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Lactoferrin Alleviated Cyclophosphamide-Induced Nephrotoxicity via Down-regulating TGF- β1/SMAD-3 Signaling Pathway

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Abstract: Cyclophosphamide (CP) is utilized for the treatment of distinctive human malignancies; however, its usefulness is complicated by the threat of developing nephrotoxicity. Oxidative stress is considered one of the main principal components for CP-induced nephrotoxicity. Lactoferrin (LF) has several pharmacological properties, comprising anti-inflammatory, antioxidant, and antiapoptotic activities The goal of the present study is to explore the possible protective effect of LF against CP-induced nephrotoxicity. To determine the LF protective properties, Sprague-Dawley rats underwent LF oral administration at 300 mg/kg per day for one week. Then, on the seventh day, animals were administered CP (150 mg/kg) through intraperitoneal injection. Treating CP-intoxicated rats with LF showed a manifested improvement in renal functions and reduced renal oxidative damage that was evidenced by the marked decrease in renal nitric oxide level with a concomitant increase in renal GSH content. Additionally, LF treatment markedly down-regulated TGF-β1/ SMAD-3 signaling pathway in CP-intoxicated animals. Kidney tissues were examined histologically to prove the renoprotective effect of LF. In conclusion, our findings specify that LF can be utilized effectively as a renoprotective agent in mitigating CP-induced nephrotoxicity.

Keywords: Cyclophosphamide; Lactoferrin; nephrotoxicity; GSH; TGF-β1; SMAD-3.

1. INTRODUCTION

Cyclophosphamide (CP) accounts for an alkylating and antineoplastic agent with potent cytotoxic effects and the ability to suppress the immune system. It is generally utilized in the management of malignant tumors, and autoimmune disorders [1-4]. CP is associated with various toxicities, including hemorrhagic cystitis, cardiotoxicity, pulmonary toxicity, and hepatotoxicity [4-9]. The main limiting reason for CP administration is its nephrotoxicity [10], which represents a main cause for drug withdrawal from the market.

Mechanisms of CP-induced nephrotoxicity are not entirely understood. However, oxidative stress represents one of the major pathogenic mechanisms by which CP induce nephrotoxicity [11]. The metabolic process of CP results in the formation of acrolein that adversely binds to reduced glutathione (GSH), leading to its depletion and finally ends with elevated reactive oxygen species (ROS) generation and, subsequently, initiation of oxidative tissue damaging[12]. As a response to oxidative stress, several signaling pathways are disrupted, including mothers against decapentaplegic homolog 3 (SMAD-3) and transforming growth factor-β1 (TGF-β1)[13], which activates the transcription of several profibrotic genes and mediating tissue fibrosis.

No effective measure has been yet discovered to protect against CP-induced nephrotoxicity. Several previous studies have indicated that antioxidants are efficient in alleviating many toxic adverse effects of anticancer drugs [14]. These facts drove our attention to the search for an antioxidant that could potentially protect against CP-induced nephrotoxicity.

Lactoferrin (LF) is a glycoprotein present in breast milk and other fluids [15]. It acts as a vital element in encouraging innate and adaptive immunity [15]. Additionally, LF possess numerous pharmacological characteristics like anti-inflammatory, anti-proliferative, and antioxidant potentials [16 -20]. Various experimental

and clinical studies have utilized LF in the treatment of different diseases [21]. Recently, preclinical studies have revealed the renoprotective activity of LF in contrast to kidney diseases [16,22].

Thus, our research aimed to report a deeper examination of the mechanisms underlying the renoprotective impact of lactoferrin in mitigating cyclophosphamide- induced nephrotoxicity including TGF-β1/ SMAD-3 signaling pathway.

2. METHODS

2.1. Animals

Male Sprague Dawley rats (180–220 g) were acquired from the animal facility at El-Nile for Pharmaceutical and Chemical Industries (Cairo, Egypt). Before the experiment, the rats underwent a one-week acclimation period. Throughout the study, the rats were kept within a room with adequate ventilation at a consistent temperature (26 °C ± 2 °C). Furthermore, the experiment upheld a regular light-dark cycle of 12 hours and maintained the humidity level according to the standard guidelines. The rats were offered water and standard rat food *ad libitum*. The experimental protocols adhered to the ethical considerations set by the Faculty of Pharmacy (Animal Ethics Committee), Al-Azhar University (Egypt), and were approved under the number 80/2021. All ethical aspects related to the handling of laboratory animals were duly taken into consideration.

2.2. Drugs and chemicals

Hygint Pharmaceuticals (located in Smouha, Alexandria) provided the lactoferrin used in the study. The National Cancer Institute (Cairo University, Egypt) graciously donated the cyclophosphamide (Endoxan vial, from Baxter Oncology GmbH, Germany). Before administration, the dry lyophilized form of CP powder underwent dissolution in sterilized water.

2.3. Experimental protocol

Rats were arbitrarily allocated in 4 groups, each with 6 rats. Control group: animals were orally administered normal saline for 7 following days, then the animals were injected intraperitoneally (i.p) with normal saline (0.25 ml/100 g) on the seventh day. In the second group, rats received oral LF (300 mg/kg/day) for a week [23,24]. Animals in the third group received saline for 6 days, and then a single CP dosage (150 mg/kg, i.p) [25] was given. The animals of the fourth group received oral LF (300 mg/kg/day) for six days, and then a single CP dosage (150 mg/kg, i.p) was given.

2.4. Animal sampling

Animal euthanasia was accomplished on day seven of the experiment through the Pentobarbital Sodium (60 mg/kg, I.P.)[26] administration. Blood samples were drawn via retro-orbital puncture; serum was isolated and used to quantify kidney functions. The cervical dislocation was performed to humanely sacrifice the rats, and subsequent collection of kidney tissues was carried out for the assessment of biochemical markers and histopathological examination of kidney tissue.

2.5. Serum biochemical analysis

Creatinine and blood urea nitrogen (BUN) concentrations (Catalog # UR 2110) were quantified according to the instructions provided by the commercial kits's manufacturer (Bio diagnostic Co).

2.6. Oxidative stress markers

Renal tissue homogenate was utilized to assess reduced glutathione (GSH) levels (Catalog # GR 25 11), following Beutler et al., 1963 [27]. The procedure includes reacting 5,5`dithiobis (2-nitrobenzoic acid) with GSH to produce a yellow product. The GSH concentration can be estimated by quantifying the reduced chromogen absorbance at a wavelength of 405 nm. The absorbance values obtained are directly correlated with the GSH concentration. To evaluate oxidative stress, nitric oxide (NO) **(**Catalog # NO 25 33) concentration was determined by measuring the total nitrite level, which serves as an indirect indicator of NO synthesis. This was accomplished using Griess reagent in an acidic environment, consisting of sulfanilamide and N1-naphthylethylenediamine dihydrochloride [28].

2.7. Enzyme linked immunosorbent assay (ELISA)

Tissue levels of TGF-β1 (Catalog #: MBS824788) and Smad3 (Catalog # MBS2516123) (MyBioSource, Inc., SanDiego, USA) were quantified through rat ELISA kits based on the guidelines provided by the manufacturer.

2.8. Histological analysis

Kidneys were put in buffered formalin (10%) and implanted within paraffin; histological preparations were prepared at that point. Tissues of the kidney were divided to 4.0 μm thick sections. The prepared formalin-fixed paraffin-implanted pieces underwent Masson's Trichrome (MTC) staining for histochemical analysis of the collagen content consistent with the method described by Bancroft and Layton[13,22]. The staining results were blue/black stain for the nuclei, red stain for the erythrocytes, muscle, and cytoplasm, and blue stain

for the collagen. The area stained blue (μm^2) was measured in five repeated fields (200 X), and the fibrotic area percentage was computed relying on the total examined area. Quantitative analysis of the collagen content in MTC-stained kidney tissue sections was completed using imaging analysis software (Image J).

2.9. Statistical analysis

Data analysis was displayed as per means ± SD, and the analysis was completed with GraphPad Prism (ISI®, USA) software (version 5). One-way analysis of variance (ANOVA) was followed by a post hoc Tukey's multiple comparison test. Comparison of means was made. $P \leq 0.05$ was considered as of statistical significance.

3. RESULTS

3.1 LF effect on CP-induced macroscopic changes, BUN, and serum creatinine within rats

As shown in Table 1; CP administration considerably increased the kidney's relative weight, approximately 147%, compared with the control group.

Pretreatment with LF markedly reduced relative kidney weight to 80% compared to the CP group. Upon assessing the effect of CP on renal function, it was found that CP induced marked renal damage, proved by the significantly increased BUN and serum creatinine (SCr) to 184% and 1017.44%, respectively, compared to the control rates. Alternatively, LF administration along with CP exhibited a noticeable decline in these biomarkers to 68% and 30%, respectively, compared to the CP group.

3.2. Impact of LF treatment on CP-induced changes in renal content of nitric oxide (NO) and reduced glutathione (GSH) in rats.

Administration of CP markedly elevated renal contents of NO to 138 % and, at the same time, reduced GSH levels in kidney tissues to 22% compared to the control rates. Meanwhile, LF administration to CP-intoxicated rats substantially decrease renal NO (84.5 %) and markedly elevated GSH content (352%) compared to the CP group (Figure 1 A&B) respectively.

Table 1. Impact of LF on CP-induced macroscopic changes, blood urea nitrogen (BUN), and serum creatinine (SCr) in rats.

Parameter / Group	kidney weight/Body weight (%)	BUN (mg/dl)	SCr (mg/dl)
Control	0.34± 0.023	48.84± 2.378	0.1720± 0.04147
LF	0.31± 0.018	38.98± 0.3962	0.1660± 0.03362
CP	0.5± 0.036*	89.88± 7.588 *	1.750± 0.2828*
LF + CP	0.4± 0.0158$^{*\#}$	61.20± 5.09$^{*\#}$	0.5240± 0.1524$^{*\#}$

Data are expressed as the mean ± SD (n=6) rats per group. ANOVA followed by post hoc Tukey's multiple comparison Test were employed for the statistical analysis. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus CP group, respectively. **LF: l**actoferrin - **CP**: cyclophosphamide. - **LF + CP:** lactoferrin+ cyclophosphamide.

3.3. LF treatment down-regulated renal TGF-β1/ SMAD-3 signaling pathway in CP-intoxicated rats.

TGF-β1 is an important moderator in the pathogenesis of tissue fibrosis [29] that activates the downstream mediator SMAD-3, which induces matrix production; as shown in Figure 2, renal fibrosis was induced by CP administration that was confirmed by the significant elevation in renal fibrotic markers TGF-β1 and SMAD-3 up to 562% and 620%, respectively, compared to the control rats. In comparison, LF-pretreated rats showed a considerable decline in renal TGF-β1 and SMAD-3 contents to 39% and 59%, respectively, compared to the CP group (Figure 2 A&B) respectively.

3.4. Histopathological examination

The extent of fibrosis was followed via using MTC staining, which indicates rise in collagen I level during fibrosis. Examination of the control group and LF group exhibited normal amount of fibrous tissue stained by MTC that was mainly detected on the glomerulus capillary tuft. Meanwhile, Masson staining showed abundant collagen deposition (blue areas) in kidney sections from CP group (2015.12%) compared to control rats, which was mainly observed in the interstitial tissue of the renal parenchyma. Pretreatment with LF significantly decreased collagen deposition (73.3%) as compared to CP group. While, LF group showed little blue area with collagen fibers that was

statistically insignificant upon comparison with that of control group (Figure 3 a-d).

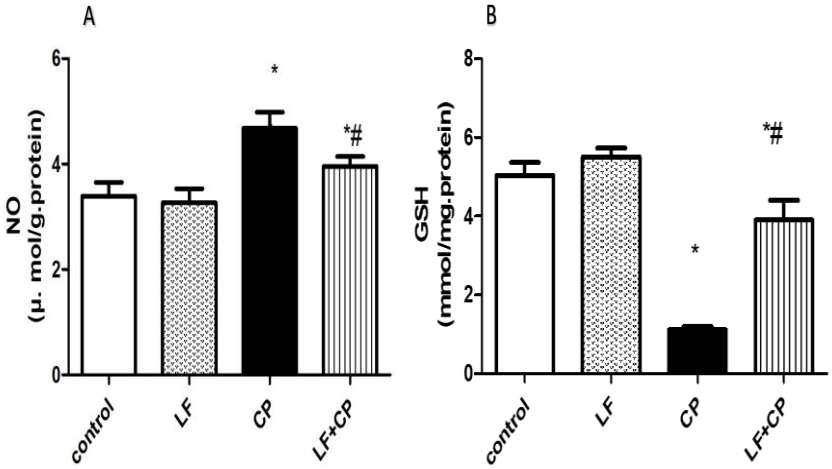

Figure 1. Impact of LF administration on CP-induced oxidative kidney damage in rats. (A): Renal nitric oxide (NO) level; (B): Renal reduced glutathione (GSH) level. Data are expressed as the mean ± SD (n=6) rats per group. ANOVA followed by post hoc Tukey's multiple comparison Test were employed for the statistical analysis. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus CP group, respectively. LF: lactoferrin - CP: cyclophosphamide. - LF + CP: lactoferrin+ cyclophosphamide.

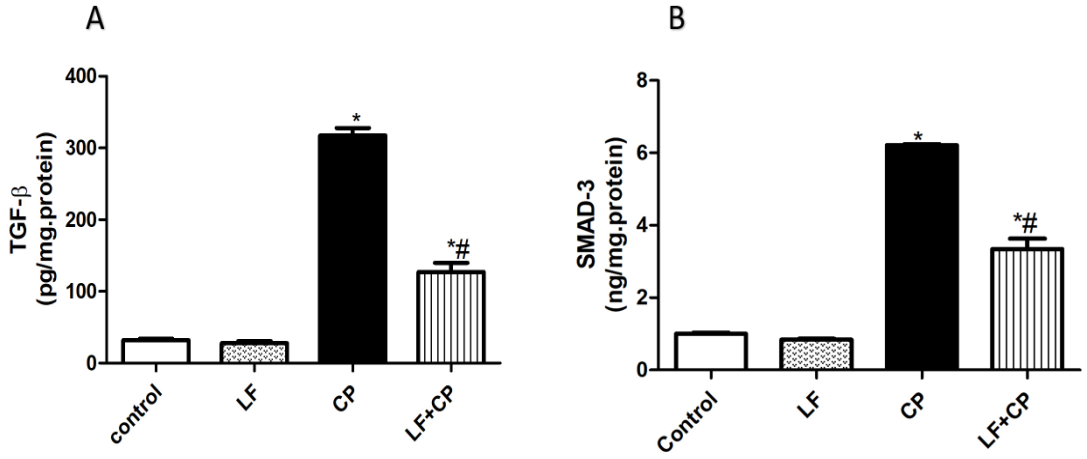

Figure 2. Impact of LF treatment on renal TGF-β1/SMAD-3 signaling pathway in CP-induced nephrotoxicity. (A): Renal transforming growth factor-β1 (TGF-β1); (B): Renal mothers against decapentaplegic homolog 3 (SMAD-3) level. Data are expressed as the mean ± SD (n=6) rats per group. ANOVA followed by post hoc Tukey's multiple comparison Test were employed for the statistical analysis. $^{*}P < 0.05$ versus the control, $^{\#}P < 0.05$ versus the CP group, respectively. LF: lactoferrin - CP: cyclophosphamide. - LF + CP: lactoferrin+ cyclophosphamide.

4. DISCUSSION

Our objective in this study is to target the devastating renal toxicity of CP. We clarified the previously unexplored protective impact of LF on kidney in contrast to CP-made nephrotoxicity via inhibiting oxidative renal damage alongside down regulating TGF-β/ SMAD-3 signaling pathway.

Acroline, the main toxic metabolite of CP, is the key player in its nephrotoxic mechanism. It initiates oxidative stress and exaggerates the production of ROS[30]. The originated ROS interrupts and initiates several signaling molecules and signaling pathways that contribute in the induction of renal damage [31]. protection against CP-induced nephrotoxicity was given by modulating these signaling cascades. Our findings are consistent with the data mentioned before, wherein CP administration elevated renal NO level (as a marker for oxidative stress) and resulted in a significant reduction in renal GSH [32]. The elevation in the levels of these markers in kidney tissue was confirmed with the apparent cellular injury shown from changed renal function.

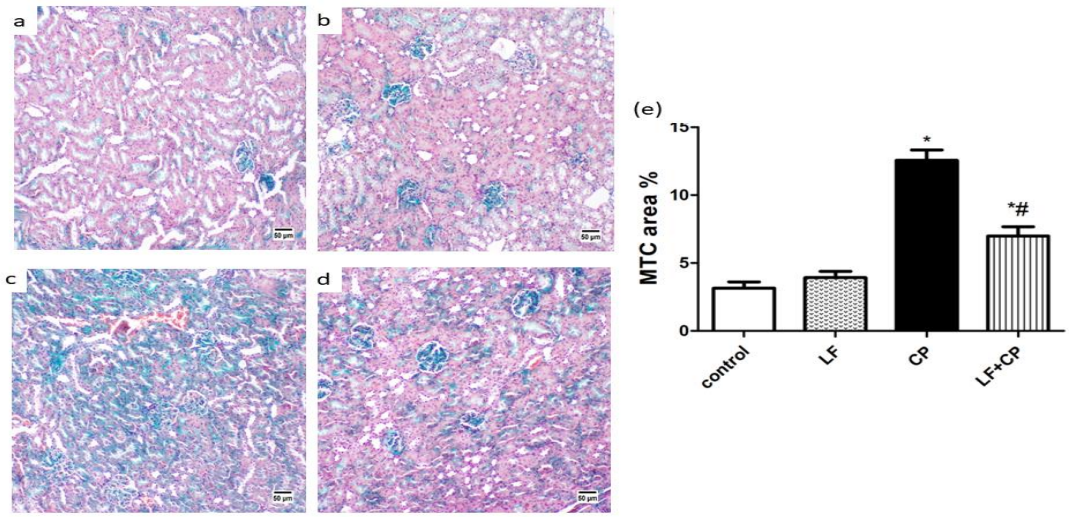

Figure 3. Representative photo-micrographs of rat kidney sections after staining with Masson's trichrome. (a): Control group displaying collagen fibers with a weak histochemical reaction, indicative of normal conditions; (b): LF group displaying collagen fibers with a weak histochemical reaction, indicative of normal conditions; (c): CP group revealing extensive fibrosis as shown from the collagen fibers, which reveal strong histochemical reaction, in the interstitial tissue of the renal parenchyma.; (d): LF+ CP group displayed moderate histochemical reaction for collagen fibers [200 X, scale bar 50µm]; and (e) Fibrotic area % in kidney tissue following treatment with CP. Data are expressed as the mean ± SD (n=6) rats per group. ANOVA followed by post hoc Tukey's multiple comparison Test were employed for the statistical analysis. $^{*}P <$ 0.05 versus the control, $^{\#}P < 0.05$ versus the CP group, respectively. LF: lactoferrin - CP: cyclophosphamide. - LF + CP: lactoferrin+ cyclophosphamide.

LF administration increased the antioxidant level (GSH) and markedly reduced renal NO level [33]. The nephroprotective impact of LF against CP-induced nephrotoxicity was accompanied by modulation in functional (BUN and SCr) improvements. These findings align with the acknowledged antioxidant and ROS scavenging activity [34] of LF in different experimental models.

It was found that TGF-β1 is highly produced through inflammatory cells, comprising eosinophils and neutrophils, and epithelium cells, fibroblasts, and smooth muscle cells [35-37]. The extreme production of TGF-β1 can encourage fibrosis to many diseased tissues such as liver, pulmonary and kidney fibrosis [36]. ROS generated by acrolein stimulates the production of TGF-β1 within the kidney. This oxidative stress could change the redox stability in renal tissues and activate latent TGF-β1[38]. Stimulation of TGF-β1/ SMAD-3 signaling pathway is a hallmark for tissue fibrosis progression. As TGF-β1/ SMAD-3 which motivates myofibroblast forming proliferation and plays a significant role in collagen synthesis and progression of tissue fibrosis [39]. The SMAD family, especially SMAD-3, facilitates responses of cells to TGF- β1 after that leads to activation of α-SMA gene expression. Finally, it causes collagen deposition[40]. TGF-β1 phosphorylates SMAD-3, promoting translocation to the nucleus, where it orchestrates the transcriptional regulation of several target genes [41]. These biochemical findings were supported by fibrosis-related pathological changes and collagen deposition shown from MTC staining of kidney tissue from CP-intoxicated rats.

Treating intoxicated animals with LF significantly reduced renal TGF-β1 and SMAD-3 levels. These findings were corroborated by the histopathological outcomes of the MTC staining, which documented that the extensive collagen fiber deposition in kidney tissues of CP-intoxicated rats was significantly amended by LF treatment. Our data are consistent with earlier recognized anti-fibrotic action of LF [42,43]. This antifibrotic impact may partly correlate to its suppressive action on TGF-β1 [43, 44].

5. CONCLUSION

Our study indicated that lactoferrin enhanced kidney damage produced by CP oxidative stress conceivably by means of its antioxidant action and down-regulating TGF-β1/ SMAD-3 signaling pathway.

Supplementary Materials:

Funding: This research did not have any funding from any source.

Conflicts of Interest: The authors declare no conflict of interest.

Ethical Statement: This study was done according to the Ethics Committee of the faculty of Pharmacy Al-Azhar University, Egypt with approval number: 80/ 2021. Unnecessary disturbance of animals, pressure and tough maneuver was avoided.

Author Contribution: All authors collaborated in each part of this research.

List of Abbreviations: CP, Cyclophosphamide; LF, Lactoferrin; GSH, reduced glutathione; NO, nitric oxide; TGF-β1, transforming growth factor-β1; SMAD-3, mothers against decapentaplegic homolog 3; i.p; Intra peritoneal; ELISA, Enzyme linked immunosorbent assay; SCr, serum creatinine; BUN, blood urea nitrogen; MTC, Masson's Trichrome staining.

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