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Synergistic Effect of L-Carnosine and EGCG in the Prevention of Physiological Brain Aging

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Abstract: The benefits of multi-target action are well established in a variety of pathological models. Many dietary supplements and nutraceuticals may be useful to slow age-related cognitive declines and the risk of developing neurodegenerative disease. L-Carnosine and EGCG are natural compounds that have received particular attention because of their potential role in modulating oxidative stress associated with aging and chronic conditions. The biological activities of these naturally occurring substances have frequently been used to prevent or reduce senile features; however they have never been evaluated as a combined treatment. In the present study we investigated the combined effect of L-Carnosine and EGCG on the activation of two stress-responsive pathways: HO-1 and Hsp72 (the inducible form of Hsp70), which play an important role in cytoprotection against oxidative stress-induced cell damage. We demonstrated that the neuroprotective effects of EGCG and L-Carnosine are achieved through the modulation of HO-1/Hsp72 systems. Furthermore, the combined action of both compounds resulted in a synergistic increase of HO-1 expression which suggests a crosstalk between the HO-1 and the Hsp72-mediated pathways. Our results indicate that the combined administration of EGCG and L-Carnosine would benefit the treatment and prevention of neurodegenerative diseases by reducing the neuronal damage caused by oxidative stress.

Keywords: Oxidative stress, synergy, nutraceuticals, antioxidants, heme oxygenase-1, heat shock protein, aging, neuroprotection.

INTRODUCTION

The concept of synergy has been explored extensively in the fields of pharmacology and Berenbaum was among the first to define pharmacological synergy [1-3]. A synergistic interaction is present if the "effect seen by a combination of substances is greater than would have been expected from a consideration of individual contributions" [4]. Examples of pharmacological synergies are increasingly widespread in the scientific literature [5, 6]. Moreover, it should be noted that many single-target drugs cannot fully correct a complex disease condition or a pathological process [7, 8].

Over the past decades scientists have been searching for novel drugs that might be used for the prevention and treatment of life-threatening or debilitating diseases, as well as for the improvement of wellbeing and quality of life in the aging population [9, 10]. In this perspective the importance of natural products for medicine and health has been enormous. Aging is associated with disruption of cerebral function and increased susceptibility to neuronal loss. Nutrition has been recognized as an important factor in the modulation of disease and longevity and several studies suggest that consumption of diets rich in functional foods may help to counteract age-related cognitive declines and the risk of developing neurodegenerative disease [11-14]. (-)-Epigallocatechin-3-gallate (EGCG) and L-Carnosine (β-alanyl-L-histidine, L-Car) are among the very few nutraceuticals able to cross the blood brain barrier and to accumulate in the brain [15, 16]. L-Car is an endogenous dipeptide found at high concentrations in glial and neuronal cells throughout the brain [17]. L-Car has been studied extensively and even though its functional role is still not completely understood many biological functions and therapeutic actions have been proposed. Several studies indicate that L-Car has useful features and significant neuroprotective actions by acting as anti-oxidant and free-radical scavenger [18, 19]. In addition to its ability to quench an excess of reactive oxygen species (ROS), L-Car has also been proposed as a metal-chelating agent with beneficial effects in the context of neurodegenerative diseases [20-22]. L-Car was suggested to be useful for preventing accumulation of aging features by interfering with the glycation processes and preventing the crosslinking of glycoxidised proteins to physiological macromolecules [23, 24]. EGCG also has been reported to have antioxidant, anti-glycating, metal-chelating, and neuroprotective activities [25-28]. EGCG is a member of the catechin family and it is a major polyphenolic constituent of green tea leaves [29]. There are numerous epidemiological studies that have emphasized the health-promoting effects of EGCG and recently this compound has attracted scientific attention as a potential nutritional strategy to counteract age related chronic disorders and to improve longevity [30]. A considerable number of intracellular signaling pathways have been described to play a central role in the neuroprotective effects of EGCG against extracellular insults, such as mitogen-activated protein kinases (MAPK) [31], protein kinase C (PKC) [32], phosphatidylinositol-3-kinase (PI-3 kinase)-Akt pathways [33]. To gain insight into the potential synergistic effect of L-Car and EGCG, we decided to investigate whether the combinations of these supplements can prevent ROS-induced neuronal cell death. More in detail, we evaluated whether the combined treatment of EGCG and L-Car synergically affect the expression and activity of two main player in the stress response pathways: the heat shock protein 72 (Hsp72) and the heme oxygenase-1 (HO-1). Hsp72 is the major stress inducible form of cytosolic Hsp70 and one of the best studied chaperones of the Hsp70 family which consists of at least 12 members [34]. Hsp72 confers a cytoprotective role against various environmental stresses [35] and several neuroprotective effects have been shown against a variety of insults [36]. A wide array of exogenous factors and physiological signals are able to induce transcription and translation of Hsp72, including inflammatory response and oxidative stress [37, 38]. During stressor exposure different intracellular signals are involved in the expression/release of Hsp72 which is coupled to activation of signal-

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ing pathways such as Toll-like receptors, adrenoreceptors, NF-kB and cAMP [39] inducing a plethora of adaptive responses.

HO-1 is one of the phase II enzymes and it is involved in the metabolism of heme for iron reutilization and oxidative stress tolerance [40, 41]. Specifically, this stress-inducible intracellular enzyme plays a pivotal role in the degradation of heme into carbon monoxide (CO), ferrous iron, biliverdin, and bilirubin [42]. The cytoprotective effect of HO-1 may be attributed to its by-products, i.e. bilirubin, CO and free iron [43]. Moreover, the products of heme degradation have strong influence on inflammatory processes and the induction of HO-1 pathway has been shown to act as a powerful defensive mechanism for tissues exposed to an oxidant challenge.

Here we show that the combination of EGCG and L-Car reduce neuronal cell death caused by oxidative stress through the induction of the endogenous antioxidant system HO-1/Hsp72.

MATERIALS AND METHODS

Cell Cultures

The embryos were extracted from a 17-day pregnant Wistar rat (Harlan-Sprague-Dawley, Indianapolis, IN, USA). The fetuses were sacrificed and the heads placed in high glucose phosphate buffered saline (PBS) (Sigma, St. Louis, MI, USA). The brains were then quickly dissected under a stereomicroscope to isolate brain cortex, which was then cut into small fragments and exposed to papain, activated in the presence of cysteine and ethylen-diamino-tethracetic acid (EDTA), for 9 minutes at 37°C. The fragments were mechanically dissociated through a fire-polished Pasteur pipette to obtain single cells suspension. The cell suspension was layered onto a gradient consisting of 2 ml Earle's balanced salt solution (Gibco, Carlsbad, CA, USA) containing 20 mg of bovine serum albumin and 20 mg of trypsin inhibitor (ovomucoid), and then centrifuged. The pellet was successively resuspended in high-glucose Dulbecco's modification of Earle's medium containing 10% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA) and antibiotics (100 IU/ml of penicillin and 100 µg/ml of streptomycin) (ICN-Biomedicals, Aurora, OH, USA). The cells were then counted and tested for viability using the trypan blue exclusion test (viability was > 99%). Finally, the cells were seeded onto 25 cm^2 T-flasks (Corning, NY, USA) previously coated with poly-D-lysine. After 48 hours, 1 µM cytosine arabinoside was added to the cells to inhibit glial cell growth. Cell cultures were incubated at 37°C in a humid 5% CO2-95% air environment. This protocol produced an enriched neuronal culture. For the cell treatment we used the following compunds: 3,4,5-Trihydroxybenzoic acid (2R,3R)-3,4-dihydro-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-2H-1-benzopyran-3-yl ester (EGCG), (TEAVIGOTM, DMS Nutritional Products, purity min. 94 %). L-Car (Sigma, St. Louis, MI, USA, purity min. 98 %).

Real Time Quantitative RT-PCR

Total RNA from cell cultures was extracted using Trizol (Sigma, St. Louis, MI, USA). Single-stranded cDNAs were synthesized incubating total RNA (1 µg) with SuperScript II RNase Hreverse transcriptase (200 U), oligo-$(dT)_{12-18}$ primer (100 nM), dNTPs (1 mM), and RNase-inhibitor (40 U) at 42 °C for 1 hour in a final volume of 20 µl. Reaction was terminated by incubating at 70 °C for 10 min. Forward (FP) and reverse (RP) primers were used to amplify HO isoforms (primer sequences are available on request). The expected amplification products for HO-1, HO-2 are 123 and 331 base pairs, respectively. Aliquots of cDNA (0.1 and 0.2 µg) and known amounts of external standard (purified PCR product, 10^2 to 10^8 copies) were amplified in parallel reactions. Each PCR reaction (final volume 20 µl) contained 0.5 µM of primers, 2.5 mM Mg^{2+} and 1 x Light cycler DNA master SYBR Green (Roche Diagnostics, Indianapolis, IN, USA). PCR amplifications were performed with a Light-Cycler (Roche Molecular Biochemicals). Quantification was performed by comparing the fluorescence of PCR products of unknown concentration with the fluorescence of the external standards. For this analysis, fluorescence values measured in the log-linear phase of amplification were considered using the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals). Specificity of PCR products obtained was characterized by melting curve analysis followed by gel electrophoresis, visualized by ethidium bromide staining, and DNA sequencing.

Cell Viability Determination

Cell viability was determined in cortical neurons treated with for 12 h with EGCG 25 µM, L-Car 25µM, or with EGCG 25 µM + L-Car 25µM, followed by incubation for 2 hours in the presence of glucose oxidase (50 mU/ml). After treatment with glucose oxidase, cells were washed and exposed to complete medium containing 1% Alamar blue for 5 hours according to manufacturers' instruction (Serotec, UK). After the incubation period, optical density in the medium of each well was measured using a plate reader (Molecular Devices, Crawley, UK). The intensity of the red color is proportional to the viability of cells, which is calculated as difference in absorbance between 570 nm and 600 nm and expressed as percentage of untreated cells.

Heme Oxygenase Activity Assay

Microsomes from harvested cells were added to a reaction mixture containing NADPH, glucose-6-phosphate dehydrogenase, rat liver cytosol as a source of biliverdin reductase, and the substrate hemin. The mixture was incubated in the dark at 37°C for 1 hour and the reaction was stopped by the addition of 1 ml of chloroform. After vigorous vortex and centrifugation, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm (e = 40 mM-1cm-1).

Western Blot Analysis for HO-1

After treatment with ECGC and/or L-Car, samples of neurons were analysed for HO- 1 protein expression using a western immunoblot technique. An equal amount of proteins (30 µg) for each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Proteins were estimated by using bicinchoninic acid reagent. The nonspecific binding of antibodies was blocked with 3% nonfat dried milk in Tris-buffered saline (TBST). Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen Victoria, BC) (1:1000 dilution in TBS-T, pH 7.4), for 2 hours at room temperature. After three washes with TBS-T, blots were visualized using an amplified alkaline phosphatase kit from Sigma (Extra-3A), and the relative density of bands was analyzed by the use of an imaging densitometer (model GS-700; Bio-Rad, Herts, UK).

Statistical Analysis

Differences in the data among the groups were analyzed by using one-way analysis of variance combined with the Bonferroni test. Values were expressed as the mean ± S.E.M., and differences between groups were considered to be significant at $p < 0.05$.

RESULTS

Synergistic Effect of EGCG / L-Car Supplementation in Preventing Neuronal Cell Death

To assess the neuroprotective activity of EGCG, L-Car and of a mixture of the two compounds, cell viability was determined in mouse cortical neurons treated with different doses (0-100 µM) of the two compounds for 12 h (Fig. **1**). A significant decrease of viability of about 25% was observed only at the 100 µM dose of EGCG. We therefore decided to use the combination of the two compounds (25 µM each) before exposing the cells for 2 hours to glucose oxydase (50 mU/ml). The glucose oxidase oxidant system

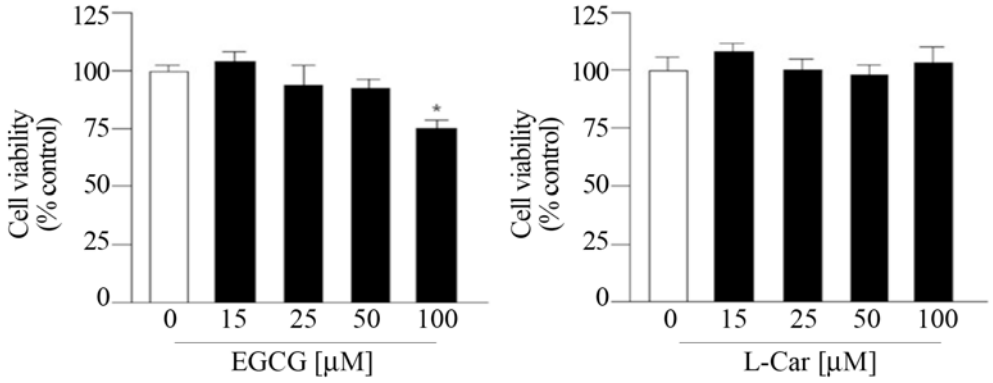

Fig. (1). Cell viability of mouse cortical neurons. The cells were treated for 12 h with 15, 25, 50 and 100 μM of EGCG or L-Car *P<0.01.

generates hydrogen peroxide at a constant rate and it is known to produce cellular injury *in vitro* [44]. Treatment of cells for 2 hours with glucose oxidase resulted in 27% of residual cell viability (Fig. **2**). Exposure of cells for 12 hours to 25 μM EGCG reduced glucose oxidase-mediated damage, rising cell viability to 51% (24% more than the glucose oxidase treatment alone). L-Car at 25 μM concentration was less effective in protecting cells from oxidative damage, giving a viability of 33% (7% more than the glucose oxidase treatment). Remarkably, the association of the two compounds protected cells in a synergistic way, giving a rate of neuronal survival of 76% (49% more than the glucose oxidase treatment, which is more than the expected additive effect of 31%).

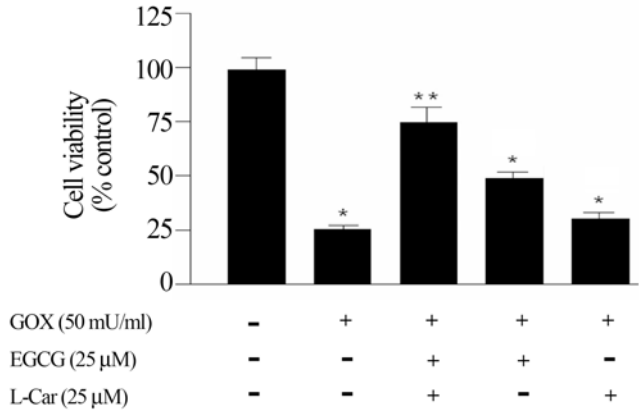

Fig. (2). Cell viability of mouse cortical neurons exposed to glucose oxidase. The cells were treated for 2 h with 50 mU/ml of glucose oxidase after exposure for 12 h with 25 μM of EGCG, L-Car or EGCG + L-Car.* P<0.01; **P<0,001.

Stimulation of the HO-1 and Hsp72 Pathways by EGCG / L-Car Supplementation

We assessed the ability of EGCG and L-Car to elicit the HO-1 and the Hsp72 pathways, by measuring HO-1 and Hsp72 gene expression through quantitative real-time PCR. HO-1 and Hsp72 mRNA steady state levels were measured following administration of increasing doses of EGCG and L-Car (Fig. **3**). Compared to the not-inducible HO-2 paralog gene, EGCG elicits a dose-dependent increase of HO-1 mRNA, which reaches the maximum (about 8 fold) at 25 μM, and decreases subsequently at 50 and 100 μM. L-Car is instead much less active in inducing HO-1 gene expression at the same concentrations, and stimulates HO-1 maximum expression at 25 μM. An opposite behavior is observed for Hsp72 gene expression which is normalized according to the levels of the paralog not-inducible gene Hsc70. EGCG administration gives a dose-dependent enhancement of Hsp72 expression at the highest concentration of 50 μM and 100μM. The last concentration is also associated to a 25% decrease in cell viability (see Fig. **1**). The 25 μM dose, known to have no effects on cell viability, does not enhance Hsp72 gene expression in a statistically significant way. L-Car at the same concentration induces an increase of Hsp72 of about 8-fold (Fig. **3**). To confirm that the increase in HO-1 gene expression l corresponded to an equivalent increase in HO-1 activity, we measured the amount of bilirubin, as indicator of HO-1 activity, after 6 and 24 hours of EGCG and L-Car treatments in presence of glucose oxidase. We observed an increase in HO-1 activity was comparable to the enhanced mRNA expression, confirming the functional significance of the data obtained by the real time PCR determinations (Fig. **4**).

L-Car Contributes to an Increase in the Levels of HO-1

To estimate the contribution of HO-1 in the synergistic neuroprotective effect of EGCG and L-Car we repeated the first experiment by adding also Tin protoporphirin IX (ZnPP), a compound known to potently and specifically inhibit HO-1 activity. As shown in (Fig. **5**), the inhibition of HO-1 activity caused by ZnPP reduced the neuroprotective effects of the combination of the two compounds by 35%, bringing it to the level obtained with EGCG alone. Determination of HO-1 by western blotting after EGCG and L-Car administration showed that EGCG strongly increased HO-1 expression while L-Car was much less effective. Similar results were already showed by quantitative real-time PCR. Interestingly their combination enhanced HO-1 protein levels in a synergistic way at 25 μM dose of treatment and decreased HO-1 expression at higher concentration (50 μM) of the compounds (Fig. **6**). Therefore, a contribution of L-Car could be observed also in terms of cooperative increase in the levels of HO-1, which is primarily induced by EGCG.

DISCUSSION

Because it is becoming evident that some drug combinations may induce a favorable clinical response in the treatment or prevention of age-related disorders, an emerging therapeutic approach involves the mixture of distinct compounds [8]. Recently, a growing number of studies have indicated that the dietary antioxidants may be beneficial for neuronal recovery and survival in neurodegenerative disorders [45]. Over the past years, the involvement of the HO-1 pathway in anti-degenerative mechanisms has received considerable attention [46, 47]. Notably, HO-1 induction occurs together with the induction of other heat shock proteins during various physiopathological conditions, generating potent protective system against brain oxidative injury [48]. In the current study, we provide for the first time evidence that the combination of EGCG and L-Car elicit two different pathways inducing synergistic neuroprotective effects and increasing viability of neuronal cells.

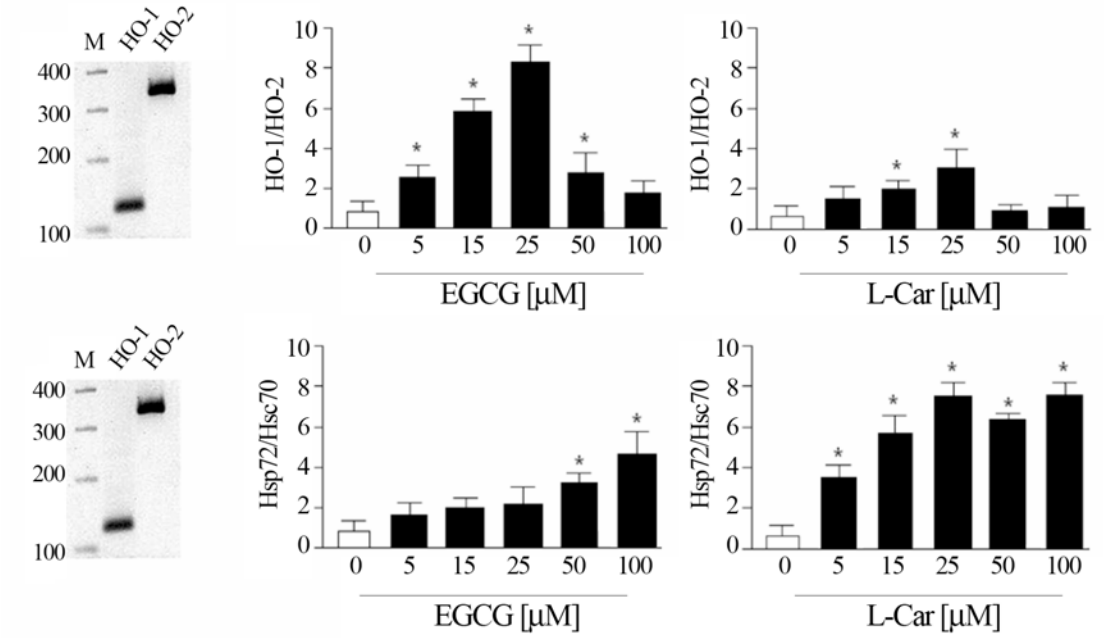

Fig. (3). HO-1 and Hsp72 mRNA steady state levels. Mouse cortical neurons were treated with increasing doses of EGCG and L-Car (5, 15, 25, 50 or 100 μM) and the levels of HO-1 and Hsp72 mRNA were measured.* P<0.01.

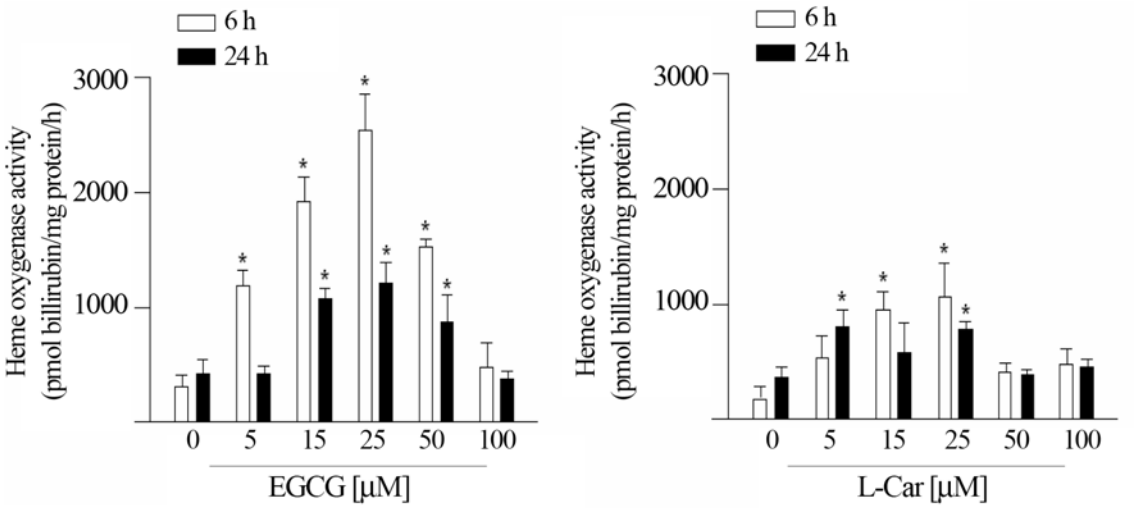

Fig. (4). Heme oxygenase activity of mouse cortical neurons. The amount of bilirubin was measured after 6 and 24 hours on cells treated with 5, 15, 25, 50 or 100 μM of EGCG or L-Car. * P<0.01

A significant cytotoxic effect of glucose oxidase on cell viability was demonstrated by using Alamar blue, a redox indicator to rapidly quantify neuronal viability (Fig. **2**). Our results demonstrated that the neuroprotective effects of EGCG and L-Car are achieved through the targeting of HO-1/Hsp72 systems. In particular, cells exposed to EGCG, have a dose-dependent induction of HO-1 expression with a peak at 25 μM, and an induction of Hsp72 expression at cytotoxic doses.

In contrast, at the same concentrations L-Car did not induce such as pronounced change in the expression of HO-1 but augmented the Hsp72 expression (Fig. **3**). We also confirmed the validity of the gene expression study evaluating HO-1 activity after short (6 hours) and prolonged (24 hours) exposure to the same concentration of EGCG and L-Car (25 μM). In accordance with the increase in expression we also registered and increase in the HO-1 activity which in turn prevents cell damage from the oxidative stress (Fig. **4**).

We finally verified the cytoprotective effect of HO-1 by treating cells with the HO-1 specific inhibitor ZnPP which increased ROS generation in glucose oxidase-stimulated cells (Fig. **5**) despite the administration of EGCG and L-Car. The results indicated that EGCG and L-Car-induced HO-1 expression serves as a protective mechanism from oxidative stress. Moreover western blot results showed that L-Car resulted less involved in HO-1 increase than EGCG (Fig. **6**). This suggests that Hsp72 mainly triggered by L-Car, provides an additional efficacy to the EGCG-elicited pathway.

Noteworthy, the effect of a combination of EGCG and L-Car was greater than the response achieved by the single compounds

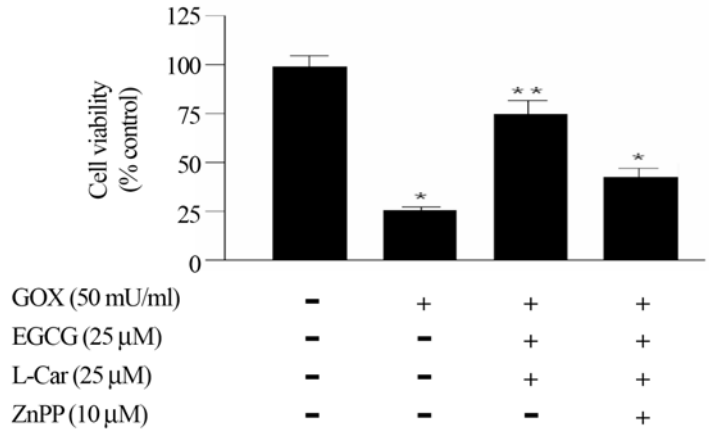

Fig. (5). Cell viability of mouse cortical neurons exposed to HO-1 inhibitor (ZnPP). The cells were exposed for 2 h into 50 mU/ml glucose oxydase after treatment for 12 h with 25 µM of EGCG, L-Car, EGCG + L-Car or 10 µM ZnPP. * P<0.01; **P<0,001.

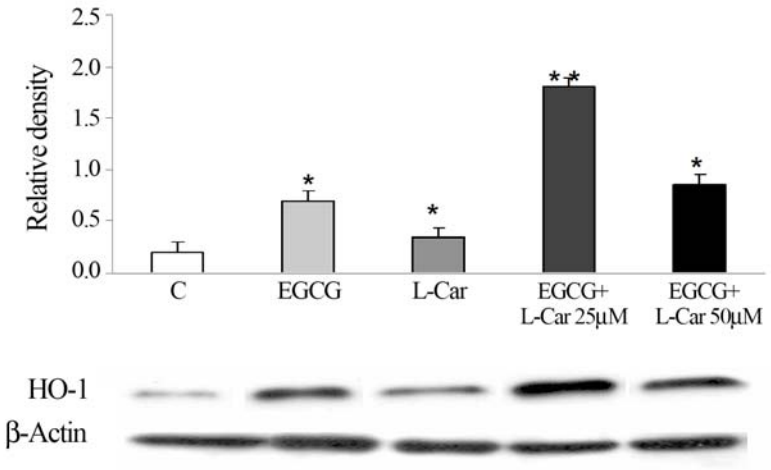

Fig. (6). Protein levels of HO-1 estimated by densitometry of bands of western blot. Neurons were treated with 25 µM of EGCG or L-Car and with, 25 or 50 µM of EGCG + L-Car. Levels of HO-1 are normalized to β-actin. * P<0.01. ** P<0.001.

alone and over the expected additive effect of EGCG and L-Car (Fig. **5**). The synergistic action of these two agents supports the idea of a crosstalk between the HO-1 and the Hsp72-mediated pathways which potentiates the efficacy of the two compounds used alone. Moreover, the hypothesis is further reinforced since we assumed that the cytoprotection obtained through the expression of Hsp72 is less effective when HO-1 is blocked with its inhibitor ZnPP.

Because of this crosstalk, combination of drugs activating the single pathways might have a more pronounced antioxidant effect in a number of physiopathological conditions where the sensing of redox status is imbalanced. These findings imply that HO-1/Hsp72 system might work as a promising therapeutic approach with potential for clinical usefulness.

CONCLUSION

To our knowledge, this is the first time that the antioxidant activities of the selected natural agents EGCG and L-Car were investigated in combinations. Our study demonstrates that combined administration of EGCG and L-Car possesses a more apparent antioxidant activity compared with each of them alone. Therefore, our findings indicate that the synergistic antioxidant effects of EGCG and L-Car may be a successful approach in the prevention of brain aging. Finally this study supports the promising therapeutic mechanism of EGCG and L-Car in protecting against oxidative stress-related diseases.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

Declared none.

ABBREVIATIONS

EGCG	=	(-)-Epigallocatechin-3-gallate
L-Car	=	L-Carnosine
ROS	=	Reactive Oxygen Species
MAPK	=	Mitogen-Activated Protein Kinases
PKC	=	Protein Kinase C
PI-3 kinase	=	Phosphatidylinositol-3-kinase
Hsp72	=	Heat Shock Protein 72
HO-1	=	Heme Oxygenase-1
ZnPP	=	Tin protoporphirin IX

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