Archives of Dermatological Research (2019) 311:491–497
https://doi.org/10.1007/s00403-019-01927-8

ORIGINAL PAPER

Carboxyethyl aminobutyric acid (CEGABA) lacks cytotoxicity and genotoxicity and stimulates cell proliferation and migration in vitro

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Received: 18 May 2018 / Revised: 12 April 2019 / Accepted: 2 May 2019 / Published online: 13 May 2019

Abstract

Cosmeceuticals are cosmetics formulated using compounds with medical-like benefits. Though the antiaging effect of carboxyethyl aminobutyric acid (CEGABA) has been discussed, its action mechanism in cosmeceuticals remains unclear. This study assessed the in vitro efficacy and safety of CEGABA. NHI-3T3 mouse fibroblast cell line was treated with two CEGABA concentrations (50 and 500 µmol/L) for 24 h, 48 h, and 72 h. Cytotoxicity and genotoxicity were evaluated by colorimetry (MTT) and the alkaline version of the comet assay, respectively. Flow cytometry and the scratch-wound assay were used to assess cell-cycle phase distributions and cell migration rates. Compared with the untreated control, CEGABA increased cell growth 1.6 times after 72 h, independent of dose. The compound also decreased cell replication time by 4 h. These findings seem to be related with the approximately 1.5-times increase in phase S cells numbers. Importantly, in vitro wound healing improved roughly 20% after treatment with CEGABA for 24 h and persisted after 48 h, indicating culture recovery. The time-dependent proliferation and migration of fibroblasts induced by CEGABA besides the fact that the compound is neither genotoxic nor cytotoxic makes it an ideal candidate in the development of cosmeceuticals in antiaging therapy.

Keywords CEGABA · Cytotoxicity · Fibroblasts · Genotoxicity · Scratch-wound assay

Introduction

Defined as a state of inhibition of cell proliferation [33], cell ageing is characterized by the progressive decrease in an organism's homeostasis capacity, leading to senescence and apoptosis [15, 21, 32, 33]. In the ageing process, the structural integrity of cells is negatively affected, manifesting as loss of collagen and deficit of elastic fiber networks due to the presence of dysfunctional fibroblasts [38].

Fibroblasts are key cells in the regulation of tissue structures and production of matrix proteins, cytokines, growth factors, and metalloproteinases that in turn are essential for tissue integrity [17, 37]. More specifically, fibroblast growth factors play a fundamental role in wound healing and cell regeneration [24]. Several skin revitalization approaches have been developed, such as the use of substances that stimulate collagen production and improve elasticity. A number of such topical formulations have been shown to induce the regeneration of the system of elastic fibers in the ageing skin [25]. However, the efficacy,

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safety, and likely interactions of some cosmeceuticals with the human organism have never been confirmed in research [28].

In this context, the search for new compounds that prevent and mitigate the effects of ageing has become a priority in the development of new active cosmetics [39]. As one of such promising compounds, carboxyethyl aminobutyric acid (CEGABA) has sparked the interest of the cosmetic industry. CEGABA is a synthetic dicarboxylated polyamine derivative structurally related to spermidine, putreanine, and isoputreanine. Originally considered a growth-promoting agent for plants [30], the compound has been identified in bovine brain and cerebrospinal fluid [8]. Preliminary observations demonstrated CEGABA's in vitro growth-promoting activity on some specialized mammalian cells, like murine and human B lymphocytes and macrophages, secretory cells from the rat seminal vesicle and testes, and hybridomas [2, 3].

In an important study on the topic [10], the authors claim that the role of CEGABA in cell metabolism remains unaffected independent of whether evaluation is carried out in vivo or in vitro. It was suggested that the compound improves skin elasticity and turgor, and indicates that CEGABA is a viable candidate in the formulation of antiaging products [34]. The efficacy of CEGABA in cosmetics is therefore related to its role in the induction and stimulation of cell metabolism without inducing any toxic effects [8, 10].

Although CEGABA is an ingredient of currently marketed cosmetics, its efficacy and cytotoxic and genotoxic potentials have not been investigated. In this scenario, the present study looked into the effect of CEGABA on proliferation, cell cycle distribution, and cell migration of a fibroblast culture. Cytotoxicity and genotoxicity of the compound were also evaluated. To our knowledge, this is the first study to examine the efficacy and safety of CEGABA in fibroblast model.

Materials and methods

Reagents, culture media, and solutions

Dulbecco's modified Eagle Medium (DMEM) and Hank's balanced salt solution (HBSS) were purchased from Invitrogen Corporation, Carlsbad, CA, US, Fetal bovine serum (FBS) was purchased form Cultilab, Campinas, SP, Brazil. Unless otherwise stated, all reagents, including CEGABA (Carboxyethyl-γ-aminobutyric acid; catalog number C1181) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Plasticware was obtained from BD Falcon (New Jersey, NJ, USA).

Cell line and culture maintenance

The standard mouse fibroblast cell line NIH-3T3 provided by the RJCB Collection (Federal University of Rio de Janeiro, RJ, Brazil) was maintained in 25-cm^2 containing DMEM supplemented with 10% FBS in a controlled environment (37 °C, 5% CO_2, and 95% minimum humidity).

Evaluation of cell proliferation and doubling population time (DPT)

Cell proliferation was analyzed inoculating cells in 24-well plates (2×10^4 cells/well) which were then stabilized for 24 h. Next, cells were treated for 24 h, 48 h, and 72 h with CEGABA 50 µmol/L, 100 µmol/L, and 500 µmol/L. These concentrations were stipulated based on a previous study [3]. The proliferative effect of CEGABA was evaluated counting the cells in a Neubauer chamber and calculating mean cell numbers per well. The DPT of the different cultures was calculated according to the following formula: log (final cell number) − log (initial cell number = $K \times T$, where K is the generation constant (0.008963) and T is time in days [12].

Cell-cycle distributions

Cell-cycle phase distributions after treatment with 50 µmol/L of CEGABA for 72 h, were evaluated by flow cytometry with propidium iodide as staining agent. Cells (5×10^5) were washed, removed from the flasks with trypsin/EDTA, and resuspended in culture medium. Cells were then centrifuged, washed in cold phosphate buffer saline (PBS), and fixed in ethanol 70%. Next, cells were centrifuged again, washed twice in PBS, and treated with a solution prepared with sodium citrate 2.4 mmol/L, propidium iodide 20 µg/mL, and RNase A 100 µg/mL for 30 min in the dark.

Cells (20,000) were assessed using a flow cytometer (Accuri, BD Biosicences). Results were expressed as percent number of cells in each cell-cycle phase [26, 35].

Evaluation of cell migration in vitro

Cell migration in vitro was assessed using the scratch-wound test as described in the literature [40, 41]. Briefly, 10^5 NHI-3T3 cells/well were incubated in triplicate in a 24-well plate under standard cultivation conditions for 24 h upon confluence as a monolayer. Then, monolayers were sectioned with the tip of a p200 pipette to slit a wound roughly the size of the well diameter and washed thoroughly with PBS 1× to completely remove cell debris produced. Subsequently, cell monolayers were treated with CEGABA 50 µmol/L and incubated again at 37 °C in a 5% CO_2 environment

during 48 h. Cell migration was recorded using a digital camera (AxioCamMRc, Carl Zeiss) with a 5-x zoom lens coupled to an inverted-phase contrast optical microscope (Axiovert 25, Carl Zeiss) and an image capture software (MRGrab 1.0.0.4, Carl Zeiss) at times 0 h, 24 h, and 48 h into treatment with CEGABA. Images were analyzed in an open-source image processing program (Image J 1.48). For quantification, the distance between the wound edges was measured at least 6 random points of four areas for each application, and the mean values were calculated (d). The percentage of wound healing (WH) was calculated as: %WH $[(d_{\text{original wound}} - d_{\text{healing}})/d_{\text{original wound}} \times 100]$.

Cytotoxicity evaluation

Cytotoxicity was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. Cytotoxicity assays were performed according to International Standards ISO 10993-6 [13, 22] with minor modifications. Cells were seeded in a 24-well plate, cultivated for 24 h, and treated on the following day with CEGABA 50 µmol/L, 100 µmol/L, and 500 µmol/L for 24 h, 48 h, and 72 h. After that, cells were incubated with MTT 150 µL per well (0.5 mg/mL MTT) for 3 h and incubated at 37 °C. The formazan crystals formed were dissolved in 100 µL dimethyl sulfoxide (DMSO). Each plate was read immediately on a microplate reader (Multiskan, Uniscence) at 540 nm. Three independent experiments were performed for each sample. Positive control (10% DMSO) and negative control (DMEM) were included in each experiment. Cytotoxic activity was performed comparing the absorbance of cells treated with CEGABA and negative control. Mean absorbance of the negative control was considered as 100% cell viability, and a reduction of cell viability by more than 30% is considered a cytotoxic effect (International Standard ISO 10993-5, 2009).

Evaluation of genotoxicity

Cells were seeded in a 24-well plate, cultivated for 24 h, and treated on the following day with CEGABA 50 µmol/L for 6 h and 24 h. The cell suspension was gently mixed with 0.75% low-melting agarose (37 °C), and the samples were spread out on a microscope slide previously embedded with 1.5% agarose. A coverslip was added, and the agarose layer was allowed to solidify at 4 °C for 15 min. After solidification, the coverslip was removed, and the slides were immersed in a lysis solution (NaCl 2.5 M, EDTA 100 mmol/L, and Tris 10 mmol/L, pH 10.0–10.5 containing freshly added 1% triton X-100 and 10% DMSO) at 4 °C for at least 1 h, protected from light. At the end of this period, the slides were placed in a horizontal electrophoresis chamber and left immersed in an alkaline solution (NaOH 300 mmol/L, EDTA 1 mmol/L, pH > 13) for 20 min. Electrophoresis was carried out for 15 min. at 25 V and 300 mA (0.7 V/cm). After electrophoresis, the slides were neutralized, fixed, and stained with $AgNO_3$ as described previously [23]. Independent experiments were performed for each treatment in quadruplicate. Cells were analyzed in an optical microscopic based on the shape of 100 randomly selected cells (for each independent treatment), totaling 400 cells per concentration. The extent of DNA damage was evaluated classifying comets into five categories based on the length of migration and/or the perceived relative proportion of the DNA in the tail to the size of head (nucleus): 0 representing undamaged cells (comets with no tail) and 1–4 representing increasing relative tail intensities and smaller head size. Two parameters, damage index (DI) and damage frequency (DF), were used to evaluate DNA damage [36]. The DI of a group could range from 0 (completely undamaged = 100 cells × 0) to 400 (maximum damage = 100 cells × 4). DF (%) was calculated for each sample based on the number of cells with tail versus those without tail.

Statistical analysis

The data obtained for the three repeats were statistically analyzed using an analysis of variance (ANOVA) followed by Dunnett's multiple comparison test ($\alpha = 0.05$) in the software Graphpad Prism version 5.01.

Results and discussion

Fibroblast growth (NHI-3T3 cell line) treated with CEGABA for 24 h and 48 h did not differ from that observed for the untreated control (Table 1; Fig. 1). However, cell growth 72 h into cultivation was 1.6 times higher compared with the untreated control, independent of the CEGABA concentration (Table 1; Fig. 1), indicating that CEGABA induced cell proliferation after 72 h exposure. This finding is in agreement with the results published previously [10, 34]. Indeed, it was demonstrated that CEGABA retains its role

Table 1 Effect of treatment with CEGABA on fibroblast NHI-3T3 proliferation after 24 h, 48 h and 72 h

	24 h	48 h	72 h
Untreated control	22.7 ± 8.9	51.9 ± 9.5	114.9 ± 18.9
50 µmol/L CEGABA	18.0 ± 5.2	49.2 ± 8.5	185.9 ± 15.0*
100 µmol/L CEGABA	21.3 ± 2.8	50.4 ± 7.3	190.1 ± 10.4*
500 µmol/L CEGABA	20.6 ± 3.4	51.0 ± 6.6	189.7 ± 14.6*

Results are expressed as number of cells × 10^4 (mean ± standard deviation; $n = 6$)

*Statically different from untreated control ($p < 0.05$)

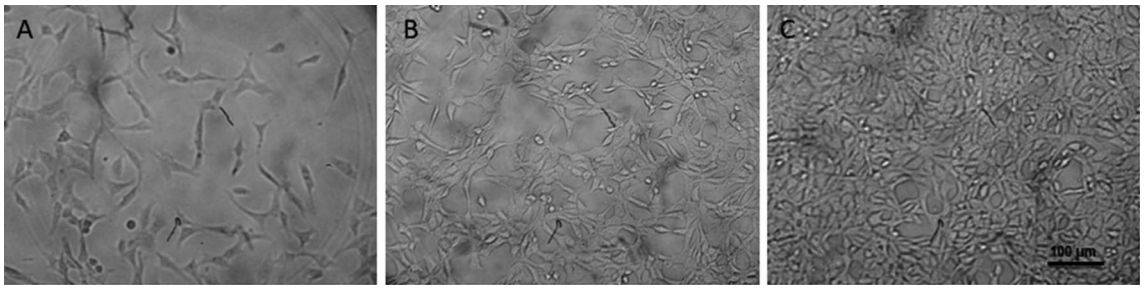

Fig. 1 Microphotographs of fibroblast line NHI-3T3. **a** Culture on the first day into the experiment. **b** Cultures not treated with CEGABA 72 h into treatment. **c** Cultures treated with CEGABA 500 µmol/L 72 h into treatment (×100 magnification)

Table 2 Effect of treatment with CEGABA 50 µmol/L on fibroblast NHI-3T3 cell distributions after 72 h

	Cell-cycle distributions		
	G0/G1(%)	S (%)	G2/M (%)
Untreated control	57.5 ± 3.8	16.2 ± 2.1	25.9 ± 1.4
CEGABA	43.1 ± 5.6*	25.3 ± 3.4*	30.8 ± 3.5*

Results are expressed as percent number of cells in each stage of the cell cycle (mean ± standard deviation; $n = 6$)

*Statically different from untreated control ($p < 0.05$)

in cell metabolism activation in vivo or in vitro. Nevertheless, in vivo use of CEGABA in these studies induced an increase in fibroblast proliferation and collagen production rates in skin [10, 34].

The results obtained also indicate that CEGABA increases cell division rate. The doubling population time (DPT) of NHI-3T3 cell line treated with CEGABA was 17.0 ± 1.0 h. This means a 4 h decrease in replication time, compared with the untreated control, for which the value was 21 h ± 1.0, (as reported for the NHI-3T3 cell line; http://www.nih3t3.com). It may therefore be suggested that CEGABA works as growth factor, which was also reported in previous studies with various cell types [2, 3, 30]. In view of the statistically similar response to treatment with CEGABA, the remaining experiments were carried out using only the 50 µmol/L concentration.

The induction of cell proliferation may be explained partly by cell cycle phase distribution. Within 72 h after CEGABA treatment, the percent of cells in S and G2 phases increased, while the percent cells in G0/G1 phase decreased (Table 2). Compared to a baseline value (16% for S phase and 25% for G2 phase) for untreated control cells, CEGABA promoted a 1.5-times increase in the amount of S phase cells and 1.2-times increase in the amount of G2 phase cells. In this line, it has been shown that cells enter the S phase to duplicate DNA in response to a growth-promoting signal (serum or growth factors) [7, 14]. Moreover, higher percentages of cells in the S and G2 phase indicate an increase in the number of cells entering the proliferation cycle and higher proliferation potential [16]. As a polyamine derivative, CEGABA is involved in a series of biological processes [19]. Polyamines play multiple functions, mostly, linked with cell growth, survival, cell proliferation, cell matrix repair, cell adhesion, and a number of signaling processes [18, 20, 27, 42]. In this sense, it is possible to suggest that CEGABA acts as a polyamine, increasing cell growth.

The evaluation of cell migration in vitro showed that wound closure started to increase at 24 h after the wound was inflicted both in the cells treated with CEGABA and the untreated control cells (Fig. 2a). Moreover, wound closure increased approximately 60% in cells treated with CEGABA versus 50% in untreated control between 0 h and 24 h treatment. After 48 h the wound inflicted was 75% closure after CEGABA treatment (Fig. 2b). Thus, we can suggest that CEGABA, beside inducing cell proliferation, can stimulate cell migration, which is also an important process in the wound healing of skin [31]. The growing similarity in cell migration data between the untreated control and cells treated with CEGABA after 48 h is due to (i) the fact that control cells continue to grow, (ii) the apparent saturation of CEGABA receptors 48 h into treatment, and (iii) the fact that CEGABA concentration was exhausted in the culture medium. This is in agreement with a study using hybridomas cells, which suggested that CEGABA gives a plateau of growth activity between 5 µg/mL and 10 µg/mL [2]. Moreover, in another study CEGABA demonstrated moderate effect on cell migration after 4 days in culture, which is a comparatively long treatment period [3].

Considering (i) that CEGABA may work as growth factor-stimulating cell proliferation [1–3, 30] and (ii) several studies have demonstrated the role of growth factors like EGF, TGFα, and FGF in cell proliferation and regeneration [4, 21, 29, 30], the results of the present study afford to suggest that CEGABA plays a role in improving migration of fibroblasts. In this line, others molecules tested for skin treatment demonstrated similar results. For instance, an extract of *Spirulina platensis* (used in skin creams) induced higher proliferation activity as well as enhanced wound healing

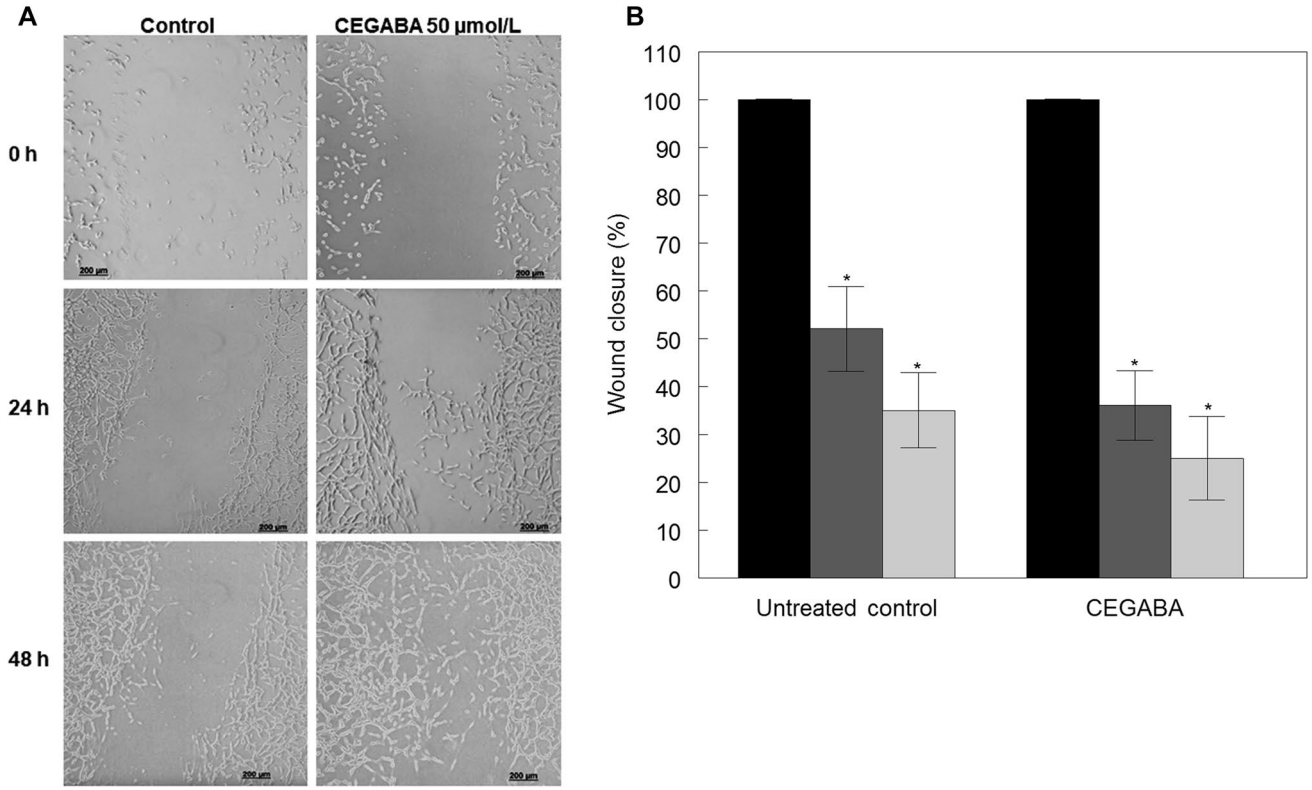

Fig. 2 **a** Microphotograph of the untreated control and cultures treated with CEGABA 50 µmol/L for 0 h, 24 h, and 48 h. **b** Quantification of cell migration in wound-healing assays after CEGABA 50 µmol/L exposure for 0 h (black), 24 h (dark grey) and 48 h (light grey). Data are presented as percentages of the recovered scratch area relative to time 0 h ($n=3$). Results are shown as mean ± SD (error bars). *statistically different from 0 h ($p<0.05$)

effect on HS2 Keratinocyte cell line when compared to culture medium [11]. There are also evidences that *Aloe vera*, today incorporated in a variety of products for skin disorders and hair repair [9], increases proliferation in fibroblasts through stimulation of FGF-2 [6].

To verify the safety of CEGABA, we performed cytotoxicity and genotoxicity evaluation. CEGABA cytotoxicity was assessed by colorimetric assay (MTT), according to the International Standards for Biological Evaluation of Medical Devices [13]. Our results show that treatment with CEGABA was not significantly cytotoxic independent of the concentration used and treatment times (Fig. 3). This is in agreement with previous study [30], which demonstrated that this compound is only slightly cytotoxic to rats. The evaluation of genotoxicity was performed using the alkaline comet assay. This assay stands as a potential tool to increase the sensitivity of an experimental system, because it also reveals damage very early (i.e., DNA double- and single-strand breaks, alkaline labile and transient repair sites, DNA crosslink, and oxidative damage) [5]. The data obtained in the present study indicate that CEGABA does not have genotoxic potential (Table 3) after 6 h and 24 h treatment, confirming that it is safe for use as cosmeceutical. To the best of our knowledge, this is the first study to look into the cytotoxicity and genotoxicity of CEGABA.

Conclusion

CEGABA promoted the proliferation and migration of fibroblast cells independent of concentration, but varying with time. The concentrations of CEGABA used were neither cytotoxic nor genotoxic, independent of treatment times. As far as we know, this is the first paper to investigate the role of CEGABA considering its cytotoxic and genotoxic potential. In view of the importance of growth factors in

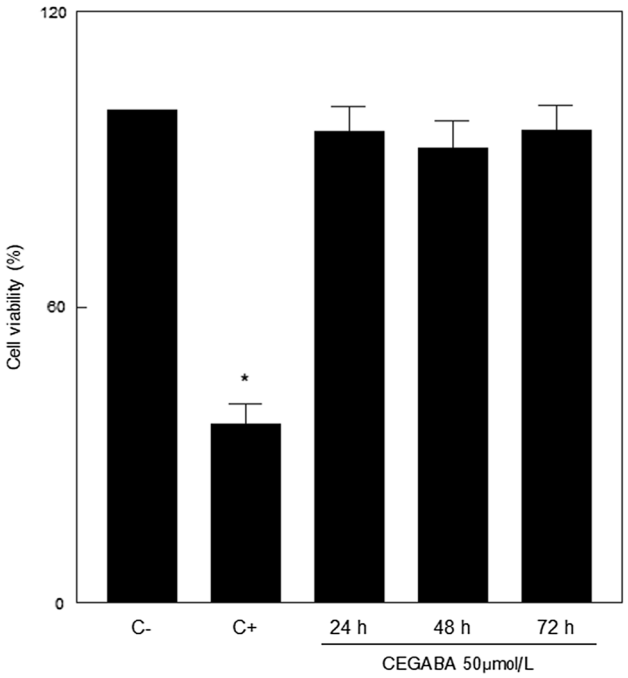

Fig. 3 Effect of CEGABA (50 µmol/L) after 24 h-, 48 h-, 72 h-exposure on cell viability. Data were plotted as the mean ± standard deviation of three different experiments. *Statistical difference of untreated control ($p < 0.05$). *C*− negative control (culture medium); *C*+ positive control (DMSO 10%)

Table 3 Evaluation of the genotoxic activity of CEGABA 50 µmol/L in fibroblast cell line NHI-3T3 using the alkaline version of the comet assay ($n = 4$)

	6 h		24 h	
	DI[a]	DF[b]	DI	DF
C−[c]	123.8 ± 22.4	77.5 ± 4.5	132.5 ± 22.3	54.3 ± 9.3
CEGABA	124.0 ± 32.8	81.8 ± 8.1	149.0 ± 16.3	64.5 ± 11.2
C+[d]	392.8 ± 8.5***	98.8 ± 1.9***	365.8 ± 31.3***	97.3 ± 4.3***

***$p \leq 0.001$ (ANOVA, Dunnett's test) compared with C−

[a]DI: damage index: varies from 0 (no damage, 100 cells × 0) to 400 (maximum damage, 100 × 4)

[b]DF (%): damage frequency: percent number of cells presenting damage

[c]C−: negative control (cells treated with culture medium)

[d]C+: positive control (cells treated ex vivo with H_2O_2 0.25 mmol/L)

the cosmeceutic industry and taking together our results, it is possible to suggest that CEGABA is safe and could be of potential value in skin cosmeceutical and biomedical applications.

Funding This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES) (Grant no. 181/2012). Finance Code 001.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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