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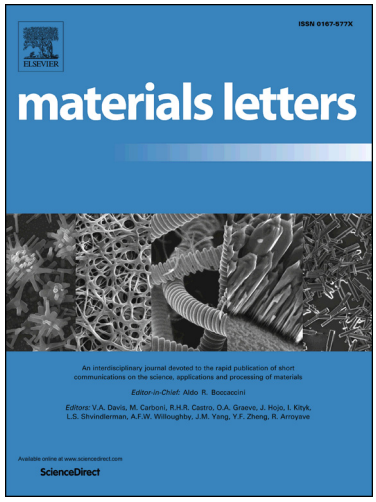

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A novel biomaterial based on salmon-gelatin and its *in-vivo* evaluation as sterile wound-dressing

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Abstract

A novel porous biomaterial based on salmon-gelatin and excipients was developed. The biomaterial was sterilized using gamma radiation. Mechanical and structural properties were studied. The biomaterial was used as wound-dressing in an animal model for four weeks. The *in-vivo* results show biosafety of the biomaterial and high potential for clinical application in patients with tissue regeneration requirements.

Keywords: Biomaterials; Polymeric composites; Polymers; Porous materials.

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1. Introduction

Gelatin, a collagen derived material, is one of the most widely used hydrocolloids in the pharmaceutical, cosmetic, medical and food industries.

In the biomedical field, a large number of applications using gelatin as the main structuring bioactive and biodegradable component have been described. Gelatin based materials have shown low toxicity and reduced antigenicity [1], being their production comparatively inexpensive relative to collagen.

Another advantage of gelatin is that can be combined with other biopolymers, such as chitosan, to make bioactive composites used as wound-dressing [2,3]. Chitosan is a bacteriostatic polymer [4] that forms a stable polyelectrolyte complex with gelatin [5].

Currently, cultural considerations limit the commercialization of products containing gelatin from mammalian origin (bovine or porcine) [6]. Additionally, there is growing concern among consumers as to whether bovine biomaterials are able to transmit pathogen vectors as prions [7]. In this sense, the use of salmon-gelatin instead of bovine compounds minimizes the risk of zoonosis.

Cold water fish gelatins, such as salmon-gelatin, present lower concentration of imino acids (proline and hydroxyproline) and lower molecular weight distribution, shows marked differences with respect to thermal behavior, rheological, viscoelastic and mechanical properties when it is compared to mammalian (bovine or porcine) and warm-water fish gelatin [8].

The physical and biological properties of the salmon-gelatin allow the development of novel materials to be used for medical applications.

2. Materials and Methods

2.1. Biomaterial preparation

Gelatin will be extracted from skins of *Salmo salar* specimens following the methodology proposed by Zhou and Regenstein [9], modified by Díaz et al [10] and used by Acevedo et al [11] to fabricate edible gelatin-chitosan composites.

Chitosan (pharmaceutical grade, 95% deacetylated, 300 kDa, derived from crab shells) was purchased from Quitoquimica (Chile). Agarose (molecular biology grade) was

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purchased from Lonza (USA). Glycerol (pharmaceutical grade) was purchased from Merck (Germany). EDC (N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride), NHS (N-hydroxysuccinimide) and MES (2-(N-morpholino)ethanesulfonic acid hydrate) were purchased from Sigma-Aldrich (USA).

The biomaterial was made using the freeze-drying method. For this purpose, a solution of salmon-gelatin was prepared. The solution was improved by using three excipients: chitosan was used for bacteriostatic properties; agarose was added as gelling agent and glycerol as plasticizer.

Three parts of salmon-gelatin aqueous solution (2%w/v in water) was mixed with one part of chitosan solution (2%w/v in 1% acetic acid), five parts of agarose solution (0.4%w/v in water) and one part of glycerol solution (1%w/v in water). Then, the formulation prepared was: salmon-gelatin 0.6%w/v, chitosan 0.2%w/v, agarose 0.2%w/v and glycerol 0.1%w/v. The solution was mixed at 50°C for 1 hour.

The solution was poured into a Petri dish adjusting the volume to obtain a height of 3 mm. Then, it was cooled at 4ºC, frozen at -80ºC and lyophilized using a freeze dry system (Liobras L101, Brazil). The dry composite was crosslinked by the use of a solution composed by EDC (30 mM) and NHS (8 mM), using MES (50 mM) as buffer and ethanol 90%v/v as solvent [12]. The resultant crosslinked composite was washed with pure ethanol, ethanol 70%v/v, ethanol 40%v/v and water, and then it was frozen and lyophilized.

The sterilization of the biomaterial was made by gamma radiation. A dose of 25 kGy was applied [13]. Irradiation was carried out at the Chilean Nuclear Energy Commission.

2.2. Physical characterization

The physical characterization of the scaffolds was made before and after gamma irradiation sterilization.

Microstructure was evaluated by scanning electron microscopy (SEM). Samples were coated with gold (10-20 nm in thickness) and examined with a Carl Zeiss SEM (EVO *MA* 10, Germany). The pore size (equivalent circular diameter) was measured using ImageJ Software (NIH, USA) with at least 100 pores per sample.

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The thermal properties, melting temperature (Tm), melting enthalpy (ΔH), glass transition temperature (Tg) and change in heat capacity (ΔCp) were determined by differential scanning calorimetry (DSC) (Mettler Toledo, DSC 1 STARe System, Switzerland) using a heat-cool regime starting from 0°C to 150°C, holding at 150°C for 1 min, cooling to 20°C, all at 10°C min^{-1}. The device was previously calibrated using indium.

Mechanical properties, Young's modulus, stress at break and strain at break, were determined from the stress-strain curves generated by tensile tests carried out using a Texture Analizer (TA.XT Plus, Surface Microsystem). The distance between the grips was set to 30 mm and extension rate was 0.17 mm/s. The length, width and thickness of the samples were ~50 mm, ~14 mm and ~1 mm, respectively.

2.3. Animal procedures

The biosafety of the biomaterial as wound dressing was tested in six rabbits (*Orictholagus cuniculus*). Animals were anesthetized with ketamine/xylasine (5 mg and 2 mg/100 g of body weight, respectively) [2,3]. A selected dorso-lumbar area was shaved and disinfected with povidone-iodine solution. A cutaneous excision wound of 3 cm in diameter was performed. Subsequently, the wound dressing was implanted on the wound. After the surgery, the evolution of the animal was followed every day. Animal growth (weight gain), physiological changes and evolution of wound cicatrization were evaluated in all individuals. Photographic evaluation of each animal wound was performed once a week.

All the *in vivo* experiments and procedures, including animal procurement, surgery, anesthesia, euthanasia and surgery facilities were approved by the Ethical Committee of the Medical School of the Universidad de Los Andes (Chile). In addition, all animal procedures were performed using the "Guide for the Care and Use of Laboratory Animals" [14].

2.4. Histological evaluation

The biopsies of each animal, where the wound-dressing was implanted, were fixed in Bouin's solution for 24 h. The histological sections of the samples were obtained on the perpendicular axis to the wound healing zone that is parallel to the cephalo-caudal axis of the rabbit. Samples were processed for routine histological technique. Histological sections

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of 5 μm were stained with a trichromic staining (Hematoxylin, Erythrosin-Orange G, Aniline Blue) [2,3].

2.5. Statistics

Statistical analysis to compare between values were made using Student t-tests and informed as significant difference when $p<0.05$.

3. Results

3.1. Biomaterial Characterization

The biomaterial made with salmon-gelatin is shown in Figure 1. The morphological analysis shows a porous microstructure, similar with other composites made with bovine-gelatin or combinations of gelatin and chitosan [12,15].

It is well known that sterilization process by gamma radiation modifies the biomaterial microstructure. Specifically, it has been reported changes in both, biological and physical properties, of gelatin-chitosan scaffolds sterilized with gamma radiation [13]. Physical properties of the scaffold before and after irradiation (25 kGy) are shown in Table 1.

The morphology of the pore was not modified notoriously by radiation, and the nanostructure of the membrane pore maintained its shape (Figure 1). However, the radiation affected significantly ($p<0.05$) the pore size average (see Table 1). It has been reported that pore size reduction by gamma radiation in gelatin-chitosan scaffolds improves biocompatibility properties [3,13]. Human fibroblasts cultured onto gelatin-chitosan scaffolds improve the cell behavior when the material is irradiated (25 kGy), increasing the proliferation and expression of growth factors [13]. Furthermore, *in-vivo* trials have demonstrated that irradiated gelatin-chitosan scaffolds are biocompatible in animals and humans when it is used as wound-dressing [3].

DSC analysis showed that irradiation did not affect significantly ($p>0.05$) Tg and ΔCp as shown in Table 1. This result would indicate that the composite molecular mobility was not affected, suggesting that the gelatin polymer molecular weight was similar after the sterilization stage. In the case of Tm, a slight reduction was observed after gamma

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irradiation but no significant variation was detected in the case ΔH, which indicated the crystalline fraction associated to triple helices content was not varied [10].

Mechanical properties were modified by gamma radiation (see Table 1). The Young's modulus, stress and strain at break increase significantly ($p<0.05$). Radiation slightly hardened the material and increased its mechanical strength. These results are in line with the literature, as sterilization by gamma irradiation has been reported to affect the mechanical properties of various biomaterials [16,17]. In the case of pure gelatin, it has been reported variation in the polymer structure when irradiated up to 10 kGy evidenced by changes in capillary viscometry and mechanical testing [16]. It was suggested the increase in intrinsic viscosity and tensile modulus was related to crosslinking over a degradation effect. When looking at the effect of gamma irradiation on a similar composite formulation as the composite tested in this work, Acevedo et al [13] showed variations in microstructure morphology of gelatin-chitosan scaffolds when irradiation energy was increased from 1 to 25 kGy. The latter was related to a reduction in porosity and increase in porous wall roughness, explaining a higher resistance to uniaxial deformation producing an increase in modulus and higher values for stress and strain at brake.

Table 1: Physical properties of the scaffold

Properties	Before Sterilization	After Sterilization (25 kGy)	Percentage Change
Pore size (μm)	185.2 (±27.1)	159.9 (±30.8)	-13.7%
Young´s modulus (Pa)	150.0 (±17.3)	170.7 (±20.0)	+13.8%
Stress at break (Pa)	316.8 (±18.4)	462.5 (±24.5)	+46.0%
Strain at break (%)	2.48 (±0.99)	3.29 (±0.01)	+32.7%
Tg (K)	318.1 (±0.5)	319.3 (±3.0)	+0.4%
ΔCp (J/g K)	0.130 (±0.030)	0.139 (±0.011)	+6.9%
Tm (K)	386.7 (±3.3)	376.5 (±2.1)	-2.6%
ΔH (J/g)	3.55± (1.18)	3.92 (±0.57)	+10.4%

3.2. Biosafety evaluation of the biomaterial as wound-dressing

The biosafety of the irradiated biomaterial as wound-dressing was tested in a rabbit animal model. The image of the Figure 2A shows the skin before and after the wound-dressing implantation. The results showed early biodegradability capacity of the biomaterial (1-2 weeks) and faster healing of the wound (3-4 weeks). In all individuals a

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granulomatous reaction occurred at the edges of the wound as healing progressed. This was not related to significant physiological changes in the animals.

The evolution with time of the wound area was similar in all animals treated (Figure 2B). After four weeks of the surgery, the wound area was close to ~7% of original size. In addition, no individual presented infection or other reactions during the experiment.

Regarding the animal growth, the weight gain (percentage) after surgery was observed in all the animals (Figure 2C), except for the rabbit 6, who presented a slightly weight loss in the first week after surgery. However, after second week it was stabilized by taking up the weight upward trend similar to the rest of the animals.

3.3. Histological analysis

The photomicrographs in Figure 3 and Supplementary Material show the implant zones four weeks after surgery. The implanted rabbits show complete wound healing with the presence of abundant hair follicles, except in the final cicatrization line. The lacking area of hair follicles is of variable extent.

The wound healing of the areas exposed to the wound-dressing presents characteristics typical of a normal process without signs of rejection. In general, it is observed a complete epithelization and granulation tissue that begins to be structured as regular connective tissue from the area of the epidermis that is still lacking of follicles but which would have developed as such in the surrounding area.

4. Conclusion

A novel biomaterial based on salmon-gelatin that serves as a wound dressing was formulated and evaluated *in-vivo.*

The biomaterial was sterilized using gamma radiation. The irradiation did not modify the main polymer configuration in the composite and seemed to improve its mechanical properties.

The *in-vivo* results showed an excellent wound healing processes without signs of rejection, confirming its biosafety.

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Figure captions

Figure 1: Microstructure of the biomaterial.

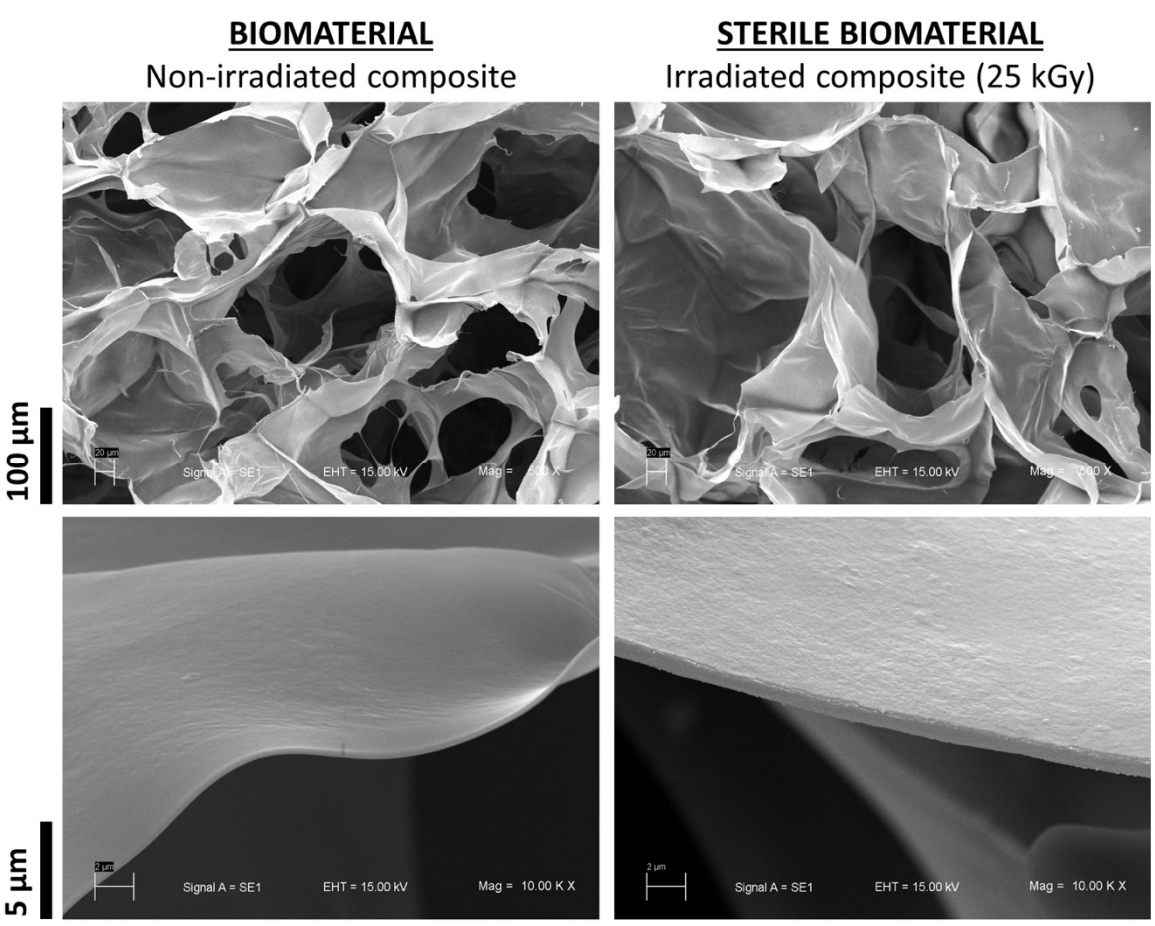

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Figure 2*: In-vivo* evaluation to test biosafety.

A, Wound-dressing implantation.

B, Evolution of the wound area for four weeks after surgery.

C, Weight gain of the animals for four weeks after surgery.

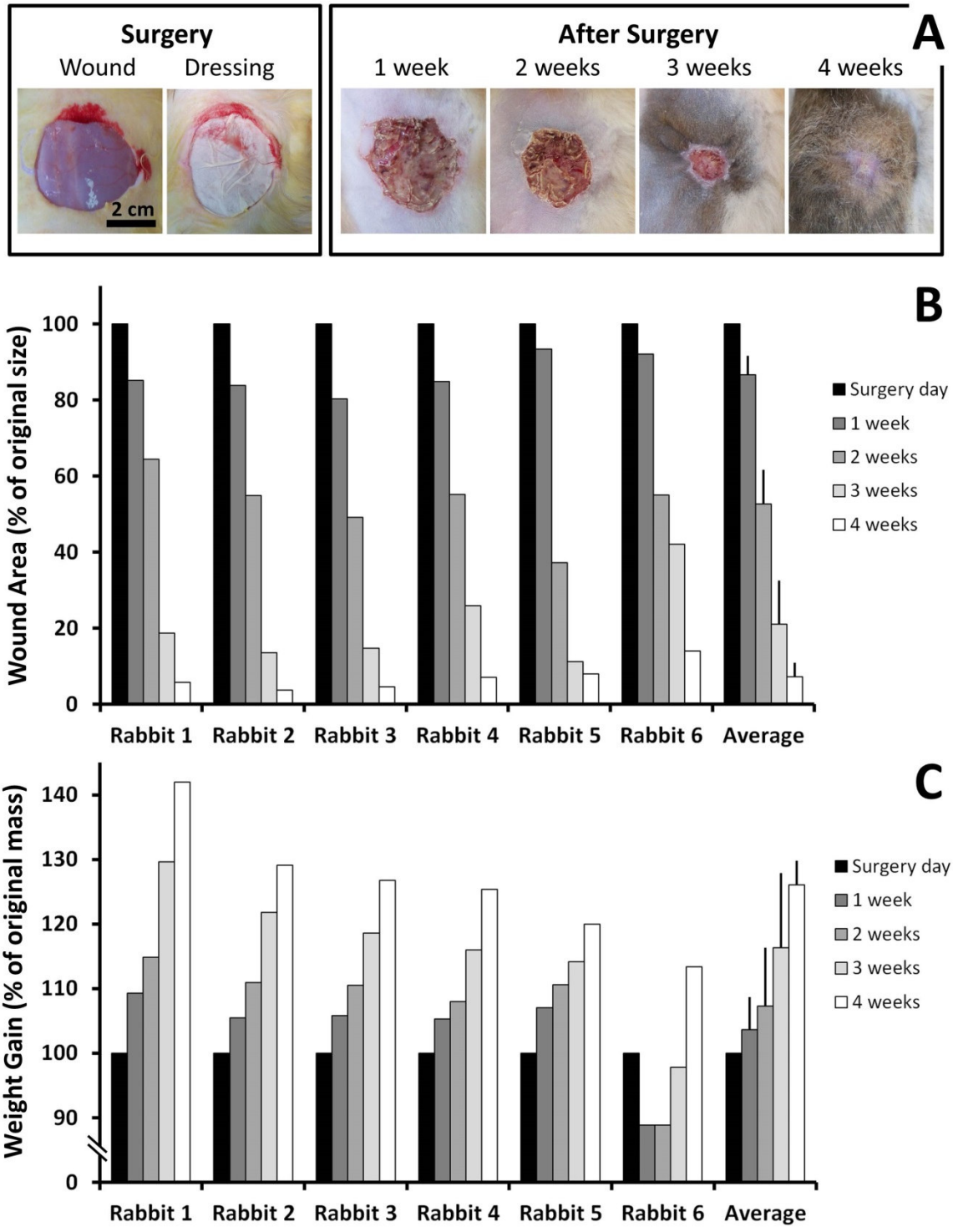

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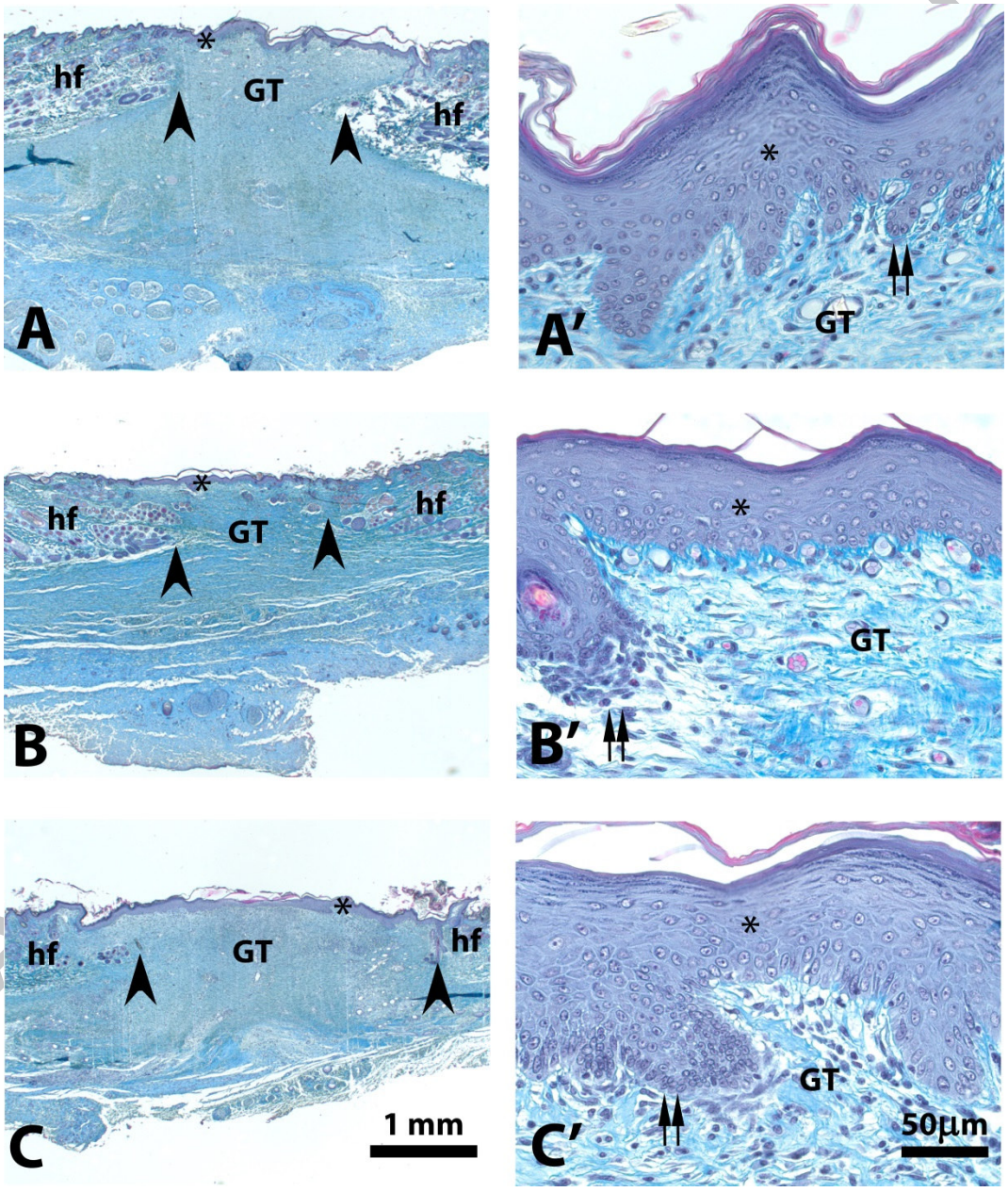
Figure 3: Histological evaluation four weeks after surgery.

A-C: Panoramic view of the wound healing area of rabbits 3, 5 and 6, respectively (animals 1, 2 and 4 are shown in supplementary material). A thick and continuous layer of epidermis (asterisks) from less to greater extension is observed (the arrowheads limit the area), which underlies the dermis of the granular tissue (GT). The hair follicles are abbreviated as hf.

A'-C ': Magnification of A-C (asterisks) showing the hyperplastic epidermal tissue and its projections (double arrows) towards the lax dermis.



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HIGHLIGHTS

Design of a novel porous biomaterial based on salmon-gelatin and excipients.

Sterilization of the biomaterial by using gamma radiation.

Structural, thermal and mechanical evaluation of the material.

In-vivo evaluation of the biomaterial as wound dressing.

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