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Lectin binding sites in the seminal vesicles of entire and castrated horse.

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Abstract

This research was undertaken to determine the glycoconjugates secreted by the glandular epithelium of the seminal vesicles in the entire and castrated horses using lectin histochemistry in combination with sialidase digestion and deglycosylation pre-treatments. The following lectins were used: Con-A, UEA-I, LTA, WGA, GSA-IB4, SBA, PNA, ECA and DBA. In the entire stallion, the glandular cells expressed the following sugar residues: α-Fuc, internal GlcNAc, terminal β- and α-GalNAc, β-D-Gal-(1-4)-GlcNAc and α-Gal included in O-linked oligosaccharides; β-Gal-(1-3)-GalNAc belonged to both O- and N-oligosaccharides whereas terminal GlcNAc to N-linked glycans. Additionally, α-Gal and β-Gal acted as acceptor sugars for sialic acid moieties. In castrated horses, the glandular epithelium showed a different lectin labelling pattern. In particular, we evidenced internal GlcNAc, α-GalNAc, β-Gal-(1-3)-GalNAc in both O- and N-linked glycoproteins whereas β-GalNAc and β-Gal-(1-3)-GalNAc in O-linked glycoproteins. The differences evidenced in the lectin profile between the stallion and castrated horse suggested an hormonal regulation of the glycoconjugate production. Additionally, the plurality of glycomolecules detected in the secretions of the stallion may be involved in spermatozoa maturation.

Key words: glycoconjugates, lectin histochemistry, horse, seminal vesicles

Introduction

The seminal vesicles are accessory sex glands present in most domestic mammals; they synthesized copious amounts of glycoconjugates that are involved in many biological activities, contributing significantly to the final volume of seminal plasma (Chan et al. 2003). These compounds are involved in the remodelling of the sperm surface, which occurs during sperm transit through the male genital tract, favouring their final maturation (Diekman 2003; Ha et al. 2003; Töpfer-Petersen et al. 2005; Kankavi et al., 2006). Indeed, in many species, the epididymal spermatozoa are immotile and become motile only during or after ejaculation with the contribution of the accessory sex glands secretions (Töpfer-Petersen et al. 2005).

Lectins are specific carbohydrate-binding proteins of non-immune origin that agglutinate cells and/or precipitate polysaccharides or glycoconjugates having glycans of appropriate complementary and, therefore, they are useful tools for investigating glycoconjugate distribution as well as cell differentiation and functional maturation (Spicer and Schulte 1992).

Various lectin histochemical works performed in the male sex accessory glands of mammals demonstrate that lectins represent suitable histochemical markers of specific secretory functions, structural components and developmental alterations of these glands (Morales et al. 2005; Töpfer-Petersen et al. 2005).

In previous studies performed with lectin histochemistry, it was demonstrated differences in the presence of glycoconjugates in adult compared to prepubertal horse (Parillo et al. 1997, 1998; Verini Supplizi et al. 2000) and alpaca (Parillo et al. 2008a; Parillo et al. 2009c,e) genital tracts, suggesting an hormonal regulation of their activity. Additionally, among the horse accessory reproductive glands, the *ampulla ductus deferentis* and the prostate have been studied histochemically, (Parillo and Verini Supplizi, 2008; Parillo et al. 2010; Parillo et al. 2008b). To our knowledge, there is a lack of information on the glycocomposition of the secretory products of the equine seminal vesicles, so, herein, we examined the chemical structure of glycoconjugates secreted

by seminal vesicles in entire and castrated horses, using lectin histochemistry combined with enzymatic digestion and deglycosylation procedures.

Material and methods

The seminal vesicles were removed from 2 stallions (8-12 years old) that were euthanized due to a fatal fracture of the humerus and from 2 castrated horses (7-10 years old) regularly slaughtered in a licensed abattoir. All the animals did not present reproductive abnormalities. The specimens were immediately fixed and processed for lectin histochemistry using procedures previously described (Parillo et al. 2009b, 2012, 2013; Zerani et al. 2012, 2013). The sections were dipped in 0.3% H_2O_2/methanol for 1 hour to inhibit endogenous peroxidase activity, washed with PBS, then incubated in a moist chamber for 1 hour at room temperature with a solution of horseradish peroxidase (HRP) conjugated lectins (Sigma-Aldrich, St Louis, MO, USA) in 0.1M PBS, pH 7.2, containing 0.1mM $CaCl_2$, $MgCl_2$ and $MnCl_2$. The sections were rinsed with PBS and the peroxidase activity sites were visualized with 3-3' diaminobenzidine (DAB kit, D.B.A. Italia s.r.l., Milano, I) for 5 minutes. Finally, all the sections were rinsed in distilled water, dehydrated and mounted in Eukitt.

Enzymatic digestion – For enzymatic pre-digestion, sections were treated overnight with 0.86 U/ml neuraminidase (sialidase type V from *Clostridium perfringens,* Sigma Chemical Co., Milano - Italy) in a solution of 0.1M acetate buffer, pH 5.5, and 10 mM $CaCl_2$ at 37 °C. Deacetylation (saponification) was performed by incubating sections with 1% potassium hydroxide solution in 70% ethanol for 15 minutes at room temperature prior to sialidase degradation. By detaching acetyl substituents, this chemical treatment renders sialic acid residues with O-acyl groups at C4 of the pyrannose ring that previously resisted *C. perfringens* neuraminidase susceptible to sialidase digestion (Parillo et al. 2009d).

Deglycosylation pre-treatments. Two chemical pre-treatments were performed: a) hydrolysis of N-linked oligosaccharides was performed with N-glycosidase F (PNGase F - Roche, Mannheim, Germany) as previously described; b) β-elimination reaction, to eliminate O-linked oligosaccharides was carried out as previously reported (Parillo et al. 2009a).

Controls - Negative controls for lectin labelling, were run either by substitution of lectin-conjugates with the respective unconjugated lectins or by preincubation of lectins with the addition of 0.2/0.4M corresponding hapten sugars (Sigma, Milano - Italy). As controls for sialidase digestion, sections were incubated in enzyme-free buffer solutions under the same conditions as described above.

All the lectins used in this research, along with their corresponding and inhibitory sugars, their optimal concentrations, are reported in Table 1.

Results and Discussion

The seminal vesicles of the stallion are considered tubuloalveolar glands; the glandular epithelium consists of a single layer of low columnar secretory and basal cells (figure 1a). In intact stallion, the glandular cells had globe shaped apical portions projecting and bulging into the lumen of the alveoli that we call apical cytoplasmic blebs (figure 1a). Their presence suggests that the equine seminal vesicles secretes the glandular products mainly in an apocrine manner. In the castrated horse, the alveoli were atrophied and the interalveolar connective tissue occupied about half of the surface area of the sections (figure 1b).

Lectin histochemistry helped us to characterized the carbohydrate moiety of glycococonjugates secreted by the seminal vesicles. The sugar composition appeared to be very heterogeneous; in particular, some of the glycans belonged to both N- and O-bound oligosaccharides such as α-Glu/Man (Con-A reactivity: figure 1c), α-Fuc (UEA-I /LTA reactivity: figure 1d,g), internal GlcNAc (WGA reactivity: figure 2a), and terminal β-D-Gal-(1-3)-D-GalNAc (PNA positivity: figure 2c). β-D-Gal-(1-3)-D-GalNAc acts also as acceptor sugar for terminal sialic acid residues

(sialidase/PNA positivity, data not shown). Conversely, terminal GlcNAc (GSA-II reactivity: figure 2e) was found only in N-linked glycans and terminal α/β-GalNAc (SBA/DBA reactivity: figure 2g-3a), β-D-Gal-(1-4)-D-GlcNAc (ECA staining: figure 3c), α-Gal (GSA-IB4 reaction: figure 3e) were only detected in O-bound glycoproteins. α-Gal residues were also found subterminal to sialyl moieties (data not shown).

In castrated horse, the glandular cells exhibited some lectin reactive sites similar to that revealed in the stallion, including Con-A (figure 1d), WGA (figure 2b), PNA (figure 2d), and GSA-IB4 (figure 3f).

Significant differences regarding either the expression of carbohydrates or the linkage type of the oligosaccharidic chains, in which the same sugar is included, has been also shown. In particular, in glandular cells of castrated animals, α-Fuc and terminal GlcNAc were not detected (negativity of UEA-I-, LTA- and GSA-II-lectins, figures 1f, 1h, 2f, respectively) whereas α/β-GalNAc (SBA/DBA reactivity: figure 2h-3b), β-D-Gal-(1-4)-D-GlcNAc (ECA reactivity: figure 3d) belonged to both N- and O-glycoproteins.

All the control staining procedures failed to disclose appreciable reactivity at any of the sites described (figure 4).

These results indicate that the differences found in the lectin feature of glandular cells, between entire and castrated horse, may depend by hormonal regulation of the glycoconjugate production. In addition, the plurality of glycomolecules detected in the horse seminal vesicle secretions seem to be involved in the series of events collectively called post-testicular sperm maturation (Töpfer-Petersen et al. 2005). Indeed, these authors demonstrated in stallion that glycoconjugates contribute to early and central steps of the fertilization process, the establishment of the oviductal sperm reservoir, modulation of sperm capacitation and gamete recognition. An important feature of the post-testicular maturation process is the remodelling of the sperm surface by specific interactions of these glycoproteins present in the seminal plasma (Töpfer-Petersen et al. 2005). In this regard in our previous work, we found differences in the lectin profile that occurred in the plasma-membrane of

equine ejaculated and ampullary sperm (Parillo and Verini Supplizi 2008; Parillo et al. 2010). In particular, we reported that the expression of some sugar residues, including GlcNAc, β-D-GalNAc and β-D-Gal detected in the acrosome of ampullary spermatozoa diminished in ejaculated sperm. This data may indicate the presence of accessory sexual gland secretions at the acrosomal level that could partially mask the above glycomolecules. These sperm modifications are probably necessary for spermatozoa transit, capacitation and may have clinical significance in the knowledge of the stallion fertility.

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enzymatic and hormonal activities in corpora lutea of pseudopregnant rabbits. Biology of Reproduction DOI:10.1095/biolreprod.112.107383.

Legends

Figures 1-3. Equine seminal vesicles. Bars = 10μm.

Figure 1a-b Haematoxylin – eosin (H.E.). Arrow = apical blebs.

Figure 1c-d Con-A staining. a) Stallion. The glandular cells and the secretions are moderately stained with this lectin; b) Castrated horse. The glandular cells and the luminal content are moderately reactive.

Figure 1e, insert UEA-I staining. Stallion. The glandular cells evidences moderate affinity for this lectin; insert) β-elimination/UEA-I. The pre-treatment determined a diminution of the reactivity.

Figure 1f UEA-I staining. Castrated horse. The glandular epithelium is unstained.

Figure 1g, insert. LTA staining. Stallion. Glandular cells display strong staining; insert) β-elimination/LTA. The cleveage of O-linked glycans abolishes the reactive sites.

Figure 1h LTA staining. Castrated horse. The glandular epithelium is unstained.

Figure 2a-b. WGA staining. a) Stallion. The secretory cells and the secretions show strong positivity; b) Castrated horse. β-elimination/WGA. The glandular cells show strong reactivity.

Figure 2c-d. PNA staining. c) Stallion. PNGase/PNA. The removal of N-linked oligosaccharides promotes weak PNA staining of glandular cells; d) Castrated horse. PNGase/PNA. The glandular cells display weak positivity.

Figure 2e. GSA-II staining. Stallion. Binding sites are moderately located in glandular cells, apical blebs (arrow) and luminal secretions.

Figure 2f GSA-II staining. Castrated horse. The glandular epithelium is unstained.

Figure 2g-h. SBA staining. g) Stallion. The glandular cells display moderate staining; h) Castrated horse. β-elimination/SBA. The pre-treatment promotes strong positivity of glandular cells.

Figure 3a, insert-b. DBA staining. a) Stallion. The glandular cells show strong staining; insert) β-elimination/DBA. The elimination of O-linked oligosaccharides abolishes DBA reactive sites; b) Castrated horse. DBA staining. The glandular cells show strong DBA positivity.

Figure 3c-d. ECA staining. c) Stallion. The reactive sites are moderately localized in glandular cells and secretions; d) Castrated horse. ECA-positive sites are moderately localized in glandular cells; insert) β-elimination/ECA. The pre-treatment causes a decrease of ECA staining.

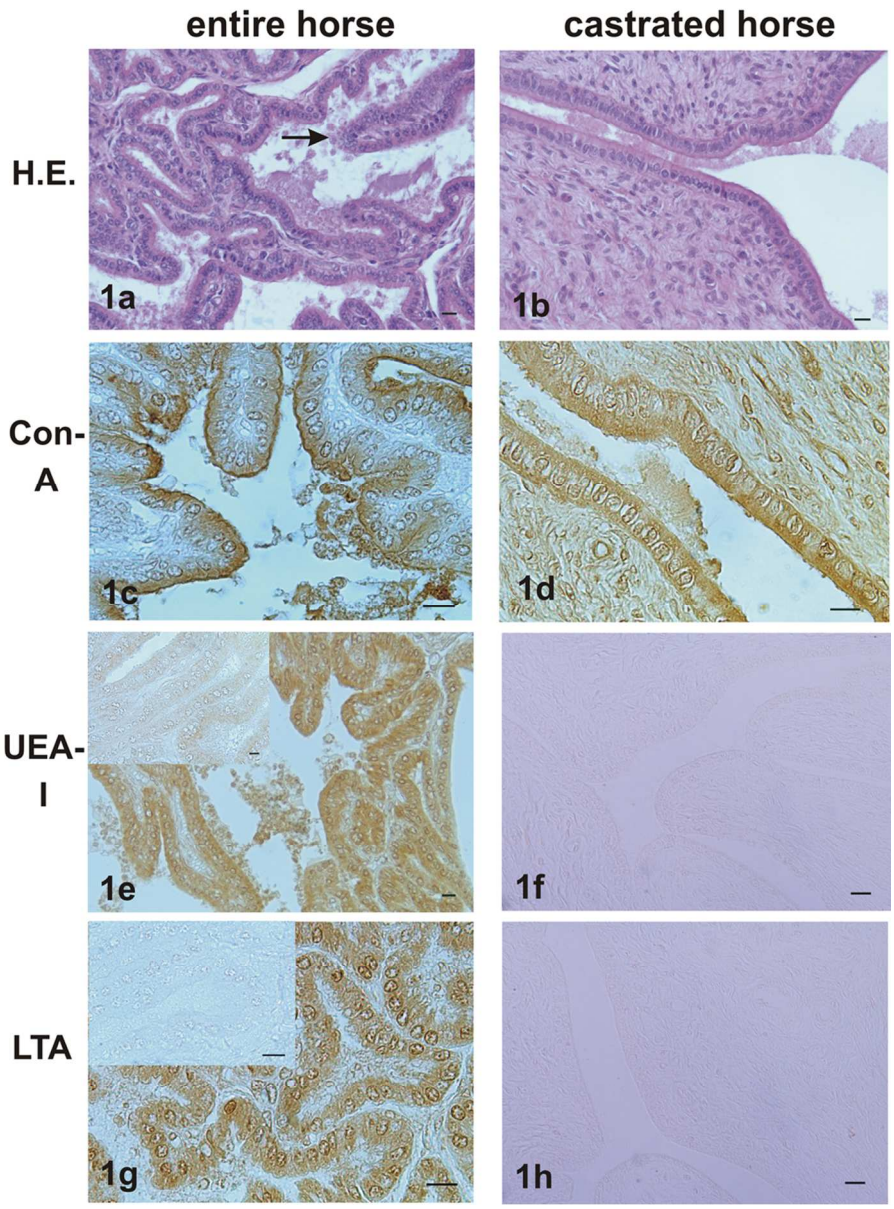
Figure 3e-f. GSA-IB4 staining. e) Stallion. GSA-IB4 signals are moderately localized in glandular cells; f) Castrated horse. The glandular cells moderately react with GSA-IB4

Figure 4 control section. Con-A- HRP with 0.4 M D-Man. Staining is completely inhibited.

Table 1. Lectins used and their carbohydrate specificities.

Source of lectin	Acronym	Carbohydrate specificity[a]	Inhibitory sugars[a]	Lectin concentration
Canavalia ensiformis	Con-A	α-D-Man>α-D-Glc	α-D-Methylman	20 µg/ml
Triticum vulgaris	WGA	GlcNAc>sialic acid	D-GlcNAc	10 µg/ml
Glycine max	SBA	α-D-GalNAc>β-D-GalNAc	D-GalNAc	10 µg/ml
Arachis hypogaea	PNA	β-D-Gal-(1→3)-D-GalNAc	D-Gal	40 µg/ml
Griffonia simplicifolia IB4	GSA-IB4	α-D-Gal	D-Gal	20 µg/ml
Erytrina cristagalli	ECA	β-D-Gal-(1→4)-D-GlcNAc	D-Gal	50 µg/ml
Dolichos biflorus	DBA	α-D-GalNAc	D-GalNAc	10 µg/ml
Lotus tetragonolobus	LTA	α-L-Fuc	L-Fuc	20 µg/ml
Ulex europaeus	UEA-I	α-L-Fuc	L-Fuc	20 µg/ml

a β-D-Gal, β-D-galactose; α-D-Gal, α-D-galactose;
D-GalNAc, D-N-acetylgalactosamine;
β-D-GalNAc, β-D-N-acetylgalactosamine;
α-D-GalNAc, α-D-N-acetylgalactosamine;
GlcNAc, N-acetylglucosamine;
α-D-Man, α-D-mannose; α-D-Glc, α-D-glucose, α-L-Fuc, α-L-Fucose;
α-D-Methylman, α-D-methylmannose



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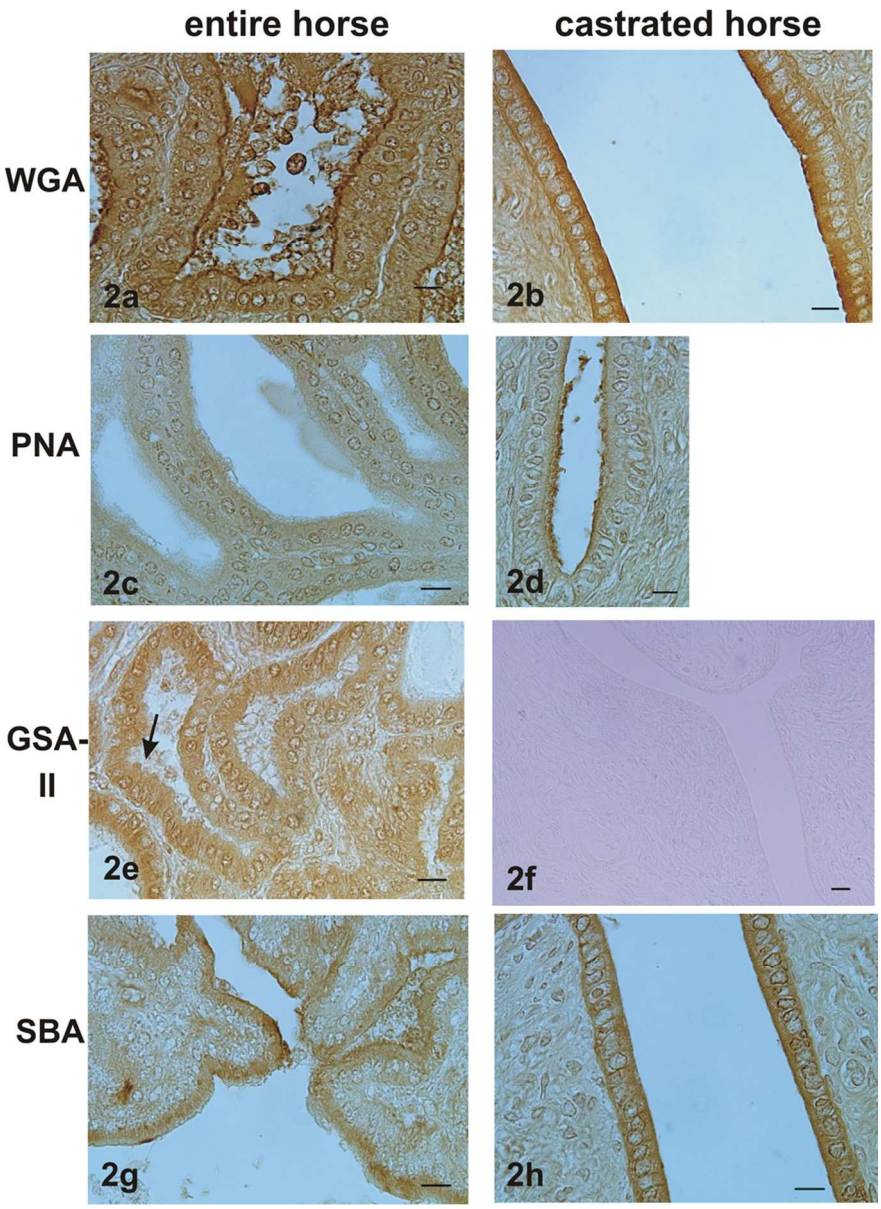

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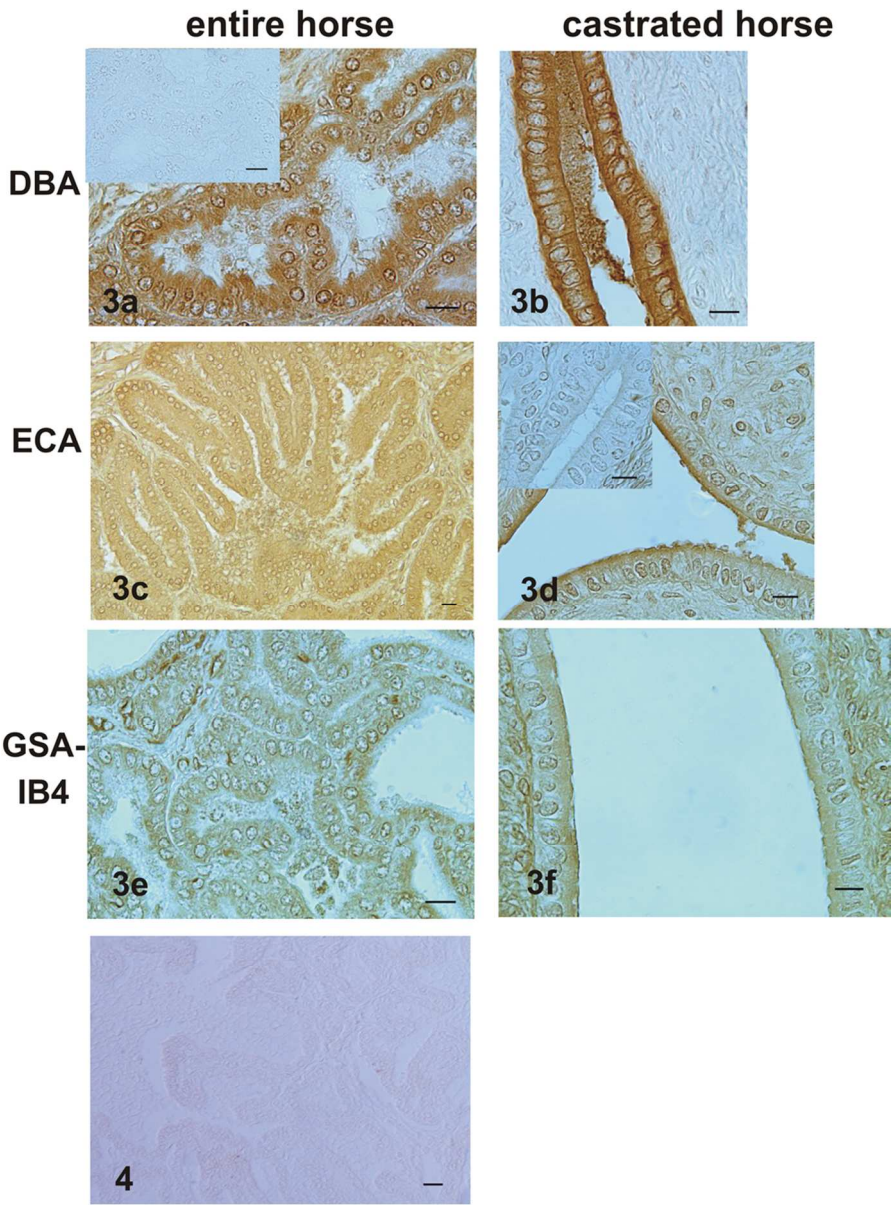

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