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Fetal brain lesions caused by cotyledon damage of *Androctonus turkiyensis* venom in pregnant rats and the protective effects of the monovalent antivenom

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Scorpion envenomation is one of the severe public health problems, particularly children and pregnant women. Increased oxidative stress in the brain during the first couple of weeks in the gestational period may limit the efficacy of antioxidants after the scorpion envenomation. High ROS activity during brain organogenesis may damage the forebrain and midbrain of fetuses. In this study, we examined cellular alterations in the fetal brain and cotyledon of *in utero* exposure to scorpion venom and antivenom during pregnancy in rats. The study focused on establishing a correlation between fetal brain and cotyledonary lesions due to altered oxidative stress. Eighteen pregnant *Wistar albino* rats were randomly divided into three groups envenomated group (EG) by *Androctonus turkiyensis* Yagmur venom, antivenom group (AVG), and physiological saline solution (1 mL, PSS: NaCl 0.85%) treated control group (CG). Pregnant rats in the EG (n=6) were injected sublethal doses of *A. turkiyensis* every day between 7-13th days of pregnancy. AVG, pregnant rats (n=6) were envenomed with *A. crassicauda* venom. After 4 h of each venom injection, these pregnant rats were administered a specific antivenom. The pregnant rats in groups were operated aseptically on the 21st day of pregnancy. Ovariohysterectomy was performed to remove the fetus with cotyledons. Fetal central nervous systems and cotyledons were examined histopathologically and immunohistochemically. To demonstrate ROS-related damages, iNOS, 8-OHdG, 4HNE and RIPK3 were quantified and analysed statistically. As a result, expressions correspondingly to venom administration were increased in EG despite of a decrease in AVG. In conclusion, it was found that the antivenom administration regarding scorpion envenomations can diminish ROS-related-cotyledonary and neuronal damages.

Keywords: Black scorpion, Immunoexpression, Oxidative stress, Pregnancy.

Scorpion envenomation is a severe public health problem in many parts of the world. Mainly the South American, Middle East, and African human populations are at risk because there are many fatal and medically important types of scorpions in these geographies[1,2]. Risk can be greater when medical care is limited. Approximately, 1.2 million cases of scorpion sting reported annually and 3250 may lost life due to this[1]. Pregnant women and children have been particularly found under greater risk[3-5]. Scorpion sting may cause fetal loss, abortion, pre-term delivery or placental abnormalities and growth retardation in pregnant women[6-8]. However, scorpion envenomation did not seem to have any effect on the development of the fetus during different pregnancy[9,10].

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The symptoms of scorpion sting in pregnant victims are clinically defined as follows: lethargy, intense pelvic pain, vaginal bleeding, eclampsia, placental and fetal abnormalities, and fetal loss is not reported as a clinical symptom in some studies[11-13]. Scorpion envenomation findings in animal models were similar to scorpion sting findings in humans[25]. In rat animal models, organogenesis initialises on the 7th to the 13th days of pregnancy and continues until the 16th day. The critical period is the 10th day of pregnancy because brain sculpture is beginning to take shape[15]. The developing brain can be exposed to toxins and be affected adversely due to the distribution of the cascade of these developmental processes in two weeks of the prenatal-critical period. Scorpion stings may release toxins in circulation and alter the sex hormones or release cytokines such as interleukins[16,17].

Ionic accumulation in cells may increase the free radicals and cause cellular distress[18,19]. Reactive oxygen species (ROS) begin to affect neurons after passing over the placenta and damage the molecular structures which play a role in neurogenesis[20-22]. This may lead to increased oxidative stress in the brain, thus limiting the efficacy of antioxidants after the scorpion envenomation during the first couple of weeks in the gestational period[23-25]. Forebrain and midbrain of fetuses can be damaged due to high ROS activity during brain organogenesis[14,27,28]. ROS activity damages the central nervous system's neurons, glia cells, myelin, the cell membrane, and DNA[29]. Some oxidative stress markers, 4-hydroxynoneal (4HNE) and 8-hydroxy deoxyguanosine (8-OHdG) are increased when damage is incurred on the central nervous system. Increased levels of the markers are frequently attributed to neurons that undergo apoptosis[30,31] but, some cells may not always drift to apoptosis. The apoptotic cascade may change to necroptosis formation in the affected cells. Receptor-interacting protein kinase 3 (RIPK3) is a pro-inflammatory and useful marker of cell death or necroptosis. RIPK3 increases drastically against the cell drifting to necrosis. RIPK3 may also regulate inflammasome-induced IL-1ß production. Activated RIPK3 in turn, phosphorylates and activates the downstream necroptosis as an executioner. RIPK3 triggers pro-inflammatory cell death termed as necroptosis[31-33].

Androctonus crassicauda, known as black scorpion and Yagmur recently, defined *A. turkiyensis*, as a new species after a comparison with *A. crassicauda* in Kashan Region (Iran)[34]. Although there are no vaccines or other effective agents against animal venoms, antivenom is still the only and unique treatment option. To the best of our knowledge, no studies have been conducted in pregnant rats on the effects of *Androctonus turkiyensis* venom over the fetal brain during organogenesis and the neuroprotective effect of monovalent antivenom.

Here, we have studied the fetal brain development and structural changes and differences in cotyledons that occur after the injection of *A. turkiyensis* venom in the organogenesis period of pregnant rats and the neuroprotective effect of specific antivenom in these animals. In addition, the relation between fetal brain and cotyledon lesions was also investigated due to altered oxidative stress.

Materials and Methods

Ethical statement

The study was approved by the Experimental Animals Ethics Committee of Gazi University (G.U.ET-20.035). Furthermore, all experimental procedures were conducted according to the ethical guidelines of the Ethics Commission on Animal Use (CEUA) and the Guide for the Care and Use of Laboratory Animals of NIH.

Scorpions

Androctonus turkiyensis scorpions were captured in Sanliurfa Province in the Southeastern region of Turkiye and kept in plastic cages with free access to food and water. Scorpions were housed in plastic boxes in Department of Zoology, University of Cankiri Karatekin Univesity, Turkiye.

Experimental animals and mating

Adult female (n=18) and male (n=5) Wistar albino rats weighing 200-250 g were used for experimental procedures. The animals were housed in polysulfone cages at 21-24℃, 40-45% humidity and under light-controlled conditions (12 h light/dark) at the Laboratory Animals Breeding and Experimental Research Center, Faculty of Pharmacy, Gazi University. The rats were fed with a standard pellet diet and water ad libitum. A male rat was introduced to the four female cages for a16 h duration. At the end of the duration, the day of the presence of the vaginal plug was designated as day 0 of pregnancy[35]. Following the diagnosis of pregnancy, the rats were taken into individual cages.

Venom

Venom was obtained from matured *A. turkiyensis* scorpions by electrical stimulation of the telson. The venom was diluted with sterile double distilled water and centrifuged at 15000 rpm for 20 min at 4°C Then, the supernatant was taken and stored at –20°C until use at Cankiri Karatekin University.

Antivenom

The *Androctonus turkiyensis* (*A. crassicauda*) antivenom was procured from the General Directorate of Public Health (former name Refik Saydam Hygiene Center), Ministry of Health. In Turkiye, it is used to treatment of all scorpion species sting cases since decades. Administering *A. turkiyensis* venom to horses and obtained plasma using enzymatically digesting and extensive dialysis procedures. The median effective dose of the antivenom is $50LD_{50}$/1 mL/s.c in mice.

Experimental design

Fourteen *A. turkiyensis* venom were dissolved in 84 mL of physiological saline solution (PSS: NaCl 0.9%) and hence the venom venom solution was prepared. The eighteen pregnant rats were randomly allocated into three groups, with six in each group. The sublethal dose of 1 mL of the scorpion venom solution was subcutaneously (s.c.) given to pregnant rats of the experimental groups (EG and AVG). The rats of the control group (CG) were administered saline solution (1 mL/s.c.). All administrations were carried out as described previously by Demirel et al. (2021)[36]. The experimental design is summarised in Fig. 1. On the 21st day of pregnancy, an ovariohysterectomy was performed to remove ovaries and the uterus under general anaesthesia (10 mg/kg xylazine hydrochloride and 50 mg/kg ketamine hydrochloride). Uterine horns of maternal rats were carefully cut and, all fetuses and their placenta were removed from uterine tissue. Following the extraction of the brains of offspring, all maternal rats and offspring died as a result of exsanguination. Fetal brains and placentas were immediately transferred into a 10% neutral buffered formalin solution for histopathological evaluation.

Histopathological evaluation

Fetal brains and cotyledons were examined for hyperemia, haemorrhagia, edema, and abnormalities. After the evaluation, the sagittal section of the fetal head, cerebrum, cerebellum, pons and medulla oblongata were taken from necropsy in all groups.

Cotyledons of dams were removed in all groups and measured separately according to groups. The important morphological changes were photographed, tissues were fixed into formalin with 10% buffer, and then taken to routine tissue follow-up (Leica, TP-1020, Germany). About 5 µm slices were taken from the tissues blocked in paraffin. For routine examinations, the hematoxylin-eosin staining method was employed first.

All the results were semi-quantitatively scored using the High Power Field (HPF) program. HPFs count was performed 10 times. In the histopathology, the average scores were calculated in the Excel spreadsheet (Microsoft Excel Program), and suitable images were monitored (Olympus BX51) and illustrated in the DP5 camera attachment. Double-blind counts were carried out by the pathologists in the department. Degenerative and necrotic cells were counted in each HPF. According to this, degeneration in neurons (migrational neuron, or neural progenitor cells in forebrain and thalamic and substantia nigra' neuron of midbrain, Purkinje cells of cerebellum, giant motoric neurons of oblangatory medulla and

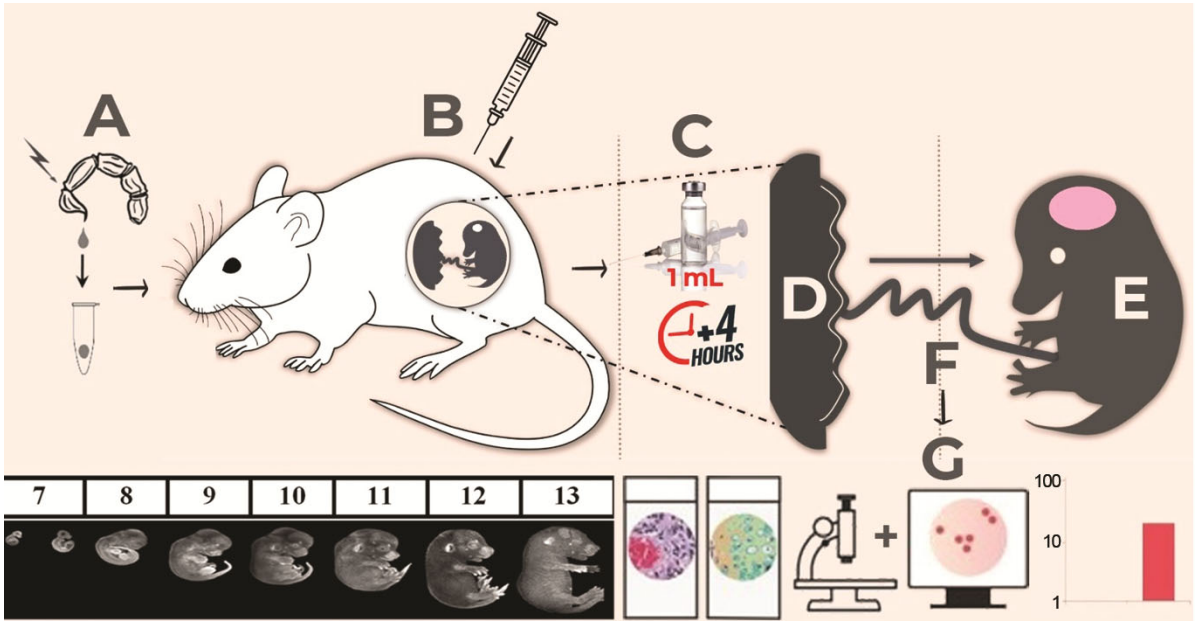

Fig. 1 — (A) *Androctonus turkiyensis* scorpion was milked via electrical stimulation. After centrifugation, the supernatant part of the venom was dissolved in PSS; (B) One mL of venom solution was injected daily during the organogenesis period (7th - 13th days of pregnancy); (C) After 4 h, 1 mL of specific antivenom was given. Pregnant rats were subjected to this procedure daily between 7 and 13 days; (D) Placenta; (E) Fetus; (F) An ovariohysterectomy was performed on the 21st day of pregnancy, and then all fetuses and their placenta were removed from uterine tissue; and (G) Analysis of immunoxpressions in fetal brain and cotyledons.

pons) and neuroglia of substantia alba were evaluated. In such kind of cells, degeneration showing cytoplasmic shrinkage and karyopyknosis or karyolysis and also necrosis were evaluated. For histomorphological studies of neonatal rat brain, atlas specified with brain localisations utilised [37]. Lateral view and sagittal sections of neonatal brain have been given in the Results section. Total average cell counts were obtained while counting degenerative and necrotic cells in several parts of the central nervous system (CNS). The scores were calculated by obtaining simple preporandence to total cell counts in the mentioned regions. The data were expressed as a percentage (%). The boundaries of scoring in histopathology were as follows. For cotyledon parts and CNS parts, the degeneration and necrotic cell counts were semi-quantitatively scored by double blind check. Negative (–): 0-10% in 10 HPFs, mild (+): 10-30% in 10 HPFs, moderate (++): 30-70% in 10 HPFs, strong (+++): 70-100% in 10 HPFs. The results were demonstrated in tabular data.

Obtaining immunoexpressions by immunoperoxidase method

The method was carried out according to the manual of Steptavidin-Biotin Complex Peroxidase (streptABC-P) kit (Novocastra, Leica, RE7120-K). Firstly, the adhesive slides including all parts of the brain were passed through xylene and alcohol series (5 min. for each) for deparaffinisation and rehydration. Antigen retrieval was performed via placing it into citrate buffer solution (pH 6.0, Bioptica, Italy) in a microwave oven at 750W for 25 min. The tissues were kept in 3% hydrogen peroxide (H_2O_2)-methanol solution for 15 min to eliminate endogenous peroxidase activity. Thereafter, the serum blockade was completed (Novocastra) and primary antibodies iNOS (Abcam, ab3523) for showing ROS activation, 4HNE, (1/500, ab46545, Abcam) for cell membrane lipid peroxidation, and 8OHdG, (1/500, ab62623, Abcam) for oxidative stress-inducing DNA damage markers in neuronal nuclei (1/400, ABIN802593, Antibodiesonline) and RIPK3 for showing necroptosis (1/200, Antibodiesonline.com, ABIN2792102) were dropped to sections and incubated in 37 centigrade degree for 60 min. Following this, the sections were incubated at 37 centigrade degree for 45 min. with biotin linked antibodies and Horse Radish Peroxidase (HRP)-marked antibodies, respectively. At the end of these steps, the sections were washed with TRIS hydrochloride (Tris-HCl)-Phosphate Buffered Saline (PBS) twice for 5 min. After that, the sections were kept under control in 3,3'-diaminobenzidine (DAB) chromogen for 5 min. At the end of this step and other previous steps, the sections were washed with TRIS hydrochloride (Tris-HCl) twice for 5 min. excluding blocking sera. For counterstaining, the sections were kept in Gill's hematoxylin for 1 min. Then, they were passed through alcohol and xylols and mounted using a mounting medium. For the negative control, PBS was used instead of primary antibodies.

During evaluation of positive reactions, neurons (migrational neuron,or neural progenitor cells in the forebrain and thalamic and substantia nigra' neuron of midbrain, Purkinje cells of cerebellum, giant motoric neurons of oblangatory medulla and pons) and neuroglia of substantia alba were evaluated. In this evaluational process, the brain localisations which specified as in neonatal rat brain atlas were utilised[37]. Lateral view and sagittal sections of neonatal brain have been given in the Results section. However, the counting was quantitatively performed by double blind check of positivities or immunoexpressions. All the immunoexpressions were quantified using an optical-light microscope at x400 magnification (High Power Field-HPF). HPFs count was performed 10 times. As in the histopathology, the average scores were calculated in the Excel spreadsheet (Microsoft Excel Program) and suitable images were monitored (Olympus BX51) and illustrated by DP5 camera attachment.

Statistical analysis of immunoexpression in fetal brain and cotyledons

The immuneexpressions were evaluated using the Graph Pad Prism 8.4.2 program (USA). The results were calculated as mean ± SD or percentage of total number in the Microsoft Excel Program. Two-way ANOVA and post-hoc Bonferroni tests in 95% confidence interval were used to compare the statistical significance of differences between groups. F values were calculated. The results with values P <0.05 were considered statistically significant.

Results

Histopathological analysis of cotyledoner zones

In the venom administration group, epithelial cells lining the yolk sac (YS) moderately degenerated in four cotyledons. Some of the cells were desquamated. Trophoblastic and syncytiotrophoblastic cells of labyrinth zone (LZ), as well as spongiotrophoblasts and trophoblastic giant cells of the basal zone (BZ),

were moderately degenerated in all cotyledons. The inner zone which was comprised of decidual cells of all was strongly affected from degeneration when compared to being the cells in upper zones. In the venom-antivenom administration group, YS epithelial cells, trophoblastic cells in the LZ and BZ, and the decidual cell were mildly degenerated or desquamated in all cases. However, in the control group, there were no outstanding histomorphological changes in the zones of all cases and results are summarised in Figs. 2 and 3.

Histopathological analysis of CNS

The central nervous system damage of cellular alterations (degeneration and necrosis) were observed in both experimental groups (EG and AVG). The cells included karyopyknotic and karyolytic nuclei as well as shrunk cytoplasm in dark pinked colour. Chromatolysis was present in cells which had undergone necrosis. General alterations were more comprised of acute cell swelling and vacuolar degeneration in the cytoplasm in these cells. Neurons, migratory cells as well as Purkinje cells were more affected by degeneration and necrosis. However, no reactive gliosis was observed against toxin exposure and glia cells were intact. In both groups, although all parts of the brain were affected by these damages, the

Localization	*Yolk sac*	*Labrynth zone*	*Basal zone*	*Decidual zone*	
Dams (n=6 for each group)	++	++	++	+++	EG
	+	+	+	+	AVG
	-	-	-	-	CG

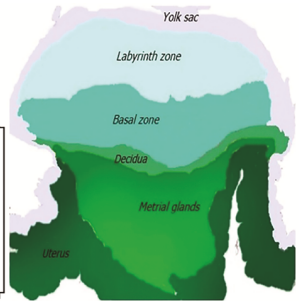

Fig. 2 — Histopathological scores according to cotyledoner zones in groups. (-): Negative, (+): Mild, (++): Moderate, (+++): Strong. The scheme at the right side illustrates the rat's cotyledoner parts.

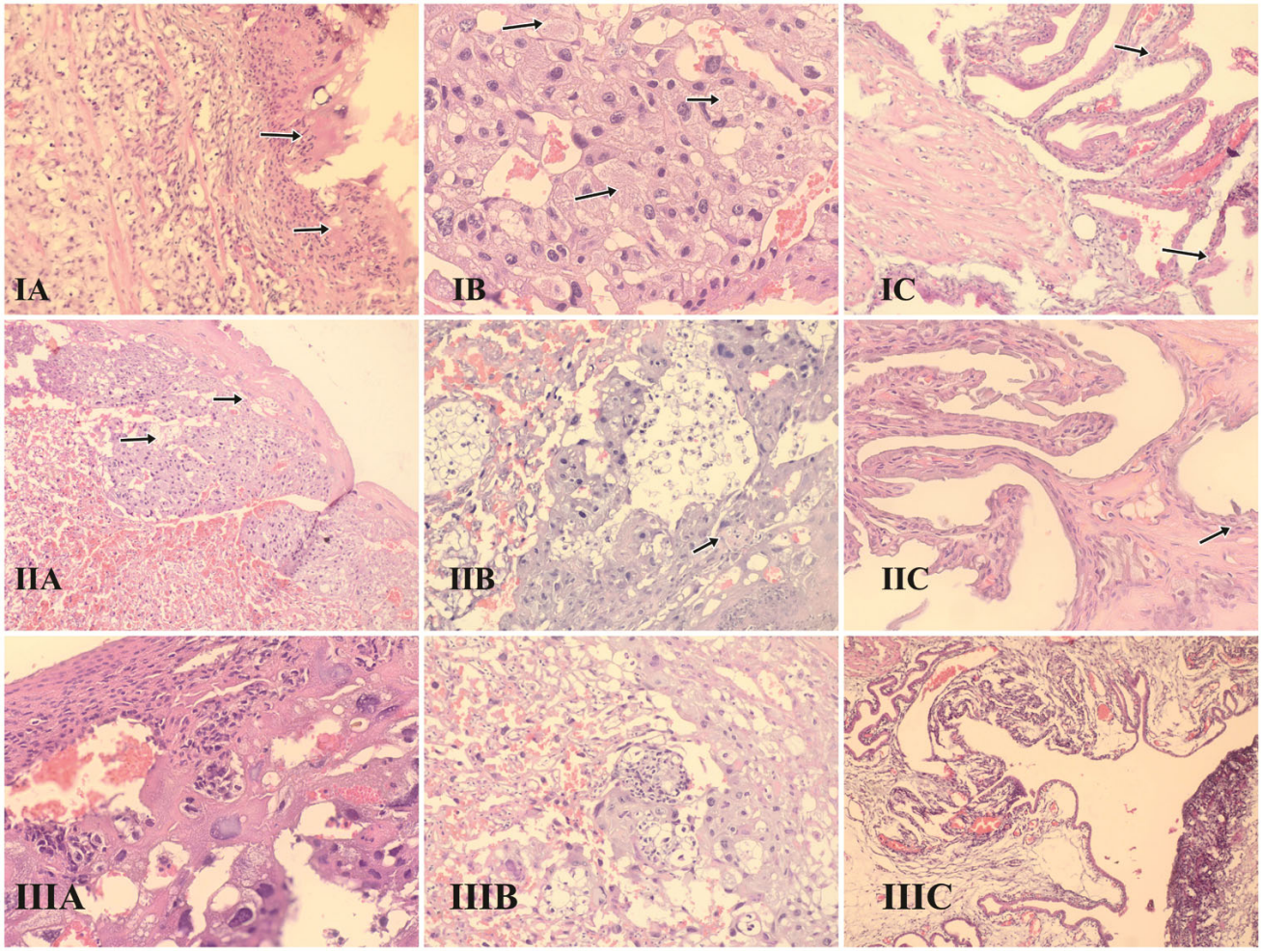

Fig. 3 — Histopathological findings in cotyledons of the envenomated group (I) antivenom group (II) and the control group (III). Degeneration of (A) epithelial cellyolk sac; (B) decidual cell; and (C) epithelial cell lining to the labrinth at arrows, 200X, H&E.

cellular destruction, predominantly necrosis was denser in the parts of the forebrain including the motoric cortex, somatosensoric cortex and lesser visual cortex. There were more degenerative changes in the midbrain parts, particularly hypocampus CA1 and CA2. Other parts of the midbrain, specifically the thalamus, substantia nigra and nucleus accumbens, these damages were milder or lesser then a mild degree. The fore- and midbrain parts of animals in EG were affected when compared with the animals of the AVG. However, giant motoric neurons in pons and oblangatory medulla and Purkinje cells in cerebellum placed in the hindbrain were less affected when compared to the fore- and midbrain in the EG and AVG., Neurons in the EG parts were more affected from degeneration compared to neurons of the hindbrain in the AVG.

Neuronal degeneration and necrosis occurance in the forebrain and midbrain in the EG, were found evidently in all offsprings. In contrast to strong alterations (degeneration and necrosis) in these parts, degeneration in neural progenitor cells (NPCs) which were found in the subgranular zone (SGZ) was milder in all of the offsprings of this group. In the remaining parts, including the hindbrain (cerebellum, pons, and obligatory medulla); degeneration and necrosis in Purkinje cells and motoric neurons were moderately observed in only four of the offsprings.

However, in the AVG, neuronal degeneration which was observed all over CNS was milder when compared to the previous group or the EG group. The alterations were found in the CNS of only of the four offsprings. But, in contrast to these experimental groups, in the CG animals, there was no meaningful neuronal degeneration or necrosis in all of the offsprings. The appearance of CNS histomorphology was intact among the CG offsprings. The histopathological scores are summarised in Fig. 4 and shown in Fig. 5.

iNOS, 4HNE, 8OHdG and RIPK3 immunoexpressions in cotyledons

iNOS expressions

In the EG, the expressions were more increased in decidual cells when compared to the epithelial cells of YS and trophoblastic cells found in other zones. The differences in decidual cells were significantly higher between those zones (P <0.05). However, in the AVG, the expression of epithelial cells of YS was more increased when compared to trophoblastic and decidual cells. The differences in YS cells were significantly increased in other zones (P <0.05). In the control group, there were some reactions, but these were evaluated as insignificant when compared to other parts (Table 1A).

Localization	Neural Progenitor Cell or NPC	Forebrain	Midbrain	Cerebellum	Pons	Oblongatory medulla
EG	+	+++	+++	++	++	++
AVG	+	+	+	+	+	+
CG	-	-	-	-	-	-

Fig. 4 — Histological scores according to the localisation in the central nervous system section of rat fetuses. (-): Negative, (+): Mild, (++): Moderate, (+++): Strong[36].

4HNE expressions

In the EG, the expressions in decidual cells were dramatically increased when compared with epithelial cells and trophoblastic cells of other zones (P <0.05). However, in the AVG, epithelial cells of YS and trophoblasts of LZ were more expressed even though there were no signs of increased significant expressions. Also, in the CG, those cells were mildly expressed in epithelial cell of YS as indicated in the AVG (Table 1A).

8OHdG expressions

In the EG, the distribution of 8OHdG-expressions in epithelial and trophoblastic cells in LZ, YS and DZ showed great similarity with iNOS expressions at each of the cotyledoner histological parts. However, in the AVG, 8OHdG expression in YS was lesser when compared to iNOS expression. In LZ, the 8OHdG expression was fairly densed when compared to iNOS expression. Although there was no statistical correlation (P >0.05) in terms of expression in YS and LZ, the differences between BZ and DZ were statistically significant and elevated among EG and AVG (P <0.05). The expressions in the CG were insignificant and the statistical correlation was increased when compared to the EG and AVG for each zones (P <0.05). However, the distribution of these positivities was the same as in the 4HNE expressions (Table 1A).

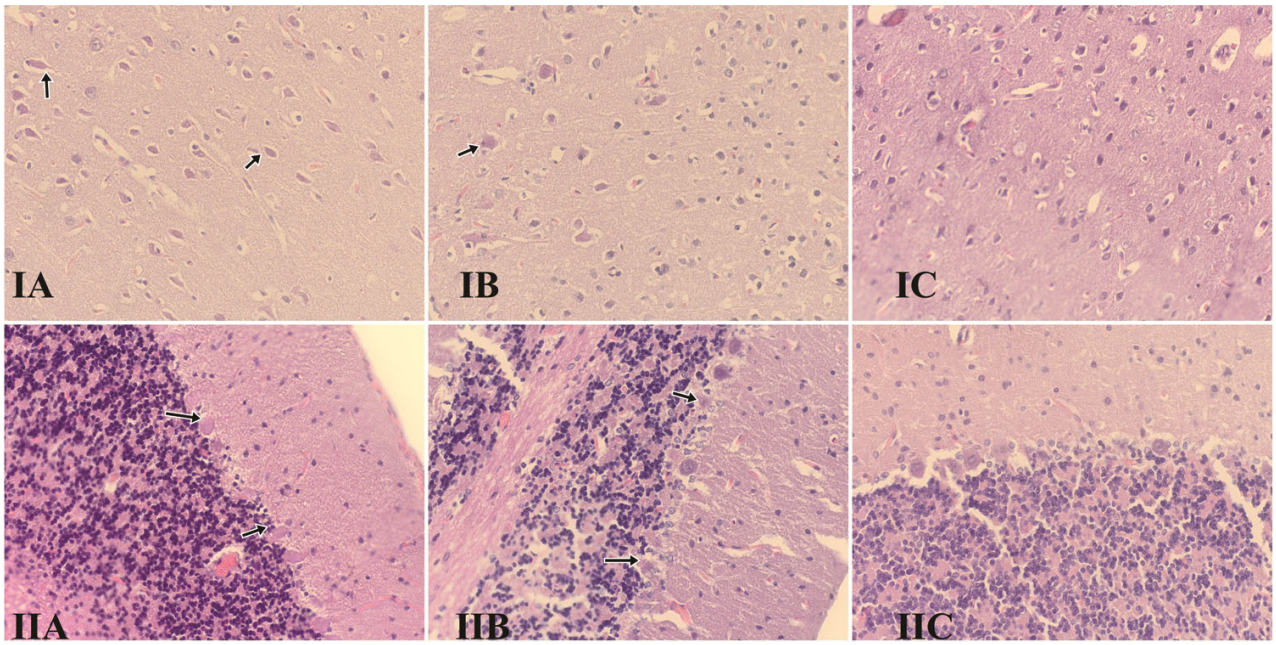

Fig. 5 — Histopathological findings in (A) the envenomated group; (B) *Androctonus turkiyensis* antivenom group; and (C) the control group. [Degeneration (arrows), intact cells (plain slide), the damages were localised in the motoric cortex region of cerebral cortex (I) in the forebrain; stratum pyramidale (Purkinje cells) substantia grisea of cerebellum (II) 200X, H&E]

Table 1 — Immunoexpressions in (A) Cotyledoner; and (B) Brain parts of rat fetuses (each group; n=6)

	cotyledoner				brain						
Localisation / Markers	Yolk sac	Labyrtnh zone	Basal zone	Decidual zone	Neural Progenitor Cell	Fore-brain	Mid-brain	Cerebellum	Pons	Oblongatory medulla	
iNOS	7±1.21	3±0.32	9±2.00	43±4.28	-	-	-	-	-	-	EG
	28±2.28	3±1.03	2±0.71	4±0.15	-	-	-	-	-	-	AVG
	1±0.64	1±0.92	1±0.48	5±1.20	-	-	-	-	-	-	CG
4HNE	9±1.12	7±1.32	9±1.12	52±1.72	0±1.12	48±1.23	26±1.16	23±1.94	24±0.76	24±1.18	EG
	11±0.32	10±0.75	2±0.41	5±1.55	9±0.34	14±1.11	8±2.31	19±0.63	19±0.42	19±1.47	AVG
	6±1.33	1±0.18	1±0.54	5±0.12	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	CG
8OHdG	12±1.12	6±1.12	10±1.32	55±1.63	1±1.21	35±2.13	68±1.22	30±3.19	27±2.17	17±2.83	EG
	11±3.19	10±2.94	2±1.28	5±1.96	10±1.32	6±2.24	1±1.15	2±1.23	1±1.86	2±2.16	AVG
	9±1.12	1±1.12	2±1.12	4±0.72	0±0.14	0±0.17	0±0.31	0±0.00	0±0.00	0±0.00	CG
RIPK3	22±1.72	2±0.42	2±0.67	14±0.90	13±1.42	32±2.14	36±3.11	14±1.20	19±1.72	20±2.29	EG
	10±0.55	6±1.23	0±1.83	2±1.47	11±1.21	3±0.83	4±0.49	2±1.15	5±0.00	3±0.00	AVG
	7±1.00	4±0.74	1±1.63	1±1.57	0±0.23	0±0.31	0±0.36	0±0.14	0±0.00	0±0.00	CG

RIPK3 expressions

In the EG, the expressions were increasingly found in epithelial cells of YS and decidual cells when compared to trophoblastic cells in other zones. However, in the AVG, the expressions generally decreased. The differences between the groups significantly increased in the YS and DZ parts of cotyledon (P <0.05) although there were higher expressions in epithelial cells of YS relative to trophoblastic and decidual cells. The distribution of expressions in the CG showed similarity to the AVG group; but, lesser in those zones (Table 1A and Fig. 6).

HNE, 8OHdG and RIPK3 immunoexpressions in fetal CNS parts

4HNE expressions

In the EG, the expressions were more elevated in terms of neurons of the forebrain and midbrain. 4HNE expressions and other expressions (8OHdG and RIPK3) in Purkinje cell and motoric neurons of the hindbrain part were found higher in degree when compared to the expressions of neural progenitor cells (NPC). However, there were more positivities in the parts of the forebrain including somatosensoric cortex and motoric cortex (P <0.05). In this context, in the EG, hindbrain expressions were decreased when

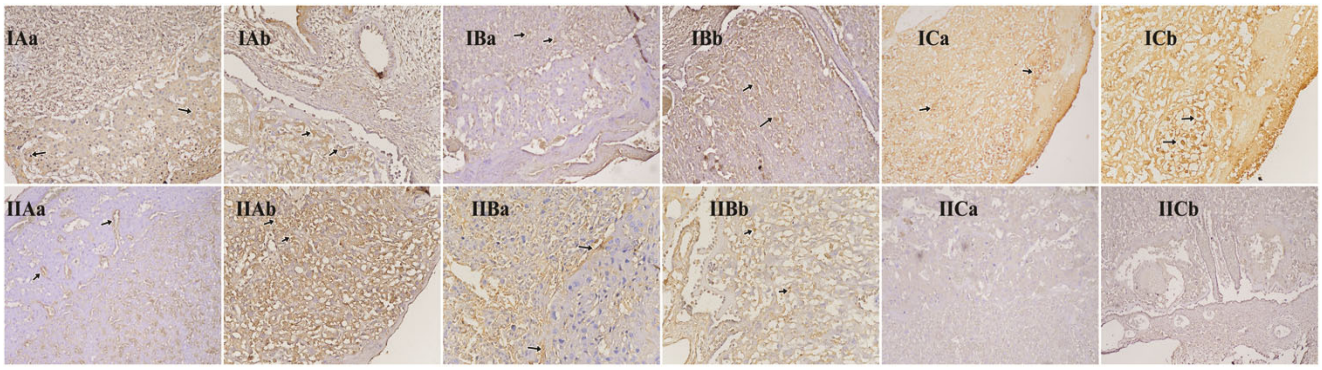

Fig. 6 — (A) 4HNE; (B) 8OHdG; and (C) RIPK3 expressions in cotyledons of the envenomated group (I) and the antivenom group (II). The reactions were demonstrated in (a) Decidual zone; and (b)Labyrnth zone. [The positivities at arrows, 200X, DAB chromogen, Strept ABC-P staining]

compared to the fore- and midbrain parts. According to other experimental groups' expression at the same parts, they were still high as many as in the fore- and midbrain expressions. In the AVG, the reactions in all of the CNS parts were lower when compared to the EG offsprings. However, NPC expressions were more increased according to the previous group. In the CG, there was almost no reaction in the neurons. Amongst the EG, AVG and CG expressions were significantly (P <0.05) increased (Table 1B).

8OHdG expressions

In the EG and AVG, the expressions showed great similarity with the 4HNE expressions in all sections over the CNS. However, in the AVG, the expression of NPC was more elevated. In this group, NPC expressions did not change and preserved stability in spite of that the expressions in the other sections of the brain were found different from the AVG. In the control group, the expressions were insignificant and there were no reaction in any parts. The EG and AVG expressions were significantly (P <0.05) found higher in comparison to the CG expressions in all brain sections (Table 1B).

RIPK3 expressions

In the EG and AVG, the distribution of expressions showed again great similarity with the previous stainings including 4HNE and 8OHdG. The same situation in NPC was observed in spite of the results which were obtained from AVG. In the CG, the expressions were insignificant. Amongst EG, AVG and CG, expressions were significantly (P <0.05) increased. For this reason, EG and AVG expressions in all sections were dramatically elevated in all parts of the brain. (Table 1B and Fig. 7).

Discussion

In this study, we investigated the possible effect of antivenom on cotyledon and brain in pregnant rats inoculated with *A. turkiyensis* venom during fetal organogenesis. As is known, the several scorpion species due to their toxins can be dangerous for humans and animals. Amongst the worst scenario, it has been observed to induce under exposure among pregnant women and children[3,7,38,39]. Although there is no damage in pregnant women, the toxin exposure can result in miscarriages, fetal resorption, placental or fetal abnormalities in the brain while fetal development continues[6,7,9,10]. Venom may be not affect placental tissues and cause direct damage to the fetus[13]. However, in such situations, increasing hypoxia by free radical production disturbs microvascular nourishment. H_2O_2 and nitric oxides (NOS) damage the cytosolic enzymes which are necessary for cellular aerobic metabolism by changing vessel permeability. Ongoing reactions continue with several activations of protein kinases, calcium ion accumulation, leukocyte and platelet activation in association with cytokine release (IL-1β and TNF-α)[17,19]. In particular, free radicals (FRs) including ROS and NOS transfers to developing embryo or fetus by means of the placenta may damage vital organs such as the brain[18,21,40]. Hence, ROS can highly affect the embryo or fetal development by the overproduction of FRs. After 2 h of the generation of FRs, ROS or NOS, this situation causes vasoconstriction and abnormal placental blood flow. FRs affect the trophoblast cells and cells in the villous stroma, damaging mitochondrial DNA. Furthermore, after the increase of FRs in both the placenta and fetal tissues, fetal vascularisation has been also disturbed. Reactive microglial cells of fetal

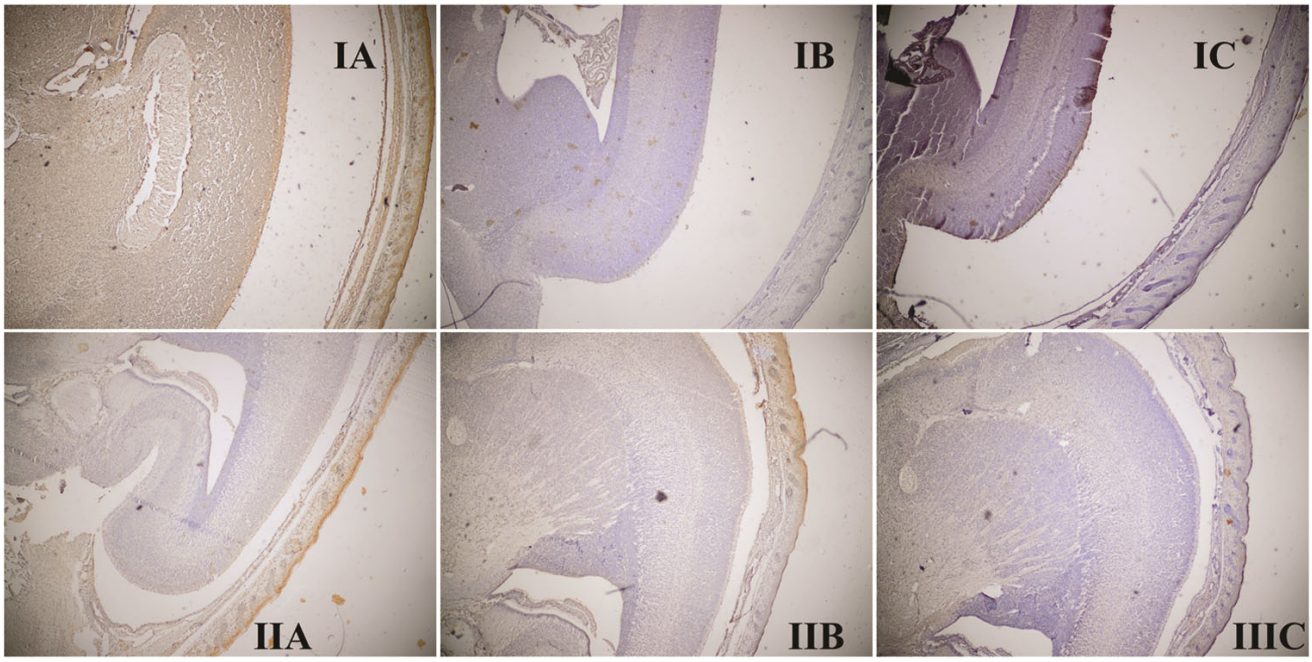

Fig. 7 — (A) 4HNE; (B) 8OHdG; and (C) RIPK3 positivities in fetal brain of the envenomated group (I) and the antivenom group (II). The brownish-colored reactions were localised among the neurons of motor cortex of the cerebrum as well as the hypothalamic region of the midbrain. [The whole fetal brain, 4X, DAB chromogen, Strept ABC-P staining]

CNS release ROS. It has been known for a long time that increasing FRs also changes the aerobic microenvironment in the fetal brain[41,42]. In our study, for showing ROS activation in cells, we utilised the iNOS marker. And also, we demonstrated increasing iNOS activation in all zones of cotyledons in parallel to histomorphological alterations (degeneration and necrosis). In the antivenom administrated group, the iNOS expressions were reduced dramatically apart from epitheliums of YS. We hypothesise that the zone is found at the upper side and it is the primary checkpoint regarding the existence of the elevated FRs. The histomorphological changes and iNOS expressions were elevated here because the epithelial cells are exposed to a harmful effect.

On the other side, it is reported that the fetal brain is vulnerable to increasing FRs due to its high rate of oxygen consumption and due to increased levels of iron and polyunsaturated fatty acids[42]. Regarding the polyunsaturation, it is reported that a chemical, 4HNE, is developed after FRs steal electrons from covalent ligands of proteins, DNA, and phospholipids containing an amino group. A polyunsaturation process in fatty acids also occur in the placenta and brain[43,44]. In our study, both the fetal brain and cotyledons reacted higher with 4HNE in the venom-administrated group. Accordingly, reactions were diminished in the antivenom administrated group. The study results show that the antivenom may have a preventive effect on the polyunsaturation process of the lipid in the brain and placenta.

Increasing ROS can be also potentially harmful to cellular DNA. In this sense, it is stated that 8OHdG is a major product formed by hydroxyl radical attack on the guanine residues of DNA. For 8OHdG, it can be suddenly increased against oxidative damage. Hence, it is a useful tool for showing oxidative stress on DNA. It has been investigated in cases including both placenta and brain as well as environmental toxicity models in rats[45-47]. In our study, elevated 8OHdG expressions were observed in neurons in almost all parts of the brain and cotyledon cells in the venom administrated group. However, the high expressions were reduced in the antivenom administrated group apart from NPCs and epitheliums of YS in the antivenom group. The reactions had similar to RIPK3 reactions in terms of distribution and positivities in the antivenom group. Hence, elevated 8OHdG and RIPK3 expressions are associated with elevated ROS activities.

Moreover, increasing oxidative stress triggers mitochondrial stress, dysregulation of calcium homeostasis as well as dysregulation in energy metabolism. As result, impairment in neurogenesis and cell death occurs by apoptotic signals. These situations ultimately alter cellular morphology[48,49]. Similar to apoptotic cell death, necroptosis can take place against

ROS activation by possible tumor necrosis factor alpha (TNF-α) activation. The molecule may create a complex with Receptor-interacting protein or RIP3[50]. In our study, some cellular alterations (degeneration and necrosis) were observed in the nuclei and cytoplasm of the neuron and glial cells as well as YC epitheliums, trophoblastic and decidual cells of the cotyledon. In great similarity with degeneration in those cells, RIPK3 reactions were increased in particularly of the fore- and midbrain organ sections of the venom administrated group. This situation shows us that cellular alterations undergo necroptosis by induction of RIPK3 expressions in cells. In contrast to that, both neuronal degeneration in histomorphology and RIPK3 expressions were diminished in the antivenom administrated group. However, elevated RIPK3 expressions did not change much in NPCs in the antivenom administrated group. Those expressions were still increasingly found in part of the brain in both experimental groups. The high RIPK3 reactions were suggested to be incorporated from other parts because the progenitor cells or NPC have high mitotic and proliferative activity. And also, the high reactions were similar to high 4HNE and 8OHdG-reactions in NPCs.

Conclusion

This study has demonstrated that ingression of scorpion toxin in the body of pregnant women could lead to increasing ROS activation in the critical organogenesis period of the fetal brain. Neuronal damage particularly within the fore and midbrain sections can occur. Increasing iNOS activation and accordingly lipid peroxidation in rich content tissues and DNA damage may be elevated after scorpion sting. However, antivenom has partly prevented the harmful effect primarily on the placenta and the developing brain.

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Conflict of Interest

Authors declare no competing interests.

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