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## ORIGINAL ARTICLE

### Effect of Local Transplantation of Bone Marrow Derived Mast Cells (BMMCs) Combined with Chitosan Biofilm on Excisional and Incisional Wound Healing: A Novel Preliminary Animal Study on Lamb

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#### Abstract

**Objective:** To determine the effects of bone marrow derived mast cells (BMMCs) on excisional and incisional wound healing in an animal model on lamb.

**Design-** Experimental Study

**Animals-** Twelve healthy male lambs

**Procedures-** Animals were randomized into four groups of three animals each. In control animals, the created wounds were left untreated receiving 100  $\mu$ L PBS. In BMMC group, the created wounds were treated with 100  $\mu$ L BMMCs ( $2 \times 10^6$  cells/100  $\mu$ L) aliquots, injected into margins of the wounds. In chitosan group the created wounds were dressed with chitosan biofilm. In BMMC/chitosan group the created wounds were treated with 100  $\mu$ L BMMCs ( $2 \times 10^6$  cells/100  $\mu$ L) aliquots and dressed with chitosan biofilm. In excisional wound model, planimetric studies were carried out to determine wound area reduction. In incisional wound model, biomechanical studies were carried out to indirectly determine structural organization of the healing wound.

**Results-** BMMC/chitosan group showed significantly earlier wound closure compared to other groups ( $p=0.001$ ). The biomechanical findings indicated that the parameters were significantly improved in the BMMC/chitosan group compared to other experimental groups ( $p=0.001$ ).

**Conclusion and clinical relevance-** BMMCs local transplantation could be considered as a readily accessible source of cells that could improve wound healing.

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## 1. Introduction

The vast majority of research with animal models today is performed in rodents and, in particular, mice. Although there are many limitations due to the lack of inbred and genetically manipulated sheep, in addition to the lower availability of appropriate reagents, sheep have many features that make them suitable for a range of studies.<sup>1</sup> Their placid nature means that they can be housed in laboratory pens or metabolic cages, which enables repeated sampling of individual animals over extended periods, not normally possible in rodent models. Their size also facilitates the evaluation of surgical techniques and devices, or cannulation experiments for studying immune responses. However, their large size can also be problematic, with few researchers having access to suitable housing facilities, plus the increased costs associated with the purchase and feeding of sheep compared with rodents. Nevertheless, compared to other large animals (e.g. monkeys and dogs) often used in research, sheep are relatively inexpensive, and using them for research raises fewer ethical issues.<sup>2</sup>

While many studies have investigated the benefits of cell therapies in animal models, clinical applications of these technologies in the form of randomized controlled trials continue to be intangible.<sup>3</sup> Cell-based therapy is a promising branch of regenerative medicine. The idea of using cultured Schwann cells, bone marrow mesenchymal cells and adipose derived nucleated cell fractions may be an attractive alternative to more aggressive therapies. If successful, the treatment may lead to functional improvement as well as shortened recovery times, avoiding the hurdles of additional surgeries.<sup>4-6</sup> In cases of severe distortion of the tissue architecture, the healing process may not lead to morphofunctional normality but result in the formation of disoriented connective tissue with a fibrous appearance.<sup>7,8</sup> This abnormal tissue architecture reduces the mechanical strength and leads to scar formation.

Biomaterials can assist the proper physiological reconstruction of the skin and reduce or prevent scar tissue formation. Chitin, chitosan, and their oligomers have been found to promote wound healing, especially in the phases of proliferation and matrix formation.<sup>9</sup> Chitosan and its oligomers are well known for their interesting biological properties, which have led to various applications. Lysozyme slowly hydrolyzes chitosan membrane and produces chito-oligomers that stimulate correct deposition,

assembly and orientation of collagen fibrils in extracellular matrix components.<sup>10</sup> Moreover, it has been indicated that chitosan membrane stimulates the migration of inflammatory cells and promotes cellular organization.<sup>11,12</sup> Employment of regenerative properties of the cells at the service of wound healing has been initiated during recent decades.<sup>13,14</sup> Mast cells are fascinating, multifunctional, bone marrow-derived, tissue dwelling cells. They can be activated to degranulate in minutes, not only by IgE and antigen signaling via the high affinity receptor for IgE, but also by a diverse group of stimuli. These cells can release a wide variety of immune mediators, including an expanding list of cytokines, chemokines, and growth factors.<sup>15</sup> Mast cells have an armamentarium of inflammatory mediators interleukins such as IL-6 and IL-8, and growth factors, such as vascular endothelial growth factor, platelet derived growth factor and proteases that are released in degranulation.<sup>16</sup> As a result of extra cellular matrix degradation and changes in the microenvironment following initial mast cell secretion, the mast cell populations may change significantly in number, phenotype and function. There is, moreover, strong evidence that mast cells significantly influence angiogenesis.<sup>17,18</sup> It has been reported that the mast cell is an ideal candidate to play a pivotal role during wound healing, due to its location, substances released and clinical associations.<sup>19</sup>

These characteristics of the mast cells has encouraged us to conduct a study to assess local BMMCs therapy in site of created wound to observe whether the cells could be of benefit in wound healing in lam. The aim of the present study was a single local transplantation of bone marrow-derived mast cells after wound healing combined with chitosan biofilm dressing.

## 2. Materials and Methods

### *Study design and animals*

In this study, 12 healthy male lambs with an average weight of  $25 \pm 1.5$  kg and 3 months of age were randomized into four groups of three animals each. Attempts were made to minimize stress in the lambs at all stages of the study. The general health status of lambs was evaluated before and during the course of the study. The lambs were fed on standard hay and the water was *ad libitum*. Lambs were given two weeks of acclimatization and weight was recorded at the beginning and end of the study. They were

kept in separate cages and no ear tags were required. All experimental procedures were approved by the Advisory Committee of the Urmia University Research Council. None of the animals used in this study were killed.

### ***Pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM)***

Spleen cells from a donor rat were cultured at a density of  $2 \times 10^6$  cells/ml in RPMI 1640 medium containing 4 mM L-glutamine,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mM nonessential amino acids (complete RPMI1640) containing lectin (8 mg/ml) and placed in 75-cm<sup>2</sup> tissue culture flasks. The cells were incubated at 37-8° C in a 5% CO<sub>2</sub> humidified atmosphere. After 5–7 days, medium was collected, centrifuged for 15min at 3200 g, filtered through a 0.22 μm Millipore filter and used as PWM-SCM.

### ***Preparation of the bone marrow derived mast cells (BMMCs)***

Bone marrow of a donor male rat was used to generate mast cells based on a method described by others.<sup>20</sup> Briefly, the animal was anesthetized, euthanized and intact femurs were removed. Sterile endotoxin-free medium was repeatedly flushed through the bone shaft using a needle and syringe. The suspension of bone marrow cells was centrifuged at 320 g for 10 min, and cultured at a concentration of  $0.5 \times 10^6$  nucleated cells/ml in RPMI 1640 with 10% FCS, 100 units/ml penicillin, 100 mg/ml streptomycin (Life technology), 10 mg/ml gentamycin, 2 mM L-glutamine and 0.1 mM nonessential amino acids (referred to as enriched medium). PWM-SCM 20% [v/v] was added to the enriched medium. Flasks were then incubated at 37° C in a 5% CO<sub>2</sub> humidified atmosphere. Non-adherent cells were transferred to fresh medium at least once a week. After 3–4 weeks, mast cell purity of >90% was achieved as assessed by toluidine blue staining and flow cytometry.

### ***Staining of the mast cells***

The granularity of the mast cells was determined by toluidine blue, alcian blue and gimsa stainings. In brief, the cells were cytospun, fixed with Carnoy's fluid, and in Toluidine blue staining specimens stained by either 2 minutes with acid toluidine blue (pH=2.7). Cells were examined by light microscopy. Staining procedure was the

same for alcian blue staining on cytospun. Briefly, slides were incubated in 3% acetic acid, 3 minutes alcian blue solution microwave: Hi power, 30 seconds and washed in running water for 2 minutes, rinsed in distilled water and counterstained in nuclear fast red solution for 5 minutes, dehydrated, cleared and coverslipped.<sup>14</sup>

### ***Excisional wound model and planimetric studies***

The dorsum of lams was calipered and under local anesthesia and aseptic surgical preparation, one square, measuring 2×2 cm one side, approximately 15 cm ipsilateral to spine was outlined using a marker. Subsequently, the full-thickness demarcated areas of skin were removed by a scalpel. In control animals, the created wounds were left untreated receiving 100 μL PBS. In BMMC group, the created wounds were treated with 100 μL BMMCs ( $2 \times 10^6$  cells/100 μL) aliquots, injected into margins of the wounds (Figure 1). In chitosan group the created wounds were dressed with chitosan biofilm. In BMMC/chitosan group the created wounds were treated with 100 μL BMMCs ( $2 \times 10^6$  cells/100 μL) aliquots and dressed with chitosan biofilm. Photographs were taken immediately after wounding (day 0) and after 3, 7, 10, 14 and 21 days by means of a digital camera while a ruler was placed near the wounds. The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc, San Jose, California, USA) and wound closure percentage was calculated using the following formula:<sup>21</sup>

$$\text{Percentage of wound closure} = (A_0 - A_t) / A_0 \times 100$$

Where,  $A_0$  is the original wound area and  $A_t$  is the wound area at the time of imaging.



**Figure 1.** Representative photo shows injection of BMMCs ( $2 \times 10^6$  cells/100 μL) aliquots into margins of the wounds.

### ***Incisional wound model and biomechanical testing***

All animals of four groups were anesthetized as mentioned above and a paravertebral long incision of 6 cm length was made through the skin, contralateral to excisional wound in each group (Figure 2). After the incision was made, the wounds were sutured at 0.5 cm intervals with 3/0 nylon. The treatments were applied the same way in the excisional wound model. The skin samples were harvested on day 10 postoperatively and fixed between frozen fixtures in a mechanical apparatus. The TA.XTPlus Texture Analyzer mechanical test device was used for the assessment (Stable Micro Systems, Surrey GU7 1YL, UK). After 5 min, the frozen fixtures were tightened to ensure that no slippage occurred during testing. The initial length was set to 20 mm. Each sample was stretched at a constant rate of 1 mm/min. The load and displacement were sampled 5 times per second. Each sample was stretched to complete tensile failure. Samples were kept moist during testing using a drop of normal saline solution.

### ***Statistical Analysis***

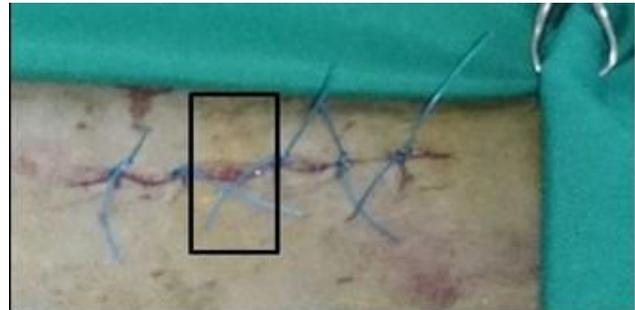
The Results were analyzed using repeated measures and a factorial ANOVA with two between-subjects factors and the Bonferroni test was used to examine the effect of time and treatments. Experimental results were expressed as means  $\pm$  SEM. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA) and the significant difference was set at  $p < 0.05$ .

## **3. Results**

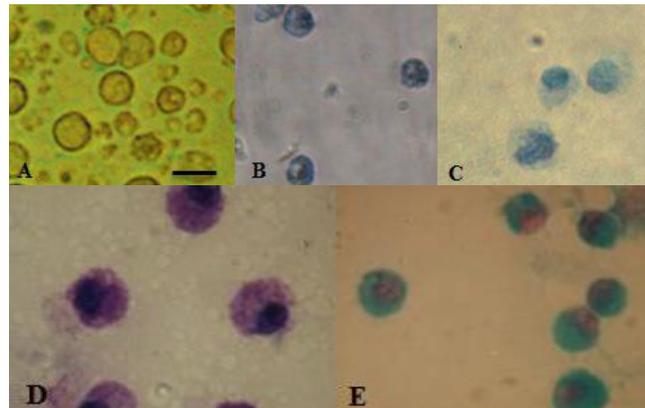
### ***Findings of mast cell staining***

Bone marrow stromal cells of the mice were successfully harvested and cultured. In the first culture of the cells both adherent and confluent cells were observed that were appeared as heterogeneous cells. Within the first week the adherent cells were observed as confluent cells. In contrary to other common culture media, the confluent cells could live longer. In the second passage, because of limited space in the smaller flasks (T25), the confluent cells were appeared densely and on days 18 and 19 the first culture cells were appeared more homogenous. A few dividing cells were also observed. Following 3 to 4 passages and change of the culture media on day 21, the cells were homogenous enough to be harvested (Figure 3A). The harvested cells were counted and their viability was

assessed using trypan blue with Neubauer method. From each flask 12,000,000 cells with viability rate of 90% were harvested. After centrifugation, the supernatant was discarded and the pellet was resuspended in a 1 mL culture media and spread on slides. The slides were air dried at room temperature. They were fixed using carnoy and stained using toluidine blue, alcian blue and gimsa stains. The granules of mast cells were purple to red where stained with toluidine blue. These cells were metachromatic. The granules were blue and the nuclei were red where stained with alcian blue and violet where stained with gimsa (Figure 3B-D).



**Figure 2.** Representative photo shows incisional wound model with black rectangle demarcation that shows the analyzed segment.



**Figure 3.** Bone marrow mast cells from rat were cultured in the medium during the third week of culturing bone marrow cells. (A) Isolated BMMCs, (B) Trypan blue staining for viability assessment, (C) Toluidine blue, (D) Gimsa and (E) Alcian blue staining. Scale bar: 10  $\mu$ m

### ***Findings of planimetric studies***

Wound closure percentage of experimental groups were measured and the findings are shown in Table 1. BMMC/chitosan group showed significantly earlier wound closure compared to other groups ( $p=0.001$ ). Furthermore, time had significant effect ( $p=0.001$ ) on wound closure of all wounds (Table 1, Figure 4).

### Findings of biomechanical testing

Analyses of biomechanical parameters including tensile strength, breaking load, maximum stored energy and

stiffness are presented in Table 2. The biomechanical findings indicated that the parameters were significantly improved in the BMMC/chitosan group compared to other experimental groups ( $p=0.001$ ).

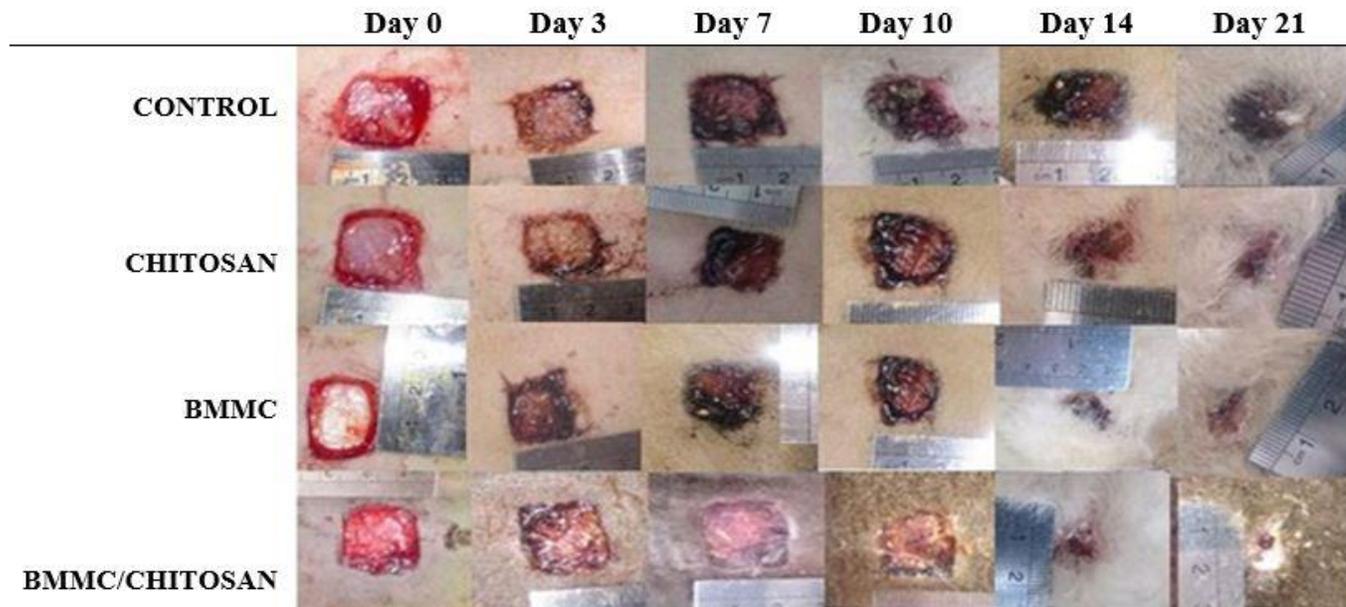


Figure 4. Serial photographs of wounds on different days in the experimental groups.

Table 1. Effect of adipose derived nucleated cell fractions combined with chitosan biodegradable film on circular excision wound contraction area ( $\text{mm}^2$ ). Values are given as mean  $\pm$  SEM.

Groups	Wound closure (%)				
	Day 3	Day 7	Day 10	Day 14	Day 21
Control	40.18 $\pm$ 1.45	34.25 $\pm$ 2.15	28.31 $\pm$ 2.15	20.51 $\pm$ 1.65	43.62 $\pm$ 1.12
Chitosan	28.26 $\pm$ 1.27	48.31 $\pm$ 1.43	29.43 $\pm$ 1.114	63.65 $\pm$ 1.38	71.82 $\pm$ 1.36
BMMC	43.23 $\pm$ 1.75	43.29 $\pm$ 1.66	25.34 $\pm$ 1.79	42.56 $\pm$ 1.25	19.73 $\pm$ 1.27
BMMC/chitosan	63.25 $\pm$ 1.17	75.34 $\pm$ 1.67	54.47 $\pm$ 1.75	62.75 $\pm$ 2.17*	37.92 $\pm$ 3.48*

\*The mean difference is significant at the .05 level vs control, chitosan, and BMMC groups

Table 2. Biomechanical parameters assessed for each of the experimental groups.

Groups	Biomechanical indices			
	MES (Kg/cm)	Stiffness (Kg/cm)	Tensile strength (Kg)	Breaking load (Kg)
Control	12.8 $\pm$ 1.14	13.3 $\pm$ 0.12	19.7 $\pm$ 0.72	12.8 $\pm$ 1.15
Chitosan	12.1 $\pm$ 0.65	58.4 $\pm$ 0.26	19.8 $\pm$ 0.14	23.9 $\pm$ 0.17
BMMC	17.9 $\pm$ 0.42	33.3 $\pm$ 0.06	24.6 $\pm$ 0.19	13.7 $\pm$ 0.75
BMMC/chitosan	27.14 $\pm$ 0.92*	68.7 $\pm$ 0.46*	14.11 $\pm$ 0.33*	12.03 $\pm$ 17*

Values are given as mean  $\pm$  SEM. MES: Maximum Stored Energy

\*The mean difference is significant at the .05 level vs control, chitosan, and BMMC groups

## 4. Discussion

The local transplantation of BMMCs improved wound healing and biomechanical indices in lambs. Several reports have demonstrated a beneficial effect of chitosan as a biologically active dressing in wound management. It has

been reported that the application of chitosan to the open wounds in dogs induced exudate, which has a high growth factor activity, and induced infiltration by inflammatory cells and granulation tissue formation accompanied by angiogenesis.<sup>22-24</sup> Chitosan-membrane-based wound products have been investigated both in laboratory animals

and humans, but are still at the early stage of development. Since 1980, chitosan and its derivatives have been used in skin and wound management products in Japan. Beschitin W, an artificial skin prepared from chitin threads, has been developed for human use and is on the market.<sup>25,26</sup>

In the present study local administration of BMMCs resulted in the enhanced biomechanical properties. To the best knowledge of the authors, the effects of local transplantation of BMMCs on wound healing and the biomechanical properties of healing skin in lamb have not previously been compared. Therefore, regarding the mechanical testing, the authors were not able to compare the results of the present study to other published studies. There are few studies on lamb using different materials in various wound models without dealing with biomechanical properties of the healing wound.<sup>13,27-29</sup> Most of the studies were focusing on the physico-mechanical aspects of leather than the healing wound.<sup>30-33</sup>

Analyzing the mechanical behavior of *ex vivo* skin can allow for a wide range of characteristics to be explored, which may not be possible *in vivo*. As the tissue sample can be removed, it is possible to conduct destructive tests to determine the failure mechanisms and tensile strength of the sample. Furthermore, the tissue layers can be separated and evaluated independently.

The most commonly used materials test performed on *ex vivo* skin samples is the tensile test.<sup>34</sup> Using this method, many have detailed the anisotropic nonlinear and viscoelastic behavior of skin, as well the effects under failure, creep, fatigue and preconditioning.<sup>35</sup> This approach has also been used to characterize the variations between animal donor groups with scarred and aged skin.<sup>36</sup> Another key advantage of using *ex vivo* skin tissue is that the effects of altering the skin's anatomical structure on the biomechanical behaviour can be evaluated. This approach has been used to vary the quantity of collagen, elastin and proteoglycans within the skin, as well the effects of blood flow and hormonal changes within rat and mouse models.<sup>37-40</sup> In addition to the benefits discussed, *ex vivo* mechanical testing of skin has vast implications for the development of new tissue engineered scaffold, as it provides a basis for comparisons between actual tissue and engineered structures.<sup>41</sup> However, examining the mechanical properties of skin *ex vivo* removes the tissue from the natural environment, thus removing the pre-stress and source of hydration, resulting in different mechanical characteristics when compared with *in vivo* analysis. Biomechanical analyses of the skin samples in this study revealed a significant improvement in tensile strength, breaking load, maximum stored energy and stiffness of healing skin in BMMC/chitosan group. This indirectly

indicated improved and sound structural organization of the healing wound.

Depending on the mast cell phenotype and stimulus, mast cells initiate the transcription, translation and secretion of a varied array of cytokines including PDGF, VEGF. It has already been shown that PDGF, VEGF Ffig.1bear beneficial effects on peripheral nerve regeneration.<sup>42-45</sup>

Mast cells have been proposed as angiogenesis promoters and the mast cell count appears to be a reliable prognostic marker in some tumors.<sup>46,47</sup> Mast cells cause neovascularization by producing angiogenic factors, such as VEGF, or substances with angiogenic properties, such as tryptase, FGF, TNF, interleukin (IL)-8, histamine and heparin.

Angiogenesis is a complex process governed by many different variables. Growth factors, including VEGF, platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), play important roles. Consequently, their generation within nerve conduits is vital to achieving positive clinical outcomes.<sup>48</sup>

This preliminary study was conducted to assess effects of *in situ* xenotransplantation BMMCs at the site of peripheral nerve injury. Since mast cells bear armamentarium of inflammatory mediators, the authors aimed to assess whether the BMMCs could positively affect the nerve repair process. The mast cells were introduced in the site of injury in the present preliminary study regarding this fact that changing the mast cell microenvironment alters significant changes in phenotype of the mast cells and they may act as growth factors packages that only degranulate *in situ* and do not induce inflammatory responses.<sup>19</sup> Study on proliferation and differentiation of the cells were not the case, as it remains to be studied in the future.

The major limitation of the present study was comparison of the cells with extracellular matrix, microtubules, fibroblasts, Schwann cells and other nerve segment constituents and conduits without giving the histological and molecular evidences for mechanism of action of BMMCs. This would be considered for further studies. Therefore, the authors stress that the current investigation was conducted to evaluate a single local dose and clinical treatment potential of BMMCs on wound healing and precise mechanisms of mechanism of action of BMMCs in transection models remain to be investigated.

The alteration in the behavior of mast cells could be favorable in cell therapy where readily accessible and instant source of cells in large quantities are required and could be taken into consideration in the emerging field of regenerative medicine and surgery. It could be considered clinically as a translatable route towards new methods to accelerate wound healing in clinical applications.

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## Conflict of Interests

There are no conflicts of interests to declare

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چکیده

تأثیر پیوند موضعی ماست سل‌های مشتق از مغز استخوان همراه با لایه نازک کیتوزان در ترمیم زخم برداشتی و برشی پوست: مطالعه اولیه جدید بر روی مدل حیوانی بره

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**هدف-** تعیین تأثیر پیوند موضعی ماست سل‌های مشتق از مغز استخوان همراه با لایه نازک کیتوزان در ترمیم زخم برداشتی و برشی پوست بر روی مدل حیوانی بره.

**طرح مطالعه-** مطالعه تجربی.

**حیوانات:** دوازده رأس بره نر سالم.

**روش کار-** حیوانات به‌طور تصادفی به چهار گروه سه‌تایی تقسیم‌بندی شدند. در گروه کنترل زخم ایجاد شده بدون دریافت هیچ‌گونه درمانی با بافر فسفات شستشو گردید. در گروه ماست سل‌های مشتق از مغز استخوان به حاشیه زخم‌ها ۱۰۰ میکرولیتر سلول به تعداد (۲×۱۰<sup>۶</sup> سلول/۱۰۰ میکرولیتر) تزریق گردید. در گروه کیتوزان زخم‌ها توسط لایه کیتوزان پوشش داده شدند. در گروه ماست سل‌های مشتق از مغز استخوان/کیتوزان به حاشیه زخم‌ها ۱۰۰ میکرولیتر سلول به تعداد (۲×۱۰<sup>۶</sup> سلول/۱۰۰ میکرولیتر) تزریق و زخم‌ها توسط لایه کیتوزان پوشش داده شدند. در مدل زخم برداشتی مطالعات پلانی متری به‌منظور تعیین مساحت زخم انجام گرفت. در مدل زخم برشی مطالعات بیومکانیکی به‌منظور نشان دادن ساختار بافت ترمیم‌یافته انجام گرفت.

**نتایج-** گروه ماست سل‌های مشتق از مغز استخوان/کیتوزان تفاوت معناداری از نظر جمع شدن زخم در مقایسه با سایر گروه‌ها نشان داد (P = 0.001). یافته‌های بیومکانیکی نشان داد که پارامترهای مورد مطالعه در گروه ماست سل‌های مشتق از مغز استخوان/کیتوزان در مقایسه با سایر گروه‌ها بهبودی معناداری را پیدا کرده بودند (p=0.001).

**نتیجه‌گیری و کاربرد بالینی-** پیوند موضعی ماست سل‌های مشتق از مغز استخوان همراه با لایه نازک کیتوزان را می‌توان به‌عنوان یک منبع سهل‌الوصول به‌منظور بهبود التیام زخم به کار برد.

**کلمات کلیدی-** التیام زخم، برشی، برداشتی، ماست سل‌های مشتق از مغز استخوان، بره