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Luteolin Abates Ifosfamide-Induced Nephrotoxicity by Downregulating Renal Oxidative DNA Damage, Inflammation, and Apoptosis in Experimental Rats

Luteolina Reduce la Nefrotoxicidad Inducida por Ifosfamida al Regular a la Baja el Daño Oxidativo del ADN Renal, la Inflamación y la Apoptosis en Ratas Experimentales

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SUMMARY: Ifosfamide (IFO) is considered as a broad-spectrum antineoplastic drug used in the management of multiple malignancies. However, its use is limited by its associated nephrotoxicity. This work aimed to examine the nephroprotective effect of luteolin against IFO-induced nephrotoxicity. Our study was divided into four groups (n=6); Control group; Luteolin group: Rats were administered luteolin at a dosage of 10 mg/kg intraperitoneally (i.p.) daily for seven days; IFO group: Rats were administered a single intraperitoneal dosage of 500 mg/kg body weight on the sixth day; and IFO+luteolin group: Rats were administered both drugs. Biochemical analyses of serum creatinine and blood urea nitrogen (BUN) were performed. Renal homogenate supernatant was used to estimate levels of the antioxidant enzyme superoxide dismutase (SOD) and the lipid peroxidation marker malonaldehyde (MDA). Furthermore, histopathological examination of renal tissues and immunohistochemical examination of nuclear factor kappa (NF-kβ), interleukin 1-beta (IL-1β), myeloperoxidase (MPO), and caspase-3 were conducted. Protein levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and 8-hydroxy-2´-deoxyguanosine (8-OHdG) were assessed by ELISA, and gene expression of Bcl-2 associated protein x (Bax), heme oxygenase-1 (HO-1), and nuclear factor erythroid 2 related factor 2 (Nrf2) were measured by RT-PCR. The results revealed that the combination of luteolin and IFO significantly decreased levels of creatinine, BUN, MDA, TNF-α, NF-kβ, IL-1β, 8-OHdG, IL-6, Bax, and caspase-3. Meanwhile, it upregulated Nrf2, HO-1, and SOD levels compared to the IFO group. Additionally, there was an improvement in renal histological morphology. These findings indicate that combined treatment with luteolin and IFO mitigates IFO-induced nephrotoxicity through its antioxidant, anti-inflammatory, and antiapoptotic properties.

KEY WORDS: Luteolin; Nephrotoxicity**;** Ifosfamide; Inflammation; Apoptosis.

INTRODUCCIÓN

Ifosfamide (IFO) is an alkylating agent classified under the oxazaphosphorine group. It is utilized in the management of ovarian carcinoma, lymphoma (both Hodgkin and non-Hodgkin), osteosarcoma, breast cancer, as well as cervical, testicular, and lung malignancies (Takimoto & Calvo, 2008). Ifosfamide possesses a structure analogous to that of cyclophosphamide. It degrades into active metabolites, primarily acrolein and chloroacetaldehyde, which are

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detrimental to the liver, heart, brain, and kidneys (Han *et al*., 2021). One of the challenges in the treatment with ifosfamide is nephrotoxicity, affecting 30 % of patients and impacting their quality of life (Skinner *et al*., 1993). Ifosfamide-induced nephrotoxicity can occur in any segment of the nephron, including the glomerulus, distal tubules, or collecting tubules (Skinner *et al*., 1993). Chronic glomerular damage associated with the treatment with IFO is manifested by elevation in the renal function tests (creatinine and urea) (Skinner *et al*., 1996). Toxic metabolites of IFO (acrolein and chloroacetaldehyde) induce cellular oxidative stress and mitochondrial dysfunction (Singh & Kumar, 2019; Wu *et al*., 2020). Oxidative stress and mitochondrial damage in renal tissue are linked to inflammation and cellular apoptosis (Duann & Lin, 2017). Consequently, the coadministration of IFO with antioxidant medicines may present a viable approach to mitigate IFO nephrotoxicity while preserving its therapeutic efficacy.

A major challenge in chemotherapy is to minimize the toxicity of chemotherapeutic agents on healthy cells (Kuzu *et al*., 2018). The application of natural compounds rich in phenolics and flavonoids, which exhibit pharmacological properties such as antioxidant, anti-inflammatory, antiapoptotic, antithrombotic, antibacterial, and antiallergic effects, may mitigate IFO-induced neurotoxicity (Rashid *et al*., 2014; Kandemir *et al*., 2017; Benzer *et al*., 2018; Caglayan *et al*., 2019). One of the well-known natural flavonoids is luteolin, which is found in many vegetables and fruits (Miean & Mohamed, 2001). Luteolin exhibits a wide spectrum of biological and pharmacological actions (Rooban *et al*., 2012), such as anti-apoptosis (Rooban *et al*., 2012; Aziz *et al*., 2018), anti-inflammation (Liu *et al*., 2017), anti-oxidative (Choi *et al*., 2008), antiallergy, and anti-cancer activities (Imran *et al*., 2019). Numerous previous studies examine the renoprotective properties of luteolin in relation to renal damage caused by nephrotoxic agents (Arslan *et al*., 2016; Oyagbemi *et al*., 2020). The aim of our research is to explore the protective effect of luteolin on IFO-induced nephrotoxicity by reducing oxidative stress, DNA damage, inflammation, and renal apoptosis.

MATERIAL AND METHOD

Drugs and Chemicals. Sterile Ifosfamide (Holoxan 1g) from Baxter Oncology GmbH, Germany, was purchased in vial form containing crystalline powder, which was dissolved in 25 ml of distilled water. Luteolin was obtained from Sigma (St. Louis, MO, USA).

Experimental animals. Twenty-four male Wistar albino rats, weighing 200-220 g, were taken from the National Central Institute Animal House, Dokki, Cairo, Egypt. The animals were divided into four equal groups and housed in separate cages, with free access to water and food ad libitum. The rats were housed in an experimental environment with a 12-hour day/night cycle and a temperature of 25 ºC for two weeks prior to the experiment for acclimatization. The study was approved by the committee of Research Ethics, Kafrelsheikh University (Approval code# KFS-IACUC/218/2024).

Experimental design. Rats were divided into four equal groups (n=6):

Control group: Rats were administered normal saline intraperitoneally (i.p.)

Luteolin group: Rats were administered 10 mg/kg of luteolin daily via intraperitoneal injection from the first day of the experiment for a duration of seven consecutive days (Arslan *et al*., 2016).

IFO group: Rats were administered IFO at a dosage of 500 mg/kg body weight (i.p) on the sixth day of the study (Al-Kharusi *et al*., 2013; Çelik *et al*., 2020).

IFO+luteolin group: Rats were administered both drugs at the aforementioned dosages for seven days. Twenty-four hours post the final drugs treatments, the rats in all groups were euthanized by injection of 300 mg/kg sodium thiopental (i.p.), followed by cervical dislocation. Blood samples were taken from the rats' abdominal aorta, and serum was stored at -80ºC for biochemical examination. Kidney samples from different groups were divided into three parts: Renal tissue was crushed and mixed with 10 mM phosphate buffer to form renal homogenate, which was centrifuged at 4 ºC for ten minutes at 3000x g. The resulting supernatant was used for biochemical analysis. Renal tissue was immersed in 10 % formalin for histopathological examination. Renal tissue was stored at -80 ºC for quantitative real-time PCR (RT-qPCR).

Biochemical Investigation. Renal function tests creatinine and BUN and oxidative stress markers (lipid peroxidation marker malonaldehyde (MDA), superoxide dismutase enzyme (SOD)) was assessed by quantitative colorimetric measurement using an ultraviolet (UV)-visible spectrophotometer and commercial kits purchased from Biodiagnostic Company, Giza, Egypt, following the manufacturer's instructions.

Histopathological and Immunohistological examinations. Formalin-fixed renal tissues were processed into 4 µm sections using a microtome (LICA RM 2135) and stained with hematoxylin and eosin (H&E) according to the

guidelines laid by Bancroft & Layton (2013). Histopathological photography was done in the Histology Department using a digital Leica photomicroscope (LEICA, DMLB, Germany). To determine the antigen site, paraffin sections of renal tissues were washed in boiling water for ten minutes after treatment with 0.03 % hydrogen peroxide. Then, renal sections were incubated with antibodies against NF-kβ, IL-1β, MPO, and caspase-3 (Cat# ab16502, MBS9140796, ab208670, MBS2536512). After washing the sections with phosphate buffered saline (pbs), they were incubated for half hour with an anti-rabbit secondary antibody conjugated with horseradish peroxidase. Finally, the sections were incubated for 10 min at room temperature with the chromogen 3,3'-diaminobenzidine tetrachloride.

Enzyme-Linked Immunosorbent Assay (ELISA). Protein levels of 8-OHdG, TNF-α, and IL-6 in renal tissues were measured using commercial ELISA kits (Cat# MBS269902, MBS175904, and MBS2021530, respectively) according to the manufacturer's instructions.

Quantitative Real-Time PCR. Renal tissues total RNA was extracted using Trizol reagent (Invitrogen, Waltham, MA, USA). The quality of the extracted RNA was verified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Reverse transcription and qRT-PCR of 1 µg of extracted RNA were performed using QuantiTect SYBR® Green PCR kits (Waltham, MA, USA) following the factory guides. The qRT-PCR with the following sequence: reverse transcription at 50 °C for 30 min, primary denaturation for 15 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C. The expression of target genes (Nrf2, HO-1, Bax) was calculated using the 2-ΔΔCt methodology. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Table I represents target genes primer sequences.

Statistical Analysis. One-way analysis of variance followed by Tukey's test was done to explore experimental groups difference by the usage of GraphPad Prism version 8 (San Diego, CA, USA), with a probability of <0.05 indicating significance. Graph illustrations were also created using GraphPad Prism. All study data were expressed as Mean ± Standard Deviation.

RESULTS

Luteolin improves ifosfamide-induced renal impairment. Single intraperitoneal injection of 500 mg/kg/b.w of IFO significantly $p<0.05$ increased renal function testes creatinine and BUN (Figs. 1A, B) in comparison to control groups. Co-treatment with luteolin significantly $p<0.05$ decreased renal degeneration by decreasing serum level of creatinine and BUN compared to IFO group.

Protective effect of luteolin against IFO-induced oxidative insult. To explore antioxidant effect of luteolin against IFO-induced renal oxidative stress, renal homogenate supernatant level of oxidative stress markers MAD, SOD, and 8-OHdG were assessed. Figures 1 C, D, E showed a significant $p<0.05$ elevation in MDA and 8-OHdG with a decrease in SOD compared to control group. Co-administration with luteolin significantly $p<0.05$ decreases MDA and 8-OHdG and increases SOD in comparison to IFO group. Meanwhile, luteolin significantly $p<0.05$ increase mRNA of antioxidant regulatory pathway Nrf-2 and HO-1 compared to IFO group (Fig. 1F, G). From all, luteolin offers a potent antioxidant power against IFO-induced oxidative damage.

Luteolin inhibited morphological changes in IFO treated rats. Figure 2 showed a normal histological picture of control and luteolin groups in the form of normal picture of renal glomeruli and tubules (Fig. 2A, B). However, renal sections of IFO group showed tubular epithelial cells hydropic degeneration with leucocytic infiltration and perivascular edema (Fig. 2C). Co-administration with luteolin significantly $p<0.05$ improved histological structure in the form of focal tubular degeneration (Fig. 2D, E).

Anti-inflammatory effect of luteolin against IFO-induced nephrotoxicity. The effect of luteolin on inflammatory markers NF-κB, IL-1β, MPO in renal tissues were investigated by immunohistochemical staining and protein level of TNF-α, and IL-6 by Elisa assay, in comparison to control rats (Figs. 3A, B, 4A, B, 5A, B), IFO significantly $p<0.05$ increases immunoexpression of NF-κB, IL-1β, MPO (Figs. 3C, G, 4C, G, 5C, G), and

Table I. qRT-PCR primer sequences of examined genes.

Gene	Forward primer	Reverse primer
Bax	5`-CACGTCTGCGGGGAGTCAC-3`	5`-TAGAAAAGGGCAACCACCCG-3`
Nrf-2	5`-GAGACGGCCATGACTGAT-3`	5`-GTGAGGGGATCGATGAGTAA-3`
HO-1	5`-ACTTTCAGAAGGGTCAGGTGTCC-3`	5`-TTGAGCAGGAAGGCGGTCTTAG-3`
GAPDH	5`-CTCTCTGCTCCTCCCTGTTC-3`	5`-CGACATACTCAGCACCAGCA-3`

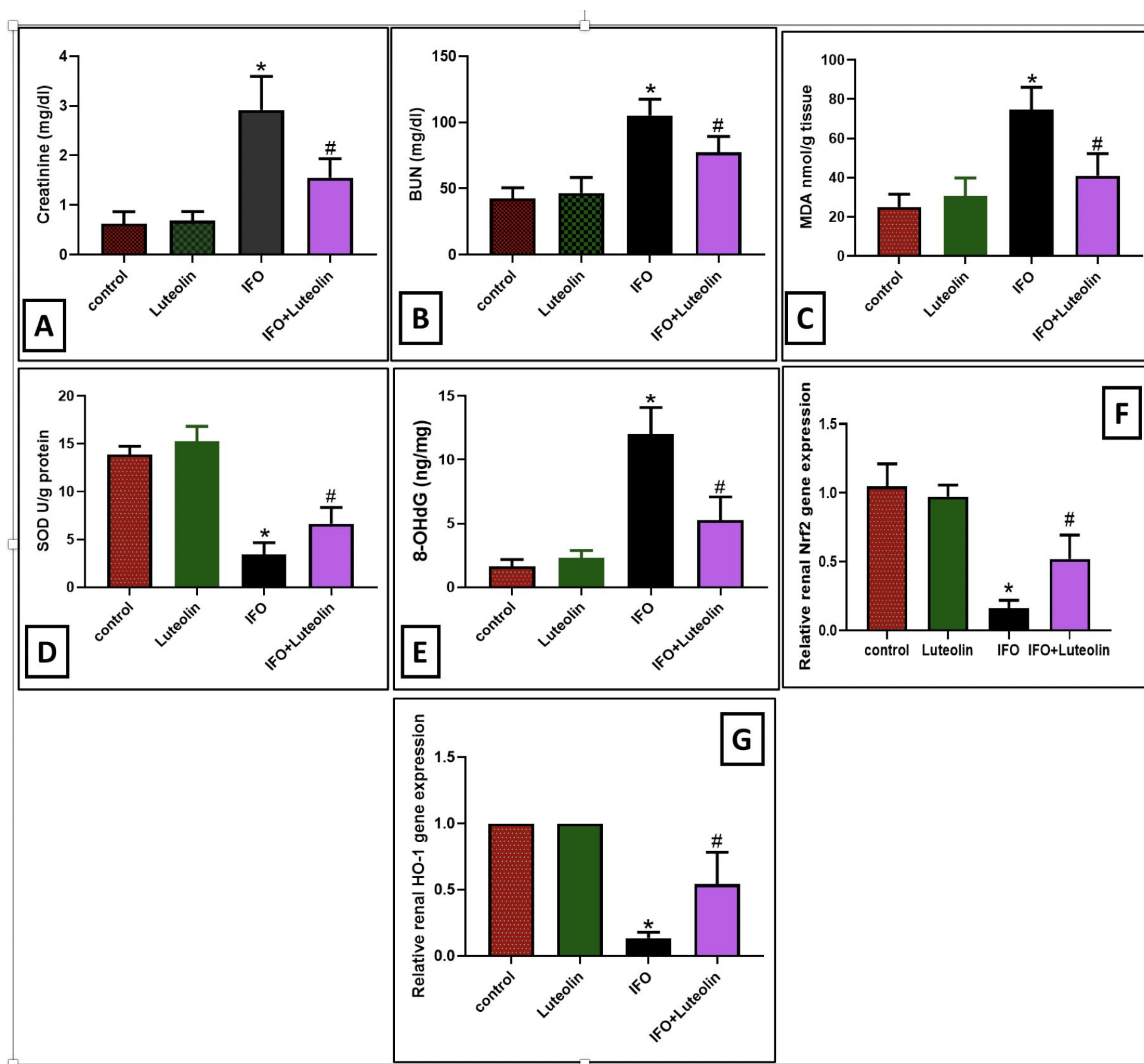

Fig. 1. Effect of luteolin on renal function testes (creatinine, BUN) (A, B) and renal oxidative stress markers malonaldehyde (MDA) and superoxide dismutase (SOD) and 8-OHdG (C, D, E). Gene expression of Nrf2 and HO-1 (F, G). Bars represent scores of lesions in renal cortices in different groups. *p versus control groups. #p versus to the IFO group ($p<0.05$). All results are reported as mean±SD.

protein level of TNF-α, and IL-6 (Figs. 3E, F). However, co-administration with luteolin significantly $p<0.05$ decreased the level of the inflammatory markers NF-κB, IL-1β, MPO, TNF-α, and IL-6 compared to IFO group. Luteolin showed a strong anti-inflammatory effect against IFO-induced neuroinflammation.

Luteolin elucidates IFO-induced renal apoptosis. Immunohistochemical examination of caspase-3 and gene expression of Bax in renal tissues indicated that, IFO intraperitoneal injection significantly $p<0.05$ increased it (Figs. 6C, E, F) compared to control group (Figs. 6A, B). Nevertheless, Co-administration with luteolin significantly $p<0.05$ decreased apoptotic markers caspase-3 and Bax in comparison to IFO group (Figs. 6D, E, F). Based on the previous result, luteolin possesses a clear antiapoptotic character against IFO-induced tubular epithelial cells apoptosis.

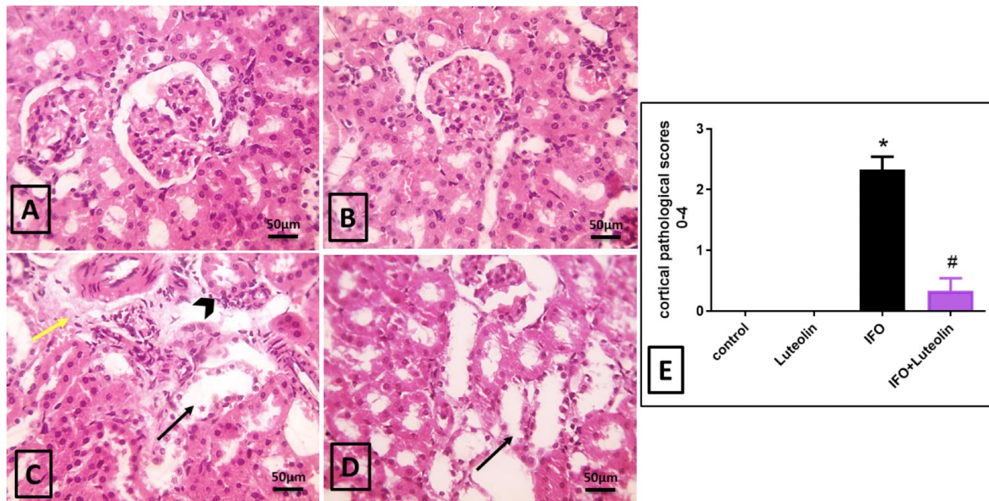

Fig. 2. H&E-stained renal sections showing normal renal morphology with normal glomeruli, tubules, blood vessels and minimal interstitial tissue in control group and luteolin group (A, B). Renal cortex from IFO group (C) showing hydroping degeneration in epithelial lining many tubules (black arrows), congested blood vessels (red arrows), mild perivascular edema & fibrosis (yellow arrows), with few leukocytic cells infiltration (arrowheads). Renal cortex from IFO+luteolin group (D) showing focal hydroping degeneration in epithelial lining few tubules (black arrows). Magnifications X: 400 bar 50. (E) Bars represent scores of lesions in renal cortices in different groups. *p means significant to control groups. #p means significant to IFO group (p<0.05). All results are reported as mean±SD.

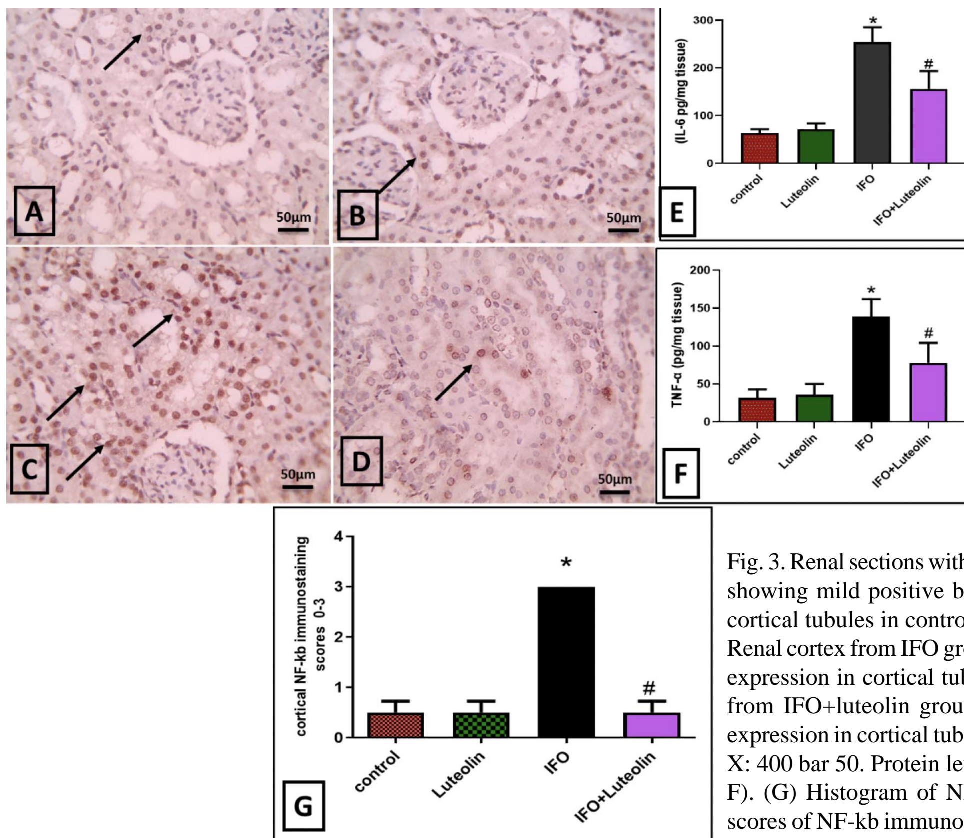

Fig. 3. Renal sections with NF-kB immunohistochemical stain showing mild positive brown expression (black arrows) in cortical tubules in control group and luteolin groups (A, B). Renal cortex from IFO group (C) showing prominent positive expression in cortical tubules (black arrows). Renal cortex from IFO+luteolin group (D) showing decreased positive expression in cortical tubules (black arrows). Magnifications X: 400 bar 50. Protein level of TNF-a, and IL-6 by Elisa (E, F). (G) Histogram of NF-kB positive area, bars represent scores of NF-kb immunostaining expression in renal cortical regions in different groups. *p means significantly different when compared to the control groups. #p means significantly different when compared to the IFO group (p<0.05). All results are reported as mean±SD.

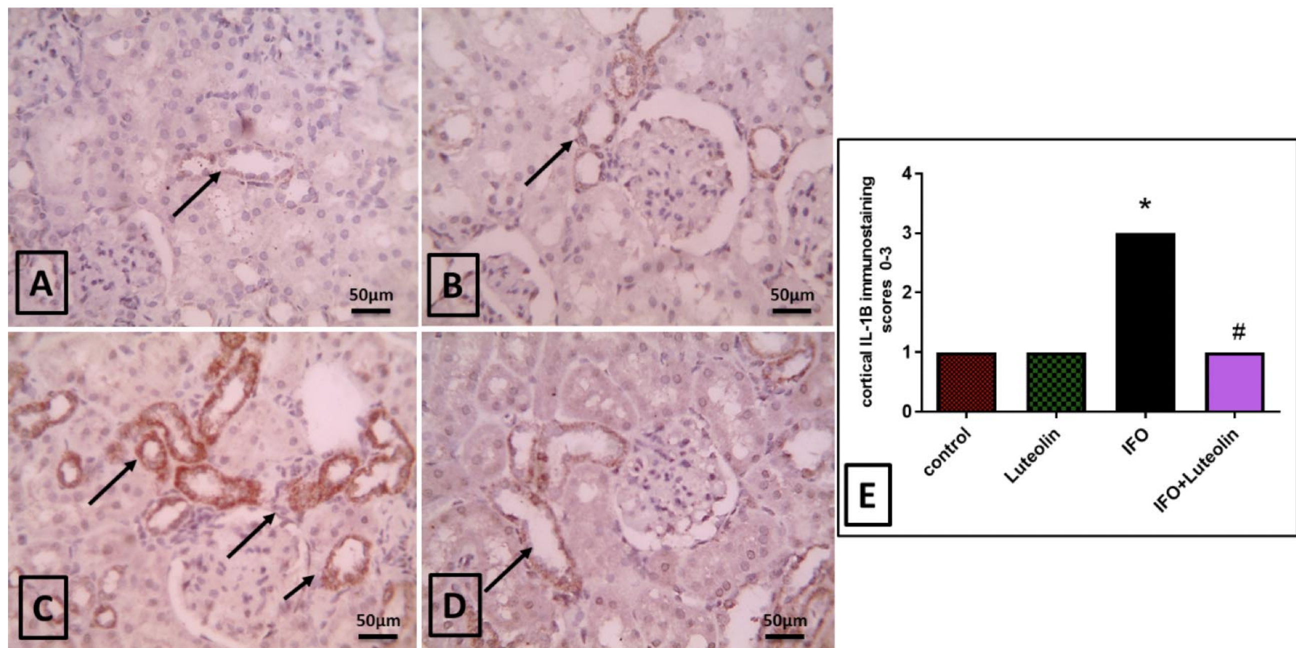

Fig. 4. Microscopic pictures of IL-1b immunostained stained renal sections showing mild positive expression (black arrows) in cortical tubules control group and luteolin groups (A, B). Renal cortex from IFO group (C) showing prominent positive expression in cortical tubules (black arrows). Renal cortex from IFO+luteolin group (D) showing decreased positive expression in cortical tubules (black arrows). Magnifications X: 400 bar 50. (E) Histogram of IL-1b positive area, bars represent scores of IL-1b immunostaining expression in renal cortical regions in different groups. *p significant to control groups. #p significant to the IFO group (p<0.05). All results are reported as a mean±SD.

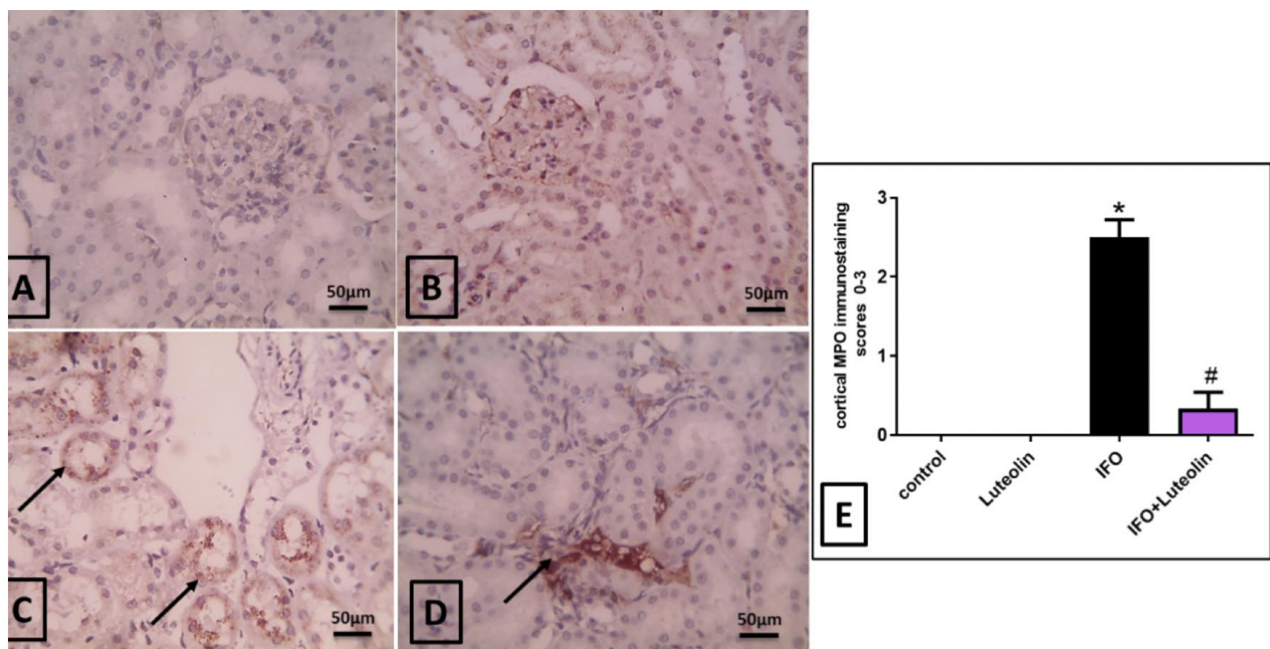

Fig. 5. MPO immunostained stained renal sections revealed a negative expression in cortical tubules in control group and luteolin groups (A, B). Renal cortex from IFO group (C) showing prominent positive expression in cortical tubules (black arrows). Renal cortex from IFO+luteolin group (D) showing decreased positive expression in cortical tubules (black arrows). Magnifications X: 400 bar 50. (E) Histogram of MPO positive area, bars represent scores of MPO immunostaining expression in renal cortical regions in different groups. *p compared to control groups (p<0.05). #p compared to IFO group (p<0.05). All results are reported as mean±SD.

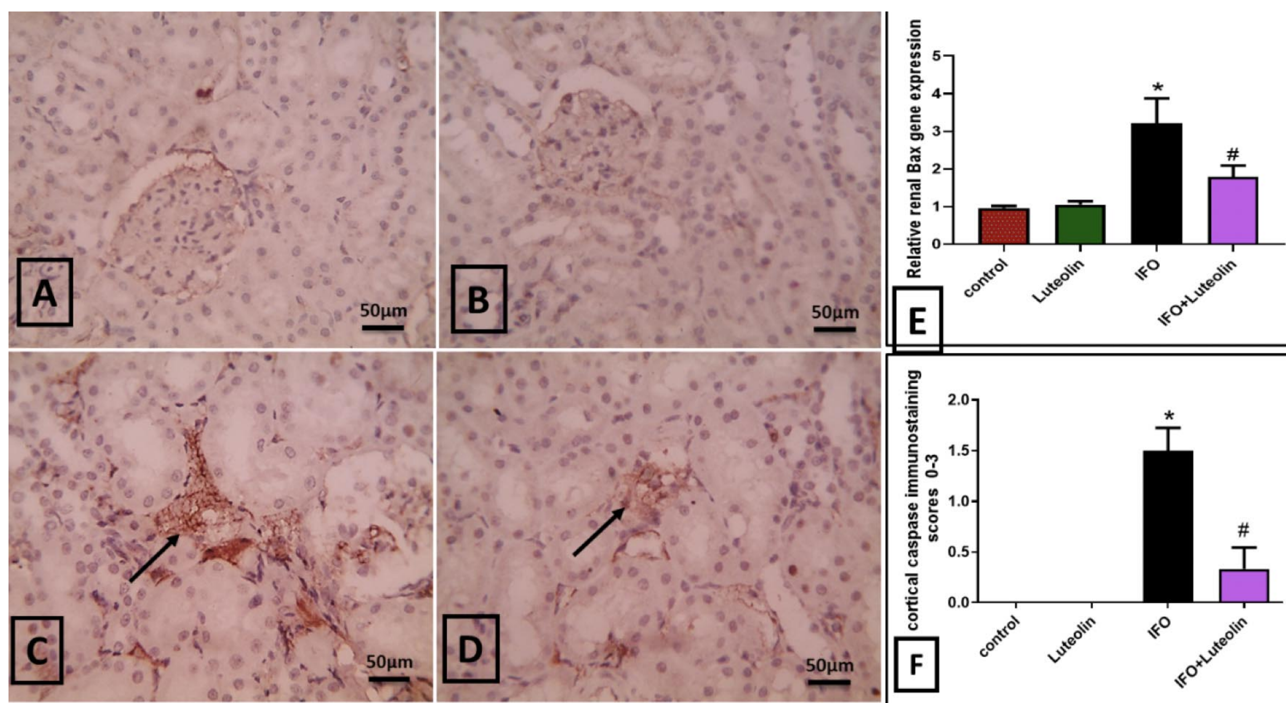

Fig. 6. Immunohistochemical stained renal sections with caspase-3 showing negative expression in cortical tubules in control group and luteolin groups (A, B). Renal cortex from IFO group (C) showing prominent positive expression in cortical tubules (black arrows). Renal cortex from IFO+luteolin group (D) showing decreased positive expression in cortical tubules (black arrows). Magnifications X: 400 bar 50. (E) gene expression of Bax in renal tissues of different groups. (F) Histogram of caspase-3 positive area, bars represent scores of caspase immunostaining expression in renal cortical regions in different groups. *p against control groups ($p<0.05$). #p against IFO group ($p<0.05$). All results are reported as mean±SD.

DISCUSSION

The primary aims of our work were to examine the nephroprotective effect of luteolin against ifosfamide (IFO)-induced renal deterioration, while exploring its possible protective mechanisms by halting oxidative stress, renal inflammation, and renal tubular apoptosis

Ifosfamide is regarded as a potent anticancer therapeutic drug employed in the treatment of many malignancies (Vermorken *et al*., 2008). Its half-life is 7 hours, but its therapeutic efficacy is significantly influenced by its dose-dependent toxicity (Gonzalez-Angulo *et al*., 2002). IFO is recognized as a prodrug, which is metabolized into ifosfamide mustard and 4-hydroxy-ifosfamide (Zaki *et al*., 2003). In addition, its non-alkalizing metabolites including acrolein and chloroacetaldehyde contribute to its nephrotoxicity, neurotoxicity, and hepatotoxicity (Kurowski *et al*., 1991). Cellular oxidative stress can be prevented by endogenous antioxidant enzymes like SOD, CAT, and GPx. Free radical production during IFO treatment results in the consumption of these enzymes, decreasing their levels and inducing oxidative insult (Sayed-Ahmed *et al*., 2012).

The results of our study revealed that a single injection of IFO significantly elevated the lipid peroxidation marker MDA and the oxidative DNA damage byproduct 8-OHdG, while concurrently reducing the levels of the endogenous antioxidant enzyme SOD and downregulating the antioxidant regulatory pathway Nrf2/HO-1. These findings correspond with the results of the study done by Cakmak *et al*. (2023). and Shabani *et al*. (2023). In contrast, co-treatment with luteolin markedly upregulated the gene expression of the Nrf2/HO-1 pathway, with a subsequent elevation in the antioxidant enzyme SOD, and decreased MDA and 8-OHdG levels. These findings are in accordance with the previous work by Chen *et al*. (2023). The antioxidant effect of luteolin can be attributed to its upregulatory effect on the Nrf2 pathway, which stimulates antioxidant enzyme production and inhibits oxidative DNA damage. The antioxidant effects of luteolin prevent IFO-induced renal damage by downregulating renal function tests (creatinine and BUN) and improving renal histology. These results supported by the study done by Albarakati *et al*. (2020).

Reactive oxygen species have a leading role in NF-κB activation, a well-known inflammatory mediator (Dos Santos Pereira *et al*., 2020). Free radicals produced in nephrotoxicity play a crucial role in the phosphorylation of NF-κB, leading to the activation of proinflammatory cytokines (Subramanian *et al*., 2015; Hassanein *et al*., 2021) MPO is an enzyme that induces oxidative stress and inflammation by enhancing the production of reactive oxygen species in infiltrating neutrophils (Chen *et al*., 2020). Our results revealed that a single injection of IFO significantly upregulated the immunoexpression of NF-κB, IL-1β, MPO, and the protein levels of TNF-α and IL-6. This is in line with study done by Çelik *et al*. (2020). Fortunately, intraperitoneal injection of luteolin combined with IFO significantly mitigates renal inflammation, as evidenced by a considerable reduction in the levels of NF-κB, IL-1β, MPO, TNF-α, and IL-6. These findings are supported by other studies, such as Boeing *et al*. (2020), which demonstrated that luteolin administration exhibited significant anti-inflammatory effects in the duodenum and colon of mice by reducing MPO activity and the levels of inflammatory cytokines in irinotecan-induced intestinal mucositis. Additionally, study by Oyagbemi *et al*. (2020), showed that luteolin exerts a protective effect against cobalt-induced nephrotoxicity by downregulating the expression of NF-κB and MPO. Therefore, the anti-inflammatory effect of luteolin can be attributed to its antioxidant properties, which impede the phosphorylation of NF-κB and the subsequent generation of inflammatory cytokines.

Caspase is considered one of the prominent cell death protease families, playing a leading role in the apoptosis process. Treatment with IFO results in the activation of caspases 3, 8, and 9 in renal epithelial cells, leading to apoptosis (Becker *et al*., 2002). The results of our study revealed that a single intraperitoneal dose of IFO causes renal damage through apoptosis, as demonstrated by the increased number of caspase-3 immunostained cells and the upregulation of Bax gene expression in the kidney. This result is consistent with previous study by Hammoodi *et al*. (2023), which reported that that IFO causes an increase in caspase-3 expression in neuronal cells, indicative of apoptosis. Conversely, luteolin mitigates renal apoptosis by decreasing the immunoexpression of caspase-3 and the gene expression of the apoptotic marker Bax. This result is corroborated by the previous work of Arslan *et al*. (2016), who documented the ameliorative effect of luteolin against colistin-induced nephrotoxicity by decreasing apoptotic cell numbers. The antioxidant and anti-inflammatory properties of luteolin elucidate its antiapoptotic action on renal tubular epithelial cells in IFO-induced nephrotoxicity.

CONCLUSIONS

The results of our study indicated that luteolin exerts a protective effect against IFO-induced nephrotoxicity. This encompasses the mitigation of renal oxidative damage through the overexpression of the Nrf2/HO-1 pathway and the downregulation of NF-kB and MPO expression, resulting in a reduction of inflammatory cytokines in renal tissues. Moreover, it reduced renal apoptosis markers.

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RESUMEN: La ifosfamida (IFO) se considera un fármaco antineoplásico de amplio espectro que se utiliza en el tratamiento de múltiples neoplasias malignas. Sin embargo, su uso está limitado por la nefrotoxicidad asociada. Este trabajo tuvo como objetivo examinar el efecto nefroprotector de la luteolina contra la nefrotoxicidad inducida por IFO. Nuestro estudio se dividió en cuatro grupos (n = 6); Grupo control; Grupo de luteolina: a las ratas se les administró luteolina en una dosis de 10 mg/kg por vía intraperitoneal (i.p.) diariamente durante siete días; Grupo IFO: a las ratas se les administró una dosis intraperitoneal única de 500 mg/kg de peso corporal en el sexto día; y Grupo IFO + luteolina: a las ratas se les administraron ambos fármacos. Se realizaron análisis bioquímicos de creatinina sérica y nitrógeno ureico en sangre (BUN). El sobrenadante de homogeneizado renal se utilizó para estimar los niveles de la enzima antioxidante superóxido dismutasa (SOD) y el marcador de peroxidación lipídica malonaldehído (MDA). Además, se realizó un examen histopatológico de los tejidos renales y un examen inmunohistoquímico del factor nuclear kappa (NF-kβ), la interleucina 1-beta (IL-1β), la mieloperoxidasa (MPO) y la caspasa-3. Se evaluaron los niveles de proteína del factor de necrosis tumoral alfa (TNF-α), la interleucina-6 (IL-6) y la 8-hidroxi-2´-desoxiguanosina (8-OHdG) mediante ELISA, y se midió la expresión génica de la proteína x asociada a Bcl-2 (Bax), la hemooxigenasa-1 (HO-1) y el factor nuclear eritroide 2 relacionado con el factor 2 (Nrf2) mediante RT-PCR. Los resultados revelaron que la combinación de luteolina e IFO disminuyó significativamente los niveles de creatinina, BUN, MDA, TNF-βα, NF-kβ, IL-1β, 8-OHdG, IL-6, Bax y caspasa-3. Mientras tanto, aumentó los niveles de Nrf2, HO-1 y SOD en comparación con el grupo IFO. Además, hubo una mejora en la morfología histológica renal. Estos hallazgos indican que el tratamiento combinado con luteolina e IFO mitiga la nefrotoxicidad inducida por IFO a través de sus propiedades antioxidantes, antiinflamatorias y antiapoptóticas.

PALABRAS CLAVE: Luteolina; Nefrotoxicidad; Ifosfamida; Inflamación; Apoptosis.

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