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The Beneficial Role of Magnetized Water on Methotrexate-Induced Acute Kidney Injury in Albino Rats

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Abstract

This research was initiated to reveal the beneficial properties of magnetized water (MW), alone or in combination with the angiotensin-converting enzyme inhibitor, Lisinopril, (Lis) on methotrexate (MTX)-induced AKI in rats, and to track the mechanisms involved in this impact. The results showed that the treatment with MW and Lis, either separately or in combination, brought about a significant reduction in serum urea and creatinine levels that were elevated upon administration with MTX. Both of MW and Lis inhibited the oxidative stress burden caused by MTX as indicated by lowering nitric oxide levels and up-regulating the total antioxidant capacity. Furthermore, both treatments decreased the increased levels of serum NAG, MIP-2 and KIM-1, which were enhanced due to MTX administration. Immunohistochemical examination of kidney tissue of both of MW- and Lis-treated groups reveal moderate and mild immune reactions for Cas-3, TNF-α, TGF-β and Bax, respectively, while the combined treatment resulted in negative immune reaction. The biochemical and immunohistochemical findings were confirmed by the histopathological investigation where both of MW and Lis exhibited moderate ameliorative effects on kidney tissue while the co-treatment produced marked improvement in the histological feature of kidney tissue. In conclusion, the co-administration of MW and Lis can synergistically attenuate the MTX-induced renal injury. Drinking MW as an adjuvant agent with Lis during the treatment of AKI can enhance the efficiency of this drug. This could be attributed to the advantage of MW as an antioxidant, anti-inflammatory and anti-apoptotic candidate.

Keywords: Acute kidney injury – Methotrexate – Magnetized water - Angiotensin-converting enzyme inhibitor – Inflammation – Oxidative stress – Apoptosis – Rats.

1. Introduction

Kidney disease is categorized into two types; acute kidney injury (AKI) and chronic kidney disease (CKD). The clinical guidelines from Kidney Disease Improving Global Outcomes (KDIGO) identifies AKI as acute kidney diseases subgroup [1]. AKI progresses rapidly with the loss of kidney function, resulting in the liberation of creatinine and toxic end products of nitrogen metabolism in the patient's blood. On the other hand, CKD takes a long duration to develop with the gradual deterioration ofkidney functions [2]. About 13 million new AKI cases appear each year worldwide [3] and about 10–15% of hospitalized patients as well as over 50% of Intensive care Unit (ICU) patients are suffering from AKI [4]. Many of these cases develop AKI, renal failure, multi-organ dysfunction, and even death due to the absence of efficient AKI treatment in clinical practice, accordingly, causing a substantial medical burden to both the society as well as the family [5]. Clinically AKI is divided to 3 classes; pre-renal, intrinsic/intra-renal as well as post-renal. Pre renal AKI is caused by external stimuli as toxins hypotension, cardiac failure, renal vascular thrombosis, hypocalcemia, the neurohumoral axis activation, as well as renal vascular resistance elevation [6]. The intra-renal/intrinsic kidney injury takes place as a result of loss of function in glomeruli, renal tubules, or interstitium. While, post renal AKI occurs as a result of a urinary tract obstruction which may be caused by kidney stones, urethral and ureter blood clots or as a result of cervix, colon or prostate cancers [7].

The AKI risk factors include age > 60 years, gender; hypovolemia, sepsis, diabetes mellitus, hypertension, pulmonary disorder, chronic heart diseases, mechanical ventilation, cancer as well as renal replacement therapy [8]. Furthermore, old as well as new chemotherapy drugs, in cancer treatment context, often lead to chemotherapy-associated nephrotoxicity [9], and about 17.5% of cancer patients are suffering from AKI, that negatively affects patients' survival [10]. In addition, drug-induced nephrotoxicity is the cause of around 60% of all AKI cases [11].

AKI is diagnosed according to KDIGO criteria by increment of Cr serum level (sCr) by ≥ 0.3 mg/dL (≥26.5 mmol/L) in 2 days or ≥ 1.5 times baseline, presumed or known to have found in the prior 7 days. According to the sCr level, AKI is classified into three stages: Stage 1: with increased the level of sCr by ≥ 0.3 mg/dL in 2 days or elevation of 1.5- to 1.9 fold change, Stage 2: with increased level of sCr from 2- 2.9 times baseline, as well as Stage 3: with increased level of sCr 3 times baseline or ≥ 4.0 mg/dL (≥ 353.6 mmol/L) [1].

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A chemotherapeutic agent, methotrexate (MTX), is broadly administered in many kinds of cancer clinical management as well as different inflammatory diseases [12]. Almost of MTX (90%) is excreted by kidneys, thus, MTX is considered as nephrotoxicin [13]. MTX-induced kidney toxicity is usually accompanied with oxidative stress, antioxidant defense system disruption as well as proinflammatory cytokines elevation that may stimulate proinflammatory cell death [14].

Water is essential for the life of living bodies as it is used in almost all body functions, including thermoregulation, lubrication, digestion, absorption, lactation, carrier, support, cushioning, and mineral balance [15]. Magnetized water (MW) is obtained via passing tap water through a magnetic field; this makes very fertile as well as active water, with increased ratio of oxygen, amino acid dissolution as well as the salts velocity [16]. The powerful antioxidant properties have been reported for MW with a great capability to easily and rapidly diffuse into body tissues [17]. Moreover, MW has been found to rescue nephrotoxicity induced by cisplatin [18], gentamicin [19], and ferric-nitrilotriacetate-induced nephrotoxicity [20].

It is well revealed that angiotensin II enhances NADPH oxidase leading to oxidative stress as well as renal damage, and that blockade of angiotensin II with angiotensin-converting enzyme inhibitors (ACEIs) protects the kidney in the cases of hypertension, diabetes and hyperlipidemia [21] due to inhibition of renal NADPH oxidase [22]. Lisinopril is a lipophilic non-sulfhydryl ACEI with antioxidant activity [23] and anti-inflammatory potential [24].
This research was designed to explore the effectiveness of magnetized water as well as Lisinopril, alone or in combination, against methotrexate-induced acute kidney injury in rats, and to uncover the implicated mechanisms underlying this effect.

2. MATERIAL AND METHODS

Drugs and chemicals
Methotrexate was purchased from Hikma Specialized Pharmaceuticals, Cairo, Egypt. Lisinopril was procured from AstraZeneca Pharmaceutical Company, Cairo, Egypt. All the other unspecified chemicals were of analytical grade**.**

Preparation of MW
Magnetized water was obtained by letting tap water pass through a static magnetic unit (produced by National Research Centre, 33 El-Behouth St., 12622 Dokki, Giza, Egypt) with a magnetized intensity of 1500 Gauss. A hand-held Gauss meter (Hirst Magnetic Instruments, Ltd, UK) with a transverse probe Brand (model Gm07, accuracy ±0.01%) was used to determine and ensure the continuous exposure of the magnetic field dose. Upon using MW in the current experiment, it was changed every day, as the shelf life of the magnetized water is 1 day [25].

Animal experiment
Animals
Forty-eight adult female albino *Wistar* rats weighing 120-140 g were obtained from the Animal Care Unit of the National Research Centre (NRC), Egypt, and housed in the Sectorial Animal Facility of the Department of Hormones, Medical Research and Clinical Studies Institute, NRC, under controlled room temperature (27±3 ℃) and a 12 hr/12 hr light/dark cycle. Animals were kept in plastic cages with wood shavings used as beddings, and provided with standard rodent chow diet and water *adlibitum.* The animals were maintained under observation for about one week before starting the experiment for acclimatization. The experimental protocol was performed as per Institutional Guidelines as the Ethics Committee Approval was obtained from the Ethical Committee for Medical Research, NRC, Egypt for all the procedures with animals in this study (Code number 5416072022).

Experimental Protocol
After the adaptation period, the rats were fasted for 12 hours and then randomly assigned into six groups (8 rats/group), as follows: Group 1: Healthy untreated negative control group (Cont+TW); this group was freely allowed to drink tap water (TW) for 4 weeks. Group 2: Healthy treated group (Cont+MW); this group was freely allowed to drink MW (1500 Gauss) instead of TW for 4 weeks. Group 3: Positive control (MTX+TW) group; this group was intraperitoneally injected with a single dose of 20 mg/kg MTX at the beginning of the experiment [26] and had free access to TW for 4 weeks. Group 4: MTX+MW group; this group was intraperitoneally injected with a single dose of 20 mg/kg MTX at the beginning of the experiment and freely allowed to drink MW (1500 G) instead of TW for 4 weeks. Group 5: MTX+TW+Lis group; this group was intraperitoneally injected with a single dose of MTX (20 mg/kg), and received 40 mg/L Lis in drinking TW [27]for 4 weeks. Group 6: MTX+MW+Lis group; this group was intraperitoneally injected with a single dose of 20 mg/kg MTX at the beginning of the experiment, and received 40 mg/L Lis in drinking MW (1500 G) for 4 weeks.

Blood and tissue sampling
At the end of the experimental period (four weeks), all rats were allowed to fast overnight and blood samples were collected from the tail vein, following light anesthesia, then the sera were separated by allowing the blood samples to clot for 30 min at a temperature of 25 ˚C and then centrifuged at 1800xg for 10 min at 4˚C. After centrifugation, the serum samples were separated into numerous aliquots and cryopreserved until further usage in biochemical evaluation. After blood collection, the rats were rapidly sacrificed and the kidney of each rat was dissected, cleaned and washed with ice-cold saline (0.9% NaCl), then, the tissue was fixed in 10% formalin saline for immunohistochemical and histopathological examinations.

Biochemical evaluation

Serum urea, creatinine (Cr), nitric oxide (NO), and total antioxidant capacity (TAC) were measured colorimetrically using kits purchased from Biodiagnostic (Egypt) following the methods described by Fawcett and Scott [28], Larsen [29], Montgomery and Dymock [30] and Koracevic *et al*. [31],respectively**.** Serum macrophage inhibiting protein-2 (MIP-2) was quantified according to the manufacturer's manual of the enzyme-linked immunosorbent assay (ELISA) kit (Cat# MBS9424870) procured from MyBioSource Co. (San Diego, USA).

Serum N-acetyl-beta-D-glucosaminidase (NAG) was estimated according to the manufacturer's recommendation of ELISA kit (Cat# MBS702746) obtained from MyBioSource Co. (San Diego, USA). Serum kidney injury molecule-1 (KIM-1) was determined according to the manufacturer's instruction of the ELISA kit (Cat# MBS355395) purchased from MyBioSource Co. (San Diego, USA).

Immunohistochemical technique

Following fixation of kidney tissue for 24 hours, the dehydration process starts by using a series of ethanol solutions of increasing concentration to remove water and formalin from the tissue. Then, the clearing agent (xylene) was used to remove the alcohol and allow infiltration with paraffin wax. After that, the tissue was infiltrated with paraffin wax and became surrounded by a large block of molten paraffin wax creating block.

The paraffin block was solidified providing a supportive matrix that allows very thin sectioning (5 µm) by using a microtome. Paraffin sections were mounted on positively charged slides by using the avidin-biotin-peroxidase complex (ABC) method. Sections of kidney tissue from each group were incubated with the following antibodies: rabbit Cas-3 polyclonal antibody (Elabscience, Cat# E-AB-63602, Dil.: 1:100), mouse apoptosis marker BCL2- associated X protein (Bax) monoclonal antibody (Elabscience, Cat# E-AB-22212, Dil.: 1:100), mouse tumor necrosis factor-alpha (TNF-α) monoclonal antibody (Elabscience, Cat# E-AB-22159, Dil.: 1:100) and rabbit transforming growth factor-beta (TGF-β) polyclonal antibody (Novusbio, Cat# NBP1-80289, Dil.: 1:100).

Then the reagents required for ABC method were added (Vectastain ABC-HRP kit, Vector laboratories). Marker expression was labeled with peroxidase and colored with diaminobenzidine (DAB, produced by Sigma, USA) to detect antigen-antibody complex. Negative controls were included using non-immune serum instead of the primary or secondary antibodies. Immunohistochemical-stained sections were examined using an Olympus microscope (BX-53, Japan)

Histopathological procedure

The acute kidney injury (AKI) was evaluated by the histological examination of the kidney tissue. The prepared solidified paraffin wax tissue blocks were sectioned by using a microtome at 5 µm thickness, and the sections were placed on glass slides, deparaffinized, and stained with hematoxylin-eosin (HE) staining for routine histopathological investigation under light microscope [32].

Data analysis

Data were statistically analyzed using the statistical software, SPSS (version 20). The results were presented as mean ± SE. Significance was determined using one-way ANOVA test, $p<0.05$ was considered significant.

3. RESULTS

Biochemical outcomes

As illustrated in **Table 1**, the control group administered MW (Cont+MW) displayed a considerable drop in serum urea level along with significant decline ($p<0.05$) in serum Cr level in comparing with the control one administered TW (Cont+TW). While, MTX administration either with TW (MTX+TW) or with MW (MTX+MW) produced significant enhancement ($p<0.05$) of urea and Cr serum levels versus the Cont+TW as well as the Cont+MW, respectively.

However, the administration of MTX with MW brought about a significant decrease ($p<0.05$) in serum urea as well as Cr levels, contrary to the MTX+TW group. Concerning Lis treatment, it triggered a significant decline ($p<0.05$) of serum urea as well as Cr levels in the MTX+TW+Lis-administered group in contrast with the MTX+TW one. Likewise, co-treatment of MTX+MW+Lis induced a significant suppression in serum urea and Cr levels compared with MTX+MW.

Results given in **Table (1)** showed that MTX administration (MTX+TW) or (MTX+MW) resulted in a significant elevation ($p<0.05$) in serum NO levels accompanied with a significant reduction ($p<0.05$) in serum TAC as compared with the Cont+TW and the Cont+MW groups, respectively. While the administration of MTX with MW led to a significant decrease ($p<0.05$) in serum level of NO in concomitant with a considerable increase in serum TAC versus the MTX+TW group.

Interestingly, treatment of Lis and MTX with TW (MTX+TW+Lis) elicited a significant inhibition ($p<0.05$) in serum NO level along with a significant increase ($p<0.05$) in serum TAC, contrary to the MTX+TW group. Similarly, there was a significant reduction ($p<0.05$) in serum NO level in concomitant with a significant increase ($p<0.05$) of serum TAC in the MTX+MW+Lis-treated group versus the MTX+MW group.

Table 1: Impact of MW and Lis either alone or in combination on serum urea, Cr, NO and TAC of rats with AKI induced by MTX

Parameters Groups	Urea (g/dl)	Creatinine(mg/dl) (Cr)	NO (µlmol/L)	TAC (mM/l)
Cont+TW	33.0 ± 0.95	0.72 ± 0.06	59.5 ± 5.2	7.7 ± 0.18
Cont+MW	29.12 ±1.91	0.41 ± 0.03[a]	57.9 ± 3.6	8.1 ± 0.29
MTX+TW	70.0 ± 5.69[a]	1.8 ± 0.11[a]	120.1 ± 8.4[a]	3.3 ± 0.36[a]
MTX+MW	45.9 ± 1.49[bc]	0.96 ± 0.11[bc]	100.7± 4.4[bc]	3.8 ± 0.24[b]
MTX+TW+Lis	37.7 ± 0.9[c]	0.96 ± 0.11[c]	76.5 ± 5.3[c]	4.7 ± 0.4[c]
MTX+MW+Lis	34.8 ± 2.39[d]	0.81 ± 0.05[d]	66.7 ± 5.57[d]	5.4 ± 0.4[d]

[a]Significant change at $P < 0.05$ comparing with the Cont+TW gp.
[b]Significant change at $P < 0.05$ comparing with the Cont+MW gp.
[c]Significant change at $P < 0.05$ comparing with the MTX+TW gp.
[d]Significant change at $P < 0.05$ comparing with the MTX+MW gp.

As depicted in Table 2, MTX with TW (MTX+TW) or with MW (MTX+MW) provoked a significant increase (p<0.05) in serum NAG, MIP-2 and KIM-1 levels as compared with Cont+TW and Cont+MW groups, respectively. However, MW administration in the control group (Cont+MW) or in the MTX group (MTX+MW) led to a significant decrease (p<0.05) of serum NAG, MIP-2 as well as KIM-1 levels versus Cont+TW and MTX+TW groups, respectively. Regarding Lis treatment, it brought about a significant decrease (p<0.05) in serum NAG, MIP-2 as well as KIM-1 levels in MTX+TW+Lis-treated group comparing with the MTX+TW group. Surprisingly, co-treatment of MTX with MW and Lis resulted in a significant decrease (p<0.05) in serum NAG, MIP-2 as well as KIM-1 concentration in contrast with MTX+TW+Lis as well as the MTX+MW groups, respectively

Table 2: Impact of MW and Lis either alone or in combination on serum NAG, MIP-2 and KIM-1 of rats with AKI induced by MTX

Parameters Groups	NAG (mlU/ml)	MIP-2 (pg/ml)	KIM-1 (pg/ml)
Cont+TW	12.15 ± 0.25	36.8 ± 0.93	62.8 ± 1.2
Cont+MW	9.17 ± 0.14[a]	27.7 ± 1.27[a]	50.5 ± 1.2[a]
MTX+TW	48.5 ± 0.62[a]	325.5 ± 2.76[a]	594.1 ± 3.4[a]
MTX+MW	31.3 ± 0.57[bc]	290.2 ± 2.28[bc]	466.0 ± 3.2[bc]
MTX+TW+Lis	21.28 ± 0.13[c]	158.9 ± 2.1[c]	203.9 ± 2.8[c]
MTX+MW+Lis	12.68 ± 0.16[de]	97.3 ± 2.2[de]	166.6 ±1.7[de]

[a]Significant change at $P < 0.05$ comparing with the Cont+TW gp.
[b]Significant change at $P < 0.05$ comparing with the Cont+MW gp.
[c]Significant change at $P < 0.05$ comparing with the MTX+TW gp.
[d]Significant change at $P < 0.05$ comparing with the MTX+MW gp.
[e]Significant change at $P < 0.05$ comparing with the MTX+TW+Lis gp

Immunohistochemical results

As illustrated in **Fig. (1)**, both of the Cont+TW and Cont+MW groups revealed negative immunohistochemical reaction of TNF-α in the renal tubules **(Fig. 1a,b)**. Regarding the MTX+TW group, it showed an intense positive reaction **(Fig. 1c)** while the MTX+MW group revealed moderate positive reaction for TNF-α in the renal tubules cytoplasm **(Fig. 1d)**.

For MTX+TW+Lis group, it showed mild positive reaction for TNF-α in the renal tubules and glomeruli **(Fig. 1e)** while the MTX+MW+Lis group revealed negative reaction for TNF-α in the cytoplasm of renal tubules **(Fig. f)**.

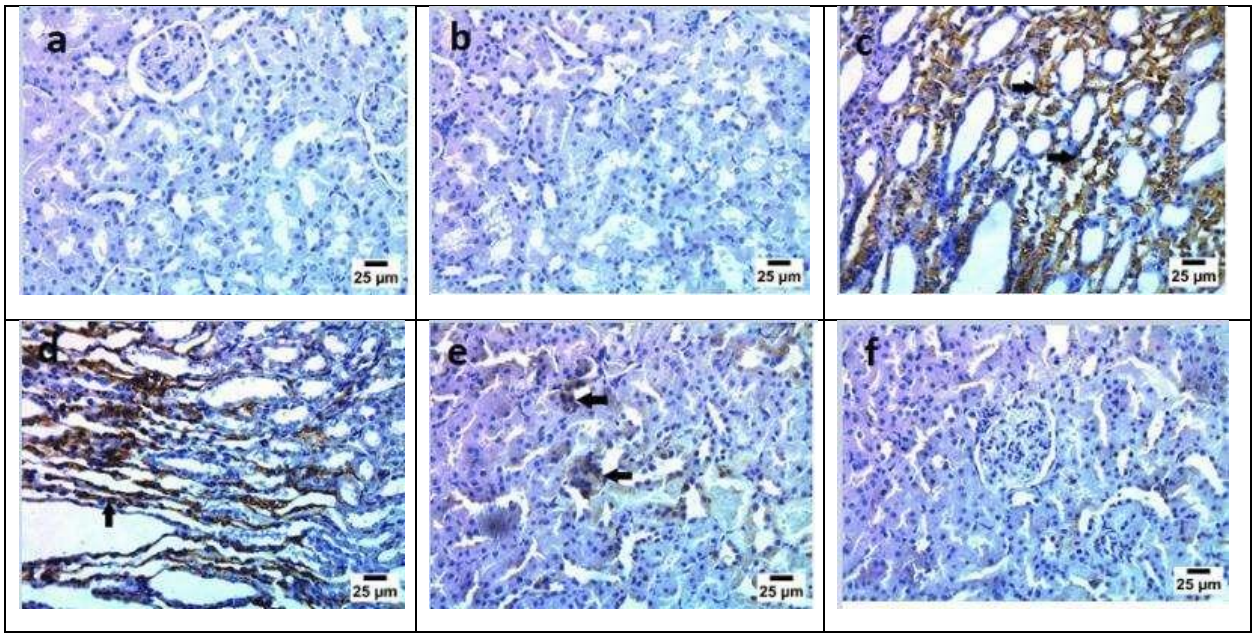


Fig.1: Immunohistochemical reaction of TNF- α in kidney tissue of rat demonstrating; (a) Cont+TW group with negative reaction for TNF-α immunostaining in the renal tubules, (b) Cont+MW group with negative immunostaining reaction of TNF-α in the renal tubules, (c) MTX+TW group with an intense postive immunostaining reaction of TNF-α in renal tubules cytoplasm (arrow), (d) MTX+MW group with moderate positive immunostaining reaction of TNF-α in renal tubules cytoplasm (arrows), (e) MTX+TW+Lis group with mild positive immunostaining reaction of TNF-α in the renal tubules and glomeruli, (f) MTX+MW+Lis group with negative immunostaining reaction of TNF-α in renal tubules cytoplasm (arrow).

As shown in **Fig. (2)**, immunohistochemical reaction in kidney tissue obtained from rats in the Cont+TW group and the Cont+MW group revealed negative reaction for TGF-β1 in the renal tubules and glomeruli **(Fig. 2a,b)**. Regarding the MTX+TW group, it showed an intense positive reaction for TGF-β1 in the cytoplasm of renal tubules **(Fig. 2c)** while the MTX+MW group revealed mild positive reaction **(Fig. 2d)**. Concerning the MTX+TW+Lis and the MTX+MW+Lis groups, both showed negative reaction for TGF-β1 in the cytoplasm of renal tubules and glomeruli **(Fig. 2e,f)**.

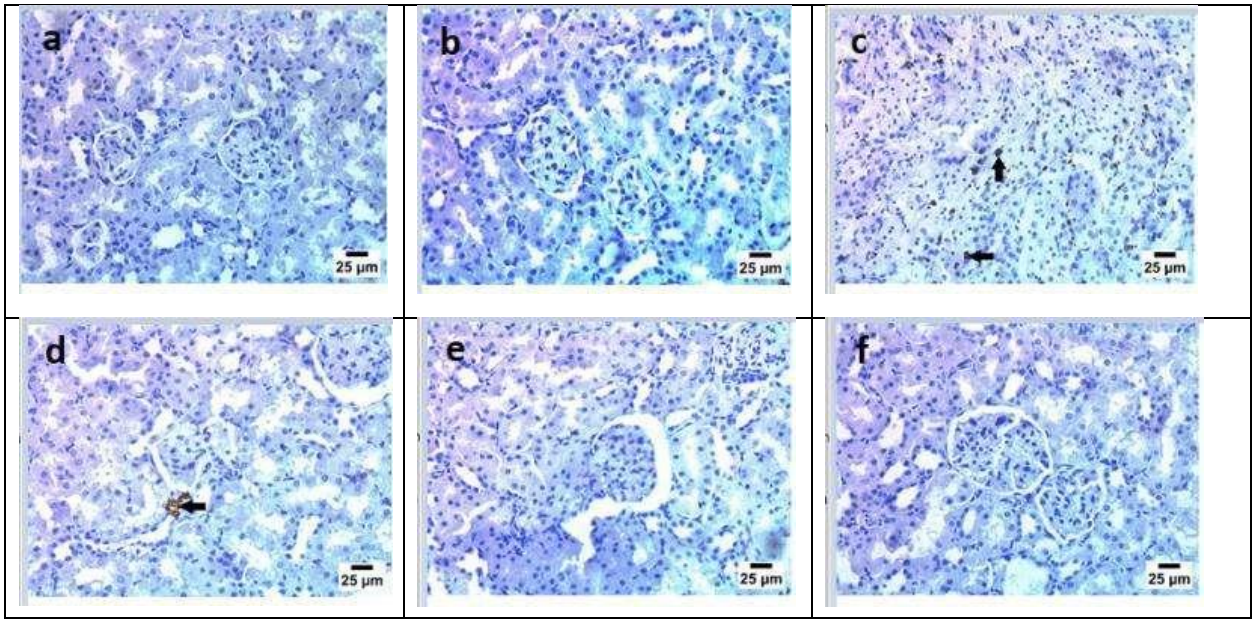


Fig.2: Immunohistochemical reaction of TGF-β1 in kidney tissue of rats illustrating; (a) Cont+TW group with negative reaction for TGF-β1 immunostaining in the renal tubules and glomeruli, (b) Cont+MW group with negative immunostaining reaction of TGF-β1 in the renal tubules and glomeruli, (c) MTX+TW group with an intense positive immunostaining reaction of TGF-β1 in the cytoplasm of renal tubules (arrow), (d) MTX+MW group with mild positive immunostaining reaction of TGF-β1 in the cytoplasm of renal tubules (arrows), (e) MTX+TW+Lis group with negative immunostaining reaction of TGF-β1 in the renal tubules and glomeruli, (f) MTX+MW+Lis group with negative immunostaining reaction of TGF-β1 in the cytoplasm of renal tubules and glomeruli (arrow).

As represented in **Fig. (3)**,the immunohistochemical examination of the kidney tissue of rats in the Cont+TW group revealed a negative immunostaining reaction for Bax in the renal tubules and glomeruli **(Fig. 3a)**. Similarly, the Cont+MW group showed negative immunostaining reaction for Bax in the renal tubules **(Fig. 3b)**. Regarding MTX+TW and the MTX+MW groups, they showed intense positive and moderate positive immunostaining reaction for Bax in the cytoplasm of renal tubules **(Figs. 3c &3d)**, respectively. Considering the MTX+TW+Lis group, it showed mild positive immunostaining reaction for Bax in the renal tubules and glomeruli **(Fig. 3e)** while the MTX+MW+Lis group showed negative immunostaining reaction for Bax in the cytoplasm of renal tubules **(Fig. 3f)**.

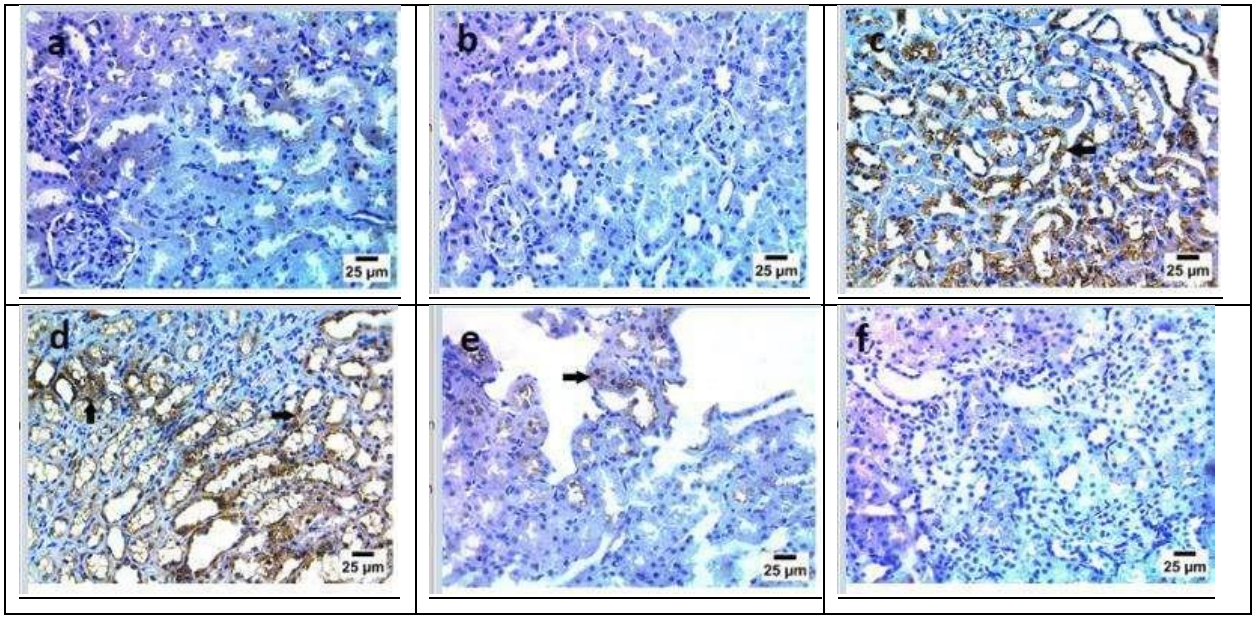
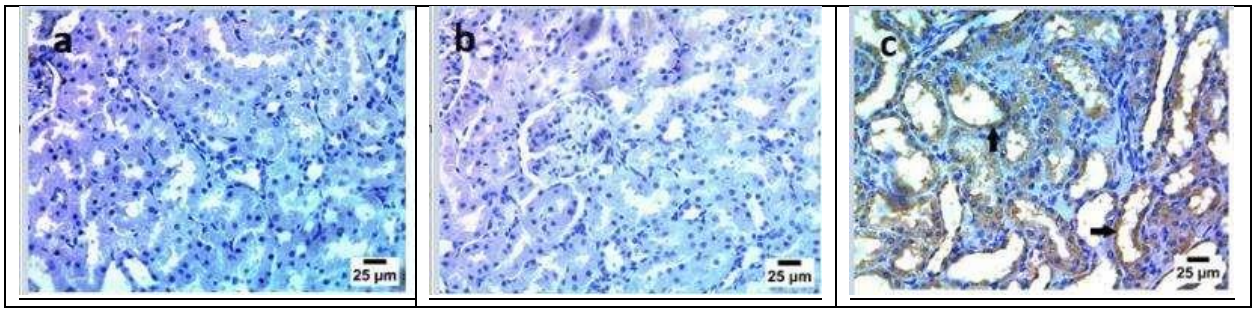


Fig.3: Immunohistochemical reaction of Bax in kidney tissue of rats clarifying; (a) Cont+TW group with negative reaction for Bax immunostaining in the renal tubules and glomeruli, (b) Cont+MW group with negative immunostaining reaction for Bax in the renal tubules, (c) MTX+TW group with intense positive immunostaining reaction for Bax in the cytoplasm of renal tubules (arrow), (d) MTX+MW group with moderate positive immunostaining reaction of Bax in the cytoplasm of renal tubules (arrows), (e) MTX+TW+Lis group with mild positive immunostaining reaction for Bax in the renal tubules and glomeruli, (f) MTX+MW+Lis group with negative immunostaining reaction for Bax in the cytoplasm of renal tubules (arrow).

As depicted in **Fig. (4)**, immunohistochemical reaction of Cas-3 in kidney tissue of rats in the Cont+TW group revealed negative reaction for Cas-3 in the renal tubules **(Fig. 4a).** Similarly,the Cont+MW group displayed negative reaction for Cas-3 in the renal tubules and glomeruli **(Fig. 4b)**. Regarding the MTX+TW group, it indicated an intense positive immune reaction for Cas-3 in the cytoplasm of renal tubules **(Fig. 4c)** while the MTX+MW group revealed moderate positive reaction **(Fig. 4d)**. For the MTX+TW+Lis group, it disclosed mild positive immune reaction for Cas-3 in the cytoplasm of renal tubules **(Fig. 4e)** while the MTX+MW+Lis group showed negative reaction **(Fig. 4f)**.



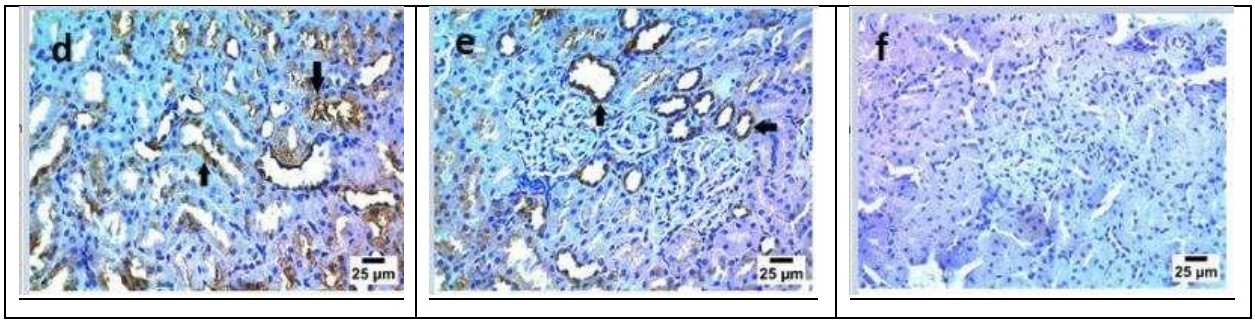


Fig.4: Immunohistochemical reaction of Cas-3 in kidney tissue of rats sections of rats explaining; (a) Cont+TW group with negative reaction for Cas-3 immunostaining in the renal tubules, (b) tissue of kidney of the Cont+MW gp with negative immunostaining reaction of Cas-3 in renal tubules and glomeruli, (c) MTX+TW group with an intense positive immunostaining reaction of Cas-3 in the cytoplasm of renal tubules (arrows), (d) MTX+MW group with moderate positive immunostaining reaction of Cas-3 in the cytoplasm of renal tubules (arrows), (e) MTX+TW+Lis group with mild positive immunostaining reaction of Cas-3 in the renal tubules, (f) MTX+MW+Lis group with negative immunostaining reaction of Cas-3 in the cytoplasm of renal tubules (arrows).

Histopathological findings

The histopathologic changes of kidney tissues assessed by using H&E staining in the different studied groups were demonstrated in **Fig. (5)**. Kidney tissue sections from rats in the Cont+TW and the Cont+MW groups manifested normal architecture of kidney tissue, glomeruli, urinary space with normal tubular structures and lining with rounded nuclei **(Figs. 5a,b)**. On the opposite side, the histopathological evaluation of kidney tissue sections stained with H&E showed that MTX group exhibits histopathological alterations compared with the control groups; the kidney tissue section derived from rat in the MTX+TW group revealed shrunken glomeruli associated with urinary spaces dilation, degeneration of tubular epithelial cell, necrosis associated with interstitial inflammatory cells and interstitial haemorrhage **(Fig. 5c)**. Regarding kidney tissue section of rat in the MTX+MW group, it displayed moderate curative effect with slight glomerular atrophy, mild urinary spaces dilation, and moderated degeneration of tubules with mild inflammatory cells **(Fig. 5d)**. Concerning the histological examination of kidney tissue section obtained from rat in the MTX+TW+Lis group, it exihibited moderate curative effect with slight glomerular atrophy, mild urinary space dilation, and tubular epithelial cells nearly normal with mild degeneration, and minor interstitial inflammatory cells **(Fig. 5e)**. In the MTX+MW+Lis group, the kidney tissue section of rat in that group exhibited noticeable ameliorative effect with slight glomerular atrophy, mild urinary space dilation, tubular epithelial cells nearly normal with mild degeneration, and minor interstitial inflammatory cells **(Fig. 5f)**.

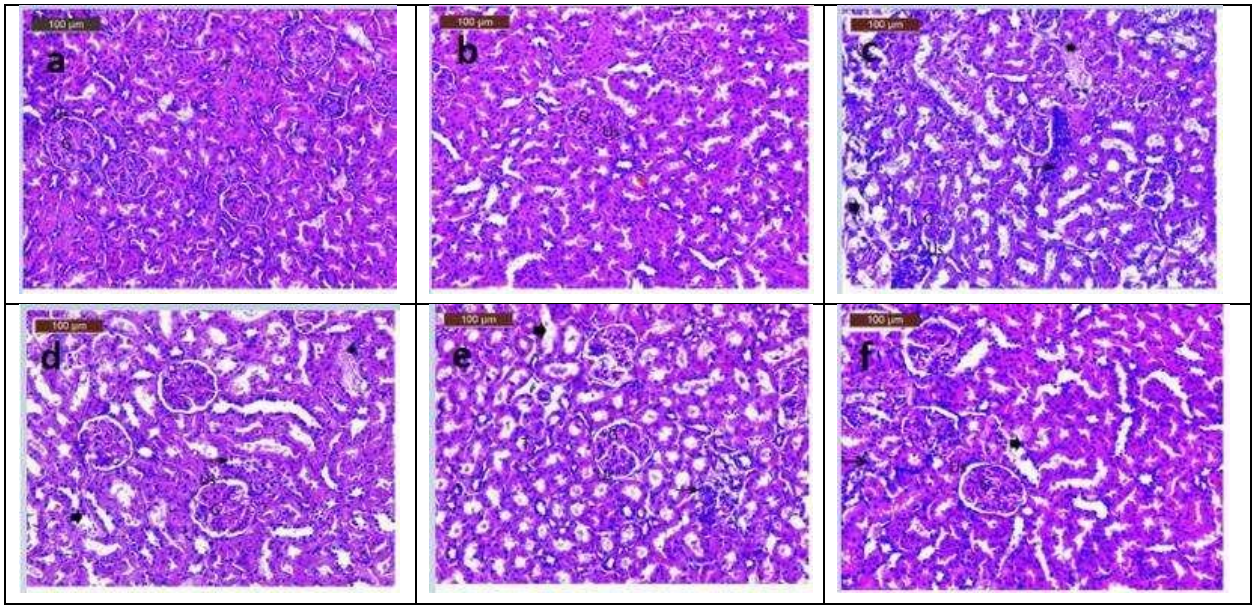


Fig. 5: Microscopic investigation of kidney tissue sections of rats in the (a) Cont+TW gp revealing the glomerulus normal structure (G), urinary space (Us) as well as tubules (T), (b) Cont+MW gp demonstrating nearly normal glomerulus structure (G), urinary space (Us) and tubules (T), (c) MTX+TW gp found shrunken glomeruli (G) with urinary space dilation (Us), degeneration of tubular epithelial cell (T), necrosis associated (arrowhead) with interstitial inflammatory (arrow) cells as well as interstitial haemorrhage (H), (d) MTX+MW gp revealing moderate curative effect with slight glomeruli atrophy (G) with mild urinary space dilation (Us), moderated tubular degeneration (arrowhead) with mild inflammatory cells (arrow), (e) MTX+TW+Lis gp found moderate ameliorative effect with slight glomeruli atrophy (G) with mild urinary space dilation (Us), tubular epithelial cell nearly normal with mild degeneration (arrowhead), and minor interstitial inflammatory cells (arrow), (f) MTX+MW+Lis gp results noticeable ameliorative effect with slight glomeruli atrophy, mild urinary space dilation (Us), tubular epithelial cell nearly normal with mild degeneration (arrowhead), and minor interstitial inflammatory cells (arrow)

4. DISCUSSION

The pharmacological activity of MTX is linked to the polymerization of MTX inside the cells, such as RBCs, to form MTX-polyglutamate (MTX-PG) [33]. Renal clearance reports up to 90% of excretion of the MTX dose as both an unchanged drug as well as its metabolite [34]**.** Theclearance of MTX possesses a long standing, well-known relationship with GFR (glomerular filtration rate), that is calculated by serum Cr as a renal clearancedelegate marker [35]. It is well-known that Cr is regularly excreted *via* the kidneys, and the impaired glomerular filtration leads to increased plasma Cr levels. Urea production occurs in the liver and it is excreted *via* the kidneys. Urea and Cr are commonly used as indices of glomerular function [36]**,** and the elevation of those blood parameters reveals kidney function deterioration [12]. In the present approach, MTX administration led to a significant increment in urea and Cr serum levels of MTX+TW and MTX+MW groups in comparison with the Cont+TW and the MTX+TW groups, respectively. This finding coincides with other studies that reported a significant elevation in serum urea as well as Cr levels in the MTX-administered group as compared with the control group [37]. The increased concentrations of urea as well as Cr in response to MTX treatment may be attributed to sharp degeneration in kidney tissues, cystic dilation of the tubular lumen as well as glomerular atrophy/hypertrophy [13].

The current research demonstrated a significant elevation in NO serum level but a significant reduction in serum TAC in the MTX+TW and the MTX+MW groups as compared with the Cont+TW and the Cont+MW groups, respectively. Our result is in harmony with the previous studies demonstrating a significant increase in NO level in MTX-intoxicated rats as compared with the controls [13]. Nitric oxide (NO), being come from induction of iNOS, may interact with superoxide anion, containing peroxynitrite that, within other effects, modifies pyrimidine as well as purine bases, leading to DNA breaks [38]**.** The upregulated NO levels can be explained by the increased levels of COX-2 and iNOS triggered by MTX administration [39]. Also, the production of ROS may intiate lipid peroxidation accompanied with inflammatory mediators releasing such as NO [40].

Consistent with our results in the current approach, Wani *et al*. [37] reported a significant depletion in TAC in kidney tissue of the MTX-administered rats as compared with the control counterparts. As revealed, MTX promoted the release of free radicals from both exogenous as well as endogenous sources. Research revealed implication of oxidative stress in the toxicity of MTX [41]. Oxidative stress can occur *via* MTX causing TAC depletion in concomitant with superoxide dismutase (SOD) as well as reduced glutathione (GSH) reduction [42]. A previous study suggested that MTX leads to rat kidney tissues oxidative stress as well as peroxidation that could be one of the causes of the nephrotoxicity induced by MTX [43].

In the current study, treatment with MW brought about a significant reduction in serum Cr level and a considerable reduction in serum urea level in the Cont+MW group as compared with the Cont+TW group. Moreover, treatment with MW resulted in a significant reduction in urea as well as Cr serum levels in MTX+MW group when compared with MTX+TW group, which indicates an improved kidney function. Our findings correlate with that of El-Hanoun *et al*. [44] who declared that geese supplied with magnetized water display signs of improved renal function as seen by attenuated serum urea and Cr levels. Additionally, more recently, Abdullaziz *et al*. [45] reported reduced Cr and blood urea nitrogen (BUN) levels in MW-treated goats as compared with the control group that was supplied with tap water. These effects of MW might be attributed to improved overall animal performance and metabolic profiles as a result of increased fluidity of MW and its higher dissolving capacity of various biological materials like minerals and vitamins which may be accompanied with improved bioavailability of the solutions to carry out their biological activity in the body [46].

It has been shown that MW has a powerful antioxidant property and possesses potent and rapidly diffusion ability into tissues [17]. In the current investigation, treatment with MW triggered a significant decrease in serum NO level while it induced a considerable elevation in TAC in serum of MTX+MW group comparing to MTX+TW group. Abdullaziz *et al*. [45]stated that drinking MW by goats for 60 days results in a significant enhancement of redox status (lower MDA levels and higher TAC) in the MW-administered group when compared with the control group. Similarly, MW could significantly enhance the antioxidant capacity and reduce oxidative stress indicated by a decrease in MDA and NO levels and an increase in SOD activity in the heart, liver and kidney in animals [47]. Consistently, El-Hanoun *et al*. [44]recorded a significant elevation in serum TAC accompanied with a significant decrease in TBARS of rabbits and geese administered MW as compared with the controls administered tap water.

ACEIs have been found to improve renal retrieval or reduce fibrotic processes after AKI [48]. In the present investigation, treatment with Lis triggered a considerable reduction in serum urea as well as Cr levels of MTX+TW+Lis group when compared with the MTX+TW group. Ajibade *et al.* [49] who stated that the treatment with Lis significantly attenuates the deleterious effects of sodium fluoride on the kidney as indicated by the reduction in BUN and Cr levels across the treated groups in comparison with the sodium fluoride-administered group. This could be attributed to the intra-renal efferent vasodilation that leads to the reduction of filtration pressure and improvement of impaired kidney function. The results of the present investigation showed that the co-treatment with MW and Lis elicited a considerable decrease of urea as well as Cr serum levels of MTX+MW+Lis group when compared with the MTX+MW group, which indicates their synergistic effect and/or accumulated effect to produce a superior influence in restoring kidney function.

In the present approach, Lis treatment elicited a significant reduction in serum NO level along with a significant elevation in serum TAC as shown in MTX+TW+Lis group comparing with MTX+TW group. Furthermore, the co-treatment with MW and Lis resulted in a significant reduction in serum NO levels in concomitant with a significant increment in serum TAC as shown in MTX+MW+Lis group comparing with MTX+MW group. Asaad *et al*. [50]found that oral administration of Lis in DOXO-treated rats resulted in a significant decline in kidney tissue oxidative stress markers (MPO and MDA) levels along with an increase in kidney tissue TAC as compared with the DOXO-treated group. Mohammed *et al*. [51]reported that the antioxidant effect of Lis could be attributed to its ability to promote the defense of antioxidant components such as GSH stores

as well as CAT activity. Finally, the reported antioxidant activity of the ACEIs may be attributed to their ability to inhibit the NADPH oxidase activity, ROS generation responsible enzyme as well as the pro-inflammatory mediators production [52]. The superior effect of Lis with MW on serum NO level and TAC in the present study could be linked with the accumulated effect of the two candidates to reduce the oxidative stress represented by the increased NO level, and induce the antioxidant defense response exemplified by TAC.

In the current research, the administration of MTX promoted a significant increase in serum NAG level in the MTX+TW and the MTX+MW groups as compared with the Cont+TW and the Cont+MW groups, respectively. This finding showed parallelism with that of Attia *et al*. [53]whoreported that MTX injection significantly increases urinary NAG levels when compared with the control group; indicating that MTX induced AKI as NAG has been considered as the fitting AKI parameter.

In the current approach, MTX triggered a significant increase in serum MIP-2 levels in the MTX+TW and the MTX+MW groups as compared with the Cont+TW and the Cont+MW groups, respectively. The chemokine (MIP-2) has been recognized to induct neutrophils [54]and researches showed that recruitment of neutrophil can be related to the progression and development of glomerular diseases. In mesangio proliferative glomerulonephritis (MPGN) rat model, glomerular NO was found to cause induction of MIP-2 expression that in turn causes recruitment of neutrophil [55]. Methotrexate administration has been demonstrated to enhance NO production in rats owing to the activation of iNOS [39]. This could explain the increased serum level of MIP-2 in the groups of rats administered MTX in the current study.

Overexpression of KIM-1 in the proximal convoluted tubule (PCT) epithelial cells indicates AKI [56]. Moreover, Alkuraishy *et al*. [57]reported that KIM-1 serum concentration is correlated with the renal proximal tubule damage during the nephrotoxicity. In the current study, MTX administration significantly increased serum KIM-1 level in the MTX+TW and the MTX+MW groups as compared with the Cont+TW and the Cont+MW groups, respectively. This finding coincides with that of Abd El-Twab *et al*. [14] who declared that MTX treatment induces renal damage status, as indicated by a significant elevation in serum KIM-1 levels in MTX-treated rats as compared with the control group. KIM-1 was found to be up-regulated due to PCT injury to inhibit apoptosis as well as tubular re-epithelization stimulation [58].

the current research showed that the control group administred MW (Cont+MW) or in the MTX group (MTX+MW) resulted in a significant decrease in serum NAG, MIP-2 and KIM-1 levels as compared with the Cont+TW and MTX+TW groups, respectively. Ebrahim and Azab [47] reported that there is an amelioration in the quality of water in case of the magnetic field exposure, with an enormous pH change, total hardness, salinity, conductivity, dissolved solids, dissolved oxygen, organic matter as well as total count of bacteria, and these changes could progress biochemical markers in the blood as well as the antioxidant status. Moreover, El-Kholy *et al*. [59] reported that all the antioxidant and renoprotective effects of MW may be attributed to mineral solubility elevation and enhancement of nutrient transfer through cell membranes, thus improving uptake as well as utilization.

In the present study, Lis treatment produced a significant depletion in NAG serum level of MTX+TW+Lis group comparing with the MTX+TW group. Also, the co-treatment with MW and Lis resulted in a significant depletion in NAG serum level of MTX+MW+Lis group comparing with the MTX+MW group. This finding agrees with that of Öktem *et al.* [60] who recorded significant decline in serum and urinary NAG after administration of Lis indicating the improvement of kidney functions. This finding could be explained by the interference of Lis with peroxynitrite-related pathways [61].

In the present investigation, Lis treatment significantly decreased serum MIP-2 level in the MTX+TW+Lis group as compared with the MTX+TW group. Likewise, the co-treatment with MW and Lis triggered a significant decrease in serum MIP-2 level in the MTX+MW+Lis group as compared with the MTX+MW group. As we mentioned above, NO plays a role in inducing MIP-2 expression in the kidney [55]. Lis has been found to inhibit iNOS expression in kidney tissue with a consequent reduction of NO level in STZ-induced diabetic nephropathy in rats [62]. This could explain the significant decrease in MIP-2 serum level in rats administered Lis in the current investigation.

In the current investigation, Lis treatment caused a significant decrease in KIM-1 level in the MTX+TW+Lis group as compared with the MTX+TW group. Correspondingly, the co-treatment with MW and Lis significantly decreased KIM-1 level in the MTX+MW+Lis group as compared with the MTX+MW group. This finding is in agreement with that of Asaad *et al*. [50] who revealed that the administration of Lis (20 mg/kg) promotes a significant decrease in kidney tissue KIM-1 as compared with the DOXO-induced group. The reduction of KIM-1 can be attributed to the blockade of the renin-angiotensin system (RAS) caused by ACEIs [63].

In the current study, the immunohistochemical investigation of kidney tissue of rats in the MTX+TW group exhibited an intense positive reaction for TNF-α while the MTX+MW group revealed moderate positive reaction. This finding corresponds with that of Elseady *et al*. [64]who indicated that MTX administration increases the immunohistochemical reaction of TNF-α in the kidney tissue demonstrating an inflammatory response. A similar result of increased TNF-α in the MTX-injected rat group as compared with the control group was reported by Wani *et al*. [37]. The increased expression of TNF-α may be attributed to inflammation which has a major function in the MTX-triggered renal damage pathogenesis. MTX-evoked generation of ROS reveals many stress signaling pathways like the nuclear factor kappa B (NF-κB) cascade as well as its downstream proinflammatory signals, such as TNF-α and cyclooxygenase-2 (COX-2) [39].

In the current study, the MTX+TW group exhibited an intense positive immunohistochemical reaction for TGF-β1 in the renal tubules [65]. Sherif *et al*. [66] recorded a remarkable renal TGF-β1 mRNA expression up-regulation after MTX treatment comparing with the normal control group. The renal tissues TGF-β1 upregulation was found in drug-induced nephrotoxicity models such as MTX [67].

In the current investigation, the immunohistochemical section obtained from rat in the MTX+MW group exhibited a moderate positive reaction for renal tubules cytoplasm TNF-α while MTX+TW group showed an intense reaction. Moreover, the immunohistochemical investigation in kidney tissue obtained from rat in the MTX+MW group revealed a mild positive reaction for TGF-β1 in the renal tubules cytoplasm while MTX+TW group showed an intense reaction. A study of Ingalagi *et al*. [68] mentioned that MW revealed an anti-inflammatory effect on Freund's adjuvant-induced arthritis rats model and that

the possible mechanism of action may be due to any of the following reasons, either single or in combination; increased blood flow at the site of injury, increased oxygen carrying capacity of blood, alteration in ion movement, i.e. migration of calcium ions away from the injury site, modulation in pH after magnetization, regulation of endocrine gland secretions, adjustment of enzyme activation and other biochemical processes, and restoring the altered metabolism. Moreover, Lindinger [69] reported that drinking MW improves blood/systemic antioxidant and inflammation profiles. Furthermore, the report of Zhou*et al*. [70] declared that the NF-κB inhibitory protein, IκB content is increased in the blood of patients with diabetic kidney disease after magnetotherapy and that the NF-κB content is reduced after the magnetic intervention.

In the current research, the kidney tissue of rats in the MTX+TW+Lis group exhibited mild positive reaction for TNF-α in the renal tubules and glomeruliwhile the MTX+MW+Lis group revealed a negative immune reaction for TNF-α in the cytoplasm of renal tubules. This finding agrees with other studies that declared that Lis exhibits an immunomodulatory effect by inhibiting the release of IL-1β [71], IL-6 and IL-8 by various cells [72], the production of reactive oxygen intermediates [73], the liberation of IL-12 and IFN-gamma production [74], and the increased of plasma concentrations of TNF-α and c-reactive protein [75]. These immunomodulatory effects of Lis could be attributed to its antioxidant effect as it inhibits ROS output and lipid peroxidation, thus reducing the induction of NF- κβ [76] which stimulates many pro-inflammatory genes expression, as those encodingTNF-α [77]. In the current work, regarding the kidney tissue obtained from rats in both of the MTX+MW+Lis and the MTX+TW+Lis groups, they revealed a negative immune reaction for TGF-β1 in the renal tubules and glomeruli. This finding is in accordance with that of Hu *et al*. [78]whoobserved that enalapril treatment can decrease kidney content of TGF-β1 levels. This effect of enalapril could be attributed to the depeltion of ANGII-mediated promotion of TGF-β1 synthesis[79].

Oxidative stress and inflammation have been found to be related to cellular damage induction as well as apoptosis. In the current study, regarding the MTX+TW and the MTX+MW groups, they displayed an intense positive immune reaction for Bax in the renal tubules. This finding concurs with other studies that reported an increased expression of the pro-apoptotic protein Bax in rat kidneys following MTX administration [14]. Abd El-Twab *et al*. [14] stated that the activation of NF-κB with subsequent stimulation of leucine-rich repeat-containing protein-3 (NLRP3) inflammasome as well as the release of inflammatory cytokines induced apoptosis in the kidney of MTX-intoxicated rats. Moreover, these investigators proved that the elevated production of ROS may be considered as the major culprit behind the activation of the kidney NF-κB/NLRP3 inflammasome signaling in MTX-intoxicated rats.

In the current approach, the kidney tissue of rats in the MTX+TW and Cont+MW groups revealed an intense positive reaction for Cas-3 in the cytoplasm of renal tubules. This finding corresponds with the results of Wani *et al*. [37] who stated that Cas-3 immunohistochemical expression is markedly elevated in MTX-treated group comparing with the control one. The elevated Cas-3 expression indicates apoptotic activity increment in MTX-intoxicated group because of oxidative stresspromotion[80]that facilitates fragmentation of DNA as well as denaturation which cause c-Jun N-terminal kinase activation and programmed cell death initiation through caspase cascades [81]**.** Additionally, Akhigbe and Ajayi [82]attributedthe considerable surge in the expression of Cas-3 gene levels induced by MTX, to the change in the mitochondrial membrane permeability and release of cytochrome c into the cytosol, which promotes the apoptosome formation, with subsequent activation of Cas-3.

In the current research, the immunohistochemical examination of the kidney tissue of rats in the Cont+TW group revealed a negative immunostaining reaction for Bax in the renal tubules and glomeruli. Similarly, the Cont+MW group showed a negative immunostaining reaction for Bax in the renal tubules. Regarding the MTX+TW and the MTX+MW groups, they showed intense positive and moderate positive immunostaining reactions for Bax in the cytoplasm of renal tubules, respectively. The renoprotective effect of MW stems from its antioxidant [59], anti-inflammatory [69] and anti-apoptotic effects [83]. It has been demonstrated that the oxidative stress elevation is considered as an inflammatory as well as apoptotic stimulus that results in cell loss [84].

In water, it was demonstrated that a magnetic field causes changes in the hydrogen protons via magnetic resonance, and the H-bond gets weaker and distorted, affecting protonation as well as hydration of ions; thus, hydrogen-rich water could scavenge ROS [82]**.** Nakashima-Kamimura *et al.* [83] observed that hydrogen water ameliorates apoptotic cell death in the kidney of mice treated with cisplatin-induced nephrotoxicity. Thus, MW in the current study could ameliorate apoptosis of kidney cells, as indicated by the decreased expression of Bax and Cas-3, through its ability to scavenge ROS and inhibit oxidative stress.

In the current investigation, the MTX+TW+Lis group presented a mild immunostaining reaction of Bax in the renal tubules and glomeruli while the MTX+MW+Lis group showed a negative immunostaining reaction of Bax in the cytoplasm of renal tuules. This agrees with Charan Sahoo *et al*. [86] who demonstrated that benazepril administration upregulates the anti-apoptotic protein Bcl-2 expression, and downregulated the pro-apoptotic protein Bax expression. Bcl-2 upregulation could be related to the heterodimers formation with Bax, leading to no/less free Bax protein obtainable for homodimerization. It is well known that if Bax homodimerizes predominantly, cell death occurs, while when Bcl-2 as well as Bax heterodimerization prevails, cells may survive [87]. In the current approach, the kidney tissue of rats in the MTX+TW+Lis group demonstrated a mild immunohistochemical reaction for Cas-3 in the cytoplasm of renal tubules while the MTX+MW+Lis group showed a negative reaction. This comes in line with the finding of Asaad *et al*. [50] which indicates that the administration of Lis (20 mg/kg) significantly depleted expression of Cas-3 with a decreased percentage of kidneys positively stained cells. Asaad *et al*. [50]attributed the significant decrease in cleaved Cas-3 of Lis- administered group comparing with the DOXO-treated group to the up-regulation of constitutive eNOS. The increased NO generation via activation of iNOS has been showed to promote production of ROS through NOX2; following activation of caspase-8 which begins mitochondrial apoptotic signaling associated a cleavage of procaspase-3, resulting in ultimately apoptosis [88]. Accordingly, Lis ability to reduce the imbalance

between eNOS/iNOS which explains its regulatory effect on NO production, as shown in the present study, could be the mechanism for Lis to inhibit Cas-3-induced renal apoptosis.

In the current study, the histopathological examination confirmed the biochemical changes in serum as well as the immunohistochemical manifestations. The kidney tissue derived from rat in the MTX+TW group revealed shrunken glomeruli with urinary spaces dilation, degeneration of tubular epithelial cells. and necrosis associated with interstitial inflammatory cells as well as interstitial hemorrhage. These observations are in line with those reported by other previous studies which revealed that MTX induced significant renal tubules alterations like large hyaline casts, tubular necrosis and dilatation, tubular epithelial desquamation as well as vacuolation and glomerulosclerosis [89, 37]. It has been found that the MTX precipitation and its metabolite, in the renal tubules results in inflammation, tubular necrosis as well as histological damage [90].

Concerning the MTX+MW group, the histopathological examination revealed moderate improvement effect accompanied slight glomerular atrophy, mild urinary spaces dilation, and degeneration of moderated tubular with mild inflammatory cells. These findings fit those of Ahmed *et al*. [91] who found that the MW protects the glomerular structure against type 2 diabetic nephrotoxicity.

The histopathological examination of the kidney tissue section of rat obtained from the MTX+TW+Lis group exhibited a moderate improvement effect with slight glomerular atrophy, mild urinary space dilation, and tubular epithelial cells nearly normal with mild degeneration, and minor interstitial inflammatory cells. These manifestations were nearly similar in the MTX+MW+Lis group with a more noticeable ameliorative effect. These observations are consistent with those of Asaad *et al*. [50]who reported that Lis oral treatment (20 mg/kg) revealed regression of the histopathological lesions caused by DOXO. These results could be due to the Lis curative effect *via* its antihypertensive, antioxidant as well as anti-apoptotic effects [50].

5. CONCLUSION

In conclusion, the current research provides scientific evidence on the synergistic effect between magnetized water and lisinopril. Thereby, one can postulate that drinking magnetized water regularly as a supportive agent with Lis during the treatment of acute kidney injury or other nephropathies may produce more efficient curative effect of this drug. This beneficial role of magnetized water in this concern may be attributed to its antioxidant, anti-inflammatory and anti-apoptotic effect.

List of abbreviations:

AKI: acute kidney injury; ACEI: angiotensin-converting enzyme inhibitor; Bax: BCL2-associated x protein; Cas-3: caspase-3; CKD: chronic kidney disease; Cont: control; Cr: creatinine; ICU: Intensive care Unit; IFN-γ, interferon-gamma; KIM-1: kidney injury molecule-1; Lis: lisinopril; MTX: methotrexate; MIP-2: macrophage inhibiting protein-2; MW: magnetized water; NAG: N-acetyl-beta-D-glucosaminidase; NO: nitric oxide; TAC: total antioxidant capacity; TGF-β: transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; TW: tap water.

Conflicts of interest

There are no conflicts to declare

6. REFERENCES

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