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Evaluating Adropine levels in kidney tissue after Methotrexate treatment in rats: a prospective experimental study

Evaluación de los niveles de adropina en el tejido renal después del tratamiento con Metotrexato en ratas: un estudio experimental prospectivo

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ABSTRACT

In this study, it was aimed to investigate Adropin levels in kidney tissues after Methotrexate (MTX) administration to identify potential changes following administration of agents with antioxidant/anti-inflammatory potential. Twenty four adult rats male albino Wistar rats were used in this study, and randomly divided into four groups. Control: These rats did not receive any treatment during the 14-day (d) experiment. N-acetylcysteine (NAC): These rats were administered 100 mg·kg^{-1}·day^{-1} NAC intraperitoneally (i.p.) for 14 d. MTX**:** A single dose of 20 mg·kg^{-1} MTX was administered i.p. at the beginning of the study. MTX+ NAC: A single dose of 20 mg·kg^{-1} MTX was administered i.p. at the beginning of the study, and the rats were given 100 mg·kg^{-1}·day^{-1} NAC i.p. for 14 d. Total antioxidant, and serum Adropin levels were found to be the lowest in the MTX group while the oxidant levels were significantly lower in the MTX group than in the MTX+NAC group (P<0.001). TUNEL positivity was similar among the groups, and no significant differences were observed. It was considered that these findings have shed light on the role of Adropin in the development of kidney failure following MTX administration.

Key words: Antioxidants; adropin protein; kidney diseases; Methotrexate; oxidative stress

RESUMEN

En este estudio, el objetivo fue investigar los niveles de adropina en los tejidos renales después de la administración de MTX para identificar cambios potenciales después de la administración de agentes con potencial antioxidante/antiinflamatorio. En este estudio se utilizaron 24 ratas adultas, ratas Wistar albinas macho, y se dividieron aleatoriamente en cuatro grupos. Estas ratas no recibieron ningún tratamiento durante los 14 días (d) del experimento. NAC: A estas ratas se les administró 100 mg·kg^{-1}·día^{-1} de N-acetilcisteína (NAC) por vía intraperitoneal (i.p.) durante 14 d. MTX: Se administró una dosis única de 20 mg·kg^{-1} de MTX i.p. al inicio del estudio. MTX+ NAC: Se administró una dosis única de 20 mg·kg^{-1} de MTX i.p. al comienzo del estudio, y las ratas recibieron 100 mg·kg^{-1}·día^{-1} de NAC i.p. durante 14 d. Se encontró que los niveles de antioxidantes totales y de adropina sérica eran los más bajos en el grupo de MTX, mientras que los niveles de oxidantes fueron significativamente más bajos en el grupo de MTX que en el grupo de MTX+NAC (P<0,001). La positividad de TUNEL fue similar entre los grupos y no se observaron diferencias significativas. Se considera que estos hallazgos han arrojado luz sobre el papel de adropin en el desarrollo de insuficiencia renal tras la administración de MTX.

Palabras clave: Antioxidantes; proteína adropina; enfermedades renales; metotrexato; estrés oxidativo

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INTRODUCTION

The kidney is one of the most important organs and takes part in several vital bodily functions, particularly blood pressure regulation and the maintenance of electrolyte balance [1]. However, its role in the concentration and excretion of drugs and toxic materials often exposes the kidney to toxic chemicals [2]. Consequently, the use of drugs, particularly chemotherapeutic agents such as Methotrexate (MTX), can lead to nephrotoxicity and the subsequent progression of acute and chronic kidney diseases (CKD). This occurs because of MTX–induced oxidative stress, which increases reactive oxygen species (ROS), leading to cytotoxicity [3].

MTX blocks Deoxyribonucleic acid (DNA) synthesis by inhibiting dihydrofolate reductase. Clinically, it is used in high doses in the treatment of various neoplastic diseases and in low doses in the treatment of autoimmune diseases [4, 5]. However, the side effects of MTX limit its clinical usefulness. MTX administration is associated with hepatotoxicity, gastrointestinal disturbances, neurotoxicity, and hematological abnormalities. Moreover, the drug is linked to acute kidney failure at an occurrence rate of 2–12%. MTX has also been reported to reduce the liver's Glutathione level and increase lipid peroxidation. This leads to defects in the antioxidant protection mechanism [6]. No precise mechanisms are currently known that explain how MTX causes kidney damage, but oxidative stress and inflammation are believed to be responsible for the occurrence of nephrotoxicity [7, 8].

MTX activates the mitochondrial pathway of apoptosis and increases the production of proinflammatory cytokines and ROS by decreasing the homocysteine remethylation rate. MTX also stimulates neutrophil recruitment by decreasing the intracellular levels of nicotinamide adenine dinucleotide phosphate (NADPH) [9, 10]. Recent studies have shown that endothelial damage is also an important factor in the toxicity that arises from MTX use [11].

Endothelial function is regulated at least in part by Adropin, a hormone expressed in the vascular endothelial cells that takes part in the regulation of lipid metabolism and in the maintenance of energy homeostasis [12]. The level of Adropin is regulated by nutrient intake and is detected in the liver as well as in the kidney glomerulus, peritubular capillaries, pancreatic tissue, and heart endocardium, myocardium, and epicardium [13, 14]. Adropin has a positive impact on the regulation of endothelial function by increasing blood flow, capillary density, and angiogenesis, but decreases endothelial permeability due to the stimulation of endothelial nitric oxide synthase (eNOS)–nitric oxide (NO) signal pathways [12]. Plasma Adropin levels have been found to be low in chronic diseases and this condition is closely related to endothelial dysfunction [15, 16].

In this study, it was aimed to investigate Adropin levels in kidney tissues after MTX administration to identify potential changes following administration of agents with antioxidant/anti–inflammatory potential, as well as to explore the potential usefulness of Adropin levels after MTX treatment in the diagnosis of kidney failure.

MATERIALS AND METHODS

All procedures included in the study were performed according to the National Institutes of Health's (NIH) Guidelines for the Care and Use of Animals. After obtaining the necessary approval (decision number: 31.03.21/2021.06) from the local animal experiments ethics committee, it was began this prospective experimental study at the Animal Laboratory of Firat University, Elazig, Türkiye, between April 2021 and December 2022. Furthermore, the PubMed and Web of Science databases were used to find studies relevant to the researches.

Animals and groups

In the current study, 24 mature male albino rats wistar (*Ratus norvegicus*) were kept in conditions of 22–25 °C with 12 h of light (7:00 am–7:00 pm) and 12 h of darkness (7:00 pm–7:00 am). Rat food pellets and water were available at all times. The rats were randomly divided into four groups of six, as follows: Group 1 (Control): These rats did not receive any treatment during the 14–day (d) experiment. Group 2 (NAC): These rats were administered 100 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ NAC (Bilim Pharmaceuticals, Kocaeli, Türkiye) intraperitoneally (i.p.) for 14 d [17]. Group 3 (MTX): A single dose of 20 $mg{\cdot}kg^{-1}$ MTX (Abdi Ibrahim, Istanbul, Türkiye) was administered i.p. at the beginning of the study [18]. Group 4 (MTX + NAC): A single dose of 20 $mg{\cdot}kg^{-1}$ MTX was administered i.p. at the beginning of the study, and the rats were given 100 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ NAC i.p. for 14 d.

At the conclusion of the experiment, all rats received Ketamine (75 $mg{\cdot}kg^{-1}$) (Alfamine 10%, Ege Vet, Türkiye) + Xylazine (10 $mg{\cdot}kg^{-1}$) (Rompun 2%, Bayer, Türkiye) i.p. and were decapitated under anesthesia. Blood samples were taken and centrifuged (NF1200R, Nuve, Ankara, Türkiye) for 5 min at 4000 G and the resulting sera were maintained at -80°C until assayed. Adropin levels, total antioxidant status (TAS), and total oxidant status (TOS) were determined using enzyme–linked immunoassay (ELISA) kits. Kidney tissues were also removed, fixed in 10% formaldehyde, and processed with routine histological procedures used for paraffin embedding.

Assessment of total antioxidant status and total oxidant status

The TAS of the rat serum samples was detected with an ELISA kit (Rat TAS; catalogue no. YLA3389RA, YL Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The kit's measuring range was 1–300 $pg{\cdot}mL^{-1}$ [the in–assay variation coefficient (CV) value was <10%, the CV value between assays was <12%, and the sensitivity was 0.54 $pg{\cdot}mL^{-1}$]. The plates were cleaned with a BioTek ELX50 automated washer (BioTek Instruments, USA). The absorbance readings were made at wavelengths of 630 nm and 450 nm with a ChroMate P4300 Microplate Reader (Awareness Technology Instruments, USA). The test findings were expressed as $pg{\cdot}mL^{-1}$.

The TOS of the rat serum samples was detected by ELISA kits (Rat TOS; catalogue no. YLA1392RA YL Biotechnology Co. Ltd., Shanghai, China) according to the manufacturer's instructions. The kit's measuring range was 0.02–60 $U{\cdot}mL^{-1}$ (the intra–assay CV value was <10%, the inter–assay CV value was <12%, and the sensitivity was 0.013 $U{\cdot}mL^{-1}$). The plates were washed, and the absorbance was measured at wavelengths of 630 nm and 450 nm, as described for TAS assays. The test results were presented in $U{\cdot}mL^{-1}$.

The serum adropin levels were measured by ELISA (Rel Assay Rat Adropin ELISA kit; catalogue no. E1069R, Mega Tip Sanayi ve Ticaret, Ltd., Gaziantep, Türkiye) following the manufacturer's instructions. The kit had an intra–assay CV value of <8%, an inter–assay CV value of <10%, and a sensitivity of 0.02 $ng{\cdot}mL^{-1}$. The plates were washed as described for TAS assays, and absorbance readings were taken using a Bio–Tek ELX800 ELISA reader (BioTek Instruments, USA) at a wavelength of 450 nm. The serum contents were determined by comparison to a standard curve prepared from known concentrations of adropin and expressed as $ng{\cdot}mL^{-1}$.

Immunohistochemistry and TUNEL assessment

Sections 4–6 µm thick were cut from kidney tissues embedded in paraffin blocks and placed onto polylysine-coated slides. The immunohistochemical study was planned and prepared with the protocol specified in a previous literature study [19, 20].

During the staining, the immunohistochemistry score was established by evaluating the prevalence (0.1: <25%; 0.4: 26–50%; 0.6: 51–75%; 0.9: 76–100%) and intensity (0: none; +0.5: very low; +1: low; +2: medium; +3: intense) of the immunoreactivity. The immunohistochemistry score equals prevalence × intensity.

Another set of 5–6 µm thick paraffin block pieces was cut and put on polylysine slides. Apoptotic cells were identified using the ApopTag Plus Peroxidase *in Situ* Apoptosis Detection Kit (Chemicon, catalogue no: S7101, USA) according to the manufacturer's instructions. TUNEL evaluation was performed using a method previously described in the literature [19, 20].

Statistical analysis

SPSS version 22 was used for statistical analysis. The quantitative measures were reported as median and minimum–maximum values. To assess the normal distribution of the data, the Kolmogorov–Smirnov test was utilized. The Mann–Whitney U test was used to compare quantitative data between two groups that did not have a normal distribution, and the Kruskal–Wallis test was used for general comparisons between more than two groups. The Bonferroni adjustment was used for paired-group comparisons. A $P<0.05$ value was considered significant.

RESULTS AND DISCUSSION

The TOS and TAS measurements in the bodily fluids were beneficial biological indicators of antioxidants' protective capacity against oxidative damage.

No significant difference was observed between Groups 1 and 2 regarding serum TAS levels ($P=0.535$). TAS levels were higher in Groups 1 and 4 than in Group 3 ($P<0.001$) (FIG. 1).

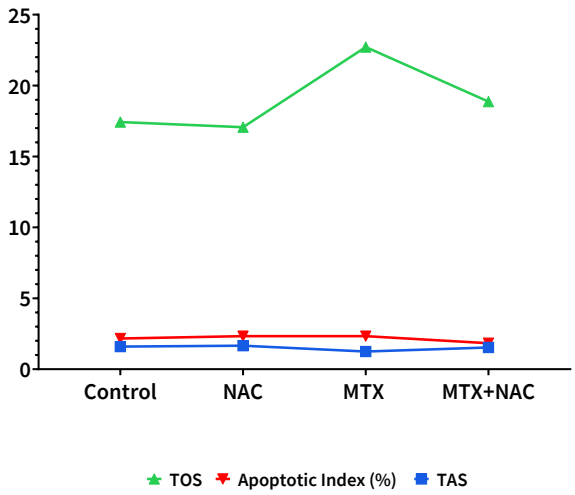

FIGURE 1. Total Antioxidant Status (TAS), Total Oxidant Status (TOS), and Apoptotic Index (%) of the groups. NAC: N-acetylcysteine, MTX: Methotrexate

No significant difference was observed in serum TOS levels between Groups 1 and 2 ($P=0.805$). TOS level was higher in Group 3 than in Groups 1 and 4 ($P<0.001$) (FIG. 1).

Serum Adropin levels were similar in Groups 1 and 2 ($P=0.151$). Group 3 was found to have significantly lower Adropin levels than Groups 1 and 4 ($P<0.008$) (FIG. 2).

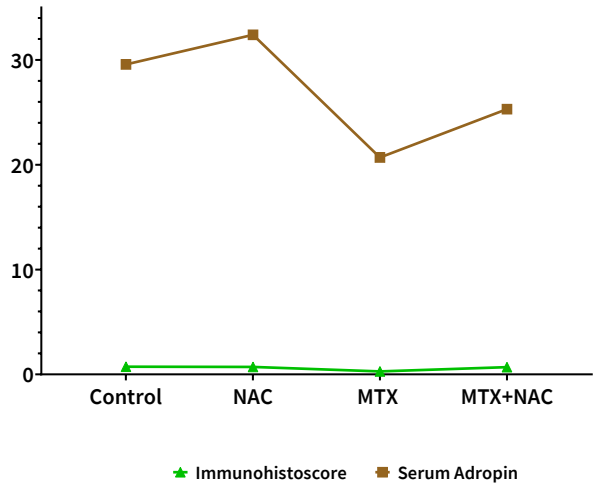

FIGURE 2. Serum Adropin levels, and Immunohistochemistry scores. NAC: N- acetylcysteine , MTX: Methotrexate

TUNEL positivity was observed in the tubular cells in the kidney tissues (black arrow). TUNEL positivity was similar among the groups (FIGS. 3a–d), and no significant differences were observed ($P=0.845$).

Adropin immunoreactivity was observed in the glomeruli of the kidney tissue (black arrow). The immunoreactivity was similar in Groups 1 and 2 (FIGS. 4a and b) ($P=0.818$) but was significantly lower in Group 3 than in Groups 1 and 4 ($P<0.002$) (FIGS. 4c and d).

MTX is predominantly used in the clinical treatment of inflammatory diseases and various malignancies. However, besides its therapeutic impact, it may also cause harm to many organs. The mechanism underlying the dysfunction caused in the kidneys is still unclear [21]; however, damage to the kidneys following MTX administration occurs in response to neutrophil infiltration and oxidative stress. MTX is known to increase the level of ROS in various organs and tissues while also decreasing the level of antioxidants, such as Glutathione, [22] an endogenous antioxidant that plays a critical role in dissipating reactive oxygen molecules. Oxidative stress decreases the level of Glutathione, but this can be reversed with NAC treatment [23]. Hence, the administration of NAC can significantly decrease the oxidative stress in kidneys caused by exposure to MTX [24]. The obtained findings agree with previous study results, as the total oxidant capacity was more pronounced in the MTX-treated *rats*(*Ratus norvegicus*) (Group 3) than in the control rats (Group 1), and the antioxidant capacity was correspondingly decreased. The total antioxidant capacity was similarly decreased after MTX treatment in Group 4 but was significantly restored after NAC treatment.

The parameters of all four groups are summarized in TABLE I.

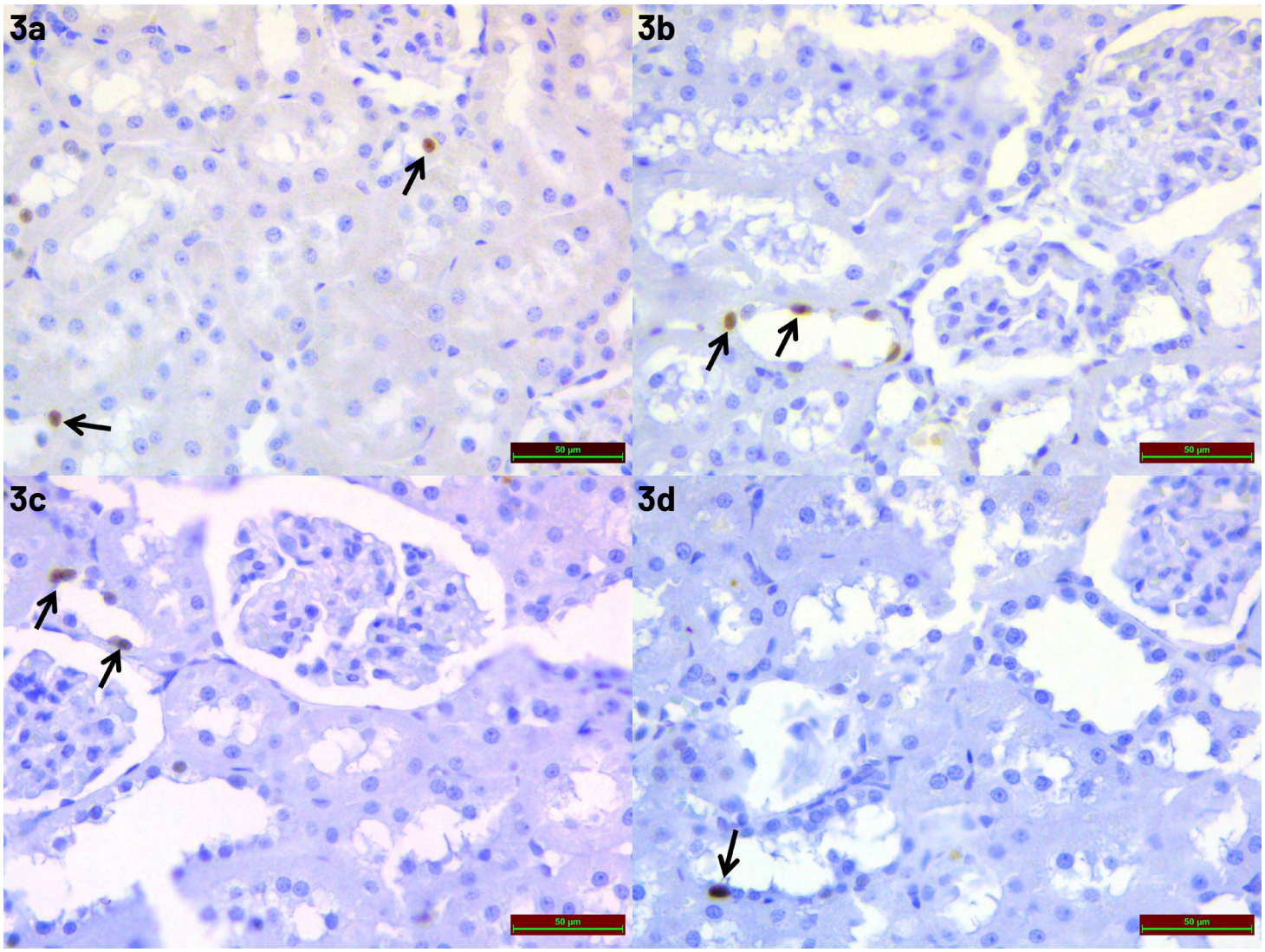

FIGURE 3. Comparison of TUNEL positivity of tubular cells in kidney tissues according to groups. a: Control group, b: NAC group, c: MTX group, and d: MTX+NAC group. TUNEL: Terminal deoxynucleotidyl transferase dUTP transferase–mediated nick end–labeling, NAC: N–acetylcysteine, MTX: Methotrexate (Mag. 400X)

TABLE I
Total Antioxidant Status, Total Oxidant Status, Apoptotic Index (%), serum adropin levels, and immunohistochemistry scores

	TAS Mean (min–max)	TOS Mean (min–max)	Apoptotic Index (%) Mean (min–max)	Serum Adropin levels Mean (min–max)	Immunohisto–chemistry score Mean (min–max)
CONTROL	1.59 (1.42–1.72)	17.42 (16.83–18.05)	2.14 (1.00–4.00)	29.56 (26.45–33.15)	0.73(0.60–0.90)
NAC	1.65 (1.31–1.91)	17.06 (15.18–18.61)	2.28 (1.00–4.00)	32.39 (29.78–34.16)	0.70 (0.45–0.90)
MTX	1.24 (1.01–1.39)[a]	22.71 (21.18–24.81)[a]	2.28 (1.00–4.00)	20.69 (19.05–22.86)[a]	0.28 (0.20–0.40)[a]
MTX+NAC	1.53 (1.35–1.66)[b]	18.84 (17.93–19.61)[b]	1.85 (1.00–3.00)	25.30 (24.56–26.23)[b]	0.69 (0.45–0.90)[b]

[a]:Compared to the control group, [b]:Compared to the MTX group (P<0.05), MTX+NAC group: NAC: N–acetylcysteine, MTX: Methotrexate

Oxidative stress plays a known role in the activation of various signal pathways, leading to transcription factor activation, gene expression, apoptosis induction, and tissue necrosis. The administration of MTX in rats increases the expression of Bax, which is an indicator of apoptosis [25]. MTX–mediated apoptosis is believed to be activated by the excess oxygen radical production that causes DNA damage. Increased oxidative stress may decrease Bcl–2 expression, regulate Bax levels, and activate the mitochondrial apoptosis pathway.

One interesting finding in the present study was that the serum TAS and TOS levels observed in the rats that were administered MTX were in line with those reported in many other studies, yet the number of

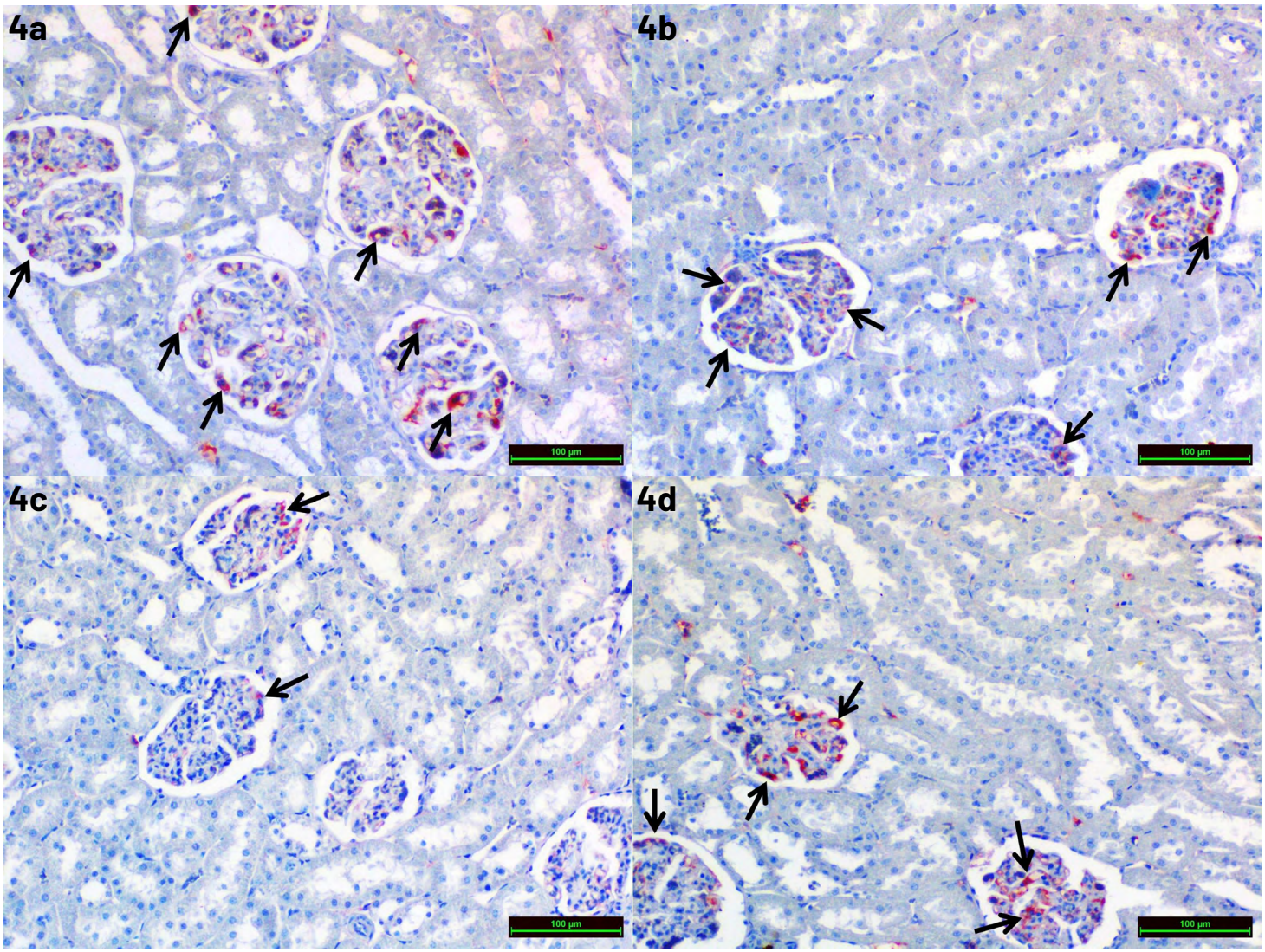

FIGURE 4. Comparison of Adropin immunoreactivity in the kidney tissues according to groups. a: Control group, b: NAC group, c: MTX group, and d: MTX+NAC group. NAC: N–acetylcysteine, MTX: Methotrexate (Mag. 400X)

apoptotic cells did not increase. This suggests that different signal pathways may trigger apoptosis, and that the sudden response to organ damage may cause a significant increase in the number of cells that undergo apoptosis. Recent studies have claimed that apoptosis induction may be a long–term process that depends on the prolonged presence of oxygen free radicals in the environment [26]. Short–term MTX use in intermittent doses is known to mediate adenosine oscillation. Adenosine effectively activates anti–inflammatory and anti–apoptotic pathways, suggesting that MTX may have indirect anti–inflammatory and anti–apoptotic effects [27].

The present findings indicate that Adropin levels may play a role in these MTX effects. Adropin is a peptide hormone coded by a gene related to energy homeostasis. Its presence has been defined in many organs, particularly in the liver, brain, and heart. In kidney tissue, Adropin immunoreactivity is localized in the peritubular interstitial cells, peritubular capillary endothelium of the cortex, and the glomeruli [20]. Lovren *et al.* [12] showed that Adropin activates vascular endothelial growth factor receptors and increases the level of eNOS. Decreasing Adropin levels would consequently decrease eNOS enzyme activity and cause a decline in NO, which acts as a vasodilator and is excreted by the endothelium. Therefore, Adropin may potentially have the capacity to protect endothelium tissues.

Current evidence now indicates that endothelium dysfunction is closely related to low plasma Adropin levels [28]. Adropin treatment has been shown to partially restore endothelial cell proliferation, migration, and capillary tube generation, and to reduce cell permeability and apoptosis induced by tumor necrosis factor alpha [12]. Experimental studies have confirmed that Adropin–mediated neural protection occurs via eNOS activation, and that Adropin decreases oxidative damage. For example, in one study, the brains of old rats showed decreasing Adropin levels, significantly increased levels of oxidative stress indicators (including gp91phox and 4–HNE), and significantly decreased levels of total and phosphorylated eNOS [29].

Chen *et al.* [30] evaluated the impact of Adropin on antioxidant reactions in preventing the progression of nonalcoholic steatohepatitis

and the clearance of ROS. They found lower Glutathione levels that did not contain Adropin in rats, as well as increased levels of oxygen free radicals, caspase-3 (an indicator of Malondialdehyde production), and apoptosis. Furthermore, they found that Adropin treatment increased the level of nuclear erythroid 2-related factor (Nrf2), which is involved in the induction of antioxidant reactions, especially Glutathione. Adropin treatment also increased the expression of antioxidative enzymes related to Nrf2, while significantly decreasing the levels of oxygen free radicals, inflammation, fibrosis, and apoptosis [30].

In this study, the serum Adropin levels were significantly lower in Group 3 than in Groups 1 and 2. It was believed that this result, in line with the literature, is related to the ability of Adropin to balance the processes involved in oxidative stress. Furthermore, the serum Adropin level and Adropin immunoreactivity level were significantly higher in Group 4 than in Group 3. It was believed that these results support the presence of an inverse relationship between the severity of oxidative stress, Adropin levels in circulation, and/or Adropin immunoreactivity. It was also considered these findings to have shed some light on the role of Adropin in the development of kidney failure following MTX administration.

CONCLUSION

In the present study supports a role for Adropin as a protective peptide against the kidney failure that occurs due to MTX-induced oxidative stress. However, this study was limited by its small sample size, and the small number of kidney tissues analyzed. Therefore, larger, and independent studies are needed to verify the obtained results.

Conflicts of interest

The authors declare no conflict of interest.

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