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Morphology and morphometry in the healing of experimentally induced wounds in rabbits treated with autologous platelet-rich fibrin and bioactive chitosan/xanthan/β-glucan dressing

Morfologia e morfometria na cicatrização de feridas induzidas experimentalmente em coelhos tratados com fibrina autóloga rica em plaquetas e curativo bioativo de quitosana/xantana/β-glucana

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Highlights

The aPRF and bioactive dressing should be used at different stages of healing.
Isolated aPRF accelerates wound contraction.
The use of biocuratives allows constant production of fibroblasts.

Abstract

Wound healing presents a dynamic and rapidly growing field of research worldwide. With technological advances, a range of dressings have been developed for different types of wounds, targeting the four phases of healing. Biopolymers, such as chitosan, are used to treat wounds owing to their biocompatibility, biodegradability, and similarity to recognized macromolecules. However, most biopolymer-based formulations have several limitations; combining them with biomaterials such as Platelet-Rich Fibrin

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(PRF) is considered a promising strategy for wound healing. Hence, in this study, we aimed to analyze the effectiveness of biomaterials, with or without the bioactive dressing comprising chitosan complexed with xanthan and β-glucan, in the healing of induced wounds in rabbits by characterizing the macroscopic and morphometric effects. Twenty-four rabbits were used to investigate the macroscopic changes in wounds that were experimentally induced and treated with autologous platelet-rich fibrin, combined with or without bioactive dressing; the morphological features associated with the healing process were studied on days 7, 14, 21, and 28. The results reflected that the tested biomaterials showed promising wound healing properties, even without presenting the expected synergistic effect; the APRF group demonstrated a higher percentage of contraction than the others, and the membrane group allowed the constant production of fibroblasts over time, which can facilitate the healing process.
Key words: Biomaterials. Biodressings. Collagenization. Tissue repair.

Resumo

A cicatrização de feridas possui um campo de pesquisa dinâmico e em rápido crescimento em todo o mundo. Com o avanço tecnológico, uma gama de curativos tem sido desenvolvida para diversos tipos de feridas, visando as quatro fases da cicatrização. Biopolímeros, como a quitosana, são utilizados no tratamento de feridas devido à sua biocompatibilidade, biodegradabilidade e semelhança com macromoléculas reconhecidas. Contudo, a maioria das formulações à base de biopolímeros apresentam diversas limitações e combiná-los com biomateriais como a Fibrina Rica em Plaquetas autóloga (FRPa) é considerada uma estratégia promissora para a cicatrização de feridas. Assim, neste estudo, objetivou-se analisar a eficácia de biomateriais, com ou sem curativo bioativo composto de quitosana complexada com xantana e β-glucana, na cicatrização de feridas induzidas em coelhos, caracterizando os efeitos macroscópicos e morfométricos. Vinte e quatro coelhos foram utilizados para investigar as alterações macroscópicas em feridas induzidas experimentalmente e tratadas com fibrina rica em plaquetas autóloga, combinada com ou sem curativo bioativo. As características morfológicas associadas ao processo de cicatrização foram estudadas nos dias 7, 14, 21 e 28. Os resultados refletiram que os biomateriais testados apresentaram propriedades promissoras de cicatrização de feridas, mesmo sem apresentar o efeito sinérgico esperado; o grupo FRPa demonstrou maior percentual de contração que os demais, e o grupo membrana permitiu a produção constante de fibroblastos ao longo do tempo, o que pode facilitar o processo de cicatrização a longo prazo.
Palavras-chave: Biomateriais. Biocurativos. Collagenização. Reparação tecidual.

Introduction

Skin is the largest organ in the body and it plays a fundamental role in various functions of an organism. The skin, composed of the epidermis and dermis, is crucial for regulating body temperature and protecting against external agents. Additionally, the dermis is composed of a layer of extracellular matrix that supports various types of cells that drive tissue regeneration, including physiological or traumatic regeneration (Adhikari et al., 2023). Fibroblasts are the most common cells in connective tissues; they are responsible for synthesizing connective tissue fibers,

extracellular matrix glycoproteins (ECM), and collagen, which are essential for tissue repair and healing (Mascharak & Longaker, 2020).

Wounds are prone to bacterial colonization generating inflammation, infection, and necrosis; in the most complicated cases, they can be fatal. Thus, selecting dressings that repair tissue damage and prevent bacterial growth and cell necrosis is a challenge in routine medical care (Budiarso et al., 2023). The wound healing process comprises three successive and interconnected phases: the inflammation phase, associated with several chemical mediators and leukocytes; the proliferation phase, which presents mononuclear cell-mediated proliferation of fibroblasts; and the remodeling/maturation phase, exhibiting a decrease in local cellular activity (Bilgen et al., 2021).

Currently, several wound healing products are commercially available; however, biomaterials have been explored aiming to the development of cost-effective biological and biodegradable alternatives with fewer side effects and minimal risk to the environment. A good biomaterial should exhibit a chemical structure modifiable according to its use and it must be biocompatible with the organs and tissues (Budiarso et al., 2023).

Biomaterials include various materials that can temporarily or permanently fuse with organs and tissues and facilitate their regeneration. Biopolymers are biodegradable and biocompatible, and they have been widely explored for application in routine biomedicine (Dec et al., 2023).

Compounds made from platelets, which are used as biomaterials, are essential in the wound healing process, and the associated growth factors aid in re-epithelialization and neovascularization during the proliferation phase of healing (Bilgen et al., 2021).

Platelet-rich fibrin (PRF), considered a new generation of platelet concentrates, supports high rates of tissue regeneration, and its slow polymerization favors the physiological architecture of tissues. Moreover, growth factors and cytokines regulate inflammatory processes and improve local angiogenesis, thus accelerating the healing phase (Herrera-Vizcaíno et al., 2019). Leukocyte–platelet-rich fibrin (L-PRF) reduces the risk of infection (Canellas et al., 2017; Castro et al., 2019).

PRF can be obtained from blood via centrifugation and is collected in a tube with no anticoagulants or gelling substances, which makes the process more advantageous concerning the preparation of PRP and rapid activation of the platelets and the coagulation cascade within a few minutes; moreover, it generates a solid clot rich in a platelet matrix and releases growth factors for a longer period (Dohan et al., 2014; Varghese et al., 2017).

Alternative therapeutic approaches include the use of dermal dressings developed from natural polysaccharides (chitin, chitosan, alginate, pectin, xanthan, cellulose, and its derivatives, methylcellulose, and carboxymethylcellulose), isolated or combined by complexation (chitosan complexed with xanthan), which offer a favorable, hydrated, and thermally insulated microenvironment, remove excess exudates, and promote gas exchange (Jayakumar et al., 2011); hence, these agents promisingly facilitate the healing processes of severe skin lesions (Bellini et al., 2012, 2015).

Chitosan, widely used in tissue repair, is a biopolymer derived from chitin; it is found mainly in the exoskeletons of crustaceans, some insects, and the fungal cell walls (Kumar et al., 2004). It efficiently contributes to biological processes, including improved immunity and healing, hemostasis, adhesion, and antimicrobial activity (Burkatovskaya et al., 2006; Ezoddini-Ardakani et al., 2011). Xanthan gum is a fermentation product of *Xanthomonas campestris*, a gram-negative strictly aerobic bacterium present in the form of motile rods. Despite being a microbial product, it is classified as a non-toxic additive for humans in the European list of permitted food additives. The *Food and Drug Administration* of the United States declared xanthan gum a safe stabilizer and emulsifier for food and pharmaceutical use, and it does not influence the development of individuals (Dzionek et al., 2022).

Furthermore, β-glucan is a polysaccharide derived from cereals, barley, oats, and some fungal cell walls. It is water-soluble and has a linear binding structure involved in the stimulation of the immune system. Studies have demonstrated its effectiveness in normalizing cholesterol and postprandial glucose levels (Mio et al., 2020).

Some advantages of biopolymers include environmental nontoxicity and their ability to undergo enzymatic or chemical transformation to form gels, films, and nanoparticles, which enhances their applicability according to the location and type of lesion (Qin & Li, 2020).

Hence, in this study, we aimed to analyze the effectiveness of biomaterials with or without the bioactive dressing of chitosan complexed with xanthan and β-glucan in the healing of induced wounds in rabbits; moreover, we characterized their effects macroscopically and morphometrically.

Material and Methods

Animals

Twenty-four clinically healthy adult male and female New Zealand rabbits (*Oryctolagus cuniculus*), with an average weight of 3.0 ± 1.0 kg, were included in this study. Individual animals were kept in separate cages at 22 ± 2ºC under a controlled photoperiod (12 h light/dark). The rabbits underwent a seven-day adaptation period before the study. Throughout the experiment, the animals were maintained under standardized conditions including water supplied ad libitum and a commercial diet. The study was approved by the Ethics Committee on the Use of Animals (CEUA) protocol no. 6115. Experiments were conducted according to the ethical standards and principles established by the Brazilian Society of Laboratory Animal Science (SBCAL) and the *Guide for the Care and Use of Laboratory Animals - National Research Council.*

Sample size

To calculate the sample size, the following equation was used:

$$n = (Z\ X \frac{S}{E})^2$$

n = number of sample elements

z = tabulated value obtained from normal distribution table Z–N (0, 1)

s = standard deviation

e = standard error

We included 24 animals in the assay. The calculation was performed considering a confidence level of 99%. Regarding the standard deviation, s=0.0469 was used, considering the standard deviation obtained in a similar study (Tetila et al., 2019).

Experimental groups

The 24 animals were randomly divided into four experimental groups (6 animals per group): control (CG), autologous Platelet-Rich Fibrin (aPRF), Membrane (MG), and autologous Platelet-Rich Fibrin and membrane (aPRFM) groups (Figure 1).

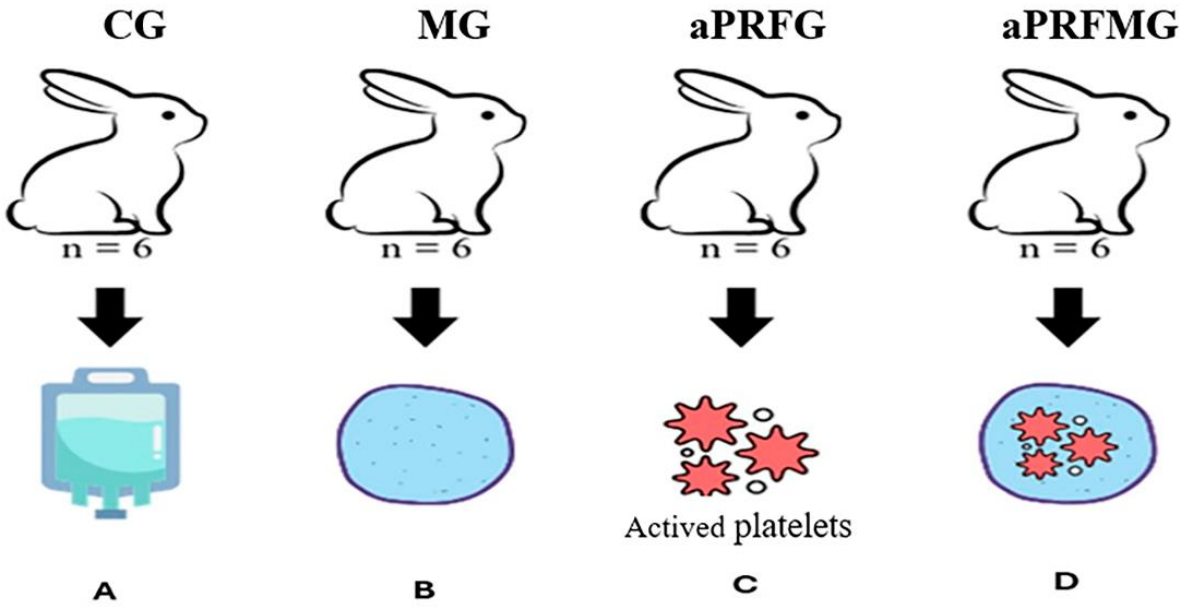


Figure 1. Experimental design scheme:
A: Control Group (CG); B: Membrane Group (MG); C: Autologous Platelet-Rich Fibrin Group (aPRFG); and D: Autologous Platelet-Rich Fibrin and Membrane Group (aPRFMG).

Anesthetic procedure

The animals were manually restrained to perform trichotomies in the right and left dorsal regions. They were then anesthetized with intramuscularly [IM] injected ketamine hydrochloride 100 mg/ml (40 mg/kg of body weight) combined with xylazine hydrochloride 2% (5 mg/kg of body weight). A local anesthetic (lidocaine hydrochloride 2% with a vasoconstrictor) was applied at the site of the injury.

Induction of lesions and treatments

The skin of the anesthetized animals was demarcated in four places using a pen, and an 8-mm punch was used to create the surgical wounds. The fragments of the tissue were removed using anatomical forceps to preserve the muscles. The lesions of the CG and aPRF groups received a saline solution and aPRF, respectively; subsequently, they were covered with rayon sterile and bandage sticker (Band-aid®). Weekly applications

of saline and aPRF, along with dressing changes, were performed for both groups. The wounds in MG and aPRFM groups were treated with membrane and aPRF + M, respectively. Treatment with biomaterial and/or saline solution was applied once.

After the surgical procedure and different treatments, the animals received the analgesic tramadol hydrochloride at a dose of 0.5 mg/kg injected (IM) twice a day for 3 consecutive days, to minimize the initial discomfort. In all the moments of the procedure, the animals were photographed using a smartphone equipped with a 12-megapixel camera (iPhone 11, Apple®), at a standardized height of 33.5 cm, and the injuries were measured with a digital caliper (DC-60 Western®).

After completing the evaluation of each moment, the animals in the group were euthanized in a CO2 chamber after dissociative anaesthesia with a combination of tiletamine hydrochloride, zolazepam (Zoletil®; Virbac, São Paulo, Brazil) 30 mg/kg IM, and 2% Xylazine (Syntec®; Syntec, São Paulo, Brazil) 0.2 mg/kg IM, and death was confirmed by the absence of heartbeats.

Preparation of autologous Platelet-Rich Fibrin (aPRF)

Blood was collected from the auricular vein of each rabbit (4 mL) using a 25G scalpel, stored in tubes without an anticoagulant, and subjected to low-speed centrifugation (200 g, Excelsa Baby 206R centrifuge) for 10 min. This procedure activates platelet and fibrin polymerization (Narang et al., 2015). The aPRF clot was removed from the tube (Figure 2), separated from the other constituents, and placed on the injured area (Camargo, 2013).

Figure 2. Preparation of aPRF:
A: Blood collection from the auricular vein; B: Centrifugation of the material; C: aPRF ready for use; D: Dressing after aPRF application.

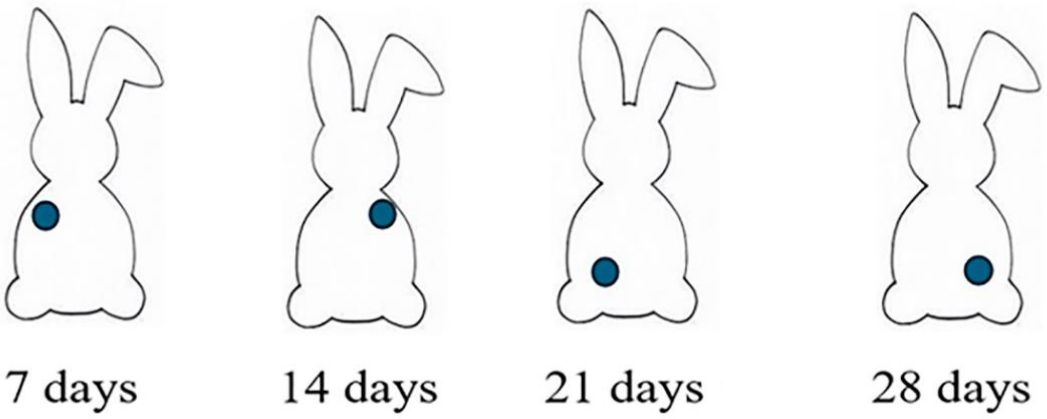
Preparation of chitosan membrane complexed with xanthan and β- glucan

The dense and porous chitosan (Q) membranes complexed with xanthan (X) were prepared at a mass ratio of X to Q equal to 1:1, using 96% deacetylated chitosan, xanthan gum, Pluronic® F-68 (Sigma-Aldrich; St. Louis, MO, USA) and glacial acetic acid (Merck; São Paulo, Brazil), with the addition of β-glucan following the procedures described by Bellini et al. (2012). To obtain the membranes, the Q solution was added to the xanthan and/or β- glucan solution through a peristaltic pump at a flow rate of 10 mL/min, with constant stirring at 1000 rpm using a mechanical stirrer with high torque. The formulations containing both the polysaccharides X and β-glucan were previously mixed by stirring constantly at 1000 rpm for 2 minutes before adding chitosan. During the preparation of the polymer complex, the temperature was maintained at 25ºC in a single jacketed glass reactor with internal and external diameters of 11 cm and 12 cm, respectively, and a total capacity of 7.5 L. After polymer complexation, the coacervate suspension was deaerated for 2 h using a vacuum pump, transferred to a 15 cm diameter polystyrene plate, and dried at 37ºC in an oven with air circulation to generate a dry membrane. Next, to neutralize the pH of the membranes, they were successively washed with deionized water and 10 mM HEPES buffer followed by further drying at 37ºC in an air circulation oven (Figure 3).

Figure 3. Membrane Processing:
A: Dilution and homogenization; B: Fractioning into Petri dishes and drying in an oven; C: Membrane ready to be hydrated and used on the lesions.

Morphometric analysis

The animals were photographed weekly, and the lesions were measured using a digital caliper. Measurements were recorded on days 7 (wound A), 14 (wound B), 21 (wound C), and 28 (wound D). While assessing the morphological characteristics of wounds, the color and margin of the wound, the presence of crust, ulcer, necrosis, and exudate as well as the characteristics of the exudate and edema were analyzed (Figure 4 and Table 1).



Scheme for macroscopic evaluation, morphometric analysis, and biopsies of the lesions.

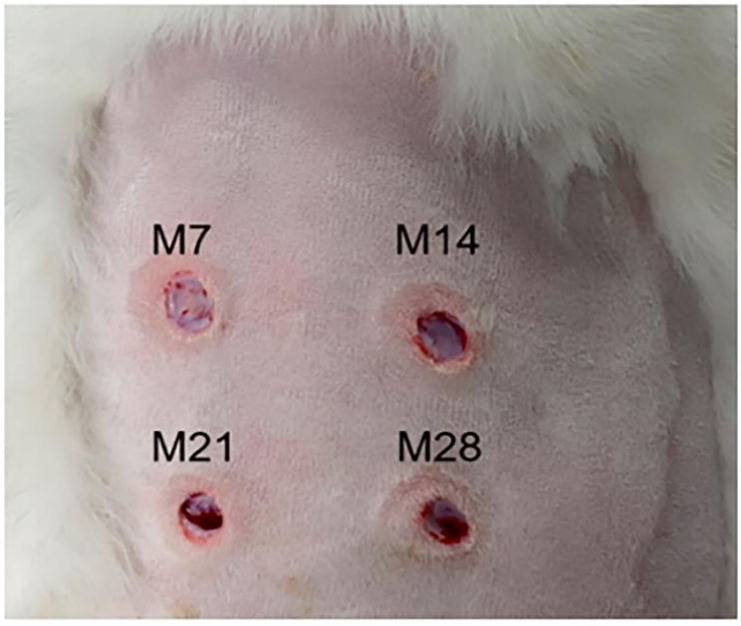


Figure 4. Photographs of the experimental lesions (Punch 6mm) and respective time points.

Table 1
Scores used in the morphological and morphometric analysis of experimentally induced wounds treated with different biomaterials in rabbits

Aspect	Scores			
Wound color	1 – Pink	2 – Yellowish	3 – Pale	4 – Cyanotic
Edges	1 – Without granulation		2 – With granulation	
Scab	1 – Without scab		2 – With scab	
Ulcer	1 – Without ulcer		2 – With ulcer	
Necrosis	1 – Without necrosis		2 – With necrosis	
Exudate	1 – Without exudate		2 – With exudate	
Characteristics exudate	1 – Serous		2 – Sanguineous	3 – Purulent
Edema	1 – Without edema		2 – With edema	
N.E	No evaluation			

The wound area was measured using a digital caliper (DC-60 Western®) at the above mentioned time points and the percentage of contraction was calculated. For each injury, the mathematical model proposed by Ågren et al. (1997) was used, in which the percentage of contraction (Pc) is equal to the initial area (Ia) minus the final area (Fa) times 100 (x100), divided by the initial area (Ouch), or it is:

$$Pc = \frac{(\text{Ia} - \text{Fa}) \times 100}{\text{Ia}}$$

Histopathological evaluation

The histopathological evaluation of each wound was performed by removing a fragment of the lesion on days 7, 14, 21, and 28 using a 6 mm punch. The same anesthetic protocol was used at the time of lesion induction. The skin samples were fixed for 24 h using a 10% formalin solution buffered at pH 7.0, followed by washing in running water for 1 h. The fragments were transferred to a 70% alcohol solution for further processing according to routine histological techniques for optical microscopy and paraffin embedding. The sections were stained using hematoxylin and eosin (HE) methods. The fibroblast count was performed by viewing the slides at 400x magnification; the average counts of 10 fields were recorded for each animal. All assessments were performed by a single blinded observer (Vendramin et al., 2010).

Statistical analysis

The variables were considered parametric, and the moments were compared within each group by repeated measures analysis of variance; we checked the assumption of sphericity of the data using Mauchly's test, contrasts via Tukey's method, and adjustment of the significance values via the Bonferroni method. All analyses were conducted using the R Program with the aid of the rstatix and PMCMRplus packages (Kassambara, 2023; Pohlert, 2023; R Core Team [R], 2024).

Results and Discussion

Morphometric aspects exhibited no significant differences in the color of the wounds, which appeared pink in all groups and time points analyzed. Additionally, no necrosis, exudates, or edema were detected in any group. The statistically significant changes are listed in Table 2 and Figure 5.

Table 2
Macroscopic and morphometric changes observed during the experiment with significant differences

Observed change	Group	Animals %	Moment
Edge with granulation	aPRF	50%	7
Ulcer in the lesion	aPRF	16%	7
Scabs	Control	100%	7
	Membrane	83%	7
	aPRF	50%	7
	aPRF + Membrane	100%	7

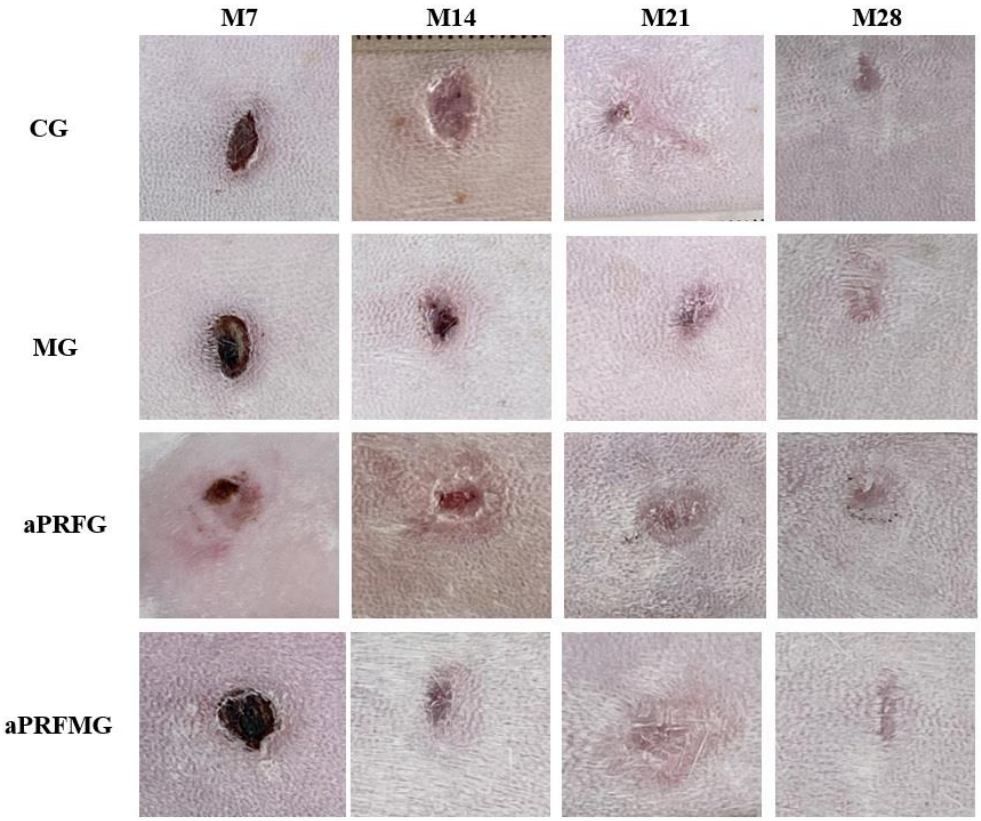


Figure 5. Macroscopy of the lesions at 7, 14, 21, and 28 days of experimentally induced wounds in rabbits.

The percentage of contraction (Figure 6) revealed no significant difference among groups (aPRF [82.25%], CG [57.04%], MG [57.17%], and aPRFM [61.65%]) and moments ($p>0.05$). This result is attributed to the fact that the release of cytokines and growth factors from PRF is greater in the first 24 h but can last up to 21 days after the injury. In this way, functions such as angiogenesis, proliferation, and cell modulation are stimulated; moreover, collagen synthesis and fibroblast recruitment are enhanced because activated platelets release granules that trigger several angiogenesis and re-epithelialization-stimulating growth factors, thus repairing the damaged tissue (Kobayashi et al., 2016; Lourenço et al., 2018; Wang et al., 2018; Bai et al., 2023).

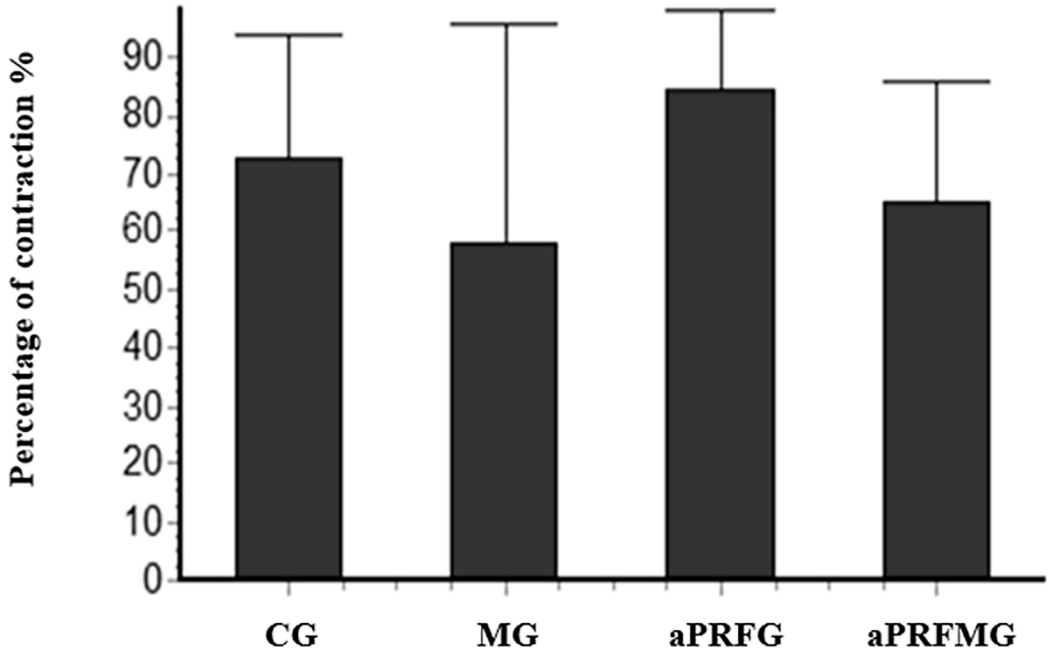


Figure 6. Percentage of wound contraction under different treatments.

The fibroblast count did not significantly differ between the treatments ($p>0.05$), however, it significantly differed ($p<0.05$) between the moments within the same treatment (Table 3 and Figure 7). The fibroblast count differed between days 7, 14, and 21 in the CG; in the membrane group, it differed between days 7 and 14; in aPRF, it differed between days 7 and 21. However, aPRFM did not exhibit a significant difference in the fibroblast count between time points.

Table 3
Mean and standard deviation of fibroblasts in rabbits treated or not with bio dressing and aPRF

Groups/days	7	14	21	28
Control	77,1±30,0A	127,6±20,6B	145,5±40,8B	139,3±39,6AB
Membrane	158,6±62,9A	196,8±50,9B	164,5±73,9AB	153,8±71,4AB
aPRF	141,5±63,2A	160,0±31,6AB	180,3±39,1B	156,8±49,6AB
aPRF+Membrane	140,1±43,4A	152,1±23,7A	159,1±51,5A	157,0±37,2A

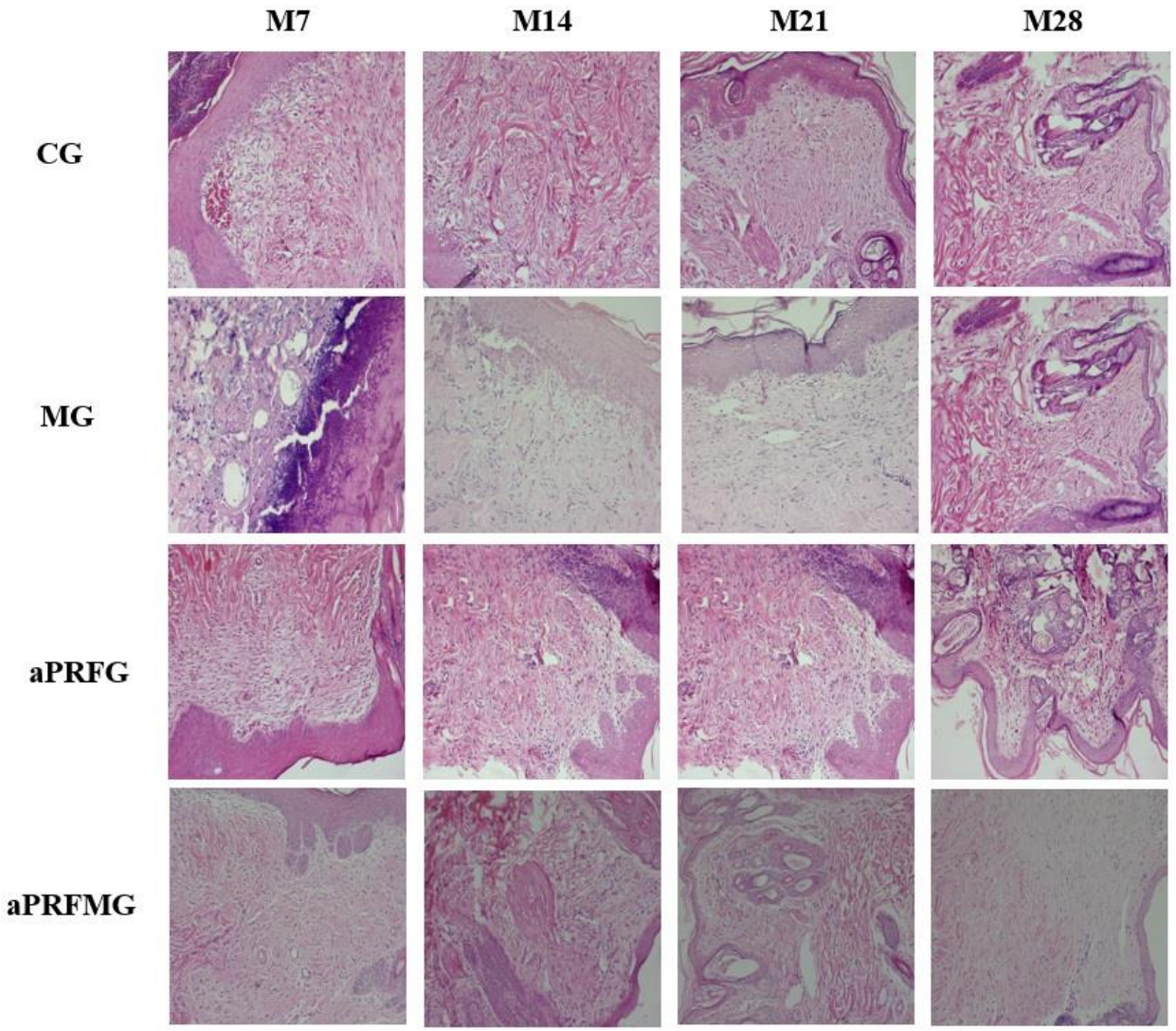


Figure 7. Fibroblast count in histological slides stained with HE (400x magnification).

According to Sezgin et al. (2021), wounds presented an inflammatory reaction in the presence of scabs on day 10, whereas, on day 21, the number of inflammatory cells decreased, which is comparable to the present study outcomes; it is potentially related to the stages of the healing phase, where the levels of inflammatory mediators and fibroblasts decreased with the progress in the wound healing period.

PRF is widely used in several fields of health, which include dental procedures, dermatology, orthopedics, cardiology, ophthalmology, and wound healing, due to its growth factors that aid in tissue regeneration and collagen synthesis (Farmani et al., 2021). Another important characteristic of PRF is its synergism with the leukocytes present in clots, which also secrete substances that stimulate tissue repair (Karimi & Rockwell, 2019).

Chitosan is the second most abundant natural polymer; this biomaterial is effective in healthcare owing to its high solubility, biodegradability, low toxicity, memory, and thermosensitivity. Chitosan- and collagen-based dressings developed by Zhang et al. (2018) inhibited bacterial proliferation and maintained good water retention at the wound site, accelerating the healing process. Choudhary et al. (2020) demonstrated that chitosan combined with graphene-silver compounds supports the control of hemorrhages caused by deep injuries through the hemostatic efficacy of the biopolymer increased by up to three times.

Moreover, Sarkar et al. (2019) reported a better hemostatic effect of chitosan in hydrogel form than that of PRF, facilitating coagulation in patients who were administered antiplatelet drugs and underwent surgical procedures. However, PRF showed better healing properties, corroborating our study outcomes. According to Cianca et al. (2020), membranes with β-glucan are thinner and have lower permeability capacity and tensile strength.

Furthermore, the incorporation of β-glucan in the membranes revealed that the number of bubbles formed on the surface of the dressing increases with increasing concentration of this biomaterial. Additionally, the high cost of purified β-glucan in the country limits the use of this component in the formulation of dressings (Nakasse et al., 2020).

Biomaterials were developed to promote tissue regeneration and wound healing considering their hemostatic and antimicrobial properties, adhesive action, and mechanical adaptation (Adhikari et al., 2023). In the present study, these properties were evident while using aPRF as well as the membrane.

Conclusion

In conclusion, the two biomaterials individually induced wound healing; however, they did not present the expected synergistic effect. The aPRF exhibited a higher percentage of contraction than the others, and the MG allowed the constant production of fibroblasts over time, which may favor the healing process.

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