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Renoprotective effect of red grape (*Vitis vinifera* L.) juice and dark raisins against hypercholesterolemia-induced tubular renal affection in albino rats

**Short running title:** Renoprotection by red grape and dark raisins in hypercholesterolemic rats

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**Abstract:**

**Background:** Red grape juice (RGJ) and dark raisins (DR) are rich in polyphenols and antioxidants. This study aimed to assess the efficacy of RGJ and DR in protecting the renal tubules against hypercholesteremic-induced pathological changes.

**Materials and methods:** Forty albino rats divided into 4 groups (n=10) were utilized in this study. They included the control, High cholesterol diet (HCD)-fed, HCD+RGJ-fed, HCD+DR fed groups. Body weight gain, food and water intake, blood and insulin levels, lipid profile and kidney functions were assessed at the start of the experiment and after 12 weeks. The right kidney was dissected out and processed for both light and electron microscopic examination. Desmin and cytokeratin antibodies were utilized as histologic markers to assess the integrity of the proximal (PTs) and distal tubules (DTs) of the kidney.

**Results:** Administration of HCD resulted in hypercholesteremia in rats as evident by the lipid profile. The PTs of hypercholesteremic rats appeared dilated with hyaline casts and mitochondria in most of the tubular cells were affected. Immunohistochemical assessment revealed affection of both PTs and DTs. Both RGJ and DR, when administrated along with the HCD for 12 weeks, improved the lipid profile, kidney functions as well as the histologic and cellular changes-induced by hypercholesterolemia in the rats. The effect of raisins was superior to RGJ which might be due to its high contents of fibers and proteins.
Conclusions: This study highlighted the importance of supplementation of red grape and raisins in protection against the harmful effects induced by deposition of fat on the renal tubules structure and function.

Key words: kidney-grapes-raisins-hypercholesterolemia-function-structure

Introduction

Hypercholesteremia became the most frequently encountered medical problem worldwide. Among its causes are the bad dietary habits and increased dependence on fast food [9]. Hypercholesteremia was reported to be linked to liver disease [42], cardiovascular diseases [28] and brain stroke [26]. Dyslipidemia could be induced in an animal model when fed high fat diet [20]. This facilitates the study of the effect of this metabolic disorder on the structure and function of different body organs and the test of the efficacy of new drugs and natural product in treating or preventing such condition.

Although a lot of medication that lower the circulating cholesterol levels are available in the market, unfortunately, they are mostly associated with many side effects [6]. The concept of the consumption of functional foods or dietary supplements to lower serum cholesterol was introduced by Kwok et al. [25].

Antioxidants from natural sources were found to have both prophylactic and protective effects against hypercholestermic-induced organ affections [7]. Red grape (Vitis vinifera L.) is rich in flavonoids, polyphenols, anthocyanins, resveratrol and other stilbene derivatives; trans-resveratrol and trans-piceatannol [14]. Those substances were reported to possess potent antioxidant effect [18]. In both fresh and dried form, the red grape (RG) provides a good source for natural antioxidants [35]. It was reported that sun-dried raisins retain the minerals and most of the phytochemicals and antioxidants of the grape, including its resveratrol. In fact, sun-drying enhances the antioxidant content of raisins. Because of the dehydration process, phytonutrients are more concentrated in raisins than in grapes [4].

Although the renal functional and structural alterations in obesity were described in many studies [24 39], few detected studies investigated the effect of high cholesterol diet on the kidney function and structure [3, 21]. In previous studies, the effect of hypercholesterolemia on the structure of the renal glomerulus in specific was assessed [1,5]. While injury of the glomeruli may precede tubular injury, it is the latter that sets into motion the irreversible process of tubulointerstitial fibrosis, thus leading to loss of kidney structure and function [31]. Therefore, this study aim to describe the effect of the High cholesterol diet (HCD) on the structure of the renal
tubules and on the renal function in an animal model of hypercholesterolemia and to assess the possible protective effect of red grape and black resins in alleviating these effects.

**Materials and Methods**

**Diet and chemicals**

The HCD utilized in this study consisted of the rat chow supplemented with 4% cholesterol powder and 1% cholic acid and was obtained from Sigma, Aldrich, St. Louis, Mo, USA. The dose of the cholesterol was calculated according to Thriuchenduran et al. [41].

The dark red grape (RG) was obtained from the local Jeddah markets (imported from Sheli). It was thoroughly washed with distilled water. The RG juice (RGJ) was prepared using a sterile blender and stored at 4°C till the time of use. The RGJ was supplied in the dose described by Castilla et al. [11].

The dark raisins (DR) were purchased from well known nut stores in Jeddah (Yemen source). They were thoroughly washed and homogenized using a sterile blender with a small amount of water then packed into small blocks of 1 g each, and stored in the refrigerator until the time of use. The dose given to the animals was determined based on the method described by Spiller et al. [38].

One hundred gram of both RGJ and DR was assessed in the Analytical Chemistry Unit at Assuit University, Egypt, using Mass Spectrometry (MS) as described by Folin-Ciocalteu procedure [45] in order to determine the composition of them. The components of each were shown in Table 1.

**Animals**

Forty adult male Wister rats were obtained from the animal unit at King Fahd Medical Research Center (KFMRC), Jeddah, Saudi Arabia. The average weight of the rats was 250-300g. They were kept for one week under the laboratory conditions for acclimatization (12/12 light–dark cycle, standard temperature (25±C) and humidity 55%). The guidelines of dealing with the experimental animals in research approved in the KFMRC were followed in this study that was also approved by the biomedical research ethics committee, King Abdulaziz University, Jeddah, Saudi Arabia.

The rats were divided into 4 groups (n=10). The rats of the control group (GI) were allowed to have free access to standard rat pellets and water ad libitum. The experimental group (GII) were fed HCD for 12 weeks according to Thriuchenduran et al., [41] while the groups GIII, GIV...
were fed HCD plus RGJ or DR through gastric gavage respectively for 12 weeks. Body weight, food and water intake were recorded for all rats at the beginning and at the end of experiments.

**Biochemical assessment**

At beginning and at the end of the experiment, rats were anesthetized by light ether and blood samples were cautiously taken from the retro-orbital vein [40]. Serum was separated from these samples at 3000 rpm for 10 minutes and stored at -80 C for assessment of the lipid profile and kidney functions.

**Histopathological assessment**

After 12 weeks all rats were sacrificed by cervical dislocation under deep ether anesthesia. The abdomen and chest were opened. Initial perfusion of the heart was done using normal saline followed by 10% neutral buffered formalin to ensure good fixation. Kidneys were dissected out and weighed. The right kidney was cut in sagittal plan via convex border into 2 halves. One half of the kidney was re-fixed in 10% neutral buffered formalin and further processed for routine histological examination using the light microscopic microscope (LM). Four microns thickness paraffin sections were obtained and stained by haematoxylin and eosin [15]. Another set of the paraffin sections were immunohistochemically stained using the standard procedure previously described [5]. Anti-desmin antibody (Dako, Trappes, France) and anti-cytokeratin (CK) (Dako Ltd., Glostrup, Denmark) were used at the dilution 1/100 as described by Petrica et al., [33] to assess the integrity of PTs and DTs respectively.

Small pieces (1 mm²) were taken from the cortex of the other half of the kidney and fixed in 2.5% glutaraldehyde in sodium cacodylate buffer and processed for obtaining epoxy resins capsules according to Johannessen [22]. These capsules were sectioned at 0.5–1 mm, stained with toluidine blue and examined by the LM to detect the orientation of the ultrathin sectioning at 500–800 Å. These sections were stained with uranyl acetate and lead citrate, examined and photographed using the electron microscope (EM) (Joel, 100 CXII) operated at 80 KV at the EM unit, Assuit University, Egypt.

**Results**

**Chemical analysis of RGJ and DR**

It was observed that both RGJ and DR contained phenolic compounds, flavones and anthocyanin compounds which are well known antioxidants compounds. The latters were present in the RGJ at higher concentrations. Both contained considerable amount of vitamins, but DR
contains much more amount of vitamin B complex. The minerals, proteins and fibers were much more abundant in DR than RGJ (Table 1).

**Body and kidney weights**

A significant increase in body weight gain was observed in rats fed HCD compared to the control. On the other hand, there was a significant decrease in the weight gain of rats received HCD along with either RGJ or DR. The kidney index was decreased in HCD-fed rats compared to the control (Table 2).

**Biochemical assessment**

As shown in Table (3), feeding on HCD resulted in a significant increase in blood glucose (p=0.0002), insulin (p<0.001), cholesterol (p<0.001), triglycerides (p<0.001) and LDL (p<0.0001) levels while HDL significantly (p<0.001) decreased compared with those at the start of the experiment (data not shown). Administration of both RGJ or DR significantly improved the lipid profile apart from the HDL, reduced the blood glucose and increased the insulin level with no significant difference between RGJ and DR. Regarding the kidney functions, it was found that creatinine, BUN (blood urea nitrogen) and urea levels were significantly decreased in HCD-fed rats along with RGJ or DR.

**Histopathological assessment**

**Light microscopy**

Proximal tubules (PTs) represent important part of the nephron, the structural unit of the kidney, as most of the urinary filtrate is reabsorbed at this level. On routine histopathological examination, the PTs and the DTs of the control group appeared intact. The PTs of HCD-fed rats appeared dilated with acidophilic casts in their lumina. The cells lining the PTs appeared shorter and some of the peritubular capillaries were dilated and congested. Although some of the renal tubules in the groups treated with RGJ and DR appeared affected, most of the other tubules appeared intact and some peritubular capillaries were congested (Figure 1). Numerous large lipid droplets existed at the basal parts of tubular cells and within the lumen. Some apoptotic cells were also observed in the renal tubules of HCD-fed rats (Figure 2).

In order to assess the integrity of the renal tubules and PTs in specific, the renal cortex was immunohistochemically stained with anti-desmin antibody. A strong desmin immunoeexpression was observed in the PTs of the control rats while those of the HCD-fed rats showed marked decrease in expression. On the other hand, most of the renal tubules of HCD-fed rats along with
RGJ or DR had strong desmin expression (Figure 2). When it came to the integrity of the DCTs, anti-cytokeratin antibody was used to assess it. Most of the renal tubules, the DCTs in specific, of the control rats as well as RGJ- and DR-treated rats showed moderate cytokeratin expression while those of HCD-fed rats showed weak expression (Figure 2).

**Electron microscopy**

Examination of the ultrastructure of the renal tubules showed that feeding the rats with HCD resulted in marked deposition of lipid droplets as well as increased number of lysosomes in the cells of both PTs and DCTs. Cells of the PTs showed smaller and darker mitochondria compared with those of the control rats. Some cells lining the DCTs appeared markedly affected with thickened basement membrane and swollen mitochondria (Figure 3, 4). Tubular cells of the rats received HCD along with RGJ or DR contained no or few lipid droplets and numerous lysosomes and most of the mitochondria appeared intact (Figure 3, 4).

**Discussion**

Hypercholesterolemia represents a common clinical problem as its complications involve many vital organs like the kidneys [29]. Animal models of hypercholesterolemia are considered valid tools to study cholesterol homeostasis and test drugs and natural products directed to control disorders in lipid or cholesterol metabolism [32]. This study aimed to assess the impact of HCD-induced hypercholesterolemia on the structure of the rat renal tubules, in specific, as well as renal functions and to assess the possible protective effect of red grape and dark resins in alleviating these effects. In the present study, feeding rats on diet containing 4% cholesterol and 1% cholic acid resulted in marked disturbance of the lipid profile parameters which was reflected on rats' body weight, kidney function and structure. Similar results regarding success of such model were reported by many investigators [23, 32].

Proximal tubules are the first parts of renal tubules where most of the filtered substances were reabsorbed back to blood. Damage of such tubules will result in loss of the valuable reabsorbed nutrients and electrolytes needed for the organism welfare [12]. In the present study, it was observed that hypercholesterolemia resulted after feeding rats on HCD affected the structure of renal tubules observed by both LM and EM. Among the observed changes was the dilation of the PTs and presence of acidophilic protein casts. These tubular casts was mostly attributed to the hypercholesterolemia-induced glomerular lesions previously described by Abdel-Hamid [1] and Ayuob [5]. Zoja et al [46] reported that damaged filtration barrier caused loss of proteins and its appearance within the lumina of the tubules.
Desmin, a cytoskeleton protein, is expressed by various types of cells (epithelial cells, mesangium, tubules, postmitotic myofibroblasts). Desmin expression is affected by certain circumstances, such as hypertension, heavy proteinuria [30]. Petrica et al. reported that desmin stains normal proximal tubular cells and is considered a sensitive marker that detected focal tubular necrosis in early stages of tubular impairment while cytokeratin is expressed in cells of distal and collecting tubular cells and is considered a sensitive marker for focal tubular necrosis [33]. Therefore they were utilized in this study as specific histologic marker of tubular necrosis. It was found that renal tubules, PTs in specific, of the HCD-fed rats showed marked decrease in desmin expression compared to the control rats. Most of the renal tubules, the DCTs in specific, of HCD-fed rats showed weak expression compared with those of the control rats. These findings indicated a negative impact of hypercholesterolemia on the integrity renal tubular cells.

Accumulation of lipid droplets in the tubular cells, evident in the present study by both LM and EM, could affect the cellular metabolism and induce oxidative stress and lipid peroxidation. The latter induced damage of membranous structures including brush borders of the tubular cells as well as the mitochondria with subsequent impairment in renal function [8]. In a more recent study, Emma et al. [16] reported that lipid accumulation could result in lipotoxicity as fatty acids act as detergents that weaken the membranous structures and enhancing cell apoptosis. Mitochondrial changes, indicated its damage, was among the changes observed in hypercholesterolemic rats in this study. These mitochondrial changes affected the Na-K pump-mediated reabsorption process in the PTs which is dependent on the ATP generated by the mitochondria and cause dysfunction of the tubules [16]. Damaged mitochondria also resulted in release of cytochrome C that set into motion apoptosis, as well as proinflammatory mediators that signal tissue damage [44]. This is an explanation of the ultrastructural signs of cellular damage evident by EM in the renal tubules in this study. Tubular epithelial cells, especially those of PTs were proved to play a dynamic role in the pathogenesis of tubulointerstitial fibrosis. Among the main mechanisms that contribute to tubule cell activation is activation by glomerular-derived cytokines that reach tubule cells via the urine space, vasa recta or diffusion through interstitial space. Another suggested mechanisms are injury by plasma proteins filtered in excess as a consequence of injury to the glomerular filtration barrier; ischemia downstream to glomerular injury and hyperfunction of remnant tubules [13, 19].

Natural products rich in antioxidants can be used as safe medicinal products for the early management of many diseases including hyperlipidemia [27]. Grape was reported to be an important source of healthy nutrients with many health benefits so can be used as supplementation supporting health and protect against oxidative stress induced disorders [37]. In addition, Bagchi et
al. proved the efficacy of raisins as excellent source of polyphenols, powerful protective antioxidants against oxidative damage [6].

Analysis of fresh red grape juice and dried raisins used in this experiment showed that they were rich in many antioxidant compounds such as phenolic compounds, flavones and anthocyanin besides considerable amounts of vitamins and minerals. The presence of high fiber content in raisins made it superior to grape juice in controlling lipid profile possibly by increasing cholesterol absorption from intestine [43]. Present study seems to support such notation.

In the present study, administration of both RGJ and DR improved the lipid profile, the elevated blood glucose level and the disturbed kidney function induced by the HCD. Some previous studies revealed contradicting findings regarding the reno-protective effect of red grape or its products. Pinheiro et al, found that grape seed extract caused an increase in serum urea in rats received methotrexate [34]. In addition, Safa et al. [36] reported that red grape seed extract failed to improve gentamicin-induced acute kidney injury. These results may point to a possible interaction between those drugs and red grape products. On the other hand, red grape consumption and its products was reported to increase serum antioxidant capacity and provide protection against many lesions induced by hyperlipidemic status. Almajwal and Elsadek [2] found that red grape seeds added as 20% to basal diet showed a significant decrease in the lipid profile, with a significant increase in level of high density lipoprotein (HDL-C). The authors attributed this effect to grape components such as procyanidins, proanthocyanidins and polyphenols. The latters were also reported by Feng et al., to be powerful free radical scavengers [17]. Bladé et al., added that the hypolipidemic effects of proanthocyanidins are attributed to their ability to decrease lipid absorption and chylomicron synthesis by the intestine beside VLDL secretion by the hepatocytes [10]. This could explained the observed improvement in the histopathological alterations in the renal tubules after administration of RGJ or DR what we named it "renoprotective effect".

In summary, this study raised the importance of supplementation of red grape and raisins in protection against the harmful effects induced by deposition of fat in the renal tubules with subsequent negative impact of the renal functions. Therefore, this study support the renoprotective effect of red grape and raisins against the high fat diet-induced nephrotoxicity.

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**Conflict of interests:** The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**References**


obesity by a mixture of red grape extract, soy isoflavone and L-carnitine: implications in cardiovascular and non-alcoholic fatty liver diseases. Food Chem Toxicol. 49(9):2453-8.


Table 1. Chemical analysis of Red grape and Raisins

<table>
<thead>
<tr>
<th>Element (g/100g)</th>
<th>Amount in RG</th>
<th>DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol compounds</td>
<td>9.4</td>
<td>6.33</td>
</tr>
<tr>
<td>Flavones</td>
<td>0.042</td>
<td>0.019</td>
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<tr>
<td>Anthocyanin</td>
<td>0.34</td>
<td>0.002</td>
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<tr>
<td>Vitamin C</td>
<td>0.0006</td>
<td>0.003</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.17</td>
<td>0.09</td>
</tr>
<tr>
<td>Vitamin B1</td>
<td>0.09</td>
<td>1.099</td>
</tr>
<tr>
<td>Vitamin B2</td>
<td>0.053</td>
<td>1.79</td>
</tr>
<tr>
<td>Vitamin B3</td>
<td>0.28</td>
<td>4.91</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.15</td>
<td>0.73</td>
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<tr>
<td>Phosphorus</td>
<td>0.06</td>
<td>0.071</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.002</td>
<td>0.012</td>
</tr>
<tr>
<td>Calcium</td>
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<td>0.04</td>
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<tr>
<td>Iron</td>
<td>0.0002</td>
<td>0.002</td>
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<tr>
<td>Protein</td>
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<tr>
<td>Carbohydrates</td>
<td>28.4</td>
<td>75.08</td>
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<tr>
<td>Cholesterol</td>
<td>0</td>
<td>0.092</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>0.05</td>
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<tr>
<td>Polyunsaturated fatty acids</td>
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<td>0</td>
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<tr>
<td>Fibers</td>
<td>0.96</td>
<td>5.9</td>
</tr>
<tr>
<td>Moist</td>
<td>80.5</td>
<td>4.92</td>
</tr>
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Table 2. Body weight, water and food intake of the studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Body weight (g)</th>
<th>Body weight gain (%)</th>
<th>Water intake (ml/d)</th>
<th>Food intake (g/d)</th>
<th>**Kidney index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At the start</td>
<td></td>
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<td></td>
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<tr>
<td>At the end</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parameter</td>
<td>Blood glucose level (mg/100ml)</td>
<td>Insulin level (ng/ml)</td>
<td>Cholesterol (mmol/L)</td>
<td>Triglycerides (mmol/L)</td>
<td>HDL (mmol/L)</td>
</tr>
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<td>-----------</td>
<td>-------------------------------</td>
<td>-----------------------</td>
<td>----------------------</td>
<td>------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Control</td>
<td>100.2±23.3</td>
<td>0.854±0.24</td>
<td>50.63±8.5</td>
<td>47.4±16.4</td>
<td>24.45±1.5</td>
</tr>
<tr>
<td>HCD</td>
<td>139.9±13.2</td>
<td>1.765±0.35</td>
<td>108.81±10.6</td>
<td>81.12±19.7</td>
<td>18.42±2.1</td>
</tr>
<tr>
<td>HCD+R GJ</td>
<td>102.6±15.2</td>
<td>0.751±0.11</td>
<td>63.12±14</td>
<td>49.7±16</td>
<td>26.81±1.5</td>
</tr>
<tr>
<td>HCD+D R</td>
<td>100.5±12.2</td>
<td>0.77±0.21</td>
<td>57.78±10.6</td>
<td>42.77±16</td>
<td>28.11±1.8</td>
</tr>
</tbody>
</table>

BUN: blood urea nitrogen

p: HCD group versus control group
p1: HCD group versus HCD+RGJ group
p2: HCD group versus HCD+DR group
p3: HC+RGJ group versus HCD+DR group

Table 3. Blood glucose, insulin level and lipid profile and kidney functions of the studied group at the end of the experiment.
Significance was considered at $p < 0.05$

**FIGURE LEGENDS**

**Figure 1.** Sections in renal cortex of rat from the control group (A, B) showing intact proximal tubules (PTs) and distal tubules (DT) with intact peritubular capillaries while those of the rats fed HCD (C, D) appear congested and dilated. The PT of these rats appear dilated with hyaline casts (star) in their lumina and the DT are lined with cells which have dark cytoplasm and dark nuclei (thick white arrow). Renal tubules of the rats received HCD+RGJ (E, F) or HCD+DR (G, H) showing same changes but at lesser extent (H&E X 600). HCD: high cholesterol diet, RGJ: Red grape juice and DR: dark resins.

**Figure 2.** Semithin section of the renal cortex of control rat (A) showing intact PT and DT while those of the rat fed with HCD (B) showing numerous large lipid droplets (thin arrows) at the basal parts of tubular cells (black arrows) and within the lumen (stars). Notice the presence of dark apoptotic cells (dotted arrows) in some tubules. Most of the renal tubules in the cortex of rats treated with RGJ (C) and DR (E) appear intact apart from few cells with dark nuclei (white dotted arrows) (Toluidine blueX1000). Renal cortex stained immunohistochemically with desmin showing strong expression in renal tubules of control rats (E) while those of HCD-fed rats (F) show marked decrease specifically in the PTs. renal tubules of rats treated with RGJ (G) and DR (H) showing preservation of desmin expression (Desmin X 600). Renal cortex stained immunohistochemically with cytokeratin showing moderate expression in renal tubules of control rats (I) while those of HCD-fed rats (J) show week expression specifically in the DTs. Most of the renal tubules of rats treated with RGJ (K) and DR (L) showing moderate expression of cytokeratin apart from few DTs (Cytokeratin X 600). PTs: proximal tubules, DTs: distal tubules, HCD: high cholesterol diet, RGJ: Red grape juice and DR: dark resins. PTs: proximal tubules, DTs: distal tubules, HCD: high cholesterol diet, RGJ: Red grape juice and DR: dark resins.

**Figure 3.** EM picture of a part of the PT of control rat (A) showing intact basement membrane (BM) microvilli (MV), mitochondria (M) and an euchromatic nucleus (N) of the cell. Cells of the PT of HCD-fed animal have numerous lipid droplets (LD) that appear compressing the nucleus and the mitochondria appear smaller and show dense matrix. Many lysosomes (L) appear in the cytoplasm. Cells of the PT of RGJ-treated rat (C) and DR-treated rat (D) showing absence of lipid droplets and numerous lysosomes. Mitochondria appear of larger size in RGJ-treated rat (C) and of
increased number in DR-treated rat (D). PTs: proximal tubules, HCD: high cholesterol diet, RGJ: Red grape juice and DR: dark resins.

**Figure 4.** EM picture of a part of the DT of control rat (A) showing intact basement membrane (BM) basal infoldins with mitochondria (M) in-between and an euchromatic nucleus (N) of the cell. Cells of the DT of HCD-fed rat either appear intact (B) with few lipid droplets (LD) or markedly affected (C) with thickened basement (BM) membrane numerous large lipid droplets (LD), swollen mitochondria (M) and small dark nucleus (N). Cells of the DT of RGJ-treated rat (D) and DR-treated rat (E) showing few lipid droplets (LD) and numerous lysosomes (L) and most of the mitochondria (M) appear intact. DTs: distal tubules, HCD: high cholesterol diet, RGJ: Red grape juice and DR: dark resins.