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Protective role of Nebivolol via Akt/Hif-1α/eNOS signaling pathway: Nephrotoxicity caused by Methotrexate in a rat model

Nasıf Fatih KARAKUYU (fatihkarakuyu03@gmail.com)
Suleyman Demirel University
Onur ERTUNÇ
Suleyman Demirel University
Mehmet BEDİR
Suleyman Demirel University
Hatice Kübra DOĞAN
Suleyman Demirel University
Rümeysa TANER
Suleyman Demirel University
Mehmet Abdulkadir Sevük
Suleyman Demirel University
Orhan Berk İMECİ
Suleyman Demirel University
Esin ERGÖNÜL
Dokuz Eylul University

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Abstract

Methotrexate (MTX) is an antineoplastic and anti-inflammatory agent which is used in serious diseases. Its use should be limited due to side effects such as nephrotoxicity, myelotoxicity and hepatotoxicity, especially during high doses in cancer treatment. Hypertension may accompany patients due to nephrotoxicity, which is shown as one of the most important side effects. Nebivolol (NBV), which is a beta blocker and used in the treatment of hypertension, also contributes to vasodilation in tissues by activating endothelial nitric oxide synthase (eNOS) enzyme. The purpose of this study is to research the effect of NBV on MTX-induced nephrotoxicity through the AKT1/Hif-1 /eNOS signaling pathway.

The rats were randomly divided into three groups of eight each. Groups were control, MTX and MTX + NBV. A single dose of 20 mg/kg MTX was given intraperitoneally to the rats on the first day of the study and 10 mg/kg NBV was given orally to the treatment group for seven days.

At the end of the study, rats' blood and kidney tissues were taken for histopathological, immunohistochemical and biochemical examinations. Total antioxidant status levels were increased and total oxidant status levels were decreased significantly in MTX + NBV group compared with MTX group. Although creatinine levels increased in the MTX group compared to the control, while decreased in the MTX + NBV group. MTX administration was significantly decreased the expression levels of AKT1, eNOS and Hif1α compared to control group and NBV treatment increased these values compared to MTX group.

In conclusion, NBV treatment ameliorated the MTX induced nephrotoxicity via AKT1/Hif-1 /eNOS signaling pathway.

Introduction

Methotrexate (MTX) is a agent with dose dependent effect used as an antineoplastic and anti-inflammatory drug in serious diseases (Cronstein and Aune 2020). Its use should be limited due to side effects such as nephrotoxicity and hepatotoxicity, especially during high doses in cancer treatment (Samdanci et al. 2018). Hypertension may accompany patients due to nephrotoxicity, which is shown as one of the most important side effects (Eki Nci-Akdemi R et al. 2018).

Some serious damage mechanisms play an important role in the basis of this kidney damage (Wang et al. 2018). Major intracellular pathways accompany the development of these mechanisms, in which inflammation and apoptosis are common, especially oxidative stress (Friedman and Cronstein 2019). Knowing these pathways that have been investigated by many researchers in the literature and affected by drugs constitute an important reason why drugs are preferred for various indications (Kanarek et al. 2019).

The AKT1/Hif-1 /endothelial nitric oxide synthase (eNOS) pathway mediates the anti-inflammatory effect in tissues due to the vasodilation by the formation of nitric oxide (Schleicher et al. 2009). Many drugs

have been tried to increase the activation of this pathway to decrease these cellular damage mechanisms (Huang et al. 2015).

Nebivolol (NBV), which is a beta-blocker and used in the treatment of hypertension, also contributes to vasodilation in tissues by activating the eNOS enzyme (Wang et al. 2017). The number of studies investigating the effect of NBV, which is known to have antiinflammatory and anti-oxidant effects, on nephrotoxicity is quite limited.

The purpose of this study is to research the effect of NBV on MTX-induced nephrotoxicity through the AKT1/Hif-1 /eNOS signaling pathway.

Materials And Methods

Animals and ethical approval

In this study, adult male Wistar Albino rats (n: 24, Age: 10–12 weeks, Weight: 250-350gr) obtained from Suleyman Demirel University Experimental Animals and Medical Research Application and Research Center were used. Permission was obtained from Süleyman Demirel University Rectorate Animal Experiments Local Ethics Committee for the study (Ethics No: 17.03.2022/03–38). Animals were housed in Euro type-4 cages within the Suleyman Demirel University Animal Experiments Laboratory under room conditions with a temperature of 22–24°C and 55% humidity, using the "Ad libitum" diet, with twelve hours of light and twelve hours of darkness. During the shelter period, no food or fluid restriction was applied to the experimental animals and the ad libitum feeding regimen was continued.

Study Design

After the adaptation period of one week, rats were divided into 3 groups. The following experimental groups were formed as; Control group (n = 8): 1 ml intraperitoneal (ip) and oral saline (SF) on the first day + 1 ml of SF was administered by oral gavage on days 2–7, MTX group (n = 8): 20 mg/kg ip MTX and oral SF on the first day + 1 ml SF was administered by oral gavage on 2–7 days (Samdanci et al. 2018), and MTX + NBV group (n = 8): 20 mg/kg ip MTX and oral 10 mg/kg 1ml NBV on the first day + same dose NBV was administered by oral gavage on 2–7 days (Arab et al. 2018). On the 7th day, 60 minutes after the last NBV administration of experiment, rats were sacrificed under anesthesia of 8–10 mg/kg Xylazine and 50–80 mg/kg Ketamine. At the end of the experiment, blood and tissues were taken for biochemical, histopathological and immunohistochemical examinations.

Biochemical Analyses

Evaluation of oxidative stress parameters

Half of kidney tissues of rats were dissolved and mixed using a vortex-mixer (Thermo, USA) for antioxidant-oxidant examination. Total oxidant status (TOS), oxidative stress index (OSI) and total antioxidant status (TAS) values were measured spectrophotometrically using a modified Erel method

(Erel 2004, Erel 2005, Savas et al. 2017). TAS and TOS results for the tissues were expressed via division by the protein value. OSI was calculated using the formula OSI = TOS/TAS (Erel 2005). TAS was measured using a novel automated colorimetric measurement method developed by Erel. In this method, anti-oxidants in the sample reduce dark blue-green colored 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical to colorless reduced ABTS form. The change of absorbance at 660 nm is related to the total anti-oxidant level of the sample. This method determines the antioxidative effect of the sample against the potent free radical reactions initiated by the produced hydroxyl radicals. The results are expressed as millimolar Trolox equivalent per liter (Erel 2004)

The TOS of tissues was measured using a novel automated colorimetric method described (Erel 2005). In this method, oxidants present in the sample oxidize the ferrous ion- dianisidine complex to the ferric ion. The oxidation reactions are enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion forms a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured spectrophotometrically, has been found related to the total amount of oxidant molecules that were already present in the sample. The assay was calibrated with hydrogen peroxide, and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H2O2 Eqv. /L) (Erel 2005).

Measurement of blood parameters

The blood of the rats was taken into suitable biochemistry tubes, the serum was separated by centrifugation at 3500 rpm for ten minutes and divided into three and portioned into eppendorf. Samples were stored at -80 ° C until they were studied. Blood urea nitrogen (BUN), creatinine (CRE) and hydroxyproline (HP) levels were measured by the spectrophotometric method with Beckman Coulter AU 5800 autoanalyzer (Beckman Coulter, USA) using the kit compatible with the device.

Total RNA isolation and Expression Analysis by Quantitative RT-PCR

Total RNA extraction and purification

Total RNA was isolated from rat kidney tissue by using Monarch Total RNA isolation kit (New England BioLabs) according to the manufacturer's instructions. RNA concentration and purity were determined by the MySPEC microvolume spectrophotometer (VWR).

cDNA synthesis

Total RNA (1 μg) was diluted with nuclease-free water to a volume of 15 μl. cDNAs were synthesis by using the iScriptTM cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, California). Reverse transcription was performed using 1 μg mRNA per sample and oligo dT primers, per the manufacturer's protocol. The reaction mix was incubated for 5 min at 25°C, for 20 min at 46°C and 1 min at 95°C.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Real-time PCR amplification was performed and monitored using the iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, California) and CFX96 Real-time PCR System (Bio-Rad Laboratories, Hercules, California). Specific primers were designed against AKT1, Hif1α, eNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primer sequences are listed in Table 1. cDNA samples were analyzed in triplicates for each PCR. Fold change for the transcripts were normalized to the housekeeping gene GAPDH. The following PCR conditions were used: denaturation at 95°C for 10 min, followed by 45 cycles of: 10 s at 95°C, 30 s at 60°C. Relative quantification of gene expression was performed using the comparative ΔΔCt method.

Table 1
Kidney primers of MTX and NBV treated groups

Gene Name		Primer Sequence
GAPDH (RAT)	Forward	CAAGGTCATC CCA GAG CTG AA
	Reverse	CATG TAG GCC ATG AGG TCC AC
eNOS (RAT)	Forward	GTGAAGGCGACTATCCTGTATG
	Reverse	CATGCTCTAGGGATACCACATC
Hif1a (RAT)	Forward	GGAAATGCTGGCTCCCTATATC
	Reverse	GTAACTGGGTCTGCTGGAATC
AKT1 (RAT)	Forward	GCTCAAGAAGGACCCTACA
	Reverse	GGGCTCAGCTTCTTCTCATAC

Histopathological Analyses

Kidney tissues were kept in 10% neutral formaldehyde solution for at least 48 hours for fixation. After the fixation was completed, the tissues were washed under running water overnight and tissue follow-up procedures were initiated. After washing, the tissues were kept for the specified periods in gradually increasing alcohol to remove the water from the tissues. After the dehydration phase, the conditions of the tissues were checked and transparent in xylol for 5–15 minutes. Afterward, the tissues were kept in the oven in liquid paraffin at 60 ° C for 1 hour, in solid paraffin for 1 night at room temperature for paraffin impregnation. Tissues in paraffin liquefied at 60°C in the oven again were embedded in blocks.

Sections 4–5 µm thickness were obtained from the paraffin blocks with the help of a microtome and transferred to the slides with the help of a hot water bath. To make histochemical evaluations, obtained sections were stained with Hematoxylin-Eosin (HE). Masson Trichrome, Periodic Acid Schiff-Asien Blue (PAS-AB) in accordance with the specified protocol.

Renal tissues obtained as a result of the experiment were examined in terms of tubular epithelial changes (vacuolization, dilatation, atrophy, cast, desquamation, necrosis), edema, interstitial inflammatory cell infiltration and glomerular changes. All histopathological parameters were graded considering four different degrees of damage. In this rating; As "(-)" did not change, "(+)" was mild unicellular necrosis, mild degenerative changes, few dilatation foci and casts, minimal inflammation and edema, "(++)" was moderate in all changes in tissue. And the evaluation criteria were considered as severe and intense changes that focused throughout the kidney tissue and "(+++)" spread to the entire kidney tissue.

Statistical Evaluation

Variables were presented as mean ± standard deviation. One-way ANOVA and post hoc LSD tests were used to compare the biochemical, histopathological and immunohistochemical scores between the groups. The calculations were made using the SPSS 20.0 program package (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was set as the significance value.

Results

Biochemical Results

TAS, TOS and OSI levels in kidney tissue

TOS levels were increased in the MTX group compare with the control group (p = 0.754) and these levels were significantly decreased in the MTX + NBV group compared with the MTX group (p = 0.045). TAS levels were significantly decreased in the MTX group compared with the control (p = 0.003) and significantly increased in the MTX + NBV group compared with the MTX group (p = 0.027), OSI levels were increased in the MTX group compared with the control (p = 0.910) and decreased in the MTX + NBV group compared with the MTX group (p = 0.904) (Table 2).

Table 2
Oxidative stress markers of kidney tissues

	TOS (μmol h_2o_2 eq./L)		**TAS** (mmol troloxeq./L)		**OSI** (TOS/TAS)	
Groups	Mean ± SD	*p* value	Mean ± SD	*p* value	Mean ± SD	*p* value
CONTROL	7,31 ± 0,89		1,10 ± 0,07		0,63 ± 0,25	
MTX	7,93 ± 1,12		0,94 ± 0,05[a]	a: 0,003	0,68 ± 0,11	
MTX + NBV	5,53 ± 2,26[b]	b: 0,045	1,07 ± 0,09[b]	b: 0,027	0,63 ± 0,26	

MTX – Methotrexate; NBV – Nebivolol; TOS – Total Oxidant Status; TAS – Total Antioxidant Status; OSI – Oxidative Stress Index; SD – Standard Deviation. Values are presented as means ± SD. The relationships between groups and the results of biochemical markers are assessed by one-way ANOVA. a: $p < 0.05$ vs CONTROL, b: $p < 0.05$ vs MTX.

Biochemical results in blood

CRE levels were significantly increased in the MTX group compared with the control group (p:0.000) and significantly decreased in the MTX + NBV group compared with the MTX group (p:0.026), BUN levels were increased in the MTX group compared with the control group (p:0.295) and decreased in the MTX + NBV group compared with the MTX group (p:0.827). HP levels were increased in the MTX group compared with the control group (p:0.462) and decreased in the MTX + NBV group compared with the MTX group (p:0.808) (Table 3).

Table 3
Biochemical parameters of kidney in the serum

	CRE (mg/dl)		**BUN (mg/dl)**		**HP (mg/dl)**	
Groups	**Mean ± SD**	p **value**	**Mean ± SD**	p **value**	**Mean ± SD**	p **value**
CONTROL	0,24 ± 0,05		17,57 ± 2,57		324,54 ± 58,56	
MTX	0,45 ± 0,04 [a]	a: 0,000	20,71 ± 3,49		355,22 ± 34,08	
MTX + NBV	0,35 ± 0,08[b]	b: 0,026	19,40 ± 5,41		338,81 ± 36,42	

MTX – Methotrexate; NBV – Nebivolol; CRE – Creatinine; BUN – Blood Urea Nitrogen; HP – Hydroxyproline; SD – Standard Deviation. Values are presented as means ± SD. The relationships between groups and the results of biochemical markers are assessed by one-way ANOVA. a: $p \leq 0.001$ vs CONTROL, b: $p \leq 0.05$ vs MTX

Relative mRNA expression results in kidney tissues

MTX treatment was significantly decreased the expression levels of AKT1, eNOS and Hif1α compared to the control group. NBV treatment was increased AKT1, eNOS, and Hif1α mRNA expression compared to the MTX group. In the MTX-NBV group, a statistically significant increase was observed in eNOS and Hif1α compared to the MTX group (Fig. 1).

Histopathological Results

In rats belonging to the control group, normal cell groups and normal glomerular and tubular area morphology were observed in the renal parenchyma as a result of light microscopic evaluations. Tubular necrosis, tubular epithelial desquamation, tubular atrophy and interstitial inflammation or fibrosis were not detected when all groups were examined. When compared with the control group (Fig. 2-A), it was observed that tubular dilatation, epithelial degeneration and hyaline (casts) damage and vacuolization in the distal tubule were observed with HE (Fig. 2-B) staining in the group receiving MTX. No necrosis or atrophy of the tubules was observed in the tissue samples examined. As a result of histopathological examinations; When the group receiving MTX and those receiving MTX-NBV were compared (Fig. 2-C), it

was found that the severity of dilatation around the tubular and glomerular decreased, and hyaline (casts) accumulation occurred at the same level. Tubular necrosis or atrophy and interstitial damage were not observed in the groups receiving MTX-NBV (Table 4).

Table 4
Relative expression levels of AKT1, eNOS, Hif-1 , NF-kB (Fold Change)

	AKT1	eNOS	Hıf-1	Nf-kB
MTX	0,459	0,233	0,363	0,806
NBV	0,901	0,996	1,117	1,019
MTX: Methotrexate; NBV: Nebivolol; Hif-1 : Hypoxia-Inducible Factor 1-Alpha; NF-κB: Nuclear Factor kappa B.				

As shown in Table 5, collagen increase or fibrosis, tubular necrosis was not detected in all tissue samples examined. No pathological finding was detected in the control group. Moderate dilatation observed around the glomeruli and tubules in the MTX group decreased in severity in the NBV groups. Besides, hyaline accumulation was found to be similar in severity and moderate in all groups receiving medication.

Table 5
Scoring of structural changes observed in experimental groups in the kidney.

EXPERIMENTAL GROUPS	Control	MTX	MTX + NBV
Glomerular circumference dilatation	-	++	+
Tubular necrosis	-	-	-
Tubular dilatation	-	++	+
Hyaline (casts) accumulation	-	++	+
Interstitial Fibrosis / Collagen increase	-	-	-
Normal tissue or no pathological findings, (+): Mild dilatation, (++): Moderate dilatation or hyaline accumulation, (+++) High degree of dilatation or hyaline accumulation			

Discussion

The folic acid antimetabolite MTX limits epithelial hyperplasia, reinforces the apoptosis of activated T cells, and inhibits the chemotaxis of neutrophils like anti-inflammatory and immunosuppressive properties (Czarnecka-Operacz and Sadowska-Przytocka 2014, Elango et al. 2014). For these reasons, it is used in some autoimmune diseases such as Crohn's disease, rheumatoid arthritis etc. Besides, MTX inhibits the activity of thymidylate synthase which causes the inhibition of growth and cell death by

inhibiting DNA synthesis (Cronstein et al. 1994). It is also used in cancer treatment due the same properties mentioned above.

Among the usage of antineoplastic reason, it leads to causing many organ toxicities by using high doses. Oxidative stress and inflammation play an important role in the background of important side effects such as nephrotoxicity (Howard et al. 2016).

The changes in oxidative stress markers of this study reflected that MTX induces oxidative stress and selected protective agent NBV ameliorated these changes by decreasing ROS levels and increasing antioxidant activities. In preventing this oxidative stress, it may have either prevented the progression of the damage by increasing the number of antioxidant enzymes, or it may have reduced stress indirectly by affecting the mechanisms of inflammation. Akgullu et al. found NBV's antioxidant properties with the treatment of hyper-homocysteinemia induced kidney injury in rats by decreasing malondialdehyde levels.

Oxidative stress may cause the release of various cytokines that cause inflammation by activating certain intracellular pathways. These released cytokines also affect their cell surface receptors and activate other intracellular pathways and cause the process to become a progressive inflammatory condition (Mahfoudh-Boussaid et al. 2014).

While AKT, which is one of the main intracellular pathways in the progression of this damage, is in contact with many other pathways and may cause aggravation of the damage, it can trigger tissue-protective effects with the NO residue synthesized due to eNOS activation by activating the Hif-1 - mediated intermediate pathway.

Hif-1 , a transcriptional activator of oxygen homeostasis, is stimulated by several factors like growth factors and NO which coordinate the expression of eNOS enzyme levels to decreased the hypoxic results. So enhancement levels of this gene resulted in vasodilation and decreased the activities of the mentioned damage mechanisms.

As known in the literature, NBV enhances the activity of the eNOS enzyme resulted in vasodilation and improvement of the defense system. For the protection of the kidney against inflammation, Akt/eNOS pathway-related NO synthesis plays a crucial role in cell survival (Mahfoudh-Boussaid et al. 2012). In this study, MTX decreased the activity of AKT1/Hif-1 /eNOS signaling and causes positive many histopathological changes. In this model of nephrotoxicity, the effect of NBV on the intracellular AKT pathway was parallel to the literature on eNOS synthesis, and this effect was shown to reduce inflammation in the tissue by histopathological responses. By cytokines, tubular dilatation, epithelial degeneration, hyaline damage and vacuolization in the distal tubule reflected the inflammatory way of MTX on the kidney. NBV treatment ameliorates these findings by using the same pathway and increasing the activity of antioxidant activity. Wang et al. studied on L-NAME –induced hypertension model on rats and they found that NBV treatment enhanced the level of NO synthesized by eNOS to reduce oxidative stress and inflammation (Wang et al. 2017).

Some markers such as BUN and CRE, which are taken from the blood, are used in routine treatment to monitor the degree of these damages in the kidney tissue. In this study enhanced levels of CRE levels by MTX were decreased by NBV reflected that NBV protects the kidney effectively.

Based on all these changes, NBV treatment ameliorated the MTX induced nephrotoxicity via AKT1/Hif-1 /eNOS signaling. So, after this stage, serial clinical studies should be conducted in humans, these activities should be proven and should be put into routine use.

Declarations

Ethical Approval

In this study, all experiments were performed under the guidelines for animal research from the National Institutes of Health and were approved by the Committee on Animal Research of Suleyman Demirel University, Isparta (Ethic No: 17.03.2022/03-38).

Consent to participate (not applicable)

Consent to publish (not applicable)

Authors Contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; NFK, RT, MAS and OBİ conducted the experiments, OE, MB, HKD and EE performed biochemical, histopathological, genetic and statictical analyzes, NFK wrote the manuscript. All authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Competing Interests

The authors report no conflicts of interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

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Figures

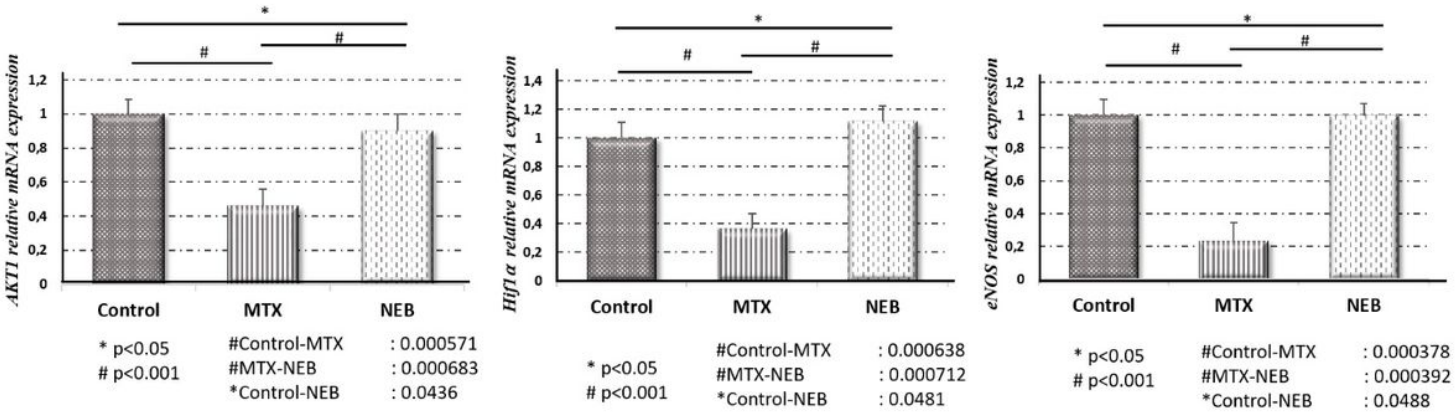


Figure 1

The effect of NBV administration on MTX-mediated altered AKT1, Hif1a and eNOS and relative mRNA expression in kidney tissue.

Values are presented as mean ± SD. MTX: Methotrexate; NBV: Nebivolol; Hif-1 : Hypoxia-Inducible Factor 1-Alpha. Relationships between groups and results of histopathological parameters were evaluated with One-way ANOVA (post hoc LSD test). *: p≤0.05, #: p≤0,001

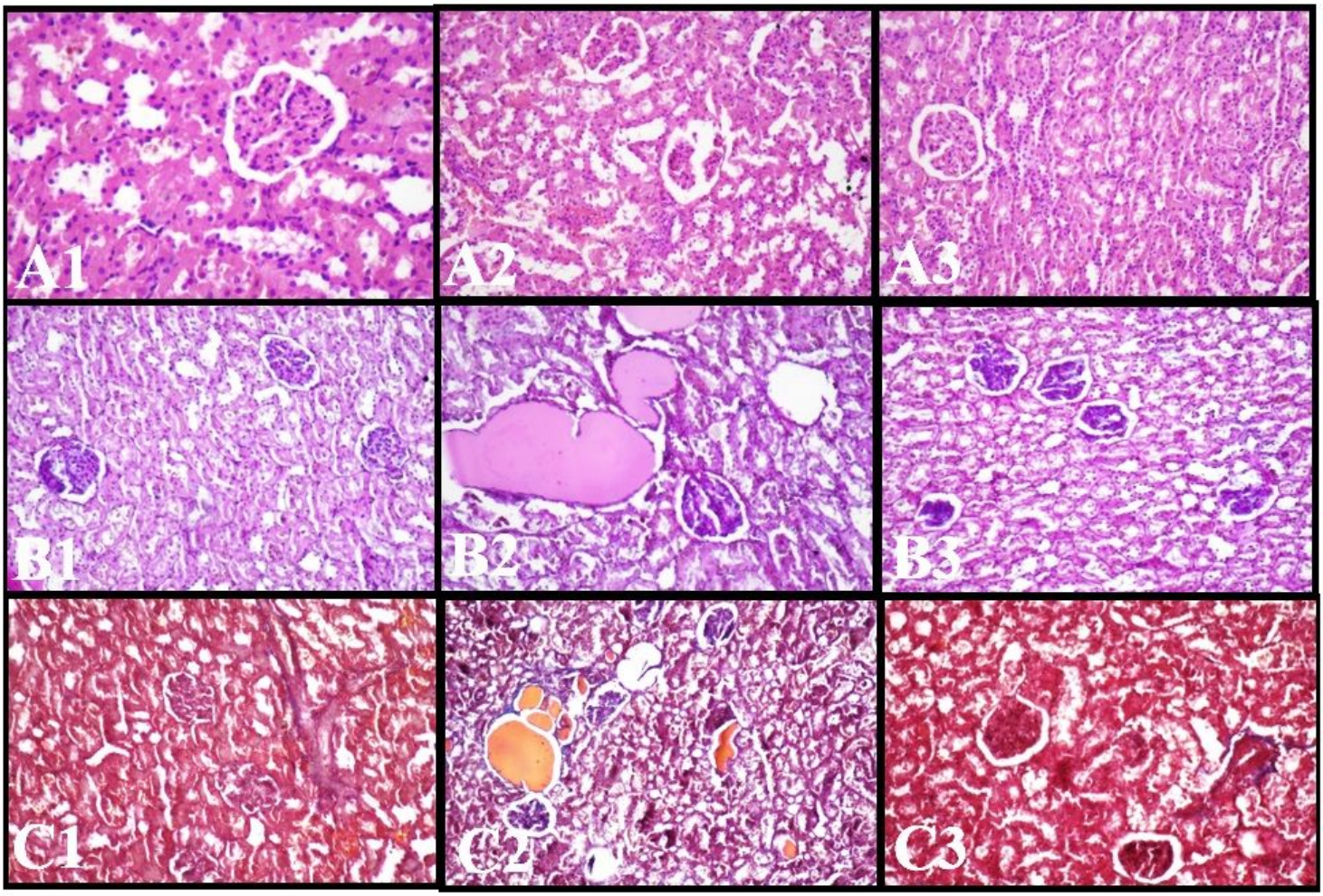


Figure 2

HE, PAS-AB and MassonTrichrome staining of renal tissues.

A1- Control group A2- Group receiving MTX A3- Comparison of MTX + NBV group with H&E Paint and Scale: 100 µm, B1- Control group B2- Group receiving MTX B3- PAS-AB Paint of the group receiving MTX + NBV and Scale: Comparison with 100 µm, C1- Control group C2- MTX group C3- MTX + NBV group comparison with Masson Trichrome and Scale: 100 µm.