Drug discovery for pain management is an important area of research given current limitations in medications for pain, including the addictive potential of opiates. While exercise may help worsen or lessen pain, the precise molecular mechanisms involved are incompletely understood. Thus, in the present study, we evaluated the effects of exercise intensity on pain via assessment of behavior and c-Fos expression. An animal model of moderate and high-intensity treadmill exercise was established. The specific nociceptive behaviors, such as flinches, favoring, lifting, and licking, were observed within 60 min following intraplantar injection of formalin. Lifting and licking times in the 1 h following formalin injection were shorter in the moderate/high-intensity exercise groups than the control group. The common pain scores in the exercise groups were significantly lower than those in the control group. There was no significant difference among the three groups. Moderate/high-intensity exercise decreased c-Fos expression in the ipsilateral dorsal horn. These results suggest that different intensities of exercise may substantially influence pain-related responses. Exercise may reduce c-Fos expression and attenuate pain-related behaviors and provide insight into how exercise may reduce pain. Further research is needed to understand the precise mechanisms by which exercise may reduce c-Fos expression as the mediating entities may represent suitable targets for medication development for pain management, including medications that might be used in lieu of or in conjunction with exercise.

INTRODUCTION

The relationship between exercise, fatigue and pain is complex. Exercise may induce or alleviate fatigue, depending on multiple factors including the intensity and duration. Similarly, exercise may induce or alleviate pain, which may relate to fatigue or be independent from it. Exercise-induced fatigue represents a specific physiological process, which may reach a homeostatic level. For these and other reasons, the integration and modulation of exercise intensity while undergoing physical training, es-

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†Abbreviations: CNS, central nervous system; G, gauge; IgG, immunoglobulin G; NGS, normal goat serum; PB, phosphate buffer; PBS, phosphate-buffered saline; SEM, Standard error of the mean.

Keywords: exercise, pain, formalin, c-Fos, rat
Liu et al.: Exercise intensity and pain

especially for athletes, has been a focus of research in exercise physiology and sports medicine [1-3]. Exercise influences biochemical processes not only within exercising muscle cells but also in other organs, including the brain. Exercise-related fatigue may have a pronounced impact upon the central nervous system, resulting in stress and pain responses, amongst others.

Multiple studies suggest an important and complex relationship between exercise and pain. Animal research has shown that responses to nociceptive stimulation change under stressful conditions [4]. Certain intensities of exercise may reduce chest pain associated with cardiovascular disease [5]. The performance of marathon runners also seemingly contrasts with their pain perception [6,7]. Specifically, some runners respond differently to stress, with faster runners showing a modest temporary reduction in pressure pain not seen in the slower runners [6]. These findings seem to resonate with those in rats indicating that fitness level may influence the perception of pain; for example, in rats bred for high and low aerobic capacities, the former have higher thresholds for pain both before and after exercise [8]. Exercise (swimming) may reduce behavioral hypersensitivity in formalin- and nerve injury-induced persistent pain in animals [9]. A study modeling neuropathic pain in rats suggested that swimming may alleviate pain related to nerve injuries [10]. Our previous study showed that exercise by forced swimming may increase the pain threshold in rats [11]. These studies suggest that the reactiveness and responses to pain relates importantly to exercise, although the precise molecular mechanisms remain unclear.

Few mechanisms underlying exercise-related changes in pain perception and behaviors have been studied at neurochemical and molecular levels. For example, the degree of lower limb pain increases following incremental load exercises, while it appears reduced during the late period of intermittent exercise [12]. Aerobic exercise may effectively alleviate pain in patients with some motility disorders (such as intermittent claudication) [13] and relieve pain after cardiac surgery [14], with static exercises perhaps being less effective in altering pain thresholds [15]. Aerobic exercise may stimulate the release of endogenous opioid peptides and increase nociceptive (i.e., pain) thresholds [16,17]. Sustained exercise may lead to a prolonged release of endogenous opioid peptides and generate decreased sensitivity to mu-opioid agonists like morphine [18,19].

It has been demonstrated that c-Fos and its protein product Fos are expressed in the spinal cords of rats subjected to peripheral noxious stimulation. As such, c-Fos expression has been described as a valuable tool in pain research [20]. Given the role of c-Fos expression in pain, it may represent an important target (either direct or indirect target) for treatment development for pain expression, as might occur in chronic pain syndromes or with persistent exercise. The purpose of the present study was to extend prior studies to examine the effects of different intensities of exercise on pain behaviors and c-Fos expression in the spinal cord, and then to determine if pain measures are correlated with exercise output. We aimed to extend prior research performed using swim tests to examine the extent to which prior findings may generalize to other models, specifically a treadmill procedure.
MATERIALS AND METHODS

Animals

Thirty-four female Sprague Dawley rats (3 months old, weighing 200 ~ 220 g, provided by the Animal Center of Xi’an Jiaotong University) were studied. The protocol and animal care were in accordance with the guidelines approved by the Committee on the Ethics of Animal Experiments of Xi’an Jiaotong University. The rats were housed in specific-pathogen-free cages (three rats/cage) on a 12-h light/dark cycle with food and water available.

Overview of Experimental Timeline

The following timeline was used in the experimental procedure, described in detail in the following paragraphs. On the day of experimentation, animals were run through the treadmill exercise experimental procedure. Within 10 minutes after running, they undertook the formalin-induced pain procedure. Over the course of 60 minutes, behavioral responses were recorded every five minutes. Within 30 minutes of the behavioral responses having been recorded, neural tissue was extracted for preparation, storage, sectioning, and staining for c-Fos. Measurement for c-Fos was then undertaken.

Establishment of the Exercise-intensity Model and Groups

The exercise-intensity model was based on rats running on small animal treadmills (Chengdu Taimeng Software Co. LTD, China) between 9:00 AM and 12:00 PM. Rats were familiarized with exercise on the treadmill by running at a speed of 10 m/min at a 5 percent inclination for 10 min per day for 3 consecutive days prior to the experiment. The purpose of this preliminary exercise was to eliminate those rats that were not able to adapt to the treadmill. Four animals were excluded in this way, and the remaining rats were divided into control, moderate-intensity, and high-intensity exercise groups (n = 10 for each group). The moderate-intensity exercise group ran at a speed of 18 m/min for 100 min. The high-intensity exercise group ran at a speed of 28 m/min for 20 min [21-23]. The levels selected to reflect moderate and high levels of exercise-induced fatigue were in accordance with general standards employed in studying sports fatigue [24]. Sound and brush stimulation were used to prevent the rats from stopping their running in the exercise process. The control group did not exercise.

Figure 2. The nociceptive behaviors including flinches (A), lifting (B), licking (C), and common pain score (D) decreased significantly in both exercise groups in the first 5 min following the ipsilateral injection of formalin. There was no significant difference between the moderate- and high-intensity groups. * P < 0.05, compared with control.
nociceptive behaviors in each 5-min cycle for 1 h after an intraplantar injection of formalin. Favoring means that the injected paw experienced little weight and rested on the floor without pressure on the footpad; during locomotion there was a definite limp. Lifting indicated that the injected paw was elevated without touching the floor. Licking indicated that the injected paw was licked or bitten. The common pain score was calculated according to the numerical scale below: 0 meaning no pain (normal weight bearing on the injected paw); 1 meaning favoring; 2 meaning lifting; 3 meaning licking. Then, a weighted specific nociceptive pain and nonspecific behavioral state score, ranging from 0 to 3, was generated by multiplying the time spent in each category with the weight; then, these products were summed and divided by the total time for each 5-min block of time.

The formula of common pain score is given by:

\[ p = \frac{\sum s_i t_i}{300} \]

Where \( p \) is the common pain score, \( s_i \) is score of each nociceptive behavior, and \( t_i \) is the time \( s_i \) lasted. The unit of time is seconds.

**Behavioral Testing in the Formalin-induced Pain Model**

Dubuisson and Dennis’s developed formalin test was used in the present experiment. It is a classic model and a validated technique that has two advantages over other pain tests. One advantage is that it allows a broad range of behavioral responses to be observed because it requires little or no restraint. A second advantage is that it delivers continuous pain, thus resembling clinical pain [25].

Behavioral observations were conducted by the same investigators from 9:00 to 11:00 AM on experimental days in the same room. The animals were habituated for 30 min before the formalin test in a clear 40 × 30 × 30-cm³ plastic box with a mirror below the surface at a 45° angle to allow an unobstructed view of the opposite paw. Formalin-induced pain is a common pain model to mimic the persistent pain induced by acute tissue damage [25-27]. Formalin (50 μL, 2.5 percent) was injected intraplantarly into the right hindpaw of the rats using a 30G needle. Then, two investigators simultaneously observed the nociceptive responses for 1 h. The specific nociceptive behaviors including flinches, favoring, lifting, and licking were recorded. The favoring, lifting, and licking behaviors were quantified by the accumulated time (in seconds) the injected hindpaw spent in specific nociceptive behaviors in each 5-min cycle for 1 h after an intraplantar injection of formalin. Favoring means that the injected paw experienced little weight and rested on the floor without pressure on the footpad; during locomotion there was a definite limp. Lifting indicated that the injected paw was elevated without touching the floor. Licking indicated that the injected paw was licked or bitten. The common pain score was calculated according to the numerical scale below: 0 meaning no pain (normal weight bearing on the injected paw); 1 meaning favoring; 2 meaning lifting; 3 meaning licking. Then, a weighted specific nociceptive pain and nonspecific behavioral state score, ranging from 0 to 3, was generated by multiplying the time spent in each category with the weight; then, these products were summed and divided by the total time for each 5-min block of time.

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**Figure 3.** The nociceptive behaviors, including flinches (A), lifting (B), licking (C), and common pain score (D) in the exercise groups in the 1 h following ipsilateral injection of formalin, showing that the time of lifting, licking, and common pain score in that 1 h decreased significantly following formalin injection. There was no significant difference between the moderate- and high-intensity groups. * \( P < 0.05 \), compared to the control group.
Liu et al.: Exercise intensity and pain

Immunohistochemistry

Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.), and the left ventricle was perfused with 100 mL warmed saline (37°C) followed by 500 mL of 4 percent paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4, 4°C) and finished within 1–1.5 h. Next, the L4/L5 spinal cord segments were removed for histochemical investigation, because the central projection of the site of the pain stimulation is in the lumbar region (L) of the spinal cord. Tissue extraction of layers I and II was performed and guided by anatomical landmarks from a rat atlas. Tissue was post-fixed for 24 h in the same fresh fixative, and cryoprotected in 30 percent sucrose in 0.1 M PB (pH 7.4) for 24–36 h.

Tissue sections (10 sections at each position) were made coronally at 40-μm thickness using a cryostat (Leica CM-1900, Germany). The sections were placed in 0.01 M phosphate-buffered saline (PBS, pH 7.4). The immunohistochemistry staining of c-Fos was performed using the avidin-biotin-peroxidase (ABC) method. After being pre-treated with 0.3 percent H₂O₂ (10 min, at room temperature) and 10 percent normal goat serum (NGS, 1 h, at room temperature), sections were incubated with primary antibody, anti-c-Fos (staining for c-Fos, 1:500; Cell Signaling Technology, Inc., Beverly, USA) in 5 percent NGS-PBS for 48 h at 4°C. Subsequently, the sections were incubated overnight with biotinylated goat anti-rabbit IgG (1:100 dilution) at 4°C, and further processed using ABC complex according to the manufacturer’s instructions (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China). The sections were incubated with 0.02 percent 3,3’-diaminobenzidine (DAB, Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) and 0.03 percent H₂O₂ in 0.05 M Tris-HCl buffer (pH 7.6) for 5 to 10 min. Between each step, the sections were rinsed 3 times in 0.01 M PBS (pH 7.4) containing 0.3 percent Triton X-100 for at least 10 min. The sections were then mounted onto gelatin-coated glass slides, air-dried, dehydrated through a graded alcohol series, cleaned with dimethylbenzene, and coverslipped with neutral balsam. One of every three sections was selected for counting.

All sections were observed under a light microscope (BX-51; Olympus, Tokyo, Japan) and images were captured using a SensiCam digital camera (SPOT-Insight QE, Diagnostic Instruments Inc., Sterling Heights, MI, USA). Images were imported and analyzed, as TIFF files, using SigmaScan Pro Image Analysis Software (SPOT-Insight QE, Diagnostic Instruments Inc., Sterling Heights, MI, USA). To discriminate positive immunostaining from

Figure 4. The time course of nociceptive behaviors including flinches (A), lifting (B), licking (C), and common pain score (D) in the 1 h following ipsilateral injection of formalin. All of the behaviors decreased significantly in the first phase following formalin injection. The time spent lifting and licking, and the common pain score, decreased significantly in the second phase following formalin injection. There was no significant difference between moderate- and high-intensity groups. * P < 0.05, compared with control.
observed in all rats 1 h following the injection of formalin. The first phase was the first 5-min period following formalin injection. Thereafter, animals became quiet and their nociceptive behaviors decreased until the second phase began 20 min following formalin injection. However, there were some minor differences in the different groups. In the control group, the most important behaviors were lifting and licking, whilst in both exercise groups the time for lifting decreased (Figure 1).

Flinches in the control group in the first 5 min were (mean ± SEM) 27.714 ± 8.228, while in the moderate- and high-intensity groups they were 14.375 ± 3.756 and 7.000 ± 0.926, respectively. Flinches in the first 5 min in the high-intensity group were significantly lower than those in the control group (P<0.05, Figure 2A). Similar with this result, the lifting and licking times in the first 5 min in the high-intensity group were also lower than in those in the control group (P<0.05, Figure 2B and Figure 2C). The common pain score in the first 5 min in the control group was (mean ± SEM) 1.902 ± 0.111, which was also significantly higher than those in the moderate- and high-intensity groups (P<0.05, Figure 2D).

The average flinches in the 1 h following formalin injection in both exercise groups were lower than those in the control group, but there were no significant differ-

Data Analysis

All data are presented as means ± SEM. The differences of average flinches and common pain scores among the different groups were analyzed by a one-way ANOVA followed by Dunnett’s testing. The differences of flinches and common pain scores in the time course following the injection of formalin among the different groups were analyzed by a two-way ANOVA followed by Dunnett’s testing. P<0.05 was considered to be statistically significant.

RESULTS

Spontaneous Nociceptive Behaviors Induced by Formalin in Different Groups

Biphasic spontaneous nociceptive behaviors were observed in all rats 1 h following the injection of formalin. The first phase was the first 5-min period following formalin injection. Thereafter, animals became quiet and their nociceptive behaviors decreased until the second phase began 20 min following formalin injection. However, there were some minor differences in the different groups. In the control group, the most important behaviors were lifting and licking, whilst in both exercise groups the time for lifting decreased (Figure 1).

Flinches in the control group in the first 5 min were (mean ± SEM) 27.714 ± 8.228, while in the moderate- and high-intensity groups they were 14.375 ± 3.756 and 7.000 ± 0.926, respectively. Flinches in the first 5 min in the high-intensity group were significantly lower than those in the control group (P<0.05, Figure 2A). Similar with this result, the lifting and licking times in the first 5 min in the high-intensity group were also lower than in those in the control group (P<0.05, Figure 2B and Figure 2C). The common pain score in the first 5 min in the control group was (mean ± SEM) 1.902 ± 0.111, which was also significantly higher than those in the moderate- and high-intensity groups (P<0.05, Figure 2D).

The average flinches in the 1 h following formalin injection in both exercise groups were lower than those in the control group, but there were no significant differ-
The Time Course of Specific Nociceptive Behaviors Following Formalin Injection

The time course of flinches in the 1 h following formalin injection is shown in Figure 4A. It showed that flinches in the 5- and 35-min marks in the control group were significantly higher than those in the moderate- and high-intensity groups \( (P < 0.05, \text{Figure } 3A) \). However, the average time for lifting and licking in the control group was significantly higher than those in the moderate- and high-intensity groups \( (P < 0.05, \text{Figure } 3B \text{ and } 3C) \). The average common pain score in the 1 h following formalin injection in the control group was significantly higher than those in the moderate- and high-intensity groups \( (P < 0.05, \text{Figure } 4A) \). It showed that the number, area, average grayscale, and optical density of the positive cells in the spinal cord ipsilateral to the noxious stimulus were also significantly lower than those of the control group \( (P < 0.01, \text{Table } 1) \). In addition, the number, average grayscale, and optical density of the positive cells in the high-intensity group were significantly lower than those in the moderate-intensity group \( (P < 0.05, \text{Table } 1) \).

DISCUSSION

In the present experiment, biphasic nociceptive behaviors were found in the control and the moderate- and high-intensity groups. Both levels of exercise intensity were associated with decreased behavioral nociceptive responses to noxious stimulation. Both levels of exercise intensity were associated with reduced c-Fos-positive cells, with the high-intensity group showing a lower number of c-Fos-positive cells as compared with the moderate-intensity group. Combining the behavioral results and the changes in c-Fos expression, our present study suggests that different intensities of exercise reduced pain-related behaviors and reduced c-Fos-positive cells in the spinal cord ipsilateral to the noxious stimulus. These findings suggest that exercise may operate in part to reduce pain-related behaviors through reduction in the spinal cord expression of c-Fos. Such a reduction may be mediated through decreased peripheral nociceptive input to the spinal cord. As such, an improved understanding of the intermediate processes may be helpful with respect not only to understanding how exercise may reduce pain, but also how medications might target pain reduction. The exercise-associated decreases in nociceptive responses may reflect several possible reasons. One possibility is that exercise may reduce pain sensation, and another possibility is that the animals exhibited an unwillingness or inability to avoid noxious stimulation because of fatigue. Fatigue may be accompanied by feelings of extreme physical or mental tiredness, resulting from either severe stress or hard physical or mental work. Exercise-induced fatigue is a main focus of sports science research and is a major problem and challenge facing athletic sports. However, it is a specific physiological state that may serve to protect the body.
Exercise intensity and pain

muscle and regulation of the central nervous system, and may be affected by psychological factors [3]. Exercise neurobiology studies have shown that neuroendocrine substances and nerve regulation factors change significantly with exercise-induced fatigue, and such changes may include an increase in nitric oxide levels and alterations in c-Fos levels in multiple brain regions [3,28].

The present study suggests moderate- and high-intensity exercise can decrease nociceptive responses, which is consistent with the results of previous studies, although the mechanism underlying these processes are unclear. In the present study, c-Fos expression in the dorsal horn decreased in the exercise groups, especially in the high-intensity group, which implies that some signal transduction pathways, including those involving c-Fos, may be involved in exercise-induced fatigue and pain. Some studies have also shown that the stress and imbalance of neurotransmitters in the CNS may be an important bridge between exercise-induced fatigue and pain [3,28]. Given that pain-related behavior was similar in the high- and moderate-intensity exercise groups whereas reductions in c-Fos were evident in the high-versus low-intensity groups, the findings raise the possibility that c-Fos expression may be a more sensitive marker than behavior.

Limitations of the present study warrant discussion. First, the study involved only female rats and future studies should examine the extent to which the findings extend to males. Second, while the sample size (n = 10) is reasonable for initial studies, the findings warrant replication in additional samples. Third, intensity-related exercise-induced fatigue may impact both exercise effects and pain sensibility. Thus, this preliminary study of exercise and pain warrants follow-up investigation to examine the complexities of the relationships between exercise, fatigue and pain in greater detail. Specifically, follow-up studies might involve the testing of mechanical thresholds prior to formalin injection in order to evaluate the possible influence of motor versus anti-nociception on the behavioral and molecular findings reported in the present study. Further studies should also test whether other activated factors or neurotransmitters (e.g. nitric oxide) may be in part responsible for mediating aspects of the perception of pain-related sensory information and potential targets for medication development to facilitate pain reduction. Such targets may be particularly relevant to pain experienced through exercise, although the findings may also have broader implications.

In summary, moderate-intensity and high-intensity exercise can decrease pain responses to noxious stimulation and may operate through c-Fos reductions in the spinal cord. Future studies are needed to replicate and extend these findings, with the goal of translating such knowledge into medication development efforts for helping to reduce pain in people.

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