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Investigation into the Experimental Effects of *Juglans regia* Cyclohexan Extract on Arginase Gene Expression (*ARG2*) in Adult Male Diabetic Rats with STZ and Healthy Rats

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ABSTRACT

Background and Aim: Diabetes type 2 is a disease with functional impairment in body. The use of medicinal herbs in the treatment of diabetes is traditional in ancient medicine. Antioxidant compounds of walnut leaf are effective in reconstructing pancreatic beta cells. This research was conducted to study the effects of walnut leaf Cyclohexanin extract on the expression of arginase gene (ARG2) in normal rats and diabetic rats.
Methodology: 50 Sprague dawley rats (200 g-250 g) were selected and divided into 10 groups. A group of rats did not receive any extracts of walnut leaf. Two normal control groups and two diabetic groups (with STZ) were gavaged with Cyclohexanin extract of walnut leaf for 6 weeks. At the end of the course, the primers of this research were designed. Real Time PCR (SYBR Green) was then used to extract and check the gene expression. To analyze Real Time PCR data, Fold change was first calculated and then the data were analyzed with REST software (ver-9).
Results: The results of the analysis show that the Arginase gene is evenly expressed in both groups of CN and CT-C and. But in the DT-C treatment groups, the Arginase gene is significantly reduced compared to the CD group. According to the P-value, which is 00/0 and the rate of Fold Change, which is 0/027, the results of the analysis showed that the gene expression in the diabetic group treated with the cyclohexan extract (DT-C)reduces significantly compared the diabetic control group (CD) and this deference is significant (P<0.05)
Conclusion: Walnut leaf has unsaturated fatty acids. These acid compounds probably inhibit the activation of the arginase enzyme gene by their effects. The inhibitory action of the gene expression by Cyclohexan extract of walnut leaf can be likely attributed to Gallic acid and Caffeoylquinic acid in the walnut leaf, which prevents gene transcription and thus turns off the gene.

Key words: Diabetes, Walnut leaf, Cyclohexanin extract, Arginase, *ARG2*

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INTRODUCTION

Diabetes is an endocrine disease which is caused by insulin functional disorders [1]. In traditional medicine, the use of herbs has a long history in the treatment of diabetes [2]. In diabetic patients, on the other hand, arginase enzyme has shown a significant increase which causes sexual dysfunctions [3]. Before the discovery of insulin as well as anti-diabetes drugs, diabetic patients were treated with medicinal herbs [4]. The main chemical compounds of all the plants reducing the blood glucose include glycoside, alkaloids, triterpenes, polysaccharides, saponins and oils [5].

Plants antioxidant compounds are effective in repairing pancreatic beta cells. The study on garlic and fenugreek showed that the number of beta cells increased significantly in the diabetic rats treated [6,7].

Walnuts

Juglans are of the *Juglandaceae* family and have three species including *J. nigra, J. regia and J. cinerea*, of which only *Juglans regia* species grows in Iran. The phenolic compounds in walnut leaf are phenolic acids and flavonoids (Table 1) [8]. Walnut leaves are an important source of flavonoids, sterols, phenolic acids and polyphenols [9-12]. The leaves of this plant are topically

used to treat, dandruff, sunburn, and also as softeners in the dermal dysfunctions [13].

Table 1: The main phenolic compounds in walnut leaf

The most important phenolic acids	The most important flavonoids
3-caffeoylquinic acid	*Juglan*
3-p-coumaroylquinic acid	Quercetin and its derivatives
	Kaempferol and its derivatives

Arginase

Arginase is an enzyme containing manganese and belongs to the family of orohydrolase. In most mammals, there are two isoforms of this enzyme: *ARG1* and *ARG2*, which are different in terms of tissue distribution, intracellular location, immunological properties, and physiological function [14]. The reaction catalyzed by this enzyme, which is the ultimate enzyme in the urea cycle, is as follows:

Arginine + H_2O → Ornithine + Urea

The second isoform, Arginase II, plays a role in regulating the concentration of arginine and ornithine in the cell. In the mitochondria of several tissues, this isoform is found with the highest frequency in the kidney and prostate and at lower levels in the macrophages, the mammary glands of the mammals and the brain [15]. Using the L-arginine amino acid, the mitochondria form produces metabolites that ultimately contribute to the production of polyamines and regulate cell growth and proliferation.

The *ARG2* gene, which encodes Type II isoform (Arginase II), is expressed with nitric oxide (NO) synthase in smooth muscle tissues, such as the smooth muscle of genitals of men and women. Inhibition of arginase with ABH or other Boronic acid inhibitors will maintain the normal level of cellular arginine and as a result, it will cause muscle relaxation and natural sexual response [16].

The physiological role of this isoform is still unknown, but it is thought to play a role in the metabolism of nitric oxide and polyamine. Because NO synthase is found in the smooth muscle of the corposum cavernosorum of penis, corpora cavernosa of clitoris and the vagina, arginase II plays an important role in the sexual process of both male and female sex. Hence, it is a potential target for the treatment of male and female sexual dysfunctions. Its chromosomal position is shown in Figure 1 below [17]:

Diabetes causes many lesions, such as cardiovascular disease, arteriosclerosis, eye diseases, kidneys, etc., but it has also some important side effects, such as sexual disorders in males and females, which affects males as an impaired erectile function. Researches have shown that there is a direct relationship between diabetes and increased activity of the arginase enzyme.

On the other hand, nitric oxide synthase enzyme has a common substrate with arginase which is produced from the L-arginine amino acid and L-citrulline in the nitric oxide production cycle. Nitric oxide is a neurotransmitter that increases the blood flow and dilation of the arteries of the penis. It has been reported that sexual malformation can be corrected by inhibiting the arginase enzyme. Therefore, the increased activity of the arginase enzyme can be related to sexual dysfunction [18].

Gene expression using the real time PCR technique

PCR Real Time is one of the important methods for examining the gene expression. Its difference with the conventional PCR is the use of a fluorescent indicator in response to tract the reaction product. These reporters are designed to produce light in the event of duplication of DNA, so more light is equivalent to the product reproduction.

In this study, DNA-bonded dyes were used such as Syber Green as the fluorescence reporters to see the reaction. This dye is bonded to the small gap of the double-stranded DNA helix. The non-bonded dyes exhibit very little fluorescence in the solution and the fluorescence clearly increases when the dye is bonded to a double-stranded DNA.

The reaction can be seen during the exponential phase by recording the amount of fluorescence emitted in the cycle. A linear relationship will be observed if a graph is plotted between the logarithm of the start of the reaction and the increase of the reporter fluorescence [19]. It should be noted that the cyber Green (SYBR® Green) or Eva green dyes do not have the ability to bond to single-stranded DNA.

Performed researches

The methanolic extract of walnut leaf at a dose of 250 mg/kg in short and long term models *in vitro* inhibited significantly the activity of α-glucosidase but showed no changes in insulin and Glut-4 expression [20]. Fukuda et al. showed the strong inhibitory effect of polyphenols and phenolic compounds of walnut, such as Casuarictin, Tellimagradin II and Tellimagradin I, on various enzymes such as glycosidase, sucrose, maltase and amylase [21].

In a study, changes in the expression of arginase gene were studied in the reproductive system of diabetic dogs

Figure 1: Arginase II chromosomal position (Cytogenetic Location: 14q24.1, which is the long (q) arm of chromosome 14 at position 24.1)

and it was found that these changes were effective in low fertility in diabetic patients [22]. Comparison of specific activity of the enzyme between diabetic and healthy dogs showed that the activity of arginase gene in liver, epididymis, prostate, corpus cavernosum and sponge tissue of diabetic dogs increase significantly [22].

METHOD AND MATERIALS

The induction of diabetics in the animals was carried out using streptozotocin (powdered in one gram vials (Sigma, USA)) at a dose of 60 mg/kg. All rates were tested for blood glucose level after 72 hours of injection and they were considered as diabetics if their fasting blood glucose were more than 250 mg/dL. Then, the diabetic animals were randomly divided into 2 groups with 10 individuals. The treatment was carried out with Cyclohexan extract of walnut leaf by gavage syringe for 6 continuous weeks (Table 2).

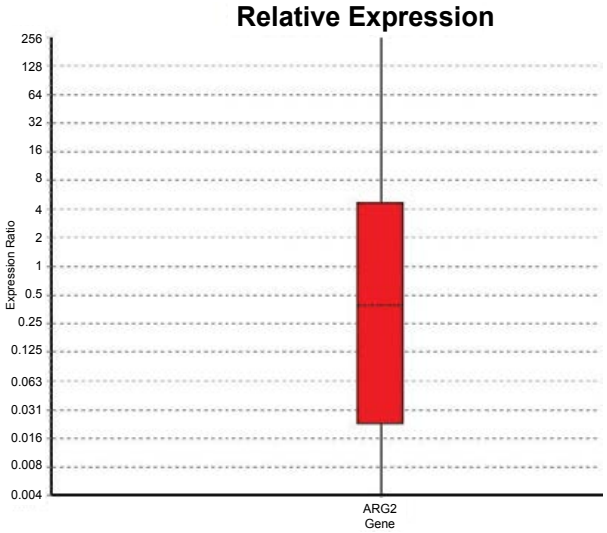
RESEARCH FINDINGS

At the end of the course, the primers of this research were designed and the sequence of the largest mRNA of the *ARG2* gene was taken from Ensembl site. Real Time PCR was then used to extract and check the gene expression. In this study, the SYBR Green method was used. To statistically analyze Real Time PCR data in this study, Fold change was first calculated using the formula $2^{-\Delta\Delta Ct}$ and then the data were analyzed with REST computer software (ver-9) p-value. This software was considered to be 0.05 or less for data analysis.

According to the P-value, which is 0.785 and the rate of Fold Change, which is 0.656, the results of the analysis show that there is no significant difference in the expression of the gene in the treated control group of cyclohexan (CT-C) compared to the healthy control group (CN) (Table 3).

In Graph 1, the amount of dispersion of the target gene expression is indicated in red (rectangle of the graph) (Graph 1). The discontinued line in the middle of the graph represents the rate of fold change and the greater dispersion of data will be equal to the longer arms of this graph. The bottom arm of this graph is for fold change indicates the amount of gene expression. The more fold change is equal to more gene expression.

25% of the samples with the least fold change. This rate of fold change for 25% of the samples is between 0.004 and 0.026. The rate of fold change for 50% of the samples is between 0.027 and about 5. The fold change



Graph 1: Relative expression (CN *via* CT-C)

Table 2: Grouping the tested animals

Groups	Group symbol	Number	Type of treatment	Dosage	Blood sampling time
Control Normal	CN	N=10	No drug treatment	60 mg/kg	The end of 6th week
Control	CN-0	N=10	Only Sesame Oil Solution	60 mg/kg	The end of 6th week
Control Therapeutic	CT-C	N=10	Cyclohexan extract of walnut leaf	60 mg/kg	The end of 6th week
Control Diabetic	CD	N=10	No drug treatment	60 mg/kg	The end of 6th week
Diabetic Treated	DT-C	N=10	Cyclohexan extract of walnut leaf	60 mg/kg	The end of 6th week

Table 3: Relative expression results (CN *via* CT-C)

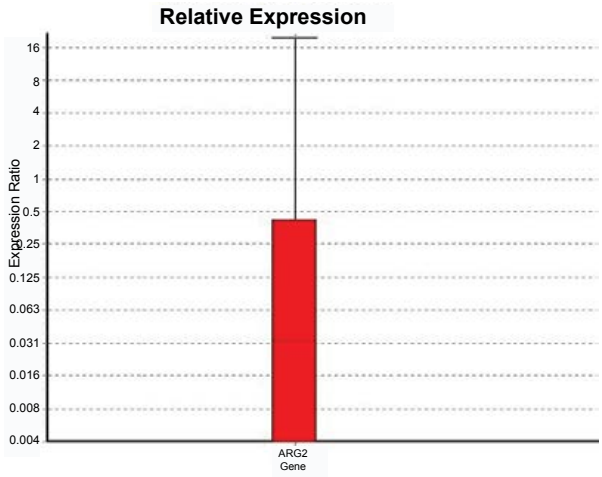
Relative Expression Results						
Parameter				**Value**		
Iterations				3000		
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P (H1) Result**
B2M	REF	1.00	2.221			
ARG2	TRG	0.95	0.656	0.009-56.972	0.001-8705.914	0.785
Act b	REF	1.00	0.45			
Interpretation			*ARG2* sample group is not different to control group. P (H1)=0.785			
Non-Normalized Results						
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P (H1) Result**
B2M	REF	1.00	5.145	0.238-116.947	0.008-951.418	0.148
ARG2	TRG	0.95	1.521	0.291-11.038	0.052-53.553	0.506
Act b	REF	1.00	1.044	0.024-35.054	0.000-71.670	0.976

Table 4: Relative expression results (CD *via* DT-C)

Relative Expression Results						
Parameter			**Value**			
Iterations			3000			
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P (H1) Result**
B2M	REF	1.00	0.447			
ARG2	TRG	0.95	0.027	0.000-1.037	0.000-11.656	0.020 DOWN
Act b	REF	1.00	2.238			
Interpretation			*ARG2* is DOWN-regulated in sample group (in comparison to control group) by a mean factor of 0.027 (S.E. range is 0.000-1.037); *ARG2* sample group is different to control group; P (H1)=0.020			
Non-Normalized Results						
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P (H1) Result**
B2M	REF	1.00	0.257	0.017-5.136	0.001-135.298	0.223
ARG2	TRG	0.95	0.016	0.002-0.110	0.001-0.594	0.000 DOWN
Act b	REF	1.00	1.289	0.041-47.413	0.019-2896.309	0.845

rate for the upper 25% of the samples, the upper arm of this graph, is between 5 and more than 128.

According to the P-value, which is 00/0 and the rate of Fold Change, which is 0/027, the results of the analysis showed that the gene expression in the diabetic group treated with the cyclohexan extract (DT-C) reduces significantly compared the diabetic control group (CD) and this difference is significant (P<0.05) (Table 4). In Graph 2, the amount of dispersion of the target gene expression is indicated in red (rectangle of the graph) (Graph 2). The discontinued line in the middle of the graph represents the rate of fold change. The bottom arm of this graph is for 25% of the samples with the least fold change (less than 0.004). The rate of fold change for 50% of the samples is between 0.004 and ~0.40. The fold change rate for the upper 25% of the samples is between 0.5 and ~16. If the data is less scattered, they will fall into a smaller range.



Graph 2: Relative expression

CONCLUSION

The results of the analysis show that the Arginase gene is evenly expressed in both groups of CN and CT-C and there is no significant difference between them. However, in the DT-C treatment groups, the Arginase gene is significantly reduced compared to the CD group. This indicates that the treatment of diabetic specimens with Cyclohexan extract of walnut leaf has a significant effect on reducing the expression of arginase gene, and therefore, in this treatment group, the average concentration of the arginase enzyme can be very low. Walnut leaf has unsaturated fatty acids. These acid compounds probably inhibit the activation of the arginase enzyme gene by their effects.

The inhibitory action of the gene expression by Cyclohexan extract of walnut leaf can be likely attributed to Gallic acid and Caffeoylquinic acid in the walnut leaf, which prevents gene transcription and thus turns off the gene.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

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