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Neuroprotective effects induced by citicoline/coenzyme Q10 fixed combination in rat CTX-TNA2 astrocytes exposed to oxidative stress

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ABSTRACT

The present study aimed to investigate the rationale and efficacy of testing endogenous substances widely used as dietary supplement such as citicoline, coenzyme Q10 (CoQ10) and a fixed combination of them in countering the oxidative stress and neurotoxicity occurring in neurological diseases. Rat CTX-TNA2 astrocytes, which have considerable antioxidant potential and could represent a key target for neurotherapies were selected as *in vitro* model to conduct the experiments. The efficacy of citicoline and coenzyme Q10 (1 nM-10 μM), with their fixed combination, were assayed in rat astrocytes either in basal condition or after challenging the cells with hydrogen peroxide in order to evaluate the biocompatibility of treatments. The gene expression of B-Cell Lymphoma protein 2 (BCL-2), BCL-2 Associated X (BAX), Superoxide dismutase 2 (SOD2), Cardiolipin Synthase 1 (CRLS1), interleukin-6 (IL-6), tumor necrosis factor α (TNFα), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) involved in neurodegenerative diseases and neuroinflammation were investigated in CTX-TNA2 cells. Furthermore, in the same condition, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was carried out to assess apoptosis in astrocytes. Neither citicoline, nor coenzyme Q10 significantly altered astrocytes cell viability; thus, suggesting the biocompatibility of single ingredients and fixed combination in the concentration range considered for the study. Moreover, each compound tested alone or in combination were effective in inhibiting the hydrogen peroxide-induced gene expression of BAX, and SOD2, in inducing the gene expression of BCL-2 and CRLS1 and in reducing apoptosis. The blunting effects induced by the above-mentioned treatments on hydrogen-peroxide induced apoptosis was also confirmed by TUNEL assay that demonstrated the capability of citicoline, CoQ10, and their combination to reduce the ratio TUNEL positive nuclei/total nuclei, a reliable marker of apoptosis. Additionally, citicoline, CoQ10, and their association were effective in inhibiting the hydrogen peroxide-induced NFkB, TNFα, and IL-6 gene. In parallel, there was an inhibition of both TNFα and IL-6 gene expression in basal condition. The co-administration of citicoline/coenzyme Q10 was overall more effective than individual ingredients. The present findings support the beneficial and synergistic effects of citicoline and coenzyme Q10 in fixed combination in reducing oxidation, and in stimulating neuroprotection in rat CTX-TNA2 astrocytes.

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1. Introduction

Neurodegenerative diseases (NDs), such as Alzheimer's and Parkinson's disease (PD), or vision disorders such as glaucoma, affect millions of people worldwide, and their incidence increases significantly with age (Scheltens et al., 2021). Although several treatments have been approved to reduce or alleviate the debilitating symptoms of NDs, there are still no effective therapies to control their progression (Lamptey et al., 2022). There is increasing interest in potential strategies with a specific neuroprotective approach for prevention, recovery, regeneration, and treatment interventions. Among the numerous compounds emerging as neuroprotective agents, citicoline and coenzyme Q10 (CoQ10) can be considered very promising molecules for their beneficial effects in some neurodegenerative diseases (Bermejo et al., 2023; Bonvicini et al., 2023; Ebrahimi et al., 2023; Jiménez-Jiménez et al., 2023). Citicoline or cytidine-5′-diphosphocholine (CDP-choline) is a natural metabolite presents in all living cells, composed of ribose, cytosine, pyrophosphate, and choline that plays a fundamental role in the synthesis of cell membranes structural phospholipids and their precursors, mainly phosphatidylcholine (Gudi et al., 2023). Unfortunately, citicoline is contained in small quantities in foods, and our body uses choline present in numerous foods such as eggs, meat, fish, dairy products, nuts, seeds, and cruciferous vegetables as a precursor of citicoline (Synoradzki and Grieb, 2019). The oral form of citicoline, commercially available for more than 50 years as an injectable nootropic drug, has recently been authorized as a functional food (USA, European Union) with benefits for neurodegenerative brain diseases (Grieb et al., 2016). Citicoline in the brain is also linked to acetylcholine synthesis as choline donor and increases levels of several neurotransmitters, such as dopamine, serotonin, norepinephrine (Adibhatla et al., 2002; Roohi-Azizi et al., 2018; Secades & Gareri, 2022). Experimental studies confirmed the neuroprotective properties of citicoline in NDs, demonstrating its positive impact in the protection or enhancement of comatous neurons (Gutierrez-Mariscal et al., 2020; Jasielski et al., 2020; Jiménez-Jiménez et al., 2023; Manzar et al., 2020; Mastropasqua et al., 2022; Papucci et al., 2003; Park et al., 2020; Piamonte et al., 2020; Zhai et al., 2017). Moreover, different clinical studies on humans demonstrated that the oral supplementation of citicoline may significantly reduce the rate of damage progression in medically controlled glaucoma (Gandolfi et al., 2020). CoQ10, also known as ubiquinone, is an endogenous lipophilic compound with a structural and functional analogy to vitamins, but since it is synthesized in the human body it is not considered a vitamin (Hargreaves et al., 2020). The main food sources of CoQ10 include oily fish, offal and whole grains, but we only obtain a small amount of CoQ10, and dietary supplementation can provide significant benefits especially in elderly subjects and in subjects with altered levels due to various diseases (Díaz-Casado et al., 2019). CoQ10 acts within mitochondria, where it participates as an electron transporter in aerobic cellular respiration converting energy into adenosine triphosphate (ATP) (Manzar et al., 2020). Thus, CoQ10 is a booster for the mitochondrial activity, but also exerts beneficial antioxidant activities by decreasing inflammation, oxidative stress, and apoptosis. This evidence supports the use of CoQ10 as an anti-inflammatory, antioxidant and pro-energetic agent, which may rescue damaged neuronal cells (Gutierrez-Mariscal et al., 2020; Papucci et al., 2003; Zhai et al., 2017). In fact, different studies confirmed the potential therapeutic role of CoQ10 in chronic NDs such as Alzheimer's, Huntington's and Parkinson's diseases, other forms of dementias, and in glaucoma (Jiménez-Jiménez et al., 2023; Lee et al., 2014; Park et al., 2020). This therapeutic role seems to be attributable to ability of CoQ10 to preserve neuronal cells from oxidative damage, thereby reducing the progression of neurodegeneration. Interestingly, recent evidence observed that the fixed combinations of citicoline and CoQ10, which are commercially available in some Countries as neuroprotectants for glaucoma, exert synergistic effects in containing oxidation and inflammation in experimental models of neuronal cells degeneration (Mastropasqua et al., 2022). Indeed, citicoline prevented the deterioration of the neuronal membrane, and inhibited the apoptosis in neurodegenerative processes (Park et al., 2020). Moreover, coenzyme Q10 as an antioxidant and neuroprotective agent can also play an important role in the treatment of neurological disorders (Bagheri et al., 2023). Based on this evidence the aims of this study were to investigate protective effects of citicoline and CoQ10 tested individually and in fixed combination on the rat astrocyte CTX-TNA2 cell line. In this context the effects of citicoline, CoQ10, and their fixed combination on the gene expression of markers of oxidative stress, inflammation, and apoptosis were investigated.

2. Materials and methods

2.1. Bioactive components

Citicoline and coenzyme Q10 were kindly provided by the company Visufarma S.p.a (Rome, Italy). Their formulation included an innovative patented delivery system (Miniactives) that enhances the bioavailability of both CoQ10 and citicoline. The dried materials of citicoline and CoQ10 have been stored at 4 °C in an cold room for a maximum of 4 weeks. The stock solutions (30 mM) were freshly prepared in dimethylsulfoxide (DMSO) and sterilized with 0.22 μm Millipore filters in sterility conditions (laminar flow hood) and immediately used. Afterwards, drug solutions were stepwise diluted in Dulbecco's modified Eagle's medium (DMEM) for the bio-pharmacological assays, as described below.

2.2. Cell Culture and treatment

The CTX-TNA2 rat astrocytes were purchased from the European Collection of Cell Cultures (ECACC), Sigma-Aldrich, Milan, Italy. Cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% of FBS and penicillin–streptomycin (100 μgmL^{-1}) (all from Euro-Clone SpA Life-Sciences-Division, Milan, Italy) and maintained in a humified atmosphere of 5% CO_2 at 37 °C. Cells were subcultivated at a ratio of 1.2–1.8 and the medium was changed every 3 days. CTX-TNA2 astrocytes were plated in 25 or 75 cm^2 flasks, according to experimental needing and cells between the 8th and the 15th passage were used. When indicated, the cells were treated with hydrogen peroxide 300 μM for 3 h and different concentrations of citicoline and coenzyme Q10 (1 nM-10 μM). The stock solutions were diluted in cell medium to the point that the concentration of DMSO in the samples was less than 0.0003%. Therefore, the addition of DMSO to the control samples was not necessary.

2.3. MTT assay

The 3 [4–dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) growth assay (Sigma-Aldrich S.r.l.) was used to assess cell proliferation. The CTX-TNA2 cells were seeded into 96-well plate at 8×10^3 cells/well and treated as described above. After 24, 48 h, and 72 h a medium containing 0.5 mg/mL MTT was added to the cells, and the cells were incubated at 37 °C for 3 h. Following a further incubation of 30 min in DMSO, the absorbance at 570 nm was measured using a Multiscan GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Gene expression analysis

The gene expression of BAX, BCL-2, CRLS1, NFkB, IL-6, TNFα, and SOD2 was conducted as previously reported (Orlando et al., 2021). Briefly, Total RNA was extracted from the astrocytes using TRI Reagent (Sigma-Aldrich, St. Louis, MO). Contaminating DNA was removed using 2 units of RNase-free DNase 1 (DNA-free kit, Ambion, Austin, TX). The RNA concentration was quantified at 260 nm by spectrophotometer reading (BioPhotometer, Eppendorf, Hamburg, Germany) and its purity

was assessed by the ratio at 260 and 280 nm readings. The quality of the extracted RNA samples was also determined by electrophoresis through agarose gels and staining with ethidium bromide, under UV light. One microgram of total RNA extracted from each sample in a 20 μL reaction volume was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Monza, Italy). Reactions were incubated in a 2720 Thermal Cycler (Thermo Fisher Scientific Inc.) initially at 25 °C for 10 min, then at 37 °C for 120 min, and finally at 85 °C for 5 s. Gene expression was determined by quantitative real-time PCR using TaqMan probe-based chemistry: Rn00667869_m1 for β-actin gene; Rn01278099_g1 for CRLS1 gene; Rn00690588_g1 for SOD2 gene; Rn01480161_g1 for BAX gene; Rn99999125_m1 for BCL2 gene; Rn01399572_m1 for NFkB gene; Rn01410330_m1 for IL-6 gene; Rn99999017_m1 for TNFα gene. PCR primers and TaqMan probes, including β-actin used as the housekeeping gene, were purchased from Thermo Fisher Scientific Inc. The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the investigated genes. Data were elaborated with the Sequence Detection System (SDS) software (version 2.3; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Gene expression will be relatively quantified by the comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.5. TUNEL assay

The images at the confocal microscope were casually taken for each experimental condition through motorized table SMC 2009 and multiple single position acquisition function (Tiles-Advanced setup) of Zen Blue software (Zen 3.0 SR, Carl Zeiss S.p.A, Italy) utilizing a Zeiss LSM800 microscope (Carl Zeiss), equipped with an inverted microscope Axioobserber D1 (Carl Zeiss) and an objective Plan-Neo 20×/0.50 DIC (Carl Zeiss). TUNEL positive nuclei were acquired using 488 nm excitation and emission detected with the filter set over 530 nm (green emission), DAPI counterstained nuclei were acquired using 405 nm excitation and detected at 445 nm (blue emission). During the acquisition the setting were maintained unchanged. The analyses of the offline pictures were executed utilizing Zen Blue software quantifying for each field acquired the total number of nuclei (DAPI-stained) and the TUNEL positive nuclei. Data are expressed as ratio of TUNEL-positive/total nuclei.

2.6. Statistical analysis

Data were means ± standard deviation (SD) of 3–5 experiments performed in triplicate. The experimental data were analyzed through the analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. The GraphPad Prism software (version 5.01; GraphPad Software Inc., California, USA) was employed for statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results and discussion

In the present study, citicoline, coenzyme Q10 (CoQ10), and their fixed combination were tested on CTX-TNA2 rat astrocytes at different concentrations (1 nM-10 μM), in basal condition and after challenging cells with hydrogen peroxide (300 μM), as reference pro-oxidant stimulus, in order to evaluate neuroprotective effects. CTX-TNA2 cells are extensively used in neurosciences for their capability to respond to inflammatory and oxidative stimuli, among which hydrogen peroxide that has been suggested to impair astrocyte glycolytic metabolism (Cantrill et al., 2012; di Giacomo et al., 2020b; di Giacomo et al., 2019).

CTX-TNA2 cells were treated with either citicoline, CoQ10, or fixed combination and the cell viability was evaluated through MTT assay.

In basal conditions, the cells tolerated all treatments in the concentration range (1 nM-10 μM; Fig. 1). Indeed, the alterations in cell viability were lower than 30%, compared with vehicle-treated cells; thus, being therefore in the range of biocompatibility (Ferrante et al., 2019).

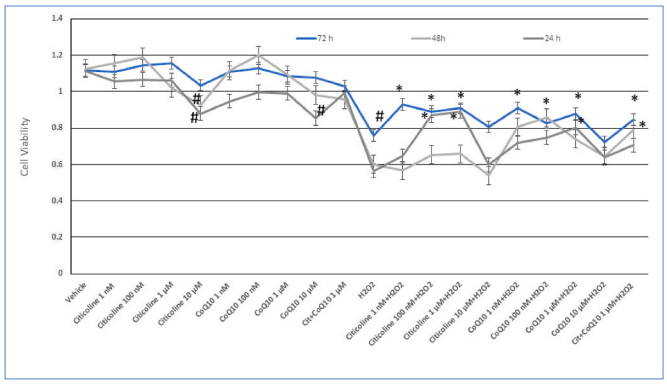


Fig. 1. Effects of citicoline, CoQ10 and their fixed association on CTX-TNA2 astrocyte viability (MTT assay) in basal condition and after astrocyte exposure to hydrogen peroxide 300 μM, at different time points: 24, 48, and 72 h $^{\#}P < 0.05$ Vs. Vehicle; $^{*}P < 0.05$ Vs. H_2O_2.

On the other hand, when cells were exposed to hydrogen peroxide the cell viability decreased under the limits of biocompatibility (≤70%). By contrast, the treatment with either citicoline, CoQ10, or their fixed combination determined protective effects against the reduction of cell viability induced by hydrogen peroxide.

Actually, the neuroprotection was overall time-dependent; in pro-oxidant conditions, the recovery of cell viability after 72 h of cell exposure was higher compared with shorter timelines, namely 24 and 48 h. This may indicate an antioxidant effect exerted on astrocytes that is corroborated, at least in part, by our previous observations of neuroprotection in neural cells exposed to the same pro-oxidant stimulus (Mastropasqua et al., 2022). It is well-known that neurons are more sensitive to oxidative stress compared with astrocytes, probably because of higher rate of oxidative metabolism (Bélanger et al., 2011; Halliwell et al., 2000). In our previous study (Mastropasqua et al., 2022), the fixed combination was particularly effective in promoting the gene expression of brain-derived neurotrophic factor (BDNF), although the effects were monitored only in the 24 h following cell treatment with the ingredients of the fixed combination.

The past and present protective effects on neurons and astrocytes, respectively, are particularly relevant if it is considered the strict interplay between them. Neurons deliver damaged mitochondria to astrocytes for degradative processes. By contrast, astrocytes transfer working mitochondria to adjacent neurons; thus, permitting the neuron recovery following cell damage (Hayakawa et al., 2016).

Moreover, recent findings highlighted a potential main role of the glia and, therefore, of the glial dysfunction-induced neuroinflammation, in the pathogenesis of glaucoma. As a consequence of the microglia and macroglia (astrocytes) dysfunction, neuroinflammation cooperates with other mechanisms of damage in promoting the retinal ganglion cells loss and, thus, in the development and progression of glaucoma (Quaranta et al., 2021).

In this context, it is worthy to underline the capability of citicoline, CoQ10, and especially their fixed combination to protect astrocytes from hydrogen peroxide-induced reduction of cardiolipin synthase 1 (CRLS1) gene expression (Fig. 2A). The stimulation of CRLS1 gene expression was concentration-dependent, with also an additive effect induced by the combination citicoline-CoQ10. By contrast, in basal condition citicoline did not alter CRLS1 gene expression, in astrocytes (Fig. 2B); thus, indicating a null effect on mitochondrial function that is also consistent with the null effect on cell viability in the same condition. Indeed, this enzyme synthesizes cardiolipin, a phospholipid representing a key component of mitochondrial membranes that therefore plays a pivotal role in mitochondrial function, including respiration and apoptosis (Quaranta et al., 2021). It is sensitive to highlight that mitochondrial

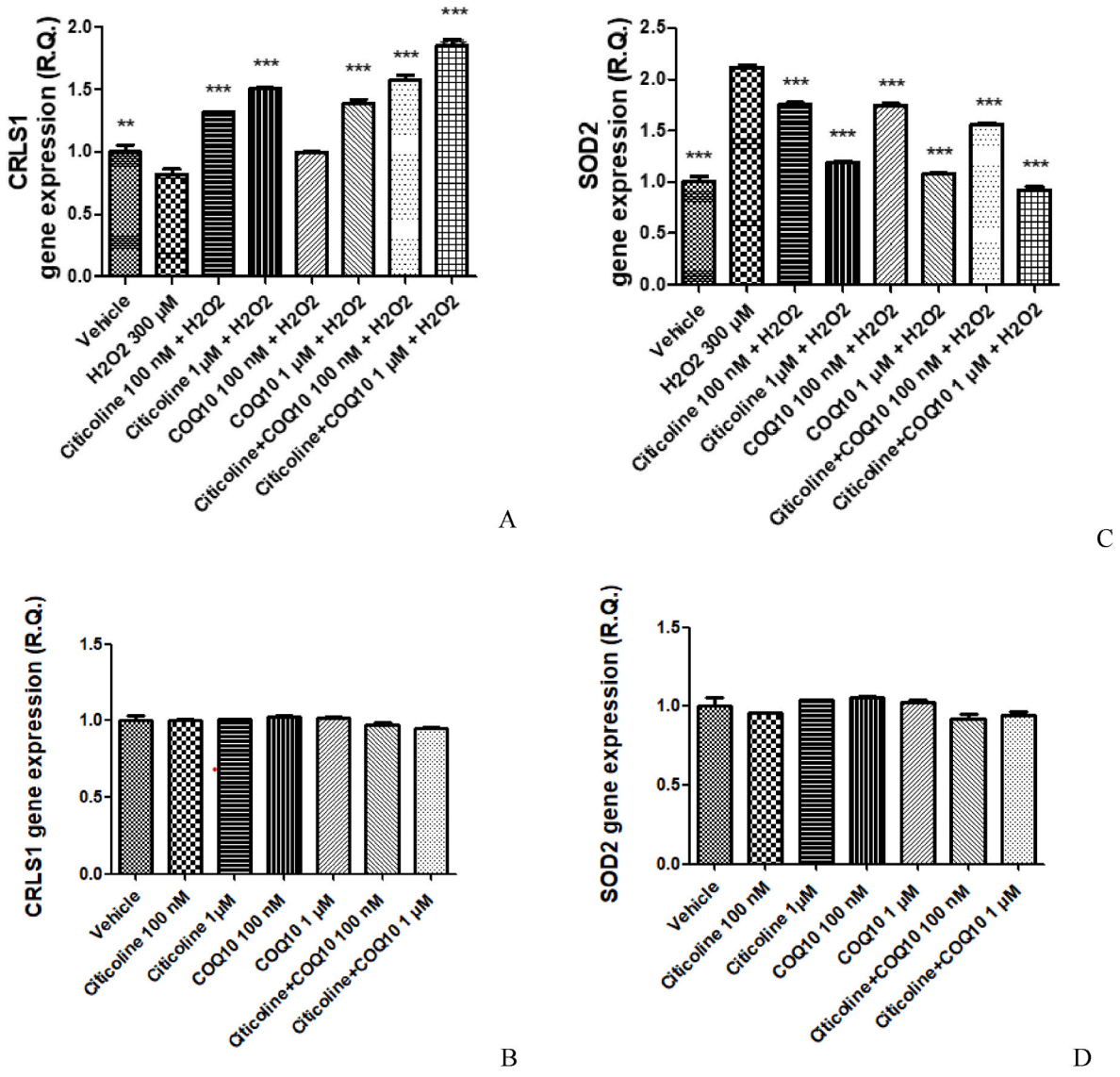


Fig. 2. **(A)** Effects of citicoline, coenzyme Q10 CoqQ10 and their fixed association on cardiolipin synthase 1 (CRLS1) gene expression in CTX-TNA2 astrocytes exposed to hydrogen peroxide. ANOVA, $P < 0.0001$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Vehicle. **(B)** Null effect of coenzyme Q10 CoqQ10 and their fixed association on cardiolipin synthase 1 (CRLS1) gene expression in CTX-TNA2 astrocytes, in basal condition. **(C)** Effects of citicoline, coenzyme Q10 and their fixed association superoxide dismutase 2 (SOD2) gene expression in CTX-TNA2 astrocytes exposed to hydrogen peroxide. ANOVA, $P < 0.0001$, $^{***}P < 0.001$ vs. Vehicle. **(D)** Null effect of coenzyme Q10 CoqQ10 and their fixed association on SOD2 gene expression in CTX-TNA2 astrocytes, in basal condition.

disfunction, besides being involved in oxidative stress and apoptosis, is also related to the onset of ophthalmological diseases, such as glaucoma. Oxidative stress seems to be associated with reduced levels of cardiolipin and downregulation of CRLS1 (Jiang et al., 2022). In this context, any treatment able in preventing the oxidative stress-induced reduction of cardiolipin levels could be effective in protecting retinal ganglion cells in glaucoma. On one side, the increased CRLS1 gene expression is consistent with the capability of citicoline to improve phosphatidylcholine pathway (Mastropasqua et al., 2022). On the other hand, it is also reasonable to hypothesize that the stimulation of CRLS1 gene expression induced by either citicoline, CoQ10, or their pharmacological association could be related to antioxidant effects (Mastropasqua et al., 2022). This hypothesis is corroborated, albeit partially, by the null effect on CRLS1 gene expression induced by the abovementioned treatments in absence of an oxidative stress stimulus challenging astrocytes (Fig. 2B). In order to confirm this hypothesis in astrocytes, the gene expression of superoxide dismutase 2 (SOD2) was measured in CTX-TNA2 cells (Fig. 2C and D). The pro-oxidant stimulus increased the gene expression of SOD2 (Fig. 2C). Whilst the treatment of cells with citicoline, CoQ10, and the fixed combination blunted the hydrogen peroxide-induced upregulation of SOD2 gene expression; thus, demonstrating antioxidant effects (Mizobuti et al., 2019). The efficacy of such antioxidant effect seems to be mostly related to CoQ10, rather than to citicoline. Indeed, the antioxidant effect of the formulation is quite similar to CoQ10 alone. This is consistent, albeit partially, with a previous study pointing to SOD-2 pathway as a possible mediator of CoQ10 antioxidant activity (Yu et al., 2023). Also in this case, the pharmacological treatments were not able to alter the pattern of gene expression in basal condition (Fig. 2D).

The effects of citicoline, CoQ10 and their fixed combination on BAX and BCL-2 gene expression was evaluated, as well. If on one side the treatments induced only a mild reduction of BAX gene expression, in astrocytes (Fig. 3B), more significant alterations in the pattern of gene expression were observed in cells exposed to hydrogen peroxide.

It is known that hydrogen peroxide-induced oxidative stress can also trigger apoptosis in both neurons and astrocytes (Chiavaroli et al., 2022; di Giacomo et al., 2020b). In this context, the gene expression of BAX and BCL-2, playing pro-apoptotic and anti-apoptotic effects respectively

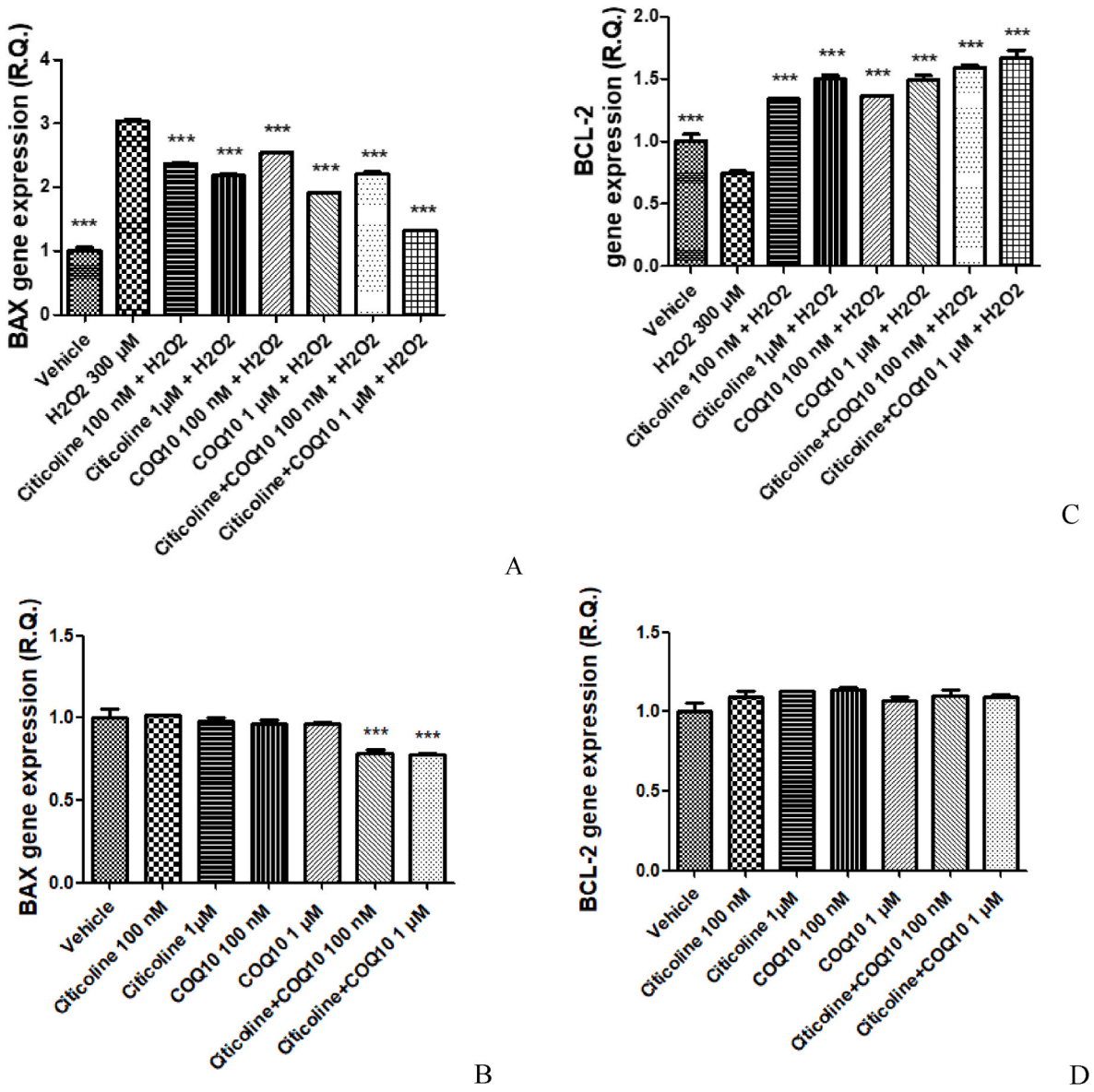


Fig. 3. **(A)** Effects of citicoline, coenzyme Q10 and their fixed association on BAX gene expression in CTX-TNA2 astrocytes exposed to hydrogen peroxide. ANOVA, $P < 0.0001$, ***$P < 0.001$ vs. Vehicle. **(B)** Inhibitory effect of citicoline, coenzyme Q10 and their fixed association on BAX gene expression in CTX-TNA2 astrocytes in basal condition. ANOVA, $P < 0.0001$, *$P < 0.001$vs. Vehicle. **(C)** Effects of citicoline, coenzyme Q10 and their fixed association on BCL-2 gene expression in CTX-TNA2 astrocytes exposed to hydrogen peroxide. ANOVA, $P < 0.0001$, ***$P < 0.001$ vs. Vehicle. (D) Null effect of citicoline, coenzyme Q10 and their fixed association on BCL-2 gene expression in CTX-TNA2 astrocytes, in basal condition.

(D'angelo et al., 2017; di Giacomo et al., 2020a), was assayed (Fig. 3A–D). Among treatments, the fixed combination citicoline-CoQ10 was the most effective in blunting the hydrogen peroxide-induced alterations on BAX and BCL-2 gene expression. Indeed, the inhibition of BAX gene expression and the increased gene expression of BCL-2 gene indicate an anti-apoptotic effect on CTX-TNA2 cells that was further corroborated by TUNEL assay (Fig. 4). This test showed a protective effect induced by the treatments against the apoptosis induced by hydrogen peroxide that was measured as TUNEL positive cells/nuclei ratio, a reliable marker of apoptosis. The capability of hydrogen peroxide in inducing apoptosis was comparable with that of the positive control, constituted by cells exposed to deoxyribonuclease. The most effective among the treatments was also in this case the fixed combination that almost completely protected CTX-TNA2 cells from the hydrogen peroxide-induced apoptosis. Actually, the protective effect was concentration-dependent, with the highest efficacy displayed by the highest tested concentration (1 μM). These results are consistent with anti-apoptotic effects induced by citicoline and CoQ10 in different experimental models of neurotoxicity and glaucoma (Kernt et al., 2013; Oshitari et al., 2002); thus, further supporting the pharmacological association of citicoline and CoQ10 as an effective tool for facing neurodegenerative diseases, with a particular interest towards glaucoma (Mastropasqua et al., 2022).

The effects of citicoline, CoQ10, and their fixed association were also evaluated on the gene expression of markers of inflammation, namely interleukin-6 (IL-6), tumor necrosis factor α (TNFα), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB). NFkB is a transcriptor factor involved in the expression of inflammatory enzymes and cytokines, among which IL-6 (Brasier, 2010; Lee et al., 2014; Mastropasqua et al., 2022; Piva et al., 2006). In the present study, citicoline, CoQ10 and their association were effective in inhibiting the hydrogen peroxide-induced NFkB, TNFα, and IL-6 gene expression (Figs. 5–7). In parallel, there was an inhibition of both TNFα and IL-6 gene expression in basal condition, that was more effective in the citicoline-CoQ10

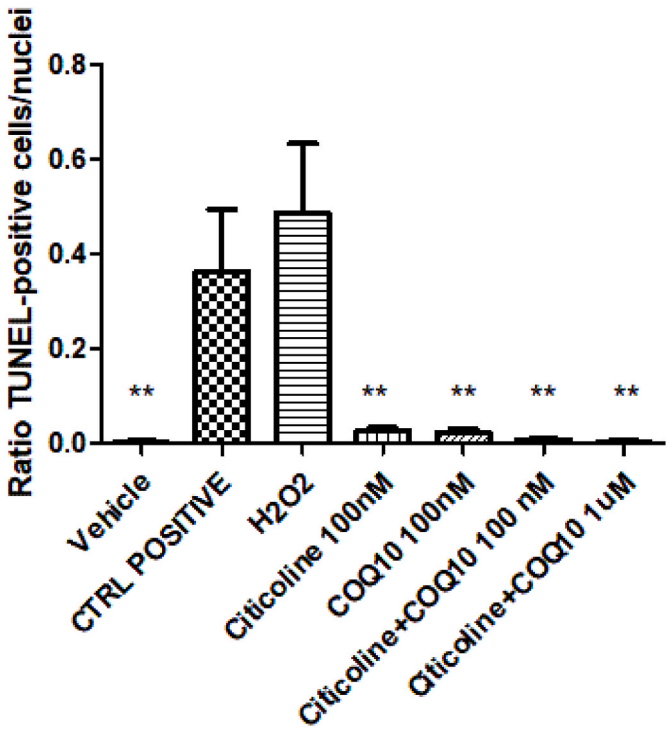
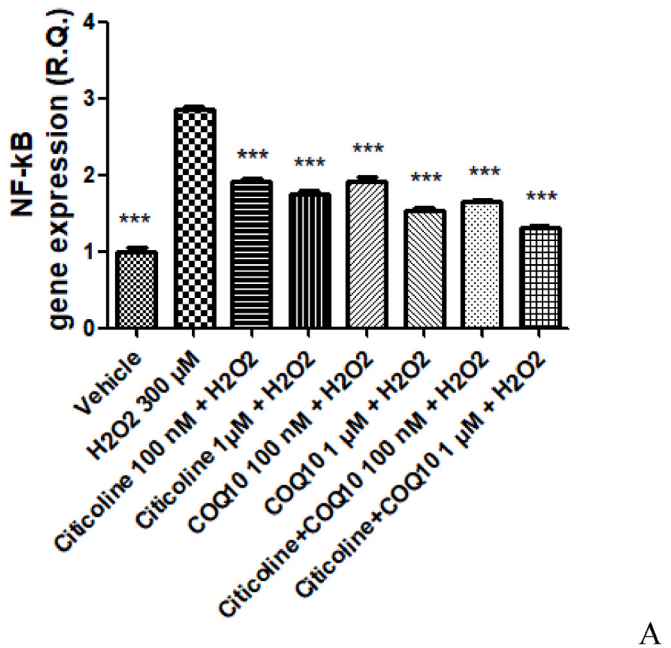


Fig. 4. Protective effects induced by citicoline, coenzyme Q10, and their fixed combination against hydrogen peroxide-induced apoptosis in CTX-NTA2 cells. The apoptosis is indicated as TUNEL-positive cells/nuclei ratio. The fixed combination was more effective compared with single ingredients in protecting CTX-NTA2 cells from hydrogen peroxide-induced apoptosis. ANOVA, $P < 0.001$, $^{**}P < 0.01$ Vs. H_2O_2.

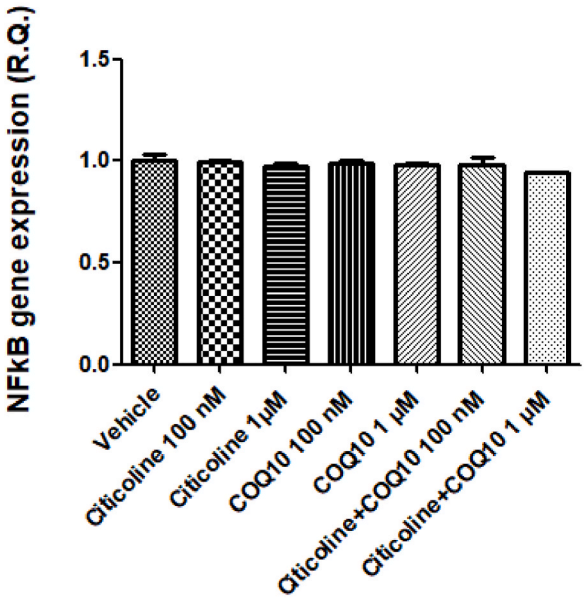
group. This could underly a protective effect induced by the formulation, in rat astrocytes; thus, further corroborating the rationale for the pharmacological association of citicoline and CoQ10 as neuroprotective agent. The present results are consistent, albeit partially, with literature. Indeed, the anti-inflammatory properties of CoQ10, mainly exerted by the inhibition of the NFkB release, represents a potential interesting strategy to contain neuroinflammation (Quaranta et al., 2021). Consistent with the inhibition of NFkB release, CoQ10 was also able to inhibit IL-6 and TNFα levels in a cerebral ischemia-reperfusion injury-induced neuronal damage model (Fakharaldeen et al., 2023). Similarly, citicoline was effective in inhibiting the gene expression of NFkB, IL-6, and TNFα *in vivo*, as well (Feng et al., 2020). Additionally, our previous study confirmed the inhibition of IL-6 and TNFα gene expression in an *in vitro* experimental model constituted by rat hypothalamic cells exposed to hydrogen peroxide (Mastropasqua et al., 2022).

4. Conclusions

Concluding, the present findings investigated the protective effects induced by citicoline, CoQ10, and their pharmacological association in rat astrocytes exposed to oxidative stress. The treatments, and particularly the fixed combination of citicoline and CoQ10 were effective in blunting the toxicity induced by hydrogen peroxide. Specifically, antioxidant, anti-inflammatory, and anti-apoptotic effects were induced, in astrocytes, as evidenced by the different experimental paradigms employed for assessing protective effects. According to *in vitro* studies, antioxidant and anti-inflammatory effects, especially by the fixed combination, could be at the basis of the protective effects induced by these substances. This was confirmed by the capability of the treatments in blunting the hydrogen peroxide-induced gene expression of all tested markers of oxidative stress, inflammation, and apoptosis. It is worthy to highlight that the fixed combination was able to decrease the gene



A



B

Fig. 5. (A) Effects of citicoline, coenzyme Q10 and their fixed association on NFkB gene expression in CTX-TNA2 astrocytes exposed to hydrogen peroxide. ANOVA, $P < 0.0001$, $^{***}P < 0.001$ vs. Vehicle. (B) Null effect of citicoline, coenzyme Q10 and their fixed association on NFkB gene expression in CTX-TNA2 astrocytes, in basal condition.

expression of IL-6 and TNFα in basal condition, as well. This may suggest a preventive effect in the onset of neuroinflammation that is strictly related to the glaucoma.

To summarize, given the role of the apoptosis, oxidative stress, neuroinflammation, and astrocytes in promoting the development and progression of NDs such as glaucoma, the present findings support the potential utility of the fixed combination of citicoline and CoQ10 in

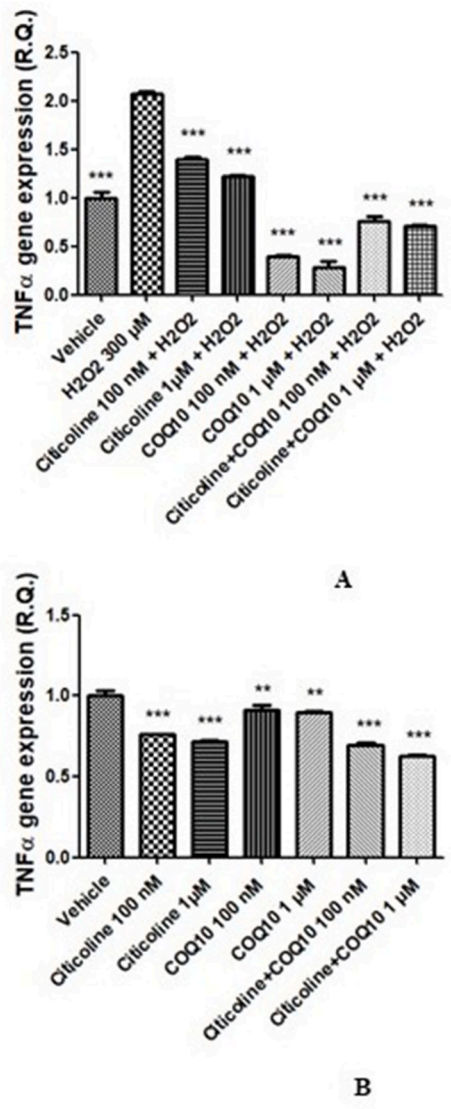


Fig. 6. (A) Effects of citicoline, coenzyme Q10 and their fixed association on TNFα gene expression in CTX-TNA2 astrocytes exposed to hydrogen peroxide. ANOVA, $P < 0.0001$, ***$P < 0.001$ vs. Vehicle. (B) Effects of citicoline, coenzyme Q10 and their fixed association on TNFα gene expression in CTX-TNA2 astrocytes, in basal condition. ANOVA, $P < 0.0001$, ***$P < 0.001$ vs. Vehicle.

containing the retinal ganglion cells loss in medically controlled glaucoma.

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Institutional review board statement

Not applicable.

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CRediT authorship contribution statement

Simonetta Cristina Di Simone: Investigation. **Maria Loreta Libero:** Investigation. **Monica Rapino:** Investigation. **Viviana di Giacomo:** Validation, Software, Formal analysis. **Amelia Cataldi:** Validation, Software, Formal analysis. **Simone Guarnieri:** Validation, Software, Investigation, Formal analysis. **Lucia Recinella:** Investigation. **Sheila Leone:** Investigation. **Luigi Brunetti:** Visualization, Supervision, Project administration. **Luigi Menghini:** Writing – review &

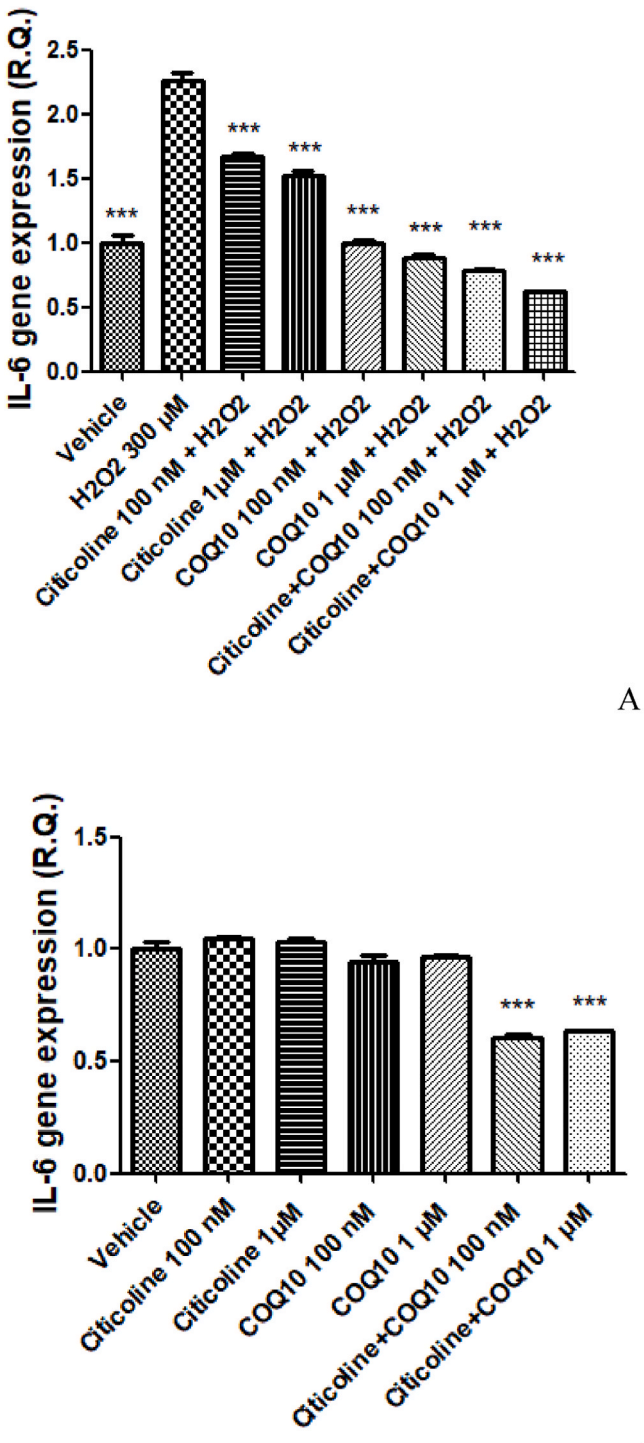

Fig. 7. (A) Effects of citicoline, coenzyme Q10 and their fixed association on TNFα gene expression in CTX-TNA2 astrocytes exposed to hydrogen peroxide. ANOVA, $P < 0.0001$, ***$P < 0.001$ vs. Vehicle. (B) Effects of citicoline, coenzyme Q10 and their fixed association on TNFα gene expression in CTX-TNA2 astrocytes, in basal condition. ANOVA, $P < 0.0001$, ***$P < 0.001$ vs. Vehicle.

editing, Writing – original draft, Data curation. **Claudio Ferrante:** Writing – review & editing, Writing – original draft, Data curation. **Luca Agnifili:** Writing – review & editing, Writing – original draft. **Gokhan Zengin:** Writing – review & editing, Formal analysis. **Giustino Orlando:** Resources, Methodology, Funding acquisition, Conceptualization. **Annalisa Chiavaroli:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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