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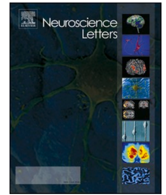

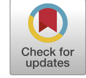

# Effect of captopril, an angiotensin-converting enzyme inhibitor, on morphine analgesia and tolerance in rats, and elucidating the inflammation and endoplasmic reticulum stress pathway in this effect

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ABSTRACT

The purpose of current study was to examine the possible involvement of captopril, an angiotensin-converting enzyme inhibitor, on nociception, morphine analgesia and morphine tolerance development involving inflammation and ER-stress pathways in rats. In this study, thirty-six male Wistar rats were used. Animals were divided into six groups: Saline, 50 mg/kg captopril, 5 mg/kg morphine, morphine + captopril, morphine tolerance and morphine tolerance + captopril. The resulting analgesic effect was measured with hot plate and tail flick analgesia tests. The dorsal root ganglions (DRG) tissues were collected for inflammation parameters, endoplasmic reticulum (ER) stress and apoptosis proteins by using ELISA. Captopril showed anti-nociceptive effect when given alone ($p < 0.05$ to $p < 0.01$). In addition, captopril increased the analgesic effect of morphine ($p < 0.05$ to $p < 0.001$) and also decreased the tolerance to morphine at a significant level ($p < 0.05$ to $p < 0.001$). However, it decreased inflammation and ER-stress when applied with single-dose morphine and tolerance induction ($p < 0.001$). Moreover, captopril decreased apoptosis proteins after tolerance development ($p < 0.001$). In conclusion, captopril has antinociceptive properties, increasing analgesic effect of morphine, and preventing tolerance development. These effects may occur by suppressing inflammation and ER-stress pathways.

## 1. Introduction

Morphine is an opiate receptor agonist and analgesic routinely administered in cases of strong and chronic pain in clinics. The duration of morphine's effect is reduced by the development of tolerance to its antinociceptive properties. Despite many studies that have been conducted to examine the development of opioid tolerance, it is still unclear as to exactly what causes this effect [1,2].

The renin-angiotensin system (RAS) was defined as a circulating humoral system. Evidence show that the brain can synthesize angiotensin peptides. All renin-angiotensin system components and enzymes needed for angiotensin peptide formation and deactivation are present in the brain [3]. Angiotensin I is cleaved from angiotensinogen enzymatically by renin, and then converted into angiotensin II (Ang II) by angiotensin-converting enzyme (ACE) [4]. Angiotensinogen exists locally in the brain, and it is considered that it is a part of the local renin-angiotensin system [5]. It was suggested that this peptide had a neurotransmitter role in central nervous system based on the existence of Ang II-immunoreactive neurons in distinct brain areas (together with the mesolimbic system) [6].

Several previous studies reported that Ang II might interact with the opioid system. In some other studies, it was demonstrated that central Ang II antagonized opioid-induced analgesia [7]. In mice, after intracerebroventricular (i.c.v) Ang II, it has a dose-dependent antinociceptive effect in acetic acid-induced abdominal writhing test [8]. On the other hand, it was suggested that these effects occurred via an opioid mechanism and activation of AT1 receptor [9], which proposes that Ang II participates in the transmission of nociceptive information and its interaction with opioid receptors [9]. After intracerebroventricular Ang II, antinociceptive effect occur, which might be blocked with naloxone pretreatment [9].

Some previous studies reported alteration in the brain ACE activity [10,11]. It was also reported based on evidence that ACE inhibitors (i.e. captopril) decreased endogenous opioid degradation, increasing the opioid level in the brain [12]. However, the mechanisms of captopril in nociception, morphine analgesia and tolerance development are still not

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clear. The current study aimed to examine the possible involvement of captopril on nociception, morphine analgesia and morphine tolerance development involving inflammation and ER-stress pathways in rats.

## 2. Materials and methods

### 2.1. Animals

Wistar Albino rats (230−250 g, n = 6 for each group, 36 rats) were obtained from Animal Center Laboratory of Cumhuriyet University (Sivas, Turkey), and were kept in standard conditions: 12-h light and dark cycle (lights on at 08:00 A.M.) with *ad libitum* food and water at constant temperature (22 ± 2 °C). All the experiments were performed between 09:00 and 17:00. The animals were handled and the procedures were carried out in line with National Institute of Health Guidelines of "Principles of Animal Laboratory Care". Sivas Cumhuriyet University Animal Ethics Committee approved the experimental protocols (Approval number: 65202830-050.04.04-369).

### 2.2. Drugs

The morphine sulfate (Sivas Cumhuriyet University Hospital, Turkey) and captopril (Sigma-Aldrich Co., St Louis, MO, USA) were dissolved in saline solution. Fresh drugs were dissolved on trial days. Morphine (5 mg/kg) was administered subcutaneously (s.c.) and captopril (50 mg/kg) intraperitoneally (i.p.) before analgesia tests.

### 2.3. Experimental protocols

Captopril and morphine's analgesic effects were evaluated at 30-min intervals (30, 60, and 90, minutes) using tail-flick and hot-plate antinociception tests. The animals were separated into six groups: Saline (S), 50 mg/kg captopril (C), 5 mg/kg morphine (M), C + M, morphine tolerance (MT), and C + MT. Saline and captopril were administered i.p. and morphine was administered s.c. at the indicated doses (volume of administration, 1 ml/kg). After analgesic tests, the animals were sacrificed by decapitation. The dorsal root ganglions (DRG) tissue (T12-L5 levels) obtained from the animals underwent assessments (Fig. 1). Before starting the experiment, three doses of captopril (25, 50, and 100 mg/kg) were chosen to evaluate the antinociceptive activity in the rats as a preliminary trial (n = 3). It was observed that there was no analgesic response at the dose of 25 mg/kg, similar to the previous study [13]. However, there were antinociceptive responses at the doses of 50 and 100 mg/kg. On the other hand, at the dose of 100 mg/kg, some adverse effects, such as lethargy and tachypnea, were observed. Therefore, 50 mg/kg was chosen as the optimal dose.

### 2.4. Antinociception tests

#### 2.4.1. Tail-flick test

Thermal nociception measurement was made with a standard tail flick device (May TF 0703 Tail-flick Unit, Commat, Turkey). The radiant heat source was focused on the distal portion of the tail at a distance of 3 cm in each measurement after the administration of saline or study drugs. Tail-flick latencies (TFL) were measured once the saline or drugs were administered. The cut-off latency time was adjusted to 15 s to prevent tissue injury. The hyperalgesic response in tail-withdrawal test was associated with central pain mechanisms [1,2].

#### 2.4.2. Hot-plate test

The antinociceptive reaction on hot-plate is thought to stem from central and peripheral mechanisms together [1,2]. Animals were placed

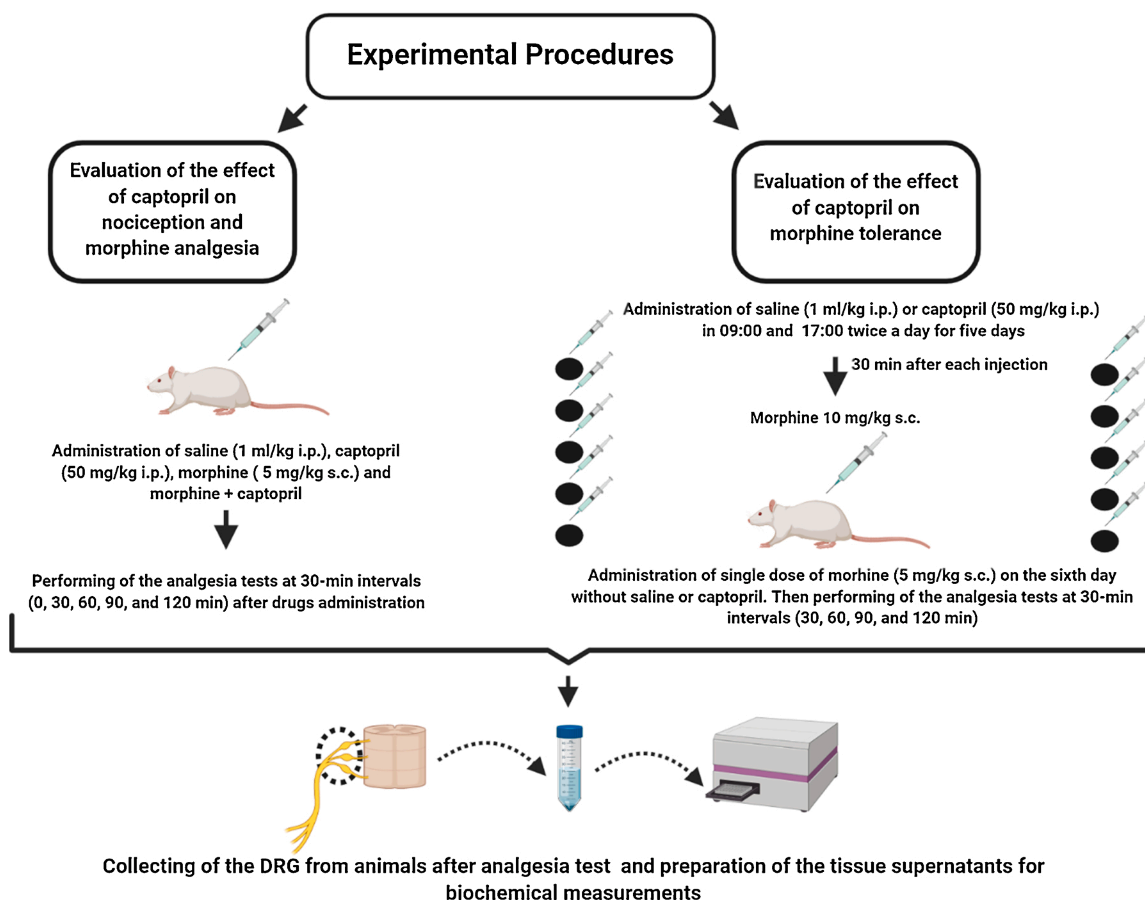


**Fig. 1.** The experimental procedures of study (created by BioRender).

on hot-plate one-by-one (May AHP 0603 Analgesic Hot-plate Commat, Turkey) at $54 \pm 3$ °C. The lag until the first paw-licking or jump reaction to avoid heat was recorded as pain threshold indicator. The cut-off time was 30 s to prevent damage to paws.

### *2.5. Morphine tolerance induction*

The method used to induce morphine tolerance was described in previous studies [14–16]. The rats in MT groups were selected randomly and treated s.c. with 10 mg/kg morphine twice a day (09:00 and 17:00) for five days for morphine tolerance induction. In the rats in C + MT groups, morphine (10 mg/kg) was applied 30 min after each captopril injection for five days to determine the impacts of captopril (50 mg/kg, i.p.) on morphine tolerance. The optimal analgesic morphine dose (5 mg/kg, s.c.) was given on the sixth day without saline or captopril to evaluate the degree of tolerance. The tail-flick and hot-plate tests were measured at 30-min intervals (0, 30, 60, and 90 min).

### *2.6. DRG tissue homogenate preparation*

The DRG tissue samples of the animals in cold phosphate buffer saline solution (pH:7.4) were homogenized using a mechanical homogenizer (Analytik Jena speedmill plus, Jena, Germany), and were then centrifuged at 4000 rpm for 10 min at a temperature of 4 °C [17]. Then, the supernatants were obtained and stored in −80 °C until biochemical analysis. Bradford protein assay kit (Merck, Germany) was used to determine the total protein levels in the samples [18].

### *2.7. Measurement of TNF-α, IL-1 β, NF-kB, GRP78, ATF-4, CHOP, caspase-3 and Bcl-2*

The levels of TNF-α, IL-1 β, NF-kB, GRP78, ATF-4, CHOP, caspase-3 and Bcl-2 from DRG supernatants were measured using rat ELISA commercial kits (YL Biont, Shanghai, China). The operation protocols were in line with the instructions of the manufacturer. In brief, standard and tissue samples were added in plate, and were incubated around 60 min at 37 °C. After the washing step, staining solutions were added, and were incubated about 15 min at 37 °C. Stop solution was added and read at 450 nm. Standard curves were plotted to determine the value of samples. The variation coefficients in and between plates were lower than 10%.

### *2.8. Analgesic tests data analysis*

To calculate the maximum antinociceptive effect percentage (% MPE), tail-flick and hot-plate lags (as seconds) were converted into antinociceptive effectiveness percentage with the following equation:

$$\% \text{ MPE} = [(\text{Post drug latency} - \text{Baseline latency}) / (\text{Cutoff value} - \text{Baseline latency})] \times 100.$$

### *2.9. Statistical analysis*

The results are given as mean ± SEM (standard error of the mean). The antinociceptive effect was measured, and mean % MPEs were calculated. Analysis of Variance (ANOVA) and post-hoc Tukey test were used in analyzing the data. The significance value was taken as $p < 0.05$.

## 3. Results

### *3.1. The effect of captopril on nociception, morphine analgesia and morphine tolerance*

To evaluate the analgesic response of captopril, analgesia tests were applied for 90 min at 30-minute intervals at 50 mg/kg captopril dose. Captopril showed anti-nociceptive effects in comparison with the saline group at 30, 60, and 90 min in both tail flick test ($p < 0.05$; Fig. 2A) and hot plate test ($p < 0.01$ to $p < 0.001$; Fig. 2B).

The findings demonstrated that captopril significantly increased the antinociceptive effect of morphine in tail-flick test at 60 and 90 min ($p < 0.05$; Fig. 2A) and hot plate test at 30, 60, and 90 min ($p < 0.05$ to $p < 0.001$; Fig. 2B) in comparison with morphine group.

The morphine group's % MPE value was statistically higher than the morphine-tolerance group in both the tail-flick test ($p < 0.01$ to $p < 0.001$; Fig. 2A) and hot plate test ($p < 0.05$ to $p < 0.001$; Fig. 2B). Captopril with morphine tolerance induction produced a significantly decreased morphine tolerance development in the tail-flick test ($p < 0.05$ to $p < 0.001$; Fig. 2A) and hot plate test ($p < 0.05$; Fig. 2B).

### *3.2. The effect of captopril on inflammation parameters (TNF-α, IL-1 β and NF-kB levels) in morphine analgesia and tolerance in DRG*

The single dose morphine administration significantly increased TNF-α, IL-1 β and NF-kB levels in DRG compared to saline ($p < 0.001$; Fig. 3A,B and C). However, captopril reduced TNF-α, IL-1 β and NF-kB levels in DRG, when combined with morphine compared to single morphine ($p < 0.001$; Fig. 3A,B and C). Moreover, morphine tolerance raised TNF-α, IL-1 β and NF-kB levels in DRG compared to both the saline ($p < 0.001$; Fig. 3A,B and C) and morphine group ($p < 0.001$; Fig. 3A,B and C). Captopril reduced TNF-α, IL-1 β and NF-kB levels in DRG together with tolerance induction when compared to only morphine tolerance group ($p < 0.001$; Fig. 3A,B and C).

### *3.3. The effect of captopril on ER-stress proteins (GRP78, ATF-4 and CHOP levels) in morphine analgesia and tolerance in DRG*

The single dose morphine raised GRP78, ATF-4 and CHOP levels in DRG compared to saline ($p < 0.001$; Fig. 4A,B and C). However, captopril reduced GRP78, ATF-4 and CHOP levels in DRG, when combined with morphine compared to single morphine ($p < 0.001$; Fig. 4A,B and C). In addition, morphine tolerance increased GRP78, ATF-4 and CHOP levels in DRG compared to saline ($p < 0.001$; Fig. 4A,B and C) and single morphine group ($p < 0.05$ to $p < 0.001$; Fig. 4A,B and C). On the other hand, captopril reduced GRP78, ATF-4 and CHOP levels in DRG with tolerance induction compared to morphine tolerance group ($p < 0.001$; Fig. 4A,B and C).

### *3.4. The effect of captopril on apoptosis (caspase-3 and bcl-2 levels) in morphine analgesia and tolerance in DRG*

The single morphine administration did not change caspase-3 levels in DRG compared to saline ($p > 0.05$; Fig. 5A). However, morphine tolerance raised caspase-3 levels in DRG compared to saline ($p < 0.001$; Fig. 5A) and single dose morphine administration ($p < 0.001$; Fig. 5A). Furthermore, captopril reduced caspase-3 levels in DRG with tolerance development compared to morphine tolerance group ($p < 0.001$; Fig. 5A).

The single dose morphine did not alter bcl-2 levels in DRG compared to saline ($p > 0.05$; Fig. 5B). Nevertheless, morphine tolerance decreased bcl-2 levels in DRG compared to the saline ($p < 0.001$; Fig. 5B) and single morphine group ($p < 0.001$; Fig. 5B). Besides, captopril increased bcl-2 levels in DRG with tolerance induction compared to morphine tolerance group ($p < 0.001$; Fig. 5B).

## 4. Discussion

In the study, the impacts of captopril on nociception, morphine analgesia, tolerance development and possible mechanisms including inflammation and ER stress pathways were investigated. Captopril showed an antinociceptive effect alone and increased the morphine

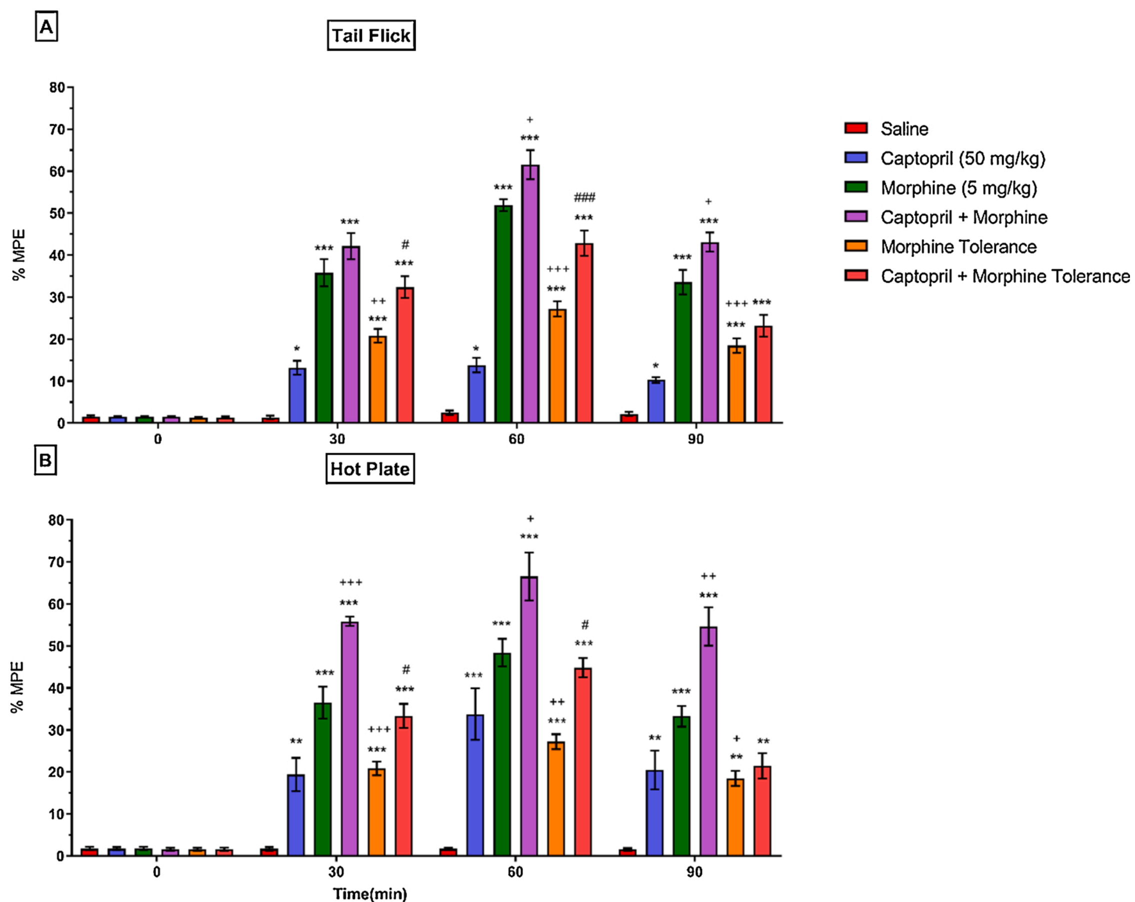


**Fig. 2.** The effect of captopril on nociception, morphine analgesia and morphine tolerance. (A) shows effect of captopril on nociception, morphine analgesia and morphine tolerance in the tail flick test; (B) shows effect of captopril on nociception, morphine analgesia and morphine tolerance in the hot plate test. Values are expressed mean ± SEM of % MPE (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to saline group. $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$ compared to morphine group. $^{\#}p < 0.05$, and $^{\#\#\#}p < 0.001$ compared to morphine tolerance.

analgesic effect. Moreover, it prevented morphine tolerance development. These effects of captopril on morphine analgesia and tolerance development may be due to the suppression of inflammatory and ER stress pathways according to our findings.

In DRG, satellite glial cells activation results in increased proinflammatory cytokine production and release (including TNF-α, IL-1 β, and NF-kB pathway), chemokines, and excitatory amino acids, all of which increase the excitability of nearby neurons [19]. Indeed, these cytokines increase in excitatory related systems such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, and decrease in inhibitory related system such as gamma-aminobutyric acid (GABA) receptors [20,21]. Glia-derived TNF-α and IL-1 β bind to receptors on astrocytes, resulting in the further release of TNF-α and IL-1 β by activating NF-kB pathway [22]. These excessive releasing blocks the analgesic actions of morphine [23]. Acute and chronic administration of morphine activates microglia and astrocytes [24], and the glial activation degree increases with opioid treatment duration [25]. Increased opioid administration and the ensuing glial activation finally lead to the rise in morphine analgesic effect and trigger morphine tolerance development [26]. Moreover, recent experimental studies have shown that morphine stimulates the proinflammatory cytokines by activating toll-like receptor 4 (TLR4) [27]. TLR4 is found on satellite glial cells and astrocytes [28]. It has been claimed that morphine binds to the glycoprotein myeloid differentiation factor-2 (MD-2) on TLR4 [29], and initiates an inflammatory response through nuclear factor kappa B (NFκB) activation and p38 mitogen-activated protein kinase (MAPK) phosphorylation [25]. Activation of the NFκB pathway results in the robust release of proinflammatory cytokines, including TNF-α, IL-1β, and interleukin 6 (IL-6) [29]. Furthermore, TLR4 activation in the spinal cord opposes the acute antinociception effects of morphine and contributes to opioid-induced hyperalgesia [30]. Besides, the systemic administration of TLR4 antagonists prevents the development of morphine tolerance [30]. In the present study, it was indicated that the inflammatory system parameters (TNF-α, IL-1 β, and NF-kB) increased in single-dose morphine and chronic morphine administration to develop tolerance in DRG. However, repeated morphine administration raised inflammatory proteins higher than single-dose morphine in DRG. These findings are consistent with the results of previous studies. Furthermore, captopril alleviated these effects of morphine. Evidence suggests that angiotensin II increases inflammation by binding to its receptors in the nervous system. Therefore, the inhibition of angiotensin II formation suppresses the inflammation [11,31]. Moreover, previous studies reported that angiotensin-converting enzyme inhibitor (SQ 14225, captopril, lisinopril) potentiates the analgesic effect of morphine, and this effect is linked indirectly through the adrenal system [13,32,33]. For this reason, inhibiting ACE and decreasing angiotensin II may be one of the possible mechanisms of captopril on morphine tolerance development.

The endoplasmic reticulum (ER) is a dynamic tubular organelle and plays a role in gluconeogenesis and lipid synthesis. In addition, polypeptides, being newly synthesized, are transferred to the ER lumen, and they obtain their proper three-dimensional conformation in the ER. Changes in intracellular homeostasis cause accumulation of unfolded and misfolded proteins in the ER lumen, which induces ER stress, and unfolded protein response (UPR) is activated to dampen this defect [34].

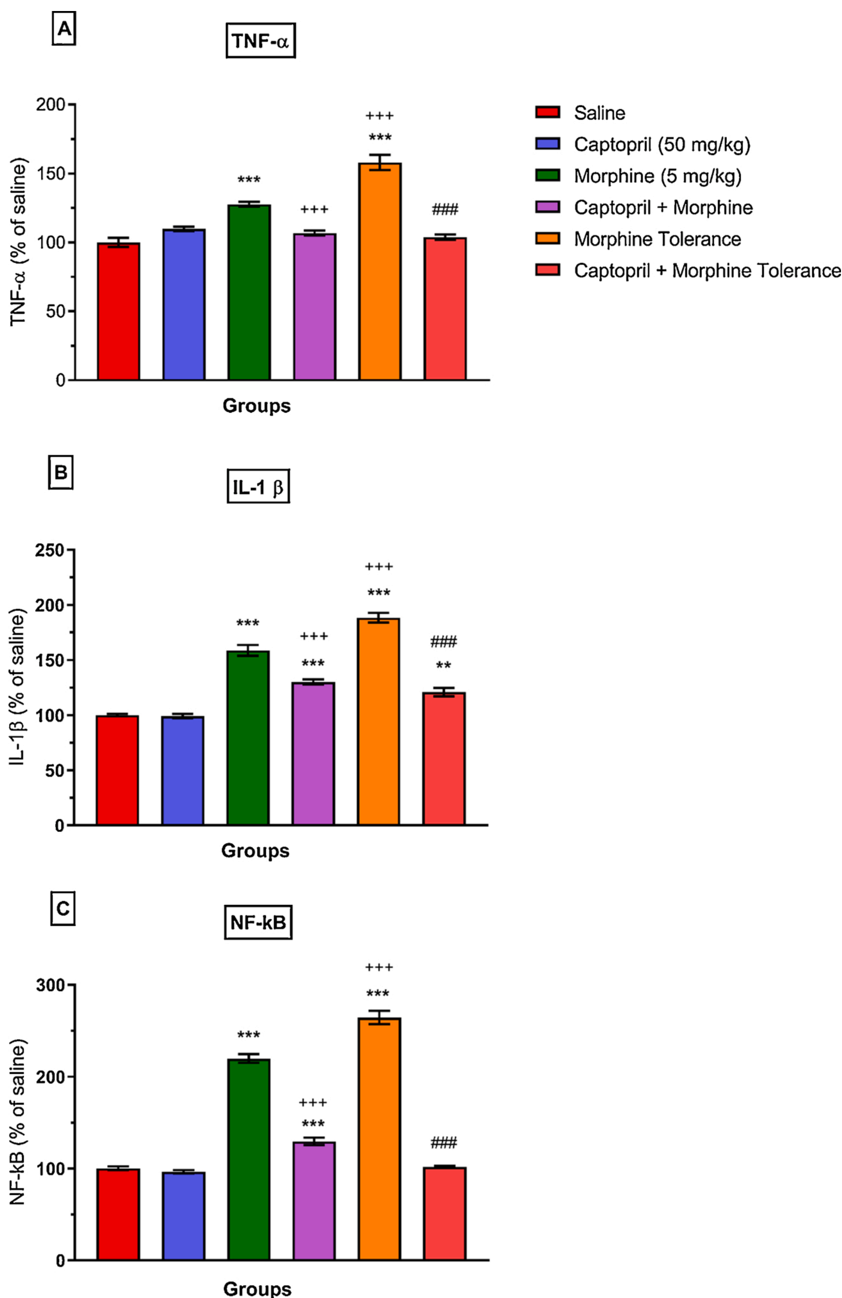


**Fig. 3.** The effect of captopril on inflammation parameters (TNF-α, IL-1 β, and NF-kB) in morphine analgesia and tolerance in DRG. The inflammation parameters were measured on the first day in saline, captopril, morphine, captopril + morphine groups, and on the sixth day in morphine tolerance and captopril + morphine tolerance groups following single-dose morphine administration, after completing the analgesia test using ELISA kits. (A) shows the effect of captopril on TNF-α levels in morphine analgesia and tolerance in DRG; (B) shows the effect of captopril on IL-1 β levels in morphine analgesia and tolerance in DRG. (C) shows the effect of captopril on NF-kB levels in morphine analgesia and tolerance in DRG. Values are expressed as the means ± SEM of % MPE (n = 6). $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to saline group. $^{+++}p < 0.001$ compared to morphine group. $^{\#\#\#}p < 0.001$ compared to morphine tolerance.

In eukaryotic cells, the UPR has three-prolonged signal-transduction pathways, including inositol-requiring kinase 1α (IRE1), pancreatic ER elF2α kinase (PERK), and activated transcription factor 6 (ATF6) [35]. Recent studies indicated that the three UPR signal transduction pathways IRE1, PERK, and ATF6 in the peripheral nervous system and spinal cord were involved in neuropathic pain [36]. GRP78, also called BIP, is a master regulator of the UPR, reducing ER stress levels and apoptosis due to enhancing the cellular folding capacity. GRP78 binds to unfolded and misfolded proteins, and activates the PERK pathway, including ATF4 protein. The ATF4 protein induces CHOP levels by increasing eukaryotic initiation factor 2 (eIF2) phosphorylation. At the end of signal transmission, CHOP stimulates the beginning of apoptosis in the neuronal cells [37]. The neuronal loss in the DRG gives rise to desensitization to morphine [38]. Besides, it has been found that ER stress increases in peripheral neurons in rats with diabetic nephropathy by activating PERK and ATF6 pathways [39]. Furthermore, some recent studies have reported that ER stress, especially the PERK pathway, is related to morphine analgesia and tolerance [14,40]. It has also been demonstrated that ER stress pathway activates in the spinal cord after chronic morphine administration that is similar to tolerance [14,40]. ER stress, particularly the IRE1 pathway, is also closely associated with inflammatory responses by activating the NF-kB pathway [41]. On the other hand, it was found that angiotensin II causes activation of ER stress preventing the formation of angiotensin II and reducing ER stress [42]. In the present study, it was found that the ER stress proteins (GRP78, ATF-4, and CHOP) increase in single-dose morphine and repeated morphine administration to develop tolerance in DRG. However, repeated morphine administration raised ER stress proteins higher than single-dose morphine in DRG. At the same time, captopril reversed these effects of morphine. Inhibition of ACE by captopril caused a decrease in the formation of angiotensin II, which prevented ER stress activation in DRG. It may be another possible mechanism of the positive effect of captopril on morphine tolerance development.

Previous studies reported that morphine tolerance causes neuronal

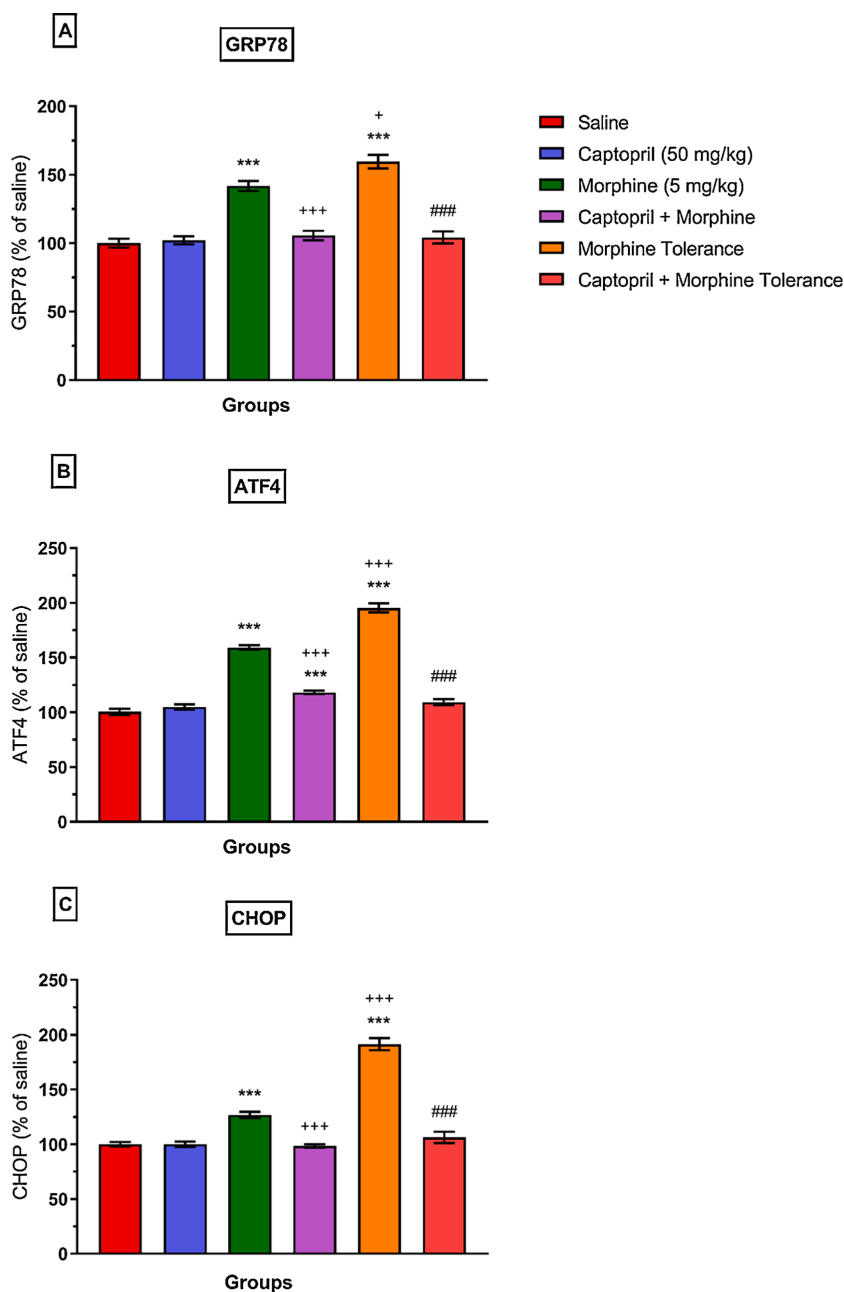


**Fig. 4.** The effect of captopril on ER-stress proteins (GRP78, ATF-4, and CHOP levels) in morphine analgesia and tolerance in DRG. The ER-stress proteins were measured on the first day in the saline, captopril, morphine, captopril + morphine groups, and on the sixth day in morphine tolerance and captopril + morphine tolerance groups following single-dose morphine administration, after completing the analgesia test using ELISA kits. (A) shows the effect of captopril on GRP78 levels in morphine analgesia and tolerance in DRG; (B) shows the effect of captopril on ATF-4 levels in morphine analgesia and tolerance in DRG; (C) shows the effect of captopril on CHOP levels in morphine analgesia and tolerance in DRG. Values are expressed as the means ± SEM of % MPE (n = 6). $^{***}p < 0.001$ compared to saline group. $^{+}p < 0.05$, and $^{+++}p < 0.001$ compared to morphine group. $^{\#\#\#}p < 0.001$ compared to morphine tolerance.

apoptosis by activating some cellular mechanisms such as inflammation and ER stress [38,43]. Our findings have shown that morphine tolerance induced apoptosis in DRG by increasing caspase-3 and decreasing Bcl-2 in DRG. It was consistent with the results of previous studies in the literature [38,43]. Although single-dose morphine increased inflammatory and ER stress proteins expression in DRG, single-dose morphine did not induce apoptosis. The explanation may be that there is a threshold for apoptosis in DRG after morphine administration. Otherwise, captopril decreased apoptosis tolerance by decreasing caspase-3 and increasing Bcl-2 in DRG. The suppressive effect of captopril on inflammation and also ER stress may shed light on the anti-apoptotic effect of captopril after morphine tolerance.

The study has potential limitations. The dose of 10 mg/kg twice a day for 5 days is considered to be adequate to create morphine tolerance development. However, it will be more effective to reveal the development of tolerance by making analgesic measurements after injection every day. However, this situation was not clear in the present study. The inflammation parameters, ER-stress proteins, and apoptosis factors were measured only with the ELISA method in the present study. The parameters measured with only ELISA method should be supported with additional methods, such as western blot and immunochemistry. This situation is another limitation of the present study.

## 5. Conclusion

The findings of this study showed that captopril has antinociceptive properties, increases morphine analgesic effect, and also prevents tolerance development against repeated administration of morphine, possibly through inhibition of inflammation and ER stress in DRG. Thus, captopril might be a potential therapeutic agent in morphine tolerance development management in the clinic.

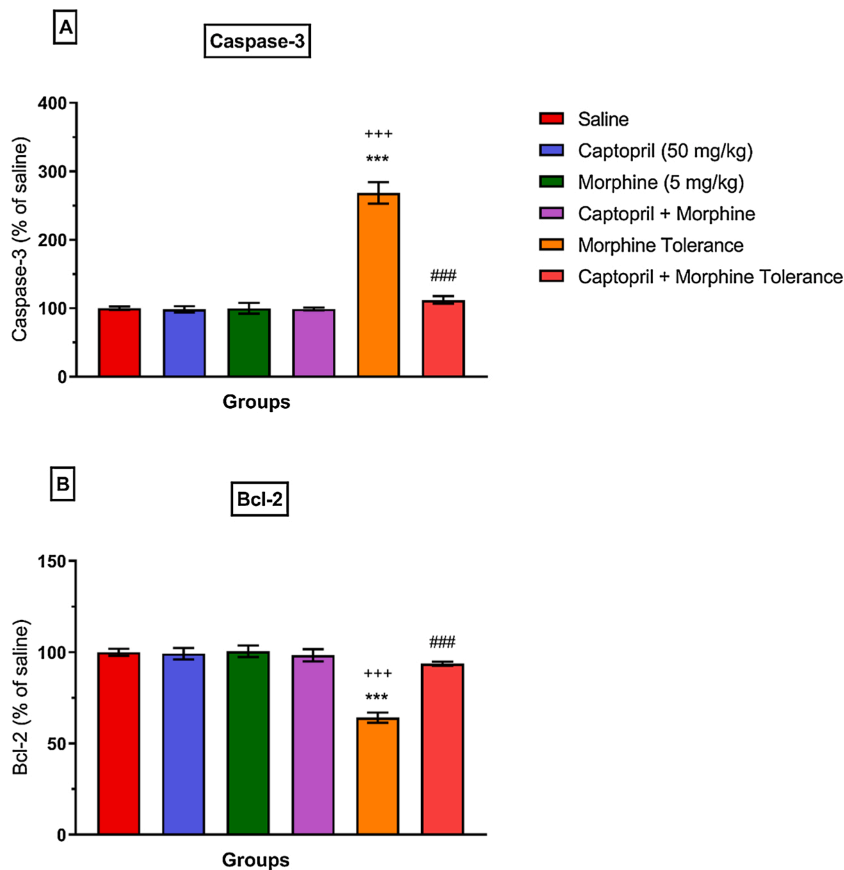


**Fig. 5.** The effect of captopril on apoptosis (caspase-3 and Bcl-2 levels) in morphine analgesia and tolerance in DRG. The apoptosis factors were measured on the first day in the saline, captopril, morphine, captopril + morphine groups, and on the sixth day in morphine tolerance and captopril + morphine tolerance groups following single-dose morphine administration, after completing the analgesia test using ELISA kits. (A) shows the effect of captopril on caspase-3 levels in morphine analgesia and tolerance in DRG; (B) shows the effect of captopril on Bcl-2 levels in morphine analgesia and tolerance in DRG. Values are expressed as the means ± SEM of % MPE (n = 6). ***$p < 0.001$ compared to saline group. $^{+++}p < 0.001$ compared to morphine group. $^{\#\#\#}p < 0.001$ compared to morphine tolerance.

## CRediT authorship contribution statement

**Ahmet Sevki Taskiran:** Investigation, Conceptualization, Methodology, Software, Writing - original draft. **Onur Avci:** Data curation, Formal analysis, Writing - review & editing.

## Declaration of Competing Interest

The authors have no conflict of interest to disclose.

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