Investigating the Effect of Hydroalcoholic Extract of *Cyperus rotundus L.* on the Expression of *Bcl-x1* Antiapoptotic Gene in Rats' Hippocampus Tissue Following Global Ischemic-Reperfusion Injury

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Abstract- Ischemia-reperfusion injury is the tissue damage happened when blood supply returns to the tissue after a period of ischemia or shortage of oxygen. This brain injury initiates an inflammatory response involving the expression of adhesion molecules and cytokines. The aim of this study is investigating the effect of hydroalcoholic extract of *Cyperus rotundus L.* on the expression of the *Bcl-x1* antiapoptotic gene in rats' hippocampus tissue following global ischemic-reperfusion injury. In the present study, attempts were made to investigate the effect of hydroalcoholic extract of Cyprus rotundus L. on the expression of the *Bcl-x1* antiapoptotic gene in rats' hippocampus tissue following global ischemic-reperfusion. To this end, eighteen male Wistar rats (250-300 g body wt) were used in this study. The animals were divided into three classes, each including 6 rats, I: Control class without ischemia-reperfusion, II: Ischemia-reperfusion class that was subjected to all surgical procedures, III: extract injection class that received *Cyperus rotundus L.* after ischemia. Seventy two hours after ischemia-reperfusion, the hippocampus was derived for studying the changes in bcl-xl gene expression. Q real-time PCR was employed for the detection of bcl-xl gene expression in ischemia and extract groups, and then their results were compared with normal samples. The results showed the generations of 0.6233, 0.23, and 0.9933 for control, ischemia, and ischemic extract groups, respectively. Moreover, it was found that the bcl-xl gene expression declined in ischemia group as compared to the extract group. A significant difference in the bcl-xl gene expression was observed in ischemia group when compared with the groups which had both injection and ischemia. These findings are consistent with anti-apoptotic properties of the bcl-xl gene. It can be concluded that this method creates a powerful tool for the investigators to study brain ischemia and the responses to the treatment which are caused by the injection of Cyprus rotundus L.

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Keywords: Apoptosis; Bcl-xl gene; Cyprus rotundus L.; Ischemia-reperfusion; Real-time PCR

Introduction

The term apoptosis (a-po-toe-sis) was first used in a paper by Kerr, Wyllie, and Currie in 1972 to demonstrate a different morphological form of cell death (1). Indeed, apoptosis is "the physiological way for a cell die", that can be seen in a variety of normal situations. It plays a critical role in the regulation of development and protection of many adult tissues, including the nervous system. Apoptosis is associated with the activation of a genetic program in which apoptosis effector genes promote cell death while repressor genes enhance cell cycle (2). Ischemia-reperfusion brain injury begins an inflammatory response involving the expression of adhesion molecules and cytokines. This injury is the tissue damage happened when blood supply returns to the tissue after a period of ischemia or deficiency of oxygen. The deficiency of oxygen and nutrients from blood during the ischemic period creates a condition in which the restoration of circulation results in inflammation and oxidative damage. Oxidative stress resulted from IR injury through the production of the reactive oxygen species, and oxidative damage causes

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DNA modification and secretion of inflammatory cytokines. This process continues and induces apoptosis. The inflammatory response partially mediates the damage of reperfusion hurt. White blood cells are carried to the damaged area by blood which releases inflammatory factors such as interleukins and tumor necrosis factor (3-4). Many studies have shown that inflammation is caused by cerebral ischemia and reperfusion after brain ischemia increments these inflammatory reactions, which can worsen neuronal injury (5–7). Attempts to help stroke patients have predominantly been concentrated on the prevention of acute cell death. The roots and rhizomes of *Cyperus rotundus L.* plant are used in the treatment of different diseases including chronic diarrhea, inflammation, skin rashes, and excess bleeding. It also has anti-estrogenic, antimicrobial, antimalarial, antihistaminic, antiemetic, antipyretic, and anti-diabetic activities (8).The present study was undertaken to investigate the neuroprotective effect of Ethanol extract of Cyperus rotundus. L, on BCLXL gene expression in rats. A study indicated that *Cyperus rotundus L.* decreases oxidative damage in rats after ischemia-reperfusion (9). In the present study, a quantitative real-time PCR assay was designed and optimized based on SYBR Green I chemistry to characterize the effect of Cyperus rotundas L. on BCLXL gene expression changes in the hippocampus after ischemia-reperfusion injury in rats.

Materials and Methods

Planting and preparation of crude extract

In order to prepare the hydro-alcoholic extract, the whole plant samples were washed, dried, and grounded. Next, 200 g of the dried powder was taken in a beaker. Then, methanol 70% was added three times (each time with 24 h interval). Finally, the extract was both filtered and concentrated by vacuum evaporator. The percentage yield was 12% (10).

Animals

In the present study, 18 male Wistar rats (250-300 g body wt) were used. The animals were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg). Their body temperature was kept between 22 and 24°C by a heating lamp. The carotid artery was closed by a vascular clamp for 20 min. After ischemia period, the clamp was removed, and the hippocampus was reperfused. The rats were divided into three groups (n = 6 in each group). I: Control group without ischemia-reperfusion, II: Ischemia-reperfusion group that was subjected to all surgical procedures, III: extract injection group that received *Cyperus rotundus L.* (200mg/kg) 60 min before and after ischemia (11). Seventy two hours after the ischemia-reperfusion, the hippocampus was taken for studying the changes in bcl-xl gene expression. After this time of injury, rats were euthanized, and their brain tissues (about 100mg) were immediately collected from their hippocampus (12).

RNA extraction, DNA digestion, and reverse Transcription

The tissues which were treated by extracted RNA were purified by using RNase Kit (Qiagen). The congestion and purity RNA were determined by spectrophotometry. High-quality RNases (A260/280≥1.8) were selected and kept at -70 °C until using for cDNA synthesis. Up to 1 µg, RNA was converted to cDNA by using Reverse Transcription Kit (Thermo Science) according to the manufacturer's instruction. To verify the integrity of the cDNA, a PCR experience was performed using GAPDH (glyceraldehydes-3-phosphate dehydrogenase) internal control primer. The primers for real-time PCR of bcl-xl and GAPDH gene expression were designed by the Primer Express and Gene Runner.

Quantitative real-time PCR with SYBR green

The selected primers underwent an extensive search using BLAST tool (www.ncbi.blast). The primers which were used in this study are listed in Table 1.

Table 1. Characteristics of the primers used in real-time PCR assay.

Rat-bcl-xl-F	GCTGGTGGTTGACTTTCTCTCC
Rat-bcl-xl-R	GGCTTCAGTCCTGTTCTCTTCG
Rat-GAPDH-F	AAGTTCAACGGCACAGTCAAGG
Rat-GAPDH-R	CATACTCAGCACCAGCATCACC

Real-time PCR was carried out in 96-well optical grade at reaction volume of 25 mL, containing 12.5 SYBR Green Master Mix, 1 μl primer, and 50 ng genomic template DNA. All samples were run in

duplicate. Thermal cycling was carried out using the Applied Rotor-Gene real-time PCR system which used following cycle conditions: 95°C for 5 sec, 40 cycles at 95°C for 5 sec, 60°C for 31 sec, and Extension72°C for 1min. Then, the temperature was ramped up from 60°C to 95°C (0.03°C/s), and fluorescence intensity data was collected continuously during the ramping phase for 20 min. Melting curve analysis was performed according to the dissociation stage data, and reactions with a single peak at expected melting temperature (Tm) were considered for further analysis.

Data analysis

Quantitative analysis was performed by measuring threshold cycle (CT) values during the exponential phase of amplification. In each assay, mCT was the main CT value of duplicate amplifications. The relative quantity of *bcl-xl* gene was determined by using CT method, and ΔCT was calculated as the difference between CT values of *bcl-xl* and the CT value of *GAPDH* gene. The results were analyzed using the following formula of Gene dosage ratio = $2^{-\Delta\Delta CT}$,$-\Delta\Delta CT = [(mCT_{target}\text{-}mCT_{reference})_{bclxl\ sample}]$ - $[(mCT_{target}\text{-}mCT_{reference})_{GAPDHsample}]$ (21).Gene dosage ratios were relative to the mean ΔCT value of these samples. Data processing was analyzed by SPSS (Social Sciences software). The graph provision was performed using Microsoft Excel 2007 (20).

Results

Cyperus rotundus L. effects on *bcl-xl* gene expression changes in the hippocampus of the rats tested after ischemia-reperfusion were compared with its effects on *bcl-xl* gene expression changes in ischemia group. As expected, there was a noticeable difference between the tested and normal samples in real-time PCR. To optimize and accredit the real-time PCR assay before using ΔCT method for gene expression and to determine the PCR efficiencies of the target and the reference genes. The input value of template DNA was plotted against the corresponding CT values. The consistency of all the PCR reactions through a wide range of template DNA concentrations (3–50 ng) was assessed by plotting ΔCT values of *bcl-xl* gene against the input value of DNA. Melting curve analysis was performed for every single reaction to exclude the amplification of non-specific products. Each valid amplification reaction showed a single peak at expected Tm (Figure 1).

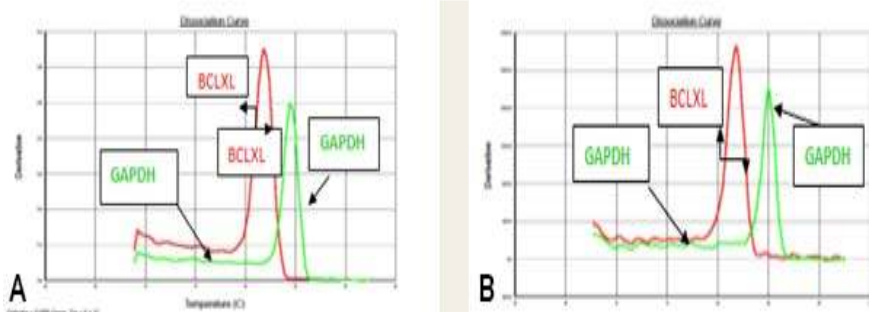

Figure 1. Amplification and melting curve analysis of real-time PCR for *bcl-xl* and *GAPDH* genes. A shows control group .B shows test group.

Furthermore, gel electrophoresis analysis of the PCR products revealed a single band with the expected size of each amplicon (Figure 2).

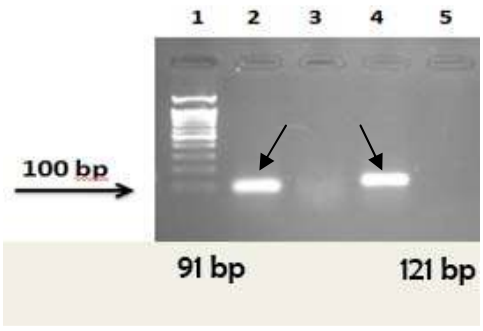

Figure 2. Results of real-time PCR analysis for the expression of *bcl-xl* gene. 1) DNA Size marker .*2) bcl-xl* .3) NTC bcl-xl , non-template

control. 4) *GAPDH*. 5) NTC GAPDH

The results indicated the gene dosage ratio of 0.623 for the control group, 0.230 for ischemia group, and 0.993 for injected groups which also had brain ischemia. The results also showed that *bcl-xl* gene expression declined in ischemia group as compared to the injected group (Figure 3).

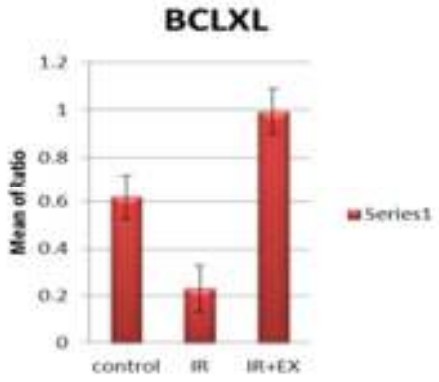

Figure 3. Results of bcl-*xl* gene expression in ischemia extract and control groups. The results also showed that the *bcl-xl* gene expression declined in ischemia group as compared to the extract and control groups

Discussion

Ischemia-reperfusion injury is the tissue damage happened when blood supply returns to the tissue after a period of ischemia or deficiency of oxygen. Tissue injury and death occur as a result of ischemic, which is determined primarily by the interruption in the blood supply. Then, subsequent damage which induced by ischemia-reperfusion brain injury initiates an inflammatory response involving the expression of adhesion molecules and cytokines. The aim of this study was to evaluate the effect of *Cyperus rotundus L. on bcl-xl* gene expression changes in the hippocampus after ischemia-reperfusion injury in rats. To this end, the quantitative real-time PCR assay was optimized by using SYBR green I technologies from Applied Biosystems. Moreover, *bcl-xl* gene, a member of the bcl-2 family which functions as an anti-apoptotic factor, was selected. Bcl2members such as bax, bak, and badly promoted apoptosis, whereas other members such as bcl-2 and bcl-xl prevented apoptosis by blocking the translocation of cytochrome c. Then caspase activation mitochondria were involved in excitotoxic injury during cerebral ischemia and the release of cytochrome c, an apoptogenic factor that advertises death signals by triggering caspases leading to cell death (14). Moreover, the coverage of the detectable *bcl-xl* gene was expanded by SYBR Green assay for *bcl-xl* gene expression. This issue could be increased in the extract group and decreased in ischemia group. Expression pattern for bcl-2 and bcl-xl at their transcriptional and translation levels in the rats' brain was subjected to transient global ischemia. While bcl-2 and bcl-xl were expressed in surviving and death neurons, their proteins were expressed early in neurons destined to survive. These results support a conceivable neuroprotective role for these two apoptosis suppressor genes in cerebral ischemic injuries (15). In the present study, the mean value of the ratios, obtained from tested and normal samples using SYBR Green assay for *bcl-xl* gene, in rats that were ischemic-reperfused, had a remarkable reduction in bcl-xl; whereas in rats injected with *Cyperus rotundus L.* before ischemic-reperfusion, the number of *bcl-xl* genes was significantly more than their number in normal ones. Accordingly, *Cyperus rotundus L.* has a protective effect on brain cells.

By usingDue to the use of real-time PCR technique, as far as the authors are concerened, This the present piece of researchattempt represents the first systematic study of simultaneous changes in bcl-2 gene expression in transient brain ischemia in Wistar rats. HoweverIn fact, many studies have focused on bcl-2 mRNA expression in ischemic brain hurt. However, conventional techniques such as Northern blot analysis, quantitative RT-PCR, and in-situhybridization are not able to detect small values of mRNA, and do not provide a quantitative measure of the value of mRNA present in the samples (18). The real-time PCR requires a very small value of mRNA. In the present study, bcl-2 promoted the transcription of mRNA in the extract drug group but prevented the transcription of mRNA in the ischemia group. In addition, bcl-2 mRNA expression significantly declined in the ischemia group as compared to drug group. These findings are consistent with anti-apoptotic properties of bcl-2 gene. Also, this methodthe method presented in this study purvey a powerful tool for the investigators to study brain ischemia and respond responses to the treatment drugs with anti-apoptotic agents.

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