Physiol. Res. 63: 351-358, 2014

Anemia in Adenine-Induced Chronic Renal Failure and the Influence of Treatment With Gum Acacia Thereon

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Received October 18, 2013
Accepted January 17, 2014
On-line February 24, 2014

Summary

Anemia frequently complicates chronic kidney disease (CKD). We investigated here the effect of adenine-induced CKD in rats on erythrocyte count (EC), hematocrit (PCV) and hemoglobin (Hb) concentration, as well as on the activity of L-γ-glutamyl transferase (GGT) and the concentrations of iron (Fe), transferrin (Tf), ferritin (F), total iron binding capacity (TIBC) / unsaturated iron binding capacity (UIBC) and hepcidin (Hp) in serum and erythropoietin (Epo) in renal tissue. Renal damage was assessed histopathologically, and also by measuring the serum concentrations of the uremic toxin indoxyl sulfate (IS), creatinine, and urea, and by creatinine clearance. We also assessed the influence of concomitant treatment with gum acacia (GA) on the above analytes. Adenine feeding induced CKD, accompanied by significant decreases ($P<0.05$) in EC, PCV, and Hb, and in the serum concentrations of Fe, Tf, TIBC, UIBC and Epo. It also increased Hp and F levels. GA significantly ameliorated these changes in rats with CKD. A general improvement in the renal status of rats with CKD after GA is shown due to its anti-inflammatory and anti-oxidant actions, and reduction of the uremic toxin IS, which is known to suppress Epo production, and this may be a reason for its ameliorative actions on the indices of anemia studied.

Key words

Rats • Anemia • Iron • Gum acacia • Adenine • Chronic renal failure

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Introduction

Anemia is known to be an early and inevitable sign in patients with chronic kidney disease (CKD), and can confer significant multiple adverse clinical consequences, morbidity and mortality (Shah *et al.* 2006) and its management is a core component of nephrology care (Atkinson and Furth 2011). The occurrence of cardiovascular and renal diseases with anemia is termed 'cardio-renal-anemia syndrome' (Jürgensen *et al.* 2010). It is caused by a relative shortage of erythropoietin (Epo) and can also be associated with disordered iron homeostasis caused by reduced iron absorption, occult blood loss and impaired iron mobilization (Ruedin *et al.* 2012), as well as chronic inflammation (Nangaku and Eckardt 2006). Among the most important factors in the pathogenesis of iron metabolism defects is hepcidin (Hp), as it maintains mammalian iron homeostasis (Pantopoulos *et al.* 2012). Various methods for treating the anemia associated with CKD in humans and chronic renal failure (CRF) in laboratory animals have been used. These include iron (Liles 2012), Epo (Gianella *et al.* 2013, Silverberg *et al.* 2010), recombinant human EPO (Nichols *et al.* 2011, Teixeira *et al.* 2010) erythropoietin-gene electrotransfer (Ataka *et al.* 2003), and some new erythropoiesis-stimulating agents such as peginesatide (Graul 2012, Locatelli and Del Vecchio 2011).

The adenine-induced CRF model in rats is a

standard method for inducing a metabolic abnormality, similar to that which occurs in humans, in which adenine is given to rats in the feed at a concentration of 0.75 %, w/w, for 4 weeks (Ali *et al.* 2013b, Yokozawa *et al.* 1986). The excretion of nitrogenous compounds in adenine-treated rats is suppressed by renal tubular occlusion because of the formation of 2,8-dihydroxyadenine crystals, leading to accumulation of various guanidino compounds (such as methylguanidine and guanidinosuric acid) and urea nitrogen in blood (Yokozawa *et al.* 1986). As far as we are aware, there is only limited published work about anemia in adenine-induced CRF in rats and its pathogenesis, or possible treatment (Hamada *et al.* 2008, Okada *et al.* 1999, Sun *et al.* 2013). In this work the aim was to verify the effect of adenine-induced CRF on anemia, and further, to investigate the status of several factors involved in the pathogenesis of anemia in adenine-induced CRF such as iron (Fe), ferritin (F), transferrin (Tf), Epo and Hp in rats with the experimental disease, and further, to test the usefulness of treatment with the natural product gum acacia (GA) thereon. The salutary effect of GA in humans with CKD (Ali *et al.* 2008, Bliss *et al.* 1996), and in rat adenine-induced CRF, and some of its consequences have been previously reported (Ali *et al.* 2010, 2011a, b, 2013a). As far as we are aware, there are no reports in the literature describing the use of natural products to ameliorate the anemia induced by adenine-induced CRF in the rat except for two publications (both in Chinese) using two local medicinal plants (Ma *et al.* 2007, Wang *et al.* 2012).

Methods

Animals

Male Wistar rats (9-10 weeks old, weighing 249±10 g) were housed in a room at a temperature of 22±2 °C, relative humidity of about 60 %, with a 12 h light-dark cycle (lights on 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. Ethical clearance was obtained from our University Animal Ethics Committee and all procedures involving animals and their care were carried out in accordance 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 a 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 (SUPERGUMTM EM 10) in the 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.

During the treatment period, the rats were weighed weekly and one day before the last day of treatment were placed individually in metabolic cages to collect the urine voided in the last 24 h. Twenty-four hours after the end of the treatment the rats were anesthetized with ketamine (75 mg/kg) and xylazine (5 mg/kg) intraperitoneally, and blood (about 3-4 ml) was collected from the anterior vena cava and placed into plain tubes (about 3 ml) and in heparinized tubes (1 ml). The first aliquot of blood and urine were centrifuged at 900 *g* at 4 °C for 15 min. The serum obtained, together with the urine specimens, were stored at −80 °C to await analysis. The two kidneys were excised, blotted on filter paper and weighed. A small piece of the right kidney was placed in 10 % neutral buffered formalin for subsequent histopathology, and the rest of the kidneys were kept frozen at −80 °C for pending measurement of Epo within a week.

Hematological methods

In the blood collected in heparinized tubes erythrocyte count (EC), hemoglobin (Hb) concentration and hematocrit (Packed cell volume, PCV) were analyzed using automated methods (COBAS MICROS, Roche, Palo Alto, CA, USA).

Biochemical methods

The concentrations of creatinine and urea, as well as that of iron (Fe), ferritin (F), transferrin (Tf) and L-γ-glutamyl transferase (GGT) in serum were measured using kits from Human GmbH (Mannheim, Germany). Creatinine clearance (CCr) was calculated as reported before (Duarte and Preuss 1993). Total iron binding capacity (TIBC) / Unsaturated iron binding capacity (UIBC) concentrations were measured in serum using commercial kits in an automated machine (Cobas Integra, Roche Diagnostics, Basel, Switzerland). Renal Epo

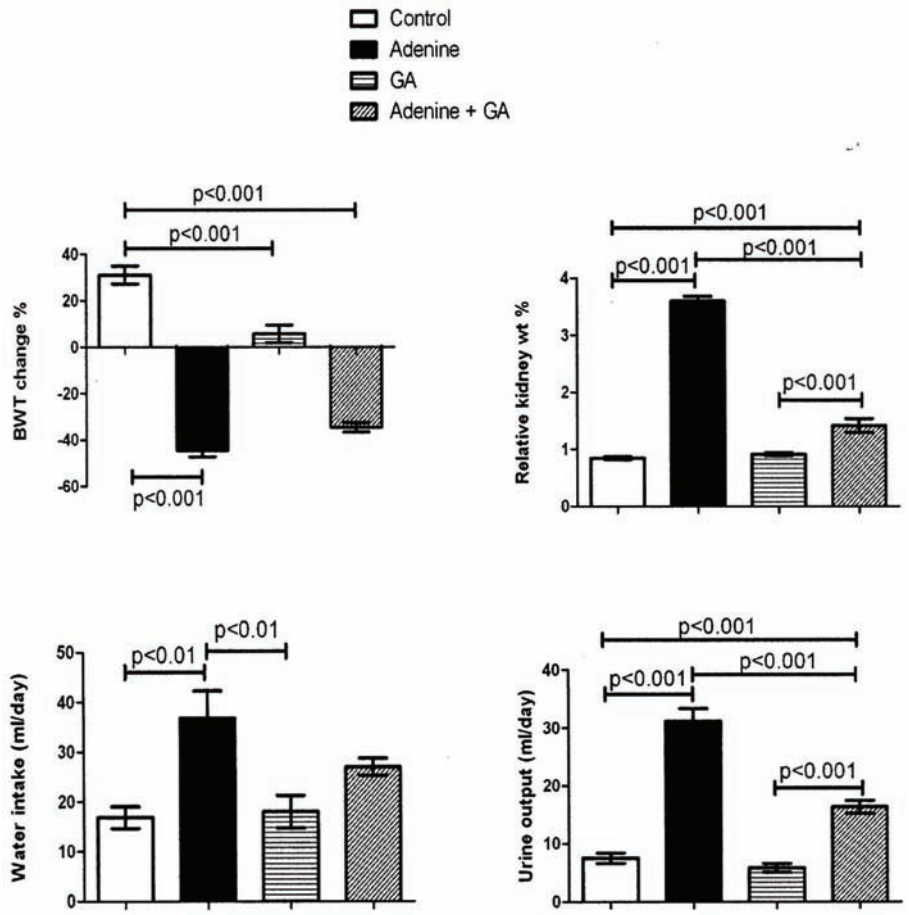

Fig. 1. Percentage body weight change (between final and initial body weight), and kidney weight (as percentage of final body weight) of control rats, rats treated with adenine (0.75 %, w/v, in feed for 28 days), and in rats treated with GA in drinking water at concentration of 15 %, w/v, with or without adenine for 28 days. Each column and vertical bar represent the mean ± SEM (n=6 rats). Statistical differences between the groups are shown.

concentration was measured by an ELISA method using commercial kits from R&D Systems, Inc. (Minneapolis, MN). Concentration of serum Hp was measured by an ELISA method using kit from Novateinbio (Woburn, MA, USA) and, plasma indoxyl sulfate (IS) concentration was measured using a validated HPLC method (Al Za'abi *et al.* 2013).

Histopathological methods

The kidneys were fixed in 10 % neutral-buffered formalin, dehydrated in increasing concentrations of ethyl alcohol, cleared with xylene and embedded in paraffin. Three micrometer sections were prepared from kidney paraffin blocks and stained with hematoxylin and eosin (H & E). The microscopic scoring of the kidney sections was carried out in a blinded fashion by a pathologist who was unaware of the treatment groups.

Chemicals

All chemicals used in this work were of the highest possible commercial grade available. Adenine was obtained from Sigma (St. Louis, MO, USA). GA (SUPERGUM^{TM} EM 10, Lot 101008, 1.1.11) was obtained from Sanwa Cho, Toyonaka, Osaka, Japan. The chemical properties of GA have been fully reviewed before (Ali *et al.* 2013a), and according to the manufacturer's data. SUPERGUM^{TM} EM 10 was characterized by size fractionation followed by multiple angle laser light scattering (GPC-MALLS) to give its molecular profile. The average molecular weight was 3.436×10^6, and the content of the arabinogalactan protein (AGP) was 26.4 %.

Aqueous solutions of both adenine and GA were prepared freshly every day just before use.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Each group consisted of at least six animals. All data are shown as means ± S.E.M. Group means were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Values of $P<0.05$ were regarded as significant.

Results

As shown in Figure 1, adenine feeding (0.75 %, w/w, for 4 weeks) caused significant decrease in body weight and a significant increase in relative kidney

Table 1. Effect of Gum Arabic (GA) on some biochemical parameters in serum of rats treated with adenine (0.75 %, w/w, 4 weeks).

Parameters/Group	Control	Adenine	GA	Adenine + GA
Urea (μmol/l)	6.4 ± 0.8	150.7 ± 2.9*	14.0 ± 1.2	26.1 ± 1.0#
Creatinine (μmol/l)	63.1 ± 4.8	205.6 ± 12.5*	54.5 ± 3.0	89.7 ± 11.3#
Creatinine clearance (ml/min)	1.1 ± 0.2	0.3 ± 0.0*	0.9 ± 0.2	0.6 ± 0.1
Indoxyl sulfate (μmol)	1.8 ± 0.8	159.6 ± 13.5*	0.0 ± 0.0	1.5 ± 0.7#
L-γ-glutamyl transferase (U/l)	4.2 ± 0.8	12.2 ± 0.3*	5.4 ± 1.0	11.5 ± 0.3*

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 GA (either alone or with adenine) was given in drinking water at 15 %, w/v. * $P<0.05$ (Control vs. all groups). # $P<0.05$ (Adenine vs. Adenine + gum)

Table 2. Adenine-induced changes in erythrocyte count, hematocrit and hemoglobin concentration in rats, and influence of gum acacia thereon (GA).

Parameters/Group	Control	Adenine	GA	Adenine + GA
Erythrocyte count (10^{12}/l)	7.0 ± 0.2	6.4 + 0.2*	7.2 ± 0.2	6.9 + 0.2
Hematocrit (%)	39.7 ± 0.8	29.5 ± 0.7*	40.1 ± 0.9	37.7 + 0.9
Hemoglobin (g/l)	127.2 ± 2.7	85.4 ± 2.9*	129.2 ± 3.0	121.2 + 6.1

Values in the table are mean ± SEM (n=6-12 rats). * Significant ($P<0.05$) difference from the control and the three other groups. No significant difference was noted between the control and GA and adenine + GA groups.

weight and in water intake and urine output ($P<0.05$). These changes were significantly but not completely antagonized by GA treatment.

As reported in the previous work on the histopathology of renal tissues (Ali *et al.* 2010, 2013a, b), here we have found that the control and the GA-treated groups of rats showed normal kidney histology (damage score of zero). The adenine-treated group showed diffuse acute tubular necrosis in about 70 % of the examined tissue areas (damage score of 4), and exhibited tubular distention with necrotic material involving loss of brush border of proximal tubules, dilatation of large number of tubules, mixed inflammatory cells infiltration of the interstitium, and focal tubular atrophy. The rats given adenine plus GA concomitantly showed improvement in the histological appearance when compared with the adenine-treated groups. There were focal areas of acute tubular necrosis involving about 30 % of examined areas, and there was also less dilatation of the tubules, less interstitial inflammatory cell infiltration, and less tubular atrophy (damage score of 1).

Adenine treatment significantly increased the concentrations of serum urea and creatinine, and significantly decreased the creatinine clearance. It caused a significant ($P<0.05$) increase in IS concentration and GGT activity (Table 1). Treatment with GA significantly abated these adenine induced actions. Adenine-induced CRF caused significant decreases ($P<0.05$) in EC, PCV, and Hb (Table 2), and in the serum concentrations of Fe, Tf, TIBC, UIBC and Hp (Table 3). It also increased Hp and F concentrations in serum. GA significantly ameliorated these changes in the adenine-treated rats. Renal Epo concentration was significantly decreased in adenine-treated rats, compared with control rats ($P<0.05$), with GA treatment having no effect on this parameter (Fig. 2).

Discussion

The global incidence of CKD is on the rise (Couser *et al.* 2011), but access to renal replacement therapy, by either transplantation or dialysis is limited in many parts of the world because of lack of financial and medical resources (Aviles-Gomez *et al.* 2006, Jha 2009). Strategies aiming at delaying the onset of dialysis or to ameliorate uremia often rely on dietary supplements (Cheu *et al.* 2013, Holden *et al.* 2012).

In the present study, we assessed the effects of adenine-induced CRF on several hematological parameters in rats, and the influence of GA thereon. The results indicated that adenine induces anemia, and that concomitant treatment with GA significantly abrogated this action.

Table 3. Effect of Gum Arabic (GA) on some serum constituents in rats treated with adenine (0.75 %, w/w, 4 weeks).

Parameters/ Group	Control	Adenine	GA	Adenine + GA
Ferritin (ng/ml)	108.0 ± 33.1	245.0 ± 53.8*	117.8 ± 16.4	160.2 ± 6.6
Iron (μg/dl)	209.6 ± 19.1	191.1 ± 17.9	215.6 ± 21.5	201.5 ± 15.5
Total iron binding capacity (μmol/l)	100.1 ± 2.2	72.1 ± 1.52*	99.28 ± 7.5	82.9 ± 3.3*
Unsaturated iron binding capacity (μmol/l)	73.4 ± 4.1	41.42 ± 3.76*	73.82 ± 8.6	55.3 ± 3.5
Transferrin (mg/dl)	115.4 ± 12.8	81.9 ± 12.4	105.4 ± 19.0	114.1 ± 19.7
Hepcidin (ng/ml)	12.4 ± 0.0	16.2 ± 1.6*	12.5 ± 2.3	10.3 ± 1.4#

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 GA (either alone or with adenine) was given in drinking water at 15 %, w/v. * $P<0.05$ (Control vs. all groups). # $P<0.05$ (Adenine vs. adenine + gum).

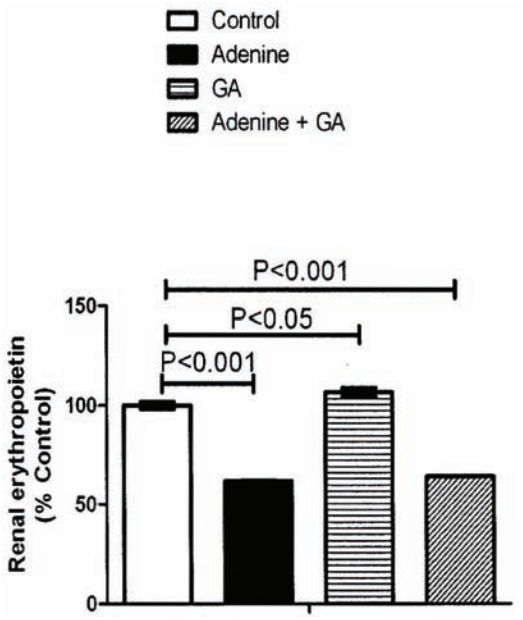

Fig. 2. Renal erythropoietin concentration (% control) of control rats, and in rats treated with adenine (0.75 %, w/w, in feed for 28 days), or GA in drinking water at concentration of 15 %, w/v, with or without adenine for 28 days. Each column and vertical bar represent the mean ± SEM (n=6 rats). Statistical differences between the groups are shown.

Insufficient production of the glycoprotein hormone Epo is one of the main causes of uremic anemia (Nangaku and Eckardt 2006). It has also been shown that IS impairs oxygen metabolism in tubular Epo-producing cells *in vitro*, and that its administration to rats suppresses renal Epo mRNA expression and plasma Epo concentrations (Chiang *et al.* 2011). This suggests a possible connection between the uremic toxin IS and the desensitization of the oxygen-sensing mechanism in Epo-producing cells, which may, at least, partly explain inadequate Epo production in hypoxic kidneys of CKD patients. In this work we have found that adenine feeding causes a significant rise in the concentration of the uremic toxin IS, confirming earlier work on adenine-induced CRF (Ali *et al.* 2010) and in rats with CRF induced by 7/8 nephrectomy (Ali and Ahmed 2006). However, we have found here that treatment with GA in adenine-treated rats significantly decreased the adenine-induced rise in IS, but this was not accompanied by any significant alteration in the renal Epo concentration when compared to that in rats treated with adenine alone (Fig. 2). Therefore, it is not possible from this work to associate the renal Epo concentration in adenine-treated rats to their serum level of IS. Further work to investigate the relationship of plasma IS and renal Epo levels, and the effect of treatment with Epo-recombinant drugs on the adenine-induced anemia are warranted. Results from previous work using Epo-recombinant drugs on anemia in rats with CKD were not conclusive (Teixeira *et al.* 2012).

GA has been reported before to increase fecal nitrogen excretion and to lower serum urea nitrogen concentration (Ali *et al.* 2009), and has since then been used in treating CKD in several developing countries such as Sudan and Iraq (Ali *et al.* 2008, Al Mosawi 2009). In addition to the increased clearance of nitrogen in CKD, GA has an additional beneficial effect on kidney function, which is related to its anti-inflammatory and antioxidative effects (Ali *et al.* 2013a). Both oxidative stress and inflammation may be involved in CRF-induced anemia (Cachofeiro *et al.* 2008), and treatment with GA may also abate the anemia through these two actions.

The liver-derived peptide hormone Hp in the

kidney has an iron-regulatory role in the renal tubular system, involving the iron transporter divalent metal transporter-1 (Kulaksiz *et al.* 2005). The concentration of hepatic Hp was shown to be increased following CRF induced by 5/6 nephrectomy (Srai *et al.* 2010) and adenine treatment (Hamada *et al.* 2008). As far as we are aware, there are no data on Hp in serum from rats with CRF, but it has been shown that in humans with CRF, Hp is elevated (Srai *et al.* 2010). Here, we have found that adenine induces a significant rise in Hp concentration in serum, and that was significantly reversed by concomitant GA treatment. The rise in serum Hp concentration was probably due to the decrease in renal Epo concentration, as addition of Epo has been shown to downregulate Hp (Babitt and Lin 2012).

In this work, serum GGT activity was measured to assess the status of the liver. The significant increase in GGT activity following adenine feeding was suggestive of hepatic damage. Although total hepatic Fe quantification has not been conducted in the work, the hepatic damage might suggest that Fe stores were reduced, and this may be a factor in adenine-induced anemia, although our current data does not support a role for the enzyme GGT in the regulation of hepatic Fe. It should also be mentioned that there are reports suggesting the implication of GGT in the development of a disturbed redox status in the kidney cortex in rats with CKD (Ceyssens *et al.* 2004), and it is also established that the pathogenesis of adenine-induced CKD involves oxidative stress in the renal tissues (Ali *et al.* 2013a). Further studies on this aspect are warranted.

In conclusion, we have shown that adenine-induced CRF is associated with the occurrence of anemia, an action that has been significantly ameliorated with concomitant treatment with GA.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was funded by the Research Council (TRC) of Oman (Project number RC/Med/Phar/10/01). We thank the staff of the Animal House of SQU for looking after the rats and I. Al-Lawati and S. Beegam for technical help in the early stages of this work.

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