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MATERNAL EXPOSURE TO SILYMARIN LEADS TO PHATOLOGICAL CHANGES IN MOUSE FOETUSES

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Abstract

Silymarin, a polyphenolic flavonoid extract, is obtained from the seeds and fruits of *Silibum marianum*. It has shown to possess anti-oxidant, anti-inflammatory and anti-carcinogenic properties. The aim of this study was to investigate the pathological effects of silymarin on Balb/c mouse embryonic heart, kidneys, lungs and cerebral.

Timed-pregnant mice received one of three doses of silymarin (50, 100 and 200 mg/kg/day) or the vehicle control during organogenesis, intraperitoneally. Then pregnant mice were sacrificed under deep anaesthesia and a Caesarean section was performed. Foetuses were removed and their brain, lungs, kidneys and heart were evaluated histopathologically.

Our findings showed that silymarinin pregnant mice caused apoptosis and oedema in foetal cells of brain in silymarin treated groups with maximal effect seen in higher dose. It also caused cardiac congestion and induced immaturity and infiltration of inflammatory cells in lungs and kidneys.

Silymarin administration to pregnant mice has pathological effects on foetal brain, kidneys, heart and lungs and the teratogenic effect of it should be considered on human.

Keywords: Apoptosis, Mice, Silymarin, Teratogen.

Introduction

Silibum marianum is a medicinal plant in Asteraceae family. It is native to the Mediterranean countries and was grown in southern Europe as a medicinal crop. Seeds of milk thistle have been used from ancient times to treat liver diseases and increasing breast milk production [1, 2].

Silymarin, a polyphenolic flavonoid extract, is obtained from the seeds and fruits of *Silibum marianum* and is largely responsible for the medical benefits attributed to this family. It is a mixture of three isomers including silybin (most prevalent and active component), isosilybin, silydianin and silychristin [3]. Silymarin has been shown to have an antioxidant [4] and anti-inflammatory activities [5] and is useful in treatment of chronic liver diseases and hepatic cirrhosis [6] in animal and human studies. Recently, silymarin and silybin has been used as a chomoprotective and anticancer agents [4]. Due to its antioxidant action, silymarin can also be valuable in the treatment and prevention of neurodegenerative, neurotoxic, gastrointestinal and cardiopulmonary problems [4-6].

Silibum marianum are prepared in a variety of forms. Due to poor water solubility, aqueous extracts or decoction is ineffective form of delivery. Most preparations are standardized to contain 70 – 80% of silymarin [6]. Complexion with phospholipids can improve its absorption and bioavailability [7-9].

Silymarin is capable of passing through the placental barrier and produce measurable amounts in fetal tissue [10]. The results of some investigations revealed that silymarin can provide some protection of fetal brain and liver from maternally ingested EtOH (ethyl alcohol) [7,8]. But there is not enough information available to support its safety in pregnancy. In our previous investigation we found that silymarin, especially in high doses can cause fetal resorption, intrauterine growth retardation and some kind of malformations (Submitted data).

The goal of this study was to investigate the pathological effects of silymarin on Balb/c mouse embryonic heart, kidneys, lungs and cerebral.

Methods

Animal and treatment

This experimental study was carried out on 24 virgin female BALB/c mice weighting 20-30 g that were approximately two months old. They were obtained from the Avicenna Research Institute, Mashhad, Iran. The mice were kept in 12-hour light/dark cycles, room temperature of 23±2 ˚C and had unlimited access to food and water. All the animal experiments were approved by the Animal Care and Ethics Committee of Mashhad University of Medical Sciences.

One male was caged with two females overnight and they were observed for the presence of a vaginal plug on the next morning. The presence of vaginal plugs has been considered as gestational day zero (GD0).The mice were divided randomly into four groups. Three groups of pregnant mice were intraperitoneally (IP) injected with silymarin at doses of 50, 100 and 200 mg/kg/ day (group I, II and III), during GD6–15 (organogenesis period). The control group received normal saline plus tween by the same route in an equivalent volume.

Tissue preparation and histopathological examination

On GD18, The pregnant mice were sacrificed under deep anaesthesia and a Caesarean section was performed. Foetuses were removed from the uterus and after cutting the umbilical cord and horizontal incision in the neck, a vertical incision was made in the skull bone embryos. Foetal brains were immediately removed and then the internal organs including heart, kidneys and lungs were isolated. The tissues were fixed in 10% formalin. After fixation and tissue processing, paraffin blocking were done. Five micron sections were prepared and stained with haematoxylin - eosin. Histopathological examination was done under a light microscope (Olympus, CX31, Japan), with 40X magnification in 10 fields on each slide. The tissue lesions were scored semi-quantitatively as following: 0: No lesion, 1: Lesion up to 25% of observed microscopic fields (mild), 2: Lesion in 25-75% of observed microscopic fields (moderate) and 3: lesion in more than 75% of observed microscopic fields (sever).

Statistics

Data was reported as mean±SEM. The pathological scores were compared using Kruskal–Wallis ANOVA test followed by Dunn's multiple comparison test. SPSS 18.0 was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

Results

Histopathological findings in different groups were expressed in each tissue separately (Table 1).

Brain

Histopathological changes of brain tissues are presented in Table1. All findings were histologically normal in the control group.

Histological changes were observed in Silymarin treated groups. Silymarin induced moderate brain injury including oedema and neuronal cell death (Figure 1). The brain lesions were significantly increased in the Silymarin treated groups compared to control group. Silymarin induced brain damage in a dose dependent manner.

Heart

The histopathological finding in silymarin treated groups was congestion. It was significantly higher in 50, 100 and 200 mg/kg groups compared to control group ($P < 0.001$ and $P < 0.01$). There was no significant difference between 50 and 100mg/kg groups.

Lungs

Histological examination revealed infiltration of inflammatory cells and immaturity after the silymarin exposure. No detectable histological signs of lung inflammation or immaturity were found in control group. The immaturity was significantly higher in silymarin treated groups compared to control**.** However, infiltration of inflammatory cells was statistically significant only in the higher-dose compared with control group ($P<0.001$).

Kidneys

Inflammatory cells and immaturity were also detected in kidneys. There was a significant difference between silymarin treated and control groups in immaturity ($P<0.01$ and $P<0.001$, respectively), but no significant difference between 100 mg/kg vs 50 mg/kg was observed ($p=0.002$). Inflammatory cells were not observed at 50 and 100 mg /kg doses. The pathological score was significantly higher in the 200 mg/kg group ($P<0.001$). The mouse kidney programme cell death has been shown in Figure 2.

Discussion

In the current study, we investigated the effects of silymarin administration on various organs (brain, kidneys, lungs and heart) of mice foetuses in different doses. Silymarin inhibits the cyclooxygenase enzyme by decreasing prostaglandin synthesis [11] and possesses non-steroid anti-inflammatory drugs (NSAIDs) properties [12]. Considering the limited number of studies investigating the effects of silymarin on the developing quantitative lung growth. In this study, supplementation with 1.0 g of prostaglandin E2 per 100 g foetus and with attention to NSAIDs-like properties, we aimed to compare the results of NSAIDs developmental toxicity studies to our findings results.

Silymarin administration to pregnant mice caused brain oedema in all doses with maximum effect in the 200 mg/kg. The results of one study showed that indomethacin increases oxygen free radical generation, membrane lipid peroxidation and cell membrane dysfunction in the hypoxic brain that causes neuronal cell death due to intracellular Ca level elevation [13]. It is also reported that administration of 4 mg/kg of indomethacin during the last three days of gestation caused an increased incidence of neuronal necrosis in the diencephalon of foetuses [10]. In humans, Baerts*et al.* showed presence of cystic brain lesions in infants born after prenatal indomethacin treatment [14]. Two studies on preterm infants have shown a relation between maternal indomethacin treatments and increased risk of intracranial haemorrhage (28%) in the neonate [15]. Iannucci [22] also showed a significantly higher incidence of intracranial haemorrhage in infants who their mothers had received indomethacin [16]. It has been reported that silymarin might cause apoptosis by preventing the hypoxic stimuli of factor-α in lung cancer cells [17]. Silymarin also activates the P-53 caspase pathway, as well as inducing apoptosis in bladder transitional-cell papilloma RT4 cells. It also increases the secretion of cyclin-kinase inhibitors (CKIs) and leads to impede the cell cycles and apoptosis in colon carcinoma (HT-29) cells by caspase activation [18]. Silymarin has been shown to have proapoptotic activity in cancerogenic cells and can decrease [13] the expression of antiapoptotic proteins, Bcl-2 and Bcl-xl in JB6 C141 cells [19,20]. We also found some brain lesions associated with silymarin administration including apoptosis and oedema.

Also, the results of aspirin instillation into the rumina of fetal lambs showed a significant increase in pulmonary arterial pressure, which was directly related to constriction of the ductus arteriosus [21]. One study has linked antenatal indomethacin to an increased risk of broncho-pulmonary dysplasia [22]. A significant decrease in alveolar luminal volume and delay in development of lamellar bodies and surfactant components after exposure to indomethacin has also been indicated [23]. Nagai et al. revealed that indomethacin administration can cause abnormal lung structure, diminished gas-exchanging surface and surface-to-volume ratio and increased septal wall thickness with reduced prostaglandin level in tissue.

However, this procedure did not alter body weight to animals treated with indomethacin reduced the

in pulmonary architecture [24]. Eronenet al. showed that the inhibition of cyclooxygenase by indomethacin, leads to an increased availability of leukotrienes with vasoconstrictor and pro-inflammatory properties [25]. These results confirm some of the findings reported in our lab including infiltration of inflammatory cells and lungs immaturity.

In the current study, silymarin also induced immaturity and an infiltration of inflammatory cells in fetal kidneys. According to Boubred et al. study, NSAIDs affect renal structure of fetuses and produce renal congenital abnormalities, including cystic dysplasia, tubular dysgenesis, ischaemic damage and a reduced nephron number [26]. Long-term indomethacin treatment during pregnancy leads to the development of renal failure and irreversible renal damage with cystic dilatation of developing nephrons in an exposed foetus. It is stated that Foetuses exposed inutero to significant amounts of NSAIDs have at birth various degrees of renal insufficiency and structural renal defects with a very high mortality. According to our results, silymarin caused cardiac congestion. A case of severe fetal congestive heart failure due to occlusion of the ductus arteriosus in a mother treated with indomethacin for polyhydramnios and premature contractions has been reported. Indomethacin administration could cause dilatation of the right and left ventricle with wall thickness and evidence of congestive heart failure including increased pericardial effusion [27,28].

Our results did not show any changes in ventricular wall thickness and ventricular mass or ventricular hypertrophy, but we also observed congestion of the fetal heart.

In conclusion, our results showed that if Silymarin administration to pregnant mice has pathological effects on foetal brain, kidneys, heart and lungs in different doses, albeit to varying degrees. It seems larger animal and human studies are necessary to investigate the mechanisms of Silymarin developmental toxicity.

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Table1. Effect of silymarin on histopathological changes in brain, heart, lung and kidney tissues of mouse foetuses.

Dose mg/kg	Brain		Heart	Lung		Kidney	
	Appoptosis	Oedema	Congestion	Infiltration of inflammation cells	Immaturity	Infiltration of inflammation cells	Immaturity
control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50	1.00±0.0[a]	1.00±0.0[a]	1.00±0.00[a]	0.0	1.00±0.0[a]	0.0	1.00±0.0[a]
100	2.00±0.0[a]	2.00±0.0[a]	1.00±0.00[a]	0.0	1.00±0.3[a]	0.0	1.00±0.3[a]
200	2.50±0.5[b]	2.33±0.5[b]	2.33±0.51[b]	0.33±0.5[b]	2.00±0.3[b]	0.33±0.51[b]	2.00±0.3[b]

Experimental and control group received 50, 100 and 200 mg/kg of silymarin and normal saline plus tween, during organogenesis, respectively. Results are shown as the mean ± S.E.M. aP<0.01 and bP<0.001 compared with control group.

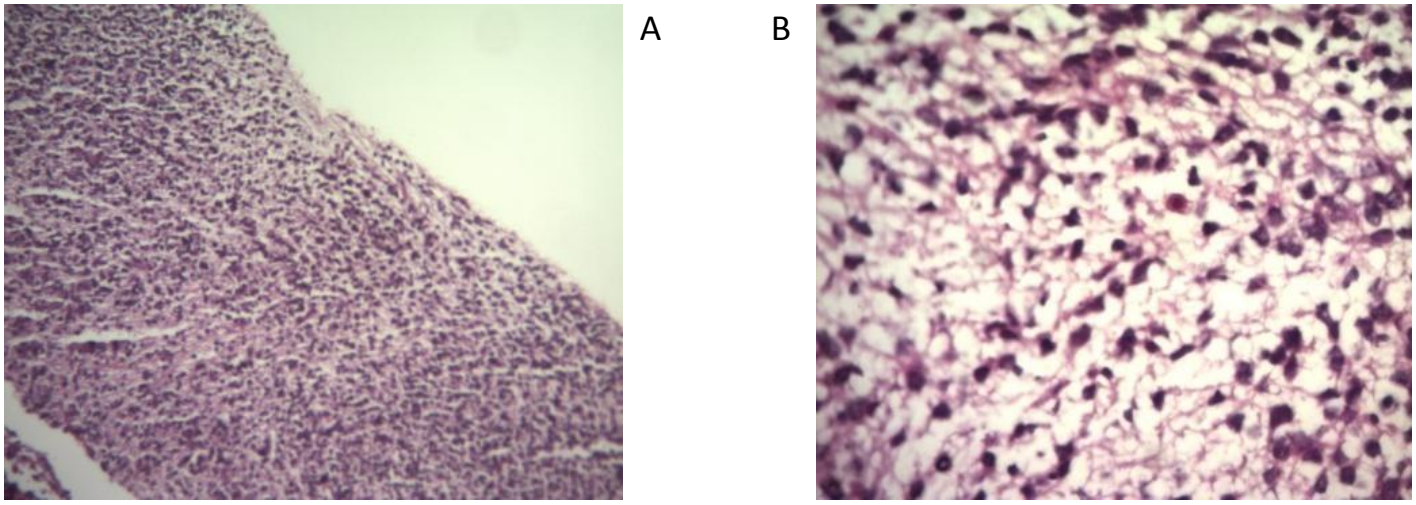

Figure 1. Light photomicrograph of hematoxylin and eosin–stained sections of embryonic brain tissue that were exposed to 50, 100 and 200 mg /kg/day of silymarin, during organogenesis period. (A) Normal brain tissue of control groups. (B) Apoptosis in background of oedema in brain sections of fetus that was exposed to 200 mg/kg silymarin.

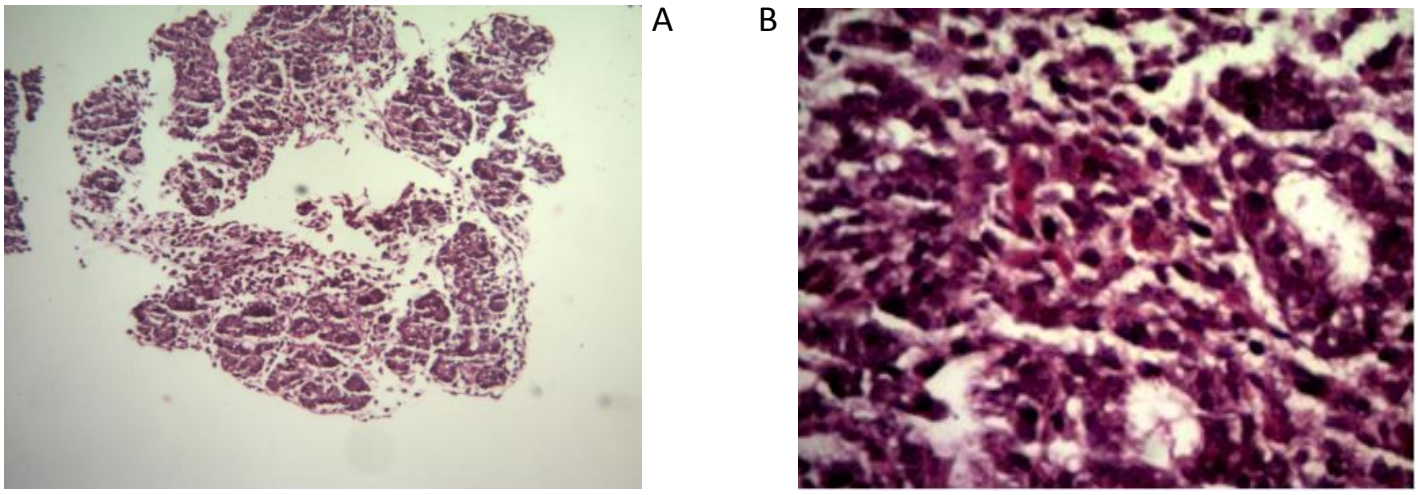

Figure 2. Light photomicrograph of hematoxylin and eosin–stained sections of embryonic kidney tissue that were exposed to 50, 100 and 200 mg /kg/day of silymarin, during organogenesis period. (A) Normal kidney tissue of control groups. (B) Apoptosis in kidneys sections of fetus that was exposed to 200mg/kg silymarin.