Original Article

The Effect of SERCA2 Transfected Stem Cells on Induced Wound in Type1 Diabetes of Male Albino Rat Model Compared to Stem Cells

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ABSTRACT

Background and Objectives: Diabetic wounds are difficult to manage. For cell proliferation, migration, differentiation and regeneration, intracellular Ca^{2+} equilibrium is crucial. Sarcoplasmic reticulum Ca^{2+} ATPase2 (SERCA2) has been shown to control Ca2+ level in keratinocytes. Adipose derived mesenchymal stem cells (AdMSCs) are essential for the repair of wounds. The therapeutic effect of SERCA2 gene modified AdMSCs was studied and compared to that of AdMSCs on experimentally induced diabetic wound type 1.

Methods and Results: 42 male rats were classified into: Donation group: three were used for stem cell culture, phenotyping, tagging and SERCA2 preparation. 1st Group I (Control Group): nine rats, 2nd Group (Wounded Group): ten rats were injected with streptozotocin (STZ) intraperitoneal (IP) 50 mg, then rats were exposed to wound induction (1x1cm skin incision). 3rd Group (AdMSCs Therapy): ten rats, injected IP with $1x10^6$ AdMSCs. 4th Group (SERCA2 modified AdMSCs Group): ten rats, injected IP with $1x10^6$ SERCA2 modified AdMSCs. By end of 1st and 3rd weeks, induced diabetic wound surface area was measured. 1st 2nd 3rd and 4th Groups were scarified three weeks after diagnosis of diabetes. Serological, biochemical, phenotypic and quantitative studies were done. In 2nd group, inflammatory, fibrotic and degenerative changes were seen in the wound area and in intact skin. In 3rd and 4th groups these changes were ameliorated, more noticed in 4th group. In the 2nd group, a significant difference was found in wound surface area. Phenotypic and quantitative changes were confirmed by serum glucose, skin Ca^{2+} concentration and western blot analysis.

Conclusions: SERCA2 gene modified AdMSCs therapy exerted a more pronounced ameliorating effect versus AdMSCs' effect.

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Key Words: Diabetes mellitus, immunohistochemistry, SCs, SERCA2, wound.

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INTRODUCTION

Diabetes wounds are chronic in nature because they regularly reoccur and heal slowly, which not only raises the cost of medical care but also significantly lowers the quality of life for diabetic patients. Clinics' current go-to treatments, as debridement, dressings and infection management, haven't produced satisfying outcomes. Therefore, it is essential to find more efficient therapeutic methods that can accelerate the healing of diabetic wounds[1]. One of the main effects of diabetes is impaired wound healing, which is characterised by persistent oxidative stress, delayed re-epithelialization and protracted inflammation[2].

Administration of stem cells of mesenchymal origin is a new treatment for chronic wound healing and repair that has gained popularity in recent years. MSCs reside in normal skin and enhance regenerative reconstruction of injured skin in diabetics[3]. Adipose-derived stromal vascular fraction, which contains MSCs, may improve healing and preserve function[4].

Skin barrier formation in cultured keratinocytes was established by restoration of the calcium level and was knocked down after treatment of cells by high glucose simulating hyperglycemia state[5]. Calcium ion (Ca^{2+}) homeostasis influences skin barrier formation and is importent for division, differentiation and development of keratinocytes junctions[6]. Sarcoplasmic reticulum Ca^{2+}-ATPase2 (SERCA2) was proved to be a key protein involved in restoration of Ca^{2+} in endoplasmic reticulum

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(ER) in keratinocytes. SERCA2 role in proliferation, cell-to-cell adhesion and microbial barrier integrity has been documented[7].

Introduction of a gene into stem cells (Transfection) in tissue cultures was authorized. Lipofectamine maintained its popularity, being simple to use, inexpensive and adaptable to throughput mechanisms[8].

The current work attempted to evaluate the therapeutic effect of SERCA2 gene modified AdMSCs and compare it to that of AdMSCs on experimentally induced wound in type 1 diabetes mellitus (TIDM).

MATERIALS AND METHODS

Experimental Animal Design

The present work was carried on 42 male albino rats, their age was3 months and their body weight ranged 180-200 grams. They were divided into the following groups and housed in accordance with Cairo University's guidelines of Institutional Animal Care and Use Committee (CU-IACUC).

Donation Group

Three rats, 2 of which were used for isolation, culture, phenotyping and tagging. In addition, a part of skin of the 3rd rat was homogenized and processed for SERCA2 preparation.

1st Group (Control Group): It constituted 9 rats that represented the experimental 2nd , 3rd and 4th groups. One IP injection of 0.5 ml citrate buffer (CB) was given to 1st 3 rats corresponding to wounded group. 2nd 3 rats were injected IP with 1ml of phosphate buffered saline (PBS). 4 days after the diagnosis of diabetes, in addition to CB. 3rd 3 rats were injected IP with 1.5 µl of lipofectamine along with citrate buffer and PBS.

2nd Group (Diabetic wounded): It constituted 10 rats. Induction of diabetes was exerted by IP injection of STZ once (Sigma Company, St. Louis, Mo, USA) as 1 g vial powder in a dose of 50 mg/kg[9]. Each dose was dissolved in 0.5 ml citrate buffer after being weighed using a digital scale. Diabetes was identified three days after the STZ injection by checking the blood glucose Three days following STZ injection, diabetes was confirmed by measuring the blood glucose level. The animals were considered diabetic If the blood glucose level was greater than 200 mg/dl, diabetes was diagnosed[10].

The rats were exposed to wound induction on the 4th day. Wound induction was performed by anaesthetizing the rats, Ketalar, (Barcelona, Spain) was injected intramuscular in a dose of 35 mg/kg. Using sterile techniques the hair was shaved on the middle part of back of rat, where a skin incision of 1x1cm was made using a sterile scalpel and 1cm2 of skin was removed[11]. Every day, the injured site was rinsed with regular saline after applying betadine to the wound.

3rd Group (Diabetic wounded and Non-transfected AdMSCs treated): It constituted ten rats. 1x106 cultivated and PKH26 tagged rat AdMSCs suspended in a tube containing 1ml PBS were injected IP the day after diabetes diagnosis[12].

4th Group (Diabetic wounded and SERCA2 gene modified AdMSCs treated): It constituted 10 rats. On the 4th day, 1x106 of cultivated and green fluorescent protein (GFP) tagged SERCA2 gene modified AdMSCs were injected IP.

Sacrifice was performed in control and corresponding experimental groups, 3 weeks after diagnosis of diabetes.

Source and culture of non- transfected rat AdMSCs[13]

Rats were cut open at the abdomen, their adipose tissues were removed, and they were then put to death with carbon dioxide (CO_2) in sterile settings. All of the adipose tissues were collected and incubated in Dulbecco's modified Eagle's medium (DMEM), (GIBCO/BRL) with 10% foetal bovine serum (FBS), after being cleaned with saline solution. Ficoll/Paque (Pharmacia) was used to separate nucleated cells using a density gradient, and the cells were then resuspended in full culture media with 1% penicillin-streptomycin (GIBCO/BRL). As a primary culture or after the formation of large colonies, cells were incubated for 12–14 days at 37° C in 5% humidified CO_2. After two PBS washes, cultures were trypsinized with 0.25% trypsin in 1 millimolar EDTA (GIBCO/BRL) for five minutes at 37° C once substantial colonies (80–90% confluence) had formed. Cells were resuspended in serum-supplemented media following centrifugation (at 2400 rpm for 20 minutes). Incubation in a 50 cm2 Falcon culture flask are considered 3rd-passage cultures. The adhering cell colonies were trypsinized and counted on day 14.

Immunohistochemical markers of nontransfected AdMSCs[14] *and flow cytometry*[15]

Using hemocytometer, 1×10^6 cells/ml were incubated with 10µl of monoclonal antibodies: CD34 and CD44 (Beckman coulter, USA) for 20 minutes. Then 2% fraction crystallizable solution (FCS) in 2 ml PBS were placed. High speed centrifugation for 5 minutes was done and supernatant was discarded. CYTOMICS Flow Cytometer 500 (Beckman coulter, USA) cell analysis was performed using CXP Software version 2.2.

PKH26 tagging of nontransfected AdMSCs[16]

4th passage collected non-transfected AdMSCs cells were tagged with PKH26 fluorescent linker dye to be able to be examined by fluorescent microscopy.

SERCA2 gene extraction[17]

Using a Polytron homogenizer skin specimens were homogenized. SERCA2 primer was processed in homogenates for ribonucleic acid (RNA) extraction. Then complementary DNA (cDNA) was synthesized by reverse transcription.

Lipid mediated gene transfection and GFP tagging of gene modified AdMSCs

Incubation in serum-free DMEM of 1.5 µl Lipofectamine 3000 reagent[18] and 1.5 µg SERCA2 gene tagged with GFP[19] was done. GFP fuses to specific loci on SERCA2. 4 hours later culture media were. To assess transfection efficiency, morphology and viability cells tagged with GFP were examined by fluorescence microscopy. Cells were incubated in 250 µL of mixture of Tryp reagent at 37°C for 10 minutes. then analyzed by flow cytometry.

Wound surface area measurements

Wound boundary was used to estimate the wound surface area. Tissue differentiation depends on different color of unhealthy tissue (Figures Ia,1C). A number of measurement techniques using images are referred to as photogrammetry. Digital photoplanimetry is the measurement of planes using digital images. These markers are positioned close to the wound and are visualized in the photo with the wound. A true scale of the image should be guaranteed, With the Sony digital camera's 12 Mega Pixels, photos of wounds were taken at a distance of 15 cm from wound. The image was afterwards processed using the image J software to determine the exact measurements (Figure Ib). The polygon selection in the tools bar was used to localize the wound and image J measured it. A second image of 1 cm2 square was obtained as a standard for calibrating wound measurements in cm2, and it had a resolution of 102704 pixels. By the ends of first and third weeks, photographs of wounds were taken. A/k2 is the formula for calculating the surface area in units of area S, where A is number of pixels and k is number of pixels per unit of distance (calibration coefficient)[20].

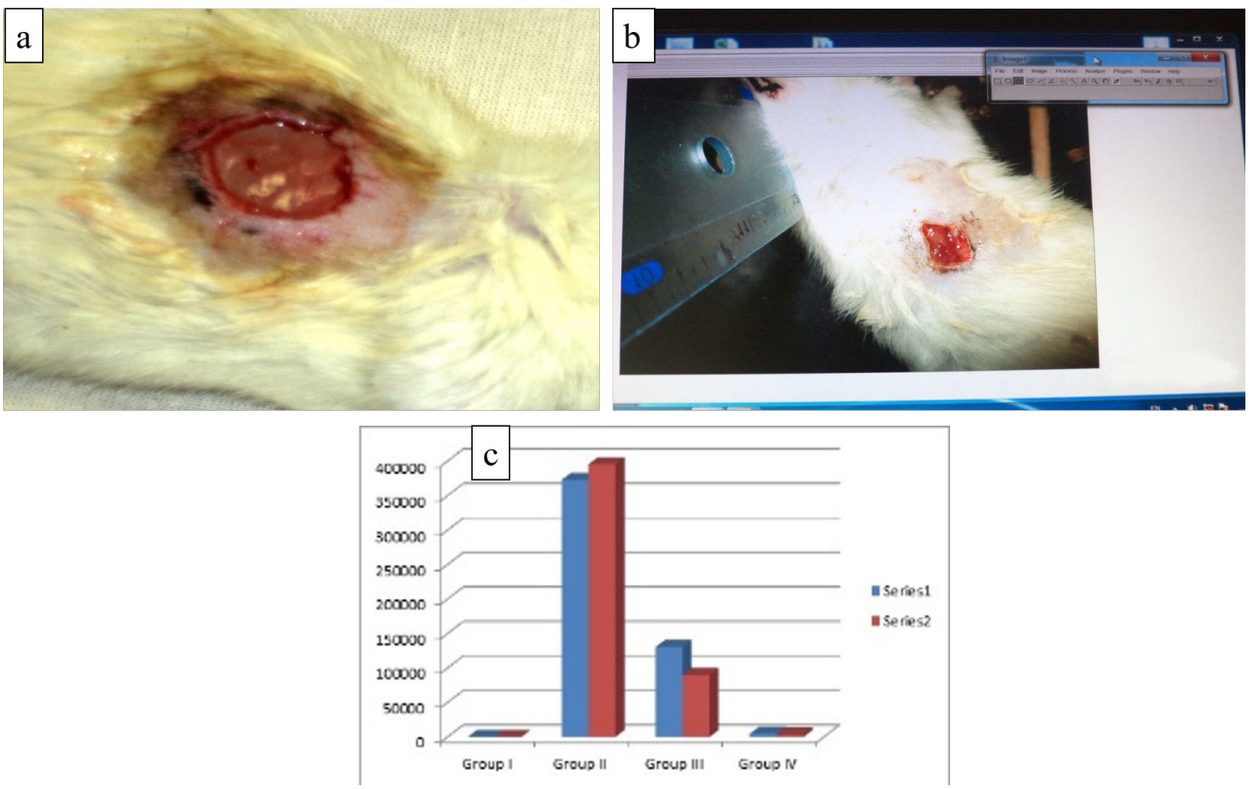

Fig. I showing (a) Wound induction (b)Tracing and measuring the wound size with image J. (c) Histogram of wound surface area measurements, series1 (post 1 week), series 2 (post 3 weeks) of treatment.

Serum glucose level

At the end of the experiment, blood samples were obtained from tail veins from control and experimental groups for serum glucose values determination.

The rats were euthanized by cervical dislocation[21]. Half the wound and 1cm of surrounding skin samples were homogenized to perform:

Skin Ca^{2+} concentration: Using a homogenizer, 1 gm of skin was homogenised in 10 ml of ordinary saline (Ortoalresa, Spain). For 15 minutes, centrifugation at 1000 x g was exerted. In epindorff tubes, the supernatant was gathered and kept in deep freezer. Ca^{2+} concentration was determined using colorimetric method. Quimica Clinica Aplicada SA (Amposta, Spain) provided the analytical kits[22].

Western blot analysis: SERCA2 protein content was estimated and normalized to actin[23].

Phenotypic Study

2nd half of samples including the wound and 1cm of surrounding skin were fixed for 48 hours using 10% formol

saline. 5µm thick sections were used for:

1. Hematoxylin and Eosin (H&E) staining[24]
2. Masson's trichrome staining[25].
3. Immunohistochemical staining[26] for:
    a. Cytokeratin (CK) 3 (MS-1447-R7, Lab Vision Corporation, USA) immunostaining, for demonstration of cytoskeletal protein.
    b. Caspase3 (RB-1197-R7, Lab Vision Corporation, USA) immunostaining, the marker for apoptosis.
    c. Proliferating cell nuclear antigen (PCNA) (Clone PC10, Lab Vision Corporation, USA) immunostaining

Prediluted 1ry mouse monoclonal CK3 antibody was added 0.1 ml for 60 minutes. The reaction is cytoplasmic. Sections of human cornea serve as +ve control. Prediluted 1ry rabbit polyclonal caspase3 antibody was added 0.1 ml for 60 minutes. The reaction is cytoplasmic. Human tonsils serve as positive control. Prediluted 1ry mouse monoclonal PCNA antibody was added 0.1 ml for 60 minutes. The reaction is nuclear. Human skin serve as +ve control. By omitting 1ry antibody addition for negative control preparation.

Quantitative Phenotypic Study

By using computer assisted Leica Qwin 500 LTD (Cambridge, UK) image analyzer, the following was assessed:

1. Distance between epidermal edges of wound using interactive measurements menu in 10 non overlapping fields using x100 magnification.
2. Area% of pale collagen fibers
3. Area% of CK3 immunoexpression (IE).
4. Area% of caspase3 IE using binary mode in 10 non overlapping fields using x200 magnification.
5. Count of PCNA +ve nuclei (N) using interactive measurements menu.

Quantitative polymerase chain reaction (qPCR)

Skin samples that were formalin-fixed and paraffin-embedded (FFPE) were used[27]. The SERCA2 primer (5′-oligonucleotide primer 5′-AAGTGCAATACCTCACTCG-3′ and 3′-oligonucleotide primer 5′- GATCAGCAGCAGACATATC-3′) was used for reverse transcription. After preparing the master mixture, adding it to RNA/primer combination, and leaving it at room temperature for two minutes. After adding 1µl RNAase it was incubated at 37°C for 20 min. Prior to usage, first strand cDNA was kept at -20°C. Gene-specific and reverse primer pairs were combined, and primer concentrations were standardised. The ABI Prism standard deviation score (SDS) 7000 was used to build up the PCR program. The software SDS 7000 was used to analyse the results.

Statistical Analysis[28]

A Bonferroni post-hoc test was employed to determine which pairs of groups were responsible for any significant ANOVA results. Statistics were considered significant for *P-values* less than 0.05. Version 16 of the Statistical Package for the Social Sciences (SPSS) was used to perform the calculations.

RESULTS

Reduced activity and increased urine output were recorded in the diabetic group. These observations improved by AdMSCs and gene modified AdMSCs therapy. Two rats died few days following STZ injection and were compensated.

Immunohistochemical and flow cytometry results of AdMSCs and gene modified AdMSCs

Spindle AdMSCs showed +ve membranous CD44 IE and -ve for CD34. 97.2 % of the cells +ve (Figures 1a,b,c). Fluorescent spindle gene modified cells were GFP tagged and represented 95.1 of cells GFP (Figures 1d,e).

Wound surface area measurements

The treated groups revealed significant decrease in surface area versus the diabetic group and in gene modified AdMSCs versus nontransfected AdMSCs treated group ($P \leq 0.05$) (Table1, Figure Ic).

Serum glucose level

The values denoted significant ($P \leq 0.05$) increase in the 2nd group versus control and treated groups (Figure 2a).

Skin Calcium Ion Concentration level

The values recorded significant decrease in the 2nd group versus other groups and in 3rd group versus 1st and 4th groups (Figure 2b).

Western Blot analysis

The mean SERCA2 protein reported significant decrease in the 2nd group versus other groups and in 3rd group versus 1st and 4th groups (Figures 2c,d).

Histological phenotyping

All control rats' sections demonstrated epidermis, dermis, hair follicles, sebaceous glands and hypodermis (Figure 3a). Closer observation showed pale nuclei and basophilic cytoplasm of the basal and spiny layers, dark basophilic granules of the granular layer and flattened non-nucleated keratinized squamous in the horny layer (Figure 3b). Sections of group II recruited wide separation of the wound edges, detached parts of the epidermis, disrupted dermal fibers and detached sebaceous glands. Follicles lined by one layer of cells and multiple micro-follicles were evident (Figure 3c). Closer observation

revealed thin wound edge at one side exhibiting vacuoles and loss of the granules in the granular cell layer (Figure 3d). Sections of group III showed less widely separated edges of the wound, micro follicles, follicles lined by multiple layers of cells and sebaceous glands (Figure 3e). Closer observation showed focal loss of granules in the granular cell layer and few vacuolated keratinocytes (Figure3f). Sections of group IV demonstrated minimal separation of wound edges, normal appearance of epidermis, multiple micro follicles, multiple sebaceous glands and fully developed hair follicles (Figure 3g). Closer observation showed normal appearance of granular cell layer and reduced thickness of the wound edge (Figure 3h).

First group recruited normal dermal collagen fibers, multiple dermal atypical and multiple pale fibers were observed in 2nd group. While, 3rd group revealed multiple pale dermal fibers, 4th group demonstrated few pale fibers (Figures 4 a,b,c,d).

In cytokeratin3 (CK3) immunostained sections, 1st group showed dense +ve IE in some and less dense IE in other epidermal areas and in the lining of follicles (Figure 5a). While in 2nd group, dense +ve IE was found in localized areas and less dense IE was observed in other areas of thin epidermis and in hair follicles with reduced lining at the wound (Figure 5b). Loss of CK3 IE was demonstrated in multiple areas of the epidermis and dense +ve IE was seen at the horny layer in some other areas of the intact skin (Figure 5c). On the other hand, 3rd group showed dense +ve IE at the horny layer, less dense IE in some areas of the epidermis IE and in the lining of the follicles. Loss of IE was noticed in other areas at the wound (Figure 5d). In addition, less dense +ve IE was detected in multiple areas besides loss of IE in localized epidermal areas and in few follicles (Figure 5e). In 4th group, dense +ve IE was demonstrated in some areas, less dense IE in multiple areas of the epidermis and in hair follicles at the wound. In addition localized loss of IE was noticed (Figure 5f). In epidermis and follicles, focal loss of IE was accidentally found, dense +ve IE in some areas and less dense IE in the rest (Figure 5g).

First group showed +ve caspase 3 IE in few surface keratinocytes (Figure 6a). While, in 2nd group +ve IE was found in many keratinocytes and dermal cells (Figure 6b). On the other hand, group III revealed +ve IE in some keratinocytes and dermal cells (Figure 6c). In group IV, +ve IE was evident in few keratinocytes and dermal cells (Figure 6d). First group showed some PCNA +ve nuclei in epidermis, few follicles and sebaceous glands (Figure 6e). While, 2nd group revealed few +ve nuclei in epidermis (Figure 6f). On the other hand, 3rd group recruited multiple +ve nuclei in epidermis (Figure 6g). In 4th group IV, many +ve nuclei were evident in epidermis and in few follicles (Figure 6h).

Multiple PKH26 fluorescent tagged cells were seen in the 3rd group and in 4th group multiple GFP fluorescent tagged cells were visualized (Figures 7a,b).

Quantitative Phenotypic results (Table 2)

4th group compared to 2nd and 3rd, as well as 3rd group compared to 2nd group, a significant decrease ($P \leq 0.05$) was reported for mean distance between epidermal margins and mean area% of pale stained collagen fibers. Compared to all groups and to 1st and 4th groups, mean area% of CK3 showed a significant decline ($P \leq 0.05$) in 2nd and 3rd groups respectively. On other hand, 2nd group showed a significant increase ($P \leq 0.05$) in mean area% of caspase3 compared to other groups, as well as 3rd group compared to 1st and 4th groups. In comparison to all groups, 4th group showed an increase ($P \leq 0.05$) in mean area% of PCNA +ve nuclei. Additionally, 3rd group showed increase ($P \leq 0.05$) versus 1st and 2nd groups. Additionally, 1st group exhibited increase ($P \leq 0.05$) versus 2nd group.

PCR results (Table 2)

A significant ($P \leq 0.05$) decrease in mean SERCA2 gene values was found in 2nd group versus all other groups and in 3rd group versus 1st and 4th groups.

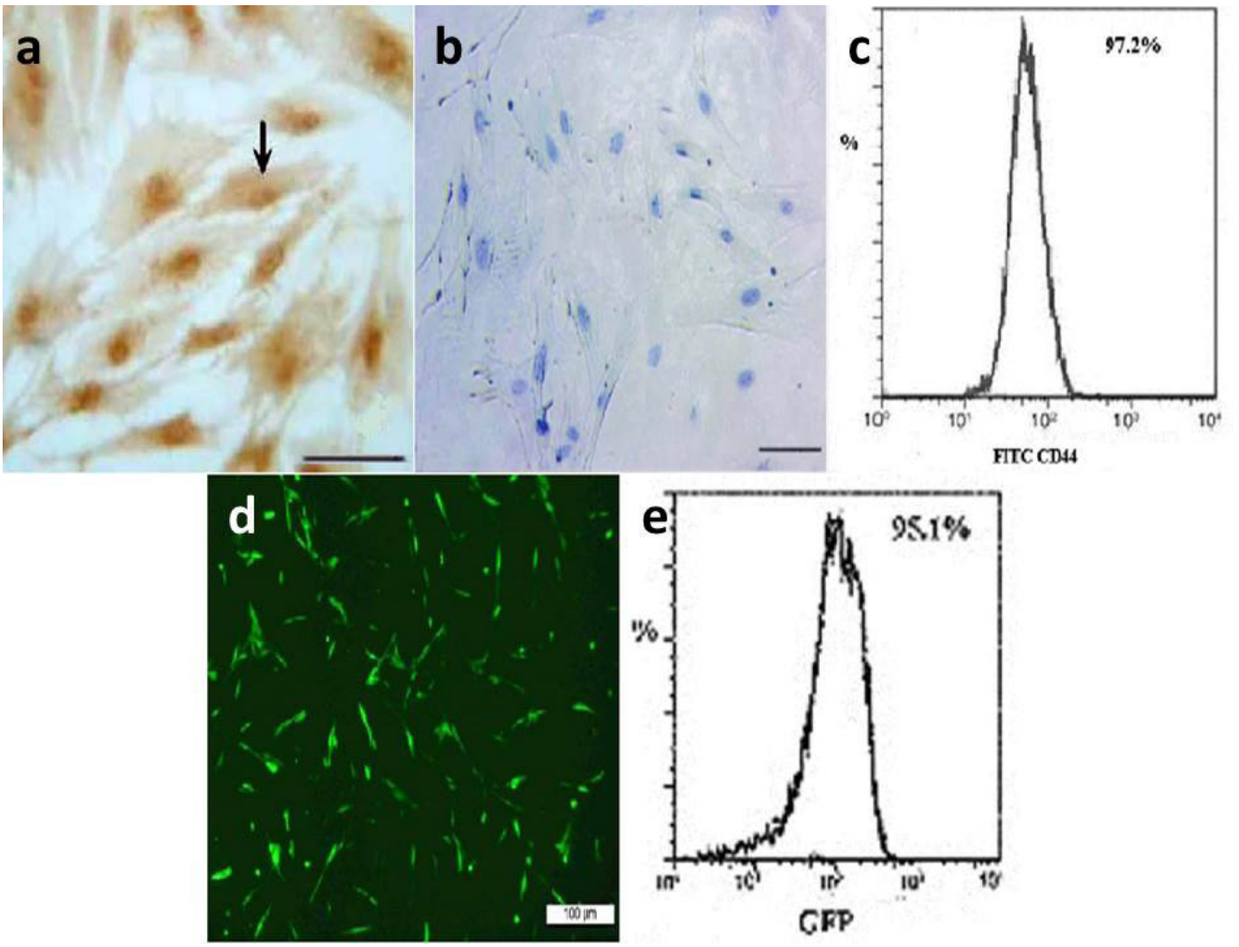

Fig. 1 showing: (a) CD44 +ve spindle cells (arrow). (b) CD34 -ve immunoreactivity (Phase contrast microscopy x 100). (c) Immunophenotyping of non-transfected AdMSCs 97.2 % are +ve for CD44 (Flow Cytometry). (d) Gene modified cells appearing mostly as spindle fluroscent tagged cells (GFP x 100) (e) Immunophenotyping of gene modified AdMSCs showed 95.1 % of the cells GFP tagged (Flow Cytometry).

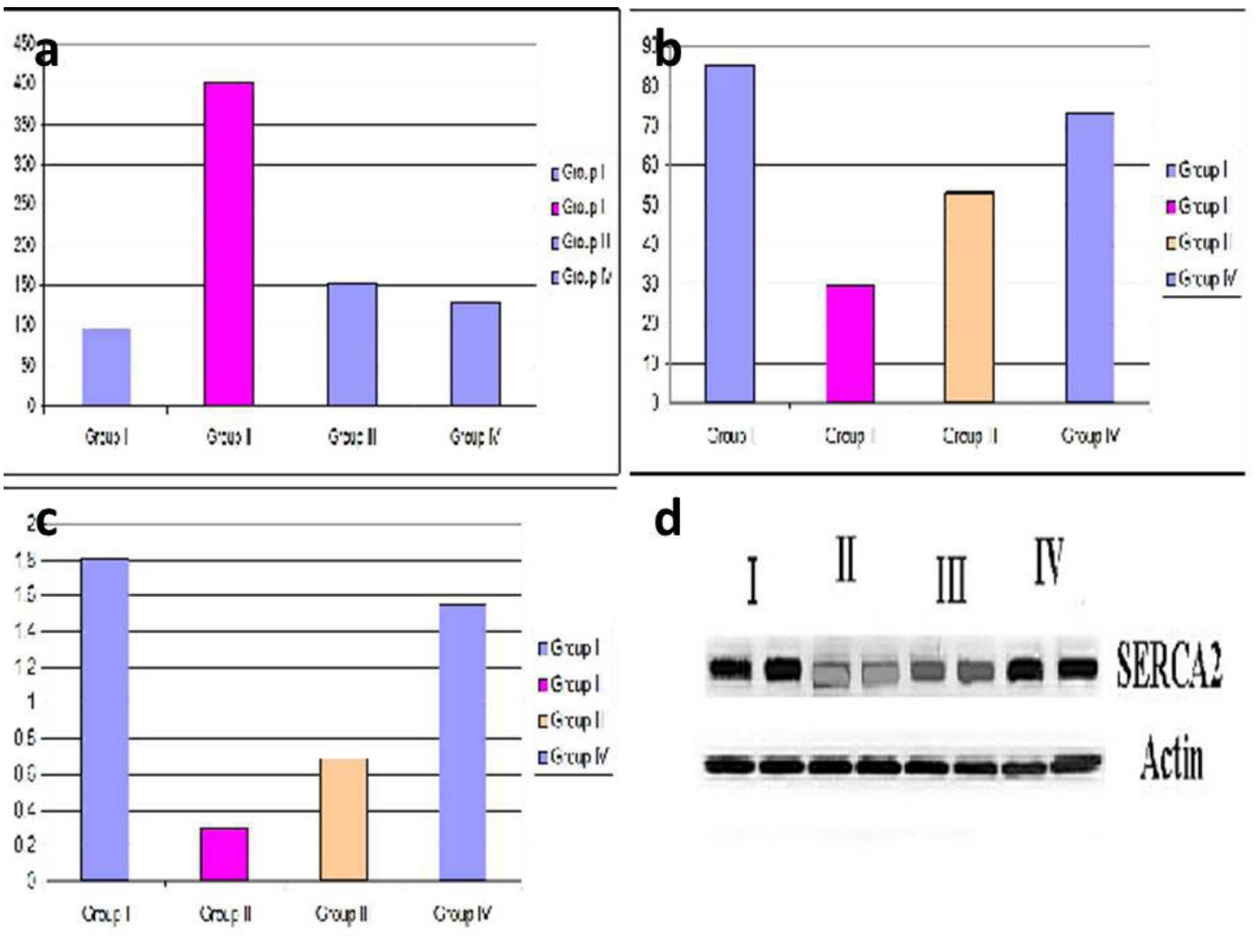

Fig. 2 showing: (a) Mean values of serum glucose level. (b) Mean Ca ion concentration values. (c) Mean Western blot values of SERCA2 protein. (d) Western blot showing SERCA2 protein detected and analyzed (normalized to actin).

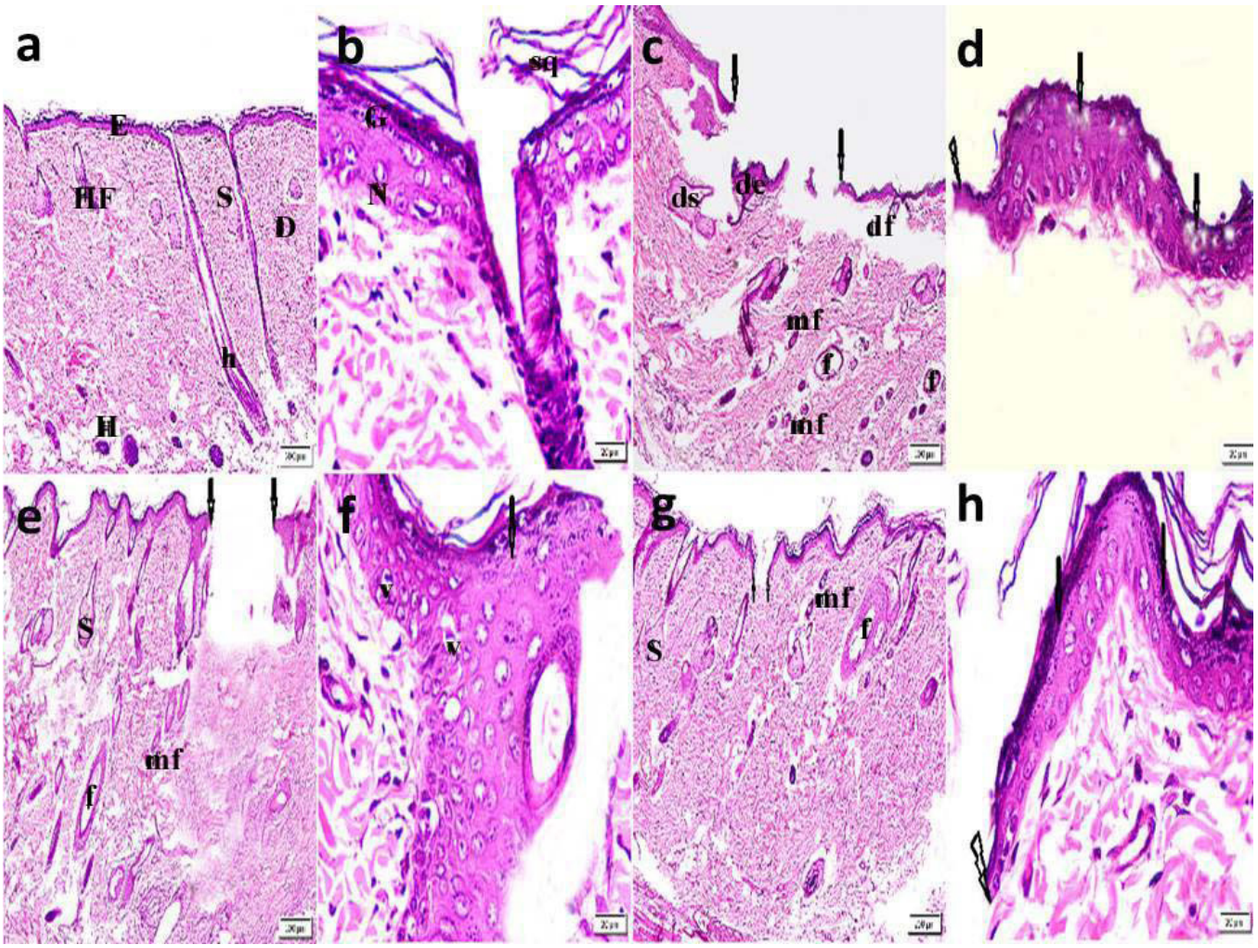

Fig. 3: skin sections showing (H&E x) (a) in 1st group epidermis (E), dermis (D), a hair follicle (HF), a sebaceous gland (S) and hypodermis (H) (x 40). (b) pale nuclei (N) and basophilic cytoplasm of basal and spiny layers, dark basophilic granules (G) of granular and keratinized squamous (sq) in horny layer (x 400). (c) in 2nd group wide separation of wound edges (arrows), detached parts of epidermis (de), disrupted fibers (df) and detached sebaceous gland (ds). Note follicles (f) lined by one layer of cells and microfollicles (mf) (x 40). (d) thinned wound edge (serrated arrow), vacuoles and loss of granules (arrows) in granular layer (x 400). (e) in 3rd group less widely separated edges of wound (arrows), micro follicles (mf), follicles (f) lined by multiple layers of cells and sebaceous glands (S) (x 40). (f) focal loss of granules (arrow) in granular layer and few vacuolated (v) keratinocytes (x 400). (g) in 4th group exhibiting minimal separation of wound edges (arrows), normal appearance of epidermis, multiple micro follicles (mf), multiple sebaceous glands (S) and fully developed hair follicles (f) (x 40). (h) normal appearance of granular layer (arrows) and reduced thickness of wound edge (serrated arrow) (x 400).

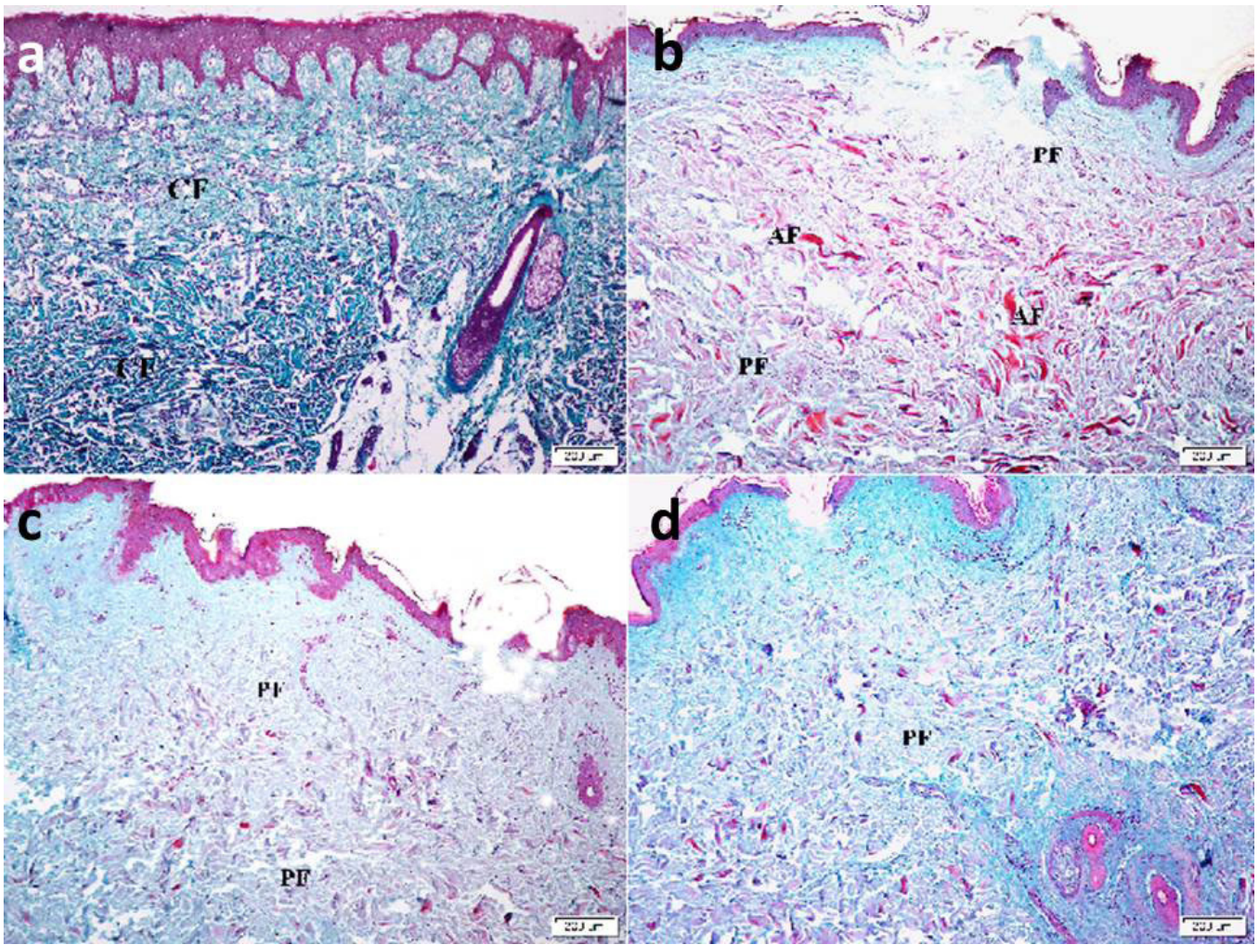

Fig. 4 showing: (a) normal collagen fibers (CF) in dermis of 1st group. (b) multiple atypical fibers (AF) and multiple pale fibers (PF) in dermis of 2nd group. (c) multiple pale fibers (PF) in dermis of 3rd group. (d) few pale fibers (PF) in dermis of 4th group (Masson's Trichrome, x 40).

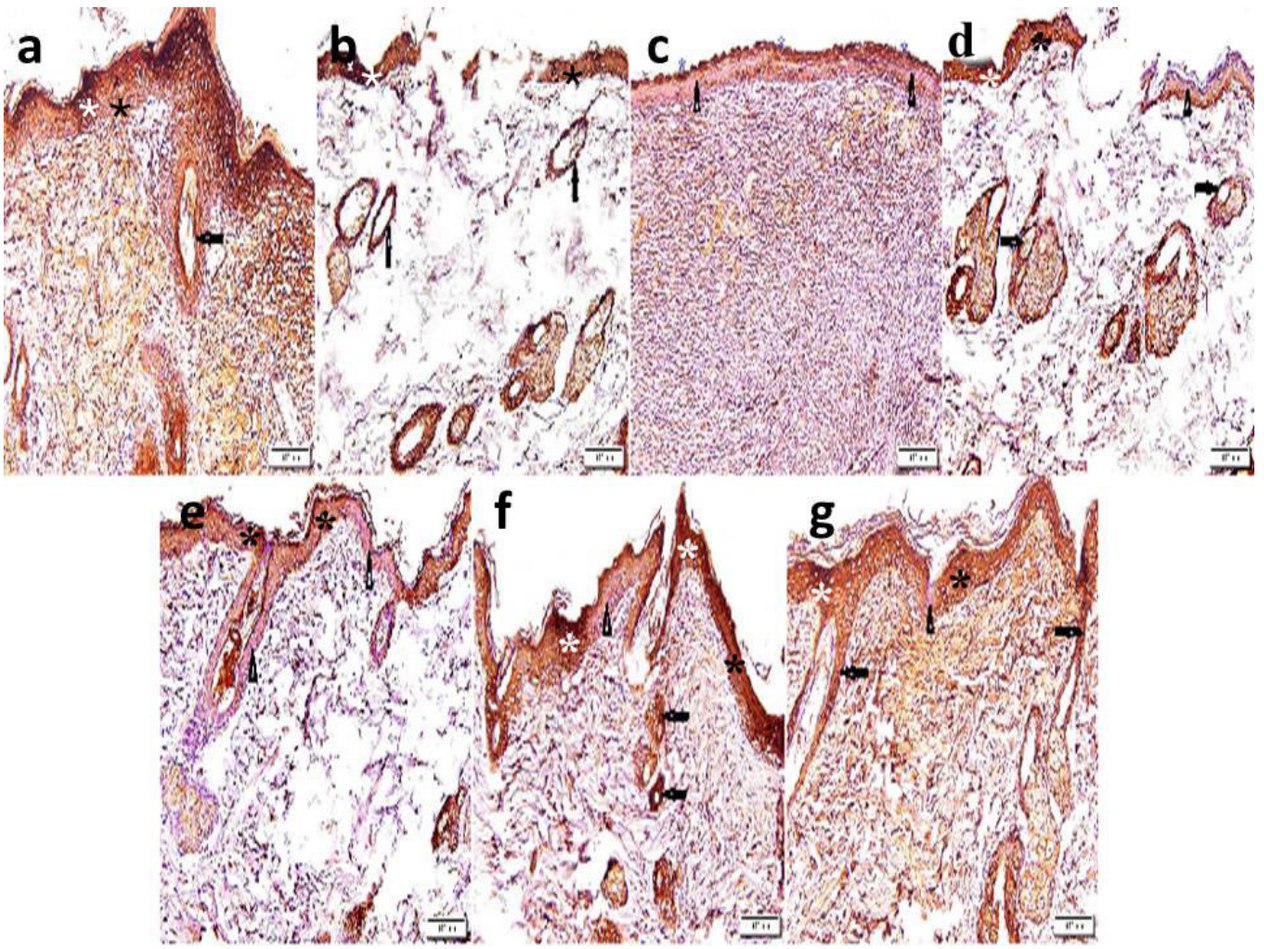

Fig. 5 showing: (a) dense +ve IE (white star) in some areas and less dense IE (black star) in other areas of epidermis and in hair follicles (arrow) of 1st group. (b) dense +ve IE (white star) in localized areas and less dense IE (black star) in other areas of thin epidermis and in hair follicles with reduced lining (arrows) at wound in 2nd group. (c) loss of IE (triangle) in multiple areas of epidermis and dense +ve IE (blue star) at horny layer in 2nd group. (d) dense +ve IE (blue star) at horny layer, less dense IE (black star) in other areas of epidermis and in follicles (arrows) at wound. Note loss of IE (triangle) in some areas in 3rd group. (e) less dense +ve IE (black star) in multiple areas and loss of IE (triangle) in localized areas of epidermis, in addition to a follicle in 3rd group. (f) dense +ve IE (white star) in some areas and less dense IE (black star) in multiple areas of epidermis and in hair follicles (arrows) at wound. Note localized loss of IE (triangle) in 4th group. (g) focal loss of IE (triangle), dense +ve IE (white star) in some areas and less dense IE (black star) in rest of epidermis and in hair follicles (arrows) in 4th group (CK3 immunostaining, x100).

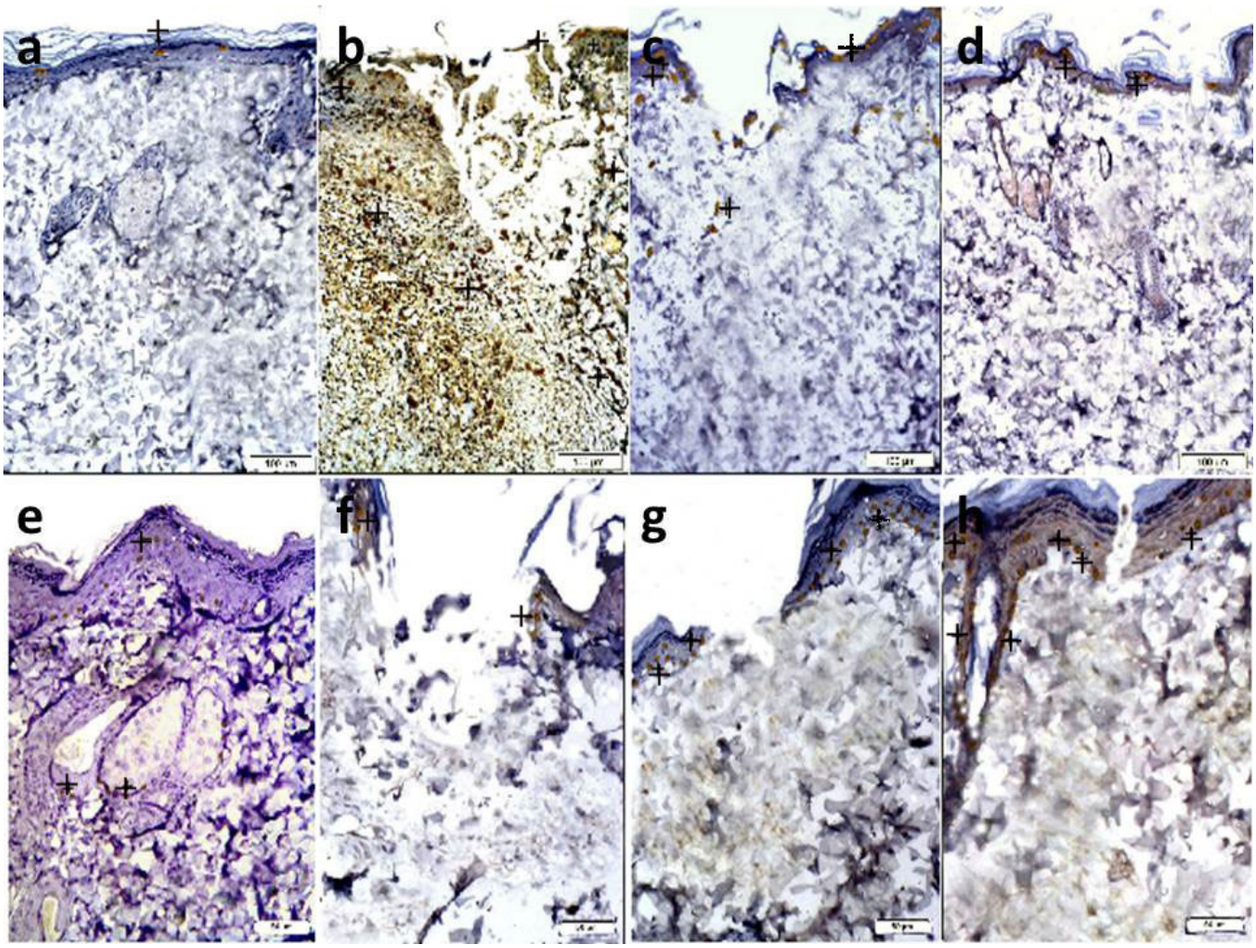

Fig. 6 showing +ve IE (+) in: (a) few surface keratinocytes in 1st group I. (b) multiple keratinocytes and dermal cells in 2nd group. (c) some keratinocytes and dermal cells in 3rd group. (d) few keratinocytes and dermal cells in 4th group (Caspase3 immunostaining, x100). (e) some PCNA +ve nuclei in epidermis and in lining of a follicle and sebaceous glands in 1st group. (f) few PCNA +ve nuclei in epidermis in 2nd group. (g) multiple PCNA +ve nuclei in epidermis in 3rd group. (h) multiple PCNA +ve nuclei in epidermis and in lining of a follicle in 4th group (PCNA immunostaining, x200).

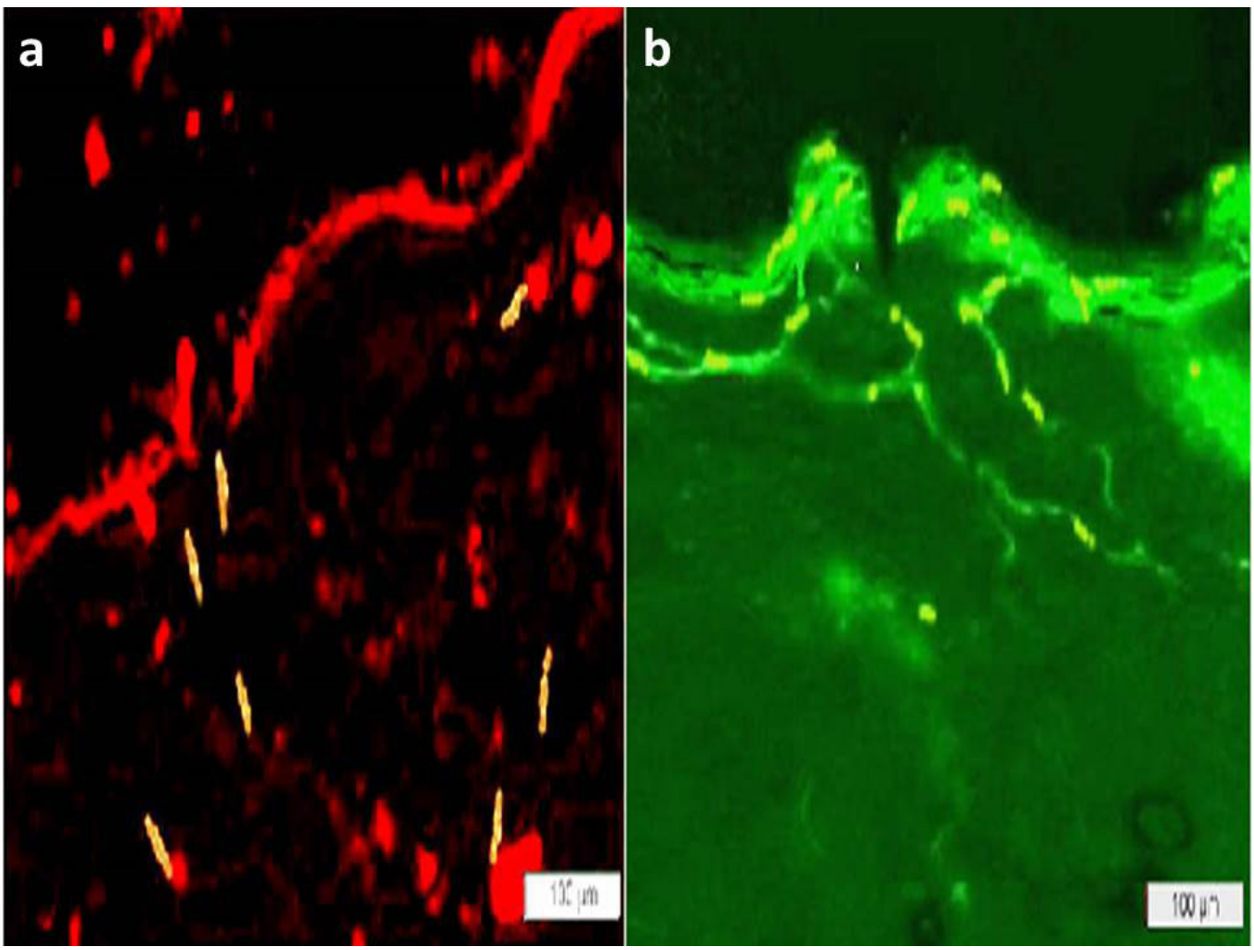

Fig. 7 showing: (a) multiple PKH26 tagged cells in 3rd group (PKH26, x 100). (d) multiple GFP tagged cells in 4th group (GFP, x 100).

Table 1: Wound surface area (Mean ± SD) at the end of 1st and 3rd weeks in different groups

Groups	1st week	3rd week
2nd Group	372842.73± 54485.501	395602.2±59779.60
3rd Group	130703.0±13740.84*	90382.6±14516.15*
4th Group	4759.6±747.10*^	3802.8±727.42*^

*significant compared to 2nd group at end of 1st and 3rd weeks
^significant compared to 3rd group at end of 1st and 3rd weeks

Table 2: Mean ± SD of the distance of epidermal margins, area % of pale stained collagen fibers, area% of CK3 +ve IE, area % of caspase3 +ve IE, area% of PCNA +ve nuclei and PCR values of SERCA2 gene in control and experimental groups

Groups	Distance of epidermal margins	Area% of pale stained collagen fibers	Area% of CK3 +ve IE	Area% of caspase3 +ve IE	Area% of PCNA +ve nuclei	PCR
1st Group	0	0	27.87±2.34	0.46±0.03	12.89±0.18e	1.45±0.25
2nd Group	59.98±5.31	76.71±11.29	6.65±12.11c	19.21±1.29c	4.18±0.43	0.19±0.03c
3rd Group	28.99±3.32a	35.04±6.89a	13.46±3.08d	5.22±1.05d	20.05±3.21f	0.46±0.07d
4th Group	12.98±2.31b	5.94±1.19b	21.32±5.23	0.61±0.04	31.98±2.97c	1.28±0.14

[a]significant compared to 2nd group II.
[b]significant compared to 2nd group and 3rd group.
[c]significant compared to other groups.
[d]significant compared to 1st group and 4th group.
[e]significant compared to 2nd group.
[f]significant compared to 1st group and 2nd group.

DISCUSSION

Diabetic wounds present a major management problem since they heal slowly, are challenging to control and may persist for weeks. Therefore, the objective of clinical treatment would be to attempt to rapidly close the skin lesions with perfect functioning results[29]. The current study compared the therapeutic effect of SERCA2 gene modified AdMSCs to non-transfected AdMSCs on induced wound in male albino rat model of TIDM.

The current work proved that stem cell therapy had an ameliorating impact on diabetic wound damage generated in the rat model. Measurements of wound surface area, phenotypic, qualitative and quantitative evaluations were confirmative.

The wound surface area can be considered a landmark between a complicated wound and a wound having high healing potential. The measurement also is crucial to assess effectiveness of treatment regimens[30]

The mean serum glucose levels in 2nd group were found to denote significant increase versus 1st, 3rd and 4th groups. In accordance, hyperglycemia represents a major factor in elevated protein glycosylation. In addition, production of free radicals 2ry to glucose oxidation that encourage cell membrane lipid peroxidation, consequently attenuating the membrane[31].

Second group demonstrated wide separation of the wound edges, detached parts of the epidermis, disrupted dermal fibers, detached sebaceous glands. Follicles lined by one layer of cells, multiple microfollicles, epidermal vacuoles, loss of the granules in the granular cell layer, multiple atypical and multiple pale fibers were observed in the dermis. Recently, Lin et al,[32] noticed hypoxia, reduced hemoglobin and tissue blood volume besides inflammatory criteria in diabetic wound that lead to delayed healing.

In 2nd group, localized dense +ve CK3 IE was found and less dense IE was observed in other areas of thin epidermis and in hair follicles with reduced lining at the wound. Loss of CK3 IE was demonstrated in multiple areas of the epidermis and dense +ve IE was seen at the horny layer in some other areas of the intact skin. A significant decrease was evidenced versus control and treated groups. Concomitantly, Sharma et al[33] documented delayed healing, epithelialization and keratinization in diabetic wounds as a consequence of excessive protease activity.

In 2nd group caspase3 IE was found in many keratinocytes and dermal cells, that was confirmed morphometrically in the diabetic rats versus all other groups. As confirmed by Liu et al[34], who stated that oxidant-nonoxidant imbalance induces apoptosis-related genes expression, stimulating cell death via caspase-3 signaling pathways. In addition, few PCNA +ve nuclei were detected in the epidermis. In accordance, it was proved that the accumulated reactive oxygen species could lead to delayed healing 2ry to cytoplasmic, nucleic acids damage and lipid peroxidation[35].

The mean Ca ion concentration, mean SERCA2 protein and mean SERCA2 gene levels recorded a significant decrease in 2nd group versus other groups and in 3rd group versus 1st and 4th groups, which can be related to lack of SERCA2 transfection in 3rd group. Concomitantly, Wu et al[36] reported a dysfunctional epithelial barrier in diabetic rats that was correlated to Ca^{2+} disturbance and could be referred to mis-handled sarcoplasmic reticulum Ca^{2+}- ATPase2 (SERCA2). Ferroni et al[37] added that the expression of genes related to recycling and stem cell potency were downregulated in diabetes.

Third group (AdMSCs treated diabetic group) revealed less widely separated edges of the wound confirmed morphometrically by distance between edges versus diabetic group. Micro follicles, follicles lined by multiple layers of cells and sebaceous glands were seen. Focal loss of granules, few vacuolated keratinocytes and multiple pale dermal fibers, confirmed morphometrically by area% versus diabetic group, were observed. The previously mentioned changes denoted amelioration of inflammatory and degenerative morphological features, consequently, favourable healing and regeneration.

Accordingly, Hilton et al[38] proved that MSCs can biomechanically affect wound closure as MSCs induced higher and more complete collagen contraction versus dermal fibroblasts. They also participate in the regulation of inflammation, and inter-cellular communications in the local tissues concerning wound repair.

In 3rd group, dense +ve CK3 IE at the horny layer, less dense IE in some areas of the epidermis and in the lining of the follicles were noticed at the wound. In addition, less dense +ve IE was detected in multiple areas besides loss of IE in localized areas of the epidermis and in the lining of few follicles. The previous results were confirmed morphometrically by the mean area% versus diabetic group. In agreement, Kanji et al,[39] recorded improved re-epithelialization, keratinization and neovascularization of cutaneous wounds in diabetic mice by hemopoeitic SCs therapy.

Less obvious caspase3 IE was found on the contrary in 3rd group which was expressed as significant decrease morphometrically versus 2nd group. In addition, multiple PCNA +ve epidermal nuclei were proved by a significant increase in mean count 1st and 2nd versus groups. Concomitantly, Zafari et al[40] stated that diabetic wound healing responded to stem cells' therapy via growth factor stimulation and down-regulation of apoptosis genes, in addition to their promising proliferative, migratory, clonogenic and immunomodulatory properties.

In 3rd and 4th groups, many PKH26 tagged and many GFP tagged cells were visualized at and around wound site. These findings confirmed migration of cultured non-transfected and gene modified AdMSCs cells to wound area, concluding enhancement of repair.

Fourth group revealed minimal separation of wound edges, normal appearance of epidermis, multiple micro follicles, multiple sebaceous glands and fully developed hair follicles. Normal appearance of granular layer and reduced thickness of the wound edge were detected. In addition, few pale fibers were evident among the normal collagen fibers in the dermis. A significant decrease of distance between wound edges and area% of pale was confirmative in 4th group compared to the other groups. This can be referred to higher efficiency of regenerative plasticity of SERCA2 modified AdMSCs and transdifferentiation into epidermal cells and appendages. In support, da Silva Vasconcelos et al[41] found that higher SERCA expression and cytosolic Ca^{2+} level are accompanied by improving structural and functional tissue capacity in diabetic animals.

In the 4th group, dense +ve CK3 IE was demonstrated in some areas, less dense IE in multiple epidermal areas and follicles at the wound. In addition localized loss of IE was noticed. Focal loss of IE was accidentally found in the rest of epidermis and in follicles, denoting reinforcement of cytoskeletal element. This finding was proved morphometrically versus diabetic and AdMSCs treated diabetic groups. Going with, massive cytoskeletal rearrangement was concluded by Kovuru et al[42] by active SERCA pumps that accumulates Ca^{2+} against its electrochemical gradient in the ER and the capacity of this Ca^{2+} store is increased by the presence of Ca^{2+} binding proteins in the lumen of the reticulum.

Concerning caspase3 IE in 4th group, few keratinocytes and dermal cells showed +ve IE and a significant decrease was reported versus 2nd and 3rd groups. In addition, multiple PCNA+ve epidermal nuclei were evident and in lining of follicles, denoting a statistically significant versus all groups. These results confirmed very minimal residual degenerative changes. Concomitantly, Britzolaki et al[43] confirmed that for cells and tissues to properly regenerate, repair, and function, intracellular Ca^{2+} homeostasis is a vital regulator. Neuropathological disorders have been linked to SERCA-mediated calcium dyshomeostasis, pointing to SERCA gene as potential innovative treatment for these and other diseases.

CONCLUSION

It can be concluded that SERCA2 gene modified AdMSCs therapy induced sterile, fast and complete wound healing more noticeable than that induced by AdMSCs therapy. The measurement of wound surface area, phenotypic and quantitative studies proved this therapeutic effect.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تأثير الخلايا الجذعية المنقولة بـجين السيركا ٢ مقارنة بالخلايا الجذعية على الجرح المستحث في مرض السكري من النوع الأول لنموذج الجرذ الأبيض الذكر

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الخلفية والأهداف: من الصعب إدارة جروح مرضى السكري. بالنسبة لتكاثر الخلايا والهجرة والتمايز والتجديد، يعد التوازن داخل الخلايا Ca٢+ أمرًا بالغ الأهمية. تبين أن الشبكة الساركوبلازمية Ca٢+ ATPase٢ (SERCA٢) تتحكم في مستوى Ca٢+ في الخلايا الكيراتينية. تعتبر الخلايا الجذعية الوسيطة المشتقة من الدهون (AdMSCs) ضرورية لإصلاح الجروح. تمت دراسة التأثير العلاجي لـ AdMSCs المعدلة جينيًا SERCA٢ ومقارنتها مع تأثير AdMSCs تجريبيًا جرح السكري من النوع ١.

الطرق والنتائج: تم تصنيف ٤٢ فأرًا ذكرًا إلى: مجموعة التبرع: تم استخدام ثلاثة منها لزراعة الخلايا الجذعية والتنميط الظاهري ووضع العلامات وتحضير SERCA٢. المجموعة الأولى I (المجموعة الضابطة): تسعة فئران، المجموعة الثانية (المجموعة الجريحة): تم حقن عشرة فئران بالستربتوزوتوسين (STZ) داخل الصفاق (IP) ٥٠ ملغ، ثم تم تعريض الفئران لتحريض الجرح (شق الجلد ١ × ١ سم). المجموعة الثالثة (علاج AdMSCs): عشرة فئران، تم حقن IP بـ ١x١٠٦ AdMSCs. المجموعة الرابعة (مجموعة AdMSCs المعدلة بواسطة SERCA٢): عشرة فئران، تم حقن IP باستخدام AdMSCs المعدل ١x١٠٦ بواسطة SERCA٢. وبحلول نهاية الأسبوعين الأول والثالث، تم قياس مساحة سطح الجرح الناجم عن مرض السكري. تم خدش المجموعتين الأولى والثانية والثالثة والرابعة بعد ثلاثة أسابيع من تشخيص مرض السكري. تم إجراء الدراساتالهستولوجيه والكيميائية الحيوية والمظهرية والكمية. في المجموعة الثانية، شوهدت تغيرات التهابية وتليفية وتنكسية في منطقة الجرح وفي الجلد السليم. في المجموعتين الثالثة والرابعة تم تحسين هذه التغييرات، ولاحظت أكثر في المجموعة الرابعة. وفي المجموعة الثانية، وجد فرق كبير في مساحة سطح الجرح. تم تأكيد التغيرات المظهرية والكمية عن طريق نسبة الجلوكوز في الدم وتركيز الجلد Ca٢+ وتحليل الكيميابيه.