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Immunolocalization of RFamide-related peptide 3 in a desert rodent *Gerbillus tarabuli* during seminiferous epithelium cycle

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ABSTRACT

Gerbillus tarabuli is a nocturnal seasonal breeder desert rodent with a main breeding season in spring and summer, and sexual quiescence in winter. This species is an interesting model for studying testis function in rodents. Therefore, the present study was performed firstly to investigate the stages of seminiferous epithelium cycle of *Gerbillus tarabuli* with a histological, morphometric and statistical study. And secondly to investigate the expression and possible variations in cellular distribution of *RFamide-related peptide-3* (RFRP-3) - the mammalian ortholog of avian *gonadotropin-inhibitory hormone* (GnIH) - during seminiferous epithelium cycle using immunohistochimestry. Our results showed for the first time that the seminiferous epithelium cycle in *Gerbillus tarabuli* comprises 14 well-defined stages according to the tubular morphology method. The seminiferous epithelium thickness showed a significant difference during the epithelium cycle, thus it was the only morphometric classification criterion of seminiferous epithelium cycle in *Gerbillus tarabuli*. The immunohistochemical study reveals, for the first time, the presence of RFRP-3 in *Gerbillus tarabuli* testes, in both testicular compartments: the tubular and the interstitial. RFRP-3 is expressed differently according to the seminiferous epithelium cycle, RFRP-3 seemed to be more expressed at the stages V-VII and XIII. RFRP-3 was detected in Sertoli cells (≈12%), spermatocytes I (≈19%), round and elongated spermatids (≈13%), and with a more important signal in Leydig cells (26.87% ± 0.07). These results indicated the importance of RFRP-3 in testicular function in *Gerbillus tarabuli*; its expression at the interstitial and germinal levels argues in favor of an involvement in androgens synthesis and in spermatogenesis specifically in meiosis and spermiogenesis. This action seems primordial from stages V-VII and XIII. Also, the study of the seminiferous epithelium cycle will enrich the histological identity of the species.

1. Introduction

Gerbillus tarabuli is a nocturnal, omnivorous desert rodent with seasonal breeding. Its range covers the desert and the semi-desert regions of North Africa (Klein et al., 1975; Le Berre, 1990). Gerbils are physiologically adapted to living in arid environments and can provide their water needs from the metabolism of their food. The weight of the adult is estimated to be above 32.8 g (Granjon et al., 1999). Their seasonal sexual cycle has a main sexual reproduction period stretching from April to November and sexual quiescence in January-February (Klein et al., 1975). Although *Gerbillus tarabuli* represents an attractive model to study testis function in rodents, there are few data regarding the reproductive biology of this species. Spermatogenesis is a cyclic highly coordinated process by which a diploid spermatogonium complete a series of events to become stream lined haploid spermatozoa capable of motility. It begins with mitotic spermatogonial proliferation, proceeds through two meiotic divisions, and is followed by spermiogenesis in which haploid spermatids develop into spermatozoa that will ultimately be released from the seminiferous epithelium via a process known as spermiation (Russell et al., 1990). The seminiferous epithelium cycle is highly organized process encompasses different cell associations or stages (Leblond and Clermont, 1952a, 1952b; Xiao et al., 2014). The stages of the seminiferous epithelium cycle might be classified according to the changes in the shape of the spermatids nuclei, the occurrence of meiotic divisions, and the arrangement of spermatids within the germinal epithelium (Berndtson, 1977). As they can be charecterized based on the development of the acrosomic system and the morphology of developing spermatids nuclei (Leblond and

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Clermont, 1952a, 1952b; Russell et al., 1990).

The vertebrate reproductive cycle is controlled by several neuropeptides/hormones coordinating the activity of the hypothalamo-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) is a key hypothalamic decapeptide hormone known to modulate the biosynthesis and release of both gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Schally et al., 1971). Up until recently, GnRH thought to be the only hypothalamic regulator of pituitary gonadotropin synthesis and release. In 2000, the discovery of Tsutsui et al., in the Japanese quail was the first to demonstrate the existence of a hypothalamic neuropeptide inhibiting gonadotropin release dubbed Gonadotropin-Inhibitory Hormone (GnIH) (Tsutsui et al., 2000). The mammalian ortholog of GnIH, RFamide-related peptide (RFRP-3) (Hinuma et al., 2000; Kriegsfeld et al., 2006), belongs to the group with the LPXRF-amide carboxyl peptide consensus sequence (X = L or Q) (Tsutsui, 2009; Tsutsui et al., 2010). GnIH regulates avian reproduction via GPR147 by decreasing gonadotropin common α-and β-subunit production and release via action on both, the GnRH system and the anterior pituitary gland (Ciccone et al., 2004; Tsutsui, 2009; Tsutsui et al., 2010; Ubuka et al., 2006). In mammals, RFRP-3 also suppresses reproductive axis activity (Kriegsfeld et al., 2006; Ducret et al., 2009) In male rats, RFRP increase due to stress, induced suppression of reproductive function via decreasing steroidogenic activity of the testis (Karami et al., 2016). Alternatively, some stimulatory effects have been reported. In male Syrian hamsters and mice, functional diversity was observed compared to mammals and birds, GnIH stimulates GnRH neurons and secretion of gonadotropins in Syrian hamsters (Ancel et al., 2012). RFRP-3 induces the expression of gene c-Fos in GnRH neurons and stimulates LH release in short photoperiod, while in long photoperiod, the effect is however inhibitory (Ancel et al., 2012). Central administration of RFRP-3 had a dose-dependent stimulatory effect on LH secretion in mice (Ancel et al., 2017), suggesting that the peptide action depends on species and physiological state (Ancel et al., 2012; Osugi et al., 2014). Few studies have focused on the function of RFRP-3 in rodents peripheral systems, however, in the testis, the expression of RFRP3 has been demonstrated in mice (Anjum et al., 2012). RFRP3 and its receptors, GPR147 et GPR74, have been investigated in the testis of Syrian hamster by semi-quantitative RT-PCR and Immunohistochemistry (Zhao et al., 2010) suggesting that GnIH/RFRP may act directly at the level of the gonad. The purposes of our study was to firstly determine the stages of seminiferous epithelium cycle of *Gerbillus tarabuli* with a histological, morphometric and statistical approaches, and secondly, to investigate the expression and the distribution of RFRP-3 during the cycle by immunohistochemical analysis.

2. Material and methods

2.1. Sample collection

The animals were collected in a desertic region in the North-Western Algerian Sahara,the Beni-Abbes region, Bechar State in Algeria (30° 4′ 48″ N, 2° 6′ 0″ W). All experiments were carried out in compliance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) following approval by the local Ethical Committee of Houari Boumediene University of Sciences and Technology, Algeria. The 5 adults used were at sexual active period (Table 1). They were weighed and sacrificed at night, regarding their nocturnal state, by dissection under a dose of anesthetic 25% Urethane (1.5 g/Kg I.P = 0.4 ml per 100 g of body weight). Testes were collected and post-fixed in the Holland's Fixative solution, then dehydrated in graded alcohols, embedded in paraffin wax and sectioned to a thickness of 7 µm. Several sets of slides were prepared for histological, morphometric, and immunohistochemical analyses. The sections were deparaffinized, hydrated then stained with Heidenhain's azan trichrome stain (Martoja and Martoja-Pierson, 1967).

Table 1
Biometric and morphometric data in sexually mature *Gerbillus tarabuli*.

Parameter (n = 5)[a]	Mean ± SEM
Body weight (g)	40.20 ± 3.1
Left Testis weight (g)	0.27 ± 0.01
Gonadosomatic index (%)[b]	0.68 ± 0.08
Lumen (µm)	33.50 ± 2.88
Tubular diameter (µm)	182.58 ± 2.49
Seminiferous epithelium height (µm)	75.04 ± 1.77

[a] Number of animals utilized n.
[b] The **gonadosomatic index**: GSI = [Gonad Weight / Total Tissue Weight] x 100 (Barber & Blake 2006). It was calculated to measure the sexual maturity of gerbils in correlation to testes development.

2.2. Seminiferous epithelium cycle identification

The seminiferous epithelium cycle stages in *Gerbillus tarabuli* were characterized based on "the tubular morphology system" method (Berndtson, 1977) which relies upon changes in the shape of the spermatid nucleus, the occurrence of meiotic divisions, the arrangement of spermatids within the germinal epithelium and the overall seminiferous epithelium composition (Roosen-Runge and Giesel, 1950). The stages were determined of six testis sections for each adult at 40x magnification.

2.3. Morphometrical analysis

The tubular and lumen diameter as well as the thickness of the seminiferous epithelium were measured at 40x magnification on a total of 20, 4 tubules of each stage for each animal (4 x each stage x 5 individuals). Digital images were obtained using a Premiere (MAX-300)® photonic microscope with a Hirocam, Premiere® (MA88-500) camera and analyzed with the ISCapture Tucsen® Imaging software.

2.4. Immunohistochemistry for RFRP-3

Immunohistochemical analysis of RFRP-3 was conducted on testis using deparaffinized and hydrated sections. Endogenous peroxidase was quenched with 3% H_2O_2 for 10 min and equilibrated in Phosphate Buffered Saline (PBS 0.1 M, pH 7.5). The nonspecific binding of the antibody was blocked using Blocking Serum (NHS; Normal Horse Serum; Vectastain Quick kit PK-8800, Vector Laboratories, Burlingame, CA) for 10 min. Sections were then incubated in primary antibody (polyclonal Goat anti-RFRP-3, Santa Cruz Biotechnology, Inc) diluted at a concentration of 1:300 in PBS 0.1 M for 1 h at room temperature. To examine the specificity of the secondary antibody, the primary antibody was omitted in the controls. Two subsequent washes in PBS 0.1 M at room temperature were followed by a 10 min incubation with biotinylated secondary antibody (polyclonal Horse IgG anti-Goat, 1:200) of the same kit. Sections were then incubated for 5 min in streptavidin–peroxydase complex. The resulting complex was visualized using 0.03% 3,3′-diaminobenzidine tetra-dihydrochloride (DAB, Sigma). Nucleus was counterstained with hematoxylin QS. Slides were then dehydrated, mounted, observed under a Premiere (MAX-300)® photonic microscope and photographed using a Hirocam, Premiere® (MA88-500) camera.

2.5. Labeling quantification

A quantitative study of immunoreactivity was carried out using Fiji Win 32 of Image J software (NIH, Bethesda, USA). The labeling was measured for 50 cells of each cellular category per individual using a ×40 objective magnification.