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Effects of thymoquinone on valproic acid-induced oxidative stress in perinatal rat brain

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Timokinonun perinatal sıçan beyninde valproik asit indüklü oksidatif stres üzerine etkileri

Abstract: Thymoquinone (TQ), bioactive molecule of black cumin, has antioxidant and neuroprotective effects. TQ's hypoglecemic effect while applied prenatally is reported. This study is aimed to find the TQ dose with maximum antioxidant and minimum side effects in valproic acid (VPA) induced oxidative stress. Pregnant Wistar rats were injected i.p. with 400 mg/kg/ml of VPA on embryonic day 12.5 (E12.5). Repeated dose groups were injected i.p. from E11.5- E14.5; RC- repeated control: did not receive TQ, R1: 0.5 mg/kg/ml of TQ, R2: 2 mg/kg/ml of TQ, R3: 4 mg/kg/ml of TQ, R4: 8 mg/kg/ml of TQ. Single dose groups were injected i.p. on E12.5; SC- single control: did not receive TQ, S1: 8 mg/kg/ml of TQ, S2: 15 mg/kg/ml of TQ. Pups were sacrificed on postnatal day 7. Glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD) levels were measured via ELISA method. Prenatal VPA exposure decreased GSH and SOD levels in RC and SC compared to naïve group. R3 group showed improved GSH and SOD levels compared to RC. No significant difference in MDA levels was found between groups. Antioxidant effects of TQ on VPA induced oxidative stress has been showed in R3 group. This dose can be used to investigate TQ's effects on other parameters that are affected by prenatal VPA exposure.

Key words: Oxidative stress, thymoquinone, valproic acid, perinatal brain, rat

Özet: Timokinon (TQ), çörek otunun aktif maddesi, antioksidan ve nöroprotektif etkileri olan bir maddedir. Prenatal dönemde uygulandığında hipoglisemi etkisi bildirilmiştir. Bu çalışmanın amacı TK'nun perinatal sıçan beyninde Valproik Asit (VPA) indüklenmiş oksidatif streste kullanımı için maksimum antioksidan ve minimum yan etkiye sahip dozunun bulunmasıdır. Gebe Wistar sıçanlara 12,5. embriyonik günde (E12.5), i.p. 400mg/kg VPA enjeksiyonu uygulanmıştır. Tekrarlayan TK doz gruplarına (R) E11,5-E14,5 arası, Tek doz TK gruplarına (S) ise E12,5'de i.p. TK uygulanmıştır. RC: tekrarlayan kontrol, TQ yok; R1: 0.5 mg/kg/ml TK; R2: 2 mg/kg/ml TQ, R3: 4 mg/kg/ml TQ, R4: 8 mg/kg/ml TQ; SC- tek doz kontrol, TQ yok; S1: 8 mg/kg/ml TQ, S2: 15 mg/kg/ml TQ. Yavrular postnatal 7. günde sakrifiye edilerek total beyin dokularında ELISA yöntemiyle glutatyon (GSH), malondealdehit (MDA) ve süperoksit dismütaz (SOD) seviyeleri ölçülmüştür. Prenatal VPA uygulaması RC ve SC gruplarında naive gruba göre GSH ve SOD miktarlarını azaltmıştır. R3 grubunda kontrol grubuna göre artmış GSH ve SOD seviyeleri ölçülmüştür. Hiçbir grupta MDA seviyelerinde farklılık görülmemiştir. TK'nun VPA indüklü oksidatif stres üzerinde yan etki olmaksızın antioksidan etkisi R3 grubunda gösterilmiştir. Bu doz prenatal VPA asit maruziyetinin olumsuz olarak değiştirdiği davranış ve beyin morfolojisi gibi diğer parametrelerin incelenmesinde kullanılmak üzere önerilmektedir.

Anahtar Kelimeler: Oksidatif stres, timokinon, valproik asit, perinatal beyin, sıçan

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1. Introduction

Valproic acid (VPA) is an effective drug that is prescribed for epilepsy, migraine and bipolar disorder. VPA is accepted as an environmental risk factor in Autism Spectrum Disorders (ASD) aetiology and is used in experimental models for this cause (Schneider and Przewlocki, 2005). Rats with in utero exposure of VPA on embryonic day 12.5 (E12.5) are reported to show autism-like behaviours and oxidative stress (Schneider and Przewlocki, 2005). These effects are related to VPA's histone deacetylate inhibitor activity (HDACi) (Dufour-Rainfray et al., 2011) and destructive effects on embryonic folate mechanism (Wegner and Nau, 1992).

ASD are complex neurodevelopmental disorders with a symptomatic spectrum that is correlated with biochemical changes, such as redox imbalance and oxidative stress related mitochondrial disruptions (Kaur et al., 2014). Experimental models are widely used to understand aetiology (Schneider and Przewlocki, 2005) and to improve symptoms (Kang and Kim, 2015).

Oxidative stress is a result of imbalance in cellular pro-oxidant and anti-oxidant mechanisms which cause an increase in reactive oxygen and nitrogen species (ROS, RON). Even though oxidative stress' effects in ASD pathogenesis is unclear, it is known that developing brain is vulnerable to oxidative damage (McQuillen and Ferriero, 2004). Compared to other organs, the brain consumes 20% of the total oxygen and is highly dependent on oxidative metabolism, it has high unsaturated lipid content with a relatively low antioxidant, activity all of which results in vulnerability to oxidative stress (Dringen et al., 2000). Blood brain barrier is not fully develop after 6 months post-birth, enabling easy transmission of ROS and RON to the brain (Adinolfi, 1985). Besides metabolic disturbances reported in people with ASD (James et al., 2006), decreased methionine and glutathione (GSH) levels and DNA hypo-

methylation (Hishida and Nau, 1998), disrupted superoxide dismutase (SOD) and increased malondialdehyde (MDA) due to lipid peroxidation (Gao et al., 2016) are reported.

Thymoquinone (TQ) is the bioactive molecule of black cumin (Ahmad et al., 2015). It is traditionally used as a miracle herb for headache, fever, rheumatism and coughing in India, the Middle East and North Africa (Burits and Bucar, 2000). TQ is reported to have gastro-protective (Kanter et al., 2006), anti-diabetic (El-Ameen et al., 2015), anti- inflammatory (Chehl et al., 2009), anti-histaminic (El-Ameen et al. 2015), anti-oxidant (Solati et al., 2014) and anti-cancer (Attoub et al., 2013) properties in preclinical studies. It is also reported to modulate oxidative stress through an increase in GSH and SOD and decrease in MDA in experimental disease models (Ince et al., 2013; Fanoudi et al., 2019).

Prenatal VPA exposure induces disrupted antioxidant defence. Several antioxidant substances are being used during the induction period to prevent or decrease the effect of VPA (Gao et al., 2016; Bambini-Junior et al., 2014). Effects of TQ supplementation before and after prenatal VPA exposure on excitatory/inhibitory balance, oxidative stress, morphological and behavioural anomalies should be studied in offsprings with prenatal VPA exposure. However, consecutive administration of TQ is reported to have negative outcomes due to its hypoglycaemic properties (Hawsawi et al., 2001) which also have teratogenic effects in pregnancy (AbuKhader et al., 2013). With known antioxidant and neuroprotective effects of TQ, a study to explore the effective dose for VPA induced models is necessary. This study is designed to find the dose of TQ administration with most effective antioxidant activity and least hypoglycaemic effect. With suggested route of administration and dose of TQ, a follow up study will be conducted to research further behavioural, morphological and biochemical parameters affected by in utero exposure to VPA.

2. Materials and Method

2.1. Animals

Wistar albino rats (bred in Bursa Uludag University Experimental Animal Research Centre) were mated (2:1) and their offspring were tested in this study. Conception was confirmed by the presence of vaginal plug and spermatozoa in vaginal smear samples (E0.5). All animals were housed in standard laboratory conditions with a 12 h / 12 h light-dark cycle, controlled temperature (20-24 °C) and humidity (40 - 60%), and access to food and water ad libitum. The study was approved by Bursa Uludag University Animal Research Ethics Committee (date: 2020— 03/ 09).

2.2. Protocol for symptomatic modelling and supplementation

According to literature and previous works of the lab, single dose of 400 mg/kg/ ml i.p. injections of VPA (Depakin, lyophilize solution, Sanofi, Aventis, Paris, France) on E12.5 is enough to constitute ASD- like symptomatic model in rats (Kim et al., 2011). TQ (Bldpharm, Shanghai, China resolved in ethyl alcohol (EtOH) and diluted to respected doses with saline) was administered according to experimental group of the animal (Hawsawi et al., 2001). All pregnant rats were handled from E0.5 to E15.5, weighed regularly. After injections, food was removed from the cages and blood glucose levels were measured by a drop of blood from the tail veins after 2 hours to detect any possible hypogliceamia (AccuCheck Glucometer, Roche, Basel, Switzerland) and dams were observed for possible side effects.

2.3. Experimental groups

Group 1 (N; Naïve, n= 8): Animals that received no injections during pregnancy.

Group 2 (RC; Control for Repeated Dose, n= 8): VPA (400 mg/kg) on E12.5 + Solvent (EtOH+ Saline, kg/ml) between E11.5- E14.5.

Group 3 (SC; Control for Single Dose, n= 7): VPA (400 mg/kg) on E12.5 + Solvent (EtOH+ Saline, kg/ml) on E12.5.

Group 4 (R1; Repeated Dose, n= 10): VPA (400 mg/kg) on E12.5 + TQ (0.5 mg/kg) between E11.5- E14.5.

Group 5 (R2; Repeated Dose, n= 6): VPA (400 mg/kg) on E12.5 + TQ (2 mg/kg) between E11.5- E14.5.

Group 6 (R3; Repeated Dose, n= 9): VPA (400 mg/kg) on E12.5 + TQ (4 mg/kg) between E11.5- E14.5.

Group 7 (R4; Repeated Dose, n= 0): VPA (400 mg/kg) on E12.5 + TQ (8 mg/kg) between E11.5- E14.5.

Group 8 (S1; Single Dose, n= 7): VPA (400 mg/kg) on E12.5 + TQ (8 mg/kg) on E12.5.

Group 9 (S2; Single Dose, n= 0): VPA (400 mg/kg) on E12.5 + TQ (15 mg/kg) on E12.5.

2.4. Postnatal evaluations

Day of birth was accepted as postnatal day 0 (P0). Offspring were allowed to be raised by their mothers. On P7, pups were sacrificed and total brain tissues were collected for further analysis.

2.5. Oxidative stress markers

GSH and MDA content and SOD activity of total brain tissues were evaluated by ELISA method according to manufacturer's protocols (Bioassay Technology Laboratory Rat ELISA Kit, Cat. No: E1101Ra; E0156Ra; E0168Ra).

2.6. Statistical analysis

Normality of distribution was assessed using Shapiro Wilk test. Comparisons between groups were analysed using one-way ANOVA (α= 0.05 in all cases) followed by post-hoc Tukey test. All statistical analyses were performed on Sigma Plot V.2 and significance was accepted as $p< 0.05$. Plots display group averages with standard errors.

3. Results

Pregnant rats were observed after injections for any side effects and blood glucose levels were measured after 2 hours (Hawsawi et al., 2001). Glucose level lower than 2 mM is accepted as moderate hypoglycaemic, and none of the rats showed a decrease to this level (Won et al., 2012). Mothers from groups R4 and S2 showed abnormal levels of blood glucose (more than 11 mM) and death in upcoming hours/days after injections. Therefore, these groups were removed from the study.

Oxidative stress markers were measured from total brain tissues of pups on P7. Comparisons of repetitive and single dose groups were made for GSH and MDA contents and for SOD activity.

For GSH, there were no significant difference between N and RC groups. When N is compared to R1, R2 and R3, there was a significant decrease in R1 and R2 in terms of GSH content with no significant difference in R3 group ($p< 0.001$; $p< 0.001$). In comparison of RC and dose groups R1, R2 and R3, there was a significant decrease in GSH content for R1 and R2 and increase for R3 ($p= 0.031$; $p< 0.001$; $p= 0.020$) (Fig 1A). Dose groups are also compared in between and there found to be a significantly lower level of GSH content was found in R2 compared to R1 and higher level in R3 compared to both R1 and R2 ($p< 0.001$; $p< 0.001$; $p< 0.001$). For single dose comparisons, SC and S1 groups displayed significant decrease in levels of GSH content compared to N group, whereas there was no difference between SC and S1 ($p= 0.033$; $p= 0.002$) (Fig 1B).

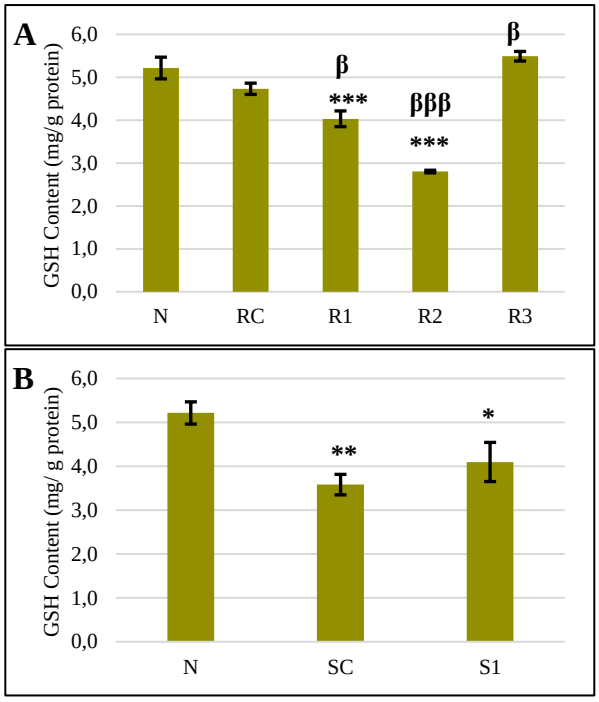

Figure 1. GSH content of total brain tissue (A) for repeated doses of TQ, (B) for single dose of TQ. Data are means ± SE of 6-9 offsprings in each group. (N: Naive group, RC; control group for repeated dose, SC; control group for single dose, R1; repeated doses of 0.5 mg/kg TQ, R2: repeated doses of 2 mg/kg TQ, R3: repeated doses of 4 mg/kg TQ, S1: single dose of 8 mg/kg TQ).

* $p< 0.05$ compared to naive group;

** $p< 0.01$ compared to naive group;

*** $p< 0.001$ compared to naive group;

β $p< 0.05$ compared to control group;

β β β $p<0.001$ compared to control group.

For MDA, there were no significant difference between N and RC groups. When N is compared to R1, R2 and R3, there was no significant difference between either group. In comparison of RC and dose groups, there was not any differences to be shown (Fig 2A). Dose groups are also compared in between and there found to be a significantly higher level of MDA content in R3 compared to R1 ($p= 0.003$) but no differences were found between R1 and R2; R2 and R3. For single dose comparisons, there was no significant difference between N, SC and S1 groups in levels of MDA content (Fig 2B).

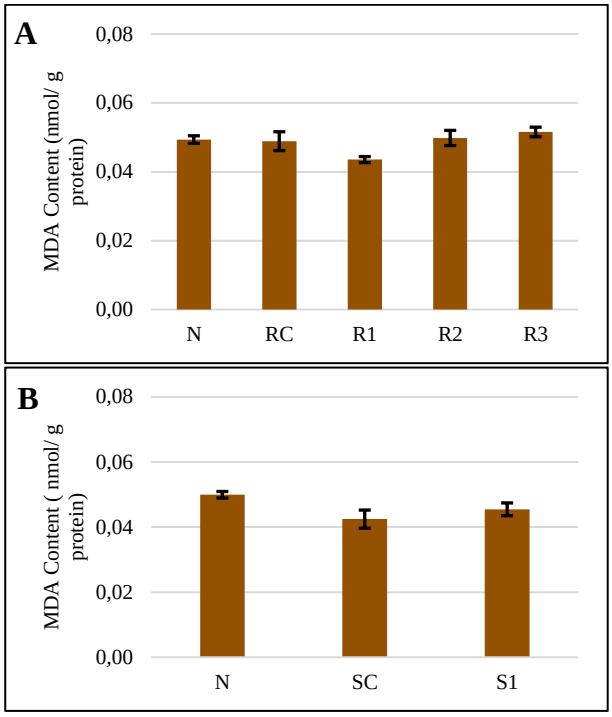

Figure 2. MDA content of total brain tissue (A) for repeated doses of TQ, (B) for single dose of TQ. Data are means ± SE of 6-9 offsprings in each group. (N: Naive group, RC; control group for repeated dose, SC; control group for single dose, R1; repeated doses of 0.5 mg/kg TQ, R2: repeated doses of 2 mg/kg TQ, R3: repeated doses of 4 mg/kg TQ, R5: single dose of 8 mg/kg TQ).

For SOD, RC showed a significant decrease compared to N ($p< 0.001$). When N is compared to R1, R2 and R3, there was a significant decrease in R1 and R3 and no difference in R2 ($p< 0.001$; $p< 0.001$). In comparison of RC and dose groups, R1, R2 and R3 groups displayed increased SOD activity compared to RC group ($p< 0.001$; $p< 0.001$; $p< 0.001$) (Fig 3A). Dose groups are also compared in between and there found to be significantly higher levels of SOD activity in R2 and R3 compared to R1 ($p< 0.001$; $p< 0.001$), and lower level in R3 compared to R2 ($p= 0.002$). For single dose comparisons, SC and S1 groups displayed significant decrease in levels of SOD activity compared to N group ($p< 0.001$; $p< 0.001$), whereas S1 group had significantly higher level compared to SC ($p< 0.001$) (Fig 3B).

4. Discussions

The developing brain is highly vulnerable to oxidative stress with partially developed blood brain barrier, higher iron concentrations and lower antioxidant defence (Panfoli et al., 2018). VPA disrupts pro-oxidant/ anti-oxidant balance due to being a HDACi (Dufour-Rainfray et al., 2011) and has a destructive effect on embryonic folate metabolism (Wegner and Nau, 1992).

In utero exposure to VPA is used to model ASD like symptoms (Schneider and Przewlocki, 2005) with its effects on disrupted excitatory/inhibitory balance (El-Ansary and Al-Ayadhi, 2014), neuronal migration (Schmitz and Rezaie, 2008) and oxidative stress (Gao et al., 2016) which are also included in ASD pathogenesis. Supplementary substances with antioxidant effects such as

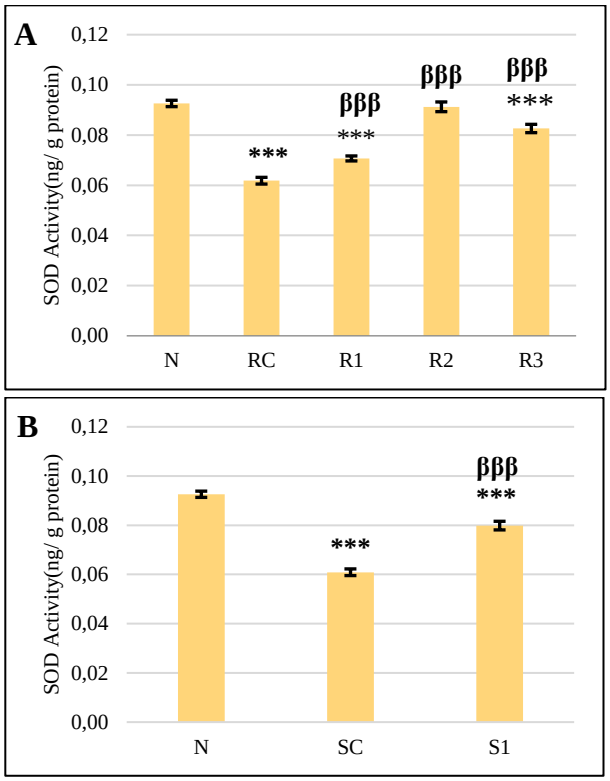

Figure 3. SOD activity of total brain tissue (A) for repeated doses of TQ, (B) for single dose of TQ. Data are means ± SE of 6-9 offsprings in each group. (N: Naive group, RC; control group for repeated dose, SC; control group for single dose, R1; repeated doses of 0.5 mg/kg TQ, R2: repeated doses of 2 mg/kg TQ, R3: repeated doses of 4 mg/kg TQ, 5: single dose of 8 mg/kg TQ).
*** p< 0.001 compared to naive group;
β β β p<0.001 compared to control group.

fingolimod (Gao et al., 2016), resveratrol (Bambini-Junior et al., 2014), curcumin (Mirza and Sharma, 2019) and S-adenosyl methionine (Al-Askar et al., 2017) are reported to regulate disrupted oxidative status and improve behavioural symptoms in VPA induced models of ASD. TQ is used in this study as it has antioxidant (Solati et al., 2014) and neuroprotective effects reported in previous studies (Ornoy et al., 2020).

Methionine is the precursor of S-adenosylmethionine, a molecule used in DNA methylation, and its concentration can be negatively affected by decreased folate content (Banerjee and Matthews, 1990). Methionine is also the precursor of major intracellular antioxidant- GSH (Finkelstein, 1998). VPA allows epigenetic changes and oxidative imbalance by hyper-acetylation and disrupted folate metabolism. Consistent with these findings, in utero exposure to VPA is reported to cause decreased methionine (Alonso-Aperte et al., 1999) and GSH content with DNA hypo-methylation (Hishida and Nau, 1998). Our study showed a decreased GSH content in VPA groups compared to naïve groups, and TQ supplementation managed to level up this decrease in R3 group. Hegazy et al. (2015) also reported decreased GSH levels in VPA model. VPA acts as an environmental factor that predisposes the organism to oxidative stress by disrupting redox/methylation balance, resulting in a decline to capacity of synchronisation in neural systems (Deth et al., 2008). Cabungcal et al. (2007), suggested that impaired GSH metabolism during development results in cognitive decline in juvenile and adult rats relative to schizophrenia.

VPA has been reported to cause an imbalance in oxidative status. Chaudhary and Parvez (2012) reported a decreased SOD activity in brain tissues of VPA exposed groups whereas Bambini-Junior et al. (2011) reported no SOD level differences in liver tissue of adult rats with in utero VPA exposure. Gao et al. (2016) used fingolimod to attenuate effects of VPA and reported increased SOD activity in supplemented groups. In our study, we showed a decrease in SOD activity in VPA-only group and increase in TQ groups for all doses compared to VPA groups, consistent with literature.

Lipid peroxidation can be measured with TBARS levels or MDA content. Chaudhary and Parvez (2012) reported increased TBARS levels and Gao et al. (2016) reported increased MDA content as a result of increased lipid peroxidation after prenatal VPA exposure. Fingolimod (Gao et al., 2016) is suggested to decrease levels of increased MDA due to VPA induction. Ornoy et al. (2020), however, reported no significant differences in levels of MDA content, as well as Bambini-Junior et al. (2011) for TBARS levels. In our study, there was no significant difference in MDA contents for either group.

TQ stimulates glucose uptake and use in tissues and cells leading to decrease in blood glucose level (Fararh et al., 2010). In our study, mothers that received 8 mg/kg for 4 days and 15 mg/kg for a day were removed from the study due to their losses in following days. During the injection days, chorionic somatomammotropin (CS) is being secreted at an optimum level (Tonkowicz et al., 1983). With CS levels high in blood, an increase in insulin levels and lipolysis would occur. All in all, hypoglycaemia caused by TQ and hyperlipidaemia caused by CS could result in acute pancreatitis (Gianfrate and Ferraris, 1998). Pancreatitis can also be caused by VPA. Acute pancreatitis is reported as a side effect of VPA in humans and it is also supported by experimental studies (Eisses et al., 2015). Role of TQ on HDACi, how and in what concentration it can contribute to VPA induced models requires further research.

In this study, several doses were used to reach the effective dose for maximum antioxidant capacity with minimum hypoglycaemic effect of TQ. Doses were determined in respect to literature. Hawsawi et al. (2001) used both *Nigella sativa* seeds and TQ in several doses to study effects on blood glucose in non- pregnant rats. They used *i.p*. injections of 0.5, 1, 2, 4, 6 and 8 mg/kg of TQ for repeated days of 1, 4, 7, 10 and 14 and reported that doses from 0.5 to 6 mg/kg were tolerable and showed no side effects. Animals injected with dose of 8 mg/kg, however, died after a week with signs of peritonitis. Al-Enazi (2007) administered TQ orally in a dose 10 mg/kg to pregnant rats from E1.5 to E19.5 and reported balanced oxidative status. AbuKhader et al. (2013) tested acute effects of TQ on pregnant rats with doses of 15, 35 and 50 mg/kg on either E11.5 (embryonic development- organogenesis) or E14.5 (early fetal development). They reported total fetal resorption in rats that received 35 or 50 mg/kg of TQ on E11.5. 15 mg/kg of TQ, however, showed no adverse effects on both E11.5 and E14.5 (AbuKhader et al., 2013). Reported LD_{50} for TQ is 57.5 mg/kg in non-pregnant rats (Kim et al., 2011). In the light of these studies, we used 0.5, 2, 4 and 8 mg/kg of i.p. TQ to be given between E11.5-E14.5, and 8 and 15 mg/kg of i.p. TQ to be given only on E12.5 to assess glycaemic state and VPA induced oxidative stress.

In conclusion antioxidant effects of TQ after VPA exposure have been studied. TQ is reported to induce hypoglycaemia which is a known teratogenic factor. Therefore, it was necessary to search for a dose with minimum hypoglycaemic and maximum antioxidant effect. Our results show that 4 mg/kg/ml of TQ given for 4 days between E11.5- E14.5 display regular blood sugar levels with highest antioxidant activity compared to the control and other dose groups. We propose that this dose can be used for further research to investigate TQ's effects on ASD-like behaviour and other parameters that are induced by in utero exposure to VPA.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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