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# Novel Facial Cream Containing Carnosine Inhibits Formation of Advanced Glycation End-Products in Human Skin

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**Keywords**
Advanced glycation end-products · Glycation · Skin aging · Anti-aging · Carnosine · Facial cream

**Abstract**
***Background:*** Accumulation of advanced glycation end-products (AGEs) in skin has been associated with skin aging. Inhibition of glycation of proteins of extracellular matrix may help skin texture and appearance. The objective of the study was to demonstrate the antiglycation activity of topically applied carnosine and novel facial cream (FC) containing carnosine in human skin explants ex vivo. ***Methods:*** Glycation was induced in human skin explants by methylglyoxal (MG) in culture media. FC containing carnosine (FC-CARN) or carnosine in aqueous solution (AQ-CARN) was applied topically on skin explants. Levels of AGEs carboxymethyl-lysine (CML) and pentosidine were determined in the epidermis and dermis of skin sections and were used to calculate antiglycation activity. ***Results:*** Exposure to MG led to increases in CML and pentosidine in skin explants. Antiglycation effect for AQ-CARN was CML: –64 and –41%, pentosidine: –48 and 42% in epidermis and reticular dermis respectively. Antiglycation effect for FC-CARN was CML: –150 and –122%, pentosidine: –108 and –136%, in epidermis and reticular dermis respectively. ***Conclusion:*** Topically applied carnosine protects against the glycation induced by MG. Novel FC-CARN significantly reduced levels of AGEs in both epidermis and reticular dermis in human skin explants.



## Introduction

Skin aging is a complex, chronic process spanning several years where external and intrinsic factors engage and interact to create diverse macroscopic, microscopic and biochemical changes that differ in extent inter-individually. Over the years, many theories have been proposed to explain the underlying causality of skin aging. However, the general consensus today is that mechanisms such as oxidative stress through free radical action, inflammatory processes, cellular senescence and epigenetic changes are non-mutually exclusive mechanisms that are part of skin aging [1].

Glycation, the non-enzymatic reaction between proteins and sugars leading to the formation and subsequent accumulation of advanced glycation end-products

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(AGEs) has been implicated in micro- and macrovascular complications in diabetes [2]. Identification of AGEs in both the epidermis and dermis in the skin has led to further investigation of the role of glycation in skin aging. AGEs have been found to be associated with extracellular matrix proteins such as collagen, vimentin and elastin. Glycation of collagen I, one of the principle structural proteins in dermis impairs its function in multiple ways. AGE-modified collagen and elastin have been reported to have modified biomechanical properties leading to loss of elasticity and increased stiffening; changes that promote the appearance of wrinkles. Glycation also modifies the interaction of collagen with cells affecting their functions such as migration, differentiation and proliferation [3]. Glycated elastin has been described to be present in photo-aged skin suggesting ultraviolet irradiation stimulates glycation of elastin [4]. Additionally, glycated extracellular matrix proteins seem to be more resistant to degradation by matrix metalloproteinases [5], thus slowing down its removal and replacement by newly synthesized and functional protein [3]. Formation of AGEs is a complex multistep process leading to the formation of a heterogeneous group of molecules numerous of which have been identified. Carboxymethyl-lysine (CML) and pentosidine are among the most prevalent AGEs in the skin.

In recent years, a greater understanding of how AGEs affect structure and functionality of the skin has brought into prominence potential of anti-AGE strategies for the development of novel anti-aging cosmeceutical compounds. Several published studies or in vitro studies have linked carnosine with a protection against glycation when taken orally [6, 7]. To our knowledge the antiglycation efficacy of topical carnosine in skin has not been published.

In the present study, we assessed (i) the ability of topically applied carnosine to inhibit glycation in human skin and (ii) the efficacy of a novel facial cream (FC) containing carnosine to reduce the formation of AGEs, that is, CML and pentosidine in human skin explants in an ex vivo study.

## Materials and Methods

*Skin Explants Preparation*

Human living skin explants were prepared from an abdominoplasty (aesthetic surgery) from a 41-year-old Caucasian woman after obtaining informed consent. Adipose tissue was removed and explants of 1 $cm^2$ were prepared using a circular biopsy punch. The explants were maintained in survival cell culture conditions at 37 °C in a humid atmosphere enriched with 5% $CO_2$ in BIO-EC's explant medium, half of which was renewed every other day. BIO-EC's explant medium is a proprietary culture medium specifically engineered by the BIO-EC Laboratory for the survival of skin explants.

**Table 1.** Identification, treatment and sampling of each batch

| Group name | Treatment | Methylglyoxal | Sampling time |
|---|---|---|---|
| T0 | – | – | Day 0 |
| Control | – | – | Day 9 |
| AQ-CARN | Carnosine | – | Day 9 |
| FC-CARN | FC with carnosine | – | Day 9 |
| MG | – | MG | Day 9 |
| AQ-CARNMG | Carnosine | MG | Day 9 |
| FC-CARNMG | FC with carnosine | MG | Day 9 |

MG, methylglyoxal; AQ-CARN, carnosine in aqueous solution; FC-CARN, facial cream containing carnosine.

*Treatment Groups*

Treatment groups included aqueous solution of 0.2% carnosine (AQ-CARN) and a face cream formulation with 0.2% carnosine (FC-CARN). Treatment regimens are indicated in Table 1. The components of the FC-CARN are listed in Table 2. Each condition of treatment and control was tested in triplicate (3 explants per batch). The products were tested by topical application of 2 mg $cm^{-2}$ on the skin explants and evenly spread with a small spatula. Treatments were performed on day 0 and every 2 days until sampling on day 9 for a total of 4 applications.

*Induction of Glycation*

On D5 and D7, methylglyoxal (Reference MG, SIGMA, St. Louis, MO, USA) was incorporated in the culture medium at a final concentration of 500 μM.

*Sampling*

On day 0, the 3 explants from the batch T0 were collected and fixed in buffered formalin. On day 9, 3 explants from each batch were collected and processed the same way as T0.

*Staining and Immunostainings*

After fixation for 24 h in buffered formalin, samples were dehydrated and impregnated in paraffin using a Leica HistoCore PEARL dehydration automat (Leica, Germany). Samples were embedded using a Leica EG 1160 embedding station. Using a Leica RM 2125 Minot-type microtome, 5-μm-thick sections were made and the sections were mounted on Superfrost® histological glass slides (Menzel Gläser, VWR, USA).

Frozen samples were cut into 7-μm-thick sections using a Leica CM 3050 cryostat. Sections were then mounted on Superfrost® plus silanized glass slides (Menzel Gläser).

*General morphology* (viability control): Masson's trichrome staining, Goldner variant was performed on formol fixed paraffin embedded skin sections. This staining, alternative to classical He-

**Table 2.** Composition of AQ-CARN and FC-CARN

| AQ-CARN | FC-CARN |
|---|---|
| Carnosine 0.2% | Carnosine 0.2% |
| Vehicle composition | Vehicle composition |
| Water | Water, Butylene Glycol, Isodecyl Neopentanoate, Propanediol, Niacinamide, Glycerin, Betaine, Caprylic/Capric Triglyceride, Silica, Dimethicone, Hydrogenated Polyisobutene, Stearyl Alcohol, Octyldodecanol, Dipentaerythrityl Hexacaprylate/Hexacaprate, Tridecyl Trimellitate, Phenylpropanol, Ammonium Acryloyldimethyltaurate/VP Copolymer, Acrylates/C10-30 Alkyl Acrylate Crosspolymer, Caprylyl Glycol, Parfum, Cetearyl Alcohol, Potassium Olivoyl Hydrolyzed Oat Protein, Glyceryl Stearate, Tridecyl Stearate, Neopentyl Glycol Dicaprylate/Dicaprate, Glyceryl Oleate, Pentylene Glycol, Sodium Hyaluronate Crosspolymer, Disodium Edta, PEG-8, Alteromonas Ferment Extract*, Hydroxypropyl Cyclodextrin, Tetradecyl Aminobutyroylvalylaminobutyric Urea, Trifluoroacetate, Tocopherol, Benzyl Alcohol, Potassium Sorbate, Sodium Benzoate, Ascorbyl Palmitate, Magnesium Chloride, Alcohol Denat., Ascorbic Acid, Citric Acid, Palmitoyl Tripeptide-38, CI 15985, CI 19140 |

* Bacterial exopolysaccharides. FC-CARN, facial cream containing carnosine; AQ-CARN, carnosine in aqueous solution.

matoxylin Erythrosine Saffron, allows a fine evaluation of the cellular and tissue morphology.

*Immunostainings* of CML and Pentosidine were carried out using, respectively, an anti-CML monoclonal antibody (clone CMS-10, ref. KH011 TransGenic) and an anti-pentosidine monoclonal antibody (clone PEN-12, ref. KH012 TransGenic), with an avidin/biotin amplifier system (Vectastain ABC Universal kit, ref. PK7200, Vector Laboratories) and revealed by VIP, a substrate of peroxidase (ref. SK-4600, Vector Laboratories).

Microscopical observations were realized using a Leica DMLB or Olympus BX43 microscope (magnification ×40). Pictures were digitized with a numeric DP72 Olympus camera with CellD storing software (Olympus). Immunostainings were assessed by microscopical observation and image analysis.

*Image Analysis*

Image analysis was carried out using the analysis module of the Olympus CellD software on 9 microscopic fields (40× magnification). Staining was detected after intensity level thresholding. The area of interest (epidermis or papillary dermis) was manually drawn and then selected. The surface covered by the staining in the area of interest was then measured and expressed as a percentage of surfaces.

*Calculation of Antiglycation Activity*

For each of the 2 parameters CML and pentosidine, the average value of percentage of surfaces of the condition without MG (aT or aAQ-CARN or aFC-CARN) was subtracted from each value ($n$ = 9) of the condition with MG (MG or AQ-CARNMG or FC-CARNMG). This delta variation of CML or pentosidine was averaged and named MG-induced Glycation (MGG). For the condition control, MGG T = MG – aT. If P was the tested product (AQ-CARN or FC-CARN), for the condition treated with P, MGG P = MGP-aP. Then, the averaged delta MGG obtained with the tested product (AQ-CARN or FC-CARN) was compared to the MGG control (MGGT). This percentage of variation reflects the antiglycation activity. The antiglycation activity was calculated according to the following formula where P is the tested product AQ-CARN or FC-CARN:

$$\text{Antiglycation activity of P} = ([\text{MGGP} \times 100]/\text{MGGT}) - 100$$

*Statistical Analysis*

Means and SDs of the surface areas positive for each of the markers in different treatment groups were calculated (individual values from triplicates) and compared using Student $t$ test with Bonferroni adjustment for multiple comparisons. The difference was stated to be significant when the value of probability "$p$" was $<0.05$.

## Results

*General Morphology*

The examination of the general morphology confirmed good cellular viability of the epidermis and the dermis for the control batch on day 0 (T0). On day 9, the cellular viability was very slightly altered in the epidermis and good in the dermis for MG-unexposed batches. The MG treatment induced no visible modification of the cellular viability compared to the MG-unexposed batches. The slight alterations observed are not likely to change the evaluations of glycation parameters in a significant way.

*CML Results*

In the epidermis, after 9 days of culture ex vivo, the CML staining (condition T) was fairly clear, similar to that observed on day 0.

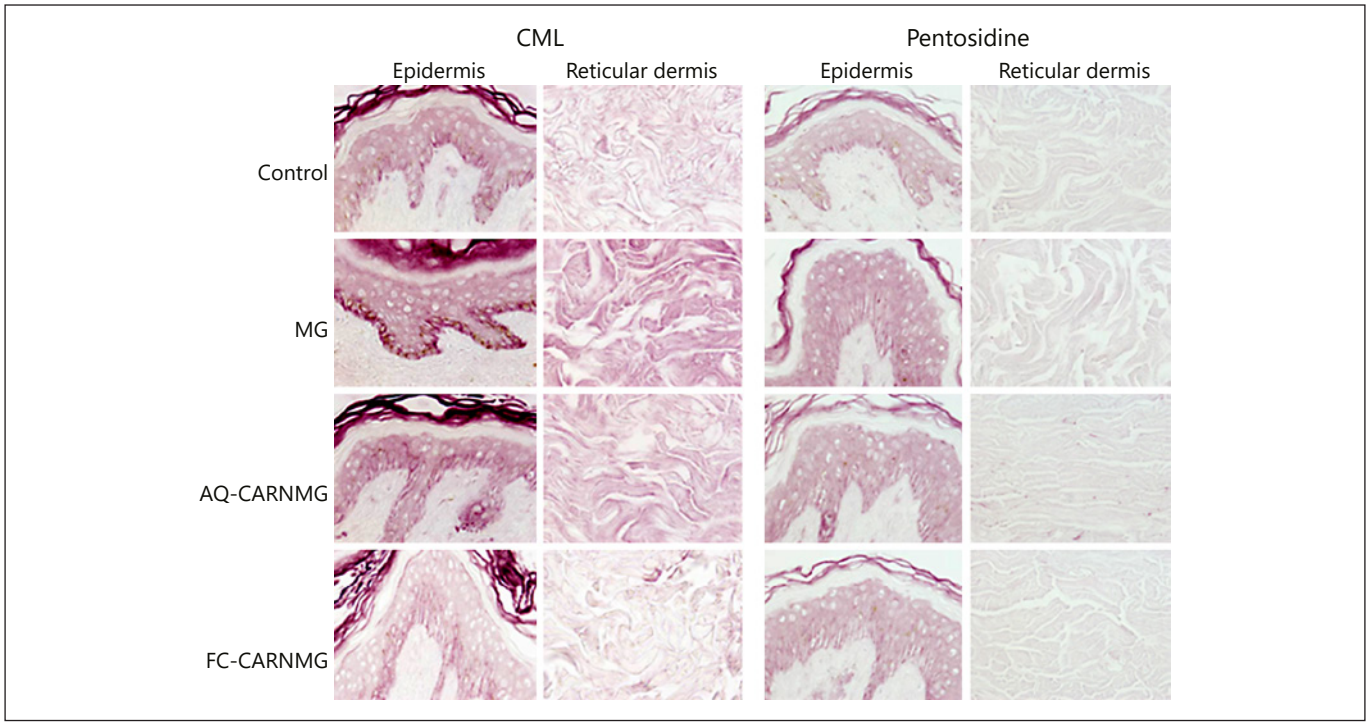


**Fig. 1.** Immunostaining of CML and pentosidine in the epidermis and reticular dermis. CML, carboxymethyl-lysine; MG, methylglyoxal; FC-CARN, facial cream containing carnosine; AQ-CARN, carnosine in aqueous solution.

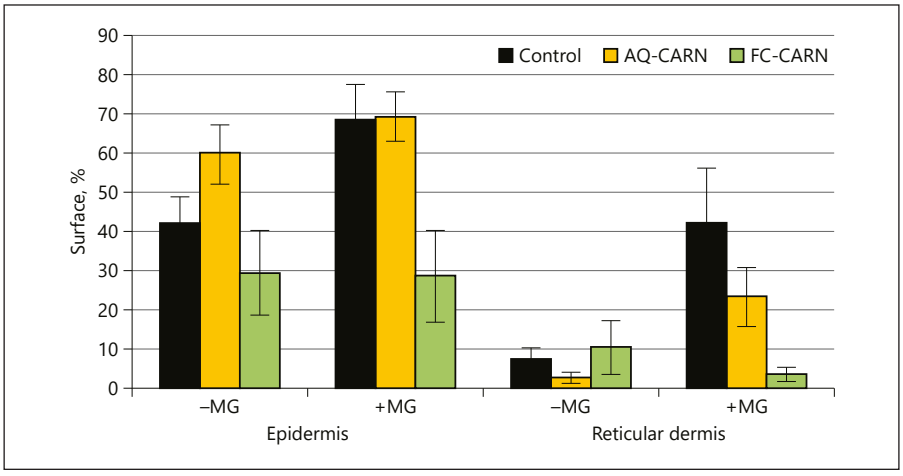


**Fig. 2.** Percentage of surface of CML covering the epidermis or reticular dermis, after immunostaining and measured by image analysis. CML, carboxymethyl-lysine; MG, methylglyoxal; FC-CARN, facial cream containing carnosine; AQ-CARN, carnosine in aqueous solution.

The tested products modified the CML expression in the epidermis: AQ-CARN induced a significant increase of CML expression by 41% ($p < 0.01$), while FC-CARN decreased the basal CML expression by 30% ($p < 0.05$; Fig. 1, 2).

The MG treatment induced a significant increase of CML formation in the epidermis by 63% ($p < 0.01$). The CML expression was non-significantly different when treated with AQ-CARN, but significantly lower when treated with FC-CARN ($p < 0.01$).

According to the basal CML level and the CML level after exposition to MG, the glycation effect of MG (MGGT) was about +26.6 ($p < 0.01$; Fig. 4; Table 3). When treated with the products, the glycation effect of MG was significantly reduced by 64% ($p < 0.01$) and 150% ($p < 0.01$) for AQ-CARN and FC-CARN respectively.

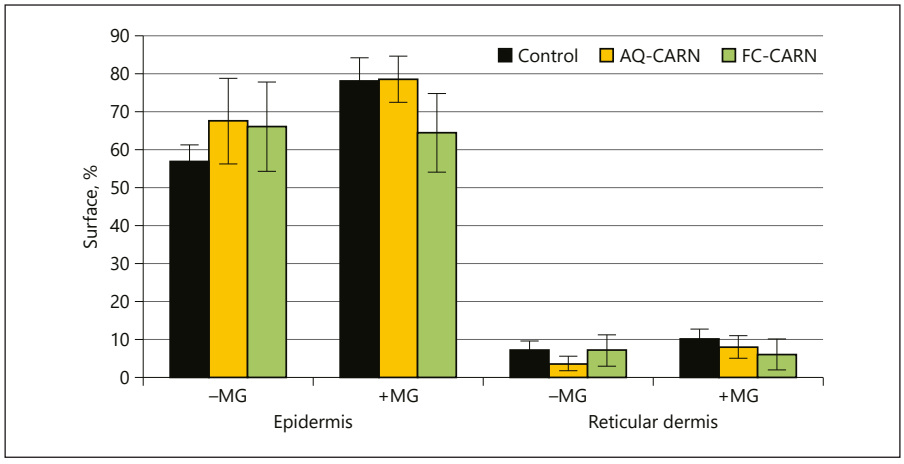


**Fig. 3.** Percentage of surface of pentosidine covering the epidermis or reticular dermis, after immunostaining and measured by image analysis. MG, methylglyoxal; FC-CARN, facial cream containing carnosine; AQ-CARN, carnosine in aqueous solution.

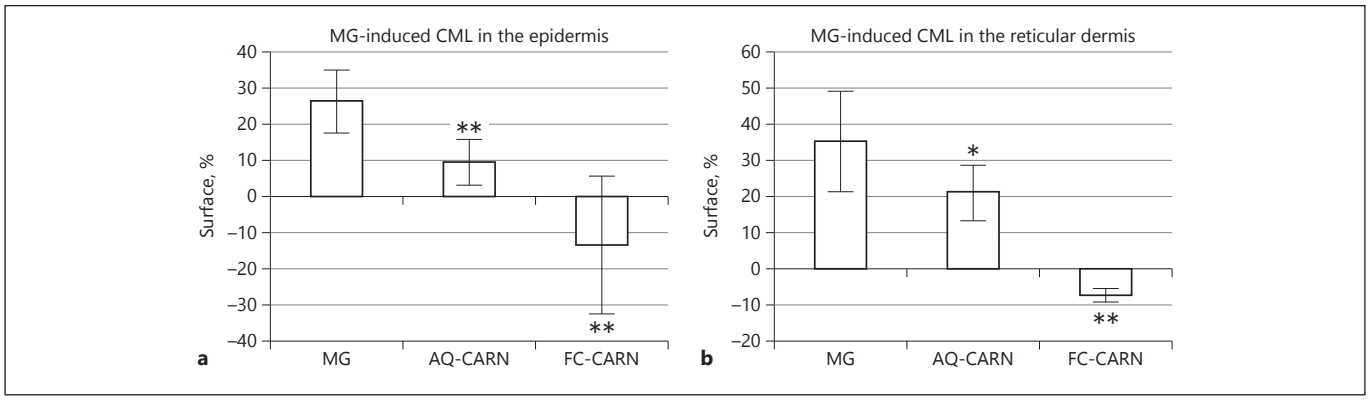


**Fig. 4.** MG-induced glycation for CML in the epidermis (**a**) or reticular dermis (**b**). * $p < 0.05$, ** $p < 0.01$. MG, methylglyoxal; CML, carboxymethyl-lysine; FC-CARN, facial cream containing carnosine; AQ-CARN, carnosine in aqueous solution.

In the reticular dermis, after 9 days of culture ex vivo, the staining of CML was weak to moderate, lower than the one observed on day 0.

The 2 tested products modified the basal CML expression in the reticular dermis. The product AQ-CARN significantly reduced the CML expression by 64% ($p < 0.01$). FC-CARN did not significantly reduce the basal CML expression (Fig. 1, 2).

The MG treatment induced a significant increase of CML formation in the reticular dermis by 459% ($p < 0.01$). The CML expression was significantly lower when treated with the products.

According to the basal CML level and the CML level after exposition to MG, the glycation effect of MG (MGGT) was about +34.8 ($p < 0.01$; Fig. 4; Table 3). When treated with the products, the glycation effect of MG was significantly reduced by 41% ($p < 0.01$), and 122% ($p < 0.01$) for AQ-CARN and FC-CARN respectively.

*Pentosidine Results*

In the epidermis, after 9 days of culture ex vivo, the staining of pentosidine was fairly clear, moderately lower than the one observed on day 0.

The tested products AQ-CARN and FC-CARN induced a significant increase of pentosidine expression by 18% ($p < 0.05$) and 15% ($p < 0.01$), respectively, in the epidermis (Fig. 1, 3).

The MG treatment induced a significant increase of pentosidine formation in the epidermis by 37% ($p < 0.01$). The CML expression was not significantly different when treated with AQ-CARN but 18% lower when treated with FC-CARN ($p < 0.01$).

According to the basal pentosidine level and the pentosidine level after exposition to MG, the glycation effect of MG (MGGT) was about +21.3 ($p < 0.01$; Fig. 5; Table 4). When treated with the products AQ-CARN and FC-CARN, the glycation effect of MG was signifi-

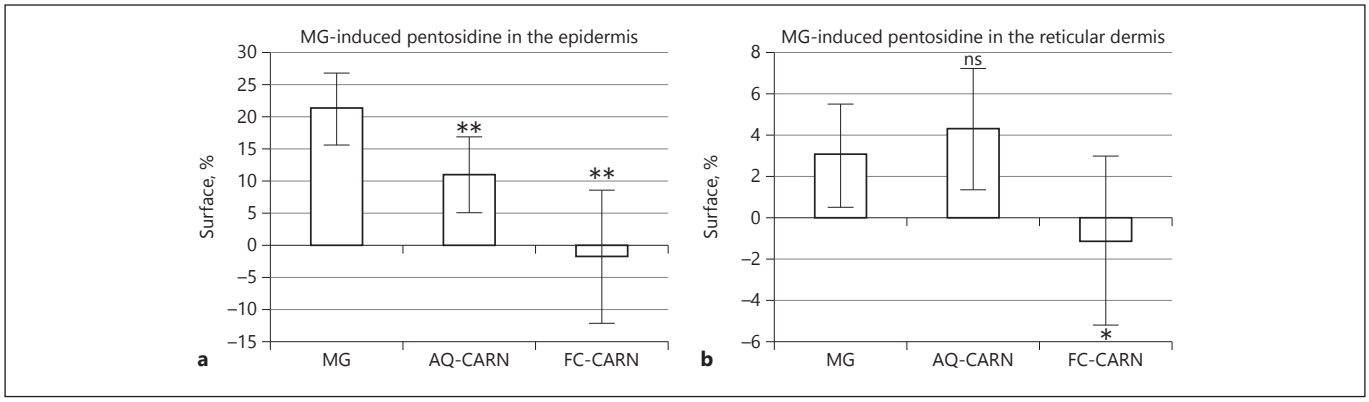


**Fig. 5.** MG-induced glycation for pentosidine formation in the epidermis (**a**) or reticular dermis (**b**). * $p < 0.05$, ** $p < 0.01$. MG, methylglyoxal; CML, carboxymethyl-lysine; FC-CARN, facial cream containing carnosine; AQ-CARN, carnosine in aqueous solution; ns, non-significant.

**Table 3.** MGG for CML in the epidermis and reticular dermis and antiglycation activity

| | | MG-induced glycation for CML | | |
|---|---|---|---|---|
| | | MG | MGG AQ-CARN | MGG FC-CARN |
| Epidermis | Mean | 26.6 | 9.6 | −13.3 |
| | SD | 8.8 | 6.4 | 19.0 |
| | Antiglycation activity (% variation vs. MG) | | −64 | −150 |
| | *p* vs. MG | | 0.0006 | 0.0002 |
| Reticular dermis | Mean | 34.8 | 20.6 | −7.8 |
| | SD | 14.0 | 7.6 | 1.9 |
| | Antiglycation activity (% variation vs. MG) | | −41 | −122 |
| | *p* vs. MG | | 0.0264 * | 0.0000 ** |

* $p < 0.05$, ** $p < 0.01$.

MG, methylglyoxal; MGG, MG-induced glycation; CML, carboxymethyl-lysine; AQ-CARN, aqueous solution; FC-CARN, facial cream containing carnosine; ns, non-significant.

**Table 4.** MGG for pentosidine formation in the epidermis and reticular dermis and antiglycation activity

| | | MG-induced glycation for pentosidine | | |
|---|---|---|---|---|
| | | MG | MGG AQ-CARN | MGG FC-CARN |
| Epidermis | Mean | 21.3 | 11.1 | −1.6 |
| | SD | 5.7 | 5.9 | 10.4 |
| | Antiglycation activity (% variation vs. MG) | | −48 | −108 |
| | *p* vs. MG | | 0.0028 ** | 0.0001 ** |
| Reticular dermis | Mean | 3.1 | 4.3 | −1.1 |
| | SD | 2.5 | 3.0 | 4.1 |
| | Antiglycation activity (% variation vs. MG) | | 42 | −136 |
| | *p* vs. MG | | 0.3607 ns | 0.0281 * |

* $p < 0.05$, ** $p < 0.01$.

MG, methylglyoxal; MGG, MG-induced glycation; AQ-CARN, aqueous solution; FC-CARN, facial cream containing carnosine; ns, non-significant.

cantly reduced by 48% ($p < 0.01$) and 108% ($p < 0.01$) respectively (Table 4).

In the reticular dermis, after 9 days of culture ex vivo, the staining of pentosidine was weak, slightly lower than that observed on day 0.

The tested products, AQ-CARN and FC-CARN, significantly reduced the pentosidine expression by 49% ($p < 0.01$) and 2% (ns), respectively, in the reticular dermis (Fig. 1, 3).

The MG treatment induced a significant increase in pentosidine formation of 41% in the reticular dermis ($p < 0.05$). The pentosidine expression was 22% (ns) and 41% ($p < 0.05$) lower when treated with AQ-CARN and FC-CARN, respectively.

According to the basal pentosidine level and the pentosidine level after exposition to MG, the glycation effect of MG (MGGT) was about +3.1 ($p < 0.01$; Fig. 5;

Table 4). When treated with FC-CARN, the glycation effect of MG was significantly reduced by 136% ($p < 0.01$).

## Discussion

The topical application of an aqueous solution of 0.2% carnosine (AQ-CARN) was protective against the increase in levels of AGEs induced by MG with a change of –64 and –41% reported with respect to CML in epidermis and reticular dermis, respectively, and –48% with respect to pentosidine in the epidermis. The antiglycating effect of AQ-CARN in the reticular dermis was not significant. Novel anti-aging FC-CARN, bacterial exopolysaccharides and niacinamide had a pronounced antiglycation activity with change of –150 and –122% with respect to CML and –108 and –136% with respect to pentosidine observed in epidermis and reticular dermis respectively. The glycation property of MG has been described extensively in in vitro studies. In our study, we confirm that MG led to the increased formation of both CML and pentosidine in both dermis and epidermis in human skin.

The facial cream FC-CARN showed a greater antiglycation activity compared to carnosine in aqueous solution. This enhanced antiglycation activity of the cream versus aqueous solution can be due to an increased cutaneous delivery of the carnosine and/or the antiglycation effects of additional ingredients of the cream such as bacterial exopolysaccharides and niacinamide related to their antioxidant or metal ion-chelating action. Future studies about the antiglycation effects of the cream without carnosine can help to understand the antiglycation activity of this novel FC.

We have previously published results for this FC obtained in a clinical study with 33 women, aged 45–65 years [8]. The study reported an improvement in the facial contour characteristics with a significant decrease in sagging jawline (7%). Additionally, an increase in hydration (12%), firmness (29%), elasticity (20%) and improved skin texture was observed after 56 days of use of the FC [8]. The results of the present study showing antiglycation effect in the epidermis and in the dermis in human skin explant, may partially explain the significant improvement of skin aging signs observed in the clinical study. The main drawback of this study is the fact that the results of the present study are derived from skin from a single donor. However, since we focused on glycation induced by MG rather than preexisting glycation level, the inter-individual variability was not considered. We chose to look at MG-induced glycation due to the large inter-individual variability we expected to find in naturally occurring glycation levels.

Carnosine, a dipeptide (β-alanyl-L-histidine), has long been associated with anti-ageing and cell longevity by suppression of cell senescence, induction of rejuvenating effects and protection against telomere shortening [9]. The mechanism through which it perpetrates this activity is thought to be a combination of its antiglycation, antioxidant, and metal ion chelating activities [10]. While oral carnosine supplementation has been shown to improve skin texture [7], we have found no published reports of antiglycation action of topically applied carnosine in literature. Results of the current study are another step in understanding carnosine's role as an anti-aging agent in skin care products.

To conclude, we report notable antiglycation properties of a novel FC with carnosine as one of the main ingredients in human skin explants. Together with results of a previously published clinical study with same product, it may be inferred that use of this cream would lead to improvement of signs linked to accumulation of AGEs in the skin such as loss of firmness, elasticity and skin tone.

## Statement of Ethics

The authors have no ethical conflicts to disclose.

## Disclosure Statement

Authors MN, CT and CG are employees of ISDIN, Barcelona, the manufacturer of facial cream tested in this study. JK is a Scientific Advisor to ISDIN.

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