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Systemic Administration of Minocycline Reduces Fos Protein Expression in Rapid Eye Movement (REM) Sleep Deprived Rat's Spinal Cord But Not Nociceptive Response After Formalin-Induced Inflammatory Pain

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Abstract: **Minocycline is a semisynthetic second-generation tetracycline, an antibiotic that capable to penetrating the blood brain barrier and believed to have anti-inflammatory and neuroprotective effects. This study want to investigates the effects of minocycline on the nociceptive response and Fos protein expression in the spinal dorsal horn of the REM sleep deprived rat after a formalin injection. REM sleep deprivation was elicited using the modified inverted flowerpot method. Minocycline (30 mg/kg) was given intraperitoneally once a day during the 72 hours of REM sleep deprivation and 30 minutes before formalin test. Fifty microliter of formalin (2.5%) was subcutaneously injected into the plantar surface of the left hind paw of the rat. The nociceptive responses were recorded for one hour. Two hours after the formalin injection, the rats were sacrificed and expression of the Fos positive cell was examined. The nociceptive responses were found not statistically significant in all phases between all groups. However, enhancement in the number of the Fos positive cell was significantly higher in the REMsd group compared to other groups but reduced after minocycline treatment. Pre-emptive administration of minocycline reduces Fos protein expression in REM sleep deprived rat's spinal cord but not the nociceptive response after inflammatory pain.**

Keywords: **Fos protein expression, Formalin test, Minocycline, Nociceptive response, REM sleep deprivation, spinal cord dorsal horn.**

I. INTRODUCTION

Minocycline is a semisynthetic second-generation tetracycline, a type of antibiotic that is capable of penetrating the central nervous system through penetration of the blood brain barrier. Minocycline is the first non-toxic drug with a proven human safety record and is shown to selectively inhibit activation of microglias in the central nervous system. Anti-inflammatory and neuroprotective effects of minocycline are postulated to be caused by its ability to suppress the microglial activation [1].

Microglial cells are emerging as possible additional players in the initiation of chronic neurodegeneration and also pain mechanism. This glial cell has close interactions with neurons; hence, it can modulate pain transmission, particularly under pathological condition [2]. Stress conditions have been shown to overactivate microglial cells and lead to the release of reactive oxygen species (ROS) [3], pro-inflammatory cytokines and chemokine such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), prostaglandin E_2 (PGE_2) and nitric oxide [4]. IL-1β and TNF-α have been reported to increase in the cerebral spinal fluid during sleep deprivation [5]. These cytokines are possibly released by microglial cells which are activated during stressful conditions.

Sleep is critical for the maintenance of health and the support of life. Sleep is an active process that not only conserves energy but also acts as a defence mechanism against oxidative stress [6], facilitating neurogenesis [7], and enhancing brain protein synthesis [8]. Sleep, in general, can be divided into two types: non-rapid eye movement (NREM) and rapid eye movement (REM) sleep [9]. Sleep deprivation has been reported to be associated with enhanced sensitivity to pain and impaired cognitive function and memory [10-12]. Studies have reported that REM sleep deprivation induces a significant increase in pain responses in many pain modalities such as mechanical, thermal and electrical [13-14] but not chemical pain stimulus in the rat.

The systemic administration of minocycline (microglial activation inhibitor) has been reported to reduce nociceptive response during the tonic phase, but not during the acute phase of the formalin test which was performed on a normal rat. In addition to that, the reduced inflammatory pain is consistent with a significant decrease in the number of Fos positive cell expression in the spinal cord dorsal horn and magnitude of paw edema after a formalin injection [15]. However, the association of minocycline with the effect of REM sleep deprivation on a nociceptive response after the formalin-induced inflammatory pain is still unknown and needs further investigation. Therefore, this study is conducted to investigate the effects of minocycline on the nociceptive response and Fos positive cell expression in the REM sleep deprived rat's spinal cord after a formalin injection.

II. METHODS

Animal preparation:

Thirty six male Sprague Dawley rats weighing 200-250g were maintained in a 12-h light dark cycle and allowed free access to food and water *ad libitum*. Animals were obtained from the Animal Research and Service Centre (ARASC), USM. All protocols were designed to minimize animal pain and discomfort and were conducted with the approval of the Universiti Sains Malaysia Animal Ethics Committee [2013/ (85) (443)].

The rats were allocated into 6 groups consisting of a control group (C) (n = 6), minocycline group (30 mg/kg i.p) (M) (n = 6), REM sleep deprivation group (REMsd) (n = 6), REM sleep deprivation + minocycline (30 mg/kg i.p) treatment group (REMsd + M) (n = 6), Wide platform control group (WP) (n = 6) and Wide platform + minocycline (30 mg/kg i.p) treatment group (WP + M) (n = 6). Minocycline was administered intraperitoneally (30 mg/kg body weight) once a day during the 72 hours of the REM sleep deprivation procedure and 30 minutes before the formalin injection. The dose for minocycline administration was based on the previous study [15, 31]. The dose of minocycline (30 mg/kg i.p) has found can attenuate significantly the nociceptive response and Fos protein expression in rat spinal cord dorsal horn in acute pain [15] and also prevented the neuropathic pain [31]. From these studies we assume that the dose of 30 mg/kg i.p of minocycline is sufficient to attenuate acute pain in this study. Therefore a single dose of minocycline (30 mg/kg i.p) was chosen in this study rather than multiple doses for dose dependent effects.

Induction of REM sleep deprivation :

The modified inverted flowerpot method was used to selectively REM sleep deprive animals for 72 hours based on our previous study [16]. Before starting the experiment, all rats except the ones from the controlled group were isolated and adapted individually in a dry tank model for 72 hours before being exposed to the REM sleep deprivation model. The controlled group of rats was adapted in their normal dry cage for 72 hours. The purpose of the adaptation was to expose and adapt the rats to the glass tank environment before being exposed to the REM sleep deprivation model. For the REM sleep deprivation procedure, two small platforms of 6.5 cm diameter, 8.5 cm height and 8 cm length between both platforms were placed in a glass tank measuring 50 cm in height, 50 cm in width and 100 cm in length. The rats were deprived of REM sleep by placing one rat at a time for 72 hours on top of one of the two platforms (6.5 cm in diameter) placed in the middle of a glass tank filled with water (the platform was 1 cm above water). REM sleep was prevented by the muscular atonia where the rats were awakened when their body came in contact with water.

For the Wide platform control group, each rat was placed in the same experimental condition as the REM sleep deprivation model rats except that the diameter of the platform was larger (14 cm, 8.5 cm height and 8 cm length between both platforms) which allowed REM sleep to occur. The purpose of this WP group was to expose them to the same aquatic environment as the REM sleep deprivation rats but allowed them to experience both NREM and REM sleep *ad libitum* [17]. This platform technique has been validated by other studies using electroencephalography and has been shown to deprive a small degree of non-REM sleep [17-18]. The temperature of the water in the tub was 30 °C. Food and water were available *ad libitum* throughout the time the rats were on the platforms.

Formalin-induced nociceptive response:

The rats were injected with 50 µl of 2.5% of formalin into the plantar surface of the left hindpaw with a 26-guage needle. All the rats were subjected to behavioral testing. The experiment was performed in a Perspex testing chamber measuring 26 cm x 20 cm x 20 cm. A mirror was placed below the floor of the chamber at a 45° angle to allow an unobstructed view of the rats' paws. The rat's behaviors were recorded with a videocam starting from the time of injection until the end of one hour. The tape was analyzed by one observer who was blinded to the treatment of each rat and the formalin test score was tabulated every minute and averaged at 5-minute intervals [19].

The scores were as follows: 0 = the injected paw is not favored (i.e. foot flat on the floor with toes splayed) indicating insignificant or no pain felt; 1 = the injected paw has little or no weight on it with no toe splaying indicating mild pain felt.; 2 = the injected paw is elevated and the heel is not in contact with any surface indicating moderate pain.; 3 = the injected paw is licked, bitten or shaken indicating severe pain.

The quantification of nociceptive response is based on the weighted scores technique described by Dubuisson & Dennis, 1997 [20]. This weighted scores technique is more likely to reflect the pain experience of the animal being tested [21].

Fos positive cell expression:

After a 2-hour formalin test, rats were sacrificed by an overdose of an intraperitoneal injection of sodium pentobarbitone. This method was used to avoid damage to the spinal cord [22]. A thoracotomy was done to expose the heart. An 18G branula was then inserted into the left ventricle and a snip was made to the right atrium for an outlet. Perfusion was performed using the gravity method with phosphate buffered saline (PBS) followed by 500 ml of cold 4% paraformaldehyde in phosphate buffer (PB) 0.1M (pH 7.4) [23].

The spinal cord was dissected out from the rats. Following an overnight cryoprotection in sucrose 20% in PB 0.1M, the spinal cord was cut using a cryostat and the lumbar spinal cord region was collected as free-floating section in PBS. The lumbar spinal cord section was incubated overnight at 4°C with primary antibodies for Fos protein (Calbiochem, USA) diluted 1: 20 000 in buffer (2% normal goat serum, 0.2% triton-X, TBS). This was followed by incubation with biotinylated secondary antibody for 1 hour. After 1 hour, the section reacted with Avidin-biotin complex (ABC) and stained with diaminobenzidine and hydrogen peroxide until a brown coloration was seen. The section was then mounted on slides, air-dried, dehydrated and put under a cover slip.

The sections were examined using an image analyzer (Leica MPS 60) at magnifications of 40X and 100X under an objective lens. At least 8-12 of the L4/L5 sections were scanned for each rat, and six sections were selected. For each rat the number of Fos positive cells on the ipsilateral side of the dorsal spinal cord were identified according to the specific laminar regions of the spinal grey matter, as follows: superficial dorsal horn (laminae I and II), nucleus propria (laminae III and IV), neck of the dorsal horn (laminae V and VI). The Fos positive cells were measured in every area unit (130 X 130 µM) in each of the specific laminar regions. The grey matter landmark was determined according to Molander *et al.* (1984) [24].

Statistical analysis:

All the data is reported as mean ± standard error mean (S.E.M). Formalin nociceptive response was analysed using repeated measurement analysis of variance (ANOVA) and when the analysis revealled a significant difference, post hoc (Bonferroni test) was used to determine the differences between the specific groups. The Fos positive cell expression on the ipsilateral side of the dorsal spinal cord was measured using One-way ANOVA and post hoc (Bonferroni test) was used to determine the differences between the specific groups.

III. RESULTS

Formalin-induced nociceptive response:

All rats showed discrete biphasic nociceptive responses which can be divided into two phases; acute and tonic phases as illustrated by Fig. 1(A). The early short lasting acute phase was observed during the initial 5 minutes response whereas the tonic phase was observed between minutes 15 to minutes 60. The early acute phase and late prolonged tonic phase was separated by a 5 minute quiescent period. No statistical significant differences were observed on the nociceptive response between all groups in the acute and tonic phases as illustrated by Fig. 1(B) and Fig. 1(C).

Fos positive cell expression:

C-Fos gene is an immediate early gene and expression of its protein product, Fos protein has been accepted as a marker to analyze nociceptive pathways [25]. Fos positive cell (CFI) expression on the ipsilateral side was evaluated in the superficial dorsal horn (laminae I-II), nucleus proprius (laminae III-IV) and the neck of the dorsal horn (laminae V-VI) of the L4-5 spinal dorsal horn 2 hours after the formalin injection (Table 1 and Fig 2). The period of two hours after the formalin injection is chosen because Fos protein expressed maximally at this time [26]. The number of CFI on the ipsilateral side of the L4-5 spinal cord dorsal horn was extensively increased in REMsd group ($p<0.001$) compared to the other groups in all laminae. However, this enhancement of CFI following the formalin injections was significantly attenuated by minocycline treatment ($p<0.001$) as showed in REMsd+M group (Table 1 and Fig 2d).

IV. DISCUSSION

In this study, Fos protein expression but not nociceptive response of the REM sleep deprived rats were seen attenuated after the formalin injection, following the minocycline treatment. The occurrence of REM sleep deprivation using the inverted flower pot technique is confirmed by the demonstration of hyperphagia with concurrent body weight loss (data not shown) which is similar to reports by Siran et al., 2014 [16]. REM sleep deprivation has been reported to increase nociceptive responses in many pain modalities such as mechanical, thermal and electrical but not chemical (formalin test) pain stimuli [13-14]. However, the formalin test is different from most models of pain. In this model, it is possible to assess the way an animal responds to moderate, continuous pain generated by injured tissues [27]. Due to this connection to tissue injury, it is believed that the formalin test provides a more valid model for clinical pain as its tonic nature makes it resemble human postoperative pain more closely than the tests with phasic mechanical or thermal stimuli [28].

Nociceptive responses after the formalin injections can be separated into two distinct phases. The acute phase starts immediately after injection of formalin and lasts for 3-5 minutes. It is due to direct chemical stimulation of nociceptors [21] and predominantly evokes activity in C and not in Aδ fibres. The tonic phase starts approximately 15-20 minutes after the formalin injection and lasts for 20-40 minutes. It is involved in the central nervous system such as in the activation of NMDA receptors as well as local inflammatory changes during the tonic phase [27]. In this study, nociceptive responses were found not significantly different in the acute and tonic phases. However previous studies have shown that systemic administration of minocycline reduces nociceptive responses during the tonic phase of the formalin test [15].

The discrepancy between these results may be due to different experimental procedure such as the duration of drug delivery, the percentage of formalin used, quantification of nociceptive response and the condition of the rats. Minocycline was given intraperitoneally 1 hour before the formalin injection and the percentage of formalin used was 5% in Cho *et al*., (2006) [15] study which was performed on normal healthy Sprague Dawley rat. While in this study, minocycline was administered during the 72 hours of the REM sleep deprivation procedure and 30 minutes before the 2.5% formalin injection. In this study, nociceptive response was quantified based on the weighted scores technique described by Dubuisson & Dennis, (1997) [20] while licking and lifting the paw was considered as nociceptive response in Cho *et al*., (2006) [15] study. Perhaps, due to different experimental procedure, especially the percentage of formalin and the procedure to quantify the nociceptive response could contribute to the lesser effects of nociceptive responses especially in REM sleep deprived rats in this study. Other studies have also demonstrated that by using 5% of formalin percentage, the third phase of the formalin test appears to be closely related to microglial activation in rat's spinal cord starting on the first to third day and peaking on the seventh day post-injection [29-30].

Previous studies also show that minocycline, an inhibitor of microglial activation, by inhibiting the release of pro-inflammatory and reducing oxidative stress, can prevent the development of neuropathic pain but not acute pain [31]. Perhaps by using 5% percentage of formalin rather than 2.5%, and quantification of licking and lifting the paw as nociceptive response rather that weighted scores technique, the effect of minocycline on nociceptive response especially in REM sleep deprived rat could be clearly seen in this study. The discrepancy also could be due to sleeping and sleepiness factor that occur in REM sleep deprived rat. The REM sleep deprived rats are under high sleep pressure condition and nociceptive response that was recorded could not represent the actual response to formalin injection due to these factors. This suggests that why the nociceptive responses in REM sleep deprived rat and the effect of minocycline in this group not statistically significant difference compared to other groups in this study. This situation has been reported by Hakki Onen *et al*., (2001) [14] in REM sleep deprived rat nociceptive response after formalin test.

The present study has demonstrated that REM sleep deprivation is responsible for the upregulation of Fos protein expression on the ipsilateral side of the spinal dorsal horn in the REMsd group. There was a significant increase in Fos positive cell expression in all laminae on the ipsilateral side of the dorsal spinal cord in the REMsd group compared to the Control group ($p<0.001$) and WP group ($p<0.05$) (Table 1). However, stress may be the contributive factor to an increase in Fos protein expression in the laminae I-II on the ipsilateral side of the dorsal spinal cord of rats in the WP group compared to the Control group ($p<0.01$) in this study (Table 1). The rat in the WP group was kept in a glass tank and exposure to this environment may increase the anxiety and stress level of those rats compared to the normal cage for the Control group.

Minocycline has been reported act as an anti-inflammatory and neuroprotection by decreasing free radical formation, inhibiting inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, inhibits apoptosis process through inhibition of caspase-1 and caspase-3 activation and enhancement of Bcl-2- derived effects [32]. In the spinal cord, minocycline has been reported to attenuate the nociceptive response with correspond to reduce Fos protein expression in the rat's spinal cord by inhibited synaptic currents of substantia gelatinosa (SG) neurons in the spinal cord dorsal horn but not affected the membrane electrical properties of dorsal root ganglion neuron [15]. The injections of formalin into the hindpaw of the rats are reported to increase Fos protein expression at L4/L5 segmental levels of the spinal cord dorsal horn [33-35]. Fos protein has been extensively used as a specific marker to map out regions in the spinal cord corresponding to noxious stimulation. It is also used as a marker for the effect of analgesic or anaesthetic agents in the treatment and/or prevention of pain [36]. In this study, the reduction of Fos positive cell on ipsilateral side of spinal cord dorsal horn is statistically significant by minocycline treatment. However, the effects of minocycline on Fos protein expression do not correspond to the nociceptive response in this study. These effects are unclear but the findings suggest that the reduction of Fos protein expression in the spinal cord dorsal horn by minocycline in this study not correspond to nociceptive response may be due to experimental procedure that created high sleep pressure condition in REM sleep deprived rat and nociceptive response that was recorded could not represent the actual response to formalin injection.

It should be noted that other studies have also reported that nociceptive responses and spinal Fos protein expression may be uncoupled under certain circumstances [37-38]. Thus, a Fos protein expression in the spinal cord dorsal horn is not always able to be used as a prediction to nociceptive responses. Therefore, the measurements of other markers such as proinflammatory and antioxidant markers and Fos protein expression in other pain pathway such as in the brainstem and thalamus are needed to give a better explanation to the effects of minocycline on the Fos protein expression in this study.

V. CONCLUSION

This study has demonstrated that the systemic administration of minocycline reduces Fos protein expression in the REM sleep deprived rats' spinal cords but not the nociceptive response after the formalin injection. The effect of minocycline on the Fos protein expression in the spinal cord dorsal horn is dissociated from the nociceptive response in the formalin test in this study probably due to experimental procedure and Fos protein expression in the spinal cord dorsal horn is not always able to be used as a prediction to nociceptive responses. Further biochemical and pharmacological investigations should be conducted to assist in the understanding of minocycline actions at the central nervous system in modulating pain mechanisms especially in stressful conditions such as REM sleep deprivation.

Authors' contribution:

Noorul Hamizah Mat and Syarina Nurrulfatin Ab Rahman carried out research experiment and data analysis. Idris Long defined the intellectual content, edited the paper, and was the guarantor for the entire experiment. All authors read and approved the final paper.

Conflict of interest:

The authors declare that there is no conflict of interests regarding the publication of this paper.

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APPENDIX – A

List of Figures:

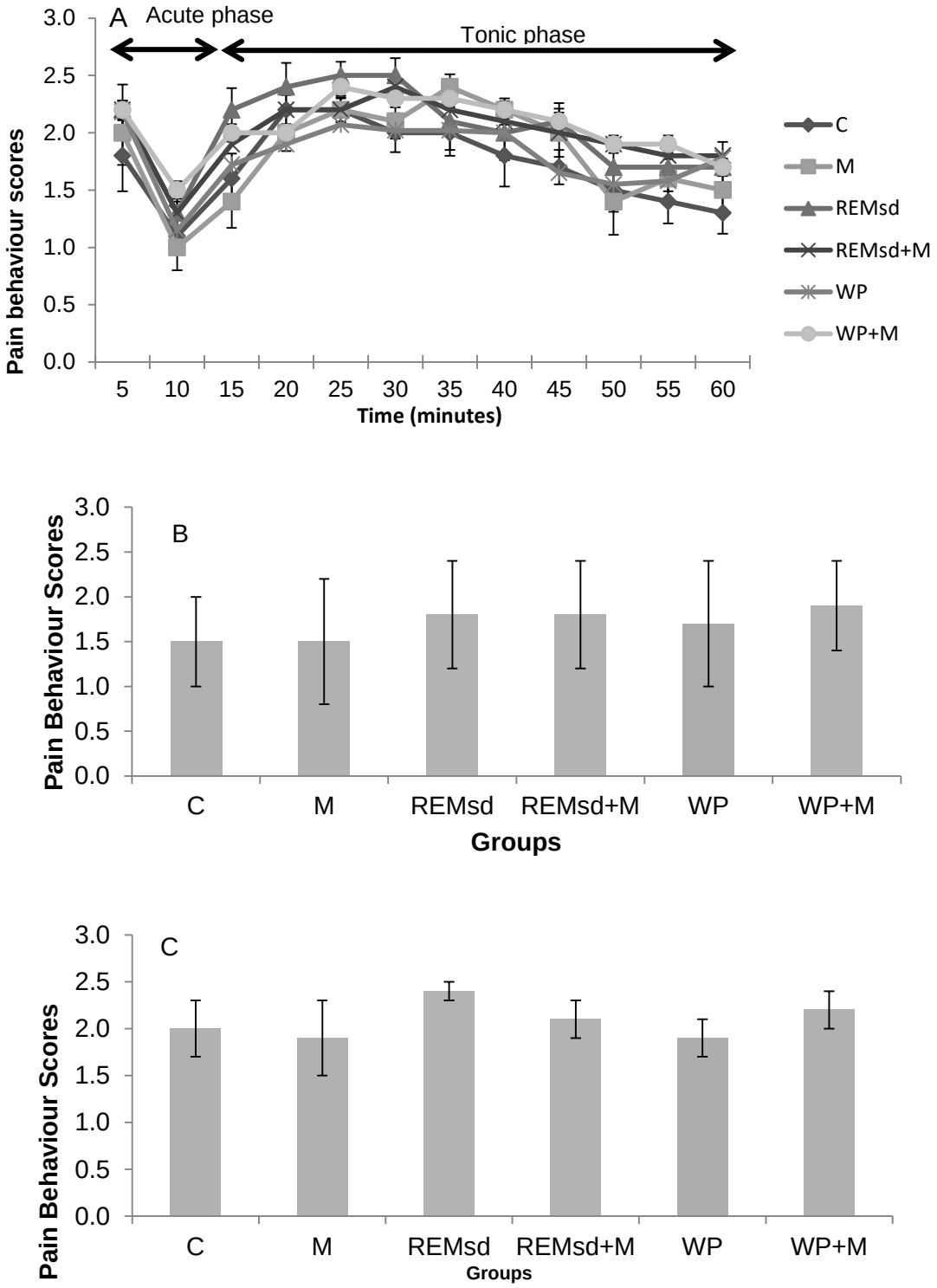

Figure 1.

Fig. 1. (A) Pain behavior response for all groups in 1-hour periods. (B) Pain behavior response during acute phase. (C) Pain behavior response during the tonic phase. Control group (C), Minocycline group (M), REM sleep deprivation group (REMsd). REM sleep deprivation + Minocycline group (REMsd+M), Wide platform group (WP), Wide platform + Minocycline group (WP+M). Values are the means ± S.E.M. n = 6 for all groups.

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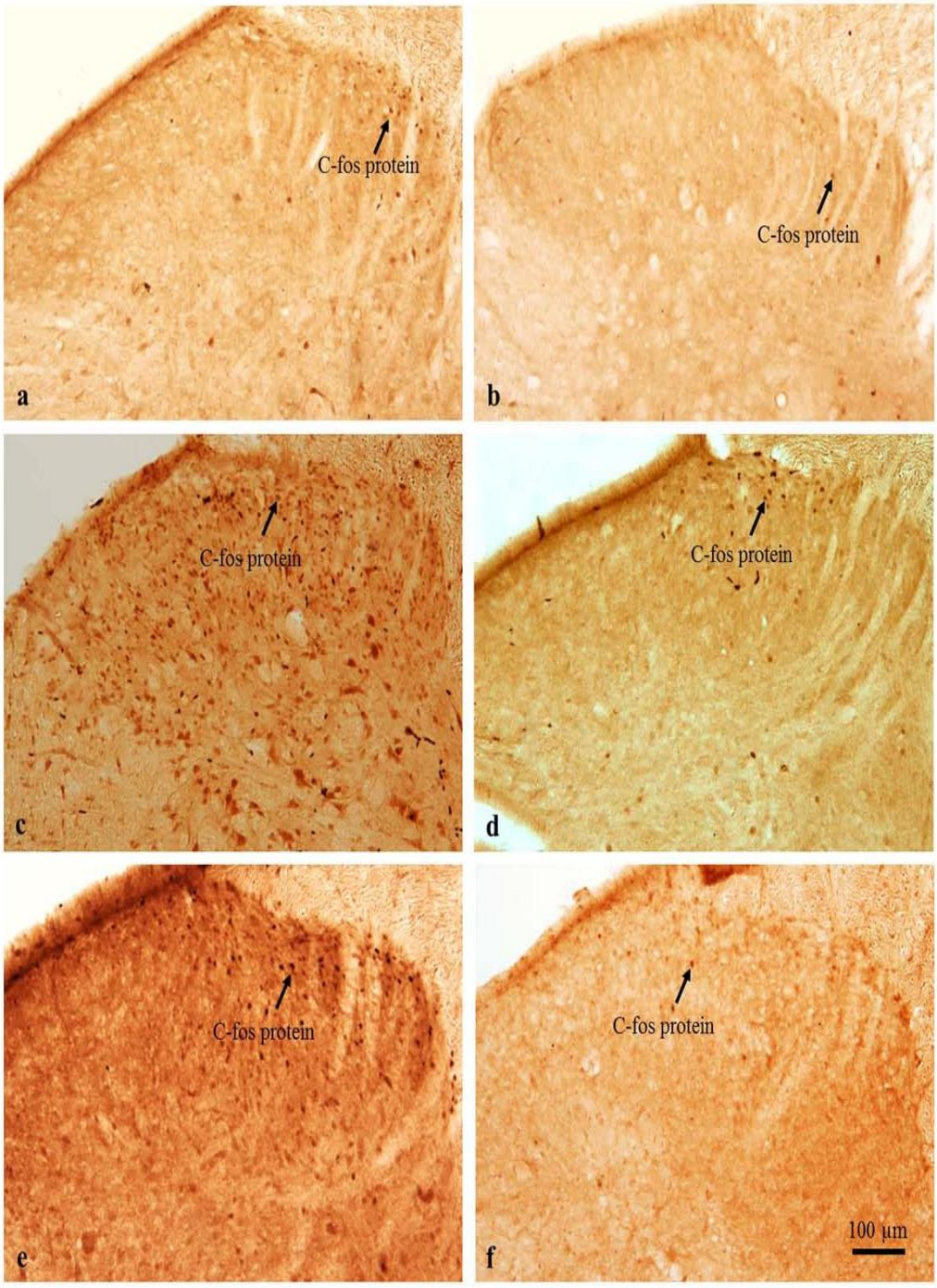

Figure 2

Fig. 2. Photomicrographs (original magnification 40× objective lens) showing Fos positive cells expression on the ipsilateral side of spinal cord sections of control group (C) (a), Minocycline group (M) (b), REM sleep deprivation group (REMsd) (c), REM sleep deprivation + Minocycline group (REMsd+M) (d), Wide platform group (WP) (e) and Wide platform + Minocycline group (WP+M) (f). The arrow indicates the dark staining of Fos positive cells.

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Table 1. Total Number of c-fos protein expression on the Ipsilateral Side of spinal cord dorsal horn for all groups according to specific laminar after formalin injection

Laminar	Treatment groups					
	C	M	REMSD	REMSD+M	WP	WP+M
Laminae I, II	10.59 ± 0.86***	4.94 ± 0.30***	20.4 ± 1.57	10.17 ± 1.22***	15.95 ± 0.80*ϒϒ	8.91 ± 0.65***
Laminae III, IV	5.33 ± 0.54	1.39 ± 0.15**	7.64 ± 0.81	4.58 ± 0.74**	5.72 ± 0.52	4.42 ± 0.45**
Laminae V, VI	9.23 ± 0.55	2.84 ± 0.19***	10.7 ± 0.80	6.22 ± 0.55***	8.61 ± 0.44	5.26 ± 0.32***

Table 1. Total number of Fos positive cells expression on the Ipsilateral Side of spinal cord dorsal horn for all groups according to specific laminar after formalin injection. Values are the means ± S.E.M. n = 6 for each group. Control group (C), Minocycline group (M), REM sleep deprivation group (REMsd). REM sleep deprivation + Minocycline group (REMsd+M), Wide platform group (WP), Wide platform + Minocycline group (WP+M). *, $p<0.05$ compared to REMsd group. **, $p<0.01$ compared to REMsd group, ***, $p<0.001$ compared to REMsd group. ϒϒ, $p<0.01$ compared to Control group.