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Could Ginger Protect Urothelium From Cyclophosphamide Toxicity? Histopathological and Ultrastructural Study

Yasser M. Elbastwisy[1&2]

[1] Anatomy and Embryology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt.
[2.]Department of Anatomy and Embryology, Faculty of Medicine, Taibah University, Madinah, KSA.
yasserbast@yahoo.com

Abstract: Background: Cyclophosphamide (CP) is a chemotherapeutic agent used in managing various neoplastic and non-neoplastic diseases. However, its clinical application is often restricted due to its baleful side effects particularly interstitial cystitis (IC). Several studies recommended the administration of a sulfhydryl compound MESNA or forced saline diuresis to reduce the CP-induced bladder toxicities, but it was not sufficient in all patients. Ginger is a flavoring spice that was proofed to have antioxidant, and anti-inflammatory properties. These properties favor its use as a possible agent to prevent CP-induced bladder toxicity. **Aim of work:** The goal of the current investigation was to clarify the CP-induced histopathological and ultrastructural effects on the urothelium of the adult male albino rats and to evaluate the possible protecting role of Ginger on such alterations. **Materials and Methods:** Forty-five adult male albino rats (200–250 g) were utilized in the existing study. They were randomly allocated into three equal groups. Group I rats served as control and were given sterile normal saline. The rats of group II were given a single intraperitoneal dose of CP dissolved in sterile normal saline. Group III animals received CP as in group II but in combination with Ginger (by gastric gavage) 7 days before and 7 days after CP injection. Urinary bladders were excised and prepared for histological and transmission electron microscope examination. **Results:** Administration of CP induced decreased thickness and desquamation of urothelial cells with hemorrhage. Inflammatory cell infiltrate was also noticed in the lamina propria. Ultrastructural examination revealed shrinkage of the nucleolar membrane, decreased nuclear volume and disturbed chromatin together with intracellular vacuolation and less prominent cytoplasmic organelles. There was also blebbing of the cell membrane with wide spaces between the cells. Administration of Ginger to the rats, the overall scene of the urothelium structure documented that there was an evident improvement so that it, more or less, resembled that of the control animals. **Conclusion:** Based on the previous data, it may be concluded that Ginger has a protecting effect on the CP-induced IC.
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1. Introduction

Interstitial cystitis (IC) is a major pathological and clinical condition characterized by chronic pelvic pain, frequency, urgency, and nocturia [1]. The etiology and pathophysiology of IC are poorly understood, and several factors have been suggested, including inflammation, autoimmune mechanisms, mast cell activation, neurogenic causes, and genetic predisposition [2]. Therapeutic strategies against IC are still limited and unsatisfactory [3].

Animal models of IC used several chemicals to induce IC with typical histopathological changes. Well-established models used potassium chloride (KCl [4], cyclophosphamide (CP) [5], and lipopolysaccharide (LPS) [6].

Cyclophosphamide is a chemotherapeutic agent that has been widely used in managing various neoplastic and non-neoplastic diseases. The commonest neoplastic diseases treated by CP are lymphoma, breast cancer, and leukemia [7]. The known non-neoplastic and autoimmune diseases treated with this drug are antineutrophil cytoplasmic antibody-associated vasculitis [8] and systemic inflammatory diseases [9-12]. Although the mechanism by which CP treats autoimmune diseases is not well known, many researchers suggested possible mechanisms as the decreased production of cytokines and adhesion molecules, B cell suppression, and subsequent decreased IgG production and induction of apoptosis [13, 14]. However, the clinical application of CP is often restricted due to its baleful side effects [15]; particularly hepatotoxicity[16], IC, and bladder cancer [17]. Interstitial cystitis occurs in 10 - 40% of patients receiving high-dose CP [15]. CP-induced IC has a wide range of severity (ranges from mild to severe) and may require a variety of treatment procedures such as hydration, clot extraction via cystoscopy, continuous bladder irrigation, or cystectomy [18]. Unfortunately, these treatment options may fail, and the condition

becomes fatal [19]. Therefore, IC prevention is a great challenge. Most CP side effects are dose-dependent. Although the doses prescribed in non-neoplastic diseases are much lower than those prescribed for cancer chemotherapy, it is often used for a longer duration due to a high rate of relapses with the subsequent increased adverse effects [20].

Several methods were tried to reduce the CP-induced bladder toxicities; concomitant administration of a sulfhydryl compound MESNA [21], reduction of the total CP dose given to a patient [22] while other studies investigated a strategy encouraging large volumes of fluids drinking by the patients [23]. Unluckily, these procedures are not always effective and cannot be applied to all patients [24]. Hence, there is a need for novel agents that could be useful in preventing adverse urologic effects during CP therapy.

Ginger (Zingiber officinale) is a known flavoring spice and medicinal herb in Southeast Asia [25]. It was proofed to be effective against nausea of different causes including that induced by chemotherapy [26]. The active constituents of Ginger were proofed to have antioxidant, anti-inflammatory [27], and antimutagenic properties [28]. It was also found to be able to lower lipid peroxidation, attenuate free radical-mediated toxic effects, and provoke the activities of antioxidants [29-32]. These properties favor the use of Ginger as a possible agent to prevent CP-induced bladder toxicity.

In this investigation, a rat model of CP-induced IC will be established to describe the histological and ultrastructural changes in urothelium and to assess the possible protective effect of Ginger.

2. Materials and methods

Chemicals

Cyclophosphamide was purchased from Baxter Oncology GmbH, Frankfurt, Germany. The Zingiber Officinale powder was obtained from the Pharmacognosy department in the Faculty of Pharmacy, Taibah University, Almadinah Almonawarah, KSA.

Animals

Forty-five adult male albino rats (200–250 g) were obtained from the Animal House of Taibah University (KSA). The animals were acclimated to the environment for 7 days by housing in well-ventilated hygienic cages under the standard conditions (12:12 light/dark cycle, room temperature of 24 - 28 C° and the humidity at 50%–60%.). They were fed a standard balanced diet and allowed water ad libitum. All animal procedures were conducted according to the principles of laboratory animal care (National Institutes of Health Guide for the Care and Use of Laboratory Animals [NIH Publication 85–23 Rev. 1985]).

Experimental design

Rats were assigned randomly into three equal groups (15 rats in each group). Group, I rats were controls injected intraperitoneally with a single dose (0.5 CC) of normal saline. Rats in group II were treated with a single intraperitoneal dose of CP (CP is dissolved in saline and given by a dose of 150 mg/kg) to induce cystitis [33]. While rats in group III received the same dose of CP (150 mg/kg intraperitoneally) together with Ginger powder dissolved in water at a measurement level of 24 mg/ml given orally by intragastric gavage (1 ml/day) starting 7 days before CP and continuing throughout the experiment [34].

Tissue sampling and preparation

Seven days after CP injection, all animals were subjected to light intraperitoneal pentobarbital anesthesia (50 mg/kg) and sacrificed. Urinary bladders were carefully dissected, removed, and fixed in a relaxed state after emptying by aspiration of urine with a 27-gauge needle [35]. The fixed bladders were divided into 2 samples; the first one is fixed in 10% neutral buffered formalin for 24 hours and processed for the usual tissue handling for paraffin blocks preparation. Sections of 3-4 µm thickness were stained with Hematoxylin and Eosin (H&E). The other tissue sample was cut into small pieces, fixed in glutaraldehyde and osmium tetroxide and processed for transmission electron microscopic examination. JEOL JEM 1200 EXII Electron Microscope (Joel CX 100 transmission electron microscope operated at an accelerating voltage of 60 kV Ltd.) was used to examine the specimens at Mansoura University, EM Unit (Egypt).

3. Results

Histological examination of hematoxylin and eosin-stained sections of the control group revealed a highly folded mucosa of the urinary bladder consisting of the transitional epithelium (urothelium) with underlying lamina propria. The urothelium is formed of multiple layers. The superficial cells are large, puffy, and dome-shaped (umbrella-like) with abundant eosinophilic cytoplasm and some binucleated cells. The intermediate cells are smaller with ample cytoplasm and oval nuclei. The basal cells are cuboidal and resting on a thin basement membrane, which separates the cells from the lamina propria. The lamina propria was formed of connective tissue and rich in blood capillaries (Fig. 1A).

A single dose of CP (group II) caused a decrease in thickness and desquamation of urothelial cells together with variable grades of hemorrhage. There is also disruption of the basement membrane and inflammatory cell infiltrate in the lamina propria (Fig. 1B).

In group III urothelial cells appeared more or less similar to the control group but, the lamina

propria still showing inflammatory infiltrate (Fig. 1C).

Ultrastructurally, the cytoplasm of the apical cells of the control group (group I) showed prominent cytoplasmic discoid vesicles. Well-formed junctional complexes (Zonula occludence, zonula adherence, and desmosome) are observed between and along the sides of the umbrella cells (Fig. 2A). The superficial layer of the transitional epithelium showed characteristic ridging. The cytoplasm contained an indented euchromatic nucleus with prominent nucleoli. Mitochondria and few lysosomes were also noticed (Fig. 2A).

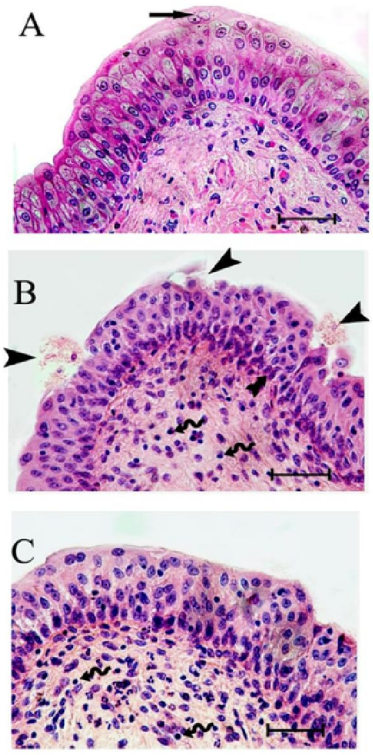

Fig. (1): A photomicrograph of the urothelium. (A) Group I: shows intact urothelium and basement membrane. The lamina propria contains blood capillaries and scattered connective tissue cells. The mucosa is folded and the urothelium has a superficial layer of large bulbous or elliptical umbrella cells, sometimes binucleated (arrow). (B) Group II: shows decreased thickness, loss of integrity, ulceration and hemorrhage (arrowhead) of the urothelium with disruption of the basement membrane (broad arrow). The lamina propria shows infiltration by inflammatory cells (curved arrow). (C) Group III: The epithelium restored its integrity but the lamina propria still contain many inflammatory cells.

CP-treated rats showed apoptotic changes in the form of shrinkage of the nucleolar membrane with a decrease in the nuclear volume and disturbed chromatin together with intracellular vacuolation and less prominent cytoplasmic organelles. There was also blebbing of the cell membrane with wide spaces between the cells. The basement membrane appeared disturbed (Fig. 2B).

Group III appeared more or less close to the control group apart from few apoptotic cells (Fig. 2C).

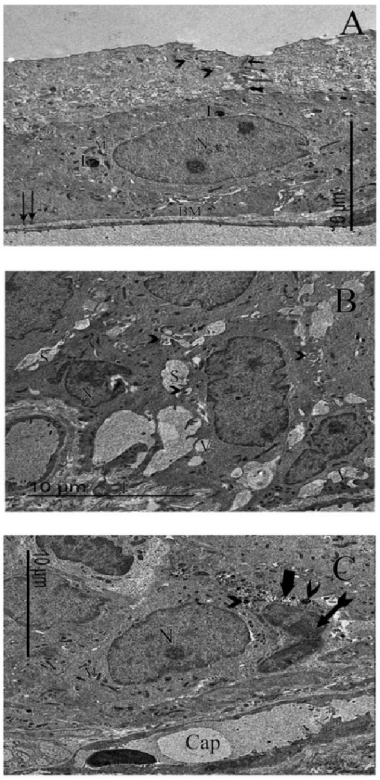

Fig. (2): An electron micrograph of the urothelium. (A) Group I: shows intact umbrella cell membrane with many discoid vesicles (arrowhead). Well-formed junctional complex between the umbrella cells (Zonula occludence [tailed arrow], zonula adherence [small thin arrow], and desmosome [tailed thin arrow]). Intact basement membrane (BM) with many hemidesmosomes (thin long arrow). Intended euchromatic nucleus (N) with prominent nucleoli. Prominent cytoplasmic organelles especially lysosomes (L) and mitochondria (M). (B) Group II: shows shrinkage of the nuclear membrane with a decrease in the nuclear volume (N) with disturbance of their chromatin. Blebbing of the cell membrane (arrowhead) with wide spaces between the cells (S). Intracellular vacuolations (V) with less prominent cytoplasmic organelles. (C) Group III: shows normal urothelial cells (N) and a small shrunken cell (thick arrow) with dark irregular nucleolus and numerous lysosomes (arrow heads).capillaries (Cap) appear in the lamina propria.

4. Discussion

CP is a widely used drug, but its side effects are still a major problem particularly with high doses. Injection of a single intraperitoneal dose of CP in rats could produce most features of IC in humans such as increasing voiding frequency, decreasing urine volume per void, and increasing permeability of the bladder wall [36]. CP-induced hemorrhagic cystitis is used as an experimental model of human diseases like urinary bladder inflammation, inflammatory infiltration, and disruption of urothelium [37].

In the present study, intraperitoneal injection of a single dose of CP caused interstitial cystitis which was demonstrated by decreased thickness and degeneration of urothelial cells together with disruption of the basement membrane. Some urothelial cells showed large vacuoles within the cytoplasm, while others showed areas of exfoliation together with variable grades of hemorrhage. Inflammatory cell infiltrate was also noticed in the lamina propria. Several studies have demonstrated histological changes like our results. Prashant et al. [36] reported urothelial ulceration, hemorrhage, and inflammatory cell infiltration. They alleged that; these findings are due to the CP pro-oxidant nature that induces oxidative stress through decreasing the activities of the antioxidant enzymes. Choi et al. [37] claimed that these findings represent the adverse effects of the toxic metabolites of CP which exert cytotoxic effects on the rapidly dividing cells. However, Sridhar et al. [38] attributed these changes to early nonapoptotic death of superficial cells, followed by apoptotic death of deeper layers. Anna et al. [39] reported similar changes in the rat genitourinary tract. They claimed that urinary bladder injury is multifactorial and involves first of all the reaction of CP metabolite (acrolein) with the urothelium with subsequent inflammatory cascade.

TEM examination of CP-treated rats demonstrated apoptotic changes in the urothelium in the form of shrinkage of the nuclei and disturbed chromatin together with intracellular vacuolation and decreased cytoplasmic organelles. Also, there were wide spaces between the cells and blebbing of the cell membrane. The basement membrane appeared disturbed. Similar findings were described by Lavelle et al, [40] who reported detachment of umbrella cells from each other, with disruption of tight junctions in rats with Feline interstitial cystitis. In addition, Jia-Fong et al, [41] ascribed umbrella cell pleomorphism and loss of tight junctions in IC patients. Jock et al, [42] described prominent widening in intercellular spaces between urothelial cells after partial bladder outlet obstruction in the rats.

These results highlight that, IC is a potentially life-threatening complication of CP therapy that necessitated searching for methods to protect against this toxicity. Previous studies recommended the administration of MESNA or forced saline diuresis, but it was not sufficient in all patients (Moy, B. et al. 2018). Therefore, there is a great need to search for new substances to prevent CP-induced toxicity.

Natural compounds, such as Ginger, or its chemically modified derivatives are in the interest of many researchers [32&43] due to their potential anti-inflammatory and antioxidant activities.

To our knowledge, no previous studies described the protective effect of Ginger on CP-induced urothelial toxicity. In the present work, Ginger administration to rats 7 days before and after CP therapy revealed evident light and electron microscopic urothelial improvement so that it resembled that of the control animals. In parallel with this, Kandemir et al. [44] reported significantly decreased apoptosis, inflammation, and histopathological alterations in CP-induced nephrotoxicity in female rats after Ginger administration. In addition, Elshiekh et al. [43] emphasized on the alleviating role of Ginger against cisplatin-induced reproductive toxicity in rats. They alleged that these protective effects are due to decreased oxidative stress. Moreover, Dina et al, [32] proposed that Ginger exerted a potential anti-proliferative activity against Diethyl nitrosamine in liver, stomach, and colon via nuclear factor-erythroid 2-related factor2 activation, whilst suppression of oxidation and inflammation. Sridhar et al, [45] postulated that Keratinocyte growth factor pretreatment blocks CP-induced urothelial apoptosis in mice, likely via protein kinase B signaling, and drives phosphorylated extracellular signal-regulated kinase-mediated keratin 5/keratin 14 cell proliferation, leading to early urothelial regeneration.

In conclusion, in light of the demonstrated protective imprints of Ginger against CP-induced urothelial injury, in the current investigation, it is advisable to widen the scale of its intake for the patients receiving CP therapy to minimize, as possible, its undesirable side effects. Eventually, further studies with different techniques are recommended to understand the underlying mechanisms of its protective effect.

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Conflict of interest

The author declares no conflict of interest.

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