Evaluation of the radioprotective activity of new green tea-collagen materials

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The aim of this study was to compare the protective effect of a green tea (*Camellia sinensis*) extract (GTE) and mixtures of GTE with collagen (COL) (GTE-COL I-V) against ultraviolet (UV) radiation using a primary culture of human dermal fibroblasts. Concentrations of GTE up to 25 μg/ml had a significant positive effect on dermal fibroblast proliferation, spectrophotometrically assayed by the MTT test. However, concentrations of GTE higher than 25 μg/ml, added in the culture medium, induced a cytotoxic effect on dermal fibroblasts. Similar results were found after UV-irradiation, when cells were cultivated in presence of the same concentrations of GTE. On the contrary, the fibroblast growth rate was similar or higher than the control, when cells were seeded on GTE-COL mixtures, even for the variants containing 50 μg/ml and, respectively, 100 μg/ml GTE. These observations were found both before and after cell irradiation. Cell adhesion data were in agreement with these results. Microscopical examination of the cells showed maintenance of the normal fibroblast phenotype, in the presence of all GTE-COL mixtures, before and after irradiation. All these data suggest that the tested GTE-COL materials could be efficient agents for skin protection against UV radiation.

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1. Introduction

Exposure of human skin to solar UV radiation may cause sunburn, immunosuppression, oxidative stress, skin cancers, as well as premature aging owing to overexpression of matrix metalloproteinases and denaturation of collagen and elastin [1]. Among the three regions of sunlight UV radiation, UV-B is particularly known to affect skin by altering cellular functions.

Camellia sinensis extracts and/or constituents are continuously studied, especially for their skin photoprotective activities [2]. Several groups have demonstrated that topic treatment or oral administration of GTE inhibited skin tumour development after UV radiation exposure of hairless mice [3,4] or humans [5]. It has been shown that epigallocatechin-3-gallate (EGCG) is the most effective polyphenolic constituent in GTE against reactive-oxygen species (ROS) [6]. Also, EGCG inhibited both intracellular release of hydrogen peroxide induced by UVB in keratinocytes and phosphorylation of epidermal growth factor receptor [7,8]. Lee *et al*. (2003) showed that EGCG was more effective than other potent antioxidants (trolox, lipoic acid, melatonin) in preventing *in vitro* lipid peroxidation [9]. A hidrophilic cream containing green tea polyphenols (GTPP) had the potential to attenuate UVB-induced oxidative stress through yet unclear mechanisms [10]. Also, *in vitro* proliferation of immortalized human keratinocyte cells (HaCaT cell line) and of primary human keratinocytes was inhibited by UVB and restored by EGCG treatment [11].

COL, the major macromolecular component of all animal connective tissues, is an important natural source for composites intended for skin regeneration [12,13] and surface coating of biomaterials for accelerating receptor-mediated cell adhesion [14].

In the present study we examined the *in vitro* radioprotective potential of both GTE and GTE-COL mixtures against UV-B radiation using spectrophotometric and light microscopy methods.

2. Materials and methods

GTE extraction. Leaves of *Camellia sinensis* were imported from China. A fixed quantity of plant material (10 g) was decocted with double distilled water (100 ml) for 3 min, at 100 °C and filtered. The filtrate was concentrated in a rotary evaporator (Heidolph, VV Micro, CE), at 55-60 °C and finally freeze-dried in a Lyophilizer (Gamma 1-16, Christ, Germany), yielding 0.88 g of total GTE. The extract was standardized by quantitative determination of total phenolic and flavonoid content [15]. The reducing power of GTE was assayed using the potassium ferricyanide assay [16] and its metal chelation activity was analyzed using the bi-pyridyl method [17].

COL isolation. Bovine tendons were purchased from the local slaughter house. Minced tissue (100 g) was treated with 1% (w/w) pepsin (E.C. 3.4.23.1) in 0.5 M acetic acid. In the resulting solution, 2.4 M NaCl was added and COL type I was isolated by centrifugation at 6000 rpm, for 20 min. Hydroxyproline (Hyp) and hexosamine content and the average molecular weight (M) for COL preparation were assayed [18].

GTE-COL mixture preparation. In each well of a 12-well culture plate, 0.5 ml of COL solution (5 mg/ml) were mixed with various quantities of GTE: 10 μg/ml (GTE-COL I), 25 μg/ml (GTE-COL II), 50 μg/ml (GTE-COL

III), 100 µg/ml (GTE-COL IV) and 200 µg/ml (GTE-COL V). The mixtures were then dried at 35 °C, for 6 h.

GTE and GTE-COL mixture's biocompatibility. A primary culture of human dermal fibroblasts was established from human skin explants, using the outgrowth technique, as previously described [19]. Only cells from passages 2 to 5 were used in the experiments. Cells ($1x10^4$ cells/ml) were placed in 12-well culture plates and cultivated for 20 h to allow cell attachment. Then, culture medium (DMEM) containing GTE (10, 25, 50, 100 and 200 µg/ml) was added and cells were incubated for 24 h and 48 h, respectively. For the control group, GTE was not added. Similarly, DMEM was added over sterilized mixtures of GTE-COL I, II, III, IV and V, placed at the bottom of 12-well culture plates. The conditioned medium was added on attached fibroblasts, and incubated for 24 h and 48 h, respectively. In both experiments, after the incubation period, the cell viability was measured by the MTT assay. A well containing cells cultivated on plastic and incubated in the same conditions was used as control.

UV irradiation. The UV-B irradiation source was a transilluminator (120 mW/cm^2) that emitted an energy peak at 312 nm. UV-B irradiation was performed in both GTE+cells and mixture+cells plates, incubated in phosphate buffered saline (PBS). After irradiation, the plates were incubated for 48 h, in the same culture conditions as described above and cell viability was measured on each plate, using the MTT assay. An UV-treated control (cells cultivated on plastic) was included on the plate and was identically cultivated.

MTT assay. The method is based on the cleavage of tetrazolium salt (MTT) by the mitochondrial succinate dehydrogenase in viable cells to form insoluble blue crystals of formazan, as previously described [20]. Briefly, the culture medium was discarded, cells were carefully washed with PBS, 500 µl of freshly prepared MTT solution (0.25 mg MTT in 1 ml DMEM) were added and the cells were incubated at 37 °C, for 3 h. Cells were then washed and the blue dye was liberated from the cells with 1 ml isopropanol. The amount of blue dye dissolved in isopropanol was measured as absorbance at 570 nm using an UV-VIS spectrophotometer (Jasco V-530, Japan). The experiment was carried out on each sample plate, after 24 h and 48 h, respectively. Results were expressed in arbitrary units, considering the control (untreated cells) as 1 unit of absorbance. All experiments were performed in triplicate.

Cell adhesion and morphology. Human dermal fibroblasts ($3x10^4$ cells/ml) were cultivated on the GTE-COL mixtures conditioned as substrates, as described above. After 48 h, the adherent cells from one plate were dispersed by collagenase type I solution (1 mg/ml) treatment, for 15 min and then stained with a 0.4% Trypan blue solution. The viable, unstained cells and nonviable, coloured cells were counted under a bright-field microscope using a Burker-Turk haemocytometer. Another identic plate was UV-irradiated 24 h after cell plating, cultivated for 48 h and then similarly processed. To analyze cell morphology, fibroblasts cultivated on GTE-COL mixtures, for 48 h were stained with Giemsa and photographed using an inversed-phase microscope (Nikon, Japan). Irradiated plates were similarly investigated for cell morphology.

Statistics. Data were expressed as means ± standard deviation of the mean (S.D.) Statistical analysis was performed using Student's test. Differences were considered significant at $p<0.05$.

3. Results

GTE antioxidant capacity. The values for the total polyphenolic and flavonoid content of GTE and its antioxidant power, assayed as ferric reducing and metal chelating activities are shown in Table 1. The GTE had a high content of polyphenolic and flavonoid compounds. Its reducing power was 4 times higher as compared to that of butylated hydroxy toluene (BHT), a standard synthetic antioxidant, because the concentration of GTE needed to reduce a certain amount of Fe^{3+} was significantly lower than that of BHT. The value calculated for GTE metal chelation activity was close to that observed for quercetin, a standard bioflavonoid with metal chelating properties (Table 1).

Table 1. Total polyphenol content, expressed as caffeic acid equivalents (CAE), flavonoid content, expressed as catechin equivalents (QE) and reducing power and metal chelation activities of GTE.

Sample	Total polyphenol content (g CAE kg^{-1} d.s.)	Flavonoid content (g QE kg^{-1} d.s.)	Ferric reducing power [a)] (µg d.s. ml^{-1})	Metal chelation activity [b)] (µg d.s. ml^{-1})
GTE	322.13 ± 2.23	9.87 ± 0.07	45 ± 1.10	5.02 ± 0.11
Trolox	N.D.	N.D.	> 2000	N.D.
BHT	N.D.	N.D.	180 ± 5.09	N.D.
Quercetin	N.D.	N.D.	N.D.	1.95 ± 0.04

Each value is mean ± S.D. of three replicate experiments. N.D., not determined. [a)]Amount to give an absorption value of 0.1. [b)]Amount to inhibit 0.1% of chromogen complex formation.

COL analysis. The biochemical parameters for the COL solution were quantitatively determined and presented in Table 2. Results indicated that COL preparation had a high content of Hyp and its molecular weight was similar to that of standard COL type I (Sigma).

Table 2. Composition and molecular weight of type I COL extracted from bovine tendon and purified by NaCl precipitation.

Sample	Hyp (%)	Hexosamine (%)	Molecular weight (Da)
COL type I	11.2	0.8	320.000
COL (Sigma)	11.5	0.7	310.000

GTE and GTE-COL mixtures' biocompatibility testing. The effect of a wide range of GTE concentrations on cell growth rate was evaluated by the MTT test. Results showed that, up to 25 µg/ml GTE, no cytotoxicity was found, since cell viability remained above the level of the control after 24 h of incubation and was close to control after 48 h of incubation (Fig. 1A). At 50, 100 and 200 µg/ml GTE, cell viability was very low compared to the control, at both incubation periods, indicating that higher concentrations of the extract are cytotoxic for fibroblast cells.

The exposure of dermal fibroblasts to various mixtures of GTE-COL I, II, III, IV and V resulted in no cytotoxic effect after 24 h and 48 h of incubation (Fig. 1B). Moreover, cells cultivated on GTE-COL III, IV and V showed a much higher absorbance (0.98, 0.92 and 0.84, respectively) than cells treated with the same GTE quantities (0.44, 0.089 and 0.052, respectively).

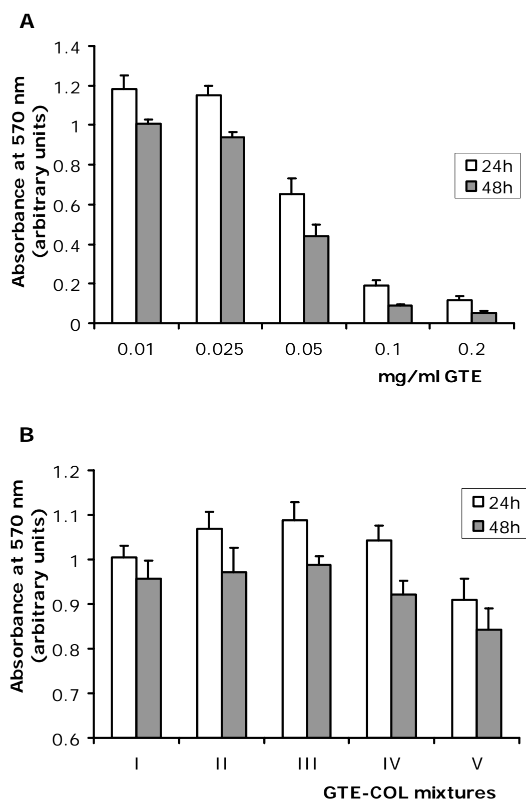


Fig. 1. Effects of GTE (A) and GTE-COL mixtures (B) on the viability of human dermal fibroblasts, assessed by the MTT assay.

In order to assess the mixtures' abilities to protect cells against UV radiation, we used the GTE-COL I-IV variants and the corresponding GTE concentrations in the following experiments.

In vitro testing of GTE and GTE-COL mixtures as radioprotective products in a dermal fibroblast culture. When dermal fibroblasts were pretreated with 10 and 25 µg/ml GTE, an increase in absorbance was observed, at 48 h after UV-irradiation. The highest value for cell survival was observed at 25 µg/ml GTE, i.e. 0.88 (Fig. 2A). At higher concentrations (i.e. above 25 µg/ml) a negative shift in cell survival was registered. On the other hand, the cells cultivated on mixtures of GTE-COL I, II, III and IV showed high values of their viability after UV-radiation. When using the mixture with the lowest GTE concentration (GTE-COL I), the cell viability value was slightly higher (no significant differences, $p>0.05$) than that of the control (irradiated cells on plastic). The mixture which provided best protection against UV radiation for dermal fibroblats was GTE-COL III (1.05, a value found to be statistically significant, $p<0.05$) (Fig. 2B).

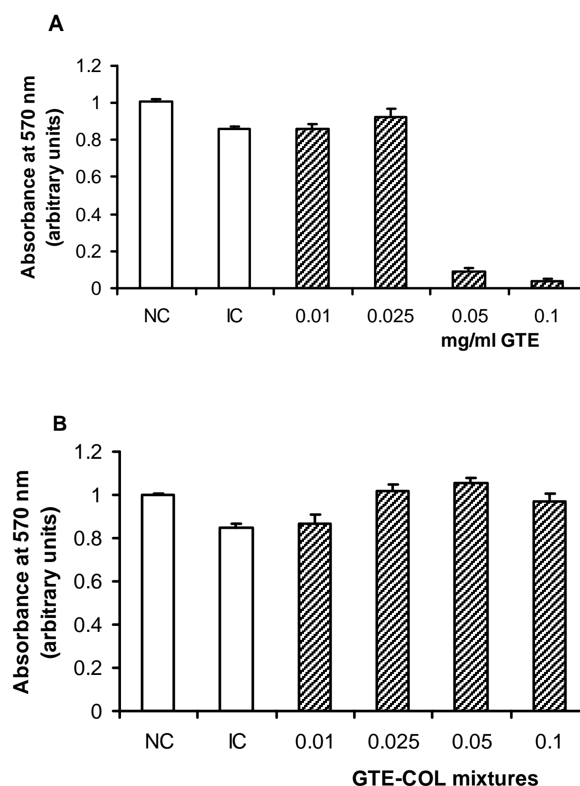


Fig. 2. Radioprotective effects of GTE (A) and GTE-COL mixtures (B) on UV-B-induced oxidative stress in primary culture of human dermal fibroblasts, assessed by the MTT assay. (NC - nonirradiated control; IC-UV-B irradiated control)

Cell adhesion and morphology on GTE-COL substrates. The results, presented in Fig. 3, show the percentage of dermal fibroblasts attached to the four different substrates (GTE-COL I-IV mixtures), at 48 h post-seeding, with and without UV-irradiation. The number of cells adhered to each GTE-COL mixture was close to control. While 100% of cells remained attached to GTE-COL II and III, after 48 h, approximately 90% (92.92% and 93.3%, respectively) remained attached to GTE-COL I and IV. UV-irradiation had a significant effect on cell attachment. Irradiated control contained only 85% viable cells attached to the bottom of the well. Over 90% viable cells remained attached to mixtures II and III, compared to 78% viable cells that remained attached to mixture I and 85% viable cells that remained attached to mixture IV.

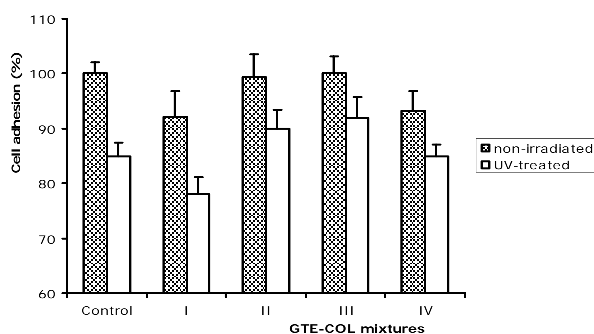
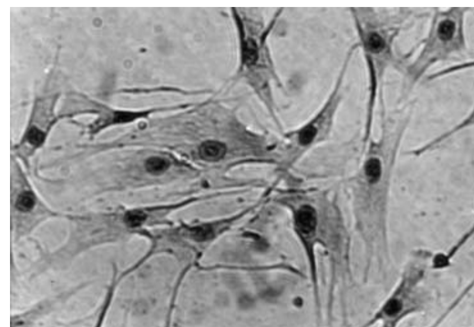


Fig. 3. Cell adhesion of human dermal fibroblasts plated onto GTE-COL mixtures, with and without UV-radiation treatment. Statistics showed a significant effect of UV-radiation ($p<0.05$) on cell adhesion to variants I-IV, compared to untreated samples.

Cell treatment with GTE-COL mixtures caused no morphological modifications of human dermal fibroblasts. Based on microscopic examinations, both control (Fig. 4A) and GTE-COL III mixture-treated cells (Fig. 4B) presented the characteristic, elongated morphology of normal fibroblasts, with one euchromatic nucleus, 2-3 proeminent nucleoli and a fine granular cytoplasm. After irradiation, both cells from control (Fig. 4C) and cells cultivated on GTE-COL III mixture (Fig. 4D) maintained the normal morphology and had a slightly reduced proliferation activity compared to non-irradiated cells. Similar results were obtained in the case of cells cultivated on the other mixture variants (data not shown).



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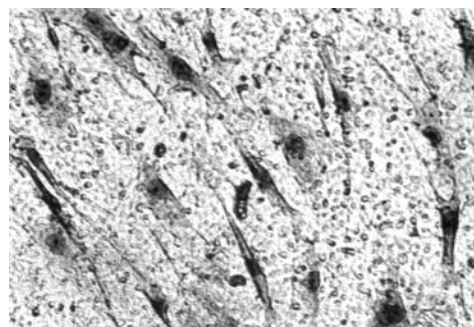

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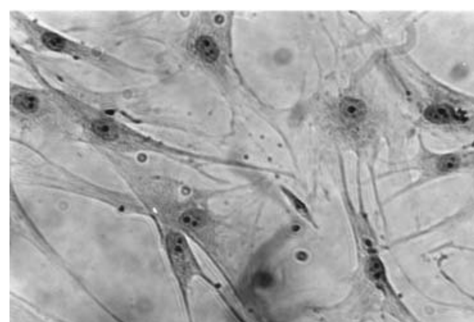

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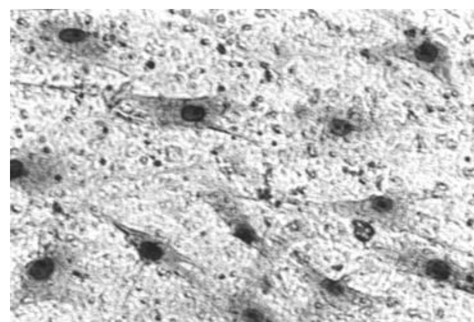

d

Fig. 4. Effects of GTE-COL mixtures on cell morphology in primary human dermal fibroblasts. The cells were cultured on plastic (A, C) and on GTE-COL III mixture (B, D) as substrate, for 3 days, before (A, B) and after UV-treatment (C, D). (Giemsa staining; x200).

4. Discussion

The present paper aims to compare the cellular radioprotective activity of different concentrations of GTE alone or mixed with a fixed quantity of COL in a normal primary cell system. To our knowledge, no studies investigating the effect of GTE-COL mixtures on *in vitro* irradiated dermal cells have been published to date.

The radioprotective activity, at cellular level, involves several pharmacological actions, such as decrease of lipid and protein oxidation, increase of cell proliferation and metal chelation, reduction of ROS generation [21]. It has been shown that increased levels of ROS generated during irradiation were effectively scavenged by ECGC from green tea as well as by other antioxidants present in plants, such as phenolic compounds (tocopherols, flavonoids and phenolic acids), nitrogen compounds (alkaloids, amines, amino acids and chlorophyll derivatives), carotenoids and vitamins, including ascorbic acid [22]. An earlier study showed that the antioxidant activity of vegetal extracts could be assigned mainly to their phenolic content [23]. Although the specific mechanisms of action remain unclear, previous studies indicated that the molecular targets for green tea polyphenols (GTPP) include proteins from the mitogen-activated protein kinase (MAPK) signaling pathway and modulation of different genes [24].

Under our *in vitro* experimental conditions, 25 μg/ml GTE alone and an 8-fold greater concentration of GTE mixed with a certain quantity of COL did not induce cytotoxicity on a dermal fibroblast culture. Cell viability was 2.2-fold higher for GTE-COL III and 10-fold higher for GTE-COL IV than the values registered for cells treated with the same concentration of GTE alone. Cell adhesion results indicated that all mixtures of GTE-COL were good substrates for dermal fibroblast cells. Moreover, cells exposed to UV-B irradiation were better protected in the case of GTE-COL mixtures than in the case of GTE alone. The high increase in absorbance observed for fibroblasts cultured on GTE-COL III could be due to both GTE high quantity which confers a better cell radioprotection and COL's presence that provides a high cellular biocompatibility.

It can be assumed that COL itself may play a role as radioprotective factor through its influence on signal transduction cascades that regulate cell cycle progression [25]. It is well known that COL provides both mechanical and chemical stimuli to cells through integrins bound to cytoskeleton. Also, cultivation on monomeric COL films results in an increase of cell proliferation and differentiation [26]. On the other hand, COL has a small content in aromatic amino acids which absorb only short-wave UV-C radiation (200-280 nm). After solar exposure, loss of water and conformational changes of COL molecule were reported, based on spectral observations of the 275 nm peak which became more shoulder-like [27]. COL degradation mechanisms were hypothesized, but the same authors found that UV-B and UV-A radiation could not break the COL chains into smaller fragments [28]. We presume that COL films containing GTE induced cooperative pathways to regulate physiologic response to UV irradiation stress in the tested cell culture.

5. Conclusions

Our experiments revealed that COL-GTE materials induced a significant increase in the proliferation and adhesion of both UV-irradiated and untreated human dermal fibroblasts. Both spectrophotometry and light microscopy results proved that these new materials are good radioprotective agents due to the sinergistic action of their constituents. Further studies designed to test the *in vivo* effect of these new compounds are necessary in order to provide additional data and a greater understanding of the mechanisms involved in the radioprotective process.

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