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RESEARCH ARTICLE

The Mechanism of Cell Death in Motor Neurons of Cultured Spinal Cord Slices; Anti-Apoptotic and Antioxidant Effects of Silymarin and Artichoke Flavonoids

Hamid Reza Momeni*, Mitra Noori, Tahereh Etemadi and Farzaneh Moshaveri

Biology Department, Faculty of Science, Arak University, Arak, Iran

***Corresponding Author:** Hamid Reza Momeni, Biology Department, Faculty of Science, Arak University, Arak, Iran, Tel.: 09183481302, E-mail: h-momeni@araku.ac.ir

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Abstract

Introduction: Oxidative stress might be one of the contributing mechanisms to motor neurons apoptosis in several neuronal diseases. Silymarin, extracted from milk thistle seeds, and artichoke flavonoids were reported as potent antioxidants with a high ability for attenuating oxidative stress.

Objective: This study was conducted to investigate the effects of silymarin and artichoke flavonoids on the apoptosis of motor neurons in cultured spinal cord slices.

The thoracic region of the spinal cord of adult mice was sliced, and divided into four groups: 1) slices at 0-hour, 2) control slices, 3) slices treated with silymarin (96.88µg/ml), and 4) slices treated with artichoke flavonoids (10µg/ml). The slices from groups 2-4 were cultured for 6 hours, then fixed and sectioned. Morphological features of apoptosis were evaluated in the motor neurons using Hoechst and propidium iodide staining. In addition, malondialdehyde (MDA) and ferric reducing antioxidant power (FRAP) were assessed to evaluate lipid peroxidation and total antioxidant capacity, respectively.

Results: After 6 hours, cultured motor neurons displayed morphological features of apoptosis. At this time point, the levels of MDA and FRAP were respectively increased and decreased compared to the 0-hour slices. The administration of silymarin and artichoke flavonoids could delay apoptosis in the cultured motor neurons. In addition, silymarin but not artichoke flavonoids compensated for the levels of MDA and FRAP compared to the control group.

Conclusion: Oxidative stress might be the mechanism involved in the apoptosis of motor neurons in cultured spinal cord slices, while silymarin and artichoke flavonoids with their antioxidant properties could delay the apoptosis of these motor neurons, introducing them as antiapoptotic compounds.

Keywords: Apoptosis; Spinal cord slices of mice; Antioxidant; Artichoke flavonoids; Silymarin

Introduction

Motor neurons degeneration can occur during the development of vertebrates [1] and/or in neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder in which motor neurons are lost in the spinal cord and motor cortex [2]. Spinal cord injury (SCI) is also known as another major cause of motor neurons degeneration [3]. Despite extensive researcher's efforts, the exact mechanisms responsible for the degeneration of the motor neurons are still unknown, and there is no universally accepted treatment for diseases in which motor neurons are destroyed. Apoptosis has been suggested to be the major cause involved in neuronal destruction in neurodegenerative diseases and spinal cord injury [4]. In this context, we have also demonstrated that the motor neurons in cultured adult mice spinal cord slices degenerated by apoptosis [5]. It has been proposed that a variety of mechanisms including glutamate excitotoxicity, caspase and calpain activation participate in the apoptosis of motor neurons in the cultured spinal cord slices. In addition, the induction of these pathways and initiation of apoptosis can be a consequence of oxidative stress in neuron cells [6].

Previous studies have illustrated that oxidative stress is a significant player in the pathology of neurodegenerative diseases [7] and SCI [8]. Therefore, scavenging free radicals and/or enhancing of the antioxidant defense system might be a possible strategy to protect neurons against oxidative stress-induced degeneration. Several pieces of evidence have reported that some antioxidants can inhibit neuronal degeneration *in vitro* and can also serve as a potential treatment option for neurological disorders [9]. Therefore, investigating the properties of antioxidants in particular antioxidants extracted from herbal medicines can clarify their potential effects on neurodegenerative diseases and even could be used as a therapeutic strategy.

Silymarin, as an effective compound derived from *Silybum marianum* and artichoke (*Cynara scolymus*), is a polyphenolic flavonoid which its therapeutic properties have been reported in several diseases [10,11]. In accordance with this, the neuroprotective effects of silymarin have also been reported in neurodegenerative disorders and brain ischemia [12]. Furthermore, it has been shown that the extracts of artichoke could protect cultured mouse hepatocytes against oxidative stress [13]. The fact that oxidative stress plays a critical role in neuronal apoptosis in neurodegenerative disorders and SCI [14], it is reasonable to assume that oxidative stress is also involved in motor neuron apoptosis in cultured spinal cord slices. Given that protecting of the motor neurons against apoptosis can be a suitable strategy for the treatment of neuronal diseases, this study, therefore, was conducted to investigate whether silymarin and artichoke flavonoids can delay apoptosis in motor neurons of adult mice spinal cord slices.

Materials and Methods

Extraction of Artichoke Flavonoids

Aerial parts of Artichoke was dried in the shade and prepared by grounding into a fine powder (300 g). The extraction was performed using a percolation method with 70% ethanol in three steps for 72 hours. All three extracts were then mixed. This initial ethanolic extract was dried using Heidolph rotary evaporator (Laborota 4000/G1) at 40°C and 40 m/sec [15]. Flavonoids in the extract were isolated and detected using two-dimensional paper and thin-layer chromatography (TLC) according to the method described by Markan and coworkers [16]. The extracted flavonoids were prepared using an acid hydrolysis method by adding hydrochloric acid (2M) and placing in a water bath at 100°C for 30 minutes. After cooling, ethyl acetate (250 ml) was added into the solution to separate the flavonoid extract from the non-flavonoids extract. The flavonoid extract was distilled off in a vacuum at 40°C and 40 m/sec. Flavonoid content (gr/Kg of dry plant matter) was measured by weight/volume method after dissolving the dried extract in 70% ethanol. Co-chromatography with standards was also performed. Flavonoid standards available for comparison during the study were Apigenin, Chrysin, Genistein, Hesperidin, Isorhamnetin, Kaempferol, Luteolin, Morin, Myricetin, Naringenin, Quercetin, Rhamnetin, Rutin, Tricine, Vitexin, and silymarin. TLC plate was viewed in UV245 nm. Each spot R_f-values and color compared to the standards helped flavonoids identification after running in BAW (n.butanol/acetic acid/water), CAW (chloroform/acetic acid/water), and Ferostal solvents [17]. The flavonoid extract was kept in dark vials and stored at 4°C un-

til further use.

Preparation of Organotypic Spinal Cord Slices

Adult female NMRI mice (23–25 gr) were purchased from Pasture Institute, Tehran, Iran. The animals were housed in plastic cages at 20C, a 12-h light/dark cycle, with water and food *ad libitum*. The experiments were approved by the local ethical committee on research animal care at Arak University, Arak, Iran. The animals were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and subsequently killed by heart puncture. The spinal cord was dissected and placed in ice-cold phosphate-buffered saline (PBS), pH 7.4. The thoracic region of the spinal cord was then sliced transversally into 500 μm-thick sections using a McIlwain tissue chopper (Stoelting, USA). The slices were divided into four groups (each group contains 6 slices from at least 8 mice): 1) slices at 0-minute, which were fixed immediately (see below), 2) control slices that received solvent of silymarin or artichoke flavonoids, 3) slices treated with silymarin (96.88μg/ml, Sigma, USA) and 4) slices treated with artichoke flavonoids (10 μg/ml). To determine an effective concentration, the effect of different concentrations of silymarin (24.12, 48.24, 72.36, 96.88, and 120.61 μg/ml) [18] and flavonoids extracted from artichoke (10, 25, 50, and 100μg/ml) [19] were tested on the motor neurons apoptosis of spinal cord slices. Our results indicated 96.88μg/ml and 10μg/ml as the most effective concentrations for silymarin and artichoke flavonoids, respectively. The slices in groups 2–4 was then placed in four well sterile plastic plates. Each well contained 450 μl culture medium composed of a mixture of 50% minimum essential medium, 25% Hanks balanced salt solution, 25% horse serum, 25 mM *N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid* (HEPES), 6gr/l glucose and 1% penicillin-streptomycin, pH 7.3–7.4. The samples were incubated at 37C in a humidified atmosphere of 5% CO_2 for 6 hours.

Fixation and Sectioning of Slices

Freshly prepared (0 hour) and the cultured slices were fixed in Stefanini's fixative (2% paraformaldehyde, 0.2% picric acid in 0.1M PBS, pH 7.2) for at least 2 hours. The fixed slices were washed in PBS (3×5 min) and incubated overnight in 20% sucrose in PBS at 4C. The slices were then cut into 10 μm-thick sections using a cryostat (Leica, Germany). The sections were collected and mounted on poly-L-lysine coated glass slides.

Assessment of Motor Neuron Apoptosis

To study morphological features of apoptosis, the combination of propidium iodide (PI, Sigma, USA, 10μg/ml in PBS, 15 minutes at room temperature) and Hoechst 33342 (Sigma, USA, 10 μg/ml in PBS, 1 minute at room temperature) was used. The slides were washed in PBS (3×5 minutes), mounted in glycerol/PBS (1:1) and cover slipped. Motor neurons could subsequently be identified by their large cell bodies and bright nuclei. Apoptotic motor neurons displayed cell shrinkage and nuclear and chromatin condensation. Digital photographs were taken with an Olympus camera attached to an Olympus fluorescence microscope (Olympus Optical Co Ltd, Japan). The photographs were also used to measure the nucleus diameter of the motor neurons using Motic image 2000 software.

Assessment of Lipid Peroxidation

Lipid peroxidation in the spinal cord slices was evaluated by measuring the levels of malondialdehyde (MDA) according to the method described by Buege and Aust [20]. The reaction of MDA with thiobarbituric acid (TBA) produces a pink complex under acidic conditions at 100°C [21]. In summary, 0.1 gr spinal cord slices were homogenized in KCl solution (1:9). Then, one volume of TBA solution (containing 15% (w/v) TBA, 0.375% (w/v) trichloroacetic acid (TCA) and 0.25 N hydrochloric acid (HCl)) was added to two volumes of the homogenate. The samples were incubated at a water bath at 95°C for 15 minutes and then chilled in ice. In the end, the samples were centrifuged at 1000 g for 10 minutes. The absorbance of the supernatant was measured by a spectrophotometer (PG Instruments T80 UV/VIS, UK) at 535 nm. The amount of MDA was calculated using its extinction coefficient (1.56×10^5 $M^{-1}Cm^{-1}$) and expressed as nmol/gr.

Assessment of Total Antioxidant Capacity (FRAP Method)

To measure the total antioxidant capacity in the spinal cord slices, the ferric reducing antioxidant power (FRAP) method, which is based on the ability of the tissue extracts to convert Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri (2-pyridyl)-1,3,5-triazine (TPTZ), was performed according to the method described by Benzie [22]. Briefly, 0.1 gr spinal cord slices were homogenized in KCl solution (1:9). Then, 50 µl of the homogenate was added to 1.5 ml of freshly prepared FRAP solution, including the acetate buffer, ferric chloride, TPTZ solution, distilled water, and standard iron sulfate solutions, in a dark place. The mixture was incubated in a water bath at 37°C for 4 minutes, and then, the absorbance was measured by a spectrophotometer (PG Instruments T80 UV/VIS, UK) at 593 nm. Different concentrations of ferrous sulfate were used for drawing the standard curve. The FRAP levels were computed using a regression equation ($y= 1.2855X + 0.0075$, $R^2= 0.9981$) obtained from the standard curve and expressed as µmol/gr.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to assess the statistical differences among means. A $p<0.05$ was considered significant.

Results

Extracted Artichoke Flavonoids

Results of two-dimensional paper chromatography showed Aglycons, Flavon *C* & *C*-/*O* glycosides, and flavonoid Sulfates in aerial part samples of artichoke. In addition, TLC data displayed the number of flavonoids such as Silymarin, Apigenin, Luteolin, Rutin, Quercetin, Naringenin, Kaempferol, and Myricetin in artichoke extract (Table 1).

Table 1: Ethanolic flavonoids of Artichoke

Artichoke flavonoids	TFN	AN	Fl C&C-/O N	FlSu N	Si	A	L	Q	Rh	M	Ru	Na	K	My
Number	8	1	4	3	+	+	+	+	-	-	+	+	±	+

TFN: Total Flavonoids Number, AN: Aglycons Number, Fl C&C-/ON: Flavon C & C-/O glycosides Number, FlSuN: Flavonoid Sulfates Number, Si: Silymarin, A: Apigenin, L: Luteolin, Q: Quercetin, Rh: Rhamnetin, M: Morin, Ru: Rutin, Na: Naringenin, K: kaempferol, My: Myricetin.

Evaluation of Motor Neurons Apoptosis

In freshly prepared slices (0 hour), the motor neurons showed large cell bodies, large nuclei, and the expected distribution of nuclear material, and no apoptotic signs could be observed within the motor neurons (Figure 1A). After 6 hours in the culture medium, many motor neurons displayed morphological features of apoptosis, including nuclear and chromatin condensation as well as cell shrinkage (Figure 1B). At this time point, the administration of silymarin (96.88µg/ml) (Figure1C) and artichoke flavonoids (10 µg/ml) (Figure1D) could considerably delay the appearance of nuclear apoptotic changes in motor neurons. In addition, slices cultured for 6 hours (control group) showed a significant ($p<0.001$) decrease in the diameter of motor neurons nucleus compared to the motor neurons from freshly prepared slices (slices at 0 hour). Administration of silymarin or artichoke flavonoids could significantly ($p<0.001$) increase the nucleus diameter of the motor neurons as compared with the control group (Figure 2).

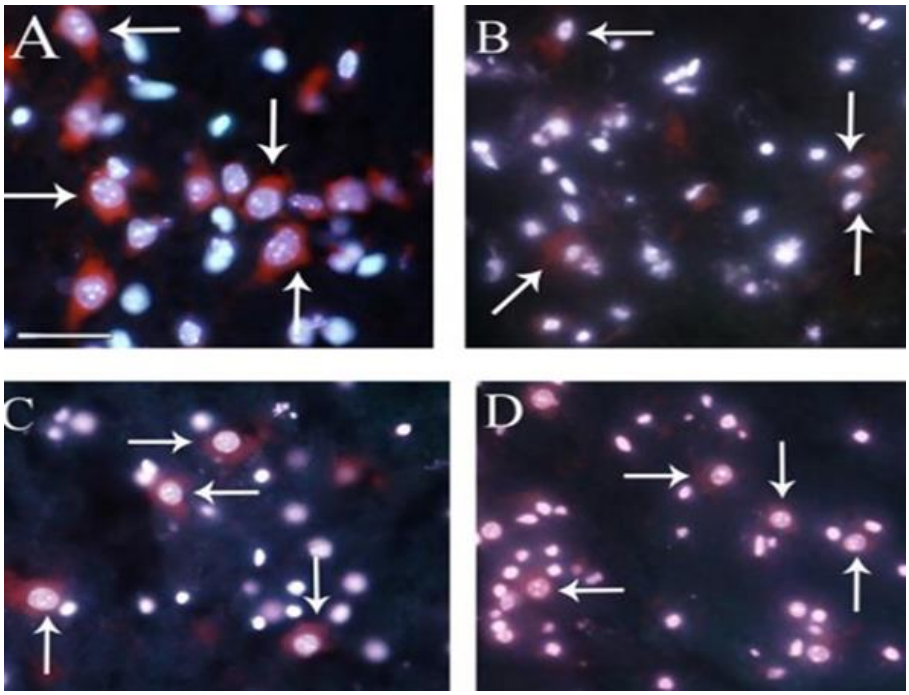

Figure 1: Neuroprotective effect of silymarin and artichoke flavonoids on apoptosis of motor neurons of spinal cord slices: A) motor neurons from freshly prepared slices (0 hour), B) motor neurons from slices cultured for 6 hours (control), C) motor neurons from slices treated with silymarin (200 µM) and (D) motor neurons from slices treated with artichoke flavonoids (10 µg/ml) for 6 hours. Arrows show motor neurons. Scale bar: 20 µm.

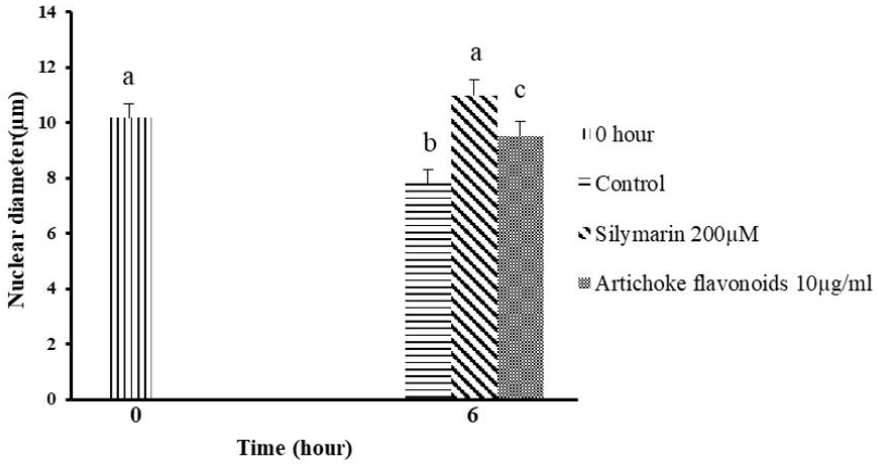

Figure 2: Effect of silymarin (200 µM) and artichoke flavonoids (10 µg/ml) on nucleus diameter of motor neurons from the spinal cord slice cultured for 6 hours. Data are presented as Means ±SD. Means with the same letters do not differ significantly (n=6, $p<0.05$)

Evaluation of Lipid Peroxidation

The levels of MDA in slices cultured for 6 hours (control group) were significantly ($p<0.05$) increased as compared with the freshly prepared slices (0-hour). At this time point, the application of silymarin (96.88µg/ml), could significantly ($p<0.001$) compensate for the levels of MDA compared to the control group. However, artichoke flavonoids (10 µg/ml) had no significant effect on MDA levels after 6 hours in comparison to the control group (Figure 3).

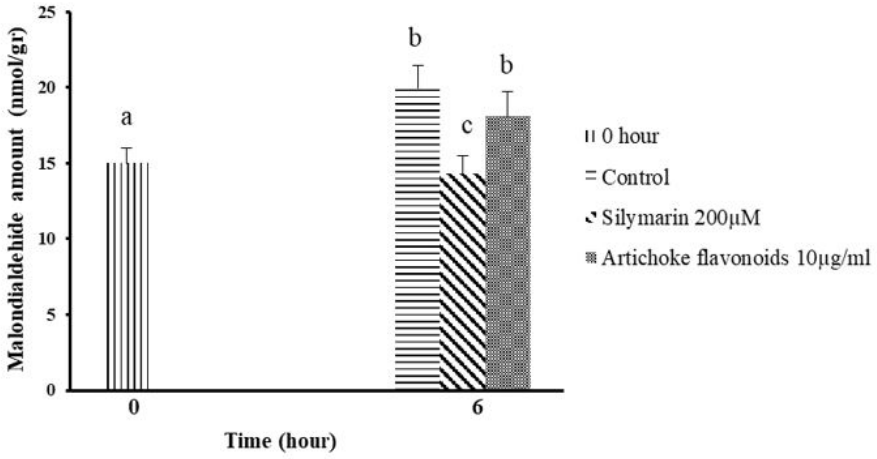

Figure 3: Effect of silymarin (200 µM) and artichoke flavonoids (10 µg/ml) on malondialdehyde levels of spinal cord slices. Data are presented as Means ±SD. Means with the same letters do not differ significantly (n=6, $p<0.05$).

Evaluation of Total Antioxidant Capacity

In the control group, the levels of FRAP were significantly ($p<0.001$) reduced after 6 hours compared to the freshly slices (0 hour). In slices exposed to silymarin (96.88µg/ml), but not artichoke flavonoids (10 µg/ml), silymarin could significantly ($p<0.001$) increase the FRAP levels compared to the control group (Figure 4).

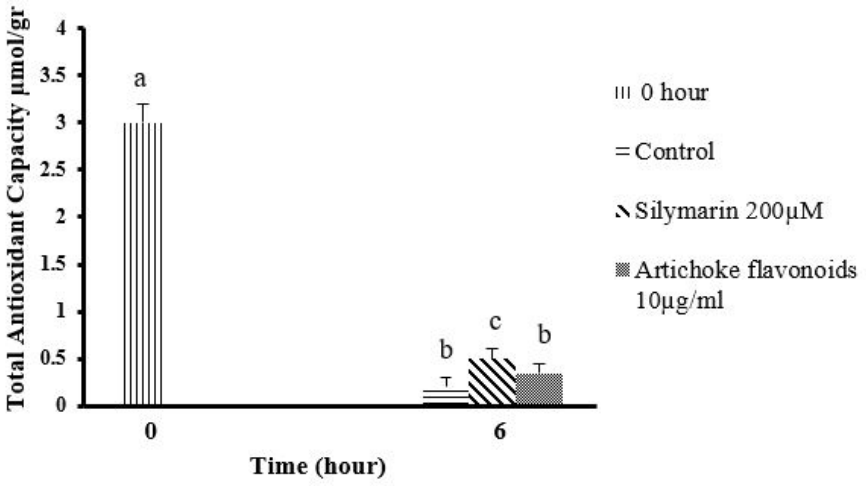

Figure 4: Effect of silymarin (200 µM) and artichoke flavonoids (10 µg/ml) on total antioxidant capacity (FRAP) of spinal cord slices. Data are presented as Means ±SD. Means with the same letters do not differ significantly (n=6, $p<0.05$).

Discussion

In this study, the culture of spinal cord slices from adult mice was used to study one of the mechanisms involved in apoptosis of the motor neurons. The results showed that oxidative stress could be the possible cause of apoptosis in the motor neurons.

Organotypic culture of adult mice spinal cord slices is a popular in vitro system not only for evaluating neuronal survival and cell death [23], but they could also be used for experimental studies of ALS and SCI [24]. Apoptosis cell death has been linked to numerous pathological conditions such as SCI and neurodegenerative disorders [25]. During apoptosis, cells undergo some sort of biochemical and morphological changes including cell shrinkage, nuclear and chromatin condensation, and membrane blabbing

[26]. In this study, we demonstrated that after 6 hours in culture medium, the motor neurons display such morphological features of apoptosis, which was consistent with our previous report [27]. Apoptosis as a complex process is induced in neurons by multiple intrinsic and extrinsic signals such as activation of caspases [28] and calpain [29], mitochondrial dysfunction [30], calcium dysregulation [31], and oxidative stress [32]. Oxidative stress-induced apoptosis has been reported in neurodegenerative diseases in numerous studies. Oxidative stress via an increase in free radical's generation and/or disruption in antioxidant defense system damages the proteins, DNA, membrane lipids and the organelles such as mitochondrial and induces neuronal apoptosis [33].

It is well documented that reactive oxygen species (ROS) play an important role in the induction of apoptosis signals in the mitochondria pathway. Oxidative stress exerted by excessive cellular levels of ROS may trigger the opening of the pores in the inner mitochondrial membrane, releasing cytochrome c and other apoptogenic proteins such as apoptosis-inducing factor (AIF) [34]. The release of cytochrome c is a key event in the activation of caspase cascades. Activated effector caspases disrupt many cytoskeletal and membrane substrates involved in maintaining cell and membrane integrity, leading to cell shrinkage [35]. Other potential targets of caspases are structural proteins in the cell nucleus. The lamins, which are located on the inner side of the nuclear membrane and are responsible for nuclear integrity, undergo proteolysis during apoptosis. Loss of nuclear integrity due to the degeneration of lamins leads to nuclear and chromatin condensation. On the other hand, released AIF from mitochondria is translocated into the nucleus to induce chromatin condensation and high molecular weight DNA fragmentation, which ultimately leads to apoptosis in a caspase-independent manner [36]. Additionally, due to the high amount of unsaturated fatty acids, the mitochondrial membrane is prone to damage by free radicals, which can alter the mitochondrial membrane potential and disrupt ATP production, leading to the intrinsic death signals induced by oxidative stress [37].

A logical association has been proven between oxidative stress and the induction of neuronal apoptosis [38]. Accordingly, oxidative stress could be a possible cause involved in the apoptosis of motor neurons. Thus, in this study, it is possible to assume that oxidative stress through direct and/or indirect pathways induced morphological changes of apoptosis in the motor neurons. If this hypothesis is true, the application of an antioxidant could prevent the adverse effects of oxidative stress on these neurons by scavenging free radicals and strengthening the antioxidant defense system. Several studies demonstrated the protective effects of silymarin and artichoke flavonoids on oxidative stress-induced neurodegenerative diseases. Silymarin is reported as a potential anti-oxidative and anti-apoptotic target in cerebral stroke [39] In addition, the artichoke flavonoids reduce the free radical's generation and increase the level of cellular glutathione and protects neurons against oxidative stress in neurodegenerative disorders [40].

Interestingly, our results have also illustrated that the application of silymarin, as a potent antioxidant, and the flavonoids extracted from artichoke containing silymarin could delay the morphological features of apoptosis in the motor neurons. The reversed changes in the level of MDA and FRAP, as oxidative stress indicators, by silymarin could further support our hypothesis about the compensated effects of silymarin for oxidative stress. Although artichoke flavonoids had no significant effect on the levels of MDA and FRAP, but their effects were considerable on neuron apoptosis. This may be due to the lower amounts of silymarin in artichoke flavonoids compared to pure silymarin. Therefore, the similarity between the results of silymarin and artichoke might be elucidated that the effects caused by artichoke flavonoids were due to the existence of silymarin in the extracted flavonoids.

Conclusion

Silymarin and artichoke flavonoids could delay the morphological feature of apoptosis in motor neurons of cultured spinal cord slices. In addition, silymarin not only could decrease lipid peroxidation but also improved total antioxidant capacity in the motor neuron. This study could suggest the application of silymarin to strengthen the antioxidant capacity of the culture media in order to improve the quality of motor neurons in pathophysiological studies. In addition, considering the potential positive effects of silymarin, it can use in some human disorders to reduce the adverse effects of oxidative stress, although this hypothesis can prove after more experimental studies.

Author Contributions

Hamid Reza Momeni contributed to the study conception and design and reviewing the manuscript. The first draft of the manuscript was written by Tahereh Etemadi. Experimental works were performed by Farzaneh Moshaveri. Some part of the manuscript was written by Mitra Nori. She also contributed to the statistical analysis of the data. All authors read and approved the final manuscript.

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