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Erythropoietin and *Hypericum perforatum* ameliorate Gentamicin–induced nephrotoxicity in rats

La eritropoyetina y el *Hypericum perforatum* mejoran la nefrotoxicidad inducida por gentamicina en ratas

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ABSTRACT

Gentamicin (GM), which causes nephrotoxicity, is an aminoglycoside antibiotic commonly prescribed to treat of gram–negative infections. Erythropoietin (EPO), which has several biological functions including neuroprotection, wound healing and nephroprotection, is a glycoprotein hormone that controls erythropoiesis. *Hypericum perforatum (HP)* is a medicinal herb with antibacterial and nephroprotective effects. The aim of this study is to demonstrate the efficacy of EPO and HP in GM nephrotoxicity using combined biochemical, histopathological and immunohistochemical evaluations together. A total of 36 male Spraque–Dawley rats were divided into as control, GM (100 mg·kg^{-1} day), GM+EPO, GM+HP, EPO (1000 IU·kg^{-1} three consecutive days apart) and HP (200 mg·kg^{-1} day) groups (n=6) and the experiment lasted for 9 days. GM–induced increased relative kidney weight and increased serum urea nitrogen (BUN), creatinine and urea levels were reduced by EPO and HP. EPO and HP reduced the level of malondialdehyde (MDA), which increased with GM application, and increased the activities of reduced glutathione (GSH), glutathione peroxidase (GSH–Px), and catalase (CAT). GM nephrotoxicity resulted in tubular degeneration, vacuolization and hyaline deposits, glomerular degeneration and interstitial mononuclear cell infiltration. EPO and HP attenuated these histopathological changes. Also, EPO and HP also reduced caspase–3 immunoreactivities, which increased with GM application. It was shown that EPO and HP have attenuating effects on GM–induced kidney injury, and especially the intense antioxidant content of HP has a regulatory effect on the negative consequences of oxidative stress.

Key words: Apoptosis; Erythropoietin; gentamicin; *Hypericum perforatum*; oxidative stress

RESUMEN

La gentamicina (GM), que causa nefrotoxicidad, es un antibiótico aminoglucósido comúnmente indicado para tratar infecciones por gram negativos. La eritropoyetina (EPO), que tiene diferentes funciones biológicas entre las que se incluyen neuroprotección, cicatrización de heridas y nefroprotección, es una hormona glicoproteica que controla la eritropoyesis. *Hypericum perforatum* (HP) es una hierba medicinal con efectos antibacterianos y nefroprotectores. El objetivo de este estudio es demostrar la eficacia de EPO y HP en la nefrotoxicidad de transgénicos utilizando evaluaciones bioquímicas, histopatológicas e inmunohistoquímicas combinadas. Un total de 36 ratas macho Spraque–Dawley se dividieron como control, GM (100 mg·kg^{-1} día), GM+EPO, GM+HP, EPO (1000 UI·kg^{-1} tres días consecutivos de diferencia) y HP (200 mg·kg^{-1} día) (n=6) y el experimento duró 9 días. La EPO y HP redujeron el aumento del peso relativo de los riñones inducido por transgénicos; mientras que provocaron un incremento de los niveles séricos de nitrógeno ureico (BUN), creatinina y urea. Así mismo, EPO y HP redujeron el nivel de malondialdehído (MDA), que aumentó con la aplicación de transgénicos, y aumentaron las actividades del glutatión reducido (GSH), la glutatión peroxidasa (GSH–Px) y la catalasa (CAT). La nefrotoxicidad de los transgénicos resultó en degeneración tubular, vacuolización y depósitos hialinos, degeneración glomerular e infiltración de células mononucleares intersticiales. EPO y HP atenuaron estos cambios histopatológicos. Además, la EPO y el HP redujeron la inmunorreactividad de la caspasa–3, que aumentó con la aplicación de transgénicos. Se demostró que la EPO y la HP tienen efectos atenuantes sobre la lesión renal inducida por transgénicos y, especialmente, el intenso contenido de antioxidantes de la HP tiene un efecto regulador sobre las consecuencias negativas del estrés oxidativo.

Palabras clave: Apoptosis; eritropoyetina; gentamicina; *Hypericum perforatum;* estrés oxidativo

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INTRODUCTION

Gentamicin (GM), a nephrotoxic agent, is an aminoglycoside antibiotic drug widely used against diseases caused by gram-negative bacteria [1]. Despite this negative side effect, it remains a powerful drug in the fight against microorganisms that are still resistant to some antibiotics [2]. The mechanism leading to GM nephrotoxicity is uncertain. It has been shown that nephrotoxic injury is associated with necrosis, which is linked to oxidative stress, inflammatory cascades, apoptosis, and lipid peroxidation [3]. After glomerular filtration, GM is thought to bind to the brush border of proximal tubule cells and is subsequently internalised by endocytosis [4]. Gentamicin, which generally accumulates in lysosomes and may cause lysosomal phospholipid rupture, is distributed to various intracellular organelles and causes their dysfunction [5]. It is specified that it reduces ATP synthesis by increasing ROS production, particularly in mitochondria, and activates the intrinsic apoptotic pathway, leading to cell death [4, 5]. Increased ROS generation and the resulting oxidative stress play a role in the pathogenesis of GM nephrotoxicity [6]. Erythropoietin (EPO) is a glycoprotein hormone that stimulates erythropoiesis [7]. EPO, which has several biological functions such as neuroprotection, wound healing and nephroprotection in addition to erythropoiesis, is produced by fibroblasts near the proximal tubules and peritubular capillaries in the kidney. EPO has been shown to exert nephroprotective effects in several models of acute and chronic renal injury [8] and to stimulate renal regeneration by acting directly on damaged tubular cells [9]. These effects are mediated by its ability to bind to EPO receptors (EPORs) found in mesangial, glomerular and tubular epithelial cells [10]. EPO has many important effects, such as reducing free radicals and pro-inflammatory cytokines [11]. This protection is generally attributed to its anti-apoptotic and antioxidant activities [12].

Hypericum perforatum (HP), known as St. John's Wort (SJW) is a plant frequently consumed around the world due to its many beneficial properties [13]. This plant contains hyperforin, hypericin, quercetin, epicatechin, catechin, resveratrol, flavonoid and xanthone derivatives. These have antioxidant activity, inhibition of lipid peroxidation and free radical scavenging properties [14]. Studies have shown that this medicinal plant has antibacterial, anti-inflammatory and nephroprotective effects [15, 16, 17]. It has been accepted that various HP extracts have no toxic effects in the dose range of 100 mg·kg^{-1} to 9 g·kg^{-1} and are safe to use [18]. Therefore, it is widely used in for the cure of diabetes, wound healing burns, nephrotoxicity [13]. The aim of this study is to demonstrate the antioxidant and antiapoptotic properties of EPO and HP in GM nephrotoxicity using combined biochemical, histopathological and immunohistochemical evaluations.

MATERIALS AND METHODS

Chemicals and animals

Gentamicin (Ibrahim Etem Menarini group, TR), EPO (Dropoetin, Drogsan, TR) and HP (St. John's Wort, Solgar, USA) were acquired from commercial companies. Thirty-six male Spraque-Dawley rats (*Rattus norvegicus*)(2 months old, weighs an average of 300 g) were acquired from Firat University Experimental Research Center (Elazig, Türkiye). The animals were sheltered (40-60% humidity, 24°C ± 3°C, and 12 hours light/dark period) and were fed free. This study was initiated with the approval of the Fırat University Animal Experiments Local Ethics Committee (Ethic no: 15.01.2020-2020/01) was carried out properly Animal Research: Reporting of In Vivo Experiments guidelines and National Institutes of Health Animal Research guidelines.

Experimental design

Animals were randomly divided into six groups (n=6). Control group: the rats received normal saline 0.5 mL·kg^{-1} day intraperitoneal (ip) for 9 days. GM group: the rats received GM 100 mg·kg^{-1} day ip for 9 days [19]. GM + EPO group: the rats received GM 100 mg·kg^{-1} day ip for 9 days and Dropoetin1000 IU·kg^{-1} ip on days 1, 5 and 9 of the study [20]. GM + HP group: the rats received GM 100 mg·kg^{-1} day ip for 9 days, and HP 200 mg·kg^{-1} day orally for 9 days [21]. EPO group: the rats received Dropoetin1000 IU·kg^{-1} ip on days the 1, 5 and 9 of the study. HP group: the rats received HP 200 mg·kg^{-1} day orally for 9 days. The animals were sacrificed under anaesthesia at the end of the experiment. Blood samples were collected for serum analysis. The kidney tissues were used for histopathological, immunohistochemical and biochemical analyses.

Serum analysis

The levels of blood urea nitrogen (BUN), creatinine and urea were determined by means of an Olympus AU 600 autoanalyzer (Optical Co., Ogaki, JAPAN).

Biochemical analysis

Malondialdehyde (MDA) and glutathione (GSH) levels and glutathione peroxidase (GSH-Px) and catalase (CAT) activities were measured spectrophotometrically (Thermo Scientific, Genesys 10S UV-VIS Spectrophotometer, USA). MDA levels were expressed as nmol·mL^{-1} [22], GSH levels as nmol·mL^{-1} [23], GSH-Px levels as IU·L^{-1} [24] and CAT levels as KU·L^{-1} [25].

Histological examination

The kidney samples fixed in 10% buffered formalin were embedded paraffin wax. Hematoxylin-eosin (H&E) staining was applied to 5 μm thick sections [26]. The slides were evaluated by histologist blindly using an AXIO LAB 5 Zeiss microscope (Carl Zeiss AG, Germany). Histopathological evaluation criteria in kidney tissues included tubular degeneration, tubular brush border loss, tubular vacuolization, hyaline cast in the tubules, glomerular degeneration, mononuclear cell infiltration. The scores values obtained from kidney tissue sections were shown as follows: (-): null, (+): mild, (++): moderate, (+++): severe [26].

Immunohistochemical analysis

Caspase-3 (E-AB-66940, dilution 1/200; Elabscience, USA) immunoreactivity in the kidney tissues were determined using the Avidin-Biotin-Peroxidase Complex (ABC) method was according to the procedure described previously [27]. Counterstaining was done with Mayer's hematoxylin. Immunoreactivity was calculated using the area × density formula (density; none (0), very little (0.5), little (1), moderate (2), severe (3) × area; 0.1(<25%), 0.4 (26-50%), 0.6 (51-75%), 0.9 (76-100%) [28].

Statistical analysis

Statistical data were analysed using IBM SPSS/PC software (version 21.0, IBM Co. USA). Data were presented as mean ± standard deviation. Differences between groups were analysed by ANOVA followed by post-hoc Duncan's test. Statistical significance was defined as $P<0.05$.

RESULTS AND DISCUSSIONS

In the fight against life-threatening infections, the use of GM, whose clinical efficacy against resistant pathogenic microorganisms is well known, is limited due to its nephrotoxic effect [29]. GM-induced nephrotoxicity is initiated by pathological mechanisms related to apoptosis, oxidative stress, inflammation, and necrosis [30]. Research to protect the kidney from off-target toxicity of GM is of great clinical importance. The use of agents with different pharmacological effects for nephroprotection is of interest in terms of safe GM practices [2].

Our results showed that relative kidney weight, BUN, creatinine and urea levels were significantly increased in the GM group compared to the control group (P<0.05).

However, there was decreased significantly in these values in the GM + EPO and GM + HP groups in comparison to the GM group (P<0.05). The relative weights of the kidneys and the levels of the biomarkers of renal function of all the groups are shown in TABLE I. The studies of GM nephrotoxicity [31] and EPO and HP used against this nephrotoxicity [7, 32] reveal the existence of findings similar to this study results. Therefore, it can be stated that the improvement in these impaired biological markers may depend on the agents used.

TABLE I
Effect of EPO and HP on GM-induced relative kidney weight and renal function biomarkers

Groups	Relative kidney weight (g)	BUN (mg·dL^{-1})	Creatinine (mg·dL^{-1})	Urea (mg·dL^{-1})
Control	0.44±0.04^{c}	15.23±1.80^{c}	0.60±0.02^{c}	20.15±0.55^{c}
GM	0.58±0.07^{a}	110.40±2.20^{a}	3.73±0.08^{a}	103.23±1.03^{a}
GM + EPO	0.48±0.03^{b}	28.33±1.78^{b}	1.36±0.04^{b}	31.11±0.93^{b}
GM + HP	0.47±0.06^{b}	25.30±1.50^{b}	1.00±0.02^{b}	30.16±0.88^{b}
EPO	0.46±0.02^{c}	17.20±1.42^{c}	0.55±0.03^{c}	19.17±0.40^{c}
HP	0.45±0.01^{c}	16. 38±1.20^{c}	0.52±0.05^{c}	18.12±0.23^{c}

^{a,b,c}: Different superscripts in the same row indicate the significant difference. GM: Gentamicin, EPO: Erythropoietin, HP: *Hypericum perforatum* and BUN: Blood urea nitrogen

Oxidative stress plays an important role in the pathophysiology of GM nephropathy [6]. Previous studies have shown that MDA levels [33], a common sign of peroxidative injury used in the definition of lipid peroxidation by extreme ROS generation, increased with GM application [32, 33].It has been explained that GM reduces GSH levels through excessive production of free radicals or depletion of sulphhydryl protein groups [34]. It has also been shown that excessive ROS production leads to the depletion of renal antioxidant enzymes such as CAT, and GM reduces CAT expression [35].

It was noted that the activity of GSH-Px, an enzymatic antioxidant that has an important role in ROS defense, also decreased in GM-induced nephrotoxicity [36]. In this study, it was determined that a significant increase in MDA levels and a significant decrease in GSH levels, GSH-Px and CAT activities in the GM group compared to the control group (P<0.05). However, it was found that there was a significant decrease in MDA levels and a significant increase in GSH levels and GSH-Px and CAT activities in the GM + EPO and GM + HP groups compared to the GM group (P<0.05). The activities and levels of oxidative stress-related parameters in all groups are shown in TABLE II. Studies on these agents used against GM nephrotoxicity support these findings [32, 37]. Another study on antioxidant agents used against GM nephrotoxicity declared that GSH level and GSH-Px and CAT activities increase [36]. In this direction, the idea that antioxidant agents such as HP reduce GM-induced oxidative stress due to their strong free radical scavenging activity is supported.

TABLE II
Effect of EPO and HP on GM-induced MDA, GSH, GSH-Px and CAT

Groups	MDA (nmol·mL^{-1})	GSH (nmol·mL^{-1})	GSH-Px (IU·L^{-1})	CAT (KU·L^{-1})
Control	2.55±0.07^{c}	0.53±0.04^{a}	287.29±3.3^{a}	399.95±3.7^{a}
GM	5.18±0.17^{a}	0.22±0.01^{c}	164.74±2.1^{c}	263.71±2.5^{c}
GM + EPO	3.85±0.09^{b}	0.41±0.02^{b}	279.14±2.7^{ab}	305.82±2.9^{ab}
GM + HP	3.68±0.08^{b}	0.45±0.0^{b}	281.22±2.5^{ab}	328.51±2.7^{ab}
EPO	2.75±0.06^{c}	0.54±0.08^{a}	291.15±2.3^{a}	374.96±3.2^{a}
HP	2.90±0.05^{c}	0.51±0.06^{a}	295.20±2.2^{a}	397.68±3.3^{a}

^{a,b,c}: Different superscripts in the same row indicate the significant difference. GM: Gentamicin, EPO: Erythropoietin, HP: *Hypericum perforatum*, MDA: malondialdehyde, GSH: glutathione, GSH-Px: glutathione peroxidase and CAT: catalase

Excessive ROS production damages cellular compounds, leading to the activation of inflammatory mediators and molecular signaling pathways that contribute to lipid peroxidation, protein denaturation, DNA damage, and cell death [6]. Additionally, GM-induced ROS can cause cell injury and necrosis by peroxidation of membrane phospholipids, disrupting the lipid structure unity and reducing antioxidant defense mechanisms. Therefore, tubular cytotoxicity in GM nephrotoxicity has also been found to be associated with cellular apoptosis and necrosis [38]. In this context, studies have reported that necrosis, degeneration, vacuolization, desquamation, hyaline eruption and mononuclear cell infiltration were observed in the proximal and distal tubules in treatment with GM [35, 37]. Besides, it has been declared that thickening of the cell membrane of glomeruli and destruction of endothelial cells, vacuolization and loss of microvilli in convoluted proximal and distal tubule cells [39]. In current study, it was demonstrated congestion, swelling and degeneration in the glomerulus in the GM group. Vacuolization, degeneration, desquamation, picnosis and necrosis in the epithelial cells of the proximal tubules, together with degenerated and desquamated cells collected in the lumen of these tubules, were noted. In addition, hyaline casts and loss of tubular brush border in the tubular lumen as well as hemorrhage and intense infiltrative cell accumulation in the interstitial region were detected. Mild histopathological lesions in the renal structure were observed in the GM + EPO and GM + HP groups compared to the GM group. Degenerative, necrotic, picnotic and infiltrative changes were alleviated, and hyaline cast was considerably reduced. The scores of the kidney tissues in all groups is exhibited in TABLE III, and the microphotographies is displayed in FIG. 1. Similarly, it has been reported by Codea *et al.* [7] that changes such as tubular degeneration, mononuclear cell infiltration, tubular necrosis and hyaline eruptions observed in GM-induced nephropathy were alleviated by the use of EPO. It can be mentioned that EPO, which is known to interact with specific receptors defined in various tissues, has nephroprotective benefits due to its various cytoprotective effects such as mitogenic, angiogenic, and apoptotic inhibition [7].

TABLE III
Effect of EPO and HP on histopathological score in GM–induced nephrotoxicity

Groups	Tubular degeneration	Loss of brush border	Tubular vacuolization	Hyaline cast in tubular lumen	Glomerular degeneration	Cell infiltration
Control	–	–	–	–	–	–
GM	+3	+3	+3	+3	+2	+2
GM + EPO	+1	+2	+1	+2	–	+1
GM + HP	+1	+1	+1	+1	–	–
EPO	–	–	–	–	–	–
HP	–	–	–	–	–	–

(–): null, (+): mild, (++): moderate, (+++): severe

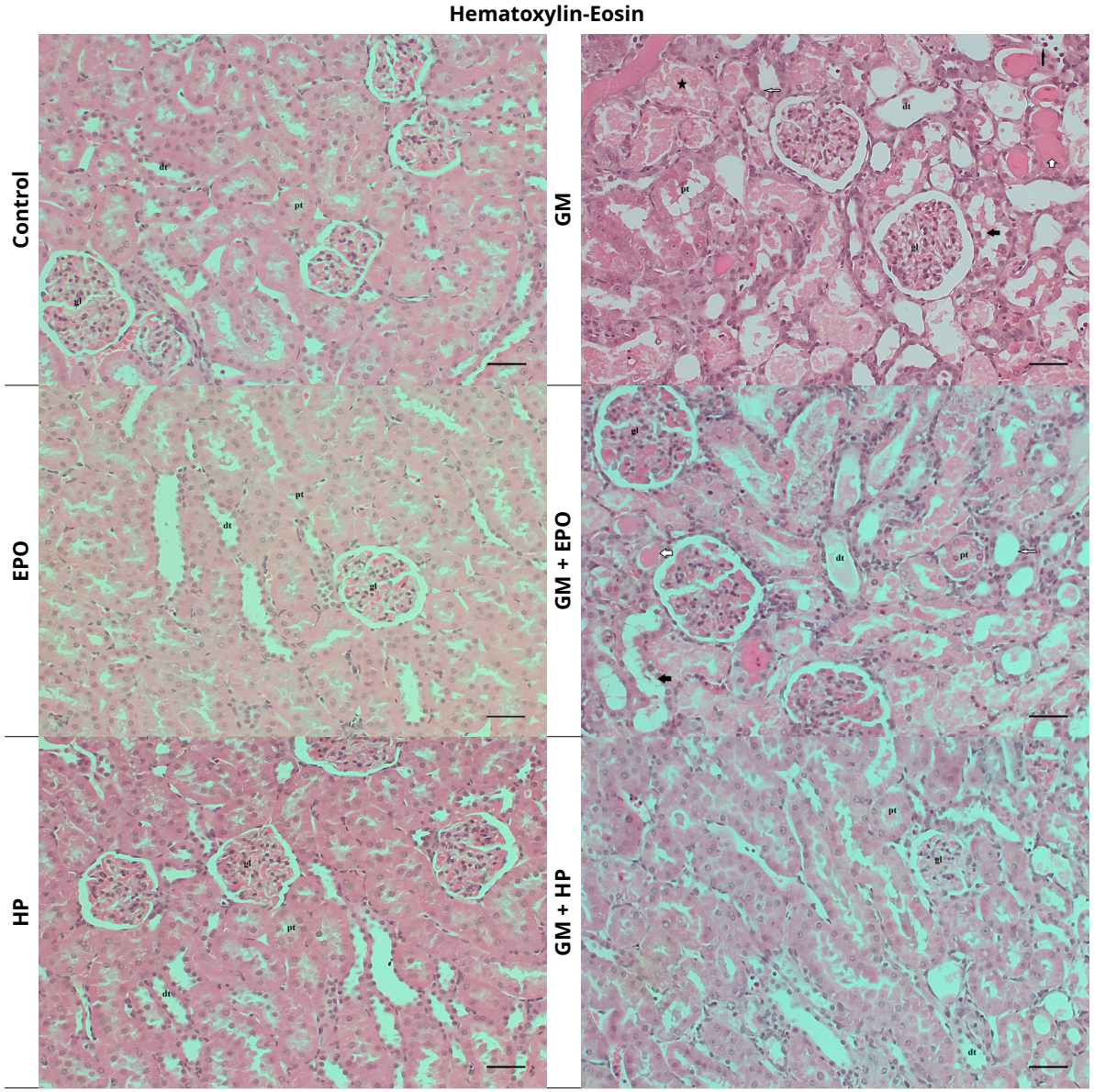

FIGURE 1. Histopathological changes of the kidney tissues in all groups, gl: glomerulus, dt: distal tubule, pt: proximal tubule, black thick arrow: vacuolization, black thin arrow: picnotic cell, white thick arrow: hyaline cast, white thin arrow: loss of brush border, black star: degenerated tubule, Bars: 100 µm

GM has been reported to increase pro-apoptotic Bax and caspase-3 levels and decrease anti-apoptotic Bcl-2 expression in renal tubular cells [40]. Similar to the literature, in this study found that caspase-3 immunoreactivity was increased in the renal tubules of the GM group in compared to the control group. However, the density of the immunoreaction was found to be reduced in the GM + EPO and GM + HP groups in compared to the GM group. The microphotographies of the kidney in all groups is displayed in FIG. 2, and the caspase-3 histoscores is exhibited in FIG. 3.

In addition, it has been found that EPO reduces caspase-3 activity against DNA damage caused by both cisplatin-induced nephrotoxicity and ischemia-reperfusion [20, 41]. We just stated that this renal damage due to GM nephrotoxicity was considerably reduced by HP administration. Izol *et al.* [32] reported that HP provided renal protection by reducing edematous damage against GM nephrotoxicity and decreased caspase-3 activity as a protective against apoptosis. In addition, some studies have shown that HP has a protective effect against renal damage in nephrotoxicity induced by different agents [17, 42]. Caglar *et al.* [42] proved that HP has a curative effect against renal damage in CCl_4 nephrotoxicity similar to our findings. Therefore, the nephroprotective activity may be due to the repair of severe and widespread kidney damage by downregulating oxidative stress biomarkers of HP.

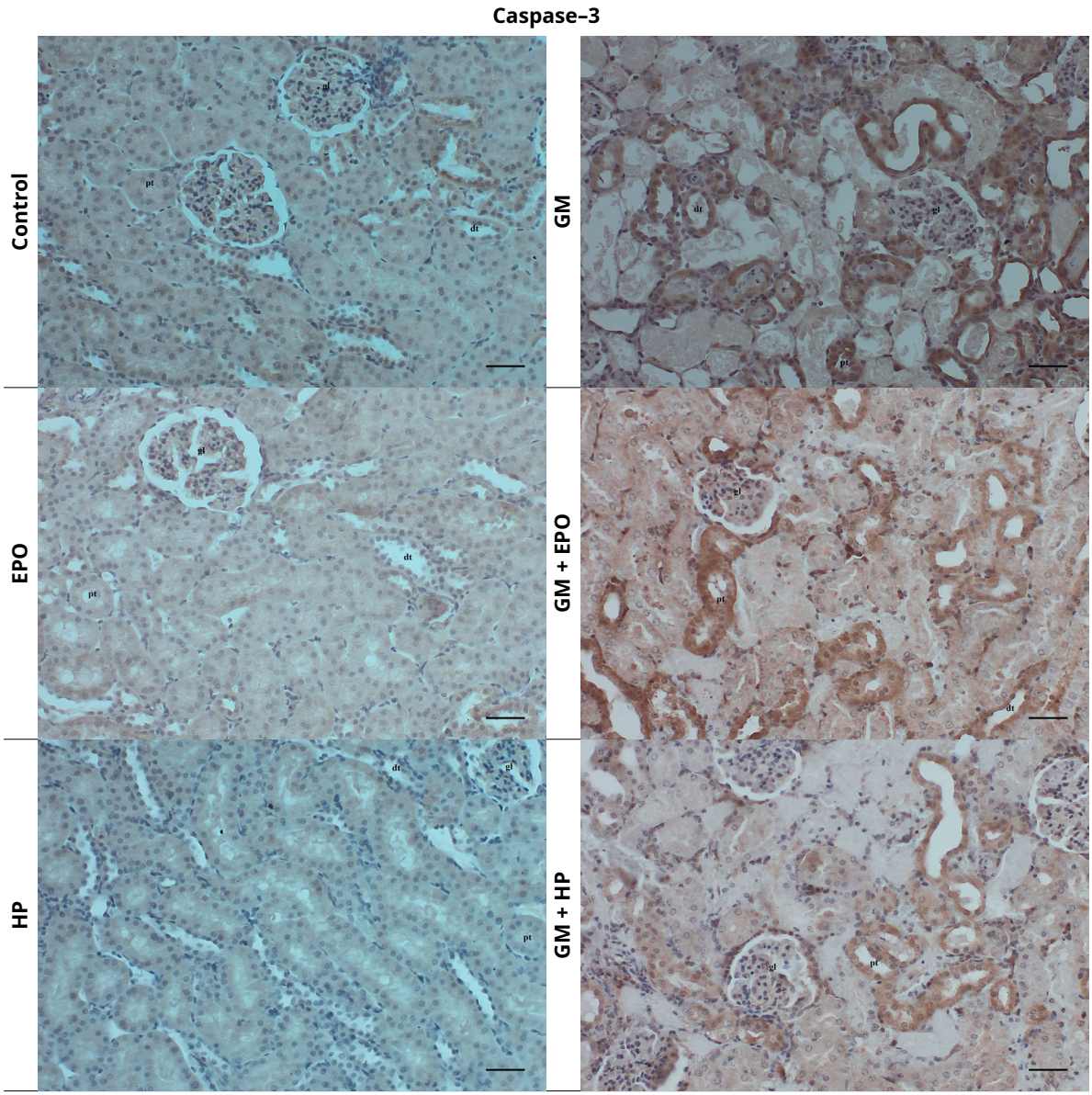

FIGURE 2. Immunohistochemical analysis of caspase-3 immunoreactivity in the kidney tissues in all groups, gl: glomerulus, dt: distal tubule, pt: proximal tubule, Bars: 100 µm

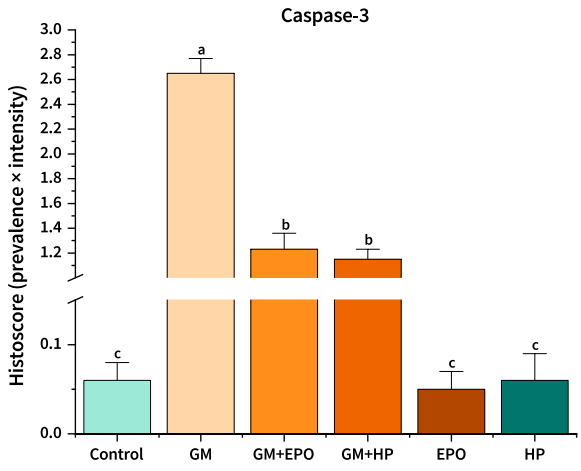

FIGURE 3. Immunohistochemical histoscore of the kidney tissues in all groups. a: *P*<0.05 for comparison between GM group and other groups, b: *P*<0.05 for the comparison between the GM group and the GM + EPO and GM + HP groups, c: *P*<0.05 for comparison between Control, EPO and HP groups and other groups. GM: Gentamicin, EPO: Erythropoietin, HP: *Hypericum perforatum*

CONCLUSIONS

Possible mechanisms of GM nephrotoxicity may be due to changes in renal function parameters, oxidative stress markers, apoptosis and histopathology. The healing impacts of EPO and HP against the renal damage reasoned by GM-induced these changes have been demonstrated in this study. It can be assumed that EPO and HP provide these restorative effects throught their antioxidant, antiapoptotic and nephroprotective properties. Further studies are needed to show that EPO and HP can be used as potential therapeutic agents to reduce the risk of nephrotoxicity.

Conflict of Interests

The authors declare that there is no conflict of interest.

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