

Behavioral and Antioxidant Profiling in a Rat Autism Model: Protective Effects of Red Grape Extract

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Abstract—Autism spectrum disorders (ASDs) are disorders of the development of the central nervous system (CNS), affecting the structure of brain regions involved in learning and memory, such as the hippocampus. Symptoms include impaired social interactions. The prevalence in Iraq is 89.40 per 10,000 children, with males being 1-4 times more likely than females. Rats are an ideal animal model for studying behavioral aspects of autism mediated by Valproic acid (VPA). Polyphenols present in red grapes *Vitis vinifera* have been shown to be antioxidant. 20 pregnant female *Albino Wistar* rats were divided into four groups. The G1 injected with normal saline (0.55 ml to 0.69 ml) according to body weight on gestational day (day 12.5) intraperitoneally (I.P). G2 was injected with 450 mg/kg valproic acid on gestational day (day 12.5E), G3 was administered orally at a dose of 400 mg/kg from day 11.5E until the end of pregnancy. G4 was administered orally at a dose of 400 mg/kg from day 11.5E until the end of pregnancy. They were then injected with 450 mg/kg (I.P) on day 12.5E. The weight and behavior of the male offspring were assessed using the three-chamber test (3CT) and indicators of oxidative stress in hippocampal tissue. G3 showed the highest weight level compared to the other groups, while the G2 recorded a lower average weight than the G1, and the G4 recorded the lowest average weight. In social interaction, the G3, G4 recorded similar high values, but their value decreased in the G2. In social Novelty, the G1, G4 showed similar high values, while the G3 recorded a higher value than the G2 and a lower value than the G1. The G2 recorded the lowest value for social novelty. The Malondialdehyde (MDA) decrease in the G3 and an increase in the G2 compared to the G1. As for the G4, the index value was close to the G1. The glutathione (GSH) in the G3 showed an increase, and it was close to the G1 level in the G4, and decreased in the G2. The G3 showed an increase in superoxide dismutase (

SOD), a decrease in the G2 compared to the G1, and close to the G1 level in the G4. In conclusion, G3 had a positive effect on weight gain, recording the highest average weights. In contrast, G2 caused a decrease in weight, while the G4 showed the lowest average weights compared to both groups. Alcoholic grape extract improves social interaction, with both groups taking it (either with VPA or alone) recording high and similar values. G2 led to a decrease in social interaction, and in social novelty, the G4 showed a value close to the G1 level. The G3 showed an improvement compared to the G2, but it did not reach the G1 level. The G4 showed an effect in reducing MDA and increasing GSH and SOD when used in combination with VPA. In contrast, G2 caused an increase in MDA and a decrease in GSH and SOD.

Keywords — Autism spectrum disorders (ASD), Valproic acid (VPA), Three-chamber test (3CT), Oxidative stress, *Vitis vinifera*.

INTRODUCTION

Autism is a developmental brain disorder characterized by impaired social interactions (1). Its symptoms range from mild to severe, and its symptoms vary from person to person (2,3). It typically appears between the twelfth and twenty-fourth month of life (4). The incidence rate in males is 1-4 times higher than in females (5). Approximately 75 million people worldwide suffer from this disorder, and its prevalence in Iraq is approximately 0.89 percent (89.4 per 10,000 children) according to the (6). Pregnant mothers with epilepsy use antiepileptic medications, which are considered an environmental factor that contributes to the development of autism (7). Valproic acid (VPA) is an antiepileptic medication. Exposure to it during pregnancy (the first three months) is related with an increased incidence of autism in offspring of mothers who take the drug (8). It causes tissue changes during pregnancy, specifically in neural tube development, and consequently, disturbances in the morphology of pyramidal and granule neurons, located in cerebral cortex and hippocampus areas of perception, memory, and learning

(9,10,11,12). Therefore, it is essential to reduce the effects of this drug. Medicinal plants, including red grapes (13), contain many biologically active compounds, such as polyphenols, which act as antioxidants, anti-inflammatory, and anticancer agents, and prevent the entry of certain drugs into the brain, thus preventing and effectively managing a (ASD) (14). The studies aimed to find out the protective effect of the red grape fruit extract in reducing autism-like behavior through the assessment of the weight of the offspring, the social behavior of animals through the three-room test, and the estimation of some indicators of oxidative stress in the hippocampal tissue.

MATERIALS AND METHODS

Animals Model

The current study was conducted at the University of Karbala, College of Education for Pure Sciences, Department of biology. used 30 adult rats, consisting of 20 females and 10 males, for insemination only. The rats weighed an average of 220–300 grams, and her age from 8–9 months. rats were placed in cages measuring 48 cm in length, 15 cm in width, and 7 cm in height. the temperature was between 25–30°C, with water and special food. to check Pregnancy we used a vaginal swab from the females, which was checked for the presence of sperm the following morning. Females with a positive result were considered pregnant, identified, and isolated (15,16). the pregnant females were then arranged into four groups: control group (G1) VPA group (G2), VPA + extract group (G3), and the extract group (G4).

Valporic acid

(VPA) was brought from outside Iraq by the american company Santa Cruz Biotechnology (SC-202378). It is available as a solid powder, the dissolved in normal saline according to the weight of rat and administered by a single intraperitoneal (IP) injection on 12.5 E dosage amount 450 mg/kg (17). amount normal saline depends on animal weight.

Red grape extract

Red grapes were purchased from local markets in Najaf Governorate. The scientific name of the plant is *Vitis Vinifera* SP. The ripe grapes were washed from dust and impurities, dried well, and cut using a home cutting machine. The slices were then dried in the sun instead of leaving them in the shade to increase the concentration of phenols. They were then ground into a coarse powder according to (18,19). The maceration method was used, where an appropriate amount of the ground sample (200) grams was weighed and ethanol was added to it at a ratio of 1:10 (500 ml of ethanol to 200 grams). The mixture was placed in a glass flask and placed on a magnetic stirrer. Then, it was stirred manually using a motor. The mixture was left for 24 hours with continuous stirring. Then, it was filtered in two stages, filtered using gauze and then filter paper to separate the liquid from the sediment. The extract was stored in a closed tube in the refrigerator at a temperature of 4°C away from light Until use (20). Then, pregnant females are given a dose of 400 mg/kg from 11.5 E

to the end of pregnancy (21).

Study of Animal Behavior

Social behavior assessment in the three-chamber test (P35-P45): Social behavior was measured in male littermates from the four groups using the three-chamber test, which consisted of a Plexiglas box measuring (60, 45, and 22) cm, divided into three open-topped chambers with retractable doors. The animals were permitted to adapt for about 10 minutes in the test box, and the beginning time of the test, the time of each phase, and the behaviors of each group were recorded according to the method used in the two studies (22,23).

Animal Sacrifice

Rats were anesthetized with 10% ketamine and 2% xylazine depending on the weight of the animals, which ranged from 45 to 75 g, by intraperitoneal (I.P) injection the dose ranged (0.10ml to 0.15ml). After the animal was anesthetized (5-10 minutes) and reached the dissection stage, the head was separated from the neck using large scissors placed at the point where the head meets the neck. Then, the fur and skin were removed, and the skull bones were cut from both sides to make a large incision to extract the brain (it is necessary to avoid tearing the layers of the brain with the skull during dissection and removing the brain). Then, the brain was isolated and placed on a flat surface covered with ice to maintain its solid consistency. A longitudinal incision was made separating the right and left brainstems so that they become separate, and they were turned over so that their lower surfaces were facing upwards. Then, the cerebrum was separated from the cerebellum and brainstem and the brain was removed. The interneuron, olfactory bulb, and thalamus of the right and left brainstem are separated, leading us to the hippocampus, The hippocampus is isolated and placed on ice to measure oxidative stress markers. The hippocampus was extracted according to (24). Freezing the brain with ice was used to maintain its rigidity and stability during dissection, instead of perfusion with formalin.

Biochemistry preparations

Hippocampus tissue squash: After collecting hippocampus tissue and storing it in a refrigerator at -80°C, we prepare the protease inhibitor solution by adding one tablet of Comprehensive Miniaturized Protease Inhibitor Mixture Cocktail (1 tablet yields a 1 mM EDTA solution in 10 ml) to 10 ml of T-PER Tissue protein extract reagent and dissolving it well (manually or using a vibrating device). Then, the tissue is placed directly on dry ice after being removed from the freezer (-80°C). We use sharp blades for cutting off a piece of tissue in the freezer, then leave it to thaw on ice. We weigh a piece of tissue, then keep it cold by placing it back on ice. Using scissors or a single-edged blade, we cut the tissue into small pieces (~1 mm), taking care to keep the tissue cold to minimize protease activity. We then place the tissue pieces in a glass masher tube and add the appropriate amount of T-PER Tissue protein extract reagent (5 ml per 250 mg of tissue). We

mash the tissue on ice using a pestle for several minutes until it disappears. The masses were then transferred to 15 ml Falcon tubes (2.5 ml per tube) and kept on ice. Then, using an ultrasonic masher on ice for 5–10 cycles until foaming occurred, the machine was set to 30% cycles and 3 output control. The sample was left on ice for 10 min to allow cell lysis. The sample was transferred to 1.5 ml Eppendorf tubes and centrifuged in a cold room at $14,000 \times g$ for 10 min to collect debris from the cells. The upper liquid was carefully collected and dispensed into PCR tubes on ice (~200 μ l per tube). Then, the samples have been stored at -80°C till utilized in subsequent analysis (25). Oxidative stress indicators were assessed as follows:

Reduced glutathione (GSH): Reduced glutathione was determined in tissues according to method (26). while Maldondialdehyde (MDA) was measured using the thiobarbituric acid (TBA) analysis method developed by (27), using a spectrophotometer & Superoxide dismutase (SOD) (Cu-Zn) operation was specified as the utilized process (28).

STATISTICAL ANALYSIS

Data were analyzed using SPSS version 27. One-way ANOVA is employed to choose statistically significant variation between various groups. s, the least significant difference (LSD) test was applied to conduct post hoc comparisons between means. Differences were considered significant when the probability value (P) was less than 0.05 ($P < 0.05$). Data were expressed as mean \pm standard error \pm SE (Mean).

RESULT AND DISCUSSION

Evaluation of male birth weights in experimental groups: When evaluating the different treatments on the average weights of males at the age of 35 days, at the P-value level (0.05) Table 1 showed the following : The group of alcoholic extract of grape fruits with the drug valproic acid recorded the highest average weight of 70.71 ± 0.359 grams, indicating that the combination of the drug VPA and alcoholic extract of grape fruits led to a considerable rise in the rat's weight comparison to other groups, while the control group recorded an average weight of 58.71 ± 0.865 grams, which is in the middle of the ranking, and is considered the standard that was compared with the control of the groups, while the VPA group recorded an average weight of 53.00 ± 0.534 grams, which is less than the control group, which may indicate the negative effect of valproic acid on the growth of male rats and reducing their weights. As for the group of alcoholic extract of grape fruits only, it recorded the lowest average weight of 51.00 ± 0.436 grams, which raises questions about the effect of Alcoholic extract of grape fruit alone affected the weight of rats, and this may need further study.

Table 1. The average weights of the four male birth groups.

Means \pm SE	Standard Groups
58.71 ± 0.865 B	Control(G1)
53 ± 0.534 C	VPA(G2)
70.71 ± 0.359 A	VPA+ Extract(G3)
51 ± 0.436 D	Extract (G4)
1.698014	LSD

(n = 5) per group. Different letters indicate significant differences vertically at the probability level of $P < 0.05$

Regarding the evaluation of weight results, the results of our study were consistent with the results of a study conducted by researchers (29). Subcutaneous injection of (RSV), a polyphenol found in grape peel, with VPA led to a significant weight increase at $p > 0.05$, because it improves the uterine environment and supports fetal growth. Therefore, an abnormal uterine environment affects the growth of offspring in its early stages. Also, recording the highest weight rate in the VPA + extract group is due to the interaction of these two compounds. Valproic acid negatively affects the indicators of oxidative stress, while polyphenols work the opposite, as they reduce free radicals and act as strong antioxidants, allowing the rat to grow better. It is worth noting that an apparent change in the curvature of the tail of offspring in groups VPA + Extract and VPA, which the extract was not able to alleviate, and that the weight loss occurred after intraperitoneal injection of valproic acid only for pregnant mothers. This was caused by immediate lower abdominal cramps, which led to temporary changes in motor behavior and breathing patterns. This discomfort disrupted feeding habits and interfered with fetal development. Although valproic acid (VPA) is rapidly absorbed, the exact mechanisms behind this phenomenon remain unclear. Females injected with saline intraperitoneally did not show any significant response. Thus, chemical gent, such as exposure to valproic acid, raise the danger of low birth weight and neurodevelopmental abnormalities in fetuses. Maternal immune activation has been linked to inflammation during pregnancy and neurodevelopmental issues in offspring, depending on the dosage. Consequently, higher concentrations of VPA are closely associated with reduced birth weight in rat fetuses, resulting in delayed growth and fewer litters. Our study's findings align with research conducted by other scientists (30), which indicated that female rats treated with grape juice and a high-fat diet experienced a reduction in body weight compared to the control group due to improved metabolism and decreased food intake, along with its effects on liver lipid enzymes.

Behavioral (3CT)

First: The Sociability Phase (SI). In this phase, our study results recorded high and similar values in the control group, the valproic acid plus grape alcoholic extract group, and the grape alcoholic extract only group (means 0.546, 0.444, and 0.5222, respectively), indicating that the addition of grape alcoholic extract improved social behavior in the valproic acid group and may have a protective effect. Conversely, the valproic acid only group showed a significant decrease in social interaction (-0.125 ± 0.125), indicating a negative effect of VPA on social behavior.

Second: Social Novelty (SNI): At this stage, the negative control group and the grape alcoholic extract group showed high and similar values (0.657 and 0.542, respectively). The valproic acid and grape alcoholic extract group recorded a value of (0.057), which, although higher than the VPA group, is still significantly lower than the control group and the grape extract group, indicating that the grape extract has a positive effect but is insufficient to fully restore social behavior to its normal level. On the other hand, the valproic acid group recorded the lowest value for social preference (-0.571 ± 0.171), confirming the effect of VPA in significantly reducing social preference.

Table 2. Behavioral experiment results.

SNI	SI	Standard Groups
0.657 ± 0.142 A	0.546 ± 0.249 A	Control(G1)
-0.571 ± 0.171 C	-0.125 ± 0.125 B	VPA(G2)
0.057 ± 0.057 B	0.444 ± 0.170 A	+VPA(G3) Extract
0.542 ± 0.173 A	0.5222 ± 0.168 A	Extract(G4)
0.420431	0.569536	LSD
0.05	0.05	P-Value

(n = 5) per group. Different letters indicate significant differences vertically at the probability level of $P < 0.05$. The Sociability Phase (SI), Social Novelty (SNI).

Regarding social interaction, our results are consistent with those from researchers (31), as grape powder contains numerous polyphenolic compounds that protect neurons from dysfunction, thereby regulating behavior and memory, maintaining oxidative stress levels, and influencing genetic mechanisms in specific neural circuits related to these behaviors. Additionally, our findings corroborate those of researchers (32), who found that prenatal exposure to valproic acid reduces indicators of social interaction. This effect stems from VPA's ability to regulate neurotransmission and gene expression by remodeling chromatin through the inhibition of histone deacetylase activity. Such molecular disruptions can lead to epigenetic changes, resulting in abnormal transcription of brain-related genes during development and subsequent neurobehavioral issues in offspring.

Furthermore, our study is compatible with a study (33). It has the potential to alter gene activity, damage DNA, disrupt mitochondrial energy metabolism, and increase oxidative stress in the fetus. Therefore, it is considered a behavioral teratogen in pregnant females. The results of our current study differ from those of (34). Male rats did not show any social impairments, which was unexpected, especially for males exposed to valproic acid. This is due to the concentration and timing of exposure and the different protocols. When injected subcutaneously on day 13 of pregnancy using a dose of 600 mg/kg, i.e., after the peak of the abnormalities had passed, it significantly affected the study results. Therefore, the dose concentration, exposure time, and gender have an important effect on the results of the study. While in the social preference stage, the outcome of our research is compatible with the results of a study conducted by researchers (35), where the control group and the RSV group showed a similar behavioral pattern, as the rats tended to prefer the familiar rat. As for the VPA group, The social preference index decreased in comparison to the control group. This difference among the experimental groups is attributed to the molecular effects of resveratrol during prenatal exposure.

Oxidative stress

First: Results of the MDA index (mmol/l): Malondialdehyde index is an indicator of lipid peroxidation in cells. the alcoholic extract of grapes was given with VPA, the MDA level decreased significantly (13.288 ± 1.056) compared to the VPA group, and became very close to the level of the control group. This indicates that the alcoholic extract of grapes has a protective effect against oxidative stress caused by VPA. On the other hand, the VPA group showed a significant increase in MDA concentration (29.794 ± 1.509) compared to the control group (13.994 ± 0.772). This result indicates that VPA causes an increase in oxidative stress. The grape extract group alone had an MDA level of 13.936 ± 0.833 , close to the level of the control group, which confirms that the grape extract itself does not cause Oxidative damage.

Second: Results of the GSH (ug/mg) index. Glutathione: the alcoholic extract of grapes with VPA significantly increased GSH levels by 82.74 ± 2.558 compared to the VPA group, returning them to normal levels close to the control group. This result demonstrates the extract's capability to promote the defense system of the body's antioxidants. Meanwhile, the group receiving only the alcoholic extract of grapes showed a GSH level of 86.574 ± 0.7421 , close to the level of the control group. Conversely, GSH levels reduced substantially in the drug-only group (58.602 ± 2.214 compared to the control group (83.498 ± 2.662). This decrease confirms the presence of oxidative stress, as GSH was consumed in an attempt to combat it.

Third: Results of the SOD index (U/ml) Superoxide. The results of our current research displays that with the addition of grape alcoholic extract, SOD activity increased in the VPA + grape alcoholic extract group (16.1 ± 0.27655) compared to the VPA group, but it did not completely return to the control group level. This indicates that the grape extract helped improve the activity of this enzyme, while the results of the

grape alcoholic extract group alone showed that the SOD enzyme activity was (29.14 ± 0.584) , i.e., close to the control group. Moreover, the VPA group displays a remarkable decrease in SOD enzyme activity (13.906 ± 0.329) compared to the control group (28.774 ± 1.469) .

Table 3. shows the results of oxidative stress markers in hippocampal tissue.

Means \pm SE			Standard Groups
MDA (mmol/l)	GSH(μ g/mg)	SOD U/ml	
13.994 0.772 \pm B	83.498 \pm 2.662 A	28.774 \pm 1.469 A	Control(G1)
29.794 1.509 \pm A	58.602 \pm 2.214 B	13.906 \pm 0.329 B	VPA(G2)
13.288 1.056 \pm B	82.74 \pm 2.558 A	16.1 \pm 0.27655 B	VPA(G3) +Extract
13.936 0.833 \pm B	86.574 \pm 0.7421 A	29.14 \pm 0.584 A	Extract(G4)
3.244827	6.549095	2.456518	LSD
0.05	0.05	0.05	P-Value

(n = 5) per group. Different letters indicate significant differences vertically at the probability level of $P < 0.05$)

Additionally, when evaluating indicators of oxidative stress, our study's findings align with those of researchers (36), who reported a significant increase in the concentration of the GSH enzyme, accompanied by a notable decrease in the MDA marker. This was observed while investigating the neuroprotective role of grape hydroalcoholic extract against toxic substances and oxidative stress in the brain. Grape fruits contain numerous polyphenols with antioxidant properties, which have been shown to enhance synaptic plasticity between neurons and cognitive functions, including resveratrol and proanthocyanidins (37). Furthermore, the results from a study by researchers (38) support our findings, indicating that certain active compounds, such as procyanidins in grape seed extract, increased SOD concentrations while decreasing MDA levels. This effect was demonstrated in their study on ethanol-induced stress in rat hippocampal neurons. Our results are also consistent with those of researchers (39), who observed that treatment with valproate led to increased MDA concentrations and decreased SOD levels. This is due to VPA stimulating p21 gene expression during the techniques connected to oxidative stress, resulting in elevated ROS levels and an increase in lipid peroxidation products like MDA. Moreover, our findings align with those of researchers (40), who noted elevated MDA levels in the VPA-treated group compared to the negative control group. However, contrary to our study, they found SOD activity to be higher in the VPA group than in the negative control group. Additionally, our results are consistent with those of researchers (41), who treated 639 rodents with VPA, leading to increased MDA levels and decreased SOD and GSH levels. VPA is known to elevate oxidative stress and

disrupt neurodevelopment, causing delays in growth, cognitive impairment, autism spectrum disorder, and neural tube defects (42).

Conclusion

From our current study the following conclusions can be drawn: (The alcoholic extract of red grapes was effective in improving fetal growth and maintaining ideal weight, while valproate (VPA) showed significant negative effects on weight loss. The polyphenols present in the red grape extract showed significant improvements in behavior and social interaction. Unlike VPA, which reduces these aspects, the grape extract had a significant effect in reducing the values of harmful indicators of oxidative stress in the body.

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N/A

Conflict of Interest

The authors declare no conflict of interest.

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