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ORIGINAL ARTICLE

Captopril exhibits protective effects through anti-inflammatory and anti-apoptotic pathways against hydrogen peroxide-induced oxidative stress in C6 glioma cells

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Abstract
Recent studies have shown that angiotensin-converting enzyme (ACE) inhibitors have reduced oxidative damage in the central nervous system (CNS). Accumulating evidence have also demonstrated that captopril, an ACE inhibitor, has protective effects on the CNS. However, its effects on hydrogen peroxide (H_2O_2)-induced oxidative damage in glial cells and interaction with the inflammatory system are still uncertain. Therefore, this study was aimed to investigate the protective effect of captopril on glial cell damage after H_2O_2-induced oxidative stress involved in the inflammatory and apoptotic pathways. The control group was without any treatment, and the H_2O_2 group was treated with 0.5 mM H_2O_2 for 24 h. The captopril group was treated with various concentrations of captopril for 24 h. The captopril + H_2O_2 group was pre-treated with captopril for 1 h and then exposed to 0.5 mM H_2O_2 for 24 h. In the captopril + H_2O_2 group, captopril at all concentrations significantly increased the cell viability in C6 cells. It also significantly increased the TAS and decreased the TOS levels which are an indicator of oxidative stress. Moreover, captopril significantly reduced the inflammation markers including NF-kB, IL-1 β, COX-1, and COX-2 levels. Flow cytometry results also exhibited that captopril pretreatment significantly decreased the apoptosis rate. Besides, captopril significantly reduced apoptotic Bax and raised anti-apoptotic Bcl-2 protein levels. In conclusion, captopril has protective effects on C6 cells after H_2O_2-induced oxidative damage by inhibiting oxidative stress, inflammation, and apoptosis. However, further studies need to be conducted to evaluate the potential of captopril as a neuroprotective agent.

Keywords Captopril · Inflammation · Hydrogen Peroxide · Oxidative Stress · Apoptosis · C6 cells

Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are produced during normal cellular metabolism, and they play a vital role in the signal transmission process of the cells (Dringen et al. 1998; Forman 2007). However, increasing the H_2O_2 production damages to the cellular components and also causes genotoxic effects (Andersen 2004; Gandhi and Abramov 2012). Moreover, excessive production of ROS results in oxidative damage leading to cellular dysfunction and cell death (Coyle and Puttfarcken 1993; Ray et al. 2012).

Due to its high metabolism and lipid composition, the brain is the most susceptible tissue to oxidative damage. Oxidative stress is also one of the fundamental causes of neurodegenerative conditions, such as Alzheimer's disease (AD) and Parkinson’s disease (PD) (Blesa et al. 2015). Glial cells are non-neuronal cells that maintain homeostasis as well as support and protect the neurons in the central nervous system (CNS) (Garden and Campbell 2016). Furthermore, activation of the glial cells causes neuroinflammation and oxidative stress that destroys the neuronal cells. Therefore, glial cells are essential to neurodegenerative disorders, particularly AD and PD (Verkhratsky et al. 2014).

Astrocytes are homeostatic cells that play a crucial role in maintaining physiological CNS function, such as providing nutrition to neurons, maintaining the integrity of the blood–brain barrier, regulating synaptic activity, and processing cell metabolites (Verkhratsky and Nedergaard 2018).

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It exerts a full antioxidant effect by promoting the decomposition and scavenging of free radicals produced by neurons and other cell types in the CNS and protects the CNS from oxidative stress damage (Tacconi 1998; Fernandez-Fernandez et al. 2012; Cabezas et al. 2012). On the other hand, under certain pathological conditions, astrocytes can act as one of the main sources of harmful ROS, and these excess free radicals can promote microglia activation or directly cause neural damage (Sofroniew and Vinters 2010).ROS, such asH_2O_2, are produced during the normal metabolic functions of the brain cells and play a role in important signaling pathways (Dringen et al. 2005). However, H_2O_2 also exerts toxicological effects because it can generate new radicals and damage major cellular components (Halliwell 2006). As an astrocyte-like cell line C6, is commonly used to investigate astrocytic function, including parameters of oxidative stress (Quincozes-Santos et al. 2013). Furthermore, C6 cell line responds promptly to external stimuli, such as H_2O_2, which can generate oxidative stress (Quincozes-Santos et al. 2010).

Renin–Angiotensin–Aldosterone System (RAAS) controls cardiovascular, renal, and adrenal functions by affecting body fluid and electrolyte balance and arterial pressure (Dielis et al. 2005). Moreover, recent reports have demonstrated that the RAAS may play a role in various neurological diseases including AD and PD (Von Bohlen Und Halbach and Albrecht 2006). Angiotensin-converting enzyme (ACE) converts inactive angiotensin I, which is formed from renin angiotensinogen to active angiotensin II. Although lung tissue has the highest ACE activity, there is also a significant amount of ACE activity in other tissues including the brain, basal ganglia, periventricular areas, hippocampus, hypothalamic neurosecretory nuclei, and cerebellum (Kurosaki et al. 2005). Neuronal levels of ACE are also increased in the brains of AD patients (Miners et al. 2009). In addition, Angiotensin II is known to promote neurodegeneration and brain aging; therefore, specific inhibition of ACE may be a promising strategy to prevent neuronal damage (Forrester et al. 2018).

Captopril is one of the ACE inhibitors and is primarily used to treat hypertension (Tastemur et al. 2020). On the other hand, recent studies have shown that captopril has different pharmacological effects on the organism. Some studies have demonstrated that captopril has anti-inflammatory properties and has positive effects on cardiac fibrosis and also lung function after lipopolysaccharide-induced (LPS) lung inflammation in rats (Abareshi et al. 2017; Boskabadi et al. 2018). Moreover, other studies have found that captopril improves learning and memory after pathological conditions such as dementia (Abbassi et al. 2016). It also has neuroprotective effects by regulating oxidative stress and nitrosative stress in diabetic rats (Paseban et al. 2019). Furthermore, several studies demonstrated that captopril modulates oxidative stress and nitrosative stress (El-Ashmawy et al. 2018; Wang et al. 2018). Besides, it has been found that captopril is related to microglial activation (Asraf et al. 2018). However, its protective effects on oxidative damage in C6 glial cells and underlying mechanisms are still unclear. In the present study, it was examined that the protective effect of captopril against H_2O_2-induced oxidative damage in C6 glial cells involved in the inflammatory and apoptotic pathways.

Materials and Methods

Cell Culture

C6 Glioma (CRL107) cell lines were obtained from American Type Culture Collection and cultured in DMEM (Thermo Fisher Scientific, Altrincham, UK) containing 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich Co., St Louis, MO, USA), 1% L-glutamine (Sigma-Aldrich Co., St Louis, MO, USA) and 1% penicillin/streptomycin (Sigma-Aldrich Co., St Louis, MO, USA). The cells were maintained at 37 °C within a 5% CO_2 humidified atmosphere. Captopril and H_2O_2 (Sigma-Aldrich Co., St Louis, MO, USA) were dissolved in DMEM, and stock solutions were prepared before treatment.

Cell Viability Assay

Cell viability was assessed using the XTT assay (Roche Diagnostic, MA, USA). C6 Glioma cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 100-µL DMEM and grown overnight before captopril. The following day, four-cell groups were prepared to evaluate the neuroprotective effect of captopril. The control group was without any treatment. Cells in the H_2O_2 group were treated with 0.5 mM H_2O_2 for 24 h. Cells in the captopril group were treated with various concentrations (0.25, 0.5, 1, 2, 4 mg/mL) of captopril for 24 h. Cells in the captopril + H_2O_2 group were pre-treated with various concentrations (0.25, 0.5, 1, 2, 4 mg/mL) of captopril for 1 h and then exposed to 0.5 mM H_2O_2 for 24 h. After incubation, the medium was removed, and wells were washed two times with phosphate-buffered saline. In the last step, 100 µL DMEM without phenol red and a mixture of 50 µL XTT labeling solution were added to all the wells, and then the plates were maintained at 37ºC for 4 h. The plates were shaken, and the absorbance was detected using an ELISA microplate reader (Thermo Fisher Scientific, Altrincham, UK) at 450 nm. All the experiments were performed three times, and the cell viability was measured as a viable cell amount percent compared to control, as untreated cells (Fig. 1).

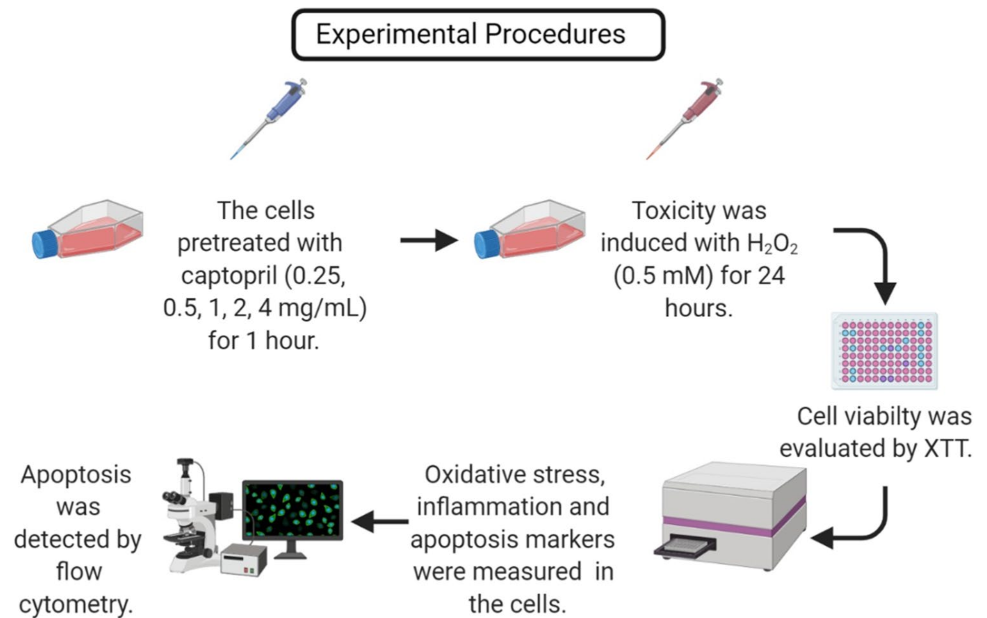

Fig. 1 Experimental design of the study (created with BioRender.com)

Preparation of cells homogenates

The cells for each group were collected by sterile tubes. They were centrifuged at 2000 RPM for approximately 10 min. The supernatants were removed. The component of cells which are under the tubes, suspended by using PBS (pH: 7.4) to dilute cell suspension to the cell concentration of approximately 1 million/ml. The cells were damaged through repeated freeze–thaw cycles to let out the inside components. They were centrifuged at 4000 rpm for 10 min at a temperature of 4ºC. Then, the supernatants were collected for biochemical analysis. Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used to determination of total protein levels in samples.

Measurement of total antioxidant status (TAS) and total oxidant status (TOS)

The TAS concentrations at the cell supernatants were determined with an automated assay (Rel Assay Kit Diagnostics, Antep, Turkey) method that was previously developed by Erel (Erel 2004). The method is based on monitoring the reaction rate of free radicals by measuring the absorbance of coloured dianisidyl radicals during free radical reactions starting with the production of hydroxyl radicals in the Fenton reaction. Antioxidants in the tissue samples should suppress colouring proportionally to their concentrations (Erel 2004). The results were expressed in micromolar Trolox equivalents per milligram tissue protein (μmol Trolox Eq/mg protein).

Tissue TOS concentrations at the cell supernatants were quantified with the automated assay (Rel Assay Kit Diagnostics, Antep, Turkey) method of Erel (Erel 2005). Because ferrous ion is oxidized to ferric ion when adequate quantities of oxidants are available in the medium, the method allows for quantifying TOS levels by measuring tissue levels of ferric ions with the use of xylenol orange. Hydrogen peroxide was used for the calibration of the assay (Erel 2005). The results of the assay were expressed in micromolar hydrogen peroxide equivalents per milligram tissue protein (μmol H_2O_2 Eq/mg protein).

Measurement of NF-kB, IL-1 β, COX-1, COX-2, Bax, and Bcl-2 Levels

The levels of NF-kB (Cat no: YLA0512HU), IL-1 β (Cat no: YLA0001HU), COX-1 (Cat no: YLA1147HU), COX-2 (Cat no: YLA1148HU), Bax (Cat no: YLA1250HU), and Bcl-2 (Cat no: YLM0188HU) in the supernatants of the cells for each group were measured using the ELISA commercial kits (YL Biont, Shanghai, China). The operation protocols were according to the manufacturer's instructions. In brief, standard and tissue samples were added to the plate and incubated for 60 min at 37 °C. After the washing step, staining solutions were added and incubated for 15 min at 37 °C. The stop solution was added and read at 450 nm. There were standard curves used to calculate for all these kits. The coefficients of variation within and between plates were less than 10%.

Annexin V Binding Assay

Apoptosis was evaluated using the Muse Annexin V/Dead Cell (Merck Millipore, Darmstadt, Germany) assay. Shortly, the cells were seeded into six-well plates and were allowed to attach overnight before treatment. The cells were then treated with captopril (1 mg/mL), H_2O_2 (0.5 mM), or their

combinations and incubated for 24 h. After incubation, the Annexin V assay was performed according to the report in our previous study (Ergul and Bakar-Ates 2019).

Statistical analysis

The results were expressed as a mean ± standard error of the mean (SEM). The data analyses were performed with SPSS Version 23.0 for Windows. The data were evaluated using a one-way analysis of variance (ANOVA). A posthoc Tukey test was utilized to identify the differences between the experimental groups, and a value of $p < 0.05$ was accepted as statistically significant.

Results

Effect of captopril on cell survival after H_2O_2-induced oxidative damage

In this study, an XTT cell proliferation assay was conducted to assess the protective effects of captopril against H_2O_2-induced oxidative damage in C6 cells. Increasing doses of captopril (0.25–4 mg/mL) were tested on cell survival in both C6 control and H_2O_2-treated cells. C6 cells were firstly treated with increasing doses (0.25, 0.5, 1, 2, and 4 mg/mL) of captopril for 1 h and incubated for the next 24 h with or without 0.5 mM H_2O_2. There was a statistically significant difference between the groups in terms of cell viability ($F_{(11,24)} = 169.52$, $p = 0.001$). The preincubation of C6 cells with H_2O_2 for 24 h significantly reduced cell survival compared to untreated-control cells ($p < 0.001$; Fig. 2). Nevertheless, the measured doses of captopril enhanced cell survival in C6 cells in comparison to H_2O_2-treated C6 cells ($p < 0.001$; Fig. 2). Captopril, at all doses, did not affect C6 cells survival compared to the untreated-control cells. ($p > 0.05$; Fig. 2). In addition, as can be seen in Fig. 2, the concentration of 1 mg/mL was not significant compared to the untreated control group, while other concentrations were significant compared to the untreated control group. Moreover, since 1 mg/mL captopril dose protects cells better both alone and when combined with H_2O_2,this dose was used in further mechanistic studies.

Effect of captopril on TAS and TOS levels after H_2O_2-induced oxidative damage

The TAS and TOS levels in C6 cells were measured using the commercial spectrophotometric kits. The cells were pretreated with single doses (1 mg/mL) of captopril for 1 h and then incubated with or without 0.5 mM H_2O_2 for the next 24 h. There was a statistically significant difference between the groups in terms of TAS levels ($F_{(3,8)} = 185.05$, $p = 0.001$). As shown in Fig. 3, the treatment of H_2O_2 significantly decreased the TAS levels in C6 cells as compared with untreated-control cells ($p < 0.001$; Fig. 3). On the other hand, captopril significantly increased TAS in C6 cells as compared with untreated-control and H_2O_2-treated C6 cells ($p < 0.001$; Fig. 3). In terms of TOS levels, there was a significant difference between groups ($F_{(3,8)} = 136.98$, $p = 0.001$). Moreover, preincubating the C6 cells with H_2O_2 for 24 h significantly raised TOS levels as compared with the

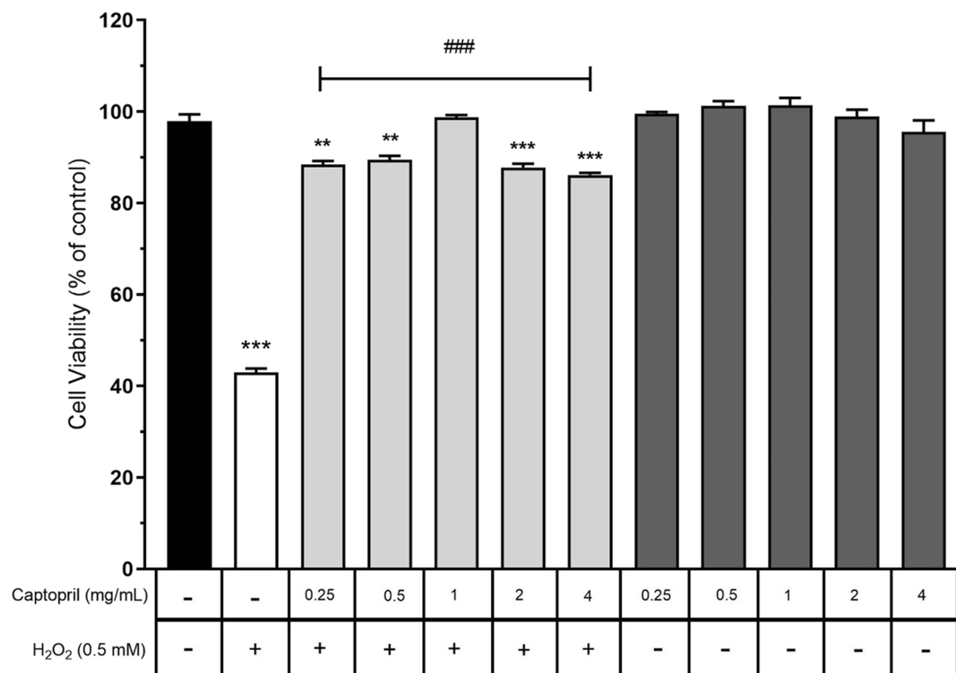

Fig. 2 The effect of captopril on C6 cell survival after H_2O_2-induced oxidative damage.The data are expressed as mean ± SEM. **$p < 0.01$ as compared to the control-untreated group, ***$p < 0.001$ compared to the control-untreated group; $^{###}p < 0.01$ compared to the H_2O_2-treated group

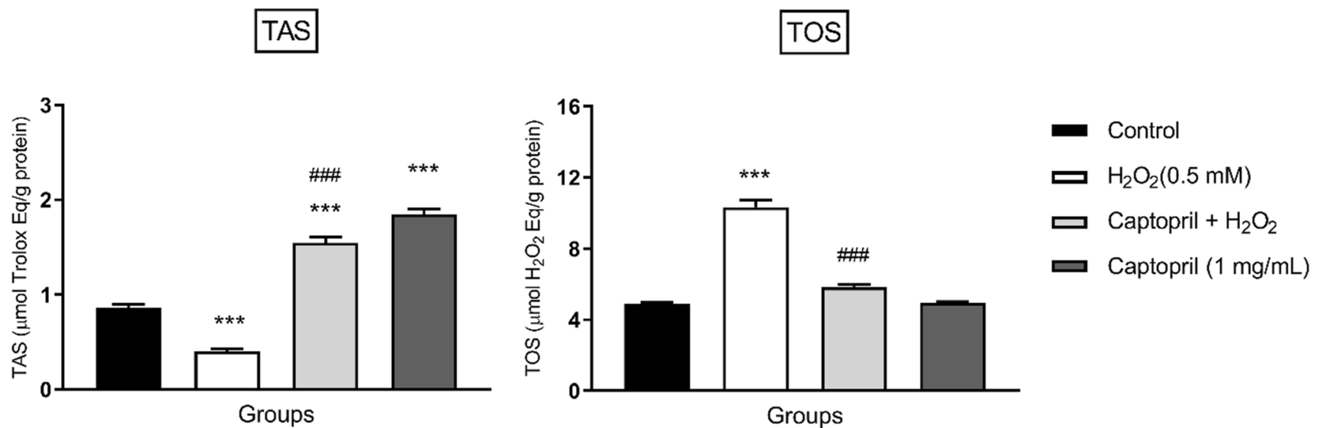

Fig. 3 Effect of captopril on TAS and TOS levels in C6 cells after H_2O_2-induced oxidative damage. The data are expressed as mean ± SEM. ***p < 0.001 as compared to the control-untreated group; ###p < 0.001 compared to the H_2O_2-treated group

untreated-control cells ($p < 0.001$; Fig. 3). Besides, captopril significantly reduced the TOS levels in C6 cells compared with the H_2O_2-treated C6 cells ($p < 0.001$; Fig. 3).

Effect of captopril on NF-kB, IL-1 β, COX-1, COX-2, Bax, and Bcl-2 levels after H_2O_2-induced oxidative damage

The ELISA measurements were performed to assess the effects of captopril on inflammatory markers after H_2O_2-induced in C6 cells. The cells were pretreated with single doses (1 mg/mL) of captopril for 1 h and then incubated with or without 0.5 mM H_2O_2 for the next 24 h. There was a statistically significant difference between groups in terms of NF-kB ($F_{(3,8)} = 353.42$, $p = 0.001$), IL-1 β ($F_{(3,8)} = 1829.11$, $p = 0.001$), COX-1 ($F_{(3,8)} = 32.36$, $p = 0.001$, and COX-2 ($F_{(3,8)} = 41.98$, $p = 0.001$) levels. Preincubating the C6 cells with H_2O_2 for 24 h significantly increased NF-kB, IL-1 β, COX-1, and COX-2 levels as compared with untreated-control cells ($p < 0.001$; Fig. 4). However, captopril significantly decreased the NF-kB, IL-1 β, COX-1, and COX-2 levels in C6 cells compared with the H_2O_2-treated C6 cells ($p < 0.001$; Fig. 4).

The ELISA measurements were also performed to evaluate the effects of captopril on apoptosis markers after H_2O_2-induced in C6 cells. The cells were pretreated with the single doses (1 mg/mL) of captopril for 1 h and then incubated with or without 0.5 mM H_2O_2 for the next 24 h. There was a statistically significant difference between groups in terms of Bax ($F_{(3,8)} = 233.52$, $p = 0.001$) and Bcl-2 ($F_{(3,8)} = 194.44$, $p = 0.001$) levels. As shown in Fig. 5, the treatment of H_2O_2 significantly increased Bax levels in C6 cells as compared with untreated-control cells ($p < 0.001$; Fig. 5). Nonetheless, captopril significantly reduced Bax levels in C6 cells as compared with H_2O_2-treated C6 cells ($p < 0.001$; Fig. 5). On the other hand, preincubating the C6 cells with H_2O_2 for 24 h significantly decreased Bcl-2 levels as compared with control-untreated cells ($p < 0.001$; Fig. 5). Besides, the captopril significantly increased Bcl-2 levels in C6 cells compared with H_2O_2-treated C6 cells ($p < 0.001$; Fig. 5).

Effect of captopril on apoptosis after H_2O_2-induced oxidative damage

Flow cytometry was performed to evaluate the anti-apoptotic effects of captopril after H_2O_2-induced oxidative damage in C6 cells. There was a statistically significant difference between groups behalf of live ($F_{(3,8)} = 1155.33$, $p = 0.001$), early apoptotic ($F_{(3,8)} = 208.91$, $p = 0.001$), late apoptotic ($F_{(3,8)} = 440.78$, $p = 0.001$) and dead ($F_{(3,8)} = 34.77$, $p = 0.001$). The results of flow cytometry analysis were given in Fig. 6, and it is exhibited that H_2O_2-treatment remarkably increased the proportion of apoptotic cells at IC_{50} concentration for 24 h when compared to untreated-control C6 cells ($p < 0.001$; Fig. 6). Furthermore, captopril pretreatment at a dose of 1 mg/mL significantly reduced the apoptotic percentage of C6 cells after H_2O_2-induced oxidative damage ($p < 0.001$; Fig. 6). However, pretreatment of captopril alone did not demonstrate a significant apoptotic effect in C6 cells ($p > 0.05$; Fig. 6).

Discussion

The present study, for the first time, evaluated the protective effect of captopril against H_2O_2-induced oxidative damage in C6 glial cells.

Oxidative stress refers to an imbalance between oxidants and antioxidant defense systems in the organism. This

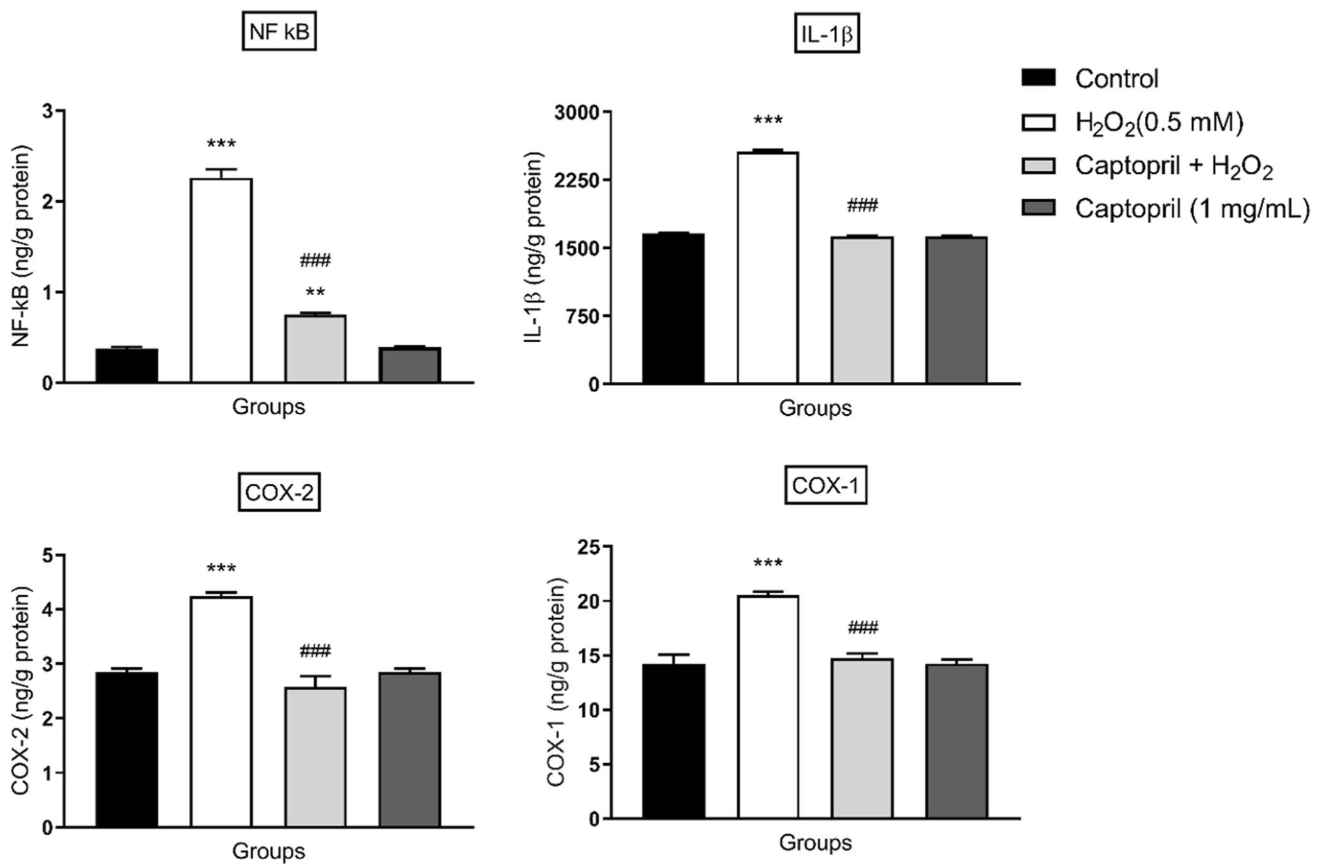

Fig. 4 Effect of captopril on NF-kB, IL-1 β, COX-1, and COX-2 levels in C6 cells after H_2O_2-induced oxidative damage. The data are expressed as mean ± SEM. ***p < 0.001 as compared to the control-untreated group; ###p < 0.001 compared to the H_2O_2-treated group

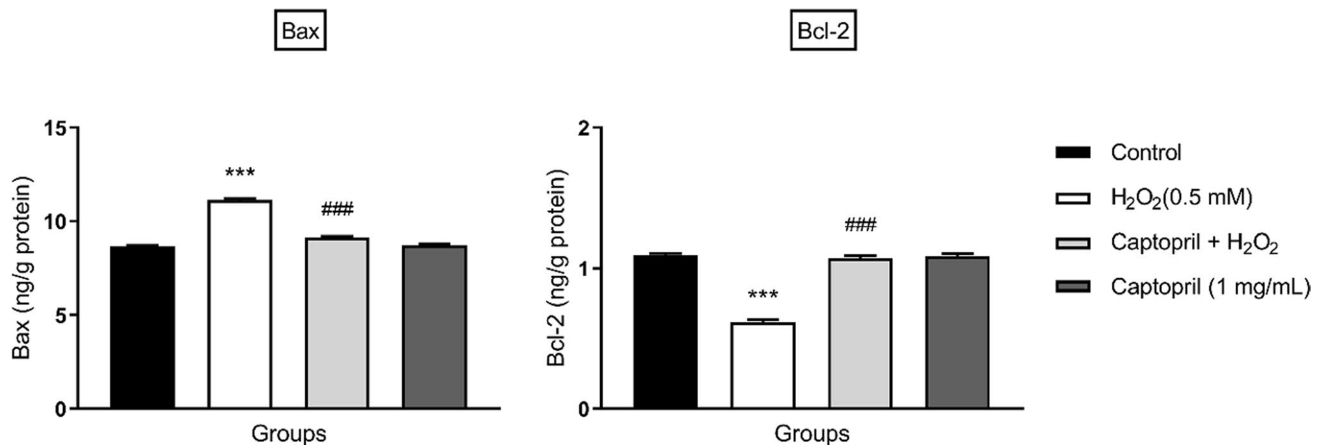

Fig. 5 Effect of captopril on Bax and Bcl-2 levels in C6 cells after H_2O_2-induced oxidative damage. The data are expressed as mean ± SEM. ***p < 0.001 as compared to the control-untreated group; ###p < 0.001 compared to the H_2O_2-treated group

imbalance in the oxidants and antioxidant systems causes excessive ROS production, which harms tissues and disturbs the physiological function of the organism (Ray et al. 2012). Moreover, evidence shows that oxidative stress involves the occurrence and progression of neurodegenerative diseases and CNS disorders (Andersen 2004). Previous studies have claimed that captopril induces antioxidant enzymes such as catalase and superoxide dismutase and protects tissues from oxidative damage by decreasing lipid peroxidation (Pourahmad et al. 2011; Tastemur et al. 2020). Consist with

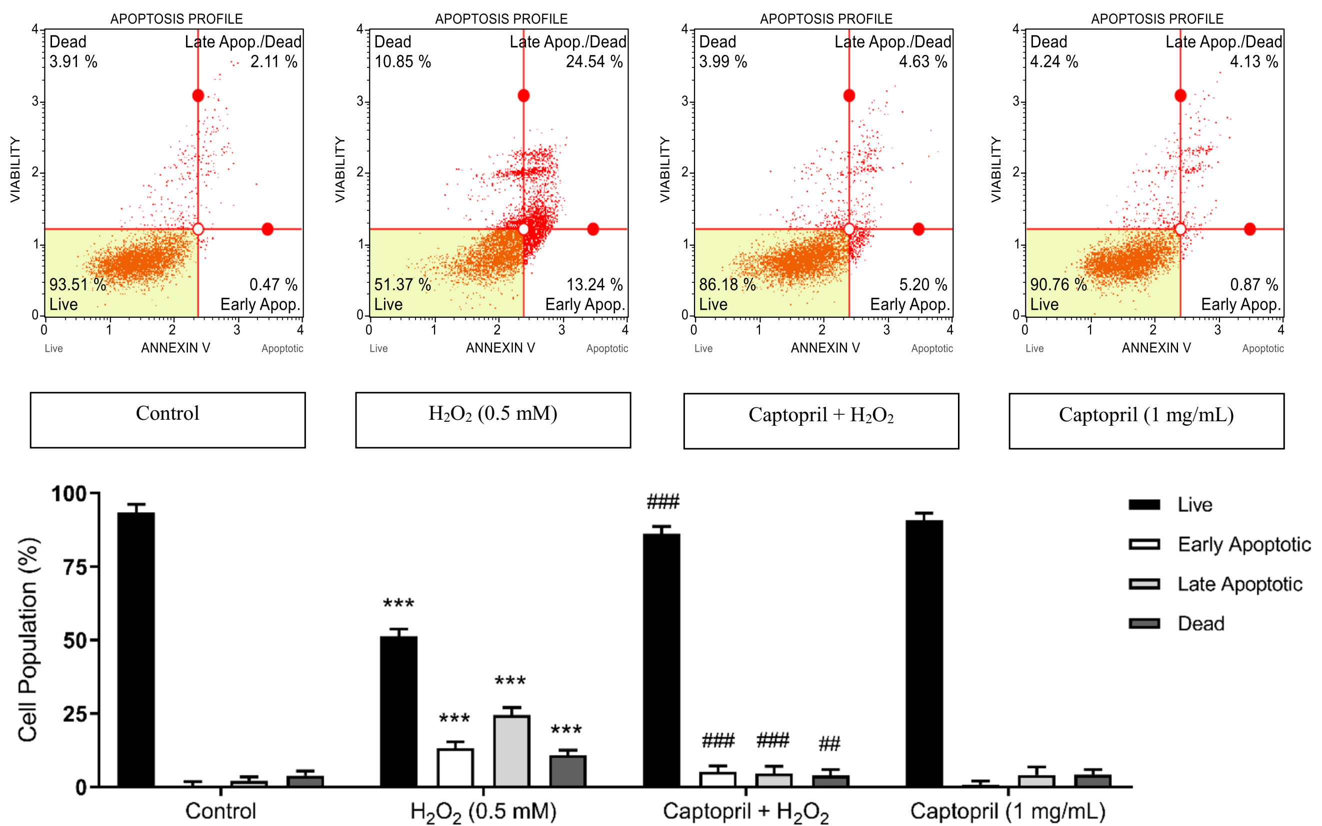

Fig. 6 Effect of captopril on apoptosis in C6 cells after H_2O_2-induced oxidative damage. The data are expressed as mean ± SEM. ***p < 0.001 as compared to the control-untreated group; $^{##}$p < 0.01 and $^{###}$p < 0.001 compared to the H_2O_2-treated group

these studies, in this study, captopril pretreatment showed antioxidant properties increasing significantly TAS levels and decreasing TOS levels which are oxidative stress markers after H_2O_2-induced oxidative damage in the C6 cells. Increasing the antioxidant system and reducing the oxidative stress could be one of the possible protective mechanisms of captopril in the C6 cells. In support of these data, this study showed that captopril significantly reduced cell death after H_2O_2-induced oxidative damage in the C6 cells.

It is known that astrocytes are extensively involved in the inflammatory response and innate immunity of the CNS (Jensen et al. 2013). In many CNS disorders, the pro-inflammatory type astrocyte activation persistently releases large amounts of inflammatory factors and exacerbates neuronal damage (Sofroniew 2009; Liddelow et al. 2017). Oxidative stress plays an important role in both astrocyte-associated inflammation and astroglial activation (Rizor et al. 2019). Free radicals can activate various inflammatory signaling pathways in astroglial cells and promote inflammatory factor release (Sofroniew 2009; Rizor et al. 2019). Thus, glial activation leads to inflammation that is closely related to the neurodegeneration in the nervous system (Takeuchi 2013). The nuclear factor-kappa light chain enhancer of activated B cell (NF-kB) is the main modulator of inflammation in the organism (Lawrence 2009). Its activation gives rise to releasing of proinflammatory cytokines, such as TNF-α and IL-1 β, chemokines, COX-1, and COX-2 (Bonizzi and Karin 2004; Ulivi et al. 2008). It has been shown that proinflammatory cytokines and COX enzymes are involved in CNS disorders by rising neuroinflammation in the brain (Glass et al. 2010; Cunningham 2013). The previous findings have found that captopril has an anti-inflammatory effect by inhibiting proinflammatory cytokines production, and it inhibits microglia activation (Asraf et al. 2018). Moreover, it has been reported that captopril decreases the proinflammatory cytokine interleukin-12 in the human peripheral blood mononuclear cells (Constantinescu et al. 1998). Furthermore, it has been claimed that other ACE inhibitor, perindopril, suppresses astrocytic and glial activation by reducing the production of TNF- α and oxidative stress (Bhat et al. 2016). Besides, in our previous study, it has been found that captopril decreases inflammatory pathway markers including NF-kB, IL-1 β, and TNF-α levels in dorsal root ganglia (Taskiran and Avci 2020). In this study, captopril pretreatment significantly reduced NF-kB, IL-1 β, COX-1, and COX-2 levels after H_2O_2-induced oxidative damage in C6 cells, consistent with previous studies. Modulating the effect of captopril in the inflammatory pathway could be another

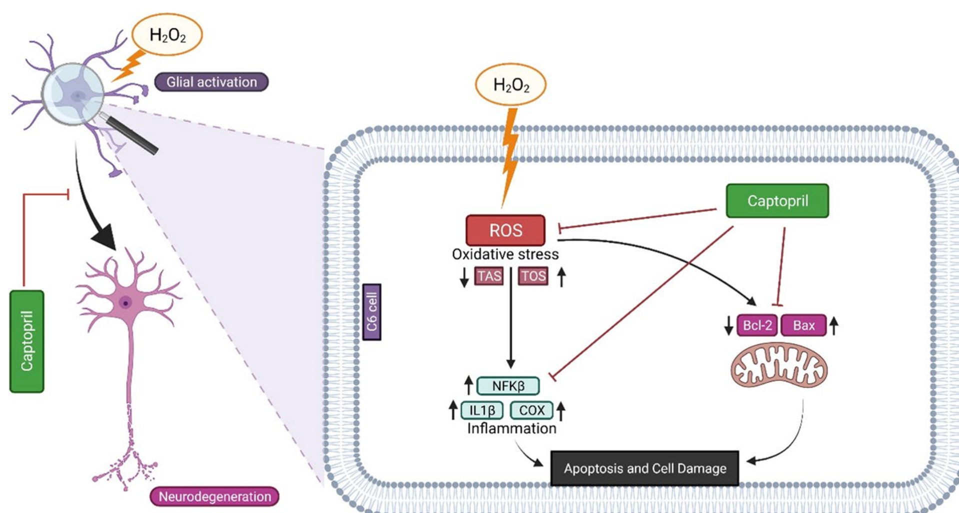

Fig. 7 A schematic diagram of the effects of captopril on C6 cells after H_2O_2-induced oxidative damage

possible protective mechanism after H_2O_2-induced oxidative damage in C6 cells.

Several studies have demonstrated that captopril has anti-apoptotic effects on various tissues including CNS (Odaka and Mizuochi 2000; Saglam et al. 2013). Previously, it has been claimed that captopril inhibits prion peptide-mediated neurodegeneration and neuronal apoptosis (Moon et al. 2019). In addition to the CNS, captopril has also been shown to have anti-apoptotic effects on liver tissue. Eid and El-Shitany (2021) reported that captopril significantly reduced apoptosis, inflammation, and oxidative stress in cisplatin-induced acutehepatic injury. It is well established that pro-apoptotic protein Bax promotes the induction of apoptosis, on the contrary, anti-apoptotic protein Bcl-2 suppresses apoptosis (Ergul and Bakar-Ates 2020). In this study, flow cytometry and ELISA assays were performed to evaluate whether captopril pretreatment suppressed apoptosis in C6 cells, and when compared with the H_2O_2 only group captopril inhibited the apoptosis significantly via decreasing Annexin V binding, Bax level and increasing the Bcl-2 level in C6 cells. Based on our results, captopril has a protective effect on glial cell survival after H_2O_2-induced oxidative damage in the C6 glioma cell line. Because glial cells are vital for neurodegenerative diseases, captopril may be a supportive therapeutic agent for the treatment of neurodegenerative diseases. However, it has to be proven by more in-depth investigations.

Conclusion

Taken together, the results of this study demonstrated that captopril decreased glial cell death after H_2O_2-induced oxidative damage. The main conclusions of this work are presented in Fig. 7, which exhibits that captopril strongly suppressed the TOS and increased TAS levels. It also significantly decreased the inflammatory pathway proteins, including NF-kB, IL-1 β, COX-1, and COX-2. Moreover, captopril treatment significantly increased expressions of Bcl-2 and the decreased expression of Bax protein levels after H_2O_2-induced oxidative damage. In conclusion, captopril seems to have meaningful protective effects via inhibition of oxidative stress, apoptosis, and inflammation markers. However, more in-depth investigation is needed to answer the questions raised about the possible mechanisms involved.

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Author contribution BS designed the study, interpreted the data, and had a major contribution in writing and revising the manuscript. ME performed the experiment, drafted the manuscript and analyzed data. All authors read and approved the final manuscript.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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

Conflict of interest All the authors declare that they have no conflict of interest.

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