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Histopathological effects on kidney of diclofenac potassium and diazepam used in an experimental epilepsy model

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ABSTRACT

Aim: To investigate the effects of diazepam, which has anticonvulsant and anxiolytic effects, and diclofenac potassium, which has anti-inflammatory, analgesic and antipyretic effects, on rat kidney tissue, used in an experimental epilepsy model.

Methods: 32 Wistar albino rats (2-4 months old, 200-250 gr) were used in the study. The rats were grouped in four as 8 rats in each group: Epilepsy, Epilepsy + Diazepam, Epilepsy + Diclofenac potassium, Epilepsy + Diazepam + Diclofenac potassium. Epileptic seizure model was created with penicillin (500.000 IU) injected intracortically under urethane anesthesia. 30 minutes later, diazepam (0.1 mg/kg) and diclofenac potassium (10 mg/kg) were administered intraperitoneally. At the end of the study, rat kidneys were removed and evaluated histopathologically in terms of inflammation, glomerular shrinkage, tubular dilatation, tubular epithelial thinning, desquame epithelium, brush epithelial loss, vacuolization, hemorrhage and congestion.

Results: No difference was found between diazepam and diclofenac potassium in terms of vacuolization, glomerular shrinkage, tubular dilatation and hemorrhage. Inflammation, congestion and tubular epithelial thinning rate were found to be lower inEpilepsy + Diclofenac potassium and Epilepsy + Diazepam + Diclofenac potassium group when compared with Epilepsy + Diazepam group. While brush epithelial loss and desquame epithelial rate was found to be lowest in the epilepsy group, these parameters were not found to show a significant difference between drug groups.

Conclusion: It was concluded that combined use of diazepam and diclofenac potassium in their effects on kidney are more useful than their single use.

Key words: Epilepsy, diazepam, diclofenac potassium, kidney histopathology, rat.

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Introduction

Epilepsy is a common neurological disease which affects many organs and systems by causing recurrent seizures and which is seen in 1% of the world population [1, 2]. It ranks the fourth in the world neurological diseases list with a prevalence of 6-7 per 1000 people throughout life. It affects all age groups and both genders and it requires long-term and sometimes life-long treatment [3].Antiepileptic drugs (AEDs) are the main choice of treatment for patients with epilepsy and two thirds of epileptic seizures can be controlled by AEDs [3]. Many factors including the effectiveness of the drug, tolerability, toxicity and cost, etc. influence the choice of AED for specific epilepsy syndrome [4].

Kidney, which is the primary excretory organ for drugs, chemical and biotransferred products, is sensitive to toxicity. Continuous clinical evaluation is required during treatment with drugs in order to avoid toxicity [5]. The exact mechanism leading to nephrotoxicity caused by antiepileptic drugs is not known [6]. Sometimes AEDs may cause serious nephrotoxicity. Since these reactions are usually unpredictable, clinicians should be careful about this possibility [7].The main focus of epilepsy is a satisfying seizure control with minimum side effects [8]. Diazepam is a benzodiazepine. It is mainly used in the acute treatment of severe seizures. Diazepam is extensively metabolized to three active metabolites. Metabolic reactions are catalyzed by CYP2C19 and CYP3A4 and less than 3% of the dose is excreted unchanged with urine [4]. In general, the renal effects of these drugs include decreased glomerular filtration rate, urinary excretion and some hemodynamic changes [9]. Current antiepileptic medications only treat the symptoms of the condition. Long-term usage of them is also accompanied by a wide range of adverse effects. Therefore, a promising area of research is the need for novel antiepileptic medications with improved compatibility. It has been demonstrated that epileptic brains amass inflammatory cells. Brain inflammation is attributed to cyclooxygenase 2 (COX-2). Hyperactive neuron networks are a result of inflammatory processes, which also contribute to the development of epilepsy [10]. Given that the function of brain inflammation in epilepsy has been repeatedly highlighted in recent years, anti-inflammatory drugs should be thoroughly explored as they may provide new therapy strategies in preventing or decreasing epileptogenesis [11].

Non-steroid anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of temporary (for exp. headache, muscle inflammation) or persistent pain (for exp. rheumatoid arthritis) [12]. Common mode of action for all NSAIDs is the inhibition of COX enzymes resulting in reduced prostanoid synthesis [13]. Diclofenac potassium is a compound with non-steroid anti-inflammatory, analgesic and antipyretic effects [14]. It is a benzene acetic acid derivative showing COX-2 enzyme inhibition. Chemical name for diclofenac potassium is 2-[(2,6-dichlorophenyl)amino]-benzene acetic acid [15]. It has a quick elimination rate and a two-hour biological half-life [14]. In this study, we aimed to investigate the histopathological effects of diclofenac potassium and diazepam on the kidneys in an experimental epilepsy model with penicillin in rats.

Materials and metods

Preparing the rats

Our study was carried out in accordance with the national health institute guidelines on the use of experimental animals with 2020/25.A-1

numbered decision and permission of Bolu Abant İzzet Baysal University Local Ethics Committee. 32 *Wistar albino* male rats, which were 2-4 months old and weighing 200-250 gr, obtained from Experimental Animals Research and Application Centre, were used in the study. The rats were kept separately in an environment of fixed temperature (19±2 ºC) and humidity on a cycle of 24-hour light/24-hour dark with access to ad libitum water and pellet food. The rats were grouped in 4 as n=8. The groups in the study were Epilepsy group created with (Ep), Epilepsy + Diazepam (Ep+Dzp), Epilepsy + Diclofenac Potassium (Ep+Dicp), Epilepsy + Diclofenac Potassium + Diazepam (Ep+Dzp+Dicp).All experiments were performed between 08.00 and 12.00.

Epileptic Seizure Induction and Drug Applications

To create an experimental epileptic seizure model, the animals which fasted for 24 hours were fixated on the operating table after being shaved from the top of their heads under urethane anesthesia. Rats' scalps were opened in the rosto-caudal direction, approximately 3 cm in length with a scalpel. The soft tissue under the left cortex scalp was removed by electrocautery. The skull bone was removed by thinning with circular movements with a micromotor. After the electrodes were placed, basal activity was recorded for 5 minutes. Afterwards, 500.000 IU penicillin (2.5 µl, icv) [16] was administered intracortically to the somatomotor cortex with a Hamilton injector (701N, Hamilton Co., Reno, NV, USA) to induce epileptic activity [17]. The injection coordinates were 2 mm lateral, 1 mm anterior, and 1.2 mm depth of the bregma line. 0.90,1 ml % saline was administered intraperitoneally to the epilepsy group for sham purposes. 30 minutes later, diclofenac potassium, diazepam and diclofenac potassium + diazepam groups were administered diazepam (0,1 ml, 5 mg/kg, i.p.) [18] and diclofenac potassium (10 mg/kg ip) [10]. Immediately following the study, sacrification was performed under the effect of urethane anesthesia, since the effect of anesthesia continued during the 125-minute recording period after the model was created (95 min after drug injection). Later, the kidney tissues obtained from the rats were fixated in 10% formaldehyde for histopathological examination.

Histopathological Evaluation

After routine histopathological procedures, kidney tissues were embedded in paraffin. 3 µm sections were taken from paraffin blocks and stained with hematoxylin-eosin. The preparations obtained were evaluated under LEICA DM 2000 LED light microscope. Kidney tissue was evaluated histopathologically in terms of glomerular shrinkage, tubular dilatation, tubular epithelial thinning, desquame epithelium, brush epithelial loss, vacuolar degeneration, lymphocyte infiltration, interstitial hemorrhage and vascular congestion. At X20 magnification, 10 different areas were examined in each preparation and scored semiqualitatively from 0 to 3 [19]. According to this scoring, absence of pathology was scored as 0, presence of mild (focal) pathology was scored as 1, presence of moderate (multifocal) pathology was scored as 2 and presence of severe (diffused) pathology was scored as 3. The sections stained with hematoxylin-eosin were photographed in different scales with Infinity 3 Analyze Release 6.5 system.

Statistical Analyses

The data were analyzed by using IBM SPSS version 21.0 program (IBM Corp., N.Y., USA). The conformity of data to normal distribution was evaluated with Shapiro-Wilk test. The data with normal distribution were shown with mean ± standard deviation, while those which did not have normal distribution were shown with median IQR. ANOVA test was conducted in the

evaluation of normally distributed data. Non-parametrical data were evaluated with Kruskal-Wallis test and Mann-Whitney U test with Bonferroni correction was performed as post-hoc test. Level of significance was considered as $p<0,05$.

Results

An epilepsy model was created in all groups. Histopathological parameters in kidney tissues of the groups are shown in Table 1 and 2. Microscopic images of the groups are shown in Figure 1a-d.

Differences were found between groups in terms of inflammation (X^2=16,953; p=0.001). When the groups were compared, significantly more intense inflammatory cells were found in Ep+Dzp group than Ep (Z=-3,276; p=0,001) and Ep+Dicp (Z=-2,816; p=0,005) group. No significant difference was found between Ep+Dicp and Ep+Dzp+Dicp groups in terms inflammation, although Ep+Dzp+Dicp group had less inflammatory cells (Z=-1,113; p=0,266). Significant difference was found between groups in terms of vascular congestion (X^2=17,237; p=0.001). Significant difference was found in Ep group, when compared with Ep+Dzp group (z=-3,303; p=0.001). There was also a significant difference between Ep and Ep+Dicp groups (z=-3,303; p=0.001). However, when Ep+Dicp and Ep+Dzp+Dicp groups were compared, no statistically significant difference was found between the groups, although fewer congestion areas were observed in Ep+Dzp+Dicp group (z=-2,227; p=0.026).

Interstitial hemorrhage showed a significant difference between the groups (X^2=23,896; $p<0.001$). In terms of hemorrhagic areas, difference was found when Ep group was compared with Ep+Dzp group (z=-3,703; $p<0.001$) and Ep+Dicp (z=-3,626; $p<0.001$) group. No significant difference was found when Ep+Dicp group was compared with Ep+Dzp group (z=-2,083; p=0,037) and Ep+Dzp+Dicp group (z=-2,412; p=0,16). However, there was a significant difference between Ep+Dzp and Ep+Dzp+Dicp groups (z=-3,289; p=0.001).
Significant difference was found between groups when compared in terms of cytoplasmic vacuolization (F:12.773; $p<0.001$). When the groups were compared, the lowest vacuolization intensity was found in Ep group ($p<0.001$). When the Ep+Dzp group was compared with Ep+Dicp and Ep+Dzp+Dicp groups, no statistically significant difference was found (p=1000), although more intense vacuolization was observed in the Ep+Dzp group.

Significant difference was found between groups in terms of glomerular shrinkage (X^2=19,578; $p<0.001$). Ep+Dzp group was found to have higher glomerular shrinkage structure than the epilepsy group (z=-3,703; p=0.001). No significant difference was found between Ep+Dzp group and Ep+Dicpgroup (z=-1,019; p=0,308). When Ep+Dicp group was compared with Ep+Dzp+Dicp group, no significant difference was found although Ep+Dicp group was found to have higher glomerular shrinkage (z=-2,221; p=0,026). Significant glomerular shrinkage observed between Ep and Ep+Dicp

Table 1. Distribution of normally distributed histopathological data to groups (mean ± SD).

Parameters	**Groups**				
	Ep (n=8)	**Ep+Dzp (n=8)**	**Ep+Dicp (n=8)**	**Ep+Dzp+Dicp (n=8)**	***p***
Vacuolization	0±0[a]	2,00 ±0,756	2,00 ±0,756	1,88 ±1,126	<0,05

a: significantly different at p=0.005 level.

Table 2. Distribution of non-normally distributed histopathological data to groups (median).

Parameters	Groups				
	Ep (n=8)	**Ep+Dzp (n=8)**	**Ep+Dicp (n=8)**	**Ep+Dzp+Dicp (n=8)**	***p***
Inflammation	0,50;(IQR:1)[a]	3;(IQR:1)[ab]	1;(IQR:1)[b]	1;(IQR:1)	0,001
Congestion	1;(IQR:0)[ab]	3;(IQR:1)[a]	2;(IQR:1)[b]	1;(IQR:1)	0,001
Haemorrhage	0;(IQR:0)[ab]	3;(IQR:1)[ac]	2;(IQR:2)[b]	0,50;(IQR:2)[c]	<0,01
Glomerular Shrinkage	0;(IQR:1)[ab]	2,50;(IQR:1)[a]	2;(IQR:2)[b]	1;(IQR:2)	<0,01
Tubular Dilation	0;(IQR:1)[a]	3;(IQR:1)[a]	2;(IQR:2)	1;(IQR:1)	<0,01
Loss of brush border	0;(IQR:1)[a]	2,50;(IQR:2)[a]	2;(IQR:1)	1;(IQR:2)	0,001
Tubular Epithelium Thinning	0;(IQR:1)[a]	2;(IQR:1)[a]	(IQR:1)	(IQR:1)	<0,01
Desquame Epithelium	;(IQR:0)[ab]	2,50;(IQR:1)[a]	1,50;(IQR:1)[b]	1;(IQR:2)	<0,01

a,b,c: significantly different at p=0.001 level (Inflammation, Congestion, Loss of brush border)

a,b,c: significantly different at p<0.001 level (Hemorrhage, Glomerular Shrinkage, Tubular Dilation, Tubular Epithelium Thinning, Desquame Epithelium)

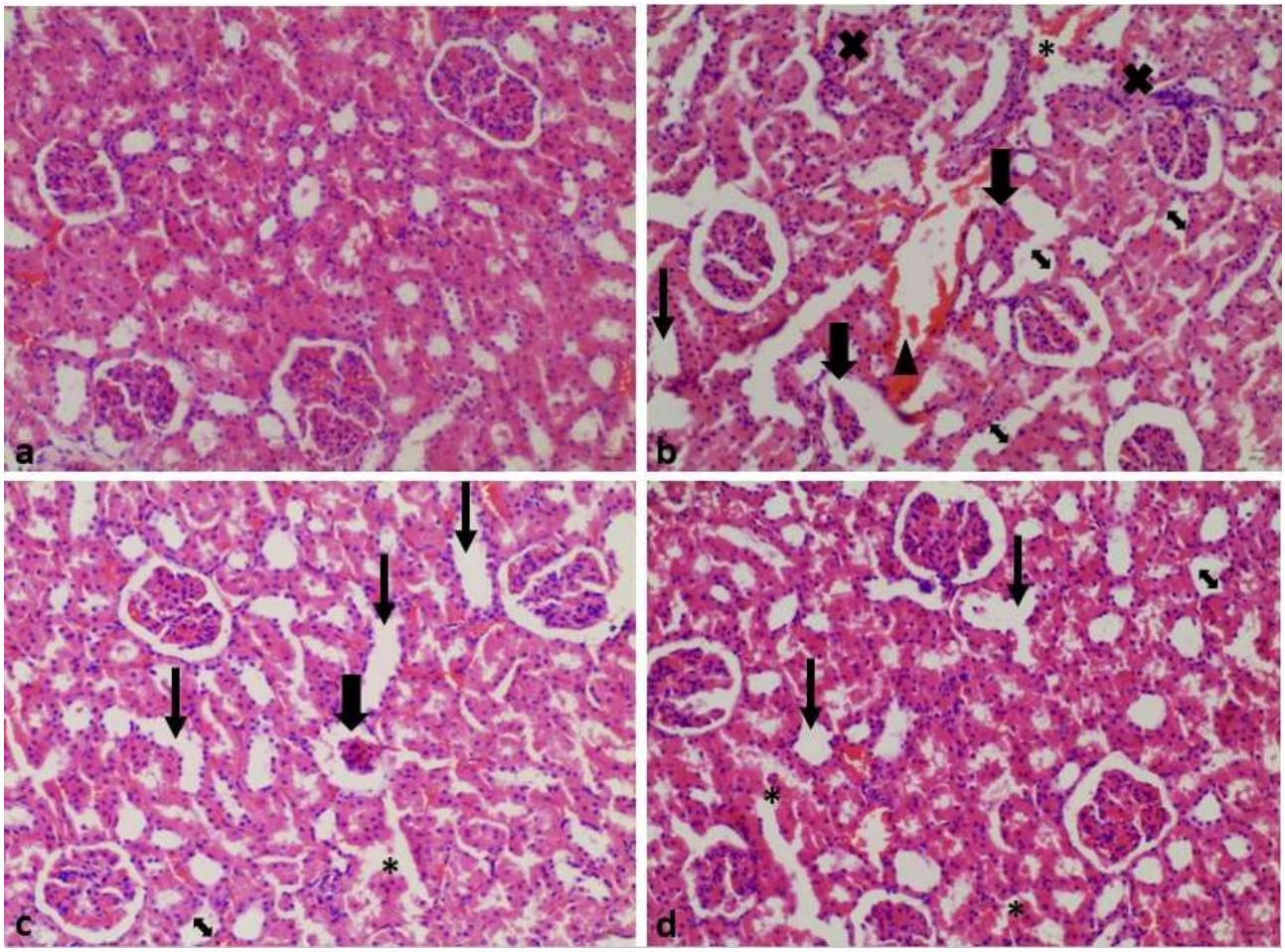

Figure 1. Kidney histopathological images. Epilepsy group, normal histological appearance (a); Epilepsy + Diazepam group (b); Epilepsy + Diclofenac potassium group, (c); Epilepsy + Diazepam + Diclofenac potassium group (d); Vascular congestion(head arrow), glomerular atrophy (thick arrow), inflammation(**X**), tubular dilation (arrow), Tubular Epithelium Thinning (double arrow), Desquame Epithelium (asterisk). H&E staining, X200 magnification.

(z=-3,289; p=0.001) was found to decrease significantly in Ep+Dzp+Dicp group (z=-2,730; *p*=0.006).

Significant difference was found between groups in terms of tubular dilatation (X^2=19,114; *p*<0.001). Significant difference was found between Ep and Ep+Dzp groups (z=-3,330; *p*=0.001). No statistical significance was found between Ep+Dzp group and Ep+Dicp group although tubular dilatation rate was higher in Ep+Dzp group (z=-1,412; *p*=0,158). In addition, a significant decrease was found in Ep+Dzp+Dicp group (z=-3,245; p=0.001).

Significant difference was found between groups in terms of loss of brush border (X^2=16,698; *p*=0.001). More loss of brush border was observed in Ep+diaz group when compared with the Epi group (z=-3,289; *p*=0.001). However, no difference was found between Ep+Dzp group and Ep+Dicp (z=-1,225; *p*=0,221) and Ep+Dzp+Dicp (z=-2,184; *p*=0.029) groups. Significant difference was found between groups in terms of tubular epithelial thinning (X^2=18,349; *p*<0.001). The highest epithelial thinning was observed in Ep+Dzp group (z=-3,390; *p*=0.001). Although there was a decreased tubular epithelial thinning in Ep+Dzp+Dicp group, no significant difference was found between Ep+Dzp group (z=-2,348; *p*=0.019). Significant difference was found between groups in terms of desquame epithelium (X^2=18,867; *p*<0.001). Significant difference was found between Ep and Ep+Dzp groups (z=-3,496; *p*<0.001). A significant difference was also found between Ep and Ep+Dicp groups (z=-2,951; *p*=0.003). No difference was found when Ep+Dzp and Ep+Dicp groups were compared (z=-1,877; *p*=0,060). Although less desquame epithelium was seen in Ep+Dzp+Dicp group, no statistical difference was found between Ep+Dicp group (z=-0,779; *p*=0,436).

Discussion

Epilepsy is a common neurological disorder affecting people of different ages [20], [3]. It affects more than 50 million people globally and it is characterized by recurrent non-provoked seizures [7]. Except a few cases that can be approached surgically, the treatment of epilepsy depends on the use of antiepileptic drugs to provide symptomatic seizure control [21]. One of the main purposes of treatment is to improve the life quality of patients by optimizing the balance between seizure control and the side effects of AEDs [7]. Antiepileptic medication side effects place a large financial burden on society and significantly reduce quality of life [11]. Some primary factors are considered in the clinical evaluation of possible toxicity that may occur as a result of side effects of drugs. These factors include the patients' age, duration of treatment, chemical structure of drugs, the number of drugs used and the condition of liver and kidney. It is a known fact that kidney is one of the primary organs open to side effects of drugs that may impair its structure and functions. Kidney performs several basic regulatory and excretory functions. These functions include protecting the acid-base balance, regulating electrolytes, disposal of nitrogenous wastes, removal of drugs and their bioconverted products. It is also responsible for the production of essential biochemicals such as renin, erythropoietin and calcitriol and reabsorption of glucose, water and amino acids [5]. Clinical and subclinical laboratory evidence of renal proximal tubular dysfunction has been reported in children with epilepsy as a side effect of some AEDs. In a study they conducted on 60 patients, Hamed et al. observed subclinical renal glomerular and proximal tubular damage in the kidneys of patients with epilepsy treated with valproate and carbamazepine [6].

Diazepam is an anticonvulsant anxiolytic, sedative benzodiazepine derivative. Diazepam causes kidney damage by destroying cells with its hemodynamic special chemical structure. By reducing blood pressure, it reduces blood flow to kidney and causes ischemia. In a study they conducted on rats, Setiawan et al. showed that diazepam (62,25 83,0 and 124,5 mg/kg) increased urea and creatinine levels and caused tubular and glomular damage, hemorrhage and congestion in kidneys histologically [22]. Grahovac et al. conducted a study which is demonstrated that the effects of diazepam (0.2 mg/kg/day for 15 days)on blood parameters and histology of target organs and they displayed that diazepam reduces blood cell production, causes an imbalance in electrolyte concentration, and lower immunity. They also proved that it causes a decrease in liver enzymes and deterioration in kidney function [23]. In a study conducted by Mousa et al. in rats, they observed that 1 mg/kg diazepam increased capillary dilatation and renal vascularization, but stated that high-dose diazepam was toxic and accumulated in rat kidney tissue [24].There are two ways of diazepam being responsible for renal injury. They are affecting the hemodynamic and destructing cells by its chemical compounds. Diazepam can decrease the blood pressure and so lower blood flow to the kidney. This case may lead to a renal ischemia which induces hypoxia condition in kidney cortex cells [25]Iqbal et al observed that the use of diazepam with another drug gave more positive results on the liver and kidney [26]. In the present study, diazepam has caused histopathological damage on the kidney, which is in line with the mentioned study.

The importance of cellular and molecular brain inflammation pathways in the etiology of epilepsy has been highlighted in recent experimental research. Significant efforts are being made to find inflammatory biomarkers linked with epilepsy in the cerebrospinal fluid and serum of affected individuals because clinical investigations have demonstrated the involvement of the brain inflammatory response in human epilepsy, including autoimmune encephalitis. For this reason, anti-inflammatory treatment can be promising in this respect. In clinical practice, NSAIDs are frequently employed as anti-inflammatory, analgesic, and antipyretic medications [11]. NSAIDs show anti-inflammatory, analgesic and antipyretic effects by inhibiting COX enzyme and suppressing prostaglandin (PG) synthesis. The kidneys and the gastrointestinal tract are significant targets for adverse clinical outcomes associated with NSAID usage. Renal side effects (for exp. kidney function, fluid and urine electrolyte excretion) vary with the extent of COX-2-COX-1 selectivity and the administered dose of these compounds [27]. The most concerning NSAID side effects are drug-induced asthmatic response, platelet inhibition, suppression of prostaglandin production necessary for proper gastrointestinal and renal function, cardiotoxicity, and hepatotoxicity. These effects are extensions of the pharmacodynamic of the drug and they may exacerbate with drug interactions. Inhibition of COX-1 suppresses prostaglandin activity, which is critical for renal cell integrity. Although COX-2 selective medications reduce the detrimental effects on the gastrointestinal system and thrombocytes, cardiac and renal concerns. [28]. Kidney function may be affected differently if one or more of these enzymes are blocked [27]. During NSAID administration, both selective COX-2 inhibitors and non-selective COX inhibitors reduce the PG level in kidneys and impair the renal hemodynamics. Renal effects of these drugs are thought to cause arterial hypertension [29]. Expression of the COX enzyme covers almost all peripheral organs of the body including lungs, liver, kidneys, stomach,

colon, thyroid gland, pancreas, uterus, testis and others [30]. This inhibition of prostaglandin synthesis is reversible, with the exception of high dose NSAID therapy, lowering the risk of renal damage [28].

Renal side effects of NSAIDs are rare, possible side effects are sometimes temporary and they are usually reversible upon discontinuation of the drug. The risk of renal side effect increase in patients with diabetes, heart failure, renal function disorder and in elderly individuals. Side effects range from electrolyte retention and decreased glomerular filtration to nephritic syndrome and chronic renal failure. These effects vary according to the dose used and exposure time [31]. In the PTZ kindling model of epilepsy, numerous studies have demonstrated that diclofenac decreased seizure severity and interlukin-6 and TNF- levels in the hippocampus. This drug suppressed cortical neuronal activity [20].

Diclofenac potassium shows analgesic, anti-inflammatory and antipyretic features. The notable acceptance of diclofenac can be attributed to being a potent COX-2 inhibitor while maintaining similar activity to some other popular non- selective NSAIDs. Tests have shown that diclofenac inhibits COX-2 enzyme with higher potency than COX-1 [14]. Since specific and robust expressions of KCNQ2/3 channels in neurons and loss of function can lead to neuronal hyperexcitability, pharmacological activation of KCNQ2/3 channels is the basis of epilepsy and pain treatment [32]. Interestingly, diclofenac can be one of the new k channel openers that activate KCNQ2\Q3 channels and this new mechanism of action can be helpful in epilepsy [33]. Anticonvulsant response by increasing KCNQ2/Q3 potassium currents makes it suitable for use in migraine epilepsy and neuropathy [15].

Al-Hayder et al. found in rats they administered naproxen and diclofenac that 50 mg/kg diclofenac caused less damage to kidneys than 50 mg/kg naproxen [34]. In a study, Störmer et al. found that 200 mg diclofenac use aggravated pre-existing kidney damage, while the use of 100 mg diclofenac for 3 days did not create a change in kidney morphology [35]. In a study they conducted, Zaruwa et al. found that diclofenac treatment caused pathological changes in soft tissues such as prostate gland and in various organs by increasing free radicals [36]. This study showed that diazepam used for the treatment of epilepsy caused histopathological damage on the kidneys. Tubular epithelial thinning was less than the other groups in the Diazepam group. Additionally; inflammatory cells, glomerular shrinkage, vascular congestion, and interstitial hemorrhage were observed less in the diclofenac potassium group, when compared with other groups. Renal histopathological damage in the Diazepam + Diclofenac potassium group was significantly less than the other groups.

This study is solely based on histopathological examination. Biochemical and other tests were not performed. This situation are seen as the limitation of our study. Therefore, it would be possible to make further interpretations with more comprehensive results.

Conclusions

As a conclusion, diazepam treatment caused negative consequences in kidney histopathological parameters. Combined use of Diazepam and low-dose Diclofenac potassium in epilepsy patients causes less renal injury than their single use. Further studies are needed for therapeutical use of these drugs. The chronic effect of Diazepam and low-dose Diclofenac potassium was not analyzed in this study.

Therefore, longer treatment is required to obtain more comprehensive data.

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Conflict of interest: *The authors declare that they have no conflict of interest.*

Ethical statement: *The study was approved by Bolu Abant İzzet Baysal University Local Ethics Committee (Number: 2020/25.A-1).*

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