STEREOLOGICAL SURVEY OF THE AMELIORATIVE EFFECTS OF SULFORAPHANE AND QUERCETIN ON RENAL TISSUE IN UNILATERAL URETERAL OBSTRUCTION IN RATS

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SUMMARY – Hydrostatic pressure, which is the result of urinary tract blockage, initiates renal injuries. The injuries are characterized by tubular dilatation and/or atrophy, tubular cell death, inflammatory process and progressive interstitial fibrosis with loss of renal parenchyma. The aim of this study was to evaluate the ameliorative effects of sulforaphane and quercetin, the two natural compounds that can be found in vegetables, in unilateral ureteral obstruction (UUO). Three groups of rats underwent surgery to induce UUO. They received distilled water, sulforaphane (500 μg/animal/day) and quercetin (50 mg/kg/day). Stereological methods were applied in order to obtain accurate, quantitative and comparable data. Less than ~4% of renal structures on average remained intact in UUO rats. After the treatment of UUO rats with quercetin, ~69%, 32%, 65%, 35% and 41% of the volume of the glomeruli, proximal and distal convoluted tubules (PCT and DCT), Henle's loop and collecting ducts remained intact, respectively ($p<0.01$). After the treatment of UUO rats with sulforaphane, ~24%, 45%, and 26% of the volume of the PCT, DCT and Henle's loop remained intact, respectively ($p<0.01$). After the treatment of UUO rats with quercetin, ~71%, 81%, 51%, and 57% of the length of the PCT, DCT, Henle's loop and collecting ducts remained intact, respectively ($p<0.01$). After the treatment of UUO rats with sulforaphane, ~42% and 41% of the length of the PCT and DCT remained intact, respectively ($p<0.01$). Changes in the length of Henle's loop and collecting ducts were not significant. In conclusion, quercetin and sulforaphane were found to be effective in preventing some structural renal damage in the direct obstruction model. Quercetin had a more ameliorative role on renal structures.

Key words: *Kidney; Quercetin; Sulforaphane; Ureteral obstruction*

Introduction

Urinary obstruction (complete or partial) is one of the important reasons of renal failure[1]. Obstruction might be observed after benign prostatic hyperplasia, bladder calculi, urethral stricture, and neoplasm of the bladder, prostate, or urethra[1]. Hydrostatic pressure, which is the result of urinary tract blockage, initiates renal injuries. The injuries are characterized by tubular dilatation and/or atrophy, tubular cell death by apoptosis and necrosis, inflammatory infiltration of leukocytes, fibroblast activation, proliferation, increase in matrix proteins, and progressive interstitial fibrosis with loss of renal parenchyma.

On the other hand, it has been shown that some natural compounds that can be found in vegetables, such as sulforaphane and quercetin, are able to ameliorate the inflammation after experimental uropathy. Sulforaphane (sulforafan) is an isothiocyanate which is found in cruciferous vegetables such as broccoli and broccoli sprouts[2-4]. It has been shown that sulforaphane is able to induce the cytoprotective enzymes and attenuate the cisplatin-induced renal dysfunction

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as well as structural damages. It has also been suggested that sulforaphane can be used as an effective adjunct for the prevention of renal oxidative insults during the ischemia-reperfusion injury.

Quercetin is a bioflavonoid and can be found in vegetables, fruits, and green tea. In addition, it has been reported that quercetin normalizes the paracetamol-induced kidney functions in rats. In another experiment, therapeutic potential of the natural antioxidant quercetin in cyclosporine A-induced nephrotoxicity has been reported[5-7].

Unilateral ureteral obstruction (UUO) is an experimental rat model of renal injury, which imitates the process of obstructive nephropathy in an accelerated manner[1]. There is evidence supporting the key role of the inflammatory process in renal structural changes after uropathy[3]. The aim of this study was to evaluate whether sulforaphane and quercetin induce ameliorative effect on the renal structure of UUO rats. Stereological survey was applied to investigate the structural changes of uropathic kidney including the volume of the cortex, medulla, glomeruli, proximal and distal convoluted tubules (PCT and DCT), Henle tubules, collecting ducts, vessels, and fibrous tissue. In addition, the length of tubules and vessels was estimated. These methods provide accurate, quantitative and comparable data.

Material and Methods

Animals

Twenty-five male rats weighing 160±30 g were selected. The animals were treated according to the standard directive as recommended and approved by the research authorities of Shiraz University of Medical Sciences. The rats were randomly divided into five groups each including five animals. Group 1 was control group and did not receive any intervention. Groups 2, 3, 4 and 5 underwent surgery to induce the UUO. Group 2 received distilled water. Group 3 received sulforaphane (500 μg/animal/day) (i.p.) for five consecutive days[8]. Group 4 received quercetin (50 mg/kg/day) dissolved in a propylene glycol vehicle given by the gavages for four weeks[9]. Group 5 received the vehicle for four weeks. All experiments were done according to the rules set by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran.

Surgical procedure

The rats were anesthetized with ketamine-xylasine (40/5 mg/kg i.p.)[10]. Then, the abdominal cavity was opened by an incision to reach the left ureter. A 3-0 silk ligature was tied around the ureter, and the incision of the skin was closed after that. After four weeks, the rats were anesthetized and their kidneys were removed[10].

Stereological study

Perirenal fat and connective tissue were completely removed from the left kidney. Then the renal pelvis was dissected out, the kidney was weighed, and primary volume, "$V_{primary}$" was measured using the immersion method[11], as briefly illustrated in Figure 1. The kidney was fixed in neutral buffered formaldehyde. As tissue deformations such as the shrinkage produced by fixation, tissue processing, and staining would affect stereological estimation, the shrinkage was estimated. The estimated shrinkage was also used for assessment of the final kidney volume to avoid consecutive sectioning, which is required for the Cavalieri method. Estimation of the shrinkage and the length of tubules and vessels requires isotropic uniform random sections[12-14]. These sections were obtained by the orientator method, as briefly illustrated in Figure 2. The entire kidney was sectioned into slabs with a blade and was placed in the direction of the isotropic uniform

Fig. 1. Immersion method. A jar with distilled water was placed on the scale and weighed (left). Then, the kidney suspended by a thin thread was immersed in the jar so that it was fully covered by water and did not touch the bottom of the jar (right). The new weight in grams minus the weight of the jar and water divided by the specific gravity of distilled water (~1.0) was the volume of the kidney in cubic centimeters (124.90-124.08=0.82/1=820 mm^3).

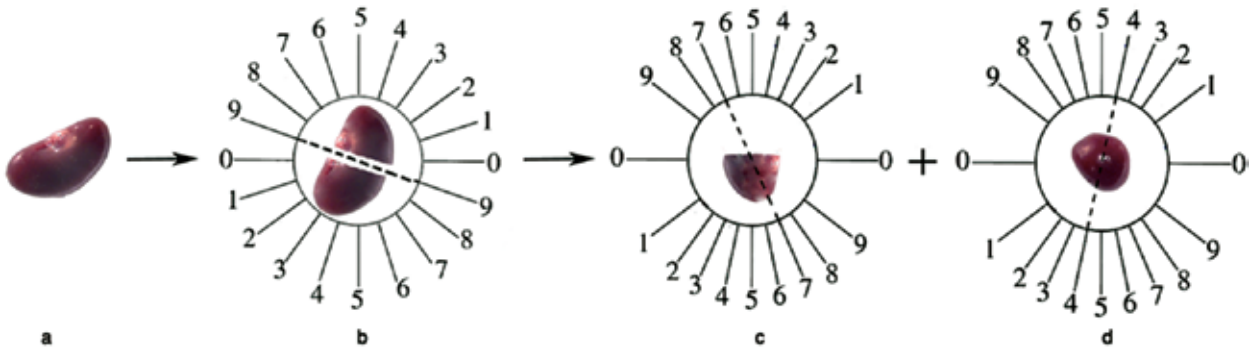

Fig. 2. Orientator method. (a) The kidney was removed and cleaned; (b) it was placed onto a circle each half of which was divided into 10 equal distances. A random number between 0 and 10 was selected and the kidney was sectioned into two halves with a blade on this direction (here 9); (c) the cut surface of one half of the kidney was then placed on the 0–0 direction of the second circle with 10 unequal sinus-weighted divisions, and the second cut was done by selecting a random number (here 7); (d) the cut surface of the other half of the kidney was placed vertically on the second circle. The second cut was done by selecting a random number (here 4).

random cut with an interval of ~0.5 mm. Then, the slabs (9-11 slabs) were collected. A circle was punched from a kidney slab by a trocar. The diameter of the circular piece and the area of the circle were estimated using the usual formula for calculating the area of a circle. The cut surfaces of all slabs and the circular piece were embedded in paraffin and sectioned (5-µm thickness) (Fig. 3). After staining with Heidenhain's trichrome azan, the area of the circular piece was measured again and the volume shrinkage was calculated using the following formula[12]:

Volume shrinkage = 1-(AA-AB)1.5,

where AA and AB represent the area of the circular piece after and before the processing, sectioning and staining, respectively. After estimating the shrinkage, the final volume of the kidney (the reference space) was corrected using the following formula:

$V_{final}=V_{primary} \times$ (1-volume shrinkage)[12].

Fig. 3. Blocking and sectioning of the slabs using a microtome. The selected slabs, the punched circle (right arrow) and the slab which the circle was punched out from (left arrow).

Estimation of the volume and length densities

Each sampled section was analyzed using a video microscopy system, which was made up of a microscope (E-200, Nikon, Japan) linked to a video camera, a computer, and a monitor to determine the parameters. Between 10 and 12 microscopic fields were examined in each kidney at equal intervals along the X- and Y-axis using a stage micrometer. This procedure was continued until all the sections had been studied. The stereological grids were superimposed on the images by means of the stereology software designed at Histomorphometry and Stereology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

The volume density of each structure (the fraction of the unit volume of the kidney which is occupied by the structure) was estimated using the point-counting method (Fig. 4). The volume fraction of the cortex and the medulla was estimated at final magnification of x375 and other parameters at x1500. The length

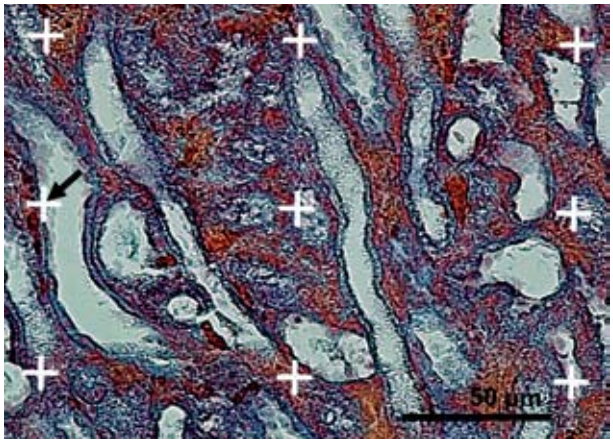

Fig. 4. Estimation of volume density. The stereological probe composed of points was superimposed upon the images of the tissue sections viewed on the monitor, and a fractional volume (V_v) of the renal cortex, the medulla, the glomeruli, the PCT, the DCT, the collecting ducts, Henle's loop, the vessels, and the connective tissue was obtained using the point-counting method and the following formula: $Vv = P_{(structure)}/P_{(reference)}$, where "$P_{structure}$" and "$P_{reference}$" represent the number of test points falling on the structure's profile and on the reference space, respectively. The arrow indicates the point which is the right upper corner of the cross.

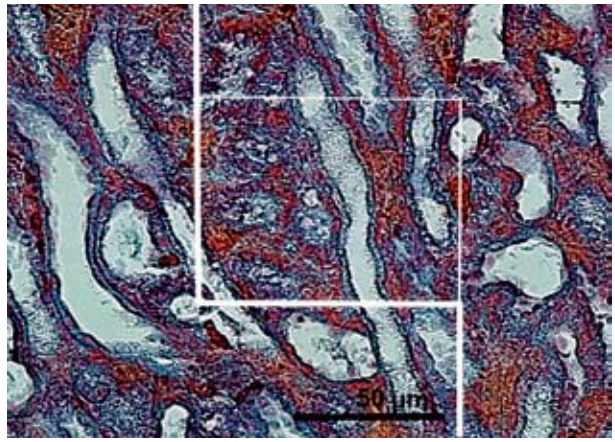

Fig. 5. Estimation of length density. The tubule profiles either completely or partly inside the counting frame, but only touching the top and the right lines were counted. The tubule profiles touching the bottom and the left lines and their extensions were ignored. The length density (L_v) of each tubule was calculated using the following formula: $Lv=2\times\Sigma Q/(a/f)\times(\Sigma f)$, where "ΣQ" denotes the total number of the tubule profiles counted per mouse kidney, "a/f" equals the area associated with a frame, and "Σf " is the total number of counted frames.

density (the length of each tubular structure in the unit volume of the kidney) of PCT and DCT, collecting ducts, Henle's loop, and vessels was estimated by randomly overlaying an unbiased counting frame with an area of 2704 µm^2 on the monitor live images (Fig. 5)[12].

Finally, the total volume of the parameters and the total length of each tubule and vessel were estimated by multiplying the fractional volume or the length density by the final volume of the kidney to prevent the "reference trap"[12-14].

Statistical analysis

Data are reported as mean and SD. Statistical comparisons between the group means were done by Kruskal-Wallis test and Mann-Whitney *U*-test. The level of significance was set at $p \leq 0.05$.

Results

Histopathologic changes

Figure 6 shows comparison of renal structures among different groups. After UUO, the histology of the kidney was disrupted. Tubular atrophy, degeneration, and interstitial fibrosis were observed. These changes were associated with destruction of all the renal parenchymal tissue. After the treatment with sulforaphane and quercetin, the histopathologic changes improved. More tubules remained identifiable. The amount of fibrotic/necrotic tissue decreased after the treatment with sulforaphane and quercetin. The result of the quercetin vehicle (propylene glycol) was the same as the UUO without treatment.

Volume of kidney structures

Data on the volume of the kidney, the cortex and the medulla, presented in Table 1, showed that there were no significant between-group differences.

Table 2 shows total volume of the glomeruli, PCT, DCT, Henle's loop, collecting duct, degenerative tubules, necrotic and fibrotic tissue in particular groups. The volume of the glomeruli, PCT, DCT, Henle's loop, and collecting ducts was reduced in UUO rats. Less than ~4% of these structures had remained intact on the average. After the treat-

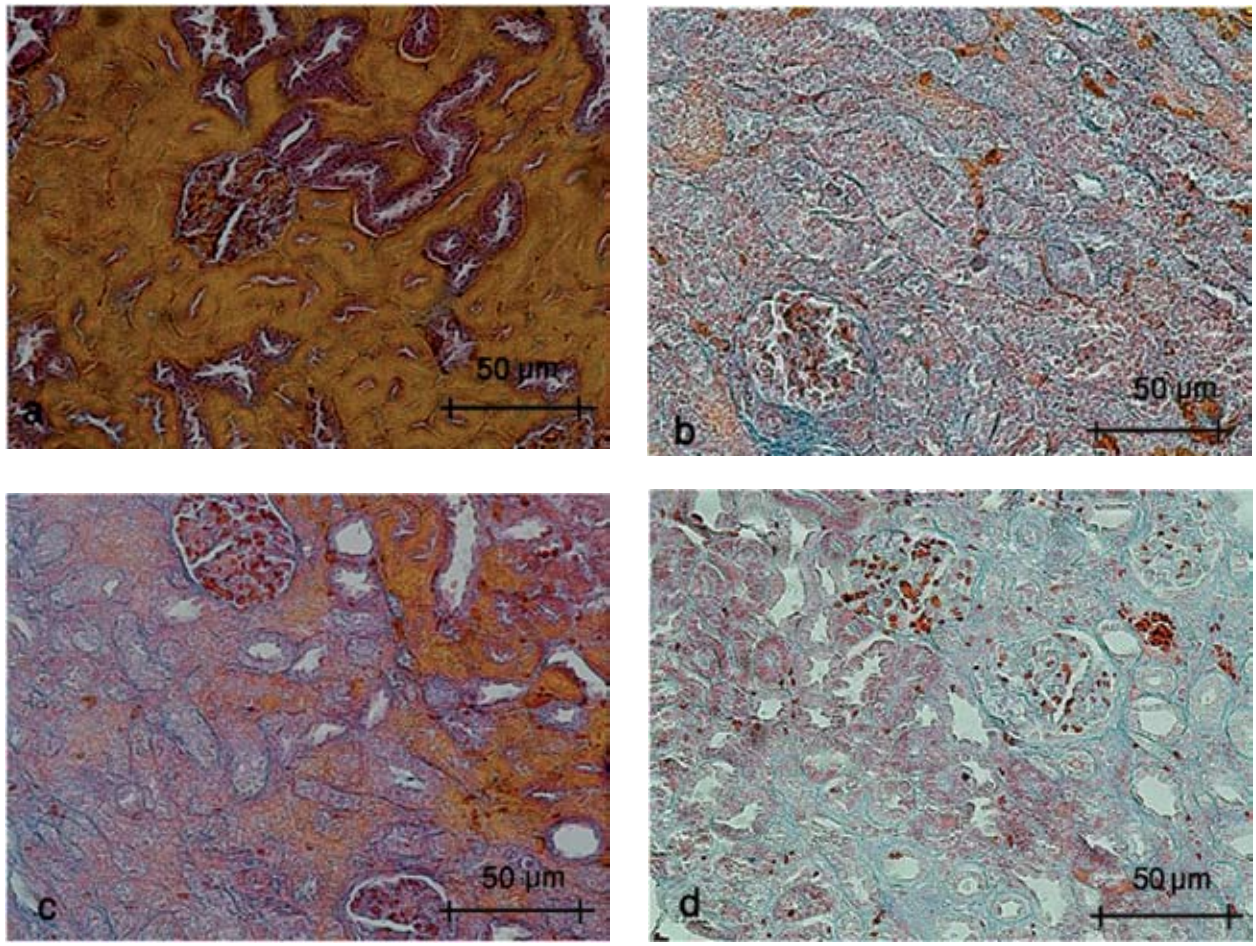

Fig. 6. Comparison of renal tissue in the groups: (a) normal tissue; (b) renal tissue after unilateral ureteral obstruction (UUO); (c) renal tissue after UUO + sulforaphane; and (d) renal tissue after UUO + quercetin

Table 1. Mean ± standard deviation of rat weight (g), kidney weight (mg), and volume (mm^3) in control, unilateral ureteral obstruction (UUO) with or without quercetin and sulforaphane treatment groups

Group	Weight		Volume		
	Animal	Kidney	Kidney	Cortex	Medulla
Control	170±13	482±93	314±55	171±28	142±32
UUO	157±21	538±134	312±12	173±5	131±10
UUO + quercetin	166±11	503±130	336±21	181±11	155±15
UUO + sulforaphane	165±4	604±137	358±20	183±14	174±5
UUO + vehicle	162±26	536±139	316±18	180±15	137±10

ment of UUO rats with quercetin, ~69%, 32%, 65%, 35% and 41% of the glomeruli, PCT, DCT, Henle's loop, collecting ducts remained intact, respectively ($p<0.01$).

Table 2. Mean ± standard deviation of the volume (mm^3) of glomeruli (GLO), proximal and distal convoluted tubules (PCT and DCT), Henle's loop (HL), collecting duct (CD), degenerative tubules (DEG), necrotic and fibrotic tissues (NFT) in control, unilateral ureteral obstruction (UUO) with or without quercetin and sulforaphane treatment groups

Group	GLO	PCT	DCT	HL	CD	DEG	NFT
Control	26±11	205±9	40±13	34±10	39±18	1±1	1±1
UUO	1±1°	1±1*	1±1*	1±1*	2±2°	5±2°	307±14*
UUO + quercetin	18±6	67±14	26±8	12±3	16±4	16±5	203±19
UUO + sulforaphane	6±5	50±8	18±2	9±8	7±4	47±11	194±13
UUO + vehicle	1±1	1±1	1±1	1±1	2±2	53±	312±19

*p<0.01, UUO *vs.* (UUO + quercetin) and (UUO + sulforaphane); °p<0.01, UUO *vs.* (UUO + quercetin)

After the treatment of UUO rats with sulforaphane, ~24%, 45%, and 26% of PCT, DCT, Henle's loop remained intact, respectively (p<0.01). The total volume of the glomeruli and collecting ducts did not change significantly.

As seen in Table 2, in the UUO groups, degenerative tubules and necrotic/fibrotic tissues increased significantly in comparison with the control group. After treating the UUO animals with quercetin and sulforaphane, the volume of degenerative tubules increased ~3- and 9-fold, respectively, in comparison with the UUO rats. After treating the UUO rats with quercetin and sulforaphane, the volume of the fibrotic/necrotic tissues decreased by ~34% and 37%, respectively, in comparison with the UUO rats without treatment (p<0.01).

Table 3. Mean ± standard deviation of the length (m) of proximal and distal convoluted tubules (PCT and DCT), Henle's loop (HL) and collecting duct (CD) in control, unilateral ureteral obstruction (UUO) with or without quercetin and sulforaphane treatment groups

Group	PCT	DCT	HL	CD
Control	83±10	58±14	39±7	52±9
UUO	1±1*	1±1*	1±1°	7±2°
UUO + quercetin	59±4**	47±2**	20±2**	30±5
UUO + sulforaphane	35±2	24±2	4±4	14±3
UUO + vehicle	1±1	1±1	1±1	8±8

*p<0.01, UUO *vs.* (UUO + quercetin) and (UUO + sulforaphane); **p<0.01, (UUO + quercetin) *vs.* (UUO + sulforaphane); °p<0.01, UUO *vs.* (UUO + quercetin)

The length of renal tubular structures

Less than ~3% of the length of these structures remained intact on the average in the UUO rats. After the treatment of UUO rats with quercetin, ~71%, 81%, 51%, and 57% of the PCT, DCT, Henle's loop, and collecting ducts remained intact, respectively (p<0.01).

After the treatment of the UUO rats with sulforaphane, ~42% and 41% of the length of the PCT and DCT remained intact, respectively (p<0.01). The length of Henle's loop and collecting ducts did not change significantly.

Discussion

The present study demonstrated the ameliorative roles of sulforaphane and quercetin in renal tissue damage after the induction of UUO in rats by observing the renal tissue using the stereological method. This quantitative method provided an accurate estimation of renal changes. The volume and the length of the normal or pathologic renal structures were compared in the animals. Reviewing Tables 2 and 3, the question why the volume and the length of degenerative tubules increased in the animals with UUO+ (sulforaphane or quercetin) treatment may arise. This is a positive point because the volume and the length of necrotic tubules and fibrous tissue decreased. It means that quercetin and sulforaphane played an ameliorative role in the process of inflammation. The findings of the present study suggest that quercetin has a more important role. The present study demonstrated the ameliorative effect of sulforaphane on renal structures. The posi-

tive effects of sulforaphane have been reported in cisplatin and ischemia-reperfusion injuries[2-4]. It has been shown that sulforaphane has a renoprotective effect on cisplatin-induced nephrotoxicity[4]. The study has demonstrated that sulforaphane is able to attenuate the oxidative/nitrosative stress, glutathione depletion, enhanced urinary hydrogen peroxide excretion and the decrease in antioxidant enzymes (catalase, glutathione peroxidase and glutathione-S-transferase). Yoon *et al.* report that sulforaphane is able to induce cytoprotective enzymes, and thereby improve the ischemia-reperfusion-induced changes in the lipid hydroperoxide, glutathione, creatinine clearance, kidney weight, and histologic abnormalities[3]. The other compound which was used in the present study was quercetin. Protective effects of quercetin on nephrotoxicity after cyclosporine or cisplatin treatment have been reported[5,6]. These authors report that due to its antioxidant properties, quercetin prevented cyclosporine-induced reactive oxygen species and consequently nephrotoxicity. In a recent article by Yousef *et al.*, it has been shown that quercetin induces a protective property after the paracetamol-induced oxidative injury of the kidney[7]. It can be concluded that sulforaphane or quercetin is effective in preventing some structural renal damages in the direct obstruction model in rats. The volume of the necrotic/fibrotic tissue was found to be less in sulforaphane or quercetin treated groups. Quercetin had a more protective role on renal structures.

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Sažetak

STEREOLOŠKA PROCJENA POVOLJNIH UČINAKA SULFORAFANA I KVERCETINA NA BUBREŽNO TKIVO KOD JEDNOSTRANE OPSTRUKCIJE URETRE ŠTAKORA

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Hidrostatski tlak, koji nastaje zbog blokade mokraćnog trakta, izaziva oštećenje bubrega. Ova oštećenja obilježena su širenjem i/ili atrofijom tubula, smrću tubularnih stanica, upalnim procesom i progresivnom intersticijskom fibrozom uz gubitak bubrežnog parenhima. Cilj ovog istraživanja bio je procijeniti povoljne učinke dvaju prirodnih spojeva koji se nalaze u povrću, sulforafana i kvercetina, kod jednostrane opstrukcije uretre (JOU). Tri skupine štakora podvrgnute su operaciji kako bi se izazvala JOU. Potom su primali destiliranu vodu, sulforafan (500 μg/životinja/dan) i kvercetin (50 mg/kg/dan). Primijenjene su stereološke metode kako bi se dobili točni, kvantitativni i usporedivi podaci. Manje od ~4% bubrežnih struktura ostalo je u prosjeku intaktno kod štakora s JOU. Nakon liječenja JOU štakora kvercetinom intaktno je bilo ~69%, 32%, 65%, 35% i 41% volumena glomerula, proksimalnih i distalnih konvolucijskih tubula (PKT i DKT), Henleovih petlja odnosno zbirnih kanalića ($p<0,01$). Nakon liječenja JOU štakora kvercetinom intaktno je bilo ~71%, 81%, 51% i 57% duljine PKT, DKT, Henleovih petlja odnosno zbirnih kanalića ($p<0,01$). Nakon liječenja JOU štakora sulforafanom intaktno je bilo ~42% i 41% duljine PKT odnosno DKT ($p<0,01$). Promjene u duljini Henleovih petlja i zbirnih kanalića nisu bile značajne. U zaključku, kvercetin i sulforafan učinkovito priječe neka strukturna oštećenja bubrega kod modela izravne opstrukcije. Kvercetin je imao povoljniji učinak na bubrežne strukture.

Key words: *Bubreg; Kvercetin; Sulforafan; Opstrukcija uretre*