## Effect of Red Grape Juice on Renal Glomeruli in Hypercholestremic Rats

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**Abstract:** Hypercholesterolemia accompanies renal disorders and is contributed to the progression of renal diseases. The aim of the present study is to investigate the possible ameliorating effects of red grape juice on renal glomeruli of adult rat fed high cholesterol diet. Sixty male albino rats were divided into three groups (n=20 each). Group I served as control (n=20) and received vehicle (saline) alone, Group II served as the high cholesterol diet (HCD) group fed with a high-cholesterol diet for 8 weeks, and Group II rats were fed HCD along with red grape juice (RGJ) for 8 weeks. Kidney was dissected out, weighted and processed for paraffin blocks. General histological and special stains were performed. Glomerular cross-sectional surface area, the capillary diameter within the glomeruli, the mean glomerular tuft area and Bowman's capsule area of each kidney were measured. Immunohistochemistry assessments for ASMA, desmin, PCNA, eNos and CD68 ; their mean intensity and area percentage of positive glomeruli were measured. HCD resulted in elevated blood glucose, insulin and all serum lipids. It induced mesangial expansion, congestion of glomerular capillaries, thickening of Bowman's capsule and foamy cells in the glomerular tuft and renal fibrosis. HCD induced mesangial-cell activation, podocyte injury, which was associated with eNOs deficiency and increased number of CD68 positive cells in glomeruli and interstitium. RGJ effectively restored most of HCD-induced deleterious effects, suggesting that adding it to diet can play a protective role against renal cortical damage and disturbed serum lipids associated with dietary hypercholesterolemia.

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Key words: hypercholesterolemia, red grape juice, kidney, rat

## 1. Introduction

Abnormalities in lipid metabolism frequently accompany renal diseases and may be important in the pathogenesis of progressive renal diseases. Several studies revealed complex interrelations between hypercholesterolemia and progression of renal damage, showing an association between hypercholesterolemia and the degree of glomerular injury [1,2]. In humans, hyperlipidaemia is considered as a factor contributing to the deterioration of renal function in patients with pre-existing nephropathies [3].

Grape phenolics, including flavonoids and related polyphenols from grape, grape fruit and grape seeds have generated significant interest based on positive reports of their antioxidant properties and ability to serve as free radical scavengers [4]. Some clinical studies have established that grape seed procyanidins and proanthocyanidins are 20 times more potent than vitamin C and 50 times more potent than vitamin E as antioxidants [5].

Previous studies were focused on the effect of hypercholesterolemia on renal tissue for long periods with the end result of focal glomerulosclerosis and proteinuria that progress rapidly to renal failure [6], however, little information is available documenting protective effects of grape on hypercholestremic rat kidney. So, the purpose of the present study is to investigate the possible ameliorating effects of red grape juice on kidney of adult rat fed high cholesterol diet.

# 2. Material and methods

Sixty male albino Wister rats weighing between (225-285 g) were purchased and maintained under the consent of ethical rules of animal house of King Fahd Medical Research Center (KFMRC) - Jeddah, Saudia Arabia, which was in accordance with the guidelines of the Canadian Council on Animal Care. The rats were maintained, with free access to food and water, at a constant temperature of 22 - 24 °C at 55% humidity, with a 12-h light/12-h dark cycle. Animals were acclimatized for one week before starting the experiment.

Rats were randomly divided into three groups. Group I served as control (n=20) and received vehicle (saline) alone, Group II served as the HCD group (n=20) fed with a high-cholesterol diet (rat chow supplemented with 4% cholesterol and 1% cholic acid-HCD), according to Thiruchenduran et al., 2011 [7]. Cholestrol powder was purchased from Sigma Aldrich, St. Louis, MO, USA. for 8 weeks, and Group III (n=20) rats were fed HCD along with red grape juice (RGJ) 50% by gavage (a solution with 50% RGJ and 50% H<sub>2</sub>O) orally for 8 weeks according to Shukitt-Hale et al., 2006 [8]. Red grape was provided from fruits stores at Jeddah and it was exported from Chili. The chemical composition of the 100 g of red grape juice (RGJ) was used in the study was analyzed

in the Analytical Chemistry Unit (ACU) and shown in Table. 1.

| Element                               | Amount | Element                   | Amount |
|---------------------------------------|--------|---------------------------|--------|
| Moist (g/100g)                        | 80.5   | Magnesium (g/100g)        | 0.002  |
| Protein (g/100g)                      | 0.46   | Phosphorus (g/100g)       | 0.06   |
| Carbohydrates (g/100g)                | 28.4   | Vitamin E (g/100g)        | 0.17   |
| Fibers (g/100g)                       | 0.96   | Vitamin B1 (mg/100g)      | 0.09   |
| Mono-unsaturated fatty acids (g/100g) | 0.05   | Vitamin B2 (mg/100g)      | 0.053  |
| Poly-unsaturated fatty acids (g/100g) | 0.03   | Vitamin B3 (mg/100g)      | 0.28   |
| Cholesterol (g/100g)                  | -      | Vitamin C (g/100g)        | 0.0006 |
| Iron (g/100g)                         | 0.0002 | Phenol compounds (g/100g) | 9.4    |
| Potassium (g/100g)                    | 0.15   | Flavones (g/100g)         | 0.042  |
| Calcium (g/100g)                      | 0.01   | Enthocyanin (mg/100g)     | 0.34   |

# **Biochemical study**

Weight gain was measured at the start and end of the experiment. Blood samples were collected under anesthesia through a glass microcapillary tube in retro-orbital region at the beginning and at the end of the experiment for biochemical assessment. Blood glucose level, insulin, lipid profile (triglycerides, cholesterol, low density lipoprotein and high density lipoprotein levels) were measured at the start, during (after 4 weeks from the beginning of the experiment) and at the end (after 8 weeks). Blood glucose level was measured by blood glucose monitoring device (Accu-Chek Active, Roche Diagnostics, Mannheim, Germany) according to Brăslasu et al., 2007 [9]. Insulin concentration was measured in serum using a rat-specific Insulin-Ak ELISA (DPC, Los Angeles, CA, USA [10]. The assay kits for lipid profile were obtained from Randox Laboratories Ltd., Ardmore, Co. Antrim, UK and assessed according to Onyeike et al., 2012 [11].

# Histological study

# General histological examination

At the end of the experiment, animals were sacrificed and kidney was dissected out, weighted and processed for obtaining paraffin blocks. Paraffin sections 5  $\mu$ m thick were stained with hematoxyline and eosin (H&E) for routine histological examination, periodic acid Schiff (PAS) and Masson trichrome [12]. Verhoeff's Van Gieson (EVG) method was used to stain elastic fibers in the wall of the blood vessels of the kidney as well the surrounding connective tissue collagen fibers. The later was appeared red while the elastic fibers appeared black [13].

# Morphometric measurements

Glomerular cross-sectional surface area  $(\mu m^2)$ and the capillary diameter  $(\mu m)$  within the glomeruli were measured in all samples, according to Langheinrich et al., 2010 [14]. The mean glomerular tuft area and Bowman's capsule area of each kidney were obtained by calculating the mean value of 100 glomeruli individual areas measured to evaluate the presence of mesangial expansion. Masson's Trichrome staining was used to tubulointerstial fibrosis quantification [15]. A total of 30 glomeruli were used to calculate the percentage of stained area of each kidney using the Pro plus image analyzer computer system (Media Cybernetics, Rockville, MD, USA).

# Immunohistochemistry

A standard immunohistochemistry staining procedure was performed on neutral buffered formalin-fixed, paraffin-embedded tissue sections (4 µm-thick) according to Pozdzik et al., 2008 & Zhao et al.,2009 [16,17]. Briefly, deparaffinization was performed using xylene and ethanol. Antigen retrieval was achieved by boiling tissue slides with 0.01 M citric buffer in a microwave power for five minutes. Hydrogen peroxide was used to quench the endogenous peroxidase activity. After blocking with 10% serum-Tris buffer, pH 7.5 for 20 minutes at room temperature, the sections were incubated with the primary antibody at room temperature for 120 minutes. Primary antibodies for ASMA, desmin and eNOS,CD68, PCNA and their characteristics were presented in Table 2. Corresponding biotinylated conjugated secondary antibody from Dako staining system was used (Code K0609). DAB staining protocol was used where; slides stained with secondary antibody only were used as negative controls. The nuclei were counterstained with hematoxylin.

# Analysis of immunhistochemistry staining

Labelling intensity was measured in 100 glomeruli in each rat kidney as follows: mean intensity and area percentage of positive glomeruli for ASMA, desmin and eNOS and number of positive cells for CD68, PCNA. Pattern of CD 68 expression

was defined as the cell membrane and cytoplasm staining. The patterns of PCNA expression were defined as nuclear staining. Images analyses were performed with Pro plus image analyzer computer system (Media Cybernetics, Rockville, MD, USA).

| Antibodies                                      | Functional significance   | Clone   | Dilution | Manufacturers                           |
|---|---|---|----------|---|
| α-SMA alpha<br>smooth muscle<br>actin (ASMA)    | α-SMA Actin of the smooth<br>muscle cells and myofibroblast<br>Marker of mesangial cell<br>activation                           | 1A4(amurinemonoclonal antibody toaNH2-terminalsynthetic decapeptide ofASMA) | 1/1000   | DakoCytomation,<br>Heverlee, Belgium    |
| Desmin  | Marker of podocyte activation   | Source: rat D33   | 1/100    | Dako, Trappes,<br>France                |
| eNOS  | the endothelial isoform of nitric<br>oxide synthase (eNOS) has an<br>important role in maintaining<br>normal renal hemodynamics | Source: rat (Polyclonal antibody)   | 1/50     | Abcam, Cambridge,<br>MA, USA            |
| CD68  | Identification of monocytes and tissue macrophages  | Source: rat ED1   | 1/300    | Dako A/S DK-<br>2600Glostrup<br>Denmark |
| PCNA<br>(proliferating cell<br>nuclear antigen) | PCNA Cofactor of polymerases<br>involved in DNA synthesis,<br>recombination, and DNA<br>damage repair processes.                | Source: rat PC10  | 1/600    | DakoCytomation,<br>Heverlee, Belgium.   |

## Table.2: Primary antibodies used in the study and their characteristics

## Statistical analysis

Data were analyzed using the Kruskal–Wallis one-way analysis of variance for non-parametric followed by a post-hoc test to analyze each pair of groups and thereby avoid multiple-comparison effect. The number of cells was expressed as the median values  $\pm$  standard error while the Mean Intensity (MI) and surface area was expressed in mean values  $\pm$ standard error. *P* value less than 0.05 was considered to be significant. Statistical analysis was performed using SPSS statistical software, version 15.0 (SPSS Inc., Chicago, IL, USA) for Windows.

#### **3.Results**

## **Biochemical findings**

Food, water and juice intake and food efficacy during the experiment were shown in Table. 3. Body weight of HCD-fed rats and HCD-fed rats plus RGJ significantly increased at the end of the experiment in comparison to control and to the starting body weight. The weight gain of the rats which received HCD was significantly higher compared to the control. RGJ significantly reduced this weight gain when compared to HCD group. It was observed that the relative weight of kidney in rats which received HCD was significantly lowered compared to both control and rats which received HCD plus RGJ. Table.3

| Table.3: Body weight, water or | grape juice and food | l intakes, weight gain | , food efficacy and | relative kidney |  |
|--------------------------------|----------------------|------------------------|---------------------|-----------------|--|
| weight over the study          |                      |                        |                     |                 |  |

| (reight over the study |                 |                           |                        |                |                             |            |                        |
|------------------------|-----------------|---------------------------|------------------------|----------------|-----------------------------|------------|------------------------|
| Groups                 | Body weight (g) |                           | *Weight gain           | Water or Juice | Food                        | Food       | **Relative weight of   |
|                        |                 |                           | (g)                    | пцаке          | штаке                       | efficacy." | кипеу                  |
|                        | At the start    | At the end                |                        | (ml/d)         | (g/d)                       |            |                        |
| Control                | 240.6±14.4      | 376±14.9                  | 56.9±11.4              | 28.5 ±0.76     | $20.4 \pm 1.1$              | 2.8        | 0.68±0.05              |
| HCD                    | 231±18.9        | 418.6±32.5 <sup>a,d</sup> | 81.8±15.8 <sup>a</sup> | $24.7 \pm 1.1$ | $18.3 \pm 0.7$ <sup>a</sup> | 4.5        | 0.56±0.01 <sup>a</sup> |
| HCD+RGJ                | 262.5±19.1      | 391.1±50.6 <sup>b,d</sup> | 62.4±12.5 °            | $33.8 \pm 0.7$ | $18.1 \pm 0.6^{b}$          | 3.4        | 0.63 ±0.08 °           |

\*Food efficacy=weight gain\food intake \*\* Relative weight of kidney= weight of the kidney/body weight.

a) control versus HCD b) control versus HCD+RGJ c) HCD versus HCD+RGJ d) weight at the start versus weight at the end Significance was considered at P < 0.05.

It was observed that the blood glucose and insulin levels of HCD-fed rats at the end of the experiment were significantly increased compared to their starting level and to the control. RGJ significantly decreased them at the end of the experiment compared to those received HCD alone Figure 1.



Figure 1: Effects of RGJ on serum glucose and insulin levels in different groups. Histograms show changes and differences in both glucose and insulin levels at the start and at the end of the experiment (after 8 weeks). HCD: high cholesterol diet, RGJ: red grape juice. a: (P < 0.05) vs starting level of HCD group, b: (P < 0.05) vs the end level of control group, c: (P < 0.05) vs the end level of HCD group, d: (P < 0.05) vs the end level of HCD group.

Plasma of HCD-fed rats showed significant increase in the cholesterol, triglycerides and LDL levels at the end of the experiment compared to their starting levels and to the control levels. Levels of these lipids was significantly decreased at the end of the experiment in rats received which HCD plus RGJ compared to those which received HCD alone Figure 2. In addition, the plasma of rats which received HCD showed significant decrease in the HDL level compared to it starting level and to the control level and its levels was significantly increased in rats which received HCD plus RGJ compared to those which received HCD alone Figure 2.



**Figure.2**: **Effects of RGJ on lipid profile in HCD rats.** Histogram shows changes and effect of HCD & RGJ in serum lipid profile, cholesterol, triglycerides, LDL and HDL at the start, during (after 4 weeks from the beginning of the experiment) and at the end of the experiments (after 8 weeks).HCD: high cholesterol diet, RGJ: red grape juice. a: (P < 0.05) vs starting level of HCD group, b: (P < 0.05) vs the end level of control group, c: (P < 0.05) vs the end level of HCD group, d: (P < 0.05) vs the end level of control group, f: (P < 0.05) vs the end level of HCD group

## **Histological findings**

The glomeruli of kidney from HCD-fed rats showed mesangial expansion, congestion of glomerular capillaries with aneurysmal dilatation of some of them as well as widening of the Bowman's space when compared to the control. Kidney of HCD-fed rats treated simultaneously with RGJ showed less mesangial expansion compared to HCDfed, rats although most of the glomerular capillaries appeared congested but not dilated (Figure 3 A-C). The small blood vessels in the kidney of HCD-fed rats showed interrupted internal elastic lamina (IEL) and few of them showed dissected aneurysm into the wall with an increase in the surrounded collagen fibers (Figure 3 E). Small renal blood vessels of HCD-fed rats treated with RGJ (Figure 3 F) resembled control group (Figure 3 D) apart from few fat cells infiltrating its wall.

Morphometric measurements showed a significant increase in both glomerular and glomerular capillaries surface areas in kidney of HCD-fed rats (P<0.5) compared to the control. These measurements were significantly decreased (P<0.5) in HCD-fed rats treated with RGJ Figure 3 G,H.



Figure 3: Effect of HCD and RGJ on the histological structure of glomeruli. Glomeruli from control kidney(A) that appears normal, while glomeulus from kidney of HCD rats (B) showing mesangial expansion, congestion of glomerular capillaries (thin arrow), aneurysmal dilatation of some of them (\*) and widening of the Bowman's space (arrow head). Kidney of HCD rats treated with RGJ. (C) showing less mesangial expansion compared to (B), most of the glomerular capillaries still appear congested but not dilated (thin arrow) (H &E). Scale bar =  $20\mu$ m

(D) showing **small blood vessel** in the renal cortex of control rat. The internal elastic lamina (IEL) appears continuous and intact (yellow arrow) and little collagen fibers (red star) was observed around the vessel. This small blood vessel from HCD kidney(E) shows interrupted IEL (yellow arrow) and dissected aneurysm (red arrow head) into the wall. The latter shows rounded fat cells (red arrow) with signet ring appearance. The surrounded collagen fibers (red star) are increased. (F) blood vessel from rat treated with grape appears like the control apart from few fat cells (red arrow) are seen in its wall Verhoeff's Van Gieson (EVG). Scale bar =  $20\mu m$ 

(G) Histogram shows significant increase of glomerular surface area of HCD kidney (\*P<0.05) compared to control. Glomerular surface area of HCD kidney treated with RGJ shows significant decrease (\*\*P<0.05) compared to HCD kidney.

(H) Histogram shows significant increase in surface area of glomerular capillaries of HCD kidney (\*P < 0.05) compared to control. Surface area of glomerular capillaries of HCD kidney treated with RGJ shows significant decrease (\*\*P < 0.05) compared to HCD kidney. HCD: high cholesterol diet, RGJ: red grape juice.

Renal glomeruli of HCD-fed rats stained with PAS showed moderate thickening of Bowman's capsule and some foamy cells in the glomerular tuft and neither of these findings are observed in kidney of HCD-fed rats treated with RGJ Figure 4 A-C. There was significant decrease (P < 0.5) in PAS stained area

in renal glomeruli of HCD-fed rats treated with RGJ compared to HCD-fed rats Figure 4 G. In sections stained with the Masson trichrome, there was an increased in amount of both peri-glomerular and peri-

tubular fibrous tissue in HCD-fed rats Figure 4 D-F. Kidney of HCD-fed rats treated with RGJ showed significant reduction (P < 0.5) in the Masson trichrome stained area compared to untreated rats Figure 4 H.



**Figure 4: Effect of HCD and RGJ on the basement membrane and fibrous content in glomeruli.** Glomeruli from control kidney (A) that appears normal, while glomeulus from kidney of HCD rats (B) showing moderate thickening of the basement membrane of Bowman's capsule (arrow head) and some foamy cells in the glomerular tuft (thin arrow) and neither of these finding are observed in kidney of HCD rats treated with RGJ (C) (PAS stain). Scale bar = 20µm

(D) Control kidney showing normal amount of fibrous tissue around the glomerulus and between the renal tubules, while kidney of HCD rats (E) showing increased amount of both periglomerular and peritubular fibrous tissue (thin arrow). Kidney of HCD rats treated with RGJ (F) showing reduced amount of these collagen fibers compared to HCD rats. Note; dilated and congested periglomerular and peritubular blood capillaries (arrow head) (Masson trichrome stain). Scale bar =  $20\mu$ m

(G) Histogram shows significant increase in PAS stained area in renal glomerli of HCD kidney (\*P < 0.05) compared to control. PAS stained area of HCD kidney treated with RGJ shows significant decrease (\*\*P < 0.05) compared to HCD kidney.

(H) Histogram shows significant increase in MT stained area in renal glomerli of HCD kidney (\*P < 0.05) compared to control. MT stained area of HCD kidney treated with RGJ shows significant decrease (\*\*P < 0.05) compared to HCD kidney. HCD: high cholesterol diet, RGJ: red grape juice.

#### Immunohistochemical findings

Renal glomeruli of the control rats showed negative expression of ASMA in its mesangial cells while those of HCD-fed rats and HCD-fed rats treated with RGJ showed strong expression of ASMA Figure 5 A-C. Glomeruli of control kidney showed weak expression of desmin, while those of the HCD rats showed moderate expression and those of rats treated with RGJ showed weak expression of desmin with a clear reaction in Bowman's capsule which not present in control group Figure 5 D-F. None of the renal glomeruli of the control, HCD-fed rats with RGJ showed positive PCNA staining, while HCD-fed rats showed weak reaction in Bowman's capsule Figure 5 G-I. Mean intensity and area percent of both ASMA and desmin in renal glomeruli were significantly decreased (P<0.5) in HCD rats treated with RGJ compared to the HCD-fed rats Figure 6 A-D.



**Figure (5) Expression of ASMA,Desmin and PCNA in rat glomeruli in different groups.** Control rats: A, D, G; HCD group: B, E, H; HCD + RGJ group: C, F, H. Glomerulus from control kidney showing negative expression of ASMA in mesangial cells (A), while glomeulus from kidney of HCD-fed rats showing strong (thin arrow) to moderate (thick arrow) expression of ASMA (B). Kidney of HCD rats treated with RGJ showing moderate (thick arrow) to weak (arrow head) expression of ASMA in the glomerular mesangial cells (C) (ASMA immunostaining). Glomerulus from control kidney (D) showing negative expression of desmin in podocytes of glomeruli, while that of HCD-fed rats (E) showing moderate expression (thick arrow) and glomeruli from HCD-fed rats treated with RGJ (F) showing weak expression (thin arrow) of desmin (desmin immunostaining). Glomeruli from control kidney (G) and HCD-fed rats treated with RGJ (I) showing negative PCNA staining while HCD-fed rats showed weak PCNA reaction in Bowman's capsule. HCD: high cholesterol diet, RGJ: red grape juice. Scale bar =  $20\mu$ m.



Figure (6): Mean intensity and area percent of ASMA and desmin in renal glomeruli. A-Histogram shows significant increase of mean intesity of ASMA (alpha smooth muscle actin) in renal glomeruli of HCD kidney (\*P < 0.05) compared to control. Mean intesity of ASMA in renal glomeruli of HCD kidney treated with RGJ shows significant decrease (\*\*P < 0.05) compared to HCD kidney. B-Histogram shows significant increase in area percent of ASMA (alph smooth muscle actin) in renal glomeruli of HCD kidney (\*P < 0.05) compared to control. Area percent of ASMA in renal glomeruli of HCD kidney treated with RGJ shows significant decrease (\*\*P < 0.05) compared to Control. Area percent of ASMA in renal glomeruli of HCD kidney treated with RGJ shows significant decrease (\*\*P < 0.05) compared to HCD kidney. C-Histogram shows significant increase in mean intesity of desmin in renal glomeruli of HCD kidney (\*P < 0.05) compared to control. Mean intesity of desmin in renal glomeruli of HCD kidney (\*P < 0.05) compared to control. Mean intesity of desmin in renal glomeruli of HCD kidney (\*P < 0.05) compared to control. Mean intesity of desmin in renal glomeruli of HCD kidney treated (\*P < 0.05) compared to control. Mean intesity of desmin in renal glomeruli of HCD kidney treated with grape shows significant decrease (\*P < 0.05) compared to HCD-fed rats kidney. D-compared to control. Area percent of desmin in renal glomeruli of HCD kidney treated with RGJ shows significant decrease (\*P < 0.05) compared to HCD-fed rats kidney. D-compared to control. Area percent of desmin in renal glomeruli of HCD kidney treated with RGJ shows significant decrease (\*P < 0.05) compared to HCD kidney. HCD: high cholesterol diet, RGJ: red grape juice.

Renal glomeruli of control rats showed strong (3+) expression of eNOS in the cytoplasm of endothelial cells of glomerular and peritubular capillaries and arterioles, while those of HCD-fed rats showed weak (1+) expression and those of treated rats with RGJ showed moderate to weak (2+ to 1+)

eNOS expression Figure 7 A-C. The mean intensity and area percent of eNOS expressed in the renal glomerular capillaries and arterioles of HCD rats treated with RGJ were significantly increased(P < 0.5) compared to the HCD-fed rats Figure 7 D, E.



**Figure (7) Experssion of eNOS in renal glomeruli.** Glomeruli from control kidney (A) showing strong (3+) expression (thin arrow) of eNOS in the cytoplasm of endothelial cells in glomeruli, while those of HCD rats (B) showing weak (1+) expression of eNOS and those of HCD rats (C) treated with RGJ showing moderate to weak (2+ to 1+) expression (arrow head). Note; arterioles and peritubular capillaries (thick head) showing strong expression of eNos in endothelial cytoplam (eNOS immunohistochemistry). Scale bar =  $20\mu m$ .

(D)-Histogram shows significant decrease of mean intesity of eNOS (endothelial nitric oxide synthetase) in renal glomeruli of HCD kidney (\*P < 0.05) compared to control. Mean intesity of eNOS in renal glomeruli of HCD kidney treated with grape shows significant increase (\*\*P < 0.05) compared to HCD kidney. (E)-Histogram shows significant decrease of area percent of eNOS (endothelial nitric oxide synthetase) in renal glomeruli of HCD kidney (\*P < 0.05) compared to control. area percent of eNOS in renal glomeruli of HCD kidney treated with RGJ shows significant increase (\*\*P < 0.05) compared to HCD kidney. HCD: high cholesterol diet, RGJ: red grape juice.

Glomeruli of control kidney showed few CD68+ve macrophages in glomeruli and periglomerular area. The mean number of these cells was significantly (P < 0.5) increased in HCD-fed rats

while, in HCD-fed rats with RGJ reduced significantly their number in both glomeruli (P < 0.5) and interstitium (P < 0.5) of HCD rats with RGJ compared to HCD rats Figure 8 A-E.



**Figure (8) Experssion of CD68 in renal glomeruli.** Glomeruli from control kidney (A) showing no CD68 positive macrophages in glomeruli and periglomerular area. CD68 positive macrophages (arrow) are seen in both glomeruli and periglomerular area of kidneys of both HCD rats (B) and HCD rats treated with grape (C) (CD68 immunohistochemistry). Scale bar =  $20\mu$ m (D)-Histogram shows significant increase of mean number of CD68 positive macrophages in renal glomeruli of HCD kidney (\*P<0.05) compared to control. Mean number of cells in renal glomeruli of HCD kidney treated with grape shows significant decrease (\*\*P<0.05) compared to HCD kidney. (E)-Histogram shows significant increase of mean number of CD68 positive macrophages in renal interstitium of HCD kidney (\*P<0.05) compared to control. Mean number of P<0.05) compared to control. Mean number of CD68 positive macrophages in renal interstitium of HCD kidney (\*P<0.05) compared to control. Mean number of CD68 positive macrophages in renal interstitium of HCD kidney (\*P<0.05) compared to control. Mean number of CD68 positive macrophages in renal interstitium of HCD kidney (\*P<0.05) compared to control. Mean number of cells in renal interstitium of HCD kidney (\*P<0.05) compared to control. Mean number of cells in renal interstitium of HCD kidney treated with RGJ shows significant decrease (\*\*P<0.05) compared to HCD kidney. HCD: high cholesterol diet, RGJ: red grape juice.

## 4. Discussion

In the present study, HCD diet induced significant increase in the weight of the rats. Supplementation HCD with RGJ could significantly reduce this weight gain. It was observed that the relative weight of kidney in HCD-fed rats was significantly lowered compared to both control and HCD-fed rats plus RGJ. [7] Previous study had similar results regarding weight gain[18],on the contrary, other study found that dietary hypercholesterolemia had no effect on body or kidney weight in rats[1].

In this study, it was observed that the blood glucose and insulin levels of HCD-fed rats at the end of the experiment were significantly increased. RGJ significantly decreased them at the end of the experiment compared to those received HCD alone. Similarly, previous study, found that of RGJ had hypoglycemic effect on HCD-fed rats [19]. This due to flavonoids content and these constituents can preserve the insulin-secreting capacity and viability of pancreatic  $\beta$  cells.

Therefore, the presence of these constituents in RGJ may explain the hypoglycemic activity. In agreement to our observations a previous study, reported increase level of glucose and insulin in hamsters received HCD which decreased by grape seed at the end of the study [20].

With regard to serum lipids in this study, the levels of triglycerides, total cholesterol, LDL cholesterol, and VLDL cholesterol were increased, whereas serum HDL-cholesterol level was decreased in HCD-fed group. These results agreed with an earlier study which, reported increased serum lipid abnormalities in HCD-fed rats [1,21]. HCD rat which received RGJ showed significant decrease in levels of these lipids and increase in HDL level.

RGJare rich in phenolic compounds which significantly ameliorated plasma lipid levels. After drinking 100 mL RGJ/day for 14 days, the concentration of cholesterol-standardized tocopherol and antioxidant capacity of plasma were significantly increased, and oxidized LDL and LDL were significantly reduced. The plasma level of HDL was also elevated [22]. In addition, ingestion of concentrated red grape juice as a polyphenolrich dietary supplement exerts hypolipidemic, antioxidant, and anti-inflammatory effects[23].

In this study, RGJ had hyoplipedimic effect which due to polyphenols provided by RGJ may interfere with cholesterol absorption [24], decreasing hepatic cholesterol concentrations, as reported with guinea pigs after supplementation with lyophilized grape powder [25]. As a result of lower hepatic cholesterol concentrations, hepatic LDL receptor expression increases to enhance cholesterol uptake from LDL, thus lowering plasma LDL-C. A reduction in plasma LDL-C was also reported with lyophilized grape powder supplementation in women [26].

This study revealed that hypercholesterolemia induced mesangial expansion, congestion of glomerular capillaries with aneurysmal dilatation and widening of the Bowman's space, a significant increase in both glomerular and glomerular capillaries surface areas, interrupted internal elastic lamina and dissected aneurysm into the wall of the small blood vessels with an increase in the surrounded collagen fibers. Consumption of RGJ by hypercholesterolemic rats resulted in less mesangial expansion and reduced dilatation of the glomerular capillaries and resulted in a significant decrease in both glomerular and glomerular capillaries surface areas. In accordance to these findings, researchers reported that ApoE null mice fed the HCD developed mesangial expansion characterized by an increase in mesangial area [27]. Also, this was accompanied by a glomerular inflammatory process as demonstrated by the presence of foam cells. On the other hand, another study, found that the glomerular tuft and Bowman's capsule areas and glomeruli number showed were not affected by hypercholesterolemia in ApoE mice [15].

In this study, hypercholesterolemia induced thickening of Bowman's capsule and foamy cells in the glomerular tuft. HCD-fed rats treated with RGJ significantly decrease in PAS stained area in renal glomeruli with no histopathological changes in glomeruli. Similar results were reported regarding hypercholesterolemia in ApoE mice induced mesangial expansion[15,28]. In the current study, hypercholesterolemia resulted in increased amount of both peri-glomerular and peri-tubular fibrous tissue in rats. RGJ intake with HCD reduced renal fibrosis. Hypercholesterolemia in ApoE mice resulted in increased in renal fibrosis [15].

In accordance with these data, [29,30] observed marked renal fibrosis in young hypercholesterolemic animals. Dyslipidemia can cause renal fibrosis due to increase in extracellular matrix deposition and reduced matrix degradation. The increase in collagen synthesis and activation of pro-inflammatory pathways by oxidized low density lipoprotein (LDL) could be responsible for increased matrix deposition.

Mesangial cells can be a target cell of hyperlipidaemia and may play a role in glomerular injury since they have a major contribution to the extracellular matrix production. The  $\alpha$ -smoothmuscle actin expression level is usually used as an index of mesangial-cell activation [27]. In this study, renal glomeruli of both HCD-fed rats with or without RGJ showed strong expression of ASMA. On contrary, another study, reported that, no mesangialcell activation as observed by  $\alpha$  -smooth-muscle actin labelling, in the hypercholesterolemic rats[27].

Desmin, an intermediate filament protein, has been long suggested as a podocyte injury indicator, the expression of which is often unregulated in various glomerular diseases in which podocyte damage is involved [31]. In the present work, glomeruli of HCD-fed rats showed moderate expression of desmin, while HCD rats treated with RGJ showed weak expression of desmin. The podocyte injury observed in this study was in accordance with previous studies, who investigated the effect of HCD diet in rats[1,32]. These observations were confirmed with measuring the mean intensity and area percent of both ASMA and desmin in renal glomeruli which were significantly decreased in rats received HCD treated with RGJ.

PCNA is cofactor of polymerases which involved in DNA synthesis, recombination, and DNA damage repair processes, maker of cell proliferation. In the present study, only HCD-fed rats glomeruli showed weak PCNA reaction in Bowman's capsule. In apoE null mice PCNA labelling did not detect any cell proliferation neither in control nor in HCD group which, indicating that the mesangial cells did not proliferate [27].

The endothelial isoform of nitric oxide synthase (eNOS) has an important role in maintaining normal renal hemodynamics which, expressed in mesangial cells [33]. Mesangial cell contractility is known to influence podocyte conformation and hence also influences podocyte stress. In the present study, renal glomeruli of HCD-fed rats showed weak expression while HCD treated rats with RGJ showed moderate eNOS expression. The mean intensity and area percent of eNOS expressed in the renal glomerular capillaries and arterioles of HCD-fed rats treated with RGJ were significantly increased compared to the HCD-fed rats. Similarly, hypercholesterolemia increased podocyte stress, which was associated with eNOs deficiency. Furthermore, hypercholesterolemia decreased renal cortical eNOS activity[32].

CD68 is used for Identification of monocytes and tissue macrophages. In the present study, the mean number of these cells was significantly increased in HCD-fed rats and fortunately RGJ could significantly reduce their number in both glomeruli and interstitium. In agreement with these findings, [1] reported that, in HCD-fed rats; glomerular macrophages and plasma cholesterol showed marked parallel increases. Glomerular macrophage accumulation is certainly an early effect of dietary hypercholesterolemia in rats [29,34]. Studies in a variety of animal models have shown that hypercholesterolemia accelerates the rate of progression of kidney disease [35].

Regarding the mechanisms by which abnormal serum lipid levels may contribute to renal disease progression, there is evidence that circulating lipids bind to and become trapped by extracellular matrix molecules [35], where they undergo oxidation increasing the formation of reactive oxygen species such as superoxide anion and hydrogen peroxide [36]. RGJ ameliorates hazards of HCD on renal structures as grape phenolic compounds had antioxidative characteristics, including scavenging of free radicals, inhibition of lipid oxidation, reduction of hydroperoxide formation [37]. Moreover, immunomodulation was the main pathway, and antioxidative action was another pathway for the antiinflammation effect of grape phenolics. Inhibition or reduction of the cytokine gene expression may be a basic pathway to antiinflammation for grape phenolics [38].

Procyanidins in grapes inhibited the increase of C-reaction protein in rat plasma induced by high fat feed. It inhibited inflammation at mRNA levels, and major health benefits brought by them involved in decreasing the risk of diseases link to high fat diets and obesity, such as cardiovascular and metabolic disorders RGJ supplementation [38].

In conclusion, in this study HCD resulted in elevated blood glucose, insulin and all serum lipid profile. It induced mesangial expansion, congestion of glomerular capillaries, widening of the Bowman's capsule, thickening of Bowman's capsule and foamy cells in the glomerular tuft and renal fibrosis. HCD induced mesangial-cell activation, podocyte injury, which was associated with eNOs deficiency and increase number of CD68 positive cells in glomeruli and interstitium. RGJ effectively restored most of HCD-induced deleterious effects, suggesting that adding it to diet can play a protective role against renal cortical damage and disturbed serum lipids associated with dietary hypercholesterolemia.

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