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Histological Evaluation of Different Types of Mastocytes in the Skin Flap Using Bone Marrow Mesenchymal Stem Cells Through Biological Growth Factor

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ABSTRACT

BACKGROUND AND OBJECTIVE: Skin flap is one of the most commonly used methods in plastic surgery. Postoperative skin flap necrosis is one of the complications of flap skin. The aim of this study was to evaluate the effect of bone marrow mesenchymal stem cells (BM-MSCs) and chick embryo extract (CEE) on mast cells in a randomized skin flap in rats.

METHODS: In this experimental study, 40 male albino Wistar rats weighing 250 – 300 g were divided into four groups of 10 (control, CEE/BM-MSCs, CEE and BM-MSCs). Skin flap (30 × 80 mm) was created behind the animals. Surgery was performed on day zero and therapeutic intervention was done on the same day. Mesenchymal stem cells were extracted from rat bone marrow and were injected. CEE was prepared from a 9-day-old embryo of Marandi chicken. On the seventh day after the surgery, samples were assessed in term of type, and the total number of mast cells (type 1 to 3) in the transfer line.

FINDINGS: The difference between the mean number of mast cells type 1 (3.67±1.91) (p=0.99), and type 3 (1.9±1.47) (p=0.384) was not significant in the study groups, but was statistically significant in type 2 (2.27±1.42) in different study groups (p=0.005). There was also a statistically significant difference between the mean total number of mast cells (2.32±0.84) in the BM-MSCs group and other experimental groups (p=0.001).

CONCLUSION: Based on the results of this study, increase in mast cell type 2, the improvement of small vessels and decrease in mast cell type 3 lead to the reduction of scarring and fibrosis.

KEY WORDS: *Biological Growth Factor, Mast Cells, Flap Surgery, CEE*.

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Introduction

One of the types of plastic surgeries is the Random Skin Flap (RSF). The flap is the most commonly used surgical treatment to treat wounds caused by burns, trauma and congenital defects (1, 2). However, incomplete or complete skin flap necrosis is one of the common problems of this type of surgery. In fact, necrosis is caused by an ischemic injury after surgery (2). Managing necrosis treatment is usually time-consuming and requires changing the wound dressing, or multiple surgeries (3).

The wound healing process is complicated and all stages of recovery are cascaded. The inflammation process is one of these steps, in which leukocytes, components of the complement system, mast cells, and immune complexes are involved (4).

Several strategies exist to reduce the healing process of wounds, including strategies for the presence of stem cells and growth factors that enhance the healing process of the wound. Significant growth factors in the repair process include: Transforming growth factor (TGF), vessels endothelial growth factor (VEGF), platelet-derived growth Factor (PDGF), basic fibroblast growth factor (BFGF) and endothelial growth factor (EGF) (5). Bone marrow mesenchymal stem cells (BM-MSCs) specifically improve the process of repairing skin ulcers, and due to their great flexibility and the ability to secrete growth factors are used as a suitable source for cell therapy in most clinical models (6).

The link between BM-MSCs improves the process of repairing diabetic wounds in rats (7). It seems that despite the positive effect of mesenchymal cell growth factors and the proper response in the wound microcontroller, the presence of exogenous growth factors can increase this effect. Chicken Embryo Extract (CEE) is a rich biological source of growth factors (8).

CEE contains many growth factors, and after fertilization, eggs become complete chicken in only twenty-one days. By adding CEE to the cell culture medium, the growth rate of various cell types increases. In addition, certain growth factors have been identified in the CEE, which are steadily increasing and provide the combined needs of cell growth in the culture medium (9,10).

The presence of CEE can increase spontaneous cell growth and alter cell morphology and cause changes in cell gene expression (9). Angiogenesis, as a delay mechanism in the healing process of the wound, causes recovery with an increase in the number of blood vessels, which itself increases the perfusion in the flap (11). There are several molecules and cells that play an important role in the wound healing process. Previous studies have pointed to the important role of mast cells in restorative processes (12). Mast cells are settled in the skin tissue. Mast cells are known as a key factor in inflammation and wound healing.

These cells contain a variety of growth factors and cytokines, which increase the number and degradation of these cells, accompanied by high expression of VEGF and eventually associated angiogenesis (13). Mast cells are involved in three major phases of wound healing, including: inflammatory reaction phase, angiogenesis, extracellular matrix uptake and reconstruction.

Much evidence suggests that mast cells play an important role in the process of angiogenesis, stimulation and regulating the growth and migration of endothelial cells and fibroblast migration (14). This study aims to investigate the effect of BM-MSCs and CEE, alone and in combination, on mast cells, degranulation of mast cells, and all types of mast cells 1, 2 and 3 in a RSF model among rats in experimental group compared to control groups.

Methods

This experimental study was approved by the Ethics Committee of Lorestan University of Medical Sciences (LUMS.REC.2016.147 – 23.8.2016), and conducted in two in vivo and in vitro steps.

In vitro environment

Extraction and marking of the cell: BM-MSCs were extracted from the femur of rats weighting 250 – 300 g. Animals were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). After disinfection of the site using surgical instruments, bone marrow contents were extracted from the bone marrow cavity using a 5cc syringe, 18G needle tip, using aspiration method. The contents were centrifuged and the bone marrow-derived cells were suspended in Dulbecco's Modified Eagle Medium (DMEM) and BM-MSCs were isolated using Ficoll-Paque density gradient (15).

In order to evaluate and validate the surface markers of mesenchymal stem cells, they were sent to the flow cytometry in Iran's Genetic Resources Center. In order to detect cells after transplantation in tissue, similar to previous research, they were incubated with

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CM-DiI fluorescent material (1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate Molecular Probes) at 37 °C for 15 minutes for marking (16,17). Then, the cells marked in 0.5cc DMEM were stored and on the day of cell transplantation surgery (day zero), 7×10^6 of these cells were injected to the experimental groups in 12 equal points on the left and right sides of the flap.

CEE Preparation Method: CEE was prepared from a 9–12–day–old embryo of the Marandi eggs from the Institute of Biological Sciences. Until performing the experiment, the eggs were kept in an incubator at 37 °C and 21% oxygen. After obtaining sterile conditions, the embryo was first scraped into small pieces and then 1ccDMEM was added to per 1 mg of embryo weight. It was then poured into a falcon and placed on the agitator for 2 hours at room temperature. The falcons were then transferred to a – 80 °C freezer within 24 hours and the next day, the falcons melted at room temperature and were centrifuged several times (at 2400 rpm and for 15 minutes) until the contents of the falcon were completely transparent.

The contents were then passed through a 50 cc syringe and stored in a freezer at a temperature of –20 °C until the time of use. Before use, CEE melted at room temperature and was passed through a filter with 0.45 µm pores. This was done for sterilization of the extract and on the day of surgery (day zero), it was injected on the flap at 1 cc in 12 points of the distal area toward proximal flap.

In vivo environment

Experimental design: 40 Albino Wistar male rats weighing 250 to 300 g were divided into four groups of 10: control, CEE, BM-MSCs, and CEE/BM-MSCs. Rats were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg).

Creating a random skin flap model: The skin of the area behind the rats was shaved, was disinfected with a betadine solution and was prepared for RSF creation. In this model, the entire skin thickness and skin muscle (Panniculus carnosus) is removed. To measure the dimensions of the flap, the iliac bone and scapula were measured and a 30×80 mm flap was made, and was then sutured using 0.4 nylon thread and Separate Model. The day of surgery was considered day zero and therapeutic interventions were done on this very day (18).

Injection of cell, extract or saline was done from the distal region toward the proximal flap, i.e. the base of the flap (Fig. 1).

Clinical evaluation: Flap tissue was evaluated in terms of color, abscess, and inflammation based on a 7– day superficial observation (19).

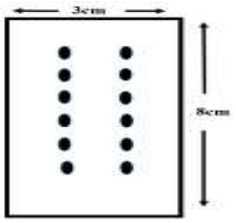


Figure 1. Schematic design of 12 injection points, dark spots, injection site of BM-MSC, CEE or saline.

Histological evaluation: Sampling was done from Transitional Line (Necrosis Initiation Area). The samples were placed in 10% formalin solution for fixation. Then, they were stained with 10% Toluidine Blue to identify all types of mast cells and degranulation.

The detection of all types of mast cells was as follows: integrated, perfectly healthy cell membrane with dark blue cell content was mast cell type 1, cells in which granules were ejected, but half of the membrane cells was preserved were mast cell type 2 and cells with complete or incomplete membrane cell damage were categorized as mast cell type 3(Fig 2)(14).

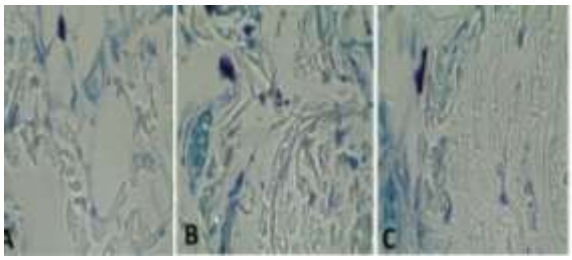


Figure 2. Three stages of mast cell degranulation in the transitional line of the flap, on the seventh day after surgery. A: mast cell type 1; B: mast cell type 2; C: mast cell type 3; (Toluidine Blue staining; 1000x magnification ×[10 µm]).

The evaluation of the total number of mast cells in the transitional line of flap was performed using a histomorphometric technique on 1033.3 mm^2 linear focus with a 100x magnification. The evaluation and counting of mast cells was done in the lattice plate (130×2140 mm^2) connected to a monitor (Fig 3).

Statistical analysis: SPSS21 software was used to analyze the data. Based on ANOVA, the difference between the mean number of mast cells and types of mast cells in the study groups was analyzed. In addition, post hoc test showed the difference between

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the study groups while $p<0.05$ was considered significant.

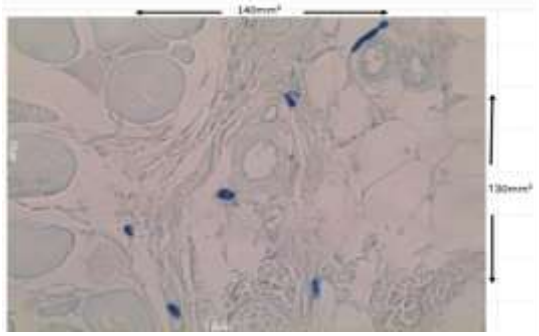


Figure 3. Counting cells on the lattice plate

Results

Clinical observation: One week after surgery, there was no inflammation, abscess, and secretion at the site of RSF, and on the seventh day after surgery, two areas were detectable. The necrosis area was observed in the distal flap part, the survived area in the proximal part and transitional lines in the studied groups (Fig 4) (21).

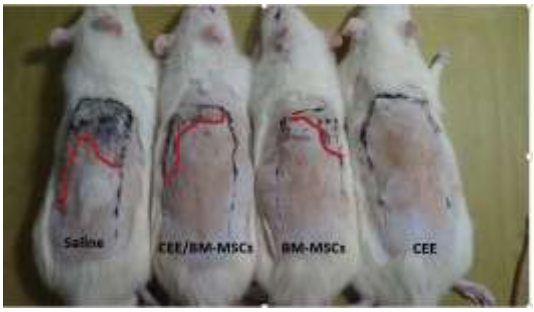


Figure 4. The macroscopic observation of the surviving part of the flap was identified on the Transitional Line (red line) on the seventh day after surgery.

Confirming the identity and stemness of BM-MSCs: Using flow cytometry, surface markers with low expression of CD45 and CD34 and high expression of CD90 and CD105 were confirmed by isotypic, mesenchymal and stem cell control (Fig. 5).

Tracing Transplanted Cells in the Tissue: On the seventh day after surgery, the cells identified by CM-DiI were observed alongside the blood vessels (Fig. 6).

Number and degranulation of mast cells: The difference between the mean number of mast cell type 1 ($p=0.099$, 3.67 ± 1.91) and type 3 ($p=0.384$, 1.9 ± 1.47) was not statistically significant between study groups. In addition, the difference between the mean number of mast cells type 2 in the study groups was statistically significant ($p=0.005$, 2.27 ± 1.42). In the Post Hoc Test, there was a significant difference between the CEE group and the BM-MSCs group ($p=0.001$, 2.85 ± 1.74) as well as between the CEE/BMMSCs group and the BM-MSCs group ($p=0.001$, 2.2 ± 0.95) and the CEE/BMMSCs group and the saline group ($p=0.032$, 1.7 ± 1.11).

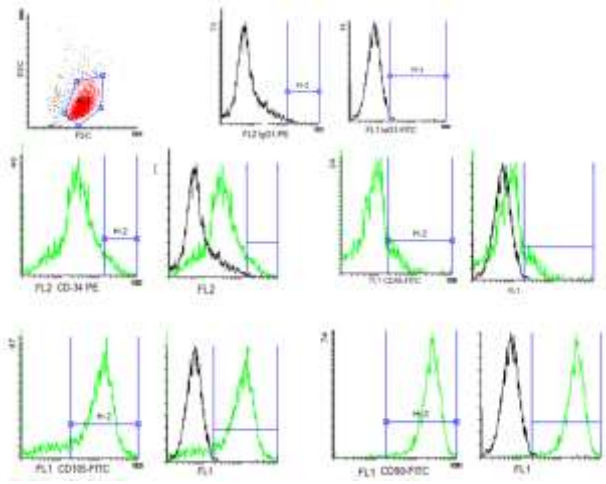


Figure 5. Comparison of Forward Scatter (FSC) and Side Scatter (SSC) characteristics shows BM-MSC scell density and two forms of control isotype in the heterogeneous sample (above charts). Different surface markers such as CD34, CD45 (middle charts), CD90 and CD105 (below charts) are displayed.

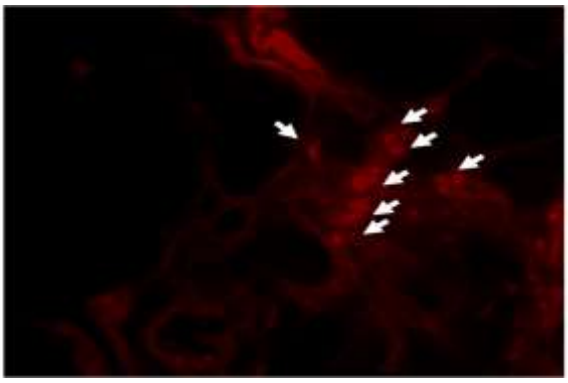


Figure 6. DiI-positive in flap tissue.

There was also a statistically significant difference between the mean number of mast cells in the BM-MSCs receiving group with the saline receiving group ($p=0.001$, 3.25 ± 0.72) (Fig. 7).

Observation of small vessels: In the qualitative observation of the small vessels in the area of the transitional line of the flaps, the distribution of the vessels in the experimental groups was higher and more developed than the control group (Fig. 8).

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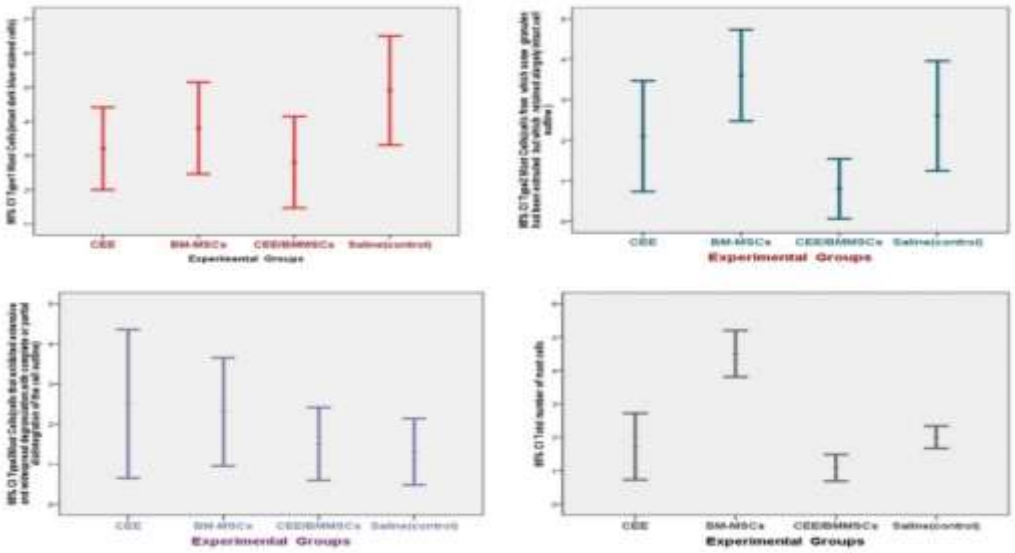


Figure 7. The following results were obtained based on ANOVA results: The difference between the mean number of mast cells type 1 in the study groups was not statistically significant (p=0.099) (up and left). The difference between the mean number of mast cells type 2 in the study groups was statistically significant (p=0.001) (up and right). The difference between the mean number of mast cells type 3 in the study groups was not statistically significant (p=0.384) (down and left). There was a statistically significant difference between the mean number of mast cells in the experimental groups compared to the control group (p=0.001) (down and right).

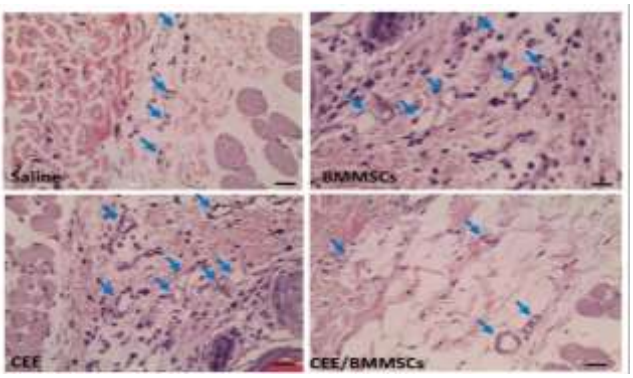


Figure 8. Distribution of blood vessels in the transitional line of the experimental and control groups with a magnification of 400x (H & E staining) (10 µm).

Discussion

In the present study, the total number of mast cells was increased in the BM-MSCs group, and among different types of mast cells, the difference between groups was only in mast cells type 2. Seventh day after surgery, mast cell type 2 had the highest value in BM-MSCs group, and after that, in CEE group compared to other experimental groups. The results of counting the number and type of mast cells in experimental groups compared with the control group showed that in the CEE/BMMSCs group, the number of mast cell types 3 was lower than other experimental groups. It seems that the presence of cell biomarkers and biological growth factor causes delay in the conversion of mast cell type 2 to type 3. Skin flaps are one of the most commonly used plastic surgeries. Ischemia, followed by necrosis, is a common problem after flap surgery (2, 13, 22, and 23). Recent studies suggest that the reduction of inflammatory components, such as mast cell degranulation, can have a positive effect on the healing process of the wound (3, 24,25).

The condition of ischemia in skin lesions is associated with a decrease in cell proliferation and an effect on collagen, which ultimately damages the process of wound healing (8). The formation of wound granulation tissue, cell proliferation, vascular and collagen growth throughout the proliferative phases and regeneration of wound healing depend on the presence of mast cells. The results of this study also indicate that an increase in the total number of mast cells is associated with an increase in the distribution of small vessels in experimental groups (26, 27). The

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process of wound healing is increased by increasing the growth factor of BM-MSCs and accelerates in the presence of the biological growth factor. In fact, in the absence or reduction of growth factor in various types of wounds, such as diabetic wounds, ischemic wounds are one of the main and important factors of causing disorder in the process of wound healing (28–30).

In a study, mast cells have been reported to contribute to the formation of scars during the wound process (31), and thus, reducing the presence of mast cell type 3 in the CEE/BMMSCs group during the wound healing process. It prevents the formation of scars and thus directly affects the proliferation of fibroblasts (27,32,33). Mast cells contain exclusive enzymes that can convert pro-collagen into collagen, and this condition causes fibrosis in abnormal cases (34, 24). Several studies have reported that in the normal healing process of human skin, the level of chemical intermediates (type 1 monocyte) is related and parallel to the use and degranulation of mast cells (33, 35). It seems that further studies are needed to determine the mechanism of action of BM-MSCs and CEE in the presence of mast cells in inflammatory conditions.

The results of this study emphasize that an increase in the total number of mast cells and an increase in the number of mast cells type 2 leads to an increase in the distribution of blood vessels in the transitional line in experimental groups, which ultimately leads to an increase in the survival rate of the flap.

Conflict of Interest: No conflicts of interest.

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