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Original Article

Short-term regular moderate exercise improved male hypothalamic-pituitary-gonadal axis function via the reduction of hypothalamic neurokinin B expression in adult rats

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ABSTRACT

Introduction: In the arcuate nucleus, kisspeptin, neurokinin-B and pro-dynorphin (KNDy) neurons control the function of gonadotropin-releasing hormone (GnRH) neurons. Early investigations indicated that exercise with various intensities affects luteinizing hormone (LH) and testosterone (T) in different ways. Meanwhile the molecular mechanisms underlying its function not yet been fully understood. Accordingly, the present study evaluated the role of alterations in the levels of KNDy mRNA upstream of GnRH neurons in conveying the effects of various short-term exercise intensities on the male hypothermic-pituitary-gonadal (HPG) axis.

Methods: Twenty-one adult Wistar rats were randomly divided into 3 groups: control, one-month regular moderate exercise (ME) and one-month regular intensive exercise (IE). In ME (22m/min) and IE (35m/min) groups, the rats were treated 5 days a week for 60min each day. Finally, we assessed serum levels of LH and T using the ELIZA technique and KNDy and *Gnrh* mRNA expression by the real-time PCR method.

Results: The results revealed that in ME group the expression of *Nkb* was reduced and the expression of *Gnrh* mRNA and the LH and T serum levels were increased. However, intensive exercise did not change the serum levels of LH and T or the relative expression of *kiss1, Nkb, Pdyn* and *Gnrh* genes.

Conclusion: The results suggested that monthly moderate exercise improved male reproductive axis function, while intensive exercise did not have an adverse effect on the reproductive axis. These various effects on the male HPG axis may be propagated by the change in hypothalamic *Nkb* gene expression.

Keywords:
Regular exercise
Kisspeptin
Neurokinin-B
Pro-dynorphin
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Reproductive axis

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Introduction

Kisspeptin is a 54-amino acid peptide, encoded by the Kiss1 gene (Dhillo et al., 2005; Kotani et al., 2001) which activates the orphan G protein-coupled receptor GPR54 (Kotani et al., 2001). Evidence suggests that the kisspeptin/GPR54 system has an important role in reproduction and kisspeptin has been introduced as an afferent neuron of the gonadotropin-releasing hormone (GnRH) neurons (Kalló et al., 2012; Lehman et al., 2010). In rodents, the kisspeptin neurons are in two separate parts of the hypothalamus, the arcuate nucleus and the anteroventral periventricular nucleus (Chaikhun et al., 2013). In addition to the kisspeptin, neurokinin-B (NKB) and pro-dynorphin (Pdyn) are co-expressed by arcuate kisspeptin neurons; because of that, they are called KNDy (Lehman et al., 2010).

Glucocorticoid, steroids, opioids (such as kappa opioid receptor) and neurokinin-B receptors are found in large numbers on KNDy neurons (Chaikhun et al., 2013; Pinilla et al., 2012). Note that only a small number of kisspeptin receptors are available on KNDy neurons (Pinilla et al., 2012). The existence of multiple receptors on KNDy neurons and the transfer of the KNDy neuronal projections to the hypothalamic-preoptic area and the zone of the median eminence indicate the pivotal role of these neurons in transmitting signals to the GnRH neurons (Lehman et al., 2010).

Previous studies have suggested that exercise can result in altered levels of luteinizing hormone (LH) and testosterone (Hackney 2020), but there are conflicting results about the effects of exercise on the reproductive axis. For example, it has been shown that testosterone or estradiol increases with acute bouts of exercise, but other evidence has shown lower testosterone in male endurance athletes (Sokoloff et al., 2016). However, 16 weeks of 150min per week of moderate aerobic exercise in premenopausal women did not significantly alter the sex hormone (Smith et al., 2011). It seems that the type and duration of exercise are important factors affecting reproductive performance. However, the molecular mechanisms of these effects are unknown (Homan et al., 2007).

Therefore, in considering the fundamental roles of the GnRH neurons in the hypothermic-pituitary-gonadal (HPG) axis, along with the necessary roles of KNDy neurons in conveying the internal and external signals to GnRH neurons and altering the reproductive axis, the present study investigated the changes in hypothalamic neuropeptide gene expression to discover the main hypothalamic pathway that influences the HPG axis functions during the one-month regular moderate exercise.

Material and methods

Animals and exercise protocol design

Twenty-one male Wistar rats (250±50g) were purchased from the Pasteur Institute, Tehran, Iran. The rats were maintained at a controlled temperature of 22±2°C in a 12-hour light/dark cycle and were given free access to food and water. All procedures for maintaining and using experimental animals were performed according to the guide for the care and use of laboratory animals (NIH Guide for Care and Use of Laboratory Animals, 8th Edition, 2010) and accepted by the Ethics Committee of the Shahrekord University of Medical Sciences (IR.SKUMS.REC.1398.195). The animals were randomly distributed into three groups (n=7 in each group): 1, control, 2, one-month regular moderate exercise (ME) and 3, one-month regular intensive exercise (IE).

After 5 days of habituation, the rats scheduled for training were exercised on a rodent treadmill (at 0 inclination) 5 days a week, for 4 weeks. The treadmill speed was 16m/min during the first week, was increased and kept at 22m/min (ME) or 35m/min (IE) for the remaining training sessions. The session duration started at 40min/day and was increased by 5min daily. During the last 3 weeks, the rats ran for 1h daily. It should be noted that all exercised rats were treated before 12AM. The control group were not exercising, but they were put on a non-moving treadmill 5 days a week for 60min/day. All tests for hormones were performed in the morning (Hesari et al., 2014; Khajehnasiri et al., 2019).

Biochemical assay and determination of the levels of LH and testosterone

Twenty-four hours after the last exercise session, all the rats were anesthetized through injection of 100mg/kg ketamine and 10mg/kg xylazine intraperitoneally (Shahidi et al., 2019; Shahidi et al., 2018). Next, blood samples were collected from the eye sinus for determination of LH and testosterone, where the serum was separated accordingly. Finally, we used Bioassay Science Laboratory's ELISA kit to assess the LH (Bioassay Technology Laboratory- E0179Ra, China) and tes-

tosterone levels (Bioassay Technology Laboratory E0179Ra, China).

Molecular assay and determination of the levels of Kiss1, Nkb, Pdyn, and Gnrh gene expressions

For evaluation molecular test, an arcuate nucleus was extracted from the brain hypothalamus based on a previous method (Khajehnasiri et al., 2018; Molaei et al., 2020). Briefly, YTzol Pure RNA buffer (Yekta Tajiz, Iran) removed total RNAs from samples. Next, the Nanodrop measured their concentration and purity. Eventually, for synthesis of cDNA a reverse transcription kit (BIONEER, Korea) was used.

The triplicate reactions utilized for estimating *Kiss1*, *Nkb*, *Pdyn*, and *Gnrh* gene expressions were conducted on cDNA specimens via gene-specific primers, as reported in Table 1. Then, real-time PCR was done by the SYBR Green PCR Master Mix (Takara Bio Inc., Japan). Note that PCRs served as the negative controls for each primer set in the absence of template cDNAs. The reaction was incubated for 5min at 95°C followed by 45 cycles of PCR for 30s at 95, 60 and 72°C for each and a final extension step at 72°C for 5min. At the end of the PCR reaction, each amplicon produced a single peak, whereas they did not present any peaks when there was no template (Schmittgen and Livak 2008).

Statistical analyses

The data related the real-time PCR analyzed via the comparative Ct procedure, while the relative expression of the target genes over reference were values determined using the arithmetic formula $2^{-\Delta\Delta CT}$. Then, the Kolmogorov-Smirnov normality test was used to see whether the collected data on the relative expression of target genes and LH and testosterone serum levels were normally distributed or not. At last, the experimental data were assayed by SPSS version 16.0. One-way ANOVA with posthoc Tukey test was utilized to compare the performance of the three groups. The significance level was considered to be $P<0.05$.

Results

Gnrh expression in response to one-month regular moderate and intensive exercise

The one-way ANOVA showed that one-month regular moderate exercise elevated the *Gnrh* mRNA levels ($P<0.05$). However, one-month regular intensive exercise treatment was not adequate to create noticeable changes in gene expression ($P=0.082$; Fig. 1).

Arcuate Nkb, Kiss1 and Pdyn gene expression in response to one-month regular moderate and intensive exercise

Next, real-time PCR analysis was used to determine the effect of one-month regular moderate and intensive

TABLE 1: The sequence of the primers used in this study

Gene	Forward	Reverse	Amplicon (bp)
Gapdh	‘CGGCCAAATCCGTTCACACCGA3‘5	‘GGCTCTCTGCTCCTCCCTGTTC3‘5	122
Kiss1	AGCCAGATAGAGGAAGCCCAGG’3‘5	CCACACAGAGGAGCAGCAG’3‘5	182
Pdyn	ACAAAGCAGCACGCAGGTCAC’3‘5	TCAGAGGGGATCACAAGGAGG’3‘5	144
Nkb	CAAGAGGAACAGCCAACCAG’3‘5	AAGGGAGCCAACAGGAGGAC’3‘5	199
Gnrh	GCCGCTGTTGTTCTGTTGACTG’3‘5	CCTCCTCCTTGCCCATCTCTTG’3‘5	131

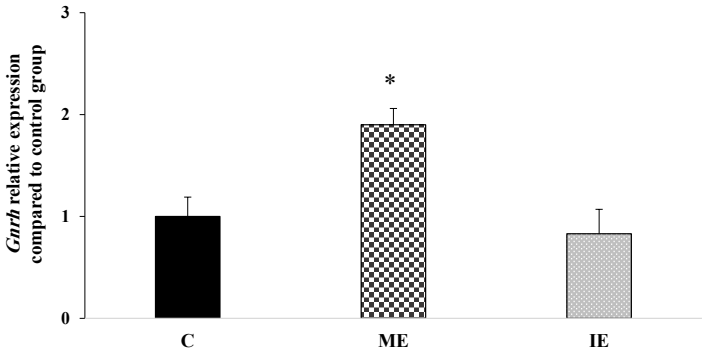

FIGURE 1. Effect of short-term moderate and intensive regular exercise on Gnrh gene expression in arcuate nucleus of hypothalamus. Control (C), one-month regular moderate exercise (ME) and one-month regular intensive exercise (IE) groups. Data are displayed as mean±SD. *$P<0.05$ compared with control group. Containing 7 rats in each group.

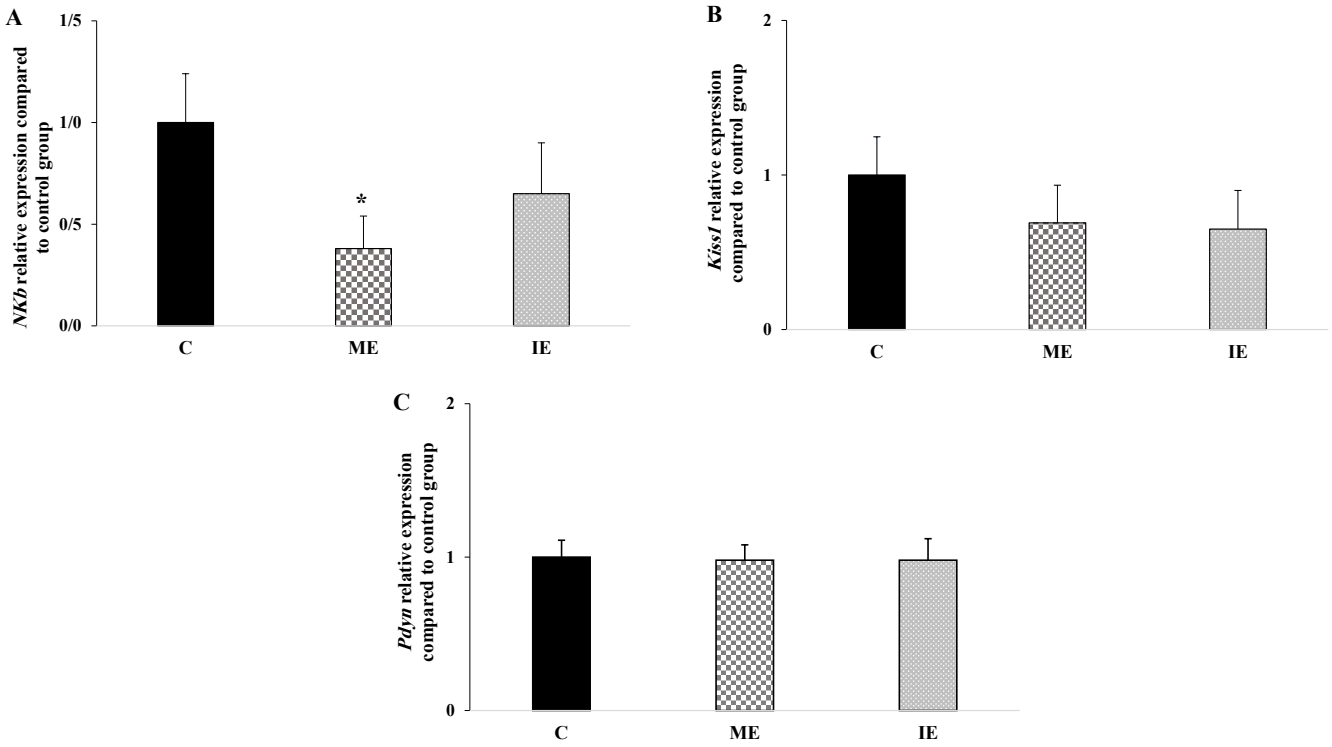

FIGURE 2. Effect of short-term moderate and intensive regular exercise on *Nkb* (A), *Kiss1* (B) or *Pdyn* (C) expression in arcuate nucleus of hypothalamus. Containing 7 rats in each group, Control (C), one-month regular moderate exercise (ME) and one-month regular intensive exercise (IE) groups. Data are displayed as mean±SD. $^{*}P<0.05$ compared with control group.

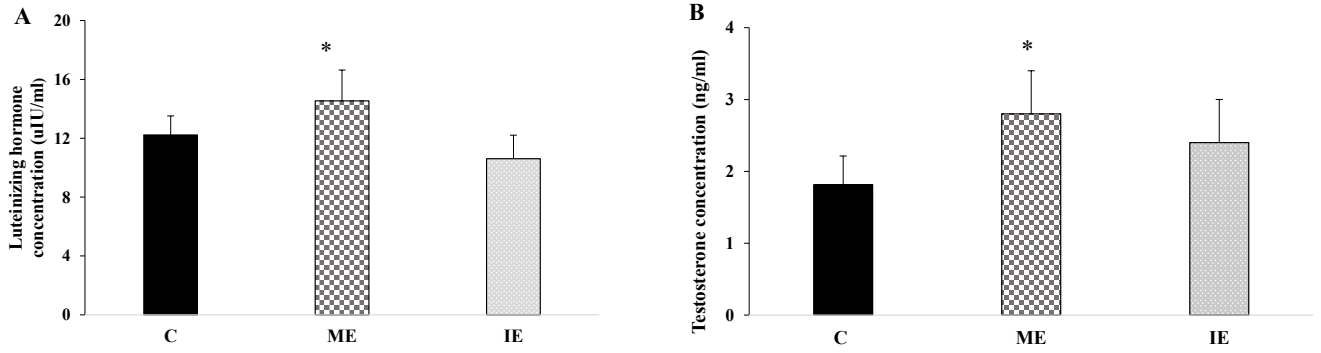

FIGURE 3. (A) Effect of short-term moderate and intensive regular exercise on luteinizing hormone and (B) testosterone serum levels. Control (C), one-month regular moderate exercise (ME) and one-month regular intensive exercise (IE) groups. Containing 7 rats in each group. Data are displayed as mean±SD. $^{*}P<0.05$ compared with control group.

exercise on arcuate *Kiss1*, *Nkb* and *Pdyn* gene expressions (known as the key HPG axis regulators). The ME group presented a significant decline in the *Nkb* mRNA level, compared to the control group (*P<0.05;* Fig. 2A). However, there was no difference in the arcuate *Kiss1* (P=0.071) and *Pdyn* (P=0.95) mRNA levels between the moderate exercise and control groups (Figs. 2B and C). No difference was observed either in *Nkb* (P=0.71), *Kiss1* (P=0.10) and *Pdyn* (P=0. 92) mRNA expression in the IE rats, compared to control (Figs. 2A-C; Table2).

LH and testosterone serum concentrations

Finally, reproductive performance indicators (LH and testosterone serum levels) were tested in response to one-month regular moderate and intensive exercise by the ELISA method. The LH (14.54±2.1 uIU/ml; P<0.05) and testosterone (2.8±0.6 ng/ml; P<0.05) serum concentrations significantly increased in ME exercised group, compared with concentrations in the control group of testosterone (1.8±0.4 ng/ml) and LH (12.22±1.3 uIU/ml, Table 3). However, no difference was observed in the LH (10.6±1.6 uIU/ml; P=0.072) and testosterone (2.4±0.0.6 ng/ml; P=0.059) serum levels between IE and control rats (Figs. 3A and B).

TABLE 2: Relative gene expressions of Gnrh, Kiss1, Nkb, and Pdyn in the control, one-month regular moderate exercise (ME) and one-month regular intensive exercise (IE). *P<0.05 between exercised groups and control rats.

	Control	ME	IE
Gnrh	1	1.9*	0.83
Kiss1	1	0.69	0.65
Nkb	1	0.38*	0.61
Pdyn	1	0.98	0.94

TABLE 3: Lutein hormone (LH) and testosterone (T) serum level in the control, one-month regular moderate exercise (ME) and one- month regular intensive exercise (IE). Data are represented as mean±SD. *P<0.05 between exercised groups and control rats.

	Control	ME	IE
LH	12.22±1.3	14.54±2.1*	10.61±1.6
T	1.81±0.4	2.80±0.6*	2.4±0.6

Discussion

Our findings revealed that the *Gnrh* mRNA level did not change following IE, but the moderate exercise induced siderable increase in the expression of the *Gnrh* gene. Note that these alterations in *Gnrh* expression coincided with changes in the serum LH and testosterone levels across all experimental groups. The early study demonstrated that several physiological factors both directly and indirectly relay their impacts on GnRH neurons; therefore, short-term regular moderate and intensive exercises may exert their effects directly or indirectly. Numerous studies have addressed the role of KNDy neurons in reproduction and have emphasized the key role of this neuropeptide in projecting the effects of various factors on releasing GnRH/LH (Amodei et al., 2020; Yeo and Colledge 2018). Accordingly, the present research studied changes in the mRNA level of the arcuate neuropeptide (upstream of GnRH neurons) in response to short-term moderate and intensive physical activity. Our results indicate that some changes occurred in the expression of genes involved in regulating the neuronal function of GnRH and subsequently in the performance of the reproductive axis, in response to short-term moderate and intensive exercise. In addition, the results provide a new perspective for understanding the reason for reproductive hormonal alteration in response to exercise. Our data show that only NKB neuropeptide was significantly altered in response to short-term regular moderate exercise; therefore, NKB had a role in conveying the effect of one-month regular moderate physical activity on the hypothalamic-pituitary-gonadal axis.

The ME decreased *Nkb* mRNA level while intensive exercise did not influence the *Nkb* mRNA level. Thus, different intensities of short-term exercise had various effects on *Nkb* mRNA levels. Accordingly, based on the findings, the arcuate *Nkb* mRNA level was mainly associated with the exercise intensity rather than its duration. Concerning the effect of moderate exercise for one month, these results are similar to the findings of our previous study, in which treatment of rats with moderate exercise for six months decreased *Nkb* mRNA expression in the arcuate nucleus (Khajehnasiri et al., 2018).

On the other hand, although recent studies in rodents have reported contradictory effects of the NKB on GnRH and LH secretions (Rance et al., 2010), most of the investigations have suggested that the NKB had an inhibitory effect on LH secretion in rodents with high levels of sex steroid hormones. The NKB agonist acted as an inhibitor of LH secretion in both rats and mice (Amodei et al., 2020; Chaikhun et al., 2013; Grachev et al., 2012).

The opposite effect of NKB has been observed under the conditions of low levels of sex steroid hormones (Salehi et al., 2012). In the current study, the *Nkb* gene expression diminished in the ME group, where this reduction was consistent with elevation of LH and testosterone serum levels. Thus, the present results

confirmed the NKB inhibitory effect on the LH secretion in rodents. Moderate exercise may reduce the inhibitory effects of the NKB on GnRH neurons, effects that were followed by an increase in the LH and testosterone hormone levels. These findings are in line with the outcomes obtained by many researchers who report that moderate exercise has beneficial effects on the pituitary-gonadal axis (Di Luigi et al., 2012; Grandys et al., 2009).

According to the obtained data, *Pdyn* gene expression was not affected by short-term moderate and intensive exercise. However, our previous study suggested that long-term intensive and moderate exercise caused enhanced *Pdyn* mRNA expression in the arcuate nucleus (Khajehnasiri et al., 2018). Consequently, based on this finding, the arcuate *Pdyn* mRNA level was mostly associated with the exercise duration rather than its intensity. Numerous studies have described the inhibitory function of *Pdyn* neuropeptide released from KNDy neurons on GnRH/ LH (Lehman et al., 2010). No change in *Pdyn* gene expression, as an inhibitory factor of reproduction, in rats after moderate exercise could be another reason for the LH- and testosterone-level evaluation.

The *Kiss1* gene expression in the regular moderate- and intensive-exercise groups showed no significant difference, compared to control rats. Khajehnasiri et al. (2018) reported that long-term physical activity can not alter *Kiss1* gene expressions, which is in line with present results. Therefore, these findings indicated that *kiss1* mRNA level was not affected by different intensity and duration of physical activity.

Conclusion

Overall, our results indicate that one-month moderate exercise improves the male HPG function through reduction of *Nkb* gene expression. On the other hand, one-month intensive exercise does not have a distractive effect on the reproduction axis as LH and testosterone serum levels remain unchanged in response to one-month regular intensive exercise.

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Conflict of interest statement

The authors state no conflict of interest.

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