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

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Research Article

Keywords: Aspirin, Diabetic neuropathy, Apoptosis, Morphine tolerance, Dorsal root ganglion

Posted Date: April 18th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1557959/v1

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. Recently, it has been stated that morphine-induced neuronal apoptosis can stimulate morphine tolerance. 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. After morphine tolerance induction, the antinociceptive effects of aspirin (50 mg/kg) and morphine (5 mg/kg) were evaluated by thermal pain tests at 15 or 30 minute intervals. Streptozotocin (STZ, 65 mg/kg) was injected intraperitoneally to induce diabetic neuropathy. To evaluate apoptosis in dorsal root ganglion (DRG) tissue, 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. Further studies are needed to elucidate these effects of aspirin on morphine tolerance.

Introduction

Neuropathic pain is one of the common complications of diabetes and affects more than 50% of patients [1, 2]. Decreased efficacy of opioid analgesic drugs such as morphine administered in patients with diabetic neuropathy is an important clinical problem [3, 4]. 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 [5]. This reduction in the antinociceptive efficacy of opioids has also been demonstrated in diabetic animal models administered μ-opioid receptor (MOR) agonists [6].

Different mechanisms have been proposed for morphine tolerance. These include nitric oxide (NO)-cGMP and mammalian target of rapamycin (mTOR) signaling pathways, glial cell and N-methyl-D-aspartate receptor (NMDAR) activation, opioid receptor desensitization, and ghrelin receptor inactivation [7–11]. Recently, morphine-induced neuronal apoptosis constitutes one of the possible mechanisms for morphine tolerance [12]. It has been reported that long-term morphine administration increases neuronal apoptosis and this leads to morphine tolerance [13–15]. Consistent with this, 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 [13].

It has been shown in different studies that the antinociceptive effectiveness of MOR agonists is decreased in painful diabetic neuropathy [4, 16]. The increase in blood glucose levels in diabetes causes

significant changes in the antinociceptive processes of the endogenous opioid system [17]. These changes in the opioid system with hyperglycemia reduce to the analgesic effect of morphine in diabetic animals [18]. This effect has been reported to be associated with loss of MOR or impaired G-protein coupling to MOR [16, 19]. 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 [20].

Clinical management of opioid tolerance includes administration of opioids in combination with adjuvants [21, 22]. In patients requiring long-term analgesic therapy, adjuvants such as gabapentin, pregabalin, ibuprofen, and aspirin (acetylsalicylic acid, ASA) are combined with opioid analgesics [22]. Aspirin is a cyclooxygenase (COX) inhibitor used to reduce pain, fever, and inflammation [23]. 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 [24]. 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 [25]. In addition, aspirin represents anti-apoptotic activity by blocking caspase-3 through Akt activation [26] and protects cells from apoptosis by preventing toxic stimuli [27, 28]. 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

Male adult Wistar rats between 9 to 10 weeks old and weighing 225 to 250 g were purchased from Cumhuriyet University Experimental Animal Center (Sivas, Turkey). 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 ± 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.). 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 saline. 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 [29, 30].

Experimental procedure

To induce morphine tolerance, morphine was administered at a cumulative dose for three days in rats [31]. 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 by administering a test dose of morphine (5 mg/kg) on the 4th day. If administration of a test dose of morphine produces a significant reduction in antinociceptive effect in rats, it is defined as morphine tolerance. The experimental protocol and study timeline are shown in Fig. 1.

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 minutes after administration of the test dose of morphine. Saline group rats were injected with the same dose of 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 into 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) times were recorded in seconds. The radiant heat source was calibrated so that the basal TFL occurs in a mean of 2.5 ± 0.5 seconds in the nociceptive test. Rats with baseline TFL less than 2.0 seconds or greater than 3.0 seconds were excluded from subsequent antinociceptive tests. The test cut-off latency was programmed to be 20 seconds to avoid tissue injury in the rat tail. Responses to the TF test are often associated with central pain mechanisms [32, 33].

In the thermal HP test, rats were placed in a test device with a plate at an average temperature of 54 ± 0.6°C. Paw licking responses to avoid heat or delay in the rat's jumping 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 [33].

Induction of diabetes

To induce diabetic neuropathy, a single dose (65 mg/kg) streptozotocin (STZ) prepared in 0.1 mol/l sodium citrate buffer (pH 4.5) was injected intraperitoneally into diabetes-induced rats [29]. One week

later, blood glucose levels were measured with GolDeal Gluco Prober (Aurum Biomedical Tecnology, 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 ≥ 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

Control and diabetic group rats were sacrificed by cervical dislocation and rapidly surgically removed dorsal root ganglia (DRGs) at L3 to S4 spinal levels. 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 minutes. 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) [34].

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 minutes. Staining solutions were added to the washed tissues and incubated for approximately 15 minutes. 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

After the antinociceptive tests were applied to the rats, anesthesia was provided with ketamine injection. To isolate the dorsal root ganglia (DRG), rats were sacrificed and DRGs of the lumbosacral spinal cord at the L3-S4 level were isolated. The resulting 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 [35]. 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 µm 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 hours 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 minutes. In the next step, neuronal tissues were exposed to PBS 2 times. After applying 50 µl 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 MgCl2, 3000 U/ml to 3U/ml in 1 mg/ml BSA) at 20–25°C was suspended for 15 minutes. TUNEL mixture solution was applied to the tissues exposed to PBS twice and incubated at 37°C for 60 minutes 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 ≤ 5% of neurons; 2, staining affecting 5–10% of neurons; 3, staining affecting 10–15% of neurons; 3, staining affecting 15–20% of neurons; 5, staining affecting ≥ 20% of neurons [36].

Samples obtained from DRG sections for hematoxylin-eosin (H&E) staining were first deparaffinized in xylol for 10 minutes. Tissues were then exposed to 95%, 80%, and 70% alcohol for 10 minutes, respectively. Neuronal tissue samples were stained with hematoxylin for 5–6 minutes 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 minutes. 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 minutes. 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:

% MPE = [(test latency-baseline) / (cutoff-baseline)]×100.

Statistical analysis

The results are presented as the mean ± 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.

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 ± 2.47; n = 8) were significantly decreased compared to pre-STZ administration ($p < 0.05$; Table 1).

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 ± 2.56	228.73 ± 2.47*	125.10 ± 2.31	389.53 ± 3.44**
The data are presented as the mean ± SEM. *$p < 0.05$ and **$p < 0.01$ compared to before STZ injection (Paired t-test).			

Blood glucose levels (389.53 ± 3.44) were significantly increased in rats with diabetes ($p < 0.01$). These glucose levels meet the criteria for diabetic rats (≥ 250 mg/dl).

Antinociceptive effects of morphine on painfull 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 2B). However, the antinociceptive effect of morphine was significantly higher in both tail flick (F3,20 = 17.85) and hot plate (F3,20 = 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 Fig. 3B) 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 attenuated morphine tolerance ($p < 0.05$). In this test, the maximum analgesic activity was obtained at 30 minutes 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 caspas-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 pycnotic structure. The cell cytoplasm is condensed and the organelles are tightly packed. Pycnosis 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 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 [37]. However, diabetes reduces antinociceptive activity in animals following systemic administration of μ-opioid receptor (MOR) agonists such as morphine [6]. 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 [38]. 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 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 [39]. 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 [40], inhibition of opioid receptor internalization [41], and activation of NMDARs [9]. However, evidences demostrate that neuronal apoptosis plays an important role in the development of morphine tolerance [15, 42]. In addition, it has been reported that chronic morphine administration leads to morphine antinociceptive tolerance by increasing neuronal apoptosis [15]. Animal studies indicate that activation of microglia plays an important role in the development of painful diabetic neuropathy and that these cells in the spinal cord are activated in the development of diabetes [43]. Microglia cells activated in the spinal cord with the development of diabetes cause neuropathic pain by increasing the release of proinflammatory cytokines [43, 44]. 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 [45].

Aspirin as an anti-inflammatory drug ameliorates lipopolysaccharide-induced brain injury and exerts neuroprotective effects by inhibiting matrix metalloproteinase-3 gene expression [46]. Aspirin has been shown to prevent NMDA-induced neuronal death by direct inhibition of protein kinase C [47]. In addition, aspirin has a direct neuroprotective activity on neurotoxicity and cerebral white matter lesions resulting from focal ischemic damage to the brain [48]. Recent studies suggest that aspirin produces direct neuroprotective effects, including inhibition of nuclear factor kappa B (NF-kB) [49]. Aspirin suppresses

neuronal apoptosis, reduces tissue inflammation and prevents astrocyte activation via the Nrf2/HO-1 signaling pathway [25]. 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 [25]. Moreover, aspirin shows anti-apoptotic activity by blocking caspase-3 through Akt activation [26]. 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 [50]. Opioid receptor desensitization and changes in neurochemical signals and function in DRG neurons cause morphine antinociceptive tolerance [51, 52]. In addition, blocking μ-opioid receptors in DRG neurons expressing transient receptor potential vanilloid-1 (TRPV1) has been shown to reduce opioid tolerance [53]. Moreover, elimination of μ-opioid receptors from DRG neurons inhibits both supraspinal and spinal antinociceptive activities of opioids [54]. From this evidence, it can be assumed that DRG neurons have a critical role in the development of anticiceptive 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 [14, 15]. 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 [14]. 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.

Declarations

Author Contributions

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.

Funding

This study was supported by Cumhuriyet University Scientific Research Project (T-856, CUBAP, Sivas, Turkey).

Data Availability

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

Conflict of interest

The authors declare no competing financial interests.

References

1. Castany S, Carcolé M, Leánez S et al (2016) The induction of heme oxygenase-1 decreases painful diabetic neuropathy and enhances the antinociceptive effects of morphine in diabetic mice. PLoS ONE 11(1):e0146427
2. Park TS, Park JH, Baek HS (2004) Can diabetic neuropathy be prevented? Diabetes Res Clin Pract 66(Suppl 1):S53–56
3. Courteix C, Bourget P, Caussade F et al (1998) Is the reduced efficacy of morphine in diabetic rats caused by alterations of opiate receptors or of morphine pharmacokinetics? J Pharmacol Exp Ther 285:63–70
4. Ohsawa M, Mizoguchi H, Narita M et al (2000) Effects of a mu-opioid receptor agonist on G-protein activation in streptozotocin-induced diabetic mice. Eur J Pharmacol 401:55–58
5. Hervera A, Leánez S, Pol O (2012) The inhibition of the nitric oxide-cGMP-PKG-JNK signaling pathway avoids the development of tolerance to the local antiallodynic effects produced by morphine during neuropathic pain. Eur J Pharmacol 685:42–51
6. Nielsen CK, Ross FB, Lotfipour S et al (2007) Oxycodone and morphine have distinctly different pharmacological profiles: radioligand binding and behavioural studies in two rat models of neuropathic pain. Pain 132:289–300
7. Ozdemir E, Bagcivan I, Durmus N et al (2011) The nitric oxide–cGMP signaling pathway plays a significant role in toleranceto the analgesic effect of morphine. Can J Physiol Pharmacol 89(2):89–95
8. Shirooie S, Sahebgharani M, Esmaeili J et al (2019) In vitro evaluation of effects of metformin on morphine and methadone tolerance through mammalian target of rapamycin signaling pathway. J

Cell Physiol 234(3):3058–3066

9. Zhang B, Wu L, Zhu Q et al (2021) Donepezil attenuates the development of morphine tolerance in rats with cancer-induced bone pain: The role of cortical N-methyl-D-aspartate receptors. Neurosci Lett 747:135678
10. Jokinen V, Sidorova Y, Viisanen H et al (2018) Differential spinal and supraspinal activation of glia in a rat model of morphine tolerance. Neuroscience 375:10–24
11. Baser T, Ozdemir E, Filiz AK et al (2021) Ghrelin receptor agonist hexarelin attenuates antinociceptive tolerance to morphine in rats. Can J Physiol Pharmacol 99(5):461–467
12. Asuni GP, Speidell A, Mocchetti I (2021) Neuronal apoptosis induced by morphine withdrawal is mediated by the p75 neurotrophin receptor. J Neurochem 158(2):169–181
13. Mao J, Sung B, Ji RR et al (2002) Neuronal apoptosis associated with morphine tolerance: evidence for an opioid-induced neurotoxic mechanism. J Neurosci 22:7650–7661
14. Hassanzadeh K, Habibi-asl B, Farajnia S et al (2011) Minocycline prevents morphine-induced apoptosis in rat cerebral cortex and lumbar spinal cord: a possible mechanism for attenuating morphine tolerance. Neurotox Res 19(4):649–659
15. Shafie A, Moradi F, Izadpanah E et al (2015) Neuroprotection of donepezil against morphine-induced apoptosisis mediated through Toll-like receptors. Eur J Pharmacol 764:292–297
16. Chen SR, Pan HL (2003) Antinociceptive effect of morphine, but not mu opioid receptor number, is attenuated in the spinal cord of diabetic rats. Anesthesiology 99:1409–1414
17. Forman LJ, Marquis DE, Stevens R et al (1985) Diabetes induced by STZ results in a decrease in immunoreactive beta-endorphin levels in the pituitary and hypothalamus of female rats. Diabetes 34:1104–1107
18. Mahajan AS, Chakrabarty K, Mishra TK et al (1997) Nociception, antinociceptive potency of morphine in streptozotocin induced diabetic rats. Indian J Physiol Pharmacol 41(4):416–420
19. Mousa SA, Shaqura M, Khalefa BI et al (2013) Rab7 silencing prevents μ-opioid receptor lysosomal targeting and rescues opioid responsiveness to strengthen diabetic neuropathic pain therapy. Diabetes 62:1308–1319
20. Zychowska M, Rojewska E, Kreiner G et al (2013) Minocycline influences the antiinflammatory interleukins and enhances the effectiveness of morphine under mice diabetic neuropathy. J Neuroimmunol 262:35–45
21. Freye E, Anderson-Hillemacher A, Ritzdorf I et al (2007) Opioid rotation from high-dose morphine to transdermal buprenorphine (Transtec) in chronic pain patients. Pain Pract 7:123–129
22. Paul AK, Smith CM, Rahmatullah M et al (2021) Opioid analgesia and opioid-induced adverse effects: A review. Pharmaceuticals (Basel) 14(11):1091
23. Zhang X, Du R, Luo N et al (2020) Aspirin mediates histone methylation that inhibits inflammation-related stemness gene expression to diminish cancer stemness via COX-independent manner. Stem Cell Res Ther 11(1):370

24. Jian Z, Tang L, Yi X et al (2016) Aspirin induces Nrf2-mediated transcriptional activation of haem oxygenase-1 in protection of human melanocytes from H2 O2-induced oxidative stress. J Cell Mol Med 20:1307–1318
25. Wei W, Shurui C, Zipeng Z et al (2018) Aspirin suppresses neuronal apoptosis, reduces tissue inflammation, and restrains astrocyte activation by activating the Nrf2/HO-1 signaling pathway. NeuroReport 29(7):524–531
26. Feng X, Lu B, Xu Y et al (2011) Aspirin reduces the apoptotic effect of etoposide via Akt activation and up-regulation of p21(cip). Int J Mol Med 4:637–643
27. Grilli M, Pizzi M, Memo M et al (1996) Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. Science 274:1383–1385
28. Kutuk O, Basaga H (2003) Aspirin prevents apoptosis and NF-kappaB activation induced by H2O2 in HeLa cells. Free Radic Res 37:1267–1276
29. Zangiabadi N, Mohtashami H, Hojatipour M et al (2014) The effect of angipars on diabetic neuropathy in STZ-induced diabetic male rats: A study on behavioral, electrophysiological, sciatic histological and ultrastructural indices. Sci World J 4:1–8
30. Micov A, Tomić M, Pecikoza U et al (2015) Levetiracetam synergises with common analgesics in producing antinociception in a mouse model of painful diabetic neuropathy. Pharmacol Res 97:131–142
31. Zarrindast M-R, Dinkoub Z, Homayoun H et al (2002) Dopamine receptor mechanisms and morphine tolerance in mice. J Psychopharmacol 16:261–266
32. Ramabadran K, Bansinath M, Turndorf H et al (1989) The hyperalgesic effect of naloxone is attenuated in streptozotocin-diabetic mice. Psychopharmacol (Berl) 97:169–174
33. Kanaan SA, Saade NE, Haddad JJ et al (1996) Endotoxin-induced local inflammation and hyperalgesia in rats, a new model for inflammatory pain. Pain 66:373–379
34. Kruger NJ (1994) The Bradford Method for Protein Quantitation. Protein Protoc. Handbook. Humana Press, New Jersey, pp 15–22
35. Wang Y, Ni H, Zhang W et al (2016) Downregulation of miR-210 protected bupivacaine-induced neurotoxicity in dorsal root ganglion. Exp Brain Res 234:1057–1065
36. Schmeichel AM, Schmelzer JD, Low PA (2003) Oxidative injury and apoptosis of dorsal root ganglion neurons in chronic experimental diabetic neuropathy. Diabetes 52:165–171
37. Dowell D, Haegerich TM, Chou R (2016) CDC guideline for prescribing opioids for chronic pain-United States. JAMA 315:1624–1645
38. Shaqura M, Khalefa BI, Shakibaei M et al (2013) Reduced number, G protein coupling, and antinociceptive efficacy of spinal mu-opioid receptors in diabetic rats are reversed by nerve growth factor. J Pain 14:720–730
39. Kou Z-Z, Wan F-P, Bai Y et al (2016) Decreased endomorphin-2 and mu-opioid receptor in the spinal cord are associated with painful diabetic neuropathy. Front Mol Neurosci 9:80

40. Zhang TJ, Qiu Y, Hua Z (2019) The emerging perspective of morphine tolerance: MicroRNAs. Pain Res Manag 2019:9432965
41. Williams JT, Ingram SL, Henderson G et al (2013) Regulation of μ-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. Pharmacol Rev 65:223–254
42. Charkhpour M, Nayebi AR, Doustar Y et al (2010) 8-OH-DPAT prevents morphine-induced apoptosis in rat dorsal raphe nucleus: a possible mechanism for attenuating morphine tolerance. Anesth Analg 111:1316–1321
43. Mika J, Osikowicz M, Rojewska E et al (2009) Differential activation of spinal microglial and astroglial cells in a mouse model of peripheral neuropathic pain. Eur J Pharmacol 623:65–72
44. Watkins LR, Hutchinson MR, Ledeboer A et al (2007) Norman Cousins Lecture. Glia as the “bad guys”: implications for improving clinical pain control and the clinical utility of opioids. Brain Behav Immun 21:131–146
45. Shen CH, Tsai RY, Shih MS et al (2011) Etanercept restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in morphine-tolerant rats. Anesth Analg 112:454–459
46. Feng D, Chen D, Chen T et al (2021) Aspirin exerts neuroprotective effects by reversing lipopolysaccharide-induced secondary brain injury and inhibiting matrix metalloproteinase-3 gene expression. Dis Markers 2021:3682034
47. Crisanti P, Leon A, Lim DM et al (2005) Aspirin prevention of NMDA-induced neuronal death by direct protein kinase Czeta inhibition. J Neurochem 93(6):1587–1593
48. Wang Z, Huang W, Zuo Z (2014) Perioperative aspirin improves neurological outcome after focal brain ischemia possibly via inhibition of Notch 1 in rat. J Neuroinflammation 11:1–10
49. Blanco A, Alvarez S, Fresno M et al (2010) Amyloid-β induces cyclooxygenase-2 and PgE2 release in human astrocytes in NF-κB dependent manner. J Alzheimers dis 22:493–505
50. Muchhala KH, Jacob JC, Dewey WL et al (2021) Role of β-arrestin-2 in short- and long-term opioid tolerance in the dorsal root ganglia. Eur J Pharmacol 899:174007
51. Li Q, Zhao X, Zhong LJ et al (2009) Effects of chronic morphine treatment on protein expression in rat dorsal root ganglia. Eur J Pharmacol 612:21–28
52. Hu XM, Cao SB, Zhang HL et al (2016) Downregulation of miR-219 enhances brain-derived neurotrophic factor production in mouse dorsal root ganglia to mediate morphine analgesic tolerance by upregulating CaMKIIγ. Mol Pain 12:1744806916666283
53. Corder G, Tawfik VL, Wang D et al (2017) Loss of μ opioid receptor signaling in nociceptors, but not microglia, abrogates morphine tolerance without disrupting analgesia. Nat Med 23:164–173
54. Sun J, Chen SR, Pan HL (2020) μ-Opioid receptors in primary sensory neurons are involved in supraspinal opioid analgesia. Brain Res 1729:146623

Figures

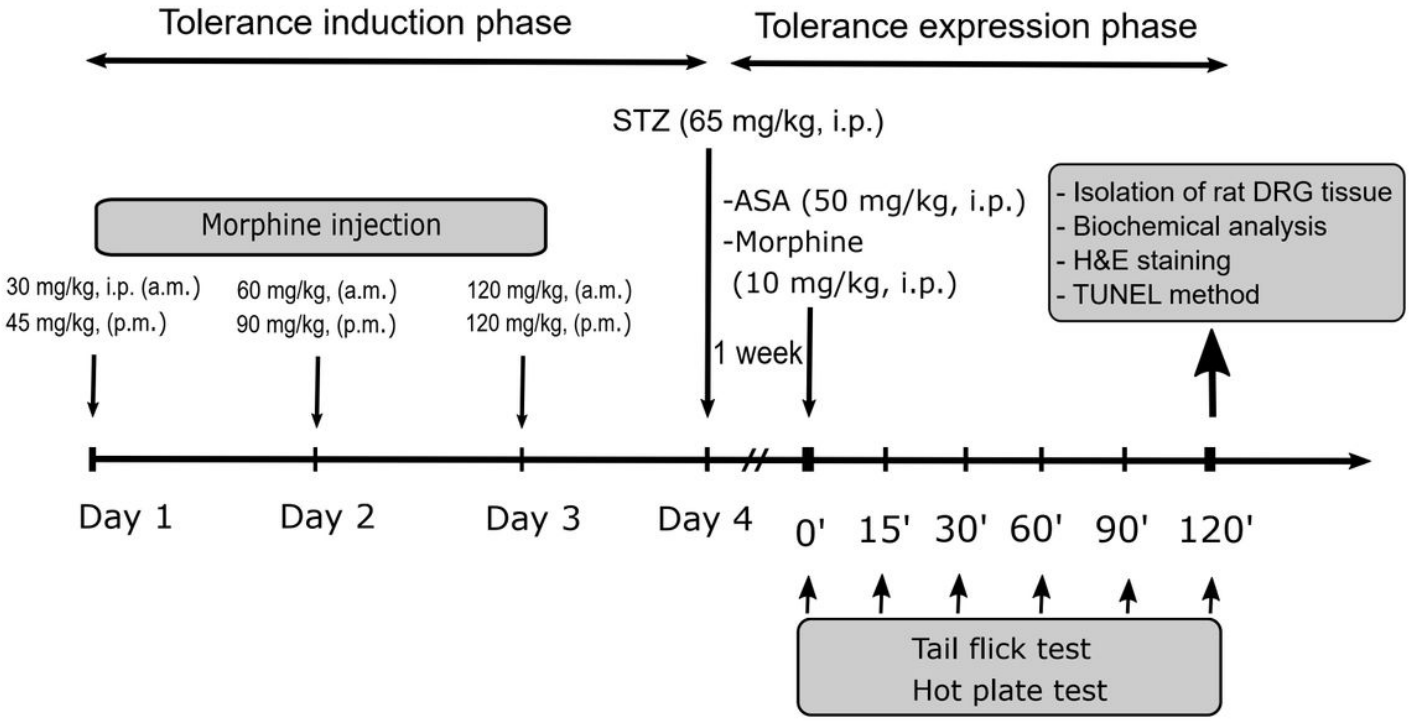

Figure 1

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

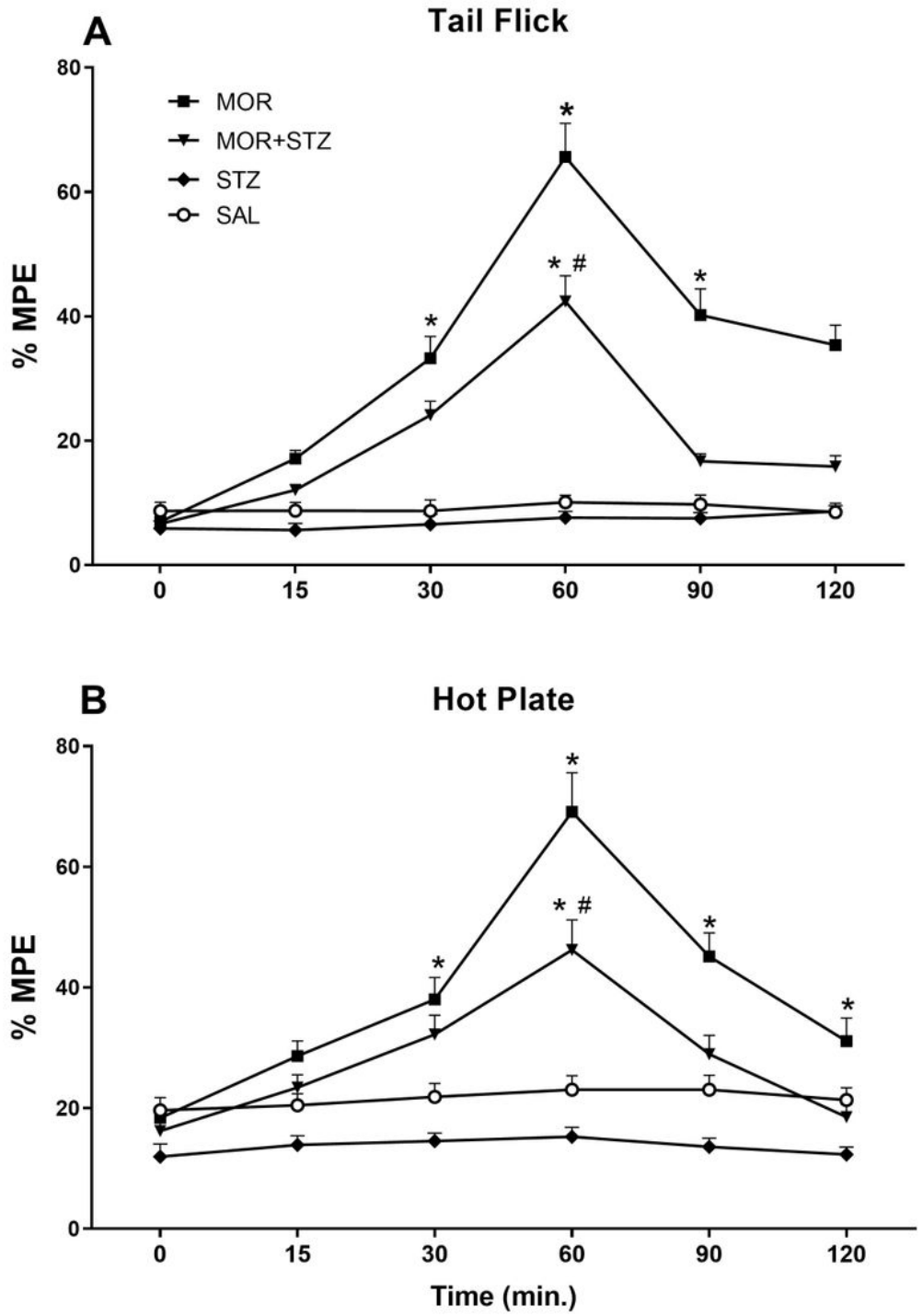

Figure 2

Antinociceptive effects of morphine on painfull diabetic neuropathy. (A) shows tail flick test data and (B) shows hot plate test data. Each point represents the mean±SEM of % MPE for 8 rats. *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

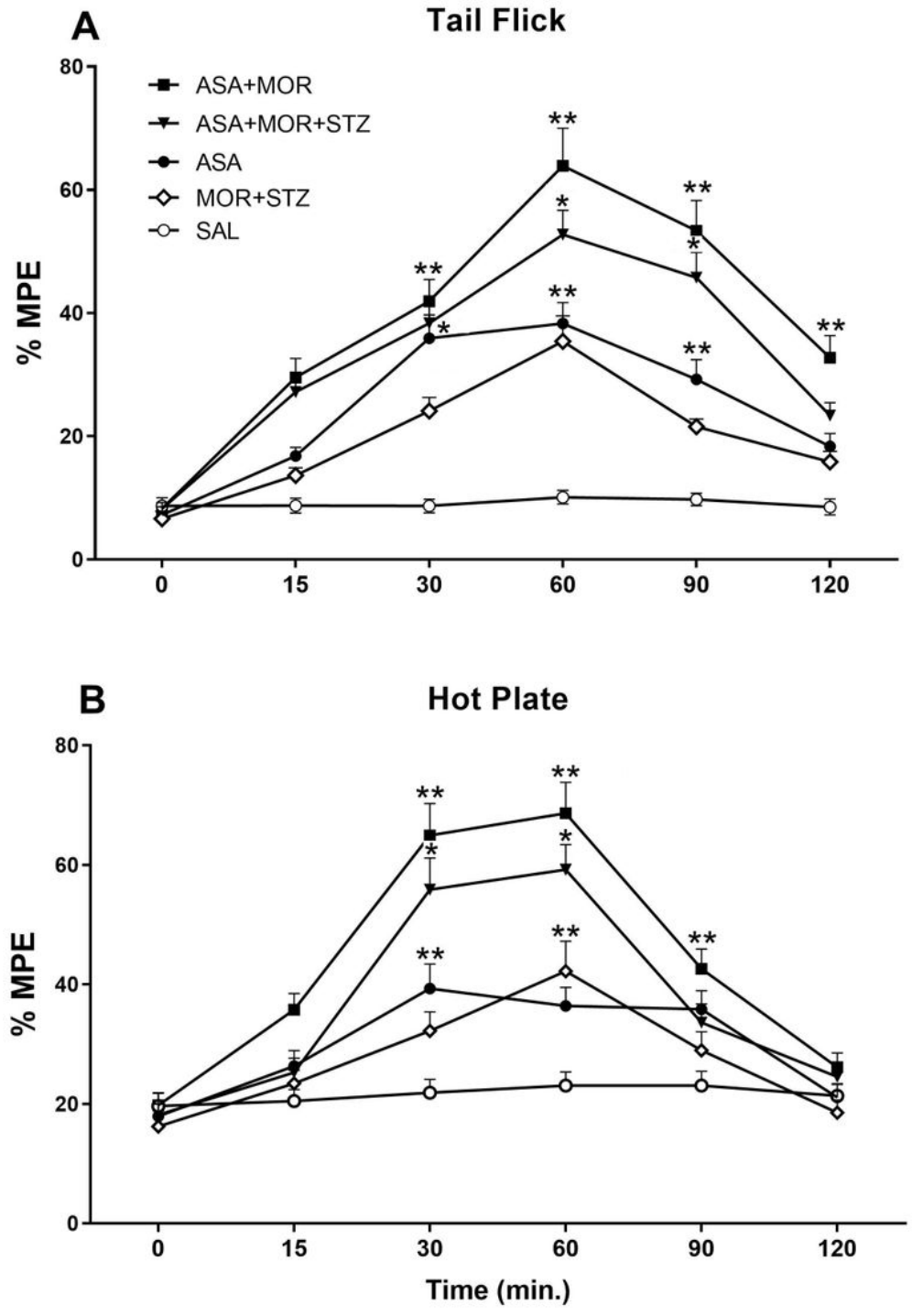

Figure 3

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±SEM of % MPE for 8 rats. *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

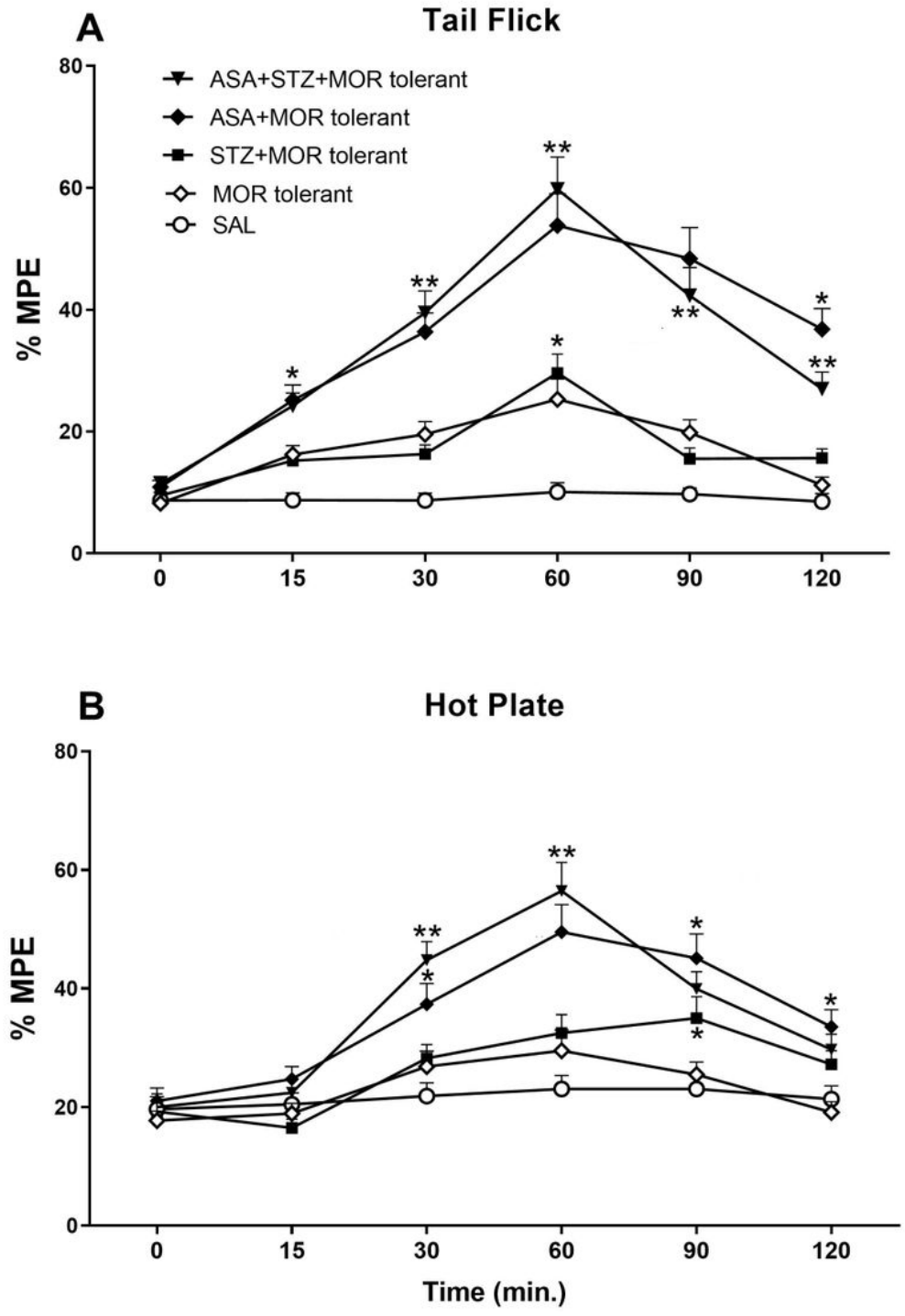

Figure 4

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±SEM of % MPE for 7-8 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

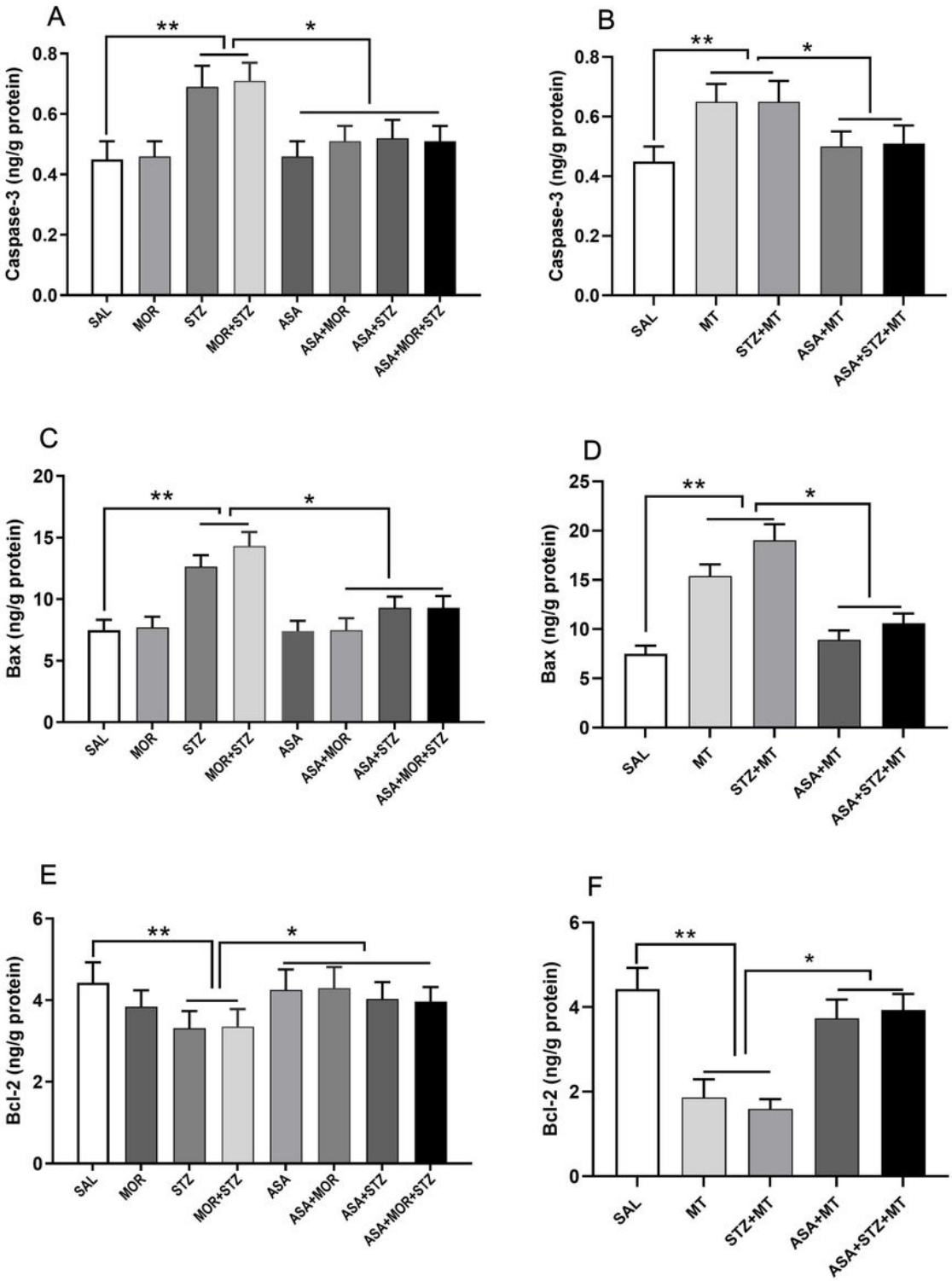

Figure 5

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. **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

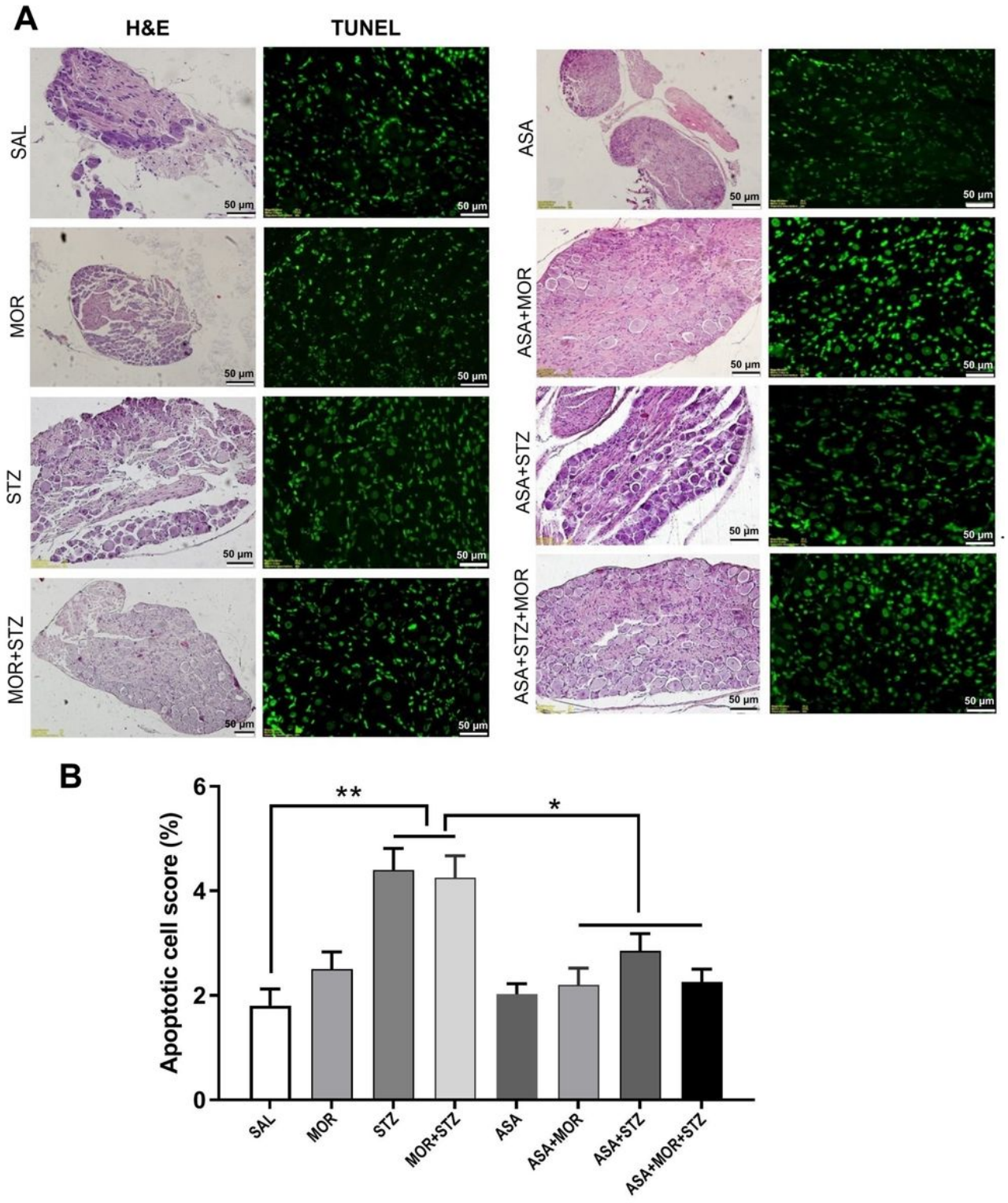

Figure 6

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 percantage 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

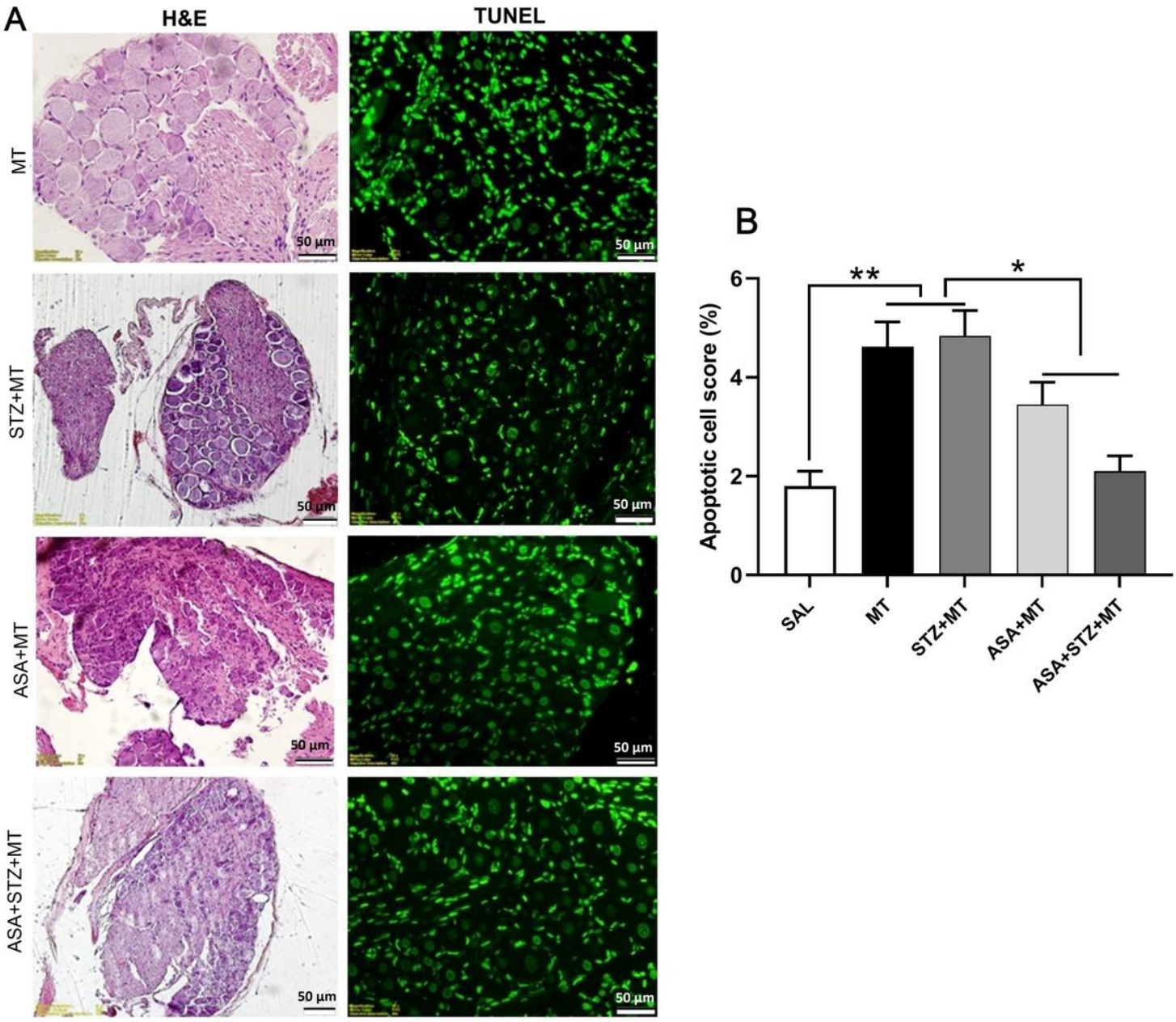

Figure 7

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). Apoptotic 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 percantage 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.