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Post-natal development of rats` offspring treated with the ethanol extract of Neem leaves (*Azadirachta indica* A. Juss) during pregnancy and lactation

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ABSTRACT. Teratogenicity and developmental abnormalities in the offspring of female rats that ingested ethanol extract of Neem plants during pregnancy and lactation period were assessed. Twenty-four female Wistar rats were randomly distributed in control group and in three experimental groups and treated during the 4th, 5th, and 6th day of pregnancy. After birth, the lactating females received, by gavage, 65, 135 and 200 mg kg^{-1} of Neem ethanol extract, during 15 days. Results show, there was no significant difference in body mass index of neonatal rats in the 4 groups evaluated, whereas mean rate of offspring survival was 79.4%. Hair growth, incisor teeth eruption, ear detachment, eyelid opening, and spontaneous ambulation were similar for all groups. Likewise, physical development and development of motor activity, ambulation, and postural reflexes were similar for all groups. The administration of Neem ethanol extract did not cause any reproductive or systemic toxicity in animals. Results show that, Neem ethanol extract safe at doses 65, 135 and 200 mg kg^{-1} in pregnant or lactating rats.

Keywords: medicinal plant, reproduction, teratogenicity, physical development, rodents.

Desenvolvimento pós-natal de proles de ratas tratadas com o extrato etanólico das folhas de nim (*Azadirachta indica* A. Juss) durante a gestação e lactação

RESUMO. Teratogenicidade e anormalidades no desenvolvimento de proles de ratas que ingeriram o extrato etanólico de folhas de nim durante a gestação e lactação foram avaliadas. Vinte e quatro ratas Wistar foram distribuídas aleatoriamente em um grupo controle e três grupos experimentais tratados no quarto, quinto e sexto dias de gestação. Após o nascimento, as fêmeas lactantes receberam através de gavagem, o extrato etanólico de nim nas doses de 65, 135 e 200 mg kg^{-1} durante 15 dias. Nenhuma diferença significativa foi observada em relação ao índice de massa corpórea das fêmeas em lactação nos grupos em todos os momentos e a taxa média de sobrevivência dos filhotes foi de 79,4%. O tempo de crescimento piloso, erupção dos dentes incisivos, descolamento de orelha, abertura palpebral e deambulação espontânea foram semelhantes em todos os grupos. O desenvolvimento físico e da atividade motora, deambulação e reflexos posturais foram semelhantes em todos os grupos. A administração não resultou em toxicidade reprodutiva ou sistêmica. Os resultados mostraram que o extrato etanólico do nim é seguro para uso nas doses de 65, 135 e 200mg kg^{-1} em ratas prenhes ou em lactação.

Palavras-chave: planta medicinal, reprodução, teratogenicidade, desenvolvimento físico, roedores.

Introduction

Azadirachta indica A. Juss, commonly known as Neem is a plant that belongs to the Meliaceae family and, possesses several important characteristics, including pesticide and therapeutic traits (KUMA; NAVARATNAM, 2013). Neem is used in traditional medicine for its antiviral (TIWARI et al., 2010), immunomodulatory (THOH et al., 2010), hypoglycemic (KHOSLA et al., 2000) and antineoplastic features (PERUMAL et al., 2012), coupled to such assets as hepaticprotector (CHATTOPADHYAY, 2003), inhibitor of gastric

secretion (MAITY et al., 2009), spermicide and anti-fertilizing, antibacterial and healing agent (ROOP et al., 2005). Neem seed oil, widely used in holistic medicine and herbal medicine, contains several components which include the insecticide azadirachtin (BOEKE et al., 2004). This oil is included in the composition of certain veterinary products for the treatment of flee infestations in cats and dogs (SUTTON et al., 2009).

Non-water based Neem extracts seem to be more toxic, with a safe dose of 0.002 and 12.5 μg kg^{-1} of body weight per day in mammals. The seed oil from non-transformed materials and water-based is less toxic. In fact, most pure compounds have a relatively low toxicity (azadirachtin, at a dose of 15 mg kg^{-1} of body weight day^{-1}). The more important toxic effect after subacute or chronic exposure seems to be the reversible effect on mammal male and female reproduction, for all preparations (BOEKE et al., 2004). Since Neem has been focused in several studies on veterinary medicine and due to the possibility that it causes systemic and reproductive changes, current research investigates, signs of toxicity, teratogenicity and developmental changes in the offspring of female rats treated with the ethanol extract from Neem leaves during the gestational and lactation period.

Material and methods

A vegetal specimen of *Azadirachta indica* A. Juss obtained from a farm in Lagoa do Carro-PE, Brazil, was identified by the Engineer Angela Maria Miranda and deposited at the herbarium of the Department of Forest Engineering of the Federal Rural University of Pernambuco (UFRPE) with the exsiccate HST-16264.

Neem leaves were dehydrated in a dry heat sterilization device at 40°C, for 72 hours, in the Animal Pathology Lab at UFRPE. The dry and pulverized powder (1.0 kg) underwent three extraction processes with 1L of ethanol (totaling 3L). The solution extracted filtered and evaporated in a rotary evaporator at 55°C under reduced pressure, produced 98.18 g of crude Neem ethanol extract and a useable percentage of 9.82%.

Stock solutions at concentrations of 20 mg mL^{-1} were prepared to be administered to experimental groups. An analytic scale was used to obtain 200 mg of the Neem crude extract, to which five drops of cremophor, 1mL of dimethyl sulfoxide (DMSO) at 10%, and distilled water were added until a volume of 10 mL was reached. The stock solution or placebo for the control group (group 1) had the same constituents except the crude Neem extract.

The experimental protocol was submitted to and approved by the Committee of Ethics in the Use of Animals at UFRPE (Protocol n. 23082.005738/2009) before the start of the study. Twenty-four Wistar rats, 90 days old, weighing 300 g, obtained from the Department of Morphology and Animal Physiology, were used in this experiment. The animals were kept in polypropylene cages with free access to water and food (Labina-Purina-Pro-rodents). Temperature was kept at 22°C ± 2°C and light intensity at 400 lux followed a 12/12 light/dark cycle. The mating system was polygamous and three females per male were paired for mating at the end of the afternoon. Gestation was determined by the presence of spermatozoids in vaginal swabs detected on the morning after mating. Colpocytological exams were performed with cotton swabs moistened with distilled water, which were introduced in the vagina in rotating movements. The collected material was transferred onto histological slides, stained with a panoptic method and studied under an optic microscope.

The females were treated orally with different doses of ethanol Neem extract at two separate periods, or rather, at the 4th, 5th and 6th day of gestation and on the first 15 days after the birth of the pups, during lactation. The rats in the control group (Group 1) received 1mL of the placebo solution and the other experimental groups (Groups 2, 3 and 4) received 1mL, 2mL and 3mL of the ethanol extract from Neem leaves at 20 mg mL^{-1}, which respectively corresponded to 65, 135 and 200 mg kg^{-1} or 19.5; 40.5 and 60 mg (300 g^{-1}) of body weight.

The rats clinical evaluation of toxicity included daily weight observations and inspection for any occurrence of abortions, bleeding, weight-loss, diarrhea, piloerection, and ataxia during the gestational and post-partum period (MANSON; KANG, 1994). The offspring were also evaluated by inspection and daily weightings, from day 1 to their 21st day of life, to assess their physical development. On the first and seventh day of life, the pups were evaluated for their postural reflex. They were placed on a flat surface, in dorsal recumbence, and reflex was evaluated in seconds until the animal was repositioned on the four limbs on the flat surface (CARLINI et al., 1988).

Ear detachment, fur growth, incisor eruption, palpebral opening, and adult walk pups start to walk without dragging their hind limbs or letting their abdomen touch the ground, were evaluation daily by observation of the pups. The days in which these

events occurred were registered so that the development between groups could be compared. On the 21st day of life, spontaneous ambulation was evaluated by employing a 30 × 30 cm square, divided into nine equal parts. Each pup was placed separately in the central square, and a total count of how many squares were invaded during one minute was calculated. In this case, invasion occurred when at least three paws were placed within a square. This test evaluated the pups motor function (CARLINI et al., 1988).

Mean body mass of the lactating rats on the 1st, 3rd, 5th, 9th, and 14th day and of the offspring on the 1st, 4th, and 21st day was evaluated by analysis of variance (ANOVA). Kruskal-Wallis test analyzed the total number of pups and the number of pups alive after 14 days. Mean rates for survival rate of the pups per rat per treatment (number of live pups/total number of pups) and evaluation of the postural reflex of the pups on the 1st and 7th day of life were also assessed by Kruskal-Wallis test, with statistically significant results at $p > 0.05$.

Results and Discussion

There were no signs of systemic or reproductive toxicity, such as piloerection, weight loss, diarrhea, stereotypes, vaginal bleeding, ataxia, coma, or death in the parents treated on the 4th, 5th, and 6th day of gestation, and from the 1st to the 15th day post-partum. Raizada and Srivastava (2007) did not report any adverse effects on the administration of azadirachtin, one of the main chemical components found in Neem leaves, fruits, flowers, and seeds, when administered at 12%, orally, in male and pregnant female rats up to 1500 mg kg^{-1} day^{-1} for 90 days. These researchers did not observe pharmacotoxic or teratogenic signs, mortality, changes in weight or changes in blood tests. Therefore, dose, 1500 mg kg^{-1} was suggested as basal to determine at which level no effects were caused by the compound and thus its safety margin warranted.

Raizada and Srivastava (2007) did not report any toxic effects in male and female rats treated with Neem oil at 5, 25, and 50 mg kg^{-1} added to the feed and offered *ad libitum* during two generations. Additionally, Kazmi et al. (2001) did not observe toxicity or adverse effects, other than absence of histological changes in the vital organs of mammals treated with products containing Neem. Gbotolorun et al. (2004) reported the toxicity effect of Neem seed on rats with a mortality rate of 40%.

In a research where rats received orally 1mg kg^{-1} of alcoholic extract from Neem flowers from the 1st to the 5th day of gestation, reports included diarrhea in all animals, and a 6.46% decrease in body mass. The same study focused on the influence of Neem alcoholic extract on the estrus cycle of rats treated with 1000mg kg^{-1} during 21 days, and found there was an increase in duration of the diestrus and, thus, a decrease in estrus frequency. Therefore, fertility decreased due to decreased ovulation (GBOTOLORUN et al., 2008).

When the body mass of the rats in lactation evaluated on the 1st, 3rd, 5th, 9th and 14th day was taken into account, no evidence of any significant decrease in body mass of the treated rats was detected when compared to females in the control group ($p > 0.05$) (Table 1). No stillborn was reported nor was any neonatal malformation observed upon external examination of the offspring from the control or experimental groups.

Table 1. Mean body mass in grams (g) of lactating rats in the control group (G1) and in the experimental groups (G2, G3 and G4) treated with ethanol extract from Neem leaves (*Azadirachta indica* A. Juss) during pregnancy and during the first 15 days of lactation at 65, 135, and 200 mg kg^{-1}, respectively.

Groups	Days				
	1st	3rd	5th	9th	14th
G1	299.63 ± 23.70	284.82 ± 28.12	294.44 ± 14.04	299.27 ± 16.18	297.13 ± 18.02
G2	318.35 ± 29.37	310.00 ± 27.36	309.04 ± 28.93	307.96 ± 31.43	310.21 ± 32.48
G3	355.19 ± 79.22	317.82 ± 33.75	318.04 ± 30.32	314.75 ± 32.01	311.23 ± 36.32
G4	279.51 ± 16.19	284.24 ± 17.87	287.18 ± 13.56	290.13 ± 17.36	290.63 ± 20.75

*Results given as mean ± standard deviation.

Raji et al. (2004) observed that intraperitoneal administration of *Azadiractha indica* (1000 mg kg^{-1} of extract) did not produce any sign of toxicity in rats, nor did it alter body or organ weight during a three week administration. However, oral administration of 3.200 mg kg^{-1} resulted in 100% mortality in rats. Nonetheless, Dallaqua et al. (2013) evaluated the effects of neem on gestation in rats treated with 1.2 mL of Neem seed oil (G1), and on another group treated orally with 1 mg mL^{-1} of an azadirachtin-based product (G2). The authors reported congenital malformations in offspring from G1, whereas no such malformations were detected in G2.

No changes were observed with regard to the physical development of the offspring in the experimental or control groups. Time for fur growth (8th day), incisor teeth eruption (9th day), ear detachment (4th day), palpebral opening (14th day) and adult walk (15th day) were the same in all four groups.

Lack of change in the offspring´s physical development corroborated results by Costa-Silva et al. (2006) in rats, when they studied the reproductive toxicity of Andiroba (*Carapa guianensis*) which, similar to the neem, is a Meliaceae.

Body mass weight for the pups of each group was obtained on the 1st, 4th, and 21st days after birth, with no statistic

al difference between the groups (Table 2). During gestation, the weight of the litter may be affected by the intrauterine capacity, litter size, and duration of gestation. After birth, besides the ability of the female, the effect of chemical substances may also compromise the pups' development.

Table 2. Mean body mass in grams (g) of the offspring of the rats in the control group (G1) and experimental groups (G2, G3 and G4) treated during gestation and for 15 days after birth with the ethanol extract from Neem leaves (*Azadirachta indica* A. Juss) at 65, 135, and 200 mg kg^{-1}, respectively.

Groups	Days		
	1st	4th	21st
G1	6.30a	9.55a	36.10a
G2	7.17a	9.78a	42.47a
G3	6.57a	7.61a	35.49a
G4	6.44a	9.39a	41.84a
Total mean	6.62	9.08	39.02
VC	13.15%	31.97%	32.55%

CV–Coefficient of Variation.

Current results demonstrate that the, pups did not have any sign of systemic toxicity, such as diarrhea, sialorrhea, shivering, piloerection, change in ambulation, decreased body mass, coma, or death, which were described by Manson and Kang (1994) in systemic toxicity with rodents.

The Kruskal-Wallis test applied to the total number of pups and number of pups alive after 14 days (Table 3) showed no difference between treatments. Survival rate of pups per rats among the groups was similar when the previously described statistical test was applied.

Table 3. Total number and survival rate of the offspring of rats in the control group (G1) and in the experimental groups (G2, G3 and G4) treated during gestation and for 15 days post-partum with Neem (*Azadirachta indica* A. Juss) leaves ethanol extract at 65, 135, and 200 mg kg^{-1}, respectively.

Groups	Number of pups			Survival %
	Dead	Alive	Total	
G1	10	33	43	76.74
G2	4	44	48	91.67
G3	23	34	57	59.65
G4	4	47	51	92.16
Total	41	158	199	79.40

In the case of the postural reflex, there was no difference in reaction time between the groups on the 1st and 7th day of the pup's life, with similar means at the two moments (Table 4).

Table 4. Mean postural reflex time in second (s) for pups evaluated on their 1st and 7th day of life from rats in the control group (G1) and experimental groups (G2, G3 and G4) treated during gestation and for 15 days post-partum with Neem (*Azadirachta indica* A. Juss) leaves ethanol extract at 65, 135, and 200 mg kg^{-1}, respectively.

Groups	ean postural reflex time	
	1st day of life	7th day of life
G1	70.00a	85.50a
G2	107.00a	61.00a
G3	57.00a	76.00a
G4	66.00a	77.50a

Since the motor activity (ambulation) was similar in all groups (Table 5), results suggested that Neem did not cause an increase in motor function, nor did it delay development of the nervous system.

Table 5. Evaluation of the motor development of the pups born to dams of the control group (G1) and experimental groups (G2, G3 and G4) treated during gestation and for 15 days post-partum with Neem (*Azadirachta indica* A. Juss) leaves ethanol extract at 65, 135, and 200 mg kg^{-1}, respectively.

Groups	Number of pups	Number of invaded squares		
		Mean	Minimum	Maximum
G1	34	13.7	5	27
G2	38	12.3	2	21
G3	41	13.6	2	26
G4	43	15.4	7	25

Rats from groups 1 and 3 did not have an adequate maternal behavior towards their offspring. Daily observations revealed that they did not nurture or feed them satisfactorily. This fact may be related with the survival rates in these groups where a certain increase in the number of neonatal deaths was observed. According to Crowell-Davis and Houpt (1986), adequate maternal behavior is necessary for the pups to develop characteristics and abilities that ensure their survival.

The development of the pups´ motor activity may be evaluated by postural reflex. When testing this reflex, the offspring of the rats in Group 3 responded faster than those in Group 2, or rather, their postural reflex was 46.7% more developed when compared to that of Group 2 pups, albeit with no statistical difference. On the 7th day, Group 2 had the lowest mean (61 seconds) and the pups from group 1 the highest mean (85.5 seconds).

On the twenty-first day after birth, the pups´ motor activity was evaluated to determine the effect of Neem on the development of their nervous system. Table 5 show that, different doses of Neem did not affect the development of the nervous system in 21-day-old pups. Costa-Silva et al. (2006) evaluated the motor activity of 21-day-old pups born from adult female rats treated with different doses of Andiroba oil, a Meliaceae similar to neem, during

the entire gestational period. The authors observed that animals treated with the smaller dose (0.375 g kg^{-1}) had a higher mean of 24.1 invaded squares, while the control group obtained a mean of 15.1. The research suggested that Andiroba oil had a central effect which increased motor activity. Current data were probably different due to evaluation time of the postural reflex being shorter than 2 minutes and to differences in phytochemical composition.

When Raizada and Srivastava (2007) investigated the effects of administering azadirachtin at 12% to rats from the 6th to the 15th day of gestation, they reported that the animals showed hyperactivity during treatment, albeit without toxicity. These results differ from those obtained in current study where treatment with ethanol Neem extract did not influence motor activity or ambulation of the offspring. There was only a slight delay in motor activity and spontaneous ambulation in the pups from the four groups, but without statistical significance, clinical repercussions, or consequences to neonatal development rate.

Tandam et al. (1995) and Sutton et al. (2009) respectively described systemic toxicity in rabbits and felines, exposed to Neem oil. In current study, the lactating rats and their offspring did not show clinical signs compatible with systemic toxicity. However, studies with larger samples, specifically calculated towards potency above 80% for each particular criterion investigated, and, if possible, with serum analysis, could result in more detailed information in this area.

Conclusion

Administration of ethanol extract from Neem leaves neither caused systemic toxicity in the studied animals, nor induced teratogenicity, nor altered the physical development or development of the nervous system of the offspring. Therefore, in doses 65, 135, and 200 mg kg^{-1} the extract appears to be safe for use during the pre- and post-natal period in rats.

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