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Neuroprotective effects of gabaergic phenols correlated with their pharmacological and antioxidant properties

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ABSTRACT

Aims: Various investigations have demonstrated the protective capacity of general anesthetics as neuroprotective agents. The effects of propofol against ischemia are known to reside in its antioxidant properties and its GABAergic activity. Other aromatic alcohols have also been reported as able to protect neurons against oxidative damage. The aim of this work is to evaluate the potential neuroprotective effect of some phenols, structurally analogues of propofol, with proven GABAergic activity. These phenols include the naturally occurring compounds thymol, carvacrol and eugenol, the synthetic product chlorothymol, and the most widely used intravenous anesthetic, propofol, as a reference compound.
Materials and methods: Taking primary cultures of cortical neurons as a suitable model to evaluate cellular protection against oxidative damage, we developed an injury model to test potential neuroprotective activity. The intracellular hydroperoxides were also determined.
Key findings: The results showed that no compound decreased cell viability at concentrations where they were active on the $GABA_A$ receptor. In neuroprotection tests, some phenols and Vit E showed a partial protective effect against the oxidative injury. These compounds induced a clear tendency to reduce H_2O_2 damage, comparing production of hydroperoxides, although these last changes were statistically non-significant.
Significance: Testing the intracellular oxidation levels suggests that this partial protection exerted by propofol, thymol and chlorothymol may be mediated in some way by their antioxidant activities. However, this neuroprotection is not completely correlated with the antioxidant capacity, but it approaches their relative pharmacological potency, which could be interpreted as a final effect that would involve both activities.

1. Introduction

The study of bioactive natural compounds has contributed significantly to the development of pharmacology and medicine through the analysis of their mechanisms of action, as well as providing models for the design of new therapeutic drugs. Numerous natural neuroactive drugs have particularly contributed to current knowledge in the field of the physiology and pharmacology of the nervous system [1]. Among the many products commonly isolated and investigated are essential oils extracted from plants and their main components such as terpenes, phenols, flavonoids, lactones, etc. In particular, many of these compounds have, among other actions, demonstrated significant activity on the nervous system as anesthetics, tranquilizers and sedatives [2–7].

The damage caused by ischemia in neurons is characterized by early death mediated by excitotoxicity or delayed death mediated by apoptosis. The vulnerability of the brain to the lack of blood supply has motivated substantial research efforts to identify pharmacological agents that can reduce brain damage [8]. Among these, general anesthetics have always been considered as logical candidates, due to their ability to reduce the metabolic rate of the brain, to antagonize glutamate-mediated excitotoxicity and enhance inhibitory synaptic transmission [9]. Potential mechanisms of neuroprotection include the inhibition of excitatory activity (as antagonists of NMDA and AMPA glutamate receptors) and the activation of inhibitory circuits (enhancing the activity of the $GABA_A$ receptor) [10–12].

It has been suggested that propofol would be an ideal anesthetic from the point of view of its possible beneficial effects on brain physiology. Various investigations have revealed the protective capacity of propofol against ischemia, which seems to lie in its antioxidant properties and its activities as an enhancer of GABA and inhibitor of glutamate release [13–15]. It has also been reported that other aromatic alcohols with intact phenolic groups, as well as various phenolic derivatives, were able to protect neurons against oxidative damage induced by

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glutamate and hydrogen peroxide because of their antioxidant properties, and that they lost this capacity when their hydroxyl groups were methylated [16,17].

The overall objective of this work is to evaluate the potential neuroprotective activity of lipophilic phenols, structurally analogues of propofol, with proven GABAergic activity [2,4]. These phenols include the naturally occurring compounds thymol, carvacrol and eugenol, the synthetic product chlorothymol, and the most widely used intravenous anesthetic, propofol, as a reference compound. The lipophilicity, membrane partition ability and "*in vitro*" antioxidant properties of all these compounds were previously studied by our group [18,19]. The correlation between their pharmacological activities and antioxidant potencies previously obtained, and the neuroprotective effects are also discussed.

2. Materials and methods

2.1. Reagents

Propofol (2,6-bis(isopropyl)-phenol), thymol (5-methyl-2-isopropyl-phenol), carvacrol (2-methyl-5-isopropyl-phenol), eugenol (2-methoxy-4-prop-2-enyl-phenol), chlorothymol (5-methyl-4-chloro-2-isopropyl-phenol), Dulbecco's minimum essential medium (DMEM), trypsin, soybean trypsin inhibitor, DNase, amino acids, bovine albumin, cytosine-arabinofuranoside, penicillin, insulin, poly-L-lysine and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and α-tocopherol (Vitamin E) were obtained from Sigma Chemical Co·(St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from PAA (Pasching, Austria). All other reagents were of the highest analytical grade.

2.2. Cell cultures

Primary cultures of cortical neurons were prepared from the cerebral cortices of 17–18 day-old Wistar rat fetuses, as previously described [2,20]. Animals were obtained from the Animal Facility of the Instituto de Investigaciones Médicas Mercedes y Martìn Ferreyra, INIMEC-CONICET-Universidad Nacional de Córdoba (Argentina). The animals were kept under controlled temperature (22–24 °C) and light cycle. Water and pelleted food were supplied *ad libitum*. All the procedures were in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Pregnant animals were killed by cervical dislocation and fetuses were extracted. Neocortices were dissected, mechanically minced, and cells then dissociated by mild trypsinization (0.02% *w*/*v*) at 37 °C for 10 min followed by trituration in a DNase solution (0.004% *w*/*v*) containing a soybean trypsin inhibitor (0.05% *w*/*v*). The cells were re-suspended in a DMEM medium (5 mM KCl, 31 mM glucose, and 0.2 mM glutamine) supplemented with insulin, penicillin and 10% FBS (DMEM-FBS). The cell suspension (1.6×10^6 cells/ml) was seeded in 96-well plates pre-coated with poly-L-lysine, and incubated for 6–7 days in a humidified atmosphere 5% CO_2/95% air at 37 °C. 20 μM cytosine arabinofuranoside was added after 36–48 h in culture to prevent glial proliferation.

2.3. Cellular injury model

Cell injury was quantitatively assessed by measuring the lactate dehydrogenase (LDH) released from damaged cells to the extracellular medium after 30 min or 24 h of treatment [2] by using LDH enzyme reagents from *Wiener Lab* (Rosario, Argentine) with an optimized UV method. The percentages of LDH release were calculated with respect to a sample exposed to triton corresponding to 100% of death. Cell cultures were treated with different concentrations of hydrogen peroxide (H_2O_2) [21,22] for 24 h to determine the harmful concentrations. The antioxidant effect of vitamin E (Vit E) (200 μM) was tested as a positive control of cell protection against oxidative injury. Cells were exposed to Vit E or phenolic compounds 30 min before contact with H_2O_2.

2.4. Determination of intracellular hydroperoxides

The intracellular production of hydroperoxides was assayed using DCFH-DA. The non-fluorescent DCFH-DA is permeable to the cell membrane and, by deacetylation to DCFH, is trapped inside the cell and oxidized by hydroperoxides to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [23]. The method followed in this work is that described by Perry et al. [24] with modifications. Briefly, cultures in 96-well plates were washed with HBSS at 37 °C and loaded with 10 μM DCFH-DA from a stock solution in methanol. After 20 min of loading at 37 °C, plates were washed again with HBSS, and 200 μl of NaOH 0.2 M was added to each well. The plates were shaken during 1 h and the fluorescence intensity was recorded in a fluorometer (*Fluoromax-3*, JobinYvon Inc., Edison, NJ, EEUU) set at 485 nm excitation / 520 nm emission. In all experiments, the fluorophore loading was previously confirmed by fluorescence microscopy, using a *Nikon Eclipse TE2000-U* (Tokio, Japan). Determination of intracellular hydroperoxides was carried out on 6–7 days cell cultures after 24 h of exposure to H_2O_2. Phenolic compounds or Vit E were added 30 min before the injury.

2.5. Statistical analysis

Data shown represent the mean ± standard error of mean (SEM) of n independent experiments. Statistical treatment of data was performed by one or two-way analysis of variance (ANOVA) as appropriate, using Fisher's LSD comparison method and Tukey's nonparametric test with $p < 0.05$.

3. Results

3.1. Phenol toxicity

To determine the effect of phenols on cell viability under the same conditions in which their GABAergic activities were proven [2,4], the cultures were exposed in the first assays for 30 min at different concentrations of each tested compound. The exposure time was selected taking into account the incubation time used in previous pharmacological studies. The results of LDH release indicated that none of the compounds showed significant effects on cell viability compared to the control ($p > 0.05$; one-way ANOVA) (results not shown).

3.2. Oxidative injury model

LDH release was determined in cell cultures exposed for 24 h to different concentrations of H_2O_2. Only the highest concentrations tested (400 and 600 μM) showed significant negative effects on cell viability (Fig. 1A). Thus, both these concentrations were chosen to test the protective effect of phenolic compounds. The positive control (Vit E) was able to protect cells from the effect of 400 μM H_2O_2, but only partially since it did not reach the basal value of the control cultures (Fig. 1B).

3.3. Neuroprotection test

Taking into account the injury model, the cells were initially treated with different concentrations of phenols for 24 h to assess their potential toxicity *per se* at longer times of exposure. No compound showed a decrease in cell viability until 24 h, showing similar values to controls (Fig. 2, white bars).

When cells were treated with harmful concentrations of H_2O_2 for 24 h in the presence of different phenols (added 30 min before injury), propofol (100, 250 and 500 μM), thymol (100, 250 and 500 μM) and chlorothymol (50 and 100 μM) demonstrated partial protective effects against damage by 400 μM H_2O_2, similar to those shown with Vit E. No treatment was able to protect cells from the effect of the highest concentration of H_2O_2 tested (600 μM) (Fig. 2, black and gray bars).

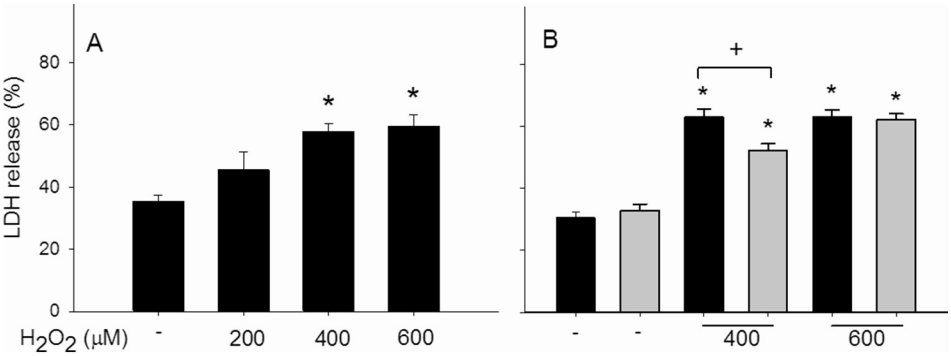


Fig. 1. Effect of H_2O_2 and Vit E on cell viability. A: Bars represent the percentage of LDH released after 24 h of exposure to different hydrogen peroxide concentrations. Values correspond to the mean ± SEM of 4 independent experiments performed in triplicate. *: $p < 0.05$ (one-way ANOVA). B: In these experiments Vit E (200 μM) (gray bars) was added 30 min before hydrogen peroxide exposure (for 24 h). Bars correspond to the mean ± SEM of 6 independent experiments performed in triplicate, *: $p < 0.05$ with respect to control, +: $p < 0.05$ between H_2O_2 400 μM in the absence and presence of Vit E (two-way ANOVA).

3.4. Intracellular hydroperoxides production

Considering the results obtained in neuroprotection tests, the comparative intracellular production of hydroperoxides was determined in the presence of Vit E, propofol (250 μM), thymol (250 μM) and chlorothymol (50 μM).

Fig. 3 shows the percentages of fluorescence intensity with respect to the control samples (without H_2O_2 and phenols). It can be observed that 400 μM H_2O_2 induced a considerable increase in fluorescence, signifying a higher intracellular content of hydroperoxides. The presence of phenols or Vit E induced a clear tendency to reduce H_2O_2 injury, matching the cell viability assays, although the changes were statistically non-significant.

4. Discussion

Propofol is a widely-used anesthetic with sedative, hypnotic, antiemetic and amnestic properties. Its clinical use is primarily for the induction and maintenance of general anesthesia in both diagnostic and therapeutic procedures [11,25]. Thymol is a naturally occurring phenolic monoterpene found as a component of many essential oils used in fragrances and flavorings with bactericidal properties [26]. Like other phenolic compounds, it has antioxidant properties, which explain its use as a stabilizer anesthetic in preparations of halothane [27]. A diet supplemented with thymol increases the antioxidant status and preserves levels of polyunsaturated fatty acids in the aged rat brain [28] and, as with carvacrol, improves the meat quality of domestic poultry intended for human consumption [29]. Carvacrol is a monoterpene phenolic product of numerous aromatic plants that has antimicrobial effects [30]. It is used on a large scale in the food and cosmetic industries and is a common ingredient of human diet [31]. Eugenol is an aromatic compound commonly contained in various types of plants, in particular medicinal herbs and spices with antimicrobial power. It is commonly used in dental procedures [32] and its nervous system effects have been widely studied ([33] and references therein). The inclusion of chlorothymol in this study is justified by its phenolic structure, similar to that of thymol, with a halogen atom (Cl) bound to the phenolic ring. It is used as a preservative in cosmetics and personal care products [34]. All these phenolic compounds have demonstrated GABAergic activity [2,4,35].

Neurons are cells that are particularly susceptible to oxidative damage due to their high consumption of oxygen, high number of mitochondria, low antioxidant enzyme activity, high concentration of polyunsaturated fatty acids (PUFAs) in membranes, their tendency to accumulate transition metals, their reduced cell regeneration capacity and the vicinity of microglial cells that produce radical oxygen species, etc. [36]. Immature and cortical cells are particularly vulnerable to oxidative stress and consequently to neurodegeneration. This makes the primary culture of cortical neurons a suitable model for evaluating cellular protection against oxidative damage, for example that induced by H_2O_2. This oxidizing agent may diffuse through membranes and alter the intracellular redox state [37].

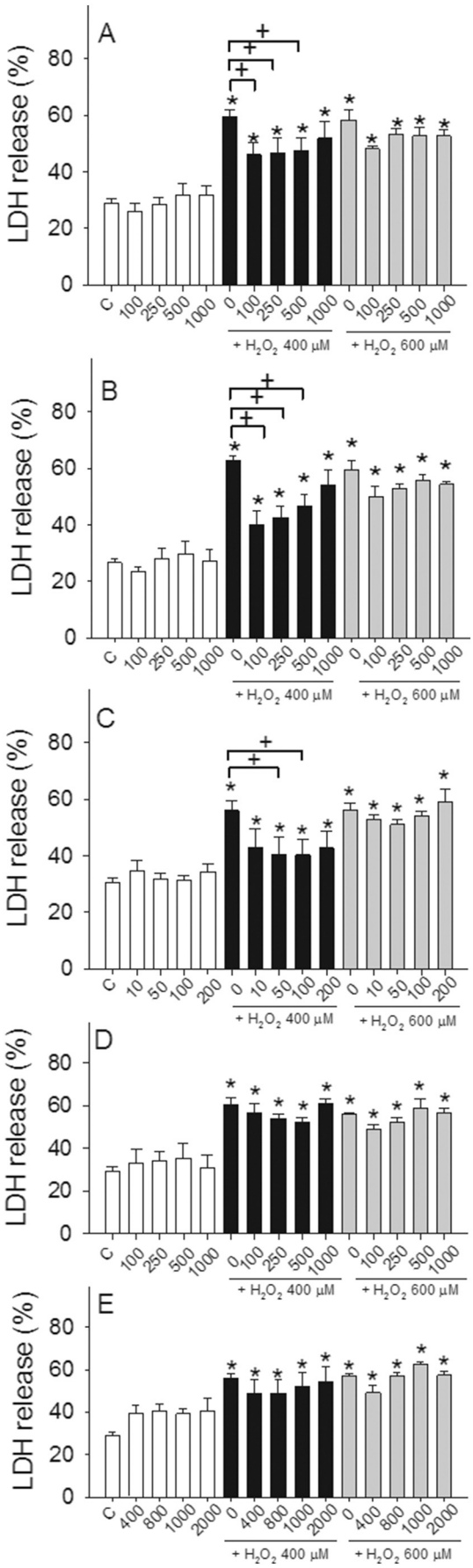
The present study demonstrated that none of the phenolic compounds tested produced cytotoxic effects on cortical neurons at the exposure times used (30 min or 24 h), at similar concentration ranges and under the same conditions as those used previously to check their GABAergic activity [2,4].

Having confirmed this lack of toxicity, an oxidative injury model by H_2O_2 was designed to evaluate their possible neuroprotective effect. This model was created from previous studies with other paradigms of H_2O_2 damage exerted on the same cell types. In these studies, exposure times of 24 h or 3 days, and H_2O_2 final concentrations between 60 and 300 μM were used in cultures, with an approximate cell density of $1.5–2 \times 10^5$ cells/cm^2 [21,22]. Considering the higher cell density of our cultures (1.6×10^6 cells/ml, equivalent to $\approx 4 \times 10^5$ cells/cm^2), the injury model we used included 24 h of exposure to varying concentrations of H_2O_2 (between 200 and 600 μM). To assess cytoprotection in these trials, cells were exposed to phenolic compounds half an hour before contact with H_2O_2, as indicated in Materials and methods [37].

The choice of Vit E as a reference antioxidant compound is because of its molecular properties, which give it a high hydrophobicity comparable to that determined for the phenolic compounds studied [19]. This property enables this compound to be partitioned into the membrane and to be located in areas close to those where the phenols would be located [38].

As described above, H_2O_2 was able to induce cytotoxicity, and this effect was partially reduced by the reference antioxidant (Vit E) and some phenols (propofol, thymol and chlorothymol). In the following paragraphs, we try to correlate these results with their pharmacological and antioxidant effects.

Considering, on the one hand, the pharmacological potencies as positive allosteric modulators of the $GABA_A$ receptor, and particularly the EC_{50} values corresponding to stimulation of the binding of [^{3}H]-flunitrazepam (a positive allosteric modulator) in similar cell preparations, the different phenols studied in this work showed the following order of activity: propofol (EC_{50}: 9 μM) [39] > chlorothymol (EC_{50}: 19 μM) [4] > thymol (EC_{50}: 131 μM) [2] > carvacrol (EC_{50}: 235 μM) [4] > eugenol (EC_{50}: 532 μM) [4]. On the other hand, if we consider their antioxidant power, determined in heterogeneous or membranous media as a more valid approach to a cellular system [18], the order of activity is: propofol ~ chlorothymol > eugenol > thymol ~ carvacrol. Taking both potency orders together, and considering the compounds that showed neuroprotective capacity here (propofol, chlorothymol and thymol), it is possible to make some correlations. Propofol and chlorothymol demonstrated comparatively greater pharmacological



potency and higher antioxidant activity, which may be related to their protective effects. However, thymol, the other compound that showed cytoprotection, exhibited lower antioxidant capacity than eugenol but comparable to that of carvacrol, but its pharmacological potency was much higher, suggesting that the neuroprotective effect would be the result of the addition of both activities: defense against oxidizing agents and improvement of the GABAergic transmission.

DCF fluorescence measurement can be used to evaluate the relative effectiveness of compounds that alter oxidative metabolism, but only in living cells or tissues, because esterase activity is necessary to remove the diacetate group [40]. In the present work, the DCFH-DA was incorporated into cell cultures after 24 h of oxidative injury and in the presence of phenolic compounds, and the reaction was stopped with cell lysis with NaOH to prevent cell activity and to measure the fluorescence intensities of accumulated DCF.

Oyama et al., who exposed cerebellar neurons to different concentrations of H_2O_2 (3–3000 μM), showed that fluorescence increases in a dose-dependent manner and obtained a peak of fluorescence intensity of DCF with 10–30 μM of DCFH-DA [41]. Other reports agree, using very similar DCFH-DA concentrations (5–20 μM) [24,42]. Sebastià et al. used flow cytometry to measure fluorescence in human neuroblastoma cell lines (SK-N-MC) cultured in DMEM with 10% FBS, in the presence of 100 μM H_2O_2. They confirmed ROS generation induced by peroxide, with a significant increase in fluorescence [40]. In another report, oxidized phenolic compounds present in black tea showed protection against damage induced by 600 μM H_2O_2 in HPF-1 cells (human fibroblasts line) and suppressed the accumulation of intracellular ROS [43].

Our study, using neuronal cultures maintained in DMEM with FBS, gave us fluorescence values that suggest the existence of an oxidative process in the presence of H_2O_2, which is probably protected by propofol, thymol, chlorothymol and Vit E. These results, although not statistically sustained, show a clear tendency which would indicate potential partial protection by intracellular ROS reduction, although further experiments are needed to confirm these differences. Nevertheless, the neuroprotection demonstrated with the three phenols in the present work, using cell viability tests, reinforce the hypothesis that, at least in part, the antioxidant activity of phenols may lead to a decrease in intracellular ROS, translated into a lower quantification of total hydroperoxides present in neurons.

The present results of cytotoxicity assays, performed in cortical neurons, demonstrated that none of the compounds tested significantly decreased cell viability, at concentrations at which they demonstrated activity on the $GABA_A$ receptor. Furthermore, some phenols showed a partial protective effect against an oxidative injury model.

The analysis of the intracellular oxidation levels suggests that this partial protection exerted by propofol, thymol and chlorothymol, may be mediated in some way by their antioxidant activities. However, this neuroprotection is not completely correlated with the antioxidant capacity for these three phenols, but approaches their relative pharmacological potency, which could be interpreted as a final effect that would involve both activities.

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Fig. 2. Cell viability in presence of different concentrations of phenolic compounds and H_2O_2. A: propofol; B: thymol; C: chlorothymol; D: carvacrol; E: eugenol. All phenols were added 30 min before hydrogen peroxide exposure (for 24 h). Bars represent the percentage of LDH released in the presence of different phenol concentrations (μM). c: control in the absence of compound or peroxide. Bars correspond to the mean ± SEM of 6 independent experiments performed in triplicate. *: $p < 0.05$ with respect to control, +: $p < 0.05$ with respect to H_2O_2 400 μM (two-way ANOVA).

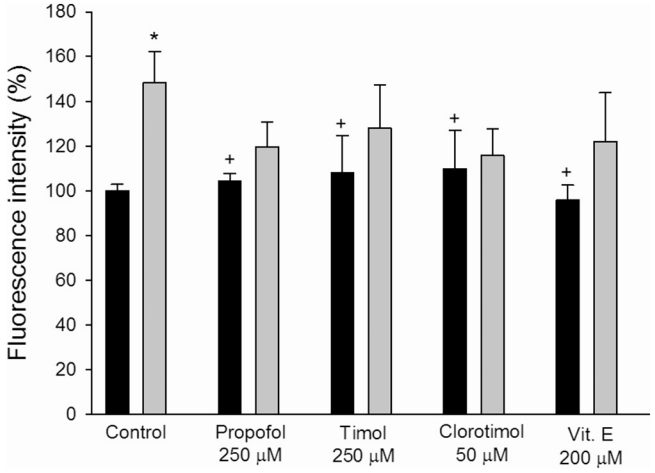

Fig. 3. Determination of intracellular oxidative activity. The cell treatments were the same indicated in previous figures. Values represent the DCF fluorescence intensity, and correspond to the mean ± SEM of 4 independent experiments performed in triplicate. Black bars: without H_2O_2; Gray bars: with H_2O_2 400 μM. $\lambda_{Em} = 520$ nm. *: $p < 0.05$ with respect to control without H_2O_2, +: $p < 0.05$ with respect to control with H_2O_2 (two-way ANOVA).

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