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

Vanessa Carla Lima da Silva¹*, Francine Maria de França Silva², Isabelle Maria Jacqueline Meunier³, Tânia Maria Sarmento da Silva⁴, Valdemiro Amaro da Silva Júnior⁵ and Frederico Celso Lyra Maia⁵

¹Programa de Pós-graduação em Ciência Veterinária, Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros, s/n, 52171-900, Recife, Pernambuco, Brazil. ²Programa de Pós-graduação em Biociência Animal, Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Recife, Pernambuco, Brazil. ³Departamento de Ciência Florestal, Universidade Federal Rural de Pernambuco, Recife, Pernambuco, Brazil. ⁴Departamento de Química, Universidade Federal Rural de Pernambuco, Recife, Pernambuco, Brazil. ⁵Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Recife, Pernambuco, Brazil. *Author for correspondence. E-mail: vcls2004@yahoo.com.br

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.

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 (TWARI et al., 2010), immunomodulatory (THOH et al., 2010), hypoglycemic (KHOOSLA et al., 2000) and antineoplastic features (PERUMAL et al., 2012), coupled to such assets as hepatic protector (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 
stereilization 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 
occurance 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 and 7th day of life were also assessed by
evaluation of the postural reflex of the pups on
(number of live pups/total number of pups) and
analyzed the total number of pups and the
pregnant female rats up to 1500 mg kg−1 day−1 for 90
when administered at 12%, orally, in male and
found in Neem leaves, fruits, flowers, and seeds,
azadirachtin, one of the main chemical components
partum. Raizada and Srivastava (2007) did not report
warranted.

by the compound and thus its safety margin
to determine at which level no effects were caused
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
of the diestrus and, thus, a decrease in estrus
duration of the diestrus and, thus, a decrease in estrus
frequency. Therefore, fertility decreased due to
decreased ovulation (GBTOLORUN 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
decline 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.

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 (GBTOLORUN et al., 2008).

Raizada et al. (2004) observed that intraperitoneal
administration of Azadirachta 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
genital 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.

Table 1. Mean body mass in grams (g) of lactating rats in the
case of five groups (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.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days</th>
<th>1st</th>
<th>3rd</th>
<th>5th</th>
<th>9th</th>
<th>14th</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td></td>
<td>299.63 ± 17.36</td>
<td>284.82 ± 20.75</td>
<td>294.44 ± 20.75</td>
<td>299.27 ± 17.36</td>
<td>297.13 ± 17.36</td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td>318.35 ± 30.94</td>
<td>310.00 ± 30.32</td>
<td>309.04 ± 30.32</td>
<td>307.96 ± 29.37</td>
<td>310.21 ± 28.93</td>
</tr>
<tr>
<td>G3</td>
<td></td>
<td>355.19 ± 28.93</td>
<td>317.82 ± 32.48</td>
<td>310.00 ± 32.48</td>
<td>314.75 ± 32.48</td>
<td>311.23 ± 32.48</td>
</tr>
<tr>
<td>G4</td>
<td></td>
<td>279.51 ± 33.75</td>
<td>284.24 ± 33.75</td>
<td>287.18 ± 33.75</td>
<td>290.13 ± 33.75</td>
<td>290.63 ± 33.75</td>
</tr>
</tbody>
</table>

*Results given as mean ± standard deviation.
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 statistically significant 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>
<thead>
<tr>
<th>Groups</th>
<th>Days</th>
<th>1st</th>
<th>4th</th>
<th>21st</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>6.30</td>
<td>9.55</td>
<td>36.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>7.17</td>
<td>9.78</td>
<td>42.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>6.57</td>
<td>7.61</td>
<td>35.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>6.44</td>
<td>9.39</td>
<td>41.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6.62</td>
<td>9.08</td>
<td>39.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>13.15%</td>
<td>31.97%</td>
<td>32.55%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CV—Coefficient of Variation.

Current results demonstrate that the pups did not show any signs of systemic toxicity, such as diarrhea, salorrhea, 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>
<thead>
<tr>
<th>Groups</th>
<th>Number of pups</th>
<th>Number of invaded squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>34</td>
<td>13.7</td>
</tr>
<tr>
<td>G2</td>
<td>38</td>
<td>12.3</td>
</tr>
<tr>
<td>G3</td>
<td>41</td>
<td>13.6</td>
</tr>
<tr>
<td>G4</td>
<td>45</td>
<td>15.4</td>
</tr>
</tbody>
</table>

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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crude ethanol extract of Neem leaves, essential for
the execution of the assay.

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