J Biosci Vol. 43, No. 4, September 2018, pp. 569–574
DOI: 10.1007/s12038-018-9782-1

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Various responses of male pituitary–gonadal axis to different intensities of long-term exercise: Role of expression of *KNDY*-related genes

NAZLI KHAJEHNASIRI[1,2], HOMAYOUN KHAZALI[1]* and FARZAM SHEIKHZADEH[3]

[1]*Department of Animal Sciences and Biotechnology, Faculty of Biological Sciences and Technology, Shahid Beheshti University, Tehran, Iran*

[2]*Department of Biological Sciences, Faculty of Basic Sciences, Higher Education Institute of Rab-Rashid, Tabriz, Iran*

[3]*Department of Animal Sciences, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran*

**Corresponding author (Email, homkhazali1@gmail.com)*

MS received 24 January 2018; accepted 21 June 2018; published online 1 August 2018

The essential role of regular physical activity has been emphasized for maintaining a healthy life. However, unfortunately, during the last few decades, the lifestyle of people has led to a decrease in physical activity. Research studies have shown that exercise of different intensities is applied on reproductive performance indices, luteinizing hormone (LH) and testosterone (T), with different effects. Nevertheless, the molecular and cellular mechanisms underlying its function are not completely understood. Therefore, this study aimed to evaluate the role of kisspeptin, neurokinin-B and pro-dynorphin (KNDY) gene-expression changes located in the upstream of GnRH neurons in transferring the effects of different long-term exercise intensities on male reproductive axis. Twenty-one adult Wistar rats were randomly divided into control, 6-month regular moderate exercise (RME-6) and 6-month regular intensive exercise (RIE-6). In moderate and intensive exercise groups, rats were treated 5 days a week for 60 min, at 22 and 35 m/min, respectively. Finally, the hypothalamic arcuate nucleus was isolated and the relative gene expression of kisspeptin (*Kiss1*), neurokinin-B (*Nkb*), pro-dynorphin (*Pdyn*) and gonadotropin-releasing hormone (*Gnrh*) genes were measured by real-time polymerase chain reaction method. The results showed that RIE-6 treatment decreased *Gnrh* and increased *Pdyn* mRNA levels in the arcuate nucleus. Furthermore, although RME-6 treatment decreased *Nkb* and increased *Pdyn* mRNA levels, the *Gnrh* mRNA was not affected. Regarding the *Gnrh* mRNA levels and serum concentrations of reproductive indices (LH and T), moderate exercise did not impose harmful effects on the hypothalamic–pituitary–gonadal axis than intensive exercise. The different impacts of diverse long-term exercise intensities on the male pituitary–gonadal axis maybe relay by the various changes in hypothalamic *Nkb* and *Pdyn* gene expressions.

Keywords. Gonadotropin-releasing hormone; hypothalamic–pituitary–gonadal axis; KNDY neuropeptides; regular intensive exercise; regular moderate exercise

1. Introduction

Today's lifestyle involves factors such as the body weight, participation in physical activity, work under stressful conditions, smoking, illicit drug use and so forth are known to affect reproductive performance (Sharma *et al*. 2013). Over the past few decades, research studies indicate that the reproductive ability and performance are affected in response to the intensity and duration of exercise differently (Du Plessis *et al*. 2011). The results of the previous studies have shown the role of moderate exercise on male fertility and acknowledged that regular–moderate exercise could improve the performance of the pituitary–gonadal axis and fertility (Sharma *et al*. 2013). However, the findings of intensive exercise often indicate the destructive and inhibitory role of this type of activity on reproductive ability, with low levels of sex hormones, hypogonadotropic hypogonadism, hypothalamic amenorrhea and infertility (Warren and Perlroth 2001; Manna *et al*. 2003; Olive 2010; Misra 2014). The term 'endurance athletes' is considered to be a fertility problem. Hence, the identification of the cellular and molecular mechanisms involved in developing changes in the hypothalamic–pituitary–gonadal (HPG) axis in response to various exercise protocols has attracted a lot of attention.

The main function of the HPG axis is related to the GnRH neurons (Pinilla *et al*. 2012). These neurons are highly affected by synapses in their soma and axonal regions. The axonal projections of the kisspeptin, neurokinin-B and pro-dynorphin (KNDY) neurons originated from the hypothalamic arcuate

nucleus involves most of these synapses. The three neuropeptides including the kisspeptin (Kiss1), neurokinin-B (NKB) and Pro-dynorphin (Pdyn) secreted from the KNDY neurons are regarded as the main proposed factors in controlling GnRH secretion (Pinilla *et al*. 2012; Clarke *et al*. 2015). The axonal projection of the KNDY neurons are drawn to the preoptic area and median eminence, and Kiss1 and NKB receptors are found at the surface of the GnRH neurons (Krajewski *et al*. 2005; Lehman *et al*. 2010), while the GnRH neurons lack the dynorphin receptor (Navarro 2012). Therefore, the two Kiss1 and NKB neuropeptides (directly), along with dynorphin neuropeptide (indirectly), affect the GnRH/LH activity.

The effective role of the Kiss1 system and its receptor has been confirmed in the natural reproductive performance of both sexes (Pinilla *et al*. 2012). On the basis of studies, Kiss1 receptor stimulates GnRH neurons and defects in the gene or receptor of this neuropeptide lead to hypogonadotropic hypogonadism (de Roux *et al*. 2003). Dynorphin, as another neuropeptide of the KNDY neurons, plays an inhibitory effect on reproductive performance (Navarro *et al*. 2009). Therefore, those dynorphin fibres leave the arcuate nucleus, enter the median eminence and cause inhibition of GnRH/LH activity (Rance *et al*. 2010). The effects of NKB on GnRH/LH activity, are not simply related to the impacts of the two neuropeptides. Since the changes in the secretion of LH in response to NKB, depending on the experimental animal model and the gonadal status are different (Grachev *et al*. 2014).

Exercise among other factors of lifestyle, can affect the performance of the HPG axis and is considered as an important indicator in health research. If different types of exercises play different roles in reproductive performance, the recognition of their cellular and molecular pathways should be emphasized. Since KNDY neuron may serve as a central node in the control of GnRH secretion, acting as conduits for a variety of intrinsic and extrinsic regulatory signals. Therefore, researchers examined the effect of various factors and hormones on KNDY-related gene expressions, upstream of GnRH, in different animal models, for determining the role of these neuropeptides in conveying the effect of these signals on HPG axis (Eghlidi *et al*. 2010; Lehman *et al*. 2010; Salehi *et al*. 2017). To the best of our knowledge, a few studies were conducted for determining the effects of long-term exercise on the molecular mechanisms, the gene expressions of Kiss1, NKB and pro-dynorphin neuropeptides, which are responsible for attenuating the reproductive function, so in this study, the gene expressions were taken into consideration.

2. Materials and methods

2.1 *Animals*

In this experimental study, 21 adult male Wistar rats (250 ± 50 g) were obtained from the animal house of the Pasteur Institute of Tehran, Iran. All rats were housed under a 12-h light/dark cycle at 22 ± 2°C temperature and were allowed free access to the standard laboratory chow and water. All procedures for the maintenance and use of the experimental rats were conducted based on the guide for the care and use of laboratory animals (*NIH Guide for Care and Use of Laboratory Animals*, 8th Edition, 2010) and were conducted with the approval of an institutional animal care and by a committee at Research and Ethics Committee of Shahid Beheshti University of Medical Sciences.

2.2 *Experimental design*

Preliminary care was taken to use the minimum number of the animals possible in each experiment, so 21 rats were randomly divided into three groups. (1) Control (n = 7), (2) 6-month regular moderate exercise (RME-6) (n = 7), (3) 6-month regular intensive exercise (RIE-6) (n = 7).

2.3 *Training protocol*

After the habituation period, the exercised rats were exposed to 5-day consecutive treadmill exercise (60 min/day) at a speed of 22 m/min (RME-6) and 35 m/min (RIE-6) (Hahn *et al*. 2007; Hesari *et al*. 2014). At the beginning of the 60-min exercise, 5 m/min was determined to warm up, the speed progressively increased to target speeds. At the end of the 60-min exercise, the speed decreased progressively to 5 m/min to cool down. The rats in the control group did not perform treadmill exercise and they were placed on a non-moving treadmill for 60 min 5 days a week, during the experimental period. The exercised rats were studied 24 h after their last exercise session (Hesari *et al*. 2014).

2.4 *Blood sampling and enzyme-linked immunosorbent (ELISA) assay*

All the rats were anaesthetized by the intraperitoneal injection of 100 mg/kg ketamine and 5 mg/kg xylazine (Hesari *et al*. 2014) between 8 and 12 PM after the treatment. Immediately, blood samples for the determination of luteinizing hormone (LH) and testosterone (T) were collected from the eye sinus, and accordingly, the serum was separated. In the next stage, LH and T levels were measured by using a rat ELISA kit from Cusabio and Bioassay Technology Laboratory. Finally, the duplicate ELISA tests were performed based on the manufacturer's protocols.

2.5 *Isolation of arcuate nucleus*

After blood sampling, the brain was immediately removed and washed with cold 0.9% normal saline, then the brain

Table 1. Sequence of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), kisspeptin (*Kiss1*), pro-dynorphin (*Pdyn*), neurokinin-B (*Nkb*) and gonadotropin-releasing hormone (*Gnrh*) primers in rat

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

with the hypothalamus facing upwards and the cerebral hemispheres downwards was placed on a foil, over dry ice. An anterior coronal section was used for diencephalon dissection, anterior to the optic chiasma and a posterior to the posterior border of the mammillary bodies. Next, the Arc was separated from the anteroventral periventricular nucleus (AVPV) with the third coronal cut in the middle of the optic tract, just rostral to infundibulum so, rostral or anterior (contains AVPV nucleus) and caudal or posterior (contains Arc nucleus). Finally, the Arc nucleus was isolated (Salehi 2013) and placed in liquid nitrogen. They were stored at −80°C for *Kiss1*, *Nkb*, *Pdyn* and *Gnrh* gene-expression analysis.

2.6 *RNA isolation and real time-polymerase chain reaction (RT-PCR)*

Total RNAs were extracted from the hypothalamus arcuate samples by using the YTzol Pure RNA buffer (Yekta Tajhiz, Iran). Then, their concentration and purity were detected by the nanodrop measurements. Finally, RNA was subjected to reverse transcription by using the reverse transcription kit (BIONEER). The triplicate reactions used for measuring *Kiss1*, *Nkb*, *Pdyn* and *Gnrh* mRNA levels were conducted on cDNA samples by using the gene-specific primers presented in table 1. Then, the relative expression of genes was evaluated on hypothalamic arcuate samples. In the next stage, RT-PCR was performed by using the SYBR Green PCR Master Mix (Takara Bio Inc). Finally, the quantitative real-time PCR data were analysed by using the comparative Ct method and the relative expression of the target mRNAs over reference values was calculated based on arithmetic formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

2.7 *Data analysis and statistics*

Normality test was used to see whether the collected data on the relative expression of target genes were normally distributed or not. One-way ANOVA plus the Tukey post-hoc test was utilized to compare the difference between groups by using SPSS statistical program (version 24). The mean ± SEM were reported in the text and $p < 0.05$ was considered as the level of significance.

3. Results

3.1 *Gnrh expression in response to RME-6 and RIE-6*

Real-time PCR was undertaken in the arcuate hypothalamus following 6-month intensive exercise treatment, which shows decreased *Gnrh* mRNA levels. However, 6-month moderate exercise treatment was not effective to induce a significant elevation in the gene expression (figure 1).

3.2 *KNDY-related gene expression in response to RME-6 and RIE-6*

Previous articles have shown the effect of exercise on HPG axis. Also, Kiss1, NKB and pro-dynorphin are known as the main regulators of HPG axis. To further investigate, effects of RIE-6 and moderate exercises on the arcuate *Kiss1*, *Nkb* and *Pdyn* mRNA levels (neuropeptides upstream of GnRH neurons) were tested by using real-time PCR analysis. RIE-6 group showed a significant increase in the *Pdyn* expression ($p < 0.05$) (figure 2). However, no difference was observed in the arcuate *Nkb* and *Kiss1* mRNA levels in the RIE-6

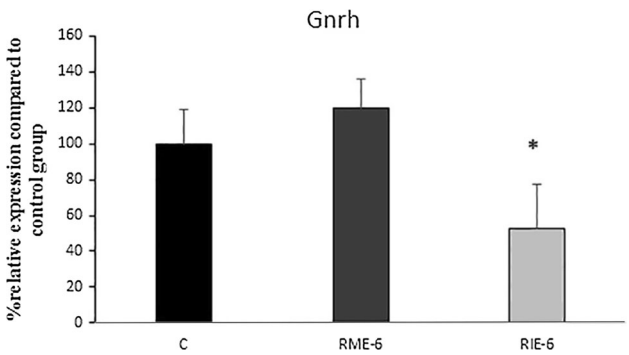

Figure 1. Relative expression of *Gnrh* in control (C), RME-6 and RIE-6 groups ($n = 7$ in each group). Data are represented as mean ± SEM. *$p<0.05$.

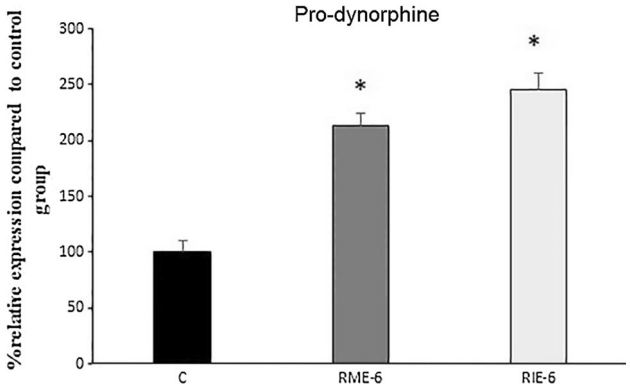

Figure 2. Relative expression of *Pdyn* in control (C), RME-6 and RIE-6 groups ($n = 7$ in each group). Data are represented as mean ± SEM. *p<0.05.

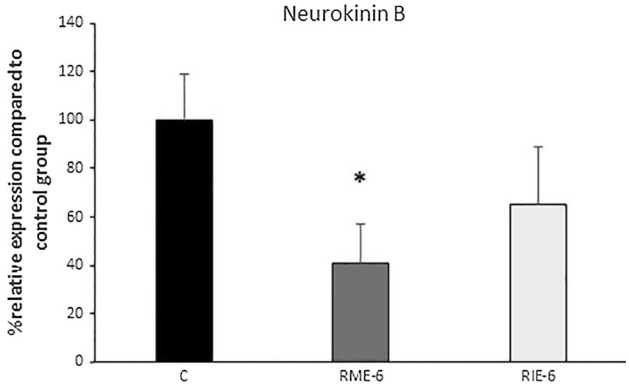

Figure 3. Relative expression of *Nkb* mRNA in control (C), RME-6 and RIE-6 groups ($n = 7$ in each group). Data are represented as mean ± SEM. *p<0.05.

group (figures 3 and 4). Also, RME-6 treatment increased the level of *Pdyn* mRNA and decreased *Nkb* mRNA in the arcuate nucleus (figures 2 and 3), whereas no difference was observed in *Kiss1* mRNA expression in the RME-6 rats (figure 4).

Finally, LH and T serum concentrations, as the reproductive performance indicator, were tested in response to RIE-6 and RME-6 by the ELISA method. The LH serum concentration was significantly suppressed in the RIE-6 group ($p < 0.05$) (figure 5a), and this suppression continued by T reduction, in the RIE-6 group ($p < 0.05$) (figure 5b). However, no difference was observed in the serum LH and T levels in the RME-6 group (figure 5a and 5b).

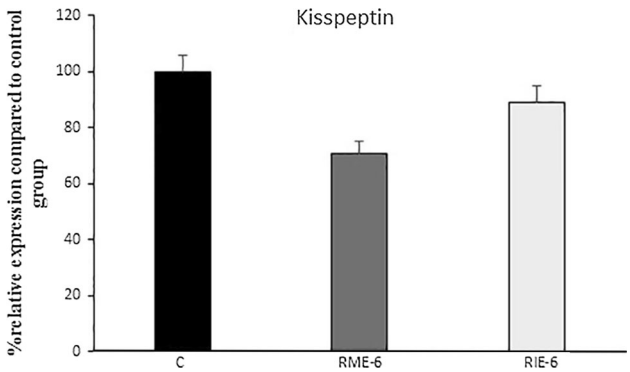

Figure 4. Relative expression of Kiss*1* mRNA in control (C), RME-6 and RIE-6 groups ($n = 7$ in each group). Data are represented as mean ± SEM. *p<0.05.

4. Discussion

This study demonstrates that *Gnrh* expression decreased following RIE-6 and interestingly the moderate exercise was not effective against inducing a significant elevation in the *Gnrh* gene expression. Also, the alteration of *Gnrh* mRNA levels was consistent with serum LH and T concentration changes, in all the studied groups.

The previous studies showed that physiological factors have their effects on GnRH neurons, directly and indirectly. This issue suggests that the effects of long-term exercise on GnRH neurons may be either direct and/or indirect. The hypothalamic arcuate nucleus is related to the presence of the KNDY neuron as one of the domain interneurons to influencing the release of GnRH/LH. Therefore, the expression of *Kiss1*, *Nkb* and *Pdyn* genes in the arcuate nucleus was investigated in response to the different intensities of the long-term exercise.

The results of changes in the *Kiss1*/*Nkb* and *Pdyn* neuropeptide gene expression revealed that only Pdyn neuropeptide showed a significant increase in response to long-term intensive exercise. Therefore, this neuropeptide can have a role in inducing the impact of this exercise on the HPG axis. However, modifying the type of exercise caused some changes in the regulatory pathway. Regular–moderate exercise reduced the *Nkb* gene expression, along with the increased *Pdyn* mRNA level. Given the evidence that many neuropeptides and neurotransmitters are involved in the neuroendocrine control of GnRH, the current finding shown may indicate the existence of a multi-neuropeptides mechanism that eventually causes the regulation of *Gnrh* gene expression in response to long-term exercise.

The long-term moderate and intensive exercise showed a significant increase in the relative expression of the *Pdyn* gene, compared to the control group. Thus, based on the results, arcuate *Pdyn* gene expression was more related to the exercise duration than the intensity. It is worth noting that, it is not the first time that the various exercise duration and intensity have demonstrated the different effects on dynorphin system. In some studies, prolonged exercise decreases the binding and activity of opioid receptor, while short-term exercise increases them (Arida *et al*. 2015). Also, another study indicated, only

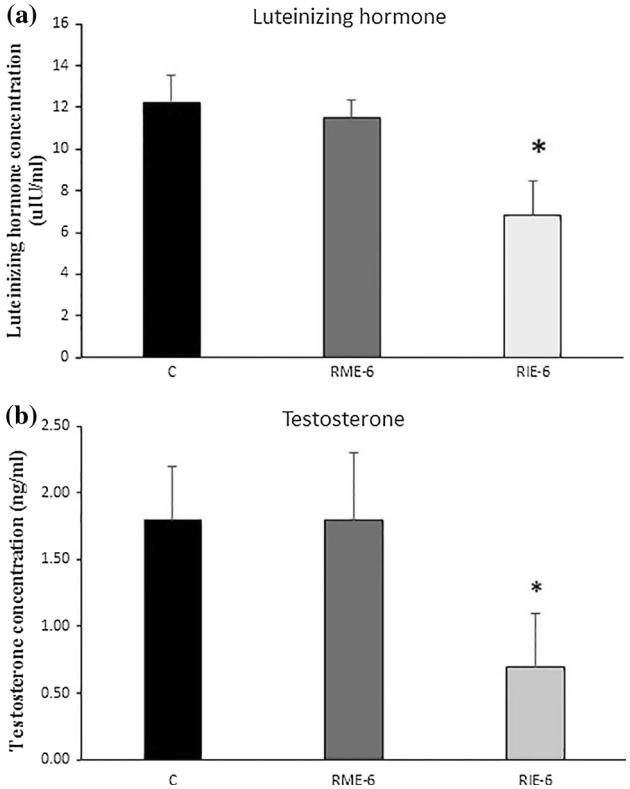

Figure 5. (**a**) LH and (**b**) T serum levels in control (C), RME-6 and RIE-6 groups (n = 7 in each group). Data are represented as mean ± SEM. *p<0.05.

intensive exercise was effective in inducing a significant elevation in the peripheral circulation of opioid levels (Bourova *et al*. 2010; Arida *et al*. 2015). This result is inconsistent with the current finding because in this study *Pdyn* gene expression significantly increases in response to intensive exercise as well as moderate exercise.

A large number of studies reported the inhibitory role of Pdyn, neuropeptide released from KNDY neurons, on GnRH / LH (Navarro *et al*. 2009; Lehman *et al*. 2010; Pinilla *et al*. 2012). It is worth noting that, the GnRH neurons lack the dynorphin receptor but, this receptor is found on the KNDY neurons (Navarro 2012). Therefore, the Pdyn neuropeptide may be either auto synaptic on KNDY neurons, which inhibits the release of GnRH/LH, by inhibiting the Kiss1 neurons or, can lead to the inhibition of GnRH neurons by inhibitory interneurons. Recent studies have shown that the distribution of the dynorphin receptor (KOR) in the KNDY neurons is negligible. This issue raises the possibility that dynorphin has its inhibitory effects on GnRH neuron through inhibitory interneuron (Urbanski 2012). In this study, the increase in *Pdyn* gene expression in RME-6 and RIE-6 groups failed to show any significant change in the *kiss1* mRNA levels. Thus, the results confirm that dynorphin may play a significant role in releasing GnRH/LH with the inhibitory interneuron pathway. Regarding the change in the expression of the *Pdyn* gene in response to long-term exercise, it is likely that a part of the changes observed in levels of LH and T hormones in the treatment groups is mediated by changes in the expression of the *Pdyn* neuropeptide gene expression.

The *Pdyn* gene expression in the regular moderate exercise group showed a statistical increase, therefore, reduction in LH and T concentration is expected. A reduction of *Nkb* along with *Pdyn* elevation may be the reason for LH and T to remain unchanged. According to the obtained data, only moderate exercise could reduce the *Nkb* gene expression. By considering the arcuate *Nkb* expressing cells interacting with GnRH neurons especially at median eminence directly and its inhibitory effect in rodents (Sandoval-Guzmán and Rance 2004; Chaikhun *et al*. 2013); therefore, long-term exercise may modulate GnRH secretion at least in part by regulating *Nkb* expression. The accumulation of two inhibitory neuropeptides alteration, NKB reduction and Pdyn evaluation, may neutralize the long-term exercise effect on GnRH neurons and kept LH and T concentrations unchanged in response to regular moderate exercise.

5. Conclusion

The results of this research study prove the role of Pdyn and NKB neuropeptides in conveying the effect of long-term moderate and intensive exercise on male HPG axis, for the first time. The results indicated that various intensities of long-term exercise play a different effect on the *Nkb* gene expression. These different *KNDY*-related gene expressions may diversely affect the male pituitary–gonadal axis.

Acknowledgements

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector. All procedures for the maintenance and use of the experimental animals were conducted in accordance with the guide for the care and use of the laboratory animals (*NIH Guide for Care and Use of Laboratory Animals*, 8th Edition, 2010) and were conducted with the approval of an institutional animal care and use committee at the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences.

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Corresponding editor: BJ Rao