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Scleral surgical repair through the use of nanostructured fibrin/agarose-based films in rabbits.

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Abstract
Scleral defects can result as a consequence of trauma, infectious diseases or cancer and surgical repair with allogeneic scleral grafts can be required. However, this method has limitations and novel alternatives are needed. Here, the efficacy of acellular nanostructured fibrin-agarose hydrogel-based substitutes (NFAH) in the repair of scleral defects in rabbits was studied. For this, scleral defects of 5-mm diameter were made on 18 adult-male New Zealand rabbits and repaired with acellular NFAH, NFAH crosslinked with genipin (NFAH-GP) or glutaraldehyde (NFAH-GA), allogeneic scleral grafts as control (C-CTR) or not repaired (negative control N-CTR) (n=3 each). Macroscopic and histological analyses were performed after 40-days. Macroscopy confirmed the repair of all defects in a comparable manner than the C-CTR. Histology showed no degradation nor integration in C-CTR while NFAH-GP and NFAH-GA biomaterials were encapsulated by connective and inflammatory tissues with partial biodegradation. The NFAH were fully biodegraded and replaced by a loose connective tissue and sclera covering the defects. This *in vivo* study demonstrated that the NFAH are a promising biocompatible and pro-regenerative alternative to the use of allogeneic cadaveric grafts. However, large defects and long-term studies are needed to demonstrate the potential clinical usefulness of these substitutes.

Keywords
Escleral surgical repair — Hydrogels — Crosslinking — Tissue engineering — Eyeball — Fibrin/agarose — Histology

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Contents

Introduction

The sclera (SC) and the cornea are the external layers of the eye globe. Both layers are joined at the corneal limbus and form the fibrous tunic of the eye [1]. The SC is a white, tough membrane representing 5/6 parts of the external eye surface (in humans) and provides insertion for the six extraocular muscles, maintaining the eye globe shape and providing biomechanical resistance to external forces and intraocular pressure [1]. Histologically, the SC is a relatively avascular layer mainly composed by a dense connective tissue which contains scattered fibroblasts immersed in a highly dense collagen-rich extracellular matrix (ECM) [1–3].

The structure and function of the SC can be affected by several conditions such as traumatic injuries, neoplasm, infections and myopia, which may compromise the vision and quality of life of affected patients [2,4]. 50% of scleral pathologies are associated to vascular systemic collagenopathies and 0.5% of rheumatoid arthritis [4]. Furthermore, scleral perforations

were reported in patients with osteogenesis imperfecta [5]. In addition, it is well-known that any alteration of the sclera ECM, is likely to lead to changes in SC shape that may dramatically affect vision [2]. Unfortunately, these injuries are difficult to treat and could lead to blindness [6].

In most cases, severe structural injuries of the human SC are treated with the use of tissue grafts [7–9]. In this context, the most frequently used graft for SC repair is the allogeneic SC obtained from cadaveric donors [10, 11]. Although it is an efficient treatment, several drawbacks have been described, including graft rejection, infectious diseases, lack of donor material, etc. [8–11]. For these reasons, other types of tissue grafts and biomaterials (e.g. amniotic membrane, Tenon grafts or dermal grafts) have been evaluated, but experimental and clinical studies are not conclusive and results are variable [8, 12, 13]. For these reasons, new efficient SC substitutes are still needed.

Over the recent years, fibrin-agarose hydrogels have been successfully used to generate diverse bioengineered tissue-like models for clinical applications [14–17]. On the one hand, fibrin provides a natural fibrillar and porous network able to support cell proliferation, adhesion and migration, differentiation and ECM synthesis. On the other hand, agarose is an inert polysaccharide whose function in the hydrogel is to protect fibrin from degradation, to prevent hydrogel contraction and to significantly improve the hydration rate and biomechanical properties of this biomaterial. All these properties are key factors in tissue regeneration [15–19]. After *ex vivo* and *in vivo* preclinical studies, cornea and skin tissue-like substitutes based on nanostructured fibrin-agarose hydrogels (NFAH) were elaborated as advanced medical products under GMP conditions for clinical use [20]. Based on the good results obtained with the use of NFAH in tissue engineering, especially in cornea [14, 21], NFAH emerges as a potential biomaterial for the surgical repair of structural defects of the SC. In addition, recent studies demonstrated the possibility to improve the structural, biomechanical and biological properties of the fibrin-agarose biomaterial by using the nanostructuration technique [18], chemical crosslinking or a combination of both [22, 23], which could improve the features of this biomaterial for use in SC repair.

The aim of this study was to evaluate the potential usefulness of NFAH and crosslinked NFAH for the repair of structural defects of the SC in a rabbit model. For this purpose, we compared the surgical efficiency and tissue regeneration of NFAH and crosslinked NFAH with allogeneic SC grafts. SC regeneration was evaluated from the surgical perspective and by using histological and histochemical approaches.

1. Methods

1.1 Laboratory animals

In this study, 19 male adult New Zealand rabbits were used. Animals were maintained during the whole study in the Experimental Unit of the University Hospital Virgen de las Nieves, Granada (Spain) with adlibitum access to water and standard rabbit chow. Animals were housed in a temperature-controlled room (21 ± 1 °C) with a 12 h light/dark cycle. The Andalusian Animal Ethics and Research Committee (CEEA) approved these studies (ref. PI 400/2016), and all procedures and protocols fulfilled the EU Directive 2010/63/EU for animal experimentation and use of laboratory animals in research.

1.2 Generation of biomaterial-based SC substitutes

Different biomaterials were used to repair scleral structural defects in rabbits: NFAH, crosslinked NFAH and cadaveric allogeneic scleral grafts (ASG).

The NFAH-based scaffolds were generated by following previously described protocols [15,16,18,22,23] . In brief, we prepared 30 ml of hydrogel by mixing 22.8 ml of pharmaceutical-degree human plasma obtained from healthy donors with 2.25 ml of phosphate buffer solution (PBS, 0.1M, pH 7.2-7.4) and 0.45 ml of tranexamic acid (Amchafibrin 500 mg). This solution was carefully mixed and then, 3 ml of 2% $CaCl_2$ and 1.5 ml of melted 2% type VII agarose (Sigma-Aldrich) were added. The mixture was immediately aliquoted in three Petri dishes and kept under standard culture conditions until complete gelation. To prevent scaffold-related technical variations, all fibrin-agarose hydrogels were generated by using a unique source of human plasma from a single healthy donor. This plasma was purified and provided by the Blood and Tissue Bank of Granada, Spain as a pharmacy degree therapeutical product. To generate a NFAH, jellified fibrin-agarose biomaterials were subjected to nanostructuration as previously described [18, 22, 23]. Briefly, biomaterials were placed between a couple of sterile nylon filters with 0.22 μm pore size (Merck-Millipore, Germany) and two pieces of Whatman absorbent paper, and an uniform compression pressure was applied by using a mass of 500 g for 2.5 min at room temperature. As a result, we obtained highly-dense NFAH of 50-60 μm thickness and around 80% of hydration [18].

Crosslinked NFAH were generated by using a chemical crosslinking agent able to improve the structural and biomechanical properties of NFAH as recently described by our group [22,23]: 0.1% genipin (NFAH-GP) and 0.5% glutaraldehyde (NFAH-GA).

In the case of ASG, these tissue grafts were obtained from one rabbit that was previously euthanatized. In this donor animal, both eyes were carefully harvested and the sclera was dissected and separated from the conjunctival and choroid layers. These SC grafts were subjected to three sequential cycles of freezing and thawing and preserved at -40 ºC until the moment of the implant in the clinical control group.

1.3 Experimental groups

Animals were deeply anesthetized by intramuscular injection of xylazine (5 mg Kg^{-1}) followed by an intramuscular injection of ketamine (25 mg Kg^{-1}). Then, a circular scleral defect of 5 mm of diameter was created in the superior-temporal region of the left eye by using a dermal biopsy punch. Defects were not transmural and only the scleral layer of the eye was removed. Scleral tissue was carefully removed until obtaining

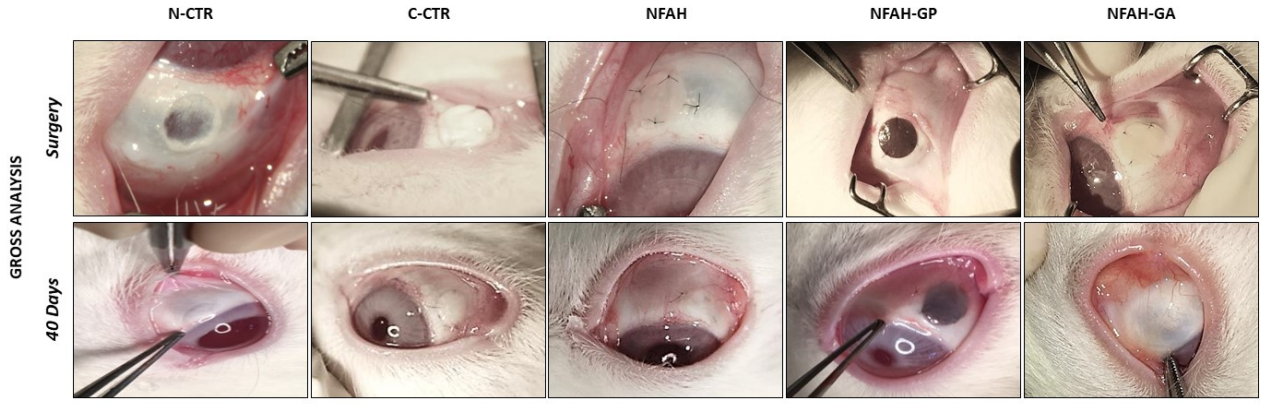


Figure 1. Representative macroscopic images of the SC defects and the surgical repair at the beginning and after 40 days *in vivo*.

the transparency of the eye wall. Animals were randomly assigned to one of the following experimental groups (n=3 each):

A) Negative control group (N-CTR): Scleral defects were not repaired.

B) Clinical control group (C-CTR): Defects were repaired by using ASG cadaveric scleral grafts of 5 mm diameter.

C) NFAH group: Defects were repaired by using NFAH.

D) NFAH-GP group: Defects were repaired by using NFAH crosslinked with 0.1% genipin.

E) NFAH-GA group: Defects were repaired by using NFAH crosslinked with 0.5% glutaraldehyde.

The native right eye of each animal was used as control (healthy controls H-CTR). In all cases, grafts were implanted by using equidistant nylon 10-0 sutures that were then covered with conjunctiva. Operated eyes were treated with topical antibiotics and the eyes were temporally closed with a palpebral suture. Palpebral occlusion was retired after one week. All animals were euthanatized 40 days after the surgery by using an overdose of anesthesia followed by intracardiac injection of KCl.

1.4 Macroscopic evaluation

The following parameters were evaluated during the surgical procedure: degree of handling of each graft, structural stability, rigidity, elasticity, adhesion and suturability. Each parameter was scored from 0 to 5 (0=lower score, 5= maximum score). After the surgery, animals were daily controlled and evaluated to determine: the presence of any kind of inflammatory reaction, rejection or infection associated to the occlusion, conjunctiva or, especially, to the implants; edema of the operated zone, defect repair or deformation, graft and biomaterial integration, degradation or other complications and side-effects.

1.5 Histology

Control and operated eyes were carefully harvested from euthanatized animals and fixed by immersion in 4% neutral buffered formaldehyde in a biopsy container with a fixative volume corresponding to 20 times the sample volume. In order to favor the fixative penetration, two 20 G needles were inserted into the posterior and vitreous chamber of each eye (far from the repaired area). After 24h, when eyes acquired certain degree of consistency, we surgically dissected the scleral region to be studied -with ample normal margins-, which was fixed for one additional day to complete tissue fixation. Finally, samples were included in paraffin, sectioned at 5 μm and subjected to routine histological analyses and histochemistry. Hematoxylin-eosin (HE) was used for general histological evaluation. Collagens were evaluated with the Masson's trichrome (MS) and Picrosirius red (PS) methods. Acid proteoglycans and glycoproteins were identified with alcian blue (AB) and Schiff periodic acid (PAS) methods, respectively. The presence of elastic fibers was studied with orcein staining (ORC). All methods were performed at the same time using the same environmental conditions following well-documented and previously optimized procedures [24].

1.6 Thickness evaluation

In this study, we analyzed the thickness of the implanted graft, the subjacent scleral wall and total scleral wall using low magnification histological images stained with HE. Quantification was performed by using the measurement function of the Image J software (National Institute of Health, USA). Ten measurements from three independent slides from each group of samples were taken perpendicular to the SC surface.

Table 1. Biomaterials properties from a surgical perspective.

Biomaterials	Handling	Structural stability	Rigidity	Elasticity	Adhesion	Suturability
N-FAH	4	3	3	4	4	3
N-FAH-GP	5	3	4	2	3	3
N-FAH-GA	4	3	4	2	3	3
C-CTR (Sclera)	5	5	5	1	2	3

Score: 0–5: 0 = lower score and 5 = maximum score.

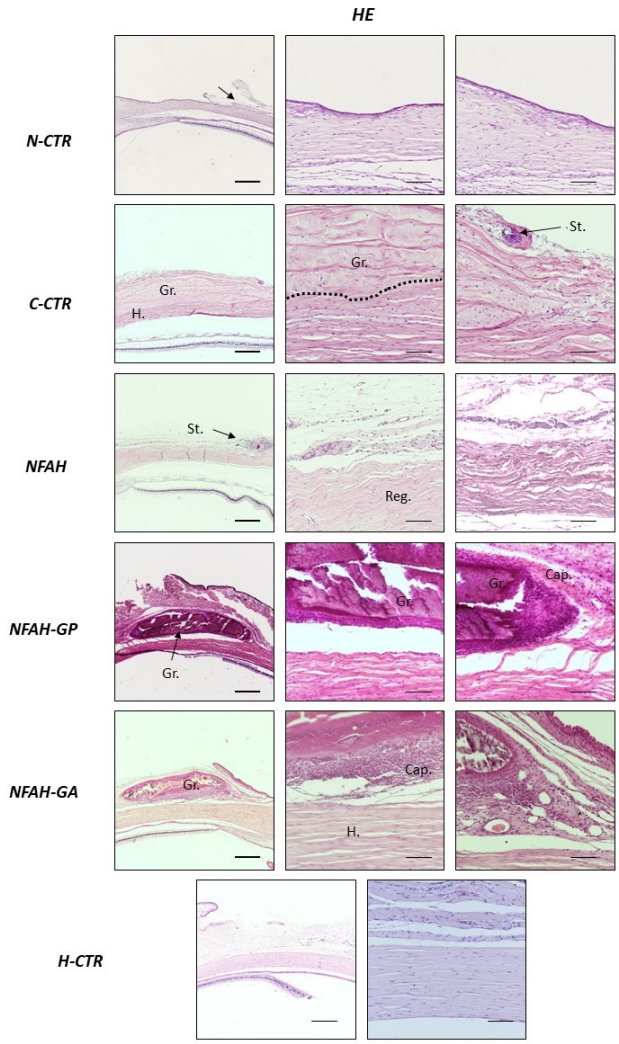


Figure 2. . Histology of SC after surgical repair with allogeneic grafts and biomaterials. Black arrow=border; Gr= Graft (sclera and biomaterials); St. arrow= suture associated inflammatory reaction; Mc. arrow= macrophages; Reg= regenerated tissue; Cap= capsule around grafted crosslinked biomaterials. Scale bar= 500 μm in low magnification images (low mag.) and 100 μm for middle portion and border image.

Then, mean and standard deviation values were calculated and statistical differences were determined with Mann-Whitney non-parametric two tailed test.

2. Results

When we macroscopically analyzed each type of tissue graft, we found that, in general, all biomaterial-based tissue-like substitutes allowed the successful repair of the created scleral defects. In the case of N-CTR group, the defect was not fully repaired, and the wound was still macroscopically evident after 40 days (Figura 1). From the surgical perspective, the use of cadaveric scleral grafts (C-CTR) resulted to be stable and easy to handle and suture without complications, although the thickness of the graft needed to be adjusted to the depth of the defect in most cases. The use of allogeneic SC grafts was able to successfully repair the defect created in the C-CTR animals after 40 days, although the borders between graft and host tissues were still visible (Figura 1). When NFAH was used, these biomaterials resulted to be elastic, stitchable and relatively easy to implant, showing good adhesive properties. After 40 days, the defects were fully repaired and the graft was not identifiable (Figura 1). Interestingly, the use of crosslinked biomaterials (NFAH-GP and NFAH-GA) showed better structural stability than NFAH, but these biomaterials were less adhesive and less elastic than NFAH and especially, than C-CTR. Both crosslinked NFAH were able to successfully repair the defect after 40 days. In the case of the NFAH-GP group, implanted biomaterials were easily identified after the follow-up period -thanks to their characteristic blue color- filling the defects and covered by a thin connective tissue layer. Table 1 summarizes the semiquantitative results of the biomaterials properties observed during implantation.

Then, the histological analysis of the different study groups using HE staining revealed important differences among the grafts evaluated in the present study. When we analyzed the N-CTR group, we found that the lesion created was partially repaired after 40 days. Indeed, a considerable reduction of the SC wall-thickness was observed at the injury site. In addition, the outer part of the defect was covered with a loose connective tissue layer instead of a typical collagen-rich scleral ECM (Figura 2). When the C-CTR group was studied, we identified the scleral allogeneic graft immersed within the scleral layers. However, the SC wall-thickness was remarkably increased and no signs of graft integration, degradation or inflammation were observed, except a localized foreign reaction associated to the non-absorbable sutures used. The ECM of the grafted sclera was characterized by longitudinally oriented lamellae and an increased inter-lamellar ECM, which resulted in the expansion of the wall-thickness (Figura

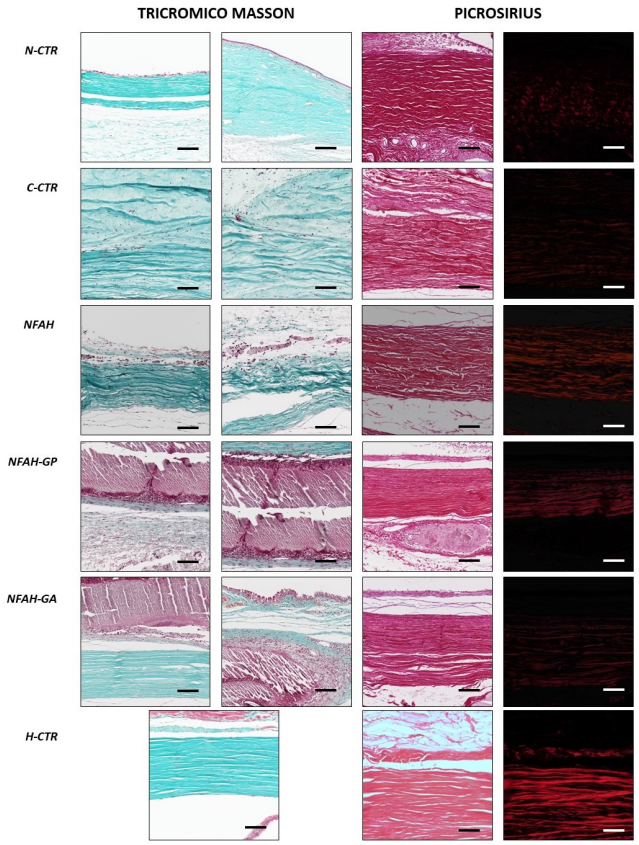


Figure 3. Histochemical analysis of collagens in healthy, none repaired and repaired sclera. Two representative images for Masson trichrome staining are shown. The picrosirius staining was analyzed at light and polarized microscopy which revealed organization pattern of collagen fibers. Scale bar= 100 μm.

2). Concerning the implantation of the NFAH, this biomaterial was able to contribute to repair the defects, resulting in a histological pattern and thickness that were comparable to the healthy controls (H-CTR group). In the outer side of the repaired zone, some macrophages and other mononuclear cells partially lined the area and no biomaterial remnants were observed. However, as observed in other groups, a foreign body reaction was identified surrounding the suture material (Figura 2). In relation to the use of crosslinked biomaterials (NFAH-GP and NFAH-GA), both grafts allowed us to repair the defect with an increase of the thickness wall after 40 days. Both biomaterials were still identifiable at this time, and an inflammatory reaction composed by macrophages lining the biomaterial surface and lympho-plasmocytes was observed (Figura 2).

The histochemical evaluation of fibrillar collagens using MS and PS showed no clear synthesis of these molecules in the defect area of N-CTR group, although the subjacent scleral wall was highly positive but slightly disorganized (PS-polarized microscopy) as compared to H-CTR (Figura 3). In the C-CTR group, histochemistry for collagens was positive at the level of the scleral graft and subjacent host sclera. In the grafted sclera, collagens were clearly stained at the lamellar level, being weakly stained in the inter-lamellar ECM. In addition, PS-polarized microscopy did not reveal a proper collagen network organization in the grafted cadaveric sclera (Figura 3). Evaluation of the NFAH group revealed a positive histochemical reaction for collagen at the newly-formed sclera, with an immature organization (Figura 3). When crosslinked biomaterials were evaluated, we confirmed the presence of a collagen-rich pseudocapsule surrounding the grafted biomaterials, which was more evident in the NFAH-GP group. No positive histochemical reaction was observed at the inner part of both implanted biomaterials, which were poorly invaded by cells and host surrounding tissues (Figura 3).

On the other hand, the histochemical analysis of elastic fibers did not reveal the presence of these ECM fibers in any of the animals included in the study (healthy or operated animals), suggesting that elastic fibers are very scarce in the SC (Figura 4). When ECM glycoproteins were analyzed by the PAS histochemical method, we found that all animals subjected to surgery were weakly positive, with native controls showing a slightly more positive signal. PAS clearly stained the macrophages observed at the surface area in the NFAH group (Figura 4). The histochemical analysis of acid proteoglycans as determined by AB revealed a positive and homogeneous staining in H-CTR, with slight changes in the distribution and intensity of these molecules among the different experimental conditions (Figura 4). In the case of N-CTR group, a weak positive reaction was observed, which clearly differed from the intense histochemical reaction observed in repaired scleral walls. In the case of C-CTR, proteoglycans were abundant around the grafted cadaveric sclera and in the inter-lamellar ECM, and were moderately positive in the newly-formed connective tissue in the NFAH group(Figura 4). When crosslinked biomaterials were used, a homogeneous reaction was observed in the subjacent sclera and around the grafts (Figura 4).

Furthermore, the morphometric analysis revealed that the control native rabbit sclera has a mean thickness of 313.85 μm at the interest. When a partially defect was created and not repaired (N-CTR), the scleral wall lost around half of its thickness, being this decrease statistically significant (p=0.000) as compared to controls (Figura 5). In contrast, the use of grafts to repair the defects resulted in a significant increase of the scleral wall thickness as compared to controls, with differences being especially significant with the N-CTR group 5. When thickness of the implanted grafts was compared in the different study groups, we found that the highest thickness corresponded to the NFAH-GA group. In contrast, we only found a thin connective tissue layer with a mean thickness of 34.09 μm in NFAH group (Figura 5). Concerning the SC tissue that remained subjacent to the implanted graft, morphometric analysis revealed a significant reduction of this tissue in the NFAH-GP and no differences in C-CTR and NFAH-GA

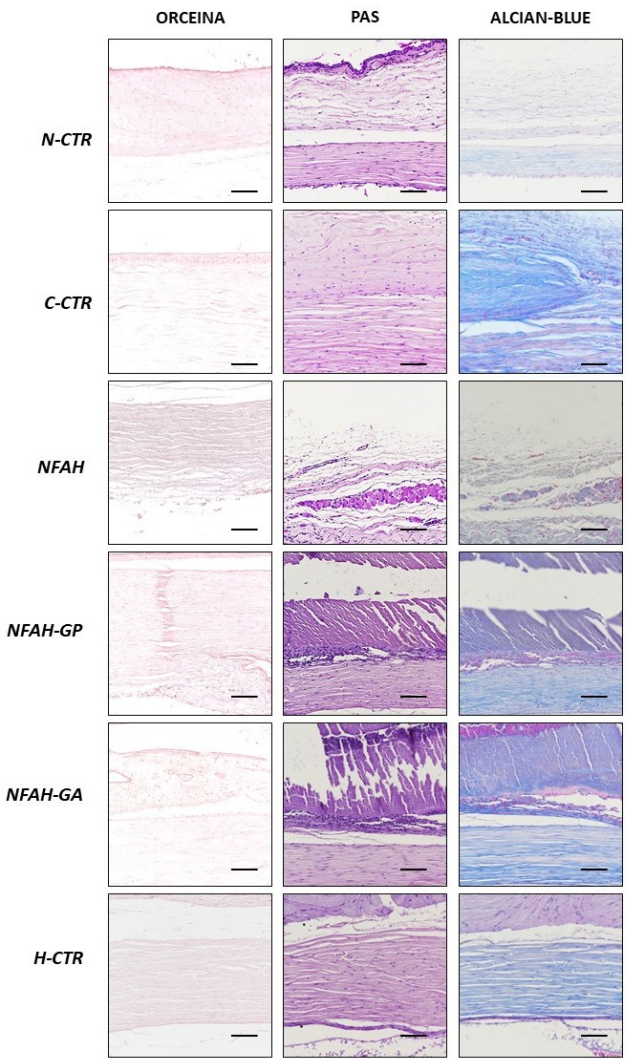
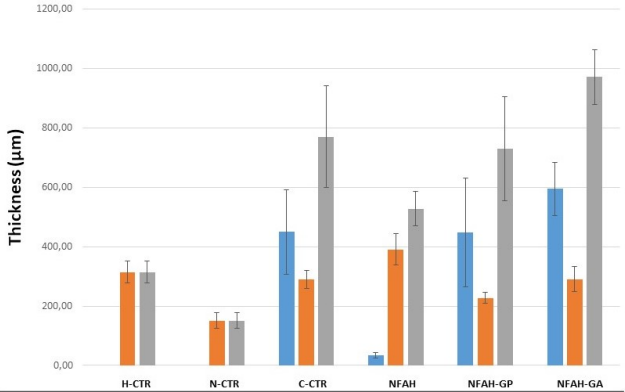


Figure 4. Histochemical analysis of elastic fibers and non fibrillar ECM molecules in healthy, none repaired and repaired sclera. No positive histochemical reaction was observed for elastic fibers with orcein. PAS staining was diffuse and did not revealed alterations of the glycoproteins, but was positive for macrophages. Alcian blue (AB) staining revealed a positive reaction for acid proteoglycans in all conditions. Scale bar= 100 μm.



	H-CTR	N-CTR	C-CTR	NFAH	NFAH-GP	NFAH-GA
IMPLANT	-	-	450.25 ± 142.18	34.09 ± 9.20 a, c	447.52 ± 183.32 d, e	595.01 ± 89.85 a, c, d
SCLERA	313. 85 ± 36.8	151.02 ± 26.11 a	289.13 ± 30.79 b	390.51 ± 53.06 a, b, c	227.17 ± 17.9 a, b, c, d, e	291.07 ± 41.84 b, d
TOTAL			770.171 ± 171.7 a, b	527.77 ± 58.55 a, b, c	729.84 ± 175.8 a, b, c, d, e	971.46 ± 92.72 a, b, c, d

Figure 5. Morphometric differences of scleral repair. Quantitative values for the thickness of implants, host sclera and whole scleral wall (implant + host subjacent sclera) are shown as mean ± standard deviation values. p¡0.05 values were considered statistically significant for Mann-Whitney non parametric test. a= significant differences between surgical groups vs H-CTR b= significant differences between graft-based repair groups vs. N-CTR c= significant differences between biomaterial-based grafts vs. C-CTR d= significant differences between crosslinked biomaterials-based grafts vs. NFAH e= significant differences between NFAH-GP vs. NFAH-GA.

groups. Interestingly, a significant increase in the scleral wall was observed in the NFAH group as compared to controls or crosslinked biomaterial-based grafts (p¡0.05).

3. Discussion

In this study, we evaluated the suitability of different biomaterial-based strategies as potential alternatives to the use of allogeneic SC grafts for the surgical reconstruction of structural defects of the SC in a novel rabbit model. Repair and tissue regeneration were assessed macroscopically and histologically.

From a surgical and biological perspective, biomaterial-based substitutes used in TE should fulfill a number of criteria that are directly related to the regeneration success rate [25, 26]. Biomaterials should be easy to handle and suture to allow surgical implantation, biocompatible and pro-regenerative, and they must respond to the biomechanical and biological demands of the tissue that must be repaired or replaced [25, 26]. In this regard, all biomaterials used in the present study demonstrated to be easy to handle, allowing their implant and suture at the site of the scleral defect in a comparable way to the allogeneic sclera. These results are in agreement with previous reports demonstrating that the use of NFAH-based scaffolds may have an important role in tissue regeneration in different models of skin, cornea, peripheral nerve and other organs [14–17, 27]. In these applications, fibrin showed excellent biocompatibility supporting tissue regeneration, and agarose was able to improve the 3D configuration and biomechanical properties of the hydrogels [19]. In the present work, we wanted to find out if these regenerative properties can be applicable to the scleral setting.

In the N-CTR group, the SC was semitransparent and partially covered by connective tissue, which confirmed that the tissue regeneration process was unsuccessful in this study group. However, this study demonstrates that all grafting strategies were able to repair anatomical defects of the rabbit SC after 40 days, although differences were observed in the biodegradation rate and/or integration of the grafts. Remark-

ably, the cadaveric sclera was not fully integrated after 40 days, and similar results were observed in the NFAH-GP group. In contrast, no remnants of NFAH or NFAH-GA biomaterials were macroscopically observed after 40 days, suggesting that a nearly-complete integration, biodegradation or replacement process of the implanted biomaterial occurred in these groups. The comparative analysis demonstrated that NFAH were biodegraded and replaced by newly-formed tissue. These results are in agreement with the biodegradation rate observed in our previous *in vivo* studies in other tissue models [15, 16]. Interestingly, implanted NFAH-GP grafts were still evident at the graft site after 40 days, suggesting that crosslinking with genipin may be a useful strategy to control the *in vivo* biodegradation rate of NFAH-based substitutes in TE. Genipin is a natural crosslinking agent usually used to modulate porosity, increase the overall biomechanical properties and reduce biodegradation of biomaterials [23, 28]. The semiquantitative evaluation of the surgical properties of biomaterials revealed some differences between NFAH and crosslinked NFAH-GP and NFAH-GA. These results are in agreement with our previous biomechanical studies showing that specific crosslinking agents are able to significantly increase stiffness of NFAH. As expected, crosslinked NFAH became significantly more resistant, but less elastic [18, 19, 22, 23]. All biomechanical parameters of crosslinked NFAH were within the expected values for use in regenerative medicine and supported handling, suturability and grafting [17–19, 23]. Finally, from the clinical and biomechanical perspectives, the biomaterials proposed in this study for SC repair combine certain degree of stiffness and elasticity, being potentially useful alternatives to the use of cadaveric SC grafts, especially when NFAH were used. Future studies should determine if the different sources of plasma may influence these results due to interpersonal differences in fibrin concentration.

In relation to the animal model, our results demonstrated that rabbits corresponding to the N-CTR group were not able to fully regenerate the scleral defect after 40 days. These results suggest that this animal model is a promising alternative to investigate novel surgical, biomaterial-based strategies or bioengineered tissue-like substitutes for SC surgical reconstruction. In this regard, histology and morphometric analysis of the negative group (N-CTR) confirmed the macroscopic anatomical findings revealing a partial repair of the defect with the formation of a loose connective tissue, which was not comparable to the histological pattern and thickness of a native sclera (H-CTR group). Although the injury created in the animals was not critical, this defect was useful to elucidate differences among experimental conditions. Furthermore, the use of rabbits in ophthalmology research is supported by studies conducted in cornea tissue engineering [14], in the development of reinforcement strategies [29] and for the analysis of scleral crosslinking for the treatment of progressive myopia [30].

Histology is crucial to elucidate cellular and molecular processes in native, regenerating and pathological tissues [15, 24]. In our study, histology revealed the fate of the grafts and biomaterials used to repair scleral defects, as well as the host tissue response after 40 days of *in vivo* follow-up. The SC is mainly composed by a highly dense collagen-rich ECM which provides the biomechanical strength and opacity needed for the normal eyeball rotation and external light isolation, respectively [31]. Histology demonstrated that the cadaveric scleral grafts (C-CTR) responded to the biomechanical and biological demands needed to successfully repair the defects, even when they were not fully integrated, but a significant increase of the scleral wall thickness occurred. Furthermore, no signs of degradation, ECM remodeling, inflammation or tissue rejection were observed after 40 days. This could be explained by the close genetic relationship of the laboratory animals used, and the poor vascularization of the SC [31]. These grafts provide a native, properly oriented collagen-rich ECM, but the success of this treatment is not related to the cells within the grafts, because they were irreversibly damaged and inactivated by the cryopreservation process [10].

Histology of crosslinked NFAH-based substitutes revealed the formation of a connective tissue pseudocapsule surrounding the biomaterial, with a variable amount of inflammatory elements. It is likely that this structure is playing a role in biodegrading the biomaterials from their surface, which could be related to the increase of the SC wall thickness that we found as compared to native sclera and NFAH group. These results are in agreement with previous studies in which crosslinking increased the resistance of biomaterials to chemical and *in vivo* biodegradation [28, 32]. Crosslinked biomaterials may be less permeable to host cell migration and biodegradation, probably due to the increase of biomechanical properties and/or modifications at the molecular level. In the case of the NFAH, histology and morphometry demonstrated the presence of a newly-formed SC tissue composed by relatively well organized collagen fibers and proteoglycans with a similar pattern and comparable thickness to native tissue. In addition, we did not observe any clear signs of inflammation, rejection or active biodegradation of biomaterials, although few and localized mononuclear macrophages were identified in the external surface. Therefore, these results suggest a good balance between NFAH biodegradation and tissue regeneration as previously observed with this hydrogel in skin [16], and peripheral nerve [15, 17]. It is true that the newly-formed SC was not fully comparable to native SC. We hypothesize that this could be related to an insufficient follow-up time for complete tissue regeneration.

In this study, the SC thickness analysis demonstrated that the surgical implantation of cadaveric SC and biomaterials was able to increase SC thickness, resulting in a successful repair process of the created defects. Interestingly, differences among groups were observed after 40 days of surgical repair. When we used allogeneic SC grafts, results showed an incomplete integration of the graft and an increase of the inter-lamellar ECM of the grafted material. Concerning the use of biomaterials, differences were clear and more favorable

results were obtained with the use of NFAH as compared to crosslinked biomaterials (p¡0.05). These differences could be explained by a biodegradation process with an active inflammatory reaction. Indeed, NFAH were completely biodegraded, supporting SC regeneration, after 40 days. In contrast, crosslinked biomaterial were partially biodegraded and associated to an active inflammatory host tissue response that significantly increased SC wall thickness. Future time-course and long-term studies should be performed to demonstrate if these biomaterials are able to support complete scleral tissue regeneration.

Currently, cadaveric scleral grafts from tissue banks or scleral autografting remain as the preferred treatment for the repair or reinforcement of the eyeball wall in many pathological conditions [10, 33, 34]. However, most patients are not candidate to scleral autografting, and there is an increase in the demand of scleral allografts grafts accompanied by a lack of donors, and allogeneic grafts have the potential risk of infectious agents transmission [10, 13]. These drawbacks stimulated researchers to find novel alternatives to treat these injuries. In this regard, fascia lata, skin, amniotic membrane, xenogeneic intestinal submucosa and many other natural materials started to be used with promising, but variable success [10, 12, 13, 35]. In this milieu, our study demonstrated that the use of NFAH is a promising alternative to repair SC defects supporting tissue regeneration in a new rabbit model of SC defect. The potential usefulness of NFAH as SC substitutes is supported by their high and well-known degree of biocompatibility and biodegradation rate and its well-demonstrated pro-regenerative properties in many tissue engineering applications [14, 16]. Besides, NFAH are currently approved for clinical use in Spain - according to the EU regulations-, plasma can be autologously obtained and prepared through a fast, easy and inexpensive procedure. All these issues are clear advantages of this biomaterial as compared to other autologous and natural biomaterials proposed for this medical application.

4. Conclusion

This *in vivo* study, conducted in a novel rabbit model of scleral defect, demonstrated for the first time that NFAH could be a promising alternative to the use of cadaveric SC in the surgical repair of SC defects. NFAH showed high biocompatibility and adequate biodegradation rate, and it was able to support tissue regeneration. However, molecular and time-course studies are in need to determine the potential clinical usefulness of NFAH in the surgical reconstruction of SC defects.

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Competing interests

All authors declare there is not any financial or personal relationship with organizations that could potentially be perceived as influencing the described research.

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