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Pyrogallol Induces Selective Cytotoxicity in SH-SY5Y and Cortical Neuron Cells

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Abstract

Objective: We aimed to evaluate whether pyrogallol (PG) has selective cytotoxic effects on neuroblastoma (SH-SY5Y) cells and cortical neuronal cells. **Materials and Methods:** SH-SY5Y cells and cortical neuronal cells were treated with PG at different concentrations for 24 h. Cytotoxicity was assessed by MTT test. Catalase (CAT), glutathione reductase (GR) activities and glutathione (GSH) level were also quantified. **Results:** Treatment of neuroblastoma cells with PG (20–200 μM) significantly decreased cell viability. It had no effect on cell viability of cortical neuronal cells at 20-80 μM. However, it decreased the viability in cortical neuronal cells at 200 μM. CAT, GR, and GSH levels were notably reduced in neuroblastoma cells treated with 200 μM of PG; but, only GSH was reduced in cortical neuronal cells at this concentration. **Conclusion:** PG had a specific cytotoxic effect in neuroblastoma cells but generally spared neuronal cells. PG decreased the antioxidant enzyme activities in neuroblastoma cells suggesting that it kills cancer cells by causing increased oxidative stress. However, having a cytotoxic effect in lower concentrations of PG without decreasing antioxidant enzyme activities suggests that PG may have alternative mechanisms for cytotoxicity in lower concentrations.

Keywords: Polyphenolic compound, pyrogallol, SH-SY5Y, cortical neuron, antioxidant enzymes

Introduction

Neuroblastoma is a malignant tumor that is commonly seen in early childhood and originates from the sympathetic nervous system (Bhoopathi, Mannangatti, Emdad, Das, & Fisher, 2021). It is a dense tumour located in the body except cranium, which is common in pediatric patients under 5 years of age with a bad prognosis (Brodeur, 2018; Qureshi et al., 2018). Neuroblastoma is a disease with very heterogeneous features, from spontaneous regression to recalcitrant tumor development (Brodeur, 2018). Although various treatment modalities, including surgery, radiotherapy, and chemotherapy, have been used; it was realized that they are generally ineffective due to the lack of selectivity, non-specific toxicities, and decreased post-recurrence survival (Zafar et al., 2021). For this reason, it is necessary to explore the effect of novel candidate drugs against neuroblastoma.

Cancer cells are hallmarked by producing excessive free radicals compared with healthy cells (Moloney & Cotter, 2018).

Moderate free radical formation is involved in physiological events such as cellular proliferation and differentiation (Bardaweel et al., 2018). However, elevated rates of free radicals production are considered toxic for cancer cells and this could potentiate the impact of agents, which elicit anti-tumourigenic (Hayes, Dinkova-Kostova, & Tew, 2020). Therefore, manipulation of cellular free radical levels could be a treatment approach to get selective toxicity against cancer cells (Schumacker, 2006).

Many plant-derived compounds have been used in cancer chemoprevention. Recently, many studies have focused on exploring natural compounds with potential anticancer activity (Atanasov, Zotchev, Dirsch, & Supuran, 2021). Natural polyphenolic compounds (NPCs) are found in abundant concentrations in fruits, vegetables, and plant-based beverages (Shahidi & Ambigaipalan, 2015). As anticancer agents, NPCs can block certain stages of carcinogenic processes, prevent cell growth, and/or induce apoptosis in tumour cell (Briguglio et al., 2020). Although anticancer effects of NPCs have been associated with several mechanisms; the most pivotal one is increased production of free radicals (Sun et al., 2019).

Pyrogallol (1,2,3-trihydroxybenzene; PG), a NPC, is known as a superoxide anion (O^{-}_{2}) generator and widely used in evaluating the role of oxidative stress in biological systems(Koo, Lee, Chung, Ko, & Kim, 2004; Yang et al., 2009). PG generates free radicals owing to its auto oxidation property, which lead to the formation of hydrogen peroxide (Siegel & Siegel, 1958). It was demonstrated that PG could stimulate O^{-}_{2} intervened death of various cancer cells; such as lung cancer, human glioma, and hepatocellular carcinoma cells (Ahn et al., 2019; Yang et al., 2009).

It was demonstrated that PG could stimulate O^{-}_{2} intervened death of various cancer cells; such as lung cancer, human glioma, and hepatocellular carcinoma cells. In this context, the precise role of PG in neuroblastoma and cortical neurons and whether it has a selective cytotoxicity in cancer cells are still obscure. Hence, the present research

was planned to evaluate the possible cytotoxicity and the underlying mechanism of action of PG on human neuroblastoma cells.

Materials and methods

Cell culture and drug application

Human neuroblastoma (SH-SY5Y) cell line was purchased from ATCC (Wesel, Germany). Cells were cultured at 37 °C under 5% CO_2 atmosphere in DMEM media containing 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 µg/mL streptomycin. Frozen rat cortical neuronal cells were received from the Pharmacology and Toxicology Department of Veterinary Medicine, Faculty of Ataturk University (Erzurum, Türkiye). Neuronal cells were resuspended in neurobasal medium containing B27, 10% FBS and 0.1% antibiotic (penicillin–streptomycin-amphotericin B). Incubation conditions were 37°C in 5% CO_2 humidified air. Human neuroblastoma cells and neuron cultures were exposed to different concentrations (20, 40, 80 and 200 µM) of PG (Sigma, Taufkirchen, Germany) dissolved in medium up-to 24 h. All experiments were conducted with permission from the Ataturk University Ethics Committee of Experimental Animals (Document number E-42190979-000-E.2200123448).

Determination of cytotoxicity

Cytotoxicity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. Neuronal and neuroblastoma cells were seeded in a flat-bottomed 96-well plate at a density of 2×10^5 and 5×10^3 cells/cm^2 respectively and then applied with PG as at the concentrations mentioned for 24h. Cells treated with MTT reagent were incubated in a humidified chamber for 4 hours. Then the medium was taken out, and the 100 µl DMSO was added to dissolve crystals. Absorbance (A) at 570 nm was assigned using Multiskan™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA). The cell viability (%) was calculated by the formula below;(Ferah Okkay et al., 2021)

Viability (%) = A of experiment well/A of control well × 100

Determination of enzyme activities

In this part, cells were added into cell culture petri dish and incubated for 24h. Then, the cells were applied with different PG concentration and incubated nightlong. Following the incubation period, cells bathed with PBS were applied with 400 mL of 1× lysis buffer that contains 1 mM phenylmethanesulfonyl fluoride (PMSF) and kept on ice for 5 min. After that, cells were thrown out, sonicated shortly, and centrifuged at 14,000 g for 10 min in a cold microfuge. Cell supernatants obtained were kept at -80 C to determine enzyme activities later. CAT activity was measured by following the decomposition rate of the substrate H_2O_2 at 240 nm. CAT enzyme activity was given as µmol of H_2O_2 conversion per minute (Habig, Pabst, & Jakoby, 1974). GR activity was measured by determining NADPH oxidation at 340 nm spectrophotometrically. The activity of the enzyme was assigned by the time-dependent

change in NADPH after the supplement of the sample. One unit of GR activity was stated as µmol of NADPH consumed per minute (Wheeler, Salzman, Elsayed, Omaye, & Korte Jr, 1990). GSH activity was measured the rate of of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation at 340 nm (Wendel, 1981). Quantitative protein determination was performed according to the Bradford method. (Bradford, 1976) Bovine serum albumin (BSA) was used as the standard.

Statistical analysis

The data are given as the mean ± SEM. Statistical differences were evaluated using t-test, one-way ANOVA, and Tukey's HSD using the SPSS 22.0 software. P less than 0.05 was accepted significant.

Results

Viability test results

The survival rates of neuronal cells and SH-SY5Y cells after exposure to 24-h PG were calculated using MTT assay test. The cell number decreased during 24 h incubation and was negatively affected by PG treatment dose-dependently in SH-SY5Y cells. Compared with control cells, treatment with 20, 40, 80, and 200 µM/mL PG for 24 h induced a significant ($P < 0.01$) decrease in cell viability by 51.9%, 48.8%, 47.5%, and 39.2%, respectively (Figure 1).

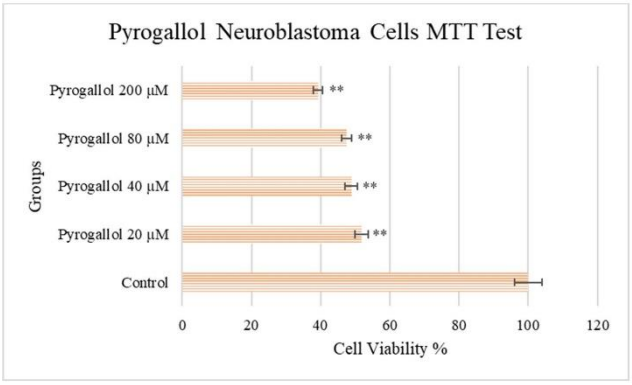

Figure 1. The effect of Pyrogallol on neuroblastoma cell viability after 24 h incubation. (Results are stated as mean ± SEM, *vs. control (untreated) cells, $P < 0.05$; **vs. control cells, $P < 0.001$.

As shown in Figure 2, treatment with 200 µM PG significantly decreased the cellular viability of neuronal cells compared with the control cells. Treatment with 200 µM PG noticeably ($P<0.05$) reduced the cell viability by 84.3%, compared to control. It has to be noted that the decreases in cell viability at concentrationsof 20, 40, and 80 µM PG were not statistically meaningful in comparison to control cells ($P > 0.05$).

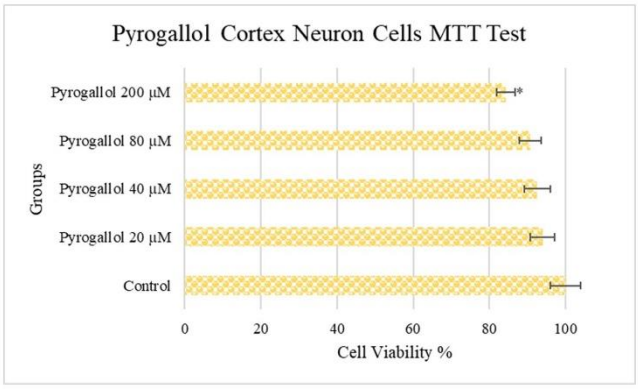

Figure 2. Effect of Pyrogallol on cortical neuronal cell viability after 24 h incubation. (Results are stated as mean ± SEM, *vs. Control cells, $P < 0.05$, **vs. Control cells, $P < 0.001$.

Biochemical measurements

Effects of PG on CAT, GR, and GSH activities in SH-SY5Y cells

In SH-SY5Y cell line, PG (200 μM) decreased CAT, GR, and GSH enzyme activities compared to controls, 20, 40, and 80 μM concentration of PG GR activity was decreased in 80 μM PG group compared to control and group treated with 40 μM PG. However, group treated with 80 μM PG had a significant decrease in GR activity compared to group treated with 200 μM PG (Table 1).

Table 1. Effects of PG treatment on CAT, GR, and GSH enzyme levels in neuroblastoma cells.

Pyrogallol (μM)	CAT (μmol/min/ mg protein)	GR (nmol/min /mg protein)	GSH (nmol/min /mg protein)
0	6.4 ± 0.6	21.4 ± 0.8	17.8 ± 0.9
20	6.1 ± 0.5	21.1 ± 0.7	18.1 ± 1.1
40	5.7 ± 0.4	20.7 ± 0.7	16.7 ± 0.9
80	5.3 ± 0.4	19.6 ± 0.6$^{\sharp}$	16.3 ± 0.9
200	3.8 ± 0.2*	15.1 ± 0.5*	12.8 ± 0.6*

*: 200 μM *vs*. 20, 40, and 80 μM; $^{\sharp}$: 80 μM *vs*. control, 40 and 200 μM (The data are given as the mean ± SEM. Data were analyzed using t-test, one-way ANOVA, and Tukey's HSD. $P<0.05$ was accepted as the level of statistical significance. CAT:catalase; GR:glutathione reductase; GSH:glutathione.

Effects of PG on CAT, GR, and GSH activities in cortical neurons

PG had no effect on CAT and GR activities, however, it significantly reduced GSH activity at a concentration of 200 μM PG compared to 20, 40 and 80 μM in cortical neurons (Table 2).

Table 2. Effects of PG treatment on the levels of CAT, GR, and GSH expressed in cortical neuronal cells.

Pyrogallol (μM)	CAT (μmol/min/ mg protein)	GR (nmol/min/mg protein)	GSH (nmol/min/mg protein)
0	7.3 ± 0.8	23.8 ± 1.2	19.2 ± 1.1
20	7.1 ± 0.6	23.4 ± 0.9	19.1 ± 0.8
40	7.2 ± 0.6	22.9 ± 0.9	19.2 ± 0.7
80	7.0 ± 0.5	22.5 ± 0.8	18.7 ± 0.9
200	6.4 ± 0.4	20.1 ± 0.4	17.8 ± 0.7*

*: 200 μM vs. 20, 40 and 80 μM (The data are given as the mean ± SEM. Data were analyzed using t-test, one-way ANOVA, and Tukey's HSD. $P<0.05$ was accepted as the level of statistical significance. CAT:catalase; GR:glutathione reductase; GSH:glutathione.

Discussion

Numerous anticancer drugs fail to selectively kill cancer cells; they damage normal cells as well cause various side effects (Blagosklonny, 2005). Limited amount of free radicals has some functions in normal cells, i.e. moderate levels of free radicals take part in the activation of transcription factor, gene expression, differentiation and proliferation in cells (Schieber & Chandel, 2014). On the other hand, cancer cells are more susceptible to free radical-modulating drugs that enhancement of free radical generation above the threshold level of redox homeostasis in cancer cells leads to cell death (Briguglio et al., 2020). Throughout the literature, a variety of

compounds were found to be selective against cancer cells without killing normal cells (Briguglio et al., 2020; Tang et al., 2018).

The effects of PG on neuroblastoma and rat cortical neuronal cells were evaluated in this study. The data indicated that application of SH-SY5Y cells with PG resulted in a considerable decrease in cell viability, whereas treatment of cortical neuronal cells with PG did not affect the cell viability significantly at 20, 40, and 80 μM. Treatment with PG (20-200 μM) significantly reduced cell viability in neuroblastoma cells (Table 1). While lower doses of PG (20, 40, and 80 μM) slightly decreased the cell viability, high dose (200 μM) significantly reduced cell viability in cortical neurons. It has been reported that PG decreased cell viability in several types of cells, such as human lymphoma cells (Saeki, Hayakawa, Isemura, & Miyase, 2000), human glioma cells (Sawada et al., 2001), and Calu-6 lung cancer cells (Han, Kim, Kim, & Park, 2008). In the meantime cytotoxic effects of PG on cancer cell line while not affecting normal cells, is in line with the findings of Yang et al (Yang et al., 2009), who showed that PG had cytotoxic effects on human lung cancer cell lines and a little impact on normal human bronchial epithelium cell line (Yang et al., 2009). These results demonstrate that the specificity of cytotoxicity not only associated with different cell types but also PG dose.

Free radicals and oxidative stress have long been related to cancer development. Increasing free radical generation is a common mechanism of most anticancer treatment strategies, such as chemotherapy and radiotherapy (Moloney & Cotter, 2018; Schumacker, 2006). Cancer cells are probably more susceptible to damage by free radical-modulating drugs that augment free radical levels over the threshold of redox homeostasis (Briguglio et al., 2020). Additionally, boosting free radical production and inhibiting cellular antioxidant enzymes would further make the cells more susceptible to cell death in tumors. GSH dependent antioxidant defense system and superoxide dismutase are the important targets of free radicals elevating anticancer agents (Schumacker, 2006; Trachootham et al., 2006). Findings from different studies have demonstrated that inhibition of cellular antioxidant defense system stimulate free radical -mediated cytotoxicity in various types of tumors (Glasauer & Chandel, 2014; Sun et al., 2019). Several chemotherapeutic agents including taxanes and alkaloids interrupt the electron transport chain in mitochondria leading to elevated O_2^-(Glasauer & Chandel, 2014). It has been known that PG produces O_2^- and stimulates death in various cancer cells (Kim et al., 2008; W. H. Park, Y. H. Han, S. H. Kim, & S. Z. Kim, 2007a; W. H. Park, Y. W. Han, S. H. Kim, & S. Z. Kim, 2007b). In this study, 200 μM PG significantly decreased the CAT, GR, and GSH enzyme activities in neuroblastoma cells (Table 1). However, PG in all concentrations did not alter the antioxidant enzyme activitiesin neuronal cells. These data suggest that the effects of PG on the activities of antioxidant enzymes can change according to the

cell types and concentrations. In line with these results, Park et al.(Park et al., 2007a) have shown that PG significantly increased O_2^- level and decreased the intracellular GSH level in As4.1 cells dose-dependently; the apoptotic effects of PG correspond well to intracellular O_2^- level. Additionally, induction of apoptosis and lowering the level of glutathione by an anticancer agent can lead to death of cancer cells (Park et al., 2007a). The study of Han et al.(Han et al., 2008) reported that exposure of Calu-6 cells to PG results in increasing O_2^- level and induces apoptosis. Adding catalase recovered Calu-6 cells from apoptosis due to PG exposure and also avoid the growth arrest by PG that was associated with downregulation of O_2- level (Han et al., 2008).

The findings of this study have some limitations. Our perception is based on the premise that PG can exert a cytotoxic effect on cancer cells while protecting normal neuronal cells. Decrease in the antioxidant enzyme activities in neuroblastoma cells with high concentration of PG suggests that it kills cancer cells by causing increased oxidative stress. However, having cytotoxic effect in lower concentrations without decreasing antioxidant enzyme activities implies that PG might have alternative mechanisms for cytotoxicity. Further studies are needed to clarify other mechanisms or signaling pathways that are activated by PG in neuroblastoma cells.

In conclusion, we have demonstrated that PG has selective cytotoxicity on neuroblastoma cells without affecting cortical neuronal cells much. As a result, PG has a specific cytotoxic effect in tumour cells but spared cortical neurons. The activities of CAT, GR, and GSH enzymes did not change after PG treatment in cortical neuronal cells, however, high concentration of PG (200 μM) significantly decreased the antioxidant enzyme levels in neuroblastoma cells. Because no information was available about the effects of PG on neuroblastoma cancer type, we anticipated that the findings of this study may offer new, potentially useful information for other studies.

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The Antimicrobial Activity of *Hippophae rhamnoides*

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Abstract

Hippophae rhamnoides is a spiny decidous shrub. The plant has sour sweet little fruits ranging from yellow to red. For this purpose we started to investigate whether the plants used in traditional medicine in Erzurum and its vicinity have antibiotic effects. Among them we investigated the fruits of Hippophae Rhamnoides, which is called as ‘’yalancı iğde’’ locally and grown in abundance in the Oltu-Tortum districts of Erzurum, Turkey. In the study, it was aimed to determine the antimicrobial activities of aqueous extraction of fruits. For the purpose, *Escherchia coli, Pseudomonas aureginosa, Staphylococcus aureus* and *Candida albicans* microorganisms were used as reference and the antimicrobial activities of fruit compared with gentamiycin (10ug) using disc diffusion method, As a negative control, DMSO used. According in the findings gentamiycin diameter of 20 mm was found an all samples,while *Escherchia coli* 11 mm, *Pseudomonas aureginosa* 10 mm, *Staphylococcus aureus* 15 mm zone diameters were observed in the aqueous extraction of fruit and there was no effect for *Candida albicans.*

As a result, it is thought that it may be an alternative to skin infections because it has a higher antimicrobial effect on *Staphylococcus aureus* which fraquently causes skin infectious.

Keywords: *H. rhamnoides,* Antimicrobial Activity, Disc Diffusion Method,

Introduction

Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus among the nosocomial infections, are important clinical pathogens. Multi-antibiotic resistance, which is responsible for multi-antibiotic-resistant infections, and it is important with its high mortality rate in infections (Arısoy, 2011). While the rate of penicillin resistance was only 1% in *Staphylococcus aureus* strains, this rate increased after 5 years. It has reached 38%, decreased sensitivity to vancomycin has begun to be mentioned (Bastürk, 2005). *Pseudomonas aeruginosa* is the most common nosocomial infection among Gram-negative bacilli is bacteria. The importance of *Pseudomonas aeruginosa* infections is itself a risk associated with death is the factor. *Escherichia coli* is mostly responsible for community-acquired urinary tract infections. However, can also be the causative agent of nosocomial infection in hospitalized patients with severe underlying disease (Bexfield et al., 2014) Chawla 2007; Kaushal,2011; Conner,1993). The pathogenicity and high invasiveness of *C. albicans*, which is one of the most well-known species, arise from a wide range of virulence factors, such as yeast-to-hyphal transformation, strong adherence to cell/tissues and easy survival in varied anatomical sites. These together allow Candida spp. to efficient evasion of host immune mechanisms (Sadowska et al.,2017).

The resistance of bacteria with multi-antibiotic resistance is clinical is increasing rapidly in the environment and society. A large number of newly developed antibiotics. Most of them are modifications of existing synthetic drugs and bacteria are rapidly becoming resistant necessitates the search for new solutions. For this purpose, superbacteria showed various herbal and animal extracts that may be effective against resistance are being investigated of these some of them are quite promising (Salem et al.,2010).

All parts of Hippophae rhamnoides, carotenoids, tocopherols, sterols, flavonoids, lipids, vitamins, tannins, minerals, etc. of a large number of bioactive compounds, including recognized as a source. Contributes to its extensive use as a natural antioxidant are available. Phenol and flavonoid content, in fruits and shells of *Hippophae rhamnoides* is rich. Phenolic compounds such as flavonoids, phenolic acid and tannin found in hippophae leaves, It is an important phytochemical group with strong antioxidant and antibacterial activity. Due to the versatile composition and low toxicity of plant essential oils and extracts, Their broad antimicrobial spectrum makes them useful in food preservation made them potential natural agents. *Hippophae rhamnoides* seed extract. *Bacillus cereus, Bacillus coagulans, Bacillus subtilis, Listeria monocytogenes* and *Yersinia enterocolitica* also has antimicrobial activity against *Hippophae rhamnoides* fruit and antibacterial activity of leaves against methicillin-resistant *Staphylococcus aureus* it has been found that it shows.

Materials and Methods

Hippophae rhamnoides Extraction

Pure extraction

Firstly, the processed and untreated leaves are one by one with a special grinding machine powdered. Then, the weight of the sample is weighed on a precision scale by taking the tare determined. Ground leaf powders of which weight is determined are placed in the vessel unit of the device given to the device.

Disc Diffusion Method

Hippophae rhamnoides leaf extracts were performed with the standard strains of "Microbiologics (France)" *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 9027, *E. coli* ATCC 25922 and Candida albicans 10231. In antimicrobial susceptibility tests, Thermo Scientific™ Oxoid™ (USA) brand blank discs and Thermo Scientific™ Oxoid™ brand (USA) gentamicin disc were used as positive and negative controls. *Hippophae rhamnoides* leaf samples were obtained from Oltu-Tortum-Erzurum region, which is among the regions where this plant is concentrated in our country.

For disc diffusion, single dropped bacteria on 18-24 hour blood agar medium. The colonies were suspended in sterile saline. Turbidity of suspension it set to 0.5 McFarland. Three loops were taken from the bacterial suspension with a sterile standard loop. Mueller Hinton agar was rubbed onto the surface of the medium. This process is done separately for each bacterium carried out. Before applying the discs, the media should be rinsed for 5 minutes to remove excess moisture kept at room temperature. Discs impregnated with plant extracts, in full contact with the agar surface with a sterile forceps, with a gap of at least 24 mm between them placed and incubated at 37°C 24 hours.

Results

According in the findings gentamiycin diameter of 20 mm was found an all samples, while *Escherchia coli* 11 mm, *Pseudomonas aureginosa* 10 mm, *Staphylococcus aureus* 15 mm zone diameters were observed in the aqueous extraction of fruit and there was no effect for *Candida albicans* (Table 1).

Table 1. Zone Diameter

Standard Bacterial Strain/Antimicrobial Agents	Zone Diameter(mm)
S. aureus ATCC 25923	15
P. aeruginosa ATCC 9027	10
E. coli ATCC 25922	11
Candida albicans 10231	None
Gentamycin	20

Discussion

Phenolic compounds such as flavonoids, phenolic acid and tannin found in hippophae leaves, It is an important phytochemical group with strong antioxidant and antibacterial activity. When examined, the leaves of Sea buckthorn dried in the shade or in the sun are mostly juicy, It is seen that ethanolic and methanolic extracts are used.

Antioxidant capacity in aqueous and methanolic extracts of processed sea buckthorn leaves and phenolic profile found in processed leaves in their study that the amount of substance is higher and the antioxidant capacity is higher than the untreated leaves shows that it is higher than.

S. aureus ATCC 25923, *E. coli* ATCC, *P. aeruginosa* ATCC 9027 and *Candida albicans* 10231 which we included in our study. The susceptibility of bacteria to H. rhamnoides was determined by disk diffusion method. It method Gupta et al. (2011) H. salicifolia D. Frost leaves of *Bacillus subtilis, Bacillus thuringirnsis, Pseudomonas fluorescens, Escherichia coli, Agrobacterium tumefaciens* and *Acinetobactor junii* were used to determine the effect.

Similarly Qadir et al. (2016), H. rhamnoides L. effectiveness of disk diffusion on MRSA strains detected by the method. Similarly, we used the disk diffusion method in our research. We used determine whether clinically pathogenic bacteria are susceptible to H. rhamnoides. It is not possible to determine precisely because there are no standards disc diffusion. The diameters of the growth zones obtained as a result of the method were used for susceptibility and resistance. Since the standard zone diameters are not known, the data obtained can only be used for further studies. It will give you a preliminary idea of where to go.

As a result of our research, our bacteria After measuring the zone diameters we see around it, Gupta et al. (2011) In the study, the specified zone diameters were taken as criteria. Although these zone diameters are standard. Although not mentioned in the guides, our results are included in a published article. It helped us think about it. These researchers planted bacteria determined the effect of H. salicifolia in the petri dishes with the zone diameters formed less than 7 mm In the inhibition zone, the bacteria are resistant, in the inhibition zone between 7 and 9 mm. Moderately susceptible to bacteria and susceptible to bacteria in an inhibition zone greater than 10 mm have stated that.

According in the findings gentamiycin diameter of 20 mm was found an all samples,while *Escherchia coli* 11 mm, *Pseudomonas aureginosa* 10 mm, *Staphylococcus aureus* 15 mm zone diameters were observed in the aqueous extraction of fruit and there was no effect for *Candida albicans*

Antibiotics today, against resistant bacteria, natural resources such as plant extracts are a source of hope is seen. Experimental animals based on the data obtained as a result of the study. Further in vivo studies are needed

Conflict of Interest: The author(s) have declared no conflict of interest.

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Carvacrol Protect Hippocampal Neurons Against Hydroxychloroquine-Induced Damage: In Vitro Study

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Abstract

The use of hydroxychloroquine, an antimalarial drug, in the treatment of Covid-19 disease, which has turned into a worldwide epidemic, was initially viewed positively. However, the lack of evidence for its use in treatment and even neuronal side effects caused hydroxychloroquine to be approached with suspicion. Carvacrol, on the other hand, is a very interesting ingredient with its anti-oxidant, anti-inflammatory, and anti-cancer properties. Primary neuron culture was prepared for our study. Carvacrol (10, 25, 50, and 100 mg/L), hydroxychloroquine (10,20,40, and 80µM), hydroxychloroquine+carvacrol groups (10µM+10mg/L, 20µM+25mg/L, 40µM+50mg/L, 80µM+100mg/L) were applied to neuron culture for 24 and 48 hours. After the application, results were obtained with MTT, TAS, TOS, Thiol analyses, and acetylcholinesterase (AChE), butyrylcholinesterase (BChE) activities. According to our MTT results, carvacrol (100mg/mL) increased neuronal viability by ~10% in the combined group compared to pure hydroxychloroquine (80µM). The same dose of carvacrol reduced the antioxidant level 1.3 times. Doses of carvacrol alone did not affect thiol levels but increased in combination with hydroxychloroquine(431µmol/L). It is now known that Covid-19 is associated with neurodegenerative diseases. Recent studies have shown that hydroxychloroquine, which is seen as a hope for the global epidemic, causes oxidative stress on neurons. In our study, we designed to both provide protection and prevent the occurrence of side effects by using carvacrol against the neurodegenerative effects of hydroxychloroquine.

Keywords: Carvacrol, Covid-19, Hydroxychloroquine, Neuron, Neuroprotective

Introduction

SARS-CoV-2, which emerged at the end of 2019, surpassed many diseases in the past in terms of both the number of infected people and the rate of spread of the epidemic (Hu et al., 2021). Complications caused by COVID-19 mainly target an immune-inflammatory pathway. Although there is still no proven cure, in vitro studies have shown that hydroxychloroquine, used to treat malaria, is effective in COVID-19 (Cortegiani et al., 2020). Effect of hydroxychloroquine on COVID-19; It manifests by interfering with the endocytic pathway, blocking sialic acid receptors, restricting pH-mediated spike (S) protein cleavage at the angiotensin converting enzyme 2 (ACE2) binding site, and inhibiting cytokine storm (Satarker et al., 2020). Although it is seen as beneficial for COVID-19, Sieb (Sieb et al., 1996) et al. showed in their studies that hydroxychloroquine negatively affects both the presynaptic and postsynaptic aspects of neuromuscular transmission. Bruinink (Bruinink et al., 1991) et al. also found that hydroxychloroquine negatively affected neuronal viability in the primary neuron cultures they used. Giovanella (Giovanella et al., 2015) et al. reported that hydroxychloroquine caused DNA damage in rat brain. While its effect for COVID-19 is still not fully proven, its effects especially on neuronal damage and oxidative stress limit the use of hydroxychloroquine.

Today, many researches are still carried out to find more effective treatment methods and natural products attract a lot of attention due to their anti-inflammatory, antioxidant and immunomodulatory properties (Gandhi et al., 2020). Carvacrol, one of the essential oil compounds, has received special attention due to its specific binding to nonstructural proteins in the viral genome (Javed et al., 2020). In fact, Abdelli (Abdelli et al., 2021) et al. reported that carvacrol can inhibit the entry of COVID-19 into the host cell by inhibiting ACE2 activity. Kulkarni (Kulkarni et al., 2020) et al found that many monoterpenoid phenols, including carvacrol, can inhibit the binding of virus spike (S) glycoprotein to the host cell. In addition, Guana (Guan et al., 2019) et al. showed that carvacrol provides protection against neuronal damage by reducing ROS production. In another study (Wang et al., 2017), carvacrol was shown to attenuate ethanol-mediated hippocampal neuronal dysfunction with antioxidative and antiapoptotic effectsIt has been reported that it exerts its protective effect by reducing neuronal oxidative stress.

The therapeutic use of essential oils in infectious, acute and chronic diseases has become clear. In our study, we aim to eliminate or improve the neuronal effects of hydroxychloroquine by using carvacrol.

Materials and Methods

This study was conducted at the Medical Experimental Research Center at Ataturk University (Erzurum, Turkiye). The Ethical Committee of Ataturk University approved the study protocol (04-2100138265/31.5.2021).

Chemicals

Hydroxychloroquine, Carvacrol, Dulbecco's Modified Eagles Medium (DMEM), Fetal calf serum (FBS), neurobasal medium (NBM), phosphate buffer solution (PBS), antibiotic antimitotic solution (100 x), L glutamine and trypsin-EDTA obtained from Sigma. (St Louis, MO, ABD).

Cell culture

Primary Neuron Culture

22 Sprague Dawley rats less than 24 hours of birth will be used to obtain cortex neurons in the study. Briefly, after the rats are rapidly decapitated, the removed cortexes will be transferred to 5 mL of Hanks' Balanced Salt solution (HBSS), macro fragmentation with the help of a scalpel, and then micro fragmentation with Trypsin-Ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin-0.02% EDTA). Then the cells will be centrifuged at 1200 rpm for 5 min. Cells settled as pellets cellular medium (88% NBM (Neurobasal medium, Gibco, USA), 10% FBS (Fetal bovine serum, Gibco, USA), 2% B-27 (Supplement, Thermo Fisher, Germany), 0.1% It will be added to the environment containing antibiotics (Penicillin-Streptomycin) and amphotericin B (Thermo Fisher, Germany). Cells will be incubated at 5% CO_2 and 37^{o}C for 10 days by changing the medium every 3 days.

Drug Preparation

Drugs were applied after reaching the desired rate (80%) of cell density in neuron culture plates. For this purpose, the platelets were treated with drugs such as hydroxychloroquine (10,20,40 and 80 µM), carvacrol (10, 25, 50 and 100 mg/L), hydroxychloroquine + carvacrol (10µM + 10 mg/L, 20µM + 25mg/L, 40µM + 50mg/L, 80µM + 100mg/L). After application, it was incubated for 24 and 48 hours (5% CO_2, 95% humidity and 37°C).

MTT Tetrazolium Assay Concept

The MTT assay was performed with a commercially available kit (Sigma Aldrich, USA). Briefly, an MTT reagent (10µL at a concentration of 5 mg/ml) was added to each well in the plated and then incubated for 4 hours (5% CO_2; 37°C) (Ali Taghizadehghalehjoughi et al., 2019). After applications, the medium was removed, and 100 µL of dimethylsulfoxide (DMSO) (Sigma, USA) was added to each well to dissolve formazan crystals. Cell viability (%) was calculated by optical density read at 570nm using the Multiskan ™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA). The control group was accepted as 100, other groups were calculated according to the formula below.

Viability Rate (%) = (O.D of groups/Control O.D.)X 100

Total Antioxidant Capacity (TAC) Assay

The antioxidant capacity was determined by inhibition of the 2-2'-azinobis (3-ethylbenzothiazoline 6-sulfonate = ABTS +) radical cation in the TAC assay (Rel Assay Diagnostics® Company (Gaziantep, Turkiye)). Briefly, to determine the TAC level, the wells are respectively; 30µL sample and 500µL Reagent 1 solution were

added, the initial absorbance was measured at 660nm. Then, 75µL Reagent 2 solution was added to the same wells, and after 10 minutes, the second measurement was made at 660nm. Absorbance values were replaced according to the formula specified in the procedure, and TAC values were calculated as Trolox Equiv/mmol L^{-1} (Aysegul YILMAZ et al., 2021).

A2-A1= ΔAbsorbance (Standard, sample, or H_2O)

$$\text{Result} = \frac{(H_2O\ \Delta Abs - \text{Sample}\ \Delta Abs)}{(H_2O\ \Delta Abs - \text{Standard}\ \Delta Abs)}$$

Total Oxidant Status (TOS) Assay

TOS assay is called the evaluation of color density with spectrophotometric properties depending on the number of oxidants in the sample (from Rel Assay Diagnostics® Company (Gaziantep, Turkiye)). For this purpose, to determine the TOS level, briefly, 500 µL of reagent1 solution was added to the wells containing 75 µL of a sample, and the initial absorbance value was read at 530nm. Then 25 µL of Reagent2 solution was added to the same well. After 10 minutes at room temperature, the second absorbance value was read. Absorbance values were replaced according to the formula specified in the procedure, and TOS values were calculated as H_2O_2 Equiv/mmol L^{-1}.

A2-A1= ΔAbsorbance (Standard or sample)

$$\text{Result} = \frac{\text{Sample}\ \Delta Abs}{\text{Standard}\ \Delta Abs} \times 10$$

The Cholinesterase Activity Assay

Following a 24-h and 48-h treatment with hydroxychloroquine, ginseng, and ginseng + hydroxychloroquine combinations drug, cells were scraped into 0,2 mL of ice-cold homogenization buffer (50 mm Tris–HCl, 1 m NaCl and 50 mm $MgCl_2$, pH 7.4 containing 1%, w/v, Triton X-100). The cells were sonicated on ice for 20 min and then centrifuged at 100.000 g at 4°C and the supernatant fraction collected. Enzymes activity have determined using a colorimetric assay employing acetylthiocholine iodide (ASChI) for AChE and butyrylthiocholine iodide (BTChI) for BChE as substrate (Ellman et al., 1961; Sáez-Valero et al. 1999; Fodero et al 2004). After a 3-min equilibration, the reaction was started with the addition of substrate (ASChI/BTChI). The substrates hydrolysis were determined by monitoring the change in absorbance at 412 nm.

Measurement of Total Thiol Amount

Total thiols were estimated according to the method of Sedlak and Raymond (Reddy et al 2004). Plasma samples (0.1 mL) were mixed with 1.5 mL of 0.2 M Tris buffer (pH 8.2) and 0.1 mL of 0.01 M DTNB. The mixture was made up to 10 mL with methanol and incubated for 30 min subsequently, it was centrifuged 15 min at 3000 rpm and assayed at 412 nm. Standard graphs were used to calculate total thiols.

Statistical Analyses

Statistical comparison between groups was calculated using One-way ANOVA and Tukey HSD

method. All calculations were performed using SPSS 20 software for statistical analysis, and P<0.05 was considered a statistically significant difference in all tests. Results are presented as mean ± standard error.

Results

MTT Tetrazolium Assay Concept

According to our MTT analysis results (figure 1), in which we measured cellular viability at the end of 24 and 48 hours, there was no significant difference even though carvacrol decreased viability with increasing dose and time. In fact, similar to the study results of Wang (Wang et al., 2017) et al., our results showed that carvacrol is protective in neuron cells (~90% protection). Hydroxychloroquine, which negatively affects viability depending on the increasing dose, was especially effective at 20, 40 and 80 µM doses. Hydroxychloroquine 80 µM dose caused a 33% decrease in viability. It was observed that carvacrol was effective even though there was a dose- and time-dependent decrease in viability in the combination groups. While pure hydroxychloroquine (80 µM) causes a 33% decrease in viability, a 24% decrease is observed when co-administered with carvacrol (100 mg/mL). This shows that carvacrol is effective in preserving neuronal vitality.

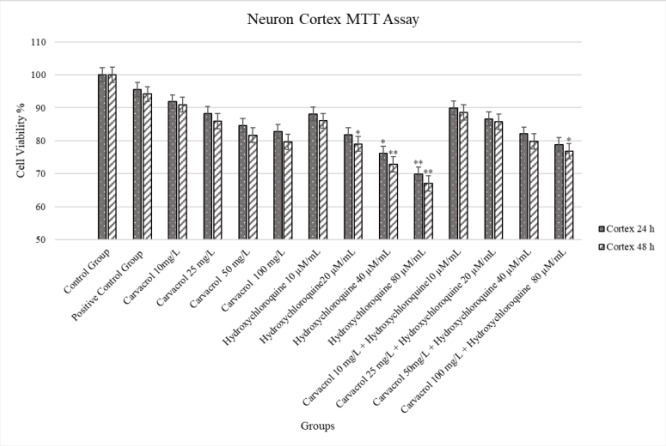
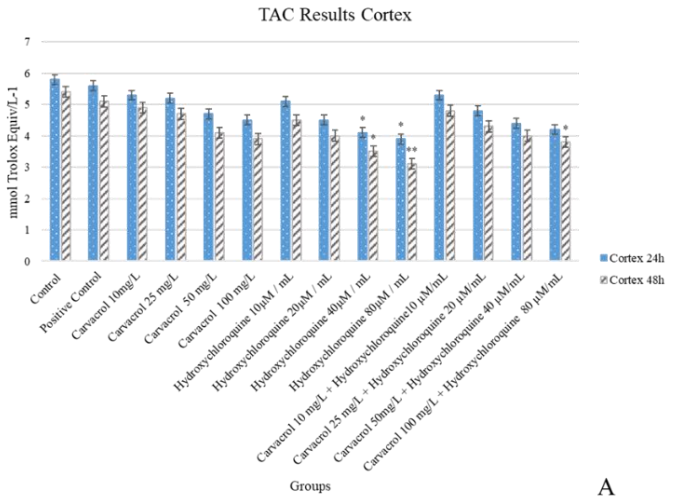


Figure 1. MTT assay results for Cortex cell line after 24 h and 48h Hydroxychloroquine, Carvacrol and combination treatment. (*Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group)

Total Antioxidant and Oxidant Analysis

When we examined the antioxidant effects of the components we used in Figure 2A, it was observed that carvacrol decreased the antioxidant effect depending on the increasing dose and time. This decrease did not make a significant difference except for the dose of carvacrol 100 mg/mL at the 48th hour. Carvacrol 100 mg/mL dose reduced the antioxidant level 1.3 times compared to the control. This decrease may be due to the fact that carvacrol provides more effective protection at low doses, as Llana-Ruiz-Cabello(Llana-Ruiz-Cabello et al., 2015) et al. In hydroxychloroquine, the antioxidant level decreases with increasing dose and time. The most significant difference occurred at the hydroxychloroquine 80 µM dose (1.7fold decrease). The combination of carvacrol 100 mg/mL + hydroxychloroquine 80 µM caused a 1.4-times decrease in antioxidant levels (3.8 Trolox

Equiv/mmol L-1). When we examined the oxidant results in Figure 2B, no significant increase was detected at other doses, except for the carvacrol 100 mg/mL dose (3.9 H_2O_2 Equiv/mmol L-1). Hydroxychloroquine, which causes an increase in the oxidant level depending on the dose and time, on the contrary of the antioxidant level, however, a significant increase was observed only at the dose of 80 µM (1.5-times increase in 24 hours; 1.6-times increase in 48 hours). In the combination groups, the most significant difference was observed at the dose of carvacrol 100 mg/mL + hydroxychloroquine µM (1.5-times increase at 48 hours; 4.1 H_2O_2 Equiv/mmol L^{-1}).



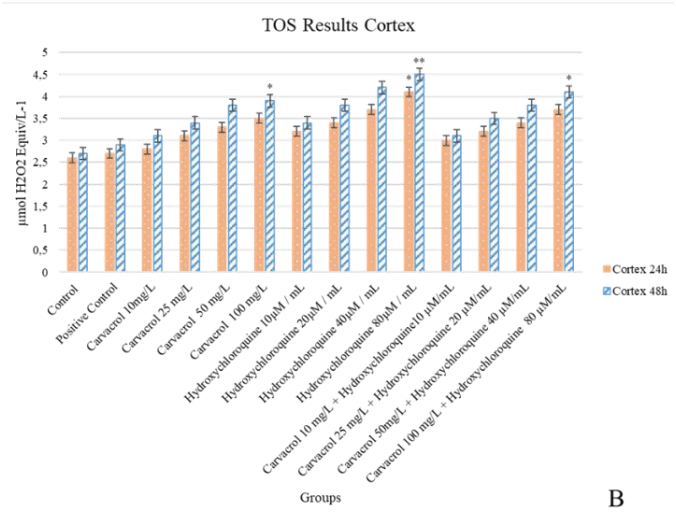


Figure 2. TAC and TOS Levels for Cortex cell line after 24 h and 48h Chloroquine, Carvacrol and combination treatment. A) Cortex cell line TAC Results, B) Cortex cell line TOS Results. (*Significant differences at $P < 0.05$ compared to control group; **Significant differences at $P < 0.001$ compared to control group).

Total Thiol Analysis

Thiol, which plays an important role in preventing the formation of oxidative stress in cells, is very effective in antioxidant defense. Disruption of dynamic thiol/disulfide balance is also an undesirable effect in antioxidant defense (Kundi et al., 2015). When we examined our results in Figure 3, it was seen that carvacrol did not affect the thiol level with increasing dose and time (excluding the dose of carvacrol 100 mg/mL). Hydroxychloroquine, on the other hand, caused an increase in thiol level with increasing dose and time. The most significant increase was seen at 48 hours at hydroxychloroquine 40 and 80 µM doses (respectively; 410 ve 456 µmol/L). In our thiol results, which also showed an increase in the combination groups, we found that the carvacrol 100 mg/mL + hydroxychloroquine 80 µM

group caused the most significant increase at the 48th hour (431 µmol/L).

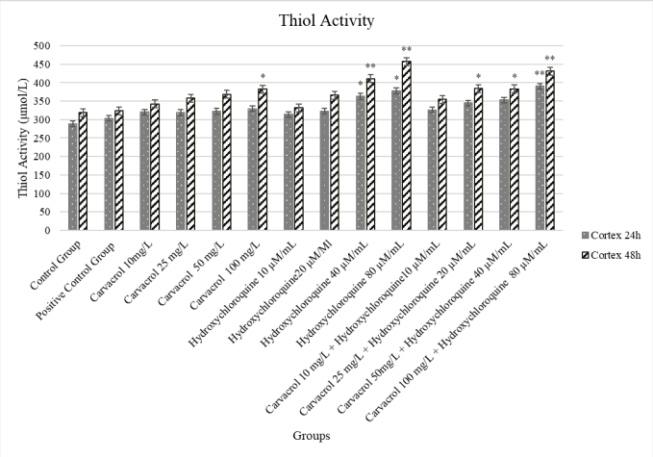


Figure 3. Total Thiol Analysis for Cortex cell line after 24 h and 48h Chloroquine, Carvacrol and combination treatment. (*Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group).

Acetylcholinesterase and Butyrylcholinesterase Activity Assays

As shown in Figure 4A, it was shown that carvacrol did not significantly affect acetylcholinesterase (AChE) activity with increasing dose and time. When we look at the effects of carvacrol on butyrylcholinesterase (BChE), we see similar results (figure 4B). Hydroxychloroquine, on the other hand, increased both AChE and BChE activity in a time- and dose-dependent manner. The most significant increase occurred in hydroxychloroquine 80 µM dose, which provided a 1.3-times increase in both activities (0,46 EU/ml AChE and 81,7 EU/ml BChE).

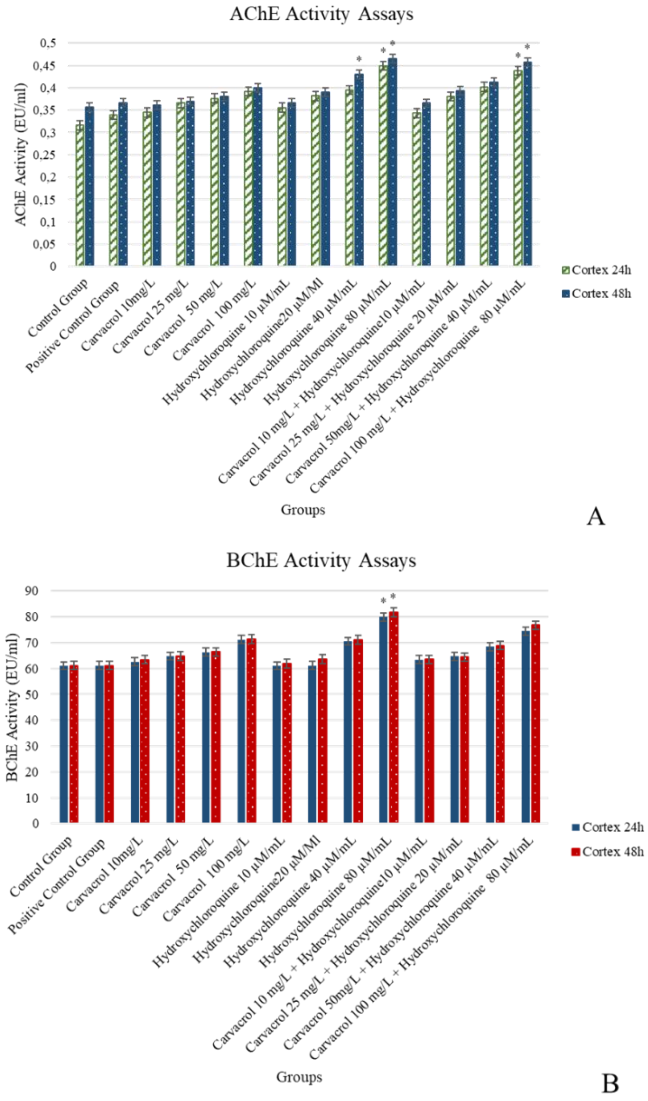


Figure 4. Acetylcholinesterase and Butyrylcholinesterase Activity Assays for Cortex cell line after 24 h and 48h Chloroquine, Carvacrol and combination treatment. A) Acetylcholinesterase activity assays for cortex cell line, B) Butyrylcholinesterase activity assays for cortex cell line (*Significant differences at P < 0.05 compared to control group; **Significant differences at P < 0.001 compared to control group).

Discussion

In general, neurodegenerative diseases are characterized by slowly progressive neuronal loss. Although the etiology of neurodegenerative diseases

has not been fully elucidated yet, oxidative stress is thought to be one of the main sources (Kim et al., 2015). Numerous studies have shown that hydroxychloroquine causes oxidative stress, especially on neurons (Fang et al., 2015; Giovanella et al., 2015; Klouda & Stone, 2020). In our study, we are investigating the effects of carvacrol against neuronal damage caused by hydroxychloroquine used for Covid-19.

Guan (Guan et al., 2019) et al., in their study with carvacrol, showed that carvacrol at 1.2mM and 2.4mM doses protects neuronal vitality, in parallel with ~90% protection in our study. In the same study, it was shown that carvacrol provides protection against neuronal damage and reduces reactive oxygen species (ROS) with an antioxidant mechanism. Guimaraes (Guimaraes et al., 2010) et al. used TBARS (thiobarbituric acid reactive species) and lipoperoxidation (oxidative damage to lipids) methods to measure the antioxidant effects of carvacrol (1µg/ml-1mg/ml). It can be shown to be similar to the result we found that carvacrol 100mg/mL reduced the antioxidant level by 1.4 times in the combined groups. Guimaraes et al. also supported our study findings by finding significant results with increasing dose. Hakimi (Hakimi et al., 2020) et al. showed that treatment with carvacrol was associated with increased thiol, SOD and CAT activity in brain tissue. They support our conclusion that 100 mg/mL dose of carvacrol causes an increase in thiol versus hydroxychloroquine with the combination of lipopolysaccharide (LPS) and carvacrol. Aazza (Aazza et al., 2011) et al. also showed that carvacrol is an important acetylcholinesterase inhibitor in their study and supports the inhibitory effect of carvacrol at low doses in our study. In their in vivo study, Bianchini (Bianchini et al., 2017) et al. observed that carvacrol (50-100mg/L) increased AChE activity in the brain, while Lopez (Lopez et al., 2015) et al. observed that compounds of the same class activated AChE at lower doses and inhibited at higher doses in vitro. According to these results, it can be said that the effects of carvacrol or, in general terms, monoterpenoids on AChE activity depend on concentration and tissue.

Conclusion

Hydroxychloroquine is one of the drugs used to protect against the Covid-19 disease, which has turned into a global epidemic, and it is one of the undesirable effects that it causes neurodegenerative diseases. It is one of the justifications for people to seek alternative ways to avoid being exposed to side effects while fighting the disease. Carvacrol is one of the components that have been used for many years and whose positive effects are known. Anti-oxidant, anti-inflammatory, anti-cancer properties have always made carvacrol interesting. In our study, we aimed to reveal the protective effect of hydroxychloroquine by drawing attention to the antioxidant system on neurons. We think that with the development and advancement of the positive results we have achieved, it can be quite effective in preventive medicine.

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Post-Traumatic Anisocoria Caused by Iris Sphincter Rupture in a Child: An Unusual Case Report

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Abstract

Anisocoria is a rare and unusual post-traumatic condition among eye pathologies. After a traumatic eye injury, the problem can often be masked by complaints of bleeding in the eye and loss of vision. Therefore, anisocoria, which may occur as a result of iris sphincter rupture, should be considered and investigated in the examination, along with other eye examination findings in patients with complaints of traumatic injury to the eye. In this case report, we present a case of a 14-year-old male patient who was investigated for the differential diagnosis of anisocoria in the eye after head trauma and was later diagnosed as iris sphincter rupture.

Keywords: anisocoria, trauma, iris sphincter rupture, family practice, primary care

Introduction

Anisocoria is a condition characterized by unequal pupils with a size difference of 0.4 mm or more (1). It may occur in 20% of the population (1), and may be completely harmless, but its sudden onset can be a sign of a serious medical problem, such as neurological emergencies (2). In this report, a patient who applied with the complaint of post-traumatic anisocoria will be presented.

Case

A 14-year-old male patient with no pre-existing disease had applied to the emergency service with the complaints of redness in the eye and loss of vision due to trauma by slippers on the right frontal region of his friend 20 days ago. In emergency service it was said that there was slight bleeding in the eye, and an eye drop medicine to be used for 3 days was prescribed and he was discharged. The patient, who stated that he could not see clearly 10 days later, applied to the eye outpatient clinic of state hospital. There he was told that he had 90% vision loss in the right eye and he was referred for further differential diagnosis for anisocoria. Due to his head trauma history, the patient was firstly consulted with neurosurgery department, underwent brain computed tomography, which was found to be normal, and then he was referred to pediatric neurology because the current clinic could not be attributed to brain trauma.

The patient was evaluated by us as Family Medicine before the pediatric neurology evaluation. In his physical examination, his general condition was good, he was conscious, oriented, cooperative, and his vitals were stable. Eye movements were normal in all directions, and not painful. However, anisocoria was observed. Direct and indirect light reflexes were found bilaterally positive. There was no ptosis and ophthalmoplegia. Visual acuity in the right eye was 1/10.

During pediatric neurology examination, deep tendon reflexes were found to be normoactive, clomus and Babinski were negative, muscle strength was 5/5 in all extremities, walking and speaking were normal. There was no sensory defect or sphincter defect. Motion induced blindness (MIB) and relative afferent pupillary defect (RAPD) were negative. Cranial nerves were found to be intact and cerebellar tests were normal. Routine laboratory tests revealed unremarkable. The patient underwent with craniocervical and orbital contrast MRI, and MRI angiography. Visually evoked potential (VEP) was reported as normal. In orbital evaluation; bulbus oculi, extraorbital muscles, retrobulbar adipose tissue and optic nerves were evaluated as normal, likewise in the evaluation of brain tissues, posterior fossa formations, bulbus, pons, 4th ventricle were evaluated as normal. No abnormality was observed in the bilateral cerebellopontine corner.

The patient, who was evaluated as normal by neurosurgeon and pediatric neurologist, was further re-evaluated by ophthalmologist, and slit-

lamp examination revealed nasal and temporal iris sphincter rupture. After diagnosing the cause of anisocoria, the patient was transferred and followed up by the ophthalmology department.

Discussion

There are several causes of anisocoria: The most common one was the physiological one. Physiological anisocoria is defined as pupillary disparity, usually 0.4 mm, rarely greater than 0.8 mm, not due to a secondary cause (1). If anisocoria is physiological, the difference in pupil sizes should remain equal in dim and bright light (3).

Another well-known anisocoria reason, Horner's syndrome, occurs as a result of lesions in the brainstem and cervical cord, along the oculo-sympathetic pathway. Clinical features include ipsilateral miosis, ptosis, anhidrosis, enophthalmos, and ciliospinal problems (4).

In the Adie's tonic pupil, caused by damage to the nerve of the iris sphincter muscle, the larger pupil cannot become as small as the other pupil. However, as the eye adapts, the pupil constricts more. This cause of anisocoria can be diagnosed with dilute pilocarpine, which causes significant constriction of the larger pupil. Other causes of a dilated pupil typically do not respond to dilute pilocarpine and help confirm the diagnosis of Adie's tonic pupil (5).

Neurological emergencies, such as stroke, intracranial aneurysm (6), demyelinating diseases, head trauma and brain tumors are the most common causes of oculomotor nerve palsy in adults. In ischemic lesions of the oculomotor nerve, pupillary function is usually preserved, while in compressive lesions the pupil is involved.

Pharmacological Anisocoria

Pharmacological agents with anticholinergic or sympathomimetic properties cause anisocoria, especially if administered to one eye. Some examples of pharmacological agents that may affect pupils include pilocarpine, opioids, scopolamine patches, inhaled ipratropium, nasal vasoconstrictors, and glycopyrrolate antiperspirants. Alkaloids found in plants, such as Jimson weed, Angel's trumpet, and blue nightshade, can also cause anisocoria (6).

An interesting pharmacological anisocoria has been reported after topical treatment of axillary hyperhidrosis with glycopyrronium, an anticholinergic resulting from accidental ocular exposure (7). Another ocular exposure was reported in an asthmatic patient who developed ipratropium bromide-related anisocoria during nebulizer therapy (8).

Traumatic new-onset anisocoria is mostly seen as a result of acute brain injuries (9). Aneurysmal or intracranial hemorrhage and stroke may accompany anisocoria after blunt brain trauma and may even reflect the severity of brain damage (10). However, anisocoria after traumatic eye injury is a rare clinical condition.

In our case, eye bleeding, vision loss and anisocoria were detected in a child secondary to eye trauma. To the best of our knowledge, there was no study in the literature on iris sphincter rupture as the cause of anisocoria as reported in our case. In traumatic iris sphincter rupture, anteroposterior compression of the eyeball leads to equatorial dilation that will actively pull along the corneoscleral junction, resulting in a sphincter tear (11). Although this sphincter tear is unusual, it should be investigated in a patient with anisocoria, especially after brain injury and other neurological causes have been excluded. As occurred in our case, somehow traumatic bleeding in an eye may overlap the anisocoria and may be neglected. Therefore, anisocoria should be kept in mind in the post–traumatic eye injury.

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Review: Glutamate, excitotoxicity and related diseases

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Abstract

Glutamate is an excitatory neurotransmitter that is abundant in many central and peripheral tissues and is essential for cell survival and body homeostasis. Although its existence has been known for many years; research still reveals that excitotoxicity plays a role in the development of new pathological conditions. For this reason, glutamate and glutamate excitotoxicity must be well understood, and glutamate-related circumstances must be clarified.

Keywords: AMPAR, EAAT, Excitotoxicity, Glutamate, Kainate, mGluR, NMDAR

1. What is glutamate?

Glutamate, along with glutamine, is the most common amino acid in the human brain, and glutamate is regarded the primary excitatory neurotransmitter in the central nervous system (CNS) due to its participation in several metabolic pathways.

Glutamate was discovered in large concentrations in the brain in the 1930s; its role as a key excitatory neurotransmitter in the CNS was described in 1984.(Krebs, 1935; Perry et al., 1971). Brain tissue contains approximately 5-15 mmol / kg glutamate, the majority of which is found in neurons. Glutamate content in glutamatergic neuron cytoplasm is about 5−10 mM, several times higher than other amino acids (Ottersen, 1989; Ottersen et al., 1992).

Glutamate has a major role in normal brain functions like learning, memory, cognition. Also, during central nervous system development, it's involved in cell migration, differentiation, formation of synapses and cell death (Danbolt, 2001; Komuro & Rakic, 1993; Mayer & Westbrook, 1987). Furthermore, because GLURs exist in both peripheral neural and non-neural tissues, it plays an important role in maintaining homeostasis in a variety of peripheral organs, including the adrenal medulla, peripheral nerves, bone and bone marrow, gut, hepatocytes, heart, lungs, kidney, spleens, ovaries, vagus, and other cholinergic nerves (Gill & Pulido, 2001; Hinoi et al., 2004).

2. Plasma membrane glutamate transporters (EAATs)

Although glutamate is required for the normal functioning of many systems, excessive glutamate stimulation causes excitotoxicity and neurodegeneration in neurons. Since there is no enzyme that can degrade glutamate, its concentration and effect are largely regulated by the rate of uptake from the

extracellular fluid. It cannot pass the blood-brain barrier and must be withdrawn from the extracellular fluid on a regular basis by glutamate transporters and presynaptic terminals in neighboring glial cells to prevent excessive receptor activation. (Bak et al., 2006; Danbolt, 2001; Vandenberg & Ryan, 2013)

Extracellular glutamate requires cellular uptake, which is mediated by excitatory amino acid transporters (EAATs) found in the plasma membranes of astrocytes and neurons (Danbolt, 2001; Featherstone, 2010; Vandenberg & Ryan, 2013). EAATs are classified into five subtypes, EAAT1–5 (O'Shea, 2002). All the five types are linked to the cotransport of 3 Na^+ and 1 H^+ followed by the counter transport of 1 K^- with one substrate molecule (Danbolt, 2001; Owe et al., 2006; Zerangue & Kavanaugh, 1996).

EAAT1 is a highly expressed and important transporter in the neocortex and cerebellum, particularly in astrocytes, as well as in a variety of other tissues including the retina, inner ear and circumventricular organs. (Berger & Hediger, 2000; Furness & Lehre, 1997; K. P. Lehre & N. C. Danbolt, 1998; Rauen et al., 1996).

All regions outside of the brain, where EAAT1 is more prevalent, contain EAAT2, which serves as the forebrain's main glutamate transporter. Although it is found in presynaptic nerve terminals, it is more abundant in astrocytes.(Mennerick et al., 1998; Niciu et al., 2012). EAAT1 and EAAT2 are the major transporters responsible for maintaining optimal glutamate in synaptic cleft while EAAT2 responsible for most of the glutamate uptake(Eulenburg & Gomeza, 2010; Knut P. Lehre & Niels C. Danbolt, 1998). According to new scientific research, a number

of disorders, including ischemia, epilepsy, Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and autism, are linked to neurotoxicity, and EAAT1/EAAT2 dysregulation may be a key factor in neuropathogenesis (Barone, 2010; Blandini, 2010; Blandini et al., 1996; Bonnet, 2000; Garcia-Esparcia et al., 2018; Li et al., 1997; Lin et al., 1998; Pajarillo et al., 2019; Scott et al., 2011; Sheng et al., 2012).

EAAT3 shows a predominantly neuronal expression, particularly at the postsynaptic terminals (Rothstein et al., 1994). It is expressed throughout the brain and is involved in maintaining low glutamate concentrations. This suggests a role in synaptic plasticity (Bjørn-Yoshimoto & Underhill, 2016). Outside of the CNS, it is thought to be the major Glu and Asp transporter in a range of cell types, including skeletal muscle and the kidney. (Hediger, 1999; Li et al., 2015).

EAAT4 is a neuron-specific glutamate transporter, has only been detected in the dendrites of cerebellar Purkinje neurons (Danbolt, 2001; Kanai et al., 2013). EAAT4 and EAAT1 are two main transporters that control glutamate levels to prevent neurotoxicity in the cerebellum (Perkins et al., 2018).

EAAT5 is selectively expressed at photoreceptors, bipolar and amacrine cells (Danbolt, 2001; Kanai et al., 2013). Its Cl- conductivity is high, therefore thought to be the glutamate-activated receptor that controls the excitability of retinal neurons (Pow & Barnett, 2000; Tse et al., 2014).

3. Glutamate Receptors

Glutamate is stored in vesicles in axon terminals, and electrical stimulation acts on glutamate receptors via Ca2+ dependent exocytosis. The receptors are

classified into two types based on their function. İonotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). The majority of nervous system cells express functioning GluR. Different forms of GluRs may be found in peripheral neuronal cells, numerous cells in non-neural tissues such as endocrine, heart, kidney, lung, ovary, testicular, bone, and most types of immune cells (Ganor & Levite, 2014; Gill & Pulido, 2001; Hinoi et al., 2004).

3.1.Ionotropic Glutamate Receptors (iGluRs)

iGluRs mediate the rapid response of the CNS. Postsynaptic membranes contain all three receptors, and each receptor has different functions in the brain. Many neurological diseases have been found to be associated with these receptors.

The receptors classified as amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate (KA), and n-methyl-d-aspartate (NMDA). (Niciu et al., 2012).

3.1.1. NMDA receptors (NMDAR)

NMDA receptors is one of the most studied glutamate receptor types as it is important for both physiological and pathological processes. They play critical roles in synaptic plasticity, a learning and memory mechanism.(Li & Tsien, 2009; Liu et al., 2004; Riedel et al., 2003; Zhu & Paoletti, 2015). They are also of significance in terms of treatment because they are effective in many neurological illnesses.

Three NMDA subunit families have been discovered: NR1, NR2A-D, and NR3A-B. NR1 and NR2 subunits combine to form heterotetramers, which are NMDA receptors (Zhu & Paoletti, 2015). With glutamate binding to NR2, glycine binding to NR1 is required for NMDA receptor opening (Johnson & Ascher, 1987). It is

hypothesized to generate triple NR1 / NR2 / NR3 tetrameric complexes in NR3-expressing cells (Sasaki et al., 2002). There are specific areas on the receptor that extracellular magnesium (Mg^{2+}) ions binds and cause blockage of the ion channel. For receptor activation, it is necessary to remove the receptor blockage caused by the Mg^{2+} ion. (Zhu & Paoletti, 2015). When ligand binding activates the receptor, ion channel opens, allowing Ca^{2+} ions to enter the cell. High permeability to calcium ions is thought to be important in synaptic plasticity (G. E. Hardingham & H. Bading, 2010; Li & Tsien, 2009; Liu et al., 2004).

In addition to roles on synaptic plasticity, NMDARs also critical for supporting neuronal survival (Hardingham, 2006; Hetman & Kharebava, 2006). Ca^{2+} levels determine cell fate; while basal NMDAR activity is required for cell survival, too much signaling produces excitotoxicity, and the absence of receptor activity induces apoptosis (Hardingham, 2006; G. E. Hardingham & H. Bading, 2010; Giles E. Hardingham & Hilmar Bading, 2010; Hetman & Kharebava, 2006; Leveille et al., 2008; Parsons & Raymond, 2014; Sattler et al., 2000). There are two models that explain the determination of whether the stimulus will be neurotoxic or neuroprotective. In the first model, the result depends on the localization of the stimulated receptor. Synaptic NMDAR activation promotes cell survival, while extrasynaptic NMDAR stimulation may cause glutamate excitotoxicity and cell death (G. E. Hardingham & H. Bading, 2010; Giles E. Hardingham & Hilmar Bading, 2010; Leveille et al., 2008; Parsons & Raymond, 2014; Sattler et al., 2000; Talantova et al., 2013). In the second hypothesis, GluN2A-NMDARs, which are linked to the

activation of pro-survival pathways including CREB and PI3K, are neuroprotective, whereas GluN2B-NMDARs, which are more associated with apoptotic signals, are excitotoxic (Liu et al., 2007; Terasaki et al., 2010).

Many CNS illnesses are characterized by NMDA receptor dysfunction. Examples include acute excitotoxic disorders like ischemic brain injury and traumatic brain injury as well as chronic neurodegenerative conditions like Parkinson's, Alzheimer's, and Amyotrophic lateral sclerosis (Beal, 1992; Hsieh et al., 2006; Koutsilieri & Riederer, 2007; Meldrum, 1994; Wang & Reddy, 2017; Zhang et al., 2019). Therefore, NMDA receptor-based approaches are also used among the treatment approaches of these diseases.

3.1.2. AMPA Receptors (AMPAR)

Among the ionotropic neurotransmitter receptors, the majority of rapid excitatory synaptic transmission in the CNS is mediated by AMPA receptors.(Palmer et al., 2005; Song & Huganir, 2002). AMPARs have intracellular pools and their numbers are regulated by endocytosis, exocytosis, and endosomal sorting. The increase or decrease in synaptic strength as a result of this dynamic regulation is widely accepted as the mechanism behind memory and learning processes, known as long-term potentiation (LTP) and long-term depression (LTD) (Diering & Huganir, 2018).

AMPA receptors are tetramers composed of four subunits (GluA1-4). Each of the subunits gives different properties to the receptor and their distribution in the brain varies regionally. GluA1, GluA2, and GluA3 subunits are found throughout the CNS, whereas GluA4 expression is limited to the cerebellum, thalamus, and brain

stem. GluA2s are seen even in the embryonic stage, while other subunits appear during development (Yadav et al., 2017).

Calcium permeability of AMPARs depends on their subunit composition. GluA2-containing receptors are Ca-impermeable, while calcium-permeable AMPARs (CP-AMPA) are involved in synaptic plasticity. The unstable state provided by calcium permeability also makes the neuron susceptible to pathological conditions. Long-term expression of CP-AMPARs has been associated with neuronal pathologies such as ischemia, drug addiction and memory deficits (Gasbarri & Pompili, 2014; Yadav et al., 2017). As a result, they are pharmacological targets for memory impairments and drug addiction illnesses, in addition to other excitotoxic ailments (Chang et al., 2012; Malinow & Malenka, 2002; Mayer, 2011).

The rapid activation and opening of the AMPA receptor, as well as sodium influx through the channels, overcomes the NMDA receptor's voltage-dependent magnesium block and improves its activation (Henley & Wilkinson, 2016; Palmer et al., 2005).

3.1.3. Kainate Receptors (KAR)

Kainate receptors found in both pre- and postsynaptic neurons so they can take role in excitatory or inhibitory neurotransmission (Huettner, 2003; Lerma, 2006). Postsynaptic Kainate participate in reducing magnesium block of NMDA while presynaptic one help to modulate release of GABA (Huettner, 2003). It’s also clear that KA receptors participate in short and long-term synaptic plasticity (Bortolotto et al., 1999).

Kainate receptors are also made up of various subunits: The GluR5-7 and KA1-2 receptors (Mayer, 2011; Mayer & Armstrong, 2004).

They allow ion flow right after glutamate, do not require different cofactors to activate [31,32]. Synapses containing KA receptors quite low in number comparison to NMDA and AMPA receptors (Marmiroli & Cavaletti, 2012).

3.2. Metabotropic glutamate receptors (mGluRs)

Metabotropic glutamate receptors (mGluRs) are a type of glutamate receptor that is commonly found in the central and peripheral nervous system and has many different functions. There are 3 types of mGluRs.

Group I, which includes mGluR1 and mGluR5, has mainly postsynaptic neuronal distribution and governs excitatory postsynaptic potentials via Gq or tyrosine kinases (Heuss et al., 1999).

Group II metabotropic receptors, which include mGluR2 and mGluR3, are coupled with G0 and Gi proteins, lower intracellular cAMP via suppression of the adenylyl cyclase/protein kinase A pathway. They found both in pre- and postsynaptic neurons (Ohishi et al., 1994).

Group III metabotropic receptors include mGluR4, mGluR6, mGluR7, and mGluR8 are associated with Gi and G0 proteins. All predominantly expressed presynaptically except mGluR6 which is mainly postsynaptic and also been observed in rod cells of retina (Mercier & Lodge, 2014). They function as auto- and hetero-receptors. (Cartmell & Schoepp, 2000; Zou et al., 2017).

4. Excitotoxicity

Glutamate excess causes excessive depolarization of neurons, which leads to neuronal death. This is known as excitotoxicity. This toxic process take place not only in acute but also chronic diseases of the CNS. While various conditions like traumatic brain injury, cerebral ischemia, and status epilepticus are thought to be caused by acute

excitotoxic nerve cell death, many neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, Huntington's disease, and lateral amyotrophic sclerosis are believed to be caused by chronic glutamate excitotoxicity (Beal, 1992; Choi & Rothman, 1990; Lau & Tymianski, 2010; Maragakis & Rothstein, 2001; Meldrum & Garthwaite, 1990; Meldrum, 1994).

4.1. Excitotoxicity Mechanisms

The mechanisms underlying neuronal necrosis include acute mitochondrial dysfunction and loss of cellular homeostasis, leading to massive energy deficiency similar to those in other cell types. When the neurotransmitter balance is disrupted in favor of excitatory stimuli, or in the presence of metabolic or oxidative stress despite normal glutamate levels, the cell is overstimulated, resulting in a cascade of damaging effects. Although many ion balances are disturbed, there is a consensus about that influx of Ca^{2+} is the main cause of excitotoxicity and NMDARs mainly responsible for calcium entry (Choi, 1985)

Overactivation of ionotropic glutamate receptors leads to opening of voltage dependent Ca^{2+} channels resulting in large amounts of Ca^{2+} influx. IP3 synthesis is promoted by activation of metabotropic glutamate receptors, and Ca2+ release from the ER into the cytoplasm is stimulated. Cytoplasmic calcium levels increase and are sequestered in mitochondria and the ER. (Thayer & Wang, 1995; Wang & Thayer, 1996).

In mitochondria, increased Ca^{2+} influx depolarizes mitochondrial membrane and results in mitochondrial permeability transition pore(mPTP) activation. This causes; leakage of accumulated Ca^{2+}; release of pro-apoptotic factor cytochrome c into the cytosol and triggering of

caspase-dependent apoptosis; inhibition of respiratory chain enzymes that causes decreased ATP synthesis and increased generation of ROS (Duchen, 2004; Orrenius, 2004; Peng & Jou, 2010; Rao et al., 2014; Yang et al., 2011).

ROS are free radicals that are normally produced during biochemical reactions within organelles such as mitochondria, endoplasmic reticulum and peroxisomes. But if it is overproduced, cannot be neutralized by cell and reacts with lipids, proteins, carbohydrates and nucleic acids. Binding to the DNA evokes its fragmentation and results in cell death (Farooqui & Farooqui, 2009; Han et al., 2001; Muller, 2000). Oxidative stress itself can potentially open mitochondrial permeability transition pores (Nicholls, 2004).

Under excitotoxic conditions, activation of NMDARs induces Ca^{2+} influx results in activation of neuronal nitric oxide synthase (nNOS) via Ca^{2+}/calmodulin signaling (Sattler et al., 1999). NO production is also increased as a result of oxidative stress. Once intracellular NO elevate to the toxic concentrations, can react with superoxide radicals and form peroxynitrite, a highly reactive oxidant that can induce lipid peroxidation, protein dysfunction, and DNA damage (Radi et al., 1991a, 1991b; Salgo et al., 1995).

Excitotoxicity also causes cell swelling and lysis as a result of the influx of Na^+ and Cl^- via AMPA-kainate receptors and Na-K-Cl cotransporter type 1 (NKCC1) (Beck et al., 2003).

Additionally, Ca^{2+} influx activate calpain that is a calcium dependent apoptotic protease and calcineurin, a Ca^{2+} dependent cysteine protease, mediate neuronal cell death (Wang, 2000).

4.2.Excitotoxicity Related Diseases

Due to their critical functions in excitatory neurotransmission, abnormal signaling in GluRs has been linked to a variety of pathologies. This has made GluRs important pharmacological targets for therapeutic applications (Bleich et al., 2003).

4.2.1. Diseases of the CNS

Because energy is required to sustain membrane potentials, abrupt decrease of blood flow and loss of energy during ischemia conditions leads to membrane depolarization. This depolarization stimukates glutamate release and extracellular accumulation.

Energy deprivation also suppresses EAAT activity and reduces glutamate reuptake. Elevated glutamate levels lead to excitotoxicity by activation of glutamate receptors, especially NMDAr's (Katsuta et al., 1995; Park et al., 1988).

4.2.2. Alzheimer's Disease

Alzheimer's disease (AD) is the leading cause of dementia, according to the World Health Organization. Alzheimer's, a chronic neurodegenerative disease of the CNS, leads to progressive cognitive impairment, memory impairment and behavioral disorders. The pathological hallmarks of AD are neurofibrillary tangles consisting of aggregated tau, senile plaques containing extracellular β-amyloid (Aβ), Gliosis, followed by neuronal cell death and brain atrophy in later stages.

The pathophysiology of AD is complex. There are both structural and functional abnormalities, and many pathways are involved in synaptic and cellular degeneration. However, evidence suggests that glutamate toxicity also plays a role in AD. Depositions act as initiating factor for neurodegenerative pathways.

Glutamat level elevation in synaptic cleft can cause neurodegeneration in AD. Aβ plaques induce extracellular accumulation of glutamate and consequently intracellular Ca^{2+} deposits in cells next to Aβ plaques (Kamat et al., 2016; Kuchibhotla et al., 2008). Increased Ca^{2+} concentration associated with dendritic spine loss and endocytosis of NMDA and AMPA receptors through calcineurin, a calcium-dependent protein phosphatase (Hsieh et al., 2006; Sheng et al., 2012)

Moreover, β-Amyloid can inhibit uptake mechanisms of glutamate. In astrocytes exposed to β-Amyloid, glutamate uptake is significantly reduced. It has been discovered that in AD, EAAT2 makes much less glutamate uptake and EAAT expression in cells is diminished (Fernandez-Tome et al., 2004; Li et al., 1997; Scott et al., 2011). Cognitive impairment and tau pathology are reduced after EAAT2 receptor function is restored with the β-lactam antibiotic Ceftriaxone (Zumkehr et al., 2015).

-Amyloid, on the other hand, has been demonstrated in multiple studies to increase the activity of NMDARs. NMDA receptor antagonists can be used to prevent this (Alberdi et al., 2010; Texido et al., 2011).

In the normal brain, there is a balance of synaptic and extrasynaptic NMDAR activity. According to a study, β-Amyloid induces glutamate release from astrocytes and selectively activates extrasynaptic NMDARs, which are thought to be responsible for glutamate's excitotoxic action (Talantova et al., 2013).

Tau protein has also been linked to AD-related excitotoxicity, either alone or in conjunction with β-Amyloid (Pallo et al., 2016; Small & Duff, 2008). Synergic action between Aβ and tau facilitates ROS

production by impairing mitochondrial function (Quintanilla et al., 2012; Rhein et al., 2009).

4.2.3. Parkinson's Disease

Parkinson's disease (PD) is a progressive and the second most common neurodegenerative disorder (Poewe et al., 2017). Clinical symptoms include motor disturbances such as rigidity, bradykinesia, postural instability and resting tremor; as well as broad spectrum of non-motor symptoms like cognitive impairment/dementia, mood disorders, pain, autonomic disturbances, psychosis and hallucinations.

The pathological hallmarks of PD include progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) which causes striatal dopamine deficiency; and Lewy bodies that are intracellular inclusions of α-synuclein (De Pablo-Fernandez et al., 2019; Dickson, 2018).

It is also known that, beyond the dopamine system, there are disruptions in other neurotransmitters involved in the pathophysiology of PD, especially in etiology of non-motor symptoms. Two of these are glutamate and γ-amino butyric acid (GABA) (Barone, 2010; Bonnet, 2000). Degeneration of dopaminergic neurons leads to glutamate overactivity in the basal ganglia, especially in substantia nigra pars compacta (SNc) (Blandini, 2010; Blandini et al., 1996). Glutamate impairs Ca^{2+} homeostasis, and increased intracellular Ca^{2+} activates L-type Ca^{2+} channels, resulting in ROS generation in mitochondria and cell death. This process is significant in the pathophysiology of Parkinson's disease because nigral dopaminergic neurons are vulnerable to oxidative stress (Cieri et al., 2017; Surmeier &

Schumacker, 2013; Surmeier et al., 2017).

Moreover, it has been hypothesized that, even in the normal glutamate levels, indirect excitotoxicity may be one of the mechanisms in PD pathogenesis. When intracellular energy levels reduced due to mitochondrial deficits; function of Na^+, K^+-ATPase cannot generate a resting potential that is necessary for NMDAR's Mg^{2+} block and removal of the barrier allows nigrostriatal dopaminergic neurons become vulnerable to indirect excitotoxicity and cell death in the SNc (Blandini, 2010; Novelli et al., 1988).

4.2.4. Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease characterized by degeneration of spinal, bulbar and corticospinal motor neurons. It is a late-onset, fatal neurodegenerative disease that leads to muscle loss, weakness, spasticity and ultimately death. More than 90% of cases are categorized as sporadic with an unknown origin, while 5-10% of cases are familial and associated with gene mutations. (Robberecht & Philips, 2013).

Despite several research to understand the mechanism, the etiology of ALS remains unexplained. Neuroinflammation, oxidative stress, mitochondrial dysfunction, nuclear abnormalities, and abnormal RNA metabolism are all thought to have a role, and excitotoxicity is still assumed to be a significant etiological factor in ALS. (Mathis et al., 2017).

Only the spinal subtype of ALS has shown significant increases in plasma glutamate levels, with no significant increase observed in the bulbar subtype; and females had greater glutamate levels than males (Andreadou et al., 2008; Plaitakis & Constantakakis, 1993). According to studies, motor neurons in the

spinal cord are vulnerable to high glutamate levels, particularly via AMPAR and Kainate mediated excitotoxicity (Saroff et al., 2000; Vandenberghe et al., 2000; Weiss, 2011). Furthermore, EAAT2 levels were shown to be lower in the motor cortex and spinal cord of ALS patients, resulting in increased extracellular glutamate levels. Excitatory glutamate impulses cause calcium influx and motor neuron activation, which initiates damaging biochemical processes within the cell, all of which are known to be significant pathophysiological events in sporadic and familial ALS (Lin et al., 1998; Trotti et al., 1999).

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