Coactivity of Mast Cells and Stem Cells on Angiogenesis and Antioxidants’ Potentials at Inflammation, Proliferation, and Tissue Remodeling Phases of Wound

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Abstract

Background Reactive oxygen species cause serious damage to the physiological function of tissues. Determination of total antioxidant capacity of skin tissue is one of the determinants of damaged tissue function. Mast cells (MCs) are one of the groups of cells that are invited to the site of injury. The healing process begins with the rapid release of various types of MCs’ intermediate factors at the site of injury. Bone marrow mesenchymal stem cell (BMMSC) production and secretion have been shown to regenerate the skin. The aim of this research was to evaluate the wound-healing and antioxidant effects of BMMSCs per MCs.

Methods Fifty-four albino Wistar male rats were divided into three groups: (1) nonsurgery, (2) surgery, and (3) surgery + BMMSCs. Groups 2 and 3 were operated with a 3 × 8 cm flap and in group 3, cell injections (7 × 10^9 cell injection at the time of surgery) were performed. After days 4, 7, and 15, percentage of the surviving tissue, histological characteristics, superoxide dismutase (SOD) activity, and amount of malondialdehyde (MDA) were measured in the groups. For results, Graph Pad Prism 8 software was used, and data were analyzed and compared by analysis of variance and Tukey test.

Results BMMSCs’ application decreased the amount of MDA, increased SOD activity and survival rate of the flaps, and improved the histological characteristics.

Conclusion This study revealed the protective effects BMMSCs alongside MCs against oxidative stress on the survival of the flaps. However, for clinical use, more research is needed to determine its benefits.

Keywords

► reactive oxygen species
► antioxidant
► superoxide dismutase
► wound healing

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The use of flap is a common procedure in plastic surgery.\(^1\) Flap is used to repair skin defects\(^2\) but in this surgery, restoring blood flow to ischemic tissue leads to tissue damage. This damage is ischemia–reperfusion (IR) injury.\(^3\) Following this damage, tissue necrosis and cell death occur. One of the mechanisms of this damage is oxidative stress, which damages cells and tissues. As blood returns to the tissue, neutrophils accumulate in the tissue. Neutrophils produce reactive oxygen species (ROS). This activated oxygen has detrimental effects on the mitochondrial membrane. As a result of a series of interactions, the mitochondrial membrane ruptures and eventually leads to cell necrosis. In addition, preapoptotic agents are released into the cytosol after the mitochondrial membrane ruptures, leading to cell apoptosis.\(^4\)–\(^8\) Many environmental stimuli catalyze the production of ROS, which may be involved in the pathogenesis of several skin disorders including flap necrosis.\(^7\) ROS induces several deleterious effects, such as deoxyribonucleic acid damage, inflammatory responses, and damage to the integrity of the extracellular matrix (ECM). Therefore, ROS production plays an important role in skin damage.\(^9\) Numerous studies have been performed to evaluate the effect of ROS using different therapeutic agents on IR phenomena, inflammatory reactions, skin diseases, and survival of skin flaps.\(^9\) These studies have shown that by reducing the rate of oxidative stress, both the rate of tissue necrosis and direct cell damage are reduced. Also, the rate of apoptosis (a type of cellular death) and apoptosis-promoting mediators is reduced.

At inflammation conditions, ROS enhances the secretion of cytokines\(^10\) and induction of matrix metalloproteinases (MMPs). MMPs are enzymes that destroy the ECM and the connections between endothelial cells.\(^11\) Also, ROS can directly or indirectly (activated by ECM proteolysis) alter or destroy proteins as well as disrupt skin fibroblasts and keratinocyte function.\(^12\) Oxidative stress and the production of ROS in tissues lead to tissue damage. The skin is no exception and if a way can be found to reduce the oxidative damage following (tissue loss of) the flap surgery, it can increase the survival rate of the tissue.

Using strategies such as preventing tissue necrosis can cause to reduce the production or elimination of ROSs as a cause of tissue damage and death. Managing ROS production by inflammatory mediators and increasing the antioxidant capacity of the tissue, and even the use of external antioxidants, can increase the survival of skin flaps.\(^13\)

One of the novel treatments for reducing the level of necrosis and improving the survival of flap is an application of stem cells.\(^14\) Bone marrow mesenchymal stem cells (BMMSCs) have a very high capacity and their transplantation in the wound matrix promotes angiogenesis and progress in the wound-healing process.\(^15\)

Different types of cells are involved in wound healing. One type of these cells is the mast cell (MC). MCs are specialized secretory cells of the immune system that originate from cluster of differentiation (CD)34\(^+\) / CD117\(^+\) bone marrow cell, and are present in large numbers in the skin.\(^16\) MCs are responsible for producing different intermediates. These intermediates are stored as premade in intracellular granules. The contents of these granules affect the repair stages of skin wounds as inflammatory intermediates.\(^17\) So, it is likely that if we can reduce necrosis and ROS by using MCs and BMMSCs, the survival of the skin flap will probably increase. On the other hand, studies have shown that due to oxidative stress conditions and reduced stem cell life, the focus on MC characteristics is increased. These cells are able to act under stress conditions as a regulator of the environment; mutations have been observed in some MC genes that damage due to oxidative stress and cannot be controlled and regulated. Therefore, the presence of two skin-tissue-supporting cells in the healing process promotes the wound-healing process, both of which are sensitive and vulnerable to oxidative stress. MCs release polypeptide growth factors including basic fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-\(\alpha\)), transforming growth factor beta (TGF-\(\beta\)), and interleukin (IL)-8. These cytokines are involved in normal as well as tumor-associated angiogenesis.\(^18\)

In this paper we assess the cellular and molecular role of MCs in the three major stages of wound healing (inflammation, proliferation, and regeneration). Also, we assess therapeutic strategies for improving wound healing by using the effects of MCs and BMMSCs on oxidative stress and necrosis of flap tissue. The present study showed the protective effects of stem cells alongside MCs on oxidative damages and histopathological changes induced by flap surgery in rat’s skin tissue.

**Methods**

**Preparation of Animals**

This study was performed according to the “guidelines for taking care of and using laboratory animals” prepared by faculty of medicine at Lorestan University of Medical Sciences (LUMS.REC.1396.254).

In this research, 54 albino Wistar male rats weighing 250 to 300 g were prepared from Elmbavaran Aftab Co., Lorestan, and tested. All animals had free access to vital facilities as well as food and water throughout the entire experiment. They were then categorized into three groups: (1) nonsurgery, (2) surgery, and (3) surgery + BMMSCs. Groups 2 and 3 were operated with a 3 × 8 cm flap and in group 3, cell injections (7 × 10\(^7\) cell injection at the time of surgery) were performed. The first group, control group (IS), did not have any surgery and completely healthy skin was examined. The animals of the third group (WH + C) and the second (WH), the animals of this group underwent flap surgery. But the animals of the third group cells also received; the wound healing process was also examined. On the surgery day (day zero), the animals in all experiments were anesthetized using ketamine 50 mg/kg and xylazine 5 mg/kg. Then the site of surgery was shaved using a Moser and Gillette before creating the flap. Next, in the WH C and WH groups, and using scalpel, random skin flap with dimensions of 3 × 8 cm was created on the back of animals, whose base was proximal...
to the animals' tail. In creating the wound, all skin layers had been elevated with panniculus carnosus muscle, such that there would be no attachment, even vascular attachment, except in the base region. Next, the tissue was elevated and returned to its original position and sutured (► Fig. 1). Before flap tissue placement in the WH+C group, $7 \times 10^9$ cells with a volume of 0.5 mL were injected at several points in the wound. After the surgery, the animals were monitored and controlled clinically and for any evidence of inflammation or bleeding. Then, the animals in all groups were sacrificed on days 4, 7, and 15 through inhaling chloroform during deep anesthesia, whereby biopsy was taken from the region of the flap between necrotic (limited by the yellow line) and survival on the red arrow for histology study (six samples at every time point).¹⁹

Clinical Investigation
On days 4, 7, and 15 postoperation, some images were taken using digital camera at the site of flap. The survival of the improved region, which was free of inflammation and necrosis, was investigated and further controlled and evaluated against the characteristics of the control group flap. At every time point of imaging, Image software (St. Peterborough, NH) was used for measuring the percentage of survived regions in relation to the entire surface of flaps.¹⁷

Evaluation by H&E Staining
For histopathological evaluation on days 4, 7, and 15, a $1.0 \times 1.0$ cm sample of tissue was obtained from intermediate zone of each flap. The specimen was immersed in 4% paraformaldehyde, and after $\sim 24$ hours the tissue was completely wrapped in paraffin and by microtome cut into 5-µm-thick sections for detection of histological indicators.

The paraffin sections were stained with hematoxylin and eosin (H&E). The histological characteristics of the flaps were examined under an optical microscope. Skin flap capillary angiogenesis was assessed. The number of new blood vessels was counted in six randomly selected high-power fields ($200 \times$). Then, we counted the number of microvessels per unit area ($/\text{mm}^2$), which was taken as the microvessel density.²⁰

Evaluation of Platelet Endothelial Cell Adhesion Molecule (Cluster of Differentiation 31, CD31) by Immunohistochemistry Technique
We used the same H&E dyeing method to make the slices, baked them in the oven at a constant temperature of 60°C, and then removed the slices from the oven and placed them in xylene and then in ethanol with a gradient concentration to hydration, digestion, and repair of antigen. After cooling naturally at room temperature, according to the instructions of the immunohistochemistry kit, we added antibodies to the slices and then observed the slices under a microscope.²⁰

Determining the Number and Extent of Activity of MCs
To assess the number of MCs and the extent of activity of these cells (degranulation), a semi-quantitative analysis was performed. When counting, the total number of MCs that were observable in different forms was determined on the skin cut. Next, the ratio of granulated MCs and degranulated MCs was counted in each of the different views through 200-µm magnification. (All images have been recorded using Leica microscope in the major magnification of 100, 200, or 400µm.)²⁰
Tracking Cells In Vivo
For short-term tracking of the cells transplanted inside the body, BMMSCs (IBRC-C10163), purchased from Genetics Reserve Center of Iran, were used. For the tracking, after transplantation in the tissue, the cells were labeled through CM-Dil (Invitrogen, Carlsbad, CA) based on the manufacturer’s instructions. Specifically, BMMSCs were incubated for 2 hours and injected in the flap on day zero of surgery. They were then observed under fluorescent microscope after sampling on predetermined days.20

Biochemical Analysis
The frozen samples from flap tissue at –80°C were homogenized in phosphate-buffered saline and centrifuged at 5°C. The supernatant was used for the analysis of malondialdehyde (MDA) and superoxide dismutase (SOD). The amount of flap tissue SOD enzyme was determined according to the method of Kakkar et al.21 To evaluate lipid peroxidation, a commercial kit for MDA was used (Asan, Khorramabad, Iran).22

Statistical Analysis
For statistical analysis of the data, Statistical Package for the Social Sciences, version 21, was used. Based on analysis of variance (ANOVA), the difference between the means of the study groups was evaluated. Also, post hoc test (Tukey) indicated the place of difference between the study groups, with p < 0.05 considered statistically significant.

Results
The Therapeutic Effects of Stem Cells for the Skin Flaps
The survival of skin flap was investigated in the rats undergoing treatment with BMMSCs or without treatment on predetermined days. The images of the skin flaps at different time points are shown in – Fig. 1A. In WH group, in the initial stages postsurgery, the skin had unsuitable status, and the level of skin survival calculated as the percentage of the survived region in relation to the entire surface of the skin flap showed low level of survival. This could suggest severe ischemia that had resulted in necrosis of the skin flap tissue and was associated with a poor clinical outcome. In the WH+C group, with BMMSCs, a better therapeutic outcome was observed for the skin flap tissue survival. Eventually, in all of the three phases of wound survival— inflammation (day 4), proliferation (day 7), and regeneration (day 15)—a significant difference was observed in the percentage of flap survival between WH+C and WH groups, where the p-values were as follows: p = 0.04, p = 0.02, and p = 0.04, respectively ( – Fig. 1B). Flap survival rates were as follows—day 4: 56.312 ± 1.98 and 68.215 ± 2.07; day 7: 76.812 ± 2.528 and 83.567 ± 1.3; and day 15: 78.1 ± 1.405 and 88.731 ± 1.35 in WH and WH+C groups, orderly.

Evaluation of H&E Staining Results
According to the results, the number of vessels in the inflammation phase on day 4 differed significantly between the study groups (p = 0.004), as found in the Tukey post hoc test ( – Fig. 2). Also, the difference in the number of vessels in the proliferation phase on day 7 was significant between the WH+C and WH groups (p = 0.03). Further, for the number of vessels in the regeneration phase, day 15, the difference between WH+C and WH groups was significant (p = 0.04). With these results, it can be stated that regeneration of vessels in both WH and WH+C groups was observed in the middle of the flap on day 7. The number of vessels in the wound on day 7 reached its maximum in WH+C rats, and then diminished considerably on day 15. On the other hand, the number of vessels in WH rats increased constantly. This increase for WH rats was sustained until the end of experiment on day 15. In both groups, angiogenesis occurred in the proliferation stage, but this value was lower in WH rats on day 7 ( – Fig. 2).

Evaluation of Immunohistochemical Results
On day 4 of the observation, we randomly selected six slices of the flap samples in each rat group for immunohistochemical staining, and measured the content of CD31 in the peripheral areas of the blood vessels, calculated the integral optical density of each slice, and then finally obtained the average values. The results showed that the average integral optical density of WH+C group was higher than that of the other two groups (WH+C group compared with the IS and WH groups), that is, p < 0.01, with statistical significance; and when WH+C was compared with WH group, p < 0.001, with higher statistical significance ( – Fig. 2).

The Number and Extent of Activity of MCs
Comparing the total number of MCs on day 4, according to the results of ANOVA, a significant difference was observed between the study groups on this day (p = 0.004). Comparison of the total number of MCs between the intact tissue (IS group) and WH (p = 0.004), and between the WH+C and WH (p = 0.003) groups showed a significant difference.
Comparing the extent of activity as degranulation of MCs on day 4, based on the results of the ANOVA, a significant difference was observed in the activity number of MCs between the study groups on this day (p = 0.001). Comparison of the extent of activity of MCs in the healthy tissue of IS and WH (p = 0.001) and between the WH+C and WH (p = 0.002) groups showed a significant difference.
In the flap wound in WH+C rats, the total number of MCs decreased significantly on day 4, and began to rise on day 7 deep in the dermis. On day 15, reduction in this group was observed. In the flap wound in WH rats, the total number of MCs increased on day 4, but it diminished on day 7. Finally, on day 15, it was close to the level of inflammation phase, day 4. A relatively low number of MCs was observed in the healthy tissues of IS rats ( – Fig. 3D).
The activity of MCs evaluated as degranulation of these cells increased throughout the entire flap wound of WH+C rats on days 7 and 15. However, on day 15, the regeneration and vascular deterioration increased. Meanwhile, in WH rats, the extent of activity on day 7 remained very low throughout the experiment, and this value was lower than days 4 and 15, representing the inflammation and regeneration phases for this.
Also, in comparing the extent of activity of WH and WH\(^+\)C groups, on days 7 and 15, a significant difference was observed, where the p-value on days 7 and 15 was reported as 0.02 and 0.01, respectively (\(\text{Fig. 3C}\)).

**Tracking the Cell Inside the Body**

On day 7 post-BMMSC transplantation, we were able to track the transplanted cells in the skin flap animal model. Fluorescent microscopy investigation of BMMSCs showed CM-Dil label alongside blood vessels of the flap tissue (\(\text{Fig. 4}\)).

**Biochemical Findings**

The MDA values were significantly reduced in the WH\(^+\)C group in comparison with WH and IS groups on day 7 (\(p < 0.0043\)). On day 15, the MDA levels in WH\(^+\)C group significantly decreased compared with WH and IS groups (\(p = 0.002\)). The SOD levels were significantly higher in the WH\(^+\)C animals compared with WH and IS groups on day 7 (\(p = 0.002\)). The SOD levels in WH\(^+\)C group significantly decreased compared with WH and IS groups on day 15 (\(p = 0.0045\); \(\text{Table 1}\)).

**Discussion**

In biological systems, the normal processes of oxidation produce highly reactive free radicals, which may continue to damage cells. Antioxidants play a housekeeping role, scavenging free radicals before they get a chance to do harm to the...
Increased ROS and oxidative stress occur frequently in malignancies and myeloproliferative diseases. PARK7, a multifunctional protein with both antioxidant function and cytoprotective activity against oxidative stress, increases with cancer progression, although the mode of PARK7 regulation has not been studied in detail. Here, we investigated the regulation of type of MCs in relation with ROS and wound healing. PARK7 levels were reduced in systematic mastocytosis with low MC burden but, in marked contrast, increased in patients with high MC burden or with advanced disease.

In addition, subtypes of SOD are expressed and secreted from stem cells. BMMSCs have a very high capacity and their transplantation in the wound matrix improves wound healing. Recent evidence has supported the protective role of BMMSCs against skin oxidative damage, most of which is mediated by secretory factors. The antioxidant function of mesenchymal stem cells was first demonstrated in dermal fibroblasts after inducing chemical oxidative stress by tert-butyl hydroperoxide. Flaps are commonly used in plastic surgery and for repair of skin defects, but in this surgery, restoring blood flow to ischemic tissue leads to tissue damage (IR injury) and necrosis. One of the mechanisms of this damage is oxidative stress. Therefore, stem cells can be used to increase survival in a skin flap. This study shows an increase in flap survival with: (1) the presence of MCs with stem cells, (2) improvement in angiogenesis, and (3) reduction in oxidative stress.

- MCs secrete substances that can inhibit the growth of keratinocytes and re-epithelialization in vitro and play a role in the homeostatic regulation of the epidermis.

Therefore, we expect that with less total number of MCs in the WH·C group, the survival of the flap will be higher than in the WH group. Therefore, we counted the number of MCs in the flap using toluidine blue staining. This study showed that wound healing and re-epithelialization in the middle of the wound in the flap were significantly different between WH and WH·C groups. Specifically, the number of MCs increased significantly in the flap wound in WH·C rats in the proliferation stage. This suggested that MCs are involved in improving wounds at the proliferation stage. Also, the number of MCs in the middle of the flap wound diminished in WH·C rats on day 4, and it was lower than the control groups. Although the mechanism is unknown, application of MC precursors to wounded regions may be activated in the late stage of wound healing after skin surgery.

The notable point is that the number of MCs in the damaged skin decreases in the primary stage, and falls after improvement in the late stage of wound healing. MCs are known to participate in three phases of wound healing: the inflammatory reaction, angiogenesis, and ECM reabsorption. The inflammatory reaction is mediated by released histamine and arachidonic acid metabolites. Growing evidence on wound healing indicates that wound repair is associated with an increased number of MCs purposefully located around blood vessels. Activated MCs control the key events of the healing phases: triggering and modulation of the inflammatory stage, proliferation of connective cellular elements, and final remodeling of the newly formed ECM.
Angiogenesis and Antioxidants at Wound Healing

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formed connective tissue matrix. MC hyperplasia is found in different pathologies such as chronic inflammatory processes, fibrotic disorders, wound healing, or neoplastic tissue transformation. BMMSC paracrine factors together with the number and periodic activity of MCs seem to be effective in the three main stages of skin wound healing. Specifically, angiogenesis, in addition to the effect of the growth factor released such as TGF-β1 from granules of MCs in a site of the wound, alongside exogenous paracrine factors such as BMMSCs’ transplantation can better represent the granulated tissue. These conditions are important in the proliferation stage and vascular deterioration in the regeneration phase. In our study, the number of MCs was lower in the inflammation phase in the group receiving cell, while in the proliferation phase, this number increased for stimulating angiogenesis alongside BMMSCs. However, the number and extent of MCs in the regeneration phase (day 15) of the wound-healing process decreased. Also, along with the deterioration of blood vessels, overcomes angiogenesis and causes transition of the tissue from granulated tissue to a tissue without vessels and rich in collagen. New blood vessels are required at the site of wound to increase blood perfusion and promote tissue healing. The course of re-epithelialization and survival of flap area were different in our study between WH and WH^C rats. Since MCs increase considerably in the inflammation and regeneration stages after damage, studying the role of MCs in this model of skin wounds has many advantages. Also, the activity of MCs in the damaged skin increased constantly in the WH^C rats.

- Since MCs aggregate in the vicinity of blood vessels and produce several strong angiogenesis stimulating factors such as FGF2, VEGF, and TGF-β1, hence MCs are involved in angiogenesis. Angiogenesis is an important factor in the survival of the skin flap, so we counted the number of vessels using H&E staining. The present study indicated angiogenic response to the wound damage in the proliferation stage of wound healing was weaker in WH compared with WH^C rats. The activity of releasing their granulation contents peaked on day 15 postsurgery in the wound tissues of WH^C rats. This finding suggested that factors such as TGF-β1, which is active in the skin wound of WH^C rats, may reach an adequately high concentration and suffice to inhibit angiogenesis during late regeneration. In addition, MCs are a powerful modifier for ECM; on the other hand, the compounds and environmental conditions of ECM are important factors for regulating angiogenesis. Overall, these findings suggested that MCs can play a key role in regulating the matrix reorganization and affect the delicate balance between proangiogenic and antiangiogenic factors in the wound tissue.

Antioxidants are one of the factors in advancing the wound-healing process that play a protective role against oxidative damage. The wound-healing process can be affected by increased oxidative damage. Due to the activity of antioxidants at the site of injury, it creates the right conditions for the wound to continue to heal. The return of blood and oxygen to ischemic tissue leads to IR damage. The production of oxygen-free radicals plays a role in causing this damage. During this oxidative stress, oxygen destroys lipids in cell membranes, resulting in cell death. The level of oxidative stress is determined by MDA (the product of lipid peroxidation). The body produces antioxidants such as SOD to repair damage when responding to oxidative stress. So, we measured the levels of MDA and SOD. In a recent study, the enzyme level of SOD in the cell-receiving groups was higher than the other groups tested, which confirms the antioxidant effect of BMMSCs. These findings suggested that factors such as TGF-β1, which is active in the skin wound of WH^C rats, may reach an adequately high concentration and suffice to inhibit angiogenesis during late regeneration. In addition, MCs are a powerful modifier for ECM; on the other hand, the compounds and environmental conditions of ECM are important factors for regulating angiogenesis. Overall, these findings suggested that MCs can play a key role in regulating the matrix reorganization and affect the delicate balance between proangiogenic and antiangiogenic factors in the wound tissue.

### Table 1
Comparison of the activities of MDA and SOD (mean ± SEM) in the tissue samples taken from three groups (six samples from each group) on days 7 and 15

| Variable name | MDA (nmol/mg protein): Day 7 | SOD (U/mg protein): Day 7 | MDA (nmol/mg protein): Day 15 | SOD (U/mg protein): Day 15 | p-Value |
|---------------|-------------------------------|---------------------------|--------------------------------|----------------------------|---------|
| IS            | 0.086 ± 0.020                 | 0.39 ± 0.06               | 0.093 ± 0.15                   | 0.73 ± 0.04                | 0.0043  |
| WH            | 0.079 ± 0.026                 | 0.93 ± 0.08               | 0.084 ± 0.022                  | 0.065 ± 0.029              | 0.002   |
| WH^C          | 0.073 ± 0.026                 | 6.94 ± 0.20               | 0.76 ± 0.05                    | 2.34 ± 0.23                | 0.0045  |

Abbreviations: IS; rats with intact skin; MDA, malondialdehyde; SEM, standard error of mean; SOD, superoxide dismutase; WH, rats with wound-healing access through flap surgical induction; WH^C, rats with wound-healing access by flap surgical induction ^C^ cells.

Fig. 4 Presence of stem cells labeled with CM-DiI, alongside the blood vessels on day 7 following flap surgery (scale bar = 50 µm).
findings indicate that the level of SOD is increased in the cell-receiving groups, which is due to the stimulation of SOD production due to the presence and antioxidant properties of BMSCs. Moreover, another protein factor, MDA, which is an important cause of tissue stress, was found to be lower in cell-receiving animals. In several studies, different cells have been used to treat wounds. In these studies, MDA and TNF-α were significantly reduced, indicating an increase in SOD and catalase (CAT) activity in response to oxidative stress. Although the present study showed a promising effect of BMSCs with MCs, through total antioxidant capacity modulation, on wound healing in rats, it is necessary to examine the data on the molecular mechanism finding from this result. The function of MCs is not provided, which can be considered as a limitation of this research.

Author Contributions
M.M. and K.A. performed research. M.N. performed research and wrote the paper. A. K. analyzed and interpreted the data. C.F. was involved in conception and design, critical revision, and final approval of the version to be published.

Animal Experiment Care Approval
This study was performed according to the “guidelines for taking care of and using laboratory animals” prepared by the faculty of medicine at Lorestan University of Medical Sciences (LUMS.REC.1396.254).

Prior Presentation
Our article is taken from a student thesis (code 493) submitted for the award of doctoral degree: Doctoral dissertation by Mr. Ahmad Khanifar.

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Conflict of Interest
None declared.

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