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The Effect of Gallic Acid on Histopathologic Evaluation of Cerebellum in Valproic Acid-Induced Autism Animal Models

Parvin Samimi and Mohmmad Amin Edalatmanesh

Department of Physiology, Islamic Azad University of Shiraz, Shiraz, Iran Corresponding Email:<u>samimiparvin@yahoo.com</u>

ABSTRACT

Autism spectrum disorder (ASD) is counted as a worldwide public health problem. The possible causes of ASD are reactive oxygen species and free radicals. So, this study is aimed to evaluate the effects of Gallic acid, as an effective antioxidant, on histopathologic disorder of the cerebellum in valproic acid-induced autism animal models. 30 pregnant female rats were randomly divided into 5 groups, including: control, autism (or VAP) and experimental 1, 2 and 3. Using a gavage needle, Gallic acid administered orally until about2 months of age. After the end of the treatment period, the rats were anesthetized with ether and their cerebellar tissues were removed for histopathologic studies. A significant decrease in the number of Purkinje and granular cells was observed in this study in VAP group compared to the control group ($P \le 0.05$). A trend toward improvement was observed in the groups received 100 and 200 mg/kg of Gallic acid ($P \le 0.05$). The results of this research revealed that Gallic acid reduces the side effects caused by valproic acid on cerebellar tissue of autistic rats. So, it should be considered for therapeutic goals.

Keywords: Autism, Gallic acid, Histopathologic, Cerebellum, Rat

INTRODUCTION

Autism is a kind of developmental disorder of social relations. It is related to nervous system and characterized by abnormal behavioral and verbal communications. Symptoms of the disorder occur before three years of age and its main cause is unknown. The disorder is more common in boys than girls. Normal growth of the brain is injured in this impairment in the area of social interactions and communication skills. Children and adults with autism have difficulty with verbal and nonverbal communications, social interactions and activities related to game. Repetitive movements (e.g. hand flapping and jumping), unusual responses to individuals, interest to objects and/or resistance to variation are observed in these patients [1]. It was also stated that autism has a series of pathological symptoms such as reduced number of cerebellar Purkinje cells and/or change in size of the brain, so that the brains of children with autism vary in size and structure from the brain of normal children [2]. According to anatomical studies on the brain of individuals with autism after their death, various areas of anatomical abnormalities in the brain of the patients have been identified. These areas include: the nervous system of the brain (limbic system), the cerebellum and the structure of the inferior olive. Increased cellular density through increase of small nerve cells is observed in limbic, hippocampus and amygdale systems and Entorhinal cortex [3]. So study on changes of the central nervous system (CNS) in individuals with autism can help to faster and more effective treatment of them.

Studies have suggested that there is no certain cure for autism; however medications prescribed for the patients with autism are often for control of their secondary symptoms such as hyperactivity, sleep disorder and stereotypical behaviors. Antioxidants prevent cell damages and cell death caused by free radicals. In case that there is no adequate antioxidant in the body, free radicals cause oxidative stress. Oxidative stress is associated with developmental delay and neurological disorders and also many other diseases processes. Recent studies have indicated that oxidative stress is higher in children with autism [4]. Other studies have showed that antioxidant nutrients can improve autism-related disorders [5]and one of them is Gallic acid antioxidant with chemical formula of $C_7H_6O_5$ that is commonly used in pharmacy. Gallic acid plays as an antioxidant and helps cells in protection from oxidative

damages. Due to its biological activity, Gallic acid has anti-bacterial, anti-viral and anti-inflammatory actions and the evaluation of its antioxidant effects have shown through tyrosinase activities [6]. The effect of Gallic acid has reported on reduction of various neurological disorders, including Alzheimer's disease associated with dementia and memory loss [7]. Earlier studies have shown that grape seed has abundant Gallic acid. Various amounts of this compoundare effective in nervous system diseases (e.g., Parkinson's disease) [7, 8]. Balu et al., in 2005 showed that memory impairment in old mice improves using antioxidant properties of grape seed extract. These properties are attributed to the antioxidant properties of polyphenols such as Gallic acid [9]. According to published statistics in recent years, increased patients with autism and their problems in family and society as well as the absence of a certain cure for them have led to perform several researches in this field to treat and prevent from the disease. Nowadays by proven of the impact of antioxidants on restoration of cells and reduction of oxidative activity in cells, we decided to review the impact of Gallic acid, which is one of the most effective antioxidants, on histopathologic disorder of the cerebellum in valproic acid-induced autism animal models.

MATERIALS AND METHODS

This study was conducted in laboratory and was completely random. The animals used in this experiment included 30 adult female rats (for pregnancy) and 30 adult male Sprague Dawley rats, which were all prepared from Breeding and Keeping Center of Laboratory Animals related to Shiraz University of Medical Sciences and then tested. Ethics, international rules and standards of Ethical Committee of Laboratory Animals were observed in all stages of working with animals. Prior to categorization, vaginal smear was prepared at first from female rats and those rats which were in estrous cycle were placed in a cage with a male rat for mating. One day after mating, the animals were examined for assurance. In case of observation of vaginal plug or presence of spermatozoa in the vaginal smear, that day was determined as zero day of pregnancy.

After initial weighing, the pregnant rats were randomly divided into 5 groups of 6 each.

Control group: this group administered no treatment and only received laboratory standard water and foods.

Sham group (VPA): female rats in this group were intraperitoneally received 500 mg/kg of valproic acid at 12.5th day of pregnancy. The animals also received normal saline (as the solvent of the medicine)via gavage with similar dose of the medicine administered to the experimental groups from 19th day of pregnancy.

The experimental1 group: female rats in this group were intraperitoneally received 500 mg/kg of valproic acid at 12.5th day of pregnancy. The animals also received 50 mg/kg of Gallic acid via gavage from 19th day of pregnancy.

The experimental2 group: female rats in this group were intraperitoneally received 500 mg/kg of valproic acid at 12.5th day of pregnancy. The animals also received 100 mg/kg of Gallic acid via gavage from 19th day of pregnancy.

The experimental3 group: female rats in this group were intraperitoneally received 500 mg/kg of valproic acid at 12.5th day of pregnancy. The animals also received 200 mg/kg of Gallic acid via gavage from 19th day of pregnancy.

10 g vial of valproic acid powder used in the study was purchased from Sigma Corporation. Injection of the medication was conducted as a single dose at 12.5^{th} day of pregnancy. It should be noted that the mothers were administered Gallic acid via gavage from 19^{th} day up to 24^{th} day of pregnancy. Newborn male and female rats were gavaged with Gallic acid from 24^{th} day of birth up to 60^{th} day.

In order to dissection process and tissue-separating, all the animals were placed within anesthesia jar containing cotton soaked in diethyl ether and anesthetized. The skull bones were separated without any damage to the brain and it was removed. In this case, the brain tissue was placed into a glass chamber containing 10% buffered formalin [10]. The tissues were then sent to pathology laboratory for preparing of slides and staining with Hematozylin and Eosin stain. To study the tissue sections, the total number of cerebellar Purkinje and granular cells was estimated in different parts of the cerebellum and in both hemispheres. Random sampling method was used in this stage and astrolite software was also used to count the number Purkinje and granular cells in folia IV, V and VI. The cells were counted in a reference frame [11, 12]. It should also be noted that detection of folia IV, V and VI in rats was performed using Paxinos and Watson rat brain atlas. The raw numbers obtained from behavioral studies and the results of histological studies were evaluated using SPSS software and analyzed through one-way analysis of variance (one-way ANOVA) and Tukey's test and compared separately and with each other. Their charts were drawn based on information obtained from analysis of the numbers. The used values are mean \pm standard deviation error (SEM) and P \leq 0.05 is considered as the significance level.

RESULTS

According to Charts (1) and (2), a significant reduction at level of P<0.0001was observed in the number of Purkinje cells in folium IV of both hemispheres and folium V of the left hemisphere in the VPA group compared to the control group. The results also showed the protective effect of Gallic acid on the number of Purkinje cells in Exerimental3 group compared to the VPA group at level of P<0.0001. However, no significant change in folia IV and V was observed the Experimental1 and 2 groups compared to the VPA group (P \leq 0.05). No significant difference was also observed at level of P \leq 0.05 between the number of Purkinje cells in folia IV and V of both hemispheres.

Chart (1): The results related to the number of Purkinje cells in folium IV in both hemispheres of the cerebellum



Chart (2): The results related to the number of Purkinje cells in folium V in both hemispheres of the cerebellum



According to the Chart (3), a significant reduction in the number of Purkinje cells in folium VI in both hemispheres was observed at level of P<0.01 in all groups received VPA (except the Experimental3 group). No significant difference between the numbers of Purkinje cells in folium VI in both hemispheres was also observed at level of $P \le 0.05$.





According to the Charts (4) and (5), a significant decline at levels of P<0.01 and P<0.001 in the number of granular cells in folia IV and V in both hemispheres was observed in VPA group compared to the control group, respectively. The number of granular cells in folium IV showed the protective effect of Gallic acid on the number of granular cells in Experimental3 group (P<0.05) in the left hemisphere as well as in Experimental3 (P<0.001) and

Experimental2 groups (P<0.05) in the right hemisphere compared to the control group. However, no significant change was observed in Experimental1 compared to the VPA group. No significant difference at level of P \leq 0.05 was also observed between the numbers of granular cells in folium IV.

The number of granular cells in folium V showed that the decline in the number of granular cells in the left hemisphere had been blocked at all received doses of Gallic acid; so that a significant difference at level of P<0.05 was only observed in Experimental3 compared to the VPA group. However, the results related to the number of granular cells in the right hemisphere were also indicated the power of Gallic acid in prevention from reduction in the number of granular cells in Experimental2 and 3 groups compared to the VPA group. No significant difference at level of P<0.05 was also observed between granular cells in folium V.



Chart (4): The results related to the number of granular cells in folium IV in both hemispheres of the cerebellum

Chart (5): The results related to the number of granular cells in folium V in both hemispheres of the cerebellum



According to the Charts (6), a significant decline at level of P<0.05 in the number of granular cells in folium VI in both hemispheres was observed in the VPA group compared to the control group. The results also indicated the protective effect of Gallic acid on the number of granular cells in Experimental3 group(P<0.05) in the left hemisphere and in Experimental2 and 3 groups (P<0.05) in the right hemisphere of the cerebellum compared to the VPA group. However, no significant difference was observed in Experimental1 group in both hemispheres compared to the VPA group. However, a significant difference was observed in Experimental1 group in the right hemisphere compared to the control group. There was also no significant difference at level of P \leq 0.05 between the numbers of granular cells in folium VI in both hemispheres.

Chart (6): The results related to the number of Purkinje cells in folium VI in the right and the left hemispheres of the cerebellum



Data is shown as Mean \pm S.E. The presence of at least one similar word in the above Chart indicates lack of difference between the studied groups. In the case that there is no similar word between the groups, it indicates the significant difference at level of $P \le 0.05$ between the groups.



Image 2: The photomicrograph of cerebellar tissue in VPA group, (H&E stain; 400 × magnification)

The reduced number of healthy Purkinje cells and their atrophy (yellow arrow) is specified in the above image. So that the nucleus is inseparable from the cytoplasm and the nucleus is displaced from the center of cell to the wall of the cytoplasmic membrane. Purkinje neurons have been actually experienced cell death. The nucleus of some of granulosa cells (red arrow) and purkinje cells have been experienced pyknosis. So, tissue damage is clearly visible.



Image 1: The photomicrograph of cerebellar tissue in the control group (H&E stain; 400 \times magnification)

The large number and accumulation of Purkinje cells (yellow arrow) is clearly observable in the above image so that no change or pathological damage is observed.



Image (4): The photomicrograph of cerebellar tissue in the autism group + 100 mg/kg of Gallic acid, (H&E stain; 400 × magnification)

The decline in the number of healthy Purkinje and granular cells has been partly prevented in above image. However, there are still some cells with pyknotic nuclei in the cerebellum tissue (red



Image (6): The photomicrograph of cerebellar tissue; counting the number of granular cells using astrolite software; (H&E stain; 400 × magnification); just available cells in the frame are counted in above image.



Image 3: The photomicrograph of cerebellar tissue in the autism group + 50 mg/kg of Gallic acid, (H&E stain; 400 \times magnification) The reduced number of healthy Purkinje cells and their atrophy is specified in above image. The nucleus of some of granulosa cells (red arrow) and purkinje cells (yellow arrow) have been experiencedpyknosis.



Image (5): The photomicrograph of cerebellar tissue in the autism group + 200 mg/kg of Gallic acid, (H&E stain; 400 × magnification) The decline in the number of healthy Purkinje cells has been prevented in above image. A sensible pathological damage in granular cells is not observed, so that their nucleus and cytoplasm are well visible.

DISCUSSION

There was a significant decrease in the number of Purkinje and granular cells in folia IV, V and VI of the cerebellum in the VAP group compared to the control group. So that reduction of the above cells was prevented in the groups treated with Gallic acid at doses of 100 and 200 mg/kg. No significant changes were observed in the group treated with Gallic acid at dose of 50 mg/kg compared with the VAP group. Therefore it can be said that Gallic acid acid acids in a dose dependent manner. Some studies have shown that this compound has a great ability to reduce oxidative stress and through this way it protects from the central nervous system [13]. Ballu et al. in 2005 showed that grape seed extract reduces memory impairment in old mice due to the presence of high amounts of polyphenol antioxidant types (e.g., Gallic acid) [9].Previous studies have also shown that grape seed extract, which contains abundant Gallic acid, in hypoperfusion ischemia disease as well as various amounts of Gallic acid in Prakinson's disease increase delay time in coming down from a platform in avoidance memory test [7, 8].These antioxidants in the brain are effective in prevention and treatment of disorders caused by oxidative damages; and possibly as a powerful

antioxidant, Gallic acid is able to improve memory and learning and to protect from cellebellar destruction and tissue damage [9]. Improvement in different parts of the cerebellum was also observed in this study in the groups received Gallic acid. This indicates the antioxidant property of the compound. Vali Zadeh in 2012 revealed that 10-day oral administration of Gallic acid can improve spatial learning and memory impairment caused by beta-amyloid injection in Alzheimer's animal models [14]. In addition, it was found that the antioxidant effect of this group of compounds is through increased level of enzymes related to antioxidant system including glutathione peroxidase; and on the other hand, these compounds are able to reduce production of end-products of lipid peroxidation (e.g. malondialdehyde) [7].

It has found in a study that was conducted by Rafei Rad et al. in 2013 that Gallic acid has impact on the central nervous system so that this compound increases the memory in healthy and sick mice [15]. Gallic acid has well explained as a scavenger of free radicals. Given to the antioxidant effect, extracts of plants containing Gallic acid have shown anti-diabetic, anti-melanogenic properties as well as reduced incidence of myocardial infarction and oxidative damage of the liver and the kidney [7, 13, 16].

Vijaya Padma et al. in 2011 conducted a study on the protective effect of Gallic acid against toxicity of lindane in rats. They have found that the compound significantly increases the concentrations of superoxide dismutase, catalase, glutathione peroxidase and glutathione s-transferase and reduces lactate dehydrogenase levels compared to the group received lindane toxin. Histological results have also indicated decreased kidney and liver damages in treated groups with Gallic acid [17]. It is also probable in this study that Gallic acid improves cerebellar tissue and reduces damages caused by valproic acid.

Ribieri et al. in 1992 showed that in addition to superoxide dismutase and catalase enzymes, glutathione peroxidase is one of the most important factors that scavenge free radicals and convert hydrogen peroxide to water [18]. Most of phenolic acids available in diet have antioxidant properties [7]. Yeh et al. in 2009 showed in their study that phenolic acids (e.g. Gallic acid) increase the activity of glutathione peroxidase and catalase in rat heart [19]. So they can prevent from tissue damage. This agrees with the results of the current study.

Li et al. in 2005 used Gallic acid in their studies to treat 9-month-old mice; not only the compound increases the expression of glutathione peroxidase and catalase, but also the levels of malondialdehyde reduce in the brain, the kidney and the liver of the mice [20]. Mansouri et al. in 2013 showed in their study that Gallic acid prevents from the brain damages caused by free radicals. The protective effects have a dose dependent manner. In other word, the study found that higher dose of Gallic acid (200 mg/kg) shows more effective impacts for treatment [7]. This is consistent with the results of the current study. These protective effects attributed in the study of Shahrzad et al. in 2001 to absorption and rapid metabolism of Gallic acid in the brain [21].

Mansouri et al. in 2013 conducted a study on neuroprotective effects of Gallic acid on oxidative stress caused by 6hydroxy-dopamine in rats. They found that Gallic acid at doses of 50, 100 and 200 mg/kg body weight increases the passive avoidance memory. In addition, this compound reduces the concentration of malondialdehyde in hippocampus and skeletal bodies and increases the concentration of glutathione peroxidase [7]. So, the results of this study showed that Gallic acid prevents from enhancement in oxidative stress by reinforcement of antioxidant defense system. The neuroprotective effects of Gallic acid have also demonstrated earlier using neurotoxic effects of beta-amyloid and nitrate and oxidative stress caused by streptozotocin in the brain [22, 23]. Another study has found that Gallic acid neutralizes the neurotoxic effects caused by 6-hydroxy dopamine in human SH-SY5Y cells [24]. In addition neuroprotective effects of Gallic acid in various neural models has identified that the compound can control oxidative stress induced by carbon tetrachloride and lindane in the liver and the kidney of rats [25, 26]. Considering the above, it can be concluded that due to antioxidant and neuroprotective properties of Gallic acid, it can prevent damage to the cerebellum caused by valproic acid.

CONCLUSION

The results of the current study showed that intraperitoneal injection of valproic acid with normal saline at 12.5th day of pregnancy to female rats caused autism in infants and reduced number of Purkinje and granular cells was observed in VPA infants, so that the above disorders had prevented in the treated groups with Gallic acid at doses of 100 and 200 mg/kg. This was probably through reduced oxidative stress in rats with autism.

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