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Role of β-Arrestin-2 in Opioid Tolerance and Opioidergic & α-2 Adrenergic Analgesic Synergism

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Abstract

Background

Fentanyl (FEN) is a synthetic opioid with high affinity for μ-opioid receptors (MOR). Tolerance development is a significant problem that limits the effective use of opioids. Dexmedetomidine (DEX) and xylazine (XYL) are α-2 adrenoceptor (AR) agonists with sedative and analgesic effects. In our previous study, we demonstrated that these agents exhibit analgesic synergy with FEN. β-arrestin-2 mediates the desensitization and internalization of G protein-coupled opioid receptors. In this study, we aimed to investigate the role of β-arrestin-2 in opioid tolerance and opioidergic-α-2 adrenergic analgesia synergism.

Methods

Wistar albino rats were divided into six groups: control, FEN, DEX, XYL, FEN + DEX, and FEN + XYL. The groups received intraperitoneal injections in the morning and evening on the first two days and only in the morning on the third day. The rats were sacrificed on the third day, and their spinal cords were removed. The tissues were subjected to immunohistochemical analysis to assess β-arrestin-2 immunoreactivity.

Results

The spinal cords of the groups appeared morphologically normal. Compared to the control group, β-arrestin-2 immunoreactivity was significantly reduced in the FEN + DEX and FEN + XYL groups. Although not statistically significant, there was a trend toward decreased β-arrestin-2 immunoreactivity in the other groups. According to our study results, β-arrestin-2 caused receptor desensitization without altering MOR levels.

Conclusions

This important protein induces tolerance by affecting MORs. It also affects α-2 ARs, affecting opioidergic-α-2 adrenergic synergism.

Introduction

Opioids are substances that induce antinociception by binding to opioid receptors located in the spinal, supraspinal ,and peripheral regions [1]. Unfortunately, tolerance can develop as a result of frequent and/or high-dose use of these substances, which provide highly effective analgesia. Once tolerance develops, higher doses of opiates are required to provide effective analgesia, and dose increases become necessary. Prolonged and repeated exposure to opioid agonists results in decreased

responsiveness of G protein-coupled opioid receptors (GPCRs) [2]. Unfortunately, tolerance can develop as a result of frequent and/or high-dose use of these substances, which provide highly effective analgesia. Once tolerance develops, higher doses of opiates are required to provide effective analgesia, and dose increases become necessary. Prolonged and repeated exposure to opioid agonists results in decreased responsiveness of G protein-coupled opioid receptors (GPCRs) [2]. The mechanisms underlying opioid tolerance remain unclear. However, the proposed mechanisms remain controversial. Chronic exposure to agonists induces a reduction in effector response (tolerance), attributed to receptor downregulation, desensitization, or changes in gene expression. After receptor phosphorylation, uncoupling from G proteins and internalization can occur. After internalization, receptors can be recycled (resensitized) or degraded [3]. In previous studies, β-arrestin-2 has been shown to play a role in desensitization and internalization of opioid receptors.

Fentanyl (FEN) is a highly potent opioid agonist with high affinity for μ-opioid receptors (MORs). It is known to be at least 100 times more potent than morphine [4]. Dexmedetomidine (DEX) is a very potent and highly selective α-2 adrenoceptor (AR) agonist with a broad pharmacological spectrum [5]. Xylazine (XYL) is one of the most commonly used α-2 AR agonists for sedation and analgesia in veterinary medicine. Both α-2 AR agonists are used in clinical and veterinary medicine to exploit their sedative and analgesic effects. Few strategies have been effective in preventing analgesic tolerance in studies aimed at preventing opioid tolerance. One of these is the result obtained from our previous research on rats. We have shown that α-2 AR agonists are effective in preventing FEN tolerance and enhancing FEN analgesia [1]. Although analgesic tolerance developed only in the opioid group, MOR immunoreactivity remained unchanged in both the opioid-tolerant group and the groups in which tolerance was prevented (with α-2 AR agonists). The fact that MOR levels did not change suggests that analgesic tolerance may be due to changes in intracellular signaling pathways. Based on this idea, we examined the role of β-arrestin-2 (a protein that causes MOR desensitization) in FEN tolerance and the synergism of opioidergic-α-2 adrenergic analgesia. To the best of our knowledge, this is the first study to address this issue.

Materials and methods

Subjects

In this study, 48 male adult Wistar-Albino rats of the same generation, weighing between 300 and 350 g, were used. Rats were kept in the experimental animal laboratory at appropriate humidity and temperature (21 ± 3°C and 50 ± 5%, respectively). The 12-hour light/dark cycle was meticulously maintained. The experimental animals were fed standard pellet feed and tap water. This study was approved by the Ege University Faculty of Medicine Animal Experimentation Local Ethics Committee. The tissues used in this study were obtained from the experimental animals used in our previous study (study number 2020-053, dated April 22, 2020). The necessary permissions were approved by the same committee, number 2021-097, dated December 22, 2021). The regulations regarding the working procedures and principles of animal experimentation ethics committees were complied with.

The regulations comply with the rules of the Central Animal Experiment Ethics Committee and the Local Animal Experiment Ethics Committee, as well as animal welfare and the 3R rule.

Drugs and groups

FEN and 0.9% NaCl used in our study were obtained from the pharmacy, DEX from Sigma-Aldrich, and ketamine and xylazine from Ege University Laboratory Animal Application-Research Center. A random distribution of eight rats was made in each group (because one animal was lost from the FEN + DEX group and two from the FEN + XYL group, the evaluations were based on forty-five animals). All groups received injections of medication or saline in the morning and evening on the first two days, and only in the morning on the third day. All groups received five intraperitoneal (*ip*) injections (Table 1).

Table 1
Experimental protocol. Drugs administered to rats, their doses, and times of administration are shown.

	DAY 1		DAY 2		DAY 3	
	Morning	Evening	Morning	Evening	Morning	
Control Group (n = 8)	0.9% NaCl	0.9% NaCl	0.9% NaCl	0.9% NaCl	0.9% NaCl	SACRIFICATION
FEN Group (n = 8)	40 µg/kg FEN	40 µg/kg FEN	40 µg/kg FEN	40 µg/kg FEN	40 µg/kg FEN	
DEX Group (n = 8)	10 µg/kg DEX	10 µg/kg DEX	10 µg/kg DEX	10 µg/kg DEX	10 µg/kg DEX	
XYL Group (n = 8)	1 mg/kg XYL	1 mg/kg XYL	1 mg/kg XYL	1 mg/kg XYL	1 mg/kg XYL	
FEN + DEX Group (n = 7)	40 µg/kg FEN 10 µg/kg DEX	40 µg/kg/ FEN 10 µg/kg DEX	40 µg/kg/ FEN 10 µg/kg DEX	40 µg/kg/ FEN 10 µg/kg DEX	40 µg/kg/ FEN 10 µg/kg DEX	
FEN + XYL Group (n = 6)	40 µg/kg FEN 1 mg/kg XYL	40 µg/kg FEN 1 mg/kg XYL	40 µg/kg FEN 1 mg/kg XYL	40 µg/kg FEN 1 mg/kg XYL	40 µg/kg FEN 1 mg/kg XYL	

Collection of tissue samples

On the 3rd day of the experiment, the rats were anesthetized with ketamine (100 mg/kg/*ip*) and xylazine (10 mg/kg/*ip*). After the depth of anesthesia was controlled by the foot pedal reflex, an abdominal incision was made in the rats. A catheter was inserted into the left ventricle of the rats, and perfusion was performed using 10% neutral formaldehyde. Dissection was performed after the perfusion. The spinal cord was carefully removed and fixed in 10% neutral formalin.

Indirect immunohistochemistry method and evaluation

After fixation, routine hematoxylin and eosin (H&E) staining was performed, and morphological evaluation was performed under light microscopy. Indirect immunohistochemistry staining was performed for immune reactivity evaluation of the samples. The sections were incubated overnight in a laboratory incubator at 60°C. The next day, the samples were kept in two different xylenes for half an hour. It was passed through a series of decreasing alcohol concentrations, from 95% to 60%. The samples were washed in distilled water with two 5 min changes. The sections were surrounded by Dacopen and washed with phosphate-buffered saline (PBS phosphate buffer solution). The cells were incubated with 1% trypsin in a 37°C oven for 10 min. After washing with PBS, the cells were treated with 3% H_2O_2 for 5 min. After washing with PBS, blocking solution (non-immune serum) was added and incubated for 1 h. After the block solution was removed, the primary antibody (Affinity Biosciences Beta Arrestin 2 Ab-DF7574) was added and kept in the refrigerator overnight. The incubated sections were washed with PBS the next day, and the secondary antibody (SignalStain Boost IHC Detection Reagent #8114) was added and kept at room temperature for half an hour. DAB chromogen was treated for 10 min to determine the visibility of the IHC reaction. After washing with distilled water in three changes of 5 min each, Mayer's hematoxylin was added for nuclear staining and kept for 5 min. After washing with distilled water for three changes of 5 minutes each, it was passed through ethyl alcohol increasing from 80% to 100%. The sections stored in xylene were covered with a mounting medium. Sections prepared for immunohistochemical evaluation were scored based on the severity of immunoreactivity. conducted an immunohistochemical evaluation, scoring it as 1 (+) for weak, 2 (+) for moderate, and 3 (+) for strong positivity. The preparations were examined under an Olympus B×43 type light microscope with a camera attachment (Olympus SC100) and photographed using a digital camera.

Data analysis

In the evaluation of immunoreactivity scores, the Chi-square and Fisher's exact tests were used to analyze the relationship between the score levels of the groups. A p-value of less than 0.05 was considered statistically significant.

Results

Morphological evaluation

The morphological structure of the hematoxylin and eosin (H&E)-stained tissues appeared normal. As shown in Fig. 1, no pathology was observed in the spinal cord tissue samples.

Immunoreactivity evaluation

After immunohistochemical analysis of the rat spinal cord, the immunoreactivity levels of the tissues were scored and are presented in Table 2. Accordingly, 25% of the control group was stained moderately and 75% strongly; of the FEN group, 25% was stained weakly, 37.5% was stained moderately, and 37.5% was stained strongly; of the DEX group, 50% was stained weakly, 12.5% was stained moderately, and 37.5% was stained strongly; of the XYL group, 12.5% was stained weakly, 62.5% was stained moderately, and 25% was stained strongly. In contrast, 28.6% of the FEN + DEX group was weakly stained, and 71.4% was moderately stained; 33.3% of the FEN + XYL group was weakly stained, and 66.7% was moderately stained. Statistical analysis revealed no significant difference between the control and FEN groups ($p > 0.05$). Similarly, compared to the control group, there was no statistical difference between the DEX and XYL groups ($p > 0.05$). However, there was a significant difference between the FEN + DEX and FEN + XYL control groups. β-arrestin-2 immunoreactivity levels were found to be significantly lower in the FEN + DEX and FEN + XYL groups ($p < 0.05$). Photographs of the groups after IHC analysis are shown in Fig. 2.

Table 2
Immunohistochemistry staining scores of the groups.

	Medulla Spinalis Region			p
	Weak	Moderate	Strong	
Control Group	0 (%0)	2 (%25)	6 (%75)	0,02
FEN Group	2 (%25)	3 (%37,5)	3 (%37,5)	
DEX Group	4 (%50)	1 (%12,5)	3 (%37,5)	
XYL Group	1 (%12,5)	5 (%62,5)	2 (%25)	
FEN + DEX Group [a]	2 (%28,6)	5 (%71,4)	0 (%0)	
FEN + XYL Group [a]	2 (%33,3)	4(%66,7)	0 (%0)	

Chi-square and Fisher's Exact Test analyses were performed. a: <0.05 is significant compared to the control group.

Discussion

Opioid analgesics are highly effective in treating moderate-to-severe pain. However, tolerance to the analgesic effects of opioids significantly limits their clinical application. Repeated or continuous use may cause undesirable side effects, such as analgesic tolerance and physical dependence. When tolerance develops, dose increases become necessary to maintain the therapeutic efficacy. MOR

belongs to the GPCR superfamily and is an opioid receptor subtype that mediates the physiological effects of opioids [6]. MORs can be regulated by multiple mechanisms, including receptor desensitization, internalization, resensitization, and downregulation [7]. Although the molecular mechanisms underlying opioid tolerance have not been fully elucidated, MOR desensitization may play a key role in the development of tolerance. A decreased response to opioid agonists has been associated with GPCR desensitization.

Arrestins are regulatory proteins for almost all known GPCRs [8] [8] and mediate their desensitization and internalization [9]. [8] and mediate their desensitization and internalization [9]. Arrestins are classified into four classes: visual arrestin, cone arrestin, β-arrestin-1, and β-arrestin-2. They play an important role in the regulation of GPCRs. β-arrestin-1 and 2 are highly expressed, particularly in nervous and lymphatic tissues, and regulate various GPCRs [10]. β-arrestins are desensitizing and receptor-modulatory proteins. β-arrestin-2, in particular, plays an important role in the opioid-induced antinociceptive tolerance mechanism [11]. β-arrestins are desensitizing and receptor-modulatory proteins. β-arrestin-2, in particular, plays an important role in the opioid-induced antinociceptive tolerance mechanism [11]. β-Arrestin-2, also called βarr-2 and arrestin-3, is a functional protein involved in GPCR signaling. This intracellular protein plays an important role in the rapid attenuation of GPCR signaling and the development of opioid tolerance [12]. β-arrestin-2 has been reported to mediate acute opioid tolerance and antinociception [11]. The primary contributing mechanism to tolerance in MORs at both the spinal and supraspinal levels is the β-arrestin-2 mediated desensitization mechanism [13]. Desensitization in MORs occurs through a series of intracellular events. When the Desensitization of MORs occurs through a series of intracellular events. When the agonist binds to the receptor, the receptor G protein is phosphorylated by kinases regulated by second messengers. After phosphorylation, a conformational change occurs in the receptors, and their affinity for cytosolic β-arrestin proteins increases. Arrestins bind to phosphorylated receptors and prevent their coupling to G proteins. Consequently, the normal activation of the secondary messenger signaling cascade is disrupted [14, 15]. G protein signaling is interrupted, and the cellular response is diminished. Signaling suppression results in receptor desensitization and endocytosis [7, 10].

β-arrestin-2 has physiological importance in opioid tolerance. Studies have aimed to clarify the contribution of β-arrestin-2 to physiological responses in desensitization mechanisms. It has been found that the analgesic effect of morphine is significantly enhanced and prolonged in mice with a deletion of the β-arrestin-2 gene [14]. n mice lacking β-arrestin-2, MOR desensitization did not occur, and antinociceptive tolerance did not develop [16]. In β-arrestin-2 knockout mice, morphine tolerance is completely reversible with a protein kinase C inhibitor [13]. It has also been shown that inhibiting β-arrestin-2 via siRNA lentivirus significantly improves analgesia [17] and delays tolerance [18]. This evidence clearly demonstrates that β-arrestin-2 plays a role in the mechanism of opioid-induced antinociceptive tolerance.

In our previous study, we demonstrated that tolerance develops to the analgesic effect of FEN, that α-2 AR agonists have the potential to prevent this tolerance, and that there is no change in MOR

immunoreactivity. The lack of change at the receptor level suggests that opioid tolerance may be due to changes in intracellular signaling pathways. Therefore, we examined β-arrestin-2 immunoreactivity in the spinal cords of experimental animals used in our previous study. The results obtained were interesting. Compared with the control group, β-arrestin-2 immunoreactivity tended to decrease in all groups. Although statistical significance emerged only in the groups receiving combined medication, we also found that strong staining was lower in the FEN, DEX, and XYL groups. These results show that the level of β-arrestin-2 circulating freely in the cytoplasm decreased in all groups, except the control group. FEN administered at high doses five times induced tolerance without causing downregulation of MORs. With the binding of β-arrestin-2 to MORs, the intracellular signaling cascade is interrupted, signaling cannot continue, and tolerance develops. Therefore, there was a decrease in the levels of β-arrestin-2 found free in the cytoplasm (although this was not statistically significant). This explains why β-arrestin-2 immunoreactivity tended to decrease in the FEN group and the tolerance that developed in this group. One of the limitations of this study is that FEN administration was limited to 3 days and 5 doses. If we had been able to perform a long-term application, we might have observed a statistically significant decrease in β-arrestin-2 immunoreactivity and MOR downregulation.

Repeated exposure to opioids has physiological significance. Prolonged stimulation of MORs and interruption of receptor signaling are called homologous desensitization. Persistent stimulation of MORs and the reduction of effector responses to GPCRs are termed heterologous desensitization. Dang et al. [19] howed that persistent stimulation of MOR first caused homologous desensitization of MORs and then caused heterologous desensitization of α-2 ARs. It has also been reported that α-2 AR desensitization occurs through β-arrestin-2. In this study, FEN, which we administered to rats, may have caused desensitization of both MORs and α-2 ARs through β-arrestin-2 protein. It is possible that the β-arrestin-2 immunoreactivity found free in the cytoplasm of both the FEN group and the groups receiving combined medication with FEN (FEN + DEX and FEN + XYL) decreased for this reason. In our previous study, we measured the latencies of responses to painful stimuli and found that analgesic potentiation tended to decrease on day 3 in the groups receiving the combined medication [1]. The decrease in analgesic potentiation on day 3 may be explained by FEN causing α-2 AR desensitization via β-arrestin-2. In other words, FEN caused desensitization of α-2 ARs; therefore, the analgesic potentiation observed on day 2 tended to decrease on day 3. This situation, reflected in analgesia tests, may be due to a decrease in β-arrestin-2 immunoreactivity levels in the cell cytoplasm, that is, desensitization. The statistical decrease in β-arrestin-2 reactivity in the combined drug groups indicates that intracellular signaling is affected and both homologous and heterologous desensitization develops.

Our study results showed that β-arrestin-2 immunoreactivity levels differed between the DEX and XYL groups (although not statistically significant). These two α-2 agonists substances we applied affected cytoplasmic β-arrestin-2 levels differently. One possible reason for this may be the doses of the medications used. Another reason may be the affinity of DEX and XYL for the α-2 AR subgroups. α-2 ARs are G protein-coupled receptors with four pharmacological subtypes (α-2A, α-2B, α-2C, and α-2D) [20]. In humans, rats, and mice, the α-2A, α-2B, and α-2C subtypes are found [21]. It is known that α-2A and α-2C ARs are expressed in the rat spinal cord [22]. It is known that α-2A and α-2C ARs are expressed in the rat

spinal cord [22]. DEX and XYL differ in their affinities for the α-2 AR subtypes. It has been shown that DEX acts only on α-2A ARs [23, 24, 25] while XYL is not selective [26] and acts by binding to both α-2A ARs [27] and α-2C ARs [28]. Arrestins also act on ARs, a family of G-protein-coupled receptors. All three α-2 ARs subtypes interact with β-arrestin-2 [8].

β-arrestin-2 expression, which has a functional role in stabilizing receptor phosphorylation on α-2A and α-2B, mediating endocytosis and desensitization, increasing the rate of ERK signaling, and increasing response sensitivity in vivo, has been shown to differentially regulate α-2 AR subtypes [8]. Arrestin expression has been reported to differentially regulate the trafficking of different α-2-AR subtypes, and the coexpression of β-arrestin-1 or β-arrestin-2 has been reported to significantly increase α-2B AR internalization. It has also been noted that α-2C AR internalization is selectively promoted by the coexpression of β-arrestin-2, while α-2A AR internalization is mildly stimulated by the coexpression of both arrestins [9]. Considering that DEX binds to α-2A ARs and XYL binds to both α-2A ARs and α-2C ARs, and that β-arrestin-2 has the potential to affect α-2 subgroups differently, the difference in β-arrestin-2 immune reactivity in the DEX and XYL groups is understandable.

Conclusion

Our data suggest that FEN, which we applied at a high dose for three days, caused β-arrestin-2 mediated receptor desensitization without causing a change in MOR level. Our study revealed the physiological importance of β-arrestin-2, which causes desensitization of both MOR and α-2 ARs. β-arrestin-2 caused FEN tolerance by affecting both MORs and affected the analgesic potentiation of DEX and XYL by affecting α-2 ARs. We support the view that one of the mechanisms underlying opioid tolerance is mediated by β-arrestin-2. Further comprehensive studies are needed to elucidate the role of β-arrestin-2 in opioid tolerance and the analgesic synergism of opioids with α-2 AR agonists.

Declarations

Author contributions Deniz Yildiz Pehlivan (D.Y.P): Conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review & editing, visualization. Ali Yücel Kara (A.Y.K): Conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing – original draft, writing – review & editing, visualization, project administration, supervision.

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Data availability The datasets generated and analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

Institutional review board This study was conducted with the ethics committee approval number 2021-097, dated December 22, 2021, from the Ege University Faculty of Medicine Animal Experimentation

Local Ethics Committee.

Competing interests The authors declare no competing interests.

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Figures

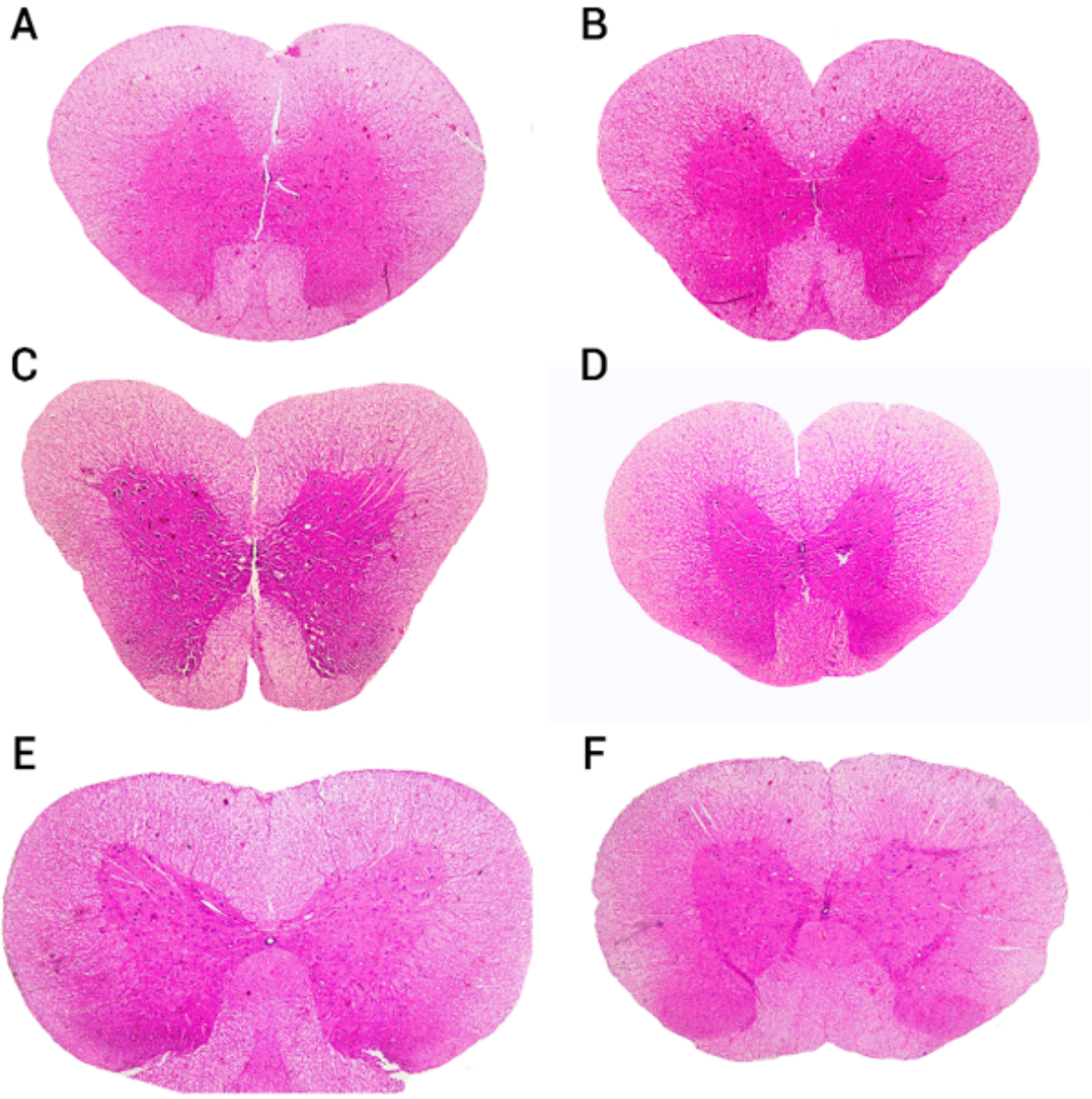


Figure 1

H&E staining images of tissue samples taken from the spinal cord (x4, bar: 200 μm). A: Control group, B: FEN group, C: DEX group, D: XYL group, E: FEN+DEX group, and F: FEN+XYL group.



Figure 2

Immunohistochemical staining images of the groups (x4, bar: 200 μm). A: Control group, B: FEN group, C: DEX group, D: XYL group, E: FEN+DEX group, and F: FEN+XYL group.