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Research Article
Human Mesenchymal Stem Cell Promotes Burn Wound Healing by Inducing FGF and VEGF in Diabetic Rat

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Abstract
Background and Objective: The hBM-MSCs have a high level of differentiation and proliferation in the healing process of cuts and burns. This study aimed to determine the role of hBM-MSCs in the formation of granulation tissue in diabetic burnt rats. **Materials and Methods:** Thirty rats were divided into two groups; phosphate-buffered saline (PBS) as a control group and a treatment group (BM-MSCs 2×10^6 cells/mL). Both groups were treated as hyperglycaemia by injecting alloxan. Rats were anesthetized using xylazine and ketamine and given full-depth burns on the dorsal using a heated plate. Rat skin tissue was excised on the 3rd, 7th and 14th day and histopathological preparations were made using immunohistochemical staining to determine the expression of the growth factors of FGF and VEGF. The results were analyzed using Tukey's t-test advanced. **Results:** The FGF level increased statistically significantly on day 3, 7 and 14 in the treatment group. Otherwise, on day 14 there was a significant difference between the control group and the treatment group ($p = 0.017$). The VEGF expression also showed an increase on days 3 and 7 but decreased on day 14. The VEGF level was not statistically significant between the control and treatment groups. **Conclusion:** The hBM-MSCs increased the formation of granulation tissue by expressing a high level of FGF which plays a role at the beginning of new blood vessel formation and VEGF affects the end of the formation of new blood vessels.

Key words: Human bone marrow-mesenchymal stem cells, fibroblast growth factor, vascular endothelial growth factor, burn wounds, diabetic rats

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The significant step of wound healing is to initiate granulation tissue to proliferate[1]. Granulation tissue is regarded as a contractile organ, consisting of fibroblast, infiltration of inflammatory cells, endothelial cells, keratinocytes and capillaries with thin wall[2]. Macrophages initiate angiogenesis of wound, promote and lead to form capillary growth[3]. Angiogenic factors (Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), angiogenin and TGF-α and TGF-β) were activated when endothelial cells emerged. The process continued to form new blood vessels; resulting in protease (unraveling of ECM), chemotaxis, proliferation, remodeling and differentiation[4].

These growth factors produce new blood vessels by initiating endothelium formation by FGF on the first day of wound healing, at the same time VEGF forms new blood vessels at the end of wound healing (granulation tissue formation process). The formation of new blood vessels is important to replace damaged capillaries, restore the supply of oxygen, blood cells and nutrients to injured tissue and assist and initiate fibroplasia. The formation of granulation tissue is very important in wound healing to accelerate the healing process of burn wounds[5].

Healing of wounds in diabetics is longer due to hyperglycemia. The hyperglycemia binds oxygen and causes hypoxia. Prolonged hypoxia reduces FGF and VEGF levels in angiogenesis of wound healing[6,7]. The hyperglycemic circumstances lead to the weakness of the immune system reacting to the inflammation and reducing the formation of granulation tissue, even to the point of a tendency for fibroblast apoptosis[8]. Burn wound treatment currently involves stem cells as a cell therapy. Stem cells can replicate, differentiate and act as immunomodulators. In recent years, various sources have proven that therapy with hMSC is a promising treatment for various diseases including skin, cuts and burns[9]. The hBM-MSCs have a great ability to differentiate the required cells, thus accelerating the healing of burn wounds and cuts. The VEGF and FGF as growth factors are liberated to initiate granulation tissue formed and angiogenesis as a form of paracrine effect[10]. The study of burn healing in diabetic conditions is not yet widely researched. This study determined the effect of hBM-MSCs in expressing FGF and VEGF as growth factors in the formation of granulation tissue in diabetic rats.

MATERIALS AND METHODS

The study was experimental with the post-test-only control design. Thirty rats were divided into 2 groups; the control group (PBS) and the treatment group (hBM-MSCs). Both groups were made hyperglycemic by injecting alloxan.

Study area: This research project was conducted from September to December, 2020 in the Laboratory of Anatomy, Faculty of Medicine, Andalas University, Padang, West Sumatra, Indonesia.

Preparation of hBM-MSC derive from human bone marrow: The hBM-MSCs originated from humans, obtained from the Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, University of Indonesia. The hBM-MSC dosage for each rat was 2×10^6 cells/mL.

Preparation of experimental rats for burns: The treatment was started by administering xylazine and ketamine (ratio 1:1) as anesthesia and shaving the back of rats. The plate was heated in boiling water for 30 min and placed on the rat's back for 20 sec to create a full-thickness burn. The PBS was injected for the control group and hBM-MSC for the treatment group. Analgesics were given after treatment. Rats were killed by inhalation with ether on days 3, 7 and 14, burnt skin tissue was harvested to determine the expression of FGF and VEGF output (R&D system) using the immunohistochemical method[11].

Immunohistochemical staining of FGF and VEGF: The burnt skin tissue of rats was fixed by deparaffinization, each slide was dipped into xylol solution three times for 5 min, rehydrated with xylol two times for 3-5 min and in graded alcohol, respectively (absolute ethanol; three times for 2 min, ethanol 70%, two times for 2 min and rinsed with distilled water three times). The specimens and slides were dried and dipped in 3% H_2O_2 in methanol for 5 min, then rinsed with distilled water and PBS three times. The cleaned slides were placed in anti-FGF and VEGF (mouse anti-rat 1:50) for 30 min at room temperature and rinsed with PBS three times for 2 min, respectively. Each slide then was soaked again in a secondary antibody (rabbit anti-mouse biotinylated antibody label) for 30 min and rinsed with PBS solution three times for 2 min, respectively. The final immersion was in labeled streptavidin horseradish peroxidase (HRP) for 30 min, then rinsed in PBS solution three times for 2 min, continued into chromogen substrate for 3-10 min and rinsed with PBS solution three times for 2 min, then rinsed with distilled water. Each slide was put in Mayer's hematoxylin for 6-15 min, rinsed with running water and mounted.

Assessing FGF and VEGF expression: The expression of FGF and VEGF appeared as a brown-colored area on immunohistochemical staining, expressed intracellularly and extracellularly in the connective tissue matrix. The brown colored area was measured on the microscopic slide using the ImageJ program (ImageJ 1.49v software, National Institute of Health, Bethesda, Maryland, USA), by isolating the brown colored area and calculating the proportion of colored area compared to the visual field area. The proportion of positively stained areas was described in percentage.

Research ethics: This study has undergone ethical clearance and received approval from the Committee of the Research Ethics, Faculty of Medicine, Andalas University with ethics number 598/UN.16.2/KEP-FK/2022.

Data analysis: The effect of hBM-MSC on the expression of FGF and VEGF levels was analyzed using Tukey's t-test advanced. A probability $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

FGF expression to administering of hBM-MSCs in diabetic rats compared with control: The treatment of hBM-MSCs to burnt skin tissue in diabetic rats showed increasing in granulation tissue formation and elevation of burn wound healing, results were described in Fig. 1.

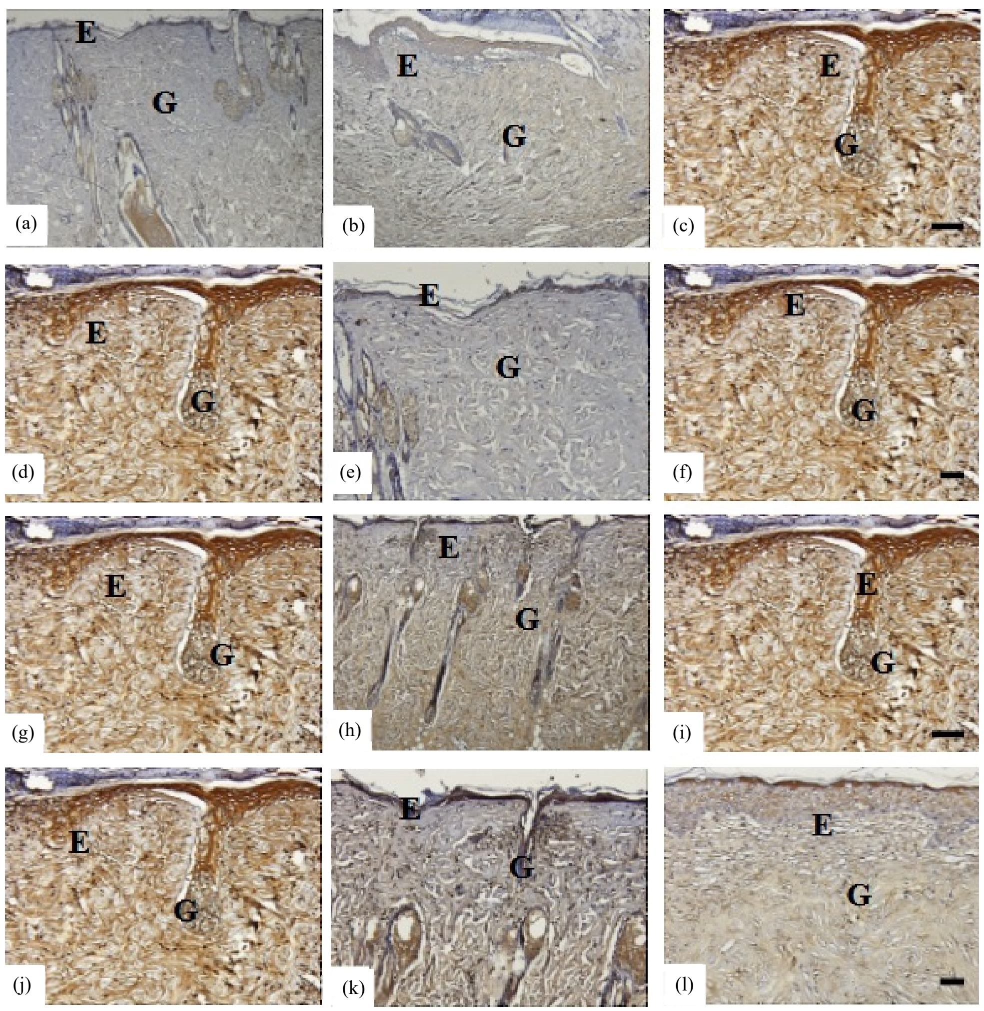

Fig. 1(a-l): FGF immunohistochemical expression in skin tissue of the (a-f) Control group (PBS injection) and (h-l) Treatment group (hBM-MSCs administration)

Epidermis epithelium (E) and dermis with post-burn granulation tissue (G) of the experimental rat were visible left panel; on day 3, middle; day 7, right day 14; positive control mice with diabetes mellitus and rats with diabetes mellitus were given hBM-MSCs. Immunoperoxidase, panels a-c and h-i objective magnification 10x, panels e-f and k-l objective magnification 20x

Table 1: FGF expression in the skin of burnt rat with diabetes on day 3, 7 and 14

	Fibroblast growth factor expression					
Group	Day 3	p-value	Day 7	p-value	Day 14	p-value
Control	13.50 (10.50-16.20)	0.900	26.00 (13.30-28.50)	0.531	31.50 (21.30-37.90)	0.017
Treatment	16.80 (10.80-23.90)		26.40 (20.50-35.80)		29.80 (18.20-29.60)	

Table 2: VEGF expression in skin of burnt rat with diabetic on day 3, 7 and 14

	Vascular endothelial growth factor expression					
Group	Day 3	p-value	Day 7	p-value	Day 14	p-value
Control	11.00 (9.10-12.60)	0.968	11.10 (8.60-12.60)	0.010	15.20 (21.30-37.90)	0.800
Treatment	13.60 (9.80-17.70)		20.00 (10.20-26.40)		16.90 (13.60-21.80)	

In Fig. 1, FGF expression was found in intracellular and extracellular epidermal cells, connective tissue cells and inflammatory cells in the dermis. The FGF expression in the treatment group (hBM-MSCs) was higher compared to the control group. The FGF expression on day 7 and 14 was higher in both treatment groups except on day 14.

Table 1 described that the control group and treatment group after hBM-MSCs treatment on day 3, 7 and 14 showed an increase in FGF expression, but there was no significant difference in the increase on day 3 and 7 between the control and treatment groups. Otherwise, on day 14 there was a significant difference between the control group and the treatment group ($p = 0.017$).

The growth factors, Fibroblast Growth Factor (FGF) and Vascular Endothelial Growth Factor (VEGF) play a role in the formation of granulation tissue. The formation of granulation tissue was the most important process of wound healing in the proliferation phase. The observation on the histopathological microscopic slide showed an increase in growth factors (FGF and VEGF) on day 3, 7 and 14 which interpreted that hBM-MSCs were involved in the formation of granulation tissue and accelerated wound healing.

The process of wound healing in the control group showed that there was a gradual elevation of FGF on day 3 and 7 and continued to increase on day 14. The pattern of expression showed an inflammatory process that gradually liven up until the last day of the biopsy. This inflammatory pattern was also in line with the histology of tissue with thin epithelialization on day 14 (Fig. 1a-f). When given hBM-MSCs there was an increase in FGF expression on day 7 compared to day 3, but there was a decrease in expression on day 14 compared to day 7. This occurrence was in line with the image of wound epithelialization and granulation, where epithelialization has completed its closure with a thicker epidermis (Fig. 1g-l).

The wound healing process was influenced by the expression of FGF by the formation of epithelial cells and by inflammatory cells in the inflammatory area, which started with an increase in cytokine expression in the early phase and then tended to decline in the final healing phase[11]. The results of this study showed several changes in the pattern of FGF expression after hBM-MSCs were administered to burn wounds of DM animals. There was an accelerated increase in FGF expression in earlier treatment followed by a decreased level of FGF in accordance with the histological wound healing process[12].

Figure 2 shows the intracellular and extracellular expression of VEGF, reflected in epidermal cells, connective tissue cells and inflammatory cells in the dermis. The expression of VEGF in the treatment group with hBM-MSCs was higher compared to the control group. The VEGF expression on day 7 was slightly higher in both groups compared to day 14. Immunoperoxidase, panels a-c and h-i) objective magnification 10x, panels e-f and k-l objective magnification 20x. f-Scale; 500 µm, l; 200 µm.

Table 2 showed that there was an increase in the expression of VEGF in the treatment group on day 3, 7 and 14 compared to the control group. There was no significant difference in VEGF levels on day 3 and 14 between the control and treatment groups. On day 7, there was a significant difference between the control group and the treatment group at $p = 0.010$.

The VEGF expression after administration of hBM-MSCs resembled the expression pattern of FGF in diabetic rats. In the burnt skin of diabetic rats, the expression of VEGF appeared tented to be low on day 3 and started to up on day 7 and still increased on day 14. This condition was in line with the skin healing process, both the closure of the epidermis and the formation of granulation tissue in the dermis leading to a normal condition (Fig. 2g-l), compared to the control group which is illustrated in Fig. 2a-f.

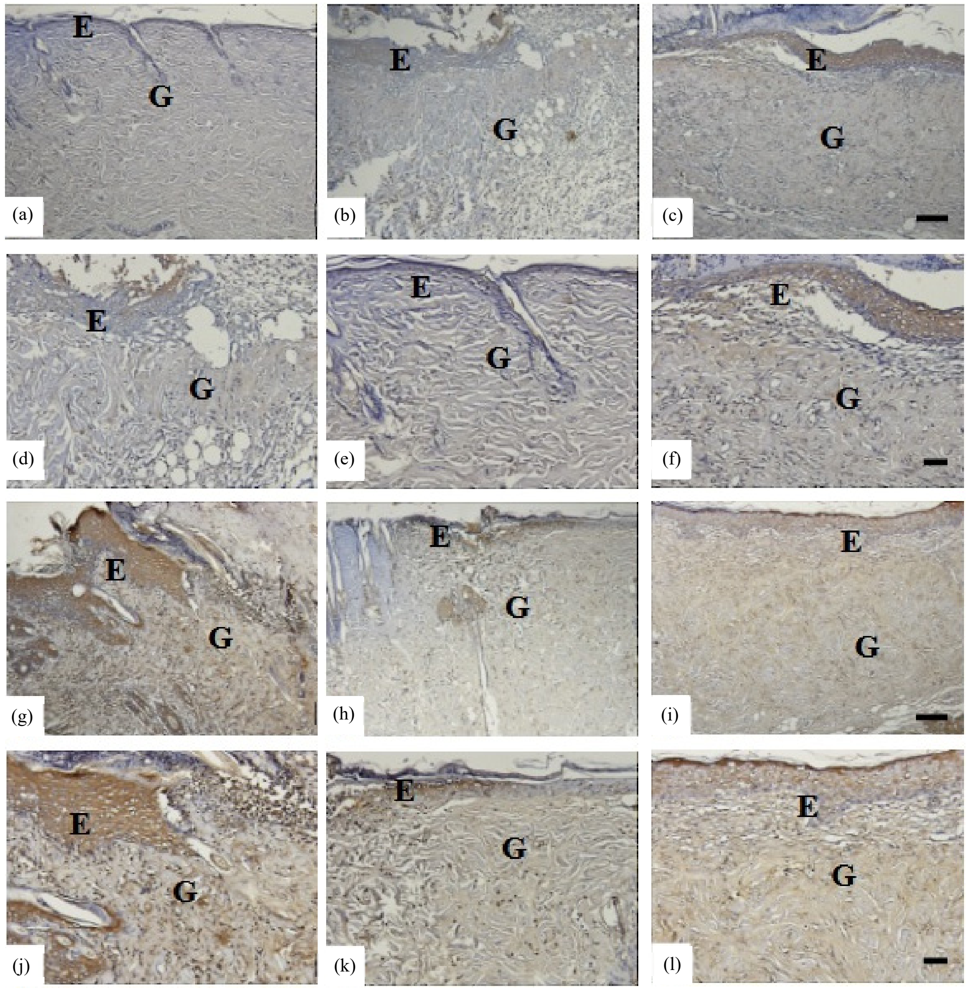

Fig. 2(a-l): Immunohistochemical expression of VEGF in the skin of rats with diabetes mellitus after hBM-MSCs treatment, (a-f) Positive control of rats with diabetes mellitus and (h-l) Rat with diabetes mellitus were given hBM-MSCs

Tissue of the experimental group showed the formation of epidermal epithelium (E) and dermis with post-burn granulation tissue (G) left panel; day 3, 7 and 14 panels

There was an increase of VEGF expression on day 3, 7 and 14 after being given hBM-MSCs but not significant on day 3 and 14 compared to the control group, whereas on day 7, there was a significant increase in VEGF expression in the treatment group compared to the control group. These occurrences showed that hBM-MSCs were able to increase the formation of VEGF as a growth factor. Previous studies conducted by researchers[13-15] found that BM-MSCs and hUC-MSCs increased the expression of VEGF in rabbit diabetic ulcer wounds, accelerated angiogenesis and played a role in the formation and thickening of granulation tissue. Increased expression of VEGF also initiated the migration of endothelial cells and the subsequent formation of new capillaries.

The implication of FGF and VEGF increasing expression levels indicated the formation of new blood vessels and tissue regeneration which were important for the healing process of burns in diabetic patients. These conditions will potentially overcome the challenges of slow wound healing in diabetic patients. Finally, the applications of this research are expected to develop cell-based therapies to improve wound healing in diabetic patients. The recommendations for further research are to extend the benefits of stem cell therapy for burns healing of diabetic patients including reviewing the administering of optimal doses, side effects testing or complications caused by this therapy and comparing this method with other therapies applied in burn healing for diabetic patients.

CONCLUSION

This study concluded that there was an increase of cytokines growth factor expression (FGF and VEGF) in granulation tissue in the healing burn wounds process of rats with diabetes mellitus by administering hBM-MSCs. There was a different pattern of increased expression of cytokine growth factors (FGF and VEGF) in the granulation tissue of burn wound healing of rats with diabetes mellitus, between experimental animals given hBM-MSCs compared to control animals. The pattern of cytokine expression resembled with histological wound healing pattern.

SIGNIFICANCE STATEMENT

The hBM-MSCs have been known to accelerate burn wound healing by increasing growth factors. The FGF and VEGF are growth factors to produce new blood vessels by initiating the formation of endothelium by FGF and VEGF forms new blood vessels at the end of healing. Hyperglycemia prolongs the burn wound healing because of the dysfunction of the inflammatory response. Previous studies revealed that hMSC was a promising treatment for various diseases including skin wounds, cuts and burns of granulation tissue and increased fibroblast apoptosis. Administering of hBM-MSCs was expected to differentiate into required cells and release VEGF and FGF as a paracrine effect, plays a role in forming granulation tissue and angiogenesis as a solution for burn wound healing for diabetic patients.

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