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Effects of intracerebroventricular administration of calcitonin gene-related peptide (CGRP) on sex hormones and sperm quality in rats

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Background: Therapeutic strategies with calcitonin gene-related peptide (CGRP) or its receptor have been investigated, but there are few studies regarding the possible harmful effects of CGRP in other body organs.
Objective: This study aimed to investigate the effect of intracerebroventricular (ICV) injection of CGRP on sex hormones and sperm quality in rats.
Methods: Twelve male rats were divided into two groups ($n = 6$ per group). The first group (control) rats were injected with 5 µl artificial cerebrospinal fluid intra-ICV; the second group rats, 5 µl (1.5 nmol) CGRP. The levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone were measured. Epididymal sperms were used to determine the sperm parameters.
Results: The levels of testosterone, LH and FSH in CGRP group was significantly lower than in artificial cerebrospinal fluid (ACSF) group ($P < 0.05$). The concentration and motility of sperm in CGRP group was significantly lower than in ACSF group ($P < 0.05$). In CGRP group live spermatozoa and intact acrosome significantly reduced compared to the ACSF group ($P < 0.05$). In addition, in CGRP group dead spermatozoa and lose acrosome significantly increased compared to the ACSF group ($P < 0.05$).
Conclusion: ICV injection of CGRP may reduce sperm quality, probably through induction of an imbalance in FSH and LH production as well as testosterone.

Keywords: CGRP, Intracerebroventricular injection, LH, FSH, Testosterone

Introduction

Calcitonin gene-related peptide (CGRP) is a neuropeptide belonging to a family of bioactive peptides that consists of a chain containing 37 amino acids produced by neurons in both the central and peripheral nervous systems. CGRP has various actions in the central and peripheral nervous system[1,2]. CGRP is increased under conditions that stimulate neuroinflammation, such that migraines and pain[3]. CGRP has been shown to be a mediator of inhibition of gonadotropin-releasing hormone (GnRH) secretion following a stressful stimulus. Investigating the direct effect of CGRP on GnRH neurons using the GT1-7 cell line has shown that CGRP decreases GnRH mRNA expression, which is reversed by the CGRP receptor antagonist, CGRP8-37[4]. In addition, it has been shown that CGRP inhibits the pulsatile secretion of LH in the anterior pituitary using µ and κ opioid receptors. Naloxone, an opioid antagonist, has been shown to stimulate the release of luteinizing hormone (LH)[5]. There is a synaptic connection between opioidergic neurons and CGRP[6]. Although CGRP is well known to directly inhibit testosterone in the testis and inhibit the release of LH in the pituitary by increasing cAMP[7], less is known the effect of CGRP while injected intracerebroventricularly (ICV). A study established the fact that ICV injection of CGRP inhibits the LH pulse in female Wistar rats[8].

CGRP has biological functions other than the nervous system. They found the role of CGRP in promoting tumour-related angiogenesis. Another recent study supports the cardioprotective role of CGRP. These functions are important considerations in therapeutic strategies with CGRP or its receptor because it may have deleterious effects in other organs or systems[9,10].

The hypothalamic-pituitary-gonadal (HPG) axis can regulate fertility[11]. Anterior pituitary gonadotropic cells play an important role in regulating the signal between GnRH in the

HIGHLIGHTS

- Intracerebroventricular injection of calcitonin gene-related peptide decrease testosterone.
- Intracerebroventricular injection of calcitonin gene-related peptide decrease LH and FSH.
- The motility of sperm in calcitonin gene-related peptide group was decrease.
- The concentration of sperm in calcitonin gene-related peptide group was decrease.

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hypothalamus and gonads. Binding of GnRH to its receptor in gonadotropic cells in the anterior pituitary gland activates the signalling pathway which ultimately leads to the release of follicle-stimulating hormone (FSH) and LH. The signalling pathway is activated as a result of the action of the HPG neuroendocrine axis[12]. Some conditions such as stress causes the release of CGRP, which in turn inhibits HPG axis[13].

In males, LH stimulates the production of testosterone hormone by Leydig cells and androgen binding protein by Sertoli cells. FSH binds to its receptors on the surface of Sertoli cells and, together with testosterone, causes the proliferation and development of spermatogonia. In the mice, deletion of the β subunit gene in LH, or its receptor, reduces the production of gonadal steroid hormones and, as a result, reduces fertility[14]. Deletion of the β-subunit in FSH decreases both testicular size and sperm production[15]. This study was conducted to investigate the effect of intra-ICV injection of CGRP on the level of serum sex hormones and sperm quality in rats.

Methods

Animals

All experiments were performed on male Sprague-Dawley rats with a mean ± SD weight of 260 ± 30 g. Food and water was freely available to the animals. Until the time of the experiment, the animals were kept at a temperature of 22 ± 2°C and darkness/light cycles of 12/12 h. All stages of this study has been reported in line with the ARRIVE criteria[16].

Study groups

Twelve male rats were divided into two groups ($n = 6$ per group). The first group rats were injected with 5 µl artificial cerebrospinal fluid (ACSF) intra-ICV; the second group rats, 5 µl (with doses of 1.5 nmol) CGRP. ICV injections of ACSF or CGRP were done 72 h before animal euthanasia (Fig. 1).

Surgery

In order to cause the least complications, animals were anaesthetised using low dose sodium pentobarbital (50 mg/kg)[17].

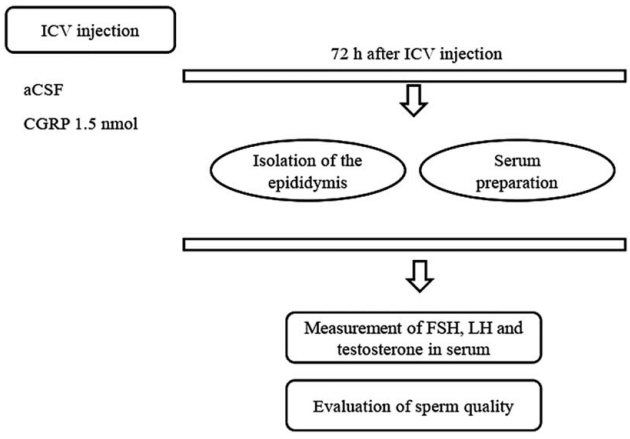

Figure 1. Schematic representation. aCSF, artificial cerebrospinal fluid; CGRP, calcitonin gene-related peptide; FSH, follicle-stimulating hormone; ICV, intracerebroventricular; LH, luteinizing hormone.

After reaching the appropriate level of anaesthesia (the animal's lack of response to environmental stimuli), the surgery was started. Thus, the animals were then fixed in the stereotaxic machine. A cannula was implanted in the left ICV (AP = -0.8, L = + 1.5, DV = -3.5) and fixed with dental cement. Breathing, body temperature, colour of the mucous membranes, and possible dehydration of the animal are checked throughout the surgery. After surgery, the animals were monitored until they regained consciousness, and their level of consciousness and body temperature were monitored. After one day, an ICV injection was performed using a Hamilton syringe. Animals were anaesthetised using CO_2. Blood sampling was done and the serums were stored at -20°C. Immediately after being euthanized, the animal testicles were removed from and the epididymis sperms were collected.

Measurement of FSH, LH and testosterone in serum

The serum concentrations of FSH, LH (Zellbio, Germany, ELISA Kit) and testosterone were measured with the ELISA method (AccuBind, ELISA Kit).

Evaluation of sperm quality

Epididymis was incubated 30 min in 1 ml Ham's F10 medium. Ten microlitres of sperm suspension was placed on the Neubauer slide and sperm concentration was calculated in five squares using a light microscope (×40 magnification).

To study the sperm motility, a drop of undiluted semen was placed on a slide at 37°C without a cover slip and examined under phase-contrast microscope (×100; Nikon, Eclipse, E200, Japan). At least 200 spermatozoa, selected at random from a minimum of four microscopic fields, were examined. The mean of all fields were recorded as the final motility score. Motility was evaluated as a percentage of progressive (slow and rapid), immotile and non-progressive spermatozoa.

Sperm suspension solution (5 µl) was mixed on a glass slide placed on a stage at 37°C; 30 s later, the mix was smeared and allowed to air dry. Smears were stained with Giemsa solution and mounted with DPX, a mixture of distyrene, a plasticizer, and xylene. Slides were then examined at ×1000 under a bright field; 200 spermatozoa from each rat were examined. The percentage of sperm with intact acrosomes, excluding the cells that showed damaged or missing acrosomes were reported.

Results

The results described mean ± SD in study groups as follows. Sperm the sperm motility in CGRP group (186.80 ± 55.32) was significantly lower than in ACSF group (256.60 ± 21.68) ($P = 0.02$) (Fig. 2).

The sperm concentrations in the CGRP group (50.80 ± 11.62) was significantly lower than that in the ACSF group (69.21 ± 4.43) ($P = 0.02$) (Fig. 3).

The number of live spermatozoa in the CGRP group was significantly ($P = 0.01$) lower than the ACSF group (Table 1). The highest number of dead spermatozoa belonged to CGRP group, significantly higher than the ACSF group ($P = 0.01$). In the CGRP group, intact acrosome was significantly ($P = 0.01$) lower compared to the ACSF group. The number of lose acrosome in the second group was also significantly ($P = 0.02$) higher than the ACSF group.

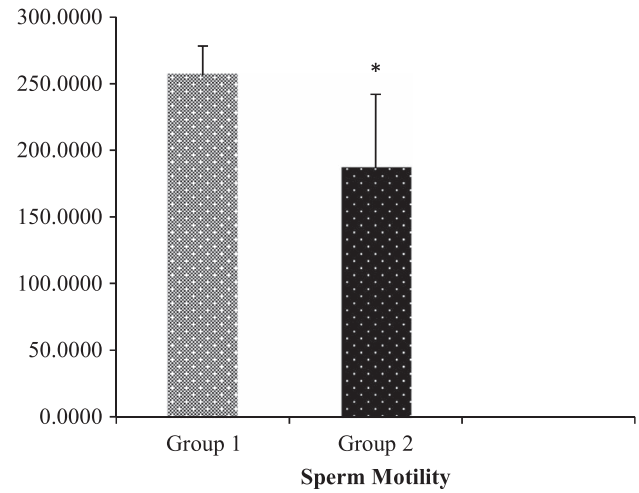

Figure 2. Sperm motility score in studied groups. Group 1: intra-intracerebroventricular (ICV) injection of 5 μl artificial cerebrospinal fluid; Group 2: intra-ICV injection of 1.5 nmol calcitonin gene-related peptide. *Significant difference between the second and first group.

The level of testosterone in CGRP group was significantly lower than in ACSF group ($P = 0.01$). The serum concentrations of LH in the CGRP group were significantly lower than those in the ACSF group. The levels of FSH in the CGRP group were significantly lower than those in the ACSF group (Table 2).

Statistical analysis

For data analysis, the software used was SPSS version 16. One-way ANOVA and Duncan's supplementary test were utilised for the analysis. A significance level of P less than 0.05 was considered as indicating a statistically significant difference.

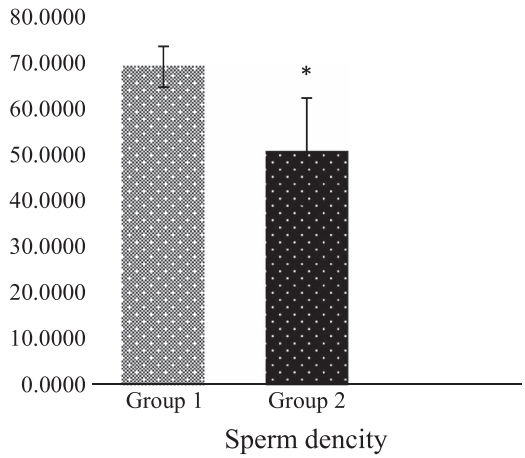

Figure 3. Sperm density in studied groups. Group 1: intra-intracerebroventricular (ICV) injection of 5 μl artificial cerebrospinal fluid; Group 2: intra-ICV injection of 1.5 nmol calcitonin gene-related peptide. *Significant difference between the second and first group.

Table 1

Mean ± SD of different forms of spermatozoa in studied groups

Group	Live spermatozoa	Dead spermatozoa	Intact acrosomes	Loose acrosome
1	37.2 ± 4.94	13.8 ± 4.94	35 ± 3.30	13.40 ± 3.97
2	26.8 ± 6.11[a]	25.2 ± 8.11[a]	17.4 ± 4.60[a]	32.81 ± 2.49[a]

Group 1: intra-ICV injection of 5 μl artificial cerebrospinal fluid (ACSF); Group 2: intra-ICV injection of 1.5 nmol CGRP; Group 3: intra-ICV injection of 1.5 nmol sCT.
ACSF, artificial cerebrospinal fluid; CGRP, calcitonin gene-related peptide; ICV, intracerebroventricular; sCT, salmon calcitonin.
[a]Significant difference between the second and first group.

Discussion

We showed that, ICV injection of CGRP decreased the serum level of LH, FSH, and testosterone. Also, CGRP significantly reduced sperm motility and density compared to the control group. CGRP reduced live spermatozoa and intact acrosome compared to the control group and increased dead spermatozoa and loose acrosome.

In a study, CGRP was found in the cauda epididymis of rat and human. It is suggested that CGRP may regulate the electrolyte and fluid secretion in the epididymis, thereby providing an optimal microenvironment for the maturation and storage of spermatozoa[18]. In the current study, intra-ICV injection of CGRP decreased concentration and motility of sperm. According to previous reports, seminal plasma calcitonin can affect sperm motility[19]. Also, CGRP caused the reduction of healthy acrosomes.

Research studies that have been conducted on calcitonin family peptides since the previous years have focused more on calcium homoeostasis. Recently, calcitonin (CT) applications on the brain, pituitary gland and gonads have been noticed[20,21]. CT receptors have been found in the brain and pituitary gland[22,23], testicular Leydig cells[24] and ovarian cells[25]. In one study, CT and CGRP were administered from the jugular vein, and blood samples were collected at 0, 10, 30, 60, 120, 180, 360, and 720 min after the injection. Plasma testosterone declined gradually from 2 to 6 h after intravenous injection of CT or CGRP. CT and CGRP also inhibit the LH release[26].

In the previous study, the potential direct action of CGRP on GnRH neurons investigated and demonstrated that CGRP downregulates expression of GnRH mRNA; in the GT1-7 cells. CGRP can act directly on GnRH neurons *in vitro* and therefore raise the possibility of a direct action on the GnRH release. ICV administration of CGRP resulted in a profound, suppression of LH pulses, which was reversed by the CGRP receptor antagonist[4]. Our results showed that intra-ICV injection of

Table 2

Mean ± SD concentration of LH, FSH, and testosterone in studied groups.

Testosterone ng/ml	FSH μu/ml	LH μu/ml	Group
3.81 ± 0.12	777.80 ± 29.40	1959 ± 89.26	1
2.61 ± 0.25[a]	605.00 ± 63.74[a]	1678.2 ± 58.81[a]	2

Group 1: intra-ICV injection of 5 μl artificial cerebrospinal fluid (ACSF); Group 2: intra-ICV injection of 1.5 nmol CGRP; Group
ACSF, artificial cerebrospinal fluid; CGRP, calcitonin gene-related peptide; FSH, follicle-stimulating hormone; ICV, intracerebroventricular; LH, luteinizing hormone.
[a]Significant difference between the second and first group.

CGRP decreased the level of serum LH, FSH and testosterone. FSH, LH and testosterone are the pivotal endocrine factors controlling testicular functions. Low levels of GnRH reduce FSH and LH, resulting in dysfunction of Sertoli and Leydig cells[27]. The absence of LH-dependent testosterone production leads to azoospermia, suggesting that testosterone is strictly required for sperm production. The role FSH in reproductive physiology in both sexes is clear. It has a synergic action with testosterone in spermatogenesis[28–30].

Inhibiting the release of CGRP or blocking its receptors during early arthritis might prevent persistent inflammation[31]. CGRP was shown to either stimulate cytokine production such as interleukins-1β, TNFα, and interleukins-6 in macrophages in previous reports[32]. Acute and chronic inflammation is associated with testosterone deficiency[33]. Therefore, intra-ICV injection of CGRP may play a role in reducing serum testosterone level by stimulating inflammatory pathways. Pro-inflammatory cytokines in Leydig cells has been shown to reduce production of testosterone[33]. It has been reported that the gene expression of GnRH decreases following inflammation. Therefore, by reducing the expression of GnRH, the level of FSH and LH hormones decreases[34].

Conclusion

The results of the current study showed that intra-ICV administration of CGRP could possibly affect sperm concentration, motility and acrosome characteristics by reducing FSH, LH and testosterone. However, generalising the study to humans requires more experiments.

Ethical approval

The protocol of this study was approved by the Ethics Committee of the Veterinary Faculty of Shahid Chamran Ahvaz University, Ahvaz, Iran. (Number: EE/1401.2.24.193086/scu.ac.ir).

Consent

All participants are required to fill out an informed consent.

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Author contribution

K.R., M.R., J.S. and S.N. participated in the study design, methodological issues, analysis, interpretation of the study, and writing of manuscript.

Conflicts of interest disclosure

The authors declare that they have no competing interest.

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Guarantor

Javad Sajedianfard is the person in charge of the publication of our manuscript.

Data availability statement

Data sharing is applicable to this article after acceptance.

Provenance and peer review

Yes.

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