Original article

Lucrative antioxidant effect of metformin against cyclophosphamide induced nephrotoxicity

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A R T I C L E   I N F O

A B S T R A C T

Cyclophosphamide is anticancer drug with a well-Known nephrotoxicity. This work was applied to study the lucrative antioxidant influence of metformin as co-therapy on the nephrotoxicity induced by cyclophosphamide in the treatment of different cancer diseases. Four groups of male Sprague Dawley rats were used; Control group (C) received single I.P. injection of 0.2 ml saline, Metformin (MET) group received daily gavage of 200 mg/kg metformin for two weeks, Cyclophosphamide (CP) group received single I.P. injection of 200 mg/kg CP, Protector group (CP.MET) received daily gavage of 200 mg/kg metformin for two weeks and single I.P. injection of 200 mg/kg CP at day 7. By day 14 rats were euthanized. Samples were collected from kidney tissues and blood for kidney function evaluation, histopathological and assessment of oxidative stress markers. The results disclosed that CP yields many functional and structural damage to the kidney, worsened oxidative stress markers and kidney function indicators. The protector group displayed better kidney tissue morphology, acceptable kidney function indicators as well as satisfactory oxidative stress markers.

In assumption, metformin could be combined with CP owing to its lucrative effect counter to CP persuaded nephrotoxicity.

1. Introduction

Cancer refer to a wide-ranging disease that can disturb diverse organs in the body (Pearson-Stuttard et al., 2018). There are about 200 diverse recognized kinds of cancers (Brown et al., 2019; de La Puente-Yagüe et al., 2018; Moses et al., 2018).

Cyclophosphamide (CP) is one of alkylating agent used expansively for several malignancies treatment as well as an immunosuppressive in case of organ transfer, multiple sclerosis, systemic lupus erythematosus and other benign diseases (Abdul Razak et al., 2019; Awad & Stuve, 2009; Kim et al., 2009; Mielcarek et al., 2016; Petri, 2004). Cyclophosphamide similar other anticancer agents produces toxicity in normal tissues as well as in malignant tissues. The dose of CP as anticancer has been stated to produces kidney damage, acute bladder inflammation and injury of liver, plus apoptosis (Ayhanci et al., 2009; Sharma et al., 2000; Shulman et al., 1980).

Cytotoxicity of CP related to one of its active metabolites; Acrolein. It adversely binds to reduced glutathione (GSH) causes increased Reactive Oxygen Species (ROS) consequently lipid peroxidation and oxidative stress on the human body, therefore initiating cell death both apoptosis or necrosis and oncosis (Kern & Kehrer, 2002).

The oxidative stress induced by CP on renal tissue was not cleared well. Studies have also proposed that antioxidant agents

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with free radical scavenger activity are effective in alleviating toxic side effects of anticancer drugs (Manda & Bhatia, 2003).

Metformin is the broadest anti-diabetic insulin sensitizer drug. There are many studies described that metformin diminishes lipid peroxidation and improves the antioxidant defense activity as stated by (Faure et al.,1999; Khouri et al., 2004; Rizk et al., 2020). Metformin weakens the mitochondrial permeability transition pore preventing oxidative stress-induced apoptosis (Jacotot et al., 1999).

Thus, this work aim to examine the possible reno-protective influence of metformin against cyclophosphamide.

2. Material and methods

2.1. Animal

Twenty-four male Sprague Dawley rats weight 180 g–220 g and aged 2–3 months old were adapted for a week before beginning the treatment. The animals were kept back in a well-ventilated room inside polycarbonate cages at a temperature of 23 °C ± 2 °C, under standard laboratory settings with 50% ± 10% humidity and a cycle of 12 h light and 12 h dark. The rats were providing standard rodent diet and water ad libitum. All ethical themes of lab animal procedures were considered wisely, and the experimental procedure was agreed by the faculty of Vet. Med. Cairo University ethical committee.

2.2. Experimental protocol

The rats were randomly allocated into four groups comprising of six animals each. Control group (C) received single I.P. injection of 0.2 ml saline, Metformin (MET) group received daily gavage of 200 mg/kg metformin for two weeks, Cyclophosphamide (CP) group received single I.P. injection of 200 mg/kg CP, Protector group (CP.MET) received daily gavage of 200 mg/kg metformin for two weeks and single I.P. injection of 200 mg/kg CP at day 7. By day 14 rats were euthanized.

2.3. Animal sampling

Animals were euthanized on day 14 of the management by injection of sodium pentobarbital (60 mg/kg, I.P.). Blood samples and kidney tissues were collected for assessment of kidney function, oxidative stress markers, and renal tissue morphology.

2.4. Clinical chemistry

The serum samples were obtained for evaluation of blood urea nitrogen (BUN) and creatinine on the word of the instruction of the commercial kits (Bio diagnostic Co.)

2.5. Oxidative stress markers

Renal tissue samples were measured for the level of reduced glutathione (GSH) as described by (Beutler et al., 1963) and for the level of peroxidation of lipid, expressed by Malondialdehyde (MDA) formation, as described by (Ohkawa et al., 1979) using commercial kits (Bio diagnostics).

2.6. Histological analysis

The rat's kidneys were fixed with formalin (10% neutral formaldehyde solution) and embedded in paraffin then routine histological preparations were followed. Renal tissues samples were sliced into 5.0 μm thick serial sections and stained with Hematoxylin-Eosin (H&E), Mallory Trichrome stain (MT) for the

inspection of renal morphology and the collagen of connective tissue (Lamar, 2011).

2.7. Immunohistochemistry

The sections were deparaffinized and rehydrated. Antigen retrieval with citrate buffer (pH6.0) was achieved at 700 W by heating the sections in a microwave for 10 min. After blocking with 3 ml/L H_2O_2 the sections were incubated. The primary antibodies directed against BAX (Thermo, Waltham, MA, USA) in dilution of the ultra-vision quanta detection system (Thermo Scientific) (Ayhanci et al., 2016).

2.8. Image analysis

The mean area percentage was evaluated randomly on ten non-overlapping microscopic fields from each slide and observed at x400 with Leica Quin 500 LTD using the software Quin 500 (England).

2.9. Statistical analysis

Data analysis displayed as means ± error of the means (SEM) and the analysis was completed with SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Student's t-test Comparison of means was performed. A value of $P \leq 0.05$ was considered statistically significant (Armitage and Berry, 1994).

3. Results

3.1. Biochemical parameters

The control group exhibited normal BUN and creatinine levels (35.33 ± 1.45 & 0.5 ± 0.02, respectively) while the CP group exhibited higher levels of the BUN and creatinine (80 ± 2.52 & 1.4 ± 0.02, respectively). The BUN and creatinine levels (mg/dl) designated a significant ($P \leq 0.05$) elevation in the CP group related to the control group. On the other hand, there were significant enhancements in BUN and creatinine levels in the protector group (44 ± 1.73 & 1 ± 0.19, respectively) as illustrated in (Table 1).

3.2. Oxidative stress markers

CP group rats expressed an increase in renal MDA level and a decrease in its GSH content. While rats of the protector group displayed a significant protective values against oxidative stress induced by CP in the kidney. The GSH content presented a significant improvement in the protector group in contrast to that of the CP group ($P \leq 0.05$) (Table 1). The protector group ensured a significant decrease in the level of the MDA in contrast to the CP group ($P \leq 0.05$) (Table 1).

3.3. Histopathological results

In this study kidney of control and metformin group showed normal histological appearance and normal size of glomeruli in the renal corpuscles (Fig. 1A & B) respectively and normal epithelial lining to proximal convoluted tubules and distal convoluted tubules (Fig. 2A & B). In addition, normal renal tubules in the renal medulla (Fig. 3A & B). While in CP group some glomeruli appeared shrunken and others appeared dilated with obliteration of urinary space and congestion in the blood vessels (Fig. 1C), lymphocytic infiltration in renal cortex (Fig. 2C & D), and renal medulla (Fig. 3D), necrosis in the renal proximal convoluted tubules

Table 1
Kidney Biochemical parameters of different groups.

	Control (C)	CP	CP.MET	MET
BUN (mg/dl)	35.33 ± 1.45	80 ± 2.52[a]	44 ± 1.73[b]	38 ± 2.08[b]
Creatinine (mg/dl)	05.33 ± 0.02	1.4 ± 0.04[a]	1 ± 0.03[a,b]	0.7 ± 0.05[b]
GSH (mmol/g. tissue)	1.23 ± 0.15	0.33 ± 0.08[a]	0.79 ± 0.15[a,b]	1.07 ± 0.15[b]
MDA (nmol/g. tissue)	7.18 ± 1.22	18.14 ± 1.13[a]	13.06 ± 1.16[b]	8.09 ± 0.68[b]

Data represented as Mean ± SE.
Abbreviations: C, control group; CP, group received cyclophosphamide only; CP. MET, group received cyclophosphamide and metformin; MET, group received metformin only
[a] Indicate significant difference from corresponding control group at $P \leq 0.05$.
[b] Indicate significant difference from corresponding CP group at $P \leq 0.05$.

(Fig. 2D), detached epithelium in the renal tubules and edema (Fig. 2E) as well as hyaline cast in renal tubules in cortex and medulla were observed (Figs. 2D & 3C). In protector group enhanced glomerular size and renal tubule epithelial lining with few congestions in the blood vessels in the cortex (Figs. 1D & 2F) and medulla (Fig. 3E).

Tissue fibrosis were examined by determination of collagen deposition in renal tissue. CP group expressed increased deposition of collagen (Fig. 4C & D), the protector group showed moderate deposition (Fig. 4E) while minimal collage deposition was observed in control and metformin groups (Fig. 4A & B).

3.4. Immunohistochemistry for detection of apoptosis

The renal cell apoptosis was determined by BAX expression in different groups. The CP group showed increased BAX immune expression reflected by strong positive Bax immunostaining (Fig. 5C & D), while the control and metformin groups exhibited significance decrease immunostaining of BAX (Fig. 5A & B). The protector group showed mild BAX immunostaining (Fig. 5E & F).

3.5. Image analysis

The control group mean area percentage of BAX expression was (0.43 ± 0.05). Which was significant to CP group (2.5 ± 0.23). In the same line, the protector group was significant to CP group with (1.2 ± 0.34) mean area percentage (Table 2, Figs. 5 & 6).

4. Discussion

The toxic-clearance role played by the kidney against various toxins in the body is conserved by a healthy kidney. The shortcomings of renal injury include the lessening of the clearance capability of the kidney (Sharma et al., 2017). The present study centers on the defending effect of metformin in contrast to the Reno-toxicity persuaded by cyclophosphamide. Cyclophosphamide is alkylating agent, used as antineoplastic drug for dealing with various malignancies (Lawson et al., 2008; Moignet et al., 2014).

In addition to the pharmacological action of CP several toxic side effects were described (Papaldo et al., 2005). Reno-toxicity is one of Cyclophosphamide chief toxic effects (Manda and Bhatia, 2003).

Cyclophosphamide has a fundamental role in the depletion of the antioxidant defense mechanism of the kidney and production of ROS and MDA (Manda and Bhatia, 2003). The production of inflammatory cytokines is the chief result of oxidative stress persuaded by cyclophosphamide in kidney tissue (Sharma et al., 2017).
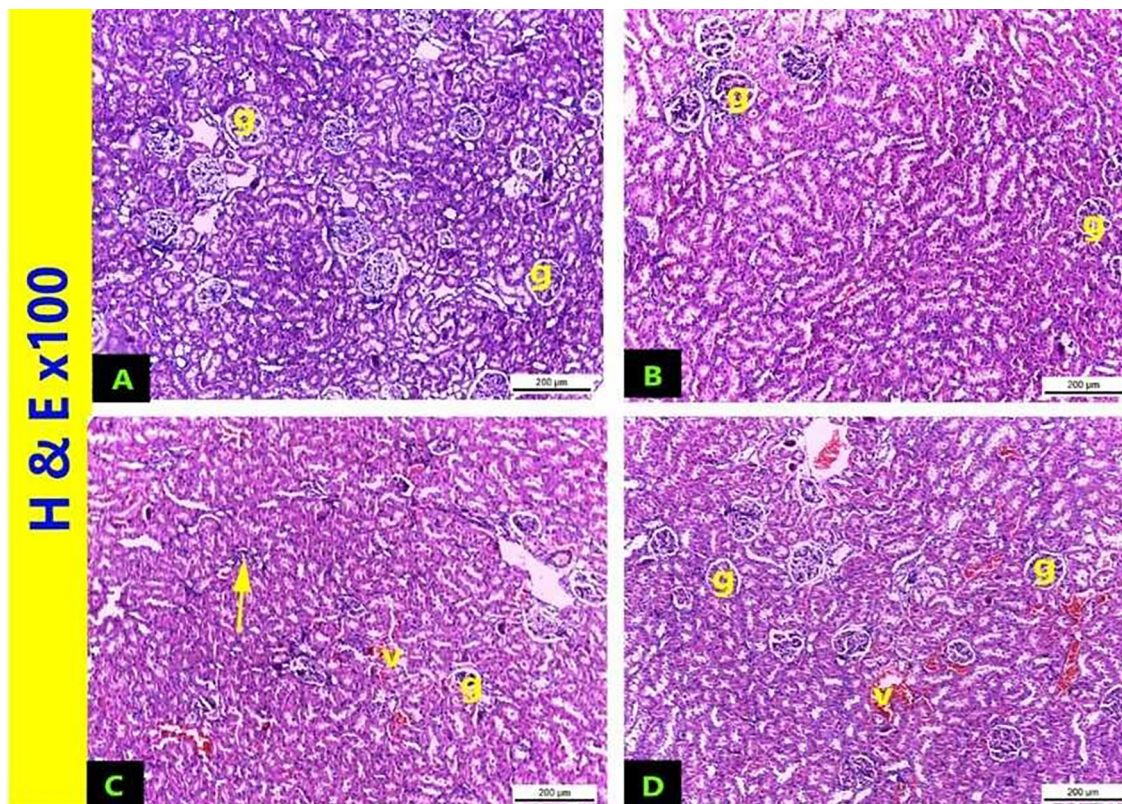

Fig. 1. Light microphotographs of sections form male Sprague Dawley rat's renal cortex showing A: normal glomeruli (g) in control group. B: normal glomeruli (g) in metformin group. C: shrunken glomeruli (g), dilated glomeruli with obliteration of urinary space (arrow) and congestion in the blood vessels (V) in CP exposed group. D: normal glomeruli (g) congestion in the blood vessels (V) in metformin plus CP treated group. (H&E X100)
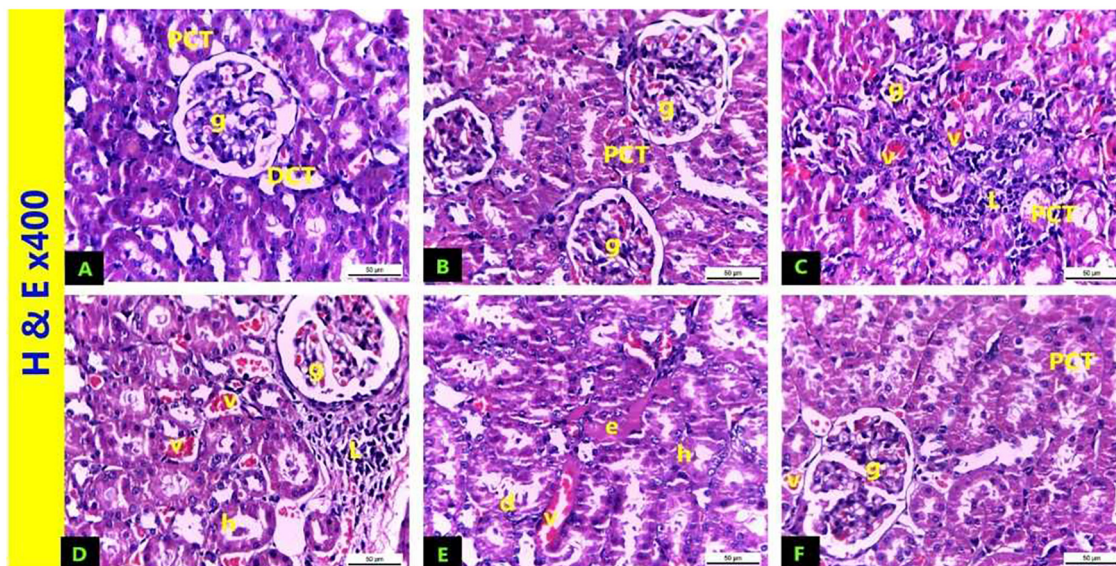
Fig. 2. Light microphotographs of sections form male Sprague Dawley rat's renal cortex showing A: normal glomeruli (g), proximal convoluted tubules (PCT) and distal convoluted tubules (DCT) in control group. B: normal glomeruli (g) and proximal convoluted tubules (PCT) in metformin group. C: dissociation of the glomeruli (g), lymphocytic infiltration (L), necrosis in the proximal convoluted tubules (PCT) and congestion in the blood vessels (V) in CP exposed group. D: shrunken glomeruli (g), lymphocytic infiltration (L), hyaline cast in the proximal convoluted tubules (h) and congestion in the blood vessels (V) in CP group. E: Edema (e), hyaline east (h) and detached epithelium in the proximal convoluted tubules (d) and congestion in the blood vessels (V) in CP exposed group. F: normal glomeruli (g) proximal convoluted tubules (PCT) and congestion in the blood vessels (V) in metformin plus CP treated group. (H&E X400)

Metformin is insulin sensitizer drug designated to type 2 diabetes mellitus treatment (Bailey, 1992). It has numerous clinical trials also for the management of cardiovascular diseases and hepatotoxicity through enhancement of mitochondrial redox state (Ling et al., 2017; Rizk et al., 2020).

Cytotoxicity evoked by anticancer drugs related to its oxidative stress. The later provoked by diminution of GSH content and increase the production of MDA (Hashem et al., 2019).

In our study, we detected an improvement in renal function parameters (BUN & Creatinine) also augmentation in redox status of the kidney in the protector group in contrast to the cyclophos-phamide group. The increase in the serum BUN & Creatinine levels in the CP group are well-suited with the report (Sharma et al., 2017).

Our results are subsequent in vivo of (Ling et al., 2017; Mansour et al., 2017); they showed the Reno-protective role of metformin in contradiction of many types of toxicity comprising anticancer drugs such as cisplatin, methotrexate, ethanol, and arsenic trioxide toxicity.

The mechanism of metformin Reno-protective mechanism counter to Reno-toxicity of anticancer drugs may be referred to it increased PPAR-γ levels and activation of AMPK. Wherever
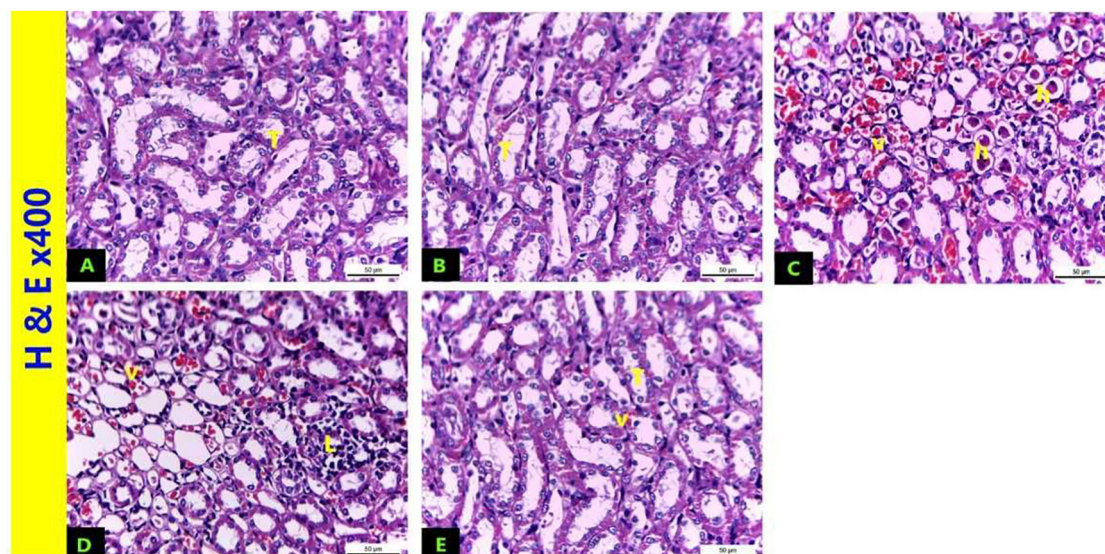

Fig. 3. Light microphotographs of sections from male Sprague Dawley rat's renal medulla showing A: normal renal tubules (T) in control group. B: normal renal tubules (T) in metformin group. C: hyaline cast in renal tubules (h) congestion in the blood vessels (V) in CP exposed group. D: lymphocytic infiltration (L), congestion in the blood vessels (V) in CP exposed group. E: normal renal tubules (T) and congestion in the blood vessels (V) in metformin plus CP treated group. (H&E × 400)
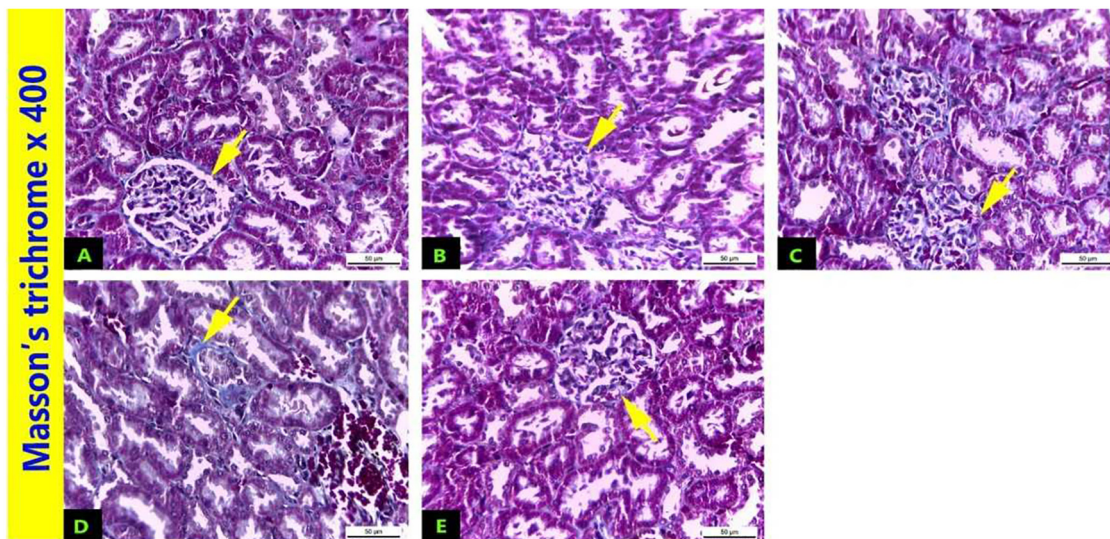
Fig. 4. Light micrograph of sections from male Sprague Dawley rat's kidney showing A: minimal collagen fibres (arrow) in control group. B: minimal collagen fibers deposition (arrow) in metformin group. C: marked increase of collagen fibers deposition (arrow) in CP group. D: marked increase of collagen fibers deposition (arrow) in CP group. E: moderate collagen fibers deposition (arrow) in metformin plus CP treated group. D: (Masson's trichrome × 400).
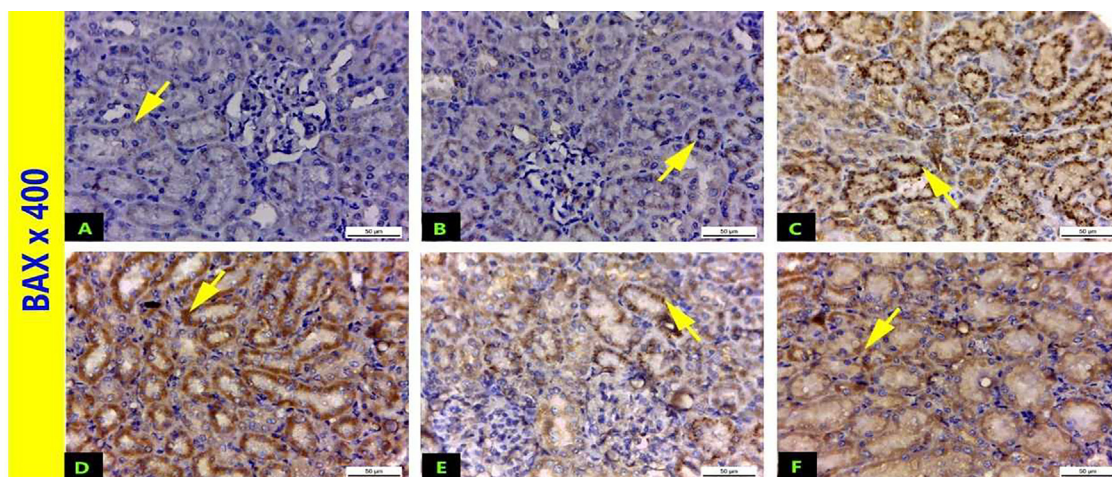

Fig. 5. Light micrograph of BAX Immunohistochemistry of kidney section from male Sprague Dawley rat showing: A: minimal immune expressions of BAX in the renal tubules in control group. B: minimal immune expression of Bax in metformin group. C: marked increase immune expression Bax in CP group. D: marked increase immune expression of Bax in CP group. E: moderate immune expression of Bax in the metformin plus CP treated group. F: moderate immune expression of Bax in the metformin plus CP treated group (BAX × 400).

PPAR-γ initiation could diminish inflammation and oxidative stress which will reflect on inhibition of apoptosis as cited by (Razavi-Azarkhiavi et al., 2016; Roohbakhsh et al., 2017); AMPK stimulation prevents TNF-α-induced apoptosis and kidney injury (Saeedi Saravi et al., 2016; Sharma et al., 2017), also governor cel-

lular apoptosis, tumor growth as well as oxidative stress and inflammatory injury (Green et al., 2014; Salminen and Kaarniranta 2012). Metformin treatment could reduce renal damage prompted by CP through restraining oxidative stress and apoptosis.

In this study, some histopathological observation noticed in the renal tissue after exposure to CP like shrunken glomeruli, dilatation of the others and congestion in the blood vessels lymphocytic infiltration, necrosis in the renal tubules detached epithelium in the renal tubules and edema and hyaline cast in renal tubules in cortex and medulla. These changes are indication of nephrotoxicity (Al-Attar et al., 2017). As cyclophosphamide, induce oxidative stress (Manda and Bhatia, 2003). Oxidative stress due to cytotoxic of reactive oxygen by products, superoxide anions and hydroxyl radicals that formed as metabolites of normal and aberrant metabolic ion processes that utilize molecular oxygen (Sies and Stahl, 1995). Oxidative stress causing lipid peroxidation, protein,

Table 2
Mean values ± SE of area percentage of BAX immunoexpression.

	Control (C)	CP	CP.MET	MET
Mean area %	0.43 ± 0.05	2.5 ± 0.23[a]	1.2 ± 0.34[a,b]	0.6 ± 0.08[b]

Data represented as Mean ± SE.
Abbreviations: C, control group; CP, group received cyclophosphamide only; CP. MET, group received cyclophosphamide and metformin; MET, group received metformin only.
[a] Indicate significant difference from corresponding control group at P ≤ 0.05.
[b] Indicate significant difference from corresponding CP group at P ≤ 0.05.
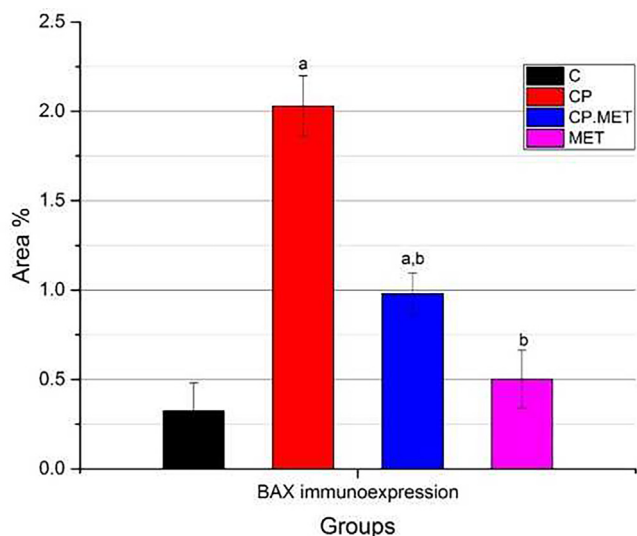
Fig. 6. Histogram showing the mean values ± SE of area percentage of BAX immunoexpression Data represented as Mean ± SEM. (a) Indicate significant difference from corresponding control group $P \leq 0.05$. (b) Indicate significant difference from corresponding CP group at $P \leq 0.05$. Abbreviations: C, control group; CP, group received cyclophosphamide only; CP.MET, group received cyclophosphamide and metformin; MET, group received metformin only.

carbohydrates oxidation and metabolic disorders (Halliwell, 1994). The MAD and 4-hydroxynonenal are products of lipid peroxidation are toxic to cells which alters membrane fluidity and allow ions such Ca to leak into the cell. The peroxyl radicals generated during lipid peroxidation attacks membrane proteins, enzymes and reinitiates lipid peroxidation (Manda and Bhatia, 2003). Liu et al. (2001), Wang et al. (2002), Kiningham et al. (2004) and Aleisa et al. (2008) recorded that ROS are implicated in several histopathological conditions like cancer, apoptosis and clastogenicity, changes in mitochondrial function and organ toxicity (Yagmurca et al 2007).

In this study, metformin was found to protect against CP toxicity indicated by enhanced glomerular size and renal tubule epithelial lining in recovery group (metformin plus CP treated group). This nephroprotective efficacy of metformin might be due to its action as antioxidant, free radical scavenging and membrane stabilizing properties of the kidney tissues. The basic mechanism of this protection might be due to its synergistic effect against inflammation and oxidative stress and its immunomodulation activity. The cellular oxidative reactions, mitochondrial dysfunction and apoptosis ameliorated by Metformin (Wang et al., 2002). neutralization of oxidative stress contributes repair of the cell membrane integrity (Manda and Bhatia, 2003).

Abd El-Hady et al. (2015) confirmed that metformin attenuates the generation of oxygen reactive species and inhibits the opening of the mitochondrial membrane permeability transition pore activated by cytosolic Ca2+ and (ROS), thereby prevents necrotic processes.

Tilly et al. (1995) demonstrated that Bax has role in the process of apoptosis and its regulation of cellular apoptosis in immune-mediated glomerulonephritis and renal fibrosis. The latter authors confirmed the importance of the Bax on caspase-3 in mediating apoptosis associated with inflammation and renal cell deletion in the process of renal fibrosis so its logic to increase collagen deposition in CP administrated group in relation to marked increase immune expression of Bax in this investigation. The relatively high level of Bax is indicator for apoptosis (Song et al., 2012).

The apoptosis had roles in the initiation and propagation of organ fibrosis via directly or indirectly pathways (Mei et al.,

2017). Directly, apoptotic cells may affect the fibroblast cells and myofibroblasts to enhance their proliferation and activation into profibrotic phenotypes (van der Veer et al., 2011). On the other hand, indirectly, apoptosis may elicit inflammation to stimulate fibrosis. Activated macrophages may secrete profibrotic cytokines and growth factors, such as interleukin (IL)-6, IL-10, tumor necrosis factor α (TNFα), and transforming growth factor β 1 (TGF-β 1) (Horowitz et al., 2006).

According to what mentioned by Baker et al. (1994) the apoptosis of excessive damaged of non-functioning renal cells and infiltrating inflammatory cells is beneficial but uncontrolled apoptosis of parenchymal cells induces a reduction of functional mass leading to renal insufficiency (Yang et al., 2001). In accordance to Mei et al. (2017) suggested that renal tubular apoptosis contributes to interstitial fibrosis.

The lucrative effect of metformin on the CP treated rats could be as a result of its antioxidant effect which was described by (Rizk et al., 2020).

5. Conclusion

The renal damages prompted by CP were point out by renal degenerative changes, elevated kidney function parameters and increased BAX staining. The Reno-protective mechanisms of metformin bring up to the prevention of GSH running down and reduce the accumulation of ROS, reduction of marked inflammatory response and apoptosis. Therefore, metformin drug might be used as co-therapy with CP in handling of diverse malignancies.

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