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An Assay of Bax and Bcl2 Expression in Mice Hippocampus Following Ischemia-Reperfusion Treatment with CoQ10

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Abstract

***Introduction*:** Preliminary studies confirmed reduction of cell death following treatment with antioxidants. According to this finding we investigated the relationship between consumption of CoQ10 and expression of bax and bcl2 in hippocampus ischemia that this expression related to cell programmed death.

***Material and Methods*:** We studied the protective role of CoQ10 against ischemia-reperfusion. Experimental design includes four groups: intact (N=7), ischemic control (N=7), sham control (N=7) and treatment groups with CoQ10 (N=7). The mice (treatment group) treated with CoQ10 as Pre-Treatment for a week. Then, ischemia induced by common carotid artery ligation and following the reduction in inflammation (a week) the treatment group post-treated with CoQ10 for a week. Nissl staining applied to counting necrotic cells of hippocampus and the western blotting performed to measurement the bax and bcl2 expression. Tunnel kit was used to quantify apoptotic cell death while to short term memory scale, we apply Y-maze.

***Results*:** Cell death was significantly lower when mice treated with CoQ10. Bax expression was significantly high in ischemic group but in treatment group was less and reversely the bcl2 expression in ischemic group was lower than treatment and vehicle groups. The memory test results were consistent with cell death results.

***Conclusion*:** Ischemia for 15 minutes induced cell death in hippocampus with more potent effect on CA1. CoQ10 intake significantly reduced cell death and decreased memory loss. with prevent of expression of bax and increase in expression of bcl2.

Introduction

Hippocampus is located in inferior horn of lateral ventricles in the brain's temporal lobe. Almost any sensory experience leads to activation at least a part of the hippocampus and the hippocampus send many signal as itself output to the anterior part of thalamus, hypothalamus and other parts of the limbic system, particularly through fornix that it's the main output pathway [1, 2]. Hippocampus and brain cortex plays an essential role in transferring information from short-term memory to permanent memory. Hippocampus plays a key role in maintaining the memory in consolidation period [3]. Hippocampus also plays a role in organizing and storing information and associated with feelings and memories [4]. Damage to the hippocampus causes amnesia and loose memory later, but earlier data are maintained and can remembers them [5]. The remaining memory can convince us that further strengthen of the memory, make data to transfer out of the hippocampus to other parts of the brain. Damage to the bilateral hippocampus can cause the subsequent amnesia or anterograde amnesia [6]. Hippocampus is involved in memory formation and spatial information processing and is among the first areas of the brain affected by degenerative diseases like Alzheimer's, Huntington's, Parkinson's disease and injuries from trauma and ischemia. Cerebral ischemia after heart attack and cancer is the third largest cause of mortality and disability in people who are older than 65 years in the world and there is no satisfactory cure for this disease [7]. Hippocampus is very sensitive to ischemia and hypoxia And hypoxia cause to reduce the ATP inhibiting synaptic potentials that is a mechanism to reduce energy consumption in hypoxic cells [8]. In this case the cells do anaerobic respiration to survive themselves for the production of ATP that this process leads to acidosis and lactate accumulation and cell death. During reperfusion, released oxygen free radicals and other oxidative cause damage to cells with greater intensity than the ischemia. Rapid medical interventions may reduce ischemic necrosis and apoptosis [9, 10]. Oxidation is a process which inside the body's oxidants can react with saturated fatty acid and with production free radicals (peroxides) cause destruction of myelin, lipids and other membrane components and can be damaged chromosomic material. However, with consumption antioxidant, free radicals cannot damage cells [11].

The usage of antioxidants can reduce cell death. Antioxidants are substances that remove free radicals preventing damage to cell membranes and DNA, and cell death. Free radicals are produced naturally in the body, but environmental factors like cigarette smoke, air pollution, and ionizing radiation are the main cause of these substances. Scientists believe that heart disease, cancer and even the aging process is associated with oxidative materials [12].

CoQ10, including substances that are usually associated with energy production in mitochondria and can be seen also as an antioxidant is well known. Antioxidants such as CoQ10 can neutralize the harmful effects of free radicals with removing them [13, 14]. In addition, an antioxidant, CoQ10 in the body, increases energy levels and strengthens the immune system. Also suggested that CoQ10 can increase fertility, improve hearing and the freshness of skin diseases and in case of AIDS and Alzheimer's are also effective. As the natural resources of coq10, fish oils and liver of animals and seeds can be noted [15, 16].

We did the experiment on the hippocampus, due to the sensitivity of hippocampal CA1 neurons against hypoxia . These neurons are extremely vulnerable against free radical accumulation, following ischemia - reperfusion in this area. The purpose of this study was investigate the antioxidant effects of CoQ10 and its health impact on reducing the mortality rate of hippocampal CA1 neurons after induction of ischemia.

Materials and Methods

Animals: Twenty-eight adult male mice (Pasteur's Institute, Tehran), weighing 30-35 g at the start of the experiment, were housed three to four per cage in a temperature-controlled colony room under a light–dark cycle with free access to tap water and standard pellet food. The experimental protocol for animal care and handling was according to the guidelines of the National Institutes of Health for the use of live animals and those of the research council of Iran University of Medical Sciences (Tehran, Iran). Principle of research ethics when working with laboratory animals was done according to the statute of Tehran University of Medical Ethics.

Experimental design: The mice were assigned as follow: 1- Intact groups: (N=7), without any induction of ischemia. 2- Ischemia control groups: (N=7). 3- Ischemia control with vehicle: (N=7), only was tacked olive oil. 4- Treatment groups: (N=7), was tacked CoQ10.

Olive oil was given as the gavages to mice 180 µl/day. It was tacked one week before ischemia induction; after one week following reduction of inflammation on ischemic zone, olive oil was given daily for a week. CoQ10 were prepared as Tablets and olive oil was used as a solvent. In order to evaluate the protective effect of Coq10 pretreatment, it was tacked as the gavages to mice one week before ischemia induction (450 mg/kg in day) (Sigma Chemical Co., Saint Louis, USA); Ischemia induced by clamping of common carotid artery (15 min) and after one week following reduction of inflammation on ischemic zone, CoQ10 was given daily for treatment group for a week. The memory test(Y-maze) performed two weeks following ischemia induction and then brains prepared for microscopic studies that involve Nissl staining, tunnel test and western blot for bax and bcl2.

Y-maze test: This working memory test is based on spontaneous exploration and alternations between arms – neither training nor food restriction are required. Three identical arms are mounted symmetrically on an equilateral triangular center.

Mice walk between the arms and we recorded the arm name in 300 sec. finally ever 3 arms name that not similar is one correct number and another are wrong number. This finding analyze with this formula:

Percent Alternation = $PA = \frac{x \times 3}{y - 2} \times 100$ (x=number of correct and y = correct + wrong number)

Anesthesia and Monitoring: Animals will be fasted overnight and anesthetized with ketamin (Sigma Chemical Co., Saint Louis, USA) (100 mg/kg, intraperitoneal injection) and xylozine (Sigma Chemical Co., Saint Louis, USA) (10 mg/kg, intraperitoneal injection) with additional ketamin as needed (30 mg/kg). A rectal temperature probe is placed for continuous monitoring of core body temperature. Core body temperature is maintained at 37±0.5ºC throughout the procedure by the intermittent use of a heating lamp. After anesthesia the mice brains prepared for microscopic studies that involve tunnel test and Nissl staining and western blot for bax and bcl2.

Nissl staining: This method is used for the detection of Nissl body in the cytoplasm of neurons. This stain is commonly used for identifying the basic neuronal structure from necrotic neurons in brain and spinal cord.

Deparaffinize sections in xylene and then hydrate. Rinse in tap water and then in distilled water. Stain in 0.1% cresyl violet solution for 3-10 minutes. Rinse quickly in distilled water. Then differentiate and dehydrate in alcohol then Clearing and finally mount with permanent mounting medium. The Nissl body will be stained purple-blue.

Tunnel test: The TUNEL Apoptosis Detection Kit is one of GenScript's newly introduced products. The kit can detect fragmented DNA in the nucleus during apoptosis. Deparaffinize sections with heater 60ºC and xylene and then hydration. Incubate in proteinase K (30 min) then blocking the endogenous peroxidase with use of H_2O_2 in methanol in dark room then wash in tris buffer and then incubate in TUNNEL reaction mixture for 60 min in moisture condition then wash in tris buffer and then detection with incubate in POD. Then washing and use of chromogen DAB (You +1'd this publicly. Undo3, 3'-Diaminobenzidine) in dark room then washing and use of hematoxylin for counter stain and dehydration and clearing and mount with permanent mounting medium.

Detection of bax and Bcl-2 by western blot technique: All steps were carried at 4ºC. Following the ischemia reperfusion, animal were sacrificed and

hippocampus tissues were removed and washed with ice-cold saline and homogenized on ice in lysis buffer containing 50 mmol/L Tris (pH=9), Deoxycolate 1% for 60 seconds each, separated by a pause for 1 minute at 4ºC. The homogenate was incubated in lysis buffer for 30 min, with vortex-mixing every 10 minutes at 4ºC. The homogenate was filtered and then centrifuged at 20,000 g for 20 minutes with a Hettich (universal 16 L-, Germany) and (Universal 16L, Hettich, Germany) the resulting supernatants (lysates) were frozen at - 80ºC for further investigation. The protein concentration was determined by Bradford's method, using bovine serum albumin as a calibrator.

Twenty micrograms of cell lysate proteins was mixed with 5 µL of loading buffer (50 mmol/L tris, 20 g/L sodium dodecyle sulphate, 100 ml/L glycerol, 100 mmol/L β -mercaptoethanol and 0.05% bromephenol blue solution, (pH=6.8) were boiled for 5 minutes and separated by 10% sodium dodecyle sulphate-polyacrylamide gel electrophoresis. The gels were transferred to nitrocellulose membranes in Tris-glycine buffer (25 mmol/L Tris, 192 mmol/L glycine, 200 ml/L methanol, pH=7.4) for 5 hours at 60 V. The nitrocellulose sheets were washed and free binding sites were saturated with 50 g/L bovine serum albumin in Tris-buffered saline buffer (50 mmol/L tris, pH=7.5, 150 mmol/L NaCl, 2 mmol/L EDTA) for 1 hour at room temperature. Then the membranes were incubated with phosphate buffer saline and mouse monoclonal anti-rat bcl2 antibody diluted 1:300 (by volume) overnight at 4ºC, then with rabbit anti-mouse IgG alkaline phosphatase conjugate diluted 1:500 for 90 min at room temperature. Finally, the membranes were incubated with BCIP/NBT (nitro-blue tetrazolium chloride) / (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) alkaline phosphatase substrate solution (at room temperature) until the developed bands were of desired intensity. Then the reaction was stopped by 200 mL of 0.5 mol/L EDTA (pH=8) and 50 mL of phosphate buffered saline. Bax and bcl2 protein band was identified by comparing with the molecular weight marker.

Statistical analysis: All data were expressed as mean FSEM. For within group and intergroup comparisons, two-tailed paired and unpaired Student's t tests were used respectively. One way ANOVA, followed by Tukey post hoc test, was used for each group at different time points. In all analyses, the null hypothesis was rejected at the level of 0.05.

Results

In this study we used the Nissl staining to compare between the necrotic and normal cells and used the tunnel kit to detect the apoptotic cell in the CA1 region. and used western blotting to measure the expression of bax and bcl2. The picture in bellow shows the result of these methods. Cresyl violet staining showed the situation of healthy and necrotic cells in tissue sections. The Animals treated with CoQ10 had less death cells and had more cell density compared to the vehicle and ischemia groups. Y-maze behavioral test showed that ischemia leads to severe damage to short-term memory. Best results achieved by treatment with CoQ10. In the vehicle group because of antioxidants in the olive oil we can see some improvemental sign. In Intact group this disorder failed to register.

Tunnel test that use for detect the apoptotic cell, showed increase of this cells in ischemic group and decrease of this cells in the treatment groups. Although in the vehicle groups the apoptotic cell was less than ischemic group but the treatment group has better position. Western blotting result for bax and bcl2 listed below. High bax expression in the ischemic group and reducing its expression in the treated group was clearly demonstrated. Anti apoptotic protein Bcl2 in the treatment groups showed much greater expression: in ischemic groups but its expression was very low.

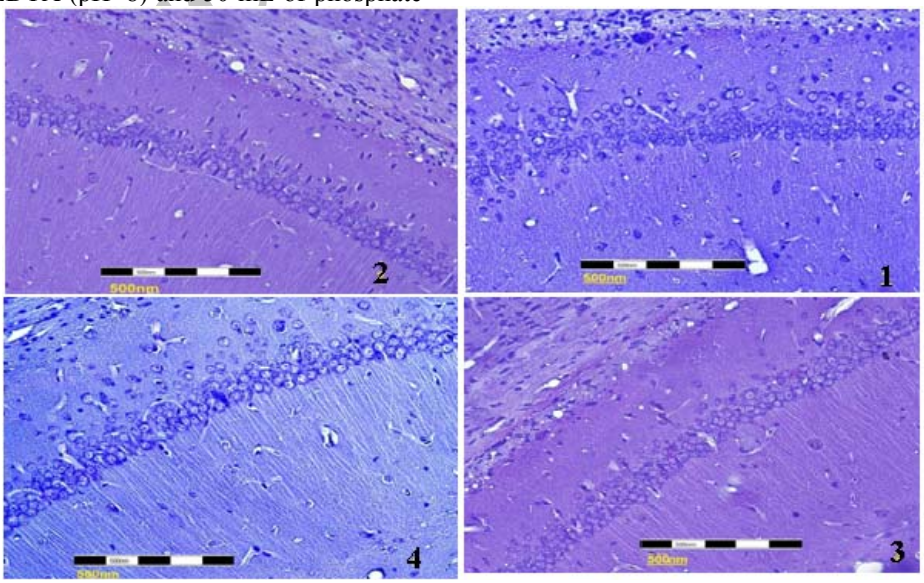

Figure 1. Nissl staining result in 4 groups. In this staining method the necrotic cells indicated with dark and compact nucleus. 1-The intact group without necrotic cell.2. The ischemic group with a lot of necrotic cells. 3. The vehicle group with less necrotic cell rather than ischemic group.4. The treatment group with infrequent necrotic cell than ischemic and vehicle groups.

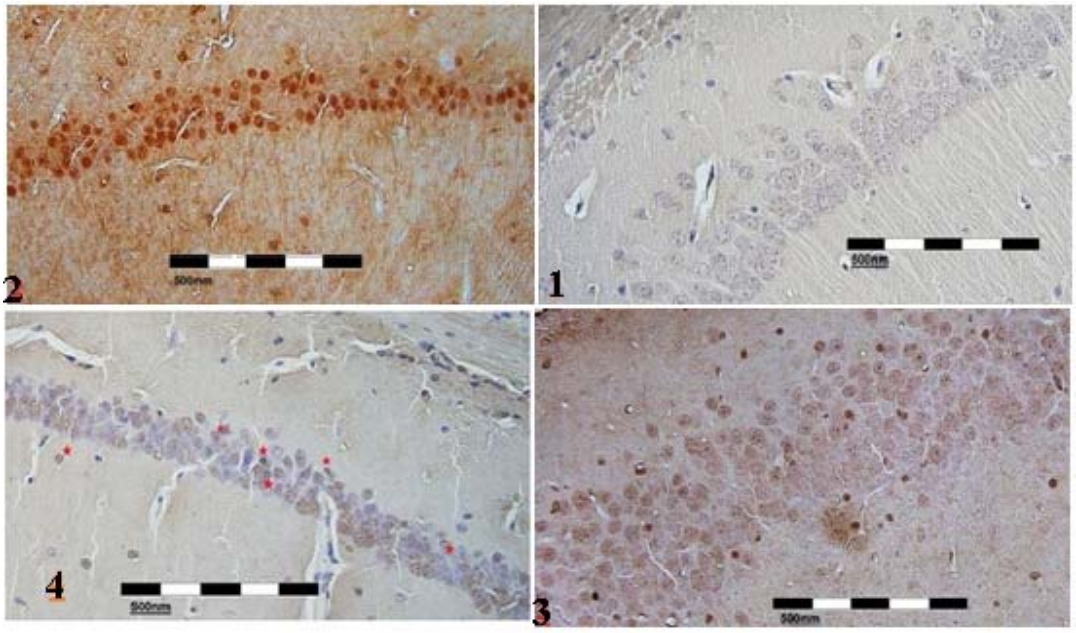

Figure 2. Tunnel test result in 4 groups. 1- The intact group without apoptotic cell. 2- The ischemic group with a lot of apoptotic cell (gray cells). 3- The vehicle group with less apoptotic cell rather than ischemic group. 4- The treatment group with the less apoptotic cell than other group (red star).

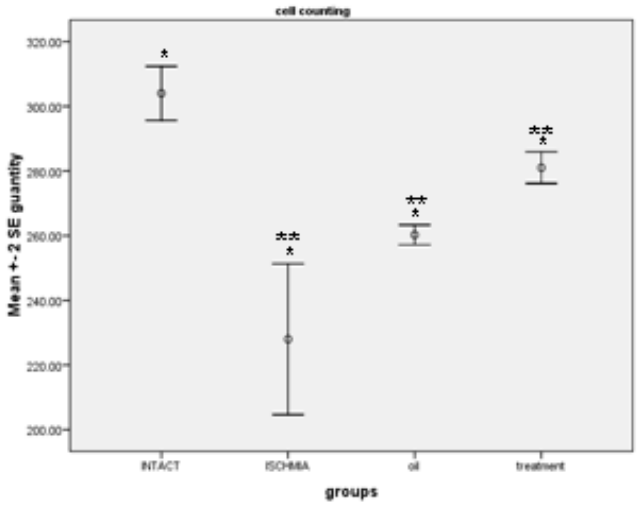

Figure 3. Comparison of the density of healthy cells in the CA1 region of hippocampus in 4 groups. Cell density of the intact group is significantly different than other groups. (* p<0.05) Cell density of the vehicle group is significantly different than ischemic group. (* p<0.05) Cell density of the treatment groups is significantly different than vehicle and ischemic groups (** p<0.001)

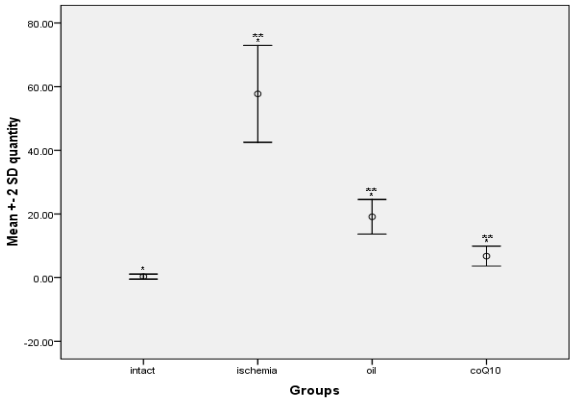

Figure 4. Comparison of the tunnel test in 4 groups. Ischemic group compared with the intact group represents significant difference (* p<0.05) Ischemic group compared with treatment groups represents significant difference (** p<0.001). The vehicle groups and treatment groups represents significant difference (** p<0.001)

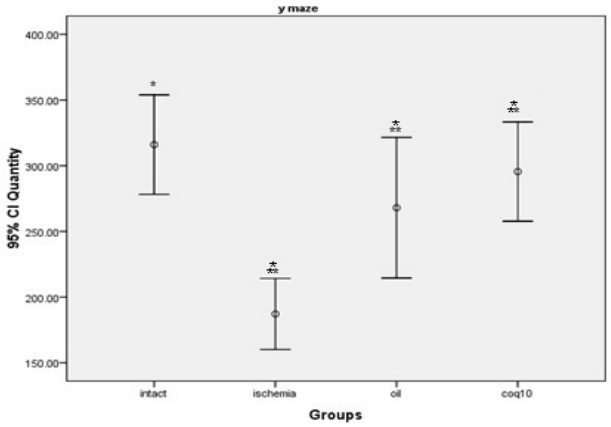

Figure 5. Comparison of the short term memory (Y-maze). Ischemic group compared with the intact group represents significant difference. (* p<0.05). Ischemic and vehicle group compared with treatment groups represents significant difference (** p<0.001)

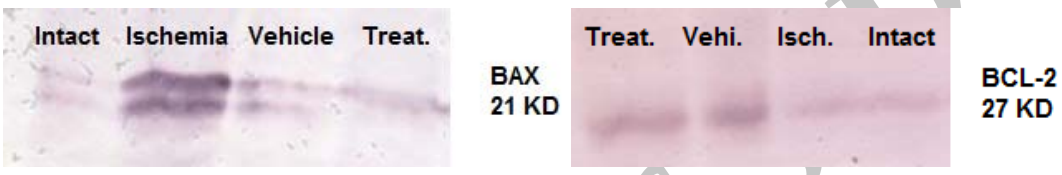

Figure 6. Western blotting method for bax and bcl2 protein. bax in the ischemic groups significantly expressed but in the other groups shows less expression. Expression of bcl2 in the vehicle and treatment groups showed the success in the treatment

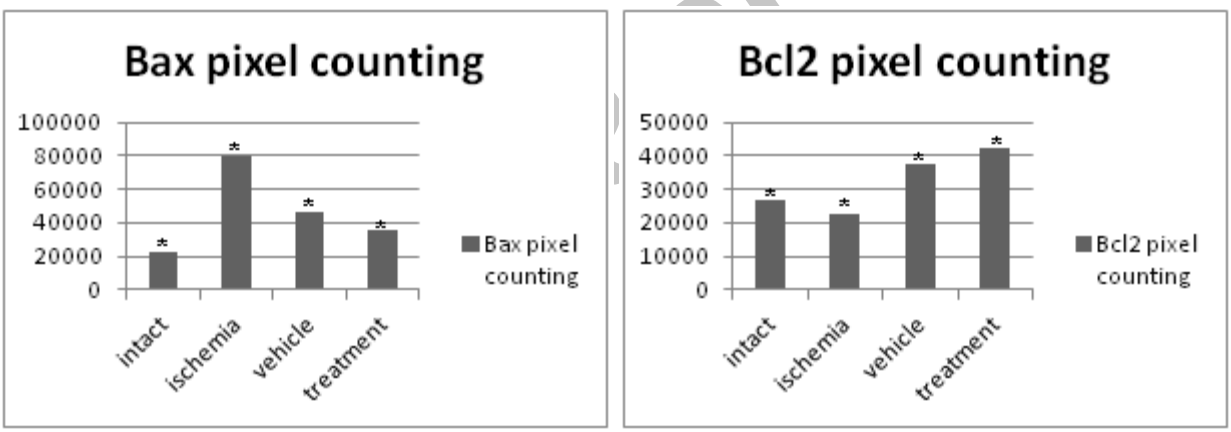

Figure 7. Pixel counting result of western blot. Bax in the ischemic group was expressed significantly than other groups and expression of bcl2 in the vehicle and treatment groups was significantly rather than other group (* p<0.05)

Discussion

In addition, high intake of saturated fats causes sediment in the artery wall and it graduals blockage creates more critical situation and increases the chance of blocked arteries and extent ischemia [17]. Thus, a structural change in the artery wall, especially in the vital organs like the brain as result formation of atheroma plaques, reduced blood supply to the brain and causes permanent damage and increases stroke risk [18-20].

To prevent free radicals from damaging the cells, the usage of antioxidants is recommended especially to lessen effects of ischemia. It is already found that the model of global cerebral ischemia leads to neurodegenerative lesions in CA1 area of hippocampus, stratum and the neocortex. It is also known that global cerebral ischemia can cause neuronal death in CA1 pyramidal hippocampus and reduces the spatial learning and memory in rats [21, 22]. We did the experiment on the hippocampus, due to the sensitivity of hippocampal CA1 neurons against hypoxia .These neurons are extremely vulnerable against free radical accumulation, following ischemia - reperfusion in this area.

The purpose of this study was investigate of the antioxidant effects of CoQ10 and its health impact on reducing the mortality rate of hippocampal CA1 neurons after induction of ischemia. In the treatment group with CoQ10 we observed decrease cell death in the hippocampus which is due to the effects of antioxidants and unsaturated fats found in oils such as polyphenols, oleic acid, linoleic acid, linoleic acid, and vitamin E. These compounds are absorbed from the gastrointestinal

tract into the blood vessel, and then some of it reaches in to the brain and past from blood-brain barrier due to being soluble in fat and dash into different parts of the brain including the hippocampus. CoQ10 consumption reduces brain ischemic mortality and protects neurons from harmful agents and thus reduces ischemic complications. Prevents the onset of enzymatic cascade that will induce apoptosis in cells and neurons in this case sufficient opportunity to repair themselves structures and can get your resume normal activities soon [23-25].

Ischemia - reperfusion reduces the oxygen level inside the cell that disrupts the normal cell metabolism and without the rapid supply of oxygen, the cells dies. Large amounts of free radicals in the reperfusion phase can invade and take it to death. The total consumption of CoQ10 with scavenge the free radicals, reduces the number of damaged cells.

This will prevent the expression of pre-apoptotic bax protein and else anti apoptotic bcl2 protein expression is increased (Fig. 3). The result of this intracellular activity is inhibition process of apoptosis and cell death that subsequent reduction of ischemic complications. In conclusion treatment with CoQ10 can be a pharmaceutical approach to lessen effects of ischemia reperfusion on hippocampus.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of interest

The authors declare no conflict of interest.

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