UNIVERSIDADE ESTADUAL PAULISTA – Unesp
Faculdade de Medicina de Botucatu

# INTERVENÇÃO COM *Azadirachta indica* (Neem) NA PRENHEZ DE RATAS DIABÉTICAS: REPERCUSSÕES MATERNO-FETAIS

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***Dissertação apresentada à Faculdade de Medicina de Botucatu – Unesp, Programa de Pós-Graduação em Ginecologia, Obstetrícia e Mastologia, Área de concentração Tocoginecologia, Área de conhecimento: Farmácia, para obtenção do título de Mestre.***

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"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis."

*Fernando Pessoa*

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# Capítulo 1

# Comprovação do efeito antioxidante de plantas medicinais utilizadas no tratamento do D*iabetes mellitus* em animais: artigo de atualização

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# Comprovação do efeito antioxidante de plantas medicinais utilizadas no tratamento do D*iabetes mellitus* em animais: artigo de atualização

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**RESUMO:** *Diabetes mellitus* (DM) é uma síndrome de etiologia múltipla caracterizada por hiperglicemia crônica. Esta hiperglicemia induz o aumento na produção de espécies reativas de oxigênio (ERO) e diminuição das defesas antioxidantes. Devido às complicações causadas pelo diabete, muitos indivíduos optam por terapias alternativas à base de plantas medicinais para amenizar seus efeitos. Sendo assim, nesta revisão de literatura, foram analisados e descritos diversos trabalhos experimentais com a utilização de animais diabéticos para comprovar os efeitos antioxidantes de algumas dessas plantas e verificar se os títulos e resumos disponibilizados nos artigos são compatíveis aos objetivos de nossa busca.

**Palavras-chave**: diabete, plantas, antioxidantes, ratos

**ABSTRACT: Evidence of antioxidant effect of medicinal plants used in the treatment of *Diabetes mellitus* in animals: an update.** *Diabetes mellitus* (DM) is a syndrome of multiple etiologies characterized by chronic hyperglycemia. This

hyperglycemia induces increased production of reactive oxygen species (ROS) and decreased antioxidant defenses. Due to complications caused by diabetes, many people choose for alternative therapies and herbal medicine to alleviate its effects. Thus, in this literature review, several experimental studies with the use of diabetic animals were analyzed to demonstrate the antioxidant effects of some plants and to verify if the titles and abstracts provided in the articles are compatible to the aims of our search.

**Key words:** diabetes, plant, antioxidant, rats

Diversas drogas são utilizadas para o controle dos níveis glicêmicos em portadores de Diabetes mellitus (DM), entretanto o perfeito controle é raramente alcançado (Damasceno et al., 2004; Takaku et al., 2006). DM é uma desordem crônica causada pela falta de produção de insulina pelas células beta (β)-pancreáticas ou pelo defeito nos receptores de insulina nas células-alvo, resultando em doença metabólica hiperglicêmica (American Diabetes Association - ADA, 2009). DM é situação clínica frequente, acomete em torno de 7% da população. Cerca de 50% dos portadores de diabete desconhecem o diagnóstico (Takaku et al., 2006; ADA, 2009). A deficiênca de insulina resulta em níveis elevados de glicose sanguínea, que, por sua vez, pode causar danos em vários sistemas do organismo (Leahy, 2005), alterando o metabolismo de carboidratos, lipídios e proteínas (ADA, 2009). A síndrome diabética pode apresentar-se com sintomas característicos, tais como sede, poliúria, visão turva, perda de peso e polifagia e, nas formas mais graves, pode ocorrer cetoacidose, que, na ausência de tratamento eficaz, ocasiona hálito acético, coma e morte (Davidson, 2001).

DM pode ser classificado em tipo 1 (DM1), tipo 2 (DM2) e gestacional. DM1 é a forma auto-imune, resulta da destruição das células β-pancreáticas por mecanismo mediado por células. No DM2, os indivíduos afetados apresentam resistência à insulina, em combinação com deficiência relativa (não absoluta) da secreção de insulina (Davidson, 2001; ADA, 2009). DM gestacional é caracterizado pelo quadro de intolerância à glicose, com primeira identificação na gravidez e pode persistir após o parto evoluindo para DM2 (Buchanan et al., 2005; 2007).

A hiperglicemia crônica no diabete provoca glicação de proteínas que, por sua vez, leva a complicações secundárias que afetam olhos, rins, nervos e artérias (Sharma, 1993). A síndrome diabética está relacionada à formação de radicais livres, que são espécies reativas de oxigênio (ERO) formadas através da redução de um ou dois elétrons de oxigênio ($O_2$) (Eriksson & Born, 1993; Reece et al., 1998; Zhao & Reece, 2005; Damasceno et al., 2006; Cimen, 2008). Em situações de estresse, nosso organismo gera desequilíbrio entre os agentes oxidantes e antioxidantes para se proteger, resultando no quadro de estresse oxidativo (Halliwell et al., 1999; Damasceno et al., 2002).

A fonte primária para formação de ERO é o aumento do influxo de piruvato e oxigênio na mitocôndria e aumento na produção de ERO (principalmente superóxido) em processos oxidativos na cadeia de transporte de elétrons (Yang et al., 1997). O radical superóxido passa para outros compartimentos da mitocôndria e citosol promovendo a formação de peróxido de hidrogênio e radical hidroxil que causará alterações na mitocôndria (Yang et al., 1995), acarretando lipoperoxidação (Wentzel et al., 1999) e danos no DNA (Lee et al., 1999).

A primeira linha de defesa contra os danos oxidativos são os antioxidantes endógenos enzimáticos, o superóxido dismutase (SOD), a catalase e o sistema de glutationas, dentre elas, glutationa redutase (GSH-Rd) e glutationa peroxidase (GSH-Px). Há também os antioxidantes não-enzimáticos que são lipossolúveis

(tocoferois, carotenos, quinonas e bilirrubinas) e hidrossolúveis (ácido ascórbico, ácido úrico e proteínas ligadas a metais) (Halliwell et al., 1999; Damasceno et al., 2002).

Embora a produção de ERO sob condições hiperglicêmicas não seja demonstrada diretamente, o estresse oxidativo é identificado por métodos indiretos, como pela peroxidação fosfolipídica, redução nos níveis intracelulares de enzimas antioxidantes (SOD, Catalase e GSH-Px) e depleção da capacidade antioxidante do meio hiperglicêmico (Trocino et al., 1995; Cederberg & Eriksson, 1997). ERO pode ser prejudicial para as células porque reagem com ácidos graxos insaturados que são encontrados nas membranas plasmáticas, produzindo peróxidos lipídicos que leva à diminuição da fluidez da membrana (Kaplan et al., 1995) e à formação de aldeídos reativos, que podem reagir com macromoléculas (Gutteridg & Halliwell, 1990; Mattson, 1998). ERO também pode reagir diretamente com proteínas, resultando em ligações cruzadas de colágeno com o DNA, causando danos em suas bases e açúcares (Imlay & Linn 1988; Elgawish et al., 1996).

Estudos realizados em humanos para explorar os mecanismos responsáveis pelas alterações causadas pelo diabete são limitados não somente por razões éticas, mas também pela multiplicidade de variáveis não controladas, como hábitos alimentares, fatores socioeconômicos, nutrição e fatores genéticos. Desta forma, existe a necessidade de reproduzir modelos experimentais para mimetizar a síndrome diabética (López-Soldado & Herrera, 2003; Souza et al., 2009; Sinzato et al., 2009). Para isso, são utilizadas drogas β-citotóxicas como *streptozotocin* (STZ) e aloxana. A aloxana induz a formação de ERO resultando em necrose das células β-pancreáticas (Lenzel, 2008), sendo assim, esta droga além de lesar o pâncreas prejudica outros tecidos, pois não apresenta especificidade somente às células β-pancreáticas. A aloxana vem sendo menos empregada pelos pesquisadores em função de efeitos adversos e a pouca margem de segurança entre a dose letal e a dose eficaz para obtenção do quadro diabético (Iessi et al., 2007). Já, o STZ inibe a síntese de pró-insulina nas células β-pancreáticas e também induz alquilação do

DNA, resultando em fragmentação e consequente morte da célula β (Lenzel, 2008 ). Desta forma, STZ não causa dano em outros tecidos do organismo (Iessi et al., 2007), com isso mostra ser mais eficaz para indução do modelo de diabete experimental.

Frente aos conhecimentos adquiridos sobre a formação de ERO e os antioxidantes presentes em nosso organismo para se defender contra os insultos oxidativos (estresse oxidativo exacerbado), diversos pesquisadores têm investigado substâncias com efeito antioxidante.

Atualmente, mais de 800 tipos de plantas são utilizados no tratamento do DM (Saxena et al., 2004) e a maioria destas apresentam amplo espectro na clínica. Sendo assim, há necessidade em se explorar o campo da fitomedicina para fornecer terapias alternativas para tratamento da síndrome diabética (Yeh et al., 2003; Suba et al., 2004).

Apesar do grande interesse no desenvolvimento de novas drogas para prevenir complicações associadas a esta doença, a comunidade científica tem interesse em avaliar o estado do produto natural isolado em estudos experimentais, sendo que alguns deles já
foram testados em seres humanos (Liu et al., 2004; Vuksan & Sievenpiper, 2005; Johnson et al., 2006; Jung et al., 2006; Matsui et al., 2006). Suplementos naturais são amplamente utilizados em todo o mundo para tratar o diabete (Liu et al., 2004). No entanto, a maioria dos produtos naturais é consumida de forma indiscriminada e grande parte deles não foi testada ou não teve efeito confirmado.

Desta forma, este estudo tem como objetivo apresentar uma atualização bibliográfica das plantas medicinais utilizadas para o tratamento do DM e que apresentaram atividade antioxidante.

Foi realizada revisão de literatura referente ao período de 1992 a 2009 (17 anos) no site do database do National Center of Biotechnology Information (NCBI –

PUB MED). Para busca dos artigos, foram empregados os unitermos Plant and antioxidant and diabetes and rats e foram encontrados 313 artigos.

Após leitura e análise dos títulos e resumos, do total de 313 artigos encontrados, 134 (42,8%) eram coerentes quanto ao título e resultados; 87 (27,8%) apresentaram título e resultados não adequados à investigação; 53 (16,9%) tinham título adequado e resultados não pertinentes e 39 (12,5%) apresentaram título não compatível à pesquisa e resultados coerentes.

A Tabela 1 mostra os resultados com relação às plantas mais conhecidas que apresentaram atividade antioxidante confirmada cientificamente dentre os artigos analisados.

**TABELA 1.** Plantas que apresentam efeito antioxidante.

| Planta | Nome Popular | Parte utilizada | Intervenção | Dose | Referência |
|---|---|---|---|---|---|
| *Aegle marmelos* | - | Fruto | Extrato aquoso | 125 e 250 mg $Kg^{-1}$ | Kamalakkannan et al. (2003) |
| *Allium cepa* | Cebola | - | Solução de *Allium cepa* | 0.4 g A. cepa/rato | Campos et al. (2003) |
| *Allium sativum* | Alho | - | Extrato metanólico | 250 e 500 mg $Kg^{-1}$ | Rajani et al. (2008) |
| *Aloe vera* | Aloe vera | Folhas | Extrato gel | 300 mg $Kg^{-1}$ | Rajasekaran et al. (2005) |
| *Andrographis paniculata* | - | Folhas | Extrato aquoso | 400 mg $Kg^{-1}$ | Dandu & Inamdar (2009) |
| *Annona squamosa* | Pinha ou Ateira | Folhas | - | - | Panda & Kar (2007) |
| *Bauhinia forficata* | Pata de vaca | Folhas | Extrato aquoso | 500, 600 e 1000 mg $Kg^{-1}$ | Volpato et al. (2008) |
| *Brassica oleracea* | Couve | Flores | Frações de MeOH, CH2Cl2, BuOH | 100 e 200 mg $Kg^{-1}$ | Cho et al. (2006) |

| | | | | | |
|---|---|---|---|---|---|
| *Camellia sinensis* | Chá verde | Folhas | Extrato aquoso | 500 mg $Kg^{-1}$ | Sabu et al. (2002) |
| *Casearia esculenta* | - | Raiz | Extrato aquoso | 200 e 300 mg $Kg^{-1}$ | Prakasam et al. (2003) |
| *Cassia fistula (Linn.)* | Chuva de ouro | Flores | Extrato aquoso | 20 g $Kg^{-1}$ | Manonmani et al. (2005) |
| *Commelina communis L* | - | - | Extrato e pó | - | Shibano et al. (2008) |
| *Coccinia indica* | - | Folhas | Extrato etanólico | 200 mg $Kg^{-1}$ | Venkateswaran & Pari (2003) |
| *Gymnema montanum* | - | Folhas | Extrato alcoólico | 200 mg $Kg^{-1}$ | Ananthan et al. (2003) |
| *Ginkgo biloba* | Ginkgo biloba | Folhas | Extrato aquoso | 200 mg $Kg^{-1}$ | Rudge et al. (2007) |
| *Gongronema latifolium* | - | Folhas | Extrato aquoso e etanólico | 100 mg $Kg^{-1}$ | Ugochukwu & Cobourne (2003) |
| *Hibiscus sabdariffa* | Quiabo-roxo; vinagreira; caruru-azedo | Flores | Extrato Etanólico | 100 e 200 mg $Kg^{-1}$ | Farombi & Ige (2007) |
| *Laminaria japonica* | Algas Kombu | Talo | Extrato aquoso | 100 mg $Kg^{-1}$ | Jin et al. (2004) |
| *Matricaria chamomilla L* | Camomila | Folhas | Extrato Etanólico | 20, 50 e 100 mg $Kg^{-1}$ | Cemek et al. (2008) |
| *Morus bombycis koidzumi* | - | Raiz | - | 200-800 mg $Kg^{-1}$ | Heo et al. (2007) |
| *Morus indica L.* | Amora | Folhas | Pó das folhas | 25% | Andallu & Varadacharyulu (2003) |
| *Panax ginseng* | Ginseng | Folhas | Extrato aquoso | 40 e 200 mg $Kg^{-1}$ | Jung et al. (2005) |
| *Phaseolus vulgaris* | Feijão-base, feijão-comum, feijoeiro | Vagens | Extrato aquoso | 200 mg $Kg^{-1}$ | Venkateswaran et al. (2002) |
| *Quercetin allievates* | - | - | - | 50 e 80 mg $Kg^{-1}$ | Mahesh & Menon (2004) |
| *Scoparia dulcis* | Vassourinha-doce | Toda a planta | Extrato aquoso | 200 mg $Kg^{-1}$ | Pari & Latha (2004) |

| | | | | | |
|---|---|---|---|---|---|
| *Syzigium cumini* | Jambolão, Jamelão, Jalão | Sementes | Extrato aquoso | 2,5 e 5,0 g $Kg^{-1}$ | Prince et al. (1998) |
| *Tinospora cordifolia* | - | Raiz | Extrato alcoólico | 100 mg $Kg^{-1}$ | Prince et al. (2004) |
| *Trigonella foenum graecum* | Feno-grego | Sementes | Extrato aquoso | 2 g $Kg^{-1}$ | Anuradha & Ravikumar (2001) |

Frente esta revisão de literatura, pode ser verificado que diferentes formas de preparo dos produtos naturais podem levar a efeitos similares como mostrado nos trabalhos realizados com extrato metanólico (250 e 500 mg $Kg^{-1}$) (Rajani et al., 2008), sumo (0,4g 100 g) (El-Demerdash et al., 2005) e o óleo de *Allium sativum* (10 mg $Kg^{-1}$) (Anwar & Meki, 2003), que observaram elevação nas atividades das enzimas antioxidantes.

Da mesma forma, estudos realizados com *Allium cepa* (cebola), utilizando diferentes preparações, mostraram que animais diabéticos tratados com o sumo (El-Demerdash et al., 2005) apresentam diminuição na concentração de TBARS e aumento das atividades das enzimas antioxidantes no plasma, fígado, cérebro e rim. Ao serem tratados com o composto isolado de *Allium cepa* (Kumari et al., 2002) também se observou aumento de enzimas antioxidantes, mas somente no plasma.

*Camellia sinensis*, conhecida popularmente como chá verde, vem despertando interesse em todo mundo. Diferentes artigos de diversos países confirmaram seu potencial antioxidante, dentre eles Juskiewicz et al. (2008), pesquisadores da Polônia, observaram aumento nas atividades de GSH-Px e de SOD no soro e diminuição na concentração do marcador de lipoperoxidação (TBARS) no rim de ratos diabéticos. Babu et al. (2006), da Índia, trataram ratos diabéticos e comprovaram redução de TBARS e aumento na atividade de GSH no coração. Nos EUA, Mustata et al. (2005) evidenciaram aumento nos níveis de GSH

e diminuição de hidroperóxidos no soro de animais diabéticos. Sabu et al. (2002), pesquisadores da Índia, também mostraram que várias doses de *Camellia sinensis* aumentaram as atividades das enzimas antioxidantes SOD e GSH e redução das concentrações dos marcadores de lipoperoxidação em animais diabéticos.

Pesquisas realizadas com diferentes tipos de extratos de *Andrographis paniculata* mostraram que o extrato etanólico (Zhang & Tan, 2000) e o extrato aquoso (Dandu & Inamdar, 2009) das folhas desta planta apresentaram aumento nas atividades de SOD e CAT, mas não apresentaram efeito significativo sobre a atividade de GSH-Px em ratos diabéticos.

O extrato aquoso de *Annona squamosa* exerceu efeito antioxidante em animais diabéticos onde observaram aumento das atividades de CAT, SOD e GSH e diminuição

nas concentrações de marcadores de lipoperoxidação (Panda & Kar, 2007; Gupta et al., 2008).

Experimentos realizados com diferentes preparações de *Aegle marmelos* para verificar o possível efeito antioxidante desta planta em diferentes tecidos evidenciaram que o extrato metanólico diminuiu a concentração de TBARS no soro e fígado e aumentou as atividades das enzimas antioxidantes CAT e GSH-Px no fígado de animais diabéticos (Sabu & Kuttan, 2004). Kamalakkannan et al. (2003), ao tratarem animais diabéticos com extrato aquoso da mesma planta, mostraram aumento das mesmas enzimas antioxidantes no pâncreas e coração.

Embora, muitos trabalhos sejam realizados para verificar os efeitos antioxidantes de plantas medicinais em animais de laboratório, diversos pesquisadores não concluem os mesmos efeitos nos animais em comparação aos obtidos na população em geral. Isto pode ser devido à diferença de sensibilidade

existente entre as espécies, tipo de preparo com as plantas, dose e via de administração utilizada.

Além disso, cabe ressaltar que os resumos não descrevem de forma satisfatória os resultados obtidos para esclarecimento do artigo como um todo, dificultando a busca por artigos coerentes à pesquisa. Desta forma, este artigo de atualização confirmou a necessidade de se redigir títulos e resumos adequados ao estudo para viabilizar a busca de informações.

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# Capítulo 2

# *Azadirachta indica* INTERVENTION IN DIABETIC PREGNANT RATS: IMPAIRED EFFECTS ON OFFSPRING AND ON MATERNAL ORGANISM

**Running title:** *Azadirachta indica* in diabetic pregnant rats

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# ABSTRACT

**Background:** Medicinal plants are widely used to control diabetes and its complications. This study aimed at evaluating the *Azadirachta indica* seed effects on maternal outcomes, oxidative stress and lipid biomarkers in the diabetic and non-diabetic pregnant rats. **Methods:** Diabetes was induced by streptozotocin at birth day (100mg/kg sc). Neem oil (1.2mL/day) and azadirachtin (1.0mg/mL/day) was orally administered to non-diabetic and diabetic rats during pregnancy. The untreated groups similarly received the vehicle. Fasting blood glucose was measured on days 0, 7, 14 and 20. GTT was analyse at day 17 of pregnancy and was used as inclusion criterion. The GTT data were used to perform area under the curve. At the end of the pregnancy, the maternal reproductive outcomes were analyzed, blood samples were collected to determine lipid parameters, antioxidant enzymes (superoxide dismutase, gluthatione peroxidase), thiol groups, malonaldehyde and maternal liver was weighed. **Results:** Of the diabetic dams, regardless treatment, 77% presented glycemic mean >100mg/dL and altered GTT and about 80% of the non-diabetic dams showed glycemic mean <100mg/dL and unaltered GTT. Diabetic rats presented decreased numbers of implantation and live fetus, and increased preimplantation loss rate, SOD, MDA and free fatty acid levels. Neem oil decreased GSH-Px, cholesterol, free fatty acid levels and increased liver weight in diabetic rats. Already non-diabetics presented increased reabsorption rate, SOD, MDA and liver weight and azadirachtin reduced live fetus number and increased preimplantation loss, SOD and MDA. **Conclusions:** The oil and the active principle (azadirachtin) extracted from seeds of *A. indica* (Neem) in pregnant rats was harmful to intrauterine development as non-diabetic as diabetic dams. The different treatments presented no benefic effect on normalization of the metabolic alterations, which were verified in the pregnant diabetic dams.



**Key-words:** *Azadirachta indica*, diabetes, pregnancy, rats, oxidative stress, lipid profile.

## INTRODUCTION

Maternal diabetes during pregnancy is known to increase the risk of reproductive anomalies, miscarriages, congenital malformation and fetal mortality and morbidity [1].

Animal models of experimentally induced hyperglycemia are helpful for specifically studying the mechanisms responsible for the diabetic changes. Experimental diabetes induced during adult life of laboratory animals causes severe diabetic clinical status that reproduces uncontrolled human type 1 *Diabetes mellitus* (DM1). In rats, severe diabetes (glycemia >300mg/dL) causes higher number of reabsorption (embryonic death) and increased rate of pre and post-implantation losses leading to decreased number of live fetuses [2,3]. These results are confirmed with clinical findings.

Several studies have been performed in our laboratory in order at evaluating the maternal, placental and fetal outcomes of mild diabetes (glycemia between 120 and 300 mg/dL) in rat pregnancy. Iessi *et al.* observed that neonatally induced mild diabetes altered fetal development, characterized by intrauterine growth restriction and the glycemic levels did not lead to any major congenital defects in the fetuses of streptozotocin-induced mild diabetic rats [4]. Saito *et al*. induced mild diabetes before (neonatal period) and at day 7 of pregnancy in rats and it was verified negative impact on maternal reproductive performance, intrauterine growth restriction and impaired fetal development [5]. Sinzato *et al*. showed that IL-10 can be used as predictor of alterations in the embryo-fetal organism and in placental development in rat with mild diabetes [6]. Damasceno *et al*. confirmed that maternal hyperglycemic environment caused metabolic alterations, including increased triglyceride and total cholesterol concentrations, and elevated oxidative stress, contributing to increase fetal visceral anomalies [7].

Pregnancy per se is a state of oxidative stress [8] arising from the increased metabolic activity and reduced scavenging power of antioxidants [9]. Reactive oxygen species (ROS) are involved in diabetic complications. Oxidative stress is described as an imbalance in the production of ROS and the ability of antioxidant defenses to scavenge them. ROS induce cellular damage by acting on proteins and lipids. In diabetes, oxygen free radicals are thought to be produced as a result of prolonged periods of exposure to hyperglycemia. Oxidative stress has also been observed in experimental studies. de Souza *et al*. reported that severe diabetes in rats led significant increase of thiobarbituric acid reactive substances (TBARS) and superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities [10]. Spada [11] using rats with mild diabetes (glycemia between 120 and 300mg/dL) observed an

increase in these markers, showing an exacerbation of oxidative stress regardless of the glycemic intensity.

Several drugs are used to control diabetes to minimize the complications of diabetes in the mother to benefit the fetus, however perfect glycemic control is rarely achieved [12,13]. Many studies have been performed using herbal medicine in the search for antidiabetic activity [2,14]. There were many plants used for diabetes treatment, such as *Azadirachta indica*, popularly known as neem. Neem is a tree originary from India and Burma which presents medicinal properties. One of the first studies performed with this plant showed that it has anti-hyperglycemic and hypoglycemic activities in female rats treated with the seed oil [15]. Neem presented a proven effect as pesticide [16], is studied against the Chagas disease [17], antibiotic [18], and against diabetes [19,20]. Due to the fact that this plant is popularly used as anti-diabetic, but as there are not any information about its effects in lipidic metabolism and in oxidative stress during pregnancy, the aim of the present study was to verify lipid profile, oxidative stress status and anti-hyperglycaemic effects of *Azadirachta indica* (Neem) in non-diabetic and diabetic female rats treated during pregnancy.

## MATERIALS AND METHODS

*Extraction of plant materials*

The oil and the alcoholic extract containing the active principle (azadirachtin) were both obtained from the washed seeds of *Azadirachta indica* and they were acquired commercially from Base Fertil Ltda (Ribeirão Preto, Brazil). The oil was obtained by first pressing of the seeds at room temperature followed by a filtration in a Whatman® filter paper. The extract containing azadirachtin was obtained by micronization of the seeds followed by solvent extraction using ethanol 70% (v/v) and the quantification of azadirachtin was performed by liquid chromatography (HPLC).

### *Experimental animals*

Female and male *Sprague Dawley* (SD) rats weighing approximately 190g and 220g, respectively, were obtained from CEMIB – Campinas State University (UNICAMP). These animals was adapted and maintained on 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 animals were cared for in accordance with the principles of the Guide for Care and Use of Experimental Animals. The local Committee of Ethics in Animal Experimentation approved all experimental procedures of this study.

## *Diabetes induction*

At day 0 of life (birth day), the diabetes was induced by streptozotocin (STZ - SIGMA Chemical Company, USA) in female newborns. STZ was dissolved in a citrate buffer (0.1M, pH 4.5) and administered by subcutaneously (sc) injection at a dose of 100 mg/kg body weight. Non-diabetic rats subcutaneously received citrate buffer [modified by 21, 22]

## *Mating procedure*

All female rats (non-diabetic and diabetic) were mated overnight to non-diabetic male. The morning when sperm was found in the vaginal smear was designated gestational day 0. 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 discarded of the study.

## *Experimental groups*

After mating period, the pregnant rats were distributed into six experimental groups (n minimum =12 rats): ND= non-diabetic non-treated; NDOil= non-diabetic treated with neem seed oil (1.2 mL/day); NDPA= non-diabetic treated with azadirachtin (1.0 mg/mL/day); D= diabetic non-treated; DOil: diabetic treated with neem seed oil; DPA = diabetic treated with azadirachtin. The treatment with oil and azadirachtin was orally given by gavage twice a day during whole pregnancy. For the oil treatment, 0.6 mL of oil was administered in the morning and 0.6 mL was given in the afternoon. The azadirachtin was reconstituted with 1mL of distilled water, then 0.5 mg/mL was administered in the morning and 0.5 mg/mL was given in the afternoon.

## *Course of pregnancy*

Blood samples were collected from the tail vein in order to determine glucose levels at days 0, 7, 14 and 20 of pregnancy by a One-Touch Ultra Johnson & Johnson® glucometer. Values were expressed in milligrams per deciliter (mg/dL). At day 14 of pregnancy, blood samples were also obtained from tail vein for insulin determination. This measurement was performed by ELISA microplates (Crystal Chemical®, USA). Oral glucose tolerance test (GTT) was performed at day 17 of pregnancy. After fasting of 6 hours, glucose solution was administered by gavage (2g/kg body weight) and blood samples were obtained from a cut tip tail for glycemic determinations at 0, 10, 20, 30 and 120 minutes [23]. GTT was applied to assess the development of altered glucose metabolism and used as a criterion fot maintain the rats in the respective groups. Non-diabetic rats that presented glycemia below 140 mg/dL in more than two time points during the test continued in the experiment into same group. For rats with STZ-induced diabetes that presented glycemia higher than 140 mg/dL in more than two time points during GTT continued in the experiment into diabetic groups. Glucose responses during the GTT were evaluated by estimation of the total area under the curve (AUC), using the trapezoidal method [24]. At day 21 of pregnancy, the rats were anesthetized by sodium thiopental (Thiopentax® - 80 mg/kg) and humanely killed. Maternal blood was then collected from the inferior cava vein. After collection, blood samples were kept in two tubes: one containing ethylenediamine tetraacetic acid (EDTA; 5% w/v, 1 drop/mL of blood) for oxidative stress status, and another without EDTA for lipid profile. The gravid uterus was weighed and dissected to count live fetuses, implantation, resorption and corpora lutea numbers. The number of implantation sites were determined by the Salewski method (1964) [25]. The rate of preimplantation loss was calculated as: No. of corpora lutea – No. of implantations x 100/ No. of corpora lutea, and post implantation loss rate was calculated as: No. of implantations – No. of live fetuses x 100/ No. of implantations [26]. Next, each maternal liver was collected and weighed to obtain relative and absolute weights of this organ.

## *Analytical methods for fatty acid profile*

Lipids were extracted from 0.20 mL of neem oil into chloroform/methanol (2:1) [27]. Fatty acids were transesterified with acetyl chloride, and fatty acid-methyl esters separated and analyzed on a Perkin-Elmer gas chromatograph (Autosystem; Norwalk, CT, USA) using a flame ionization detector and capillary column Omegawax 30 mx 0.25 mm. Fatty acids

results were expressed as a percentage (% w/w) and concentration of all detected fatty acids, with a chain length of 12–24 carbon atoms in the sample [28,29].

## *Assay for lipid profile*

The blood samples without anticoagulant were kept at the low temperature for 30 minutes (min) and then centrifuged at 3,500 rpm for 10 min at 4ºC. The supernatant was collected as serum and stored at –80ºC for determination of the total cholesterol, high density lipoprotein (HDL) and triglycerides by Wiener® assay kit (Rosario, Argentina). The free fatty acid concentration was performed by methodology of Moura *et al*. [30]

## *Assay for oxidative stress status*

Other portion of blood put into anticoagulant tubes was centrifuged at 1,200 rpm for 10 min at 4°C for assay of oxidative stress status. The oxidative stress biomarkers estimated were superoxide dismutase (SOD), thiol groups (SH), glutathione peroxidase (GSH-Px) and malonaldehyde (MDA). All parameters were estimated in the washed erythrocytes. MDA was used as index of lipid peroxidation. This marker was analyzed using the thiobarbituric acid (TBA) by spectrophotometry. Briefly 1.0 mL of washed erythrocytes were added to the test tube containing 1.0 mL of 3.0% sulphosalicilic acid, agitated for 10 sec, centrifuged at 11,000 rpm for 3 min and kept in rest for 15 min. The sample was diluted to 500μl of 0.67% TBA solution. The mixture was heated to 80ºC for 30 min and the absorbance measured at a wavelength of 535 nm. The results were expressed as nM of MDA per gram of hemoglobin (nM/g Hb) [10].

Superoxide dismutase (SOD) activity was determined from its ability to inhibit the auto-oxidation of pyrogallol. The reaction mixture (1.0 mL) consisted of 5.0 mM Tris (hydroxymethyl) aminomethane (pH 8.0), 1.0 mM EDTA, bidistilled water and 20 μL of the sample. The reaction was initiated by the addition of pyrogallol (final concentration of 0.2 mM), and the absorbance was measured by a spectrophotometer with a wavelength of 420 nm (25ºC) for 5 min. Enzymatic activity unit was defined as SOD units able to produce 50% of pyrogallol oxidation inhibition. All data were expressed in units of SOD per miligram of hemoglobin [10].

Thiol groups content (SH) was enzymatically determined using 5,5′- dithio-bis (2-nitrobenzoic acid) (DTNB) and glutathione reductase in the presence of a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), forming 2-nitro-5-thiobenzoic acid.

A mixture consisting of 1290 μL of distilled water, 200 μL of Tris/HCl buffer (1M, pH 8.0, 5mM EDTA), 200 μL of 10 UI/mL glutathione reductase (GSH-r) (SIGMA, St. Louis, USA), 200 μL of 2 mM NADPH (SIGMA, St. Louis, USA) and 100 μL of 12 mM of DTNB (SIGMA, St. Louis, USA) was added to 10 μL of the sample. Activity was measured at 412 nm on a spectrophotometer. One unit of activity was equal to the micromolar of substrate reduced per gram of hemoglobin [10].

Glutathione peroxidase content (GSH-Px) was performed by monitoring NADPH oxidation. The mixture consisted in the addition of 1.300 μL of distilled water, 200 μL of Tris/HCl buffer (EDTA 1M; pH 8,0; 5mM), 200 μL de 10 UI/mL of glutathione reductase (GSH-Rd), 200 μL of NADPH (2mM), 40 μL of GSH (0,1 M) to 40 μL of hemolysate. The mixture was agitated in a vortex mixer for 10 sec. Next, 20 μL of T-Buthyl hydroperoxide (7mM) were added and maintained at 37ºC for 10 min. Absorbance was determined by a spectrophotometer with a wavelength of 340 nm. GSH-Px activity was expressed in enzymatic activity units per milligram of hemoglobin (UI/mg Hb) [10].

### *Statistics*

The data were presented as mean ± standard deviation. For determination of insulin, malonaldehide, superoxide dismutase, glutathione peroxidase, total cholesterol and maternal liver weight, Test of Multiple Comparison of Tukey was used. For rate of rats with glycemic mean inferior or superior to 100 mg/dL, Fisher Exact Test was performed. The data presented no normal distribution, the tests of log Binomial Distribution (for free fatty acids concentration), Poisson Distribution (for implantation and live fetus numbers), and Normal Gamma Distribution (for GTT and pre and postimplantation loss rates) were used. Statistical significance was considered as $p < 0.05$.

## RESULTS

Table 1 shows the profile of fatty acids of the oil extracted from the *A. indica* seed, and the profile of these fatty acids in the olive oil. There was higher presence of saturated fatty acids (14:0= Myristic Acid; 16:0= Palmitic Acid; 18:0= Stearic Acid; 22:0=

Docosanoic; 24:0= Lignoceric) and unsaturated fatty acids [18:1 (ω - 9)= Oleic Acid; 18:2 (ω - 6)= Linoleic Acid; 20:5 (ω - 3)= Eicosapentaenoic] in neem oil (Table 1).

From 54 female rats that received citrate buffer, 81% mated. From 122 female rats that received streptozotocin (STZ) in the neonatal period, 38% mated in adult life (data not shown).

During pregnancy, 0% of the non-diabetic female rats presented glycemia higher than 100 mg/dL, and 92.3% of the diabetic female rats presented glycemic mean higher than 100 mg/dL. Treatments performed with the oil and the active principle (azadirachtin) in non-diabetic and diabetic animals did not interfere ($p>0.05$) in glycemic means during pregnancy as compared to their respective controls (Table 2).

Figure 1 shows glycemic curves for oral glucose tolerance test (GTT) at day 17 of pregnancy. Experimental groups (ND, NDOil, NDPA, D, DOil, DPA) presented an increased significant glycemia in times 10, 20 and 30 minutes as compared to time points zero and 120 minutes (except DOil group in time points 20 and 30 minutes as compared to 120 minutes). Figure 2 presents the comparison of the GTT curves among different experimental groups. An increase ($p<0.05$) was verified in the glycemia (> 140 mg/dL) at time points 10, 20 and 30 minutes post glucose overload in group D in comparison with group ND (Figure 2A). The GTT curves of ND, NDOil and NDPA groups showed similar results (Figure 2B). In group DOil a decreased glycemia was observed ($p<0.05$) at time points 10 and 20 minutes, and an increased glycemia at time points 30 and 120 minutes in relation to the values presented in the group D (Figure 2C). The glycemic values at the different moments of GTT in group DPA presented differences at time points 30 and 120 minutes ($p<0.05$), as compared to group D (Figure 2D).

The figure 3 represents the area under the curve (AUC) of the GTT from the different experimental groups. It was observed the AUC from NDOil and NDPA rats did not show differences ($p>0.05$) as compared to ND group. The DOil and DPA dams presented higher AUC in relation to that from D group.

No significant statistically difference was evidenced in the number of corpora lutea among different experimental groups. The rats from group D presented a decrease ($p<0.05$) in the number of implantations and live fetuses as compared to ND group. The NDOil dams presented increased ($p<0.05$) number of embryonic deaths (reabsorption) in comparison with ND group. The NDPA dams had a decrease ($p<0.05$) in the number of live fetuses, and an increase in the pre-implantation loss percentage in relation to ND group. An increase ($p<0.05$) in the post-implantation loss percentage in D group was evidenced as compared to

group ND (Table 3). At day 14 of pregnancy, there was not statistically significant difference in the maternal insulin concentrations among experimental groups (Table 3). It was observed increased ($p<0.05$) means of liver absolute and relative weights in the NDOil and DOil dams as compared with ND and D groups, respectively (Table 3).

Table 4 shows the oxidative stress characterization. NDOil, NDPA and D dams presented an increase ($p<0.05$) in the superoxide dismutase enzymatic activity (SOD) and in the malonaldehyde (MDA) concentration in relation to ND group. DOil dams presented a reduced ($p<0.05$) glutathione peroxidase enzymatic activity (GSH-Px) in relation to D group (Table 4).

The rates of total cholesterol, triglycerides, HDL and free fatty acids from NDOil and NDPA groups were not statistically different ($p>0.05$) as compared to those from ND group. An increase ($p<0.05$) was verified in the free fatty acid rates in D rats in comparison with ND group. DOil dams presented a reduction ($p<0.05$) in the total cholesterol and fatty acid levels in relation to the D dams. The biomarkers of lipid profile from DPA group did not differ ($p>0.05$) of those from D group (Table 5).

## DISCUSSION

In the present study, it was verified that streptozotocin (STZ)-induced dams in the neonatal period, without any type of treatment during pregnancy, presented a diabetic status. This fact was confirmed because the diabetic dams presented glycemic means higher than 100 mg/dL during pregnancy. These results were compatible with the clinical findings verified in women with clinical and/or gestational diabetes and with mild gestational hyperglycemia at our diabetes and pregnancy medical service [31]. The treatments with the oil and with azadirachtin did not present hypoglycemic and anti-hyperglycemic effects in non-diabetic and diabetic dams, respectively. Radwan *et al*. evidenced that the sub-acute exposure of azadirachtin increased glycemia in normoglycemic rats [32]. In contrast, when Khosla *et al*. treated diabetic rabbits with oil and extract from neem leaves, they observed hypoglycemic effect [33]. Corroborating these results, Dixit *et al*. treated rats only with the oil and observed similar effects [34]. A study performed in patients with type 2 diabetes showed that neem seeds in high doses presented hypoglycemic effect [19]; however, studies with this type of intervention in pregnant women were not performed. By this way, the

literature results are controversial related to the hypoglycemic and anti-hyperglycemic activities of the neem.

The non-diabetic rats treated with the oil and those treated with azadirachtin did not present glycemic alteration in the glucose tolerance test (GTT), showing the GTT curves of these dams were similar to those from non-diabetic dams, according to the results found in the area under the curve (AUC) from these animals. However, the diabetic dams presented altered GTT, i.e., showed more than two altered times points (glycemia > 140mg/dL) and, consequently higher values in the AUC, confirming glucose intolerance. In the presence of the oil and azadirachtin treatment in these animals, there was not contribution these to improve the insulin concentration and it was verified an exacerbation of the glucose intolerance status, showing that *A. indica* (Neem) did not present benefic effect on the glycemic levels. In contrast, Bhat *et al*. evidenced that diabetic mice treated with neem, extracted by methanol and by chloroform, presented improvement in the glucose tolerance status [35]. The divergent results between our study and that of Bhat *et al*. is due to the fact that there are differences between the mild diabetes induction methods, plant species and extraction methodologies [35].

Our results showed the non-treated diabetic dams presented a decreased number of implantations and increased reabsorption rates (embryonaric deaths). These findings corroborate those observed in the literature, since as uncontrolled diabetes during pregnancy causes an increased intra-uterine death rate and abortions in women [1,36] and in laboratory animals [4]. Maternal hyperglycemia can compromise the viability of embryos in early development stage and/or alter maternal decidua, interfering in the embryo fixation process on the maternal endometrium, thus impairing embryonaric implantation [37]. Besides, it was observed that diabetic dams without treatment presented a reduction in the number of viable fetuses and, consequently an increased loss rate after embryonic implantation, reflecting the reproductive disturbances caused by hyperglycemia [38]. Non-diabetic rats treated with the oil during pregnancy presented increase in the number of reabsorptions, corroborating with Boeke *et al.* [39]. When these authors treated normoglycemic female rats with neem oil at the beginning of pregnancy evidenced abortive effect in comparison with female rats that were treated only at the end of pregnancy [39]. While, non-diabetic animals treated with azadirachtin also presented increase in embryo loss rates before implantation and reduction in the number of live fetuses, showing that this treatment negatively interfered in the embryofetal development, similarly as in diabetic rats. These results differ of Srivastava & Raizada, who 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 [40]. It was also observed an increase of pre-implantation loss in non-diabetic female rats treated with azadirachtin, demonstrating that the neem isolated active principle exacerbated the losses before implantation. The different experimental groups treated with the oil and with azadirachtin presented exacerbation in the post-implantation loss rate. Then, the different treatments did not protect the embryos and fetuses from diabetes-induced damage.

The increased oxidative stress caused by hyperglycemia is already well described in the literature [1]. A lipoperoxidation exacerbation was evidenced in this study, confirmed by the increase of MDA concentrations in diabetic dams, even with the increased antioxidant defense system. Similarly, non-diabetic and diabetic dams treated with the oil and with azadirachtin had an increased oxidative stress, characterizing that these treatments impaired the oxidant/antioxidant status in non-diabetic dams, and absence of protection in the diabetic animals. These data do not corroborate the study of Gupta *et al*., who observed a beneficial effect (decreased lipoperoxides) by different parts of the seed in males [41]. Other studies also showed that the vegetal extract of *Azadirachta indica* in diabetic rats was effective in the prevention of diabetes and oxidative stress [42,43].

The diabetic condition leads alterations in the lipid profile as verified by Boden, who evidenced an increase of free fatty acids concentration in patients with type 2 diabetes (DM2) [44]. In the present study, increased fatty acid concentrations were observed in diabetic rats. The oil and azadirachtin did not interfere in the lipid profile of non-diabetic dams, but the treatment with oil caused a reduction of total cholesterol and free fatty acids rates in the presence of diabetes. Besides, it was verified that the oil treatment contributed to increase the liver weight in non-diabetic and diabetics dams. It is suggested that the increased liver weight in non-diabetic and diabetic dams treated with oil was a consequence of the increased fatty acid concentrations in this organ due to neem oil is rich in fatty acids, mainly oleic acid (18:1 ω-9), linoleic acid (18:2 ω-6), stearic acid (18:0) and palmitic acid (18:0). These data may be confirmed by Jourdan *et al.* [45]. These authors showed that linoleic acid causes carbohydrates and lipids metabolism alteration and induced liver steatosis is partly due to an increase in hepatic lipogenesis. Besides, Boeke *et al*. verified an increased ALT and AST enzymatic levels, which are hepatic alteration markers in rats treated with the active principle isolated from neem (azadirachtin) [39].

## CONCLUSION

The oil and the active principle (azadirachtin) extracted from seeds of *A. indica* (Neem) in pregnant rats was malefic to intrauterine development as non-diabetic as diabetic dams. The different treatments presented no benefic effect on normalization of the metabolic alterations, which were verified in the pregnant diabetic dams. All these results show that the use of this plant was malefic to the maternal organism. Therefore, this study shows the importance of raising the population's knowledge, especially pregnant women about indiscriminate use of natural products without the due knowledge of their effects.


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Table 1. Fatty acid profile of the oil extracted from seeds of *A. indica*, and profile of similar fatty acids found in olive oil.

| | Fatty acid (FA) profile Oil neem | | | | Fatty acid (FA) profile Olive Aceite | | | |
|---|---|---|---|---|---|---|---|---|
| | (%) | | (mg FA/g of aceite) | | (%) | | (mg FA/g of aceite) | |
| **FA** | **mean** | **SE** | **mean** | **SE** | **mean** | **SE** | **mean** | **SE** |
| **14:O** | 0.17 | 0.02 | 1.52 | 0.17 | n.d. | n.d. | n.d. | n.d. |
| **14:I** | n.d. | n.d. | n.d. | n.d. | 0.06 | 0.00 | 0.52 | 0.03 |
| **15:O** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **15:I** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **16:O** | 15.92 | 0.12 | 139.62 | 4.77 | 11.50 | 0.08 | 106.08 | 1.42 |
| **16:1 (w-7)** | 0.16 | 0.00 | 1.42 | 0.07 | 0.88 | 0.02 | 8.11 | 0.19 |
| **17:I** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **18:O** | 16.68 | 0.03 | 146.29 | 4.25 | 3.37 | 0.01 | 31.09 | 0.31 |
| **18:1 (w-9)** | 42.68 | 0.26 | 374.23 | 10.90 | 77.02 | 0.03 | 710.29 | 4.49 |
| **18:2 (w-6)** | 21.41 | 0.07 | 187.66 | 4.93 | 5.89 | 0.06 | 54.29 | 0.17 |
| **18:3 (w-6)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **18:3 (w-3)** | 0.72 | 0.00 | 6.28 | 0.17 | 0.63 | 0.00 | 5.80 | 0.02 |
| **20:O** | 1.40 | 0.00 | 12.26 | 0.32 | 0.36 | 0.01 | 3.34 | 0.11 |
| **20:1 (w-9)** | 0.08 | 0.01 | 0.74 | 0.06 | 0.22 | 0.00 | 2.07 | 0.03 |
| **20:2 (w-6)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **21:O** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **20:3 (w-6)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **20:4 (w-6)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **20:3 (w-3)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **20:5 (w-3)** | 0.16 | 0.06 | 1.40 | 0.55 | n.d. | n.d. | n.d. | n.d. |
| **22:O** | 0.29 | 0.01 | 2.54 | 0.10 | n.d. | n.d. | n.d. | n.d. |
| **22:1 (w-9)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **22:2 (w-6)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **23:O** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **22:4 (w-6)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **22:3 (w-3)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **22:5 (w-6)** | 0.08 | 0.01 | 0.74 | 0.05 | 0.07 | 0.02 | 0.62 | 0.22 |
| **22:5 (w-3)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **24:O** | 0.24 | 0.00 | 2.14 | 0.05 | n.d. | n.d. | n.d. | n.d. |
| **22:6 (w-3)** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

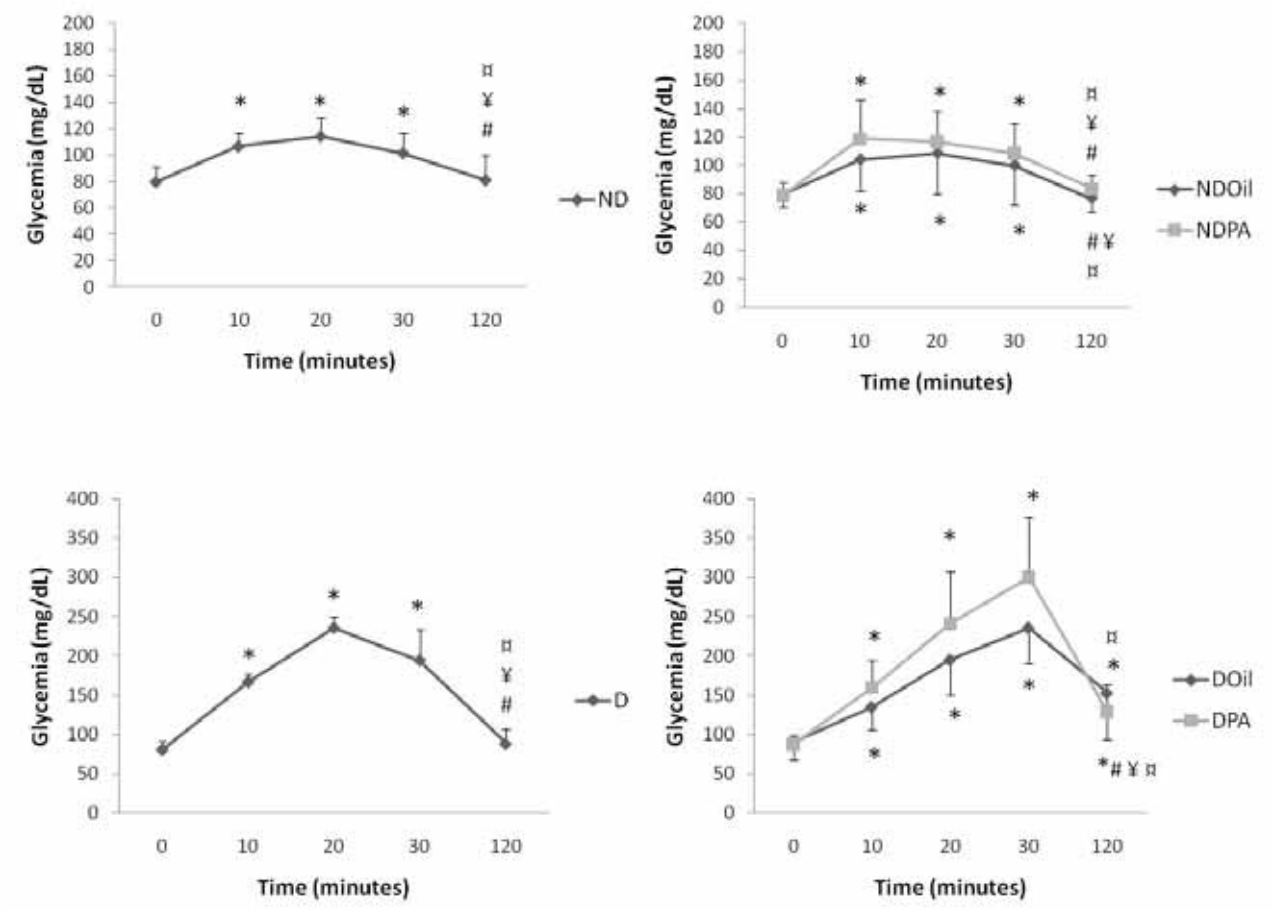
Values are expressed as mean ± standard error (SE) and as percentage (%) of total fatty acids and concentration (mg fatty acid /g oil).

Table 2. Proportion of rats with alteration in glycemic mean during pregnancy.

| | **Glycemia < 100mg/dL (%)** | **Glycemia > 100mg/dL (%)** |
|---|---|---|
| **ND** | 100 | 0* |
| **NDOil** | 91.67 | 8.33* |
| **NDPA** | 81.81 | 18.18* |
| **D** | 7.69 | 92.31* |
| **DOil** | 16.67 | 83.33* |
| **DPA** | 23.07 | 76.93* |

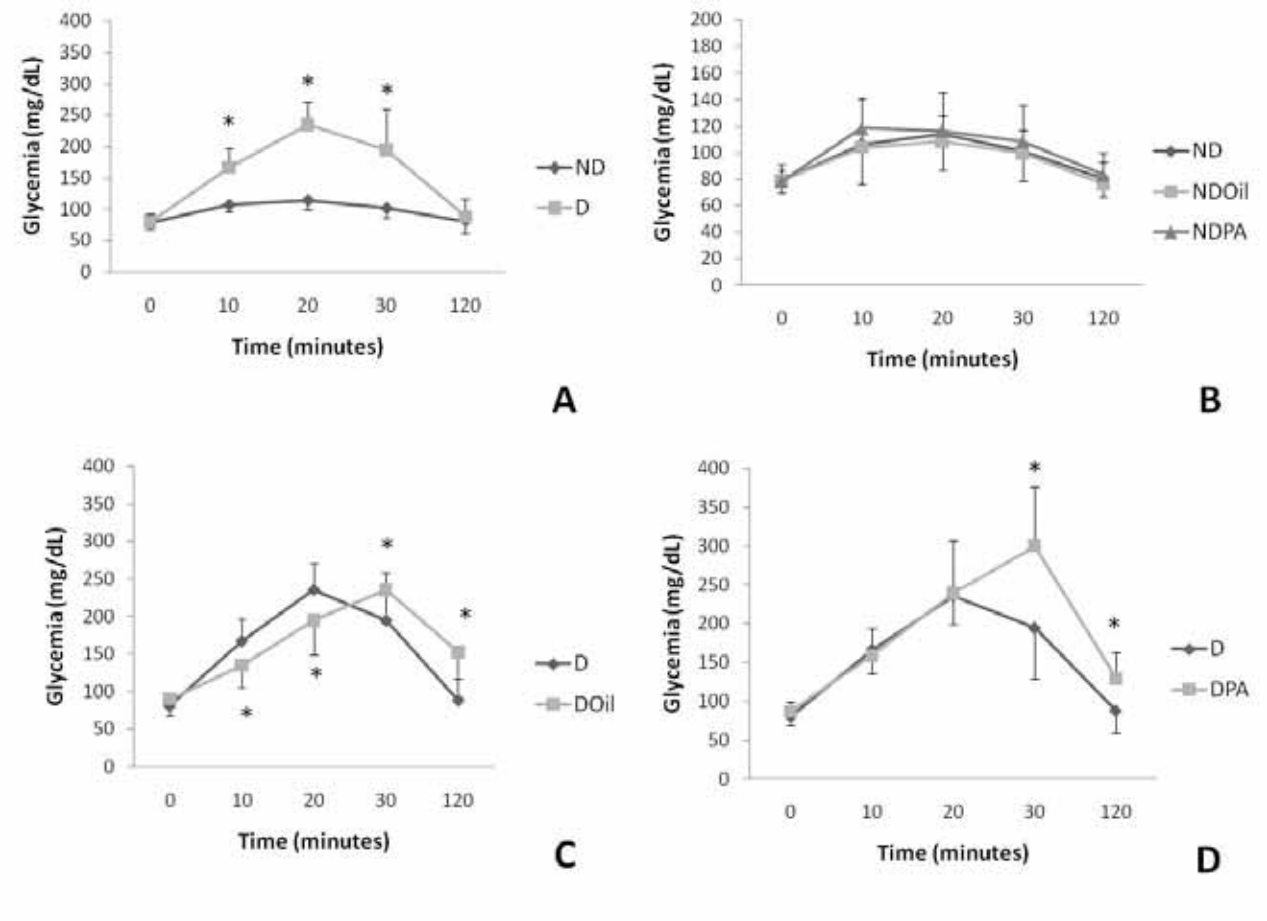
*$p < 0.05$ – Significant statistically difference (Fisher Exact Test)

Figure 1. Plasma glucose levels at different time points after oral glucose overload (2g/kg body weight) at day 17 of pregnancy in non-diabetic non-treated (ND), non-diabetic treated with seed oil neem (NDOil), non-diabetic treated with azadirachtin (NDPA), diabetic non-treated (D), diabetic treated with seed oil neem (DOil), diabetic treated with azadirachtin (DPA) rats.



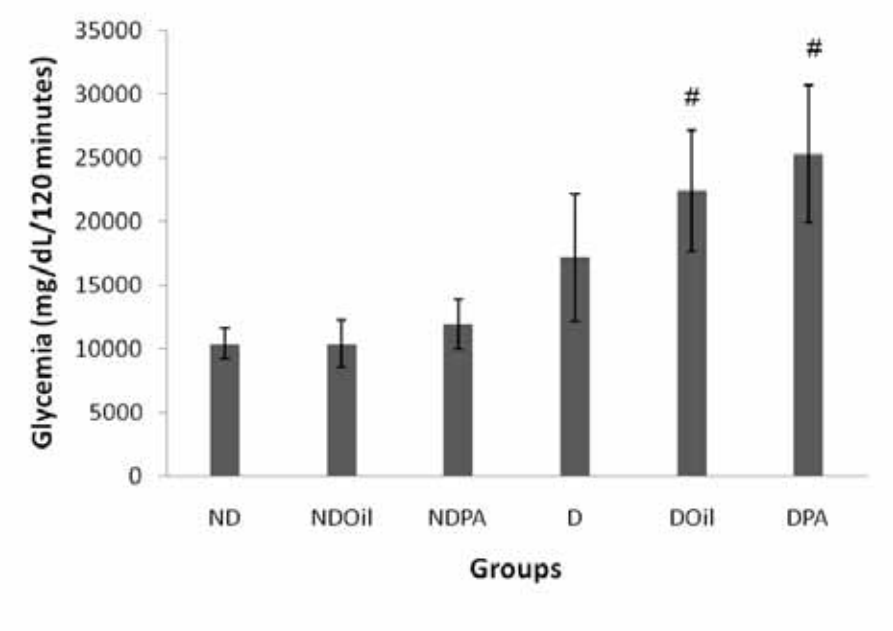

*p<0.05 - Significant statistically difference – Normal Gamma Distribution;
#p<0.05 - Significant difference compared to 10 minutes after oral glucose overload (T10);
¥p<0.05 - Significant difference compared toT20;
¤p<0.05 - Significant difference compared toT30;

Figure 2. Plasma glucose levels at day 17 of pregnancy among different experimental groups into the same time point after oral glucose load (2g/kg body weight).




*p<0.05 – Significant statistically difference (Normal Gamma Distribution)

Figure 3. Area under the curve (AUC) from non-diabetic non-treated (ND), non-diabetic treated with seed oil neem (NDOil), non-diabetic treated with azadirachtin (NDPA), diabetic non-treated (D), diabetic treated with seed oil neem (DOil), diabetic treated with azadirachtin (DPA) rats at day 17 of pregnancy.




#p<0.05 Significant statistically difference compared to D (Test of Multiple Comparison of Tukey).

Table 3. Maternal reproductive performance, liver weight (absolute and relative) and insulin determination from non-diabetic non-treated (ND), non-diabetic treated with seed oil neem (NDOil), non-diabetic treated with azadirachtin (NDPA), diabetic non-treated (D), diabetic treated with seed oil neem (DOil), diabetic treated with azadirachtin (DPA) rats at term pregnancy.

| | **ND** | **NDOil** | **NDPA** | **D** | **DOil** | **DPA** |
|---|---|---|---|---|---|---|
| **No. of corpora lutea** | 14.36 ±2.44 | 14.50 ±2.39 | 14.36 ±1.96 | 14.00 ±3.41 | 16.17 ±3.04 | 15.69 ±2.14 |
| **No. of implantations sites[a]** | 13.26 ±2.82 | 13.75 ±2.77 | 11.09 ±4.30 | 11.44 ±2.99* | 12.25 ±2.26 | 13.15 ±4.32 |
| **No. of live fetuses[a]** | 12.82 ±2.91 | 12.58 ±3.23 | 10.09 ±4.04* | 9.78 ±3.40* | 9.78 ±3.40 | 10.46 ±4.50 |
| **Reabsorption (%)[b]** | 11/33 (33) | 28/40* (70) | 6/12 (50) | 5/11 (45) | 10/12 (83) | 10/13 (76) |
| **Preimplantation loss (%)[c]** | 8.60 | 5.48 | 24.08* | 17.42* | 17.42 | 16.94 |
| **Post-implantation loss (%)[c]** | 3.29 | 8.33 | 8.21 | 16.37* | 16.37 | 19.24 |
| **Liver absolute weight (g)[d]** | 14.70 ±2.05 | 18.75 ±1.91* | 15.61 ±1.80 | 15.30 ±1.53 | 18.46 ±1.32# | 15.31 ±1.84 |
| **Liver relative weight (g/100g maternal corporal weight)[d]** | 3.70 ±0.15 | 4.87 ±0.47* | 4.03 ±0.41 | 4.03 ±0.44 | 5.35 ±0.72# | 4.32 ±0.55 |
| **Insulin (ng/mL)[c]** | 1.27 ±0.15 | 1.25 ±0.30 | 1.64 ±0.52 | 1.50 ±0.88 | 1.52 ±0.65 | 1.07 ±0.41 |

Values are expressed as mean ± Standard deviation (SD). *Significant statistically difference compared to ND; #Significant statistically difference compared to D. [a]$p<0.05$ – Poisson distribution; [b]$p<0.05$ – Fisher Exact Test; [c]$p<0.05$ – Normal Gamma Distribution; [d]$p<0.05$ – Test of Multiple Comparison of Tukey.

Table 4. Oxidative stress profile in blood sampled from non-diabetic non- treated (ND), non-diabetic treated with seed oil neem (NDOil), non-diabetic treated with azadirachtin (NDPA), diabetic non-treated (D), diabetic treated with seed oil neem (DOil), diabetic treated with azadirachtin (DPA) rats at term pregnancy.

| | **ND** | **NDOil** | **NDPA** | **D** | **DOil** | **DPA** |
|---|---|---|---|---|---|---|
| **Hb (g/dL)** | 0.87 ±0.12 | 0.89 ±0.09 | 0.87 ±0.07 | 0.84 ±0.09 | 0.86 ±0.08 | 0.88 ±0.09 |
| **SOD (UI/mg Hb)** | 8.60 ±3.67 | 11.45 ±0.95* | 11.32 ±0.55* | 12.50 ±5.43* | 11.54 ±0.13 | 11.56 ±0.14 |
| **Thiol groups (µM/g Hb)** | 3.64 ±1.30 | 4.17 ±0.74 | 4.38 ±0.51 | 5.11 ±2.17 | 4.39 ±0.59 | 4.30 ±0.52 |
| **GSH-Px (UI/mg Hb)** | 0.30 ±0.16 | 0.22 ±0.09 | 0.26 ±0.06 | 0.69 ±0.71 | 0.22 ±0.08# | 0.25 ±0.06 |
| **MDA (nM/g Hb)** | 166.54 ±85.59 | 501.2 ±172.7* | 542.94 ±95.06* | 582.94 ±219.32* | 641.83 ±152.58 | 501.18 ±138.03 |

Values are expressed as mean ± SD. *Significant statistically difference compared to ND; #Significant statistically difference compared to D. $p<0.05$ – Test of Multiple Comparison of Tukey.

Table 5. Lipid profile in blood sampled from non-diabetic non- treated (ND), non-diabetic treated with seed oil neem (NDOil), non-diabetic treated with azadirachtin (NDPA), diabetic non-treated (D), diabetic treated with seed oil neem (DOil), diabetic treated with azadirachtin (DPA) rats at term pregnancy.

| | **ND** | **NDOil** | **NDPA** | **D** | **DOil** | **DPA** |
|---|---|---|---|---|---|---|
| **Cholesterol (mg/dL)**[a] | 128.46 ±36.20 | 119.93 ±31.20 | 109.46 ±21.52 | 155.44 ±43.32 | 113.17 ±28.99# | 128.48 ±24.91 |
| **Triglycerides (mg/dL)** | 246.82 ±182.20 | 329.21 ±187.00 | 481.03 ±208.11 | 398.28 ±251.48 | 303.91 ±196.16 | 452.68 ±214.20 |
| **HDL (mg/dL)** | 45.95 ±10.00 | 46.30 ±9.78 | 39.67 ±10.04 | 44.96 ±12.47 | 54.67 ±11.35 | 50.71 ±17.49 |
| **Fatty acid (mEq NEFA/L)**[b] | 0.43 ±0.16 | 0.63 ±0.40 | 0.65 ±0.40 | 1.46 ± 1.18* | 0.43 ±0.30# | 0.45 ±0.21 |

Values are expressed as mean ± SD. *Significant statistically difference compared to ND; # Significant statistically difference compared to D. [a]$p<0.05$ – Test of Multiple Comparison of Tukey; [b]$p<0.05$ – Test of log Binomial Distribution.

# Anexos

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Criada através da Portaria DFM nº 10 de 26/04/99

# Certificado

*Certificamos que o Protocolo n.º 788 sobre o projeto de pesquisa "Intervenção com Azadirachta indica (Neem) na prenhez de ratas diabéticas: repercussões materno-fetais", de autoria de Bruna Dallaqua, orientada pela Profª. Drª. Débora Cristina Damasceno, co-orientada por Tiago Rodrigues, com a participação de Aline Bueno, Felipe Hiroshi Saito, Iracema de Mattos Paranhos Calderon, Isabela Lovizutto Iessi, Marilza Vieira Cunha Rudge, Marcelo Broggio Casarri e Silvana Barroso Corvino, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva de que os "ratos", são provenientes de Biotério convencional, sem condições de atestar a Sanidade dos mesmos.*

*Projeto de Pesquisa aprovado em reunião da CEEA em 25/03/2010.*

*Profª Drª Regina H. Garcia Martins*
*Presidente da CEEA*

*Alberto Santos Capelluppi*
*Secretário da CEEA*

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