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Theriogenology 78 (2012) 49–56

Theriogenology

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Nitric oxide (NO) expression during annual reproductive activity in buffalo epididymis: A histochemical and immunocytochemical study

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Received 6 June 2011; received in revised form 19 January 2012; accepted 19 January 2012

Abstract

The buffalo is one of the few domestic animals that has a seasonal mating cycle, influenced by the photoperiod. It is known that the photoperiod regulates gonadal function probably via the pineal and/or hypothalamus-pituitary axis. Moreover, the hypothalamus (melatonin) and gonads influence the production of the signaling transmitter nitric oxide (NO), suggesting that the NO may have an important role in the regulation of gonadotropin-releasing hormone secretion. This further suggests the hypothesis that NO in the epididymis has an important role in the maturation of spermatozoa and their motility and posterior fertilization capacity. The aim of the present study is to investigate the seasonal variations in the morphology of the epididymis by means histochemical and immunocytochemical techniques. We used the NADPH-d, nitric oxide synthase (NOS) I and NOS III to clarify the relationship between epididymis function and NO signaling activity. The results of this work show that NO is present in the caput of epididymis during short photoperiods, i.e., periods of maximum gonadal activity (winter) and absent during long photoperiods, i.e., periods of gonadal regression according to the previously described role of NO in spermatozoa capacitation and motility in the caput epididymis.

Keywords: Buffalo; Epididymis; Seasonality; NADPH-d; Immunogold labeling

1. Introduction

The buffalo is a domesticated ruminant species of peculiar biological interest because it is one of the few domestic animals that has a seasonal mating cycle. Mating activity only takes place from autumn to winter, and there is a long non-mating season from late spring to the beginning of the autumn. It is well-known that the photoperiod plays an important role in influencing the sexual activity of the adult male buffalo. This period regulates gonadal function, probably via the pineal and/or hypothalamus-pituitary axis by melatonin production. A growing body of recent evidence suggests that the hypothalamus (melatonin) and gonads influence the production of the signaling transmitter nitric oxide (NO) [1,2]. Aydogan, et al. [3] reported that melatonin inhibits the activity of NO synthase in mammals. The anatomical localization of NO neurons is in close proximity to the gonadotropin-releasing hormone neurons in the hypothalamus. This suggests that NO may have an important role in the regulation of gonadotropin-releasing hormone secretion [4]. In fact, NO has been shown to modulate gonadal and adrenal functions in Japanese quail [5]. This further suggests the hypothesis that NO in the epididymis has an important role in the maturation of spermatozoa, including their acquisition of progressive motility and posterior fertil-

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doi:10.1016/j.theriogenology.2012.01.017

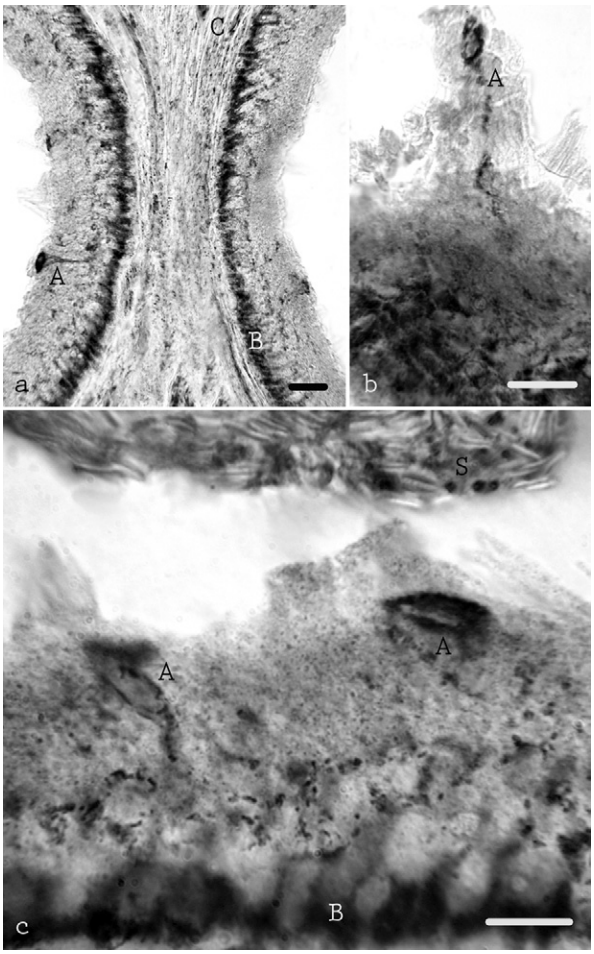

Fig. 1. LM buffalo epididymis in the winter. Intense NADPH-d staining of the caput epididymis. (a) epididymis duct; (b) narrow cell that protrudes in lumen of epididymis duct; (c) nuclei of the narrow cells. A = narrow cell; B = basal portion of the principal cells; C = blood capillary; S = spermatozoa. Scale bars = 10 μm.

ization capacity. In addition, it is also well-known that NO plays a decisive role in regulating the functions of the male reproductive system, where it is produced from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS) and various cofactors, such as oxygen, nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterina, calmodulin and calcium [6,7]. In mice, NO was observed on the acrosome and tail of non-capacitating spermatozoa suggesting a role for NO in capacitation [8]. Lewis, et al. 1996 and Donnelly, et al. 1997 [9,10] have reported that respectively low concentrations of NO improve and maintain human sperm motility. Moreover, Meiser, et al. 2003 and Leal, et al. 2009 [11,12] have reported that NO is involved in control of sperm motility and spermatozoa capacitation bull. In addition, Herrero, et al. 1999 [13] reported that NO is produced by capacitating human spermatozoa and acting as a cellular messenger by modulating the cAMP pathway and protein tyrosine phosphorylation. Sperm capacitation by protein tyrosine phosphorylation was also observed in buffalo spermatozoa [14,15], although the buffalo epididymis has often been studied to define the morphostructural characteristics that effect reproduction of this species. Scala, et al. 2002 [16] investigated the microvasculature of the buffalo epididymis and found fenestrations, located in the ovoid area of the endothelium post-capillary venules, that appeared to play an important role in the absorption and secretion processes of the epididymal epithelium. There are a few studies on the morphostructural characteristics of mammals epididymis, and buffalo in particular, in relation to seasonal reproduction. Schon and Blottner 2009 [17] reported in *Capreolus capreolus* only seasonal variations of the epididymis. Nitric oxide is currently one of

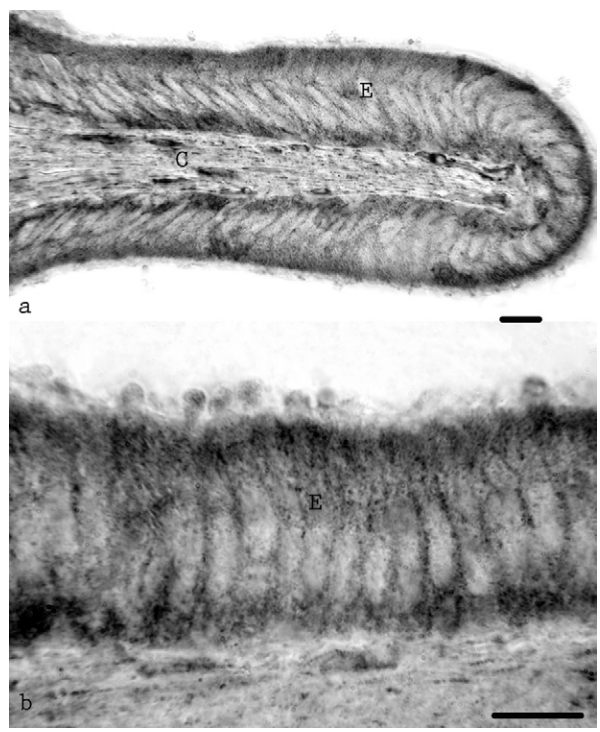

Fig. 2. LM buffalo epididymis in the winter. (a) NADPH-d staining of the corpus epididymis; (b) NADPH-d staining of the cauda epididymis. C = blood capillaries; E = epithelium of duct epididymis. Scale bars = 10 μm.

the most studied molecules, in biomedical sciences, because of the multiplicity of roles it plays in regulating multiple functions within the male reproductive system. In this study histochemical and immunocytochemical techniques were used to investigate the seasonal NO variations in the epididymis during three different photoperiods of the seasonal cyclic reproduction (spring, summer and winter) in the adult male buffalo to achieve a better understanding of NO action in the epididymis.

2. Materials and methods

2.1. Animals

The epididymis were collected immediately after slaughter from thirty (n = 30) adult male buffalos (*Bubalus bubalis*): ten (n = 10) in the winter, ten (n = 10) in the spring, and ten (n = 10) in the summer. The epididymis were divided into three parts (caput, corpus and cauda), and from these were obtained the specimens that were studied using different techniques.

2.2. Histochemistry

The specimens were washed in 0.1 M PBS, transferred into a graded series of saccarose (10%, 20%, 30%), immersed in Tissue Teck OCT compound (Sigma http://Chem.Co, St. Louis, MO, USA), frozen in liquid nitrogen, and sectioned by a cryostat. To measure NADPH-d activity, sections were incubated with 0.25 mg/mL nitro blue tetrazolium, 1 mg/mL NADPH, and 0.5% Triton X-100 in 0.1 M Tris-HCl buffer, pH 7.3, at 37 °C for 10 to 15 min in a dark box or at room temperature for 30 min. The reaction was stopped by sample immersion in 0.1 M Tris-HCl buffer. Finally, sections were mounted on cover slips, examined under a light microscope (Orthoplan, Leitz, GmbH, Wetzlar, Germany), and photographed. Control sections included incubation in media in which substrate was omitted, and preincubation with the sulfydryl inhibitor, 5.5'-Dithio-bis-2-nitrobenzoic acid. Neither of these controls produced positive immunosignals.

2.3. Immunogold labeling SEM analysis

For the immunogold labeling SEM analysis, the specimens were immediately immersed in PBS for 1 h. The samples were incubated for 2 h with a solution containing normal goat serum (X 0907, Dako Italia s.p.a., Milano, Italy) diluted 1:10 in PBS, and next with primary polyclonal rabbit antibodies directed toward NOS I (AB5380, Chemicon, Temecula, CA, USA), and NOS III (AB16301, Chemicon, Temecula, CA, USA) diluted both 1:1500 in PBS, and incubated overnight at 4 °C. After washing in PBS, the samples were incubated with gold-conjugated goat anti-rabbit IgG (E.M-.GAR10, Agar Scientific, Limited, Stansted, UK) diluted 1:200 in PBS, for 1 h at room temperature. The secondary antibody was conjugated with gold particles of different size, namely 5 and 15 nm after washing in PBS, samples were fixed by 2.5% glutaraldehyde in 0.1 M cacodilate buffer containing $CaCl_2$, pH 7.2, for 30 min. After the fixation step and washing with distilled water, samples were subjected to silver enhancement (British Biocell International, Cardiff, Wales, UK). The enhancement process enables the use of antibodies conjugated to smaller (1–5 nm) gold particles, preserving

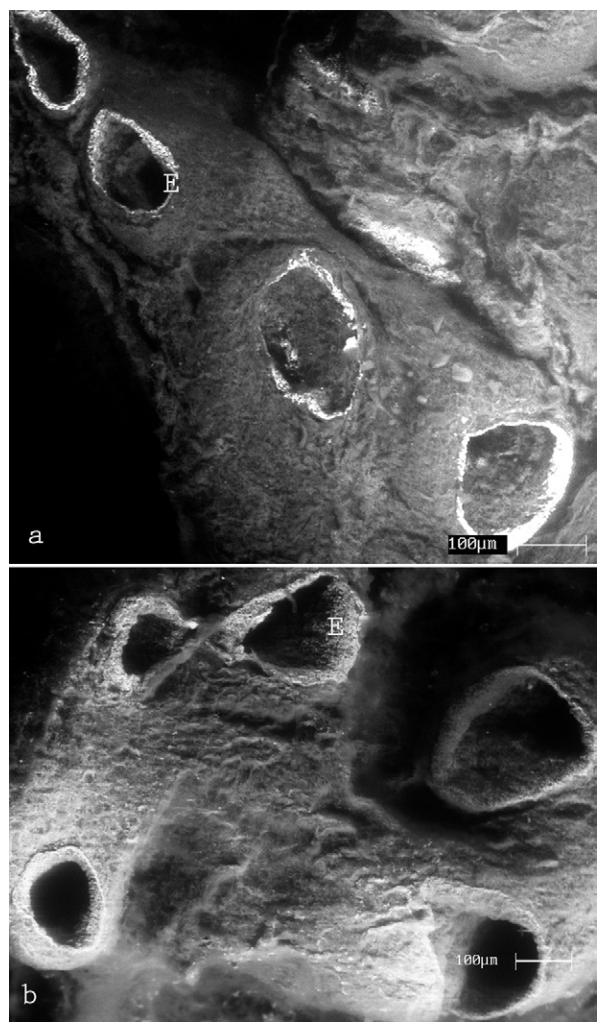

Fig. 3. SEM immunogold labeling of the buffalo epididymis in the winter. (a), NOS I immunoreactivity in the specimens of the caput epididymis; (b) NOS III immunoreactivity in the specimens of the caput epididymis. E = epithelium of the epididymis duct.

the advantage of faster penetration and higher labeling efficiency [18]. Next, samples were dehydrated through an ethanol series, and dried to the critical point. The specimens, mounted on stubs, were examined under a LEO 435 VP scanning electron microscope at variable pressure (80–120 Pa) in the backscattered electron mode, which allows detection of gold particles associated with cells even if they are located intracellularly [19]. The samples had not been coated by gold–palladium, so the only conjugated gold deriving from immunocytochemical reaction was observed under SEM and photographed.

2.4. Western blotting

The specimens were homogenized by an Ultraturrax L-407 at 4 °C with 5 ml/1.5 g tissue of buffer containing 50 nM Tris-HCl, pH 7.4 150 nM NaCl, 1 nM ethylenediaminetetraacetic acid, 10 nM NaF, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1% nonited P-40, 1 N m phenylmethylsulfonyl fluoride, 0.1 U mL^{-1} aprotinin, 10 mg/mL leupeptin, and 1 mM Na3VO4. Homogenates were centrifuged at 15 000 – g for 20 min at 4 °C. Supernatants were divided into small aliquots and stored at −80 °C until used. The amount of total proteins in each sample was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Samples containing equal amount of proteins were boiled for 5 min in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% b-mercaptoethanol), and run on a 10% SDS/polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose using a Mini transblot apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions. Membranes were blocked for 1 h at room temperature with TBS-T buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% Tween 20) containing 5% milk. The blots were incubated overnight with a rabbit polyclonal antibody raised against a peptide mapping the 724 to 739 amino acid sequence of human brain NOS I (AB1632, Chemicon International, Inc.) diluted 1:1000 in TBS-T containing 2.5% milk. After incubation, the membranes were washed three times with TBS-T and incubated for 1 h with horseradish peroxidase conjugated anti-(rabbit IgG) Ig (Sigma Chemical) diluted 1:3000 in TBS-T containing 2.5% milk. The proteins were visualized by enhanced chemiluminescence (ECL; Amersham Little, Chalfont, UK). To ensure specificity, preabsorption of NOSI antibody with its control peptide (AG591; Chemicon International, Inc.) was performed before western blotting. The same blots were stripped and reprobed using anti-tubulin monoclonal antibody (MAB 1637; Chemicon International, Inc.) to confirm equal loading of proteins in all lines.

2.5. Specificity of the immunoreactivity

The specificity of the immunoreactivity was tested by successively substituting PBS for either the antibodies used and/or substituting the gold-conjugated anti rabbit IgG. The cross-reactivity of the primary antibodies was tested by incubating the specimens with antibodies that had been preincubated with excessive

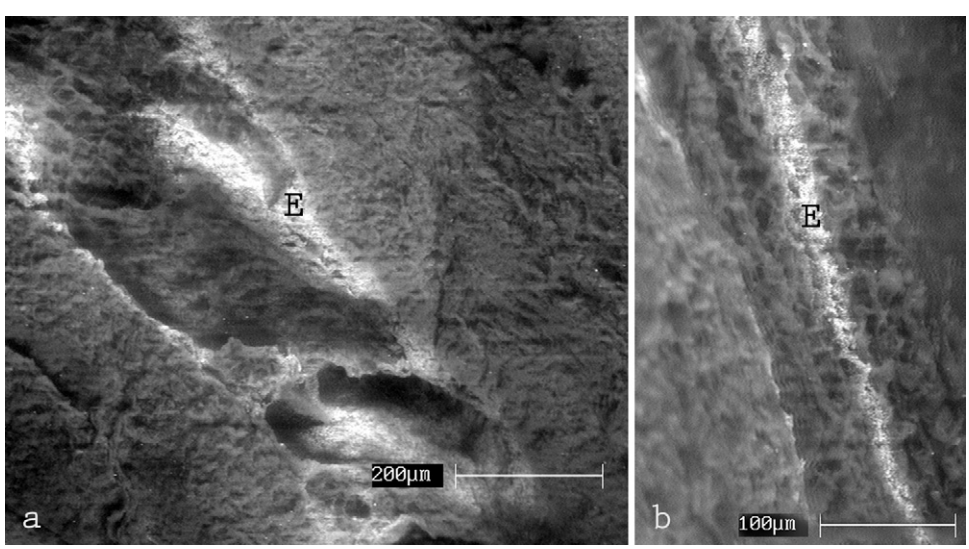

Fig. 4. SEM immunogold labeling of the buffalo epididymis in the winter. (a) NOS I immunoreactivity in the specimens of the corpus epididymis; (b) NOS III immunoreactivity in the specimens of the corpus epididymis. E = epithelium of the epididymis duct.

amounts of their homologous (50 μg/mL) and heterologous (100 μg/mL) antigens. The control peptides were AG591 (AB1632 Chemicon Int., Inc) and AG593 (AB1630 Chemicon Int., Inc) for NOS I and NOS III proteins, respectively.

All nomenclature in this work was adopted from *Nomina Anatomica Veterinaria, Nomina Histologica* and *Nomina Embryologica Veterinaria* (World Association of Veterinary Anatomists, 2005).

3. Results

3.1. Histochemistry (LM)

3.1.1. Winter

The examination of the caput epididymis specimens revealed intense NADPH-d staining in the basal portion of the principal cells and narrow cells of the epithelium and endothelium, and in the tunica media of the blood vessels: 1) the basal portion of the principal cells showed intense NADPH-d staining, and formed a continuous line at the base of the epididymal duct (Fig. 1). No cells of the corpus and cauda of the epididymis were stained by NADPH-d (Fig. 2); 2) in the epithelium of the epididymal duct, staining was intense in the narrow cells located between the principal cells, and their nuclei were ovoidal, located in apical position, and protruded into the epididymis lumen. No cells of the corpus and cauda of the epididymis were stained by NADPH-d; and 3) the endothelium and tunica media of the blood vessels were intensely stained by NADPH-d in the caput, corpus and cauda.

3.1.2. Spring

The NADPH-d staining decreased in the basal portion of the principal cells, and was not evident in the narrow cells. The specimens of the corpus and cauda of the epididymis revealed no evident NADPH-d staining. The endothelium and tunica media of the blood vessels maintained their intense NADPH-d staining with respect to winter.

3.1.3. Summer

There was little NADPH-d staining in the epithelium of the three portions of the epididymis. In the blood vessels the staining was unchanged with respect to spring.

3.2. Immunogold labeling SEM analysis

3.2.1. Winter

Intense NOS I and NOS III immunoreactivity were detected in the specimens of the caput epididymis. The immunoreactive images of the NOS I and NOS III were similar in all specimens, specifically, an intense, non-uniform immunoreactivity was located in the caput of the epididymis epithelium (Fig. 3). The corpus specimens of the epididymis were less intense in immunoreactivity to NOS I, and NOS III (Fig. 4). The cauda specimens of the epididymis showed no immunoreactivity to NOS I, and NOS III (Fig. 5).

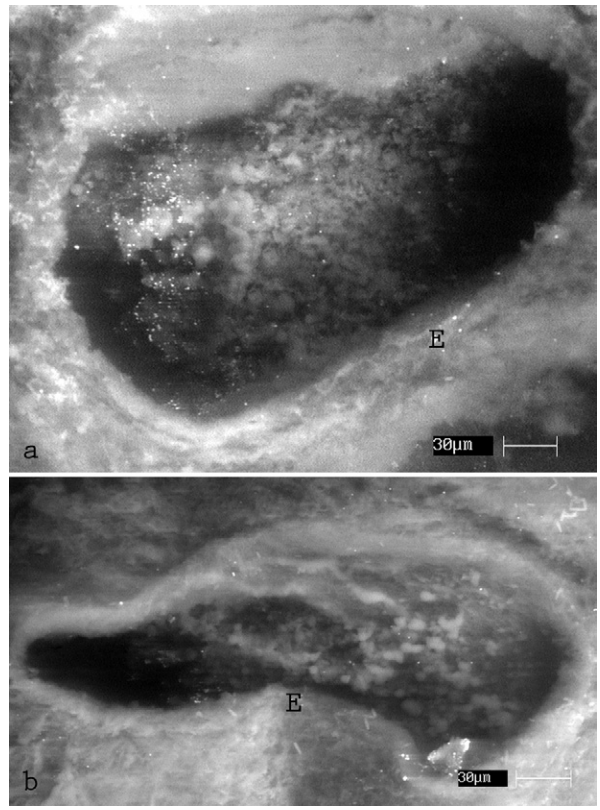

Fig. 5. SEM immunogold labeling of the buffalo epididymis in the winter. (a), NOS I immunoreactivity in the specimens of the cauda epididymis; (b) NOS III immunoreactivity in the specimens of the cauda epididymis. E = epithelium of the epididymis duct.

3.2.2. Spring

The immunoreactivity to NOS I and NOS III of the caput specimen epididymis were less intense than in winter (Fig. 6). In particular, the immunoreactivity to NOS I and NOS III were scattered throughout the epididymis epithelium. The specimens of the cauda and corpus epididymis showed no immunoreactivity to NOS I and NOS III.

3.2.3. Summer

The caput, corpus and cauda epididymis specimens showed no immunoreactivity to NOS I and NOS III (Fig. 7).

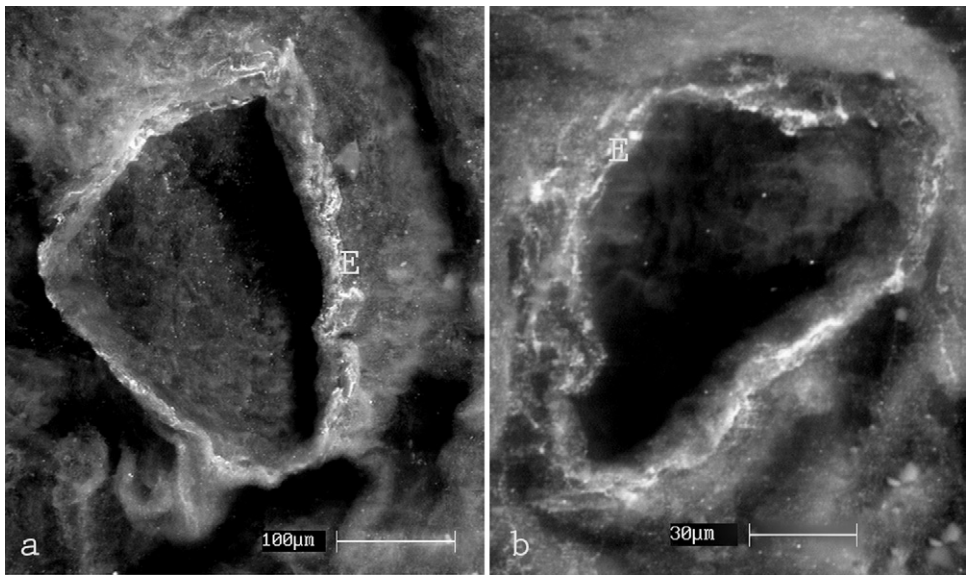

Fig. 6. SEM immunogold labeling of the buffalo epididymis in the spring. (a), NOS I immunoreactivity in the specimens of the caput epididymis; (b) NOS III immunoreactivity in the specimens of the caput epididymis. E = epithelium of the epididymis duct.

3.3. Western blotting

Expression of NOS I was found in the epididymis epithelium using a specific rabbit polyclonal antibody raised against human brain NOS I. Moreover, the presence of the protein was detected in the mouse brain homogenate, which was used as positive control (Fig. 8).

4. Discussion

Adult males buffalo exhibit an annual reproductive cycle [20,21] with periods of maximum gonadal activity (winter) and of gonadal regression (spring, summer) regulated from the photoperiod. The results of the present paper demonstrate that NO is one of the factors that changes during the annual reproductive cycle of the male adult buffalo, and that it is located in the complex testis-epididymis. In particular, the caput represents the segments most sensitive to change for photoperiod action. In fact, NO is mainly present in the caput epididymis during short photoperiods, i.e., period of maximum gonadal activity and it is absent during long photoperiods, i.e., period of gonadal regression. This results are in agreement that the photoperiod regulated the activity of epididymal epithelium data observed in other animal species [22,23]. It seems, therefore, that NO plays a decisive role in regulating multiple functions of the male reproductive system. Further confirmation of this decisive role is found in previous studies of NO, which correlate NO to sperm motility [24]. In particular, NO is derived from L-arginine by the enzymes constitutive NOS's, i.e., NOS I, and NOS III. These enzymes are found on the acrosome and tail region of non-capacitated spermatozoa [9]. At low concentrations in the epididymis spermatozoa of mice and humans, NO improved sperm motility and enhanced capacitation [25].

The results of the present work show that NO is present in the caput of the epididymis during short photoperiods, i.e., maximum gonadal activity, and absent during long photoperiods, i.e., periods of gonadal regression. This is consistent with the results of many previous studies that have described the role of NO in two basic functions of the epididymis, namely, spermatozoa maturation-involved in the late sperm capacitation- and motility. In the epididymis there is sperm maturation, not sperm capacitation, although maturation affects the later capacitation in female reproductive organs.

4.1. Capacitation

Nitric oxide has been shown to induce potent sperm capacitation in buffalo [26]. Inversely, the addition of NOS inhibitors at the onset of the incubation period causes a decrease of human capacitation, indicating that the endogenous NO was necessary for spermatozoa to display their full fertilizing ability. Furthermore, because NO reflects on the levels of tyrosine phosphory-

lation of two (p81 and p105) human sperm proteins [27,28], it is consistent that tyrosine phosphorylation was absent in non-capacitated sperm in mouse [29] and hamster [30] spermatozoa.

4.2. Motility

The propulsion of the immotile spermatozoa from the caput, corpus, and cauda of epididymis occurs by means of spontaneous phasic contractions of the surrounding smooth muscle layers (*Tunica muscularis*). The transport of the sperm through the buffalo epididymis is characterized by pendular movements providing stirring of intraluminal content [31]. The effects of NO on smooth muscle are believed to be solely mediated by its action on a soluble guanylate cyclase, which, in turn, activates the cyclic guanosine monophosphate (cGMP)-dependent pathways [32].

In conclusion, the buffalo produces an abundance of constitutive NOS (NOS I and NOS III) in the epithelium of the caput epididymis during the winter period only. The capacitation and motility of the spermatozoa probably can be modified the presence of NO in the caput epididymis during winter season.

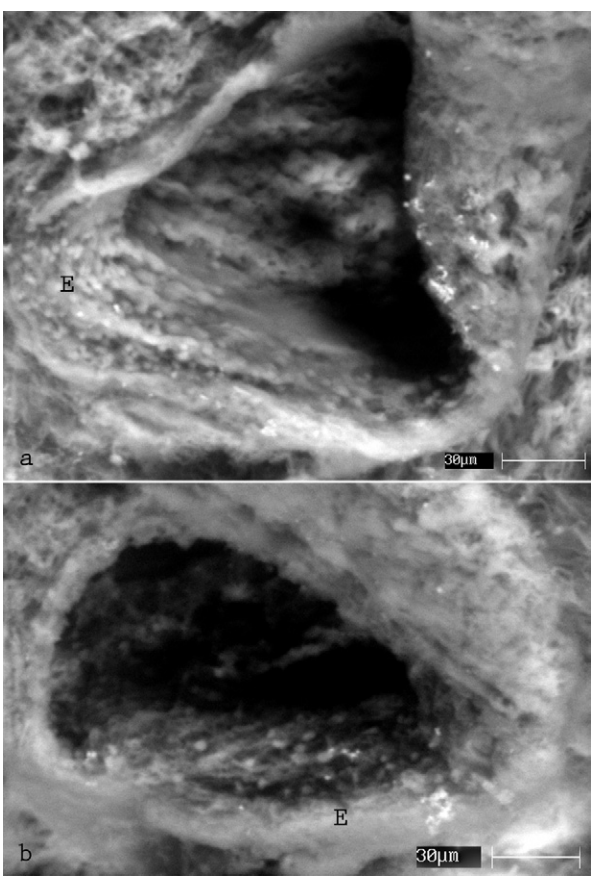

Fig. 7. SEM immunogold labeling of the buffalo epididymis in the summer. (a), NOS I immunoreactivity in the specimens of the caput epididymis; (b) NOS III immunoreactivity in the specimens of the caput epididymis. E = epithelium of the epididymis duct.

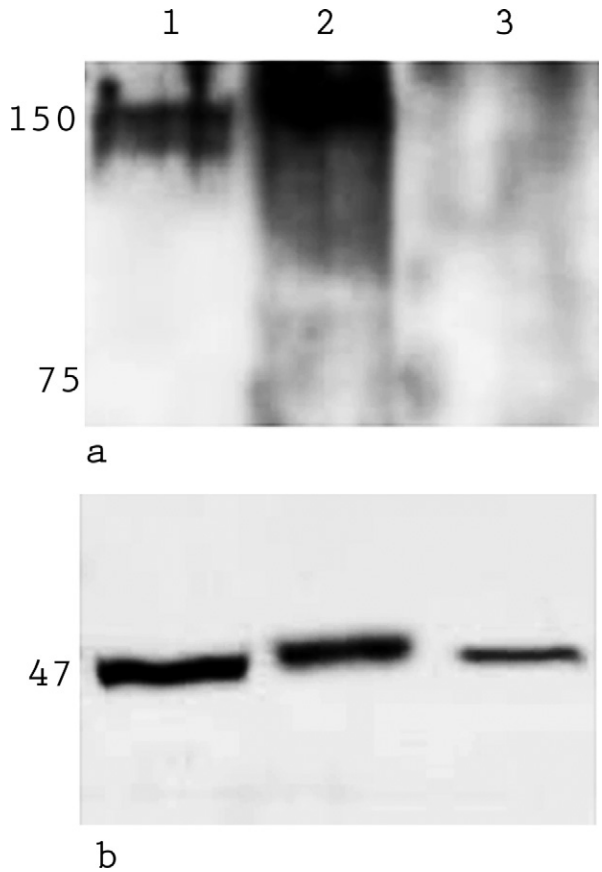

Fig. 8. Expression of NOS I in the caput epididymis of the buffalo in the winter. (a) Western blotting analysis performed using antiserum directed against humans NOS I (molecular mass 125–160 kDa) on tissue homogenate: Lane 1, homogenate of the buffalo caput epididymis; line 2, homogenate from a mouse caput epididymis (positive control); Lane 3, homogenate of the buffalo caput epididymis treated with antiserum directed against human epididymis NOS I preabsorbed with its control peptide (negative control). (b) The blot was stripped and reprobed with anti-tubulin monoclonal antibody to ensure equal loading of the proteins in all lanes. Molecular mass markers are indicated on the left.

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