Original Article

A Study on the Alteration of Endoplasmic Reticulum Stress-related Proteins in Cyclophosphamide-induced Damage to Urothelium

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ABSTRACT

Background: Cyclophosphamide is widely prescribed as an anti-cancer drug and used as an immunosuppressant. Hemorrhagic cystitis is one of the common complications of cyclophosphamide intake. We hypothesized that endoplasmic reticulum stress-related proteins could be altered in urothelium treated with cyclophosphamide.

Objectives: We checked the effect of cyclophosphamide on the expression of various endoplasmic reticulum stress-related proteins in Vero cells.

Methods: We treated Vero cells with varying doses of cyclophosphamide and observed its viability in flow cytometry using propidium iodide staining. We looked for changes in the expression of endoplasmic reticulum stress-related proteins in Vero cells treated with cyclophosphamide by western blot technique.

Results: Cyclophosphamide at higher doses caused more death in Vero cells that could be attributed to an increase in apoptosis as evidenced by the changes in the morphology of cells and increased expression of endoplasmic reticulum specific caspase-12 proteins. Growth arrest/DNA damage 153 (GADD 153), one of the key transcription factors involved in the mediation of endoplasmic reticulum stress and apoptosis, was upregulated in Vero cells treated with cyclophosphamide. The protective effect of glucose-regulated protein GRP 78 against apoptosis was lost in Vero cells treated with a higher dose of cyclophosphamide, which is corroborated by decreased expression of GRP 78 in Vero cells treated with higher doses compared to Vero cells treated with lower doses of cyclophosphamide. Expression of disulfide isomerase protein, which guides misfolded proteins to fold properly, was downregulated in Vero cells treated with cyclophosphamide.

Conclusion: To summarize, our study showed an alteration in the expression of key endoplasmic reticulum stress-related proteins in Vero cells treated with cyclophosphamide.

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Introduction

Cyclophosphamide is widely used to treat solid malignancies (breast and ovarian cancer) [1, 2], hematological malignancies (chronic lymphocytic leukemia and non-Hodgkin's lymphoma) [3, 4], and childhood malignancies (acute lymphoblastic leukemia and retinoblastoma). With its immunosuppressant effect, cyclophosphamide is used for hematopoietic stem cell transplantation and treating autoimmune diseases like multiple sclerosis, systemic lupus erythematosus, scleroderma, and rheumatoid arthritis [5]. Cyclophosphamide is an inactive prodrug, which upon metabolism in the liver [6], converts to phosphoramide mustard and acrolein [7]; the former compound is responsible for the cytotoxicity of cancer cells through DNA cross-links, and the latter compound is responsible for hemorrhagic cystitis seen in patients taking cyclophosphamide.

Though cyclophosphamide has a far outreaching therapeutic effect on various disease conditions, its associated toxicities are a major limiting factor in using this drug. Intake of cyclophosphamide is associated with bone marrow suppression leading to pancytopenia, cardiotoxicities like arrhythmia and myocarditis, gonadal failure in women, and hemorrhagic cystitis [8].

Hemorrhagic cystitis is the most common urological complication of cyclophosphamide, and its incidence ranges between 12% and 40% in those on cyclophosphamide [9]. Mild hemorrhagic cystitis could be managed with hydration, whereas moderate and severe forms of hemorrhagic cystitis warrant invasive interventions like clot extraction using cystoscopy or could even lead to cystectomy. Acrolein, a byproduct of cyclophosphamide metabolism, accumulated in the bladder and was thought to be the key player in initiating hemorrhagic cystitis. Acrolein was shown to increase the generation of reactive oxygen species (ROS), thereby causing cell death. Acrolein increases nitric oxide production by activating inducible nitric oxide synthase (iNOS) [10]. Acrolein favors peroxynitrate formation through ROS and nitric oxide; peroxynitrates can damage proteins, lipids, and DNA [11].

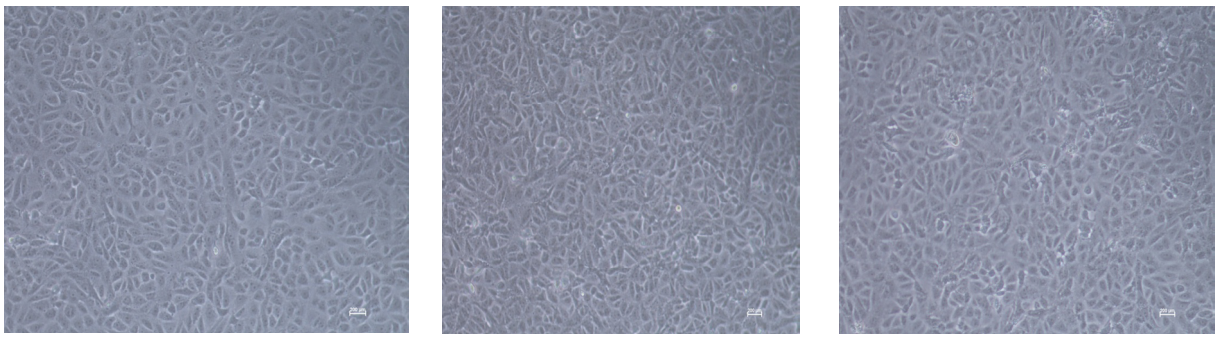
Mesna (sodium-2-mercaptoethanesulfonate) was touted as a promising agent to prevent cyclophosphamide-induced hemorrhagic cystitis. Metabolite of mesna, i.e. dimesna, when acted upon by glutathione dehydrogenase, generates free sulfhydryl groups, facilitating the excretion of acrolein from the bladder [12]. However, some studies clearly show that hyperhydration has similar effects in preventing hemorrhagic cystitis compared to mesna administration in patients on cyclophosphamide [13]. Mesna was also shown to have no uroprotective effect in a study where mesna was given along with cyclophosphamide in rheumatic disease patients [14]. In addition, mesna administration has side effects, like cutaneous and systemic hypersensitivity reactions [15]. There is a strong need for novel therapeutic agents to prevent the emergence of hemorrhagic cystitis in patients on cyclophosphamide regimens.

Lack of complete understanding of the molecular level changes in urothelium during cyclophosphamide administration is part of the failure to develop a drug molecule that can prevent hemorrhagic cystitis. Our previous research findings demonstrated that cyclophosphamide enhanced the production of reactive nitrogen species [16]. Endoplasmic reticulum stress-related proteins are often activated in conditions with increased production of reactive oxygen species [17]. Since cyclophosphamide administration increases ROS, we hypothesized that cyclophosphamide administration would alter the expression of endoplasmic reticulum stress-related proteins in the urothelium. In this study, we were interested in seeing the expression of endoplasmic stress-related proteins in the urothelial cells (Vero cell line) exposed to varying doses of cyclophosphamide.

Materials and Methods

Cell culture

Vero cells obtained from American Type Culture Collection, USA, were grown as a monolayer in minimum essential medium, supplemented with 10% fetal bovine serum, sodium bicarbonate, HEPES, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 25 ng/mL of amphotericin-B. Cells were cultured under 5% CO_2 in a humidified incubator at 37°C. The medium was changed every 3-4 days. When the cells reached >90% confluency, they were subcultured using 0.25% trypsin-EDTA (Gibco). Cyclophosphamide was obtained from Sigma Chemicals. Cells were plated at 8000 cells/cm^2 on a polystyrene culture dish coated with poly-L-lysine. Cells were incubated with various concentrations of cyclophosphamide (1.2, 2.4, 4.8, 9.6, and 12 mg/mL) for 24 h at 37°C. The cells were visualized, and their morphology was studied using a phase-contrast microscope.



A) Control cells B) 1.2 mg of CP C) 2.4 mg of CP

D) 4.8 mg of CP E) 9.6 mg of CP F) 12 mg of CP

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Figure 1. Morphology of Vero cells exposed to various doses of cyclophosphamide (CP)

A) Phase contrast microscopy image of untreated control cells, B) cells treated with 1.2 mg of cyclophosphamide showing fewer apoptotic cells, C) cells treated with 2.4 mg of cyclophosphamide showing fewer apoptotic cells, D) cells treated with 4.8 mg of cyclophosphamide showing changes in morphology and more apoptotic cells, E) cells treated with 9.6 mg of cyclophosphamide showing more rounded and apoptotic cells, and F) cells treated with 12 mg of cyclophosphamide showing more apoptotic and fragmented cells.

Cell viability determination by flow cytometry

Cells treated with different doses of cyclophosphamide for 24 h were trypsinized and collected in a culture medium. Cells were then resuspended in propidium iodide dissolved in phosphate buffer solution and incubated at room temperature in the dark for 15 minutes. Propidium iodide-stained cells were then analyzed in a flow cytometer using the excitation wavelength of 488 nm. Unstained and untreated cells were also analyzed at 488 nm of wavelength.

Western blot analysis

For protein analysis, 3×10^5 cells were seeded in 6-well dishes, and the cells were harvested 24 h following cyclophosphamide treatment with different doses as described above. Vero cells were washed with cold PBS, and the cells were resuspended in 200 µL of lysis buffer (10 mM Tris pH7.4, 150 mM NaCl, 5 mM EDTA pH=8, 0.1% Triton-X, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, and 0.2% protease inhibitor cocktail [Sigma]), and incubated on ice for 30 min. The cell lysates were centrifuged for 30 min at 12000 g. Protein concentrations in the supernatants were determined by Lowry's method. For western blot analysis, 50 µg of lysate proteins were resolved over 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF transfer membranes (Whatman, UK). The membranes were blocked with 5% nonfat dry milk in buffer (10 mM Tris-HCl [pH=7.6], 100 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature and then incubated with the desired primary antibody anti-protein disulfide isomerase (PDI) (1:1000), anti-growth arrest/DNA damage 153 (GADD153) (1:500), anti-caspase-12 (1:1000), anti-glucose-regulated protein 78 (GRP 78) (1:1000) from (Sigma), and anti-β-actin (1:2000) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody anti-rabbit or mouse (Thermo, Waltham, MA) at 1:2000 dilution for 2 h at room temperature. The representative immunoreactive bands of proteins were visualized with ECL West Dura substrate, and their intensity was quantitated using a chemiluminescent imaging system (FluorChem TM SP, Alpha Innotech). The levels of proteins were normal-

ized to that of β-actin. Western blot analysis of the proteins was done in three independent experiments.

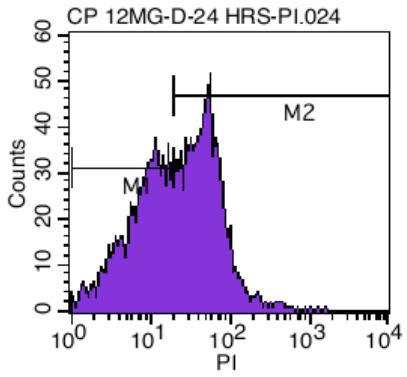
Statistical analysis

Statistical analysis was done using RStudio Team (2021) (RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com). The normality of the data was assessed by the Shapiro-Wilk test. Normally distributed data were analyzed by ANOVA test. The Kruskal-Wallis test analyzed data that are not normally distributed to assess the difference between the various treatment groups, followed by post hoc Bonferroni correction for identifying the significant difference between individual groups. $P<0.05$ was considered statistically significant.

Results

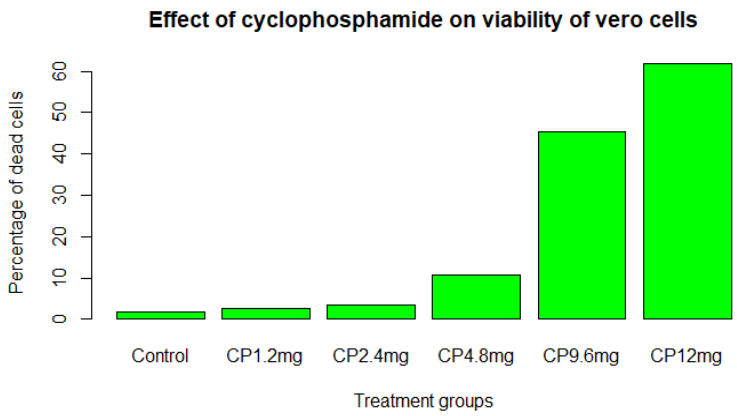
Dose-dependent effect of cyclophosphamide on the viability of Vero cell line

Vero cells treated with cyclophosphamide for 24 h exhibited a dose-dependent effect on viability; with an increase in cyclophosphamide dosage, the cells increasingly showed features of apoptosis, such as more rounded and fragmented cells (Figure 1A-1F).



Marker	Events	% Gated	% Total
All	8886	100.00	88.86
M1	4025	45.30	40.25
M2	4884	54.96	48.84

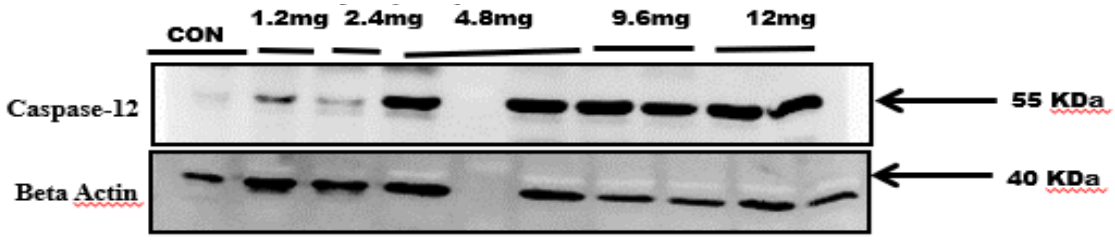
A. Propidium iodide staining



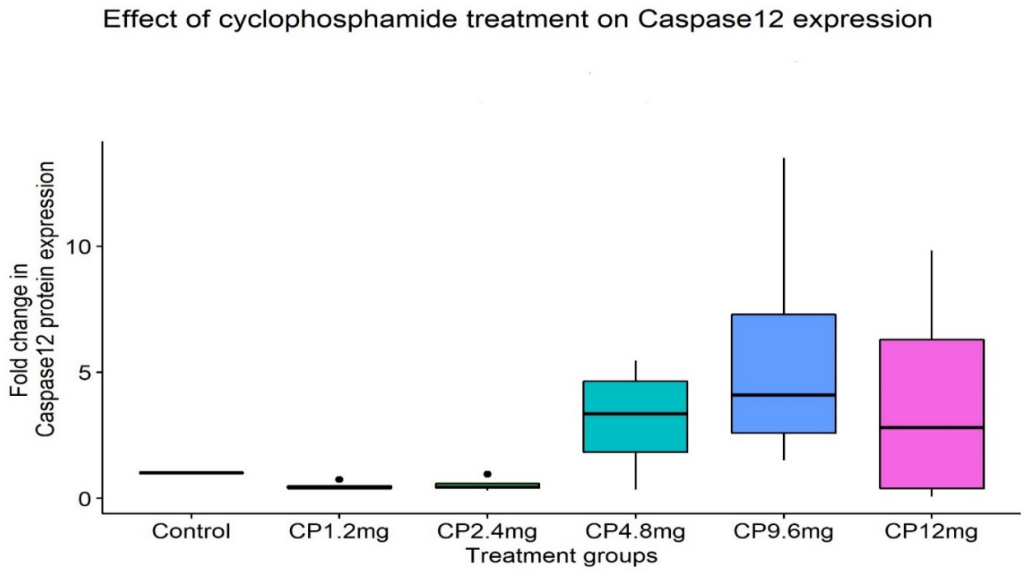
B. Vero cells exposed to different doses of CP

Figure 2. Effect of 24 h exposure to cyclophosphamide (CP) on the viability of Vero cells

A) Viability of the cells was assessed by propidium iodide staining and subsequent analysis in a flow cytometer at 488 nm, and B) Percentage of dead cells as assessed by flow cytometer in Vero cells exposed to different doses of cyclophosphamide.



A. Western blot image for Caspase-12 protein in Vero cells treated with cyclophosphamide



B. Quantification of Caspase-12 bands in western blot

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Figure 3. Expression of Caspase-12 protein in cyclophosphamide-treated Vero cells

A) Western blotting image of caspase-12, with β-actin was used as a loading control, and B) Fold change in expression of caspase-12 as compared to control and the difference in expression of caspase-12 between the groups was analyzed by Kruskal-Wallis test (P=0.06). Quantification value expressed as median and interquartile range.

The viability of the Vero cells treated with increasing doses of cyclophosphamide was also checked using propidium iodide staining, which preferentially stains dead cells due to membrane damage seen specifically in dead cells (Figure 2A). There was a dose-dependent increase in the death of Vero cells; with increasing dosage of cyclophosphamide, the proportion of dead cells increased in Vero cells (Figure 2B).

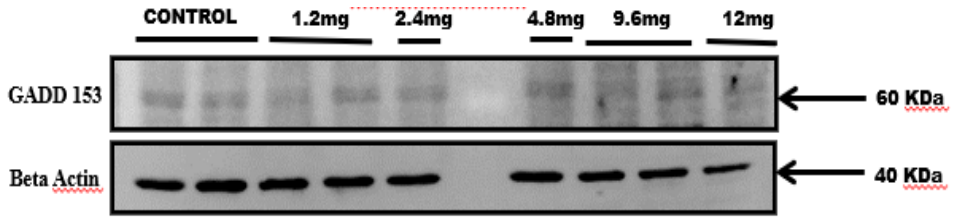
We checked whether apoptosis was the cause of death in the cyclophosphamide-treated cells. Caspase-12 overexpression is associated with increased apoptosis. Caspase-12, particularly seen in the endoplasmic reticulum, is upregulated in conditions that cause endoplasmic reticulum stress and is known to induce apoptosis [18]. Caspase-12 expression was upregulated in Vero cells treated with higher doses of cyclophosphamide (Figure 3A, 3B). The difference between the cyclophosphamide treatment groups regarding caspase-12 expression was not statistically significant but closer to significance (P=0.06).

Effect of cyclophosphamide on endoplasmic reticulum stress-related proteins GADD 153

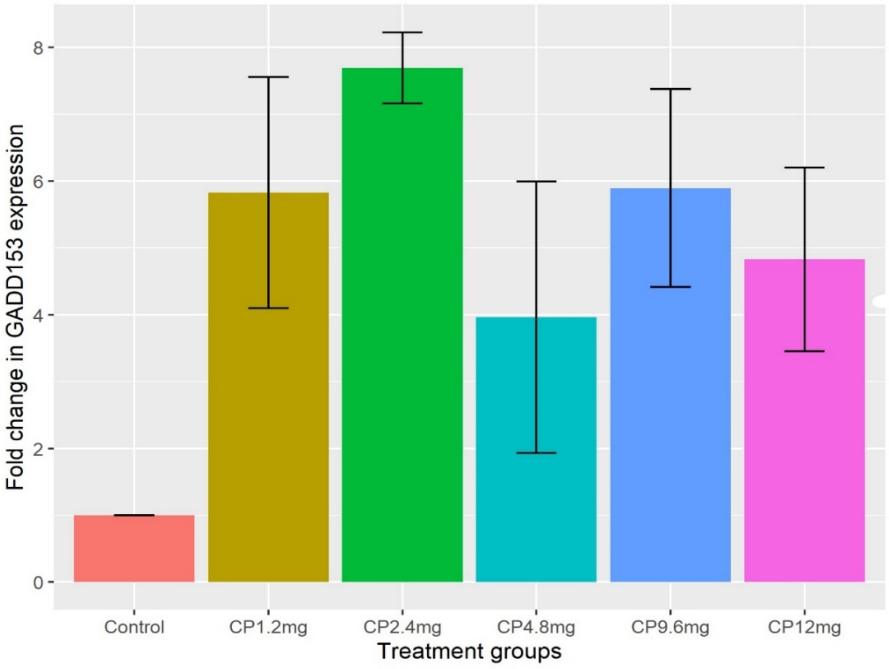
GADD 153 expressions induced cell death by down-regulating Bcl2 expression and depleting thiols in the cell [19]. Vero cells treated with different doses of cyclophosphamide for 24 hours led to increased expression of GADD 153 proteins across all the groups compared to the untreated control cells (Figure 4A, 4B). The difference in the expression of GADD 153 proteins among the groups treated with different doses of cyclophosphamide approached closer to statistical significance (P=0.07).

Glucose regulated protein (GRP 78)

Glucose-regulated proteins (GRP) are chaperones produced in response to endoplasmic reticulum stress.



A. Western blot image for GADD 153 protein in Vero cells treated with cyclophosphamide



B. Quantification of GADD 153 bands in western blot

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Figure 4. Expression of growth arrest/DNA damage 153 (GADD 153) protein in cyclophosphamide-treated Vero cells
A) Western blotting image of GADD 153, with β-actin used as a loading control, and B) Fold change in expression of GADD 153 expression compared to control and the difference in expression of GADD 153 between the groups were analyzed by ANOVA test (P=0.07). Quantification value expressed as mean±SE.

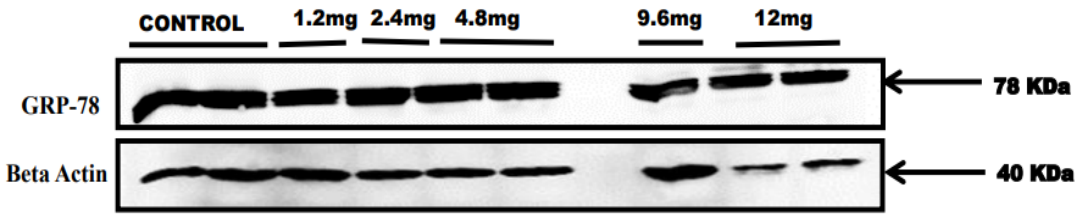
Treatment of Vero cells with different doses of cyclophosphamide for 24 hours leads to a decrease in the expression of GRP 78 at higher doses (Figure 5A, 5B). There was a statistically significant difference (P=0.028) in the expression of GRP 78 proteins among the groups treated with different doses of cyclophosphamide. However, post hoc Bonferroni correction did not show a difference in the expression of GRP 78 protein between any two treatment groups.

Protein disulfide isomerase

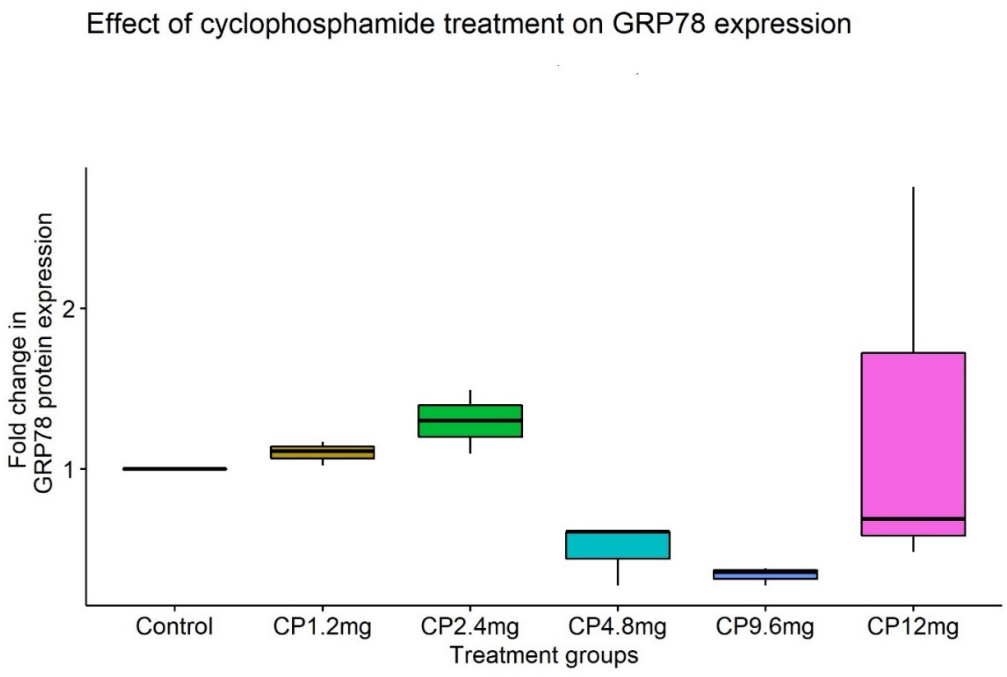
PDI, along with GRPs, helps in the proper folding of misfolded proteins, improving cells' survival. PDI protein expression was downregulated in cyclophosphamide-treated Vero cells compared to the untreated control groups (Figure 6A, 6B). The difference in the expression of PDI among the groups treated with different doses was found to be closer to statistical significance (P=0.0547). Exposure of cyclophosphamide to Vero cells downregulated the expression of cell-protective PDI, whose natural role is to improve the viability of cells.

Discussion

Endoplasmic reticulum stress activates unfolded protein response (UPR) to promote cell survival. However, prolonged activation of UPR could lead to cell death [20]. Toxic elements like cadmium increased cell death in renal epithelial cells by activating the endoplasmic reticulum stress pathway [21]. Recently it was shown that Tilapia (Oreochromis niloticus), when injected with



A. Western blot image for GRP 78 protein in Vero cells treated with cyclophosphamide



B. Quantification of GRP 78 bands in western blot

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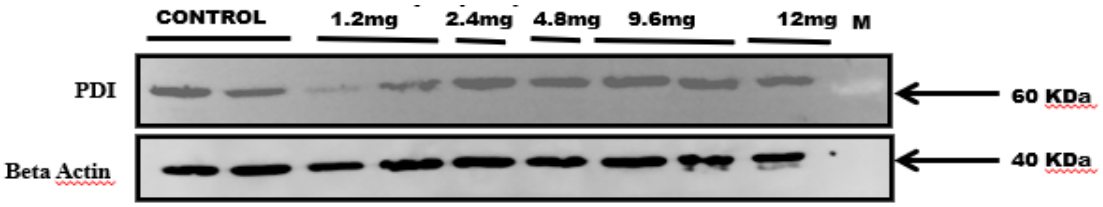
Figure 5. Expression of glucose-regulated proteins (GRP) 78 protein in cyclophosphamide-treated Vero cells

A) Western blotting image of GRP 78, with beta-actin used as a loading control, and B) Fold change in expression of GRP 78 as compared to control and the difference in expression of GRP 78 between the groups were analyzed by the Kruskal-Wallis test (P=0.028, with no significance in post hoc Bonferroni correction). Quantification value expressed as median and interquartile range.

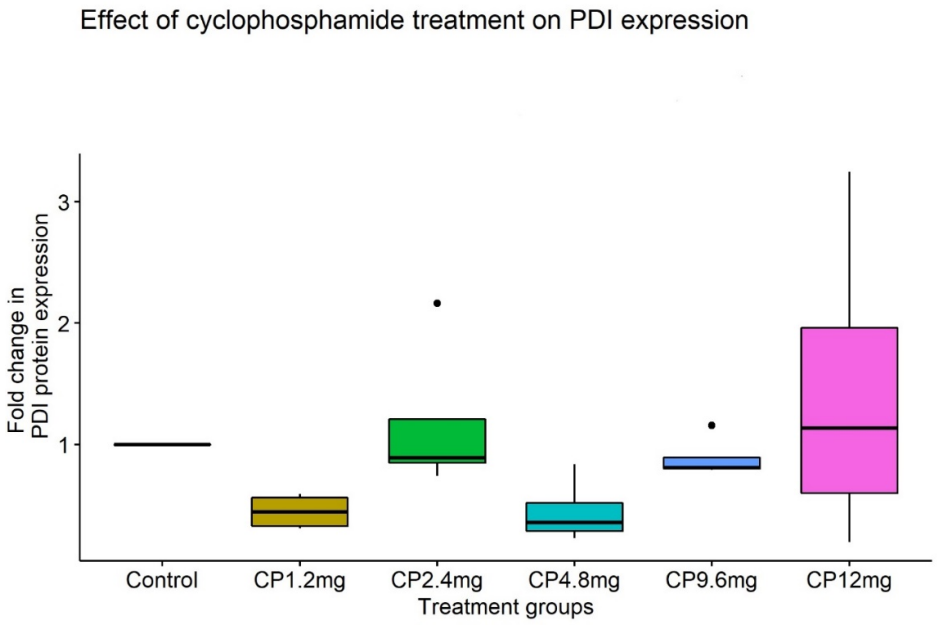
cyclophosphamide, developed liver injury by increasing the expression of endoplasmic reticulum stress-related genes [22]. Reduction of endoplasmic reticulum stress-related protein by inhibiting Brd4 resulted in apoptosis blockage and is considered a therapeutic approach to ischemia/reperfusion injury in the kidney [23]. Cyclophosphamide is shown to activate endoplasmic reticulum stress, and endoplasmic reticulum stress, in turn, can lead to the death of renal epithelial cells; we thought it would be interesting to see whether there is a link between cyclophosphamide usage and endoplasmic reticulum stress in the urothelium.

Recently endoplasmic reticulum stress inhibitors have been tried for their beneficial effect in various conditions. 4-Phenylbutyrate, a known endoplasmic reticulum stress inhibitor, alleviates intestinal injury by inhibiting ER stress-related proteins [24]. Neuronal apoptosis mediated by endoplasmic reticulum stress is one of the crucial factors in initiating neurodegenerative disease in diabetes. ER stress inhibitor 4-phenylbutyrate was protective against neurodegenerative disease by inhibiting endoplasmic reticulum stress in a mouse model [25].

The key to successfully using cyclophosphamide in various clinical scenarios is reducing its toxic effects. Hemorrhagic cystitis is one of the common complications that arise out of the usage of cyclophosphamide. This study showed that an increased dosage of cyclophosphamide causes increased cell death in Vero cells,



A. Western blot image for PDI protein in Vero cells treated with cyclophosphamide



B. Quantification of PDI bands in western blot

Figure 6. Expression of protein disulfide isomerase (PDI) protein in cyclophosphamide treated Vero cells

A) Western blotting image of PDI, with beta-actin used as a loading control, and B) Fold change in expression of PDI as compared to control and the difference in expression of PDI between the groups were analyzed by the Kruskal-Wallis test (P=0.0547). Quantification value expressed as median and interquartile range.

possibly by inducing endoplasmic reticulum-specific caspase-12. This caspase is present specifically in the endoplasmic reticulum, and its upregulation is shown to induce apoptosis in cells. GADD 153 (Chop) is a transcription factor expressed at low levels in normal and higher levels in conditions leading to endoplasmic reticulum stress [26]. We observed that the expression of GADD 153 protein was upregulated across treatment groups with different doses of cyclophosphamide, which could be one of the reasons for cell death. GADD 153, maintained at a low concentration within the cell, gets elevated when there is endoplasmic reticulum stress, which could promote cell death. GRPs help the cell deal with the endoplasmic reticulum's misfolded proteins. GRP78 decreased cell death in renal epithelial cells [27]. Expression of GRP78 was downregulated in Vero cells treated with higher doses of cyclophosphamide, thus losing its protective effect against cell death. GRP78, being a chaperone, is essential for the cell with endoplasmic reticulum stress to properly fold misfolded proteins. A decrease in GRP78 expression in Vero cells treated with higher doses of cyclophosphamide could contribute to increased death. Protein disulfide isomerase is located in the endoplasmic reticulum, which catalyzes the formation, breakage, and rearrangement of disulfide linkage, thereby playing an important role in the folding of proteins [28]. Protein disulfide isomerase was downregulated in Vero cells upon exposure to cyclophosphamide. Thus, the protective effect of GRP78 and PDI are lost in Vero cells treated with a high dose of cyclophosphamide, resulting in increased cell death. In summary, cyclophosphamide causes damage to urothelium probably by altering the endoplasmic stress-related proteins. Our study suggests that endoplasmic reticulum stress-related proteins could partly be responsible for causing damage to the urothelium. Our study does not establish a causative

role for endoplasmic reticulum stress-related proteins in cyclophosphamide-induced hemorrhagic cystitis. Further studies are warranted to explore the clinical utility of endoplasmic reticulum stress inhibitors in mitigating the side effects of cyclophosphamide intake.

5. Conclusion

Endoplasmic reticulum stress-related proteins are altered in Vero cells treated with cyclophosphamide. Upregulation of GADD 153 in cyclophosphamide-treated Vero cells could cause cell death in Vero cells. Proteins protecting against endoplasmic stress-related death and misfolding, such as GRP 78 and protein disulfide isomerase, are downregulated in Vero cells treated with cyclophosphamide.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Institutional Review Board (IRB) of Christian Medical College, Vellore, Tamil Nadu, India.

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Authors' contributions

Conceptualization and supervision: Premila Abraham; Methodology: Hemalatha R; Data collection: Hemalatha R and Muthuraman N; Data analysis:Muthuraman N and Sandya Rani; Writing original draft: Muthuraman N; Review and editing of draft: All authors; Fund acquisition and Resources: Premila Abraham.

Conflict of interest

The authors declared no conflict of interest.

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