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EFFECTS OF ERYTHROPOIETIN AND *Hypericum Perforatum* ON GENTAMICIN-INDUCED AGT, β-catenin, iNOS AND eNOS IMMUNOREACTIVITIES

ABSTRACT

Gentamicin (GM) is an antibiotic used in the treatment of acute infections caused by gram- negative microorganisms. Erythropoietin (EPO) is a cytokine that regulates cell proliferation and differentiation and has physiological roles in tissue protection. *Hypericum perforatum* (HP) is a phytochemical antioxidant with free radical scavenging and cell rejenerative properties. This study aimed to examine the effects of EPO and HP on GM-induced angiotensinogen (AGT), β-catenin, inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) immunoreactivities. 36 male Spraque-Dawley rats were divided into control, GM, GM+EPO, GM+HP, EPO and HP groups. It was determined that EPO and HP decreased the GM-induced increased AGT, β-catenin, iNOS immunoreactivities and increased the decreased eNOS immunoreactivity. In conclusion, it is thought that the renoprotective effects of EPO and HP can regulate the immunohistochemical changes caused by GM in the kidney.

Keywords: Gentamicin, Erythropoietin, *Hypericum perforatum*, Kidney, Immunohistochemistry

1. INTRODUCTION

Gentamicin (GM) is an effective antibiotic used in the treatment of acute and life-threatening infections caused by gram-negative microorganisms [1]. Especially after long-term treatment, 10-30% of patients exposed to this drug have a risk of nephrotoxicity pathology [2]. Despite its adverse side effects such as nephrotoxicity and ototoxicity, GM is still used to combat microorganism species that develop resistance to some antibiotics [3]. Although the mechanisms underlying GM nephrotoxicity are unclear, research suggest that gentamicin nephrotoxicity is a complex and multifaceted process in which gentamicin triggers cellular responses involving multiple pathways that culminate in renal damage and necrosis [1 and 4]. Therefore, the use of agents that control these cellular responses resulting from GM-induced tissue damage may be promising in the development of an effective preventive treatment [5]. Erythropoietin (EPO) is known as a hematopoietic hormone produced in the kidney and fetal liver in response to hypoxia, inflammation and cell death [6]. It has been stated that EPO which is known to have important physiological roles on general tissue protection, performs this protection via transmembrane receptors (EPO-R) expressed in different tissues [7 and 8]. It has been reported that EPO-R is found in tubular epithelial and endothelial cells, especially in the kidney, and has a protective effect in acute kidney injury [9]. Therefore, as a

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therapeutic agent, EPO has been shown to have a nephroprotective effect in various experimental models of kidney injury [10]. *Hypericum perforatum* (HP), known as St. John's Wort (SJW), is a perennial herb with phytochemical properties used in traditional medicine all over the world [11]. This plant is known to contain bioactive compounds such as hyperforin, quercetin, resveratrol, and flavonoid and xanthone derivatives [12]. It has been reported that HP which has been reported to have an anti-inflammatory effect and to prevent ROS-induced DNA damage and apoptosis, is widely used for the treatments of nephrotoxicity [11 and 13].

2. RESEARCH SIGNIFICANCE

Some of the nephrotoxicity that occurs in response to kidney-damaging drugs or toxins has been attributed to nephrotoxic drugs. Drug-induced toxicity is a common problem in clinical medicine, and these drugs can damage the kidney through a variety of mechanisms, including structural and functional changes. GM is a potent, broad-spectrum antibiotic used to defend against infections triggered by gram-negative microorganisms. However, GM, which is frequently used due to its low cost and sustained effect, has limited therapeutic efficacy as it triggers nephrotoxicity. It is known that some plants used for medicinal purposes and therapeutic agents with high antioxidant content have protective effects on GM-induced cell damage. Particularly considering that these agents prevent or ameliorate GM nephrotoxicity, this study aimed to examine the regulatory effect of EPO and HP on the immunohistochemical changes resulting from this nephrotoxicity.

Highlights:

- Induction of nephrotoxicity by GM in rats
- Use of EPO and HP on immunohistochemical changes caused by GM-induced nephrotoxicity
- Determination of the regulatory effects of EPO and HP on altered immunoreactivity due to GM-induced nephrotoxicity.

3. EXPERIMENTAL METHOD-PROCESS

3.1. Chemicals

GM (160mg/2ml, Ibrahim Etem Menarini group, Istanbul, TR), EPO (Dropoetin, 4000IU/0.4ml, Drogsan, Istanbul, TR) and HP (St. John's Wort, 300mg/kg, Solgar, Leonia, USA) were obtained from various companies and other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

3.2. Animals

In this study, 36 male Spraque-Dawley rats, 6-8 weeks old, weighing 280-300g, were used. The rats were obtained from Firat University Experimental Research Center (Elazig, Turkey). During the study period, the rats were housed under standard laboratory conditions (40%-60% humidity, 24±3°C temperature, and 12 hours light-12 hours dark cycle), food and water was provided *ad libitum*. All the experiments were approved by the local ethics committee of the Firat University (Ethic no: 15.01.2020-2020/01) followed by Animal Research: Reporting of In Vivo Experiments guidelines and National Institutes of Health Animal Research guidelines.

3.3. Experimental Design

The rats were divided into 6 groups, 6 in each group, and the study continued for 9 days. In the control group, 0.5ml isotonic saline was administered daily intraperitoneally (ip). In the GM group,

at a dose of 100mg/kg/BW/day Gentamicin was administered ip with 0.5ml isotonic saline [14]. In the GM+EPO group, at a dose of 100mg/kg/BW/day Gentamicin was administered ip with 0.5ml isotonic saline and at a dose of 1000IU/kg/BW Dropoetin was administered ip on the 1st, 5th and 9th days of the study period [15]. In the GM+HP group, at a dose of 100mg/kg/BW/day Gentamicin was administered ip with 0.5ml isotonic saline and at a dose of 200mg/kg/BW/day HP was administered by gavage with 0.5ml isotonic saline [16]. In the EPO group, at a dose of 1000IU/kg/BW Dropoetin was administered ip on the 1st, 5th and 9th days of the study period. In the HP group, at a dose of 200mg/kg/BW/day HP was administered by gavage with 0.5ml isotonic saline. All rats were sacrificed under ether anesthesia 24 hours after the last administration. Then, kidney tissues were taken by laparotomy and they were placed in fixation solution for immunohistochemical examinations.

3.4. Immunohistochemical Analysis

The Avidin-Biotin-Peroxidase Complex (ABC) procedure was applied to the tissue sections [17]. In this method, it was used that as primer antibodies angiotensinogen (AGT) polyclonal antibody (Catalog no: PA5-33340, dilution ratio 1/200; Invitrogen, USA), β-catenin polyclonal antibody (Catalog no: 71-2700, dilution ratio 1/200; Invitrogen, USA), inducible nitric oxide synthase (iNOS) polyclonal antibody (Catalog no: bs-22924R, dilution ratio 1/200; Bioss, USA)and endothelial nitric oxide synthase (eNOS) polyclonal antibody (Catalog no: bs-0163R, dilution ratio 1/200; Bioss, USA). The staining was performed with the immunohistochemistry kit (IHC kit, Catalog no: TP-015-HD, UltraVision Detection System, Anti-Polyvalent, HRP/DAB; Thermo Fisher Scientific Co., USA) used for the other steps according to the manufacturer's instructions. The staining was completed with 3,3′-diaminobenzidine (DAB) chromogen and counterstained with Mayer's hematoxylin. Immunohistochemical staining was calculated with a numerical score of 0–3, with 0=negative, 0.5=trace, 1=mild, 2=moderate, and 3=intense. Negative, <25%, 26%–50%, 51%–75%, and 76%–100% areas were assigned values of 0, 0.1, 0.4, 0.6, and 0.9, respectively. Final histoscore calculation was performed using the following formula Histoscore=AreaxDensity [18].

3.5. Statistical Analysis

Statistical data were analyzed with the IBM SPSS/PC (Version 21.0, IBM Co., North Castle, New York, USA) software program. Data were presented as mean ± standard deviation. Differences between groups were analyzed using one-way analysis of variance (ANOVA) and the posthoc Duncan test. Statistical significance was determined as $p<0.05$.

4. RESULTS AND DISCUSSION

The immunohistochemical histoscores of the kidney tissues in all groups are presented in Table 1.

Table 1. Effect of EPO and HP on GM-induced AGT, β-catenin, iNOS and eNOS immunoreactivities

Groups	AGT	β-catenin	iNOS	eNOS
Control	0.05 ± 0.01^{c}	0.06 ± 0.03^{c}	0.05 ± 0.08^{c}	1.10 ± 0.15^{ab}
GM	2.70 ± 0.13^{a}	2.67 ± 0.11^{a}	2.70 ± 0.10^{a}	0.83 ± 0.08^{b}
GM + EPO	1.19 ± 0.11^{b}	1.19 ± 0.18^{b}	1.20 ± 0.14^{b}	1.66 ± 0.11^{a}
GM + HP	1.17 ± 0.09^{b}	1.18 ± 0.12^{b}	1.30 ± 0.12^{b}	1.72 ± 0.17^{a}
EPO	0.06 ± 0.03^{c}	0.08 ± 0.07^{c}	0.06 ± 0.03^{c}	1.22 ± 0.10^{ab}
HP	0.05 ± 0.02^{c}	0.07 ± 0.01^{c}	0.05 ± 0.01^{c}	1.25 ± 0.09^{ab}

Data are given as mean ± standard deviation for each group. [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*, AGT: angiotensinogen, iNOS: inducible nitric oxide synthase and eNOS: endothelial nitric oxide synthase. AGT immunoreactivity was observed to be similar in the control, EPO and HP groups. Compared to the control group, this immunoreactivity in the GM group was found to be increased especially in the proximal and distal tubules. On the other hand, it was determined that the immunoreactivity in the tubules was decreased in the GM+EPO and GM+HP groups compared to the GM group. The immunohistochemical analysis of AGT immune reaction in the kidney tissues in all groups is shown in Figure 1.

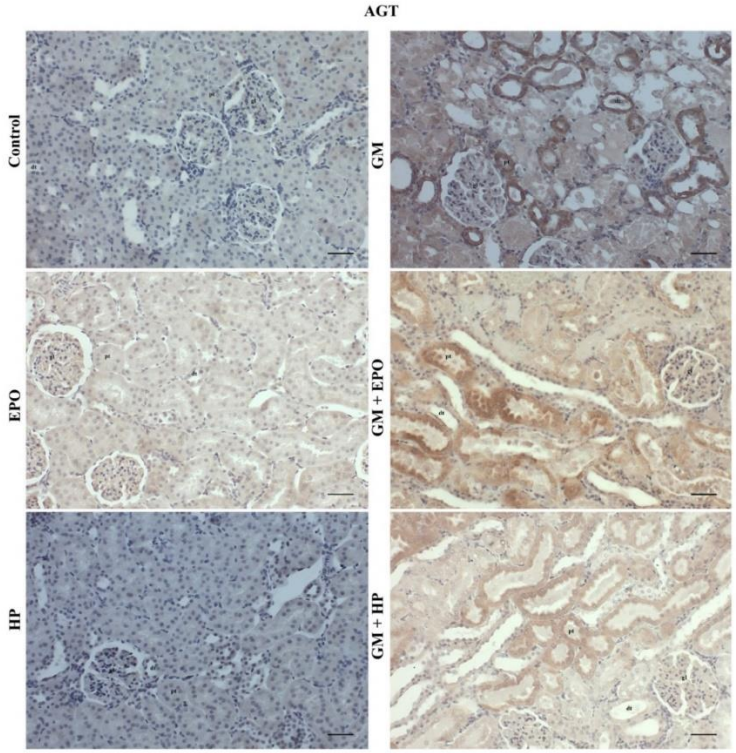

Figure 1. Immunohistochemical analysis of AGT immune reaction in the kidney tissues in all groups, GM: Gentamicin, EPO: Erythropoietin, HP: *Hypericum perforatum*, AGT: Angiotensinogen, gl: glomerulus, dt: distal tubule, pt: proximal tubule, Scale bar: 100µm

β-catenin immunoreactivity was observed to be similar in the control, EPO and HP groups. Compared to the control group, this immunoreactivity in the GM group was found to be increased especially in the glomerular areas. However, it was noted that the immunoreactivity in the GM+EPO and GM+HP groups was decreased compared to the GM group. The immunohistochemical analysis of β-catenin immune reaction in the kidney tissues in all groups is shown in Figure 2.

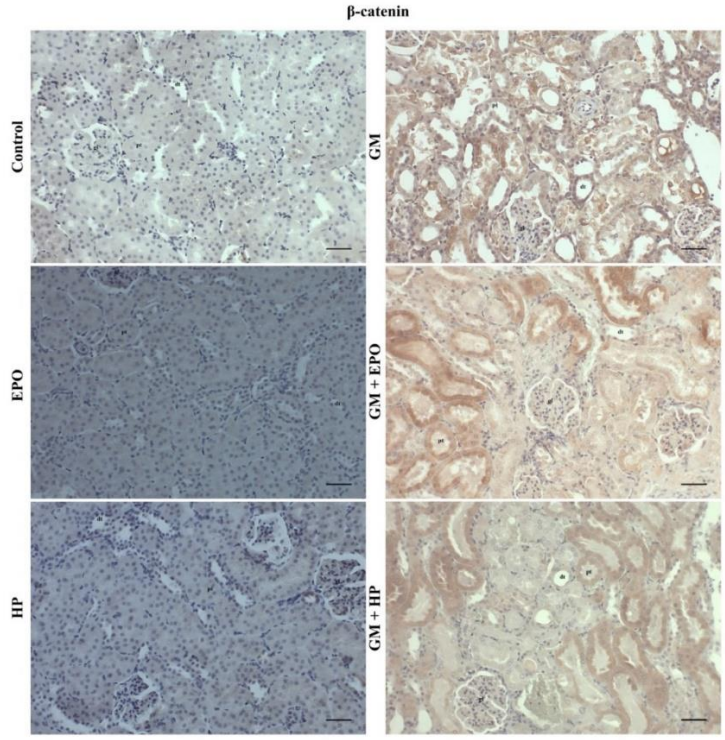

Figure 2. Immunohistochemical analysis of β-catenin immune reaction in the kidney tissues in all groups, GM: Gentamicin, EPO: Erythropoietin, HP: *Hypericum perforatum*, gl: glomerulus, dt: distal tubule, pt: proximal tubule, Scale bar: 100µm

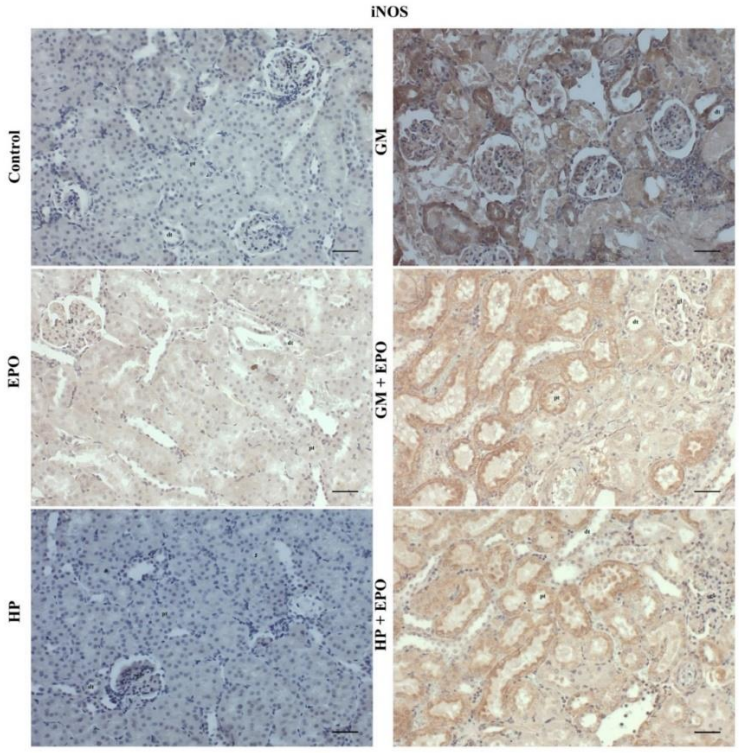

Figure 3. Immunohistochemical analysis of iNOS immune reaction in the kidney tissues in all groups, GM: Gentamicin, EPO: Erythropoietin, HP: *Hypericum perforatum*, iNOS: inducible nitric oxide synthase, gl: glomerulus, dt: distal tubule, pt: proximal tubule, Scale bar: 100µm

iNOS immunoreactivity was observed to be similar in the control, EPO and HP groups. This immunoreactivity was observed to be increased in the glomerular, intertubular and tubular regions in the GM group compared to the control group. The intensity of immunoreactivity in these regions was seen to be decreased in the GM+EPO and GM+HP groups compared to the GM group. The immunohistochemical analysis of iNOS immune reaction in the kidney tissues in all groups is shown in Figure 3.

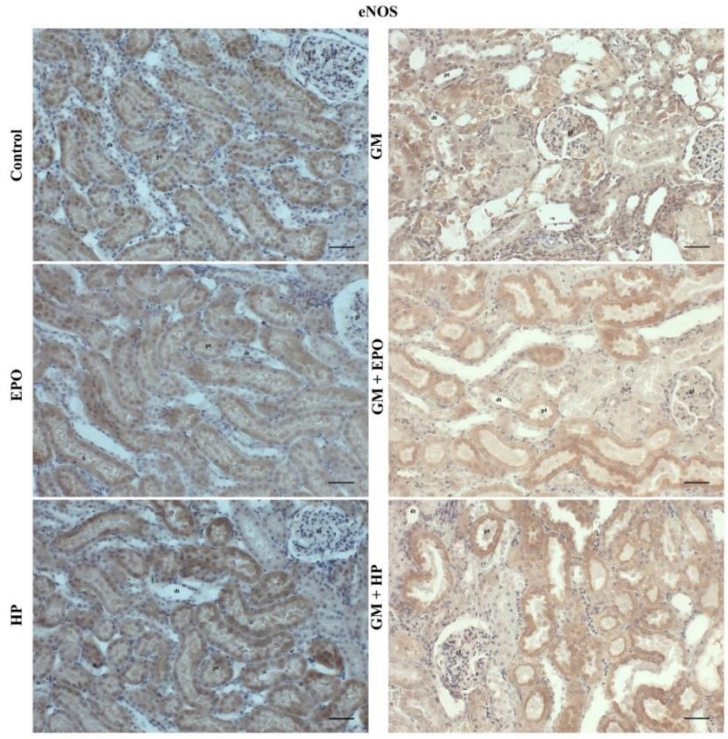

Figure 4. Immunohistochemical analysis of eNOS immune reaction in the kidney tissues in all groups, GM: Gentamicin, EPO: Erythropoietin, HP: *Hypericum perforatum*, eNOS: endothelial nitric oxide synthase, gl: glomerulus, dt: distal tubule, pt: proximal tubule, Scale bar: 100µm

eNOS immunoreactivity was observed to be similar in the control, EPO and HP groups. It was noted that this immunoreactivity was decreased in the glomerulus and renal tubules of the GM group compared to the control group. However, it was determined that the intensity of immunoreactivity increased in the GM+EPO and GM+HP groups compared to the GM group. The immunohistochemical analysis of eNOS immune reaction in the kidney tissues in all groups is shown in Figure 4.

It is known that intrarenal RAS expression is significantly correlated with markers of acute tubular injury and may be an indicator of the severity of acute tubular necrosis [19]. The presence of AGT reflecting this expression in the proximal tubules and its secretion into the lumen have been associated with the severity of chronic kidney injury [20]. In a study, it was stated that strong AGT immunoreactivity especially in the proximal and distal tubules in overexpression of the intrarenal RAS system determines the severity of acute tubular necrosis [19]. In our study, we first identified intense AGT immunoreactivity, especially in the proximal and distal tubules, due to GM nephrotoxicity. In this context, the strong localization of AGT in necrotized tubules in our study may suggest that this substrate may be a biomarker candidate in GM nephrotoxicity. It has been

reported that inhibition of RAS activity is renoprotective and can slow or even stop the progression of nephropathies [21]. EPO, whose renoprotective effects are known, has been reported to have beneficial effects on the RAS and aldosterone system (RAAS). Although the mechanism of these effects has not been fully determined, it has been stated that EPO mediates the suppression of RAS and aldosterone [22]. In addition, the effects of treatment with EPO on the RAAS system at the molecular level in rats were evaluated and it was reported that both renin mRNA and AGT mRNA levels in the kidney were increased by EPO [23]. In the light of this information, we observed that EPO reduced the increase in AGT immunoreactivity in GM-induced nephrotoxicity. Therefore, it can be said that EPO exhibits a nephroprotective effect against kidney damage through inhibition of RAS. In addition, AGT mRNA/protein expression, which is the only substrate of renin, known as the rate-limiting enzyme of the RAS system, is known to exist in the proximal tubule cells of the kidneys [24]. In addition, studies in which excessive activation of the RAS system was inhibited by various angiotensin-converting enzyme (ACE) inhibitors were reported to have a renoprotective effect of RAS blockade [25]. It has been reported that HP has an antihypertensive effect in the treatment of some cardiovascular diseases, especially with its newly discovered ACE inhibitor activity [26]. In our study, we found that HP reduced the increase in GM-induced AGT immunoreactivity. In the light of this information, it can be predicted that HP reduces the systemic adverse effects of RAS through its ACE inhibitor effect.

As a signaling protein, β-catenin, which plays a role in important physiological processes including organ development, tissue homeostasis and damage repair, has been reported to be important for Wnt signal-dependent nephron formation at the renal level [27 and 28]. While the Wnt/β-catenin signaling mechanism is low in normal kidneys, this mechanism is upregulated in rat models with acute and chronic kidney injury [29 and 30]. It has been determined that this signal plays a role in the proliferation and in the regulation of cell cycles of intact renal tubular cells after injury [31]. It has been reported that β-catenin-dependent signaling pathway is effective in GM-induced nephrotoxicity and β-catenin levels increase with this nephrotoxicity [32]. We first identified the increased β-catenin immunoreactivity in GM-induced nephrotoxicity in our study. Therefore, it can be said that the β-catenin signaling pathway is activated to support cell survival against tubular damage caused by GM, and its localization increases accordingly. It is stated that the regenerative effects of EPO are due to the stimulation of angiogenesis, cell proliferation and cell differentiation, which reduces cell damage, promotes repair and regulates physiological functions [33 and 34]. In a study on EPO used against GM nephrotoxicity, it was reported that β-catenin positivity decreased in nephrogenic bodies and tubules [35]. Similarly, in our study, we found that increased β-catenin immunoreactivity in GM-induced nephrotoxicity was reduced by EPO treatment. According to the data of ours and other studies, it can be said that EPO, which has a wide range of regenerative effects, regulates the activation of β-catenin especially in GM nephrotoxicity. In addition, it has been reported that activation of Wnt/β-catenin signaling pathway plays an important role in adaptive repair of acute kidney injury and attenuation of regeneration [29 and 36]. In a recent study, it was proven that anticarcinogenic hyperforin from the HP plant leads to inhibition of canonical Wnt/β-catenin signaling, an oncogenic pathway that contributes to tumorigenesis. It has also been demonstrated that hyperforin through inhibition of this pathway reduces cell

proliferation and anchorage-independent growth [37]. In our study, we determined that increased β-catenin immunoreactivity in GM-induced nephrotoxicity decreased with HP administration. Based on this, it can be assumed that HP, known for its widespread reparative effects, regulates GM-induced β-catenin activation.

It is known that nitric oxide (NO) production is usually carried out by iNOS [38] and a small amount of eNOS [39]. It has been reported that excessive NO production is associated with oxidative stress [40] and is due to increased iNOS expression induced by GM [41]. Increased iNOS-mediated NO concentration has been reported to lead to DNA damage and apoptosis and thus trigger renal failure as a result of iNOS induction and tubular cytotoxicity [42]. Studies have shown that iNOS expression increases and kidney damage is induced in GM-induced nephrotoxicity [43 and 44]. Conversely, eNOS expression was found to be decreased in GM-induced nephrotoxicity [45]. In this study, we determined increased iNOS and decreased eNOS immunoreactivity in GM-induced nephrotoxicity. As a result of GM application, it can be said that NO production can be regulated by up-regulation of iNOS expression and down-regulation of eNOS expression. There are studies showing that the inhibition of NO production in GM-induced nephrotoxicity can be improved by using various renoprotective agents [5, 43, 44 and 46]. In particular, it has been reported that the decrease in NO level may be due to the decrease in iNOS level. However, it has been reported that the low amount of NO produced by eNOS and its synthesis in large amounts by iNOS cause the eNOS level to remain high [39]. It is known that EPO, which has a cytoprotective effect, achieves this effect through the regulation of EPO-induced NO production and especially eNOS [47 and 48]. It has also been stated that EPO also increases NO bioavailability by upregulating its expression through transcription and activation of eNOS [47 and 49]. We found that increased iNOS immunoreactivity decreased and decreased eNOS immunoreactivity increased with EPO treatment in GM-induced nephrotoxicity. Accordingly, it can be thought that the cytoprotective effect of EPO occurs by modulation of iNOS and eNOS expressions. In addition, some antioxidant flavonoids [50] contained in HP have been reported to inhibit NOS activity [51]. In a study, the inhibitory effect of HP on iNOS was demonstrated [52]. Basically, it is known that the decrease in iNOS level is associated with the decrease in NO production, but the eNOS level, which contributes less to NO production, remains high [39]. It has been reported that NO produced by iNOS has a pathogenic role in GM-induced nephrotoxicity [53] and HP application reduces the increase in GM-induced iNOS [54]. We determined that increased iNOS immunoreactivity decreased and decreased eNOS immunoreactivity increased with HP administration in GM-induced nephrotoxicity. In light of these results, it can be thought that treatment of increased iNOS and decreased eNOS expressions with HP may reduce GM-induced immunohistochemical changes.

5. CONCLUSION AND RECOMMENDATIONS

GM-induced kidney damage may cause changes in the expression of some immunohistochemical markers. In this study, the regulatory effects of EPO and HP, which have nephroprotective properties, on AGT, β-catenin, iNOS and eNOS immunoreactivities resulting from GM-induced kidney damage were revealed. Since HP's damage-regulating effect is more pronounced, it can be stated that its use as a natural product suitable for daily nutrition will be particularly attractive. As a result, we think that alternative uses of these pharmacological agents in various kidney injuries, especially GM-induced, can be demonstrated by applying different parameters and techniques.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

FINANCIAL DISCLOSURE

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DECLARATION OF ETHICAL STANDARDS

This experimental study was performed with approval from the Animal Experiments Local Ethics Committee of the Firat University (Approval Number: 15.01.2020-2020/01).

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