Chemico-Biological Interactions 252 (2016) 36–46

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Polyacrylic acid attenuates ethylene glycol induced hyperoxaluric damage and prevents crystal aggregation *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 25 December 2015
Received in revised form
4 March 2016
Accepted 20 March 2016
Available online 24 March 2016

Keywords:
Polyacrylic acid
Anionic polymer
Hyperoxaluria
Urolithiasis
Nephroprotection

ABSTRACT

The study explores calcium oxalate crystal inhibiting characteristic of polyacrylic acid (pAA), an anionic polymer in *in vitro* and *in vivo*. Animals were divided into 5 groups where group 1 served as control, group 2 were made hyperoxaluric by supplementing with Ethylene glycol (EG) 0.75% (v/v) for 30 days. Group 3, 4 & 5 were also given with EG and treated simultaneously with 2.5, 5 & 10 mg of pAA/kg of body weight, respectively. Urine, serum and tissue analyses along with histological studies were performed at the end of the 30 days study. *In vitro* crystallization was significantly inhibited by pAA and further it was supported by particle size analyses, XRD and FT-IR studies. Toxicological analyses showed that pAA was safe to use in animals at concentrations below 100 mg/kg BW. *In vivo* anti-urolithic study showed significant improvement in urinary lithogenic factors (calcium, oxalate, phosphate, citrate & magnesium) and renal function parameters (creatinine, urea and protein). Tissue analyses on anti-oxidant enzyme activity and lipid peroxides showed maintenance of tissue antioxidant status in the pAA supplemented rats and histological studies demonstrated the nephroprotection offered by pAA and were concurrent to the biochemical analyses. Supplementation of pAA not only reduces the crystal aggregation but also regulates the expression and localization of crystal inhibiting proteins and gene expression of inflammatory cytokines in experimental animals. In summary, pAA is a potent anti-urolithic agent in rats and we can propose that 10 mg/kg body weight is the effective dosage of pAA and this concentration can be used for further studies.

1. Introduction

Kidney stone formation (urolithiasis) is one of the common kidney diseases that can be attributed to multiple factors. The complexity in lithogenesis and recurrence are the major concerns [1]. Stone formation starts with supersaturation, which is the major event involved in crystal formation, while hyperoxaluria, hypercalciuria, hypocitrateuria and membrane damage are the pathophysiological features that aid in disease progression [2]. Urinary macromolecules (predominantly urinary proteins) are highly anionic in nature and are considered to be the inhibitors of stone formation [3]. However, in the metastable urine, the structural modifications of these proteins lead to protein aggregation and drive the crystal aggregation process to occur at faster rate [4]. Thus, a slight alteration in structural or ionic properties of crystal inhibitors can be considered as another major risk factor nephro/urolithiasis [5].

Unconvincing drug interventions and invasive surgical techniques are the major concerns in management of urolithiasis [6]. Thiazides and alkalizing agents are still being prescribed by physicians for treatment of different types of stones. But the primary risk factors will remain unaltered even after the medication and recurrence becomes very frequent [7]. In order to avoid calcium oxalate stone recurrence, here we used bio-compatible and anionic polymer since that can mimic the anionic urinary macromolecules with diverse applications in medical and industrial field.

Polymers possess diverse physical, chemical and mechanical

Abbrevations: pAA, Polyacrylic acid; EG, Ethylene Glycol; XRD, X-ray Diffraction; FT-IR, Fourier Transform-Infra red; BW, Body Weight; HEPES, 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid; JCPDS, Joint Committee on Powder Diffraction Standards; COM, Calcium Oxalate Monohydrate; IAEC, Institutional Animal Ethical Committee; PBS, Phosphate Buffered Saline; SOD, Superoxide Dismutase; THP, Tamm-Horsfall Protein.

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http://dx.doi.org/10.1016/j.cbi.2016.03.020
0009-2797/

properties, which makes them a valuable component in biomedical research arena [8]. Some of these polymers have been used as drugs, as they are readily bioavailable and biocompatible. Grohe et al. (2009) demonstrated that ionic polymers completely made of aspartic acid or glutamic acid residues have a significant effect on crystallization processes. It was experimentally demonstrated that highly anionic molecules tend to inhibit crystal growth while crystal aggregation can be checked by molecules that are weakly anionic [9–11]. Polyacrylic acid (pAA), is one such anionic polyelectrolyte, which is highly responsive to the change in environmental factors like temperature, pH which makes it a desirable candidate drug that targets urinary environment, since, pH is one of the major risk factors in lithogenic process [12].

A study performed by Kleinman et al. (2008) demonstrated that carboxyl groups in pAA are highly potent in inhibiting calcium oxalate crystal formation and they also reported that intravenous administration of pAA 5.1 prevented calcium oxalate crystal deposition in Sprague-Dawley rats [13]. Though pAA was reported to reduce calcium oxalate deposition in rats, the biochemical alterations that happened during the disease processes and treatment regimen were not testified. In the present study, pAA 5.1 was used for preventing stone formation by oral administration and we hypothesize that calcium oxalate crystallization in Wistar rats will be inhibited by pAA with its ability to mimic the urinary anionic, crystal inhibiting proteins. We have established a complete urine and serum biochemistry of urolithic and pAA treated groups in this study, along with tissue localization of NF-κB, Tamm-Horsfall protein, osteopontin and transferrin were investigated. Apart from this, gene expression of pro-inflammatory markers (NF-κB, TNF-α, IL-6 & MCP-1) were analysed to demonstrate change in inflammatory events after pAA administration.

2. Materials and methods

2.1. Materials

Polyacrylic acid 5.1 (pAA 5.1) was purchased from Sigma-Aldrich, India. Solvents and other standard chemicals were purchased from Sisco Research Laboratories, Pvt. Ltd., India. The reagents used for the estimation of calcium, urea, creatinine and protein were purchased from Span Diagnostics Ltd., India. X-ray diffraction was performed using Powder X-Ray Diffractometer, D8 Advance model, Bruker, Germany. Studies were carried out with the aid of ALPHA-T spectrometer, Bruker, Germany. Crystal aggregation study of pAA was performed using Master sizer 2000, Malvern Instruments Pvt. Ltd, United Kingdom.

2.2. In vitro experiments

2.2.1. In vitro calcium oxalate crystallization study

The assay was performed as described by Khan et al. (2011) [14]. Stock solutions of $CaCl_2$ (5 mM) and $Na_2C_2O_4$ (5 mM), containing 200 mM NaCl and 10 mM sodium acetate were adjusted to pH 5.7. Effect of pAA (50, 100, 250 & 500 mM) on calcium oxalate crystallization was studied by time course measurement of turbidity changes at 620 nm, due to the crystal nucleation and aggregation after mixing metastable solutions of calcium (Ca^{2+}) and oxalate (Ox). All the values were obtained after three independent experiments. Regression analyses was performed for each curve and the slopes were used to calculate the percentage inhibition by the formula given below,

$$\text{Percentage inhibition } (\%) = (1 - (\text{Sm}/\text{Sc})) \times 100$$

Where Sm is slope in the presence of pAA and Sc is slope of the control experiment (without pAA).

2.2.2. Calcium depletion assay

Assay was carried out with the methodology described by Chutipongtanate and Thongboonkerd (2011) with slight modifications [15]. The reduction in calcium depletion in the presence of pAA will directly correlate with the reduction in crystal formation [16]. Briefly, the assay was performed by mixing 5 mM $CaCl_2$ and $Na_2C_2O_4$ in a buffer containing 200 mM NaCl and 10 mM sodium acetate adjusted to pH 5.7. The contents were incubated in the presence and absence of pAA at different concentrations for 12 h. Supernatant (20 μl) from each sample were estimated for calcium content by Arsenazo III reagent (1 ml). The samples were gently mixed and then incubated for 5 min at 25 °C. The colour formed was measured at 620 nm and the amount of Ca^{2+} was calculated using the standard curve that was generated by using $CaCl_2$. All the values were obtained after three independent experiments.

2.2.3. Particle size analyses

Crystal aggregation study of pAA was performed using the method already established by Wesson et al. (2005) [17]. The change in crystal size was measured as R_D which is the ratio of size of the crystal in the presence of pAA to size of the crystal in the absence of pAA. All the values were obtained after three independent experiments.

$$R_D = \text{Size of crystal with additive}/\text{Size of seed crystal}$$

2.2.4. X-ray diffraction and FT-IR analyses

The crystal formed after an hour of incubation with 100 mg pAA was centrifuged. The pellet was dried before submitting for X-ray diffraction and FT-IR analyses. FT-IR was performed at a spectral range of 400–4000 cm^{-1} with a 4 cm^{-1} resolution. The peaks obtained were compared with the standard peaks of COM seed crystal obtained from JCPDS database for X-ray diffraction. FT-IR peaks were compared with various literatures provided with standard COM peaks.

2.3. In vivo experiments

2.3.1. Animals

Wistar rats of 8 weeks, weighing 200–250 g were used for the study. All animal experiments and maintenance were carried out according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (Registration No-1333/c/10/CPCSEA; Approval number-VIT/IAEC/8th/13). Animals were housed in polypropylene cages and maintained under standard conditions of 12 h dark/light cycle at 27 ± 1 °C. The rats were supplied with regular pellets and water *ad libitum*.

2.3.2. Sub-acute toxicity study

Forty eight Wistar rats (24 male & 24 Female) were divided into 4 groups with each group containing 6 male and female animals. Group 1 received saline and was designated as control animals; group 2, 3 & 4 animals received pAA at a concentration of 20, 60 & 100 mg/kg body weight respectively by oral administration with intra-gastric tubes. The food and water consumption of the animals and the changes in their body weight were monitored on a daily basis. At the end of 30 days, the animals were sacrificed and blood was collected by cervical decapitation under anaesthetic condition. Blood collected with anticoagulant was used for haematological analyses, while the blood without anticoagulant was incubated at room temperature and allowed to clot completely. Then it was