Improvement of Chronic Wound Healing by Pre-activated Bone Marrow Cells with Sodium Nitroprusside in Rabbits

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ABSTRACT

Aim of study This study investigated whether pre-activated bone marrow cells with sodium nitro prusside have effectiveness in the inhibition of diabetic wound healing in diabetic rabbits. In diabetic skin disorders and conditions involved redox state disturbances. The aim was to determine the effect of two minimum dosages of sodium nitro prusside, and its' potential with bone marrow cells for chronic wound healing in-vivo.

Methods Full-thickness skin dorsal wounds were created on diabetic rabbits. The effects of two minimum concentrations of sodium nitro prusside solution with bone marrow cells on wound healing were studied. The useful combination of sodium nitro prusside with bone marrow cells on wound repair may be attributed to its functional influences on inflammation, angiogenesis, cell proliferation, matrix deposition, and remodeling.

Results The in-vivo experiments confirmed that pre-activated bone marrow cells contributed to wound healing by alleviating oxidative stress, increasing proliferation and migration, decreasing apoptosis. In histological results, improved collagen deposition, enhanced re-epithelization, angiogenesis, and decreased inflammatory infiltration were also detected in wound biopsies.

Conclusions For the treatment of chronic wounds, cell-based therapy was an attractive approach. Bone marrow cells have a low ability to differentiate various types of cells or late healing without pretreatment. So it was needed to increase their potency of differentiation. The transplantation of pretreated bone marrow cells with a prime quantity of sodium nitro prusside solution improved chronic wound healing with a greater level of growth factors and a minimum level of oxidative stress.

Introduction

Diabetic wound healing (DWH) is a primary clinical problem and is one of the foremost causes of lower extremity amputation. DWH is a complex process involving a highly regulated cascade of events, initiated by interactions between many soluble factors, cell types, and matrix components [1]. Globally around 15–20 % of diabetic patients suffer from long-term delayed wound healing impairments during their lifetime [2].

The absence or delay of healing of wounds is usually due to lack of nitric oxide (NO) release [3], deficient secretion of growth factors, an insufficient supply of oxygen, leading to reduce angiogenic activity and thus wound healing process [4]. Wound healing components are highly affected by diabetes, including hemostasis, inflammation matrix deposition, and angiogenesis [5]. There have been altered blood flow, impaired neutrophil antimicrobial activity, and a dysfunctional inflammatory state associated with abnormal chemokine expression in chronic wounds [6]. Bone marrow (BM)

is the main reservoir for adult organ-specific stem cells, including endothelial progenitors and hematopoietic stem cells. Many chemokines or cytokines trigger endothelial progenitor cells (EPC) secreted through induction of metalloproteinase 9 in BM and NO was also linked to this all process [7]. Sodium Nitroprusside (NaNP) is a NO donor, investigated for its intravascular effects on ischemia-reperfusion injury [8]. NaNP plays a role in collagenous protein biosynthesis in a dose-dependent manner [9]. Due to its small size and uncharged nature, NO is a small free-radical gas that can easily traverse the cell membrane and is involved in many biological events in all living systems. NO is produced by the nitric oxide synthase (NOS) enzyme, which catalyzes the conversion of L-Arginine (L-Arg) to L-citrulline. L-Arg supports in stimulating Protein synthesis via the activation of the mTOR (Thr 2446)/p70S6K signalling pathway in an NO-dependent manner [10]. Asymmetric di-methyl arginine (ADMA) has been produced and raised in patients with type 2 DM [11]. There is a new paradigm of NO that participates in specific signal transduction pathways; in signalling and cell communication processes. The key steps are regulating and modifying NO production that governs various biological mechanisms, both in physiological and pathophysiological conditions [12]. NO either suppress or stimulates cellular movements and or proliferation. NO has played a role in the stimulus of cellular migration and proliferation of endothelial cells [13]. The biomaterials with NO-releasing ability receive greater attention as an ideal wound dressing [14]. By up-regulation of eNOS, NO has been produced via VEGF. The protection from apoptosis, proliferation of endothelial cells, and mediated VEGF production have been stimulated by NO. Formation of granulation tissue with angiogenesis, recruitment of inflammatory cells, the proliferation of fibroblasts, and migration of keratinocytes contribute to restoring of functional and anatomical integrity [15]. The wound healing process is also dependent on systemic signals, such as growth factors, chemokines, cytokines, and proteolytic enzymes. All these interrelated events determine the speed of wound healing [16]. Diabetic wound healing exhibits reduced chemotactic ability to convert inflammatory cells into damaged tissues, creating an attenuated inflammatory response. After correlating with the extent of prolonged or insufficient healing, these deficiencies lead to decreased angiogenesis due to dysfunctional endothelial progenitor cells that can't proliferate and differentiate. Collagen matrixes that are severely affected in diabetic wounds also contribute to reducing wound healing [17]. The bone marrow (BM) harbors a subset of cells capable of endothelial differentiation. Endothelial cell proliferation is linked to angiogenesis [18]. Different cytokines and inflammatory stimuli induce NO production by primary cultures of BMCs and that this mediator may play a major role in the regulation of BMCs development and growth. In vitro, adult BMCs have the potency to trans-differentiate into the pancreatic lineage and provide a pool of cells for the treatment of DM [19].

Transplanting the pretreated BMCs help in functional recovery and suppresses other inflammatory and immune responses [20]. The efficacy and efficiency of BMCs therapy are limited because very few transplanted cells survive and are home to injured tissues and functional recovery often is inadequate. The low homing rate and poor survival of transplanted cells restrict the clinical potential of this therapeutic approach [21, 22]. Aimed to target this problem, our recent study focused on two minimum doses of NaNP with BMCs for DWH. The combined effect of NaNP minimum concentrations in BMCs plays a vital difference in DWH, angiogenesis, collagen formation, and vascularization. NO signalling positively affects the diabetes wound and induces swift healing.

Material and Methods

Preparation of Stock Solution Sodium nitroprusside solution was prepared in (PBS) Phosphate Buffer Solution. An exact 0.05 g/50 mL solution was prepared. The Sample bottle was covered with aluminium foil because the sample was photosensitive. Rabbits were sacrificed and extracted BM from the tibia and fibula.

Animals Used

The animal study was performed following the Guide for the care and use of Laboratory Animals published by the US national institutes of Health (NIH Publication No. 85–23, revised 2011). The performed protocol was permitted by the institutional Research Animal Ethics committee of Mirpur University of Science and Technology, Pakistan. All surgeries were conducted in the animal laboratory of Akson College of Pharmacy. 70 healthy rabbits were used (6 in each group). Adult New Zealand White rabbits of 6–8 months bearing a weight 3–3.5 kg were used in this study, encaged separately. The rabbits were housed in a room with controlled temperature (23 ± 2 °C) and humidity (60 ± 10 %) under a 12–12 h light-dark cycle and allowed access to a rabbit-specific diet and water ad libitum. Concentrated feed (mixed cereal grains) was 120–150 g per rabbit per day and green fodder consumption was 400–600 g per rabbit per day. The rabbits were anaesthetized with Ketamine (Alcon Lab U.K limited) and Xylazine Injection (Bimeda Inc U.S). Once under anaesthesia, the level of anaesthesia was monitored and maintained by a veterinarian. Surgery was performed using aseptic techniques.

Diabetes Induction

Diabetes was induced in rabbits using a single intra-venous injection of alloxan monohydrate at 90 mg/kg body weight dissolved in sodium citrate buffer (pH 4.5) as used to induce diabetes in anaesthetized rabbits. A glucometer was used to record the blood glucose level after the treatment of rabbits. After one_week, the blood glucose level was stabilized in the treated group and was recorded to be 250–420 mg/dl. The blood glucose level of normal animals to 75–175 mg/dl.

Preoperative Procedure

The preoperative procedure was conducted in the laboratory of an animal care centre. The Left lower backside of the tested animals was completely shaved. The specific area was traced by outline. 2 % lignocaine was used for local anaesthesia. Forceps, scissors, and surgical blades gave a full-thickness wound of about 20 mm square [23].

Grouping of Wounds

Animals were divided into six groups, every group comprised of six members in each (n = 6).

Groups 1 and 2 are control groups (Negative and Positive). 3 and 4 are Vehicle Control groups

(BMCs and NaNP (50 µL). 5 and 6 are treatment groups BMCs (NaNP 50 µL) and BMCs (NaNP 100 µL).

Group I: (Negative) was left untreated. Superficially, it was covered with a bandage, which remains under observation until the end of healing.

Group II: (Positive) applied topically to wound area with Polyfax (0.05 mg) after 2 days intervals.

Group III: Injection of 10^4 suspended BMCs in 1 mL PBS, administration on wound area, after 2 days interval till the end day.

Group IV: (Administration of 50 µL of NaNP injection to wound area, after 2 days interval, until complete healing).

Group V: (administration of injection including 10^4 BMCs with 50 µL NaNP, on wound area after 2 days interval till the end day).

Group VI (administration of injection including 10^4 BMCs with 100 µL NaNP, on wound area after 2 days interval till the end day.

Wound creation and Sampling

After the wound creation, the wound was washed with a saline solution immediately. The animals were kept in individual cages and back to their standard situations (Temperature 23 ± 3) ˚C. All dressing and animal maintenance were followed by the ethical rules of standard surgical processes [24]. Skin samples were quickly removed including the whole wound and healthy edges around each wound. With the help of scissors and forceps, thick-sized wounds of 20 mm square width were created on both sides of the body at the site of the mid-dorsal line of the animal.

Measurement of the wound closure area

Photographs of the wounded area for the closure of wounds and physical appearance were taken using a digital camera (DSA-W320 Sony: Sony Corp., Tokyo, Japan) on 0, 5, 10, and 15 days posing vertically to middle of the wound with a distance of 6 cm. The efficacy of treatment was reflected in the amount of reduction in the wounded area. With the help of a transparent paper millimetre scale, the wound area was retracted and measured on 0, 5, 10, and 15 days. To minimize errors in the measurement of wound area as well as achieve statistically sound results, wound healing percentage was replaced with wound area and calculated as follows (▶ **Fig. 2**).

$$\text{Wound healing percentage in nth day} = \frac{\text{Wound area in the first day} - \text{wound area in nth day}}{\text{Wound area on the first day}} \times 100$$

Isolation of BMCs White New Zealand healthy rabbits was selected for bone marrow (BM) isolation. Accordingly previously reported [25].

Pretreatment of B.M cells Added NaNP as an induction factor to BMCs suspension for the increase of differentiation potential of a cell, about 50 µL & 100 µL from the stock solution [26].

Extraction of RNA

0.75 mL TRIzol reagent was added to 0.25 mL of a sample of RNA in a Cell pellet, lysed cells were suspended by vortex vigorously for 10 minutes. Then added 200 µL of chloroform, vortex for 10 minutes followed the incubation on ice. RNA was taken from an aqueous phase and then added in a separate tube with 0.5 mL of isopropanol and incubated for 10 minutes on ice. Centrifugation was done for 10 min at 1200 rpm and the supernatant was removed. After air-drying of a tube, 20 µL of RNAase-free water was used to dissolve RNA.

cDNA Synthesis

cDNA was synthesized using the total RNA extracted (cDNA kit by Thermos Fisher Scientific: K2668) from wound biopsies. The tube should be kept on ice, then added RNA 5 µL, nuclease-free water 12 µL, Oligo (dt) 18 primer 1 µL. Mixing of the solution was done gently and incubated at 65 ℃ for 5 minutes. After that put on ice for 10 minutes, then 5x -reaction buffer 4 µL,10 mmol dNTPs 2 µL, RNase inhibitor (20 U/ µL, RT Primer 1 µL were added.

Gene Expression Analysis

To evaluate the effect of treatment at the molecular level, the expression of BCl2, eNOS, and VEGF was measured through reverse transcriptase PCR. Total cellular-RNA was taken out from sample tissues on day 15 with the help of TRIzol (Invitrogen) following the manufacturers' guidelines. Online software Primer3 was used for the designing of Gene-specific primers. (http://frodo.wi.mit.edu/primer3/). Sequences were taken from NCBI. The sequences of these genes are related to growth factors and the proliferation of WH.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed by using (Thermos Fisher Scientific kit: K1891). The BCl_2 (B cell lymphoma-2), VEGF (Vascular endothelial growth factor), and eNOS (endothelial nitric oxide synthase) were carried out and normalized against β-Actin. At first, the expression of these genes was analyzed through RT-PCR. The scheme applied for amplification for VEGF, eNOS, BCl_2, and β-Actin was initial heat shock at 95 ℃ for 5 min, denaturation at 95 ℃ for 30 sec, primer annealing at 57 ℃ for eNOS and 58 ℃ for VEGF, 55 ℃ for BCl_2 at 30 sec, and extension at 72 ℃ for 30 sec, Final extension for 10 min at 72 ℃.

5 µl of each cDNA sample with PCR master reaction mixture. (dH_2O 12 µL, Primer R + F 2 µL, h PCR buffer 2.5 µL, $MgCl_2$ 12 µL, 10 mm dNTPs 1 µL, Taq DNA polymerase 0.5 µL) were used (▶ **Table 1**). The PCR products were then size separated on 1 % agarose gel with the help of EtBr.

Determination of wound nitric oxide concentration

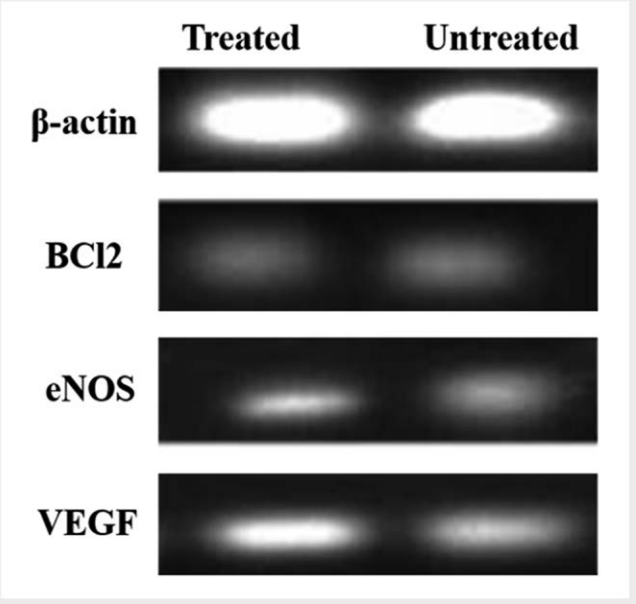
The concentration of nitric oxide in the wound was estimated by using the Griess reagent. An equal volume of sample (homogenate tissue/media) and Griess reagent (5 % phosphoric acid containing 0.1 % NEDD, 1 % Sulfanilamide) should be mixed and incubated in the dark for 10 min at room temperature. The NO concentration was checked in all wounds. Tissues were cut, washed with PBS, and minced properly with Griess reagent. Incubate the tubes at 37 °C, 5 % CO_2 for 48 h. Centrifuged 1000 rpm for 5 min. The supernatant of the samples was checked. The absorbance of the reaction mixture was measured at 540 nm [27]. Sodium nitrite (NaNO2) dilutions from 0–100 µM were used as a standard to accurately determine NO concentration in the media.

Cell Proliferation

For the extraction of BMCs in the experiment, tibia and fibula sections (bones of Diabetic rabbits) were used. Saline solution was used to aspirate the BMCs. Cells were centrifuged at room temperature at 1200 rpm for 10 min., cell debris was used, and the supernatant was discarded. 1 × 10^4 cells were seeded in 96 well plates

▶ **Table 1** Primer sequence.

Gene	Primer Sequence	Product Size(Kb)
BCl2-F	5'-CGACTTTGCAGAGATGTCCA-3'	223
BCl2-R	5'-ATGCCGGTTCAGGTACTCAG-3'	223
eNOS-F	5'-TGACCCTCACCGATACAACA-3'	123
eNOS-R	5'-CTGTACAGCACAGCCACGTT-3'	123
β-Actin-F	5'-GCTGTGTTGTCCCTGTATGC-3'	106
β-Actin-R	5'-GAGCGCGTAACCCTCATAGA-3'	106



▶ **Fig. 1** The Gene expression of VEGF (proangiogenic growth factor) and eNOS (endothelial Nitric oxide synthase) increased with many folds in the treated group (with 50 µL NaNP) than the untreated group (without pretreatment). BCl2 (a cytoprotective gene) has increased in the untreated group indicating little apoptosis had occurred.

for 6 groups. i. e, group 1. No treatment group as a negative control. Group 2. Add 0.05 mg Poly-fax to cells as a positive control. Group 3. Add NaNP 50 µL. group 4. Add Normal BMCs (1×10^4). Group 5. Preactivated BMCs (1×10^4) with 50 µL NaNP. Group 6. Add Preactivated BMCs (1×10^4) with 100 µL NaNP. 800 µL serum-free medium and 80 µL 3-(4, 5-Dimethylthiazol-2-YI)-2,5-Diphenyltetrazolium Bromide (MTT) solution (5 mg/mL in PBS) were put into cell culture wells with different samples separately. Followed by incubation at 37 °C for MTT formazan formation. The medium and MTT were rinsed with PBS and MTT were performed; 350 µL/well of MTT (0.5 mg/mL) was used to elute the ice crystals of MTT and the absorbance of the solution was measured at 570 nm using a spectrophotometer (Thermo Spectronic, Genesys 10UV scanning). The extent of in-vivo cell viability was expressed as a percentage of cell proliferation [28].

ELISA assays

With the help of the ELISA Test, different samples were assessed for their content of specific growth factors. Collagen type 1 (CoL-1) and VEGF were measured per manufacturer's instructions: (kits from R &D System, Abcam, and My BioSource), on days 5, 10, and 15 [26].

Histopathological studies

Rabbit's wound skin was excised after three weeks. Rabbits were anaesthetized on days 5, 10, and 15. The wounded area with a periphery of about 5 mm^2 of ambient un-wounded skin biopsy was taken. In 10 % formalin via graded alcohol series samples were fixed, clearly in xylene, and embedded in paraffin wax. Thin tissue sections of 5 µm thickness as made using Microtome and stained with hematoxylin and eosin (H & E). The stained sections were observed using a light microscope fitted with a camera about neovascularization, epidermis, scar, and granulation tissues, and images were taken.

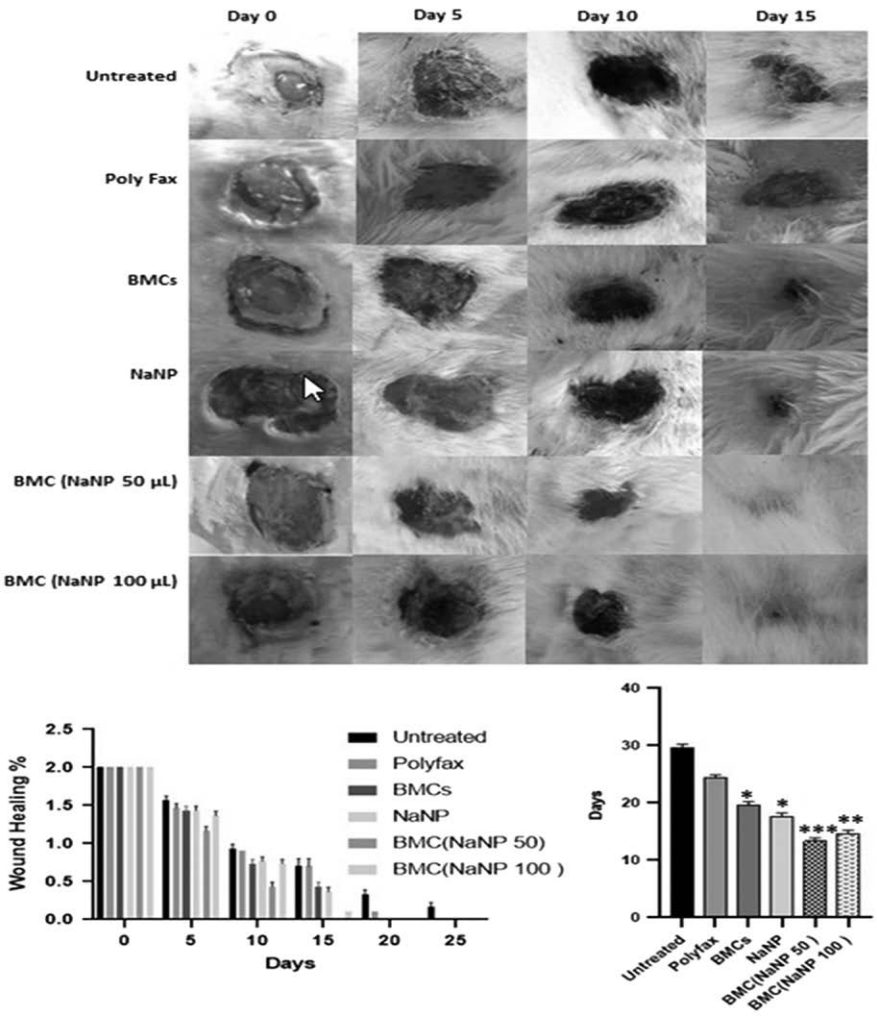
Results

RT-PCR

The expression of pro-angiogenic, cell proliferating and NO-responsible genes were assessed via RT-PCR. The resultant bands are shown in (▶ **Fig. 1**) which shows the gene expression of VEGF BCL2 and eNOS in normal BMCs and pre-activated BMCs. Pretreatment of BMCs supported epithelization and differentiation because NaNP was NO donor and eNOS expression level indicated the main role in healing.

Wound Healing Evaluation

The wound area was exact 20 mm^2 in this type of metabolic disease; the wound was later healed. The diabetic wound was not easy to heal till the 5th day. In the initial days the growth rate among wounds was slowed; later on, it was necessary to clean the wound with saline solution before being examined. Both wounds of BMCs (NaNP 50) and BMCs (NaNP 100) were applied. NaNP, BMCs, and Poly-fax groups didn't show any progress, as compared to other wound groups i. e. applied with BMCs pretreated. Before day 20, all wounds were healed except the untreated and poly-fax group in diabetic rabbits. In the study, pretreatment of BMCs, wounds represented tremendous healing specifically in group BMCs (NaNP 50) than BMCs (NaNP 100). A high concentration of NO donors led to the generation of reactive oxygen species (ROS) that related to delayed WH in BMCs (NaNP 100). The Healing pattern of BMCs (NaNP 50 µL) was very smooth compared to another group. The BMCs healing in the starting days was very quick but with time it healed late. But the preconditioning aspect increases healing capacity. The healing pattern was earlier in a pretreated group with NaNP. The significant difference between the Polyfax group with NaNP and BMCs was * $p<0.05$. The surface of BMCs was not smooth in a pattern of healing. The wound surface of the untreated group has a loose pattern of healing until the 12th day. Moreover, the NaNP group in the starting days examined and was less healing area and loose pattern. The pretreatment aspect played a vital role in the proliferation, growth, and migration of cells, and resisted the DWH conditions. The BMCs provided all the basic needs or potency for the enhancement of proliferation and division in the early days



▶ **Fig. 2** (**a**) Study of in-vivo diabetic wound analysis. The size of the wound was about 20 mm square and every wound healed according to the applied sample. Wound samples were measured at day 0, 5, 10, and 15 posts operational. Wound closure rate as plotted as the reduction potential percentage of original wound area over time. Among different wound groups; Two-way Anova was used to evaluate the statistical difference. (**b**) The comparative study of wound healing mean ± SD of all experimental groups on day 0, 5, 10, and 15 were analyzed by using two-way ANOVA. The Wound area of all groups decreased from 0 days towards the 15th day. (**c**) By using ANOVA, we evaluated the statistical difference (* $p<0.05$, * * $p<0.01$ and * * * $p<0.001$) among groups.

(▶ **Fig. 2**). Day of epithelization was varied among groups. On the end day of the complete healing of the wound, the rabbit was sacrificed. The significant difference among experimental groups was * $p<0.05$. A Two-way variance of analysis was carried out for the evaluation of different groups. Poly-fax (positive control) was used as a control; to evaluate the significant difference with other experimental groups. At day 10, day-wise significance comparatively for BMCs, NaNP and BMCs (NaNP 50 µL) was * $p = 0.04$, * * $p = 0.016$ and * 0.03, respectively. The p-value was at day 15 of BMCs and BMCs (NaNP 50 µL) (▶ **Fig. 2b**). Among the varied groups, the day of epithelization was carried out by using one-way ANOVA. In wound closure percentage, the wound area of all groups decreased from day 0 to day 15. The significant differences between control with NaNP and BMCs were * $p = 0.05$ and * $p = 0.03$. The significant difference for BMCs (NaNP 50 µL) was * * * $p<0.001$ and BMCs (NaNP 100 µL) was * * $p = 0.003$ (▶ **Fig. 2c**).According to the data, complete wound healing was observed in pretreated diabetic wound groups. Moreover, the variance analysis for the wound area designated that the pretreatment of BMCs with NaNP had been more successful throughout the experiment.

Determination of Nitric oxide Concentration

In the wound tissue of diabetic animals, there were significant decreases in NO levels. The group of diabetic rabbits indicated decreased concentration of NO. Due to that concentration, the WH was abrupt. There was a significant difference in the nitric oxide concentration among different groups. The determination of nitrite produced due to the reaction of nitric oxide (NO) with oxygen was evaluated with the help of the Griess assay. Results showed that the level of NO was significantly different among groups. Polyfax was used as a control with other experimental groups. The p-value of BMCs (NaNP 50 µL) and BMCs (NaNP 100 µL) was * $p<0.02$ * * $p<0.01$

respectively, at day 5. On day 10, the significant difference among groups was * p<0.05 for the BMCs, NaNP, and BMC (NaNP 50 µL) group. At day 15, the significant difference was * p<0.05 for NaNP group, * * p<0.01 for BMC (NaNP 50 µL) (▶ **Fig 3**). So, NO signalling Is an important aspect of diabetic wound healing.

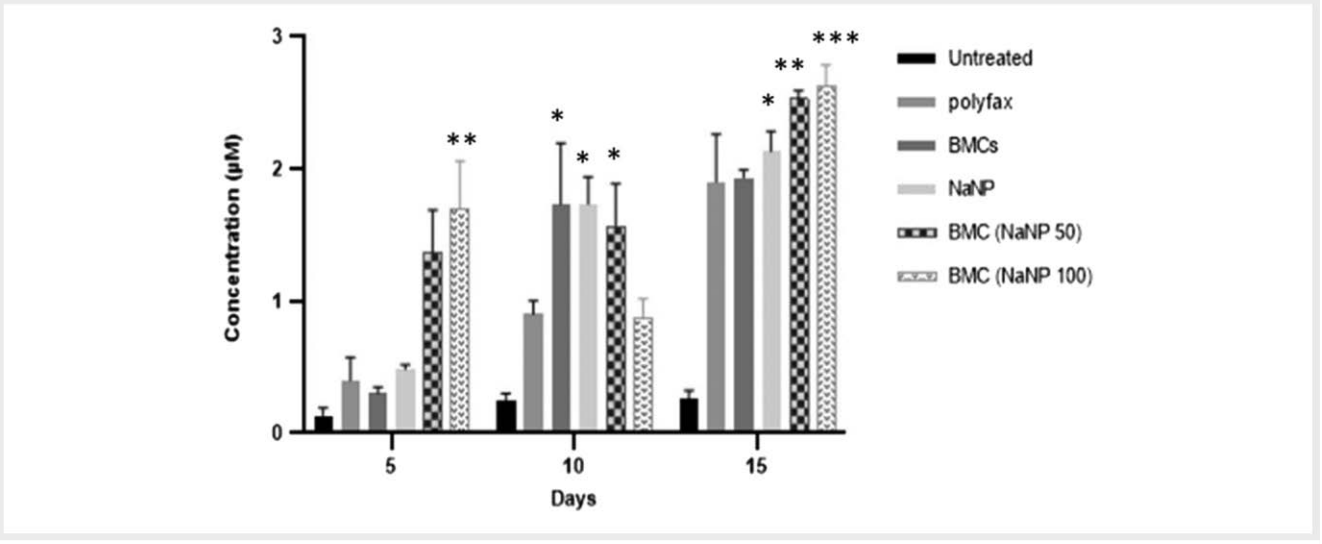
Determination of cell proliferation

In-vitro cell proliferation of rabbit bone marrow cells was evaluated. For the cell proliferation rate of BMCs, approximately 1×10^4 of BMCs were seeded per well of a 96-well culture plate and divided into 6 groups. Therefore for the evaluation of proliferating cells, used MTT assay. During incubation, checked viable cells in different intervals of time. On day 10, the cell proliferation was examined, the absorbance of the solution was measured at 570 nm using a spectrophotometer. The extent of in-vivo cell viability was expressed as the percentage of cell proliferation. Significantly the difference among groups was evaluated. Significantly, the p-value of different groups was evaluated by using one-way ANOVA. The significant difference for BMCs, NaNP and BMC (NaNP 100 µL) was * p<0.05 and for BMC (NaNP 50 µL) * * p<0.01. Results indicated that NaNP can activate BMC and enhance proliferative and regenerative ability (▶ **Fig. 4a**).

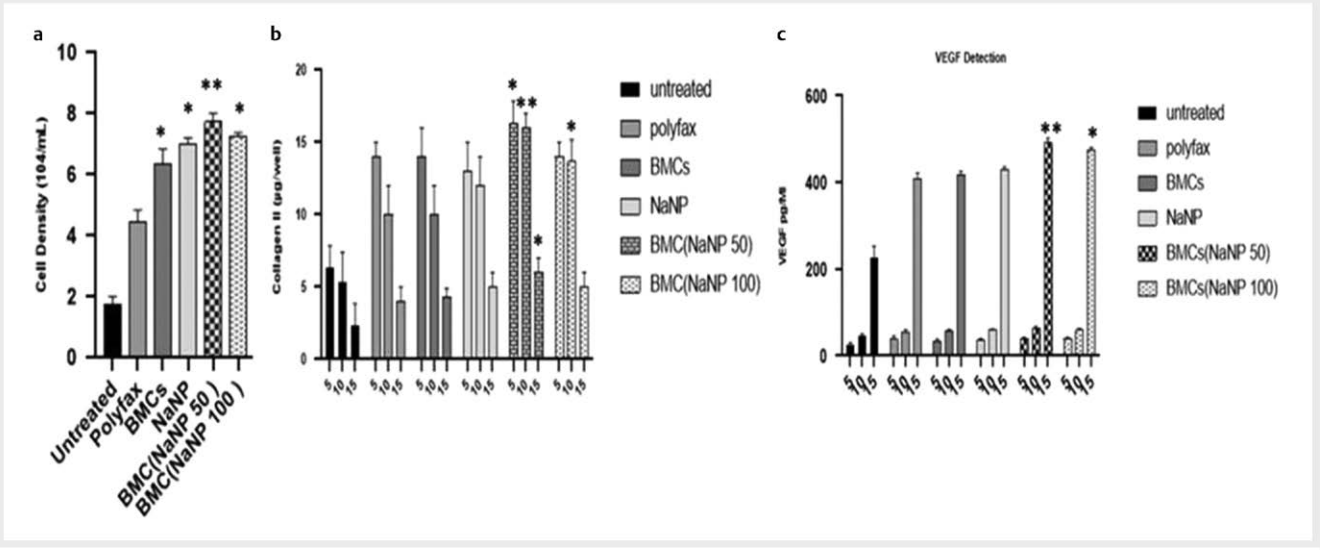
Levels of VEGF and CoL 1

Levels of VEGF and CoL-1 were evaluated significantly. Elisa measurements of the secreted level of Collagen and VEGF among different samples of the wound were quantified.

The relationship between these growth factor concentrations and the observed response in terms of wound closure kinetics, the characteristics of regenerated skin, and the healing process were



▶ **Fig. 3** Evaluation of statistical difference in NO concentration among different groups at days 5, 10, and 15, by using Two-way ANOVA.



▶ **Fig. 4** (**a**) Cell proliferation ability among (MMT Assay; Cell density) 6 groups. (**b**). Elisa measurement of secreted levels of Collagen I among different samples from the wound (**c**). Secreted Level of VEGF among different samples of the wound.

examined. In the Pre-activated experimental groups, the ratio of these growth factors was varied in contrast to other groups. Wound healing depends on the extent of collagen deposition and angiogenesis. Earlier wound healing also depends on the collagen1 formation. A T-test was used to evaluate statistical differences among groups about levels of collagen and VEGF and a poly-fax was used as control. For CoL-1 level detection statistically, the p-value at days 5, 10, and 15 were evaluated. On day 5, the statistical difference for group BMC (NaNP 50 µL) was * $p<0.05$. At day 10, the value was * * $p<0.01$ for BMC (NaNP 50 µL) and * $p<0.05$ for BMC (NaNP 100 µL). On the 15th day, the statistical difference was * $p<0.05$ (▶ **Fig 4b**). The VEGF level was significantly varied among groups. Significantly, the p-value between poly-fax with BMC (NaNP 50 µL) and BMC (NaNP 100 µL) was * * $p<0.01$ and * $p<0.05$ respectively at day 15. Results indicated the useful role of preconditioning with two minimum concentrations of NaNP.

Inside CWH, there was a harsh environment so many cells were not migrating to the wound sites or cells were remaining to undergo dysfunction and apoptosis [29]. Therefore, BMCs treatment can help in the migration and regeneration of skin cells (▶ **Fig. 4c**).

Histopathological studies

Diabetic wound histological studies represented the healing pattern in different samples via H & E staining and Masson's Trichome.

H & E staining

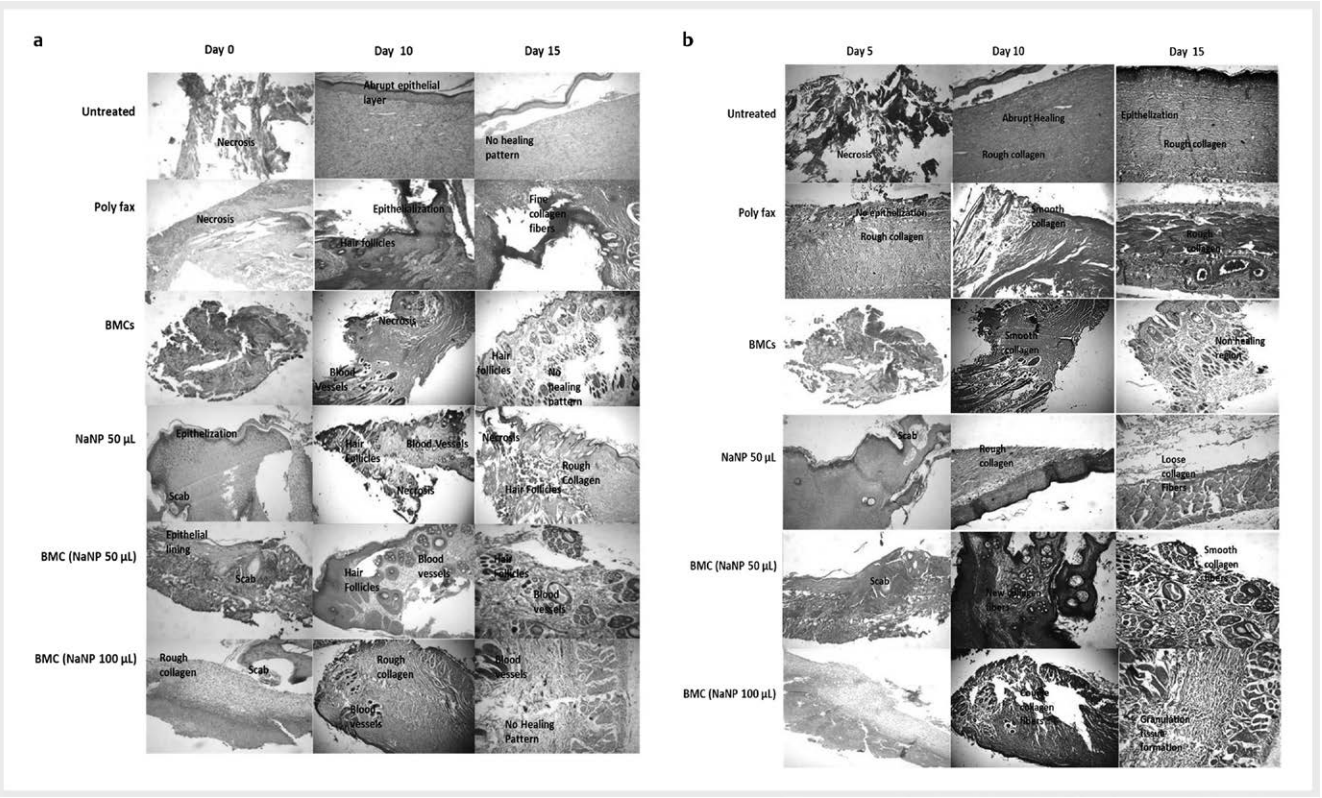
Represented biopsies indicated the comparative study of day 5, 10, and 15 wounds **(▶ Fig. 5a)**. On days 5, 10, and 15 the untreated wound sample showed a loose healing pattern of healing with fibrosis. In Poly-fax wound group; the necrotic area was seen at day 5, but at day 10 and 15 some area was healed properly but some patches of the unhealed wound were also seen. For BMCs wound group; at day 5, necrosis with no wound pattern as seen but at day 10, there was the formation of small blood vessels at the wound corner and at day 15 mostly area having mature cell bodies but some area of wound sample had blank patches indicated that BMCs groups showed healing activity at later stages. In the NaNP group at day 5, epithelial lining and scab were determined. On day 10 small blood vessels, abrupt epithelial lining with necrosis was seen. In BMC (NaNP 50 µL) wound group; some loose healing pattern region was observed on day 10 small blood vessels with proper epithelial lining were seen. Moreover, on day 15 wound was covered properly with skin cells, mature blood vessels, hair follicles with the proper healing pattern were seen. Besides this, in BMC (NaNP 100 µL) wound group on day 5, a rough healing region and scabs were found. On day 10 some blood vessels with the proper healing pattern were seen but on day 15 the healing pattern was abrupt. The wound had blank patches that illustrated not proper healing. Thus, by observation, ample evidence that the wound with more proliferating bodies and certain nuclei with less inflammatory issues revealed that the wound group with BMC (NaNP 50) provided a synergistic acceleration potential of wound healing in an induced diabetic wound rabbit model.

Masson Trichome's staining

In Masson Trichome's staining ▶ **Fig. 5b**, at day 5 only necrosis was seen, at day 10 less collagen with the abrupt healing region was seen but at day 15, the untreated wound group represented less healing area, no epithelial layer was present in the slide and other bodies and rough collagen fibres formation were observed. The poly-fax wound group was observed at day 5; rough collagen was seen but at day 10 smooth collagen formation was seen with no cell body, at day 15; the formation of some basal layer, rough collagen formation with some dermal bodies was formed but as compared to other experimental groups, in BMCs group; the formation of disrupted healing pattern large spaces and fewer collagen fibres were formed until day 15 with the non-healing region. In NaNP group indicated the formation of dermal bodies but not the formation of an epithelial layer at day 10, but loose collagen fibres were seen till day 15. In BMCs (NaNP 50 µL) on day 5, a loose healing pattern was seen. On day 15 there was showed proper healing. Complete fine collagen fibres were observed with organized healing patterns and the formation of blood vessels with proper dermal bodies was examined in wound biopsy. In BMCs (NaNP 100 µL the wound had smooth healing with tough collagen fibres, but within the wound rough collagen fibres patches were observed, which represented incomplete healing **(▶ Fig. 5b)**.

Discussions

Poor wound healing is a foremost complication in diabetic patients and a consequence of death and morbidity. Less concentration of NO-donor has been related to increased angiogenesis and collagen formation. Inhibition of COX-2 has been associated with greater cutaneous wound healing pressure ulcers in mice via reduction of iNOS expression [30]. Moreover, the impaired healing of cutaneous wounds in diabetes is accompanied by hypoxia [31], which has amplified the early inflammatory response by increasing the levels of inflammatory mediators and oxygen radicals. The Hyperglycemic condition has led to oxidative stress when the ROS production exceeds the antioxidant capacity [32]. ROS accumulation is related to widespread cellular damage and poor wound neovascularization [33]. Wound healing has improved by VEGF by applying topically, that upregulate growth factors essential for tissue repair and by systemically mobilizing bone marrow-derived cells, including a population that supports blood vessel formation. In addition, these recruited nearby wounds and accelerate repair mechanisms. So, VEGF therapy has been considered useful in diabetic complications [34]. Not only angiogenesis but bone-marrow-derived cells including endothelial progenitor cells have supported new vessels, also responded to trauma or ischemia, and can be able to assist in tissue repair [35]. By tropic support and cell substitution method, the BM helps in tissue repairing and functional revitalization [36]. According to molecular and cellular bases of wound healing in diabetes that the NO is the main regulator of cell response in both vascular and inflammatory infections, allergens, and wound [37]. NO availability has been detected during wound repair in-vitro is further maintained by the proliferation and angiogenesis, via greater effect with a low concentration of NO-donor in-vivo [38]. The growth factors have the potency to differentiate the different levels of wound healing. Growth factors are dependable for mobilizing, stimulating, and activating of SCs from the BM. In our previous study, the regeneration of specific tissue in that area depends on the specific growth factor and optimum doses of NaNP with BMCs



▶ **Fig. 5** **a** Comparative study of H & E-stained slide of Diabetic wound healing of 6 different groups; Untreated, Poly-fax, BMCs, NaNP, BMCs (NaNP 50), and BMCs (NaNP 100). Scale bar 100 µm. BMC (Bone marrow cells), NaNP (Sodium nitroprusside). **b** Comparative study of Masson Trichome's stained slide of Diabetic wound healing of 6 different groups; Untreated, Poly-fax, BMCs, NaNP, BMCs (NaNP 50), and BMCs (NaNP 100). Scale bar 100 µm. BMC (Bone marrow cells), NaNP (Sodium nitroprusside).

that enhance WH [26]. So the SC research tells about the proper sequence of growth factors i. e. needed for specific tissue regeneration [39]. The present study was designed to produce skin layers or increase its epithelization activity by introducing pre-treated BMCs into CW. In wound healing, neovascularization is the crucial step. The pretreatment factor affects the potential of BMCs that increases the regenerative ability of cells; it becomes a revolution in biochemical research. A limitation of our study was we only use the two minimum dosages of NaNP with BMCs for DWH. The study revealed only the optimum dosage of this combined effect.

Conclusion

The development of two minimum doses of NaNP, and the mutual effect of BMCs and NaNP represented the protection against skin regeneration and gave no scar formation in diabetic rabbits. BMC itself was a low capability to discriminate various types of cells or late healing without pretreatment. So it was needed to enhance their potential to distinguish the cells. The transplantation of pretreated BMCs with the optimum quantity of NaNP solution enhanced WH with an increased ratio of growth factors and decreased level of oxidative stress. The pretreatment aspect can enrich the skin with collagen deposition, and reepithelization, so the improved Chronic WH suggested the benefits of pretreatment BMCs with NaNP in clinical practice.

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Conflicts of interest

The authors declare no conflict of interest. The author alone is responsible for the content and writing of the paper

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