They are based to evaluate its safety,

determine a safe dosage range and identify the side effects. This phase provides the approximate profile of safety and tolerance of the product. For instance, it would not be ethical to conduct a gene therapy trial on healthy human volunteers. While in Phase I primary endpoints must respond to safety and efficacy, surrogate endpoints may check for the efficacy. Phase II studies include initial clinical research treatment effect. Clinical trials of this phase are carried out on patients with the clinical entity of interest. Their main objective is getting to know the dose with the best risk/benefit profile. Phase III studies or pivotal trials are designed to evaluate the safety and efficacy of the experimental drug trying to reproduce the conditions of common use and considering the therapeutic alternatives available for the disease studied. Phase IV convenes pharmacovigilance and additional efficacy studies and corresponds to post marketing/post authorization studies with marketed medicines. The objective at this stage is to study the detection of long-term side effects and possible effects of drug on the disease itself or studies of morbidity and mortality (Fig. 1).

Clinical application of sCTMP should be subjected to the same regulatory principles as for any other biotechnological medicinal product for human use,[50] although their technical requirements for quality, safety and efficacy must be more specific.[51]

Requirements to conduct clinical trials in the EU are provided by Directive 2001/20/EC.[37] Clinical trials have to be designed and performed according to the overarching principles and ethical requirements laid down in GCP as laid down in Directive 2005/28/EC,[52] where principles and detailed guidelines for GCP as regards investigational medicinal products for human use are described.

GCPs are a set of ethical and scientific requirements of internationally recognized quality, which must be met in planning, conducting, recording and reporting of clinical trials involving humans. Their compliance ensures the protection of human rights, safety and welfare will of trial subjects and the reliability of clinical trial results. These studies have to be made respecting the principles of the Helsinki Declaration prepared by the Assembly of World Medical Congress, Helsinki, Finland in 1964 and revisited periodically with the main goal of protecting patient rights. GCP stipulates the clinical trial process, including protocol and Case Report Form design, analyses planning, as well as analyzing and preparing interim and final clinical trial/study reports. In contrast to small molecule clinical trials, sCTMP cannot be given to volunteers to demonstrate safety and feasibility. Therefore, 'proof-of-concept' pilot studies consist in testing the cellular medicine in a small group of patients with an untreatable disease or condition and demonstrate safety and feasibility while observing efficacy. Phase II and III may be randomized double blinded with placebo group. These clinical trials focus on

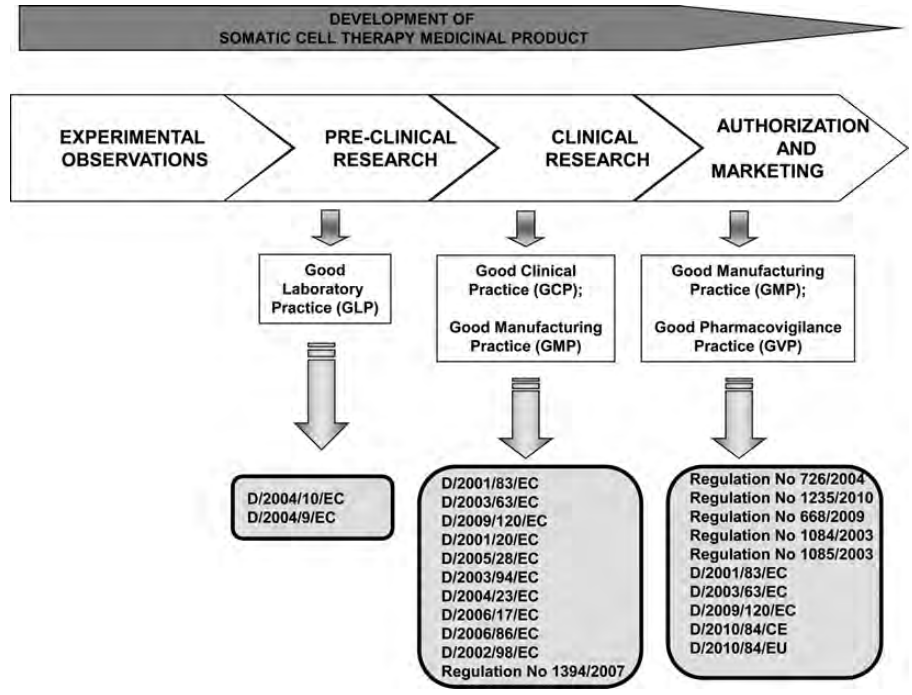

Fig. 1 Regulatory issues in the development of a cellular medicine in the EU.

the study of the cellular function, their distribution, dose, effect and above all safety. A recent position paper by the European Science Foundation has made a proposal for a revision of the 'Clinical Trial Directive (2001/20/EC)' and other recommendations to facilitate clinical trials (December 2011).

Clinical trials on the use of sCTMP are underway for a wide variety of diseases; however, different cell types are used, most of them are badly defined. Bone marrow mononuclear cells are a heterogeneous mixture of cells,[53] umbilical cord blood[54] is mostly but not only hematopoietic and mesenchymal[55] stromal cells from different origins are selected by their capacity to adhere to the culture plastic dish, etc. Most clinical trials in cell therapy currently ongoing are Phase I/II studies, and ~15 562 studies are registered in www.ClinicalTrials.gov.

Manufacturing cell-based medicinal products

Technical requirements for sCTMP are based primarily on quality, safety and efficacy aspects. In contrast to traditional medicines, sCTMP have different and more specific characteristics. The risks analysis of the whole manufacturing process, the quality of manufacturing

aspects and nonclinical and clinical development are aspects that should be taken into account when manufacturing a sCTMP.[35]

It is also necessary to conduct a risk analysis covering the entire process. Risks associated with a sCTMP are highly dependent on the biologic characteristics of the product. Risk factors may include, among others, origin of cells, level of manipulation, combination of cells with bioactive molecules or materials structural, management or use mode,[12,14] etc. Recommendations of the risk analysis is part of the dossier for a marketing authorization application, described in EMA/CHMP/CPWP/708420/2009,[40] guideline on the risk-based approach according to annex I, Part IV of Dir. 2001/83/EC applied to ATMP. Regarding safety and efficacy of follow up risk management the EMEA/149995/2008 guideline was published.[56]

All batches of a sCTMP manufactured for the clinical phase should be carried out under GMP standards. sCTMP in clinical phase is considered to be an investigational medicinal product, then it should be in compliance with the principles of GMP (Directive 2003/94/EC).[38] Therefore, requirements for installations, staff, equipment, documentation, production, quality control, batch release, labeling, etc., must comply with the requirements of GMP guidelines.

In practical terms, these standards are described in EudraLex-Volume 4 GMP guidelines and consist of: Part I—Basic requirements for medicinal products (nine chapters), Part II—Basic Requirements for active substances used as starting materials and Part III—GMP-related documents and 20 Annexes.[57] The manufacture of sCTMP must be carried out as described in the nine chapters of Part I, in addition to that referred in Annexes: 1—Manufacture of sterile medicinal products, 2—Manufacture of biologic medicinal products for human use, 8—Sampling of starting and packaging materials, 13—Manufacture of investigational medicinal products, 14—Manufacture of products derived from human blood or human plasma, 15—Qualification and validation, 16—Certification by a qualified person and batch release, 17—Parametric release and 19—Reference and retention samples. Likewise, detailed clinical guidelines for GCP have been described in EudraLex-Volume 10 that contains six guidance documents applying to clinical trials.[58]

The manufacturing process of a sCTMP for clinical use must be consistent and reproducible, providing sufficient quality to the final product for patient safety. GMP applies to both production and quality control of the medicinal product. These guidelines ensure that medicinal products are consistently produced and controlled to the quality standards appropriate to their intended use and according to the requirements of the product specification.[59]

Concerning quality control requirements, it is important to check that the active substance of sCTMP is viable, so at the end of the process the

final product for the patient should not include terminal sterilization, purification steps and/or viral removal. Therefore, the quality of the starting materials derived from animal or human origin should be analyzed. These materials comprise excipients and the donor of biologic sample for the active substance. The quality criteria required by the European regulation for the characterization of the final product require considerations such as the identity of the cellular and noncellular components, cell purity, impurities of product or related process, impurities as adventitious agents, potency and tumorigenicity.[35]

The manufacturing process of sCTMP should be carefully designed and validated to ensure product consistency and repeatability of the cell culture process. These products are highly heterogeneous due to their origin, starting material, degree of *in vitro* manipulation and manufacturing process.[60]

During the manufacture of a sCTMP considering an aseptic procedure, all manufacturing processes should be validated, including validation of sterility, validation of aseptic process, validation of microbiologic environmental monitoring and validation of cleaning process.[61] Thorough study of each process involved in the manufacture of sCTMP should be made to ensure their safety in terms of quality.

Authorization, registration and marketing

The evaluation of the medicines for their authorization and commercialization is regulated by Directive 2001/83/EC and Regulation (EC) No. 726/2004,[62] amended by Regulation (EU) No. 1235/2010,[63] as regards pharmacovigilance of medicinal products for human use. This regulation develops and improves European procedures for the authorization, supervision and pharmacovigilance of medicinal products for human and veterinary use.

There are three procedures to approve new medicinal products: the centralized procedure generating a single marketing authorization valid throughout the EU, the decentralized procedure in which the application is submitted to member states selected by the applicant and the mutual recognition procedure when the reference member state has already issued a marketing authorization. However, the centralized authorization procedure is compulsory for medicinal products manufactured by biotechnological methods and for ATMP such as sCTMP.

The CAT is involved in all scientific advice on ATMPs and in the regulatory procedures of the classification and the certification procedures. CAT is responsible for the evaluation of the marketing authorization applications for sCTMP.[19] Following the CHMP scientific assessment, the European Commission issued a decision that is

published in the Official Journal of the European Community, which is valid for all EU countries.

The marketing authorization is valid for 5 years and may be renewed. Once it has been renewed, it is valid indefinitely, unless the Commission chooses not to validate again for another 5 years. The approval decision will be taken based on the scientific criteria of quality, safety and efficacy in evaluation. These three criteria allow evaluating the risk–benefit balance of all medicines.

On the other hand, to help micro-, small- and medium-sized enterprises, EMA published the Regulation (EC) No. 668/2009,[64] with regard to the evaluation and certification of quality and nonclinical data relating to ATMP developed (described in two guidelines, EMA/CAT/486831/2008/corr[65] and EMA/CAT/418458/2008/corr.),[66] implementing Regulation (EC) No. 1394/2007.

Post authorization

Once the marketing authorization is requested and approved, EMA may request post authorization studies, the authorization may be conditionally granted. Post authorization studies focus on pharmacovigilance, and it is the science for the assessment, awareness and prevention of any side effects or possible adverse reactions associated with medicines. This phase is regulated by Directive 2010/84/EU[67] that defines a new concept of post authorization safety studies (PASS). PASS have the aim at collecting data to study for the assessment of the safety and efficacy of the sCTMP with the purpose of generating additional information on the effects of medicines with usual conditions of clinical practice and to complete the information obtained during Phases I, II and III.

PASS can be a noninterventional trial or observational and a clinical trial (Phase IV). With sCTMP, PASS are carried out with the aim of identifying, characterizing or quantifying a safety hazard of cell medicine, confirming the safety profile of these products or of measuring the effectiveness of risk management measures.[67]

Post authorization phase shall be carried out in conformity with the provisions related to GVP. GVPs are a set of measures drawn up to facilitate the performance of pharmacovigilance in the EU. The guidelines on GVP are divided into 16 modules, each of which covers 1 major process in pharmacovigilance.[39] It released modules III and X for consultation in June 2012. Each of the seven modules released covers 1 major process in the safety monitoring of medicines. The full set of 16 final modules is scheduled to be available by early 2013. After marketing authorization has been granted, any change to the data in the dossier shall be submitted to the competent authorities in

accordance with the requirements of Commission Regulations (EC) No. 1084/2003[68] and (EC) No. 1085/2003.[69]

Discussion

Application, administration or implantation of human cells is opening new avenues in the search for the treatment of critical diseases, some of which are as yet incurable.[70] In the last years, encouraging observations at the preclinical level have promoted the development of cell therapies that are in distinct clinical phases. Several if not all clinical trials use cells from adult origin, most of them mesenchymal-like cells. When compared with hundreds of clinical trials with other medicines, only a limited number of sCTMP are translated into products for clinical development and marketing authorization (Table 2).

The development and manufacture of conventional medicines is performed by physical and chemical techniques with consistency and robustness. However, sCTMP development is a technique that is still far to reach such strength. sCTMP should take into account the variability of the production process, the risk of exogenous contamination (microbiologic, viral, etc.)and variability of quality control techniques. Because active principals are active substances and not chemical compounds.

The European framework for the development of sCTMP aims to provide an increased international competitiveness of these products, and ensure that all patients receive treatment with good quality drugs in the field of advanced therapies.

We anticipate that a deeper knowledge of regulatory issues among clinical investigators and small biotech enterprise will expand this field that holds the promise for so far untreatable diseases.

Since 2003, the cells and/or cellular component are considered as medicines, so since then, public institutions such as hospitals and universities and private companies have been forced to change their methods of clinical and nonclinical research and their development. All this change has been accompanied by the update on the EU regulatory field. The legal and technical requirements necessary for the development of new medicines as sCTMP are framed by common guidelines for the entire EU.

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Table 2 Examples of ATMP marketed in the EU, USA, Japan and Canada

Country	Product (company)	Cell types	Description
EU	ChondoCelect® (TiGenix NV)	Autologous cultured chondrocytes	Indicated for the repair of cartilage lesions of the cartilage of the knee in adults. Used only in case of injury of the femoral condyle cartilage of the knee (end of the femur)
USA	Carticel® (Genzyme Corporation)	Autologous cultured chondrocytes	A cell therapy medicine product indicated for the repair of symptomatic cartilage defects of the femoral condyle, caused by acute or repetitive trauma of the knee of adults who have not responded to a prior arthroscopic or other surgical repair procedure
	Epicel® (Genzyme Corporation)	Cultured keratinocytes	A sheet of autologous keratinocytes used to replace the epidermal or top layer of skin on severely burned patients. Human keratinocytes are grown on a layer of irradiated mouse cells (xenogenic)
	Provengene® (Dendreon)	Autologous CD54+ cells activated with PAPGM-CSF	An autologous cellular immunotherapy indicated for the treatment of asymptomatic or minimally symptomatic metastatic castrate-resistant (hormone refractory) prostate cancer
	Apligraf® (Organogenesis)	A collagen matrix and living keratinocytes and fibroblast cells	Used to heal ulcers such as diabetic foot and venous leg ulcers
Japan	Laviv® (Fibrocell Science)	Autologous cultured fibroblasts	Antiaging treatment; fibroblasts are then reinjected into wrinkles
Canada	Prochymal® (Osiris)	Mesenchymal stromal cells	A cellular suspension for the treatment of acute GvHD in children. Prochymal is made up of bone marrow stem cells derived from an adult donor and is designed to control inflammation, promote tissue regeneration and prevent scar formation.

GvHD, graft versus host disease.
For detailed information, access http://www.tigenix.com, http://www.genzyme.com, http://www.dendreon.com, http://www.organogenesis.com and http://www.fibrocellscience.com;http://www.osiris.com.

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III.4. ISOLATION AND CHARACTERIZATION OF THE ENVIRONMENTAL BACTERIAL AND FUNGI CONTAMINATION IN A PHARMACEUTICAL UNIT OF MESENCHYMAL STEM CELL FOR CLINICAL USE

El uso clínico de MSCs como tratamiento alternativo en numerosas patologías debe llevarse a cabo bajo estándares de calidad predeterminados por su consideración de medicamento. Su fabricación debe regirse por las normas GMP, en concreto por todos los aspectos descritos en la Parte I y en el Anexo I de medicamentos estériles.

Una de las principales características de un CTMP es que las células que constituyen el activo farmacológico deben ser viables. Esto implica que el producto final obtenido no pueda ser esterilizado por métodos físicos o químicos ya que podrían dañar la integridad de la célula. Con el fin de obtener un producto estéril y seguro desde el punto de vista de calidad farmacéutica es necesario realizar la fabricación en condiciones asépticas.

En este capítulo se llevó a cabo la puesta a punto de un plan de monitorización ambiental en una sala blanca que permitiera la fabricación aséptica del producto celular a estudiar. Se definieron los protocolos de control microbiológico tanto bacteriológico como fúngico para el aire y superficies de trabajo, según estándares GMP. El plan de monitorización se llevó a cabo durante un año y en paralelo con los primeros lotes de producto celular con hMSCs. Los resultados permitieron elaborar un programa de gestión de riesgos de contaminación ambiental.

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Isolation and characterization of the environmental bacterial and fungi contamination in a pharmaceutical unit of mesenchymal stem cell for clinical use

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ABSTRACT

Design and implementation of an environmental monitoring program is vital to assure the maintenance of acceptable quality conditions in a pharmaceutical manufacturing unit of human mesenchymal stem cells. Since sterility testing methods require 14 days and these cells are only viable for several hours, they are currently administered without the result of this test. Consequently environmental monitoring is a key element in stem cell banks for assuring low levels of potential introduction of contaminants into the cell products.

The aim of this study was to qualitatively and quantitatively analyze the environmental microbiological quality in a pharmaceutical manufacturing unit of human mesenchymal stem cells production for use in advanced therapies.

Two hundred and sixty one points were tested monthly during one year, 156 from air and 105 from surfaces. Among the 6264 samples tested, 231 showed contamination, 76.6% for bacteria and 23.4% for fungi.

Microbial genuses isolated were *Staphylococcus* (89.7%), *Micrococcus* (4.5%), *Kocuria* (3.2%) and *Bacillus* (2.6%). In the identification of fungi, three genuses were detected: *Aspergillus* (56%), *Penicillium* (26%) and *Cladosporium* (18%).

The origin of the contamination was found to be due to personnel manipulation and air microbiota. For all sampling methods, alert limits were set and corrective measures suggested.

1. Introduction

Advances in biotechnology have led to a new concept of personalized medicine, providing new promising therapeutic challenges. Medicinal products for advanced therapies are new concepts of medical treatments comprising cell therapy, gene therapy and tissue engineering. Cell therapy consists of infusion of live cells into an organism as a alternative to ineffective drug therapies in chronic diseases or illnesses that require tissue repair or restore, because of loss or defective functions [1–4]. Cell therapy uses mainly stem cells, due to their indefinite capacity for self-renewal and wide potential for differentiation into many cell types [5]. Despite the worldwide tremendous increase in research activity aiming to achieve clinical application of stem cells, the actual number of cell therapy medicinal products (CTMP) available in the market is very low [6]. Transferring research findings to potential clinical therapies requires prior accomplishment of high biosafety standards [7] established by the regulatory authorities [8]. The success of these approaches will be highly dependent upon the development and standardization of protocols [9] for Good Manufacturing Practice (GMP) and product safety including environmental microbial contamination assessment. Maintaining a controlled environment for the manufacture of CTMP minimizes the risk of contamination [10,11] because quality assurance is important in all aspects of these cellular products [12]. It is imperative that for clinical use the prevention of fungi and bacteria infection, and not only in cell cultures, but also the patient receiving the cells [13,14]. Currently these cells are administered without the results of its sterility test since this last procedure requires 14 days [15], and cells are only fully viable (90–99%) for several hours [16]. This fact makes essential processes standardization, environmental programs implementation and quality control methods [17,18] to

Abbreviations: CTMP, cell therapy medicinal products; GMP, good manufacturing practice; MSCs, mesenchymal stem cells; TSA, trypcase soja agar; SDC 1, sabouraud dextrosa cloramphenicol.

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prevent contamination that could invalidate the administration of these cells. In addition, after each mesenchymal stem cells (MSCs) batch manufactured a quality control is performed by several tests, among them, endotoxine detection test and mycoplasma detection test.

The aim of this study was to perform a qualitative and quantitative environmental analysis of microbiological quality in a pharmaceutical manufacturing unit of human mesenchymal stem cells (MSCs) for use in clinical trials to ensure safe and reliable cells products. The cellular production unit (cell factory) evaluated in the present work is located in Seville (Spain), at the Center for Molecular Biology and Regenerative Medicine, it has been the first laboratory authorized by the Medicines and Health Products Spanish Agency for the production of stem cells as drugs in Andalucía (Spain).

Monitoring of this unit was carried out weekly over a 12-month period (January 2010–December 2010) in order to evaluate the microbial contamination coming from air and surfaces, during the manufacturing process of the MSCs (under normal working conditions) [19]. Contamination levels were compared with levels established by the European Union and GMP guidelines. The study evaluated whether the microbiota found might or might not damage the quality of the product. Besides, it allowed the establishment of alert limits to determinate the existence of a microbiological environment suitable for production of MSCs with use in advances therapies in humans.

The monitoring program carried out included: definition of sampling points, sampling frequency, sampling methods, microorganism identification, alert limits and corrective measures establishment.

2. Materials and methods

2.1. Cell lines and their origins

This study was performed in the context of a clinical phase I/II single-center trial under GMP conditions for autologous MSCs infusions in patients.

MSCs were isolated from subcutaneous adipose tissue from human donors in hospital. All subjects gave their informed consent, which was approved by our institutional committee on human research. Tissues were transferred to the cell factory, where they were digested with collagenase from Roche-Farma (Madrid, Spain) and centrifuged to obtain the stromal cells. The isolation of the MSCs was based on their ability to adhere to the plastic, contrary to the stromal cells that remain floating in the media.

Adherent cells are attached to the surface of culture flasks using proteins secreted by the cells which form bonds, these peptide bonds must be broken through trypsinization by trypsin–EDTA (Gibco®, Grand Island, NY, USA) in the incubator at 37 °C for no longer than 3–5 min. Trypsin (proteolytic enzyme) breaks proteins at specific places and EDTA allows trypsin to work more efficiently by engaging certain metal ions that may inhibit its activity. The trypsin was inactivated by adding Dulbecco's Modified Eagle's medium containing 10% Fetal Bovine Serum. After, the cells detached from the culture surface were collected by means of centrifugation.

Large-scale expansion (the number of passes to perform depends on the final dose but no longer to passage 6) cultures of MSCs were carried out in irradiated culture flasks at a seeding concentration of $3.5–5.0 \times 10^3$ cells/cm^2 in GMP conditions (the final doses would be ranging $10–100 \times 10^6$ cells depending on the specific treatment assigned to the patient). The expansion medium used was low glucose Dulbecco's Modified Eagle's Medium, supplemented with 10% Fetal Bovine Serum, 100 U/mL Penicillin–100 μg/mL Streptomycin and 2 mM L-glutamine without the addition of growth factors. All reagents were purchased from Sigma–Aldrich (Madrid, Spain). However, concerns over the safety of FBS have been raised because they can trigger adverse responses, the supplier certify that the FBS is compliance with the criteria described in the current version of the monograph products with risk of transmitting agents of animal spongiform encephalopathies n° 1483 of the European Pharmacopoeia. MSCs were maintained in culture in CO_2 incubator (37 °C, 5% CO_2 and 95% relative humidity).

2.2. Area selected for microbiological tests

There are three rooms for cell processing in this laboratory classified as grade B, inside which three laminar flow cabinets (grade A) are placed, a clean clothing (grade B), a dirty clothing (grade C) and entry and store area (grade D). All these areas were selected for microbiological tests.

2.3. Active air sampling

All samples were taken using a MAS-100® Eco air monitoring system (EMD Chemicals Inc. Gibbstown, NJ, USA), a high-performance instrument based on the principle of the Andersen air sampler [20], drawing air through a perforated plate. The particle-bearing airflow is directed onto a standard Petri dish containing agar-media. After finishing the collection cycle, the plates were incubated and the total colony count determined. The MAS-100® Eco system, operated with a high-performance suction device. Annually calibration checking is performed. The aspirated volume was 100 L per minute, allowing up to 1000 L of air to be collected per 10 min cycle. This performance enabled sampling to be carried out in line with the strict demands of monitoring in clean rooms, avoiding at the same time the dehydration of the agar surface. The impaction velocity (speed at which airborne microorganisms hit the surface of the agar) was approximately 11 m/s, equivalent to stage 5 of the Andersen Sampler, guaranteeing that all particles >1 μm were collected.

2.4. Passive air sampling

Open agar plates were exposed directly to the ambient air or placed it under a laminar flow hood for 4 h. The microorganisms transported by particles were deposited on the surface of the agar medium with an average deposition value of 0.46 cm/s [21].

2.5. Surface sampling

A direct contact method of the surface to be tested with the agar during 10 s was used to collect samples from dry surfaces. The convex agar meniscus allowed its direct application to test surfaces, such as walls, floors, utensils or personnel. Subsequently, the surfaces from which samples were collected were adequately cleaned for hygiene monitoring.

2.6. Microbiological culture medium and incubation conditions

Environmental microbiological monitoring of air and surfaces was carried out by standard testing in accordance with the European Union standards: EudraLex – Volume 4 GMP guidelines [22].

Active and passive air samples were incubated in solid media Petri 90 mm plates. For detection of bacteria, 2 days incubation at 35 °C in Trypcase Soja Agar (TSA) was employed (bioMérieux, Madrid, Spain). The mixture of peptones that contains in this medium promotes the growth of most microorganisms. For detection of fungi, samples were incubated for 5 days at 22 °C in

Sabouraud Dextrosa Cloramphenicol Agar (SDC) (bioMérieux, Madrid, Spain). The high concentration of glucose optimizes the growth of fungi whereas its pH and the addition of 50 mg/L of chloramphenicol improve the selectivity of medium avoiding bacterial growth.

For surface samples contact plates or replicate organism detection and counting (RODAC) plates (55 mm diameter) were used to facilitate an easy and reproducible surface microbial testing. In order to inactivate residual disinfectants, plates containing TSA or SDC were supplemented with lecithin and polysorbate 80.

All media were prepared following the formulae described in the harmonized chapters of the European Pharmacopoeia: B Medium and C Medium (European Pharmacopoeia, 2008). Each batch of ready-prepared medium was tested by growth promotion test: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus niger*, *Bacillus subtilis*, *Candida albicans* from BioBall SingleShot (bioMérieux Madrid, Spain). Reagents used: Irradiated Trypcase Soy 3P Agar, Irradiated Sabouraud Dextrose Chloramphenicol Agar,Irradiated Count-Tact 3P Agar, Count-Tact Sabouraud Dextrose Chloramphenicol Neutralizers Irradiated Agar, all these from bioMérieux (Madrid, Spain).

Daily calibration of the incubators used for environmental monitoring tests was carried out using a Data Logger Escort Junior device (Escort Data Logger Inc., Buchanan, VA, USA).

2.7. Microbial identification

After incubation, each sample was tested to check the presence/absence of colony forming units (cfu). Bacteria identification was performed by Gram's staining and morphological characterization followed by pertinent biochemical tests, with an API system (bioMèrieux, Madrid, Spain). Fungi were identified by colony and hyphal morphology after staining with lactophenol blue (Sigma–Aldrich, Madrid, Spain) [23]. For fungi identification, a comparison with the Atlas of clinical fungi was also utilized [24].

2.8. Sample collection frequency and number of measurement points

The monitored areas were defined according to our microbiological environmental monitoring program, summarized in Table 1. A total of 261 points (156 corresponding to air and 105 to surfaces) were analyzed monthly.

For each area, sampling was conducted on the basis of critical points chosen for their proximity to the MSCs work area. Critical areas require more frequent monitoring than those in which there is no contact with the product, therefore grade A and B areas were more often controlled. Surface sampling and passive air sampling in grade C and D areas were dismissed from the study based on the negative results obtained in a preliminary study.

For grade A and B areas, active air sampling locations were entrance zone and zone where personnel were frequently present. Passive air sampling and surface sampling were taken from inside and under biological safety cabinets, work surfaces, CO_2 incubators, centrifuges, inside airlocks, walls and grilles. For each point, two plates, one for bacterial growth (TSA) and a second one for fungi (SDC) were used.

This study was carried out from 1 January 2010 to 31 December 2010 with a sampling frequency of once per week. The total number of samples tested was 6264 (261 points/moth × 12 months × 2 plates (TSA and SDC)), from which 3744 belonged to air and 2520 to surfaces.

The results for each area were interpreted according to the annex I-GMP. Recommended limits proposed for microbiological monitoring of the cell factory are reported in Table 2, [25].

Table 1
Number of sampling points per month in a cell factory for each grade.

Test	Grade A	Grade B	Grade C	Grade D
Active air sampling	20	20	4	12
Passive air sampling	48	52	0	0
Surface sampling	60	45	0	0

3. Results

3.1. Qualitative analysis of the samples

The microbiological quality of all sampled points was evaluated for the presence/absence of bacteria and fungi cfu. Two hundred and sixty one points per month were studied. As shown in Table 3, from 6264 samples obtained, bacterial growth was observed in 177 samples (76.6%) and fungi growth in 54 samples (23.4%). Results were expressed as cfu/plate for surface, as cfu/m^3 for active air sampling and as cfu/4 h for passive air sampling.

The individual analysis from the active air sampling revealed microbiological growth in all samples taken except in those from grade A area, the most critical zone in contact with cell cultures of MSCs, where no bacterial or fungi growth was observed. In grade B area, bacterial growth was observed in 38 samples (21.8%) and fungi growth in 2 samples (3.8%). The number of samples where microbiological growth was observed on agar-plates increased for grade C and even further for grade D areas: 45 samples (25.9%) showed bacterial growth and 7 samples (13.2%) fungi growth in grade C area, and in grade D, 91 samples (52.3%) with bacterial growth and 44 samples (83%) for fungi were observed.

Passive air sampling results were negative for all areas studied. No microbiological growth was detected in any of the agar plates.

In samples from surfaces, only those taken during months of May and June showed microbiological growth. On May, fungal growth was detected in a sample taken from the floor of the clean clothing area (grade B). On June, bacterial growth was detected in three plates, corresponding to samples taken from the return grille of a production hall (grade B).

3.2. Quantitative analysis of the samples

The monthly quantitative analysis (Figs. 1 and 2) showed that for all points evaluated the number of cfu was below the limits allowed by Annex I of GMP guidelines (Table 2). No differences were detected in cfu when the summer months were compared with the same winter period, due to the constant humidity and temperature inside the laboratory. In agreement with above described data, the samples taken from grades C and D presented a higher number of cfu when compared to grade B. This fact is even more obvious for fungi cfu.

3.3. Identification of microorganisms

The analysis of all the bacterial colonies isolated from the cell factory (grades B, C and D) revealed that all of them were Gram-positive. Gram-negative bacteria were not found in any sample.

Table 2
Recommended limits for microbiological contamination monitoring according to the rules governing medicinal products in the European Union Annex 1.

Grade	Air sample (cfu/m^3)	Settle plates (cfu/4 h)	Contact plates (cfu/plate)
A	<1	<1	<1
B	10	5	5
C	100	50	25
D	200	100	50

Table 3
Temporal and area description of the samples studied indicating the presence/absence of microbiological growth. All environmental monitoring methods: active air sampling, passive air sampling and surface sampling are detailed individually. Each value represents the number of plates that show growth in TSA/SDC media. For each sampling point, 1 plate of each medium per week was used.

Method	Grade	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Active air sampling (cfu/m^3)	A	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	B	3/0	2/0	3/1	1/0	3/0	5/0	0/0	4/0	7/01	5/0	2/0	3/0
	C	4/0	4/0	3/1	4/2	3/0	3/1	4/0	4/1	4/0	4/1	5/1	3/0
	D	9/1	7/2	8/2	7/3	8/1	7/6	10/5	9/5	8/5	6/6	7/6	5/2
Passive air sampling (cfu/4 h)	A	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	B	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	C	–	–	–	–	–	–	–	–	–	–	–	–
	D	–	–	–	–	–	–	–	–	–	–	–	–
Surface sampling (cfu/plate)	A	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	B	0/0	0/0	0/0	0/0	0/1	3/0	0/0	0/0	0/0	0/0	0/0	0/0
	C	–	–	–	–	–	–	–	–	–	–	–	–
	D	–	–	–	–	–	–	–	–	–	–	–	–

Staphylococcus was found to be the predominant bacterial genus (89.7%). *Micrococcus* was also found to be abundant (4.5%) Other bacterial genuses found were *Kocuria* (3.2%) and *Bacillus* (2.6%). For fungi identification three genuses were detected: *Aspergillus* (56%), *Penicillium* (26%) and *Cladosporium* (18%). Fig. 3 shows the percentage of the different genuses of microorganisms observed in the cell factory during all the study.

In order to evaluate the bacterial and fungal surface contamination, some representative surfaces potentially exposed to microbial contamination were identified. In this way, three different species have been identified with the same percentage of incidence: *Staphylococcus epidermidis* (33.3%), *Staphylococcus hominis* (33.3%) and *Staphylococcus haemolyticus* (33.3%). Also the presence of environmental fungi was detected: *Aspergilus niger*, but in unrepresentative amount and frequency (Table 4).

In active air sampling four genuses were identified: *Staphylococcus* (89.5%), *Micrococcus* (4.6%), *Kocuria* (3.3%) and *Bacillus* (2.6%). Among the *Staphylococcus* genus the following species were identified: *S. epiderminis*, *S. hominis*, *Staphylococcus cohnii*, *Staphylococcus auricularis*, *Staphylococcus capitis*, *Staphylococcus warneri*, *Staphylococcus saprophyticus*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, *Staphylococcus xylosus*, *Staphylococcus lentus*, *Staphylococcus lugdunensis* and *Staphylococcus hyicus*. Only one micrococcus genus was identified: *Micrococcus luteus*. The isolated species of *Kocuria* genus were *Kocuria varians* and *Kocuria kristinae*. *Bacillus* genus predominant species were *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Bacillus cereus* and *Bacillus megaterium*.

In active air sampling, the isolated fungi genuses were *Aspergillus* (55.1%), *Penicillium* (26.5%) and *Cladosporium* (18.4%) (Table 5). Fig. 4 shows the images obtained from microscopic identification of isolated fungal species.

To define alert limits for the microbiological analysis in the working area, the limits given by the GMP guidelines (Table 2) were subdivided into several groups (Tables 6 and 7 upper row). The alert limit was determined experimentally by performing a quantitative study of the microbiota in this area. The observed results at each sub-interval are detailed in Tables 6 and 7 (lower row) indicating the number of sampled points in each grade area throughout the study period. Each value corresponds to cfu counts for bacteria and fungi. These values are considered as optimum for our cell factory. Higher number of cfu per point would set the alert limit. If the alert limit values would be reached, corrective measures should be taken.

Table 6 shows the analysis and alert limits calculation for microbiological control of surfaces. In grade A area, no microbiological growth was observed. Sampled points in grade B showed increased microorganisms growth; there were 534 points with growth between 0 and 1 cfu. For the interval 1–5, only 6 points showed growth. Table 7 shows analysis and alert limits calculation for air microbiological control. In grade A area, any tested point showed microbiological growth. Sampled points in grade B showed greater growth (208 points) of microorganisms between 0 and 1 cfu. For interval 1–5, there were 32 points in which microbiological growth was observed. In grade C area, until 100 cfu are allowed. In the air monitoring, the highest frequency of growth was between 1 and 25 cfu. The number of tested points with microbiological growth ranging between 25 and 50 cfu were 6. Equally in grade D, the majority of points with growth were in the interval 1–25 cfu, between 25–50 only 7 points showed growth.

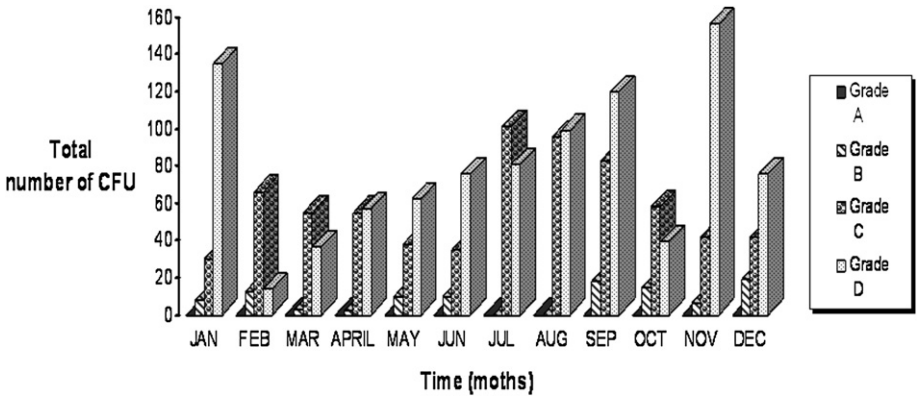

Fig. 1. Bacterial quantification. Quantification of cfu obtained from an active air sampling for bacteria during each month of the study showing different grades.

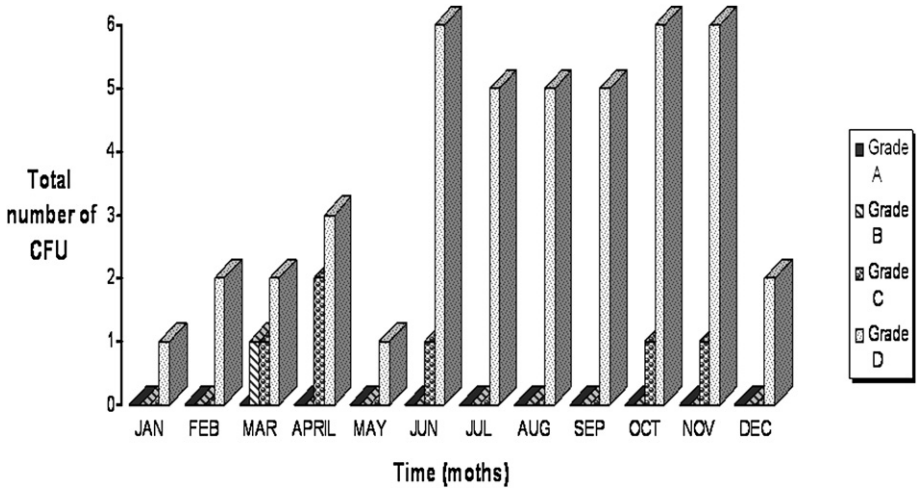

Fig. 2. Fungal quantification. Quantification of cfu obtained from an active air sampling for fungi during each month of the study.

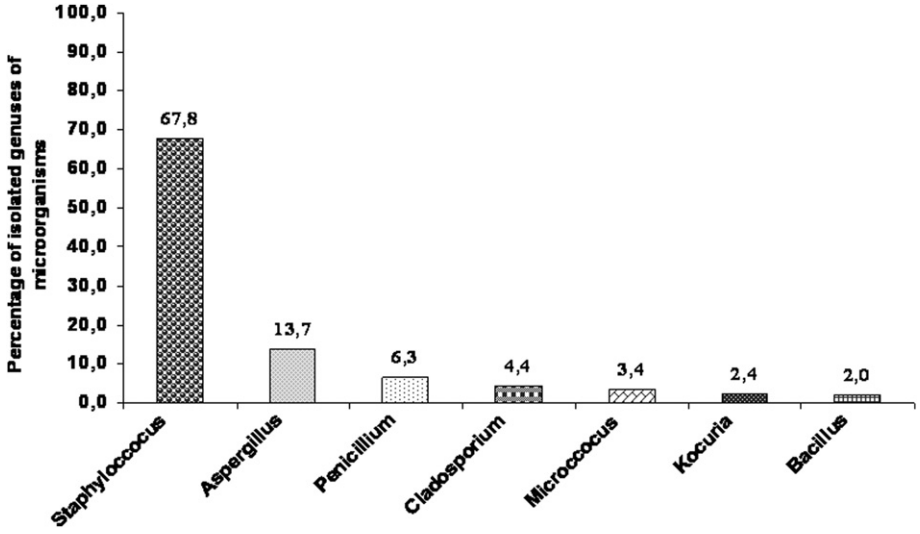

Fig. 3. Isolated genuses. Percentage of isolated genuses of microorganisms observed in the cell factory from the environmental monitoring program.

4. Discussion

MSCs are ubiquitous adult stem cells with capacity for self-renewal and differentiation into a variety of cell as osteoblasts, chondrocytes, myocytes, adipocytes, tenocytes and pancreatic islets beta-cells [26,27]. MSCs were initially characterized by plastic adherence, fibroblastoid cells with capacity to generate different organs and tissues [28]. From a therapeutic perspective, based on their easy culture and immunosuppressive properties, MSCs are emerging as an extremely promising therapeutic agent for cell therapy. MSCs have been also shown to play a role in tissue repair and regeneration in both preclinical and clinical studies [29,30]. Moreover MSCs migrate to sites of injury, increasing their ability to correct inherited tissue disorders to facilitate tissue repair *in vivo* [31].

Based on these properties, this laboratory is focused on their use for cell regeneration therapy in the syndrome of chronic critical limb ischemia in diabetic patients, involved in the development of a clinical trial phase I–II (EudraCT number 2008-001837-88). A cell product for application in the human body must be viable, genotypically stable, endotoxin-free and sterile. However, the obtained cells are not a conventional drug, and cannot be sterilized by physical or chemical methods, since these cells must retain its viability. Sterility test of MSCs is parametric and results cannot be obtained up to 14 days [15]. Therefore a quality assurance programme including the principles of good manufacturing practice and a quality control system over cleanliness, personnel, facilities, materials, intermediate and final product [32] is necessary to keep the cell culture, free of microbiological contamination [33]. A contaminated culture can spread contamination to other cell cultures in the cell factory [34]. Environmental microbiological monitoring (air and surface) in a cell factory helps to ensure that

Table 4
Isolated microorganisms in surface sampling. Percentage values are referred to each genus.

Methods	Microorganisms	Species	Number of isolated/percentage
Surface sampling	Bacteria *Coagulase negative*	*Staphylococcus epidermidis*	1/33.3
		Staphylococcus hominis	1/33.3
		Staphylococcus haemolyticus	1/33.3
	Fungi	*Aspergillus niger*	1/100

Table 5
Isolated microorganisms in active air sampling. Percentage values are referred to each genus.

Methods	Microorganisms	Species	Number of isolated/ percentage
Active air sampling	Bacteria *Coagulase negative*	*Staphylococcus epidermidis*	52/38.2
		Staphylococcus hominis	19/14
		Staphylococcus cohnii ssp. *cohnii*	13/9.6
		Staphylococcus auricularis	13/9.6
		Staphylococcus capitis	12/8.8
		Staphylococcus warneri	8/5.9
		Staphylococcus saprophyticus	5/3.7
		Staphylococcus chromogenes	4/2.9
		Staphylococcus haemolyticus	3/2.2
		Staphylococcus sciuri	2/1.5
		Staphylococcus xylosus	2/1.5
		Staphylococcus lentus	1/0.7
		Staphylococcus lugdunensis	1/0.7
		Staphylococcus hyicus	1/0.7
		Micrococcus luteus	7/100
		Kocuria varians	3/60
		Kocuria kristinae	2/40
	Bacteria *Bacillus* spp.	*Lactobacillus plantarum 1*	1/25
		Lactobacillus acidophilus 3	1/25
		Bacillus megaterium	1/25
		Bacillus cereus 1	1/25
	Fungi	*Aspergillus niger*	27/55.1
		Penicillium spp.	13/26.5
		Cladosporium spp.	9/18.4

Table 6
Study and determination of alert limits for the surfaces microbial monitoring in a cell factory.

Grade	A		B			
Ranges (cfu)	0	≥1	0–1	1–5	>5	>10
Points with mg[a]	720	0	534	6	0	0

[a] mg: Microbiological growth.

cells are manufactured in optimal conditions [35] allowing their application in clinical trials [36,37]. Therefore, a good designed monitoring program will prevent potential risks of contamination [38], avoiding potential risks to patients.

In this study all the results were within the standards required by the GMP guidelines. Specifically, 261 points were sampled for 12 months, obtaining a total of 6264 samples (TSA / SDC). Among these samples, only 231 showed microbiological growth. The quantification of the microbiological growth determined that all areas of the cell factory (grades A, B, C and D) have an optimal number of cfu, below the limits established in the regulations. In previous studies reported by *Ottria* et al. [37] and *Caorsi* et al. [8] greater number of cfu were obtained in grade B areas. In passive air sampling the presence of microorganism was not detected.

From surface sampling, the presence of microorganism was not detected in grade A areas. However in grade B areas, the presence of bacteria and fungi was observed but only on June and May. These data are not considered representative. These points were located in places where the proportion of clean filtered air was lower, namely a grid (area of dirty air return) and clean room clothing (costume change area). Both points were found to be the dirtiest environment areas.

Results obtained from the active air sampling provided us more details about the existing microbiota in the cell factory. In grade A areas presence of microorganism was not detected. On the other hand, in grade B areas bacterial growth was detected, and in grades C and D areas an increasing number of samples with microbiological growth was observed, being in grade D where the highest microbiological growth was detected, concretely the zone closest to the outside. In all areas analyzed the sampled points with microbiological growth corresponds to points near the floor, doors and center of the room, where all air streams converge and where the mobility of the personnel is greater.

Our results indicated that the resident microbiota of the cell factory belonged mainly to gram-positive bacteria. Other study analyzing the microorganisms present in a cell bank [12] showed that the most frequently found microorganisms belonged to *Staphylococcus* spp. and *Micrococcus* spp. This is in agreement with our results where these two genuses are the most abundantly detected in our samples. Nevertheless in our study the total number of microbial species found was lower, suggesting a cleaner environment in the cell factory.

Detailed analysis of the species belonging to the *Staphylococcus* genus showed that the most frequent specie was *S. epidermidis*. *S. hominis* was also found (albeit in varying quantities) confirming the human origin of this contamination. Other identified bacterium was *M. luteus*. This microorganism is part of the normal flora of human skin. When it is isolated in clinical samples, skin or mucosal surfaces are often considered to be involved in the contamination [39]. Within *Kocuria* genus, two species were identified: *K. varians* and *K. kristinae*. These two bacteria are generally considered harmless saprophytes of skin and mucous membranes [40]. This type of contamination of human origin is frequent, taking into account the microorganism emission capacity of a person; it can reach hundreds and sometimes thousands of bacteria per minute in air [41]. Therefore the

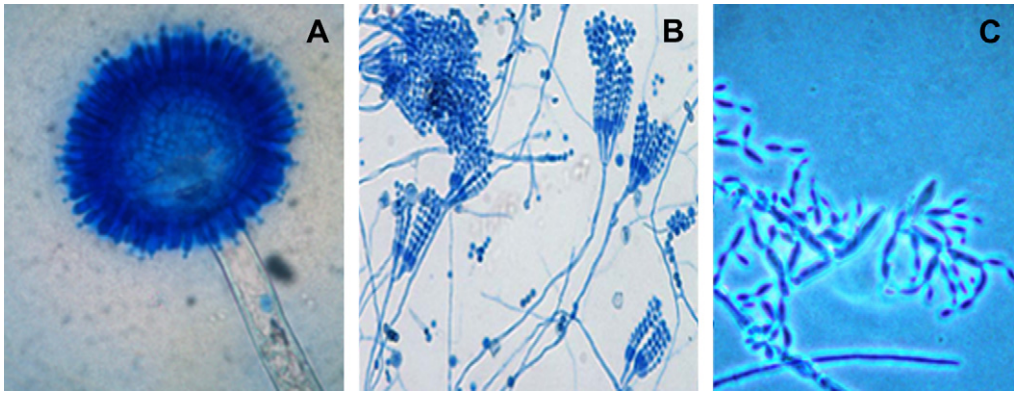

Fig. 4. Microphotographs. Microscopic observation of lactophenol blue staining (A) *Aspergillus*; (B) *Penicillium*; and (C) *Cladosporium*.

Table 7
Study and determination of alert limits for active air microbial monitoring in a cell factory.

Grade	A		B				C					D				
Ranges (cfu)	0	≥1	0–1	1–5	5–10	>10	0–1	1–25	25–50	>50	>100	0–1	1–25	25–50	50–200	>200
Points with mg[a]	240	0	208	32	0	0	5	37	6	0	0	39	98	7	0	0

[a] mg: Microbiological growth.

microbiota found is likely derived from work practices carried out by the personnel, input materials from areas without controlled environment and the different air conditions allowed in each specific grade, being strict in grade B and less strict in grade D. The skill training and attitude of personnel involved in the production of CTMP are important factors for quality in the manufacturing process.

The less abundant microbial genus identified was *Bacillus*. Their main habitat is soil although they are also widely spread in the air [42]. Some of them are often part of the normal flora of human.

The microscopic fungi identification detected the presence of three genuses belonging to the soil microbiota and the environment: *Aspergillus*, *Penicillium* and *Cladosporium*. *Aspergillus niger* and *Penicillium* spp. They grow with very low humidity conditions whereas *Cladosporium* spp. need more water [43]. The presence of fungi in a cell factory suggests environmental contamination inside the critical area. This can be originated by personnel, or by input or output of waste materials.

Finally, an important function of environmental microbiological monitoring is to identify trends in the microbial load of a cell factory, defining an alert system to indicate a loss of the environment quality or the breakdown of aseptic practices. For all sampling methods the following alert limits were established: grade A (1 cfu), grade B (>5 cfu), grades C and D (>50 cfu). Corrective measures should be taken if these limits would be reached, firstly checking the high efficiency particulate air filters of ventilation system (H14, F9 and G4), and secondly disinfection of the area by spraying with hydrogen peroxide [44]. After these actions new environmental monitoring should be done to verify its environmental microbiological quality.

Biological safety cabinets (grade A) were lower-risk areas in which there was no microbiological growth. The results obtained confirm the suitability of input and output circuit air conditioning. The analysis of the microbiota of the cell factory provides data on its origin, mainly being human and environmental. Both types of contamination are studied in detail during the entire cell production by the environmental monitoring program designed. The development of this program of environmental monitoring has allowed us to know the general microbiota that can be found in a cell factory, and set alert limits that will prevent future contaminations affecting the MSCs production that can lead to health problems for patients.

In conclusion, a formal program of environmental monitoring should be maintained in a pharmaceutical manufacturing unit of human mesenchymal stem cells for use in clinical trials to specify and assess key factors and their influence on the microbiological quality of the process and product. After weekly performing quantitative and qualitative analysis in the Center for Molecular Biology and Regenerative Medicine during a 12-month period (January 2010–December 2010) to evaluate its specific needs and establish appropriate monitoring procedures to assure the quality of preparation of advanced medical therapy products.

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III.5. STANDART REQUIREMENT OF A MICROBIOLOGICAL QUALITY CONTROL PROGRAM FOR THE MANUFACTURE OF HUMAN MESENCHYMAL STEM CELLS FOR CLINICAL USE

La fabricación de un CTMP conlleva la expansión *ex vivo* de células madre. Durante este periodo pueden aparecer contaminaciones causadas por contaminación cruzada con otras líneas celulares o con microorganismos. Por tanto una vez definido el protocolo de fabricación y las condiciones ambientales en las que debe llevarse a cabo la producción de hMSCs para uso clínico, se definieron los controles de calidad microbiológicos de todos los materiales y reactivos implicados en el proceso de fabricación, junto con los del producto intermedio (subcultivos) y producto final (CTMP).

Los ensayos de control calidad se basaron en el estudio de esterilidad, endotoxinas, micoplamas y personal (monitorización en proceso). Cada uno de ellos fue validado acorde a las recomendaciones de la Farmacopea Europea.

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ORIGINAL RESEARCH REPORT

Standard Requirement of a Microbiological Quality Control Program for the Manufacture of Human Mesenchymal Stem Cells for Clinical Use

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The manufacturing of human mesenchymal stem cells (hMSCs) as cell-based products for clinical use should be performed with appropriate controls that ensure its safety and quality. The use of hMSCs in cell therapy has increased considerably in the past few years. In line with this, the assessment and management of contamination risks by microbial agents that could affect the quality of cells and the safety of patients have to be considered. It is necessary to implant a quality control program (QCP) covering the entire procedure of the ex vivo expansion, from the source of cells, starting materials, and reagents, such as intermediate products, to the final cellular medicine. We defined a QCP to detect microbiological contamination during manufacturing of autologous hMSCs for clinical application. The methods used include sterility test, Gram stain, detection of mycoplasma, endotoxin assay, and microbiological monitoring in process according to the European Pharmacopoeia (Ph. Eur.) and each analytical technique was validated in accordance with three different cell cultures. Results showed no microbiological contamination in any phases of the cultures, meeting all the acceptance criteria for sterility test, detection of mycoplasma and endotoxin, and environmental and staff monitoring. Each analytical technique was validated demonstrating the sensitivity, limit of detection, and robustness of the method. The quality and safety of MSCs must be controlled to ensure their final use in patients. The evaluation of the proposed QCP revealed satisfactory results in order to standardize this procedure for clinical use of cells.

Introduction

CELL-BASED THERAPY has led to the development of new biological medicines to repair, replace, or recover the biological function of damaged tissues and organs [1]. Among cell types used for this propose, human mesenchymal stem cells (hMSCs) are considered as cell-based therapy medical product (CTMP) and should be handled with appropriate controls to ensure their safety, quality, and efficacy as a final medicine [2–6].

The manufacture of hMSCs involves an ex vivo expansion for a relatively long period of time, which leads to a risk of contamination by microbiological agents that could affect the quality and safety of the cells [7]. Contamination of a CTMP can cause adverse reactions in patients (eg, fever, chills, infections, and irreversible septic shock) and even death [7,8]. Therefore it will be necessary to standardize and validate all procedures and analytical techniques involved in the manufacture of CTMP [9], posing a quality control program (QCP).

A QCP should ensure that cells have been manufactured in aseptic conditions, under GMP conditions to minimize the contamination risk of the cell medicine and, thus, to ensure the safety of patients and the quality of the medicine. This program will comprise the whole process of ex vivo expansion, starting from type of cells, source of materials, reagents, and intermediate products (subcultures), to CTMP, the final cellular medicine [10,11]. Chiefly, because the cells must be viable for their administration and should not be sterilized by physicochemical methods, in this scenario a risk analysis must be performed to determine the possibilities of microbiological contamination before designing a QCP. For a QCP applied to a CTMP, each analytical technique should be justified, and the amount and type of evidence required for microbiological quality control were defined according to the different pharmacopoeias, as well as, the guidelines issued by regulatory agencies and International Conference on Harmonisation (ICH), in particular, quality guidelines [12,13]. Validation studies must be performed for each analytical

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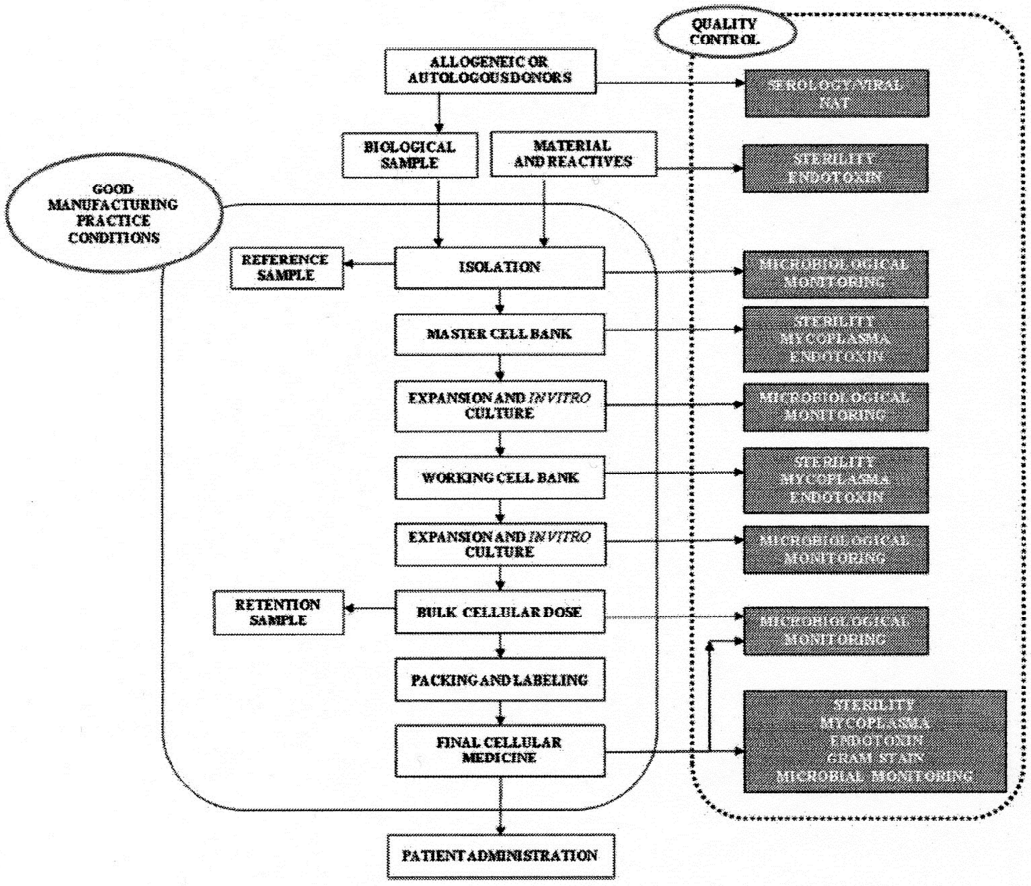

FIG. 1. Scheme of manufacturing process of autologous stem cell mesenchymal as a cell therapy medicinal product.

technique to demonstrate and verify that the procedure adopted at each site laboratory does not alter the method and consequently the result [14].

The aim of this study was to develop a microbial QCP of a CTMP (Fig. 1), for the long-term expansion of human adipose-derived MSCs. In particular, the manufactured medicine was an injectable cell suspension, elaborated by suspending the active principle (hMSCs) and other additives (culture medium or packing medium), packaged in a suitable container to be administered parenterally (intramuscular, intravenous, and intra-arterial). Contamination by bacteria, fungi, and mycoplasma and bacterial endotoxin concentration were analyzed in line with QCP proposed in different phases, such as Master Cell Bank (MCB), Working Cell Bank (WCB), and in the final cellular medicine. Each analytical technique was validated on three different cultures.

Materials and Methods

This study was performed in the quality control unit of the CABIMER's GMP facility authorized by the Spanish Agency of Medicines and Medical Devices and regularly inspected by the Spanish competent authorities.

Isolation and culture of hMSCs

Autologous hMSCs were isolated from abdominal adipose tissue biopsies of patients enrolled in a Phase I/II clinical trial. All donors provided informed consent that was formerly approved by local and regional medical research ethics committees. Each patient was appropriately screened and tested for human pathogens. In particular, the presence of human immunodeficiency virus (HIV), hepatitis B virus, and hepatitis C virus was analyzed. On the other hand, all starting materials and reagents required for the expansion were analyzed to certify that they were sterile and endotoxin free.

Cells were isolated from human adipose tissue by enzymatic digestion with collagenase (2 mg collagenase/1 g adipose tissue; Roche Farma, Reinach, BL, Switzerland), centrifuged at 400 *g* for 10 min, and filtered and washed with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) to obtain the stromal cells. These cells were suspended and plated at medium density of $12–20 \times 10^4$ cells/cm^2 in culture flasks (Nunc, Roskilde, Roskilde-DK4 000, Denmark) with expansion medium composed by Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% of 10,000 IU/mL

penicillin and 10 mg/mL streptomycin, 2% of 200 mM L-alanine solution, and 1% of 200 mM L-glutamina (all from Sigma-Aldrich). After 24 h, nonadherent cells were removed by replacing the expansion medium. The medium was replaced every 2 or 3 days a week. Cells were harvested upon reaching 80% confluence, and subcultured using 0.25% trypsin (Gibco, Invitrogen, Grand Island, NY) in expansion medium, and plated at medium density, 3500–5500 cells/cm^2. Cells were cultured under 95% relative humidity, at 37°C, and 5% CO_2. Three different processes of ex vivo expansion of hMSCs were carried out (named 1, 2, and 3) from passages 3–4 to analyze their microbiological quality. For the final product, cells were packed at concentration of 1×10^6 cells/mL with 1 mL of packaging medium composed by 50% of glucose 5%, 45% of lactated Ringer's solution, and 55% of albumin 20% (Grifols, Barcelona, Spain). hMSCs were packed in 10-mL Luer-lock syringes (Becton Dickinson and Company, Franklin Lakes, NJ).

Sterility test and Gram stain

The test was carried out in different phases (MCB, WCB, and final cellular medicine) on three different cultures, by direct inoculation of 1 mL of intermediate product (supernatant) or final suspension in the microbiology medium, so that volume of the product is not more than 10% of the volume of the medium but not less than 1 mL [15]. Two media were used: Thioglycollate Penase Broth 125 mL (TPB; BDH Prolabo, Mexico D.F., Mexico), to detect anaerobic and aerobic bacteria, and Tryptic Soy Penase Broth 125 mL (TSPB; BDH Prolabo), which is a soybean casein digest medium to detect fungi and aerobic bacteria. For each media (TPB and TSPB), both sterility test and growth promotion test of aerobes, anaerobes, and fungi were verified before testing; the strains used in the growth promotion test are indicated in the Table 1. A negative control was included inoculating 1 mL of 0.9% sterile NaCl (bioMérieux, Marcy l'Etoile, France) for each medium. The inoculated media were incubated for 14 days at 35°C and 22°C for TPB and TSPB, respectively. If microbial growth appears after 14 days, then the medium will show turbidity.

Sterility test was performed as soon as possible after sample collection, which was stored at 5°C ± 3°C for up to 24 h [16] to avoid phagocytosis of microorganisms by cells present in the sample. This assay was performed in aseptic conditions with an isolator HPI-4PI-S (Esco Technologies, Inc., Hatboro, PA).

Table 1. Microbial Strains Needed to the Growth Promotion Test and the Validation of Sterility Test

Culture medium	*Microorganisms*	*Strain*
Thioglycollate Penase Broth	*Clostridium sporogenes*	ATCC 11437
	Pseudomonas aeruginosa	ATCC 9027
	Staphylococcus aureus	ATCC 6538
	Aspegillus niger	ATCC 16404
Tryptic Soy Penase Broth	*Bacillus subtilis*	ATCC 6633
	Candida albicans	ATCC 10231

ATCC, American Type Culture Collection.

Additionally, in each final cellular medicine for the three cultures, the presence/absence of colony forming unit (CFU) was examined by standard procedure of Gram staining [17]. Spanish Agency of Medicines and Medical Devices recommends that Gram staining should be performed before releasing the medicine to verify that there is no contamination. If the Gram stain is positive, then the cells will not be administered.

Pictures of Gram stains were taken using brightfield microscope (Zeiss Axioskop 40; Carl Zeiss Mediatec AG, Jena, Germany). The stains were photographed by a microscope Zeiss Axioskop 40 (Carl Zeiss Mediatec AG), data not shown.

Detection of mycoplasma

The test was carried out in different phases (MCB, WCB, and final cellular medicine) on three different cultures, cells were centrifuged and resuspended at concentration of 1×10^6 cells/mL with 1 mL of expansion medium, and the detection of mycoplasma in the final cellular medicine was as previously described [18].

To perform this assay, 5×10^5 cells in 500 µL of supernatant were used. Briefly, the sample was introduced in the dry-block thermostat (TDB-120; Biosan Ltd., Riga, Latvia) to inactivate DNAses at 95°C for 10 min to avoid mycoplasma DNA degradation. Then, genomic DNA extraction was performed by the method of MB DNA (Minerva Biolabs, Berlin, Germany), which is sensitive for mycoplasma genomes. DNA was amplified by the nucleic acid amplification technique (NAT) with a PCR mycoplasma detection kit (VenorGeM; Minerva Biolabs). The DNA polymerase used for the amplification was MB Taq DNA polymerase (Minerva Biolabs). The PCR device utilized was a Life Express thermal cycler (Bioer Technology Co., Hangzhou, P.R. China). The amplification products were analyzed by gel electrophoresis in the FlashGel® DNA System containing ethidium bromide (Lonza, Walkersville, MD). The bands of any PCR amplicons were visualized by UV transillumination. They were identified by comparing them with the bands visible in the positive control and the negative control reaction. The presence of mycoplasma was indicated by an amplification product at ~267 bp. A 191-bp band in every lane was indicated a successfully performed PCR without polymerase inhibition. The samples were analyzed before and until 24 h after their collection, which after inactivation were stored for 6 days from 2°C to 8°C [19].

Endotoxin assay

The bacterial endotoxin test (BET) method was used to detect the endotoxin unit (EU) by the gel-clot technique limulus amebocyte lysate (LAL; Pyrogent Ultra; Lonza) [20,21] from the sample in different phases (MCB, WCB, and final cellular medicine on three different cultures). For the endotoxin assay in the final cellular medicine, cells are centrifuged and the resulting pellet is resuspended at concentration of 1×10^6 cells/mL with 1 mL of expansion medium. A 1/10 dilution of sample was necessary (100 µL of sample in 900 µL of water for BET); this dilution did not exceed the maximum valid dilution (MVD). Then, 100 µL of LAL and 100 µL of the diluted sample were mixed in a pyrogen-free tube (Lonza). Each sample also included a

negative control (100 µL of LAL and 100 µL water for BET), and two different positive controls (100 µL of LAL and 100 µL of 2λ/diluted test solution or 2λ/water for BET). Each dilution was assayed in duplicate. All tubes were incubated at 37°C for 1 h in a water bath (Grant Instruments Ltd., Cambridge, United Kingdom). Each tube was examined to observe the presence/absence of gelation. The storage conditions of the samples in this assay are not defined, so each laboratory should define and validate those conditions.

Storage stability testing. To determine sample storage conditions, a study was performed using a purified standard control of endotoxin from *Escherichia coli* (ATCC 12014; 10 ng/vial) with a potency of 4 EU/ng (Lonza). The samples were prepared adding 200 µL of standard control and 1800 µL of sample obtaining a solution of 0.8 EU/mL. About 2 mL of solution was stored at 4°C and −20°C for 6 months in duplicate, and analyzed at different times (24 h, 48 h, 7 days, 14 days, 28 days, 2 months, 3 months, 4 months, 5 months, and 6 months). For each assay the storage sample was vigorously vortexed for 15 min. Then, 100 µL of stored sample was mixed with 900 µL of water for BET obtaining a dilution of 1:10. Equally, a negative control was evaluated in each assay.

Microbiological monitoring in process

An environmental microbiological monitoring plan was carried out to control the air, surfaces, and staff in accordance with the European Union standards: EudraLex—Volume 4 GMP guidelines.

The environmental and staff monitoring were conducted during the manufacturing process and the sterility assay in work areas (laminar-flow cabin and isolator, respectively) with settle plates. Two media were used: Trypcase Soja Agar (TSA; bioMérieux) for detection of bacteria and Sabouraud Dextrose Chloramphenicol (SDC; bioMérieux) for fungi. The plates were incubated for 2 days at 35°C for TSA and 5 days at 22°C for SDC [22].

For environmental monitoring, two settle plates (TSA and SDC) were exposed during each handling near the activity for a maximum of 4 h. For staff monitoring, when work shift finished, the operator gloves' print (five fingers) was monitored in each media (TSA and SDC).

Analytical method validation

Analytical methods of sterility, mycoplasma, and endotoxin are "limit tests" analytical procedures, for this, an investigation of specificity; limit of detection (LOD) should be conducted during their validation according to ICH Q2 [23]. In the development phase of an analytical procedure, robustness should also be considered GMP [24].

The validation of sterility test was carried out in the intermediate product (cells with expansion medium, phase of MCB) and in the final cellular medicine (cells and packaging medium), each in triplicate (cultures 1, 2, and 3).

Validation detection of mycoplasma and endotoxin assay was carried out with three samples of intermediate product (phase of MCB) of each culture (1, 2, and 3).

Validation of sterility test. Validation was carried out to verify whether the antimicrobial activity of the intermediate product and final cellular medicine had been satisfactorily eliminated under the conditions of the test [15,16]. Specificity for sterility test was analyzed by absence of false positive results [25]; to determine LOD, a visual evaluation of turbidity was performed [15] and the robustness of these methods was studied by the reproducibility of the procedures.

The test was validated with respect to LOD accepted by Pharmacopoeia, with an inoculum not more than 100 CFU (BioBall SingleShot; bioMérieux). BioBall is an accredited reference material that contains a precise number of between 28 and 30 CFU with a standard deviation of 3 CFU [15]. The validation was based on the inoculation, in a laminar-flow cabinet BIOII-A (Telstar S.A., Madrid, Spain), of six different viable microorganisms, in each medium (TSB and TSPB) with 1 mL of supernatant or final suspension. The microorganisms used for the validation are indicated in Table 1.

All media were incubated for 3 days for bacteria (*Clostridium sporogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis*) and 5 days for fungi (*Aspegillus niger* and *Candida albicans*) at 35°C and 22°C for TPB and TSPB, respectively. For each microorganism a Gram stain was performed at the end of incubation to confirm microorganism identity as well as a growth promotion test as a positive control.

Validation of mycoplasma detection test. Demonstration of specificity requires the use of a suitable range of bacterial species other than mycoplasmas. Bacterial genera with close phylogenetic relation to mycoplasmas are most appropriate for this validation, for example, Lactobacillus [18]. To determine the LOD for mycoplasma assay, the performance and reproducibility of the analytical procedure was demonstrated with three reference strains known as *Mycoplasma orale* (NCTC 10112), *Acholeplasma laidlawii* (NCTC 10116), and *Mycoplasma fermentans* (NCTC 10117) [26]. The LOD studied was 10 CFU/mL for each strain [18]. Each sample (5×10^5 cells by 500 µL of supernatant) was inoculated by *M. orale*, *A. laidlawii*, and *M. fermentans* with a final concentration of 10 CFU/mL. All reference strains were obtained from European Directorate for the Quality of Medicines (EDQM). Briefly, in the inoculated samples, the mycoplasma assay was carried out eight times for each sample in the same PCR assay. This procedure was repeated three times on different days to analyze the variability and robustness [27].

Validation of endotoxin assay. To validate the analytical procedure used for endotoxin assay, two studies were conducted: confirmation of the labeled lysate sensitivity and test for interfering factors.

Specificity was demonstrated by the absence of interfering factors; for determination of LOD, a visual evaluation of the gelation was performed with a lysate sensitivity of 0.03 EU/mL and four replicates of each concentrations equivalent to 2λ, λ, 0.5λ, and 0.25λ by diluting the standard endotoxin with test solution and the robustness was studied by the reproducibility [20].

Confirmation of the labeled lysate sensitivity: This study was performed in three different batches of LAL (I, II, and III) to confirm the labeled lysate sensitivity (λ), which must be 0.03 EU/mL [17]. λ was the antilog_{10} of this mean log endpoint (the last positive result in the series of endotoxin decreasing concentration).

For each batch, four endotoxin standard dilutions were used (2λ, 1λ, 0.5λ, and 0.25λ). In a pyrogen-free tube with

100 μL of LAL, 100 μL of each standard dilution was added. Each dilution was assayed in quadruplicate. All tubes were incubated at 37°C for 1 h in water bath. Then, the presence/absence of gelation in each tube was observed, and the endpoints were determined. Each endpoint was converted to $\log_{10}$ and the mean and the antilog$_{10}$ were calculated.

Test for interfering factors: This study was performed in three samples of intermediate product (phase of MCB, WCB, and final cellular medicine on three different cultures) to check the presence of interfering factors. Test solution was used at a dilution 1:10 (less than the MVD). Four solutions were necessary, namely, A, B, C, and D. Solution A (four replicates of test solution) was the diluted sample in water for BET (1:10). Solution B (B consisted of four replicates of each concentrations equivalent to 2λ, λ, 0.5λ, and 0.25λ by diluting the standard endotoxin with test solution) was prepared concentrations of 2λ, 1λ, 0.5λ, and 0.25λ. 2λ was elaborated with 100 μL standard solution of 20λ and 900 μL of diluted sample. 1λ was manufactured with 100 μL solution of 2λ and 100 μL of diluted sample. 0.5λ was made up with 100 μL solution of λ and 100 μL of diluted sample. 0.25λ was produced with 100 μL solution of 0.5λ and 100 μL of diluted sample. Solution C (2 replicates of each concentrations equivalent to 2λ, λ, 0.5λ, and 0.25λ by diluting the standard endotoxin with water for BET) was standard concentrations of 2λ, 1λ, 0.5λ, and 0.25λ. Solution D (two replicates of water for BET) was the water for BET.

In a pyrogen-free tube, 100 μL of each solution (A, B, C, and D) was added with 100 μL of LAL. All tubes were incubated at 37°C for 1 h in water bath. Solutions A and B were assayed in quadruplicate and solutions C and D in duplicate. Then, the presence/absence of gelation was observed.

Results

Isolation and culture of MSCs

Three different cultures (named 1, 2, and 3) of MSCs from human adipose tissue were analyzed according to the QCP proposed (as shown in Fig. 1) for the manufacturing of a cellular medicine under GMP conditions. Three samples of adipose tissue were processed to carry out the ex vivo expansion. The donor of the biological sample both autologous and allogeneic should be studied from the point of view serological, to know whether there is any infectious diseases that can cause quality problems and conservation for ex vivo expansion [28]. For each biological tissue sample, a serological report was required. The results of the three samples were negative for immunoglobulin M (IgM) A hepatitis, surface B antigen, IgG C hepatitis, and HIV, and positive for anti-HBc and anti-HBs. All materials and reagents necessary for the elaboration of the hMSCs were controlled, their sterility was analyzed, and all results were negative; no microbiological growth was shown in any media (TSB and TSPB). Also, the endotoxin assay was performed and the results demonstrated that all materials and reagents were endotoxin free.

For ex vivo expansion, different critical points have been defined: MCB, WCB, and final cellular medicine; in each point, the analytic techniques required to ensure the microbiological quality of the culture were proposed. Throughout the process a qualitative and quantitative environmental analyses of microbiological quality of air and surfaces were carried out; the results were within the standards required by the GMP guidelines as previously described [22].

Sterility test and Gram stain

Before carrying out the sterility test of samples, the effectiveness of two isolation media (TSB and TSPB) was demonstrated by growth promotion test with reference strains (*C. sporogenes*, *P. aeruginosa*, *S. aureus*, *A. niger*, *B. subtilis*, and *C. albicans*). Turbidity was observed in all media. On the other hand, sterility test of each medium was also satisfactory.

After verification of the culture media, they were inoculated with the test sample of the different phases and, after 14 days of incubation, none showed turbidity, so there was no evidence of microbial growth and the sterility of the cells was accepted for the three cultures. All negative control samples with NaCl were negative after the required incubation period.

From the results of Gram stains, no CFU was observed, in none of the three final cellular medicines studied.

Detection of mycoplasma

The presence of mycoplasma in each sample was analyzed by DNA amplification and the presence of 267-bp amplicon; none of the samples of the different phases showed presence of this band (data not shown). A successful PCR was performed without polymerase inhibition. Internal control DNA was demonstrated by a 191-bp band in the gel, which verified extraction, reverse transcription, amplification, and detection that were carried out since the internal control was added to the sample before isolating the nucleic acid.

Endotoxin assay

The threshold of endotoxin was calculated using the formula endotoxin limit (EL) = K/M [20]. Where K is the threshold pyrogenic dose of endotoxin per kilogram of body mass in 1-h period and M is the maximum recommended dose of the product per kilogram of body mass in 1-h period. For intravenous administration, the suggested value of K by the EP is 5.0 EU/kg·h and M was 1 mL/kg·h for the final cellular medicine manufactured in our laboratory, thus

$$\mathrm{EU} = [5.0\ \mathrm{EU/kg \cdot h}]/[1\ \mathrm{mL/kg \cdot h}];\ \mathrm{EU} = 5.0\ \mathrm{EU/mL}$$

The threshold of endotoxin was defined for each sample as 5.0 EU/mL. LAL used for the endotoxin assay had a sensitivity of 0.03 EU/mL, which is lower than EL calculated in-house, 5.0 EU/mL, thereby, preventing false negatives. For the preparation of sample, MVD was calculated according to the formula:

$$\mathrm{MVD} = \mathrm{EL}/\lambda = (5\ \mathrm{EU/mL}/0.03\ \mathrm{EU/mL}) = 166.7$$

The sample could be diluted 166.7 times; in our method, the selected dilution was 1:10, far below the permitted MVD. In each phase for ex vivo expansion, diluted samples were tested and including negative control no sample showed gelation except the two positive controls.

In addition, a study of storage stability was performed; the concentration selected for this study (0.08 EU/mL) was based on the preparation of a concentration similar to positive control. The positive control was calculated according to the formula

$$2\lambda = 2 \times 0.03 \text{ EU/mL}$$

The positive control was 0.06 EU/mL. All samples showed gelation except the negative control samples for the duration of the study (6 months) at 4°C and −20°C. The gelation demonstrated the presence of endotoxin from *E. coli*. And therefore, the storage conditions of a sample in a QCP defined for endotoxin assay were at 4°C or −20°C for a maximum of 6 months.

Microbiological monitoring in process

Results were interpreted according to the annex I-GMP (Grade A: <1 CFU/4 h and <1 CFU/glove, for environmental and staff monitoring, respectively) [29].

The effectiveness of the aseptic procedures has to be continuously evaluated in order to guarantee the safety of the medicine and to identify foci of contamination risk. The results obtained for environmental and staff monitoring during the ex vivo expansion of the three cultures were within the recommended specifications for microbial contamination in TSA and SDC.

Validation of the analytical method

Analytical methods described in pharmacopoeia monographs are considered validated; however, the laboratory must also confirm the in-house procedure with the samples for study in each technique initially, ensuring that there is no interference.

Validation of sterility test. No antimicrobial activity was observed in the three cultures studied of any of the microorganisms tested. The media inoculated (with the supernatant and the final cellular medicine) and the microorganisms presented a similar growth as in the promotion test, proving that the antibiotics present in the intermediate product or the excipients of the final suspension do not interfere with the results of the sterility test. Gram stains of these media inoculated evidenced the microorganisms added.

In the study of cell culture sterility, the presence of antibiotic in the expansion medium as microbial growth inhibitory substance must be taken into account. The antibiotic concentration in 1 mL of supernatant was 10,000 IU/mL of penicillin and 10 mg/mL of streptomycin, and was inactivated with 10^3 IU/mL of penicillinase contained in the medium (TPB and TSPB).

Absence of false positive results confirmed the specificity of the test. The LOD was shown because all the microorganisms were grown at a concentration of 30 CFU/125 mL. The robustness was demonstrated satisfactorily by three replicate testing.

Validation of mycoplasma detection. The studied specificity by the kit manufacturer did not detect any of the phylogenetically related microorganisms: *Clostridium*, *Lactobacillus*, and *Streptococcus*.

To evaluate LOD, *M. orale* was selected in terms of the most possible source of contamination along with *A. laidlawii* and *M. fermentans*. The Fig. 2 shows the results to *M. orale* (Fig. 2). The required LOD of 10 CFU/mL was reached in all conditions ($n=24$ results: eight test replicates for each of the three samples with each strain) using different combinations of different reagents and reagent lots at different working dates by different analysts. On different days and with different samples, the same results were obtained; the three strains of tested mycoplasma were detected with equal certainty. The results presented demonstrate the robustness of mycoplasma PCR-based test. ◀F2

Validation of endotoxin assay. Confirmation of labeled lysate sensitivity: Three batches of Pyrogent Ultra Gel Clot with labeled sensitivity of 0.03 EU/mL were evaluated with standard solutions. The standard solution potency had been previously established using the current FDA Reference Standard Endotoxin. The results of these studies are described in Table 2. The results from this study confirmed the ◀T2

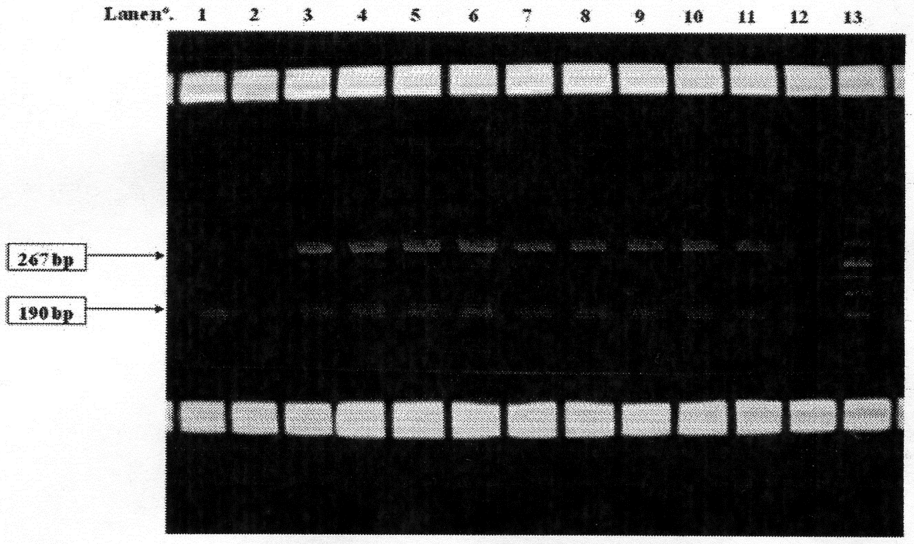

FIG. 2. Detection of PCR products by gel electrophoresis of 1 mL sample with 10 CFU *Mycoplasma orale*. Lane 1: negative control; Lanes 3–10: replicates 1–8 (10 CFU/mL); Lane 11: positive control; Lane 13: size marker 50 bp. The presence of mycoplasma in the sample was indicated by an amplification product at ~267 bp. A successfully performed PCR without polymerase inhibition was indicated by a 191-bp band (internal control DNA). Three individual sample batches were tested with eight replicates for each one. On different days the same results were obtained; *M. orale* was detected with equal certainty. The detection limit (10 CFU/mL) was confirmed.

TABLE 2. RESULTS OF CALCULATED LYSATE SENSITIVITY (LAL)

	Endotoxin standard dilution (EU/mL)							
Batch of LAL	*0.06*	*0.03*	*0.015*	*0.0075*	*Endpoint (EU/mL)*	*Log_{10} endpoint*	*Mean*	*$Antilog_{10}$ mean*
I	+	+	−	−	0.03	−1.52	−1.29	0.05
	+	−	−	−	0.06	−1.22		
	+	−	−	−	0.06	−1.22		
	+	−	−	−	0.06	−1.22		
II	+	+	−	−	0.03	−1.52	−1.37	0.04
	+	+	−	−	0.03	−1.52		
	+	−	−	−	0.06	−1.22		
	+	−	−	−	0.06	−1.22		
III	+	−	−	−	0.06	−1.22	−1.29	0.05
	+	+	−	−	0.03	−1.52		
	+	−	−	−	0.06	−1.22		
	+	−	−	−	0.06	−1.22		

λ. The sensitivities measured were not <0.5λ (0.015 EU/mL) and not more than 2λ (0.06 EU/mL).

Test for interfering factors: The results of the assay are shown in Table 3. Solution A was negative for three samples, which indicated no detectable endotoxin. The sensitivity was determined with solution B in the three cultures, which was not <0.5λ (0.015 EU/mL) and not >2λ (0.06 EU/mL). The test solution did not contain interfering factors under the experimental conditions used. The sensitivity of the LAL was confirmed with solution C, and the negative control (solution D) was confirmed.

For endotoxin test absence of interfering factors validated the specificity. The LOD was confirmed for an amebocyte lysate sensitivity of 0.03 EU/mL and the robustness was demonstrated by three times satisfactory testing. The three analytical procedures (sterility, mycoplasma, and endotoxin) satisfied the required characteristics for a limit test: specificity, LOD, and robustness.

Discussion

The quality of any pharmaceutical product, including biological medicines as a CTMP, is established by the European Medicine Agency guidelines in conjunction with GMP guidelines and general chapters of the EP [30–32]. In addition, each laboratory should establish a quality system tailored to its own process and the characteristic of the cellular medicine it is producing.

Medicines are generally subjected to end-product batch testing as a means of quality control; in the case of a biologic medicine using stem cell, for clinical use, this control must be amplified due to limitations inherent in the cells, such as viability, genetic stability related to extended culture time, and

TABLE 3. TEST RESULTS OF INTERFERING FACTORS

Studied cultures (phase MCB)	*Solutions*	*Endotoxin dilution (EU/mL)*				*Endpoint (EU/mL)*	*Log_{10} endpoint*	*Mean*	*$Antilog_{10}$*
		0.06	*0.03*	*0.015*	*0.0075*				
1	B	+	+	−	−	0.03	−1.52	−1.37	0.04
		+	+	−	−	0.03	−1.52		
		+	−	−	−	0.06	−1.22		
		+	−	−	−	0.06	−1.22		
	C	+	+	−	−	0.03	−1.52	−1.37	0.04
		+	−	−	−	0.06	−1.22		
2	B	+	+	−	−	0.03	−1.52	−1.29	0.05
		+	−	−	−	0.06	−1.22		
		+	−	−	−	0.06	−1.22		
		+	−	−	−	0.06	−1.22		
	C	+	+	−	−	0.03	−1.52	−1.52	0.03
		+	+	−	−	0.03	−1.52		
3	B	+	+	−	−	0.03	−1.52	−1.29	0.05
		+	−	−	−	0.06	−1.22		
		+	−	−	−	0.06	−1.22		
		+	−	−	−	0.06	−1.22		
	C	+	+	−	−	0.03	−1.52	−1.37	0.04
		+	−	−	−	0.06	−1.22		

Solution A, solution of the preparation being examined that is free of detectable endotoxins; Solution B, test for interference; Solution C, control of the labeled lysate sensitivity; Solution D, negative control (water for bacterial endotoxin test).

microbiological contamination [33,34]. Microbiological contamination is one of the major risks associated with the administration of a CTMP. Therefore, it will be necessary to establish minimum standards of microbiological quality during the manufacture of these medicines through a QCP as proposed in this article, encompassing microbiological analysis of biological sample, materials, reagents, intermediate, and final products, as well as the environmental microbiological quality of air, surfaces, and staff [35]. Regarding all the reagents used for clinical-scale manufacturing of MSCs, they should be clinical grade. The use of reagents of animal origin must be justified and approved by the appropriate regulatory agencies. In the case of FBS, it is one of the most widely used reagents in the expansion of MSCs as cell culture supplement but the FBS presents a high variability between different batches as well as risk of contamination. As alternatives to this supplement, human factors from serum, plasma, or platelet derivatives have been suggested [36,37]. All of them must be included in the QCP, with stringent criteria of donor eligibility as well as sensitive viral NAT testing and serology, ensuring safety of the starting material for MSC manufacturing [37].

In this study, our results showed no microbiological contamination in any of the phases of cultures studied according to the QCP. In each of the three cultures studied, quality of adipose tissue sample was analyzed based on its serology and the existence of cellular pathogens and exogenous contaminants after the process of ex vivo expansion. Further, all starting materials and reagents involved in the expansion process were tested for sterility and endotoxin and their traceability was followed [34, 38].

A CTMP must be viable for its administration, besides being sterile [39, 40]. So that the manufacture of these medicines should be carried out under aseptic conditions, in a clean room and under GMP conditions, preventing contamination of the medicine and ensuring its quality, which requires identifying and controlling the critical aspects of ex vivo expansion [41].

A sterility test may be defined as a critical; the traditional method described in EP [15, 41] for the sterility test takes 14 days, although other rapid methods have been published and supported in the last annex II of the GMP [42], which entail 7 days [43], the "shelf-lives" of cells do not exceed 48 h. Therefore, the final cell medicine must be released parametrically [44], being necessary to provide all additional quality controls described in QCP to ensure that the medicine is free of contaminating microorganisms at the time of release [45]. Through sterility tests prior to release, in the intermediate phases (MCB and WCB) and on the starting material and reagents, besides endotoxin and mycoplasma assays, Gram stain and management of the monitorization process in the final cellular medicine, the absence of viable and active microorganisms can be ensured [6].

That sterility validation must demonstrate that the presence of antibiotics does not interfere with the results of sterility test. On the other hand, the use of antibiotics should be controlled to avoid resistances in patients after infusion of final cellular medicine, so the cellular pool should be washed with PBS three or four times, eliminating any trace of antibiotics.

Regarding the contamination risk of a biologic medicine, mycoplasma contamination is a major problem [46,47]. It can produce a myriad of different effects with a dramatic alteration of biological characteristics of the contaminated cells: alteration of proliferation characteristics, immune reactions, viruses' proliferation, chromosomal aberrations, and more besides. These organisms are resistant to most antibiotics commonly employed in cell cultures [16]; therefore, it is essential to analyze both the intermediate products and the final medicine in the development of a CTMP to control disturbances not only to the cells, but also to the inhibition of cell growth and hence the difficulty to obtain the final dose. Although the incidence of mycoplasma infection in the cultures is low [48], in most cases it has a human origin, making staff the major source of contamination, particularly *M. orale*. Accordingly, staff should receive regular training for the aseptic manipulation of therapeutics [22]. Also, both animal serum products and environment are possible sources of contamination [49].

Endotoxin assay is another requirement for parenteral medicines. The endotoxin effects are different depending on the cell types. It can induce contractile dysfunction, and increased production of immunoglobulin light chains. Equally, the absence of bacterial endotoxins in a product implies the absence of pyrogenic components.

We performed three successful validation runs to demonstrate the sensitivity, LOD, and robustness of the method through the analytical technique validation [50]. The validation parameters of different assays involved in the proposed QCP (sterility, mycoplasma, and endotoxin) demonstrated that the reagents used in each analytical technique were free of inhibitory factors and verified the suitability of each assay [51], based on the presence or absence of a detected analyte [52] by turbidity appearance, gelation after incubation, and/or detection of mycoplasma as band from PCR amplification.

In conclusion, this work proposes a control plan to analyze the safety of hMSCs as cellular medicine for clinical use, based on the evaluation of bacteriological agent contamination throughout the manufacturing process.

The efficiency of the proposed QCP in this article, carried out during the ex vivo expansion of three different cultures of hMSCs for the elaboration of autologous cell medicine under GMP conditions, has been premised on the standardization and validation in situ of each analytical technique, to ensure that the products are manufactured in a reliable and safe manner. Minimum controls of microbiological quality of the cells to be administered parenterally to patients have been established, encompassing both control samples and starting, intermediate, and final materials or products. Based on the QCP proposed, it was observed that the contamination risk could be prevented.

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Author Disclosure Statement

No competing financial interests exist.

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III.6. STUDY OF THE STABILITY OF PACKAGING AND STORAGE CONDITIONS OF HUMAN MESENCHYMAL STEM CELL FOR INTRA-ARTERIAL CLINICAL APPLICATION IN PATIENT WITH CRITICAL LIMB ISCHEMIA

El estudio de células madre en la terapéutica actual implica profundizar en el diseño de nuevas formas de dosificación en las que el principio activo sea sustituido por células viables. Por tanto es fundamental el diseño y estudio de formulaciones del medicamento celular, tanto desde el punto de vista tecnológico, físico-químico y biológico con el objeto de permitir, facilitar y/o mejorar la supervivencia de la célula en su acondicionamiento final hasta ser administrada.

En este capítulo se estudiaron diferentes excipientes para el diseño de la suspensión celular final, seleccionando aquel que mantenía las células viables durante más tiempo (48 h). El CTMP elaborado con hMSCs junto con los excipientes seleccionados fue sometido a un estudio de estabilidad térmica determinando que el producto final debe mantenerse a 4 ºC.

Una vez definida la formulación y las condiciones de almacenamiento y envasado, se realizó una caracterización biológica, morfológica, microbiológica, inmunofenotípica, genética y físico-química de las hMSCs antes de su envasado y una vez trascurridas 48 h, verificando que ninguna de las características inherentes a la célula había sido alterada. Asimismo se demostró la idoneidad de este medicamento para su administración intra-arterial.

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Research paper

Study of the stability of packaging and storage conditions of human mesenchymal stem cell for intra-arterial clinical application in patient with critical limb ischemia

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ABSTRACT

Critical limb ischemia (CLI) is associated with significant morbidity and mortality. In this study, we developed and characterized an intra-arterial cell suspension containing human mesenchymal stem cells (hMSCs) for the treatment of CLI. Equally, the stability of cells was studied in order to evaluate the optimal conditions of storage that guarantee the viability from cell processing to the administration phase. Effects of various factors, including excipients, storage temperature and time were evaluated to analyze the survival of hMSCs in the finished medicinal product. The viability of hMSCs in different packaging media was studied for 60 h at 4 °C. The best medium to maintain hMSCs viability was then selected to test storage conditions (4, 8, 25 and 37 °C; 60 h). The results showed that at 4 °C the viability was maintained above 80% for 48 h, at 8 °C decreased slightly, whereas at room temperature and 37 °C decreased drastically. Its biocompatibility was assessed by cell morphology and cell viability assays. During stability study, the stored cells did not show any change in their phenotypic or genotypic characteristics and physicochemical properties remained constant, the ability to differentiate into adipocytes and osteocytes and sterility requirements were also unaltered. Finally, our paper proposes a packing media composed of albumin 20%, glucose 5% and Ringer's lactate at a concentration of 1×10^6 cells/mL, which must be stored at 4 °C as the most suitable to maintain cell viability (>80%) and without altering their characteristics for more than 48 h.

1. Introduction

The recent advances of biomedical research and biotechnologies have opened new promising therapeutic strategies, and human mesenchymal stem cells (hMSCs) are attracting increasing interest for potential application in cell therapies for the treatment of several human diseases [1,2]. hMSCs offer considerable therapeutic potential through the development of different cell therapy medical products (CTMP) for clinical use [3], due to their regenerative and immunoregulatory capacities [4,5], which have made them one of the most promising candidates for cell therapy success including regenerative and immune therapies where other current conventional treatments are inadequate.

Critical limb ischemia (CLI) is one of the diseases most studied in the field of cell therapy, in particular in diabetic patients, CLI of the leg develops earlier and more intensely, avoiding revascularization [6,7]. CLI is a syndrome manifested by ischemic rest pain, non-healing ulcers, tissue loss and gangrene. The incidence of CLI is estimated to be approximately from 500 to 1000 patients per million and year [8]. Diabetic patients with CLI are at acute risk of amputation, which leads to a low quality of life, and severe morbidity and mortality, resulting in a significant social and economic impact [9].

Currently, the latest advances in this pathology have led to the development of new medicines with stem cells as an alternative to surgical and pharmacological treatment. hMSCs transplantation is one of the most studied therapeutic alternatives in preclinical and clinical stages, due to its paracrine, immunomodulatory, and differentiation effects [8,10,11].

hMSCs encompass a broad range of anchorage dependent fibroblast-like cells which can be obtained from bone marrow aspirates,

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skeletal muscle connective tissue, human trabecular bones, adipose tissue, periosteum, fetal blood and liver, and umbilical cord blood [12]. These cells are characterized by being able to adhere to plastic. They can proliferate *ex vivo* and exhibit multilineage differentiation capacity being capable to give rise to diverse cells like osteoblasts, chondrocytes, adipocytes, myocytes, tenocytes and vascular cells and to express several common cell surface antigens [13].

Adipose tissue-derived stem cells have emerged as a new and promising type of stem cells because adipose tissue is an abundant source of stem cells, it lacks donor limitation and it is possible to obtain by a minimally invasive method [14].

Conducting cellular therapeutics is a complex undertaking, and both safety and efficacy measures shall be considered in the establishment of the manufacturing process of a finished medicinal product for cell therapy [15] among all steps involved, research phase, translational phase for scaling-up the protocol for the clinical requirements, establishment of standard operation procedures (SOP), validation runs, regulatory registration, storage and transportation [16]. The use of hMSCs for clinical application requires a high number of cells, which entails the *ex vivo* expansion in a certified laboratory under good manufacturing practice (GMP) conditions [17]. After culturing and having obtained the necessary number of cells, hMSCs must be loaded in a suitable dosage form for their administration. hMSCs can be formulated into liquid and semisolid dosage forms. Current methods of cell delivery involve the use of injections and microencapsulation. The use of several biomaterials for microencapsulation results in an impenetrable membrane to cells, and requires strong mechanical disturbances such as pressurized nozzles, emulsification, or stirring during droplet generation leading to cell degradation [18]. A cell suspension can be parentally administered directly in the damaged organ/tissue whilst offering medical devices use possibility. Attempts of CLI treatment with intra-arterial hMSCs have been associated with significant therapeutics benefits [10].

On the other hand, the administration of the cells to the patient is not. Following the obtention of cells, formulation of the finished medicinal product is carried out, all quality checks should be performed before approving its release, hence cells must be stored and transported in the best conditions to maintain stability [19]. The finished medicinal product of a CTMP includes an active ingredient (hMSCs) and the selected excipients (packaging medium), which are different from the expansion media. The cell stability of the finished medicinal product is determinant for its therapeutic applications in clinical use since baseline characteristics of cells should be maintained [15].

The stability information should include biological (sterility including mycoplasma, endotoxin and adventitious viral agents, identity, purity and potency) and physicochemical tests including those related to the design of the dosage form such as cell sedimentation rate and resuspension [20]. Alternatively, these products are likely to have a short shelf-life, which often means that these products are administered to patients before current sterility test results are available [17]. Due to cells are highly fragile and sensitive to their surrounding environment, and in order to maintain their quality, their environment needs to be strictly controlled during the time gap between cell harvesting and administration. Therefore, for the formulation of a cell suspension, important key factors must be taken into account, such as selection of the excipients of the packing medium, which must be protein-free to avoid inflammatory responses affecting the efficacy and safety [21]. Temperature and time conditions of storage as well as transport should be also studied.

Even though characterization of hMSCs has been extensively studied for their *ex vivo* expansion, there are not data dealing with the cell characterization in the finished medicinal product. The present work studies the stability of MSCs from human adipose tissue elaborated in a cell suspension for intra-arterial application.

The choice of the packing medium and storage conditions for a hMSCs suspension have been studied through stability studies, with the purpose of formulating a finished medicinal product that assured the maintenance of the characteristics more similar as possible to those of native hMSCs. hMSCs have been characterized before and after formulation, studying cell viability, immunophenotypic and genotypic characterization, differentiation, microbiology and physicochemical properties.

2. Materials and methods

Clinical application of hMSCs requires a concentration approximately between 1 and 2×10^6 cells/kg [22], therefore hMSCs should be expanded in long-term culture that can affect their characteristics [23,24], specially their immunogenicity or a lack of safety of the medium components, resulting in chromosomal aberrations [25–28].

For intra-arterial administration of hMSCs the formulation of a suitable and safe finished medicinal product is a need absolutely vital. The storage condition study is a key factor to assure the viability of cells and hold their properties in the moment of administration [29–31]. For this reason, different excipients at different temperatures were studied in the finished medicinal product.

2.1. GMP environment

This study was performed in the context of a clinical trial, phase I/II under GMP conditions. All procedures were performed in a certified clean room at the Center for Molecular Biology and Regenerative Medicine (CABIMER), it was the first laboratory accredited by the Spanish Agency for Medicines and Medical Devices for production of stem cells as medicines in Andalucía (Spain).

All starting materials and reagents required for this study were according to GMP guidelines. All equipment was validated.

2.2. Human mesenchymal stem cell isolation and culture from adipose tissue

The donor source of the hMSCs was appropriately screened and tested for human pathogens. Procedures were performed at the San Lazaro Hospital (Spain). The therapeutic protocol was approved by the hospital ethics committee in accordance with Spanish law. All patients signed a detailed informed consent form before intervention and gave their consent for publication of the study results. This study was conducted in accordance with the ethical standards of the Helsinki Declaration (1975). In particular, the presence of Human Immunodeficiency Virus (HIV), hepatitis B and hepatitis C virus was analyzed. On the other hand, all starting materials and reagents required for the expansion were analyzed to certify that they were sterile and endotoxin-free.

Autologous hMSCs were isolated from adipose tissue by enzymatic digestion with collagenase (2 mg collagenase/1 g adipose tissue) (Roche Farma, Reinach, BL, Switzerland). Briefly, the sample was centrifuged at 400*g* for 10 min, filtered and washed with Phosphate Buffered Saline (PBS) (Sigma–Aldrich, St. Louis, MO, USA). The isolated cells were suspended and plated at medium density (passage 0) of $12–20 \times 10^4$ cells/cm^2 in culture flasks (Nunc, Roskilde, Denmark) with expansion medium composed by Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 1% of 10.000 IU/mL Penicillin, 10 mg/mL Streptomycin, 2% of 200 mM L-alanine solution and 1% of 200 mM L-glutamine, (all from Sigma–Aldrich, St. Louis, MO, USA). After 24 h non-adherent cells were removed by replacing

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the expansion medium. Cells were harvested upon reaching 80% confluence, and subcultured using 0.25% trypsin (Gibco, Invitrogen, Grand Island, NY, USA) on expansion medium, and plated at medium density of 3500–5500 cells/cm^2. Cells were cultured in a 95% relative humidity, incubator at 37 °C in 5% CO_2. The medium was completely replaced every 2 or 3 days a week.

2.3. Cell viability and counting assay

This study was conducted before and after the preparation of the finished medicinal product, in the supernatant and in each packaging media.

Cell viability was determined by trypan blue dye exclusion staining [32] and posterior cell counting in a Neubauer chamber. Each sample was counted three times and the average was calculated. The percentage of viability was calculated using the following formula:

$$\text{Cell viability}(\%) = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100 \quad (1)$$

2.4. Assessment of stability

2.4.1. Influence of the packaging medium

hMSCs from passage 4 were used. Cells were harvested using 0.25% trypsin (Gibco, Invitrogen, Grand Island, NY, USA) and centrifuged at 400*g* for 10 min with expansion medium. The cell pellet was resuspended at concentration of 1×10^6 cells/mL with 1 mL of packaging medium. For this study, different excipients (Grifols, Barcelona, Spain) were selected to analyze the hMSCs viability during the storage period of the finished cell medicinal product. Four packaging media were prepared, the excipients for 50 mL of each such media is reported in Table 1.

hMSCs were packed in 10 mL-Luer-lock syringes (Becton Dickinson and Company, Franklin Lakes, NJ, USA). 11 syringes for each medium were prepared and stored at 4 °C in a normal atmosphere. Every 6 h for 60 h, cell viability was tested.

2.4.2. Influence of the temperature of storage

hMSCs were packed in twenty-four 10 mL-Luer-lock syringes (Becton Dickinson and Company, Franklin Lakes, NJ, USA) at a concentration of 1×10^6 cells/mL with 1 mL of the most stable packing medium after the study described above. Samples were stored at different temperatures: 4 °C, 8 °C, 25 °C and 37 °C in normal atmosphere (6 syringes each temperature). Cell viability was tested every 12 h for 60 h.

2.5. hMSCs immunophenotypic characterization

At passage 4 and after stability study of the finished medicinal product, immunophenotyping study of hMSCs was performed in order to identify the presence of specific surface antigens. Between 2.5 and 5×10^5 cells were separated in 1.3 mL expansion medium. The following markers were analyzed: CD13-PE, CD29-PE, CD90-FITC, CD105-PE, CD31-FITC, CD34-PE, CD45-FITC and HLA-II-FITC (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Mouse antibodies served as control: Isotype-FITC IgG1-k, Isotype-FITC IgG2a-k, Isotype-PE IgG1-k (all from Becton Dickinson and Company, Franklin Lakes, NJ, USA). 100 μL of cell suspension were prepared with 5 μL of each of the following reagents: fluorescein isothiocyanate (FITC), phycoerythrin (PE) antibody and control, and incubated at 4 °C for 20 min in the dark. Then, 3 mL of PBS (Sigma–Aldrich, St. Louis, MO, USA) were added to each cell suspension and centrifuged at 400*g* for 10 min. Finally each cell pellet was diluted in 300 μL of PBS and 5000 labeled cells were acquired and analyzed using a FACSCalibur analyzer flow cytometer system running CellQuest Pro software (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the percentage of viable cells positive for each marker were determined.

2.6. Ex vivo osteogenic and adipogenic differentiation

The differentiation of hMSCs into osteoblasts and adipocytes was analyzed on passage 4 and after stability study of the finished medicinal product. For osteogenic and adipogenic induction, cells were seeded at 16×10^4 cells/plate in two 35 mm plastic Petri dishes (Nunc, Roskilde, Denmark) and cultured for 1 week with expansion medium. Briefly, the medium was removed and Osteogenic Differentiation Medium and Adipogenic Induction Medium (Lonza, Walkersville, MD, USA) were added in each plate respectively [22]. For adipogenic differentiation, 2 weeks after the Adipogenic Induction Medium was changed by Adipogenic Maintenance Medium (Lonza, Walkersville, MD, USA). New medium was replenished every 3 to 4 days. After 21 days cells were fixed in 4% paraformaldehyde (VWR International, Radnor, PA, USA) for 5–10 min at room temperature and stained with Oil Red-O adipocytes (Sigma–Aldrich, St. Louis, MO, USA) and Alizarin Red S sodium salt osteoblast (Alfa Aesar, Ward Hill, MA, USA). Images were captured using a microscope (Olympus 1×71, Tokyo, Japan) and a CCD camera (Olympus DP70).

2.7. hMSCs karyotyping analysis

The hMSCs were analyzed before and after the stability study. Karyotype analysis was performed by G band techniques [33]. In order to obtain chromosomal preparations the hMSCs were treated with 0.8 μg/mL colchicines (KaryoMAX®-Colcemid™; Gibco, Invitrogen, Grand Island, NY, USA) and incubated at 37 °C for 1.15 h. Briefly, cells were washed with 1 mL trypsin (Gibco, Invitrogen, Grand Island, NY, USA) twice. Then, 2 mL trypsin was added at 37 °C for 2 min and centrifuged at 400*g* for 10 min with 1 mL FBS. The pellet was suspended in 5 mL 75 mM KCl (Merck, Darmstadt, Germany) at 37 °C for 20 min and centrifuged at 400*g* for 10 min. The cells were fixed with 5 mL methyl alcohol–acetic acid mixture (3:1 v/v) (Merck, Darmstadt, Germany) and centrifuged at 400*g* for 10 min, this process was repeated twice more. For each karyotype 30 metaphases were analyzed. The final result was described to account the recommendations from the International System for Human Cytogenetic Nomenclature [34]. Chromosomal aberrations were considered when at least two metaphases showed the same alteration (additions, deletions, inversions and translocations).

Table 1
Composition (mL) of different packaging media (50 mL final volume).

Excipients	Lactated Ringer's solution	Glucose 5%	Albumin 20%	Sodium Chloride 0.9%
Medium 1	22.5	2522.5	2.5	–
Medium 2	47.5	2.5	–	–
Medium 3	50	–	–	–
Medium 4	–	–	–	50

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2.8. Microbiological studies

Microbiological analysis was based on the study of the sterility of the cells before being packaged and sterility of the finished medicinal product after stability study. The test was carried out as described in the European Pharmacopoeia [35], by direct inoculation of 1 mL of sample (supernatant of the cells in the last passage and the final cell suspension with the selected packaging medium) in the microbiological medium to test for the growth of yeast, fungi, aerobic, and anaerobic bacteria. Two microbiological media were used: Thioglycollate Penase Broth 9 mL (TPB), to detect anaerobic and aerobic bacteria and Tryptic Soy Penase Broth 9 mL (TSPB) (VWR International, Radnor, PA, USA), which it is a soybean casein digest medium to detect fungi and aerobic bacteria. For each media (TPB and TSPB), sterility test and growth promotion test of aerobes, anaerobes and fungi were previously verified. The inoculated media were incubated for 14 days at 35 °C and 22 °C for TPB and TSPB respectively. After 14 days, if there had been microbial growth, the medium would have shown turbidity. Negative controls were established by inoculating 1 mL of 0.9% sterile NaCl (bioMérieux, Marcy l'Etoile, France) in duplicate for each medium. The inoculated media were incubated for 14 days at 35 °C and 22 °C for TPB and TSPB respectively. After 14 days, if there had been microbial growth, the medium would have shown turbidity. This assay was performed in aseptic conditions with an isolator HPI-4PI-S (Esco Technologies, Inc., Hatboro, PA, USA).

2.9. Physicochemical characterization of the finished medicinal product

2.9.1. Rheological studies

The rheological characterization was conducted in order to evaluate changes induced by cells. Properties of the formulations such as viscosity, a parameter closely related with physical and structural stability were studied, allowing us to estimate the behavior of the formulations.

The rheological characterization of the formulations was performed at 25 °C using a rotational rheometer HAAKE Rheostress 1 (Thermo Fisher Scientific, Karlsruhe, Germany) equipped with a parallel plate geometry set-up with a fixed lower plate and an upper plate (Haake PP60 Ti, 6 cm diameter). Different gaps between plates were tested and a separation of 0.105 mm was selected. The rheometer was connected to a computer provided with the software HAAKE Rheowin® Job Manager V. 3.3 to carry out the test and Rheowin® Data Manager V. 3.3 (Thermo Electron Corporation, Karlsruhe, Germany) to carry out the analysis of the obtained data. Viscosity curves and flow curves were recorded for 1 min during the ramp-up period from 0 to 100 s^{-1}, 1 min at 100 s^{-1} (constant share rate period) and finally 3 min during the ramp-down period from 100 to 0 s^{-1}. All determinations were performed in triplicate.

Data from the flow curve (when resulted to be non-Newtonian) were fitted to different mathematical models using the Prism® V. 3.00 software (GraphPad Software Inc., San Diego, CA, USA). Eqs. (2)–(6) correspond to the fitting models respectively [36]:

$$\tau = \tau_0 + \eta_p \times \gamma \quad \text{Bingham} \tag{2}$$

$$\tau = k \times (\gamma)^n \quad \text{Ostwald—De-Waele} \tag{3}$$

$$\tau = \tau_0 + k_1 \times (\gamma)^n \quad \text{Herschel—Bulkley} \tag{4}$$

$$\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{k1 \times \gamma} \quad \text{Casson} \tag{5}$$

$$\frac{\eta - \eta}{\eta_0 - \eta_\infty} = \frac{1}{(1 + (\lambda\gamma)^m)} \quad \text{Cross} \tag{6}$$

where τ is the shear stress (Pa), η is the viscosity (Pa s), γ is the shear rate (1/s), τ_0 is the yield shear stress (Pa), η_p is the consistency index (Pa s), k is the consistency (s), λ is the time constant calculated from the point on the viscosity versus shear rate curve where the flow changes from the lower Newtonian region to the Power law region, η_0 and η_∞ are asymptotic values of viscosity at zero and infinite (very low and very high shear stress), respectively, m is a dimensionless exponent with a typical range from 2/3 to 1, and n is the flow index, the different values of n indicate the fluid behavior. For a Newtonian fluid, $n = 1$. If $n < 1$, the fluid is called pseudoplastic; if $n > 1$, the fluid is dilatants [37].

2.9.2. Morphological analysis

Morphological characteristics of hMSCs in each passage and after stability study were observed by a microscope (Olympus 1 × 71, Tokyo, Japan), and captured by a CCD camera (Olympus DP70). Also cell suspension of finished medicinal product was observed at 4 °C, 8 °C, 25 °C and 37 °C, each 12 h for 48 h in the medium more stable to analyze to cell aggregation.

2.9.3. pH measurements

pH test was conducted on the supernatant before hMSCs packaging, and on the finished medicinal product stored at 4 °C at times 0 h and 48 h. pH values were measured in triplicate by immersing the probe directly into the sample using a digital pH-meter Basic 20 (Crison Instruments S.A., Barcelona, Spain) with the electrode for liquid samples.

2.9.4. Optical characterization of the stability

Light scattering methods are often used to study the stability of suspensions; an analysis of multiple dispersion of light was used to predict and confirm the physical stability of the cell suspensions by using the TurbiScanLab® (Formulaction, L'Union, France). The light source is a pulsed near infrared light source (λ = 880 nm). Two synchronous optical sensors receive respectively light transmitted through the sample (0° from the incident radiation, transmission sensor), and light back-scattered by the sample (135° from the incident radiation, backscattering detector). Each undiluted formulation (20 mL) was placed and kept on a cylindrical glass measuring cell which was completely scanned by a reading head.

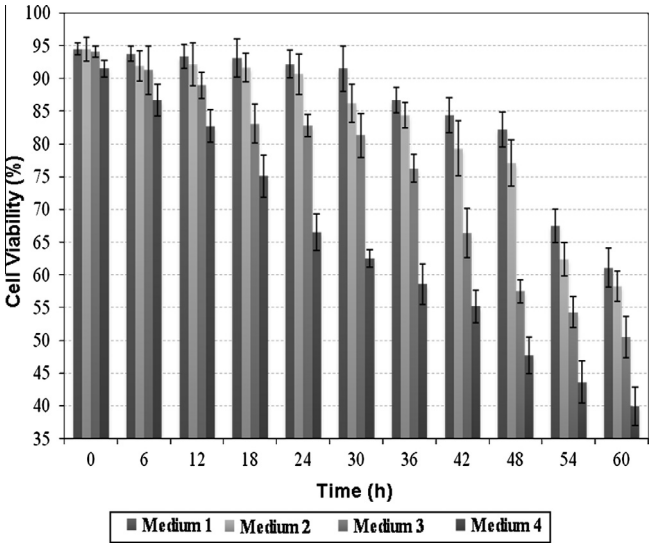

Fig. 1. hMSCs viability (%) packed in different media and stored at 8 °C, from 0 h to 60 h. Data represent mean ± SD.

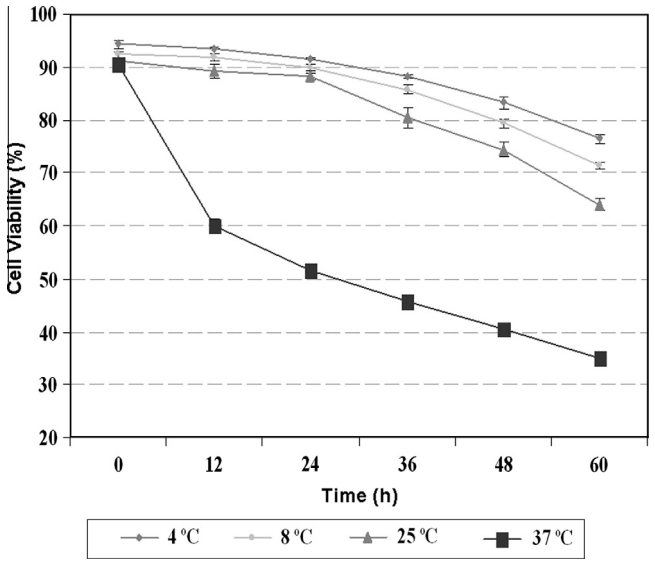

Fig. 2. hMSCs viability (%) packed in medium 1 and stored at different temperatures, from 0 h to 60 h. Data represent mean ± SD.

2.10. Statistical analysis

Tests for significant differences between means were performed by Student's *t*-test or one-way ANOVA using the Prism®, V. 3.00 software (GraphPad Software Inc., San Diego, CA, USA). Differences where $p < 0.05$ were considered statistically significant. Experiments were repeated on three different samples and the results were expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Assessment of stability

3.1.1. Influence of the packaging medium

On passage 4, the average viability of the hMSCs cultured before to be packed was 98.1 ± 2.1%. The same cells were packed in four different media and the average viability decreased throughout the assay. Viability measurements are indicated in Fig. 1.

Different effects of packing media were observed on cell viability. Medium 1 showed that viability was greater than 80% for 48 h after packaging (82.2 ± 2.7%). Media 2, 3 and 4 maintained the viability above 80% for less time. Medium 2 for 36 h (84.4 ± 1.9%), medium 3 until 30 h (81.3 ± 3.3%), and medium 4 maintained viability of cells only for 12 h (82.7 ± 2.5%).

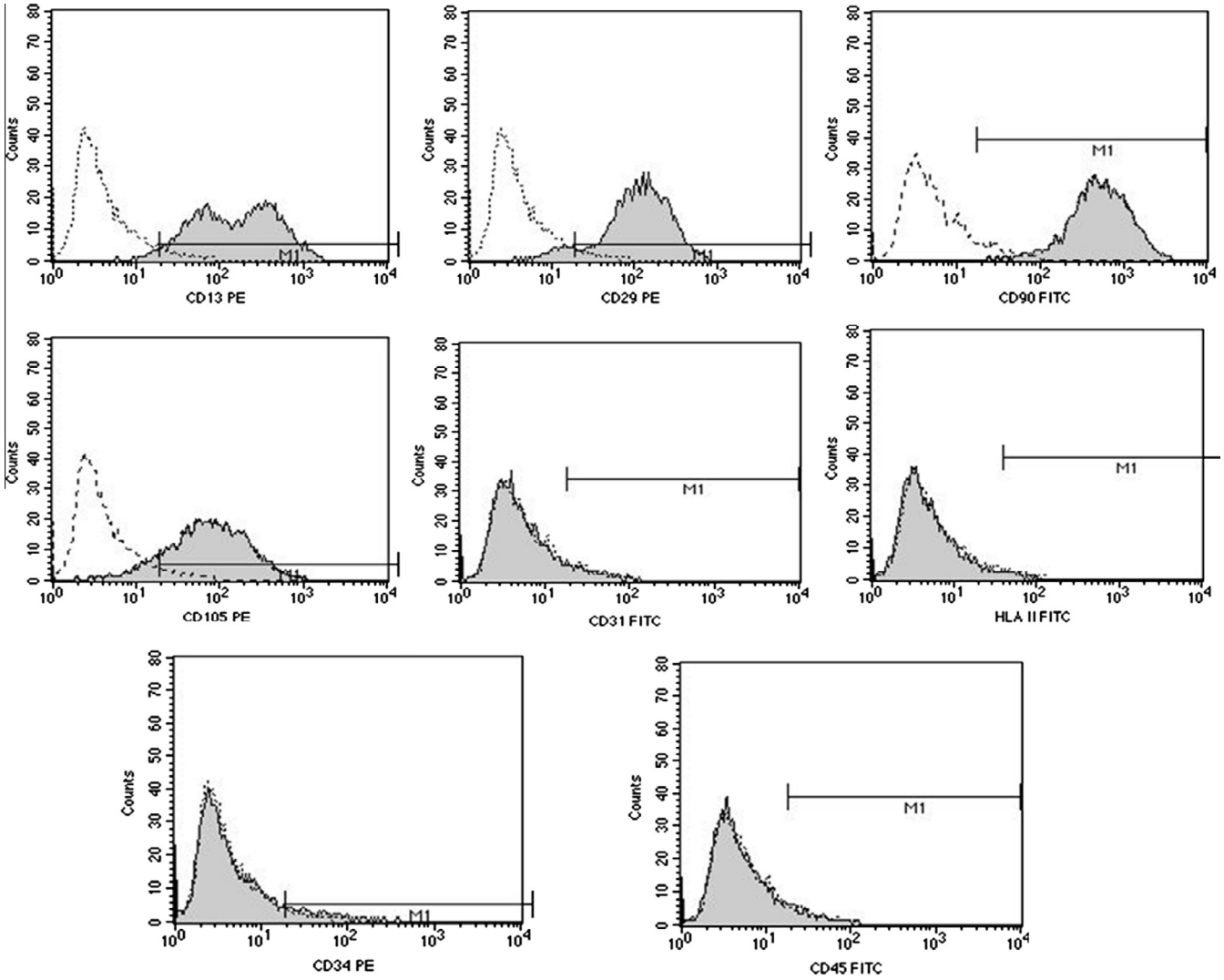

Fig. 3. Immunophenotypic characterization of the hMSCs after being packed in medium 1 and stored at 4 °C for 48 h. Both of the MSCs were CD13+, CD29+, CD90+, CD105+ and CD34−, CD45−, CD31−, HLA-II−.

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Although generally NaCl 0.9% is the vehicle of choice for any parenteral suspension, our study demonstrated that it was the medium in which viability decreased, and thus the least suitable. These results indicated greater cell viability in media with albumin and nutrients. Whilst, media 3 and 4 were solutions lacking of essential nutrients causing a rapid and significant decreased of cell viability.

In general there was a progressive loss of viability but the results indicated that hMSCs had better survival rate when they were packed in medium 1 as compared to the packaging media 2, 3 and 4.

3.1.2. Influence of the temperature of storage

Taking into account results above described, the effect of storage temperature was tested on medium 1. The hMSCs were maintained in culture until passage 5, their average viability before the study was 95.1 ± 2.4%. All syringes were stored at 4 °C, 8 °C, 25 °C and 37 °C, for 60 h and their viability was calculated each 12 h, results are depicted in Fig. 2. Our results indicated that during storage, the viability of hMSCs had decreased considerably at 37 °C compared to other temperatures analyzed, decreasing from 90.5 ± 0.3% at 0 h to 60.1 ± 1.1% at 12 h. Among 4 °C, 8 °C and 25 °C the viability mean values were similar throughout the study, approximately 80% up to 36 h at three temperatures (88.3 ± 0.4%, 85.7 ± 0.8% and 80.4 ± 1.9% respectively). However, at 4 °C the viability of hMSCs remained above 80% (83.4 ± 1.1%) up to 48 h, being the temperature most suitable for maintaining the viability of the cells for longer.

3.2. hMSCs immunophenotypic characterization

hMSCs packed in medium 1 and stored at 4 °C after 48 h were analyzed by a flow cytometer. The results were compared with previous storage test results (cell on passage 4). Analysis revealed that both populations (before and after packaging) were positive (>95%) for mesenchymal markers (CD13, CD29, CD90 and CD105) and were negative (<10%) for endothelial and hematopoietic lineage markers (CD34, CD45, CD31 and HLA-II) as is shown in Fig. 3. Fluorescence cytometry between hMSCs in both cultures and finished medicinal product showed no differences when the hMScs were stored at 4 °C for 48 h in medium 1.

3.3. Ex vivo osteogenic and adipogenic differentiation

hMSCs were seeded and cultured in osteogenic and adipogenic media again to confirm if hMSCs packed and stored in the medium 1 at 4 °C after 48 h could differentiate into osteoblasts and adipocytes. The results were compared with differentiation conducted on passage 4, before the stability test had been performed (Fig. 4). The differentiation was confirmed following the standard protocols and no difference was observed among differentiated cells indicating that storage at 4 °C for 48 h in the medium 1 did not affect the differentiation capability of hMSCs.

3.4. hMSCs karyotyping analysis

Cells were tested for genomic stability using a conventional analysis by G band techniques to check whether the hMSCs maintained their normal karyotype during cultivation to passage 4 and during their packing and storage before their administration phase. The results showed that on passage 4, the hMSCs were normal diploid karyotype (46, XX). After packaging and storage phases in medium 1 at 4 °C for 48 h, a sample contained in a sterile syringe Luer-lock, was taken to carry out another karyotype test. The results of these test showed no karyotype changes in the finished medicinal product, obtaining a normal diploid karyotype (46, XX) (Fig. 5).

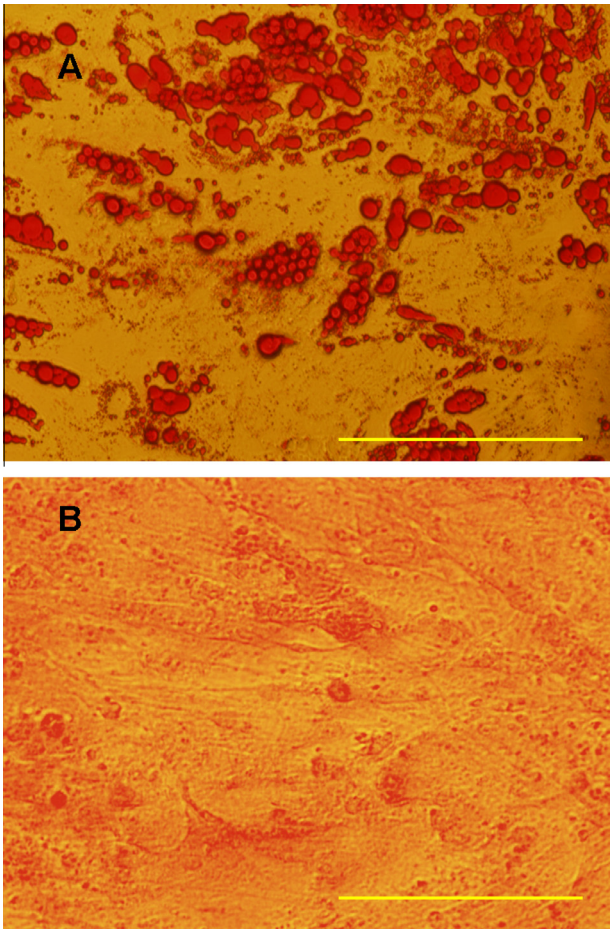

Fig. 4. Cell culture differentiation of adipocytes (panel A) and osteoblasts (panel B) stored in medium 1 after storage at 4 °C for 48 h. Bar length 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Microbiological studies

Sterility testing was performed on the hMSCs during culture (passage 4) and packing after stability study to ensure no contamination. When the incubation period had finished all tested samples were observed and no turbidity was exhibited, and so completely free of contaminating microorganisms. These results showed that not only the expansion procedure maintained asepsis, but also package and storage in medium 1, for 48 h at 4 °C, preserved the sterility of the finished medicinal product until administration phase. All negative control tubes were negative after the required incubation period.

3.6. Physicochemical characterization of the finished medicinal product

3.6.1. Rheological studies

Rheological disturbances can play an important role in the parenteral administration of cell suspensions. In fact the viscosity of suspensions is strongly influenced by factors such as aggregation of hMSCs. Thus, the rheological properties of the finished cell suspension are fundamental in the parenteral administration in micro- and macro-vessels.

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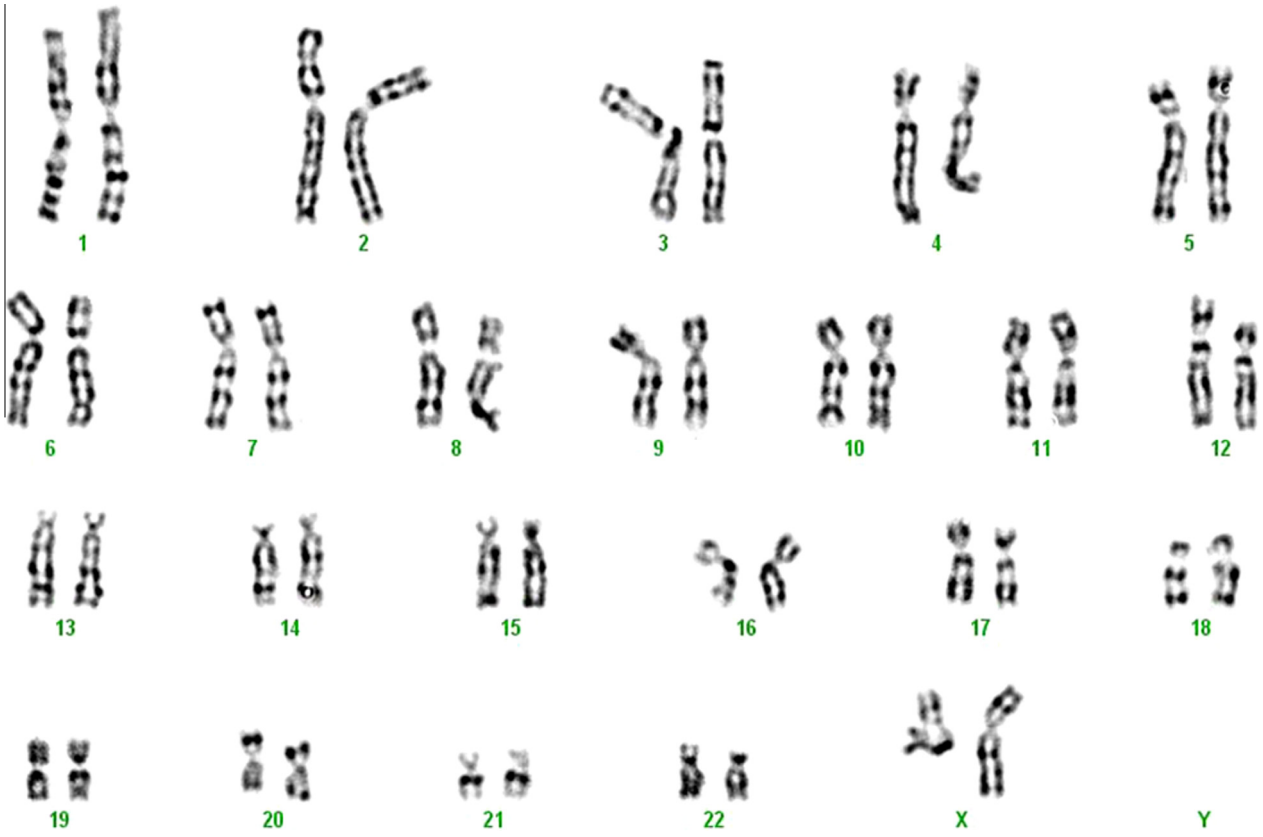

Fig. 5. Karyotyping analysis of human MSCs. The image shows the result of G-banding karyotyping of a metaphase after to study stability of the finished medicinal product. The karyotype shows a female normal karyotype (46, XX). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

For this reason the rheological characteristics of unstirred cell suspension were also studied. The sample exhibited Newtonian behavior and has lower viscosity value (1.11 ± 0.06 mPa s), which may be an indication of the migration of aggregates/flocculates. To distinguish both alternatives suspensions were studied after being shaken. Most aggregates were separated as consequence of shear forces, which will be sufficient to break-up the weak reversible flocculation. Viscosity value of the finished cell suspension in this case was 1.575 ± 0.043 mPa s. This value is similar to viscosity of blood plasma of 1.2 mPa s at 37 °C [38].

The flow and viscosity curves of the suspensions (shear stress versus shear rate in red, and viscosity versus shear rate in blue) are shown in Fig. 6. When the shear rate increases, the viscosity values decrease, and this tendency is exhibited in all samples. This rheological behavior could be described as typical for a non-Newtonian, pseudoplastic fluid. The hysteresis area (thixotropy), a pseudoplastic natural characteristic, was observed in the obtained rheogram. Rheological parameters were fitted to mathematical models in order to identify the model that provided the best overall match of the experimentally observed rheological data based on the highest correlation coefficient of the linear regressions (r) and the lowest chi-square value. The model that best fitted the experimental data was Cross in all cases, showing that flow behavior was not influenced by time and/or temperature with an r^2 of 0.995 and chi-square of 0.0015, the Newton model provided the worst overall prediction of rheological behavior. Cross-model is a rheological model that combines four parameters and covers the entire shear rate range [39]. In general, the four parameter models are difficult to apply because there is seldom enough data to allow good model fitting. However, they represent the best results in predicting the behavior of non-Newtonian fluids. These results may have important implications for the administration because the success of the injection is dependent upon limiting shear forces of the needle wall causing cell lysis mainly produced by turbulent flow.

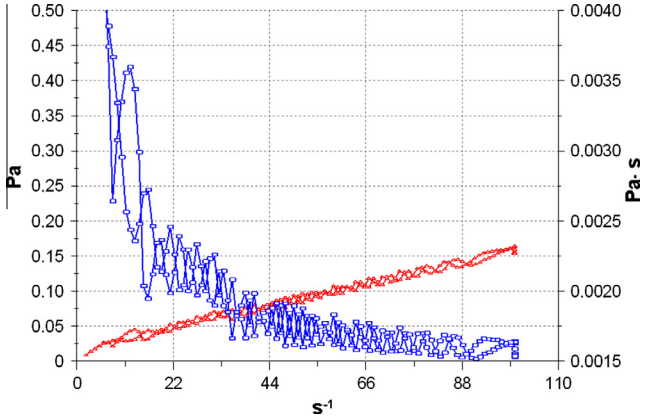

Fig. 6. Cell suspension rheogram. It shows the shear stress (Pa) (in red) and the viscosity (Pa s) (in blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6.2. Morphological analysis

To determine whether the hMSCs cultures maintained their morphologic characteristics, they were tested in each passage and before packing. The adherent cultured hMSCs exhibited a homogeneous population with a fibroblast-like morphology when observed under a light microscope before packaging. Following the stability test, no morphological differences were observed when the cells were plated again.

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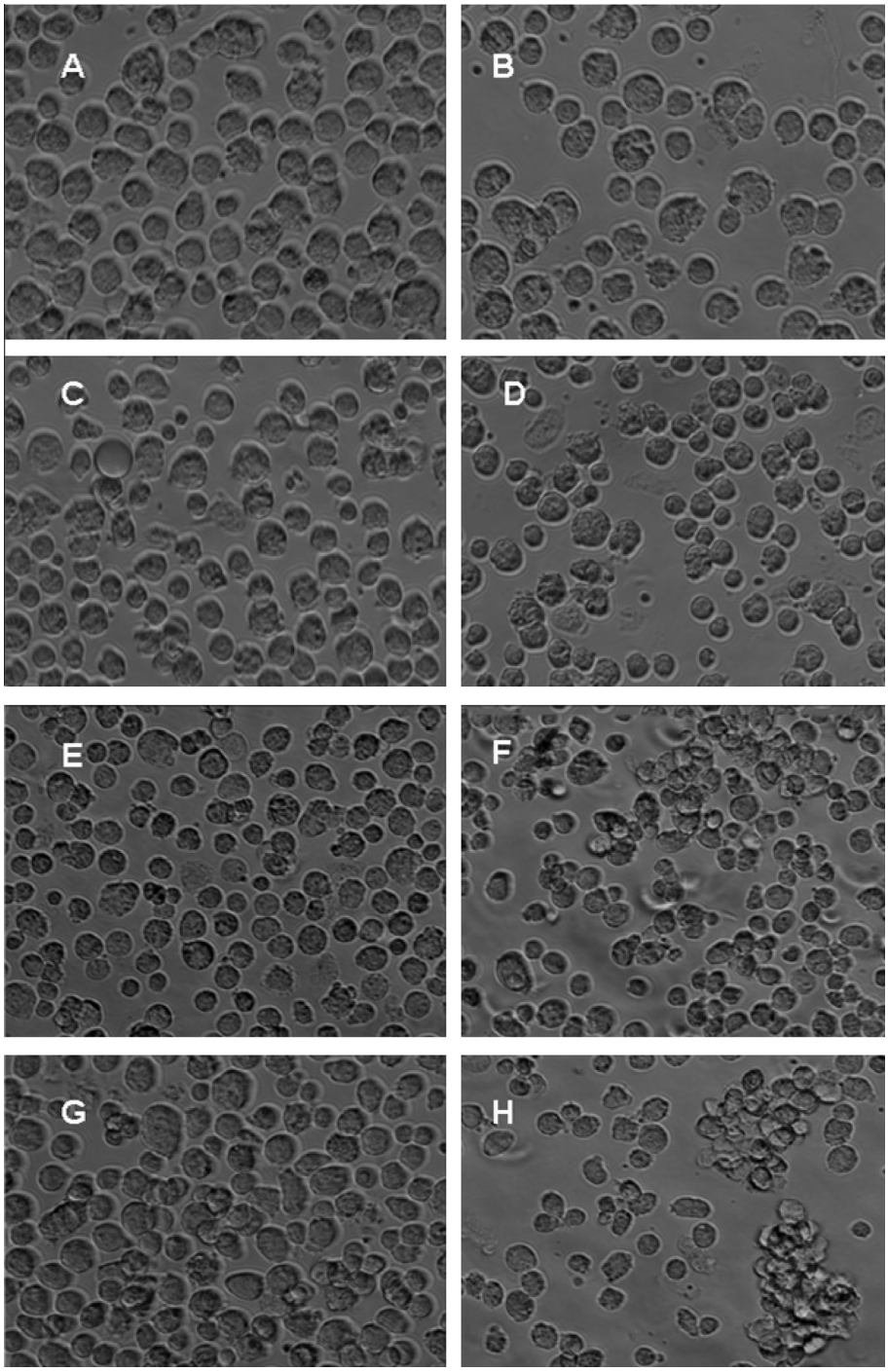

Fig. 7. Morphology of hMSCs for stability study at different storage temperatures and times. Panel A 0 °C and 12 h; panel B 0 °C and 24 h; panel C 4 °C and 12 h; panel D 4 °C and 24 h, panel E 8 °C and 12 h, panel F 8 °C and 24 h; panel G 25 °C and 12 h; panel H 25 °C and 24 h.

On the other hand, the packed hMSCs in medium 1 at different temperatures (4, 8, 25 and 37 °C) for 48 h were observed each 12 h to determine if cell aggregation may have occurred. Imagines showed no cell aggregation at 4 and 8 °C at any time (Fig. 7). However, at room temperature and 37 °C, after 24 h cell aggregate signs could be seen. Therefore it can be concluded that the packaged finished medicinal product in medium 1, at 4 °C for 48 h did not exhibited hMSCs aggregation in suspension and was safe to be administrated.

3.6.3. pH measurements

pH for intra-arterial administration should be equal to the blood pH, slightly alkaline to prevent potential stinging, burning, pain, irritation or tissue damage [40]. pH in the supernatant was 7.82 ± 0.02 on passages 4 and 5. After packaging, at time 0 h pH was 7.91 ± 0.01 and 48 h was 6.86 ± 0.01. These results demonstrated that although pH values of the finished medicinal product decreased over time, at 48 h continued to be acceptable for parenteral administration.

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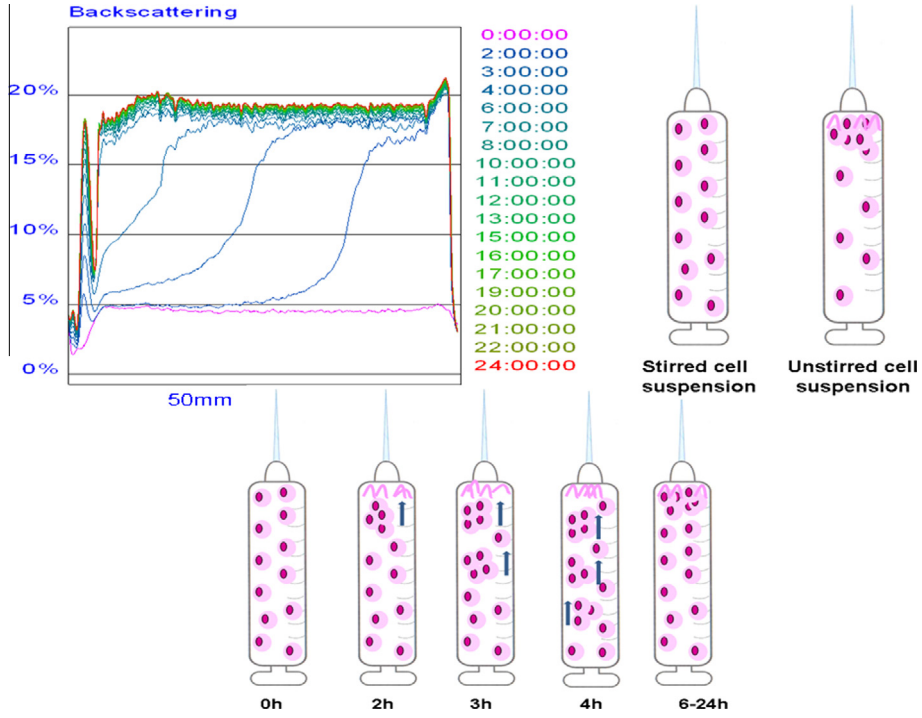

Fig. 8. Backscattering profiles of suspensions. The left side of the curve corresponds to the bottom of the vial, whereas the right side corresponds to the sample behavior on the top. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6.4. Optical characterization of the stability

Stability evaluation is generally a crucial point in the scenario of advanced therapies. The long-term physical stability of cell suspensions was tested by evaluating the photon backscattering profiles of the various samples. Turbiscan®Lab is considered as a device which predicts the stability, being able to detect destabilization before than the classical stability methods (microscopy, spectroscopy or turbidity). Moreover, it provides real-time information on the destabilization process based on the variation in backscattering. When sedimentation process occurs (migration of cells from the top to the bottom), a backscattering increase versus time at the bottom of the sample is observed. When the sample suffers a creaming process, an increase in transmission versus time on the top of the vial is observed. If the destabilization phenomenon occurs due to aggregation (migration of cells from the bottom to the top), a backscattering increase versus time can be observed over the whole height of the sample [41]. If backscattering profiles have a deviation of $\leqslant \pm 2\%$ it can be considered that there are no significant variations. Variations up to ±10% indicate instable formulations. Fig. 8 shows backscattering profile of the final product containing hMSCs corresponding to measurements on different hours. The left side of the curves corresponds to the bottom of the vial, whereas the right side corresponds to the top.

The region below 4 mm marks the metal base and the strong decay of backscattering above 38 mm the beginning of the free surface of the sample.

For cell suspension both creaming and flocculation mechanisms were involved. It can be observed that the initial dispersion presented a backscattering value about 5% and there were no changes in backscattering for the first 2 h. Between 2 and 6 h, a progressive backscattering increase was observed. This phenomenon could be attributed to the sample flocculation, possibly due to the formation of aggregates, as it was previously observed in microscopy study. These result confirmed the natural preference of hMSCs to form aggregates [42]. In our samples, flocculation started at different times.

The peak that appeared at the top of the tube was indicative of creaming, presumably caused for migration of cellular components as lipid membranes from dead cells. Finally, after 7 h approximately there was no evolution in backscattering, indicating that the suspension was starting to stabilize.

In this way, it could be concluded that before the future administration, the injectable cell suspension should be shacked just before using in order to get a homogeneous hMSCs suspension, avoiding thrombotic or thromboembolic events.

4. Conclusion

In the present study, the stability of a CTMP with hMSCs was studied to be applied in diabetic patients with critical limb ischemia in clinical trial phase I–II, based on the study of the viability of the cells before and after being packaged and stored. Our results clearly showed that the viability of the hMSCs in a packaging medium comprising albumin 20%, glucose 5% and Ringer's lactate at a concentration of 1×10^6 cells/mL and stored at 4 °C for more than 48 h is maintained above 80%, therefore acceptable viability was obtained for a potential clinical use. Our results also demonstrated that hMSCs processed and packaged under GMP conditions environment and subsequently stored maintained their phenotypic, genotypic and physicochemical characterization. Besides, the ability to differentiate into adipocytes and osteoblast was also preserved, as well as, all sterility requirements. Thus a safe medicine for clinical application was developed.

There are several papers dealing with stability studies of stem cells for clinical use. The best storage conditions for preservation of hematopoietic progenitor function have been reported to be at 4 °C for 24 h [43]. In the case of MSCs, Muraki et al. [44] demonstrated that viability of MSCs in PBS was maintained above 80% but only for 24 h. However, there is no information about which would be the best packaging medium or storage conditions (time and temperature) to maintain the viability for clinical application.

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For the foreseeable future, cell therapy development will be based on not only in the search for new therapies but also in the study and design of these medicines, in terms of stability and safety, to expand the margins of use over time until administration.

In conclusion, the stability of a medicine is a function of storage conditions and chemical properties of the active ingredients. The conditions used in the stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Our stability studies in the final medical product were based on showing that hMSCs characteristics remained unchanged until administration.

This is the first study to examine the stability and viability of hMSCs with different excipients for cell suspension packaging at various temperatures, but further studies will be needed to improve the stability of cells for developing a cell medicine.

Acknowledgments

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III.7. DEVELOPMENT AND CHARACTERIZATION OF GLUCOSE MICROSPHERES FOR ENHANCING VIABILITY OF A HUMAN STEM CELL SUSPENSION WITH CLINICAL APPLICATION

Uno de los factores limitantes en la traslación clínica de la terapia celular es la corta vida de las células madre en la forma de dosificación final. A fin de mejorar dicha viabilidad la bibliografía actual describe el uso de diferentes estrategias a modo de vehículos tipo "andamios" y técnicas de encapsulación. En consonancia con estas líneas de investigación, el principal objetivo del presente estudio fue el desarrollo, caracterización y estudio de la influencia de microesferas cargadas de glucosa en la viabilidad de las hMSC formuladas en suspensión con potencial aplicación clínica.

Para la obtención de las microesferas de alginato cargadas con glucosa se empleó de forma satisfactoria el método de emulsificación/gelificación interna. Del estudio de viabilidad se dedujo un aumento de la carga celular activa durante las primeras 30 h gracias a la liberación de glucosa de forma exponencial desde las microesferas.

Así mismo estudios de microbiología, inmunofenotipo y físico-química demuestran la inocuidad de las microesferas sobre el CTMP.

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Abstract: A critical limiting factor of cell therapy is the short life of the stem cells in the final medicinal product. The use of different excipients scaffolds and encapsulation techniques have been studied to increase cell viability for the drug to be developed.
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Development and characterization of glucose microspheres for enhancing viability of a human stem cell suspension with clinical application.

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Abstract

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In this study, microspheres were developed and characterized to create a glucose sustained release system and study their effect on the viability of human mesenchymal stem cells in the final medicinal product. The microspheres of glucose were satisfactory elaborated with alginate by emulsification/internal gelation method resulting in 86.62 µm mean particle size with unimodal distribution, spherical shape and –32.54 mV zeta potential, assuring optimal stability and encapsulation efficiency. FTIR spectra graphs showed that glucose did not induce subsequent modifications in the structure of alginate. *In vitro* release of glucose followed an exponential model. Cell viability was determined by trypan blue dye exclusion staining and posterior counting of cells in a Neubauer chamber. The results indicated that hMSCs had better survival rate when they were packed with glucose microspheres as compared to the rest of media. Microspheres maintained the aseptic conditions of the final cell suspension. No rheological disturbances where induced by the inclusion of glucose microspheres on the cell suspension. Equally, no morphological or immunophenotypic changes were observed in cells after microspheres incorporation in the final product. Finally, the injectable cell suspension should be re-dispersed just before using by gentle shaking in order to get a homogeneous hMSCs suspension, avoiding thrombotic or thromboembolic events.

Keywords: Mesenchymal stem cells; cell viability; alginate; microspheres; internal gelation.

1. Introduction

Human Mesenchymal Stem Cells (hMSCs) are a potential resource with utility in preclinical studies, screening and development of new drugs and clinical applications as regenerative medicine [1,2] . Their anti-inflammatory, angiogenic, antithrombogenic and antiapoptotic properties make them valuable tools for potential application in cell therapy [3,4]. Current investigation focuses on the search for effective treatments for a range of diseases such as diabetes [5], heart disease [6], neurodegenerative diseases [7], tissue regeneration and repair [8], etc. However, substantial barriers to clinical translation still exist and must be overcome to realize full clinical potential. These

barriers span processes including cell isolation, *ex vivo* expansion, differentiation, quality control, shelf-life of the stem cell in the final product for being marketed and therapeutic efficacy and safety [9,10].
The use of hMSCS as drugs is characterized in that cells must be viable at the time of administration [11], specifically viability should be greater than 80% [12], for this reason, in order to improve cell viability the study of optimal delivery systems is of great interest.
Notwithstanding the advent of current strategies to reach a better control of hMSCs viability, such as encapsulation of cells [13,14], cell injection therapy is still the most commonly used scaffold-free delivery format to treat a variety of diseases, including: immunodeficiencies, myocardial defects, cartilage damage, peripheral arterial disease, diabetes, and neurological disorders [15]. Long term clinical success will in part be dependent on the cells that remain viable and that assume correct functionality post-administration. In this line, limited impact of cell therapy and failure to generate long term effects has been attributed largely to extensive cell death [16]. Thus, cell delivery in injectable hydrogels [17], adhered to microcarriers [18], cell sheets [19] or new packing media [20] have been developed with important influence on the cell viability rate [21]. In spite of interesting and significant advances in the field already achieved, some challenges still remain unsolved. Optimal shelf-life and thus prolong the cell-loaded system upon *in vivo* implantation seems to be a pending issue.
As a result of the short shelf life of hMSCs many donors are needed to maintain the minimum number of units available for implantation at any given time. Furthermore, sets certain limits for the current viability methods of cellular products as can be the terminal sterility procedures. This short shelf life also causes organizational problems since it is necessary to coordinate a multidisciplinary professional team, including the patient in a few hours, because of decreasing cell viability over time.
The use of a scaffold material enables implementation of additional excipients to improve cell viability by secreting a specific molecule or protein. [22,23]. This technology is based on the immobilization of ingredient within a polymeric matrix surrounded by a semipermeable membrane for the long-term release [24]. Natural polymers as alginates are the most studied and characterized for cell encapsulation technology [25]. Alginate is a biomaterial that has found numerous applications in biomedical science, particularly in the areas of wound healing, drug delivery, *in vitro* cell culture, and tissue engineering due to its favourable properties, including biocompatibility and ease of gelation. Alginate is intrinsically non-fouling, not cleavable by natural enzymatic digestion and does not support cell adhesion [26].
It has been shown that reduced levels of glucose and oxygen combined with other environmental conditions (i.e., decreased pH, cell aggregation) decreases the anabolic activity of cells [27]. Contrary, high extracellular glucose or glycolytic intermediate concentrations can maintain cell viability to some extent whilst stimulating lactate production [28,29] , but a few studies, however, have examined the influence of nutrient factors and excipients on the survival of stem cells, particularly in a cellular suspension. Therefore, the present study aimed to develop biodegradable alginate microspheres secreting glucose to enhance and prolong the functionality of an hMSC suspension. Viability of cells was analysed. Furthermore, a detailed evaluation of the elaboration and characterization processes has been performed to guarantee particle uniformity, reproducibility and optimum cell viability. To our knowledge, this is the first time that such strategy to enhance cellular shelf-life has been reported.

2. Materials and methods

2.1. Synthesis of glucose loaded alginate microspheres

Glucose loaded microspheres elaboration method was based on the well-known emulsification/internal gelation methodology with modification [30]. The W/O emulsion was performed with a sodium alginate aqueous solution (Fagron Iberica, Terrassa, Spain) purified by filtration, extraction and precipitation [31]. Calcium carbonate (Panreac Quimica, Barcelona, Spain) and glucose anhydrous (Guinama, Alboraya, Spain) were utilized as the internal phase, and vegetable oil (Guinama, Alboraya, Spain) as the external phase.

Briefly, 0.2 g of calcium carbonate was added to 40 mL solution of 50% (w/w) glucose and 5% (w/v) sodium alginate. After homogenization, the suspension was dispersed in 100 mL of vegetable oil (continuous phase) containing Span® 80, 2% (w/v), (Guinama, Alboraya, Spain). The mixture was stirred at 700 rpm for 10 min to form uniform W/O emulsion. Under stirring, 20 mL of vegetable oil containing 0.850 mL of glacial acetic acid (Panreac Quimica, Barcelona, Spain) was added to the W/O emulsion, to complete the calcium carbonate solubilisation. After 10 min under stirring, pregelified microspheres were separated from the oil dispersion by mixing with calcium chloride solution 5% (w/v) (BDH Prolabo, Barcelona, Spain). The supernatant was discarded and the alginate microspheres were centrifuged and washed two times with double distilled water. After this, particles were collected and washed again in 100 mL double distilled water by vacuum filtration and stored at 4º C in Petri dishes. Glassware and reagents used in the experiments were sterilized before used.

2.2. Cell isolation and culture

Autologous hMSCs were isolated from abdominal adipose tissue biopsies. All donors provided informed consent that was formerly approved by local and regional medical research ethics committees. Each patient was appropriately screened and tested for human pathogens. In particular, the presence of Human Immunodeficiency Virus (HIV), hepatitis B and hepatitis C virus were analysed.

Cells were isolated from human adipose tissue by enzymatic digestion with collagenase (2 mg collagenase/1 g adipose tissue) (Roche Pharma, Basel, Switzerland), centrifuged at 400 g for 10 min, filtered and washed with Phosphate Buffered Saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) to obtain the stromal cells.

These cells were suspended and plated at medium density of $12–20 \times 10^4$ cells/cm^2 in culture flasks (Nunc A/S, Roskilde, Denmark) with expansion medium composed by Dulbecco´s Modified Eagle´s Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 1% of 10,000 IU/mL penicillin, 10 mg/mL streptomycin, 2% of 200 mM L-alanine solution and 1% of 200 mM L-glutamine, (all from Sigma-Aldrich, St. Louis, MO, USA). After 24 hours, non-adherent cells were removed by replacing the expansion medium. The medium was replaced every 2 or 3 days a week. Cells were harvested upon reaching 80% confluence, and subcultured using 0.25% trypsin (Invitrogen, Grand Island, NY, USA) in expansion medium, and plated at medium density, 3500–5500 cells/cm^2. Cells were cultured under 95% relative humidity, at 37 °C and 5% CO_2. Three different processes of *ex vivo* expansion hMSCs were carried out (named 1, 2 and 3) from passages 3–4 to analyse their microbiological quality. Cells were finally packed at concentration of 1×10^6 cells/mL with 1 mL of packaging medium consisting of 50% glucose 5%, 45% lactated Ringer's solution and 55% albumin 20% (Grifols, Barcelona, Spain). hMSCs were packed in 10 mL Luer-lock syringes (Becton Dickinson & Co., Franklin Lakes, NJ, USA) at 4 ºC [20].

2.3. Physicochemical characterization of microspheres

2.3.1. Morphological and particle size analysis

Morphology of hydrated microspheres was first monitored by optical microscopic observation using an optical microscope Olympus BX40 microscope equipped with a calibrated eyepiece micrometer and camera Olympus SC35 (Tokyo, Japan) under a magnification of 100×.

Particle size distribution was confirmed by light diffraction (LD) using a LS 13 320 analyser (Beckman coulter Inc., Brea, CA, USA) with a size range from 0.04 to 2000 μm yielding the volume distribution of the particles. Characterization parameters were the diameters $LD_{0.1}$, $LD_{0.5}$ and $LD_{0.9}$, namely the particle diameters determined at the 10th, 50th and 90th percentile of the undersized particle distribution curve. A diameter $LD_{0.5}$ of 1 μm means that 50% of all particles possess a diameter of 1 μm or less. Equally, mean diameter over the volume distribution ($LD_{4.5}$) and polydispersity expressed as the Span factor were also calculated [32].

The shape, surface texture and internal structure of the microspheres were examined by scanning electron microscopy (SEM) using a Zeiss DSM 950 microscope (Carl Zeiss AG, Oberkochen, Germany).

2.3.2. Zeta potential

The surface electrical properties of cell and microspheres suspension ~ 0.1% (w/v), were analysed by electrophoresis measurements in double distilled water using a Zetasizer 2000 (Malvern Instruments Ltd., Malvern, UK) electrophoresis device. Final zeta potential (ζ) values calculated from the electrophoretic mobility by the Helmholtz–Smoluchowski equation were average values from nine measurements made on the same sample at 25.0 ± 0.5 ºC.

2.3.3. Fourier transforms infrared spectroscopy measurement (FTIR)

Studies of infrared spectra of glucose, raw polymer, and microspheres were conducted with a FTIR UATR Two N 92052 spectrometer (Perkin Elmer, Waltham, MA, USA). Each sample was scanned over a wave number region of 400 to 4.500 cm^{-1}. The characteristic peaks were recorded.

2.3.4. Determination of percentage yield, loading capacity and encapsulation efficiency

The percentage yield (PY), loading capacity (LC) and encapsulation efficiency (EE) was calculated based on the dry weight of glucose and the alginate. EE was determined for all samples after the manufacturing process. Total glucose utilized was considered as the sum of encapsulated and non-encapsulated. Determination was performed as follows, 0.5 g of particles at 25 ºC was added to phosphate buffer (0.1 M, pH 7); the mixture was liquefied by gently shaking for 30 min at room temperature. After that, all samples were filtered through a 0. 22 μm filter and analysed with the kit for the detection of glucose (Spinreact, Girona, Spain), the coloured reaction was measured in triplicate at 505 nm in a Lambda 40 spectrophotometer (Perkin Elmer, Waltham, MA, USA). Equally, for determination of the amount of glucose adsorbed in the microsphere surface, 0.5 g of each microcapsule was added to 50 mL double distilled water and maintained at 25.0 ± 0.5 ºC under mechanical stirring (50 rpm) for 48 h. Then solution was filtered through a 0. 22 μm filter and analysed in triplicate.

2.4. *In vitro* glucose release

In vitro release test was performed by suspending 1.25 g of glucose microspheres in 50 mL of the packaging medium in closed glass vials. Then vials were stored at 4 ºC for 72 h, sink conditions were maintained during the study. At each time (24, 48 and 72 h),

three vials were collected and vortexed, the content was filtered through a 0. 22 µm filter (Teknokroma®, Barcelona, Spain) and the concentrations of the released glucose determined in triplicate by the kit as described above. In order to evaluate the *in vitro* release kinetics, first order polynomial and exponential growth mathematical models were used. The adequacy of the delivery profiles to the mathematical models was based on the highest correlation coefficient value (r^2) and the smallest Akaike's Information Criterion (AIC), [AIC = n × lnSSQ + 2p], as indicator of the model's suitability for a given dataset [33]. Where *n* is the number of pairs of experimental values, *SSQ* is the residuals sum of squares and *p* is the number of parameters in the fitting function. The study was conducted using the software Prism®, v. 3.00 (GraphPad Software Inc., San Diego, CA, USA).

2.5. Determination of the effect of glucose microspheres on cell viability

MSCs from passage 3 were used. Cells were harvested using 0.25% trypsin (Invitrogen, Grand Island, NY, USA) and centrifuged at 400 g for 10 min with expansion medium. The cell pellet was resuspended at concentration of 1×10^6 cells/mL with 1 mL of packaging medium. Four packaging media were prepared, medium A, containing glucose microspheres; medium B, microspheres without glucose (unloaded); medium C, glucose microspheres with phosphate buffer solution (0.1 M, pH 7); and medium D, control medium without microspheres. The excipients (Grifols, Barcelona, Spain) for 50 mL of each such media are reported in Table 1.

Table 1
Composition (mL) of different packaging media (50 mL final volume).

Ingredients	Medium A	Medium B	Medium C	Medium D
Lactated Ringer's solution (mL)	22.5	22.5	21.5	22.5
Glucose 5% (mL)	25	25	25	25
Albumin 20% (mL)	2.5	2.5	2.5	2.5
Glucose loaded microparticles (g)	1.25	–	1.25	–
Unloaded microparticles (g)	–	1.25	–	–
Phosphate buffer solution (mL)	–	–	1	–

MSCs were packed in 10 mL Luer-lock syringes (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and stored at 4 ºC in a normal atmosphere. The stability study was based on the MSCs viability every 6 h for 60 h.
Cell viability was determined by trypan blue dye exclusion staining and posterior counting of cells in a Neubauer chamber [34]. Each sample was counted three times and the average (%) was calculated [% = Number of viable cells/number of cells ×100].

2.5.1. Kinetic evaluation of stability

A kinetic evaluation of the cell viability was performed on the base of the obtained results. Experimental data were fitted to five different kinetic models: zero order, first order polynomial, second order polynomial, dual with shoulder and plateau decay zero, to find out the mechanism that statistically best represented our findings.
A nonlinear least-squares regression was performed using the Prism®, v. 3.00 software (GraphPad Software Inc., San Diego, CA, USA). The best model to describe the stability profile was selected based on the r^2 and AIC.

2.6. Microbiological studies

Microbiological analysis was based on the study of the sterility of the cells before being packaged and after stability study with microspheres (60 h). The test was carried out by direct inoculation in accordance with the European Pharmacopoeia [35]. 1 mL of each medium (A, B, C and D) was inoculated in two microbiological media: Thioglycollate Penase Broth 9 mL (TPB) to detect anaerobic and aerobic bacteria, and Tryptic Soy Penase Broth 9 mL (TSPB) (VWR International, Radnor, PA, USA) , a soybean casein digest medium to detect fungi and aerobic bacteria. For each media (TPB and TSPB), sterility test and growth promotion test of aerobes, anaerobes and fungi were previously verified. The inoculated media were incubated for 14 days at 35 ºC and 22 ºC, respectively. After 14 days, if there had been microbial growth, the medium would have shown turbidity. Negative controls were established by inoculating 1 mL of 0.9% sterile NaCl (bioMérieux, Marcy l'Etoile, France) in duplicate for each medium. The inoculated media were incubated for 14 days at 35 ºC and 22 ºC (TPB and TSPB respectively). After 14 days, if there had been microbial growth, the medium would have shown turbidity. This assay was performed in aseptic conditions with an isolator HPI-4PI-S (Esco Technologies, Inc., Hatboro, PA, USA).

2.7. hMSCs immunophenotypic analysis

At passage 4, after stability study of the MSCs with glucose microspheres (Medium A) and with control medium (Medium D), immunophenotyping study of hMSCs was performed in order to identify the presence of specific surface antigens. Between 2.5 and 5×10^5 cells were separated in 1.3 ml of expansion medium. The following markers were analysed: CD13-PE, CD29-PE, CD90-FITC, CD105-PE, CD31-FITC, CD34-PE, CD45-FITC and HLA II-FITC (Becton Dickinson and Co., Franklin Lakes, NJ, USA). Mouse antibodies served as control: Isotype-FITC IgG1-k, Isotype-FITC IgG2a-k, Isotype-PE IgG1-k (all from Becton Dickinson and Co., Franklin Lakes, NJ, USA). One hundred µl of cell suspension were prepared with 5 µl of each of the following reagents: fluorescein isothiocyanate (FITC), phycoerythrin (PE) antibody and control, and incubated at 4 °C for 20 min in the dark. Then, 3 ml of PBS (Sigma-Aldrich, St. Louis, MO, USA) were added to each cell suspension and centrifuged at 400 g for 10 min. Finally each cell pellet was diluted in 300 µl of PBS and 5000 labeled cells were analysed using a FACSCalibur analyser flow cytometer system running CellQuest Pro software (Becton Dickinson and Co., Franklin Lakes, NJ, USA), and the percentage of viable cells positive for each marker were determined.

2.8. Physicochemical characterization of the MSCs –microsphere suspension

2.81. Morphological analysis

Morphological characteristics of hMSCs were observed in each medium for the stability study by optical microscopy equipped by camera (Olympus, Tokyo, Japan). Each suspension (A, B, C and D) was observed at 4 ºC, each 24 h for 48 h to analyse the morphology and the cell aggregation.

2.8.2. Rheological studies

The rheological characterization was performed at 25 ºC using a rotational rheometer HAAKE Rheostress 1 (Thermo Fisher Scientific, Karlsruhe, Germany) equipped with a parallel plate geometry set-up with fixed lower and upper plate (Haake PP60 Ti, 6 cm diameter). Different gaps between plates were tested and a separation of 0.105 mm was selected. The rheometer was connected to a computer provided with the software HAAKE Rheowin® Job Manager V. 3.3 to carry out the test and Rheowin® Data Manager v. 3.3 (Thermo Electron Corporation, Karlsruhe, Germany) to carry out the

analysis of the obtained data. Viscosity curves and flow curves were recorded for 1 min during the ramp-up period from 0 to 100 s^{-1}, 1 min at 100 s^{-1} (constant share rate period) and finally 3 min during the ramp-down period from 100 to 0 s^{-1}. All determinations were performed in triplicate.
Data from the flow curve (when resulted to be non-Newtonian) were fitted to different mathematical models, Bingham, Ostwald-De-Waele, Herschel-Bulkley, Casson and Cross, using the Prism®, v. 3.00 software (GraphPad Software Inc., San Diego, CA, USA).

2.8.3. Optical characterization of the stability

Light scattering methods are often used to study the stability of suspensions; an analysis of multiple dispersion of light was used to predict and confirm the physical stability of the cell suspensions using the TurbiScanLab® (Formulaction, L'Union, France). The light source is a pulsed near infrared light source (λ = 880 nm). Two synchronous optical sensors receive respectively light transmitted through the sample (0 º from the incident radiation, transmission sensor), and light back-scattered by the sample (135 º from the incident radiation, backscattering detector). Each undiluted formulation (20 mL) was placed and kept on a cylindrical glass measuring cell which was completely scanned by a reading head.

2.9. Statistical analysis

Tests for significant differences between means were performed by Student's *t*-test or one-way ANOVA using the Prism®, v. 3.00 software (GraphPad Software Inc., San Diego, CA, USA) followed by a Tukey's multiple comparison test. Differences where $P < 0.05$ were considered statistically significant. Experiments were repeated at least three times and results expressed as the mean ± standard deviation (SD).

3. Results

3.1. Microspheres elaboration

An emulsification/internal gelation method is proposed for producing small diameter alginate microspheres. Importantly, alginate purified by a multi-step extraction procedure to a very high purity did not induce any significant foreign body reaction when implanted into animals [36]. Gelation was achieved by gentle acidification of the alginate solution containing an insoluble calcium salt with an oil-soluble acid that causes calcium ion release, resulting spherical shaped and small sized alginate microspheres. Thus, it is a suitable procedure to encapsulate materials such as glucose which was first confirmed by optical microscopy (Fig. 1A-B).

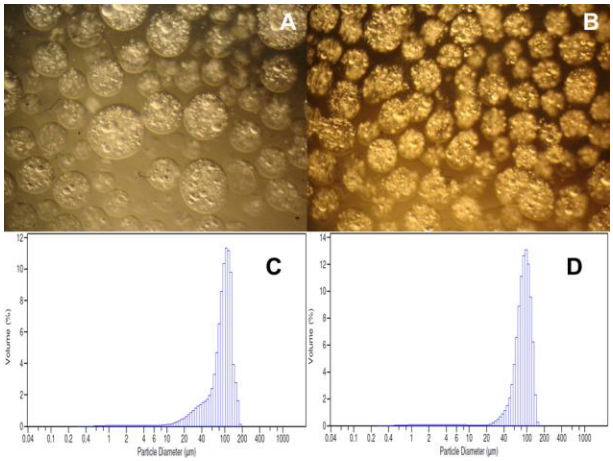

Fig. 1. Optical micrographs and volume distribution of alginate microspheres. Optical micrograph of unloaded microspheres (A), optical micrograph of glucose loaded microspheres (B), particle size distribution of unloaded microspheres (C) and particle size distribution of glucose loaded microspheres (D).

3.2. Physicochemical characterization of microspheres

Particle size analysis is important factor in the characterization of the physical stability of colloidal dispersions. LD measurements were performed to investigate the influence of glucose incorporation on the microspheres dispersion. Size distribution values of microspheres are presented in Table 2, as well the Span factor. Both types of microspheres, unloaded and glucose loaded presented unimodal distribution with no secondary peaks (Fig. 1C-D). The mean particle sizes ($LD_{4.5}$) were 94.03 and 86.62 μm for unloaded and glucose loaded microspheres, respectively. Increasing the internal phase with glucose led to a decrease on microspheres size, which agrees with other studies. In this line, it was verified that increasing internal phase ratio resulted in a slight decrease in mean size it was verified, during haemoglobin encapsulation [37].

Table 2
Particle size distribution, span and zeta potential of microspheres. K is the release rate constant, y_0 is the initial percentage drug released at the beginning of the experiment and doubling timing is the time it takes to double the % of glucose released.

Parameter		Glucose microspheres	Unloaded microspheres
Volume distribution	$LD_{4.3}$ (μm ± SD)	86.62 ± 27.19	94.03 ± 37.49
	$LD_{0.1}$ (μm)	53.19	37.19
	$LD_{0.5}$ (μm)	87.89	97.90
	$LD_{0.9}$ (μm)	120.50	140.70
Span factor		0.77	1.06
Zeta potential (mV± SD)		–32.54 ± 1.91	–31.29 ± 1.74
Encapsulation efficiency (%)	Surface	5.43 ± 0.32	–
	Inside	25.11 ± 5.51	–
Loading capacity (%)		20.45 ± 3.74	–
Percentage yield (%)		70.29 ± 2.61	–
Release parameters	y_o (%)	4.056	–
	K (h^{-1})	0.044	–
	Doubling timing (h)	15.57	–

In this study, using the same methodology, these differences were further evidenced. During the elaboration method, an increase of viscosity solution is coupled with a reduction of microsphere size by the decreasing surface tension and emulsion stability enhancement [38]. In concordance, the inclusion of the glucose in the alginate solution would result in such viscosity augmentation, and glucose loaded microspheres exhibited significantly smaller size. Moreover, the same effect was observed in the polydispersity with Span factor values of 1.06 and 0.77 for unloaded and loaded microspheres, indicating a slightly broader particle size distribution in unloaded microspheres.
The morphological evaluation of the microspheres by SEM (Fig. 2) showed a homogeneous aspect with sphericity, equally no aggregation was observed. The particle shape observed by SEM was more irregular than optical microscopy. This is due to the

oil in the microspheres leaked out of shell which led to the shrinking and deformation of the microspheres [39]. Unloaded microspheres showed slightly smoother surface (Fig. 2C) than glucose loaded (Fig. 2D). It could be justified by an increase in the frequency of collisions during emulsification process, resulting in a rough surface, due to a high viscosity of the internal phase, and thus a need of higher share force to separate the oil phase into droplets. However, the agitation used to prepare the particles was the same in alginate and in glucose loaded microspheres.

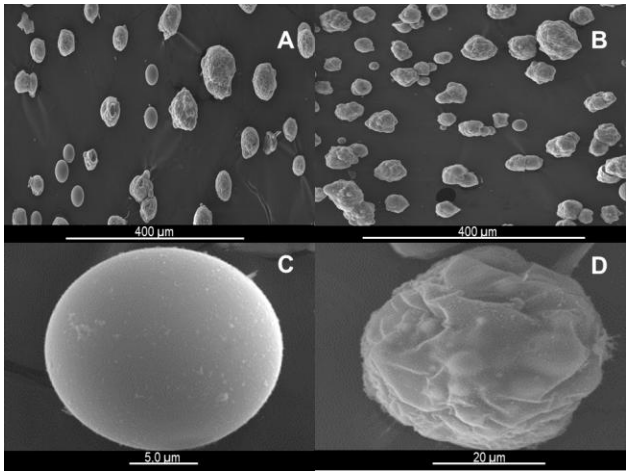

Fig. 2. Scanning electron microscope micrographs of unloaded alginate microspheres (A and C) and glucose loaded alginate microspheres (B and D).

Measurements of ζ derived from photon correlation spectroscopy are shown in Table 2. Both types of microspheres exhibited negatives ζ values, –31.29 and –32.54 mV for unloaded and glucose microspheres. The inclusion of the glucose did not significantly alter ζ. This negative charge could be attributed to the presence of polymeric terminal carboxylic groups provided by the acidic groups of alginate on the microsphere surface. A ζ less than −30 mV or higher than +30 mV can be an indicator to assure the stability of particulate systems. This ζ value could explain that aggregation phenomenon with cells was easily preventable, due to the electrostatic repulsion force between microsphere surface and cell (−12.07 mV).

Fig. 3 shows the FTIR spectra graphs of glucose, unloaded microspheres and glucose loaded microspheres. Glucose displays the following absorption bands: *v* (O-H) between 3570 and 3120 cm^{-1}, *v*(C-O) between 1230 and 1000 cm^{-1}, and *v*(C-O-C) between 1275 and 800 cm^{-1}. The two latter bands, the C-O region, are known to be the most specific of this molecule [40].

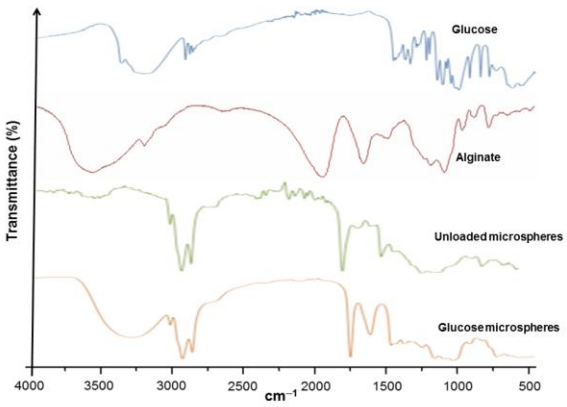

Fig. 3. FTIR spectra of glucose, alginate, unloaded alginate microspheres and glucose loaded alginate microspheres.

Alginate show different peaks: an antisymmetric stretch at 1596 cm^{-1} and a symmetric stretch at 1412 cm^{-1} due to the carboxylate group [30]. Cross-linking of alginate by Ca^{+2} was shown by a decrease in the wave number of the carbonyl peak from 1650 to 1465 cm^2. Alginates present different proportions or alternating patterns of guluronic and mannuronic units. The presence of these acids can be identified from their characteristic bands: while the guluronic units originate a band at approximately 1025 cm^{-1}, the mannuronic units originate a band at approximately 1100 cm^{-1}. Thus, the guluronic/mannuronic concentration ratio can be inferred from the relative intensity ratio of the 1025 and 1100 cm^{-1} bands. As it can be seen in Fig. 3 the alginate used in this study has higher proportion of guluronic. High mannuronic content alginates are immunogenic and approximately 10 times more potent in inducing cytokine production compared with high guluronic alginates [41]. The spectra showed that the characteristic bands of unloaded microspheres are maintained in the loaded microspheres indicating that glucose did not induce subsequent modifications in the structure of alginate.
The values of PY, LC and EE of several batches of glucose loaded microspheres are listed in Table 2. Concretely, the main factors of the vehiculization of the glucose were 71.73 %, 21.37 % and 30.54 %, respectively. Compared to surface adsorption onto the preformed microspheres, the glucose entrapment into the polymeric shell resulted in a much better loading.

3.3. *In vitro* release of glucose from microspheres

Glucose loaded microspheres were subjected to *in vitro* release studies in the presence of packaging media. Fig. 4 shows the release pattern, within 24 h, 13.81 ± 4.61% of glucose was released from the microspheres. This release rate increased after 24 h and 48 h from 31.63 ± 11.22% to 100.45 ± 25.34%. It is expected that glucose release from cross-linked alginate microspheres occurred as a result of swelling and erosion of the polymer. Based on the above described experimental findings, various mathematical approaches were used to describe the observed *in vitro* release rates, exhibiting an increase in growing velocity as time increases. The exponential growth model, defined as the mathematical equation [$y = y_0 \times e^{K \cdot x}$] was the most appropriate to describe the kinetics of glucose release from elaborated microspheres with a r^2 of 0.9991 and AIC 14.17, where K is the release rate constant (h^{-1}) and y_0 is the initial percentage glucose released at the beginning of the experiment (%).

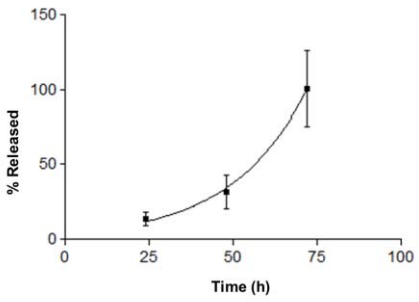

Fig. 4. Percentage glucose cumulative release from alginate microspheres (error bars ± SD, n = 3).

Pharmacokinetics parameters are reported in Table 2, in this context, doubling timing is the time it takes to double the percentage of glucose released (h). The mathematical model shows an initial percentage of glucose released of 4.056 ± 1.022, which can be attributed to the surface associated glucose (5.43 ± 0.32), followed by an exponential release. This means that glucose release from microspheres is exponentially proportional to the released amounts from the dosage form offering fast release of glucose. Two main factors may affect the control of the glucose release, the porous structure and hydrophilic character of polymer. The hydrophilicity characteristics of the polymeric particles also contribute to the degradation rate, and hence, drug release.

3.4. Effect of glucose loaded microspheres on cell viability

On passage 3, the average viability of the hMSCs cultured before to be packed was 95.1 ± 1.2%. The same cells were packed in four different media (A, B, C, D) and the average viability decreased throughout the assay. Viability measurements are represented in Fig. 5A.

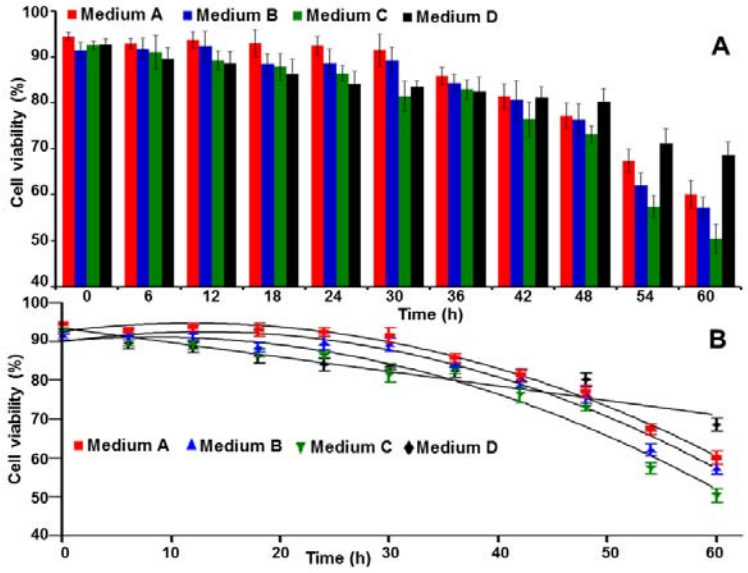

Fig. 5. Cell viability in different media (A). Mathematical model graphs after fitting experimental data of cell viability over time (B).

Medium A showed that viability was greater than 90% for 30 h after packaging (91.5 ± 3.5%), however after 48 h viability declined below 80% (77.2 ± 2.7) as medium B (76.3 ± 3.5%). Medium C maintained the viability above 80% for less time, up to 36 h (82.9 ± 2.1%). Medium D showed that viability was greater than 80% for 48 h after packaging (80.2 ± 2.85%), however decreased more rapidly from 12 h. Although medium D showed better results at 48 h, our study demonstrated that it was the medium in which viability decreased more rapidly during the first hours, and thus the least suitable. These results indicated greater cell viability in medium A in which glucose microspheres were included. Whilst, medium C was the least effective, buffer solution caused a rapid and significant decrease on cell viability. Statistically, medium A exhibited significant differences for the first 24 h when compared with other media. From 24 h up to 36 h, medium A presented better cell viability than C and D media ($P \leq 0.01$). After 36 h no significant differences were observed among A, B and D, being C the worst assayed medium.

In general, there was a progressive loss of viability but the results indicated that hMSCs had better survival rate when they were packed in medium A until 30 h as compared to the packaging media B, C and D. Although the control medium is suitable as vehicle for MSCs, our study demonstrated greater cell viability in media with glucose microspheres for the first 30 h. Contrary, in medium C, with phosphate buffer, a greater decrease of cell viability during the first 36 h was observed when compared with A (without buffer). Thus, the results showed that the buffer solution caused a rapid and significant decrease of cell viability.
According to the smallest AIC and r^2 value closest to 1, it was observed that second order polynomial model was the best to describe statistically the kinetic viability of medium A, B and C. On the other hand, first order polynomial was the most suitable for medium D. As shown in Fig. 5B. Then the almost constant maintenance of cell viability was more evidenced in the parabolic than linear behaviour, in this case, cell viability followed a downward trend from the first minutes. Contrary, the quadratic behaviours provided a constant cell viability, among them, medium A in a more stable and sustainable manner.

3.5. Microbiological studies

Sterility testing was performed on the hMSCs during culture (passage 3) and after stability study to ensure no contamination. When the incubation period had finished all tested samples were observed and no turbidity was exhibited, confirming the absence of contaminating microorganisms. These results showed that microspheres maintained the aseptic conditions of the final cell suspension.

3.64. hMSCs immunophenotypic analysis

Analysis revealed that all populations (before and after packing with glucose microspheres) were positive (> 95%) for mesenchymal markers (CD13, CD29, CD90 and CD105) and negative (< 10%) for endothelial and hematopoietic lineage markers (CD34, CD45, CD31 and HLA-II) as is shown in Fig. 6. Fluorescence cytometry between MSCs before to be packed, and in both cultures, with and without microspheres showed no differences when the MSCs were stored at 4 ºC for 48 h.

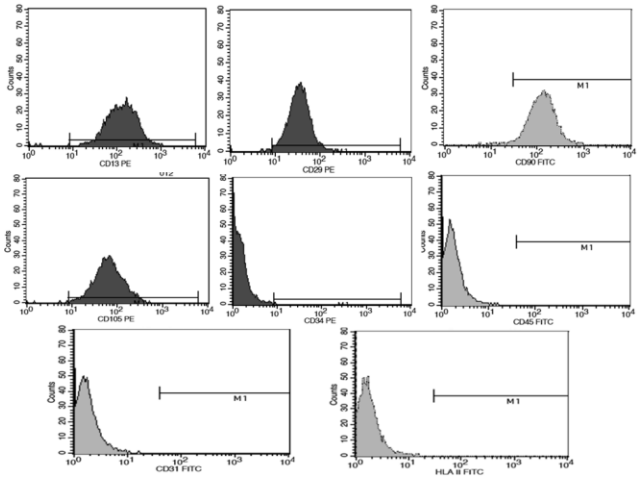

Fig. 6. Inmunophenotipic characterization of the hMSCs suspension after incorporating glucose microspheres (Medium A) for 48 h at 4 ºC.

3.7. Physicochemical characterization of the final MSCs –based medicine

3.7.1. Rheological studies

The rheological characterization was conducted in order to evaluate changes induced by the inclusion of glucose microspheres on the cell suspension. Rheological disturbances can play also an important role in the intramuscular administration of the final cell suspension. In fact, the viscosity of suspensions is closely related with physical and structural stability. Viscosity is also strongly influenced by factors such as aggregation of hMSCs. For this reason the rheological characteristics of unstirred cell suspension were also studied. The flow and viscosity curves of the suspensions (shear stress versus shear rate, and viscosity versus shear rate) are shown in Fig. 7.

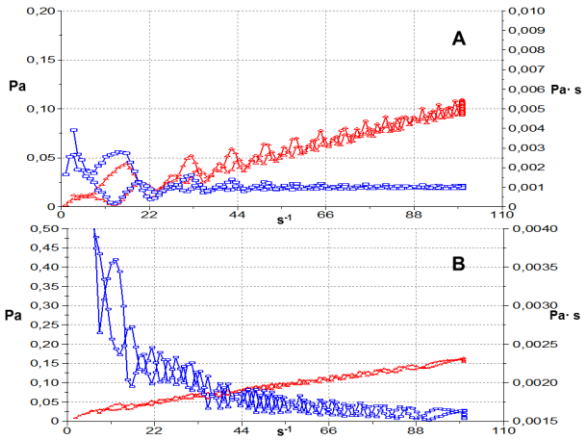

Fig. 7. Cell suspension rheograms. It shows the shear stress (Pa) (in red) and the viscosity (Pa·s) (in blue). Unstirred sample (A), and stirred sample (B).

The sample unstirred (Fig. 7A) exhibited Newtonian behaviour and had lower viscosity value (1.011 ± 0.05 mPas), which indicated that there was migration of aggregates/flocculates.

Sample analysed after been shaken (Fig. 7B) exhibited an pseudoplastic behaviour, where most aggregates were separated as a consequence of shear forces, which was sufficient to break-up the weak reversible flocculation. Viscosity value of the finished cell suspension in this case was 1.533 ± 0.044 mPas. Rheological data of this sample were fitted to mathematical models. Based on the lowest chi-square value and the r^2 closest to 1 (0.0021 and 0.9926, respectively), Cross model was statistically the best, showing that flow behaviour was not influenced by time and/or temperature. However, Newton model provided the worst overall prediction of rheological behaviour, 0.0088 (chi-square) and 0.9699 (r^2). Cross model is a rheological model that combines four parameters and covers the entire shear rate range [42]. As a rule, the four parameter models are difficult to apply because there is seldom enough data to allow good model fitting. However, they represent the best results in predicting the behaviour of non-Newtonian fluids. These results may have important implications for the administration because the success of the injection is dependent upon limiting shear forces of the needle wall causing cell lysis mainly produced by turbulent flow [43].

3.7.2. Morphological analysis

To determine whether the hMSCs suspension with microspheres maintained their morphologic characteristics, they were tested after 48 h storage the in each medium at 4 ºC and before packing. The adherent cultured hMSCs exhibited a homogeneous population with a fibroblast-like morphology when observed under a light microscope before to be packed (Fig. 8). Equally, no morphological differences were found when the cells were plated packed.

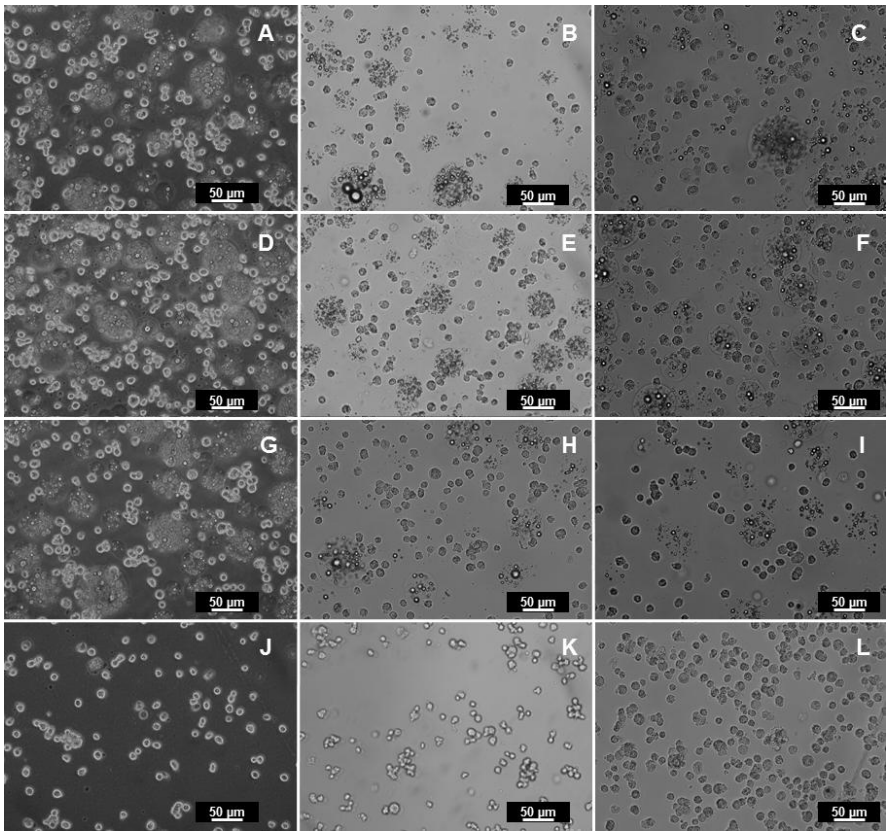

Fig. 8. Morphology of hMSCs for stability study in four media after different storage period at 4 ºC. Panel A, 0 h in medium A; panel B, 24 h in medium A; panel C, 48 h in medium D; panel D, 0 h in medium B; panel E, 24 h in medium B; panel F, 48 h in medium B, panel G, 0 h in medium C; panel H, 24 h in medium C; panel I, 48 h in medium C, panel J, 0 h in medium D; panel K, 24 h in medium D; panel L, 48 h in medium D.

As regard cell aggregation phenomena, imagines showed no overall cell aggregation. However, in medium D after 24 h slightly cell aggregate signs could be seen. Therefore it can be concluded that the packaged finished medicinal product in medium A (containing glucose microspheres) did not exhibit hMSCs aggregation in suspension ensuring a good acceptance.

3.7.3. Optical characterization of the stability

Stability evaluation is generally a crucial point in the scenario of advanced therapies. The long-term physical stability of cell-microspheres suspensions was tested by evaluating the photon backscattering profiles of the various samples. Turbiscan®Lab is considered as a device which predicts the stability, being able to detect destabilization before than the classical stability methods (microscopy, spectroscopy or turbidity). Moreover, it provides real-time information on the destabilization process based on the variation of backscattering. When sedimentation process is produced (migration of cells or microspheres from the top to the bottom), a backscattering increase versus time at the bottom of the sample is observed. When the sample suffers a creaming process, an increase of transmission versus time on the top of the vial is observed. If the destabilization phenomenon occurs due to aggregation (migration of cells from the bottom to the top), a backscattering increase versus time can be observed over the whole height of the sample [44]. If backscattering profiles have a deviation of $\leq \pm 2\%$ it can be considered that there are no significant variations. Variations up to $\pm 10\%$ indicate instable formulations. Fig. 9 shows backscattering profile of the final product containing

hMSCs and glucose microspheres corresponding to measurements on different hours. The left side of the curves corresponds to the bottom of the vial, whereas the right side corresponds to the top.

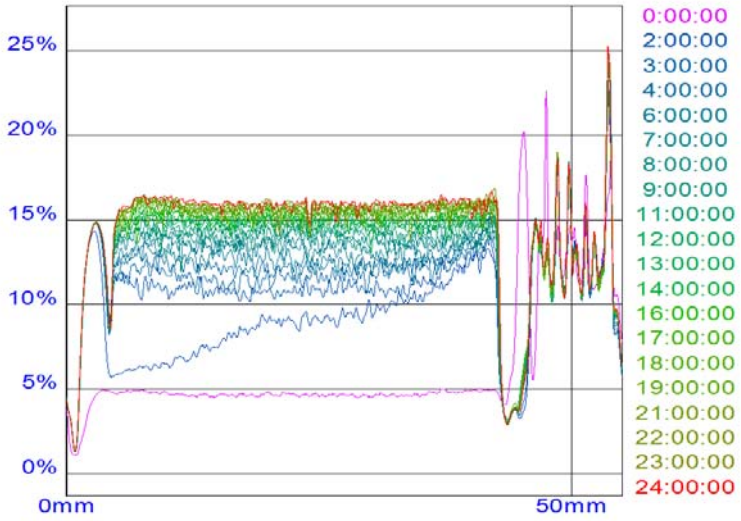

Fig. 9. Backscattering profile of the final cell product. The left side of the curve corresponds to the bottom of the vial, whereas the right side corresponds to the sample behaviour on the top.

The region below 2 mm marks the metal base and the strong decay of backscattering above 48 mm the beginning of the free surface of the sample. Both creaming and flocculation mechanisms were involved. It can be observed that the initial dispersion presented a backscattering value about 5% and there were no changes in backscattering for the first 2 h. Between 2 and 24 h, a progressive backscattering increase was observed and suspension developed a “delayed” or “hindered” sedimentation. This phenomenon could be attributed to the sample flocculation, possibly due to the formation of cells aggregates. The sedimented volume is high (the aggregates extend to roughly the whole volume of the suspension), it might be due to the particle size of the microspheres.

The open flocculi will settle slowly, and a long time will thus be needed for producing relatively compact sediments. Furthermore, because of the open structure of the aggregates, they will enclose comparatively large volumes of the supporting medium. Hence, the average particle–particle, cell-cell or particle-cell distances will be rather large, and the particles can get separated even after a mild shaking, so the suspensions will be easily re-dispersible. The peak that appeared at the top of the tube was indicative of creaming, presumably caused for migration of cellular components as lipid membranes from dead cells. Finally, after 11 h approximately there was no evolution in backscattering, indicating that the suspension was starting to stabilize.

It could be concluded that before the future administration, the injectable cell suspension should be re-dispersed just before using by gentle shaking in order to get a homogeneous hMSCs suspension, avoiding undesirable effects.

4. Conclusion

For the foreseeable future, cell therapy development will be based on not only in the search for new therapies but also in the study and design of these medicines. Particular

attention will be paid to stability, safety and to expand the margins of use over time until administration.
The present study covers a comprehensive technological development of a packaging media. The media is composes of a nutrient (glucose) encapsulated into alginate microspheres. These particles are able to enhance the functionality of an hMSC suspension. For this reason, this strategy could be broadly applicable to various therapeutic approaches in which prolonged viability of cells is necessary. However future works are claimed to improve the stability of cells during a longer period of time.

Acknowledgments
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CAPÍTULO IV.
DISCUSIÓN

Las investigaciones científicas de las últimas décadas dirigen el campo de la Medicina Regenerativa hacia una nueva era, donde las terapias convencionales se apoyarán en los avances biotecnológicos cuyo máximo exponente son las Terapias Avanzadas.

En concreto, la Terapia Celular presenta las células madre como nuevos activos, los cuales deben ser producidos y controlados conforme a la legislación vigente, al igual que la fabricación de medicamentos clásicos, en sus diferentes formas de dosificación. Las células deben mantener sus características biológicas intrínsecas además de ser viables, seguras y eficaces hasta el momento de su administración al paciente.

Este trabajo propone el desarrollo y estudio de un CTMP basado en hMSCs derivadas de tejido adiposo, para el tratamiento de CLI en pacientes diabéticos tipo II. La investigación se ha centrado en el estudio y desarrollo de todos los procesos de fabricación, calidad y caracterización del producto final, incluyendo los aspectos legales y regulatorios que le son de aplicación a nivel europeo y nacional.

La primera etapa de este trabajo comprende una revisión exhaustiva sobre los últimos avances biotecnológicos destinados al desarrollo de nuevos medicamentos. Para ello se describen los fundamentos y campos de actuación de la Terapia Celular, Génica y la Nanomedicina.

La **Terapia Celular** se basa en la utilización de células madre cuyas principales características son su capacidad de autorrenovación y su potencialidad a diferenciarse a células y/o tejidos especializados (Brignier y Gewirtz; 2010), haciendo de ellas una herramienta crucial para el desarrollo de nuevos tratamientos frente a múltiples patologías: disfunciones hormonales (DM tipo I o déficit en la hormona del crecimiento) (Zhao y Mazzone, 2010; Perl y cols., 2010), lesiones cardiovasculares (insuficiencia cardíaca congestiva, enfermedad arterial periférica, infarto de miocardio, pacientes trasplantados) (Lara y cols., 2008; Cook y cols., 2009; Schoenhard y Hatzopoulos, 2010), enfermedades neurodegenerativas (Parkinson, Alzheimer, Corea de Huntington) (Park y cols., 2010), lesiones osteoarticulares (Teo y Vallier, 2010; Lee y cols., 2010), distrofias musculares o lesiones en las que hay que regenerar el epitelio, como la fístula perianal (Meregalli y cols., 2009). Los principales avances científicos se han llevado a cabo mediante HSCs, MSCs, ESCs y células iPS (González y Bernad, 2012; Keller y cols., 2012).

La **Terapia Génica** consiste en transferir al organismo receptor (paciente) material genético modificado y/o corregido con la finalidad de prevenir o tratar una enfermedad (Muñoz- Ruiz y Regueiro, 2012). La administración del gen terapéutico se puede llevar a cabo por vía sistémica *in vivo* o *ex vivo,* a través del aislamiento de las células y corrigiendo la mutación para ser administradas al paciente (Fischer y cols., 2002).

La **Nanomedicina** se basa en el control, la reparación y la mejora integral de todos los sistemas biológicos humanos, trabajando a nivel molecular con dispositivos de ingeniería y nanoestructuras para lograr beneficios en áreas de diagnóstico y pronóstico, protección de tejidos trasplantados y en farmacocinética (Desai, 2002; Freitas, 2005; Álvarez-Salas, 2008).

Los avances en el área de Terapia Celular, Terapia Génica, junto con la Ingeniería Tisular han dado lugar a las llamadas **Terapias Avanzadas** y con ello a los Medicamentos de Terapias Avanzadas (ATMP) que incluyen medicamentos a base de células, genes, tejidos y la combinación de todos ellos con productos sanitarios (CTMP, GTMP, TEP y PC).

Para el desarrollo del medicamento celular final basado en hMSCs objeto de este trabajo ha sido necesario definir los **requerimientos legales de un CTMP**.

Los tres organismos reguladores más importantes a nivel internacional que regulan el desarrollo de los medicamentos para uso humano son: la Food and Drug Administration (FDA) en Estados Unidos, el Ministerio de Salud, Trabajo y Bienestar (MHLW del inglés Ministry of Health Labour and Welfare) de Japón y la Agencia Europea de Medicamentos (EMA del inglés European Medicines Agency).

La armonización de los requisitos técnicos en el plano internacional se logra a través de la Conferencia Internacional sobre Armonización de los requisitos técnicos para el registro de productos farmacéuticos para uso humano (ICH del inglés International Conference on Harmonization). Su principal misión es lograr la armonización en las directrices técnicas sobre la calidad, seguridad y eficacia de los nuevos medicamentos (Dixon, 1998). Actualmete en el área de las Terapias Avanzadas la FDA y la EMA trabajan conjuntamente para establecer directrices similares con una regulación equiparable.

En Europa un CTMP está regulado de forma específica por la Directiva 2001/83/CE del Parlamento Europeo y del Consejo del 6 de noviembre de 2001 por la que se establece un código comunitario sobre medicamentos para uso humano, modificada posteriormente por las Directivas 2003/63/CE de la Comisión de 25 de junio de 2003 y 2009/120/CE de la Comisión del 14 de septiembre de 2009 por la que se establece un código comunitario sobre medicamentos para uso humano, en lo que se refiere a los medicamentos de terapia avanzada.

En el año 2007 las Directivas publicadas en este área fueron ampliadas por el Reglamento (CE) Nº 1394/2007 del Parlamento Europeo y del Consejo del 13 de noviembre de 2007 sobre medicamentos de terapia avanzada y por el que se modifican la Directiva 2001/83/CE y el Reglamento (CE) no 726/2004.

Para el asesoramiento y control específico de un ATMP, la EMA ha creado un Comité de Terapias Avanzadas (CAT del inglés Committee for Advanced Therapies). Se trata de un comité multidisciplinario que incluye la participación de los mejores expertos de todos los estados miembros de la Unión Europea (EU del inglés European Union). Su principal responsabilidad es emitir un informe al Comité de Medicamentos de Uso Humano (CHMP del inglés Committee for Medicinal Products for Human Use) valorando el ATMP para su autorización centralizada final, evaluando los datos de calidad, seguridad y eficacia presentados. Otra de sus funciones es el asesoramiento y la definición del producto de interés, ya que en ocasiones es fácil saber que se trata de un ATMP, pero no de como clasificarlo en base a si es un CTMP, GTMP, TEP o PC.

Un CTMP se define como aquel producto que contiene células madre viables sometidas a una modificación sustancial y/o cuya función biológica es distinta en el donante y receptor. Por tanto el medicamento a desarrollar en este trabajo debido a que las hMSCs sufren un cambio sustancial al ser expandidas *ex vivo*, debe considerarse CTMP.

Un producto celular que no cumpla las características de CTMP, se considerará Terapia Celular pero no medicamento. Estos productos son considerados trasplantes y están regulados por la Directiva 2004/23/CE del Parlamento Europeo y del Consejo del 31 de marzo de 2004 relativa al establecimiento de normas de calidad y de seguridad para la donación, la obtención, la evaluación, el procesamiento, la preservación, el almacenamiento y la distribución de células y tejidos humanos, por la Directiva

2006/17/CE de la Comisión del 8 de febrero de 2006, por la que se aplica la Directiva 2004/23/CE del Parlamento Europeo y del Consejo en lo relativo a determinados requisitos técnicos para la donación, la obtención y la evaluación de células y tejidos humanos y la Directiva 2006/86/EC de la Comisión del 24 de octubre de 2006, por la que se aplica la Directiva 2004/23/CE del Parlamento Europeo y del Consejo en lo que se refiere a los requisitos de trazabilidad, la notificación de las reacciones y los efectos adversos graves y determinados requisitos técnicos para la codificación, el procesamiento, la preservación, el almacenamiento y la distribución de células y tejidos humanos. Estas directivas se aplican a los productos clasificados como no ATMP.

Para un ATMP, estas directivas son de aplicación únicamente en relación con la donación, obtención y evaluación de las muestras biológicas (biopsia) de las cuáles se obtendrán las células, genes y/o tejidos.

El diseño y desarrollo de un CTMP es complejo, debiéndose realizar múltiples estudios en cada una de sus fases. Las principales fases de desarrollo son: investigación básica, investigación preclínica, investigación clínica, fabricación, autorización, registro, comercialización y post-autorización.

De todas ellas, las más relevantes son la fase preclínica en la que se deben realizar estudios de toxicidad y actividad biológica en animales, junto con la fase clínica en la que se estudiará la seguridad y eficacia en humanos (Ruiz y Abad-Santos, 2010) así como la fabricación del producto celular como medicamento. Estas etapas deben llevarse a cabo bajo estándares de Buenas Prácticas de Laboratorio (GLP del inglés Good Laboratory Practice), Buenas Prácticas Clínicas (GCP del inglés Good Clinical Practice) y Normas de Correcta Fabricación (GMP).

Este trabajo estudia el diseño y desarrollo de los procesos de **fabricación de un CTMP** con hMSCs, en concreto se estudia la instalación, personal, proceso de producción, calidad, estabilidad y los requerimientos galénicos de la formulación.

La fabricación de células madre como medicamentos debe llevarse a cabo bajo estándares GMP, debiendo cumplir los nueve capítulos de la Parte I- Requisitos básicos para medicamentos, Parte II- Requisitos básicos para sustancias activas usadas como materiales de partida y los siguientes Anexos: Anexo I- Fabricación de medicamentos

estériles, Anexo 2- Fabricación de sustancias activas biológicas y medicamentos biológicos para uso humano, Anexo 8- Toma de muestras de materiales de partida y de almacenamiento, Anexo 13- Fabricación de medicamentos en investigación, Anexo 14- Fabricación de productos derivados de sangre o plasma humano, Anexo 15- Cualificación y validación, Anexo 16- Certificación por una persona cualificada y liberación, Anexo 17- Liberación paramétrica, Anexo 19- Muestras de referencia y muestras de retención y Anexo 20- Gestión de riesgos para la calidad (EudraLex vol. 4).

Además es de aplicación la Guideline on Human Cell-Based Medicienal Products, del 21 de mayo del 2008 (CHMP/410869/2006) de la EMA, en la que se tiene en cuenta la heterogeneidad de los productos basados en células humanas.

La primera fase en la fabricación de un CTMP es el diseño, construcción y validación de la infraestructura (laboratorio de producción celular) y del equipamiento requerido. La principal característica de la instalación es que debe propiciar una fabricación aséptica. Estas instalaciones se denominan salas blancas, salas GMP o salas limpias, en ellas se concentran tres requerimientos: protección del producto, protección del personal y protección del medio ambiente (Yufit y cols., 2013; Sensebé y cols., 2013).

Una sala blanca es una sala especialmente diseñada para obtener bajos niveles de contaminación. Estas salas deben tener los parámetros ambientales estrictamente controlados: partículas en aire, temperatura, humedad, flujo de aire, presión interior del aire e iluminación (Favero y cols., 1966). En todos los sistemas de acondicionamiento de aire, el sistema de filtración a seleccionar, debe prever la retención apropiada de las partículas procedentes del exterior. El riesgo de contaminación cruzada debe ser necesariamente evaluado para diseñar correctamente una sala blanca (Nelson-Rees y cols., 1981; Doblhoff-Dier y cols., 2009).

Las salas blancas para la fabricación de productos estériles se clasifican en diferentes entornos según las características requeridas del aire:

Grado A: zona local donde se realizan operaciones específicas de alto riesgo tales como la zona de llenado, de cultivo, de cambios de medio, de envasado y de realización de operaciones asépticas; Grado B: entorno para la zona de grado A en el caso de

preparación y llenado aséptico; Grados C y D: zonas limpias para realizar fases menos críticas de la fabricación de productos estériles.

Las instalaciones de una sala blanca deben presentar los siguientes compartimentos mínimos: zona de entrada, almacén, vestuario sucio, vestuario limpio, distribuidor estéril, sala de producción, área acondicionamiento, laboratorio de control de calidad, área de criopreservación y muestroteca. Las zonas más importantes por ser las más cercanas al producto son aquellas de grado A (cabinas de flujo laminar y aisladores) y grado B que engloba las sala de producción, distribuidor y vestuario limpio.

Una vez diseñada la sala blanca y equipada con todo lo necesario (cabinas de flujo laminar, incubadores, microscopio, centrífugas, etc.) se procede a la expansión *ex vivo*, para lo cual es necesario validar cada uno de los procesos implicados en la fabricación de hMSCs como medicamentos. En concreto deben validarse los procesos de control ambiental, limpieza, esterilización de materiales y agua, desinfección de materiales, producción de MSCs, mediafill o validación aséptica de la producción celular, esterilidad y transporte de la muestra biológica y producto final.

El proceso de fabricación de un CTMP es complejo, su primer factor limitante es la cantidad de materia prima biológica (biopsia) disponible, ya que se trata de una materia prima finita. Las células madres son obtenidas de una biopsia del paciente (autólogo) o del donante (alogénico), y una contaminación del cultivo celular implicaría una nueva biopsia lo cual es agresivo y poco ético, por lo que han de extremarse todas las medidas posibles para evitar dicha contaminación (Coecke y cols., 2005; Stacey, 2011).

Por otro lado debido a las características de las células, estas no pueden ser esterilizadas en la fase final, por métodos químicos y/o físicos ya que podrían afectar a su viabilidad y a sus características intrínsecas, por lo que la fabricación aséptica de las mismas es crucial para asegurar su esterilidad. Además existe otro inconveniente referido a la esterilidad de las células, el cual compete a la técnica. El ensayo de esterilidad requiere 14 días para el dictamen final (Ph. Eur 2.6.1), sin embargo las células sólo son viables durante horas, debiendo llevar a cabo una liberación paramétrica del producto final basado en un plan de monitorización ambiental previo, junto con un plan de control de calidad y una buena gestión de riesgos.

En cuanto a la instalación y personal se llevó a cabo un **estudio de la contaminación ambiental bacteriana y fúngica en una sala blanca,** del Centro Andaluz de Biología Molecular y Medicina Regenerativa en Sevilla, con el fin de verificar que la producción de hMSCs podía llevarse a cabo en condiciones asépticas y minimizar por tanto el riego de contaminación del producto final.

Este estudio consiste en el estudio cualitativo y cuantitativo de la calidad del aire de dicha sala blanca durante la fabricación simultánea de hMSCs (derivadas de tejido adiposo) incluyendo: definición de los puntos de muestreo, frecuencia de muestreo, métodos de muestreo, identificación de microorganismos, identificación de los límites de alerta y las medidas correctivas para evitar la contaminación microbiana.

El ensayo se llevó a cabo durante un año (enero - diciembre del 2010), evaluando las áreas de grado A, B, C y D. El estudio microbiológico ambiental se realizó a través de un muestreo activo y pasivo del aire, además del control de las superficies de trabajo.

El control activo se llevó a cabo con placas de sedimentación y un muestreador de aire, aspirando un volumen de aire de 100 L/min (Andersen, 1958). Para el muestreo pasivo se utilizaron placas de sedimentación, que fueron colocadas en los puntos de muestreo durante 1 h (White, 1995). El control de superficies se realizó con placas de contacto durante 10 s. Los medios de cultivo fueron agar triptona soja (TSA) para baterías y agar sabouraud dextrosa cloranfenicol (SDC) para hongos, incubándose a 35 ºC durante 2 días y a 22 ºC durante 5 días, respectivamente. Tras el período de incubación, se determinó la presencia o ausencia de unidades formadoras de colonias (ufc) y se caracterizaron morfológicamente con una tinción de Gram. Las bacterias fueron identificadas por test bioquímicos (sistema API) y los hongos se compararon con el atlas of clinical fungi (Hoog y cols., 2000, 2 ed).

En concreto, 261 puntos fueron muestreados durante doce meses, obteniendo un total de 6.264 muestras (TSA/SDC), de las cuales sólo 231 presentaron crecimiento microbiológico. Los resultados cuantitativos mostraron que en grado A no creció ninguna ufc, pero sí en B, C y D, siendo el número de ufc mayor conforme se disminuyó de grado. Por lo que el grado D fue la zona donde se detectó mayor crecimiento de ufc, siendo la zona de menor riesgo para la fabricación de las células.

Los resultados cuantitativos determinaron que la especie encontrada en mayor porcentaje fue Staphyloccocus (67.8%), concretamente *S. epidermis* (38.2%).

Respecto al análisis cualitativo, *S. epidermis* es de origen humano, y su presencia se debe al personal de la sala, por lo que serán necesarias medidas higiénicas y un vestuario específico para evitar la contaminación del producto celular por esta bacteria a pesar de que no representa riego para el producto (White y Bailey, 1985; Sheraba y cols., 2010). Todos estos resultados concluyeron que la sala blanca es idónea para la fabricación aséptica, ya que a pesar de existir crecimiento microbiano, éste se encuentra dentro de los límites descritos en el Anexo I de las GMP.

Una vez definidas las condiciones asépticas de la instalación para la fabricación de hMSCs como medicamento, se llevó a cabo el **diseño de un programa de control de calidad** que abarca todos los materiales de partida incluyendo el material biológico (biopsia), los productos intermedios o subcultivos generados y el producto final.

La fabricación de hMSCs implica una expansión *ex vivo* durante un largo período de tiempo que puede llegar hasta los dos meses y que dependerá del número de células final (dosis) a fabricar (Santos y cols., 2011; Fekete y cols., 2012). Esta expansión conlleva un riesgo de contaminación por agentes microbiológicos que podrían afectar a la calidad y la seguridad del CTMP pudiendo causar reacciones adversas en el paciente (fiebre, escalofríos, infecciones, shock séptico irreversible) e incluso la muerte (Guo y cols., 2011). Por tanto es necesario estandarizar y validar todos los procedimientos y técnicas analíticas que aseguren el control de calidad durante la fabricación de un CTMP lo que justifica el diseño de un Programa de Control de Calidad (QCP del inglés Quality Control Program).

A lo largo de todo el proceso de fabricación de un CTMP se deben definir los puntos críticos y los controles de calidad a realizar con el fin de obtener un medicamento seguro desde el punto de vista de calidad microbiológica.

El proceso de fabricación de las hMSCs, consta de varias etapas: (i) biopsia del tejido adiposo y transporte; (ii) preparación de todos los materiales de partida (fungible y reactivos); (iii) aislamiento de las células y obtención del cultivo primario; (iv) obtención del Banco Celular Maestro (MCB del inglés Master Cell Bank); (v)

subcultivo (pases); (vi) obtención del Banco Celular de Trabajo (WCB del inglés Work Cell Bank); (vii) subcultivo y (viii) envasado y acondicionamiento del producto final. En concreto, la recepción de materiales y su aprobación, como la obtención del MCB, WCB y producto final son las etapas más críticas debido a su implicación en el proceso.

El QCP se basó en el estudio microbiológico de bacterias, micoplamas y endotoxinas en diferentes fases: MCB, WCB y producto final. Las técnicas analíticas llevadas a cabo incluyeron el estudio de esterilidad, tinción de Gram, detección de micoplasma, detección de endotoxinas y monitorización en proceso. Cada técnica analítica fue validada por triplicado.

Respecto a los materiales de partida, todos ellos deben ser controlados con el fin de evitar la contaminación del producto celular. Es necesario establecer unas especificaciones internas para la muestra biológica, reactivos y fungibles. En el QCP diseñado, a cada muestra biológica de tejido adiposo se le realizó una serología, determinando la presencia del VIH, virus de la hepatitis B y de la hepatitis C. Por otro lado, se analizaron los reactivos y fungibles necesarios para la expansión y envasado de las hMSCs, acorde a un ciclo de calidad concertada con el proveedor, en el que se analiza un lote de cada tres lotes recepcionados, certificando y verificando que son estériles y libres de endotoxinas.

El **ensayo de esterilidad** es uno de los test más cruciales debido a que se requieren 14 días para la lectura del resultado. Por ello es determinante realizar esta prueba en las fases más críticas del proceso de fabricación celular como son la obtención del MCB y del WCB; obteniendo por tanto resultados previos a la liberación del producto final. Además este ensayo debe llevarse a cabo en el producto final, no pudiendo liberarse definitivamente el producto hasta 14 días después, por lo que es necesaria una liberación paramétrica o temprana debido a la escasa viabilidad de las células. Esta liberación paramétrica debe ser avalada por los resultados de esterilidad realizados en las fases previas y por las validaciones llevadas a cabo anualmente (mediafill) que aseguran la asepsia del proceso.

El test de esterilidad se realizó por inoculación directa de 1 mL de suspensión celular en dos medios de cultivo microbiológicos (Ph. Eur 2.6.1): caldo de tioglicolato y penasa (TPB del inglés Thioglycollate Penase Broth), para la detección de bacterias aerobias y,

caldo de penasa soja y triptona (TSPB del inglés Tryptic Soy Penase Broth) para la detección de bacterias anaerobias y hongos; incubándolos durante 14 días a 35 ºC y 2 ºC respectivamente. La lectura de los resultados se realizó observando la turbidez del medio comparándolo con un medio control de cloruro sódico.

La validación del test de esterilidad es la validación más importante de todo el proceso de producción celular, verificando que ninguno de los medios ya sea de expansión o de envasado, ni los distintos reactivos y materiales, interfieren en el test, pudiendo originar falsos negativos.

La validación se realiza en dos tipos de suspensión celular: hMSCs en subcultivo con medio de expansión y hMSCs en medio de envasado (producto final). La validación se basó en el límite de detección (LD) aceptado por la Farmacopea Europea, inoculando 30 ufc de seis microorganismos (*Clostridium sporogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Aspegillus niger y Candida albicans)* en cada uno de los medios a estudiar (TPB y TSPB) adicionando 1 mL de la suspensión con hMSCs.

Los resultados obtenidos demuestran que ninguno de los medios de suspensión interfieren en el test de esterilidad, a pesar de que el medio de expansión incorpora antibióticos (penicilina-estreptomicina), los cuales han sido previamente inactivados con penasa. Todo ello asegura las condiciones asépticas del proceso de fabricación, de la técnica y la validez del resultado de esterilidad obtenido.

La **detección de micoplasma** se lleva a cabo debido a su grave implicación en procesos infecciosos. Los micoplasmas representan a los microorganismos de vida libre autorreplicables más pequeños hasta ahora descritos. Presentan características fenotípicas y genotípicas particulares como son la ausencia de pared celular, formas pleomórficas, un genoma reducido pero con información genética capaz de sintetizar diversos tipos de enzimas que les han permitido destacar como parásitos exitosos (Razin y cols., 1998). La contaminación de un cultivo celular por micoplasma puede modificar las características biológicas de las células contaminadas, alterando la proliferación, inestabilidad genética, etc. Por otro lado, estos organismos son resistentes a la mayoría de los antibióticos adicionados comúnmente a los cultivos celulares (PIC/S, 2013), por

todo ello es esencial analizar si existe contaminación durante el proceso de fabricación de un CTMP.

Para su análisis se llevó a cabo la extracción y amplificación del ADN por PCR. Las bandas de amplificación fueron estudiadas por electroforesis y comparadas con un control positivo y otro negativo (Ph. Eur 2.6.7). Para la validación de este test se determinó el LD, el rendimiento y la reproductibilidad del procedimiento analítico con tres cepas de referencia *Mycoplasma orale* (NCTC 10112), *Acholeplasma laidlawii* (NCTC 10116) y *Mycoplasma fermentans* (NCTC 10117) (Dabrazhynetskaya y cols., 2011).

Los resultados mostraron que no existió contaminación por micoplasma durante la expansión *ex vivo.* No obstante se realizó un análisis de riesgo para la validación, determinando que aunque la incidencia de la infección por micoplasma en los cultivos celulares es baja, cuando sucede, tiene un origen humano (*Mycoplasma orale)* en la mayoría de los casos (Barile y cols., 1998; Young y cols., 2010). Por lo tanto es crucial que el personal este bien formado y cumpla medidas de contención higiénicas, además de unas buenas prácticas de laboratorio para evitar posibles contaminaciones.

El **ensayo de endotoxina** es imprescindible en cualquier análisis y control de un medicamento parenteral. Las endotoxinas bacterianas son principalmente lipopolisacáridos que se localizan exclusivamente en la membrana externa de bacterias gram negativas como *Escherichia coli*, *Salmonella spp., Shigella spp., Neisseria spp.* entre otros patógenos, (Bryans y cols., 2004; Brandenburg y cols., 2009). Dado que concentraciones demasiado altas de lipopolisacáridos bacterianos constituyen un grave problema de salud, es de suma importancia llevar a cabo un ensayo fiable para la determinación de la concentración de endotoxinas en el producto celular final.

El método de ensayo de endotoxina bacteriana se llevó a cabo por la técnica del lisado de amebocitos (LAL del inglés Limulus Amebocyte Lysate) (Ph. Eur 2.6.14) de la muestra en diferentes fases (MCB, WCB y producto final) de los tres cultivos ensayados.

La selección adecuada de la sensibilidad del reactivo LAL, y el límite de endotoxina concreto del producto celular a evaluar permite obtener resultados más fiables (Kaca,

1996). Por lo tanto para la validar el procedimiento analítico del ensayo de endotoxina, se llevaron a cabo dos estudios: la confirmación de la sensibilidad del lisado (LAL) y la prueba de factores de interferencia.

Los resultados obtenidos durante la validación confirmaron la sensibilidad del lisado para el CTMP analizado, mediante una evaluación visual de la gelificación; se obtuvo una sensibilidad del lisado de 0.03 UE/ml, verificando la detección de endotoxinas en una suspensión con hMSCs. Por otro lado se demostró que no existían factores de interferencia en la detección de endotoxinas en el CTMP, lo cual demuestra la especificidad de la técnica para el producto celular.

Por último el QCP incluyó la **monitorización en continuo del aire y personal** durante la manipulación del cultivo en cabina y durante el ensayo de esterilidad en aislador, con objeto de verificar las condiciones asépticas del ambiente y del personal, y poder descartar ser la causa de un resultado positivo en alguna de las otras técnicas descritas.

Las principales directrices que regulan los ensayos de calidad de un medicamento han sido redactadas por la EMA como "guidelines", además de lo descrito en las GMP, en la Farmacopea Europea, PIC´s, etc. Sin embargo cada laboratorio debe establecer un sistema de calidad adaptado a su propio proceso y a las características del medicamento a fabricar. En el trabajo aquí descrito se realizó la adaptación (en base al equipamiento, instrumental, reactivos, fungibles y productos a analizar) de los requerimientos básicos de calidad a la fabricación del CTMP objeto de estudio.

Los resultados no mostraron contaminación microbiológica en ninguna de las fases del proceso de producción de los tres cultivos estudiados. La estandarización y validación in situ de cada técnica analítica del QCP garantiza la calidad del producto final.

El QCP diseñado define los controles mínimos de calidad microbiológica de las hMSCs para ser administrados por vía parenteral, demostrando la idoneidad de cada una de las técnicas.

Otro aspecto importante en la fabricación de un CTMP es el **desarrollo galénico de la forma de dosificación.** Actualmente son diversas las formas farmacéuticas diseñadas para un CTMP, incluyendo suspensiones, bases sólidas deshidratadas, microcápsulas y

scaffolds biodegradables (Ruiz-Salmerón y cols., 2011; Baek y cols., 2012; Chlapanidas y cols., 2013; Elder y cols., 2013; Moshaverinia y cols., 2013; Levit y cols., 2013).

Como ya se ha comentado previamente, un CTMP se caracteriza porque las células deben ser viables en el momento de la administración, en concreto deben presentar una viabilidad superior o igual al 80% (Brinchmann, 2008). La viabilidad de las células disminuye en horas, siendo este uno de los principales inconvenientes de la Terapia Celular y por tanto una de las principales áreas de investigación actuales. De aquí que las últimas investigaciones se hayan focalizado en la búsqueda de sistemas o formulaciones que permitan mantener viables las células durante el mayor tiempo posible facilitando que dicho tratamiento llegue a un mayor número de pacientes.

Estudios de seguridad y eficacia han determinado que dosis celulares de hMSCs para administración parenteral de 1 x 10^6 células/kg paciente son adecuadas para obtener el efecto terapéutico buscado (Ra y cols., 2011). Por tanto la producción celular y posterior diseño galénico deberá tener en cuenta que el medicamento celular final estará constituido por 60-80 x 10^6 células.

Tras determinar la concentración celular del producto final se realizó un estudio con diferentes excipientes para seleccionar el mejor medio de envasado para las células, en base a la viabilidad de las mismas. La viabilidad celular se determinó mediante tinción de tripan blue (Louis y Siegel, 2011) y el recuento posterior en cámara de Neubauer.

Las hMSCs fueron envasadas en cuatro medios e introducidas en jeringas Luer-lock. Todas las jeringas fueron almacenadas en frío a 4 ºC durante 60 h. Cada 6 h la viabilidad celular de cada muestra fue estudiada. Los resultados demostraron que el mejor medio fue el compuesto por 50% de Glucosa 5%, 45% of Ringer Lactado y 55% de Albúmina 20% (Grifols, Barcelona, España), con una viabilidad de 82.2 ± 2.7%, alcanzadas las 48 h.

Definida la concentración y el medio de envasado, se realizó un estudio de la estabilidad de la suspensión celular final para el CTMP. En concreto se llevó a cabo un estudio de estabilidad térmica a 4 ºC, 8 ºC, 25 ºC y 37 ºC analizando la viabilidad celular de cada muestra cada 12 h durante 60 h. Los resultados de este estudio determinaron que las células mantiene una mejor viabilidad a 4ºC.

Así mismo se estudia el inmunofenotipo, la capacidad de diferenciación a adipocitos y osteocitos, estabilidad genética y esterilidad. Los resultados fueron comparados con un cultivo en pase 5, previo al envasado, para determinar si las condiciones de envasado (medio de envasado, tiempo y temperatura) y almacenamiento afectan a las características intrínsecas de las hMSCs, manteniendo o no la calidad y seguridad final.

La **caracterización inmunofenotípica** de las hMSCs antes y después de ser envasadas se llevó cabo por citometría de flujo laminar. Los resultados no mostraron ningún cambio, manteniéndose un fenotipo positivo (>95%) para los siguientes antígenos de superficie: CD13, CD29, CD90 and CD105 y negativos (<10%) para CD34, CD45, CD31 y CMH-II.

La **capacidad de diferenciación** *ex vivo* de las hMSCs a osteocitos y a adipocitos fue estudiada induciendo a las células a su diferenciación a través de medios de inducción: medio de diferenciación osteogénica y medio de inducción adipogénica (Lonza, Walkersville, MD, EE.UU.) (Pittenger y cols., 1999). La capcidad de diferenciación se confirmó tanto en las células no envasadas como en aquellas que conforman la suspension final.

La **estabilidad genética** se basó en el estudio del cariotipo por análisis de bandeo G (Bayani y Squire, 2004). Los resultados mostraron que las células en cultivo y posteriormente envasadas durante 48 h mantenían un cariotipo diploide, estable y normal (46, XX).

También se realizaron estudios de **esterilidad**, analizando si existe riesgo de contaminación de las células durante las 48 h en las que pueden ser almacenadas hasta su administración. Los resultados determinaron que las hMSCs mantenían su esterilidad durante todo el tiempo de almacenamiento.

Por otro lado se procedió a la **caracterización físico-química** de la formulación final. Con tal fin se realizaron estudios reológicos, morfológicos, medida de pH y estudios sobre la migración o agregación de las células en suspensión.

Los **estudios reológicos** son importantes en la administración parenteral de una suspensión celular, ya que la viscosidad de la suspensión puede estar influenciada por la agregación de las células. La caracterización reológica se llevó a cabo antes y después

de agitar la suspensión con el fin de evaluar los cambios inducidos por las células en el producto final. Las muestras no agitadas presentaron un comportamiento Newtoniano y un valor de viscosidad de 1.11 mPas. En cambio la suspensión agitada mostró un comportamiento no Newtoniano tipo pseuplástico y un valor de viscosidad de 1.575 ± 0.043 mPas, similar a la viscosidad del plasma de la sangre de 1,2 mPa s a 37 º C (Skalak y cols., 1989).

Las **características morfológicas** de las hMSCs se estudiaron por observación de la suspensión final cada 12 h a 4 ºC, 8 ºC, 25 ºC and 37 ºC, durante 48 h con un microscopio. Los resultados mostraron que la morfología fibroblastoide de las hMSCs se mantenía homogénea durante 48h a cualquier temperatura. Sin embargo la agregación celular se ve afectada por la temperatura de conservación, de forma que cuando la suspensión se mantiene a 37 ºC se aprecian agregaciones a las 24 horas, en cambio cuando la suspensión se conserva a 4 ºC no se observa agregación alguna, manteniéndose este efecto hasta las 48 h.

El **pH** de la suspensión celular final debe ser similar al pH de la sangre para su administración intra-arterial (Van Slyke y cols., 1966). Las medidas de pH fueron tomadas antes y después del envasado de las células (48 h) a 4 ºC. Los resultados mostraron que el pH de la suspensión celular se mantenía alcalino hasta las 48 h, pudiendo ser administrado por vía parenteral.

Finalmente y con objeto de predecir la estabilidad física de la formulación se llevó a cabo una **caracterización óptica de la suspensión celular** mediante dispersión múltiple de la luz. Los resultados obtenidos tras 24 h de ensayo muestran fenómenos tales como cremado y floculación, probablemente debidos a la migración de restos celulares y agregación de células, respectivamente. Estos hallazgos corroboraron los perfiles reológicos obtenidos en los ensayos anteriores.

De todos ellos se deduce que para una adecuada administración del producto final, la suspensión celular debe ser agitada hasta su completa homogenización, para evitar posibles acontecimientos trombóticos o tromboembólicos.

El desarrollo de nuevas formulaciones con células madre está siendo objeto de estudio por numerosos grupos de investigación, dirigidos a aumentar la estabilidad de las células en el producto final en base a su viabilidad, mediante sistemas como

microcápsulas o scaffolds (geles, matrices, láminas, etc.) (Orive y cols., 2002; Chen y cols., 2013; Shahini y cols., 2014).

Una vez seleccionado el medio de envasado más idóneo (glucosa, albúmina y ringer lactado) y establecidas las condiciones de conservación de las hMSCs en el CTMP (4 ºC/48 h), se estudió la influencia que puede tener la liberación controlada de uno de los nutrientes sobre la viabilidad celular. Con este objetivo fueron elaboradas **microesferas como sistemas transportadores de glucosa**. Los ensayos realizados revelan la idoneidad del método de gelificación interna.

Como polímero formador de las microesferas se empleó el alginato dada su demostrada biodegradabilidad e inocuidad *in vivo* (Orive y cols., 2002).

La **caracterización de las microesferas** se basó en el estudio de su morfología, tamaño, potencial Z, espectrofotometría de infrarrojos (FTIR), capacidad de vehiculización de la glucosa (expresada como capacidad de carga, eficacia de encapsulación y rendimiento) y cinética de liberación de la glucosa desde las micropartículas.

Las microesferas obtenidas presentaron un tamaño medio de 86.62 µm, una distribución unimodal, forma esférica, un potencial Z de − 32.54 mV, una óptima estabilidad y eficiencia de encapsulación. Los espectros FTIR mostraron que la glucosa no indujo modificaciones posteriores en la estructura del alginato de las micropartículas. La liberación *in vitro* de la glucosa siguió un modelo de crecimiento exponencial.

Tras la caracterización de las microesferas y demostrada su idoneidad desde el punto de vista tecnológico, se estudió el efecto de éstas en la **viabilidad celular de las hMSCs.** Para ello se prepararon cuatro medios de envasado (Medio A: hMSCs-Microesferas de glucosa; Medio B: hMSCs-Microesferas blanco; Medio C: hMSCs-Microesferas de glucosa en tampón fosfato; Medio D: hMSCs) en jeringas Luer-lock a una concentración de 1 x 10^6 células/mL. La composición de todos los medios fue 50% de Glucosa 5%, 45% of Ringer Lactado y 55% de Albúmina 20% (Grifols, Barcelona, España) (Galvez - Martin y cols., 2013). La viabilidad celular se determinó mediante tinción de tripan blue (Louis y Siegel, 2011) y el recuento posterior en cámara de Neubauer cada 6 h durante 60 h.

La viabilidad celular en el medio D se ajustó a una cinética de primer orden polinomial describiendo una línea recta entre el % de células viables y el tiempo; mientras que el resto de medios con microesferas de glucosa se ajustaron a una cinética de segundo orden polinomial, describiendo una parábola y por tanto una viabilidad más sostenida durante las primeras horas de conservación.

Concretamente, los resultados demostraron mejor tasa de supervivencia para las hMSCs conservadas en el Medio A (91.5 ± 3.5%) que cuando fueron conservadas en el medio D (83.5±1.3%) ($p<0.01$), durante las primeras 30 h. Sin embargo no se observaron diferencias estadísticamente significativas entre el medio seleccionado en estudios anteriores (medio control) con una viabilidad de (80.2 ± 2.8%) y el mismo medio junto a microesferas cargadas de glucosa, para el resto de tiempos muestreados durante el estudio de viabilidad.

Las microesferas mantuvieron las condiciones asépticas de la suspensión celular. No hubo alteraciones reológicas inducidas por la inclusión de microesferas de glucosa en la forma de dosificación final. Igualmente, no se observaron cambios morfológicos en las células o inmunofenotípicos. De los estudios de estabilidad óptica acelerada se dedujo la necesidad de resuspender la formulación antes de ser administrada.

CAPÍTULO V.
CONCLUSIONES

El objetivo principal de esta Tesis Doctoral ha sido el diseño, desarrollo, caracterización y estudio de estabilidad de un medicamento celular con hMSCs de origen autólogo derivadas de tejido adiposo, destinado al tratamiento de la CLI en pacientes diabéticos tipo II. Fruto del trabajo realizado durante esta etapa, se han publicado seis artículos, cuatro proceedings, ocho comunicaciones orales y doce comunicaciones en forma de póster. Asimismo parte de este trabajo fue objeto del premio: "Premio Puleva Biotech Exxentia", a la mejor comunicación científica en la I Reunión de Jóvenes Farmacólogos de Andalucía, Granada 2009: "Células Mesenquimales como Nueva Terapia Celular para la Diabetes". Todos estos méritos recogen los objetivos planteados en esta Tesis y que concluyen con las siguientes consideraciones:

1. La Terapia Celular no sólo implica el trasplante de células a un paciente sino también toda una serie de tecnologías relativas al aislamiento, caracterización, cultivo, diferenciación, calidad y diseño galénico del medicamento celular. En este trabajo se han definido los requisitos necesarios para su diseño, las normas de fabricación en condiciones asépticas, las instalaciones requeridas para dicha fabricación, la cualificación mínima del personal implicado y todos los procesos de validación necesarios para la puesta a punto del protocolo de fabricación del CTMP. Asimismo se han descrito y discutido las diferentes etapas en la fabricación de un CTMP en base a la normativa Europea.

2. Paralelamente se han normalizado todas las tecnologías implicadas en la fabricación de hMSCs, obteniendo la aprobación de la AEMPS como medicamento en investigación.

3. Se han puesto a punto diferentes protocolos para la obtención de hMSCs, estableciéndose las condiciones óptimas de cultivo bajo estándares GMP, asegurando la reproducibilidad y seguridad del CTMP. Éstos fueron: cultivo celular, limpieza del área estéril, control de calidad, control ambiental, desinfección, esterilización y transporte.

4. La fabricación de hMSCs implica una expansión *ex vivo.* Esta expansión conlleva un riesgo de contaminación por agentes microbiológicos que podrían afectar la calidad y/o la seguridad del CTMP, y por tanto a la salud del paciente.

Para evitar posibles efectos adversos se llevaron a cabo: un programa de monitorización ambiental y un programa de control de calidad.

5. Con el programa de monitorización ambiental se ha estudiado la contaminación bacteriana y fúngica en la sala blanca. Tanto el aire como las superficies de trabajo presentan resultados microbiológicos inferiores a los aceptados por los estándares GMP, para la fabricación de medicamentos estériles. Este estudio ha permitido identificar los puntos más sensibles a la contaminación, siendo la zona D la que presentó mayor crecimiento de ufc. Los resultados aquí obtenidos han permitido además definir un sistema de alerta que establece los límites de los grados A, B, C y D así como algunas medidas correctivas referentes al personal, limpieza y ventilación.

6. El programa de control de calidad (QCP) ha mostrado ser adecuado para estudios de calidad microbiológica en la producción de hMSCs como medicamento. La asepsia se mantuvo durante la obtención del MCB, WCB y producto final. Del análisis de esterilidad, micoplasma y endotoxinas se obtuvieron resultados negativos. En cada una de las validaciones se demostró que el producto celular no interfería en los resultados, asegurando la idoneidad de cada una de las técnicas para el análisis de hMSCs. Este programa puede ser utilizado para analizar las características microbiológicas de un CTMP, determinando su seguridad para la administración en seres humanos.

7. De entre todos los medios ensayados, la suspensión celular compuesta por (50% de Glucosa 5%, 45% of Ringer Lactado y 55% de Albumina 20%) mantiene las células con una viabilidad mayor del 80% hasta las 48 h, permitiendo aumentar el tiempo que transcurre desde la liberación del medicamento hasta su administración al paciente.

8. Los datos aportados por el estudio de estabilidad ponen de manifiesto la influencia de la temperatura de conservación sobre la viabilidad y agregación de las células. Así las muestras conservadas a 4 ºC poseen una viabilidad superior que aquellas mantenidas a 8 ºC, 25 ºC y 37 ºC durante 48 h.

9. Los excipientes seleccionados no interfieren en la caracterización inmunofenotípica de las hMSCs, ni en su capacidad de diferenciación, además de mantener la estabilidad genética de las células.

10. La concentración celular utilizada presenta fenómenos de agregación fácilmente solventables con la agitación del inyectable. Las características reológicas y fisicoquímicas del producto final son adecuadas para su administración intra-arterial.

11. La incorporación de microesferas de glucosa al medio y condiciones de almacenamiento seleccionadas anteriormente suponen una mejora de la viabilidad celular en las primeras 30 h. De forma que el CTMP formulado con microesferas cargadas de glucosa mantiene las células con una viabilidad mayor del 90%, permitiendo aumentar la dosis celular activa en el caso de que el tratamiento se produzca en las 30 h posteriores a su envasado, presentando también adecuadas características microbiológicas, reológicas, morfológicas e inmunofenotípicas.

CAPÍTULO VI.
CONTRIBUCIÓN Y PERPECTIVAS FUTURAS

La aportación principal de la presente Tesis Doctoral, en el campo de la investigación y el desarrollo de medicamentos celulares, es el diseño, caracterización y estudio de estabilidad de un inyectable de administración intra-arterial para el tratamiento de la CLI en pacientes diabéticos tipo II.

Las células madre mesenquimales humanas (hMSCs) poseen características inmunoreguladoras y angiogénicas que las hace susceptibles de ser empleadas como activos farmacológicos. Sin embargo su uso clínico debe ser estandarizado como medicamento celular (CTMP). Este campo de investigación es complejo y se encuentra en constante crecimiento, por lo que consideramos que nuestro trabajo de investigación podría contribuir en los siguientes aspectos:

Análisis y definición de las principales fases de desarrollo. Estas son: investigación básica, investigación preclínica, investigación clínica, fabricación, autorización, registro, comercialización y post-autorización.

Validación y establecimiento de los protocolos de producción celular, limpieza de área estéril, control de calidad, control ambiental, desinfección, esterilización y transporte.

Los resultados obtenidos representan un avance en la estandarización de la fabricación de células madre para el desarrollo de medicamentos biotecnológicos basados en Terapia Celular. Las células madre como activo farmacológico deben ser estériles y viables para ser administrados al paciente. Para garantizar estos requisitos se han diseñado dos programas, por un lado un programa de monitorización ambiental y por otro, un programa de control de calidad. Se han establecido los controles mínimos necesarios para garantizar la asepsia del proceso de fabricación y en consecuencia la esterilidad del medicamento.

Por otro lado, el estudio galénico de las suspensión celular objeto de esta Tesis Doctoral, ha permitido mejorar los resultados de viabilidad de las hMSCs, obteniendo un tiempo de vida media de 48 h frente a las 24 horas conseguidas por otros investigadores para el mismo tipo de células. Los excipientes seleccionados para el medio de envasado, además de mantener las células viables han demostrado no alterar las características intrínsecas de la célula, respecto a su inmunofenotipo, diferenciación, estabilidad genética y morfología. Asimismo la forma de dosificación elaborada

presenta adecuadas características microbiológicas y físico-químicas para su administración intra-arterial.

En cuanto a la incorporación de microesferas de alginato cargadas de glucosa, éstas aumentan la dosis celular activa durante las primeras 30 h, no influyendo negativamente en las características microbiológicas, morfológicas, inmunofenotípicas ni reológicas del CTMP.

Pese a que en la actualidad se están llevando a cabo diferentes ensayos clínicos, pocos son los trabajos que se han publicado sobre las pautas, normativa y aspectos tecnológicos en el desarrollo de este tipo de medicamentos. Por tanto las revisiones, protocolos y resultados derivados de este trabajo servirán como fuente de información a fin de garantizar que hospitales, laboratorios e industria farmacéutica puedan producir un CTMP de forma eficaz y segura. Siendo, además, especialmente útil y de aplicación a otras células madre de uso clínico.

Nuestras investigaciones futuras se centrarán en ampliar el tiempo de viabilidad de las células, mediante el empleo de sistemas de vehiculización como microesferas, geles o scaffolds con el fin de seguir optimizando los resultados aquí obtenidos.

CAPÍTULO VII.
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ANEXO I.

OTRAS PUBLICACIONES: PROCEEDINGS

ANEXO I.1. AUTOLOGOUS BONE MARROW MONONUCLEAR CELL THERAPY OF CRITICAL ISCHAEMIA OF THE LIMBS IN DIABETIC PATIENTS.

R. Ruiz Salmerón, A. de la Cuesta Diaz, M. Constantino Bermejo, I. Perez Camacho, F. Marcos Sanchez, P. Gálvez Martín, M. Punzano Teruel, D. Rodríguez Rodríguez, A. Hmadcha, and B. Soria. Autologous Bone Marrow Mononuclear Cell Therapy of Critical Ischaemia of the Limbs in Diabetic Patients. Human Gene Therapy. 2009; 20 (9):1081.

active in MSCs. The inhibition of endogenous BMP signaling by treatment with dorsomorphin reduced the proliferation and survival rates of MSCs, and consequently produced a drastic decrease of the cell recovery. On the contrary, the addition of low doses of exogenous BMP4 induced stimulation of MSC proliferation and survival, and maintenance of the expression of key transcription factors (Nanog and Oct4) essential to the pluripotent and self-renewing phenotypes of undifferentiated stem cells. In addition, we demonstrated that BMP-4 pretreatment did not affect the MSC differentiation potential.

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155 METABOLIC AND AUTOIMMUNE DISEASES

Autologous Bone Marrow Mononuclear Cell Therapy of Critical Ischaemia of the Limbs in Diabetic Patients

R. Ruiz Salmeron, A. de la Cuesta Diaz, M. Constantino Bermejo, I. Pérez Camacho, F. Marcos Sánchez, P. Gálvez Martín, M. Punzano Teruel, D. Rodríguez Rodríguez, A. Hmadcha, and B. Soria

Andalusian Center for Molecular Biology and Regenerative Medicine (CABIMER) and Hospital Virgen Macarena-San Lázaro

This study aimed to evaluate the safety and efficacy of intra-arterial transplantation of autologous bone marrow mononuclear cells on the treatment of lower limb ischemia in diabetic patients (Stage III-IV Fontaine (CLI and diabetic foot) with an ulcerated limb in whom all previous therapeutic strategies failed). In this Phase I/II single-center clinical trial, 20 type II diabetic patients (with critical limb ischemia were submitted to an iliac crest bone-marrow aspiration (30 ml) performed under local anesthesia and therefore received transplantation of autologous bone marrow mononuclear cells by intra-arterial injection into the affected limb at popliteal artery by blocking antegrade perfusion during 3 minutes.

Results: The efficacy/safety of this therapy was assessed by using several endpoints such as (a) prevention of amputation (no patient needed major amputation, (b) wound healing (improvement), (c) degree of angiogenesis-vasculogenesis (improved as reported from METAMORPH quantification and ANOVA analysis of angiographic follow-up), (d) transcutaneous oxygen pressure (improved 50%) and (e) clinics (rest pain improved), ABI-ankle brachial index (improved 60%), limb temperature and walking test (improved), etc. Surprisingly, 6 out of 20 patients reported a decrease in the insulin needs.

Conclusion: Transplantation of autologous bone marrow mononuclear cells is a simple, safe and effective method for the treatment of lower limb ischemia in diabetic patients, and shows promising results that makes possible that more patients benefit from the stem cell treatment.

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157 STEM CELLS

Characterization of Equine Mesenchymal Stem Cells: Differentiation Capacity and Immunophenotype

B. Ranera, J. Lyahyai, L. Ordovás, A. Romero, F. Vázquez, C. Cons, F. Fernandes, M.L. Bernal, R. Osta, P. Zaragoza, I. Martin-Burriel, and C. Rodellar

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Adult stem cell therapies are a promising tool to the treatment of equine illnesses, which are consequences of athletic endeavor. Osteoarthritis and tendinopathy are the most common disorders of the locomotor system observed in working horses. The aim of this work was to characterize the differentiation potential of equine MSC derived from two different sources, bone marrow (BM) and adipose tissue (AT). Equine MSCs were isolated from bone marrow aspirates obtained from the sternum and adipose tissue of the supragluteal subcutaneous area of 4 donor horses. All horses were clinical patients. As shown by the alkaline phosphatase activity and specific staining of the extracellular matrix, BM-MSC and AT-MSC displayed osteogenic capacity. Time course of gene expression of 2 osteogenic markers in cultured cells was measured by quantitative RT-PCR. Regarding adipogenic differentiation, although a wide battery of adipogenic media was used, only BM-MSC presented the capacity to differentiate into adipocytes. Besides, a great variability between individuals was observerd. Adipogenic capacity was quantified using real-time RT-PCR. Finally, to characterize equine MSCs, antigen-specific markers have been analyzed by RT-P-CR and flow cytometry. Gene expression was positive for CD105, CD73, CD166 and negative for CD45, CD31. CD34 displayed different expression depending on the origin source. Moreover, CD105 immunophenotype was confirmed by flow cytometry. This work indicate equine MSCs are a hopeful cell source for tissue engineering and gene therapy for the treatment of locomotor diseases.

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ABSTRACT WITHDRAWN

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160 NEURODEGENERATIVE DISEASES

Carotid Body GDNF Expression Is Differentially Regulated by Chronic Hypoxia and Intrastriatal Grafting Along Aging

ANEXO I.2. DEVELOPMENT OF PROTOCOLS REQUIRED FOR APPROVAL OF A LABORATORY OF CELL THERAPY.

Galvez P, Martin MJ, Gonzalez M, Ruiz MA, Clares B. Development of protocols required for approval of a laboratory of cell therapy. Histology and Histopathology. 2011; 26 (1): 363-364.

in the MMP/TIMP imbalance, strongly suggesting a paracrine mediated action of ASC.

Conclusion. Our results shown that ASC increase perfusion and reverse adverse remodeling in a clinically relevant model of I/R. ASC thus constitute an attractive candidate for the treatment of myocardial infarction.

Keywords. stem cells, Regenerative Medicine, Cardiac ischemia/reperfusion, Angiogenesis, Heart remodeling

(43.O3) ADIPOSE DERIVED STEM CELLS (ASC) FOR TREATMENT OF CROHN'S FISTULA

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Complex perianal fistulas are extremely difficult to manage due to the limitations of currently available treatments. The use of ASC therapy could represent an alternative treatment. We performed an initial phase I clinical trial of nine implants with autologous ASCs in five patients with Crohn's disease. Eight weeks after treatment, signs of repair were apparent and 75% of treated fistulas had closed and were considered healed. After these promising results a phase II clinical trial was designed to evaluate the safety and efficacy of this novel stem cell therapy. Forty-nine patients with complex perianal fistulas of cryptoglandular origin (n = 35) or associated with Crohn's disease (n = 14) were recruited and randomized for treatment with autologous ASCs (dose 20 – 60 million cells) in combination with fibrin glue or fibrin glue alone. The proportion of patients who achieved fistula healing was significantly higher with autologous ASCs than with fibrin glue (17 (71%) versus 4 (16%) respectively, risk ratio = 4.43 (95% confidence interval 1.74, 11.27); $p < 0.001$). A phase III multicenter clinical trial to evaluate the safety and efficacy of autologous ASCs to treat complex perianal fistulas not associated with Crohn´s disease is currently being completed. However, although the data of this phase III are not yet published the trial was complicated by the unexpected finding that the clinical outcomes of patients getting fibrin glue alone were better than anticipated, making it harder to show the effectiveness of autologous ASCs as demonstrated in phase II studies. In the near future we will know whether or not the treatment is effective. A further three clinical trials in phase I/II are ongoing to evaluate ASCs for the treatment of fistulas associated with Crohn`s disease: One with autologous ASCs, one with allogeneic ASCs and one with allogeneic ASCs to treat specifically recto-vaginal fistulas. Although the efficacy of ASCs in the treatment of fistulas has yet to be clarified; the results obtained in the treatment of nonhealing wounds with MSC therapies in experimental models continue to arouse great expectations in the clinical practice.

Keywords. stem cells, Regenerative Medicine, Terapia Celular

(43.O4) INDEPENDENT MEDICAL TRIALS ON REGENERATIVE MEDICINE SUPPORTED BY A BIOMEDICAL INSTITUTION

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In the last few years the ISCIII has provided funds to the Spanish Network for Regenerative Medicine to support the construction and validation of the platform of GMP facilities that provided the required scientific and regulatory expertise to translate of stem cell based therapeutics to the clinic. This measure has been very important after the 2003 European community law that considered expanded cells as drugs and to be produced under pharmacological regulations.

On those grounds, the unit in Valladolid pioneered as the first GMP facility for expanded bone marrow derived mesenchimal stem cells produced in Valladolid (MSV) for cardiac regeneration on the NOGA trial.This start was follow by the use of MSV on different osteo-articular pathologies on inter-vertebral disc incompetence and grade II gonartrosis. Consolidated protocols like engineered skin ad limbar stem cells expanded over amniotic membrane, that were successfully used by several clinic groups in Spain, were also validated in our GMP unit as pharmaceutical products an are used now days on several clinical trials.Preliminary results of those trials will be presented in the symposium.

Keywords. Regenerative Medicine, stem cells, terapia cellular

(43.P1) DEVELOPMENT OF PROTOCOLS REQUIRED FOR APPROVAL OF A LABORATORY OF CELL THERAPY

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Introduction. In the field of the biotechnology, there is included the use of the cellular therapy as a new biomedical science capable of offering new treatments to diseases or human dysfunctions that up to the moment do not have treatments or the existing ones are not effective. The cellular therapy is defined as the use of alive cells, mature cells, progenitors or mother cells in order to restore or improve the function of damaged organs as consequence of traumatic injuries, degenerative chronic diseases or tumour diseases. This study shows the validation protocols requiring the production of cell therapy products.

Material and Methods. In the translation of basic research to the clinic, attended by several factors: production rules, facilities, personnel and procedures. The owner of a marketing authorization for advanced therapy medicinal products should ensure a system. Its components can be examined during the process of procurement, manufacturing, packaging, storage, transport and delivery.

Results. 1. Production Processes: It is required that the part of those processes are properly validate. 2. Validation of aseptic processing. Demonstrate the

cleanliness of the environment, staff and material. 3. Analytical Methods: The analytical methods are beyond the scope of these requirements. 4. Cleaning Procedures: To check to ensure the absence of cross-contamination. 5. Environmental Control Systems: Means those environmental control systems to ensure that the environmental conditions of temperature, pressure differential and control of microbiological and particulate loads are adequate. 6. Machinery and Equipment: This section includes all the equipment and machinery that affect or operate directly in production processes or quality control.

Conclusion. Validation processes required for the manufacture of cellular medicine have been conducted in the unit cell production Cabimer (Centro Andaluz de Biologia Molecular y Medicina Regeneraativa), from obtaining results within the range of GMP.

Keywords. Cell, validation, good manufactures production, cell medicine

(43.P2) DEDIFFERENTIATED SMOOTH MUSCLE CELLS NEXT TO INTESTINAL NERVOUS TRUNCKS

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Introduction. The dedifferentiation of smooth muscle cells is a natural repair process that includes the elimination of contractile apparatus and nucleus activation to prepare the cell toward proliferation or redifferentiation. The dedifferentiation process causes the cell regress to an embryonic state. Smooth muscle cells (SMCs) and Interstitial Cells of Cajal (ICCs) in the small intestine emerge during embryonic period from Kit + mesenchymal precursor cells. Previous studies suggested that Kit + cells adjacent to myenteric neurons might decide to become IC-MY (Interstitial Cells-Myenteric plexus) because enteric neurons express stem cell factor, the ligand for Kit (Torihashi S. et al 1996, 1997). This process could be reversible in the presence of certain microenvironmental factors.

Materials and Methods. Human biopsies and different mammalian vertebrate specimens were analyzed by electron microscopy.

Results. In our results we have observed the dedifferentiation process characterized by an increase in the number of organelles next to the nucleus, a clear disorganization of the cytoskeleton, the appearance of vesicles that fuse together until the vesicle membrane breaks, and cytoplasmic fragments are detached from the SMC. This dedifferentiation process is often found next to nerve trunks.

Conclusions. We suggest dedifferentiation of smooth muscle cells as an homeostatic repair process characterized by the regression to an embryonic state mediated by the influence of signalling pathways from enteric neurons.

Acknowledgements. This research received financial support from Aragon Institute of Health Sciences (I+CS) (PIPAMER 001/11) and the European Social Fund (ESF), DGA (B83).

Keywords. Dedifferentiation; intestinal smooth muscle cell; Interstitial Cells of Cajal

(43.P3) DIABETIC ULCER HEALING IS STIMULATED BY OTR4120

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Introduction. Heparan sulfate (HS) regulates the bioavailability of HS-bound polypeptides and maintains the balance between tissue integrity and tissue disruption allowing the cellular tissue components to unfold their natural mechanism to achieve tissue homeostasis. In chronic wounds, HS is disrupted in highly proteolytic environment. OTR4120 is a heparan sulfate (HS) mimetic. It replaces the degraded HS and takes its role in restoring tissue homeostasis. Previously, we showed that the OTR4120 improved pressure ulcer healing in non-diabetic rats. This study describes the profound effects of OTR4120 on diabetic wound healing.

Materials and Methods. Experimental diabetes was induced by an intra-peritoneal injection of streptozotocin (STZ). Six weeks after STZ-diabetes induction, rats were subjected to pressure ulcer formation by external clamping a pair of magnet disks on the dorsal skin for a single ischemic period of 16 h. Immediately after magnet removal, rats received an intramuscular injection of OTR4120 weekly for up to one month.

Results. Compared to the untreated non-diabetic rats, ulcer healing was impaired in untreated diabetic rats. However, ulcers in OTR4120-treated diabetic rats healed significantly more rapid than wounds in untreated diabetic rats. OTR4120 treatment reduced inflammation, reduced matrix metalloproteinase expression and increased collagens synthesis. Furthermore, the increased ratio of collagen type III to I in diabetic ulcers was reversed to normal in OTR4120-treated ulcers. Also short and long-term restoration of ulcer biomechanical strength was significantly enhanced following OTR4120 administration.

Conclusion. OTR4120 treatment is beneficial to improve diabetic ulcer healing.

Acknowledgments. The authors thank Prof. D.W. van Bekkum (LUMC, Leiden, the Netherlands) for his assistance in the development of the pressure ulcer model, and Prof. D. Barritault (OTR3, SAS, Paris, France) for providing OTR4120. This research was supported by a grant from the Nuts Ohra Foundation (the Netherlands), Grant No. SNO-T-0-0501-159.

Keywords. diabetes, pressure ulcers, matrix, Heparan sulfate

44. THE USE OF MAGNETIC NANOPARTICLES FOR TAGGING, TRACKING AND ACTIVATION IN REGENERATIVE MEDICINE

Chair: Alicia El Haj
Co-chair: Gerjo Van Osch
Keynote speaker: E. Sykova
Organizer: Alicia El Haj

ANEXO I.3. ENVIRONMENTAL MICROBIOLOGICAL QUALITY IN A CLEANROOM DURING THE EXPANSION OF MESENCHYMAL STEM CELL TO BE USED IN CLINICAL TRIALS.

Gálvez P, Bermejo M, Gálvez M, Del Rio JM, Rodriguez MV, Jimenez VE, Soria B. Environmental microbiological quality in a cleanroom during the expansion of Mesenchymal Stem Cell to be used in clinical trials. Histology and Histopathology. 2012; 27 (supplement 1): 68-69.

P-021. INFLUENCE OF THE EFFECTS OF ENDOREM® ON ENCAPSULATED AND UNENCAPSULATED PASCS IN PROLIFERATION AND CELL VIABILITY

AUTHORS
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Introduction: Stem cell transplantation is being investigated as a novel approach to regenerate heart tissue and enhance cardiac function. Adipose tissue-derived stem cells (ASCs) are similar to bone marrow derived cells (BMCs) and they can secrete various growth factors with therapeutic potential. Experimental evidence indicates that administration of ASCs can effectively improve left ventricular and cardiac function and decrease MI size by promoting angiogenesis and myogenesis in animal models. However, the low rate of engraftment and survival of the transplanted cells is an important drawback in myocardial cell therapy. Large proportions of the injected cells are lost from the myocardium within the first few minutes post-injection. Encapsulation of therapeutic stem cells may help to increase their retention in the heart tissue while promoting the local and continuous release of proteins and growth factors. Following the application of cell therapy after MI, many studies have used MRI (magnetic resonance imaging) to assess changes in function and remodeling. Direct cell labeling is the most straightforward approach to visualize transplanted cells in living subjects. Superparamagnetic iron oxide (SPIO) nanoparticles, have been widely used to label and follow the cells in vivo by MRI. We investigated the effects of Endorem® on pASCs (porcine adipose stem cells) and the potential use of microcapsules as a carrier and scaffold to increase cell survival and retention. Additionally, these microcapsules allowed us to localize and to monitor the cells non-invasively in real time by MRI. **Material and Methods:** The labeling with SPIO was evaluated in vitro for 21 days comparing cellular activity of encapsulated and unencapsulated pASCs. The viable cell number was determined by a sensitive colorimetric assay (Cell Counting Kit-8 allows CCK-8), using WST-8 (2-(2-methocy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methosy PMS. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. The resulting orange formazan produced is directly proportional to the number of living cells and this product that is soluble in tissue culture medium was read 5 min later on a microplate reader at 450 nm with 650 as reference wavelength. **Results:** The metabolic activity was analyzed in vitro over the course of 21 days. The pASCs immobilized in both microcapsules and magnetocapsules showed similar viabilities over the 21 days assay period. The results of this study of the metabolic activity evidenced no significant differences between Endorem® labeled and unlabeled cells. **Conclusion:** Results from the present study reveals that pASC appeared to be unaffected by magnetic labeling and viability was not changed in comparison with unlabeled cells, suggesting that ASCs could be successfully marked in vitro using commercially and clinically approved Endorem®. Similarly, in vitro comparison of the pASCs immobilized in magnetocapsules and in conventional microcapsules (unlabeled capsules) did not reveal differences in cell viability.

KEYWORDS ENCAPSULATED PORCINE ASCS, SPIO/ ENDOREM®, CELL VIABILITY

P-022. ENVIRONMENTAL MICROBIOLOGICAL QUALITY IN A CLEANROOM DURING THE EXPANSION OF MESENCHYMAL STEM CELL TO BE USED IN CLINICAL TRIALS

AUTHORS
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Introduction: The production and characterization of cellular medicaments (human mesenchymal stem cells, MSCs) for clinical use in compliance with good manufacturing practice (GMP) includes the design of process free of microbiological contaminants. Ex vivo growing of stem cells faces three important obstacles: contamination by other cell lines (cross-contamination), chromosomal instability and contamination by microorganisms. The presence of microorganisms in the air and surfaces in a cleanroom must be controlled with a monitoring programme that points to the risk of microbial contamination, and the subsequent possible contamination of the cell product. The aim of this work is to determine the microbiological quality of air in the cleanroom for the production of MSCs and its subsequent release, transport and administration to the patient. **Material and Methods:** The selected area for microbiological tests was the cleanroom of the CABIMER. Sampled points were: three in grade D

area, one in grade C area, five in grade B area and five in grade A area. During one year (January 2011-December 2011, except August) these 14 points were tested weekly. Contamination levels were compared with those established in the Annex 1-GMP guidelines (EudraLex - Volume 4 GMP, 2009). Microorganisms counting was performed by the method of impact with the air sampler MAS-100 Eco (1 m3 of air at a rate of 100L/min). The airflow was directed onto a solid media plate: Agar Trypcase Soja (TSA) for bacteria and Agar Sabouraud Dextrosa Cloramfenicol (SDC) for fungi. Incubation conditions: 2 days at 35°C for TSA and 5 days at 22°C for SDC. The identification of the bacteria was performed by Gram staining and biochemical tests (API bioMèrieux). The fungi were identified by morphological studies and staining with lactophenol. **Results:** From the 616 samples, 160 showed contamination; 17.21% for bacteria and 7.31% for fungi. The study showed that in all points sampled, the number of CFU was below the limits allowed GMP guidelines. The results were negative for all points studied in grade A. In grade B, 5.45% of points showed bacterial growth, and no point showed fungi growth. In grade C, 79.55% of points were positive for bacterial and 11.36% for fungi. In grade D, 44.70% of points showed bacterial growth and 30.30% fungi growth. Identification of the colonies isolated in the cleanroom showed that the predominant bacterial genus found was Staphylococcus spp. (55.6%) and Penicillium spp. (18.1%) for fungi. (Figure 1) **Conclusion:** In this study all the results are within the standards required by the GMP. The results show prevalence of bacteria over fungi. The main focus of the bacterial contamination is human skin (S. epidermis).Therefore the potential risk of contamination in the cleanroom is by the staff. The results obtained confirm the suitability of environmental microbiological quality of the air. Therefore the contamination by microorganisms is controlled during production of MSCs.

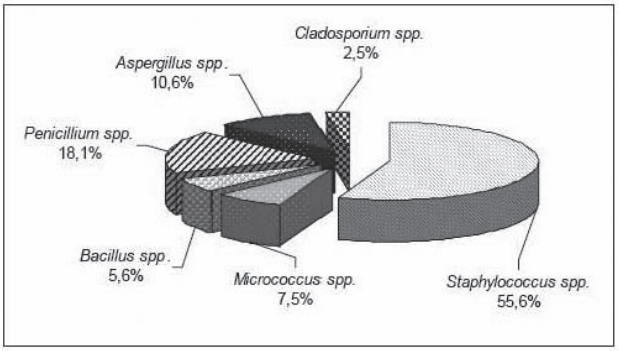

Figure 1. Frequency of the different genera of microorganisms observed in the cleanroom.

KEYWORDS MESENCHYMAL STEM CELL, ENVIRONMENTAL MICROBIOLOGY, CLEANROOM

P-023. VALIDATIONS OF CELL MANUFACTURE PERFORMED IN THREE YEARS IN THE GMP FACILITY OF THE UNIVERSITY HOSPITAL OF SALAMANCA

AUTHORS
LOPEZ VILLAR, OLGA / VILLARON, EVA / GARCIA MONTES, TERESA / LORENZO IGLESIAS, EVA / HERRERO MARTIN, MANUEL / ORTEGA, REBECA / MUNTION, SANDRA / SANCHEZ-GUIJO, FERMIN / DEL CAÑIZO, CONSUELO

Introduction: The number of procedures to be validated in a GMP facility varies depending on the advanced-therapy medicinal products manufactured and the major changes required. In the past three years, since the GMP facility of the University Hospital of Salamanca was approved by the National Agency, we had to validate different procedures. In this work we describe the cell manufacture validations performed and their results. **Methods-Results:** In 2009, the Hematology department of our institution participated in a clinical trial for the treatment of graft versus host disease with bone marrow derived mesenchymal (MSC) stem cells. The MSC were prepared in another hospital, they shared the IMPD with us, so we could prepare our expansion protocols. Three bone marrow samples were obtained from healthy donors, the expansion procedure was followed and the quality controls were performed with a positive result. Late that year, a new clinical trial was proposed to us. In this case, the MSC were to be administered in patients undergoing vertebral column surgery. This was a single center clinical trial. In this case we had to ask to the National authorities whether new procedure validations were required. Since cells were mixed prior to their administration with a carrier, the MSC were considered to be a different product, so the validations were performed. The MSC expansion was performed without any protocol deviation. In 2011 a new clinical trial was proposed in collaboration with other hospital. The administration of MSC for patients with knee arthritis is to be tested. In this case, the IMPD was prepared by this hospital and shared with us. Since MSC were resuspended in saline solution, this was considered to be a different product. The three expansions required were performed. In the beginning of 2012, a collaborative trial was proposed, in this case with adipose derived MSC. The IMPD is being prepared by another institution. Prior to this validations, some expansions were performed in the research laboratory in order to gain manufacture skills with this type of cells. After 4 expansions in the research lab, and having the guideline of the procedure, we could finally perform the validations, with satisfactory results. For each ATMP, the aseptic processing validation

ANEXO I.4. MULTICENTER CLINICAL TRIAL PHASE I/II RANDOMIZED, PLACEBO-CONTROLLED STUDY TO EVALUATE SAFETY AND FEASIBILITY OF THERAPY WITH TWO DIFFERENT DOSES OF AUTOLOGOUS MESENCHYMAL STEM CELLS IN PATIENTS WITH SECONDARY PROGRESSIVE MULTIPLE SCLEROSIS WHO DO NOT RESPOND TO TREATMENT.

Fernández O, Izquierdo G, Guerrero M, Navarro G, Pozo D, León A, Pinto-Medel MJ, Páramo MD, Gálvez P, Leyva L. Multicenter clinical trial phase I/II randomized, placebo-controlled study to evaluate Safety and feasibility of therapy with two different doses of autologous mesenchymal stem cells in patients with secondary progressive multiple sclerosis who do not respond to treatment. Histology and Histopathology. 2012; 27 (supplement 1): 72-73.

The primary endpoint will be to evaluate the safety and tolerability of the intravenous infusion of 3 doses of autologous AdMSC in ALS patients, assessed by the absence of:
1. complications at the infusion site,
2. emergence of a new neurological deficits not attributable to the natural progression of the disease;
3. severe adverse reactions attributable to treatment.

Secondary endpoints
1) Assessment of the clinical efficacy of IV administration of 3 doses of AdMSC in patients with ALS, as measured by:
• Changes in the rate of disease progression; the degree of muscle strength; forced vital capacity; muscle bulk, estimated by MRI, and circumference of the upper and lower extremities; neurophysiological, neuropsychological and quality of life parameters; measures of spasticity and pain
• Need and time to tracheostomy or permanent assisted ventilation, and/or to gastrostomy
• Overall survival
2) Evaluation of the immunomodulatory effects of the 3 doses of AdMSC assessed by regulatory cell populations affecting the inflammatory and tolerogenic status and immunological control mechanisms mediated by TLRs
3) Adquisition of metabolomic profiling data as an alternative tool for monitoring and identification of new markers in ALS

This work is granted by ISCIII, Ministerio de Sanidad y Consumo.

KEYWORDS AMYOTROPHIC LATERAL SCLEROSIS (ALS), MESENCHYMAL STEM CELLS, SAFETY

P-028. MULTICENTER CLINICAL TRIAL PHASE I/II RANDOMIZED, PLACEBO-CONTROLLED STUDY TO EVALUATE SAFETY AND FEASIBILITY OF THERAPY WITH TWO DIFFERENT DOSES OF AUTOLOGOUS MESENCHYMAL STEM CELLS IN PATIENTS WITH SECONDARY PROGRESSIVE MULTIPLE SCLEROSIS WHO DO NOT RESPOND TO TREATMENT

AUTHORS
FERNÁNDEZ, ÓSCAR / IZQUIERDO, GUILLERMO / GUERRERO, MIGUEL / NAVARRO, GUILLERMO / POZO, DAVID / LEÓN, ANTONIO / PINTO-MEDEL, MARIA JESÚS / PÁRAMO, MARÍA DOLORES / GÁLVEZ, PATRICIA / LEYVA, LAURA

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), resulting in destruction of myelin. Oligodendrocytes and nerve fibers are also affected. It is initially treated with immunomodulatory drugs, to reduce disease activity and disability progression. Non-responder patients are treated with immunosuppressive drugs, that have a higher efficiency, about 60%, but they are less secure. Especially aggressive cases can be treated with autotransplantation of hematopoietic lineage stem cells. But despite all therapeutic attempts, there are patients in whom therapy fails and clinical activity and lesions on magnetic resonance imaging (MRI) persist. Cell replacement strategies to promote repair in neurodegenerative diseases are very promising areas of research. The transplantation of Autologous Mesenchymal Stem Cell (SC) has been effective in many experimental models, providing important evidence of remyelination. In humans, SC treatment has been evaluated positively in neurological diseases in general, specifically in MS. Autologous Mesenchymal SC are found in multiple organs and can migrate to other places to perform their regulatory activities and regeneration. Their local differentiation is driven by the micro and macro-environment of the organs where SC migrate. Although the experience of the application of these cells in CNS diseases is poor, the international experience in the use of SC mesenchymal in multiple clinical trials suggests that its use is safe, at least in the short to medium term.

AIM:
Multicenter clinical trial phase I/II randomized, placebo-controlled study to evaluate safety and feasibility of therapy with Autologous Mesenchymal Stem Cells from adipose tissue.

STUDY DESIGN:
Patients: 30 patients with secondary progressive multiple sclerosis (SPMS) and treatment failure, divided into three arms: control group (no intervention), experimental group 1 (low dose of autologous mesenchymal cells) and experimental group 2 (high dose of autologous mesenchymal cells).

Cells: Autologous Mesenchymal SC from adipose tissue, developed at the Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER)
N° injected cells: 2 different doses: 1x106 cells/Kg and 4x106 cells/Kg
Route of administration: intravenous
Study variables:
Safety parameters: Number, intensity and relationship to study drug of adverse and serious adverse events in all study visits.
Feasibility parameters: clinical and paraclinical variables
Functional analysis of the immune response: Evaluation of the immunomodulatory effect of cell therapy before cell therapy and at 3, 6 and 12 months after cell infusion.
Original primary outcome measures: Evaluation of safety and tolerability related to the intravenous infusion of autologous mesenchymal stem cells.
Original secondary outcome measures: Evaluation of the effects on MS disease activity measured by: clinical imaging immunological, neurophysiological,, neuropsychological and quality of life variables.
Acknowledgements: this clinical trial was supported by Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo.

KEYWORDS MESENCHYMAL STEM CELLS, MULTIPLE SCLEROSIS, IMMUNOMODULATION

P-029. CELLULAR CHARACTERISTICS OF THE EXTRA-ADRENAL ORGAN OF ZUCKERKANDL

AUTHORS
FERNANDEZ, EMILIO / TUNEZ, ISAAC / TASSET, INMACULADA / GARCIA, JOSE MANUEL

The organ of Zuckerkandl or abdominal paraganglion is the most important extra-adrenal tissue, and it is considered as a chromaffin organ, with a functional role secondary to that of the adrenal medulla. Their chromaffin cells have been used for cell therapy on Parkinsonian animal models. We have studied the cytohistological structure and neurochemical characteristics of this organ in three-month-old Wistar rats. The findings revealed that this organ is a medullo-cortical paraganglion surrounded by a capsula and with a hilum for vessels and nerve fibers. There are two differentiated zones: a cortical zone that is rich in lymphocytes, and a medulla containing both chromaffin cells and lymphocytes. All cells are separated by a connective reticulum. Hence, it seems that this organ is a lymphoid-chromaffin tissue rather than a pure chromaffin one. Chromaffin cells have the typical rounded morphology with 12-15 ƒÊm diameter, most of them are TH+/DBH+ cells (noradrenergics), and they also express the trophic factors GDNF and TGFbeta1. These cells are arranged forming cell clusters, where dopaminergic and adrenergic cells can also be observed. ELISA measurements revealed the presence of noradrenaline, adrenaline and very low levels of dopamine within the tissue. Catecholaminergic levels were very low relative to adrenal medulla, indicating the secondary adrenergic role of this extra-adrenal organ. Lymphocytes were found to be arranged forming typical niches, and all of them expressed CD20 marker. Hence they are type B lymphocytes distributed throughout the paraganglion. No clear evidence of the presence of stem cells was obtained, and only some dispersed BrDU+ and Ki27+ cells could be observed in the whole tissue, with a similar morphology to that of lymphocytes.

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KEYWORDS CHROMAFFIN, ZUCKERKANDL, EXTRA-ADRENAL

P-030. THERAPEUTIC EFFECT OF HUMAN DECIDUAL STROMAL CELLS IN THE MURINE MODEL OF RECURRENT SPONTANEOUS ABORTION (CBA/JXDBA/2)

AUTHORS
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Introduction: The human decidua or gestating endometrium in close contact with trophoblast (fetal tissue) formed the maternal-fetal interface, in which immune interrelations are important for development of embryo. Decidual Stromal Cell (DSC) the main cellular component of the human decidua originated from the proliferation and differentiation (decidualization) of a fibroblast like

ANEXO II.
OTROS MÉRITOS

Anexo II.1. PROYECTOS DE INVESTIGACIÓN

Uso del óxido nítrico para generar líneas celulares a partir de células madre y progenitores de origen adulto (EU-CELL). Subprograma INNPACTO 2011. Ministerio de Ciencia e Innovación - (IPT-2011-1615-900000).

Terapia Celular de la Isquemia Crítica del Miembro Inferior en pacientes diabéticos tipo II insulinizados: estudio de las necesidades de insulina. CetMad/ICPD/2008. Instituto de Salud Carlos III.

Ensayo clínico multicéntrico fase I/II aleatorizado y controlado con placebo, para evaluación de seguridad y factibilidad de la terapia con dos dosis distintas de células troncales mesenquimales autólogas de tejido adiposo (CetMad) en pacientes con esclerosis múltiple secundariamente progresiva, que no responden adecuadamente a los tratamientos registrados. CMM/EM/2008. Instituto de Salud Carlos III - EC08/00224.

Uso de las células troncales mesenquimales de tejido adiposo (CetMad) como terapia de regeneración celular en el síndrome de isquemia crónica de miembros inferiores en pacientes no diabéticos. CeTMAd/ICC/2009. Ministerio de Sanidad y Política Social.

Ensayo clínico fase I/II multicéntrico, abierto, aleatorizado y controlado para el estudio del uso de las células madre como terapia celular en isquemia crítica del miembro inferior en pacientes diabéticos tipo II insulinizados: estudio de las necesidades de insulina. CeTMMoTa/ICPDI/2010. Ministerio De Sanidad Y Política Social / Dirección General de Terapias Avanzadas (TRA-120).

Anexo II.2. COMUNICACIONES ORALES

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