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The effects of intracerebroventricular infusion of apelin-13 on reproductive function in male rats

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HIGHLIGHTS

- Infusion of central apelin-13 can suppress LH pulse.
- Apelin-13 decreases serum testosterone levels.
- Apelin-13 may cause infertility in male rats.

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ABSTRACT

Apelin is a novel bioactive peptide as the endogenous ligand for APJ. Apelin and APJ have also been identified in the testis, hypothalamic nuclei such as arcuate, supraoptic and paraventricular nuclei, implicating roles in the control of reproduction.

Therefore, the present study was designed to investigate the effects of chronic central infusion of apelin-13 on LH, FSH and testosterone levels and testis morphology. 21 Wistar–Albino male rats received continuous intracerebroventricular infusion via Alzet osmotic mini pumps filled artificial cerebrospinal fluid (vehicle) or apelin-13 at concentrations of 1 or 10 nmol (10 μl/h) for seven days. At the last 90 min of the infusion period, the blood samples were collected at 15 min intervals (0–90 min) for LH and FSH analyses. At the last sampling point, the blood samples were analyzed for testosterone levels.

Infusion of high dose apelin-13 significantly suppressed LH release compared with the vehicle values at 30, 60 and 75 min ($p < 0.05$). However, FSH levels did not significantly differ among the groups. Serum testosterone levels in high dose apelin-13 group were statistically lower than the control group ($p < 0.05$). In addition, histological examination showed that infusion of high dose apelin-13 significantly decreased the number of Leydig cells compared with the control and lower dose apelin-13 groups ($p < 0.05$, $p < 0.01$).

Our results suggest that apelin-13 may play a role in the central regulation and decreases testosterone release by suppressing LH secretion. Thus, antagonists of the apelin receptor may, therefore, be useful for pharmaceuticals in the treatment of infertility.

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1. Introduction

Gonadotropin-releasing hormone (GnRH) is a neuropeptide utilized as messenger molecules in the systems of nervous and neuroendocrine to control many physiological processes containing reproduction and sexual behavior. The secretion of GnRH from the hypothalamus is necessary for the synthesis and secretion of ideal gonadotropin (luteinizing hormone; LH and follicle stimulating hormone; FSH), and finally for normal reproductive behavior [18,21,27]. Reproductive functions start with the secretion of GnRH from the hypothalamus; this process is followed by releasing the hormones of LH and FSH from the anterior pituitary [12]. Testosterone secretion from the cells of Leydig in the testis improves with LH stimulation [36]. In addition to the system of central GnRH, there

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is available evidence for the peripheral production for GnRH in testicular cells [9], ovarian granulosa cells [26], and in the pituitary gland [2].

In 1993, described to APJ as a putative receptor protein related to AT1 in a human gene [23]. Further evidence suggested important roles for these receptors in the regulation of fluid homeostasis [28,32] and cardiovascular function [14]. Successively, apelin, endogenous ligand for APJ, was identified in human and bovine tissue that it is derived from a 77-amino-acid precursor [17,19,30].

The varying forms of the apelin peptide including apelin-13, apelin-17 and apelin-36 have been shown in different tissues in order to activate the APJ. Apelin-13 is the main circulatory form, and its biological activity is greater than apelin-17 and 36 [33,35]. It has been shown that apelin and its receptor APJ are widely expressed in several tissues such as lung, heart, kidney, ovary, testis, mammary gland, gastric mucosa, adipose tissue and central nervous system [15,19,20].

There are studies showing both large number of apelin positive cells and large amount of mRNA expression of APJ in the hypothalamus area in the rat brain, especially in the paraventricular (PVN) and the supraoptic (SON) nuclei [7,8,22]. The intense labeling in anterior and intermediate lobes of the pituitary has also been exhibited [8].

The presence of APJ in the brain areas related to reproduction such as hypothalamus and hypophysis suggests that apelin can play a specific role in the control of LH and FSH release from the adenohypophysis. In addition, distribution of APJs in the ovary and testicles brings to mind that apelin may affect regulation of reproduction through hypothalamus–hypophyseal–gonadal axis.

Therefore, the current study was designed to investigate the effects of central infusion of apelin-13 on plasma LH, FSH and serum testosterone levels and testis morphology in the male rats.

2. Materials and methods

2.1. Animals and experimental design

In the present study, was used to 21 adult Wistar–Albino male rats (weighing 300–350 g), and all protocols were approved by the local ethics committee of Inonu University. Rats were housed in a temperature controlled room at $22 \pm 1\,^{\circ}C$ with a 12:12 h light/dark cycle and they were fed as ad libitum. Twenty one rats were randomly divided into three groups, control (vehicle), low (1 nmol) and high (10 nmol) doses of apelin-13 group ($n = 7$ in each group).

Animals were anesthetized with ketamine and xylazine and placed in a sterotaxic frame (Harvard Apparatus, USA) for intracerebroventricular (icv) injection. Right lateral ventricle coordinates were decided from the rat brain stereotaxic coordinates (1.4 mm lateral, 0.8 mm posterior and 4.8 mm vertical from bregma) [25]. The brain infusion kits were placed in right lateral ventricle and fixed by dental cement. Subsequently, an Alzet osmotic mini pump was implanted by insertion under the skin.

All rats received continuous icv infusion via osmotic mini pumps filled artificial cerebrospinal fluid (aCSF in mmol/l: KCl 3.4, NaCl 133.3, $MgCl_2$ 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 32.0, NaH_2PO_4 0.6 and glucose 3.4, pH 7.4) [13] for control (10 μl/h) or apelin-13 at concentrations of 1 or 10 nmol (10 μl/h) for experimental group for seven days.

Rats were re-anesthetized and implanted a catheter into femoral vein after seven days from implantation of osmotic mini pumps. Blood samples were collected at the 15 min intervals (0, 15, 30, 45, 60, 75 and 90 min) for LH and FSH analysis. At the last sampling point (90 min), blood samples were analyzed serum testosterone levels. The testis were taken for histological examination after the rats sacrificed.

2.2. LH, FSH and Testosterone enzyme immunoassays

The levels of LH and FSH were analyzed based on Pappa et al. with some alterations [24]. 96-well immunoplates (Nunc, Denmark) were coated with rat LH or rat FSH. Standards or plasma samples were preincubated with primer antibodies and were then transferred into coated plates for competition with antigens on the solid phase. Plates were washed, and the secondary antibodies conjugated to streptavidine peroxidase was added into each well, and color was developed by using tetramethylbenzidine as a substrate. Plates were read at 450 nm using a plate reader (Tecan Spectra III, Austria). Rat LH, rat FSH and primer antibodies (rabbit anti-rat LH and rabbit anti-rat FSH) were achieved from Dr. A.F. Parlow (NIDDK, NIH, USA) and secondary antibodies (goat anti-rabbit IgG) conjugated to streptavidin peroxidase was bought from Sigma (Sigma–Aldrich, Germany). Serum testosterone levels of rats was identified by EIA (Testosterone EIA kit; Cayman Chemical Company, USA), in pursuance of the manual.

2.3. Histological examination

For histological study, testis were fixed in formalin%10, dehydrated in ethyl alcohol, cleared in xylol and embedded in paraffin wax. 5 μm thickness sections were cut, and stained with haematoxylin-eosin. We measured the diameter of the seminiferous tubule (hundred tubules for each testis). The Leydig cells from twenty diverse interstitial regions of each testis were counted. The sections were examined using a Leica Q Win plus Image Analysis System (Leica Micros Imaging Solutions Ltd., UK), respectively, at 10X and 100X.

2.4. Statistical analysis

Statistical analysis was performed using SPSS software (version 22.0). The experimental results were reported as mean ± SEM (standard error of means). Normality assumption was confirmed by Kolmogorov Smirnov test. One-way analysis of variance (ANOVA) was employed to compare the experimental groups with the controls. Multiple comparisons were carried out using post hoc Tukey HSD test. In addition, histological results were compared with Kruskal–Wallis variance analysis. Where differences among the groups were detected, groups were compared using the Mann–Whitney U with Bonferroni correction. Values of $p < 0.05$ were considered significant.

3. Results

3.1. The effect of icv apelin-13infusion on LH and FSH release

After the icv apelin-13 infusion, the plasma LH and FSH levels changes in the groups were shown in Figs. 1 and 2, , respectively. The LH levels of animals showed an increase in all groups that apelin-13 was administered. However, infusion of both low and high dose apelin-13 significantly suppressed LH release ($p < 0.05$) compared with the vehicle values at 30, 60 and 75 min (Fig. 1). However, FSH levels did not significantly differ among the groups (Fig. 2).

3.2. The effect of icv apelin-13 infusion on serum testosterone levels

Effects of icv apelin-13 administration on serum testosterone levels were shown in Fig 3. Low dose apelin-13 suppressed serum testosterone, but this was not statistically significant. In contrast,

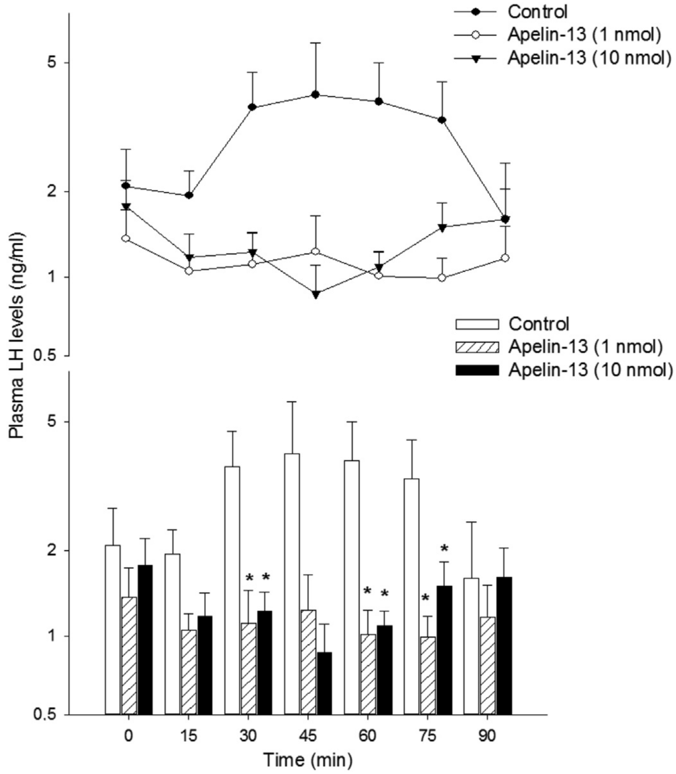

Fig. 1. Plasma LH release after apelin-13 infusion (*$p < 0.05$); the experimental results were reported as mean ± SEM.

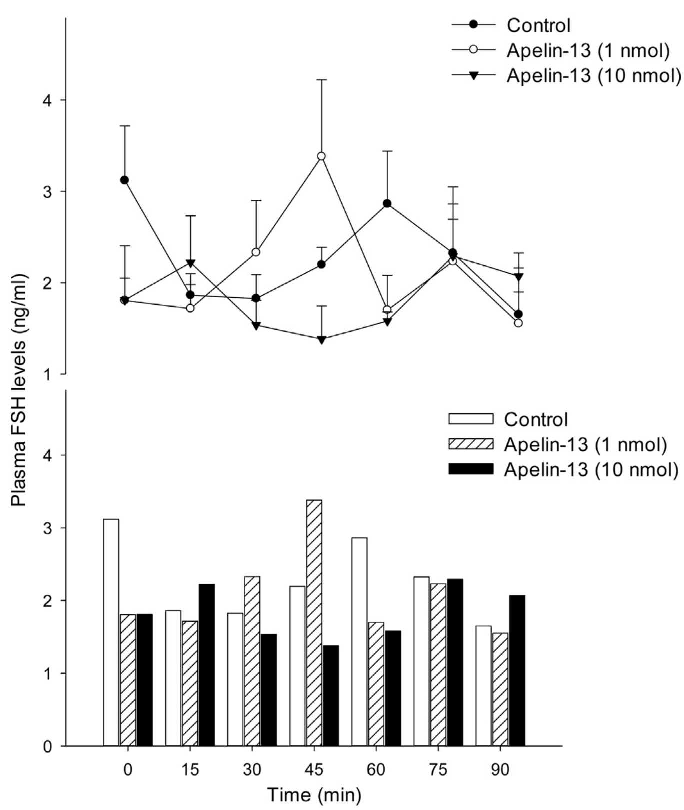

Fig. 2. Plasma FSH release after apelin-13 infusion; the experimental results were reported as mean ± SEM.

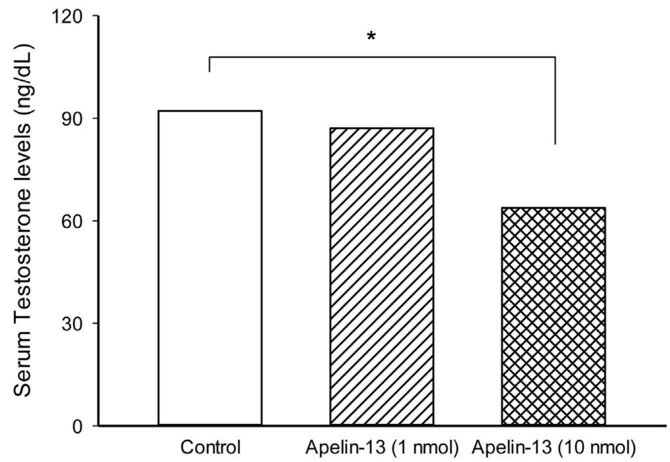

Fig. 3. Serum testosterone levels after apelin-13 infusion (*$p < 0.05$); the experimental results were reported as mean ± SEM.

serum testosterone levels in high dose apelin-13 group were statistically lower than the control group ($p < 0.05$).

3.3. Histological results

In many seminiferous tubules germ cells organized in concentric layers and seminiferous tubules containing all stages of spermatogenesis in sections of all groups. However, in some tubules slight tubular atrophy was present such as spermatogenic arrest and disorganization of germinal cells in the all groups (Fig. 4).

Leydig cells were located between seminiferous tubules and in close vicinity to blood capillaries. Euchromatic round-shaped nuclei of the cells condensed chromatin on the inner part of the nucleolemma. Histologic appearance of Leydig cells was indistinguishable in all groups (Fig. 5).

Nonetheless, Leydig cell numbers in high dose apelin-13 group significantly decreased according to the control group (5.4 ± 3.01 vs 6.4 ± 2.9, respectively, $p = 0.01$). High dose of apelin-13 administration significantly reduced the Leydig cell numbers. On the other hand, the number of Leydig cell in the low dose apelin-13 group were found to be increased when compared to control group (7.6 ± 3.1 vs 6.4 ± 2.9 respectively, $p = 0.001$). Spermatogonia, spermatocytes and spermatids cells of the germinal epithelium were less affected. Mean seminiferous tubule diameter decreased in the low and high dose apelin-13 groups when compared to control group; but no significant difference was found ($p = 0.3$). Moreover, mean seminiferous tubule diameter in low dose and high dose apelin-13 groups were almost similar. Diameters seminiferous tubules and Leydig cell numbers were given, respectively, Figs. 4 D and 5 D.

4. Discussion

It is important for the continuum of the generations that reproductive behavior and the central and peripheral control mechanisms of this behavior are known. Hormonal network that is responsible for the reproductive control interacts over the hypothalamus–hypophyseal–gonadal axis. This axis is composed of three big compartments: hypothalamus where the GnRH synthesis is carried out; frontal pituitary where LH and FSH are synthesized; gonads where the sex steroids and other hormones are produced [6]. GnRH is released especially from ARN, PVN, SON and medial preoptic nuclei of hypothalamus [37].

In studies carried out in mammalia, it has been demonstrated that pulsatile LH secretion from the frontal pituitary accompanies

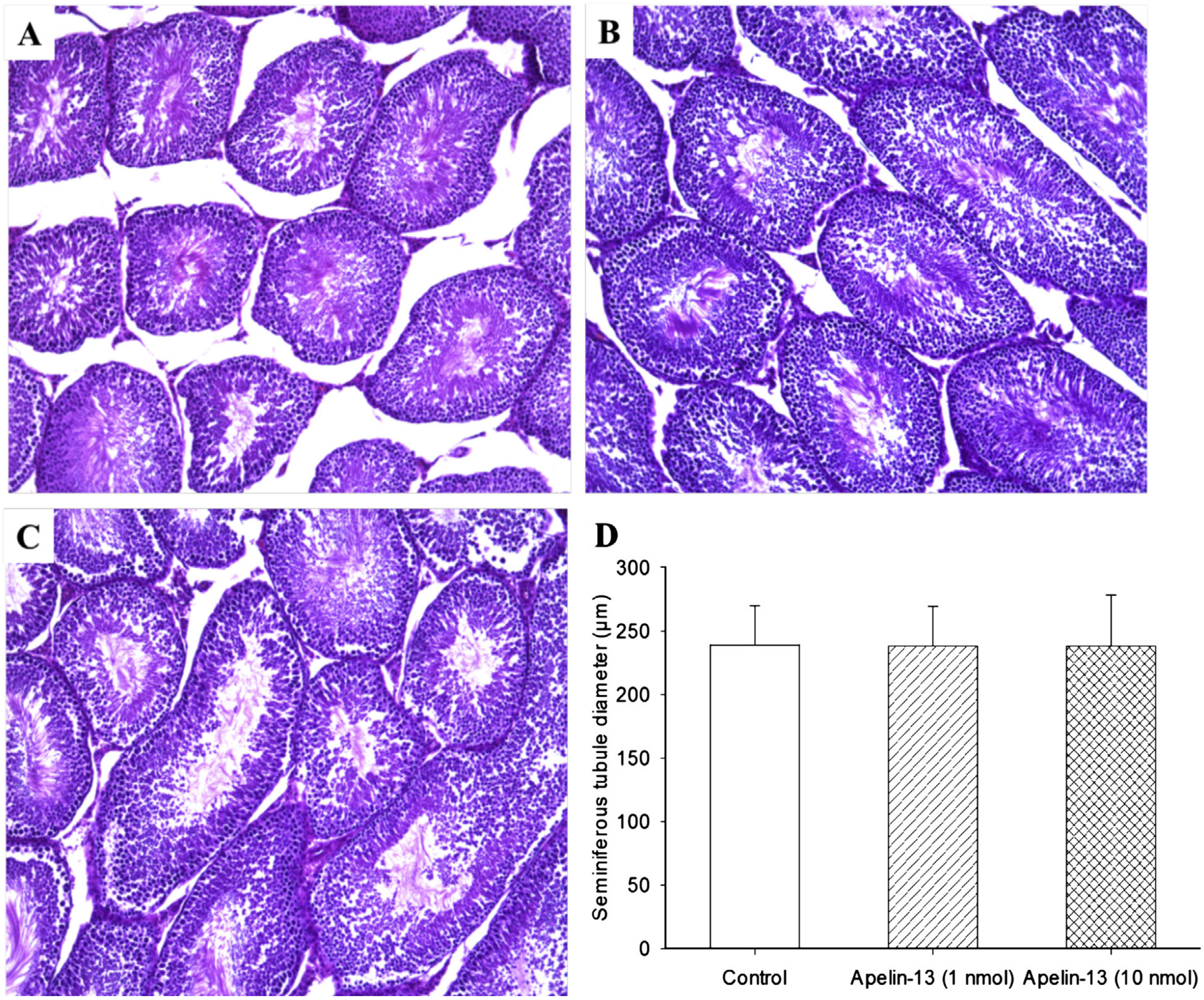

Fig. 4. Histological appearance of seminiferous tubules in (A) control, (B) 1 nmol and (C) 10 nmol apelin-13 groups, H–E X10. (D) The experimental results were reported as mean ± SEM.

the pulsatile GnRH release from hypothalamus [1,34]. LH secretion of frontal pituitary causes testosterone release over Leydig cells in testis [3]. Testosterone, that is added to the circulation by being secreted from the Leydig cells, can stop the GnRH release via a negative feedback mechanism [10]. FSH, another gonadotropin released in frontal pituitary with the effect of GnRH, is known to start spermatogenesis by stimulating sertoli cells in testis [4].

Presence of high amount of apelin and APJ in brain sections where GnRH is released (especially in SON and PVN) [5,29] and reproductive organs such as testis and ovaries [15,22] may indicate that apelin may have important effects on the reproductive system. Some of the reproductive processes may be regulated with the help of apelin and APJ. A study reported that LH could increase the levels of apelin and APJ mRNA in theca cells [30]. In another study, it has been revealed that apelin inhibits the human uterine contractions [11]. The results of our study identified that LH release from the groups with apelin-13 treatment was lower in all sampling times compared to control; however, this suppression was statistically significant only in 30, 60 and 75 min. The decrease in the levels of LH at only 30, 60 and 75 min can emerge from the release LH pulse. In parallel, it has been noted that serum testosterone levels from the high dose apelin-13 group was significantly lower. There was not a difference observed in plasma FSH levels in either time interval among the groups. There has been no study regarding the long term infusion of apelin on male reproduction system; therefore, this study is the first in this aspect. However, in a study carried out on rats, following an icv (single injection) apelin-13 treatment in different doses (3, 10, 30 nmol), it was reported that plasma LH and FSH levels decreased in 10 nmol apelin-13 given group compared to control [31]. In regards to plasma LH level, although the results of the aforementioned study were similar to our results, single application and LH analyses obtained from blood samples taken from a single time point were not appropriate with the pulsatile nature of LH release. Plasma FSH levels in the study were different from our results. Differences in apelin treatment time and study follow-up time are the main reasons for such a result. Following the histological examination of the testis that are the last part of hypothalamus–hypophyseal–gonadal axis, in high dose apelin treatment group, although Leydig cells were decreased (except 1 nmol apelin-13 treated group), there were no differences among groups in spermatogenesis steps and seminiferous tubule diameters. In a study, it was demonstrated that blockage of GnRH release and LH stimulus from hypothalamus caused a decrease in the volume of Leydig cells and stop of testosterone production in rats [16]. Depending on the apelin dose, the decrease caused in the number of Leydig cells and in testosterone levels was not accompanied by the seminiferous tubule diameters and spermatogenesis steps. Thence, this result directs us to think that there was not a direct effect of apelin on testis, rather it does result from the suppression caused in LH release.

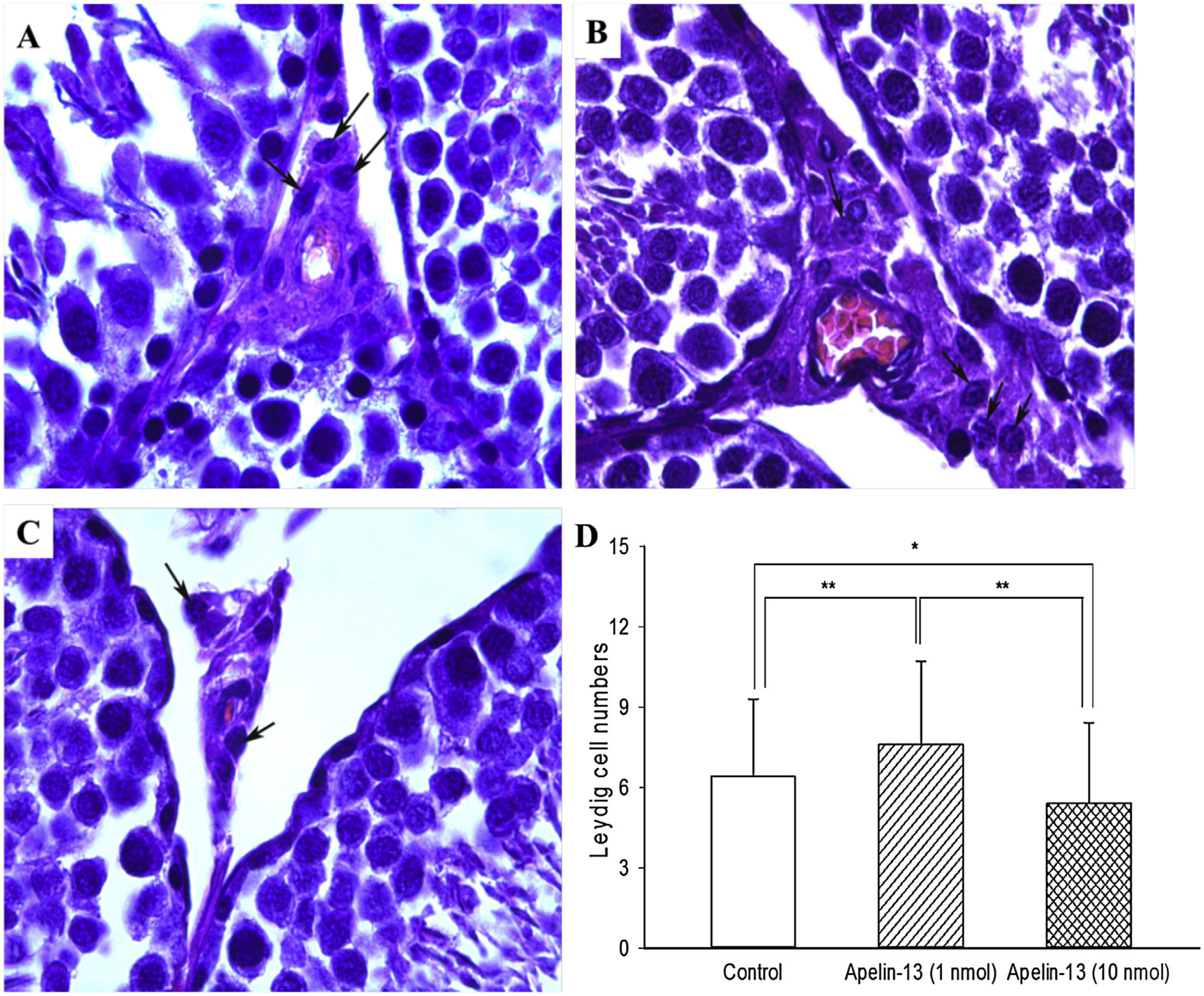

Fig. 5. Leydig cells located in the vicinity of vessel capillary (arrows) in (A) control, (B) 1 nmol and (C) 10 nmol apelin-13 groups. Notice condensed chromatin on the inner part of the nuclear envelope, H–E X100. (D) The experimental results were reported as mean ± SEM (*$p < 0.05$, ** $p < 0.01$).

Consequently, our results suggest that apelin-13 negatively affects the male reproductive system by suppressing LH pulse in spite of the unclear of the cellular mechanisms.

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References

[1] A. Bachelot, Z. Chakhtoura, G. Plu-Bureau, M. Coudert, C. Coussieu, Y. Badachi, J. Dulon, B. Charbit, P. Touraine, Influence of hormonal control on LH pulsatility and secretion in women with classical congenital adrenal hyperplasia, Eur. J. Endocrinol. 167 (2012) 499–505.
[2] T.W. Bauer, C.M. Moriarty, G.V. Childs, Studies of immunoreactive gonadrotropin releasing hormone (GnRH) in the rat anterior pituitary, J. Histochem. Cytochem. 29 (1981) 1171–1178.
[3] M.C. Beattie, L. Adekola, V. Papadopoulos, H. Chen, B.R. Zirkin, Leydig cell aging and hypogonadism, Exp. Gerontol. 68 (2015) 87–91.
[4] M. Bergendahl, W.S. Evans, J.D. Veldhuis, Current concepts on ultradian rhythms of luteinizing hormone secretion in the human, Hum. Reprod. Update 2 (1996) 507–518.
[5] G.C. Brailoiu, S.L. Dun, J. Yang, M. Ohsawa, J.K. Chang, N.J. Dun, Apelin-immunoreactivity in the rat hypothalamus and pituitary, Neurosci. Lett. 327 (2002) 193–197.
[6] W.H. Colledge, H. Mei, X. d'Anglemont de Tassigny, Mouse models to study the central regulation of puberty, Mol. Cell. Endocrinol. 324 (2010) 12–20.
[7] K. Czarzasta, A. Cudnoch-Jedrzejewska, The role of the apelinergic and vasopressinergic systems in the regulation of the cardiovascular system and the pathogenesis of cardiovascular disease, Kardiol. Pol. 72 (2014) 122–125.
[8] N. De Mota, Z. Lenkei, C. Llorens-Cortes, Cloning, pharmacological characterization and brain distribution of the rat apelin receptor, Neuroendocrinology 72 (2000) 400–407.
[9] L. Di Matteo, M. Vallarino, R. Pierantoni, Localization of GnRH molecular forms in the brain, pituitary, and testis of the frog, Rana esculenta, J. Exp. Zool. 274 (1996) 33–40.
[10] C. Foresta, P. Bordon, M. Rossato, R. Mioni, J.D. Veldhuis, Specific linkages among luteinizing hormone, follicle-stimulating hormone, and testosterone release in the peripheral blood and human spermatic vein: evidence for both positive (feed-forward) and negative (feedback) within-axis regulation, J. Clin. Endocrinol. Metab. 82 (1997) 3040–3046.
[11] M.P. Hehir, J.J. Morrison, The adipokine apelin and human uterine contractility, Am. J. Obstet. Gynecol. 206 (2012) 359, e351–e355.
[12] J.M. Jin, W.X. Yang, Molecular regulation of hypothalamus-pituitary-gonads axis in males, Gene 551 (2014) 15–25.
[13] S. Kagiyama, M. Fukuhara, K. Matsumura, Y. Lin, K. Fujii, M. Iida, Central and peripheral cardiovascular actions of apelin in conscious rats, Regul. Pept. 125 (2005) 55–59.
[14] S.D. Katugampola, J.J. Maguire, S.R. Matthewson, A.P. Davenport, [125I]-(Pyr1) Apelin-13 is a novel radioligand for localizing the APJ orphan receptor in human and rat tissues with evidence for a vasoconstrictor role in man, Br. J. Pharmacol. 132 (2001) 1255–1260.
[15] Y. Kawamata, Y. Habata, S. Fukusumi, M. Hosoya, R. Fujii, S. Hinuma, N. Nishizawa, C. Kitada, H. Onda, O. Nishimura, M. Fujino, Molecular properties of apelin: tissue distribution and receptor binding, Biochim. Biophys. Acta 1538 (2001) 162–171.
[16] D.S. Keeney, R.L. Sprando, B. Robaire, B.R. Zirkin, L.L. Ewing, Reversal of long-term LH deprivation on testosterone secretion and Leydig cell volume, number and proliferation in adult rats, J. Endocrinol. 127 (1990) 47–58.

[17] M.J. Kleinz, A.P. Davenport, Emerging roles of apelin in biology and medicine, Pharmacol. Ther. 107 (2005) 198–211.
[18] L.Z. Krsmanovic, S.S. Stojilkovic, K.J. Catt, Pulsatile gonadotropin-releasing hormone release and its regulation, Trends Endocrinol. Metab. 7 (1996) 56–59.
[19] D.K. Lee, R. Cheng, T. Nguyen, T. Fan, A.P. Kariyawasam, Y. Liu, D.H. Osmond, S.R. George, B.F. O'Dowd, Characterization of apelin, the ligand for the APJ receptor, J. Neurochem. 74 (2000) 34–41.
[20] A.D. Medhurst, C.A. Jennings, M.J. Robbins, R.P. Davis, C. Ellis, K.Y. Winborn, K.W. Lawrie, G. Hervieu, G. Riley, J.E. Bolaky, N.C. Herrity, P. Murdock, J.G. Darker, Pharmacological and immunohistochemical characterization of the APJ receptor and its endogenous ligand apelin, J. Neurochem. 84 (2003) 1162–1172.
[21] S.M. Moenter, A.R. DeFazio, G.R. Pitts, C.S. Nunemaker, Mechanisms underlying episodic gonadotropin-releasing hormone secretion, Front. Neuroendocrinol. 24 (2003) 79–93.
[22] A.M. O'Carroll, S.J. Lolait, L.E. Harris, G.R. Pope, The apelin receptor APJ: journey from an orphan to a multifaceted regulator of homeostasis, J. Endocrinol. 219 (2013) R13–R35.
[23] B.F. O'Dowd, M. Heiber, A. Chan, H.H. Heng, L.-C. Tsui, J.L. Kennedy, X. Shi, A. Petronis, S.R. George, T. Nguyen, A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11, Gene 136 (1993) 355–360.
[24] A. Pappa, K. Seferiadis, M. Marselos, O. Tsolas, I.E. Messinis, Development and application of competitive ELISA assays for rat LH and FSH, Theriogenology 51 (1999) 911–926.
[25] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, London, UK, 2007.
[26] C. Peng, N.C. Fan, M. Ligier, J. Väänänen, P.C. Leung, Expression and regulation of gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells, Endocrinology 135 (1994) 1740–1746.
[27] D.D. Rasmussen, Episodic gonadotropin-releasing hormone release from the rat isolated median eminence in vitro, Neuroendocrinology 58 (1993) 511–518.
[28] A. Reaux, N. De Mota, I. Skultetyova, Z. Lenkei, S. El Messari, K. Gallatz, P. Corvol, M. Palkovits, C. Llorens-Cortes, Physiological role of a novel neuropeptide, apelin, and its receptor in the rat brain, J. Neurochem. 77 (2001) 1085–1096.
[29] A. Reaux, K. Gallatz, M. Palkovits, C. Llorens-Cortes, Distribution of apelin-synthesizing neurons in the adult rat brain, Neuroscience 113 (2002) 653–662.
[30] T. Shimizu, N. Kosaka, C. Murayama, M. Tetsuka, A. Miyamoto, Apelin and APJ receptor expression in granulosa and theca cells during different stages of follicular development in the bovine ovary: involvement of apoptosis and hormonal regulation, Anim. Reprod. Sci. 116 (2009) 28–37.
[31] S. Taheri, K. Murphy, M. Cohen, E. Sujkovic, A. Kennedy, W. Dhillo, C. Dakin, A. Sajedi, M. Ghatei, S. Bloom, The effects of centrally administered apelin-13 on food intake, water intake and pituitary hormone release in rats, Biochem. Biophys. Res. Commun. 291 (2002) 1208–1212.
[32] K. Takayama, H. Iwazaki, M. Hirabayashi, K. Yakabi, S. Ro, Distribution of c-Fos immunoreactive neurons in the brain after intraperitoneal injection of apelin-12 in Wistar rats, Neurosci. Lett. 431 (2008) 247–250.
[33] K. Tatemoto, M. Hosoya, Y. Habata, R. Fujii, T. Kakegawa, M.X. Zou, Y. Kawamata, S. Fukusumi, S. Hinuma, C. Kitada, T. Kurokawa, H. Onda, M. Fujino, Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor, Biochem. Biophys. Res. Commun. 251 (1998) 471–476.
[34] R. Tsutsumi, N.J. Webster, GnRH pulsatility, the pituitary response and reproductive dysfunction, Endocr. J. 56 (2009) 729–737.
[35] A. Valle, N. Hoggard, A.C. Adams, P. Roca, J.R. Speakman, Chronic central administration of apelin-13 over 10 days increases food intake, body weight, locomotor activity and body temperature in C57BL/6 mice, J. Neuroendocrinol. 20 (2008) 79–84.
[36] W.H. Walker, Non-classical actions of testosterone and spermatogenesis, Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365 (2010) 1557–1569.
[37] C.M. Whitelaw, J.E. Robinson, P.M. Hastie, V. Padmanabhan, N.P. Evans, Effects of cycle stage on regionalised galanin, galanin receptors 1-3, GNRH and GNRH receptor mRNA expression in the ovine hypothalamus, J. Endocrinol. 212 (2012) 353–361.