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Original Research

The effect of the ginger on the apoptosis of hippochampal cells according to the expression of BAX and Cyclin D1 genes and histological characteristics of brain in streptozotocin male diabetic rats

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Abstract: Diabetes is the most common endocrine disorder in humans with multiple complications including nervous system damages. The aim of the present study was to determine the effect of ginger extract on apoptosis of the neurons of hippocampus, via evaluation of BAX and Cyclin D1 and also histological analysis, in male diabetic rats. In this experimental study, 60 Wistar rats (220 ± 30gr) were conducted in 5 groups as follow: diabetic group treated with saline (group 1), normal group treated with saline (group 2), diabetic group treated with ginger (group 3), diabetic group treated with ginger-insulin (group 4), diabetic group treated with insulin (group 5). STZ (60 mg/kg) was intraperitoneally used to induce the diabetes. Expression levels of BAX and Cyclin D1 were examined using Real-Time PCR technique and the normality of neurons was evaluated using H&E staining method. The results showed that blood glucose level significantly decreased in group 4 when compared to group 1. In molecular analysis, there was no significant difference between groups regarding the expression of BAX gens, while, the expression of Cyclin D1 were significantly decreased in group 4 compared with group 1. Histological analysis revealed that pathological symptoms were lower in group 4 than the other diabetic groups. The results of present study showed that the ginger in addition to lowering blood sugar level, changes the expression of Cyclin D1 gene and histological characteristics in a positive manner. This means that the ginger may protects neurons of the hippocampus from apoptosis in diabetic patients.

Key words: Ginger, hippocampus, diabetes, BAX and Cyclin D1.

Introduction

Diabetes is the prevalent metabolic disease which leads to lipids, proteins and carbohydrates metabolisms disorders (1). Free radicals and its corresponded oxidative stress are the main cause of pathogenesis and complications in diabetes (1). It has been reported that in addition to common complications such as vasculopathy, nephropathy and retinopathy, some damages to nerve system (neuropathy) is also plausible (2). Several complications are associated with neuropathy including damages to hippocampus region of central nerves system (CNS) which results in recognition, learning and memorial disabilities (3).

It has been demonstrated that some drugs including herbal and antidepressant drugs, neurotrophic agents and non-drug agents such as antioxidants can be considered as suitable treatment strategies for regeneration of neurons in dentate gyrus of hippocampus (4, 5). The neuron regeneration induces differentiation of hippocampus dentate gyrus neurons to mature neurons which is the main cause of improvement of learning, memory and recognition in diabetes (4, 5). Based on the fact that herbal drugs have either a suitable effects or lower side effects than chemical drugs (6), hence, recent studies have tried to examine the efficacy of herbal drugs for treatment of diabetes and its complications including neuropathy.

Zingiber Officinale (ginger) is introduced as an herbal drug that is used for treatment of diabetes in several countries as traditional medicine (7). Ginger extraction has several antioxidants including gingerol, shogaol and some phenolic ketone factors which results in elimination of anion superoxidase and OH free radicals (8, 9). Although, ginger has several anti-tumor functions, via its antioxidants and also effects on down-regulation of cancer related molecules such as BCL2, NF-κB and STAT3, but its effects usually result in decreasing in inflammation (10). The herbal drug not only has anti-inflammatory effects, but also has anti-pain effects which demonstrate the effects of ginger on hippocampus (11, 12).

Apoptosis is a normal response of cell system to avoid pathologic conditions which is seen in several tissues (13). Ectopic induction of apoptosis is a pathologic condition which is happened in the central nerve system (CNS) during metabolic diseases including diabetes (14). Several intracellular molecules participate

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in the apoptosis such as BCL-2 family members (15). BAX is a pro-apoptotic member of BCL-2 family which are expressed in the neurons during diabetes dependent damages to neurons (16). Additionally, Cyclin D1 is an intracytoplasmic molecule which induces cell survival and proliferation (17). The molecule also induces differentiation of cells from G1 to S phase (17). According to the fact that ginger has several functions including reducing blood glucose and inhibition of ectopic apoptosis as well as anti-inflammatory effects (18), hence, it has been hypothesized that it may suppress apoptosis in CNS. Hippocampus is a region of CNS which is the corresponded of memory, recognition, pain and learning which are disrupted during diabetes dependent neuropathy (3). Accordingly, it is proposed that ginger may has protective effects on the neurons of hippocampus. Thus, the main aim of this study was to evaluate the effects of ginger extraction on the expression of BAX and Cyclin D1 in hippocampus neurons and also the histological features of the neurons in a rat diabetic model.

Materials and Methods

Animals

This experimental study has been performed on 60 Wistar male rats which had 6-7 weeks and 220 ± 30 gr body weights. The animals were prepared from Rafsanjan Medical University Animal House and kept in standard condition including 22 ± 2ºC for 12 hours in dark and light as well as fed by *ad libitum*. The animals were divided to 5 groups randomly including diabetic controls (group 1), healthy controls (group 2), diabetic rats treated with ginger extract (group 3), diabetic rats treated with ginger extract plus insulin (group 4) and diabetic rats treated with insulin (group 5). According to the previous investigations, treatments were started 7 days after induction of diabetes (21). Ginger extract was administrated 200 mg/kg intraperitoneally every other day for 6 weeks for group 3 and 4. Insulin (4-6 units/kg) was injected to groups 3 and 4 daily (14 and 22). Finally, in a standard and ethical condition, the animals were killed (24 hrs after the latest treatment) and, then blood samples as well as their brain tissues were collected to measure biochemical factors (blood glucose, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) and mRNA levels of BAX and Cyclin D1 as well as histological analysis, respectively.

Diabetes induction

Diabetes has been induced in the animals using a standard protocol (19), using Streptozotocin (STZ). Briefly, STZ was dissolved in suitable buffer (citrate buffer solution, 0.01 M, pH: 4.5) and was administrated intraperitoneally (60 mg/kg). Diabetes signs were illustrated after 7 days in the animals by blood glucose increasing more than 220 mg/dl and also polyuria and polydipsia (21).

Ginger extraction

Hydroalcoholic extract of ginger was prepared based on the Cao study (20). Briefly, ginger powder (Isfahan Herbarium Company Iran) was solved in equal amounts of absolute ethanol. The mixture was incubated in 70ºC for 15 hours and then centrifuged at 2500g for 15 minutes to separate supernatant. The separated supernatant were incubated at water bath (40-50ºC) to evaporate ethanol.

Table 1. Primer sequences using in the study.

Gene name	Sequences
BAX F	GATGGCAACTTCAACTGGGG
BAX R	AGCCACCCTGGTCTTGGAT
Cyclin D1 .F	CAAGTGTGACCCGGACTGC
Cyclin D1 .R	CACATCTCGCACGTCGGT
Betta-Actin F	CTGTGCTGCTCACCGAGG
Betta-Actin R	CGGAGTCCATCACAATGCCT

Evaluation of blood sugar

Blood glucose, cholesterol, triglyceride, serum glutamic oxaloacetic transaminase (SGOT), serum glutamate-pyruvate transaminase (SGPT), alkaline phosphatase (ALP), low density lipoprotein (LDL) and high density lipoprotein (HDL) levels were examined using Pars Azmoon biochemical kits (Pars Azmoon, Tehran, Iran) according to the manufacture`s guidelines.

Evaluation of BAX and Cyclin D1 mRNA levels

Total RNAs were extracted from hippocampus using a commercial kit from Cinnaclon Company (Tehran, Iran) and then, the concentration of extracted total mRNA was measured using spectrophotometry (260/280 nm). The protocol of cDNA synthesis and Real-Time PCR conditions and programs (for amplification of BAX, Cyclin D1 and β–actin, as housekeeping) were described in our previous investigation (21), except primer sequences which is illustrated in table 1.

Histological analysis

Histological analyses were performed to evaluate neurons normality, plausible inflammation and leukocyte infiltration. The protocols of histological analyses were described in our previous study (21).

Statistical analysis

One Way ANOVA followed by Post-Hoc Tukey test under SPSS software (version 17) was used to analyze the raw data obtained from evaluation of biochemical factors, histological parameters and also mRNA expression of BAX and Cyclin D1. The results are presented as mean ± SD and the differences between groups were considered significant at $P<0.05$.

Results

The results demonstrated that, although, mRNA levels of BAX were altered in the groups 3 (p= 0.996), 4 (p= 0.998) and 5 (p= 0.999) in comparison to group 1, but the differences were not significant (Figure 1).

Evaluation of expression levels of Cyclin D1 showed that mRNA levels of the molecule were significantly increased in group 4 in comparison to group 1 (p= 0.049). There were no significant differences between groups 3 (p= 0.990) and 5 (p= 0.965) when compared with group 1 (Figure 2).

Histological analysis demonstrated that there are several neurons with pyknotic nucleus and a white marginal around the neurons which are associated with

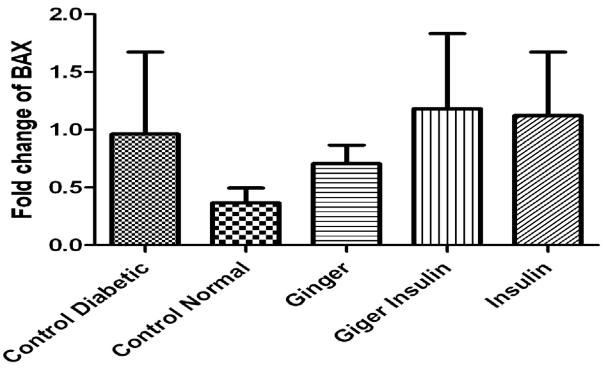

Figure 1. mRNA levels of BAX in the hippocampus of evaluated groups. The figure illustrates that mRNA levels of BAX in the hippocampus of evaluated groups were not significantly differ. Accordingly, as the figure is showing, mRNA levels of BAX in the hippocampus of group 4 were decreased but the differences were not significant. Group 1: Diabetic rats treated with saline. Group 2: Non-diabetic rats treated with saline. Group 3: Diabetic rats treated with ginger. Group 4: Diabetic rats treated with ginger and insulin. Group 5: Diabetic rats treated with insulin.

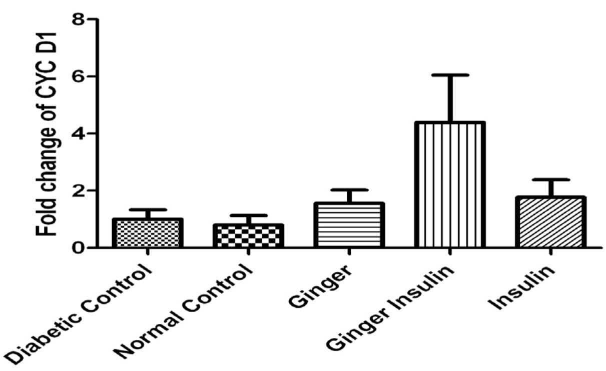

Figure 2. mRNA levels of Cyclin D1 in the hippocampus of evaluated groups. The figure illustrates that mRNA levels of Cyclin D1 in the hippocampus of group 4 were significantly increased in comparison to group 1, but the differences between other groups in comparison to group 1 were not significant.
*Increased Cyclin D1 in group 4 in comparison to group 1 (p= 0.049).
Group 1: Diabetic rats treated with saline. Group 2: Non-diabetic rats treated with saline. Group 3: Diabetic rats treated with ginger. Group 4: Diabetic rats treated with ginger and insulin. Group 5: Diabetic rats treated with insulin.

empty spaces between neurons in group 1. The histological features were also seen in the hippocampus of other groups with lower rates, but the neurons had normal vesicular nucleus (Figure 3).

Data revealed that serum levels of blood sugar decreased significantly in the groups 3, 4 and 5 when compared to group 1 (p< 0.001). Serum levels of cholesterol (p= 0.979), TG (p= 0.228), SGOT (p= 0.103), SGPT (p= 0.302), ALP (p= .056), HDL (p= 0.271) and LDL (p= 0.827) were not significantly differ in groups 3, 4, and 5 when compared with group 1. Table 2 illustrates the data in details.

Discussion

It has been demonstrated that ginger is able to reduce blood glucose (18) and also other diabetic complications like hear abnormality (22), blood pressure (23) and so on. Ginger also has hepatoprotective, anti-obesity, hypolipidemic and anti-oxidant effects (24). Based on the fact that neuropathy is an important complication of diabetes, a complication which is associated with damage to neurons, hence, it is important to evaluate effects of ginger on the neurons of hippocampus which is corresponded for several behaviors including memory, learning and recognition (25). The results of current

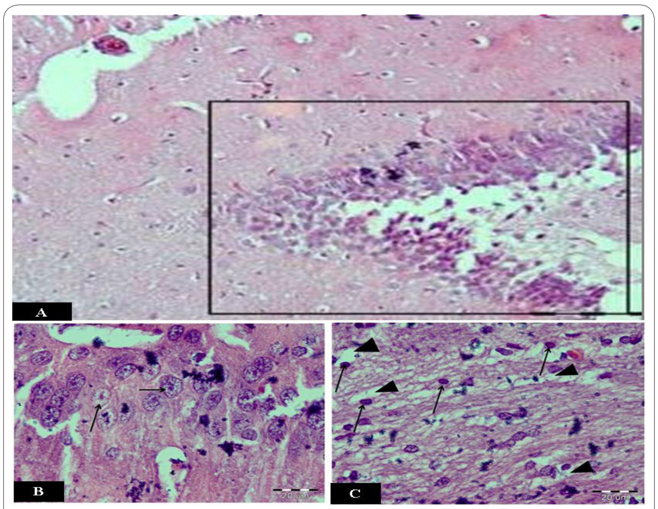

Figure 3. Histological sections from hippocampus after 6 weeks treatment using H&E staining. A. Mice hippocampus (showing dentate gyrus with 20X power). B. Hippocampus dentate gyrus of rats treated with ginger extraction and insulin (group 4) which shows the normal vesicular nucleus of neurons (point of a spear) with 40X power. C. Hippocampus dentate gyrus of diabetic rats (group 1) which shows neurons with pyknotic nucleus and a white marginal around the neurons which are associated with empty spaces between neurons (40X power).

Table 2. Serum levels of blood sugar, cholesterol, TG, SGOT, SGPT, ALP, HDL and LDL in evaluated groups.

Groups / Variable	Diabetic controls	Normal controls	Ginger	Ginger plus insulin	Insulin	P value
Blood sugar	298 ± 27.48	95 ± 1	190.67 ± 13.29	199.75 ± 5.97	202.50 ± 14.84	< 0.001*
Cholesterol	65.00 ± 3	64.00 ± 1	63.66 ± 6.56	66.50 ± 4.19	66.75 ± 3.66	0.979
Triglyceride	64.66 ± 6.38	39.00 ± 1.00	45.33 ± 7.88	45.25 ± 9.15	42.50 ± 5.00	0.228
SGOT	193.67 ± 28.90	284.00 ± 1.00	205.33 ± 26.08	199.25 ± 15.83	158.75 ± 4.97	0.103
SGPT	103.00 ± 15. 87	167.00 ± 2.00	131.33 ± 51.54	102.00 ± 11.56	84.25 ± 14.59	0.302
ALP	430.33 ± 93.93	352.00 ± 2.00	584.33 ± 24.47	538.50 ± 63.57	655.50 ± 54.61	0.056
HDL	41.33 ± 1.33	46.50 ± 1.50	36.33 ± 4.17	39.25 ± 2.46	42.25 ± 2.05	0.271
LDL	33.66 ± 6.88	35.50 ± 0.50	36.66 ± 4.25	38.00 ± 3.58	32.25 ± 2.05	0.827

*Table illustrates that serum levels of blood sugar significantly decreased after treatment with all components (ginger, ginger + insulin, insulin).

study confirmed the roles played by ginger on the reducing blood glucose in accompaniment with insulin. The results demonstrated that ginger is able to reduce the blood glucose in short time consumption, when insulin was unable to reduce it significantly. Previous investigations showed that ginger reduces blood glucose via interaction with serotonin receptors on pancreas betta cells to increase secretion of insulin (26). Thus, it seems that ginger can be considered as a suitable supplementary herbal drug for treatment of diabetes. The results also demonstrated that ginger can increase the survival of neurons via up-regulation of Cyclin D1. Previous investigations reported that ginger extraction is containing several anti-oxidants (22). Oxidants are produced following elevated blood glucose and can induce some damages to neurons including hippocampus neurons. The ginger anti-oxidant can neutralize the oxidant, so, it may be considered as an important mechanism for up-regulation of Cyclin D1. Additionally, Lim et al., showed that ginger effectively suppress Amyloid-β accumulation in hippocampus which is the main responsible of memorial dysfunctions during Alzheimer (27). It may be hypothesized that ginger also up-regulate Cyclin D1 in hippocampus through suppress Amyloid-β accumulation. The histological analysis also confirmed the conclusion and revealed that ginger reduce neuron damages and leads to regeneration of neurons in the hippocampus. Interesting Lim and colleagues reported that ginger improves recognition and memory through nerve growth factor (NGF)-induced extracellular-signal-regulated kinase (ERK)/cyclic AMP response element-binding protein (CREB) activation in the hippocampus of the mouse (28). Cyclin D1 has a link with ERK/CREB pathway for survival and proliferation of cells (29). Accordingly, it seems that ginger induces several molecular pathways in the neurons which protect them from apoptosis and necrosis which is happened during diabetes. The results identified that ginger was unable to reduce BAX as a pro-apoptotic molecule. To the best of our knowledge, there are not studies regarding the roles of ginger on expression of anti/pro-apoptotic molecules in hippocampus of diabetic patients or animals, while, some studies on tumors revealed that ginger leads to down-regulation of anti-apoptosis molecules like BCL2 in the cancer cells (30). Therefore, it seems that effects of ginger on cells are dependent of the body conditions, so ginger can decrease and increase apoptosis in neurons during diabetes and tumors, respectively. It appears that more investigations are needed to shed lights the main mechanisms played by ginger to survival and repair of neurons.

Based on the results it seems that ginger extract can help insulin to reduce blood glucose and also reduce neuron damages during diabetes via up-regulation of Cyclin D1. Accordingly, it may be concluded that ginger can be used for treatment of human diabetes in accompaniment with other routine therapeutic strategies.

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