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.

Abbreviations:

EPL: Early pregnancy loss

EqTM: Equine trophoblast media

Amnio: AmnioChrome™ Plus

Chang: Chang D ®

AR: androgen receptor

SRY: sex determining region Y

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.

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 24-72 hours 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. 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 minutes. 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 trophoblast medium (EqTM) is a medium used in our laboratory for the routine culture of equine trophoblast cells derived from normal early pregnancies [10]. Cells were incubated at 37°C 8% CO_2 in a small volume of medium to promote adhesion to the culture vessel. Depending on the amount of tissue available, treatment with each medium was replicated by placing 3 mls in a T25 flask and 0.8 mls in 3 wells of a 6 well plate, one of which contained a circular glass coverslip. After 24-72 hr, an extra 2 mls of medium was added to the T25 flasks and an extra 0.5 mls added to each well. The time at which the top up media were added varied depending on the level of tissue adherence seen. Media were subsequently changed every 3-4 days as required. Supplementary methods provide additional details on the freezing and thawing of the cells.

Coverslips were removed when cells reached confluency and fixed in ice cold acetone for 10 minutes and stored at -20° C. Immunohistochemistry was performed as previously described [9] using an equine anti-trophoblast (102.1) [11] monoclonal antibody that has been shown to label all equine trophoblast tested [11], an anti-equine endoderm/mesoderm (102.5) monoclonal antibody as a isotype control [11] and a goat anti mouse Ig peroxidase conjugate (Invitrogen, Paisley, UK) as a secondary antibody. Four randomly selected images were collected for each labelling reaction using a Leica DM4000B microscope and Leica DC500 camera (Leica, Milton Keyne, UK). The number of cells labelled with 102.1 and 102.5 was counted using Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015).

Cell number and viability were measured from 1 well of each medium using trypan blue exclusion after 10 days of culture. Medium was removed and 1ml of PBS used to wash residual medium. Cells were trypsinised by adding 300 µl of trypsin (Gibco) and incubating at 37°C for 5 minutes. Trypsin was inactivated using 300 µl of the appropriate medium (pre heated to 37°C). Cells were stained with 0.4% trypan blue by mixing equal volumes of cells and trypan blue. Cells were then

added to the microcytometer and the number of viable cells counted. Two repeat counts were performed and a mean taken.

At dissection, allantochorion, chorion, chorionic girdle and fetal hindlimbs were snap frozen in liquid nitrogen before storing at -80°C. Genomic DNA was extracted using Qiagen DNeasy Blood and Tissue Kit as described by the manufacturer and quality assessed using a NanoDrop spectrophotometer. As controls, genomic DNA was also isolated from PBMC isolated as previously described [12] from a gelding and mare. PCR was performed on gDNA using sex specific primers for sex determining region Y (SRY) (Y chromosome) and androgen receptor (AR) (X chromosome) as previously described [13]. See supplementary methods for primer sequences.

The effect of variable factors on culture success was assessed using Mann-Whitney U and Fisher's exact tests. Media types were compared using Friedman and Wilcoxon tests. Statistical analyses were performed in GraphPad Prism 6.0 and significance level set at $P<0.05$.

Results and Discussion

Conceptus material was received from 30 failed pregnancies of gestational age 14 - 65 days at time of pathology detection. Clinical parameters of the pregnancies are described in Table 1. A definitive diagnosis of the cause of the loss was not achieved in any of the clinical cases included in this study. No complications were observed secondary to uterine lavage.

Culture was not attempted for 6/30 pregnancies due to limited or contaminated material or time constraints. Allantochorion and chorion cells were successfully grown successfully in 75% (18/24) of cultures attempted and in all three media (Fig. 1A) both immediately following isolation and after freezing and thawing cells. Therefore cells from 60% (18/30) of all failed pregnancies received in the laboratory were successfully cultured in at least one media type. These figures compare favourably to the 72.3% culture success rate of material from first trimester losses in women [3]. Gestational age, time from flushing to processing in the laboratory and vasculature exsanguination

did not influence culture success (Fig. 1B). The wide time frames allowing culture success make this a viable method for application in a clinical setting. Fifteen of the 30 mares in the study had both endometrial swabs collected at the time of the conceptus removal and trophoblast culture attempted. Cells were cultured successfully in 9/9 with a negative endometrial swab but in only 3/6 with a positive endometrial swab. Therefore, the presence of bacterial endometritis had a negative effect on ability to culture cells ($p=0.044$) (Fig. 1B). The high rate of culture success in the absence of endometrial infection make this a viable method to assist in diagnosing cause of loss when endometritis has been excluded.

Cells grown in Amnio showed a significant increase in both number of viable cells ($p=0.041$) and time to reach 100% confluency ($p=0.020$) compared to Chang or EqTM (Fig. 1C). Thus, Amnio is the preferred medium for genetic studies such as karyotyping which require rapidly proliferating cells to produce high quality metaphase spreads. However, proliferative factors that may be included in this medium may be detrimental to cell functionality and so EqTM medium may be better suited for studies that assess gene expression or cell function, providing a known, controlled environment [14]. Further, EqTM has been used successfully to culture and study the functionality of chorionic girdle trophoblast cells isolated from clinically normal early equine conceptuses [9, 10].

The majority of cells grown in all media had epithelial-like morphological characteristics consistent with trophoblast cells (Fig. 1D). Immunohistochemistry using an antibody against equine trophoblast confirmed 83.7 % cells (CI: 79.3-87.4%) in culture were trophoblast (n=5 conceptuses) (Fig. 1D). No cells labelled with the 102.5 isotype control.

Genomic DNA was extracted successfully from 29/30 conceptuses (Table 1) with higher total gDNA isolated from conceptuses that were also cultured successfully (Fig. 1E). PCR for sex determination of conceptuses using androgen receptor (AR) and sex determining region Y (SRY) primers showed this material to be suitable for sexing the conceptuses (Fig. 1E), and downstream applications such as SNP genotyping or sequencing.

Whilst the culture of placental cells isolated from healthy, terminated equine pregnancies has previously been described [9, 10, 15], to the best of the authors' knowledge, this is the first demonstration of a method to isolate and culture placental cells from failed early pregnancies in domestic species. This material will allow genetic characterisation and detection of further aetiologies associated with EPL in the horse and may be applied to a range of species to increase our understanding of this common and economically important condition.

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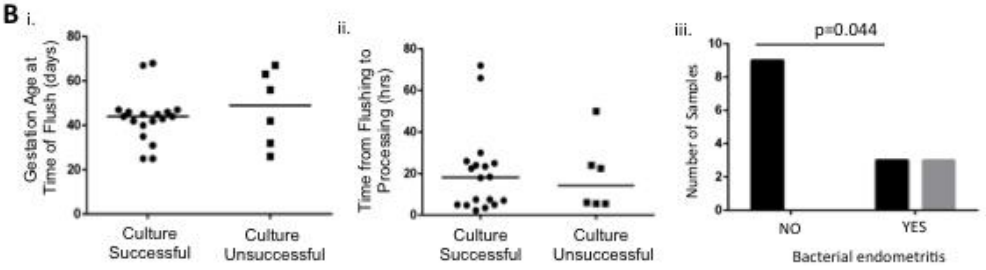
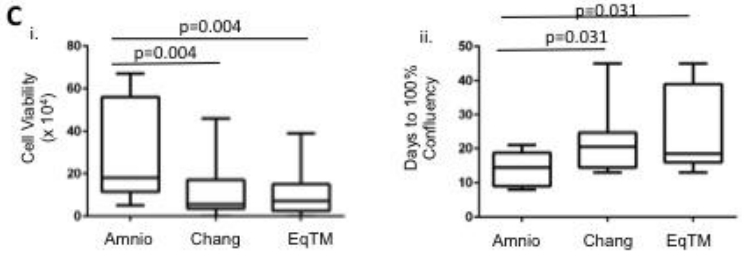
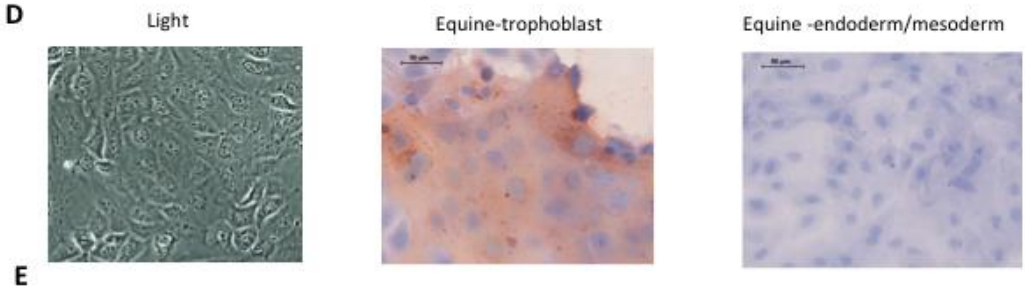
Figure 1: Rose, et al.

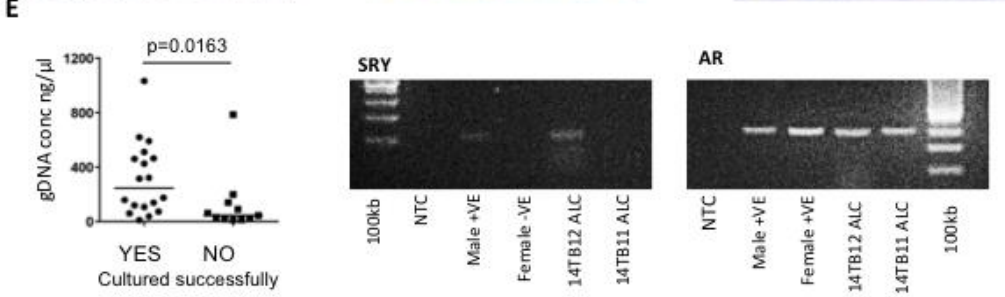
A

	Allantochorion	Chorion
Chang D® (+ amphoterecin and kanamycin)	16/21	4/10
AmnioChrome Plus ™ (+ amphoterecin)	16/21	5/10
Equine Trophoblast Medium	15/21	5/10









A. Proportions of allantochorion and chorion from which cells were successfully grown when put into culture with three different media.

B. Culture success rates compared to (i) gestational age of pregnancy at time of flush, (ii) time from sterile uterine flush to arrival in laboratory, (iii) presence of bacterial endometritis at the time of sterile uterine flush. Bars in i and ii represent median value; in iii black bar represents cells successfully cultured, grey bar is cells failed to grow.

C. Effect of media on (i) 10 day viability count and (ii) time to 100% confluency. Box and whisker plots represent median and 5–95 percentile.

D. (i) Cells from pregnancy 14TB09 at 100% confluency after 14 days growth in Chang D ® media. Cultured allantochorion cells (Amnio media) from conceptus 14b13 labelled with antibody against equine trophoblast (102.1) (ii) and an anti-equine endoderm/mesoderm antibody (102.5) as a control (iii)

E. Genomic DNA was extracted from allantochorion or chorion tissue (see Table 1) as well as peripheral blood mononuclear cells (PBMC) from a gelding or mare as previously described as male and female controls. Concentration of gDNA isolated from conceptuses in which cells were successfully cultured (YES) or not cultured (NO) (left panel). Sex determination of conceptuses using PCR and SRY (Y chromosome) (middle panel) and AR (X chromosome) (right panel) primers on allantochorion tissue from 14TB01 to 14TB02, male and female control gDNA and water only (NTC = no template control).

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

Mare ID	Mare Age (yrs)	Gestation day pathology detected	Gestation day pregnancy flushed	Embryo present	Placental vasculature present	Exsanguin-ation 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

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

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

Supplementary Materials and Methods:

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Freezing cells

Once cells had reached 80 % confluency, cells were harvested using 3 mls of trypsin and incubating at 37°C for 5 minutes. A cell scraper was then used to ensure all cells had lifted and 6 mls of the appropriate medium added to deactivate the trypsin. Cells were centrifuged at 250 G at 21°C for 5 minutes. The supernatant was removed and the cell pellet resuspended in freezing medium. This consisted of the appropriate medium type plus 20% fetal bovine serum (GE Healthcare) and 5% dimethyl sulfoxide (Sigma). The cell pellet was dislodged and then freezing medium (1 ml) was added to the appropriate cell pellet, the cells resuspended and placed in a 1.5 ml cryogenic vial. The vial was wrapped in paper towel to ensure cells cooled slowly and stored at -80°C overnight before transferring to liquid nitrogen for storage.

Sex Determination using PCR

Genomic DNA was isolated from snap frozen allantochorion or chorion (when allantochorion was not available) using a Qiagen DNeasy Blood and Tissue Kit following the manufacturers guidelines (Qiagen, UK). A polymerase chain reaction was performed on gDNA using sex specific primers for sex determining region Y (SRY) (Y chromosome) and androgen receptor (AR) (X chromosome) [13].

Primer Sequences:

SRY_F 5’ TGCATTCATGGTGTGGTCTC

SRY_R 5’ ATGGCAATTTTTCGGCTTC

AR_F 5’AGCAGCAACAGGAGACCAG

AR_R 5’ TGCTTAAGCCTGGGAAAGTG

Firepol ® 5x Master Mix Ready to Load (Solis BioDyne) was used to create the following PCR mix with a 10µl reaction volume:

- Firepol ® 5x Master Mix Ready to Load 1x
- Forward primer (10pmol/µl) 0.3 µM
- Backward primer (10pmol/µl) 0.3 µM
- Sample DNA 50 ng
- Nuclease free water

Water was used as a negative control. Genomic DNA was isolated from Peripheral Blood Mononuclear cells (PBMC) as previously described [13]. PBMC from a gelding acted as a positive control for both PCRs and gDNA isolated from PBMC from a mare acted as a negative control for SRY and positive control for AR PCR reactions.

The following thermal cycle was used: 95°C for 1 minute, 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, (last three steps for 30 cycles), 72°C for 10 minutes, Hold at 10°C

PCR products were loaded onto a 2 % agarose gel (Invitrogen) with a 100kb ladder (Invitrogen) and run for 40 minutes at 100 volts.