

Full Length Research Paper

Riparin III effect on the development of neural tube embryos of *Gallus gallus*

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The aim of this study is to demonstrate the teratogenicity of Riparin III in embryos of *Gallus gallus* (domestic) at an early stage of development. This study is unprecedented in the literature. Chicken eggs were used to investigate the morphometry. All the groups were incubated at $37.2 \pm 0.1^\circ\text{C}$ and $60 \pm 5\%$ relative humidity for 24 h. The embryonic disc was identified and the eggs of the control group were administered 0.1 ml of saline tamponade (PBS, pH 7.0) and those of the other group were administered Riparin III in 25, 50 and 100 $\mu\text{g/ml}$ doses. The eggs were closed with sterile adhesive strips and incubation was continued for another 24 h. All the eggs were then reopened and the embryos dissected from embryonic membranes and evaluated histopathologically with haematoxylin eosin dye for 15 s and washed with distilled water. An embryological development within twelve stages as classified by Hamburger and Hamilton (1951) was obtained. When compared with the control group, the width of the neural tube of the embryos was modified with all Riparin III concentrations. Longer somites resulted from the 50 and 100 $\mu\text{g/ml}$ Riparina III concentrations. The function of the structures was preserved. This drug can potentially be used in pregnant women as an anxiolytic and for treating depression.

Key words: Aniba Riparia, depression, Riparin III, chicken embryo, neural tube defect.

INTRODUCTION

The family, Lauraceae encompasses 52 genera and 3,000 species spread throughout the world. *Aniba riparia* (Nees) Mez. (Lauraceae) is found in Amazonia and the Guianas, west of the Andes. Alkamide alkaloids were isolated from the green fruit of this plant, and were called riparin I (methyl ether of N-benzoyl tyramine) and riparin III (methyl ether of N-2,6-dihydroxy-benzoyl tyramine)

(Thomas et al., 1994; Santo et al., 2011). Riparins I and III, when administered orally or intraperitoneally in mice, showed anxiolytic effects, yet without any sedative or muscle relaxing effects, thus eliminating the common side effects associated with classic benzodiazepines (Melo et al., 2006; Sousa et al., 2004). There was an antidepressant effect and increased brain-derived

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neurotrophic factor (BDNF) levels in the mouse hippocampus (Barbosa-Filho et al., 2015). The inflammatory response was attenuated by modulation of neutrophil migration (Silva et al., 2015). Riparin II is a molecule with interesting anti-inflammatory activity, possibly due to its ability to decrease TNF- α and IL-1 β production and its histamine antagonism (Carvalho et al., 2013). Riparin A, the core structure of all Amazonian riparins, presented weak antioxidant activity, showed moderate activity against colon carcinoma, leishmanicidal activity in promastigotes of *L. amazonensis* (Araújo et al., 2016), and did not produce a sedative effect, loss of motor coordination or muscle relaxation (Nunes et al., 2015).

The object of embryological studies, the embryos have been described in terms of the length of incubation time, and this arbitrary method is still in general use, except for the first three days of incubation, during which more detailed characteristics are applied, such as the numbers of somites (Hamburger and Hamilton, 1951). Fonseca et al. (2013) provided a description of macro and microscopic aspects of the ectoderm-derived structures in chicken embryos/poultry fetuses (*Gallus gallus domesticus*) from the 1st to 19th day of incubation and concluded that this species has a pattern of development similar to other avian species. Neural tube defects (NTD) are congenital malformations of the central nervous system and they occur because of incomplete closure of the neural tube between the 3rd and 4th weeks of pregnancy (Au et al., 2010; Lo et al., 2014). The worldwide incidence of NTD ranges from 1.0 to 10.0 per 1000 births (Lo et al., 2014). Of the 300 thousand children that are born every year with NTD, survivors are left with lifelong disabilities (Pandey and Upadhyay, 2012). An NTD which is a consequence of abnormal neurulation can cause serious medical problems, which sometimes even lead to death in the prenatal and postnatal periods (Unlu, 2002; Cetinkal et al., 2010). In this study, *G. gallus* embryos (domestics) were used to evaluate the effect of Riparin III on anatomical and morphological aspects after 24 h of incubation. Riparina III may be an option for pregnant women, as long as it does not cause damage to embryonic development.

MATERIALS and METHODS

The experiments were performed at the Tissue Culture Laboratory of the Department of Histology and Embryology, Federal University of Pernambuco (UFPE). Riparin III, a synthetic product of *Aniba riparia* (Nees) Mez (Lauraceae), belonging to the chemical class Benzoyl - tyramine and discovered by the Laboratory of Pharmaceutical Technology (LPT - UFPB) was used.

Purchase and cleaning of eggs

The eggs were purchased from Granjita (Pombos City/ PE State), with one day of fertilization before the experiments. They were

cleaned with a paper towel and lightly moistened with 70% ethanol. Only eggs weighing about 70 g were numbered with black pencil to identify them with respect to the experimental groups.

Experimental group

In this study, 70 embryos were handled. According to Berger (1971 apud SCHATZ, 2003), the species used in this study presents the following taxonomic classification: Class Aves; Subclass Neornithes; Superorder Neognathae; Order Galliformes; Phasianidae family. The embryonated eggs were incubated for 24 h at a temperature of 37°C with atmospheric moisture retained by a container with 2000 ml of water replaced every two days. Diaz et al. (2016) shows that increasing the incubation temperature by 2°C during the first 18 days for Araucana hen eggs (*Gallus inauris*) increases the rate of embryonic mortality and decreases the hatch rate. Every day, the incubator was opened for three minutes to renew the air. The embryonic disc was identified using the candling technique. The solutions were injected into the center of the yolk through the air chamber in a volume of 100 μ l. The eggs were incubated for another 24 h. They were divided into two groups: (1) the control group, which was injected with saline (PBS, pH 7.0); (2) the experimental group, which was injected with the riparin III solutions in different concentrations (25, 50 and 100 μ g/ml); 10 fertilized eggs were used for each concentration. To administer the doses, a needle was used with an outer diameter of 0.70 mm (size: 25 x 7) to pierce the egg shell in order to facilitate the insertion of an insulin needle used to inject the solutions. After the solutions were injected, the holes made in the eggs were sealed with tape and the eggs were incubated.

Processing of incubated eggs

After the 24-h incubation, the eggs were broken with the aid of scissors and fine tip forceps, and the embryonic disc was cut out and transferred with the aid of a brush to a Petri plate containing PBS to remove the excess vitellus.

Histologic procedure: Total assembly

Twenty-four hours after treatment, the eggs were opened (Figure 1) and embryonic disks cut with the aid of scissors and tweezers. They were transferred to a Petri plate containing PBS. To remove the excess vitellus, the embryos were washed in PBS. A rectangular filter paper with a diamond window was fitted in the central region of the embryo to keep it distended and immersed in the fixative solution. Then, the embryos were fixed in Carnoy fixative for 24 h at room temperature in a covered Petri plate. After that, they were washed in distilled water and placed in 70% ethanol, where they remained for 12 h. For staining, the embryos were hydrated in distilled water for 10 min (two five minute immersions) and placed in haematoxylin-eosin dye for 30 s and washed with distilled water. After staining, they were dehydrated in ascending alcohol (ethanol 80, 90% for 10 min during each immersion and 100% for 20 min) and then diaphanized in xylene for 10 min. To mount the permanent slides, Intellan® was used and they were left to dry. After making the slides, the analysis was performed using an optical microscopy system (LABOMED LX 400) camera for capturing and recording images.

Histologic procedure for morphometric analysis

The evaluation of the morphological features of the embryos.

Table 1. The control group and distribution of the three experimental groups.

	Control group	Experimental groups (Riparin III- $\mu\text{g/ml}$)		
		25	50	100
Number	20	16	17	17
Normal development	12	10	10	10
Insufficient development	6	6	6	5
Lost through histological procedure	2	-	1	2

Table 2. Quantitative of slides and stages identified according to Hamburger and Hamilton (1951).

Groups	Slides			
	Stage 9	Stage 10	Stage 11	Stage 12
Control	1	5	6	---
Riparin III 25 $\mu\text{g/ml}$	---	6	3	1
Riparin III 50 $\mu\text{g/ml}$	2	4	4	---
Riparin III 100 $\mu\text{g/ml}$	1	7	2	---

(*G gallus*) was carried out using common optical microscopy. Embryos at 24 h. were fixed in buffered formalin (10%) and were subsequently dehydrated in alcohol (70 to 100%), and diaphanized in xylene. The embryological preparations were mounted between the slide and cover slip using synthetic resin (Entellan®). After assembly, the embryological preparations were photographed with the help of a video-microscopy system (LABOMED LX 400 Microscope, with a Moticam 1000 1.3M Pixel-USB 2.0 digital camera, QUIMIS and MOTIC Images Plus 2.0 software). The authors evaluated the regions related to the development of the neural tube (the precursor structures of the central nervous system), measuring the neural tube and width and length of the somites.

Determination of embryo stage

The developmental stage of the embryos was determined according to Hamburger and Hamilton (1951). This research has the approval of the Ethics Committee to work with experimental animals (No. 23076.022496/ 2015-89).

Statistical analysis

The data were analyzed using the One Way test. Probability values of $p < 0.05$ were considered to indicate a significant difference. The SigmaStat 3.5 software was used for the analysis. All data were evaluated in a blinded fashion.

RESULTS

At the time of injection, each embryo should have at least four pairs of somites, neural folds of the future midbrain and a portion of the hindbrain, corresponding to the criteria for a healthy egg and normal biological development. After 24 h of post-injection incubation,

32.85% of the embryos showed insufficient development (prior to stage 8), while another 7.14% were lost through the histological procedure (Table 1).

The stages of embryonic development were classified between 9 and 12. In all the groups, we identified 9.52% of the embryos at stage 9, 52.38% at stage 10, 35.71% at stage 11, and 2.39% at stage 12 (Table 2). According to Hamburger and Hamilton (1951), the characteristics of stage 9 involve primary optic vesicles being present. At stage 10, the first somite becomes dispersed and the three primary brain-vesicles are clearly visible. At stage 11, there is slight cranial flexure and five neuromeres of the hindbrain are distinct. Finally, at stage 12, the following can be observed: a head turning to the left, anterior neuropore closure, an identifiable telencephalon, primary optic vesicles and a well-established optic stalk, a deep but wide open auditory pit, and a slightly S-shaped heart (Figure 2). The variation found among the stages is a natural variation between different embryos and does not necessarily have anything to do with the drug.

The control and experimental groups of Riparin III 100 $\mu\text{g/ml}$ lost 2 embryos each by incorrect histological procedure while the experimental group of Riparin III 50 $\mu\text{g/ml}$ lost only 1 embryo. Insufficient development means prior to stage 08 according to Hamburger and Hamilton (1951).

When the chick embryos were examined for general morphological condition, we were able to observe differences between the experimental groups involving varying somite lengths and neural tube width. In the group with Riparin III 25 $\mu\text{g/ml}$ (RIP 25), the somite lengths were very close to those of the control group (PBS), except for the width of the neural tube.



Figure 1. Open egg showing the embryonic disc.

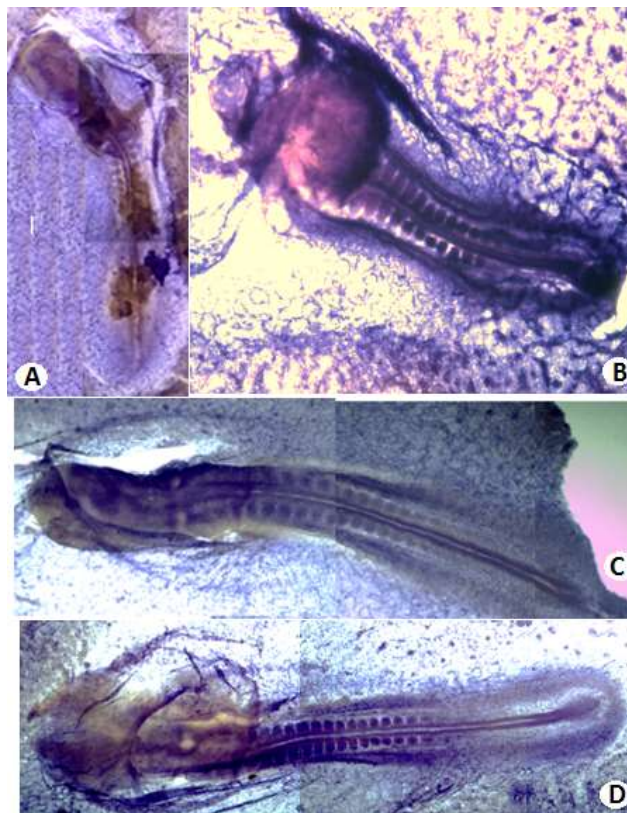


Figure 2. An example from the morphological examination showing normal stage 9 in the control group (A), stage 10 in experimental group Riparin III 50 $\mu\text{g/ml}$ (B), stage 11 in experimental group Riparin III 100 $\mu\text{g/ml}$ (C), and stage 12 in experimental group Riparin III 25 $\mu\text{g/ml}$ (D), for the development of the chick embryo (Microscopic Examination, 4x, Hematoxylin-Eosin).

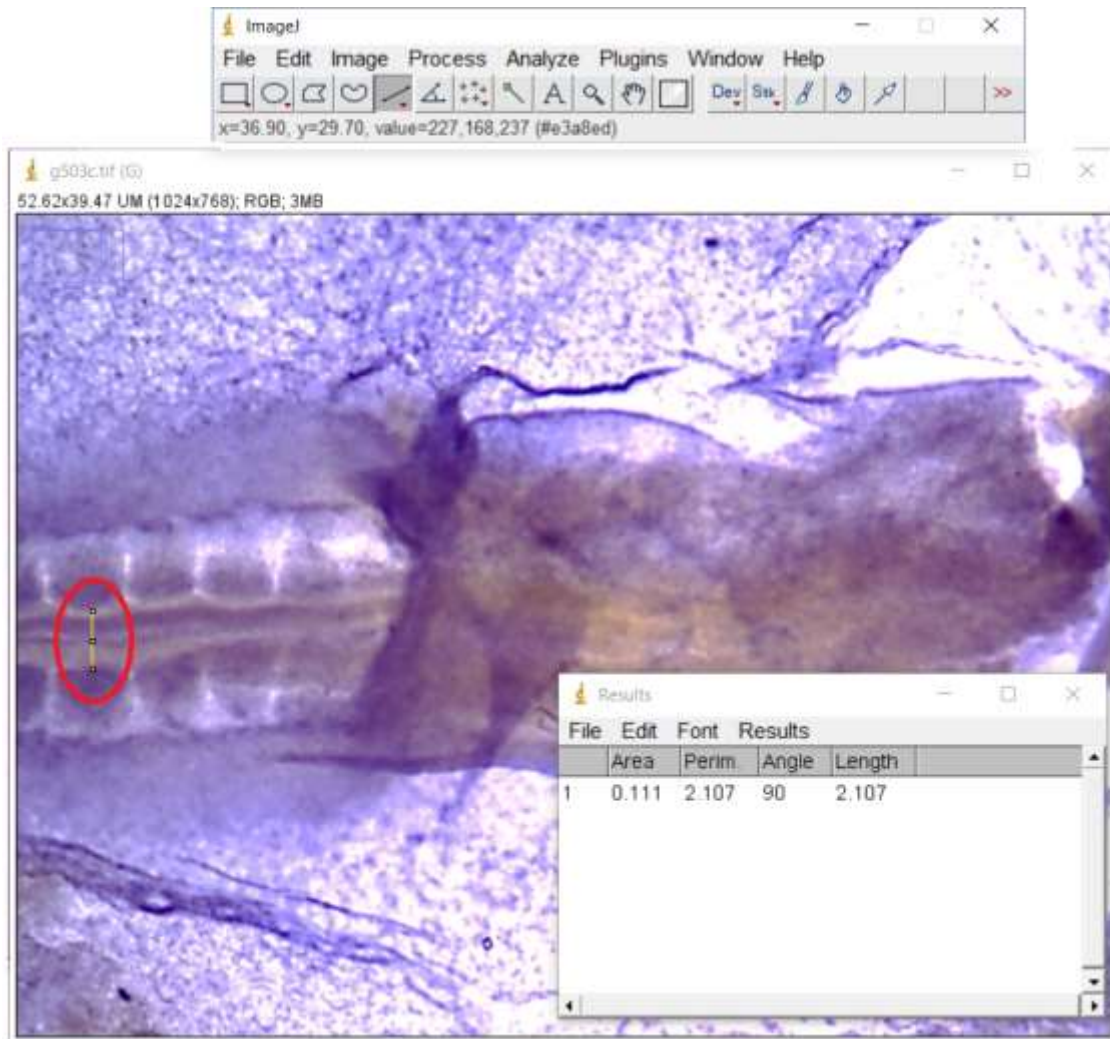


Figure 3. Example of measurement of neural tube width (μm): red circle. "Embryo n°03" of the Experimental Group Riparin III 50 $\mu\text{g}/\text{ml}$.

The somite length increased statistically with dosages of Riparin III 50 (RIP 50) and 100 $\mu\text{g}/\text{ml}$ (RIP 100) ($df = 3.41$, $p \leq 0.001$, $F = 37.14$, $t = 7.919$) (Figure 4). The neural tube width was statistically significant between the groups ($df = 3.41$, $p \leq 0.001$, $F = 12.478$, $t = 2.184$ with RIP 25, $t = 2.822$ with RIP 50 and $t = 3.401$ with RIP 100) (Figure 5).

It was found that the development of all the embryos was consistent with their stages. Neural tube defects were not detected in any embryo group. The somite and neural tube measurements were obtained using ImageJ Software (Figure 3).

DISCUSSION

This study demonstrated that the administration of Riparin III was able to morphologically alter the structures

that make up the chick embryos, such as the lengths of the somites and width of the neural tube. On the whole, the function of the structures was preserved.

Neural tube defects (NTD) are congenital anomalies of the central nervous system. The period between the appearance of neural plaque to closure of the palate, that is, the period between the 18th and 60th day of pregnancy, is the period when the possibility of congenital anomaly is highest. These anomalies originate from an insufficiency in neural tube formation (Guvenc et al., 2013, 2016) or re-opening after formation of the neural tube (Gardner, 1960). Environmental factors that would influence fetus development may cause congenital anomalies during this period. The best known risk factor for fetal NTD is maternal folate deficiency (Au et al., 2010; Lo et al., 2014). Other risk factors for NTD development include a positive family history, smoking and air pollution (Bedford and Clarke, 1972; Pandey and

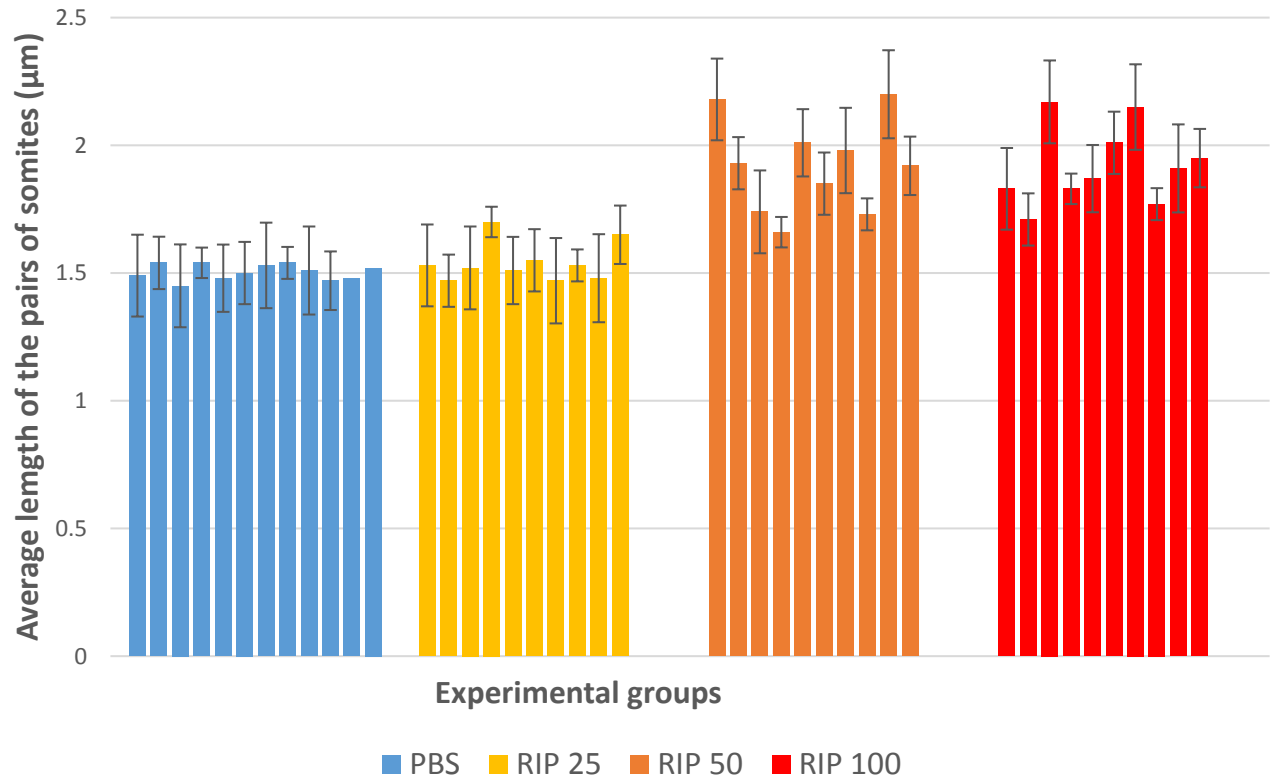


Figure 4. Comparing the length of the somites and experimental groups. PBS (Control Group), RIP 25 (Riparin III 25 µg/ml), RIP 50 (Riparin III 50 µg/ml), RIP 100 (Riparin III 100 µg/ml).

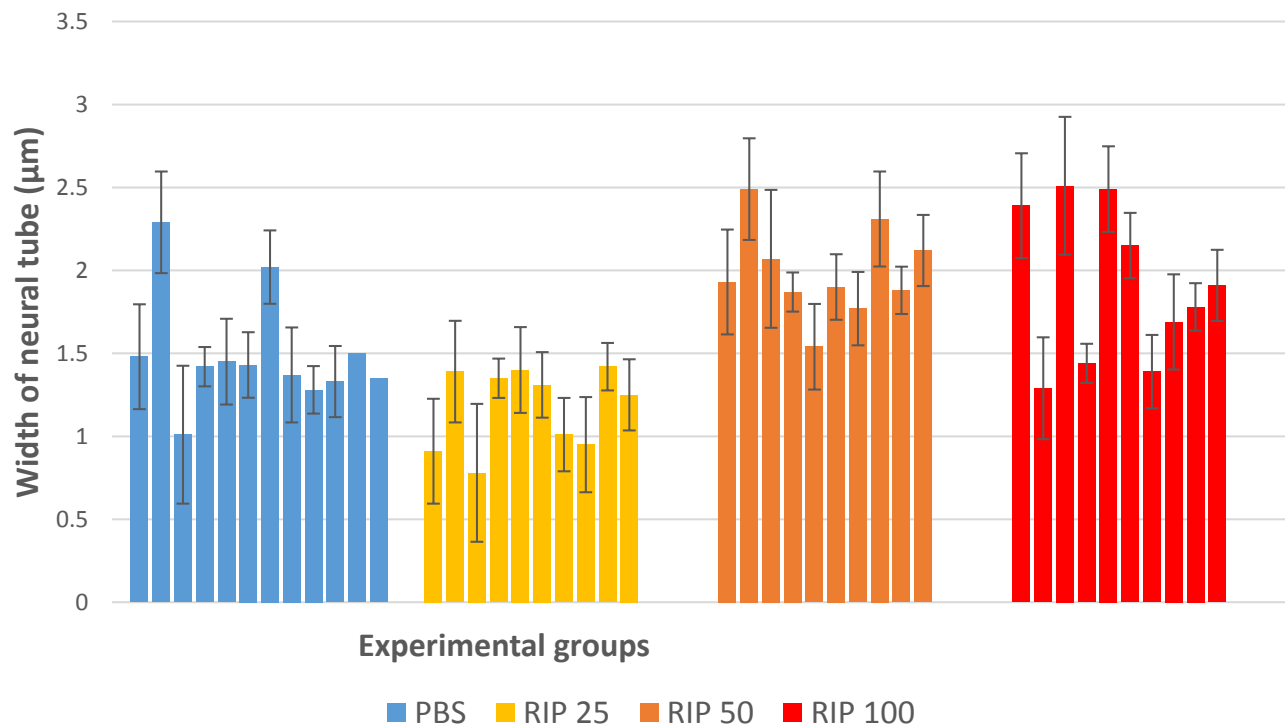


Figure 5. Width of the neural tube according to the experimental groups. PBS (Control Group), RIP 25 (Riparin III 25 µg/ml), RIP 50 (Riparin III 50 µg/ml), RIP 100 (Riparin III 100 µg/ml).

Upadhyay, 2012). Additionally, NTDs may be related to maternal socioeconomic and socio-cultural status as well as physical condition (Au et al., 2010; Bateman et al., 2004). Many studies have been done on chicken embryos to investigate the etiology of NTD. They evaluated the effects of environmental factors and drugs (Cetinkal et al., 2010; Dalgic et al., 2009; Guvenc et al., 2013; Simsek et al., 2012; Temiz et al., 2009; Whitsel et al., 2002).

In this study, it was observed that the width of the neural tube was altered with the Riparin III concentrations evaluated. However, no physiological changes were observed. High fever in the mother during the first month of pregnancy, alcohol use during pregnancy, and use of some antiepileptic drugs can be specified in the etiology of neural tube closure defects (Honein et al., 2001; Tuncbilek et al., 1999). It has been demonstrated in experimental studies looking at early stage chick embryos that ethanol, high dose meloxicam, high dose progesterone, the folic acid antagonist methotrexate, and cotinine in cigarettes, causes neural tube closure defect (Barutcuoglu et al., 2001; Cetinkal et al., 2010; Dalgic et al., 2009; Erdinciler et al., 2006; Vatanserver et al., 2003). Glatiramer acetate affects spinal cord development through Forkhead box protein P1 (FOXP1) in the chick embryo model at high doses (Taskapilioglu et al., 2015). Levetiracetam, a new antiepileptic drug that especially affects calcium ion concentrations, leads to defects in midline closure in embryos, depending on the dose (Ozgural et al., 2014). The width of the neural tube in all embryos in the fourth pair of somites was measured, where it represents the largest opening of the developing neural tube. No neural tube defect was detected in any embryo.

Emon et al. (2014) showed that sodium benzoate as one of the widely used food preservatives has no effect on neural tube defect development in chicken embryos, even at high doses. The early chick embryo model is an ideal model that corresponds to the first month of embryonic development in mammals and is well suited for investigating the effect of chemicals on the development of embryos. Numerous chemical agents such as caffeine, phenytoin, diazepam and local anesthetics are known to cause neural tube defects in chick embryos (Erdinciler et al., 2006; Guney et al., 1999; Lee and Nagele, 1985). Stage eight embryos were generally chosen for these investigations since developing neural tissues exhibit a gradual variation in the degree of opening along their length, which provides an excellent opportunity for studying the effect of chemical agents on neural tube closure. These findings are in agreement with data from the scientific literature, which support the anxiolytic and antidepressant actions of Riparin III in basic studies (Melo, 2012; Sousa et al., 2004). A previous study, using models of acute depression, verified normalization of depressive behavior and an additional increase in monoamine levels

(Melo et al., 2013). The same study concluded that Riparin III was able to increase the levels of monoamines NA, 5-HT, and DA in the striatum and prefrontal cortex, and NA and 5-HT in the hippocampus, while decreasing their metabolites in the striatum and prefrontal cortex (Melo et al., 2013). Riparin III can probably be used as an anti-depressant and anxiolytic in women during pregnancy. The somites increased in length with dosages of Riparin III 50 and 100 µg/ml but this does not mean that they have malformations or serve as evidence for adverse reactions.

CONCLUSION

Neural tube defects were not observed when Riparin III was given to chick embryos. However, a larger number of subjects and further studies involving its use in high doses are needed to show the mechanism of embryonic damage and the mechanisms of its teratogenic effects. Riparin III can be a safe candidate drug, with regards to neural tube defect, for use in depressive women during pregnancy in doses within the therapeutic range of these studies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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