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The effects of icariin on stereological parameters in mice kidneys treated with acrylamide

Running title: The impact of icariin on renal complications caused by acrylamide

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ABSTRACT

Introduction: Acute kidney injury, a leading cause of kidney failure, can be triggered by toxic substances such as acrylamide. Herbal medicines, known for their antioxidant properties, may help reduce the harmful effects of acrylamide.

Objectives: This study aims to investigate the protective antioxidant effects of icariin, a natural plant compound, against acrylamide-induced stereological changes in kidney tissue in mice.

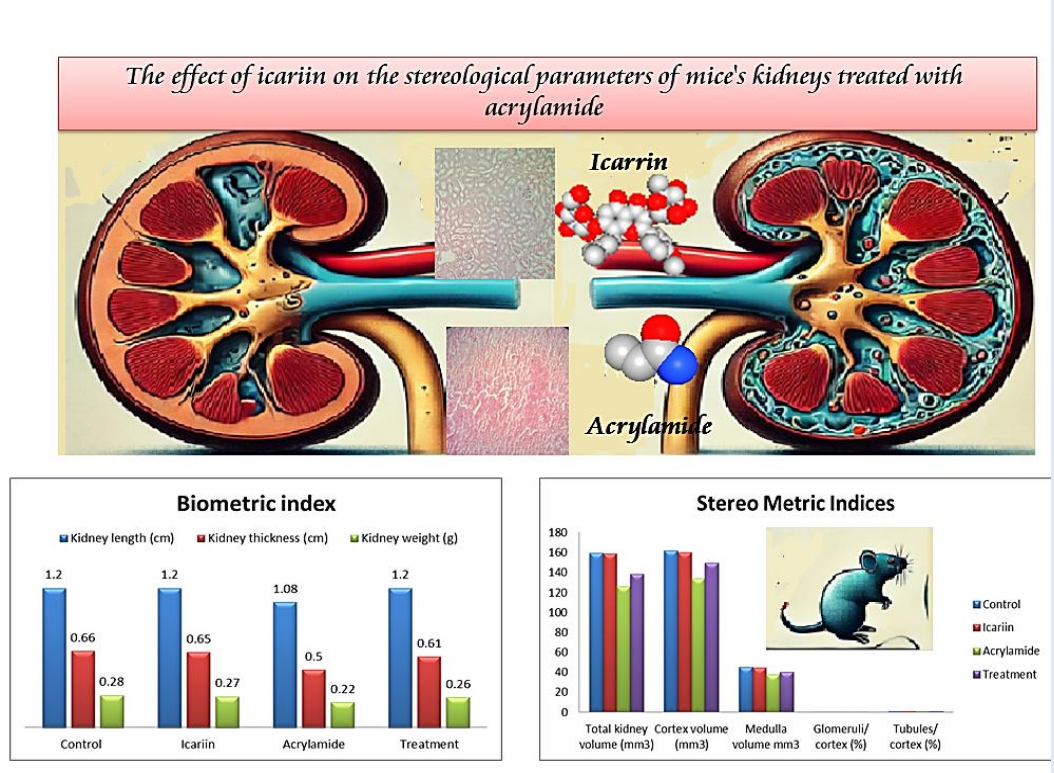
Materials and Methods: In an experimental study, 32 male mice were randomly divided into four groups: intact control, icariin control (dose (100mg/kg/day), acrylamide control (dose 10mg/kg/day), acrylamide treatment group (10mg/kg/day), and icariin treatment group (with a dose of 100mg/kg/day). All substances were administered orally for five weeks. At the end of the fifth week, the animals were anesthetized and ethically euthanized, and their kidneys were subsequently removed. Biometric indicators such as kidney length, changes in kidney thickness, and weight were examined before the kidneys were fixed in formalin. Tissue sections were prepared and analyzed using the H & E method

after staining. Stereological indices, including changes in kidney volume, cortex volume, medulla volume, the ratio of glomerulus to cortex surface, and the ratio of renal tubules to cortex surface, were then calculated. The data was analyzed using SPSS version 21 statistical software.

Results: Biometric indicators such as kidney length and thickness, as well as stereological indicators like kidney volume, cortex volume, and medulla volume, showed a statistically significant decrease in the acrylamide group compared to the control and treatment groups (P<0.05). These findings were consistent with the histopathological results observed in the studied groups.

Conclusion: Acrylamide leads to a reduction in both biometric and stereological indicators of kidney health, while the use of icariin potentially mitigates these negative effects. Further studies are necessary to determine the efficacy of icariin in alleviating renal complications caused by acrylamide.

Keywords: Icariin, Acrylamide, Kidney, Stereological parameters, Biometric parameters



Graphical Abstract: The effects of icariin on stereological parameters in mice kidneys treated with acrylamide

INTRODUCTION

Different chemicals and drugs have varying effects on kidney tissue. One chemical known to cause numerous side effects on the body is acrylamide. Acrylamide, also known as 2-propanamide or acrylic acid amide, is an unsaturated amide with the molecular formula C3H5NO that exists as a colorless or white solid crystal [1]. Acrylamide is produced industrially by combining water

with acrylonitrile and is commonly used to synthesize polyacrylamides for water purification and waste removal [2].

Due to its proper absorption through the skin, acrylamide is used as a basic ingredient in some skin creams [3]. However, the primary way acrylamide enters the body is through food absorption [4]. When cooking foods with protein, a approximately 50-50 µg/kg of acrylamide is produced. On the other hand, cooking carbohydrate-rich foods like potatoes, corn, and wheat results in high levels of acrylamide (150-4000 micrograms per kilogram). In plant-based foods, the presence of the amino acid asparagine leads to increased acrylamide production [5]. During the *Maillard reaction*, asparagine reacts with a reducing sugar at temperatures above 120°C, forming acrylamide [6]. Acrylamide enhances the activity of lipid peroxidases, increasing oxidative reactions of fatty acids and leading to elevated levels of free radicals. Oxygen radicals can damage cellular macromolecules and cause cellular degeneration [7].

In *vivo*, acrylamide is metabolized, and its byproducts are excreted through urine. Due to these properties, it can have harmful effects on the kidneys during the excretion process [8]. On the other hand, acrylamide initially combines glutathione, which is commonly present in the human diet. As an ingredient in food, the varying levels of GSH in food items can impact the concentration of acrylamide in different food products. Additionally, GSH acts as a detoxifier for acrylamide in the body. Acrylamide is oxidized by cytochrome P450 2E1 and converted into glycidamide, an epoxide derivative. GSH reacts directly with acrylamide and glycidamide, then undergoes further metabolism before being excreted through urine. This process helps protect proteins and DNA from harmful electrophiles [9]. Research has shown that acrylamide is neurotoxic, genotoxic, reproductive toxic, hepatotoxic, and immunotoxic in rodents [10].

Bioactive compounds play a pivotal role in the development of functional foods. These compounds, naturally found in food, exert positive effects on physiological functions and help prevent chronic diseases. According to scientific definitions, functional foods not only meet nutritional needs but also offer additional health benefits beyond basic nutrient provision. Examples include antioxidants, polyphenols, and probiotics, each contributing uniquely to improving health outcomes [11] The Quantum and Tempus Theories emphasize the role of timing and metabolic interactions in enhancing the effectiveness of functional foods. These theories highlight the importance of consumption timing and the modulation of biological processes to optimize the efficacy of bioactive compounds [12].

Icariin (C33H40O15; 676.668 g·mol−1), an important flavonoid isolated from *Herba epimedin* [13], affects smooth muscles by binding to cGMP. Like sildenafil, icariin inhibits phosphodiesterase (PDEs) and prevents the breakdown of cGMP [14]. As a result, icariin promotes vasodilation and increases blood supply to organs, particularly sexual ones [15]. Icariin can cross the blood-brain barrier, reducing oxidative stress and inflammatory mediators in the brain [16]. Several studies have highlighted the protective effects of natural antioxidants against kidney damage caused by oxidative stress. For instance, the antioxidant properties of bioactive compounds like polyphenols have been shown to reduce inflammation and oxidative stress, potentially improving kidney function [17]. Icariin has shown potential to improve urinary protein excretion and kidney injury in hypertensive pregnant rats by affecting angiotensin II expression. Icariin can decrease the production of reactive oxygen species by inhibiting the activity of NADPH oxidase. This reduces the vasoconstriction effect of AngII-induced hypertension in rats [18].

Considering that acrylamide is excreted through the kidneys and one of the primary mechanisms of kidney damage involves the creation of free radicals, and since Icariin is a natural antioxidant, we hypothesized that it could prevent significant kidney damage caused by acrylamide-induced free radicals. In this study, we investigated the effect of icariin on the complications caused by acrylamide, focusingon biometric, stereological and histopathological changes in kidney tissue in mice.

MATERIAL AND METHODS

Animals*:* Thirty-two male C57Bl/6 mice weighing between 25±5 grams, and two months old, were purchased from the Shiraz Animal Breeding Center. The animals were housed in standard conditions with 12 hours of light and 12 hours of darkness, at a temperature of 21±1 °C throughout the experiment. They had free access to drinking water and standard food (Livestock and Poultry Joint Stock Company). After a two-week adaptation period, the mice were randomly divided into four groups.

Intact control, icariin control (dose 100mg/kg/day); acrylamide control (dose 10mg/kg/day); Acrylamide treatment group (10mg/kg/day + Icariin with a dose of 100mg/kg/day); all substances were administered orally for five weeks. Icariin and Acrylamide were purchased from Sigma-Aldrich, Germany. The procedures used in this study adhered to the ethical guidelines of Shahid Sadoughi University for the use of animals in experimental studies which are in line with the Helsinki ethics guidelines.

Tissue Preparation: Following the conclusion of the animal maintenance period on day 35, all mice were anesthetized with chloroform and euthanized in accordance with laboratory animal ethics. The ventral area of the mice was then laparoscopically accessed, and both kidneys were carefully removed. After being rinsed with physiological saline solution and weighed (using a digital scale) and measured (using a caliper), the kidneys were fixed in 10% formalin.

Preparation of tissue sections: After the kidneys were fixed in formalin, they were molded in a tissue processing device (Shandon, Germany, LEICATP1010). The tissue processing steps included dehydration (passing through different dilutions of 90%, 80%, and 70% alcohol for one hour each, followed by three passes in 100% alcohol for one hour each), clarification (replacing ethanol with xylene by immersing the samples in two xylene containers for one hour each), immersion in paraffin (placing the samples in two containers of melted paraffin at 65°C for one hour), paraffin embedding and tissue block preparation. Tissue sections were then prepared using a microtome (MicroHM320, Germany). At least 10 tissue slices with a thickness of 10 microns and an interval of 50 slices were prepared from each sample.

Hematoxylin-eosin staining: H&E staining involved several steps: deparaffinization (sections were immersed in xylene three times for 15 minutes each), dehydration (slides were dipped in alcohol starting from 100% and decreasing to 70% for 10 seconds each), rinsing with running water, staining with Hematoxylin for 8 minutes, rinsing again, treating with acid-alcohol, rinsing once more, immersing in eosin for two minutes, and a final rinse with running water. Following staining, the slides were sequentially dipped in alcohol concentrations increasing from 80% to 100% (80% once, 90%, and 100% twice each) for 10 seconds each, then passed through xylene three times for 10 seconds each. Lastly, the slides were mounted with DPX glue in preparation for stereological examination.

STEREOLOGICAL CALCULATIONS

Calculating the total volume of kidney, medulla and cortex: To determine the total volume of the kidney, including the medulla and cortex, sections were prepared with a thickness of 10 µm and an interval of 500 µm

between them. After staining the tissues, the volume was measured using an optical microscope and stereological software following the Cavalieri method [19].

Photographs were taken of the slides using a stereomicroscope with a magnification of two. The collision points were counted using the cross grid randomly placed on the photo. Points above and on the right side of the cross were included in the count. The area of the points, which was 10 square millimeters, was also factored into the formula. The total kidney volume, medulla volume, and cortex volume were then calculated using the formula V= ∑p.a/p.t where, "∑P" is the total points on the area of samples, "a/p" represents the area of each point, and "t" denotes the interval between the sections.

Calculating the ratio of the surface area of the glomerulus and tubule to the surface area of the cortex: Slides prepared from kidney tissue were photographed using a 200x magnification light microscope. Then, a checkerboard grid used to count the number of glomeruli, and a cross grid was used to count the number of tubules. This allowed us to determine the number of points related to these two structures.

$$AA\left(\frac{struct}{sect}\right) = \left(\frac{\sum P(tubular)}{\sum P(cortex)}\right)$$
$$AA\left(\frac{struct}{sect}\right) = \left(\frac{\sum P(glomerol)}{\sum P(cortex)}\right)$$

Statistical Analysis: The data was entered into SPSS software. One-way analysis of variance was used to compare the measured parameters between the groups, and Tukey's post hoc test was used to identify significant differences. A p value <0.05 was considered statistically significant.

RESULTS

Biometric indicators: The study yielded three main results regarding biometric indicators. Specifically, there was a statistically significant difference between the groups in terms of kidney length and thickness. The acrylamide group exhibited a significant decrease in kidney length and thickness compared to the control and treatment groups (P<0.05). However, there was no statistically significant difference in kidney weight among the different groups (see Table 1).

Table 1. The results of the biometric index among the groups (n=8)

Biometric indices	Control [a]	Icariin [b]	Acrylamide [c]	Treatment* [d]
Kidney length cm	1.2± 0.07	1.2± 0.07	1.08± 0. 7d	1.2± 0.01c
Kidney thickness cm	0.66± 0.05	0.65± 0.05	0.5± 0.06 d	0.61± 0.09c
Kidney weight g	0.28± 0.06	0.27± 0.04	0.22± 0.04	0.26± 0.05

*Treatment: Acrylamide + Icariin

Stereo metric Indicators: In all stereo metric indicators, the acrylamide control group showed a significant decrease compared to the control and icariin groups. Additionally, the acrylamide group exhibited a significant decrease in certain stereological indicators (such as total kidney volume, cortex volume, medulla volume, and the ratio of ducts to cortex surface) compared to the treatment group (P<0.05) (Table 2).

Table 2. The results of stereo metric indices among the studied groups

Stereo metric indices	Control [a]	Icariin [b]	Acrylamide [c]	Treatment [d]
Total kidney volume mm^3	159.65±1.2	158.9±1.1	126.12±1.9d	138.72±1.1c
Cortex volume mm^3	162.22±8.1	160.42±4.01	134.35±3.5d	149.56±1.2c
Medulla volume mm^3	45.3± 1.7	44.67±1.5	38.05±1.1d	40.05±0.5c
Glomeruli/ cortex %*	0.059±0.0004	0.06±0.002	0.056±0.003	0.059±0.005
Tubules/ cortex %**	0.912±0.04	0.905±0.02	0.9±0.02	0.908±0.01

*Glomeruli/ cortex: The ratio of glomeruli to the surface of the cortex
**The ratio of tubules to the surface of the cortex

Kidney Histopathological Results: Histopathological changes in the kidneys of different groups were consistent with biometric, stereo metric, and stereological findings. The groups exposed to acrylamide showed significant pathological changes in kidney tissue, but the presence of icariin had improved this process to some extent. In the acrylamide control group, dilated and hyperemic vessels, indistinct nuclei, fibrosis, and necrosis were observed in both the tubules and glomeruli. The glomerular tissue structure was compressed, with cellular disorganization. The tubule tissue was disrupted, with unclear and sometimes small, pyknotic nuclei, indicating necrosis, The tissue showed signs of fibrosis, and inflammatory cells with small, round nuclei were scattered within the tubules. Pink hyaline compounds and sporadic vacuolar changes were also present. In the treatment group, some tubule disarray and hyperemia were observed, but necrotic changes were minimal, and no inflammatory cells were detected. See Figure 1.

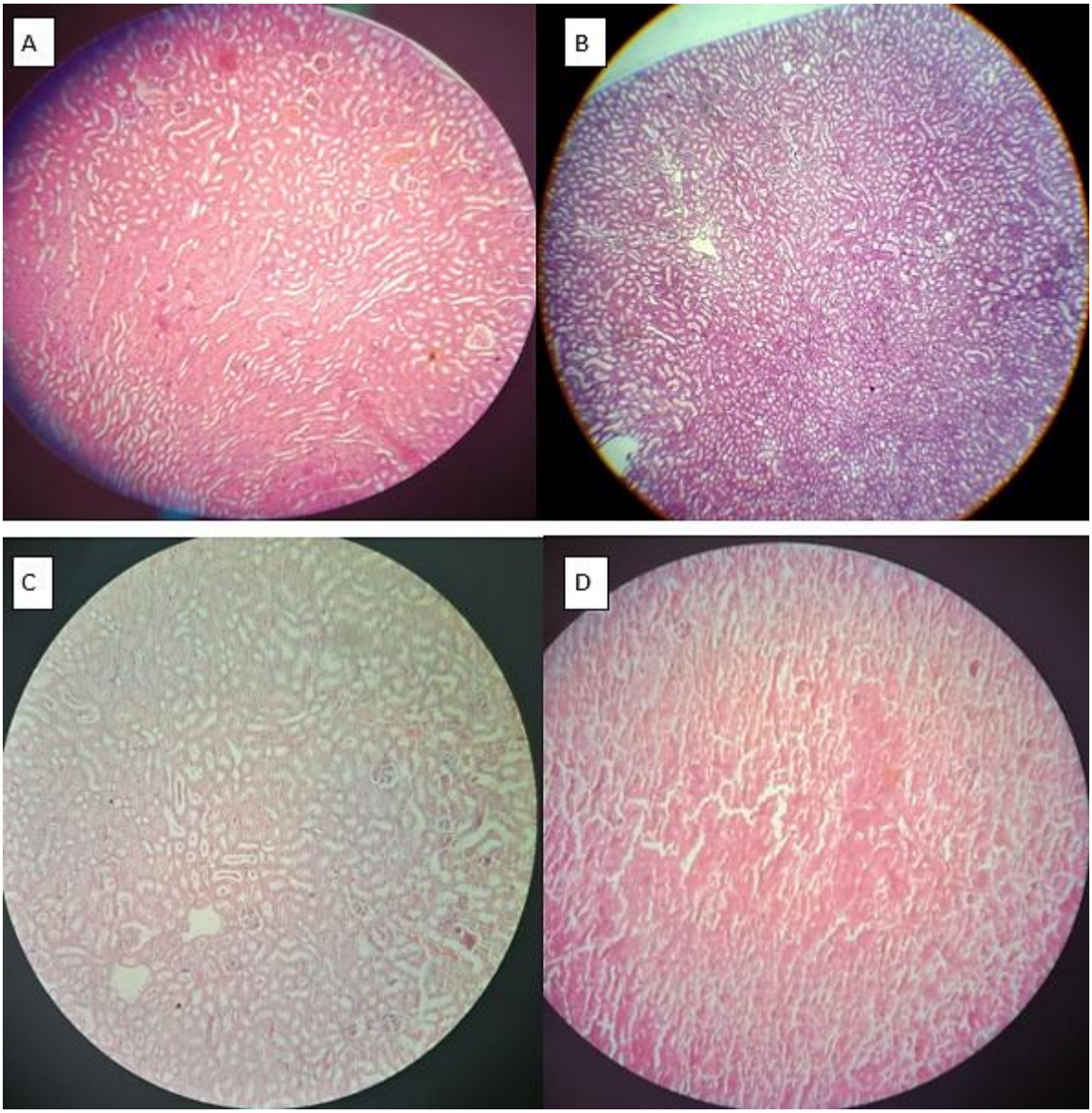


Figure 1. Microscopic images of kidney tissue in different groups. A (intact control) and B (icariin control), the tissue has a normal appearance,Glomeruli have normal size and normal cells, necrotic and inflammatory changes are not seen; C (acrylamide control) the tissue structure of the glomeruli is compressed and cellular disorganization is seen; D (acrylamide+icariin) Disarray of the tubules and hyperemia are seen to some extent, there are few necrotic changes, but inflammatory cells are not seen. Hematoxylin-Eosin staining, 100X magnification

DISCUSSION

In an experimental study, we investigated the histopathological effects of acrylamide on mice kidney tissue. In this regard, we examined the impact of icariin on the pathological changes in the kidneys exposed to acrylamide using stereological and histopathological biometric indicators. The results of the study indicate that the pathological effects of acrylamide can be observed in the kidneys of mice from biometric, stereological, and histopathological perspectives. The beneficial role of icariin in mitigating these pathological effects is significant.

Acrylamide is a small organic molecule with high solubility in water. This molecule contains an electrophilic vinyl group that can be attacked by nucleophiles. This characteristic is utilized in the production of various polymers. However, it also allows acrylamide to readily react chemically with biological molecules, leading to potential damage [20]. Conjugation of acrylamide or its metabolite, glycidamide, with the glutathione S-transferase enzyme causes cell dysfunction and cell death [21]. It has also been noted that the depletion of glutathione reserves stimulates the production of reactive oxygen species, which in turn triggers signaling cascades in the MAPK protein kinase family, such as JUN kinases. These kinases play a crucial role in regulating important intracellular processes like apoptosis [22]. The increase in free radicals and the depletion of glutathione reserves disrupts the balance between the oxidant-antioxidant system, leading to a reduction in antioxidant reserves and the initiation of the lipid peroxidation process [23].

There is a possibility that acrylamide, with its toxic properties, causes oxidative stress and, consequently, damage to the kidney tissue in the groups that received acrylamide. This damage leads to a decrease in the volume of the kidney, cortex, and medulla, as well as the thickness of the kidney. Aligning with the results of our study, Dortaj et al. (2014) demonstrated that groups exposed to acrylamide showed reduced kidney volume, cortex, and medulla volume, indicating kidney tissue damage caused by acrylamide [24]. Davoudi Moghadam et al reported that chronic acrylamide consumption can lead to pathological changes in kidney tissue and unfavorable alterations in serum urea, creatinine, TAC, and MDA levels [25]. This difference can be caused by the different breeds of mice, which is a rat in the mentioned study.

Recent research has demonstrated that bioactive compounds, including probiotics and antioxidants, play a key role in reducing oxidative stress and inflammation caused by toxic substances such as acrylamide. For instance, Lactobacillus probiotics not only regulate gut microbiota but also contribute to weight management and metabolic improvements. These findings align with the protective mechanisms of icariin against kidney damage [26]. Additionally, the role of bioactive compounds in promoting overall health and preventing diseases through mechanisms such as inflammation regulation and cellular protection has been well-established. These concepts corroborate the findings of this study on icariin's protective effects on kidney tissues [11-12]

We investigated the effect of icariin on kidneys that were exposed to acrylamide, various studies revealed icariin has a protective role on animal kidneys by inhibiting pathological matrix. The results of our study are aligned with findings from similar research on the role of plant-derived antioxidants. For example, Ajwa dates have been shown to ameliorate nephrotoxicity caused by oxidative stress, supporting the hypothesis that antioxidants can reduce kidney damage through structural recovery in proximal tubules [27]. Additionally, rose hip demonstrated antioxidative and anti-inflammatory properties in a murine model of glomerulonephritis, further confirming the therapeutic potential of plant-based antioxidants in kidney health [17].

Chen et al (2019) investigated whether icariin treatment could improve renal fibrosis associated with chronic kidney disease (CKD). In a unilateral ureteral obstruction (UUO) mouse model, mice were treated with icariin for 17 days. Results showed that icariin significantly reduced pathological changes, collagen deposition, and elevated levels of fibrotic and inflammatory factors in the kidneys while increasing antioxidant enzyme expression. Thus, icariin protects against CKD-associated renal fibrosis through its antifibrotic and anti-inflammatory properties [28]. Zang et al showed that icariin improved kidney function in diabetic rats suffering from nephropathy. Icariin inhibits epithelial mesenchymal transition of renal tubular epithelial cells via regulating the mir-122-5p/foxp2 axis in diabetic nephropathy rats [29]. Shen et al. (2016) mentioned that icariin, like sildenafil, reduces collagen type IV and fibronectin in mesangial cells of glomerular vessels, thus increasing blood supply to kidney tissue [30]. In 2017, Zhang et al. explored the effect of icariin on kidney tissue damage caused by gestational hypertension. They found that icariin has protective effects on nephropathy caused by hypertension during pregnancy due to its antioxidant properties and impact on blood vessels. The study showed that icariin injection reduced urine protein, kidney tissue damage, glomerular lesions, and renal interstitial fibrosis in rats with gestational hypertension [31].

Histopathological results of the kidney tissue in the group receiving acrylamide + icariin revealed that in some areas, tubule disarray and hyperemia were less compared to the acrylamide group. Additionally, the volume of glomeruli in this group increased, urinary space decreased, and necrotic changes decreased. These findings suggest that icariin has the potential to reduce histopathological lesions caused by acrylamide (resulting from oxidative stress) to some extent through its properties.

This study is the first to investigate the protective effects of icariin, a plant-derived product with antioxidant properties, on kidney damage caused by acrylamide in mice. While previous research has primarily focused on acrylamide toxicity and its effects on various bodily systems, this research demonstrates novelty by integrating stereological, biometric, and histopathological methods for a comprehensive assessment of kidney damage. Furthermore, the study introduces icariin as a potential therapeutic agent to mitigate oxidative stress and inflammation-induced kidney damage, providing a new perspective for treating acrylamide-induced complications.

CONCLUSION

The findings from this study highlight the protective role of icariin against acrylamide-induced renal damage in mice. Acrylamide exposure led to significant reductions in key biometric and stereological parameters of the kidneys, including the cortex and medulla volumes, indicating substantial tissue damage. The administration of icariin not only mitigated these adverse effects but also improved renal health by reducing necrotic changes and preserving kidney structure. These results suggest that icariin, with its strong antioxidant properties, has potential therapeutic value in alleviating kidney complications caused by acrylamide exposure. Further studies are warranted to explore its precise mechanisms of action and potential clinical applications for human health.

Abbreviations: PDEs: phosphodiesterase; TAC: total antioxidant capacity, MDA: malondialdehyde; CKD: chronic kidney disease; UUO: unilateral ureteral obstruction

Competing interest: There are no conflicts of interest to declare.

Author contribution: MH analyzed data and edited and revised the manuscript. MP, MR, and FR designed the study. FR obtained funding, designed the study, performed the experiments, analyzed data, wrote the manuscript, and provided overall supervision. All authors have read and approved the final manuscript.

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