Original Article

Ameliorative Potential of Lactoferrin on Methotrexate-Induced Nephrotoxicity in Rat: A Histological and Immunohistochemical Study

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ABSTRACT

Introduction: Methotrexate (MTX) is a commonly used chemotherapeutic and immunosuppressant drug. Nevertheless, due to its numerous side effects, clinical use is restricted. Lactoferrin (Lf) a natural milk glycoprotein with anti-viral, anti-inflammatory, and antioxidant characteristics are among the various pharmacological effects.

Aim of the Study: Assessment of the reno-protective potential of Lf in experimental MTX-induced nephrotoxicity and renal damage.

Material and Methods: A total of twenty-eight adult male albino rats were allocated into four groups: control group, Lf treated group received Lf (300mg/kg/day) orally for 2 weeks; MTX treated group received MTX by intraperitoneal route (20mg/kg) once; and Lf +MTX treated group. Kidney functions, redox status, interleukin (IL)-1β level and tumor necrosis factor (TNF)-α expression were assessed. Moreover, histological characteristics were assessed by light and electron microscopic study.

Results: MTX nephrotoxicity was evidenced by significantly impaired renal functions, increased renal malodialdehyde (MDA) level, IL-1β level, and TNF-α protein expression along with significant decreases in renal GSH and antioxidant enzymes activities. The biochemical results were confirmed by histopathology results that showed disturbed architecture, glomerular atrophy and congestion, damaged tubular cells, interstitial bleeding and inflammatory infiltration with significant increase in collagen fibers and decreased PAS staining. Ultrastructure study revealed; degenerated mitochondria, cytoplasmic vacuoles, and basement membrane damage in renal tubules. Additionally, renal filtration barrier was disrupted. Meanwhile, Lf treatment improved renal function, redox state, inflammation, histology and ultrastructural characteristics.

Conclusion: Lf has the potential to protect the kidneys against MTX-induced nephrotoxicity and cytotoxicity. Results herein suggested that Lf, as an adjuvant therapy, may be valuable in preventing the occurrence of renal damage in patients on MTX or other reno-toxic drugs.

Received: 21 June 2021, **Accepted:** 30 September 2021

Key Words: Lactoferrin (Lf); methotrexate (MTX); renal injury; tumor necrosis factor (TNF)-α; redox status.

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ISSN: 1110-0559, Vol. 46, No.1

INTRODUCTION

As kidney is involved in drug concentration and excretion, it is considered the common site of drug toxicity. Owing to this role, drug-induced kidney injury and toxicity is a rapidly growing health concern with socioeconomic consequences. The key underlying mechanisms are oxidative damage and inflammation[1]. Methotrexate (MTX) a chemotherapeutic medication inhibits dihydrofolate reductase enzyme which is important for synthesis of activated folate, DNA replication and cell division. MTX is extensively utilized in the treatment of cancer, autoimmune and inflammatory diseases with an effective therapeutic power[2]. The use of MTX has significant side effects as nephrotoxicity, hepatotoxicity and bone marrow suppression, which often limits its therapeutic use. The kidney represents the main excretory organ for MTX, consequently nephrotoxicity is the most concerning issues that limits its therapeutic utility. Severity of nephrotoxicity is determined by the dose and frequency of its usage. In an experimental model, MTX-induced renal injury can be induced by single dose[3,4]. In both low and high doses, MTX and its 7-hydroxy metabolite cause nephrotoxicity via precipitation in the renal tubules, inducing tubular necrosis and lowering the glomerular filtration rate. Although the exact mechanism of MTX-induced renal injury is unknown, mounting evidence has implicated mitochondrial malfunction, oxidative stress, inflammation and apoptosis as possible underlying mechanisms in MTX-induced nephrotoxicity[1,2].

Oxidative stress is the imbalance between oxidant and antioxidant defense mechanisms causes tissue lipid, protein, nucleic acid and cell membrane damage. Moreover, reactive oxygen species (ROSs) activates nuclear factor-kappa B (NF-κB), a master regulator of oxidative stress and inflammation, resulting in the production of pro-inflammatory cytokines as tumor necrosis factor (TNF)-α

 DOI: 10.21608/ejh.2021.80881.1507

and interleukin (IL) 1 β, which may play a crucial role in the development and progression of acute and chronic kidney injury, as well as in monitoring the severity of inflammation during renal damage[1,5]. Preceding reports have shown that natural compounds with antioxidant properties could mitigate the oxidative damage caused by MTX without compromising its anticancer action. Lactoferrin (Lf) is an 80-kD iron-binding glycoprotein present in milk, external secretions as saliva and tears and granules of neutrophils[6].

Several investigators have revealed that Lf has many implications as being anti-inflammatory, anti-oxidant, anti-cancer and immune-regulatory agent. Lf was found to be expressed in renal tissues, demonstrating that it may have an important role in shielding the kidney from oxidative damage[7]. Lf has also been reported to diminish hypoxia-induced lung and kidney injury by declining the production of ROSs and regulating the inflammatory response[8]. However, the potential role of Lf in MTX-induced nephrotoxicity has not studied yet. Hence, the current study marveled to investigate the effects of Lf in MTX-induced kidney injury through exploring its anti-inflammatory, antioxidant and anti-cytotoxic potentials.

MATERIAL AND METHODS

MTX was provided by Maylan company in the form of vials (2ml each ml contains 50 mg). Lf (Pravotin) was acquired in the form of sachets, each sachet containing 100 mg, from hygint pharmaceuticals, Egypt.

Animals

Twenty-four adult male Wister albino rats, weighting 150-180 g, and aged twelve weeks were used. The animals were placed in clean well ventilated cages, under conventional laboratory settings and allowed 2 weeks of adaptation before the beginning of the experiment. The study was done in compliance with the National Institutes of Health's guidelines for the care and use of laboratory animals and accordance to Ethical Committee of Medical Research, Faculty of Medicine, Tanta University' guiding principles (approved code: 34706).

The experimental design

The animals were allocated into equal four groups. **Group I (control):** injected with normal saline (vehicle) intraperotineally (i.p.) (0.5 ml /rat once in the 1st day then administrated orally via oral gavage with 1ml distilled water daily for 14 days. Group II (Lf group): received Lf in a dose of 300 mg/kg /day once daily by oral gavage for 14 days[9].

Group III (MTX or nephrotoxic group): injected i.p. with a single dose of MTX (20 mg/ kg) at the first day of the experiment[10]. Group IV (MTX + Lf group): injected i.p. with a single dose of MTX ((20 mg/ kg) at the first day of the experiment then they were orally administered Lf (300 mg/kg) daily for 14 days. At the end of the experiment, the weights of rats in each group were assessed to estimate the final body weights. Additionally, relative kidney weight was estimated (organ ratio (%) = organ weight (gm) × 100/ body weight (gm)).

Blood and tissue sampling

Rats were anaesthetized with ether and then killed, at the end of the experiment. Blood was collected for serum separation then stored until utilized for the study of kidney function. Kidneys were removed, rinsed in ice cold saline then chilled on ice before being cut into pieces. Left kidney from each rat was homogenized at 4°C in lysis buffer. Homogenate was centrifuged at 11000 g for 20 minutes at 4°C, and supernatant was kept at −80°C till utilized for biochemical study. Estimation of Protein level was done as previously reported[11].

Biochemical assessment of renal' impairment, inflammation and redox state

Serum urea and creatinine as a measure for renal impairment were assessed using commercial kit (Diamond Diagnostic, Egypt). Renal malondialdehyde (MDA) level a marker of lipid peroxidation; glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities and reduced glutathione (GSH) level as a measure of the anti-oxidant defense were estimated by commercial kit (Biodiagnostic, Inc., Egypt). Renal IL-1β level was assessed by ELISA (Sunred Biological Technology Co., Ltd. Shanghai, China) in line with the manufacturer' instructions by ELISA Reader (Star fax 2001).

Histological and Immunohistochemical studies

The right kidney from each rat was cut longitudinally and processed for light and transmission electron microscopic studies.

Light microscopic study

For histological study, Specimens were kept in 10% of neutral buffered formalin then were processed and embedded in paraffin wax and serial sections of 4-6 µm thickness were cut and stained with Hematoxylin and eosin (H&E) stain, Masson's trichrome stain and Periodic acid Schiff reaction (PAS)[12].

For immunohistochemical staining, briefly, 4µm renal sections were de-paraffinized and handled for immunohistochemical staining as described before by Ramos-Vara *et al*., 2008[13]. In this study, the diluted primary Anti-TNF-α antibody (ab6671 Abcam, Massachusetts, USA) was used to evaluate the inflammatory reactivity. Positive TNF-α –immunostaining was revealed by the presence of brown cytoplasmic staining.

Transmission electron microscopic study

Renal specimens were sliced and fixed in phosphate buffered glutaraldehyde and ultrathin sections were obtained for ultrastructure analysis as described previously[14] using JEM-1400plus JEOL at the lab of EM in the Faculty of Science, Alexandria University

Morphometric analysis

Ten separate non-overlapping fields in each slide were chosen randomly and were examined using the computer system for image analysis (Leica Qwin 500 C Image analyzer computer system; Leica Imaging System LTD., Cambridge, England), at magnification power of 400. Histopatholgical scoring to renal damage was done in line with Colbay *et al*., 2010; the intensity of the renal damage was scored according to dilatation of Bowman capsule, a focal glomerular necrosis, damage of tubular epithelial, inflammatory interstitial infiltration, and congestion. The score used is as follow: no damage = 0, damage <25% = 1, damage from 25 to 50% = 2 and damage > 50% =3[15]. Moreover, mean area % of masson trichrome stained sections, mean area % of PAS stained sections, mean area % and mean color intensity of TNF-α immunohistochemical reaction were assessed.

Statistical analysis

Results were statistically analyzed as mean ± standard deviation by using the statistical package for social sciences (SPSS) version 21.0 (IBM Corp., Armonk, NY, USA). For multiple comparisons, one-way analysis of variance (ANOVA) was used, followed by post-hoc test to test the statistical significance between experimental groups. Pearson test was used to examine correlations. *P value* less than 0.05 was considered significant.

RESULTS

Effect of MTX and Lf on body, kidney, and relative kidney weighs and renal toxicity indices

Animals treated with MTX (group III) revealed significant (P<0.05) decrease in body and kidney weighs and relative kidney weight as comparison to control group. Lf, on the other hand, guarded against the loss in body, kidney and relative kidney weighs in Lf+MTX group (group IV) in comparison with group III (Table 1). Furthermore, MTX treatment (group III) significantly raised serum urea and creatinine levels (p<0.05) in comparison with controls. Nevertheless, Lf significantly (P<0.05) improved renal function in Lf +MTX group (group IV) in comparison with group III. Additionally, non-significant (P>0. 05) difference in body, kidney, and relative kidney weighs and renal toxicity indices were found in group IV in comparison with controls (Table 1).

Effect of MTX and Lf on redox and inflammatory status

Intoxication with MTX (group III) caused impaired redox and inflammatory states as evidenced by significant (p<0.05) reduction in the antioxidant enzymes, GSH-PX and SOD, activity and GSH level. On the other hand, MDA and IL-1β levels were significantly raised in comparison with controls. Meanwhile, Lf improved redox state as evidenced by significant increase in the antioxidant enzymes activity, and GSH level, however, MDA and IL-1β levels were significantly reduced in MTX+ Lf group (group IV) in comparison with group III (Table 2).

Histological results

Light microscopic results

Results from Hematoxylin and Eosin stained sections

Hematoxylin and Eosin (H&E) stained sections of renal cortex in control and Lf treated groups (group I, II respectively) revealed that renal corpuscles, proximal and distal convoluted tubules all had normal histology. The renal corpuscles were made up of glomerular capillaries enclosed by Bowman's capsule made up of outer parietal and inner visceral layers separated by a clear Bowman's space. The proximal convoluted tubules with simple high cuboidal epithelium and the distal convoluted tubules with low cuboidal epithelial lining (Figure 1 A,B) were seen. In MTX group (group III), the renal cortex features were disturbed, renal corpuscles revealed congested glomerular capillaries and atrophy of the glomerulus with widening of the Bowman's space, lining epithelium of tubules showed degenerated cells and vacuoles with pyknotic nuclei, casts and detached epithelial cells in lumen of some tubules, and hemorrhage with mononuclear infiltration in the interstitial tissue (Figure 1 C). On the other hand, there was noticeable preservation of the normal renal tissue, renal corpuscles, proximal convoluted tubules and distal convoluted tubules except for few cells with pyknotic nuclei and degenerated vacuoles were still seen in MTX &Lf treated group (group IV), (Figure 1 D). The histopathological scoring of the damage of renal cortex was recorded in (Table 3).

Results from Masson Trichrome stained sections

The amounts of collagen fibers around renal tubules and in the glomerulus were minimal in Masson trichrome stained sections of renal cortex in control and Lf treated groups (group I, II respectively) (Figure 2 A,B). MTX treatment in group III revealed that the amount of collagen fibers was increased especially in the interstitial tissue (Figure 2 C), however, Lf treatment decreased the amount of collagen fibers in MTX+Lf treated group (group IV) (Figure 2 D). Mean area % of Masson trichrome stained sections from MTX treated rats (group III) revealed significant (P<0.05) increase in comparison with controls. While, Lf treatment showed significant (P<0.05) decrease in MTX & Lf treated group (group IV) as compared with group III (Table 3).

Results from peroidic acid schiff (PAS) stained sections

PAS stained sections of renal cortex featured a strong PAS-positive reaction in the apical brush borders of the proximal convoluted tubules, in basement membrane of the renal tubules, glomerular capillaries and parietal layer of Bowman's capsule in control groups (group I, II respectively) (Figure 3A,B). In MTX group (group III), the apical brush borders of the proximal convoluted tubules, basement membrane of the renal tubules and glomerular capillaries, and in parietal layer of Bowman's capsule were partially disrupted (Figure 3 C). On the other hand, in MTX

&Lf treated group (group IV) the apical brush borders of the most proximal convoluted tubules appeared intact, basement membrane of many tubules appeared regular, glomerular capillaries and parietal layer of Bowman′s capsule could be noticed (Figure 3 D). Morphometric measurements of the mean area percentage of PAS stained sections exhibited significant (P<0.05) increase in MTX treated rats (group III) in comparison with controls (group I& II), however, a significant (P<0.05) decrease was revealed in MTX & Lf treated group (group IV). On the other hand, group IV revealed non-significant (P>0.05) difference in comparison with controls (Table 3).

Results of TNF-α immunohistochemical stained sections

Immun-stained sections from renal cortex of group I, II (control and Lf groups respectively) revealed weak positive cytoplasmic immunoreaction for TNF-α (Figure 4 A,B), while, sections from group III (MTX group) displayed strong positive Immunoreaction in both tubular and glomerular cells expressed by brownish coloration (Figure 4 C). On the other hand, treatment of rats with MTX & Lf in group IV depicted slight cytoplasmic TNF-α immunoreaction in some tubular epithelial cells and in glomeruli (Figure 4 D). Morphometric analysis of the mean color intensity and area % of TNFα immunoreactivity exhibited significant (P<0.05) expression of TNF-α in rat renal cortex in group III in comparison with controls. Nevertheless, MTX & Lf treated group (IV) revealed significant (P<0.05) decrease in TNF-α expression in comparison with MTX only treated group (Table 3).

Results of Transmission Electron Microscopic study

Ultra-thin sections of renal cortices depicted that cells of the proximal convoluted tubules lining epithelium exhibited oval central euchromatic nucleus, characteristic long apical microvilli, elongated basal mitochondria, basal infoldings, regular basement membrane, and multiple electron dense lysosomes (Figure 5 A,B) in control groups (group I, II). However, In MTX treated group (group III), the luminal membrane was destructed with partial loss of apical microvilli, many cytoplasmic degenerated vacuoles, disorganized shaped of mitochondria with some were ruptured and others are fused, basally located nucleus, loss of basal infoldings and multiple lysosomes could be noticed (Figure 5 C). Nevertheless, in MTX &Lf treated group (group IV) the proximal convoluted tubules epithelial lining showed more or less normal structure in the form of normal long apical microvilli, basal elongated mitochondria, basal infoldings and lysosomes except for a few degenerated vacuoles that were still seen (Figure 5 D).

Ultra-thin sections of renal cortices depicted that the cells of the distal convoluted tubules lining epithelium in control groups (group I, II) revealed large oval apical euchromatic nucleus, characteristic basal vertically oriented mitochondria with basal infoldings in-between as well as the cells were rested on well-developed thick regular basement membrane (Figure 6 A,B). While, in MTX treated group (group III), the lining epithelium of the distal convoluted tubules revealed small central dark nucleus, cytoplasm revealed degenerated vacuoles, disrupted mitochondria, basal infoldings and basement membrane was split (Figure 6 C). On the other hand, sections from MTX &Lf treated group (group IV) showed near normal ultra-structure of the epithelial lining of the distal convoluted tubules, the nucleus appeared large and oval, The cytoplasm showed basal infoldings with vertically oriented mitochondria, and the basement membrane had a consistent thickness. However, only a few vacuoles were found in the cytoplasm. (Figure 6 D).

Ultra-thin sections of renal cortices depicted that filtration barrier formed of arranged three layers from outside inwards; foot processes of podocyte, glomerular basement membrane and fenestrated capillary endothelium. In control groups (group I, II), the filtration barrier displayed regular, uniform foot processes and basement membrane, and fenestrated capillary endothelium (Figure 7 A,B). While, in sections from MTX treated group (group III), the filtration barrier was disturbed, some podocyte's foot processes were fused together and some were detached, irregular capillary fenestrated endothelium and glomerular basement membrane where some areas were thin and others were very thick. (Figure 7 C). On the other hand, in sections from MTX &Lf treated group (group IV) the filtration barrier was more or less similar to the control, and the glomerular basement membrane and the capillary fenestrated endothelium were regular except few foot processes were still fused together (Figure 7 D).

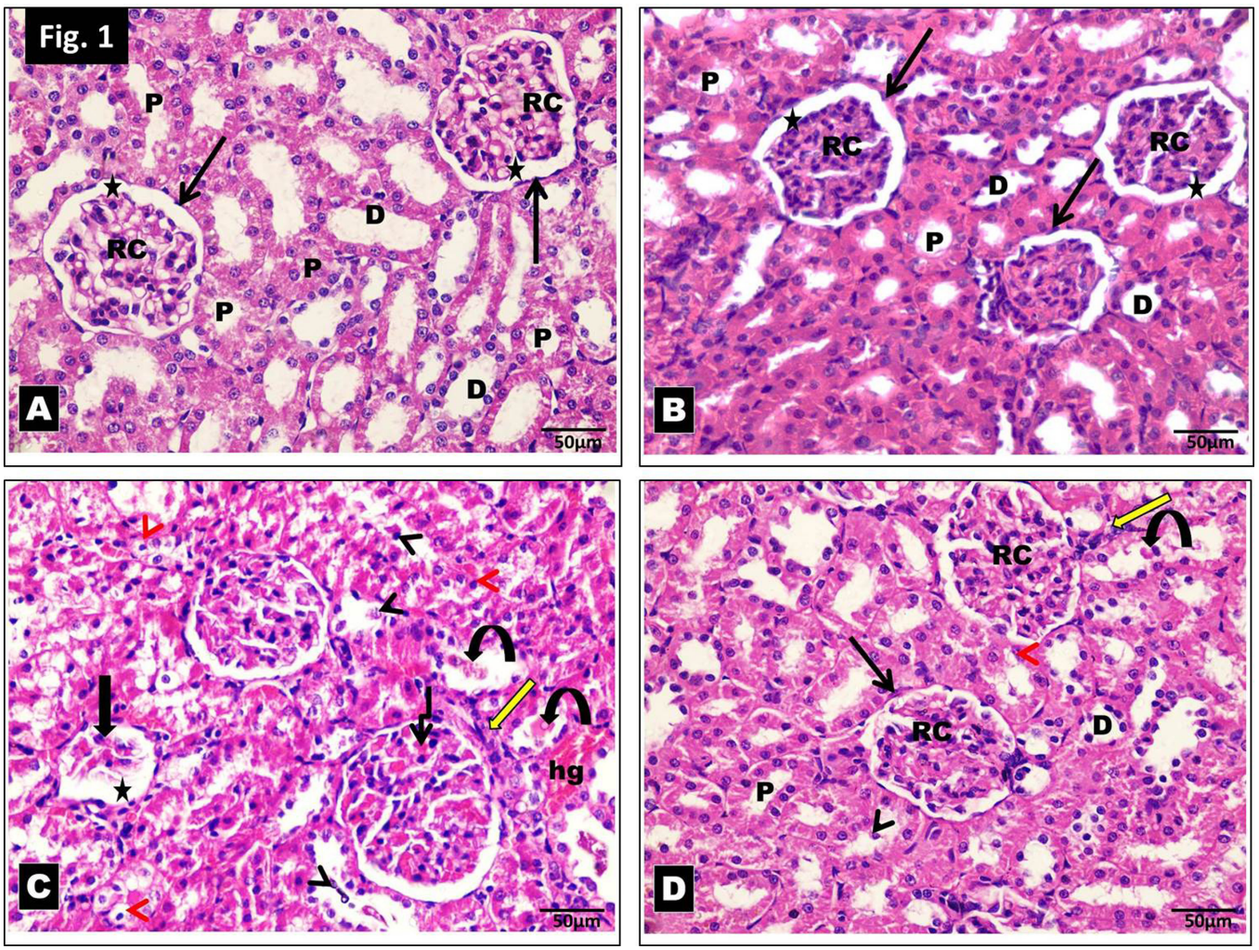

Fig. 1: Photomicrographs of Hematoxylin and Eosin stained sections of the studied groups' renal cortex; A & B) In group I & II (control & Lf group respectively) showing renal corpuscle (RS) is made up of glomerular capillaries that are surrounded by a two-layered Bowman's capsule (arrows) with an obvious clear Bowman's space (star) in between. Simple high cuboidal epithelium lines the proximal convoluted tubules (P). Low cuboidal epithelium lines the distal convoluted tubules (D). C) In group III (MTX group) displaying a distorted renal cortex. The renal corpuscles show congested glomerular capillaries (zigzag arrow) and one of them appears shrunken (thick arrow) surrounded by wide Bowman's space (star). The renal tubular epithelial lining shows dark stained pknotic nuclei and degenerated cytoplasmic vacuoles (red arrow heads). Some of these cells become detached (black arrow heads) and appear in the renal tubular lumina. There are interstitial haemorrhage (hg), an inflammatory infiltrate (yellow arrow) and acidophilic casts (curved arrows) inside some tubular lumina. D) In group IV (MTX & Lf treated group) showing a normal renal cortex with a normal renal corpuscle (RS) surrounded by Bowman's capsule (arrow) and normal proximal (P) and distal (D) convoluted tubules. There are only a few tubular cells with vacuolated cytoplasm (red arrow head), intraluminal detached cellular debris (black arrow head), an intraluminal cast (curved arrow), and an interstitial inflammatory infiltrate (yellow arrow). Magnifications: x400.

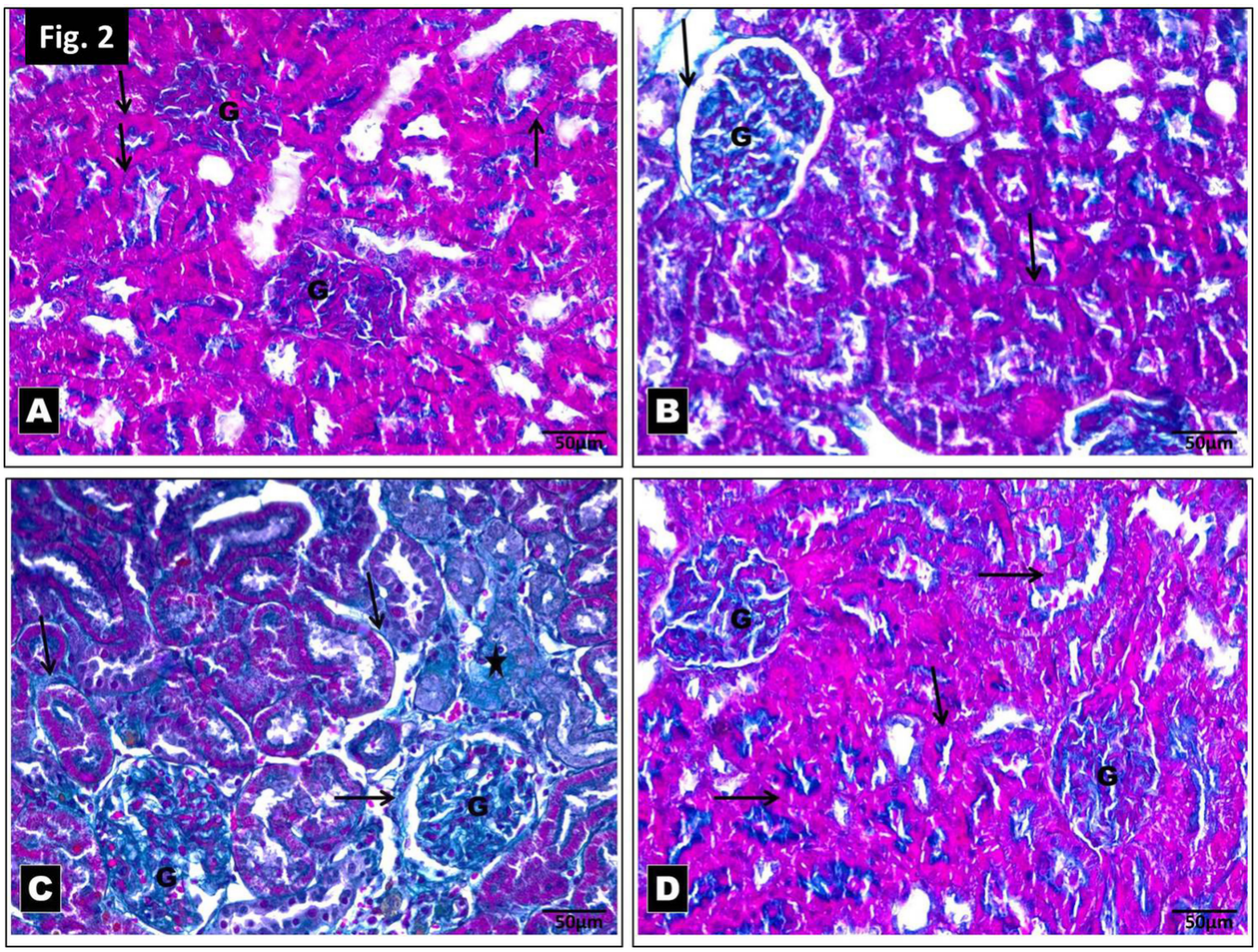

Fig. 2: Photomicrographs of Masson trichrome stained sections of the studied groups' renal cortex; A & B) In group I & II (control & Lf group respectively) demonstrating minimal collagen fibres deposition (arrows) around the tubules and between the glomerular capillaries (G). C) In group III (MTX group) demonstrating massive collagen fibres deposition (arrows) around tubules and between glomerular capillaries (G). There is significant interstitial deposition of collagen fibres (star). D) In group IV (MTX & Lf treated group) showing few collagen fibres (arrows) around the tubules, but there are moderate collagen fibres in between the glomerular capillaries (G).Magnifications: x400.

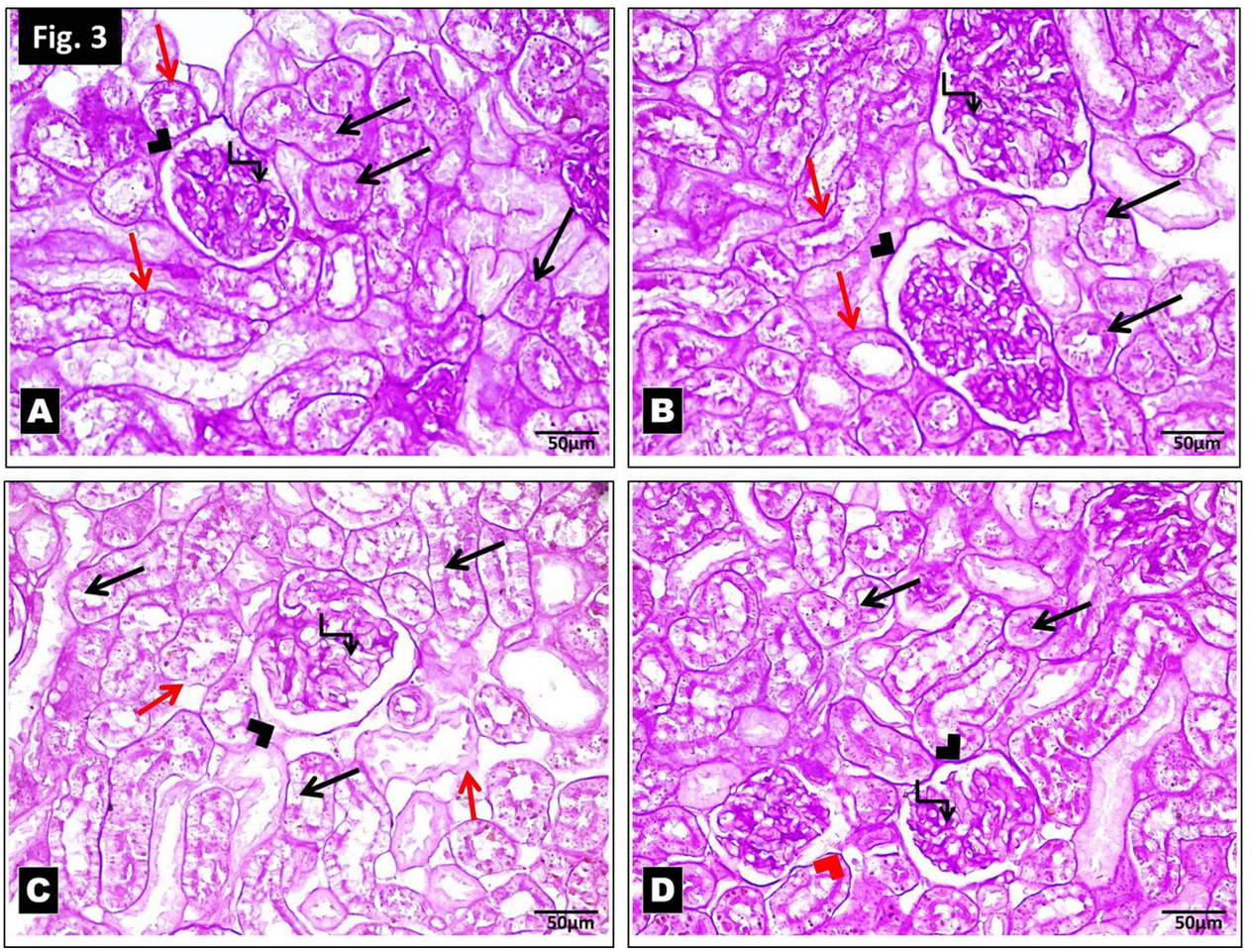

Fig. 3: Photomicrographs of PAS-stained sections of the studied groups' renal cortex; A & B) In group I & II (control & Lf group respectively) demonstrating that apical brush borders of the proximal convoluted tubules (black arrows), the basement membrane of the renal tubules (red arrows) and glomerular capillaries (zigzag arrow), and the parietal layer of Bowman's capsule all showing a strong PAS-positive reaction (arrow head).C) In group III (MTX group) showing weak PAS-positive reaction in the apical brush borders of the PCTs, which was partially interrupted (black arrows). There was partial loss of the basement membrane of the renal tubules (red arrows) and glomerular capillaries (zigzag arrow), as well as the parietal layer of Bowman's capsule (arrow head). D) In group IV (MTX & Lf treated group) showing a strong PAS-positive reaction in the apical brush borders of the majority of proximal convoluted tubules (black arrows), basement membrane of renal tubules (red arrows) and glomerular capillaries (zigzag arrow), and parietal layer of Bowman's capsule (black arrow head). However, it appears to be interrupted in a few places (red arrow head). Magnifications: x400.

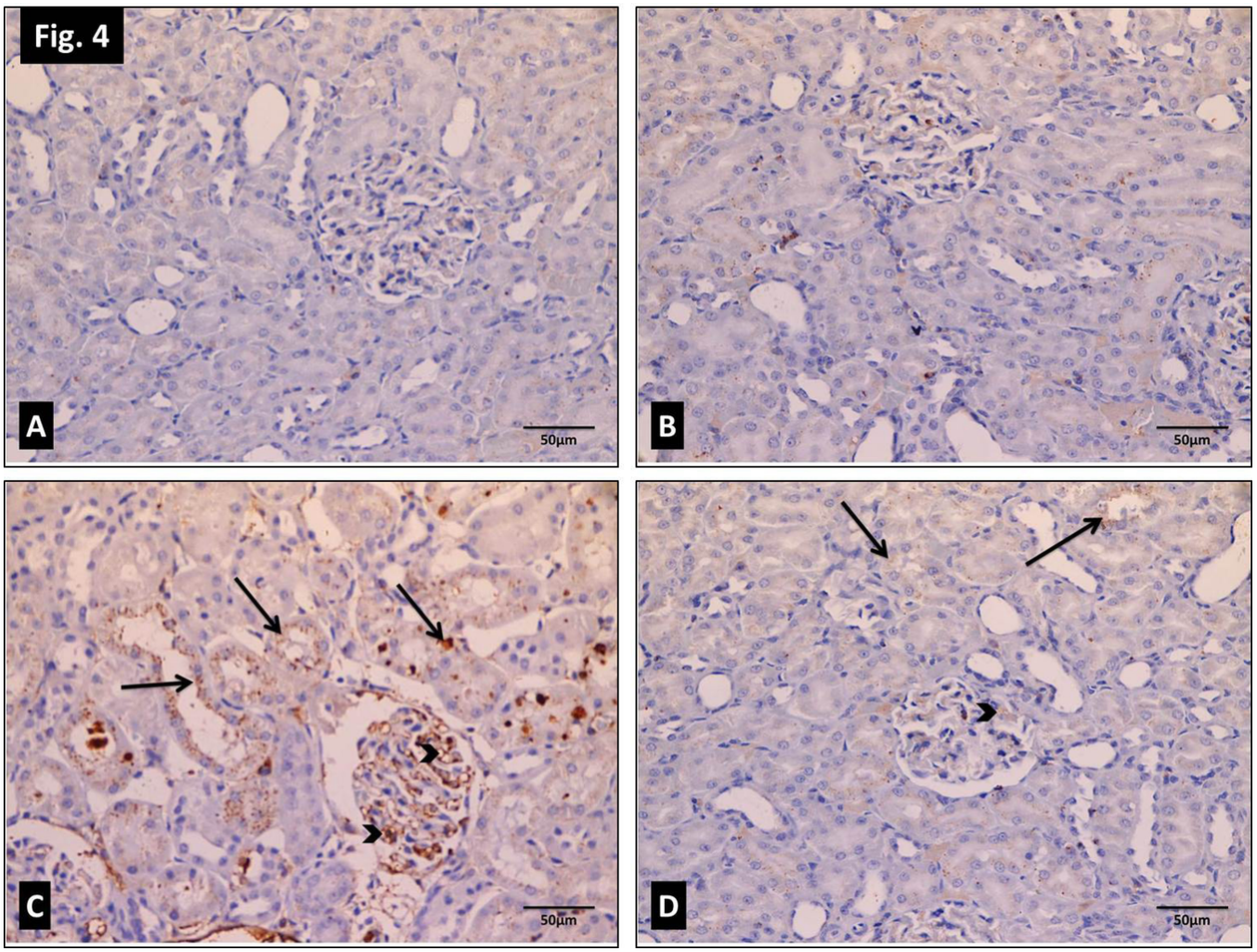

Fig. 4: Photomicrographs of TNF-α immunoreaction of the studied groups' renal cortex; (A& B): group I & II (control & Lf groups respectively), exhibiting a weak positive TNF- immunoreaction, (C): group III (MTX group), TNF-α expression is highly positive in tubular epithelium (arrows) and glomeruli (arrow heads) (D): in group IV(MTX & Lf treated group), some tubular epithelial cells (arrows) and glomeruli (arrow heads) show a slight immunoreaction. Magnifications: x400.

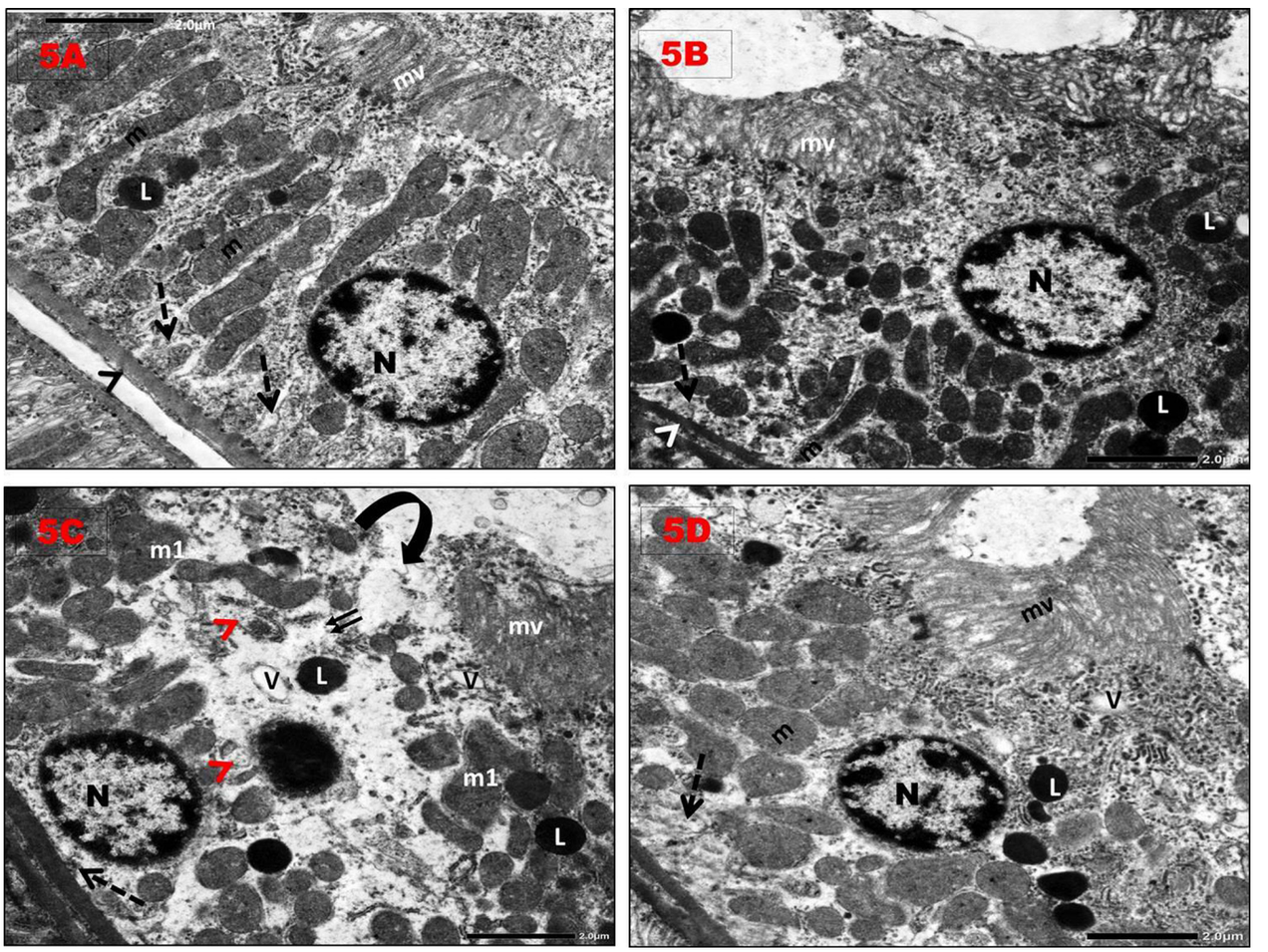

Fig. 5: Transmission electron micrographs of ultrathin sections of the adult albino rat renal cortex showing the epithelial lining cells of the proximal convoluted tubules in the studied groups: A & B) In group I & II (control & Lf group respectively) showing central oval euchromatic nucleus (N), characteristic long apical microvilli (mv), basal elongated mitochondria (m), basal infoldings (dashed arrow), regular basement membrane (arrow head) and multiple electron dense lysosomes (L). C) In group III (MTX group) demonstrating a damaged luminal membrane (curved arrow) and partial loss of apical microvilli (mv). The cytoplasm reveals degenerated vacuoles (V), as well as rarified areas (double arrows). The mitochondria appear distorted; some are degenerated and ruptured (arrow heads), while others are fused (m1). The nucleus (N) reverts to basal position. There may be a loss of basal infoldings (dashed arrow) and multiple lysosomes. D) In group IV (MTX & Lf treated group) demonstrating distinct preservation of normal structure in the proximal tubular epithelial cell; normal long apical microvilli (mv), basal elongated mitochondria (m), basal infoldings (dashed arrow), and lysosomes (L). However, only a few degenerated vacuoles (V) are visible. Magnifications: x3000.

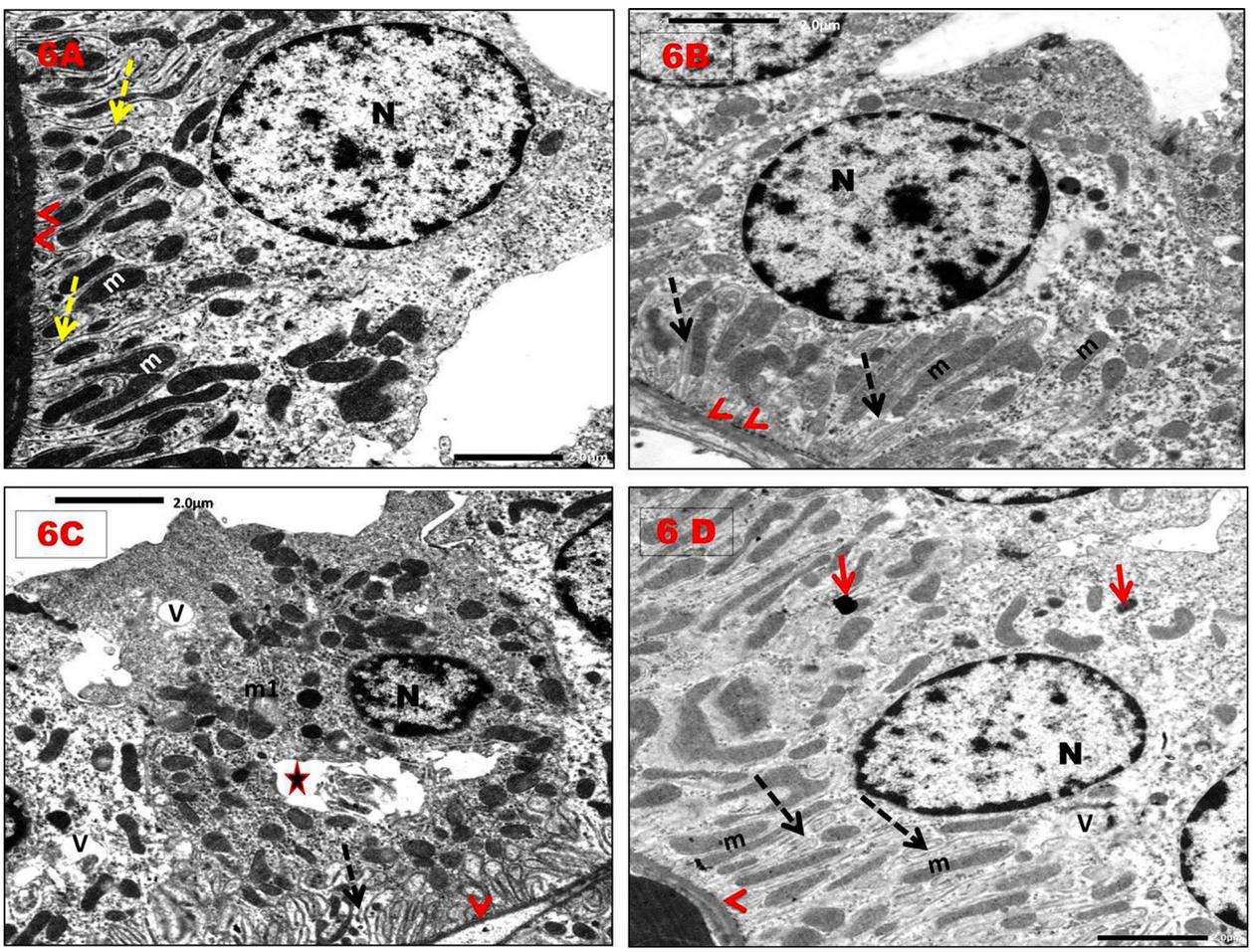

Fig. 6: Transmission electron micrographs of ultrathin sections of the adult albino rat renal cortex showing the epithelial lining cells of the distal convoluted tubules in the studied groups: A & B) In group I & II (control & Lf group respectively) showing large oval apical nuclei (N), distinctive basal infoldings (dashed arrows), elongated basally arranged mitochondria (m) in-between, and regular basement membrane thickness (arrow heads).C) In group III (MTX group) demonstrating a small nucleus (N), disrupted basal infoldings (dashed arrows), and disorganised and degenerated mitochondria (m1). Multiple degenerated vacuoles (V) and rarified areas can be seen in the cytoplasm (star). The basement membrane appears to be separated (arrow head). D) In group IV (MTX & Lf treated group) revealing near normal structure in the distal tubular epithelial cell; normal oval nucleus (N), basal infoldings (dashed arrows) with vertically oriented mitochondria (m), and normal basement membrane thickness (arrow head). However, there are few vacuoles (V) and electron dense bodies (arrows) visible in the cytoplasm. Magnifications: x 3000.

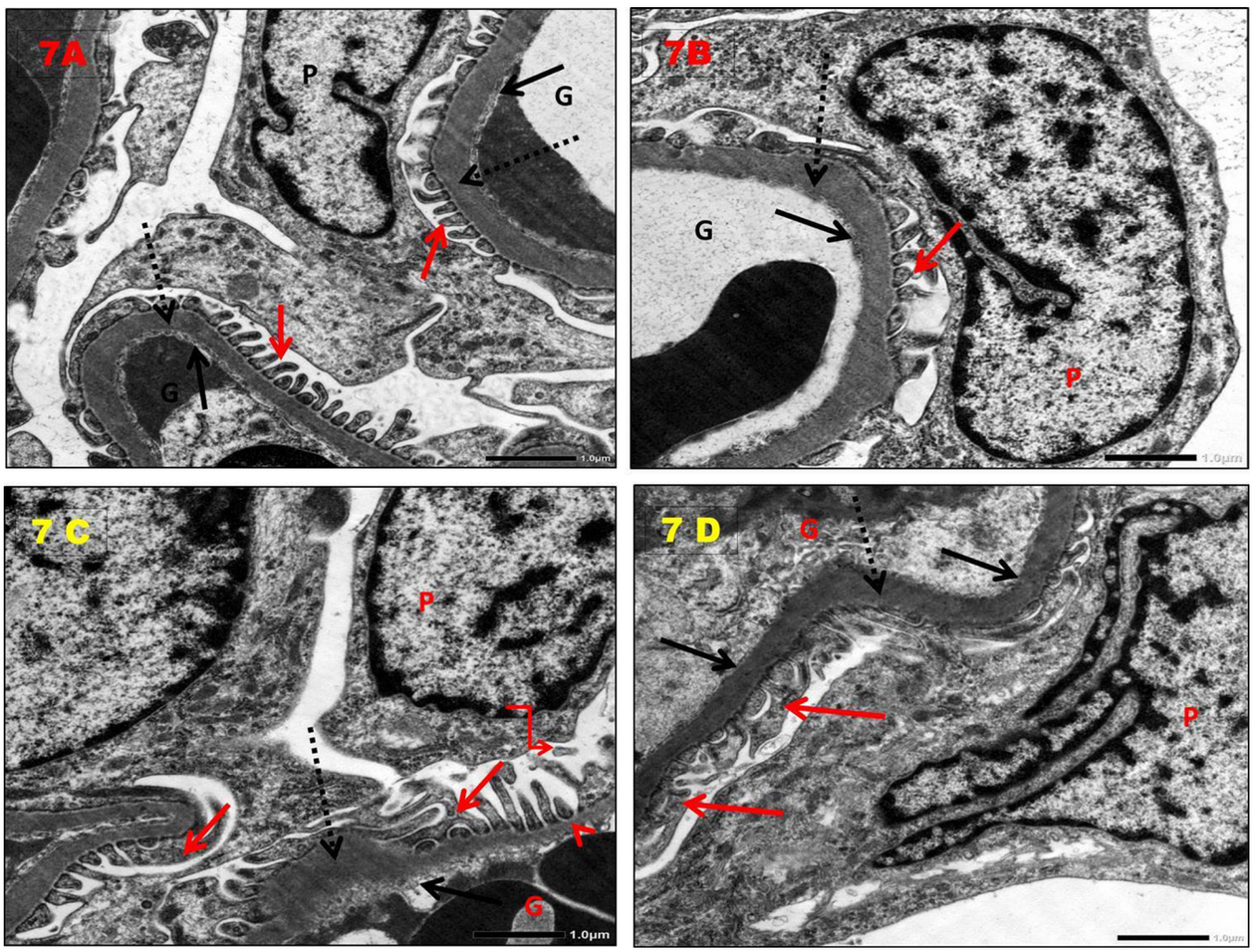

Fig. 7: Transmission electron micrographs of ultrathin sections of the adult albino rat renal cortex showing the renal glomerular corpuscle in the studied groups: A & B) In group I & II (control & Lf group respectively) showing the normal filtration barrier formed by podocyte foot processes (red arrows); the outer layer, regular and uniform glomerular basement membrane (dashed arrows); the middle layer and fenestrated endothelium (black arrows) of the glomerular capillaries (G); and the inner layer. The podocyte's characteristic indented nucleus (P) could be seen. C) In group III (MTX group) reveals disturbance in the filtration barrier The foot processes appear to be disorganised, with some fused together (red arrows) and others detached (zigzag arrow). The thickness of the glomerular basement membrane appears irregular; areas are thin (arrow head) and areas are very thick (dashed arrow). The fenestrated endothelium (black arrow) is erratic. Parts podocyte (P) and glomerular capillary (G) are visible. D) In group IV (MTX & Lf treated group) revealing a filtration barrier that is more or less normal. The glomerular basement membrane (dashed arrow) and fenestrated endothelium (black arrow) appear to be in place. However, a few foot processes (red arrows) remain fused together. Notice, the indented nucleus of the podocyte (P) and a portion of the glomerular capillary (G).Magnifications: x5000.

Table 1: Effect of methotrexate and lactoferrin on body, kidney and relative kidney (%) weights, and kidney functions in the different studied groups

Parameters/ Groups	Group I[a] (control group) (n=7)	Group II[b] (Lf control) (n=7)	Group III[c] (n=15) (MTX- intoxicated) (n=7)	Group IV[d] (MTX-intoxicated+ Lf treated) (n=7)
Body weight (g)	237.1±5.7[c]	235.7±4.1[c]	198.7±11.7[a,b,d]	227.22±10.1[c]
kidney weight(g)	0.87±0.06[c]	0.88±0.05[c]	0.57±0.05[a,b,d]	0.81±0.0[4c]
Relative kidney weight (%)	0.37±0.029[c]	0.37±0.027[c]	0.29±0.03[a,b,d]	0.4±0.03[c]
Serum creatinine level (mg/dl)	0.8±0.08[c]	0.84±0.09[c]	2.6±0.5[a,b,d]	0.97±0.26[c]
Serum urea level (mg/dl)	27.6±4.2[c]	27.8±5.2[c]	46.909±6.5[a,b,d]	31.5±2.9[c]

a–d Significant difference between groups at $p < 0.05$ calculated by one-way ANOVA test followed by Tukey's post hoc test. a denotes significance from group I; b denotes significance from group II; c denotes significance from group III; d denotes significance from group IV. MTX:Methotrexate; Lf: lactoferrin.

Table 2: Effect of Methotrexate and lactoferrin on redox and inflammatory status in the studied groups

Parameters/ Groups	Group I[a] (control group) (n=7)	Group II[b] (Lf control) (n=7)	Group III[c] (MTX- intoxicated) (n=7)	Group IV[d] (MTX-intoxicated+ Lf treated) (n=7)
Renal SOD activity (U / min./ mg protein)	39.8±3.7[c,d]	40.2±2.765[c,d]	14.2±2.650[a,b,d]	32±2.1[a,b,c]
Renal IL-1B(pg/mg protein)	46 ±3.01[c,d]	44.2±3.03[c,d]	129.1±2.2[a,b,d]	65.3±3.02[a,b,c]
Renal MDA level (nmol/mg protein)	1.03±0.17[c,d]	0.95±0.1[c,d]	6. 2±0.7[a,b,d]	2.5±0.5[a,b,c]
Renal GSH-Px activity (umol/ min./ mg protein)	133.6±2.3[c,d]	132.8±2.1[c,d]	54.7±3.01[a,b,d]	103.9±4.3[a,b,c]
Renal GSH(mg /g wet tissue)	3.8±0.6[c]	3.9±0.5[c]	1.4±0.4[a,b,d]	3. 4±0.8[c]

a–d Significant difference between groups at *$p < 0.05$ calculated by one-way ANOVA test followed by Tukey's post hoc test. a denotes significance from group I; b denotes significance from group II; c denotes significance from group III; d denotes significance from group IV. MTX; Methotrexate; Lf; lactoferrin; GSH: reduced glutathione; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase; MDA: malondialdehyde.

Table 3: Effect of methotrexate and lactoferrin on morphometric study parameters in the different studied groups

Parameters/ Groups		Group I[a] (control group) (n=7)	Group II[b] (Lf control) (n=7)	Group III[c] (MTX- intoxicated) (n=7)	Group IV[d] (MTX-intoxicated+ Lf treated) (n=7)
Scoring of the renal injury		1±0.6[c]	2.2±0.8[a,b,d]	0.6±0.5[c]	0.5±0.5[c]
Area percentage% of Masson trichrome		16.851±7.8[a,b,c]	42.2±8.9[a,b,d]	10.7±1.[9c,d]	10.4±1.7[c,d]
Area percentage% of PAS		33.3±10.1[c]	14.6±2.7[a,b,d]	36.7±7.9[c]	35.6±5.5[c]
TNF-α immune-reactivity	Color intensity	17.3±4.1[a,b,c]	30.4±4.3[a,b,d]	10.5±1.3[c,d]	10.9±2.5[c,d]
	Area percentage%	13.3±1.7[a,b,c]	36.8±2.8[a,b,d]	111±1.2[c,d]	10.2±1.3[c,d]

a–d Significant difference between groups at *$p < 0.05$ calculated by one-way ANOVA test followed by Tukey's post hoc test. a denotes significance from group I; b denotes significance from group II; c denotes significance from group III; d denotes significance from group IV. MTX: Methotrexate; Lf: lactoferrin

DISCUSSION

Chemotherapeutic drugs have a wide range of efficacy in the treatment of cancer. Nonetheless, kidney is one of the organs most vulnerable to their negative effects. MTX an inhibitor dihydrofolate reductase enzyme acts by inhibiting DNA synthesis[16].

Asci *et al*., 2017, previously reported that MTX-induced nephrotoxicity via oxidative injury and inflammation with sequel of tubular injury and decreasing glomerular filtration rate[17]. To induce nephrotoxicity, we employed a single dose of MTX, as described in the literature. Despite the fact that there is a lot of evidence that MTX could induce renal damage, competent therapies hindering this severe complication aren't available till now. Hence then, it becomes crucial to find a novel treatment options to prevent and/or cure MTX-induced renal damage[1]. Lf is a natural glycoprotein of transferrin family present in milk with minimal adverse immune reaction[18]. Lf was used in the present study to explore its potential effect in mitigating nephrotoxicity induced by MTX.

In the current study, MTX significantly decreased body, kidney and relative kidney weighs the results that may be due to excessive loss of water, salts and proteins as a result of renal injury with subsequent dehydration and weight loss and were in line with those reported previously[2,19]. Nonetheless, treatment of the nephrotoxic group with Lf protected against the loss in body and kidney weighs induced by MTX. These results were in accordance with reported previously by Hsu *et al*., 2020[20].

Nephrotoxicity induced by MTX, was confirmed by the histological alterations. Sections from MTX nephrotoxic group in the present study revealed atrophic glomeruli with congested capillaries, renal architecture was disturbed, renal tubules depicted vacuolation, pyknosis of the nuclei and detachment of its epithelial lining, and the interstitial tissue revealed hemorrhage and inflammatory infiltration. These histopathological findings were in line with Asci *et al* 2017, and Ahmed *et al*., 2020 results[17,21]. However, treatment of the nephrotoxic group with Lf had a cytoprotective effect in the form of normally appeared kidney with few degenerated vacuoles and slight interstitial inflammatory infiltration that may be due to its antioxidant and anti-inflammatory effects. The results herein were in agreement with Hegazy *et al*., 2016 who studied the effect of Lf on Chromium-Induced acute kidney injury[9]. Arab *et al*., 2018 reported that camel milk protected against MTX-induced renal histopathological changes[3].

In the present study, Masson Trichrome stained sections of renal cortex of MTX group revealed increased amount of collagen fibers. Similar changes were observed by Fu *et al*., 2021[22]. The results may be due to increased transforming growth factor (TGF)-β and kidney injury molecule-1 (KIM-1) expression with excessive accumulation of extracellular matrix and subsequent tubular injury and fibrosis[22,23]. On the other hand, the results of the present study depicted significant reduction of the amount of collagen fibers upon treatment of nephrotoxic group with Lf. The results that was in coherence with Hsu *et al*., 2020 who reported that Lf recovered renal function and suppressed renal fibrosis through inhibiting TGF-β1-induced renal fibrosis and

pro-fibrogenic genes expression, inhibition of apoptosis and the induction of autophagy and finally recommended the use of Lf as a potential therapeutic target for the prevention of the acute to chronic kidney injury transition[20].

In present results, PAS stained sections of renal cortex of MTX treated group revealed partial disruption of the proximal convoluted tubules' apical brush borders, basement membrane of the renal tubules, glomerular capillaries and Bowman's capsule. These results coincide with those obtained previously by Gronroos *et al*., 2006[24]. However, treatment of the nephrotoxic group with Lf revealed intact apical brush borders of the most proximal convoluted tubules. The results were in coherence with those obtained by Abdel-Aal1 *et al*., 2016 who reported that camel milk protect against tramadol-induced nephrotoxicity through preservation of renal structure and function[25].

Ultra-structural results of the current study confirmed the light microscopic findings. MTX treated nephrotoxic group demonstrated many destructive effects on the renal structure in the form of damaged luminal membrane with loss of apical microvilli in the proximal convoluted tubules, degenerated mitochondria, degenerated vacuolated cytoplasm and disturbed basal infoldings in the proximal and distal convoluted tubules. Furthermore, the renal filtration barrier experienced changes such as detachment of some podocyte foot processes and fusion of others, irregularity of the glomerular basement membrane, and capillary fenestrated endothelium. These current findings were in agreement with Nasr, 2013 who reported an evidence of degenerative and atrophic changes in the ultra-structure of renal cortex in rats that were treated with cisplatin, antineoplastic drug[19].

Du *et al*. 2004 assumed that the microvilli of brush-border have a mechano-sensory function in fluid dynamics and modulates Na absorption in the proximal tubules[26]. So, the distortion of brush border and completely disorganized microvilli of renal tubules, described in the current study, may lead to dysfunction of salt and water reabsorption, and might result in kidney failure. Nagata, 2016 stated that Podocytes preserve the glomerular filtration barrier by production of glomerular basement membrane components, slit membrane and maintain endothelial cell viability[27]. But excessive stress leads to loss of integrity and dysregulation of cellular metabolism. In oxidative stress, Podocytes produce ROSs as a response to stimuli that are harmful and cause malfunction[28]. Fatty acid oxidation and production of ATP are performed mainly by mitochondria that play an important role in producing the energy-dependent ion gradients that force the renal tubular reabsorption. Also the mitochondria perform an important role in apoptosis regulation[29]. In the present study, Lf treatment in nephrotoxic group resulted in a distinct improvement in ultrastructural findings. These findings were in agreement with Hassan *et al*., 2021 who found that treatment with Lf in Chromium-induced nephrotoxicity improved brush borders integrity, basal infoldings and normal shaped mitochondria[30].

In the present study, the nephrotoxic effect of MTX was also confirmed by assessment of kidney function, redox and inflammatory state. The results herein revealed a significant impairment in kidney function with depletion of the antioxidant defense system in MTX treated group. Meanwhile, significant increase in MDA level was found indicating impairment in redox state. The obtained results may be due to cystic dilation of the renal tubules, severe inflammatory infiltrates, atrophy and hypertrophy in some glomeruli[31]. MTX treatment caused persistent ROSs production which caused exhaustion of the antioxidant defense mechanisms and oxidative damage of enzyme protein decreasing their activities in addition to decreased their synthesis at the molecular level[2]. Furthermore, ROSs cause cell injury through oxidizing lipid and proteins, loss of membranes integrity via changing its fluidity and permeability, inactivating the antioxidant enzymes, and provoking DNA damage[32]. The results obtained here were in line with previous reports obtained by Hassanein *et al*, 2019 and Shalaby *et al*, 2019[16,33].

On the other hand, treatment of the nephrotoxic group with Lf revealed significant improvement in kidney function, and redox status. Given the importance of ROS and oxidative stress in the development of MTX-induced nephrotoxicity, promoting the antioxidant mechanisms and cytoprotective enzymes is crucial for protecting against various redox assaults. The results obtained herein may be due to its role in impeding ROSs production and further lipid peroxidation[34]. Arab *et al*., 2018 reported that Camel milk which contains Lf attenuated MTX-triggered impairment in kidney function, redox status and renal histopathological changes[3]. Hassan *et al*., 2021 reported that after inducing chromium nephrotoxicity, Lf played a significant role in restoring kidney function[30].

MTX, via boosting ROSs production and promoting oxidative stress, causes inflammation and cell death through activating multiple stress signaling pathways, such as NF-κB, which promotes the expression of pro-inflammatory cytokines as TNF-α, IL-1β, mitochondrial malfunction and apoptosis[35]. MTX increased immunohistochemical gene expression TNF-α and IL-1β level in kidney tissue in the present study demonstrating an inflammatory response. The results were in coherence with previously obtained ones[17,1,35]. Cakir, 2015 and Kandemir, 2017 attributed the pathogenesis of MTX nephrotoxicity to the release of inflammatory cytochines as TNF-α IL-1β which activate inflammatory cells with the large amount of toxic ROSs production leading to cellular damage by different mechanisms including peroxidation of membrane lipids and oxidative damage of DNA and proteins with vicious circle of inflammation and oxidative stress[36,10].

Meanwhile, treatment of the nephrotoxic group with Lf revealed significant decrease in TNF-α expression and IL-1β level. Legrand *et al*., 2005, reported that Lf bind free Fe+++ ions as well as pro-inflammatory compounds reducing their effects on the immune cells' activation and recruitment beside its ability to stimulate the release

of anti-inflammatory cytokines and suppressed the po-inflammatory cytokines synthesis[37]. Hegazy *et al*., 2016 reported the protective role of Lf in modulating chromium-induced nephrotoxicity, via its antioxidant, anti-inflammatory, and anti-proliferative effects[9].

CONCLUSION

Lf is a potential nephroprotective and cytoprotective agent against MTX-induced nephrotoxicity and renal damage through its antioxidant, anti-inflammatory and ant-fibrotic properties. Our findings suggested that Lf could be beneficial as an adjuvant therapy for cancer and autoimmune disease patients on MTX to prevent kidney injury.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

القدرة التحسينية للاكتوفيرين على السمية الكلوية التي يسببها الميثوتريكسات في الجرذ: دراسة نسيجية وكيميائية مناعية

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الخلفية: الميثوتريكسات هو دواء شائع في العلاج الكيميائي ومثبط للمناعة. ومع ذلك ، فإن استخدامه السريري محدود بسبب العديد من الآثار الجانبية مثل السمية الكلوية والتسمم الكبدي. اللاكتوفيرين هو بروتين سكري طبيعي بالحليب له العديد من الخصائص الدوائية كمضاد للفيروسات ومضاد للالتهابات ومضاد للأكسدة.

هدف الدراسة: تقييم التأثير الوقائي للكلى للاكتوفيرين في السمية الكلوية التجريبية والضرر الكلوي التي يسببها الميثوتريكسات.

المواد و الطرق: تم تقسيم ثمانية وعشرون ذكرا من الجرذان البيضاء بشكل عشوائي إلى أربع مجموعات: المجموعة الضابطة ، تلقت المجموعة المعالجةلاكتوفيرين (٣٠٠ مجم / كجم / يوم) بالفم لمدة أسبوعين. تلقت المجموعة المعالجة بالميثوتريكسات جرعه واحده عن طريق الحقن البريتونى(٢٠ ملجم / كجم) ؛ والمجموعة المعالجة بالميثوتريكسات + لاكتوفيرين. و قد تم قياس وظائف الكلى ، حالة الأكسدة والاختزال. بالإضافة إلى ذلك ، تم تقييم إنترلوكين (IL-١B) والتعبير عن عامل نخر الورم (TNF-α) عن طريق الكيمياء المناعية. علاوة على ذلك ، تم تقييم الخصائص النسيجية بواسطة الميكروسكوب الضوئى و الالكترونى.

النتائج: تم إثبات السمية الكلوية للميثوتريكسات من خلال الزيادة الكبيرة في وظائف الكلى في الدم ، ومستوى (Malodialdehyde (MDA ، ومستوى IL-١B، وتعبير بروتين TNF-α جنبًا إلى جنب مع انخفاض ملحوظ في نشاط إنزيمات GSH الكلويه وأنزيمات مضادات الأكسدة. تم تأكيد النتائج البيوكيميائية من خلال نتائج فحص الانسجه التي أظهرت اضطراب في التركيب البنائى، وضمور الكبيبات واحتقانها ، وخلايا أنبوبية متحللة ذات انويه ضامره متكدسة الكروماتين، ونزيف خلالي ، وتسلل التهابي مع زيادة ملحوظه في ألياف الكولاجين مع انخفاض فى صبغة PAS. كما كشفت دراسة التركيب المجهرى الدقيق للخلايا عن وجود متقدرات منتفخة ومتحللة ، فجوات سيتوبلازميه، تمزق الغشاء القاعدي في الأنابيب القريبة والبعيدة. ذلك بالاضافه الى خلل فى حاجز الترشيح الكلوي. وفي الوقت نفسه ، أدى علاج لاكتوفيرين إلى تحسين وظائف الكلى وحالة الأكسدة والاختزال والالتهاب والخصائص النسيجيةو التركيب الدقيق للخلايا .

المستخلص: لاكتوفيرين لديه القدرة على حماية الكلى ضد السمية الكلوية والسمية الخلوية التي يسببهاالميثوتريكسات. كما تشير نتائجنا إلى أن لاكتوفيرين قد يكون مفيدًا ضد إصابة الكلى كعلاج مساعد لمرضى السرطان وأمراض المناعة الذاتيةمن مستخدمى عقارالميثوتريكسات