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Silymarin protects the structure of kidney in the neonatal rats exposed to maternal cadmium toxicity: A stereological study

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Abstract

The present study aimed to investigate the protective effect of silymarin on maternal cadmium toxicity complications in the kidney of neonatal rats. Forty adults Wistar female rats were selected and placed with male rats for copulation. The pregnant animals were randomly divided into five groups (n = 8) including control, sham, silymarin, cadmium, and silymarin + cadmium. The animals received 400 mg L^{-1} cadmium and 100 mg kg^{-1} silymarin (subcutaneously, three days per week, three weeks). Two-day neonates were dissected and their right kidneys were fixed in 10.00% buffered formalin solution and processed by standard paraffin embedding. Tissue sections were stained by hematoxylin and eosin and analyzed histologically and stereologically. The data were statistically analyzed by SPSS using a one-way ANOVA test and Tukey's post-hoc. The results showed that silymarin significantly increased the neonatal rats' weight compared to the control group. Cadmium significantly decreased the weight of neonatal rats' kidneys. The results of histological studies indicated that cadmium caused subacute glomerulosclerosis, severe damage to urinary tubules such as tubular necrosis, and severe hyperemia in the medulla, but silymarin could preserve these complications. Stereological results revealed that cadmium decreased the total volume of kidney, medulla, and proximal and distal tubules and increased interstitial tissue and indicated the protective effects of silymarin on maternal cadmium toxicity complications in the kidney tissue of neonatal rats. It can be concluded that the administration of silymarin during pregnancy may be used as a useful and effective way of protecting the maternal cadmium toxicity complications in the kidney tissue of neonatal rats.

Introduction

Although the advancement of technology and industry in the world has brought welfare to human societies, the increasing development of industry, besides its benefits, has also led to numerous problems including the threat to human health. One of these risk factors is industrial pollutants that often contain heavy metals such as cadmium. Cadmium can enter the body through food and accumulate in various organs including the liver and kidneys, causing acute toxicity for them.[1] Neonates are more sensitive in this respect.[2] Cadmium is one of the most toxic metals and one of the main industrial pollutants found everywhere, with the greatest negative effects on organs such as the kidneys and liver. Cadmium can be found in industrial products such as nickel-cadmium batteries, some plastic, and ceramic products, glass, and stain. The presence of this heavy metal in soil, water, air, cigarettes' smoke and a variety of industrially manufactured foods has also been reported.[3]

Cadmium can cause certain structural and functional damages in various tissues of the body.[4] Because of its ability to accumulate in animal tissues, this element can also cause poisoning in humans and animals through food.[5] Previous studies have shown that cadmium causes toxicity and damage to target organs including kidneys by increasing the production of reactive oxygen species (ROS).[6] The results of various reports indicate that cadmium can

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also transfer to the fetus. Therefore, its entry into the body of mothers during pregnancy can have detrimental effects on different organs of a developing fetus, particularly its kidneys, and cause various anomalies.[7] Cadmium binds to the red blood cell membrane and plasma albumin after absorption into the body and bloodstream. Then, it accumulates in the liver and binds to its carrier, metal-lothionein I. Finally, this compound following reaching the kidneys is filtered and accumulates in the proximal convoluted tubule due to the presence of cadmium carriers causing its functional impairment.[8] This metal can also cause some autoimmune diseases of the kidney including glomerulonephritis.[9] Also, the detrimental effects of $CdCL_2$ on the kidney cortex, cell membrane, brush border, and proximal and distal tubules have been proven in exposed rats.[10] The accumulation of this metal in kidney cortex is thought to lead to impairment in the normal functions of kidneys.[11] Some studies have also shown that prolonged exposure to Cd^{2+} in pollutants may lead to dysfunction in E-cadherin proteins of intercellular connections in epithelial cells.[12] It should be noted that the half-life of this element in the kidney has been reported to be more than 30 years.[13] This compound is also known as a carcinogenic substance that can cause renal tumors.[14] Cadmium toxicity or exposure to industrial pollutants containing this element during pregnancy can have adverse effects on mother and fetal health,[15] thus, it is necessary to pay more attention to this issue. The most important step in eliminating this concern is to find a way of reducing the damaging effects of this element on the vital organs of the fetus and infants of mothers who are unconsciously exposed to cadmium during pregnancy. One of these methods can be the use of herbal drugs that neutralize the effects of various toxins by their antioxidant effects.[16]

Mary thistle with the scientific name of *Silybum marianum* is a 2-year-old, pale green, and barbed plant with pink flowers. Its fruit contains an active ingredient called silymarin, which is a mixture of flavonolignans of silibinin, isosilibinin, silychristin and silydianin. Silydianin forms a major part (70.00%) of silymarin.[17,18] Silymarin has long been used as an antioxidant and anti-inflammatory agent as well as a very strong cell-protective agent.[19] Its antioxidant effect has been reported to cause glutathione increase and lipid peroxidation inhibition.[18] This substance is sold in the pharmacopeia of many countries under the trademarks of Legalon™ and Hepatron™.[20] Silymarin can be used as a nephroprotective drug because of its safety profile.[21] Some studies have shown that pre-treatment with silymarin prevents cellular absorption of toxic substances or removes toxic substances from the cell before they affect it.[22,23] The use of this substance during pregnancy and lactation is not only allowed, but it can also prevent the toxicities and oxidative damages in mother and fetus during pregnancy through its antioxidant effect and ability to be transferred to the fetus via placenta.[24]

Considering these points and the lack of scientific information about the effects of silymarin on the histological and stereological structure of kidney in infants that their mothers have been exposed to cadmium toxicity during pregnancy, the present study aimed to investigate the effects of this compound as a potent antioxidant on reducing or even compensating the cadmium side effects in the kidney of neonatal rats exposed to maternal cadmium toxicity.

Materials and Methods

Chemical agents. Silymarin was purchased from Sigma-Aldrich (St. Louis, USA) and cadmium, ketamine, and xylazine were provided from Merck (Darmstadt, Germany).

Animals and experimental design. Female Wistar rats in the 8 weeks of age and with the mean weight of 200.00 ± 23.00 g which had been purchased from the Laboratory Animals Center of Pasteur Institute, Tehran, Iran were used in this study. The animals were divided into four-member groups after they were transferred to the laboratory and one week of adaptation and kept in standard cages in an environment with a temperature of 23.00 ± 2.00 ˚C, the relative humidity of 45.00 ± 5.00% and the 12/12 hr light-dark cycle. During the experimental period, the animals were fed with standard pellet diet and water *ad libitum*. At the end of the adaptation period, a male rat and two female rats were placed in the cage for mating. Twenty-four hr later, the vaginal plaque was examined and the first day of the pregnancy was also determined. Finally, 40 pregnant rats were selected and randomly divided into five groups (n = 8) including control, sham (solvent), silymarin, cadmium, and cadmium + silymarin groups.

During the entire period of the experiment, the animals of silymarin and cadmium + silymarin groups received silymarin at a dose of 100 mg kg^{-1} in hydro-alcoholic solvent three times a week for three weeks.[20] The animals of the sham group received only an equal volume of the hydro-alcoholic solvent of silymarin (ethanol + distilled water). The animals of the cadmium and cadmium + silymarin groups also received only an aqueous solution of 0.40% cadmium (400 mg L^{-1}) through drinking water.[25] Two days after the end of pregnancy and the birth of neonates, two male neonates were randomly selected from each mother. After euthanizing by intraperitoneal injection of 80.00 mg kg^{-1} ketamine (Alfasan, Woerden, Netherlands) and 10.00 mg kg^{-1} xylazine (Alfasan), the animals were dissected and their right kidneys were removed and fixed in a 10.00% buffered formalin solution after macroscopic examination and weighing by a sensitive scale. All experimental procedures followed the guidelines of the Care and Use of Laboratory animals according to the international recommendations about

clinical and laboratory animals researches and approved by the Ethics Committee of Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran (NO: FVMUT/2017/108).

Histological and stereological studies. For microscopically studies, after tissue fixation, various stages of tissue processing including dehydration with ascending concentrations of alcohol, clearing with xylene, and impregnation with paraffin were carried out using a histokinette device (Leica, Wetzlar, Germany). Then, tissues were blocked using the standard paraffin embedding method and serial sections in 5.00 µm (for histological studies) and 20.00 µm (for stereological studies) thickness were prepared using a rotary microtome (Leica). The minimum intervals distance between the sections was determined by the results of the pilot studies. The systematic uniform random sampling method was considered at all steps of the stereological analysis, in such a way that the first 20.00 µm thick section was determined and selected using a random number table and after 480 µm (24 sections with 20.00 µm thickness and four sections with 5.00 µm thickness), the next 20.00 µm thick section was selected again (Fig. 1). This process was continued until the end of the tissue sample in the paraffin block. From each paraffin block, 20.00 to 25.00 thick sections were selected for stereological studies and 10 thin sections were selected for histological studies and stained with hematoxylin and eosin staining method by a standard protocol and evaluated under light microscopy BX60; Olympus, Tokyo, Japan) and photomicrographs were captured by the digital camera (DP12; Olympus). All microscopically analyses were carried out by expert evaluator who was blinded to the groups and previously described score applied to grade histopathological lesions of the kidney.[7,8]

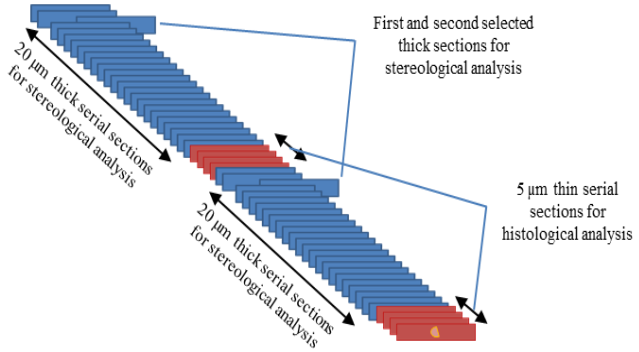

Fig. 1. The systematic uniform random sampling method for the selection of tissue sections in the stereological analysis.

Each block was cut into 24 thick serial sections and then four thin serial sections using a rotary microtome. Finally, based on the systematic random sampling protocol, the first section was chosen randomly using a random number table. For example 3, thus sections 3, 31, 59, 87, … were chosen for stereological analysis.

To obtain the tissue shrinkage level, a circular part of the kidney tissue was removed using a trocar and the mean of the two perpendicular diameters in this circle was determined using a caliper (r_{before}). This parameter was recalculated after the tissue processing, blocking, and staining (r_{after}). Finally, the tissue shrinkage estimation was calculated using the following formula:[26]

$$Shrinkage\ coefficient = 1 - \left(\frac{r^2_{after}}{r^2_{before}}\right)^{3/2}$$

We used this coefficient obtained from above formula to correct the estimated volume by Cavalieri method.[27]

All stereological studies were conducted under the principle of systematic uniform random sampling and using stereo-investigator system (version 9.0; MBF Bioscience; Micro Bright Field, Inc., Hanover, Germany). This system consists of a standard microscope, a motorized stage, a digital camera and a software application. The program commands automatic XY displacements of the microscope stage which allows the systematic randomized sampling of tissue microscopic fields. The software program creates point grids, dissectors, and nucleators, superimposed on the tissue samples visualized on a monitor. A total volume of the right kidney was estimated by the Cavalieri's principal and point counting method using the following formula:[27]

$$V = T \times (a/p) \times \sum_{i=1}^{n} Pi$$

where, T represents the distance between the selected sections, a/p is the area of each point in the grid used and P_i represents the number of counted points corresponding to the ith section of the tissue.

To estimate the volume of renal structures including cortical and medullary parts of the kidney, renal corpuscle, urinary space, glomerulus, proximal (PT) and distal tubules (DT) and the interstitial tissue, at the first step, the volume density (relative volume) of each structure was calculated by point counting technique at the appropriate magnification and point grade with following formula:[28]

$$V_{V(structure/kidney)} = P_{(structure)} \times P_{(kidney)^{-1}}$$

where, the $P_{(structure)}$ represents the number of points meeting each structure, and $P_{(kidney)}$ represents the number of points meeting the entire kidney tissue. Finally, the absolute volume of each structure was calculated by multiplication of its volume density by the absolute volume and shrinkage coefficient.

To estimate the lengths of PT and DT, assuming that the tubules are completely cylindrical, at the first step, the length density of the tubules was calculated using an unbiased counting frame with dimensions of 400 × 400 µm and at 200× magnification with the following formula:[26]

$$L_{V(structure/ref)} = 2\sum Q \times (\sum f \times a/f)^{-1}$$

where, $\sum Q$ represents the total number of sections evaluated in each kidney, $\sum f$ is the total number of frames examined and a/f is the area of each frame.

Finally, the total length of the tubule was calculated by removing the shrinkage effect from the following formula:[26]

$$L_{(structure, ref)} = L_{V(structure, ref)} \times V_{(kidney)}$$

To estimate the epithelium height of the PT and DT, the selected tubules in the unbiased counting frame were used and estimated by a linear probe and at 400× magnification with the following equation: $H = V_V \times S_V^{-1}$, where V_V represents the volume density of the epithelium calculated using the point-counting method and S_V represents the surface density (related to the inner surface) of the epithelium calculated by the following formula:[29]

$$S_{V(structure/ref)} = \frac{2 \sum I}{\sum p \times l/p}$$

where, I represent the number of collisions of the desired surface with the lines of the grid, P represents the number of points in the reference space and l/p represents the length of each line in the grid. Finally, the total surface area was estimated by eliminating the shrinkage effect.[26]

Statistical analysis. In all cases, the data were reported as mean ± SEM and statistically analyzed using SPSS (version 21.0; SPSS Inc., Chicago, USA). The coefficient of variation and the coefficient of error of each data were also calculated using the Gundersen *et al*. method.[30] The Kolmogorov-Smirnov test was used to investigate the normality of the data and one-way ANOVA and Tukey's post-hoc used with the significance level of α = 0.05 to compare the stereological data among the groups.

Results

Body weight of neonates. As shown in Figure 2, the mean body weight of the neonates at the birth time in sham and cadmium groups was lower than that in the control group, but this decrease was not statistically significant. However, the mean body weight of the neonates at the birth time was increased in the silymarin and cadmium + silymarin groups in comparison with the control group. The results of statistical analysis showed that these changes were significant only in the silymarin group compared to the control and sham groups and in the silymarin and cadmium + silymarin groups in comparison with the cadmium group ($p < 0.05$).

The weight of the right kidney. Figure 3 shows a comparison of the wet weight of the neonatal rats' right kidneys in different groups. The results showed that the weight of the right kidney of the neonatal rats had an increase in the silymarin group compared to the control group, but this increase was not statistically significant. The results also indicated a decrease in the wet weight of the right kidneys in the neonates of the sham, cadmium and cadmium + silymarin groups in comparison with the control group, of which only the weight of the right kidneys of the neonates in the cadmium group showed a significant decrease compared to the control and sham groups ($p < 0.05$).

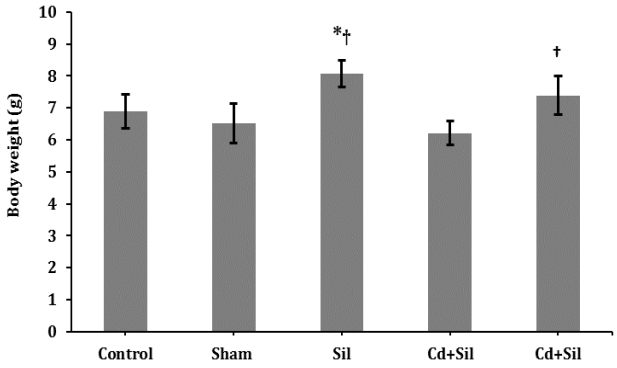

Fig. 2. The body weight of the neonates in different groups at the birth time (Cd = Cadmium, Sil = Silymarin). * represents significance in comparison with the control and sham groups, and † indicates significance in comparison with the cadmium group.

The weight of the right kidney. Figure 3 shows a comparison of the wet weight of the neonatal rats' right kidneys in different groups. The results showed that the weight of the right kidney of the neonatal rats had an increase in the silymarin group compared to the control group, but this increase was not statistically significant. The results also indicated a decrease in the wet weight of the right kidneys in the neonates of the sham, cadmium and cadmium + silymarin groups in comparison with the control group, of which only the weight of the right kidneys of the neonates in the cadmium group showed a significant decrease compared to the control and sham groups ($p < 0.05$).

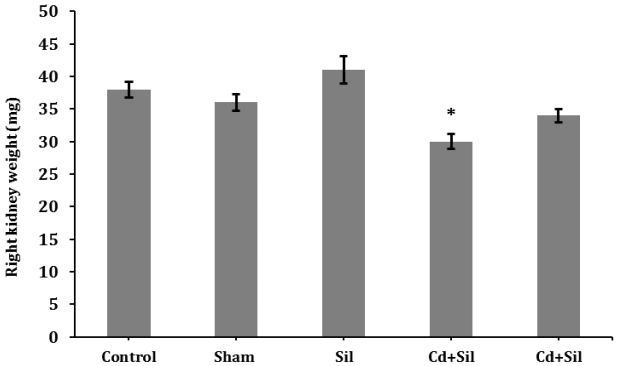

Fig. 3. The wet weight of the right kidneys of the neonates in different groups (Cd = Cadmium, Sil = Silymarin). * represents significance in comparison with the control and sham groups.

Histological analysis. The histological analysis of the renal tissue in the neonates of the cadmium group showed subacute glomerulosclerosis, glomerular shrinkage and glomerulocystic changes with capillary tuft congestion and severe damages such as tubular necrosis and degeneration as well as tubulorrhexis in urinary tubules of the cortical part of the kidney (Fig. 4C). Relative nephrosis associated with severe vascular hyperemia was also demonstrated in the medullary part of the kidney (Fig. 4D), whereas the tissue structure was almost normal in the other groups and no distinct tissue damage was observed. In these groups, the structure of the urinary tubules especially PT and DT in the cortical and medullary parts and the renal corpuscle and glomeruli structures in the cortical part of kidneys were quite preserved in comparison with cadmium group. The details of the microscopic structure of the right kidney tissue in neonates are shown in Fig. 4.

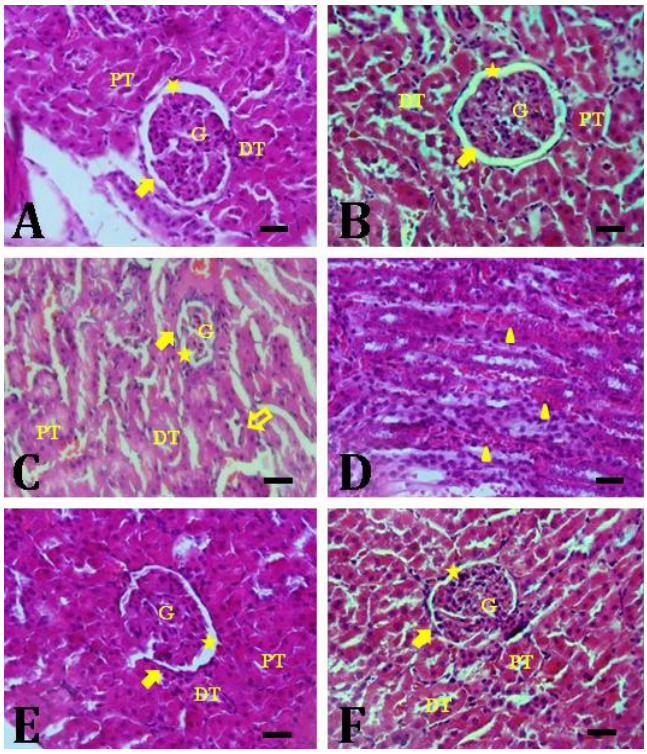

Fig. 4. Micrographs of right kidney tissue structure in different groups. Micrographs show the cortical part of the right kidney in the **A)** control and **B)** sham groups, where the structure of the renal corpuscle (arrow), urinary space (asterisk), and the urinary tubules (PT and DT) is quite normal and also in **C)** cadmium group, where characterized by glomerulosclerosis with capillary congestion of glomerulus, glomerular shrinkage (G) and severe damages such as necrosis and tubulorrhexis (hollow arrow). **D)** It shows the medullary part of the kidney in the cadmium group, with relative nephrosis associated with severe hyperemia in interstitial tissue (arrowhead). The renal corpuscle and urinary tubules structures in **E)** silymarin group and **F)** cadmium + silymarin groups are preserved in comparison with the cadmium group (C and D). (H & E; Scale bars = 10.00 µm).

Cortex and medulla volume and total volume of the right kidney. The mean total volume of the right kidney of the neonates indicated a significant decrease in cadmium group in comparison with control (p = 0.001), sham (p = 0.023) and silymarin (p = 0.005) groups (Table 1). Besides, a significant decrease was observed in the mean of the renal volume of the neonates of cadmium + silymarin group (p = 0.009) compared to the control group. Although the volume of the cortical part had decreased in all groups compared to the control group, there was no statistically significant difference among the different groups, while the volume of the medullary part was significantly lower in the cadmium group than that in the control, sham and silymarin groups (p < 0.05). Furthermore, there was a significant decrease in the volume of a medullary part of the cadmium + silymarin group in comparison with the sham (p = 0.001) and silymarin (p = 0.000) groups.

Table 1. The mean of medulla, cortex, and total volume (mm^3) of the right kidney.

Groups	Cortex	Medulla	Total
Control	$42.80 \pm 0.66^{b*}$ CE=0.04, CV=0.26	27.80 ± 0.58^{cde} CE=0.05, CV=0.19	72.10 ± 0.52^{c} CE=0.04, CV=0.08
Sham	40.80 ± 0.97^{ab} CE=0.04, CV=0.11	28.60 ± 0.24^{de} CE=0.05, CV=0.08	70.86 ± 0.91^{bc} CE=0.06, CV=0.15
Silymarin	40.80 ± 0.58^{ab} CE=0.04, CV=0.09	29.20 ± 0.37^{e} CE=0.04, CV=0.23	71.42 ± 0.51^{bc} CE=0.05, CV=0.17
Cadmium	40.60 ± 0.75^{ab} CE=0.03, CV=0.26	24.60 ± 0.51^{a} CE=0.04, CV=0.18	67.07 ± 0.77^{a} CE=0.06, CV=0.18
Cadmium + Silymarin	40.00 ± 0.71^{ab} CE=0.05, CV=0.08	26.20 ± 0.20^{abc} CE=0.05, CV=0.22	68.06 ± 0.59^{ab} CE=0.05, CV=0.26

CE= Coefficient of error; CV= Coefficient of variation.
Same letters in each column indicate that there is no significant difference among the groups (α = 0.05).

Renal corpuscle, glomerulus, urinary space, and interstitial tissue volume. The mean volume of the renal corpuscle and urinary space showed no significant differences among the neonates of the different groups (p > 0.05). Although the volume of glomerulus was relatively lower in the cadmium group than that in the control group, none of these changes were statistically significant (p > 0.05). The interstitial tissue volume of the cadmium group showed a significant increase in comparison with the control, sham, and silymarin groups (p < 0.05). Also, there was a significant increase in interstitial tissue volume of cadmium + silymarin group compared to control (p = 0.001), sham, and silymarin (p = 0.000) groups (Table 2).

Total volume, the volume of lumen and epithelium, height of the epithelium, and length of the PT. The results of the stereological analysis of PT indicated a significant decrease in the total volume of PT in the cadmium group compared to the control, sham, silymarin, and cadmium + silymarin groups. This study showed that although the lumen volume of the PT showed a significant decrease in the silymarin group (p = 0.023) in comparison with the control group, this change was not statistically

Table 2. The mean volume of the renal corpuscle, glomerulus, urinary space, and interstitial tissue (mm^3) of the right kidney.

Groups	Renal corpuscle	Glomerulus	Urinary space	Interstitial tissue
Control	$4.59 \pm 0.03^{a*}$ CE=0.06, CV=0.13	3.20 ± 0.04^{a} CE=0.05, CV=0.19	1.09 ± 0.02^{a} CE=0.04, CV=0.27	3.39 ± 0.45^{ab} CE=0.05, CV=0.12
Sham	4.56 ± 0.01^{a} CE=0.05, CV=0.011	3.18 ± 0.02^{a} CE=0.04, CV=0.09	1.09 ± 0.02^{a} CE=0.06, CV=0.24	2.62 ± 0.37^{a} CE=0.04, CV=0.19
Silymarin	4.56 ± 0.02^{a} CE=0.05, CV=0.19	3.19 ± 0.02^{a} CE=0.05, CV=0.17	1.07 ± 0.03^{a} CE=0.04, CV=0.19	3.22 ± 0.49^{a} CE=0.04, CV=0.11
Cadmium	4.32 ± 0.06^{a} CE=0.06, CV=0.25	2.98 ± 0.05^{a} CE=0.04, CV=0.29	1.05 ± 0.02^{a} CE=0.03, CV=0.16	7.66 ± 0.50^{d} CE=0.05, CV=0.21
Cadmium + Silymarin	4.48 ± 0.04^{a} CE=0.04, CV=0.19	3.09 ± 0.03^{a} CE=0.04, CV=0.23	1.09 ± 0.02^{a} CE=0.05, CV=0.09	6.10 ± 0.40^{cd} CE=0.05, CV=0.11

CE= Coefficient of error; CV= Coefficient of variation.
Same letters in each column indicate that there is no significant difference among the groups ($\alpha = 0.05$).

significant compared to the other groups ($p > 0.05$). It was also found that the epithelium volume of the PT was significantly increased in the sham and silymarin groups in comparison with the control and cadmium groups. Also, the epithelium volume of the PT significantly decreased in the cadmium group compared to the control, sham, silymarin, and cadmium + silymarin groups. According to stereological results, there was no significant difference ($p > 0.05$) in the height of epithelium and the length of PT among the different experimental groups (Table 3).

Total volume, the volume of lumen and epithelium, height of the epithelium, and length of the DT. The results of the stereological analysis of the DT showed that the mean volume of these tubules in the neonates of the cadmium group decreased significantly compared to the control, sham, and silymarin groups ($p < 0.05$). Also, the mean volume of DT showed a significant decrease in the cadmium + silymarin group compared to the control and sham groups and a non-significant increase compared to the cadmium group. As shown in Table 4, the lumen volume of DT showed a significant decrease in the silymarin group in comparison with the control, sham, cadmium, and cadmium + silymarin groups. Also, there was a significant increase in the lumen volume of the cadmium group compared to the sham and silymarin groups ($p < 0.05$). The volume of the epithelium of DT showed a significant increase in the silymarin group compared to the control, sham, cadmium, and cadmium + silymarin groups. There was a significant decrease in the volume of the epithelium of DT in the cadmium group in comparison with the other groups. On the other hand, the cadmium + silymarin group showed a significant increase in the epithelium volume of DT in comparison with the cadmium group and a significant decrease in that in comparison with the silymarin group. The results of the statistical analysis of the stereological data of DT indicated that there is no significant difference in the height of epithelium and the length of DT among the different experimental groups ($p > 0.05$).

Discussion

Today, heavy metals especially cadmium have different uses in various industries including galvanization, electroplating, coloring industry, the television screen, and laser batteries,[31] however, there is a lot of evidence for its toxic and carcinogenic effects.[32] Cadmium has been recognized by the International Agency for Research on Cancer of the United States to be classified into the first category of carcinogenic substances.[33] Some studies have shown that long-term exposure of pregnant mothers to cadmium has bad side effects on the fetus.[34] The knowledge that cadmium at any dosage can transfer to the fetus via the placenta helps understand the effects of

Table 3. The mean of total volume, lumen, and epithelium volume (mm^3), the height of the epithelium (μm), and length (m) of the proximal tubule (PT) in the right kidney.

Groups	Total volume	Lumen volume	Epithelium volume	Epithelium height	PT length
Control	$40.00 \pm 0.84^{bc*}$ CE=0.04, CV=0.11	12.60 ± 0.87^{d} CE=0.04, CV=0.09	27.40 ± 0.93^{bc} CE=0.04, CV=0.27	9.34 ± 0.02^{a} CE=0.03, CV=0.13	153.60 ± 2.77^{ab} CE=0.05, CV=0.11
Sham	43.40 ± 0.87^{c} CE=0.04, CV=0.13	11.40 ± 0.51^{bcd} CE=0.05, CV=0.19	32.00 ± 0.55^{d} CE=0.04, CV=0.21	9.16 ± 0.04^{a} CE=0.05, CV=0.11	170.28 ± 7.79^{b} CE=0.04, CV=0.15
Silymarin	41.60 ± 0.81^{bc} CE=0.05, CV=0.11	10.20 ± 0.37^{abc} CE=0.04, CV=0.23	31.40 ± 0.51^{d} CE=0.03, CV=0.11	9.24 ± 0.10^{a} CE=0.04, CV=0.13	166.57 ± 6.04^{b} CE=0.04, CV=0.19
Cadmium	30.40 ± 0.87^{a} CE=0.04, CV=0.19	8.80 ± 0.37^{a} CE=0.04, CV=0.18	21.60 ± 0.68^{a} CE=0.05, CV=0.08	9.22 ± 0.06^{a} CE= 0.04 CV=0.10	156.38 ± 7.19^{ab} CE=0.06, CV=0.22
Cadmium + Silymarin	40.40 ± 0.93^{bc} CE=0.04, CV=0.26	10.60 ± 0.51^{abcd} CE=0.06, CV=0.18	29.80 ± 0.49^{cd} CE=0.04, CV=0.11	9.32 ± 0.06^{a} CE=0.04, CV=0.13	155.01 ± 5.52^{ab} CE=0.04, CV=0.19

CE= Coefficient of error; CV= Coefficient of variation.
Same letters in each column indicate that there is no significant difference among the groups ($\alpha = 0.05$).

Table 4. The mean of total volume, lumen, and epithelium volume (mm^3), the height of the epithelium (μm), and length (m) of the distal tubule (DT) in the right kidney.

Groups	Total volume	Lumen volume	Epithelium volume	Epithelium height	DT length
Control	$8.29 \pm 0.07^{cd*}$ CE=0.03, CV=0.08	2.91 ± 0.35^{bc} CE=0.03, CV=0.12	5.38 ± 0.05^{de} CE=0.04, CV=0.21	8.28 ± 0.06^{a} CE=0.03, CV=0.18	49.99 ± 1.13^{a} CE=0.05, CV=0.11
Sham	8.23 ± 0.02^{cd} CE=0.04, CV=0.15	2.85 ± 0.03^{b} CE=0.04, CV=0.09	5.37 ± 0.03^{de} CE=0.04, CV=0.18	8.24 ± 0.05^{a} CE=0.04, CV=0.17	50.74 ± 0.91^{a} CE=0.04, CV=0.11
Silymarin	8.09 ± 0.03^{bc} CE=0.05, CV=0.17	2.50 ± 0.08^{a} CE=0.05, CV=0.10	5.59 ± 0.06^{f} CE=0.03, CV=0.26	8.28 ± 0.11^{a} CE=0.04, CV=0.11	49.03 ± 1.45^{a} CE=0.04, CV=0.09
Cadmium	7.77 ± 0.72^{a} CE=0.04 ,CV=0.09	3.07 ± 0.03^{c} CE=0.04, CV=0.11	4.70 ± 0.07^{a} CE=0.03, CV=0.19	8.26 ± 0.13^{a} CE=0.05, CV=0.09	48.57 ± 0.52^{a} CE=0.06 ,CV=0.24
Cadmium + Silymarin	7.93 ± 0.04^{ab} CE=0.05 ,CV=0.11	2.97 ± 0.03^{bc} CE=0.04 ,CV=0.25	4.96 ± 0.04^{b} CE=0.04 ,CV=0.18	8.30 ± 0.06^{a} CE=0.05, CV=0.19	49.33 ± 0.96^{a} CE=0.05, CV=0.17

CE= Coefficient of error; CV= Coefficient of variation.
Same letters in each column indicate that there is no significant difference among the groups (α = 0.05).

mothers' exposure to cadmium and its harmful effects on the fetus.[35] However, the level of cadmium accumulated in fetal tissues increases when its dosage increases during the advanced stages of pregnancy.[7] With the advancement of pregnancy, cadmium is condensed in the placenta and transported to the fetus after it exceeds its tolerable threshold and the fetus is thus exposed to toxicity. Fetuses that are exposed to cadmium in the mother's uterus show a higher risk of developing hypertension and kidney impairment in adulthood.[36]

The main way of exposure to cadmium was through inhalation (cigarette smoke) and swallowing [31,37], and half-life of the body's clearance is estimated to be about 25 years.[31] The liver and kidney are the main organs for cadmium accumulation.[1] However, the most sensitive organ that is damaged by cadmium toxicity is the kidney.[38] The mechanism of cadmium toxicity in the kidney is due to the production of ROS and disturbance of balance in the body's antioxidant system, which is known as oxidative stress.[7,32,33,38] This is why antioxidant treatments can be considered as one way for inhibition of the toxic effects of cadmium.[38] Therefore, considering the lack of documented scientific data about the effects of silymarin as a strong antioxidant on the side effects of cadmium in the kidney tissue of fetuses exposed to maternal toxicity, this study was designed to investigate these effects on the histological structure of kidney by stereological techniques.

The results of the present study showed that the mean weight of the neonates at the birth time was lower in the cadmium group than that in the control group, but this decrease was not statistically significant. Other studies have also confirmed the effect of cadmium on weight loss.[39] Also, the mean wet weight of the right kidney of the neonates in the cadmium group was significantly lower than that in the control and sham groups. However, no similar studies have been conducted on the effects of maternal cadmium toxicity on the wet weight of the neonates' kidneys.

The results of the histological analysis of kidney tissue in the present study showed that there were a subacute glomerulonephrosis and relative tubulointerstitial nephrosis associated with severe hyperemia in the medullary part of kidney in the cadmium group. The other studies investigating the kidney structure of cadmium-exposed adult rats have indicated structural changes such as degeneration of glomerular capsules, pyknotic nuclei with focal necrosis in the urinary tubules especially PT, multiple foci of hemorrhage in renal tissue and dilation and congestion of blood vessels.[7]

The results of the stereological analysis of this study showed a significant decrease in the total volume and medullary part volume of the right kidneys in the cadmium group. In addition, the volume of interstitial tissues of their kidneys was significantly increased, but the mean of total volume, volume of lumen and epithelium and height of the epithelium of PT showed a significant decrease in the cadmium group compared to the control group, but in the DT, only the mean of the total volume of the tubule and the volume of the epithelium were significantly decreased in comparison with the control group. It seems that more changes in the stereological indices of PT than those of the DT may be due to toxic effects such as oxidative stress and the reduction of trans-epithelial resistance[36,38,40] resulting from the concentration of the Cd-MT1 complex in the PT.[41] No similar studies have been carried out yet, but a study by Rafati *et al*., on adult male rats exposed to acute cadmium toxicity, indicated an increase in the renal volume and total volume of the glomerulus and a significant decrease in PT and DT volumes.[40] Previous studies have shown that various mechanisms are involved in the process of Cd-induced nephrotoxicity, but the main pathway of kidney damage is through the production of ROS and oxidative stress;[32,38] therefore, therapies that can strengthen the antioxidant system of the body can prevent the harmful effects of cadmium toxicity.[38]

Plants have long been considered as one of the main sources of medicine for the treatment of a large number of diseases and poisonings.[42] Some herbal medicines have very good antioxidant effects and are extensively used for the prevention of nephrotoxic effects of a large number of toxins and drugs.[16]

Silymarin is one of the most widely used herbal medicines. It belongs to the family of flavonoids and is produced from the extract of the seeds and fruits of the milk thistle, *S. marianum*. The main chemical compounds of silymarin include silibinin, isosilibinin, silicristin, and silidianin.[43] This substance is sold in the pharmacopeia of many countries under the trademarks of Legalon™ and Hepatron™.[20] Silymarin can be used as a nephroprotective drug because of its safety profile.[21] The nephroprotective properties of this compound have been proven to counteract the effects of nephrotoxic drugs such as cisplatin,[16] gentamicin,[21] and cyclophosphamide.[42] No adverse and harmful effects of silymarin on the recommended therapeutic doses have been reported so far.[43,44] Studies have also shown that this substance lacks any embryotoxic effects.[45] In support of this claim, the United States Food and Drug Administration and numerous studies have confirmed the safety of the use of silymarin with the recommended dose.[32,46] One of these studies showed no adverse and side effects of the silymarin administration on mother and fetus at a dose of 560 mg per day for 16 days in pregnant women involved with intra-hepatic cholestasis.[43]

Due to its high antioxidant properties, silymarin can be used as a cytoprotectant against the harmful effects of various toxins.[22,23] The antioxidant effects of this substance are due to different reasons such as eliminating the free radicals, inhibiting the production of free radicals by inhibiting the enzymes involved in the production process of these radicals, or because of maintaining the integrity of the electron transport chain in the inner mitochondrial membrane. It also increases the expression and activation of enzymatic and non-enzymatic antioxidants by regulating the *Nrf2* and *NFκB* pathways and ultimately maximizes the antioxidant response by increasing the expression of protective molecules such as heat shock protein, thioredoxin, and sirtuin.[18,47] As mentioned earlier, silymarin also has a nephroprotective effect against many toxins and medications.[16,21,42,48] Oxidative stress can reduce the glomerular filtration ratio due to ROS production, but the use of silymarin can reduce the serum creatinine levels.[47] Previous studies have shown that silymarin has the potential to cross the placental barrier[24] and not only does not damage the fetus but also has protective effects on the brain and liver of the fetus.[24,43]

The results of the present study showed that the mean weight of neonates in the silymarin group increased significantly. Moreover, this index showed a significant increase in the silymarin + cadmium group compared to the cadmium group but did not reach the level of the control group. The results of histological studies also indicate that the right kidney tissue structures including the cortical and medullary tubules, renal corpuscle, and glomerulus have been quite normal in the neonates of the silymarin and silymarin + cadmium groups. The results of the silymarin + cadmium group indicate that silymarin has greatly prevented the negative side effects of cadmium on the tissue structure of the kidney. A study conducted by Abouzeinab showed that administration of silymarin at a dose of 50 mg kg^{-1} two hr before exposure to cisplatin inhibited the nephrotoxic effects of this substance on the tissue structure of glomeruli and tubules in the cortical and medullary parts of the kidney indicating the good protective effect of silymarin on the tissue structure of the kidneys.[16] Also, Alcaraz-Contreras *et al*. have shown that silymarin has good protective effects on the histological structure of the renal tissue of rats exposed to chronic toxicity with lead acetate.[49] Previous studies have shown that vitamin C and ginger extract have also good protective effects in preventing the side effects of cadmium toxicity on renal tissue structure. These two substances are good examples for comparison with the nephroprotective effects of silymarin because the protective effects of vitamin C is due to its antioxidant properties and the protective effect of the ginger extract is not only due to its antioxidant properties but also due to its regulatory properties in metabolism and the effects of cadmium toxicity in the body.[7,50]

The stereological analysis of the present study showed that the use of silymarin did not only significantly change the stereological indices, but also its administration in the silymarin + cadmium group could improve most of the stereological indices. These results are completely new and there are no reports of a stereological study similar to this research. The newest stereological methods deal with the quantitative and three-dimensional investigations of the size, shape, and other tissue indices in the whole structure, which is a good confirmation and a golden index in the qualitative studies such as histological and histopathological studies conducted on the considered tissue structure. In line with the beneficial effects of administration of silymarin on the renal structure of all neonates who their mothers exposed to cadmium toxicity during pregnancy, the results of previous studies have shown that silymarin inhibits lipid peroxidation and renal damage due to its antioxidant activity and prevents inflammation in the kidney by controlling the *NFκB* pathway and thus can have appropriate supportive effects in kidney damage prevention.[47]

Considering the results of this study and reviewing the previous studies, we can conclude that the exposure of the pregnant mothers to cadmium even at low doses can have adverse effects on their neonates' body tissues, especially kidneys, and the use of the appropriate dosage of silymarin and similar compounds during pregnancy can help to inhibit the renal damage in these neonates. Although we need precise studies to determine the mechanism of the silymarin effects, it seems that this treatment needs to be continued after birth to obtain better and more effective results from this protocol.

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Conflict of interest

The authors declare that there is no conflict of interest.

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