REPRODUCTION
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Consequences of conceptus exposure to colony-stimulating factor 2 on survival, elongation, interferon-τ secretion, and gene expression

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Abstract

Exposure of bovine conceptuses to colony-stimulating factor 2 (CSF2) from days 5 to 7 of development can increase the percentage of transferred conceptuses that develop to term. The purpose of this experiment was to understand the mechanism by which CSF2 increases embryonic and fetal survival. Conceptuses were produced *in vitro* in the presence or absence of 10 ng/ml CSF2 from days 5 to 7 after insemination, transferred into cows, and flushed from the uterus at day 15 of pregnancy. There was a tendency ($P=0.07$) for the proportion of cows with a recovered conceptus to be greater for those receiving a CSF2-treated conceptus (35% for control versus 66% for CSF2). Antiviral activity in uterine flushings, a measure of the amount of interferon-τ (IFNT2) secreted by the conceptus, tended to be greater for cows receiving CSF2-treated conceptuses than for cows receiving control conceptuses. This difference approached significance when only cows with detectable antiviral activity were considered ($P=0.07$). In addition, CSF2 increased mRNA for *IFNT2* ($P=0.08$) and keratin 18 ($P<0.05$) in extraembryonic membranes. Among a subset of filamentous conceptuses that were analyzed by microarray hybridization, there was no effect of CSF2 on gene expression in the embryonic disc or extraembryonic membranes. Results suggest that the increase in calving rate caused by CSF2 treatment involves, in part, more extensive development of extraembryonic membranes and capacity of the conceptus to secrete IFNT2 at day 15 of pregnancy.

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Introduction

Colony-stimulating factor 2 (CSF2) is an important regulator of embryonic development. In the cow, it improves the proportion of cultured conceptuses that develop to the blastocyst stage (de Moraes & Hansen 1997, Loureiro *et al.* 2009), increases the number of cells in the inner cell mass (Loureiro *et al.* 2009), and decreases the percentage of blastomeres undergoing apoptosis in response to heat shock (Loureiro *et al.* 2011). Moreover, addition of CSF2 to culture medium from days 5 to 7 after insemination improved the competence of *in vitro* produced conceptuses to establish pregnancy after transfer to a recipient female while also reducing the probability of fetal loss after days 30–35 of pregnancy (Loureiro *et al.* 2009).

The objective of this study was to evaluate possible mechanisms by which CSF2 acts during days 5–7 of development to improve embryonic and fetal survival. Conceptuses treated with or without CSF2 from days 5 to 7 after insemination were transferred to recipients and then flushed from the reproductive tract at day 15 of gestation. At this time in gestation, the conceptus is undergoing elongation of the trophoblast and secretes interferon-τ (IFNT2) to block uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release and prevent luteolysis (Robinson *et al.* 2008). Failure of the embryo to elongate and secrete sufficient IFNT2 results in failure of the embryo to block uterine release of $PGF_{2\alpha}$. One hypothesis tested in the current experiment was that CSF2 would enhance conceptus elongation and increase the secretion of IFNT2. Indeed, CSF2 has been shown to stimulate the production of IFNT2 by sheep trophoblast (Imakawa *et al.* 1993, 1997, Rooke *et al.* 2005) and a bovine trophoblast cell line (CT1; Michael *et al.* 2006). It was also tested whether CSF2 would affect the survival of the embryonic disc because a large proportion (~25%) of

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in vitro produced conceptuses that survive to days 14–15 of gestation lose the embryonic disc (Fischer-Brown *et al.* 2004, Block *et al.* 2007). Finally, the gene expression of elongated conceptuses was evaluated to determine whether the increased ability of CSF2-treated embryos to avoid loss after days 30–35 is associated with altered expression of genes in the embryonic disc or extraembryonic membranes at day 15 of pregnancy.

Results

Conceptus survival after transfer

Results are presented in Table 1. A total of 80% of cows receiving a control conceptus and 93% of cows receiving a CS2F-treated conceptus had a corpus luteum (CL) detected by ultrasound. For the other cows, the CL was either absent or small and regressing. Cows without a CL represent those that were not successfully synchronized, so that the presumed day 15 was actually later in the estrous cycle, or cows in which the CL had undergone luteolysis by day 15. When all cows were considered, including those without a large CL, there was a tendency ($P=0.07$) for the proportion of cows with a recovered conceptus to be greater for those receiving a CSF2-treated conceptus (35% for control versus 66% for CSF2). The same trend was apparent when only cows with a CL were considered (44% for control versus 71% for CSF2) but the difference did not approach significance.

One cow in the control group did not have a detectable conceptus but there was abundant antiviral activity in uterine flushings (59 049 IU IFN/ml). This cow either lost its pregnancy after the conceptus initiated large-scale IFNT2 secretion or the conceptus was not recovered in the flushing. When this cow was considered pregnant, differences in survival between control and CSF2 groups remained but did not approach significance (Table 1).

Table 1 Estimates of effect of colony-stimulating factor 2 (CSF2) on embryonic survival at day 15 after expected ovulation.

	Control	CSF2	*P* value
Number of cows receiving conceptuses	20	15	
Number of cows (percentage) with CL on day 15	16/20 (80%)	14/15 (93%)	NS
Number (percentage) of cows with a recovered conceptus, of all cows	7/20 (35%)	10/15 (66%)	0.07
Number (percentage) of cows with a recovered conceptus, of cows with a CL	7/16 (44%)	10/14 (71%)	NS
Number (percentage) pregnant[a], of all cows	8/20 (40%)	10/15 (67%)	NS
Number (percentage) pregnant[a], of cows with a CL	8/16 (50%)	10/14 (71%)	NS

[a]Cows were considered pregnant if there was a detectable embryo or antiviral activity in the flushing.

Embryonic growth and development

Five of seven (71%) control conceptuses and eight of ten (80%) CSF2-treated conceptuses were filamentous. The embryonic disc was visible in six of seven (83%) control conceptuses and six of ten (60%) CSF2-treated conceptuses. Differences between treatments in these two variables were not significant.

As illustrated in Fig. 1, there was great variability in conceptus length, even if the dataset was restricted to conceptuses that were filamentous. Although there was no significant effect of treatment, CSF2-treated conceptuses tended to be longer than control conceptuses regardless of whether all conceptuses were considered, only conceptuses that were recovered intact were considered (four conceptuses were recovered in pieces and were likely to be larger than measured), or only filamentous conceptuses were considered. Overall, the average length was 39 mm for control conceptuses and 62 mm for CSF2-treated conceptuses.

Antiviral activity in uterine flushings

Antiviral activity was also highly variable, ranging from non-detectable amounts (one cow in each group) to almost 9 000 000 IU IFN/ml (Fig. 1). The correlation between embryo length (for intact conceptuses) and antiviral activity was low ($r=0.002$) For all cows, there was a non-significant trend for antiviral activity to be greater for cows receiving CSF2-treated conceptuses than for cows receiving control conceptuses. This difference was $P=0.07$ when only cows with detectable antiviral activity were considered.

Expression of IFNT2, KRT18, *and* PAG2 *in the extraembryonic membranes*

The expression of *IFNT2*, keratin 18 (*KRT18*), and pregnancy associated glycoprotein 2 (*PAG2*) in the extraembryonic membranes of all recovered conceptuses was analyzed by quantitative PCR (qPCR; Fig. 2). *IFNT2* was 22.4-fold greater ($P=0.08$) and *KRT18* was 297.4-fold higher ($P<0.05$) in the extraembryonic membranes of conceptuses treated with CSF2 when compared with the controls.

Across treatment, the correlation between *IFNT2* mRNA ($\Delta\Delta C_T$) and antiviral activity in uterine flushings was -0.61 ($P<0.02$). Among intact conceptuses, the correlation between *IFNT2* mRNA ($\Delta\Delta C_T$) and embryo length was -0.62 ($P<0.04$) and the correlation between *KRT18* mRNA ($\Delta\Delta C_T$) and embryo length was -0.58 ($P=0.06$).

There was no difference in the expression of *PAG2* between extraembryonic membranes from control and CSF2 conceptuses (0.7-fold).

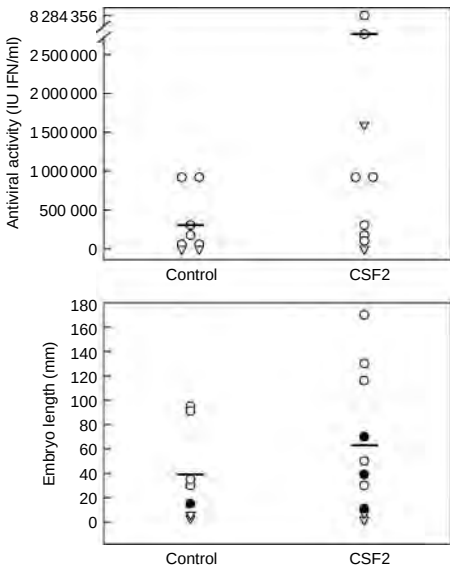

Figure 1 Individual values of antiviral activity in uterine flushings (top) in pregnant cows and length of recovered conceptuses (bottom). Triangles represent tubular conceptuses and circles represent filamentous conceptuses. In the bottom panel, conceptuses that were not recovered intact are represented by closed circles. The horizontal bars represent the mean value for each treatment. Antiviral activity was not affected by treatment. However, when considering only those cows in which detectable antiviral activity was present, there was a tendency for antiviral activity to be greater for cows receiving CSF2-treated conceptuses ($P=0.07$). Conceptus length was not significantly affected by treatment.

Changes in the transcriptome of embryonic disc and extraembryonic membranes

To test the hypothesis that CSF2 alters the transcriptome of the filamentous conceptus, gene expression was evaluated by microarray hybridization in two tissues – embryonic disc (which also contains adjacent extraembryonic membranes) and extraembryonic membranes. All 43 803 probes on the array produced signal above background. These probes represent 19 500 distinct genes or 88% of the bovine genome (Bτ 4.0). Hierarchical analysis indicated that the eight embryonic disc samples formed a distinct cluster separated from the extraembryonic membranes. There was, however, no such distinction between samples from control and CSF2-treated conceptuses. Using a false discovery rate (FDR)$\leq$0.01 and a twofold difference as criteria, there were no genes affected by treatment or the treatment $\times$tissue-type interaction. When a more liberal threshold was used (FDR$\leq$0.05), only a single gene was found to be differentially expressed between the control and the CSF2-treated embryos. This was a non-annotated gene (Genbank EE242708.1) that was 2.1-fold lower in CSF2-treated embryos.

There was, however, a total of 627 genes that were differentially expressed between embryonic disc and extraembryonic membranes and 576 of these could be annotated. A list of the differentially expressed genes is provided in Supplementary File 1, see section on supplementary data given at the end of this article. Of the annotated genes, 538 genes were upregulated in embryonic disc and 38 genes were upregulated in extraembryonic membranes. One would expect a preponderance of differentially regulated genes that were upregulated in the embryonic disc, and not in the extraembryonic membranes, because the former tissue contains some extraembryonic membranes, whereas the extraembryonic membranes sample was free of embryonic disc tissue. Ingenuity software was used to identify pathways that were significantly overrepresented in the set of genes that were differentially expressed between embryonic disc and extraembryonic membranes. A total of 31 canonical pathways were identified as above the threshold ($P<0.05$; Supplementary Table 1, see section on supplementary data given at the end of this article) with the highest score being for embryonic stem cell pluripotency.

It is likely that many of the genes that are differentially expressed between embryonic disc and extraembryonic membranes will prove useful in future studies for identifying embryonic disc and extraembryonic membranes. Two different criteria were used to identify some of the 576 differentially expressed genes that might be particularly useful as markers. The first criterion was to identify genes with the highest fold change between embryonic disc and extraembryonic membranes. The 15 genes with the highest fold difference for genes overexpressed in embryonic disc and the 15 genes with the highest fold difference for genes overexpressed in extraembryonic membranes are shown in Supplementary Table 2, see section on supplementary data given at the end of this article. The gene that had

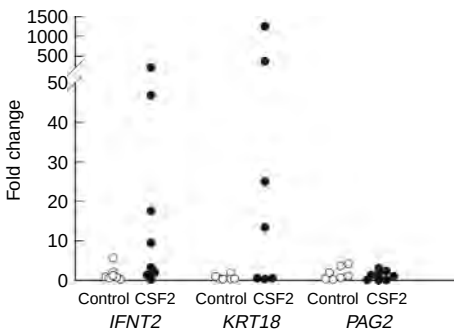

Figure 2 Fold change by quantitative PCR of *IFNT2*, *KRT18* and *PAG2* in extraembryonic membranes of control and CSF2-treated day 15 conceptuses. *IFNT2* was 22.4-fold higher ($P=0.08$) and *KRT18* was 297.4-fold higher ($P<0.05$) in the extraembryonic membranes of conceptuses treated with CSF2 when compared with the controls. There was no difference in the expression of *PAG2* between extraembryonic membranes from control and CSF2 conceptuses (0.7-fold).

the highest fold-increase in embryonic disk was fatty acid binding protein 1. The second approach to identify candidate gene markers was to identify the differentially expressed genes that were the most abundant. The rationale was that abundant genes, or their protein products, would be more easy to identify using a variety of molecular and immunochemical procedures. A list of the 15 most abundant genes for embryonic disc and extraembryonic membranes, based on signal intensity on the microarray, is presented in Supplementary Table 3, see section on supplementary data given at the end of this article. The most abundant mRNA for the genes overexpressed in the embryonic disc were stathmin 1, tubulin, α 1a, and follistatin.

Validation of microarray results using qPCR

A total of 11 genes that were differentially expressed between embryonic disc and extraembryonic membranes based on microarray analysis, were subjected to the analysis by qPCR. For all of the 11 genes, the fold change for embryonic disc relative to extraembryonic membranes was in the same direction for qPCR as the fold change as determined by microarray hybridization. Differences between embryonic disc and extraembryonic membranes were significant for ten of the 11 genes (Supplementary Table 4, see section on supplementary data given at the end of this article). There was no effect of treatment (control versus CSF2) or treatment×tissue interaction on mRNA abundance as determined by qPCR.

Discussion

Exposure of bovine conceptuses to CSF2 from days 5 to 7 of development has profound effects on the subsequent developmental fate of the conceptus after transfer. In particular, a greater percentage of conceptuses result in pregnancies at days 30–35 of pregnancy and fewer of the pregnancies established at that point are lost thereafter (Loureiro *et al*. 2009). The purpose of this experiment was to understand the mechanism by which CSF2 increases conceptus survival as measured at days 30–35 and reduces fetal mortality thereafter. Results suggest that higher pregnancy rates at days 30–35 represent, in part, a greater capacity of the conceptus to elongate and secrete IFNT2 at day 15. Analysis of gene expression in filamentous conceptuses indicates little difference in transcription among this subset of conceptuses that survived to day 15 and elongated successfully. Therefore, the reduction in embryonic and fetal loss after days 30–35 caused by CSF2 is probably not a direct reflection of altered gene expression at day 15.

Several lines of evidence support the idea that CSF2 increased the capacity of the conceptus to elongate and produce IFNT2. Conceptus length and antiviral activity in the uterus (a measure of IFNT2 bioactivity; Alexenko *et al*.1997) were highly variable, as is typical at this stage of pregnancy (Short *et al*. 1991, Mann & Lamming 2001, Block *et al*. 2007), but the largest conceptuses were in the CSF2 group. Antiviral activity tended to be greater for cows receiving CSF2 conceptuses and *IFNT2* gene expression was 22 times greater for CSF2 conceptuses. In addition, expression of *KRT18*, a marker of trophoblast differentiation (Winger *et al*. 2007), was highly upregulated in conceptuses treated with CSF2. The fact that there was no effect of CSF2 on *IFNT2* or *KRT18* expression among filamentous embryos as determined by microarray analysis and that mRNA abundance for these genes was correlated with conceptus length implies that the treatment effect on these genes is related to effect of CSF2 on conceptus elongation.

Failure of the conceptus to block luteolysis is one cause of pregnancy loss because administration of a PG synthesis inhibitor increased pregnancy rate in heifers (Guzeloglu *et al*. 2007). The larger size and greater IFNT2 secretory activity of the CSF2-treated conceptus should improve its ability to prevent luteolysis and allow continuation of pregnancy. Secretion of $PGF_{2\alpha}$ was not attenuated in pregnant cows at day 16 of gestation if embryos were not elongated and IFNT2 in uterine flushing was non-detectable (Mann & Lamming 2001).

The mechanism by which CSF2 treatment from days 5 to 7 results in greater conceptus growth and IFNT2 secretion at day 15 is not known. Treatment with CSF2 can increase expression of *IFNT2* in sheep trophoblast (Imakawa *et al*. 1993, 1997, Rooke *et al*. 2005) and bovine trophoblast cells *in vitro* (Michael *et al*. 2006). In addition, conceptuses treated with CSF2 at day 5 had altered expression of genes involved in developmental processes at day 6 characterized by an increase in expression of genes involved in mesoderm, mesenchyme and muscle formation and a decrease in genes involved in neurogenesis (Loureiro *et al*. 2011). Perhaps, effects of CSF2 on differentiation at the blastocyst stage lead to increased growth of trophoblast between days 7 and 15 of development.

In contrast to the effects of CSF2 on *IFNT2* and *KRT18* expression, there was no effect of CSF2 on expression of another trophoblast gene, *PAG2*, which is the most abundant member of a large family of inactive aspartinly proteinases expressed by the trophoblast (Telugu *et al*. 2009). It is likely that the regulation of this gene is distinct from that of *IFNT2* and *KRT18*.

A relatively large number of conceptuses at days 14–15 do not have a detectable embryonic disc (Fischer-Brown *et al*. 2004, Block *et al*. 2007). Such conceptuses eventually die (Fischer-Brown *et al*. 2004). Despite its effects on inner cell mass number (Loureiro *et al*. 2009), however, CSF2 did not affect the incidence

of conceptuses without a disc. Numerically, the percentage of conceptuses without a disc was reduced in the CSF2 group. It can be concluded from these observations that CSF2 is not improving conceptus survival by affecting survival of the embryonic disc.

There was some evidence, though non-significant, that CSF2 improved conceptus survival between days 7 and 15 of gestation. In particular, the proportion of transferred conceptuses that were recovered at day 15 was twice as high for cows receiving CSF2 conceptuses. Numbers of animals in the experiment were low and additional experiments involving larger number of animals are warranted to verify effects of CSF2 on survival between days 7 and 15.

Earlier, it was observed that CSF2 treatment reduced the proportion of pregnancies lost after days 30–35 of gestation (Loureiro *et al.* 2009). In the present experiment, there was no effect of CSF2 on the expression of genes in either the embryonic disc or the extraembryonic membranes of filamentous conceptuses at day 15. Thus, CSF2 has no discernable effect on gene expression at day 15 for the subset of conceptuses that has developed normally up to that point. It is unlikely, therefore, that the reduction in pregnancy fetal loss caused by CSF2 later in gestation is the result of global changes in transcription at day 15. Instead, perhaps, altered trajectory of development caused by CSF2, as characterized by changes in expression of developmentally important genes at day 6 (Loureiro *et al.* 2011), can lead to formation of a fetus that is less likely to experience a developmental defect leading to pregnancy loss after day 35. It is also possible that CSF2 causes epigenetic changes in the developing conceptus that result in altered gene expression at time points later than studied here.

One benefit of the transcriptomal analysis was that a large number of genes that are overexpressed in embryonic disc were identified. The most highly expressed of these genes (measured as fold change or absolute magnitude) represent good candidates for markers of the embryonic disc. These genes, as well as genes reported to be differentially expressed between embryonic disc and extraembryonic membranes in the cow by Degrelle *et al.* (2005) and in the pig by Isom *et al.* (2010), can be used to characterize developmental status and function of the embryo during the period of elongation and attachment.

In conclusion, results support the idea that the increased survival of conceptuses exposed to CSF2 from days 5 to 7 of development is the result, in part, of a greater capacity of the conceptus to elongate and secrete IFNT2 at day 15. The reduction in embryonic and fetal loss after days 30–35 caused by CSF2 is probably not a direct reflection of altered gene expression at day 15.

Materials and Methods

Production of conceptuses

In vitro production was performed as described earlier (Loureiro *et al.* 2009) with modifications described below. Cumulus–oocyte complexes from ovaries from a mixture of beef and dairy cattle were collected in tissue culture medium-199 (TCM-199) with Hank's salts without phenol red (Hyclone, Logan, UT, USA) supplemented with 2% (v/v) bovine steer serum containing 2 U/ml heparin (Pel-Freez, Rogers, AR, USA), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocytes were allowed to mature for 20–22 h in groups of ten in 50 μl microdrops of TCM-199 (Invitrogen) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/ml estradiol-17β, 20 μg/ml bovine FSH (Folltropin-V, Belleville, Ontario, Canada), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate, and 1 mM glutamine. Matured oocytes were then washed in HEPES–TALP (Parrish *et al.* 1986; Caisson, Sugar City, ID, USA) and transferred in groups of 50 to four-well plates containing 600 μl of IVF-TALP supplemented with 25 μl PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% (w/v) NaCl), and fertilized with 30 μl Percoll-purified spermatozoa ($\sim 1 \times 10^6$ sperm cells). Sperm were prepared from a pool of frozen–thawed semen from three different bulls; a different set of bulls was used for each replicate. Fertilization proceeded for 18–20 h at 38.5 °C in a humidified atmosphere of 5% (v/v) CO_2 in humidified air. Putative zygotes were removed from fertilization plates, denuded of cumulus cells by vortexing in HEPES–TALP, and placed in groups of 30 in 45-μl microdrops of KSOM-BE2 (Soto *et al.* 2003).

Conceptuses were cultured at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 (v/v). Cleavage rate was assessed at day 3 after insemination. At day 5 after insemination, 5 μl of KSOM-BE2 or 5 μl of KSOM-BE2 containing 100 ng/ml recombinant bovine CSF2 (a gift from Novartis) were added to each drop to achieve a final CSF2 concentration of 0 or 10 ng/ml.

Transfer into recipients

Morula, blastocyst, and expanded blastocyst stage conceptuses (six, nine and five in the control group and five, five and five in the CSF2 group) classified as Grade 1 (Robertson & Nelson 1998) were harvested on day 7 after insemination and loaded into 0.25 ml straws in 250 μl HEPES–TALP supplemented with 10% (v/v) FCS and 50 μM dithiothreitol (Sigma–Aldrich). Straws containing selected conceptuses were then placed horizontally into a portable incubator (Cryologic, Mulgrave, Victoria, Australia) at 38.5 °C and transported to the farm.

Totally, 35 multiparous lactating Holstein cows were used as recipients. Cows were housed in a free-stall barn equipped with fans and a sprinkler system at a commercial dairy in Bell, FL, USA (29.75578°N, 82.86188°W). A total of four replicates were completed between June and October 2009 with 5–11 recipients per replicate. For each replicate, eligible cows were synchronized for embryo transfer as described previously (Loureiro *et al.* 2009). Day 0 was considered the day of expected ovulation. Hormone treatments consisted of 100 μg

GNRH (Merial, Duluth, GA, USA) i.m. on day −10; 25 mg $PGF_{2\alpha}$ (Pfizer, New York, NY, USA), i.m. on day −3; and 100 μg GNRH i.m. 56 h after $PGF_{2\alpha}$. Cows were diagnosed for the presence of a CL at day 7 after anticipated ovulation using an Aloka 500 ultrasound equipped with a 5 MHz linear array transducer. Cows diagnosed with a CL were given epidural anesthesia (5 ml of 2% (w/v) lidocaine) and a single conceptus transferred to the uterine horn ipsilateral to the ovary via the transcervical route.

Conceptus recovery and evaluation

At day 15 after expected ovulation, the ovary ipsilateral to the uterine horn that received the conceptus was examined using ultrasonography to confirm the presence of the CL. Cows that did not have a visible CL were not flushed. Conceptuses were recovered transcervically with a 20 French Foley catheter inserted with a stainless steel stylet and held in position by inflating the cuff at the end of the catheter. The uterine horn ipsilateral to the CL was flushed with Dulbecco's PBS (DPBS; Sigma–Aldrich) with 1% (v/v) polyvinyl alcohol (PVA). Flushing involved multiple injections and recovery of 60 ml DPBS using a 60 cc syringe attached to the Foley catheter. The procedure was continued until either a conceptus was identified in the flush or a 180–240 ml of DPBS–PVA had been flushed. The flushings recovered from the first 60 ml flush were centrifuged at 1000 ***g*** for 10 min and stored at −20 °C for analysis of antiviral activity.

Following recovery, each conceptus was assessed for length, stage, and the presence or absence of an embryonic disc by light microscopy using a stereomicroscope. Stage of development was classified based on shape as ovoid, tubular, or filamentous. After all measurements were recorded, conceptuses were washed once in DPBS–PVA and the conceptus was dissected to produce a piece of tissue containing the embryonic disc and some nearby extraembryonic membranes (termed here as embryonic disc) and two pieces of extraembryonic membranes that were on either end of the embryonic disc (Fig. 3). Tissues were immediately snap frozen separately in liquid nitrogen.

Antiviral assay

The quantity of biologically active IFNT2 in uterine flushings was determined indirectly using an antiviral assay based on the inhibition of vesicular stomatitis virus-induced lysis of Madin–Darby bovine kidney cells. The assay was performed as described by Michael *et al*. (2006). All samples (from cows with and without a conceptus) were analyzed in duplicate. Antiviral units were converted to IFN (IU/ml) using a standard curve with known amounts of human IFN-α standard included in the assay (EMD Biosciences, San Diego, CA, USA).

Microarray hybridization

Microarray analysis was performed using a subset of eight samples of embryonic disc and eight samples of extraembryonic membranes from filamentous conceptuses (four per treatment). Tissues were disrupted by vortexing for 30 s and passing through a 20 gauge needle. Samples were homogenized using a QIAshredder (Qiagen, Inc.). Total cellular RNA was extracted with the RNeasy Plus Micro kit (Qiagen, Inc.) following manufacturer's instructions. Concentration of the input RNA was determined by Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA integrity was determined by use of the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples that showed high RNA integrity (RIN > 7) were used for the microarray hybridization and qPCR analysis. Extracted RNA was stored at −80 °C until microarray analysis.

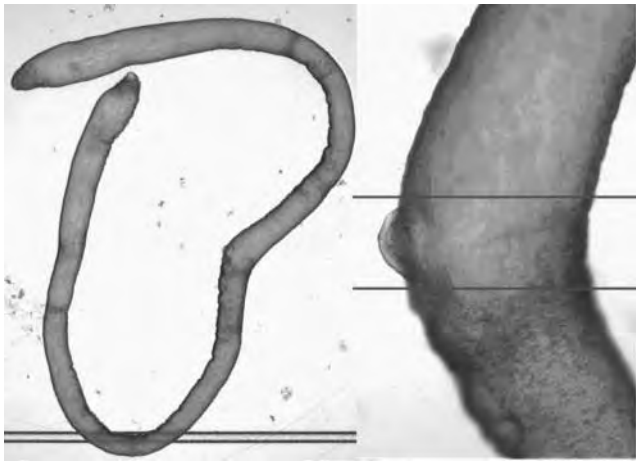

Figure 3 Separation of a day 15 conceptus into embryonic disc and extraembryonic membranes. The left panel shows an entire day 15 elongated conceptus and the right panel shows an enlargement of the same conceptus to visualize the embryonic disc. The conceptus was bisected along the lines.

Procedures were performed by the University of Florida Gene Expression Division of the Interdisciplinary Center for Biotechnology Research. Labeled amplified RNA was generated using Amino Allyl MessageAmp II Amplification kit (Ambion, Inc., Austin, TX, USA). First strand cDNA was synthesized from 425 ng total RNA. Half of the first strand cDNA was used to generate labeled aRNA according to the manufacturer's protocol and the remainder was reserved for qPCR verification. The microarray analysis was performed using the *Bos taurus* Two Color Microarray Chip from Agilent v 2 (Agilent Technologies). The 16 samples were distributed between the two microarray chips and the two dyes to avoid location biases. A total of 825 ng labeled RNA per sample was used for the hybridization. Hybridization and washing were performed according to the manufacturer's protocol using the Gene Expression Hybridization kit and Gene Expression Wash Buffers (Agilent). Arrays were scanned using a dual-laser DNA microarray scanner (Model G2505C, Agilent).

Analysis of microarray data

Microarray images were first analyzed with Agilent Feature Extraction Software v10.1 (Agilent Technologies, Inc.). Spot signal intensities were adjusted by subtracting local background and normalizing using within-array lowess approach for dye-bias correction. The quantile approach was then used

for between-array normalization. Statistical tests were performed using BioConductor statistical software (http://www.bioconductor.org/), which is an open source and open development software project for analysis of microarray and other high-throughput data primarily based on the R programming language (Gentleman *et al*. 2004). Expression values were deposited in the Gene Expression Omnibus repository as series number GSE26842 (www.ncbi.nlm.nih.gov/geo/).

Differentially expressed genes were identified using Limma, a software package that implements linear models for microarray data (Smyth *et al*. 2005). In Limma, *P* values are obtained from moderated *t*-statistics or *F*-statistics using empirical Bayesian methods. The primary analysis comparing differential expression was a 2×2 factorial design with tissue (embryonic disc versus extraembryonic membranes) and treatment (control, CSF2) as main effects and the tissue×treatment interaction as another effect. Pairwise comparisons of interest were also performed to test for significant differential expression. The Benjamini–Hochberg procedure (Benjamini & Hochberg 1995) was used to control FDR at the 0.01 level. Genes meeting this statistical threshold and showing a fold change equal or greater than 2 were considered as differentially expressed.

Ingenuity pathway analysis software (Ingenuity Systems, Redwood City, CA, USA) was used to identify canonical pathways associated with differentially expressed genes. Fisher's exact test was used to determine the probability that the association between the genes and the pathway was explained by chance alone.

Confirmation of microarray results with quantitative real-time PCR

Real-time qPCR analysis of 11 differentially-expressed genes and one housekeeping gene (*GAPDH*) was performed to confirm microarray results. In addition, qPCR was performed in the extraembryonic membranes of all conceptuses recovered for *IFNT2*, *KRT18*, and *PAG2*. Specific primers (Supplementary Table 5, see section on supplementary data given at the end of this article) were obtained from the literature (*IFNT2*; Cooke *et al*. 2009) or were designed using Integrated DNA technologies software (http://idtdna.com). The SsoFast EvaGreen Supermix (Bio-Rad Laboratories) reaction chemistry and a CFX 96 Real Time PCR Detection System (Bio-Rad) were used to quantify mRNA concentrations. After an initial activation step (95 °C for 30 s), 45 cycles of a two-step amplification protocol (95 °C for 5 s; 60 °C for 5 s) were completed.

The amplification of a single product was verified by performing dissociation curve analysis (65–95 °C) in the thermocycler. In additional, agarose gel electrophoresis of the PCR product was performed. Identity of the amplicon was confirmed by sequencing the PCR product of the band product. The DNA was extracted from the gel fragment using the PureLink Quick gel Extraction and PCR Purification Combo kit (Invitrogen). First, the gel with the DNA fragment was dissolved using solubilization buffer for 15 min at 50 °C. The gel mixture was centrifuged for 1 min at 10 000 ***g*** in a PureLink clean up spin column. After a wash, the DNA was extracted from the tube membrane by centrifuging the column with elution buffer for 1 min at 10 000 ***g***. The purified DNA was sequenced by the University of Florida Genomics Division of the Interdisciplinary Center for Biotechnology Research.

Each sample was analyzed in duplicate reactions. All C_T values for genes of interest were normalized to the housekeeping gene *GAPDH* using the ΔC_T method. The $\Delta\Delta C_T$ for each sample was calculated by subtracting the average ΔC_T of the gene of interest for control conceptuses from the value for each individual conceptus. Fold change was determined by solving for $2^{-\Delta\Delta C_T}$.

Statistical analysis

Categorical data were analyzed by logistic regression using the LOGISTIC procedure of the statistical analysis system (SAS 9.1.3, SAS Institute, Cary, NC, USA) with backward selection ($P=0.2$). The statistical model included replicate, stage of the conceptus at the time of transfer, and treatment. Effects on continuous variables were analyzed by least squares ANOVA using the general linear models procedure of SAS. Various mathematical models were utilized. All included treatment, replicate, stage of the conceptus at the time of transfer, stage of the conceptus at day 15 and, for conceptus length, whether the conceptus was intact. Data for conceptus length and antiviral activity were subjected to log transformation before analysis to account for heterogeneity of variance. Data on transcript abundance determined by real-time PCR were analyzed by least squares ANOVA. Data analyzed were the $\Delta\Delta C_T$ values but results are presented as fold difference relative to average values for control embryos.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-10-0511.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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