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Silk fibroin nanoparticles enhance quercetin immunomodulatory properties in DSS-induced mouse colitis

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic and idiopathic inflammatory disorder affecting the gastrointestinal tract. The pharmacological treatments used currently for its treatment lack efficacy, so new therapeutic strategies should be developed. In this context, flavonoids loaded in biopolymeric nanoparticles can be considered as novel promising candidates. The aim of the present study was to evaluate the intestinal anti-inflammatory effects of quercetin when is administered loaded in silk fibroin nanoparticles (QSFN) in the dextran sulphate sodium experimental model of mouse colitis, which displays some similarities to human IBD. Previously characterized quercetin-loaded silk fibroin nanoparticles (QSFN). QSFN showed a reversible aggregation profile induced by the acidification of the solution but did not affect the loaded quercetin. Daily administration of QSFN significantly reduced disease activity index values compared to the control colitic group. This beneficial effect was not only corroborated by the histological examination of the colonic specimens but also the improvement of the colonic expression of the different proinflammatory cytokines (*Tnf-α*, *Il-1β*, *Il-6*, *Mcp-1*, *Icam-1*, *Nlrp3* and *iNOS)*. Therefore, these data suggest that QSFN could be a promising alternative to current treatments as a drug delivery system for IBD treatment.

1. Introduction

Inflammatory bowel disease (IBD) comprises chronic and idiopathic inflammatory disorders affecting the gastrointestinal tract, mainly ulcerative colitis and Crohn's disease (Sairenji et al., 2017). Its prevalence in the general population is up to 0.3%, but it is increasing and has

Abbreviations: ATR-FTIR, Attenuated Total Reflectance Infrared Spectroscopy; DSS, dextran sulphate sodium; DAI, disease activity index; DLC, drug loading content; DLS, dynamic light scattering; EE, encapsulation efficiency; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; H&E, haematoxylin and eosin; *iNos*, inducible nitric oxide synthase; IBD, inflammatory bowel disease; *Icam-1*, intracellular cellular adhesion molecule-1; *Mcp-1*, monocyte chemotactic protein-1; ANOVA, one-way analysis of variance; PdI, polydispersity; Q, quercetin; QSFN, quercetin-loaded silk fibroin nanoparticles; SF, silk fibroin; SFNs, silk fibroin nanoparticles; SGF, simulated Gastric Fluid; TNF-α, tumour necrosis factor α.

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become a worldwide problem (Ng et al., 2017). Although the aetiology is unclear, genetic and environmental factors are involved, including alterations of the intestinal microbiota composition and/or function (termed as dysbiosis), as well as the immune response (de Souza et al., 2017). IBD is characterized by an up-regulation of different proinflammatory mediators, such as cytokines, nitrogen and oxide reactive species, eicosanoids and platelet-activating factor, together with an impairment of the intestinal epithelial integrity, thus leading to a chronic inflammation of the gut (Vezza et al., 2016). The current pharmacological strategies for the management of IBD, such as aminosalicylates, glucocorticoids, immunomodulators or biologicals, like antitumour necrosis factor α (TNF-α) antibodies (Seyedian et al., 2019), fail to induce and maintain remission in some patients and are associated with a high incidence of adverse effects, which can limit its administration (Abraham and Quigley, 2020). In consequence, there is a need for safer and more effective treatments that could complement or substitute the current available drugs. In this context, flavonoids, low-molecular-weight polyphenolic phytochemicals found in vegetables and fruits, can be considered as promising candidates (Filipe-Ribeiro et al., 2018). They have extensively shown anti-inflammatory, antioxidant and immunomodulatory properties, without presenting important toxic or side effects (Panche et al., 2016). After ingestion, flavonoids can be absorbed or directly act on gut epithelial, endocrine and immune cells, strengthening the intestinal barrier integrity and modulating the immune response. Moreover, they can also suffer biotransformation either by intestinal epithelial cells or the microbiota, and their metabolites can also exert further beneficial biological actions (Oteiza et al., 2018).

Among dietary flavonoids, quercetin is the most ubiquitous, being largely present in vegetables, fruits, tea, wine and olive oil (Lakhanpal, 2007). It is mainly found as glycosylated forms, such as quercitrin or rutin (Xiao and Lee, 2018). Quercetin has been reported for its anti-inflammatory, anti-oxidative, antihypertensive and anti-thrombotic properties for the treatment of different conditions, including cardiovascular diseases, obesity, diabetes and even cancer (Batiha et al., 2020). When considering the impact of quercetin on intestinal inflammation, the studies reveal inconsistent results. Kwon et al. (2005) failed to observe beneficial effects in the dextran sulphate sodium (DSS) model of mouse colitis while more recent studies have reported intestinal anti-inflammatory effects of quercetin in the same experimental model (Dong et al., 2020; Ju et al., 2018). However, quercetin glycosides, including quercitrin or rutin, have shown intestinal anti-inflammatory properties in various experimental models of colitis (Camuesco et al., 2004; Comalada et al., 2005; Kwon et al., 2005), being their effects attributed to quercetin, which is released after hydrolysis in the colon (Comalada et al., 2005).

Therefore, the fact that quercetin can be absorbed in the stomach and small intestine, or suffer intense degradation and rapid metabolism, may prevent its access to the colon at effective concentrations to display local anti-inflammatory and antioxidant effects, thus limiting its potential use for the management of these intestinal conditions (Berkel KaŞIkci and Bağdatlıoğlu, 2016; Crespy et al., 2002). Nevertheless, different quercetin delivery systems have been developed, in order to avoid extraction procedures and enhance their pharmacokinetic properties (Cai et al., 2013). For instance, quercetin-loaded microcapsules have been reported to ameliorate experimental colitis, showing higher efficacy than quercetin (Guazelli et al., 2013). It is acknowledged that nanoparticles have the potential to improve the stability and solubility of encapsulated cargos, facilitate the crossing through biological barriers and prolong circulation times. Thus, they are able to increase safety and efficacy, and reduce limitations of free drugs (Blanco et al., 2015; Kou et al., 2018; Davoudi, et al 2021). It is important to note that nanoparticles, increase the contact surface between the particle and the surrounding area, thus increasing its absorption and effectiveness (Khadka et al., 2014). Indeed, mucosal inflammation is closely related to loss of the inner adherent and the outer mobile mucus-gel layers, and impairment of the epithelial barrier integrity due to enterocyte damage. These alterations facilitate accumulation and uptake of nanoparticles by both enterocytes and macrophages, and increase exposure of the inflammatory receptors to nanoparticles (Hua et al., 2015). Silk nanoparticles have emerged, among other biopolymeric nanoparticles, as good candidates for drug delivery in medicinal applications because they are constituted by highly versatile, non-toxic, biocompatible and biodegradable proteins (Crivelli et al., 2018; Pham and Tiyaboonchai, 2020; Zhao et al., 2015; Lamboni et al., 2015; Tomeh et al., 2019; Ma et al., 2020). Silk fibroin nanoparticles have already been used in nanomedicine as a bioactive reversible carrier of macromolecules (Hofmann et al., 2006; Liu et al., 2017; Mottaghitalab et al., 2015) and small polyphenolic biomolecules such as resveratrol (Lozano-Pérez et al., 2014) or curcumin (Crivelli et al., 2019; Montalbán et al., 2018), among others. In a previous study our group demonstrated quercetin-loading efficiency of silk fibroin nanoparticles, which improved the stability and bioavailability of the compound (Lozano-Pérez et al., 2017). Then, <40% of the loaded quercetin was released in the gastrointestinal tract, where it could exert local beneficial effects, while the remaining could reach the colon, unmodified, which augments its anti-inflammatory effect in the damaged areas. Besides, silk fibroin nanoparticles should not be considered as mere inert carriers since they have also been reported to have intestinal anti-inflammatory properties in experimental models of colitis (Montalbán et al., 2018) and periodontitis in rats (Gimenez-Siurana et al., 2020). Moreover, a recent work has proven safety for oral use of silk derived from *Bombyx mori* cocoons at doses up to 500 mg/kg body weight/day, assessing toxicological and food allergy concerns, using well-established *in vitro* and *in vivo* models, and bioinformatics evaluation methods (Yigit et al., 2021).

We, therefore, hypothesized that silk fibroin nanoparticles would effectively deliver quercetin to the damaged intestinal cells improving quercetin intestinal wound healing capacity.

Thus, the aim of the present study was to evaluate the intestinal anti-inflammatory effects of quercetin when it is administered loaded in silk fibroin nanoparticles in the DSS experimental model of mouse colitis, which resembles human IBD considering its pathogenesis and therapeutic response (Yigit et al., 2021).

2. Materials and methods

2.1. Reagents

Silk fibroin (SF) was obtained, as previously described by our group (Lozano-Pérez et al., 2017), from cocoons of white silkworm (*Bombyx mori*) reared in the sericulture facilities of IMIDA (Murcia, Spain) and fed a diet of fresh *Morus alba* L. leaves. Quercetin and all chemicals were purchased from Sigma Aldrich Química, S.A. (Madrid, Spain), unless otherwise stated. Silk fibroin nanoparticles (SFNs) were prepared following the methodology described before (Lozano-Pérez et al., 2017), according to an adapted version of the method reported by Zhang *et al.* (Zhang et al., 2007). Ultrapure water (18.2 MΩ·cm^{-1}) produced by an ELGA Purelab Flex 2 (Veolia, High Wycombe, UK) was used throughout.

2.2. Preparation of the quercetin loaded silk fibroin nanoparticles

Quercetin-loaded silk fibroin nanoparticles (QSFN) for the *in vivo* assays were prepared in one batch as described before by a simple incubation of quercetin (Q) and SFNs at Q/SFN 1:25 (w/w) ratio, which produced the highest drug loading content according to previous results (Lozano-Pérez et al., 2017). Briefly, 40 mL of a solution of quercetin 0.5 mM in ethanol (6.4 mg) was mixed with an aqueous suspension of SFNs at 10 mg/mL (160 mg) and stirred for 3 h protected from light. QSFNs were recovered by centrifugation, washed with ultra-pure water (3x), and finally freeze-dried. QSFNs were obtained as a light green dry powder and kept at room temperature protected from light until use. Non-loaded SFNs were used in the experiments and considered as

controls.

2.3. QSFN characterization and stability

The characterization of the nanoparticles was performed using common techniques such as UV–Vis spectroscopy, Dynamic Light Scattering (DLS), and Attenuated Total Reflectance Infrared Spectroscopy (ATR-FTIR), in order to verify the characteristics of the nanoparticles in terms of hydrodynamic size, protein conformation and quercetin loading, as previously described in more detail (Lozano-Pérez et al., 2017).

Briefly, the spectrophotometric quantification of the drug loading content (DLC) and encapsulation efficiency (EE) of quercetin were performed by using a Synergy MX UV–Vis spectrometer (BioTek Instruments Inc; Winooski, VT, USA). The hydrodynamic characteristics of the QSFN, including mean diameter (Z-average), Polydispersity (PdI) and Zeta potential (ζ), were determined as described previously (Lozano-Pérez et al., 2017) by DLS using a Malvern Zetasizer Nano ZSP (Malvern Instruments Ltd, Malvern, UK).

Additionally, in order to confirm the suitability of the nanoparticles for the *in vivo* experiments, stability tests in different digestive fluids were performed. Briefly, 1 mg of QSFNs were suspended in 40 mL of Simulated Gastric Fluid (SGF) without pepsin (pH 1.2) prepared in accordance with the International Pharmacopeia methods (Lei et al., 2020), and incubated at 37 °C for 2 h under mild agitation. After incubation time, samples were neutralized with NaOH 0.1 M to pH 6.8. Unloaded SFNs were used as controls in the same conditions.

2.4. Dextran sulphate sodium (DSS) model of mouse colitis

This study was carried out in accordance with the 'Guide of the Care and Use of Laboratory animals' as promulgated by the National Institute of Health and the protocols approved by the Ethic committee of Laboratory Animal of the University of Granada (Spain) (Ref. No. 17/09/2019/156). Male C57BL/6J mice (7–9 weeks old) obtained from Janvier labs (St. Berthevin Cedex, France) were housed in makrolon cages, maintained in an air-conditioned atmosphere with a 12 h light/dark cycle and provided with free access to tap water. They were randomly assigned to five groups of 10 animals each. Colitis was induced by adding 3% w/v DSS (36–50 KDa, MP biomedicals, Ontario, USA) in the drinking water for 5 days. Mice were daily treated orally using an esophageal catheter with quercetin, SFNs or QSFNs at a dose of 5 mg of quercetin/kg in 200 μL of PBS solution. Non-colitic and non-treated DSS-colitic groups were included as reference and received PBS solution (200 μL/mouse/day). The treatments were maintained for three days after DSS removal, and then, mice were sacrificed. Every day, animal body weight, presence of gross blood in the faeces and stool consistency were individually evaluated in a blind manner. A score was assigned to each parameter according to a previously described criterion, which were used to calculate the average daily disease activity index (DAI) (Table 1) (Cooper et al., 1993). After sacrifice, the colon was aseptically removed, and the luminal content was collected. A representative piece of whole gut specimens (0.5 cm from the anus) was taken for histological studies. The remaining colonic tissue was minced, aliquoted and kept at −80 °C until RNA isolation.

Table 1
Scoring of disease activity index (DAI).

Score	Weight loss	Stool consistency	Rectal bleeding
0	None	Normal	Normal
1	1–5%		
2	5–10%	Loose stools	
3	10–20%		
4	> 20%	Diarrhoea	Gross bleeding

DAI value is the combined scores of weight loss, stool consistency, and rectal bleeding divided by 3. Adapted from Cooper et al. (1993) (Cooper et al., 1993).

2.5. Histological studies

The gut specimen taken for histological studies was fixed in formaldehyde and embedded in paraffin. 5 μm sections were stained with haematoxylin and eosin (H&E), and alcian blue. The histological damage was evaluated by a blinded pathologist observer, according to a previously reported histological score (Camuesco et al., 2012), taking into account presence of ulceration, infiltration, oedema and crypts condition.

2.6. Analysis of gene expression by RT-qPCR

TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to extract total RNA from colon tissue following the manufacturer's instructions. RNA was transcribed using olig(dT) primers (Promega, Southampton, UK), and the resulting cDNA (20 ng) was amplified on optical grade 48-plates in an EcoTM Real time PCR system (Illumina, San Diego, CA, USA) using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA). The specific primers used for each gene are shown in Table 2. To normalize mRNA expression, the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was measured for comparative reference. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Statistics

Results are expressed as the mean ± SEM unless otherwise stated. Statistical analyses were performed using GraphPad Prism 7 software (Graph-Pad Software Inc., La Jolla, CA, USA) and SPSS Statistics 17.0 Software Package (SPSS Inc., Chicago, IL, USA). Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. Non-parametric data (score) are expressed as the median (range) and were analysed using the Mann–Whitney *U* test. Differences between proportions were evaluated using the Chi-square test. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Characterization of the nanoparticles

The DLS measurements showed that QSFN used in this assay presented a monomodal nanometric size distribution (Zaverage = 175.8 ± 0.9 nm), low Polydispersity (PdI = 0.153 ± 0.009) and negatively

Table 2
Primer sequences and annealing temperatures used in real-time PCR assays in colonic tissue.

Gene	Organism	Sequence 5′- 3′	Annealing T °C
Gapdh	Mouse	FW: CCATCACCATCTTCCAGGAG RV: CCTGCTTCACCACCTTCTTG	60
Icam-1	Mouse	FW:AGGAGGTGAATGTATAAGTTA RV: GGATGTGGAGGAGCAGAG	60
IL-1β	Mouse	FW:TGATGAGAATGACCTCTTCT RV: CTTCTTCAAAGATGAAGGAAA	55
Il-6	Mouse	FW: AGTCCTTCCTACCCCAATTTCC RV: TTGGTCCTTAGCCACTCCTTCC	60
iNOS	Mouse	FW:GTTGAAGACTGAGACTCTGG RV GACTAGGCTACTCCGTGGA	56
Mcp-1	Mouse	FW:CAGCTGGGGACAGAATGGGG RV: GAGCTCTCTGGTACTCTTTTG	62
Nlrp3	Mouse	FW: ATGCTGCTTCGACATCTCCT RV: AACCAATGCGAGATCCTGAC	59
Tnf-α	Mouse	FW: AACTAGTGGTGCCAGCCGAT RV: CTTCACAGAGCAATGACTCC	60

charged surface (ζ = −24.5 ± 4.1 mV) (see Table S1 and Figs. S1–S3 of the supplementary information file for details).

The ATR-FTIR spectrum of QSFN, not only confirmed the presence of Q in the nanoparticles, but also the β-sheet conformation of the SF in the nanoparticles, which confers high stability in aqueous media (see Figs. S4 and S5 of the supplementary information file for details).

QSFN also showed a DLC of 0.72 ± 0.09% (7.2 ± 0.9 μg/mg QSFN), representing a quercetin encapsulation efficiency of 18.12 ± 0.24%, which agrees with previous reports for these loading conditions (Fig. S6) (Lozano-Pérez et al., 2017).

In order to use QSFN orally *in vivo* experiments, additional stability characterization tests were carried out. Thus, the stability tests performed in the different digestive fluids confirmed the suitability of the nanoparticles for the *in vivo* experiments. QSFN dispersed in SGF without pepsin at pH 1.2, and incubated at 37 °C, presented a fast aggregation due the protonation of the carboxylate groups of the aspartic and glutamic residues of the fibroin, showing a ζ = 3.62 ± 0.38 mV. Fig. 1 represents the evolution of the particle's size and their aggregation along the experiment. Size increased from 175.8 ± 0.9 nm, when they were dispersed in ultrapure water at t = 0 min, to 1285 ± 93 nm at t = 5 min, 1972 ± 144 nm at t = 10 min, 2408 ± 236 nm at t = 15 min, reaching 6697 ± 414 nm at t = 2 h of incubation time in the SGF (pH = 1.2). As it is known, ζ is another important parameter that directly affects the stability of nanosuspension (Grumezescu, 2019). Thus, low values of ζ implies small repulsion forces among nanoparticles which lead to the formation of aggregates due to the high affinity of the surfaces of the nanoparticles. After raising the pH of the suspensions to 6.8, the size of the aggregates was reduced to 4366 ± 356 nm in comparison with the one at lower pH, although still bigger than in water. Interestingly, the size distribution of the nanoparticles still showed their original characteristics even at pH higher than 7.5. SFNs, prepared as controls, displayed the same reversible aggregation profile after incubation in SGF at pH 1.2 and neutralization at pH 6.8. Thus, the presence of the quercetin did not affect aggregation or integrity of the nanoparticles

3.2. Dextran sulphate sodium (DSS) model of mouse colitis

Mice were given 3% DSS in drinking water which resulted in body weight loss, diarrhoea and presence of blood in the faeces. As a result, DAI values increased daily in control colitic mice during the course of the experiment. When the treated colitic mice were considered, only those mice administered QSFN showed significantly reduced DAI values at day 6 compared to the control colitic group as seen in Fig. 2. This beneficial effect was corroborated when the histological examination of the colonic specimens was performed (Fig. 3). DSS colitis is characterized by an important number of histological features, including mucosal erosions, mucin-depletion in the goblet cells, altered crypt architecture and intense inflammatory cell infiltration in the mucosa and lamina

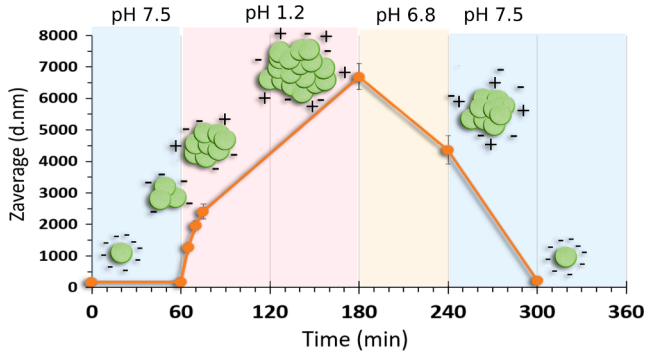

Fig. 1. Size evolution of the particles and their aggregates along the experiment as a function of the pH of the media. Error bars represent standard deviation of the mean (n = 3).

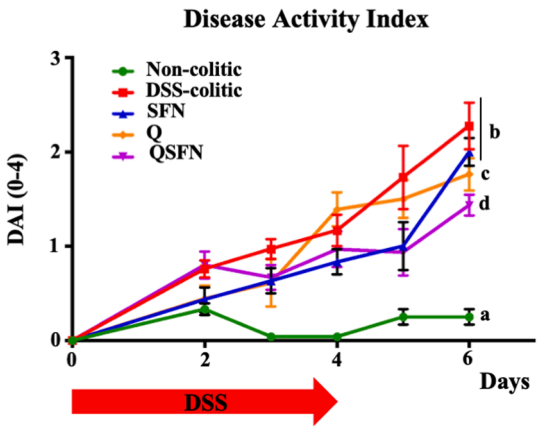

Fig. 2. Effects of QSFN treatment in DSS-induced colitis. Mice were exposed to 3% DSS for 4 days, with the exception of one group (Non-colitic). Body weight loss and clinical score (DAI) are shown (n = 8 per group). Values are expressed as means ± SEM. Different letters are significantly different ($p < 0.05$).

propria. Thus, untreated colitic mice exhibited marked unstructured lamina propria mucosa, greater distortion/damage to crypt architecture and severe inflammatory cell infiltration (Fig. 3C) in comparison to the non-colitic mice (Fig. 3B). However, SFN, Q and QSFN mice showed less severe pathological inflammation than the colitis control mice, and a partial recovery of the mucosa (Fig. 3D-F). However, only the QSFN treatment significantly improved tissue morphology, showing a significant amelioration of the damage in the mucosal barrier, with the presence of goblet cells replenished with their mucin content, together with a reduction of the inflammatory cell infiltration (Fig. 3F).

The intestinal inflammatory process induced by DSS was also associated with an altered expression of different inflammatory markers. In fact, the colonic expression of the pro-inflammatory cytokines (*Tnf-α*, *Il-1β* and *Il-6)*, the chemokine monocyte chemotactic protein-1 (*Mcp-1*), and the intracellular cellular adhesion molecule-1 (*Icam-1*) was significantly increased in untreated colitic mice in comparison with non-colitic mice (Fig. 4). The administration of QSFN or SFNs to colitic mice significantly reduced the expression of *Tnf-α*, *Il-1β* and *Il-6*; however, only QSFN significantly lowered the expression of *Mcp-1* or *Icam-1* (Fig. 4). Moreover, the colonic expression of the enzyme inducible nitric oxide synthase (*iNos*) was significantly upregulated in untreated colitic mice when compared to non-colitic mice and was only significantly reduced in those colitic mice treated with QSFN (Fig. 4). The DSS-induced colonic inflammation was associated with an increased expression of *Nlrp3*, one of the components of the inflammasome, and the administration of QSFN or quercetin to colitic mice showed a trend to decrease its expression, although no statistical differences were observed in comparison with untreated control colitic group (Fig. 4).

4. Discussion

Quercetin is a flavonoid ubiquitously found in nature in crude vegetables, foods and beverages, with interesting biological properties, like antioxidant and anti-inflammatory activities, which could be of potential use in the management of different human conditions (Ulusoy and Sanlier, 2020). Unfortunately, this therapeutic potential is conditioned by its low bioavailability after oral intake, due to low solubility and stability, being this is of special relevance in intestinal conditions, like IBD (Cai et al., 2013). In fact, previous preclinical studies have proposed that quercetin needs to be administered as a glycoside, like quercitrin or rutin, to show intestinal anti-inflammatory effects (Camuesco et al., 2004; Comalada et al., 2005; Kwon et al., 2005; Mascaraque et al., 2014). In an attempt to overcome these limitations, several strategies have been considered. Among these, different nanotechnology-based systems have been developed in order to obtain the delivery of natural

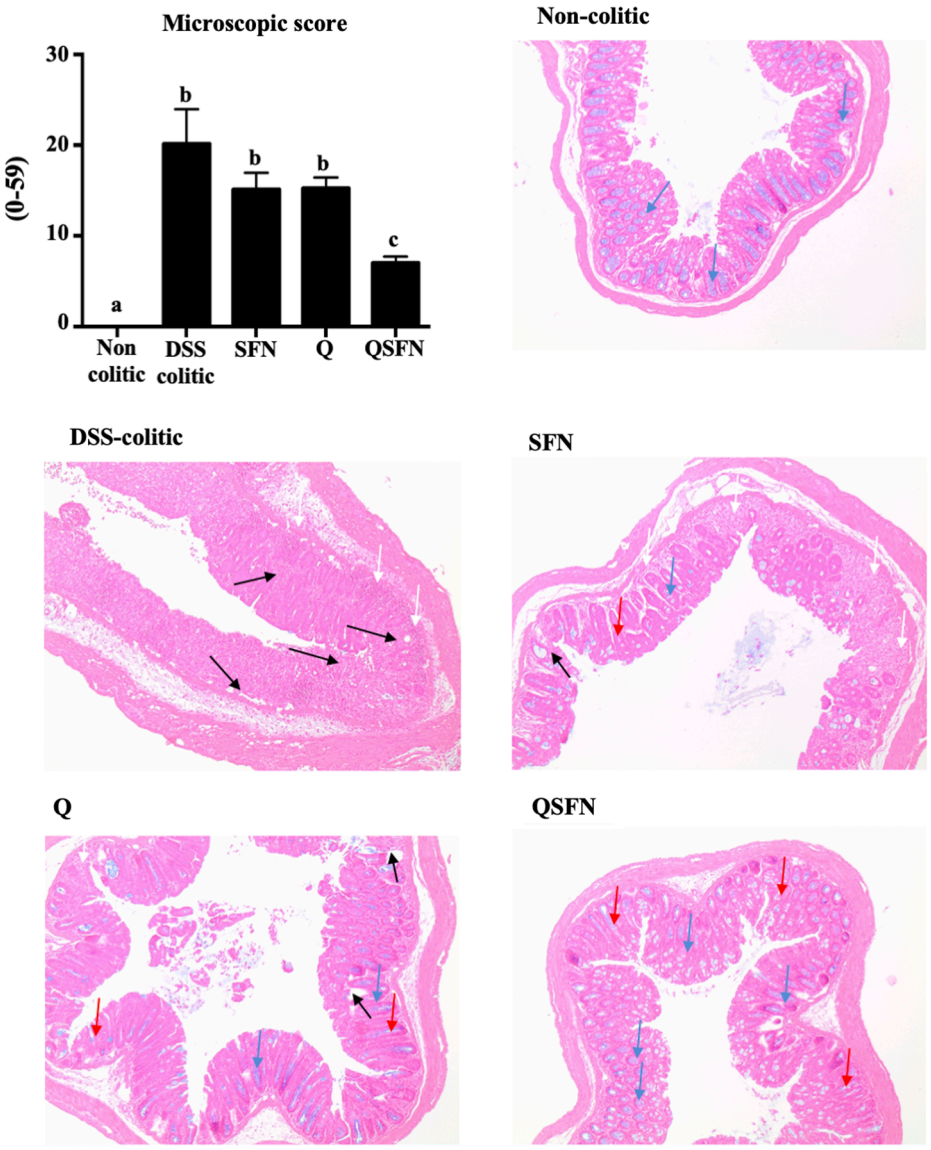

Fig. 3. Relative histology score and representative H&E stained images per each group. Values are expressed as means ± SEM. Statistical significance among groups was evaluated by one-way ANOVA. Bars with a different letter differ statistically ($p < 0.05$). Arrows indicate crypts/regeneration of crypts (red), goblet cells (blue), epithelium surface erosion (black) and inflammatory cells infiltration (white).

antioxidants, including quercetin, thus improving its bioavailability and then, obtain a higher efficacy in clinical practice (Mukhopadhyay and Prajapati, 2015; Vaiserman et al., 2019). Therefore, the effects of previously characterized SFN loaded with quercetin (Lozano-Pérez et al., 2017) were evaluated in the DSS experimental model of colitis in mice.

The results obtained in this study have demonstrated the intestinal anti-inflammatory effects of QSFN in DSS colitis, improving the efficacy of the single components, both SFN and quercetin. This effect was evidenced in the course of the experiment, when DAI values were daily evaluated for each experimental group. DAI comprises different factors associated with intestinal inflammation, including weight loss, decreased stool consistency and blood in faeces, which are assumed to be the determinants of the severity of the DSS-induced intestinal inflammatory process (Hasannejad-Bibalan et al., 2020), similarly to human IBD. Moreover, the beneficial effect was also corroborated by the histological evaluation of the colonic samples. QSFN administration to colitic mice ameliorated the DSS-induced damage which included the preservation of the normal crypt architecture including the recovery of the mucin content of the goblet cells as well as a lower inflammatory cell infiltration in comparison with untreated colitic mice. On the contrary, colitic mice treated with quercetin or SFN did not show a significant beneficial effect when these parameters were considered. The significant histopathologic changes produced by QSFN could be associated with its intestinal anti-inflammatory effect. Actually, QSFN was able to reduce the colonic expression of different proinflammatory cytokines, probably as a result of the lower infiltration and activation of immune cells involved in the inflammatory response induced by the DSS. Besides, QSFN significantly reduced the colonic expression of the chemotactic protein *Mcp-1* and the adhesion molecule *Icam-1* in treated colitic mice, which have been reported to participate in lymphocyte migration to the inflamed tissue (Dotan et al., 2020). Of note, the infiltration of monocytes in the damaged colonic tissue and the subsequent differentiation to macrophages seem to play a crucial role in the intestinal inflammatory response. Macrophage activation leads to production of different proinflammatory cytokines, including TNF-α, IL-1β and IL-6. TNF-α is considered a central mediator in intestinal inflammation that generates a cascade of intracellular events leading to the activation of death signals, mitogen activated protein kinases or transcription factors like NF-kB (Ruder et al., 2019). IL-1β can act in combination with TNF-α and IL-6 to increase the severity of inflammation (Mao et al., 2018). In

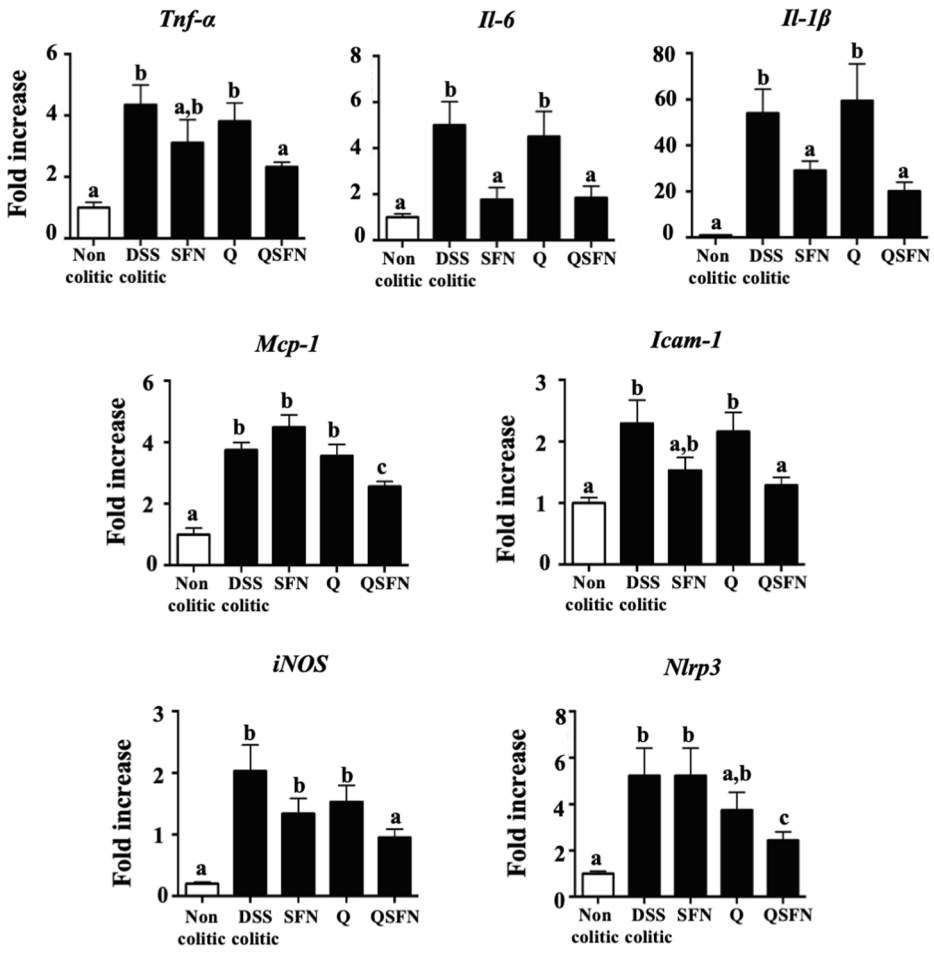

Fig. 4. Biochemical evaluation of the effects of QSFN treatment; mRNA expression of different mediators; *Tnf-α, Il-6, Il-1β, Mcp-1, Icam-1, iNos, Nlrp3,* was quantified by real-time PCR, and fold changes are expressed as means ± SEM. Statistical significance among groups was evaluated by one-way ANOVA. Bars with different letters are significantly different ($p < 0.05$).

particular, the binding of TNF-α and IL-1β to intestinal immune cells amplifies the immune response by enhancing T cell proliferation, promoting leukocyte infiltration and facilitating cell–cell signalling (Neuram, M.G., 2004). Interestingly, the inhibition of cytokine expression exerted by QSFN is similar to that obtained when SFN was administered to colitic mice, thus indicating that the carrier also contributed to lessen the intestinal inflammation, probably due to its antioxidant properties, and thus confirming our previous observations in other experimental models of colitis (Gou et al., 2019; Lozano-Pérez et al., 2017; Lozano-Pérez et al., 2014; Rodriguez-Nogales et al., 2016). On the contrary, the oral administration of quercetin was devoid of any significant effect on all these biochemical markers, thus confirming the absence of intestinal anti-inflammatory effect of the aglycone previously reported (Kwon et al., 2005). These results support those obtained in the histological evaluations. Finally, IL-6 is a mediator of the acute phase response and T cell differentiation, activation and resistance against apoptosis (Tanaka et al., 2014; Waldner and Neurath, 2014) as well as participates on the onset of inflammation in colitis (Mitsuyama et al., 1995; Wine et al., 2013). Our results indicated that QSFN and SFN supplementation restored significantly the expression of *Il-6*, however, quercetin did not show significant changes in its expression. Considering all these, it is obvious that the carrier has a positive impact on the inflammatory process. These results may be relevant since the expression of these proinflammatory cytokines has been described to be increased in human IBD, and the effect of these cytokines on the intestine has been directly related to the induction of tissue injury and/or destruction (León et al., 2009).

In addition, NLRP3 inflammasome has been proposed as a crucial regulator of intestinal homeostasis, and it has been implicated in the pathogenesis of IBD through the production of pro-inflammatory cytokines, including IL-1β (Zhen and Zhang, 2019). Thus, colonic inflammation was associated with increased expression of *Nlrp3*, which was significantly ameliorated after administration of QSFN to colitic mice. Previous *in vitro* and *in vivo* studies have shown the ability of quercetin to inhibit NLRP3 inflammasome activation in different experimental conditions (Domiciano et al., 2017; Xue et al., 2017). However, in the present study, quercetin administration to colitic mice only showed a significant trend to decrease the colonic expression of *Nlrp3* when is loaded in the nanoparticles, which would support the fact that quercetin needs its incorporation into SFN to exert an intestinal anti-inflammatory effect.

Finally, overproduction of NO is a feature of intestinal inflammation that correlates with the severity of the symptoms in IBD patients (Kamalian et al., 2020). The main source of NO in these inflammatory conditions is the iNOS enzyme, whose expression is upregulated by proinflammatory cytokines and different immunogenic stimuli. The activation of iNOS and the subsequent overproduction of NO facilitate the amplification of the inflammatory response by chemotaxis of neutrophils, natural killer cells and macrophages. Moreover, in an inflammatory environment there is oxidative stress with excessive production of reactive oxygen species, and nitric oxide can react with superoxide anions to promote the production of reactive nitric oxygen species, which act as cytotoxic agents contributing to perpetuate mucosal injury and inflammation (Kolios et al., 2004). When colonic iNOS expression

was evaluated in colitic mice, only those receiving QSFN showed a significant reduction in comparison with control colitic mice, thus contributing to its intestinal anti-inflammatory effect. It is important to note that, although flavonoids have been reported to modulate nitric oxide pathways that would justify their beneficial effects against inflammatory conditions (Kamalian et al., 2020), the oral administration of quercetin to colitic mice did not result in a significant reduction of the expression of iNOS, thus confirming the key role that the carrier SFN may play in the beneficial effects observed in the present study.

5. Conclusion

The treatment with QSFN has displayed intestinal anti-inflammatory properties in the DSS model of colitis in mice. Taking into account the results, it could constitute an attractive and innovative strategy for the management of the inflammatory bowel conditions, even more considering that it overcomes the low bioavailability of quercetin due their solubility properties, which restrains its use in clinical practice. These results reinforce the previous knowledge about the ability of the silk fibroin nanoparticles acting as an bioactive carrier for different natural molecules, displaying antioxidant and anti-inflammatory properties, which supports their future application in nanomedicine.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2021.120935.

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