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C6 Glial Hücre Hattında Hidralazinin Hidrojen Peroksit (H_2O_2) Kaynaklı Oksidatif Hasara Karşı Koruyucu Etkisi

Ahmet Şevki Taşkıran[1]*, Mustafa Ergül[2]

[1]Sivas Cumhuriyet Üniversitesi, Tıp Fakültesi, Fizyoloji Ana Bilim Dalı, Sivas, Türkiye
[2]Sivas Cumhuriyet Üniversitesi, Eczacılık Fakültesi, Biyokimya Ana Bilim Dalı, Sivas, Türkiye

ÖZET:
Amaç: Güncel çalışmalar hidralazinin sinir sistemi üzerinde olumlu etkileri olduğunu ortaya koymuştur. Fakat, glial hücrelerde hidrojen peroksit kaynaklı oksidatif stres üzerindeki etkisi hala belirsizdir. Bu çalışma, hidralazinin C6 glial hücrelerinde hidrojen peroksit kaynaklı oksidatif hasar sonrası glial hasar üzerindeki etkisini ve bu etkide proinflamatuar sitokinlerin rolünü incelemek için tasarlanmıştır. **Gereç ve Yöntem:** Bu çalışmada, C6 rat glioma hücre hattı kullanılmıştır. Hidralazinin hidrojen peroksitle oluşturulan oksidatif hasar üzerine etkisini değerlendirmek için dört farklı hücre grubu oluşturuldu. Kontrol grubu herhangi bir uygulama yapılmamıştır. H_2O_2 grubundaki hücreler 24 saat boyunca 0.5 mM H_2O_2 ile muamele edildi. Hidralazin grubundaki hücreler, 24 saat boyunca farklı konsantrasyonlarda (12.5, 25, 50 ve 100 µM/mL) hidralazin ile muamele edildi. Hidralazin + H_2O_2 grubundaki hücreler, 1 saat süreyle farklı konsantrasyonlarda (12.5, 25 50 ve 100 µM/mL) hidralazin ile ön işleme tabi tutuldu ve ardından 24 saat süreyle 0.5 mM H_2O_2'ye maruz bırakıldı. Oksidatif stres indüksiyonu tamamladıktan sonra, hücre canlılığı XTT analizi ile değerlendirildi. Hücrelerdeki total antioksidan durum (TAS), total oksidan durum (TOS), tümör nekroz faktörü-alfa (TNF-α) ve interlökin-1 beta (IL-1 β) seviyeleri ticari kitlerle ölçüldü. **Bulgular:** Hidralazin 50 ve 100 µM/mL konsantrasyonlarında, hidrojen peroksit ile indüklenen oksidatif hasara sonrası C6 hücrelerinde hücre canlılığını önemli ölçüde artırdı ($p < 0.001$). Ayrıca, hidralazin hidrojen peroksit kaynaklı oksidatif hasar sonrası artan TOS seviyelerini düşürürken ($p < 0.001$), azalan TAS seviyesini arttırdı ($p < 0.01$). Bununla birlikte, hidralazin C6 hücrelerinde oksidatif hasara bağlı artan TNF-α ve IL-1 β seviyelerini düşürdü ($p < 0.001$). **Sonuç:** Hidralazin, antioksidan sistemi aktive ederek ve proinflamatuar sitokinleri inhibe ederek C6 hücrelerinde hidrojen peroksit kaynaklı oksidatif hasara bağlı glial hücre ölümünü azaltmaktadır.
Anahtar Kelimeler: *Hidralazin, Oksidatif Stres, Hücre Ölümü, Proiflamatuvar Sitokinler, C6 Gliom*

The Protective Effect of Hydralazine against Hydrogen Peroxide (H_2O_2)-Induced Oxidative Damage in C6 Glial Cell Line

ABSTRACT:
Purpose: Recent studies have shown that hydralazine has positive effects on nervous system. However, its effect on hydrogen peroxide-induced oxidative damage in glial cells is still unclear. The current experiment was designed to examine the effect of hydralazine on glial damage after hydrogen peroxide-induced oxidative damage in C6 glial cells involving proinflammatory cytokines. **Material and Methods:** In this study, the C6 glioma cell line was used. Four cell groups were prepared to evaluate the effect of hydralazine on glial cell death after hydrogen peroxide-induced oxidative damage. 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 hours. Cells in the hydralazine group were treated with various concentrations (12.5, 25, 50, and 100 µM/mL) of hydralazine for 24 hours. Cells in the hydralazine + H_2O_2 group were pre-treated with various concentrations (12.5, 25 50, and 100 µM/mL) of hydralazine for 1 hour and then exposed to 0.5 mM H_2O_2 for 24 hours. After completing oxidative damage induction, the cell viability was evaluated XTT assay. Total antioxidant status (TAS), total oxidant status (TOS), tumor necrosis factor alpha (TNF-α), and interleukin-1 beta (IL-1 β) levels in the cells were measured by commercial kits. **Results:** Hydralazine at the concentrations of 50 and 100 µM/mL significantly increased the cell viability in C6 cells after hydrogen peroxide-induced oxidative damage ($p < 0.001$). It also significantly decreased the levels of TOS ($p < 0.001$) whereas rising TAS levels ($p < 0.01$) after hydrogen peroxide-induced oxidative damage. Moreover, hydralazine reduced TNF-α and IL-1 β levels in C6 cells after hydrogen peroxide-induced oxidative damage ($p < 0.001$). **Conclusion:** Hydralazine decreases glial cell death after hydrogen peroxide-induced oxidative damage in C6 cells by activating antioxidant system and inhibiting proinflammatory cytokines.
Keywords: *Hydralazine, Oxidative Stress, Cell Death, Proinflammatory Cytokines, C6 Glioma*

**Corresponding author: Ahmet Şevki Taşkıran, email: a.sevkitaskiran@gmail.com*

INTRODUCTION

In the normal cellular metabolism, reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are generated. They play vital roles in the process of signal transmission (Dringen et al., 1998; Forman, 2007). However, increasing in the H_2O_2 production damages to the cellular component and also causes genotoxic effects (Andersen, 2004; Gandhi and Abramov, 2012). Besides, excessive production of ROS leads to oxidative damage, which results in cellular dysfunction and cell death (Coyle and Puttfarcken, 1993; Ray et al., 2012). The brain is the most vulnerable tissue to oxidative damage because of its high metabolism and lipid structure. Therefore, oxidative stress is one of the main factors for neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases (Blesa et al., 2015; Lu et al., 2014). Glial cells are non-neuronal cells and they have important functions in the central nervous system (CNS) like maintaining homeostasis, supporting, and protecting of the neurons (Jessen, 2004). Therefore, the attenuation of oxidative stress in glial cells is critical for neurodegenerative disorders.

Hydralazine is primarily used to treatment severely hypertension in the clinics (Magee, 2003). However, recent studies have been reported other pharmacological properties of hydralazine. On the one hand, in vivo studies have shown that hydralazine has positive effects on heart failure and ischemia/reperfusion injury via its antioxidant and anti-inflammatory properties (Dulce et al., 2013; Li et al., 2019). On the other hand, in vitro studies have found that hydralazine has a potent radical scavenger and reduces pro-inflammatory cytokines and the activation of macrophage (Daiber et al., 2005; Leiro et al., 2004). It also has been demonstrated that hydralazine suppresses carbonyl precursors during the lipid peroxidation processes and prevents cell death by inhibiting secondary reactions (Galvani et al., 2008; Kaminskas et al., 2004). However, its effect on oxidative stress in the C6 glial cells and underlying mechanisms are still unclear. In the present study, it was examined that the effect of hydralazine against hydrogen peroxide-induced oxidative stress in C6 glial cells involving in proinflammatory cytokines.

MATERIAL 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 5% CO2 humidified atmosphere. Hydralazine 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 1×104 cells per well in 100-µL DMEM and grown overnight before hydralazine. The following day, four cell groups were prepared for evaluate the protective effect of hydralazine. 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 hours. Cells in the hydralazine group were treated with various concentrations (12.5, 25, 50, 100 µg/mL) of hydralazine for 24 hours. Cells in the hydralazine + H_2O_2 group were pre-treated with various concentrations (12.5, 25, 50, 100 µg/mL) of hydralazine for 1 hour and then exposed to 0.5 mM H_2O_2 for 24 hours. 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 was added to all the wells and then the plates were maintained at 37ºC for 4 hours. 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 viable cell amount percent compared with control, as untreated cells (Figure 1).

Preparation of cells homogenates

The cells for each groups were collected by sterile tubes. They were centrifuged at 2000 RPM for

approximately 10 minutes. The supernatants were removed. The component of cells which are in 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 minutes at a temperature of 4ºC. Then, the supernatants were collected for biochemical analysis of total antioxidant status (TAS) and total oxidant status (TOS) by using TAS and TOS commercial kits (Rel Assay Kit Diagnostics, Antep, Turkey). Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used to determination of total protein levels in samples (Kruger, 1994).

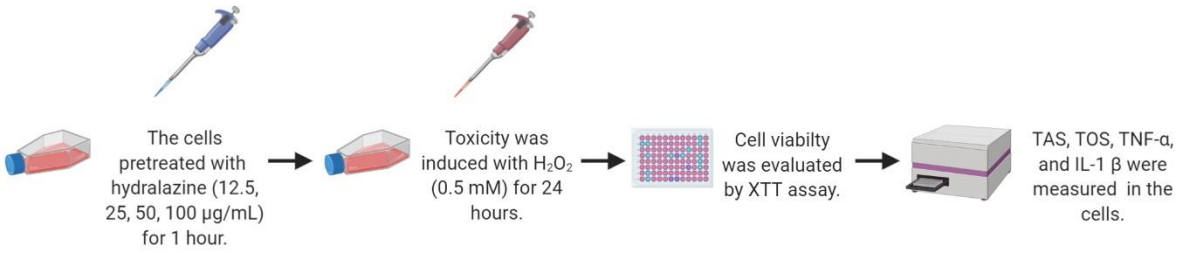

Figure 1. Experimental design of study (created by BioRender).

Measurement of TAS and TOS

The TAS concentrations at the cell supernatants were determined with an automated assay 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 colored dianisidyl radicals during free radical reactions starting with the production of hydroxyl radicals in Fenton reaction. Antioxidants in the tissue samples should suppress coloring 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 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 TNF-α and IL-1 β

The levels of TNF-α and IL-1 β from the cells supernatants for each groups were measured using rat ELISA commercial kits (YL Biont, Shanghai, China). The operation protocols were according to manufacturer's instructions. In brief, standard and tissue samples were added in plate and incubated for 60 minutes at 37°C. After washing step, staining solutions were added and incubated for 15 minutes at 37°C. Stop solution was added and read at 450 nm. Standard curves were plotted to determine the value of samples. The coefficients of variation within and between plates were less than 10%.

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) and a postdoc 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 hydralazine on cell survival after H_2O_2-induced oxidative damage

In this study, it was tested that increasing doses of hydralazine (12.5–100 µM/mL) on cell survival in both control and H_2O_2-treated C6 cells. The cells were initially treated with increasing doses (12.5, 25, 50, and 100 µM/mL) of hydralazine for 1 hour and then incubated with or without 0.5 mM H_2O_2 for the next 24 hours. As shown in figure 2, preincubating the C6 cells with H_2O_2 for 24 hours significantly reduced cell survival as compared with control-untreated cells ($p < 0.001$; Figure 2). However, the tested doses of 50 and 100 µM/mL hydralazine increased cell survival in C6 cells as compared with H_2O_2-treated C6 cells ($p < 0.001$; Figure 2). Furthermore, hydralazine, in all doses, did not change C6 survival compared with control-untreated cells ($p > 0.05$; Figure 2).

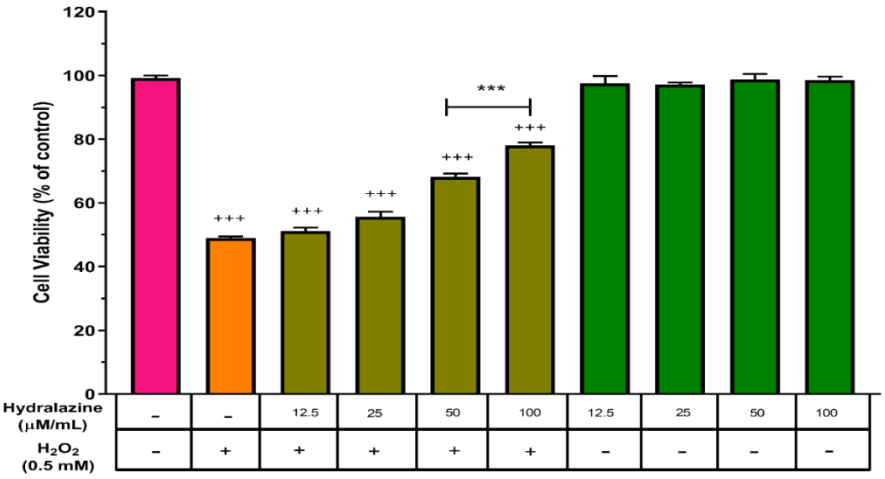

Figure 2. Effect of hydralazine on cell survival in C6 cells after H_2O_2-induced oxidative damage. The data are expressed as mean ± standard error mean. +++$p < 0.001$ as compared with control-untreated group; ***$p < 0.001$ compared with H_2O_2-treated.

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

The cells were treated with the single doses (100 µM/mL) of hydralazine for 1 hour and then incubated or not incubated with 0.5 mM H_2O_2 for the next 24 hours. As shown in figure 3, the treatment of H_2O_2 decreased TAS levels in C6 cells as compared with control-untreated cells ($p < 0.001$; Figure 3A). On the other hand, the hydralazine significantly increased TAS in C6 cells as compared with control-untreated ($p < 0.01$; Figure 3A) and H_2O_2-treated C6 cells ($p < 0.001$; Figure 3A). Moreover, preincubating the C6 cells with H_2O_2 for 24 hours significantly raised TOS levels as compared with control-untreated cells ($p < 0.001$; Figure 3B). Besides, the hydralazine significantly reduced TOS levels in C6 cells compared with H_2O_2-treated C6 cells ($p < 0.001$; Figure 3B).

Effect of hydralazine on TNF-α and IL-1 β levels after H_2O_2-induced oxidative damage

The cells were treated with the single doses (100 µM/mL) of hydralazine for 1 hour and then incubated or not incubated with 0.5 mM H_2O_2 for the next 24 hours. Preincubating the C6 cells with H_2O_2 for 24 hours significantly increased TNF-α and IL-1 β levels as compared with control-untreated cells ($p < 0.001$; Figure 4A and 4B). However, the hydralazine significantly decreased TNF-α and IL-1 β levels in C6 cells compared with H_2O_2-treated C6 cells ($p < 0.001$; Figure 4A and 4B).

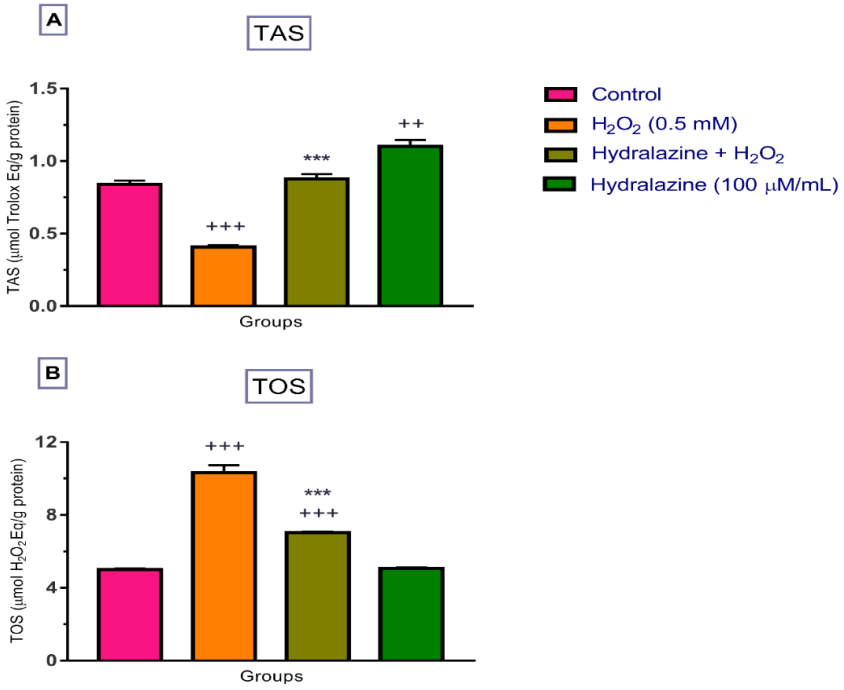

Figure 3. Effect of hydralazine on TAS and TOS levels in C6 cells after H_2O_2-induced oxidative damage. The data are expressed as mean ± standard error mean. ++p < 0.01 and +++p < 0.001 as compared with control-untreated group; ***p < 0.001 compared with H_2O_2-treated.

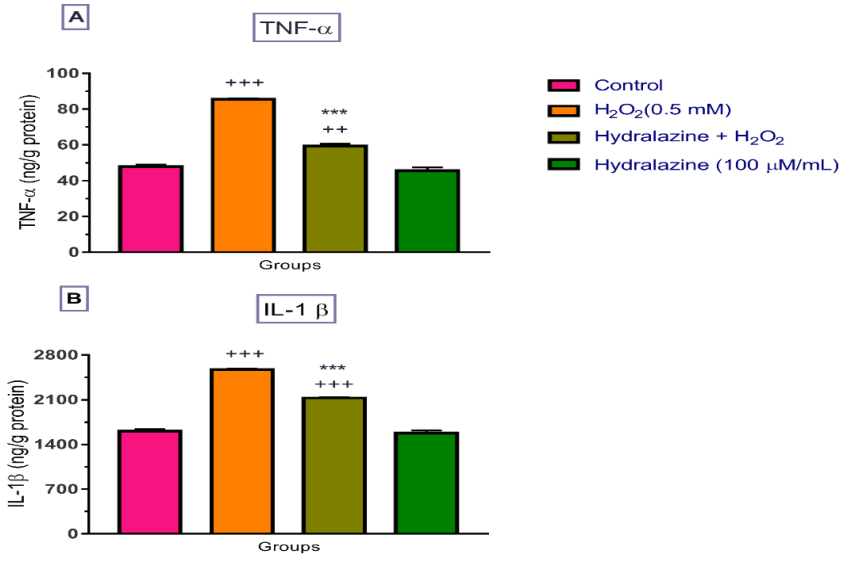

Figure 4. Effect of hydralazine on TNF-α and IL-1 β levels in C6 cells after H_2O_2-induced oxidative damage. The data are expressed as mean ± standard error mean. ++p < 0.01 and +++p < 0.001 as compared with control-untreated group; ***p < 0.001 compared with H_2O_2-treated.

DISCUSSION

The present study, for the first time, evaluated the effect of hydralazine against hydrogen peroxide-induced oxidative stress in the C6 glial cells. Here it has shown that pretreatment with hydralazine reduced toxic changes of hydrogen peroxide in the C6 cells. Furthermore, hydralazine pretreatment increased in TAS levels and decreased in TOS levels after hydrogen peroxide-induced oxidative stress in the C6 cells. Besides, hydralazine pretreatment

reduced proinflammatory cytokines TNF-α and IL-1 β after hydrogen peroxide-induced oxidative stress. Several studies have demonstrated that hydralazine protective effects on CNS disorders. In one in vitro study, it has been claimed that hydralazine suppresses Aβ fibril formation and prevents Alzheimer's formation (Maheshwari et al., 2010). Moreover, in another in vitro study, hydralazine protects SH-SY5Y neuroblastoma cell 6-hydroxydopamine-induced Parkinson's model by modulating oxidative stress and inflammatory pathways (Mehrabani et al., 2020). Furthermore, line on these studies, in this study, it was found that hydralazine increased cell viability in XTT assay after H_2O_2-induced oxidative damage in C6 cells by activating antioxidant system and inhibiting of oxidative stress, 2 enzymes.

Oxidative stress is defined as an imbalance between oxidants and antioxidant defense systems. This imbalance 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 plays a vital role in the occurrence of neurodegenerative diseases and CNS disorders (Andersen, 2004). Previous studies have claimed that hydralazine has antioxidant properties and protect tissues from oxidative damage by increasing total glutathione and increasing lipid peroxidation (Hamann, Nehrt, Ouyang, Duerstock, and Shi, 2007; Mehrabani et al., 2020). Consist with these studies, in this study, hydralazine pretreatment showed antioxidant properties increasing TAS levels and also decreased oxidative damage reducing TOS levels after H_2O_2-induced oxidative damage in the C6 cells.

TNF-α and IL-1 β are proinflammatory cytokines that released from immune cells. It has been reported that TNF-α and IL-1 β are involved in the CNS disorders by rising neuroinflammation in the brain (DiSabato et al., 2016; Ransohoff, 2016). The previous findings have shown that hydralazine has anti-inflammatory properties through the inhibition of macrophage activation, leukocyte adhesion molecule expression and pro-inflammatory cytokines production (Daiber et al., 2005; Dulce et al., 2013; Li et al., 2019).In this study, hydralazine reduced TNF-α and IL-1 β levels after hydrogen peroxide-induced oxidative damage in C6 cells consist with previous studies. According to our findings, hydralazine plays important role in the glial cell survival. Because glial cells are critical for neurodegenerative diseases, hydralazine could be a supportive therapeutic agent to the treatment of neurodegeneration related diseases. However, it is needed to be proven by further investigations.

The study has potential limitations. This study was performed in C6 rat glioma cells rather than primer glial cells. Our methods are not enough to illuminate all mechanisms of hydralazine protective effect on C6 glial cells. Further methods such as immunohistochemical and calcium imaging studies are needed to clarify underlying mechanisms of hydralazine on glial cells.

CONCLUSION

The findings of this study showed that hydralazine reduced oxidative damage against hydrogen peroxide-induced oxidative damage in C6 glial cells. These effects may occur possibly through inhibition of oxidative stress and proinflammatory cytokines. Therefore, hydralazine could be protective effect in the CNS. However, further investigation is required to answer the questions raised about the probable mechanisms involved.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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