Original Paper

Renal and Myocardial Histopathology and Morphometry in Rats with Adenine - Induced Chronic Renal Failure: Influence of Gum Acacia

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Key Words

Gum acacia • Adenine • Renal failure • Heart remodeling • Left ventricle • Rat

Abstract

Background/Aim: Chronic kidney disease (CKD) is associated with increased occurrence of cardiovascular system dysfunction. Previous studies have revealed a number of alterations in the kidneys and heart during CKD. However, unbiased quantitative studies on these structures in this disease have so far not been addressed. **Materials and Methods:** We induced CKD in rats by feeding adenine (0.75% w/w, four weeks) and using unbiased stereological methods, investigated the effect of the ensuing CKD on the kidneys and left ventricular structure. Since gum acacia (GA) has previously been shown to ameliorate the severity of CKD in humans and rodents, we investigated the effect of giving GA (15% w/v in the drinking water concomitantly with adenine) on the kidneys and left ventricular structure using the above model. **Results:** The CKD was confirmed by standard biochemical indices in plasma and urine and by accumulation of the uremic toxin indoxyl sulfate. Additionally, it increased blood pressure. In rats with CKD absolute volume of left ventricle was significantly increased, and the volume density and absolute volume of myocardial capillaries were decreased, whilst the same parameters of myocardium and interstitial tissue were increased. Renal morphometry demonstrated significant increase in kidney volume and interstitial tissue in adenine- treated rats. Similarly, glomerular Bowman's capsule was significantly thickened. The myocardial and renal changes were significantly mitigated by GA treatment. **Conclusions:** These results add to our existing knowledge of the pathophysiology of adenine – CKD and provides plausible histopathological and morphometric evidence for the usefulness of GA in CKD.

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Introduction

In chronic kidney disease (CKD), the incidence of cardiovascular complications, including hypertension, left ventricle (LV) hypertrophy, both systolic and diastolic dysfunctions, autonomic neuropathy and arrhythmia, is on average about 20 times higher compared with the normal population [1, 2]. Cardiovascular diseases (mainly heart failure, ischemic heart disease or sudden cardiac death) are the leading causes of death in CKD patients [2]. Both structural and functional changes in the heart have been described due to experimental CKD [3].

Some pathological processes are known to affect the left ventricular function. For example the impact of experimental diabetic cardiomyopathy on electrophysiological characteristics of the rat heart or the negative effect on the contractility of the end-stage heart failure in spontaneous hypertensive rats are more expressed in the LV [3, 4]. With regards to CKD, the majority of the experimental studies were focused on LV function, while only minimal attention was paid to quantitative structural studies. An unbiased stereological study on the LV in CKD models is, to the best of our knowledge, so far lacking.

Ali et al. [5] have recently demonstrated that gum acacia (GA) treatment was effective in ameliorating several biochemical, physiological and histopathological indices, and motor and behavioral changes in adenine-induced CRF [6]. However, the effect of adenine-induced CKD on cardiac microstructure in rats, and the possible protective effects of GA thereon have not been investigated.

Therefore, in this study, we report on an unbiased stereological approach to quantify the myocardial structural changes that occur in adenine-induced CKD in rats and whether treatment with GA would mitigate these changes.

Materials and Methods

Animal experiments

Male Wistar rats (9–10 weeks old, weighing 270 g in average) were housed in a room at a temperature of 22–23°C, relative humidity of about 60%, with a 12 h light–dark cycle (lights on at 6:00), and free access to standard pellet chow diet containing 0.85% phosphorus, 1.12% calcium, 0.35% magnesium, 25.3% crude protein and 2.5 IU/g vitamin D3 (Oman Flour Mills, Muscat, Oman) and water. The study was approved by the University Animal Ethical Committee and was conducted in conformity with international laws and policies (EEC Council directives 86 / 609, OJL 358, 1 December, 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publications No. 85-23, 1985).

Experimental Design

After an acclimatization period of one week, rats ($n = 24$) were randomly divided into four equal groups and treated for four consecutive weeks. The first group continued to receive the same diet without treatment until the end of the study (control group). The second group was switched to a powder diet containing Adenine (0.75% w/w in feed). The third group was given normal food and GA (Supergum EM 10) in drinking water at a concentration of 15% w/v . The fourth group was given adenine in the feed as in group two, plus GA in drinking water at a concentration of 15% w/v . The dose of adenine was chosen from the original method by Yokozawa et al. [7] and the dose of GA was chosen as was reported earlier [8]. During the treatment period, the rats were weighed weekly. For the collection of urine, they were placed individually in metabolic cages for 24 h, after the 28 days treatment period. On the morning after the metabolic sampling, the rats were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5 mg/kg), and blood (about 3.5 mL) was collected from the anterior vena cava and placed into plain or heparinized tubes. The blood and urine were centrifuged at 900 g at 4°C for 15 min. The serum or plasma obtained, together with the urine specimens, was stored at - 80°C to await analysis within 4 weeks after the end of the treatment.

Biochemical Methods

The concentrations of urea, creatinine, protein, calcium and phosphorus in plasma, serum and/ or urine were estimated spectrophotometrically using commercial kits (Human GmbH, Mannheim, Germany). Creatinine clearance (CCr) was calculated as reported before [9]. Plasma nitrite/nitrate concentrations were measured by an ELISA method using a commercial kit (R&D, Oxon, UK). Plasma indoxyl sulfate concentration was measured by an HPLC method [10].

Perfusion fixation and tissue sampling

After induction of anesthesia, the abdomen was opened via a midline incision. The inferior vena cava and abdominal aorta were cannulated. Before perfusion fixation, anticoagulant sodium heparin (1000 units) was injected through the inferior vena cava, and followed one minute later by a bolus injection of 1.5 mL 2% procaine in physiological saline to arrest the heart in diastole and abolish vascular tone. The heart was then perfusion-fixed with 100 mL 10% formaldehyde, injected through the aortic cannula at a pressure of 160mm Hg. Thereafter, the kidneys were removed and placed in 10% neutral-buffered formalin pending analysis. The heart was excised, and the LV free wall plus septum was dissected out for the determination of its mass (LVM) and absolute volume (LVV_A), and for tissue sampling.

The tissues were dehydrated in increasing concentrations of ethanol, cleared with xylene and embedded in paraffin. Three micrometer sections were prepared from renal and myocardial paraffin blocks and stained with hematoxylin and eosin (H & E), periodic acid-Schiff (PAS) and Masson's trichrome stains. The slides were evaluated by a histopathologist in a blinded fashion.

Stereological analysis

After weighing, the LVV_A was determined using the Cavalieri method [11]. In brief, a series of parallel sections were cut through the LV or the whole kidney at fixed thicknesses (T). This generated a series of slabs, which were all laid the same way up. Traditional point counting was used to estimate the area (A) of each of the cut surfaces of all slabs. Absolute volume, LVV_A was then calculated by the equation:

$$LVV_A = A \times T$$

Tissue sampling was performed according to the orientator method [12]. Briefly, a slab was randomly selected from those used for evaluating V_A, and processed via a series of cuts accordingly [13]. The last cut surface was considered to be uniformly isotropic. One block of tissue was made from each ventricle, and was embedded in paraffin wax. Using a Leica microtome (Leica RM2165; Heerberg, Switzerland), a three-micron-thick section was obtained from each block and were stained as described above. Two random sections were obtained from each block of tissue. A systematic uniform random sample of six fields was obtained from each section and was digitally stored using a camera attached to an Olympus microscope (BH2; Olympus Optical Co, Tokyo, Japan).

Stereological measurements of volume density (V_D) measurements of left ventricle components (capillaries V_{DC}, muscle V_{DM}, and interstitium V_{DI}), kidney components (interstitium and nephron) and thickness of glomerular Bowman's capsule, BC_T, were performed with stereology software (Histometrix, MIL 6 Version 1; Kinetic Imaging Ltd, Nottingham, UK) installed on a personal computer. Only micro-vessels without an apparent smooth muscle wall in the heart were included. Absolute volumes of capillaries (V_{AC}), myocardium (V_{AM}) and interstitial tissue (V_{AI}) in left ventricle were calculated using the mathematical equation;

$$V_{A(C,M,I)} = V_{D(C,M,I)} \times LVV_A$$

Statistical analysis

Data were expressed as means ± SEM, and were analyzed with GraphPad Prism Version 4.01 for Windows software (Graphpad Software Inc., San Diego, USA). Comparisons between the four groups were performed by analysis of variance ANOVA, followed by Newman-Keuls multiple-range tests. *P* values <0.05 are considered significant.

Table 1. Effect of Gum Acacia (GA, 15% ^{w/v} 4 weeks) on some physiological parameters in rats treated with adenine (0.75% ^{w/w} 4 weeks). Values in the table are mean ± SEM (n=6). Adenine was added to the feed at a concentration of 0.75% ^{w/w} for 4 weeks, and gum Acacia (GA) was given at a concentration of 15% ^{w/v} for 4 weeks. **P* < 0.05, compared to Control group; #*P* < 0.05, compared to Adenine-treated group

Parameters/Group	Control	Adenine	GA	Adenine+GA
Body weight change %	31.13 ± 3.83	44.52 ± 2.73*	5.76 ± 3.79*	34.55 ± 2.04
Kidney weight (% body weight)	0.85 ± 0.02	3.60 ± 0.07*	0.91 ± 0.02	1.41 ± 0.12#
Water intake (ml/day)	16.83 ± 2.18	36.80 ± 5.02*	18.00 ± 3.26	27.00 ± 1.71
Urine output (ml/day)	7.50 ± 0.88	31.16 ± 2.16*	5.83 ± 0.74	16.33 ± 1.11#

Table 2. Effect of Gum Acacia (GA, 15% ^{w/v} 4 weeks) on blood pressure and heart rate in rats treated with adenine (0.75% ^{w/w} 4 weeks). Values in the table are mean ± SEM (n=6). Adenine was added to the feed at a concentration of 0.75% ^{w/w} for 4 weeks, and gum acacia (GA) was given at a concentration of 15% ^{w/v} for 4 weeks. **P* < 0.05, compared to Control group; #*P* < 0.05, compared to Adenine-treated group

Parameters/Group	Control	Adenine	GA	Adenine + GA
Systolic blood pressure (mm Hg)	127 ± 4.77	164 ± 6.03*	129 ± 4.96	133 ± 5.17#
Diastolic blood pressure (mm Hg)	69 ± 4.65	115 ± 8.02*	64 ± 2.46	68 ± 2.16#
Heart rate (BPM)	360 ± 9.97	325 ± 20.41	358 ± 11.27	348 ± 13.78#

Table 3. Effect of Gum Acacia (GA, 15% ^{w/v} 4 weeks) on some biochemical parameters in rats treated with adenine (0.75% ^{w/w} 4 weeks). Values in the table are mean ± SEM (n=6). Adenine was added to the feed at a concentration of 0.75% ^{w/w} for 4 weeks, and gum Acacia (GA) was given at a concentration of 15% ^{w/v} for 4 weeks. **P* < 0.05, compared to Control group; #*P* < 0.05, compared to Adenine-treated group

Parameters/Group	Control	Adenine	GA	Adenine + GA
Plasma urea (μmol/l)	6.36 ± 0.80	150.71 ± 2.89*	13.97 ± 1.23	26.14 ± 0.98#
Plasma creatinine (μmol/l)	63.09 ± 4.76	205.57 ± 12.45*	54.53 ± 3.02	89.71 ± 11.31#
Creatinine clearance (ml/min)	1.06 ± 0.20	0.29 ± 0.04*	0.94 ± 0.19	0.55 ± 0.06
Urinary protein (g/l)	18.4 ± 1.94	98.25 ± 5.77*	23.02 ± 2.50	57.65 ± 5.47#
Plasma Calcium (mmol/l)	3.18 ± 0.14	1.52 ± 0.24*	2.87 ± 0.16	2.9 ± 0.21#
Serum Phosphorus (mg/dl)	14.35 ± 5.86	30.73 ± 12.54*	15.47 ± 6.31	8.40 ± 3.43#
Plasma nitrate (μmol/l)	55.79 ± 26.02	284.22 ± 44.22*	54.60 ± 16.75	222.19 ± 38.90
Plasma nitrite (μmol/l)	60.27 ± 14.77	230.42 ± 38.28*	36.64 ± 2.50	113.45 ± 18.30#
Indoxyl sulfate (μmol)	1.79 ± 0.81	159.55 ± 13.46*	0.00 ± 0.00	1.48 ± 0.70#

Results

Physiological effects

Table 1 shows the changes in body weights, relative kidney weights, as well as water intake and urine output in the four experimental groups. Adenine feeding alone significantly decreased body weight by about 44% when compared with the initial body weight. Rats

Fig 1. A representative micrograph showing kidney histomorphology in control rat (A) and rats treated for 4 weeks with adenine (0.75% ^{w/w} in the feed) (B), gum acacia (GA, 15% ^{w/v} in drinking water) (C) or adenine + GA simultaneously (D), using H & E (a), Trichrome Masson (b) and PAS (c) stains. No changes were seen in either untreated or gum acacia-treated controls (A and C). Marked expansion and hypercellularity of the interstitial area were noted in adenine-treated rats, along with increased amounts of collagen-like material (B). These abnormalities were markedly attenuated in adenine-treated rats that also received gum acacia (D). No glomerular or vascular injury was detected in any of the groups. Bowman's capsule (arrows), polymorphonuclear cell nuclei (arrowhead) are shown.

treated with adenine alone drank significantly more water and voided more urine compared to the control group, and the relative kidney weight in the former group was increased three-fold. These effects were significantly reversed with the addition of GA to the adenine-treated rats.

The data concerning SBP, DBP and HR at the end of the 4-week experimental protocol are presented in Table 2. Diastolic BP showed a significant increase in adenine-treated group when compared with the control or GA groups. ($P < 0.05$). GA treatment has no significant effect on either the SBP or the DBP when compared to the corresponding values in the controls. In rats given adenine and GA, there was a significant reduction in both SBP and DBP when compared to the adenine-treated group. There was no significant difference in the heart rates between all four experimental groups.

Fig 2. A representative micrograph showing left ventricle histomorphology in control rat (A) and rats treated for 4 weeks with adenine (0.75% ^{w/w} in the feed) (B), gum acacia (GA, 15% ^{w/v} in drinking water) (C) or adenine + GA simultaneously (D), using H & E and PAS stains. Capillary (black arrow), fibrosis (white arrow) are shown.

Fig 3. cortical IN thickness (INT) in control rat (A) and rats treated for 4 weeks with adenine (0.75% ^{w/w} in the feed) (B), gum acacia (GA, 15% ^{w/v} in drinking water) (C) or adenine + GA simultaneously (D).

Biochemical measurements

Table 3 shows the biochemical parameters in each of the four experimental groups. Adenine feeding (0.75% ^{w/w} for 4 weeks) caused significant increases ($P < 0.05$) in the concentrations of plasma urea and creatinine, and a significant decrease in the creatinine

Fig 4. Absolute volumes (in μm^3) of kidneys (A), and of left Kidney components (B & C) in control rats and rats treated for 4 weeks with adenine (0.75% w/w in the feed), gum acacia (GA, 15% w/v in drinking water) or adenine + GA simultaneously). Each column and vertical bar represent mean $\pm$ SEM (n=6). $P < 0.05$ was considered significant.

clearance ($P < 0.05$) compared to control group. As a further marker of kidney injury and established uremia, proteinuria and plasma concentration of indoxyl sulfate (a major uremic toxin) were analyzed showing a significant increase of urinary protein and plasma indoxyl sulfate in adenine-treated rats compared to controls. Most of these biochemical changes found in the adenine - treated rats were significantly abated in rats treated with adenine plus GA. There was no significant difference in any of the biochemical parameters measured between control rats and rats treated with GA alone.

Histopathological changes

Histopathological features of the kidney parenchyma stained with Hematoxylin and eosin (H&E), Trichrome Masson and PAS are illustrated in Figures 1 a, b and c, respectively.

No changes were seen in either untreated or gum acacia-treated rats (A and C, respectively). Marked expansion and hypercellularity of the interstitial area were noted in adenine-treated rats, along with increased amounts of collagen-like material (B). These abnormalities were markedly attenuated in adenine-treated rats that also received gum acacia (D). No glomerular or vascular injury was detected in any of the groups.

Histopathological features of the left ventricle myocardium stained with H&E and PAS are illustrated in Figure 2. Control rats and rats treated with GA only (A and C, respectively)

Fig 5. Absolute volumes (in μm^3) of left ventricle (A) and its components: muscle, (A) interstitium (B) capillaries (C) in control rats and rats treated for 4 weeks with adenine (0.75% w/w in the feed), gum acacia (GA, 15% w/v in drinking water) or adenine + GA simultaneously). Each column and vertical bar represent mean $\pm$ SEM (n=6). $P < 0.05$ was considered significant.

showed normal myocardium histology. The only obvious qualitative feature in adenine-treated rats was the presence of diffuse fibrosis and myocardial hypertrophy as compared to control rats.

Stereological changes

Quantitative morphometric kidney data are illustrated in Figures 3 and 4. Mean absolute volume of left kidney was doubled in adenine-treated rats as compared to control group. This increase was reversed in rats treated with adenine + GA. There was no significant difference in left kidney volume between control and GA-treated rats. Analysis of the cortical IN thickness (INT) showed that it was significantly increased in adenine-treated rats when compared to the controls and the GA-treated rats. The action was reversed but not completely when GA was given to the rats that received adenine (Fig 3). The increase in kidney volume is illustrated in interstitial fibrous tissue accounted for most of the increase in kidney volume in adenine-treated rats with volume of glomeruli and tubules actually decreased. These changes were significantly reversed with addition of GA to adenine-treated rats.

Quantitative myocardial data are illustrated in Figure 5. The mean absolute volume of the left ventricle was significantly increased in adenine-treated rats. This increase was mainly contributed by the myocardium and interstitial tissue with the capillary bed actually decreased. These changes were not observed in rats treated with adenine plus GA.

Discussion

In this work we reported on the structural and morphometric alterations in the renal and myocardial tissues that occur subsequent to adenine-induced CKD. The changes in the renal structure and in the vascular function in rats with adenine-induced CKD have recently been investigated, [8, 9, 14, 15] but as far as we are aware, there are no reports on similar morphometric analysis of the myocardium in this animal model.

Adenine feeding (0.75% ^{w/w} for four weeks) induced CKD as judged by the histopathological alterations in the kidneys, and by the significant increases in water intake and urine production, and in proteinuria, accumulation of the uremic toxin indoxyl sulfate, creatinine and urea concentrations, and the decrease in creatinine clearance, as well as in body weight. As a consequence of the adenine-induced CRF, blood pressure was significantly raised, and this has been recently reported [16]. Also, in confirmation of an earlier work, [6, 9] the co-administration of the natural product GA was effective in ameliorating all the above adenine-induced changes.

Our results on renal morphology further support earlier findings demonstrating glomerulosclerosis in adenine-induced CKD [9]. Previous studies on adenine-induced renal changes have been qualitative in nature [5]. In this paper, we provide innovative quantitative data that supports above previous observations. Thickness of Bowman's capsule is linked to the deposition of fibrous tissue usually secondary to chronic inflammation. Glomerular mesangial tissue cellularity further indicates infiltration with inflammatory cells thereby linking it with later development of glomerulosclerosis. The functional correlates of these quantitative changes include the deterioration in creatinine clearance, the significant increases in water intake and urine production, and the increases in creatinine and urea concentrations observed in adenine-CKD. Accumulation of the uremic toxin (as was shown here) is known to cause, among other actions, several structural changes in the kidneys such as inhibition of tubular epithelial cell proliferation, probably mediated by stress in the endoplasmic reticulum.

The proposed mechanism by which GA may have produced a salutary action on the kidney has been discussed earlier [8, 17, 18, 19]. Briefly, GA exerts both anti-inflammatory and anti-oxidant actions, and these may counteract the generation of free radicals and the inflammation that is associated with adenine-induced CKD. Also, we have recently shown that adenine-treated rats showed by Western blotting a detectable caspase-3 cleavage, which may be considered a possible mechanism involved in the adenine-induced CKD. The induced apoptosis was almost totally suppressed by GA in rat kidney tissue [5]. Recently, it has been reported that the complex inflammatory phenomena associated with adenine-induced CKD at the molecular level, may depend, at least partially, on NF- κ B activation [20].

We have shown in a previous similar work, [14] adenine-induced CKD causes a significant rise in systolic and diastolic blood pressure, with variable and insignificant effect on heart rate. GA was effective in ameliorating these actions. The possible mechanisms for this finding have been discussed [15]. Similarly, structural remodeling of the heart characterized by left ventricular hypertrophy (LVH) and expansion of interstitial tissue has been reported in uremic patients [21] and rats with partial renal ablation [22]. In the latter model, an antioxidant (dl- α -tocopherol) has been found to abrogate the renal insult. In this study we present quantitative morphometric characteristics of the LVH supporting the observed physiologic changes and that addition of GA has significantly reversed those changes.

As far as we are aware, there is no previously published work on the effect of GA on the heart in CKD. However, a single report by has shown that GA protects against doxorubicin cardiotoxicity in rats [23]. Some of the possible mechanism by which GA reverses the remodeling of the LV could involve its effects on the metabolism of some minerals such as phosphate and/or its antioxidant action.

Disturbances of the mineral metabolism are early and frequent occurrence in CKD [24]. The progressive loss of renal function leads to the lower production of calcitriol and phosphorus retention leading to hypocalcemia, as well as hyperphosphatemia. Moreover,

recent studies have shown the association of hyperrphosphatemia with LVH as well as with increased mortality in CKD patients [25]. It could well be that the beneficial effects of GA in reducing LVH and fibrosis is linked to its action in improving renal handling of serum calcium and phosphate. In fact, we did find that adenine-induced CRF causes hyperphosphatemia and hypocalcaemia.

Oxidative stress has been established to be a constant feature in CKD, and may be the basis of the resulting hypertension and other consequences [26]. Several authors have reported that cardiac remodeling characterized by LVH and coronary atherosclerosis is seen in uremic patients [27]. GA has been shown in previous studies to cause a decrease in the concentrations of several pro-inflammatory cytokines in plasma and kidneys of rats suffering from adenine-induced CRF [8]. Similarly, GA has been shown to decrease adenine-induced superoxide production and DNA damage in CKD model [8]. These anti-inflammatory and antioxidant capacities of GA may therefore add to the explanation of its effects in reversing the myocardial changes.

In conclusion, this study presents renal and myocardial morphometric data that supported the observed physiologic changes and correlated well with documented renal and cardiac remodeling seen in CRF. We add in this paper a further structural characterization of this remodeling to be myocardial hypertrophy and interstitial fibrosis, as well as myocardial capillary reduction.

Acknowledgements

This work was supported by a grant from the Research Council of Oman (RC/Med/Pharm/10/01). Thanks are due to the staff of the SQU Small Animal House for looking after the animals. GA was a kind gift from Sanwa-Cho, Toyonaka, Osaka, Japan

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