

Pre-Differentiated Skin-Like Cells can Alleviate *In Vitro* Burn Injury

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Abstract

Considering importance of stem cell based therapies for wound healing, we have previously established protocols for successful differentiation of stem cells (isolated from placenta) into skin-like cells (keratinocytes and fibroblasts). In the current study we aim to evaluate the effect of pre-differentiated skin-like-cells on wound healing potential using an *in vitro* burn injury model. The amniotic epithelial cells (AECs) and umbilical cord mesenchymal stem cells (UC-MSCs) obtained from amniotic membrane and umbilical cord tissue; respectively, were differentiated into skin-like cells (keratinocyte and fibroblasts respectively). In order to make an *in vitro* burn injury model, keratinocytes and fibroblasts were isolated from rats and insulted with high temperature (up to 50°C). The optimal temperature for burn injury models was determined using viability assay, cytotoxicity level, proliferation, and expression of pro-apoptotic genes. In order to assess effectiveness of pre-differentiated skin like cells, the keratinocyte- and fibroblast-like cells were co-cultured with injured keratinocytes and injured fibroblasts, respectively. Results indicated that optimal temperature for the induction of heat injury for both keratinocytes and fibroblasts was 50°C. At this temperature both types of cells (keratinocytes and fibroblasts) showed modified morphology, drastic cellular injury, least viability, higher expression of pro-apoptotic markers and lower expression of proliferation genes. Co-culturing of pre-differentiated cells leads to an increase in viability and proliferation concurrent with decreased apoptosis. We have established successfully an *in vitro* model of burn injury that might be helpful to evaluate the wound healing potential of cells. Further, pre-differentiated skin-like cells are a potential source for the treatment of severe burn injuries.

Keywords: Burn injury; Injury models; Keratinocytes; Fibroblasts; Pre-differentiated cells

Abbreviations: AECs: Amniotic Epithelial Cells; UC-MSCs: Umbilical Cord Mesenchymal Stem Cells; DMEM: Dulbecco's Modified Eagle Medium; KGF: Keratinocyte Growth Factor; FGF: Fibroblast Growth Factor; PCR: Polymerase Chain Reaction; CK: Cytokeratin; BAX: BCL2-Associated X Protein; FADD: FAS Associated Death Domain; PCNA: Proliferating Cells Nuclear Antigen; VEGF: Vascular Endothelial Growth Factor

Introduction

Skin being the largest and most exposed part of the body has highest chances of damage due to dehydration, trauma, radiation exposure, severe temperature changes and other physical injuries. Burn injuries are major cause of morbidity and mortality all over the world including Pakistan. Burn injuries are the most common cause of skin damage. Healing of severe burn injuries is difficult and time taking process that exposes the patient to even high risk of infections. Such patients are left with few viable treatment options such as skin grafting or stem cell based therapy. Due to limitations in using a skin graft, stem cell based therapies are gaining robust attention of researchers and clinicians. Considering the importance of such cell based therapies, we have previously optimized protocols for making the skin-like cells using stem cells from human placenta [1].

Although there are significant advances in terms of novel techniques for burn injuries (such as use of stem cell based therapies) [2] and knowledge of burn care, the progress of field is slow as it is challenging to analyze the changes at molecular and cellular level. Therefore, in the current study we aim to develop *in vitro* model of skin injury (using keratinocytes and fibroblasts) in order to efficiently evaluate the effect of pre-differentiated skin-like cells on wound healing. The *in vitro* injury models are cost effective, and can provide reproducible and prompt results. In addition, there are no ethical concerns in using such models as may be the case for use of animals and humans.

In the current study, *in vitro* thermal injury models were established for both keratinocytes and fibroblasts separately and the effect of pre-differentiated skin-like cells was evaluated using these models. The results of the current study indicated that keratinocytes and fibroblasts could be used to make *in vitro* injury model. Further the established *in vitro* models were successfully used to evaluate the wound healing effect of pre-differentiated cells. In addition, pre-differentiated keratinocyte- and fibroblast-like cells showed cytoprotective effect as indicated by increased viability, low cytotoxicity and up-regulation of survival genes concurrent with low gene expression of pro-apoptotic genes.

Methods

Isolation of cells from placenta

Prior to collection of placentas, informed, written consent was obtained from all the donors. The protocols used in the study were approved by IRB (institution review board) at the "National Center of Excellence in Molecular Biology (CEMB)", University of The Punjab Lahore, Pakistan. Human placentas (n=15) were obtained after uncomplicated, full-term, elective cesarean deliveries. Amniotic epithelial cells (AECs) and mesenchymal stem cells (UC-MSCs) were isolated from amniotic membrane and umbilical cord tissue by explant culture as described by our group previously [1].

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Differentiation of AECs and UC-MSCs into skin-like cells

AECs and UC-MSCs were induced to differentiate into keratinocyte- and fibroblasts-like cells respectively, as described [1]. Briefly, at passage 2, AECs and UC-MSCs were cultured in a 6-well plate in respective expansion media. AECs were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma Aldrich, USA) while UC-MSCs were propagated in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma Aldrich, USA). When cells attained their regular morphology, expansion medium was replaced with keratinocytes [(DMEM+HAM F12 (3:1), supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.5 mg/ml hydrocortisone, 1% insulin transferrin (SigmaAldrich, USA) and 15 ng/ml keratinocytes growth factor (Invitrogen, USA)] and fibroblasts [(DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 5 ug/ml insulin and 1 ng/ml basic fibroblast growth factor (Sigma Aldrich, USA)] differentiation medium [1]. Differentiation medium was replaced after every 3-4 days for 15 days.

Isolation and culturing of keratinocytes and fibroblasts

Keratinocytes and fibroblasts were isolated from the skin pieces obtained from Sprague Dawley rats (weighing 250-350 g). These cells (keratinocytes and fibroblasts) were subsequently used for preparing *in vitro* burn injury models. For the isolation of keratinocytes and fibroblasts, rat was euthanized with an access dose of ketasol and xylazine (1:10, 1 ul/g body weight), hairs were shaved and a piece of skin (1-2 inches) was removed using scissors. Skin piece was washed immediately with PBS, and muscular tissues and fat were removed.

Culturing of keratinocytes: Keratinocytes were isolated using enzymatic method as described previously [3]. Briefly, rat skin was cut into small pieces of (1-2 cm²) and digested using Dispase II solution (Roche diagnostics, Germany) at 37°C. After 15 minutes, epidermis was removed by scrapping and dispase II solution was removed. Pieces of epidermis were taken in a fresh tube and incubated with tryipsn-EDTA (0.05%) at 37°C for another 15 min with periodic agitation. Cell suspension was passed through a 100 um cell strainer (Sterilin, UK) and centrifuged at 1,200 rpm for 10 min. Supernatant was discarded and pellet was re-suspended in keratinocyte expansion medium [i.e., DMEM+HAM F12 (3:1), supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.5 mg/ml hydrocortisone, 1% insulin transferrin (Sigma Aldrich, USA) and 15 ng/ml KGF (Invitrogen, USA)]. Cells were cultured on irradiated 3T3 cell culture (irradiated at 6,000Rad using Cobolt 60) at a density of 1×10^5 . Plated cells were incubated at 37°C, 5% CO₂ in humidified incubator until used.

Culturing of fibroblasts: To isolate dermal fibroblasts, de-epithelized rat skin (as described above) was digested using collagen type II (2 mg/ml) overnight at 37°C with continuous agitation. Cell suspension was taken in a new 15 ml tube and centrifuged for 10 min at 1,200 rpm. Supernatant was discarded and cell pellet was suspended in fibroblast expansion medium [DMEM supplemented with 10% FCS (Sigma Aldrich, USA)]. 1×10^5 cells were cultured in 25 cm² culture flask at 37°C and 5% CO₂ in humid environment.

In Vitro Burn injury model of keratinocytes and fibroblasts

Keratinocytes and fibroblasts at a density of 1×10^4 cells (at passage 2) were grown in separate 35 mm petri plates. After about 48 h when cells are 80-90% confluent, they were subjected to various temperatures (37°C to 50°C) in a digital water bath to optimize temperature for induction of thermal injury. Briefly, when the desired temperature was

attained, paraffin wrapped cell culture plates were placed in water bath for 10 min. Culture plates were taken and assays were performed to assess cell viability, LDH release and apoptosis.

Assessment of burn injury to keratinocytes and fibroblasts

Cell viability assay: Viability of heat treated cells was determined using a trypan blue exclusion method [4]. Briefly, the cells (heat induced keratinocytes and fibroblasts) were trypsinized using trypsin-EDTA. After centrifugation, the cell suspension was mixed thoroughly and an equal volume of trypan blue (Sigma Aldrich, USA) was added. The positively stained cells were counted using a haemocytometer under a microscope (Olympus, USA) and percentage of viable cells was determined by using following formula [5]:

Viable cells (%)=(number of viable cells counted/number of total cells counted) × 100

Lactate dehydrogenase (LDH) assay: Cytotoxicity levels in heat induced cultures of keratinocytes and fibroblasts were measured with a commercially available lactate dehydrogenase kit (sigma Aldrich, USA) according to the instructions of manufacturers. Briefly, cell free medium was collected from each high temperature treated group and centrifuged at 2,000 g for 10 min and filtered using 0.22 µm filters (Millipore, USA). 100 µl of LDH assay mixture (dye, substrate and co-factor) was added to 50 µl of collected sample medium and incubated for 30 min at room temperature. Reaction was stopped by adding 10 µl of 1 N HCL to each well. Absorbance was measured at 490 nm by using a spectrophotometer (Spectramax PLUS 384, molecular devices, USA). For each sample, the assay was performed in triplicate.

Reverse transcriptase polymerase chain reaction (RT-PCR): Passage 2 cells were grown in six well plates for RNA extraction. Briefly, cells were washed with PBS and RNA was extracted using Trizol reagent (Invitrogen, USA). RNA was quantified with ND-1000 spectrophotometer (NanoDrop Technologies, USA) and 1.5 ug was used for cDNA synthesis using a commercially available kit (Invitrogen, USA) as per manufacturer's instructions. PCR was performed in a thermocycler (AB Applied biosystems, USA) using a mixture of dNTPs, Spermin, primers (forward and reverse), cDNA, Taq polymerase and nuclease free water. The reaction cycle of PCR consisted of initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C-60°C (depending on primers) for 45 s and extension at 72°C for 45 s, followed by a final extension step at 72°C for 10 min. PCR products were visualized on 2% agarose gel. Bands were quantified using Image J software (<https://imagej.nih.gov/ij/>) according to instructions given in user guide. Primer sequences are listed in Table 1.

Co-culturing of pre-differentiated skin-like cells with injured keratinocytes and fibroblasts

To assess the protective effect of pre-differentiated skin-like cells, a trans-well culture system was used. Briefly, the heat-injured keratinocytes and fibroblasts were cultured separately in a six well plate (1×10^4 /well) while the AECs derived keratinocyte-like and UC-MSCs derived dermal fibroblast-like cells were cultured at 1:1 (5,000 cells of each type) on trans-well inserts in such a way that the insert bottom is in contact with the medium in six well plate. This system of cultured cells was incubated at 37°C, 5% CO₂ in humidified incubator for 48 h. Same transwell culture system was used for non-differentiated AECs and UC-MSCs. The experiments were divided into following groups:(a) normal keratinocytes/fibroblasts only (group 1), (b) injured keratinocytes/fibroblasts only (group 2), (c) injured keratinocytes/

No.	Genes	5'-3' sequence	Size (bp)
1	BAX	GAGAGGTCTTTTCCGAGTGG (F)	95
		CAGTTTGCTGGCAAAGTAGAAA (R)	
2	FADD	CTGCGGGAGTAGTTGGAAGT (F)	241
		GGAAATGGGACAAAACATCCT (R)	
3	PCNA	CATCTCAAGAAGGTGTTGGAG (F)	112
		CAAAGAGACGTGGGACGAGT (R)	
4	VEGF	TGGTGAAGTTCATGGATGTCTATC (F)	114
		CACAGGATGGCTTGAAGATGTA (R)	
5	Beta actin	CGCATGGGTCAGAAGGATTC (F)	137
		TAGAAGGTGTGGTCCAGATT (R)	

Table 1: Primer sequences.

fibroblasts co-cultured with non-differentiated AECs and UC-MSCs (group 3) and (d) injured keratinocytes/fibroblasts co-cultured with pre-differentiated Keratinocyte-like and fibroblast-like cells (group 4).

Assessment of protective effect of pre-differentiated skin-like cells

To assess the protective effect of pre-differentiated skin-like cells, viability, cytotoxicity level, expression of pro-survival and pro-apoptotic genes were measured using cell viability assay, LDH assay and quantitative real time PCR; respectively, as described above. In addition, real time RT-PCR was performed using SYBER Green PCR super Mix (BioRad Laboratories, USA). β -actin was used to normalize the gene expression.

Results

Placenta derived cells can differentiate into skin-like cells

We have previously established protocols for the isolation and differentiation of placenta derived stem cells into skin like cells [1]. AECs were positive for CK8, CK16, CK18, CK19 and E-Cadherin while UC-MSCs were positive for mesenchymal lineage markers CD29, CD44, CD49, CD73 and CD90 [1]. After induction, AECs were differentiated into keratinocytes-like cells as indicated by typical polygonal morphology [6] and expression of CK1, CK10, CK14 and E-Cadherin determined by RT-PCR analysis. Similarly, UC-MSCs showed triangular shape [7] in induction medium and high mRNA expression of collagen-3, desmin, FGF-7 and vimentin [1].

In vitro burn injury model using keratinocytes and fibroblasts

In vitro injury models were established by exposing separately the cultured keratinocytes and fibroblasts at 45°C and 50°C in a water bath. As compared to normal keratinocytes (Figure 1A) and fibroblasts (Figure 1B), thermally injured keratinocytes (Figure 1C) and fibroblasts (Figure 1D) showed destructive morphology at 50°C. This temperature (50°C) was selected as optimal temperature for subsequent experiments on the basis of following results.

Cell viability assay

The percentage viability of both keratinocytes and fibroblasts was significantly reduced with increasing temperature from 37°C to 50°C. For keratinocytes least viability was observed at 50°C as compared to 45°C and 37°C [26.3% \pm 4.16% (50°C) vs. 62.7% \pm 6.03% (45°C) vs. 88.0% \pm 2.00% (37°C)] as shown in Figure 2A. Similarly, in fibroblast cultures, there were 22.33% \pm 3.21% viable cells at 50°C, 68.00% \pm 3.00% viable cells at 45°C, as compared to 88.33% \pm 2.08% at 37°C (Figure 2B).

LDH release assay

Cytotoxicity levels in heat injured keratinocytes and fibroblasts were

estimated using LDH release assay performed at different temperatures. The percentage of LDH release for keratinocytes was highest at 50°C (26.09 \pm 0.93%) as compared to 45°C (15.01 \pm 0.70%) and 37°C group (7.187 \pm 0.255%) (Figure 2C). Similarly, in fibroblast cultures, thermal injury at 50°C resulted in maximum release of LDH (28.43 \pm 0.52%) as compared to at 45°C (17.29 \pm 0.73%) and 37°C (6.09 \pm 0.89%) (Figure 2D).

Expression of pro-apoptotic, pro-survival and proliferative genes

Expression of pro-apoptotic, pro-survival and proliferative genes was also analyzed for the optimization of temperature for thermal injury for both keratinocytes and fibroblasts. Expression of FADD and Bax which are apoptotic genes was significantly up-regulated with increasing temperatures. In keratinocytes, the expression of BAX was 32.33 \pm 1.52% at 50°C vs. 21.0 \pm 2.00% at 45°C vs. 15.67 \pm 2.51% at 37°C. Similarly, FADD expression was 35.00 \pm 7.00% vs. 22.67 \pm 1.52% vs. 19.33 \pm 1.52% at 50°C, 45°C and 37°C; respectively. Contrary to this, the expression of PCNA (8.66 \pm 3.51% at 50°C vs. 22.0 \pm 3.60% at 45°C vs. 15.33 \pm 2.51% at 37°C) and VEGF (2.33 \pm 1.52% at 50°C vs. 6.33 \pm 4.04% at 45°C vs. 17.67 \pm 3.05% at 37°C) was significantly down-regulated at higher temperatures (Figures 3A and 3B).

In fibroblasts, heat injury produced similar pattern of mRNA levels of pro-apoptotic, pro-survival and proliferative genes. In response to higher temperature, the expression of BAX (31.33 \pm 3.51% at 50°C vs. 19.67 \pm 5.50% at 45°C vs. 10.33 \pm 2.51% at 37°C) and FADD (38.00 \pm 4.58% at 50°C vs. 29.67 \pm 1.52% at 45°C vs. 1.00 \pm 0.21% at 37°C) was significantly up-regulated at 50°C. Levels of mRNA of PCNA and VEGF down-regulated at 50°C as compared to other groups. PCNA gene expression was 4.66 \pm 3.51% vs. 20.33 \pm 2.08% vs. 12.67 \pm 4.50%, while VEGF gene expression was 4.66 \pm 1.15% vs. 7.00 \pm 2.00% vs. 6.33 \pm 2.51% at 50°C, 45°C and 37°C, respectively (Figures 3C and 3D).

Co-culturing of injured keratinocytes and fibroblasts with pre-differentiated placental cells helps in revival of injured cells

Heat injured (at 50°C) keratinocytes and fibroblasts were co-cultured separately with either control (i.e., untreated AECs and

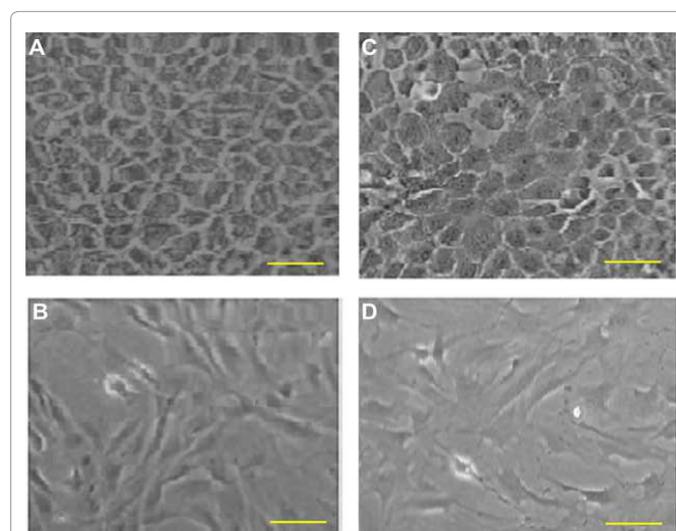


Figure 1: Morphological changes after thermal injury (50°C). Control keratinocytes (A), Injured keratinocytes (C). Control fibroblasts (B) and Injured fibroblasts (D).

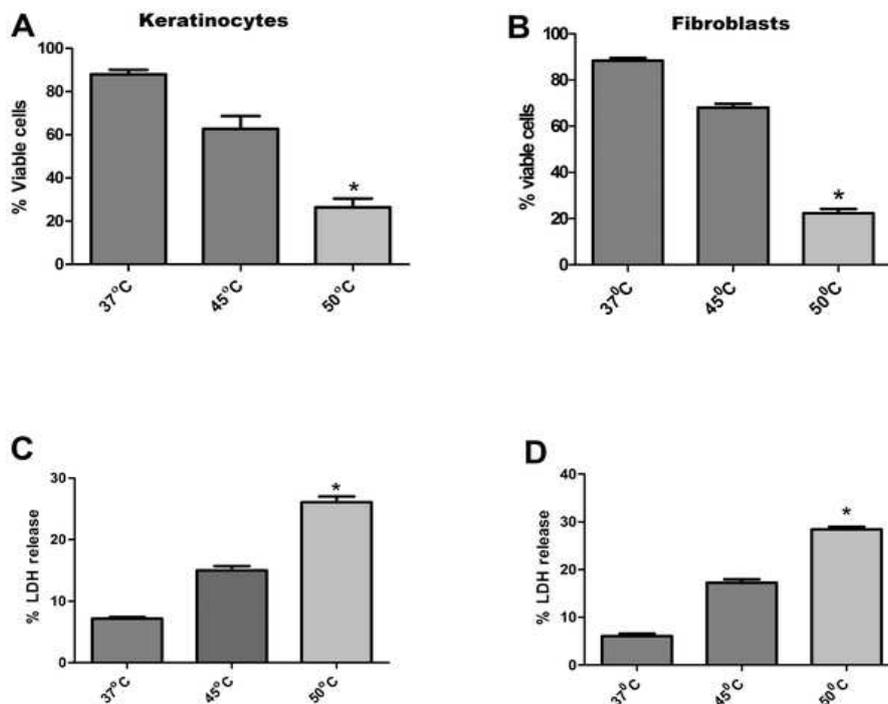


Figure 2: Viability and cytotoxicity levels after thermal injury. The number of viable cells after heat injury was significantly reduced. Viability of keratinocytes (A) and fibroblasts (B) after thermal injury which was significantly reduced at 50°C. Data was expressed as mean \pm SD (* p <0.05 vs. control). Percentage LDH release was increased at higher temperatures in both types of cells. The cytotoxicity levels as indicated by measurement of LDH release in keratinocytes (C) and fibroblasts (D). Data was expressed as mean \pm SD (* p <0.05 vs. control).

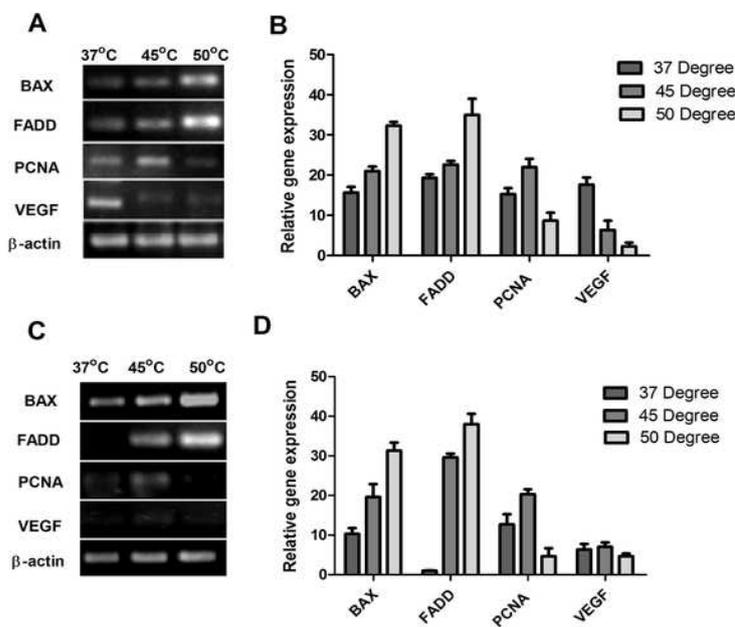


Figure 3: Expression of pro-apoptotic genes (BAX and FADD), proliferation gene (PCNA) and vascular endothelial growth factor (VEGF) in keratinocytes (A) and fibroblasts (C). Relative quantification of gene expression at 37°C, 45°C and 50°C for keratinocytes (B) and fibroblasts (D). Data was expressed as mean \pm SD (* p <0.05 vs. control).

UC-MSCs mixed in 1:1) or treated (i.e., differentiated keratinocytes and fibroblasts in 1:1) placental cells. After 48 h of co-culturing, cell viability, cytotoxicity and gene expression analysis was performed to evaluate recovery of heat injured keratinocytes and fibroblasts.

Co-culturing of pre-differentiated cells enhance viability: Cell viability in all experimental groups was assessed using trypan blue exclusion method. Results showed highest number of viable cells in Group 4 (69.00 \pm 2.64%) as compared to group 3 (60.33 \pm 2.51%) and

group 2 ($31.33 \pm 3.51\%$) in keratinocytes (Figure 4A). Further, the experiments with fibroblasts also showed similar pattern, with highest number of viable cells in group 4 ($70.33 \pm 2.51\%$) as compared to group 3 ($57.00 \pm 2.64\%$) and group 2 ($21.67 \pm 3.05\%$) as shown in Figure 4B).

Cytotoxic levels were reduced after co-culturing with pre-differentiated cells: Recovery from thermal injury was estimated after co-culturing of pre-differentiated cells with heat injured keratinocytes and fibroblasts. Cytotoxic levels were estimated by measuring LDH release in different experimental groups. Results showed significant decrease in percentage of LDH release from injured keratinocytes in group 4 ($10.73 \pm 1.31\%$) as compared to group 3 ($18.21 \pm 0.77\%$) and group 2 ($26.75 \pm 1.01\%$) as shown in Figure 4C. Similarly, Figure 4D shows low levels of LDH release in heat injured fibroblasts in group 4 ($13.17 \pm 1.86\%$) as compared to group 3 ($26.42 \pm 1.55\%$) and group 2 ($34.54 \pm 0.73\%$).

Effect of co-culturing on gene profiling of heat injured keratinocytes and fibroblasts: Quantitative real time PCR show that co-culturing of pre-differentiated cells with heat injured keratinocytes and fibroblasts decreases expression of pro-apoptotic genes (BAX, FADD) while increases expression of proliferative (PCNA) gene and the growth factor (VEGF).

Co-culturing with heat injured keratinocytes: A significant decrease in the expression of both FADD and BAX was observed in group 4 in which heat injured keratinocytes were co-cultured with pre-differentiated skin-like cells. In group 3, the expression of FADD and BAX although was lower as compared to group 1 and 2 but was higher as compared to group 4. Contrary to this the expression of PCNA and VEGF genes was significantly up-regulated as a result of co-culturing with pre-differentiated cells (Figure 5A).

Co-culturing with heat injured fibroblasts: In heat injured fibroblasts, the co-culturing experiments showed similar pattern of mRNA levels of BAX, FADD, PCNA and VEGF genes. The expression of FADD and BAX was lowest in group 4 as compared to other groups. On the other hand, the expression of PCNA and VEGF was significantly increased when heat injured fibroblasts were co-cultured with pre-differentiated skin-like cells (group 4) as compared other groups (Figure 5B).

Discussion

In humans, skin represents approximately one-tenth of the body mass and any damage to this major organ has serious consequences. An intact skin surface is vital for the preservation of thermoregulation, body fluid homeostasis and protection against infection. However, burn injuries may create a breach in the surface and thus result in severely compromised function of skin. Healing of skin wounds involves a cascade of events that aid in restructuring of the damaged tissue to restore it morphologically [8]. Patients with severe burn injuries are usually treated by implanting a skin graft; however, the use of graft is limited in case of severe burn injuries (when donor area for grafts is not available). The ideal treatment option for such patients is stem cell based therapies by using readily available sources of cells. Placenta is one such source that is abundant, readily available, rich in stem cells and has no ethical concerns. Further, placental stem cells have immunosuppressive properties that make it even ideal for allogenic stem cell based therapies [9].

The main findings of this study are; 1. Skin derived keratinocytes and fibroblasts could be used for the establishment of *in vitro* skin injury models, 2. Skin injury models can be used to assess effect of pre-differentiated skin like cells, and 3. Pre-differentiated skin like

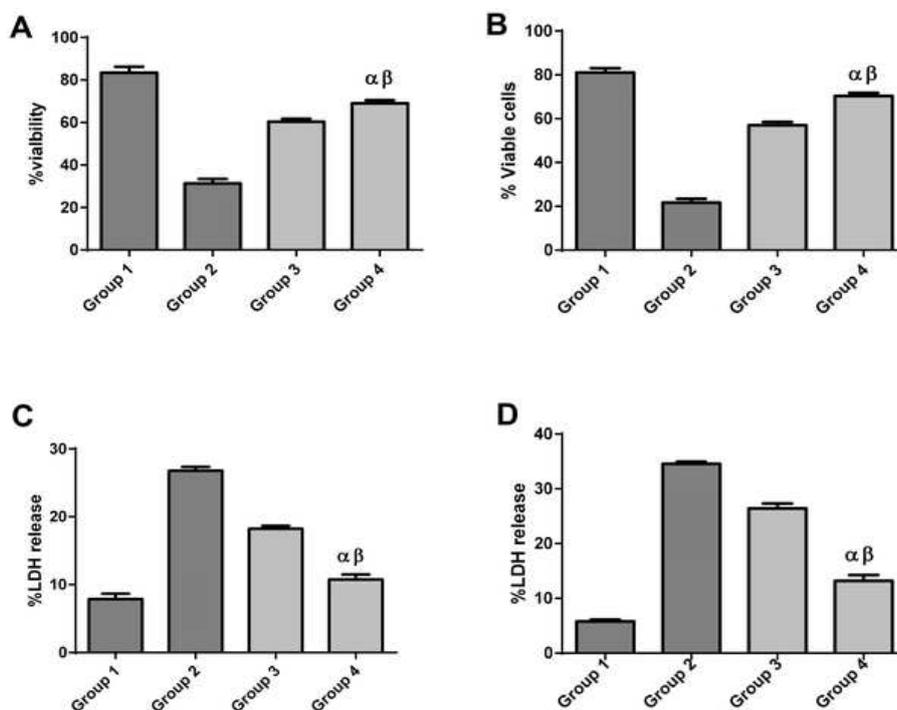
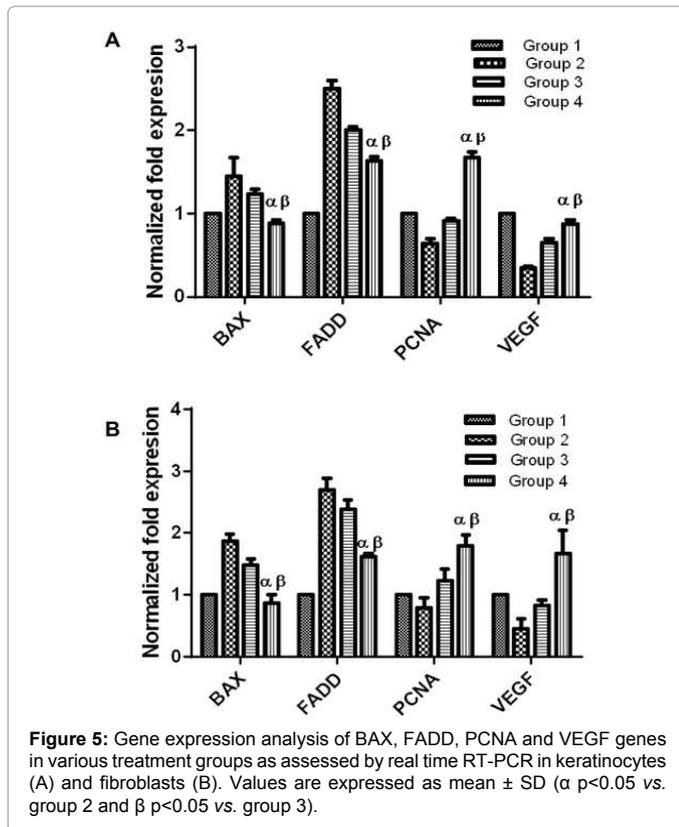


Figure 4: Co-culturing of pre-differentiated cells enhances viability of injured keratinocytes and fibroblasts: Assessment of percentage of viable cells in different treatment groups of keratinocytes (A) and fibroblasts (B) after co-culturing with pre-differentiated cells. (α $p < 0.05$ vs. group 2 and β $p < 0.05$ vs. group 3). Percentage analysis of LDH release by different experimental groups of keratinocytes (C) and fibroblasts (D) after co-culturing pre-differentiated cells (α $p < 0.05$ vs. group 2 and β $p < 0.05$ vs. group 3).



cells have protective effect on injured keratinocytes and fibroblasts. We had previously established the protocols for successful differentiation of placenta derived cells into skin-like cells, i.e., keratinocytes- and fibroblast-like cells [1]. Differentiation of placenta derived cells into keratinocytes and fibroblasts was confirmed by observing morphological changes, using immunostaining and PCR analysis of lineage specific genes. Cells differentiated into keratinocytes showed expression of CK1, CK5, CK10, CK14, involucrin, loricrin and E-Cadherin. Results of this study are in accordance with other published reports that indicate upregulation of these genes in cells undergoing differentiation into keratinocytes [10]. Similarly, fibroblasts after differentiation showed expression of collagen-3, desmin, FAP- α , procollagen-1, FGF-7 and vimentin. Our results were in accordance with other studies [11].

Wound healing and cytoprotective effects of new modalities are difficult to study using humans and even animals. *In vitro* models may prove valuable tools for the study of wound healing and skin pathophysiology because these models are convenient, exclude systemic variables and are reproducible. Previously keratinocytes [12], endothelial cells or [13] fibroblasts [14] were used for such studies, however, in the current study, the burn injury models consisted both of skin derived keratinocytes or fibroblasts. Both keratinocytes and fibroblasts were exposed separately to higher temperatures to select optimal temperature for inducing *in vitro* injury. Based on morphological analysis, cytotoxicity assay and expression of pro-survival and pro-apoptotic genes, we found maximum compromised activity of both keratinocytes and fibroblasts at 50°C. The purpose of using high temperature was to damage the cells at a level where they exhibit mutilation but could be recovered if provided with suitable conditions (potential mimicry of burn injury). Morphological analysis indicated that the cells at 50°C showed more destructive morphology

as compared to cells heated at 45°C and 37°C. In addition, high temperature resulted in least viability of cells (both keratinocytes and fibroblasts) while cytotoxicity as determined by LDH release assay was significantly increased in both keratinocytes and fibroblasts. These results are in accordance with previously published reports that indicate reduced viability at high temperature [15]. Further, there was an up-regulation in the expression of apoptosis markers FADD and BAX [16,17] while decrease in the expression of PCNA and VEGF [18] at 50°C for both keratinocytes and fibroblasts. These findings are in line with other reports that indicate increased apoptosis and reduced proliferation at high temperatures [15]. So at this temperature (50°C) both the keratinocytes and fibroblasts showed severely compromised function and this temperature were selected to induce burn injury.

The established injury models were used to determine the effect of pre-differentiated skin-like cells (keratinocyte- and fibroblasts-like cells) in a co-culture system. Pre-differentiated cells were co-cultured either with heat injured keratinocytes or fibroblasts. Co-cultured experiments indicated that pre-differentiated skin-like cells augment the parameters that were compromised with heat injury. Viability of heat injured keratinocytes and fibroblasts were significantly improved after co-culturing with pre-differentiated cells. Reduction of cytotoxicity as evidenced by low levels of LDH release in injured cells co-cultured with differentiated skin like cells was also observed. Quantitative real time PCR analysis further confirmed these findings. The expression of pro-apoptotic genes (BAX and FADD) was significantly down-regulated while expression of PCNA and VEGF genes was up-regulated in injured keratinocytes and fibroblasts co-cultured with pre-differentiated skin-like cells. These findings indicated that placental cells differentiated into skin like cells have ability to recover cells with burn injury.

In conclusion, in this study we have described that keratinocytes and fibroblasts could be used as burn injury model. The pre-differentiated cells are a potential source of stem cells for severe burn injuries. Overall, the results of this study indicated pre-differentiated cells can alleviate the burn injury.

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