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Özgün Araştırma / Research Article

Metotreksat kaynaklı böbrek hasarı ve irisin immünoreaktivitesine karşı benfotiaminin terapötik potansiyeli

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Theraupeutic Potency Of Benfotiamine Against Methotrexate-Induced Kidney Injury And Irisin Immunoreactivity

Özet

Amaç: Benfotiamine (BFT), antioksidatif etkilere sahip etkili bir ajandır. Bu çalışmada, BFT'nin metotreksat (MTX) kaynaklı böbrek hasarı ve irisinin immünoreaktivitesi üzerindeki etkileri araştırılmıştır. **Yöntem:** Toplam 28 sıçan dört eşit gruba ayrıldı: Kontrol (herhangi bir tedavi uygulanmadı), BFT (50 mg / kg BFT oral gavaj ile verildi), MTX (20 mg / kg MTX intraperitoneal yolla verildi) ve MTX + BFT. Çalışmanın sonunda, alınan böbrek dokuları rutin histolojik takip serilerinden geçirildi ve parafin bloklara gömüldü. Histopatolojik inceleme için hematoksilen & eozin, irisin ve kaspaz 3 için streptavidin-biotin-peroksidaz kompleks yöntemi parafin bloklardan alınan kesitlere uygulandı. Toplam antioksidan seviyesi (TAS) ve toplam oksidan seviyesi (TOS) Rel Assay kitleri ile belirlendi. **Bulgular**: MTX'in histopatolojik hasara, irisin ve kaspaz-3 immünoreaktivitesinde önemli bir artışa neden olduğu gözlendi. Biyokimyasal olarak, MTX verilen hayvanlarda toplam oksidan seviyesinde (TOS) önemli artış ve toplam antioksidan seviyesinde (TAS) düşüş belirlendi. BFT tedavisinin histopatolojik hasarı iyileştirdiği, TOS ve kaspaz-3 immünoreaktivitesini önemli ölçüde azalttığı, TAS düzeylerini artırdığı ve irisin immünoreaktivitesini önemsiz ölçüde azalttığı bulundu. **Sonuç:** Sonuç olarak BFT, MTX'in neden olduğu böbrek hasarını önlemede koruyucu etkiler sergilemiştir.

Anahtar Kelimeler: benfotiamin, irisin, böbrek, methotrexate

Abstract

Objective: Benfotiamine (BFT) is an effective agent with anti-oxidative effects. In this study, the effects of BFT on methotrexate (MTX)-induced kidney injury and the immunoreactivity of irisin were investigated. **Method:** A total of 28 rats were separated into four equal groups. Control (no treatment was performed), BFT (50 mg/kg BFT was applied by oral gavage), MTX (20 mg/kg MTX was applied via intraperitoneal route), and MTX+BFT. At the end of the study, the removed kidney tissues were passed through routine histological follow-up series and embedded in paraffin blocks. For histopathological examination hematoxylin & eosin, irisin and for caspase 3 streptavidin-biotin-peroxidase complex method was applied to sections taken from paraffin blocks. Total antioxidant status (TAS) and total oxidant status (TOS) status were determined with Rel Assay Kits. **Results:** MTX was found to have caused histopathological damage and a significant increase in irisin and caspase-3 immunoreactivity. Biochemically, significant increases in total oxidant status (TOS) and decreases in total antioxidant status (TAS) were determined in the animals given MTX. BFT treatment was found to ameliorate histopathological damage, significantly decrease TOS and caspase-3 immunoreactivity, increase TAS levels, and insignificantly decrease irisin immunoreactivity. **Conclusion:** Consequently, BFT exhibited protective effects in preventing kidney damage caused by MTX.

Key words: benfotiamine, irisin, kidney, methotrexate

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INTRODUCTION

Methotrexate (MTX) is an extensively used anticancer drug that is applied for the treatment of some malignant and autoimmune diseases. MTX is principally used for its cytotoxic, anti-inflammatory, and anti-proliferative properties (Shen et al. 2012). In contrast its usage has been associated with various adverse effects in different tissues and organs with a wide range of severity (Tabassum et al. 2010).

Nephrotoxicity is admittedly one of the mostly adverse effects of MTX because it is principally (90%) drained by the kidney (Jahovic et al. 2003). In MTX nephrotoxicity, damage occurs directly from the toxic effects of MTX or by the deposition of MTX and/or metabolites into the tubules of the kidneys (Chabner and Longo 2010). MTX causes mitochondrial dysfunction (Heidari et al. 2018) and enhances the generation of reactive oxygen species (ROS) by reducing the intracellular nicotinamide adenine dinucleotide phosphate (NADPH) levels. (Vardi et al. 2010). Oxidative damage and neutrophil infiltration might be the cause of MTX-induced nephrotoxicity (Kolli et al. 2009). Apoptosis occurs by way of oxidative stress that causes DNA damage (Herman et al. 2005).

Thiamine, riboflavin, and pyridoxal have preventive potencial in terms of drug-caused nephrotoxicity (Bello and Chika 2009). Thiamine, also known as vitamin B1, is a fundamental nutrient, member of the antioxidant system and has role in energy metabolism (Dhir et al. 2019) which has been declared as one of several fundamental cofactors for adenosine triphosphate (ATP) generation; however, its effects on the cellular and molecular levels are not fully understood yet (Hernandez-Vazquez et al. 2016). Reduced dietary intake of thiamine results in a defective energy metabolism and raises levels of cellular oxidative stress (Raj et al. 2018). Benfotiamine (BFT) is a lipophilic thiamine pro-drug with a much higher bio-availability than thiamine (Frank et al. 2000). It has direct ROS scavenging capacity and prevents oxidative damage (Schmid et al. 2008). Schupp et al. (2008) suggested that BFT reduces DNA damage by increasing antioxidant capacity. The anti-inflammatory effect of BFT is achieved through the synthesis of prostaglandins and leukotrienes (Shoeb and Ramana 2012).

Irisin, first reported in the muscles as an exercise-related hormone (Bostrom et al. 2012), plays a role in energy regulation (Aydin 2014) and has many different metabolic effects (Patil et al. 2018). In addition to being primarily secreted by muscle tissue, irisin is also secreted by the liver, pancreas, kidney, skin, salivary gland, and sweat glands (Kuloglu and Aydin 2014). It has been reported that irisin has antioxidant and anti-inflammatory effects (Bosma et al. 2016). Circulating irisin plays an important role in the mechanism of many diseases (Park et al. 2013). Since its discovery, the physiopathological role of irisin has been investigated in many studies (Park et al. 2013; Zhang et al. 2014; Kuloglu and Aydin 2014; Sarioglu et al. 2016; Erdogan and Yalcin 2018; Patil et al. 2018; Keles et al. 2019). If the working mechanism of irisin is well determined, it may play an important role in overcoming many clinical disorders (Gouveia et al. 2016).

As no study has been published about the protective effects of BFT in MTX-induced renal injury and irisin immunoreactivity, we aimed to investigate the histopathological, immunohistochemical, and biochemical effects of BFT treatment against MTX-induced renal injury using a rat model.

MATERIALS AND METHODS

A total of 28 male Wistar strain albino rats (200-220 g, 8-10 weeks of age), supplied from Adiyaman University Experimental Animal Production and Research Center, were randomly separated into four groups (n=7). After 15 days of conditioning, the experimental rats were maintained with an ad libitum standard rodent pellet diet and water in a 12 hour light / 12 hour dark cycle at room temperature (25 ± 30C). The control group received no treatment during the 14 day experimental period. The BFT group was administered a daily 50 mg/kg dose of BFT by oral gavage (o.g). The MTX group was administered a single 20 mg/kg dose of MTX (Methotrexate, Kocak Farma Pharmaceuticals and Chemical Industry Inc. Istanbul-Turkey) via the intraperitoneal (i.p.) route on the first day of the experiment. The MTX+BFT group was administered a single dose of MTX at 20 mg/kg ratio via the i.p. route on day 1 and 50 mg/ kg BFT by o.g. daily throughout the experimental period. At the end of the experiment, the rats were decapitated under anesthesia: ketamine (75 mg/kg) and xylazine (10 mg/kg). Kidney tissues were removed and placed in 10% formalin. Some fresh tissues samples were stored at -20°C for total antioxidant status (TAS) and total oxidant status

(TOS) measurements. The animals were processed according to suggested ethical guidelines for the care of laboratory animals throughout the experiment, (Laboratory Animal Care Committee of Adiyaman University, protocol number: 2018/019).

Measurement of TAS and TOS levels

Kidney tissues were washed with cold phosphate buffer saline (PBS) and kept at - 20°C until the analysis for evaluating tissue TAS and TOS levels. The kidney tissues were homogenized in ice with a 0.1 M, phosphate buffer at a ratio of 1/10 at a pH of 7.4 and centrifuged at 1800 rpm for five minutes. TAS and TOS levels were measured by using Total Antioxidant Status Assay Test Kit (Rel Assay Diagnostic, Gaziantep, Turkey) and a Total Oxidant Status Assay Test Kit (Rel Assay Diagnostic, Gaziantep, Turkey) respectively with an auto analyzer (Olympus AU2700) to judge the degree of oxidant damage according to the method of Erel (2004) and (2005) respectively.

Tissue preparation and histopathologic examination

Formalin-fixed kidney tissue samples were embedded in paraffin after routine procedures, then sectioned to a thickness of four to six µm and stained with hematoxylin-eosin (HxE). The stained sections were blindly analyzed under a light microscope (Leica DM500 attached Leica DFC295 Digital Image Analyze System).

Immunohistochemistry for irisin and caspase-3

Irisin and caspase-3 expression was evaluated within kidney tissue sections. For this purpose 5 µm thick tissue sections obtained from the paraffin blocks were stained with primary antibody of irisin (Irisin Rabbit Polyclonal H-067-17, Phoenix Pharmaceuticals, Inc., California, USA) according to a previous study (Erdogan and Yalcin 2018) and of caspase-3 (Caspase-3, Rabbit polyclonal IgG, Abcam, ab2302, London, UK) according to Yalçın and Pekmez 2020. Slides were photographed on a Leica DM500 microscope (Leica DFC295). The histopathological score was determined with respect to the prevalence (0.1: < 25%, 0.4:26–50%, 0.6: 51–75%, 0.9: 76–100%) and intensity (0: absent, +0.5: very low, +1: low, +2: moderate, +3: severe) of immunoreactivity. The histopathological score was determined as prevalence (X) intensity.

Statistical analyses

Statistical analyses were performed with SPSS 15.0 according to Üçkardeş et al. (2013). The normal distribution of the groups of TAS and TOS, and the immune variables were evaluated by the Kolmogorov Smirnov test. A one-way analysis of variance was used in group the comparisons of TAS, TOS, and immunoreactivity variables. Levene statistics were used for homogeneity testing of the variances. The Tukey HSD multiple comparison test was used to define the differences between groups of significant variables. Results were given as a mean ± SD. Significance level was accepted as $p<0.05$.

RESULTS

TAS and TOS Results

In the biochemical analysis of the tissue samples, the control and the BFT groups had similar TAS and TOS levels (Table 1). A statistically significant decrease of TAS levels and increase of TOS levels was observed in the MTX group ($p<0.05$) compared to the control group. In terms of TAS level, an insignificant increase was observed in the MTX+BFT group ($p>0.05$), while a significant decrease in TOS levels was observed ($p<0.05$) compared to the MTX group.

Table 1. Tissue levels of TAS and TOS in the kidney

Groups (n:7)	TAS	TOS
Control	$1,38^{a}\pm0,08$	$15,32^{a}\pm0,79$
BFT	$1,40^{a}\pm0,06$	$15,38^{a}\pm0,711$
MTX	$0,93^{c}\pm0,20$	$20,28^{c}\pm0,81$
MTX+BFT	$1,14^{bc}\pm0,06$	$16,72^{b}\pm0,61$
P* values	<0.001	<0.001

TAS and TOS levels are given as a mean ± standard deviation compared with the control group.
^{abc} Means within the same column with differing superscripts are significantly different (P<0.05)
**:One Way Anova*

Histopathological Results

HxE stained kidney tissues are shown in Figure 1. In the histopathological examination, the control (A) and the BFT group (B) had normal glomerulus, proximal tubul and distal tubul. The MTX group (C) exhibited marked glomerular atrophy, tubular dilatation and leukocyte infiltration compared to the control group. A significant decrease of tubular dilatation and glomerular atrophy were observed in the MTX+BFT group (D). In addition, leukocyte

infiltration, which was prominent in the MTX group, was not observed in this group.

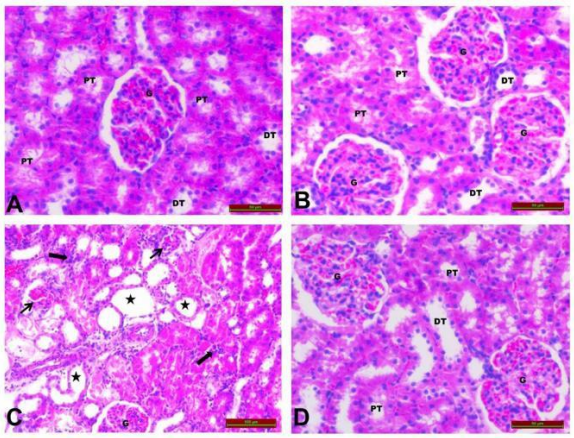

Figure 1. *A-D: H-E stained kidney tissues. The scale bars represent 50 µm for figure A,B,D and 100 µm for figure C. A- Control group, normal histological structure of glomerulus (G) proximal tubul (PT) and distal tubul (DT). B- BFT group, normal histological structure of glomerulus (G) proximal tubul (PT) and distal tubul (DT). C- MTX group, glomerular atrophy (thin black arrow), dilated tubules (asterisk), leucocyte infiltration (thick black arrow). D- MTX+BFT group, similar histologic appearance to the control group*

Immunoreactivity Results For İrisin

Irisin immunoreactivity was observed in the epithelial cells of the renal tubules (Figure 2). The control group (0.714±0.254) and the BFT group (0.886±0.227) exhibited similar reactivity. Irisin immunoreactivity was observed to be significantly increased ($p<0.05$) in the MTX group (1.257±0.411) compared to the control group. In contrast an insignificant decreased in irisin immunoreactivity was determined in the MTX+BFT group (1.114±0.363) compared to the MTX group as shown in Table 2.

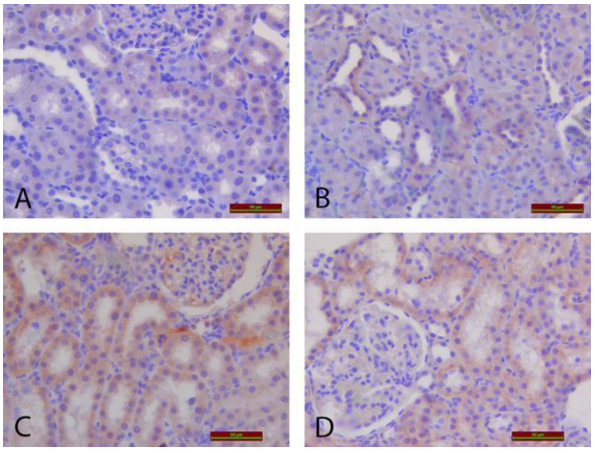

Figure 2. *A-D. Kidneys stained with Streptavidin biotin peroxidase complex method with Mayer's Hematoxylin counterstain for irisin immunoreactivity. The scale bars represent 50 µm in AEC chromogen used for visualization. A - Control group, irisin immunoreactivity (brownish-red) B - BFT group, irisin immunoreactivity (brownish-red) C - MTX group, significantly increased irisin immunoreactivity (brownish-red) D - MTX+BFT group, insignificantly decreased irisin immunoreactivity (brownish-red).*

Immunoreactivity Results For Caspase-3

Caspase-3 immunoreactivity was observed in the epithelial cells of the renal tubules (Figure 3). In the control (0.500±0.17) and the BFT (0.400±0.19) groups, caspase-3 was minimally expressed and no statistical differences were observed between these groups ($p>0.05$). Adversely in the MTX group (2.25±0.49), caspase-3 reactivity was significantly increased when compared to the control group. In contrast caspase-3 expressions were observed to reverse in the MTX+BFT group in a significant manner compared to MTX (1.30±0.33) as shown in table 2.

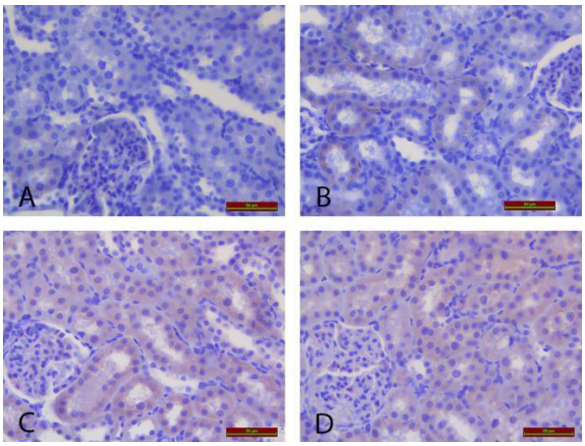

Figure 3. *A-D: Kidneys are stained with the Streptavidin biotin peroxidase complex method with Mayer's Hematoxylin counterstain for caspase-3 immunoreactivity. The scale bars represent 50 µm in AEC chromogen used for visualization. A - Control group, caspase-3 immunoreactivity (brownish-red). B - BFT group, caspase-3 immunoreactivity (brownish-red). C - MTX group, significantly increased caspase-3 immunoreactivity (brownish-red). D - MTX+BFT group, significantly decreased caspase-3 immunoreactivity (brownish-red).*

Table 2. Histoscore of irisin and caspase-3 immunoreactivities in kidney tissue

Groups (n:7)	Irisin	Caspase-3
Control	$0,714^{b}$±0,254	$0,500^{c}$±0,17
BFT	$0,886^{ab}$±0,227	$0,400^{c}$±0,19
MTX	$1,257^{a}$±0,411	$2,25^{a}$±0,49
MTX+BFT	$1,114^{ab}$±0,363	$1,30^{b}$±0,33
P* values	<0.001	<0.001

Irisin and caspase-3 immunoreactivities are given as a mean ± standard deviation compared with control group.
abc Means within the same column with differing superscripts are significantly different (P<0.05)
**:One Way Anova*

DISCUSSION

The kidneys are responsible for retaining homeostasis and eliminating toxins and drugs; moreover, they have an outstanding role in basal metabolism (Ferguson et al. 2008). Nephrotoxicity is one of the major adverse effects of MTX therapy (Widemann and Adamson 2006). Oxidative stress, inflammation, and apoptosis are major known pathways in the pathogenesis of MTX-induced nephrotoxicity (Chabner and Longo 2010; Asvadi et al. 2011; Morsy et al. 2013).

In previous studies, atrophic glomeruli with enlarged capsular cavities, mostly dilated tubules and intra-tubular cellular casts (El-Sheikh et al. 2015), as well as renal tubular cell necrosis and tubular dilatation (Hafez et al. 2015) have been reported in MTX-induced renal injury in rats. Ulusoy et al. (2016) reported that MTX causes tubular dilatation, epithelial desquamation, and congestion of the peritubular vessels and glomerular capillaries. In this study, similar to previous studies, we observed tubular dilatation, glomerular atrophy, and leukocyte infiltration in MTX-induced kidney injury.

Apoptosis may affect renal function and is frequently encountered in acute renal failure (Jo et al. 2001). In previous studies (El-Sheikh et al. 2015; Hafez et al. 2015), an increase of caspase-3 immunoreactivity was reported in the kidney tissues of MTX-treated rats. Similar to these investigators, we observed a significant increase in apoptosis in the MTX group. In contrast, BFT administration showed an anti-apoptotic effect with the decrease of caspase-3 expression. This finding is compatible with previous studies reporting BFT-mediated suppression of caspase-3 in diabetic mice (Gadau et al. 2006; Jung et al. 2014).

MTX-induced tissue damage is manifested by oxidative damage with its free radical generation and lipid peroxidation. This results from a breakdown of the balance between oxidants and antioxidants in favor of oxidants. If the antioxidant/oxidant balance cannot be maintained in the tissues, some pathological changes in cellular damage may occur (Akbulut et al. 2014). A significant increase of TOS levels and significant decrease of TAS levels was reported in the rats administered with MTX (Bozkurt et al. 2014; Erdogan and Yalcin 2018) as a sign of oxidative stress. In the present study, MTX-induced oxidative stress in the kidney tissues of rats was evaluated by analyzing TAS and TOS levels as biochemical markers. Compared to the control group, a statistically significant decrease in TAS levels and increase in TOS levels was determined in the MTX group compatible with the previous data (Bozkurt et al. 2014; Erdogan and Yalcin 2018). In contrast, BFT was found to improve biochemical parameters by significantly decreasing TOS and insignificantly increasing TAS levels in kidney tissue. This ameliorating effect of BFT could be related to its direct ROS scavenging activity (Schmid et al. 2008).

In both in vitro and in vivo studies antioxidant (Bozic et al. 2015; Yilmaz et al 2015) and theraupetic potential (Manzardo et al. 2013; Pan et al. 2016) of BFT have been reported. Bozic et al. (2015) notified that BFT had increased glutathione and both activity and expression of the main antioxidant enzymes: superoxide dismutase, catalase, and glutathione peroxidase. Ustuner et al. (2017) reported that extremely pronounced necrotic tubules (in the renal cortex) and hyaline cast accumulation (in the medullar tubules of animals dosed with gentamicin) were significantly improved with BFT treatment. In another study on rats (Harisa 2013), cisplatin caused renal damage (evident due to renal dysfunction) and it was suggested that BFT could be evaluated as a preventing agent in cisplatin-induced nephrotoxicity by inhibiting the formation of reactive oxygen and nitrogen species. BFT was found to be effective in preventing MTX-induced liver damage (Akbulut et al. 2014). Similarly, in the present study, BFT application exhibited restorative effects on MTX-induced nephrotoxicity in terms of histopathological damage, oxidant state, and apoptosis. These ameliorative effects of BFT are mostly mediated via its antioxidant, anti-inflammatory, and anti-apoptotic properties.

Increased levels of irisin have been reported in different pathological events, such as, during ischemia reperfusion (Fan et al. 2019), acute myocardial infarction (Sarioglu et al. 2016), MTX-induced liver damage (Akbulut et al. 2014), and metabolic syndrome (Rizk et al. 2016). Provatopoulou et al. (2015) reported that serum irisin levels were significantly lower in breast cancer patients compared to controls, and the increase in one unit of irisin decreased the probability of breast cancer by 90%. A positive correlation has been observed between irisin levels and renal function as well as insulin resistance (Ebert et al. 2014). Irisin has been reported to alleviate endothelial dysfunction in type 2 diabetes (Zhu et al. 2015), and in cultured human umbilical vein endothelial cells (Deng et al. 2018). It can also have a cardiac protecting role against ischemia-reperfusion injury (Wang et al. 2017). Systemic administration of irisin may be protective against endothelial injury and ameliorated atherosclerosis in diabetic mice (Lu et al. 2015). It has been demonstrated that irisin is an essential antioxidant and anti-inflammatory myokine and can protect cells from oxidative damage through the activation of the antioxidant mechanism (Mazur-Bialy et al. 2018).

In the current study, the kidney tissue of the MTX administered animals showed a significant increase in irisin immunoreactivity compared to the control group. This increase was insignificantly reduced in the MTX+BFT group compared to the MTX group. It remains to be determined whether this increased irisin immunoreactivity is a result of kidney damage or a protective mechanism against MTX-induced cellular stress related with its antioxidant and anti-inflammatory (Bosma et al. 2016; Mazur-Bialy et al. 2018) / energy regulation role (Aydin 2014).

The effects of BFT on MTX-induced renal injury in rats were investigated in this study and BFT was shown to be promising in preventing MTX-induced oxidative stress and histopathological damage. Although not statistically significant, increased irisin immunoreactivity in response to MTX-induced oxidative stress decreased as a result of BFT administration.

In conclusion BFT may be used as a pharmacological agent to prevent side effects against MTX-induced kidney damage. Further studies are expected to explain the importance and role of increased irisin in the pathogenesis and mechanism of action.

Declaration of interest

The authors declare that they have no conflict of interest.

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