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## THE INFLUENCE OF THE WATER-SALT EXTRACT OF THE MEDICINAL LEECH *HIRUDO VERBANA* CARENA, 1820 ON THE GENERAL COURSE OF EMBRYOGENESIS IN RATS AFTER INTRAPERITONEAL ADMINISTRATION

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**Background.** The increase in infectious and non-infectious diseases occurrence during pregnancy prompts scientists to search for substances that can fight these ailments. This especially applies to natural substances that have virtually no side effects. These include substances obtained from medicinal leeches. These substances have a wide range of therapeutic effects; as to their practical use, all preclinical studies must be completed. This particularly refers to the presence of an embryotoxic effect. The aim of this work was to investigate the effect of the water-salt extract of the medicinal leech *Hirudo verbana* Carena, 1820 on the general course of embryogenesis in rats when administered intraperitoneally.

**Materials and Methods.** The work was performed on female non-linear white rats. Two experimental groups of animals were injected intraperitoneally with a water-salt extract of the medicinal leech at a concentration of 5 µg/g of the animal, in a volume of 0.5 mL, three times before mating and three times during pregnancy every 3 days. Two control groups were injected intraperitoneally with physiological saline according to the same scheme as the experimental groups. Subsequently, one of the control and experimental groups underwent euthanasia, dissection of the abdominal cavity and uterine horns on the 20th day of pregnancy. We analyzed the total number of corpora lutea of pregnancy, the number of live and dead fetuses. Fetuses were weighed, examined for the presence of skin hematomas, mutilations, and external abnormalities in the development of internal organs. The other two groups of control and experimental animals were left until the offspring were born. Then the females of both groups were weighed, and their general condition was assessed. Newborn rats were examined, weight and body length were determined, mortality was recorded from the beginning of birth to 15 days,



the presence of skin hematomas, mutilations, and external anomalies of the development of internal organs were checked.

**Results and discussion.** The study established that the intraperitoneal injection of a water-salt extract of a medicinal leech before and during pregnancy in female rats does not cause any toxic effect during embryogenesis and at the early stages of post-embryonic ontogenesis. During the external examination of fetuses and offspring in both groups, no developmental anomalies were noted, all organs in both groups were of normal topography and size. The number of subcutaneous hematomas in the experimental group of fetuses compared to the control group decreased by an average of 50%, postnatal mortality decreased by an average of more than 3 times, as well as an increase in body weight in females, an increase in body weight and body length in the fetus and offspring in the early post-embryonic period ontogenesis ( $p < 0.05$ ).

**Conclusion.** Intraperitoneal administration of a therapeutic dose of a water-salt extract of the medicinal leech to female rats before and during pregnancy, after every three days, does not have any toxic effect on the course of embryogenesis and later in early post-embryonic ontogenesis.

**Keywords:** medicinal leeches, embryotoxicity, teratogenicity, rats

## INTRODUCTION

The increased amount of infectious and non-infectious diseases in the last decade, especially in newborn animals and humans who are more susceptible to their influence encourages scientists to look for different methods and ways to fight them. Various methods of treatment are used including diet therapy, vitamin therapy, chemotherapy, physiotherapy, pharmacotherapy, reflexology, balneotherapy and climate therapy, manual and radiation therapy, and phytotherapy. New synthetic drugs are created, and various natural substances are obtained from bacteria, fungi, plants, and animals (Aminov, 2022).

Nowadays preference is given to natural substances that have virtually no side effects or contraindications. These include substances obtained from medicinal leeches that have a wide range of therapeutic effects (Aminov *et al.*, 2022; Davoodi *et al.*, 2021; Amani *et al.*, 2021; Yang *et al.*, 2021; Aminov *et al.*, 2021). These substances have shown effectiveness in the prevention and treatment of a significant number of diseases in animals and humans (Fattakhov *et al.*, 2021; Trenholme *et al.*, 2021; Amani *et al.*, 2021; Afroz *et al.*, 2020; Akalin *et al.*, 2020; Curcio *et al.*, 2020). For example, in veterinary medicine and agriculture: for the treatment of mastitis, vascular diseases, diabetic nephropathy, wound healing, hematomas, inflammation, fever, ataxia, arthritis, myositis, eczema, abscesses, neuritis, osteoarthritis, and others (Aminov, 2022; Davoodi *et al.*, 2021; Amani *et al.*, 2021; Yang *et al.*, 2021; Trenholme *et al.*, 2021). In medical practice: for leiomyoma of the skin, psoriasis, heart failure, hypertension, infectious myocarditis, angina pectoris, glaucoma, acute sensorineural deafness, acute and chronic diseases of the middle and inner ear, radiculopathies, arthritis, osteochondrosis, arthrosis, cerebrovascular diseases, endometriosis, uterine myoma, chronic inflammation of the appendages, nephritis, diabetic foot, diabetes, in the treatment of thrombophlebitis, boils in goats, stomatitis, pulpitis and many others (Aminov, 2022; Huang *et al.*, 2021; Akalin *et al.*, 2020; Rehman, 2020; Sudhadevi *et al.*, 2020; Afroz *et al.*, 2020). There is evidence of their positive effect in cancer, tuberculosis, and Covid-19 treatment (Aminov, 2022; Fattakhov *et al.*, 2021; Ojo *et al.*, 2018).

There are many disputes among scientists about the effectiveness of the above-mentioned substances in veterinary and medicine since preliminary preclinical research is a necessary condition for the use of new therapeutic agents. This especially concerns their use by pregnant women and children. Thereby, the study of the embryotoxic and teratogenic effects of substances is an important precondition for their practical use (Shatorna *et al.*, 2022; Grytsaj *et al.*, 2022; Shamelashvili *et al.*, 2021; Shatorna *et al.*, 2020; Nefodova *et al.*, 2018).

Therefore, the aim of this work was to investigate the effect of the water-salt extract of the medicinal leech *Hirudo verbana* Carena, 1820 on the general course of embryogenesis in rats under intraperitoneal administration.

## MATERIALS AND METHODS

The study of the embryotoxic and teratogenic effects of the water-salt extract obtained from medicinal leeches *Hirudo verbana* Carena 1820 was carried out in the laboratory of cellular and organismal biotechnology of Zaporizhzhya National University. The extract was obtained according to our improved method (Aminov *et al.*, 2018). The studies were conducted on 60 female non-linear white rats aged 6 months with an average weight of  $210 \pm 13.6$  g. The animals were divided into four groups: 15 individuals in each group. The first two groups were control groups who were injected intraperitoneally with a saline solution in the volume of 0.5 ml three times before mating and three times during pregnancy, every three days. The other two groups were experimental ones, they were administered 0.5 ml of medicinal leech water-salt extract intraperitoneally in a concentration (5  $\mu$ g/g of animal) three times before mating and three times during pregnancy every three days. The selected concentration of the substance was previously experimentally tested *in vitro* in our previous studies and was chosen as a non-toxic therapeutic concentration (Aminov *et al.*, 2018). At the preparatory stage, the estrous cycle of females was studied by the method of vaginal smears, which made it possible to determine the duration of the cycle and individual phases, the presence of all phases of the cycle and the rhythmicity of their alternation in each female. Females with a stable rhythm of the estrous cycle of proestrus and estrus stages were identified and mated with intact males according to the 3:1 scheme. Determination of the first day of pregnancy of females was determined by the presence of spermatozoa in vaginal smears. Pregnant rats were kept in separate cages and provided with the necessary litter for nesting.

From the first day of pregnancy, the animals were observed: their condition and behavior were monitored; the dynamics of body weight changes, the duration of pregnancy, and the course of childbirth were recorded. In parallel, control groups of animals were observed.

Subsequently, one of the groups, from both control and experimental female rats, was euthanized under light ether anesthesia by dislocation of the cervical vertebrae on the 20th day of pregnancy (15 animals in each group). In these female rats, abdominal cavity and uterine horns were dissected; the number of corpora lutea of pregnancy, and the number of live and dead fetuses were analyzed (Shatorna *et al.*, 2022; Shamelashvili *et al.*, 2021; Shatorna *et al.*, 2020).

The embryotoxic effect of the studied substances was evaluated according to the following indicators (Shatorna *et al.*, 2022; Shamelashvili *et al.*, 2021; Shatorna *et al.*, 2020):

1. Total embryonic mortality (TEM):

$$\text{TEM} = \frac{B - A \cdot 100}{B},$$

where  $A$  is the number of living fetuses;  $B$  – the number of corpora lutea of pregnancy.

2. Preimplantation mortality (PIM):

$$\text{PIM} = \frac{B - (A + C) \cdot 100}{B},$$

where  $A$  is the number of living fetuses;  $B$  – the number of dead fetuses;  $C$  – the number of corpora lutea of pregnancy.

3. Postimplantation mortality (PostIM):

$$\text{PostIM} = \frac{B \cdot 100}{A + B},$$

where  $A$  is the number of living fetuses;  $B$  – the number of dead fetuses.

Fetuses were subjected to a morphometric study of body parameters, the presence of hematomas on the skin, mutilations, and external anomalies of the development of internal organs.

The other two groups of control and experimental female rats (15 animals in each group) were kept until the birth of offspring. After the birth of rat pups, in females of both groups the body weight was determined, the general condition was assessed, the gestation period and the total number of living and dead newborn offspring were recorded. From both groups of female rats, all newborn rat pups were analyzed on the first day and the 15th day (130 rat pups from each group). The body weight and length of the rat pups were determined, mortality was recorded from the beginning of birth to the 15th day, the presence of hematomas on the skin, mutilations, and external anomalies of the development of internal organs. The teratogenic effect of the substance was noted visually. Animal manipulations were carried out in accordance with the regulated norms and rules for the treatment of laboratory animals: bioethical principles, legal norms, and requirements according to the provisions of the “European Convention for the Protection of Vertebrate Animals Used for Research and Scientific Purposes” (Strasbourg, France, 1986), Law of Ukraine “On the Protection of Animals from Cruelty Treatment”, the Procedure for Conducting Research and Experiments on Animals by Scientific Institutions, Regulations on the Committee on Ethics (Bioethics), and the protocol of the meeting of the commission on bioethics of the Faculty of Biology of the Zaporizhzhia National University for the planned research, protocol No. 1 dated March 22, 2018.

Statistical processing of the obtained data was carried out using parametric statistical methods (Student’s  $t$ -test) using Microsoft XP Excel application program package and IBM SPSS Statistics 21.0 (USA), after previously checking the samples for normality of distribution. The values in the tables are presented in the form of  $X \pm SE$ , where  $X$  is the sample mean,  $SE$  is the standard error of the mean. Differences were considered significant at the level of  $p < 0.05$ .

## RESULTS AND DISCUSSION

In the experiment, all females survived, were active and consumed food and water well. The state and behavior of the experimental group were not different from the control group. Cases of abortion were not registered in either of the groups of animals. We noted a more pronounced weight gain in the experimental group of females on the 20th

day of pregnancy by  $30.2 \pm 3.2$  g compared to the control group by  $18.2 \pm 2.4$  g ( $p < 0.05$ ). An increase in body weight can be due to the stimulation of morphogenetic function of the immune system by the action of biologically active substances of medical leeches (Aminov *et al.*, 2022; Aminov, 2018; Frolov *et al.*, 2010). Similar data were obtained by other authors in domestic animals and pets under hirudological influence (Trenholme *et al.*, 2021; Abdisa, 2018; Sobczak *et al.*, 2014; Frolov *et al.*, 2010). For example, in goats after courses of hirudological attachments, body weight increased during the milking period, milk yield increased without complications of mastitis, and in the reproductive period, 100 % fertilization was noted, as well as the birth of twins with increased weight compared to the control group of animals (Frolov *et al.*, 2010). In rats, after the indirect hirudological influence, an increase in body weight and length was noted, the percentage of stillbirths decreased, and hematological and immunological indicators of blood increased (Aminov *et al.*, 2021).

After the dissection of females on the 20th day of pregnancy in both groups, the number of corpora lutea of pregnancy, and the number of live and dead fetuses did not differ statistically (**Table 1**). All organs of females of both groups were of normal topography and size.

**Table 1. Effect of water-salt extract of medicinal leech on embryogenesis ( $\bar{X} \pm SE$ )**

Parameters	Group of animals	
	Control	Experimental
The number of pregnant females	15	15
The number of fetuses per female	$8 \pm 1.07$	$8 \pm 0.7$
The total of the studied fetuses	120	120
The number of corpora lutea of pregnancy	$10.1 \pm 0.59$	$10.6 \pm 0.45$
The number of dead fetuses	$0.16 \pm 0.07$	$0.12 \pm 0.09$
The number of living fetuses	$9.42 \pm 0.51$	$9.94 \pm 0.36$
Fetus weight, mg	$2590.11 \pm 1.81$	$2596.30 \pm 1.65^*$
Fetus length, mm	$35.52 \pm 0.33$	$37.01 \pm 0.23^*$
The presence of subcutaneous hematomas	$0.40 \pm 0.04$	$0.20 \pm 0.09^*$
The number of fetuses with anomalies, mutilations	there are not	there are not

**Note:** \* –  $p < 0.05$  compared to the control group

The analysis of the weight and length of embryos in both groups did not reveal any delay in fetal growth. In the experimental group, these indicators are higher, which may be the result of a positive hirudological influence ( $p < 0.05$ ) (**Table 1**). During the external examination, no developmental abnormalities were noted in either of the groups. The position, shape of the limbs, and number of fingers in experimental and control embryos were within normal limits. There were no signs of pigmentation disorders of the skin. It should be noted that the number of subcutaneous hematomas in the experimental group compared to the control group was lower by an average of 50 % ( $p < 0.05$ ). It can be a manifestation of the well-known hemostatic effect of medicinal leeches (Trenholme *et al.*, 2021, Afroz *et al.*, 2020). For example, after sessions of hirudological exposure, skin hematomas quickly disappeared and the physiological state was restored in pets and domestic animals (Nowicki, *et al.*, 2021; Trenholme *et al.*, 2021; Abdisa, 2018;

Canpolat *et al.*, 2004). Our results on the reduction of skin hematomas in fetuses after hirudological influence coincide with results of other researchers. Also, foreign and Ukrainian scientists have discovered many other therapeutic hirudological effects on animals, without toxic side effects (Trenholme *et al.*, 2021; Frolov *et al.*; 2010, Sobczak *et al.*, 2014). For example, medicinal leeches are widely used in the treatment of arthritis and osteoarthritis, postoperative wounds, eczema, ataxia, neuritis, dysplasia, myositis, mastitis, tendinitis, sprained ligaments (Trenholme *et al.*, 2021; Sobczak *et al.*, 2014).

Indicators of pregnant females in experimental groups corresponded to similar indicators of control groups (**Table 1**). All organs of both groups were of normal topography and size.

When analyzing indicators of general embryotoxic mortality, pre-implantation mortality, and post-implantation mortality, we noticed that they were significantly lower in the experimental group compared to the control group (**Table 2**).

**Table 2. Dynamics of embryonic development parameters ( $X \pm SE$ )**

Parameters	Group of animals	
	Control	Experimental
Total embryonic mortality,%	6.73±0.02	6.23±0.02*
Preimplantation mortality, %	5.15±0.02	3.11±0.02*
Postimplantation mortality, %	1.67±0.01	1.17±0.01*

**Note:** \* –  $p < 0.05$  compared to the control group

The obtained experimental data indicate the absence of an embryotoxic effect, which was also confirmed by other authors in relation to medicinal leech substances (Shatorna *et al.*, 2022; Grytsaj *et al.*, 2022; Shamelashvili *et al.*, 2021; Shatorna *et al.*, 2020; Nefodova *et al.*, 2018). It should also be noted that body weight and length of animals in the experimental group increased within the physiological norms for this age ( $p < 0.05$ ). An increase in body morphometric indicators may be a manifestation of the well-known stimulating morphogenetic effect of medicinal leech substances, which was noted by other authors and in our previous experiments at the early stages of post-embryonic ontogenesis (Aminov *et al.*, 2021; Trenholme *et al.*, 2021; Abdisa, 2018; Sobczak *et al.*, 2014; Frolov *et al.*, 2010).

The next stage of the study was the analysis of the offspring at the early stages of postnatal ontogenesis: newborns and on the 15th day for the absence of developmental defects. Since, in most cases, the negative effect of a drug can manifest itself at birth. The results are presented in **Table 3**.

It was established that the terms of childbirth in both groups were the same. The number of offspring did not differ statistically. It should be noted that in the experimental groups, an increase in body weight and length was observed in both newborns and 15-day-old rats in comparison with the control groups **Table 3** ( $p < 0.05$ ). An increase in body weight in early post-embryonic ontogeny after the hirudological influence was observed in our previous works and the results of other authors (Aminov *et al.*, 2022; Davoodi *et al.*, 2021; Amani *et al.*, 2021; Yang *et al.*, 2021; Trenholme *et al.*, 2021) as a morphogenetic effect of the substances of medicinal leeches. During the external examination, no developmental abnormalities were noted in either of the groups. All organs of animals of both groups were of normal topography and size. It should be

noted that for the first time, the influence of the water-salt extract of the medicinal leech at the intraperitoneal injection of a therapeutic concentration (5 µg/g of the animal) on the general course of embryogenesis in rats was experimentally investigated, and no toxic effect was detected. This suggests future prospects for the application of the studied substances to pregnant animals for treatment and prevention of many diseases after a thorough preclinical study.

**Table 3. Parameters of rats during early postnatal development under the influence of water-salt extract of medicinal leeches ( $X \pm SE$ )**

Parameters	Group of animals	
	Control	Experimental
Pregnancy period of females, days	22±2	23±2
The body weight of non-linear white female rats, g	219.10±10.6	262.23±13.2*
The number of pregnant females	15	15
The number of rats born per female	9.5±1.1	10.6±1.3
Postnatal mortality, %	0.94±0.01	0.32±0.005*
Body weight at birth, g	6.23 ±0.17	6.74 ±0.20*
Body length at birth, cm	4.91 ± 0.16	5.16±0.14
Body weight on the 15th day, g	21.12 ± 0.34	24.68±0.42*
Body length on the 15th day, cm	7.99 ± 0.29	8.26±0.19
The presence of subcutaneous hematomas	0.14±0.03	0.11±0.02
The number of rats with anomalies, mutilations	there are not	there are not

**Note:** \* –  $p < 0.05$  compared to the control group

## CONCLUSION

In the experimental female rats, no mortality, cases of abortion, changes in behavior, disturbance of the general condition, terms of pregnancy and number of offspring were observed. The indicators of the total embryotoxic mortality, pre-implantation mortality and post-implantation mortality were significantly lower in the experimental group compared to the control group ( $p < 0.05$ ). No developmental abnormalities or pigmentation disorders were detected in the fetuses and offspring in either of the groups. It should be noted that the number of subcutaneous hematomas in the experimental group of fetuses was lower by an average of 50 %, and postnatal mortality was lower by an average of more than 3 times compared to the control group. Besides, an increase in body weight of females, as well as in body weight and body length of the fetuses and offspring during early post-embryonic ontogenesis was observed ( $p < 0.05$ ).

## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest:** There were no any commercial or financial relationships that could be interpreted as a potential conflict of interest.

**Human Rights:** The article does not contain any investigations with human subjects.

**Animal studies:** All international, national and institutional guidelines for the care, maintenance and use of laboratory animals were followed.

## AUTHOR CONTRIBUTIONS

The author has read and agreed to the published version of the manuscript.

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## ВПЛИВ ВОДНО-СОЛЬОВОГО ЕКСТРАКТУ МЕДИЧНОЇ П'ЯВКИ *HIRUDO VERBANA CARENA*, 1820 НА ЗАГАЛЬНИЙ ХІД ЕМБРІОГЕНЕЗУ ЩУРІВ У РАЗІ ВНУТРІШНЬОЧЕРЕВНОГО ВВЕДЕННЯ

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**Обґрунтування.** Зростання ймовірності інфекційних та неінфекційних захворювань під час вагітності спонукає вчених до пошуку речовин, здатних боротися з цими недугами. Особливо це стосується природних речовин, які практично не дають побічних ефектів. До таких належать речовини, які отримують із організму медичних п'явок. Ці речовини мають широкий спектр терапевтичної дії, однак для їхнього практичного використання потрібно пройти всі доклінічні дослідження. Особливо це стосується наявності ембріотоксичного ефекту. Мета роботи – дослідити вплив водно-сольового екстракту медичної п'явки *Hirudo verbana Carena*, 1820 на загальний хід ембріогенезу щурів у разі внутрішньочеревного введення.

**Матеріали та методи.** Робота виконана на самицях нелінійних білих щурів. Дослідним двом групам тварин внутрішньочеревно вводили водно-сольовий екстракт медичної п'явки в концентрації 5 мкг/г тварини, в об'ємі 0,5 мл, тричі перед спаровуванням і тричі під час вагітності, через кожні 3 доби. Контрольним двом групам внутрішньочеревно вводили фізіологічний розчин за тією ж схемою, як і дослідній групі. У подальшому одну з контрольних і дослідних груп піддавали евтаназії, розтину черевної порожнини та рогів матки на 20-ту добу вагітності. У них аналізували загальну кількість жовтих тіл вагітних, кількість живих плодів і мертвих плодів. Плоди зважували, оглядали на наявність шкірних гематом, каліцтва, на зовнішні аномалії розвитку внутрішніх органів. Інші дві групи контрольних і дослідних тварин залишали до народження приплоду. Потім самиць обох груп зважували, оцінювали загальний стан. Новонароджених щурят оглядали, визначали масу та довжину тіла, реєстрували смертність від початку народження до 15-ї доби, наявність шкірних гематом, каліцтва, зовнішні аномалії розвитку внутрішніх органів.

**Результати.** Внаслідок експериментального дослідження встановлено, що внутрішньочеревне введення водно-сольового екстракту медичної п'явки до вагітності та під час вагітності самицям щурів не спричиняє токсичного ефекту під час ембріогенезу та на ранніх етапах постембріонального онтогенезу. Під час зовнішнього огляду плодів і приплоду в обох групах аномалії розвитку не відмічено, всі органи в обох груп були звичайної топографії й розмірів. Кількість підшкірних гематом у дослідній групі плоду, порівняно з контрольною групою, зменшилася в середньому на 50 %, постнатальна смертність зменшилася в середньому більш ніж утричі, а також збільшилася вага тіла у самиць, збільшилися вага і довжина тіла у плоду та приплоду на ранньому постембріональному онтогенезі ( $p < 0,05$ ).

**Висновки.** Внутрішньочеревне введення самкам щурів терапевтичної дози водно-сольового екстракту медичної п'явки до та під час вагітності через кожні три доби не впливає токсично на хід ембріогенезу та надалі в ранньому постембріональному онтогенезі.

**Ключові слова:** медичні п'явки, ембріотоксичність, тератогенність, щури