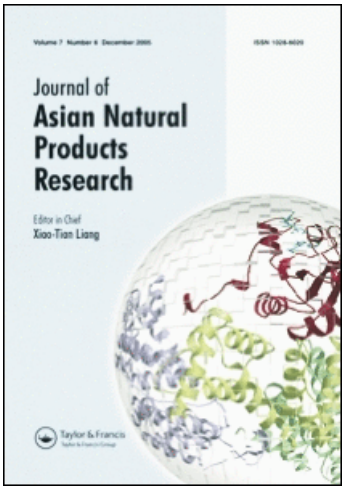

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The effect of silybin on passive avoidance learning and pathological changes in hippocampal CA1 and DG regions in male Wistar rats offspring

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Silybin, an extract from seeds of milk thistle (*Silybum marianum*), is known to have hepato-protective, anticarcinogenic, and estrogenic effects. Given that estrogen effects on memory have been reported, silybin may cause structural changes in the hippocampal CA1 and dentate gyrus (DG) neurons and as a result it may enhance learning and memory. Wistar rats were provided with silybin (from day 7 of gestational age up to 4 weeks after birth) with 2 dosages of 18 mg/kg in the experimental group 1 (Exp1) and 9 mg/kg in the experimental group 2 (Exp2). Offspring memory retention was compared by duration of step-through latency in passive avoidance apparatus. Furthermore, histological changes were investigated in experimental groups and control group (CG). Both the experimental groups showed significantly longer step-through latency than CG ($p < 0.001$ for Exp1 and $p < 0.01$ for Exp2). The average number of pyramidal cells in hippocampal CA1 and granular cells in hippocampal DG was remarkably higher in Exp1 and Exp2 compared with CG. The difference was significant between Exp1 and Exp2 for pyramidal cells ($p < 0.05$) but not for granular cells. Silybin administration during pregnancy resulted in histological changes in hippocampus and better memory function. These data may lay the ground work using silybin in memory impairment diseases.

Keywords: silybin; passive avoidance learning; Wistar rat; hippocampus; CA1; dentate gyrus

1. Introduction

Silybum marianum, commonly known as "Milk thistle" (Family: Asteraceae/Compositae), is originally a native of southern Europe through to Asia, and it is now found throughout the world. The medicinal parts of the plant are the ripe seed. Silymarin is comprised of six major flavonolignans: silybin (A and B isomers), isosilybin (A and B), silychristin, and silydianin. Silybin is its major constituent (50–70%; Figure 1). Antioxidant [1], anti-inflammatory/immunomodulatory [2], and antifibrotic [3] properties of silymarin and its flavonolignans have been demonstrated in various *in vitro* and animal models.

Intensive attention has been paid to the estrogenic effects (non-steroidal estrogen, phytoestrogens) of silybin. Similarity of silybin chemical structure with that of steroid allows it to bind and activate estrogen receptor (ER) of mammalian target cells. Its activity has been reported

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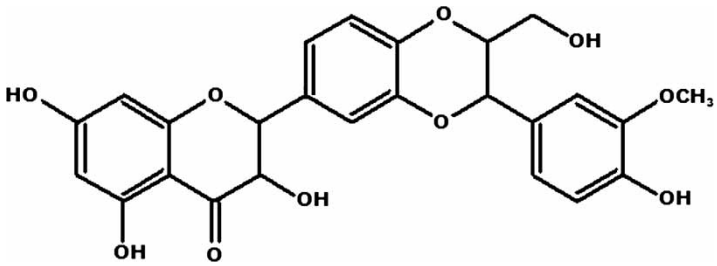

Figure 1. Structure of silybin.

to be near 10^{-2} of 17β-estradiol biological activity [3,4]. The structural similarity of silybin to steroid hormones is believed to be responsible for protein synthesis [5]. Silybin can enter inside the nucleus and act on RNA polymerase enzymes resulting in the increasing of ribosomal formation [6]. This in turn hastens protein and DNA synthesis [5]. Silybin B is probably responsible for a majority of this activity [4]. On the other hand, estrogen effects on memory have been reported in animal models and in studies on humans [7,8]. Estrogen treatment increases dendritic spine density on CA1 pyramidal neurons [9].

Both silymarin and silybin have been reported to have neuroprotective and neurotropic activities [10]. Several mechanisms of their cytoprotective effects in central nervous system have been identified but other mechanisms are still unknown. It has been shown that silymarin inhibits nuclear factor kappa B activation and reduces production of inducible nitric oxide (NO) synthesis [10]. In addition, silybin showed to have protective effects on primary hippocampal neurons in oxidative stress conditions that induce apoptosis and neuromodulatory action in persistent viral infections leading to encephalitis [11]. Further on, an extract from silymarin enhances the differentiation of cultural neural cells (i.e., rat PC-12 pheochromocytoma cell line). Moreover, the extract prevents apoptosis following nerve growth factor withdrawal and protects primary hippocampal neurons against oxidative stress-induced apoptosis [12].

It is now clear that hippocampus plays a central role in many memory formation processes, including spatial learning, locating objects in the environment and consciously recalling facts, episode, and unique events [13]. Learning needs some instruments for information storage and information maintenance mechanisms which resemble memory. On the other hand, memory is always accompanied by learning. The dentate gyrus (DG) and CA1 are two important parts of hippocampal formation. The DG contains granule cells that are the principal excitatory neurons of the DG.

Given that silybin has been described to be an antioxidant with anti-inflammatory, estrogenic, and cytoprotective effects, we hypothesized that silybin may cause structural changes in the hippocampal CA1 pyramidal neurons and granule cells in DG. As a result it may enhance learning and memory.

2. Results and discussion

The time rats spent in light compartment before passing the guillotine door of passive avoidance apparatus (step-through latency) was compared between the experimental groups and control group (CG). The step-through latency of rats of experimental group 1 (Exp1; offspring of mothers treated with 18 mg/kg of silybin) was significantly more than CG (125.2 ± 14.1 s for Exp1 compared with 24.9 ± 12.3 s for CG, $p < 0.001$). In a similar manner, it takes significantly more time for offspring of rats treated with

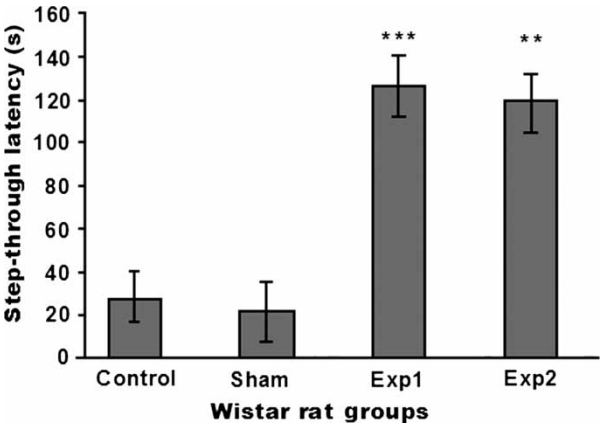

Figure 2. Step-through latency in four groups. Treatment with high dose of silybin has caused an increase in the duration of step-through latency which indicates an increase in memory retention. Exp1, offspring treated with 18 mg/kg silybin during gestational age and then until 4 weeks of age. Exp2, offspring treated with 9 mg/kg silybin during gestational age and then until 4 weeks of age. Sham, offspring of rats treated with saline. Control, intact group. $***p < 0.001$; $**p < 0.01$.

9 mg/kg of silybin to pass the guillotine door and enter the dark compartment (118.6 ± 15.3 s for experimental group 2 (Exp2) compared with 24.9 ± 12.3 s for CG, $p < 0.01$; Figure 2). Furthermore, the time of step-through latency was significantly higher in Exp1 compared with Exp2 ($p < 0.05$; Figure 2).

The histological changes were examined to assess the potential effects of administration of silybin to pregnant rats and then to their offspring on pups' hippocampus (Figures 3 and 4). The average number of pyramidal cells in hippocampal CA1 in the CG was 99.2 ± 1.2 per 100 μm compared with 136.2 ± 2.7 in Exp1 and 118.9 ± 2.2 in Exp2. This difference was significant for both Exp1 ($p < 0.01$) and Exp2 ($p < 0.05$; Figures 3 and 5(A)–(D)). In addition, the difference was significant when Exp1 compared with Exp2 ($p < 0.05$).

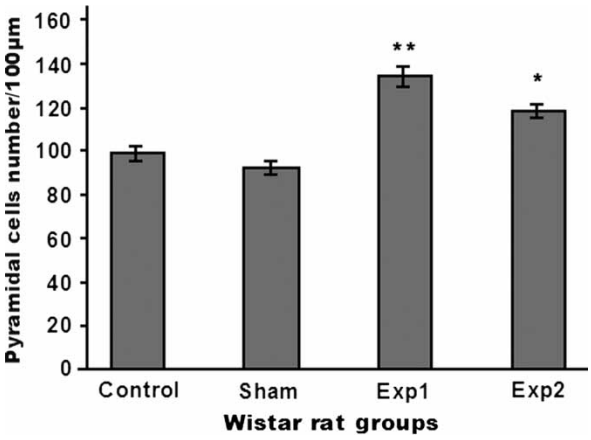

Figure 3. Treatment with silybin has increased pyramidal cells of CA1 hippocampus. Exp1, offspring treated with 18 mg/kg silybin during gestational age and then until 4 weeks of age. Exp2, offspring treated with 9 mg/kg silybin during gestational age and then until 4 weeks of age. Sham, offspring of rats treated with saline. Control, intact group. $**p < 0.01$; $*p < 0.05$.

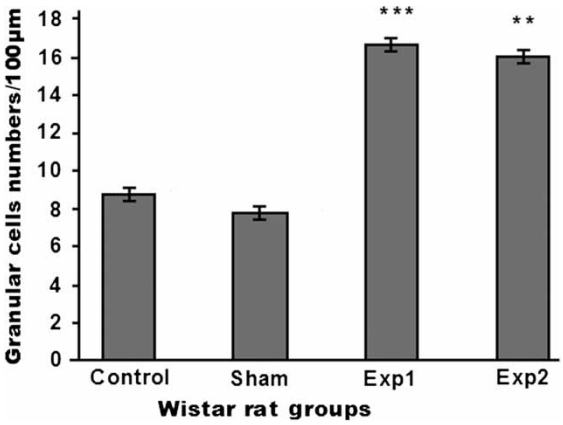

Figure 4. Treatment with silybin has increased granular cells in hippocampal DG. Exp1, offspring treated with 18 mg/kg silybin during gestational age and then until 4 weeks of age. Exp2, offspring treated with 9 mg/kg silybin during gestational age and then until 4 weeks of age. Sham, offspring of rats treated with saline. Control, intact group. $^{**}p < 0.01$; $^{***}p < 0.001$.

The average number of granular cells in CGs hippocampal DG was 8.9 ± 0.25 (per 100 μm). In both Exp1 and Exp2, granular cells were remarkably higher compared with CG [16.3 ± 0.34 cells per 100 μm in Exp1 ($p < 0.001$) and 15.1 ± 0.26 cells per 100 μm in Exp2 ($p < 0.01$)] (Figures 4 and 5(A),(B),(E), and (F)). The difference was not significant between two experimental groups.

Hippocampus plays a central role in many memory formation processes, including spatial learning, locating objects in the environment, and consciously recalling [13–16]. It is reported that hippocampal CA1 lesions produce a deficit in the acquisition of the water maze task and significant memory impairment on the passive avoidance task [13,14]. Moreover, learning and memory deficits have been observed following granule cell degeneration in DG [15]. Further on, enhancement of these two parts of hippocampus has been shown to increase spatial memory function and step-through latency [16].

This study showed that administration of silybin from day 7 of pregnancy in Wistar rat and then until 4 weeks of age to the offspring causes higher nerve cell density in hippocampal CA1 and DG. Furthermore, it enhances step-through latency in offspring. These data support one another, and they are in line with several other investigations. First, Bayer [17] pointed out that days 13–19 of fetal life are the principal time of neurogenesis in the hippocampus. Second, silybin has been shown to cross the placental barrier [18,19]. Moreover, Reid *et al.* [18] compared the social recognition scores in 90 days of age of pups whose mothers received ethanol with others who received ethanol in addition to silymarin during pregnancy. Social recognition scores recorded for the ethanol pups were significantly poorer than those observed in silymarin/ethanol group. At the end, as reported by Neese *et al.* [19], addition of silybin to the ethanol liquid diet of pregnant rats appears to ameliorate the ethanol-induced learning deficits.

The present study does provide for the first time evidences that perinatal administration of silybin (from day 7 of pregnancy until 4 weeks of age) in Wistar rats can improve pups' memory retention, and it may be related to the structural changes in hippocampus generated by silybin. The mechanisms have not been fully understood. However, neutralization

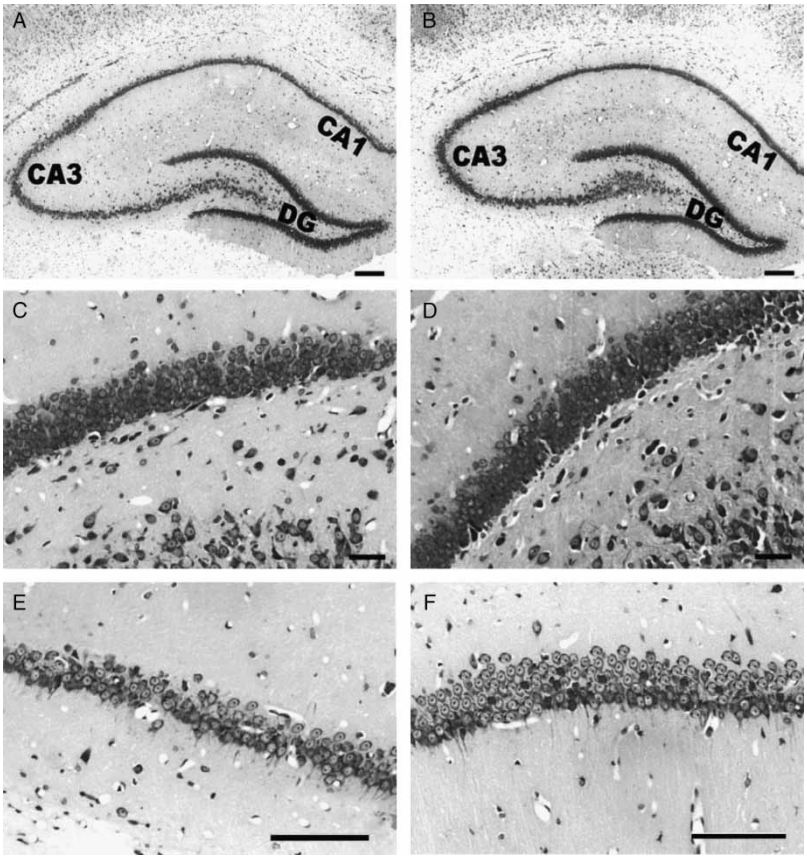

Figure 5. Distribution of nerve cells in the rat hippocampus of the silybin treated and CG. (A) Rat hippocampal nerve cells in CG. (B) Rat hippocampal nerve cells in Exp1 group (administration of 18 mg/kg silybin during gestational age and then until 4 weeks of age). (C) Rat hippocampal granular cells in DG of the CG. (D) Rat hippocampal granular cells in Exp1 group. (E) Rat hippocampal pyramidal cells in CA1 of CG. (F) Rat hippocampal pyramidal cells in CA1 of Exp1 group. H&E staining, scale bar is 100 μm for (A) and (B), 25 μm for (C) and (D), and 100 μm for (E) and (F).

of free radicals and replenishing the glutathione levels by silybin may be involved. These mechanisms are specially effective in oxidating conditions. These are more important in the brain and nervous system according to their more susceptibility to free radical damage than other tissues [20]. According to the recent findings, the previous theory that an increase in NO during the critical period of brain maturation may have good long-lasting consequences on the organization of the adult brain has been falsified [21]. In contrast, excessive NO production from neuroglial cells in the brain has been correlated with neurotoxicity. It has been shown that silybin reduces NO release from astrocyte cells [22]. The other mechanism that can explain silybin effects on hippocampus structural changes can be the increases in protein synthesis by silybin which results in promotion of regenerating processes [5,6]. Additionally, silybin stabilizes cell membranes. It can occupy receptors on the cell membrane and therefore cause a partial blocking of immunologic reactions and can protect cell membrane by conserving glutathione

in the cells [23]. Furthermore, possible binding of silybin to an estradiol-binding site situated on a subunit of RNA polymerase-I, which would stimulate the synthesis of ribosomal RNAs, may restore structural proteins and damaged enzymes in the cell's nucleus [4].

It is well accepted that estrogen has positive effects on memory and learning. Recent data demonstrate that acute and chronic estrogen deprivation disrupts dynamic synaptic plasticity processes in the hippocampal CA1 region and this disruption is ameliorated by chronic estrogen replacement [24]. *In vitro* and animal studies have shown that estrogens possess antioxidant activity, protect cells from the cytotoxic effect of β-amyloid peptides, and decrease the amyloidogenic processing of the amyloid precursor protein. Estrogen also increases acetylcholine and dendritic spine density on CA1 pyramidal neurons in the hippocampus within 24–72 h after acute administration [9], and it can induce cyclic changes in synaptic formation and spine density of the hippocampus [9,25]. Furthermore, presence of ERs immunoreactivity in hippocampus and its role in behavior have been proved [26]. Recently, a growing body of evidences focused on the estrogenic effects of silymarin and silybin [4,5,27]. Silybin B is probably responsible for a majority of the ER-mediated activity of silymarin [4]. Ziolkowska *et al.* [27] investigated the effects of silymarin on the secretory and proliferative activity of rat adrenocortical cells *in vitro*. They reported that 24-h exposure to silymarin (10^{-10} and 10^{-8} M) lowers basal but not ACTH-stimulated corticosterone secretion. The present study for the first time demonstrated that administration of silybin can enhance memory and learning in Wistar rat offspring. This can be strongly related to its estrogenic effects. Further investigation is needed to assess this theory. Whether this effect is brought about by silybin's estrogenic effects or its direct effect on hippocampal or due to other mechanisms remain to be elucidated experimentally.

In this study, silybin was continuously administered until 4 weeks of age to the pups, i.e., the experimental rats were under influence of silybin for a period of 6 weeks. It is well known that aging can reduce function and the number of pyramidal cells in CA1 and granular cells in DG [28]. This study showed a significant difference between these cell counts in the experimental groups that received silybin and CG. According to Kempermann *et al.* [29], the newly generated cells in the DG and CA1 must take four important steps to become functional neurons, including differentiation, maturation, migration, and formation of new synapses. As silybin administration causes the increase in step-through latency in experimental groups, we concluded that silybin enhanced all of these steps. In other words, the new generated cells were functional and enhancement of hippocampal neuronal dendritic arborization by silybin was predictable. Since it is established that the newly generated cells in DG need 4 weeks to perform the aforesaid four steps, the recovery of passive avoidance learning function accruing in the 5th week in this study is consistent with established findings [29].

Nowadays, silymarin is successfully used in hepatic diseases and certain kinds of cancer. The presented data may lay the ground work using silybin for memory impairment diseases such as Alzheimer's disease. Further investigations are required to prove this theory.

3. Experimental

3.1 Animals

Twenty-four female Wistar rats and 12 male rats were brought from Pasteur Institute of Iran. They weighed 200–250 g. Animals were maintained in a 12-h light/dark cycle at 22–24°C with air humidity nearly 60% and had free access to standard pellet food and tap water.

Two female rats were housed with one male rat per cage. Observation of vaginal plug was assumed as "day 1" of pregnancy. Pregnant rats were divided into four groups, two experimental groups, a sham group and a CG, and kept in different cages. Subsequently, pregnant rats of experimental groups received silybin (18 mg/kg per day: Exp1 and 9 mg/kg per day: Exp2) from day 7 of pregnancy throughout the gestational period. Each female had 8–16 births and 4–9 of them were male. Twenty male pups were selected randomly and kept with birth mothers until separation. Daily intake of liquid diet across mothers was 70 ml ± 7 ml/day. They received silybin as follows during this period:

- Group 1 (Exp1): Oral administration of 18 mg/kg of silymarin ($n = 20$).
- Group 2 (Exp2): Oral administration of 9 mg/kg of silymarin ($n = 20$).
- Group 3 (sham): Oral administration of saline ($n = 20$).
- Group 4 (control): Received standard rodent diet and tap water.

Once the pups reached 21 days of age they were weaned and placed in an individual standard laboratory cage. Subsequently, the treatment of silybin in addition to standard food and diet continued until 4 weeks of age.

The tested substances were administered by gavage dissolved in saline. The animals were firmly restrained (they were grasped by the loose skin of the neck and back) to immobilize the head and maintained in an upright (vertical) position. The gavage needle was passed through the side of the mouth, and advanced towards the esophagus into the stomach. After the needle was passed to the correct length, silybin was injected.

3.2 Passive avoidance apparatus

The passive avoidance apparatus consisted of a light (plexiglass) and dark (black) compartment of the same size (20 × 20 × 30 cm each), separated by a guillotine door (7 × 9 cm). The floor of the dark compartments was made of stainless-steel rods (2.5 mm diameter) separated by a distance of 1 cm. Intermitted electric shock (50 Hz, 1 mA, and 5 s) was delivered to the gird floor of dark compartment from an insulated stimulator.

3.3 Training

The rats were allowed to habituate to the laboratory environment and apparatus for 1 h before each of the training and testing experiments done at the age of 5 weeks. All the tests were conducted between 08:00 and 14:00 h. Each animal was gently placed in the light compartment for 5 s, after which the guillotine door was lifted and the latency with which the animal crossed to the dark (shock) compartment was timed. If an animal waited more than 300 s to cross the other side, it was eliminated from the experiment. Once the animal crossed with all four paws to the next compartment, the door was closed and the rat was taken into the home cage. The habituation trial was repeated after 30 min and followed by the same interval by the acquisition trial during which the guillotine door was closed and a foot shock (50 Hz, 1 mA, and 5 s) was delivered immediately after the rat entered the dark compartment. After 20 s, the rat was removed from the apparatus and placed temporarily into home cage. Two minutes later, the rat was re-tested in the same way as before, if the rat did not enter the dark compartment in 120 s, successful acquisition of a passive avoidance response was recorded. Otherwise, when the rat entered the dark compartment, again the door was closed and the rat received the same shock as above.

3.4 Retention test

Twenty-four hours after training, a retention test was performed to evaluate

long-term memory. Each animal was placed in the light compartment for 5 s, the door was opened and the step-through latency for entering into the dark compartment was measured. The test session was ended when the animal did not enter the dark compartment after 300 s (criterion for retention). During these sessions, no electric shock was applied. Increase or decrease in step-through latencies indicated an increase or decrease in memory retention, respectively.

3.5 Histology

At the end of experiments, animals were decapitated with ether anesthesia and the brain removed for histological assessments. First, the brains were fixed in 10% formalin and 2 weeks later processed them for embedding with paraffin, after embedding serial sections in 7 μm of thickness were taken. For staining of pyramidal and granular cells, hematoxylin and eosin method was used. Morphometric measurement was carried out with an Olympus Dp 12 digital Camera and BX 51 microscope. Seven microscopic fields in a random process were selected from each animal. The person who counted the cells was blind to experimental procedures of this research.

3.6 Drugs

Silybin was purchased from Darou Pakhsh Company (Tehran, Iran). Its purity was measured by radiochemical high-purity liquid chromatography, and determined to be $>98\%$. Because of poor solubility, a suspension of silymarin was prepared for intra-gastric administration [30].

3.7 Data analysis

Analysis of variance followed by Tukey constant test was used to evaluate the data. The criterion for statistical significance was $p < 0.05$.

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