Maternal exposure to *Bothrops jararaca* snake venom: effects in mice offsprings

Exposição maternal ao veneno de Bothrops jararaca: efeitos na prole de camundongos

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Abstract

Objective – *Bothrops jararaca* snake is involved in almost 90% of all reports in the State of São Paulo, Brazil. Little is known about the effect of maternal exposure to *B. jararaca* snake venom [*BjV*] on fetal development. This study was designed to investigate the effect of a moderate dose of the venom (1.2 mg/kg sc on either gestation day GD5 or GD12), in pregnant mice and their offspring. **Methods** – In dams, during pregnancy, it was observed the body weight gain, food and water consumptions. In the last day of pregnancy, dams were submitted to a cesarean and the reproductive performance was measured. Thus, the fetuses body weight, the number of live and dead fetuses as wells as the external, visceral or skeletal alterations were assessed. **Results** – Results showed that the venom injection on GD5 did not change the dams weight and reproductive parameters, the fetuses weight, but it was observed high incidence of skeletal anomalies such as incomplete skull ossification and supernumerary ribs relative to controls. Dams treated in GD11 showed decreased food ingestion in the day after treatment. Their offspring presented a high incidence of skeletal anomalies such as vertebrae anomalies, sternebrae anomalies and incomplete skull ossification, which might be a sign of craniostenosis, than controls. **Conclusions** – In conclusion, subcutaneous administration of 1.2 mg/kg *BjV* to pregnant mice either at GD4 or GD12 produced subtle maternal toxicity but a clear fetotoxicity. Whether these observations represent a reaction to treatment and, if so, the underlying mechanisms and their toxicological impact remain to be examined further in future studies.

Descriptors: Snake venoms; *Bothrops jararaca*; Pregnancy; Congenital abnormalities

Resumo

Objetivo – O veneno de *Bothrops jararaca* está envolvido em quase 90% dos casos de envenenamento do Estado de São Paulo, Brasil. Pouco se sabe sobre o efeito no desenvolvimento fetal quando no caso de envenenamento materno. Este estudo examinou, em camundongas prenhes e sua prole, os efeitos do envenenamento por uma dose moderada do veneno de *B. jararaca* (1,2 mg/kg sc no 5° (GD5) e 11° (GD11) da gestação. **Métodos** – Anotou-se durante a gestação o ganho de peso corporal e o consumo de água e comida materno; no último dia de gestação, as mães foram submetidas a uma cesariana e a performance reprodutiva foi avaliada. Para tanto, anotou-se o peso dos fetos, o número de fetos vivos e mortos, assim como examinou-se a presença de alterações externas, esqueléticas, e viscerais. **Resultados** – Os resultados mostraram que a injeção do veneno no GD5 não alterou o peso materno, o consumo de água e ração, o peso dos fetos e os parâmetros reprodutivos, tendo sido observada alta incidência de anomalias esqueléticas tais como ossificação do crânio incompleta e costelas supranumerárias em relação aos controles. O envenenamento no GD11 promoveu decréscimo na ingestão de alimentos no dia subsequente ao tratamento. Neste caso, a prole apresentou alta incidência de anomalias esqueléticas tais como anomalias vertebrais, do esterno e ossificação incompleta do crânio, ou seja, craniostenose. **Conclusões** – O envenenamento moderado pelo veneno da *B. jararaca* em camundongas prenhas tanto no GD5 como no GD11 produziram efeitos sutís ao nível materno, porém nos fetos, apareceram severas alterações. Estudos futuros deverão ser feitos para entender os mecanismos subjacentes a este envenenamento durante a gestação.

Descritores: Venenos de serpentes; *Bothrops jararaca*; Gravidez; Anormalidades congênitas

Introduction

Bothrops jararaca snake venom (BjV) is a complex mixture of proteins with several activities, such as coagulant, proteolytic and hemorrhagic ones and contain factors capable of activating the blood coagulation such as X, II and I that together induce clotting of fibrinogen[1-4]. *BjV* also a source of zinc-dependent metalloproteinases and hemorrhagins, responsible for its hemorrhagic activity. Reports of snakebite accidents, including accidental Bothrops envenomation[5-7], caused high fetal wastage, high morbidity or mortality rates among pregnant women[8-12].

Women represent 20% of snakebite accidents[13] among them 5% are pregnant women[10,14]. During pregnancy, snakebites could be a risk to gestation maintenance[7,10-11] which depends on maternal envenomation degree, the pregnancy period as well as the time to the beginning of envenomation. Obstetrical consequences such as maternal death (10%) and fetal wastage (21,4% to 43%) can occur in some cases[12].

Although *BjV* is involved in almost 90% of all reports in the State of São Paulo, Brazil, little is known about the effects of prenatal exposure to its venom, and no information is available to aid the rational treatment of victims stung during pregnancy. Some experimental and clinical data suggest that snakebite accidents at the beginning of pregnancy have unfavorable prognostic[11]. Pardal *et al.*[10] (1997) could observe that the accidents were more frequent in the first trimester of pregnancy (75% of the cases).

One study in laboratory mice[15] showed that intramuscular injection of *BjV* (0.24 mg/kg body weight) on day 8 of pregnancy produces severe injuries at maternal and fetuses levels. Thus, it is not clear whether disturbances during the development of pregnancy are due to a direct effect of venom on uterus/fetus or to homeostatic changes in dams or both of them. In spite of snakebite envenomation during pregnancy not being completely elucidated, the exposure to those venom during this period could lead to teratogenic effects, fetal growth retardant and mutation[11]. Experimental records about the *BjV* teratogenic effect during pregnancy do not exist in literature. This study was designed to investigate if the *BjV* administered on implantation (GD5) and organogenic (GD12) periods of mice pregnancy in a dose that

causes did not induces severe envenomation may lead to deleterious effects in their offspring. In this sense, no reports of envenomation cases in the 3º trimester of pregnancy. Therefore, we investigated the effects of the venom administered to pregnant mice on GD5 or GD12.

Methods

Animals and treatments

The experiments followed the guidelines of the Bioethics Commission of the School of Veterinary Medicine of University of São Paulo, Brazil and were approved by the Commission protocol. Female Swiss adult male and female mice were used throughout this study. They were maintained in a temperature and light-controlled room (24 ± 2ºC, 12h light/dark), with water and food *ad libitum*. Lyophilised venom of *B jararaca* was obtained from the Laboratory of Hepertology of Butantan Institute, São Paulo, Brasil, and stored at –20ºC. *BjV*, 1.2 mg/kg, was dissolved in sterile physiological saline in the moment of use and administered by subcutaneous route (sc). After acclimation for 1 week, two female mice were placed together with one male in the afternoon. The next morning, females showing evidence of mating (vaginal plug) were housed in groups of two in plastic cages measuring 40 cm x 50 cm x 20 cm and covered with metal lids. This day is designated as GD0. Males and non-pregnant females were euthanized and discarded. Twenty four pregnant female mice were randomly assigned to 2 groups: one used to implantation period treatment and the other to organogenic period treatment. The venom dose employed was 1.2 mg/kg. Other two groups, control groups with 12 pregnant female mice each, received the sterile saline solution 0,9% on GD5 or GD12, respectively. The subcutaneous route was chosen because this is the way by which the venom is injected in accidents.

The pregnant mice were weighted every other day until treatment day. Food and water consumption were recorded on the same days weighing. Other parameters, as weight gain and physical alterations (edema, tissue necrosis, local bleeding), were also observed to investigate the maternal toxicity. On the GD19 the dams were anesthetized with halothane and their uterus removed. The unopened uterus were weighted and used to calculations of maternal real weight (maternal weight on GD19 minus unopenend uterus weigh). The fetuses and its respectively placentas were macroscopic examined and individually weighted. The ovaries were also removed and the *corpora lutea* were counted. The rate of preimplantation loss was calculated as: nº of *corpora lutea* – nº of implantation x 100/ nº of corpora lutea, and postimplantation loss rate was calculated as: nº of implantation[16] – nº of live fetuses x 100/ nº of implantation[16]. If the fetus development or visible implantation sites did not occur, the female uterine horns were removed and tested to resorptions sites by Salewsky test[17].

The fetuses were examined macroscopically for external abnormalities, and the following parameters were observed: skull form, ears, eye, mouth and palate implantation, tail and foot conforming and anal drilling. Following, sexing and weighing of each fetus were done as well as its placentas. Half of each litter was fixed in Bouin's solution for subsequent visceral examination by Wilson's serial section method[18], and the other half was stained with Alizarin red by the technique of Staples and Schnell[19] to identify skeletal alterations.

Statistical analysis

Maternal toxicity, reproductive performance and ossification centers data were analyzed by Tukey-Kramer test[20]. The external, skeletal and visceral malformations/abnormalities was analyzed by the Fisher test[21]. The level of significance was set at $p < 0,05$.

Results

Maternal effects of the BjV administration on GD5 or GD12

The total weight gain, weight gain one day after both *BjV* administrations (GD4 to GD5 or GD12) and the real weight gain were not statistically different between all groups (data not show). Relative to control group, also the food and water consumptions of GD5 treated females did not show statistical differences (data not show). The GD12 treated females had a lower food consumption than controls on GD12 (control = 7.88 ± 1.61; experi-

Table 1 – Maternal performance of pregnant mice treated or not with 1,2 mg/kg Bothrops jararaca venom at the 5th or 12th day of pregnancy: Control: females treated with saline (sc); experimental: females treated with *Bothrops jararaca* venom (sc)

	5th		12th	
	Control	**Experimental**	**Control**	**Experimental**
Corpora lutea Total number (mean ± SD)	167 13,92 ± 1,98	155 12.92 ± 2,11	150 12.50 ± 1.62	150 12.50 ± 1.98
Implantation (mean ± SD)	13,00 ± 1.95	12,00 ± 2.30	11.38 ± 1.19	11.38 ± 1.95
Live fetuses (means ± SD)	11.38 ± 1.34	11.00 ± 2.56	10.67 ± 1.56	9.92 ± 2.81
Dead fetuses (means ± SD)	2	0	1	1
Resorptions (means ± SD)	1.00 ± 1.28	1.00 ± 0.85	1.08 ± 1.24	1.83 ± 1.59
Preimplantation (%)	6.40	14.00	4.81	5.18
Postimplantation (%)	8.17	8.80	9.78	17.07
Unopened uterus weight (g) (mean ± SD)	19.18 ± 2.54	17.81 ± 3.79	17.14 ± 2.38	15.81 ± 3.50
Fetal weight (mean ± SD)	1.12 ± 0.08	1.12 ± 0.08	1.19 ± 0.09	1.17 ± 0.11
Placenta weight (mean ± SD)	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Females (means ± SD)	5.50 ± 1.78	5.25 ± 1.60	4.50 ± 1.45	5.17 ± 2.44
Males (mean ± SD)	6.33 ± 2.35	5.75 ± 1.77	6.17 ± 1.34	4.75 ± 1.60

n = 12 animals/group. No signficant difference was found between all groups ($p > 0.05$; ANOVA)

mental = 5.46 ± 1.08, $p < 0.01$, ANOVA followed by the Tukey-Kramer test), whereas the water consumption was not statistically different (data not show).

The maternal performance of both treated groups did not presented differences in relation to its respective control group. Thus, no statistical differences were detected in the number of *corpora lutea*, implantations number, live fetuses, resorptions, preimplantation and postimplantation losses, unopened uterus weight, fetal weight, placental weight, number of male and female (data not show). Also, no macroscopically abnormalities of placenta were found in all groups (data not show).

Offspring effects of the BjV administration on GD5 or GD12

On GD5 malformation was found in one fetus (exencefalia), but no statistic differences were detected in relation to the control group. Results show a high incidence of anomalies on experimental fetuses such as incomplete skull ossification and 14th rib presence, compared with control group (Table 1). Visceral malformation and anomalies were found in all groups but without statistically difference (Table 2).

On GD12 external anomalies occurred in just one fetus of control group while no external malformations and skeletal malformation and/or skeletal anomalies were not observed in all groups. Vertebral anomalies and lower incidence of incomplete skull ossification were observed in experimental group (Table 1). No visceral malformations or anomalies were detected in both control and treated groups (Table 3).

Discussion and Conclusions

Present results showed that *BjV* injection on GD5 did not change the dams weight and reproductive parameters, the fetuses weight, but it was observed high incidence of skeletal anomalies such as incomplete skull ossification and supernumerary ribs relative to controls. Dams treated in GD12 showed a decreased food ingestion in the day after treatment. Their offspring presented a high incidence of skeletal anomalies such as vertebrae anomalies, sternebrae anomalies and incomplete skull ossification, which might be a sign of craniostenosis, than controls.

The schedule of treatment using a single dose of the venom was choosing because repeated contact with the venom is rare in humans. In addition, the 1.2 mg/kg dose was used in the present experiments because was unable to induce hemorrhage and 3.6 times smaller than the 50% lethal dose[22].

The venom was injected at two different stages of pregnancy, i.e., in GD5, the end of the period of implantation of the blastocyst in the uterus and in GD12, the mice is in the middle of the period of organogenesis, to study if the *BjV* induces abortion or teratogenesis, respectively.

GD5 exposure to *BjV* induced several envenomation signs in dams (edema, local bleeding and pain), but those did not interfered on water and food consumption neither on weight gain of all animals. Similar data were observed in GD12 treated mice, except in food consumption at GD12, one day after envenomation, suggesting a greater sensitivity to venom in this period than in GD5. Thus maternal homeostasis during pregnancy periods examined was slightly modified by the venom exposure[23].

To evaluate interference on fetal development produced by the venom, fetal weight, unopened uterus weight and ossification centers were recorded on GD5 and GD12 treated mice. Alterations on the placenta also can lead to lower development of the concept because it transports glucose, oxygen, essential aminoacids and lipids[24]. Also the percentage of female and male fetuses could involved in maternal envenomation[25]. By this way, the placentas were observed macroscopically and individually weighed and results did not show differences between controls and experimental dams.

The envenomation effects on incidence of external, visceral and skeletal malformation/anomalies were evaluated by inspection after euthanasia.

On GD5 treatments, experimental groups showed the presence of external malformation, however, it occurs in just one fetus what could not be related to venom exposure. On GD5 and GD12, skeletal malformations were not observed in all groups; skeletal anomalies were observed in all groups, but the experimental group showed statistic differences when compared to control group. Particularly, incomplete skull ossification was increased in both experimental groups. Experimental and control groups showed higher rates of supernumerary ribs. Supernumerary ribs is usually associate to stress during the treatment[26]. The visceral malformations and anomalies were observed in all groups, but were not statistically different.

Experimental data show that fast during 24 hours in pregnant mice can induce fetuses vertebral defect[25]. Based on this, reduction on food consumption after venom treatment on 12th day of pregnancy it could be associated to the higher rate of vertebral defects. In addition, the same thing were not observed in fetuses of dams treated

Table 2. Incidence of external and skeletal malformations/anomalies and ossification centers from litters treated or not with 1,2mg/kg *Bothrops jararaca* venom at the 5th or 12th day of pregnancy: Control: females treated with saline (sc); experimental: females treated with *Bothrops jararaca* venom (sc)

	5th		12th	
	Control	**Experimental**	**Control**	**Experimental**
Number of fetuses	71	65	65	56
Number of litters	12	12	12	12
External malformations				
Affected fetuses	0	1	1	0
Affected litters	0	1	0	0
Skeletal malformations				
Affected fetuses	0	0	0	0
Affected litters	0	0	0	0
Skeletal anomalies				
Affected fetuses	56	**65a**	59	51
Affected litters	12	12	12	12
Incomplete skull ossification	20	**44a**	39	**64a**
Esternebrys anomalies	37	31	38	46
Vertebral anomalies	#	#	0	**4b**
14th rib	41	**56a**	50	41
Incomplete pelvic ossification	8	8d		

a: statistically different from respective control group ($p<0.01$, Fisher test)
b: statistically different from respective control group ($p<0.05$, Fisher test)

Table 3. Incidence of visceral malformations/anomalies and ossification centers from litters treated or not with 1,2mg/kg *Bothrops jararaca* venom at the 5th or 12th day of pregnancy: Control: females treated with saline (sc); experimental: females treated with Bothrops jararaca venom (sc)

	5th		12th	
	Control	**Experimental**	**Control**	**Experimental**
Visceral malformations				
Affected fetuses	23	23	22	21
Affected litters	10	12	12	12
Ectopic kidney	21	20	22	18
Hypoplasic kidney	8	5	6	7
Hypoplasic testis	0	1	#	#
Visceral anomalies				
Affected fetuses	26	30	23	25
Affected litters	8	11	11	11
Gonadal ectopic	26	29	23	24
Peritoneal bleeding	0	1	2	2
Cervical vertebrae	7.0	7.0	7.0	7.0
	(7.0 ; 7.0)	(7.0 ; 7.0)	(7.0 ; 7.0)	(7.0 ; 7.0)
Phalanges previous	8.0	8.0	8.0	8.0
	(6.0 ; 8.0)	(6.0 ; 8.0)	(6.0 ; 8.0)	(4.0 ; 8.0)
Metacarpal	8.0	8.0	8.0	8.0
	(8.0 ; 8.0)	(8.0 ; 8.0)	(8.0 ; 8.0)	(6.0 ; 8.0)
Esternebrys	6.0	6.0	6.0	6.0
	(6.0 ; 6.0)	(6.0 ; 6.0)	(6.0 ; 6.0)	(6.0 ; 6.0)
Metatarsal	10.0	10.0	10.0	10.0
	(10.0 ; 10.0)	(10.0 ; 10.0)	(10.0 ; 10.0)	(10.0 ; 10.0)
Phalanges subsequent	8.8	8.4	8.9	8.3
	(6.0 ; 10.0)	(8.0 ; 10.0)	(6.0 ; 10.0)	(0.0 ; 10.0)
Caudal vertebrae	4.8	4.5	5.0	4.7
	(4.0 ; 5.0)	(3.0 ; 5.0)	(4.0 ; 5.0)	(3.0 ; 5.0)
Total	52.2	52.4	52.7	52.0
	(45.0 ; 54.0)	(49.0 ; 54.0)	(45.0 ; 54.0)	(37.0 ; 54.0)

No significant difference was found between all groups (p>0.05; ANOVA)

at the GD5, a period not critical to vertebrae development (Table 3). The litters from experimental group treated on GD12 also presented reduction on incidence of incomplete skull ossification that was significantly different when compared with control group. The increase of appearance of 14th rib on litters from the females treated at GD5 could be due to stress induced by the venom treatment[26].

Visceral malformation was present in two fetuses on control group that could be associated with natural incidence of it. Other visceral malformations such as ectopic kidney, hypoplasic kidney and anomalies as ectopic gonadal, peritoneal bleeding were observed in all groups, but did not present statistic differences between control and experimental groups.

The incidence of alterations observed on fetuses from experimental group in both treatment periods are the same presented in others toxicological tests[27], which indicate that those alterations are due to maternal physiological disturbance and not to the venom.

All data showed that *BjV* did not alter reproductive performance of mice at implantation and organogenic periods, but increased skeletal anomalies on litters due to maternal envenomation; anomalies that could be signs of embryotoxicity.

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