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Effects of serum addition to culture medium on gene expression in day-7 and day-14 bovine embryos

Jaime Manuel Angulo Campos

Louisiana State University and Agricultural and Mechanical College

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EFFECTS OF SERUM ADDITION TO CULTURE MEDIUM ON GENE EXPRESSION IN DAY-7 AND DAY-14 BOVINE EMBRYOS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University
and Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

In

The Interdepartmental Program in
the School of Animal Sciences

by

Jaime Manuel Angulo Campos

M.V., Universidad Nacional Agraria, 2005

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ABSTRACT

The addition of serum to embryo culture media may alter gene expression and trigger development of Large Offspring Syndrome. The objectives of this study were to determine gene expression levels in embryos cultured in the absence or presence of 5% calf serum and compare these expression patterns to in vivo derived embryos (IVD), and to determine the effects of serum on the length of day-14 embryos. Abattoir derived oocytes were fertilized and cultured in mSOF_{aa}. At 72 hours post-insemination (hpi), embryos were randomly allocated into two treatments: mSOF_{aa} without and with 5% calf serum. Embryos were then cultured to 168 hpi and blastocyst rates were assessed. In experiment 1, blastocysts from each treatment were pooled and stored at -80°C. In experiment 2, blastocysts (n=5-10) from each treatment were transferred into synchronized recipients, and were recovered 7 days post-transfer. Embryos were photographed, measured, and immediately stored at -80°C. Isolation of mRNA, reverse transcription and quantitative PCR were performed to determine transcript abundance for COX6A, IFNT1a, PLAC8, IGF2R and GAPDH for each sample. In both experiments, blastocyst development rates were higher in embryos cultured with serum compared to the no-serum treatment (14.9 and 7.4% respectively, P<0.001). In experiment 1, no differences were found in the expression of COX6A, IFNT1a, IGF2R and PLAC8; however upregulated expression of IGF2R, COX6A and IFNT1a were observed in some samples in both IVP treatments. In experiment 2, lengths of elongated embryos from the serum and no-serum culture treatments differed from the IVD treatment. Mean expression levels for COX6A, IFNT1a, PLAC8 and IGF2R did not differ across treatment groups. However, in the serum treatment 3 of 11

embryos over-expressed IFNT1a, 4 of 11 over-expressed IGF2R and 2 of 11 over-expressed PLAC8, over-expression being defined as two standard deviations above the mean of the IVD treatment for each respective gene. While mean expression levels were not affected by culture with serum under these conditions, very high expression of IFNT1a, IGF2R and PLAC8 in experiment 2 and IGF2R and IFNT1a in experiment 1 was observed in some embryos cultured with serum, but not in embryos cultured without serum or in in vivo derived embryos.

CHAPTER I

INTRODUCTION

Large sized calves have been observed with different assisted reproductive technologies (ART) such as embryo transfer, somatic cell nuclear transfer (SCNT) and in vitro produced calves (Kruip and den Daas, 1997; van Wagtendonk-de Leeuw et al., 2000; Rooke et al., 2007). In general, large size calves is an undesirable trait, which increases the incidence of dystocia and costs of production. The observation of a syndrome known as large offspring syndrome (LOS), more recently referred to as abnormal offspring syndrome (Farin et al., 2010) has been observed in cows and sheep derived from ART that use in vitro culture.

Although, the most obvious sign of LOS is large sized calves, other characteristics have been documented such as: weaker calves, breathing difficulties, high stillbirth rates, high prenatal loss rates (first third of gestation), sudden perinatal death, increased dystocia incidence, congenital malformations, organomegaly, placental abnormalities and skeletal abnormalities.

There are different factors that may trigger LOS, such as high urea diets (McEvoy et al., 1997; Sinclair et al., 1998b), asynchronous embryos transfers (Sinclair et al., 1998a), high levels of exogenous progesterone (Kleemann et al., 1994), somatic cell nuclear transfer (Constant et al., 2006; Everts et al., 2008), co-culture of embryos with somatic cells (Sinclair et al., 1998b) and culture of embryos with serum (Sinclair et al., 1998b; Rooke et al., 2007). The mechanism by which each of these factors cause LOS is unknown. It is possible that the mechanism or mechanisms that cause LOS can vary according to the nutritional and environmental conditions to which embryos are

exposed. Substantiation of this theory can be found in the differential gene expression of SCNT, in vivo derived (IVD) and in vitro produced (IVP) embryos obtained in genomic microarray analysis, which showed that genes expressed by SCNT embryos and IVP embryos are different, even when both groups were subjected to the same culture conditions (Ushizawa et al., 2004; Smith et al., 2009). Although there are several factors that may trigger LOS, the studies presented herein focus on the effects of serum addition during in vitro culture of bovine embryos.

Embryo culture is a technique used in both SCNT and in vitro production of embryos. These techniques are in commercial high demand. Data from the international embryo transfer society (IETS) indicate that in 2006 approximately of 291,845 of IVP embryos were transferred (Thibier, 2007). Demand for IVP embryos, compared to IVD embryos, may increase in the future especially in emerging countries like Brazil, China and South Korea (Thibier, 2007). Serum is frequently added to culture media during in vitro production to increase blastocyst rates and stimulate faster blastulation; however, serum addition has been linked to LOS.

CHAPTER II

LITERATURE REVIEW

Introduction to Culture Media

Culture Media

Initially embryo culture was performed in traditional cell culture media, which were found to not be appropriate conditions for embryo development. The addition of serum to these media was the standard for cell culture as well as for co-culture of embryos with somatic cells, an example of these media is tissue culture media 199 (TCM-199). Later, other media such as Synthetic Oviduct Fluid (SOF), Charles Rosenkrans medium (CR1) and potassium simplex optimized medium (KSOM) were developed specifically for culture of mice, ovine and bovine embryos. The development of these media were based on the simplex optimization method (Lawitts and Biggers, 1991) or by experimental changes in media based on previous studies (Rosenkrans et al., 1991; Gardner et al., 1998, 1999). These methods basically consisted of changing the concentrations of certain components of the medium and observing embryo development under the altered conditions. More recently, embryo culture media can be classified as non-sequential and sequential media. Sequential media like G medium (Gardner, 1999) were developed with the premise that in vivo nutritional requirements change as embryos migrate from the oviduct to the uterine environment.

Currently, there are several culture media available with some variation between media or a particular medium can be modified according to the needs of the laboratory or researcher. A good example of this is the synthetic oviduct fluid medium, which has

been extensively modified compared to its original formulation (Tervit et al., 1972; Takahashi and First, 1992; Gardner et al., 1994; Holm et al., 1999; Sinclair et al., 1999; Gandhi et al., 2000). Generally, medium additives and components used are standardized and come from the same commercial sources. However, medium modifications can have either beneficial or detrimental effects on embryo development. For instance, phosphate inhibits early cleavage of preimplantation embryos (Schini and Bavister, 1988), glucose above 0.56 mM inhibits early cleavage (Warzych et al., 2007; Hasler, 2010) and sperm capacitation (Parrish et al., 1989), and sodium chloride content above 95 mM can be detrimental for embryo development due to hyperosmolarity of the medium. Variations between media formulations in some of the most used culture media are presented in Table 1.

Table 1. Composition of culture media used for IVP of bovine embryos (molar weight)

Comp.	¹ SOF _{aa}	² KSOM _{aa}	³ CR1 _{aa}	⁴ G1	⁴ G2
Na	126.15	130.2	143.8	126.15	121.3
Cl	119.26	100.9	117.08	99.18	99.18
K	8.35	2.85	3.1	5.5	5.5
PO ₄	1.19	0.35	-	0.25	0.25
Ca	1.71	1.7	5	1.8	1.8
HCO ₃	25.07	25	26.2	25	25
Mg	0.49	0.2	-	1	1
SO ₄	-	0.2	-	1	1
Pyruvate	0.33	0.2	0.4	0.32	0.1
Lactate	3.3	10	2.5	10.5	5.87
Glucose	1.5	0.2	-	0.5	3.15
Amino acids	yes	yes	yes	yes	yes
EDTA	-	0.01	-	0.01	No

¹(Tervit et al., 1972)

²(Lawitts and Biggers, 1991)

³(Rosenkrans Jr and First, 1991)

⁴(Lane et al., 2003)

Protein Sources

The sources of protein added to culture media are serum, bovine serum albumin (BSA), and essential and non-essential amino acids. These sources not only provide the amino acids necessary for embryo development, but also function as chelators, pH buffers, molecule carriers, and increase surface tension of culture media (Gardner, 1998, 2008; Hasler, 2010).

The amount and the time of inclusion of a protein source play an important role in stimulating embryo development. For instance, the addition of non-essential amino acids before the 8-cell stage stimulates cleavage, increases cell numbers and enhances early embryo development; but the addition of essential amino acids during this same stage does not have positive effects on early embryo development. Moreover, it has been reported that essential amino acids reduce embryo cell numbers (Gardner, 2008). In contrast, the addition of essential amino acids after the 8-cell stage stimulates the number of inner cell mass (ICM) cells (Steeves and Gardner, 1999; Gardner, 2008).

Besides containing amino acids and other protein sources, serum and BSA also provide usually unquantified growth factors and energy sources that may stimulate embryo development. Additionally, the use of these animal derived sources may increase the risk of disease transmission. Therefore, a recent tendency is to use little, if any animal-derived products. In order to solve this problem, replacements for serum and BSA, known as macromolecules, have been introduced in the market, which can be from natural and synthetic sources. Examples of macromolecules are polyvinyl alcohol, polyvinyl pyrrolidone, hyaluronic acid and other serum replacers (Wrenzycki et al., 1999; Yaseen et al., 2001; Moore et al., 2007b; Warzych et al., 2007)

Serum Sources

There are many kinds of sera available in the market today, which differ in processing and source. Generally, serum is heat inactivated, but can also be purified by charcoal treatment. It has been shown that type of serum, timing of serum addition and serum processing method have an impact in embryo development during in vitro culture (Pinyopummintr and Bavister, 1994; Thompson, 1997). The most commonly used sera for in vitro culture are calf serum, new-born calf serum and fetal calf serum. Some researchers have used estrous cow serum, steer serum and human serum. However, the presence of human serum during in vitro culture has been associated with several developmental abnormalities, such as larger blastocysts with more lipid droplets, large sized calves and high prenatal loss (Gardner et al., 1994; Thompson et al., 1995). Some studies that have showed that the addition of serum to in vitro culture media may increase the incidence of LOS (Rooke et al., 2007; Young et al., 1999). However, other studies found no difference between embryos produced in the absence or presence of serum. One factor that might be related to the tendency for abnormal development and may explain the variation between these studies, is the amount of ammonium in sera. It has been shown that supplementation of urea increase ammonium concentration in blood, which affects embryos development (McEvoy et al., 1997; Sinclair et al., 1998b).

Energy Sources

The most frequently used energy sources are sodium pyruvate, sodium lactate, glucose, fatty acids found in serum and bovine BSA fraction V. It has been demonstrated that pyruvate and lactate are the preferred energy source for murine embryos (Leese and Barton, 1984). Similarly, Takahashi and First (1992) stated that

lactate as well as pyruvate can support bovine embryo development in the absence of glucose. In this study lactate was the preferred energy source for early (2-8 cell stages) bovine embryos. Lactate levels in the ovine oviductal fluid range between 2-4 mM (Takahashi and First, 1992).

In early embryo culture studies, researchers used cell culture media which contained high levels of glucose (4.5-5.6 mM), which is comparable to glucose concentrations found in the blood of monogastric species and to that of fetal calf serum. However, blood glucose concentration of adult ruminants is much lower (3-3.5 mM) than that of monogastric species (Barcelo-Fimbres and Seidel, 2007a). Furthermore, glucose levels in the reproductive tract of the cow are significantly lower than in the peripheral circulation. For example, glucose levels in the bovine oviductal fluid ranged from 0.05 to 0.2 mM (Takahashi and First, 1992), but the uterine glucose levels tend to be higher. It is also important to remember that adding serum to culture media provides an additional source of glucose to the formulation. For instance, the addition of 5% calf serum can provide between 0.15 to 0.22 mM of glucose to the medium. Several authors have observed that glucose has detrimental effects on sperm capacitation (Parrish et al., 1989) and embryo development prior to the 8-cell stage (Schini and Bavister, 1988; Ellington et al., 1990; Takahashi and First, 1992). However, glucose has favorable effects on embryo development when added after the 8-cell stage (Robl et al., 1991).

Other energy sources have been examined as a replacement for glucose. The addition of fructose at a concentration of 1.5 to 2 mM increased the number of blastocysts and cell numbers compared to the same culture medium when glucose was used as the energy source (Kwun et al., 2003; Barcelo-Fimbres and Seidel, 2007a, b).

Sodium acetate also has been also added to culture media (0.61mM) in addition to lactate and pyruvate for culture of bovine embryos with good results (Moore and Bondioli, 1993). Similarly, Holm et al., (1999) increased lactate and pyruvate concentrations (5.35mM and 7.27mM, respectively), and replaced glucose with sodium citrate and myo-inositol (0.34 and 2.77 mM, respectively). These changes resulted in similar blastocyst rates as serum-supplemented medium for bovine embryos.

Role of Serum in Embryo Culture

Serum and Embryo Development

Several authors (Thompson et al., 1998; Rizos et al., 2003; Rooke et al., 2007) have observed a dual effect of serum in in vitro culture, which is commonly known as the “biphasic effect” of serum. This effect consists of the inhibition of cleavage and embryo development (first cleavages through the 4-cell stage) when zygotes are exposed to serum, and stimulation of embryo development after the 8-cell to 16-cell stage.

The addition of serum (5 - 20% concentration) in the culture medium accelerates blastocyst development compared to embryos produced with high (16 mg/ml) or low (3 mg/ml) levels of BSA fraction V (Rizos et al., 2003). Data obtained in our laboratory corroborates this tendency, in which embryos cultured with 5% calf serum have higher blastocyst rates than those cultured with 6 mg/ml of BSA (Puerpera et al., 2007). Similarly, IVP embryos cultured with serum had faster developmental rates than embryos cultured in oviducts of synchronized ewes (Enright et al., 2000). However, when serum is added during later stages, such as day 5 post-insemination, blastocyst

rates were greater (40.1% vs 21.6%) than the serum-free control (Pinyopummintr and Bavister, 1994; Thompson et al., 1998).

Although day-7 blastocyst rates increased when serum was added to medium during the 4 to 16-cell stage compared to BSA-supplemented medium, the cumulative blastocyst rates at day 9 were similar between serum and BSA treatments (Rizos et al., 2003). These investigators also showed that the capability of a fertilized oocyte to develop into a blastocyst is innate to the oocyte, and culture medium conditions will determine blastocyst quality. Therefore, oocyte quality (molecular, cytoplasmatic and meiotic maturation accomplished) is the key factor determining embryo development (Rizos et al., 2002b).

It has been observed that embryos reaching the blastocyst stage by day 7 of in vitro culture are more competent and have higher cell numbers than those becoming blastocysts after day 7 (Hasler et al., 1995). Only day-7 and day-8 blastocysts are normally used for embryo transfer (Hasler et al., 1995; Sommerfeld and Niemann, 1999) because these blastocysts tend to yield higher pregnancy rates and have higher cell numbers than embryos that become blastocysts after day eight of culture (Byrne et al., 1999; Enright et al., 2000). Furthermore, embryos that cleave faster are generally the embryos that become blastocysts sooner, and have fewer apoptotic blastomeres than those with slower cleavage rates (Byrne et al., 1999).

Serum and Embryo Morphology

Several authors (Thompson et al., 1995; Thompson, 1997; Ferguson and Leese, 1999; Crosier et al., 2000, 2001; Rizos et al., 2002a) have demonstrated that the

exposure of in vitro cultured embryos to serum can alter embryo metabolism, morphology and biochemistry. Doubts exist on the effect of serum in in vitro culture on embryo cell numbers. Some studies suggest that embryos cultured in vitro with serum have higher total cell numbers than in vivo counterparts (Marquant-Le Guienne et al., 1989; Lazzari et al., 2002). Other studies indicate that there is no difference in cell numbers between embryo culture with serum and without serum (Thompson et al., 1998; Enright et al., 2000; Kubisch et al., 2001). Others (Carolan et al., 1995; Van Langendonck et al., 1997) have observed higher cell numbers in IVP embryos cultured with serum than those cultured in the absence of serum. On the other hand, other authors (Gardner et al., 1994; Byrne et al., 1999) found reduced total cell number of IVP embryos cultured with serum compared to in vivo embryos cultured without serum. Similarly, Byrne et al., (1999) demonstrated that embryos with low cell numbers had more apoptotic cells than embryos with higher cell numbers, and vice versa. This finding suggests that these differences in cell number may be influenced by variations in culture media conditions and formulations between different laboratories and studies. Although, there are contradictory reports about the effect of serum on total cell number, most authors concur that addition of serum accelerates blastocyst formation and increases the size of the blastocyst compared to in vitro produced (IVP) without serum and in vivo derived (IVD) embryos.

One possible explanation for faster blastocyst formation after serum addition to the culture medium may be high content of lipids in serum, possibly due to preferential trophoblastic cell development by lipid stimulation. This finding can be supported by the observation that embryos cultured with serum had lower ICM:Trophectoderm cell ratio

than IVD blastocysts (Iwasaki and Nakahara, 1990; Du et al., 1996). It is probable that this faster blastulation could be due to the overgrowth of trophoblastic cells and increased apoptosis of ICM cells (Byrne et al., 1999). As stated by Thompson (1997) the result of serum addition is large diameter blastocysts with reduced cell numbers.

It has been shown (Prather and First, 1993; Plante and King, 1994; Thompson, 1997) that IVP morulas have a reduced cell coupling (gap junctions) and are poorly compacted compared to IVD embryos. Non-physiological conditions found in culture media, and possibly the addition of serum, may alter the expression of some genes responsible for morula compaction (Wrenzycki et al., 1999; Enright et al., 2000; Wrenzycki et al., 2001).

Embryonic cell compaction and blastocyst formation are important processes of early embryonic development leading to differentiation of the ICM and trophectoderm cells, which eventually give rise to the embryo proper and extraembryonic membranes, respectively. Similarly, IVP embryos cultured with serum contained more apoptotic blastomeres than IVP embryos cultured in serum free medium (Byrne et al., 1999; Pomar et al., 2005). In addition, Byrne et al., (1999) observed that the majority of apoptotic cells were localized within the inner cell mass and not in the trophectoderm (TE).

Embryos produced in vitro with or without serum showed an increased proportion of lipids compared to in vivo derived embryos (Crosier et al., 2000, 2001). Likewise, Thompson et al (1995) found more lipid droplets in ovine embryos cultured with human serum than those embryos cultured without serum using synthetic oviduct fluid medium

with aminoacids (SOFaa) + BSA. Based on Nile red staining pattern, embryos produced with 10% serum had higher lipid content than those produced with 0.3% BSA (Leroy et al., 2005). Similarly, Farin et al., (2001) observed a higher density of lipids in IVP embryos cultured with serum regardless of the time of exposure to serum (from day 1 to 7 of culture or from day 3 to 7 of culture) compared to IVD embryos. Ferguson and Leese (1999) demonstrated that triglyceride content of day-7 in vitro serum-supplemented embryos was 36% (45 ng/embryo) greater than that of in vivo derived and no-serum supplemented IVP embryos (33 ng/embryo). Also in this study, triglyceride content was measured from the oocyte stage to the blastocyst stage. Initially, there was no difference in triglyceride content between oocytes matured in vitro and in vivo; however, at the 8 to 16-cell stage, embryos cultured with serum began to produce and store triglycerides. Even though triglyceride content does not account for the total lipid content in embryos, lipids tend to increase when serum is added to culture medium.

Other morphological effects of serum addition have been described in elongated early conceptus. There are some studies in which the length of elongated preimplantation bovine embryos have been documented at different stages of development (Menezes et al., 1982; Bertolini et al., 2002; Carter et al., 2008; Rodriguez-Alvarez et al., 2009; Rodriguez-Alvarez et al., 2010a; Rodriguez-Alvarez et al., 2010b). However, there are few studies that compare the length of IVP and IVD embryos (Table 2).

Table 2 Length of elongated embryos

Stage	IVD (mm)	IVP (mm)	Cloned (mm)	Reference
Day-12	0.6-2.5			(Menezo et al., 1982)
Day-13	0.4-2			(Menezo et al., 1982)
Day-13	1.1±0.6			(Carter et al., 2008)
Day-14	0.8-2.5			(Menezo et al., 1982)
Day-14		5.4±5.5		(Block et al., 2007)
Day-16	75.0	37.3		(Bertolini et al., 2002)
Day-17	174±50		91.8±45.8	(Rodriguez-Alvarez et al., 2010b)

Serum, Lipids and Cryopreservation

Cryopreservation of IVD embryos has allowed the expansion of embryo transfer worldwide, opening new markets for the cattle industry. However, IVP embryos have reduced cryotolerance, survival after thawing and subsequent pregnancy rates, limiting the use of IVP embryos to fresh (immediate) transfer. Nevertheless, freezing and post-thaw in vitro culture of IVP embryos can be used as an evaluation method to quantify development capacities of IVP embryos.

IVP embryos cultured with serum have high lipid content (Pollard and Leibo, 1994; Ferguson and Leese, 1999) and low survival rate after cryopreservation (Enright et al., 2000). For example, Leibo and Loskutoff (1993) observed that 80% of IVD embryos survived and hatched in culture media after cryopreservation and warming compared to only 20% for similarly treated IVP embryos. Likewise, Barcelo-Fimbres and

Seidel (2007b) observed that IVP embryos cultured in Chemical Define Medium (CDM) without serum had a higher survival rate after cryopreservation (thawed and then cultured) than IVP embryos cultured in CDM with 10% fetal calf serum (84.9% vs. 60.2%, respectively). In this same study, embryos cultured in medium supplemented with serum and Phenazine Ethosulfate (PES), a chemical that reduces lipid production and accumulation in IVP embryos, had a higher survival rate after cryopreservation and post-thaw embryo culture than embryos cultured with serum-supplemented medium alone. The comparison of these treatments suggests that embryo cryotolerance can be increased by reducing the accumulation of lipids in embryos or by avoiding the use of serum during in vitro culture. Nevertheless, despite a reduction in the lipid content and an improvement in cryotolerance, pregnancy rates accomplish with these treatments are still reduced compared to IVD embryos (De La Torre-Sanchez et al., 2006; Barcelo-Fimbres and Seidel, 2007b). This evidence suggests that factors other than lipid accumulation during in vitro culture cause developmental gaps between IVP and IVD embryos. An inappropriate concentration of phosphate (Schini and Bavister, 1988; Lawitts and Biggers, 1991), increased osmolarity of culture media to non-physiological conditions (Hasler, 2010), use of non-physiological concentrations of energy and protein sources (Schini and Bavister, 1988), absence of amino acids and growth factors (Rosenkrans Jr and First, 1991; Moore and Bondioli, 1993; Gardner, 2008; Hansen et al., 2010), content of ammonium in serum (Sinclair et al., 1998b) and oocyte competence and quality (Enright et al., 2000; Khurana and Niemann, 2000; Rizos et al., 2002b) have all been implicated as factors impacting the developmental competence of IVP embryos.

Some reports demonstrate that independently of the oocyte source (in vivo or in vitro matured oocytes), culture conditions determine embryo morphology and gene expression (Enright et al., 2000; Rizos et al., 2002b). Rizos et al., (2002b) stated that “the intrinsic quality of the oocyte is the main factor affecting blastocyst yields, while the conditions of embryo culture have a crucial role in determining blastocyst quality”. Lipid content affects the buoyant density of embryos. In vivo derived embryos tend to sink (higher embryo density) at a specific density, while, IVP embryos cultured with serum tend to float (lower embryo density) in the same density solution (Leibo and Loskutoff, 1993). Given that the specific gravity of lipids is less than water, this observation was attributed to a higher lipid concentration in IVP embryos cultured with serum. Breed also affects lipid content of oocytes and embryos. Brahman and Jersey IVD embryos have greater lipid content, and decreased survival and subsequent pregnancies after cryopreservation compared to non-cryopreserved IVD Brahman embryos (Ballard et al., 2006; Pryor et al., 2007).

Although, there are several differences between IVP and IVD embryos in morphology and composition, the optimum freezing rate (0.6°C/minute) from -7°C to -35°C for both IVP and IVD embryos is the same (Leibo and Loskutoff, 1993). However, the developmental stage at which IVP embryos are frozen plays an important role in embryo survival post-thaw and during culture (0% and 63.5%, embryo survival for morulas and blastocyst, respectively) (Pollard and Leibo, 1993). Conversely, blastocysts cultured in SOF medium had a low survival after being frozen-thawed and cultured for 72 hours compared to IVD embryos (Enright et al., 2000).

Pregnancy Rates of IVP Embryos

Table 3. Pregnancy rates of in vitro produced embryos by different culture conditions

Medium	Treatment	Pregnancy rate	Source
Co-culture	Slow frozen	38% (312/866)	(Kajihara et al., 1992)
SOF	Fresh transfer	37.5% (21/56)	(Enright et al., 2000)
SOF	Fresh transfer	41.8% (367/877)	(Van Langendonck et al., 1997)
SOF	Slow frozen	35% (156/446)	(Van Langendonck et al., 1997)
Co-culture	Fresh transfer	56% (1064/1884)	(Hasler et al., 1995)
Co-culture	Slow frozen	43% (10/23)	(Sommerfeld and Niemann, 1999)

Serum and Large Offspring Syndrome (LOS)

Manipulation and exposure of gametes and pre-implantation embryos to non physiological environments cause perturbations in the later development of the conceptus (Jacobsen et al., 2000; Rooke et al., 2007). One such disturbance is the Large Offspring Syndrome (LOS), which is a pathological combination of features. Although increased fetal and calf size is the most notable characteristic of this syndrome, other features have been recognized such as: longer gestation periods, weaker calves, respiratory difficulties, high stillbirth rates, high embryo and early fetal loss rates, sudden perinatal death, increased dystocia incidence, congenital malformations, increased sized of certain organs (heart, liver and kidney), plantaris muscle enlargement, placental (edema, hydrallantois, reduce number, but larger

cotyledons, decreased feto-maternal contact) and skeletal abnormalities (Young et al., 1998; Farin et al., 2001; Constant et al., 2006).

Risk factors that may contribute to the occurrence of LOS have been identified, but the exact mechanism or mechanisms which cause LOS are still unknown (Thompson, 1997; Young et al., 1998; Farin et al., 2001). These factors may be the presence of serum, somatic cell co-culture, growth factors, free radicals, ammonia during embryo culture and exogenous progesterone. The present study focuses on the effects of serum and its components on the expression of genes.

The poor understanding of the mechanisms that generate the appearance of LOS, as well as the presence of more than one of the factors that have been proposed to trigger LOS during in vitro maturation, fertilization and culture have not allowed the elucidation of the degree of influence any one of these factors have on the occurrence of large offspring syndrome. For instance, co-culture of pre-implantation embryos with somatic cells appears to stimulates the occurrence of LOS (Sinclair et al., 1997; Sinclair et al., 1998b; Jacobsen et al., 2000; van Wagtendonk-de Leeuw et al., 2000); however, co-culture is typically performed in the presence of a high proportion of serum (10-20%) (Sinclair et al., 1998b). This leaves open the question of whether co-culture, serum or the combination of the two conditions triggers LOS.

Similarly, embryos produced by SCNT are likely to develop LOS (Constant et al., 2006). It has been observed that the range of calf birth weight of nuclear transfer and IVP embryos are similar (Kruip and den Daas, 1997; Young et al., 1998). LOS has been observed in ovine and bovine species, but has not been observed in humans and mice.

This could be due to the variations in the in vitro protocols between species. In the mouse, it is speculated that this phenomenon is due to mice gametes and embryos not being exposed to serum. Similarly, in human in vitro production systems, the exposure of embryos to non-physiological culture conditions is very brief because human embryos are generally transferred at the 2 to 4-cell stage. Thus, human embryos experience embryo genomic activation, morula compaction and blastulation in vivo rather than in vitro as do ovine and bovine embryos during the extended in vitro culture period (5 to 7 days).

Rooke (2007) observed that even though serum was absent during in vitro culture of in vivo fertilized ovine zygotes, there was a high rate of large size fetuses after embryo transfer, but was reduced when serum-free culture conditions were utilized (Rooke et al., 2007). In this study, and in the majority of studies with ewes, maturation and fertilization are performed in vivo; the remainder of the culture is performed in vitro for 5 to 6 days. This research demonstrated that in addition to the effects of serum on the occurrence of LOS, there are other, as yet undefined, factors in the in vitro culture system, which could be responsible for the overgrowth of offspring.

Genes Potentially Altered by Culture in the Presence of Serum

Gene expression of preimplantation embryos can be altered by culture media (Sagirkaya et al., 2006) and protein sources within culture media (Wrenzycki et al., 2001; Yaseen et al., 2001; Warzych et al., 2007). Therefore, the present study focused on the effects of the presence of serum in SOF medium on the expression of candidate

genes, which previously have been implicated in the development of LOS (Bertolini et al., 2002; Niemann and Wrenzycki, 2000; Young et al., 2001).

Interferon tau (IFNT)

Interferon tau is a polypeptide secreted by trophectoderm cells of bovine and ovine embryos (Roberts et al., 1992). Production of IFNT starts at the blastocyst stage and continues until day 25 to 36 of gestation (Godkin et al., 1988; Farin et al., 1990; Hernandez-Ledezma et al., 1992). In the cow, IFNT production peaks around day 15 to 16 of pregnancy (Farin et al., 1990; Hernandez-Ledezma et al., 1992; Roberts et al., 1992; Wrenzycki et al., 1999; Lonergan et al., 2003). The antiluteolytic effect of IFNT is due to inhibition of Cyclooxygenase-2 pathway and by down-regulation of the endometrial and oxytocin receptor, thereby inhibiting oxytocin-induced secretion of prostaglandins by the endometrium. IFNT also stimulates the production of endometrial proteins such as histotroph, which nurture and stimulate embryo development.

In a recent study, all elongated embryos (day 17) expressed IFNT; however, the expression of this gene varied when bovine blastocysts were cultured in a homologous (bovine) or in a heterologous uterus (ewe or goat) (Rodriguez-Alvarez et al., 2009). Based on this finding, it was recommended that the study of gene expression in elongated embryos be performed in a homologous environment (with respect to the embryos specie) because non-physiological conditions can alter transcripts of IFNT. IFNT may subsequently affect (positive or negative) the expression of other genes. It has been suggested that IFNT expression is mediated through other genes, such as caudal type homeobox 2 (CDX2) (Farin et al., 2010). Similarly, other authors have

observed that all embryos that expressed IFNT also expressed CDX2 (Rodriguez-Alvarez et al., 2009). Also, IFNT can influence transcription levels (up or down-regulation) of several genes in preimplantation embryos and in the endometrium (Satterfield, 2008; Mansouri-Attia et al., 2009a; Mansouri-Attia et al., 2009b).

In vitro produced 6 to 8-day bovine blastocysts cultured in SOF medium with serum or BSA have higher expression of IFNT than IVD embryos and embryos cultured with serum expressed greater IFNT than those cultured with BSA (Wrenzycki et al., 2001). Based on these data, it has been suggested that over-expression of IFNT may be an indicator of poor embryo development. Furthermore, up-regulation of IFNT in IVP embryos is associated with poor developmental competence (Kubisch et al., 1998). Similarly, higher transcript levels of IFNT were observed in elongated (day 17) cloned bovine embryos compared to IVP embryos, despite the same culture medium being used for each kind of embryo (Rodriguez-Alvarez et al., 2010b).

IVP embryos reaching the blastocyst stage before day 8 of culture, express significantly lower amounts of IFNT compared to day-9 and day-10 blastocysts (Kubisch et al., 1998; Kubisch et al., 2001), and were more competent than embryos developing to blastocysts after 9 days of culture (Hasler et al., 1995). Conversely, Kubisch et al., (2001) did not find a difference in IFNT production between IVP embryos that become blastocysts on day 7 or 8 of culture compared to in IVD embryos. Reports show that IFNT expression varies between embryos regardless of origin, with larger and more elongated embryos producing more IFNT (Rodriguez-Alvarez et al., 2009; Rodriguez-Alvarez et al., 2010a; Rodriguez-Alvarez et al., 2010b). It is important to note that

differences exist in IFNT secretion of embryos which are derived from different oocyte batches, independent of vitro production protocol (Kubisch et al., 2001).

Kubisch et al., (2001) did not find a difference in IFNT transcripts between blastocysts cultured in SOF with serum or BSA. In contrast, (Rizos et al., 2003) found that IFNT was down-regulated in blastocysts cultured with serum compared to embryos produced with BSA. Likewise, Bertolini et al., (2002) stated that IVP embryos have lower transcripts levels of IFNT than day-7 IVD blastocysts. On the other hand, (Lonergan et al., 2003) found an up-regulation of IFNT in embryos cultured in SOF with serum compared to embryos cultured in ewe oviducts at the blastocyst stage. Using microarray analysis, Ushizawa et al., (2004) found an up-regulation of IFNT of day-14 embryos compared to day-7 in vivo derived embryos, indicating that IFNT increased from day-7 to day-14 as mentioned before.

Insulin-like Growth Factor 2 receptor (IGF2R)

IGF2R is an imprinted gene that is expressed from the maternal allele, and has been implicated in the control of fetal and placental overgrowth (Niemann and Wrenzycki, 2000). Studies in mice support the relationship between down-regulation of IGF2R and fetal overgrowth, but in cattle, there is no clear information that corroborates this conclusion.

Up-regulation of IGF2R in IVD blastocysts from *Bos taurus* and *Bos indicus* donors, compared to their IVP counterparts, has been observed (Bertolini et al., 2002; Nasser et al., 2008). IGF2R was down-regulated in tissues from ovine fetuses affected with LOS at 80% gestation, and there was no altered IGF2R expression at normal

weight fetuses by this method of embryo production (Young et al., 2001). This observation suggests that not all offspring produced in vitro in the presence of serum will develop LOS, but those affected with LOS display altered gene expression.

Bertolini et al., (2002) described a differential pattern of transcripts with stage of development, in which IGF2R was higher at the blastocyst stage and decreased in day-16 elongated embryos (transferred on day 7 and subsequently collected at day 16 of gestation). The size of individual embryos was inversely correlated with lower IGF2R and higher insulin-like growth factor 2 (IGF2) expressions. High levels of IGF2R alone do not trigger overgrowth; however, high levels of IGF2 and low levels of IGF2R may lead to the development of LOS (Lau et al., 1994). In a recent study, there was no significant difference of transcript levels of IGF2R and IGF2 at the blastocyst stage between IVD and IVP embryos, although the IGF2R transcripts had a tendency to be higher in IVD blastocyst pools (Moore et al., 2007a). Other authors did not find a difference in the expression of IGF2R between culture in SOF with and without serum or IVD blastocysts (Lazzari et al., 2002). In another study transcript levels of IGF2R varied between IVP and IVD blastocysts, but did not differ between day-16 IVP and IVD elongated embryos (Bertolini et al., 2002)

Although there is abundant information about IGF2R transcripts at the blastocyst stage, there is little information about the effect of presence or absence of serum during embryo culture at the blastocyst stage and subsequently in elongated embryos.

Warzych et al., (2007) reported that IGF2R transcription in blastocysts was influenced by the protein source (free fatty acid BSA or fetal calf serum) used in the media for in vitro maturation. Likewise, the addition of polyvinyl alcohol (Yaseen et al., 2001) and

polyvinyl pyrrolidone (Warzych et al., 2007) to culture medium may affect IGF2R transcriptions levels.

It was suggested that IGF2R can bind IGF1 as well as IGF2, albeit with different efficiencies (Warzych et al., 2007). Alternatively, Farin et al., (2010) proposed that the mechanism responsible for triggering LOS could be the abundance of IGF binding proteins and the effects of differential demethylation of imprinted regions that change the abundance of IGF2R during embryonic development.

Cytochrome c oxidase subunit VIa (COX6A)

According to Everts et al., (2008), COX6A was upregulated five fold in placentomes from IVP (cultured without serum) pregnancies compared to placentomes derived from IVD or SCNT pregnancies. It is suggested that this is due to the effects of in vitro fertilization, as SCNT and IVD placentomes did not over express COX6A (Everts et al., 2008).

Placenta-specific factor 8 (PLAC8)

The exposure to non-physiological progesterone levels during early gestation may trigger the development of LOS as well as promoting upregulation of IFNT (Wilmut and Sales, 1981; Garrett et al., 1988; Kleemann et al., 1994; Carter et al., 2008). PLAC8 was upregulated in day-12 pre-implantation embryos from ewes receiving 25 mg of progesterone from days 2 to 12 of gestation (Satterfield, 2008), and PLAC8 expression is mediated through the expression of IFNT (Mansouri-Attia et al., 2009a). These studies suggest that high levels of progesterone as well as the expression of IFNT may mediate the expression of PLAC8 by the trophoblastic layer of the pre-

implantation embryos. On the other hand, PLAC8 is expressed in caruncular tissue during early stages of gestation and at implantation (Mansouri-Attia et al., 2009a; Mansouri-Attia et al., 2009b). No difference was observed in the level of expression of PLAC8 between IVD and IVP blastocysts (Nasser et al., 2008), and no difference in PLAC8 transcripts levels were detected between female and male IVP embryos at day 15 of gestation (Dode, 2009).

Interestingly, embryo density during culture in CR1aa increased the transcript levels of PLAC8 (Hoelker et al., 2009). PLAC8 expression was greater when cultured singly in groups of 50 and 16 embryos compared to IVD blastocyst and IVP embryos cultured in a system known as well of the well (WOW). The number of cells also increased when embryos were cultured at higher densities (Hoelker et al., 2009).

CHAPTER III

EFFECTS OF SERUM ADDITION TO CULTURE MEDIUM ON GENE EXPRESSION OF DAY-7 AND DAY-14 BOVINE EMBRYOS

Introduction

During the last four decades development of techniques for in vitro production of embryos has facilitated the understanding of embryo development. Recently, modifications to improve media to more closely mimic the reproductive tract environment have increased the efficiency of in vitro production techniques and developmental competence of in vitro produced embryos. However, considerably progress is needed to produce IVP embryos equal in competence to IVD embryos, which are considered as the “gold standard”. In some countries the availability of recipients and cattle genetics have stimulated the use of follicle aspiration and in vitro production of embryos in order to obtain as many offspring as possible from valuable donor cows. Similarly, the use of embryo IVP from domestic species can be used as for research purposes in general, and as research models to study diseases and syndromes that may affect humans and other species, and preservation of endangered species. These assisted reproductive techniques are an alternative to multiple ovulation and embryos transfer techniques (MOET), which have been reliable during the past 40 years.

The addition of serum has been extensively applied in in vitro culture media to favor embryo development. However, serum addition to culture media has been implicated as a cause for abnormal offspring from cows and sheep. These offspring are characterized by weaker calves, respiratory difficulties, high

stillbirth rates, high embryonic and early fetal loss, sudden perinatal death, increased dystocia incidence, congenital malformations, organomegaly, placental abnormalities and skeletal abnormalities.

The study of large offspring syndrome (LOS) is a difficult task because, in addition to serum addition, there are other factors in culture media that may lead to LOS. Also, in order to obtain significant differences in the effects of culture conditions on LOS, large number of IVP embryo transfers are needed. Research stations do not typically maintain the large numbers of recipients necessary to perform embryo transfer. From the welfare standpoint, these trials are difficult to obtain approval from Animal Care and Use Committees due to the incidence of dystocia. Thus, the application of molecular techniques as polymerase chain reaction (PCR) allows examination of gene expression from embryos cultured under specific conditions. Embryo transcript levels of genes of interest can be analyzed to discern the possible effects of culture conditions on gene expression. Such an approach can reduce the number of samples, increase the accuracy of results, and reduce the costs, and allow results to be obtained in a shorter period of time.

Material and Methods

Experimental Design

Experiment 1

Pools of in vitro produced day-7 blastocyst (5-10 blastocysts per pool) were produced for each treatment. Pools were also generated from *in vivo* derived

blastocysts collected from superovulated cows, which were inseminated with the same semen used to generate the in vitro produced embryos. For IVP embryos, IVF was performed and at 18 hours post-insemination, presumptive zygotes were cultured in mSOF_{aa}. At 72 hours post-insemination, embryos were allocated to two treatments (mSOF_{aa} and mSOF_{aa} with 5% calf serum). At day 7 post-insemination, blastocyst rates were assessed for each treatment. Embryos were moved and pooled into 1.5 ml vials with 3 μ l of PBS containing 0.1% polyvinyl alcohol (PVA), and immediately stored at -80°C. In order to collect IVD blastocyst, cows were non-surgically flushed on day 8 after artificial insemination (AI). IVD embryos were pooled, and processed in the same manner as IVP embryos. mRNA was isolated, reversed transcribed to cDNA, and gene expression was analyzed by quantitative PCR (Q-PCR) from all embryo pools.

Experiment 2

In vitro produced day-14 embryos were produced for each treatment. Also in vivo derived day-14 embryos were generated from superovulated cows, which were inseminated with the same semen used to generate the IVP embryos. For IVP embryos, IVF and IVC was performed as described in Experiment 1. At day 7 post-insemination blastocyst rates were assessed for each treatment. Blastocysts were transferred into synchronized recipients, and were recovered 7 days after transfer. The IVD embryos were collected on day 15 after AI. At embryo recovery, all IVP and IVD elongated embryos were photographed, measured and stored independently in a minimum volume (30-60 μ l) of PBS + 0.1% PVA into a 1.5 ml vial, and were stored at -80°C. mRNA was isolated, reversed transcribed to cDNA, and gene expression analyzed by Q-PCR.

In Vitro Production of Embryos

In vitro matured (IVM) oocytes were shipped overnight from a commercial supplier (Trans Ova Genetics, Long Prairie, MN, USA) in a Biotherm™ portable incubator (CryoLogic, Victoria, Australia) at 38.5°C, in 1.5 ml vials containing IVM medium (TCM-199, 10% fetal bovine serum (FBS), 0.1% Penicillin/Streptomycin (P/S), Na pyruvate, L-glutamine, 50 ng/ml of epidermal growth factor, FSH, LH and Estradiol) and cumulus oocyte complexes (COCs). At arrival, vials were transferred into a CO₂ incubator for a total elapse time of IVM of 22-24 hours, at which point IVF was performed. Later, COCs were washed twice in HEPES-TALP (tyrode albumin lactate pyruvate), then washed twice in IVF-TALP and finally transferred into fertilization wells with 425 µl of IVF-TALP. Fertilization was performed with a single Holstein bull with a history of proven fertility in our IVF program. One straw of semen was thawed at 37°C for 30 seconds in a water bath. The semen was layered on top of the Isolate® (Irvine Scientific) density gradient and centrifuged for 12 min at 700 x g, isolating motile spermatozoa in the bottom of the tube. Following aspiration of the supernatant, motile spermatozoa were washed with 5 ml of Sperm-TALP and re-centrifuged for 5 min at 700 x g. Sperm concentration was assessed (with an hemocytometer) and a fertilization suspension of IVF-TALP and sperm was prepared at a concentration of 1x10⁶ sperm/ml. During fertilization 20 µl of PHE (1 mM epinephrine, 10 µM hypotaurine, 20 µM penicillamine), 20 µl of heparin (2 µg/ml of heparin) and 20 µl of sperm suspension (1x10⁶ sperm/ml) were added to each fertilization well containing COCs. Fertilization was performed at 39°C in a humidified atmosphere of 5% CO₂ in air.

Eighteen hours post-insemination, presumptive zygotes were denuded by vortexing at maximum speed for 2 min in 2 ml of HEPES-TALP. Embryos were washed twice in HEPES-TALP and four times in mSOF_{aa}. Fifteen presumptive zygotes were cultured in a 30 µl drop until 72 hours post-insemination, at which point cleavage rate was assessed. Fifty percent of the embryos were allocated to treatment 1 (mSOF_{aa}) and the remainder to treatment 2 (mSOF_{aa} + 5% calf serum) until 168 hours post-insemination.

In Vivo Derived Embryos

A group of Angus cows were examined by transrectal ultrasonography in order to select donor cows. Selection was based on the presence of an active corpus luteum, the presence of a dominant follicle and at least 8 antral follicles. Selected cows received a superstimulation protocol which began with the insertion of a CIDR-B, and 100 µg of GnRH, IM (Cystorelin[®] Merial Canada Inc.). Thirty six hours later, a new follicular wave was enhanced by administration of 200 mg of Follicle Stimulating Hormone (400 mg Folltropin, Bioniche Animal Health) every 12 h for 4 consecutive days in decreasing doses. Dinoprost Tromethamine (Lutalyse[®] Pfizer, USA) was administered with the last two doses of FSH, and the CIDR was removed with the last FSH dose (Table 4).

Table 4 Superstimulation protocol used to produce in vivo derived embryos.

Day	Treatment	Time
1	CIDR + GnRH	PM
3	FSH (36 mg)	AM
	FSH (36 mg)	PM
4	FSH (30 mg)	AM
	FSH (30 mg)	PM
5	FSH (22 mg)	AM
	FSH (22 mg)	PM
6	FSH (12 mg) + PG (25 mg)	AM
7	CIDR + FSH (12 mg) + PG (25 mg)	PM

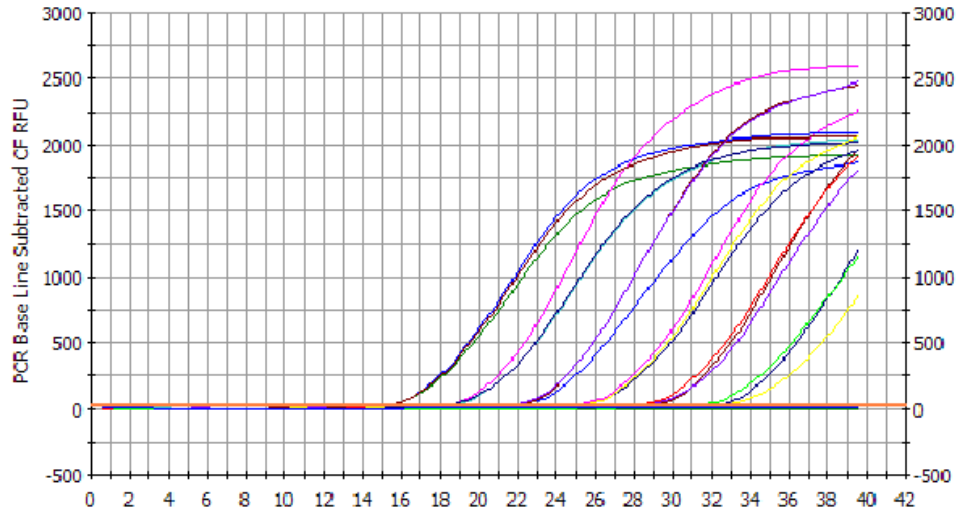
Estrus was detected using an electronic system (HeatWatch® system CowChips, LLC Manalapan, NJ). Cows were artificially inseminated with 2 straws at 12 h after first standing heat and another AI at 24 h (1 straw). The semen of the same bull was used for IVF.

mRNA Isolation and Reverse Transcription

Poly(A) RNA was isolated from day 7 and day 14 embryos using a commercial isolation system (Dynabeads® mRNA Direct Kit™, Invitrogen, Carlsbad, CA, USA) as described previously (Wrenzycki et al., 2001). Messenger RNA was immediately used for reverse transcription (RT). Reverse transcription was performed in a total volume of 20 µl using a commercial transcription system (iScript™ cDNA Synthesis Kit, Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to manufacturer's instructions. The reaction mix contained 4 µl of iScript reaction mix, 1 µl of reverse transcriptase, 4 µl of nuclease-free water and 11 µl of mRNA.

PCR Validation

A day-14 IVD embryo was used to obtain amplicon of each gene of interest, which was electrophoresed on a 2% agarose gel and sequenced to confirm the amplification of the proper product (see product length in table 5 and Figure 2). To demonstrate that the primers amplified only cDNA and not genomic DNA, 1 ng of genomic DNA was used as a template for the amplification of the target genes. No products were recovered after RT-PCR. The amplicon of each gene was purified using a commercial kit (PureLink™ PCR Purification Kit, Invitrogen). To determine if the primers amplified a single product in a quantitative manner, cDNA at six serial dilutions from the PCR product (0.4, 0.04, 0.004, 0.0004, 0.00004, 0.000004 pg/ μ l) and from each gene were analyzed by Q-PCR (Figure 1). All the target genes led to a PCR efficiencies between 88.3-100.3% and correlation coefficients between 0.98-0.999.



Correlation Coefficient: 0.999 Slope: -3.343 Intercept: 13.266 $Y = -3.343 X + 13.266$
 PCR Efficiency: 99.1 %

□ Unknowns
 ○ Standards

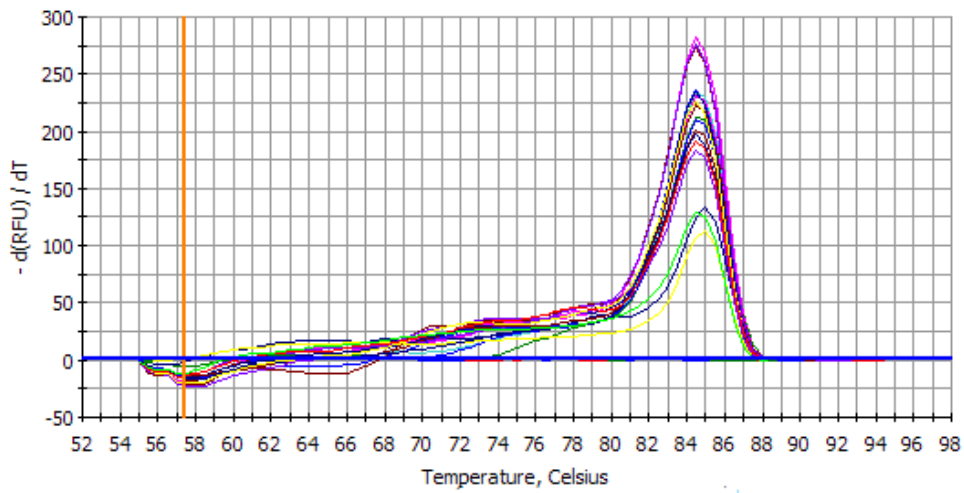
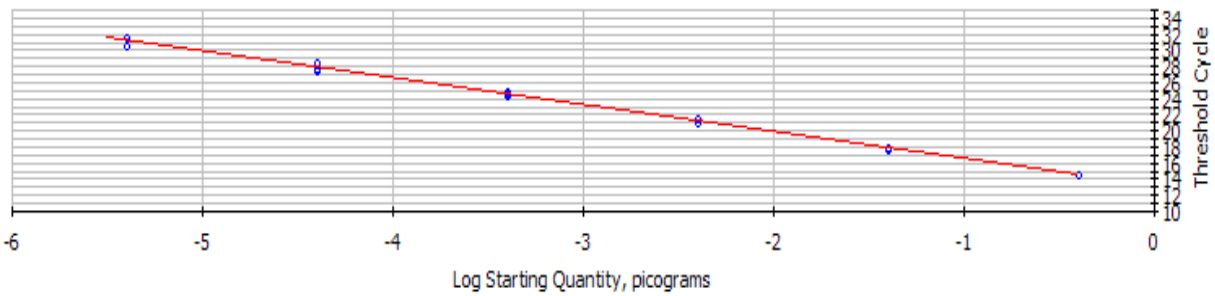


Figure 1. Standard curve, correlation and melting curve of serial dilutions for the gene COX6A

Quantitative-PCR

The following PCR primers COX6A1, IFNT1, IGF₂R, PLAC8 and GAPDH (Bosnakovski et al., 2005) were designed from bovine gene sequences using a commercial primer design software (Beacon Designer 6.8, PREMIER Biosoft International, Table 4). cDNA was amplified using SsoFast™ EvaGreen® Supermix 2X (Bio-Rad Laboratories, Inc., Hercules, CA, USA, Cat. No. 172-5202). The final reaction volume was 20 µl consisting of 10 µl of 2X supermix, 2 µl of cDNA, 6 µl water, and 2 µl of forward and reverse primer pairs (10 pmol/µl) for each gene (Experiment 1).

A reaction mix was formulated for the samples and for a control reaction. In the case of day-7 blastocyst pools and day-14 ovoid embryos, the cDNA was used directly in the Q-PCR. For the elongated day-14 embryos, the cDNA was diluted with 20 µl of DEPC-treated water for a final reaction volume of 40 µl. The thermocycler used was a Bio-Rad MyiQ (Bio-Rad Laboratories, Inc., Hercules, CA.), and the program used for the amplification of all the genes consisted of an enzyme activation cycle of 30 seconds at 95°C, 40 cycles of PCR (denaturation at 95°C for 5 sec, and annealing/extension at 55°C for 30 sec), a melting curve consisting of 95°C for 1 min followed by 55°C for 1 min, a step cycle starting at 55°C for 10 sec with a 0.5°C/sec transition rate, and cooling at 4°C.

Final quantification was done using the relative standard curve method. Standard curves were constructed for each individual gene, using six ten-fold serial dilutions (beginning at 0.4 pg/ µl) of its respective PCR product. Amplicon concentrations were calculated using a spectrophotometer (Nanovue, General Electric, USA). Transcript concentrations of each gene within each sample were provided by My IQ software

(BioRad, USA) and were normalized by comparing the transcript concentration of the internal standard gene, GAPDH. The relative expression of each gene was obtained by dividing the quantity of each gene by the quantity of the standard gene.

Table 5 Primers used for PCR analysis

Gene	Accession N°	Primers	Amplicon Length (bp)
COX6A1	NM_001077831	Forward CGGCTATGAAGACGAATAAAG Reverse AATGGTCCTCAAGTGTAAATGG	158
IFNT1A	M31557	Forward CAGTGATGGGAGAGAAAGAC Reverse GGTGGTTGATGAAGAGAGG	167
IGF ₂ R	NM_174352	Forward ATTCAGAGTAGCATCACCTTC Reverse GTCGTCCACCAAGTAAGC	225
PLAC8	NM_001076987	Forward CTGATATGAATGAATGCTGTCTG Reverse AAGTGCGATTGGCTCTCC	177
GAPDH	U85042	Forward CCTTCATTGACCTTCACTACATGGTCTA Reverse TGGAAGATGGTGATGGCCTTTCCATTG	127

Bovine sequences were used to design the primers

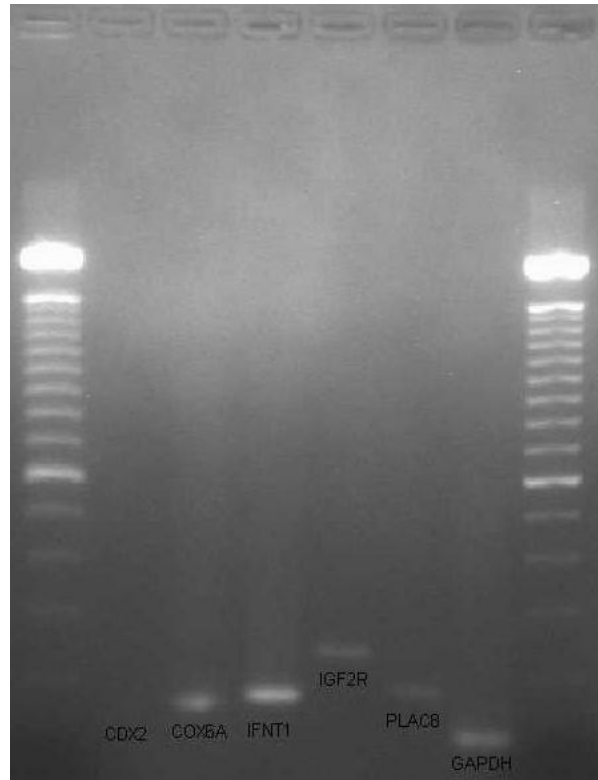


Figure 2. Agarose gel showing the expression and product length of each gene of interest

Statistical Analysis

The analysis of difference in the expression of the genes was performed using one-way ANOVA. Gene expressions of IVP embryos cultured with serum, without serum and IVD embryos at two different stages, blastocyst and day-14 embryos, were compared. Descriptive statistics were used to determine embryos that were upregulated or downregulated above two standard deviations from the mean of IVD treatment in each gene of interest. In order to accomplish this, a 95% confidence interval was constructed for in vivo derived embryo expressions for each gene of interest. If the relative expression of a sample (gene of interest/ GAPDH expression) did not fall within

the confidence interval for the *in vivo* embryos for each gene of interest, the sample was considered either significantly upregulated or downregulated.

Blastocyst rates for IVP treatments were analyzed using chi square. The length of day 14 embryos of each treatment was analyzed using ANOVA. Pearson correlation was performed in order to observe any relationship between genes of interest. Variance in gene expression between stages was performed using one-way ANOVA. Statistical analysis was run using SAS software (SAS Institute Inc.). Differences of $P \leq 0.05$ were considered to be significant.

Results

In Vitro Production of Embryos

In vitro culture results from both experiments are summarized in Table 6. The addition of 5% calf serum to the culture medium at 72 hours post-insemination increased blastocyst rates compared to embryos that were cultured in mSOF_{aa} only ($P < 0.001$). In every replicate, an approximately 50-100 oocytes were used for other procedures. Based on that information, the maturation rate of the total number of oocytes was 68.2 percent. Although, it is not the maturation rate of the fertilized oocytes, this data could give an approximate maturation rate for oocytes that were fertilized due to the fact that all oocytes were treated under the same conditions.

Table 6 Day-7 blastocyst rates for embryos cultured in mSOF_{aa} in the absence or presence of calf serum

Treatment	Oocytes	Blastocyst	Blastocyst (%)
No-serum	1939	143	7.4% ^a
Serum	1054	157	14.9% ^b

^{ab} Values within row with different superscript are significantly different (P<0.001)

Experiment 1

The numbers of day-7 blastocyst pools obtained per treatment were 7 pools for serum treatment, 6 pools for no-serum and 5 pools for IVD. Serum treatment averaged 9.4 blastocysts per pool, no-serum average 6.3 blastocysts per pool and IVD pools average 5.8 blastocysts per pool. The number of blastocysts per pool did not influence gene expression due to the fact that relative expression was calculated as a ratio of the genes of interest and GAPDH transcripts of the same sample.

There was no difference in the mean expression for COX6A, IFNT1a, IGF2R and PLAC8 among serum, no-serum and IVD day-7 blastocyst pools (P≥0.21; Figure 3). Mean relative levels may not be the best method of analysis of gene expression data, in particular when sample size is small, the mean reported values are low, and the distribution of expression is well spread. Therefore, in order to observe the incidence of abnormal expression in each treatment and gene of interest a confidence interval based on expression of IVD embryos was constructed, which relative expression either two

standard deviations above or below the mean was considered as altered expression pattern.

With this descriptive statistical method, the expression of COX6A, IFNT1a and IGF2R were upregulated in some samples of the IVP treatments (serum and non-serum) (Figure 4, 5, 6 and 7; Table 7). The expression of PLAC8 in all samples of all treatments was considered as normal expressions. Also a significant correlation was observed among PLAC8 and IFNT1a across all treatments ($r \geq 0.84$; $P \leq 0.03$).

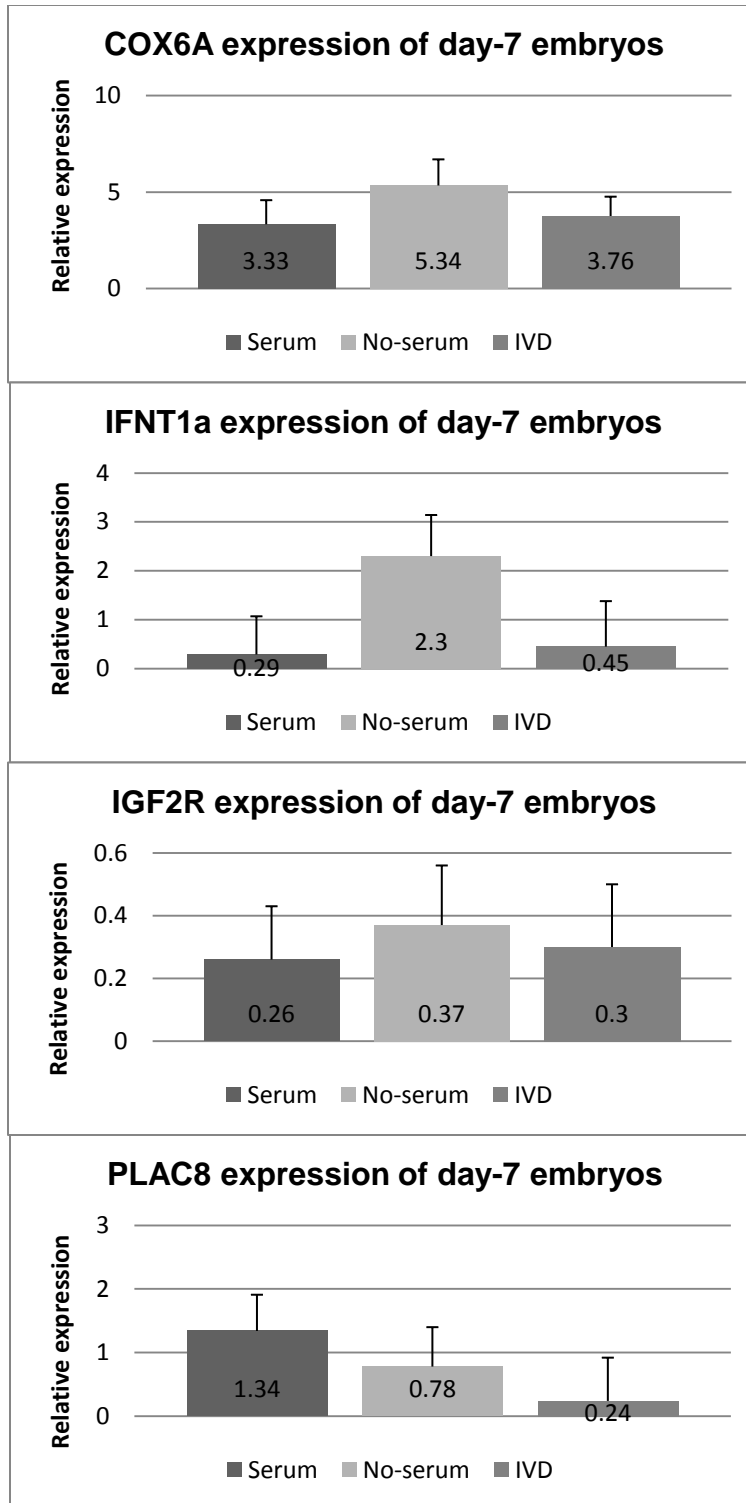


Figure 3 Relative expression of COX6A, IFNT1a, IGF2R and PLAC8 in each treatment in day-7 blastocyst pools (LSM ± SE)

Table 7 Day-7 blastocyst pool expression based on IVD 95% confidence interval for each gene of interest

Gene	Expression*	Serum	No-serum	IVD
COX6A	Upregulated	0	1	0
	Normal	7	5	5
	Downregulated	0	0	0
IFNT1a	Upregulated	1	2	1
	Normal	6	4	4
	Downregulated	0	0	0
IGF2R	Upregulated	1	1	0
	Normal	6	5	5
	Downregulated	0	0	0
PLAC8	Upregulated	0	0	0
	Normal	7	6	5
	Downregulated	0	0	0

*Upregulated and downregulated samples are those that are two standard deviations above or below the of the IVD mean for each gene of interest, respectively

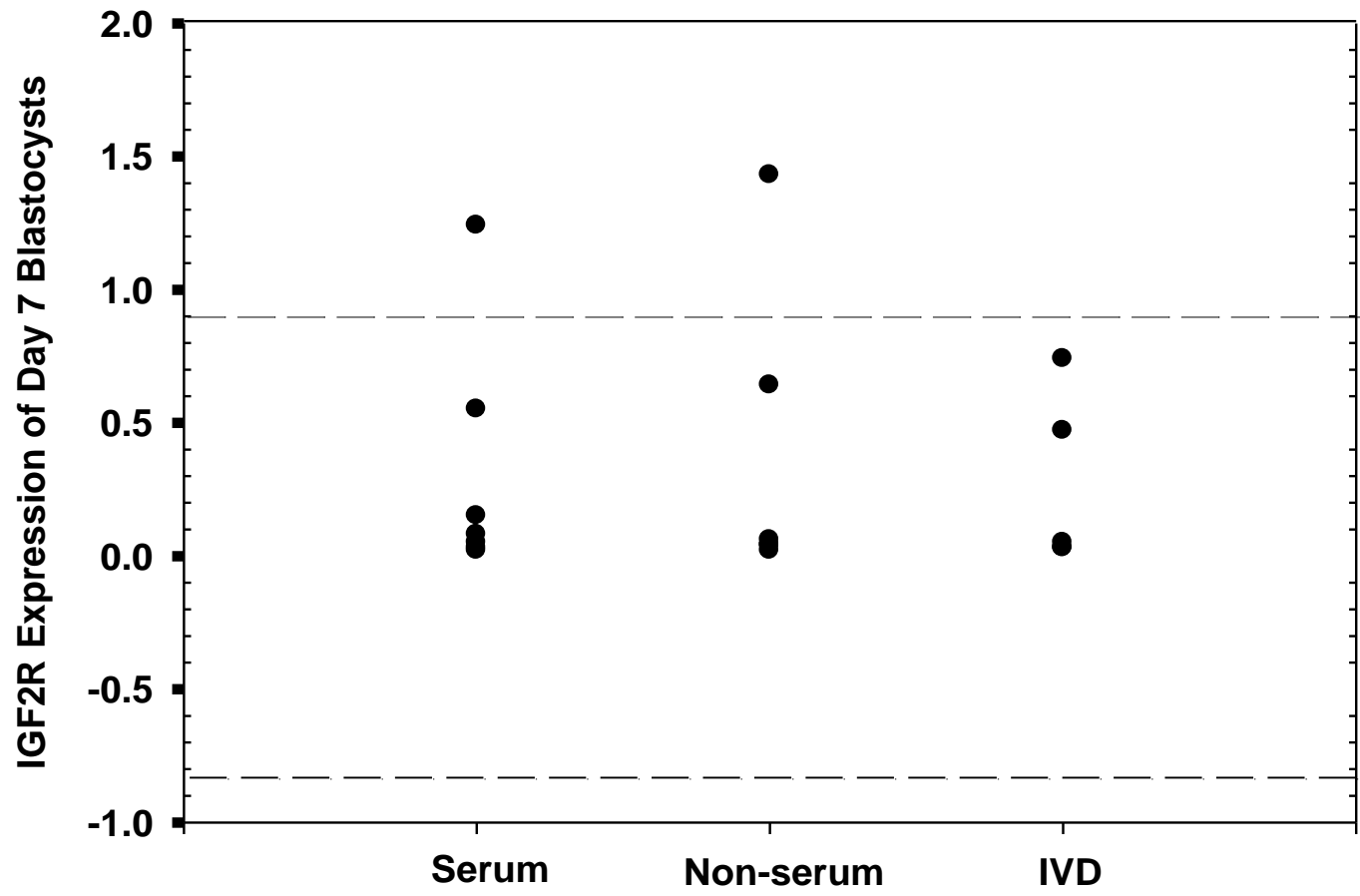


Figure 4 Distribution of IGF2R expression levels and 95% confidence interval for day-7 blastocyst pools (-0.39 to 0.93)

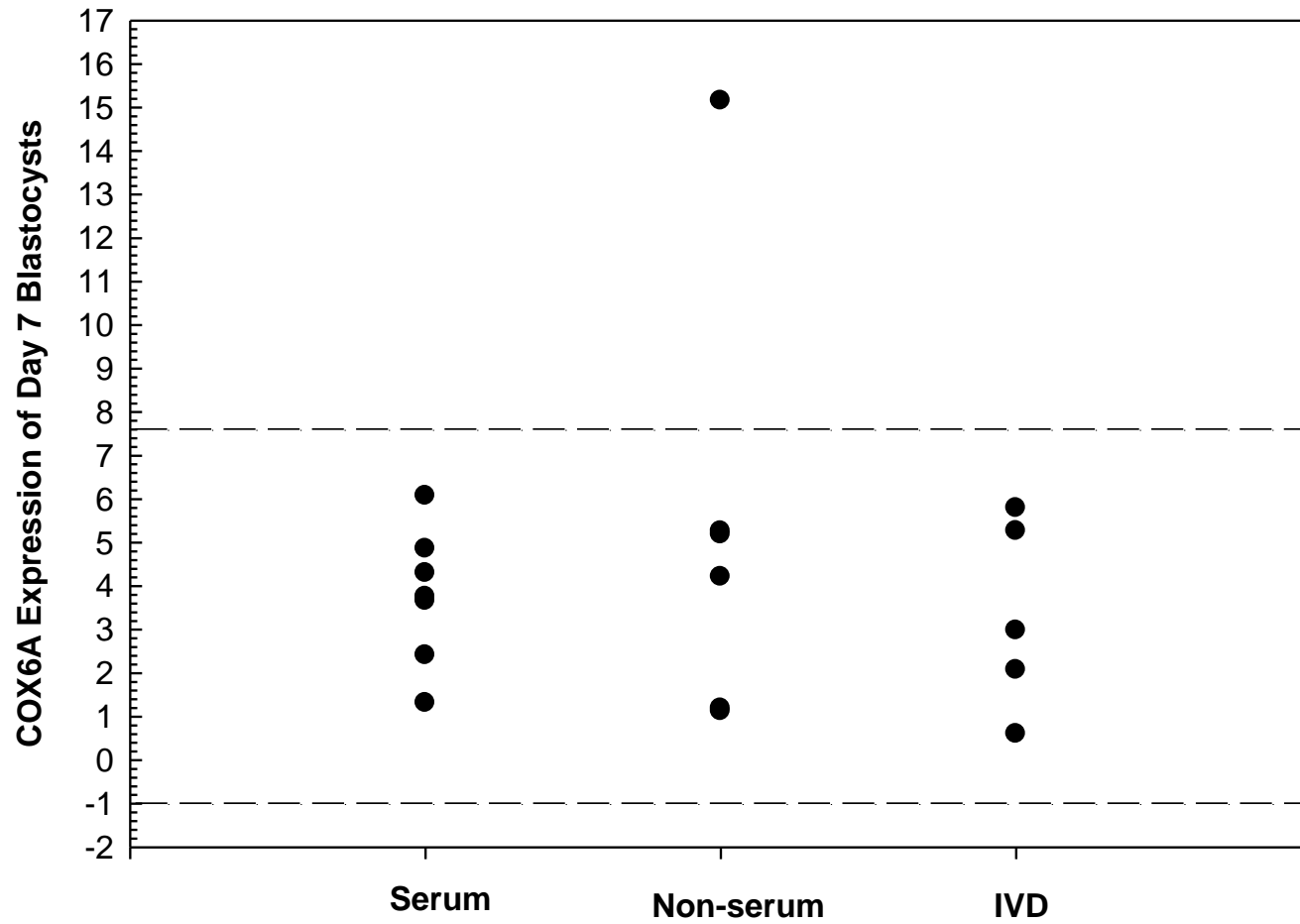


Figure 5 Distribution of COX6A expression levels and 95% confidence interval for day-7 blastocyst pools (-1.02 to 7.7)

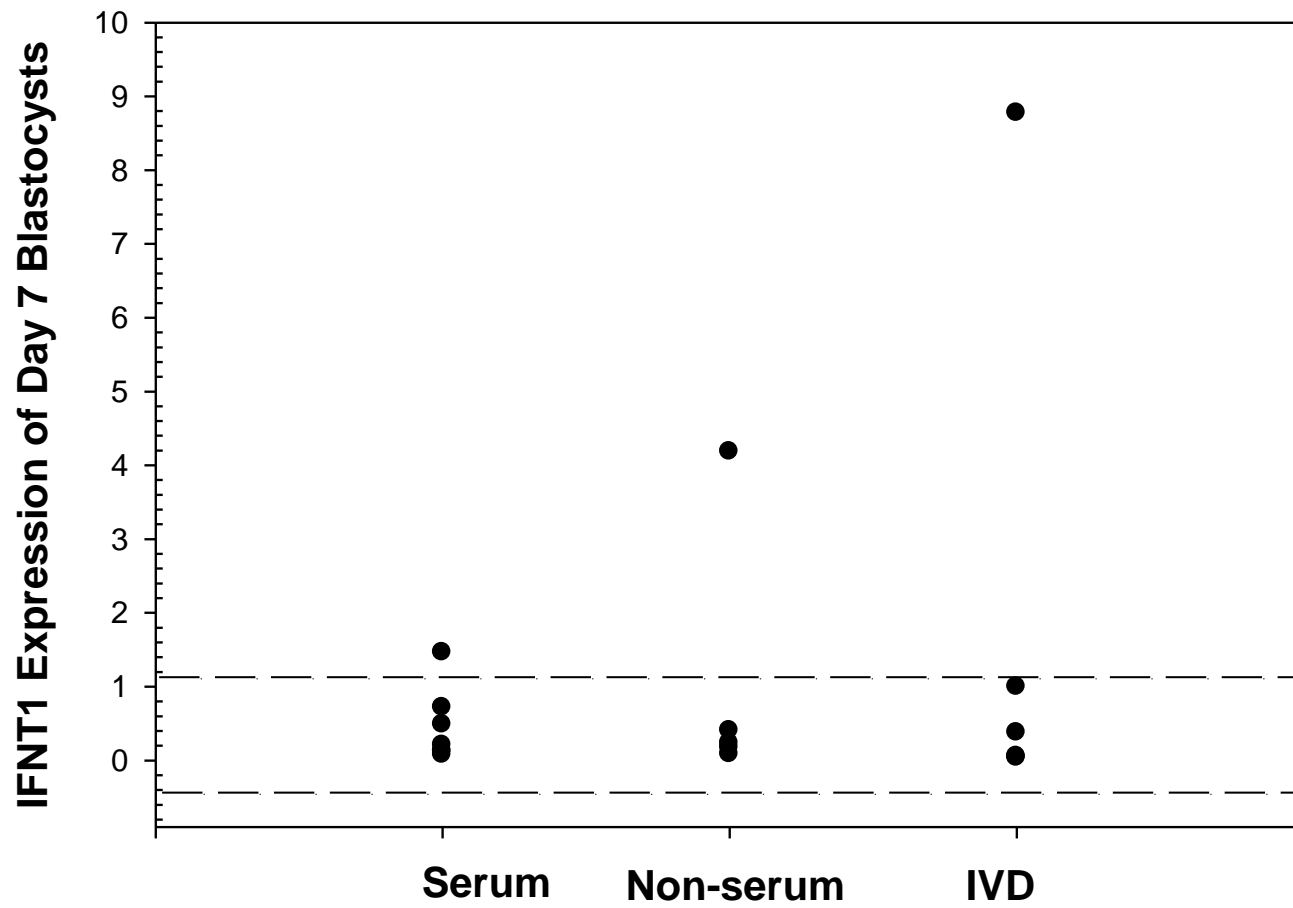


Figure 6 Distribution of IFNT1a expression levels and 95% confidence interval for day-7 blastocyst pools (-0.53 to 1.11)

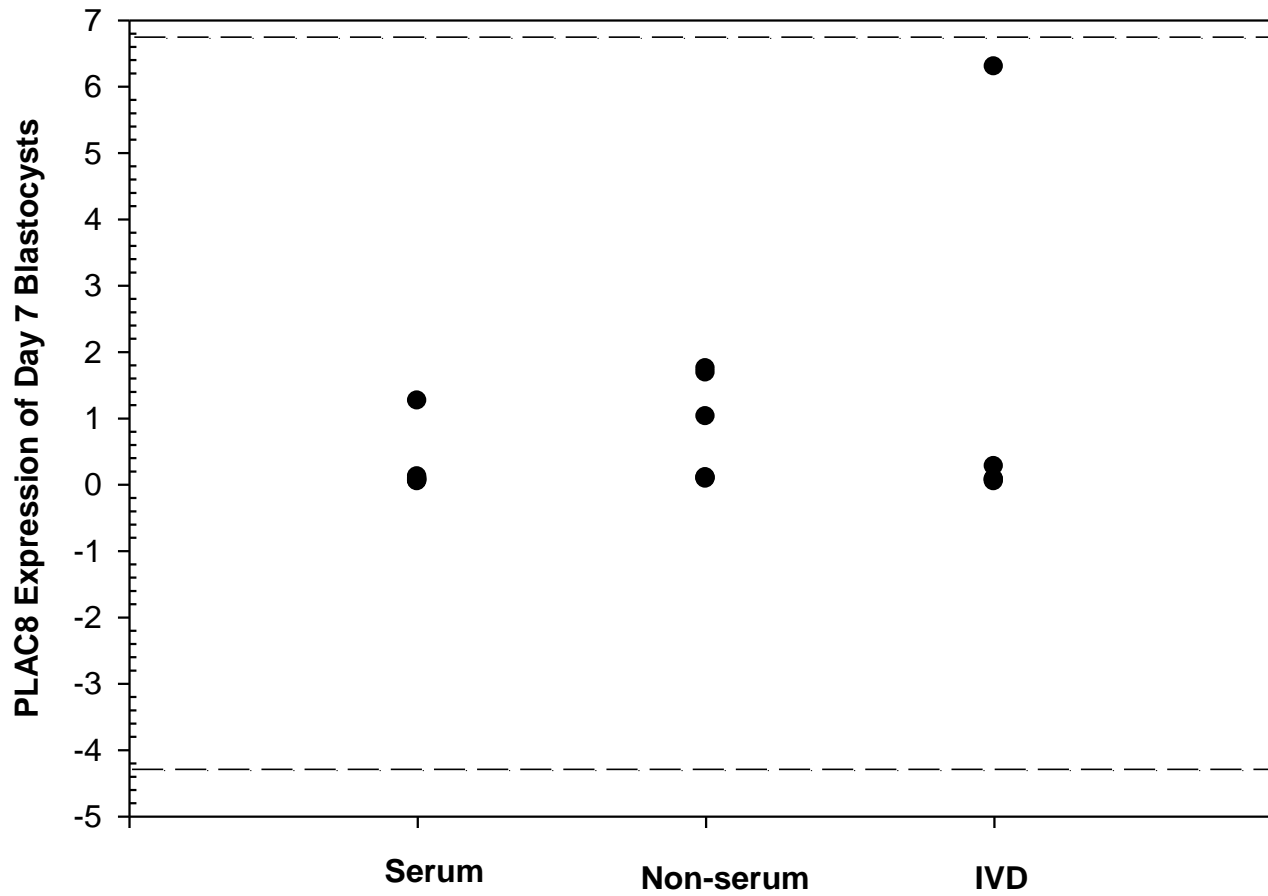


Figure 7 Distribution of PLAC8 expression levels and 95% confidence interval for day-7 blastocyst pools (-4.19 to 6.89)

Experiment 2

Day-14 embryos were classified as elongated and ovoid according to their stage of development. Initially, the serum treatment consisted of 22 embryos (16 elongated and 6 ovoid), the no-serum treatment consisted of 10 embryos (5 elongated and 5 ovoid) and the IVD group consisted of 11 embryos (6 elongated and 5 ovoid). However, after evaluating photographs taken to all embryos and the expected length at this stage, it was decided to exclude all the ovoid embryos from all treatments. Similarly, five elongated embryos from the serum treatment were withdrawn from gene expression analysis because the mRNA was isolated by a different method. See figure 8 and 9 to observe the difference in shape and length between these two stages of development. There was no difference ($P=0.19$) in the length of all day-14 embryos (Table 8); however, there was a significant difference ($P<0.002$) of embryo length between elongated IVD embryos and both treatments groups of IVP elongated embryos (Table 9). No significant difference were observed between recovery rate for embryos culture with and without serum ($P = 0.194$). Mean expression for COX6A, IFNT1a, IGF2R and PLAC8 did not differ among treatments ($P\leq 0.32$; fig.10). The same method previously described was used in treatment 2 to observe gene expression distribution of the genes of interest. In this way, altered expressions can be more easily observed. In the IVP serum treatment, 3 out of 11 samples had upregulated IFNT1a expression over two fold standard deviation above the mean of IVD embryos (fig.12). In PLAC8 expression, 2 out of 11 samples were upregulated two fold above IVD mean (fig.13). In IGF2R expression of serum treatment 4 out of 11 samples were above 2 standard deviations above IVD

mean (fig. 14). However, there was not any COX6A abnormal expression at this stage (fig.11).

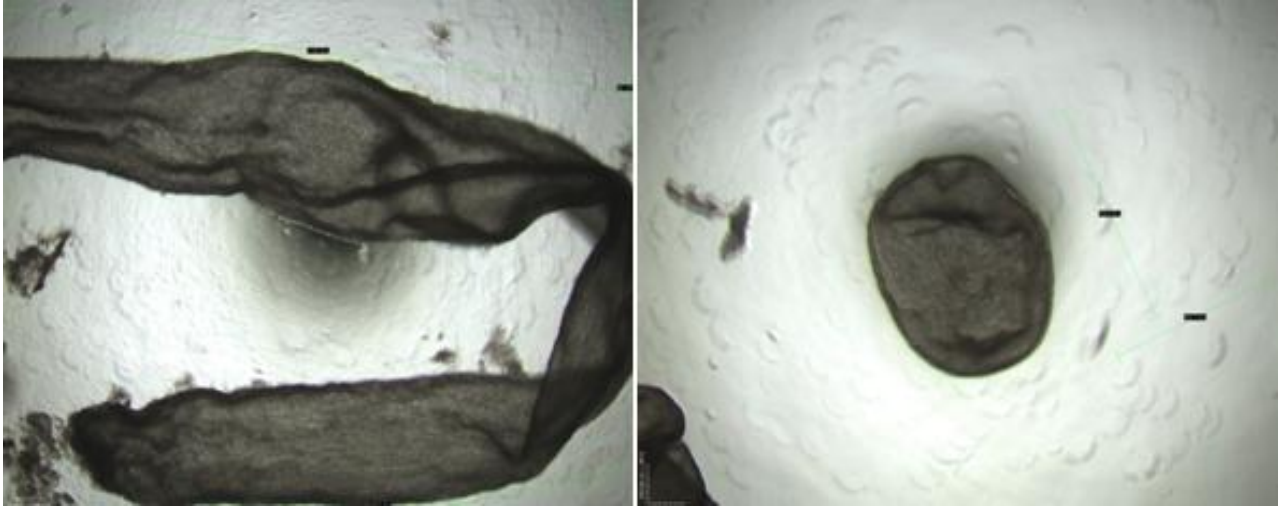


Figure 8 Day-14 elongated and ovoid embryos from IVP with serum treatment

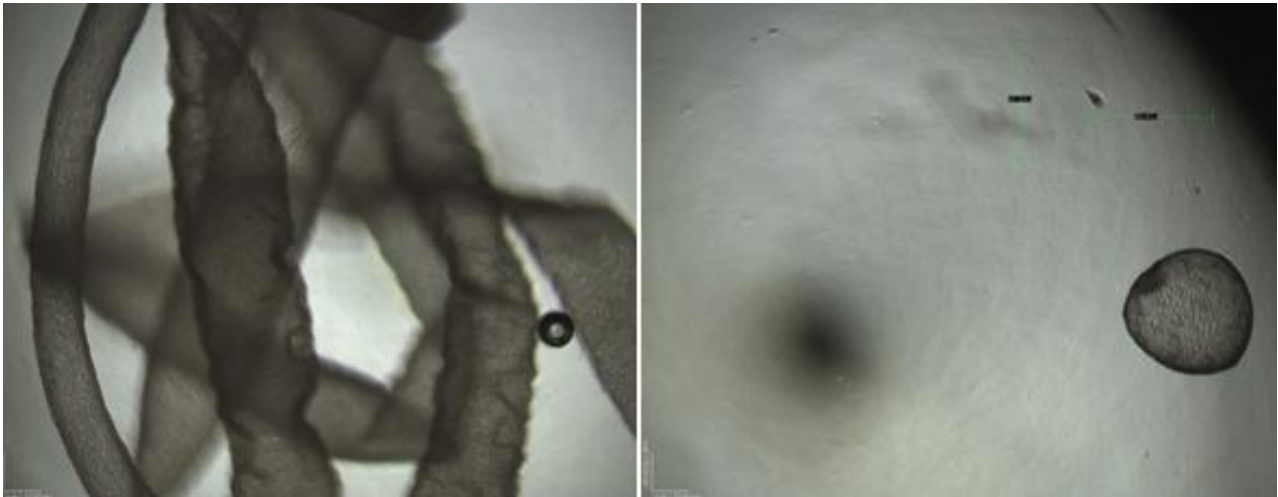


Figure 9. Day-14 elongated and ovoid in vivo derived embryos

Table 8 Length of all day-14 embryos (ovoid and elongated)

Treatment	Embryos	Length (μm)	SE (μm)
No Serum	11	1761.1 ^a	716.3
Serum	22	2663.7 ^a	482.9
In vivo	11	3595.0 ^a	682.9

^a Means with different subscripts are statistically significant

Table 9 Length of elongated day-14 embryos

Treatment	Embryos	Length (μm)	SE (μm)
No Serum	5	2784.8 ^a	741.8
Serum	16	3395.3 ^a	414.7
In vivo	6	6297.7 ^b	677.2

^{ab} Means with different superscript are statistically different ($P < 0.002$)

Table 10 Embryos transferred on day 7 and recovered on day 14 of gestation

	Serum	No-serum
Transferred	28	30
Recovered	16	11
Recovery rate (%)	57 ^a	37 ^a

^a Percentages with different subscripts are statistically significant

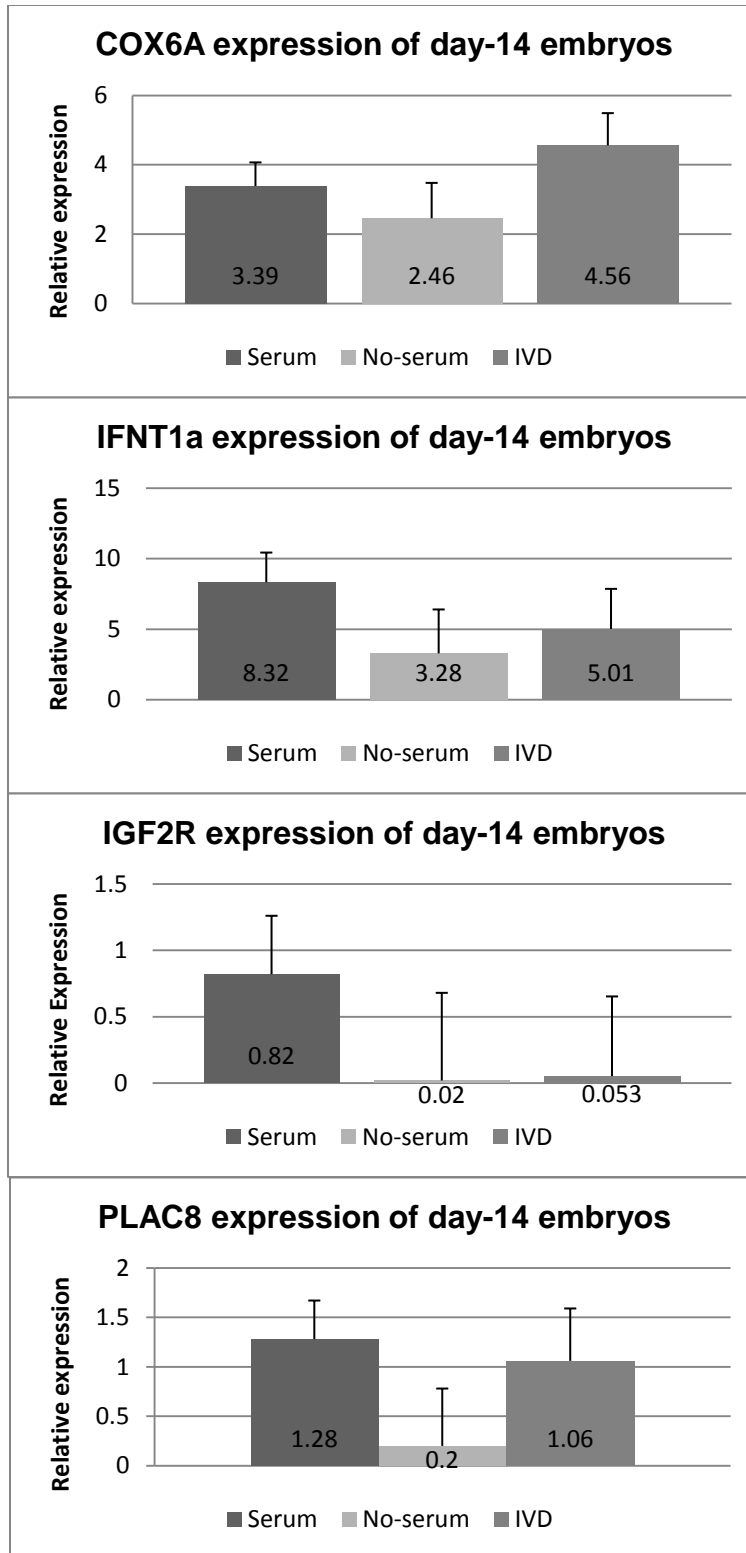


Figure 10 Relative expression of COX6A, IFNT1a, IGF2R and PLAC8 in each treatment in day-14 elongated embryos (LSM ± SE)

Table 11 Elongated embryos expression based on IVD 95% confidence interval for each gene of interest

Gene	Expression*	Serum	No-serum	IVD
COX6A	upregulated	0	0	0
	normal	11	5	6
	downregulated	0	0	0
IFNT1a	upregulated	3	0	1
	normal	8	5	5
	downregulated	0	0	0
IGF2R	upregulated	4	0	0
	normal	7	5	6
	downregulated	0	0	0
PLAC8	upregulated	2	0	0
	normal	9	5	6
	downregulated	0	0	0

* Upregulated and downregulated samples are those that are two standard deviations above or below the of the IVD mean for each gene of interest, respectively.

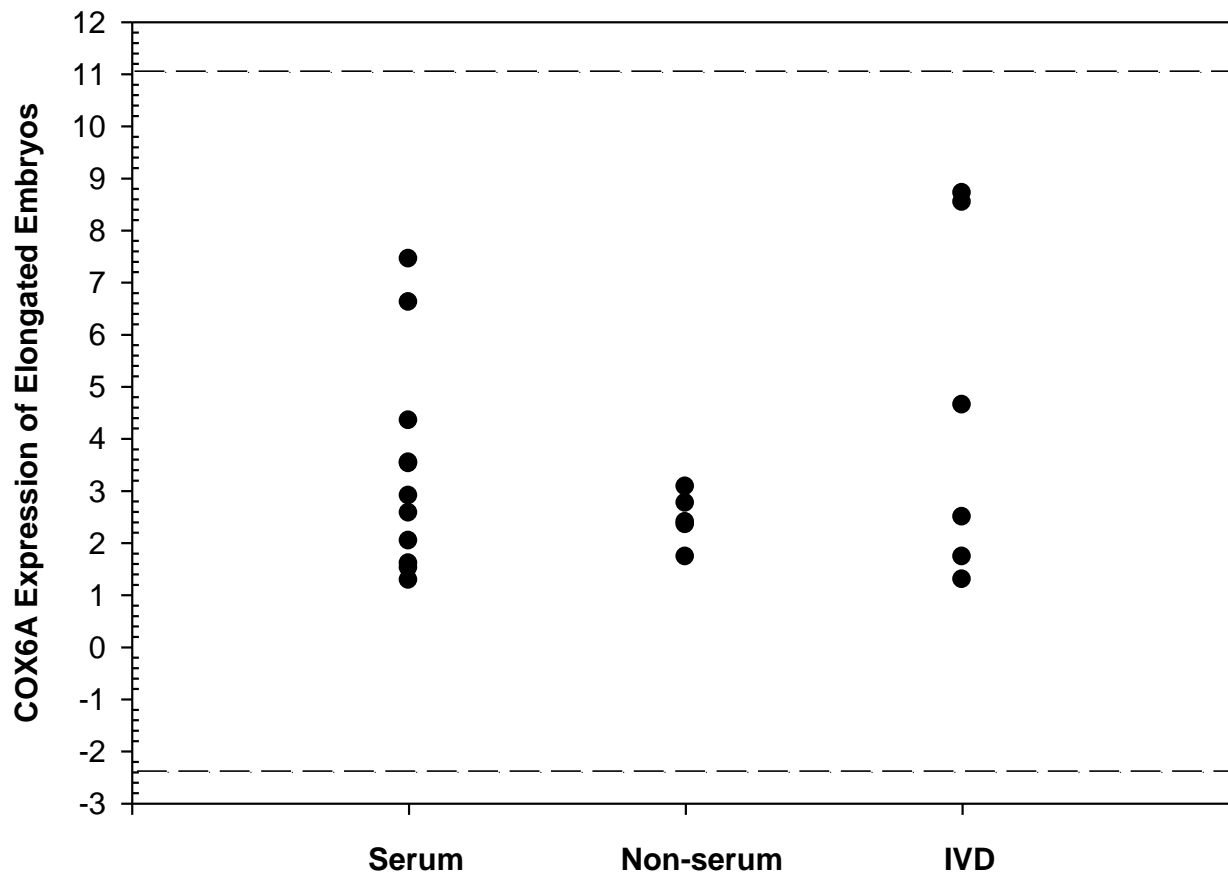


Figure 11 Distribution of COX6A expression levels and 95% confidence intervals for elongated embryos (-2.13 to 11.25)

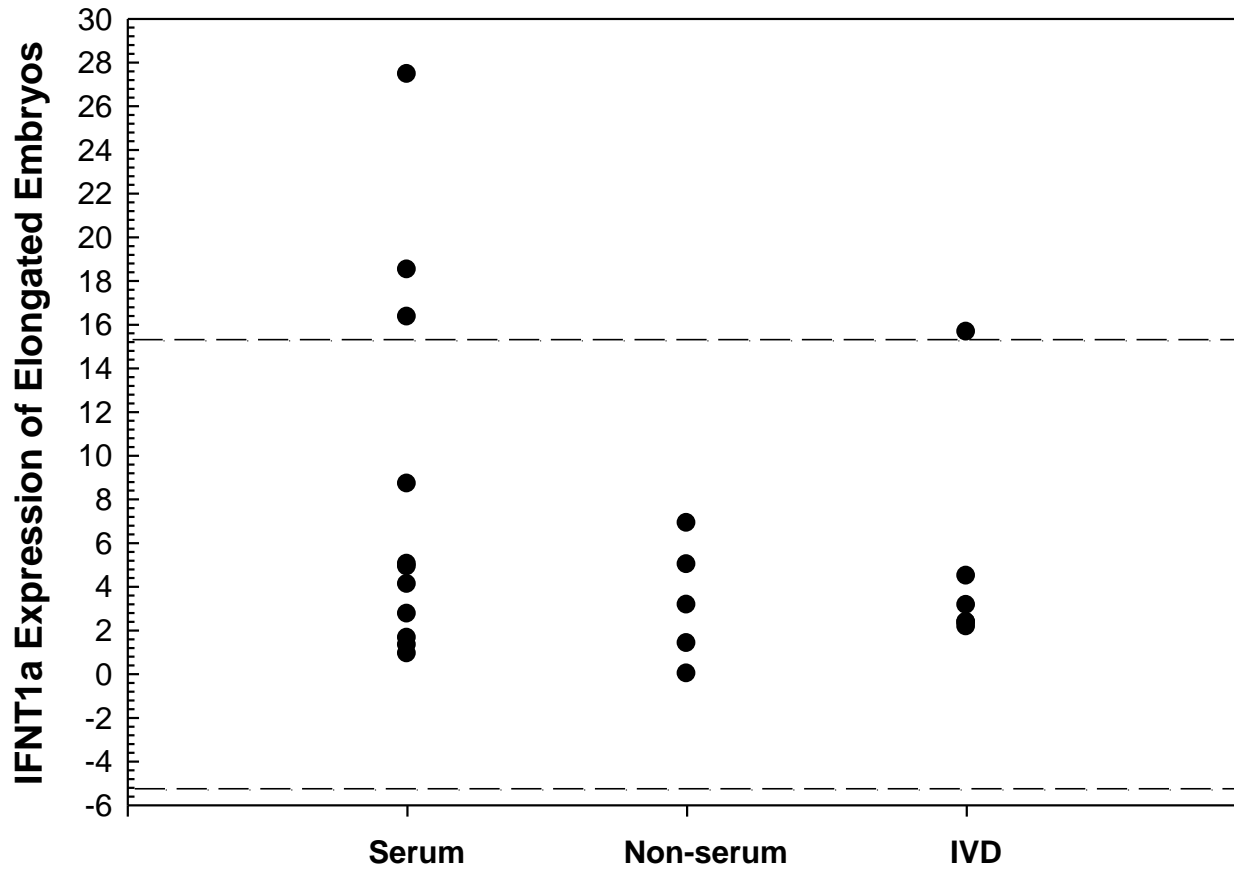


Figure 12. Distribution of IFNT1a expression levels and 95% confidence intervals for elongated embryos (-5.53 to 15.55)

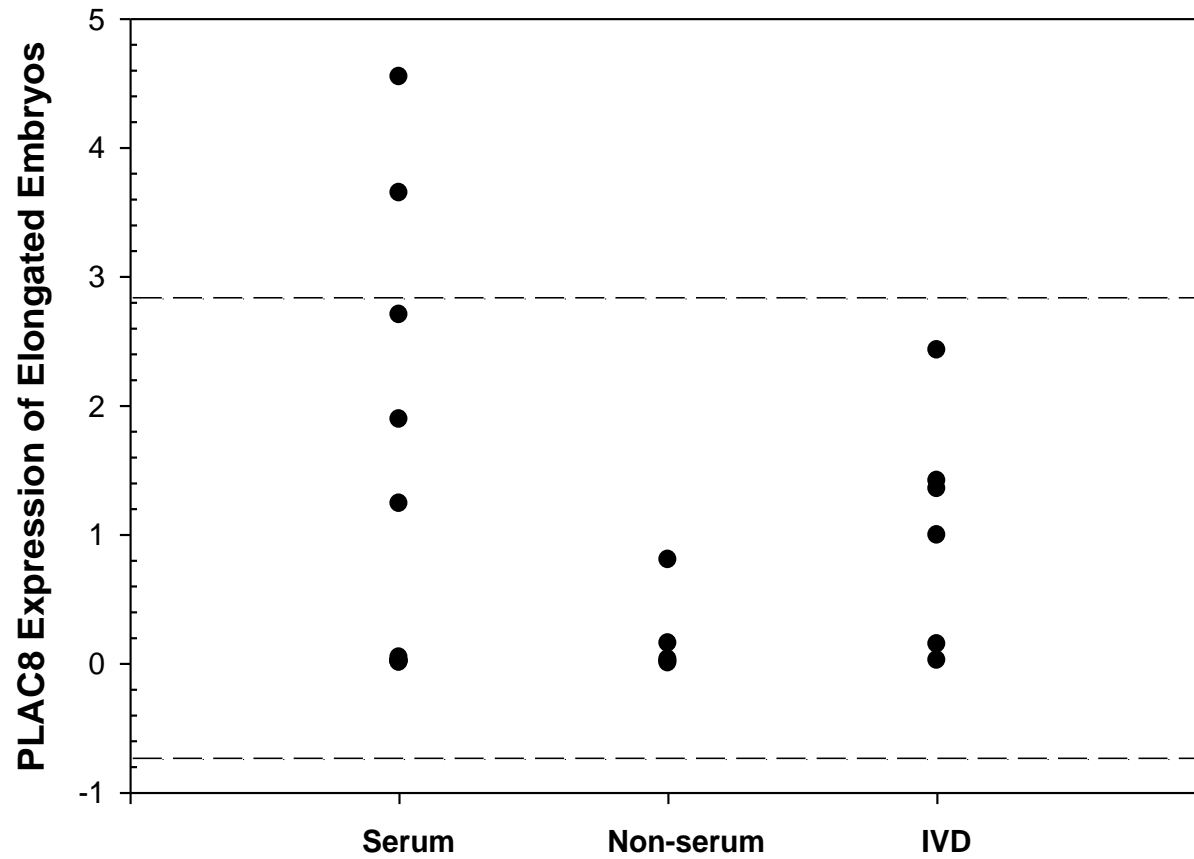


Figure 13. Distribution of PLAC8 expression levels and 95% confidence intervals for elongated embryos (-0.73 to 2.85)

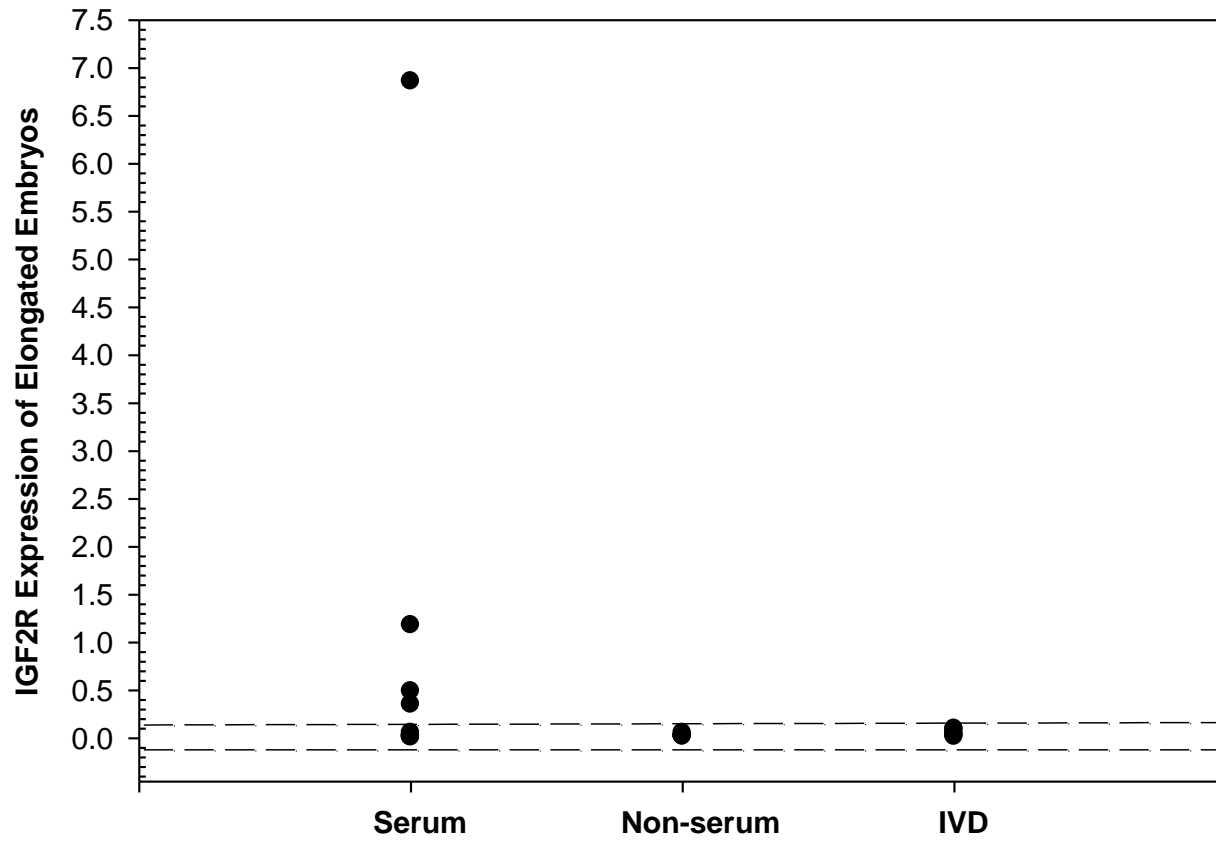


Figure 14. Distribution of IGF2R expression levels and 95% confidence intervals for elongated embryos (-0.02 to 0.125)

Pattern of IFNT1a

IFNT transcripts increased significantly between day 7 pools and day 14 elongated embryos across all treatments ($P < 0.004$; Table 12), but the other genes analyzed did not differ. No treatment interaction was found across stages.

Table 12 Transcript levels of IFNT1 at two developmental stages

Stage	Mean	SE
Day 7	1.02 ^a	1.14
Day 14	6.27 ^b	1.27

^{ab} Means with different superscripts are different ($P < 0.004$)

Discussion

There is abundant information about the short term effects of the addition of serum to culture media. These short term effects could be beneficial or detrimental depending on the time of inclusion and the dose. It has been demonstrated that early addition of serum and high levels of glucose inhibits early cleavage, but the addition of these components to culture medium will stimulate embryo development at later stages (Schini and Bavister, 1988; Pinyopummintr and Bavister, 1991; Takahashi and First, 1992; Thompson et al., 1998; Rizos et al., 2002b; Rooke et al., 2007). For these reasons, the culture medium used in the present study was modified in order to fulfill embryo nutritional requirements by excluding the use of serum and low levels of glucose (0.4 mM) during the first 72 hours of culture. After 72 hours post-insemination glucose level was increased to 1.5 mM in all treatments. Under these conditions, the

addition of 5% calf serum increased the proportion of day-7 blastocysts compared to mSOF_{aa} without serum.

A favorable effect has been observed when serum is added from 8-cell to the early morula stage in terms of blastocyst rates (Khurana and Niemann, 2000; Rizos et al., 2002b; Rooke et al., 2007). Although in this study blastocyst rates for day-8 and day-9 embryos were not recorded and analyzed. Other authors have observed that either serum or no-serum culture treatments yield similar cumulative blastocyst rates from day 7 to day 9 of culture; however, when only day-7 blastocyst rates are taken into account, blastocyst rates tend to be greater with serum treatment than with no-serum treatment (Enright et al., 2000; Rizos et al., 2003). Therefore, it was concluded that greater blastocyst rates obtained with serum treatments were due to a faster blastulation, which does not imply that addition of serum improves embryo development because, blastocyst derived from culture system with serum may have altered embryo metabolism, morphology and biochemistry (Thompson et al., 1995; Thompson, 1997; Ferguson and Leese, 1999; Crosier et al., 2000, 2001; Rizos et al., 2002a). However, embryos that become blastocysts earlier during in vitro culture have higher cell numbers and less apoptotic cells than embryos that become blastocyst after day 9 of culture (Hasler et al., 1995; Byrne et al., 1999; Enright et al., 2000). Nevertheless, the addition of serum can cause alterations in embryo morphology and metabolism, such as increased lipid droplets, increased size blastocysts, increased number of apoptotic cells and alteration in mitochondria distribution (Thompson, 1997; Byrne et al., 1999; Crosier et al., 2000, 2001).

The shape of day-14 embryos have been described to be spherical, ovoid and elongated (Alexopoulos and French, 2009). These findings are in agreement with embryo shapes observed in the present study. However, the day-14 ovoid embryos collected were excluded from the present study due to they were considered as degenerated embryos because according to previous studies the length of day-14 embryos should be at least 0.85 mm in length (Block et al., 2007; Menezo et al., 1982). The length of day-14 IVD elongated embryos differed ($P < 0.002$) from either serum or no-serum treatments; although there were no significant difference between both IVP treatments, embryos cultured with serum tend to be larger than embryos cultured without serum (3395.3 vs 2784.8, for serum and no-serum, respectively). Results concur with those presented by Bertolini et al. (2002), who observed that IVD embryos were larger than IVP embryos at day-16 after fertilization.

The expressions of COX6A, IFNT1a, IGF2R and PLAC8 were detected in all of the single embryos or embryo pools analyzed in the present study, but no differences were found in transcript levels between serum, no serum and IVD embryos in either blastocyst pools or elongated embryos. These results are in agreement with the results obtained by other authors (Kubisch et al., 2001). On the other hand, conflicting gene expression differences have been observed between in vitro produced embryos with serum, no-serum and IVD embryos (Kubisch et al., 1998; Wrenzycki et al., 2001; Lonergan et al., 2003; Rizos et al., 2003). However, the majority of these studies tend to show that embryos cultured with serum have higher transcripts of IFNT1a and lower transcript levels of IGF2R than no-serum and IVD embryos.

When low relative expression with high variance is obtained during gene expression studies, it is difficult to find differences between treatments using the mean relative expression level method. Therefore, a confidence interval was utilized in order to observe altered expressions (upregulated or downregulated) of individual samples in each treatment. Several upregulated samples were observed at the two different stages, but none of the genes of interest were downregulated throughout the study. In day-7 embryos, two IGF2R, one COX6A and three IFNT1a upregulations were observed. In day-14 elongated embryos, upregulated expressions of IFNT, IGF2R and PLAC8 on elongated day-14 embryos produced with serum compared to the no-serum and IVD elongated embryos were observed.

IFNT is secreted predominantly by trophoblastic cells, it is possible that embryos cultured with serum may produce more IFNT because embryos cultured with serum have an increased ratio of TE:ICM cells (Iwasaki et al., 1990; Du et al., 1996). However, no difference was found in IFNT1a expression between treatments in this study. On the other hand, a higher proportion of elongated embryos cultured with serum were considered to be upregulated compared to no-serum and IVD embryos, and the relative levels of IFNT1a differed between day-7 and day-14 embryos across all treatments ($P < 0.004$); there was not treatment interaction, and all treatments and developmental stages showed the same trend. This data confirms that transcript levels of IFNT1a increased from the blastocyst stage to the day-14 embryos, which is in agreement with previous data (Bertolini et al., 2002; Ushizawa et al., 2004; Rodriguez-Alvarez et al., 2009; Rodriguez-Alvarez et al., 2010a; Rodriguez-Alvarez et al., 2010b). These results and the methods used to analyzed gene expression in the present study

suggest that the secretion of IFNT1a increase per cell base from the blastocyst stage to the elongated stage, regardless of the cell number increment.

No differences in IGF2R were observed at the blastocyst stage and at the elongated stage, but more frequent abnormal transcript levels of IGF2R were observed in the IVP treatments. This was evident at the elongated stage were 3 out of 11 embryos had upregulated expression. Similar results have been obtained by others; however, more abnormal expressions were found in IVP treatments (Moore et al., 2007a). On the other hand, some studies have observed that IGF2R was downregulated in IVP embryos cultured with serum compared to IVD embryos (Young et al., 2001; Bertolini et al., 2002; Nasser et al., 2008), and fetuses with overgrowth have been associated with low levels of IGF2R and high levels of IGF2 (Lau et al., 1994; Bertolini et al., 2002). It has been suggested that in IGF2R expression studies, IGF2 and IGF2BP expression should be examined in order to obtain a stronger analysis base on these correlations (Lau et al., 1994; Young et al., 2001; Farin et al., 2010).

Transcript levels of COX6A did not differ across treatments and between stages (day-7 and day-14), just one sample was upregulated in the no-serum treatment at the day-7 stage. However, Everts et al., (2008) observed 5-fold higher expression of COX6A in placental tissues derived from artificial insemination compare to IVP placental tissues from close to term pregnancies. It is possible that expression levels of COX6A differ between IVP and IVD until late developmental stages of pregnancy, and it may not differ or have abnormal expressions between IVP and IVD at the preimplantation embryonic stages.

Similar to previous studies (Nasser et al., 2008), transcript levels of PLAC8 did not differ between IVD and IVP embryos. It also has been observed that either IVP and IVD embryos that progressed to term gestation expresses similar PLAC8 expression levels at the blastocyst stage (Tesfaye et al., 2009). Although in the present study day-14 elongated embryos did not differ between treatments, 2 out of 11 embryos were consider to have upregulated expression of PLAC8; however, no PLAC8 upregulations were found in the IVP without serum and IVD elongated embryos. Even though IVD embryos were larger and have more TE cells, the IVP embryos cultured with serum had 18% of its samples upregulated.

At the blastocyst stage, a correlation among PLAC8 and IFNT1a was observed, which suggests that the activation or secretion of these two trophoctoderm-originated proteins may be activated at the same developmental time or that they are regulated by the same mechanism or mechanisms. This finding agrees with previous studies that showed that IFNT can affect the transcription levels of other genes in the preimplantation embryos and in the endometrium (Satterfield, 2008; Mansouri-Attia et al., 2009a; Mansouri-Attia et al., 2009b). However, PLAC8 and IFNT1a correlation was not observed at the elongated stage. This suggest that even though these genes may both be activated blastocyst stage, the PLAC8 transcripts of day-14 elongated embryos did not increased from the blastocyst to the elongated stage as occur with IFNT transcripts.

CHAPTER IV

CONCLUSIONS

In the present study, serum addition stimulated earlier blastulation as has been showed by previous studies. At day-14 of gestation two shapes of embryos can be recovered ovoid and elongated, these two kinds of embryos were observed in both IVP treatments and IVD embryos. This finding suggests that even though IVD embryos are considered as the “gold standard”, it is possible to find non-competent embryo even when they are in vivo derived. Thus, caution should be excercised when embryos are selected for gene expression analysis.

At day 14, IVD embryos tended to be larger and looked more uniform than IVP embryos. Although no significant differences in gene expression were observed either at the blastocysts stage or at the elongated stage, some upregulated samples were observed in IVP treatments, specifically for the IGF2R, PLAC8 and IFNT expression of day-14 embryos cultured with serum. This suggests that culture with serum may increase the frequency of abnormal gene expression at elongated stages. It is probable that some of the effects of non-physiological maturation, fertilization and culture conditions may not occur immediately, but that they may occur at later developmental stages.

The increased IFNT transcript levels from the blastocyst stage to the elongated stage observed in this study is in agreement with previous studies. However, other proteins of trophoblastic origin like PLAC8 may not increased in the same manner as IFNT. Although PLAC8 and COX6A have been associated with developmental

competence in other studies, in the present study no difference in gene expression or gene pattern was observed.

In this study, no significant differences in gene expression were observed between treatments at the blastocyst and at the elongated stage. However, IGF2R expressions of IVP embryos cultured with serum have more upregulated samples (embryos) than IVD embryos; meanwhile, previous studies have showed an association between IGF2R downregulation and overgrowth. The upregulation of some samples cultured with serum warrant further studies in this area.

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APPENDIX A: PROTOCOLS

BOVINE IVF PROTOCOL

Preparations:

1. Prepare and label IVF-TALP, Sperm-TALP and HEPES-TALP (Appendix B) in advance, but the same day that fertilization will be performed
2. Move two centrifuge carriers to oven (39°C).
3. Make fertilization plates
 - a. Prepare a washing and a fertilization plate (4 wells Nunc® plate) with 425 µl of IVF-Talp per each well.
 - b. Equilibrate in CO₂ incubator (39°C) at least 3 hours.
4. Move the tube containing IVF-TALP medium to the CO₂ incubator (loose cap).
5. Fill 1 conical tube with 5 ml Sperm-TALP from the previously prepared Sperm-TALP
6. Transfer the 20 ml HEPES-TALP (cap tight) and 5 ml SP-TALP (cap tight) to the 39°C oven.
7. Prepare Isolate density gradient:
 - a. Label 1 conical tube “Isolate sperm gradient” and fill the tube with 1.5 ml of Isolate lower layer (90%) and very carefully and slowly dispense the 1.5 ml of Isolate upper layer (50%)
8. Carefully, transfer the Isolate gradient to the pre-warmed centrifuge carrier within the oven.

9. Move PHE (100 μ l) (Appendix B) and heparin (100 μ l) (Appendix B) from freezer to oven (39°C) with 15 minutes before starting the procedure. PHE should be covered with aluminum foil (light sensitive).

Procedures:

1. At 22-24 hours post-maturation thaw 1 straw of semen in water at 39°C for 30 seconds. When getting semen straws out of the liquid nitrogen tank, make sure not to raise anything above the frost line. Use semen forceps.
2. Dry a straw, hold it in a kimwipe to keep it warm and dark, cut the sealed end off and slowly layer thawed semen on top of the Isolate gradient by gently pushing the plug in the straw with a metal rod. Place the conical tube back into the centrifuge carrier and centrifuge at 1200 rpm for 12 min at 37°C.
3. Check viability of the thawed semen by placing a drop remaining in the straw on a slide. View at 40X magnification to assure that motile sperm are present.
4. While centrifuge is running, pour 2 ml of HEPES-TALP (from conical tubes in oven) into Petri dish (35 mm). Remove oocytes from maturation medium (plate/vial) and transfer to a separate corner in the HEPES-TALP. Thoroughly wash oocytes through 2 dishes of HEPES-TALP to remove any glucose from the maturation medium, which is detrimental to fertilization.
5. Transfer 50 oocytes to each well with 425 µl in a 4-well dish (first in washing plate and later move them to the fertilization plate) return IVF plate back to incubator when finished. *You only have 15 min to wash and transfer all oocytes to IVF 4-well plates. Set a timer and ask for help if necessary.
6. After centrifuge stops, carefully remove carrier with the Isolate gradient from centrifuge. There should now be a sperm pellet, if not you must start completely over with new gradient and semen.
7. Within the laminar flow hood and a sterile pasteur pipette, aspirate the Isolate down to the sperm pellet. Slowly add the 5 ml of pre-warmed Sperm-TALP to the conical

tube containing the sperm pellet. Put the tube into a second pre-warmed centrifuge carrier and centrifuge at 1,200 rpm for an additional 5 min.

8. After the centrifuge stops, aspirate the Sperm-TALP down to the sperm pellet. Return the conical tube with the sperm pellet to the oven.

9. Determine sperm pellet concentration

A. Gently swirl the sperm pellet to mix the sperm with any remaining medium. Use a clean pipette tip to transfer 5 μ l of sperm into 95 μ l of water, pipetting gently to mix. Label this vial as "hemocytometer"

B. Clean the hemocytometer and coverslip by washing with water followed by 70% EtOH; dry with a Kimwipe.

C. Using a new pipette tip, transfer 10 μ l of diluted sperm into each chamber (each side) of the hemocytometer.

D. Use 40X magnification to count sperm cells in the 5 squares arranged diagonally across the central square on one side of the hemocytometer. Use an event counter to keep track of how many cells are counted. Record the count on the "Sperm Dilution Work Sheet" (see below)

E. Continue counting on the second side of the hemocytometer counting 5 diagonally arranged squares to obtain the total hemocytometer count. If the count of one side varies more than 10% from the other side, then the diluted sample was not properly mixed. Repeat procedure starting at step 1. When the count is consistent, record the total count and continue the procedures.

F. Clean hemocytometer and coverslip with water followed by EtOH.

10. Preparing sperm suspension for insemination (See "Explanation of Sperm Suspension")

Note: The final sperm suspension used to IVF is composed of fertilization medium and sperm pellet produced by Isolate separation gradient. A worksheet is attached and can be duplicated and used to assist in calculating sperm suspensions (see below).

A. Calculations are based on the following parameters:

- a. 300 μ l of final sperm suspension will be prepared
- b. 1×10^6 sperm/ml is desired in the final fertilization medium
(this concentration can be adjusted if needed using Step 3 below)

B. Calculate the volume of sperm pellet needed per 300 μ l of final sperm suspension using the formula:

$7,500/X = \mu$ l of sperm pellet to make 300 μ l of final sperm suspension when inseminating with 1×10^6 sperm/ml

Where X is the average hemocytometer count (total hemocytometer count divided by 2)

C. Adjust for desired sperm concentration: If a concentration other than 1×10^6 sperm/ml is desired; To adjust this volume perform the following calculation:

Divide the average hemocytometer count calculated by the adjustment factor to yield the volume sperm pellet needed to prepare 300 μ l of final sperm suspension at the desired concentration.

Example: If a bull requires are 2×10^6 sperm/ml rather than 1×10^6 sperm/ml = adjustment the conversion factor to $15,000/X$ in step 10

- D. Calculate volume of fertilization medium needed in the final sperm suspension: Subtract the volume found in Step 10C from 300 μ l
- E. Place the calculated amount of fertilization medium (D) into an Eppendorf microcentrifuge tube. Then add the calculated amount of sperm pellet (C) to the tube. Sperm stick to plastic, so add the fertilization medium to the tube first. Mix gently by pipetting up and down several times within the tube. Immediately begin fertilizing each well since the pH of this solution will change rapidly.

Fertilization

1. Add 20 μ l heparin (for a final concentration of 2 μ g/ml of heparin in the fertilization medium), 2 μ l of PHE and 2 μ l of final sperm suspension to each well.
2. Record time and date on each fertilization dish.
3. Incubate for 18 h at 39°C in a humidified atmosphere of 5% CO₂

Culture

1. Make five 30 μ l drops of culture medium (SOF_{aa}) in a 35 mm Petri dish. Cover the drops with equilibrated oil. Make sure to equilibrate the culture medium for at least 30 minutes in the CO₂ incubator before preparing the culture and washing plate.

Note: The culture (five 30 μ L drops of SOF) and washing (four 70 μ l drops of SOF) plates

should be prepared after fertilization (between 15 and 18 hours in advance to moving the embryos into culture medium) and put them in the CO₂ incubator.

2. Thaw one vial of hyaluronidase (1 mg/ml). Place the solution in a 15 ml tube and vortex at maximum speed for 2 minutes.
3. Rinse the tube with HEPES-TALP and transfer the oocytes to a 35mm Petri dish.
4. Rinse the presumptive embryos two times in HEPES-TALP in a 35mm Petri dish
5. Wash the oocytes in every 70 μ l drop of SOF_{aa}
6. Move 15 presumptive zygotes in every culture drop (30 μ l SOF_{aa})

BLASTOCYST POOLS mRNA ISOLATION PROTOCOL

1. Store pools of embryos (5-10 blastocyst per pool for Experiment 1 in approximately 3µl of PBS plus 0.1% polyvinyl alcohol in 1.5 ml siliconized tubes.
2. Bring Dynabeads mRNA® DIRECT™ Kit (Dynal Biotech, Inc., Lake Success, NY, USA, Cat No. 610.11) to room temperature.
3. Lyse the the blastocyst pool in 50 µl of lysis/binding buffer (100 mM Tris HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulfate, 5 mM dithiothreitol) and vortex for 10 seconds.
4. Centrifuge the samples at maximum speed for 15 seconds and incubate at room temperature for 10 minutes.
5. Add 10 µl of pre-washed oligo dT Dynabeads (dT25) to the sample. Pre-wash beads In lysis/binding buffer.
6. Incubate the Dynabeads and sample by rotating on a mixer or roller for 10 min at room temperature.
7. Place the tubes in a Dynal MPC-E-1 magnetic separator for 2 minutes.
8. After removal of the supernatant, wash the beads once with 100 µl of buffer A (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA, 0.1% lithium dodecylsulfate) and three times with 100 µl of buffer B (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA).
9. Elute the RNA from the beads by adding 11 µl of sterile water and heating the sample at 70°C for 2 minutes.

10. Use the sample directly for reverse transcription.

- Protocol from Wrenzycki et al., (1999, 2001)

DAY-14 EMBRYO mRNA ISOLATION PROTOCOL

1. Store day-14 embryos a minimal volume of PBS plus 0.1% polyvinyl alcohol in 1.5 ml siliconized tubes.
2. Bring Dynabeads mRNA® DIRECT™ Kit (Dynal Biotech, Inc., Lake Success, NY, USA, Cat No. 610.11) to room temperature.
3. Lyse the day-14 embryo in 150 µl of lysis/binding buffer (100 mM Tris HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulfate, 5 mM dithiothreitol) and vortexing for 10 seconds.
4. Centrifuge the samples at maximum speed for 15 sec and incubate at room temperature for 10 minutes.
5. Add 50 µl of pre-washed oligo dT Dynabeads to the sample. Pre-wash beads In lysis/binding buffer.
6. Incubate the Dynabeads and sample by rotating on a mixer or roller for 10 min at room temperature.
7. Place the tubes in a Dynal MPC-E-1 magnetic separator for 2 minutes.
8. After removal of the supernatant, wash the beads once with 100 µl of buffer A (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA, 0.1% lithium dodecylsulfate) and three times with 100 µl of buffer B (10 mM Tris HCl (pH = 8.0), 150 mM LiCl, 1 mM EDTA).
9. Elute the RNA from the beads by adding 11 µl of sterile water and heating the sample at 75°C for 2 minutes.

10. Use the sample directly for reverse transcription.

- Protocol from Wrenzycki et al.,(1999, 2001)

cDNA SYNTHESIS PROTOCOL (iSCRIPT)

1. Mix 4 μ l of iScript reaction mix, 1 μ l of reverse transcriptase, 4 μ l of nuclease-free water (Appendix B) (Bio-Rad Laboratories, Inc., Hercules, CA, Cat No. 170-8891).
2. Make master mixers when possible.
3. Add 11 μ l of mRNA sample.
4. Extra mix should be prepared for the no mRNA template negative control.
5. Total volume mix should be 20 μ l.
6. Place the mix in the thermocycler.
7. Run the thermocycler at 25°C for 5 minutes, 42°C for 30 minutes, denaturation at 85°C for 5 minutes, and a final hold at 4°C.
8. Label and store the cDNA samples in a -80°C freezer.

RT-PCR PROTOCOL

1. Prepare a RT-PCR Mix of 19 μ l auclaved water, 2 μ l cDNA sample and 25 μ l JumpStart™ REDTaq® ReadyMix™ Reaction Mix for PCR (Sigma-Aldrich, Inc., St. Louis, MO, Cat. No. P-0982) Make a bigger volume for multiple samples when necessary (Master Master-MM; Appendix B).
2. Add 1 μ l of both sense and antisense primers (20 μ mol) to the RT-PCR Mix of each gene.
3. Place the 96-well plate in the thermocycler.
4. Run one cycle of 1 minute at 95°C; 35 cycles of PCR (95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds); followed by 72°C for 4 minutes; with a final hold at 4°C.

Q-PCR PROTOCOL

1. Prepare a Q-PCR Mix with 10 μ l of SsoFast™ EvaGreen® Supermix 2X (Bio-Rad Laboratories, Inc., Hercules, CA, Cat. No. 172-5200) and 6 μ l of water (Appendix B)
2. Make sure to prepare enough Q-PCR Mix per cDNA sample/Calibrator/H₂O for the 6 genes).
3. Prepare a Q-PCR Master Mix by adding 1 μ l of each primer (20 pmol of sense and antisense) (Appendix B) for each gene.
4. Prepare a Q-PCR Reaction Mix by adding cDNA/Calibrator/ H₂O.
5. Place the sample in it respective well and put the plate in the thermocycler
6. Run one cycle of 3 minutes at 95°C; 40 cycles of PCR (95°C for 10 seconds and 55°C for 45 seconds); a melting curve consisting of 95°C for 1 minutes followed by 55°C for 1 minute, a step cycle with 80 repeats starting at 55°C for 10 seconds with a +0.5°C/seconds transition rate; and a final hold at 4°C.

GENE EXPRESSION QUANTIFICATION PROTOCOL

1. Use a mix of cDNA from an elongated day-14 in vivo derived embryo was used as a calibrator for the target genes. The same calibrator mix should be used throughout all the experiments and plates.
2. Use GAPDH as the endogenous control gene.
3. Use the signal of the reference gene GAPDH to normalize the target gene signals of each sample.
4. Calibrate the ΔCT for gene transcription against the sample used as calibrator.
5. Report gene quantification as relative transcription or the n-fold difference relative to a calibrator.
6. Calculate the relative linear amount of target molecules relative to the calibrator by using the following equation.

$$\text{n-fold difference} = \frac{\text{Efficiency Target Gene}^{\Delta CTT}}{\text{Efficiency Reference Gene}^{\Delta CTR}}$$

7. Efficiencies are obtained via a 10-fold dilution standard curve performed prior to analysis.
8. Calculate the ΔCTT value by subtracting the sample CT value of the target gene from the calibrator CT value of the target gene.
9. Calculate the ΔCTR value by subtracting the sample CT value of the reference gene (GAPDH) from the calibrator CT value of the reference gene.

IN VIVO EMBRYO PRODUCTION – SYNCHRONIZATION AND SUPEROVULATION

1. Administer an injection of 15 mg progesterone and 2.5 g estradiol-17 β intramuscular (IM) at CIDR insertion (day 0), or start of treatment to each donor.
2. Administer IM Follitropin-V (Bioniche Animal Health, ON) FSH injections (20 mg/ml) to each donor as follows:
 - Day 4: A.M. 1.8 ml
P.M. 1.8 ml
 - Day 5: A.M. 1.5 ml
P.M. 1.5 ml
 - Day 6: A.M. 1.1 ml
P.M. 1.1 ml
 - Day 7: A.M. 0.6 ml + PGF₂ α
P.M. 0.6 ml + PGF₂ α

Total FSH Dose: 10 ml
3. Administer Lutalyse PGF₂ α injections (5 mg/ml) IM to each donor in the morning and night of day 7 of treatment.
4. On the P.M. of day 7 of treatment remove the CIDR inserts.
5. Check for estrus (AI is performed 12 and 24 hours after the onset of standing estrus).
6. Nonsurgically collect embryos from donors on day 8 post-AI

APPENDIX B

MEDIA FORMULATIONS AND STOCK SOLUTIONS

SPERM – TL¹

Component	Product Number	Company	Final (mM)	mg/100 ml
NaCl	S-5886	Sigma	100	582
KCl	P-5405	Sigma	3.1	23
NaHCO ₃	S-5761	Sigma	25	209
NaH ₂ PO ₄	S-5011	Sigma	0.29	3.48
HEPES	H-3375	Sigma	10	238
Lactic acid	L-7900	Sigma	21.6	183.4 µl
Phenol red	P-0290	Sigma	1 µl/ml	100 µl
*CaCl ₂ ·2H ₂ O	C-7902	Sigma	2.1	29
*MgCl ₂ ·6H ₂ O	M-2393	Sigma	1.5	31

¹Add NaCl, KCl, NaHCO₃, NaH₂PO₄, HEPES, lactic acid, and phenol red into a beaker. Bring volume to 90ml with ddH₂O and dissolve completely.

*CaCl₂·2H₂O and MgCl₂·6H₂O should be dissolved in a small amount of ddH₂O before added to other ingredients. Adjust volume to 100ml with ddH₂O. Vacuum-filter into a plastic bottle. Date, label "SP-TL", and store at 4oC for one month.

IVF – TL¹

Component	Product Number	Company	Final (mM)	mg/100 ml
NaCl	S-5886	Sigma	114	666
KCl	P-5405	Sigma	3.2	23.5
NaHCO ₃	S-5761	Sigma	25	210.4
NaH ₂ PO ₄	S-5011	Sigma	0.34	4.08
Lactic acid	L-7900	Sigma	10	84.92 µl
Phenol red	P-0290	Sigma	1 µl/ml	100 µl
*CaCl ₂ ·2H ₂ O	C-7902	Sigma	2	30
*MgCl ₂ ·6H ₂ O	M-2393	Sigma	0.5	10

¹Add NaCl, KCl, NaHCO₃, NaH₂PO₄, lactic acid, and phenol red into a beaker. Bring volume to 90ml with ddH₂O and dissolve completely. *CaCl₂·2H₂O and MgCl₂·6H₂O should be dissolved in a small amount of ddH₂O before added to other ingredients. Adjust volume to 100ml with ddH₂O. Vacuum-filter into a plastic bottle. Date, label “IVF-TL”, and store at 4°C for one month.

HEPES – TL¹

Component	Product Number	Company	Final (mM)	mg/500 ml
NaCl	S-5886	Sigma	114	3330
KCl	P-5405	Sigma	3.2	120
NaHCO ₃	S-5761	Sigma	2	84
NaH ₂ PO ₄	S-5011	Sigma	0.34	20.4
HEPES	H-4034	Sigma	10	1200
Lactic acid	L-7900	Sigma	10	424.6 µl
Phenol red	P-0290	Sigma	1 µl/ml	500 µl
*CaCl ₂ ·2H ₂ O	C-7902	Sigma	2	150
*MgCl ₂ ·6H ₂ O	M-2393	Sigma	0.5	50

¹Add NaCl, KCl, NaHCO₃, NaH₂PO₄, HEPES, lactic acid, and phenol red into a beaker. Bring volume to 480 ml with ddH₂O and dissolve completely.

*CaCl₂·2H₂O and MgCl₂·6H₂O should be dissolved in a small amount of ddH₂O before added to other ingredients. Adjust volume to 500 ml with ddH₂O. Vacuum-filter into a plastic bottle. Date, label “HEPES-TL”, and store at 4°C for one month.

IVF – TALP¹

Component	Product Number	Company	Amount
BSA, EFAF	A-6003	Sigma	60 mg
IVF-TL	-	-	9.8 ml
Na pyruvate (20 mM stock)	-	-	100 µl
Pen/Strep	15140-122	Gibco	100 µl

¹pH should be ~7.4 – Sterile-filter. Date, label “IVF-TALP”, and store at 4°C for one week.

HEPES – TALP¹

Component	Product Number	Company	Amount
BSA, Fraction V	A-4503	Sigma	60 mg
HEPES-TL	-	-	20 ml
Na pyruvate (20 mM stock)	-	-	200 µl
Pen/Strep	15140-122	Gibco	200 µl

¹pH should be ~7.4 – Sterile-filter. Date, label “HEPES-TALP”, and store at 4°C for one week.

SPERM – TALP¹

Component	Product Number	Company	Amount
BSA, Fraction V	A-4503	Sigma	60 mg
SPERM-TL	-	-	9.5 ml
Na pyruvate (20mM stock)	-	-	500 µl
Pen/Strep	15140-122	Gibco	100 µl

¹pH should be ~7.4 – Sterile-filter. Date, label “SP-TALP”, and store at 4°C for one week.

SOF STOCK¹

Component	Product Number	Company	Final (mM)	mg/100 ml
EDTA (add first)	E-5134	Sigma	0.01	100 µl stock
NaCl	S-5886	Sigma	107.7	629.4
KCl	P-5405	Sigma	7.16	53.38
KH ₂ PO ₄	P-5655	Sigma	1.19	16.2
NaHCO ₃	S-5761	Sigma	25.07	210.6
DL-lactic acid	L-7900	Sigma	3.3	47.33 µl
*MgCl ₂ ·6H ₂ O	M-2393	Sigma	0.49	9.96
*CaCl ₂ ·2H ₂ O	C-7902	Sigma	1.71	25.14
Phenol red	P-0290	Sigma	1 µl/ml	100 µl

¹Add all components except CaCl₂·2H₂O and MgCl₂·6H₂O to 90 ml ddH₂O and dissolve completely. *Separately, dissolve CaCl₂·2H₂O into ~5ml ddH₂O and then combine with other ingredients. Separately, dissolve MgCl₂·6H₂O into ~5ml ddH₂O and then combine with other ingredients. Bring pH to 7.3 (Osmolarity of ~270 mOsm) and volume to 100 ml. Vacuum filter stock solution into a plastic bottle; store at 4°C for up to one month.

SOFaa MEDIUM¹

Component	Product Number	Company	Amount
SOF Stock	-	-	4.542 ml
L-glutamine	G-8540	Sigma	25 µl stock
Na pyruvate	P-4562	Sigma	82.5 µl stock
Glucose	G-7021	Sigma	150 µl stock
BSA EFAF	A-6003	Sigma	15 mg
Pen/Strep	15140-122	Gibco	50 µl
BME Essential			
Amino acids, 50X	B-6766	Sigma	100 µl
MEM Nonessential			
Amino acids, 100X	M-7145	Sigma	50 µl

¹Made medium on day of use; medium should be pink color. Sterilize the solution by filtration and store at 4°C for one week.

HEPARIN STOCK¹

Component	Product Number	Company	Amount
Heparin	H-3149	Sigma	1 mg
0.9% Saline	-	-	20 ml

¹Sterile filter and aliquot 100 µl into sterile 0.5 ml microcentrifuge tubes. Store at -20°C indefinitely.

PHE STOCK¹

Component	Product Number	Company	Amount
1 mM Hypotaurine	H-1384	Sigma	5 ml
2 mM Penicillamine	P-4875	Sigma	5 ml
250 mM Epinephrine	E-1635	Sigma	2 ml
0.9% Saline	-	-	8 ml

¹Prepare primary stocks of 1 mM hypotaurine (Sigma H-1384) (1.09 mg/10 ml saline), 2 mM penicillamine (Sigma P-4875) (3 mg/10 ml saline) and 250 mM epinephrine (Sigma E-1635) [1.83 mg/ 40 ml of the following solution (165 mg 60% Na lactate syrup, 50 mg Na metabisulfite (Sigma S-9000) and 50 ml water]. Epinephrine is easily oxidized by direct light so take precautions to avoid this problem (wrap in aluminum foil or place in dark container). Sterile filter and aliquot 100 μ l into sterile 0.5 ml microcentrifuge tubes. Store in a light resistant container at -20°C indefinitely.

EDTA STOCK¹ (0.1 mM)

Component	Product Number	Company	Amount
EDTA	E-5134	Sigma	3.8 mg
ddH ₂ O	-	-	1 ml

¹Do not filter. Make fresh each time.

Na PYRUVATE STOCK¹ (20 mM)

Component	Product Number	Company	Amount
Pyruvic acid	P-4562	Sigma	22 mg
ddH ₂ O	-	-	10 ml

¹Sterile filter into aluminum foil wrapped 15 ml tube. Store at 4°C for two months.

HYALURONIDASE SOLUTION¹

Component	Product Number	Company	Amount
HEPES-TALP	-	-	10 ml
Hyaluronidase	H-3506	Sigma	10 mg

¹Filter and aliquot 1 ml into 1.5 ml tubes. Solution may be stored at -80°C indefinitely.

L- GLUTAMINE STOCK¹ (200 mM)

Component	Product Number	Company	Amount
L-glutamine	G-8540	Sigma	2.923 g
0.9% Saline	-	-	100 ml

¹Sterile filter into 100 µl aliquots in sterile 0.5 ml microcentrifuge tubes. Store at -20°C indefinitely.

GLUCOSE STOCK¹ (50 mM)

Component	Product Number	Company	Amount
D-glucose	G-7021	Sigma	90.08 mg
ddH ₂ O	-	-	10 ml

¹Sterile-filter into 15 ml tube. Store at 4°C for 2 months.

iSCRIPT RT MIX

Component	Product Number	Company	Amount
iScript Rxn Mix	170-8891	BioRad	4 µl
Reverse Transcriptase	170-8891	BioRad	1 µl
mRNA	-	-	11 µl
H ₂ O	170-8891	BioRad	4 µl

RT-PCR MIX (M)

Component	Product Number	Company	Amount
H ₂ O ¹	-	-	19 µl
Jump Start™ ReadyMix™	P-0982	Sigma	25 µl
cDNA sample	-	-	2 µl

¹Autoclave the water before use.

RT-PCR REACTION MIX (RM)

Component	Product Number	Company	Amount (Per Sample)
RT-PCR M	-	-	46 µl
Primer 1	-	Invitrogen	2 µl
Primer 2	-	Invitrogen	2 µl

Q-PCR MIX

Component	Product Number	Company	Amount (Per Well)
SsoFast™			
EvaGreen® Supermix	172-5201	BioRad	10 µl
H ₂ O ¹	-	-	6 µl

¹Autoclave the water before use.

Q-PCR MASTER MIX (MM)

Component	Product Number	Company	Amount (PerSample/Pool) ¹
Q-PCR M	-	-	16 µl
Primer 1	-	Invitrogen	1 µl
Primer 2	-	Invitrogen	1 µl

¹Analyzing 6 genes; Multiplied per well values by 6.2 to ensure enough.

Note: To make a mix for all pools of embryos, leave out the cDNA/Calibrator/H₂O and multiply by the number of pools. Again overcompensate for loss in pipetting. For example, if analyzing 12 pools, multiply by 12.5.

Q-PCR REACTION MIX (RM)

Component	Product Number	Company	Amount (PerSample/Pool)
Q-PCR MM	-	-	18 µl
cDNA/Calibrator/ H ₂ O	-	-	2 µl

VITA

Jaime Manuel Angulo Campos was the third child born from Armando Angulo and Ada Ligia Campos in Boaco, Nicaragua, on March 20th, 1982. He attend La Salle Primary School and High School. After graduating he attended Universidad Nacional Agraria (UNA) from March 2000 to November 2005, where he earned her doctorate in veterinary medicine.

After graduation he worked in an animal hospital in Managua for 2 years. In 2008, he was award with a Fulbright scholarship to enter Graduate School to study Reproductive Physiology in Louisiana State University under the direction of Dr. Kenneth Bondioli, and is now a candidate for the degree of Master of Science in reproductive physiology in the Department of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.