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Original Research Article

ADIPOSE-DERIVED STEM CELLS (ADSCS) PRETREATED WITH VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) PROMOTED WOUND HEALING IN RAT SKIN BURN MODEL

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Abstract: *Stem cells are extensively used for regenerative purposes as they are unspecialized cells capable of renewal and differentiation. Growth factors like vascular endothelial growth factor (VEGF) play a crucial role in enhancing the regenerative potential of stem cells. The present study was designed to elucidate the effects of VEGF preconditioning in accelerating the regenerative potential of adipose-derived stem cells (ADSCs) for wound healing. In vivo study was carried out using female Sprague Dawley (SD) rats randomly divided into three groups, i.e., VEGF preconditioned ADSCs transplanted group (Pre-Tx), normal ADSCs transplanted group (N-Tx) and control group. ADCS were isolated from female SD rats and treated with VEGF for the Pre-Tx group. At 21st day post-transplantation, the wound in the Pre-Tx group was completely closed. However, the wound was not fully healed in the N-Tx group and the Control group. For further analysis, the experimental area of skin tissues was taken from all groups and examined histologically. The cellularity and granulation were thicker in the pre-Tx group and thicker in the pre-Tx group and thicker, indicating rapid wound recovery. Furthermore, the polymerase chain reaction results also confirmed the down-regulation of apoptotic marker caspase3, which indicates less cell death at the injury site and up-regulation of certain growth factors like IGF, sdf1α and some other markers like E cadherin and vimentin. RT-PCR analysis revealed significant up-regulation of all these factors in the pre-Tx group compared to other groups. These results suggest that VEGF pre-conditioning improves the reparative potency of ADSCs by increasing their survival rate and stimulating the secretion of various growth factors having crucial involvement in angiogenesis and recovery of damaged tissue.*

Keywords: VEGF, Adipose-derived stem cells, Pre-conditioning, Burn wound, Apoptosis, Angiogenesis

Introduction

Adipose-derived stem cells (ADSCs) are multipotent cells serving as a promising remedy in regenerative medicine because of their ample availability and ease of harvest by less invasive methods from adipose tissue (Kamda *et al.*, 2008). ADSCs can be differentiated into adipogenic, chondrogenic, osteogenic and myogenic lineages by important inducing factors. Adipose-derived stem cells secrete different vital growth factors and cytokines, which are very significant and critical for tissue repair and regeneration (Zuk *et al.*, 2001; Riaz, 2021). Burn lesions cause mortality and disability everywhere in the world. Multiple organ failure has been observed in both animals and humans, enduring the preliminary insult of severe burn injury (Adeteye *et al.*, 2011). Depth and percentage of the total body surface area define the severity of the burn. Third-degree burn destroys a major part of the epidermis and dermis layers of skin. Hair follicles and sweat glands get involved in addition to subcutaneous fat tissue. Third-degree burn causes charred, leathery and depressed skin texture compared to adjacent tissue. Unexpectedly, third-degree burns are generally not painful as the injury destroys nerve endings (Moore and Darley, 2006). Chronic third-degree burns are very frequent, but their treatment options are inadequate and mostly ineffective. Cell therapy using stem cells was very promising for skin wound restoration (Altman *et al.*, 2008; Kim *et al.*, 2007). Briefly, ADSCs facilitate wound healing by secretory factors, which is further boosted by hypoxia (insufficient oxygen level). Hypoxia augments the paracrine effects of ADSCs by inducing the secretion of certain growth factors (Rehman *et al.*, 2004; Kinnaird *et al.*, 2004). Transplantation of ADSCs results in tissue rejuvenation by promoting the growth of blood vessels (Lopatine *et al.*, 2011).

Vascular endothelial growth factor (VEGF) is a process vital for angiogenesis (Zingg *et al.*, 2012). Several previous studies have demonstrated fundamental role of VEGF in the regulation of

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angiogenesis and have demonstrated fundamental role of VEGF in the regulation of angiogenesis. It has been documented that even a single allele loss of VEGF results in lethality at the embryonic level due to a unique role offered by this growth factor in the development and differentiation of the vascular system. Moreover, VEGF-induced angiogenesis resulted in a healing outcome in animal models of coronary or limb ischemia and a human patient affected by critical leg ischemia (Carmeliet *et al.*, 1996). This study was conducted to explore the effects of VEGF pre-conditioned ADSCs on burn wound injuries. Preconditioned ADSCs were found to improve wound skin histology, enhanced process of wound skin histology, and also enhanced tissue regeneration by decreasing level of apoptosis and concomitant increasing expression of various significant factors having a therapeutic role in wound healing.

Materials and Methods

Animals

3-4 months old female Sprague–Dawley (SD) rats were selected for the *in vivo* experiments. The 12-hour light/dark cycle and access to food and water were sustained in a controlled environment for the rats. Animals were kept according to guidelines of the institutional review board (IRB) at the National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan.

Isolation and expansion of Adipose stem cells (ADSCs) from adipose tissue

Adipose tissue of rats was isolated from the lower abdomen of SD Rats weighing between 150-200g. The rat was euthanized in chloroform (Merck Cat No. 8.22265.25000). Adipose tissue was digested with collagenase 1 (Sigma–Aldrich, USA) following the previous protocol (Meric et al., 2013). Isolated cells were cultured in DMEM (Sigma, Aldrich) low glucose supplemented with 15% fetal bovine Serum (Sigma, Aldrich). Exhausted media was replaced on alternate days. Cells were sub-cultured when they reached 70–80% confluency. All the succeeding experiments were performed in passage 3 (P3).

Preconditioning of ADSCs with Vascular Endothelial Growth Factor

ADSCs at passage 3 were preconditioned with VEGF.50ng/ml of VEGF (Millipore. USA) in serum-free DMEM media was supplemented to the ADSCs containing flask and incubated at 37ºC and 5% CO_2 for 1 hour.

Animal model of the burn wound

Female SD rats, 3–4 weeks old, were housed under controlled environment conditions. Different experimental groups were designed (n=8 rats/group), as explained in Table 1. A burn wound model was established by putting a hot metal bar for 15 seconds on the dorsal side of rat's shaved skin was excised the next day.

Cell Transplantation

PKH26 Red Fluorescent Cell Linker Kit (Sigma Aldrich, USA) was used to label ADSCs, and VEGF pre-conditioned ADSCs. Cells were transplanted subcutaneously into four sides of the wound at a concentration of 1×10^6 cells per 50 µl phosphate buffer saline (PBS) per animal. After transplantation, the animals were housed individually. All animals were sacrificed on 21st day of cell transplantation.

Macroscopic and histological analysis

For fixation, skin tissues were kept in 10% buffered formalin overnight. After fixation, tissues were dehydrated in ascending grades of ethyl alcohol. Later, tissues were cleared by xylene solution. Samples were implanted in fresh molten paraffin. 5 µm thin sections were prepared and mounted on glass slides.

Assessment of cell homing and skin regeneration.

Paraffin was removed from the skin sections and stained with hematoxylin solution. Slides were washed with distilled water and counterstained with eosin and 95% alcohol in 1:4 ratios, respectively. To check the homing of transplanted cells, deparaffinized slides were stained with 4, 6-diamidino-2-phenylindole (DAPI) (MP Biomedicals Cat No. 157574) and mounted with fluorescent mounting media vectashield (Vector laboratories Inc. H1000). Samples were then visualized under a fluorescence microscope IX51 microscope (Olympus, USA).

In vivo gene expression analysis

Trizol reagent (Invitrogen; Cat No.15596-018) was used for the isolation of total RNA from animals' skin (n= 6 rats in each group) following manufacturer protocol. cDNA was made using 1 µg total RNA by using cDNA Synthesis Kit (Fermentas, Carlsbad, CA, USA). cDNA samples were kept at -20°C for further analysis.

Table 1. Different experimental groups of *in vivo* studies

Group	Group detail
Ctrl	Normal control rats
N-Tx	Burn model +ADSCs transplanted
Pre-Tx	Burn model + VEGF pre-conditioned transplanted

Table 2. List and sequence of primers used for gene expression analysis

Serial No.	Primer name	5'-3' Sequence	Product size (bp)	Tm (ºC)
1	β-Actin- F	GCTGTGTTGTCCCTGTATGC		
	β-Actin -R	GAGCGCGTAACCCTCATAGA	106	57

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2	caspase3- F	ACAGAGCTGGACTGCGGTAT		
	caspase3 -R	TGCGGTAGAGTAAGCATACAGG	110	57
3	Igf- F	GCTGAAGCCGTTCATTTAGC		
	Igf- R	CCACCCAGTTGCTATTGCTT	160	55
4	E cadherin -F	ACGTATCAGGGTCAAGTGCC		
	E cadherin -R	CCTGACCCACACCAAAGTCT	150	60
5	vimentin- F	ACGAGTACCGGAGACAGGT		
	vimentin- R	TCCAGCAGCTTCCTGTAGG	120	60
6	sdf1α- F	AGCCAGTCAGCCTGAGCTAC		
	sdf1α -R	GGCACAGTTTGGAGTGTTGA	104	57

Results

VEGF improves kin texture

Gross morphological features of various transplanted groups were compared with the control group. Maximum wound closure, smooth and even skin texture was observed in VEGF treated ADSCs transplanted group. ADSCs transplanted group also showed a reduced wound size, but less than VEGF treated group. Transplantation of ADSCs and preconditioned ADSCs resulted in better skin regeneration than the control group.

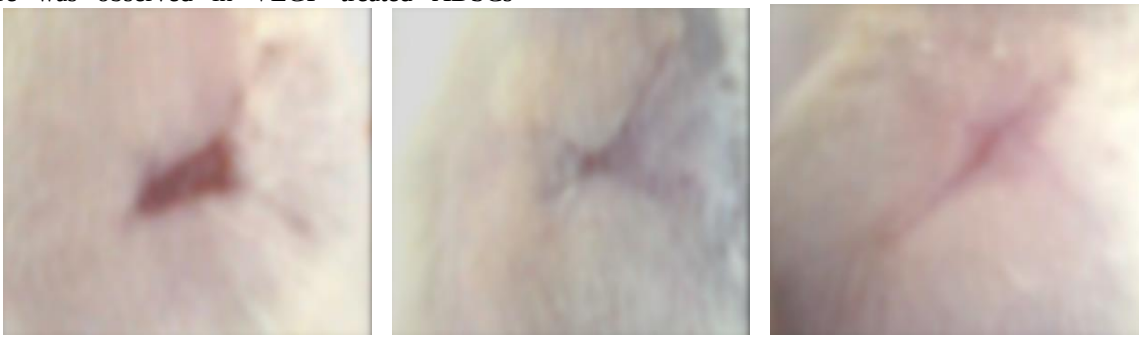

Figure 1. The figure shows the pattern of wound healing in PBS (1X) transplanted model "Ctrl", ADSCs transplanted "N-Tx", and VEGF treated ADSCs transplanted "Pre-Tx" at day 21st.

VEGF treatment enhanced homing and commitment of ADSCs

Homing of transplanted ADSCs in skin observed by fluorescent microscopy described that percentage of PKH-26 labelled ADSCs was noticeably high in the VEGF treated ADSCs group as compared to the untreated ADSCs group. This indicated more engraftment and survival of VEGF-treated ADSCs in the stress environment, which led to better re-epithelization and regeneration of burnt skin

VEGF triggeredReepithelization

Histological examination of the wounds disclosed that VEGF treated ADSCs group had enhanced cellularity, and granulation appeared thicker than in another group. In addition, reepithelization appeared to be increased in the VEGF treated cell group.

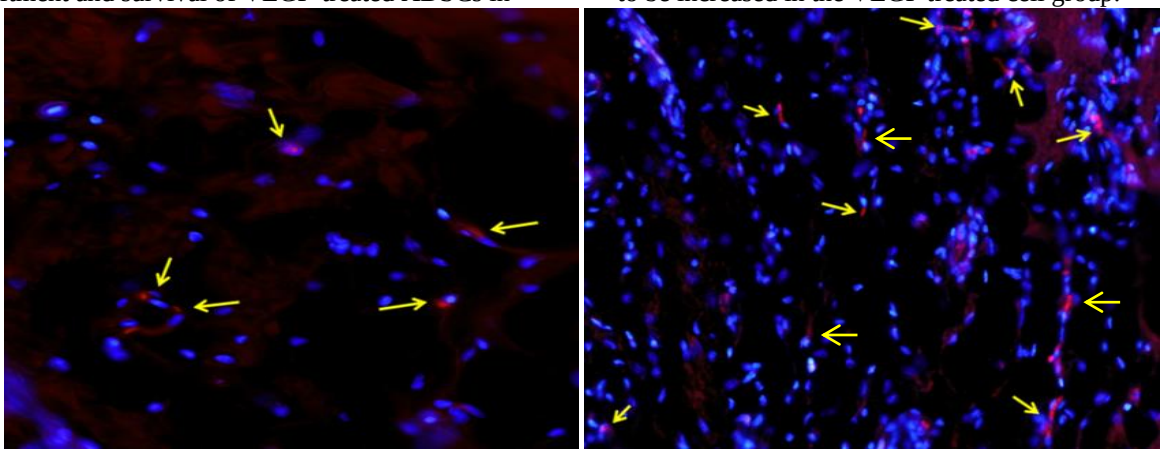

Figure 2. Homing of transplanted cells in experimental animals were checked by staining the sections with DAPI. Under a fluorescent microscope, PKH26-labeled cells appeared red with a blue nucleus.

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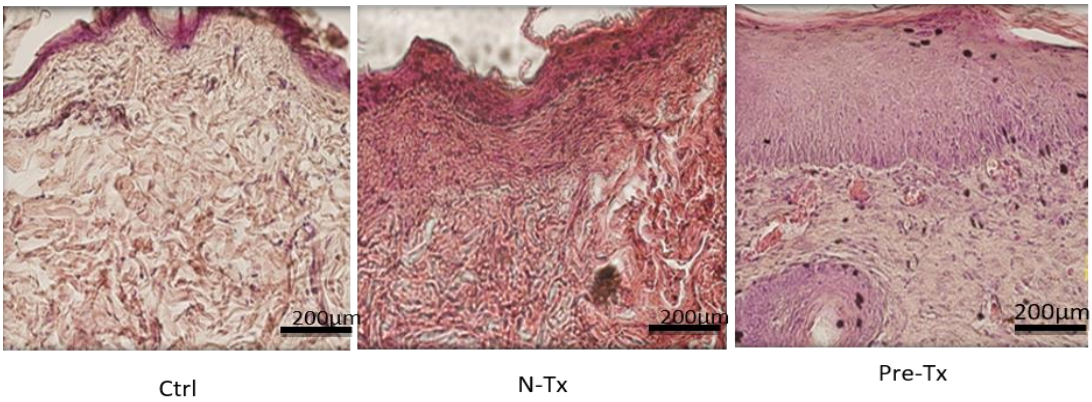

Figure 3. The figure shows more cellularity, thick granulation, and re-epithelialization in VEGF preconditioned ADSCs(Pre-Tx)group compared to the control and ADSCs (N-Tx) transplanted groups.

Gene expression analysis

Gene expression profiling was performed in VEGF-treated ADSCs or ADSCs alone in transplanted groups. Gene expression levels of growth factors (E cadherin, vimentin, igf1, sdf1α) and apoptotic marker (caspase 3) were performed through semi-quantitative real-time polymerase chain reaction (PCR). β actin mRNA expression levels were used to normalise these makers mRNA expression values.

Expression of E cadherin

Gene expression of E cadherin revealed a significant upregulation in the VEGF preconditioned ADSCs transplanted group (Pre-Tx) compared to control and ADSCs transplanted (N-Tx) groups. These results suggest that VEGF preconditioning improved the cell adhesion in the Pre-Tx group compared to other groups representing a boosted cell to cell bond which led to accelerated wound healing in the preconditioned group.

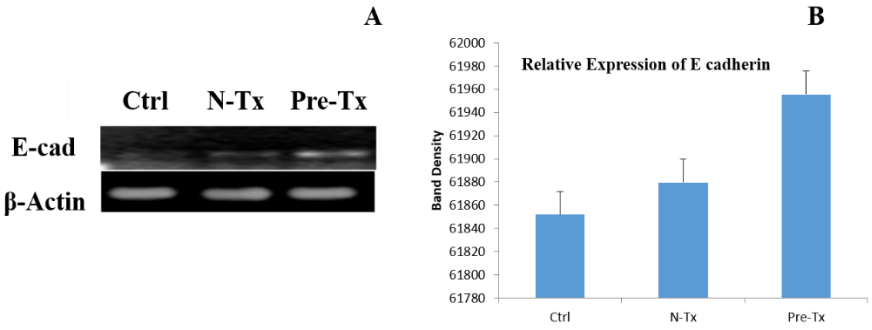

Figure 4.(a). Expression of E-Cad in different groups. VEGF preconditioned ADSCs transplanted group (Pre-Tx) showed enhanced expression compared to other groups.(b) Plot of E cadherin gene expression quantified by Image J software. Data is significant with 'p' value less than 0.05.

Expression of vimentin

A prominent increase in the expression of vimentin was detected in the VEGF preconditioned transplanted group (Pre-Tx) compared to control and ADSCs transplanted groups which assisted in repairing damaged skin.

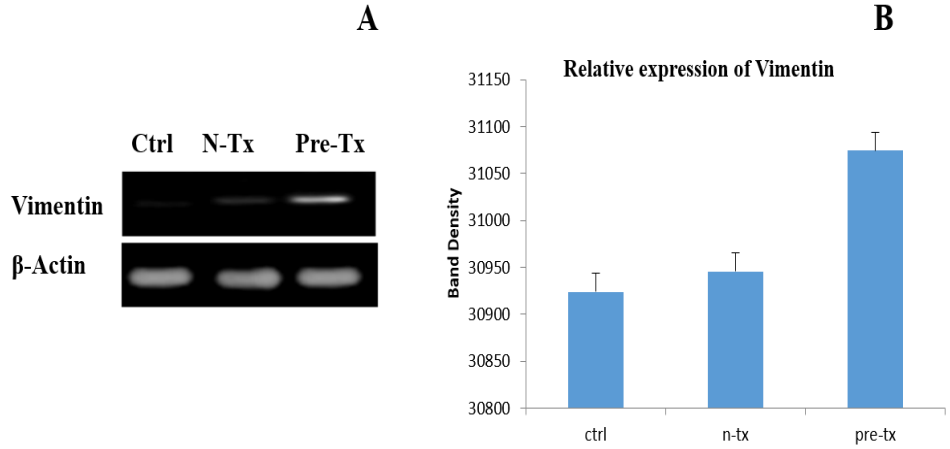

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Figure 5. Expression of Vimentin in different experimental groups. VEGF preconditioned ADSCs transplanted group (Pre-Tx) showed enhanced the expression as compared to control and ADSCs transplanted (N-Tx) groups. (b) Plot of vimentin gene expression quantified by Image J software. Data is significant with 'p' value less than 0.05.

Expression of insulin growth factor

Gene expression of Insulin Growth Factor1 (igf1) was analyzed in all three groups. ADSCs preconditioned with VEGF resulted in an augmented gene expression of igf1 in contrast to the non-preconditioned ADSCs and Control groups.

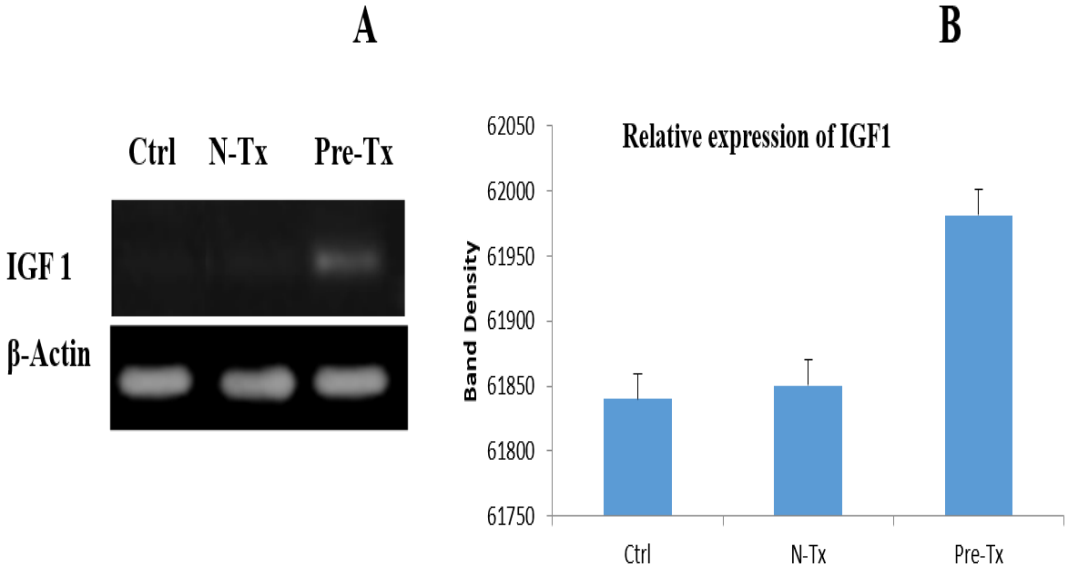

Figure 6. (a). Expression of IGF 1 in different groups. (b) The plot of IGF 1 gene expression quantified by Image J software. Data is significant with 'p-value less than 0.05.

Analysis of expression of stromal-derived factor1α

ADSCs preconditioned with VEGF revealed significant upregulation of stromal-derived factor1α as compared to Control, and ADSCs transplanted groups. It helped in cell proliferation and survival of transplanted ADSCs more in the Pre-Tx group than in the N-Tx group.

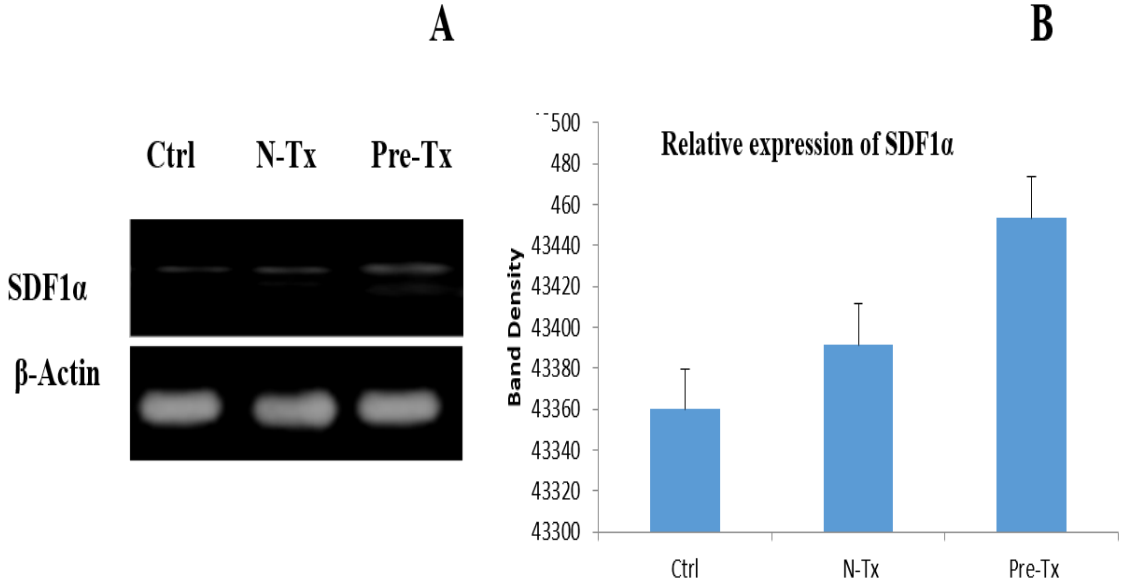

Figure 7. (a). Expression of SDF1α in different groups. (b) Plot of SDF1α gene expression quantified by Image J software. Data is significant with 'p' value less than 0.05.

Expression of caspase3

ADSCs preconditioned with VEGF decreased caspase 3 apoptotic marker expression compared to the control and ADCSs-only group. This decreased expression led to an increased survival and to home of the transplanted Preconditioned ADSCs at the injury site and aided in skin rejuvenation.

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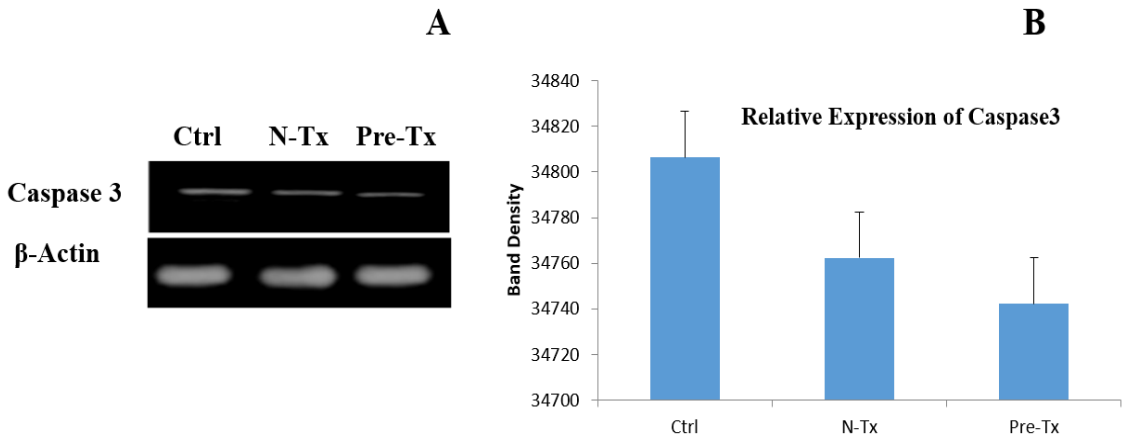

Figure 8. (a). Expression of caspase3 in different groups. (b) The plot of caspase3 gene expression quantified by Image J software. Data is significant with ' p-value less than 0.05.

Discussion

The combination of biological and molecular events takes place to heal the cutaneous wounds. These activities are interlinked with each other and support the regeneration process. Many processes like cell migration and proliferation, extracellular matrix (ECM) deposition, angiogenesis, and remodelling are necessary for the complex process of healing wounds (Kim et al., 2009; Masson-Meyers et al., 2019). However, several chronic disorders, like diabetes, impede this normal evolution (Falanga, 2005). Significant advancement has been achieved in last past 20 years in understanding the physiological process of wound healing, and new regenerative approaches have also been created. Enhancing wound healing, though, is still a problem that faces the disciplines of plastic and reconstructive surgery frequently (Ko et al., 2011; Trevor et al., 2020). The interaction of dermal and epidermal cells and the release of chemical mediators from inflammatory cells, fibroblasts, and keratinocytes are necessary for the healing of cutaneous wounds. The granulation tissue is filled with macrophages and mesenchymal cells, which replace the cutaneous defect and act as substrates and inducers for re-epithelialization. Various cell-based therapies offer to promise therapeutic approaches to enhance wound healing in healthy and pathological circumstances because different cell types engage in the wound-healing process (Swindon et al., 2011; Kanji et al., 2017).

BMSCs have previously been reported to trigger the healing process by mediating dermal regeneration as they can differentiate into the skin's epidermis (Wu et al., 2007; Zheng et al., 2015). Additionally, numerous studies have shown that the rate of wound healing is enhanced following the transplantation of BMSCs, mesenchymal stem cells, or ADSCs (Badiavas et al., 2003; Huo et al., 2018). (Jung et al., 2011; Ebrahimian et al., 2009). However, adipose tissue can be obtained for much less money than bone marrow, with a less intrusive procedure, and in larger quantities. Since isolated adipose tissue has a higher rate of stem cell growth than BMSCs, clinically significant stem cell quantities can be recovered from it (Cowan et al., 2004; Toyserkani et al., 2015). Furthermore, irrespective of cell type and ease of isolation, the most challenging factor in stem cell transplantation success is their survival against the harsh microenvironment. This can be countered through preconditioning stem cells (Haider et al., 2010). Despite other growth factors (such as epidermal growth factor, transforming growth factor, and the FGFs) serving redundant, overlapping functions, VEGF is one of many cytokines released during tissue repair. Though it is commonly believed that VEGF is only significant for promoting angiogenesis, a recent study shows that VEGF also improves endothelial cells and fibroblasts activation and their cross-talk (Nissen et al., 1998; Belvedere et al., 2022). Previous research has shown that up-regulation of endogenous VEGF is linked to the control of oxidative damage or suppression of the receptor for advanced glycation end products, which improves wound healing in diabetic mice. Additionally, neutralising antibodies that block VEGF hamper tissue repair, while VEGF treatment speeds up recovery in non-diabetic ischemic wounds (Howdieshell et al., 2001; Okizaki et al., 2016). Clinical validation of this experimental research has come from identifying decreased VEGF activity in chronic human wounds (Lauer et al., 2000). Together, these findings suggest that targeted VEGF supplementation may be beneficial and that VEGF is essential for repair in circumstances of poor healing (Zingg et al., 2012).

The present study investigated the in vivo effects of vascular endothelial growth factors preconditioned adipose adipose-derived stem cells against burn injury wounds. The data obtained from hematoxylin and eosin staining of sections revealed an increased granulation and re-epithelialization in the preconditioned cells group compared to normal

ADSCs and the control group (Fig 3). Our hematoxylin and eosin staining results align with the previous study of using multiple ASCs injections to accelerate burn wound healing in rat models (Zhao et al., 2019). We also examined the extent of ADSCs homing and found a greater number of cells incorporated in the skin tissue of Pre-Tx group as compared to ADSCs group after 21 days of cell transplantation. (Fig. 2). This directed that VEGF-ADSCs are more capable of homing in the skin tissue than untreated ADSCs. Furthermore, VEGF preconditioned ADSCs showed better survival in the hostile environment of burnt skin. Our results are following our previously reported data on knee joints (Bhatti et al., 2017).

Cadherins are a type of protein that plays important roles in cell adhesion, ensuring that cells within tissues are bound together (Maître et al 2013). Different members of the cadherin family are found in different locations, including epithelial tissue (Bhatt et al., 2013). The increase in its expression level (Fig.4) affirms that VEGF preconditioning enhanced the cell adhesion in the preconditioned group as compared to other groups indicating an enhanced cell-to-cell adhesion, improved cellularity and granulation, which resulted in early wound healing in preconditioned group. A similar high expression of Cadherin was previously reported in burn wound healing via targeting E-cadherin through RUNX2 (Li et al, 2018). vimentin is an important type III intermediate filament (IF) protein expressed in mesenchymal cells (Ise et al., 2019. vimentin expression has also been reported in skin repair (Yao et al., 2020). Therefore, vimentin is often used as a marker of mesenchymal-derived cells (Lee et al., 2014). The enhanced expression of vimentin in the preconditioned cells group (Fig. 5) shows that many mesenchymal cells have successfully homed at the injury site. Previous results also reported enhanced wound healing using exosomal vimentin isolated from adipocyte progenitors (Parvanian et al., 2021). Similarly, SDF1α is a chemokine of the CXC subfamily involved in cell proliferation and survival and possesses chemotactic activity. It also plays an important role in the angiogenesis and results in the induction of capillary vessel formation in the kidney (Zhou et al., 2002). Moreover, IGF1 is a multifactorial growth factor and promotes cell growth and proliferation (Singh et al., 2006). The current data showed an increase in the expression of sdf 1α (Fig.7) and igf (Fig.6) in the preconditioned group. The upregulation of sdf1α and igf managed rapid angiogenesis, cell survival, proliferation, and cellular and functional recovery that resulted in an increased homing of the transplanted cells to the injury site and thus participates in the recovery process. conversely, caspase3 plays an important role in the apoptosis pathway (Jiang et al., 2020). It is involved in many proteolytic processes in apoptosis (Shalini et al., 2015). A low expression level of caspase3 was observed in VEGF pre-conditioned ADSCs transplanted group (Pre-Tx), suggesting a decrease in apoptosis in this group, as shown in Fig 8. The VEGF preconditioning increased the survival of ADSCs against burn injury by lowering the caspase 3 expression. The similar study reports an upregulation of VEGF and downregulation of Caspase 3 has been reported in a mouse model of burn injury (Khan et al, 2020)

Conclusion

This study demonstrated that preconditioning of ADSCs with VEGF plays an imperative role in attenuating apoptosis and enhancing the proliferation under harsh microenvironment at the injury site by up-regulating the expression of anti-apoptotic and angiogenic cytokines and down-regulating cytopathic factors. Hence VEGF pre-conditioning may be an alternate strategy to overcome the cellular loss of transplanted stem cells due to the adverse effects of the injury.

Declaration

The authors declare no conflict of interest regarding the publication of this paper.

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