The Effect of Walnut Leaf Aqueous Extract (Juglans Regia L.) On The Embryonic Testicles Evolutionary Trend in Streptozotocin-Induced Rats with Type I Diabetes

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ABSTRACT

Introduction: Diabetes is one of the most important endocrines diseases. The current research aims at the survey of the effect that walnut leaf aqueous extract might exert on the embryos' testicles of the diabetes-induced mothers.
Study Method: 42 female adult Wistar rats were assigned to seven 6-individual groups, including a control group, a group receiving walnut leaf extract for 200 mg, a group receiving walnut leaf extract for 400 mg, a diabetic group receiving 20 IU/kg insulin, a diabetic group receiving 200 mg walnut leaf extract and a diabetic group receiving 400 mg walnut leaf extract. On the day eighteen of the pregnancy, their fetuses were extracted and their testicles were removed out of which serial tissue cross-sections were prepared and colored by the use of Hematoxilina-Eosina paints so as to undergo histological studies. The results were analyzed in P≤0.05 significance level by taking advantage of ANOVA statistical tests.
Findings: The mean number of the spermatogonic, leydig, cells and the mean annulus of the seminiferous tubule showed a significant decrease in the diabetic group as compared with the control group (P≤0.05). The mean number of the spermatogonic cells and the mean inner diameter of the seminiferous tubule exhibited a significant increase in diabetic groups that received 200 and 400 mg/kg walnut leaf extract in contrast to the diabetic group that received 20 IU/kg insulin (P≤0.05). The mean number of the leydig cells in diabetic group that had received 400 mg/kg walnut leaf extract demonstrated a significant increase in respect to the diabetic group that received 20 IU/kg insulin (P≤0.05).
Conclusion: Administering walnut leaf extract to the diabetic pregnant mothers exerts a protective effect on the fetuses' testicles of the diabetic mothers and causes an improvement in the testicles' qualitative and quantitative parameters due to it possessing antioxidant ingredients that reduce free radicals.

Key Words: diabetes, walnut leaf, rat, evolution, testicles.

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INTRODUCTION

Diabetes is one of the most important endocrines diseases common in the human communities that is spreading due to the changes in the methods of living and the nutrition types (world health organization). The disease is known to be accompanied with certain side effects such as nephropathy, retinopathy, neuropathy,

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cardiomyopathy [1, 2&3] as well as damage to the adults' reproduction system such as reduction in the fertility rate [4&5], the decrease in the ovary diameter [6], vaginal inflammation, menstrual cycle disorders, delay in oocytes' maturation, reduction in yellow matter and increase in atretic follicle [7], disorders in erection, ejaculation, reduction in sperm stimulation, the increase in abnormal sperm shapes, decrease in the number of the leydig cells, change in the appearance of leydig cells' nucleus and the decrease and transformation of the sertoli cells [8&9].

Pregnancy diabetes occurs in more than 8% of the pregnancies [10]. In pregnancy diabetes, the increase in glucose and other nutritional materials transfer through placenta to the fetus leads to an increase in the production of insulin and restoring fat as well as the increase in a series of unfavorable pregnancy outcomes like fetal macrosomia, shoulder dystocia and mortalities at delivery [11]. Also, the diabetes emergence risk is higher in the offspring of the mothers that have non-insulin associated diabetes [12]. Leaving pregnant mothers' diabetes uncontrolled causes congenital disorders, especially during the early stages of organ production [13]. It is well-clarified that the prevalence rate of the congenital deformities in the fetuses of the diabetic mothers is 2 to 4 times the fetuses of the non-diabetic mothers and since the pregnancies subjected to diabetes are at high risk of inauspicious consequences at delivery-close time and the development of Type II diabetes in future, therefore treatment intervention is necessary [14]. Although, the most common medication used for curing diabetes is insulin nutritional approaches towards diabetes' treatment has been found featuring numerous advantages in developing countries [11].

There are many plants that are frequently applied in the traditional medicine by various nations in treating diabetes. A great many of these medicinal herbs have been investigated for their hypoglycemic effects [15, 16] and there are identified more than 1200 medicinal herbs with positive effects on the reduction of the blood glucose and/or the reduction of the diabetes-driven symptoms up to now [17]. Walnut with its scientific name Juglans regia L. is from the family Juglandaceae and it is a plant that possesses anti-diabetic properties in its many parts including its leaves and shells [18, 19]. The results of the studies undertaken by Daveband et al have also indicated that administering walnut leaf aqueous extract for four weeks causes a considerable and significant reduction in rats' blood glucose [18]. Also, the investigations carried out by Fathi Azad et al have suggested the reduction in blood glucose as a result of the consumption of various dosages of walnut leaf extract in rats [19].

The existence of numerous ingredients in walnut leaf has been ascertained including glycopeptides and flavonoids. The suggested processes for these ingredients are stimulating glycogenesis, controlling the potassium channels in pancreas's beta cells and the regulation of glucose absorption from the intestines all of which justify the anti-diabetic effects of the walnut leaf [18].

In addition, it has been made clear that walnut leaf extract contains polyphenolic antioxidants that can be of great effectiveness in diabetes treatment [20]. Therefore, according to the anti-diabetic and antioxidant features attributed to the walnut leaf, the present study aims at the survey of the effect exerted by walnut leaf aqueous extract on the testicular tissue of the diabetic rats' fetuses.

STUDY METHOD

The protocol used in the present study has been implemented corresponding to the standard methods of handling laboratory animals and via acquiring permits from Ethics Committee of Jahrom's Medical Sciences University (Ethical Code: IR.JUMS.REC.1394.174). In the present empirical study, 30 Wistar rats with weights ranging from 180 g to 200 g were selected and in order to get them accustomed to the new conditions the animals were kept for one week in the animal breeding room in Jahrom's Medical Sciences University. The study specimens were kept under controlled conditions in terms of light (12 hours day/night), temperature (within a range of 23 ± 1) and humidity (within a range from 50 to 55 percent).

Sufficient amount of walnut leaves were procured for taking extract. The leaves were rinsed by distilled water and they were placed in 37-degree-centigrade oven to completely dry; then the leaves were evenly and completely grinded in porcelain mortar. Every time, 50 g of the obtained powder was solved in one liter of distilled water and the concoction was placed in magnetic stirring device for 24 hours so as to acquire a completely even solution; then, the solution obtained via the foresaid procedure was passed through filtering paper and dried out under appropriate conditions (inside a 37-degree-centigrade oven) [18]. In the end, the animals were fed with sufficient amount of the dried powder of the extract according to the prescribed amount of daily consumption in the form of a solution and via a special oral tube.

The animals were randomly assigned to five 6-individual groups. The first group (non-diabetic control group) received no drug or extract and it was given only ordinary food and water. The second group members were not induced with diabetes and plus the ordinary food and water, they received 400 mg/kg walnut leaf extract solved in distilled water orally by means of a syringe one time in the morning, at ten o'clock, on a daily basis. The third group (controlled diabetic group) was induced with diabetes and, like the first group, received only ordinary food and water. The fourth group was induced with diabetes and besides the ordinary food and water it was fed on 200 mg/kg walnut leaf extract solved in distilled water which was administered orally by syringe once a day

in the mornings. The fifth group was induced with diabetes and in addition to ordinary food and water it was given 400 mg/kg walnut leaf extract solved in distilled water which was fed orally once a day in the mornings [18]. The intraperitoneal injection volume of streptozotocin was 2.0 cc and a 0.5-cc volume of walnut leaf extract was also injected.

Streptozotocin was used in order to induce the female rats with diabetes. The drug was injected intraperitoneally in a dosage of 50 mg/kg [30]. Blood tests were conducted before injection and 24 hours post-injection so as to confirm diabetes and also ten days after injection so as to assure the diabetes stability. The blood samples were taken through the rats' tails via creating a small incision on the surficial veins of the ending section of the animals' tails; moreover, blood glucose was measured by a glucometer device, Acuq Check-Alman. After the stability of diabetes was ascertained (the increase in blood glucose and urination volume), the diabetic rats were copulated with male rats and then the female ones were examined for vaginal plaque formation and the day it was confirmed positive was considered as the day zero for the animal.

According to the fact that the formation of reproduction system in rats is commenced after the day 13 of the embryonic stage, therefore, on the day twentieth before delivery the mother rats from all the groups were put to anaesthesia and their fetuses were extracted and their ovaries and testicles were removed and finally serial tissue cross-sections (in a consecutive manner) were prepared from the testicles and ovaries in all the study groups so as to undergo histological examinations. The lamellas prepared in this way were colored by Hematoxilina-Eosina paints.

Histomorphometric studies were carried out based on two standard micrometry methods (via graded ocular lamellas and lenses) as well as by taking advantage of a microscope, Olympus BX51 and the software named Olysia.

To analyze the information, one-way variance analysis, ANOVA, was applied. Statistical calculations were conducted in SPSS software in a 5% significance level.

FINDINGS

The mean number of the spermatogonic cells in group 4 (Diabetic group) showed a significant decrease in P≤0.05 level in contrast to the control group. But, the mean number of spermatogonic cells in groups 2, 3, 5, 6 and 7 did not indicate any significant increase in P≤0.05 level (Table 1).

The mean number of the sertoli cells was not found reflecting any significant difference in any of the study groups in comparison to the control group (Table 1).

The mean number of the leydig cells in the group 4 (diabetic group) and group 6 (diabetic plus 200 mg/kg walnut leaf extract) displayed a significant decrease in P≤0.05 level (Table 1). The mean number of leydig cells in groups 2, 3, 5 and 7 demonstrated a significant increase in P≤0.05 level as compared to the control group (table 1).

The mean diameter of somniferous tubule in group 4 (diabetic group) showed a significant decrease in P≤0.05 level in contrast to the control group (table 1).

The mean diameter of the sperm-making tube in groups 2, 3, 5, 6 and 7 indicated a significant increase in P≤0.05 level.

The mean thickness of the Tunica Albuginea plus the visceral layer of tunica vaginalis pouch did not show any significant difference in any of the study groups as compared to the control group (table 1).

The mean weight of the testicles was not suggestive of any significant difference in any of the study groups in comparison to the control group (table 1).

The mean fetus body weight in the group 4 (diabetic group) and group 6 (diabetic plus 200 mg/kg walnut leaf extract) showed a significant increase in P≤0.05 level in respect to control group (table 1). The fetus mean body weight in groups 2, 3, 5 and 7 did not indicate a significant difference in contrast to control group (Table 1).

Table 1: a comparison of the study parameters in various groups

Group VII (Diabetic + WLAE 400 mg/kg)	Group VI (Diabetic + WLAE 200 mg/kg)	Group V (Diabetic + Insulin 20 IU/kg)	Group IV (Diabetic)	Group III (WLAE 400 mg/kg)	Group II (WLAE 200 mg/kg)	Group 1 (Control)	Groups / Parameters
2342.61±147.23 a	2267. 25± 132.18 a	2439.17±167.46 a	1891.38±137.14 b	2521.68±175.16 a	2509.32±168.28 a	2482.41±181.22 a	Number of Spermatogonies (n/mm^2)
632.15±32.81 a	613.39± 29.92 a	634.91±37.35 a	607. 17±32.27 a	671.43±41.12 a	661.37±35.14 a	673.24±48.62 a	Number of Sertoli (n/mm^2)
587.36±40.66 a	522.26±38.25 b	633.23±47.69 a	448.65±39.22 b	668.11±50.27a	654.23±41.16 a	641.39±44.13 a	Number of Leydig (n/mm^2)
65.93±8.73 ab	61.86±7.65 ab	71.25±11.04 a	53.27±8.33 b	75.19±11.24 a	73.41±9.18 a	74.38±10.94 a	Diameter of Semeniferous tubul (µm)

34.61±2.35 a	33.11±1.78 a	34.72±2.08 a	31.76±1.83 a	35.28±2.67 a	34.69±1.43 a	35.64±2.92 a	Diameter of Tunica albuginea + Visceral layer of tunica vaginalis
27.02±2.18 a	27.38±2.24 a	25.81±1.98 a	28.01±2.61 a	26.18±1.89 a	26.11±2.04 a	25.31±1.48 a	Testicular weight (mg)
4.53.12 ab	4.69±.10 b	4.58±.17 ab	4.73±.15 b	4.38±.16 a	4.35±0.23 a	4.41±0.11 a	Embryo's weight (gr)

- Based on Duncan's test, the existing means featuring at least one common letter in every row are not indicative of any significant difference in 5% level.
- The means are presented in the form of mean ± SEM.
- P<0.05 has been considered statistically significant.
- WLAE=Walnut Leaf Aqueous Extract, kg= kilogram, IU= International Unit, n= number, mm^2=millimeter square, mg= milligram, g= gram, µm = micrometer

DISCUSSION AND CONCLUSION

In the current research paper, a significant decrease was figured out in the number of spermatogonic cells and leydig cells as well as the diameter of sperm-making tubes of the fetuses whose mothers had been induced with diabetes as compared to the fetuses of the mothers from control group (fetuses of healthy mothers). The reduction in the number of spermatogonic cells, leydig cells and the decrease in the diameter of sperm-making tubules of the fetuses from diabetic mothers are suggestive of the idea that the mothers' diabetes can impede and disrupt spermatogenesis process in the fetuses [21, 22]. Guneli et al reported that the changes in testicle's tissue in diabetes mellitus include apoptosis, sperm-producing tubules' atrophy, decline in tubules' diameter and the overall reduction of the spermatogonic cells systems [23]. Vignon et al, as well, reported that diabetes causes an increase in the thickness of sperm-producing tubules' basement membrane which is accompanied by a reduction in sperm production as well as a decline in the number of sertoli cells that finally lead to the decrease in the number of spermatogonial cells [9].

Balster et al reported that diabetes can cause disruptions in spermatogenesis function through a FSH-associated mechanism which finally ends in sperm production reduction [24]. On the other hand, it has been demonstrated that the increase in glucose directly manifests its effect in mitochondria as well as smooth endoplasmic reticulum damages on leydig and sertoli cells of the diabetic rats [25].

The results of the present study are also reflective of the significant increase in the weights of the fetuses belonging to diabetic mothers as compared to the fetuses born by healthy mothers (control group). The increase in the weights of the diabetic mothers is a result of the increase in glucose as well as other nutritional materials transfer from the mothers to the fetuses via placenta [26]. Under such circumstances, a great deal of extra fat is restored in the infants' shoulders and body [27].

The increase in the number of spermatogonial cells, leydig cells as well as the thickening of the sperm-producing tubules' annulus in the fetuses of the diabetic mothers subject to walnut leaf extract medication was found statistically significant in contrast to diabetic control group members. Sperm-producing tubules' atrophy and the decline in the number of the spermatogonic cells are morphological symptoms of spermatogenesis processes' disorders. The thickening of the sperm-producing tubules' basement membrane plays an important role in diminishing sperm production [28]. Diabetes increases the basement membrane thickness and causes a reduction in the production of sperm [22]. Thus, walnut leaf extract has been found capable of reducing the number of spermatogonic as well as leydig cells in the fetuses of the rats induced with diabetes.

Under chronic diabetes and hyperglycemia conditions, ROS (reactive oxygen species) production rate increases. The results of the studies have shown that ROS plays a substantial role in STZ-induced diabetes mellitus [29]. Additionally, it has been made clear that oxygen free radicals play an active part in harming the sexual cells. On the one hand, hyperglycemia increases the production of oxygen free radicals and, on the other hand, it causes a reduction in the production of antioxidants in the cells and it is via the weakening of their related protective mechanisms that oxygen free radicals exacerbate the damage resulting from oxidative stress [30]. Also, it is reported that the antioxidants' concentrations significantly decrease under hyperglycemia conditions in the embryonic cells [31]. The results of the present study indicate that the use of walnut leaf extract as an herbal antioxidant can somewhat decrease the hyperglycemic effects in diabetes-induced rats' fetuses but it does not perfectly neutralizes them. Walnut leaf is rich in antioxidant ingredients such as phenolic compounds. Phenolic acids and flavonoids comprise two substantial groups of phenolic compounds existent in walnut leaf. The most important phenolic acids present in walnut leaf are Caffeoylquinic acid and chlorogenic acid and the most important flavonoid residing therein is Quercetin [32&33]. The results of the studies show that flavonoids cause reduction in

plasma glucose level [34]. Quercetin, a flavonoid compound, controls glucose absorption in the intestines. Also, chlorogenic acid brings about a reduction in blood glucose through specifically controlling glucose 6-phosphatase enzyme that plays a key role in the blood glucose rate regulation and the amount of the glucose outputted from the liver [35].

GENERAL CONCLUSION

in the current research paper, the comparison between treatment group, diabetic group and healthy control group was indicative of the positive effects of walnut leaf aqueous extract on preventing diabetes from influencing the testicles' evolutionary trend in the fetuses from diabetes-induced rats. Such effects can be attributed to the existence of antioxidants therein.

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