Bulgarian Chemical Communications, Volume 52, Special Issue D (pp. 13-17) 2020

Effects of pioglitazone on the hippocampal oxidative status of rats with prenatal valproic acid-induced autistic-like symptoms

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Received February 28, 2020; Accepted May 12, 2020

Valproic acid (VPA) is known as a potent teratogen for offspring of pregnant human females and is commonly used in animal models to environmental triggering of various behaviors, biochemical profiles and neuroanatomical features similar to those of patients with autistic spectrum disorder (ASD). The underlying molecular mechanisms often involve neuroinflammation and enhanced levels of oxidative stress. The hippocampus plays a key role in cognition, mood regulation, learning and memory functions, and is particularly vulnerable to oxidative damage. The aim of the current study was to investigate the effect of pioglitazone on the hippocampal oxidative status of rats with prenatal VPA-induced autistic-like symptoms. The male offspring of pregnant Wistar rats, treated or not with valproic acid during the pregnancy period, were separated from their mothers on the 23rd postnatal day and were divided into four groups: 1. Control; 2. Control, treated with pioglitazone; 3. With experimental autism; 4. With experimental autism, treated with pioglitazone. Our results demonstrated that the prenatal application of VPA is associated with alteration of the hippocampal reducing ability in the experimental groups. Treatment with pioglitazone demonstrated modulating activity on the oxidative status and beneficial effect on the proinflammatory parameters in this model of VPA-induced autistic-like features and its role may be suggested in the complex therapy of ASD.

Keywords: antioxidant capacity; valproic acid; autistic-like features; pioglitazone

INTRODUCTION

Autism spectrum disorder (ASD) is a complex and pervasive neurodevelopmental disorder, diagnosed by the age of three, upon clinical presentation of impaired social-communication skills and stereotypical, repetitive behaviors. In addition, most patients with ASD have attention deficit and cognitive, and intellectual challenges. Although genetic, environmental, inflammatory and metabolic conditions appear to interact as risk factors, the exact etiology of ASD remains unknown [1, 2].

Valproic acid (VPA) is an anti-epileptic drug, known as a potent teratogen for offspring of pregnant human females and is commonly used in animal models for environmental triggering of various behaviors, biochemical profile and neuroanatomical features, similar to patients with ASD [3, 4]. The offspring of VPA-treated rats is known to display disruption in their brain neuroplasticity (loss of neuronal cells, neuronal disorganization and structural connectivity alteration) [5, 6], as well as signs of neuroinflammation like reactive gliosis, elevation of inflammatory cytokines and altered immune responses [7, 8]

Oxidative stress is considered to play an important role in the etiopathogenesis of ASD [9, 10]. Studies reported participation of both central and peripheral markers of oxidative status disruption in the underlying molecular mechanisms of neuropsychiatric disorders [11, 12]. Also, there have been shown abnormal levels of detoxifying agents and antioxidants in ASD [9]. Since oxidative stress has been previously demonstrated as crucial in the neurodegeneration during development, it has been suggested as a possible molecular pathway by which VPA exerts teratogenicity [13]. Recent study has linked prenatal VPA exposure to impairment of cerebellum functions and dysregulation of motor, cognitive and social behavior in rats [14]. In addition, the hippocampus plays a key role in cognition and mood regulation, and is particularly vulnerable to oxidative damage [15].

Pioglitazone is an agonist of the peroxisome proliferator activated receptor gamma (PPAR-γ) and was initially designed as an anti-diabetic drug due to its insulin-sensitizing effect through changing expression of genes that influence carbohydrate and lipid metabolism [16].

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Consequently, PPAR-γ were found to be widely distributed in the central nervous system - in neurons, microglia, astrocytes, adipocytes, macrophages, etc. [17]. Pioglitazone has also been proved to possess anti-inflammatory and anti-proliferative effects [16, 18]. Strong evidence was demonstrated of pioglitazone neuroprotective activity against inflammation and oxidative stress in CNS and neuropsychiatric disorders [19-21]. Clinical studies have suggested the therapeutic potential of PPAR-γ agonists in ASD [22].

The aim of this study was to investigate the effect of pioglitazone on the hippocampal oxidative status of rats with prenatal VPA-induced autistic-like symptoms.

MATERIALS AND METHODS

The experiments were carried out in accordance with the Bulgarian regulations on animal welfare, in conformance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the approval of Bulgarian Food Safety Agency (BFSA license №188/13.11.2017).

Animals and VPA model

Pregnant Wistar rats (220±30 g body weight) were maintained under standard laboratory conditions (12 h light-dark cycle, temperature 20±0,5°C, humidity 65±1%). On gestational day 12.5 the rats were intraperitoneally treated with either physiological solution - control conditions or valproic acid (VPA) (600 mg/kg) to develop the experimental model of autism. The male offspring rats were separated from their mothers on the 23rd postnatal day and according to the treatment that followed they were divided into 4 groups (n=5): 1. Control (physiological solution, p.o.); 2. Pioglitazone "Pio" (pioglitazone 2 mg/kg, p.o.); 3. Experimental VPA model "VPA" (physiological solution, p.o.); 4. Experimental VPA model + pioglitazone "VPA+Pio" (pioglitazone 2 mg/kg, p.o.).

Substances and reagents

Sodium valproate, pioglitazone hydrochloride and rat IL-6 ELISA kit were of finest grade and were obtained from Sigma-Aldrich.

Pioglitazone was administered to the animals for 21 days (from 23rd to 43rd postnatal day). At the end of this period the animals were sacrificed and their blood and brains were collected. Blood sera were extracted and hippocampi were dissected for assessment of biochemical parameters.

Reagents for antioxidant capacity determination

Ferric chloride hexahydrate, 2,4,6-tri-(2-pyridil)-s-triazine (TPTZ), 2,2'-azinobis (3-ethylbenzotiazolin-6-sulfonic acid diammonium salt (ABTS) and potassium persulfate were obtained from Sigma-Aldrich. All other used chemicals were of reagent grade and were used without further purification.

Assessment of antioxidant capacity

For this part of the experimental work we used the ABTS test and the Ferric reducing ability assay. The former is based on the ability of the antioxidants in the studied samples to decrease the concentration of the preformed stable ABTS radical (estimating a decrease of the absorbance measured at 734 nm due to the capability of the sample antioxidant to eliminate the stable ABTS radical) and the other on the reduction of the ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) complex, determined as increased absorbance at 593 nm. Both methods are characterized by operational simplicity and possibility to be automated.

ABTS assay

Experiments were done according to Re *et al.* [23], In order to obtain the stock ABTS radical solution 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and potassium persulfate were mixed in buffered distilled water to a concentration of 7mM and 2.45 mM, respectively. The obtained mixture was left overnight in the dark to form the stable ABTS cation radical (approximately 16 h before use). A working ABTS solution was prepared by diluting the stock until we obtained a solution with absorbance of 0.700 ± 0.005 at 734 nm. The ten times diluted brain homogenate supernatants were reacted with the ABTS radical working solution in a 96-well plate. Two groups of samples were prepared: controls where hippocampal homogenate was omitted and the samples contained the tested substance. The absorbance of the samples was measured using Epoch 2, BioTek reader at 734 nm after 60 min incubation in the dark. The radical scavenging activity of the tested samples was calculated on the base of the obtained results, using the following formula:

$$RSA\ \% = \left(\frac{A_{control} - A_{\ sample}}{A_{control}}\right).\ 100.$$

FRAP assay

The reducing ability of the biological samples was estimated by the Ferric reducing antioxidant power (FRAP) assay of Benzie and Strain [24]. The

FRAP reagent was prepared by mixing 25 ml of 300 mmol/L acetate buffer (pH 3,6), 2,5 ml of 10 mmol/L TPTZ (in 40 mM HCl) and 2,5 ml of 20 mmol/L $FeCl_3.6H_2O$ (proportion of 10:1:1) at 37°C. The homogenate supernatants were allowed to react with the FRAP solution in the dark for 30 min. The absorbance of the obtained blue colored product (ferrous tripyridyltriazine complex) was checked at 593 nm. Reagent blank reading was also taken at 593 nm.

Assessment of IL-6 levels

The estimation of serum interleukin-6 (IL-6) levels was performed by following the Sigma-Aldrich® Rat ELISA kit protocol: sandwich *in-vitro* enzyme-linked immunosorbent assay (ELISA) for the quantitative estimation of rat serum IL-6 on a 96-well plate reader at 450 nm. The values were expressed in pg/ml.

RESULTS

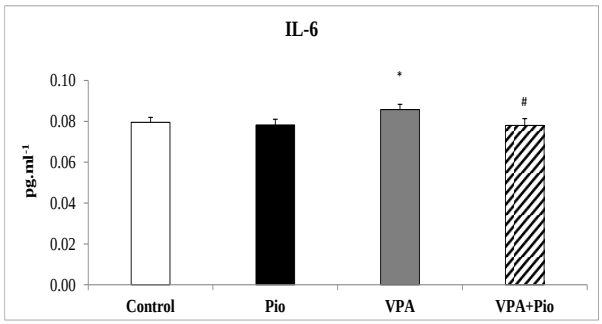

Figure 1. Effect of pioglitazone on serum IL-6 levels in experimental model of autistic like features. * $p<0.05$ *vs* control group; # $p<0.05$ *vs* VPA group.

Figure 1 demonstrates that the serum IL-6 levels in the "VPA" group are higher: 0.086±0.003 $pg.ml^{-1}$ in comparison to control group: 0.080±0.002 $pg.ml^{-1}$, $p<0.05$. This result shows that the VPA model induces elevation of pro-inflammatory cytokine and suggests peripheral inflammatory changes.

Administration of pioglitazone did not change serum level of IL-6 in the control condition but decreased it in the "VPA+Pio" group to a level comparable to the baseline: 0.078±0.003 $pg.ml^{-1}$, $p<0.05$, and indicated an anti-inflammatory effect of pioglitazone.

The results from the total antioxidant capacity (TAC) estimation assays are presented on Figure 2 for the FRAP assay and Figure 3 for the ABTS assay.

The FRAP assay established increased absorbance values from 0.424±0.023 to 0.502±0.032, $p<0,05$ in the samples containing hippocampal homogenate of the "VPA" group of animals in comparison to the control group. Application of pioglitazone attenuated the effect of VPA pretreatment on the absorbance values: 0.460±0.020.

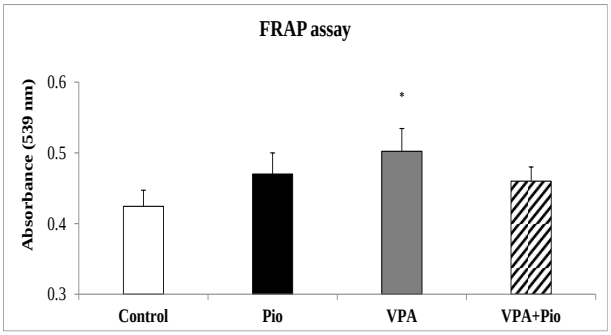

Figure 2. Absorbance values determined using FRAP assay in rat hippocampus homogenate. Each column represents the average ± SEM of five values corresponding to five animals, each value being the mean of triplicate assays. * $p<0.05$ *vs* control group.

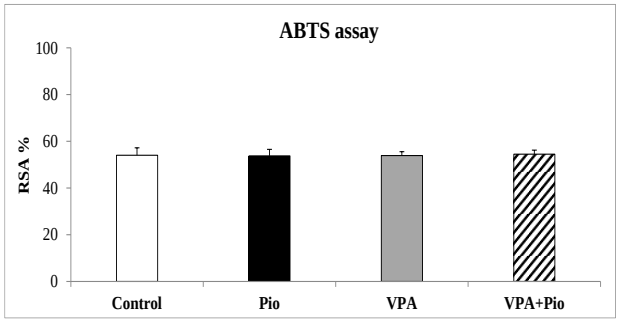

Figure 3. Radical scavenging activity (RSA) of hippocampal homogenates, estimated through the ABTS assay. Each column represents the average ± SEM of five values corresponding to five animals, each value being the mean of triplicate assays.

The ABTS assay did not demonstrate statistically significant changes in the absorbance values of the hippocampal supernatants of the studied groups. The calculated radical scavenging activities in the investigated groups were in the range from 53.73 to 54.50%.

DISCUSSION

Our present investigation demonstrates that prenatal VPA application induced increased serum IL-6 levels in the experimental animals, which suggests inflammatory changes. PPAR-γ agonist pioglitazone did not change the IL-6 levels in the animals that were not treated prenatally with VPA but decreased the IL-6 levels in the treated ones, thus proposing anti-inflammatory properties of pioglitazone in the model animals (Fig. 1).

This is in agreement with numerous studies for long-term neuroanatomical and biochemical abnormalities in the offspring after administration of VPA during pregnancy [25]. VPA exposure within the critical period for rodent brain organogenesis has

led to disrupted brain plasticity and neuroinflammatory changes, along with various behaviors, similar to autistic patients [26]. In addition, chronic neuroinflammation was shown to be present in ASD brains, including activation of microglia, increased levels of pro-inflammatory cytokines and other systemic inflammatory biomakers [27].

Recent clinical studies presented beneficial effects of pioglitazone on the stereotype behaviors and social interaction of autistic children [22, 28]. Furthermore, Kirsten *et al.* suggested that pioglitazone might eliminate autistic-like behavior through inhibition of IL-6 cascade in immune activation-based model of autism [21]. This finding was subsequently supported by experimental data for positive impact of pioglitazone on immune disruption in prenatal VPA-induced autistic features [26], and was confirmed by our present results.

Brain oxidative stress has been linked to autistic-like features in VPA-prenatal rodent models [29]. Oxidative status imbalance was associated with significantly elevated levels of oxidative stress markers of lipid peroxidation [30], as well as compromised antioxidant status - abnormal glutathione redox status and methionine cycle, and decreased activity of superoxide dismutase and catalase [31]. Oxidative stress due to excitotoxicity and deficiency of endogenic antioxidants was linked to abnormal brain development in cerebellum, frontal lobe, dorsal prefrontal cortex, orbitofrontal cortex, etc., that are key regions for social interaction, cognitive development, behavioral and communication functions, disrupted in ASD.

In consent with the abovementioned data, our results demonstrated imbalance in the hippocampal oxidative status, particularly the data from the FRAP assay concerning the rats prenatally treated with VPA. Various methods have been cited in the literature for estimation of TAC in biological samples and different approaches showed diverse values which many authors claimed to be because biological sample antioxidants comprised different reactivity against the indicators, used in the systems. The results from the present ABTS assay did not demonstrate statistically significant changes between the absorbance values of the hippocampal supernatants (Fig. 3). However, the FRAP assay established considerable differences between the hippocampal homogenates of the control and the "VPA" group of animals. Interestingly, increased ferric reducing activity in the supernatants of the VPA model rats was observed. Furthermore, application of pioglitazone diminished the effect of the VPA pretreatment on the redox imbalance in the hippocampal homogenates (Fig. 2). Several academic reports have also demonstrated contradictory results about the VPA impact on the oxidative status in animal models, as well as about antioxidant properties of some substances on the TAC of biological samples. There are no data for the hippocampal TAC, studied through a FRAP method in the present experimental model. On the other hand, there is inconclusive clinical and experimental evidence for the modulating activity of valproic acid on the endogenous antioxidant capacity [32].

One possible explanation of our data inconsistency is that the FRAP assay might be more sensitive at high altitude to the changes of the different types of antioxidants presented in the tested samples compared to the ABTS method. Previous investigation has established correlation between the results obtained in the ABTS system and the levels of endogenous glutathione whereas the measured activity in the FRAP assay was mainly associated with the levels of ascorbic acid, uric acid and α-tocoferol [33].

Moreover, the FRAP system is more biologically plausible. Iron is the most abundant metal in the body, and within the brain it shows an uneven distribution. Iron is essential for fundamental processes associated with the normal physiological brain functions like oxygen transportation, neurotransmitter synthesis and metabolism of myelin production. It is a well-known fact that brain tissue is highly vulnerable to oxidative stress. Reactive oxygen species (ROS) mediate iron toxicity *via* the iron-catalyzed Haber-Weiss reaction, and are known to interfere with cytochrome c- and caspase-3-dependent apoptotic pathways and subsequent neuronal damage.

Taken together, the literature data and our results suggest that treatment with pioglitazone of rats in an experimental model of autistic-like features reduced inflammatory changes *via* PPAR-gamma anti-inflammatory potential. Moreover, pioglitazone displayed modulating effects on the total antioxidant capacity and on the redox balance determined *via* the presented assays in this prenatal VPA-induced experimental model.

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