Decrease in the activities of lysosomal enzymes may contribute to the urotoxicity of cyclophosphamide in the rat

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Vol. 18, No. 2 (2007-05 - 2007-08)

Biomedical Research 2007; 18 (2): 129-134

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Key words: Cyclophosphamide; urotoxicity; lysosomal enzymes; protein content; rat

Accepted April 17 2007

Abstract

One of the most common adverse side effects of cyclophosphamide, a potent anticancer drug which limits its clinical utility is its urotoxicity. The mechanism of cyclophosphamide induced urotoxicity (cystitis) is poorly understood. In the present study the possible role of lysosomal enzymes in cyclophosphamide induced hemorrhagic cystitis was investigated using rat model.

Adult female Wistar rats weighing 200-250 gm were used for the study. The rats were administered single intraperitoneal injection of cyclophosphamide at the dose of 150 mg/ kg body wt and sacrificed at various time intervals 6 hr, 16 hr or 24 hr after the dose of cyclophosphamide. The control rats were administered saline alone. The urinary bladder was removed, weighed and used for histological examination and assay of lysosomal enzymes namely acid phosphatase, β glucuronidase and N acetyl glucosaminidase.

The weight of the urinary bladder (g/ 100 g body wt) of the drug treated rats was significantly higher compared to that of the untreated rat (0.12± 0.01 Vs 0.064 ± 0.005, P≤ 0.004). Histologically, all the characteristic features of hemorrhagic cystitis and hematuria were observed in the drug treated rats. The extent of damage to the bladder increased with time after treatment. A highly significant increase in protein content (mg/ g wt) was observed in the bladders of the cyclophosphamide treated rats as compared with the control (16.4± 3.0 Vs 6.5 ± 1.7, P ≤0.009). A significant drastic decrease in the activities of the entire lysosomal enzyme studied was observed in the bladders of drug treated rats at 16 hrs and 24 hrs. Acid phosphatase activity was decreased by 437% (P≤ 0.004); N acetyl glucosaminidase activity was decreased by 403% (P≤ 0.05) and glucuronidase activity by 392% (P≤ 0.009) as compared with the control.

Decrease in the activities of lysosomal enzymes in the urinary bladder of cyclophosphamide treated rat may contribute to the urotoxicity of cyclophosphamide.

Introduction

Cyclophosphamide (CYP) is a drug with a wide spectrum of clinical uses, and it has been proved to be effective in the treatment of cancer and nonmalignant disease states. However, this drug may induce acute inflammation of the urinary bladder (cystitis) and therefore the therapeutic use of CYP is limited [1, 2]. Cystitis is manifested by marked congestion, edema, extravasation of the urinary bladder, as well as marked desquamative damage to the urothium, severe inflammation in the lamina propria and focal erosions.

Although there are some preliminary clinical studies and animal studies [3-5], there does not appear to be an extensive examination of mechanism of cystitis induced by cyclophosphamide. It is important to find out the mechanism by which CYP produces cystitis in order to prevent/ treat the patients who develop cystitis as a result of CYP therapy.

Cyclophosphamide is cytotoxic to the transitional epithelium lining the urinary bladder, and also causes hemorrhage from the sub epithelial blood capillaries. Under normal conditions, the main function of the transitional epithelium is to act as protective lining to the bladder. The normal epithelium contains variable number of lysosomes, which are most numerous in the superficial cells. By electron microscopy, vesicular or heterogeneously dense lysosomes have been demonstrated in rat and human epithelial cells; it has also been suggested that epithelial cells actively form autophagolysosomes [6]. Electron microscopic investigations of the cyclophosphamide-induced lesions of the urinary bladder of the rat suggest that lysososomes may be involved in its urotoxic effect [7]. Acid phosphatase activity [8, 9] and glucuronidase activity [10] have been demonstrated in these lysosomes and their normal function in the superficial cells is to breakdown surplus or damaged plasma membrane [9].

Lysosomes contain hydrolases that breakdown unwanted macromolecules including proteins. Therefore decrease in the activities of lysosomal enzymes can result in the accumulation of abnormal amounts of proteins and other macromlecules within the bladder causing damage.

Lysosomes are reported also to play a role in cell death and tissue damage due to drugs e.g. paracetamol or gentamicin [11-14] and toxins [15, 16]. We hypothesised that cyclophosphamide induced urotoxicity may be due to alteration in function of the lysosomal enzymes. Thus far there are no studies reported on the role of lysosomal enzymes in cyclophosphamide induced urinary bladder damage to the best of our knowledge.

The rat is a suitable model for the study of urinary bladder toxicity of the chemotherapeutic agent because after the administration of CYP, the rats present with histological evidence of urinary bladder injury similar to that seen in humans [17, 18].

Therefore, in the present study we investigated whether there is any alteration in the activities of the lysosomal enzymes in the urinary bladder of rats after treatment with cyclophosphamide.

Materials and Methods

Cyclophosphamide (Endoxan) was purchased from Baxter oncology GmbH, Germany. All the other chemicals were obtained from Sigma Chemical Company U, S, A.

Animals: Adult Female Wistar rats (180-220g) were used for the experiments. The study was approved by animal ethics Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India. The guidelines were followed.

Animal treatment

Dosage and route of administration of cyclophosphamide were determined from those described in literature as causing consistent cystitis in normal rats [17]. The rats were divided into two groups and treated as follows.

Group I: The rats in this group (n = 21) received single intraperitoneal injection of cyclophosphamide in saline at the dose of 150 mg/ kg body weight.

Group II: The rats in this group (n=6) received saline alone. Rats were killed 6 hours, 16 hours or 24 hours after the dose of CYP or vehicle.

Tissue procurement

Rats were killed by exsanguination. The urinary bladder was removed and blotted dry before weighing. A part of the bladder was used for biochemical assays and another part for histological assessment.

Histology

The dissected bladder were fixed overnight in buffered neutral formalin, processed to paraffin wax, sectioned at 3 to 4 μm, and stained with haematoxylin and eosin for examination by light microscopy.

Biochemical assays

Urinary bladder homogenates was prepared as described by Kyaw et al. [19]. Portions of urinary bladder were homogenized in 10 ml of ice cold 0.25 M sucrose-1mM Na2EDTA in a Potter-Elvehjem homogeniser. The homogenate was centrifuged at 4 °C at 11,000 x g for 30 minutes to remove unlysed particles. The supernatant was used for the assay of glucuronidase [20], N acetyl glucosaminidase [20], acid phosphatase [21] and protein content [22].Enzyme activities are expressed as milliunit per gm weight of the bladder.

Statistical Analysis

The results are expressed as mean ± S.D. Comparison between groups was done using Mann-Whitney U test. A P value of ≤ 0.05 was considered as statistically significant.

Results

Histology:

Figure 1 shows the histology of the urinary bladder of control rats and cyclophosphamide treated rat. In control animal, the urinary bladder had normal morphology (Fiigure 1a).

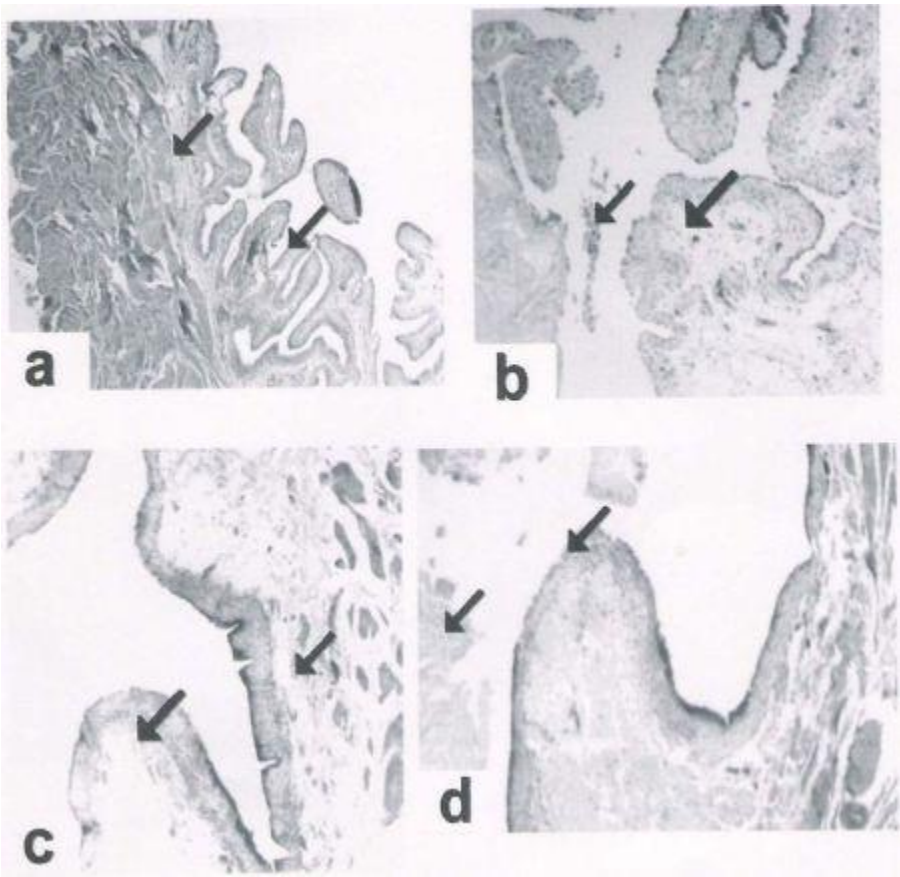

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Figure 1: shows histological appearance of the urinary bladder at various time intervals after treatment with cyclophosphamide.
a) Control animal. Normal appearance. Arrow points to tight packing of cells and dense connective tissue in lamina propria. Subepithelial crypts are also seen in this figure. (H & E, Magnification × 100).
b) Six hours after treatment with cyclophosphamide only minor damage was observed. Arrows point to edematous lamina propria and cellular and fluid exudates in the lumen. (H amp; E, Magnification × 100).

c) Sixteen hours after treatment with cyclophosphamide. Arrows point to hemorrhage in the epithelium and in the lamina propria. The lamina propria is edematous. (H amp;E, Magnification × 100).
d) Twenty four hours after treatment with cyclophosphamide. The specimen shows ulceration of epithelium (arrow). (H amp; E, Magnification X 100).

Contrary to the normal picture presented above, the bladder wall in treated animals showed damages, which became severe with increased time after treatment with the drug (Fig 1). In six-hour case the mucosa become edematous and the cells of the urothelium were not compact. There seemed to be cellular exudates in the lumen. Mucosal content formed follicular cystitis .However hemorrhage was not seen in this group (figure 1b). The condition became worse in sixteen hours, where edema of lamina propria with epithelial and subepithelial hemorrhage was seen (arrows in figure 1c). In animals twenty four hours after treatment with the drug, epithelium showed patches of ulceration (arrow in figure 1d). The lamina propria was edematous and there were exudates in the lumen (arrow in figure 1d).

Biochemical Parameters

The weights of the urinary bladder, protein content, and activities of the lysosomal enzymes in the CYP treated rat and control rat is shown in the figures 2-6. Since the protein content in the bladders of CYP treated rats were much higher than that of control rats, the activities of the enzymes are expressed here as mU or U per gm weight of the urinary bladder.

The weight of the urinary bladder of the drug treated rats was increased by 172% ($P \leq 0.004$) at 16 hours and by 185% ($P \leq 0.004$) at 24 hours as compared to that of the control rat (Fig.2).

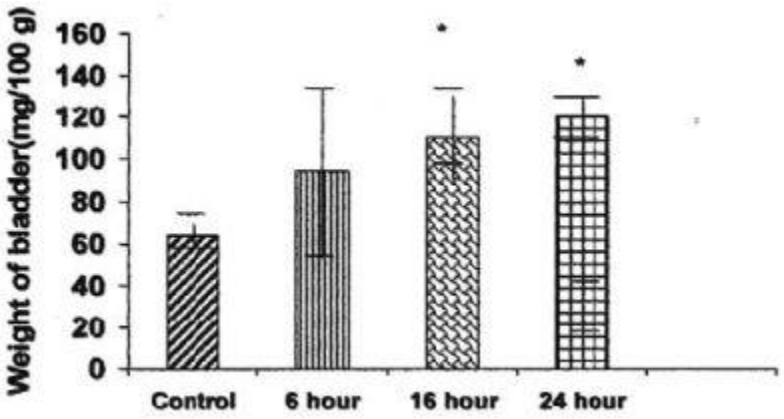

Fig. 2: Weight of urinary bladder in the control rats and at different time intervals in the cyclophosphamide treated rats. N= 7in each group. * <0.004 compared with control

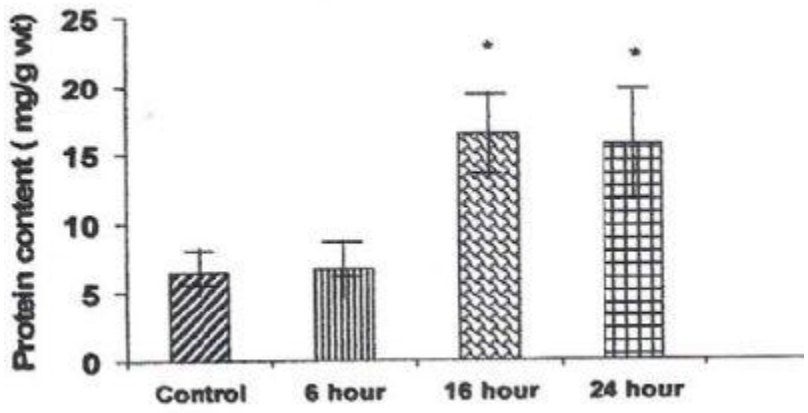

Fig. 3: Protein content in the urinary bladder control rats and at different time intervals in in the cyclophosphamide treated rats. N= 7in each group. * <0.004 compared with control

A highly significant increase in protein content was observed in the bladders of CYP treated rats at 16 hours and 24 hours after treatment. Protein content was increased by 252% at 16 hour and by 240% at 24 hour (Fig.3).

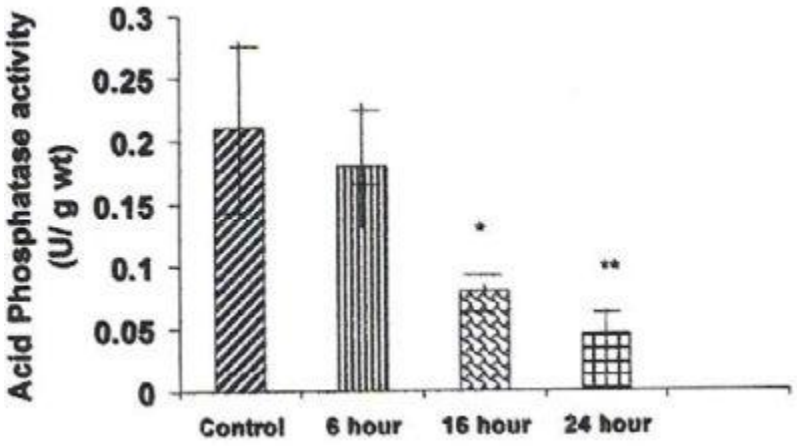

Fig. 4: Acid phosphatase activity in the normal control rats and at different time intervals in the cyclophosphamide treated rats. N= 7 in each group. * P<0.05, ** P<0.004 as compared with control.

A significant decrease in the activities of lysosomal enzymes was observed in the bladders of drug treated rats at 16 hrs and 24 hrs. Acid phosphatase activity was decreased by 263% at 16hr ($P \leq 0.05$) and by 437% ($P \leq 0.004$) at 24 hr after treatment with CYP (Fig. 4). With regard to glucuronidase activity, the activity was decreased by 253% ($P \leq 0.009$) at 16 hours and by 51% at 24 hours by as compared with the control (Fig.5).

N acetyl glucosaminidase activity was decreased by 403% ($P \leq 0.05$) at 16 hours and by 275%($P \leq 0.05$) at 24 hours (Fig. 6). An increase in the activities of N acetyl glucosaminidase and glucuronidase was observed in the urinary bladders of cyclophosphamide treated rats 6 hour after treatment, but the increase was not statistically significant.

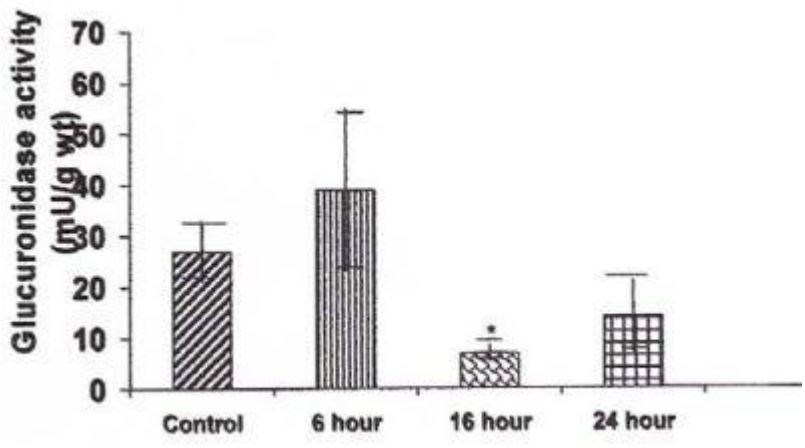

Fig. 5: Glucuronidase activity in the control rats at different time intervals in in the cyclophosphamide treated rats. N= 7in each group. * <0.004 compared with control

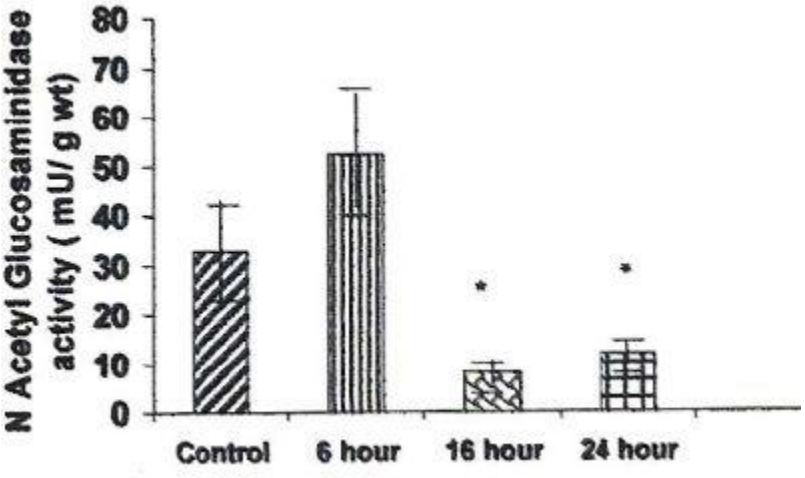

Fig. 6: N-acetyl glucosaminidase activity in the control rats and at different time intervals in the cyclophos-phamide treated rats. N= 7 in each group. * P<0.005 as compared with control

Discussion

Significant increase in weight of the urinary bladder and protein content was observed in the cyclophosphamide treated rats. In general, the levels of proteins within the cells are determined not only by rates of synthesis, but also by rates of degradation. Therefore,

increased protein content in the urinary bladder may be due to a decrease in cell protein degradation or an increase in protein synthesis. Normally proteins are degraded by two major path ways1. Lysosomal proteolysis and 2. ubiquitin-proteosome pathway. Lysosomes which are major machinery for recycling intra and extracellular material have long been regarded as "suicide bags" [23]. In the present study, protein accumulation in the urinary bladder is associated with significant decrease in the activities of all the lysosomal enzymes that were studied at 16 hours and 24 hours after treatment with cyclophosphamide, suggesting that lysosomal dysfunction may contribute to accumulation abnormal amounts of proteins and hence cyclophos-phamide induced damage to the urinary bladder.

Cyclophosphamide treatment results in hemorrhage from sub epithelial capillaries, and many erythrocytes which pass into the epithelium are phagocytosed by the epithelium [24]. In tissues where erythrophagocytois normally takes place, e.g. the spleen, liver , bone marrow and lymph nodes [25], the red blood cells are engulfed by the phagocytic reticuloendothelial cells and subsequently digested by them [26,27]. Two methods of digestion have been described in these tissues: the first one is by fusion of lysosomes with the phagocytic vacuole with the subsequent lysis of the erythrocytes [25] and the second one by fragmention of the ingested red blood cells followed by the subsequent breakdown of the fragments [26]. The lysosomal pathway of digestion of red blood cells plays a main role in the clearance of engulfed red blood cells. Therefore, the increased red blood cells observed in the urinary bladder of cyclophosphamide treated rats of the present study may be due to lack of the degrading enzymes of lysosomal origin.

The mechanism of cyclophosphamide induced urotoxicity appears to be different from that of cyclophosphamide induced hepatotoxicity. Cyclophosphamide induces progressive damage to hepatocyte membranes by the destabilization of lysosomal membrane and activation of lysosomal enzymes [28] while in the urinary bladder it appears to cause decrease in the activities of lysosomal enzymes, as has been revealed in the present study.

We suggest that the urotoxicity of cyclophosphamide may be attributed at least in part to the decrease in the activities of lysosomal enzymes thereby (1). Decreasing the degradation of proteins leading to accumulation of proteins in abnormal amounts (2). Decreasing digestion of the red blood cells in the urinary bladder transitional epithelium.

It is concluded that cyclophophamide induced damage to the urinary bladder may be due at least in part to the decreased activities of the lysosomal enzymes. In future we intend to study the mechanism by which cyclophosphamide causes decrease in the activities of lysosomal enzymes in the urinary bladder and, once the mechanism is elucidated it will pave way for the prevention of the damage. The results of the present study may be useful for researchers who are interested in preventing cystitis a common side effect of cyclophosphamide therapy in patients on cyclophosphamide therapy.

Acknowledgements

The funding by CMC fluid research committee is acknowledged.

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