



# Aspirin attenuates morphine antinociceptive tolerance in rats with diabetic neuropathy by inhibiting apoptosis in the dorsal root ganglia

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## Abstract

Morphine is a drug used in chronic pain such as diabetic neuropathy, but the development of tolerance to its antinociceptive effect is an important clinical problem. Aspirin is an analgesic and antiapoptotic drug used in combination with morphine as an adjuvant in diabetic neuropathy. Our aim in this study was to investigate the effects of aspirin on morphine-induced neuronal apoptosis and analgesic tolerance in rats with diabetic neuropathy. The antinociceptive effects of aspirin (50 mg/kg) and morphine (5 mg/kg) were evaluated by thermal pain tests. Streptozotocin (65 mg/kg) was injected intraperitoneally to induce diabetic neuropathy. To evaluate apoptosis, ELISA kits were used to measure caspase-3, Bax and Bcl-2 levels. Apoptotic cells were detected histologically by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. Study results indicate that prior administration of aspirin to diabetic rats significantly increased the antinociceptive efficacy of morphine compared to morphine alone. Thermal pain tests showed that aspirin significantly reduced morphine tolerance in rats with diabetic neuropathy. Biochemical analysis revealed that aspirin significantly decreased the levels of pro-apoptotic proteins, caspase-3 and Bax, while increasing the anti-apoptotic Bcl-2 in DRG neurons. Semiquantitative scoring demonstrated that aspirin provided a significant reduction in apoptotic cell counts in diabetic rats. In conclusion, these data suggested that aspirin attenuated morphine antinociceptive tolerance through anti-apoptotic activity in diabetic rat DRG neurons.

**Keywords** Aspirin · Diabetic neuropathy · Apoptosis · Morphine tolerance · Dorsal root ganglion

## Introduction

Neuropathic pain is one of the common complications of diabetes and affects more than 50% of patients (Castany et al. 2016; Park et al. 2004). Decreased efficacy of opioid analgesic drugs such as morphine administered in patients with diabetic neuropathy is an important clinical problem (Cooper et al. 2017; Gupta et al. 2021). It is necessary to use high doses of morphine to prevent neuropathic pain, but this

produces numerous side effects such as sedation, respiratory depression and morphine tolerance (Boorman and Keay 2021). This reduction in the antinociceptive efficacy of opioids has also been demonstrated in diabetic animal models administered  $\mu$ -opioid receptor (MOR) agonists (Nielsen et al. 2007).

Different mechanisms have been proposed for morphine tolerance. These include nitric oxide (NO)-cGMP (Ozdemir et al. 2011) and mammalian target of rapamycin (mTOR) signaling pathways (Shirooie et al. 2019), glial cell and N-methyl-D-aspartate receptor (NMDAR) activation (Zhang et al. 2021), opioid receptor desensitization (Jokinen et al. 2018), and ghrelin receptor inactivation (Baser et al. 2021). Recently, morphine-induced neuronal apoptosis constitutes one of the possible mechanisms for morphine tolerance (Mao et al. 2002). It has been reported that long-term morphine administration increases neuronal apoptosis and this leads to morphine tolerance (Mao et al. 2002; Hassanzadeh et al. 2011; Shafie et al. 2015). Consistent with this,

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prolonged administration of morphine up-regulated the proapoptotic proteins Bax and caspase-3 while down-regulated the anti-apoptotic Bcl-2 in the dorsal horn of the spinal cord (Mao et al. 2002).

It has been shown in different studies that the antinociceptive effectiveness of MOR agonists is decreased in painful diabetic neuropathy (Ohsawa et al. 2000; Chen and Pan 2003). The increase in blood glucose levels in diabetes causes significant changes in the antinociceptive processes of the endogenous opioid system (Forman et al. 1985). These changes in the opioid system with hyperglycemia reduce to the analgesic effect of morphine in diabetic animals (Mahajan et al. 1997). This effect has been reported to be associated with loss of MOR or impaired G-protein coupling to MOR (Chen and Pan 2003; Mousa et al. 2013). However, some evidence has shown that microglia cell activation in the spinal cord contributes to the attenuated antinociceptive effect produced by morphine in diabetic pain. Consistent with this, administration of minocycline, a microglia inhibitor, increased the antinociceptive activity of morphine in mice with diabetic neuropathy (Zychowska et al. 2013).

Clinical management of opioid tolerance includes administration of opioids in combination with adjuvants (Freye et al. 2007; Paul et al. 2021). In patients requiring long-term analgesic therapy, adjuvants such as gabapentin, pregabalin, ibuprofen, and aspirin (acetylsalicylic acid, ASA) are combined with opioid analgesics (Paul et al. 2021). Aspirin is a cyclooxygenase (COX) inhibitor used to reduce pain, fever, and inflammation (Khan and Lee 2011). Aspirin exerts cytoprotective effects on many diseases, alleviates tissue inflammation, prevents astrocyte activation by activating nuclear factor-erythroid factor 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway, and inhibits neuronal apoptosis after spinal cord injury (Jian et al. 2016). Aspirin treatment has been shown to significantly attenuate the expression of pro-apoptotic proteins cleaved caspase-3, caspase-3 and Bax in the spinal cord (Wei et al. 2018). In addition, aspirin represents anti-apoptotic activity by blocking caspase-3 through Akt activation (Feng et al. 2011) and protects cells from apoptosis by preventing toxic stimuli (Grilli et al. 1996; Kutuk and Basaga 2003). Although aspirin is used together with morphine as an adjuvant in diabetic neuropathy, its effects on morphine antinociceptive activity and tolerance have not been fully elucidated.

In the light of all this information, we aim to investigate the effects of aspirin on morphine-induced apoptosis and tolerance in diabetic rats by using thermal antinociceptive tests, biochemical and histological methods.

## Materials and methods

### Animals

In this study, 88 male adult Wistar rats 9–10 weeks old (225–250 g weight) purchased from Cumhuriyet University Experimental Animal Center (Sivas, Turkey) were used. The experimental protocols were approved by the Animal Ethics Committee of Cumhuriyet University (Ethic no: 2019/181). The rats were housed under 12-h/12\_h light/dark conditions (lights on at 7:00 a.m.) in a room with controlled temperature ( $23 \pm 2$  °C) and a humidity of 50–55%. Animals had free access to food and water and were used after a minimum of 7 days acclimatization to the housing conditions. All experiments were carried out blindly between 9:30 (a.m.) and 4:30 (p.m.). Rats were housed in sterile plexiglass cages (80 × 50 × 50 cm) and in groups of four. The guidelines in the “Manual for the Care and Use of Laboratory Rats” were followed in the housing and administration of the rats (Institute of Laboratory Animal Resources, Commission on Life Sciences 2011).

### Drugs

Acetylsalicylic acid (ASA, Aspirin; 50 mg/kg) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and administered intraperitoneally (i.p.) after dissolving in dimethyl sulfoxide (DMSO). Morphine HCl (10 mg/kg; i.p.) was obtained from Cumhuriyet University Research Hospital (Sivas, Turkey) and dissolved in sterile 0.9% saline. After purchasing streptozotocin (STZ) from Sigma-Aldrich, a fresh solution was produced by dissolving in citrate buffer (pH 4.5) and injected at a dose of 65 mg/kg (i.p.) to induce diabetic neuropathy. Selection of drug doses was based on data obtained from pre-tests and previous studies (Zanjiabadi et al. 2014; Micov et al. 2015).

### Experimental groups

The control (saline) and drug groups rats were randomly selected (no randomization method was used in the selection of animals) and divided into eleven groups of 8 animals in each group ( $n = 8$ ) as follows: Group 1 (Saline, SAL) rats were injected with saline (i.p.) at the same dose as the drug. Group 2 (Diabetes, STZ) rats were administered with a single dose of STZ (65 mg/kg, i.p.). Group 3 (Morphine, MOR) rats were administered 5 mg/kg (i.p.) morphine as a trial dose. Group 4 (Aspirin, ASA) animals received aspirin (50 mg/kg) intraperitoneally. Group 5 (ASA+MOR) aspirin and morphine were administered together to the rats. Group 6 (STZ+MOR) morphine (5 mg/kg) was administered to diabetic rats. Group 7 (ASA+MOR+STZ) aspirin

and morphine were administered to diabetic rats together. Group 8 (MOR Tol) morphine was administered at a cumulative dose for three days in rats. Group 9 (ASA+MOR Tol) aspirin was administered to morphine-tolerant rats. Group 10 (STZ+MOR Tol) morphine tolerance was induced in diabetic rats. Group 11 (ASA+STZ+MOR Tol) aspirin was administered to morphine tolerant diabetic rats. Two of the diabetic rats died during the experiment. In addition, the number of groups (n) was determined as 6 in biochemical and histological measurements. First, diabetes was established after morphine tolerance was induced in several groups of rats. Then, all group animals were subjected to behavioral tests. Finally, DRG tissues were isolated and biochemical and histological analyzes were performed. In this study, no statistical method was used to determine the sample size.

### Experimental procedure

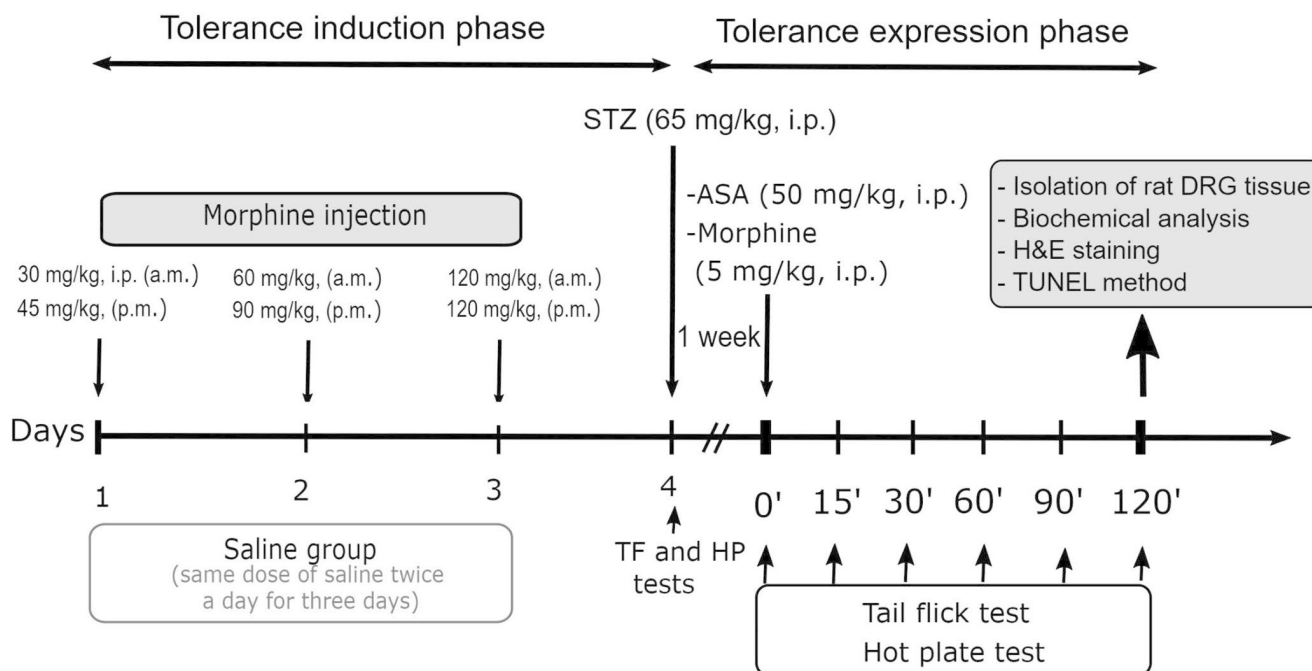
To induce morphine tolerance, morphine was administered at a cumulative dose for three days in rats (Altun et al. 2015). The treatment schedule consisted of twice daily doses of morphine administered at 30 mg/kg (i.p.) (a.m.) and 45 mg/kg (p.m.) on day 1, 60 and 90 mg/kg on day 2, and 120 mg/kg twice on day 3. The development of tolerance to morphine in rats was evaluated by antinociceptive tests on day 4. The experimental protocol and study timeline are shown in Fig. 1. If administration of a test dose (5 mg/kg) of morphine produces a significant reduction in antinociceptive

effect in rats, it is defined as morphine tolerance. Initially, tail flick (TF) and hot plate (HP) thermal antinociceptive tests were performed for each rat to determine basal latency times. In the next step, post-drug latency times for each rat were measured by TF and HP tests at 15, 30, 60, 90 and 120 min after administration of the test dose of morphine. Saline group rats were injected with saline twice a day for three days as in the drug groups.

### Behavioral tests

Thermal antinociceptive tests, TF test (May TF 0703 TF Unit; Commat, Ankara, Turkey) and HP test (May AHP 0603, Analgesic Hot Plate; Commat) were used to measure antinociceptive behaviors in rats. In the thermal TF test, after the drug was injected to the rats, the radiant heat source was applied to the 2 to 2.5 cm distal part of the rat tail and the tail flick latency (TFL) was recorded in seconds. The radiant heat source was calibrated so that the basal TFL occurs in a mean of  $2.5 \pm 0.5$  s in the nociceptive test. Rats with baseline TFL less than 2.0 s or greater than 3.0 s were excluded from subsequent antinociceptive tests. The test cut-off latency was programmed to be 20 s to avoid tissue injury in the rat tail. Responses to the TF test are often associated with central pain mechanisms (Ramabadran et al. 1989; Kanaan et al. 1996).

In the thermal HP test, rats were placed in a test device with a plate at an average temperature of  $54 \pm 0.6$  °C. Paw licking responses to avoid heat or delay in the rat's jumping



**Fig. 1** Flowchart of the experimental procedure. DRG, dorsal root ganglion; ASA, acetylsalicylic acid (aspirin); STZ, streptozotocin; i.p., intraperitoneal; H&E, hematoxylin and eosin

behavior were considered an index of the pain threshold. In this antinociceptive test, the cut-off time was programmed as 30 s to prevent injury to the rat paw. It is stated that antinociceptive responses in HP test result from a combination of peripheral and central mechanisms of pain (Kanaan et al. 1996).

### Induction of diabetes

Rats were injected intraperitoneally with a single dose (65 mg/kg) of streptozotocin (STZ) prepared in 0.1 mol/l sodium citrate buffer (pH 4.5) to induce diabetes (Zangiabadi et al. 2014). One week later, blood glucose levels were measured with GolDeal Gluco Prober (Aurum Biomedical Technology, Hsinchu City, Taiwan) in rats that developed diabetic neuropathy. A few drops of blood from the animal's tail vein were poured onto the instrument strip. In the next step, blood glucose levels were read from the instrument monitor. Animals with a blood glucose level of  $\geq 250$  mg/dl were considered diabetic. After diabetes induction, the effect of aspirin on morphine antinociception and tolerance was evaluated in diabetic rats.

### Homogenate preparation of the dorsal root ganglia

After experimentation, animals were anesthetized by injection of 50/20 mg/kg ketamine/xylazine solution to minimize suffering and dorsal root ganglia (DRGs) at spinal levels L3 to S4 were rapidly surgically removed. DRG tissue samples in phosphate buffered saline solution (pH 7.4) were homogenized with a mechanical homogenizer device (Analytik Jena speedmill plus, Jena, Germany). Then, the tissues were centrifuged at 4 °C and 4000 rpm for 10 min. Supernatants formed by centrifugation were stored at -80 °C for predetermined biochemical analysis. Bradford protein assay kit was used to analyze total protein levels in tissue samples (Merck KGaA, Darmstadt, Germany) (Kruger 1994).

### Measurement of bax, caspase-3 and Bcl-2 levels

All commercial rat enzyme-linked immunosorbent assay (ELISA) kits (YL Biont, Shanghai, China) were used to measure Bax, caspase-3 and Bcl-2 levels from DRG tissue samples. Measurements were performed in accordance with the manufacturer's application guide. In summary, DRG tissue samples and standard were added to the plate and incubated at 36.5 °C for approximately 60 min. Staining solutions were added to the washed tissues and incubated for approximately 15 min. After addition of the stop solution, the reading was performed at 450 nm. Standard curves were drawn to determine the value of the samples, and the

coefficients of variation within and between plates were found to be less than 10%.

### Detection of apoptotic cells

Extracted DRG tissues were quickly transferred to a glass petri dish containing a stable fixative solution (4% paraformaldehyde in 0.1 M phosphate-buffered saline, PBS) at 4 °C (Wang et al. 2016). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was used to evaluate apoptosis in DRG tissue. The TUNEL method is based on detecting DNA fragmentation by labeling the 3'-hydroxyl ends of double-stranded DNA breaks produced during apoptosis.

In situ cell death detection kit (fluorescein) was used to assess neuronal apoptosis (Roche Diagnostics GmbH, Mannheim, Germany). The procedures were performed in accordance with the kit manufacturer's application manual. First, DRG tissues were fixed in 10% neutral buffered formalin and then washed for histological examination. In the next stage, the preparations were passed through increasing concentrations of alcohol (70%, 80%, 90% and 100%) and triple xylene series and embedded in paraffin. 3–4  $\mu$ m sagittal and horizontal serial sections were taken from paraffin-embedded neuronal tissue samples with a microtome (Leica SM 2000R, Heidelberg, Germany). Tissue sections left on slides were kept in the oven for 12 h and exposed to chemical deparaffinization in xylol twice. To increase the water content of the tissue, ethanol, which was reduced 2 times, was first passed through distilled water and then through PBS. Then, DRG tissues were incubated in proteinase K solution in a dark and humid environment at 21–37 °C for 30 min. In the next step, neuronal tissues were exposed to PBS 2 times. After applying 50  $\mu$ l of kit label solution to the negative controls, the enzyme solution was mixed with the label solution and incubated. Before applying the resulting mixture to positive controls, for detection of DNA breaks, in DNase 1 recombinant solution (50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 3000 U/ml to 3U/ml in 1 mg/ml BSA) at 20–25 °C was suspended for 15 min. TUNEL mixture solution was applied to the tissues exposed to PBS twice and incubated at 37 °C for 60 min in a dark and humid environment. Each neuronal tissue sample was washed 3 times with PBS-Triton-X-100. Detection of apoptotic cells was examined semi-quantitatively under fluorescence microscopy (Olympus BX51). Photographs of DRG sections were recorded using a microscope (Leica DM2500, Nussloch, Germany). A semi-quantitative scoring system was used to evaluate apoptotic cells visible by TUNEL staining. DRG cells that appeared bright and green in nuclei due to chromatin aggregation were considered apoptotic cells. Apoptotic cell counts were expressed as a percentage and graded. The

grading is as follows: 1, light staining and affects only  $\leq 5\%$  of neurons; 2, staining affecting 5–10% of neurons; 3, staining affecting 10–15% of neurons; 4, staining affecting 15–20% of neurons; 5, staining affecting  $\geq 20\%$  of neurons (Schmeichel et al. 2003).

Samples obtained from DRG sections for hematoxylin-eosin (H&E) staining were first deparaffinized in xylol for 10 min. Tissues were then exposed to 95%, 80%, and 70% alcohol for 10 min, respectively. Neuronal tissue samples were stained with hematoxylin for 5–6 min and then washed with water. Washed tissue samples were immersed in acid alcohol to turn pale blue within a few seconds. In the next step, neuronal tissues were exposed to the eosin solution for 3–4 min. In order to remove excess eosin in the preparation, the tissues were passed through 70%, 80% and 95% absolute alcohol, respectively, and treated with xylol 3 times for 10 min. Synthetic resin was used to bond the preparations extracted from xylol and then the preparation was left to dry.

### Data analysis

The latency times measured by the hot plate test and the tail flick test were calculated as a percentage of the maximum possible effect (% MPE) with the following equation:

$$\% \text{MPE} = [(\text{test latency} - \text{baseline}) / (\text{cutoff} - \text{baseline})] \times 100.$$

### Statistical analysis

The results are presented as the mean  $\pm$  standard error of the mean (S.E.M.). Normal distribution was assessed in accordance with the Shapiro Wilk's test. A paired t-test statistical analysis was used for the means of the two groups. Behavioral and biochemical data were analyzed via two-way repeated-measures analysis of variance (ANOVA) and multiple comparisons determined by Tukey test using SPSS computer program (version 22.0 for windows Chicago, IL, USA). In all groups,  $p < 0.05$  was considered statistically significant.

**Table 1** Body weight and blood glucose levels of STZ-induced diabetic rats

Body weight (g)		Blood glucose (mg/dL)	
Before STZ injection	End experiment	Before STZ injection	End experiment
244.18 $\pm$ 2.56	228.73 $\pm$ 2.47*	125.10 $\pm$ 2.31	389.53 $\pm$ 3.44**

The data are presented as the mean  $\pm$  SEM. \* $p < 0.05$  and \*\* $p < 0.01$  compared to before STZ injection (Paired t-test)

## Results

### Body weight and blood glucose levels in diabetic rats

Before behavioral testing, blood glucose levels and body weights of animals were measured before and after diabetes induction. Body weights of diabetic animals ( $228.73 \pm 2.47$ ;  $n = 8$ ) were significantly decreased compared to pre-STZ administration ( $p < 0.05$ ; Table 1). In addition, blood glucose levels ( $389.53 \pm 3.44$ ) were significantly increased in rats with diabetes ( $p < 0.01$ ). These glucose levels meet the criteria for diabetic rats ( $\geq 250$  mg/dl).

### Antinociceptive effects of morphine on painful diabetic neuropathy

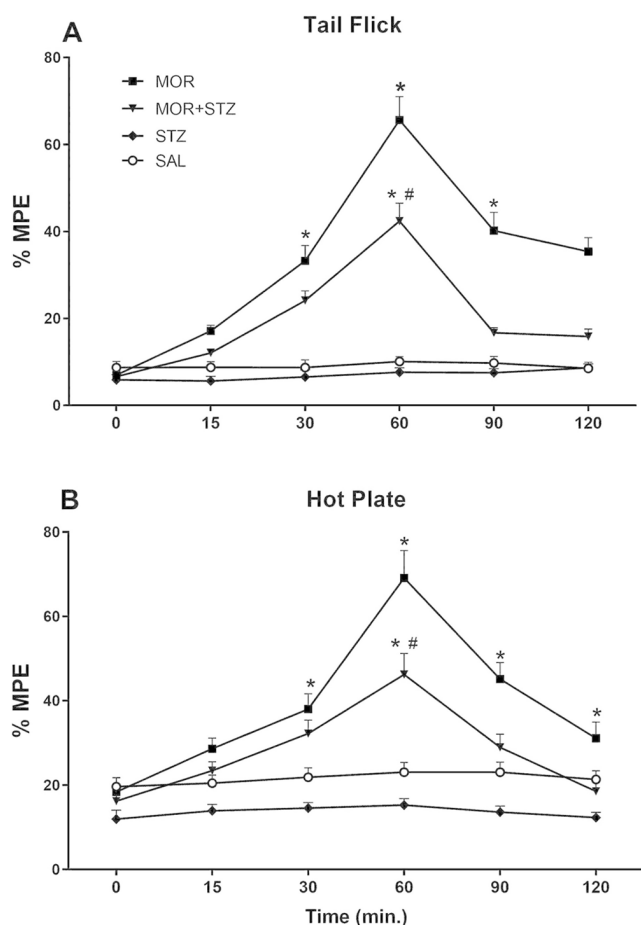
Thermal antinociceptive test results showed that the antinociceptive effect of morphine in the diabetic rats was significantly reduced in both the tail flick ( $F_{3,20} = 15.32$ ;  $p < 0.05$ ) and hot plate ( $F_{3,20} = 13.45$ ;  $p < 0.05$ ) tests compared to the morphine group rats (Fig. 2A and B). However, the antinociceptive effect of morphine was significantly higher in both tail flick ( $F_{3,20} = 17.85$ ) and hot plate ( $F_{3,20} = 21.35$ ) tests compared to the saline group ( $p < 0.01$ ). The maximal antinociceptive effect (% MPE) was observed at 60 min after the administration of morphine (10 mg/kg) in antinociceptive tests of all group rats.

### Effects of aspirin on morphine antinociception in diabetic rats

Co-administration of aspirin (50 mg/kg) with morphine (Fig. 3A and B) showed a significant increase in antinociceptive effect compared to morphine administered alone in diabetic rats ( $p < 0.05$ ;  $F_{4,25} = 18.87$  for TF test and  $F_{4,25} = 19.34$  for HT test). However, maximal analgesic activity was observed in normal rats in which aspirin was combined with morphine in behavioral tests. In addition, these data suggested that aspirin alone has a significant antinociception in both TF and HP tests compared to the saline group rats ( $p < 0.01$ ).

### Effects of aspirin on morphine tolerance

Administration of aspirin to morphine-tolerant rats significantly reduced morphine tolerance (MPE increased) in both TF ( $p < 0.05$ ; Fig. 4A) and HP test experiments ( $p < 0.05$ ; Fig. 4B) compared to morphine tolerant animals ( $F_{4,25} = 23.21$  for TF test;  $F_{4,25} = 25.32$  for HP test). Similarly, administration of aspirin to diabetic rats significantly



**Fig. 2** Antinociceptive effects of morphine on painful diabetic neuropathy. (A) shows tail flick test data and (B) shows hot plate test data. Each point represents the mean  $\pm$  SEM of % MPE ( $n=8$ , number of animals per group). \* $p<0.01$  vs. saline group and # $p<0.05$  vs. morphine group (two way ANOVA followed by Tukey HSD post hoc test). MOR, morphine; STZ, streptozotocin; SAL, saline

attenuated morphine tolerance ( $p<0.05$ ). In this test, the maximum analgesic activity was obtained at 30 min of measurements.

### Effects of aspirin on caspase-3, bax and Bcl-2 levels in DRG

Biochemical analysis (ELISA) results showed that caspase-3 levels were significantly increased in the DRG of morphine-treated diabetic rats compared to the saline group ( $p<0.01$ ; Fig. 5A). However, administration of aspirin to rats in this group caused a significant decrease in caspase-3 levels ( $p<0.05$ ). In addition, administration of aspirin to morphine-tolerant rats significantly decreased the level of caspase-3 in ganglion tissue ( $p<0.05$ ; Fig. 5B). The data demonstrated that the Bax protein level was higher in rats with painful neuropathy than in the saline group ( $p<0.01$ ; Fig. 5C). Similarly, injection of aspirin into

morphine-tolerant rats significantly decreased Bax protein levels ( $p<0.05$ ; Fig. 5D). There was a significant decrease in antiapoptotic Bcl-2 protein level in diabetic rats compared to the saline group ( $p<0.01$ ; Fig. 5E). Administration of aspirin to morphine-tolerant rats resulted in a significant increase in Bcl-2 levels ( $p<0.05$ ; Fig. 5F).

### Effect of aspirin on morphine-induced apoptosis

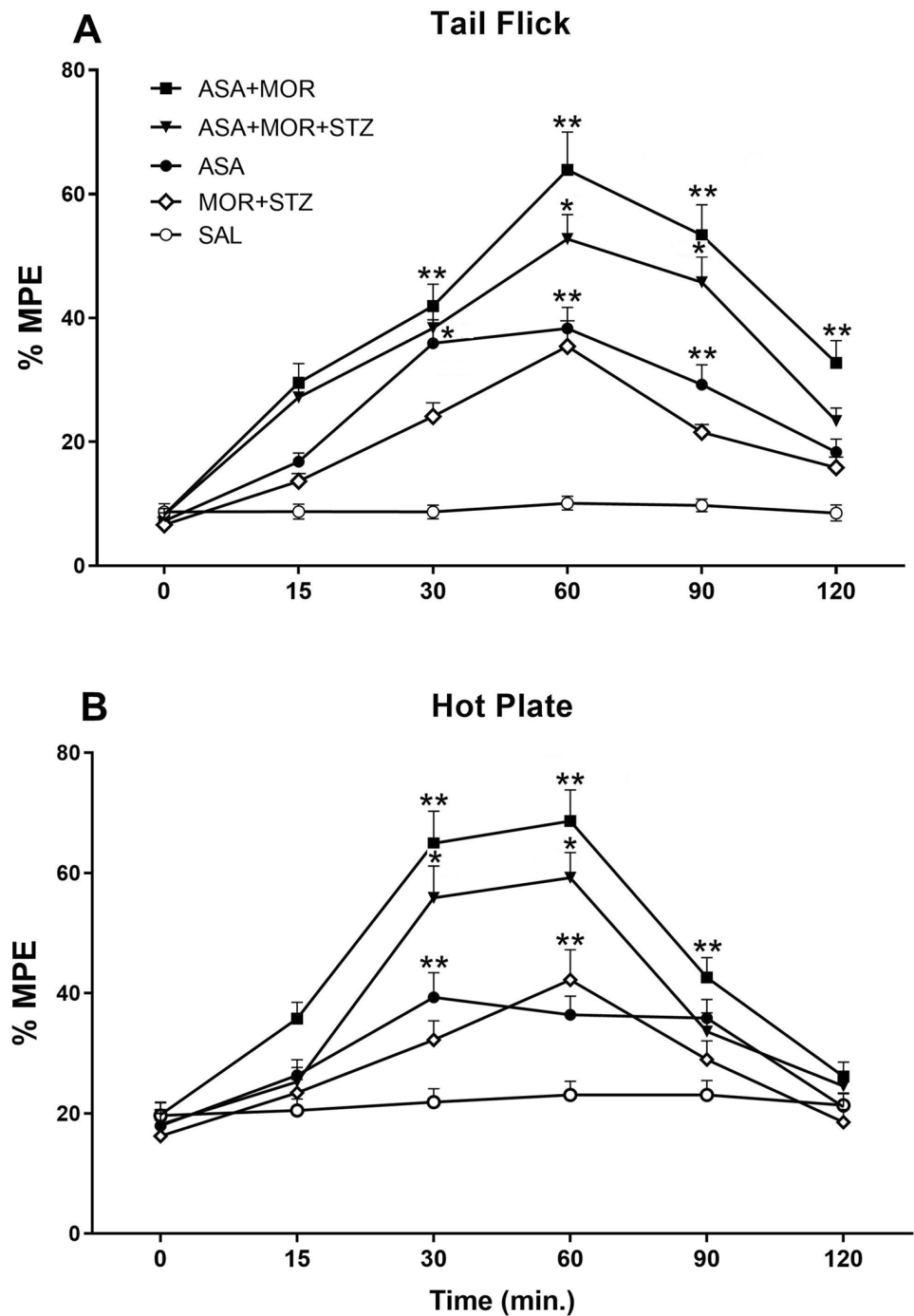
Hematoxylin-eosin (H&E) staining shows that the ganglion cells are located linearly along the axons and the satellite cell layer surrounds the ganglia in the morphine, aspirin and saline groups (Fig. 6A). Significant improvements in the ganglion cell morphology and structural integrity of the connective tissue and a decrease in the number of satellite cells were observed in the aspirin-administered groups. It is seen that ganglion cell diameters decrease and take a pyknotic structure. The cell cytoplasm is condensed and the organelles are tightly packed. Pyknosis by chromatin condensation is the most characteristic feature of apoptosis. Apoptotic cells appear as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments. TUNEL staining revealed important results regarding apoptotic cell numbers in DRG tissue. A semi-quantitative scoring system was used to evaluate apoptosis. The percentage of apoptotic cells in the diabetic group was significantly higher than in the saline group ( $p<0.01$ ; Fig. 6B). Moreover, administration of aspirin to diabetic rats significantly reduced the number of apoptotic cells ( $p<0.05$ ;  $n=6$ ).

In H&E staining of DRG sections, ganglion cell number decreased and there was a significant increase in satellite cells in morphine-tolerant rats. Furthermore, structural deterioration is observed in the connective tissue between ganglion and satellite cells (Fig. 7A). There was a significant increase in the percentage of apoptotic cells in the morphine tolerant group compared to the saline ( $p<0.01$ ; Fig. 7B). However, administration of aspirin to morphine-tolerant rats resulted in a significant reduction in the number of apoptotic cells ( $p<0.05$ ).

### Discussion

The results of the present study indicated that intraperitoneal administration of aspirin to rats with diabetic neuropathy provides a significant reduction in morphine-induced tolerance. In addition, co-administration of aspirin with morphine increased the antinociceptive activity of morphine in diabetic rats. Biochemical analysis revealed that aspirin decreased the levels of the pro-apoptotic proteins caspase-3

**Fig. 3** Effects of aspirin on morphine analgesia in diabetic rats. (A) shows tail flick test and (B) shows hot plate test data. Each point represents the mean  $\pm$  SEM of % MPE (n = 8). \* $p < 0.05$  vs. morphine + STZ group and \*\* $p < 0.01$  vs. saline treated group (two way ANOVA followed by Tukey HSD post hoc test). ASA, acetylsalicylic acid (aspirin); STZ, streptozotocin; MOR, morphine; SAL, saline

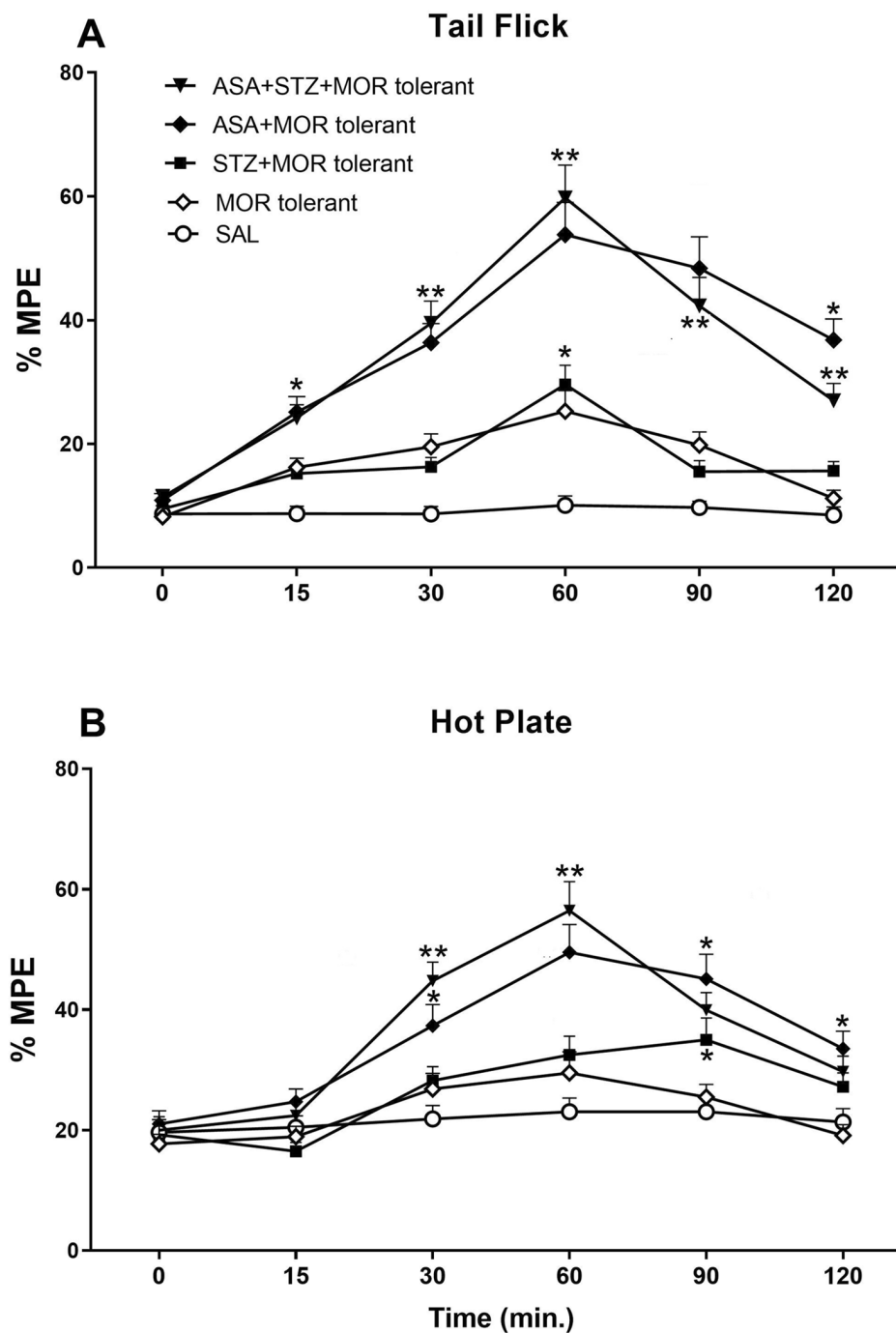


and Bax in DRG neurons, but increased the anti-apoptotic Bcl-2.

Opioids such as morphine are one of the important therapeutic drugs in severe neuropathic pain conditions and provide fast pain relief (Rosenblum et al. 2008). However, diabetes reduces antinociceptive activity in animals following systemic administration of  $\mu$ -opioid receptor (MOR) agonists such as morphine (Nielsen et al. 2007). It has

been observed that a decrease in the concentration of opioid receptors in diabetic animals leads to impaired opioid antinociception. In addition, it has been shown that opioid receptor density in the spinal cord tissue of animals with diabetes is markedly reduced (Shaqura et al. 2013). Consistent with others, our study results indicated that the antinociceptive effect of morphine was attenuated in diabetic rats and that co-administration of aspirin with morphine

**Fig. 4** Effects of aspirin on morphine tolerance. The effects of aspirin are demonstrated in (A) tail flick test and (B) hot plate test. Each point represents the mean  $\pm$  SEM of % MPE for 8 ( $n=7$ , for STZ groups) rats. \* $p<0.05$  vs. saline, \*\* $p<0.01$  vs. STZ + MOR tolerant group (two way ANOVA followed by Tukey HSD post hoc test). ASA, acetylsalicylic acid (aspirin); STZ, streptozotocin; MOR, morphine; SAL, saline

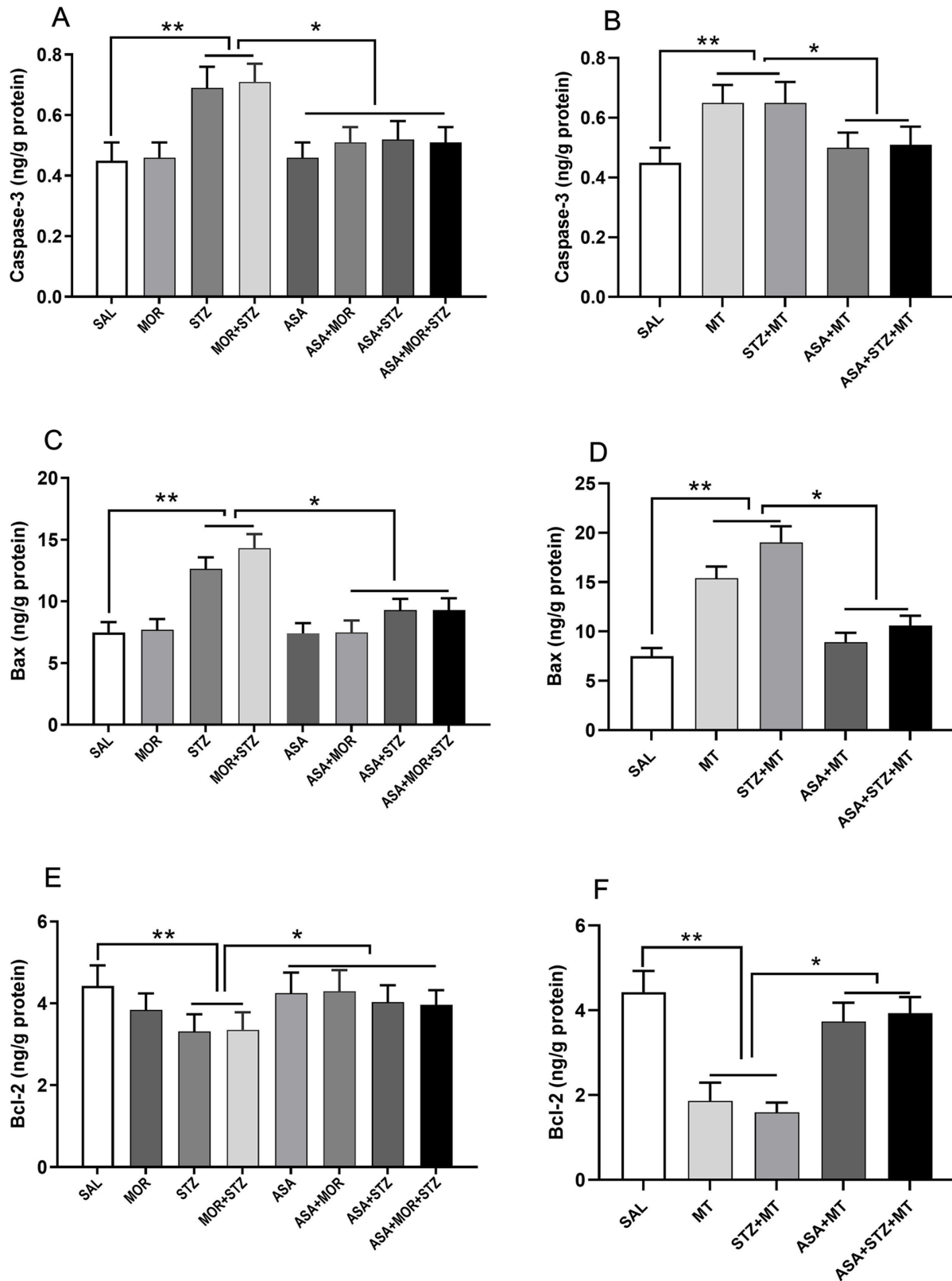


increased the antinociceptive effect in morphine-tolerant rats. Evidence suggests that the antinociceptive efficacy of morphine is decreased with a decrease in MOR density in diabetic rats (Kou et al. 2016). Nevertheless, high doses of morphine are required to reduce neuropathic pain, but this causes tolerance to morphine.

Several mechanisms have been suggested for the development of morphine tolerance, including downregulation of MOR (Zhang et al. 2019), inhibition of opioid receptor

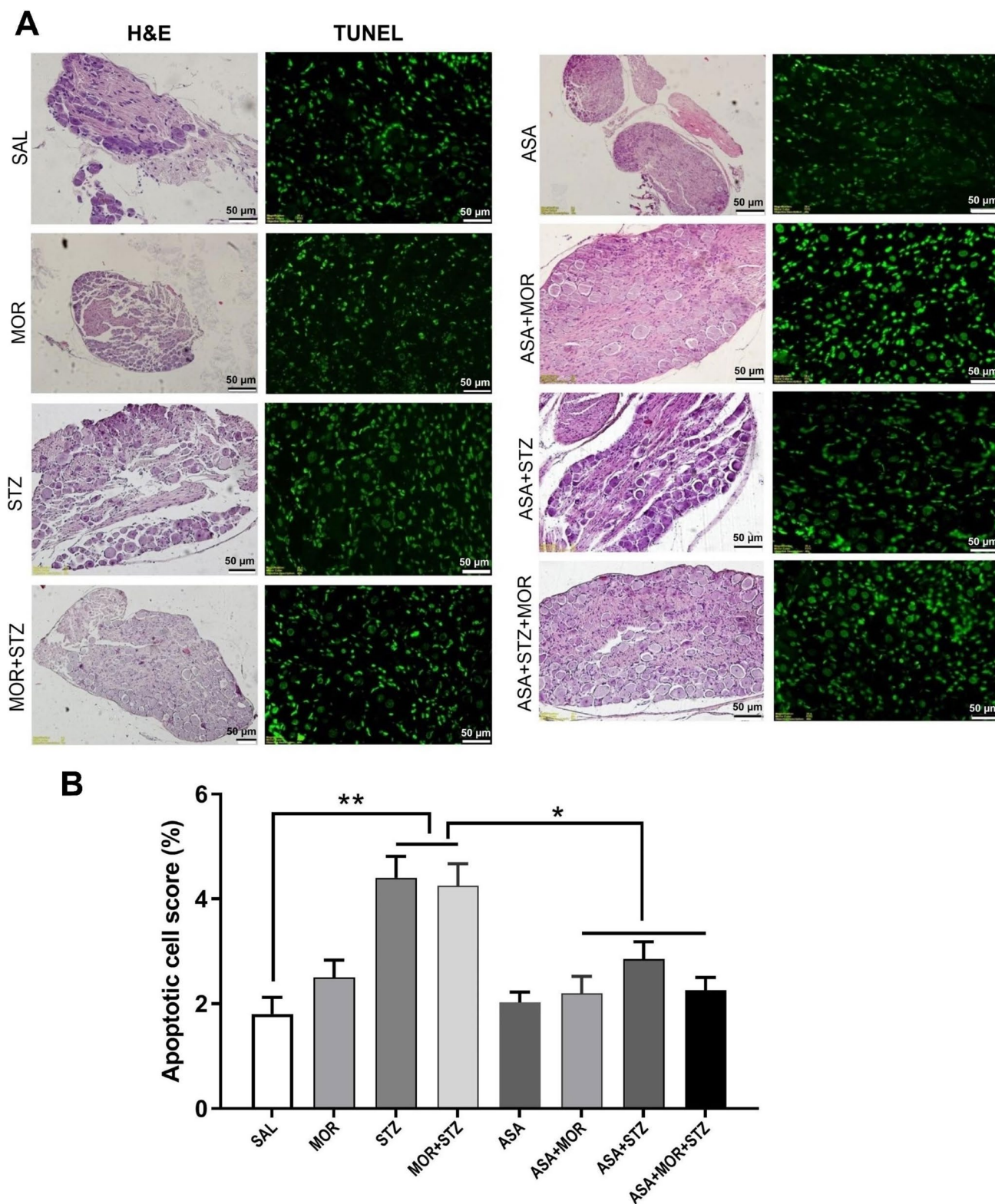
internalization (Williams et al. 2013), and activation of NMDARs (Zhang et al. 2019). However, evidences demonstrate that neuronal apoptosis plays an important role in the development of morphine tolerance (Shafie et al. 2015; Charkhpour et al. 2010). In addition, it has been reported that chronic morphine administration leads to morphine antinociceptive tolerance by increasing neuronal apoptosis (Shafie et al. 2015). Animal studies indicate that activation of microglia plays an important role in the development of





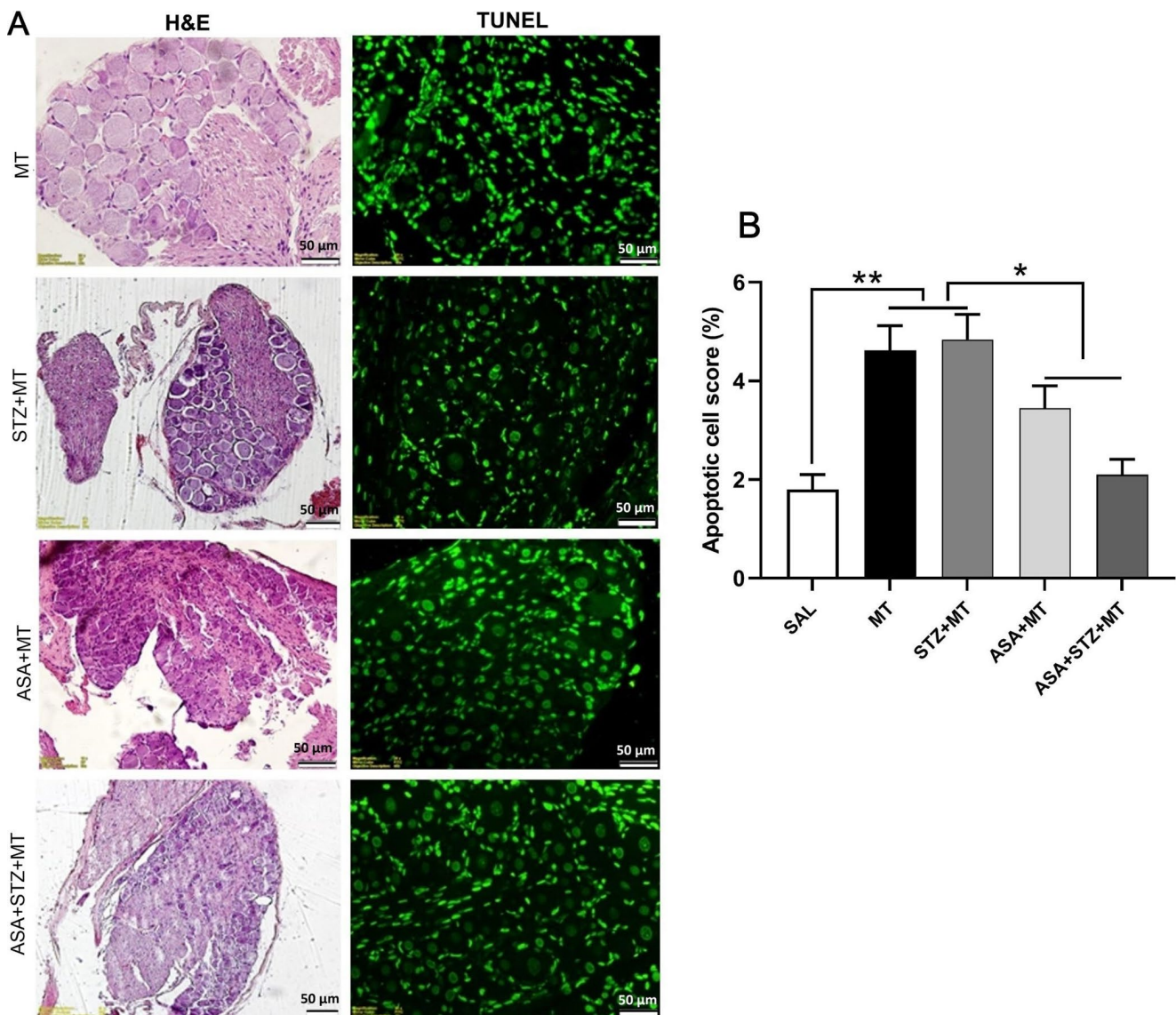
**Fig. 5** The effect of aspirin on caspase-3 (A), Bax (B), and Bcl-2 (C) levels in morphine and morphine tolerant groups. Each point represents the mean ± SEM, n = 6 (number of animals per group). \*\*p < 0.01

vs. saline group and \*p < 0.05 vs. MOR + STZ group or MOR + MT group. ASA, acetylsalicylic acid (aspirin); STZ, streptozotocin; MOR, morphine; MT, morphine tolerant; SAL, saline



**Fig. 6** Effect of aspirin on cell morphology and morphine-induced apoptosis in diabetic rats. Tissue sections were prepared from rat dorsal root ganglia. H&E staining in tissue sections was used to evaluate cell morphology. H&E stained cell nuclei and tissue elements are seen in blue and cytoplasmic elements in light pink. Slides were analyzed with a light microscope (X100 objective). Apoptosis was tested with cell death detection kit (fluorescein). Apoptotic cells scattered throughout

the tissue section were prominently stained (fluorescent) with TUNEL (X100 objective). (A) shows hematoxylin-eosin and TUNEL staining in DRG tissue, (B) shows percentage of apoptotic cells by semiquantitative scoring system.  $n=6$ . \* $p<0.05$  vs. MOR+STZ group and \*\* $p<0.01$  vs. saline group. ASA, acetylsalicylic acid (aspirin); STZ, streptozotocin; MOR, morphine; SAL, saline



**Fig. 7** Effect of aspirin on cell morphology and morphine-induced apoptosis in morphine tolerant rats. Tissue sections were prepared from rat dorsal root ganglia. H&E staining in tissue sections was used to evaluate cell morphology. H&E stained cell nuclei and tissue elements are seen in blue and cytoplasmic elements in light pink. Slides were analyzed with a light microscope (X100 objective). Neuronal apoptosis was tested with cell death detection kit (fluorescein). Apop-

totic cells scattered throughout the tissue section were prominently stained (fluorescent) with TUNEL (X100 objective). (A) shows H&E and TUNEL staining in DRG tissue (X100), (B) shows percentage of apoptotic cells by semiquantitative scoring system ( $n=6$ ). \* $p<0.05$  vs. STZ+MT group and \*\* $p<0.01$  vs. saline group. ASA, acetylsalicylic acid (aspirin); STZ, streptozotocin; SAL, saline; MT, morphine tolerant

painful diabetic neuropathy and that these cells in the spinal cord are activated in the development of diabetes (Mika et al. 2009). Microglia cells activated in the spinal cord with the development of diabetes cause neuropathic pain by increasing the release of proinflammatory cytokines (Mika et al. 2009; Watkins et al. 2007). Furthermore, experimental studies show that chronic administration of morphine to animals activates microglia cells in the spinal cord and induces morphine tolerance by increasing proinflammatory cytokines (Shen et al. 2011).

Aspirin as an anti-inflammatory drug ameliorates lipopolysaccharide-induced brain injury and exerts neuroprotective effects by inhibiting matrix metalloproteinase-3 gene expression (Feng et al. 2021). Aspirin has been shown to prevent NMDA-induced neuronal death by direct inhibition of protein kinase C (Crisanti et al. 2005). In addition, aspirin has a direct neuroprotective activity on neurotoxicity and cerebral white matter lesions resulting from focal ischemic damage to the brain (Wang et al. 2014). Recent studies suggest that aspirin produces direct neuroprotective

effects, including inhibition of nuclear factor kappa B (NF- $\kappa$ B) (Blanco et al. 2010). Aspirin suppresses neuronal apoptosis, reduces tissue inflammation and prevents astrocyte activation via the Nrf2/HO-1 signaling pathway (Wei et al. 2018). Caspase-3, Bax, and Bcl-2 proteins play an important role in the regulation of neuronal apoptosis. Administration of aspirin significantly attenuates the expression of the pro-apoptotic proteins caspase-3 and Bax in injured rat spine (Wei et al. 2018). Moreover, aspirin shows anti-apoptotic activity by blocking caspase-3 through Akt activation (Feng et al. 2011). In our study, biochemical results showed that aspirin produced anti-apoptotic activity by decreasing the levels of pro-apoptotic proteins caspase-3 and Bax and increasing anti-apoptotic Bcl-2 in DRG neurons. All this evidence suggests that the apoptotic signaling pathway can be suppressed by aspirin.

It was formerly believed that opioid-induced antinociception and tolerance are a function of the central nervous system. However, recent evidence has revealed that nociceptive DRG neurons have an important role in expressing antinociception and inducing opioid tolerance (Muchhala et al. 2021). Opioid receptor desensitization and changes in neurochemical signals and function in DRG neurons cause morphine antinociceptive tolerance (Li et al. 2009; Hu et al. 2016). In addition, blocking  $\mu$ -opioid receptors in DRG neurons expressing transient receptor potential vanilloid-1 (TRPV1) has been shown to reduce opioid tolerance (Corder et al. 2017). Moreover, elimination of  $\mu$ -opioid receptors from DRG neurons inhibits both supraspinal and spinal antinociceptive activities of opioids (Sun et al. 2020). From this evidence, it can be assumed that DRG neurons have a critical role in the development of antinociceptive tolerance to morphine. Several anti-apoptotic agents released from the central nervous system alleviate opioid tolerance by inhibiting neuronal apoptosis. However, prolonged administration of morphine in rats induces apoptosis due to an increase in pro-apoptotic Bax and a decrease in anti-apoptotic Bcl-2 proteins (Shafie et al. 2015; Hassanzadeh et al. 2011). In this study, biochemical data revealed that aspirin decreased apoptotic proteins caspase-3 and Bax levels in DRG tissue, while increasing antiapoptotic Bcl-2 levels, preventing apoptosis. Administration of minocycline, an antibiotic of the tetracycline group, prevents the development of tolerance to morphine by reducing the number of apoptotic cells in TUNEL staining of the spinal cord in rats (Hassanzadeh et al. 2011). Consistent with this, the TUNEL staining data of our study showed that aspirin decreased apoptotic cell numbers in DRG neurons of diabetic rats. This study revealed important results in terms of the effects of aspirin on morphine antinociceptive activity and tolerance in animals with diabetic neuropathy.

## Conclusion

In summary, these study results showed that co-administration of aspirin with morphine enhanced the antinociceptive activity of morphine and attenuated morphine tolerance in antinociceptive tests. In addition, aspirin prevented morphine-induced apoptosis by decreasing pro-apoptotic caspase-3 and Bax and increasing anti-apoptotic Bcl-2 levels in DRG of diabetic rats. Aspirin caused a significant reduction in the number of apoptotic cells in DRG neurons. Further studies are needed to elucidate possible mechanisms for the modulatory role of aspirin on morphine antinociception in diabetic rats.

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**Author Contribution** EO planning, statistical analysis, reviewing and writing original draft. OA planning, editing and writing work. AST investigation, writing, review and editing. ZDSI histological analysis and writing. HG performing behavioral tests and taking tissue samples. SG conceptualization, supervision, formal analysis.

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**Data Availability** The data presented in this study are available on request from the corresponding author.

**Code Availability** Not applicable.

## Declarations

**Ethics approval** The experimental protocols were approved by the Animal Ethics Committee of Cumhuriyet University (Ethic no: 2019/181). All animal housing and experiments were conducted in strict accordance with the institutional guidelines for care and use of laboratory animals.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing financial interests.

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#### Declarations.

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