Bull. Pharm. Sci., Assiut University, Vol. 46, Issue 2, 2023, pp. 1453-1474.

Bulletin of Pharmaceutical Sciences
Assiut University
Website: http://bpsa.journals.ekb.eg/
e-mail: bullpharm@aun.edu.eg

POTENTIAL PROTECTIVE EFFECTS OF MESNA AND VITAMIN D SEPARATELY OR CONCOMITANTLY ON CYCLOPHOSPHAMIDE INDUCED HEMORRHAGIC CYSTITIS IN RATS

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Background: *Hemorrhagic cystitis (HC) is the most common adverse effect of cyclophosphamide (CP). We assessed the protective role of Mesna and vitamin D against CP induced HC.* ***Methods:*** *This experiment was performed on 64 rats; CON group (control), CP group (sham cyclophosphamide), M group (Mesna), CL group (low dose calcitriol), CH group (high dose calcitriol), VD group (cholecalciferol), CM group (calcitriol & Mesna), and VDM group (cholecalciferol & Mesna).* ***Results:*** *Mesna and vitamin D revealed a significant decrease in visceral pain score, bladder index, malondialdehyde (MDA) and tumor necrosis factor alpha (TNF-α) levels, caspase expression, and a significant increase in total antioxidant capacity (TAC) versus the CP group. Also, the VD, CH, CM & VDM groups revealed a significant decrease in TNF-α level and caspase, microtubule-associated protein light chain 3-II (LC3-II), and sequestosome-1 (SQSTM1/p62) expression versus the M group. The CH group showed a significant increase in serum calcium versus other groups.* ***Conclusion:*** *Vitamin D increased the uroprotective effect of Mesna, which is attributed to its ability to restore autophagy flux.*

Keywords: *Hemorrhagic cystitis, cyclophosphamide, Mesna, calcitriol, and cholecalciferol*

INTRODUCTION

Hemorrhagic cystitis (HC) is defined as an inflammation of the urinary bladder with urothelial damage and a complex of urinary dysfunctional manifestations. Urinary retention and sever hematuria are serious complications of cystitis. HC may be combined with nephropathy that needs endovesical intervention[1,2] and It is one of the main reasons for fatalities as it induces bladder constriction, hydronephrosis down to renal failure [3].

HC etiology ranges mainly between the two most common causes, chemotherapy, and radiation. Oxaphosphorines (cyclophosphamide, ifosfamide) are the chemical agents that are mainly responsible for HC which is considered an iatrogenic complication[4,5] However, it may be secondary to viral infection[6] or idiopathic[7]. HC was represented in 20-25% of patients treated by cyclophosphamide and in 75% of patients treated by it after bone marrow transplantation[8,9]. Cyclophosphamide (CP) is an alkylating agent that induces cross-linkages between two adjacent DNA strands, leading to apoptosis. Acrolein is a CP metabolite that is responsible for its cytotoxic adverse effects without any antitumor activity[10]. It is excreted in the urine and concentrated in the bladder. It induces an inflammatory reaction and apoptosis of the urothelium, leading to ulceration. Its cytotoxicity is mainly through reactive oxygen species generation leading to an increase in the expression of transcription factors such as nuclear factor kappa B (NF-kB) and inflammatory cytokine as tumor necrosis factor alpha (TNF-α)[11,12].

CP can induce HC via induction of autophagy, as proved by the detection of microtubule-associated protein light chain 3 (LC3-II) in urinary bladder tissue, which is a key regulatory protein in the autophagy initiation step and yields autophagosome

Received in 16/7/2023 & Accepted in 1/9/2023

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membrane[8]. CP also decreased the expression of autophagy receptor sequestosome 1 protein (SQSTM1/p62), which is an indicator of complete autophagy flux and degradation of the autophagosome and its contents[13].

Mesna is 2-mercapto-ethane-sodium sulphonate, which is a sulfhydryl donor and binds to acrolein in the urinary bladder, decreasing its cytotoxic effects. It also has antioxidant, anti-inflammatory, and anti-apoptotic effects[14]. It reduces the incidence of HC by up to 50%. However, HC is still a critical, life-threatening adverse effect of CP that needs hospitalization and may lead to death. It is documented that 66.7% of Mesna-treated cases were diagnosed with gross bladder damage and 100% of cases suffered from urothelial injuries[4]. Therefore, it is urgent to discover a new prophylactic approach to increase Mesna's effectiveness.

Vitamin D is fat soluble secosteroid. It is considered a steroid hormone that is responsible for bone mineralization [15]. Vitamin D and its derivatives have pleiotropic effects that maintain cellular viability. It has a reliable antioxidant[16] and anti-apoptotic effects [17]. Moreover, vitamin D has an immune and anti-inflammatory effect through T cell function regulation and reduction of inflammatory cytokines[18]. In addition, it induces autophagic activity[19] that aborts apoptosis and oxidative stress and can attenuate inflammation and activate host immunity[20]. Notably, how vitamin D affects the recuperation of functional tight junction and increases the expression of its proteins e.g., occludin, zonula occludens-1 (ZO-1), and clandin-1. The proper tight junction acts as a barrier and host defense against any inflammation[21,22].

The best that we can tell is there is no research on the protective effect of Mesna and vitamin D concomitantly on CP induced HC. Consequently, our study was created to explore the potential protective effect of Mesna and vitamin D each alone or in combination on CP induced hemorrhagic cystitis.

MATERIAL AND METHODS

Animals

Strain: Female Wister rats weighing 160-200 gm obtained from Tanta University Animal House.

Housing: One week before the experiment, the rats were housed in groups (total: n = 64 for this experiment) in polyethylene cages with unlimited access to food and water ad libitum. The Faculty of Medicine, Tanta University, Egypt's institutional "Research Ethics Committee, REC" adopted all experimental techniques including the treatment of animals (Approval no. #34196/10/20).

Drugs

Cyclophosphamide (Endoxan vial 1000 mg) is a product of Baxter healthcare company, Hong Kong. Mesna (Uromitexan ampoule 400 mg / 4 ml) is a product of Baxter healthcare company, Hong Kong. Cholecalciferol (Vidrop oral drops 2800IU/ml) is a product of Medical Union Pharmaceutical (MUP). Calcitriol (Calcidoveros oral drops 1mcg/ml) a product of Averroes Pharma (AVS).

Experiment design

Eight groups, each with seven rats, were created by randomly assigning habituated animals to the groups **(Fig. 1)**:

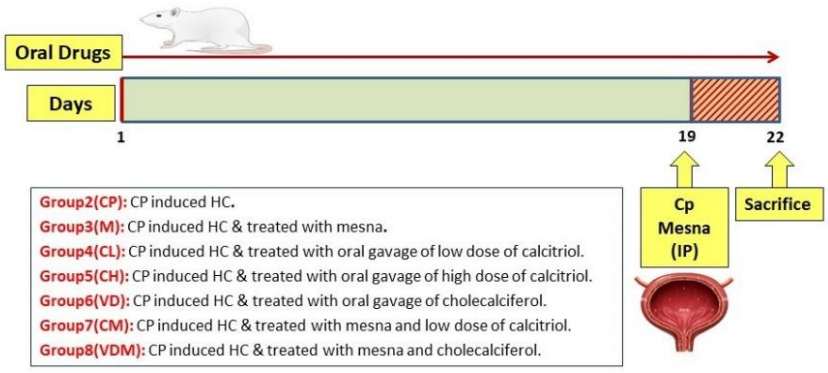

Fig. 1: Study design and grouping.

- **Group (CON):** received 0.5 ml saline (i.p) once, in addition to distilled water daily by oral gavage for 21 days.
- **Group (CP):** received cyclophosphamide in a dose of 200 mg/kg via single i.p. injection [23]on the 19th day; these served as the untreated HC group.
- **Group (M):** rats received Mesna were administered in three equal i.p. doses of 120 mg/kg each, 20 minutes before and 4, 8 hours after the cyclophosphamide injection[24]. Cyclophosphamide was injected i.p. in a dose of 200 mg/kg on the 19th day.
- **Group (CL):** rats received a low dose of calcitriol by oral gavage in dose 1 mcg/Kg/d [25] 5 times per week for 3 weeks. Cyclophosphamide was injected i.p. in a dose of 200 mg/kg on the 19th day.
- **Group (CH):** rats received a high dose of calcitriol by oral gavage in dose 3 mcg/Kg/d [25] 5 times per week for 3 weeks. Cyclophosphamide was injected i.p. in a dose of 200 mg/kg on the 19th day.
- **Group (VD):** rats received cholecalciferol by oral gavage in dose 1000 IU/Kg/d[26] for 21 days. Cyclophosphamide was injected i.p. in a dose of 200 mg/kg on the 19th day.
- **Group (CM):** rats received a low dose of calcitriol by oral gavage in dose 1 mcg/Kg/d 5 times per week for 3 weeks plus Mesna was administered in three equal i.p. doses of 120 mg/kg each, 20 minutes before and 4, 8 hours after the cyclophosphamide injection. Cyclophosphamide was injected i.p. in a dose of 200 mg/kg on the 19th day.
- **Group (VDM):** rats received an inactive form of vitamin D (cholecalciferol) by oral gavage in dose 1000IU/Kg/d for 21 days plus Mesna was administered in three equal i.p. doses of 120 mg/kg each, 20 minutes before and 4, 8 hours after the cyclophosphamide injection. Cyclophosphamide was injected i.p. in a dose of 200 mg/kg on the 19th day.

At the end of the experiment (22nd day), the weight of each rat was recorded and then anesthetized with i.p. pentobarbital sodium (50 mg/kg)[27] and Each animal's blood was drawn by an intracardiac puncture. Drawn blood was centrifuged for 20 minutes at 4000 rpm and serum was taken for assay calcium and vitamin D level.

Test for visceral pain assessment

Immediately after the cyclophosphamide injection, each rat was placed in an observation box individually. The rats were observed every 30 minutes for 4 hours after CP injection. Intraperitoneal injection of CP induced changes in respiratory rate, eye-opening and postural position of rats describing visceral pain that occurs with hemorrhagic cystitis. Then, these changes were scored. For the respiratory rate, every 10 cycles/min was scored as 1 and the control value is 140 cycles/min nearly. For the opening of the eye, 0 represents full opening, 10 represents full closure, 5 represents half closure, and 2 and 7 represent, respectively, the intermediate positions between full opening and half closure. When there was a rounded back or full limpness, the postural behavior received a score of 10. When no particular posture was seen, a score of 0 was recorded[28,29].

Tissue harvest

Urinary bladders were harvested, washed with phosphate-buffered saline, weighed, and normalized to body weight and expressed as urinary bladder index and calculated according to the formula: Tissue index = urinary bladder weight (g)/total body weight (g) × 100%. Then bladders were cut into 3 parts. 1st and 2nd parts were processed for histological and electron microscope assessment; respectively and 3rd part was homogenized in 150 mM KCl (pH 7.4) at a dilution of 1:10 (w/v) of the total homogenate. The homogenates were centrifuged for 30 min. at 18.000 rpm g and 4oC. To measure tumor necrosis factor-alpha, total antioxidant capacity, and malonaldehyde, the supernatant was taken.

Oxidant and antioxidant assays

Malonaldehyde (MDA) (nmol/ml) and total antioxidant capacity (TAC) (mM/L) (catalog number MD 2529, TA 25 13

respectively), Biodiagnostic company according to the method described by *Ohkawa et al.,* (1979)[30] and *koracevic et al*.,(2001)[31] respectively.

Tumor necrosis factor alpha (TNF-α) assay

TNF-α was quantitatively estimated in the supernatant of tissue homogenate using enzyme-linked immunosorbent assay kits according to the manufacturer`s protocol**.** (Catalog number: 201-11-0765), Biodiagnostic Company. The values were expressed as ng/L.

Histopathological examinations of the tissue

Tissues were preserved in 10% formalin for examination under a light microscope. The formalin-fixed tissue was cut, dried in ethyl alcohol in increasing concentrations, and then embedded in paraffin wax. A microtome was used to cut paraffin slices (3-5), which were then stained with hematoxylin and eosin and evaluated under a light microscope for histological alterations [32].

Immunohistochemistry examinations of the tissue

Immunohistochemical expression of caspase-3, Microtubule-associated proteins 1A/1B light chain 3B (LC3-II), and SQSTM1/p62 protein was done via rabbit polyclonal antibody purchased from Master Diagnostica, Sun Red Bio laboratories, and ABclonal technology; respectively. The immunohistochemistry staining was assessed by counting the positive cells [urothelial cells and inflammatory cells] per total 1000 cells in 10 HPF after subtraction of background using image analysis software. Nuclear with or without cytoplasmic staining is considered positive and categorized as negative (0-<10%) or positive and scored as the overall proportion of cells (Weak, 10-25%), (moderate 26%-50%) and (strong >50%).

Transmission Electron microscopic (TEM) examination of the tissue

The urinary bladder tissues were first fixed for three hours at 4°C in 2.5% buffered glutaraldehyde (pH 7.2), followed by a postfixation step in 1% osmium tetroxide (pH 7.3), dehydration in escalating concentrations of alcohol, passage through propylene oxide, and lastly embedding in epoxy resin. Samples were divided into 90 angstrom-thick pieces and laid out on grids before being stained for 5 minutes with uranyl acetate and 2 minutes with lead citrate[33]. At the Electron Microscopic Unit, Faculty of Science, Alexandria University, the grid was examined and photographed using a transmission electron microscope (JEOL-JEM-100, Tokyo, Japan).

Serum vitamin D and calcium assay

Serum level of 25 hydroxy vitamin D was measured by automated fluorescence immunoassay analyzers (Tosoh AIA 1800 ST, Tokyo, Japan)[34]. Serum calcium (mg/dl) (catalog number CA 12 10), Biodiagnostic company according to the method described by Gindler and king, (1972)[35].

Statistical Analysis

GraphPad Prism, version 8 for Windows, was used to tabulate and statistically analyze all of the collected data. The distribution's normality was tested using the Shapiro-Wilk formula. The one-way ANOVA and post hoc Tukey honestly significant difference test was used to depict the parametric data as mean ± standard deviation of the mean. The significance was considered at values of $P<0.05$**.** Visceral pain assessment was represented using two-way ANOVA.

RESULTS AND DISCUSSION

Results

Amelioration of visceral pain score by both Mesna and vitamin D therapy

CP group revealed a significant increase in visceral pain score versus the CON group. M, CL, CH, VD, CM, and VDM groups revealed a significant decrease in visceral pain score versus the CP group. CH, CM, and VDM groups revealed a significant decrease in visceral pain score versus the M group. CL and VD groups revealed a significant increase in visceral pain score versus the CH group. CL and VD groups revealed a significant increase in visceral pain score versus the CM group. CL, CH, VD, and CM groups revealed a significant increase in visceral pain score versus the VDM group **(Fig. 2).**

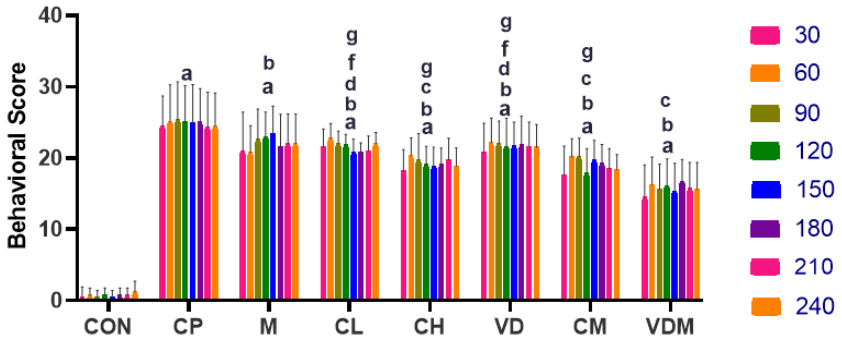

Fig. 2: The visceral pain score in different groups. Data were represented as mean ± standard deviation (n=7). ns—non-significant ($p > 0.05$), a—Significant ($p < 0.05$) as compared with CON group, b—significant ($p < 0.05$) as compared with CP group, c—significant ($p < 0.05$) as compared with M group, d—significant ($p < 0.05$) as compared with CH group, e—significant ($p < 0.05$) as compared with VD group, f —significant ($p < 0.05$) as compared with CM group, g—significant ($p < 0.05$) as compared with VDM group.

Amelioration of bladder index by both Mesna and vitamin D therapy

CP group revealed a significant increase in bladder index versus the CON group. M, CL, CH, VD, CM, and VDM groups revealed a significant decrease in bladder index versus the CP group. The VDM group revealed a significant decrease in bladder index versus the M group **(Fig. 3).**

Amelioration of oxidative stress by both Mesna and vitamin D therapy

CP group revealed a significant increase in MDA level versus the CON group. M, CL, CH, VD,, CM and VDM groups revealed a significant decrease in MDA level versus the CP group. CL and VD groups revealed a significant increase in MDA level versus the M group. CL and VD groups revealed a significant increase in MDA level versus the CH group. CL and VD groups revealed a significant increase in MDA level versus the CM group. CL, CH, and VD groups revealed a significant increase in MDA level versus the VDM group. CP group revealed a significant decrease in TAC versus the CON group. M, CL, CH, VD, CM, and VDM groups revealed a significant increase in TAC versus the CP group. CL and VD groups revealed a significant decrease in TAC versus the M group, but the VDM group revealed a significant increase in TAC versus the M group. CL, CH, and VD groups revealed a significant decrease in TAC versus the CM group and VDM group **(Fig. 4).**

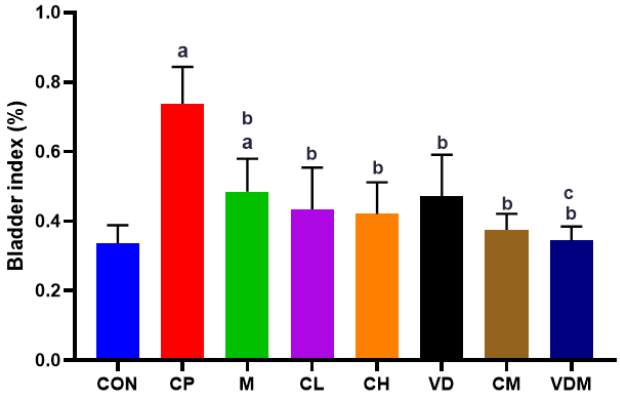

Fig. 3: The bladder index in different groups. Data were represented as mean ± standard deviation (n=7). ns—non-significant ($p > 0.05$), a—Significant ($p < 0.05$) as compared with CON group, b—significant ($p < 0.05$) as compared with CP group, c—significant ($p < 0.05$) as compared with M group, d—significant ($p < 0.05$) as compared with CH group, e—significant ($p < 0.05$) as compared with VD group, f —significant ($p < 0.05$) as compared with CM group, g—significant ($p < 0.05$) as compared with VDM group.

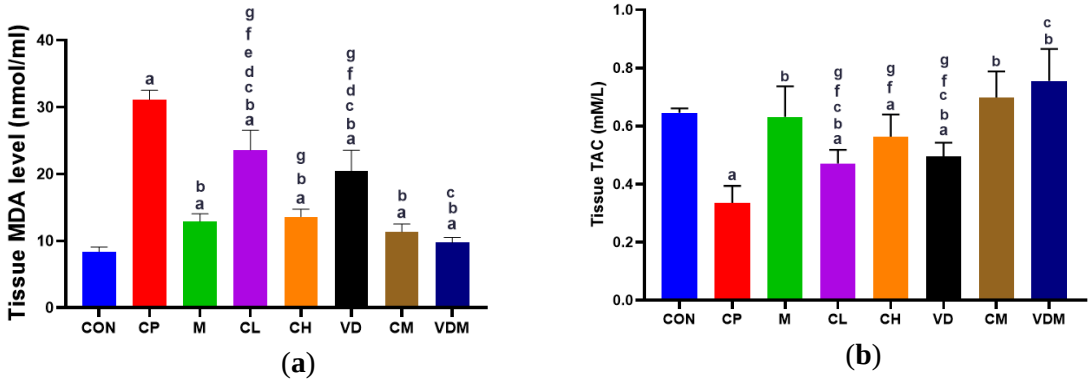

Fig. 4: The oxidant and antioxidant assay in different groups (a) MDA and (b) TAC. Data were represented as mean ± standard deviation (n=7). ns—non-significant ($p > 0.05$), a—Significant ($p < 0.05$) as compared with CON group, b—significant ($p < 0.05$) as compared with CP group, c—significant ($p < 0.05$) as compared with M group, d—significant ($p < 0.05$) as compared with CH group, e—significant ($p < 0.05$) as compared with VD group, f —significant ($p < 0.05$) as compared with CM group, g—significant ($p < 0.05$) as compared with VDM group.

Amelioration of inflammation by both Mesna and vitamin D therapy

CP group revealed a significant increase in TNF-α versus the CON group. M, CL, CH, VD, CM, and VDM groups revealed a significant decrease in TNF-α versus the CP group. CH, CM, and VDM groups revealed a significant decrease in TNF-α versus the M group. CL and VD groups revealed a significant increase in TNF-α versus the CH group. CL and VD groups revealed a significant increase in TNF-α versus the CM group. CL, CH, VD, and CM groups revealed a significant increase in TNF-α versus the VDM group **(Fig. 5)**.

Recovery of bladder tissue architecture

Hematoxylin and eosin-stained sections from the CON group revealed the urinary bladder's normal morphology and architecture. In the CP group, sections revealed ulcerated surface epithelium lined with ulcerated granulation tissue with inflammatory cells infiltration, vascular congestions and extravasated RBCs, and atrophic mucosa with underlying massive edema. This pathology was ameliorated by Mesna and vitamin D treatment in M, CL, CH, VD, CM, and VDM groups **(Fig. 6)**.

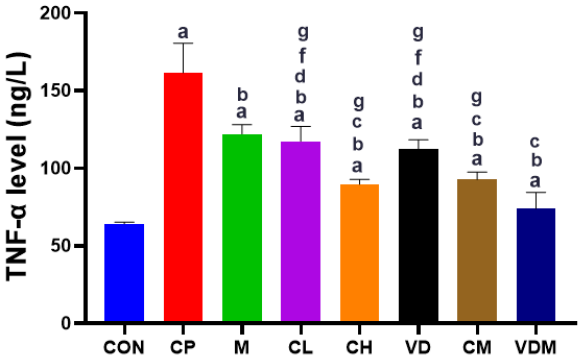

Fig. 5: TNF-α in different groups. Data were represented as mean and mean difference (n=7). ns—non-significant ($p > 0.05$), a—Significant ($p < 0.05$) as compared with CON group, b—significant ($p < 0.05$) as compared with CP group, c—significant ($p < 0.05$) as compared with M group, d—significant ($p < 0.05$) as compared with CH group, e—significant ($p < 0.05$) as compared with VD group, f —significant ($p < 0.05$) as compared with CM group, g—significant ($p < 0.05$) as compared with VDM group.

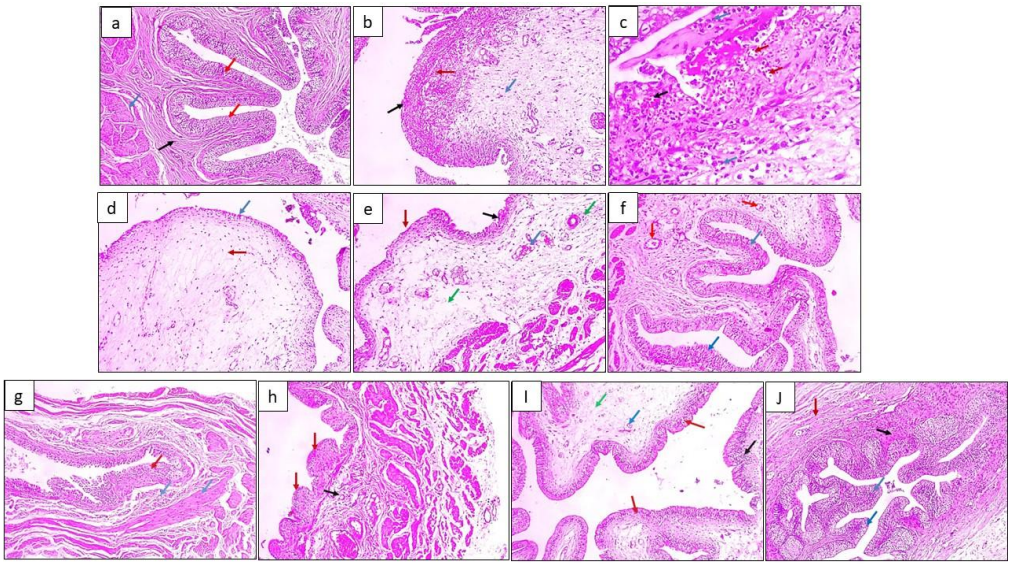

Fig. 6: Hematoxylin and eosin sections **(a)** CON group: Hematoxylin and eosin sections showing normal urothelium (red arrows) surrounded by normal submucosa (black arrow) and musculosa (blue arrow) (X 100 magnification). **(b)** CP group: ulcerated surface (black arrow) lined with granulation tissue with inflammatory cells (red arrow) and marked edema (blue arrow) (X 100 magnification). **(c)** CP group: Higher magnification of hematoxylin and eosin sections showing ulcerated granulation tissue with inflammatory cells (blue arrows) vascular congestions (red arrows) and extravasated RBCs (black arrow) (X 400 magnification). **(d)** CP group: Hematoxylin and eosin sections showing atrophic mucosa (blue arrow) with underlying massive edema (red arrow) (X 100). **(e)** M group: Hematoxylin and eosin sections showing alternating ulcerated (red arrow) and atrophic mucosa (black arrow) with moderate submucosal congested vessels (blue arrow), mild inflammation and moderate edema (green arrows) (X 100). **(f)** CL group: Hematoxylin and eosin sections showing moderate thickness of urothelium (blue arrows) surrounded by mild congested submucosal vessels (red arrows), no edema or inflammation (X 100). **(g)** CH group: Hematoxylin and eosin sections showing focal normal thickness (red arrow) surrounded by normal submucosa and musculosa (blue arrows) without congestion, inflammation, or edema (X 100). **(h)** VD group: Hematoxylin and eosin sections showing thin mucosa with single layer of transitional epithelium without ulceration (red arrow) with mild submucosal congested vessels (black arrow), no edema or inflammation (X 100). **(i)** CM group: Hematoxylin and eosin sections showing no surface ulceration with mild thickness of urothelium [not the normal thickness] (red arrows) with mild submucosal congested vessels (blue arrow), mild inflammation (black arrow) and moderate edema (green arrow) (X 100). **(j)** VDM group: Hematoxylin and eosin sections showing normal thickness of urothelium (blue arrows) surrounded by normal submucosa (black arrow) and musculosa (red arrow) without ulceration, congestion, inflammation, or edema (X 100).

Amelioration of cyclophosphamide-induced apoptosis

Immunohistochemically stained sections revealed a significant increase in caspase-3 expression percentage in the CP group versus the CON group. M, CL, CH, VD, CM, and VDM groups revealed a significant decrease in caspase-3 expression percentage versus the CP group. CL, CH, VD, CM, and VDM groups revealed a significant decrease in caspase-3 expression percentage versus the M group. CL and VD groups revealed a significant increase in caspase-3 expression percentage versus the CH group. CL group revealed a significant increase in caspase-3 expression percentage versus the CM group, but the CH group revealed a significant decrease in caspase-3 expression percentage versus the CM group. CL and VD groups revealed a significant increase in caspase-3 expression percentage versus the VDM group **(Fig. 7)**. **Fig. 8** demonstrates caspase immunohistochemistry slides of the urinary bladder of the different groups.

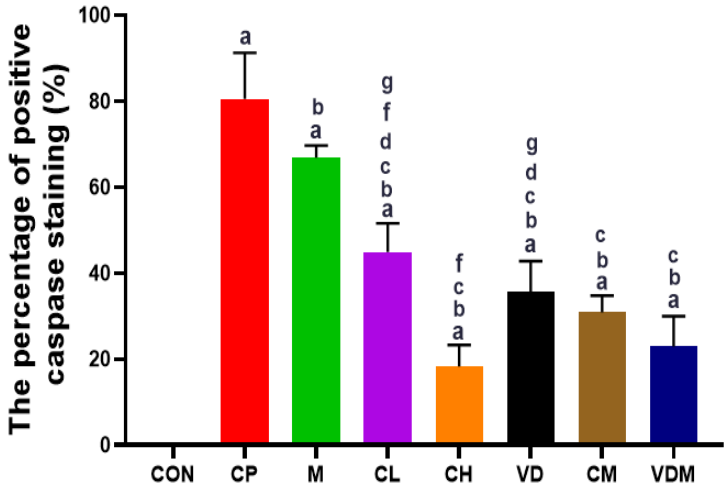

Fig. 7: Immunoexpression percentage of caspase-3 was assessed in different groups. Data were represented as mean ± standard deviation (n=7). ns—non-significant ($p > 0.05$), a—Significant ($p < 0.05$) as compared with CON group, b—significant ($p < 0.05$) as compared with CP group, c—significant ($p < 0.05$) as compared with M group, d—significant ($p < 0.05$) as compared with CH group, e—significant ($p < 0.05$) as compared with VD group, f —significant ($p < 0.05$) as compared with CM group, g—significant ($p < 0.05$) as compared with VDM group.

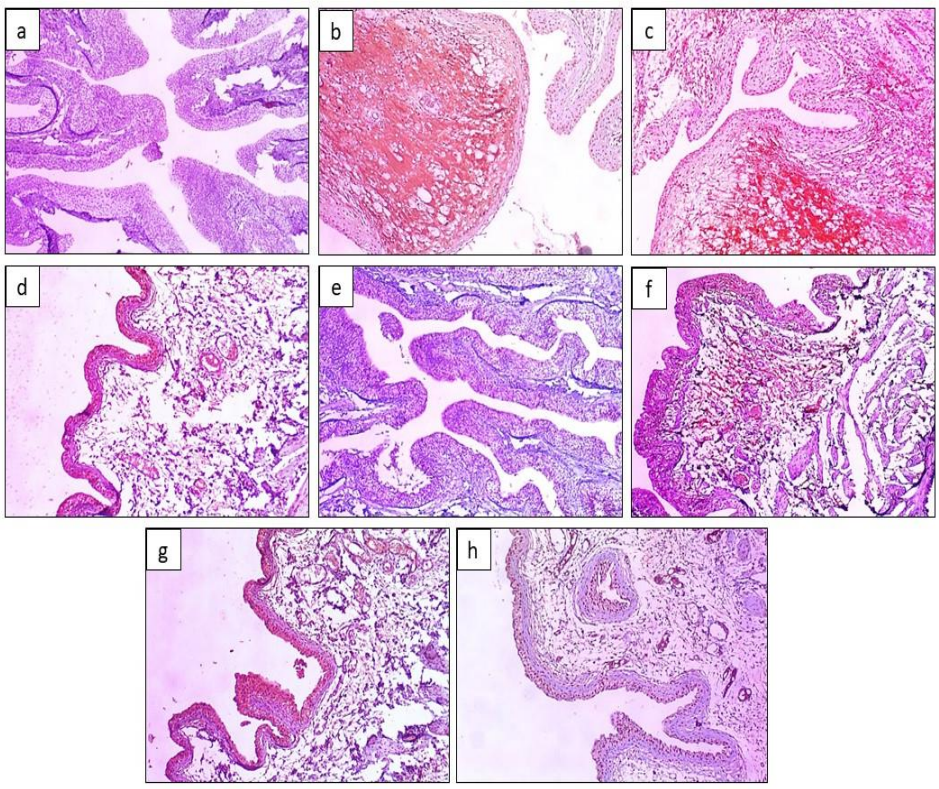

Fig. 8: Caspase immunohistochemical expression of the urinary bladder tissue in different groups (X 100). **(a)** negative expression of caspase in CON group (0.16%). **(b)** strong expression of caspase in CP group (85.2%). **(c)** strong expression of caspase in M group (66%). **(d)** Moderate expression of caspase in CL group (43.23%). **(e)** weak expression of caspase in CH group (16.45%). **(f)** moderate expression of caspase in VD group (35.6%). **(g)** moderate expression of caspase in CM group (30.8%). **(h)** weak expression of caspase in VDM group (22.2%).

Promotion of the autophagic activity

LC3-II expression

Immunohistochemically stained sections revealed a significant increase in LC3-II expression percentage in the CP group versus the CON group. CL, CH, VD, CM, and VDM groups revealed a significant decrease in LC3-II expression percentage versus the CP group, but the M group revealed a non-significant difference versus the CP group. CL, CH, VD, CM, and VDM groups revealed a significant decrease in LC3-II expression percentage versus the M group. CL and VD groups revealed a significant increase in LC3-II expression percentage versus the CH group. CL group revealed a significant increase in LC3-II expression percentage versus the CM group, but CH and VD groups revealed a significant decrease in LC3-II expression percentage versus the CM group. CL, VD, and CM groups revealed a significant increase in LC3-II expression percentage versus the VDM group **(Fig. 9)**.

SQSTM1/p62 expression

Immunohistochemically stained sections revealed a significant increase in SQSTM1/p62 expression percentage in the CP group versus the CON group. CL, CH, VD, CM, and VDM groups revealed a significant decrease in SQSTM1/p62 expression percentage versus the CP group, but the M group revealed a non-significant difference versus the CP group. CL, CH, VD, CM, and VDM groups revealed a significant decrease in SQSTM1/p62 expression percentage versus the M group. CL and VD groups revealed a significant increase in SQSTM1/p62 expression percentage versus the CH group. CL group revealed a significant increase in SQSTM1/p62 expression percentage versus the CM group, but CH and VD groups revealed a significant decrease in SQSTM1/p62 expression percentage versus the CM group. CL, VD, and CM groups revealed a significant increase in SQSTM1/p62 expression percentage versus the VDM group, but the CH group revealed a significant decrease versus the VDM group **(Fig. 9)**.

Fig. 10 and **Fig. 11** demonstrate LC3-II and SQSTM1/p62 immunohistochemistry slides of the urinary bladder of different groups and an illustration of an electron microscope examination of the urinary bladder of the different groups is shown in **Fig. 12**.

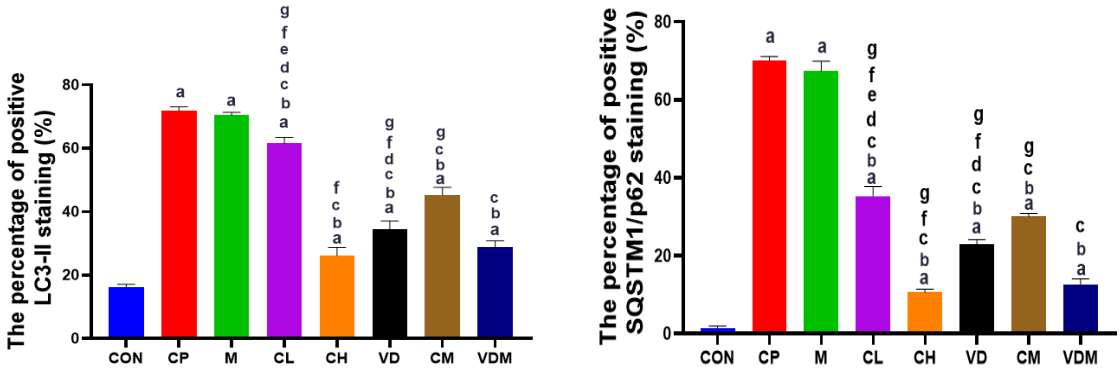

Fig. 9: Immunoexpression percentage of the urinary bladder tissue. (a) LC3-II was assessed in different groups. (b) SQSTM1/p62 was assessed in different groups. Data were represented as mean ± standard deviation (n=7). ns—non-significant ($p > 0.05$), a—Significant ($p < 0.05$) as compared with CON group, b—significant ($p < 0.05$) as compared with CP group, c—significant ($p < 0.05$) as compared with M group, d—significant ($p < 0.05$) as compared with CH group, e—significant ($p < 0.05$) as compared with VD group, f —significant ($p < 0.05$) as compared with CM group, g—significant ($p < 0.05$) as compared with VDM group.

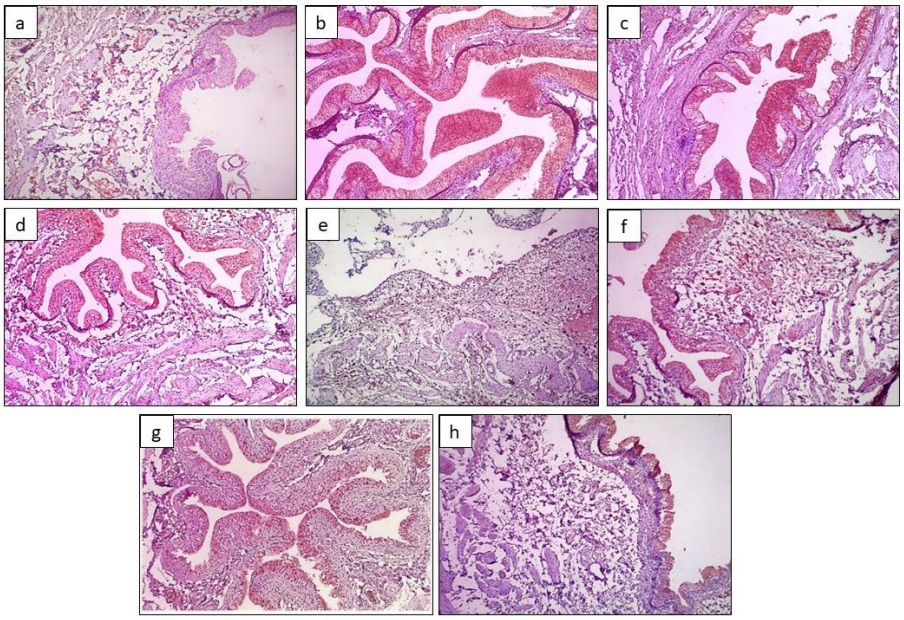

Fig. 10: LC3-II immunohistochemical expression of the urinary bladder tissue in different groups (X 100). **(a)** weak expression of LC3-II in CON group (16.12%). **(b)** strong expression of LC3-II in CP group (73.24%). **(c)** strong expression of LC3-II in M group (70.72%). **(d)** strong expression of LC3-II in CL group (64.27%). **(e)** moderate expression of LC3-II in CH group (26%). **(f)** moderate expression of LC3-II in VD group (34.72%). **(g)** moderate expression of LC3-II in CM group (45.18%). **(h)** moderate expression of LC3-II in VDM group (28.12%).

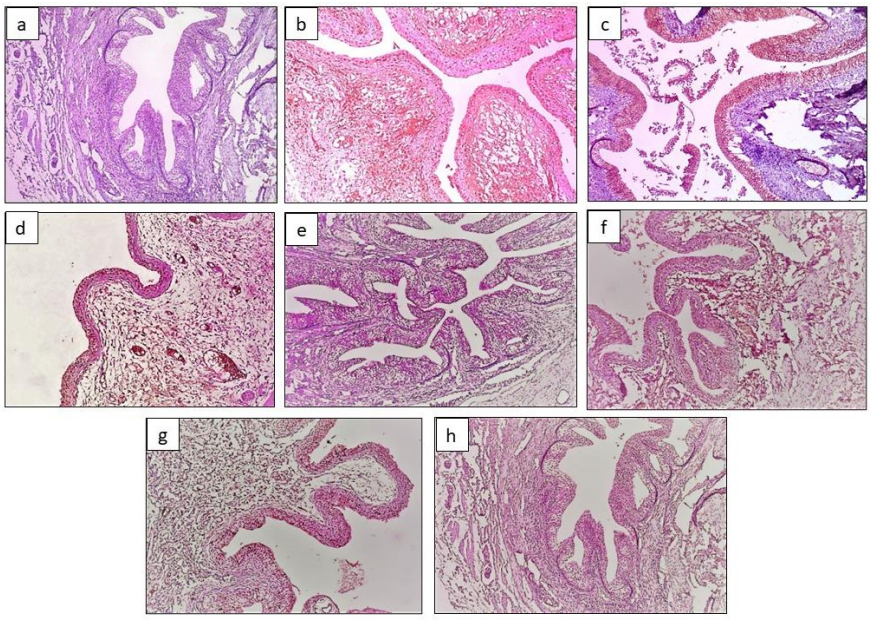

Fig. 11: SQSTM1/P62 immunohistochemical expression of the urinary bladder tissue in different groups (X 100). **(a)** negative expression of SQSTM1/P62 in CON group (1.4%). **(b)** strong expression of SQSTM1/P62 in CP group (70.7%). **(c)** strong expression of SQSTM1/P62 in M group (68.6%). **(d)** moderate expression of SQSTM1/P62 in CL group (35.66%). **(e)** weak expression of SQSTM1/P62 in CH group (10.2%). **(f)** weak expression of SQSTM1/P62 in VD group (21.3%). **(g)** moderate expression of SQSTM1/P62 in CM group (30.7%). **(h)** weak expression of SQSTM1/P62 in VDM group (12.4%).

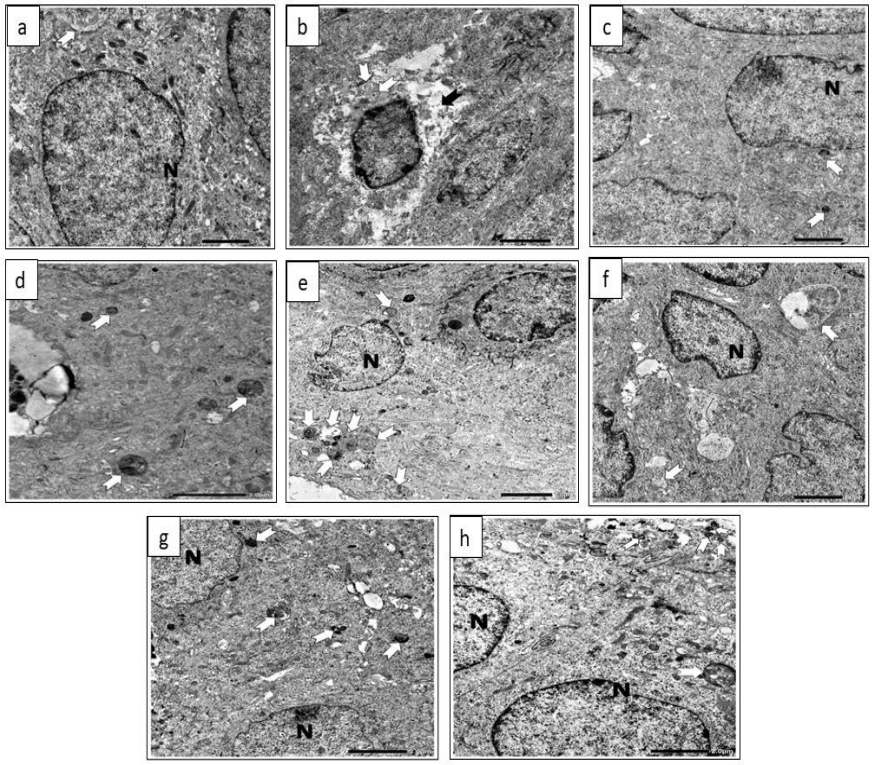

Fig. 12: Illustration of transmission electron microscope of the urinary bladder tissue in different groups (X 100). **(a)** An ultrathin section in the urothelium of CON group showing a normal euchromatic nucleus (N) and autophagic vacuole (white arrow). magnification × 3000. **(b)** An ultrathin section in the urothelium of CP group showing a cytoplasmic rarefaction (black arrow) and autophagic vacuoles (white arrow). magnification × 3000. **(c)** An ultrathin section in the urothelium of M group showing a slight intended nucleus (N) and autophagic vacuoles (white arrow). magnification × 3000. **(d)** An ultrathin section in the urothelium of CL group showing autophagic vacuoles (white arrow). magnification × 3000. **(e)** An ultrathin section in the urothelium of CH group showing an indented nucleus (N) and autophagic vacuoles (white arrow). magnification × 3000. **(f)** An ultrathin section in the urothelium of VD group showing a slight indented nucleus (N) and autophagic vacuoles (white arrow). magnification × 3000. **(g)** An ultrathin section in the urothelium of CM group showing nearly normal nucleus (N) and autophagic vacuoles (white arrow). magnification × 3000. **(h)** An ultrathin section in the urothelium of VDM group showing nearly normal nucleus (N) and autophagic vacuoles (white arrow). magnification × 3000.

Serum 25 hydroxyvitamin D and serum calcium level

CP group showed a non-significant difference in serum 25 hydroxyvitamin D versus the CON group. M, CL, CH, and CM groups showed a non-significant difference in serum 25 hydroxyvitamin D versus the CP group. VD and VDM groups showed a significant increase in serum 25 hydroxyvitamin D versus CP and CON groups. The VD group showed a significant increase in serum 25 hydroxyvitamin D versus the CH group. The VD group showed a significant increase in serum 25 hydroxyvitamin D versus the CM group. CL, CH, and CM groups showed a significant increase in serum 25 hydroxyvitamin D versus the VDM group. CP group showed a non-significant difference in serum calcium versus the CON group. M, CL, VD, CM, and VDM groups showed a non-significant difference in serum calcium versus the CP group. The VD group showed a significant decrease in serum calcium versus the CM group. The CH group showed a significant increase in serum calcium versus other groups **(Fig. 13)**.

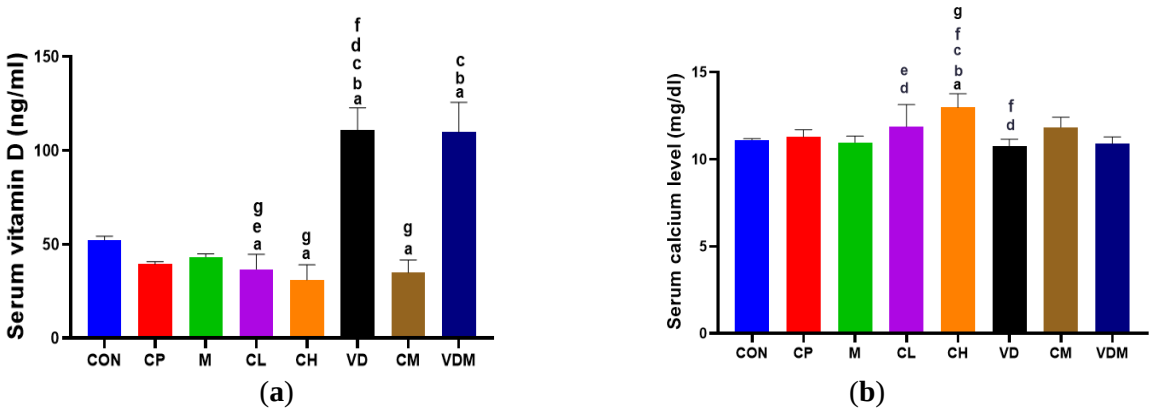

Fig. 13: (a) serum 25 hydroxyvitamin D level was assessed in different groups. (b) serum calcium level was assessed in different groups. Data were represented as mean ± standard deviation (n=7). ns—non-significant ($p > 0.05$), a—Significant ($p < 0.05$) as compared with CON group, b—significant ($p < 0.05$) as compared with CP group, c—significant ($p < 0.05$) as compared with M group, d—significant ($p < 0.05$) as compared with CH group, e—significant ($p < 0.05$) as compared with VD group, f —significant ($p < 0.05$) as compared with CM group, g—significant ($p < 0.05$) as compared with VDM group.

Discussion

CP induced HC incidence is on a continuous increase and affects around 23% of individuals who get treatment for it, especially those who undergo hematopoietic stem cell transplantation. [36]. CP is one of the most often used chemotherapeutic medications that is involved in the treatment of numerous malignant tumors and used as an immunosuppressive agent in autoimmune diseases and post-hematopoietic stem cell transplantation[10]. Acrolein is the leading agent for the induction of HC. Prophylactic administration of Mesna can reduce the occurrence of hemorrhagic cystitis in patients treated with CP due to its cytoprotective effect[37]. Unfortunately, Mesna is the only authorized protective measure for CP-induced HC, but its administration may cause adverse effects such as hypersensitivity reactions, especially in young age and children [38]. Therefore, there are always several experimental studies to find a new method that reverses or protects against CP induced HC.

Previous research proved that CP induces HC through direct damage to urothelium and inflammation[39,40]. The urothelium damage is induced via several mechanisms such as oxidative stress with lipid peroxidation[41], inflammation induced by different inflammatory mediators[42], apoptosis[43], and recently there is compelling evidence proposed that autophagy has a paramount role in CP induced HC[13,44].

Hence, in the present study, cyclophosphamide 200 mg/kg by single intraperitoneal injection was utilized to induce HC according to previous studies[23,45], it was chosen as an animal model of CP induced HC to evaluate the potential protective effect of Mesna and active (calcitriol) or inactive (cholecalciferol) forms of vitamin D each alone or in combination against CP induced HC which is the main goal of the present study.

The current study demonstrated that a single dose of CP (200 mg/kg) induced HC that was manifested by a statistically significant increase in visceral pain score as compared to the CON group. These results agree with Boucher et al., (2000)[29], Dornelles et al., (2014)[28], and González-Cano et al., (2020)[46] who suggested that CP administration induces marked inflammation that results in visceral pain sensation, strongly predicting HC. This inflammation was confirmed via a significant increase in TNF-α level in the CP group versus the CON group which is in accordance with Amanat et al., (2022)[47] and Juszczak et al., (2022)[48]. This inflammation is associated with edema and a subsequent significant increase in bladder index in CP group versus CON group which is in accordance with Merwid-Ląd et al., (2021)[49]. All these results were supported by the histopathologic examination of urinary

bladder sections in the CP group as it showed ulcerated granulation tissue with inflammatory cell infiltration, hemorrhage, and atrophic mucosa with massive edema which correlates with Abdelaziz et al., (2019) [14]. Several studies have pointed out that oxidative stress is a cornerstone in the pathogenesis of CP induced HC [47,49,50] and our current study substantiates this idea. Animals in the CP group presented a state of oxidative stress that is confirmed by a significant decrease in TAC and a significant increase in MDA as compared to the CON group. This result correlates with Abdelrahman et al., (2023)[51] and Akbaş et al., (2022)[52].

CP induces apoptosis in tissue [53.] Caspase is an essential protease enzyme in apoptotic cascade[54]. In our present study, the untreated HC group (CP group) exhibited a significant increase in caspase-3 immunohistochemical expression percentage in the urinary bladder tissue as compared to the CON group. This proves that apoptosis is a stepwise mechanism in HC pathogenesis, these results agree with Luo et al., (2020)[55] and Engin et al., (2022)[56].

The pathology of CP induced HC is not fully described via the oxidative inflammatory apoptotic pathway, although it is a main initiating pathway. Autophagy recently has a crucial role in the pathogenesis of HC[8]. Autophagy is a cytoprotective mechanism that is related to cellular stress exposure to maintain cellular homeostasis and deport apoptosis[57]. It also can abort reactive oxygen species (ROS) aggravation with a subsequent decrease in oxidative stress. However, If the cellular stress is continuous and more than the capacity of autophagy to restore cellular homeostasis, the apoptotic cascade will be activated[58]. Therefore, CP initiates the autophagy process in the urinary bladder, but leads to impairment of autophagic flux represented by increased the level of LC3-II and SQSTM1/p62[13,44] that yields to upregulation NF-kB transcription with subsequent increase in other proinflammatory cytokines such as TNF-α [59]. Our study is in harmony with this research point that was proved by a significant increase in LC3-II and SQSTM1/P62 expression percentage as compared to the CON group and the presence of autophagic vacuoles analyzed by TEM and correlates with Liu et al., (2020)[13].

Acrolein is a leading factor in HC pathogenesis. Conjugation of acrolein with glutathione is an important step to protect against CP induced HC[60]. As Mesna is a sulfhydryl donor[61], it can ameliorate CP induced HC. Our results confirmed this concept, as Mesna ameliorated the oxidative stress through a significant increase in TAC and a significant decrease in MDA level compared to the CP group which agrees with Mahmoudi et al., (2018)[62]. The preventive role of Mesna against HC is not restricted to its antioxidant effect but extends to anti-inflammatory and anti-apoptotic effects63. Our results of the M group when compared to the CP group revealed a significant decrease in TNF-α level which agree with Almeida de Oliveira et al., (2022)[64]. Also, the M group when compared to the CP group revealed a significant decrease in caspase expression percentage which agrees with Elrashidy and Hasan, (2021)[8]. Mesna offered an improvement of HC via its antioxidant, anti-inflammatory, and antiapoptotic effects that led to a significant decrease in bladder index and visceral pain score as compared to the CP group. These results agree with Merwid-Ląd et al., (2021)[49] and Boeira et al., (2011)[65]. In our results, we investigated the autophagic activity of Mesna, but it insignificantly increased autophagic activity represented by a non-significant difference in LC3-II and SQSTM1/P62 expression percentage as compared to CP group which correlates with Elrashidy and Hasan, (2021) [8].

The choice of vitamin D, being an essential vitamin. It has a pleiotropic effect that maintains cellular viability. It has a reliable antioxidant, anti-inflammatory [66,] and anti-apoptotic effects[67]. When it comes to the treatment and prevention of many diseases, vitamin D is a crucial therapeutic component[66,68]**.** Vitamin D3 receptors are expressed in bladder tissue [69]. It can attenuate urinary tract infection by promoting epithelial integrity[70] and can ameliorate the severity of chronic interstitial cystitis via its anti-inflammatory effect 71. Cholecalciferol is an inactive form of vitamin D that is metabolized by 25 hydroxylase enzymes and 1 α hydroxylase enzyme in the liver and kidney respectively giving calcitriol which is the active form of vitamin D[72].

Regarding visceral pain score and bladder index, vitamin D-treated HC groups either

cholecalciferol group (VD group) or calcitriol groups (CL group and CH group) showed a significant decrease when compared to untreated HC group and amelioration of inflammation that expressed in histopathological findings. All these results are believed to be a result of the antioxidant, anti-inflammatory, and antiapoptotic effects of vitamin D that proved by a significant decrease in MDA, TNF-α tissue levels, caspase-3 expression, and a significant increase in TAC, these findings are consistent with previous finding of Abood et al., (2021)[73], Elseweidy et al., (2023)[74] and Salem et al., (2023)[75].

Regarding the autophagy activity, our results of vitamin D-treated HC groups when compared to the CP group showed a significant difference in LC3-II and SQSTM1/P62 expression percentage which correlates with Cui et al., (2021)[25] who supposed that vitamin D not only initiated the autophagy and induced the formation of autophagosomes but also imposed their degradation that represented by decreasing the expression of LC3-II and SQSTM1/P62 as evidence of autophagic flux occurrence.

Meanwhile Jang et al., (2014)[76] revealed in their study that vitamin D promoted autophagy and increased LC3-II expression. The shorter duration of vitamin D treatment employed in the earlier study may be to blame for this dispute, so vitamin D only initiated autophagy and induced autophagosome formation without induction of the autophagy flux.

Our current study showed that vitamin D protection against CP induced HC has dose-dependent manner as there was a significant decrease in visceral pain score in the CH group as compared to the CL group attributed to a dose-dependent significant difference in oxidative stress and inflammation. It was proved by a significant decrease in tissue MDA and TNF-α levels and a significant increase in tissue TAC in the CH group as compared to the CL group. These results agree with Kianian et al., (2019)[77] and Li et al., (2021)[78].

Herein, the anti-apoptotic effect of vitamin D and its induction of autophagic activity had been proved through a significant dose-dependent difference in caspase-3, LC3-II, and SQSTM1/P62 expression percentage in the CH group and CL group. These results are in harmony with the previous finding of Jang et al., (2014)[76], Lyu et al., (2020)[79], Niu et al., (2021)[19] and Fang et al., (2023)[80] which point to the suppressive effect of autophagy on the apoptosis in spinal cord injury model through inhibition of apoptotic mitochondrial cascade.

According to our vitamin D results, we also showed that there was more improvement in the high-dose calcitriol (active form) treated HC group (CH group) than cholecalciferol treated HC group (VD group) -which is an inactive form of vitamin D- in a manner dependent on pharmacological activity represented by a significant increase in visceral pain score that attributed to a significant difference in oxidative stress and inflammation that was proved by a significant increase in tissue MDA and TNF-α level, and a significant decrease in tissue TAC in VD group versus CH group. These results correlate with Tsujino et al., (2019)[81]. This significant difference may justify a significant difference in the antiapoptotic activity and the autophagic activity between the CH group and VD group that had been represented by a significant difference in caspase-3, LC3-II, and SQSTM1/P62 expression percentage.

Despite the pleiotropic effect of vitamin D and its clinical benefits, the hazard of hypercalcemia is still a serious complication that impedes calcitriol systemic administration[82]**.** However, with the significant improvement of the CH group in our study, there was a significant increase in serum calcium in the CH group versus the CON group and other treated groups. These results agree with Anisiewicz et al., (2020)[83], but disagree with Takeda et al., (2010)[84] who use higher doses of calcitriol without significant alteration in serum calcium level. We can attribute this to the number of doses administrated per week in our study being more than that used in the study of Takeda et al., (2010)[84].

While the low dose calcitriol treated HC group (CL group) showed a non-significant difference in serum calcium level versus the CON group. This result agrees with Jiang et al., (2014)[85]. Also, the cholecalciferol-treated HC group (VD group) showed a non-significant difference in serum calcium level versus the CON group. This result agrees with Fleet et al., (2008)[86] who postulated that different doses of

cholecalciferol even higher doses than our dose do not alter serum calcium levels.

In our study, Vitamin D forms either cholecalciferol or a high dose of calcitriol treated HC group versus M group showing more attenuation in the inflammation and the apoptosis that proved by a significant decrease in tissue TNF-α level and caspase expression percentage. It may be attributed to the autophagic activity of vitamin D. While Mesna did not show a significant change in autophagic activity as compared to the untreated HC group (CP group).

The combination of calcitriol and Mesna (CM group) when compared to monotherapy groups either CL group or M group showed a significant difference in visceral pain score, tissue level of TNF-α, and caspase-3, LC3-II and SQSTM1/P62 expression percentage. CM group didn`t show an advantage in oxidative stress when compared with the M group represented by insignificant differences in tissue MDA level and TAC. There was a significant increase in MDA level and a significant decrease in TAC in the CL group when compared with the CM group.

While the combination of cholecalciferol and Mesna (VDM group) when compared to monotherapy groups either the VD group or M group showed a significant difference in visceral pain score, bladder index, tissue level of TNF-α, MDA, and TAC, and percentage of caspase-3, LC3-II and SQSTM1/P62 expression.

Herein, Mesna and vitamin D therapy cannot completely reverse HC as there was a significant difference between all treated HC groups and the control group in visceral pain score, tissue MDA and TNF-α level, and percentage of caspase, LC3-II, and SQSTM1/P62 expression.

These findings supposed that vitamin D can increase the uroprotective effect of Mesna. Since both drugs have antioxidant, anti-inflammatory, and antiapoptotic activities. Both drugs together can diminish free radicals' release. Moreover, vitamin D plays a role in the restoration of autophagic flux.

It is worth noting that the combination of cholecalciferol and Mesna showed more uroprotection than the combination of calcitriol and Mesna regarding restoring autophagic flux and amelioration of visceral pain and inflammation without risk of hypercalcemia.

Conclusion

Hence, in our study, it is concluded that vitamin D boosts the uroprotective effect of Mesna against CP induced HC. The combination of Mesna and cholecalciferol has a protective effect superior to a combination with low-dose calcitriol. Moreover, a high dose of calcitriol has also a protective role against CP induced HC but it carries the risk of hypercalcemia.

Therefore, our ongoing studies will examine whether this combination could also potentiate the anticancer effect of CP, beyond amelioration of the urotoxicity. Besides, our findings should be verified in further human clinical studies.

Author Contributions

"conceptualization, Mennatallah Elkady, Heba Abd Elgalil Aly Mahmoud, Samia Abou-El-Seoud and Mahmoud Saeid; methodology, Mennatallah Elkady; writing—original draft preparation, Mennatallah Elkady; writing—review and editing, Heba Abd Elgalil Aly Mahmoud, Samia Abou-El-Seoud and Mahmoud Saeid; supervision, Heba Abd Elgalil Aly Mahmoud, Samia Abou-El-Seoud and Mahmoud Saeid.

Acknowledgments

I would also want to thank Dr. Aya Abd Elmoneim and Prof. Dr. Yomna Zamzam for their assistance with the histological evaluation, photo-imaging, and the completion of this study.

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Bull. Pharm. Sci., Assiut University, Vol. 46, Issue 6, 2023, pp. 1453-1474.

نشرة العلوم الصيدلية
جامعة أسيوط

BULL. PHARM. SCI. Assiut Univ.

التأثير الواقي المحتمل لمادة الميسنا وفيتامين د كلا على حدة أو معا على التهاب المثانة النزفي المستحث بالسيكلوفوسفاميد في الجرذان البيضاء

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ان التهاب المثانة النزفي هو أحد الآثار الجانبية الأكثر شيوعا للسيكلوفوسفاميد. و قد قمنا بتقييم الدور الوقائي للميسنا وفيتامين (د) ضد التهاب المثانة النزفي الناجم عن السيكلوفوسفاميد.

الطرق: أجريت هذه التجربة على ٦٤ فأرا. مجموعة ١:الضابطة العادية. و قد تم حقن الجرذان بـ ٢٠٠ مجم / كجم من السيكلوفوسفاميد مرة واحدة عن طريق الحقن بالبطن في اليوم التاسع عشر في المجموعات ٢ و ٣ و ٤ و ٥ و ٦ و ٧ و ٨. مجموعة ٢ (المجموعة الغير معالجة) ، مجموعة ٣ (عولجت بالميسنا) ، مجموعة ٤ ومجموعة ٥ (عولجت بالكالسيتريول) ، مجموعة ٦ (عولجت بالكوليكالسيفرول) ، مجموعة ٧ (عولجت بالكالسيتريول و الميسنا) ، و مجموعة ٨ (عولجت بالكوليكالسيفرول والميسنا).

النتائج: أظهر الميسنا وفيتامين د انخفاضا ذو دلالة احصائية في درجة الألم الحشوي ، ومؤشر المثانة ، و مستوى المالوندهيد وعامل نخر الورم ألفا في الانسجة ، ونسبة نشاط الكاسبيز٣ في التقييم المناعي، و كانت هناك زيادة ذات دلالة احصائية في القدرة الكلية المضادة للأكسدة مقارنة بالمجموعة الغير معالجة. بينما أظهرت مجموعات ٥ و ٦ و ٧ و ٨ انخفاضا معنويا في مستوى وعامل نخر الورم ألفا في الانسجة ، ونسبة نشاط الكاسبيز٣ و نسبة بروتين ال سي ٣-٢ وبروتين اس كيو اس تي ام / بي ٦٢ في التقييم المناعي مقارنة بمجموعة ٣. و قد أظهرت مجموعة ٥ زيادة ذات دلالة احصائية في مستوى الكالسيوم في الدم مقارنة بالمجموعات الأخرى.

الاستنتاج: هذه النتائج تثبت أن فيتامين د يزيد من التأثير الوقائي للميسنا ضد التهاب المثانة البولي الناجم عن السيكلوفوسفاميد و يرجع هذا إلى تأثيره على الالتهام الذاتي.