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Effect of Dermal Fibroblast Conditioned Medium on Keratinocytes Irrespective of Age Group

(Kesan Medium Terkondisi Fibroblas Kulit pada Keratinosit Tanpa mengira Kumpulan Umur)

NURUL 'IZZAH ABDUL GHANI[1], MANIRA MAAROF[1,*], SHIPLU ROY CHOWDHURY[1], AMINUDDIN BIN SAIM[2], RUSZYMAH BINTI HAJI IDRUS[1,3] & FAUZI MH BUSRA[1]

[1]*Centre for Tissue Engineering and Regenerative Medicine, Faculty of Medicine, Universiti Kebangsaan Malaysia, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Federal Territory, Malaysia*
[2]*KPJ Ampang Puteri Specialist Hospital, 1 Jalan Mamanda 9, 68000 Ampang, Kuala Lumpur, Federal Territory, Malaysia*
[3]*Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Federal Territory, Malaysia*

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ABSTRACT

Skin aging causes delayed re-epithelialisation and impaired wound healing. Thus, supplementation of wound healing mediators and extracellular matrix (ECM) components may be a potential treatment strategy for age-related impaired wound healing. Fibroblasts secrete wound-healing factors and can be collected from used medium, i.e., dermal fibroblast conditioned medium (DFCM). In this study, we elucidated the effect of DFCM on the *in vitro* wound healing of keratinocytes isolated from different age groups (≥18–35, 36–54, ≥55 years) via cell attachment, growth rate, and wound healing rate assays. The DFCM was prepared by culturing confluent fibroblasts with serum-free keratinocyte-specific (DFCM-KM) and fibroblast-specific (DFCM-FM) medium. The cell attachment efficiency decreased with the increase of age. However, keratinocyte attachment was enhanced in the DFCM-KM group, where it was 1.24, 1.27, and 1.32 times higher of cells concentration for the ≥18–35-, 36–54-, and ≥55-year age groups, respectively, as compared to the control group. The keratinocytes from each age group demonstrated a similar growth profile for all culture conditions, where the DFCM-KM group exhibited a comparable growth rate with the control group whilst the DFCM-FM group exhibited a significantly lower growth rate compared to the other groups. In contrast, the DFCM-FM group demonstrated a significantly higher healing rate in all age groups as compared to the DFCM-KM and control groups. However, there was no significant difference between the healing rates of the DFCM-KM and control groups. In conclusion, DFCM-KM enhanced keratinocyte attachment while DFCM-FM enhanced the keratinocyte healing rate irrespective of donor age, which indicated the potential application of DFCM in wound healing in aged skin.

Keywords: Aging; conditioned medium; re-epithelialisation; wound

ABSTRAK

Penuaan kulit menyebabkan kelewatan dalam pengepiteliuman semula dan penyembuhan luka. Oleh itu, penambahan faktor penyembuhan luka dan komponen matriks ekstrasel (ECM) merupakan strategi rawatan yang berpotensi bagi penyembuhan luka yang terjejas akibat faktor usia. Fibroblas merupakan sel yang merembeskan faktor pertumbuhan yang terlibat dalam proses penyembuhan luka dan boleh dikumpul daripada medium terpakai, iaitu medium terkondisi daripada fibroblas kulit (DFCM). Kajian ini bertujuan untuk mengkaji kesan DFCM pada penyembuhan luka secara *in vitro* bagi sel keratinosit daripada kumpulan umur yang berbeza (≥18-35, 36-54 dan ≥55 tahun) melalui pelekatan sel, kadar pertumbuhan dan penyembuhan luka. DFCM disediakan daripada pengkulturan fibroblas di dalam media tanpa serum khusus keratinosit (DFCM-KM) dan khusus fibroblas (DFCM-FM). Tahap pelekatan sel berkurangan dengan peningkatan umur. Walau bagaimanapun, pelekatan keratinosit menunjukkan peningkatan dengan tambahan DFCM-KM berbanding kumpulan kawalan yang masing-masing 1.24, 1.27 dan 1.32 kali lebih tinggi kepekatan sel bagi umur ≥18-35, 36-54 dan ≥55 tahun. Keratinosit daripada kumpulan umur yang berbeza menunjukkan profil

pertumbuhan yang sama untuk semua keadaan kultur, dengan DFCM-KM menunjukkan kadar yang setanding dengan kumpulan kawalan manakala DFCM-FM adalah jauh lebih rendah berbanding kumpulan lain. Sebaliknya, kumpulan DFCM-FM menunjukkan kadar penyembuhan yang jauh lebih tinggi dalam semua kumpulan umur berbanding DFCM-KM dan kumpulan kawalan. Walau bagaimanapun, tiada perbezaan yang ketara dalam kadar penyembuhan luka untuk keratinosit dengan tambahan DFCM-KM dan kumpulan kawalan. Kesimpulannya, tanpa mengira umur penderma, DFCM-KM meningkatkan pelekatan keratinosit manakala DFCM-FM meningkatkan kadar penyembuhan keratinosit, menunjukkan potensi penggunaan DFCM dalam penyembuhan luka pada kulit pesakit yang sudah tua.

Kata kunci: Luka; medium terkondisi; pengepiteliuman semula; penuaan

Introduction

The skin is the first barrier of the human body that protects against the outer environment, water loss, and microorganism infection. Organ aging, including that of skin, begins at birth in humans and continues throughout life (Zhang & Duan 2018). The most remarkable physiological and histological changes in aged skin are epidermis thinning and decreased surface area interaction between the dermis and epidermis, which causes low nutrient supply and subsequently decreased basal cell proliferation (Makrantonaki & Zouboulis 2007). The complex extracellular matrix (ECM) network at the dermal–epidermal junction is important to connect the epidermis and dermis. The junction also provides structural support to the skin cells, keratinocytes, and fibroblasts for cellular renewal and regeneration (Roig-Rosello & Rousselle 2020). However, the normal skin biological and physiological structure is affected by intrinsic and extrinsic factors and change gradually with aging (Wang & Dreesen 2018).

Aging is a major factor that affects wound healing, where normal wound healing is impaired in aging and photo-aged skin. Moreover, diseases such as diabetes influence wound healing, and these diseases are prevalent among the elderly. Impaired wound healing is characterised by the overexpression of proteolytic enzymes such as elastase and matrix metalloproteinase, the abundance of disorganised and degraded collagen fibres, reduced collagen synthesis, and diminished expression of wound healing mediators such as growth factors, chemokines, and cytokines (Gosain & Dipietro 2004). Consequently, skin cell proliferation, migration, and maturation decrease and result in delayed re-epithelialisation and impaired wound contraction (Gosain & Dipietro 2004). Thus, the supplementation of wound healing mediators and ECM components may be a potential treatment strategy for age-related impaired wound healing.

Growth factors, cytokines, and chemokines are specialised multifunctional proteins that play a vital role in promoting skin cell proliferation and migration and stimulating collagen formation (Spiekstra et al. 2007). In native skin tissue, fibroblasts are responsible for producing these factors, which include fibroblast growth factor-10 (FGF10), KGF, GM-CSF, and IL-6 and the ECM, which includes collagen type I and III, fibronectin, and laminin (Desjardins-Park, Foster & Longaker 2018). Fibroblasts secrete factors that affect keratinocyte growth and differentiation (Chowdhury, Aminuddin & Ruszymah 2012; Chowdhury et al. 2019). The development of cell culture technology facilitated dermal fibroblast cultivation in the laboratory, which led to fibroblast-secreted factors and ECM being collected in abundance.

Fibroblasts are typically cultured using 1:1 F12 medium:Dulbecco's modified Eagle's medium (F12:DMEM [FD]) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich, USA). Previously, we used serum-free medium to prepare serum-free FD (designated FM) and a defined medium containing growth supplements (EpiLife™ medium, Gibco, USA; designated KM). The collected dermal fibroblast conditioned medium was designated DFCM-FM and DFCM-KM, respectively (Chowdhury et al. 2019; Maarof et al. 2018). Serum-free fibroblast medium is a basal medium that contains essential amino acids, organic and inorganic salts, and buffers and does not contain growth factors. In contrast, EpiLife™, i.e., KM, is typically used for culturing keratinocytes. The growth supplements contained in KM are recombinant insulin-like growth factor-1, hydrocortisone, bovine transferrin, and human EGF, which are essential for enhancing cell proliferation or supporting rapid cell growth (Tsao, Walthall & Ham 1982). EGF and insulin are also essential for inducing protein secretion by fibroblasts (Lee, Yang & Park 2007; Xie et al. 2008).

Previously, we successfully evaluated dermal fibroblast secretory proteins in DFCM-FM and DFCM-KM. The conditioned medium facilitated *in vitro* re-epithelialisation by enhancing keratinocyte attachment and wound healing *in vitro* and *in vivo* (Chowdhury et al. 2019; Maarof et al. 2020, 2019, 2016). However, the effect of DFCM on skin cells from donors of different ages has not been evaluated. Thus, in this study, we used attachment efficiency, growth rate, and wound healing rate to determine the effect of DFCM on the *in vitro* re-epithelialisation of keratinocytes isolated from different age groups.

Materials and Methods

This study was approved by the Universiti Kebangsaan Malaysia Medical Research and Ethics committee (approval numbers UKM 1.5.3.5/244/FF-232-2013 and UKM FPR/4/244/FF-2015-204). The skin samples were collected as redundant tissue. Informed consent was obtained from the patients or their legal guardians before surgery.

Skin sample collection, processing, and culture

Redundant skin samples were obtained from consenting patients (age groups: ≥18–35 years, 36–54 years, ≥55 years; n = 3 per group). Table 1 lists the demographic data of the samples. The samples were processed within 24 h following surgery according to a protocol described elsewhere (Ishak et al. 2019). The skin was cleaned, washed with Dulbecco's phosphate-buffered saline (DPBS; Gibco), and cut into small pieces. Then, the skin was digested using 0.6% collagenase type I solution (Worthington, USA) in a shaker incubator for 4-5 h at 37 °C. Subsequently, the skin was treated with trypsin-EDTA (TE; Worthington) for 8-10 min. The digested sample containing both fibroblasts and keratinocytes was centrifuged and the pellet was suspended in co-culture medium ((FD (Gibco) containing 10% FBS (Gibco) and KM (Gibco)). The cells were seeded in 6-well plates (Greiner Bio-One, USA) and grown at 37 °C in a 5% CO_2 incubator. The culture medium was replaced every 2 days until the cells were 80-90% confluent.

TABLE 1. Demographic data of the skin samples collected for the study

Age Group	Patient	Age (Years)	Sex	Type of surgery
≥18 - 35 years	1	27	Female	Amputation
	2	34	Female	Abdominoplasty
	3	28	Male	Amputation
36 - 54 years	4	43	Female	Facelift
	5	45	Female	Facelift
	6	40	Female	Abdominoplasty
	7	55	Female	Abdominoplasty
≥55 years	8	65	Male	Amputation
	9	61	Female	Amputation

Separation of fibroblasts from co-culture

Differential trypsinisation was performed to remove or detach the fibroblasts when the co-cultured cells were 80–90% confluent according to a protocol described elsewhere (Maarof et al. 2020). The co-cultured cells were treated with TrypLE Select (TS; Gibco) for 3-5 min to detach the fibroblasts. The cell suspension was collected and centrifuged at 5000 rpm for 5 min. The cell pellet was suspended in FD+10% FBS and subcultured in T75 flasks (Greiner Bio-One) separately until passage 3 (P3). The keratinocytes remaining on the culture plates were washed with DPBS and supplemented with KM for further expansion.

Preparation of DFCM

To prepare DFCM according to the protocol described by Maarof et al. (2018), P3 human dermal fibroblasts

were cultured in FD+10% FBS (seeding density: 5000 cells/cm^2) until confluent. The waste medium was discarded and the cells were washed twice with DPBS to remove excess medium, then KM and FM were added to the fibroblast culture. The cells were incubated at 37 °C in 5% CO_2 atmosphere for 3 days and the waste medium was collected as DFCM and designated DFCM-KM or DFCM-FM, respectively. To avoid donor-to-donor variation, the DFCM was prepared using fibroblasts from three donors and pooled, and was used throughout the experiment.

KERATINOCYTE BIOLOGICAL PROPERTIES
CELL ATTACHMENT AND GROWTH RATES

P2 keratinocytes from the different age groups were trypsinised and seeded (seeding density: 1×10^4 cells/cm^2) on 12-well plates (Greiner Bio-One) to analyse cell attachment and proliferation. Three technical replicates per age group were tested per sample (n = 3). The cells in the control culture were supplemented with KM only. For the test sample, the DFCM was supplemented with KM in a 1:3 ratio. To evaluate cell attachment and growth, the keratinocytes were incubated at 37 °C in a 5% CO_2 incubator mounted on a Nikon A1R-A1 microscope (Tokyo, Japan) (Chowdhury et al. 2019; Maarof et al. 2020). Five random positions per well were selected and images were captured every 20 min for up to 72 h. At 24 h, the cells were counted manually to determine the concentration of adherent cells using equation (1):

$$\text{Concentration of adherent cells} = \frac{\text{Average cell count}}{\text{Area of the image in cm}^2} \quad (1)$$

The keratinocyte growth rate was evaluated by counting the cells at 24 h and 72 h. The growth rate was evaluated using equation (2):

$$\text{Growth rate } (h^{-1}) = \frac{\text{Ln (Cell concentration at 72 h/Cell concentration at 24 h)}}{(72\text{ h} - 24\text{ h})} \quad (2)$$

SCRATCH ASSAY

The scratch assay was performed to determine the rate at which the cells repopulated the scratch area. Confluent monolayer keratinocytes (P3) from each age group were scratched with a 10-µl pipette tip to create a wound area. After washing with DPBS, the cells were supplemented with culture medium with or without DFCM. Images were captured every 20 min for 48 h using a time-lapse imaging system (Maarof et al. 2020). Three technical replicates were performed per condition per sample (n = 6). The wound area was analysed using NIS Element AR 3.1 software and the healing rate under each condition was evaluated using equation (3):

$$\text{Rate of healing} = \frac{\text{Initial area } (\mu m^2) - \text{Final area } (\mu m^2)}{\text{Observation time (h)}} \quad (3)$$

STATISTICAL ANALYSIS

All quantitative data are presented as the mean ± standard error of the mean (SEM) and were analysed using the Statistical Package for the Social Sciences (SPSS, version 20.0). Statistical differences were evaluated using one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis for ≥3 groups. Differences were considered significant at $p \leq 0.05$.

RESULTS

EFFECT OF DFCM ON KERATINOCYTE ATTACHMENT

The keratinocytes from each age group were seeded at a density of 1×10^4 cells/cm^2 and cultured for 24 h in keratinocyte culture medium (control condition) or keratinocyte culture medium containing DFCM-KM and DFCM-FM at a 3:1 ratio to evaluate the keratinocyte attachment efficiency. In all culture conditions, the cell attachment efficiency decreased as the age increased (Figure 1). However, in comparison to the control, DFCM-KM enhanced keratinocyte attachment, which was 1.24, 1.27, and 1.32 times higher for the ≥18–35-, 36–54, and ≥55-year age groups, respectively. The DFCM-KM enhancement of keratinocyte attachment in the ≥55-year age group was significantly greater than that of the control. In contrast, DFCM-FM reduced the keratinocyte attachment efficiency in all age groups in comparison with the control and DFCM-KM.

GROWTH RATE

To investigate the keratinocyte growth properties, the cells were cultured for 9 days in the presence or absence of DFCM. The keratinocytes in each age group did not exhibit different growth rates in all culture conditions (Figure 2). DFCM-KM did not affect the keratinocyte growth rate. However, DFCM-FM significantly reduced the growth rate in all age groups as compared to DFCM-KM and the control, which was supported by the keratinocyte growth profile (Figure 3). In all age groups,

the keratinocyte concentration at all culture times was significantly lower with DFCM-FM than with DFCM-KM and the control. In contrast, DFCM-KM yielded a similar growth profile for all age groups compared to that of the control.

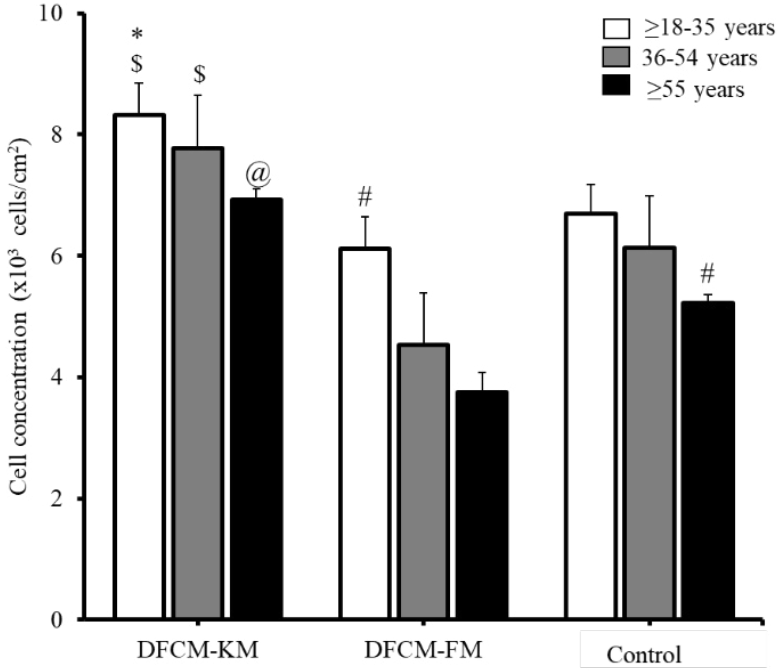

FIGURE 1. Efficiency of attachment of keratinocytes, isolated from different age groups, with or without supplementation of DFCM. Here, *, indicates significantly higher compare to ≥ 55 years in the same condition; #, indicates significant different between other donor age in the same condition; @, indicates significantly higher compared to DFCM-FM and control of same age group; and $, indicates significantly higher compare to DFCM-FM of same age group ($p \leq 0.05$); n=3

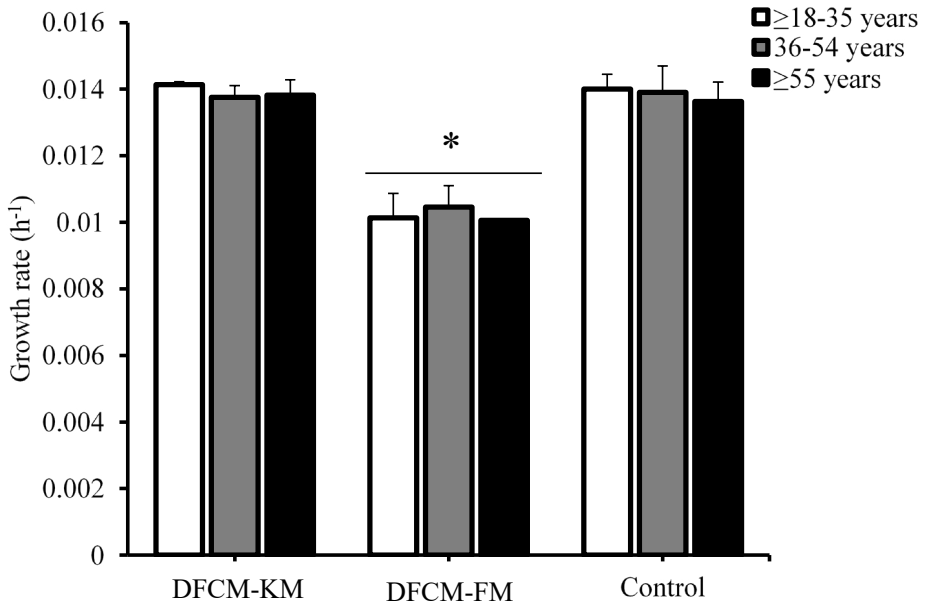

FIGURE 2. Growth rate of keratinocytes from different age groups with or without supplementation of DFCM. Supplementation of DFCM-KM had no effect on the growth rate of keratinocytes. However, supplementation of DFCM-FM significantly reduced the growth rate for all age groups compared to DFCM-KM and control. Here,*, indicates the significantly lower compare to DFCM-KM and control ($p \leq 0.05$); n=3

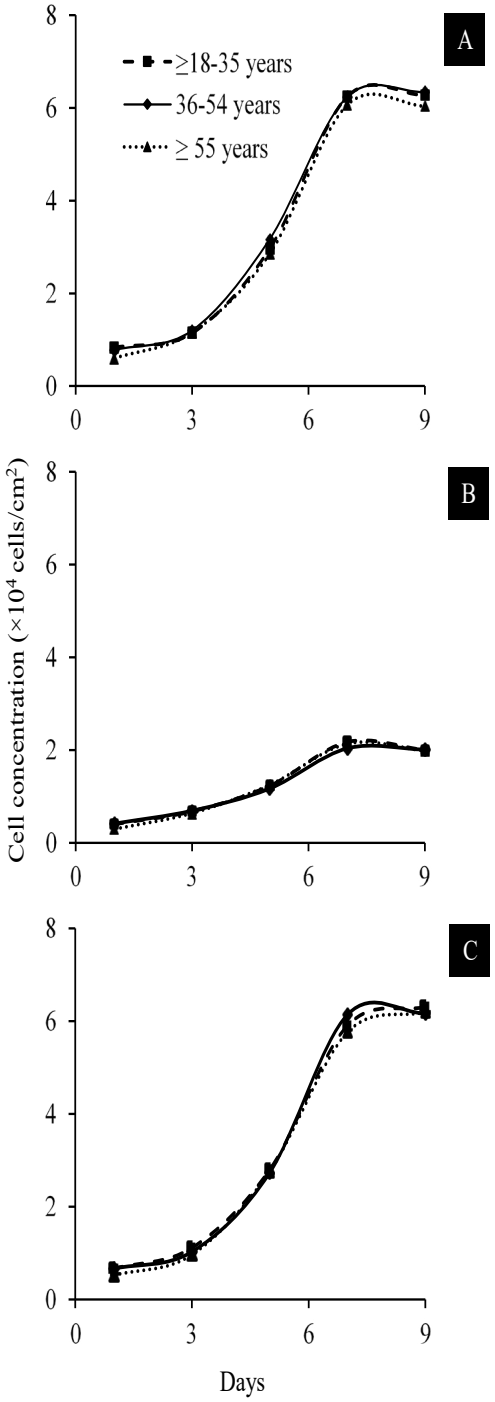

FIGURE 3. Growth profile of keratinocytes with or without DFCM for different age group samples (A: DFCM-KM, B: DFCM-FM, C: Control). The concentration of keratinocytes supplemented with DFCM-FM was significantly lower than DFCM-KM and control for all age groups at all culture time. In contrast, supplementation of DFCM-KM demonstrated a similar growth profile for all groups, as the control. n = 3

RATE OF HEALING

To investigate wound healing efficiency, a scratch was made on confluent keratinocytes and the scratch healing was observed via live imaging in the presence or absence of DFCM. In all age groups, DFCM-FM yielded a significantly higher healing rate compared to DFCM-KM and the control (Figure 4). However, the healing rates in the DFCM-KM and control were not significantly different. Nevertheless, the healing rate efficiency decreased as age increased in all culture conditions.

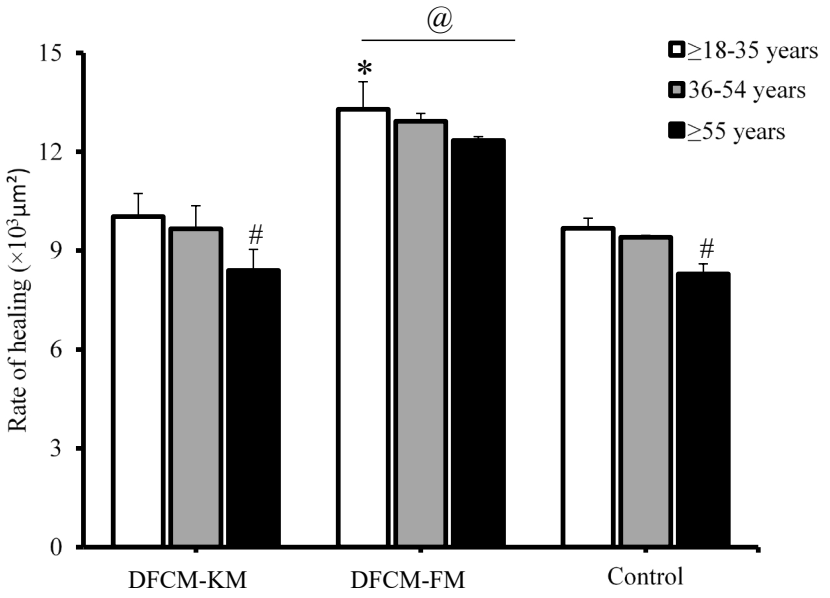

FIGURE 4. Rate of healing of keratinocytes from different age groups with or without supplementation of DFCM. Here, *, indicates significantly higher compare to ≥ 55 years in the same condition; #, indicates significant different between others donor age in the same condition; and @, indicates significantly higher compared to other conditions ($p \leq 0.05$), n = 3

DISCUSSION

Physiologically aged and photo-aged skin demonstrate structural and functional changes. In aging animals, the rate of healing was delayed by 20–60% as compared to that of a young animal (Gosain & Dipietro 2004). Aged skin typically experiences changes in the basal cell layer via decreased keratinocyte, fibroblast, and melanocyte proliferative ability. This process is termed cellular senescence, where the expression of the senescence marker β-galactosidase increases in aged skin as its levels are higher in senescent cells (Zhang & Duan 2018). Senescent cells are identified based on the loss of their proliferative ability, apoptosis resistance, and increased secretion factors that promote inflammation and tissue deterioration (He & Sharpless 2017). This cellular senescence is caused by the intrinsic and extrinsic factors typically associated with DNA damage, telomere shortening and dysfunction, or oncogenic stress (Wang & Dreesen 2018). In intrinsically aged skin, procollagen production and expression are reduced due to the downregulation of TGF-β–Smad signalling (Quan et al. 2010). In addition to collagen, the ECM components (elastin, fibril, oligosaccharides) are decreased (Naylor, Watson & Sherratt 2011). With these metabolic changes that occur with aging, it is reasonable to expect that older adults have a high risk of developing chronic or impaired wound healing (Alam, Hasson & Reed 2021).

Multiple studies have demonstrated the beneficial effect of growth factors in the healing process in both animal models and patients with different wound healing disorders (Werner & Grose 2003). Considering the complexity of the healing process and the involvement of multiple factors, supplementation with CM, which contains myriad amounts of wound healing mediators,

was recently tested and significantly improved healing (Jayaraman et al. 2013). Previously, we demonstrated enhanced *in vitro* re-epithelialisation via DFCM supplementation (Chowdhury, Aminuddin & Ruszymah 2012; Maarof et al. 2020). In the present study, we demonstrated that DFCM enhanced keratinocyte attachment and wound healing *in vitro* irrespective of donor age.

Aging reduces keratinocyte proliferation and migration *in vivo* (Chowdhury et al. 2019; Gosain & Dipietro 2004). As cells have a limited life span, it was expected that aging would affect the keratinocyte properties during *in vitro* culture. However, our results demonstrated that donor age had no effect on the keratinocyte growth rate. On the contrary, the keratinocyte attachment and healing rate decreased with the increase in donor age. Compared with the control, the DFCM modulation of keratinocyte properties was independent of donor age, which indicated that DFCM may be a possible supplement for enhancing wound healing efficiency in patients of all ages.

Our results demonstrated that compared to the control, DFCM-KM increased keratinocyte attachment while DFCM-FM decreased it irrespective of donor age. In contrast, irrespective of donor age, DFCM-FM reduced the keratinocyte growth rate as compared to the control, while DFCM-KM did not alter the keratinocyte growth rate. The decreased growth rate following DFCM-FM supplementation was due to the reduction of keratinocyte proliferative potential and the induction of keratinocyte differentiation (Chowdhury et al. 2019). The attachment and growth of many cell types are dependent on secretory factors and ECM components. While some cells, such as fibroblasts, can synthesise these components, other cells require an exogenous source, particularly when grown in serum-free culture (Smola, Thiekötter & Fusenig 1993).

Compared to the control, DFCM-FM enhanced the keratinocyte healing rate while no change was observed for DFCM-KM irrespective of donor age. The healing process is influenced by factors present in the local wound environment, such as the ECM component composition and cytokines, chemokines, and growth factors (Fivenson et al. 1997; Metcalfe & Ferguson 2007; Riha, Maarof & Fauzi 2021). Fibroblast-secreted factors are essential for promoting keratinocyte migration during wound healing (Maarof et al. 2020, 2018; Werner & Grose 2003). Here, DFCM-FM supplementation resulted in keratinocyte morphological changes and keratinocytes demonstrated collective migration during healing due to the presence of calcium or other small molecules. Collective migration is essential in cellular biological processes and wound healing (Shim, Devenport & Cohen 2021). The collective migration of keratinocytes was responsible for the faster healing in the DFCM-FM group as compared to the single cell migration in the DFCM-KM and control groups. However, further study is required to identify the specific components in DFCM-FM and their influence on regulating the migratory property of keratinocytes during healing.

Our results demonstrated the comparable beneficial effect of DFCM on *in vitro* re-epithelialisation in samples from all ages. However, the inability to achieve any distinct difference in keratinocyte properties during culture rendered it difficult to derive a clear conclusion. The challenges and limitations of this study are the difficulties in obtaining samples from older age groups (≥55 years) and inconsistent cell performance. Moreover, the keratinocytes were only supplemented with 25% DFCM without standardisation of the protein concentration in each batch production of DFCM. Therefore, considering future clinical applications, lyophilised DFCM or concentrated DFCM would be preferable to determine the optimal dose for a better outcome.

Conclusions

Irrespective of donor age, DFCM-KM enhanced keratinocyte attachment while DFCM-FM enhanced the keratinocyte healing rate, which indicated the potential application of DFCM for impaired re-epithelialisation or wound healing, especially in the elderly. Further investigation of soluble factors and their signalling pathways would provide valuable information that will improve knowledge on the interaction between DFCM and cells from different age groups.

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*Corresponding author; email: manira@ukm.edu.my