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A lectin histochemical study to detect variation in glycosylation at the feto-maternal interface in three interbreeding equine species

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Introduction: In this study, we compare glycosylation at the fetomaternal interface in 3 equine species: horse, donkey and zebra, all of which can interbreed to produce hybrids, to assess their glycan similarities and differences.

Methods: Sections cut from 3 specimens of horse (*Equus caballus*) placenta (50, 200 and 280 days gestation), one donkey (*Equus asinus*) placenta (65 cm crown-rump length) and 5 specimens of zebra (*Equus quagga*) placentae (81–239 days gestation) were stained with a panel of 24 biotinylated lectins using an avidin-peroxidase revealing system.

Results: There were only slight quantitative differences in the lectin histochemistry at the feto-maternal interface between all three specimens; zebra placentae expressed more α2,6-linked sialic acid, with α1,2fucosyl residues at the microvillous interface. However, zebra trophoblast showed histological differences from the other two species, with polarised cells, prominent supranuclear Golgi bodies, and fewer intracellular granules.

Discussion: Our findings appear to confirm the hypothesis that closely related, interbreeding species with epitheliochorial placentae express similar glycans at the feto-maternal interface, thereby supporting the existence of a placental glycocode. We also observed intraspecies evolutionary diversion to be associated with a different histological architecture and the absence of significant intracellular granules.

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1. Introduction

We have previously examined glycosylation of the feto-maternal interface in the horse (*Equus caballus*) and donkey (*Equus asinus*), which can interbreed [1], and found a general similarity of glycan expression in the two species [2]. This is in contrast to the situation in the Old World dromedary camel (*Camelus dromedarius*) and the New World camelids which cannot freely interbreed and show a different glycosylation pattern at their placental interface [2]. The opportunity to examine placentae of the Common or Plains zebra (*Equus quagga*, formerly *Equus burchelli*), allowed comparison of zebra placental glycosylation with that of the horse and donkey, all three of which can interbreed [2,3]. It also enabled us to test our previous postulate [4,5] that glycosylation at the feto-maternal interface is regulated by a glycocode which prevents attachment and interdigitation of the trophoblast with an inappropriate maternal epithelium and, hence, hybridisation or extragenetic embryo transfer.

2. Materials and methods

2.1. Animals

As described previously [6], the gravid uteri of 5 adult female Common or Plains zebra ranging in estimated gestational age from 81 to 239 days (see Table 1), and culled for management reasons in Zimbabwe, were dissected 90 - 120 min after death and pieces of the placental interface were fixed in 10% neutral buffered formalin (NBF). The fixed tissues were transported to the UK where they were post-fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4 for 3 h at room temperature. After washing 3 times

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over 24 h in 0.1M sodium cacodylate buffer containing 1 mM $CaCl_2$, the tissues were trimmed and embedded in epoxy resin for lectin histochemistry.

Three resin-embedded specimens of horse placenta at 50, 200 and 280 days gestation (term = 329-342 days) were obtained courtesy of Dr Peter Wooding (University of Cambridge, UK). The 50 day specimen had been immersion-fixed in 4% (v/v) glutaraldehyde; the placenta at 200 days was fixed by immersion in 3% (w/v) paraformaldehyde with 1% (v/v) glutaraldehyde and the 280 day placenta was perfused from both maternal and fetal sides with the same fixative.

One specimen of donkey placenta from a fetus of 65 cm crown-rump length (see Table 1), kindly provided by V. Dantzer (Royal Veterinary and Agricultural University, Denmark) was also used in this study. It had been perfusion-fixed with 3% (v/v) glutaraldehyde in 0.1M phosphate buffer, pH 7.3, with added 2% (w/v) polyvinylpyrrolidone via a small branch of the uterine artery on the maternal side and a branch of the umbilical artery on the fetal side. After immersion fixation for a further two hours it was processed into epoxy resin.

2.2. Lectin histochemistry

Semithin sections (0.75 μm) were cut on a Reichert OMUIII ultramicrotome and mounted on APES-coated slides. Resin was removed using saturated sodium ethoxide diluted 1: 1 with absolute ethanol, after which the sections were stained with a carefully selected panel of 24 biotinylated lectins as described previously [7], followed by an avidin-peroxidase revealing system; some were treated with Type VI neuraminidase (Sigma, from *Clostridium perfringens*) in 0.2M acetate buffer, pH 5.5, with 1% (w/v) calcium chloride at 37 °C for 2h [8] to cleave off terminal sialic acid. Controls were as described previously. All the specimens were stained together with selected lectins to avoid any day-to-day variation and to enable between-species comparisons to be made with confidence. See Table 2 for lectin binding specificities and their sources.

Sections were assessed using a ranking system of analysis whereby staining intensity was graded from 0 (negative) to 4 (intensely stained) and intracellular granule density was graded from + (sparse) to +++ (densely packed). With the zebra trophoblast and endometrial epithelium, the amount of staining around vesicular structures was similarly assessed and graded.

3. Results

3.1. General histology

After apposition and attachment, the trophoblast and maternal uterine epithelium interdigitate with each other via their surface microvilli to form an undulating placental membrane which folds so that trophoblast villi are accommodated within maternal endometrial crypts. Surprisingly, the trophoblast of the zebra placenta differed from that of the horse and donkey in that the trophoblast lacked the presence of large, irregular granules described previously in later gestation in the horse [9] and tended to be more polarised, with cuboidal cells bearing long microvilli and sub-microvillous granules that were visible when staining with some lectins. Supranuclear Golgi bodies were often detected and the cytoplasm appeared to be composed of a sponge-like substructure with hollow vesicles and occasional solid granules. Due to the inevitable delay in obtaining tissue from culled animals, the fixation was often suboptimal, especially in the early gestation specimens where the trophoblast had retracted away from the maternal epithelium and the villous core was sometimes lost completely.

The maternal uterine epithelium was generally more flattened and it contained some granules; darkly staining structures adjacent to the nuclei were observed occasionally which may have been Golgi bodies. The microvilli of these maternal epithelial cells were much shorter than those of the trophoblast. The horse and donkey placentae were similar in appearance to one another and in both species the granules in the trophoblast cells seemed randomly dispersed and lacked the polarity seen in the zebra. Some large inclusions were also present, as has been described previously [9,10]. Fig. 1 shows typical fields of the placental barrier of horse, donkey and zebra stained with WFA to show the general characteristics.

Table 1
Details of the material used in this study.

Species	Days of gestation	CR length (cm)	Weight of embryo (g)
Horse	50	~43[a]	N/A
Horse	200	~53[a]	N/A
Horse	280	~75[a]	N/A
Donkey	N/A	65cm	N/A
Zebra	81	10	14.3
Zebra	117	24	230
Zebra	195	58	2790
Zebra	230	72	5580
Zebra	239	72	6590

CR = crown-rump; N/A = not available.
[a] Estimated from [26].

3.2. Lectin histochemistry

In general, there was little change in glycan expression over the period of gestation studied. In many cases the uterine epithelium showed apical microvillous staining when the cytoplasm was unstained, suggesting the possibility that this had resulted from trophoblast microvillous glycan adhering to the epithelium, rather than being synthesised by it.

3.3. Trophoblast (see Table 3)

Trophoblast bound several lectins strongly, especially at the earliest stage examined (day 81). The cytosol had a vesiculated appearance (Fig. 2A) and component granules were not easily detectable, even though the vesicular structures bound many lectins more strongly than the background cytosol. The apical membrane, which was clothed in microvilli that were longer than those on the uterine epithelium, was stained intensely by all the lectins used in the study apart from MAA, though it is possible that with UEA-1 some of the staining was to uterine glycan adhering to microvilli upon post-mortem loosening of the interdigitation (Fig. 2B). The cytosol varied from virtually unstained (MAA), to very pale (lPHA, UEA-1, DBA, VVA, SBA), to more strongly stained as with most other lectins, and invariably the intensity of stain increased considerably just under the microvillous surface. In the later stages of gestation, populations of small, discrete granules could only be detected with certainty when using PSA, ALA (Fig. 2C), GNA and LEA, particularly in the apical regions of the cell, and supranuclear foci of staining, probably Golgi bodies judging by their shape and location, were particularly prominent with ALA, BSA-1B_4, PAA and WGA staining, the latter two both with and without neuraminidase pretreatment (Fig. 2D). This feature was most prominent at the later stages of gestation studied. Cell membranes were stained particularly strongly, especially at the apical tips of the microvilli, with ePHA, MPA (Fig. 2E), DSA, STA, HPA, AHA and ECA (+/-N), WFA, SNA-1 and PAA (+/-N).

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