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Research article

An original amino acid formula favours *in vitro* corneal epithelial wound healing by promoting Fn1, ITGB1, and PGC-1α expression

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ABSTRACT

Corneal disorders are frequent, involving most diabetic patients; among its manifestations, they include delayed wound healing. Since maintenance of mitochondrial homeostasis is fundamental for the cell, stimulation of mitochondrial biogenesis represents a unique therapeutic tool for preventing and treating disorders with a deficit in energy metabolism. We have recently demonstrated that a branched-chain amino acid (BCAA)-enriched mixture (BCAAem) supported mitochondrial biogenesis in cardiac and skeletal muscle, reduced liver damage caused by alcohol, and prevented the doxorubicin-dependent mitochondrial damage in cardiomyocytes. The present study aimed to investigate a new amino acid mixture, named six amino acids (6AA), to promote corneal epithelial wound healing by regulating mitochondrial biogenesis. A murine epithelium cell line (TKE2) exposed to this mixture showed increased mitochondrial biogenesis markers, fibronectin 1 (Fn1) and integrin beta 1 (ITGB1) involved in extracellular matrix synthesis and cell migration. Most importantly, the 6AA mixture completely restored the wound in scratch assays, confirming the potential of this new formula in eye disorders like keratopathy. Moreover, our results demonstrate for the first time that peroxisome proliferator-receptor γ coactivator 1 α (PGC-1α) is expressed in TKE2 cells, which controls mitochondrial function and corneal repair process. These results could be relevant for the treatment mainly focused on corneal re-epithelialisation.

1. Introduction

Corneal diseases are frequent, and recent research has demonstrated that they affect up to 70% of diabetic patients examined (Abdelkader et al., 2011; Vieira-Potter et al., 2016). They are an indication of peripheral neuropathy (Bikbova et al., 2016), whose manifestations include delayed wound healing, compromised barrier function, persistent epithelial defects and ulcerations, decreasing corneal sensitivity, epithelial edema, neurotrophic corneal ulcers, and stem cell dysfunction (Chen et al., 2009; Herse, 1988; Wang et al., 2014).

Corneal epithelial stem cells, located in the limbal area, play essential roles in maintaining normal corneal homeostasis and corneal wound healing. Stem cell deficiency is the pathological process of diverse corneal diseases, such as chemical burns (Castro-Muñozledo, 2013; Ramos et al., 2015). Scientists and ophthalmologists found corneal stem cell preparation, mechanisms, and transplantation exciting research topics (Shaharuddin et al., 2013; Eberwein and Reinhard, 2015). A murine corneal epithelial progenitor cell line, called TKE2 (Kawakita et al., 2008), was developed in the past few years by Kawakita and coworkers, and it manifests characteristics of stem cells; for example, cell self-renewal and the expression of stem cell-associated markers, such as ATP-Binding Cassette Transporter G2 (ABCG2) and N-cadherin (Kawakita et al., 2008; Kubota et al., 2010). TKE2 has become an excellent model to investigate corneal epithelial stem/progenitor cells (Wang et al., 2009).

Fibronectin 1 (FN1) belongs to the extracellular matrix (ECM) glycoprotein family. It functions in the ECM process and contributes to cellular adhesion, polarity, migration and tissue remodeling (Ma et al., 2014; Hanahan and Weinberg, 2011). Besides, FN1 also executes its functions in infection resistance and microvascular integrity maintenance (Mammoto et al., 2009). Integrins are a family of heterodimeric extracellular matrix receptors; they act on cell-matrix adhesion, intracellular signalling and modulate cell proliferation, differentiation, motility and survival (Maschler et al., 2005). Cells interact with their

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ECM primarily through integrin receptors composed of heterodimeric proteins of α and β subunits. Integrins mediate cell adhesion and mechanical stress, serving as a bidirectional channel for signals originating inside and outside the cell (Rozario and Desimone, 2010). Integrin 1 is known, for example, to have a role in maintaining the lens epithelial phenotype (Simirskii et al., 2007).

The mitochondrial life cycle (*e.g.*, mitochondrial biogenesis, mitochondrial dynamics, and mitochondrial removal by mitophagy) is arranged to generate cellular energy efficiently. It has been suggested that defective cellular energy metabolism is a significant cause of metabolic and age-related diseases, and obesity, diabetes, and aging share mitochondrial impairment to be a common pathophysiological mechanism (Wlodek and Gonzales, 2003: Boudina et al., 2005; Sparks et al., 2005; Wisloff et al., 2005). Therefore, activation of mitochondrial biogenesis is essential for preventing and treating energy metabolism and age-related diseases. Previously, we have demonstrated the efficacy of the branched-chain amino acid (BCAA)-enriched mixture (BCAAem) in several pathologies: supplementation of this mixture was able to prevent muscular damage induced by rosuvastatin through preservation of mitochondrial efficiency and ameliorating the control of oxidative stress in the statin-treated mice and muscle cells (D'Antona et al., 2016). Moreover, we demonstrated that dietary supplementation of the BCAAem mixture prevented fat accumulation and mitochondrial dysfunction in hepatocytes of alcohol-consuming rats (Tedesco et al., 2018). Recently, our results demonstrated that a new amino acid (AA) formula named α5 prevented the doxorubicin-dependent mitochondrial damage and oxidative stress better than the previous BCAAem mixture (Tedesco et al., 2020).

In the present investigation, we compared the different responses of TKE2 cells to treatment with diverse AA mixtures to identify a novel combination able to promote fibronectin and integrin expression and mitochondrial function with an efficiency higher compared to the classic BCAAem formula. To this aim, we studied the α5 and the new formula called 6AA. The α5 mixture is integrated with the tricarboxylic acid cycle (TCA) intermediates—citrate, succinate, and malate (ratio 8:2:2) (Table 1)—whereas 6AA was enriched with sodium hyaluronate (HA) (Table 1). Thus, we have analyzed gene expression of the principal mitochondrial biogenesis markers, including peroxisome proliferator-receptor γ coactivator 1 α (PGC-1α), mtDNA transcription factor A (Tfam), and cytochrome *c* (Cyt *c*). Moreover, we have evaluated the expression of genes implied in the ECM production, like Fn1 and ITGB1 and gene expression of transporters/receptors of HA, succinate and citrate (*i.e.*, RHAMM, SCNR1, and Scl25a1, respectively). Also, protein levels of PGC-1α, Cyt *c* and ITGB1 were analyzed. We performed a scratch assay to study further the role of 6AA in corneal epithelial cell motility and migration. The 6AA mixture increased mitochondrial biogenesis markers, FN1, and ITGB1 expression in corneal TKE2 cells. Most importantly, 6AA completely restored the wound in scratch assays, confirming the potential of this new formula in corneal trauma.

Table 1
Composition of the mixtures.

Mixture	BCAAem (%)	α5 (%)	6AA (%)
L-Leucine	30.01	31.09	3.23
L-Lysine (chlorhydrate)	19.58	16.90	2.54
L-Isoleucine	15.00	10.36	–
L-Valine	15.00	10.36	12.92
L-Threonine	8.40	7.25	–
L-Cysteine	3.60	3.11	–
L-Histidine	3.60	3.11	–
L-Phenylalanine	2.40	2.07	–
L-Methionine	1.20	1.04	–
L-Tyrosine	0.72	0.62	–
L-Tryprophan	0.48	2.07	–
Vitamin B1 (thiamine chlorhydrate)	–	0.004	–
Vitamin B6 (piridoxine chlorhydrate)	–	0.004	–
Citric acid	–	8.00	–
Malic acid	–	2.00	–
Succinic acid	–	2.00	–
Glycine	–	–	23.23
L-Proline	–	–	17.46
L-Alanine	–	–	17.54
Sodium Hyaluronate	–	–	23.07

2. Materials and methods

2.1. Cell culture and treatment

TKE2 is a murine limbal/corneal epithelium-derived progenitor cell line. TKE2 cells were maintained in a defined keratinocyte serum-free medium (Stemline Keratinocyte Medium II, Sigma-S0196, Italy) supplemented with Stemline Keratinocyte Growth Supplement (Sigma-S9945, Italy) provided by the manufacturer until use. Cell cultures were incubated at 37 °C, under 95% humidity and 5% CO2. Cells were treated with 0.01%, 0.1%, 0.5%, or 1% of the mixtures (Table 1 reported the composition) for 24 h, 48 h, or 72 h, with or without 5 mM citric, malic, and succinic acids (CMS) at 8:2:2 ratio, as previously studied for the α5 formula and reported in Tedesco et al. (2020). Untreated cells were plated as controls. Every 24 h, media were replaced. At the end of the experimental treatments, cells were used for mRNA extraction, protein levels analysis, cell viability assay, and the *in vitro* scratch injury model.

2.2. Cell viability assay

Viable cell number was determined using the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. For the treatments (24 h or 48 h), we used 10^4 cells/well in 96 wells plate in 100 μL of the medium. The purple formazan crystals were dissolved overnight at 37 °C in 5% SDS/0.1M HCl (100 μL/well). The absorbance was recorded on a microplate reader at a dual-wavelength of 570 nm/655 nm.

2.3. In vitro scratch injury model

Cells were grown in 24-well plates. After the TKE2 cultures reached confluence, the cells from the centre of the culture well were scraped using a standard 200 μL pipet tip to create a scratch injury and washed with PBS to remove detached cells and debris. The cell cultures were then incubated in control or media enriched with the 6AA mixture with or without CMS for 72 h. The microscopy images (n = 6/group) were then collected by a person blinded to the images' details at 0, 24, 48, or 72 h. The percentage of the wound healing area was calculated using ImageJ software.

2.4. Total RNA extraction and gene expression analysis

RNA was isolated from TKE2 cells using the RNeasy Mini Kit (Qiagen, Segrate, Italy), and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative RT-PCRs were performed as previously described (Vettor et al., 2014; Tedesco et al., 2008) with the iQ SybrGreenI SuperMix (Bio-Rad; Segrate, Italy) on an iCycler iQ real-time PCR detection system (Bio-Rad). After RNA extraction from TKE2 cells, cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Primers were designed with Beacon Designer 2.6 software from Premier Biosoft International (Palo Alto, CA, USA) and are shown in Table 2. We compared the cycle number at which the various transcripts were detectable (threshold cycle, CT) to TBP, referred to as ΔCT. The relative gene level was expressed as 2-(ΔΔCT), in which ΔΔCT equals the difference between the DCT of either treated TKE2 cells and the ΔCT of the untreated TKE2 cells.

2.5. Western blot analysis

Protein extracts were obtained from TKE2 cells in M-PER

Table 2
Primers for qRT-PCR.

Gene	Primer Sense (5′-3′)	Primer Antisense (5′-3′)	PCR Product (bp)	T_a (°C)
Cyt c	ATAGGGGCATGTCACCTCAAAC	GTGGTTAGCCATGACCTGAAAG	172	60
Fn1	AGATTGGCGACAAGTGGAGG	GGTAGGGCTTTTCCCAGGTC	172	60
ITGB1	CTGGTCCCGACATCATCCCA	CCGTGTCCCACTTGGCATTC	167	60
PGC1-α	ACTATGAATCAAGCCACTACAGAC	TTCATCCCTCTTGAGCCTTTCG	148	60
RHAMM	CCTTGCTTGCTTCGGCTAAAA	CTGCTGCATTGAGCTTTGCT	190	60
Scl25a1	TCGGGAACAAGGGCTAAAGG	CCCGTGATCAGTGGGTTCAT	157	60
SUCNR1	ACAGAAGCCGACAGCAGAAT	GCACAGGAAAGCAAAGTCAG	229	50
TBP	ACCCTTCACCAATGACTCCTATG	TGACTGCAGCAAATCGCTTGG	186	60
Tfam	AAGACCTCGTTCAGCATATAACATT	TTTTCCAAGCCTCATTTACAAGC	104	60

mammalian protein extraction reagent (Pierce, ThermoScientific, Rockford, USA), as indicated by the manufacturer, in 1 mM $NaVO_4$, 10 mM NaF and a cocktail of protease inhibitors (Sigma-Aldrich, Milan, Italy). The bicinchoninic acid protein assay was performed to determine the protein content (BCA, Pierce, Euroclone, Milan, Italy). We separated 30 μg of the protein extract under reducing conditions (SDS-PAGE). Then, the proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Segrate, Italy) (Vettor et al., 2014). To detect the proteins of interest, we used these specific antibodies: anti-Cyt c (cytochrome complex, Cell Signaling Technology Cat# 4280), anti-PGC-1α (proliferator-activated receptor γ coactivator 1α, Cell Signaling Technology Cat# 2178), anti-ITGB1 (GeneTex Cat# GTX128839), and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Cell Signaling Technology Cat# 2118) at 1:1000 dilution each. Immunostaining was performed using horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin for 1 h at room temperature. We used SuperSignal substrate (Pierce, Euroclone, Milan, Italy) to detect the proteins and quantified them by densitometry with ImageJ image analysis software.

2.6. Statistical analysis and data presentation

Statistical analysis was performed with unpaired Student's t-test for two-group analysis or one-way ANOVA with Tukey correction for multiple group comparisons. Data are presented as the means ± standard deviations (SD) unless otherwise specified. A statistically significant difference was accepted at $P < 0.05$.

3. Results

3.1. 6AA mixture is more effective than BCAAem and α5 in upregulating Fn1, ITGB1, PGC1-α, Tfam, and Cyt c in TKE2 cells

As shown in Table 1, the α5 mixture's composition is different from BCAAem. First, the Leucine: Isoleucine: Valine ratio of α5 is 3:1:1, whereas the stoichiometric ratio of BCAAem mixture is 2:1:1; secondly, in α5 mixture, the TCA cycle substrates (citric acid, succinic acid, and malic acid) were added. The α5 mixture also includes vitamins B1 and B6. 6AA mixture has been created especially for the conservation and nourishment of fibroblast and skin. 6AA mixture contains six amino acids: glycine, proline, leucine, lysine, valine and alanine. Furthermore, this mixture is enriched with HA. The optimal ratios of the six amino acids for collagen and elastin production were previously identified (De Servi et al., 2018). Moreover, the retinal pigment epithelium uses proline for several purposes, like to fuel mitochondrial metabolism, synthesize AA, build the EM and fight against oxidative stress (Du et al., 2021).

To compare the efficacy of the new amino acid formula, we first analyzed the *Fn1* and *ITGB1* gene expression in TKE2 cells. As shown in Figs. 1 and 24 h of 1% 6AA treatment significantly increased both *Fn1* and *ITGB1* mRNA levels. BCAAem and α5, instead, decreased *Fn1* expression, while *ITGB1* was not affected by both mixtures.

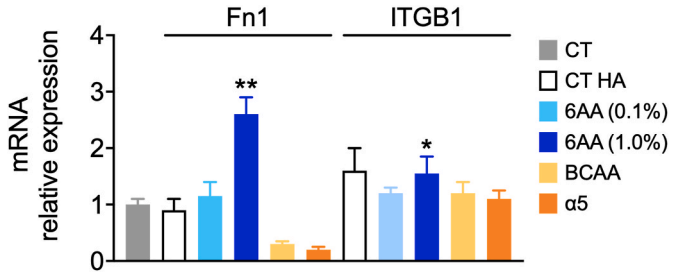

Fig. 1. Fn1 and ITGB1 expression in TKE2 cells treated with 0.1% or 1% 6AA or 1% BCAA/α5 mixtures for 24 h. Relative mRNA levels of Fn1 and ITGB1 normalized to TBP (n = 3 in triplicate, mean ± SD). *P < 0.05 and **P < 0.01 *vs.* untreated cells, espressed as 1.0.

Similar results were also obtained with the addition of citric, malic, and succinic (CMS) acids (Fig. 2), suggesting that these TCA intermediates do not potentiate the AA mixture on Fn1 and ITGB1 gene expression.

We then studied the effects of the 6AA mixture on mitochondrial biogenesis in TKE2 cells by analysing the gene expression of mitochondrial biogenesis markers. After 24 h treatment with 6AA mixture plus CMS, we observed a significant increase of PGC-1α, Tfam, and Cyt *c* mRNA levels than controls (Fig. 3).

However, and in line with Fn1 and ITGB1 data, BCAAem and α5 were ineffective. 6AA mixture increased mRNA levels of all the mitochondrial biogenesis markers; in particular, PGC-1α was increased 16-fold when TKE2 cells were treated with 1% of 6AA plus CMS (Fig. 3), indicating a higher stimulation of mitochondrial biogenesis. Without CMS, 1% 6AA treatment upregulated the mitochondrial biogenesis markers to a lesser extent (data not shown). These data suggest that the 6AA formula, particularly with CMS, is a potent inducer also of mitochondrial biogenesis markers.

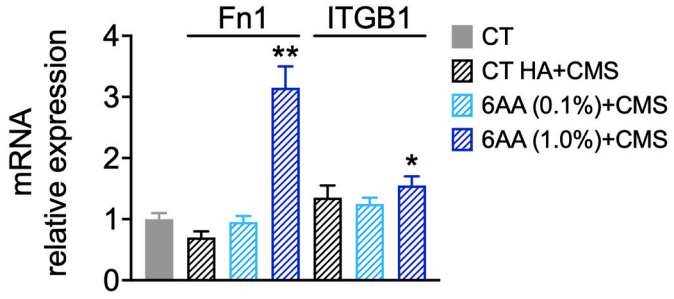

Fig. 2. Fn1 and ITGB1 expression in TKE2 cells treated with 0.1% or 1% 6AA + 5 mM CMS for 24 h. Relative mRNA levels of Fn1 and ITGB1 normalized to TBP (n = 3 in triplicate, mean ± SD). *P < 0.05 and **P < 0.01 *vs.* untreated cells, espressed as 1.0.

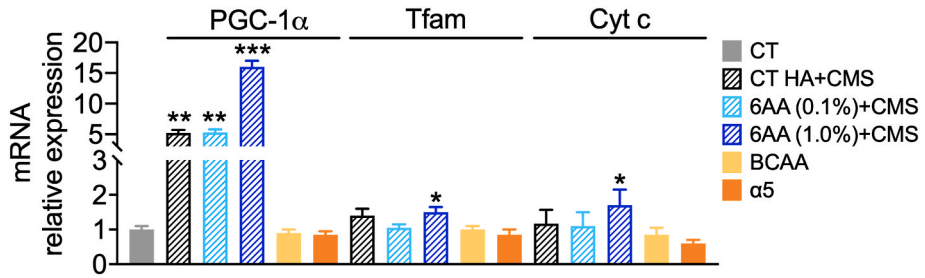

Fig. 3. Mitochondrial biogenesis markers in TKE2 cells treated with 0.1% or 1% 6AA + 5 mM CMS or 1% BCAA/α5 for 24 h. Relative mRNA levels of PGC-1α, Tfam, and Cyt *c* normalized to TBP (n = 3 in triplicate, mean ± SD). *P < 0.05, **P < 0.01, and ***P < 0.01 *vs.* untreated cells, espressed as 1.0.

3.2. 6AA mixture increased ITGB1, PGC-1α and Cyt c protein levels in TKE2 cells

We next investigated the protein levels of ITGB1, PGC-1α and Cyt *c* in TKE2 cells treated with 6AA with or without CMS. We observed the protein level of PGC-1α in TKE2 cells for the first time, and we confirmed the upregulation of this mitochondrial biogenesis marker observed as mRNA by this mixture. In particular, at 1% of 6AA for 48 h, the PGC-1α protein level was increased by 40% when the cells were treated with CMS (Fig. 4A). Cyt *c* protein levels were increased by 80 and 95%, respectively, after 6AA 0.1% and 6AA 1% with CMS (Fig. 4B). Furthermore, 1% 6AA increased ITGB1 protein levels by 30% (Fig. 4C). We obtained similar results when TKE2 cells were treated without CMS. These data suggest that the upregulation of the mitochondrial markers PGC-1α and Cyt *c* and the extracellular matrix ITGB1 were also evident at the protein level.

3.3. 6AA mixture increased TKE2 cell viability

To measure the viable cell number after 6AA treatment, we tested the response of TKE2 cells to different concentrations of 6AA, with or without CMS, after 24 h or 48 h by the MTT assay. We observed an increase of viability at 0.01% and 0.1% of 6AA whereas, at higher concentrations (0.5% or 1%) and we revealed significant inhibition of TKE2 cell growth (more than 50% after 48 h at 1% Fig. 5), probably due to more elevated concentration of HA.

3.4. Citrate, malate, and succinate acids increase the succinate receptor gene expression in TKE2 cells

Citrate, malate, and succinate mitochondrial metabolites, usually considered only energy-generating TCA intermediates, can act as signalling molecules by modulating gene expression and protein translational modifications (Frezza, 2017). We then investigated if the up-regulation of Fn1 and ITGB1 and the high mitochondrial biogenetic capacity seen with 6AA could be accompanied by changes in the expression of genes involved in HA, succinate, or citrate transport into corneal epithelial cells. We assessed the mRNA levels of receptors for HA-mediated motility (RHAMM), succinate receptor (SUCNR1), and citrate membrane transporter (Scl25a1) in TKE2 cells treated with 6AA mixture, with or without CMS for 24 h. As shown in Fig. 6A, we observed only a trend to higher RHAMM, SUCNR1, and Scl25a1 mRNA levels

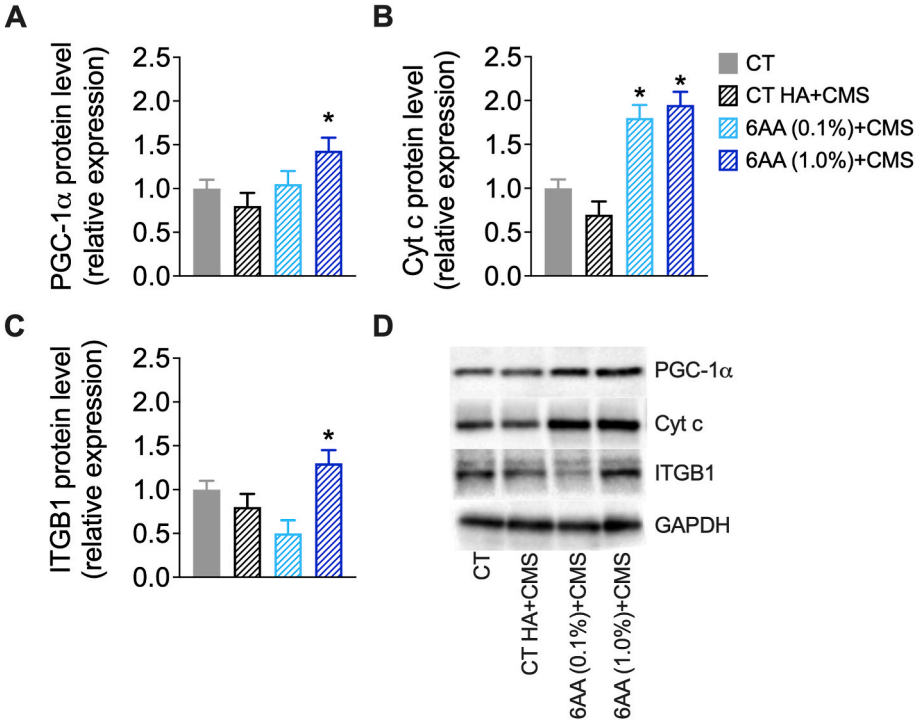

Fig. 4. PGC-1α (A), Cyt *c* (B) and ITGNB1(C) protein levels were determined by Western blot analysis. (D) One experiment representative of five reproducible ones. The relative values were determined by densitometric analysis relative to GAPDH levels. The TKE2 were treated with 0.1% or 1% 6AA + CMS for 48 h. Values for untreated TKE2 cells were taken as 1.0 (n = 3, mean ± SD). *P < 0.05 and **P < 0.01 *vs.* untreated cells, expressed as 1.0.

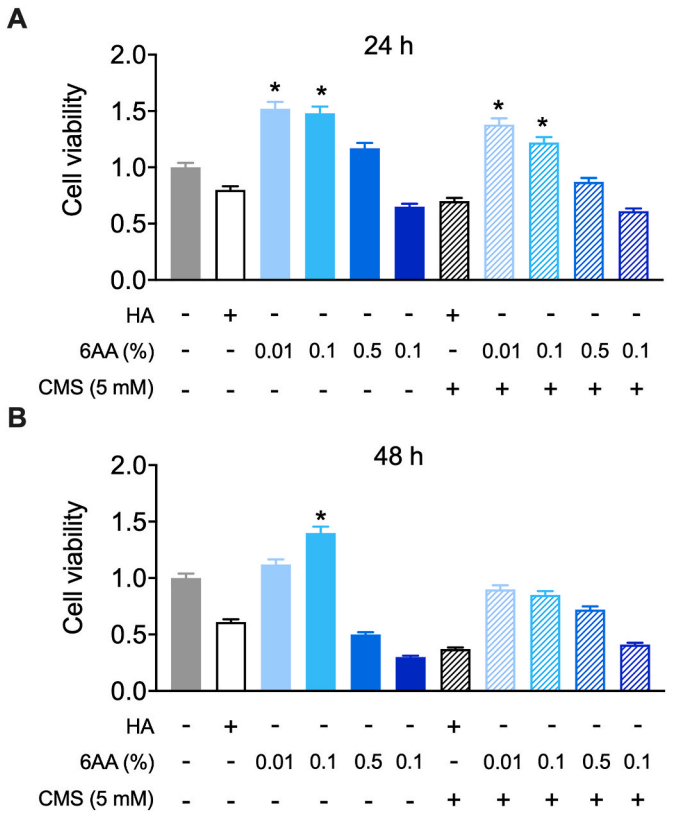

Fig. 5. Cell viability was evaluated by the MTT assay. A) After 24 h treatments with 6AA at different concentrations ± CMS. B) After 48 h treatments with 6AA at different concentrations ± CMS. *P < 0.05 *vs.* untreated cells, expressed as 1.0.

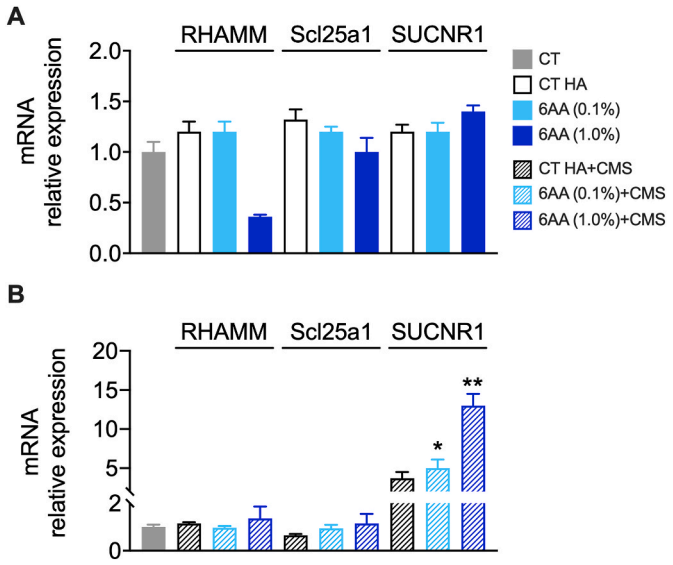

Fig. 6. *RHAMM*, *SUCNR1* and *Scl25a*1 mRNA in TKE2 fibroblasts treated with 0.1–1% 6AA for 24 h (A) and plus CMS (B). Relative mRNA levels were normalized to TBP (n = 3 in triplicate, mean ± SD). *P < 0.05 and **P < 0.01 *vs.* untreated cells, expressed as 1.0.

compared to those in untreated TKE2 cells. However, CMS's addition significantly increased the SUCNR1 mRNA levels to a greater extent than 6AA alone (Fig. 6B). These results suggested that the enrichment of the 6AA mixture with CMS could affect gene expression in corneal epithelial cells. We thus decided to investigate this fact in more detail; in particular, we analyse the effects of the 6AA mixture in the presence of each TCA cycle intermediate. Fig. 7 shows that only citrate promoted gene expression when present alone, while the intermediates variously potentiated the effect of the 6AA formula with the maximal efficacy when combined altogether.

3.5. 6AA mixture promotes the TKE2 cell wound healing

We thus executed a scratch assay to study further the role of the 6AA and CMS combination in corneal epithelial cell motility and migration. The presence of 6AA enhanced motility and migration of corneal epithelial cells compared to the control cells (Fig. 8A). During this assay, the 6AA-treated TKE2 cells filled the scratch area much faster than the untreated control cells, similarly in the presence or absence of CMS (Fig. 8B). After three days, 1% 6AA closed the wound completely. The scratch injury test demonstrated a significant increase in the healing rate of the cells treated with 6AA compared to control cells. The supplementation of the mitochondrial metabolites was unable to improve this effect significantly; on the contrary, in some cases, these metabolic intermediates seemed to impair the amino acid efficacy.

4. Discussion

Corneal wounds represent the most common form of damage to the eye, and the majority of these injuries are corneal epithelium abrasions. Corneal wound healing is a complex process whose mechanisms and genetic control are not fully understood. We know that different cells' growth factors, cytokines and proteases are involved. In corneal epithelial wound healing, a topically administered drug via eye drops is the most common method to accelerate wound closure. However, this approach shows limitations like low bioavailability and repeated application (Maulvi et al., 2015; Urtti, 2006).

The present study shows a relevant effect of the 6AA mixture created to conserve and nourish fibroblasts on mouse corneal epithelium wound healing. This mixture contained beyond amino acids, HA. HA has a gripping biological action on cell adhesion, proliferation and migration, cytokine release, and synthesis of extracellular matrix proteins in connective tissues. Indeed, it has been shown that the biological action of HA can be synergistically enhanced by adding small active molecules as AAs, thus increasing the possible application of this polymer or revitalization of connective tissue in particular (Colella et al., 2009; Corsetti et al., 2010). Moreover, HA demonstrated the ability to enhance rabbit corneal epithelial wound healing *in vivo* in different scraping models (Nakamura et al., 1994), and various scientists have shown that HA may play a crucial role in corneal epithelial development, wound healing, and inflammation in several *in vitro* models (Miyauchi et al., 1990; Nishida et al., 1991; Inoue and Katakami, 1993; Gomes et al., 2004; Pauloin et al., 2009). We found that the 6AA mixture increased *Fn1* and *ITGB1* gene expression. A higher abundance of ITGB1 in 6AA-treated TKE2 cells may be noted. This integrin protein plays an essential role in cell-ECM adhesion, migration, and tissue repair (Liu et al., 2010; Rankin et al., 2013), and its appearance in the cornea was correlated with corneal epithelial wound repair (Murakami et al., 1992). Moreover, our results demonstrate an increased expression of mitochondrial biogenesis markers, PGC-1α and Cyt *c* protein levels. In particular, for the first time, the expression of PGC-1α has been observed in corneal epithelium cells, and its up-regulation by 6AA treatment seems to implicate it, in addition to the extracellular matrix, in the corneal repair process.

It is well-known that citrate is a crucial metabolite linked to many critical metabolic pathways. The most known citrate function is its role in the TCA cycle. It represents the starting point for generating reduced equivalents NADH and FADH2 and then generating ATP. All the multiple biological functions that require citrate occur in the cytoplasm except for its role in the TCA. This event necessitates the transfer of citrate from the mitochondrial matrix into the cytoplasm. A specific

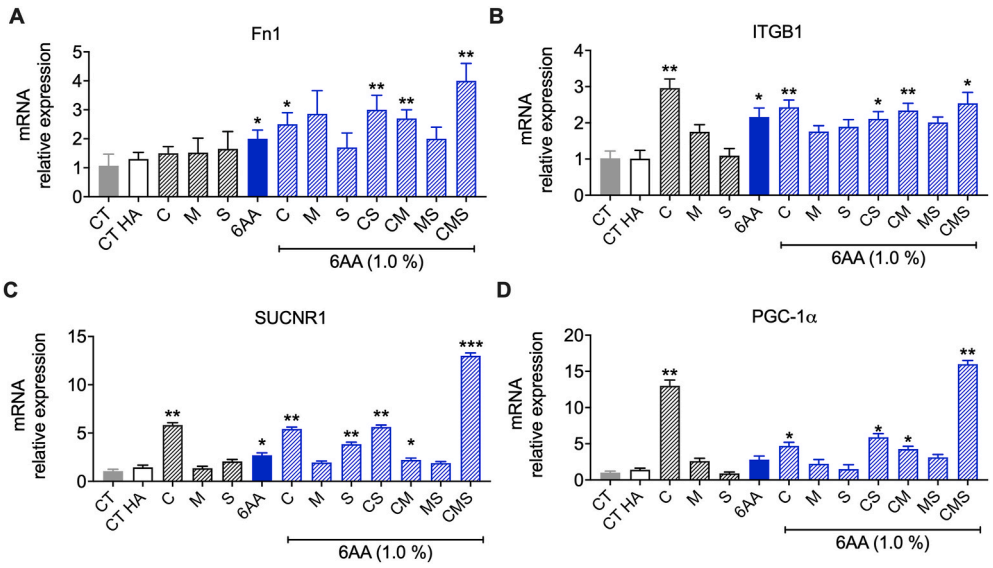

Fig. 7. *Fn1* (A), *ITGB1* (B), *SUCNR1*(C) and *PGC-1α* (D) mRNA in TKE2 fibroblasts treated with 1% 6AA with or without TCA cycle intermediates for 48 h. Relative mRNA levels were normalized to TBP (n = 3 in triplicate, mean ± SD). **P < 0.01 and * P < 0.05 *vs.* untreated cells, expressed as 1.0.

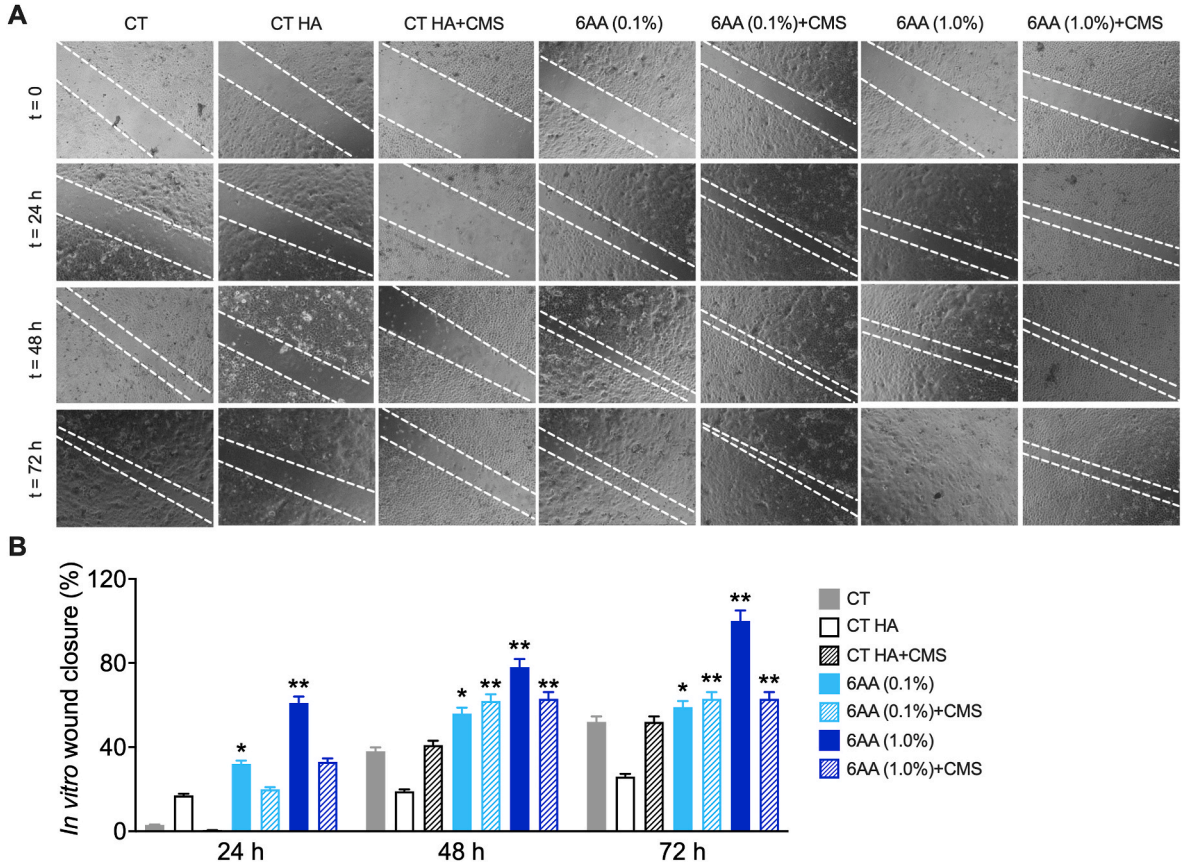

Fig. 8. The *in vitro* scratch assay of TKE2 cells with control, HA, 6AA 0.1% and 6AA 1% +/− CMS. A) Representative pictures (n = 6 images/group) of TKE2 cells cultured in the presence or absence of 6AA with or without CMS. B) Extent of wound closure in scratch assays after 24 h, 48 h, and 72h. *P < 0.05 and **P < 0.01 *vs.* untreated cells.

transporter present in the inner mitochondrial membrane (Slc25a1) mediates this efflux of citrate from the matrix into the cytoplasm in exchange for malate from the cytoplasm into the matrix. As part of the citric acid cycle in the mitochondrial matrix, succinate is formed from succinyl-CoA synthetase and converted by succinate dehydrogenase to generate fumarate. Succinate usually is present in the mitochondria; however, it can be released to the extracellular space due to local energy metabolism (Krebs, 1970) through the succinate receptor (SUCNR1), belonging to G protein-coupled receptors (GPCR) family. HA resides in the interstitial collagenous matrices to augment viscosity and hydration;

HA binds to a "link molecule motif" of HA-binding proteoglycans (i.e., CD44) and link proteins. In most tissues, native HA has a high molecular mass of 1000–10000 kDa, with size lengths of 2–20 μm. The receptor for HA-mediated motility (RHAMM) was first identified as a 60–63 kD soluble binding partner of the extracellular matrix component in chick fibroblast cultures (Turley, 1982). Also, the receptor for HA-mediated motility (RHAMM) belongs to the GPCR family.

We observed in the present work an increase of *SUCNR1* mRNA levels after 6AA treatment of TKE2 cells supplemented with either CMS combination or each TCA intermediate. This upregulation doesn't seem to correlate to the increased *Fn1*, *ITGB1*, and *PGC*-1α gene expression or wound healing because the *SUCNR1* induction in the presence of CMS doesn't change markedly the effects observed with the 6AA alone. Given that it has been demonstrated that the SUCNR1 levels increase in the presence of hypoxia and the stimulation of succinate receptors in the eyes induces the production of vascular epithelial growth factor A to sustain vascularization after injury (Saoieha et al., 2008), the increase of SUCNR1 might be necessary to the regeneration processes after injury. However, high levels of SUCNR1 expression were found in patients with Mooren's ulcer (Li et al., 2018). This ulceration is a rare disease (incidence of 0.03% in China) (Chen et al., 2000), characterized by a painful peripheral corneal ulcer, of unknown aetiology—autoimmunity seems to contribute to its pathogenesis with possibly genetic and environmental factors (e.g., infection, trauma, or systemic disease) (Taylor et al., 2000). Although SUCNR1 signaling has been found in inflammatory and metabolic disorders (Li et al., 2016; Liu et al., 2020; Macias-Ceja et al., 2019), high levels of succinate are reported under physiological conditions, such as exercise and postprandial states (Astiarraga et al., 2020). The discovery of cognate receptors for such energy metabolite (He et al., 2004) offers a potential regulatory mechanism, through which succinate has a hormone-like function acting in various organs and tissues, including the cornea, where it controls carbohydrate and lipid metabolism and regulate cellular processes (Blad et al., 2012). Therefore, investigating nutrient metabolism in degenerative ocular disorders might contribute relevant understanding and therapy suggestions.

In summary, our data suggest that 6AA is significantly more active than BCAAem and α5 formulas in promoting markers involved in the extracellular matrix process, like Fn1 and ITGB1 and markers of mitochondrial biogenesis. Moreover, 6AA restored wound healing in TKE2 cells in 72 h. For the first time, we detect PGC-1α protein expression in TKE2 cells. Thus, this study shows essential evidence of the 6AA mixture's ability to repair corneal epithelial wounds, justifying more appropriate clinical trials to confirm its benefits in humans. The principal limitation of this work is the lack of *in vivo* experiments; we have shown only *in vitro* results. Additional *in vivo* studies will be needed to affirm the efficacy of this new amino acid formula in animal models of corneal trauma and understand the SUCNR1 role in this disorder. In addition, whether or not the results apply only to amino acid-deficient environments, or if they are evident in the presence of other potentially damaging chemicals — including advanced glycosylated end products or excess free radicals, both of which are common to the diabetic phenotype — remain to be investigated.

Author contributions

L.T. and F.R. performed experiments; L.T., F.R. and E.N. analyzed data; L.T. and C.R. prepared the figures; L.T. and E.N. wrote the manuscript; all authors made suggestions, read and approved the final version.

Declaration of competing interest

All authors declared no conflicts of interest, financial or otherwise.

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