Journal of Ethnopharmacology 150 (2013) 1109–1113

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Azadirachta indica treatment on the congenital malformations of fetuses from rats

Bruna Dallaqua [a], Felipe Hiroshi Saito [a], Tiago Rodrigues [b], Iracema Mattos Paranhos Calderon [a], Marilza Vieira Cunha Rudge [a], Gustavo Tadeu Volpato [c], Débora Cristina Damasceno [a,*]

[a] Graduate Program in Gynecology, Obstetrics and Mastology, Botucatu Medical School, UNESP_Univ. Estadual Paulista, Botucatu, Department of Gynecology and Obstetrics, Laboratory of Experimental Research on Gynecology and Obstetrics, São Paulo State, Brazil
[b] Federal University ABC, São Paulo, São Paulo State, Brazil
[c] Institute of Biological and Health Sciences, University Center of Araguaia, Mato Grosso Federal University (UFMT), Barra do Garças, Mato Grosso, Brazil

ARTICLE INFO

Article history:
Received 24 June 2013
Received in revised form
22 October 2013
Accepted 23 October 2013
Available online 31 October 2013

Keywords:
Azadirachta indica
Neem
Pregnancy
Rat
Anomaly

ABSTRACT

Ethnopharmacological relevance: *Azadirachta indica* A. Juss, popularly known as neem, presents medicinal and insecticide properties. However, the repercussions of the neem maternal treatment on fetal development should be investigated. Thus, the aim of this study was to evaluated the effects of *Azadirachta indica* (neem) on the frequency of congenital malformations in fetuses from rats.
Materials and methods: Pregnant rats were randomly distributed into three experimental groups: NT=non-treated; TOil=treated with neem seed oil (1.2 mL/day); TAP=treated with active principle of *Azadirachta indica* (azadirachtin—1.0 mg/mL/day). The neem oil (1.2 mL/day) or azadirachtin (1.0 mg/mL/day) treatments were orally administered throughout pregnancy. Blood samples were collected on days 0, 7, 14 and 20 of pregnancy. Oral glucose test tolerance (OGTT) was performed at day 17 of pregnancy for estimation of total area under the curve (AUC). At term, the fetuses were collected and external and internal (visceral and skeletal) malformations were analyzed.
Results: The data showed that the dams treated with neem seed oil and Azadirachtin had no significant change in glucose levels and AUC. It was also verified that neem oil treatment contributed to increase the frequency of malformation/variation, in particular the visceral in their fetuses, while neither significant result was observed in TAP group.
Conclusion: In conclusion, neem seed oil treatment administered during pregnancy caused abnormalities in rat fetuses, showing teratogenic effect but the Azadirachtin (active principle) presented no impairment in the fetuses.

© 2013 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Azadirachta indica A. Juss, commonly known as neem, is one of the most versatile medicinal plants that has gained worldwide importance due to medicinal (hypolipidemic, hypoglycemic, immunostimulant, hepatoprotective) and insecticide properties (Bopana et al., 1997; Khosla et al., 2000; Raizada et al., 2001; Gupta et al., 2004; Othman et al., 2012). There are several studies showing the effects of neem in experimental and clinical models. Sheep that ate neem leaves resulted in nervous symptoms with dyspnoea, increased body temperature and hepatic failures. These symptoms lasted for 12 h and were followed by the death of the animals (Ali and Salih, 1982). In a clinical study, young children after received small amounts of neem seed oil presented toxic encephalopathy (vomit, drowsiness, tachypnoea), leukocytosis and metabolic acidosis (Lai et al., 1990). Rats treated with azadirachtin presented increased serum SGOT (serum glutamic oxaloacetic transaminase) and SGPT (serum glutamic pyruvic transaminase) activities and pathological changes in the liver in terms of congestion, hydropic degeneration and necrosis (Abdel Megeed et al., 2001).

Azadirachtin is an active major ingredient isolated from seed neem, which is known to disrupt the metamorphosis of insects (Tomlin, 1997), whereas the neem oil may be used as spermicide and contraceptive (Riar et al., 1990). Studies have been observed that female rats treated with Praneem (a purified extract of *Azadirachta indica*) from day 8 to 10 at a dose of 0.6 ml presented a complete

* Correspondence to: Departamento de Ginecologia e Obstetrícia, Faculdade de Medicina de Botucatu - Unesp, Distrito de Rubião Júnior, s/n, CEP. 18603.970, Botucatu, São Paulo, Brazil. Tel.: +55 14 38801631.
E-mail addresses: brunadallaqua@gmail.com (B. Dallaqua), pi_saito@hotmail.com (F.H. Saito), trodrigues.ufabc@gmail.com (T. Rodrigues), calderon@fmb.unesp.br (I.M.P. Calderon), marilzarudge@gmail.com (M.V.C. Rudge), gtvolpato@yahoo.com (G.T. Volpato), damascenofmb@gmail.com (D.C. Damasceno).

0378-8741/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2013.10.046

resorption of embryos on day 15 of pregnancy in every animal treated (Mukherjee and Talwar, 1996). Another study performed using baboon monkeys verified that the treatment with Praneem for six days after establish the pregnancy caused abrogation/abortion. (Mukherjee et al., 1996). Previously, Upadhyay et al. (1990) demonstrated that when a single dose of neem seed oil (100 μL) is administrated directly into uterine horns of Wistar rats, these rats remained infertile for variable periods ranging from 107 to 180 days even after repeated mating with males of proven fertility. Riar et al. (1991) verified that pre and post coital application of the oil intravaginally prevented pregnancy in rhesus monkey, showing that this product performed as a good spermicidal agent.

People from all over the world use indiscriminately natural products, such as neem. This indiscriminate use during pregnancy can lead to deleterious effects for both mother and fetus. Dallaqua et al. (2012) verified that, both neem seed oil and azadirachtin, did not modify the maternal glycemia, caused impaired maternal outcomes and altered antioxidant/oxidative status during rat pregnancy. However, there are no reports about neem influence on the fetal organism and occurrence of congenital malformations in rats. Thus, in the present study, we aimed at evaluating the effect of *Azadirachta indica* (Neem) on the frequency of congenital malformations in fetuses from rats.

2. Materials and methods

2.1. Extraction of plant materials

The alcoholic extract containing the active principle (azadirachtin) and the oil were both obtained from the washed seeds of *Azadirachta indica* and they were acquired commercially from Base Fertil Ltda (Ribeirão Preto, Brazil). According to the company, the oil was obtained by first pressing of the seeds at room temperature followed by filtration with a Whatman® filter paper. Dallaqua et al. (2012) verified that the neem oil presented a higher proportion of fatty acids, especially linoleic acid (18:2, $\omega-6$). The alcoholic extract containing azadirachtin was obtained by micronization of the seeds followed by solvent extraction using ethanol 70% (v/v) and the amount of azadirachtin was quantified by liquid chromatography (HPLC). This analysis indicated 1.430 ppm (mg/kg) of azadirachtin in alcoholic extract. Seed samples were authenticated as those of *Azadirachta indica* A. Juss by the Mogiense Herbarium, University of Mogi das Cruzes, São Paulo State, Brazil.

2.2. Experimental animals

Female and male *Sprague Dawley* (SD) rats weighing approximately 190 g and 220 g, respectively, were obtained from CEMIB (Multidisciplinary Center for Biological Research)—Campinas State University (UNICAMP). These animals were adapted and maintained in Vivarium of Laboratory of Experimental Research on Gynecology and Obstetrics, Unesp, and were maintained under standard laboratory conditions (22 ± 3 °C, 12 h light/dark cycle), with pelleted food (Purina rat chow, Purina®, São Paulo State, Brazil) and tap water ad libitum. The local Committee of Ethics in Animal Experimentation approved all experimental procedures of this study.

2.3. Mating procedure

All female rats were mated overnight to male rats. The morning when sperm was found in the vaginal smear was designated as day 0 of pregnancy. The mating procedure consisted for 15 consecutive days, which comprises approximately three oestral cycle, however non-mated female rats in this period were considered infertile and removed of the study (Damasceno et al., 2012).

2.4. Experimental groups

After mating period, the pregnant rats were distributed into three experimental groups (minimum $n=11$ rats): NT=non-treated with neem; TOil=treated with neem seed oil (1.2 mL/day); TAP=treated with active principle of *Azadirachta indica* (azadirachtin—1.0 mg/mL/day). The NT group received only vehicle (filtered water). The treatment with oil and azadirachtin was orally given by gavage twice a day during whole pregnancy. After previous test experiment to evaluate the neem concentration, Dallaqua et al. (2012) established for the oil treatment 0.6 mL of oil administered in the morning and 0.6 mL given in the afternoon. The azadirachtin was reconstituted with 1 mL of distilled water, then 0.5 mg/mL was administered in the morning and 0.5 mg/mL was given in the afternoon.

2.5. Course of pregnancy

Blood samples were collected from the tail vein in order to determine glucose levels on days 0, 7, 14 and 20 of pregnancy by One-Touch Ultra Johnson & Johnson® glucometer. Values were expressed in milligrams per deciliter (mg/dL). Oral glucose tolerance test (OGTT) was performed at day 17 of pregnancy for estimation of total area under the curve (AUC). After fasting of 6 h, glucose solution was administered by gavage (2 g/kg body weight) and blood samples were obtained from a cut tip tail for blood glucose levels at 0, 10, 20, 30 and 120 min (de Mello et al., 2001). OGTT was applied to assess the development of altered glucose metabolism during treatment. At day 21 of pregnancy, the rats were lethally anesthetized by sodium thiopental (Thiopentax®—50 mg/kg). Then, laparotomy was performed to remove the uterine horns and the fetuses were collected.

2.6. Analysis of external and internal (visceral and skeletal) malformations

The fetuses were weighed and analyzed for the presence of external malformations (Damasceno et al., 2008). After this analysis, half of the fetuses were fixed in Bodian's solution and serial sections prepared as described by Wilson (1965) for visceral examination. The remaining fetuses were prepared for skeletal examination by the staining procedure of Staples and Schnell (1964). Besides the skeletal analyses, the counting of the ossification sites was performed according to methodology proposed by Aliverti et al. (1979), which determines the degree of fetal development. The question whether a fetal abnormality is classified as malformation, variation or anomaly is much discussed. The agreed definition for malformation was as follows: Malformation is a permanent structural change that is likely to adversely affect the survival or health of the species under investigation. Variation is a change that occurs within the normal population under investigation and is unlikely to adversely affect survival or health. This might include a delay in growth or morphogenesis that has otherwise followed a normal pattern of development (Chahoud et al., 1999).

2.7. Statistical analysis

The data were presented as proportions (%) and mean ± standard deviation. For blood glucose levels, area under curve (AUC), ossification site comparison, *t* test was applied. Fisher´s exact test was used for fetal malformation/variation frequency and proportion the test of multiple comparison of Tukey was used. Statistical significance was considered as $p<0.05$.

3. Results

The effect of neem seed oil and active principle treatment on blood glucose levels is shown in Fig. 1. Both treatments caused no hypoglycemic effect in rats during pregnancy.

The Fig. 2 represents the area under the curve (AUC) of the OGTT from rats treated with neem seed oil and active principle. The AUC of rats from TOil and TAP groups showed no differences ($p > 0.05$) as compared to NT group.

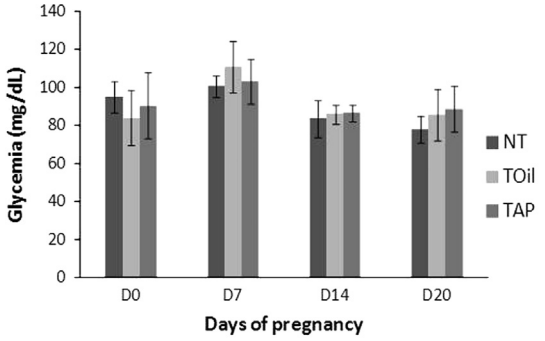
Fig. 3 shows a relationship among fetuses presenting no malformation/variation (normal fetuses) and fetuses with malformation/variation (malformed fetuses).

NT dams had 54 normal fetuses and 18 fetuses presented variations, and TOil dams had 11 malformed fetuses and 53 fetuses with variations. In the TAP group, it was verified 34 fetuses and these showed no malformations, only variations.

A total of 12 dams treated with neem seed oil, only one dam had fetuses with external malformations, i.e., seven fetuses presented exoencephaly and one of them had macroglossia (Fig. 4). All other experimental groups showed no external malformations in fetuses.

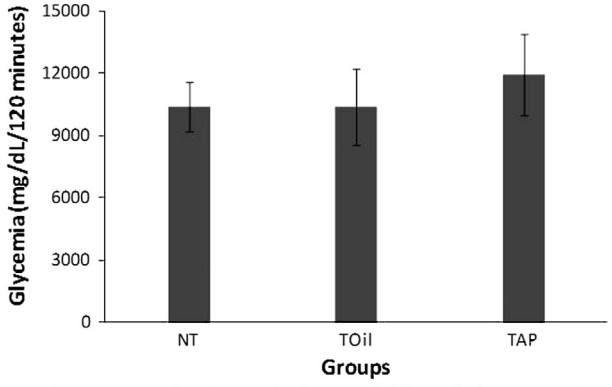
The frequency of visceral and skeletal malformations in fetuses is shown in Table 1. Fetuses from TOil rats presented higher incidence of anophthalmia (11.86%) compared with fetuses from NT rats (0.00%). Both treatments with oil and active principle increased the incidence of enlarged trachea (13.56 and 23.08%, respectively) as compared to fetuses from non-treated rats (NT). Moreover, the dams treated with neem oil (TOil) presented fetuses with increased frequency of abnormally shaped sternebrae (32.76%) as compared to those from NT rats (13.51%).

Fetuses from rats treated with neem oil or azadirachtin showed no altered ossification sites as compared to those from non-treated rats (Table 2).



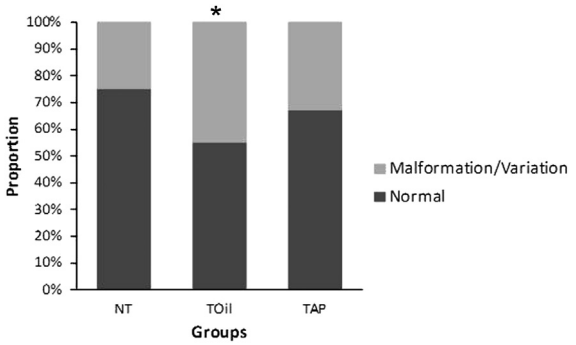
Legend: NT = non-treated; TOil = treated with neem seed oil (1.2 mL/day); TAP = treated with azadirachtin (1.0 mg/mL/day).

Fig. 1. Mean blood glucose levels at days 0, 7, 14 and 20 of pregnancy of rats non-treated and treated with neem seed oil and azadirachtin.



Legend: NT = non-treated; TOil = treated with neem seed oil (1.2 mL/day); TAP = treated with azadirachtin (1.0 mg/mL/day).

Fig. 2. Area under de curve (AUC) at day 17 of pregnancy of rats non-treated and treated with neem seed oil and azadirachtin.



Legend: NT = non-treated rats; TOil = rats treated with neem seed oil (1.2 mL/day); TAP = rats treated with azadirachtin (1.0 mg/mL/day).
**p<0.05 – significant difference compared to ND group (Test of Multiple Comparison of Tukey)*

Fig. 3. Proportion of normal and malformed fetuses from rats treated with neem seed oil and active principle.

4. Discussion

Medicinal plants are widely spread in the populations of underdeveloped countries, which have limited access to medical assistance.

In this study, the treatment with neem seed oil and active principle (azadirachtin) caused no significant effect in blood glucose levels from pregnant rats. Similarly, Dallaqua et al. (2012) using the same methodology observed the same results in pregnancy rats. In contrast, Dixit et al. (1986) observed lower blood glucose levels in male and female rats treated with neem seed oil (petroleum ether extraction). Perez-Gutierrez and Damian-Guzman (2012) demonstrated that sub-acute exposure of one new *Azadirachta indica* tetranortriterpenoid-derived, called by authors as meliacinolin, administrated in mice showed significant reduction in blood glucose. In another study, Radwan et al. (2001) demonstrated that sub-acute exposure to azadirachtin increased glycemia in rats. The divergent results observed might be due to the part of the plant and the extraction technique used significantly influence the results obtained.

The treatment with neem oil caused higher proportion of fetus with malformation/variation. The dams treated with neem seed oil during pregnancy presented fetuses with external (macroglossia and exoencephaly) and internal (anophthalmia, enlarged trachea and abnormally shaped sternebrea) malformations, showing the deleterious effect of this plant on the fetal development. The exact mechanisms involved in the neem-induced abnormalities need be more investigated.

In general, there is evidence that the major causal factor to teratogenic processes in embryonic tissues is oxidative stress (Eriksson, 2009), which is defined as an imbalance between prooxidants and ROS-scavenging enzymes (Poston et al., 2011). In our laboratory, a previous study using similar treatment methodology in rats observed higher levels of malonaldehyde (MDA—lipid peroxidation marker) at term pregnancy in rats treated with neem seed oil and active principle (Azadirachtin) (Dallaqua et al., 2012). In addition, it was verified that there was a significant positive correlation between maternal MDA levels and number of malformed fetuses in TOil group (data not shown). These results show that neem oil led to increase in lipoperoxidation, i.e, exacerbated oxidative stress status, contributing to an

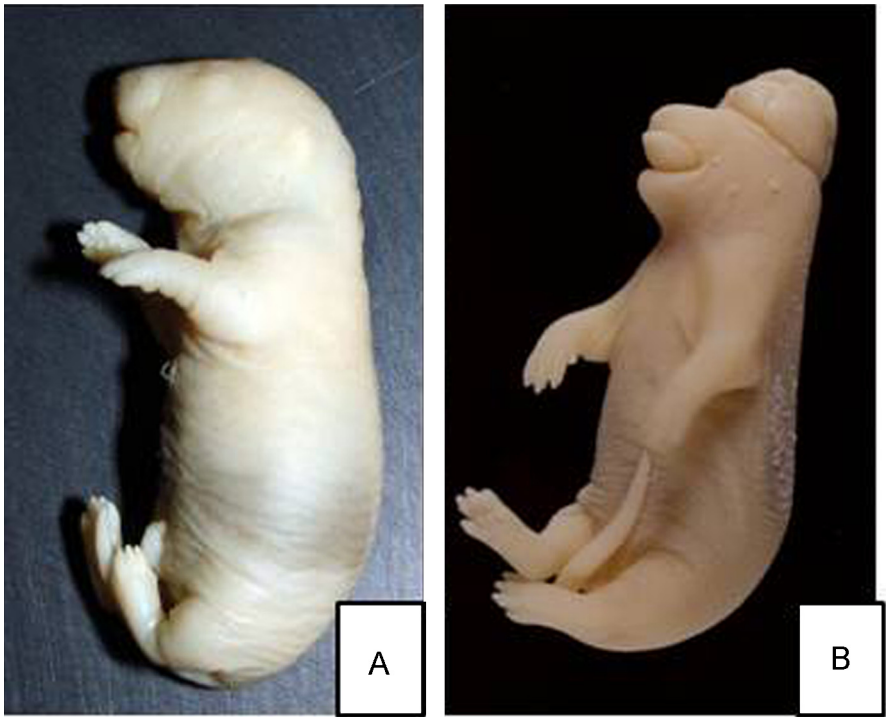


Fig. 4. Photography of fetuses of rats treated with neem oil presenting external malformations. (A) Normal fetus and (B) Fetus presenting macroglossia (large tongue) and exencephaly (brain is located outside of the skull).

Table 1
Frequency of malformations in fetuses from rats treated with neem seed oil and active principle.

	NT	TOil	TAP
Total number of pregnant rats	13	12	12
Number of rats with abnormal fetuses	8	10*	10*
Total number of fetuses examined	138	117	82
Visceral Malformations			
Number of fetuses examined	64	59	39
Anophthalmia	0 (0.0)	7 (11.9)*	0 (0.0)
Enlarged renal pelvis	0 (0.0)	1 (1.7)	2 (5.1)
Hydroureter	12 (18.7)	10 (16.9)	12 (30.8)
Sinuous ureter	10 (15.6)	9 (10.2)	6 (15.4)
Enlarged nasal cavity	0 (0.0)	6 (11.8)	2 (5.1)
Enlarged trachea	0 (0.0)	8 (13.6)*	9 (23.1)*
Enlarged esophagus	0 (0.0)	1 (1.7)	0 (0.0)
Enlarged bronchia	0 (0.0)	3 (5.1)	0 (0.0)
Skeletal Malformations			
Number of fetuses examined	74	58	43
Abnormally shaped sternebrae	10 (13.5)	19(32.7)*	8 (18.6)
Incomplete ossification of sternebrae	0 (0.0)	2 (3.5)	0 (0.0)
Unilateral supranumerary rib	8 (10.8)	4 (6.9)	5 (11.6)
Bilateral supranumerary rib	2 (2.7)	0 (0.0)	2 (4.6)
Bipartite ossification vert centrum	2 (2.7)	5 (8.6)	1 (2.3)
Abnormal ossification vert. centrum	2 (2.7)	9 (15.5)	2 (4.6)

NT=non-treated; TOil=rats treated with neem seed oil (1.2 mL/day); TAP=rats treated with azadirachtin (1.0 mg/mL/day).

* $p < 0.05$—significant difference compared to ND group (Fisher's exact test).

Table 2
Ossification sites from rats treated with neem seed oil and active principle.

Ossification sites	ND	NDOil	NDPA
Anterior phalanges	3.96 ± 0.10	3.98 ± 0.07	4.00 ± 0.00
Metacarpus	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
Posterior phalanges	3.38 ± 0.63	3.30 ± 0.92	3.51 ± 0.61
Metatarsus	4.93 ± 0.12	4.95 ± 0.14	5.00 ± 0.00
Caudal vertebrae	5.48 ± 0.33	5.05 ± 0.77	5.40 ± 0.70
Sternebrae	6.00 ± 0.00	5.98 ± 0.07	6.00 ± 0.00
Total	27.75 ± 0.97	27.26 ± 1.59	27.91 ± 1.10

NT=non-treated; TOil=rats treated with neem seed oil (1.2 mL/day); TAP=rats treated with azadirachtin (1.0 mg/mL/day).

unfavorable intrauterine environment, which impaired fetal development. Differently of our results, Srivastava and Raizada (2007) verified that female rats treated with a diet supplemented with azadirachtin (100, 500 and 1000 ppm) presented no alterations in the maternal reproductive performance and in the post-natal development in two generations.

Another factor contributing to an increased malformation rate in fetuses from dams treated with neem seed oil is found in study of Dallaqua et al. (2012). These authors observed that neem seed oil presented a higher proportion of linoleic acid (18:2, ω-6). Linoleic acid is polyunsaturated fatty acids (PUFAs) derived from the diet, and dietary intake influences their availability (Weber et al., 1986). During its metabolism is converted to gamma (γ)—linoleic acid and then arachidonic acid (AA). AA is converted to 2-series eicosanoids, such as prostaglandins (PGs) E_2 and $F_{2\alpha}$. (Jungheim et al., 2013). Studies have been shown that eicosanoids derived from AA are generally proinflammatory agents (Poudyal et al., 2011, 2012). It is well-known that PUFAs play an important role in animal reproduction, manifested by alternating the composition of PUFAs in membrane phospholipids during reproductive processes (Wathes et al., 2007). In the early development, the proinflamatory action is important to play a key role at the time of embryo implantation (Mor et al., 2011). Therefore, an increase in the PUFAs balance at the time of implantation may enhance endometrial inflammation and encourage embryo implantation (Jungheim et al., 2013).

Some studies attribute the protective effects of PUFAs, while others disagree on the long-term implications of recommended dietary intake of linoleic acid because of the proinflammatory effects of eicosanoids derived from arachidonic acid, the major metabolite of linoleic acid (Russo, 2009). A study performed by

Weiss et al. (2012) showed that the dietary intake of linoleic acid was associated with increased odds of gastroschisis (OR: 1.72; 95% CI: 1.08, 2.74; $p=0.02$). The authors concluded that the mechanism by which this occurs may be via inflammatory processes and oxidative stress leading to a vascular disruption. This fact might explain the increased rate of fetal malformation found in fetuses from rat treated with neem oil related to an increased intake of "oxidizable" and "proinflammatory" PUFAs.

5. Conclusion

In conclusion, neem seed oil treatment administered during pregnancy caused abnormalities in rat fetuses, showing teratogenic effect, but the Azadirachtin (active principle) presented no impairment in the fetuses.

Acknowledgments

The authors are grateful to Talísia Moreto, technician of the Laboratory of Experimental Research on Gynecology and Obstetrics and to the Research Support Center (RSC) of Universidade Estadual Paulista_UNESP, for their valuable contribution in study design and statistical analysis. This study was supported by grants from FAPESP/Brazil (Grant Number 2009/14147-8), under the coordination of Dr. Débora C. Damasceno.

References

Abdel Megeed, M.I., Radwan, U.M., Hindy, A.Z., El Zarook, A., 2001. Liver functions under stress of certain common pesticides residue used on fruits and vegetables orally administrated. Ann. Agr. Sci. Cairo 46, 383–404.

Ali, B.H., Salih, A.M., 1982. Suspected *Azadiracta indica* toxicity in a sheep. Vet. Rec. 111, 494.

Aliverti, V., Bonanomi, L., Giavini, E., 1979. The extent of fetal ossification as an index of delayed development in teratogenic studies on the rat. Teratology 20, 237–242.

Bopana, K.N., Kannan, J., Gadgil, S., Balaram, R., Rathod, S.P., 1997. Antidiabetic and antihyperlipidaemic effects of neem seed kernel powder on alloxan diabetic rabbits. Indian J. Pharmacol. 29, 162–167.

Chahoud, I., Buschmann, J., Clark, R., Druga, A., Falke, H., Faqi, A., Hansen, E., Heinrich-Husch, B., Hellwig, J., Lingk, W., Parkinsin, M., Paumgartten, F.J.R., Pfeil, R., Platzek, T., Scialli, A.R., Seed, J., Stahlann, R., Ulbrich, B., Wu, X., Yasuda, M., Younes, M., Solecki, R., 1999. Classification terms in development toxicology: need for harmonization. Reproductive Toxicol. 13, 77–82.

Dallaqua, B., Saito, F.H., Rodrigues, T., Calderon, I.M., Rudge, M.V., Herrera, E., Damasceno, D.C., 2012. Treatment with Azadirachta indica in diabetic pregnant rats: negative effects on maternal outcome. J. Ethnopharmacol. 143, 805–811.

Damasceno, D.C., Kempinas, W.G., Volpato, G.T., Consoni, M., Rudge, M.V.C., Paumgartten, F.J.R., 2008. Anomalias Congênitas: Estudos Experimentais, 1° ed. Coopmed, Belo Horizonte.

Damasceno, D.C., Sinzato, Y.K., Bueno, A., Dallaqua, B., Lima, P.H., Calderon, I.M., Rudge, M.V., Campos, K.E., 2012. Metabolic Profile and Genotoxicity in Obese Rats Exposed to Cigarette Smoke. Obesity (Silver Spring), 10.1002/oby.20152.

de Mello, M.A., de Souza, C.T., Braga, L.R., dos Santos, J.W., Ribeiro, I.A., Gobatto, C.A., 2001. Glucose tolerance and insulin action in monosodium glutamate (MSG) obese exercise-trained rats. Physiol. Chem. Phys. Med. NMR 33, 63–71.

Dixit, V.P., Sinha, R., Tank, R., 1986. Effect of Neem seed oil on the blood glucose concentration of normal and alloxan diabetic rats. J. Ethnopharmacol. 17, 95–98.

Eriksson, U.J., 2009. Congenital anomalies in diabetic pregnancy. Semininars in Fetal & Neonatal Medicine 14, 85–93.

Gupta, S., Kataria, M., Gupta, P.K., Murganandan, S, Yashroy, RC, 2004. Protective role of extracts of neem seeds in diabetes caused by streptozotocin in rats. J. Ethnopharmacol. 90, 185–189.

Jungheim, E.S., Frolova, A.I., Jiang, H., Riley, J.K., 2013. Relationship between serum polyunsaturated fatty acids and pregnancy in women undergoing in vitro fertilization. J. Clin. Endocrinol. Metab. 98, E1364–E1368.

Khosla, P., Bhanwara, S., Singh, J., Seth, S., Srivastava, R.K., 2000. A study of hyperglycemia effects of *A. indica* (Neem) in normal and alloxan diabetic rabbits. Indian J. Physiol. Pharmacol. 44, 69–74.

Lai, S.M., Lim, K.W., Cheng, H.K., 1990. Margosa oil poisoning as a cause of toxic encephalopathy. Singapore Med. J. 31, 463–465.

Mor, G., Cardenas, I., Abrahams, V., Guller, S., 2011. Inflammation and pregnancy: the role of the immune system at the implantation site. Ann. N.Y. Acad. Sci. 1221, 80–87.

Mukherjee, S., Lohiya, N.K., Pal, R., Sharma, M.G., Talwar, G.P., 1996. Purified neem (*Azadirachta indica*) seed extracts (Praneem) abrogate pregnancy in primates. Contraception 53, 375–378.

Mukherjee, S., Talwar, G.P., 1996. Termination of pregnancy in rodents by oral administration of praneem, a purified neem seed extract. Am. J. Reproductive Immunol. 35, 51–56.

Othman, F., Motalleb, G., Peng, Lam Tsuey, Rahmat, S., Basri, A., Pei Pei, C., R., 2012. Effect of neem leaf extract (*Azadirachta indica*) on c-Myc oncogene expression in 4T1 breast cancer cells of BALB/c mice. Cell J. 14, 53–60.

Perez-Gutierrez, R.M., Damian-Guzman, M., 2012. Meliacinolin: a potent α-glucosidase and α-amylase inhibitor isolated from *Azadirachta indica* leaves and in vivo antidiabetic property in streptozotocin-nicotinamide-induced type 2 diabetes in mice. Biol. Pharm. Bull. 35, 1516–1524.

Poston, L., Igosheva, N., Mistry, H.D., Seed, P.T., Shennan, A.H., Rana, S., Karumanchi, S.A., Chappell, L.C., 2011. Role of oxidative stress and antioxidant supplementation in pregnancy disorders. Am. J. Clin. Nutr. 94, 1980S–1985S.

Poudyal, H., Panchal, S.K., Diwan, V., Brown, L., 2011. Omega-3 fatty acids and metabolic syndrome: effects and emerging mechanisms of action. Prog. Lipid Res. 50, 372–387.

Poudyal, H., Panchal, S.K., Ward, L.C., Brown, L., 2012. Effects of ALA, EPA and DHA in highcarbohydrate, high-fat diet-induced metabolic syndrome in rats. J. Nutr. Biochem. http://dx.doi.org/10.1016/j.jnutbio.2012.07.014.

Radwan, M.U., Hindy, Z.A., Megeed, M.A., 2001. Residual activity of orally adimistrated pesticides used in fruits and vegetables on rat blood parameters behavior. Ann. Agr. Sci. Cairo 46, 365–382. (abstract).

Raizada, R.B., Srivastava, M.K., Kaushal, R.A., Singh, R.P., 2001. Azadirachtin, a neem biopesticide: subchronic toxicity assessment in rats. Food Chem. Toxicol. 39, 477–483.

Riar, S.S., Devakumar, C., Ilavazhagan, G., Bardhan, J., Kain, A.K., Thomas, P., Singh, R., Singh, B., 1990. Volatile fraction of neem oil as a spermicide. Contraception 42, 479–487.

Riar, S.S., Devakumar, C., Sawhney, R.C., Ilavazhagan, G., Bardhan, J., Kain, A.K., Thomas, P., Singh, R., Singh, B., Parshad, R., 1991. Antifertility activity of volatile fraction of neem oil. Contraception 44, 319–326.

Russo, G.L., 2009. Dietary n-6 and n-3 polyunsaturated fatty acids: from biochemistry to clinical implications in cardiovascular prevention. Biochem. Pharmacol. 77, 937–946.

Srivastava, M.K., Raizada, R.B., 2007. Lack of toxic effect of technical azadirachtin during postnatal development of rats. Food Chem. Toxicol. 45, 465–471.

Staples, R.E., Schnell, V.L., 1964. Refinements en rapid clearing technic in the KOH-alizarin red S method for fetal bone. Stain Technol. 39, 61–63.

Tomlin, C., 1997. The Pesticide Manual. British Crop Protection Council, 11th Edition, Famham, Surrey GU9 7PH, United Kingdom.

Upadhyay, S.N., Kaushic, C., Talwar, G.P., 1990. Antifertility effects of neem (*Azadirachta indica*) oil by single intrauterine administration: a novel method for contraception. Proc. Biol. Sci. 242, 175–179.

Wathes, D.C., Abayasekara, D.R., Aitken, R.J., 2007. Polyunsaturated Fatty Acids in Male and Female Reproduction. Biol Reprod. 77, 190–201.

Weber, P.C., Fischer, S., Von Schacky, C., Lorenz, R., Strasser, T., 1986. The conversion of dietary eicosapentaenoic acid to prostanoids and leukotrienes in man. Prog. Lipid Res. 25, 273–276.

Weiss, L.A., Chambers, C.D., Gonzalez, V., Hagey, L.R., Jones, K.L., 2012. The omega-6 fatty acid linoleic acid is associated with risk of gastroschisis: a novel dietary risk factor. Am. J. Med. Genet. 158A, 803–807.

Wilson, J.C., 1965. Methods for administering agents and detecting malformations in experimental animal. In: Wilson, JC, Warkany, J. (Eds.), Teratology: Principles and Techniques. Univ. of Chicago Press, Chicago.