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Effect of the Bone Marrow Mesenchymal Stem Cells on the Wound Healing Mediators of the Full Thickness Scald Injury

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ABSTRACT

Full thickness thermal injury causing severe tissue destruction resulting in excess scarring and disfigurements, so its therapy represents agreat challenge.The severity of this type of wounds could be ameliorated through controlling the wound healing mediators which could be achieved through using of bone marrow mesenchymal stem cells (BM-MSCs) as a type of regenerative therapy. 90 albino rats were divided into 3groups; group I (control), group II (scald model) the animals were exposed to water at 100^c for 10 sec under anesthesia, and group III (the scalded animals subcutaneously injected with BM-MSCs (2×10^6 cells/ mL),they were clinically observed and sacrificed at different time intervals and skin samples were collected for histopathologicalexamination, and determiningthe expression rate of different wound healing mediators *via* quantitative polymerase chain reaction (qPCR). Therapy with BM-MSCs leading to significant down regulation of interleukin-6 (IL-6), tumer necrotic factor-α (TNF-α), metalloproteinase-9 (MMP-9), andmicroRNA-21 (miR-21),and marked up-regulation of transforming growth factor-β (TGF-β), andheat shock protein-90α(HSP-90α) specially in late stage, whilst the inflammatory cells and the scab were still detected until 14 day post treatment.

In conclusion: BM-MSCs have the ability to regulate the expression rate of the mediators that incorporated in the healing process of full thickness scald and enhancing the wound to progress to the inflammatory phase not to return to the chronic phase.

Key words
BM-MSCs; Full thickness scald; Histopathological;qPCR; Inflammatory mediators;Metalloproteinase-9;microRNA-21;heat shock protein-90α

Introduction

Skin damage from hot liquids exposure usually hot water (Knight, 1996) or molten rubber, tar, oil, liquid chemicals, hot gases, or steam called scald (Wohlsein*et al*., 2016). Owing to the long recovery period of scald injuries, they consider one of the most expensive traumatic injuries (Sánchez *et al*., 2007),Death from scald may be occur in all ages, but very young people and elderly are at a higher risk than other age groups (Marshall, 1998), whereas 7% TBSA scald may lead to 22% mortality (Alden *et al*. 2007). In severe cutaneous burn injury, the natural healing process and the endogenous source of SCs in the basal layer are scanty for ideal repairing the extensive deep damage (Kanji and Das, 2017).

Treating of burns *via* cells has been established in 1975 *via* Green, starting with epithelial autografts and followed by dermal and dermal-epidermal bio-engineered cultured skin substitutes till emerging of SCs (Leclerc *et al*., 2011). Introduction of SCs in the cellular therapy aims to mediation of the cellular replacement and protection, adjusting the microenvironment of the injury and enhancing the self-regenerative and reparative processes (Pati*et al*., 2015). Treatment of burn injury with iPSCs, allogeneic MSCs or immunomodulatory T-cells causing modulation of the post-burn immunosuppression, hypermetabolic response, and ARDS (Butler *et al*., 2010).

Primarily, BM-MSCs and embryonic fibroblasts have been used to treat burn injury in rats, in which BM-MSCs caused significant reduction in the wound cellular infiltration and enhancing new blood vessels and granulation tissue genesis than the embryonic fibroblasts (Shumakov*et al*., 2003). MSCs have the ability to mingle with sebaceous glands, blood vessels, and hair follicles of skin after their engraftment into injuries (Lewis, 2013), helping in wound bed reconstruction and regulation of tissue repair (Dash *et al*., 2009).

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In relation to the affected layers of skin from the applied heat, severity of burns is categorized into different degrees ranging from first to forth degree (Merck and Miller, 2013), in which the developed lesions vary from mild erythema to severe necrosis (Munro and Munro, 2008).The current study aims to determine the efficiency of BM-MSCS in treating the full thickness scald in a rat model**.**

2. Material and methods

2.1. Chemicals:

DMEM (Dulbecco's modified Eagle's medium), Fetal bovine serum (FBS), penicillin streptomycin solution, and trypsin/EDTA, were purchased from Lonza, Belgium. Sodium hydrogen carbonate was purchased from LOBA Chemie, India. Culture consumables and culture flasks were purchased from Greiner Bio-One (Germany).

2.2. Isolation of BM-MSCs:

2.2.1. Preparation of complete culture medium:

According to Sun e*t al.* (2007) solution of penicillin streptomycin, FBS, and sodium hydrogen carbonate were added to 89% DMEM for preparation of the complete medium.

2.2.2. Isolation and culture of BM-MSCs:

For isolation,4-6 weeks old male albino rats were sacrificed *via* decapitation, followed by complete sterilization of the whole body surface through spraying ethyl alcohol (70%), then the tibiae and femurs bones were dissected out and the whole surrounding tissue removed. Under complete a septic conditions in a Biobase vertical laminar flow cabinet (Biobase, Model: BBS V1300; NO-51, South Gongye Road, Jinan, Shandong Province, China), the epiphysis of the previous dissected bones was cut off just below the growth plate by using sterile fine scissors, for harvesting of the cells, the bone marrow was flushed with DMEM, and the solution was collected in a Falcon tube (15 mL), which centrifuged for 5 min at 3000 rpm for dislodgement and separation of the cells, the supernatant was discarded and the cell pellet was washed with phosphate buffer saline (PBS) rapidly, and suspended in the previously prepared complete culture medium. The viable and dead cells were counted post staining with trypan blue solution (0.2%) which detect the viability of the cells, counting were done *via* the hemocytometer at 100X magnification (the number of the viable cells was relative to the total number of the cells).

In T-25 cm_2 sterile Greiner cell culture flasks with canted neck, 2.5×10^6 cells were seeded at the cell density of 1×10^6 cells/ cm_2 area, then placed in 5% CO_2-humidified incubator (Biobase, Model: BJPX-C50; South Gongye Road, Jinan, Shandong Province, China) at 37°C. Floating and non-adherent cells, as well as dead cells, were removed after 4 days from incubation, and through 7-10 days of culturing and incubation, the adherent cells were washed twice with sterile PBS (pre-warmed at 37°C), and trypsinized with 1 to 2 mL of trypsin (0.25%)/EDTA (1 mM) (pre-warmed at 37°C) for 2-3 min. The detachment of the adherent cells was warranted *via* examiningthe cells under an inverted biological microscope (Novel, Model: NIB-100; Jiangsu, China). Adding 3-5 ml complete culture medium for stopping the action of trypsin, which followed by collection of cells and centrifugation for 5 min at 3000 rpm, the cells were re-suspended in the culture medium after washing two times an incomplete DMEM. After that the cells were counted and viability was assessed by adding of an equal volume of trypan blue (0.2%).The procedures of isolation and culturing were done according to Chaudhary and Rath. (2017) with some modifications.

2.3. Animals:

Ninety healthy male albino rats (*Rattusnorvegicus*), about 100-150 g, were used. Animals were maintained under observation for about 7 days, for ensuring the absence of to any infections. In animal house department, the animalswere housed in plastic cages covered with stainless steel cover, at normal temperature, with enough food and water *ad-libitum.* All animal procedures were conducted according to the standards set forth guidelines for the care and use of the experimental animals by the Animal Ethics Committee of Zoology Department,Faculty of Science,

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Beni-Suef University (Approval number is BSU/FS/020-110).

2.4. Experimental design:

Animals were divided into 3 groups (6 animals /time interval) as follows: Group I (control group) animals were not exposed to burn. Group II (scald group) Rats were anesthetized by intraperitoneal (IP) injection of (80 to 100 mg/kg) ketamine and (12 to 10 mg/kg) xylazine, and hair of the dorsal abdominal area was removed using hair removal cream. For induction of a full thickness scald wound, the animal's back was immersed in water at 100°C for 10 sec which covering 20% TBSA. Immediately after scald injury, the animals were IP resuscitated with saline (50 mL/kg) (Yang *et al*., 2012).Group III (scalded animals treated with BM-MSCs), the isolated BM-MSCs were suspended in an incomplete DMEM with viability greater than 95% and rapidly injected subcutaneouslyin the burned animals at a dose of (2×10^6 cells/mL) (Revilla *et al*., 2018).The clinical course of skin lesions was evaluated for 14 sequential days. The wound retraction was determined using a caliper in the tested time intervals. Wound contraction was expressed as reduction in percentage of original wound size through using the following formula

The Contracture rate = wound size in the specific day /wound size in the original state × 100% (Galiano*et al*., 2004).

At the end of each tested time intervals (zero, 2, 6, 12 &24hr, 7 and 14 day),animals underwent anesthesia *via* a mixture of alcohol, chloroform, and ether (ACE) for sacrification, thenskin samples were collected , which immediately divided into two parts, in which parts of 1 mm^3 thick were fixed in 10% neutral buffered formalin for 24 hr, and then transferred into 70% ethyl alc. to carry out the histopathologicalexamination, while others were immediately snap frozen in liquid nitrogen and then preserved under -80^oC for determining the expression rate of variable wound healing mediators.

2.5.Histopathological examination:

The fixed skin specimens were dehydrated, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) (Bancroft and Gamble 2002).

2.6. Quantitative assay of the m-RNA levels of the pro-inflammatory cytokines (IL-6 and TNF-α) and TGF-β genes *via* Polymerase chain reaction (PCR):

Total RNA was extracted *via* the RNA easy Extraction Kit (Qiagen) according to the manufacturer's instructions. Both yield and purity were assessed at 260 and 280 nm respectively using Nano drop ND-2000 spectrophotometer (Thermo Electrom). A total amount of 1 ug RNA was used for cDNA synthesis by Viva 2- steps RT-PCR Kit according to the manufacturer's guidelines. Quantitative PCR using Thermo Scientific Verso 1-Step RT-PCR Ready-Mix kit (Applied Bio systems, Foster City, CA, USA) was performed to analyze the mRNA rate of the target genes. The primers used for amplification were as follows:

B-actin,

F: 5- d TCCCTGAAGTACCCCATGGAG-3′,

R: 5′- d TTGGCCTTGGGGTTCAGGGGG-3,

IL-6

F: 5′-GCCT TCTTGGGACTGATG-3,

R: 5′-TGGTCTGTTGTGGGTGGT-3′,

TNFα

F: 5′-GCTGAGGTTGGACGGATAAA-3′,

R: 5′-AAAATCCTGCCCTGTCACAC -3′,

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and TGF-β F: 5′-TGGCGTTACC TTGGTAACC- 3′,

R: 5′- GGTGTTGAGCCCTTTCCAG- 3′

2.7. Quantitative assay of the m-RNA levels of HSP-90α, MMP-9, and miR-21 genes *via* Quantitative Real time-PCR (RT-qPCR):

RNA was extracted *via* nucleic acid extraction kit (NucleoSpin®) purchased from Macherey- Nagel GmbH & Co. KG- Germany (REF 740955.50) according to the manufacturer's protocol. The purity (A260/A280 ratio) and the RNA concentration were obtained using spectrophotometry (dual wave length Beckman, Spectrophotometer, USA).cDNA Synthesis *via*Vivantis, ViPrimePLUS One Step Taq RT-qPCR Green Master Mix I with ROX (SYBR Green Dye) (cat no #QLMM14-R) kit according to the manufacturer's protocol.The prepared reaction mix samples were applied in real time PCR (Step One Applied Biosystem, Foster city, USA) was done to analyze the mRNA expression rate of the target genes. The primer sets used for amplification as follow,

β-actin F:TGACAGGATGCAGAAGGAGA,

R:TAGAGCCACCAATCCACACA,

HSP-90 F:TGTTGGGACCAGCAACTCAA,

R: TTTGAGGCTCAGTGGTAGCC,

MMP-9 F:GGCAGCTTCAACAACCATCA,

R: GGATGGACTAGATCGGAGCC,

6UB F: AACGCTTCACGATTTGCGT,

R: CTCGCTTCGGCAGCACA ,

and miR- 21 F:TAGCTTATCAGACTGATGTTGA,

R: GAATCGAGCACCAGTTACGC.

The RQ of each target gene is quantified according to the calculation of delta-delta Ct (ΔΔCt). We calculated the RQ of each gene by taking $2^{-\Delta\Delta Ct}$.

2.8. Statistical analysis:

All data analyses were performed using SPSS version 9.0 (SPSS, Inc., Chicago IL).

3. Results

3.1. Clinical evaluation:

Immersing of the animal's back in boiling water at 100°C for 10 sec causing slight cutaneous redness and blistering for 2 hr after exposure, which become pale with time and the ruptured blisters was developed at 6, 12 & 24hr in both treated and non-treated animals, but the number of ruptured blisters was reduced in the treated animals. A thin crust or thick crust in some animals was observed after 7 days from the immersing which sloughed and the underlying granulation tissue was appeared at 14 day. The most obvious observations that noticed in the treated animals with BM-MSCs, are the developing of bluish coloration at 12 hr post immersing and the thick stiff crust which detected at 7 days still continued until the end of the experiment (at 14 day) (Fig. 1).

Concerning the percentage of the wound contraction rate in scald animals (G2), it's markedly decreased at 14 day, while in comparison with the treated animals (G5) the rate was increased significantly at 12 hr (Fig. 2).

3.2.Histopathological findings:

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Skin sections of scalded male albino rats (G2) were showed severe necrosis of the epidermal and dermal layers additionally, severe degenerative changes and necrosis of hair follicles, sweat, and sebaceous glands with the presence of coagulative acidophilic fused collagen fibers (Fig. 3 A, B &C), and (Fig. 4 A, B, C & D).
Skin sections of the treated animals with BM-MSCs (G5) were showed the similar pathological lesions in both epidermal and dermal layers including sweat gland, sebecous glands and hair follicles without any improvement (Figs. 3 a, b &c) and (Fig.4 a, b, c &d).

3.3.mRNA expression rate of TGF-β and the pro-inflammatory cytokines (IL-6 & TNF-α):

The TGF-β mRNA expression rate in all-time intervals of scald healing was significantly down regulated in comparison to the control group (G1), In group (3) in which the scald animals treated with BM-MSCs, animals showed noteworthy down regulation in the expression rate at zero time, 2, 6 & 24 hrand the significant up-regulation was detected at 12 hr, 7 & 14 day(Fig. 4). Figure (5) was illustrated that the expression rate of IL-6 was remarkably up-regulated at at all-time intervals in comparison to the control group (G1).In contrast, S/C injection of BM-MSCs in the scalded animals (G3) causing significant down regulation in IL-6mRNA expression rate at all-time intervals.

In comparison to the control group (G1) the expression rate of TNF-α in the scalded animals was salient down regulated at acute intervals of wound healing (zero time, 2, 6, 12 & 24 hr),after treatment with BM-MSCs (G5), the expression rate was significantly down regulated in all-time intervals (Fig. 6).

3.5.mRNA expression rate of HSP-90α, MMP-9 and miR-21 genes:

Significant down regulation of HSP-90α expression rate was detected in the animals subjected to scald (G2) in comparison to the control group (G1). On the other hand, treating of the immersed animals with BM-MSCs (G3) leading to significant up regulation of HSP-90α expression rate in 6&24 hr and 7 &14 day (Fig. 7). While the expression rate of MMP-9 was markedly up-regulated at all-time intervals in comparison to the control group (G1). In contrast, the rate was significantly reduced at 6&24hr, and 7&14 day after the S/C injection of BM-MSCs (G3)(Fig. 8). Figure (9) was revealed significant up-regulation of miR-21 expression rate in all-time intervals of the scald wound (G2) in comparison to the control group (G1). Whilst, the expression ratewas significantly down regulated at 6& 24 hr, and 7 & 14 day.

4. Discussion

Hemostasis, inflammation, proliferation, and remodeling are well-orchestrated supportive dynamic processes which responsible for repairing the cutaneous injury (Reinke and Sorg, 2012). MSCs have a significant role in all phases of wound repair by varying grades. They could stimulate the wound to proceed towards the inflammatory stage not to fall back to the chronic wound state (Aggarwal and Pittenger, 2005), in the current studythe inflammatory cells still reported till 14 day which agreed with Caliari-Oliveira *et al*. (2016). The immunomodulation property of MSCs enable them to attenuate the inflammatory response directly through inhibition the pro-inflammatory cytokines production as TNF-α and IFN γ with simultaneous elevation of the anti-inflammatory cytokines secretion such as IL-10 and IL-4 (Aggarwal and Pittenger, 2005), besides their inhibitory effect on neutrophil infiltration and IL-6 (Abbas *et al*., 2018), and the successful wound healing is achieved through resolution of inflammation (Xu*et al*., 2012) which recorded in the current study in which the expression rate of IL-6 and TNF-α were significantly down regulated,while the expression rate of TGF-β was significantly up-regulated 14 day that come in accordance with Gilbert *et al*. (2016).The wound closure has been accelerated in response to MSCs or MSC-conditioned medium, because of their ability to stimulate the dermal fibroblasts which produce huge amount of collagen type I and alter the gene expression (Smith *et al*., 2010).

Li *et al*. (2012) reported that growth factors not the only factors responsible for wound closure. But also, Hsp90α has a significant role in this process *via* promoting the cellular survival and motility (Dong *et al*., 2016). Moreover, it was responsible for the migration of human epidermal and dermal fibroblasts (Cheng *et al*., 2008).

The expression rate and the activity of gelatinase MMPs (MMP9 and MMP2) were significantly up-regulated through TGF-β, in which it's expression rate markedly down-regulated with inhibition of miR-21 (Liu *et al*., 2016), other results reported that the miR-21 was in-dispensable for TGF-β-driven keratinocyte migration *in vitro* and

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could enhance the process of re-epithelialization during wound healing in mice (Yang *et al*., 2011).

The Paracrine function of BM-MSCs causing up-regulation of TGF-β3 and MMP-9, leading to increasing the formation of granulation tissue with inhibition ECM deposition during the proliferative phase (Gilbert *et al*., 2016). MSCs have the ability to inhibit the regression of collagenous matrix and maintaining the matrix with promotion the regeneration of fibroblast through down regulation the expression rate of (MMP)-1 (Jeon*et al*., 2010), hence, they providing favorable conditions for ECM remodeling during the healing process (Lee *et al*., 2012).

Recent study by (Jiang *et al*., 2020) revealed that the activation of TGF-β receptors on MSCs may be up-regulated or suppressed the expression level of miR-21, according to the concentration of TGF-b1 in the wound bed, while over activation of TGFβ receptors on MSCs at high TGF-β1 concentrations down-regulates miR-21, so no inhibition of Smad7 translation occurred *via* miR-21 and ultimately production of TGF-b1 from MSCs was suppressed. Thus the signaling between TGF-β receptor, miR-21, Smad7 and TGF-β1 regulates the release of MSC-derived TGF-b1 production according to the wound site demands (Jiang and Scharffetter-Kochanek, 2020). In the current study the expression rate of MMP-9 and miR-21 was down regulated significantly at 6&12hr and 7&14 day in the treated animals BM-MSCs.

5. Conclusion

Therapy of full thickness scald wound with BM-MSCs leading to significant down regulation of the expression rate of the pro-inflammatory cytokines (IL-6 and TNF-α), miR-21,and MMP-9, marked up-regulation of TGF-β, and HSP-90α specially at late phases of wound healing, while no improvements were recorded at the level of clinical evaluation and histopathological examination.

Conflict of interest

The authors declare no potential conflict of interest.

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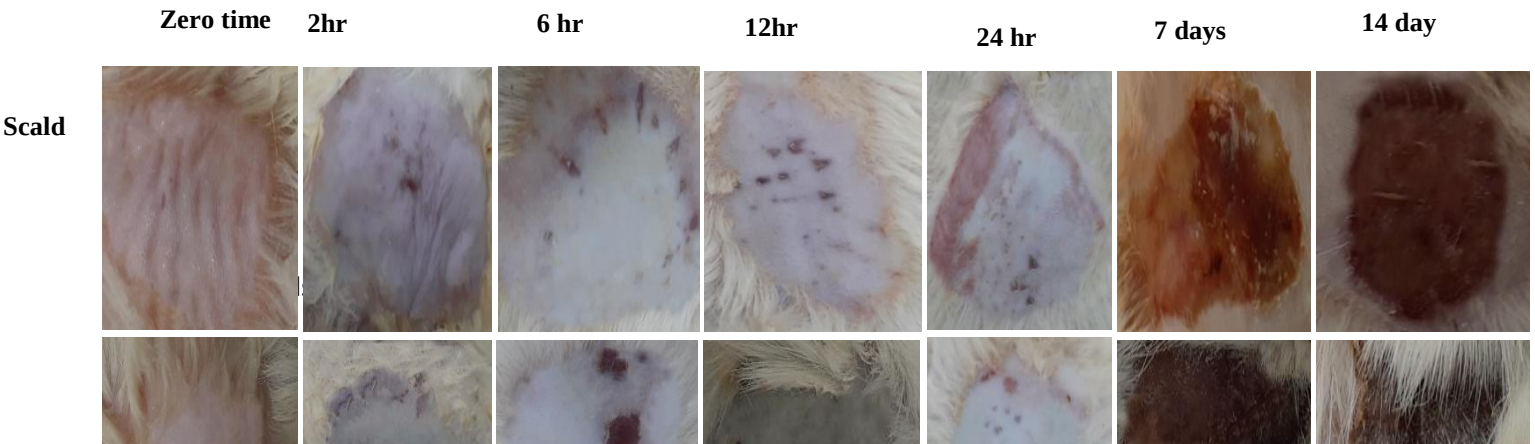

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Scald&
BM-
MSCs

Fig. (1): Macroscopic appearance of scald wound and the treated wound with BM-MSCs.

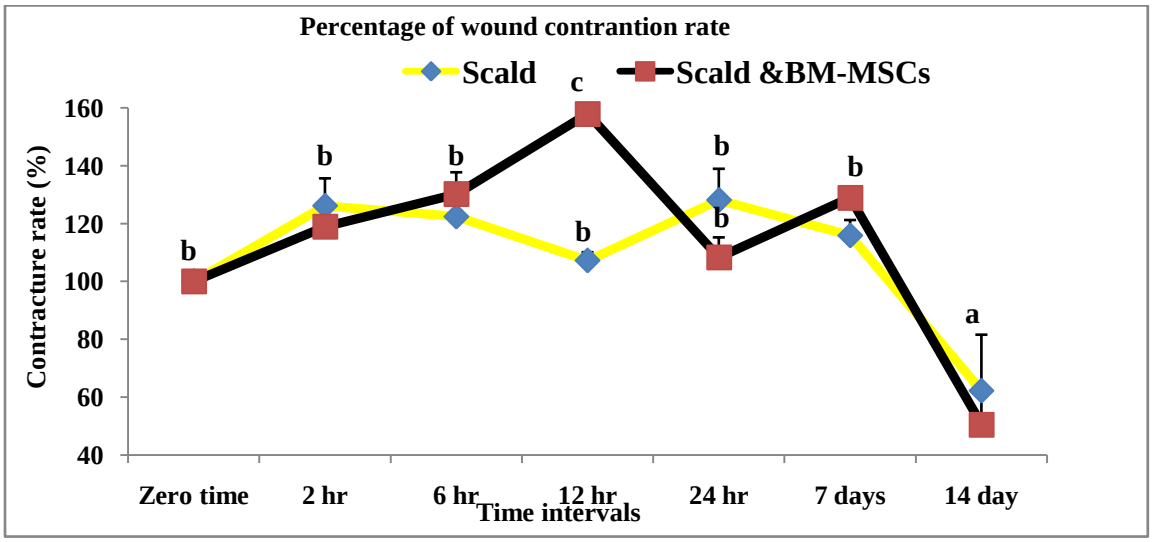

Fig. (2): Percentage of the contracture rate of scald wound and the treated wound with BM-MSCs.

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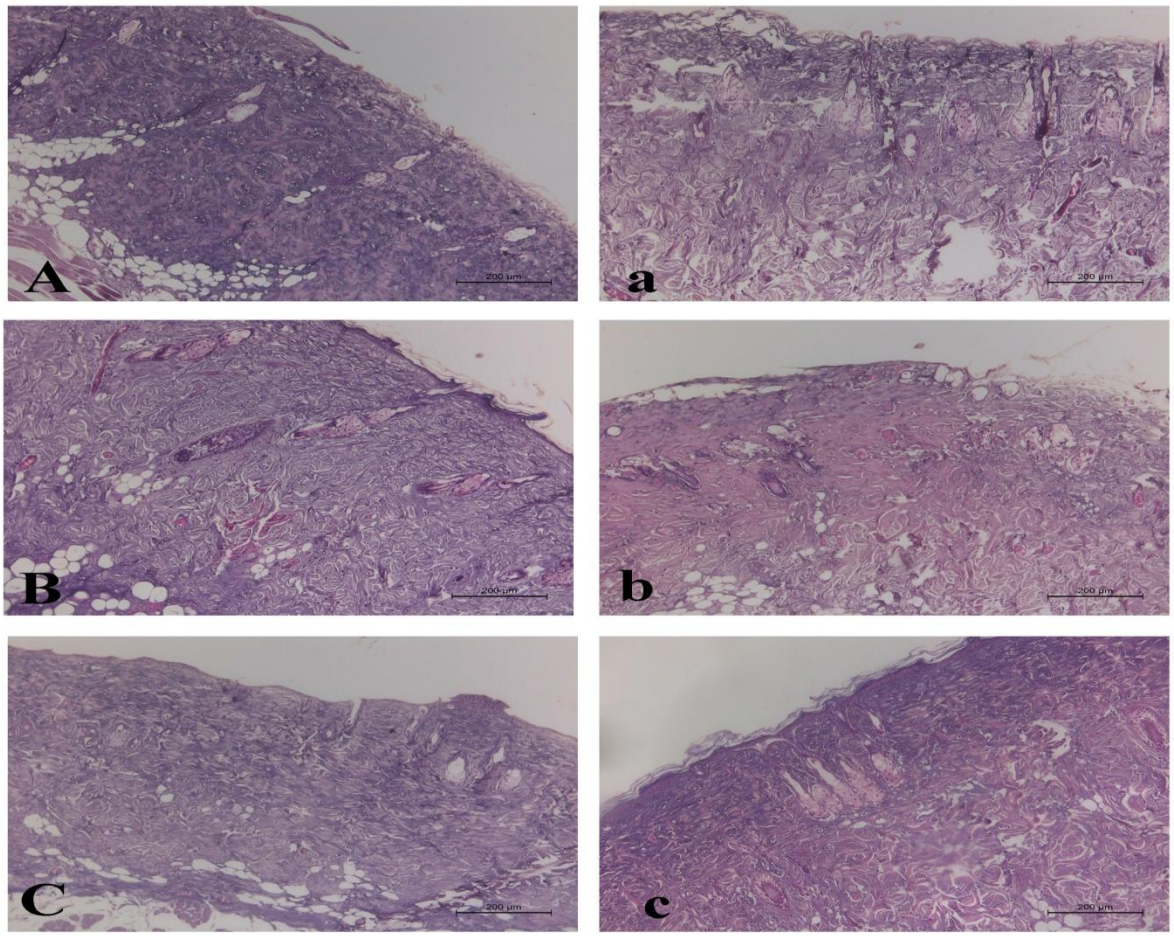

Figure (3): Histopathological alterations of the scald wound including zero time, 2 &6 hr in A, B & C respectively, and the treated wound with BM-MSCs for the same corresponding hours in a, b&c respectively.

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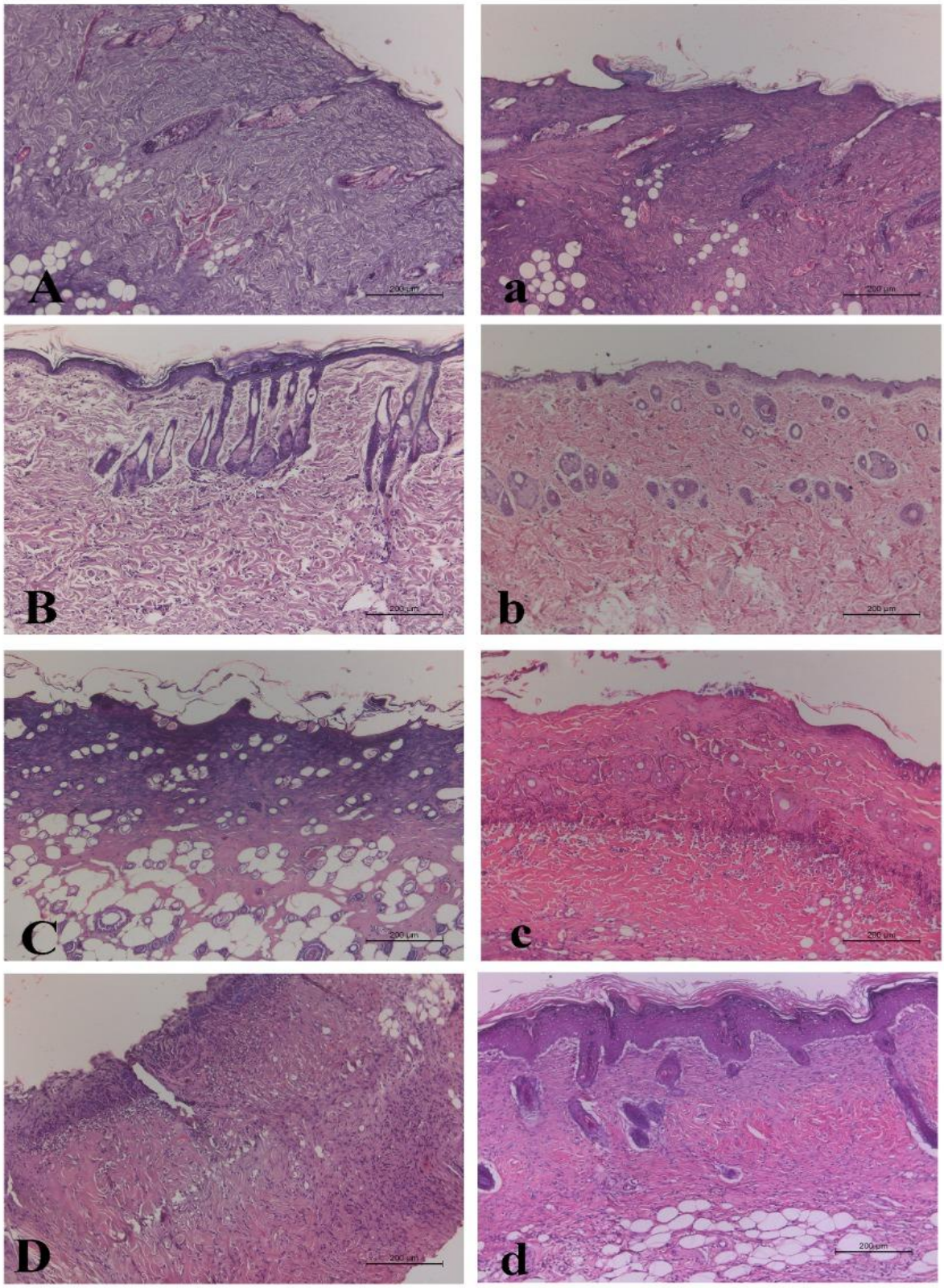

Figure (4): Histopathological alterations of the scald wound including 12&24hrand 7 &14 day in A, B, C&D respectively, and the treated wound with BM-MSCs for the same corresponding hours in a, b,c&drespectively.

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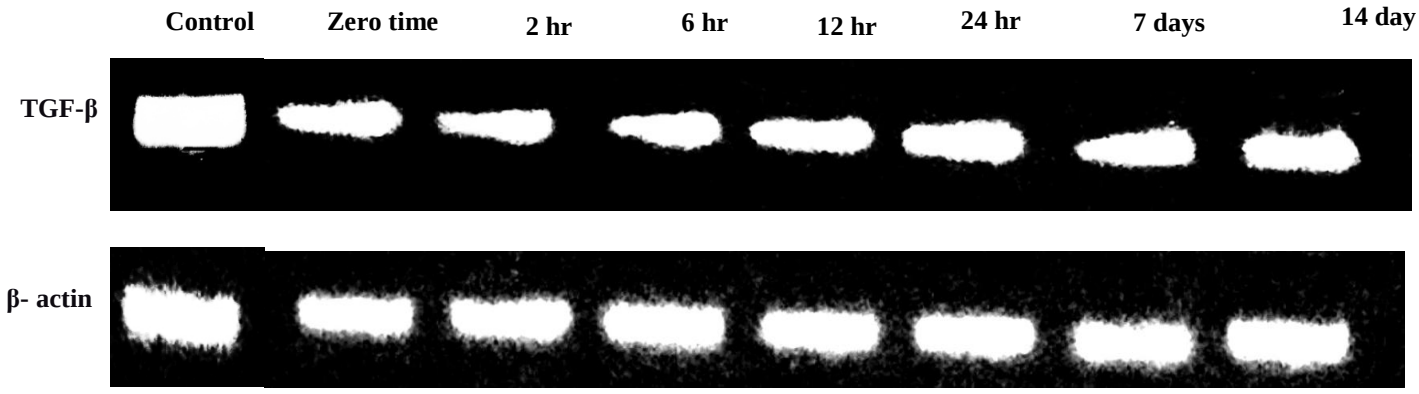
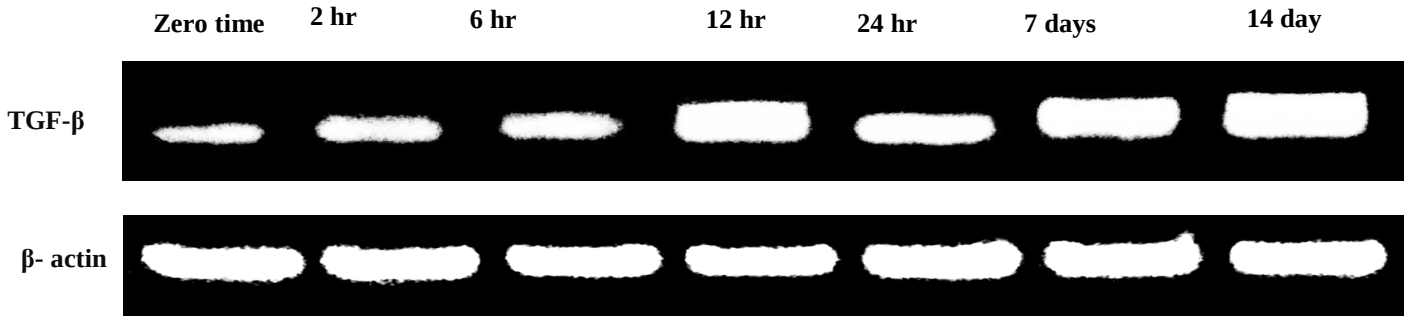
A: Gel photographshowing PCR products of TGF-β in scald



B: Gel photograph showing PCR products of TGF-β in scald& BM-MSCs



C: Desinometric analysis of PCR products of TGF-β

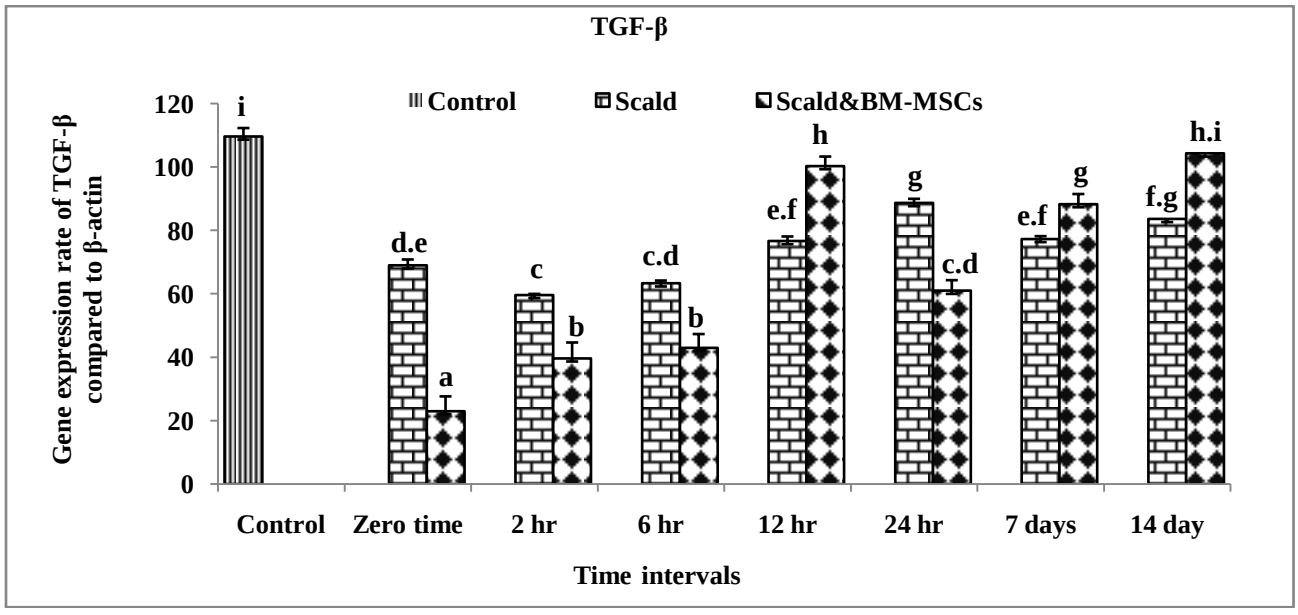


Fig. (4):mRNA expression rate of TGF-β in scald wound and the treated wound with BM-MSCs.

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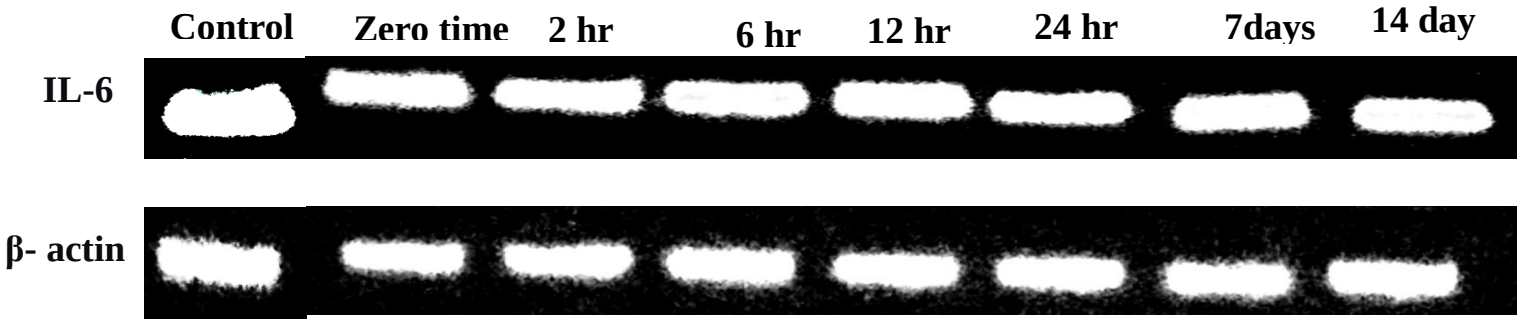
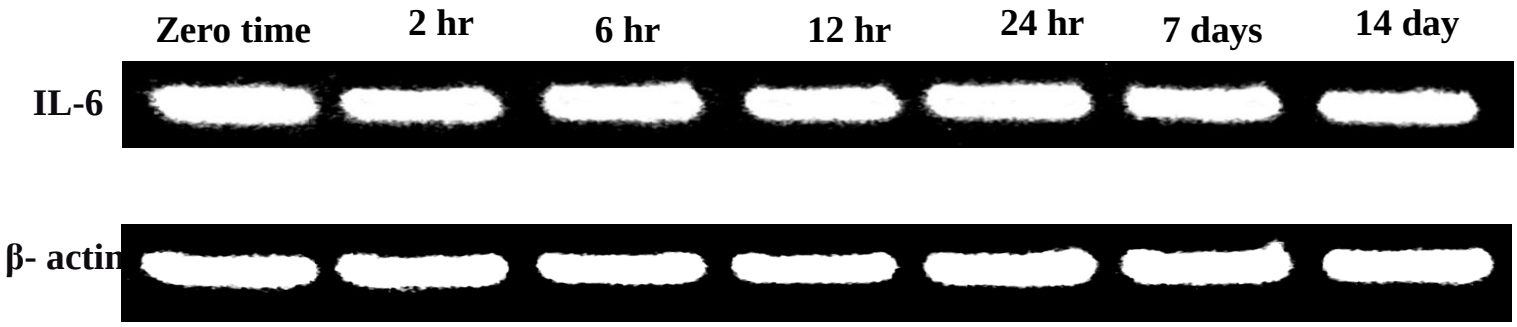
A: Gel photograph showing PCR products of IL-6 in scald



B: Gel photograph showing PCR products of IL-6 in scald& BM-MSCs



C: Desinometric analysis of PCR products of IL-6

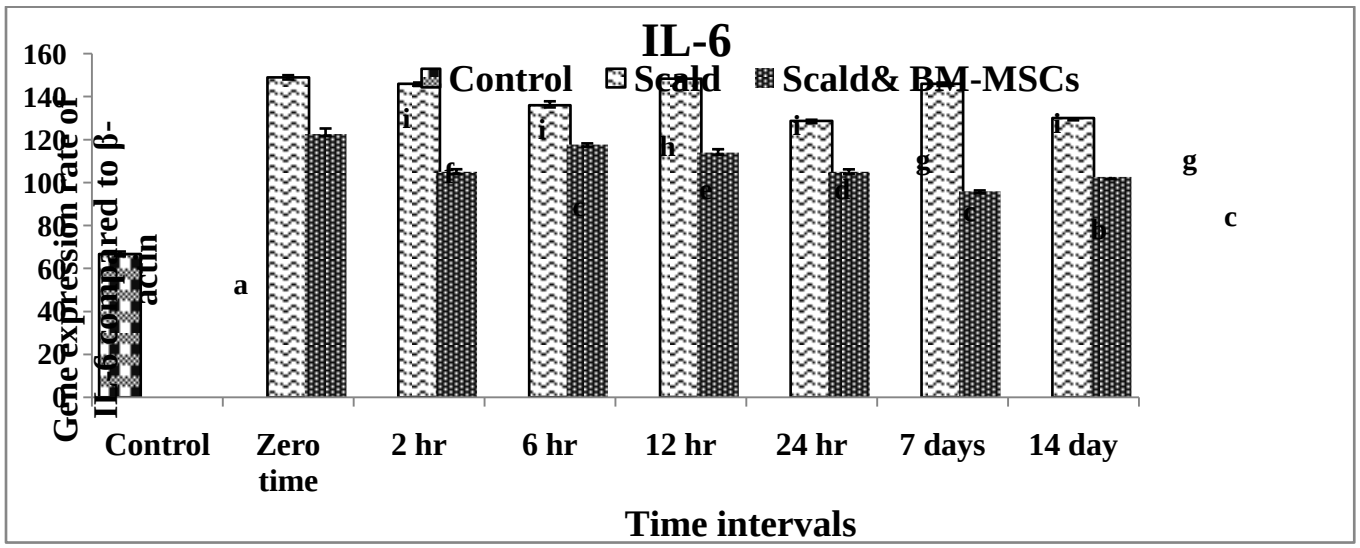


Fig. (5):mRNA expression rate of IL-6 pro-inflammatory cytokine in scald wound and the treated wound with BM-MSCs.

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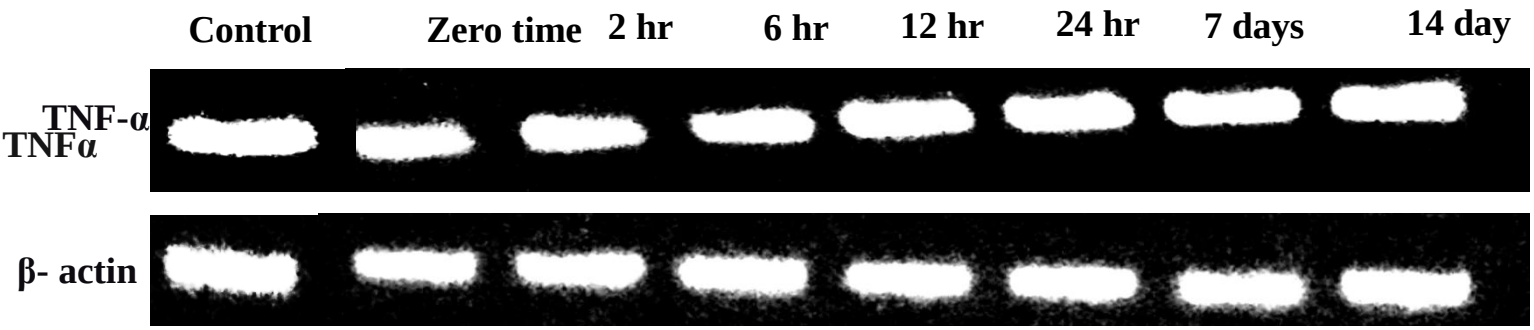
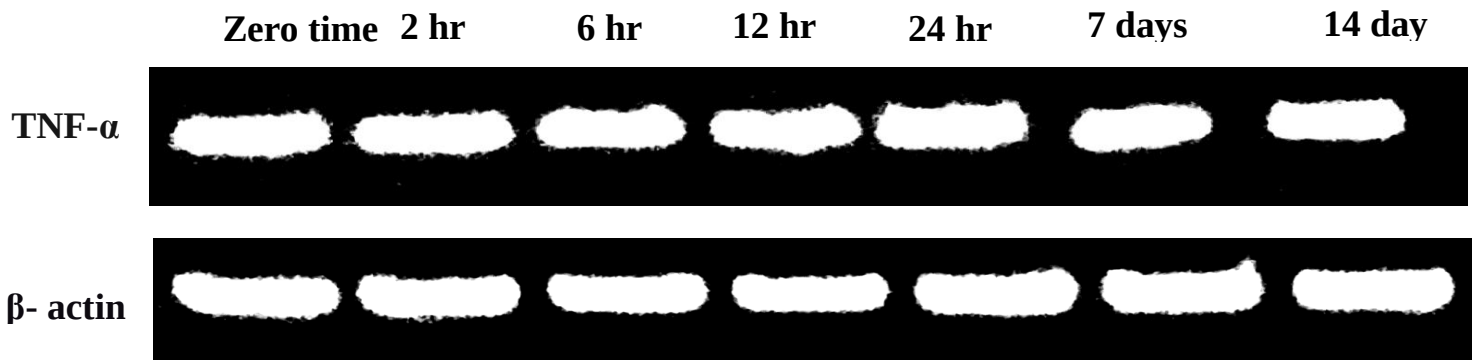
A: Gel photograph showing PCR products of TNF-α in scald



B: Gel photograph showing PCR products of TNF-α in scald& BM-MSCs



C: Desinometric analysis of PCR products of TNF-α

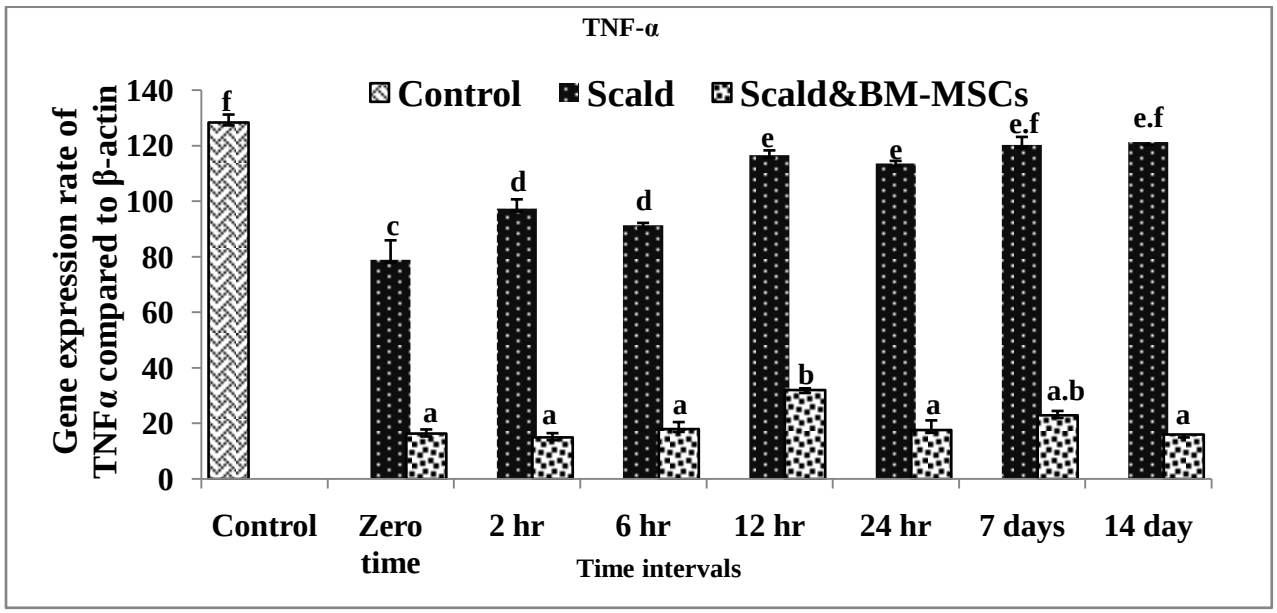


Fig. (6):mRNA expression rate of TNF-α pro-inflammatory cytokine in scald wound and the treated wound with BM-MS

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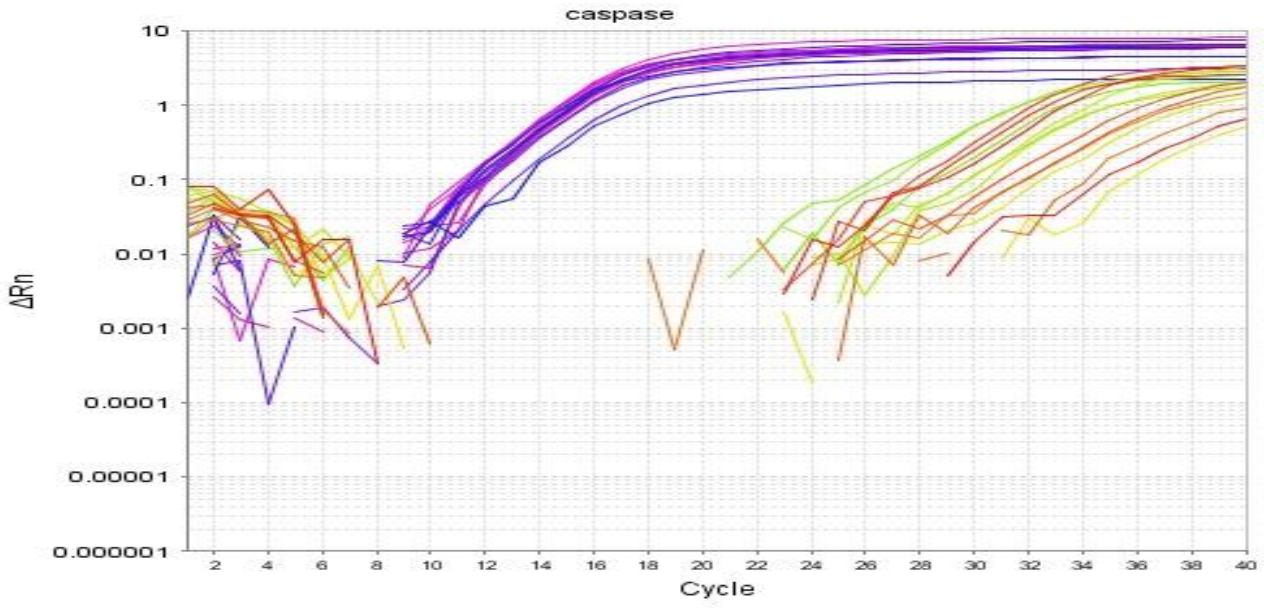

Amplification blot curves for RT-qPCR of HSP-90αgene vs. β-actin in scald wound and the treated wound with BM-MSCs.

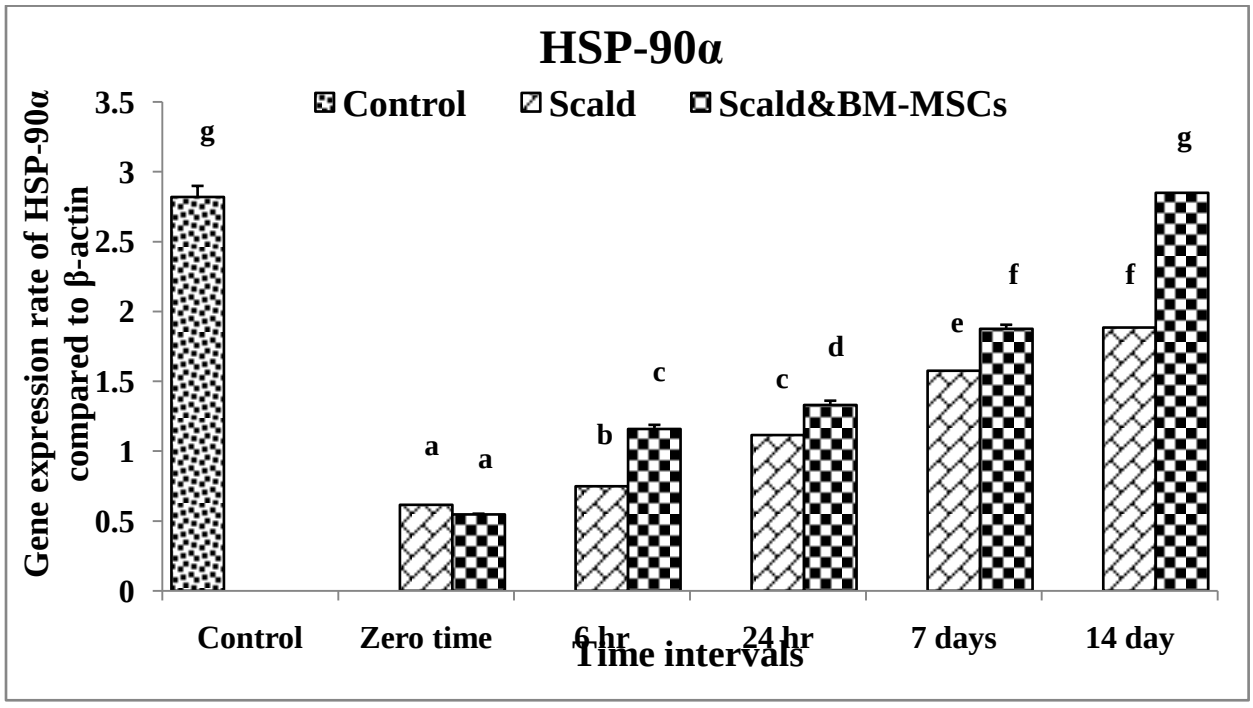

Fig. (7): mRNA expression rate of HSP-90α gene in scald wound and the treated wound with BM-MSCs.

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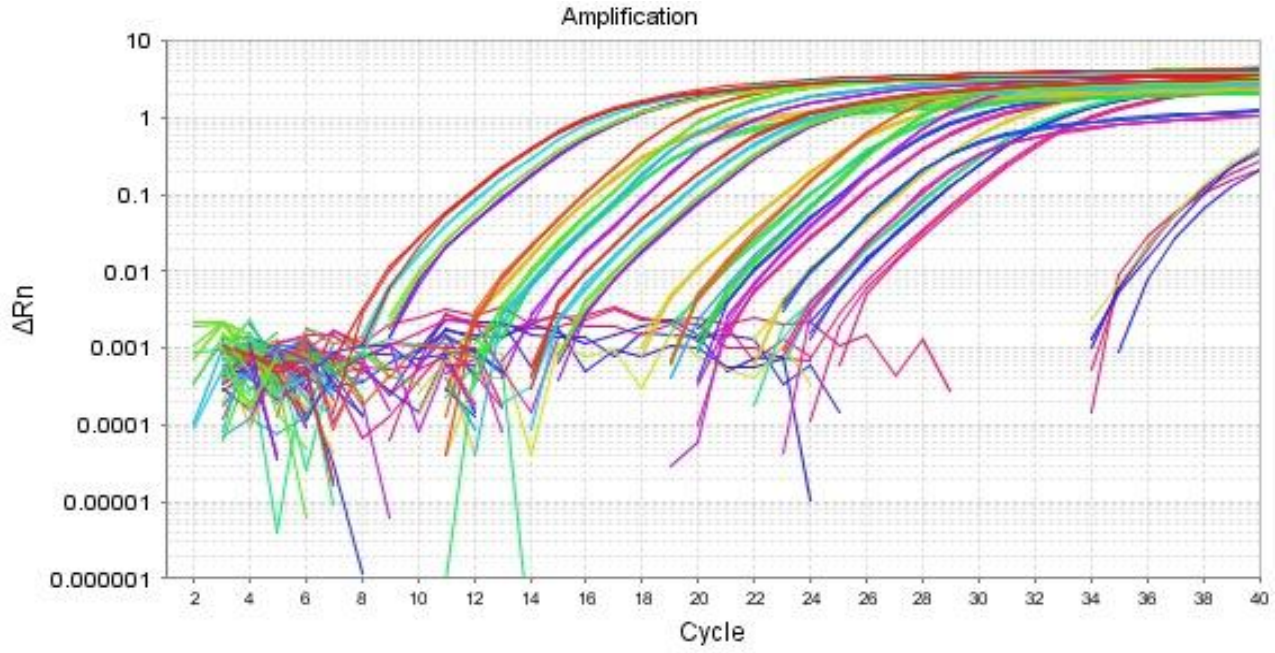

Amplification blot curves for RT-qPCR of MMP-9 gene vs. β-actin in scald wound and the treated wound with BM-MSCs.

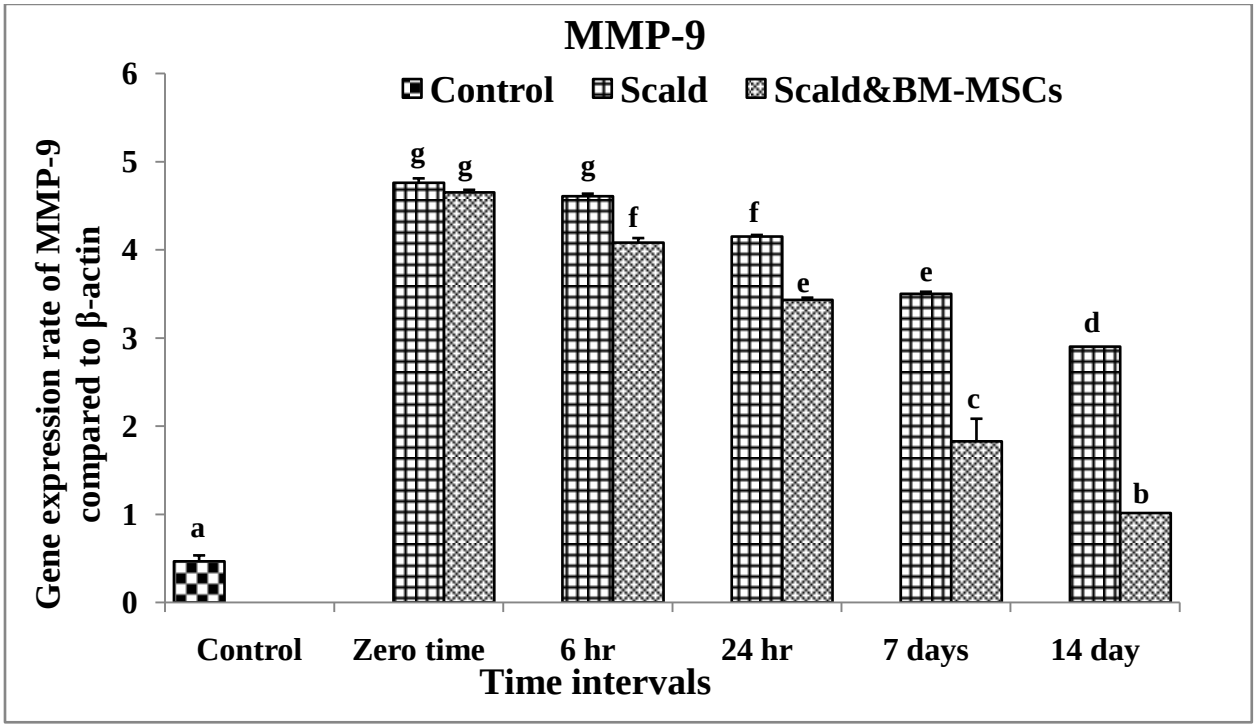

Fig. (8):mRNA expression rate of MMP-9 in scald wound and the treated wound with BM-MSCs.

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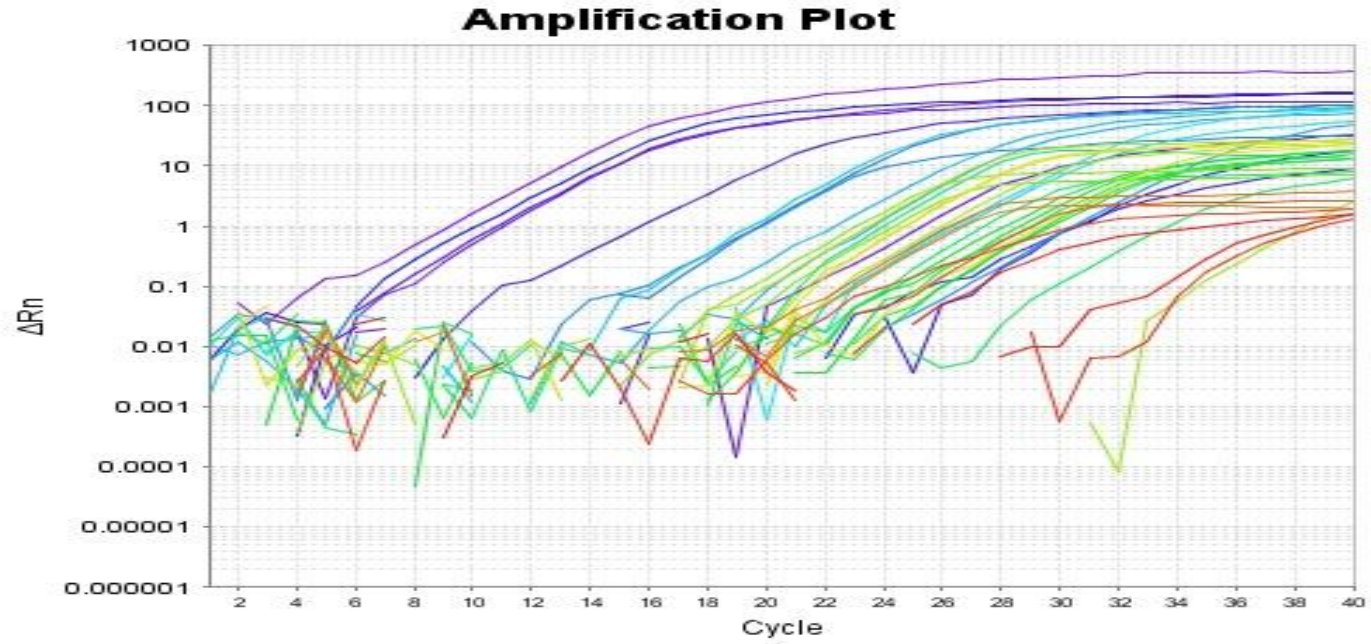

Amplification blot curves for RT-qPCR of miR-21gene vs. 6 UB in scald wound and the treated wound with BM-MSCs.

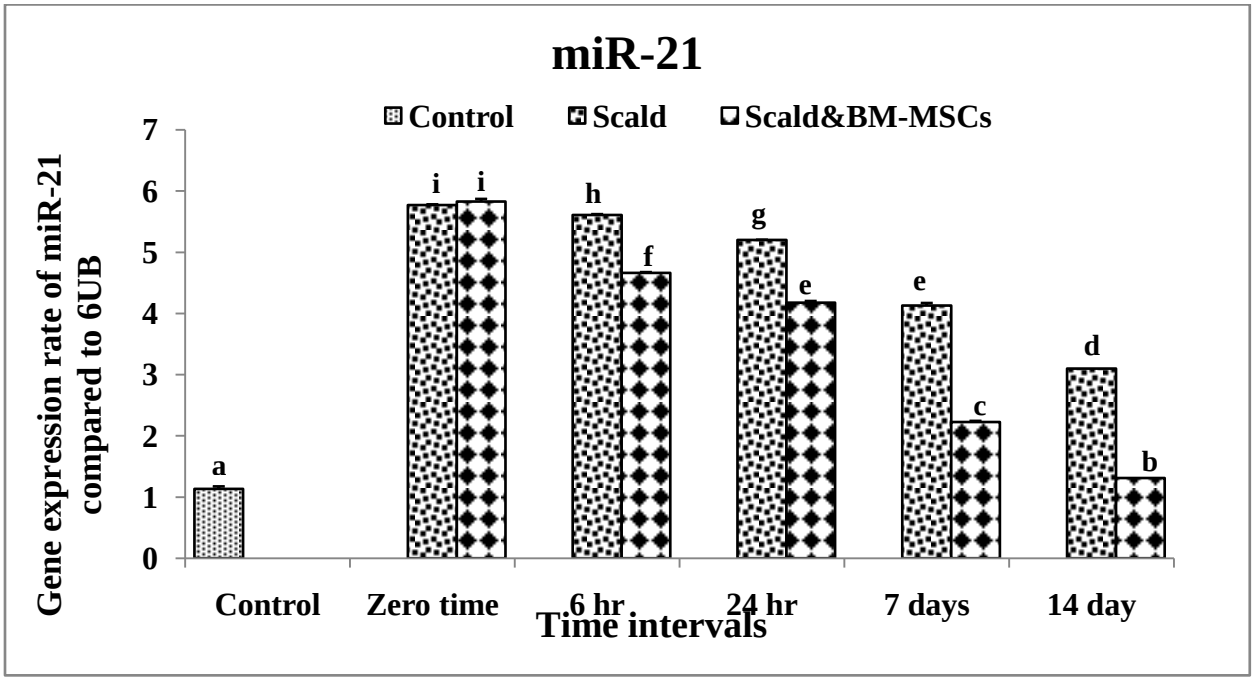

Fig. (9):mRNA expression rate of miR-21 gene in scald wound and the treated wound with BM-MSCs.