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Research Article

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Morphometric study of the effect of Walnut (*Juglans regia)* leaf extract on cerebrum malformation in fetuses of diabetic rats

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ABSTRACT

Hypoglycemic effect of Juglans regia leaves has been was reported in other previous researches. This study investigated the effect of Juglans regia leaves ethanolic extract on Cerebrum in 18 and 20 day old fetus of diabetic mother. A total of 32 Sprague-Dawley female rats became diabetic by intraperitoneal injection of streptozotocin (50 mg/kg). The animals were divided randomly into four groups. Rats in all groups became pregnant by natural mating. After formation of the nervous system, eight fetuses were obtained after anesthezing animals on the 18th and 20th gestational days in each group; the animals were euthanized, their birth weights recorded and cerebrum samples were taken and fixed. Tissue sections were prepared by routine procedures and different histological parameters were examined. Results revealed a significant decrease in number of cells in gray matter and white matter, ratio of gray matter to white matter and thickness of gray matter at 18 and 20 days in cerebrum of diabetic rats fetus as compared with other groups. The body weight of diabetic control was significantly (P<0.05) more than that of the other group. The present study demonstrates that alcoholic extract of walnut leaves possesses significant antihyperglicemic properties, thus suggesting its beneficial effect in the treatment of maternal diabetes.

Key words: Juglans regia leaves extract, Maternal diabetes, fetuses, Cerebrum.

INTRODUCTION

Diabetes mellitus is a group of metabolic alterations characterized by hyperglycemia resulting from defects in insulin secretion, action, or both. It has already been established that chronic hyperglycemia resulting from diabetes is associated with long term damage, dysfunction, and eventually the failure of organs, especially the eyes, kidneys, nerves, heart and blood vessels [1]. In diabetic mothers during pregnancy, placental transport of glucose and other nutrients will be increased, due to an increased availability at the maternal site, resulting in their increase in fetal and neonatal Macrosmia [2]. The risk for diabetes is significantly higher in the offspring of mothers who have non-insulin-dependent diabetes [3]. In addition, maternal diabetes increases the risk of hypoglycaemia and other chemical imbalances such as low calcium and magnesium levels [4]. One of the mammalian systems that is clearly impaired in diabetes is nervous system. Diabetes leads to a lack of sensation at the nerve endings [5]. Atherosclerosis in brain is one of the prominent changes in diabetes. Studies have shown that obstruction of feeding vessels of nerves due to diabetes causes nerve bundles death and myelin destruction [6]. An increased number of malformations occur in infants born from mothers with maternal diabetes involving the central nervous system (CNS), the spinal column, the ribs and the urinary tracts [7].

Herbal drugs are gaining popularity in the treatment of diabetic mellitus [8]. The Juglans genus (family Juglandaceae) comprises several species and is widely distributed throughout the world [9]. Green walnuts, shells, kernels and seeds, bark and leaves have been used in the pharmaceutical and cosmetic industries [10]. Walnut leaves are considered a source of healthcare compounds, and have been intensively used in traditional medicine for treatment of venous insufficiency and haemorrhoidal symptomatology, and for their antidiarrheic, antihelmintic,

depurative and astringent properties [11,12,13,]. Keratolytic, antifungal, hypoglycaemic, hypotensive, anti-scrofulous and sedative activities have also been described [14,15]. Walnut (Juglans regia) has been widely used as an herbal medicine in the treatment for diabetes [16].

In walnut leaves, naphtoquinones and flavonoids are considered as major phenolic compounds [13]. Juglone (5-hydroxy-1,4-naphthoquinone) is known as being the characteristic compound of Juglans spp. and is reported to be found in fresh walnut leaves [12,13,15,17]. Nevertheless, because of polymerization phenomena, juglone only occurs in dry leaves in vestigial amounts [13].

The purpose of this investigation is the effect of Walnut (Juglans regia) leaf extract to evaluate possibility of congenital cerebral malformation in offspring of diabetic rats at day 18 and 20 of pregnancy.

EXPERIMENTAL SECTION

2.1. Ethanolic Extract of Juglans regia Leaves (JRLEE)

Leaves of *Juglans regia* L. (Juglandaceae) were collected from Fars province. The leaves were cleaned, shed dried at 25°C and ground with a blender. The powdered leaves of J. regia (1000g) were then allowed to soak for about 24 h in 70% ethanol and re-extracted three times with fresh 96% ethanol at room temperature for 24 h. The ethanolic phases were pooled and the residue was removed by filtration. The ethanolic extract was concentrated at 40°C by rotary evaporator and then lyophilized to obtain a powder (JRLME). The powder was stored in the dark at 4°C for subsequent experiments**.**

1.2. Animals

Thirty two adult female Sprague-Dawley rats (200-250g weight and 3-4 months old) were acclimatized in an environmentally controlled room (temperature, 22±2^{o}C, and 12h light/12h dark). Food and water were given *ad libitum*. In this study all experiments conducted on animals were in accordance with the guidance of the ethical committee for research on laboratory animals of Shiraz University. Animals were divided into four equal groups.

1.3. Induction of diabetes mellitus

Adult rats were rendered hyperglycemic by a single intraperitoneal (I.P.) injection of B.W. of stereptozocin (Sigma Chemical Co., USA) (50 mg/kg body weight) (18). Diabetes was identified by polydipsia, polyuria and by measuring non–fasting serum glucose concentration 48h after the injection of STZ, rats with a blood glucose level over 250 mg/dl were considered to be diabetic.

1.4. Experimental design

Animals were divided into four identical groups as follows:
(1) Normoglycemic control group (NC): normal rats which received distilled water
(2) Normoglycemic treated group (NJRLE): normal rats which received the Walnut leaf extract (JRLE, 250 mg/kg B.W).
(3) Diabetic control group (DC): Diabetic rats treated with distilled water.
(4) Diabetic treated group (DJRLE): diabetic rats receiving the Walnut leaf extract (JRLE, 250 mg/kg B.W)

Female animals of four groups in oestrus stage were caged with male rat for mating. Mating was confirmed by vaginal plug observation (19). Each group included 8 rats and animals were given the extract orally by an intragastric tube once daily for 21 days. The stock solution was prepared for multiple groups, such that 1 mL of extraction was administered per day for each animal.

At days 18 and 20 of pregnancy, four rats of both groups were killed. Fetuses were immerged in appropriate fixative (buffered formaline 5% for light microscope and gluthardehyde 2% for electron microscope). Then, the cerebrum was collected from offspring of all rats and the weight of the neonates was measured.

1.5. Histomorphometric study

All tissue samples were fixed in 5% buffered formalin fixative for histolopathological investigations and subsequently embedded in paraffin. Sections (5 microns thickness) were stained with H&E and Green Masson's trichrome techniques. Sections were observed with Olympus BX51 microscope for evaluation of histomorphometrical parameters such as:
1) Thickness of gray matter (μm). 2) Thickness of white matter (μm). 3) The number of cells in the gray matter per unit (mm^2). 4) The number of cells in white matter per unit (mm^2). 5) The ratio of gray matter to white matter.

Thicknesses of gray matter and white matter were measured by ocular micrometer and Olympus BX51 light microscope using Olysia software. The number of cells per unit (mm^2) in both white and gray matters and the ratio of gray matter to white matter were counted by ocular graticule and Olympus BX51 light microscope using Olysia software.

1.6. Statistical analysis

All values were expressed as mean ± standard deviation (SD). Significant differences among the groups were determined by One way analysis of variance (ANOVA) followed by Duncan's test to analyze the difference. The Statistical Package for Social Sciences (SPSS) 13.0 software package program was used. Values of $P \leq 0.05$ were taken as statistically significant.

RESULTS

The fetal body weight changes of four groups have been shown in Table 1. The mean of body weight in the fetuses of diabetic mothers (FDM) was significantly ($P<0.05$) more than that of the other groups.

Table 2 demonstrates different parameters of cerebrum from offspring of four groups at 18 days old. Table 3 demonstrates different parameters of cerebrum from offspring of the four groups at 20 days old. The thickness of gray matter was decreased significantly ($p<0.05$) in diabetic rat fetuses as compared to that of other groups at days 18 and 20 days old. The number of cells in gray matter and white matter was significantly ($p<0.05$) decreased in diabetic rat fetuses compared with other groups.

The thickness of white matter was decreased in diabetic rat fetuses compared to other groups and this reduction was not significant, while ratio of gray matter to white matter was significantly ($p<0.05$) decreased in diabetic rat fetuses compared with other groups.

DISCUSSION

In recent years, various plant extracts have been claimed to be useful for the cure of diabetes mellitus, but few of them have been tested for their effects on body tissue of diabetic patient. In the present study, we investigated the antidiabetic effect of walnut leaves Ethanolic Extract (JRLME) on embryo of ovaries structure in STZ- induced female diabetic rats. STZ is a compound commonly used for the induction of type I diabetes in experimental rats. STZ caused diabetes by rapid depletion of β cell in pancreas Langerhance Island, which leads to a reduction of insulin release [18]. In our studies, oral feeding of walnut leaves reduced blood glucose level. The hypoglycemic activity of walnut leaves was reported by previous studies [20]. Walnut leaves constitute a good source of antioxidant compounds, namely phenolics, suggesting that it could be useful in prevention of diseases in which free radicals are implicated. Phenolic acid and flavonoid are two major groups of phenolic compounds in walnut leaves. The most important phenolic acid in walnut leaf is caffeoylquinic acid and the main flavonoid is quercetin [17, 21, 22]. The period of these treatments was similar to the present study.

In diabetic pregnancy, maternal glucose transport to fetal blood via the placenta [4] and increase in fetal blood glucose may result in diabetic neuropathy in fetus, as diabetes leads to neuropathy in adult [23]. Hyperglycemia effectively makes more substrate available for aerobic glycolysis in the brain, leading to acidosis [24] and enhanced oxygen free radical formation by reduction in levels of protective endogenous antioxidants [25].

Therefore, maternal diabetes results in malformation of this region. Neuropathy of numerous nerves like sciatic nerve has been reported in diabetic rats fetus [23,26]. Malformations in this region of brain may occur due to neuropathy [27, 28]. Diabetes mellitus is associated with moderate cognitive deficits and neurophysiological and structural changes in the brain, a condition that may be referred to as diabetic encephalopathy. Maternal diabetes leads to white matter hyperintensities and gray matter density changes in fetus [29]. Maternal diabetes leads to fetal hyperbilirubinemia [30], that could result an encephalopathy which is named Kernicterus [31]. The mean number of cells in gray matter and white matter increased in diabetic rats fetus that were fed walnut leaf extract compared to diabetic rats fetus group, indicating the extract has a beneficial effect in the treatment of maternal diabetes. Some studies have shown that flavonoids are able to decrease plasma glucose level [32]. Quercetin inhibits glucose transporter (GLUT2), so diminishes glucose intestinal absorption [33]. Caffeoylquinic acid (chlorogenic acid) is a specific inhibitor of glucose-6-phosphate translocase and reduces hepatic glucose production [34], and thus decreases blood glucose level and HbA1C [35]. Plant antioxidants are able to restore and regenerate pancreatic β cells.

Table1: Comparison of means and standard error of the body weight of fetuses of rats at 18 and 20 days of pregnancy

Group	NC	DC	NJRLE	DJRLE
18	3.13±0.11	3.51±18.95	3.15±0.21	3.25±0.21
20	4.01±0.58	5.18±0.46*	4.02±0.43	4.31±0.53

Table 1, Values are demonstrated with mean± SD. Significant difference between DC and other groups demonstrated with*sign (P<0.05).

Table2: Comparison of means and standard error of the cells number and dimension Cerebrum at 18 days pregnancy

Days	**18**		**18**	
Group	**NC**	**DC**	**NJRLE**	**DJRLE**
TGM(μ)	463.31±21.33	428.14±12.36*	462.38±18.95	442.92±21.46
TWM(μ)	262.49±12.19	281.76±13.23	261.84±15.76	266.32±12.87
NGM(n/mm^2)	26731.56±744.02	24185.72±735.64*	26532.24±849.51	25891.28±863.67
NWM(n/mm^2)	12135.28±525.19	10631.68±519.01*	12133.15±524.76	11985.54±515.96
GWR	1.83±0.07	1.59±0.09*	1.81±0.08	1.75±0.06

Table 2, TGM (Thickness of gray matter), TWM(Thickness of white matter), NGM (Number of cells in gray matter), NWM (Number of cells in white matter), GWR (Ratio of gray matter to white matter), Values are demonstrated with mean± SD. Significant difference between DC and other groups demonstrated with*sign (P<0.05).

Table 3: Comparison of means and standard error of the cells number and dimension Cerebrum at 20 days pregnancy

Days	**20**		**20**	
Group	**NC**	**DC**	**NJRLE**	**DJRLE**
TGM(μ)	483.14±35.83	405.88±32.04*	481.94±33.88	465.44±26.94
TWM(μ)	305.41±22.39	316.17±31.25	305.98±23.29	309.29±22.86
NGM(n/mm^2)	25734.62±840.58	22203.52±728.41*	25717.86±664.34	24516.32±625.75
NWM(n/mm^2)	10871.88±376.16	9871.71±336.48*	10845.14±373.48	10598.72±355.99
GWR	1.76±0.09	1.43±0.03*	1.75±0.11	1.71±0.08

Table 3: TGM (Thickness of gray matter), TWM(Thickness of white matter), NGM (Number of cells in gray matter), NWM (Number of cells in white matter), GWR (Ratio of gray matter to white matter), Values are demonstrated with mean± SD. Significant difference between DC and other groups demonstrated with*sign (P<0.05).

CONCLUSION

In conclusion, our data determine the use of the *Juglans regia* leaves was effective on Cerebrum malformation in offsprings of diabetic rats, thus suggesting its beneficial effect in the treatment of diabetes.

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