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Neuroprotective role of chrysin against bupivacaine induced apoptosis and oxidative stress in SH-SY5Y cell line

İlknur Çınar Ayan[1]*, Ebru Güçlü[1]

[1]Necmettin Erbakan University, Meram Faculty of Medicine, Depertman of Medical Biology, 42090, Konya, Türkiye.

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Corresponding Author
Tel.: +90 534 971 11 35
E-mail: ilknrcnar@gmail.com

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Abstract

Chrysin, a natural flavonoid, has a strong neuroprotective effect in many neurodegenerative diseases. Therefore, we aimed to investigate the neuroprotective effect of chrysin against bupivacaine-induced neurotoxicity in SH-SY5Y cells. According to the results of XTT analysis, the non-toxic concentration of chrysin was determined and the cells were treated with bupivacaine alone and together with this determined chrysin dose. According to the results of RT-qPCR analysis, the level of caspases increased in the group treated with only bupivacaine compared to the control group, while the expression of antioxidant enzymes decreased. When compared with the group treated with bupivacaine alone, it was determined that while the expression of caspases decreased in the group in which bupivacaine and chrysin were treated together, the expression of antioxidant enzymes increased. According to the ELISA results, SOD and CAT activities were decreased in the group treated with bupivacaine alone compared to the control group. SOD and CAT activities increased in the presence of chrysin treated with bupivacaine compared to the group treated with bupivacaine alone. The obtained data showed that chrysin may play a neuroprotective role by inducing the expression of antioxidant enzymes while inhibiting apoptosis against bupivacaine-induced neurotoxicity in SH-SY5Y cells.

Introduction

Regional anesthesia is the temporary elimination of nerve conduction and pain sensation in certain parts of the body without causing loss of consciousness (Ardon et al., 2019). Local anesthetics are used in blocking the nerves going to the area to be operated and in the management of post-operative pain (Yu et al., 2017). Bupivacaine is the most toxic local anesthetic widely used in clinical practice for epidural anesthesia, nerve blockade, and postoperative analgesia (Zhao & Wang, 2020; Kendall et al., 2018). In studies conducted so far, it has been determined that long-term and high-dose treatment with local anesthetics is highly effective as an analgesic, but it also causes neurotoxic damage and serious neurological complications (Wang et al., 2021; Niu et al., 2014). Bupivacaine treatment triggers a series of signaling pathway cascades that cause neuronal apoptosis-mediated neurotoxicity (Li et al., 2013). Increased reactive oxygen species, decreased antioxidant response systems, caspase activation, and mitochondrial dysfunction are among the markers of apoptosis induced by local anesthetics (Bouderba et al., 2012).

Oxidative stress occurs when redox homeostasis associated with cell survival is disrupted. When the level of ROS (reactive oxygen species) produced as by-products of oxygen metabolism in the body exceeds the antioxidant capacity, it causes oxidative stress called cellular redox imbalance and this induces cellular biomolecular damage (Su et al., 2013; Nirmaladevi et al., 2014). Cellular antioxidant systems directly remove free

radicals and maintain the intracellular redox state by converting ROS into more stable molecules such as H_2O and O_2 through antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Sukprasansap et al., 2020; Reddy, 2008). Oxidative stress caused by the cumulative increase of ROS also plays an important role in many physiological processes including cellular apoptosis and in the pathologies of neurodegenerative disorders (Wang et al., 2018). Although the mechanisms underlying bupivacaine-induced neurotoxicity are not fully understood, it is widely believed that activation of the apoptotic pathway can induce nerve damage and neurotoxicity (Werdehausen et al., 2009; Ji et al., 2015). Considering that the use of anesthetics in surgical procedures is inevitable, it becomes very important to develop new neuroprotective strategies against bupivacaine-induced neurotoxicity (Zhao & Wang, 2020).

Chrysin (5,7-Dihydroxyflavone) is a natural phytochemical flavonoid abundant in honey, propolis, and blue passion flower, which have great economic and medicinal value, as well as various mushrooms, and plants (Mani & Natesan, 2018). In vivo, in vitro, and clinical studies carried out so far chrysin has been shown to have many pharmacological activities such as antioxidant, anticancer, antibacterial, anti-inflammatory, antidiabetic, and antidepressant (Karthikeyan et al., 2013; Xiao et al., 2014; Ahad et al., 2014; Filho et al., 2015; Song et al., 2016). It has also been determined that chrysin has a neuroprotective effect in many neurodegenerative disorders due to neuron damage (Sathiavelu et al., 2009; Zhang et al., 2015; Souza et al., 2015). In this study, we aimed to investigate the anti-apoptotic and antioxidant response-mediated neuroprotective potential of chrysin against bupivacaine-induced neurotoxicity in SH-SY5Y neuroblastoma cells.

Materials and Methods

Cell culture and treatment

SH-SY5Y (Human Neuroblastoma cells) purchased from ATCC were cultured in DMEM-F12 medium (Sigma-Aldrich, USA) supplemented with 10 % fetal bovine serum (FBS) (Capricorn, Germany) and 100 U/ml penicillin-streptomycin (10 mg/mL) at 37°C in a humidified atmosphere containing 5 % CO_2. Chrysin was obtained from Sigma-Aldrich (D7321 Merck; Germany) and dissolved at a final concentration of 0.1 % DMSO to prepare a stock solution. The stock solution was stored at -20°C until used in the experiments and diluted with a culture medium to prepare different concentrations.

Cell viability assay

The cytotoxic effects of bupivacaine and chrysin on SH-SY5Y cell viability were evaluated by XTT cell proliferation assay (Biological Industries, 20-300-1000). SH-SY5Y cells were seeded into 96-well plates at a density of 2x104 cells/well. After 24 hours (h) of incubation, cells were treated with different concentrations of chrysin (5-10-15-20-40-50-75-100 µM) and bupivacaine (200-400-500-750-1000 µM) for 24 h. XTT assay was carried out to determined IC50 value of bupivacaine and chrysin. To assess the therapeutic efficacy of chrysin against bupivacaine toxicity, cells were pretreated with different concentrations of chrysin (5-10-15-20-40-50-75-100 µM) for 6 h and then incubated with the bupivacaine of effective IC50 dose (500 µM) for 24 h. According to the results of XTT analysis, the non-toxic concentration of chrysin was detected 10 µM at 24th hours. XTT solution was added to each well and incubated for 4 h at 37 °C. After incubation, the OD (optical density) absorbance values of the wells were measured at a wavelength of 450 nm and 630 nm (reference absorbance) on an ELISA microplate reader (BioTek, Epoch). In subsequent analyses, cells were co-treated with 10 µM chrysin, 500 µM bupivacaine as a combination dose. The detail of the cell viability assay has been described in our previous study (Çınar Ayan et al., 2022).

RT-qPCR

To evaluate the apoptosis pathway and antioxidant enzymes mediated protective effect of chrysin against bupivacaine-induced neurotoxicity in SH-SY5Y cells, real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed. First, SH-SY5Y cells were seeded in 6-well plates at a density of 2,5x104 cells/well and incubated for 24 h at 37 °C. After dose treatment to the cells for 24 h, RNA isolation was performed with RiboEx reagent (GeneAll, 301–001) from the sample wells of the dose and control group. The concentrations and quality of the isolated RNA samples were determined by reading the absorbance at 260 and 280 nm with a nanodrop instrument, a UV spectrophotometer. DNase I enzyme (Thermo Scientific, USA) was used to remove possible DNA contamination from RNA samples. Purified RNAs were reversed to cDNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad, 170–8891) according to the manufacturer's instructions. The primer sequences of CASP3, CASP7, CASP8, CASP9, SOD1, SOD2, SOD3, CAT genes associated with the apoptosis pathway, and antioxidant response enzymes were designed with IDT PrimerQuest (https://eu.idtdna.com/Primerquest/Home/Index). RT-qPCR was performed using SYBR on an Applied Biosystems thermocycler. The RT-qPCR conditions were as follows: 95°C for 4 min, 40 cycles of amplification (95°C for 10 sec, 60°C for 60 sec, and 72°C for 4 min). The detail of the RT-qPCR assay has been declared in our previous study (Güçlü et al., 2022).

Superoxide dismutase (SOD) activity assay

The SOD enzyme activity was measured by using a Superoxide dismutase (SOD) activity assay kit (BioVision-K335-100) (ELISA based) according to the manufacturer's protocol. For this analysis, cells were seeded into a 6-wells plate at a density of 5x105

cells/well and incubated overnight. Afterwards, cells were treated with dose groups (Bupivacaine and chrysin alone and together) for 24 h. Cells were lysed in ice-cold 0.1 M Tris/HCl, pH 7.4 containing 0.5 % Triton X-100, 5 mM β-ME, 0.1 mg/ml PMSF. The obtained cell lysate was centrifuged at 14000 x g for 5 min at 4 °C. The collected supernatant contains total SOD activity from cytosolic and mitochondria. Sample, blank 1, blank 2, and blank 3 wells were determined in a 96-well plate. While 20 µl of Sample Solution was added to the sample and blank 2 well, 20 µl H_2O was added to Blank 1 and Blank 3 well. Afterwards, 200 µl of WST Working Solution was added to all wells. 20 µl of dilution buffer was added to blank 2 and blank 3 wells, and 20 µl enzyme working solution was added to the sample and blank 1 wells and mixed well. After the plate was incubated for 20 min at 37 °C, the absorbance of each well was measured at 450 nm using a microplate reader.

Catalase (CAT) activity assay

Catalase is an antioxidant enzyme that catalyzes the conversion of hydrogen peroxide, an undesirable by product of aerobic respiration, into water and oxygen. The catalase enzyme activity was measured by using a CAT activity assay kit (BioVision-K335-100) (ELISA based) according to the manufacturer's protocol. Afterwards, cells were treated with dose groups (Bupivacaine and chrysin alone and together) for 24 h. Cells were homogenized in 0.2 ml cold assay buffer and centrifuged at 10000 x g for 15 minutes at +4 °C, then the phase of supernatant was collected for assay. The total volume in each well was made up to 78 µl, and 50 µl of sample supernatant and 28 µl of assay buffer were added to the wells. In separate wells, 50 µl of sample supernatant and 28 µl of assay buffer were added to prepare sample high control (HC), making the total volume to 78 µl. To inhibit the catalase activity in the samples, 10 µl of stop solution was added to the sample HC and incubated at 25 °C for 5 min. For the H_2O_2 standard curve, stock 0.88 M H_2O_2 was diluted with distilled water and a 1mM H_2O_2 solution was prepared. Afterwards, an H_2O_2 standard was created at different concentrations of 0-2-4-6-8-10 nmol/well in 96 plate from a 1 mM H_2O_2 solution. To initiate the catalase reaction, 12 µl of 1 mM H_2O_2 was added to each well and incubated at 25°C for 30 min. After incubation, 50 µl developer buffer containing 46 µl of Catalase assay buffer, 2 µl of OxiRed Probe, and 2 µl of HRP solution were added to each well, mixed, and incubated at 25°C for 10 min. The absorbance (OD) of each well was measured in a microplate reader at a wavelength of 570 nm.

Statistical analysis

Data were repeated in triplicate. All results were presented as mean ± SD (standard deviation). Cell viability analysis, comparison of control versus treatment groups, and comparison between groups were performed with GraphPad Prism version 8.0.2 using Student's t-test and one-way ANOVA test. P<0.05 values were considered statistically significant.

Results & Discussion

SH-SY5Y human neuroblastoma cells are widely used to investigate the neurotoxicity of local anesthetics because they can mimic the biological properties of neuron cells. Therefore, SH-SY5Y cells were used as an in vitro neuronal damage model. In this study, we aimed to investigate the protective role of chrysin mediated by apoptosis and oxidative stress against bupivacaine-induced neuronal damage in SH-SY5Y cells.

Firstly according to the XTT cell viability test, it was observed that bupivacaine inhibited SH-SY5Y cell proliferation in a dose-dependent manner. And the IC50 dose, which was half maximal inhibitory concentration (killed about half of the cells), was determined as 500 µM at 24 h (Figure 1). Therefore the dose for bupivacaine was used as 500 µM in subsequent experiments. In previous studies in the literature, the neurotoxic IC50 dose (about 50% cell growth inhibition) of bupivacaine in SH-SY5Y cells was found to be in the range of 500-1000 µM (Wen et al., 2013; Wang et al., 2019; Zhao & Wang, 2020). With our cell viability test results, we have demonstrated that bupivacaine can cause neuronal cell death by inducing neurotoxicity, in agreement with previous studies (Dhanalakshmi et al., 2015; Zhang et al., 2019).

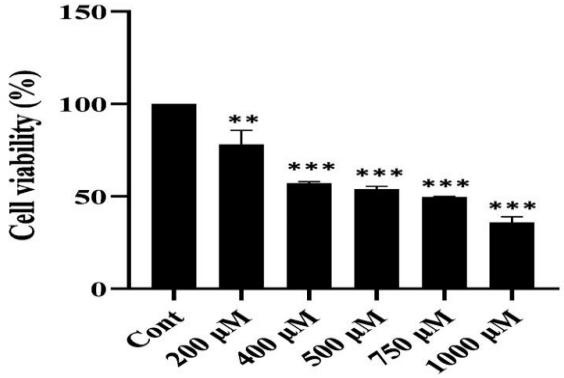

Figure 1. Cytotoxic effects of bupivacaine on SH-SY5Y cells. Different concentrations of bupivacaine were treated in SH-SY5Y cells for 24 h dose dependent manner. Each group was subjected to least three independent experiments. Cell viability assay determined by XTT cell proliferation assay (*P <0.05, **P <0.01, and ***P<0.001).

It was also determined that cell viability decreased in a dose-dependent manner when cells were treated with chrysin alone (Figure 2). SH-SY5Y cells were treated for 24 h in combination with bupivacaine (500 µM) after 6 hours of pretreatment with different concentrations of chrysin. Chrysin significantly inhibited bupivacaine-induced cell death, resulting in an increase of 45.1078, 41.6888, and 44.0577 % in cell viability at 5 µM, 10 µM, and 15 µM concentrations, respectively. When the cytotoxic effects of both chrysin alone and in combination with bupivacaine on cell viability were

evaluated together, the non-toxic chrysin dose was determined as 10 µM for 24 h (Figure 3).

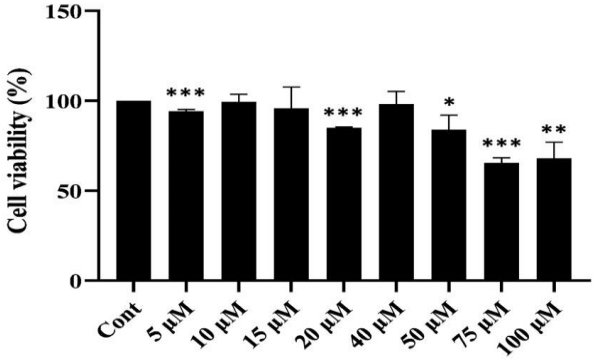

Figure 2. Antiproliferative effects of chrysin on SH-SY5Y cells in a dose-dependent manner. Cells were treated with different concentrations of chrysin for 24 h. Each group was subjected to least three independent experiments. Cell viability was determined by XTT assay and presented as a percentage relative to untreated control cells (100%) (*P <0.05, **P <0.01, and ***P<0.001).

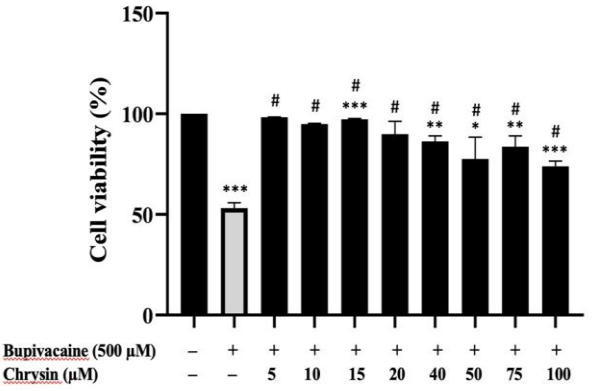

Figure 3. Effects of different concentration of chrysin against bupivacaine-induced oxidative damage in SH-SY5Y cells using XTT assay. Each group was subjected to least three independent experiments. After pretreatment with various concentrations of chrysin (5, 10, 15, 20, 40, 50, 75 and10 µM) for 6 h, cells were treated with 500 µM bupivacaine for 24 h (*P<0.05, **P<0.01, and ***P<0.001 versus control group; #p <0.05 versus bupivacaine group).

It is known that foods rich in antioxidants have protective effects against cancer, cardiovascular diseases, and neurological degeneration (Wollgast & Anklam, 2000). Medicinal plant-based natural phenolic compounds induce apoptosis of many cancer cells by targeting multiple pathways involved in cell death. Flavonoids are phenolic compounds commonly found in natural plant structures (Lim et al., 2018). Chrysin is a naturally occurring flavonoid, especially in honey and propolis. In a study, it was determined that chrysin decreased cell proliferation by inducing apoptotic cell death in the PC-3 human prostate cancer cell line (Samarghandian et al., 2011). In another study, it was found that chrysin inhibited cell proliferation by inducing apoptosis in malignant glioma U87-MG and U-251 cells in a dose-dependent manner (Parajuli et al., 2019).

Against the triggered ROS production, the expression level of Nrf2, an important transcription factor to initiate antioxidant mechanisms, is increased. After Nrf2 activation, it separates from the Nrf2-Keap1complex and translocates to the nucleus. Activation of the Nrf2/ARE signaling pathway increases the expression of antioxidant enzymes (SOD, CAT, GSH, and GST), thus preventing cellular damage caused by oxidative stress (Zhang et al., 2021). Oxidative stress results from the disruption of the redox balance between ROS production and antioxidant mechanisms that remove ROS. Thus, the amount of ROS in the cell increases cumulatively (Lin & Beal, 2006). Increased ROS in the cell causes neuronal damage by inducing apoptosis (Moldogazieva et al., 2018). Previous studies have shown that chrysin exerts a neuroprotective effect against neuronal damage induced by different agents by decreasing the level of ROS in the cell and increasing the level of antioxidant enzymes (El-sisi et al., 2017; Belli et al., 2019; Khezri et al., 2020). It has been determined that chrysin can affect oxidative stress by increasing the intracellular expression of antioxidant enzymes such as SOD, CAT, and GPx (Vedagiri & Thangarajan, 2016). In addition, it has been demonstrated that chrysin has neuroprotective effects through different pathways such as antioxidant, anti-inflammatory and antiapoptotic mechanisms (Mishra et al., 2021).

In this study, SOD and CAT activity in control and treatment groups were evaluated at the protein level by ELISA assay. According to the results obtained, it was determined that CAT activity decreased significantly in the group treated with bupivacaine alone (4.81 mU/mL) compared to the control group (8.74 mU/mL), and increased in the group treated with bupivacaine in the presence of chrysin (8.04 mU/mL) compared to the group treated with bupivacaine alone (4.81 mU/mL) (Figure 4B). Similar to the results of CAT activity, it was identified that SOD activity decreased significantly in the group treated with bupivacaine alone (66.57%) compared to the control group (103.92%), and increased in the group treated with bupivacaine in the presence of chrysin (80.88%) compared to the group treated with bupivacaine alone (66.57 %) (Figure 4A).

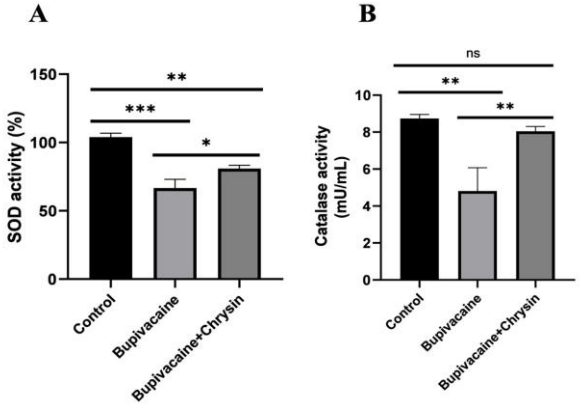

Figure 4. Antioxidative effects of chrysin against bupivacaine-induced oxidative stress in SH-SY5Y cells. The activity of SOD and CAT were measured using ELISA (Colorimetric based)

assay. Each group was subjected to least three independent experiments. Bupivacaine (500 µM) treatment significantly decreased the levels of SOD (A) and CAT (B) activity as compared to control cells, while chrysin (10 µM) pretreatment significantly increased the levels of SOD (A) and CAT (B) as compared to bupivacaine alone treated cells (*P<0.05, **P<0.01, and ***P<0.001).

According to the RT-qPCR results obtained, it was determined that CASP8 and CASP9 gene expression associated with apoptosis increased 8.3 and 6.24 times in the group treated with only bupivacaine, respectively, while the expression of SOD2, SOD3 and CAT associated with oxidative stress decreased 1.79, 4.7, 2.95 times, respectively. It was determined that SOD2, SOD3 and CAT gene expression in the group treated with bupivacaine in the presence of chrysin increased by 6.81, 3.67 and 4.34 times, respectively, compared to the group treated with bupivacaine alone, while the expression of CASP8 and CASP9 genes decreased by 12.23 and 9.14 times, respectively (Figure 5).

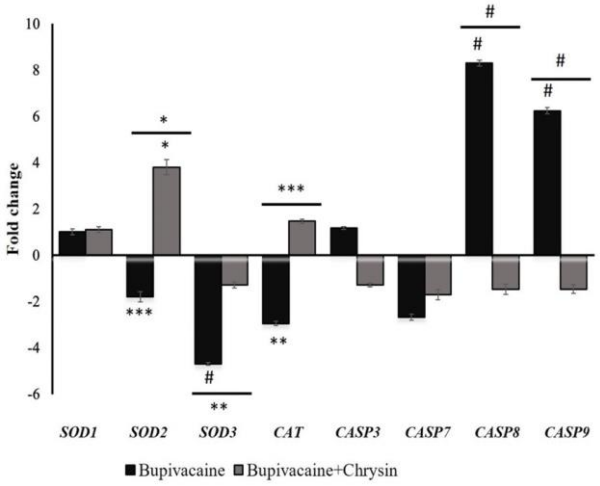

Figure 5. Neuroprotective effect of chrysin against bupivacaine-induced SH-SY5Y neuronal cell death on level of genes expression associated with apoptosis and antioxidant enzymes (*P<0.05, **P<0.01, ***P<0.001 and #P<0.0001). Each group was subjected to least three independent experiments.

Conclusions

Our results revealed that the pretreatment of SH-SY5Y cells with chrysin exerted a protective effect on bupivacaine-induced neurotoxicity. In this study, it was determined that chrysin suppressed oxidative stress by inducing antioxidant enzyme expressions and could resist neuronal cell death mediated by the bupivacaine-induced apoptosis pathway. Therefore, it shows that the mechanism underlying the neuroprotective effects of chrysin is due to the inhibition of oxidative stress and apoptosis pathway. Our findings from this study suggest that chrysin may be a potential neuroprotective agent candidate against bupivacane-induced neurotoxicity if it can be performed in neuron cells in vivo.

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