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ANTIEPILEPTIC DRUGS CAUSE LETHAL, SUBLETHAL, TERATOGENIC EFFECTS AND MORPHOMETRIC PARAMETERS ON EMBRYOS AND LARVAE OF ZEBRAFISH (*DANIO RERIO*)

MEDICAMENTOS ANTIEPILÉPTICOS CAUSAM EFEITOS LETAIS, SUBLETAIS, TERATOGÊNICOS E PARÂMETROS MORFOMÉTRICOS EM EMBRIÕES E LARVAS DE ZEBRAFISH (DANIO RERIO)

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ABSTRACT: In the present study, we evaluated the toxic effects of antiepileptic drugs gabapentin (GAB), phenobarbital (PB), oxcarbazepine (OX), and lamotrigine (LTG) the lethal, sublethal, teratogenic effects, and morphometric parameters on embryos and larvae of zebrafish (Danio rerio). An acute toxicity test was performed using *D. rerio* embryos according to OECD 236 (2013) guidelines. The acute toxicity test revealed that the mortality of animals increased among the time (24 to 96 hpf) on animals exposed to GAB, PB, OX, and LTG. The sublethal effects had a significant ($p < 0.05$) variation in the number of heartbeats, not a dose-dependent manner. It was also possible to observe variation in the pigmentation in ll animals and the presence of pericardial edema in some groups. The teratogenic effects revealed the malformation of the head only in the animals exposed to GAB. In addition, tail deformation, spine deformation, yolk sac edema, and inflated swim bladder were observed in all animals. The morphometric parameters had a decrease ($p <0.05$) in body length, head-width, ocular distance, the distance between eyes, and eye diameter in all groups in the 96 hpf. It has been observed that gabapentin, phenobarbital, oxcarbazepine, and lamotrigine induce lethal, sublethal, teratogenic effects, and morphometric alterations on zebrafish.

Keywords: Gabapentin, Phenobarbital, Oxcarbazepine, Lamotrigine, Toxicity.

RESUMO: No presente estudo, avaliamos os efeitos tóxicos das drogas antiepilépticas gabapentina (GAB), fenobarbital (PB), oxcarbazepina (OX) e lamotrigina (LTG), os efeitos letais, subletais, teratogênicos e parâmetros morfométricos em embriões e larvas de peixe-zebra (Danio ri). Um teste de toxicidade aguda foi realizado usando embriões de D. rerio de acordo com as diretrizes OECD 236 (2013). O teste de toxicidade aguda revelou que a mortalidade de animais aumentou com o passar do tempo (24 a 96 hpf) em animais expostos a GAB, PB, OX e LTG. Os efeitos subletais tiveram uma variação significativa ($p <0,05$) no número de batimentos cardíacos, não de forma dependente da dose. Também foi possível observar variação na pigmentação em todos os animais e a presença de edema pericárdico em alguns grupos. Os efeitos teratogênicos revelaram malformação da cabeça

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apenas nos animais expostos ao GAB. Além disso, deformação da cauda, deformação da coluna, edema do saco vitelino e bexiga natatória inflada foram observados em todos os animais. Os parâmetros morfométricos tiveram uma diminuição (p <0,05) no comprimento corporal, largura da cabeça, distância ocular, distância entre os olhos e diâmetro do olho em todos os grupos no 96 hpf. Foi observado que gabapentina, fenobarbital, oxcarbazepina e lamotrigina induzem efeitos letais, subletais, teratogênicos e alterações morfométricas no peixe-zebra.

Palavras-chave: Gabapentina, Fenobarbital, Oxcarbazepina, Lamotrigina, Toxicidade.

INTRODUCTION

Antiepileptic drugs are among the most common teratogenic drugs prescribed to women of childbearing age (Shihmanab, et al., 2019; Li et al., 2020). Approximately 1 million women of childbearing age have epilepsy, being important to highlight that the continued use of antiepileptic drugs is recommended to reduce the maternal and fetal trauma associated with seizures in the USA (Patel and Pennell, 2016; Kim et al., 2019; Cho et al., 2020). However, prenatal exposure to antiepileptic drugs can cause growth retardation, major congenital malformations, and intelligence deficits in the developing fetus (Kim et al., 2019; Patel and Pennell, 2016; Bhakta et al., 2015). The estimated prevalence of major congenital malformations, such as facial clefts, hypospadias, cardiac defects, and neural tube defects, in the children of epileptic women, is 4–10 %, which represents a two- to fourfold increase compared to the general population (Bhakta et al., 2015). In Brazil, drugs with central nervous system performance were responsible for 59.9% of the occurrences according to Toxicology Information Center in women of childbearing age, especially antiepileptics (21.2%) (Takahama et al., 2014). Gabapentin (GAB), Phenobarbital (PB), Oxcarbazepine (OX), and Lamotrigine (LTG) has been widely used in the treatment of epilepsy, but to the best of our knowledge, few studies on the ecotoxicity of GAB, PB, OX, and LTG can be found in the literature. One way to remedy this need would be to understand the mechanisms of interference of these drugs on evidence-based aquatic organisms. However, understanding these mechanisms encounters problems as evaluating the changes induced by the drugs GAB, PB, OX, and LTG at developmental levels.

Tons of medicines components (i.e., drugs and excipients) including antiepileptics are produced annually worldwide to be consumed by humans or animals. Knowledge of environmental contamination by those compounds grew (Shi et al., 2019). Thus, compelling the scientific community to consider this contamination type as a potential issue meriting concern. The drugs were designed to target specifically to resist inactivation. However, these same properties are responsible either for bioaccumulation and toxic effects in aquatic and terrestrial ecosystems (Santos

et al., 2010). GAB is eliminated from the systemic circulation by renal excretion (FDA, 2011), and intestinal excretion (Prasad, 2019) as unchanged drug and is not appreciably metabolized in humans (FDA, 2011), a quarter of the administered dose of PB is excreted unchanged in the urine in neonates and adults (Pacifici, 2014), and intestinal excretion (Yumiko et al., 2019), 2% of OX is excreted unchanged by the body, as well as carbamazepine and intestinal excretion (Bahlmann et al., 2014). Renal excretion of unchanged LA accounts for less than 10% (Dickins and Chen, 2002). In addition, the conventional effluents treatment process was found to be invalid for its removal (Martinez et al., 2011; Doll and Frimmel 2005). In Minnesota, researchers sampled 24 sewage treatment plants effluents, and many kinds of drugs were detected, among which the concentration of GAB varied in the ng/L range (Writer et al., 2013). The low removal efficiency in these effluents leads to its frequent detection in surface waters worldwide. In the UK and America, GAB has been detected at the concentration level of μg/L and ng/L (Petrie et al., 2015; Deo, 2014). In Vidy Bay, Switzerland, GAB has been even detected at a concentration of 400 ng/L in raw drinking water (Morasch, 2010). The frequent occurrence of GAB in aquatic systems raises concerns about its ecotoxicity. Besides, studies were not found with the environmental concentrations of PB, OX, and LTG. As already reported above, the toxic effects of these antiepileptics on aquatic biota are not known and, therefore, further studies must be carried out to reveal this knowledge.

Regarding animal model to study ecotoxicological effects like as zebrafish (*Danio rerio*), a very limited number of articles describes the effect of GAB, PB, OX, and LTG. Therefore, ecological relevance can be underestimated due to the joint effects are different from the predicted sum of individual effects leading to the necessity to develop equivalent concentration and equitoxic ratio tests (Wang et al., 2017). The zebrafish has emerged as an important model for toxicology studies since it is an animal that is susceptible to intoxication by toxic agents. For some types of anti-epileptic drugs, the extensive evolutionary conservation of the sequences of their protein targets means that even unicellular eukaryotes can be employed for drug screening and mechanism-of-action studies (Cunliffe et al., 2015). Network mechanisms in higher non-mammalian systems, such as zebrafish that generate seizures are likely to be similar to those that underlie seizures in mammals. Many other non-mammalian organisms exhibit phenotypes with seizure-like characteristics when the balance between excitatory and inhibitory neurotransmission within the CNS is perturbed (Cunliffe et al., 2015).

Therefore, the aim of the present study was to analyze whether antiepileptic drugs cause lethal, sublethal, teratogenic effects and morphometric parameters on embryos and larvae of zebrafish (*Danio rerio*).

2 Material and methods

2.1 Chemicals and Reagents

Gabapentin (GAB), Phenobarbital (PB), Oxcarbazepine (OX), and Lamotrigine (LTG) was purchased from a commercial supplier. All other reagents utilized were of analytical grade.

2.2 Preparation of stock solutions

GA in capsules were broken down, and their contents were also diluted in dimethyl sulfoxide 1% of DMSO solution with a nominal concentration of GAB was 3.0 x10^6 μg/L. PB, OX, and LTG drugs were macerated with 1 mL of the 1% DMSO solution and then diluted in 100 mL of distilled water. The nominal concentration was 1.0 x10^6 μg/L of PB, 3.0 x10^6 μg/L of OX, 2.5x10^5 μg/L of LTG. Then, the solutions were centrifuged with 1512 *g* for 15 minutes. Stock solutions of GAB, PB OX, LTG were prepared by the dissolution of supernatants in the distilled water to obtain the final nominal concentrations of 3.0x10^4, 1.0x10^5, 3.0x10^4, and 12.5x10^4 μg/L of GAB, PB, OX, LTG, respectively. The solutions prepared were stored under refrigeration (4 °C) for further use. These solutions were renewed daily (Lammer et al., 2009; Yang et al., 2016; Silva et al., 2019; Johannes et al., 2019).

2.3 Fish maintenance

The protocols used in this study were approved by the Ethics Committee in the use of animals of the same University, protocol number 112/2018. The experiments were carried out in the *Laboratório de Ecofisiologia e Comportamento Animal – LECA, Universidade Federal Rural de Pernambuco* – UFRPE. The adult animals used were obtained from a commercial supplier and put in confinement to detect and corroborate the absence of pathogens or diseases. Adult animals were housed for 15 days, kept in the aquariums (80 L) in dechlorinated water with artificial aeration (11 mg/L DO), the temperature of 26 ± 1 °C, pH 7.5 ± 0.5 with a density of one animal per liter. Nitrite, ammonia, and nitrate were also continuously monitored. The animals were fed twice a day with extruded marketable fish feed (40% crude protein). All experiments were conducted under a constant artificial dark/light cycle of 10/14 h (OECD 236, 2013).

2.4 Egg production

The day before a test, males, and females in a ratio of 2:1 is placed in breeding chambers immediately before the onset of darkness. The eggs were collected, rinsed in water, and transferred

to Petry dishes and for determination of the overall egg number and viability (i.e. fertilized) (Silva et al., 2019; Lammer et al., 2009).

2.5 Selection of eggs

Eggs were collected and assessed for fertilization success, where the spawning groups with the highest number of fertilized eggs (> 90%) were selected for testing (OCDE 236, 2013). To identify fertilized eggs, a microscope (BIO2B SSI with an LED bulb) was used. Eggs with overt anomalies (asymmetries, the formation of vesicles) or damaged membranes were discarded (Lammer et al., 2009; Johannes et al., 2019). For the toxicity test, only fertilized eggs with 1 hpf (hour post-fertilization) were used.

2.6 Modified fish embryo acute toxicity test (OECD 236)

The assay was based on the OECD 2036 guidelines for the Fish Embryo Acute Toxicity (FET) Test (OECD, 2013). All groups were exposed to the antiepileptics at 1 hpf. Animals were divided into groups where each concentration was tested in triplicate (10 embryos per replicate). The study with antiepileptic drugs used a control group and four experimental groups with animals exposed to gabapentin, phenobarbital, oxcarbazepine, and lamotrigine. The control group was exposed to DMSO 1%, a safe concentration for the use of the DMSO as solvent (Zhu et al., 2012) in the toxicity tests. Also, for each compound described above, the experiments were conducted increasing concentrations of each antiepileptic at nominal concentrations of 10, 100, and 1000 μg/L. These concentrations were based on the environmental concentration of GAB of 0.1 to 10 μg/L (Li et al., 2018) and the environmental concentration of LTG of 1 to 10 μg/L (Chefetz et al., 2019). However, the environmental concentrations for PH and OX were not found and we consider the same concentrations of GAB and LTG for the experiments. Finally, the increasing concentrations (100 and 1000 times greater than the environmental concentration) for each compound were used to simulating possible concentrations to be found in future years or a large amount of disposal in the environment. During all experiments, water renewal was performed daily with the replacement of antiepileptic concentrations according to OECD 236 (OECD, 2013) guidelines. The endpoints evaluated were lethal, sublethal, teratogenicity, and morphometric parameters.

2.7 Analysis of the embryonic development

The analysis of the embryonic development was checked under an optical microscope BIO2B SSI with an LED bulb. The lethal endpoints analyzed were coagulation (Cg), tail not

detached (Tnd), no somite formation (Nsf), no heartbeat (Nhb), lack of hatching (Lh), and mortality (Mo) (%) (adaptation from Lammer et al., 2009). The dead animals were removed every day (OECD, 2013). The sublethal developmental endpoints analyzed were the formation of somites (Fs), development of eyes (De), heartbeat/blood circulation (Hbc), heartbeat frequency (Hf), increased pigmentation (Ip), pericardial edema (Pe), Number of heartbeats (Nh) (bpm/min) (adaptation from Lammer et al., 2009). The heart rate of the embryos was measured manually by counting the number of heartbeats under an optical microscope (400x, 1000x) (adaptation from Lammer et al., 2009; Yang et al., 2016). Lastly, the endpoints of teratogenicity were malformation of the head (Mhd), malformation of the heart (Mh), tail deformation (Td), spine deformation (Sd), yolk sac edema (Yse), inflated swim bladder (Isb), growth retardation (Gr) and coagulation points (Cp) (adaptation from Lammer et al., 2009). The photographic records of the embryos aided the identification of possible toxic effects during development at 24, 48, 72, and 96 hpf. The score determination used in this article is a modification of the data presenting method proposed by Lammer et al. (2009) and OECD 236 (2013). The endpoints collected by dichotomous (binary) response (presence or absence) was based on the severity as well as the number of animals out of the total in which changes were observed with plus (+) = effects until 25% of animals (mild); (++) = effects in more 25% until 50% of animals (moderate); (+++) = more 50% until 75% of animals (severe); (++++) more 75% of animals (very severe).

2.8 Morphometric parameters

The morphometric parameters were used to evaluate de toxicity of antiepileptics. Digital images were made of the dorsal aspect of surviving larvae. The images were captured using a Hayear Mod. HY-2307 digital camera microscope and S-EYE 1.4.2.474 software. We calibrated and took measurements from the images using Image J (version v1.52k, 2019, National Institutes of Health, MD). The body length (Bl) (μm), head-width (Hd) (midbrain) measurement (μm), ocular distance (OD) using the distance between the inner edge of the two eyes (μm) (similar to the inner intercanthal distance in humans) and eye diameter (ED) (μm) were evaluated (CADENA et al., 2020).

2.9 Data management and statistical analysis

Acute fish and embryotoxicity data were collated in EXCEL spreadsheets. The statistical analysis for the assays with embryos was showed in authentic triplicate (OECD 236, 2013). In the statistical analyses, all data are given by mean ± SD. Morphometry and heartbeats were investigated

by one-way ANOVA. When the difference was significant, means were associated with the Tukey test with $p < 0.05$. Statistical analyses were performed using the Origin Pro Academic 2015 (Origin Lab. Northampton, MA USA).

3. Results

3.1. Gabapentin

The acute toxicity test revealed that the mortality of animals increased among the time (24 to 96 hpf). All deaths occurred due to the presence of coagulation (Table 1, Figure 1). The sublethal parameters (Table 2) revealed that there was a significant ($p < 0.05$) variation in the number of heartbeats, but not dose-dependent manner at 24-72 hpf. However, there was an increase ($p < 0.05$) in the heartbeats at 96 hpf. It was possible to observe the increase in pigmentation in embryos and larvae. The pericardial edema was observed in all groups in embryos and larvae in a dose-dependent manner. No changes were observed in the formation of somites, development of eyes, spontaneous movement, and heartbeat/blood circulation in the animals exposed to GAB.

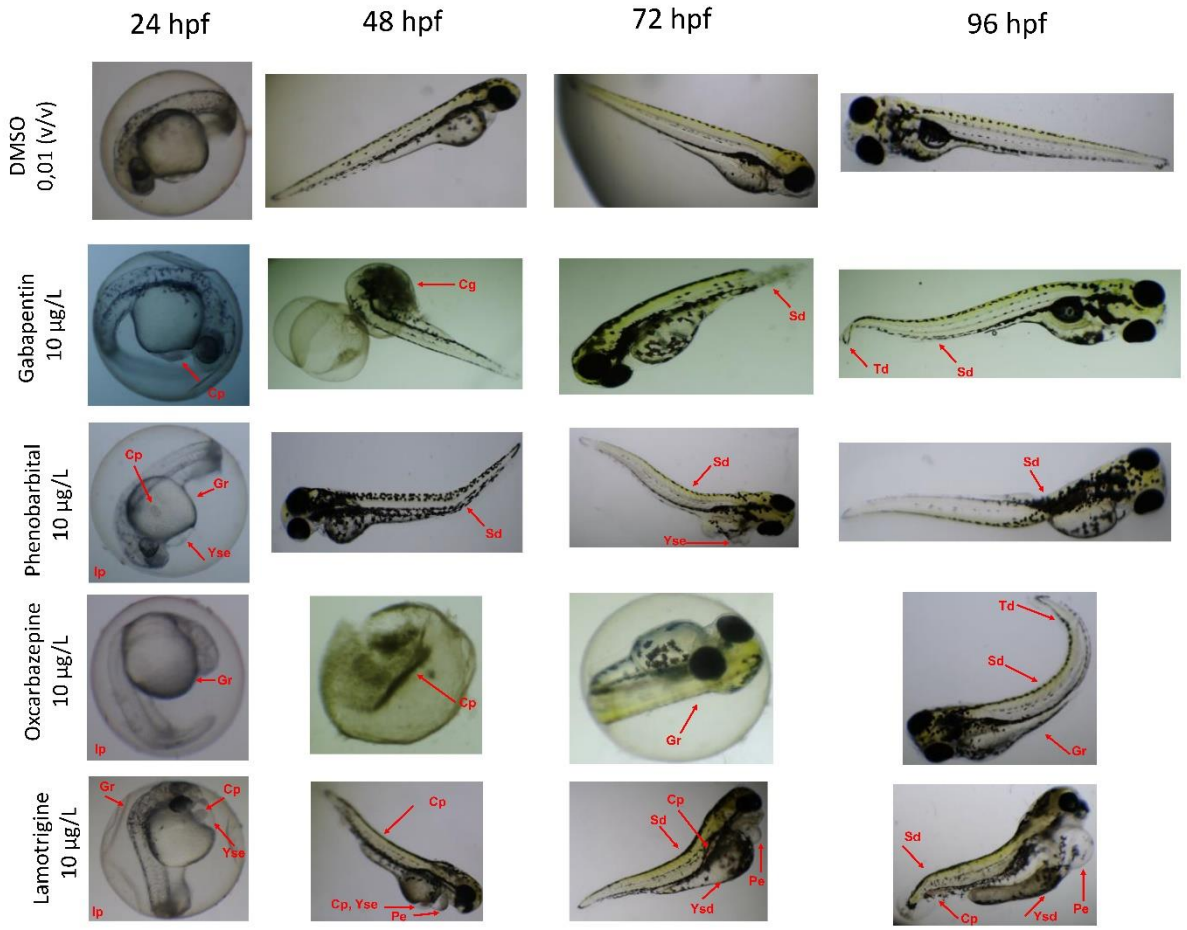

Figure 1

The endpoints of teratogenicity revealed that there was malformation of the head visualized in all groups at 96 hpf (Table 3). The tail deformation was present at 48 and 72 hpf only in the animals exposed to 1000 μg/L of GAB. Besides, in the 96 hpf, this teratogenicity was present in all groups. The spine deformation was present in all groups at 48, 72, and 96 hpf. However, in the 96 hpf, the effect is more severe. The yolk sac edema and inflated swim bladder were absent in all groups. The growth retardation was present in all groups in the 24 - 72 hpf, being absent in all groups in the 96 hpf. The coagulation points were present in all groups at 24 - 96 hpf. No changes were observed in the malformation of the heart. From the morphometric parameters, it was a significant ($p < 0.05$) variation in the body length, head-width, ocular distance, the distance between eyes, and eye diameter in all groups (Table 4).

3.2. Phenobarbital

The acute toxicity test revealed that the mortality of animals increased among the time (24 to 96 hpf). All deaths occurred due to the presence of coagulation (Table 1). The sublethal parameters (Table 2) revealed that there was a significant ($p < 0.05$) variation in the number of heartbeats, but not dose-dependent manner at 24-96 hpf. It was possible to observe an increase in the pigmentation of animals at 24 - 72 hpf. However, there was an absence of the increased pigmentation in all groups at 96 hpf. The increase in the presence of pericardial edema was observed in all groups in the embryo stage (24 and 48 hpf). However, in the larvae stage, there was a reduction at 72 to 96 hpf. No changes were observed in the formation of somites, development of eyes, spontaneous movement, and heartbeat/blood circulation in the animals exposed to PB. The endpoints of teratogenicity revealed that there was no tail deformation (Table 3). The spine deformation was present at 48 hpf; however, in the larvae stage, there an increase of severity at 100 to 1000 μg/L in 72 and 96 hpf. The yolk sac edema and inflated swim bladder were present in all groups at 24, 48, and 72 hpf, but it was not observed in all groups at 96 hpf. The growth retardation was only observed in the embryo stage at 24 and 48 hpf. The coagulation points were present in all groups at 24 to 96 hpf. No changes were observed in the malformation of the heart. Embryos are protected by the chorion and larvae are not. Therefore, larvae are most affected. From the morphometric parameters, it was a significant ($p < 0.05$) variation in the body length, head-width, ocular distance, the distance between eyes, and eye diameter in all groups (Table 4) as same the GAB.

3.3. Oxcarbazepine

The acute toxicity test revealed that the mortality of animals increased among the time (24 to 96 hpf). The presence of coagulation explains it in the 10 and 100 μg/L, but in the 1000 μg/L no heartbeat was also observed at 48 hpf (Table 1). The sublethal parameters (Table 2) revealed that there was a significant decrease ($p < 0.05$) in the number of heartbeats at 24 – 72 hpf, but this decrease was only observed in the 1000 μg/L at 96 hpf. No changes were observed in the formation of somites, development of eyes, spontaneous movement, and heartbeat/blood circulation in the animals exposed to OX. The endpoints of teratogenicity revealed that there was no tail deformation in all groups exposed at 24 and 48 hpf (Table 3). However, they were present in the animals exposed to the concentrations of 100 and 1000 μg/L in the 72 and 96 hpf. The spine deformation was present in the animals exposed to 100 and 1000 μg/L of OX at 48 and 72 hpf. Also, all the groups exhibited the endpoint at 96 hpf. The yolk sac edema was observed in the high concentrations in the animals after 48 hpf. The inflated swim bladder, growth retardation, and coagulations points were present in all groups. Malformation of the head and malformation of the heart were not observed. From the morphometric parameters, it was a significant ($p < 0.05$) variation in the body length, head-width, ocular distance, the distance between eyes, and eye diameter in all groups (Table 4) as same the GAB and PB.

3.4. Lamotrigine

The acute toxicity test revealed that the mortality of animals increased among the time (24 to 96 hpf). No heartbeat explains it at 48 hpf and the presence of coagulation at 96 hpf (Table 1). The sublethal parameters (Table 2) revealed that there was a significant ($p < 0.05$) variation in the number of heartbeats, but not dose-dependent manner at 24-96 hpf. The increased pigmentation was only observed at 24 hpf in the embryo stage but is more severe in the larvae stage at 96 hpf. The pericardial edema was only observed at 24 hpf. The formation of somites, development of eyes, spontaneous movement, and heartbeat/blood circulation were not observed in the animals exposed LTG. The only teratogenic effect observed is points of coagulation in the embryo stage (Table 3). However, in the larvae stage, it was observed the tail deformation and spine deformation. The animals did not present growth retardation, malformation of the head, and malformation of the heart. From the morphometric parameters, it was a significant ($p < 0.05$) variation in the body length, head-width, ocular distance, the distance between eyes, and eye diameter in all groups (Table 4) as same the other antiepileptics.

Table 1: Lethal endpoints observed during experiments on zebrafish embryos after exposure to individual compounds in different concentrations. No effects were observed in the animals of the control (DMSO < 0.1%) group. According to Lammer et al. (2009) and OECD 236 (2013).

		Chemicals											
Time	*Lethal Endpoints*	GAB (10 µg/L)	GAB (100 µg/L)	GAB (1000 µg/L)	PB (10 µg/L)	PB (100 µg/L)	PB (1000 µg/L)	OX (10 µg/L)	OX (100 µg/L)	OX (1000 µg/L)	LTG (10 µg/L)	LTG (1000 µg/L)	LTG (1000 µg/L)
24 hpf	Coagulation	+	+	+	+	+	+	+	+	+			
	Tail not detached												
	No somite formation												
	No heart-beat												
	Lack of hatching												
	Mortality (%)	6.7	16.7	10.0	13.3	6.6	3.3	10.0	3.3	3.3			
48 hpf	Coagulation	+	++	+	+	+	+	+	+	+			
	Tail not detached												
	No somite formation												
	No heart-beat					+				+			
	Lack of hatching												
	Mortality (%)	10,0	26,6	16,7	13.3	6,6	13,3	10.0	3.3	10.0			
72 hpf	Coagulation	+	++	++	+	+	+	+	+	+			
	Tail not detached												
	No somite formation												
	No heart-beat										+		
	Lack of hatching												
	Mortality (%)	16.7	26.6	30.0	13.3	6,6	16,6	10.0	3.3	13.3	3.3		
96 hpf	Coagulation	+	++	++	+	++	+	+	+	+	+		+
	Tail not detached												
	No somite formation												
	No heart-beat												
	Lack of hatching												
	Mortality (%)	16.7	43.3	30.0	16.6	30.0	13.3	20.0	20.0	16.6	3.3		3.3

Legend: GAB: gabapentin PB: phenobarbital, OX: oxcarbazepine and LTG: lamotrigine. Score determination was based on the severity as well as the number of animals out of the total in which changes were observed with plus (+) = effects until 25% of animals (mild); (++) = effects in more 25% until 50% of animals (moderate); (+++) = more 50% until 75% of animals (severe); (++++) more 75% of animals (very severe).

Table 2: Sublethal developmental endpoints observed during experiments on zebrafish embryos after exposure to individual compounds in different concentrations. No effects were observed in the animals of control (DMSO < 0.1%) group. According to Lammer et al. (2009) and OECD 236 (2013).

Legend: Nh - number of heartbeats. GAB: gabapentin PB: phenobarbital, OX: oxcarbazepine and LTG: lamotrigine. Score determination was based on the severity as

			Chemicals											
Time	*Sublethal developmental endpoints*	DMSO (< 0.1%))	GAB (10 µg/L)	GAB (100 µg/L)	GAB (1000 µg/L)	PB (10 µg/L)	PB (100 µg/L)	PB (1000 µg/L)	OX (10 µg/L)	OX (100 µg/L)	OX (1000 µg/L)	LTG (10 µg/L)	LTG (1000 µg/L)	LTG (1000 µg/L)
24 hpf	Formation of somites													
	Development of eyes													
	Spontaneous movement													
	Heartbeat/blood circulation													
	Heartbeat frequency													
	Increased pigmentation		++++	+++	+	+	+++	+	++++	++++	++++	+	+	+
	Pericardial edema		+	++	+++	+	+	+	+	+	+	+	+	+
	Nh (bpm/min)	114.2±10.9	121.7±21.4	94.7±46.7*	112.9±18.4*	114.3±11.8	125.1±21.9*	127.2±12.3*	51.0±14.0*	56.7±10.2*	60.2±7.1*	110.5±8.2	122.3±6.1*	136.1±10.8*
48 hpf	Formation of somites													
	Development of eyes													
	Heartbeat/blood circulation													
	Heartbeat frequency													
	Increased pigmentation				+		+	+	++	+	++++			
	Pericardial edema		+	++	+++	++	+++	+++	+	+	+			
	Nh (bpm/min)	148.5±11.4	157.3±15.0*	190.7±21.2*	157.6±9.4*	150.9±15.3	166.4±8.9*	113.3±18.4*	137.6±16.5*	140.3±12.9*	137.5±6.5*	152.5±14.6*	165.9±14.9*	161.3±11.1*
72 hpf	Formation of somites													
	Development of eyes													
	Spontaneous movement													
	Heartbeat/blood circulation													
	Heartbeat frequency													
	Increased pigmentation		+			+++	++	+	+	+	+		+	+++
	Pericardial edema		+	++	+++		++	++	+	+	+			
	Nh (bpm/min)	207.6±17.1	205.9±41.7*	200.8±17.3	205.6±9.5*	210.4±6.7	166.4±8.8*	113.4±18.1*	177.3±7.4*	191.0±11.1*	190.6±10.3*	194.0±12.0*	202.8±15.4*	205.6±15.2*
96 hpf	Formation of somites													
	Development of eyes													
	Spontaneous movement													
	Heartbeat/blood circulation													
	Heartbeat frequency													
	Increased pigmentation		+++	+++	++							+++	+++	+++
	Pericardial edema		+	++	+++				+	+	+			
	Nh (bpm/min)	218.4±3.2	208.1±23.9*	226.6±14.2*	314.3±38.7*	218.3±3.2	217.6±20.2	228.4±9.3*	193.0±26.6*	193.7±28.3*	192.8±10.3*	220.1±4.1*	231.8±13.6*	214.4±15.7*

well as the number of animals out of the total in which changes were observed with plus (+) = effects until 25% of animals (mild); (++) = effects in more 25% until 50% of animals (moderate); (+++) = more 50% until 75% of animals (severe); (++++) more 75% of animals (very severe). * Significative difference ($p < 0.05$) to control (DMSO) group according to Tukey Test.

Table 3: Endpoints of teratogenicity observed during experiments on zebrafish embryos after exposure to individual compounds in different concentrations. No effects were observed in the animals of the control (DMSO < 0.1%) group. According to Lammer 2009 and OECDE (2013).

		Chemicals											
Time	Endpoints of teratogenicity	GAB (10 µg/L)	GAB (100 µg/L)	GAB (1000 µg/L)	PB (10 µg/L)	PB (100 µg/L)	PB (1000 µg/L)	OX (10 µg/L)	OX (100 µg/L)	OX (1000 µg/L)	LTG (10 µg/L)	LTG (1000 µg/L)	LTG (1000 µg/L)
24 hpf	Malformation of the head												
	Malformation of heart												
	Tail deformation												
	Spine deformation												
	Yolk sac edema				++	+	+						
	Inflated swim bladder							+	+	+			
	Growth retardation	+++	+	+		+		++++	++++	++++			
	Coagulation points	++	++	++	+	+	++	+	+	+	++	++	++
48 hpf	Malformation of the head												
	Malformation of heart												
	Tail deformation			+									
	Spine deformation	+	+	+		+			+	+			
	Yolk sac edema				+	+	+	+					
	Inflated swim bladder							+	+	+			
	Growth retardation	+++	+++		+	+	+	++++	++++	++++			
	Coagulation points	++	++	++	+++	+++	+++	++	+	+	++	++	+
72 hpf	Malformation of the head												
	Malformation of heart												
	Tail deformation			+					+	+	+	+++	+
	Spine deformation	+	+	+		+	++		+	+	+		+
	Yolk sac edema				+	+	++			+			
	Inflated swim bladder							+	+	+			
	Growth retardation	+++	+++					++++	++++	++++			
	Coagulation points	++	++	++	++	+	+	++	+	++			
96 hpf	Malformation of the head	+	+	+									
	Malformation of heart												
	Tail deformation	++	+++	+++					+	+	+		+
	Spine deformation	++	+++	+++		+	++	+	+	+	+		+
	Yolk sac edema								+	+			
	Inflated swim bladder							+	+	+			
	Growth retardation							++++	++++	++++			
	Coagulation points	++	++	++	++	+	+	++	+	++			

Legend: GAB: gabapentin PB: phenobarbital, OX: oxcarbazepine and LTG: lamotrigine. Score determination was based on the severity as well as the number of animals out of the total in which changes were observed with plus (+) = effects until 25% of animals (mild); (++) = effects in more 25% until 50% of animals (moderate); (+++) = more 50% until 75% of animals (severe); (++++) more 75% of animals (very severe).

Table 4: Morphometry of *Danio rerio* larvae with 96 hpf exposed to antiepileptics.

	Chemicals												
Morphometric Analysis	DMSO (< 0.1%)	GAB (10 µg/L)	GAB (100 µg/L)	GAB (1000 µg/L)	PB (10 µg/L)	PB (100 µg/L)	PB (1000 µg/L)	OX (10 µg/L)	OX (100 µg/L)	OX (1000 µg/L)	LTG (10 µg/L)	LTG (100 µg/L)	LTG (1000 µg/L)
Bl	3.918.5 ± 44.9	3549.5 ± 161.6*	3685.7 ± 61.8*	3575.5 ± 95.2*	3355.8 ± 106.9*	3357.1 ± 94.9*	3388.0 ± 25.6*	3374.1 ± 54,8*	3923.3 ± 59.9*	3426.8 ± 95.2*	3690.4 ± 57.5*	3883.8 ± 114.7*	3872.1 ± 43.5*
Hd	515.7 ± 4,0	477.1 ± 13.8*	499.7 ± 9.6*	492.3 ± 16.8*	477.1 ± 13.8*	499.7 ± 9.6*	492.3 ± 16.8*	442.3 ± 20.8	444.7 ± 18.7*	452.1 ± 8.7*	510.9 ± 20.1	526.9 ± 18.9*	526.8 ± 31.6*
Od	128.3 ± 1.5	109.7 ± 16.1*	97.1 ± 12.7*	94.3 ± 18.9*	77.5 ± 9.6*	98.9 ± 11.6*	94.7 ± 18.1*	117.4 ± 14.2*	110.9 ± 19.2*	106.4 ± 10.5*	84.3 ± 19.1*	90.6 ± 34.1*	108.9 ± 24.9*
Ed	324.1 ± 101.6	288.1 ± 35.7*	330.7 ± 20.1*	311.4 ± 16.2*	337.1 ± 64.8	306.6 ± 13.6*	308.2 ± 9.6*	304.5 ± 37.1*	298.4 ± 24.4*	311.2 ± 23.2*	334.7 ± 15.1*	330.5 ± 20.4*	337.2 ± 12.4*

Legend: Bl: Body Length; Hd: Head-Width; Od: Ocular distance, distance between eyes; Ed: Eye diameter; LA: lamotrigine, GA: gabapentin PH: phenobarbital and OX: oxcarbazepine. * Significant statistical difference in relation to the control ($p < 0.05$) by the Tukey test.

5 Discussion

The attitudes of society towards the disposal of indiscriminate drugs can cause serious damage to the environment and the population itself. The lack of understanding of the lethal, sublethal, teratogenic, and morphometric effects caused by the disposal of Gabapentin (GAB), Phenobarbital (PB), Oxcarbazepine (OX), and Lamotrigine (LTG) also makes prevention difficult. In this work, it was analyzed the lethal, sublethal, teratogenic, and morphometric effects caused by the disposal of Gabapentin (GAB), Phenobarbital (PB), Oxcarbazepine (OX), and Lamotrigine (LTG) on the development of zebrafish.

The lethal endpoints analyzed were coagulation (Cg), tail not detached (Tnd), no somite formation (Nsf), no heartbeat (Nhb), lack of hatching (Lh), and mortality (Mo) (%) (Lammer et al., 2009). The most recent works that drug the number of heartbeats at 48 hpf is frequently used as an indicator to assess adverse effects of pollutants are important to highlight being (Li et al., 2018; Fraysse, Mons and Garric, 2006). The heart is the first organ formed in the zebrafish. The heart can be visualized quickly due to the transparent development of this animal. Its beat is regularized between 36 and 48 hpf and this parameter is directly linked to the temperature (Li et al., 2018). Carbamazepine and valproic acid significantly decrease the heartbeat of zebrafish embryos according to the dose at which the embryos are exposed (Pruvot et al., 2012). However, the use of gabapentin increased the frequency of zebrafish, as well as mammals suggesting GABA activated the circulatory system of zebrafish and this might be caused by the mechanism of GAB to the nervous system of zebrafish. Furthermore, GABA toxicity tests show children who have become hyperactive and have aggressive behavior (Wolf et al., 1996). This strongly indicates GA has adverse effects on the development of zebrafish.

Sublethal developmental endpoints analyzed were the formation of somites (Fs), development of eyes (De), heartbeat/blood circulation (Hbc), heartbeat frequency (Hf), increased pigmentation (Ip), pericardial edema (Pe), Number of heartbeats (Nh) (bpm/min) (Lammer et al., 2009). Pericardial edema is the most popular indicator in zebrafish toxicity tests (Li et al., 2018; Fraysse, Mons, and Garric, 2006).

Endpoints of teratogenicity were malformation of the head (Mhd), malformation of the heart (Mh), tail deformation (Td), spine deformation (Sd), yolk sac edema (Yse), inflated swim bladder (Isb), growth retardation (Gr) and coagulation points (Cp) (Lammer et al., 2009). A reducing tendency was observable on the body length of zebrafish by effect of GA (Li et al., 2018). Carbamazepine induced an increase in body length. However, exposure to

GAB caused a concentration-dependent decrease in the body length of zebrafish at 72 hpf (Li et al., 2018). The tail size is also an important indicator to assess the toxicity of chemicals. Tail size decreases due to exposure to pollutants (Fraysse, Mons, and Garric, 2006). Normal hatching was observed, and the morphology was not altered in the animals exposed to PH and LA in concentrations up to 100 μM (Martinez et al., 2018). Among the antiepileptic drugs analyzed LA is the most appropriate candidate since it performs well as an antiepileptic drug at low doses and it does not present teratogenic or neurotoxic effects (Martinez et al., 2018).

Some studies address the development of the zebrafish cardiovascular system in a detailed way (Li, et al., 2003; Berndt, et al., 2014), correlating as changes to oxidative stress (Ni, et al., 2020), crest cell neural migration (Li, et al., 2003; Berndt, et al., 2014), lipid metabolism, receptor-mediated biotransformation (Antkiewicz, Peterson, Heideman, 2006), early differentiation of cardiomyocytes (Roi, et al., 2016), or retinoic acid metabolism (Sarmah and Marrs, 2013; Wu, et al., 2013).

However, few studies address this (eco) toxicological theme (Manjunatha et al., 2020). In animals with mild edema, they resemble the enlarged pericardium, however, severe cases can be taken as thin-walled cavities around the heart. It can develop outside the yolk sac or concentrate in cavities inside the egg. Edema can appear throughout the body, found among the vacancies in the present study, being classified as nonspecific. The edema observed in the development of zebrafish embryos appears to be reversible if it does not occur in severe form.

6 Conclusion

In this work, we studied lethal, sublethal, teratogenic effects, and morphometric alterations on embryos and larvae of zebrafish of the antiepileptic drugs GAB, PB, OX, and LTG. The present study found that the acute toxicity test revealed that the mortality of animals increased among the time in animals exposed to GAB, PB, OX, and LTG. We demonstrated that the sublethal parameters revealed that there was significant variation in a number of heartbeats concentrations in animals exposed to GAB, PB, OX, and LTG.

It was possible to observe variation in pigmentation and the presence of pericardial edema. The endpoints of teratogenicity revealed that there was malformation of the head visualized only in the animals exposed to GAB.

It was possible to observe the presence of tail deformation, spine deformation, yolk sac edema and inflated swim bladder in the animals exposed to GAB, PB, OX, and LTG. From the morphometric analysis, it was observed a decrease in body length, head-width,

ocular distance, the distance between eyes, and eye diameter in all groups. Based on results, the OX is the more toxic compound studied and LTG is the less toxic.

These results draw our attention to possible adverse effects of GAB, PB, OX, and LTG in the environment and help scientists as well as regulators to prepare measures to reduce the toxic effects of these compounds.

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Captions for figures

Figure 1. The teratogenic effects are: Main teratogenic effects are signaled as Cg: coagulation, Cp: Coagulations points, Gr: Growth retardation, Ip: Increased pigmentation, Pe: pericardial edema, Sd: spine deformation, Td: Tail deformation, Ysd: Yolk sac deformation and Yse: yolk sac edema.