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The Effects of High-Intensity Interval Training with Supplementation of Flaxseed Oil on BDNF mRNA Expression and Pain Feeling in Male Rats

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ABSTRACT

Background. It seems that based on the independent effects of physical activity and flax oil on pain, it is possible the interactions of these two effects reduces or mitigate the impact of pain or strengthen it. **Objectives.** This study investigates the effect of high-intensity interval training (HIIT) and flaxseed oil supplementation on hippocampal BDNF expression and pain feeling in male rats. **Methods.** Twenty adult Wistar rats were randomly divided into four groups (five in each group) including control-saline (CS), training-saline (TS), control-flaxseed oil (CO), and training-flaxseed oil (TO). The training groups were given HIIT program (10 weeks, five sessions in week) on a rodent treadmill at 90–95% of VO2 max and supplement groups also received flaxseed oil (100 mg/kg per cage). Pain threshold was assessed by the hot plate test at a temperature of 55 ± 0.5 °C five days after the last session training. Then rats were sacrificed, their hippocamp tissue frizzed, and sent to laboratory to determine the BDNF gene expression. **Results.** The results showed that training significantly induced higher hippocampal BDNF mRNA expression (P=0.001) and lower pain threshold (P=0.02). Training-flaxseed oil combination group induced significant increase in BDNF expression (P=0.04). There were no significant differences between the other groups. **Conclusion.** The present study showed the useful role of flaxseed oil supplement and HIIT program in increasing an important factor for Alzheimer's disease (BDNF gene). It also showed that, although HIIT may be accompanied with reduced pain threshold and increased pain feeling, it has beneficial effects on memory enhancement gene (BDNF).

KEY WORDS: ***Supplementation, Pain Threshold, Unsaturated Oil, High-Intensity Interval Training, BDNF.***

INTRODUCTION

Alzheimer's disease is one of those problems of modern life that are not only limited to old age, but also affect young people. Today, researchers know that Alzheimer's disease, like heart diseases, spreads due to sedentary lifestyle as a result of modern mechanization. Thus, factors such as cholesterol levels, hypertension, obesity, depression, education, nutrition, sleep, mental conditions, and social and physical activities affect it (1-6). Regardless of mechanization of life, pain resulting from daily activities limits mobility and activity.

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Neurotrophins are a family of growth factors that protect neuronal survival (7, 8). The family is composed of at least four mammalian proteins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophins-4/5 (NT 4/5), which mainly form the activities of the nervous system and affect the central and peripheral nervous systems. In the present study, BDNF has been investigated as it mediates in a variety of actions such as neuronal survival, neurogenesis, cell death, axon growth, continuity, and plasticity. Moreover, studies have shown that it can have a relationship with the feeling of pain (9), which has not been investigated clearly. Therefore, change in BDNF may play an important role in the prevention of Alzheimer's disease (10) and the feeling of pain. Stressful factors can cause changes in BDNF. Previous studies have shown that physical activity is effective as a disruptive factor for causing changes in BDNF. Animal studies have shown that daily physical activities cause the release of different neurotransmitters in the brain such as dopamine, norepinephrine, and especially the release of BDNF, which is why the release of neurotripin is related to an increase in learning speed and protects it better after a week (11). In this regard, researchers have said that exercises lead to increasing survival and resistance against brain damages and increase neuron growth in hippocampus (11). Long-term stress can be a factor for reducing pain (12). Previous studies have shown that pain perception is different in various conditions of stress (13-16). One of the first studies in this field shows that soldiers who were in stressful conditions felt less pain and that they needed significantly less pain medication (17, 18). However, physical activity as well as stress and disruption can cause a change in the feeling of pain (19-21). Studies show that 'athletes who are doing hard and exiting sports, they have less pain feeling', which strengthens the hypothesis that extreme sports may reduce the sensation of pain through simulation opioid system activity of growth and corticotropin (22). On the other hand, many studies have examined the effect of drugs on the feeling of pain. Most of these studies have focused on morphine and alcohol, and have shown a reduction in pain during the course of medication (23-25). It has been shown that these drugs have harmful side effects (26, 27); therefore, a trend has emerged towards the use of non-pharmaceutical methods and compounds acquired from plants. Many studies of the analgesic effects of plants have shown that components in plants can reduce injury, inflammation, and pain (28-30). Also, studies have shown that the use of natural materials can change gene expression (31, 32). Flaxseed in Mediterranean traditional medicine has been in use as a drug for centuries. The oil obtained from the seedling contains a major portion of the properties and benefits of flaxseed. The plant contains many microelements such as dietary fibre, manganese, vitamin B1, and essential fatty acid alpha- Linoleic, which is also known as omega-3 (33, 34). So far, there has not been a direct study about the effect of flaxseed oil on induced pain and BDNF. Studies have shown that flaxseed oil, owing to the vitamins and beneficial fatty acids that it contains, have anti-inflammatory and pain-relieving effects (migraine, menstruation) (33, 35-38). However, as mentioned above, the effect of flax oil on pain is unclear and needs to be investigated. On the other hand, as explained previously, physical activity may reduce pain, but whether intense interval exercise (HIIT) may have beneficial effects on pain or not needs more study. Besides the independent effects of physical activity and flax oil on pain, it is possible that the interactions of these two effects reduces or mitigate the impact of pain or strengthen it. But this case has not been investigated and needs to be studied in detail. With respect to BDNF, regardless of numerous reports about the positive impact of physical activity on BDNF, to date no study has investigated the effect of interactive HIIT and use of flax oil on the gene expression of hippocampus BDNF in rats. Thus, the present research will examine the effects of HIIT program and flaxseed oil supplement on the hippocampus BDNF gene expression and pain induced in male rats.

MATERIALS AND METHODS

Animals. Twenty adult male Wistar rats were selected randomly on a statistical sample. The animals were kept in animals houses at the Baqiyatallah University of Medical Science at a

temperature (± 2) 22 degrees, humidity 45–50 %, and lighting–dark cycle (12 hours light, 12 hours darkness) in special cages made of plastic fasters with metal caps and their palms were covered with clean wood chips. Special compressed food for laboratory rats manufactured by Behparvar of Karaj and urban filtered water in bottles of 500 ml were used for the rats (39). Rats were divided randomly into four groups (five rats in each group) including control-saline (CS), training-saline (TS), control-flax oil supplement (CO), and training-flax oil supplement (TO). This study was conducted in accordance with NIH publication and all ethical principles were considered regarding handling of laboratory animals including the availability of water and food, appropriate storage conditions, non-refoulement, and ill-treatment. The present study was conducted with the written permission of the deputy of research of Baqiyatallah University. Five days after completing the experimental stages of research, the rats were anesthetized using a combination of Xylazine and Ketamine. Hippocampus tissue was isolated and frozen in liquid nitrogen and the samples were kept until they were sent to the laboratory at - 20° C.

Flaxseed oil and its preparation method. Fresh flaxseed was collected from growing areas in Mehriz City, Yazd Province, and the oil was extracted using an oil-making machine after approval by the Department of Biology (Department of Botany) at Baqiyatallah University. The oil was fed to the rats based on weight at a dose of 100 mg kg to the relevant groups, and for the same effect of gavage, saline was fed to other groups. The supplement using oral gavage was fed to the rats before the exercise.

Training protocol. The orientation of the rats with HIIT was determined using protocol with 10 practice sessions in two weeks, meaning that on the first day of training the rats were placed on a treadmill with all the precision and comfort, and they began to practice with a low and even speed. In the next sessions, the rats were made familiar with the interval protocol that was used with low speeds of intervals. During the next two weeks, increased time followed until the real time of training session reached 18 minutes. After two weeks, without any kind of problem, the main training protocol for 10 weeks began and completed. It should be noted that the slope of the treadmill during the whole sessions was zero degree and familiarity was conducted for non-trained groups. At the end of two weeks' familiarity, the maximum oxygen consumption (VO_2max) was measured in the rats, and according to the training protocol, they began to practice based on the percentage of maximal oxygen consumption (which was converted to metre per minute). The speed in the measurement protocol of maximum oxygen consumption in rats for reaching exhaustion was considered 100% and the rest of intensity was considered as a percentage of the speed. At the end of every two week, the maximal oxygen consumption was estimated and the new speed of exercise was applied for the next two week.

Each session consisted of 30 minutes of physical activity of HIIT, as shown in Table 1. The programme included three intensive and low intensity intervals. Intensive intervals were performed for 90–100% of maximum oxygen consumption for four minutes and low-intensity intervals were performed for 50–60% of maximum oxygen consumption for two minutes. It should be noted that 50–60% of maximum oxygen consumption was considered for each of the warm-up and cool-down parts (40). At this moment, a control group for the standardization of the effect of stress was placed for 15 minutes on the treadmill at a speed of two metres per minute. The protocol continued for five days before sacrificing the rats.

The method of calculating VO_2max. Owing to the lack of direct access to tools such as the device for respiratory gases analysis and based on recent research conducted by Hoydal et al. (41), indirect protocol was used very carefully in the following manner: At first, 10-minute warm-up was performed at low speed (10 metres per minute). After the warm-up, the test started with the rats running at 15 metres per minute for two min. The treadmill speed was then increased every two minutes at a rate of 0.03 metre per second (1.8–2 metres per minute) so the animals were unable to run. The speed in exhaustion stage was considered as 100% of maximum oxygen consumption and the least oxygen consumption was calculated as a percentage of this speed.

Table 1. Schema of HIIT protocol

HIIT steps	Warm up	The main body (Three alternates)		Cool down
		High intensity intervals	Low intensity intervals	
Time (min)	6	4	2	6
Intensity (VO_2max)	50 to 60 %	90 to 100 %	50 to 60 %	50 to 60 %
The first & second week (m/min)	16 to 19	28 to 31	16 to 19	16 to 19
Third & fourth week (m/min)	19 to 22	33 to 37	19 to 22	19 to 22
Fifth & sixth week (m/min)	21 to 25	38 to 41	21 to 25	21 to 25
Seventh & Eight week (m/min)	27 to 32	48 to 53	27 to 32	27 to 32
Ninth & tenth week (m/min)	30 to 36	53 to 59	30 to 36	30 to 36

Evaluation of hippocampus BDNF gene expression. RNA extraction was done using a specific kit based on the manufacturer's instructions (Total RNA Extraction Kit, Yekta Tajhiz Azma, Cat NO: YT 9065). CDNA manufacturing was performed using a special kit according to the manufacturer's instructions (Yekta Tajhiz Azma, Cat NO: YT 4509). cDNA was made at -20 ° C and was kept to be used for real-time PCR reactions. Real-time PCR was performed using the Corbet machine. For the measurement of each gene, 1 ml SRBR Green Master (SRBR Green Master Mix, Yekta Tajhiz Azma Cat NO: YT 2551) was mixed with five microlitres of RNAse-free water. One microlitre forward initiator and one microlitre reverse initiator with one microlitre of cDNA were then added to the mixture. Then, tubes were kept in the real-time PCR system at 95 ° C for five minutes. After that, the test was performed for 30–45 times at 90 ° C for five minutes and at 60 ° C for 10 seconds. Quantity determination in real time was done by measuring the increase in fluorescence radiation due to double-stranded DNA attached to the SRBR Green to double-stranded DNA at the end of each reproduced cycle. At the end of the PCR, the breaking action of DNA and the melting curve slowly started, heating the samples from 72 to 95 ° C. There was a continuous decrease of registration in fluorescence as a result of differences between the two strands of DNA. The threshold cycle (CT) recorded the increase in fluorescence as recognizable for the first time and was determined for each sample. The level of BDNF mRNA, based on the PCR efficiency and CT deviation as an unknown sample to control, was estimated to be $2^{\wedge-\Delta\Delta CT}$, βeta actin mRNA was used as the reference gene. In this method, the difference of CT genes from βeta actin CT was obtained to get the value of ΔCT. Then by reducing Δ_{CT} related to control-saline from ΔCT of other groups, $\Delta\Delta_{CT}$ was calculated. After that, $2^{\wedge-\Delta\Delta CT}$ was calculated (42). Therefore, the gene expression of all groups was expressed relatively than the control group.

Table 2. The specific primer reference used in the stage of real-time PCR

Name	Reference
BDNF	NM _001048142.1
β-actin	NM_031144

Evaluation method of pain threshold. Acute pain assessment was performed by the hot plate test. In this test, the rats were placed slowly on a screen with a temperature of 55±0.5 and the time spent on the screen from the moment of exposure to movements that measure pain threshold was recorded. The criterion for reaching the pain threshold was licking behaviour or jumping out of the hot plate. The maximum time for keeping the rats on a hot plate was 40 seconds (43). This test was performed five days after the last training session for all rats.

Statistical analysis. For classification and determining the distribution, we used descriptive statistics. It was used as a Kolmogorov–Smirnov test to detected data distribution. For investigating changes between groups, there were two-way analysis of variance, a post hoc LSD test, and the relationship was a used Pearson correlation coefficient. All significant levels were considered <0.05 (by using SPSS, 19).

RESULTS

Data analysis using two-way analysis of variance showed significant differences in BDNF gene expression in hippocampus between control and trained groups (F= 43.799, P= 0.001). Owing to the training, hippocampus BDNF gene expression independently increased in rats (Figure 1). Data analysis showed that, this supplement flaxseed oil has no significant changes in the expression of BDNF in consumer groups (F=2.664, P= 0.12) (Figure 1). Data results in interactive group showed significant increase in hippocampus BDNF gene expression in rats (F =4.918, P=0.04) (Figure 1). The use of two-way analysis of variance showed significant difference in pain threshold between control and training groups (p=0.01, F= 8.420). Pain threshold reduced in training than control (Figure 2). However, using two-way analysis of variance did not show any significant difference between supplement and non- supplement (saline) (F=1.351, p=0.26) (Figure 2). Moreover, pain threshold did not have significant differences in the interactive group' training-supplement compared to the independent groups (F= 0.116, P=0.73) (Figure 1). The results of the study did not show a significant relationship between pain threshold and hippocampal BDNF expression in any of the study of research groups (Table 3).

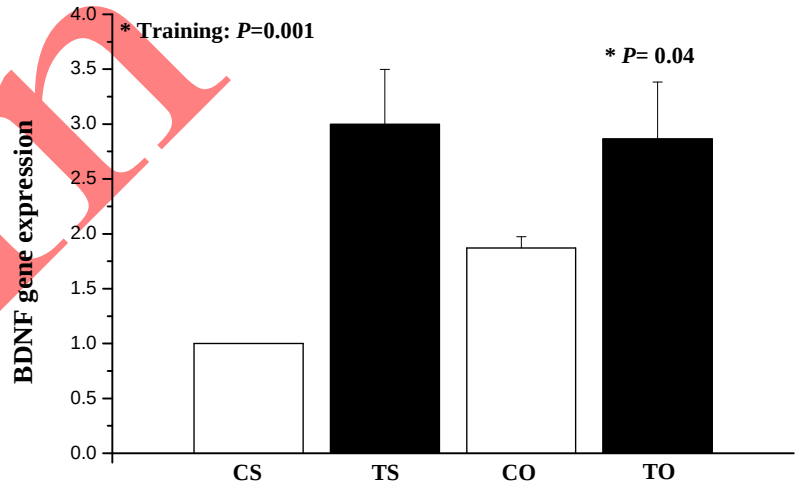

Figure 1. BDNF/ βeta-actin gene expression in the hippocampus

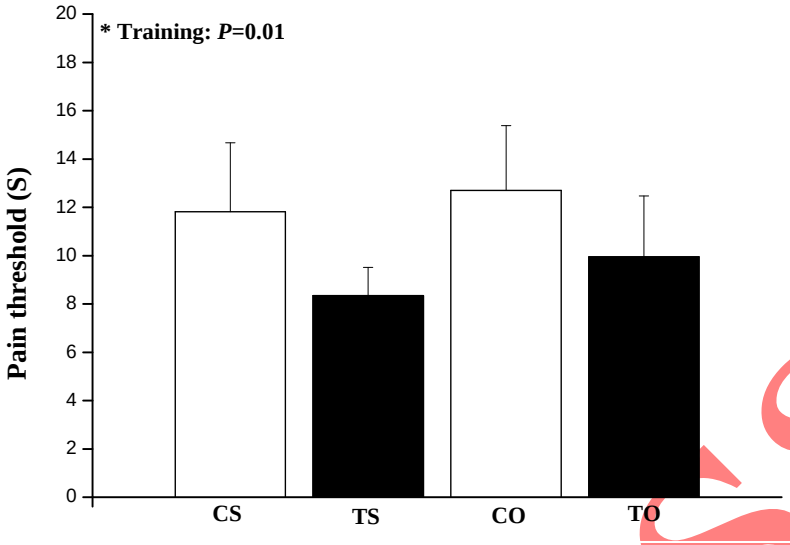

Figure 2. The pain threshold (s) in different study groups

Table 3. The correlation between pain threshold and expression of BDNF in the hippocampus

Index	BDNF	
	The significance level	The correlation coefficient
Pain threshold	0.17	-0.315

DISCUSSION

Previous studies have shown that exercise and nutrition causing changes in stress hormones and other factors can have an effect on BDNF. For BDNF and physical activity, studies have been shown the increase in BDNF (44-46). Research has investigated the effects of acute and chronic activities on hippocampus BDNF, showing positive effects of exercise on BDNF. Johnson et al. (2003) investigated the effect of voluntary running on BDNF of rats and found the following pattern of running for seven nights in an experimental group, which showed a significant increase in BDNF. The results showed that neuronal plasticity due to exercise increases potentially (47).

Generally, physical activity may provide rich conditions that affect nerve neurogenesis (48), synaptic potentiation (49), noradrenaline (50), spatial memory (48), and influence on other parameters of neurochemical (51). That reduced mortality and brain damage followed two weeks' exercise is shown in the cerebral ischemia of the rats. They reported possible mechanism for potential of increase in BDNF and claimed that BDNF leads to increase in resistance in brain injury. Therefore, the up-regulation of BDNF may play a significant role in normal processes in brain and nerve protection (52). However, regardless of the type of exercise, several studies have shown that intensity of exercise is one of the factors affecting BDNF performance. Luo et al. (2008) showed that exercise intensity affects neurogenesis and BDNF mRNA expression, N-methyl-D-aspartate receptor of type 1 (NMDA R1), vascular endothelial growth factor (VEGF), and foetal liver Kinase 1 (FLK-1) in the hippocampus of rats for five weeks. A week's exercise on treadmill with low or moderate intensity increases neurogenesis activity in the dentate gyros in the hippocampus region. Low-intensity patterns cause significant increase in the expression of BDNF, NMDARI, and FLK-1 mRNA. Levels of gene expression in the exercise group with low intensity compared to exercise group with high intensity was greater for four molecules (45). Soya et al. (2007) evaluated induced C-fos (is an indicator for neuronal activity) and BDNF expression after cute exercise on running with different intensities. Even running with low intensity (15 metres per minute) set up to increases C-fos

mRNA, while it seems that induction is strongly related to activity. On the other hand, the increase in BDNF mRNA and protein was observed only in running with low intensity. In running with moderate intensity (25 metres per minute), when the levels of corticosteron and blood lactate became high, BDNF mRNA induction reduced but BDNF protein did not reduce. The results of this study showed that moderate exercise compared with a variety of hard exercises has more benefit for hippocampal function (44). Exercise with moderate duration and intensity led to significant impact on the performance of BDNF compared to low and high duration. Another study also showed that BDNF protein levels increase progressively in longer periods in response to both types of daily exercise (every day) and exercise on alternate days (every other day). It is interesting that, while everyday practice ensures a more rapid increase in BDNF levels, periodic exercise is also effective. This suggests that protein induction has a time element that has a relationship with interactional exercise. In addition, protein levels even after three months of daily exercise continue to increase (11). Unlike studies that observed beneficial effects of moderate-intensity exercise, the present research indicated that high intensity workout can have beneficial effects on BDNF. For justifying this difference, we consider stress factors on BDNF. Stress and stress hormones are also effective on BDNF. Running on treadmill maybe considered a stressful factor for rats. Corticosteroids are more susceptible to damage neurons and reduce long-term potentiation (LTD), and temporarily BDNF mRNA is set for reduction in hippocampus (53, 54). Animals run due to fear or shock, which increases the amount of adrenal hormones (55). It inhibits the down-regulation of the glucocorticoid receptors in hippocampus (56) and shows an increase in corticosteroids and stress. In a research by Ploughman et al. (2005), both optional and mandatory running increased serum corticosterone (57). By increasing voluntary optional movement, serum corticosteron levels increase energy and stress (57). In animal specimens that experiment became traumatic brain injury, optionally running led to a reduction in BDNF. When running interference delayed for 14 days after a brain injury, BDNF went up and runner animals in recovery period showed better performance (58). This suggests that stress of running due to increase in metabolic demand causes more damage to tissue and have temporary harmful effects after injury. However, it is important to note that many researchers have reported an increase in BDNF after exercise. They did not mention the interval between the end of exercise and killing rats (57). Schaaf et al (1998) found that BDNF by increasing in corticosterone concentration reduced but, its effect is temporary and return to first position after 24 hours. Shaw et al. (2003) found that serum corticosterone returns to baseline levels after 60 minutes (1 hour) (59). In this study, rats were killed after five days in the last exercise session. It seems that due to reduced corticosteron levels, BDNF also increased. Moreover, long-term training (10 weeks) seems to reduce stress and ensures consistency in rats.

Neurotransmitter systems are known to regulate BDNF by activity in hippocampus, acetylcholine systems, GABA, serotonin, and norepinephrine. The main source of acetylcholine and GABA to hippocampus is septum input brain, which is an important natural regulator and excitability of hippocampus. During physical activity, it guides the activity of GABA in medial septum of neuronal activity. In addition, exercise causes the flow of acetylcholine increase in hippocampus as acetylcholine regulates BDNF gene expression. Acetylcholine appears as a good representative for increasing BDNF gene expression after physical activity. Studies have shown that, although acetylcholine regulates BDNF gene expression in hippocampus, it is not a key regulator in depending on activity. If cholinergic neurons in the medial are damaged, it cuts off all cholinergic input and output to the hippocampus. Complete damage to cholinergic, despite reduction in the basic level of BDNF gene expression, did not damage BDNF induced by exercise (60). In addition to the cholinergic, neurotransmitters and monoamine signals may also participate in the regulation of BDNF gene expression. Following the antidepressant effects, it increases monoamine neurotransmitter serotonin and norepinephrine levels. Physical activity increases norepinephrine in several areas

of brain including hippocampus and may increase neurotransmitters serotonin levels. It is suggested that physical activity and antidepressants can be synchronized in hippocampus and change the expression of BDNF (61). Damaging neurotransmitters can damage noradrenergic, serotonergic, and other neurotransmitters of brain. Owing to damaged noradrenergic, sports cannot increase hippocampus BDNF gene expression. However, the basic levels of BDNF cannot be affected by noradrenergic and serotonergic, and they have little effect on the regulation of sports in BDNF. These results indicate that norepinephrine and serotonin are not very effective in the regulation of BDNF in hippocampus, whereas noradrenaline is very important in the regulation of BDNF, which is dependent on exercise. However, it is considered as another mechanism and it seems that HIIT exercise can influence BDNF.

Generally, the increase in BDNF may be regulated by different mechanisms. For example, absorption exercise like insulin-1 growth factor induced in the brain is essential to increase BDNF mRNA following exercise (62). Moreover, oestrogen is necessary to increase protein BDNF in hippocampus in rats due to exercise (63), while stress hormones reduce hippocampus BDNF (64, 65). In the short term (2–4 running at night), there was a positive correlation between running distance BDNF mRNA levels in hippocampus (66). On the other hand, neurotrophin 3 (NT3) may weaken BDNF signalling pathways in the hippocampus neurons that is cultured. Down-regulation 3NT after exercise may facilitate the effects of BDNF on the synaptic function of hippocampus (67) .In the present research, NT3, oestrogen, cortisol, insulin, and epinephrine were not measured and need further research. All possible mechanisms are discussed as an idea for future research.

Most studies have examined the effect of physical activity on thermal pain sensitivity in animal specimens, but there is no study to investigate very periodic intense exercise on pain. Bodnar et al. showed that three minutes of swimming in water at 2 ° C increased the pain threshold in rats (68). Christie et al. examined the effect of swimming for three minutes at 32 ° C on female rats (69). Yao et al. indicated that 60-minute muscle simulation increases the pain threshold in male rats (70). Tierney et al. indicated the increase in pain threshold after swimming in different times in water at 20–22 ° C in female rats (19). Generally, analgesia or increase in pain threshold is investigated by running, cycling, and swimming. By examining these studies, we find that a change in pain threshold could happen for light and medium types of activities, but it is a little more complicated in intense activity. Intense exercises may increase with possible mechanism in the activity of opioid system, growth, and corticorteinon after exercise. They cause analgesia during exercise (22). On the other hand, the production of free radicals and reduction in antioxidant defence cause inflammation and pain (71). With low testosterone due to high intensity, activity reduces pain threshold (36). In the present study, the effects of exercise decreased the pain threshold, which may be due to a number of reasons. In the present study, it was considered that these possible mechanisms and levels are not measured, and it requires further study in this area by considering these factors. This study showed that flax oil did not have a significant effect on pain sensation. This is clearly shown by Figure 2. In this case, pattern changes in the pain threshold were significantly reduced in training groups and non-significant increase in flaxseed groups. There is no direct study on the impact of flax oil on the pain threshold and the present study is the first to examine that. However, previous studies guide us on the anti-inflammatory and anti-inflammatory properties of flax oil (72). However, there is a potential mechanism for the effects of oil on pain and vitamins that form oil. Studies have shown that flax seed oil is rich in vitamin E, and due to the analgesic effects of vitamins, it can reduce pain (33, 37, 38). It has been shown that supplementation with Alpha-Tocopherol has anti-inflammatory and analgesic effects. In a study, the use of vitamin D for four weeks reduced CRP level significantly (inflammatory marker) (73, 74). In another study, healthy and diabetic individuals were given natural Tocopherol (vitamin E) for three months, and there was a significant decrease in plasma concentration of CRP and IL-6 monocytes (73).

This oil is rich in omega-3 and omega-6 (75, 76). Omega-3 and omega-6 are polyunsaturated fatty acids that have been shown to be effective on pain and inflammation (77-79). However, present study showed that the pain threshold did not show any significant change after the use of flax oil, but according to the above studies, we found that they have used more active ingredients in flaxseed oil and high doses. Thus maybe a higher dose should have been used in the present study. However, future studies should investigate these effective factors.

CONCLUSION

Overall, the present study showed that periodic intense exercise, despite reducing the pain threshold, can increase BDNF expression as one of the factors facilitating the improvement of Alzheimer's disease.

APPLICABLE REMARKS

- High intensity interval training (HIIT) increases hippocampal BDNF gene expression that is benefit for trained subjects.
- It is recommended that trained subjects consume the flaxseed oil supplement with HIIT; because this combination compared with HIIT alone, has more effect on hippocampal BDNF gene expression that trained subjects derive a benefit from this over-expression.

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