Research Article

Role of Bone Marrow (Non-hematopoietic Stem Cells) on Random Skin Flaps in A Rat Model. Experimental Study

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Abstract

Background: Random skin flap is one of the most widely used tools in the repair of tissue defects. Partial flap necrosis is a common problem. Improvement of random flap survival is a crucial issue in reconstructive surgery. Different pharmacological or surgical methods were used to improve skin flap survival. Stem cell therapy has aroused an interest in improving flap survival. The aim of this study was to evaluate the effect of born marrow Non-hematopoietic Stem Cells (BM-MSCs) in improvement of random skin flap survival in a rat model. **Material and methods:** It is an experimental study, was done in Medical Research Center at Ain Shams University Hospitals. 88 male albino Wistar rats were used, 40 rats as donor model and 48 rats as flap model. Flap model rats were divided into three groups [A, B& C]. Each group consists of 16 rats. The first group (A): was injected by Normoxic Bone marrow stem cell. The second group (B): was injected by hypoxic Bone marrow stem cell. The third one (C): was a control group, which was injected by the medium only. Each group was subdivided into two subgroups according to the time of injection (1: at time of flap elevation & 2: one week before flap elevation). The flap was assessed clinically, by histology for vascular density and by ELISA assay for VEGF level. **Results:** The mean survival percentages of the flaps were; 60.9%±2.2 for (subgroup **A1**), 28.8%±2.1 for (subgroup **A2**), 73.3%±2.2 for (subgroup **B1)**, 28.6% ±2.4 for (subgroup **B2**), 28.7%±2.1 for (subgroup **C1**), 28.5%±2.2 for (subgroup **C2**). The mean numbers Capillaries/ Field by histological examination were: **A1** (18.4±1.9), **A2** (7.5±2.1), **B1** (24.1±2.0), **B2** (8.4±2.1), **C1** (7.3±2.2) and **C2** (7.4±2.1). The mean VEGF level in subgroups was: **A1** (186.3±28.8), **A2** (25.7±2.5), **B1** (246.3±38.5), **B2** (25.5±2.2), C1 (25.3±2.4) and **C2** (23.3±2.1). **Conclusion:** Random skin flap viability could be enhanced through local administration of BM-MSCs simultaneously with flap elevation and the flap viability was maximized when the cells were cultured under hypoxic conditions and injected simultaneously with flap elevation.

Introduction

Random-pattern skin flap is one of the most widely used tools in the repair of tissue defects. Partial flap necrosis is a common problem encountered postoperatively. This flap necrosis occurs as a result of inadequate blood supply resulting from poor surgical techniques and handling, peripheral vascular disease, smoking, diabetes mellitus and others[1]. Improvement of random flap survival is a crucial issue in the practice of reconstructive surgery. Many studies were done to validate different pharmacological or surgical methods to improve skin flap survival, however, surgical delay is time consuming and requires more procedures and the pharmacological agents exhibited major drawbacks such as; the need for systemic application at relatively high doses to get significant improvement of flap vascularity, with subsequent possible side effects [2,3] . Therapeutic angiogenesis is an important mean used to preserve the integrity of tissues subjected to ischemia[4]. Growth factors such as; basic Fibroblast Growth Factor (bFGF), Platelet Derived Growth Factor (PDGF), Transforming Growth Factor-β (TGF-β) and Vascular Endothelial Growth Factor (VEGF), can activate angiogenesis and promote microcirculation in a skin flap

with ischemic injury[5]. However, growth factors delivery is limited by their rapid diffusion, poor biostability and short half-lives in vivo, thus requiring high doses or multiple injections, which could lead to excessive uncontrolled vascular formation and other side effects[6]. The stem cell therapy has aroused an interest in improving flap survival. MSCs mainly Bone Marrow Mesenchymal Stem Cells (BM-MSCs) and Adipose Derived Stem Cells (ADSCs) provided a new modality in promoting angiogenesis and tissue regeneration[1,7]. BM-MSCs have many advantages in comparison to other stem cells including; abundance in the bone marrow and easy isolation. BM-MSCs are also heterogenous population of cells, which have been shown more beneficial than homogenous composition of other stem cell sources. In addition BM-MSCs can be transplanted directly without in vitro expansion[8, 9]. Oxygen concentration is an important factor in the differentiation, maintenance and function of stem cells, Hypoxic preconditioning of BM-MSCs has a significant influence on the production of growth factors involved in promoting angiogenesis[10]. Hypoxic preconditioning of Bone Marrow Stem Cells (BMSCs) can lead to their acquiring the ability to survive for a long time under ischemic condition. Thus, more living BMSCs can promote angiogenesis efficiently and improve the ischemic flap vascularity[11]. The aim of this study is to evaluate the effect of born marrow Non-hematopoietic Stem Cells in improvement of random skin flap survival in a rat model by comparing the effect of hypoxic and normoxic bone marrow Stem Cells.

Material and methods

An experimental study was done in Medical Research Center at Ain Shams University Hospitals after the approval of Ethical Committee of Faculty of Medicine, Ain Shams University (Diary no. FMASU (1393-2012) 88 male albino Wistar rats were used in this study. 40 rats were used as donor model and 48 rats were used as flap model.

Isolation & Culture of Bone Marrow Derived Stem Cells:

Two-week old Forty Albino Wistar rats were used as cell donors. The used protocol was a modification of Yang et al.. 2010 & Wang et al., 2011 *as* recommended by Ain Shams Medical Research Center Team.

Rat was anaesthetized by intramuscular injection of 0.3 mL of ketamine (50 mg/kg). Rat's hind limb was shaved sterilized with Povidone iodine 10%.The femur and tibia of each rat was harvested under complete aseptic conditions. Harvested bones are then transferred to the laminar flow hood where the procedure would continue. Bones were opened from both ends. Bone marrow is flushed with 5 ml of medium consists of low glucose Dulbecco's modified Eagle's medium *(DMEM-LG. Lonza, Germany)* using a blunt-end hypodermic needle with a syringe. Marrow plugs were disaggregated by multiple passages through 18 or 22 gauge needles. Cells were isolated by centrifugation at 2000 rpm for 5 minutes then resuspended in medium solution. Cell pellet was formed at the bottom of the falcon tube. The supernatant was carefully removed by pipette leaving the pelleted SVF. 5 ml of Phosphate Buffered Solution (PBS) was added to the pelleted SVF and was re-centrifuged at 2000 rpm/min for 5 minutes. The supernatant was removed and cell pellet was resuspended in a complete culture medium formed of DMEM *(*Lonza, Verviers, Belgium). Cells were counted and then plated into culture flasks in DMEM solution. Culture flasks were examined with inverted microscope which was done prior to the start of cell culture.

Culture of Bone Marrow-derived Stem Cells:

The cell suspension was put in the culture flasks and incubated at 37^{o} C and 5 % CO2. There are two methods to prepare BMSCs according to the percentage of oxygen applied in the incubator. They are the normoxic and hypoxic types.

Normoxic Bone Marrow-derived Mesenchymal Stem Cells: (nBM-MSCs):

The incubation conditions were adjusted at 37 $^{\circ}$C, 20% O2 and 5% CO2. Medium was replaced every 3 days where nonadherent and hematopoietic cells were discarded by

washing using PBS (Lonza, Germany). Cultures were observed with an inverted microscope to assess expansion and cell morphology. BM-MSCs expansion was followed up by the examination with the inverted microscope. To prevent differentiation, cells were harvested at 80% to 90% confluence. At confluence; the cultured cells were dissociated from the culture dishes with 0.25% trypsin-EDTA solution (Lonza, Verviers, Belgium), neutralized with equal amount of culture medium, and collected by centrifugation at 2000 rpm for 5 minutes. Cells were calculated and examined for their viability by light microscopy using the haemo-cytometer. Fifty microliter of the PBS containing the cells were mixed with the same volume of 0.4% trypan blue (Sigma-Aldrich, USA). Calculation of total number of cells per ml was done. The BMSCs were suspended in 1 ml PBS in a final concentration of $4x10^6$ / ml for injection into each random pattern skin flap by insulin syringe.

Hypoxic Bone Marrow-derived Mesenchymal Stem Cells (hBM-MSCs):
Bone marrow stem cells were cultured as the normoxic cells till the cells expand to nearly 70% confluence then the incubation conditions were adjusted as follows; 37°C, 1% O_2 and 5% CO_2 for 48 hours before transplantation.

Random Pattern Skin Flap Model:
A total of 48 inbred albino Wistar rats weighting between 250 and 300 g were randomly divided into three groups [A, B, C]. Each group consists of 16 rats. The first group (A): was injected by **nBM-MSCs** .The second group (B): was injected by **hBM-MSCs**. The third one (C): was a control group, which was injected by the medium only. Each group was divided into two subgroups according to the time of injection.

Experimental Groups:
In Group A, the rats were injected subcutaneously with $4x10^6$ nBM-MSCs suspended in 1ml PBS. While in In Group B rats were injected with 4x106 hBM-MSCs suspended in 1ml PBS. In Group C: rats were injected by the medium only. In subgroups A1, B1 & C1, injection was done at time of flap elevation. In subgroups A2, B2&C2: it was one week before flap elevation. At the end of the experiment, the animal was euthanized with cervical dislocation.

Surgical Procedure:
All Rats were anaesthetized by intramuscular injection of 0.3 ml ketamine (50 mg/kg). After shaving and sterilization, the rat was placed in prone position. Cranially based random pattern skin flap2 cm x 9 cm was elevated on the dorsal trunk under sterile conditions, then sutured back to its original position with 4/0 polypropylene suture. After recovery, the rats were returned to their individual cages and received intramuscular injection of ceftriaxone (3mg/100gm body weight/ 24 hrs.) and Ketoprofen (5mg/kg/24hrs).

Flap Evaluation Methods:
At day 7 postoperatively, the survival area of each flap was grossly determined based on its appearance, color and texture. The animal was reanaesthetized as previously described. Digital images of each flap were recorded with Nikon P350 (Nikon, China), and the area of survival was determined *by image J 1.48 (NIH, USA).*Results were expressed as percentage of survival in relation to the total surface area of the flap.
After measurement of the survived area of the flap, tissues were biopsied for histological evaluation of the vascular density and enzyme-linked immune-sorbent assay (ELISA) for VEGF level.

For histological evaluation, ten tissue specimens (1cm x 1cm) from each subgroup were collected 1cm proximal to the demarcation of the survived area. Tissue specimens were fixed in formalin solution followed by dehydration, clearing and embedded in paraffin then serial sections of 6 µm thickness were cut then stained using the haematoxylin and eosin stain.

Neovascularization was assessed by counting the number of capillaries in the subcutaneous layer in five fields using Leica Qwin V.3 Image Analysis Software (Leica Microsystems, Wetzlar, Germany) installed on computer in Histology department Minia University, the computer was connected to Leica DM 2500

microscope under 100 x magnifications. The mean number of capillaries was calculated in each slide. The slides were examined blindly as regards the related groups.

For VEGF level measuring, ten samples (0.5g) taken from each subgroup were collected from the middle part of the viable flaps, minced in 0.5 ml tissue protein extraction reagent, and then homogenized by ultrasonic homogenizer and centrifuged at 4^0C at 2000 rbm. The supernatant was collected and assessed for VEGF using Rat VEGF ELISA kit (Sun Long Biotech Co., China, Cat No: SLO 740Ra) according to the manufacturer's instructions; the optical density (O/D) values of absorbance were read on a micro plate reader (Stat fax-2100, USA).

Statistical analysis

The statistical software program SPSS [version 20] was used for data entry and analysis. Quantitative data were presented by mean and standard deviation. One-way analysis of variance (ANOVA) was used to compare multiple means. Student t-test was used to compare two means values. The probability of less than [0.05] was used as a cut off point for all significant tests.

Results

Skin flap survival was evaluated in the main three Groups [A, B, C] collectively & the six subgroups [A1, A2, B1, B2, C1, C2] individually. The evaluation was done by the seventh postoperative day.

Evaluation methods include; estimation of percentage of flap survival, histological evaluation of vascular density & ELISA assay of VEGF expression.

1- Percentage of Flap Viability:

As regard the percentage of flap survival in the main three groups collectively (A, B and C) it was significantly higher in both groups (A&B) when compared to the control group C, ($p< 0.001$) (Figure 1). However, no significant difference was observed between group A and group B, ($P< 0.409$).

Results showed that the mean survival percentages of the flaps in the subgroups were; 60.9%± 2.2 for (subgroup A1), 28.8%±2.1 for (subgroup A2), 73.3%±2.2 for (subgroup B1), 28.6% ±2.4 for (subgroup B2), 28.7%±2.1 for (subgroup C1), 28.5%±2.2 for (subgroup C2), the mean survival percentages of the flap were improved when stem cells were injected at time of flap elevation subgroups [A1&B1], ($p<0.001$) for both subgroups, with the highest mean when the stem cells were injected at time of flap elevation under hypoxic preconditioning (subgroup B1). These results revealed that flap vascularity was enhanced when BM-MSCs were injected at time of flap elevation. However, no alteration of the flap survival was observed when the flaps were injected one week before flap elevation (subgroups A2&B2) when compared to the control group C (Figure 2, 3).

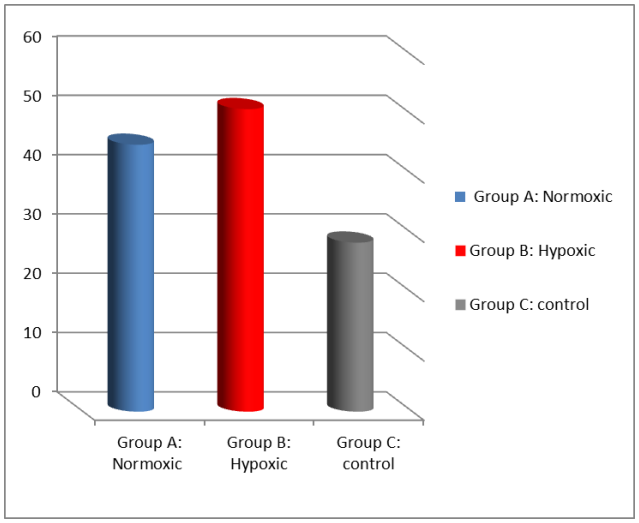

Figure 1: graph for Comparison of Percentage of flap survival between Studied Groups.

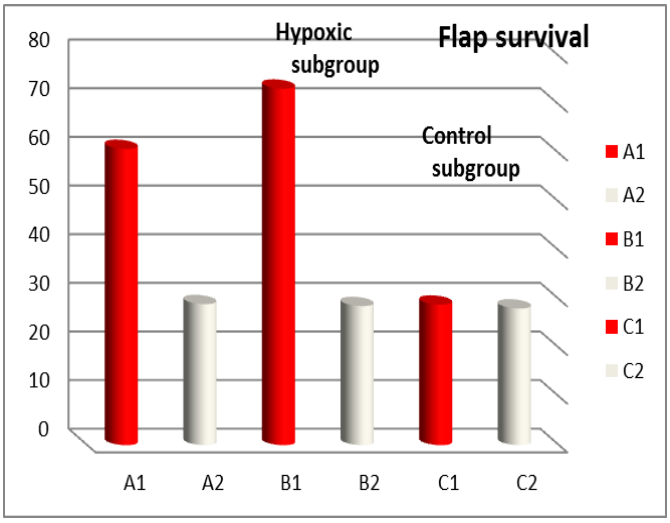

Figure 2: Graph for comparison of percentage of flap survival Between studied subgroups

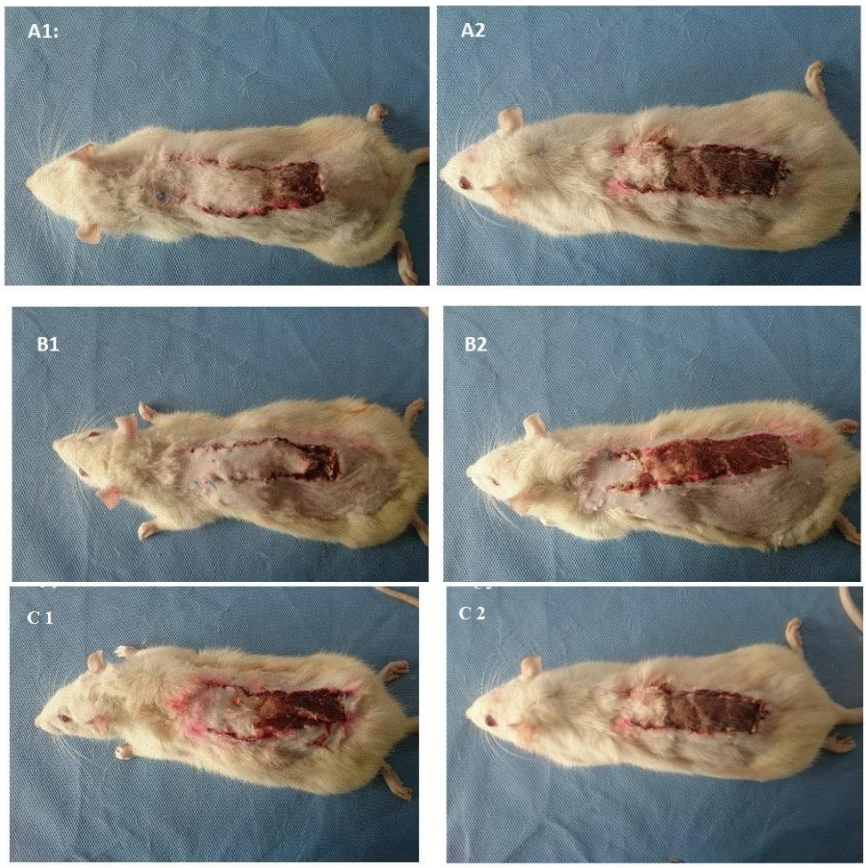

Figure 3: Photo showing flap survival by postoperative day 7 with the area of necrosis is clearly demarcated in the subgroups (A1, B1 & C1: Stem cells were injected at time of flap elevation. A2, B2 &C2: Stem cells were injected one week before flap elevation).

2- Histological Evaluation of Vascular Density:

Histological study showed improvement of the vascular density as evidenced by the increased number of capillaries/field, in both groups (A & B), when compared to group C. ($P<0.001$) (Figure 4). However, no significant difference was observed between group A and group B ($P=0.237$). The number of capillaries/field in the subgroups

was; 18.4±1.9 for (subgroup A1), 7.5±2.1 for (subgroup A2), 24.1±2.0 for (subgroup B1), 8.4±2.1 for (subgroup B2), 7.3±2.2 for (subgroup C1), 7.4±2.1 for (subgroup C2), the mean number of capillaries/field was increased when stem cells were injected at time of flap elevation subgroups [A1&B1], ($p<0.001$) for both subgroups, with the highest mean when the stem cells were injected at time of flap elevation under hypoxic preconditioning (subgroup B1). These results revealed that the vascular density was enhanced when BM-MSCs were injected at time of flap elevation. However, no alteration of the vascular density was observed when the flaps were injected one week before flap elevation (subgroups A2&B2) when compared to the control group C (Figure 5, 6).

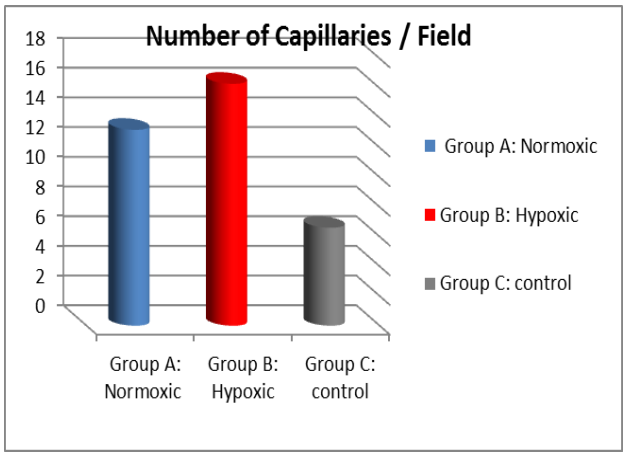

Figure 4: Graph showing Comparison of Number of Capillaries/Field between Main Groups Capillaries/ Field

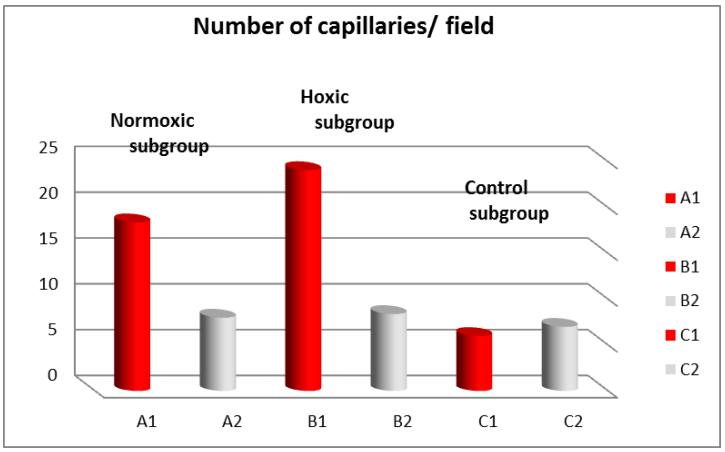

Figure 5: Graph showing Comparison of Number of Between Studied Subgroups

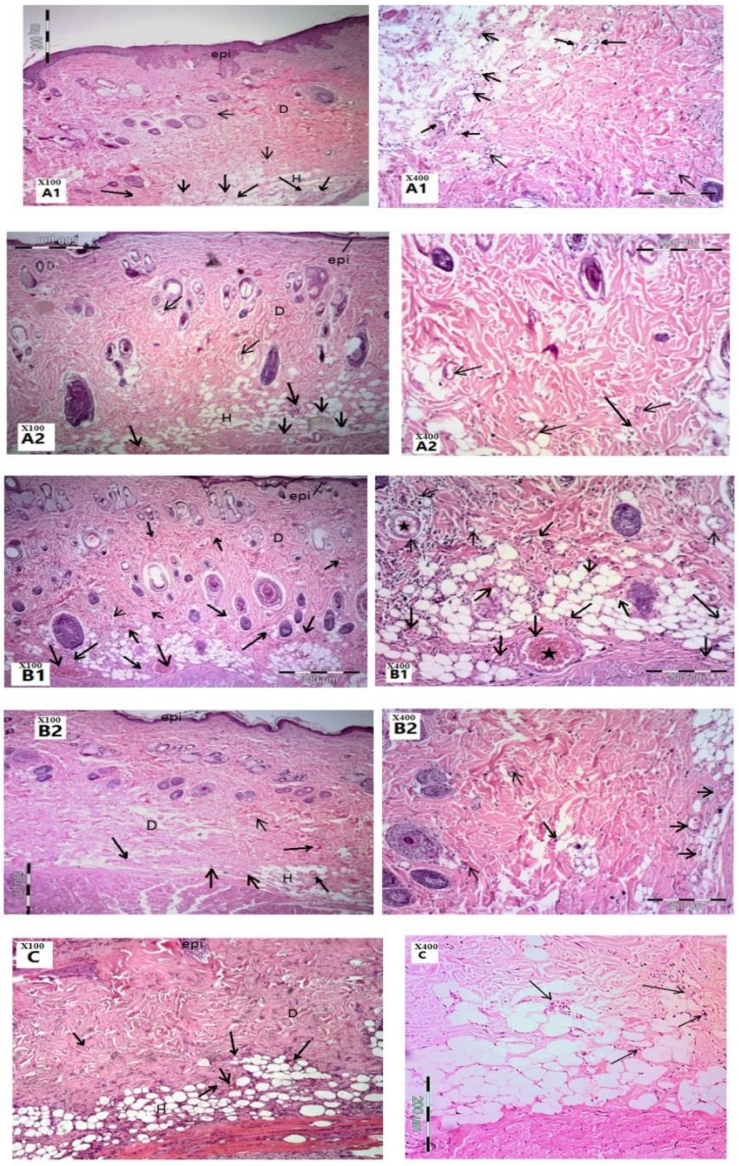

Figure 6: Photomicrograph showing the section from rat skin flap of subgroups on postoperative day 7, showing the number of subcutaneous capillaries with flat endothelial lining (↘) with magnification **(X 100)** on the left & **(X400)** on the right**.**

3- ELISA assay of VEGF expression:

VEGF in flaps was measured using ELISA. We found that VEGF level was generally significantly higher in both groups (A & B**),** when compared to group C (P< 0.001) (Figure 7). However no significant difference was observed between group A & group B (P = 0.423). The VEGF levels in the subgroups were; (186.3± 28.8) pg/ml for subgroup A1, (25.7±2.5) pg/ml for subgroup A2, (246.3±38.5) pg/ml for subgroup B1, (25.5 ±2.2) pg/ml for subgroup B2, (25.3±2.4) pg/ml for subgroup C1, (23.3±2.1) pg/ml for subgroup C2, the VEGF level was increased when stem cells were injected at time of flap elevation subgroups [A1&B1], (p<0.001) for both subgroups, with the highest mean when the stem cells were injected at time of flap elevation under hypoxic preconditioning (subgroup B1). These results revealed that the vascular density was enhanced when BM-MSCs were injected at time of flap elevation. However, no alteration of the VEGF level observed when the flaps were injected one week before flap elevation (subgroups A2&B2) when compared to the control group C (Figure 8).

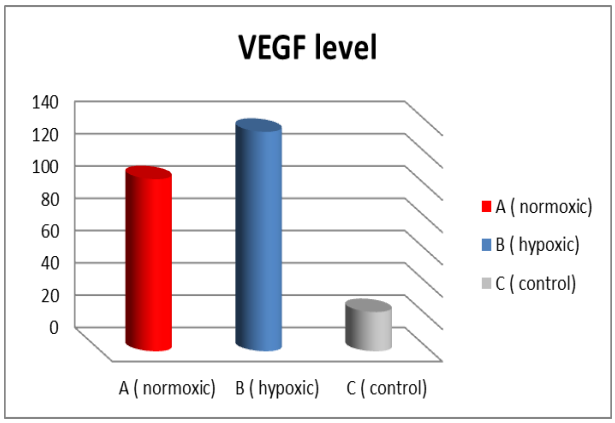

Figure 7: Comparison of VEGF Level (Pg. / ml.) Between Studied Groups

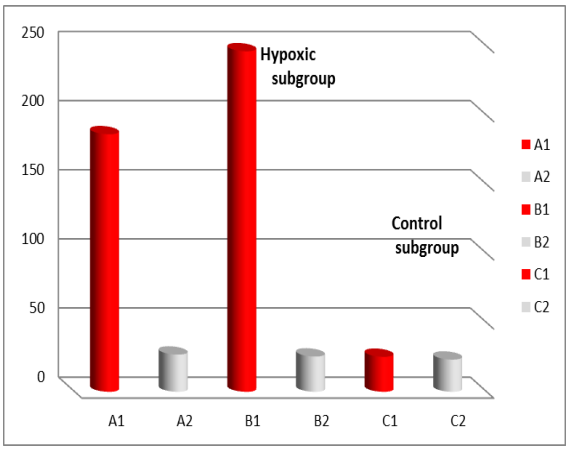

Figure 8: graph for comparison between studied Subgroups as regards VEGF level (Pg/ml).

Discussion

Random-pattern skin flap is one of the most widely used tools in the repair of tissue defects in plastic surgery. However, its utility is limited by unpredictable blood supply, which sometimes results in partial flap necrosis, especially in the distal part. Improvement of random flap survival is a crucial issue in practice of reconstructive surgery.

Many studies were conducted to validate different pharmacological or surgical methods to improve skin flap survival. The method of surgical delay and ischemic preconditioning was proved to improve skin flap survival in clinical practice. However the delay procedure needs an additional surgical procedure and longer time. Many researchers have focused on drug therapy as an alternative method for augmenting blood flow rather than the time consuming surgical delay. The use of sympatholytic drugs, vasodilators and calcium channel blockers, could improve ischemia of skin flaps. However, applying high doses of these drugs can lead to many side-effects[12].

Stem cells were introduced as one of the modalities used for promoting angiogenesis and tissue regenerations. MSCs are adult stem cells which can be isolated from human and animal sources. They can be isolated from; bone marrow, adipose tissue, amniotic fluid, umbilical cord, peripheral blood, endometrium, dental tissues and others[13-19].

Optimization and understanding stem cell growth and function requires knowledge of the specific microenvironment conditions in vivo. Oxygen tension in the bone marrow of mice is significantly lower than other tissues[20]. Hypoxia may be used as a stimulus to promote differentiation into various cell lines. And the production of growth factors. So the preconditioning of the cells through hypoxic culturing, prior to clinical use, may maximize their efficacy in clinical use[10].

We aimed in this research to study the effect of BM-MSCs on random pattern skin flap survival. Our study was planned to work on both; cultured BM-MSCs under normal oxygen tension (normoxic) and BM-MSCs under low oxygen tension (hypoxic). The influence of different timing of BM-MSCs injection on flap viability was studied too.

Results showed that the mean survival percentages of the flaps were; 60.9%± 2.2 for (subgroup A1), 28.8%±2.1 for (subgroup A2), 73.3%±2.2 for (subgroup B1), 28.6%±2.4 for (subgroup B2), 28.7%±2.1 for (subgroup C1), 28.5%±2.2 for (subgroup C2), the mean survival percentages of the flap were improved when stem cells were injected at time of flap elevation (subgroups A1&B1), with the highest mean when the stem cells were injected at time of flap elevation under hypoxic preconditioning (subgroup

B1). These results revealed that flap vascularity was enhanced when BM-MSCs were injected at time of flap elevation. However, no alteration of the flap was observed when the flaps were injected one week before flap elevation (subgroups A2&B2).

Few studies have focused on the effect of BM-MSCs in preventing ischemic tissue damage of flap surgery. In addition, the optimal time for BM-MSCs administration was not clear.

Chehelcheraghi et al., and xu et al., injected stem cells into random pattern skin flap at the same time of flap elevation. They found that stem cell injection increased survival of the flaps with considerable improvement in capillary density[21,22].

Yang et al., injected the BM-MSCs into random skin flap two days before flap elevation. They found that preoperative treatment with BM-MSCs transplantation could promote neovascularization and improve flap survival via expression of VEGF& bFGF. They explained their preference of preoperative administration of stem cells based on their observation of the tissue inflammatory reaction. They found that when the stem cells were administered on the same day of flap elevation, this might affect the survival of the injected stem cells. In our study we aimed to inject the stem cells not only two days before flap elevation but one week before, to get the maximum effect of stem cells in enhancing vascularization and improving flap survival via expression of VEGF[9].

Simman et al., studied the combined effect of BM-MSCs and VEGF in two groups. The first group had the injection one week before flap elevation, while the second group had the injection at the same day. Their results showed that administration of stem cells one week before flap elevation had better good results. This makes the comparison with our results inconsistent[1].

At this study we also compared the effect of BM-MSCs when cultured under normoxic conditions group A, and when cultured under hypoxic conditions group B, to the control group C. The results showed that the mean survival percentages of the flaps were improved at both groups (A&B), when compared to group C ($P<0.001$). However, no significant difference was observed between group A and group B ($P=0.409$).

Histological study showed improvement of angiogenesis in both groups (A& B), as evidenced by the increased number of capillaries. Capillary density was significantly higher in both groups (A and B), when compared to group C. ($P<0.001$). However, no significant difference was observed between group A and group B ($P=0.237$). These results are in accordant with the previous study done by Wang et al.,.

The result of capillary density is in the agreement with the macroscopic observation in which injection of BM-MSCs improves tissue survival of our random-pattern skin flap when injected at time of flap elevation.

The angiogenic effect of BMSCs has been hypothesized to be the combined result of their ability to produce angiogenic growth factors and their ability to differentiate into endothelial cells; this effect is maximized when stem cells are injected at time of flap elevation under hypoxic condition[23].

Simman et al., founded that combined injection of BM-MSCs and VEGF led to a significantly greater area of flap survival when injected one week before flap elevation[1].

During our study in an attempt to understand the role of BMCs in improving skin flap viability, we examined the expression of VEGF in the flaps. We found that VEGF level was generally significantly higher in group A and group B when compared with group C ($P< 0.001$), however no significant difference

was observed between group A & group B (P = 0.423) but it was highest in Subgroup B1 (when stem cells were injected at time of flap elevation under hypoxic preconditioning). These results are in agreement with Wang et al., study[11].

The overall results of our study showed that Group A and Group B have significantly improvement in the survival of the random pattern skin flap with no significant difference was observed between group A & group B as regard percentage of flap survival, capillary density & VEGF level. But there was significant difference between subgroups A1& B1 as regard percentage of flap survival, capillary density & VEGF level. These results were unpredicted as we were predicting to find statistical difference between groups which actually not found this due to small sample size of the subgroups and also due to the results of the subgroups which were at the extremes that neutralized the effect of each other's. the highest mean flap viability percentage combined with high mean capillary density and high levels of VEGF was observed in subgroup B1 when stem cells were injected at time of flap elevation under hypoxic condition. These results are consistent to other findings by Hung et al., Abdollahi et al., and wang et al., who found that hypoxic MSCs induced angiogenesis and thus improved the flap viability significantly. The hypoxic conditions help BMSCs to secrete more angiogenic factors[10,11,23]. That is why the transplantation of mesenchymal stem cells in vivo is usually performed at the time of surgery. The flap elevation leads to hypoxia at the skin flaps, and this stimulates the mesenchymal stem cells in the most efficient way[24]. Thus, the injection of the BMSCs one week before flap elevation might decrease their effectiveness and concentration due to the absence of the trigger at the time of application.

When we constructed our research we thought that the application of BMSCs one week before flap elevation was an adequate time to differentiate and to increase the vascularization of the skin flap in preparation for the ischemic insult, however our results did not show any improvement in the skin flap survival when we injected the stem cells one week preoperatively.

Park et al., founded that most of the applied stem cells died within one week of injection and therefore to develop successful stem cell therapies, it was necessary to transplant the stem cells in combination with growth factors or hypoxic preconditioning of stem cells just before application to develop successful stem cell therapies[25], this is consistent with the results of our study, while, Simman et al., founded that administration of stem cells alone was incapable of improving flap survival, addition of VEGF led to a significantly greater area of flap survival when injected one week before flap elevation. This is inconsistent with our study as regard the optimal time for injection of the stem cells[1].

Conclusion

Random skin flap viability could be enhanced through local administration of BM-MSCs simultaneously with flap elevation and the flap viability was maximized when the cells were cultured under hypoxic conditions and injected simultaneously with flap elevation.

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