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ORIGINAL ARTICLE

Preclinical evaluation of collagen type I scaffolds, including gelatin-collagen microparticles and loaded with a hydroglycolic *Calendula officinalis* extract in a lagomorph model of full-thickness skin wound

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Abstract Previously, we have developed collagen type I scaffolds including microparticles of gelatin-collagen type I (SGC) that are able to control the release of a hydroglycolic extract of the *Calendula officinalis* flower. The main goal of the present work was to carry out the preclinical evaluation of SGC alone or loaded with the *C. officinalis* extract (SGC-E) in a lagomorph model of full-thickness skin wound. A total of 39 rabbits were distributed in three groups, of 13 animals each. The first group was used to compare wound healing by secondary intention (control) with wound healing observed when wounds were grafted with SGC alone. Comparison of control wounds with wounds grafted with SGC-E was performed in the second group, and comparison of wounds grafted with SGC with wounds grafted with SGC-E was performed in the third group. Clinical follow-ups were carried in all animals after surgery, and histological and histomorphometric analyses were performed on tissues taken from the healed area and healthy surrounding tissue. Histological and histomorphometric results indicate that grafting of SGC alone favors wound healing and brings a better clinical outcome than grafting SGC-E. In vitro collagenase digestion data suggested that the association of the *C. officinalis* extract to SGC increased the SGC-E cross-linking, making it difficult to degrade and affecting its biocompatibility.

Keywords Collagen type I · Scaffolds · Gelatin-collagen microparticles · *Calendula officinalis* L. flowers extract · Full-thickness wounds

D. Millán and R. A. Jiménez contributed equally to this work.

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Introduction

Our group has developed collagen type I scaffolds and artificial connective tissue seeding primary autologous fibroblasts into the collagen scaffolds. The performance of both the scaffolds and the autologous tissue has been evaluated using a lagomorph model of oral wound [1–3]. The data obtained demonstrated that grafting autologous tissue leads to a better wound healing than to graft the scaffold without cells. However, it was shown that the healing of wounds grafted with scaffold was better than wounds closed by secondary intention [2]. Our work and others have demonstrated that bioactive acellular scaffolds of different biomaterials can promote regeneration of both oral mucosa and skin tissue [2–10]. Thus, not all oral mucosa or skin wounds require grafting natural or artificial tissue in order to close when they cannot heal by first intention.

In various traditional medicines, *Calendula officinalis* L (Linn. Family Asteraceae) has been used to treat skin lesions such as wounds, infections, burns, and ulcers. Nevertheless, not many studies have been conducted in order to obtain scientific evidence supporting the efficacy of traditional uses of

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this plant. Studies that provide information regarding pharmacological activity of extracts or individual components have been mainly obtained in vitro or in pre-clinical studies [11–20]. Although the clinical evidence is scarce and often not conclusive, data from human studies support the topical use of this plant as described by traditional medicines. In a randomized study including 111 women, topical application of an ointment significantly improved episiotomy healing over the routine procedure used by the hospital [21]. A preliminary study that included 34 patients evaluated the efficacy of an ointment containing an extract of this plant in the treatment of leg ulcers versus a control group treated with saline solution dressings. The data obtained suggested that epithelialization in the *C. officinalis*-treated group was better than in the control group [22]. A randomized trial that studied the benefit of applying topically an ointment of *C. officinalis* on irradiated skin showed it is effective in preventing acute dermatitis grade 2, or higher, during post-surgical irradiation in breast cancer patients [23].

Pharmacological studies have also suggested that *C. officinalis* extracts have anti-inflammatory [24, 25], antibacterial [25, 26], anti-viral, and anti-tumor [25, 27] properties, among others. Flowers and florets are the main parts of the plant used in the treatment of skin pathological conditions on account of their high content of antioxidant compounds [25], among which are flavonoids, carotenoids, triterpenes, and triterpenoids [25].

With the aim of improving the healing properties of collagen type I scaffolds that we have developed, in a previous work, we modified them by including microparticles of gelatin-collagen type I (GC) and the subsequent loading of a hydroglycolic extract of *C. officinalis* flowers [28]. Scaffolds incorporating GC microparticles (SGC) were intended to be used in skin wounds and designed to sustain a release of the load throughout the time lapse that goes with the in vivo skin healing process. The aforementioned was done to ensure the presence of compounds with an antioxidant activity that modulates inflammation [29] during wound closure. Results of our work showed that the developed system controlled the release of the extract during the study period and pointed towards the need for preclinical studies in an animal model of cutaneous wound. In the present study, a lagomorph model of full-thickness skin wound was developed and used to evaluate the in vivo performance of SGC and SGC loaded with an extract of *C. officinalis* flowers.

Materials and methods

Calendula officinalis extract

Hydroglycolic *C. officinalis* flower extract was acquired from Neyber (Bogotá, Colombia). Full details on batch number, content, preparation method, and quality control of this extract are presented in the Supplementary Material.

Ethical considerations

Animal experimentation was performed following the guidelines of animal research of the Colombian Ministry of Health (resolution no. 008430, 1993; Law 84 December 27th, 1989). Qualified personnel under the supervision of a veterinary doctor and a plastic surgeon carried out all animal procedures.

Loading of the collagen type I scaffolds incorporating GC microparticles with a hydroglycolic extract *of C. officinalis*

Collagen type I scaffolds (4 cm^2) including GC microparticles were made following a procedure already described [28]. Briefly, 250 mg of microparticles made with a mixture of gelatin (225 mg)-collagen type I (25 mg) were added to 50 mL collagen type I suspension (5 mg/mL), magnetically stirred for 1 h and poured into polypropylene dishes, 10 cm diameter (Corning Inc., NY, USA). Samples were degasified, frozen (−20 °C), lyophilized for 48 h, and cross-linked with 0.02 % of glutaraldehyde for 24 h. Cross-linked scaffolds were washed extensively with water until no glutaraldehyde was detected (Brady's reagent 2,4 dinitrophenylhydrazine). Thereafter, they were freeze-dried as described above and sterilized with ethylene oxide. For extract loading, cross-linked scaffolds were immersed for 2 h at 37 °C in 2 mL of 1 % *v/v* hydroglycolic extract of *C. officinalis* flowers in rabbit autologous plasma adjusted to the same protein concentration (1.45 mg/mL), which was measured with the bicinchoninic acid assay [30].

In vivo study

A total of 39 New Zealand male rabbits (approximately 3 months of age and 2.5 kg) were distributed in three groups, of 13 animals each. The first group was used to compare wound healing by secondary intention (control) with wound healing observed when wounds were grafted with SGC alone pre-wetted in 2 mL of autologous plasma. Comparison of control wounds with wounds grafted with SGC loaded with the extract (SGC-E) was performed in the second group. Comparison of wounds grafted with SGC with wounds grafted with SGC-E was performed in the third group.

Animals were anesthetized (intramuscular ketamine, 50 mg/kg; xylazine, 5 mg/kg; local lidocaine, 20 mg/mL; and epinephrine, 0.02 mg/mL). Each animal upper dorsal area was shaved, washed with povidone-iodine, and marked with a surgical marker; and the marked area was anesthetized using lidocaine (2 %). Two identical contralateral full-thickness skin excisions (4 cm^2) were made at a distance of 3 cm between them. The grafted scaffolds (SGC or SGC-E) were fixed with

Vicryl®4–0 sutures (Ethicon, Inc., Johnson & Johnson Company, West Somerville, NJ, USA), one in each corner of the implant. Tie-over bolster dressing with non-adherent gauze was used to immobilize the graft at the wound bed.

Clinical follow-ups

A plastic surgeon carried out the follow-ups of the healing process. At the seventh day post-surgery, all wounds were debrided and new SGC and SGC-E were grafted, while the controls were left to heal by secondary intention. Animals were sacrificed 4 weeks post-surgery using sodium pentobarbital and sodium diphenylhydantoin. Digital images of the wounds were taken the day of the surgical procedure and at the time of sacrifice (4 weeks post-surgery).

Histological analysis

Full-thickness biopsies of both the grafted and the control areas, including healthy surrounding tissue, were taken to perform the histological and histomorphometric analysis. Samples were fixed with 10 % formaldehyde in PBS (pH 7, 4; 4 °C; 24 h), washed with PBS, embedded in paraffin, cut (5 mm sections), stained with hematoxylin-eosin (H & E), and observed under a light microscope (Nikon ECLIPSE 55i, Nikon, Tokyo, Japan). Stained tissue from wounded (grafted or control) and healthy surrounding area of each animal was analyzed. Panoramic images of biopsied tissue were composed by over imposing photographs of the different sections. The arrangement of collagen fibers in the dermis was determined by observing fiber orientation (uni- or multi-directional orientation) in five fields. All animals from the three groups were analyzed. A pathologist who was not informed about the samples' sources performed these histological analyses.

Histomorphometric analysis

Five fields of analyzed tissue (treated and control wounds) were observed at random for histomorphometric analysis. Connective tissue thickness, number of keratinocytes, number of inflammatory cells, and number of blood vessels per field were determined and averaged. The average values obtained were expressed relatively to the data from healthy surrounding tissue as follows: average of connective tissue thickness, number of keratinocytes, number of blood vessels, and number of inflammatory cells in both treated control wounds/average of connective tissue thickness, number of keratinocytes, number of blood vessels, and number of inflammatory cells in surrounding healthy tissue. The software NIS ELEMENTS F2.20 (Nikon, Chiyoda-Ku, Tokyo, Japan) and the free software ImageJ [http://imagej.nih.gov/ij/] were used to perform all the histomorphometric analysis that was carried out by a pathologist who did not know about the nature of the samples.

In vitro assessment of collagenase digestion of SGC and SGC-E

Fragments (1×1 cm) of SGC and SGC-E were digested with 1 mL of collagenase I (1 μg/mL) for 6 and 24 h. After each digestion time, images of the scaffolds were taken and analyzed using the software NIS ELEMENTS F2.20. Additionally, the supernatant was removed and replaced by fresh collagenase (1 mL). In each sample gelatin, the byproduct of the collagenase digestion was quantified using the bicinchoninic acid (BCA) method.

Statistical analysis

The number of animals required to ensure 95 % accuracy and confidence level for this study was calculated with Epi Info 6 StatCalc. The data distribution was assessed with the Shapiro-Wilk test. Thereafter, the nonparametric Spearman's rank correlation coefficient was used to analyze the relationship between the variables studied here. Statistical differences were significant at $p<0.05$.

Results

Evaluation of SGC and SGC-E integration into the skin wound bed

Clinical observations of day 8 post-surgery show differences in the integration to the wound bed of SGC and SGC-E (Fig. 1). At that moment, SGC was partially degraded and replaced with an uneven red granulation tissue. Exudation was low and odorless, and wound-raised edges, inflammations, infections, or necrosis were not seen (Fig. 1a). Histological analysis of samples taken from the SGC facing the wound shows the presence of cellular infiltrate into the collagen fibers of the scaffold (Fig. 1b). Conversely, SGC-E did not integrate into the wound bed that was covered by dry coagulum, as well as free of exudation and infection (Fig. 1c). The SGC-E removed was evaluated histologically also, finding that the wound facing area was covered with inflammatory cells as if a foreign body reaction had occurred. Interestingly, the scaffold remained undergraded, and its characteristic porous structure was lost (Fig. 1d).

Follow-ups of treated and control wounds

All the wounds were closed 4 weeks after surgery. Representative images of all the treatments and their controls are shown in Fig. 2. Wounds that were treated with SGC-E closed with a hypertrophic, star-shaped, red-colored scar, similar to the scar seen in wounds that are closed by secondary intention used as controls (Fig. 2a). On the contrary, wounds

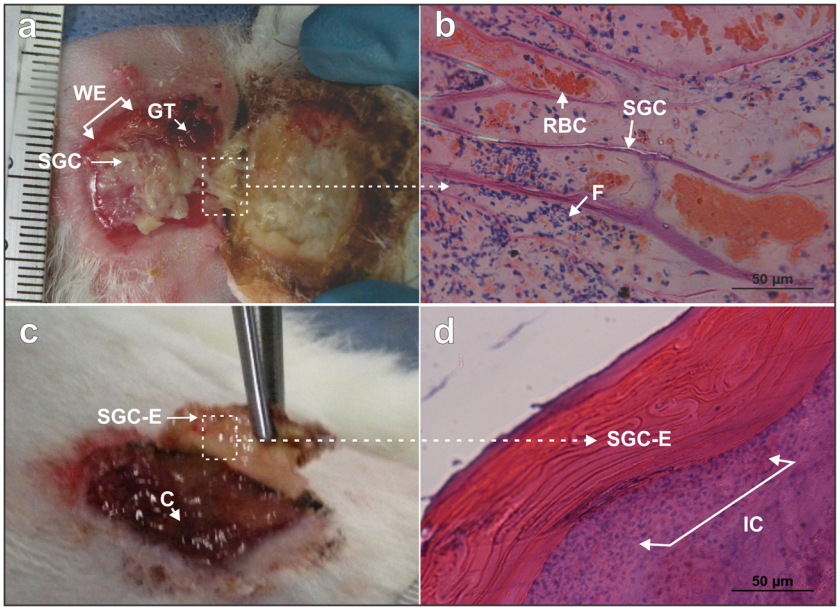

Fig. 1 Integration of SGC and SGC-E into rabbit full-thickness skin wounds. **a** Wound treated with SGC and appearance of the surface of SGC facing the wound. **b** Histology of a sample taken from the surface of the SGC facing the wound. **c** Appearance of a wound treated with SGC-E. **d** Histology of a sample taken from the surface of the SGC-E facing the wound. *SGC* collagen scaffold including microparticles of gelatin-collagen, *SGC-E* SGC loaded with a hydroglycolic extract of *Calendula officinalis*, *WE* wound edges, *GT* granulation tissue, *C* coagulum, *IC* inflammatory cells, *RBC* red blood cells, *F* fibroblasts, *H & E* hematoxylin and eosin stain

grafted with SGC alone showed small or no hypertrophic scar with round-shape, which had the same color of the healthy surrounding skin and different appearance than the controls (Fig. 2b). The comparison of the wounds grafted with SGC or SGC-E confirmed that healing was better in the group treated with SGC than in the one treated with SGC-E (Fig. 2c).

Histological analysis

Representative panoramic images of histological samples taken from the grafted and control wounds, including surrounding healthy tissue, at 4-week post-surgery are presented in Fig. 3. Wounds treated with SGC showed epidermis regeneration with normal stratum corneum and mature keratin as well as mild vacuolization of cells of the basal layer. In the dermal-epidermal junction, capillaries and tissue with a moderate number of cells were observed; also, hair follicles with slight perifollicular inflammatory reaction at the lesion-healthy tissue interphase. The newly formed connective tissue exhibited zones of randomly oriented fibers and zones of unidirectional fibers parallel oriented; in all cases, its thickness looked very similar to the one of surrounding healthy tissue (Fig. 3a). The tissue formed in wounds treated with SGC-E also showed epidermis with normal stratum corneum and mature keratin, capillaries, and a moderate number of fibroblasts arranged in parallel in the dermal-epidermal junction. The reticular dermis presented fibroblasts and capillaries parallel oriented and a mild inflammatory perivascular reaction, and the connective tissue formed seemed thinner than the healthy connective tissue (Fig. 3b). The epidermis and connective tissue formed in the wounds that healed by secondary intention were like the wounds grafted with SGC-E; however, it was evident the presence of hemorrhagic and severe perivascular inflammation foci in the papillary region of the dermis. The new connective tissue was thinner than the surrounding healthy connective tissue (Fig. 3c).

Representative images of dermal tissue taken from wounds grafted with SGC or SGC-E, and their contra-lateral control wounds healed by secondary intention are shown in Fig. 4. In each case, an image of the surrounding healthy tissue was also included. Wounds treated with SGC had collagen fibers of moderate density and few high-density fibers, all multi-directionally oriented; non-elongated fibroblasts randomly oriented were also observed. The contralateral wound healed by secondary intention (control) showed collagen fibers of low density, uni-directionally oriented, and parallel to the epithelium; elongated and uni-directionally aligned fibroblasts were also seen. The healthy surrounding dermis had high-density fibers with non-elongated fibroblasts randomly oriented (Fig. 4a). The zones treated with SGC-E showed collagen fibers of moderate density, uni-directionally oriented, and parallel to the epithelium covering the dermis. Their control wounds showed collagen fibers of low density, uni-directionally oriented, and parallel to the epithelium. In both, neo-formed dermal tissues elongated and uni-directionally aligned fibroblasts were seen. Conversely, the healthy surrounding dermis showed collagen fibers of high density, multi-directionally oriented with non-elongated fibroblasts randomly oriented (Fig. 4b). The representative images of stained tissue from animals grafted in one side with SGC and in the other with SGC-E are shown in Fig. 4c. In the wounds treated with SGC collagen fibers of moderate and high density, multi-directionally oriented as well as non-elongated fibroblasts randomly oriented were observed. Conversely, wounds treated with SGC-E showed collagen fibers of moderate density, uni-directionally oriented, and

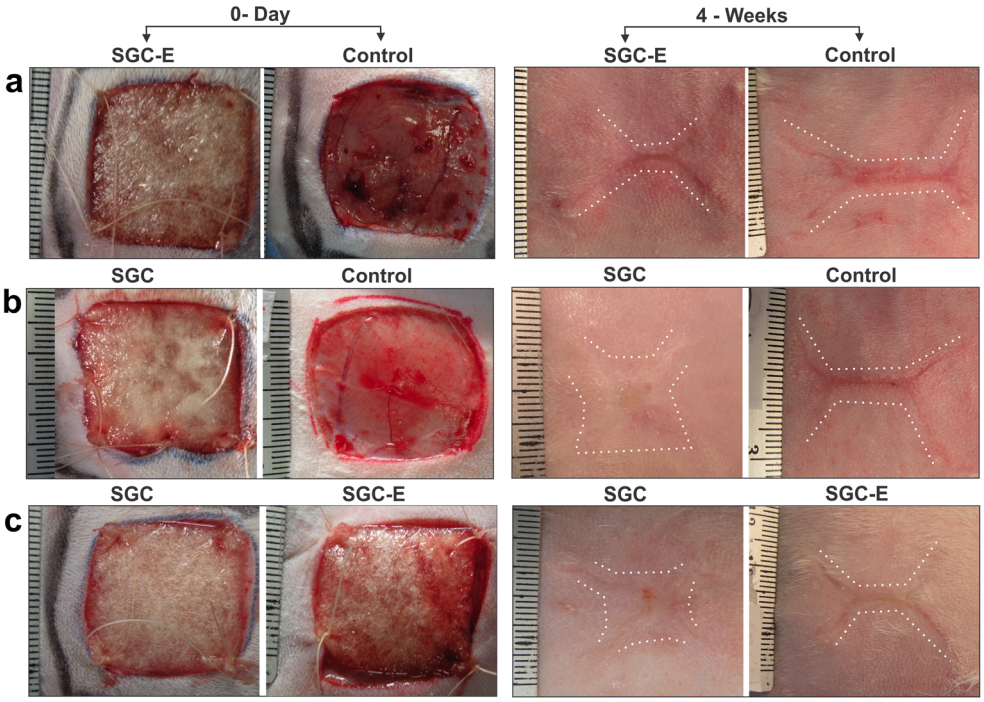

Fig. 2 Clinical follow-ups of treated and control wounds at 0 day and 4 weeks. Representative images of the different wounds are shown. **a** Wound treated with SGC-E and its control wound. **b** Wound treated with SGC alone and its control wound. **c** Wounds treated with SGC or SGC-E. *SGC* collagen scaffold including microparticles of gelatin-collagen, *SGC-E* SGC loaded with a hydroglycolic extract of *Calendula officinalis*. Closed wounds are delimited with *dotted lines*

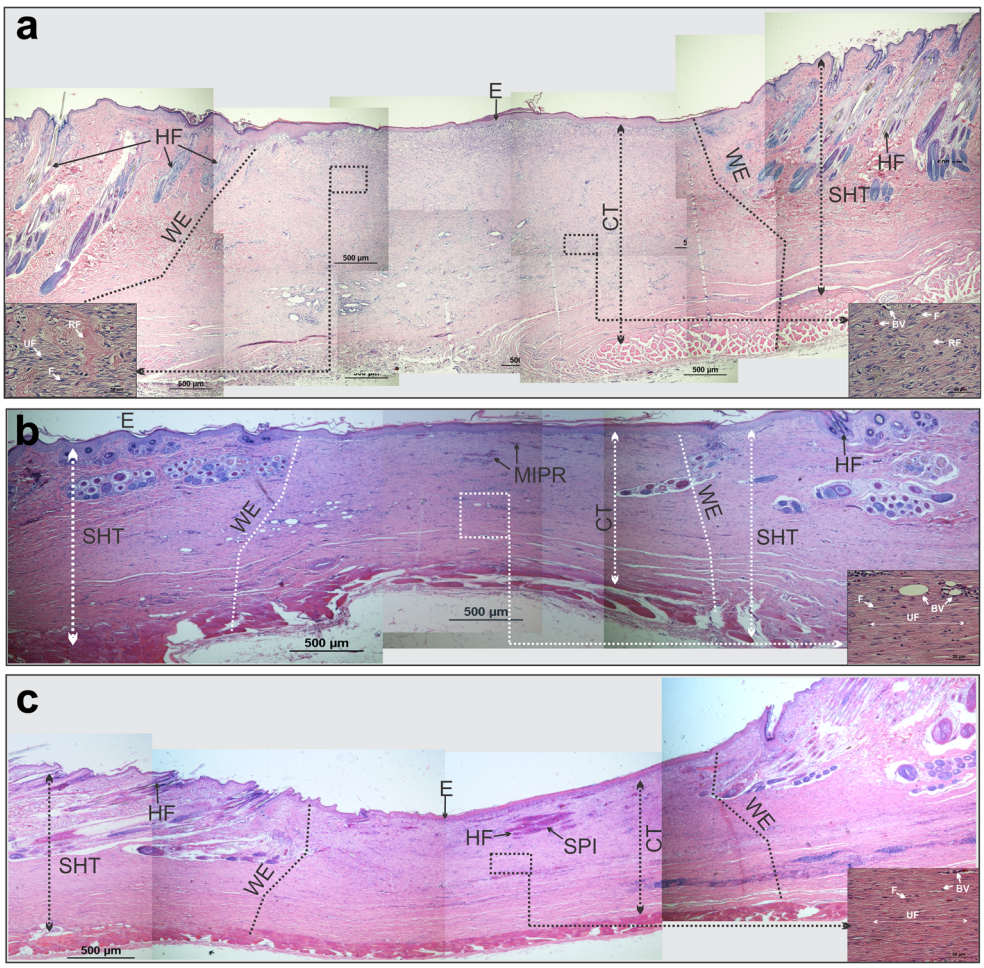

Fig. 3 Panoramic images of biopsied tissue. Representative images of tissue samples composed by over imposing photographs of the different sections (treated and control wounds, healthy surrounding tissue). **a** Tissue formed into wounds grafted with SGC. **b** Tissue formed into wounds grafted with SGC-E. **c** Tissue formed into control wounds closed by secondary intention. *CT* connective tissue, *SHT* surrounding healthy tissue, *WE* wound edges, *HF* hair follicles, *E* epidermis, *BV* blood vessels, *F* fibroblasts, *RF* randomly oriented fibers, *UF* uni-directional oriented fibers, *MIPR* mild inflammatory perivascular reaction, *SPI* severe perivascular inflammation, *HF* hemorrhagic foci

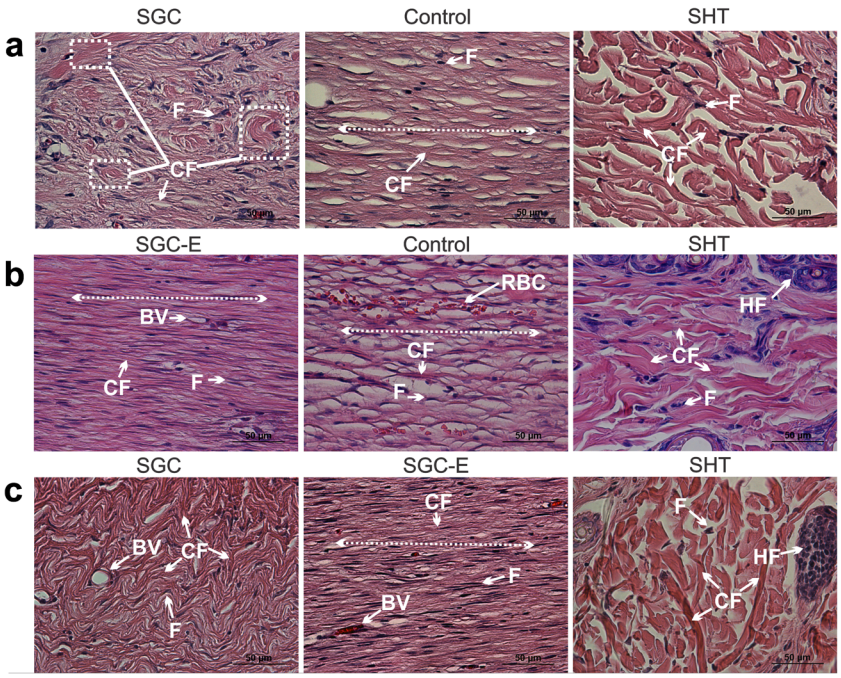

Fig. 4 Histological analysis of collagen density and fiber orientation. **a** Biopsies taken from wound grafted with SGC, control wound, and surrounding healthy tissue. **b** Biopsies taken from SGC-E grafted wound, control wound, and surrounding healthy tissue. **c** Biopsies taken from SGC, SGC-E grafted wounds, and surrounding healthy tissue. *BV* blood vessels, *CF* collagen fibers, *F* fibroblast, *HF* hair follicle. *Dotted lines* show multi-directional and high-density collagen fibers

parallel to the epithelium. The healthy surrounding dermis showed collagen fibers of high density, multi-directionally oriented with non-elongated fibroblasts randomly oriented.

Histomorphometric analysis

Average values of connective tissue thickness measurements, number of keratinocytes, number of blood vessels, and number of inflammatory cells in treated and control wounds relative to the same average values determined in surrounding healthy tissue are shown in Fig. 5. Box plots showing the ratios of connective tissue thickness are presented in Fig. 5a. The comparison of control and SGC-E grafted wound ratios showed that there were non-significant differences (p=0.202) between both groups (Fig. 5a (i)). On the contrary, there was a significant increase in the ratio of wounds grafted with SGC compared to their control wounds (p=0.007), being this value approximately 1 (Fig. 5a (ii)). The same was observed in animals grafted in one side with SGC-E and in the contra-lateral side with SGC; a significant increase (p=0.013) was seen in the latest group of wounds compared to the former (Fig. 5a (iii)). Comparison among the ratios of the other evaluated parameters (average number of keratinocytes, number of inflammatory cells, and number of blood vessels) indicates that there were non-significant differences between control and SGC-E (p=0.153), control and SGC (p=0.698), and SGC versus SGC-E (p=0.260) wounds (Fig. 5b–d, respectively).

In vitro assessment of collagenase digestion of SGC and SGC-E

Collagenase digestion assay indicates that SGC has the fastest degradation rate of both scaffolds. Comparison of the scaffolds appearance suggested that SGC-E degrades slower than SGC. As seen in Fig. 6a, b, at 6 and 24 h of enzymatic digestion, SGC-E apparently remained the same while degraded collagen fibers were observed on the SGC surface. Likewise, gelatin percentage was higher in the SGC collagenase digestion samples than in the SGC-E ones (Fig. 6c).

Discussion

In a previous work, we modified collagen type I scaffolds by including microparticles of gelatin-collagen type I (GC) and the subsequent loading of a hydroglycolic extract of *C. officinalis* flowers to develop a dermal substitute with improved healing features. Scaffolds incorporating GC microparticles (SGC) were designed to load and sustain the release of this flower extract to ensure the presence of compounds with antioxidant activity throughout the lapse of time that goes with the in vivo skin healing process. The system developed controlled the release of the extract during the 15 days of the study and pointed to the need for preclinical studies in an animal model of full-thickness cutaneous wound [28].

In the present work, a rabbit model of full-thickness skin wound was developed and used to evaluate the in vivo performance of the SGC and SGC loaded with an extract of

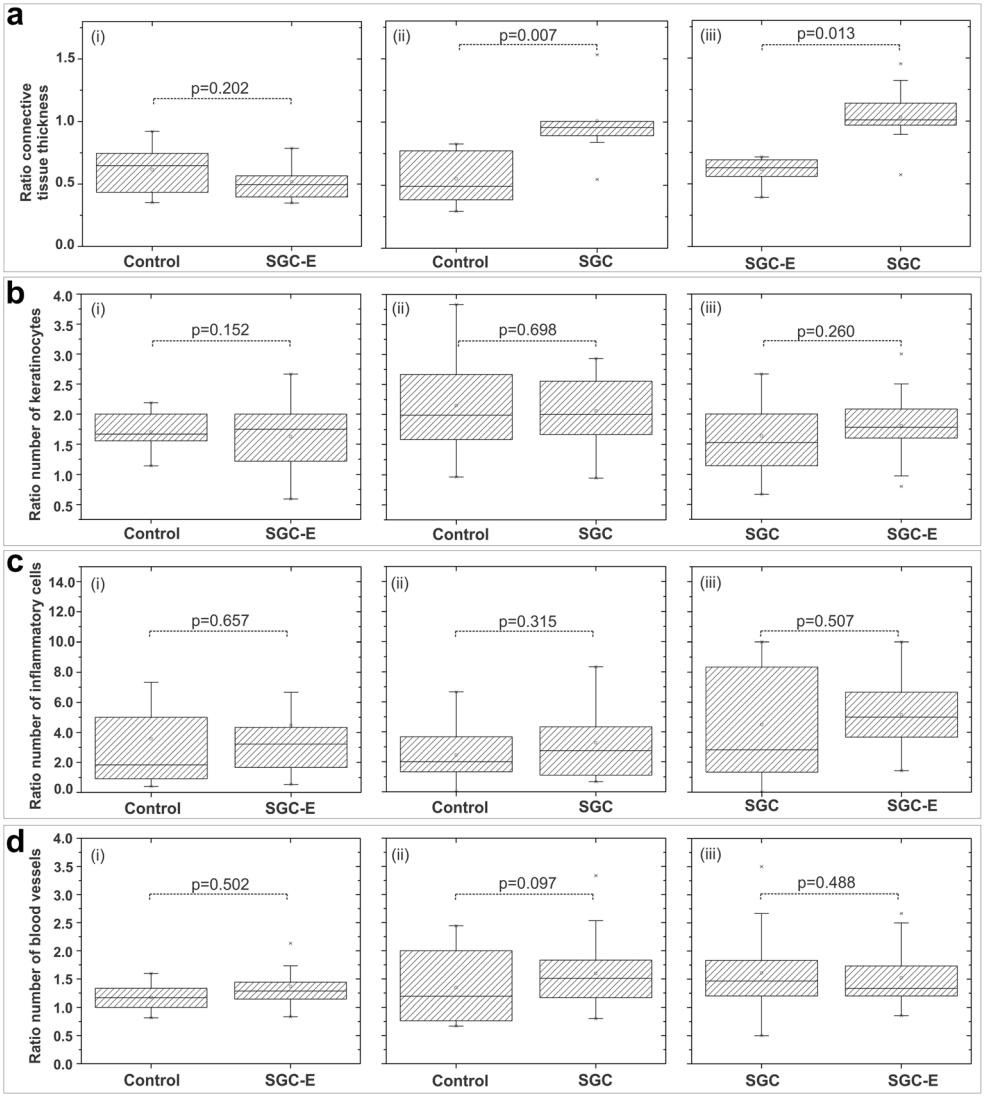

Fig. 5 Box plot of histomorphometric analysis of newly formed tissue. **a** Ratio of connective tissue thickness obtained in each of the three studied groups. **b** Ratio of number of keratinocytes obtained in each of the three studied groups. **c** Ratio of the number of inflammatory cells obtained in each of the three studied groups. **d** Ratio of vascularization obtained in each of the three studied groups. (*i*) Wounds grafted with SGC-E versus control. (*ii*) Wounds grafted with SGC versus control. (*iii*) Wounds grafted with SGC-E versus wounds grafted with SGC. *p* values are indicated in each graph

C. officinalis flowers (SGC-E). Rabbits belong to the family Leporidae of the order Lagomorpha [31]; despite differences with human skin, their back offers a suitable area for creating two critical size full-thickness defects in the skin [32]. This animal model provided us a clear scenario to assess clinical, histological, and histomorphometric differences between wounds grafted with SGC or SGC-E and those closed by secondary intention.

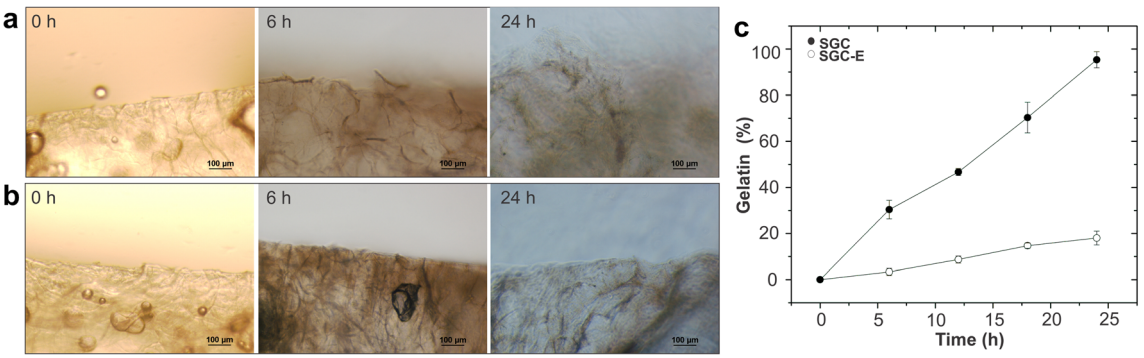

Fig. 6 In vitro assessment of SGC and SGC-E collagenase degradation. **a** Microscopic images of SGC at 0, 6, and 24 h of collagenase digestion. **b** Microscopic images of SGC-E at 0, 6, and 24 h of collagenase digestion. **c** Scaffold percentage of degradation vs. time. Data represent the average of three independent determinations ±SD

C. officinalis flowers have a high content of antioxidant compounds [25]. Antioxidants modulate inflammation by controlling the oxidative stress that kills cells, damages tissues, and leads to chronic inflammation [33–35]. Therefore, we aimed to determine whether or not the controlled release of an extract of *C. officinalis* flowers with antioxidant activity favored wound healing and tissue regeneration over repair. Surprisingly, it was found that when SGC was grafted, the healing of full-thickness skin wounds was better than when SGC-E, the extract loaded scaffold, was used. Healing of the SGC-E-treated wounds was comparable, also, to the one observed in the wounds left to heal by secondary intention. Our data clearly demonstrated that in wounds treated with SGC, the newly formed tissue seemed more like the surrounding healthy tissue in comparison with wounds treated with SGC-E or with controls. Furthermore, scar contracture was mild, and new connective tissue presented collagen fibers similar in density and orientation to the fibers of the healthy surrounding tissue. A balance toward tissue regeneration, rather than repair in SGC-treated wounds, was also suggested by the thickness of the newly formed connective tissue, which was similar to the one of healthy surrounding connective tissue. On the contrary, wounds treated with SGC-E healed with more contracture and exhibited clinical and histological characteristics similar to the ones of control wounds closed by secondary intention. Additionally, capsule formation was observed at the interface scaffold-tissue in all the SGC-E-treated wounds. In all cases, histological observations on connective tissue thickness were confirmed by the histomorphometric analysis. Altogether, these data indicate that grafting of SGC favors wound healing and brings a better clinical outcome than grafting SGC-E.

Seeking to explain the in vivo results, we hypothesized that the association of the *C. officinalis* extract to SGC increased the SGC-E cross-linking, making it difficult to degrade, favoring capsule formation at the tissue-scaffold interface and affecting its biocompatibility. This assumption was made considering that there were no chemical or microstructural differences between the two studied scaffolds besides the loaded hydroglycolic extract of *C. officinalis* flowers [28]. The aforesaid hypothesis was supported by our findings that in vitro enzymatic degradation of the scaffolds loaded with the extract is less than the degradation of the scaffolds without extract. These data might indicate that the *C. officinalis* flower extract loaded into the SGC enhances the scaffold cross-linking. Our preceding Fourier transform infrared spectroscopy (FTIR) data showing that spectra of the scaffolds unloaded or loaded with the extract were different [28] strongly support the aforementioned suggestion.

Several precedents exist for the cross-linking effect of plant extracts and plant-isolated compounds on collagen type I fibers. Data already published have demonstrated that they can affect the structural and mechanical properties of proteins rich in proline residues, such as collagen type I, and elastin [36, 37]. Genipin, an aglycone isolated from extracts of *Gardenia jasminoides Ellis* flowers, has been used as a natural cross-linker of collagen, gelatin, and chitosan, among others [38]. Collagen fibers treated with extracts of *Myrica rubra*, known as Chinese bayberry, increase their resistance to denaturation [39]; and polyphenols, such as procyanidin, present in many vegetables and fruits, stabilize, and cross-link collagen type I fibers [40]. Likewise, it has been reported that polyphenols increase resistance to degradation by elastase of scaffolds made from decellularized porcine carotid arteries [36]. *C. officinalis* flower extracts are rich in polyphenols, such as catechin a flavan-3-ol, that in vitro have proved to stabilize collagen at temperatures around 70 °C and to increase its resistance to enzymatic degradation and to the denaturing action of compounds such as urea [41, 42]. In vitro assays have shown that catechin and epigallocatechin gallate, polyphenols ubiquitously found in many plants, are competitive inhibitors of collagenase activity on collagen type I [43, 44]. Our novel in vitro and in vivo data demonstrating low degradation and encapsulation of SGC-E strongly support an increase in scaffold cross-linking by loading *C. officinalis* flower extract. The resulting increased resistance of SGC-E to enzymatic degradation might negatively influence its biodegradability and biofunctionality hampering its in vivo performance as dermal substitute. However, the results presented here are insufficient to suggest inhibition of collagenase activity by the flower extract.

Previously, it was demonstrated using in vitro assays that the SGC-E platform we have developed sustained the release of polyphenols during 15 days [28]. It was out of the scope of the present work to show that SGC-E scaffolds release these antioxidants into the wounds upon grafting, neither it was to quantify the polyphenols that remained in the encapsulated SGC-E scaffolds after they were removed from the wounds. This research aimed to assess whether or not loading of a *C. officinalis* hydroglycolic extract into the SGC scaffolds improved their performance as a grafting material rather than to demonstrate a direct pharmacological effect of the flower extract. In this frame, the data presented here show that biodegradation of SGC scaffolds, loaded or not with the hydroglycolic extract, is a key component of the healing effect they have upon grafting in rabbit total thickness skin wounds.

The work presented here demonstrated that incorporation of *C. officinalis* flower extract into the SGC platform does not facilitate wound healing. Nonetheless, it also suggests the possibility of using this flower extract to in vivo modulate collagen type I scaffold biodegradability and bioactivity. Ongoing experiments are looking into determining the relationship between various concentrations of the *C. officinalis* flower extract and the cross-linking degree and susceptibility to collagenase digestion of SGC. Once the cross-linking activity of the extract has been proved, it can be used as a natural, non-toxic, and low-cost cross-linker of collagen type I scaffolds.

Conclusion

The present preclinical study clearly demonstrates that performance of collagen type I scaffolds incorporating GC microparticles as grafting material in rabbit full-thickness skin wounds was better than the performance of SGC scaffolds loaded with a hydroglycolic extract of *C. officinalis* flowers (SGC-E), which upon grafting were encapsulated rather than biodegraded by the studied animals. Low biodegradability of the latter might be explained by a cross-linking effect of the extract on the SGC-E, which is suggested by in vitro collagenase digestion results presented here.

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Compliance of ethical standards All institutional and national guidelines for the care and use of laboratory animal were followed.

Conflict of interest Diana Millán, Ronald A. Jiménez, Luis E. Nieto, Itali Linero, Manuel Laverde, and Marta R. Fontanilla declare that they have no conflict of interest. The authors declare no competing financial interests.

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