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Note

Desialylation of core type 1 *O*-glycan in the equine embryonic capsule coincides with immobilization of the conceptus in the uterus

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Abstract—During the second and third weeks of pregnancy, the equine conceptus expands rapidly while it is enclosed within a glycan capsule. Around day 16 of gestation, the conceptus loses its mobility in the uterus by a process termed 'fixation', coinciding with various changes in the capsule. Here, we compared the structure of the carbohydrate moieties expressed by the capsule during pre- and post-fixation periods. The glycan structures were studied by chemical analyses in combination with mass spectrometry. Capsule material from conceptuses collected before fixation (days 13–16) was observed to carry a sialylated core type 1 O-linked glycan, Neu5Ac-(2→3)-Gal-(1→3)-GalNAc-(1→Ser/Thr. By comparison, analysis of post-fixation capsules (days 17–19) revealed a desialylated core type 1, Gal-(1→3)-GalNAc-(1→Ser/Thr. The equine embryonic capsule also furnished 4-substituted GlcNAc, 4-substituted Glc and 2,3,4,6-tetrasubstituted Glc residues, the concentrations of which did not change between pre- and post-fixation stages. The loss of sialic acid from the sialylated core type 1 in the capsule appears to be directly related to successful fixation of the conceptus, and thus critical to the continuance of pregnancy in horses.

Keywords: *O*-Glycan; Sialic acid; Core type 1; Embryonic capsule; Equine pregnancy; Mass spectrometry

Pregnancy maintenance in the mare depends upon the transitory appearance of a mucin-like glycoprotein capsule around the blastocyst as it expands in the uterus during the second and third weeks of gestation.[1–4] This acellular capsule (Fig. 1) is produced, at least in large part, by the trophoblast[5] and is thus part of the conceptus (embryo proper, the embryonic membranes and their contained fluid), forming an interface between its cellular wall and the lumen and lining of the uterus (the endometrium). For the first two-thirds of its existence, the capsule is resilient and favours extensive migration of the spherical conceptus within the uterus until, at about day 16–17 (ovulation = day 0), migration ceases and the conceptus becomes 'fixed' at the site of subsequent placentation.[6] Pre-fixation mobility is essential for pregnancy maintenance,[7] allowing signals to be exchanged between the conceptus and the entire endometrium. Coincidently with fixation, the conceptus becomes flaccid and the capsule changes in composition.[8] Fixation coincides with a reduction in sialic acid in the capsule,[4] and with alterations in several capsule-bound proteins including uterocalin/p19.[9–12] Pregnancy failure due to embryonic loss is of great economic importance in horse breeding and most of the losses are incurred during the period that the capsule is present.[13–15]

Changes in the capsular glycans during fixation might influence permeability to nutrients or noxious substances, or contribute to a physical interaction between the capsule and the endometrium where placentation eventually becomes established. The fine structures of

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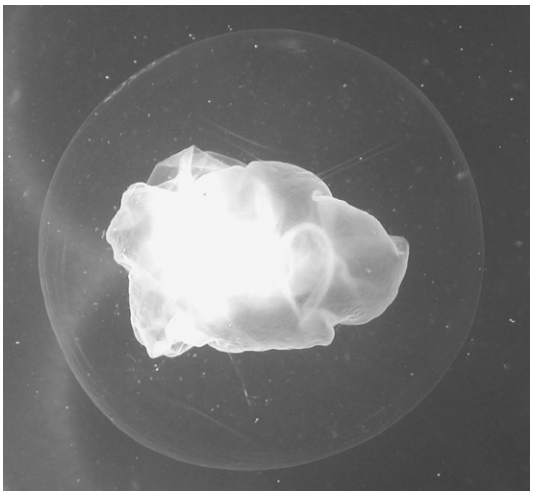

Figure 1. An equine conceptus collected 17.5 days after ovulation in which the yolk sac (white cellular tissue in the centre) ruptured and collapsed during the procedure thereby revealing the still-intact acellular capsule (the outer circle).

normal capsular glycans have not been determined, but previous studies showed that it is composed of mostly of sialic acid (Neu5Ac), galactose (Gal), *N*-acetyl-galactosamine (GalNAc) and minor amounts of glucose (Glc) and *N*-acetyl-glucosamine (GlcNAc).[3,16] These sugar analyses revealed that in the normal conceptus sialic acid was present during the pre-fixation period (before day 16), but was lost after day 16 during the post-fixation stage.[3,16] However, this decrease in sialic acid did not occur after day 16 when pregnancy failure was initiated experimentally.[16] These findings suggested that loss of sialic acid has a role in normal fixation of the conceptus. Thus, an understanding of the structures of the normal and abnormal capsule glycans might help to explain how pregnancy succeeds or fails during the critical fixation period. Accordingly, we determined here the fine structures of the O-linked glycan moieties expressed by the capsule during pre- and post-fixation periods.

Structural characterization of capsule O-glycan expressed during pre-fixation. Monosaccharide composition analysis carried out on the intact capsule materials ($n = 14$) by the alditol acetate procedure showed that the capsule (pre-fixation) contained Gal and GalNAc as the major components, with Glc and GlcNAc in minor concentrations. Sugar linkage analysis (Fig. 2a) performed on the intact capsule preparations ($n = 14$) by the characterization of permethylated alditol acetates by gas liquid chromatography–mass spectrometry (GLC–MS) revealed that the capsule (pre-fixation) expressed 3-substituted Gal [→3)-Gal-(1→] and 3-substituted GalNAc [→3)-GalNAc-(1→] as the main residues and, in lesser amounts terminal Gal [Gal-(1→], 4-substituted GlcNAc [→4)-GalNAc-(1→], 4-substituted Glc [→4)-Glc-(1→] and 2,3,4,6-tetrasubstituted Glc residues [→2,3,4,6)-Glc-(1→].

Fine structural information was obtained by fast atom bombardment mass spectrometry (FAB-MS) of the methylated material. The FAB-MS spectrum (Fig. 3a) of the methylated intact capsules from pre-fixation period showed primary A type glycosyloxonium ions of defined composition at *m*/*z* 376 (and 344 from β-elimination of methanol) for terminal $[Neu5Ac]^+$, and 825, which in line with the sugar composition and linkage analyses could be attributed to the linear triglycosyl $[Neu5Ac\text{-}(2\rightarrow3)\text{-}Gal\text{-}(1\rightarrow3)\text{-}GalNAc]^+$. Also observed was a strong molecular ion $[MH]^+$ at *m*/*z* 857, which corresponded to the methyl glycoside (formed during the methylation procedure by virtue of cleavage of GalNAc from Ser/Thr by NaOH) of the above described trisaccharide [Neu5Ac-(2→3)-Gal-(1→3)-GalNAc-(1→O-Me] (Fig. 3a). The FAB-MS spectrum of the pre-fixation methylated capsule (Fig. 3a) also showed a weaker glycosyloxonium ion at *m*/*z* 464 for $[Gal\text{-}(1\rightarrow3)\text{-}GalNAc]^+$ and the corresponding molecular ion $[MH]^+$ at *m*/*z* 496 for the diglycosyl methyl glycoside [Gal-(1→3)-GalNAc-(1→O-Me]. The capsule material was treated with 1% acetic acid at 100 °C for 1 h to selectively cleave the sialic acid moiety and subsequent linkage analysis revealed that the concentration of terminal Gal increased and 3-substituted Gal decreased to trace amounts whilst the 3-substituted GalNAc remained unchanged. These results underlined the fact that sialic acid was connected to the 3-substituted Gal residue.

Structural characterization of capsule O-glycan expressed during post-fixation. Similarly to the capsule pre-fixation, monosaccharide composition analysis carried out on the intact preparations ($n = 15$) showed that the capsule post-fixation contained Gal and GalNAc as the major components, with Glc and GlcNAc in minor concentrations. Sugar linkage analysis (Fig. 2b) on the intact material revealed that the capsule post-fixation carried terminal Gal [Gal-(1→] and 3-substituted GalNAc [→3)-GalNAc-(1→] as the main residues with now small amounts of 3-substituted Gal [→3)-Gal-(1→]. Also detected in minor amounts were 4-substituted GlcNAc [→4)-GalNAc-(1→], 4-substituted Glc [→4)-Glc-(1→] and 2,3,4,6-tetrasubstituted Glc [→2,3,4,6)-Glc-(1→] residues. The concentration of these GlcNAc and Glc units did not change between pre- and post-fixation capsule materials.

The FAB-MS spectrum (Fig. 3b) of the methylated intact capsules from post-fixation period showed strong A type primary glycosyloxonium ions at *m*/*z* 464 for $[Gal\text{-}(1\rightarrow3)\text{-}GalNAc]^+$ and the respective molecular ion at *m*/*z* 496 for [Gal-(1→3)-GalNAc-(1→O-Me]. *m*/*z* Ions of lesser intensity were also observed at 376 for $[Neu5Ac]^+$, 825 for $[Neu5Ac\text{-}(2\rightarrow3)\text{-}Gal\text{-}(1\rightarrow3)\text{-}GalNAc]^+$ and $[MH]^+$ at 857 for [Neu5Ac-(2→3)-Gal-(1→3)-GalNAc-(1→O-Me]. In order to confirm that *m*/*z* 464 belonged solely to the Gal-(1→3)-GalNAc moi-

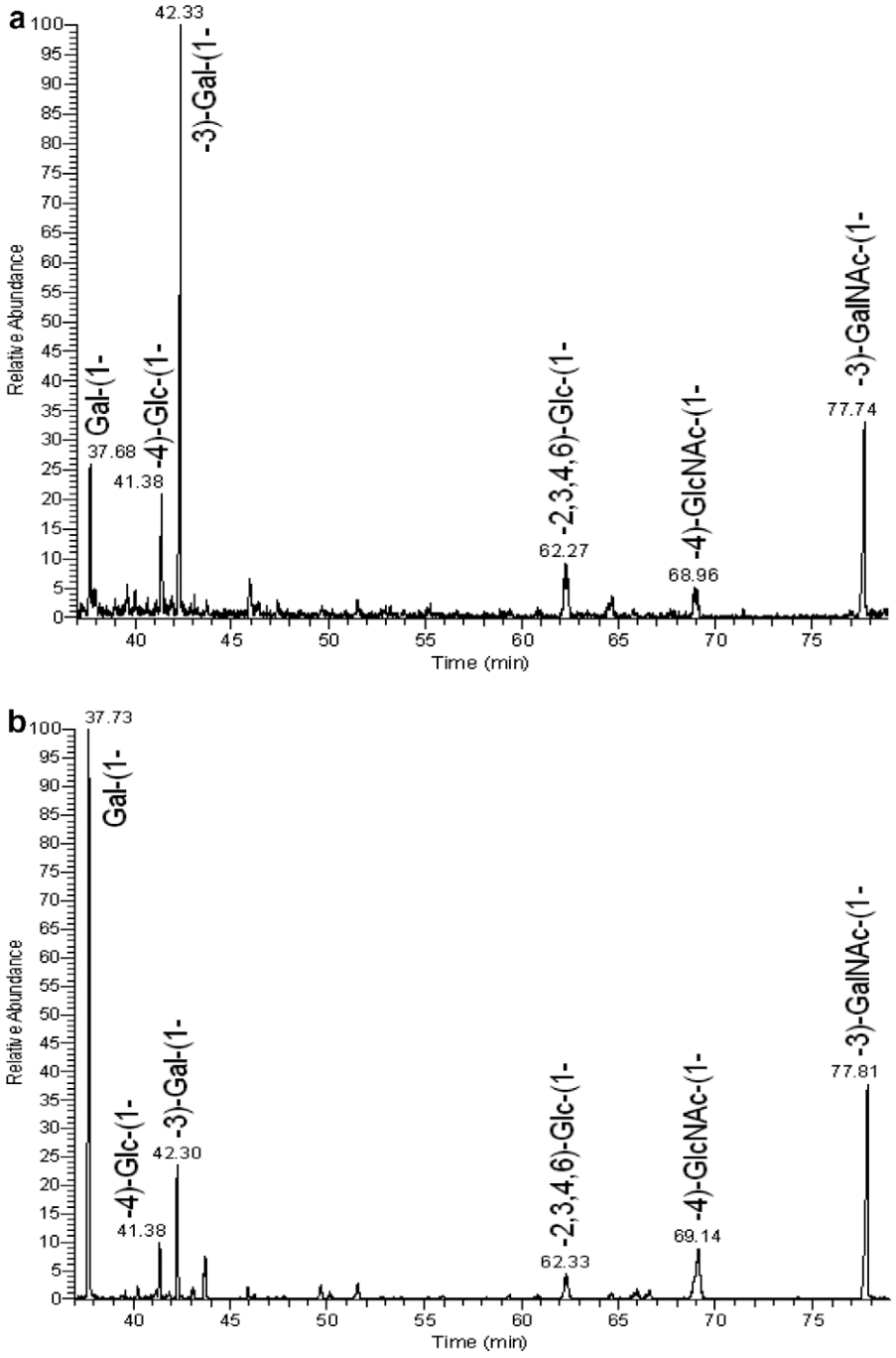

Figure 2. Gas liquid chromatogram profile of permethylated alditol acetate derivatives obtained from (a) pre-fixation intact capsule material (day 15) showing 3-substituted Gal and 3-substituted GalNAc as the major components, and terminal Gal, 2,3,4,6-tetrasubstituted Glc and 4-substituted GlcNAc as minor substituents; and from (b) post-fixation intact capsule material (day 17) showing terminal Gal and 3-substituted GalNAc as the major components. The expression of 2,3,4,6-tetrasubstituted Glc and 4-substituted GlcNAc remained constant.

ety, and not from a possible Gal-(1→4)-GlcNAc, a product ion (parent ion) MS/MS experiment (Fig. 4) was carried out on *m*/*z* 464, which furnished as the sole secondary ion *m*/*z* 228 that corresponded to the loss of Gal-OH through β-elimination from O-3 of GalNAc.

Collectively, the structural data obtained from chemical analysis and MS revealed that the capsule during pre-fixation (mobile stage) expressed as the main glycan moiety a sialylated core type 1, Neu5Ac-(2→3)-Gal-(1→3)-GalNAc, typically found as O-linked glycans linked to Ser or Thr in mucin-like glycoproteins,[17] and as a minor constituent the O-linked core type 1 moiety, Gal-(1→3)-GalNAc. Conversely, the capsule post-fixation (non-mobile stage) was found to be decorated mainly by the desialylated core type 1, Gal-(1→3)-GalNAc, with only traces of the sialylated form, Neu5Ac-

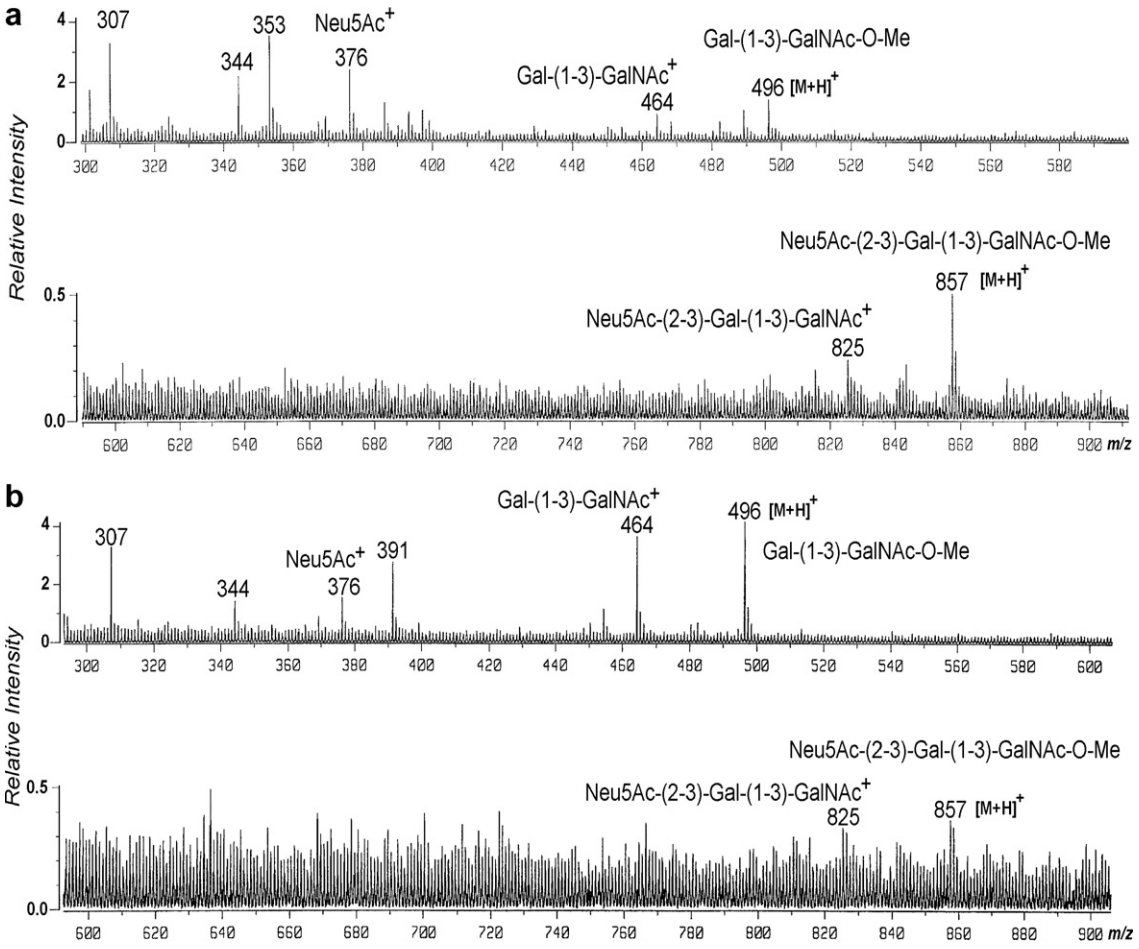

Figure 3. (a) The FAB-MS spectrum of the methylated capsule pre-fixation period showing that the major species was a sialylated core type 1; (b) the FAB-MS spectrum of the methylated capsule post-fixation period showing that the major species was a desialylated core type 1.

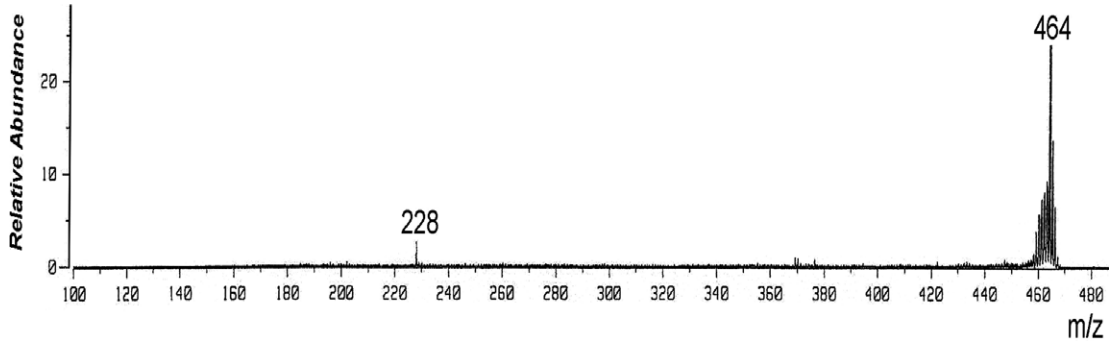

Figure 4. The FAB-MS spectrum of parent ion MS/MS experiment carried out on the A-type primary ion *m*/*z* 464, which yielded a secondary ion at *m*/*z* 228 from β-elimination of Gal-OH that confirmed that Gal was attached to position O-3 of GalNAc.

(2→3)-Gal-(1→3)-GalNAc, being detected. The capsule also expressed in lesser concentrations, 4-substituted GlcNAc, 4-substituted Glc and 2,3,4,6-tetrasubstituted Glc residues. The relative concentration of these units did not change between pre- and post-fixation capsule materials.

The observed desialylation of O-linked core type 1 in the equine embryonic capsule might represent the initial stage of degradation of the capsule that occurs before the yolk-sac wall is free to make cell-to-cell contact with the endometrium around day 21. Desialylation is the initial step in degradation of various sialylated glycans including ganglioside glycolipids.[18] Alternatively, the loss of sialic acid might contribute directly to the still unexplained mechanism by which the encapsulated conceptus becomes 'fixed' in the uterine location at which

placentation will occur later. One possibility is that the exposed desialylated core type 1 Gal-(1→3)-GalNAc might be a functional ligand for an endometrial adhesion receptor.

The rapid and virtually complete loss of capsular sialic acid at the time of fixation suggests a programmed physiological event during early pregnancy in the mare. This might require the activity of an extracellular sialidase. Reversible sialylation is well documented as a regulator bio-mechanism in cell adherence and mobility during embryogenesis and malignant growth.[18] The structural results presented here strongly suggest that control of the degree of sialylation of *O*-glycan core type 1 in the embryonic capsule is vital to the success of equine pregnancy.

1. Experimental

1.1. Collection of conceptuses

Standardbred and thoroughbred mares from the research herd of the University of Guelph were used to recover conceptuses under conditions approved by the University Animal Care Committee. The timing of ovulation was monitored by transrectal ultrasonography at daily intervals and was deemed to have occurred on day 0, midway between examinations showing an intact follicle and a corpus luteum. Thus, ages of conceptuses were subject to an error of ±0.5 days. Conceptuses were collected from mares by transcervical uterine lavage, with Dulbecco's phosphate-buffered saline, pH 7.4, using a technique based on that described by Sirois and Betteridge.[19] The mares were sedated with xylazine hydrochloride (Rompun: Bay-Vet, Etobicoke, ON), administered i.v., usually 0.3 mg/kg body weight. The flush fluid (1000 mL) was infused by gravity flow and drained into a beaker to recover the conceptus.

1.2. Collection of capsule

Capsules were separated from cellular tissue of the conceptus under a dissecting microscope and each was stored frozen in 0.5 mL PBS pending analysis. Before being used in the present study, each capsule had been subjected to elution of proteins by boiling for 5 min in 200 μL of 1% SDS, 4% mercaptoethanol, 12% glycerol in 0.1 M Tris–HCL pH 6.8 (SDS treatment buffer) for parallel studies.[12] The capsule structure remained intact throughout the elution procedure.

1.3. Sugar composition analysis and linkage analysis

Monosaccharide composition analysis was performed by the alditol acetate method.[20] The glycosyl hydrolysis was carried out directly on the intact capsule preparations ($n = 14$) with 4 M-trifluoroacetic acid at 105 °C for 5 h followed by reduction in H_2O with $NaBD_4$ overnight at room temperature, and subsequent acetylation was accomplished by acetic anhydride treatment with residual sodium acetate as the catalyst at 100 °C for 2 h. The alditol acetate derivatives were analyzed by GLC using a Varian 3400 gas chromatograph equipped with a 30-m DB-17 capillary column [210 °C (30 min)-240 °C at 2 °C/min], and by GLC–MS in the electron impact mode, which was recorded using a Hewlett Packard 5890 mass spectrometer. Sugar linkage analysis was performed on the intact capsule materials by the methylation procedure[21] ($NaOH/Me_2SO/CH_3I$) and with characterization of permethylated alditol acetate derivatives by GLC–MS in the electron impact mode (DB-17 column, isothermally at 190 °C for 60 min). To ensure complete solubility, the capsules were stirred in Me_2SO overnight before methylation. NaOH was added the next morning and allowed to stir for 2 h at room temperature before the addition of CH_3I.

1.4. Fast atom bombardment-mass spectrometry (FAB-MS)

A fraction of the methylated sample was used for positive ion FAB-MS, which was carried out on a Jeol JMS-AX505H mass spectrometer with glycerol/thioglycerol (1:3) as the matrix. A 6-kV xenon beam was used to produce pseudomolecular ions that were accelerated to 3-kV and their mass analyzed. Product ion scan (B/E) was performed on metastable ions created in the first free field with a source pressure of 5×10^{-5} torr. The interpretations of the positive ion mass spectra of the methylated material were as described previously by Dell.[22] For the FAB-MS experiments the pre- and post-fixation samples were pooled from day-13 to day-16 and from day-17 to day-19 conceptuses, respectively.

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