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Protective effect of resveratrol on acrylamide induced renal impairment

Running title: Resveratrol, acrylamide induced renal impairment

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Abstract

Background: Acrylamide (ACR) has a wide range of uses. It possesses a renal impairment effect. The work aimed to study the possible protecting role of resveratrol (RVS) over the ACR-mediated renal impairment in rats. The suggested underlying mechanisms participating in such protection were investigated.
Materials and methods: Thirty Sprague-Dawley adult albino rats were divided into 3 groups, control, ACR, and RVS. After 4 weeks, the kidney was removed, and prepared for histological, immunohistochemical, and biochemical studies. The activity of tissue oxidative (MDA) and anti-oxidative (GSH) markers were assessed.
Results: ACR induced glomerular renal affection in the form of shrinkage and distortion of the glomeruli with wrinkling of their basement membranes and widening of the urinary spaces. Degenerative tubular changes were markedly present in the PCT. The necrotic tubular cells exhibited cytoplasmic vacuolation with desquamated epithelial cells within the tubular lumen. ACR increases the deposition of collagen fibers in the basement membrane of the glomerular capillaries and induced thickening of the basement membranes of the renal corpuscles and renal tubules. The administration of RVS affords high protection to the kidney. The glomeruli and renal tubules were nearly normal. The content of collagen fibers and the PAS reaction of

the basement membrane of the renal tubules were 70 % and 19% lower linked to the ACR group. The creatinine and urea levels decreased by 51%, 47%. RVS induced such a protective role through its antioxidant effect as the MDA level decreased by 45%, while the GSH level increased by 83% compared with the ACR group.
Conclusions: ACR displays the structural and functional affection of the kidney. It induces kidney affection through oxidative stress and apoptosis. With the use of RVS, normal kidney architecture was preserved with little structural affection. Adding, functional kidney test became normal. RVS exerts its protective effect through its anti-apoptotic and antioxidant features.
Key words: resveratrol, acrylamide, kidney

INTRODUCTION

Acrylamide (ACR) is a well-known environmental pollutant that exerts a range of systemic toxic effects on people after both occupational and dietary exposure [2,22]. It possesses a variety of harmful properties; carcinogenicity, genotoxicity, neurotoxicity, and reproductive toxicity [5,7]. CR and its analogs are widely used in various chemical and environmental applications and are produced by heating plant tissue-derived biological material [25]. ACR formation occurs during food processing due to exposure of carbohydrates to temperatures above 200° C [12]. High levels of ACR have been found in foodstuffs commonly consumed, in particular; potato chips and bread [31].

ACR is absorbed from the gastrointestinal tract and dispersed widely in body fluids and stored in the liver and kidney [28]. The ACR is known to cause structural and functional changes in many organs. The renal tubular cells undergo degenerative vacuolar changes, inflammatory cell infiltration and periglomerular edema [28]. Furthermore, ACR administration in rats raises the levels of serum urea, creatinine, uric acid, and renal proinflammatory cytokine [1].

The metabolism of acrylamide triggers the release of free radicals (ROS), which initiates oxidative stress leading to an imbalance in the development and degradation of ROS [19]. It also induces lipid peroxidation and DNA harm [1].

The effects of many antioxidants and anti-inflammatory compounds such as olive oil, vitamin E and 5-aminosalicylic acid were studied to prevent and treat ACR-induced renal impairment [9,19].

Resveratrol (RVS) is a phytoalexin found in at least 72 species of plants, many of which are eaten by humans, including mulberries, peanuts, and grapes [8]. RVS has anti-inflammatory, antiplatelet, antioxidant, and anti-carcinogenic activity, as well as the ability to reduce kidney damage caused by chemical compounds [8,10]. However, it is unclear whether RVS can defend against ACR-induced renal impairment or not. Therefore, we studied the oxidative and apoptotic damaging effect of the ACR over the kidney and investigated the protecting role of RVS over such renal impairment in rats.

MATERIALS AND METHODS

Animals

Thirty Sprague-Dawley adult albino rats weighing 170-200 g were encompassed in our study. The animals were maintained in spacious wire mesh cages in a special room with direct daylight and natural ventilation. The rats had free access to standard rat chow and water.

All the animals were treated according to the standard guidelines for the care and use of laboratory animals. The study was permitted by the Ethics Committee, Faculty of Medicine, Cairo University. The procedures followed were following the ethical standards of the responsible organization and with the Helsinki Declaration of 1975 as revised in 1983.

Experimental design

The rats were distributed into three groups (ten in each group): control (given distilled water at a dose of 1ml), ACR group, and RVS group (concomitant ACR + RVS).

Chemicals

ACR was obtained in a container of powder purchased by Biostain company UK weighing 500 g. It was dissolved in distilled water, in a concentration of 10 g/Liter. It was given at a dose of 1 ml of distilled water containing 40 mg/kg/day orally via gastric gavage [9].

RVS (purity, > 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). It was dissolved in DMSO and diluted in 0.9% physiological saline. It was given at a daily dose of 20 mg/kg/day orally via gastric gavage [18].

By the end of the experiment (after 4 weeks), each animal was weighed, and a blood sample was withdrawn from the tail vein using a fine heparinized capillary tube. The kidney was extracted, washed with saline, and left to dry on a plot paper.

Light microscopic study

Kidney specimens were fixed in formalin 10%, dehydrated in ethyl alcohol, cleared in xylol, and embedded in paraffin wax. Sections of five micrometres thickness were cut and mounted on glass slides. Other sections were mounted on positively charged slides for immunohistochemistry. The sections were subjected to the following:

I. **Hematoxylin & eosin (H &E) and Masson's trichrome stained sections** were prepared according to Suvarna et al. [24].

II. **Histochemical evaluation: Periodic acid Schiff (PAS) stain:** PAS stained sections were prepared according to Suvarna et al. [24].

III. Immunohistochemistry analysis of BAX [20]

Paraffin sections were prepared. Then, a suitable quantity of serum was added to the sections for 30 min. Endogenous peroxidase was inactivated with a methanol solution containing H2O2 (1:50) for 10 min and washed with PBS. The tissue sections were blocked with 1.5% serum for 30 min. The sections were incubated with the primary antibody Bax (anti-human BAX protein, DakoCytomation, Denmark), followed by the secondary antibody (biotinylated link universal from the commercial kit LSAB: DakoCytomation, Denmark). Subsequently, samples were incubated with AB enzymes for 30 min and rinsed in PBS. Positive signals were detected using peroxidase chromogenic substrates. The negative control included PBS instead of the secondary antibody.

Image analysis and morphometric measurements

Using the Leica LAS V3.8 image analyzer computer system (Switzerland), the following parameters were assessed: the diameters of the renal glomeruli and proximal convoluted tubules, the width of the renal space, and the height of the lining proximal tubular epithelium. Adding, the number of structurally altered glomeruli was

assessed as a percentage of the total number of glomeruli. The content of the collagen fibers was also assessed.

In PAS-stained histological sections, the optical density of the basement membrane of the proximal convoluted tubules and the parietal layers of Bowman's capsules was additionally determined.

In the BCL2 immunohistochemical staining area percentage of the immune reaction was also measured.

Biochemical study

Blood samples withdrawn from the rats before sacrifice were used for biochemical assessment in the Biochemistry and Molecular Biology Department, Faculty of Medicine, Cairo University.

1- **The serum urea and creatinine levels** were estimated by the conventional colorimetric method using Quanti Chrom TM Assay Kits (DIUR- 500 and DICT-500), based on the improved Jung and Jaffe methods, respectively [32]. The mean values of these biochemical parameters were calculated and subjected to statistical analysis.

2- **Tissue level of malondialdehyde (MDA), and reduced glutathione (GSH)**

The renal tissue was homogenized in 5–10 ml cold buffer (50mM potassium phosphate, pH 7.5. 1mM EDTA) per gram tissue then it was centrifuged at 100,000 x g for 15 minutes at 4°C. The supernatant was removed for assay and stored on ice.

MDA assay was performed with thiobarbituric acid (TBA) test in the supernatant, according to the method suggested by Buege and Aust [4]. MDA reacts with TBA to give a red compound absorbing at 535 nm.

Measurement of glutathione (GSH) was based on the reduction of 5,5dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione to produce a yellow compound [6].

Statistical analysis

The mean values of relative kidney weight, histomorphometric measurements, and biochemical levels were analyzed using SPSS version 22. Statistical estimation was done using ANOVA followed by Bonferroni pairwise comparisons.

RESULTS

I. Light microscopical evaluation

The examination of the control group showed intact architecture of the renal cortex. The renal cortex is formed of renal corpuscles, and proximal and distal convoluted tubules (Fig 1A).

The examination of the renal cortex of the ACR group revealed marked structural changes. The renal glomeruli showed moderate to marked shrinkage, distortion, segmentation, and vacuolation with widened urinary spaces. The renal tubules were dilated with marked diminution of their epithelial height and widening of their lumina. Their lining cells showed cytoplasmic vacuolation, cellular fragmentation, and intraluminal cast formation were observed in many convoluted tubules. The interstitium showed congested blood vessels with intimal thickening and massive cellular infiltration could be also seen (Figs 1 B-D).

Examination of the RVS group revealed the almost normal appearance of most of the glomeruli and the tubules. Minimal interstitial inflammatory cellular infiltration was encountered (Fig1E).

II. Content of collagen fibres

The content of the fibers was minimal in the control group. The content increased around the parietal layers of Bowman's capsules and the basement membranes of the renal tubules in ACR (nine-fold) and RVS (Two-fold) groups when related to the control group. The content in the RVS group was 70 % lower when related to the ACR group (Fig 2 A-C, Tab 1).

III. Histochemistry of the kidney

In the control group, the basement membranes of the renal tubules and the parietal layers of Bowman's capsules demonstrated a weak PAS reaction. Adding, the apical brush borders of the proximal convoluted tubules (PCT) were intact and partially occluding the tubular lumina (Fig 3A).

The basement membranes of the renal tubules and the parietal layers of Bowman's capsules of the ACR group showed an intense PAS reaction (42% higher than the control group). Adding, the apical brush borders of the PCT were attenuated (Fig 3B). With the use of RVS, the PAS reaction became comparable to the control group and was 19% lower than the ACR group (Fig 3 C, Table 1).

IV. Immunohistochemical staining BAX

BAX showed a weak reaction in the control group (Fig 4A). The area % of BAX immunopositive cells increased 4.5-fold in the ACR group matched to the control group (Fig.4 B-C, Table 1). With the use of RVS, the area % of the immunopositive cells decreased 56% equated to the ACR group; however, the area % in the RVS group was 1.4-fold higher than the control group (Fig. 4D, Table 1).

V. Biochemical and oxidative/antioxidative markers

The serum creatinine and urea levels increased in the ACR group by 2.75-fold, 1.9-fold linked to the control group. With the concomitant use of RVS, the levels of creatinine and urea decreased by 51%, 47% allied to the ACR. Nevertheless, the levels of creatinine and urea in the RVS group were 83%, 55% higher than the control group (Table 1).

The values of the oxidative marker (MDA) in the ACR group increased by 1.4-fold, while the anti-oxidative marker (GSH) decreased by 1.3-fold compared with the control group. With the use of RVS, the MDA level decreased by 45%, while the GSH level increased by 83% compared with the ACR group. Still, the level of both markers was away from the control group (Table 1).

VI. Morphometric glomerular and PCT changes

The percentage of the affected glomeruli in the ACR group increased 8.8-fold matched to the control group. With the use of RVS, the % of the affected glomeruli decreased 71% equated to the ACR group; however, the percentage in the RVS group was 1.6-fold higher than the control group (Table 2).

In the ACR group, the glomerular diameter decreased 58% with an increase in the width of the urinary space 2.4-fold matched to the control group. With the use of RVS, the glomerular diameter increased 1.26-fold with a decrease in the width of the urinary space 57% equated to the ACR group. The glomerular diameter and width of urinary space in RVS and control groups were alike (Table 2).

In the ACR group, the diameter of the PCT increased 31%, while the height of their lining epithelium decreased 62% compared to the control group. With the use of RVS, the diameter of the PCT decreased 18%, while the height of their lining epithelium increased 1.4-fold equated to the ACR group. Both parameters were comparable in RVS and control groups (Table 2).

DISCUSSION

ACR induced glomerular renal affection in the form of shrinkage and distortion of the glomeruli with wrinkling of their basement membranes and widening of the urinary spaces. Adding, degenerative tubular changes were markedly present in the PCT. The necrotic tubular cells exhibited cytoplasmic vacuolation with desquamated epithelial cells within the tubular lumen. Adding, ACR induced massive inflammatory cellular infiltration with congestion of glomerular blood vessels.

ACR induced fibrosis that was established by a nine-fold increase deposition of collagen fibers in the basement membrane of the glomerular capillaries. Such collagen fibers can be the result of the epidermal growth factor that stimulates fibroblast proliferation and collagen synthesis [14].

The basement membranes of the renal corpuscles and renal tubules of the ACR group showed an intense PAS reaction (42% higher than the control group). Thickening of the tubular basement membrane is a common feature of atrophy and may be associated with hyalinosis [14]. Thickening is also a possible cause of reduced active transport in PCT causing micro- albuminuria [14].

The brush border of the PCT in the ACR group was interrupted. Loss of the brush border is the earliest morphological sign of impaired proximal tubular function [17,27]. Furthermore, loss of the brush border affects the reabsorptive power of the PCT with loss of glucose, salts, and large amounts of water in urine [17,27]. This mostly explains the observed serological changes (the elevated serum levels of urea and creatinine). The diameter of the PCT increased 31% in the ACR group which mostly is a compensatory mechanism to conserve the renal function [15].

Oxidative stress is the main pathogenic mechanism through which ACR induces renal damage. Oxidative stress is a shift in the balance between oxidants and antioxidants in favor of oxidants [3]. Many researchers proved the oxidative stress role of ACR over the kidney [23]. The values of the oxidative marker (MDA) in the ACR group increased by 1.4-fold, while the anti-oxidative marker (GSH) decreased by 1.3-fold. Oxidative stress creates oxygen free radical (ROS) that reacts with numerous biomolecules in the cell, leading eventually to oxidative damage [16]. ROS is scavenged by several cellular defense mechanisms involving non-enzymatic (GSH). GSH peroxidase proteins convert hydrogen peroxide to water and lipid peroxides to their respective alcohols [30]. The prolonged use of ACR decreased the activities GSH. This consequence in augmented production of the O_2^- and H_2O_2 that outcomes the production of OH^- [11]. Many researchers believed that MDA's level is sufficient

proof of oxidative stress [13] and higher value of MDA revealed an increase in lipid peroxidation.

Apoptosis is also another pathogenic mechanism through which ACR induced renal affection [23]. BAX reaction increased 4.5-fold in the ACR group. BAX exerts proapoptotic activity [29].

RVS, as one of the flavonoids, affords a high protection to the kidney as the glomeruli and renal tubules were nearly normal. Compared to ACR group, the content of collagen fibres and the PAS reaction of the renal tubules decreased by 70, 19%. Adding, the levels of creatinine and urea decreased by 51, 47%.

Many researchers recorded the exogenous antioxidant protective effect of RVS over the kidney [21]. RVS induced such protective role through its antioxidant effect as the MDA level decreased by 45%, while the GSH level increased by 83% compared with the ACR group. RVS prevents superoxide production from uncoupled endothelial nitric oxide synthase and increases the expression of various antioxidant enzymes [30]. The antioxidant activity of many flavonoids is due to direct scavenging of oxygen-free radicals or excited oxygen species as well as inhibition of oxidative enzymes producing ROS [26].

Another protective mechanism to RVS is through its anti-apoptotic effect. With the use of RVS, the area % of the immunopositive BAX cells decreased 56% equated to the ACR group

In conclusion, ACR displays structural and functional affection of the kidney. It induces kidney affection through oxidative stress and apoptosis. With the use of RVS, the normal kidney architecture was preserved with a little structural affection. RVS exerts its protection through its anti-apoptotic and antioxidant features.

Conflict of interest: None

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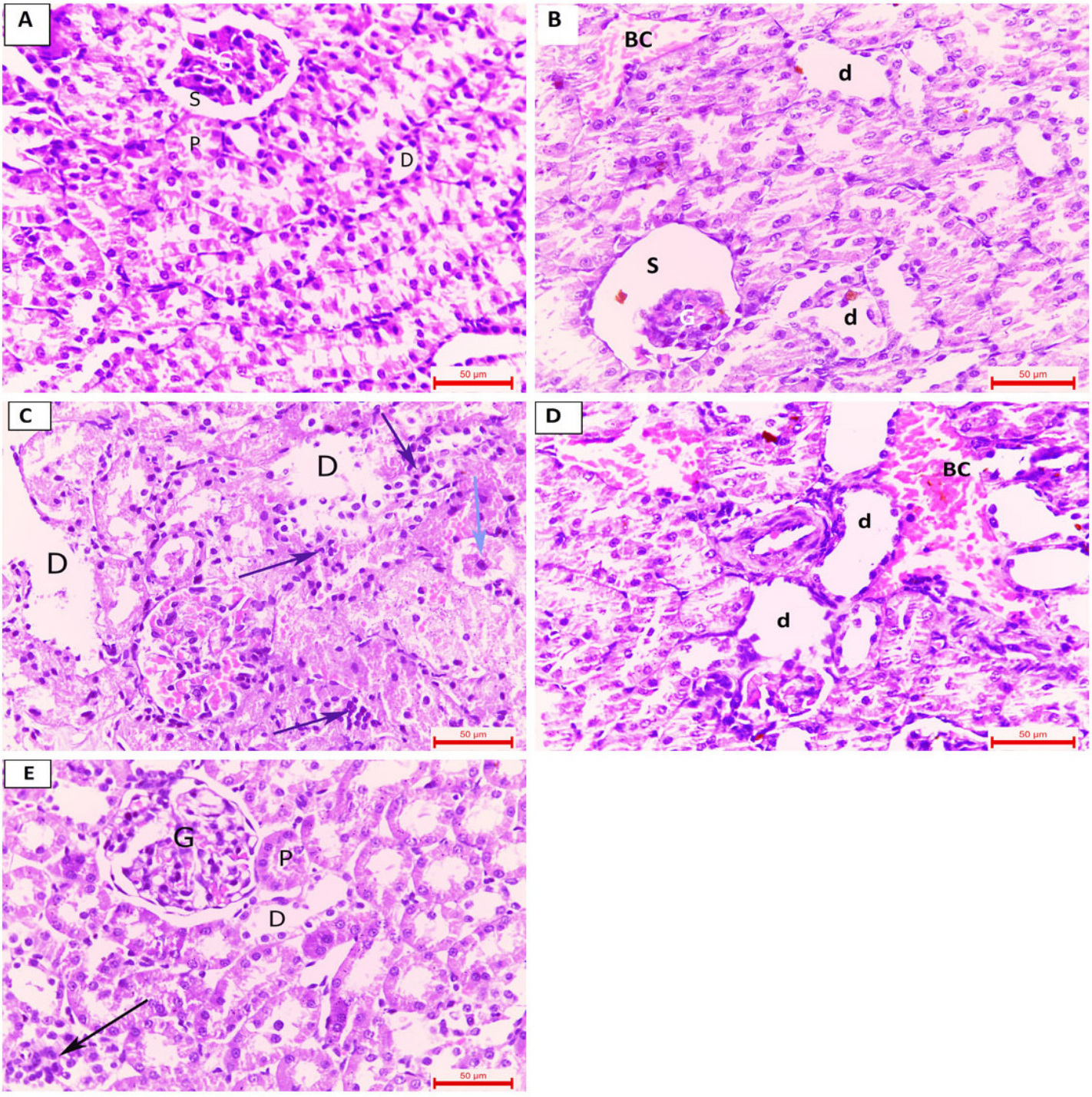
Figure 1. A: Normal renal architecture in the control group showing normal architecture with normal renal glomeruli (G), Bowman's spaces (S), PCT (P), and DCT (D). **B-D:** shrinkage and distortion of renal glomeruli (G) with glomerular congestion and interstitial cellular infiltration (arrow) in the ACR group. Note vacuolation and degenerated nuclei of the PCT (P). **E:** Normal appearance of renal glomeruli (G), Bowman's spaces (S), PCT (P), and DCT of the RVS group. Note minimal interstitial cellular infiltration (arrow) (Hx. & E.; × 400).

Figure 2. A: The control group with normal basement membranes of renal tubules (arrows) and parietal layers of Bowman's capsules (arrow). **B:** ACR group with thickened basement membranes of the renal tubules (arrows) and parietal layer of Bowman's capsules (arrowheads). Note augmented interstitial collagen fibers (arrow). **C:** RVS group with normal appearance of renal tubules (arrows) and parietal layer of Bowman's capsules (arrow). Note a slight increase of interstitial collagen fibers (Masson's trichrome; X400).

Figure 3. A: Apical brush borders (arrowheads) of PCT of the control group occluding the tubular lumina. Note weak PAS reaction of parietal layers of Bowman's capsules and basement membranes of PCT. **B:** intense PAS reaction of parietal layers of Bowman's capsules and the basement membranes of the renal tubules (arrows) of the ACR group. **C:** Moderate PAS reaction of the RVS group (PAS; X400).

Figure 4. A: a negative immunoreactivity of the control group. **B-C:** a positive immunoreactivity of the ACR group. **D:** a negative immunoreactivity of the RVS group (BAX; 400).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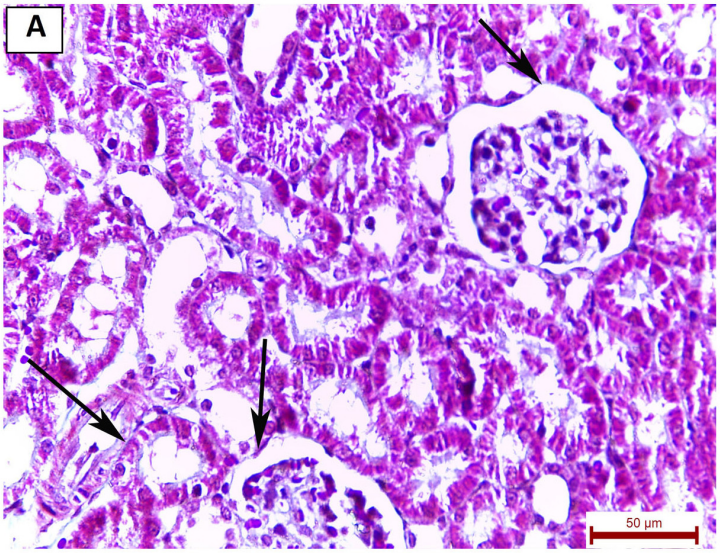
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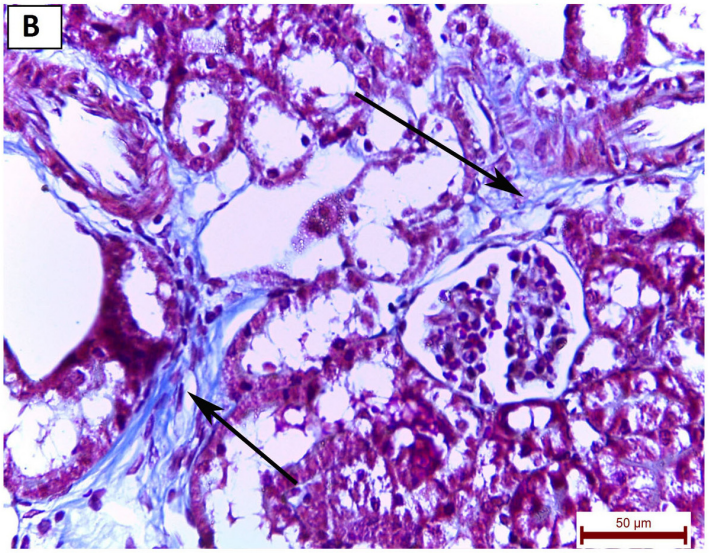
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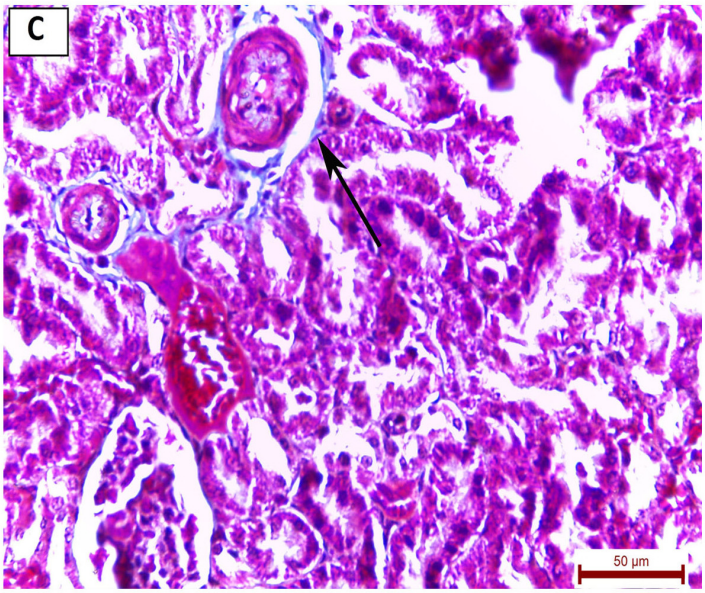
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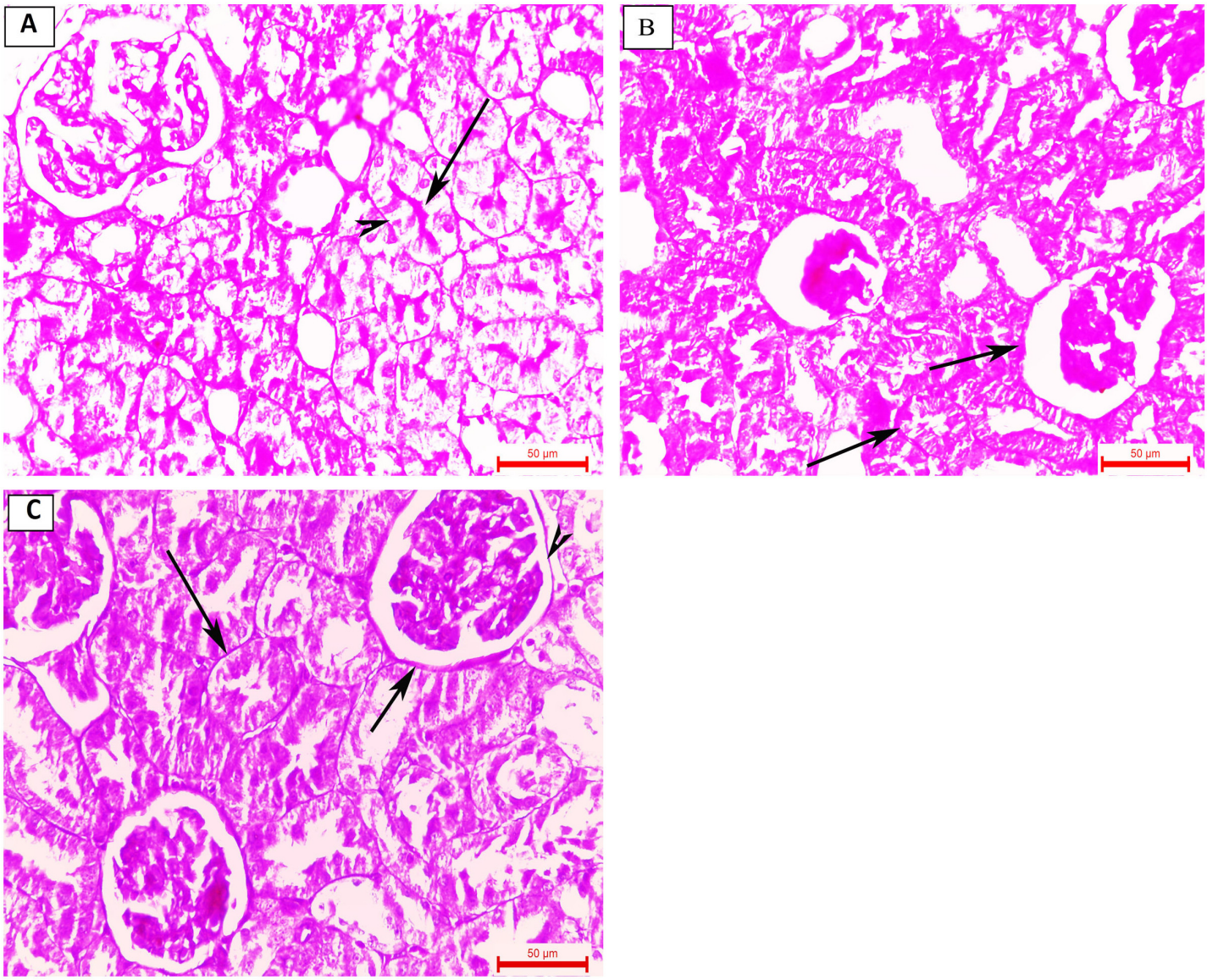
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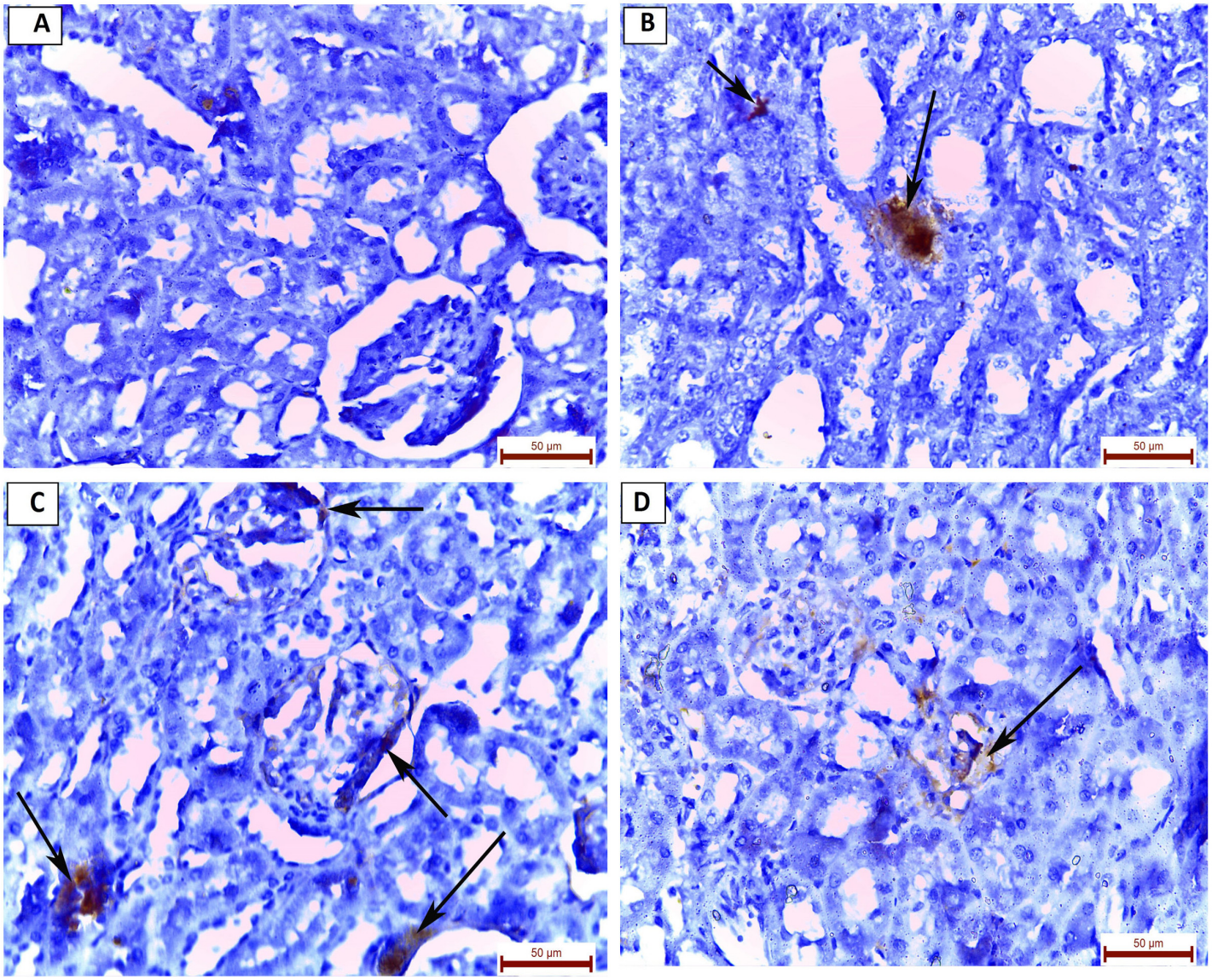
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Table (1): Content of collagen fibres, PAS reaction, apoptotic, biochemical and oxidative/antioxidative markers

Group		Content of collagen fibres	Optical density of PAS reaction	Area % of BAX immunopositive cells	Serum creatinine (mg/dl)	Serum urea (mg/dl)	MDA (nmol/g protein)	GSH (µmol/g protein)
Control	Mean ±SD	**3.51±0.46**	**0.47±0.01**	**1.35± 0.15**	**0.12±0.01**	**36.1±2.0**	**17.52± 2.4**	**0.28±0.02**
ACR group	Mean ±SD	**30.46±0.50**	**0.67±0.01**	**7.45 ± 0.37**	**0.45±0.03**	**106.6±2.2**	**42.18 ±3.6**	**0.12 ±0.02**
	Versus control group	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
	Versus RVS group	0.003*	0.005*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
RVS group	Mean ±SD	**9.63± 0.60**	**0.54± 0.01**	**3.24 ± 0.21**	**0.22±0.03**	**56.6± 2.4**	**23.16 ±1.8**	**0.22 ±0.01**
	Versus control group	0.002*	0.144	0.052	0.007*	< 0.001*	< 0.001*	0.259
	Versus ACR group	0.003*	0.005*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*

*= p-value significant

Table (2): Morphometric glomerular and PCT changes

Group		Percentage of the affected glomeruli	Glomerular diameter (µm)	Width of urinary space (µm)	PCT diameter (µm)	PCT epithelial height (µm)
Control	Mean ±SD	**5.23± 1.78**	**353.29±1.39**	**24.93±1.6**	**180.38±1.24**	**134.59±2.21**
ACR group	Mean ±SD	**49.15± 2.7**	**146.45±2.09**	**83.40±2.05**	**237.13±1.64**	**50.80±2.77**
	Versus control group	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
	Versus RVS group	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*
RVS group	Mean ±SD	**13.2±2.2**	**330.74±1.12**	**35.69± 1.38**	**193.46± 1.35**	**120.64± 3.01**
	Versus control group	0.003*	0.01*	0.01*	< 0.001*	< 0.001*
	Versus ACR group	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*

*= p-value significant