MORPHOMETRIC EVALUATION OF THE ISLETS OF LANGERHANS OF DIABETIC RATS TREATED WITH EXTRACTS OF Azadirachta indica (NEEM) AND STREPTOZOTOCIN 6C

AVALIAÇÃO MORFOMÉTRICA DAS ILHOTAS DE LANGERHANS DE RATOS DIABÉTICOS TRATADOS COM EXTRATOS DE Azadirachta indica (NEEM) E ESTREPTOZOOTOCINA 6 CH

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SUMMARY

The effects of aqueous and hydro-alcoholic extracts of *Azadirachta indica*, A. Juss and streptozotocin 6 CH on the morphometrics of the islets of Langerhans in streptozotocin-induced diabetic rats were evaluated. The morphometric results indicate that the area and the perimeter of the control group have higher means compared to the other groups of diabetic animals, which did not differ among themselves. The minimum diameter of the group treated with aqueous extract of *Azadirachta indica*, A. Juss 10% was larger than the control group. There were no significant differences between treated groups and diabetic control group. Therefore, we concluded that the treatments studied do not induce the regeneration of endocrine pancreas of diabetic animals because they had no significant effect on the morphometrics of pancreatic islets during the period of this study.

KEY-WORDS: Diabetes mellitus. Herbal medicine. Homeopathy. Hypoglycemic agents.

RESUMO

Foram avaliados os efeitos dos extratos aquoso e hidroalcoólico de *Azadirachta indica,* A. Juss e da estreptozootocina 6 CH sobre a morfometria das ilhotas de Langerhans de ratos com diabetes mellitus induzida por estreptozootocina. Os resultados morfométricos indicam que área e o perímetro do grupo controle branco possuem valores médios maiores quando comparados aos demais grupos, formados por animais diabéticos, os quais não diferiram entre si. O diâmetro mínimo do grupo tratado com extrato aquoso de *Azadirachta indica*, A. Juss a 10% revelou-se com maior valor confrontado ao grupo controle branco. Não se observou diferenças significativas entre os todos os grupos tratados e o grupo controle branco diabético. Portanto, conclui-se que os tratamentos analisados não tiveram efeito significativo quanto à morfometria das ilhotas pancreáticas, ou seja, não induziram a regeneração do pâncreas endócrino em animais diabéticos durante o intervalo de tempo deste estudo.

PALAVRAS-CHAVE: Agentes hipoglicemiantes. Diabetes mellitus. Fitoterapia. Homeopatia.

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INTRODUCTION

The pancreatic hormones are synthesized in rounded clusters of epithelial endocrine cells called islets of Langerhans (SCHOSSLER, 2004). The insulin secreting β-cells account for 70% of the islet (JUNQUEIRA & CARNEIRO, 2004) and occupy its central region (JOHNSON, 2000). Inside the cells, the hormones are stored in granules (BERNE et al., 2000). Insulin lowers glucose, fatty acid and amino acid blood concentrations and promotes the conversion of these compounds to its storage form as well (CUNNINGHAM, 1999). Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose due to insulin deficiency and/or resistance that brings several serious complications such as macrovascular problems, microangiopathy, retinopathy, nephropathy and neuropathy (RANG et al., 2004), mainly as a consequence of hyperglycemia, which should be strictly controlled (COTRAN et al., 2000). In the islets, it causes a reduction of their number and size, leukocyte infiltration, degranulation of β-cells by depletion of the stored insulin and amyloid replacement in type II diabetes (COTRAN et al., 2000). As for inducing diabetes mellitus, Furlan (2001) reported that most of the changes that appear after the treatment with streptozotocin result from their toxic action on β-cells when they accumulate in the central portions of the pancreatic islets. Marles & Farnsworth (1999) suggested that streptozotocin stimulates the production of free radicals, which leads to destruction and disconnection of the β-cells.

The *Azadirachta indica,* known as Neem, is a plant native from India (CHOPRA, 1958; SAXENA, 1993) that was introduced in Brazil in 1993 by the EMBRAPA (CNPAF) in Goiânia,GO. Currently, it is known to have a large concentration of terpenoids that are responsible for its therapeutic action (RAGASA et al., 1997). Several studies indicate beneficial effects of the plant in the treatment of diabetes mellitus. Comparison of the hypoglycemic action of several medicinal plants showed that the extract from the leaves of *Azadirachta indica* was the most potent (ALAM et al., 1990). Chattopadhyay (1999) reported that the hypoglycemic effect of the leaf extract is possibly due to the blocking of the inhibitory effect of serotonin on the release of insulin-mediated glucose. Parshad et al. (1999) found significant improvement in weight and mortality reduction of rats with streptozotocin-induced diabetes treated with aqueous extract of *Azadirachta indica*. Khosla et al. (2000) confirmed its hypoglycemic effect on alloxan diabetic rabbits treated with leaf extract and oil from the seeds. Biswas et al. (2002) reported that the aqueous extract from its leaves, when given orally, reduces blood glucose in healthy and diabetic rats, possibly due to the presence of the flavanoid, quercetin.

Significant hypoglycemic effect was also observed in healthy and alloxan-induced diabetic rabbits. The fasting rabbits were fed oil of *Azadirachta indica*, leaf extract and oil from the seeds as well. Hussain (2002) showed that the aqueous treatment with *Azadirachta indica* has a favorable effect on blood glucose levels and glucose tolerance, it reduced serum lipids and body weight, and completely reversed retina abnormalities and limb inflammation. Mahdi et al. (2003) concluded that *Azadirachta indica* and the other species studied are not useful to control the levels of lipid peroxidation, but effective to enhance the antioxidant potential in streptozotocin-induced diabetic rats.

According to Gupta et al. (2004) oral treatment of streptozotocin-induced diabetic rats with almond extracts together with seed husks of *Azadirachta indica*, during 28 days, prevented the oxidative stress caused by streptozotocin on the heart and erythrocytes. Waheed et al. (2006) reported that *Azadirachta indica* administered in large doses during 14 days had hypoglycemic activity on type II diabetic patients and could be combined successfully with oral hypoglycemic agents in cases where diabetes can not be controlled with these drugs alone. Chandra et al. (2007) reported that aqueous extracts of certain plants, including *Azadirachta indica*, have antidiabetic and antioxidant action and that their adequate use in the diet can help minimize complications related to this disease. Mostofa et al. (2007) reported that extracts of *Catharanthus roseus*, *Azadirachta indica* and *Allium sativum* increased body weight and decreased blood glucose in streptozotocin-induced diabetic rats compared to the drug glimeprida. Ebong et al. (2008) while treating diabetic rats with extract of *Azadirachta indica*, showed that a single dose causes a peak reduction of blood glucose one hour after being administered. Its extract alone or combined with extract of *Vernonia amygdalina*, reduced glucose levels after the treatment. Atangwho et al. (2010) concluded that blood glucose decreased in healthy and diabetics rats treated with extracts of *Vernonia amygdalina* and *Azadirachta indica*, seven days after initiation of treatment and even more rapidly when administered together. Moreover, the change of blood glucose levels was similar to that of rats treated with insulin, however more constant, a trend that was also observed in relation to animal body weight, but on the opposite direction.

Kumari (2010) reports that the powdered seeds *Azadirachta indica* reduced fasting and postbrandial glucose levels in human patients of diabetes type II and average blood lipid levels as well. However, Rosa et al. (2010) considered doubtful its use to control glucose levels of streptozotocin-induced diabetes in rats, since blood glucose levels of the animals treated with the aqueous and hydro-alcoholic extracts were higher than the control group.

Homeopathy is a therapeutic science based on natural law of healing *Simila similibus curentur,* that is, "let like be cured by like". Thus, any substance capable of producing a morbid condition in a healthy individual, however sensitive, would be equally capable of, in appropriate doses, to cure this individual with a similar disease, except when irreversible. The fact that the process of sequential dilution and succussion acquires crescent dynamic power made the terms dilution, potentization and dynamization to be

used interchangeably from the practical point of view (KOSSAK-ROMANACH, 1993). The rat is the model more commonly used to elucidate the therapeutic effects of substances diluted and potentiated (VAN WIJK et al., 2009). In preparations where ultra high dilutions are used, a substance is sequentially diluted 1:100 C and undergoes succussion every stage. The letter C stands for the centesimal scale created by Hahnemann correspond to diluting by a factor of 100 at each stage and the number that precedes the letter C is the number of stages (CARVALHO, 2000). Regarding the homeopathic treatment of *diabetes mellitus*, Santos (1990) reported the hypoglycemic action of alloxan 6C in diabetic rats. Rosa et al. (2010) reported that rats with *diabetes mellitus* induced by streptozotocin, when treated with streptozotocin 6C showed on the 30th day of therapy the lowest glucose levels compared to control and diabetic groups and to the animals treated with aqueous and hydro-alcoholic extracts of *Azadirachta indica*.

MATERIAL AND METHODS

Two extracts of *Azadirachta indica*, one aqueous and the other alcoholic, were prepared with leaves grown and supplied by EMBRAPA-CNPAF, which were previously stabilized under the shade in a ventilated environment. Subsequently, the 70% (w/w) ethanol extract was prepared by percolating the dried ground leaves until the active ingredient was depleted, at a speed of 8 drops per minute which was then concentrated to obtain a soft extract. An aqueous extract using distilled water was also prepared and used in the percolation until depletion of the active ingredient and, subsequently this aqueous extract was lyophilized.

The ultra high dilutions were prepared in dynamised systems (streptozotocin 6C) using streptozotocin (Sigma) as a starting point and ethanol solutions at different concentrations (30% or 70%) as vehicle. The dilutions by a factor of 100 were carried out mechanically. The succussion was performed by vigorous, continuous and rhythmic movements where the bottom of the flask was struck against an elastic body. Six 30-mL flasks were placed on the bench and to reach the desired dynamisation, 19.8 mL of the 70% ethanol solution was added to each flask (V/V). To the first flask, 0.2 g of streptozotocin was added and subsequently, homogenized and diluted 100 times using a mechanical arm to obtain 1C. From this dilution, an identical procedure was repeated subsequently until the desired dynamisation of 6C was reached. The 6C preparation was made in 30% ethanol solution (V/V) and the resulting liquid was stored in amber glass jar, tightly closed, protected from heat and direct light (FARMACOPÉIA, 1997).

Twenty five male albino rats, of the Wistar breed, weighing between 200 and 250 gr were used. The rats were supplied by the Bioterio Central of the UNESP, Botucatu, SP. They were divided in groups of five animals each, placed in cages in the Bioterio of the Departamento de Morfologia e Fisiologia Animal, Faculdade de Ciências Agrárias e Veterinárias, UNESP, in Jaboticabal, SP. Temperature was controlled and the dark-light cycles lasted 12 hours, ration and water were supplied *ad libitum* during five days.

After the adjustment period, the animals were left to fast during 14 to 16 hours and then, blood samples (1 mL) were collected via infraorbital artery to determine glucose levels following the method of King & Garner (1947), after light anesthesia by ether inhalation. Subsequently, 35 mg/kg of streptozotocin diluted in sodium citrate buffer (pH 4.5) was administered intravenously in the coronary sinus of the penis of 20 rats that were still anesthetized. Another five rats formed the control group. After five days, blood was collected to determine blood glucose levels following the same methodology. After hyperglycemia was confirmed, the rats were separated into 5 groups with five animals each that received oral treatment once a day, with different extracts of *Azadirachta indica* and streptozotocin in ultra high dilutions and dynamised systems (streptozotocin 6C). The control group was formed by 5 non-diabetic rats.

All animals were treated orally daily (0.2 mL/100 g of animal weight) by gavage, namely: the control and the control diabetic group received water; the second group was treated with aqueous extract of *Azadirachta indica* 10%; the third with hydro-alcoholic extract (70%) of *Azadirachta indica* 10%; and the last group with streptozotocin in ultra high dilutions in dinamised systems (streptozotocin 6C). This procedure lasted 30 days.

On the 31st day the animals were euthanized and the pancreas was removed. After that, the pancreas was fixed in Bouin solution during 24 hours and routinely processed for paraffin embedding. After the semi-serial microtomy at 110-μm intervals, the 7-μm thick histological cuts were stained with hematoxylin-eosin (TOLOSA et al., 2003) and photomicrographed using a Leica DM 5000B photomicroscope. Five slides with 5 histological cuts were analyzed per animal, a total of 25 cuts. All islets of Langerhans of each histological cut were analyzed. The morphometric analysis determined the area (μm^2), perimeter (μm), maximum (μm) and minimum (μm) diameter, which are respectively the maximum and minimum diameter measured for each islet and the shape factor. The shape factor, as well as the other parameters are programmed in the Image Pro-plus program. The lowest value is 1 (one), meaning that the shape of the cytoplasm and/or nucleus shows the shape of a perfect circle. When this factor is greater than 1, it is understood that the shape of the cytoplasm and/or core is roundish but not perfectly circular. These analyzes were performed using low magnifying objective (5X), by image analysis program (LEICA).

The experimental design was completely randomized with 5 treatments and 5 repetitions. The means were compared by Tukey test at 5% probability according to Pimentel Gomes (2000).

Table 1- F values, variance coefficient (VC), means and standard deviation (DP) for the islets of Langerhans parameters measured in the pancreas of albino Wistar rats, control and diabetic groups.

Treatment		Area ($x10^3$ µm²)	Maximum Diameter ($x10^3$ µm)	Minimum Diameter ($x10^3$ µm)	Perimeter ($x10^3$ µm)	Shape factor
Control	Mean	8.58 a	0.62 a	0.44 a	0.36 a	2.19 a
	DP	0.87	0.08	0.03	0.04	0.32
Diabetic control	Mean	4.29 b	0.71 a	0.49 ab	0.24 b	2.24 a
	DP	0.49	0.03	0.02	0.02	0.77
***Azadirachta indica* aqueous extract**	Mean	3.22 b	0.71 a	0.53 b	0.24 b	1.92 a
	DP	0.27	0.06	0.07	0.02	0.31
***Azadirachta indica* 70% alcoholic extract**	Mean	3.70 b	0.63 a	0.49 ab	0.25 b	2.11 a
	DP	1,22	0.01	0.04	0.04	0.28
STZ 6CH	Mean	3.51 b	0.65 a	0.49 ab	0.22 b	1.57 a
	DP	0.34	0.06	0.04	0.01	0.29
Test F		45.17**	2.75**	2.70**	17.65**	1.71**
VC		15.14	8.51	9	10.98	21.09

**: significant at 1% probability
Means followed by the same letter in the column do not differ by Tukey at 5%.

RESULTS AND DISCUSSION

The morphometric variables determined for the pancreatic islets of albino Wistar rats are presented in Table 1. The results show significant differences ($p<0.01$) among treatments.

The area and perimeter of the control group displayed higher mean values ($p<0.05$) compared to the other groups, which did not differ ($p>0.05$). This result suggests that diabetes mellitus caused a decrease in these measures, agreeing with the results reported by Cotran et al. (2000).

The minimum diameter of the group treated with aqueous extract of *Azadirachta indica*, A. Juss 10%, was higher than the control group, while the other groups did not differ ($p>0.05$).

The maximum diameter, shape factor and volume displayed similar mean values ($p>0.05$) for all groups. The shape factor, in addition to being similar for all groups, it was also higher than 1 for the control and diabetic groups, thus indicating that the islets have a round contour that is morphologically normal for this part of the pancreas. Therefore, it is possible to conclude that diabetes mellitus did not change the format of the islets of Langerhans.

The results determined by the morphometric analysis of all measured parameters indicated that there were no significant differences between the

control and the diabetic groups. Therefore, it is clear that the treatments performed at the time interval of one month did not improve the changes caused by diabetes mellitus in the parameters measured for the islets of Langerhans. This conclusion may be justified by the short time during which the experiment run.

CONCLUSIONS

It was concluded that the results from the analysis of pancreatic islets showed no significant effect on the morphometric parameters during the experimental period of this study, that is, there was no regeneration of the endocrine pancreas in diabetic rats treated during one month.

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