Prenatal exposure to oxcarbazepine increases hippocampal apoptosis in rat offspring

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ABSTRACT

This study assessed apoptosis in the offspring of rats exposed to oxcarbazepine (OXC) from day 7 to 15 of gestation. Three groups of pregnant Wistar rats were used: 1) Control, treated with saline solution; 2) treated with 100 mg/kg OXC; 3) treated with 100 mg/kg of carbamazepine (CBZ, as a positive control for apoptosis); the route of administration was intragastric. Apoptosis was detected at three postnatal ages using the TUNEL technique in the CA1, and CA3 regions of the hippocampus and in the dentate gyrus (DG); neurogenesis was assessed in the DG using an antibody against doublecortin. The litter characteristics were recorded. OXC increased apoptosis in all regions (p < 0.01) at the three ages evaluated. Lamination disruption occurred in CA1 and CA3 due to the neuron absence and to ectopic neurons; there were also malformations in the dorsal lamina of the DG in 38% and 25% of the pups born from rats treated with OXC and CBZ respectively. CBZ also increased apoptosis. No clear effect on neurogenesis in the DG was observed. The size of the litter was smaller (p < 0.01) in the experimental groups. Nineteen-day OXC fetuses had low weight (p < 0.01), but 21 and 30 postnatal days old CBZ and OXC pups were overweight (p < 0.01). The results demonstrate that OXC administered during gestation is pro-apoptotic, alters the cytoarchitecture of the hippocampus, reduces litter size, and probably influences postnatal weight. We provide evidence of the proapoptotic effect of CBZ when administered early in gestation.

1. Introduction

Epilepsy is the most common chronic neurological disorder affecting people of all ages. Approximately 50 million people are affected by this disease worldwide (World Health Organization, 2014), 25% of whom are women of childbearing age (Fricke-Galindo et al., 2015; Bruno et al., 2013; Ngugi et al., 2010). It has been estimated that one in every 200 pregnant women has epilepsy, and that the frequency of seizures increases by 30% during the first trimester of gestation (Arteaga-Vazquez et al., 2012; Adab, 2006). Although most patients with epilepsy have normal pregnancies, deliveries and offspring (Martinez-Ferri et al., 2016; Motamedi and Meador, 2006; Meador et al., 2006, 2007), exposure to some antiepileptic drugs (AEDs) has been associated with congenital malformations in offspring, psychomotor retardation, language impairment, hyperactivity, and long-term cognitive impairments of various degrees (Pennell, 2016; Gedzelman and Meador, 2012; Perucca, 2005; Wide et al., 2002). The main hypothesis employed to explain the mechanism behind behavioral/cognitive dysfunction involves AED-induced apoptosis and altered synaptogenesis (Velez-Ruiz and Meador, 2015; Turski and Ikonomidou, 2012; Olney et al., 2002a).

Oxcarbazepine (OXC) is a second-generation anticonvulsant drug analogous to Carbamazepine (CBZ), with similar efficacy. Carbamazepine (CBZ) is an older AEDs, widely prescribed worldwide; it is among the four most commonly used AEDs for treating pregnant epileptic women (Åberg et al., 2013), even though it is considered a human teratogen that can cross the placenta and accumulate in fetal tissues (Bath and Scharfman, 2013; Nie et al., 2016; Ahmed and EL-
Gareib, 2017). Furthermore, there are many inconclusive studies about the safety of using CBZ during pregnancy (Manna et al., 2017; Forcelli et al., 2011).

OXC can be used to replace CBZ, even abruptly, replacing all doses (Tecoma, 1999; Shorvon, 2000). OXC is used to treat partial seizures; although its safety has not been sufficiently established, it is used during pregnancy (Martinez-Ferri et al., 2016). It has been associated with congenital cardiac and renal malformations, as well as dental agenesis in children exposed to it during gestation (de Jong et al., 2016; Jacobsen et al., 2014; Rolnitsky et al., 2013). Some indications suggest that OXC causes important congenital malformations, but the effects of exposure to OXC during pregnancy on the offspring’s brain, and its possible effects during the postnatal period, especially with respect to cognitive dysfunction, are still unknown. In experimental epilepsy studies in adult rats, the administration of OXC significantly reduced the onset time of kainic acid-induced seizures; it did not exert a neuroprotective effect and did not prevent hippocampal neurons from dying (Gonzalez-Maciel et al., 2000). The neuronal death induced by the administration of OXC alone was similar to that caused by the administration of kainic acid (Ayala-Guerrero et al., 2008). Since OXC, like other antiepileptic drugs, can cross the placenta (Myllynen et al., 2001), we hypothesized that exposure to OXC during gestation increases apoptosis in the offspring. The present study aimed to evaluate the effect of administering OXC during gestation on hippocampal apoptosis in the offspring of rats. We also assessed the presence of neurogenesis, the cytoarchitecture of the hippocampus and the physical characteristics of the pups.

2. Materials and methods

2.1. Experimental animals

All animal procedures were approved by the ethics committee of the National Institute of Pediatrics in accordance with the provisions of the National Institute of Health (NOM-062-ZOO, 1999) and Olff et al. (1993). We used the minimum number of animals needed according to the bioethical and statistical criteria provided by Festing (1994).

Thirty-five Wistar rats of reproductive age (80–90 days old) were obtained from the biotherium of the National Institute of Paediatrics. The animals were housed in plexiglass boxes under standard vivarium conditions: 12/12 light/dark cycle, 40% humidity, and controlled temperature (23 ± 3 °C), with free access to food and water. To obtain offspring, of the 35, a total of 30 pregnant rats were randomly divided into three experimental groups of 10 animals each: Control group, OXC group and CBZ group. For breeding, one male rat was placed in a cage containing 3 or 4 females for no more than 7 days. Daily vaginal smears were taken to determine the onset of gestation; the presence of sperm was considered as day zero. Each pregnant rat was placed in a cage where it was kept alone. The body weight was monitored during the entire pregnancy period.

2.2. Experimental procedure and dosing

A total of 30 pregnant rats were randomly divided into three groups of 10 animals each. The rats in the control group were given saline solution at 0.9% (0.5 ml/day); the OXC group was treated with 100 mg/kg/day of oxcarbazepine (Trileptal 60 mg/1 ml solution, Novartis); the CBZ group was given a dose of 100 mg/kg/day of carbamazepine (Tegretol 100 mg/5 ml solution, Novartis). The doses used for OXC and CBZ groups were adjusted according to the weight of the rats, which were weighted weekly. The administration route for the three groups was intragastric. The treatment began at day 7 and ended at day 15 of gestation before and during the neurogenesis period (Ikonomidou and Turski, 2010; Ikonomidou et al., 2007). In the present work, we used CBZ as a positive control for apoptosis and to obtain information about apoptosis in the hippocampus. It is known that CBZ induces apoptosis at doses greater than 50 mg/kg (Kaushal et al., 2016; Åberg et al., 2013; Kim et al., 2007). Apoptosis induced by CBZ has been demonstrated in cultures of hippocampal and cerebellar cells (Gao and Chuang, 1992; Gao et al., 1995; Pierna et al., 1998; Ambrósio et al., 2000; Ahmed and El-Gareib, 2017; Manna et al., 2017), and in vivo in postnatal animals (Pierna et al., 1998), but not as a result of exposure during pregnancy.

2.2.1. Litter characteristics

Since rats turn to cannibalism when pups are born with defects, the pups were extracted through caesarean section. On day 19 of gestation, control and experimental pregnant rats were anesthetized (3/10 in the control group and 4/10 in the experimental groups) and the fetuses were removed. The days of gestation and the weight of the body and brain of each pup were recorded at birth, either through cesarean section at day 19 of gestation or carried to term, of the six pregnant females of each group, at birth and at 14, 21 and 30 days of postnatal age. The results are shown in the tables.

2.2.2. Tissue analysis

We examined tissue samples from the offspring of six rats of each group. Each rat gave birth to an average of 10 pups, so there was an average of 60 ± 3 pups per group. The assessment was performed when the postnatal age of the pups was 14, 21 and 30 days. Eight male pups were randomly chosen to assess apoptosis, neurogenesis and the cytoarchitecture of the dorsal hippocampus at each age/group (Graphic Abstract). It was recently proposed that antiepileptics administered during pregnancy might act as fetal immune-neuroendocrine disruptors producing complex and mosaic interactions during the prenatal period (Ahmed and El-Gareib, 2017). Using only males eliminates the sex variable in the offspring. At the ages indicated above, the pups were deeply anaesthetized with sodium pentobarbital (55 mg/kg of body weight). They were then transcardially perfused with a cold solution of phosphate buffered saline (PBS), 0.1 M pH 7.4, for 3 min, followed by a cold fixative solution containing paraformaldehyde 4% dissolved in PBS. The brains were removed and post-fixed in the same fixative for 24 h. Afterwards, the brains were embedded in paraffin, and 5 μm thick coronal sections were obtained with a microtome and collected on charged slides. Serial sections of the dorsal hippocampus (1800 μm) were obtained in rostral-dorsal direction from the bregma (~2.5 to 3.8 ± 0.15 mm), following the atlas of Paxinos and Watson (1998), (Fig. 1). After being deparaffinized with xylene, the sections were rehydrated with ethanol at graded concentrations of 100–70% (v/v), followed by washing with water. Four sections per animal/age/group were randomly selected for each technique (histology, apoptosis and neurogenesis). Each section was separated from the others by a space of 50 μm to avoid of counting the same cells (samples of a total of 1800 μm).

2.3. Histology

For histological examination, the sections were stained with Cresyl Violet (0.1%) (Prophet et al., 1992), dehydrated with alcohol, cleared with xylol and mounted with Entellan resin (Merck, Darmstadt, Germany).

2.4. Apoptosis

To assess cell death, histological sections were processed using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to label apoptotic cells (In Situ Cell Death Detection Kit, Roche, Mannheim, Germany) according to the manufacturer’s protocol (Umemura et al., 1996; Negoescu et al., 1998). Deparaffinized sections were rehydrated and then in cubated with proteinase K (100 μg/mL), rinsed, incubated in H2O2 (3%), permeabilized with Triton X-100 (0.5%), rinsed again, and incubated in the TUNEL reaction mixture. The

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sections were then rinsed and visualized using a Converter-AP (alkaline phosphatase) with 3, 3′-diaminobenzidine (0.03%) (DAB). Nuclear red (DAKO, Glostrup, Denmark) was used as a counterstain, and the sections were mounted with Entellan resin.

2.5. Neurogenesis

To assess neurogenesis, we used an anti-doublecortin (DCX) antibody. DCX has been employed as a reliable marker for new neurons in regions such as the dentate gyrus, where persistent neurogenesis occurs (Srikandarajah et al., 2009). Antigens were recovered with 0.1 M citrate buffer at pH 6.0, and endogenous peroxidase activity was blocked using hydrogen peroxide (1.6% in methanol). The sections were incubated for 2 h in normal goat serum (10%) (Vector Laboratories, Inc., Orton Southgate, United Kingdom), followed by incubation with guinea pig-DCX (1:1000, Merck Millipore), the antibody was diluted in PBS (0.1 M, pH 7.4). Subsequently, the avidin-biotin-peroxidase method was used (ABC kit, Vector Laboratories, Inc., Orton Southgate, United Kingdom), the reaction was revealed with chromogen 3′3′- diaminobenzidine (Sigma Aldrich, St. Louis, USA). The samples were counterstained with haematoxylin (H), and the sections were then mounted in resin. As negative control for the immunochemical procedures, the primary antibody was omitted.

2.6. Definition of anatomical regions

Fig. 1 shows the regions that were subjected to quantitative analysis. Apoptotic cells were counted in the pyramidal cell layers of regions 1 and 3 of the Cornus Ammonis (CA1 and CA3), and in the dorsal (DGD) and ventral (DGV) layers of granule cells of the Dentate Gyrus (DG). DCX-immunopositive cells (DCX +) were counted in the DG.

2.7. Quantitative analysis

The tissues were observed using an Axioskop 2 Plus microscope coupled to an image analysis system. The number of apoptotic and doublecortin-positive cells (DCX +) in a constant area (10,000 μm²; at minimum of 128 frames, 40X) was estimated using Zen 2.3 program. In 4 fields per Section (4) for each animal (8), age and group (4 × 4 × 8); the neurons were marked by a click of the mouse on their micrografs on the micrographs (fig. 1).
the monitor ad automatically counted; neurons with a third of their soma within the frame were counted. Photomicrographs were taken of CA1, CA3 and the dorsal (DGD) and ventral (DGV) blades of the dentate gyrus (DG), at 40X magnification. A well-trained observer, blinded to the samples, performed all assessments and assigned a numerical code to each slide.

2.8. Statistical analysis

The statistical analysis was performed using the statistical program in ersl 13. We first calculated the mean and standard deviations; normality was determined by Shapiro Wilks test, and homogeneity was performed using Levene’s test. We applied the parametric procedure (ANOVA) and comparison of means using Tukey’s test; considers the differences among variances of the variables of interest – litter characteristics, neurogenesis and apoptosis in control and experimental subjects. Statistical significance was assumed at p < 0.05. Data are expressed as mean values ± SD.

3. Results

3.1. Histology

The cytoarchitecture of the hippocampal formation in the offspring of the Control group showed a normal arrangement in layers or laminae, with a compact and defined band formed by pyramidal neurons in CA1 and CA3, where the layers are wider (Fig. 2). The size of the neurons in CA1 and CA3 had known values: 20–40 μm and 40–60 μm, respectively. In the dorsal blade of the dentate gyrus (DDG) the granular cell layer had a thickness of 8 cells in columns, and a normal cell size of 10–15 μm (Shepherd, 2004).

Four animals (50%) of the OXC group and three (37%) of the CBZ...
group (Fig. 2) had CA1 and CA3 regions where the typical arrangement in layers had been lost; the neurons were scattered and disorganized, and there were large spaces without neurons (Fig. 2). In the DDG, the cells forming the columns of granular neurons were disorganized and reduced in number (Fig. 2).

3.2. Apoptosis

The number of apoptotic cells in the Control group was similar at 14 and 21 days after birth, but it decreased at day 30 (Fig. 3). This behavior was observed in the CA1 and CA3 areas of the hippocampal formation (Table 1) and in the dorsal and ventral dentate gyrus (Table 2). The offspring of rats that received OXC during gestation showed a significant increase in the number of apoptotic cells (p < 0.01) in all the regions analyzed (Fig. 3); the same behavior was observed in both CAs and in the DG, with persistent increase at 30 days after birth (Tables 1 and 2). In the offspring of the rats that received CBZ during gestation, there was also an increase in apoptosis (Tables 1 and 2), besides there was also a decrease in CA1 and DDG at 14 day and in VDG at days 14 and 30 (Table 2). In CA1 and CA3, the behavior of apoptosis was similar to that observed in OXC pups (Fig. 3). In the DDG and VDG, at day 30, the apoptosis was lower compared with the offspring of OXC mothers (Fig. 4). The nuclei were stained with an intense blue color using the TUNEL technique and were found distributed throughout all the hippocampal regions (CA1 and CA3, Fig. 5); (DDG and VDG, Fig. 6).

3.3. Neurogenesis

Neurogenesis behaved similarly in all three groups (Fig. 7). It decreased from day 14 to 21 postbirth, and from day 21 to day 30. There was only a significant increase at day 21 in the CBZ group. DCX-positive cells stained in brown were observed in the subgranular zone (SGZ) of the dentate gyrus (Fig. 8), their dendritic processes were observed extending through the granule cell layer (GCL) towards the molecular layer.

3.4. Litter characteristics

Treating pregnant rats with OXC or CBZ did not cause any deaths. The weight gain of the OXC and CBZ rats was lower from gestational day 7 to day 19, compared to Controls, but only on day 14 of gestation the weight was significantly lower (p < 0.05) in the CBZ group (Table 3). No malformations were detected. Table 4 summarizes the litter characteristics of the three groups. The gestation period (days) for the three groups was within the expected range (Min 21, max 23 average 22). The litter size decreased (p < 0.01) in the OXC and CBZ groups. Fetuses with 19 days of gestation in the OXC group showed a 58% reduction in body weight (Table 5; Fig. 9) and a 70% reduction in brain weight (Table 6). At 14 days postbirth, the OXC pups had 16% more body weight than Control pups, but 10% less at the age of 30 days. The pups in the CBZ group had 34%, 21% and 31% more body weight than control pups at 14, 21 and 30 days after birth, respectively, and lower brain weight at the age of 30 days (Tables 5 and 6).

| Table 1 |
| --- |
| Apoptosis in hippocampus. |
| Days old | C | OXC | CBZ |
| CA1 | CA1 | CA3 | CA3 |
| 14 | 130 ± 12 | 230 ± 18 * | 110 ± 40 * | 140 ± 15 | 230 ± 17 * | 170 ± 18 * |
| 21 | 140 ± 30 | 210 ± 34 * | 180 ± 30 * | 130 ± 18 | 190 ± 22 * | 210 ± 10 * |
| 30 | 110 ± 30 | 230 ± 10 * | 230 ± 22 * | 108 ± 15 | 198 ± 30 * | 200 ± 43 * |

Mean and standard deviation of TUNEL-positive cells in the CA1 and CA3 regions of the hippocampus. * = significant increase or decrease (↓), p < 0.01. Control: C, oxcarbazepine: OXC, carbamazepine: CBZ.

| Table 2 |
| --- |
| Apoptosis in the Dentate Gyrus. |
| Days old | C | OXC | CBZ |
| DDG | DDG | DDG | DDG |
| 14 | 130 ± 26 | 230 ± 17 * | 110 ± 32 * | 140 ± 15 | 250 ± 35 * | 110 ± 11 * |
| 21 | 120 ± 20 | 270 ± 47 * | 270 ± 50 * | 140 ± 24 | 290 ± 29 * | 240 ± 13 * |
| 30 | 109 ± 28 | 380 ± 68 * | 150 ± 45 * | 100 ± 11 | 310 ± 29 * | 85 ± 21 * |

Mean and standard deviation of TUNEL-positive cells in the dorsal dentate gyrus (DDG) and ventral dentate gyrus (VDG). * = significant increase or decrease (↓), p < 0.01. Control: C, oxcarbazepine: OXC, carbamazepine: CBZ.
4. Discussion

In this study, we investigated the effect of administration of OXC during gestation on hippocampal apoptosis in the offspring of rats. We also assessed the presence of neurogenesis, the cytoarchitecture of the hippocampus and the physical characteristics of the pups. Our findings demonstrated that prenatal exposure to OXC, an antiepileptic commonly used during pregnancy, induces an increase of apoptosis in hippocampal CA1, CA3 and the dentate gyrus. It was also found that exposure to this drug can modify the cytoarchitecture of the hippocampus, interrupting the lamination of the pyramidal cells due to the absence or ectopic neurons, and the malformation of the dorsal blade of the dentate gyrus. The changes observed in the pyramidal neurons of CA1 and CA3, whose neurogenesis occurs between days 15 and 20 of gestation (Shepherd, 2004); and those detected in the granular cells of the dentate gyrus, which originate from day 19 of gestation and during the postnatal stage (Shepherd, 2004), show that OXC affected neural development during gestation throughout the postnatal stage. The increase in apoptosis persisted at the three ages evaluated in all regions; the dispersion of neurons and the malformation of the dentate gyrus suggest that neuronal migration was also affected. These changes could disrupt the complex organization of the neural network (Manent et al., 2007; Rice and Barone, 2000) and could even cause postnatal cognitive impairment (Velez-Ruiz and Meador, 2015); the hippocampal granule and pyramidal cells are critical for learning and memory (De Bruyckere et al., 2018; Schmitz et al., 2002). With the only exception of CA1 at the

Fig. 5. Representative images of cells labeled with the assay TUNEL of offspring at 30 days old. TUNEL positive cells have complete dark staining of the nucleus; only some are indicated (arrows). Counterstain with nuclear fast red. 40×; scale bar = 20 μm.

Fig. 6. Representative images of cells of 30 days old rat pups labeled with the TUNEL assay. Dorsal layer granule cells (DLGC); ventral layer granule cells (VLGC). TUNEL positive cells have complete dark staining of the nucleus; only some are indicated (arrows). Counterstain with nuclear fast red. 40×; scale bar = 20 μm.
The difference in the effect of the drugs on CA and DG was probably due to heterochrony in the development of the different regions of this structure (Díaz-Cintría et al., 2007; Dupuy-Davies and Houser, 1999; Seress and Ribak, 1988). There have been few morphological studies using experimental models on the effects of carbamazepine during pregnancy, and many of them reached inconclusive results (Manna et al., 2017). In this work we found that just as OXC, CBZ induces apoptosis.

It has been reported that when OXC is administered during the postnatal day 5, equivalent to late third trimester early life in humans (Dobbing and Sands, 1979), it increases apoptosis in cerebral cortex and hippocampus (Song et al., 2018), determined that the signaling pathways involved in apoptosis are that of caspase 3 and Bax/Bcl-2. Similarly, when CBZ is administered during the postnatal day 7, it increases apoptosis in the thalamus, striatum and frontal cortex (Kim et al., 2007), but not in white matter areas such as the corpus callosum, the cingulum (Kaushal et al., 2016; Ikonomidou et al., 2007).

A study in mice exposed to CBZ in the diet from one week before mating until the birth of the offspring found a 50% reduction in the number of mature hippocampal neurons (CA1 and CA3) in newborn pups, and 20% in five week- old; no effects were observed on the dentate gyrus (Aberg et al., 2013). It is difficult to compare the results of that study with the results obtained here due to the methodological differences. They estimated the number of neurons in general, without differentiating between apoptotic or newly generated neurons. The absence of an effect on the dentate gyrus may be explained by their use of a CBZ dose that was lower than the optimal therapeutic dose (Aberg et al., 2013). The results of longitudinal studies of children born to women with epilepsy and treated with CBZ (Velez-Ruiz and Meador, 2015; Karataş et al., 2014; Wide et al., 2002; Scolnik et al., 1994), were also contradictory, but the prevailing notion is that cognitive damage should not be attributed to this drug (Velez-Ruiz and Meador, 2015).

The usual dose of OXC in monotherapy is 900–1,200 mg for adults and 25–30 mg/kg/ day for children. (Shorvon, 2000; Beydoun, 2000;
The brain weight of the OXC offspring is significantly lower (*p < 0.01) at day 19 of gestation. At day 30 of gestation, the brain of both the OXC and CBZ pups had lower weight. *p < 0.01; PN = postnatal day.

The results of the present study suggest that when the development of the neuronal circuits of the hippocampus is disturbed, the result is abnormal neuronal circuits (Rodier, 1995; Rice and Barone, 2000; Turski and Ikonomidou, 2012). The similarity of the effect between OXC and CBZ on CA3s (neurons scattered, disorganized, spaces without neurons), DDG malformation, and the incidence of apoptosis in the DG suggest that both drugs affect the development of the hippocampus.

OXC and CBZ are carboxamide derivative AEDs and their primary mechanism of action is to blockade of voltage-gated sodium channels (Livingston et al., 1967; McLean and Macdonald, 1986; Shorvon, 2000; Kuo, 1998; Vreugdenhil and Wadam, 1999; Gierbolini et al., 2016). Another antiepileptic mechanism attributed is its benzodiazepine-like activity that acts on GABA<sub>A</sub> receptor, enhanced activation of potassium channels and inhibition of voltage-dependent calcium channels (Marangos et al., 1983; Zona et al., 1990; Schirrmacher et al., 1993; Huang et al., 2008). It is also known that OXC and CBZ reduce synaptic transmission in the hippocampus by direct inhibition of ionotropic glutamate receptors (Giustizieri et al., 2008; Booker et al., 2015).

The target ion channels, neurotransmitters and second messenger systems in the brain (Ikonomidou and Turski, 2010), the same tagets, GABA and Glutamate neurotransmitter systems regulate proliferation of neural stem cells, neuroblasts, and glioblasts, modulate migration, induce differentiation, axonal arborization, synaptogenesis, synaptic plasticity (Emerit et al., 1992; Bashir et al., 1993; Retz et al., 1996; Levitt et al., 1997; Nguyen et al., 2001). If these drugs act on these processes it is plausible to assume that the administration of OXC and CBZ during pregnancy can influence this processes in the context of brain morphogenesis, network formation and widespread neuronal apoptosis (Rice and Barone, 2000; Ikonomidou and Turski, 2010). Apoptosis is characterised by a number of characteristic morphological changes in the structure of the cell, together with a number of enzyme-dependent biochemical processes; involving an energy dependent cascade of molecular events (Elmore, 2007). It has recently become clear that there exists a number of subtypes of apoptosis (D’Arcy, 2019); the main apoptotic pathways are the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. There is evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (Martinvelet et al., 2005).

Several mechanisms are involved in the proapoptotic effect of antiepileptics in the developing brain have been described. One is the change in the synthesis of brain-derived neurotrophic factor, particularly of neurotrophins 3 and 4. Another is the decrease in the levels of extracellular signal-regulated protein kinases such as ERK1/2 and the protein kinase B/AKT. ERK1/2 and AKT are key for the survival pathways MEK-ERK 1/2 and PI3 kinase-AKT, which are activated by the binding of growth factors to tyrosine receptors. These changes disturb the mechanisms of neuroprotection and neurodevelopment of the developing brain (Bittigau et al., 2002; Olney et al., 2002b; Hardingham and Bading, 2003; Hansen et al., 2004). On the other hand, some mechanisms involved in the proapoptotic action of anticonvulsant, sedative and oxygen drugs are shared, including the decrease in neurotrophic expression, the inactivation of survival signaling proteins, the activation of inflammatory cytokines and the oxidative stress (Ikonomidou, 2009). It has been shown that the administration of non-

Table 5

| Age in days | C       | OXC     | CBZ     |
|------------|---------|---------|---------|
| Day 19 of gestation | 2.08 ± 0.29 | 0.33 ± 0.01 | 2.02 ± 0.19 |
| PN-1       | 6.20 ± 0.12 | 6.30 ± 0.18 | 6.20 ± 0.14 |
| 14         | 19.3 ± 0.74 | 22.30 ± 0.35 | 25.9 ± 0.09 |
| 21         | 27.3 ± 2.2  | 31.30 ± 2.5 | 33.0 ± 1.00 |
| 30         | 58.2 ± 3.8  | 52.50 ± 3.0  | 76.1 ± 4.10 |

The brain weight of the OXC offspring is significantly lower (*p < 0.01) at day 19 of gestation. At day 30 of gestation, the brain of both the OXC and CBZ pups had lower weight. *p < 0.01; PN = postnatal day.

Table 6

| Age in days | C       | OXC     | CBZ     |
|------------|---------|---------|---------|
| Day 19 of gestation | 0.13 ± 0.010 | 0.04 ± 0.007 | 0.13 ± 0.007 |
| PN-1       | 0.27 ± 0.130 | 0.27 ± 0.006 | 0.27 ± 0.050 |
| 14         | 1.10 ± 0.040 | 1.10 ± 0.060 | 1.20 ± 0.060 |
| 21         | 1.40 ± 0.040 | 1.40 ± 0.040 | 1.40 ± 0.070 |
| 30         | 1.50 ± 0.100 | 1.40 ± 0.130 | 1.40 ± 0.060 |

The brain weight of the OXC offspring is significantly lower (*p < 0.01) at day 19 of gestation. At day 30 of gestation, the brain of both the OXC and CBZ pups had lower weight. *p < 0.01; PN = postnatal day.

In the case of CBZ, the starting dose is 100–200 mg once or twice a day. The dose of OXC is different in animal models because it is toxic to rats and dogs, mainly because it causes hepatic alterations, increased liver weight, centrilobular macrocytosis and progressive nephropathy (Degen et al., 1994; Tecoma, 1999). The doses of OXC and CBZ used in the present study fell within the anticonvulsant dose range for rats (Kubová and Mares, 1993; Tecoma, 1999; Gonzalez-Maciel et al., 2000; Kim et al., 2007; Ayala-Guerrero et al., 2008; Gürgen et al., 2012; Erisman and Tekelioğlu, 2017). The dose of CBZ (100 mg/kg) was equivalent to a human dose of 900 mg per day (Ahmed and El-Gareib, 2017). In the present work, we used CBZ as a positive control for apoptosis and to obtain information about apoptosis in the hippocampus. It is known that CBZ induces apoptosis at doses greater than 50 mg/kg (Kaushal et al., 2016; Åberg et al., 2013; Kim et al., 2007). Apoptosis induced by CBZ has been demonstrated in cultures of hippocampal and cerebellar cells (Gao and Chuang, 1992; Gao et al., 1995; Piersma et al., 1998; Ambrósio et al., 2000; Ahmed and El-Gareib, 2017; Mannà et al., 2017), and in vivo in postnatal animals (Piersma et al., 1998), but not as a result of exposure during pregnancy.
steroidal anti-inflammatory drugs to pregnant rats reduces the number of neurons in the hippocampus and dentate gyrus (Gökçmen et al., 2007; Türkmen et al., 2016; Yurt et al., 2018). During the aerobic metabolism of the brain a large amount of free radicals is produced (Halliwell and Gutteridge, 1985), the hippocampus is one of the regions of the brain that produces more reactive oxygen species (ROS). Free radicals act as second messengers in various cellular processes. The treatments with oxidizing drugs increase the production of reactive species, these produce toxic agents for proteins, lipids and DNA contributing to pathological states (Hill and Switzer, 1984; Cecere et al., 2010). Oxidative stress regulates redox transcription factors such as: mitogen activated protein kinases (MAPK), advanced glyca tion products (AGE), poly-ADP-ribose polymerase (PARP), protein kinase (PKC), nuclear factor erythroid related factor-2 (Nrf2), nuclear factor kappa light chain enhancer of B cells (NF-kB) (Ganesh-Yerra et al., 2013; Kaplan et al., 2018). Oxygen-free radicals alter DNA and DNA-bound proteins by producing lipid peroxidation of cell membranes. They also lead to cell death due to apoptosis or necrosis by inhibiting sodium-potassium ATPase (Kwon et al., 2004; Singh et al., 2006; Sullivan et al., 2007; Visavadiya et al., 2016; Hayta and Elden, 2018). It is still necessary to prove that these pathways are involved in the pro-apoptotic action of OXC and CBZ in prenatal stage.

Neurogenesis is high at birth and declines in the first few months of postnatal life until reaching a low level in a stable number of neurons (Amrein et al., 2014, 2011; Ben Abdallah et al., 2010; Kronenberg et al., 2006; Rao et al., 2005; Heine et al., 2004). In adult rats, this stable number constitutes the pool of progenitor neuronal cells (Lois and Alvarez-Buylla, 1993; Kuhn et al., 1996; Eriksson et al., 1998; Kukekov et al., 1999). The neurogenesis results obtained in the present study reflected the behavior described above, both in the control group and in the groups treated with OXC and CBZ. The CBZ group showed a greater number of doublecortin-positive neurons at the three ages studied, with a significant increase at day 21. With the exception of this increase, no change was found in the neurogenesis process. A possible explanation of the unclear effect on neurogenesis may be the period of treatment, from day 7 to 15. The DG develops from progenitor cells of the so-called dentate neuroepithelium (DNE). The hippocampal neurons are produced from the DNE from embryonic day (E) 13.5, and by E17.5, the precursor cells of the dentate migrate and accumulate within the fissure thus forming the layer of neural stem cells (NSC). In the area adult subgranular (SGZ), will differentiate and become neurons of the granular cell layer (GCL) (Kozareva et al., 2019; Nicola et al., 2015; Urbán and Guilllemot, 2014). The time of origin can have important consequences for the neurogenic permissive environment that emerges after childbirth (Kozareva et al., 2019; Urbán and Guilllemot, 2014). With respect to NSC, the neurogenic niche, it has also been suggested that they are generated perinatally in the ventral region of the dentate gyrus (VDG) and subsequently migrate to the dorsal subgranular region of the dentate gyrus (DDG) (Kozareva et al., 2019; Berg et al., 2018; Nicola et al., 2015; Li et al., 2013).

In rats, neurogenesis and apoptosis reach their peak from days 7 to 14 post birth (Oboiriah et al., 2015; Ikonomidou and Turski, 2010; Rice and Barone, 2000). The DDG of CBZ pups showed decreased apoptosis at the age of 14 days, and an increase at the age of 21 days. It cannot be ruled out that the increase in neurogenesis and the decrease and increase of apoptosis reflects a delay in the process of programmed cell death (Ikonomidou and Turski, 2010), but more studies are needed to clarify the temporal sequence of these cellular events in the DG. The persistence of apoptosis at the age of 30 days in the CAis of both OXC and CBZ pups makes it necessary to assess the neuronal populations of the hippocampus at later ages (Ben Abadallah et al., 2010) to determine whether apoptosis continues to increase, if the cytoarchitecture of the hippocampus is affected further, and how does the increase in apoptosis affects the cognitive process.

As for the characteristics of the litter, a study in which rats were administered several doses of OXC before and four days after being inseminated, demonstrated that it caused no toxic effect on the mothers and no alterations in embryonic development (Guerra et al., 2000).

In the present study, we found a slight but statistically significant decrease in the average litter size due to the administration of both OXC and CBZ during pregnancy; low weight in 19-day-old OXC fetuses and increase in average body weight suggest can probably affect postnatal weight.

There are a number of factors that may determine number of pups delivered in rats. Strain, and age of female are the most important. Wistar rats deliver an average of 11 ± 1 pups in the second and third mating; in this study we use females in their second mating (Ohi et al., 2004; Baker, 1979). Another possibility would be the nutritional status of mothers, an indicator is the mother’s body weight during pregnancy. In this regard, we found a decrease on day 14 of pregnancy only in the CBZ group; the weight evaluated in the CBZ group in the other ages during pregnancy had no significant difference, the possibility that this decrease may be the cause of the litter size decrease for this group cannot be ruled out. We found few experimental works that mention a decrease litter size by administration of antiepileptics during pregnancy, or refer increase of resorption or abortion. CBZ (600 mg/kg) administered by gavage in corn oil on days 7–18 of gestation, significantly increased resorptions, reduced live fetal weight (51.6% less than controls), and increased skeletal and visceral abnormalities (Vorhees et al., 1990). Lamotrigine (LTG) administration at higher-doses (50–200 mg/kg/day, i.p., on E7 or E8) reportedly increased the incidence of maternal mortality, abortion, embryonic lethality, congenital malformations, and intrauterine growth retardation, compared with controls (Padmanabhan et al., 2003). In a review of the literature about current knowledge of prenatal and early postnatal antiepileptic exposure, from both clinical and basic research aspects (Fujimura et al., 2017) it is proposed that fetal exposure to antiepileptics during pregnancy may alter the in utero environment during the earliest stages of fetal development. Especially when these environmental changes cause heritable changes to DNA or chromatin structures that affect the gene expression profiles not based on the nucleotide sequences, they are called “epigenetic” changes (Wolfe and Matzke, 1999). The epigenetic mechanisms include DNA methylation, histone acetylation, and noncoding RNA, which are reported to affect cell proliferation/differ entiation characteristics in developing mammalian tissues (Li et al., 1992; Tate et al., 1996; Tou et al., 2004; Fu et al., 2013). Indeed, prenatal exposure to valproic acid (VPA), CBZ, LTG, and Levetiracetam (LEV) has been reported to decrease the level of DNA methylation in umbilical cord blood cells in human neonates (Smith et al., 2012; Hauser et al., 1996).

OXC and CBZ pups were overweight after birth. Data from clinical trials, retrospective and cross-sectional studies have quantified the metabolic changes associated with long-term use of AEDs. AEDs can be associated with weight gain or weight loss, although there is still controversy is this regard (Mikken et al., 2005; Hamed et al., 2009; Rauchenzauner et al., 2010; Verrotti et al., 2011; Cansu et al., 2011; Hamed, 2015). In longitudinal investigation of weight and serum blood glucose, insulin, cortisol, leptin, NPY, ghrelin and galanin levels in prepubertal and pubertal epileptic children commencing OXC monotherapy, no significant differences in any of these levels were determined (Cansu et al., 2011). In other research, they found that the administration of OXC for eight months also results in a significant increase in body weight (Garoufli et al., 2016) and combined with the effects of OXC on known cardiovascular risk factors such as lipids, homocysteine, insulin, and uric acid levels, could provoke endothelial dysfunction and pre-mature atherosclerosis (Emeksziz et al., 2013; Kim et al., 2013).

Although no mechanism of action of OXC has been suggested to induce weight gain, this is an adverse effect of postnatal treatment with CBZ and other antiepileptics, and it has been suggested that the tumor necrosis factor-alpha cytokine system (TNF) is the pathophysiological mechanism involved (Podgorac et al., 2016; Yilmaz et al., 2014; Ness-
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Cytoskeleton P450 2C19 polymorphisms and VPA induced weight gain (Nooi et al., 2016). So far we have not found experimental studies that report weight gain by administration of an antiepileptic during pregnancy. In contrast with our results, other studies on the administration of AEDs during gestation have found delayed motor development and lower body weight of the pups in the postnatal stage (Haddad et al., 2009; Mach et al., 2006). It is possible to suggest that the TNF or that cytochrome P450 2C19 polymorphisms can be modified by OXC and CBZ during pregnancy, however, further studies are necessary to clarify this point. The results of the present study show a great increase of apoptosis and damage in the hippocampal cytoarchitecture; both for offspring of rats exposed to OXC and CBZ during pregnancy. The probable change in the chronology of development, in the establishment of circuits, in the production of neurotransmitters and possibly myelination (Rodier, 1995; Glier et al., 2004; Ikonomidou, 2009; Ikonomidou and Turski, 2010; Turski and Ikonomidou, 2012) in the postnatal stage, makes it possible to suggest that normal children born to mothers treated during pregnancy with OXC may present cognitive damage in the postnatal stage (Pennell, 2016; Velez-Ruiz and Meador, 2015; Gedzelman and Meador, 2012; Perucca, 2005; Wide et al., 2002). We present strong evidence of the apoptosis-inducing effect of CBZ. To our knowledge, the present study is the first to demonstrate proapoptotic effect of CBZ when administered early in gestation. A study in humans associated the use of OXC during gestation with cardiac and renal malformations, the development of withdrawal syndrome and hyponatremia (Roinitsky et al., 2013). In combination with another antiepileptics, OXC was found to induce a three-fold increase in dental agenesis in children (Jacobsen et al., 2014). When administered alone during the first trimester of pregnancy, it has been associated with congenital anomalies (de Jong et al., 2016). In vitro studies have shown that OXC and its metabolites have cytotoxic and genotoxic potential on peripheral blood lymphocyte cultures (Atli Şekeroglu et al., 2017). Our results demonstrated that his administration of OXC during pregnancy has a pro-apoptotic effect on the hippocampus. Even though studies in non-human animal models provide information of the species employed, some characteristics are shared with human in similar conditions. However accurate extrapolation to human patients has been difficult due to the interspecies differences and it has to be recommended carefully (Meador et al., 2007; Bortolotto and Collingridge, 1993; Adams et al., 1990; Vorhees et al., 1990). Thus, more studies focusing on the findings found in this work are needed to make a careful prescription of the AEDs evaluated for pregnant women.

5. Conclusion

The safety of the use of OXC and CBZ during pregnancy was tested and it is clear that both have adverse effects.

Ethical statements

The project was approved by the Research Committee of the National Institute of Pediatrics.

All animal procedures were approved by the ethics committee of the National Institute of Pediatrics in accordance with the provisions of the National Institute of Health (NOM-062-ZOO, 1999) and Olert et al. (1993). We used the minimum number of animals needed according to the bioethical and statistical criteria provided by Festing (1994).

CRediT authorship contribution statement

A Gonzalez-Macieł: Conceptualization, Funding acquisition, Formal analysis, Writing - review & editing, Writing - original draft. RM Romero-Velázquez: Methodology. A Alfaro-Rodríguez: Formal analysis. P Sanchez Aparicio: Methodology. R Reynoso-Robles: Funding acquisition, Formal analysis, Methodology, Writing - review & editing.

Declaration of Competing Interest

None of the authors has any conflict of interest to disclose.

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