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Technical note

A method for isolating and culturing placental cells from failed early equine pregnancies

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ABSTRACT

Early pregnancy loss occurs in 6–10% of equine pregnancies making it the main cause of reproductive wastage. Despite this, reasons for the losses are known in only 16% of cases. Lack of viable conceptus material has inhibited investigations of many potential genetic and pathological causes. We present a method for isolating and culturing placental cells from failed early equine pregnancies. Trophoblast cells from 18/30 (60%) failed equine pregnancies of gestational ages 14–65 days were successfully cultured in three different media, with the greatest growth achieved for cells cultured in AmnioChrome™ Plus. Genomic DNA of a suitable quality for molecular assays was also isolated from 29/30 of these cases. This method will enable future investigations determining pathologies causing EPL.

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1. Introduction

Early pregnancy loss (EPL) remains a significant cause of reproductive wastage in mammals [1–5] but the underlying aetiologies are often unknown [6,7]. A retrospective study in horses showed a speculative cause for EPL was found in just 16% of cases [8]. The absence of suitable methods to (i) obtain viable EPL conceptus material and (ii) isolate and culture trophoblast cells from these conceptuses, has limited investigations to determine further causes. The objective of this study was to develop methods to isolate tissue and placental cells from failed early equine pregnancies to enable future investigations determining pathologies causing EPL.

2. Materials and methods

Material was collected during the 2013–2014 breeding seasons from pregnant thoroughbred mares under approval from the Royal Veterinary College Ethics and Welfare Committee (URN 2012/1169). For inclusion, mares had a positive day 14–16 pregnancy scan which showed ultrasonographic evidence of failure (no heartbeat, collapsing vesicle, anembryonic vesicle) at a subsequent routine evaluation. Conceptuses were collected by the attending veterinary surgeon using non-invasive sterile uterine lavage. In some instances the failed conceptus was flushed at the time of detection and in other cases prostaglandin F2a analogues (Estrumate, Merck Animal Health or Lutalyze, Zoetis) was administered and the conceptus flushed between 24 and 72 h later. Isolated tissue was placed into transport medium containing Hank's Balanced Salt Solution (Sigma, Poole, Dorset, UK), 5% HyClone™ Donor Serum (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK), 2.5 µg/ml Amphotericin B preparation (Sigma), Penicillin-Streptomycin, 10 units/ml penicillin and 100 µg/ml streptomycin (Gibco, Paisley, Renfrewshire, UK) and 100 µg/ml Kanamycin (Gibco). Conceptuses were then stored at 4 °C until transport on ice to the laboratory.

Abbreviations: EPL, early pregnancy loss; EqTM, equine trophoblast media; Amnio, amnioChrome™ Plus; Chang, Chang D ®; AR, androgen receptor; SRY, sex determining region Y.

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Endometrial swabs to detect for the presence of microorganisms were collected at the time of flush using amies charcoal swabs. Swabs were cultured on blood agar and MacConkey agar at Newmarket Equine Hospital Laboratory or Beaufort Cottage Laboratory (both Newmarket, Suffolk, UK).

Conceptus material was dissected under a dissecting microscope in a sterile petri dish containing PBS with 10 units/ml penicillin and 100 μg/ml streptomycin. Dependent on the developmental stage, 2–9 cm^2 allantochorion and chorion were dissected free of surrounding tissue. Tissue was finely chopped in a petri dish with 3 ml of equine trophoblast medium (EqTM) [9].The material was split evenly between three tubes and centrifuged at 250 RCF at 10 °C for 5 min. All media were removed and the remaining cell pellets resuspended at a concentration of 0.2 cm^2 tissue/ml medium in either (i) AmnioChrome™ Plus (Amnio) (Lonza, Slough, Berkshire, UK) supplemented with 2.5 μg/ml amphotericin B, (ii) Chang D ® (Chang) (Irvine Scientific, Newton, Co. Wicklow, Ireland) supplemented with 2.5 μg/ml amphotericin B and 100 μg/ml kanamycin sulphate or (iii) EqTM containing Dulbecco's Modified Eagle's Medium (Gibco), 10% HyClone Fetal Bovine Serum, 1% L-glutamine 100x concentrate (Gibco), 10 units/ml Penicillin-Streptomycin and 100 μg/ml streptomycin [9]. AmnioChrome™ Plus (Amnio) and Chang D ® (Chang) are commercial media developed for the primary culture of amniotic fluid and chorionic villi cells for the purpose of karyotyping. Both have previously been shown to be successful for this purpose [3]. Equine

Table 1
Details of failed conceptuses submitted to the laboratory in 2013 and 2014.

Mare id	Mare age (yrs)	Gestation day pathology detected	Gestation day pregnancy flushed	Embryo present	Placental vasculature Present	Exsanguination occurred	Gross appearance of conceptus	Endometrial swab result^ (taken at conceptus flush)	Successfully cultured	gDNA# Conc. ng/μl	gDNA 260/280
13TB01	16	40	43	No	Yes	Yes	Very friable tissue	No growth	Yes	73	2.11
13TB02	18	42	42	Yes-autolysed	Yes	Yes	Friable	Staphylococcus spp	No	30	2.09
13TB03	17	56	56	Yes	Yes	Yes	Extensive membranes	BHS	No	18	2.06
13TB04	5	25	25	No	Yes	No	Good vasculature	BHS	Yes	427	2.07
13TB05	20	46	46	Yes	Yes	No	External contamination Flocculent material in media	Staphylococcus aureus	Yes	323	2.07
13TB06	7	35	35	Remnants	Yes	No	Friable Fibrin deposits Contamination of transport media	No growth	Yes	177	2.06
13TB07	6	64	68	Yes	Yes	No	Small inflammatory plaques on chorion Fibrin deposits	No growth	Yes	12	1.99
13TB09	5	41	45	Remnants	Yes	No	Normal	No growth	Yes	39	1.98
13TB11	9	42	47	Yes	Yes	Yes	Some autolytic changes	No growth	Yes	60	1.57
13TB12	10	42	42	Yes	*	*	Contamination	No growth	CNA	61	2.03
13TB13	6	42	45	Yes	Yes	Yes	Pale membranes No fibrin deposits	No growth	Yes	513	2.05
13TB14	4	58	63	Yes	Yes	No	Enlarged skull	Not taken	No	786	1.97
13TB15	5	42	42	No	Yes	Yes	Friable membranes	Not taken	Yes	593	2.00
13TB16	14	65	67	Yes	Yes	*	Thickened membranes	Not taken	No	15	1.94
13TB17	8	43	47	Yes	*	*	Cell aggregates within membranes	Not taken	Yes	462	2.00
13TB18	15	42	47	No	Yes	No	No obvious tissue structures	No growth	CNA	45	2.04
13TB19	*	28	35	Yes	Yes	Yes	*	*	CNA	199 (C)	2.08
14TB02	19	42	42	Yes	Yes	Yes	Normal	No growth	Yes	119	2.08
14TB03	5	45	47	Yes	Yes	Yes	Slightly thickened/ opaque membranes	No growth	CNA	23	2.18
14TB04	13	30	31	No	Yes	No	Normal appearance	No growth	Yes	140	2.00
14TB05	18	28	32	No	Yes	Yes	Normal appearance	E.coli	No	12	1.96
14TB06	9	42	44	Yes	Yes	Yes	Normal appearance	Not taken	Yes	465	2.04
14TB07	*	14	26	No	No	N/A	Small inflammatory nodules in chorion	Not taken	No	141 (C)	2.1
14TB08	12	18	24	Yes	No	N/A	Normal appearance	No growth	CNA	¥ (C)	¥
14TB09	12	40	40	Yes	Yes	No	Slightly thickened membranes	No growth	Yes	1033	2.06
14TB10	13	41	44	No	Yes	Yes	Normal appearance	Enterobacter aerogenes	Yes	160	2.08
14TB11	7	41	46	Yes	Yes	Yes	Slight opacity in allantochorion	Not taken	Yes	621	2.07
14TB12	12	23	25	No	No	N/A	Multiple small inflammatory nodules	Not taken	Yes	107 (C)	2.19
14TB13	14	65	67	Yes	Yes	No	Normal appearance	Not taken	Yes	316	2.01
14TB14	15	33	55	No	No	N/A	Normal	No growth	CNA	89 (C)	1.83

* refers to data not available. N/A no vasculature detected so exsanguination not applicable. CNA = culture not attempted. ^ cultured aerobically and anaerobically for bacterial and fungal pathogens. BHS = beta haemolytic Streptococcus, #Allantochorion tissue. When this was not available, chorion (C) was used as indicated adjacent to values. ¥ insufficient tissue for gDNA isolation.

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