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Original Paper

Bone Marrow Mesenchymal Stem Cells Promote Repairing the Bruised Tissue via Regulating mRNA Expression of Molecular Biomarkers and the Apoptotic Rate

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Key Words
Bruises • BM-MSCs • Skin • mRNA expression rate • Apoptotic rate.

Abstract
Background/Aims: Bruise is the extravasation of blood that may be mild or severe. Bone marrow mesenchymal stem cells (BM-MSCs) are one of the most promising cells used in regenerative medicine for treating many disorders. We aimed to evaluate the efficiency of BM-MSCs in treating cutaneous bruises. ***Methods:*** 78 male albino rats were equally divided into 3 groups, control group (G1), bruise wound group (G2) and Bruised animals treated with BM-MSCs group (G3). The sequences of color changes were recorded. Animals were sacrificed and skin samples were collected for histopathological examination and analyzing the mRNA expression rate of transforming growth factor- β (TGF-β), interleukin-6 (IL-6), tumor necrotic factor-α (TNF-α), Heat shock protein-90 α (HSP-90α), Metalloprotiens-9 (MMP-9), and microRNA-21 (miR-21), which incorporated in the healing process and the apoptotic rat. ***Results:*** Subcutaneous injection of BM-MSCs reduced the color intensity of the bruised skin, with statistically significant upregulation of TGF-β, TNF-α, and HSP-90α, significant down-regulation of MMP-9 and miR-21 mRNA expression rate, and significant reduction of the apoptotic rate and the inflammatory cells. ***Conclusion:*** BM-MSCs have a promising improvement in the healing process of bruises by regulating the expression rate of TGF-β- IL-6- TNF-α- HSP-90α- MMP-9- miR-21 and reducing the apoptotic rate and inflammatory cell infiltration.

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Introduction

Bruises, contusions, or ecchymoses are a common type of mechanical injury [1], in which blood extravasates in the surrounding cutaneous connective tissue or deeper in the underlying tissues [2]. Blood extravasation occurs due to shredding the walls of veins and small arteries [3]. It appears as a non-raised, irregular, or rounded patch with blue or purplish coloration [4].

Trauma, surgery, bleeding disorders, scurvy, and cosmetic procedures considering different insults to the skin resulting in ecchymosis, which is bleeding under the which may result in combination with proteins called clotting factors to form a clot and considered a sign of bleeding disorders as hemophilia, liver disease or medical treatment with Aspirin and antibiotics, Moreover, bruises, represent a later stage of various pathophysiological processes accompanied with vascular permeability adjacent to the venules of the skin or papillary dermis capillary kinks [5].

Disseminated intravascular coagulation, Ehlers-Danlos syndrome, Meningococcemia infections, inflammatory or autoimmune disorders such as Gardner-Diamond syndrome and Henoch-Schönlein purpura, neuroblastoma and leukemia malignancy, deficiency of vitamin C and K are medical complaints combined with bruising [6, 7]. During bruises evaluation the coagulative illnesses (inherited or acquired) should be taken into consideration, [8] like von Willebrand disease, hemophilia, and abnormalities of blood platelet [9], besides the non-traumatic skin diseases and other findings as phytophotodermatitis, eczema, erythema multiforme, striae, hemangiomas, and tattooing [6].

The forensic significance of bruises is its observation in cases of violence such as vulnerable adult abuse, sexual assault, and intimate partner violence [10]. It is identified to be the most prevalent injury of physical child abuse [11]. Elderly patients with minimal repair mechanisms and those with thin and delicate skin are more susceptible to bruising and a long period of recovery [4].

The presence of different colors on the body surface reflects the presence of multiple bruises in different stages of healing; this condition may refer to long-term physical abuse [12]. In some cases of child abuse, assault, and sexual assault, forensic experts are called up for bruises and age determination, in which their comments have a medico-legal consequence [13]. In which the determined time point by the expert, can confirm or exclude the presence of a suggested perpetrator [14].

Bruises could be treated through the application of local compression, [15] such as cold compress, topical application of vitamin K, arnica, or bromelain, or using laser therapy in case of persistent bruising [16].

MSCs act as foundational elements in the newly emerging discipline of regenerative therapy; in which they could be isolated from variable sources, [17] capable of fascinating cellular biology, [18] strongly possess stemness potency, and developed rapidly for clinical applications on a large scale with minimal ethical concerns rather than embryonic stem cells (ESCs) [17].

BM-MSCs are one of the most multipotent progenitor stem cells; they could be distinguished into variable cell types as the endothelial cells and epithelial cells, fibroblasts, myofibroblasts, neurons, adipocytes, chondrocytes, osteoblasts [19]. Besides their powerful ability to regenerate the whole structures of the cutaneous tissue [20], they are also, isolated from other marrow cells easily, and have a high propensity to attach to the plastic surface of the flask of culturing [21].

When they are administrated systemically to humans or animals from an exogenous source, they tend to migrate to the inflamed damaged tissues [22]. After their engraftment into cutaneous injuries, they are mingled with the sebaceous glands, blood vessels, and hair follicles [23], helping in wound bed reconstruction and regulation of tissue repair [24]. On the level of angiogenesis, they promote this process *via* elevating the proliferation capacity of the endothelial cell, the vascular permeability [25], the capillary density, formation of new blood vessels, wound strength [26], secretion of the soluble factors [27], as well as,

formation of blood vessels in the smooth muscle that conjugating the endothelial layer of the vascular wall [28]. Transplantation of the dermis-derived multipotent cells systemically has the superiority for curing the rat of cutaneous injuries that resulted from irradiation than their topical applications [29].

Multilineage differentiation of MSCs into different cell types [30], cytoprotective and anti-apoptotic effects, angiogenic capacity [31], immunomodulatory, and low immunogenicity [32] are unique properties [33] making them the most promising cells for regenerative medicine, cell therapy, and tissue repairment [34]. They have been identified as a curative agent for skin regeneration and rejuvenation in recent years [35].

Therefore, the purpose of the present study was designed to evaluate the potency of BM-MSCS in the therapy of bruised cutaneous tissue as a type of regenerative medicine through analysis of the expression rate of certain genetic markers that regulate and control the healing process *via* quantitative polymerase chain reaction (qPCR) and determining the histopathological improvements and the apoptotic rate.

Materials and Methods

BM-MSCs isolation

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

Complete culture media preparation. To obtain complete culture media, 89% DMEM was supplemented with 15% FBS, 1% penicillin-streptomycin solution, and 0.36% sodium hydrogen carbonate [36].

Culturing of BM-MSCs. 4-6 weeks male albino rats were sacrificed through decapitation, followed by spraying 70% (vol/vol) ethyl alcohol for complete body surface sterilization. The tibiae and femurs were dissected out and the attached muscles were cleaned out, then their epiphysis was cut off with sterile fine scissors, and the flushed bone marrow cells with DMEM were collected in a falcon tube (15 mL) in a Biobase vertical laminar flow cabinet (Model: BBS V1300; NO-51, South Gongye Road, Jinan, Shandong Province, China). The collected cells were centrifuged for 5 min at 3000 rpm, then the cell pellet was washed rapidly with phosphate buffer saline (PBS) and suspended in a complete culture medium, the obtained cells were stained with trypan blue solution (0.2%) for assessing the cell viability that means the number of viable cells relative to the total number of cells by using the hemocytometer at (100X).

In sterile Greiner cell culture flasks with a canted neck (T-25 cm2), 2.5× 10^6 cells were cultured at the cell density of (1×10^6 cells/ cm^2 area), and incubated in a 5% CO_2-humidified incubator (Biobase, Model: BJPX-C50; South Gongye Road, Jinan, Shandong Province, China) at 37°C. 4 days post-incubation, the dead, non-adherent, and floating cells were removed. Subsequently, through 7-10 days of culturing (the cells have spindle shape) (Fig. 1), the adherent cells were washed with pre-warmed sterile PBS at 37°C twice and trypsinized with 1-2 mL of pre-warmed trypsin (0.25%)/EDTA (1 mM) for 2-3 min. The detachment of the adherent cells was ensured *via* examination under an inverted biological microscope (Novel Model: NIB-100; Jiangsu, China). 3-5 ml complete culture medium was added to stop the trypsin action and the cells were harvested and centrifuged for 5 min at 3000 rpm. The obtained pellet was washed with incomplete DMEM and centrifugation, following 2 times of washing; the cells were resuspended in incomplete DMEM, counted, and checked for viability by adding an equal volume of trypan blue (0.2%).

The suspended BM-MSCs (viability > 95%) were injected S/C in the bruised rats quickly at a dose of (2 × 106 cells/mL) [37]. The procedures of isolation and culturing were conducted according to the guidelines of Chaudhary and Rath [38] with some modifications.

Characterization of BM-MSCs. BM-MSCs were isolated from the bone marrow cells of albino rats (*Rattus norvegicusa*) under complete condition of sterilization and characterized *via* determination of the gene expression rate of CD34 & CD 45 (as negative markers), and CD73 and CD105 (as positive markers) through PCR (Table 1).

Animals

Healthy seventy-eight male albino rats (*Rattus norvegicus*), about 100-150 g, were maintained under observation for about 7 days before the beginning of the study to exclude any infections. They were placed in cleaned plastic cages with stainless steel covers in the animal house department at normal temperature, supplemented with food and water, and a standard 12 hr. light-dark cycle. All animal procedures followed the standards set 4th guideline for the care and use of experimental animals regarding their handling, housing, feeding, cleaning, temperature, humidity, anathesia, medications, sacrification, and pain reduction, by the Animal Ethics Committee of the Zoology Department, Faculty of Science, Beni – Suef University (Approval number is BSU/FS/020-110).

The design of the experiment

The animals were divided into the following 3 groups (6 animals /time interval), Group I (control) was not subjected to injury. Group II (bruise injury) animals were anesthetized *via* Isoflurane (0.5-2 %) which was brought through an oxygen flow system. Then the abdominal hair was removed using hair removal cream and pinched by a small pair of Spencer-Well forceps lightly and actually traumatized [39]. Group III (bruised animals treated with BM-MSCs) the bruised animals were inoculated with BM-MSCs (2×10^6 cells/ mL) S/C [37]. Animals were observed for detecting the different colors of bruises throughout the experiment. After each interval (3, 6, 12, 24, 48, and 72 hr.), the animals underwent anesthesia *via* mild diethyl ether (ACE) mixture for scarification, which was followed by the collection of skin samples, which snapped frozen at once in liquid nitrogen and then well kept under -80 oC for analysis mRNA expression rate of variable genes and determining the rate of apoptosis, the other parts were preserved in neutral buffer formalin (10%) for the histopathological examination.

Fig. 1. Spindle-shaped plastic-adherent BM-MSCs.

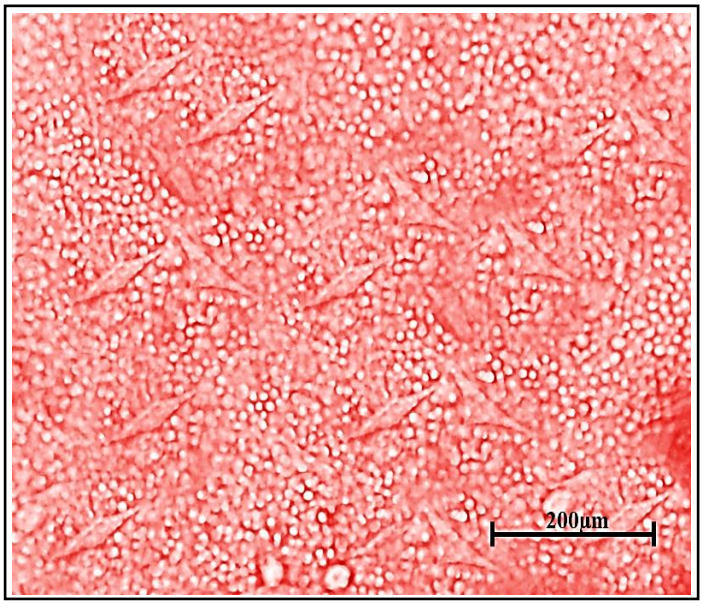

Table 1. Sequence of primers used in the characterization of BM-MSCs

mRNA species	Primer sequence
CD34	F:AGCCATGTGCTCACACATCA
	R: CAAACACTCGGGCCTAACCT
CD 45	F: TTGCTCCCCATCCGATAAGAC
	R: AGCGTGGATGAAAAACCATCG
CD73	F: TGCATCGATATGGCCAGTCC
	R: AATCCATCCCCACCGTTGAC
CD105	F: ACTGAGTTGCACATCTGGGG
	R: TTCCGAAGTGGTGGTAAGCC

Histopathological examination

The fixed skin specimens in neutral buffer formalin were undergoing dehydration, embedded in paraffin wax, and sectioning at 5 µm for staining with hematoxylin and eosin stain (H&E) [40].

Quantifiable assays of the mRNA expression rate of TGF-β, IL-6, and TNF-α through PCR

The RNA Easy extraction Kit (Qiagen Inc.) was utilized for total RNA extraction from the skin samples from both traumatized and treated traumatized tissue, following the manufacturer's instructions. Then the

RNA yield and purity were assessed *via* Nanodrop ND-2000 spectrophotometer (Thermo Electron) at 260 and 280 nm correspondingly. Viva 2-step RT-PCR Kit was utilized for the synthesis of cDNA from a total amount of 1 ug RNA following its guidelines. Quantitative PCR using Thermo Scientific Verso 1-Step RT-PCR Ready-Mix kit (Applied Biosystems, Foster City, CA, USA) was performed to assess the mRNA expression rate of the studied genetic markers (Table 2).

Quantifiable assays of the mRNA expression rate of HSP-90α, MMP-9, and miR-21 genes via RT-qPCR

The nucleic acid extraction kit (NucleoSpin®) was used for the extraction of RNA obtained from Macherey- Nagel GmbH and Co. KG- Germany (REF 740955.50) following the producer's guidelines. The spectrophotometry (dual Wavelength, Beckman Spectrophotometer, USA) was used for detecting the RNA purity (A260/A280 ratio) and concentration. Synthesis of the cDNA was done *via* Vivantis, ViPrimePLUS One Step Taq RT-qPCR Green Master Mix I with ROX (SYBR Green Dye) (cat no #QLMM14-R) kit following the manufacturer's instructions. Samples of the prepared reaction mix were placed in real-time PCR (Step One, Applied Biosystem, Foster City, USA). ViPrime PLUS One Step Taq RT-qPCR Green Master Mix I with ROX Kit was compatible with three-step cycling (Table (3), performed for analyzing the mRNA expression rate of the target gene (Table 4).

The RQ of each studied gene is calculated according to the calculation of delta-delta Ct (ΔΔCt). We determined the RQ for each gene by taking 2-ΔΔCt.

Determination rate of apoptosis

For determination of the cutaneous apoptotic rate, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunostaining was done using the Apo-BrdU *In Situ* DNA Fragmentation Assay Kit (Catalog #K401-60) in relation to its instructions.

The frozen tissue was sectioned and fixed with 4% formaldehyde/ PBS in a Coplin jar followed by incubation and washing with PBS. Each section was covered with Proteinase K Solution followed by washing with PBS and fixation with 4% formaldehyde, then washing and drying the slide. About 50 µl of the DNA Labeling Solution was placed on the cells and covered with a plastic coverslip, and then incubated in a dark, humidified 37°C incubator for 60 minutes, the coverslip was removed and the slides were transferred to a fresh Coplin jar filled with PBS for rinsing then drying occur, and 100 µl of the Antibody solution was placed for incubation for 30 minutes at room temperature after that the solution was removed and 100 µl of Propidium Iodide/ Rnase A solution was added with incubation, finally, the slides washed with ddH_2O in a fresh Coplin jar and incubated at room temperature for

Table 2. Sequence of primers used in amplification

mRNA species	Sequences
TGF- β	F: TGGCGTTACC TTGGTAACC
	R: GGTGTTGAGCCCTTTCCAG
IL- 6	F: GCCTTCTTGGGACTGATG
	R: TGGTCTGTTGTGGGTGGT
TNF- α	F: GCTGAGGTTGGACGGATAAA
	R: AAAATCCTGCCCTGTCACAC
β- actin	F: TCCCTGAAGTACCCCATGGAG
	R: TTGGCCTTGGGGTTCAGGGGG

Table 3. The thermal profile cycling of RT-qPCR

Step	Cycle	Temp.
Reverse Transcription	1	55°C
Enzyme activation	1	95°C
Denaturation	40	95°C
Annealing and extension		60°C

Table 4. The primer sets used for amplification

Gene symbol	Sequences	Gene bank
HSP- 90α	F: TGTTGGGACCAGCAACTCAA R: TTTGAGGCTCAGTGGTAGCC	NM_021576.2
MMP- 9	F: GGCAGCTTCAACAACCATCA R: GGATGGACTAGATCGGAGCC	XM_032900290.1
β- actin	F: TGACAGGATGCAGAAGGAGA R: TAGAGCCACCAATCCACACA	NM_031144.3
miR- 21	F: TAGCTTATCAGACTGATGTTG R: GAATCGAGCACCAGTTACGC	MN_753178.1
6UB	F: AACGCTTCACGATTTGC R: CTCGCTTCGGCAGCACA	XR_003710259.1

5 minutes. The slides were viewed using FITC and rhodamine filters. Apoptotic cells will exhibit strong nuclear green fluorescence. All cells should be stained with PI and exhibit strong red counterstaining.

Statistical analysis

The results are represented as the mean values ± the Standard Error (SE). Continuous variables were analyzed with the one-way analysis of variance (ANOVA), followed by the Duncan post hoc test. A *P* value of < 0.05 was regarded as statistically significant. All data analyses were performed *via* SPSS version 9 [41].

Results

Characterization of MSCs

Results illustrated in Table 5 and Figure 2 revealed marked-down regulation of the mRNA expression rate of CD 34 & CD 45, and significant up-regulation of CD 73 and CD 105.

The color changes of the bruised skin were observed as follows: Red color was developed after 3 hours of pinching the skin, and its intensity reduced with time and became slightly red at 6 and 12 hours. A slight bluish coloration appeared after 24 hours, while the yellow coloration was detected at 48 hours and became prominent at 72 hours. The chronology of the previous colors was noted following therapy, but their intensity markedly reduced, especially at 72 hours (Fig. 3).

Histopathological findings

Histopathological examination of H&E-stained skin sections of the bruised animals (G2) and the treated animals (G3) revealed degenerative changes of the squamous epithelium lining, presence of leukocytic infiltrations mainly neutrophils in the dermal layer associated with congested blood vessels till 12 hr post-traumatic injury Fig. 4 (A, B, and C) and (a, b, and c). At 24 hr, the neutrophilic infiltration was extended to the muscular layer (Fig. 5A), which increased significantly at the treated corresponding hour (Fig. 5a). Then the leuckocytic infiltration of the dermal layer was reduced until 72 hr, Fig. 5 (B and C). Moreover, in the corresponding treated hours, minimal leuckocytic infiltration in the dermal layer can be found in Fig. 5 (b and c).

Table 5. mRNA expression rate of CDs compared to β-actin. *a- b, Means which have the same superscript symbol(s) are not significantly different. Data are expressed as mean ± SE (n=6) A P value of (< 0.05)

CDs	CD/ β-actin ×10²
CD34	528.3±116[a]
CD45	553.3±102[a]
CD73	2820.3±762.9[b]
CD105	2950±709[b]

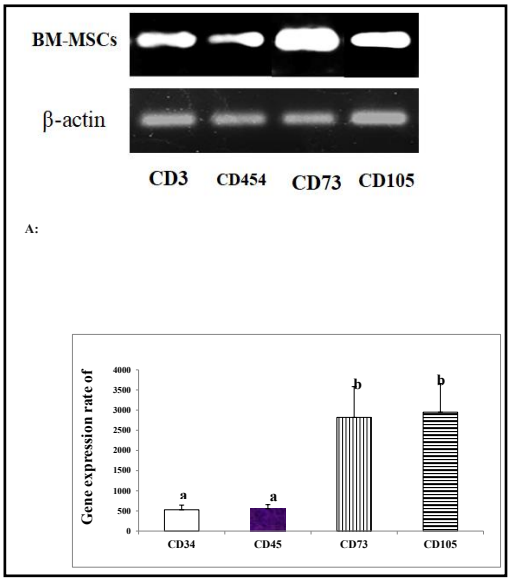

Fig. 2. mRNA expression rate of CDs of BM-MSCs. B: Desinometric analysis of PCR products of CDs A: Gel photograph showing PCR products of CDs in BM-MSCs.

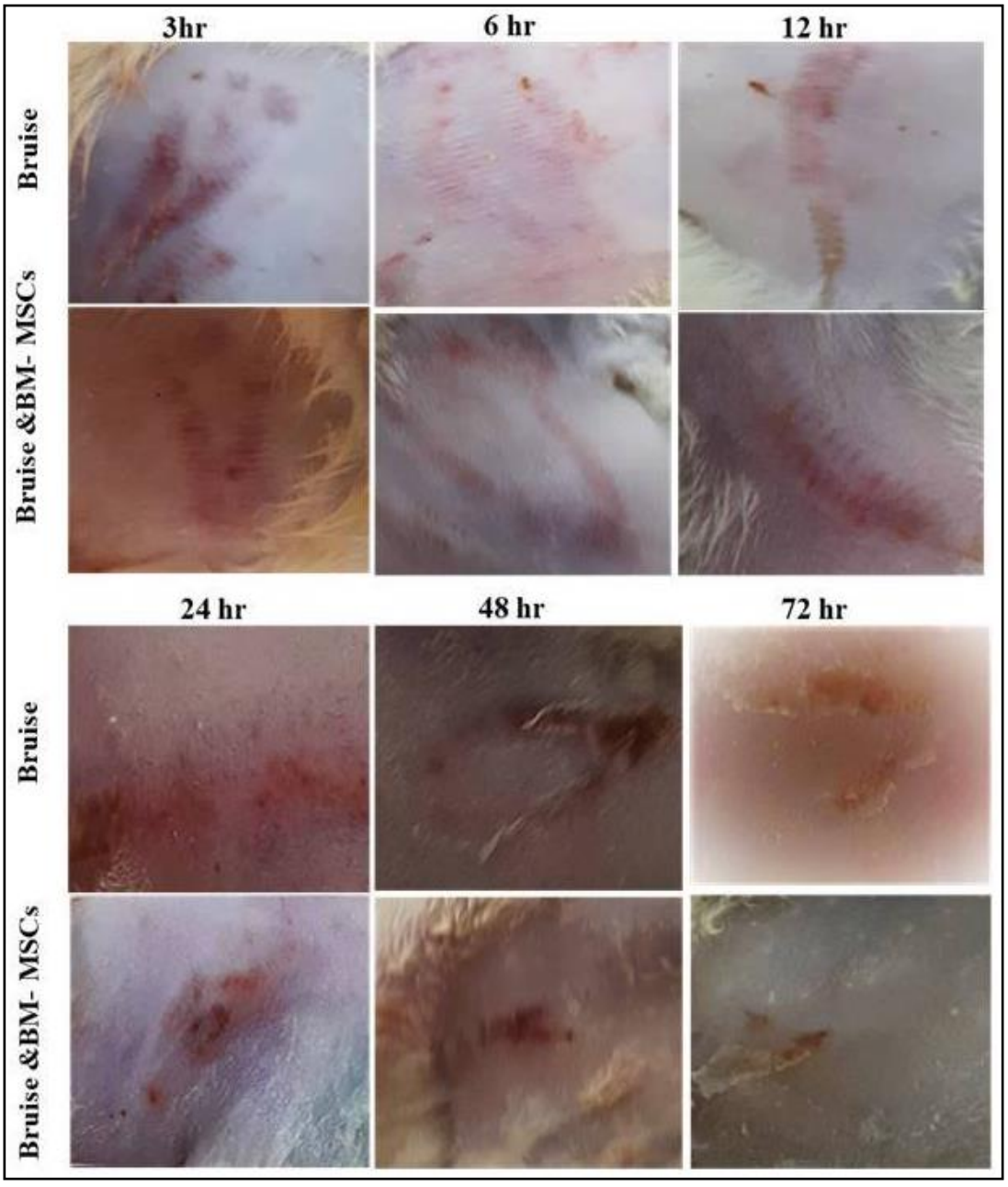

Fig. 3. Effect of BM-MSCs on the color changes of bruised tissue.

The mRNA expression rate of TGF- β, IL- 6, and TNF- α

Injection of the bruised skin with BM-MSCs resulted in significant upregulation of TGF-β mRNA expression at 3, 6,12 and 48 hr. with marked reduction at 72 hr. (Fig. 6). In contrast, a significant downregulation in the IL-6 expression rate was determined at 6 and 24 hr., While a significant upregulation, was detected at 48 and 72 hr. following therapy with BM-MSCs (Fig. 7). Concerning the mRNA expression rate of the mRNA expression rate of TNF-α was upregulated significantly posttreatment with BM-MSCs (Fig. 8, Table 6).

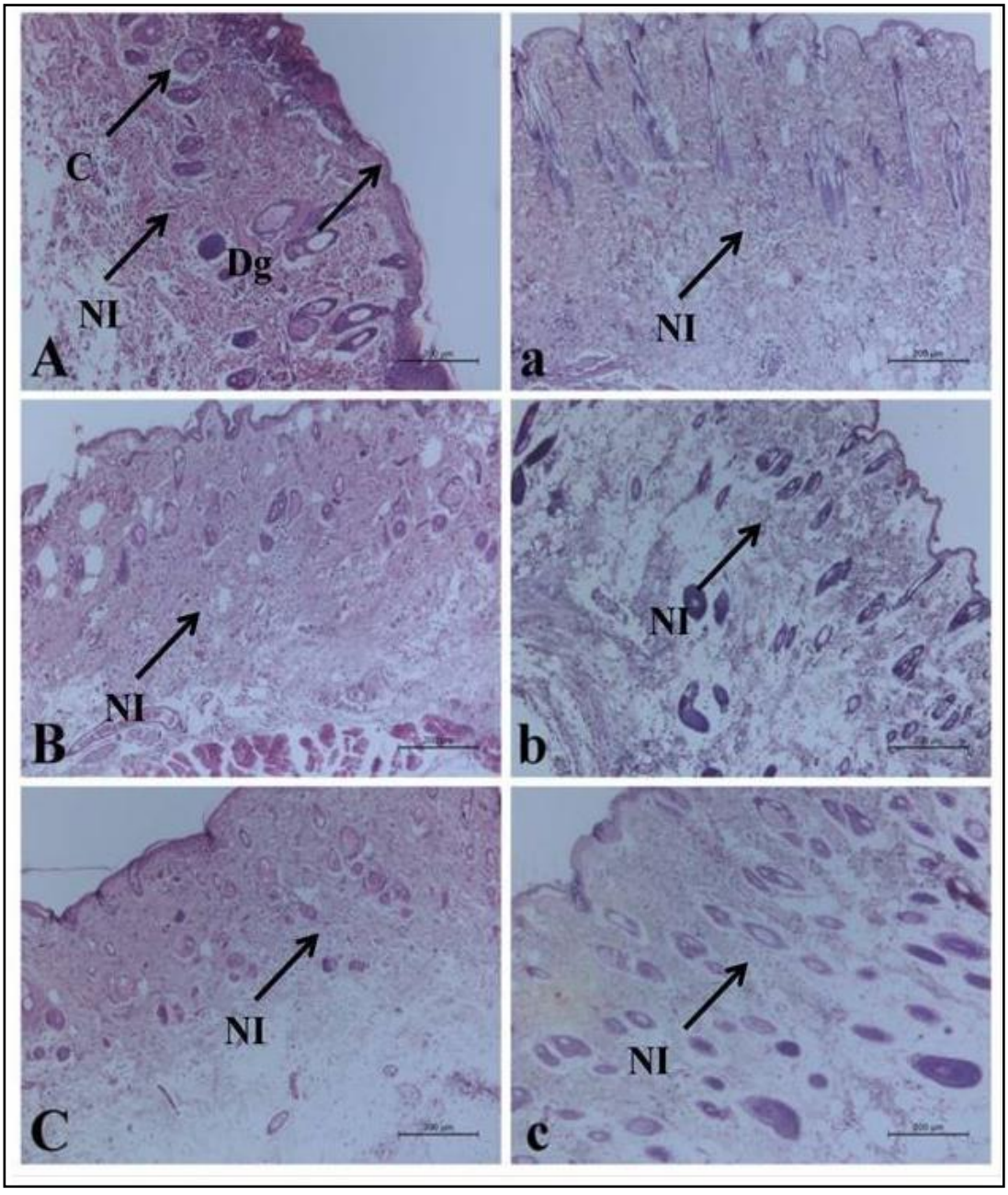

Fig. 4. Histopathological alterations of the bruise wound at (3, 6, and 12 hr) in A, B, and C respectively, and the treated wound in (a, b, and c) respectively, including degenerative changes (Dg), congestion (C) and neutrophilic infiltration (NI). (H&E).

The mRNA expression rate of HSP- 90α, MMP- 9, and miR- 21 genes

The HSP-90α expression was markedly upregulated in the bruised animals treated with BM-MSCs, in all-time intervals (Fig. 9). Regarding MMP-9 and miR-21 expression rates, the treated animals with BM-MSCs showed significant down-regulation as illustrated in Fig. 10 and Fig. 11 respectively Table 7.

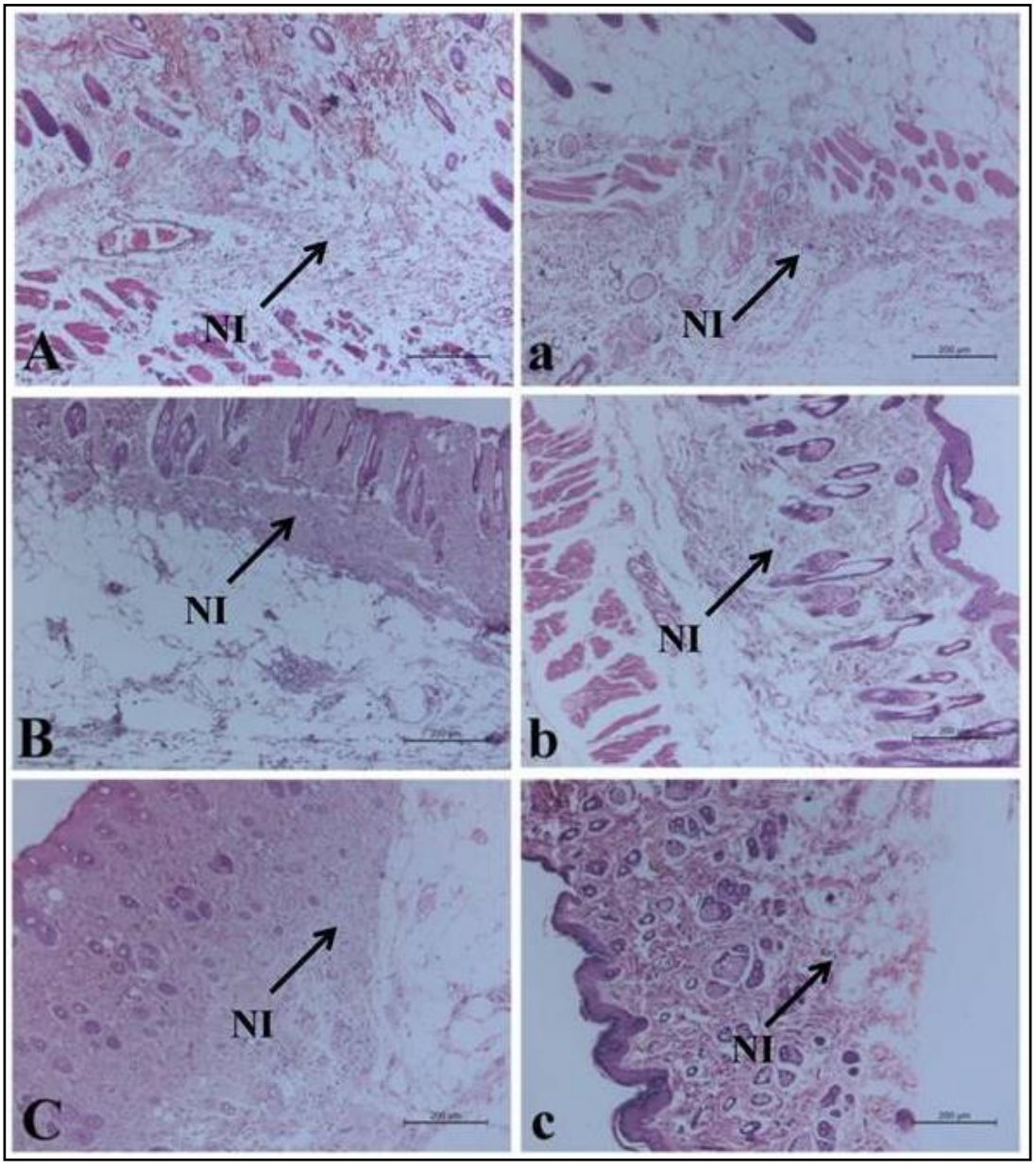

Fig. 5. Histopathological alterations of the bruise wound at (24, 48 and 72 hr) in (A, B, and C) respectively, and the treated wound in (a, b and c) respectively including neutrophilic infiltration (NI). (H&E).

The rate of apoptosis

The obtained data in Figure (12) and Table (8) revealed that treating the traumatic animals with BM-MSCs causing decreased the apoptotic rate at all time intervals significantly compared to the traumatic ones.

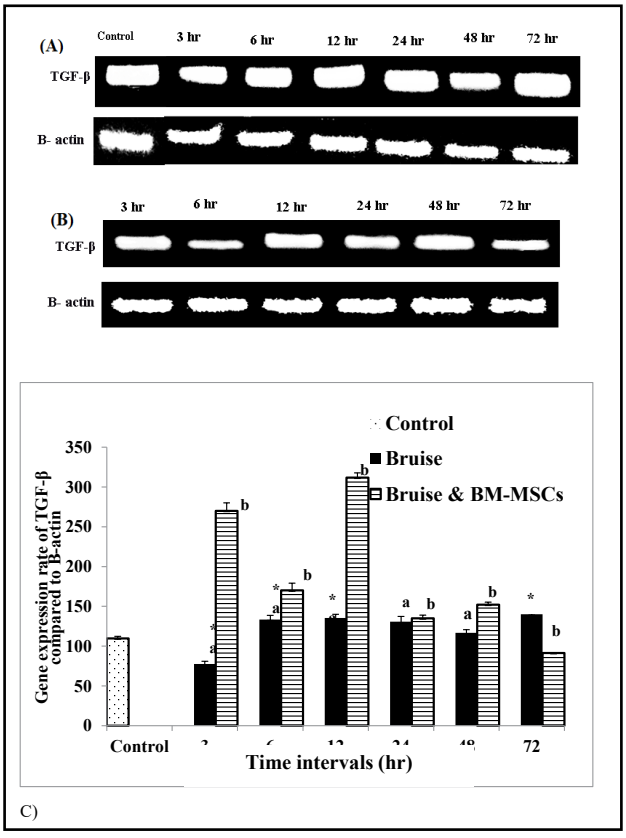

Fig. 6. mRNA expression rate of TGF-β. A: Gel photograph demonstrating PCR products of TGF-β in bruise, B: Gel photograph demonstrating PCR products of TGF-β in bruise and BM MSCs and C: Desinometric analysis of PCR products of TGF- β. * Means significance, data are expressed as mean ± SE (n=6) A P value of (< 0.05).

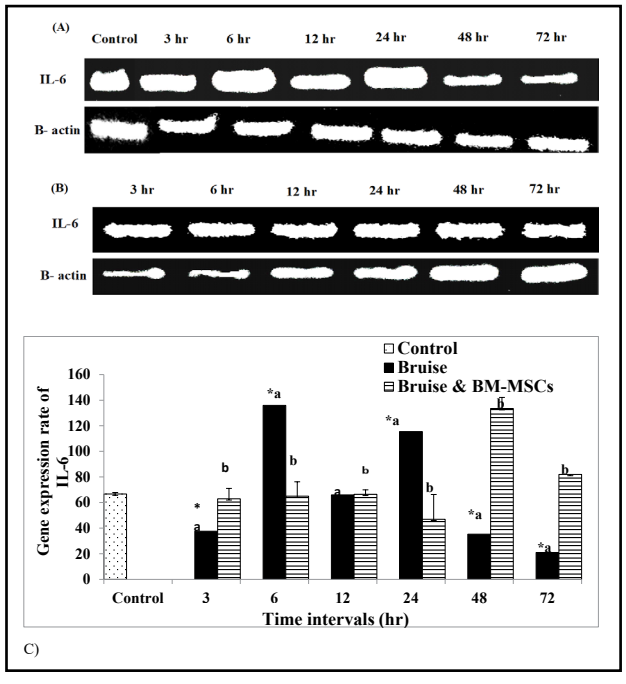

Fig. 7. mRNA expression rate of IL-6 pro-inflammatory cytokine A: Gel photograph demonstrating PCR products of IL-6 in bruise, B: Gel photograph demonstrating PCR products of IL-6 in bruise and BM MSCs and C: Desinometric analysis of PCR products of IL-6. * Means significance, data are expressed as mean ± SE (n=6) A P value of (< 0.05).

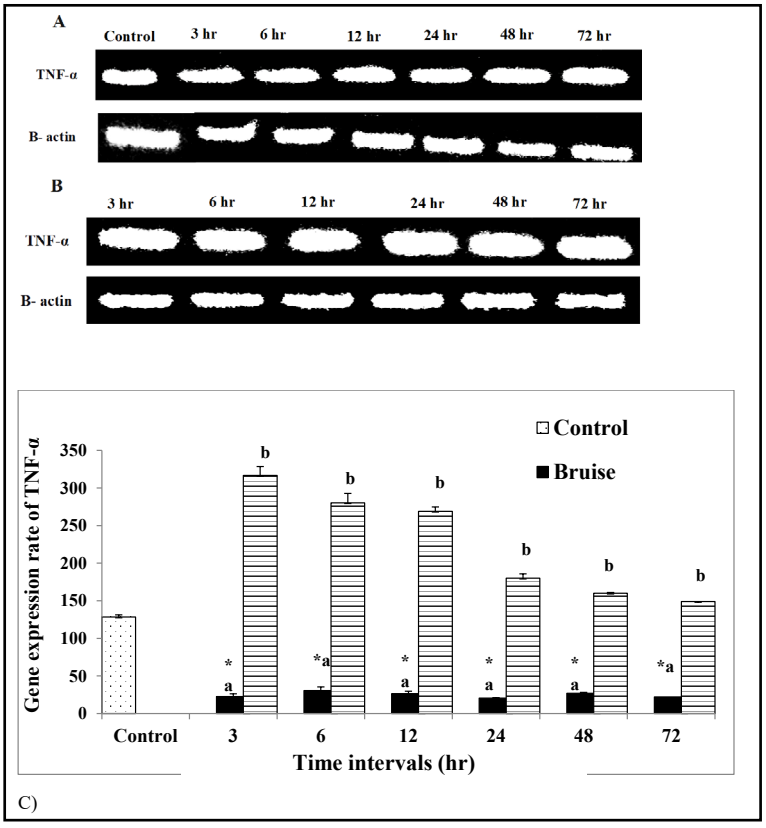

Fig. 8. mRNA expression rate of TNF-α pro-inflammatory cytokine A: Gel photograph demonstrating PCR products of TNF-α in bruise, B: Gel photograph demonstrating PCR products of TNF-α in bruise and BM MSCs and C: Desinometric analysis of PCR products of TNF-α.* Means significance, data are expressed as mean ± SE (n=6) A P value of (< 0.05).

Table 6. mRNA expression rate of TGF-β and the pro-inflammatory cytokines (IL-6 &TNF-α). *a- b, Means which have the same superscript symbol(s) are not significantly different. Data are expressed as mean ± SE (n=6) A P value of (< 0.05). The different superscript letters indicated a significant difference between groups at level p< 0.05

Parameters / Groups		TGF-β/β-actin ×10²	IL-6/β-actin ×10²	TNF-α / β-actin ×10²
Control (G1)		109.7±2.7	66.7± 1.2	128.3±2.9
Bruise (G2)	3 hr	77. 7±3.7 *a	37.7±6.1 *a	22.5±2.6 *a
	6 hr	133.3±3.3 *a	136±9.2 *a	30.5±3.8 *a
	12 hr	135.3±5.3 *a	66±9.2 a	26.5±4.9 *a
	24 hr	130. 7±4.7 a	115.5±6.1*a	20.5±3.1 *a
	48 hr	116. 7±6.7 a	35.3±5.5 *a	27±0.6 *a
Time (hr)	72 hr	140±4 *a	21±3.5 *a	22±1.2 *a
Bruise &BM-MSCs (G3)	3 hr	270±10 b	63±8.1b	316. 7±15.1b
	6 hr	170±10 b	65±8.1b	280.3±11.8b
	12 hr	311. 7±9.2b	66.5±11.3b	269±12.4b
	24 hr	135±6 b	47±3.5b	180±5.8b
	48 hr	152±4 b	133.5±19.3b	160±5.8 b
	72 hr	91.3±3.3 b	82±8.7b	149±1 b
P-Value			P< 0.05	

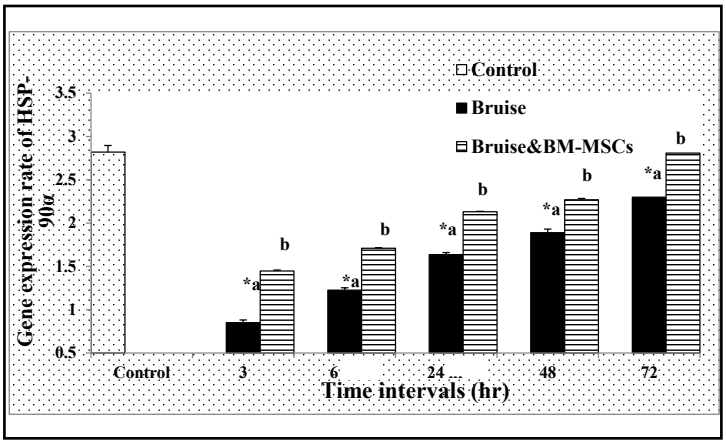

Fig. 9. The mRNA expression rate of HSP-90α. * Means significance, data are expressed as mean ± SE (n=6) A P value of (< 0.05).

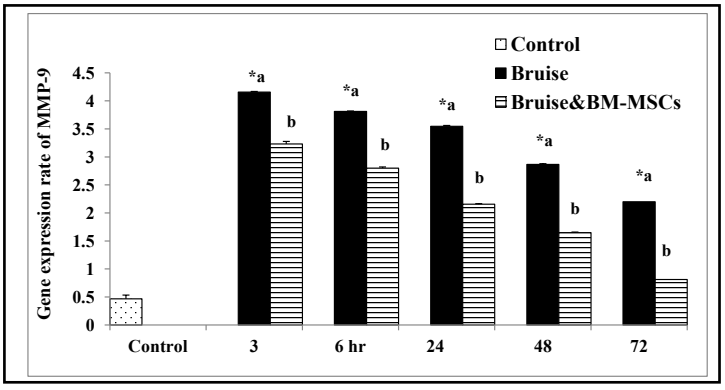

Fig. 10. The mRNA expression rate of MMP-9. * Means significance, data are expressed as mean ± SE (n=6) A P value of (< 0.05).

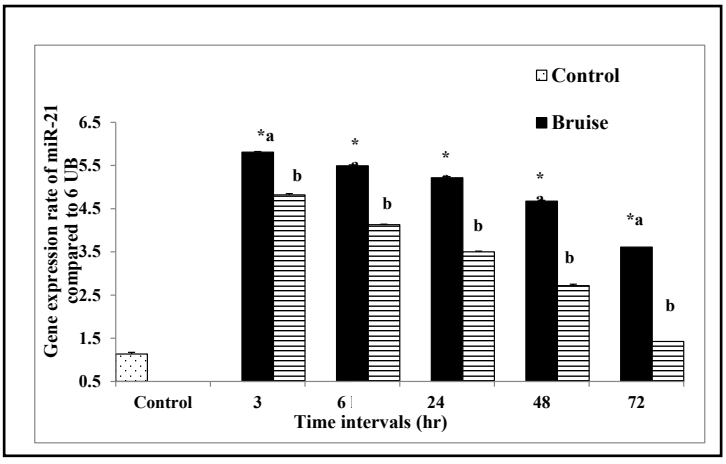

Fig. 11. The mRNA expression rate of miR-21 gene. * Means significance, data are expressed as mean ± SE (n=6) A P value of (< 0.05).

Table 7. mRNA expression rate of HSP-90α, MMP-9, and miR-21 genes in bruise wound and the treated wound with BM-MSCs. *a- b, Means which have the same superscript symbol(s) are not significantly different. Data are expressed as mean ± SE (n=6) A P value of (< 0.05)

Parameters Groups		HSP-90α/β-actin ×10²	MMP-9/β-actin ×10²	miR-21/ 6 UB ×10²
Control (G1)		2.82±0.07	0.47±0.07	1.1±0.04
Bruise (G2)	3	0.85±0.05 *a	4.16±0 *a	5.81±0.02 *a
	6	1.23±0.03 *a	3.81±0.01 *a	5.49±0.01 *a
Time (hr)	24	1.64±0.03 *a	3.55±0.01 *a	5.22±0.02 *a
	48	1.89±0.02 *a	2.87±0.01 *a	4.68±0.03 *a
	72	2.3±0.04 *a	2.2±0.01 *a	3.61±0.02 *a
Bruise & BM-MSCs (G3)	3	1.45±0.01 b	3.23±0.03 b	4.82±0.02 b
	6	1.71±0.01 b	2.8±0.05 b	4.13±0.03 b
	24	2.13±0.01 b	2.16±0.02 b	3. 5±0.01 b
	48	2.27±0.01 b	1.65±0.01 b	2.72±0.02 b
	72	2.81±0.01 b	0.81±0.01 b	1.43±0.03 b
P-Value			P< 0.05	

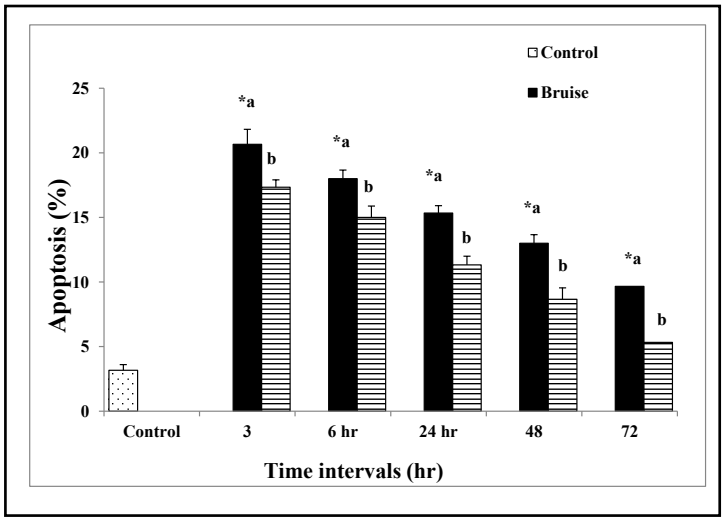

Fig. 12. The rate of apoptosis. * Means significance, data are expressed as mean ± SE (n=6) A P value of (< 0.05).

Table 8. The rate of apoptosis. [*a- b], Means which have the same superscript symbol(s) are not significantly different. Data are expressed as mean ± SE (n=6) A P value of (< 0.05)

Parameter Groups		Apoptosis
Control (G1)		3.17 ± 0.4
Bruise (G2)	3	20.67± 0.9 *a
	6	18 ±1.15 *a
	24	15.3 ± 0.7 *a
	48	13 ± 0.6 *a
	72	9.67±0.7 *a
Bruise & BM-MSCs (G3)	3	17.3 ±0.9 b
	6	15 ±0.6 b
	24	11.3 ±0.9 b
	48	8.67±0.7 b
	72	5.3 ±0.9 b
P-Value		P< 0.05

Discussion

Bruising is distinctly a source of pain, [42] it has resulted from an extreme mechanical force sufficient for crushing the small vessels within the skin and the underlying tissues [43]. After the blood leaked into the tissues, the inflammatory response was stimulated to break and remove it [44]. In which the activated macrophages phagocytose RBCs and hemoglobin undergo degradation, [45] through a biochemical process, which catalyzes *via* Haem-oxygenase generating an iron atom, carbon monoxide, and biliverdin (green) [46]. Biliverdin was metabolized rapidly to bilirubin (yellow) [44]. whilst the created iron constituting complex with ferritin to yield the hemosiderin (brown), so bruises assume variable colors through healing [44]. In the existing study, the color intensity of the bruised skin was markedly reduced after therapy with BM-MSCs especially at 72 hr., as MSCs cause up-regulation of haemoxygenase, [46] which has anti-apoptotic, anti-inflammatory, and antioxidant properties [47].

MSCs ameliorate the tissue repair process *via* paracrine signaling and differentiation mechanisms; whereas their differentiating capacity participates in regenerating damaged tissues and paracrine signaling orchestrates the local cellular responses to injury resulting in regression of inflammation, and promotion of angiogenesis by stimulating cell migration and proliferation [48]. Hence, the histopathological examination of the treated bruised tissues revealed that the neutrophilic infiltration was increased significantly in the muscular layer at 24 hr., While, at 48 and 72 hr., minimal leukocytic infiltration in the dermal layer could be found.

MSCs' paracrine function means that MSCs have the ability to modulate and incorporate in the inflammatory, proliferative, and remodeling wound healing process through their secretion of variable bioactive molecules as chemokines, growth factors, cytokines, and

exosomes [49]. The excreted exosomes bind to the vascular endothelial cell receptors resulting in stimulation of the downstream signaling pathways as the EGFR, ERK1/2, PI3K/Akt and subsequently modulating their proliferation, migration, and differentiation with activation of neovascularization [50].

Healing of mechanical injury starts with platelet arrival for hemostasis, mediation, and secretion of various vasoactive substances and mediators such as prostaglandins, fibroblast growth factor (FGF) platelet-derived growth factor (PDGF), TGF-β, serotonin, b-thromboglobulin, epidermal growth factor (EGF), bradykinin, thromboxane, and histamine, which acts as a stimulator for the healing process [51]. TGF-β can trigger the early response of pro-inflammatory cytokines secretion, involving IL-6, IL-1b, and TNF-α [52]. Therefore, the level of IL-6 was markedly increased in the current study under the influence of TGF-β; also, MSCs secreted IL-6 as an immunomodulatory effect causing inhibition of lymphocyte apoptosis [53].

Modulation of the inflammatory microenvironment *via* BM-MSCs could be done under the effect of secreted TSG-6 that suppresses the stimulation of the TLR2/NF-κB signaling pathway [31].

Triggering of vigorous anti-inflammatory and immunosuppressive mechanisms has been recorded following apoptotic cell engulfment by activated macrophages at the wound site which secretes IL-10 (anti-inflammatory cytokine) and inhibits TNF-α secretion, [54] which may be the reason for the inhibited TNF-α expression rate, while the rate was up-regulated with BM-MSCs therapy in the current study, as MSCs could become pro-inflammatory to stimulate the early tissue repair [55].

The latest study by Jiang *et al* [56]. recorded that the activation of TGF-β receptors on MSCs may upregulate or suppress the expression level of miR-21, according to the concentration of TGF-b1 in the wound bed, while overactivation of TGF-β receptors on MSCs at extreme TGF-β1 concentrations down-regulates miR-21, so inhibition of Smad7 translation not happened *via* miR-21 and ultimately production of TGF-b1 from MSCs was suppressed. Thus, the signaling between the TGF-β receptor, Smad7, miR-21, and TGF-β1 regulates the production of MSC-derived TGF-b1 production according to the wound site needs [57]. These results were identified in the current study where the rate of both TGF-β and miR-21 were down-regulated after treatment with BM-MSCs.

The paracrine function of BM-MSCs causes upregulation of MMP- 9 and TGF-β3, leading to increasing granulation tissue formation with inhibition of extracellular matrix (ECM) deposition during the proliferation phase [58]. Furthermore, MSCs have the ability to inhibit the regression of collagenous matrix and maintain the matrix with the promotion of the regeneration of fibroblasts through downregulating the expression rate of MMPs [59] hence, they provide favorable conditions for ECM remodeling during the healing process [60], which reported in our study in which BM-MSCs causing significant down-regulation of MMP-9.

In addition, their paracrine actions downregulated nucleic acid and protein metabolism, apoptotic MSCs enhance the process of wound healing by suppressing inflammation, accelerating angiogenesis, and stimulating fibroblast migration and collagen production *via* their paracrine actions [61], and upregulated homeostasis and antiapoptotic genes, they can synthesize the granulocyte-macrophage colony-stimulating factor (GM-CSF), that suppress the cellular apoptosis and maintain the tissue homeostasis [62]. So the apoptotic rate was significantly reduced after therapy with BM-MSCs in the present study.

Multiple mechanisms have been reported to be incorporated in MSCs anti-apoptotic effects such as prevention of the dysfunctional MMPs [63], inhibition of cellular death signals as caspase-3 [64], or activation of survival signaling pathways such as phosphorylation of STAT3 and Akt [65], secretion of exosomes and growth factors [66].

A plenty amount of Hsp90α was preserved in all cell types of the skin and secreted into the extracellular space under stress conditions, such as tissue injury leading to disruption of the vessel integrity with rapid consumption of oxygen by the cells, resulting in a hypoxic microenvironment that stimulated Hsp90α production that influenced by HIF-1α and consequently enhanced skin cell migration through LRP-1 to initiate the wound healing

[67]. In our study, a dramatic increase in the Hsp90α level has been observed following treatment by BM-MSCs till the level reached its level in normal state, which may be due to the production of Hsp-90α from cells in the wound bed or penetrated immune cells or increased Hsp90α expression intracellular [68].

One of the facts that helps in the scalability of BM-MSCs widely is their easy collection and isolation from the bone marrow, [69] but many issues faced the Conveying MSCs from the laboratory to clinical application such as quality control and discrepancies insufficiencies in many aspects as immunocompatibility, stability, heterogeneity, differentiation, and the noted migratory capacity in the clinical trials [70]. Despite these challenges, Wu et al. stated that MSCs will become the backbone of wound healing in the future. Based on that we suggest a flow up studies in the same concept of healing the bruised or contused wound either cutaneous type or other internal types that may threaten life or other studies regarding blood vascular disorders.

Conclusion

BM-MSCs proved to be efficient in therapy the bruised type of wounds through modulation of the wound healing mediators, which resulted in improvement in the healing process that reflected morphologically and restored the normal skin histological structure, this provided a great insight into the therapy of related conditions of skin destruction or disorders related to vascular conditions. Moreover, BM-MSCs may be promising therapeutic approaches for other mucosal healing and augment the integrity of the gut barrier, suggesting their potential to treat inflammatory bowel disease. However, extensive clinical trials and assessment of the long-term impact of cell therapy are required for additional work.

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Statement of Ethics

All animal procedures followed the standards set 4th guideline for the care and use of experimental animals regarding their handling, housing, feeding, cleaning, temperature, humidity, anathesia, sacrification, and pain reduction by the Animal Ethics Committee of the Zoology Department, Faculty of Science, Beni – Suef University (Approval number is BSU/FS/020-110).

Author Contributions

DA, MA, RR, and WM conducted the laboratory studies, participated in the sequence alignment, and drafted the manuscript. DA, KA, WM, and DA carried out the assays. WM and KA performed the histopathological examination. KA, WM, SA, HM, and DA participated in the study design and performed the statistical analysis. DA, WM, RR, HA, SA, HM, and KA, contributed to the study design and coordination, supported the interpretation and discussion of the results, and assisted in drafting the manuscript. All authors read and approved the final manuscript.

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