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ISSN: 0015-5659

e-ISSN: 1644-3284

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DOI: 10.5603/FM.a2017.0046

Article type: ORIGINAL ARTICLES

Submitted: 2013-11-17

Accepted: 2014-01-02

Published online: 2017-05-16

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Immunolocalization of NOS isoforms in the *ductuli efferentes* and epididymis of prepubertal and adult alpaca (*Lama pacos*)

Running head: NOS isoforms in the alpaca genital tract

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ABSTRACT

The present research used immunohistochemistry to analyse the detection and localization of NOS isoforms in the *ductuli efferentes* and epididymis of prepubertal and adult alpaca. In the *ductuli efferentes* and epididymis of prepubertal and adult animals, nNOS and eNOS were similarly expressed in epithelial lining cells, conversely differences were observed in the immunopresence of iNOS. Our data provide evidence that NOS isoforms may have roles in reproductive functions and in the developmental processes of the excurrent duct system in the alpaca.

Key words: immunohistochemistry, NOS isoforms, genital tracts, alpaca

INTRODUCTION

Nitric oxide (NO) synthase (NOS) is the enzyme that convert L-arginine to NO and L-citrulline; NO acts as an intracellular messenger in many physiological and pathophysiological events, in numerous cell types, including those of the reproductive system [6, 7]. NO functions as an atypical neurotransmitter in the central nervous system and as a non-adrenergic and non-cholinergic mediator in the control of different reproductive tract organs [2]. NOS exists in two forms: constitutive, Ca²⁺-dependent forms that are rapidly activated by agonists that elevate intracellular free Ca²⁺, including neuronal NOS (nNOS) and endothelial NOS (eNOS); and a Ca²⁺-independent inducible form (iNOS) [8]. Both constitutive nNOS and eNOS are normally expressed in cells other than the neuronal and endothelial cells in which they were respectively first detected; additionally, they produce low levels of NO. Conversely, iNOS generates large amounts of NO in various cell types when expression is activated; it can be induced after several hours of immunological

stimulation and is detectable in macrophages, neutrophils and endothelial cells [9]. The presence of NOS has been demonstrated by immunohistochemical and enzyme studies in female reproductive tissues, including the ovary, oviduct, and uterus showing the involvement of NO in a number of reproductive processes such as ovulation, implantation and embryo development and uterine contractility [1, 22, 16, 18, 26]. In male, NO regulates the mating behaviour and is involved in sexual performance through its direct involvement in penile erection and contractility of seminal vesicles; additionally, it is implicated in different sperm functions including motility, metabolism, acrosome reaction and biosynthesis and secretion of steroid hormones [23, 22].

The male alpaca (*Lama pacos*), a South American camelid species, has unique reproductive characteristics, which cause their poor breeding performance compared with other domestic species [21]. There are some basic descriptions of the histology, histochemistry and immunohistochemistry of the male genital tracts of alpacas [10, 11, 12, 14, 19] and also some aspects of the male reproductive physiology have been examined [3, 13, 24].

In order to add new data about the male reproductive biology of this camelid, the present research was designed to evaluate the cellular localization of NOS isoforms in the *ductuli efferentes* and epididymis of prepubertal and adult alpaca.

MATERIALS AND METHODS

Animals and tissue collection

Five normal male alpacas of the farm “Maridiana” (Umbertide, Umbria, Italy), ranging in age from 4 to 8 years, were used. These animals were castrated, following the description provided by Fowler [5], to prevent aggressive behaviour or to allow housing with non-pregnant females. Immediately after castration at the Veterinary Teaching Hospital of the University Camerino, the testes and epididymides were promptly removed, trimmed of excess tissue, and weighed separately.

Immunohistochemistry of nNOS, eNOS and iNOS

Ductuli efferentes and epididymides (divided into *caput*, *corpus*, *cauda*) were processed for immunohistochemical investigation according to procedures previously described [25, 27]. The slides were incubated with the following primary rabbit polyclonal antibodies (Abcam, Cambridge): anti-nNOS (1:250), anti-eNOS (1:10) and anti-iNOS (1:200) [15, 17]. Then, the slides were incubated with biotinylated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, CA, USA), exposed to avidin-biotin complex (ABC kit, Vector Laboratories) and the peroxidase activity sites were visualized using the DAB kit (Vector Laboratories) as chromogen [20, 4]. In some cases, the sections were counterstained with Mayer’s haematoxylin. Tissue sections in which the primary

antibody was omitted or substituted by rabbit IgG were used as negative controls of non-specific staining.

RESULTS AND DISCUSSION

In this research, we have demonstrated a variable presence of NOS isoforms in the *ductuli efferentes* and in the epididymal tracts of the prepubertal and adult alpaca.

In the *ductuli efferentes* of prepubertal animals, immunosignals for nNOS are localized in the nucleus and cytoplasm of non ciliated and ciliated cells (Fig. 1A) whereas, in adult alpaca, they were observed only in non ciliated cells (Fig. 1B). Immunoreactivity of eNOS was evidenced in the nucleus and cytoplasm of epithelial lining cells of the *ductuli efferentes* of both prepubertal (Fig. 1C) and adult animals (Fig. 1D), conversely, immunosignals for iNOS were detected in cytoplasm and nucleus of ciliated and non ciliated cells only in prepubertal alpaca (Fig. 1E). The smooth muscle cells, neuronal fibers, endothelial cells, and fibroblasts were immunostained with n- and eNOS in prepubertal and adult animals (Fig. 1A-D) whereas with iNOS only in prepubertal ones (Fig. 1E).

In all tracts of epididymis, immunostaining for nNOS is localized in the nucleus and cytoplasm of principal and basal cells in prepubertal (Fig. 2A, C, E) and adult (Fig. 2B, D, F) animals. In particular, in the adults, it was evidenced the intense immunoreactivity of the supranuclear cytoplasm (probably corresponding to the Golgi zone) in the principal cells of the epididymal *corpus* (Fig. 2D) and *cauda* (Fig. 2F). The apical rich mitochondria cells (AMRCs), that we previously described in the alpaca epididymis [10], expressed nNOS in the *caput* (Fig. 2A) and *corpus* (Fig. 2C) epididymis of prepubertal animals and in the *caput* epididymis of adults (Fig. 2B). Smooth muscle cells, neuronal fibers, endothelial cells and stromal fibroblasts displayed immunosignals for nNOS in all epididymal regions of both prepubertal and adult alpaca (Fig. 2A-F).

The immunopresence of eNOS was observed mainly in the nucleus of epithelial lining cells of epididymal *caput* (Fig. 3A), *corpus* (Fig. 3C) and *cauda* (Fig. 3E) of prepubertal animals whereas it was detected in the Golgi zone of principal cells of all the epididymal tracts in the adults (Fig. 3B, D, F). The AMRCs expressed eNOS in all epididymal tracts only in prepubertal alpaca (Fig. 3A, C, E); smooth muscle cells, neuronal fibers, endothelial cells and stromal fibroblasts were immunostained with eNOS in all epididymal regions but only in prepubertal animals (Fig. 3A, C, E).

Inducible NOS immunoreactivity was evidenced in the epithelial cells of the epididymal *corpus* and *cauda* and it was localized in the cytoplasm and nucleus of these cells in prepubertal alpaca (Fig. 4B, D) and in their cytoplasm in adults (Fig. 4C, E). The AMRCs expressed iNOS in all

epididymal tracts of prepubertal alpaca (Fig. 4A, B, D). Smooth muscle cells, neuronal fibers, endothelial cells and stromal fibroblasts showed immunosignals for iNOS in prepubertal epididymal *corpus* (Fig. 4B) and *cauda* (Fig. 4D) whereas, in adults, they were always unreactive (Fig. 4C, E).

On the basis of the widespread distribution of NOS isoforms in the genital tracts of the alpaca, we suggest the potential role for NO in the mediation of various reproductive functions such as sperm transit, storage and maturation [6, 22]. Additionally, NO may also act locally within the epithelial lining cells affecting their functions. The greatest immunopresence of i-NOS detected in prepubertal *ductuli efferentes* and epididymis respect to the adult animals, suggests that this enzyme could be involved in the developmental processes of the excurrent duct system in the alpaca [13].

However, further functional studies are warranted to clarify the exact role of NO in the male reproductive tracts of this specie.

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LEGEND TO FIGURES

Figure 1. Immunoreactivity of NOS isoforms in the ductuli efferentes of prepubertal (A, C, E) and adult (B, D) alpaca. A) nNOS; B) nNOS (counterstained with hematoxylin): the ciliated cells are unreactive (arrows); C) eNOS; D) eNOS (counterstained with hematoxylin); E) iNOS. Smooth muscle cells (S), neuronal fibers (N), endothelial cells (E) and fibroblasts (F). Bars = 10 μ m.

Figure 2. Immunoreactivity of nNOS in the caput, corpus, and cauda epididymis of prepubertal (A, C, E, respectively) and adult (B, D, F, respectively - counterstained with hematoxylin) alpaca. AMRCs (arrows), smooth muscle cells (S), neuronal fibers (N), endothelial cells (E) and fibroblasts (F). Note in fig. 2D the AMRCs (arrow) is unreactive. Bars = 10 μ m.

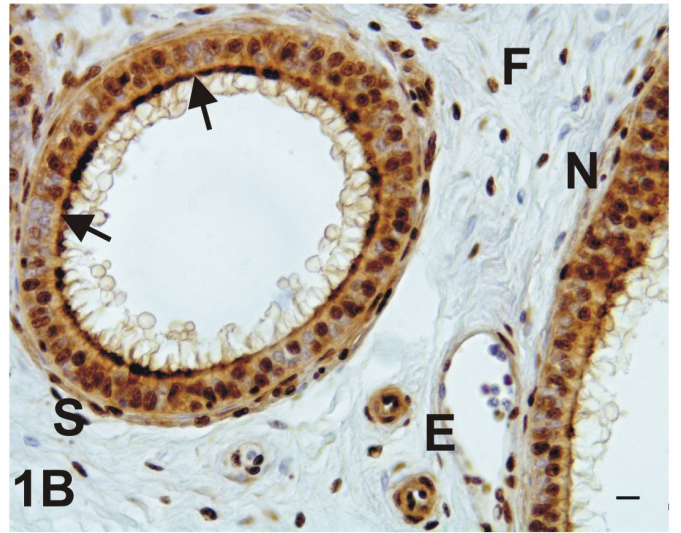
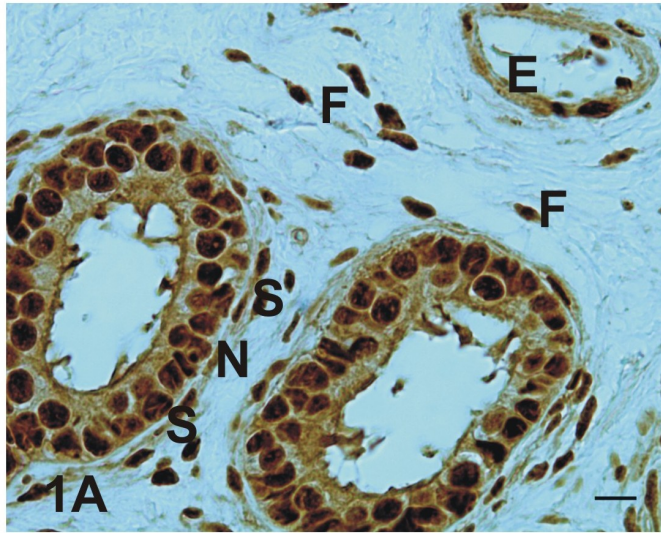
Figure 3. Immunoreactivity of eNOS in the caput, corpus, and cauda epididymis of prepubertal (A, C, E, respectively) and adult (B, D, F, respectively - counterstained with hematoxylin) alpaca. AMRCs (arrows), smooth muscle cells (S), neuronal fibers (N), endothelial cells (E) and fibroblasts (F). Bars = 10 μ m.

Figure 4. Immunoreactivity of iNOS in the caput, corpus, and cauda epididymis of prepubertal alpaca (A, B, D, respectively) and in the corpus and cauda of adult animals (C, E, respectively - counterstained with hematoxylin). AMRCs (arrows), smooth muscle cells (S), neuronal fibers (N), endothelial cells (E). Bars = 10 μ m.

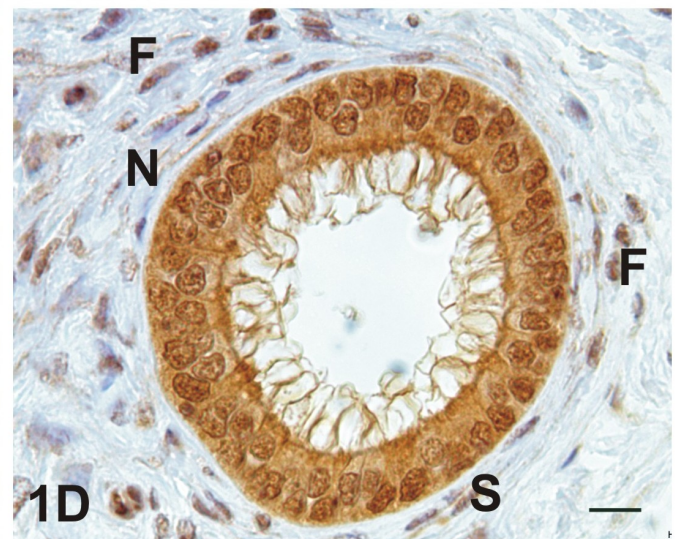
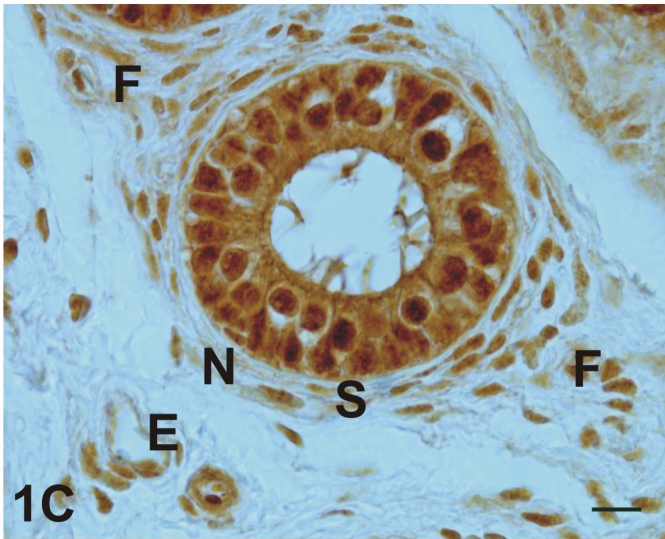
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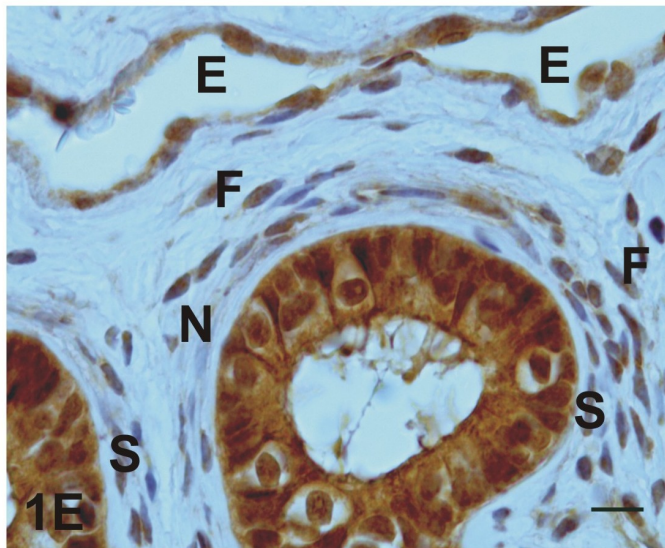
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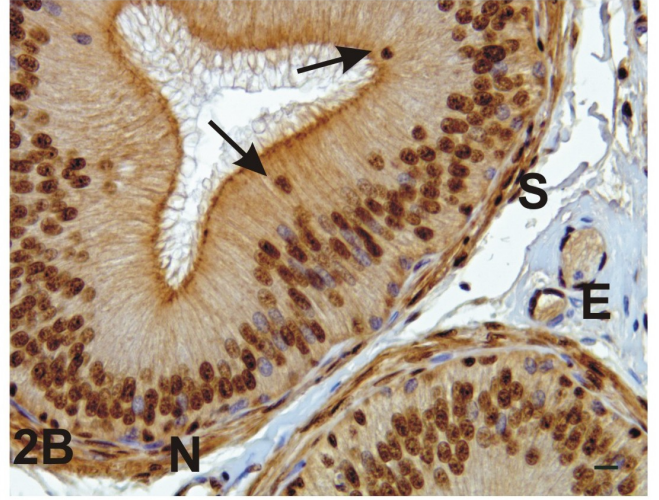
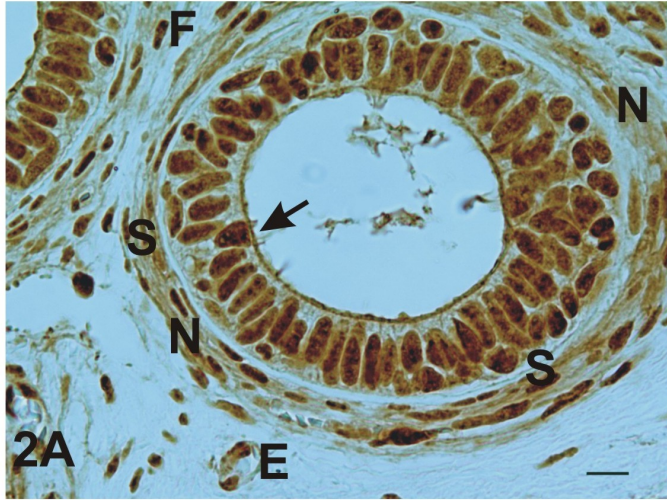


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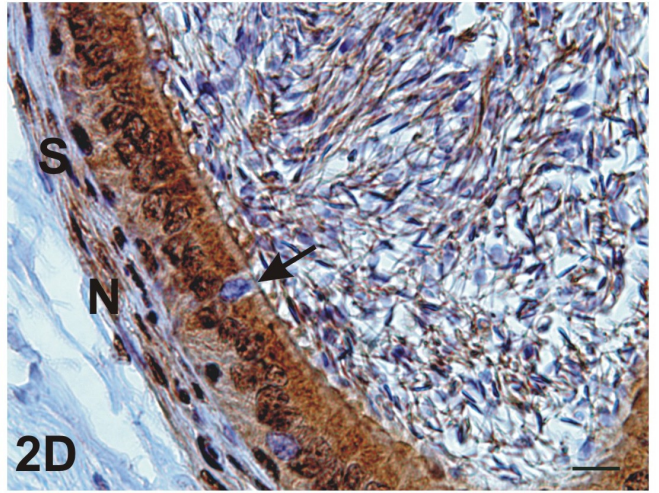
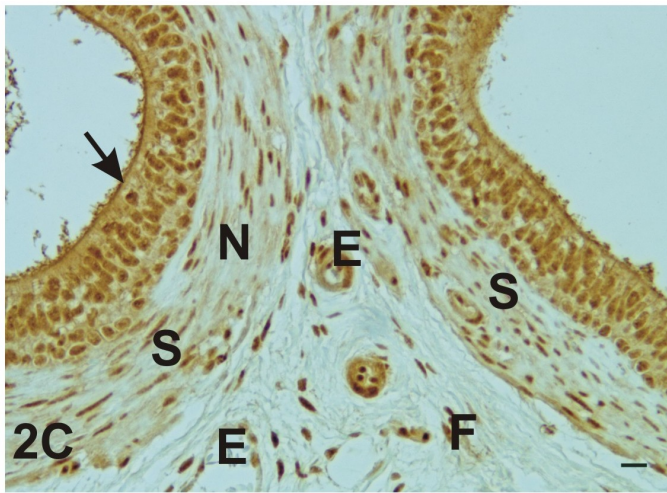
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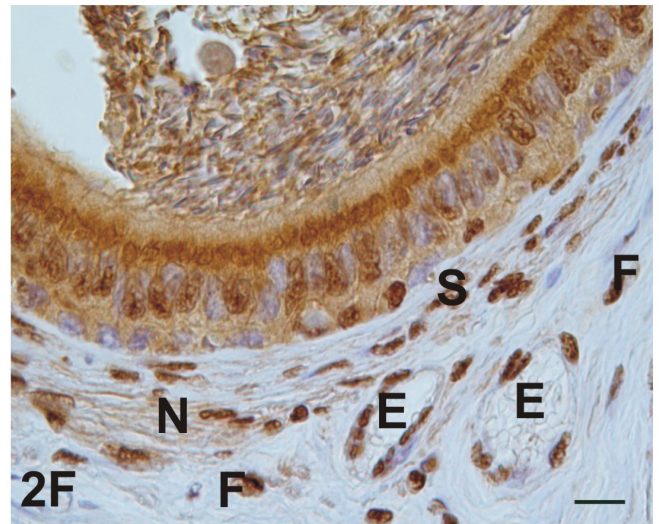
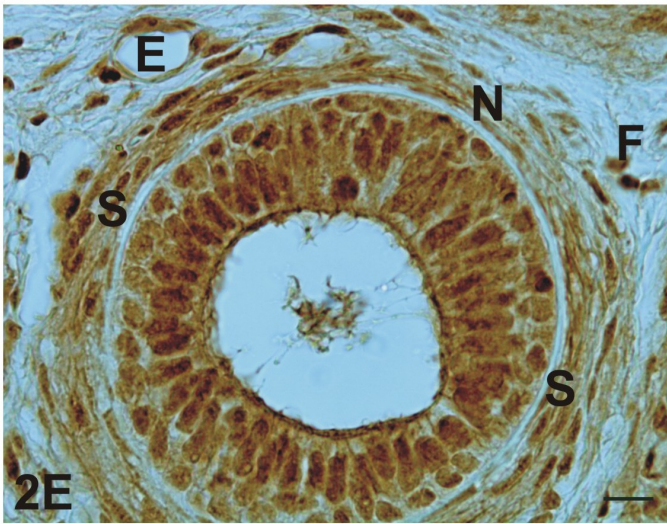
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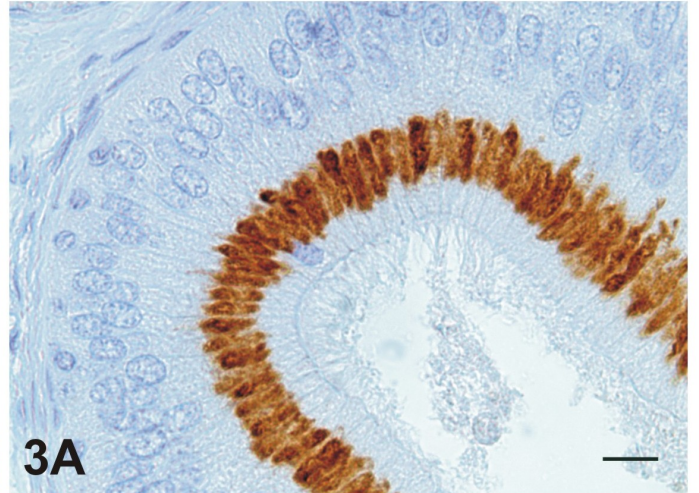
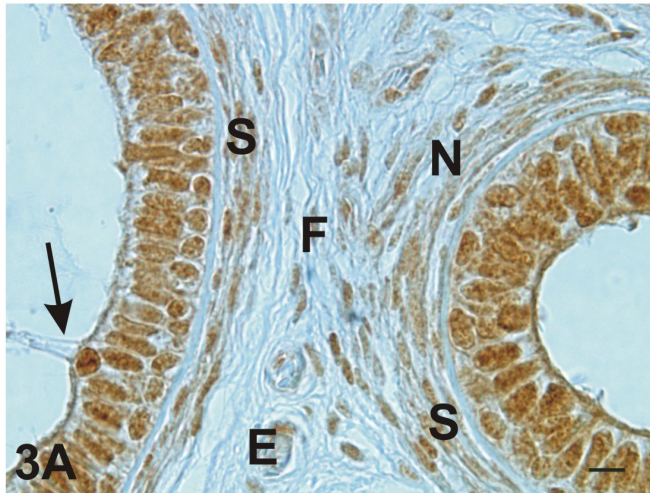


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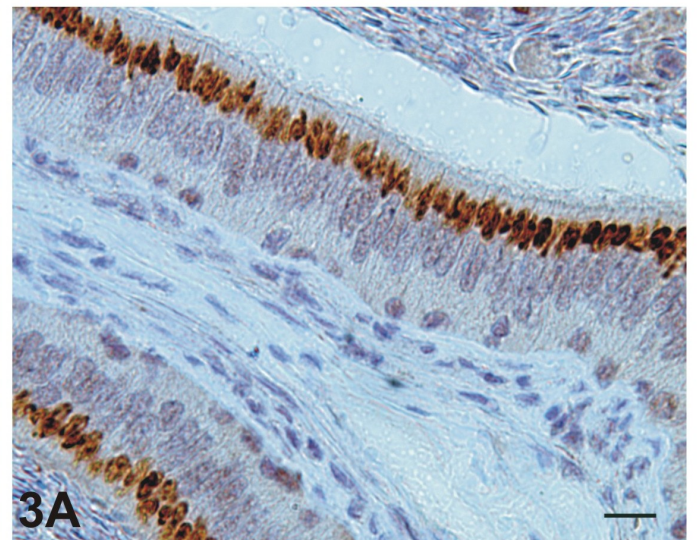
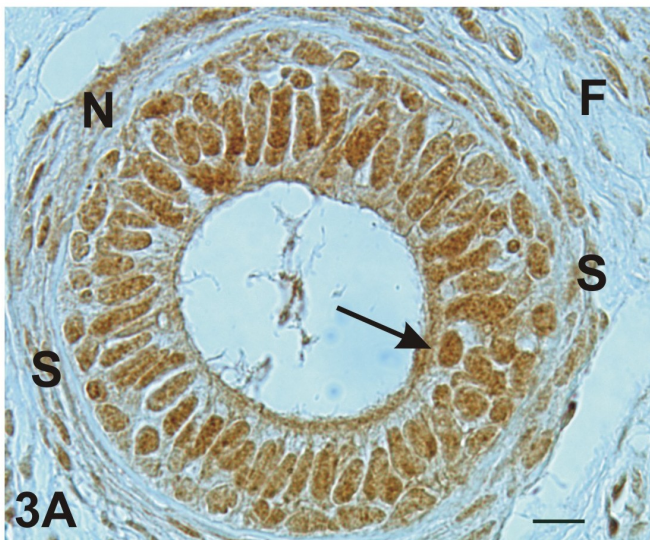
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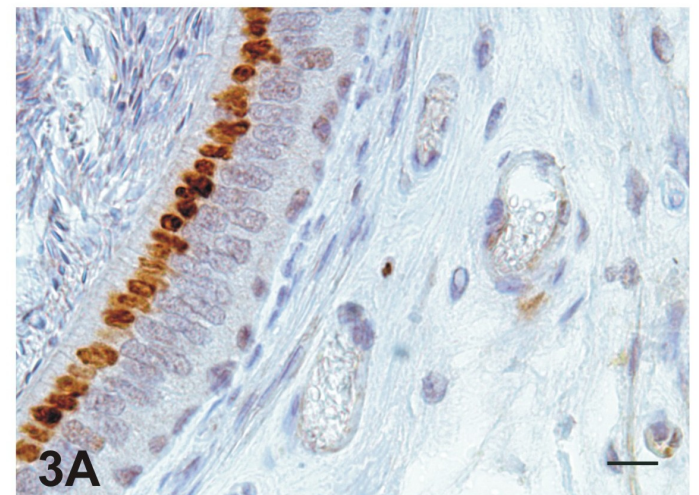
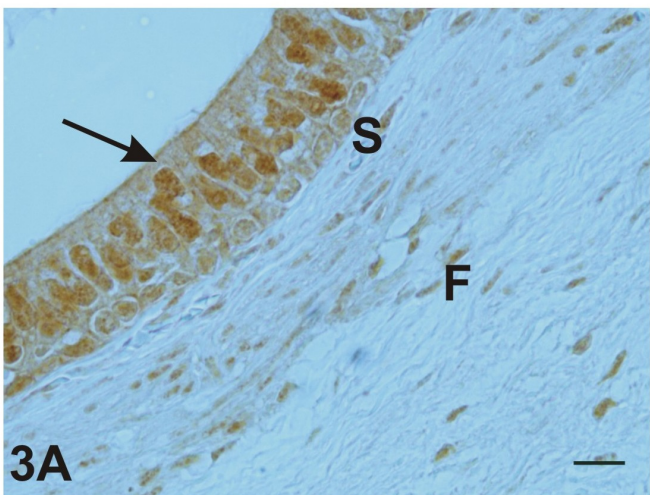
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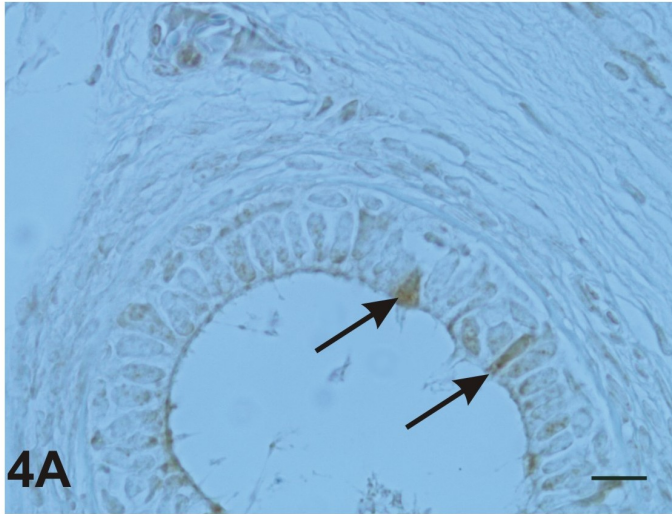


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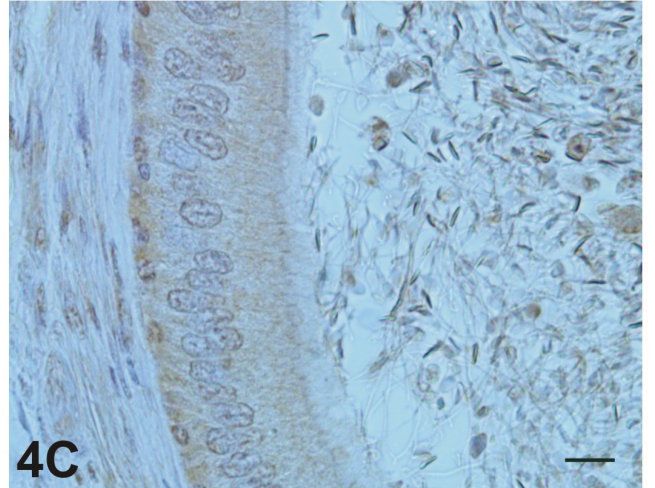
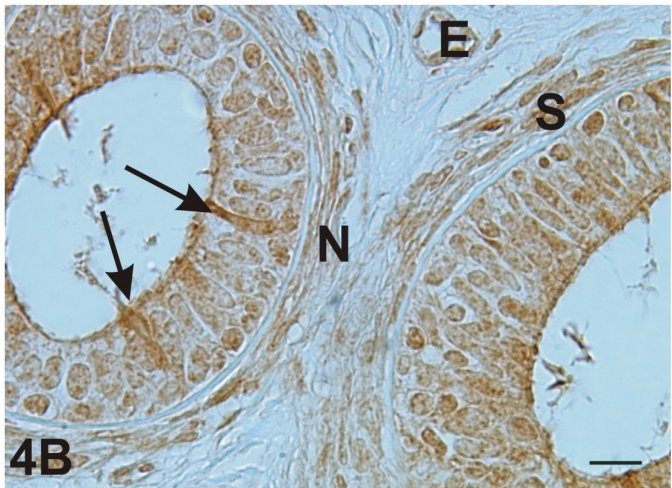
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