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PHARMACOLOGY

ROLE OF L-, N- AND T-TYPE CALCIUM CHANNELS IN ACHIEVING MAXIMUM ANALGESIC AND MINIMUM TOXIC EFFECT OF TRAMADOL

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Abstract: The aim of the study was to investigate the effects of lacidipine, amlodipine, and benidipine on the analgesic activity and toxicity of tramadol. Rats (n = 6/each group) were divided into five groups as control (CG), tramadol (TRD), lacidipine+tramadol (LTRD), amlodipine+tramadol (ATRD), and benidipine+tramadol (BTRD). Tramadol was administered once at a dose of 50 mg/kg and lacidipine, amlodipin, benidipin were administered at a dose of 4 mg/kg orally, once a day for 10 days. After the animals were sacrificed, malondialdehyde (MDA) and total glutathione (tGSH) levels were measured in brain, heart, liver, and kidney tissues. Troponin I (Tp I), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine levels were determined in serum. Paw pain thresholds were measured one hour before and after drug administration**.** The analgesic effect of tramadol was increased the most by benidipine, while amlodipine and lacidipine increased it equally. Lacidipine failed to suppress MDA increase and tGSH decrease in brain tissue. Benidipine suppressed MDA increase and tGSH decrease in brain tissue better than amlodipine. All three drugs suppressed cardiac tissue oxidative stress and the release of TP I into the circulation. Lacidipine and amlodipine could not prevent tramadol-induced oxidative stress in liver and kidney tissues, whereas benidipine prevented excessive MDA increase and tGSH decrease. Benidipine significantly suppressed the increase of ALT, AST, BUN, and creatinine. Analgesic activity was 35.1% in TRD, 43.7% in LTRD, 50.4% in ATRD, and 67.5% in BTRD. Study results show that benidipine and tramadol co-treatment is better than co-treatment with lacidipine or amlodipine in achieving minimal toxic effects and maximum analgesia.

Keywords: amlodipin, analgesic activity, benidipin, lacidipin, toxicity, tramadol

Abbreviations: CG control; TRD: tramadol only; LTRD: lasidipine + tramadol; ATRD: amlodipine + tramadol; BTRD: benidipine + tramadol; HG: healthy; MDA: malondialdehyde; TP I: troponin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; tGSH: total glutathione

Tramadol was first introduced in Germany in 1977 and was approved in the United States in 1995 as a synthetic opioid analgesic [1]. Tramadol is effective in relieving moderate and severe pain conditions such as osteoarthritis, neuropathic pain, cancer pain, and post-operative pain [2, 3]. The mechanism of action is suggested to involve stimulation of μ opiate receptors and inhibition of norepinephrine and serotonin reuptake [4]. Since tramadol cannot bind to μ receptors as strongly as morphine, high or repeated doses may be required to get a satisfactory analgesic response. However, high doses of tramadol remain challenging due to serious side effects [5, 6]. A previous study reported that high doses of tramadol cause an increase in oxidants such as malondialdehyde (MDA) and a decrease in endogenous

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antioxidants such as glutathione (GSH), superoxide dismutase, and catalase [7]. The investigations also reported that neurotoxic, hepatotoxic, and nephrotoxic effects become evident in direct proportion when the tramadol dose is increased [8]. Faria et al. reported that tramadol causes oxidative and inflammatory damage to the heart even at analgesic doses [9]. Thus, different combinations of tramadol with other drugs were investigated to increase its analgesic activity and reduce its toxic effects [10].

Papers argue that Ca_2^+ channel blockade plays a role in analgesic activity [11]. Modi et al. showed that L and N-type Ca_2^+ channels blocker, amlodipine, significantly increased the analgesic effect of tramadol [12]. The selective L-type Ca_2^+ channel blocker lacidipine alleviates experimentally induced hyperalgesia [13]. Furthermore, clinical and preclinical studies reported that benidipine, which blocks T-type Ca_2^+ channels in addition to L- and N- types, causes a decrease in pain scores [14]. However, there is a lack of information in the literature investigating the relationship between Ca_2^+ channel blockers and the toxic consequences of tramadol. The current study aimed to investigate the effects of lacidipine, amlodipine, and benidipine (LAB) on the analgesic activity of tramadol and their possible impact on tramadol-induced neurotoxicity, cardiotoxicity, hepatotoxicity, and nephrotoxicity in rats.

EXPERIMENTAL

Animals

A total of 30 albino male Wistar rats weighing between 260 and 285 grams were used in the experiment. All of the experimental animals were obtained from Erzincan Binali Yıldırım University Experimental Animals Application and Research Center. In order to adapt to the environment, the animals were housed and fed for one week in a laboratory environment at normal room temperature (22°C) with a 12-hour light-dark cycle. The protocols and procedures were approved by the local Animal Experimentation Ethics Committee (Date: 27.04.2023, Decision no.: 15).

Chemicals

Thiopental sodium was obtained from IE Ulagay (Turkey), lacidipine from Glaxo Smith Cline (Turkey), amlodipine from Pfizer (Turkey), benidipine from Deva (Turkey), tramadol from Abdi İbrahim İlaç Sanayi (Turkey), MDA and tGSH assay kit Cayman Chemical Company (USA), Commercial kits Roche Diagnostics (Germany), VIDAS TP I Ultra kit, bioMérieux (İskandinav).

Experimental Groups and Procedure

Experimental animals were divided into five groups as CG, TRD, LTRD, ATRD, and BTRD. The LTRD (n = 6), ATRD (n = 6), and BTRD (n = 6) groups were administered lacidipine (4 mg/kg), amlodipine (4 mg/kg), and benidipine (4 mg/kg), respectively, by oral route with a catheter (1 cc). The same volume of distilled water was given orally to the CG (n = 6) and TRD (n = 6) groups. Animals were administered LAB and tramadol once daily for 10 days. One hour after the drugs and distilled water were administered, 50 mg/kg of tramadol was administered orally to the animals in the LTRD, ATRD, BTRD, and TRD groups. Paw pain thresholds of the rats in the whole group were measured one hour before drug administration and at the first hour after administration using Basile Algesimeter (Cat. No. 37215, Ugo Basile, Italy). Analgesic activity was calculated according to the formula: analgesic activity = 100 - (paw pain threshold measurement value of the CG group X100/paw pain threshold value of the treated group) [15]. Then, the animals were euthanized with thiopental sodium (50 mg/kg) and brain, heart, liver, and kidney tissues were removed. MDA and tGSH levels were measured in tissues. Tp I, ALT, AST, BUN, and creatinine levels were determined in blood samples taken from tail veins 1 cc before the animals were sacrificied.

Biochemical Analysis

Preparation of samples

A total of 0.2 grams of tissues (brain, heart, liver, kidney) taken from the rats were washed thoroughly with NaCl (0.9%), and homogenised with liquid nitrogen. Next, 2 mL of 1.15% potassium chloride buffer solution (pH 7.4) was added to the prepared homogenisation and centrifuged at 10.000xg for 15 min at 4°C. The obtained supernatants were used for biochemical analyses, such as malondialdehyde (MDA) and total glutathione (tGSH).

Determination of MDA and GSH

The determination of MDA and tGSH in tissues was performed following the instructions of each assay kit (item no. 10009055, 703002 Cayman Chemical Company, respectively).

MDA measurement principle in the kit: TBARS Assay Kit provides a simple, reproducible, and standardized tool for assaying lipid peroxidation in plasma, serum, urine, tissue homogenates, and cell lysates. The MDA- TBA adduct formed by the reaction of MDA and TBA under high temparature (90-100°C) and acidic conditions is measured

colorimetrically at 530-540 nm or fluorometrically at an excitation wavelenght of 550 nm.

GSH measurement principle in the kit: GSH Assay utilizes a carefully optimized enzymatic recycling method. Using glutathione reductase, for the quantification of GSH. The sulfhydryl group of GSH reacts with DTNB (5,5'-dithio-bis-2-(nitrobenzoic acid), Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). Measurement of the absorbance of TNB at 405-414 nm provides an accurate estimation of GSH in the sample.

Determination of TP I

TP I levels were measured with a VIDAS TP I Ultra kit using the ELFA (Enzyme-Linked Fluorescence Assay) technique in the plasma obtained from the animals. The analysis was performed automatically on a VIDAS (Marcy-I'Étoile, France) instrument using reagents from the kit.

Determination of ALT and AST

Tubes without anticoagulant were used for venous blood samples. After coagulation, centrifugation was performed and the serum fraction was collected and stored at 80°C until analysis. Cobas 8000 autoanalyzer (Roche Diagnostics GmBH, Mannheim, Germany) and commercial kits (Roche Diagnostics) were used for serum Alanine Aminotransaminase ALT and Aspartate Aminotransferase AST analysis. In both measurements, enzyme activity was evaluated according to a decrease in NADPH absorbance in the medium. The decrease in NADH absorbance measured at 340 nm is directly proportional to the serum activity of ALT and AST.

Determination of BUN

To determine the serum urea level, the spectrophotometric method was studied on a Roche brand Cobas 8000 autoanalyzer (Roche Diagnostics GmBH, Mannheim, Germany) The test principle of the study: Urea is hydrolyzed by urease to form ammonium and carbonate. In the second step, 2 oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and coenzyme NADH to form L glutamate, and serum urea concentration is obtained by a decrease in NADH absorbance measured at 340 nm.

Creatinine determination

Serum creatinine levels were determined by spectrophotometric measurement in a Roche brand Cobas 8000 autoanalyzer (Roche Diagnostics GmBH, Mannheim, Germany) according to the Jaffe method (16).

Statistical Analysis

Statistical analyses were performed in the IBM SPSS Statistics 22.0 program. The GraphPad Prism 9.0.0 program was used for graphics. The Shapiro-Wilk test was used to ensure the data conformed to normal distribution. One-way ANOVA was preferred for analysis, since we determined the data were normally distributed. In the pairwise comparison of the groups, the Games-Howel test or Tukey HSD were preferred according to the Levene test results. Data are presented as mean ± standard deviation($X \pm SD$) and 95% confidence intervals for mean (lower bound-upper bound). A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The present study was conducted to investigate the effects of lacidipine, amlodipine and benidipine (LAB) on the analgesic activity of tramadol and their possible impact on tramadol-induced neurotoxicity, cardiotoxicity, hepatotoxicity and nephrotoxicity in rats.

Results of Paw Pain Threshold Measurements

Tramadol increased the paw pain threshold values in the first hour compared with the control animals ($p < 0.001$). In the benidipine, amlodipine, and lacidipine groups, paw pain thresholds were increased compared with the tramadol only group ($p < 0.001$). The highest paw pain threshold values were obtained in the benidipine + tramadol group, while lacidipine and amlodipine data were similar ($p = 0.070$). There was a difference between the tramadol and the control group in terms of differences in paw pain threshold values before and after treatment ($p < 0.001$). The difference in paw pain threshold values was greater in LTRD, ATRD, and BTRD compared with TRD ($p < 0.05$). The largest difference was obtained in the benidipine group, while the difference between the two measurements was similar in the lacidipine and amlodipine groups ($p = 0.051$). Analgesic activity was 35.1% in TRD, 43.7% in LTRD, and 50.4% in ATRD. The best analgesic activity belonged to BTRD with 67.5%. Results are presented in Table 1.

Analysis of MDA and tGSH Levels in Brain, Heart, Liver, and Kidney Tissues

An increase in MDA (Figure 1A) levels and a decrease in tGSH (Figure 1B) levels were detected in the brain tissues of the tramadol group animals compared with the control animals ($p < 0.001$). It was determined that lacidipine treatment could

Table 1. Results of paw withdrawal threshold analysis.

Groups n = 6/each group	Paw pain threshold (gram)			Analgesic activity (%)
	Before treatment	One hour after treatment	Difference between before and after treatment	
CG	52.33 ± 3.88^{ns} (48.26-56.41)	50.50 ± 3.73 (46.59-54.41)	-1.83 ± 1.60 (-3.51(-)-0.15)	-
TRD	54.33 ± 3.56^{ns} (50.60-58.07)	83.67 ± 4.50* (78.94-88.39)	29.33 ± 7.39* (21.57-37.09)	35.1
LTRD	54.17 ± 4.22^{ns} (49.74-58.59)	96.33 ± 2.16* (94.07-98.60)	42.17 ± 4.71* (37.23-47.11)	43.7
ATRD	51.50 ± 5.17^{ns} (46.08-56.92)	103.83 ± 5.71* (97.85-109.82)	52.33 ± 6.53* (45.48-59.19)	50.4
BTRD	49.00 ± 3.10^{ns} (45.75-52.25)	150.67 ± 6.15*.** (144.21-157-13)	101.67 ± 3*.** (93.66-109.68)	67.5
F(4,25)/p values	1.755/0.170	361.543/0.001	237.534/0.001	

*; $p < 0.001$ vs CG, Statistical analysis was done with one way ANOVA, followed by Tukey HSD. Data were presented as mean ± standard deviation (X ± SD) and 95% confidence intervals for mean (lower bound-upper bound).

not prevent a tramadol-induced MDA increase ($p = 1.000$) and tGSH decrease ($p = 0.736$). When the amlodipine and benidipine groups were compared with the tramadol group, a difference was found in terms of the MDA and tGSH values ($p < 0.001$). On the other hand, we found that benidipine better suppressed the MDA increase and the tGSH decrease than amlodipine ($p < 0.001$). The MDA and tGSH values found in the BTRD and the CG groups were similar ($p > 0.05$).

In the heart tissues of the TRD group, an increase in MDA (Figure 1C) and a decrease in tGSH (Figure 1D) were found compared with CG ($p < 0.001$). Lacidipine, amlodipine, and benidipine were able to inhibit a tramadol-induced MDA increase and tGSH decrease in the heart tissue

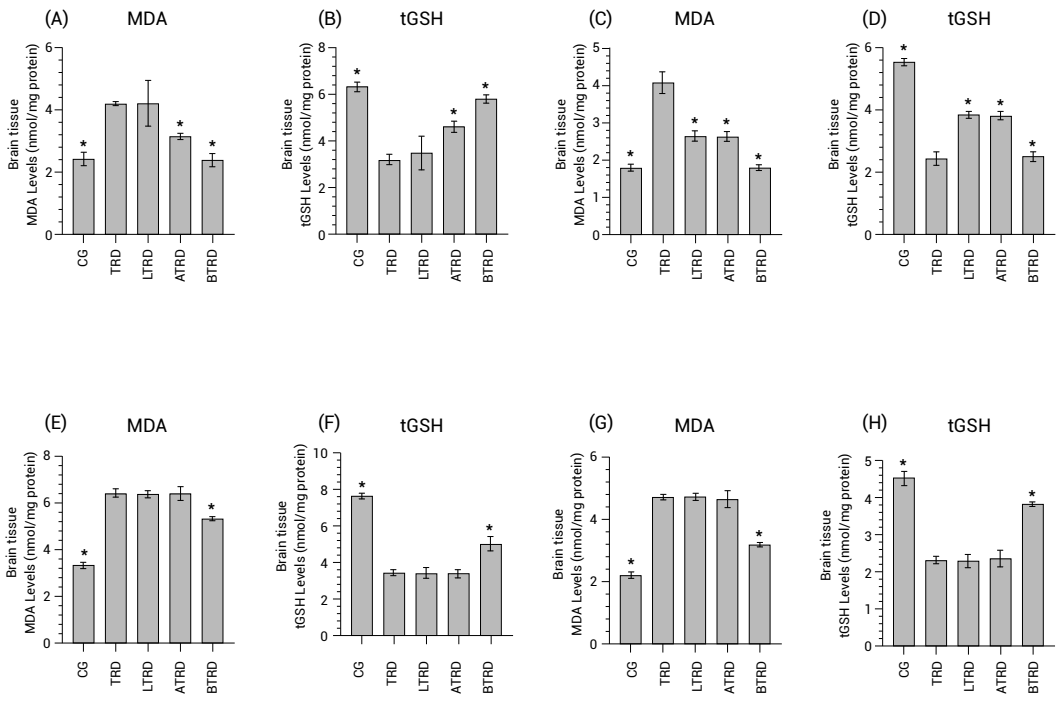

Figure 1. Analysis results of MDA and tGSH data in brain, heart, liver, and kidney tissues obtained from experimental groups.
*; $p < 0.001$ vs TRD.

($p < 0.001$). Heart tissue MDA and tGSH levels were similar in the lacidipine and amlodipine groups ($p > 0.05$). In addition, the MDA values of the BTRD and CG groups were similar ($p = 1.000$).

Likewise, in the liver tissues, the TRD group MDA (Figure 1E) values increased compared with the control animals, and the tGSH (Figure 1F) levels decreased ($p < 0.001$). While lacidipine and amlodipine treatment could not prevent the tramadol-induced liver tissue MDA increase and tGSH decrease ($p > 0.05$), benidipine treatment prevented the deviation of these parameters and reduced oxidative stress ($p < 0.001$).

Tramadol caused an increase in MDA (Figure 1G) and a decrease in tGSH (Figure 1H) in kidney tissue ($p < 0.001$). MDA and tGSH results in TRD, LTRD, and ATRD groups were close to each other ($p > 0.05$). However, benidipine suppressed the tramadol-induced MDA increase and the decrease in tGSH ($p < 0.001$).

Analysis of Troponin (TP I), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Blood Urea Nitrogen (BUN), and Creatinine Levels in Serum

Tramadol administration increased serum TP I (Figure 2A), ALT (Figure 2B), AST (Figure 2C), BUN (Figure 2D), and creatinine (Figure 2E) levels ($p < 0.001$). While lacidipine and amlodipine treatments suppressed an increase in TP I ($p > 0.001$), they could not prevent an increase in ALT, AST, BUN and creatinine ($p > 0.05$). TP I, ALT, AST, BUN, and creatinine levels were lower in the benidipine group compared with the TRD ($p < 0.001$). TP I, BUN, and creatinine values in the BTRD and CG groups were similar ($p > 0.05$).

Information obtained from the literature suggests that Ca2+ channel blockers may potentiate the analgesic effect of tramadol and also alleviate its toxic effects [9, 12, 17].

Our experimental results showed that amlodipine and lacidipine equally enhanced the analgesic effect of tramadol. In addition, benidipine increased the analgesic effect of tramadol the most. Currently, one of the goals of pain management is Ca_2^+ channel blockade (18). In clinical practice, the combination of Ca_2^+ channel blockers and opioids provides more potent analgesia [11]. For instance, N-type Ca_2^+ channels were found in pain-related nerve fibers and the blockade of these channels produces analgesia by inhibiting nerve conduction (19). In the current study, the combination of benidipine-tramadol showed more robust analgesia than the combination of lacidipine-tramadol and amlodipine-tramadol, which may be attributed to the fact that it blocks L, T, and N-type Ca_2^+ channels.

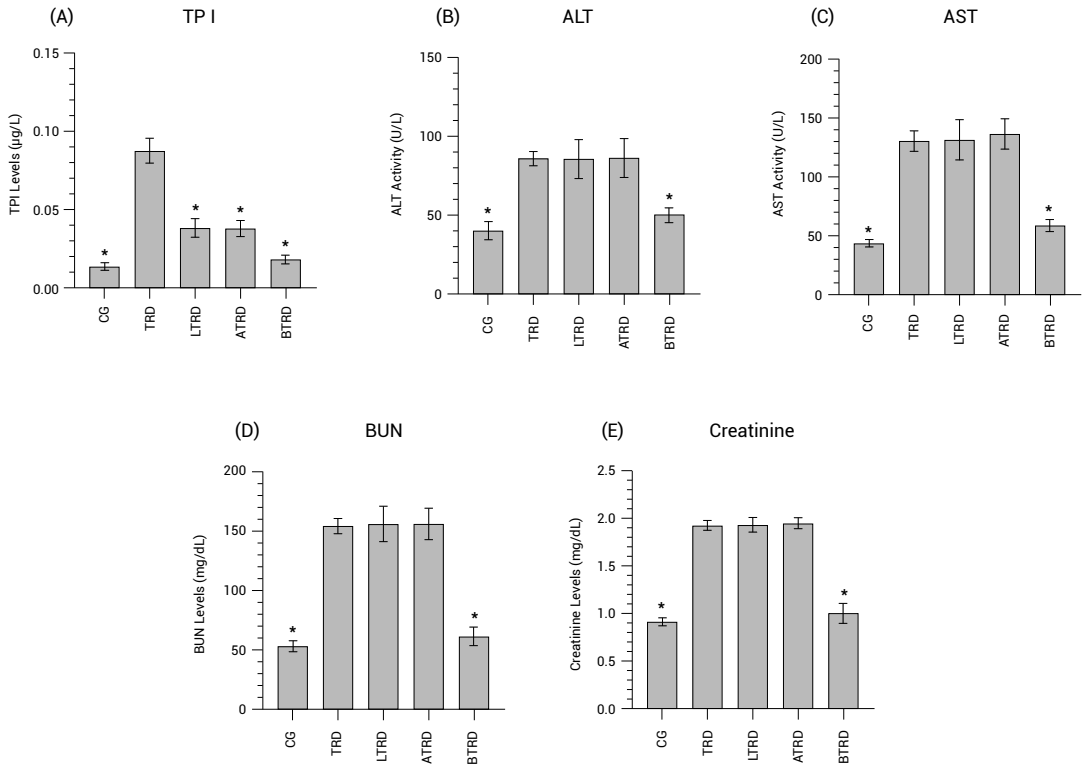

Figure 2. Analysis results of serum TP I, ALT, AST, BUN, and creatinine data of the experimental groups. *; $p < 0.001$ vs TRD.

Recent papers reported tramadol-related toxicity parallel to increased use [9]. Previous animal studies stated that reactive oxygen species (ROS) production and oxidative stress were the main causes of tramadol toxicity [20]. As is known, the overproduction of ROS leads to oxidative stress [21]. Excessive ROS formation increases Ca_2^+ leakage into the cell. High intracellular Ca_2^+ levels also exaggerate the activation of ROS-producing enzymes and free radical formation [22]. Among the many biological targets of oxidative stress, membrane lipids are the most common class of biomolecules [21]. Peroxidation (LPO) of these lipids leads to the formation of highly toxic products such as MDA [23]. Mohamed et al. [20] revealed that MDA levels increased in rat brain tissue treated with tramadol. In addition, they showed that an increase in MDA levels is associated with a decrease in GSH reduced glutathione, which is an essential non-enzymatic antioxidant in mammalian cells. GSH acts directly as an antioxidant to protect cells against ROS and pro-oxidants [24]. Similarly, in the present study, the high MDA and low tGSH levels in the brain tissue of the tramadol-treated group suggest that oxidative stress developed in the brain.

The present study results showed that tramadol increased MDA heart tissue levels and decreased tGSH levels. Tramadol caused heart and aortic damage by inducing oxidative stress and inflammation [25]. Faria et al. reported that tramadol increased MDA levels in rat heart tissue [9]. We also measured the blood serum TP I levels to support the development of oxidative stress in the heart tissue. TP I, a cardiac marker, is increased in oxidative stress as well as in coronary events [26]. An increase in oxidants and decrease in antioxidants in the heart tissue result in high levels of circulating TP I [27]. According to our experimental results, there was an increase in TP I released into the circulation in parallel with increased oxidative stress in heart tissue. Consistent with the present study, Bekr et al. [25] and El-Shamarka et al. [28] revealed serum TP I levels increased in rats treated with tramadol.

The present study showed that the tramadol treatment resulted in high MDA and low tGSH levels in liver tissue in addition to increased ALT and AST levels in the blood serum. Owoade et al. also stated that oxidant levels increased in liver tissues of rats treated with tramadol [29]. In addition, serum liver function markers were found to be significantly higher in patients who used tramadol for a long time compared with the control group who did not use it [8, 30]. A study conducted on rats reported that tramadol increased serum ALT and AST levels [31]. High serum ALT and AST activities are considered indicators of oxidative liver cell damage [32].

In the present study, tramadol also changed the oxidant-antioxidant balance in favor of oxidants in kidney tissue. Similarly, Owoade et al. found that oxidants increased in kidney tissues of rats treated with tramadol for 28 days [29]. Studies reported that tramadol can increase creatinine and BUN levels—indicators of kidney function in humans and animals [30, 31]. While evaluating the effects of oxidative stress on kidney function, studies were based on increased serum BUN and creatinine levels [33]. In the current study, an increase in MDA and decrease in tGSH levels in kidney tissue indicated that tramadol caused oxidative tissue damage and kidney dysfunction.

The present study also evaluated the effects of LABs on tramadol-induced brain, heart, liver, and kidney toxicity. According to our results, lacidipine could not suppress tramadol-induced oxidative stress in brain tissue. The inability of lacidipine to prevent tramadol-related oxidative stress in brain tissue may be due to its selective blocking of only L-type Ca_2^+ channels. Previous studies have shown the benefit of N-type Ca_2^+ channel blockade in brain damage treatment [34]. Consistently with previous studies, in the present study, amlodipine prevented tramadol-related oxidative stress in brain tissue. However, a study investigating the effects of various Ca_2^+ channel blockers against LPO in rat brain homogenates determined that the antioxidant effect of amlodipine was lower than that of benidipine [35]. Studies reported that T-type Ca_2^+ channels play a role in the maturation and repair of various tissues, such as brain and heart tissues [36]. In line with the literature data, in the present study, benidipine suppressed the increase in MDA and decrease in tGSH in brain tissue more significantly than amlodipine.

In this study, benidipine prevented tramadol-related heart tissue oxidative damage and TPI leakage into the circulation more significantly than lacidipine and amlodipine. Khan et al. reported that lacidipine protects heart tissue from oxidative stress [37]. However, another study showed that the beneficial anti-oxidative effects of drugs that simultaneously block L-, T-, and N-type Ca_2^+ channels are superior to selective L-type blockers [38]. A strong correlation was found between the blockade of T-type Ca_2^+ channels and the prevention of oxidative damage [39]. This information from the literature explains why the cardioprotective effects of benidipine are more pronounced than those of lacidipine and amlodipine.

The present study demonstrated that benidipine was effective against tramadol-induced oxidative

liver injury, while lacidipine and amlodipine were ineffective. We did not encounter any studies that investigated the effect of LABs on tramadol-induced oxidative damage in liver tissue. Hassan et al. determined the beneficial effects of benidipine by suppressing the release of ALT and AST into the serum [40]. Our experimental results are consistent with a previous study showing that benidipine exerts a hepatoprotective effect by suppressing an excessive increase in ALT, AST, and oxidant parameters [41]. The protective effect of benidipine against tramadol-induced oxidative liver damage may be due to the blockade of T-type Ca_2^+ channels in the liver [42]. However, amlodipine was insufficient to prevent an increase in oxidant and liver enzymes with tramadol. Conversely, studies reported that amlodipine increased liver enzymes and caused hepatotoxicity [43]. Contrary to our biochemical findings, it was shown in a previous study that lacidipine suppressed an increase in LPO and liver markers and had a hepatoprotective effect [44].

Based on the current study results, lacidipine and amlodipine could not prevent tramadol-related oxidative kidney damage and dysfunction. However, benidipine significantly suppressed the MDA increase and tGSH decrease and managed to keep serum creatine and BUN levels at normal levels. Perico et al. stated that lacidipine does not have a protective effect on the kidneys; conversely, it may cause damage [45]. While T- and N-type Ca_2^+ channels are found in both afferent and efferent arterioles in the kidney, L-type is found only in afferent arterioles. Therefore, L-type channel blockade may cause kidney damage by increasing intraglomerular pressure [44]. Kocaturk et al. also showed that benidipine attenuated bevacizumab-induced oxidative kidney damage and had protective effects on kidney function and structure [17]. According to our experimental results, the fact that creatinine and BUN levels were high in the lacidipine and amlodipine groups and low in the benidipine group suggests that T-type channel blockade is effective in renoprotection.

CONCLUSIONS

In conclusion, while the analgesic effect was obtained with tramadol administration, the redox balance in the brain, heart, liver, and kidney tissues was disturbed in favor of oxidants and serum TP I, ALT, AST, BUN, and creatinine levels increased. The addition of Ca_2^+ channel blockers to tramadol treatment increased the analgesic activity and showed benidipine, amlodipine, and lacidipine in potency, respectively. While lacidipine did not protect brain tissue, amlodipine showed a mild protective effect, while benidipine provided a significant protective effect. LABs were effective in terms of the cardioprotective effects. Benidipine was more effective than lacidipine and amlodipine at protecting against tramadol-related heart damage. In addition, benidipine was able to protect liver and kidney tissues against oxidative damage of tramadol, but lacidipine and amlodipine did not. The protective effect of benidipine on the brain, liver, and kidneys compared with other CCBs may be due to blocking the L-type, N-, and T-type Ca_2^+ channels. Based on these findings, benidipine co-treatment with tramadol is a better option than lacidipine and amlodipine in achieving minimal toxic effects and maximum analgesia.

Authors' Contributions

Conceptualization, R.M. and H.S.; Methodology, M.Y.; Software, R.M.; Validation, M.Y. and D.A.; Formal Analysis, S.B.; Investigation, B.S.; Resources, H.S.; Data Curation, R.C.; Writing – Original Draft Preparation, M.Y.; Writing – Review & Editing, T.A.C.; Visualization, S.B.; Supervision, B.S.; Project Administration, H.S.; Funding Acquisition: all authors.

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