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Vitamin E preconditioning alleviates in vitro thermal stress in cultured human epidermal keratinocytes

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ABSTRACT

Aims: Thermal burns are the most common type of skin injuries. Clinically, the deteriorating thermal wounds have been successfully treated with skin cell sheets, suspensions or bioengineered skin substitutes. After thermal injury, oxidative microenvironment prevalent in the burnt tissue due to imbalance between production of free radicals and antioxidants defense aiding to destruction of cellular or tissue components. However, depleted antioxidant content particularly vitamin E after heat injury challenges efficient regenerative and healing capacity of transplanted cells. Thus, aim of current study was to pretreat human epidermal keratinocytes with vitamin E in order to enhance their survival rate and therapeutic ability under oxidative microenvironment induced by in vitro heat stress.

Main methods: Keratinocytes were treated with 100 μM vitamin E at 37 °C for 24 h followed by thermal stress at 51 °C for 10 min. Cell viability and cytotoxicity assays, gene expression analysis and paracrine release analysis were performed.

Key findings: Vitamin E preconditioning resulted in significantly improved cell morphology, enhanced viability and reduced lactate dehydrogenase release. Furthermore, Vitamin E preconditioned cells exposed to thermal stress showed significant down-regulated expression of BAX and up-regulated expression of PCNA, BCL-XL, vascular endothelial growth factor (VEGF), involucrin, transglutaminase 1 (TGM1) and filaggrin (FLG) escorted by increased paracrine release of VEGF, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF).

Significance: Results of the current study suggest that clinical transplantation of vitamin E preconditioned keratinocytes alone or in combination with dermal fibroblasts in skin substitutes for the treatment of thermally injured skin.

1. Introduction

Keratinocytes are predominant cell component (95%) of skin epidermis. Differentiated keratinocytes maintain epidermal integrity and have an important role in mechanical barrier function of epidermis, thus preventing water or electrolytes loss and invading pathogens [1,2]. Barrier function of the skin can be disturbed by thermal injury.

Thermal and scald burns account for majority of skin burns. Every year, there are an estimated 180,000 deaths caused due to burns and a vast majority is reported in low-middle income countries [3]. These injuries can be divided into superficial, partial thickness and full thickness skin burns depending on depth of injury. In solely epidermal (superficial) or partial dermal injuries, re-epithelialization from proliferated keratinocytes starts within hours of injury and wounds are healed by regeneration without scarring within 3–5 days. During wound healing, the interaction of epidermal keratinocytes and dermal fibroblasts through release of growth factors and cytokines are critical. Usually, normal wound healing mechanism is enough to heal the skin wounds [3–5].

On the other hand, full-thickness burns, due to extensive tissue injury and impaired skin functions, require surgical intervention for tissue restoration. Keratinocytes play important role especially in proliferative phase of wound healing that leads to restoration of epithelialization and vascularization. Wounds could not close without epithelialization. Rapid epithelialization is required to restore skin barrier function. Thus, keratinocytes are considered as first-rate cell transplantation candidate

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to accomplish faster re-epithelialization [3,4].

Numerous approaches have been used for delivery of epidermal keratinocytes to burn wounds previously. First development was the application of autologous keratinocytes sheet, afterward administration of a single cell suspension of pre-confluent keratinocytes, uncultured keratinocytes and allogeneic neonatal keratinocytes to burn wound by a syringe or using a spray device. However, in the case of full-thickness wounds, cultured keratinocytes are transplanted along with dermal fibroblasts in the form of bioengineered skin substitute to ensure complete and improved restoration of skin tissue after injury [4,6].

Free radicals, generally in the form of reactive oxygen species (ROS), are constantly generated in all aerobic organisms during normal cellular respiration. In order to maintain the homeostasis of ROS, mitochondria of cells are equipped with non-enzymatic antioxidants (e.g. vitamin E, vitamin C) and enzymatic antioxidants (e.g. glutathione, superoxide dismutase) to scavenge and neutralize the generated free radicals. Low levels of ROS play a critical role in stimulating normal wound healing. They seem to be important in coordinating the recruitment of immunocytes and lymphoid cells to the wound bed and are involved in effective tissue repair. ROS also possess the ability to regulate angiogenesis and optimum blood perfusion at wound site. However, after thermal injury, the consequent inflammation and ischemia trigger the release of ROS while depletion of antioxidant contents of skin especially vitamin E that further increases the production of free radicals. This results in oxidative stress that is an imbalance between ROS generation and antioxidant (enzymatic and non-enzymatic) defense system due to undue generation of free radical species. These extensive free radicals oxidize cellular molecules such as proteins, lipids and nucleic acids, and result in cellular damage and impaired wound healing [7–10]. Despite different delivery methods, oxidative microenvironment of burnt wound compromises the regenerative and healing capacity of transplanted keratinocytes. Therefore, Vitamin E supplement therapy against oxidative stress is important for rescue of burn patients [9,11].

Vitamin E, a lipid-soluble antioxidant in skin tissue, prevents lipid peroxidation or cellular dysfunction, and protects degradation of endogenous epidermal antioxidant content [12,13]. There are no percutaneous specific transport proteins for vitamin E. It first accumulates in the sebaceous glands before it is transported to the stratum corneum via sebum. Due to lipophilic nature of vitamin E, its topical applications permeate into all underlying layers of skin. In humans, the rate of vitamin E absorption through skin and factors that impact its penetration are mainly unidentified. It is commonly presumed that < 0.1% solutions of vitamin E can enhance its concentration in the skin. Vitamin E accumulates in cell membranes as well as in the lipid rich extracellular matrix of the stratum corneum, where vitamin E plays its part in antioxidant defense system [14–16]. In cells, vitamin E modulates gene expression pathways in redox dependent or redox independent manner [12,13].

In the light of above, we investigated the effect of vitamin E preconditioning in protecting epidermal keratinocytes from subsequent thermal injury.

2. Materials and methods

2.1. Collection of human skin biopsy

Normal skin biopsies along with patient's data as informed consent forms were collected from Jinnah hospital, Lahore, Pakistan. The donor patients were HBV or HCV negative.

2.2. Isolation and culturing of keratinocytes

Keratinocytes from human skin were isolated by using previously published protocol [17]. In short, epidermal tissue was minced and enzymatically digested with 1 × trypsin/ethylenediaminetetraacetic acid (T/E; Gibco, USA) solution at 37 °C with periodic agitation. After every 15 min, T/E was replaced with a fresh enzymatic solution. The process was repeated until maximum tissue digestion. Then, isolated keratinocytes in culture medium (Dulbecco's Modified Eagle's Medium High Glucose/Ham's F-12 Medium in 3:1 ratio, Sigma life science, USA) supplemented with 10% fetal bovine serum (FBS, Sigma life science, USA), epidermal growth factor (EGF; 10 ng/ml, MP Biomedicals, USA), insulin (5 μg/ml, Sigma life science, USA) and hydrocortisone (0.4 μg/ml, Sigma life science, USA) were seeded on irradiated (at 6000 radiations for 10 min) monolayer of 3T3 cell line (Sigma life science, USA) and cultured at 37 °C in 5% CO_2, 70-80% humidified incubator till 80-90% confluency (approximately for 15-20 days). Every 3rd day, the spent medium was replaced with fresh one. Keratinocytes at passage 2 (without feeder layer) were used for succeeding experiments.

2.3. Characterization of keratinocytes

Characterization of epidermal keratinocytes was done by immunocytochemistry followed by fluorescent microscopic analysis. Immunocytochemical analysis of keratinocytes was performed by using involucrin (Abcam, USA), loricrin (Abcam, USA) and cytokeratin 5 (CK5; Abcam, USA) antibodies using protocol as reported previously [18] and images were captured using Olympus IX51 microscope (Olympus, Japan).

2.4. Keratinocytes treatments

Passage 1 keratinocytes were trypsinized and plated at the density of 3×10^5 cells in 25 cm^2 flasks. The preconditioning of keratinocytes was performed when plated cells became 80% confluent. These cells were randomly assigned to four groups: Control group: untreated cells incubated in serum free medium for 24 h and unexposed to heat stress; Vitamin E group: cells incubated in 100 μM vitamin E containing serum free medium for 24 h and unexposed to heat stress; Heat group: untreated cells incubated in serum free medium for 24 h and exposed to 51 °C for 10 min; Vitamin E + Heat group: cells incubated in 100 μM vitamin E containing serum free medium for 24 h and exposed to heat injury of 51°C for 10 min. Before and after vitamin E treatment, cells were carefully washed with 1 × phosphate buffer saline once.

2.5. Morphological analysis

After completion of all treatments, at least 10 images (per group) of different treatment groups of keratinocytes were taken using fluorescent microscope (Olympus, Japan). Changes in cellular morphology in the term of average size (arbitrary unit, AU) were analyzed using “Analyze particles” tool of Image J software. For this, pixel area size (zero to infinity) and circularity values (zero to one) were set for ensuring precision of calculation.

2.6. Cell viability and cytotoxicity assays

The viability and cytotoxicity of various experimental groups of keratinocytes were assessed by 2,3-bis (2-methoxy-4-nitro-5-sulfoxyphenyl)-2H-tetrazolium 5-carboxyanilide inner salt (XTT) kit (Roche, Mannheim, Germany) and lactate dehydrogenase (LDH) kit (Sigma life science, USA) respectively using manufacturer's protocols.

2.7. Gene expression analysis by real time PCR (qPCR)

Semi-quantitative expression analysis of BAX, PCNA, BCL-XL, vascular endothelial growth factor (VEGF), involucrin, transglutaminase 1 (TGM1) and filaggrin (FLG) genes in different treatment groups of keratinocytes were studied by qPCR using protocol as reported previously [19]. Primer sequences, annealing temperatures and product sizes are given in Table 1. GAPDH was used for normalization of

Table 1
Primer sequences, annealing temperatures and product sizes.

Primer name	Sequences (5′–3′)	Annealing temperature (°C)	Product size (bp)
GAPDH-R	TGCTGTAGCCAAATTCGTTG	58	250
GAPDH-F	AACGTGTCAGTGGTGGACCT		
BAX-R	AAGTCCAATGTCCAGCCCAT	58	163
BAX-F	GAGAGGTCTTTTTCCGAGTGG		
BCL-XL-R	GGGCCTCAGTCCTGTTCTCT	58	95
BCL-XL-F	GGAGCTGGTGGTTGACTTTCT		
PCNA-R	GTATCCGCGTTATCTTCGGC	58	244
PCNA-F	AGGCACTCAAGGACCTCATC		
VEGF-R	CTGCGCTGATAGACATCCATG	56	137
VEGF-F	CTGTCTTGGGTGCATTGGAG		
FLG-R	TGCTTTCTGTGCTTGTGTCC	58	187
FLG-F	GGCAAATCCTGAAGAATCCA		
TGM1-R	AACCACTGCTGCTCCCAGTA	60	287
TGM1-F	GCACAGTGAAACTGCACCTC		
Involucrin-R	TGGGTATTGACTGGAGGAGG	58	134
Involucrin-F	TCTGCCTCAGCCTTACTGTG		

expression. Fold change was calculated.

2.8. Paracrine release analysis by enzyme linked immunosorbent assay (ELISA)

The paracrine release potential of different treatment groups of keratinocytes was analyzed by performing sandwich ELISA using VEGF (Santa Cruz, USA), epidermal growth factor (EGF; Abcam, USA) and basic fibroblast growth factor (bFGF; Santa Cruz, USA) antibodies by using protocol reported previously [20].

3. Results

3.1. Characterization of keratinocytes

The phase contrast images showed the hexagonal shape of pure keratinocytes and immunocytochemical analysis indicated the positive expression of involucrin, loricrin and CK5 in keratinocytes (Fig. 1A and B).

3.2. Vitamin E preconditioning and cellular morphology

Morphologically, thermal injury caused the shrinkage of cells as numerically demonstrated by reduced average cell size in heat injured group (30.02 ± 3.05 AU) vs. control group (66.14 ± 6.43 AU) as shown in Fig. 2A and B. Vitamin E preconditioning protected the morphology of keratinocytes from thermal injury by preserving the average cell size (51.09 ± 6.08 AU) in vitamin E preconditioned heat injured group vs. heat stress group. Improvement in morphology of non-heat stressed vitamin E pretreated cells vs. untreated control group was also observed, although not much prominent (Fig. 2A and B).

3.3. Vitamin E preconditioning and cell viability

The results depicted in Fig. 2C revealed that only vitamin E preconditioning remarkably enhanced the viability of keratinocytes (121.57 ± 7.34% in vitamin E preconditioned group vs. 100 ± 0.0% in non-preconditioned group). Vitamin E preconditioning alleviated the deteriorating effects of thermal stress as demonstrated by enhanced viability of vitamin E preconditioned heat injury group (107.06 ± 9.54%) as compared to heat injury group (48.59 ± 8.01%).

3.4. Vitamin E preconditioning and cytotoxicity

The results of LDH assay as shown in Fig. 2D illustrated the significantly reduced release of LDH in non-heat exposed vitamin E preconditioned group (49.2 ± 4.2%) with reference to non-preconditioned group (100 ± 0.0%) and in vitamin E pretreated heat stress group (99.04 ± 17.7%) with reference to only heat injured group (195.7 ± 30.5%). Thermal injury damaged the cell membrane of keratinocytes as revealed by increased LDH release in heat injury group vs. rest of the groups.

3.5. Vitamin E preconditioning and gene expression analysis

Vitamin E preconditioned keratinocytes showed the significantly decreased expression of BAX (0.26 ± 0.004 fold) and remarkably up-regulated expression of BCL-XL (1.53 ± 0.09 fold), PCNA (1.921 ± 0.131 fold), VEGF (1.32 ± 0.03 fold), FLG (1.15 ± 0.023 fold), TGM1 (1.14 ± 0.02 fold) and involucrin (1.85 ± 0.05 fold) in only vitamin E pretreated cells vs. untreated control cells (1 ± 0.0 fold).

Vitamin E preconditioning significantly down-regulated the expression of BAX in vitamin E pretreated heat injured cells (1.29 ± 0.09 fold) as compared with untreated heat injured cells (3.06 ± 0.05 fold). Whereas under heat stress, the significantly up-regulated expression due to vitamin E preconditioning was observed for BCL-XL (1.42 ± 0.17 fold in vitamin E preconditioned heat injured group vs. 0.21 ± 0.01 fold in heat injured group), PCNA (1.66 ± 0.19 fold in vitamin E preconditioned thermally injured group vs. 0.14 ± 0.01 fold in thermally injured group), VEGF (1.06 ± 0.05 fold in vitamin E preconditioned thermally injured cells vs. 0.16 ± 0.01 fold in heat stressed cells), FLG (0.85 ± 0.05 fold in vitamin E pretreated thermally injured group vs. 0.09 ± 0.01 fold in thermally injured group), TGM1 (1.02 ± 0.01 fold in vitamin E preconditioned heat stress group vs. 0.38 ± 0.04 fold in heat injured group), involucrin (0.98 ± 0.03 fold in vitamin E pretreated heat injured cells vs. 0.12 ± 0.003 fold in only heat injured cells) as shown in Fig. 3A–G.

3.6. Vitamin E preconditioning and paracrine release potential

The non-heat stressed vitamin E pretreated cells showed increased release of EGF (308.5 ± 8.1 pg/ml in vitamin E preconditioned group vs. 210 ± 6.4 pg/ml in untreated control group), bFGF (298.23 ± 15.03 pg/ml in vitamin E preconditioned group vs. 219.45 ± 12.5 pg/ml in untreated control group) and VEGF (301 ± 11.8 pg/ml vitamin E preconditioned group vs. 206.5 ± 9.5 pg/ml in untreated control group).

Vitamin E preconditioning abrogated the thermal injury induced decrease in paracrine release potential of keratinocytes as demonstrated by significantly enhanced concentration of EGF (287 ± 12.3 pg/ml in vitamin E preconditioned heat injured group vs. 139.5 ± 9.01 pg/ml in heat injured group), bFGF (310.120 ± 19.6 pg/ml in vitamin E pretreated heat injured group vs. 98.1 ± 11.4 pg/ml in heat injured group) and VEGF (209.09 ± 17.09 pg/ml vitamin E pretreated heat injury group vs. 107.22 ± 13.2 pg/ml in only heat injury group) as shown in Fig. 4A–C.

4. Discussion

Vitamin E has its protective role in the body through its free radical scavenging (antioxidant) and gene expression modulation (non-antioxidant) attributes. The present study reports these protective effects of vitamin E on cultured human epidermal keratinocytes against thermal injury. Previously we have already published the results conferring the cytoprotective effects of vitamin E on human skin fibroblasts against thermal injury [18].

In the present study, keratinocytes were characterized (Fig. 1A and

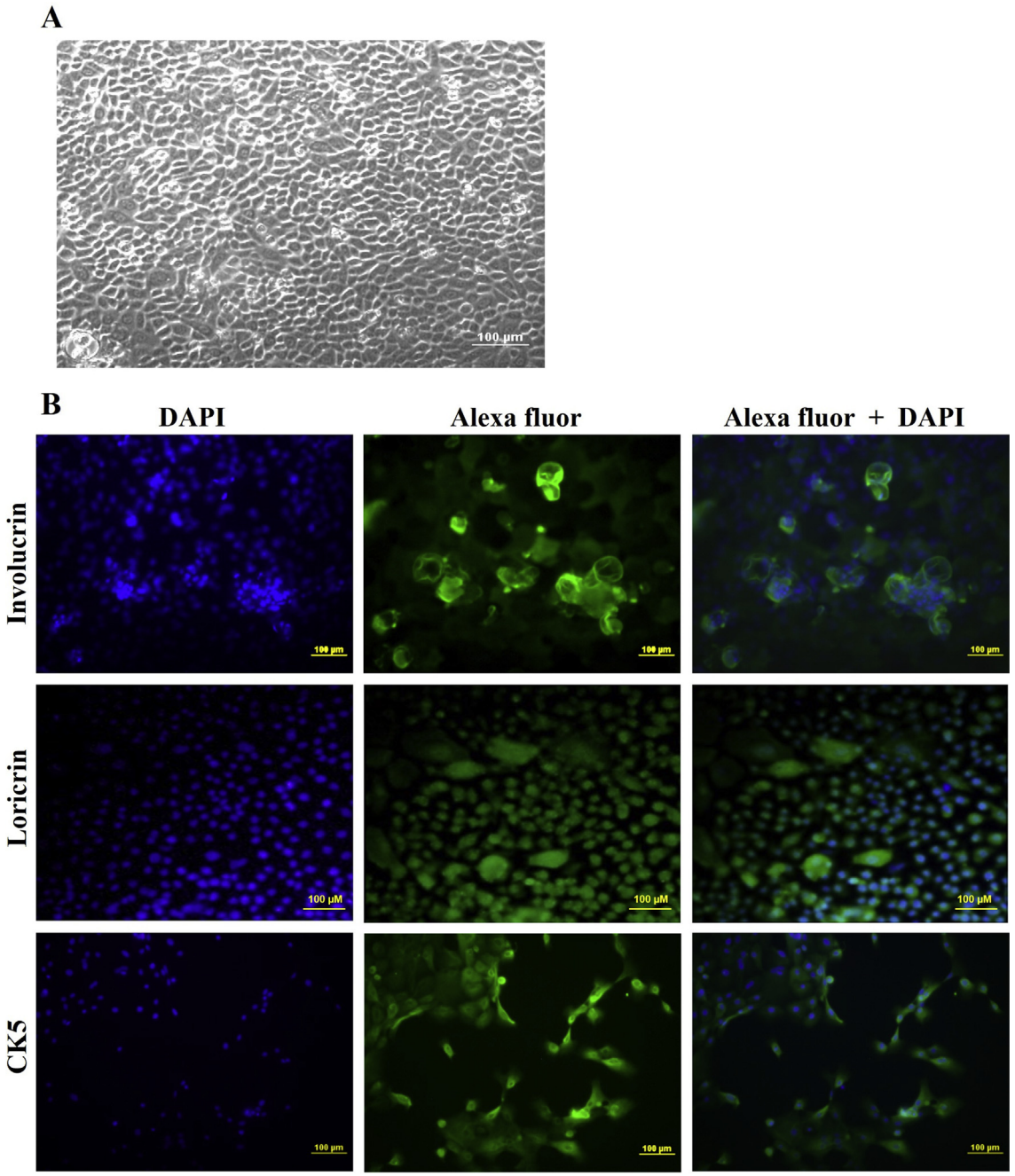

Fig. 1. Characterization of keratinocytes. Hexagonal shape of confluent keratinocytes **(A)**. Alexa Fluor fluorescence micrographs of keratinocytes stained with involucrin, loricrin and CK5 **(B)**. 200× magnification, scale bar: 100 µm.

B) on the basis of hexagonal shape and positive expression of their proliferation (CK5) and differentiation (involucrin and loricrin) specific markers. The results in Fig. 1B suggest that cultured keratinocytes display partial stratification in culture. These results were in line with previous reports. Keratinocytes grow as a typical hexagonal architecture in the keratinocyte culture medium [21]. The proliferative basal keratinocytes express CK5 whereas differentiated keratinocytes express involucrin and loricrin [22–24].

Morphological analysis of different treatment groups of keratinocytes in present study (Fig. 2A and B) demonstrated that thermal stress resulted in shrinkage of keratinocytes. Maximum number of cells with shrunken morphology was observed in heat injured group (Fig. 2A and B) as compared with rest of the groups. Vitamin E pretreatment preserved the shape and membrane integrity of cells even after their exposure to thermal injury. Previous studies also reported that vitamin E abrogates the oxidative stress induced morphologic destruction in MG-63 osteoblast-like cells [25], primary cultured cortical neurons [26], human gingival fibroblasts [27], and pheochromocytoma cell line (PC12) [28,29]. Similarly, vitamin E has also been shown to suppress the 2,4-dinitrochlorobenzene or lauroylsarcosine induced keratinocyte damage [30].

The results of XTT assay (as assessment of cell viability) and LDH assay (as function of cell membrane damage) shown in Fig. 2C and D in the current study illustrate that thermal injury affected the viability of keratinocytes and reduced it to almost half while concomitantly it doubled the release of LDH from thermally injured cells when compared to untreated control cells. On the other hand, vitamin E conferred its protective effects in counteracting deteriorative effects of thermal

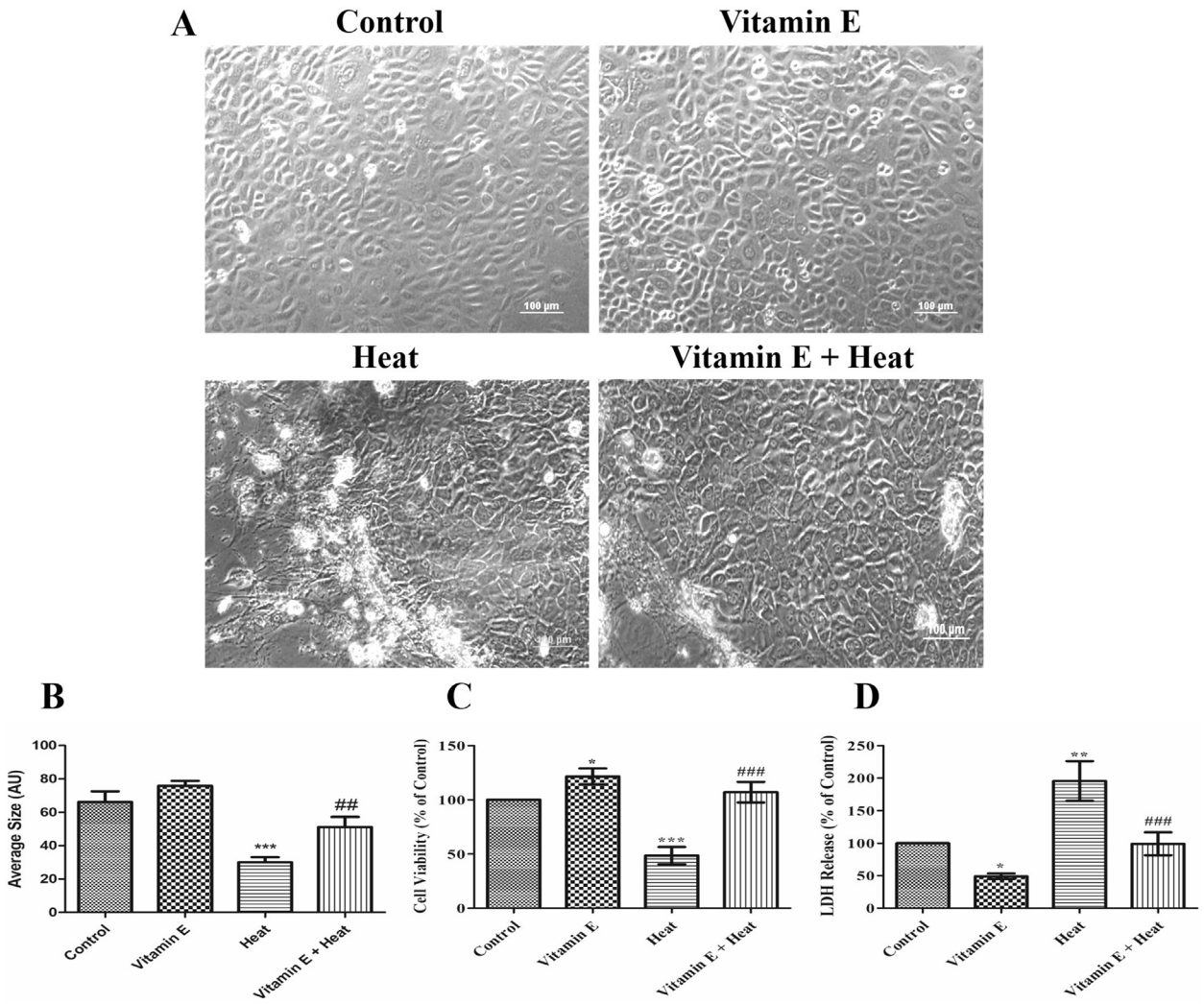

Fig. 2. Impact of vitamin E on cellular morphology, cell viability and cytotoxicity. Morphological analysis of keratinocytes; 200× magnification, scale bar: 100 µm **(A)**, graphical representation of average cell size (Arbitrary unit, AU) **(B)**, assessment of cell viability **(C)** and LDH release **(D)** in different treatment groups of keratinocytes. Data is presented as mean ± standard deviation. Untreated keratinocytes were represented as 100%. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control group. $\#\#\#P < 0.001$ vs. heat group.

injury. All the results were in good correspondence to that of observed cell morphology in various treatment groups (Fig. 2A and B). Vitamin E has been previously reported to protect keratinocytes against UV-induced morphological damage, decrease in viability and increase in apoptosis [31]. Similar role of vitamin E on survival and apoptosis or LDH release has been observed previously against oxidative or ischemic stress conditions in fibroblasts [18,27,32,33], H9c2 cardiomyocytes [34], neuronal cells [35], rat dental follicle stem cells and osteoblast like cells [25,36], cortical neuronal cells [26], rat bone marrow mesenchymal stem cells [19,37], mouse embryonic lung cells [38], human proximal tubular cells [39] and astrocytes [40].

In the present study, gene expression analysis of epidermal keratinocytes by qPCR (Fig. 3A–D) revealed the proliferative, antiapoptotic and angiogenic properties of vitamin E on cells as shown by down-regulated expression of BAX, whereas remarkably up-regulated expression of BCL-XL, PCNA and VEGF in vitamin E preconditioned cells unexposed or exposed to in vitro thermal stress (Fig. 3A–D). These results corroborated to previously published effects of vitamin E on proapoptotic gene (BAX), antiapoptotic gene (BCL-XL), proliferative gene (PCNA) and proangiogenic gene (VEGF) in various cell or tissue types exposed to oxidative stress [18,19,41–45].

Involucrin, FLG and TGM1 gene expression analysis in current investigation showed the up-regulated mRNA level of these genes in vitamin E pretreated thermal stress cells (Fig. 3E–G). These results suggest that vitamin E may play not only antioxidant role by protecting keratinocytes against heat induced oxidative stress, but also non-antioxidant role by modulating expression of keratinocytes specific genes. Involucrin, FLG and TGM1 are known to be expressed in terminally differentiated keratinocytes and involved in structural integrity of skin [23]. It has been previously studied that vitamin E up-regulates the protein expression of TGM1 in normal human keratinocyte cell line unexposed or exposed to tetradecylthioacetic acid [46]. Moreover, vitamin E restores the expression of FLG and TGM1 in skin derived keratinocytes exposed to diesel particulate extract or its vapor [23]. Sodium dl-α-tocopheryl-6-O-phosphate, a vitamin E derivative, induces the differentiation of human skin derived keratinocytes by increasing the gene expressions of TGM1 and involucrin [24].

During wound healing, keratinocytes and fibroblasts exhibit paracrine communication with each other by producing relatively high quantities of growth factors such as EGF, VEGF and bFGF to repair the wounded skin [47,48]. The data in the present study illustrated markedly significant release of VEGF, EGF and bFGF in aspirated medium of vitamin E pretreated epidermal cells (Fig. 4A–C). While thermal injury induced decrease in paracrine release of these factors suggesting the impairment in wound healing ability of keratinocytes. These detrimental effects of thermal injury were well preserved in vitamin E

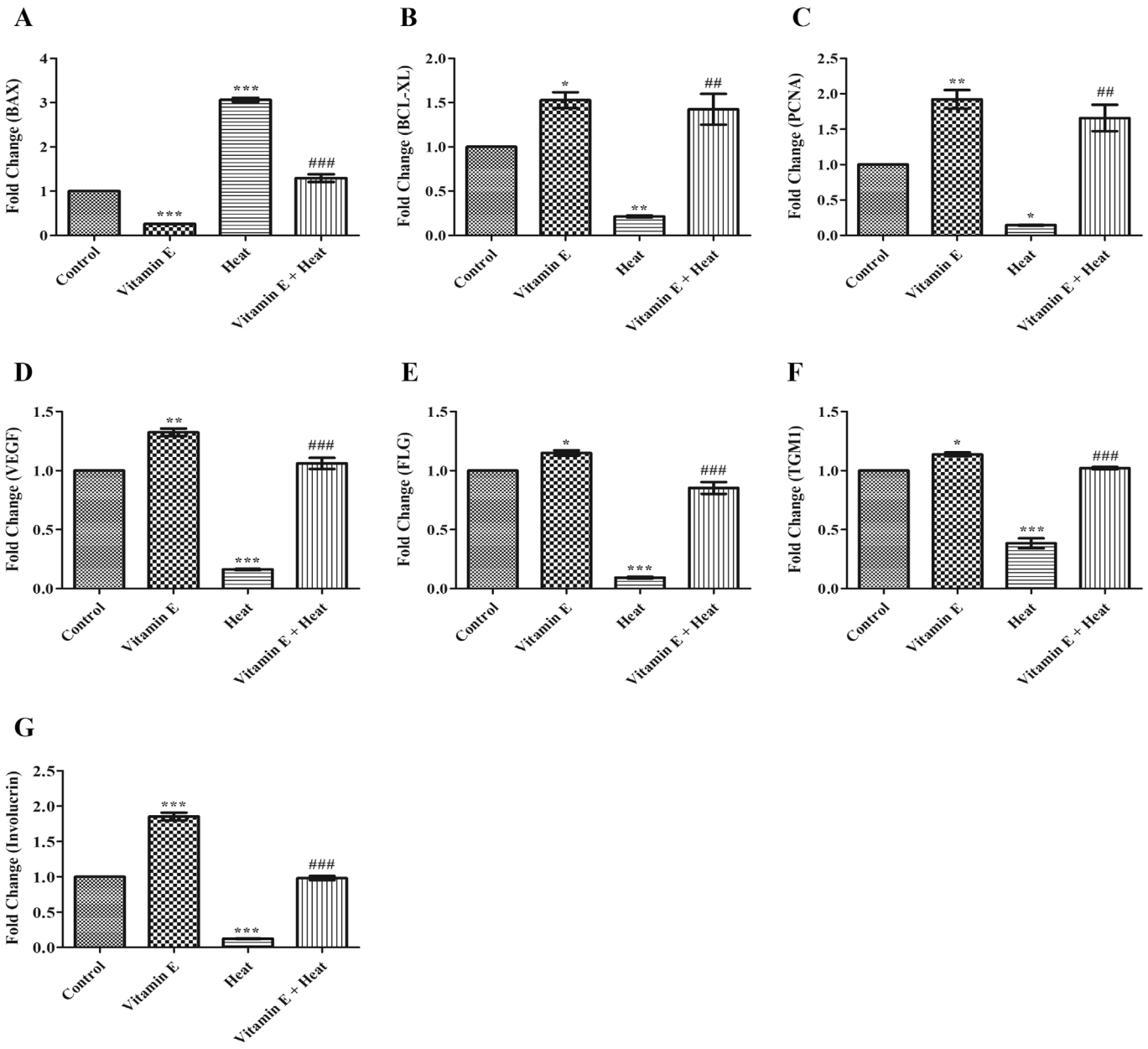

Fig. 3. qPCR of BAX **(A)**, BCL-XL **(B)**, PCNA **(C)**, VEGF **(D)**, FLG **(E)**, TGM1 **(F)** and involucrin **(G)** in different treatment groups of keratinocytes. Data is presented as mean ± standard deviation. Untreated keratinocytes group was represented as 1.0 fold. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs. control group. $\#\#P < 0.01$ and $\#\#\#P < 0.001$ vs. heat group.

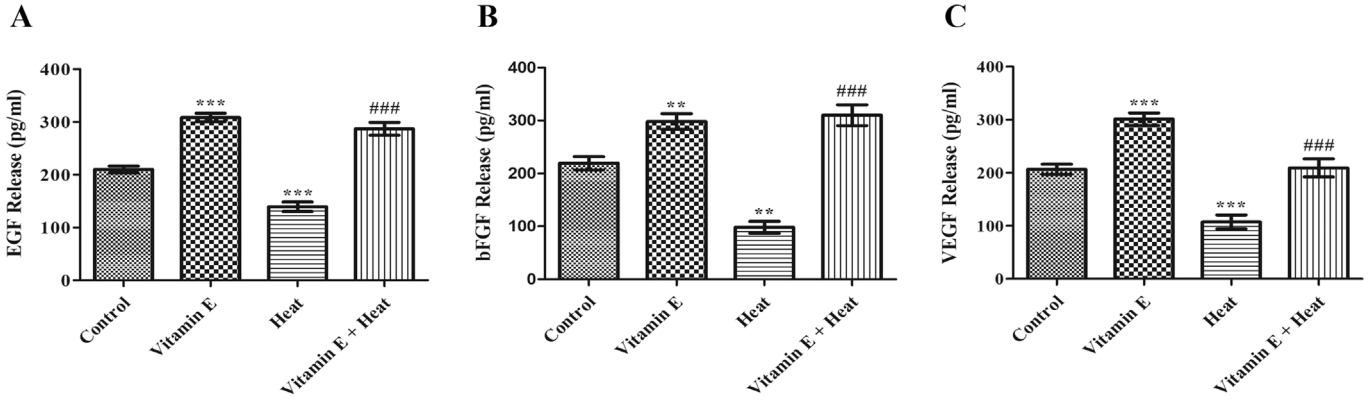

Fig. 4. Quantitative assessment of paracrine release of EGF **(A)**, bFGF **(B)**, VEGF **(C)** in different treatment groups of keratinocytes. Data is presented as mean ± standard deviation. $**P < 0.01$ and $***P < 0.001$ vs. control group. $\#\#\#P < 0.001$ vs. heat group.

pretreated cells. This corroborates with our previous report showing amelioration of heat injury induced reduction in paracrine release of VEGF, bFGF and EGF by human skin fibroblasts due to vitamin E pretreatment [18]. It is also in line with a previous report illustrating gastroprotective effects of vitamin E against water immersion restraint stress induced ulcers in rats via up-regulation of VEGF and bFGF [49].

5. Conclusions

The current study demonstrated that vitamin E preconditioning vitally protects human epidermal keratinocytes against in vitro heat induced oxidative stress. This study in conjunction with our previous study on human skin fibroblast encourage the preclinical evaluation and also suggest the clinical utility of vitamin E preconditioned skin derived cells, alone or as part of composite skin or bioengineered skin substitute, for rapid healing and regeneration of burnt skin.

Declaration of competing interest

No conflict of interest.

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Abbreviations

bFGF: Basic fibroblast growth factor
CK5: Cytokeratin 5
DMEM-HG: Dulbecco's Modified Eagle's Medium - high glucose
EGF: Epidermal growth factor
ELISA: Enzyme linked immunosorbent assay
FBS: Fetal bovine serum
FLG: Filaggrin
LDH: Lactate dehydrogenase
qPCR: Real time PCR
ROS: Reactive oxygen species
SDF-1α: Stromal derived factor-1alpha
T/E: Trypsin/ethylenediaminetetraacetic acid
TGM1: Transglutaminase 1
VEGF: Vascular endothelial growth factor
XTT: 2,3-bis(2-methoxy-4-nitro-5-sulfoxyphenyl)-2H-tetrazolium 5-carboxyanilide inner salt