Iranian Journal of Basic Medical Sciences

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Improved viability of random pattern skin flaps with the use of bone marrow mesenchymal-derived stem cells and chicken embryo extract

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Jan 2, 2015
Accepted: Jul 9, 2015

Keywords:
Bone marrow
Chicken
Stem cells
Surgical flaps
Survival rate
Wistar rats

ABSTRACT

Objective(s): Covering tissue defects using skin flaps is a basic surgical strategy for plastic and reconstructive surgery. The aim of this study was to evaluate the effects of chicken embryo extract (CEE) and bone marrow derived mesenchymal stem cells (BM-MSCs) on random skin flap survival (RSF) in rats. Using chicken embryo extract can be an ideal environment for the growth and proliferation of transplanted cells.

Materials and Methods: Forty albino male Wistar rats were divided into 4 groups; each group consisted of 10 rats. BM-MSCs and CEE were transplanted into subcutaneous tissue in the area, where the flap would be examined. On the 7th postoperative day, the survival areas of the flaps were measured by using digital imaging with software assistance, and tissue was collected for evaluation.

Results: Survival area was 19.54±2 in the CEE group and 17.90±2 in the CEE/BM-MSC group when compared to the rates of the total skin flaps, which were significantly higher than the control group (13.47±2) (P<0.05). The biomechanical assessment showed a slight difference, although there was no statistically significant difference between the experimental groups and the control group (P>0.05).

Conclusion: The findings from this study demonstrated that in operative treatment with BM-MSCs and CEE transplantation could promote flap survival, but the biomechanical parameters were not contrasted with a saline injection.

► *Please cite this article as:*
Chehelcheraghi F, Eimani H, Sadraie SH, Torkaman G, Amini A, Shemshadi H, Alavi Majd H. Improved viability of random pattern skin flaps with the use of bone marrow mesenchymal-derived stem cells and chicken embryo extract. Iran J Basic Med Sci 2015; 18:764-772.

Introduction

Surgical skin flaps are frequently used in plastic and reconstructive surgery to cover acquired or congenital defects. Either partial or total skin flap loss is a common complication, as survival of the skin flap is determined by tissue ischemia because of insufficient vascularity. To address this issue, a number of strategies have been described to enhance the blood supply and to increase skin flap survival (1-3). Despite the numerous advances in endovascular procedures, surgical bypass approaches, and wound healing researches, ischemic non-healing wounds continued to be a clinical challenge and impaired tissue-levels and neovascularization remain as serious unsolved problems (4). Therefore, various therapeutic approaches have recently been pursued to prevent ischemic reperfusion induced tissue damage, by implementing unspecific local or systemic physical stressors and pharmacological agents (5-9).

Formation of a functional vessel network in wound tissue, via angiogenesis, represents a crucial and critical step in the wound healing process (10, 11). Effective angiogenesis occurs because of the proliferation of pre-existing endothelial progenitor cells (EPCs) in response to stimuli, release in the wound bed; however may also involve the direct or indirect action of EPCs. The direct incorporation of EPCs (from different sources) into neo-vessels in wounds has been demonstrated, but it is now increasingly accepted that EPCs, and other cells recruited in response to systemic signals, may promote neo-angiogenesis during wound healing via the production of paracrine signals released at the wound site (12-14). The paracrine growth factors released by cells have been demonstrated to play an important role in angiogenesis, according to previous studies (15-17). The cells produce bioactive substances that seem to accelerate the regeneration process of the wound (18).

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This is demonstrated in the transplantation of mesenchymal stem cells (MSCs), and contributes to improving ischemia through angiogenesis in ischemic vascular disease (19, 20). Bone marrow derived mesenchymal stem cells (BM-MSCs), which are also referred to as stromal progenitor cells, are self-renewing and expandable stem cells. The transplantation of *ex vivo* expanded BM-MSCs improves repair of the infracted heart and brain in animals (21). They have been widely used for tissue engineering and regenerative medical study (22). On the other hand, the recent concept of therapeutic angiogenesis by the local administration of angiogenic growth factors has emerged as an attractive approach (23), to enhance the blood supply and perfusion in compromised tissues, thus improving flap survival (23). The angiogenic response to tissue ischemia is a complex process, involving the coordinated inter play of a variety of soluble factors, controlling new blood vessel formation (23).

Growth factors are members of a large functional group of polypeptide regulatory molecules that influence the biological activities of responsive cells (24, 25). In the last decade, the use of a variety of growth factors as therapeutic agents to improve wound healing and the viability of ischemic skin flaps have aroused considerable interest (24, 25). A critical requirement for the growth of cells in culture is the presence of appropriate growth factors (26-28). Further investigations have documented the positive effect of rescuing ischemic skin flaps by increasing tissue perfusion. Significant rise in growth factors, such as the vascular endothelial growth factor (VEGF) as well as fibroblast growth factor (FGF), has been reported after stem cell therapy in rodent models (26-28).

Chicken growth factors identified in chicken embryo extract (CEE) increase constantly, and the combinational needs of the cells grown in culture are being investigated (29). Large numbers of proteins are in CEE (30). Additionally found in these extracts are two specific proteins, one of high molecular weight and one of low molecular weight, both of which must be present to produce full mitogenic activity (31-33). Considering these characteristics of CEE and BM-MSCs, the aim of this work was to study the effects of the application of CEE and BM-MSCs in survival rate of rats random skin flaps (RSFs), by measuring the surviving parts of the flaps, and the biomechanical examination of the wound incision of the flap, 7 days after flap elevation.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Penicillin/Streptomycin, and red fluorescent dye carbocyanine 1,1-dioctadecyl-1 conjugated to 3,3,30,30-tetramethylidocarboyanine perchlorate (CM-DiI, Molecular Probes) were purchased from Invitrogen, Germany. Trypan blue, phosphate-buffered solution (PBS), and hematoxylin and eosin (H&E) staining materials were purchased from Sigma-Aldrich, Germany. In addition, chloroform and glycerol 98% were purchased from Merck, Germany.

Animals

A total of 70 male albino Wistar rats, acquired from the Pasteur Institute, Tehran, Iran, were used. Of these, 40 adult rats weighing 280 to 300 g and 30 rats weighing up to 40 g were used for inducing RSF, and as cell donors, respectively. All animals were kept in appropriate cages, which were conventional and separately ventilated*, Post hoc- ad hoc –ad libitum*. All experimental protocols were approved by the Guide for the Care and Use of Laboratory Animals, the medical ethics committee of the Research Council of Baqyiatallah University of Medical Sciences (protocol number: February 10, 2014, No. 399).

Harvest and preparation of cells

The isolation and preparation of the BM-MSCs were performed according to the method described by Azizi *et al* (34). Briefly, rats were euthanized using an overdose of ketamine, and the femurs and tibias were exposed. The marrow was then extruded with 10 ml of DMEM. All procedures were performed under sterile conditions. The BM-MSCs used for the transplantation were harvested and labeled with molecular probes, CM-DiI (1:100), and suspended in 0.5 ml DMEM media for transplantation. Before transplantation, the cells were incubated in DMEM with CM-DiI at 37 °C for 15 min, and then subjected several times to centrifugation in PBS in order to remove the excess DiI dye (35).

Viability of BM-MSCs

Trypan blue staining was used to discriminate between viable and non-viable cells. The diluted cell samples in normal saline were prepared in a 1:1 (vol:vol) dilution of the suspension used before. We mixed 20 µl of 0.04% trypan blue with 20 µl cell suspension and following proper pipetting, 10 µl of solution was loaded on a hemocytometer slide. After that, the cells were incubated for 1-2 min at room temperature. Then they were counted under a microscope in all four squares (1×1 mm) of the hemocytometer chambers, and the average number of cells per square was determined. The concentration of the cells per ml was 10^9. The injections were performed in the same manner for the control group, but saline was used instead (3).

Preparation of chicken embryo extract plus dulbecco's modified Eagle's medium

CEE was isolated from 9-day-old embryos, according to the method by Spafas, Lancaster, PA,

and under sterile conditions. The extracts were rinsed 3 times in PBS before homogenization through a 50 ml syringe. The homogenization was diluted in DMEM at a 1:1 (vol:vol) ratio (29), and the extract was put in a shaker at room temperature for 2 hr. Then it was put in a centrifuge (6000x g) at 4 ˚C for 15 min to pellet out.

The pellet of the CEE was re-suspended in 5 ml of deionized H_2O and re-pelleted by centrifugation (6000× g) at 4 ˚C for 15 min. The supernatant was filtered through a 0.45 mm HT Tuffryn membrane filter (Pall, East Hills). Sodium chloride (5 M) was added to the supernatant at a 1:30 (vol:vol) ratio to restore the physiological salt concentration, and was combined with the original CEE. The protein concentration of the CEE was estimated according to the method of Bradford, using bovine serum albumin as a standard. The CEE protein was determined from the absorbance reading at 595 nm (34), and the CEE was frozen at -20 ˚C and thawed prior to use (29). When used, the samples were incubated at 37 ˚C for 15 min, and then centrifuged at 6000x g for 10 min at 4 ˚C. The CEE was used with the BM-MSCs for the transplantation in the CEE/BM-MSCs experimental group and used alone in the CEE experimental group. The CEE/BM-MSCs and CEE solutions were subcutaneously injected into four points of the central axis of the flaps, from proximal to distal of the flap area. The injections were performed in the same manner for the control group by saline solution.

Random skin flap model and experimental design

The flaps were made on the standard McFarlane-type dorsal rat flap model, using 40 adult albino male Wistar rats weighting 280 to 300 g. Samples were randomly divided into 4 groups of 10 rats in each, as follows: group 1, CEE injection; group 2, BM-MSC transplantation; group 3, CEE/BM-MSC; and group 4, the control (saline) group. All surgical procedures, injections, and transplantations were performed under sterile conditions. All of the animals were anesthetized with the IP injection of ketamine hydrochloride (50 mg/kg) along with diazepam (5 mg/kg). The rat´s skin was shaved and cleaned with povidone-iodine.

RSFs consisted of the entire thickness of the skin and skin muscle (panniculus carnosus). The base of the RSF was located at the distal end of the animal, on a horizontal line between the crests of the iliac bones. The dimensions of the flaps were 30×80 mm. After elevation, the flaps were immediately replaced using interrupted 4/0 nylon sutures. The day of surgery was considered to be day zero. Seven days after flap elevation, the rats were euthanized by chloroform inhalation in a closed space, and the survival areas of the flaps were measured (Figure 1).

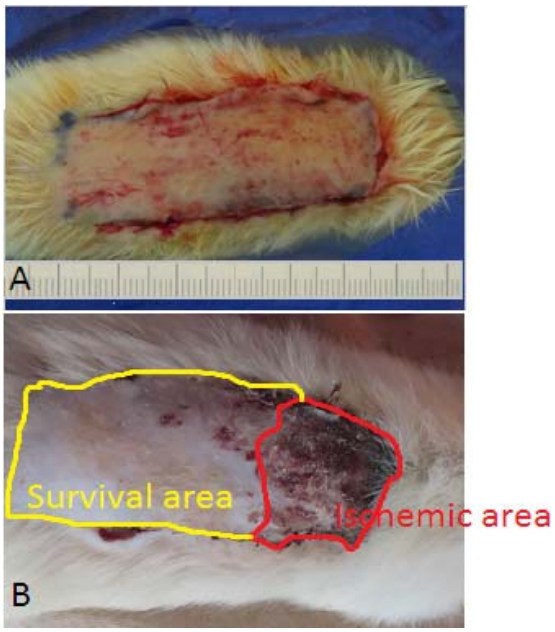

Figure 1. Design of random pattern dorsal skin flap (RSF) model. (A) each flap was 8×3 cm. One rectangular flap was drawn on the rat dorsum. Surgery days (zero) CEE, BM-MSCs, CEE/BM-MSCs, and saline (control) were injected at four sites on each flap and the flaps were elevated as random pattern flaps, (B) the flaps were immediately sutured back and the survival area was measured 7 days after flap elevation

Measuring the flap on day zero

At day zero, after flap elevation, the flap area was measured. The total area of the skin flap was soft and covered with normal skin, based on the clinical observation of the surgical site.

The flaps were photographed as digital images, and the total areas of the flaps were traced and analyzed using Adobe Photoshop CS6 extended software (Adobe Systems, Inc., San Jose, CA, USA). The surface areas of the flaps were compared among the groups.

Measurement of the flap survival on day seven

Seven days after flap elevation, the rats were euthanized by the inhalation of chloroform and the flap survival areas were measured. The distal areas of the skin flap that were rigid and enclosed with crust were judged to be necrotic. The survival area compared to the total skin flap area at day zero was calculated as survival rate (37). The flaps were photographed as digital images at days 0 and 7, and the total surface area of the flaps was traced, calculated, and analyzed using Adobe Photoshop CS6 extended software (Adobe Systems, Inc., San Jose, CA). This surface area was compared between the studied groups on both day 0 and day 7.

Sampling and biomechanical flap testing

The biomechanical flap properties of the samples were evaluated as in our previous study (8). Briefly, the rats were euthanized by the inhalation of

chloroform at the end of day 7, and skin samples were taken (15 mm of length × 35 mm of breadth). The sampling location was in the center of the distal part of the flap site (incision of the sample passed) (Figure 2). The samples were then placed in a piece of gas packing wound impregnated with saline solution and maintained at -20 ˚C. Prior to conducting the tensiometry test, the samples were slowly thawed at room temperature. We applied a material testing instrument (Zwickau/Roll Z 2-5-PH 1 F, Germany) for the tensiometry, which was calibrated for each use.

Each end of the samples was fixed in both the movable and stable clamps of the device. Data on the thickness and width of the sample and speed of the moving clamps were transferred to a computer connected to the material testing instrument. The mobile clamps were vertically moved away from the stable clamp at a rate of 20 mm per min, and the computer recorded the load-deformation curve for each sample. To evaluate the biomechanical properties, we measured the ultimate tensile strength (UTS) (N), stiffness (N/mm), deformation at F_{max} (mm), and absorbed energy up to F_{max} (Nmm) (Figure 2).

Statistical analysis

All values are expressed as the mean±standard error of the mean (SEM). The normal distribution of the data was analyzed using the one-sample Kolmogorov-Smirnov test (K-S test). All data were analyzed using the one-way analysis of variance (ANOVA), followed by the Tukey test (SPSS, version 22). *P-value*<0.05 was considered to be statistically significant.

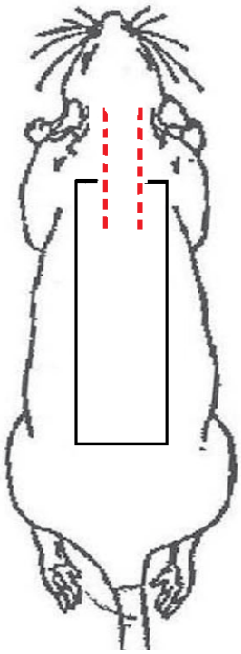

Figure 2. Diagram of flap sample site for biomechanical test

Results

Gross observation

All of the rats tolerated the treatment and skin flap procedures without any complications, and did not show any signs of swelling and/or exudation, or total necrosis at the surgical site during the experimental periods. The flaps showed congestion at the distal end on day 7, after the flap elevation. A demarcation line between the surviving and necrotic areas was clearly observed on day 7. The survival area of the flaps appeared to be pink and normal in texture, whereas the necrotic area was dark brown and rigid, and no bleeding was observed when it was cut with a scalpel (Figure 3).

Measurements of surface area of flaps on days 0 and 7

The results of total flap area measurements on day 0 showed that there was no significant difference between the studied groups (mean±SEM, 20.77±0.35; *P-value* = 0.586; Figure 4). The effect of BM-MSCs, CEE, CEE/BM-MSCs, and saline on survival flaps was evaluated 7 days post flap surgery. The results showed a significant difference in surviving area between the flaps treated groups and the control group (mean±SEM, 16.630±0.73; *P-value*= 0.013). The effect of CEE/BM-MSCs on survival rate of the flaps was significantly higher than that observed for the other groups (CEE group, *P-value*= 0.815; BM-MSCs group, *P-value*= 0.569; and saline group, *P-value*= 0.095; Figure 4 and 5).

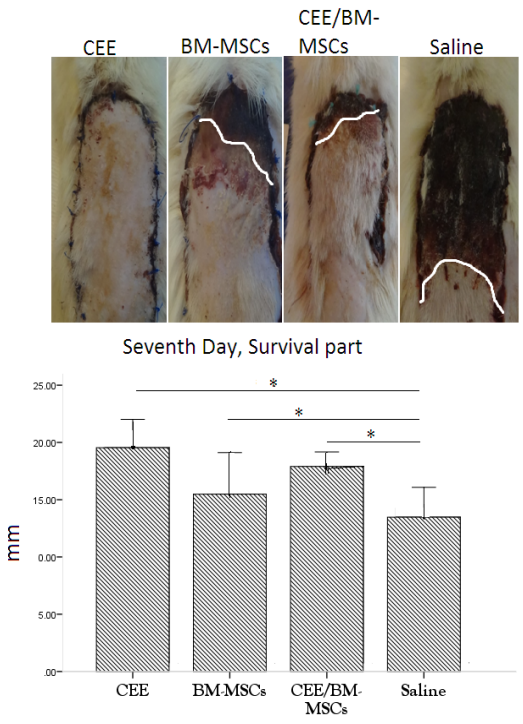

Figure 3. Mean±SEM of the rate of the surviving parts on day 7. Analysis of variance in all of the studied groups showed a significant difference between the flap size on day 7, *P-value* <0.05*

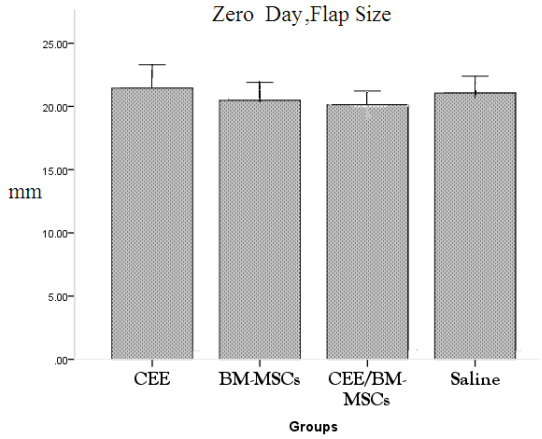

Figure 4. Mean±SEM of the rate of the sizing parts of the random-pattern dorsal skin flap (RSF) in the surface area of the flap on day 0. Analysis of variance in all of the studied groups showed no significant difference between the flap sizes on day 0. *P-value*>0.05

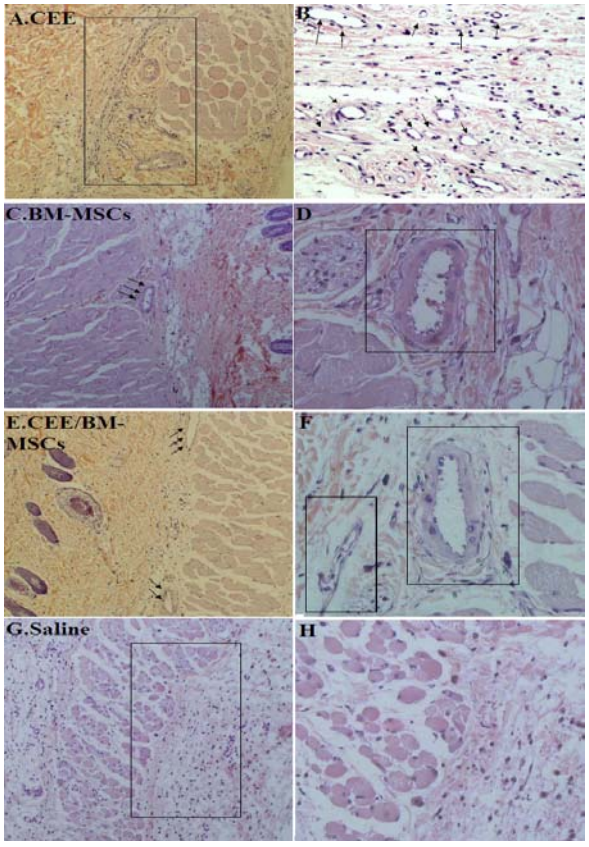

Figure 5. Histopathological appearance of flap tissues. CEE (A-B), BM-MSCs (C-D), CEE/BM-MSCs (E-F), and saline (G-H). Arrows and black rectangle indicate the formation of new or developing capillaries (by H&E, 200×, 50× magnification)

The tracking of transplanted cells

At the 7th day after surgery, the CM-DiL-labeled BM-MSCs could still be found in the subcutaneous tissue of the flaps. Some of the transplanted BM-MSCs were found in close proximity to the vascular vessels in the fluorescence slices (Figure 6).

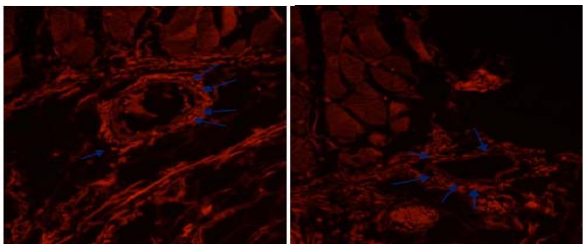

Figure 6. DiI-fluorescence in skin flap. Evidence of DiI fluorescent BM-MSCs in tissue area with high nuclear density (DiI); tissues of the transplanted areass on day 7 after flap elevation. Note the close position of the BM-MSCs to the microvessels in the skin flap tissue (magnification ×100)

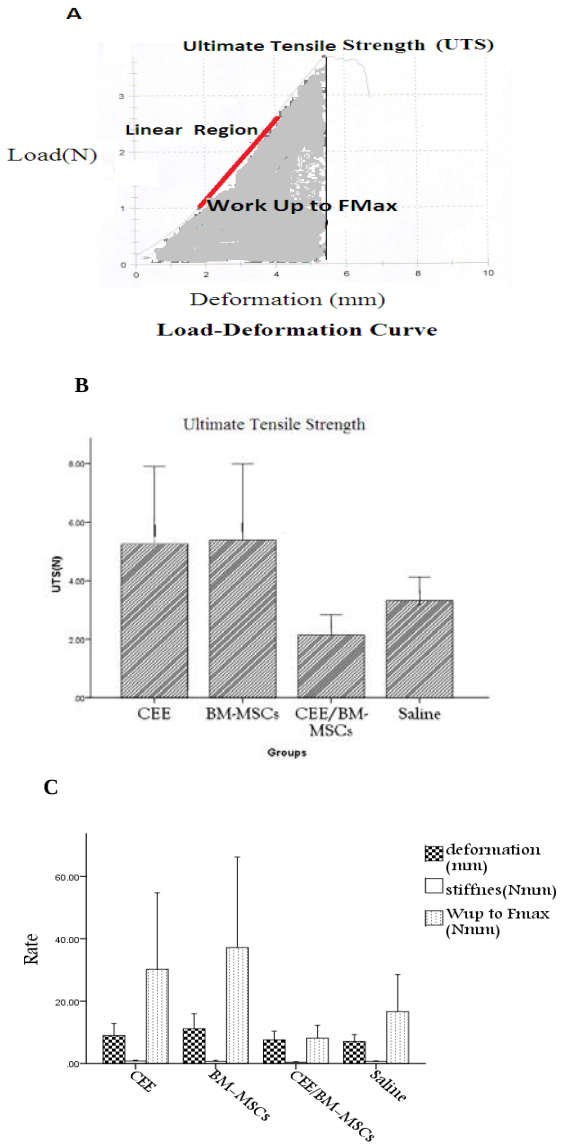

Figure 7. Mechanical properties of flaps 1 on day 7. (A) A typical load-deformation curve. The linear region of the load-deformation curve was used to calculate the flap stiffness, and the maximum point of the curve was deemed as the UTS of the flaps. (B) The UTS of the wounds on the flap margins of the control and experimental groups, *P-value* =0.103. (E) Deformation stiffness and work up of Fmax was of the wounds on the flap margins of the control and experimental groups, *P-value*> 0.05. For all determinations, the sample size was n=10 and all values were represented as the mean±SEM

Protein level assay in CEE

The protein level was 22.105 µg/ml in the CEE. It was isolated from 9-day-old embryos. Consistency in the survival area rate increased significantly in the CEE group, when compared to the control group.

Biomechanical flap properties

The ultimate tensile strength (UTS) in the CEE groups was higher than the other groups, but there was no significant difference between the studied groups (mean±SEM, 14.019±0.51; *P-value* =0.064). There were no significant differences in the work up of the F max (mean±SEM, 23.04±4.62; *P-value*=0.106), stiffness (mean±SEM, 0.62±0.061; *P-value*=0.208), and the deformation (mean±SEM, 8.690±0.80; *P-value*=0.278) of the flaps between the studied groups (Figure 7).

Discussion

Distal skin ischemic necrosis is a common complication of skin flap surgery. Many methods have been devised to increase skin flap perfusion in order to reduce the risk of ischemic skin necrosis (38). We hypothesized that application of CEE and BM-MSCs can increase the surviving part of the flap in RSF. We chose a treatment with CEE, combined with BM-MSCs, and on day zero the treatment was well tolerated in rats. The measurement of the surface necrosis is used most often for the assessment of flap survival (39); therefore, we measured that too. The results from 7 days postoperatively indicated enhancement of the skin flap survival in the experimental groups, especially in the CEE group. A slight improvement of the biomechanical tests results in this study was similar to the results obtained from a previous study that showed no significant progress of the biomechanical tests (8). This evident agreement in the result can be explained by the fact that the extension of surface survival does not correlate with the biomechanical parameters of the wounds (40, 41). Furthermore, these findings are in accordance with recent studies that reported significant flap survival in different animal models after stem cell therapy or using growth factors (4, 42-45). Since CEE and BM-MSCs do not have their own unique mechanisms of action, we have applied them unaccompanied, as well as both of them in one experimental group (45-47).

Several approaches have been made to improve skin flap survival; one of these methods is the use of drugs (8). The pharmacological manipulation mimics the surgical delay, and thereby increases the skin's blood flow, so has been promoted as a method for improving the skin flap survival. Our previous study found pentoxifylline (PTX) preferable to the other agents administered for the treatment of RSFs. Its application could lead to effective therapeutic tissue levels, without significant side effects (8).

Increasing evidence has confirmed that BM-MSCs can contribute to wound healing *in vivo*, and can differentiate into skin cells *in vitro*, including keratinocytes, vascular endothelial cells, sweat gland cells, etc. (48). Another researcher also demonstrated that linear incisional abdominal wounds in rats injected intradermally with allogenic BM-MSCs increased granulation tissue formation (49). This suggested that MSCs can serve as universal repair cells in adult tissues as they undergo regeneration or remodeling (50, 51).

It has been recently revealed that BM-MSC transplantation supports the neovascularization by virtue of increasing capillary angiogenesis, and augmenting blood flow perfusion (52). These results were accordant with new study (52). BM-MNCs are also a heterogeneous population of cells, which have been shown to be more beneficial than the homogenous composition in therapeutic vascularization (52).

Other exmainers demonstrated that BM-MSCs promote angiogenesis (45); however, BM-MSCs were not found in the vascular structures but in close proximity (45). They found that the BM-MSC conditioned medium promoted endothelial tube formation, and that the BM-MSCs expressed high levels of VEGF-α and Ang-1, but not Ang-2. Notably, the BM-MSC treatment resulted in significantly increased amounts of Ang-1 and VEGF-α in wounds. VEGF plays a key role in angiogenesis by stimulating endothelial cell proliferation, migration, and organization into tubules (45). That study systemically confirmed the beneficial effects of BM-MSCs in cutaneous regeneration and wound healing in non-diabetic and diabetic mice through differentiation and paracrine effects (45). The administration of allogeneic BM-MSCs or BM-MSC derived molecules may represent novel therapeutic approaches in the treatment of chronic wounds and other conditions (53, 54). Following the concept of therapeutic angiogenesis, several factors, including the transforming growth factor, basic fibroblast growth factor (bFGF), and VEGF have demonstrated marked abilities to improve skin flap survival (38,54). Experimental studies have demonstrated that the administration of exogenous VEGF can induce flap angiogenesis, improve the survival of the random extensions of axial-pattern skin flaps, and accelerate flap prefabrication (54).

Several laboratories have explored the possibility of promoting skin flap neovascularization by using recombinant VEGF proteins (55). In most of these studies, with some exceptions, the recombinant VEGF provided a beneficial effect on flap survival (55); although the exact mechanisms have not always been specifically addressed (55). The effect of CEE on improving the survival area in RSF may be partially due to angiogenic property of CEE. CEE contains an extraordinary concentration of various growth factors, and other condensed bioactive proteins (29, 30). Bioactive proteins have been shown to augment tissue and regeneration processes (29, 30), and the effects of these various growth factors on neovascularization have been examined (56).

Increased growth factor rate has been achieved by treating with 50 µg/ml for 40 passages, and then with CEE with down-growth factors for 40 passages. This study observed contacts between the myogenic cells of the somites and primitive axons growing from the neural tube as early as 35 hr of incubation in CEE (29). According to their results neural influence is required for both the initial differentiation and the subsequent maintenance of muscle cells *in vitro* (29).

We believe that there are multiple reasons for the effectiveness of skin flap survival by using the growth factors of CEE in ischemic skin flap rat model. It has been confirmed that applied CEE growth factors in combination with BM-MSCs, is an important and new alternative to previous strategies in treating ischemic skin flaps (8). Our group will promote the present study.

Conclusion

This study demonstrated the beneficial effects of the local administration of CEE or BM-MSCs on the survival rate of the RSF. The use of CEE alone, and with BM-MSC, may improve the survival of ischemic parts; even though the results of the bio-mechanical parameters did not show an increase. This may be attributed to the endothelial differentiation of transplanted cells and growth factors of CEE. In clinical flap surgery, topically applied BM-MSCs with CEE may become a novel therapeutic approach to improve the blood supply in ischemic compromised tissue. Larger doses or better methods of factor delivery at critical times are necessary to result in a clinically significant improvement.

Acknowledgment

The results described in this paper were part of a student thesis. We wish to thank the Vice Chancellor of Research at Baqyiatallah University, MS for financial support. We also thank Dr Assadi for technical support. This work was supported by (grant code: No. 399, February 10, 2014) the Medical Faculty, Baqyiatallah University of Medical Sciences, Tehran, Iran.

Conflict of interest

The authors declared no conflict of interest.

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