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Seasonal variation of peptidase activities in the reproductive tract of *Crotalus durissus terrificus*

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ABSTRACT

Seasonal quantitative patterns of acid (APA), basic (APB), puromycin-sensitive (APN-PS) and puromycin-insensitive neutral (APN-PI), cystyl (CAP), dipeptidyl IV (DPPIV), type-1 pyroglutamyl (PAP-I) and prolyl-imino (PIP) aminopeptidases and prolyl oligopeptidase (POP) activities in soluble (SF) and solubilized membrane-bound (MF) fractions from ductus deferens, vagina and uterus were studied to evaluate their relationships with the reproductive cycle and the extensive long-term spermatozoa storage (LTSS) of the Neotropical rattlesnake *Crotalus durissus terrificus*. APB, PIP and POP were detected only in SF, while other peptidases were detected in SF and MF. APB, APN-PI and APN-PS were predominant in most tissues in all seasons. Peptidase activities had a common pattern of increment during the dry season (winter/autumn), which coincides with the mating period (autumn) and LTSS in the female (winter), as well as the reduction of spermatozoa motility and maintenance of fertilization capacity of spermatozoa. The high CAP activity in the soluble fraction of the vagina during winter, compared to summer (time of parturition) and spring, coincides with the relaxation of this tissue. In the soluble fraction, the low PAP-I activity of the ductus deferens coincided with its high activity in the vagina during the winter; and the inverse occurred in summer, which is consistent with the physiological process of preserving spermatozoon viability. In conclusion, the studied peptidase activities had seasonal and tissue-specific characteristics, which suggest a relevant role in the reproductive physiology of *C. d. terrificus*.

1. Introduction

Many vipers (Serpentes: Viperidae), as an obligatory component of their reproductive cycle, show remarkable long-term sperm storage (LTSS) with maintenance of fertilization capacity (Schuett, 1992; Isogawa and Kato, 1995; Almeida-Santos and Salomão, 1997; Almeida-Santos et al., 2004b). In the South American rattlesnake (*Crotalus durissus terrificus*), spermatogenesis occurs in spring (austral), peaks in summer and spermatozoa are stored in the ductus deferens until mating, which occurs during mid autumn. Post-mating storage of sperm in females occurs in the posterior region of the oviducts (posterior uterus) throughout winter until ovulation and fertilization in spring (Schuett, 1992; Almeida-Santos and Salomão, 1997). LTSS in the female has been considered as an adaptation of snakes from temperate regions (Shine, 1977). It is thus intriguing that a rattlesnake species from the Neotropics exhibits a pattern of sperm storage (males and females) similar to that of snakes from temperate regions (Schuett, 1992; Almeida-Santos et al., 2004b).

The male reproductive tract of *C. d. terrificus* is a paired system (i.e., two testes, two ductus deferens, two epididymis, two hemipenes). An additional system includes the paired kidneys, and contains the unique sexual segment of the kidneys (SSK) that is located in the posterior region of nephron tubules (Sever et al., 2002). Differently from mammals, the epididymis of reptiles does not participate in sperm maturation and storage (Sever et al., 2002). However, the present study aims to focus on the ductus deferens owing to their role as sperm storage organs in male reptiles. The ductus deferens connect the testicle with the genital papilla in the cloaca, near the basis of the hemipenis, since reptiles do not have penile urethra (Aldridge et al., 1990; Vasse, 1994). Three regions of the ductus deferens can be distinguished: proximal (testicular), medial and distal (cloacal). Along the year, spermatozoa of *C. d. terrificus* can be found in the ductus deferens (Salomão and Almeida-Santos, 2002). The number of spermatozoa is increased in summer and autumn (which is related to the mating in the middle of autumn), compared to winter (post-mating) (Almeida-Santos et al., 2004b). During the mating (autumn), the percentage of total

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spermatozoa defects of *C. d. terrificus* is lower compared to the winter, as well as the serum levels of testosterone is lower in winter than in summer (Zacariotti, 2004). As in other squamate reptiles, the female reproductive tract of *C. d. terrificus* is also a paired system (i.e., two ovaries, two oviducts), which are bilaterally and asymmetrically positioned (e.g., Ghelman, 1998). Regions of the oviduct are the infundibulum, uterus (anterior and posterior) and vagina (Almeida-Santos and Orsi, 2002). In many rattlesnakes, the ovarian cycle consists in two vitellogenic phases: primary (quiescent period) and secondary (the active phase of the follicle development) (Aldridge, 1979; Almeida-Santos and Orsi, 2002). In *C. d. terrificus*, primary vitellogenesis occurs early in the year, while secondary vitellogenesis occurs mainly in autumn and winter (Almeida-Santos and Salomão, 2002). In this snake it has been assumed that the LTSS in the female could be related to the uterine contraction, which occurs in the post-mating, promoting a chronic muscular constriction that narrows the uterus lumen, thus retaining the semen within it until the ovulation and subsequent fertilization (Almeida-Santos and Salomão, 1997) and/or blocking the entrance of other spermatozoa in the posterior portion of the uterus (Andrén et al., 1997). However, the role of chronic muscular constriction as a mechanism for LTSS has been under criticism based on the presence of traditional anatomical structures currently associated with sperm storage, such as sperm storage tubules, in Cottonmouth snake, *Agkistrodon piscivorous* (Siegel and Sever, 2008). Anyway, the stimulus to the long-term uterine contraction of *C. d. terrificus* has been reported to be dependent of the direct action of vasotocin (AVT), which is modulated by estradiol and progesterone during vitellogenesis (Almeida-Santos et al., 2004a). In this turn, the maintenance of this long-term uterine contraction depends on the high levels of estradiol and low levels of vasotocinase, which is an enzyme activity that is downregulated by estradiol (Yamanouye et al., 2004). In mammals, this activity corresponds to the cystyl aminopeptidase enzyme, CAP (EC 3.4.11.3), which hydrolyses oxytocin (OXT) and vasopressin (AVP). The functional role of CAP in regulating these peptides is well established during pregnancy in primates (Davison et al., 1993). Circulating CAP activity varies seasonally in females of *C. d. terrificus* (Almeida-Santos et al., 2004a). In mammals, fluctuation of other aminopeptidase activities, related to peptides that are important to the reproductive physiology, according to the reproductive cycle, is well described (de Gandarias et al., 1988; Mitchell and Denker, 1991). This class of enzymes also includes acid aminopeptidase (APA, EC 3.4.11.7), which hydrolyses angiotensin (Ang) I[1] and Ang II (Kugler, 1982); basic aminopeptidase (APB, EC 3.4.11.6), which hydrolyses bradykinin (BK), kallidin, methionine-enkephalin and somatostatin (Barret et al., 1998); neutral aminopeptidases puromycin-sentitive (APN-PS, EC 3.4.11.14) and -insensitive (APN-PI, EC 3.4.11.2), which hydrolyze enkephalin (Fernández et al., 2002) and promote the formation of Ang IV from Ang III (Kugler, 1982) and BK from kallidin (Mizutani et al., 1993); dipeptidyl-peptidase IV (DPPIV, EC 3.4.14.5), which hydrolyses substance P and endorphin-2 (Barret et al., 1998); type-1 pyroglutamyl aminopeptidase (PAP or PAP-I, EC 3.4.19.3), which hydrolyses the luteinizing-hormone releasing hormone (LHRH), thyrotropin-releasing hormone (TRH) and the fertilization promoting peptide (FPP) (O'Cuinn et al., 1990). Another important peptidase which hydrolyses Ang II, substance P, BK, OXT, AVP, LHRH (Barret et al., 1998), TRH and FPP (Siviter and Cockle, 1995) is the prolyl oligopeptidase (POP, EC 3.4.21.26).

The main goal in our study was to evaluate, in males and females of *C. d. terrificus*, seasonal activity levels of APA, APB, APN-PS, APN-PI, CAP, DPPIV, PAP-I, POP and prolyl-iminopeptidase (PIP) in soluble and solubilized membrane-bound fractions of tissues in which semen passes through or is stored (e.g., ductus deferens, posterior uterus and vagina).

2. Material and methods

2.1. Animals

The use of snakes for this research was approved by the Ethics Committee of the Instituto Butantan (CEUAIB), protocol 193/04, in agreement with the Ethical Principles for Experiments on Animals of the Brazilian Council Directive (COBEA). In the present study, adult snakes (*C. d. terrificus*, Serpentes, Viperidae, Crotalinae) were captured from natural environment in the states of São Paulo and Minas Gerais, and identified by the Laboratory of Herpetology of the Instituto Butantan. Snakes were housed individually in wood cages (inside length × width × height of 35 × 26 × 22 cm) and acclimated to controlled conditions of temperature (25 °C), humidity (65.3 ± 0.9%) and photoperiod (12 h light: 12 h dark–lights on at 6:00 am) in a restricted-access room for a period of 10 days.

In all procedures snakes were anesthetized with CO_2 exposure for 3 h. The snout vent-length—SVL (cm), tail length—TL (cm) and body wt (g) of each specimen were recorded. After the ventral dissection, the reproductive tract was examined macroscopically. Pregnant snakes were discarded. The male ductus deferens and the female uterus and vagina were collected.

2.2. Collection of tissues and the fractionation procedure

The ductus deferens, uterus and vagina were removed by laparotomy (Langlada et al., 1994) from anesthetized snakes. Considering that different areas of the ductus deferens and vagina could have distinct physiological roles, these entire organs were taken systematically to assure similar components for each analysis. Only the posterior portion of the uterus was used, considering its importance, since after mating the spermatozoa are maintained in this region until ovulation and fertilization (Almeida-Santos and Salomão, 1997). These organs were opened, stretched out and elongated on polystyrene plates. To remove mucus, spermatozoa and other fluids, a compression with a cell scraper (TPP—Techno Plastic Products AG, Switzerland) over the whole extension of the tissues was applied. These organs were then washed in 10 mM Tris–HCl buffer, pH 7.4, at a slow infusion rate using a syringe, in order to assure the complete withdrawal of secretions that could remain in the lumen.

In order to obtain soluble (SF) and solubilized membrane-bound (MF) fractions, individual samples of ductus deferens and uterus were homogenized in 10 volumes and vagina in 5 volumes (wt [g]/volume [mL]) of 10 mM Tris–HCl buffer (pH 7.4), with a Teflon pestle in a glass potter (2 min at 800 rpm) and ultracentrifuged (100,000 *g* for 35 min) (Hitachi model HIMAC CP56GII). The resulting supernatants corresponded to the SF. To avoid contamination with the SF, the resulting pellet was washed three times with the same buffer and was then homogenized (2 min at 800 rpm) in 10 mM Tris–HCl buffer plus 0.1% Triton X-100 (Calbiochem, USA) (pH 7.4), and then ultracentrifuged (100,000 *g* for 35 min). The resulting supernatants corresponded to the MF. All steps were carried out at 4 °C. After the fractionation procedure, SF and MF were

[1] *Abbreviations used:* Ang, angiotensin; APA, acid aminopeptidase; APB, basic aminopeptidase; APN-PI, puromycin-insensitive neutral aminopeptidase; APN-PS, puromycin-sensitive neutral aminopeptidase; AVP, arginine vasopressin; AVT, arginine vasotocin; BK, bradykinin; BSA, bovine serum albumin; CAP, cystyl aminopeptidase; DMSO, dimethyl sulfoxide; DPPIV, dipeptidyl-peptidase IV; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MF, solubilized membrane-bound fraction; FPP, fertilization promoting peptide; SF, soluble fraction; GHRH, growth hormone releasing hormone; LDH, lactate dehydrogenase; LHRH, luteinizing-hormone releasing hormone; LTSS, long-term sperm storage; NADH, β-nicotinamide adenine dinucleotide reduced form; OXT, oxytocin; PAP or PAP-I, type-1 pyroglutamyl aminopeptidase; PIP, prolyl iminopeptidase; POP, prolyl oligopeptidase; SSK, sexual segment of the kidneys (SSK); SVL, snout vent-length; TL, tail length; TRH, thyrotropin-releasing hormone.