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From Molecular Characterization to Clinical Validation: Unveiling – New Science of Skin Glycation and the Skin Lightening Effect of Dimethylmethoxy Chromanol Mediated *via* an Anti-Glycation Mechanism

Ka-Heng Lee, Yi-Peng Ng, Ursula Rho-Wan Chong, Kah-Huong Chong, Fang-Yee Yeo, Yi-Fan Teah, Sek-Yan Tan, Yeak-Fong Chen, Norsyariza Abd-Aziz, Khan-Loon Ng, Mong-Sah Toh
Wipro Skin Research & Innovation Centre, No. 7, Persiaran Subang Permai, Taman Perindustrian Subang, 47610 Subang Jaya, Selangor, Malaysia

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Abstract

Skin glycation is a nonenzymatic reaction of a sugar molecule and the functional group of a protein to form an advanced glycation end product, which is one of the considerable factors involved in accelerating skin aging intrinsically. The aim of this study was to develop an in vitro skin glycation model to characterize the impact of glycation stress on skin physiology and further to be used for identifying potent antiglycation molecules. As a result, a new skin model based on the presence of N^{ε}-(carboxylmethy)lysine as glycation biomarker in glyoxal-challenged human reconstituted skin was developed. From microarray profiling, skin glycation was found to affect multiple skin biological activities, including epidermis keratinization, skin lipid degradation, dermis extracellular matrix and hemidesmosome disassembly, the trigger point for skin oxidative stress and inflammatory responses. Intriguingly, skin glycation was shown to be highly correlated with skin darkening without involvement of melanocyte activity. We could show that dimethylmethoxy chromanol demonstrated potent antiglycation activity even through it was known for its antioxidant property. However, it is highly reactive and prone to be degraded in formulations. Therefore, a nanoemulsion formulation was designed to improve its stability and its topical anti-glycation activity was validated subsequently. Further, a clinical trial with a dimethylmethoxy chromanol encapsulated nanoemulsion demonstrated significant improvement in skin complexion (ITA°), reduction of skin redness and antihyperpigmentation efficacy. In summary, a profound understanding of skin glycation stress at the molecular level was established and it is also highly associated with skin darkening. In addition, dimethylmethoxy chromanol could be used as the lead molecule in cosmeceutical applications to further improve overall skin complexion and hyperpigmentation via its antiglycation effects.

INTRODUCTION

Glycation is a nonenzymatic reaction of a sugar molecule and functional group of a protein to form an advanced glycation end product (AGE) which has been linked to the development of various age-related disorders [1]. Interestingly, glycation is highly associated with a skin aging process in which elevated levels of N^{ε}-(carboxylmethy)lysine (nonfluorescent AGE) and pentosidine (fluorescent AGE) were found to correlate with an older age group in both diabetic and nondiabetic patients [2]. Moreover, a recent large-scale population study comparing the skin AGE levels of both Caucasian and Asian skin showed that the skin AGE levels of both populations correlated well with age [3]. With increasing evidence of a link between skin glycation and skin aging a strong interest has developed in exploring the role of glycation in skin at the molecular level.

A recent review by *Pageon et al.*, (2017) highlighted the critical role of glycation stress on acceleration of skin aging, whereby most of the work was based on results from monolayer cells and findings on molecular changes in intact skin was rather limited [4]. In addition, identification of topical lead compounds with an anti-glycation property has been of interest in the personal care industry. Therefore, the objective of this study was to develop a skin glycation model to facilitate characterization of molecular changes by stimulating actual human skin and study the topical anti-glycation activity of a for-

mula. Ultimately, we aimed to develop a cosmeceutical formula with potent topical anti-glycation activity that helps to improve overall skin health.

EXPERIMENTAL

Human reconstituted skin culture and treatment

Human reconstituted full-thickness skin (EFT-400) was cultured as per the manufacturer's guidelines (Mattek, Ashland, Massachusetts, USA). Preparation of skin glycation was carried out according to the previously described protocol [5]. Briefly, EFT-400 skin was treated with culture media containing 1 mM of glyoxal and cultured at 37 °C, 5 % CO_2 for 3 days, whereby the glyoxal media was refreshed every day. For the control group, the same culture conditions were applied but normal culture media without glyoxal was used. For the formula efficacy validation experiments, EFT-400 skin tissues were challenged with glyoxal and treated topically with formula (four hours per day) for three consecutive days concomitantly. Lastly, treated skin tissues were harvested for histological analysis, skin tone measurement and DNA extraction for microarray gene profiling.

RNA extraction and quality check

Total RNA of the skin tissues was extracted using a Total NucleoSpin RNAII isolation kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). RNA quantity and quality were assessed spectrophotometrically (BioSpec-Mini, Shimadzu, Kyoto, Japan) and RNA integrity was assessed using a Bioanalyzer 2100 (Agilent, Santa Clara, California, USA). Isolated RNAs were stored at -80 °C prior to microarray analysis.

RNA labeling and microarray hybridization

RNA labeling and microarray hybridization were performed according the recommended protocol of GeneChip® Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, California, USA). Briefly, 100 ng of purified RNA were reverse transcribed to create a double-stranded cDNA template. This was followed by cDNA amplification *via in vitro* transcription (IVT) to produce cRNA.Then, IVT-generated cRNA was further purified and subjected to a second cycle single-stranded sense cDNA synthesis which was later fragmented, labeled, and hybridized to a GeneChip® array for 16 hours at 45 °C with rotation at 60 rpm. After being washed, the hybridized arrays were scanned using a GeneChip® 3000 7G Scanner (Affymetrix, Santa Clara, California, USA).

Microarray and gene ontology enrichment analysis

Microarray data were extracted and analyzed using Transcriptome Analysis Console (TAC) Software v3.0 (Affymetrix, Santa Clara, California USA). Differentially expressed mRNAs were established through fold-change filtering by setting fold change at ±4 with 1-way ANOVA statistically significant (p-value < 0.05). The generated list of differentially expressed genes (DEGs) was subjected to Gene Ontology (GO) Enrichment analysis using the open source DAVID Bioinformatics database [6-7].

Immunohistochemistry analysis of CML and carbonyl protein

The treated skin tissues were fixed in paraformaldehyde (Merck, Darmstadt, Germany) and embedded in paraffin. This was followed by sectioning at 15 μm for histology analysis. The section was stained with hematoxylin and eosin (H&E) for general morphological analysis. For immunohistochemical analysis, the goat anti-N^{ε}-(carboxymethyl)lysine polyclonal antibody (Cell Biolabs Inc., San Diego; California, Usa) was used as the primary antibody, followed by biotin-XX rabbit anti-goat IgG (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Alexa Fluor-488 Streptavidin (Thermo Fisher Scientific, USA) as secondary antibodies. The cell nuclei were counterstained with DAPI (Nacalai Tesque, Kyoto, Japan). For carbonyl protein detection, immunostaining was conducted following the protocol recommendation from the OxyIHC Oxidative Stress Detection Kit (Merck, Darmstadt, Germany). The immunostained specimens were viewed under an Eclipse 50i fluorescence microscope (Nikon, Tokyo, Japan) and the expression level was analyzed using NIS-Elements software version 3.2 (Nikon, Tokyo, Japan).

Nanoemulsion preparation

The nanoemulsion containing dimethylmethoxy chromanol (Lipotech, Barcelona, Spain) was prepared using a high pressure homogenizer (Microfluidics, Westwood, Massachusetts, USA). The particle size and zeta potential of the nanoemulsion were measured with a Zetasizer Nano (Malvern Instrument, Worcestershire, UK). Then the nanoemulsion was compounded into a formulation and subjected to the *in vitro* skin anti-glycation test and a clinical trial.

HPLC analysis of dimethylmethoxy chromanol

The dimethylmethoxy chromanol (DMMC) in formulation was extracted by a liquid extraction method with methanol and water. Then the clean-up samples were subjected to HPLC analysis (Waters, Milford, Massachusetts, USA) using C-18 column (Merck, Darmstadt, Germany) and acetonitrile/water as the eluent with a detection wavelength of DMMC at 295 nm. A peak high of the standard compound concentration calibration was used for absolute quantification of the active in the formulation.

Clinical trial

A single blinded clinical study was conducted in a group of 11 healthy females aged from 40 to 50 with inclusion of dull skin tone and obvious facial skin hyperpigmentation (dark spot). The product was applied to the face twice daily for a duration of 60 days. Then skin tone and erythema index were measured by a Chromameter CR400 (Konica Minolta Sensing Inc., Tokyo, Japan) and a Mexameter MX18 (Courage-Khazaka Electronic GmbH, Köln, Germany), respectively. The images of the volunteer were acquired using a VISIA Complexion Analysis System CR2.2 (Canfield Scientific, Parsippany, New Jersey, USA).

Statistical Analysis

For *in vitro* experiments, results were expressed as the mean ± standard error of the mean (S.E.M.) of two to three independent experiments with each experiment performed at least in duplicate and statistical analyses were performed either using Student's t-test or one ANOVA followed by Dunnett's multiple group comparison

test. For the active recovery test, results were expressed as the mean ± standard deviation (S.D.) of the single experiment and statistical analyses were performed using two-way ANOVA followed by Dunnett's multiple group comparison test. For the clinical trial, results were expressed as the mean ± S.E.M. of total volunteers (n=11) and statistical analyses were performed using the Wilcoxon Matched Pairs Signed Rank Test. For all experiments, statistical significance of differences between group was accepted at a p value < 0.05.

RESULTS AND DISCUSSION

Establishment of a new glycated skin model

In this experimental skin model glyoxal was shown to be a potent accelerator to induce skin glycation based on the presence of N^{ε}-(carboxymethyl)lysine (CML) as the prominent glycation biomarker in skin [8]. From the immunohistochemistry analysis ***(Figure 1)***, CML was found mainly localized in the epidermis, where anti-CML (green fluorescent) stained strongly from the basal to granular layer as well as in the cytoplasm of fibroblasts, but stained weakly in the extracellular matrix protein in dermis, closely resembling the *in vivo* staining of skin advanced glycation end products (AGEs) [8-9]. It was previously reported that intracellular intermediate filament proteins, such as cytokeratin-10 in keratinocytes and vimentin in fibroblasts, are major targets for glycation modification, which was further supported by our observation of the specific CML localization in skin [8, 10]. The extracellular matrix in the dermis layer was found less susceptible to the glycation process which aligned with results of an earlier study that demonstrated a higher rate of AGE accumulation intracellularly than in extracellular matrix components [11]. Despite signs of epidermal detachment from the dermis layer in glyoxal-treated skin, no significant cytotoxicity effect was observed based on the MTT and Alamar Blue assay [5]. Taken together, these results show that a new glycated skin model was developed to facilitate the molecular characterization of skin glycation.

Association of skin glycation and skin darkening

Interestingly, the macroscopic view of glycated skin revealed a yellowish skin tone compared with normal skin ***(Figure 1A)***, which was validated quantitatively by an increase in darkening skin based on a reduction in the ITA° value and an increase in the b-value ***(Figure 1B)***. This observation was in agreement with a study conducted by *Yokota et al*. (2016), which also showed the appearance of a yellow tone in the glyceraldehyde-induced hairless mouse skin model concomitantly with an increase in the AGE level [9]. Compared with a previous study, which demonstrated a smaller b-value increase in an acellular dermis model while treating skin with a relatively high concentration of glyoxal (10 mM), our skin model showed better efficiency in inducing development of a yellowish tone. This can be explained by the presence of full-thickness skin including the epidermis layer where a relatively high abundance of CML was observed compared with the dermis layer [12]. Besides, there was an increasing trend of carbonyl protein found in glycated skin that could result in reduction of light transmission of the stratum corneum that further reduced the skin transparency [13]. Nevertheless, AGE protein was also shown to promote melanogenesis in human skin explants *via* interaction of surface receptors of AGE (RAGE) and activation of the CREB-MITF-tyrosinase pathway in the melanocyte cell [14]. However, this is actually the first time that an association of skin glycation with development of skin darkening without involvement of melanocytes could be demonstrated, as the developed glycated skin model was devoid of melanocytes. Taken together, it would be of great interest to further elucidate the exact mechanism of skin darkening mediated by skin glycation.

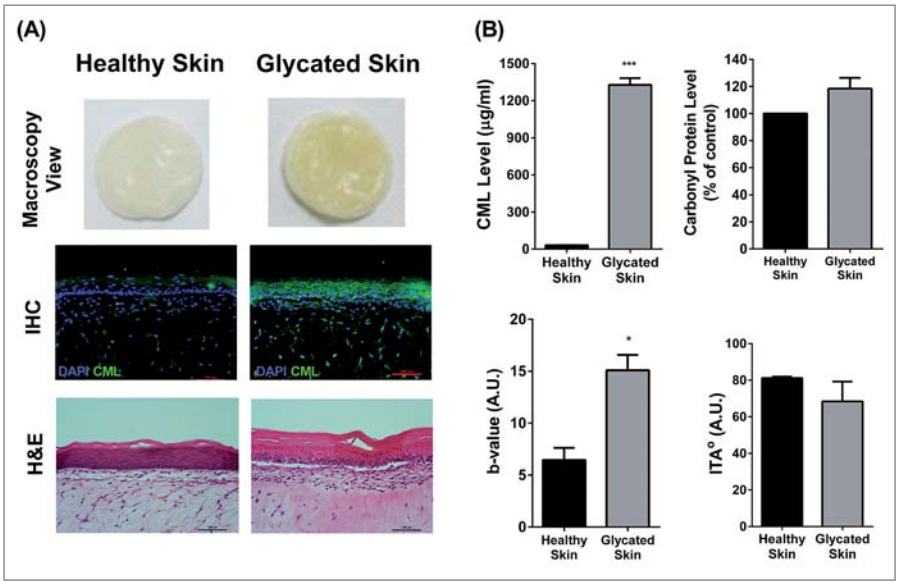

Figure 1 Establishment of *in vitro* human skin glycation model.
(A) Macroscopic view of healthy reconstituted human skin and glyoxal-induced glycated skin, general morphology by H&E staining and CML level by immunohistochemistry analysis. (B) Effects of skin glycation on CML and carbonyl protein level as well as development of skin darkening reflected from increase of b- value and reduction of ITA°. Data are expressed as mean ± S.E.M. of two independent experiments (n=2). *P<0.05, ***P<0.001, significant difference between healthy and glycated skin. The figure was partly adopted and modified from *Lee. et al.,* (2017) [5].

Acceleration of skin aging by glycation

In order to understand the molecular changes of skin glycation, a microarray was performed and 283 genes were found to be differentially regulated (98 genes upregulated and 185 genes downregulated) compared with normal skin. Using principle component analysis (PCA), one healthy skin control was identified as an outlier and excluded from the gene profiling analysis ***(Figure 2A)***. The mRNA expression profiling was displayed in a heat map, whereby hierarchical clus-

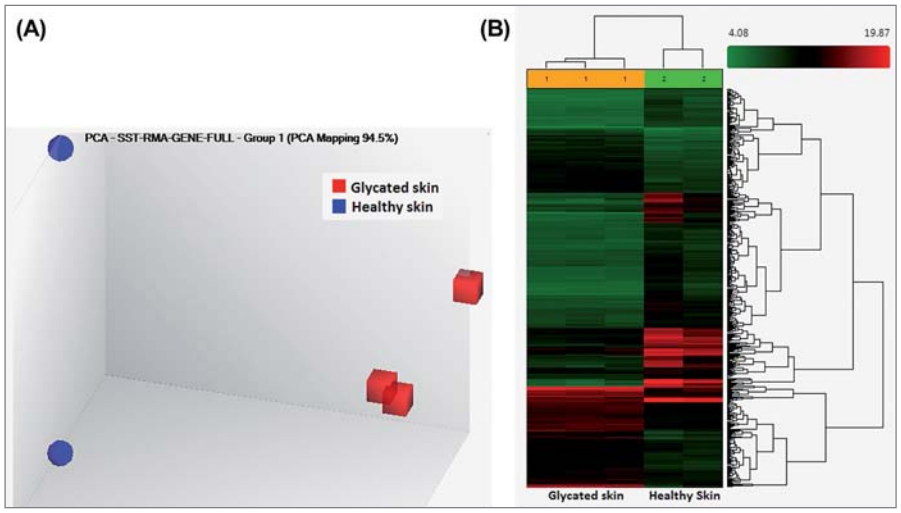

Figure 2 Molecular characterization and gene expression profiling of skin glycation. (A) Comparison of the principal component analysis of mRNA expression profiles of healthy and glycated skin. (B) Heat map and hierarchical clustering of the differentially expressed genes (DEGs) of healthy skin and glycated skin using Transcriptome Analysis Console (TAC) software. All data are derived from three independent experiments (n = 3) with exclusion of one outlier in the healthy skin group.

tering clearly separated the glycated from and control skin ***(Figure 2B)***. The differentially expressed genes (DEGs) were further classified based on a biological process (BP) and gene ontology (GO) enrichment analysis, which found that the DEGs of skin glycation related to the biological process of epidermis keratinization, skin lipid degradation, extracellular matrix disassembly, hemidesmosome disassembly, trigger point of skin oxidative stress and inflammatory responses ***(Table I)***.

Epidermal keratinization was the most prominent biological process induced by glycation stress, with the majority of critical epidermal structural proteins, including filaggrin, involucrin, transglutaminase-1 and various isoform of small proline rich proteins, being dramatically downregulated. The downregulated structural proteins are mainly linked with immature cornified envelop development and led to impairment of skin barrier function [15]. Previous studies have also confirmed impairment of skin barrier function in glycated skin, which was reflected by an increase in transepidermal water lost (TEWL) and a high permeability of hydrophilic compounds in glycated skin [16-17]. In addition, glycation induced upregulation of various forms of matrix metalloproteinases genes (MMP-3, MMP-8 and MMP-10) and downregulation of hemidesmosome genes (COL17A1, ITGA6 and ITGB4) that favor dermis extracellular matrix disassembly, all of which are hallmarks of skin aging. Moreover, the downregulation of hemidesmosome genes that was found had weakened the functionality of the dermal-epidermal junction, which could

Table I Gene Ontology (GO) Analysis for the Genes Differentially Regulated in Glycated *versus* Healthy Skin.

CLUSTERS	GO TERMS (BIOLOGICAL PROCESS)	ENRICHMENT SCORE	REGULATED GENES
Keratinization and epidermis development	GO:0031424 GO:0030216 GO:0008544 GO:0018149	12.56	ABCA12, S100A7, BVES, CDH3, CASP14, CERS3, COL17A1, CNFN, CSTA, DSP, FABP5, FLG2, FLG, GRHL1, HNRNPM, IFFO2, IVL, JUP, KRT1, KRT10, KRT14, KRT16, KRT17, KRT2, KRT5, KRT6A, KRT6C, KRT75, LCE3C, LCE3D, LCE3E, MAP2, SCEL, PPL, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR3D, SPRR2E, SPRR2F, SFN, TP63, TGM1
Hemidesmosome assembly	GO:0031581	4.81	LYPD3, COL17A1, DSP, EPPK1, EXPH5, ITGA6, ITGB4, JUP, KRT14, KRT5,
Protease inhibitor	GO:0010951	3.43	RAB5C, A2ML1, ARG1, CSTA, IL1B, PANX1, PANX1, PI3, SLPI, SPINK5, SERPINB12, SERPINB13, SERPINB3, SERPINB4, SERPINB5, TFP12
Desmosomal cadherins and cell-cell adhesion	GO:0007156	2.07	CDH3, DSC1, DSC3, DSG1, DSG2, MPZL2, PCDH18
Regulation of inflammatory response	GO:0006955 GO:0006954	1.69	CCL2, CXCL3, CXCL5, CD36, S100A8, S100A9, TNFAIP6, TNFRSF10D, CSF3, DEFB1, IL1B, IL1RL1, IL18, IL24, IL7R, ILF2, KRT16, RIPK2, SLPI
Lipid digestion and degradation	GO:0044241	1.51	SENP1, CPA4, LIPK, LIPM, LIPN, PLA2G4D, PLA3G4E, TMPRSS11D, TMPRSS11F
Oxidation-reduction process	GO:0055114	1.38	NQO1, STEAP1, ADH1B, AKR1B1, ALOX12B, CP, CYP4F22, DUOX1, EGLN3, PTGIS, RDH13, RRM2B, SCD, TXNRD1, TDO2
Proteolysis, extracellular matrix disassembly	GO:0022617	1.27	SENP1, ANPEP, CAPNS2, CPA4, CASP14, CLCA2, DCN, MMP10, MMP3, MMP8, NRIP3, TMPRSS11D, TMPRSS11F

Up-regulated gene, down-regulated gene

explain an earlier observation of morphology defeat (epidermal detachment from dermis) in glyoxal-treated skin.

It is known that glycation can trigger skin inflammatory responses *via* interaction with the receptor for advanced glycation end products (RAGE) [18]. In the present study an inflammatory response was triggered in glycated skin and dramatic elevation of related inflammatory gene expression (CCL2, CXCL5, IL-24, TNFAIP6 and IL-1B) was evident. In line with this finding a recent study revealed that AGEs stimulate the production of pro-IL-1B *via* activation of NLRP3 inflammasome signaling in human placenta [19]. Moreover, the result of elevated CCL-2 expression (also known as MCP-1) was in accordance with an investigation conducted by *Pageon et al.* (2015) that had confirmed the increase in MCP-1 secretion in a human reconstituted skin model constructed from CML or glycoxidized pentosidine-rich collagen matrix [20]. In addition, MCP-1 secretion in glycated skin was previously reported [5]. We hypothesized that the glycation-driven inflammatory response could be an upstream factor which partly contributes to skin hyperpigmentation, specifically an increase in MCP-1 for inflammatory macrophage recruitment leading to post-inflammatory hyperpigmentation.

Anti-glycation activity of encapsulated DMMC in a nanoemulsion (En-DMMC) formula

Dimethylmethoxy chromanol (DMMC) is a powerful antioxidant that is widely used in cosmetic applications due to its strong capability to scavenge various radical species, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) [21, 22]. In addition to its radical scavenging property, we discovered that DMMC also exhibits potent, dose-dependent glyoxal scavenging and topical anti-glycation activity (data not shown). Despite its promising anti-glycation activity, DMMC is highly reactive and easily oxidized or degraded. Thus, the efficacy of DMMC may be diminished upon product aging and severe product discoloration occur. To address the instability of DMMC, a nanoemulsion was designed to encapsulate DMMC to improve its stability with an average particle size of 120 nm and zeta potential range of -69mV ***(Figure 3)***. HPLC analysis showed the non-encapsulated DMMC in the control formula to be degraded in a time-dependent manner under accelerated stability conditions at an elevated temperature (45 °C) and a significant improvement in the active stability of the encapsulated DMMC in the nanoemulsion formulation (En-

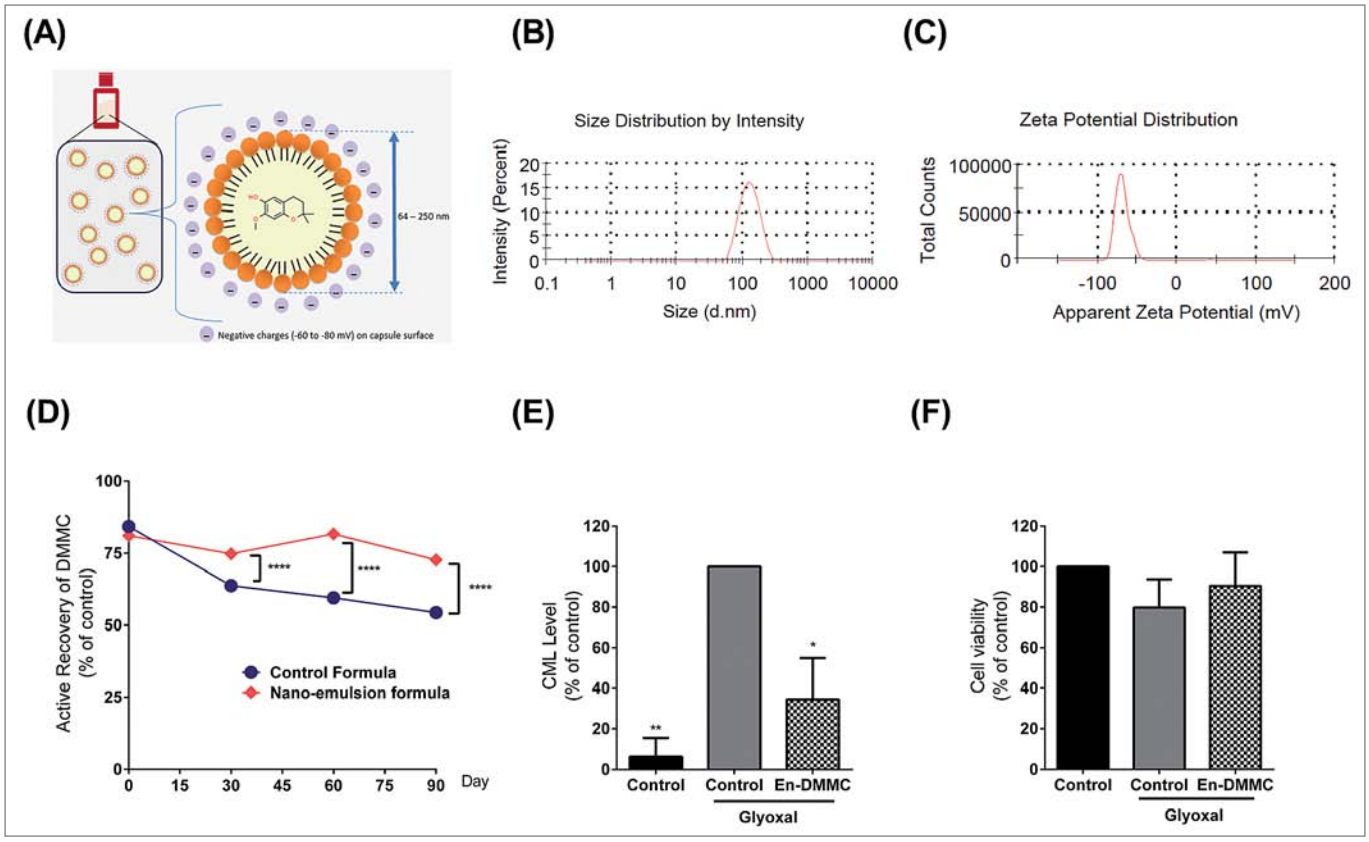

Figure 3 Development and anti-glycation activity of stabilized dimethylmethoxy chromanol in nano-emulsion formulation.
(A) Illustration of the encapsulated dimethylmethoxy chromanol (en-DMMC) nanoemulsion formula. (B) Particle Size and (C) Zeta potential distribution of the en-DMMC nanoemulsion measured by Zetasizer. (D) Stability and recovery of DMMC in the control formula and nanoemulsion formulation evaluated by HPLC analysis. Data are expressed as the mean ± S.D of duplicates (n = 2). ****P < 0.0001, significant difference between control and en-DMMC formula. (E) Anti-glycation effects of the stabilized en-DMMC nanoemulsion formulation. (F) Effects of various treatments on tissue viability by MTT assay. Data are expressed as the mean ± S.E.M. of two independent experiments (n = 2). *P < 0.05, **P < 0.01, significantly different from group of glyoxal-induced skin (second column).

DMMC) relative to that of the DMMC in the control formula. Lastly, the topical anti-glycation activity of the En-DMMC formula was further subjected to validation using the developed skin glycated model. The result showed that En-DMMC significantly attenuated glyoxal-induced CML formation in the skin glycated model. Moreover, the false positive finding of its anti-glycation activity was eliminated, as a high tissue viability (>80 %) was observed in all treated skin.

Clinical skin lightening and anti-hyperpigmentation efficacy of the En-DMMC formula

In view of the strong association of skin darkening and glycation stress, it was of interest to investigate the skin lightening efficacy of En-DMMC formula, as its topical anti-glycation activity had been confirmed. As expected, En-DMMC showed progressive improvement of skin tone, which was reflected by the increase in ITA° throughout the treatment duration *(Figure 4)*. Interestingly, the decreasing trend of yellowish skin tone based on the b-value (Δb-value of 0.27 between D0 and D60, p value of 0.123) was observed, although statistically insignificant. We believed that one of the primary skin lightening activities of the En-DMMC formulation was mediated *via* its anti-glycation activity and complemented by its free radical scavenging activity to prevent further development of skin darkening. One of the interesting observations was the reduction of skin redness, whereby the erythema index and a-value were significantly reduced after En-DMMC treatment. Moreover, a significant improvement in facial dark spots was also evident. A recent investigation revealed a high level of dermis CML in lesioned areas of solar lentigo [23]. Therefore, it is not surprising that the anti-glycation activity of En-DMMC plays a vital role in diminishing facial hyperpigmentation. Taken together, the En-DMMC formula may exert its anti-inflammatory and anti-hyperpigmentation activity indirectly, at least partly mediated *via* suppression of skin glycation. Although the present clinical trial focuses on skin lightening performance, noticeable improvement in the nasolabial fold of selected volunteers was observed (data not shown), showing that the anti-aging effects of En-DMMC can be another potential area to be explored in future investigations.

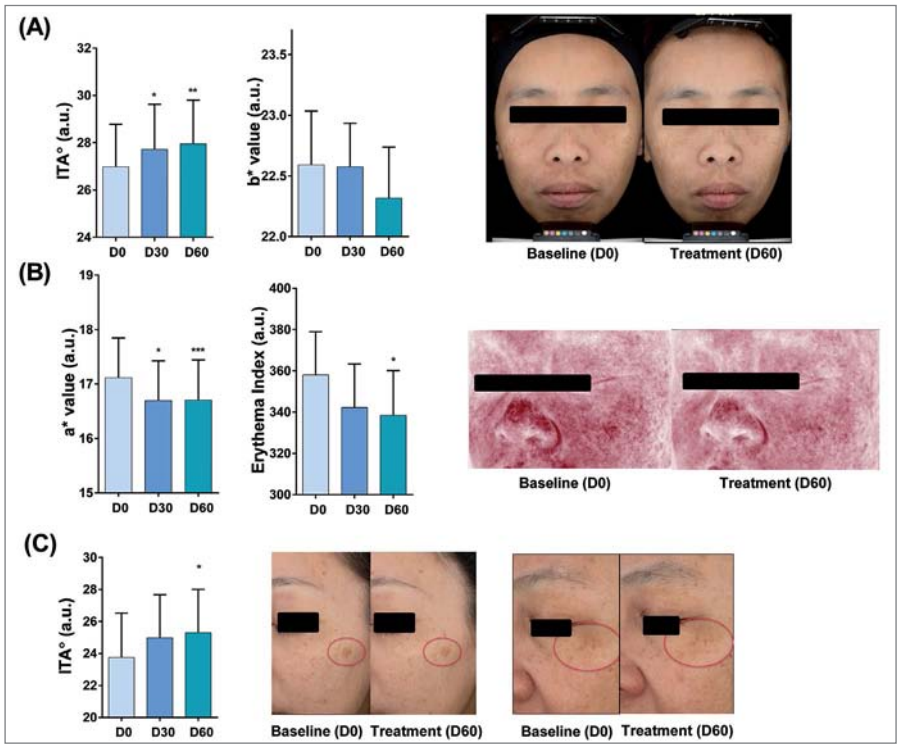

Figure 4 Clinical trial of the stabilized dimethylmethoxy chromanol nano-emulsion formula. (A) Improvement in overall skin tone based on measurement of the ITA° and b-value, respectively. (B) Improvement in skin redness based on measurement of the a-value and erythema index, respectively. (C) Improvement in skin hyperpigmentation based on measurement of the ITA° after 2 months of product usage. Data are expressed as mean ± S.E.M. of total volunteer (n = 11), *P < 0.05, ** P < 0.01.

CONCLUSION

In summary, we developed a human reconstituted skin glycation model which provides a new technology dimension for understanding the skin glycation and topical anti-glycation activity of a cosmeceutical formula. In addition, an in-depth understanding of skin glycation stress was obtained at the gene level in regulation of the biological process related to epidermal skin barrier impairment, dermis extracellular matrix and hemidesmosome disassembly, triggering of skin inflammatory response and skin oxidative stress. Moreover, glycation was found to be one of the leading factors in inducing skin darkening without direct involvement of the melanogenesis pathway. Lastly, a DMMC encapsulated nanoemulsion was developed and shows potential for use in cosmeceutical applications for overall skin complexion improvement and anti-hyperpigmentation *via* its anti-glycation activity as one of the primary mechanisms.

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Corresponding Author
Lee Ka Heng, PhD
Wipro Skin Research
and Innovation Centre
Subang Jaya
Selangor Darul Ehsan
Malaysia
lee.kaheng@wipro-unza.com