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Research Article

Preparation, Characterization and Wound Healing Effect of β Chitosan and Gelatin Hydrogels from *Sepia Officinalis*: *In Vivo* Study

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ABSTRACT

Wound healing is a fundamental process through which the body repairs itself following damage to the skin's integrity. This process is intricate and involves multiple biological mechanisms. The objective of this study is to assess the wound healing properties of β-chitosan and gelatin hydrogels. Twenty-five rats were divided into five groups to facilitate the evaluation of wound closure rates and histopathological changes. Upon analyzing the results, we noted a decrease in the initial surface area of all wounds examined. The extent of contraction varied depending on the type of hydrogel used. By day 14, the contraction was most significant in the hydrogel (ch + gel) group (97.30%), followed closely by β-chitosan (96.72%) and gelatin (96.63%), as well as the cicatryl-bio treatment (92.76%).

Key Words: β Chitosan, Gelatin, Hydrogel, Wound Healing, *Sepia Officinalis*, Os, Skin.

INTRODUCTION

Wound healing is a dynamic and intricate process that encompasses inflammation, re-epithelialization, granulation tissue formation, neovascularization, wound contraction, and remodeling of the extracellular matrix, ultimately restoring tissue integrity and homeostasis [1]. This process is highly regulated and consists of four distinct phases: hemostasis, inflammation, proliferation, and maturation or remodeling. Successful repair requires the coordinated action of various cells, growth factors, and cytokines.

Hydrogels possess ideal properties for tissue engineering, such as facilitating moist wound healing, effective fluid absorption, and exceptional water retention [2]. Their porous structure can absorb wound exudate, minimize infection risk, and create an environment conducive to accelerating wound healing. Additionally, the use of natural-synthetic polymer composites in hydrogel production enables the creation of porous structures that exhibit favorable biocompatibility due to the presence of natural polymers.

Chitin is a promising biopolymer that is abundantly available in nature and has been extensively studied for its biocompatibility. Most research on chitin focuses on readily accessible forms, characterized by an antiparallel arrangement of the main chain and strong intermolecular hydrogen bonding. However, β-chitin, as noted by Kurita et al. [3], demonstrates significantly higher reactivity and versatility compared to standard chitin due to its parallel main chain arrangement and weaker intermolecular hydrogen bonding. This makes it a suitable candidate for novel biomedical applications. Despite this, β-chitin faces solubility challenges, as it requires strong acidic solvents—such as formic acid—that can irritate human tissues. In contrast, chitosan, which is derived from the deacetylation of chitin, can be dissolved in water with a small amount of acetic acid, allowing for hydrogel preparation under milder conditions. This positions chitosan as a strong candidate for hydrogel applications in the biomedical field. Furthermore, chitosan can be hydrolyzed by lysozyme, an enzyme found abundantly in animal tissues [4].

Beta-chitosan (CS), obtained from the deacetylation of chitin predominantly found in squid pens, has gained attention due to the increasing production of squid. It offers unique advantages, including simpler preparation, a higher affinity for solvents, and enhanced biological activity compared to CS derived from crab and shrimp shells [5,6].

Gelatin is a protein-rich compound that is commercially sourced from the skins and bones of cattle and pigs. It has a wide range of applications in the food, pharmaceutical, and photographic industries [7].

As a bio-based polypeptide polymer derived from collagen, gelatin exhibits a less ordered structure due to the absence of triple helices. It is known for its biodegradability and biocompatibility, as well as its excellent solubility in water and capacity to form strong hydrogen bonds. However, its main limitations for medical use include low mechanical strength and rapid degradation. Consequently, it is often used in combination with other materials in mixed formulations [8].

In light of this, the present study aims to evaluate the healing potential of β-chitosan–gelatin hydrogel for full-thickness skin wounds in Wistar rats.

MATERIALS AND METHODS

Preparation of cuttlebone (*sepia officinalis*)

Cuttlebone (*Sepia officinalis*) was collected from Mostaganem, Algeria, in April 2021. The cuttlebone

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was thoroughly washed several times with deionized water to eliminate impurities and salts. After washing, it was dried in an oven at 60 °C for 48 hours. The dried cuttlebone was then ground, and the resulting particles were sorted using sieves.

Extraction of β chitin and conversion into β chitosan

β Chitin was isolated from the cuttlebone of S. officinalis through a series of processes including demineralization, deproteinization, and decolorization, as outlined by Takiguchi [9]. Initially, 100 grams of cuttlebone powder was submerged in 1000 ml of a 10% (w/w) hydrochloric acid solution at ambient temperature (25 °C) for 24 hours. After this period, the mixture was filtered, and the remaining solid was thoroughly washed with distilled water until neutral. Next, the residue was treated with 1000 ml of a 10% (w/w) sodium hydroxide solution at 60 °C for another 24 hours to remove proteins. The filtrate was discarded, and the residue was again washed with distilled water to achieve neutrality. This procedure was repeated two additional times. To eliminate ethanol-soluble compounds from the crude β chitin and to dehydrate it, the residue was sequentially treated with 250 ml of 95% and absolute ethanol. Finally, the β chitin (200 g) was dried in an air oven at 50 °C overnight. The isolated β chitin was then converted to β chitosan through deacetylation using a 40% aqueous sodium hydroxide solution and a microwave technique, as described by Sahu et al. [10].

Preparation of β chitosan

To prepare crude chitosan, 10 grams of β chitin was placed in a ceramic mortar and treated with 100 ml of 50% sodium hydroxide solution at 100 °C for 120 minutes. The ceramic mortar was then positioned in the center of a microwave oven, where it was irradiated for 15 minutes at a power setting of 1000 W. After irradiation, the mixture was filtered, and the remaining solids were washed with distilled water until neutral. The residues were subsequently dried in a hot air oven at 40 °C until they reached a constant weight, and then stored for further analysis. The crude β chitosan was obtained by drying 5 grams of the material in an air oven at 50 °C overnight.

Gelatin extraction

Preparation of cuttlefish skin

Sepia officinalis were sourced from the marine processing industry in Mostaganem City, Mostaganem. The samples were packed in polyethylene bags and placed on ice with a sample-to-ice ratio of approximately 1:3 (w/w) for transport to the research laboratory, which took about 30 minutes. Upon arrival, the samples were washed twice with water to remove the dark ink, composed of melanin granules suspended in a viscous, colorless medium. The cuttlefish by-products were then sorted, and only the outer skin was collected. This skin was stored in sealed plastic bags at 20 °C until it was needed for gelatin extraction and analysis.

Extraction of gelatin from cuttlefish skin using the fish protease-aided process

Gelatin was extracted from cuttlefish skin following the methods described [11,12]. The skin was immersed in a 0.05 M sodium hydroxide solution at a skin-to-solution ratio of 1:10 (w/v), with gentle stirring at room temperature. The solution was replaced every hour over a total duration of 6 hours to eliminate non-collagenous proteins. After alkali treatment, the skin was rinsed with distilled water until the wash water reached a neutral pH.

The prepared skin was then bleached using a 5% hydrogen peroxide solution, with a sample-to-solution ratio of 1:10 (w/v) for 48 hours at 4 °C. Following the bleaching process, the skin was washed three times with ten volumes of water.

To extract gelatin, the bleached skin was treated with distilled water at 60 °C for 12 hours, using a sample-to-water ratio of 1:2 (w/v). The mixture was stirred continuously during extraction. Afterward, the extract was centrifuged at 8000 g for 30 minutes at room temperature in a refrigerated centrifuge to separate insoluble materials. The supernatant was collected and freeze-dried, resulting in a product referred to as 'gelatin powder,' which was then subjected to hydrolysis.

A. Preparation of B-chitosan hydrogel

One gram of β-chitosan was dissolved in 7 ml of 1% (v/v) acetic acid. To sterilize the gel and preserve its structure, 0.5 ml of 25% (w/v) glutaraldehyde (GA) was added. The mixture was stirred mechanically for 24 hours. However, some fine particles remained after thorough stirring, so the solution was filtered before use.

B. Preparation of gelatin hydrogel

Gelatin was mixed with acetone in equal proportions (1:1) to precipitate the gel. Following this, 25% (w/v) glutaraldehyde (GA) was added to help maintain the protein structure.

C. Preparation of B-chitosan and gelatin hydrogel

Chitosan hydrogel and gelatin were mixed and shaken for 12 hours at 37 °C to create a chitosan-gelatin mixture. All hydrogels were then poured into polystyrene tissue culture plates (Falcon), which had been pre-frozen at -20 °C for 12 hours. The samples were subsequently lyophilized at -81 °C.

In vivo wound healing activity

Animal model

For this experiment, 25 male Wistar albino rats, each weighing between 100 and 120 g, were obtained from the Pasteur Institute in Algiers, Algeria. The rats were housed in steel cages at room temperature, maintained on a 12-hour light/dark cycle. The cages were cleaned biweekly, and the rats had unrestricted access to water and a standard laboratory diet. Following a two-week acclimatization period, the animals were grouped into five test batches.

Wound creation

Experiment protocol

The dorsal region of each rat was shaved while under anesthesia (50 mg/kg body weight of ketamine) and subsequently disinfected with alcohol. A circular wound with a diameter of 2 cm was then created on the nape of the rats using a round punch, following the method outlined by Morton et al. [13].

Grouping of animals

The animals were allocated into five groups, each consisting of five rats: Group 1 received a topical application of β chitosan hydrogel; Group 2 was treated with gelatin hydrogel; Group 3 received a topical application of β chitosan-gelatin hydrogel; Group 4 served as the untreated negative control; and Group 5 was treated topically with Cicatryl-Bio as the positive control.

Evaluation of the Wound Healing Activity

The wound-healing process was assessed by monitoring the progression of each wound excision in both treated and untreated animals every three days.

Evaluation of Wound contraction

The percentage of wound contraction and the epithelialization period were assessed on a daily basis, while histopathological analysis of the

granulation tissue was conducted at the conclusion of the experimental phase. Wound contraction percentage was determined by measuring the wound area.

$$\% \text{ of wound closure} = \frac{\text{Wound area on day 0} - \text{wound area on day n}}{\text{Wound area on day 0}} \times 100$$

n: number of days (4^{th} and 8^{th})

Histological study

At the end of the experiment, all rats were anesthetized with ketamine at a dose of 150 mg/kg body weight. Wound tissue samples were fixed in 10% formalin solution for more than 6 hours, dehydrated with acetone, cleared with xylene, and embedded in paraffin. A 5 µm section was stained with hematoxylin and eosin. Histopathological changes were examined using an optical microscope.

RESULTS AND DISCUSSION

Wound healing activity

Macroscopic examination of the rats throughout the experimental period showed the formation of a scar covering the wound, with healing occurring beneath it. Epithelialization started from the wound edges, gradually leading to the detachment of the scar's margins, while the center remained firmly attached to the wound until the epithelialization progressed. In the treated groups, scars softened rapidly following the topical application of treatments. These natural, oily products helped maintain moisture in the excision area, accelerating the separation of necrotic tissue, which was then easily removed.

Body weight evolution

Rats were weighed on the day of skin excision (D0), with subsequent weights recorded every three days throughout the experiment. No significant differences in body weight were observed in the treated groups. Moreover, normal weight gain was noted over time, indicating that the tested formulations did not interfere with the rats' growth.

Wound contraction

The initial wound size was similar across all groups, but different rates of wound size reduction were recorded. Rats in the treated groups showed better wound progression compared to the control group. Notably, wounds treated with β-chitosan and gelatin hydrogel healed faster than those treated with Cicatryl-bio®. It is well-known that free radicals can prolong wound formation, and thus, antioxidant compounds can aid the wound healing process. The antioxidant and free radical-scavenging abilities of β-chitosan and gelatin are attributed to the presence of phenolic acids and flavonoids, which are also known for their antimicrobial and anti-inflammatory properties. Additionally, polyphenols such as tannins and proanthocyanidins, due to their astringent properties, may enhance wound contraction and facilitate the re-epithelialization process. We observed a reduction in the surface area of all wounds studied, though the rate of contraction varied depending on the hydrogel used. By day 14, the contraction was nearly complete for β-chitosan + gelatin hydrogel (97.30%), β-chitosan (96.72%), gelatin (96.63%), and Cicatryl-bio® (92.76%).

Histopathological study

The histological evaluation of excision wounds is summarized in table 2. In this study, the initial increase in wound surface during the first few days of healing is histologically linked to the inflammatory phase.

Three days post- surgery

Microscopic examination of control wounds revealed minimal epithelial tissue regeneration. Infiltration of inflammatory cells and superficial necrotic regions were observed within the first three days of healing. Additionally, the cut edges of the epidermis were thickened, and a demarcation line, formed by polymorphonuclear leukocytes (PMNL), separated the vital tissue from necrotic areas. In wounds treated with chitosan hydrogel, inflammatory cell infiltration was also noted, but granulation tissue formed more quickly compared to the control group.

Table 1: Macroscopic observation of rats.

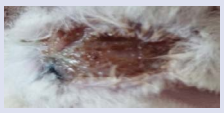
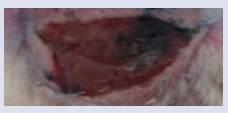
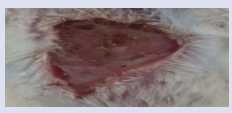
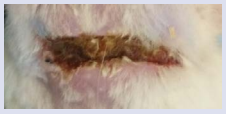
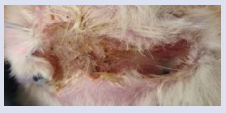
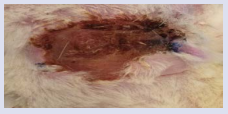
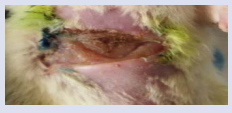
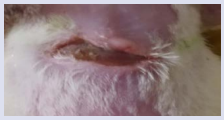
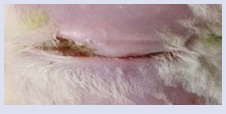
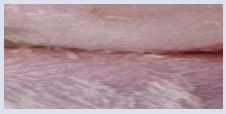
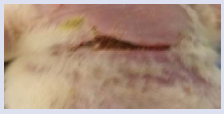
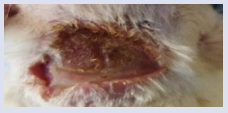
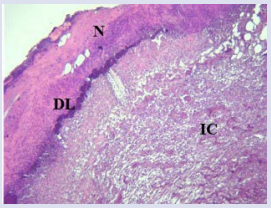
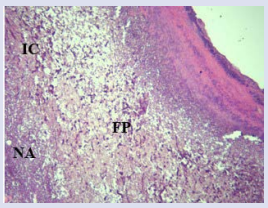
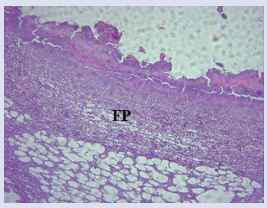
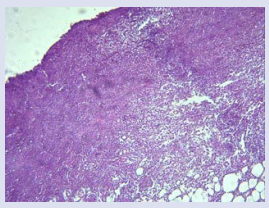
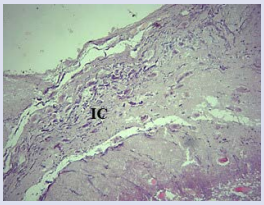
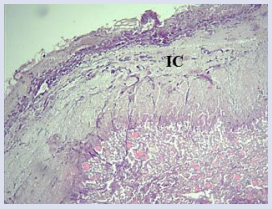
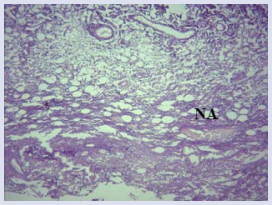
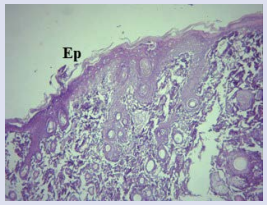
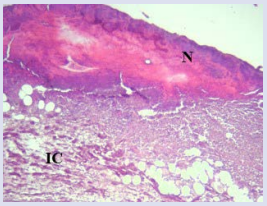
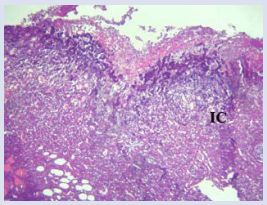
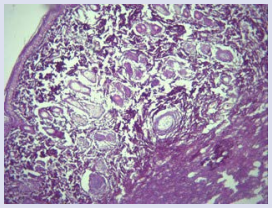
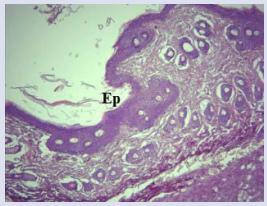
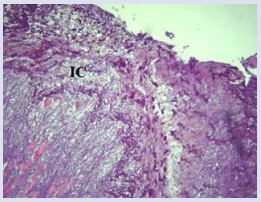
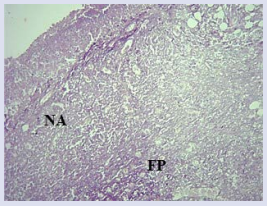
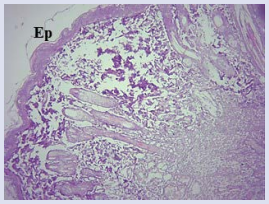
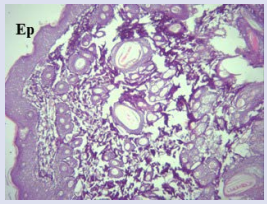
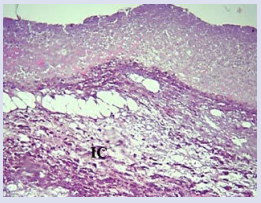
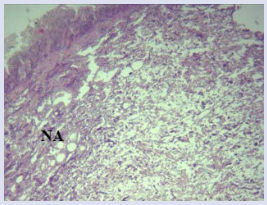
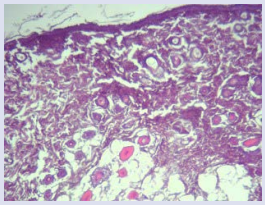
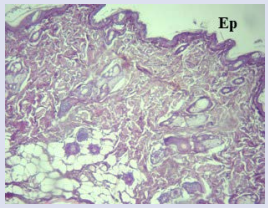
	chitosan	gelatin	Chitosan + gelatin	negative control	Cicatryl-bio
J0					
J3					
J6					
J9					
J12					

Table 2: Histology section of skin tissue (stained with H&E, X100) HE). A: control group, B: Chitosan hydrogel-treated group, C: gelatin hydrogel-treated group, D: chitosan and gelatin hydrogel-treated group, E: cicatryl-bio treated group, DL: demarcation line, Ep: epithelization, FP: fibroblasts proliferation, IC: inflammatory cells, N: necrosis, NA: Neo-angiogenesis.

	Day 03	Day 06	Day 09	Day 12
A				
B				
C				
D				
E				

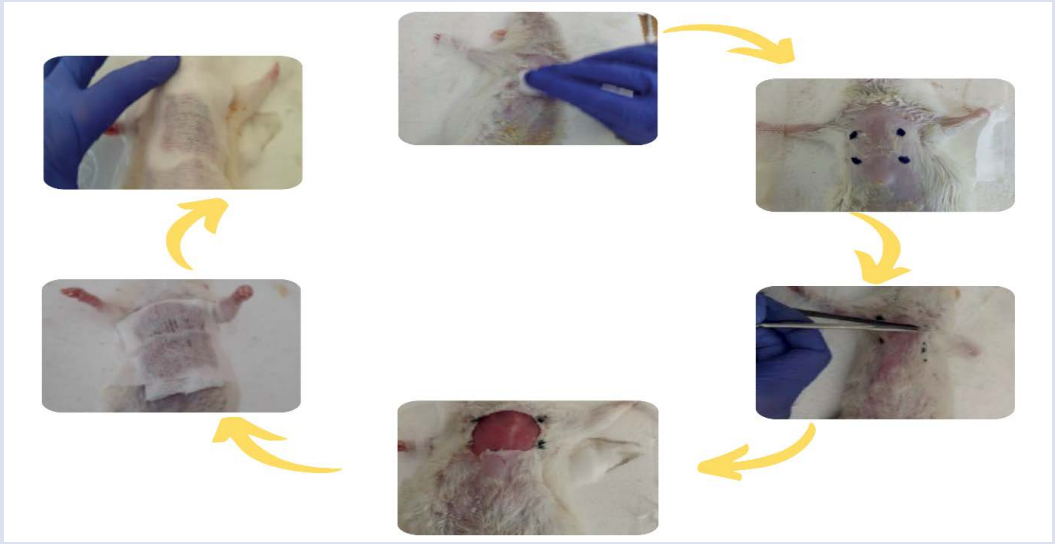


Figure 1: Wound creation

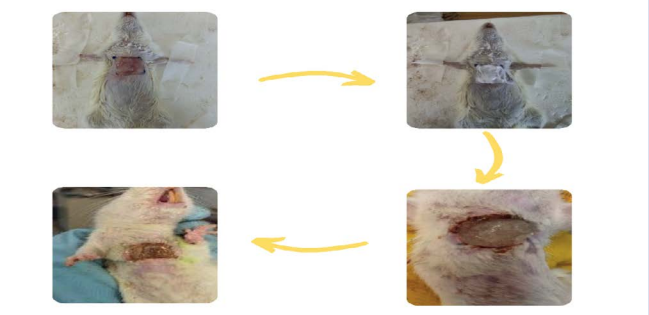


Figure 2: Treatment of rats

Figure 3: Wound tissue

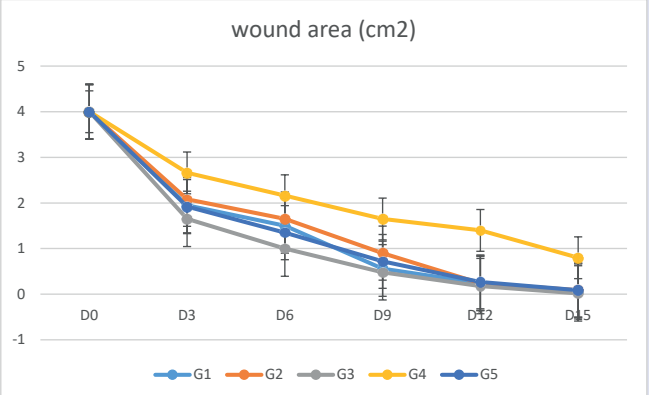


Figure 4: Evolution of wound area excisions at days 0, 3, 6, 9 and 12.

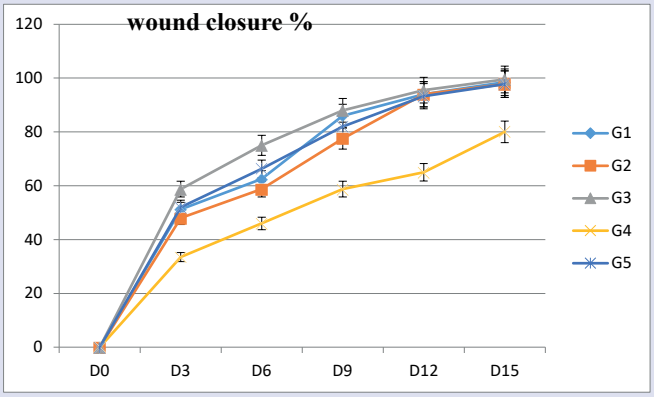


Figure 5: Percent closure of excisions at days 0, 3, 6, 9 and 12.

Similar to the control group, the gelatin hydrogel-treated wounds showed some necrosis at the epidermal edges, with granulation tissue containing inflammatory cells and congested blood vessels. In the group treated with chitosan and gelatin hydrogel, only a few inflammatory cells occupied the granulation tissue, while signs of neo-angiogenesis and fibroblast proliferation were visible in the epidermis.

In the Cicatryl-bio® group, the wound area was covered by regenerated epidermis, with granulation tissue infiltrated by inflammatory cells and congested blood vessels. In all groups, the granulation tissue was insufficient, and the wound areas remained concave.

Six days post- surgery

Moderate dermal inflammation persisted in the control wounds, though fibroblast proliferation and early signs of neo-angiogenesis were evident. Both the chitosan and gelatin hydrogel groups showed regenerated epidermis covering the wound area, with granulation tissue forming through fibroblast migration and proliferation. The group treated with chitosan and gelatin hydrogel exhibited the fastest wound healing, with nearly complete epidermal regeneration six days after surgery. Inflammation had subsided compared to the first three days, and granulation tissue had regenerated. In the Cicatryl-bio® group, a few inflammatory cells remained in the granulation tissue, and new blood vessels were forming in the epidermal layer.

Nine to twelve days post-surgery

In the untreated group, wound healing progressed more slowly, with wound closure taking more than 12 days. The wound area showed reduced size and was covered by epithelial tissue with initial keratin formation, though inflammatory cells were still present in the granulation tissue.

By day 11 post-surgery, wounds in the chitosan and gelatin hydrogel groups had healed faster than the control group and showed healing similar to the Cicatryl-bio® group. Most necrotic tissue in the gelatin-treated group had been replaced by new granulation tissue, and inflammatory cells were absent in the regenerated epidermis. In the group treated with chitosan and gelatin, granulation tissue without inflammatory cell infiltration was observed as early as day 9, with the wound area healing almost perfectly and granulation tissue showing a predominance of collagen fibers. Cicatryl-bio® treatment accelerated the healing process, achieving wound closure in 12 days, compared to the control group. No diffuse inflammatory cells were present in the granulation tissue.

DISCUSSION

Wound healing is a complex and dynamic process that is still not fully understood. It involves a series of biosynthetic and degradative reactions that occur in a coordinated and organized cascade of molecular and cellular events. This process includes inflammation, which plays a crucial role in preventing infection and initiating the proliferation phase necessary for tissue repair [14]. Inflammation, characterized by the rapid migration of neutrophils and macrophages to the wound site, must happen quickly to support the following stages: granulation tissue formation, fibrogenesis, neovascularization, wound contraction, and, in the later stages, epithelial resurfacing to close the wound [15,16].

Wound healing is an injury response focused on restoring damaged tissue. This process requires the precise coordination of connective tissue repair, re-epithelialization, and angiogenesis to form new tissue and close the wound. It also involves increased fibroblast proliferation and the production of various extracellular matrix proteins and growth factors [17,18].

Healing of full-thickness skin wounds occurs through the formation of granulation tissue, wound contraction, and epithelialization [19].

Epithelialization is achieved as undamaged epidermal cells from the wound edges migrate across the granulation tissue [20]. Supplementing with exogenous collagen has been shown to accelerate the migration of cells involved in skin wound healing. Due to its molecular nature, exogenous collagen integrates seamlessly with wound tissue, supporting endogenous collagen in vivo and promoting cell attachment, migration, and proliferation at the wound site [21,22].

Wound contraction serves as an indicator for monitoring the healing process, with wound area gradually decreasing as healing advances. The fibroblast, a key cell in this process, reaches its peak around day 7 post-injury and plays a crucial role in initiating angiogenesis, epithelialization, and collagen formation. In the control group, complete healing occurred within 28 days, resulting in a significant scar due to extensive contraction. In contrast, the β-chitosan and gelatin group achieved full healing within 14 days, showing minimal contraction and leaving a smaller scar compared to the control. Wound contraction refers to the inward movement of wound edges, facilitating closure after injury.

This process is driven by myofibroblasts, which contain α-actin derived from smooth muscle and are responsible for generating contractile forces within the granulation tissue at the wound site [23]. The wound healing rate is measured by the overall epithelialization of the wound bed, while wound contraction is evaluated based on the percentage of the original wound area that remains [24].

Moderate exudation was observed at the wound site up to day 6 in both chitosan-treated and control wounds, likely due to the inflammatory response triggered by surgical trauma. [25] reported a notable reduction in exudation in full-thickness wounds treated with small intestinal submucosa compared to untreated wounds in a rat model. Additionally, [26] found an inverse correlation between the survival area of skin grafts and the level of exudation in the graft bed. Other studies have also documented decreased exudate levels in full-thickness wounds treated with acellular matrices in rabbit and rat models as healing progressed [27].

The formation of healthy granulation tissue, closely linked to angiogenesis, plays a crucial role in wound healing [28]. Moderate levels of granulation tissue with new blood vessels were observed at the site of contact with the chitin sponge on days 7 and 14 following subcutaneous implantation of a sponge-like chitin in dogs [29]. Similar findings were also noted in group 3 of this study.

Chitosan is biocompatible, biodegradable, hemostatic, and anti-infective, and, most importantly, it accelerates wound healing [30]. Porous chitosan scaffolds offer a promising approach for tissue engineering applications [31]. In experimental animal models, chitosan has been shown to affect all stages of wound repair [32]. Its hemostatic properties are particularly evident during the inflammatory phase.

It also plays a role in modulating the migration of neutrophils and macrophages, contributing to repair processes such as fibroplasia and epithelialization [30, 31]. Derivatives of chitin and chitosan are well-tolerated and serve as effective adjuncts, showing significant potential for clinical applications [33]. These findings support our own observations that medical-grade, sterilized chitosan powder is a valuable biomaterial that accelerates wound healing. Histological analysis was performed to assess the biological response factors [34].

The wound healing process is influenced by several factors, including inflammation, angiogenesis, epithelialization, contraction, collagen synthesis, and remodeling. Numerous clinical and experimental studies have demonstrated the effectiveness of chitosan in promoting wound healing. In comparison to a previous histological study, our results show that beta chitosan hydrogel derived from *Sepia officinalis* accelerates wound healing. This has also been reported for alpha chitosan by various authors. Additionally, studies have indicated that the topical use of gelatin hydrogel can enhance the wound healing process. Our study further demonstrated that combining beta chitosan with gelatin hydrogel significantly accelerates wound healing, consistent with findings from Shamloo et al. [35].

Hydrogels contribute to wound healing by creating an optimal environment, reducing the risk of infection, and facilitating the absorption of exudate 2.

Furthermore, the rheological properties of hydrogels are crucial in determining their adhesive qualities and their effectiveness in skin regeneration [36].

ETHICAL NOTE

All experiments were conducted in accordance with Algerian law (Law No. 95–322/1995) on the protection of animals used for scientific and experimental purposes, following the guidelines of the Algerian Association of Experimental Animal Sciences (AASEA, authorization number 45/DGLPAG/DVA/SDA/14) for the ethical treatment of animals in research.

DECLARATIONS

Credit Authorship Contribution Statement

Amina DOUBBI BOUNOUA: Investigation, Methodology, Formal analysis, Validation, Writing -original draft; **Mokhtaria Yasmina BOUFADI and Soumia KEDDARI**: Conceptualization, Investigation; **Karima BOUGUEROUA**: participated in Animal experiments.

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Ethics approval

All rat experiments, were approved by the local animal care, Ethics committee (Abdelhamid ibn Badis University, Mostaganem, Algeria).

Declaration of competing interest

All the authors confirms that there is no financial or other conflicts of interest in this study.

Conflicts of Interest

The authors declare no conflicts of interest.

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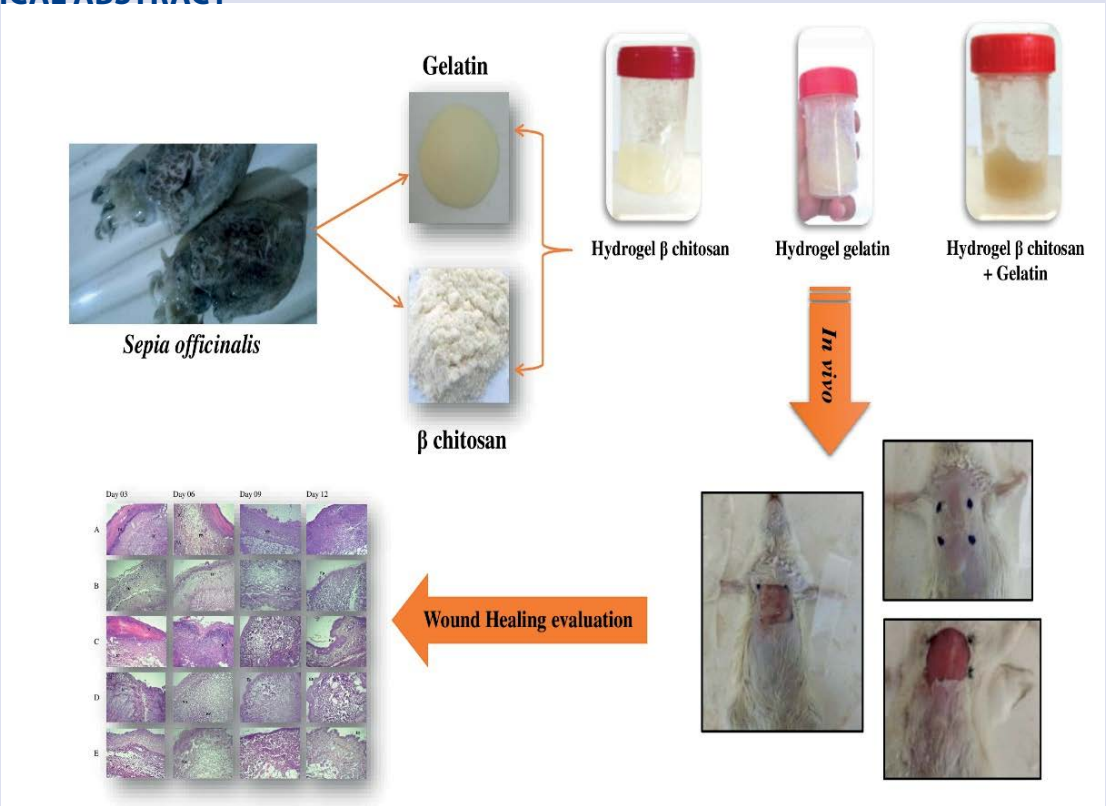
GRAPHICAL ABSTRACT



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