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In vivo study of impact transplantation hematopoietic progenitor cells on induced cutaneous wound healing in rabbits model

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Abstract

The goal of this study was to assess the effects of the Hematopoietic Progenitor Cells (HSCs) on full-thickness cutaneous wounds healing in rabbits. Twenty clinically healthy adult New Zealand White rabbits were used in this study; under aseptic technique and general anesthesia full-thickness excisional cutaneous wound; 20 mm x 20 mm were made on the back. The rabbits were randomly partitioned into two. In first group considered as control group, the wounds were treated with 3 ml of saline solution. In second group (Hematopoietic Progenitor Cells group); wounds were treated with 5x106 hematopoietic progenitor cells. The result of molecular evaluation along the interval of following-up recorded a significant difference of the level Fibroblasts Growth Factor (FGF) and Vascular Endothelial Growth Factor (VEGF) recorded at 7, 14 and 35-day post-surgical operation in hematopoietic progenitor cells group compare than control group. These outcomes were parallel with the finding of the clinical and histopathological assessment showed that hematopoietic progenitor cells group reduces inflammation and promotes epithelialization during 3th weeks with increased vasculature than those in untreated wounds. This study confirms that local implantation of hematopoietic progenitor cells leads to enhance and develop of cutaneous wound healing in rabbit model.

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Introduction

Skin wound recuperating is a dynamic and diverse interaction including a progression of procedures including inflammation; dermal reconstruction; re- epithelization; remodeling and wound contraction (1). Cutaneous wound healing is constrained by interacting signals that direct a considerable of the cellular and molecular proceeding (2). Different cell types, like cytokines, chemokines, and extracellular matrix molecules at the injury site interact with numerous foundational factors platelets, coagulation cascade and humoral cell component which promote the healing (3). However, mesenchymal stem cells were originally described as plastic-adherent fibroblast-like cells and can differentiate into adipocytes, chondrocytes and osteoblast (4). Mesenchymal undifferentiated cells can be disconnected from different locales, including bone marrow, fat tissue; and cord blood (5). Furthermore, mesenchymal stem cells are (multipotent stromal progenitor cells) that contribute to regeneration therapies by participating in the proliferative process, provocative, homeostatic, and rebuilding periods of tissue healing (6). Hematopoietic progenitor cells (HSCs) can facilitate tissue healing by growth factors, cytokines, and promoting the migration of other cells (7,8). Walter *et al.* have shown that mesenchymal stem cells are involved in reconstituting dermal fibroblast populations and healing wounds and confirmed that mesenchymal stem cells have the same favorable effects on wound healing, in addition mesenchymal stem cells which have immunomodulation and antiapoptotic activity (9). Therefore, the goal of this study was to assess the healing ability of HSCs transplantation via topical treatment to experimentally skin wounds in rabbit's.

Materials and methods

Experimental animals

All creatures were performed in accordance with conditions arrangement by the Ethics Committee of Faculty of the College of Veterinary Medicine, Al- Qasim Green University, along of the period from 10 November 2020 to 10 April of 2021. A total of twenty clinically healthy adult New Zealand White rabbits weighting 2.2±2.5 kg and 10-12 month of ages were utilized in this study. All creatures were accustomed; full- excisional cutaneous wounds 20*20 mm were made on the back of each rabbit. The skin wounds were randomly partitioned into two gatherings. In control group which treated with 3ml of saline solution. In second group; which treated with $5x10^6$ HSCs. Cutaneous wounds were left without suturing and bandaged which had been changed two times a week and wounds have been gently cleaned. The rabbits were euthanized 7^{th}, 14^{th}, 21^{st}, and 35^{th} post-operative days for assessment molecular and histopathological findings.

Hematopoietic stem cells - isolation and culture

Hematopoietic Progenitor Cells were gotten by described ways strategies Frascoli *et al.* (10). HSCs were created in a laboratory setting (issue Culture Unite, Department of Biotechnology, Al-Nahrain University. Briefly, a syringe was used to collect 1.5 ml of bone marrow from the ilium under aseptic condition, summarized in (Figure 1), and then centrifuges the conical tube at 1,500 rpm for 5 minutes, carefully removing the supernatant. Make sure not to disturb the cell pellet and then suspend and wash cells in 15 mL fetal bovine serum, and centrifuge the conical tube at 1,500 rpm for 5 min. Suspend the pellet in three ml of Dubleccos modified Eagles medium with 30 percent fetal bovine serum; penicillin G 10 U/ml, streptomycin 10 U/ml, amphotericin B 25 mg/ml; 1 percent insignificant amino corrosive and sodium pyruvate 100 ng/ml, all provided by (GIBCO/USA). In a 75 cm^2 flask, three milliliters of FBS were placed; after three minutes, the mixed media containing BM was added to the flask. At that point, the cells were cultured for three days at 37°C in 5% CO_2 in air, and after 72 hours, the non-follower cells were gathered and the medium was replaced. At day 12, the way of life reached the combination stage; monolayer cells were rinsed twice with 2 ml of PBS at pH 7.2. The way of life was next treated with two ml of 0.20 percent trypsin 0.02% ethylenediamine-tetraacetic corrosive (EDTA Sigma/USA) and applied to the exterior of the layer for two minutes. DMEM with 10% FBS was added to the medium, which was then delicately tapped to extract cells. Taping the medium from the carafe and washing with PBS yielded the cells. Then, washing with trypsin and replacing with 10 ml DMEM; medium and cells were then collected in sterile test tubes and centrifuged at 2000 rpm for five minutes. The detached hastened pellets were combined with 1ml of DMEM. To guarantee the base tally of $5*10^6$ mesenchymal stem cells, cells were examined using a hematocytometer.

Figure 1: shows initial step of isolation hematopoietic stem cells from rabbit.

Differentiation potential of mesenchymal stem cells

Following 21 days of differentiation MSC, the medium from tissue culture flask was removing. Then, the adipocytes suspension fixed with 4% paraformaldehyde 45 min at 37°C. The stem cells were flushed 3 times, for 15 min each with PBS. Then added 1 ml of Oil Red O, solution to cover each well and, incubated at 37°C for 60 min. then removal of the solution, the stem cells in the wells were flushed 3 times with 6 ml of tap water. The stem cells nuclei were stained with 0.5 ml hematoxylin for 15 minutes, the osteocytes suspension was fixed with 70% ethanol 60 min at 37°C. After aspiration of alcohol, the stem cells were flushed twice for 5 min with tap water. Then added Alizarin Red S solution for staining with incubated at 37°C for 30 min. The osteocytes containing calcium deposits stained orange red by the Alizarin Red staining solution. All these protocol was performed according to Frascoli *et al.* (10). Then, the stem cells n was examined under an inverted microscope at 200X amplification.

Creation of the wound

Prior all surgical procedures, the animals were first tranquilized with Diazepam at a dose of 1 mg/kg, a mixture of xylazine hydrochloride at a dose of 5 mg/kg with ketamine hydrochloride 35 mg/kg was directed by intramuscularly (2). The skin was prepared for aseptic surgical condition. Full thickness excisional cutaneous wounds 20*20 mm were created on the back of each rabbit (Figure 2) was performed according to (11). In control group the wounds were treated with 3ml of saline solution. While, treatment group; wounds were treated with $5x10^6$ HSCs. A clinical estimate was performed in all rabbits inside the defect area; a daily macroscopic follow-up was completed during the whole period of study. During initial five days' post-implantation, the animals were administration daily intramuscularly with a combination of penicillin and streptomycin in a dose of 10.000 IU and 10 mg/kg B.W.

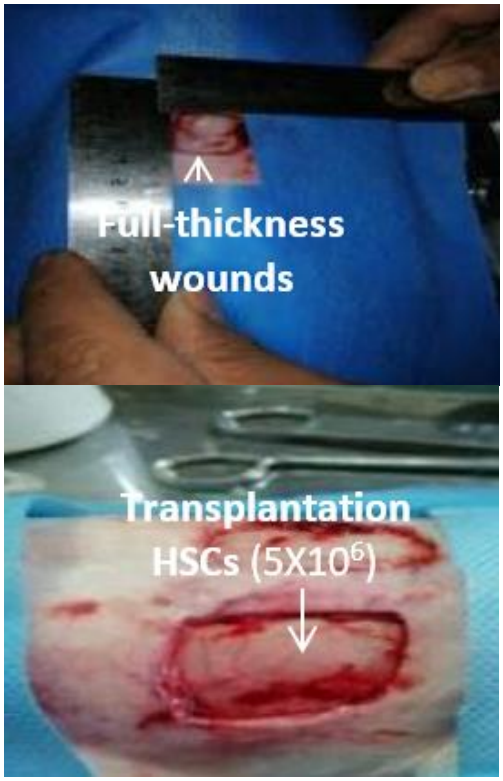

Figure 2: shows the size of induced wound and the distance between wounds with transplantation of HSCs.

Molecular evaluation of wounds healing

In each group at 7, 14 and 35-day post-surgical operation, the biopsies collection from native skin tissue and treatment wounds after surgical -operation. One gram of local tissue was gotten from each site and afterward, the examples were utilized for FGF and VEGF quality expression as referenced underneath; these biopsies were immerged in Trizol Regent, and kept in freezing, by then evaluated using Real Time Polymerase chain response procedure for identification VEGF and b-FGF quality articulation, RNA extraction as following the assembling convention. The quality and grouping of the separated RNA were surveyed utilizing a nanodrop spectrophotometer. Tests with ODA260/A280 from 1.8 to 2.0 were utilized. The uprightness of all extricated RNA was affirmed by 1.5% ethidium bromide-stained agarose gel in 1x Tris-acetic acid derivation EDTA cradle, pH 8.0. The gel picture was envisioned utilizing UV transilluminator. cDNA blend and ongoing qPCR RT-qPCR examination of relative mRNA articulation of VEGF and b-FGF as the housekeeping quality was performed utilizing rodent explicit groundworks recorded in (Table 1). cDNA amalgamation from 2 µg of the absolute RNA was performed utilizing the Intron-Power cDNA union pack following the maker's convention. At that point, the cDNA was utilized as the format for RT-qPCR. Relative quantitative RT-PCR was performed to every one of the inspected tests in three-fold utilizing green in the Mx3005P Real-time PCR. The overall changes in quality expression were determined utilizing edge cycle (CT) values that were first standardized to those of the New Zealand White bunnies house-keeping quality and utilizing ΔCT worth of control tests as calibrator utilizing the 2−ΔΔCT as indicated by the recently distributed conventions (11). In the first place, standardize the Ct of the reference quality to that of the objective quality, for calibrator test: ΔCt calibrator = Ct ref, calibrator - Ct target, calibrator. Second, standardize the Ct of the reference 3 quality to that of the objective quality, for the test Sample: ΔCt Test = Ct ref, test - Ct target, test; ΔΔCt= ΔCt test - ΔCt calibrator fold change = 2-ΔΔCt Ratio reference/target = 2Ct reference - Ct target. Along these lines, the general expression was partitioned by the expression worth of picked calibrator for every expression proportion of test.

Table 1: Shows the details of primers used in the study

Primer	Sequence	Size
GAPDH	GCAAGTTCCACGGCACAGTC CCCACTTGATGTTGGCAGGA	219
FGF	GCAGAGTGGGCATCGGTTT CTGAACTTGCAGTCATC	211
VEGF	GTGCGGGGGCTGCTGTAATGA TCACCAGGAAAGACTGACACA	213

Histopathological evaluation

On the 7th, 14th, 21st, and 35th post-operative days, routine histology procedures were done on the biopsies. Fixed biopsies with 10% neutral-buffered formalin, and then embedded in a paraffin solution, and full-thickness biopsies cut transversely by a microtome into 5 µm. Tissue sections were stained with hematoxylin-eosin (H&E) and using light microscopy examined for possible histopathological changes.

Statistical analysis

To determine the effect of different components in study parameters, the SAS application was employed in the current investigation, the least significant difference test was utilized to make a significant comparison between means.

Results

Morphology of hematopoietic progenitor cells

After 48-72 h, small heterogeneous cell colonies were seen; the cells were round shape, flattened and started to connect at the coated surface of plastic culture flask with the development of primary colonies (Figure 3A). On 10th days, the colonies expanded particularly and colonies were continuously expanded in size to form a monolayer of adherent cells and flasks were confluent (Figure 3B). On 12th days of culture at passage 1 these cells showed spindle shaped and large flatted morphology (Figure 3C). On P2 following two days post culture mesenchymal stem cells showed high conversion of axle molded cells and the cells

adjusted themselves along their longitudinal pivot. Following, 5 days, the mesenchymal stem cells at P3 subculture exhibited combination of huge, level, axle formed; round and polygonal-molded cells (Figure 3D), Following, 5 days, the mesenchymal stem cells at P3 subculture exhibited mixture of large, spindle shaped; flat, and polygonal-shaped cells, (Figure 3E), at P4, exhibited fibroblastic morphology spindle- shaped with numerous nucleoli which are a typical morphology of mesenchymal stem cells (Figure 3F).

Characterization of hematopoietic stem cells

After 14 days, mesenchymal stem cells adipogenic cells had developed, as evidenced by intracellular accumulation of red-stained lipid vacuoles when stained with Oil Red O solution (Figure 4A). After 14 days of differentiation, the osteogenic lines of mesenchymal stem cells revealed mineral deposition typical of adult osteocytes, as seen under Alizarin Red solution (Figure 4B), also deposition of calcium was determining by Alizarin Red S staining (Figure 4C).

Clinical evaluation

During the current study, no evidence of clinical complications such as infection, ulceration and no rejection of implantation stem cell. Additionally, all wounds were healed without signs of complications in all rabbits. Treatment injuries were showed up on ordinary healing process, with predominance with HSCs and decreasing of the total wounds size than untreated wound which watched all the more plainly in 21 days' post treatment and proceeded until end of the examination on 35th days (Figure 5).

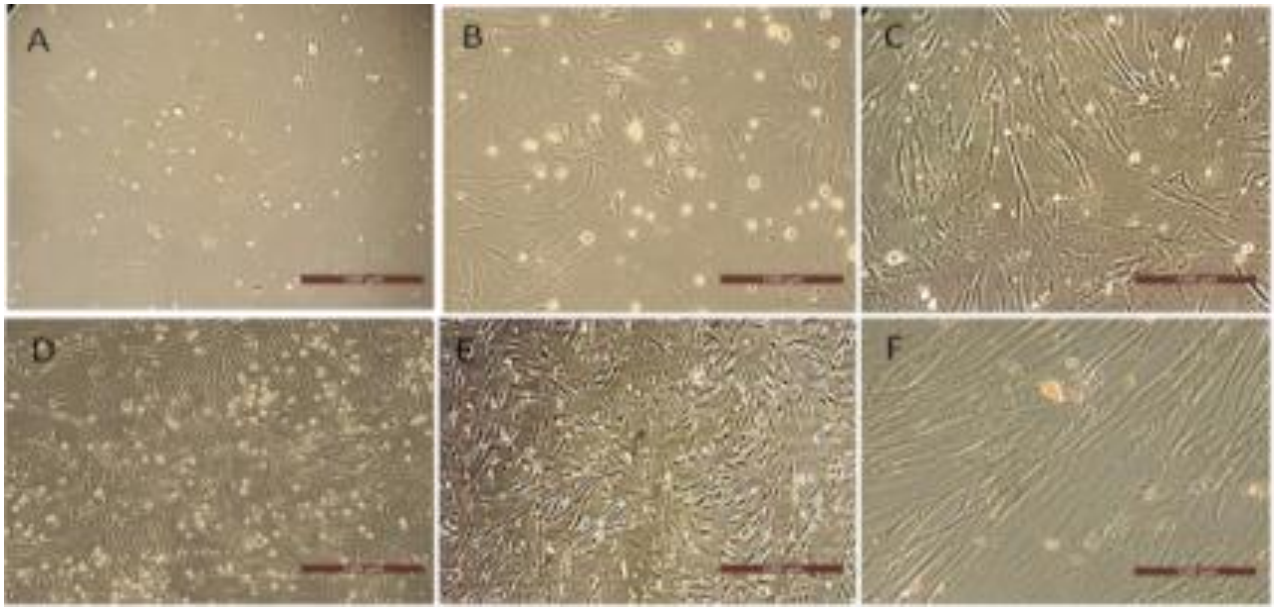

Figure 3: Stem Cells in primary cultures of HSCs growing in a monolayer under *in vitro* condition under inverted microscope; Scale bar: 100 µm.

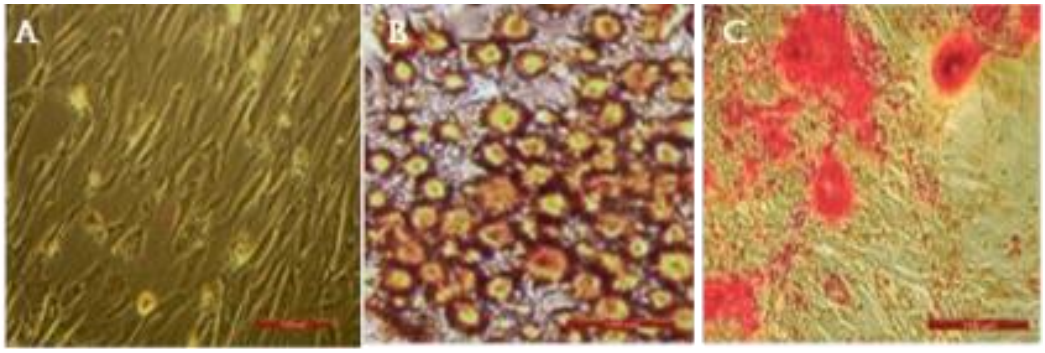

Figure 4: Multilineage differentiations of HSCs. (A) Undifferentiated mesenchymal stem cells (B) Accumulation of intracellular lipid droplets was determine by Oil Red O staining. (C) Deposition of calcium was determining by Alizarin Red S staining (Scale bar: 100 µm).

Figure 5: show differences in total wound healing between two groups at, 21st and 35th day's post-operative.

Molecular evaluation

The molecular assessment along the time of following-up recorded a significant up-regulation of the degree of both growth factors in treated contrast control groups was summarized in (Table 2). The degree of genes of VEGF was specific for angiogenesis activity. It was demonstrated significant differences of the mean values of gene of VEGF, which was higher mean in treatment groups compared to normal tissue along the period of experimental study. The mean of value was high in treated animals along of the period study contrasted to control animals, it was recorded 3.87±0.58, 3.45±1.31 and 1.85±1.74 on 7th, 14th and 35th days respectively, while in control group the mean value was 2.56±0.42, 2.12±0.14 and 1.64±6.12 on 7th, 14th and 35th days respectively. Moreover, the degree of genes of b-FGF was specific for the presence and activity of fibroblasts of the tissues in the wound site, as demonstrated in (Table 3). It referred significant differences of the mean values of gene of b-FGF, which was higher mean in treatment groups contrasted to normal tissue along the period of experimental study. The mean of value was high in treated animals along of the period study 4.12±0.63, 6.13±2.44 and 3.85±2.28 on 7th, 14th and 35th days respectively.

Table 2: Shows the degree level of VEGF gene post operation

Group	7 Day	14 Day	35 Day
Normal	1.00±0.16 Ac	1.00±0.16 Ac	1.00±0.16 Ac
Control	2.56±0.42 Ab	2.12±0.14 Bb	1.64±6.12 Cb
HSCs	3.87±0.58 Aa	3.45±1.31 Ba	1.85±1.74 Ca
LSD	0.3334		

Table 3: Shows the degree level of b-FGF gene post operation

Group	7 Day	14 Day	35 Day
Normal	1.57±0.25 A b	1.57±0.25 A c	1.57±0.25 A b
Control	3.87±0.15 Aa	4.28±2.73 Ab	2.88±4.78 Aab
HSCs	4.12±0.63 AB a	6.13±2.44 Aa	3.85±2.28 Ba
LSD	2.288		

Histopathological evaluation

The histopathological assessment of tissue biopsies demonstrated the main differences between treatment and control groups with more progression of wound healing in treatment group along of period study. In control group on 7th days showed, hemorrhage and inflammatory cells infiltration, fibrin network and small amount of granulation tissues (Figure 6A). In another section in HSCs group at same period showed, granulation tissue extends to injury site with inflammatory cells (Figure 6B). However, on 14th days, remanding of epithelialization the reorganization of the collagen high and deposition of fibrous tissue (Figure 7A), while, in HSCs group showed thick epidermal area with rete ridge over the mature granulation tissue and numerous hair follicles were reformed (Figure 7B). On 21st day, thick epidermal layer with short rete ridge over the granulation tissue with short rete ridge (Figure 8A), in HSCs group showed normal epidermal area covers mature granulation tissue with fully joined tissues (Figure 8B), on 35th day post-operative in HSCs group showed, collagen restructuring, keratinization of the skin, and the return of hair follicles a reorganization of collagen with keratinization of the skin and re appearance of hair follicles and sebaceous gland in the injury site, (Figure 9B) as well as, poor scarring was seen with advanced fibrosis in wounds in control group (Figure 9A).

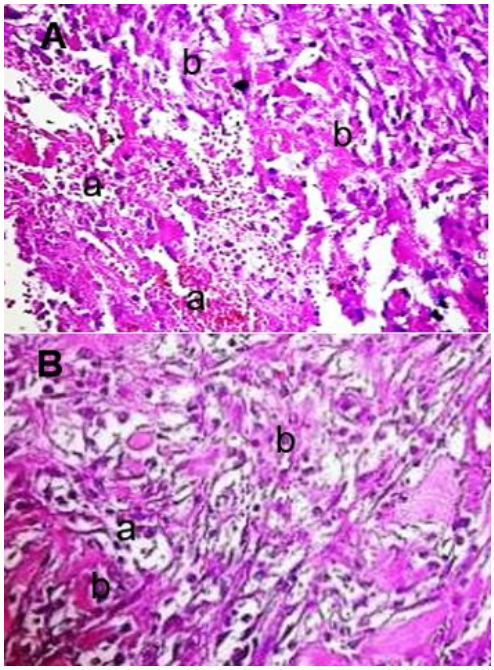

Figure 6: Representative histological sections at 7th day's post-operative (A) Control group shows hemorrhage and inflammatory cells (a) small amount of granulation tissues (b). (B) HSCs group shows inflammatory cells (a) granulation tissue extend to (injury site) (b) (H&E 20X).

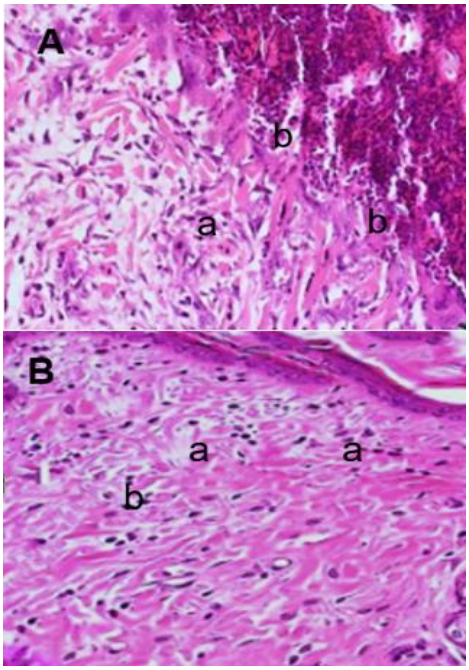

Figure 8: Representative histological sections at 21th day's post-operative (A) Control group show granulation tissue (a) with short rete ridge (b). (B) HSCs group shows apparently normal epidermal area covers mature granulation tissue (a) hair follicles sebaceous and gland (b). (H&E 20X).

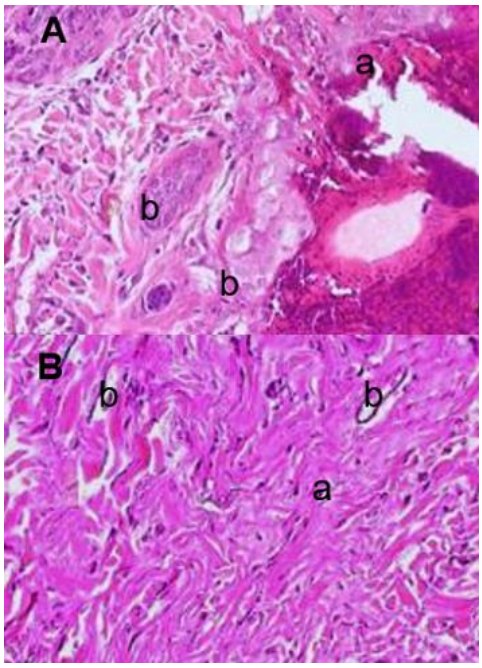

Figure 7: Representative histological sections at 14th day's post-operative (A) Control group shows hemorrhage (a) The remanding epithelialization (a) deposition of fibrous tissue (b). (B) HSCs group shows mature granulation tissue (a) hair follicles were reformed (b) (H&E 20X).

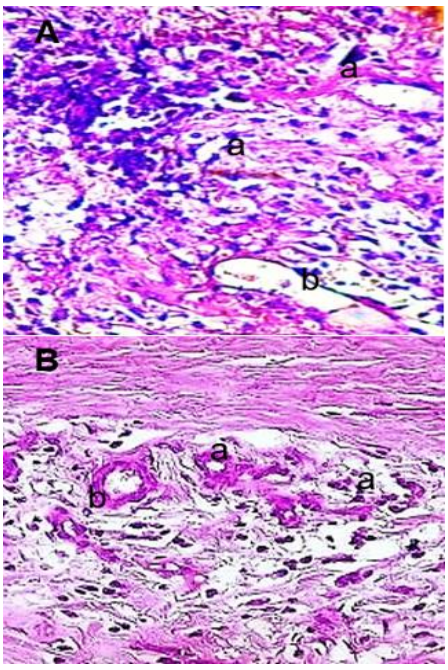

Figure 9: Representative histological sections at 35th day's post-operative (A) Control group show mature granulation tissue consisting of congested blood vessels (a) presence of collagen fibers (b). (B) HSCs group shows newly formed hair follicle (a) sebaceous gland in the injury site (b). (H&E 20X).

Discussion

In the current investigation, transplantation of HSCs was no adverse complications and no immune rejection along of period study. These findings were observed by Hadad *et al*. (12) demonstrated that the implantation mesenchymal stem cells into cutaneous wounds may prove to be beneficial, accurate placement, retention of cells and minimizing iatrogenic trauma and not provoke any signs of rejection. Morasso *et al*. (13) who showed that mesenchymal stem cells have immunosuppressive impact and there are a variety of mechanisms for this effect. Furthermore, Satoh *et al.* (14) suggested that mesenchymal stem cells modulate the activation, multiplication and function of T-cells, B-cells, natural killer cells and other immune cells like dendritic cells. These immunosuppressive impacts are halfway interceded by PGE2, IL-10, human lymphocyte Antigen-G and nitric oxide. The consequence of molecular assessment along the time of following-up recorded a significant differences of the degree FGF gene specific for the presence of fibroblasts and collagen deposition in HSCs group contrast to saline group. This outcome concurred with (15,16) which were evaluated the potential therapeutic effects of HSCs on wound healing in laboratory animals, they have been found that the implantation of mesenchymal stem cells be effective in increased level of many growth factors which lead to supporting and acceleration of cutaneous healing. Chen *et al.* (17) that alluded to VEGF as a growth factor answerable for the enlistment of angiogenesis and maximal VEGF fixation level which was found somewhere in the range of 3 and 7 days after full-thickness injuring, during the time of granulation tissue arrangement. Qi *et al.* (18) who showed that the VEGF is the important tissue factor liable for angioplasty differentiation. However, the histopathological evaluation of treated wound appeared a high incidence of new blood vessels; mature granulation tissue and myofibroblasts, in contrast at same time, few myofibroblasts were scattered through fibrous connective tissue containing congested blood vessels were appeared in the sections of control group. Likewise, the treated injuries showed a highly contraction with less scar tissue formation, comparison to the untreated injuries. The histopathological outcomes are authenticating to the findings gotten by previous studies (19,20) they referred that the advantageous effects mesenchymal stem cells on wound healing through the direct and indirect application of these cells to the wound. mesenchymal stem cells have incendiary in the inflammatory, Chen *et al*. (17) recommended that adult stem cells can partake in the repairing and regeneration of tissues damaged through two distinct unmistakable; direct contribution by trans differentiation and creation of tissue ECM or indirect commitment by creation of bioactive proteins of healing tissue, Similar conclusion have been gotten by a study in a murine excisional injury by Duscher *et al*. (20) who revealed that mesenchymal stem cells promotion wound healing by increasing re-epithelization and angiogenesis as well as, mesenchymal stem cells express multiple growth factors VEGF, EGF, TGF, HGF and FGF-2 in a full thickness wound excisional, which direct cells signaling events and tissue repair through stimulate the dermal fibroblasts migration and proliferation, which provide tissue strength and resiliency collagen and elastin and creation of ECM, just as, the significant enhancing of neovasculogenesis withdrawal in any inflammatory process and then promotion the time of healing (21-23).

Conclusion

That utilizing of transplantation of hematopoietic stem cells improves and enhanced skin wounds healing into enhances cell migration, makes a prior differentiation of fibroblasts to myofibroblasts and hence allows earlier wound remodeling and improves the healing wound.

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Conflict of interest

The authors declare that no conflict of interest exists.

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دراسة تأثير الخلايا الجذعية المكونة للدم على التئام الجروح الجلدية المستحدثة في الأرانب

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[١]قسم التقنيات الحيوية الجزيئية والطبية، جامعه النهرين، بغداد، [٢]فرع الجراحة والتوليد، كلية الطب البيطري، جامعة القاسم الخضراء، بابل، العراق

الخلاصة

صممت هذه الدراسة لتقييم تأثير زرع الخلايا الجذعية على التئام الجروح الجلدية، تم استخدام ٢٠ من الأرانب الذكور البالغة في هذه الدراسة. تحت تأثير التخدير العام، تم استحداث جرح بأبعاد ٢٠×٢٠ ملم على جانبي الظهري من الجانب الصدري الجانبي لحيوانات التجربة. تم تقسيم الحيوانات بشكل عشوائي إلى مجموعتين متساويتين عشرة أرانب لكل مجموعة. في المجموعة السيطرة؛ تم علاج الجروح لمجموعة السيطرة بحقن ٣ مل من المحلول الملحي. بينما في المجموعة المعالجة (مجموعة الخلايا الجذعية) تم العلاج بخلايا الجذعية بجرعة ٥×١٠٦. وقد سجلت نتيجة التقييم الجزيئي على طول فترة المتابعة فروق معنوية لمستوى عامل نمو الخلايا الليفية في مجموعة الخلايا الجذعية على النقيض من مجموعة السيطرة في نهاية الدراسة. كذلك أظهرت ارتفاعا ملموسا في مستوى الجين التقليدي المولد للأوعية الدموية في المجموعة المعالجة الخلايا الجذعية بفروقات مهمة إحصائيا مقارنة ب مجموعة السيطرة. هذه النتائج تناغمت مع نتائج دراسة المقاطع النسجية المرضية حيث أظهرت أن المجموعة المعالجة باستخدام الخلايا الجذعية في علاج الجروح الجلدية خلال الأسابيع الثلاثة الأولى قد ساعدت في تسريع وتحسين التفاعل الخلوي، وزيادة الأوعية الدموية مقارنة مع تلك الموجودة في الجروح غير المعالجة. نستنتج أن العلاج بالخلايا الجذعية قد أدى إلى تعزيز وتسريع التئام الجروح الجلدية.