Mitigating Effect of Resveratrol on the Structural Changes of Mice Liver and Kidney Induced by Cadmium; A Stereological Study

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ABSTRACT: Exposure to cadmium (Cd) has harmful effects on the liver and kidney. Resveratrol (RES) is an herbal substance that functions as a protective mediator. This study aimed to investigate the effects of RES on the histology of liver and kidney in Cd-exposed mice. Male mice were divided into 4 groups daily receiving normal saline (1 mL normal saline/d), Cd (1 mg/kg/d), RES (20 mg/kg/d), and Cd plus RES, respectively. After 4 weeks, the liver and kidney components were evaluated using stereological methods. The total volume and number of hepatocytes, and volume of fibrous tissue were respectively increased by 34%, 58%, and a 3-fold in the Cd-exposed mice in comparison to the control animals ($P < 0.03$). On the other hand, the volume of the main vasculature (sinusoids and central veins) was decreased by 36% in the Cd group compared to the control mice ($P < 0.03$). Considering the kidney, the results showed a 3-fold increase in the total glomeruli volume and a 7-fold increase in fibrous tissue in the Cd-treated group compared to the control mice ($P < 0.03$). After Cd treatment, a 32% reduction was observed in the volume and length of the proximal and distal convoluted tubules. RES-treatment alone did not induce any structural changes. In comparison to the Cd group, an increase in the normal components of the liver and kidney and a decrease in the formation of the fibrous and degenerated tissues were observed in the Cd+RES-treated mice ($P < 0.03$).

Keywords: cadmium, resveratrol, liver, kidney, quantitative

INTRODUCTION

Cadmium (Cd) is a toxic metal commonly used in electroplating, industrial paints, manufacturing of some types of batteries, shipyard employment, construction industry, and agricultural industry. In addition to its industrial application, Cd is also found in soil, air, water, vegetables, aquatic foods, and industrial workplaces (1-3). Tobacco and cigarette smoke are some of the most public origins of Cd. Therefore, there is a high risk of possible exposure to Cd. Release of Cd into the environment resulting from its use poses a potential danger to the general population and represents major health hazards. Exposure to Cd has harmful effects on the cells and tissues in a variety of organs, including the liver and kidney (4). Acute and chronic contacts with Cd lead to collection of the metal in the liver, inducing hepatotoxicity (5,6). The liver is the first organ to drug detoxication, and the liver structure can be detrimentally al-

tered by injury resulting from exposure to toxicants (7). Therefore, liver injury due to Cd exposure can cause liver dysfunction. Hyder et al. (8) evaluated the effects of chronic Cd exposure on liver disease and liver-related mortality. They showed the association between urinary Cd levels and hepatic necroinflammation, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, and liver-related mortality in the United States general population. They also reported that individuals in the top quartile of urinary Cd had over a 3-fold increased risk of liver disease mortality. Cd also accumulates largely in the kidney and following long-term exposure to Cd, nephropathy occurs. Nephropathy is characterized by various signs of tubular dysfunction and chronic interstitial nephritis (9-12). Hence, it is important to find a protective agent that can be easily used by the population.

Resveratrol (RES) is an herbal substance, which can be found in a number of fruits and nutritional sources.

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RES is a stilbenoid, a type of natural phenol, and a phytoalexin made naturally by numerous plants in response to damage or when the plant is embattled by pathogens, such as microorganisms. Up to now, different beneficial effects of RES have been reported, including anticancer and neuroprotective properties and cardiovascular protection. This substance functions as an antioxidant and anti-inflammatory mediator and changes the pro-inflammatory cytokines in rats' hepatocytes (13- 16). RES is known to be associated with grapes, mulberries, groundnuts, and peanuts. Therefore, consumption of RES can be easily recommended to the population. The importance of RES is known to many people, and a large proportion of the population uses RES for the prevention or treatment of atherosclerosis and balancing cholesterol levels.

Considering the important role of liver and kidney structures, the present study aims to investigate the effects of RES on liver and kidney cytoarchitecture in mice receiving Cd for 28 days. Therefore, the aim of this study is to answer the following questions using quantitative stereological methods: how much does the total volume of the liver, hepatocytes, sinusoidal space, central vein lumen, fibrous tissue, and portal triad change after Cd-exposure? How much does the population of hepatocytes' nuclei change by Cd exposure? How much does the volume of the renal histological structure [glomeruli, proximal and distal convoluted tubules (proximal convoluted tubule, PCT; distal convoluted tubule, DCT), Henle loop, collecting ducts, and fibrous tissue] change after Cd-treatment? How much does the length of the tubular structure of the kidney change after Cd-exposure? Can co-treatment of RES and Cd prevent the structural changes? Stereological techniques were applied to obtain the answers.

MATERIALS AND METHODS

Animals

In this study, 24 male BALB/c mice at 12 weeks of age weighing 31.8 ± 1.4 g were divided into 4 groups (N=6), receiving normal saline (1 mL normal saline/d), Cd (Cd chloride, 1 mg/kg/body weight/d, Sigma-Aldrich, Munich, Germany) (17), RES (20 mg/kg/body weight/d, Sigma-Aldrich) (18), and Cd plus RES through intraperitoneal injection, respectively.

The dose of Cd was selected according to the literature. After evaluating the lethal effects of different doses of Cd, Paul et al. (17) showed that 1.0 mg/kg/body weight was considered as the suitable sub-lethal dose for experimental studies. The general safety of RES has been reported in several *in vivo* studies. RES was harmless and well tolerated in rodents from low doses (20 mg/kg/d)

to high doses (up to 750 mg/kg/d) in 28- or 90-d studies (18). Only at a very high dose of RES (3,000 mg/kg/d), which is at least 30 times higher than the routine human dose (the dose as high as 7.5 g/d has been recommended for humans, which is equal to 100 mg/kg/d for a 75 kg individual), the rats did not display clinical signs of toxicity after 4 weeks of RES consumption (18).

The mice were treated according to the standard directive as recommended and approved by the University (approval No. HSRC-93-01-12). After 4 weeks, the histological parameters of the liver were quantified using stereological methods.

Estimation of the volume and shrinkage

At first, the liver and kidney were weighed and the volume, "V (liver and kidney)", was measured using the immersion method (19,20). Briefly, a container with normal saline was placed on the scale and weighed. Then, the liver or kidney hung by a thin thread was put deep in the container. The new weight in grams minus the weight of the container and water divided by the specific gravity of normal saline (1.0048) was the volume of the organ in cubic centimeters. Tissue shrinkage was estimated before fixating in neutral buffered formaldehyde for 1 week. Isotropic uniform random sections were obtained by applying the orientator method on the organ (19,20). Briefly, each lobe of the liver and the whole kidney were located on a circle divided into 10 equal parts. Then, a random number between 0 and 10 was selected and the organ was sectioned in that direction. The cut surface of each half of the organ was then located on the second circle with 10 unequal sinus-weighted divisions and the second cut was done. Afterwards, each organ was sectioned into slabs parallel to the direction of the second cut with an interval of 1 mm. Then, 8~12 slabs were collected randomly from each organ. A circle was punched from the organ slab by a trocar with 5 mm diameter. The cut surfaces of the slabs and the circular piece were embedded in a paraffin block, sectioned (4 and 26 μ m thicknesses), and stained with Heidenhain's azan. The area of the circular piece was measured again, and the degree of volume shrinkage [d(sh)] of the tissue was estimated using the following formula (19,20):

$$d(sh) = 1 - (AA/AB)^{1.5}$$

Where "AA" and "AB" were respectively the area of the circular piece after and before tissue processing, sectioning, and staining.

Each 4 μ m sampled section was analyzed using a video microscopy system consisting of a microscope (E-200, Nikon, Tokyo, Japan) and a computer. Overall, 10~14 microscopic fields were studied in each mouse's liver or

kidney, which were sampled at equal intervals along the X- and Y-axis, using a stage micrometer at a final magnification of 1,500 $\times$. A point probe (composed of 25 points) was superimposed upon the images of the tissue sections viewed on the monitor, and the volume density (V_v) and total volume of the liver components (including hepatocytes, sinusoids, central veins, portal triad, and fibrous tissue) and kidney components (including glomeruli, PCT, DCT, Henle loop, collecting duct, and fibrous tissue) were obtained using the point-counting method and the following formula (19,20):

$$V(\text{component}) = V_v(\text{component}) \times V(\text{liver or kidney})$$

$$V_v = P(\text{component}) / P(\text{reference})$$

where $P(\text{component})$ and $P(\text{reference})$ were the number of points falling on the component's profile and on the reference space, respectively.

Estimation of the number of the hepatocytes nuclei

To estimate the total number of hepatocytes and their nuclei, 26 μm thickness sections were used. In doing so, a high numerical aperture oil immersion lens (60 $\times$, NA=1.4) was used. The number of the hepatocyte nuclei was estimated using the optical disector method at the final magnification of 3,500 $\times$. An unbiased counting frame with inclusion (right and upper) and exclusion (left and lower) borders was overlaid on the tissue images. The optical section was progressed downwards the Z-axis. A microcator (Heidenhain MT-12, Dr. Johannes Heidenhain GmbH, Traunreut, Germany) measured the Z-axis scanning. Any nucleus which came into maximal focus within the optical section (height of disector) was selected if it was placed in the counting frame or touched the inclusion borders and did not touch the exclusion borders of the frame. The disector's height was set according to plot of the Z-axis distribution of the nuclei (21,22). According to the plot, the first and last 4 μm of the sections were ignored (the guard zones). The numerical density (N_v) and the total number of the hepatocytes' nuclei were estimated using the following formula:

$$N_v = \Sigma Q^- / (\Sigma A \times h) \times (t/BA)$$

$$N(\text{hepatocytes}) = N_v \times V(\text{liver}) \times d(\text{sh})$$

where ' ΣQ^- ' denoted the total number of the nuclei coming into focus in each liver, ' ΣA ' denoted the total area of the unbiased counting frame in all fields (area of each frame=1,400 μm^2), ' h ' was the height of the disector (12 μm), ' t ' was the mean section thickness in all fields measured by the microcator (20 μm), and ' BA ' was the block advance of the microtome set at 26 μm (21,22).

Estimation of length of the renal tubules

Length density is the length of the tubules per unit volume of the renal tissue. Length density (L_v) of the renal tubular structure was estimated by overlying a counting frame on the live histological image of the kidney. The number of the profiles of each tubule, which was in the counting frame and did not touch the left and lower borders of the frame, was counted. The length (L) was estimated using the following formula (19,20):

$$L = 2 \Sigma Q / [\Sigma p \times (a/f)] \times [1 - (\text{Sh})^{2/3}] \times V(\text{kidney})$$

where ΣQ was the total counted profiles of the each tubule (an average of 150 profiles in each animal), a/f was the area per frame (here 2,700 μm^2), and Σp was the total number of the counted frames in each animal. The total volume of the parameters as well as the total length of the tubules was estimated by multiplying the fractional volume or the length density by the final volume of the kidney to prevent the "reference trap" (19,20).

Estimation of the mean glomerulus volume

Point-sampled intercept was applied to estimate the volume-weighted mean volume of the glomerulus using the following formula (19,20):

$$\text{Mean volume} = \frac{\pi}{3} \times \overline{l_0^3}$$

where l_0 was the intercept length of the glomeruli, which was sampled using a point grid in each animal.

Statistical analysis

The study data were entered into the SPSS statistical software (IBM corporate, Chicago, IL, USA) and were analyzed using the Kruskal-Wallis and Mann-Whitney U tests. $P < 0.03$ was considered as statistically significant.

RESULTS

Volume of the liver

The liver volume was increased by 18% on average in the Cd-treated animals in comparison to the control group. However, the volume was not restored after co-treatment of the mice with RES+Cd in comparison to the Cd group ($P < 0.03$) (Fig. 1).

The total volume and number of the hepatocytes

The total volume and number of the hepatocytes were respectively increased by 34% and 58% on average in the animals exposed to Cd in comparison to the control

Fig. 1. Scatter plots of the total volume of the liver (A), hepatocytes (B), sinusoids (C), central veins (D), and connective tissue or fibrosis (E) and number of the hepatocytes' nuclei (F) in the control group (CON) and cadmium group (Cd) with or without resveratrol (RES) treatment. The differences are indicated on each plot.

mice ($P < 0.03$). The results revealed a significant difference between Cd- and RES+Cd-treated groups regarding these components ($P < 0.03$) (Fig. 1).

Volume of the sinusoids and central veins

The volume of the sinusoids and central veins were respectively decreased by 32% and 40% in the Cd group in comparison to the control mice ($P < 0.03$). The volume of the sinusoid and central vein also increased, but to a lesser extent (13% and 19%, respectively), in the mice treated with RES+Cd compared to the control mice. A significant difference was observed between Cd- and RES+Cd-treated groups regarding these components ($P < 0.03$) (Fig. 1).

Liver fibrous tissue

Treating the mice with Cd increased the fibrous tissue by a 3-fold compared to the control group ($P < 0.03$). The study findings showed a significant difference between

Cd- and RES+Cd-treated groups in this regard ($P < 0.03$). Also, RES-treatment alone did not induce any structural changes in the liver (Fig. 1).

Qualitative study of the liver

Qualitative histological study of administration of RES to the mice's liver induced by Cd compared to the control, Cd, RES, and RES+Cd groups is presented in Fig. 2.

Volume of the kidney

The findings showed a 7% increase in the kidney volume in the Cd-treated animals in comparison to the control. However, it was increased by 54% in the Cd+RES group compared to the Cd group ($P < 0.03$) (Fig. 3).

Volume of the kidney components

The total glomeruli volume was increased by 3-fold in the Cd group compared to the control animals ($P < 0.03$) (Fig. 3). The mean glomerulus volume also showed a

Fig. 2. Photomicrograph of the mice's livers in the control (A), cadmium (Cd) (B), resveratrol (RES) (C), and RES+Cd (D) groups stained with Heidenhain's azan trichrom stain. A larger number of hepatocytes' nuclei, lesser sinusoidal space, and prominent bridge of the fibrous tissue can be seen in the Cd-treated mice (B). Perisinusoidal fibrosis and narrowing of the channels can also be seen in the Cd-treated livers. No structural changes were detected in the mice treated with RES. (E) and (F) respectively show Cd and RES+Cd at higher magnification. A smaller number of hepatocytes' nuclei, lesser accumulation of the fibrous tissue, and larger sinusoidal space can be observed in the mice treated with RES+Cd.

24% increase in the Cd group compared to the control mice ($P < 0.03$) (Fig. 4). On the other hand, the total volumes of PCT and DCT were respectively reduced by 29% and 34% in the Cd group compared to the control animals ($P < 0.03$) (Fig. 3). In addition, the volume of the fibrous tissue was 7-fold higher in the Cd group compared to the control mice ($P < 0.03$) (Fig. 3). The amount of the degenerated glomeruli and tubules was also considerable in the Cd-treated mice (Fig. 3).

In the Cd+RES animals, the changes in the glomeruli, PCT, DCT, and fibrous and degenerated tissues were

improved in comparison to the Cd group ($P < 0.03$) (Fig. 3 and 4).

Length of the tubular structures

The total lengths of PCT and DCT were respectively reduced by 32% and 18% in the Cd group compared to the control animals ($P < 0.03$) (Fig. 5). Besides, the length of the intact DCT and DCT was larger in the Cd+RES animals than in the Cd group ($P < 0.03$).

Fig. 3. Scatter plots of the total volume of the kidney (A), glomeruli (B), PCT (C), DCT (D), Henle's loop (E), and collecting duct (F) in the control group (CON) and cadmium group (Cd) with or without resveratrol (RES) treatment. The differences are indicated on each plot.

Qualitative study of the kidneys

In the Cd-treated animals, prominent changes were observed in PCT and DCT. Hypertrophy and degenerative changes were also detected in the epithelial cells of the renal tubules in these animals. Dilatation of the lumen of PCT and DCT was also apparent (Fig. 5). The glomeruli also underwent a variety of changes, including increased mesangial matrix, glomeruli swelling, and increased glomerular urinary space in the Cd-treated group (Fig. 5). In addition, a marked protection in renal structure was apparent after Cd+RES treatment. Moreover, the fibrous tissue was increased in the mice treated with Cd. In the mice co-treated with Cd+RES, changes in the tubules, glomeruli, and fibrous tissue were ameliorated extensively. However, treatment of the mice with RES alone induced no significant changes in the renal structures (Fig. 6).

DISCUSSION

The present study quantified the structure of the liver and kidney after Cd exposure and showed the mitigating effects of RES. The first part of the current study showed a significant increase in the total volume and number of hepatocytes and fibrous tissue in the animals exposed to Cd. According to Cupertino et al. (23), increases in liver weight, liver edema, and necrosis could be found in animals exposed to high doses of Cd. Marcano et al. (24) also reported cellular necrosis of the liver after treatment of the rats with Cd. Moreover, Siddiqi et al. (25) indicated that Cd fluoride caused an increase in the liver collagen content. The results of the present study showed an increment in the total volume and number of the hepatocytes, indicating hyperplasia of the cells after Cd-exposure. When functional liver cells are injured due to different agents, including heavy metals, the immune system is activated to restore the damage. In the next

Fig. 4. Scatter plots of the mean glomerulus volume (A), volume of the connective tissue or fibrosis (B), degenerated glomeruli (C), and degenerated tubules (D) in the control group (CON) and cadmium group (Cd) with or without resveratrol (RES) treatment. The differences are indicated on each plot.

Fig. 5. Scatter plots of the length of PCT (A), DCT (B), Henle's loop (C), and collecting duct (D) in the control group (CON) and cadmium group (Cd) with or without resveratrol (RES) treatment. The differences are indicated on each plot.

steps, cirrhosis might occur. Cirrhosis is characterized by regenerative nodules that are separated from one another by bridges of fibrosis. At this stage, hepatocytes are lost through a process of hepatocellular damage and

inflammation. This injury triggers a regenerative response in the remaining hepatocytes. The fibrotic scars limit the extent to which the normal structure can be re-established as the scars isolate groups of hepatocytes.

Fig. 6. Comparison of the renal tissue among different groups. Normal renal cortical tissues are observed in the controls (A) and resveratrol (RES)-treated mice (B). Hypertrophy, degeneration, and dilatation of the tubules and enlargement of the glomeruli and fibrous tissue (the arrows) are evident in the cadmium (Cd)-treated animals (C). A marked protection is apparent in renal structure after Cd+RES treatment (D).

This results in formation of regenerative hepatocytes nodules. Therefore, the increase in hepatocytes' nuclei seen in this study might be due to the above-mentioned triggering regenerative response (26).

The volume of the normal vascular structure was reduced after Cd-exposure in the present study. Vascular channels are narrowed by fibrotic tissue. This finding is in agreement with that of the study by Kim et al. (27), demonstrating that concentrations of Cd caused abnormal sinusoids formation.

Generally, it has been explained that Cd-absorption in the liver stimulates the synthesis of metallothionein, a protein that has a high affinity for Cd. Cd-metallothionein complex is stored in the liver (28). When the total amount of Cd in the liver exceeds the binding capability of metallothionein, the non-metallothionein-bound Cd ions can induce oxidative stress that, in turn, can cause damage to these tissues (28).

The second part of the current study evaluated the renal structure after Cd-exposure. The animals exposed to Cd showed severe changes in the renal tubules and glomeruli. In fact, glomerulus enlargement was detected in the Cd-treated animals. Tripathi and Srivastav (12) reported glomerular swelling at the initial stages and shrinkage and degeneration of the glomerulus at the later stages of Cd-treatment. They concluded that lesions depended upon the dose and duration of Cd treatment. According to Renugadevi and Prabu (9), after exposure of the rats to 1 mg/kg CdCl₂ for 30 days, increased amounts of mesangial matrix, swelling of the glomeruli, and thickening of glomerular capsular were evident.

Lakshmi et al. (11) also reported glomerular congestion after treatment of the rats with 3 mg/kg/week CdCl₂ for 4 weeks. In contrast, the quantitative study conducted by Barrouillet et al. (29) on the isolated glomeruli showed their size reduction and contraction. In this study, the total and mean volume of the glomeruli increased after Cd exposure.

The findings of the present study also indicated a decrease in the volume and length of the renal tubules in the Cd-treated animals, indicating atrophic changes in the renal cortex. Renal tubules are sensitive to many toxic substances. As Chargui et al. (30) stated, Cd is a powerful inducer of endoplasmic reticulum stress as well as autophagic course in tubular cells resulting from their hypersensitivity. They suggested that the renal cortex adapted to a subtoxic Cd dose by starting autophagy, a housecleaning procedure that ensures disrepair of damaged proteins. Increased volume of the fibrous tissue in the Cd-treated animals indicates replacement of the damaged renal tissue with the connective tissue. It has been reported that low levels of Cd exposure caused oxidative stress, alteration of the signaling cascades, cell adhesion, and damaged proteins in the PCT. Moderate levels of injuries also induced cell-detachment, autophagy, and apoptotic cell death. Moreover, severe damage resulted in breaking down of the cell-cell and cell-substrate adhesions, PCT necrosis, and irreversible damage (30,31). Prozialeck et al. (31) explained that Cd acted as a catalyst in production of reactive oxygen species (ROS). It also increased lipid peroxidation, promoted production of inflammatory cytokines, and depleted anti-

oxidants.

The third part of the current research focused on the mitigating effects of RES on the liver of the Cd-exposed animals. The present research showed that RES diminished fibrosis progression and protected the existing liver damage. Eybl et al. (32) reported that RES pre-treatment prevented the Cd-induced lipid peroxidation in the liver. In addition, they showed that RES could prevent Cd-induced inhibition of glutathione peroxidase activity (a defending enzyme against oxidative damage) in the liver (32). RES was also effective against Cd-induced inhibition of catalase activity (32). Catalase is a very important enzyme in protecting the cells from oxidative damage by ROS.

The forth part of current study evaluated the beneficial effects of RES on kidney structure. RES effects have also been shown in both *in vitro* and *in vivo* studies after renal damages. The *in vitro* study by Morales et al. (33) showed that the gentamicin-induced reduction in contracted mesangial cells surface area could be protected by incubation of the cells with RES. Furthermore, Chander and Chopra (34) reported that pretreatment of the rats with RES noticeably attenuated renal dysfunction and morphological changes after ischemia-reperfusion injury. Chander and Chopra (35) also showed that pretreatment of the animals with RES 60 min prior to glycerol injection markedly attenuated the changes in renal structure and function. Cisplatin-induced nephrotoxicity (36) and streptozotocin-induced diabetic nephropathy (37) were other examples of the advantages of RES treatment after renal damages.

The present study showed that after Cd and RES co-treatment, the volume and length of the tubules were increased even more than in the control group. This can be a reason for alleviation of tubular damage by RES. As mentioned earlier, moderate damage can lead to tubular cell proliferation. In other words, Cd caused a severe injury and cell death followed by tubular volume reduction. The increased volume and length is a clear evidence of hypertrophy and proliferation of the damaged tubules after RES treatment.

This study had some limitations including the length of the study and the dosage of RES. As noted in the "Materials and Methods" section, up to 750 mg/kg/d RES was given to rodents in 28- or 90-day studies (18). In the current study, only one dose (20 mg/kg) was used for 4 weeks. Thus, different doses and study periods should be considered in future studies.

CONCLUSION

The present quantitative stereological study revealed severe structural changes of liver, including increased hep-

atocytes number, volume, and fibrosis and reduced vasculature volume after treatment of the mice with 1 mg/kg/d Cd for 4 weeks. The kidney also underwent structural changes, including enlarged glomeruli, increased fibrous issue, and reduced tubular volume and length. Yet, RES (20 mg/kg/d) could mitigate most of the changes, including fibrous tissue formation and cellular degeneration in the liver and kidney.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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