Stereological Survey of the Effect of Vitamin C on Neonatal Rat Kidney Tissue Treated With Acrylamide

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KEYWORDS	ABSTRACT

ABSTRACT

***Background and Objectives*:** Acrylamide (ACR) is a known carcinogenic chemical agent found in some foods at considerably high concentration. The aim of this study was to investigate the protective effects of vitamin C as an antioxidant on kidney tissue in rats treated with ACR.

***Methods*:** Twenty female Wistar rats (200-220 grams) were divided into 4 groups (n=5) of control group, ACR group, vitamin C group and ACR+vitamin C group. Pregnant rats were orally administered 10 mg/kg ACR and/or 200 mg/kg vitamin C. Six infants at day 21 after birth were randomly selected and weighted with hematoxylin and eosine and periodic acid staining and studied using stereological methods. Data was analyzed using one way ANOVA and LSD test and means difference were considered significant at P<0.05.

***Results*:** Mean body weight, kidney weight, kidney volume, the volume of cortex and medulla, number of glomeruli and thickness of medulla significantly decreased in ACR group compared to the controls (P<0.001). The thickness of cortex also decreased in ACR group compared to the control group (P<0.05). In vitamin C group, body weight, kidney volume and number of glomeruli increased compared to the control group (P<0.001). In vitamin C group, increased kidney weight, thickness of medulla, volume of cortex and glomeruli were observed compared to the control (P<0.05). In ACR+vitamin C group, this reduction was less significant compared to the ACR group.

Conclusion: Vitamin C as an antioxidant can protect the kidneys from ACR induced tissue damage.

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Introduction

In recent years, harmful effect of acrylamide (ACR) in foods has attracted much attention (1). ACR (C3H5NO) is a white, odorless, crystalline solid at room temperature (2). It has small hydrophilic vinyl monomer molecules with many chemical and industrial applications (3). The International Agency for Research on Cancer classified ACR as a probable human carcinogen on the basis of its carcinogenicity in rodents (4). Before 2002, exposure to ACR had occurred mainly by exposure in workers, furthermore by smoking and expenditure of water and consumption of cosmetic products (5, 6). However in 2002, scientists reported its presence in carbohydrate-rich foods produced at high temperatures (higher than 200°C), especially when asparagines react with sugar (4, 7). ACR can undergo oxidative biotransformation by cytochrome P450 (8). The eventuate metabolite is an epoxide formation like glycidamide that is more passive for proteins and DNA than the original compound ACR (9). ACR also crosses placenta to

developing fetus significantly, leading to direct prenatal and postnatal anomalies (10). ACR is existent in foods at extremely higher condensation up to other popular food carcinogens (11). ACR quickly metabolizes in body and its metabolites are mostly excreted through the urine (12). The antioxidant and free radical scavenger ascorbic acid (vitamin C) has a protective effect against drug-induced nephrotoxicity in animals (13, 14). In humans, nephrogenesis happens within embryonic period and stops after geniture. However, in rats it happens within the latter term of pregnancy and follow out since geniture (15). Interference within the third trimester embryonic development has been demonstrated to modify nephron development (16). Kidney volume and the thickness of cortex is clinically important because kidney size gives insight into kidney function (17). The volume of biological structures can be obtained by the cavalirie principle. Methods of stereological counting are used for quantitative analysis of three-dimensional structures and can analyze cell number and size at microscopic level (18). Number estimation of kidney structures may have great impact to study development, growth and transformation of the kidney (19). The present study was conducted to evaluate the histopathological effect of ACR treatment on development of kidney in rat and also to determine the effect of vitamin C as an antioxidant.

Material and Methods
Animals and Treatment

We used 20 female and 10 male Wistar rats (50-60 days old, 200-220 grams). The male rats were used for fertilization of female ones. Gestation was authenticated by perception of vaginal plaque. Female pregnant rats were randomly divided into 4 groups as control group, ACR group, vitamin C group and ACR+ vitamin C group. Rats in the control group were preserved under normal condition of diet and water. In the experimental group, we used 10mg/kg/day ACR and 200 mg/kg/day vitamin C orally from 7th day of gestation and continued up to 21 days after delivery.

Surgical Procedure

At 21th day, 5 neonates were selected from each group and their weight measured. The rats were then anesthetized under ether and retrograde perfusion trough the left ventricle of heart. The solution for perfusion contained 4% formaldehyde and 1% glutaraldehyde in 0.1 phosphate buffer for 5 minutes. Right kidneys were released from their fatty covering connecting tissue and gently removed. The kidneys were then irrigated with physiologic serum. The weight of the kidneys was measured using analytic scale. Then, the collected kidneys were kept in 10% buffered formaldehyde solution for 10 days (20). All procedures used in this study were approved by the Ethical Committee of Shahid Sadoughi University of Medical Sciences Yazd – Iran.

Volume of cortex, medulla and glomeruli

Following routine histological processing, paraffin kidney blocks were collected and sectioned in identity with systematic random sampling. Depending on the size of kidneys, 11-13 5μ-thick samples were obtained, and every tenth section selected from all kidneys. All samples were stained with hematoxylin and eosine (H&E) method. A calibrated micrometer eyepiece of microscope was used to measure the thickness of cortex and medulla. The whole kidney was viewed on a light microscope at a magnification of 10× with the image projected on a computer monitor. Volume calculations were performed using Cavalieri's principle. A point grid with 100μ distance between two points was overlaid on the images. Hitting points on the cortex and medulla were counted and then the volume of each component was calculated using the following formula:

$V= t \times a(p) \times \Sigma P / M^2$

Where V refers to volume component of interest, t is the section thickness, a(p) is the area of one point (1000μ), ΣP is the total number of point counted in the component of interest, and M is the linear magnification (21).

Numerical density of glomerul and total number of glomerul

Selection of the physical dissector pairs was performed as described by Stereo. Based on the findings obtained from a pilot study, the first

chosen section and its adjacent section, called a dissector pair, were separated by a distance of 30μ as a rule of physical dissector. According to this rule, the distance between the sections pairs must be about 30–40% of the average projected height of the object of interest (in this study glomeruli) to be estimated. In this way, approximately 15– 20 section pairs were obtained and evaluated. Two consecutive sections were mounted on each slide. Photographs of adjacent sections were taken with a digital camera at a magnification of 400×. An unbiased counting frame was placed on the reference and the look-up sections on the screen of the PC, to perform counting according to the dissector method. The bottom and the left-hand edges of the counting frame were considered to be the forbidden (exclusion) lines together with the extension lines. Other boundaries of the frame that are the top-right edges were considered to be inclusion lines, and any particle that hit these lines or was located inside the frame was counted as a dissector particle. The size of the unbiased counting frame was adjusted to count 100-200 glomeruli from each sample. The dimension of the counting frame on the PC screen was 10×10cm.

Glomeruli seen in the reference section but not in the look-up section were counted.

The mean numerical density of glomeruli was estimated using the following formula:

$$NV = \Sigma Q / t \times a(p)$$

Where ΣQ is the total number of glomerul counted in the reference section, t is the mean section thickness (5 m) and a (p) is the area of the unbiased counting frame.

The total number of glomerul in a whole rat kidney was estimated by the following equation:

$$T_n = N_V \times V_{KIDNEY}$$

Where N_V is the numerical density of glomerul, Tn is total number of glomeruli in the whole kidney calculated using the kidney volume results estimated by the Cavalieri method (22, 23).

Statistical Analysis:

All values expressed as mean ± standard deviation (SD). One way ANOVA and LSD test were used to evaluate the results. Differences with P<0.05 were considered significant.

Results

The results of body and kidney weight, cortex and medulla's thickness for each group are presented in Table 1.

Table1: Comparison between control and experimental groups regarding body weight, kidney weight, cortex and medulla thickness.

Group	Control	Acrylamide	Vitamin C	Acrylamide+Vitamin C
Body Weight (gr)	35.75±6.021	31.25±7.136[*]	38.65±5.737[**]	34.25±7.455
Kidney Weight (gr)	0.302±0.007	0.248±0.115[**]	0.323±0.015[**]	0.268±0.005[**]
Cortex Thickness (mm)	62.25±4.203	57.5±2.082	63±3.742[**]	60.25±1.708[*]
Medulla Thicknes (mm)	148.5±4.203	130.25±5.123	154±4.6900[**]	133.5±4.509[**]

The results are shown as mean±SEM. P<0.05 is indicated by"*" and P<0.001 by"**".

Histopathological observations:

Comparative histopathological analysis of tissue samples from each group was performed by light microscope studies, to evaluate the effects of ACR and vitamin C treatment. In control and vitamin C groups, glomeruli had normal structures. In ACR group, hyalinized glomeruli and degeneration was seen. Damage to the kidney tubules was observed as vacuolated cells. These changes were associated with destruction of all the renal parenchymal tissue in ACR group. In ACR+vitamin C group, these changes caused by ACR did not decrease (Figures 1, 2, 3).

Volume of kidney structures:

Stereological investigation in the four groups showed that total volume of the kidneys in ACR group was significantly decreased compared to the controls (P<0.001). The mean volume of kidneys in vitamin C group was significantly higher than the control group (P<0.001). In ACR+vitamin C group, compared to control, mean volume of the kidneys decreased but not significant (P=0.1) (Figure 4).
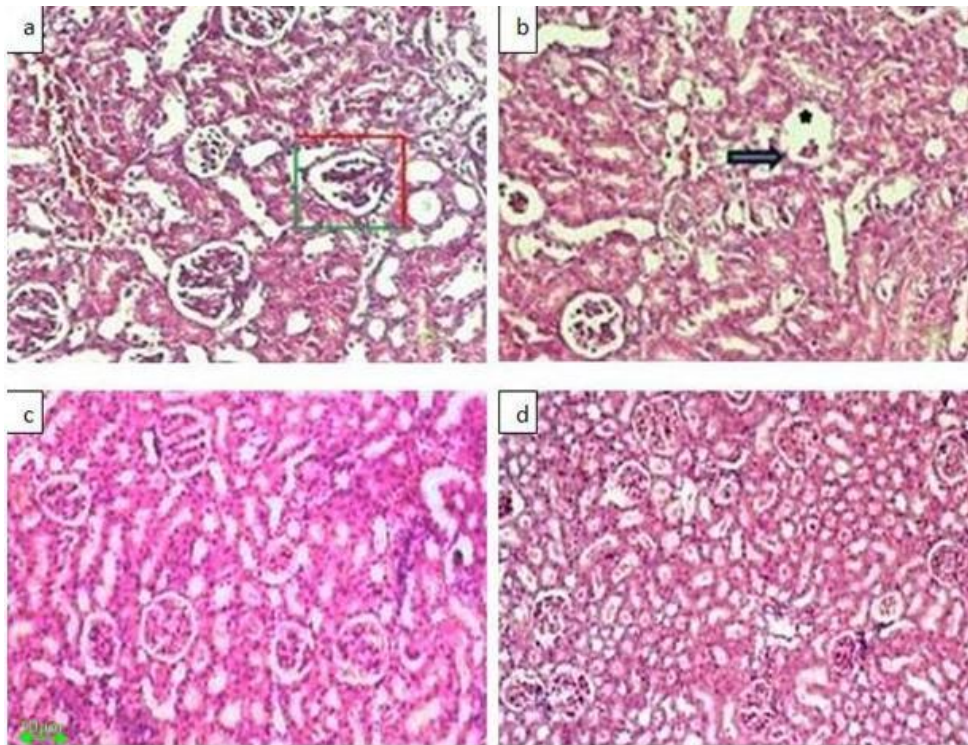
Figure1: Light photomicrograph ACR changes and estimation of the number of glomeruli using grid and physical dissector method. Forbidden sides and allowed to count (red and green) are indicated. There were no histological changes in the control group (a), but in ACR group (b) the glomeruli demonstrated fibrosis, and shrinkage resulting in crowding of hyalinised glomeruli (arrow) and widening of the Bowman's space (star). Vitamin C (c) glomeruli had normal structures. In ACR+ vitamin C, number of glomeruli increased compared to ACR group (d). H&E×400. Scale Bar:20μm
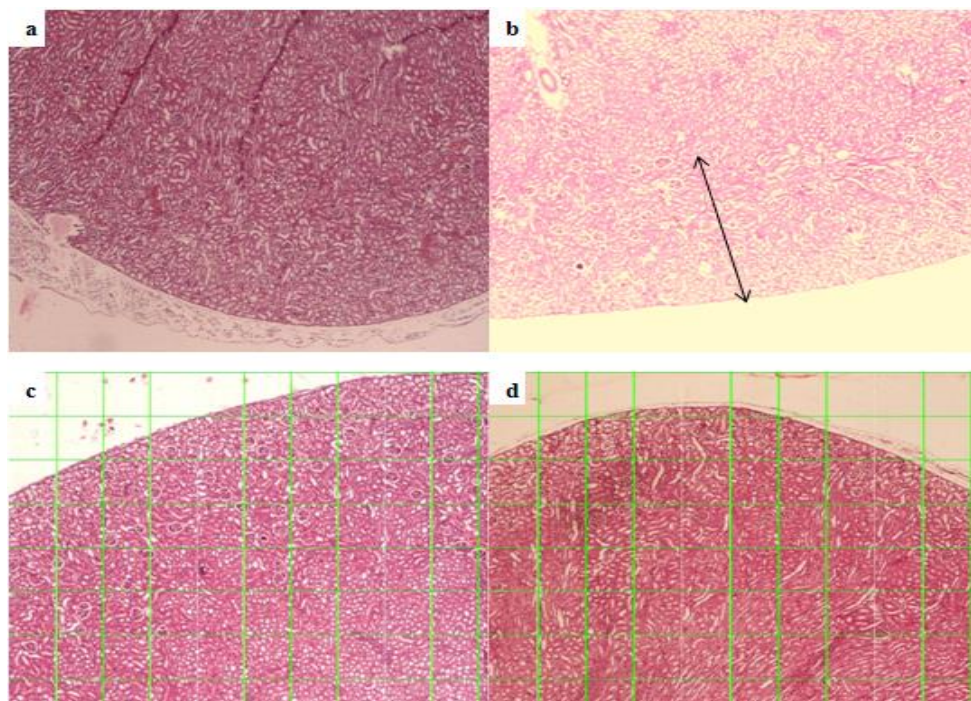
Figure2: light photomicrographs estimating volume cortex, medulla and whole kidney by grid and physical dissector method. Cortex and medullar volume in ACR group decreased and in vitamin C group increased compared to the control. In ACR+vitamin C group this reduction was less significant compared to ACR group. Thickness of cortex is shown by arrow. a)control ,b) ACR, c)vitamin C, d) ACR+ vitamin C. H&E×400. Scale Bar:20μm

Estimated volume of cortex by stereological analysis in the four groups revealed that cortex volume in ACR and ACR+vitamin C group were less than the control group (P<0.001), while cortex volume in vitamin C group was significantly higher than the control (P<0.05). (Figure 5)

Estimated volume of medulla by stereological analysis demonstrated that mean medullar volume in ACR group was less than the control group (P<0.001). Medullar volume in ACR+vitamin C group was also decreased but it was P<0.05. In vitamin C group, medullar volume was more than the control group but the difference was not significant (P= 0.2). (Figure 6)

Stereological investigation for estimating the volume of glomeruli showed that mean glomeruli volume in ACR group was significantly decreased compared to the control group (P<0.001). It was also decreased in ACR+vitamin C group but not significant (P= 0.4). In vitamin C group, mean glomeruli volume was more than control (P<0.05). (Figure 7)
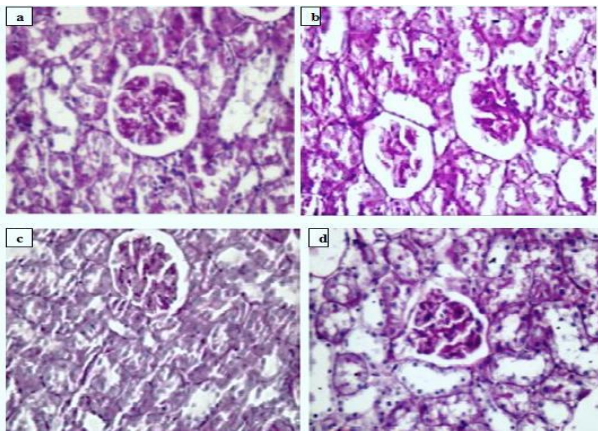
Figure3: Light photomicrograph of different groups. a) control: normal glomerulus and kidney tissue, b)ACR: tubular injury with thickened basement membrane, c) vitamin C: normal basement membrane and glomerulus, d)ACR+ vitamin C: reduces shrinkage of glomerulus after treatment. (PAS staining x400). Scale Bar: 25μm.

Estimated number of glomeruli;

The number of glomeruli in ACR (P<0.001) and ACR+vitamin C(P<0.05) groups were decreased compared to the control. Glomerular count in vitamin C group was significantly higher than the control group (P<0.001). (Figure 8)
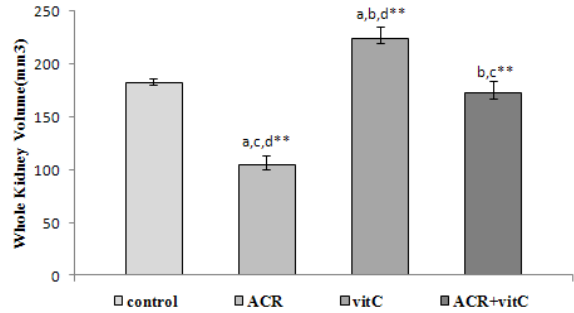
Figure4: Mean whole volume of kidneys (mm^3). Comparison between controls and the other groups (a), comparison between vitamin C group with other groups (b), comparison between ACR+ vitamin C with other groups (c) and comparison between ACR and other groups (d). P<0.05 is indicated by"*" and P<0.001 by"**".

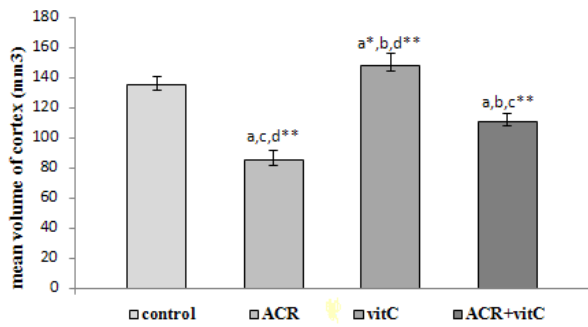**Figure5:** Mean volume of cortex (mm^3). Comparison between control and other groups (a), comparison between vitamin C group and other groups (b), comparison between ACR+ vitamin C and other groups (c) and comparison between ACR and other groups (d). P<0.05 is indicated by "*" and P<0.001 by"**".

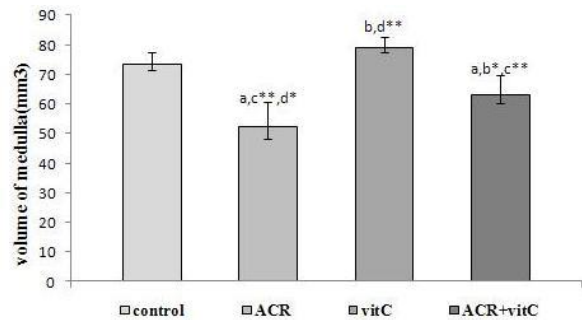**Figure6:** Volume of medulla (mm^3). Comparison between control and other groups (a), comparison between vitamin C groups and other groups (b), comparison between ACR+ vitamin C and other groups (c) and comparison between ACR and other groups (d). P<0.05 is indicated by "*" and P<0.001 by"**".
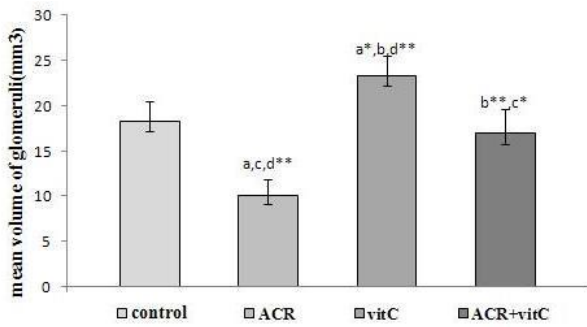
Figure7: Mean volume of glomeruli (mm³). Comparison between control and other groups (a), comparison between vitamin C group and other groups (b), comparison between ACR+ vitamin C and other groups (c) and comparison between ACR and other groups (d). $P<0.05$ is indicated by "*" and $P<0.001$ by"**".
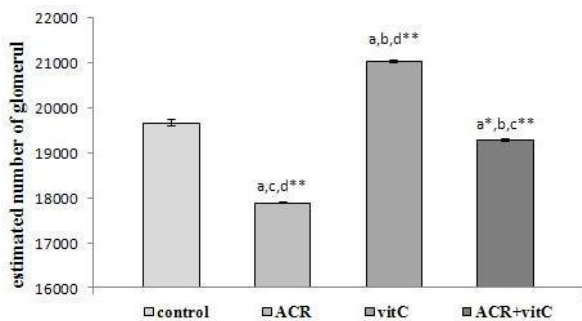

Figure8: Estimated number of glomeruli. Comparison between controls and other groups (a), comparison between vitamin C group and other groups (b), comparison between ACR+ vitamin C and other groups (c) and comparison between ACR and other groups (d). $P<0.05$ is indicated by "*" and $P<0.001$ by"**".

Discussion

In the present study, ACR reduced body weight, kidney weight, thickness of cortex and medulla and volume of kidney. Vitamin C alone increased these parameters compared to the control. Also in ACR+vitamin C group, vitamin C significantly decreased these parameters caused by ACR. ACR is listed by the World Health Organize (WHO) as an eventual human carcinogen (24). According to previous studies, it is supposed that exposure to low doses of ACR could increase the risk of cancer (8). In this study, we demonstrated acute nephrotoxicity supported by histological sections and stereological analysis. The estimated volume obtained from stereological method provided unbiased evidence concerning ACR induced

nephrotoxicity and vitamin C protective effect. Eman M and colleagues reported that 40% to 70% of ACR that attain into blood vessels is excreted via urine within 24 hours in male albino rats (25). This theory may infer that kidney can perform its function to several limits within ACR toxicity. Our research would provide a discernment to verification and collation of hypertrophy, atrophy and formation of tumor in the kidney tissue. Kidneys of rats in ACR group showed inflammatory cells and vascular degenerative changes and necrosis. These findings might have been due to the truth that kidneys are the excretion gateway of ACR and its metabolites. These results were similar to that reported by Mahmood SA et al. (26). WHO reported that in rats, biotransformation of ACR occurs via glutathione expenditure and decarboxylation and at least four urinary metabolites have been found in rats urine (27). Vitamin C as an antioxidant agent can prevent chain reactions of free radicals or the reactive oxygen species before reaching their renal targets (28). Antioxidant are able to reduce the incidence of fragmentation and subsequent rearrangement induced by pesticides (29) Thus, the results of the present study suggest that vitamin C improving effects on kidneys are mediated by prevention of free radicals generation and/or free radical cleaning activity. It is concluded by biochemical and pathological alterations that ACR has toxic effect even at low doses. It is suggested for other researchers to investigate the association between dietary ACR intake and renal, bladder and prostate cancer risk.

Conclusion:

Acrylamide is a nephrotoxic substance and vitamin C as an antioxidant can protect kidneys from ACR induced tissue damage.

Conflict of Interest

Authors declared no conflict of interest.

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