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Sulfated glycosaminoglycans in bladder tissue and urine of rats after acute exposure to paraoxon and cyclophosphamide

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ABSTRACT

Glycosaminoglycans (GAGs) in the urine of Wistar rats, and on the surface of the epithelium and lamina propria of the bladder, were quantitatively assessed before and after acute intoxication with paraoxon or cyclophosphamide. Paraoxon was administered subcutaneously (s.c.) twice with an interval of 1 h to a final dose of 275 mg/kg; cyclophosphamide was administered intraperitoneally (i.p.) with a single dose of 100 mg/kg or to a final dose of 240 mg/kg (three times per 80 mg/kg every 12 h). GAGs on the surface of the epithelium and lamina propria of the urinary bladder of rats were quantitatively determined by Alcian blue dye staining. GAGs in the urine were determined spectrophotometrically with 1-9-dimethyl methylene blue. By 48–96 h after intoxication with either paraoxon or cyclophosphamide, statistically significant increases in the concentration of GAGs were obtained both for the tissues of the bladder and the urine of rats. Cyclophosphamide, in contrast to paraoxon, caused the development of hemorrhagic cystitis in the animals. The synthesis of GAGs is considered to be compensatory response to the toxic xenobiotics.

1. Introduction

Many xenobiotics adversely affect the functioning of the urinary system of mammals (Wang et al., 2014). Paraoxon and cyclophosphamide are organophosphorus compounds and the mechanistic actions of such compounds can differ fundamentally. We have previously noted their toxic effects on the kidneys and urinary tract (Shmurak et al., 2012; Anikin et al., 2014), which aroused our interest in specifying the effects of these compounds in order to reveal new markers of kidney damage.

Paraoxon (diethyl 4-nitrophenyl phosphate) is a product of the metabolism of organophosphate parathion. It is an extremely toxic organophosphate (OP) and one of the most potent inhibitor of acetylcholinesterase (AChE), with around 70% of the potency of the nerve agent sarin (Nurulain et al., 2015). The cholinergic effects of paraoxon on the bladder were first described in 1961 as increased urinary frequency and incontinence (Verhulst and Page, 1961). A single per oral administration to mice of methyl parathion in a dose of 0.28 mg/kg led to several histological changes in the kidneys: glomerular atrophy, dilated renal tubules, haemorrhage, oedema and necrosis. Methyl parathion treated animals showed positive reaction to Bax in glomerulii and renal tubules as compared with controls (Abdel-Rahman et al., 2014).

Cyclophosphamide (CP), or (*RS*)-*N*,*N*-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide, is a derivative of bis-β-chloroethylamine and is an alkylating cytostatic drug. The drug was synthesized in 1958 in the laboratory of Norbert Brock and is one of the most popular anti-tumor and immunosuppressive drugs in human and veterinary medicine (Brock, 1996; Emadi et al., 2009). A common feature of all alkylating agents is their high biochemical reactivity. When entering the human or animal body, 75% of the injected dose is activated at the initial stage and undergoes a series of metabolic changes, while the remainder is not metabolized. In the last stages of this process aldofosfamid spontaneously splits by β-elimination into acrolein and phosphoramide mustard, which are the end products of the CP metabolism (Zhang et al., 2005). Acrolein formation is the cause of urotoxic side effects of CP, associated in particular with aseptic hemorrhagic cystitis (Talar-Williams et al., 1996).

Glycosaminoglycans (GAGs) are polysaccharides with varying degrees of sulfation, molecular weight and biological activity. In humans and animals they are covalently bound to the protein moiety of the proteoglycans, and as a rule do not occur in the free

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form. The main classes of GAGs are heparins, heparans, chondroitins, keratans, and dermatans (Hook et al., 1984). The biological functions of GAGs in mammals are regulation of hemostasis, vascular protection and regulation of permeability. Macromolecules of heparan sulfate proteoglycans (HSPGs) are associated with the cell membrane and extracellular matrix; in particular, they are principal components of the glomerular basement membrane (GBM) (Miner, 1999). These molecules are synthesized by endothelial cells and podocytes (Pyke et al., 1997; Sorensson et al., 2003) and localized in *laminae rarae* (Stow et al., 1985). HSPGs have a variety of functions, including the formation of negative charges of GBM. They also act as contact points for the podocytes and endothelial cells (Taipale and Keski-Oja, 1997), and stabilize the GBM by binding to laminin, collagen IV and enactin/nidogen (Miner, 1999). Furthermore, HSPGs bind and sequester cytokines, antithrombin III and growth factors which determine development of kidneys (Kanwar and Rosenzweig, 1982; Pyke et al., 1997).

Although there are a large number of published studies on pathophysiology of acute kidney injury, the roles of heparan sulfate GAGs in the glomerular capillary wall are still not entirely resolved (McCarthy and Wassenhove-McCarthy, 2012). GAGs are homeostatic factors providing a protective function in the urinary tract exposed to harmful agents, so can be regarded as biomarkers in biological fluids and tissues in response to toxic substances and their metabolites. Due to the damaging effect of paraoxon and CP on the kidneys and bladder tissue, it is interesting to speculate that the level of GAGs provide the local homeostasis. The purpose of the research reported on here was to compare the toxic effects of paraoxon and CP in the urinary tissue of rats, by determining the levels of urinary GAGs excretion and on the surface of the epithelium and tissues of the bladder.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats (180–220 g) were kept in standard plastic T2 type cages, at 21–23 °C and light conditions of 12 h day – 12 h night (*12*C:*12*T). Before the experiment, rats were acclimated to the forthcoming conditions for 96 h. The rats were fed with standard pelleted feed and water *ad libitum*. The non-pathogenic food for laboratory animals "Chara" was provided by Laboratorkorm (Russia). The drinking water was purified according to recommendations of the Institute for Laboratory Animal Research (ILAR) and contained no more than 1 CFU/100 mL. All experiments were conducted in accordance with National guidelines for the welfare of experimental animals and with the approval of the Bioethics Committee of the Research Institute of Hygiene, Occupational Pathology and Human Ecology (RIHOPHE). Selection and randomization of animals were carried out with comparative adaptive randomization method, and the instruments of the Graph Pad software (https:www.graphpad.com/quickcalcs).

2.2. Paraoxon and cyclophosphamide intoxication

Cyclophosphamide was purchased as a medicine for injections, Endoxan (Baxter©), containing 1000 mg of sterile powder per bottle. The solution was prepared ex tempore with physiological saline to final concentration of CP 20 mg/mL. Paraoxon-ethyl was purchased from Sigma-Aldrich (Cat. No. D9286) as a yellowish oil with purity ≥90%.

Rats of group 1 were intact controls. Animals of group 2 were injected intraperitoneally (i.p.) with CP at a dose of 100 mg/kg b.w. Rats of group 3 were treated with CP i.p. three times per 80 mg/kg at 12 h intervals, to a final dose of 240 mg/kg. Rats of group 4 were administered with ethyl paraoxon (Sigma, USA) to a final dose of 275 mg/kg (150 + 125 mg/kg s.c. with an interval of 1 h). Urine samples were collected from CP intoxicated rats before exposure, and 48 and 96 h after exposure. For paraoxon intoxicated rats, urine samples were collected before exposure, and 24, 48, 72, 96 and 168 h after exposure. Bladder tissue samples for histochemical studies were withdrawn 96 h after the exposure.

2.3. Histochemical method for quantitative assessment of Alcian blue concentration in the bladder tissue of rats

Preparation of Alcian blue calibration standards was according to advice from Dr. Silantyeva in our laboratory, in which calibration standards of methyl green dye and pyronine solutions were used to identify nucleic acids (Silantyeva, 2010). We used Alcian blue dye for histochemical identification of GAGs. Saturated aqueous solutions of Alcian blue (Biovitrum©, Russia) were prepared at 37 °C, in a 20% colloidal solution of gelatin (Table 1). The standards were polymerized at +4 °C over 24 h and cut into 5 × 5 × 5 mm blocks prior to freezing in liquid nitrogen to prevent the crystallization of water. The frozen blocks were placed on a chilled microtome cryostat object holders (Slee Mainz, Medical GmbH©, Germany) and slices (7 and 9 μm thickness) were obtained at −20 °C. The sections were mounted onto glass slides with the final concentrations of stain indicated, then air dried and embedded under coverslips.

The sections prepared to contain calibration standards of Alcian blue were photographed at magnifications of x100, x200 and x400. Then, a minimum of 10 areas were randomly selected in a digital imaging program (VideoTest size 5.0, Akond, Russia) using the "circle" tool, in which absorbance was estimated in arbitrary units. The relative absorbances of the calibration standards were used to construct calibration curves for dye concentration in the 7 and 9 μm sections (Fig. 1).

Bladder cryostat sections of 7 and 9 μm were obtained on a cryostat microtome (Slee MEV, Medical GmbH©, Germany). Sections were stained with hematoxylin-eosin and Alcian blue at pH 2.5 (Biovitrum©, Russia). Staining of the histological sections with Alcian blue involved placing in a solution of Alcian blue for 3 min, with subsequent washing in distilled water for 2 min. For additional visualization of the tissue, the sections were stained with Harris' hematoxylin solution for 2 min, then washed twice in distilled water for 2 min.

The sections were dehydrated in alcohols of increasing strength, then clarified with *ortho*-xylene for 2 min and embedded into a Biomaunt© medium under coverslips. The stained histological sections were photographed at magnifications ×103, ×213 and

Table 1
Preparation of calibration standards of Alcian blue dye.

##	The dye/water ratio	Dye, μL	Water, μL	Concentration, g/L[a]
1	1:400	2.5	997.5	0.0125
2	1:200	5	995	0.025
3	1:100	10	900	0.05
4	1:50	20	980	0.1
5	1:10	100	900	0.5
6	1:5	166	834	1.0
7	1:1	500	500	2.5
8	2:1	666	333	3.3
9	4:1	800	200	4.0
10	10:1	900	100	4,5
11	1:1	1000	1000	5.0
12	2:1	1335	665	6.6
13	4:1	1600	400	8.0

[a] The final concentration in a volume of 2 mL after mixing with a 20% gelatin solution. The latter was prepared as following: Gelatin (Sigma, USA), 4 g, was dissolved in 15 mL of deionized water at 37C. The final calibrating standard of the dye was obtained by mixing 1 mL of the 20% gelatin solution with 1 mL calibrating standard of Alcian blue.

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