Original Research Article

Acute effect of cyclophosphamide on rat’s urinary bladder and the possible protective role of sulforaphane: a histological and ultrastructural study

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ABSTRACT

Background: Cyclophosphamide disturbs the oxidant and antioxidant balance that is associated with several unwanted toxic effects and induction of secondary cancers. The aim of this study was to test the protective effects of Sulforaphane on the cyclophosphamide toxicity of rat urinary bladder.

Methods: 32 male albino rats were divided into 4 groups, 8 animal each (n=8). Group I received saline intraperitoneal, group II received 5 mg/kg sulforaphane for 5 days and then saline, group III received 0.9% saline intraperitoneal for consecutive 5 days and a single dose of cyclophosphamide 200 mg/kg on the six-day, group IV received sulforaphane at a dose of 5 mg/kg for consecutive 5 days and a single dose of cyclophosphamide 200 mg/kg on the six day. On the seventh day of the experiment, the animals were sacrificed, and the urinary bladder samples were dissected for histopathological and immunohistochemical investigations and electron microscopic studies.

Results: the mucosa of the urinary bladder of Sulforaphane treated group showed normal architecture while that of cyclophosphamides treated group showed features of degenerated and ulcerated lesions of the epithelial lining associated with hemorrhage. Theses lesions markedly decreased in the mucosa of urinary bladder of cyclophosphamides and sulforaphane treated group.

Conclusions: The use of sulforaphane reduces the cyclophosphamide toxicity on the urinary bladder in the form of decreased vacuolation with decreased degeneration of the epithelial lining.

Keywords: Cyclophosphamides, Sulforaphane, Antioxidants and cystitis

INTRODUCTION

Cyclophosphamide (CP) is a sort of nitrogen mustard drug which exerts its effects through the alkylation of DNA. It is capable of inhibiting protein synthesis through DNA and RNA crosslinking.¹ The cyclophosphamide is used mainly in the treatment of malignant lymphomas stages III and IV. Also used in the treatment of breast cancer, disseminated neuroblastomas, retinoblastoma. Multiple studies have found cyclophosphamide useful in the treatment of autoimmune diseases such as multiple sclerosis. Cyclophosphamide has also been prescribed pretransplant as an
immunosuppressant to prevent transplant rejection and graft-vs-host complications.\(^5\)6

One of adverse effects of cyclophosphamide is hemorrhagic cystitis. It developed at lower doses and shorter durations if taken intravenous rather than oral administration.\(^5\) Acrolein is the main molecule responsible of this side-effect and mesna (2-mercaptoethane sulfonate) is the commonly used preventive agent. Mesna binds acrolein and prevent its direct contact with uroepithelium.\(^5\)

Several transcription factors and cytokines, free radicals and non-radical reactive molecules, as well as poly (adenosine diphosphate-ribose) polymerase (PARP) activation have roles in its pathogenesis. In addition to many cytokines such as tumor necrosis factor (TNF) and the interleukin (IL) family and transcription factors such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) contribute to its pathogenesis.\(^6\)

So, pathogenesis of CP-induced bladder toxicity occurs in three steps; first entrance of acrolein rapidly into the uroepithelial cells; then it activates intracellular reactive oxygen species and nitric oxide production forming peroxo-nitrite; finally, high level of peroxo-nitrite damages lipids, proteins and DNA causing activation of PARP resulting in the depletion of oxidized nicotinamide–adenine dinucleotide and adenosine triphosphate, and consequently in necrotic cell death.\(^7\)

Herbal medicines widely used as a source of curative treatment, because chemical components of plants promote health and prevent diseases and plants are important sources of new drugs.\(^5\)9 Sulforaphane which is one of isothiocyanates. Naturally occurring isothiocyanates (IST) are enzymatically degraded products of glucosinolates (GST), found abundantly in cruciferous vegetables, i.e., cauliflower, cabbage, broccoli, and brussels sprouts. When plant cells are ruptured by food processing or damaged by microbial infection, the myrosinase enzyme hydrolyzes the inactive GST into active IST.\(^10\) Sulforaphane (SFN) is the most studied naturally occurring IST, a hydrolysis product of glucoraphanin, predominantly found in broccoli. However, cooked broccoli contains no myrosinase, but gut microbiota can convert GST to SFN, as they possess myrosinase-like activity.\(^11\)

Sulforaphane has antioxidant, anti-diabetic, antineoplastic and anti-inflammatory properties as shown by previous studies. Sulforaphane has function as suppressor of the MALP-2-induced inflammatory response, not only by inhibiting the expression of cytokines and induction of hemeoxygenase-1 (HO-1) but also by diminishing NF-kB activation in cultured monocytes and the lungs of mice.\(^12\)

Sulforaphane can correct the side effects of cyclophosphamide as it decreased the serum TNF-α level when given before the alkylating agent and regulated both the hypokalemia and hyponatremia induced by cyclophosphamide.\(^13\) In addition, it decreased cyclophosphamide-induced elevated myeloperoxidase activity. Sulforaphane also reduced urinary concentration of nitric oxide possibly through inhibition of inducible nitric oxide synthase. Prior administration of sulforaphane before cyclophosphamide increased the bladder content of adenosine triphosphate. This effect could possibly emerge because of increased adenosine triphosphate synthesis.\(^14\) We aimed in study to investigate effect of sulforaphane versus cyclophosphamide in regenerating urinary bladder mucosa.

**METHODS**

**Animals**

The present study was carried out using 32 male albino rats weighing 200-250 gm. Animals were housed in metal cages at room temperature and good ventilation. Rats were fed with standard pellets, containing all nutritive elements. They were provided free access to tap water and were acclimatized for 1 week prior to treatment. The animals were kept at temperature of 25±1°C and a relative humidity of 55% with a regular 12 hr light/12 hr dark cycle.

**Experimental design**

Rats were randomly divided into four groups, 8 animals each. Treatment regimens were as such: Group I (control group): received a single injection of 0.9 % NaCl (normal saline) (pH: 7.4) through intraperitoneal injection and olive oil daily in equal volumes. Group II (sulforaphane group): the rats were administered sulforaphane at a dose of 5 mg/kg for consecutive 5 days and then 0.9% saline injection through intraperitoneal injection.\(^15\) Group III (cyclophosphamide group): the rats received 0.9% saline injection through intraperitoneal injection for consecutive 5 days and a single dose of cyclophosphamide (200 mg/kg) on the sixth day.\(^16\) Group IV (Sulforaphane and cyclophosphamide): the rats received sulforaphane at a dose of 5 mg/kg for consecutive 5 days and a single dose of cyclophosphamide 200 mg/kg on the sixth day. On the seventh day of experiment, the animals were sacrificed under anesthesia and urinary bladder samples were dissected for histopathological, immunohistochemical investigations and electron microscopic studies.

**Drugs**

*Cyclophosphamide*: Cyclophosphamide (Cytoxan) or Endoxan® 500 mg vial, (Eczacıbaşı Baxter, Turkey) is a nitrogen mustard derivative was supplied as vials from Baxter oncology (Düsseldorf, Germany) (Sigma, St, Louis, Mo, USA).
**Sulforaphane**: Sulforaphane (Glucosinolate) was purchased as orange-yellow powder from Fluka chemical Co. (Steinheim, Germany) dissolved or diluted in 0.9% sterile saline.

Animals were sacrificed by deep anesthesia using isoflurane inhalation. Urinary bladder was dissected out for the different investigations which described below.

**Histopathological investigation and immunohistochemical study**

The removed bladder was fixed in 10% neutral buffered formaldehyde for 24 h before the histopathological study, followed by dehydration by passing through an increasing concentration of ethyl alcohol series (70%, 80%, 90%, and 96%, respectively), after they had been washed under tap water. Once they became light permeable in xylol, the tissues were blocked by being passed through liquid paraffin. Serial sections of 4-5 µm thickness were taken with the aid of microtome, their histopathological examination being performed under hematoxylin-eosin. Thus, a few digital images representing the groups were obtained for assessment of urinary bladder tissue morphology and observation of inflammatory cell invasion. Stained tissue sections were examined under light microscope at 20X magnification.16

For immunohistochemistry (IHC) staining, the sections were dewaxed (xylene, 4×3 min), rehydrated (100% ethanol, 4×1 min, 70% ethanol, 1 min, distilled water, 3×1 min), and then heat-induced antigen retrieval was used as previously reported.17 Then endogenous peroxidase was blocked with 3% hydrogen peroxide at room temperature (10 min), and sections were washed with distilled water (2×5 min) and PBS (5 min). The sections were then incubated with 200-300 µL of blocking solution (5% bovine serum albumin, BSA) in a room-temperature humidity cabinet (1 h). Immediately after removing the blocking solution, the sections were incubated with primary antibody for Caspase3 at 4°C overnight. The sections were washed with PBS (3×5 min) the next day and treated with secondary antibodies for 30 min. Immediately after washing the sections with PBS (3×5 min), they were stained with diamobenzidine and then washed with distilled water (5 min) and counterstained with hematoxylin.18

**Electron microscopic studies**

According to Kim et al transmission electron microscopy was used to examine the urinary bladder ultrastructure.19 The urinary bladder tissues biopsy specimens were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C for 1 hour, then subsequently post-fixed with 1% osmium tetroxide/0.1 M cacodylate buffer for 1 h at room temperature. After dehydration in graded ethanol, the tissues were washed 3 times in propylene oxide, and embedded in in Spurr’s resin. Ultrathin sections of 70-80 nm were cut on a Leica Ultracut R ultramicrotome, collected on formvar-coated single slot grids, and examined. The obtained ultra-thin sections (60-80 nm thick) were mounted on copper grids, stained with uranyl acetate and lead citrate, and inspected with JEM-2200 FS transmission electron microscope (JEOL-JEM-100 SX electron microscope, Japan, at electron microscope unit, Mansoura faculty of medicine). Urothelium cell layer numbers, the integrity of umbrella cells, and anchoring junctions were investigated.

**RESULTS**

**Light microscopic study**

Histopathological investigation of sections of control animals and those treated with sulforaphane showed normal architecture of mucosa of the urinary bladder. It was lined by a multilayered transitional epithelium (urothelium) and composed of tightly packed cells arranged in 4–5 layers. Thickened plasma membrane of the luminal surface of the superficial cells was noted. The basal cells rested on basement membrane which separated cells from lamina propria. Lamina propria was formed of dense connective tissue (Figure 1A and B).

The mucosa Urinary bladder of animal treated cyclophosphamides (CP) showed features of degenerated and ulcerated lesions of the epithelial lining associated with hemorrhage. Some superficial cells showed cytoplasmatic vacuolization cells. The lamina propria showed dilated congested blood capillaries surrounded by perivascular cellular infiltration (Figure 1C). While the mucosa of urinary bladder of animal treated with both cyclophosphamides and sulforaphane showed marked decrease of the degenerative and vascular lesions of the epithelial lining (Figure 1D).

**Immunohistochemical study**

In control group and sulforaphane injected group, active mild caspase-3-positive cells were detected within the epithelial lining (Figure 2A and B). While in Urinary bladder of animal treated with cyclophosphamides there was marked caspase 3 expression with positive cells appearing within the ulcerated epithelial lining (Figure 2C), animal treated with both cyclophosphamides and sulforaphane showing marked decrease of caspase 3 expression within the epithelial lining (Figure 2D).

**Electron microscopic study**

The bladder urothelium in group I (control) was compact and made up of a superficial cell layer facing the lumen of the bladder with large furrowing nucleus and numerous fusiform vacuoles. Also 1 or 2 layers of intermediate cells with intact organelles were seen (Figure 3A). While in sulphophane treated group, degenerated mucosal cells with degeneration of cellular organelles was also detected. The mitochondria were...
vacuolated with loss of tight junction. Also, basal cells were necrotic with pyknotic nucleus (Figure 3B).

**In cyclophosphamide treated group:** TEM of urinary bladder mucosa showed degenerated swollen intermediate cells with dilation and loss of many cytoplasmic organelles. There was necrotic mucosal urinary bladder cell with pyknotic, shrunken nucleus and damage. Vacuolation of most cytoplasmic organelles with loss of desmosomes or tight junction were also seen (Figure 3-C1-3).

While the mucosa of urinary bladder of animal treated with both cyclophosphamides and sulforaphane TEM showed sloughing of the mucosal cell membrane. Most mucosal cells appear intact with nearly normal nucleus and intact organelles (Figure 3D).

**Figure 1:** (A) A photomicrograph of a control rat urinary bladder, of normal multilayered transitional epithelium (arrowhead), thickened irregular plasma membrane of luminal surface of superficial cells is seen (arrow). The lamina propria (LP) is a dense connective tissue layer. (B) A photomicrograph of sulforaphane treated rat urinary bladder showing normal transitional epithelial lining, (arrowhead), and thickened irregular plasma membrane of the luminal surface of superficial cells is seen (arrow). The lamina propria (LP) is a dense connective tissue layer (C) A photomicrograph of cyclophosphamides-treated rat urinary bladder showing features of degenerated and ulcerated lesions of the epithelial lining (star) associated with hemorrhage (arrowhead). Some superficial cells show cytoplasmic vacuolization cells (thin arrows). Note the dilated congested blood capillaries (V) and perivascular cellular infiltration (dotted arrow) in the lamina propria (LP). (D) A photomicrograph of a cyclophosphamides and sulforaphane treated rat urinary bladder of marked decrease of degenerative and vascular lesions of the epithelial lining (arrowhead), H and E staining, X200.

**Figure 2:** (A) A photomicrograph of control rat urinary bladder showing mild caspase 3 expression within the epithelial lining. (B) A photomicrograph of sulforaphane treated rat urinary bladder showing mild caspase 3 expression within the epithelial lining. (C) A photomicrograph of cyclophosphamides-treated rat urinary bladder showing marked caspase 3 expression within the ulcerated epithelial lining (arrow). (D) A photomicrograph of a cyclophosphamides and sulforaphane treated rat urinary bladder showing marked decrease of caspase 3 expression within the epithelial lining. Cleaved caspase 3 antibody, X200.
**DISCUSSION**

Cyclophosphamide (CYP) is an alkylating agent that is widely used as an antineoplastic and immunosuppressive medications but causes oxidative systemic harm. One of them is hemorrhagic cystitis, causing dysuria, urinary frequency, urgency, and abdominal pain.\(^{2,20}\)

In our study, histopathological features of the urinary bladder mucosa of cyclophosphamides treated animal (CP) showed degenerated and ulcerated lesions of the epithelial lining associated with hemorrhage. Some superficial cells showed cytoplasmic vacuolization cells. These results agreed with Zirak, et al who found severe edema and hemorrhage in the bladder as well as hematuria.\(^{21}\) In addition, after an acute CYP administration at a dose of 200 mg/kg Wróbe et al observed an increased urothelium thickness and bladder edema (measured by bladder permeability).\(^{22}\)

In the present study, the lamina propria showed dilated congested blood capillaries surrounded by perivascular cellular infiltration. This in a line with Auge et al who detected edema in the lamina propria and focal urothelial the urinary bladder injury after administration of CYP.\(^{23}\)

Our findings are generally consistent with observations made by other authors, who showed that CYP given to rats only once caused an increase in urinary bladder weight, urinary bladder wall thickness, edema, hemorrhage.\(^{24}\)

Although the mechanism of cyclophosphamide-induced hemorrhagic cystitis is not understood, it has been shown that oxidative stress plays an important role in this pathology.\(^{25}\)

Several lines of evidence have shown that inflammation plays a central role in the pathophysiology of hemorrhagic cystitis triggered by cyclophosphamide. Cyclophosphamide increased the pro-inflammatory cytokines identified by Zirak et al.\(^{21}\) Such as TNF-α and oxidative stress marker like MDA in the bladder.

Its cytotoxic metabolites: phosphor-amide mustard and acrolein may be attributed to the mechanism through which CYP induces tissue harm. Phosphor-amide mustard has antineoplastic effect whereas acrolein, is a highly reactive metabolite that induce the urotoxic and immunotoxic effects of CYP to cause oxidative stress.\(^{26}\)
First the direct adverse effect of acrolein to tubular epithelium and urinary bladder epithelium and secondly the increased development of free oxygen radicals by intracellular phosphor-amide mustard are the cellular mechanisms for CYP toxicity from Abraham et al.25

In the present study the mucosa of urinary bladder of animal treated with both cyclophosphamides and sulforaphane showed marked decrease of the degenerative and vascular lesions of the epithelial lining. This improvement may be due to antioxidant effect of sulforaphane as mentioned by Ruhee et al who reported the antioxidant defense system of sulforaphane through the Nrf2/HO-1 signaling pathway, on acute exhaustive exercise-induced inflammation.23

Khaleel et al suggested that Nrf2 activation plays a critical role in mediating the protective effect of sulforaphane.28

Sulforaphane (SFN) is an isothiocyanate abundant in cruciferous vegetables and, for a variety of diseases based on previous in vivo and in vitro studies, has been shown to be antioxidant via Nrf2 and can indirectly act as an antioxidant.29,30 Furthermore, in Helicobacter pylori-infected mice and in cultured cells Yanaka observed the protective effects of SFN against oxidative stress in GI cells.31

In addition, Wakabayashi et al and Fahey et al have thoroughly researched the mechanisms for the detoxification effects of SFN by induction of cytoprotective, antioxidant, and anti-inflammatory enzymes via the transcription factor Nrf2, which activates the genes that control these endogenous protective responses.32,33

Imbalance of Apoptosis is associated with the pathogenesis of many diseases.34 Shie et al found increase bladder epithelial apoptosis in case of BPS/IC have urinary bladder.35 Caspase-3 antibodies serve as excellent biomarkers to monitor induction of apoptosis.36

In the present study there was marked expression of caspase 3 with positive cells appearing within the ulcerated epithelial lining in urinary bladder of animal treated with cyclophosphamides. In agreement, with Luo et al who found that expression of two pro-apoptotic markers was significantly higher in the bladder of the IC group.38 In addition, Ayhanci et al detected high caspase-3 levels in transient epithelium of the bladder treated with cyclophosphamide.16 This cellular necrosis may be due to protein denaturation, oxidative DNA damage through changes of mitochondrial membrane potential, release of cytochrome C, and activation of the caspase cascade.37

TEM of urinary bladder mucosa in cyclophosphamide treated group showed degenerated swollen intermediate cells with dilation and loss of many cytoplasmic organelles. There was necrotic mucosal urinary bladder cell with pyknotic, shrunken nucleus and damage. Vacuolation of most cytoplasmic organelles with loss of desmosomes or tight junction were also seen.

These results were coincided with Jezernik et al who found that apoptotic cells were appearing in the basal, intermediate and superficial layer of the urothelium from 12 h until 24 h after CP administration.38

The result of this study was also in agreement with Taha et al who observed necrosis, widening of the intercellular gaps with poorly formed junctional complexes, cytoplasmic vacuolization of superficial cells was also noticed in the superficial epithelial cells in CP treated group.39

In The present study, animal treated with both cyclophosphamides and sulforaphane showing marked decrease of caspase 3 expression within the epithelial lining and TEM showed sloughing of the mucosal cell membrane. Most mucosal cells appear intact with nearly normal nucleus and intact organelles. This indicates the protective properties of SFN and it has anti apoptotic effect as mentioned by Tortorella et al.40

Guerrero-Beltrán et al found that SFN decrease the proapoptotic pathways and inflammation and by promoting activation of prosurvival mechanisms.41

On the other hand, a study in mesenchymal stem cells found that SFN, at low concentrations, protected cells against induced oxidative stress. While high SFN concentrations caused cytotoxicity and DNA damage.42

CONCLUSION

The present study revealed that CP induced destruction of rat urothelium accompanied not only by necrosis but also by apoptosis. These pathological changes can be prevented by SFN who has antioxidant, anti-inflammatory and antiapoptotic effect which prevent complication of the chemotherapy.

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