DISSERTATION

ENERGY SUBSTRATES, METABOLIC REGULATORS, AND LIPID ACCUMULATION DURING CULTURE OF IN VITRO-PRODUCED BOVINE EMBRYOS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MOISES BARCELO-FIMBRES ENTITLED ENERGY SUBSTRATES, METABOLIC REGULATORS, AND LIPID ACCUMULATION DURING CULTURE OF IN VITRO-PRODUCED BOVINE EMBRYOSBE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY.

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ABSTRACT OF DISSERTATION

ENERGY SUBSTRATES, METABOLIC REGULATORS, AND LIPID ACCUMULATION DURING CULTURE OF IN VITRO-PRODUCED BOVINE EMBRYOS

The main objective of this experiment was to optimize in vitro culture conditions for bovine embryonic development, using alternative energy sources and metabolic regulators. Replacing glucose with fructose in culture medium consistently increased blastocyst production per oocyte and decreased lipid content in bovine embryos. The use of phenazine ethosulphate (PES) or fetal calf serum (FCS) supplementation did not affect embryonic development; however, PES consistently decreased, and FCS increased lipid content of embryos compared to the control. There was no effect of glucose or fructose on survival of embryos after cryopreservation by slow freezing or vitrification; however, embryos-treated with PES to reduce lipid content resulted in improved cryotolerance, and FCS decreased cryotolerance compared to the control. Transfer of embryos treated with PES during in vitro culture did not affect pregnancy rates, conceptus losses, or fetal or post-natal development in calves born normally. The sex ratio of calves born was skewed toward males. This effect likely was due to a toxic effect of glucose to female embryos cultured in vitro. Therefore, the more expanded day 7 blastocysts were mostly male embryos.

A new, objective and less time consuming technique to quantify lipid accumulation using fluorescence of Nile red dye was validated. The progression of the early to expanded blastocyst resulted in decreased lipid content; also, the inner cell mass

accumulated more lipids than the trophoblast compartment. Embryos were treated with various lipolytic agents. Forskolin reduced lipid content of embryos relative to controls, but caffeine and epinephrine did not affect lipid content of embryos at the doses tested. None of the lipolytic agents affected embryonic development except that high doses of caffeine were detrimental. A higher a percentage of oocytes derived from cow than post-pubertal heifer ovaries developed into blastocyst in vitro; however, more good quality oocytes were recovered per heifer ovary.

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DEDICATION

To GOD, to allowed me write this dissertation.

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CHAPTER I

INTRODUCTION

In the last 10 years great improvements have marked in vitro production of embryos (IVP) (Hasler et al., 1995), but further improvement is required to apply this technology commercially. In vitro-produced embryos are abnormal compared with in vivo derived embryos in morphology, metabolism (Khurana and Niemann, 2000), gene expression (Lazzarri et al., 2002), chilling and freezing sensitivity (Leibo and Loskutoff, 1993) and post transfer developmental competence (Hasler, 2000). Despite international efforts, procedures are not available to produce in vitro embryos that are as healthy as in vivo embryos.

In vitro culture (IVC), is an important part of the process of in vitro production of embryos, which usually starts with in vitro oocyte maturation, followed by fertilization of the oocyte, and ending at the blastocyst stage (7-8 d). IVC is characterized by important metabolic and genomic changes. The change of low metabolism of the zygote to the high metabolism of the implanted embryo is, according to Brinster (1974), like comparing metabolism of bone to that of brain. The relevance of IVC is that during this time important events must happen, including genome activation (Memili and First, 2000), compaction of the morula with formation of cell to cell contacts, and blastocyst

formation, which implies blastocoele formation and the differentiation to two cell types, the trophoblast and ICM.

The intrinsic quality of the oocyte determines blastocyst rates, because in vivo matured oocytes result in more morulae and blastocysts than those matured in vitro (Behboodi et al., 2000). Approximately 35% of oocytes matured and fertilized in vitro reach this stage, while up to 70% of in vivo matured and fertilized oocytes, developing either in in vivo or in vitro culture, go to the blastocyst stage (Lonergan et al., 2001, Rizos et al., 2002c). Therefore, the origin of the blastocyst is important. On the other hand, in vitro-produced blastocysts develop better in vivo in the sheep oviduct than with in vitro culture in synthetic oviduct fluid (SOF) media (Lonergan et al., 2001); also culturing in vivo blastocysts 72 h in vitro produced lactate at similar levels to that in vitro (Khurana and Niemann, 2000). In some way, culture in vitro modifies the metabolism of the embryos. Futhermore, gene expression during culture in vitro can be altered by choice of media (Natale et al., 2000, Khosla et al., 2001a, Lee et al., 2001, Wrenzycki et al., 1996), so the culture system is a major determinate of blastocyst production.

The main objective of this dissertation was to optimize in vitro culture conditions for bovine embryos, exploring new alternatives of energy and metabolic regulators to produce embryos as healthy as in vivo embryos. Understanding the physiology of embryo culture and the factors affecting it, and finding alternatives to resolve these problems, will allow better commercial application of this technology.

CHAPTER II

LITERATURE REVIEW

2.1 Embryo metabolism during In vitro culture

2.1.1 Metabolic changes in the embryo

In vivo, the oviduct is site of fertilization and early embryo development, but it also can support development up to blastocyst stage (Enright et al., 2000). In vivo culture of in vitro-produced embryos resulted in more similarity to normal embryos than embryos produced in vivo by superovulation. (Fair et al., 2001). However, IVP is less efficient than in vivo procedures, with less zygote development to blastocysts.

Oviductal fluid is more complex than the media currently used for IVC in different species. Most media are based on reproductive tract fluids, such as uterus (Summers and Biggers, 2003), oviduct (Tervit et al., 1972) or a combination of oviduct-uterine fluids according to the embryo stage (Gardner and Lane, 1998). Systems that

mimic changes in these fluids, also called sequential media, contain different energy substrate levels similar to oviductal and the uterine fluids (Gardner et al, 1996). Differences in substrate use between early and late cleaved embryos have been detected, with elevated oxygen and glucose consumption as the embryo advances to the blastocyst stage (Hardy et al., 1989; Houghton et al., 1996). After genome activation mammalian embryos experience a critical switch from pyruvate oxidation via the tricarboxylic acid (TCA) cycle to the use of hexoses as the main substrate via glycolysis (Leese and Barton, 1984; Gardner and Leese, 1986); in bovine embryos from the zygote to the 8-16 cell stage, pyruvate and lactate are the preferred energy sources (Leese and Barton,1984), and from embryonic genome activation (8 to 16 cells) to blastocysts, embryos prefer glucose as the main source of energy for compaction and blastulation (Gardner,1998; Khurana and Niemann, 2000). These metabolic changes of low utilization of glucose precompaction to high utilization postcompaction have led to development of sequential media of differing composition (Gardner, 1998; and Gardner and Lane, 2002). Bovine embryos produced by sequential media system have equivalent glucose metabolism to embryos developed completely in vivo (De La Torre et al., 2004). Embryos cultured in a single medium will not experience those energy substrate changes that occur in the oviduct and uterus.

2.1.2 Energy metabolism

Energy in cells is obtained by production of adenosine triphosphate (ATP), which is produced by two main metabolic pathways: glycolysis and the tricarboxylic acid cycle (TCA), manifest by substrate level oxidation and reduced coenzyme NAD^+ and FAD^+

that feed the respiratory chain, resulting in producing ATP by oxidative phosphorylation. During in vitro culture, energy is required for blastocyst formation due to an increase in protein synthesis (Thompson et al., 1998), and also for blastocoele cavity formation, because considerable ATP is required for function of Na^+/K^+-ATPase (Betts et al., 1998; Watson, 1992).

There are some important issues to discuss related to embryo metabolism; for example, under anaerobic conditions, pyruvate is converted to lactate, which is an energetically inefficient pathway, because just 2 ATP are obtained, instead of 12 ATP per pyruvate molecule metabolized through the TCA (Lane and Gardner, 2005). Besides, this pyruvate-lactate conversion uses the reduced coenzyme $NADH^+$, which then is not used to produce energy by oxidative phosphorylation.

Species-specific differences can exist for energy production in embryos. For example mouse embryos are totally dependent on oxidative phosphorylation through preimplantation development. Therefore, they are very sensitive to inhibition of oxidative phosphorylation (Leese, 1991), even at 2-cell or blastocyst stage (Thomson, 1967); pig embryo are similarly sensitive at the 1-cell stage (Macháty et al., 2001). Most other mammalian species are not totally dependent of oxidative phosphorylation, as reported for postcompaction embryos of rats (Brison and Leese, 1991), pigs (Macháty et al., 2001), cattle (Thompson et al., 2000), sheep (Thompson et al., 1993), and humans (Gott et al., 1990).

2.2 Hexoses in the reproductive tract

2.2.1 Glucose

Glucose is the energy source most used for culture of tissues and embryos, usually at blood serum concentration of 5.6 mM (Biggers, 1998), which is close to the concentration found in monogastric species like pigs (Nichol et al., 1992), mice and humans (Lee et al., 1978; McDonald, 1980). However, the glucose concentration in ruminant blood is lower, for cattle 3.0 to 3.5 mM (Meier et al., 2003), and sheep 3.6 to 4.8 mM (Maas et al., 2001).

Glucose concentration in the reproductive tract differs among species, differs in different components of the reproductive tract such as follicular (FF) and oviductal fluid (OF) (Table 1.1), and also differs throughout estrous cycle. In general glucose concentration in follicular fluid is higher than oviductal fluid, except in the mouse. Apparently cattle have less glucose in the oviduct than the other species. (Table 1.1).

There are reports that stage of the estrous cycle affects glucose concentration. For example the FF glucose concentration in mares is 2.3 to 7 mM and tends to decrease during the preovulatory maturation of the dominant follicle (Gérard, 2000); in OF of cattle the concentration is between 0.04 to 0.2 mM during ovulation (metestrus) and early cleavage stages of embryo development (early diestrus) (Carslon et al; 1970; Parrish et al., 1989); in pigs it is 0.59 mM, which decreases tenfold after ovulation (Nichol et al., 1992, 1998); and humans glucose was 3.1 mM in the follicular phase, 0.50 mM midcycle

and subsequently increased to 2.32 mM in the luteal phase (Gardner et al., 1996; Kenny et al., 2002).

Table 1.1. Glucose concentration in follicular (FF) and oviductal fluid (OF) of different species of mammals

Species	FF (mM)	References	OF (mM)	References
Cattle	2-2.85	Leroy et al., 2004	0.04 to 0.2	Carslon et al., 1970 Parrish et al., 1989
Sheep	---		1.57 to 1.76	Hammer, 1973
Goat	1.4	Herrick et al., 2005	---	
Pig	1.03-1.81	Brad et al., 2003	0.59	Nichol et al., 1992, 1998
Mouse	0.46	Harris et al., 2005	3.4-5.19	Gardner and Leese, 1988
Human	3.29-3.78	Leese and Lenton, 1990 Sucha et al., 2002	0.5-3.11	Gardner et al., 1996 Kenny et al., 2002
Horse	2.3-7	Gérard, 2000	---	
Rabbit	2-6	Leese and Jeffries, 1977	1.46	Leese and Jeffries, 1977

Most systems for producing mammalian embryos in vitro use glucose as an energy source in the media despite putative toxic effects (Schini and Bavister, 1988; Takahashi and First,1992) and metabolic disturbances including high lactate production (Khurana and Niemann, 2000). However glucose also has beneficial qualities, including a direct source of energy for post compaction embryo development, blastocoele formation (Rieger et al., 1992, Gardner and Lane, 2002) and its biosynthetic role (Khurana and Wales, 1989; Wales and Hunter, 1990; Wales and Du, 1993).

2.2.2 Fructose

Fructose is the main exogenous source of energy for the spermatozoa in the male reproductive tract of most mammals (Mann, 1964); however, spermatozoa can also metabolize glucose (Mann and Lutwak-Mann, 1951). In the female reproductive tract,

fructose is present in many species including the reproductive tract of rabbits (Gregorie and Gibbon, 1965), pigs (Haynes and Lamming, 1967), cattle (Suga and Masaki, 1973), sheep (Spilman et al., 1970), roe deer (Aitken, 1976), and the oviduct of ovoviviparous salamandra (Greven and Baldus, 1984), and humans (Casslen and Nilsson, 1984; Sucha et al., 2002) (Table 1.2).

Table 1.2. Fructose concentration in follicular (FF) and oviductal fluid (OF) of different species of mammals

Species	FF (µg/mL)	References	OF (µg/mL)	References
Sheep	---		0.52	Spilman et al., 1970
Human	12.37	Sucha et al., 2002	---	
Roe deer	---		2.03	Aitken, 1976

The presence of the fructose transporter 'glucose transporter-5' (GLUT-5) in bovine embryos (Agustin et al., 2001) and the detection of aldose reductase and sorbitol dehydrogenase in the rat ovary, oviduct, and uterus, which are enzymes that catalyze the conversion of glucose to fructose via sorbitol (Kaneko et al., 2003), suggests an important role for this hexose. Also fructose is the predominant reducing sugar present in the fetal plasma and placental fluids in cattle (Hugget and Nixon, 1961, Nixon, 1963).

Fructose as an energy source has been used in different mammals for in vitro culture, including bovine (Guyader-Joly et al., 1996; Chung et al., 2000; Kwun et al., 2003; Kimura et al., 2005), hamster (Ludwig et al., 2001), human (Hwang et al., 2004), pig (Wongsrikeao et al., 2005), and mouse embryos (Sakkas et al., 1993). Fructose supported embryonic development effectively in these studies.

2.2.3 Galactose

There are few reports of the presence of galactose in the reproductive tract, but galactose is found in porcine follicular fluid that surround the oocyte during maturation (Yanagishita et al., 1979), and also in the oviduct of the salamandra (Greven and Baldus, 1984). Galactose has been used for production of embryos in vitro with null or very low embryo development: pigs (Wongsrikeao et al., 2005), cattle (Kwun et al., 2003), and hamster embryos (Ludwig et al., 2001).

2.3 Hexoses and embryo metabolism

Some differences in metabolism of hexoses through glycolysis are important. Glucose enters glycolysis in two different phases, the first in the steps for the conversion of glucose to fructose 1,6 biphosphate (FBP), and the second, the conversion of FBP to lactate with the production of energy and reduced NAD^+. However, glucose also is converted to glucose 6 phosphate (G6P) by the enzymes hexokinase or glucokinase, and this metabolite can feed directly to the pentose phosphate pathway (PP) pathway by the rate limiting enzyme glucose 6 phosphate dehydrogenase (Filosa et al., 2003). In contrast fructose is directly converted to FBP below the entry of glucose, going directly through glycolytic pathway and getting around the PP pathway. In some ways, fructose metabolism requires less energy consumption and less enzyme activity to be metabolized by glycolysis; fructose can also feed the PPP through riibose5-phosphate without the production of NADPH. Fructose metabolism is not under similar hormonal control as glucose with insulin (Froesch, 1976). On the other hand, galactose requires specific

enzymes to go through to the glycolytic pathway, with the conversion of galactose to glucose (Stryer, 1995); however, this mechanism probably is insufficient or does not exist in the oocyte or mammalian embryo, because negative effects of galactose have been observed in different experiments (Ludwig et al., 2001; Wongsrikeao et al., 2005). Some of the needed machinery needed for galactose utilization could exist in somatic cell nuclear transfer (SCNT) bovine embryos, where galactose was able to achieve a better blastocyst rate than the control without galactose (Kwun et al., 2003).

Glycolysis is thought to be important for providing a "dynamic buffer" of metabolic intermediates for biosynthesis of macromolecules (Newsholme and Newsholme, 1989). For example glucose 6-phosphate is required for DNA and RNA synthesis. When glycolytic flux through the pentose phosphate (PP) pathway increases (Katz and Wood. 1963), this produces primary products ribose-5-phosphate (R5P) and NADPH; R5P is an important component in the generation of ATP, NAD^+, flavine adenine dinucleotide (FAD) and coenzyme A (Stryer, 1995). While these are important for the use and storage of energy, R5P also is precursor of 5-phosphoribosyl-1-pyrophosphate, which makes up ribose phosphate, an essential component of the nucleotides, constituting of RNA and DNA. Furthermore, NADPH is utilized by the cell to convert oxidized glutathione to its reduced form, which is important for detoxification of hydrogen and organic peroxides. Therefore PP pathway activity provides a balance of energy sources, increases the ability of the cell to protect itself against oxidative damage, and makes precursors to synthesize purines and pyrimidines.

After genome activation mouse embryos undergo an increase in TCA metabolites and fructose 1,6 biphosphate, and a decrease of glycerol-3 phosphate (Chi et al., 2002). Glucose uptake and metabolism increases after compaction in bovine (Rieger, 1992; Rieger et al., 1992, 2002), and ovine embryos (Gardner et al., 1993). Glucose is metabolized primarily to lactate anaerobically (Thompson et al., 1996; Harvey et al., 2002); the accumulation of the TCA metabolites is thought to be due to the low metabolic activity, as shown in rats, wuth less than 1% of glucose consumed at the blastocyst stage oxidized via the TCA (Dufrasnes et al., 1993). The regulation of TCA cycle flux depends in part on the availability of pyruvate and NAD^+, but high levels of glucose would increase the pyruvate which is mostly converted to lactate; in addition increased fructose 1,6 biphosphate levels would stimulate TCA flux and slow glycolysis (Chi et al., 2002).

Fructose 1,6 biphosphate, is a potent activator of the enzyme phosphofructokinase (PPK), a regulatory enzyme of glycolysis. The protective role of fructose 1,6 biphosphate likely is due to maintenance of ATP levels (Bickler and Buck, 1996), regulation of glucose metabolism (Markov et al., 2000), and recently to the down-regulation of free radical production through MAPK/ERK pathway, at least in neurons (Park et al., 2004), ERK is involved in cell death via ERK 1/2 signaling pathway (Rothstein et al., 2002). Homozygous mutants of the enzyme fructose-2,6-bisphosphatase are lethal at the embryo stage in mice (Chesney et al., 2005). An X-linked isoenzyme with dual activity (PPK/fructose 1,6-biphosphatase) called PFK-2, has been identified in mouse embryos (Winger et al., 2001).

Elevated glucose metabolism, mainly by glycolysis is seen as an embryo stress response (Leese et al., 1998; Leese, 2002); on the other hand grade one embryos have grater pregnancy rates, and they metabolize more glucose than grade two embryos (De La Torre et al., 2004). It appears that embryos do not like environmental culture changes, and when switching embryos between in vivo and in vitro in a sequential media system, the vivo-vivo and vitro-vitro metabolized more glucose than vivo-vitro and vitro-vivo (De La Torre et al., 2004).

2.4 Toxicity of glucose during IVP

Several studies and mechanisms have been proposed to explain the toxicity attributed to glucose pre and post compaction in vitro. The use of 5.6 mM glucose is not appropriate for development of early embryos in monogastric mammals such as hamsters (Schini and Bavister, 1988), mouse (Lawitts and Biggers, 1991; Scott and Whittingham, 2002), rat (Kishi et al., 1991; Miyoshi et al., 1994), and human (Conaghan et al., 1993; Quinn, 1995), or in ruminats including cattle (Peippo et al., 2001), and sheep (Thompson et al., 1992).

This toxic effect at precompaction stages is attributed to the "Crabtree effect" (Crabtree, 1929), defined as a stimulation of glycolysis, which competes for phosphate, thus reducing energy generation; glucose may also repress specific metabolic genes transcribed in response to changes in the availability of different carbon sources (Brown, and Whittingham. 1992). Some research suggests that these toxic effects can also be related to interactions with other compounds such as phosphates in mouse (Scott and

Whittingham, 2002), and hamster embryos (Seshagiri and Bavister, 1991), leading to mitochondrial disruption (Barnett et al., 1997), reduced respiration (Seshagiri and Bavister, 1991), and arrest of embryonic development (Seshagiri and Bavister, 1989). Use of phosphates and use of fructose instead of glucose in the medium, removes the 2-cell block and promotes blastocyst formation in mice (Menezo and Khatchadourian, 1990).

High glucose levels used post compaction produce further detrimental effects in blastocysts (Colton et al., 2002), and in mouse embryos leads to a significant decrease in postimplantation fetal development compared with a low dose (Ludwig et al., 2001). Mouse blastocysts exposed to high glucose concentrations of 50 mM increased TCA metabolites, pyruvate and decrease glycolytic metabolites compared with 0.2 mM glucose, causing a decrease in glucose transport (Chi et al., 2002).

2.5 Hexoses and transporters

Differences in transporters have been identified for glucose and fructose, which may result in differences in hexose uptake (Augustin et al., 2001). Two different mechanisms of glucose transporters have been identified in cattle: active transport is a sodium-dependent (SGLT), which couples glucose uptake with Na^+ influx against a concentration gradient; and passive, energy independent transport by glucose transporters (GLUT). In cattle mRNA's for glucose transporters GLUT-1, GLUT-3, GLUT-8 and SGLT are expressed in all embryonic stages from oocyte to blastocyst. GLUT-5 presence starts at the 8-16 cell and GLUT-4 at the blastocyst stage (Augustin et al., 2001). There

are species-specific differences; for example: GLUT-3 expresion starts in the mouse at the 4 cell stage.

The existence of the SGLT was controversial in the past, because glucose uptake was not inhibited by a specific inhibitor phlorizin (Chi et al., 1993), not in Na^+ free media (Gardner and Leese, 1988); however, in another study the transporter was localized in mouse early cleavage stage embryos using inmunocitochemistry (Wiley et al., 1991), and later Augustin et al. (2001) confirmed the existence of SGLT in all the stages. Is been proposed that SGLT participates in blastomere polarity during compaction in mouse embryos (Wiley et al., 1991), but its utility at the blastocyst stage is questioned, because glucose concentration in the blastocoel fluid is half that of the external environment (Brison et al., 1993). Besides the glucose transport, SLGT can act as water pump independently of glucose concentrations (Loike et al., 1996; Loo et al., 1999).

GLUT-1 is found in the lateral membrane of trophectoderm cells (Augustin et al., 2001); therefore, it could have a more intracellular role rather than glucose uptake from the outside environment. In mouse embryos Glut-1 can be detected even in the unfertilized oocyte (Aghayan et al., 1992). GLUT-3 is the main transporter for providing glucose from the external environment, and has an important role in glucose uptake in the blastocyst stage (Pantaleon et al., 1997). Two insulin regulated transporters, GLUT-4 and GLUT-8, are found in the bovine embryo; the GLUT-4 transporter starts beeing expressed at the blastocyst stage, but GLUT-8 is expressed through all stages (Augustin et al., 2001). GLUT-5 is a transporter with higher affinity for fructose than glucose,

which is first transcribed at 8 to 16 cells, coinciding with the time of genome activation (Augustin et al., 2001); this indicates that it is possible to use fructose as energy substrate during early embryo development. Possibly fructose supplied by this transporter coincides with the change in the PP pathway towards production of ribose-5-phosphate, which is an essential precursor for nucleotide synthesis.

2.6 Additives in culture media

2.6.1 Metabolic regulators

Metabolic regulators have been used to regulate embryonic development such as ethylene diamine tetracetic acid (EDTA), 2,4-dinitrophenol (DNP), sodium azide (NaN_3), pyrroline-5-carboxylate (p5C) and phenazine ethosulphate (PES). EDTA has beneficial effects during precompaction, by a putative reduction of glycolysis due to sequestering magnesium (Olson and Seidel, 2000). DNP is a lipophilic weak acid that binds protons, and is used to uncouple oxidative phosphorylation, which down regulates mitochondrial ATP production sub-acutely by separating electron transport from proton flux, even when ATP production is inhibited, and TCA function and electron transport continues (Voet and Voet, 1990). DNP favors glucose metabolism without toxic effects in bovine embryos via increased glucose uptake (Thompson et al., 2000; De la Torre-Sanchez et al., 2006). NaN_3 inhibits oxidative phosphorylation, inhibiting cytochrome oxidase 3, which thus inhibits electron transport in the mitochondrial membrane. Thompson (1967) suggested that partial inhibition of mitochondrial ATP production leads to more balanced glycolysis-mitochondrial ATP production, and a redox state in the embryo that favors

metabolism via glycolysis (Machaty et al., 2001). PES and p5C are electron acceptors for NADPH, producing NADP, which causes conversion of glucose-6-phosphate to 6-phosphogluconate and stimulates the PPP. PES also and decreases lipid production (De la Torre-Sanchez et al., 2006) indirectly because NADPH is unavailable for lipid production.

2.6.2 Fetal Calf Serum (FCS)

Blood serum is one of the most widely used supplements for in vitro culture, and is a rich source of nutrients and anti-oxidants (Catt, 1994), growth factors, hormones, which can provide an environment for development of embryos (Gardner, 1994). Also, FCS can prevent the toxic effect of acetate in 8 d blastocysts (Diez et al., 2000), stimulates blastocyst production at day 6 (Pinyopummintr and Bavister, 1991; Van Langendonckt et al., 1997; Rizos et al., 2003), and increases cell number at day 8 of culture in bovine embryos (Van Langendonckt et al., 1997).

The use of FCS during in vitro culture has variable results. There are multiple reports of negative effects of the use of FCS during in vitro culture. In embryos, serum inhibits the first cleavage division and blastocyst production in mouse (Fernández-Gonzalez et al., 2004), and cattle embryos (Pinyopummintr and Bavister, 1991), produces premature blastulation at day 6 (Holm et al., 2002; Rizos et al., 2003), and decreases hatching rates (Rizos et al., 2003). Serum also alters metabolism (Gardner et al., 1994) and increases glycolytic rates (Krisher et al., 1999); reduces the inner cell mass (ICM):trophectoderm ratio by increasing apoptosis of ICM cells (Byrne et al., 1999);

increases occurrence of lipid droplets in morulae and blastocysts (Abe et al., 2002, Crosier, et al., 2000; Thompson et al., 1995; Gardner et al., 1994); generates darker and less compact morulae (Abe and Hoshi, 2003; Shamsuddin and Rodriguez-Martinez, 1994); produces organelles abnormalities such as mitochondrial degeneration (Dorland et al., 1994), decreases volume density of mature and total mitochondria (Crosier, et al., 2000), and increases vacuoles (Thompson et al., 1995). After cryopreservation, FCS decreases embryo survival (Rizos et al., 2002b).

There is evidence that culture of embros in vitro can produce specific anormalities during fetal and postnatal development (Bavister, 1995; McEvoy, 2003), including abnormal offspring syndrome (AOS), which has been related to the use of serum in embryos of cattle (Young et al., 1998) and sheep (Thompson et al., 1995; Holm et al., 1996; Sinclair et al; 1998). Serum also, reduces pregnancy rates (Massip et al., 1995; Abe and Hoshi, 2003), postimplantation embryo viability (Fernández-Gonzalez et al., 2004), number of normal fetuses (Caro and Trounson, 1984, Arny et al., 1987), and increases calf mortality and weight in cattle (Abe and Hoshi, 2003), and gestation length and birth weight in lambs (Thompson et al., 1996). In mice serum alters postnatal development, producing larger hearts, hypoactivity, fatter females, and behavioral consequences, such as sex-dimorphic hyperactivity and low anxiety state of young (Fernández-Gonzalez et al., 2004).

Staining in vitro-produced embryos with the lipophilic dye Sudan Black B, reveals that culturing embryos with FCS increases the number of sudanophilic lipid

droplets of all sizes (Abe and Hoshi, 2003), but particularly the large size lipid droplets. Histochemical quantification corroborates that culturing embryos with FCS containing medium significantly increases sudanophilic lipid droplets from the morula to the blastocyst stage (Abe et al., 2002).

FCS can support embryo development; however, some fractions (<1000 and >30, 000 MW) contain embryo growth inhibitors (Ogawa et al., 1987). It has been postulated that FCS misregulates embryo development (Walker et al., 1996) or cause aberrant epigenetic modification in the genome (Reik et al., 1993), which can affect the gene expression in later stages of embryo development (Dean et al., 1998, Moore and Reik, 1996). FCS may also affect mRNA expression of imprinted genes (Rizos et al., 2002a), and is reported to produce abnormally high levels of methylation in the fetus (Khosla et al., 2001b). Also, FCS increases the expression of MnSOD, SOX, Bax, LIF, and LR-β mRNA, but decreases expression of Cx 43 and INF-τ (Rizos et al., 2003).

An additional issue is evolving international regulations to eliminate animal products in media when embryos are imported; FCS is known to be source of pathogenic viruses (Blake et al., 2002), and it is proposed that serum can create osmotic problems for embryos (Ferguson and Leese, 1999).

2.7 Lipids in bovine oocytes and embryos

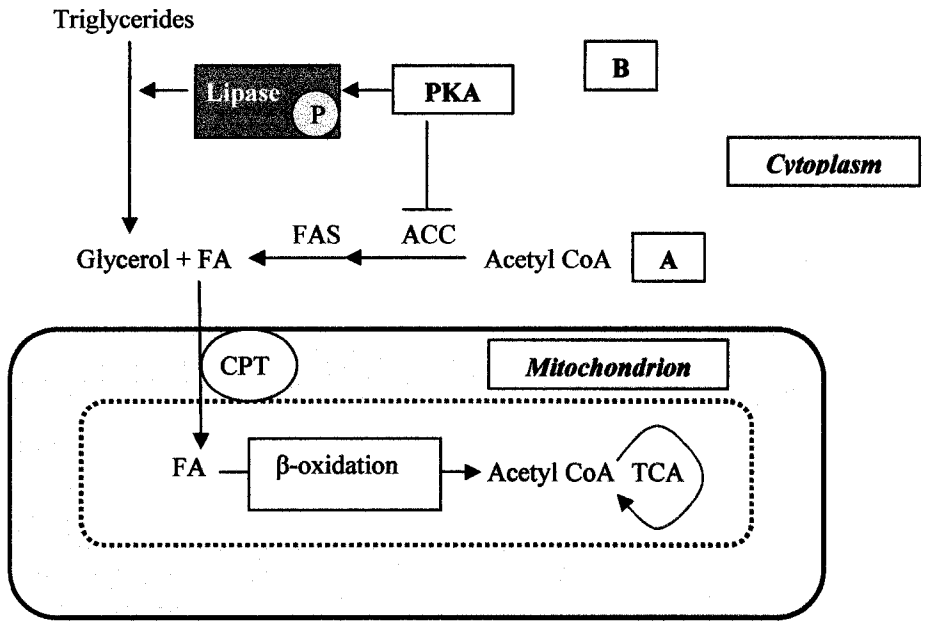
2.7.1 Lipid metabolism

Triglycerides are made of fatty acids (FA) and glycerol. In mammalian cells, the synthesis of FA is mediated by two enzymes, acetyl-CoA carboxylase (ACC) and FA synthase (FAS); however, catabolism is more complex, and involves both the cytoplasmic and mitochondrial compartments. A kinase is present in the cytoplasm that acts as a metabolic master switch: AMP-activated protein kinase gamma 1 (PKA), which activates lipase and inactivates ACC during lipid catabolism (Ouchi et al., 2005); activated lipase translocates to the lipid droplet and hydrolyzes triglycerides as the primary target and the first step in their catabolism (Londos et al., 1999; Holm, 2003). However lipase is a non-specific enzyme that also hydrolyzes mono- and diacylglycerols, cholesterol esters, and others (Kraemer and Shen, 2002). For FA import and cytoplasmic transport, there are two main proteins, FA translocase (FAT or CD36), and FA transport protein (FATP1) (Schaffer, 2002). Moreover, protein carnitine palmitoyltransferase I (CPT-I) transports FA into the mitochondria for oxidation. ACC is an inhibitor of CPT-1, so inactivation of ACC by PKA results in increased fatty acid transport and subsequent oxidation (Figure 1.1). Transcripts of mRNA for these proteins have been identified in oocytes and blastocysts of cattle (Algriany et al., 2007).

Lipids appear to be an important source of energy during oocyte maturation, fertilization, and early embryo cleavage in bovine embryos (Ferguson and Leese, 1999; Kim et al., 2001; Sinclair et al., 2002; Ferguson and Leese, 2006). Endogenous lipids in the embryo in the form of triglycerides can be use as an energy source in mouse embryos, but they support development for only short times (4-5 h) (Leese, 1991). Triglycerides are the main class of lipid synthesized in oocytes and embryos of cattle (Ferguson and

Leese, 1999, McEvoy et al., 2000; Sinclair et al., 2002), and are reported to be essential for hatching in cattle (Renard et al., 1980). In vitro-produced bovine blastocysts have darker cytoplasm as a consequence of higher lipid accumulation than in vivo-derived embryos (Pollard and Leibo, 1994; Abd El Razek et al., 2000). This is associated with impaired embryonic quality (Hill and Kuehner, 1998; Reis et al., 2003; Rizos et al., 2003), and reduced cryotolerance (Pollard and Leibo, 1993; Hill and Kuehner, 1998; Abe et al., 2002).

Figure 1.1. Lipid metabolism in the cytoplasmic and mitochondrial compartments (**A.** Synthesis of fatty acids, **B.** PKA activates Lipase and transport of fatty acids to the mitochondria for oxidation).



Increased lipid complexity may directly affect lipid content and the biophysical and biological properties of the cell membrane, and the ability to undergo mitosis (Horwitz et al., 1974). A report showed that triglyceride content in embryos from 4 to

eight cells with and without zonae is not different, which indicates that lipids are not detectable in the zona at those stages (Ferguson and Leese, 1999).

2.7.1 Lipid quantity in embryos

Before maturation triglycerides per oocyte are quiet different from species to species: in sheep 25 ng (of 89 ng total lipid) (Coull et al., 1997), cattle 59 ng (Ferguson and Leese, 1999), and pigs 75 ng (McEvoy et al., 1997) (Table 1.3). For in vivo-produced cattle embryos, the amount of total fatty acids remain stable from 7 to 10 d (1.5 to 4.2 μg per embryo), which represents approximately 0.2% of the total embryonic fresh weight; by day 13, FA increases in complexity and accumulation with 13 μg per embryo (Menezo at al., 1982).

The triglycerides levels in embryos during in vitro culture also vary. Mouse embryos contain less lipids than other species, containing just 4 ng of total lipid content (Lowenstein and Cohen, 1964); in vivo-derived cattle embryos from the 2-cell stage to hatched blastocyst contain very similar amounts of lipids (33 ng); in the presence of FCS 4 to 8-cell embryos remained at 33 ng lipid; however, from 8 cell to hatched blastocyst FCS increased lipid significantly from 37 ng to 62 ng (Ferguson and Leese, 1999); in pig embryos lipid content of 10 and 11 d embryos was 113 and 141 ng (Youngs et al., 1994) (Table 1.3).

Table 1.3. Amount of triglycerides (TG), and total lipid content (TLC) of mammalian oocytes and embryos (ng / embryo).

Species	Stage	Produced	TG (ng/embryo)	TLC (ng/embryo)	References
Cattle	oocyte	In vivo	59	--	Ferguson and Leese, 1999
Cattle	2-cell-BL	In vitro	33	--	Ferguson and Leese, 1999
Cattle	BL+FCS	In vitro	62	--	Ferguson and Leese, 1999
Sheep	oocyte	In vivo	25	89	Coull et al., 1997
Pig	oocyte	In vivo	75	156	McEvoy et al., 1997
Pig	BL: 10-11d	In vitro	113-141	--	Youngs et al., 1994
Mouse	BL	In vitro	--	4	Lowenstein and Cohen, 1964

BL= blastocyst, FCS= fetal calf serum, TLC=tTotal lipid content -- data not available.

2.7.2 Fatty acids supplementation in vitro

Addition of different fatty acids in vitro has different effects on bovine oocyte maturation; oleic acid had no effect, but palmitic and stearic acids had a negative effect (Leroy et al., 2005b). Studies in rat embryos have demonstrated that development to blastocysts is promoted by addition of unsaturated fatty acids to media (oleic, linoleic and arachidonic), but not the saturated palmitic acid (Khandoker and Tsujii, 1999). The combination of palmitic and oleic acid was better than either single FA in promoting the formation and hatching of mouse blastocysts from the one-cell stage (Quinn and Whittingham, 1982).

Culturing in vitro-produced blastocysts with 10% FCS resulted high levels of palmitic, stearic and oleic acid in embryos; saturated fatty acids were higher (72%) than monounsaturated and polyunsaturated fatty acids (14.7 and 13.3%, respectively) (Lapa et al., 2005). Similarly, bovine embryos supplemented with FCS during culture had higher levels of saturated fatty acids such as palmitic, stearic and palmitoleic (13.5, 8.1, and 12.6%, respectively) than the controls; for unsaturated fatty acids oleic acid increased

(8.1% of fatty acids), but linoleic and linolenic were not different from the non serum-supplemented control (Sata et al., 1999).

2.7.3 Extrinsic Factors affecting oocyte and embryo lipid accumulation

Recent studies have shown that the origin of the oocytes or embryos are important factors to consider in terms of lipid content and embryonic development; differences occur due to in vivo vs. in vitro-derivation (Pollard and Leibo, 1994; Abd El Razek et al., 2000), donor breed (Adamiak et al., 2004; Leroy et al., 2004; Visintin et al., 2002), lactational stage (Leroy et al., 2006), and nutritional management (Fouladi-Nashta et al., 2007; Adamiak et al., 2004),

Bovine embryos produced in vitro have darker cytoplasm and lower buoyant density than in vivo–produced embryos (Pollard and Leibo, 1994) as a consequence of higher lipid content and different lipid composition (Abd El Razek et al., 2000). Lipid accumulation is associated with embryo darkness (Sata et al., 1999; Abe and Hoshi, 2003; Leroy et al 2005a), impaired embryonic quality (Hill and Kuehner, 1998; Reis et al., 2003; Rizos et al., 2003), and reduced cryotolerance (Pollard and Leibo, 1993; Hill and Kuehner, 1998; Abe et al., 2002). Also, in vivo-derived dark embryos showed poor freezability, quality and pregnancy rates (Hill and Kuehner, 1998).

Differences of in vivo derived embryos based on the donor breed have been found. Holstein cows had higher levels of cholesterol but low triglycerides in serum, and more embryos per flush with darker appearance and lower quality than Belgian blue

donor cows (Leroy et al., 2004). Hill and Kuehner (1998) also reported a significant correlation between embryos with dark color and Holstein donors with high serum cholesterol. Another study found that Holstein embryos have more intracellular lipids, containing larger and greater numbers of lipid droplets than embryos of Nelore cows (Visintin et al., 2002).

Lactational stage may affect lipid accumulation in embryos; one study showed that embryos from high producing dairy cows contained 45% more lipids compared to embryos of non-lacting animals, as determined by staining with Nile red (Leroy et al., 2006).

Molecular and metabolic variations may exist in embryos derived in vivo and developed in donor heifers on nutritional regimens differing in type and quantity (Wrenzycki et al., 2000). High nutrition planes in heifers are detrimental to oocyte quality, resulting in low embryonic development postfertilization (Nolan et al., 1998; Yaakub et al., 1999). Adamiak et al. (2005) found that oocyte quality after OPU and IVF is dependent on the body condition of heifers; high levels of feeding were beneficial to oocytes from animals of low body condition, but detrimental to oocytes of moderately high body condition.

Nutritional management of oocyte donors, especially fat supplementation, may be an important factor to consider in terms of oocyte or embryo lipid content and embryonic development. Modifying oocyte donor carbohydrate metabolism during ovarian

stimulation prior to OPU can influence the IVP outcome in a complex manner dependent on body composition; a high starch diet reduced blastocyst yield in thin heifers and cleavage rates in fat heifers compared to a high fiber based diet. However, adding protected lipids (calcium soaps of fatty acids) to diets increased plasma fatty acids, which was reflected in an increase in total fatty acids within the oocyte and also increased blastocyst yield (Adamiak et al., 2004).

Varying results in follicular dynamics have been obtained in terms of fat supplementation of diets, possibly due to differences in fat composition of the diet. Fat supplementation of diets increased the size of preovulatory follicles and numbers of follicles in the ovary (Lucy et al., 1993; Mattos et al., 2000). Feeding lacting dairy cows with a high fat diet reduced number of small and medium size follicles and decreased mean numbers oocytes collected per cow, but no differences in oocyte quality were observed compared with a low fat diet (Fouladi-Nashta et al., 2007). Likewise another study showed that fat supplementation in lacting dairy cows had no effect on quality of OPU recovered oocytes, but feeding a high linoleic acid diet tended to result in oocytes with a lower embryonic development than feeding high oleic acid (Bilby et al., 2005).

Fouladi-Nashta et al. (2007) evaluated developmental competence of OPU recovered oocytes of lacting dairy cows fed with a low and high in fat diet; they found no differences in cleavage rate, but an increase in blastocyst production and more ICM and trophectoderm cells, compared to a low fat diet. Interestingly, milk yield, dry matter

intake, metabolizable energy intake, starch intake, and growth hormone concentration were negatively related to blastocyst yield in the low fat but not the high fat group.

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CHAPTER III

EFFECTS OF EITHER GLUCOSE OR FRUCTOSE AND METABOLIC REGULATORS ON BOVINE EMBRYO DEVELOPMENT AND LIPID ACCUMULATION IN VITRO

3.1 Summary.

Our objective was to determine if replacing glucose with fructose would decrease cytoplasmic lipid accumulation during culture of embryos with or without regulators of metabolism. In vitro-produced bovine zygotes were cultured 60 h in chemically defined medium-1 (CDM-1) plus 0.5% BSA and 0.5 mM fructose or glucose in expt 1, and glucose in expt 2. In both experiments, 8-cell embryos were next cultured 135h in CDM-2 plus 2 mM fructose or glucose in factorial combination with 5 treatments: (expt 1: control, 10% FCS, 0.3 μM phenazine ethosulfate (PES), 30 μM dinitrophenol (DNP), and PES+DNP), and (expt 2: control, PES, PES+DNP, and 1 and 3 μg/mL cerulenin (C1 and C3)). Day 7.5 blastocysts were stained with Sudan Black B to quantify cytoplasmic lipid droplets as small (SD, <2 μm), medium (MD, 2-6 μm), or large (LD, >6 μm). Blastocyst rates per oocyte were 22% (expt 1) and 15% (expt 2) higher ($P<0.05$) for fructose than glucose. For expt 1, numbers of MD were lower for PES, DNP, and PES+DNP than

control and FCS ($P<0.05$). LD were lower for PES and DNP than control, and higher for FCS than all other treatments ($P<0.05$). For expt 2, MD were lower ($P<0.05$) for PES, and PES+DNP than C1, C3 and control. For LD, PES was lower ($P<0.05$) than control, C1 and C3, but not different from PES+DNP. The only effect of hexose on lipids was that fructose resulted in fewer MD ($P<0.01$) in expt 2. In conclusion, fructose produced more blastocysts than glucose, and PES reduced lipid accumulation.

3.2. Introduction.

Over the past decade, there have been a number of improvements concerning in vitro production of embryos (IVP) (Bavister, 1995; Biggers, 1998; Thompson and Peterson, 2000; Gardner and Lane, 2002; De La Torre-Sanchez et al., 2006a), but further improvements would facilitate commercial application of this technology. In vitro-produced embryos are abnormal compared with in vivo derived embryos in morphology, metabolism (Khurana and Niemann, 2000), gene expression (Lazzari et al., 2002), chilling and freezing sensitivity (Leibo and Loskutoff, 1993), and post transfer developmental competence (Hasler, 2000). Despite international efforts, procedures are not available to produce embryos in vitro that are as healthy as in vivo embryos.

From the zygote to the 8- to 16-cell stage, pyruvate and lactate are the preferred energy sources (Leese and Barton, 1984), and from embryonic genome activation (8 to 16 cells) to blastocysts, embryos prefer glucose as the main source of energy for compaction and blastulation (Gardner, 1998; Khurana and Niemann, 2000). The metabolic changes of low utilization of glucose to high utilization have led to

development of sequential media of differing composition (Gardner, 1998; Gardner and Lane, 2002).

Most systems for producing mammalian embryos in vitro use glucose as an energy source in the media despite putative toxic effects (Schini and Bavister, 1988; Takahashi and First, 1992). Metabolic disturbances in embryos have been reported, particularly by including glucose in culture media at concentrations in blood (Kim et al., 1993). High lactate production (Khurana and Niemann, 2000) associated with "aerobic glycolysis" also reduces embryo viability and development (Gardner, 1999). However, glucose also has the following beneficial qualities: direct source of energy, energy reserve in the form of glycogen (Rieger, 1992), and as a biosynthetic molecule (Khurana and Wales, 1989; Wales and Hunter, 1990; Wales and Du, 1993). Glucose metabolized via the pentose phosphate pathway (PPP) can generate triacylglycerols; glycoproteins (Wales and Hunter; 1990); ribose-5 phosphate required for nucleic acid synthesis (Khurana and Wales, 1989); and nicotinamide adenine dinucleotide phosphate (NADPH), which is involved in lipid biosynthesis (Wales and Du, 1993) and inhibiting peroxidation by reducing intracellular glutathione (Harvey et al., 2002).

Glucose, fructose and galactose are monosaccharide hexoses capable of entering the glycolytic pathway, and fructose is the main exogenous source of energy for spermatozoa in the male reproductive tract (Mann, 1964). Fructose also is present in the female reproductive tract in many species including rabbits (Gregorie and Gibbon, 1965), pigs (Haynes and Lamming, 1967), cattle (Suga and Masaki, 1973), sheep (Spilman et

al., 1970), roe deer (Aitken, 1976), salamanders (Greven and Baldus, 1984), and humans (Sucha et al., 2002). The presence of fructose and the fructose transporter, 'glucose transporter-5' (GLUT-5), in bovine embryos (Augustin et al., 2001) suggests an important role for this hexose.

Fructose has been used as an energy source in media for in vitro culture of embryos in many species including cattle (Guyader-Joly et al., 1996; Chung et al., 1996; Kwun et al., 2003; Kimura et al., 2005), hamsters (Ludwig et al., 2001), humans (Hwang et al., 2004), pigs (Wongsrikeao et al., 2005), and mice (Sakkas et al., 1993), supporting embryonic development effectively. On the other hand, galactose has been used for in vitro production of embryos with null or very low embryo development in pigs (Wongsrikeao et al., 2005), cattle (Kwun et al., 2003), and hamsters (Ludwig et al., 2001).

Chemicals are often used to regulate metabolism to improve embryo development. Examples includes: ethylene diamine tetracetic acid (EDTA) (Olson and Seidel, 2000) to reduce glycolysis; sodium azide to inhibit cytochrome oxidase 3, which improves development of bovine embryos (Thompson et al., 2000; Macháty et al., 2001; De La Torre-Sanchez and Seidel, 2002); 2,4-dinitrophenol (DNP), a lipophilic weak acid that binds protons which sub-acutely down regulates mitochondrial ATP production, but with continued electron transport which uncouples oxidative phosphorylation (Voet and Voet, 1995) (Figure 5.1); this favors glucose metabolism without toxic effects (Thompson et al., 2000; De La Torre-Sanchez et al., 2006b). Electron acceptors for

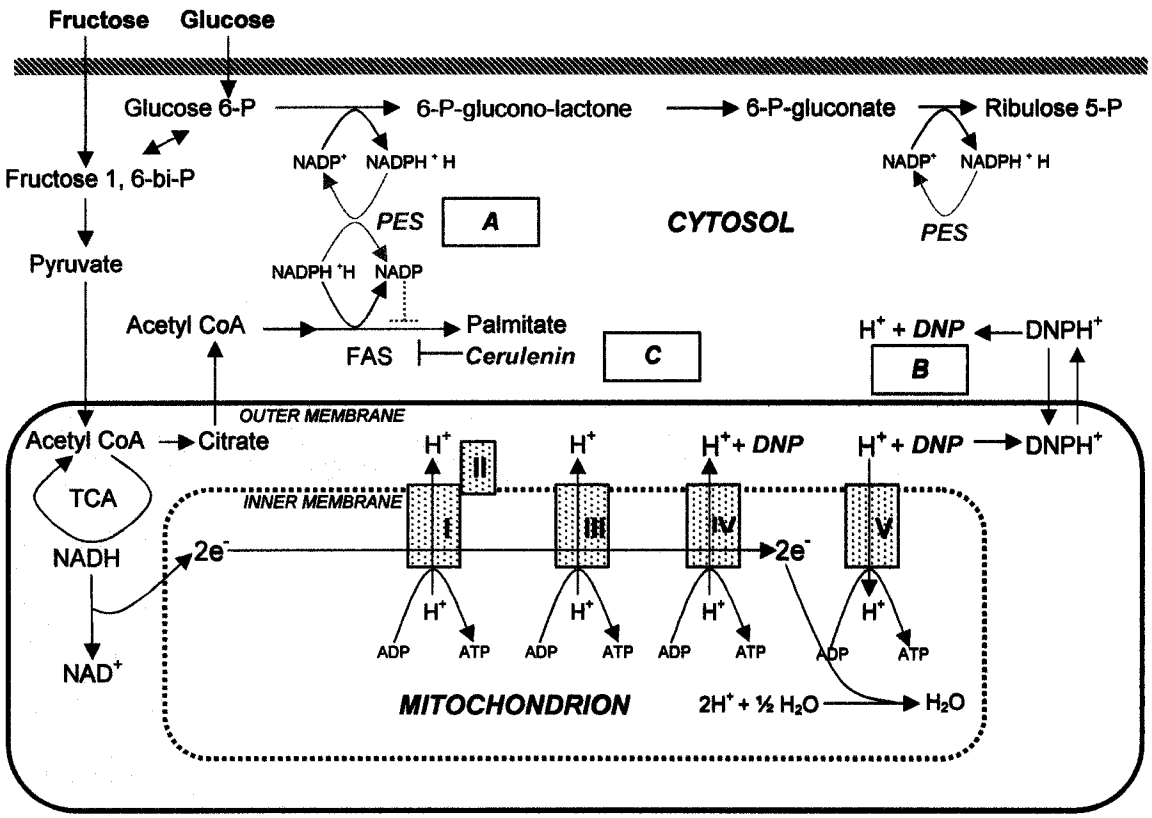
NADPH such as pyrroline-5-carboxylate and phenazine ethosulphate (PES) also have been used, which produces NADP. These have been used for mouse oocytes (Downs et al., 1998) and cattle embryos (De La Torre-Sanchez et al., 2006b), causing conversion of glucose-6-phosphate to 6-phosphogluconate, which stimulates the PPP, decreasing lipid production (De La Torre-Sanchez et al., 2006a) indirectly because NADPH is unavailable for lipid production (Figure. 3.1).

Cerulenin is a mycotoxin that inhibits fatty acid synthase (Kauppinen et al., 1988) (Figure 5. 1), decreasing lipid production as shown in neurons (Landree et al., 2004). The importance of high lipid accumulation is that such embryos are more susceptible to oxidative damage (McEvoy et al., 2001) and have reduced cryotolerance (Lonergan et al., 2003; Pollard and Leibo, 1994; Rizos et al., 2003).

Fetal calf serum (FCS) is one of the most widely used supplements for in vitro culture; it contains many compounds and is a rich source of nutrients and anti-oxidants (Catt, 1994), growth factors, and hormones that can provide a stimulatory environment for development embryos (Gardner, 1994; Hasler et al., 1995). However, there is evidence that culture in vitro with FCS can produce specific abnormalities during fetal and postnatal development (Bavister, 1995; McEvoy, 2003) in cattle (Young et al., 1998) and sheep (Thompson et al., 1995; Holm et al., 1996). Furthermore, FCS during culture in vitro can reduce developmental potential (Bavister, 1995; Fernández-Gonzalez et al., 2004), and can cause abnormal metabolism (Gardner et al., 1994), abnormal structure of

embryos (Abe et al., 2002), and decreased embryo survival after thawing (Rizos et al., 2002).

Figure. 3.1. Mechanism of action of PES, DNP and Cerulenin. (*A*. PES accepts H+ from NADPH, by converting $NADPH^+$ to $NADP^+$, PES stimulates PPP rate. *B*. DNP dissipates the H+ gradient formed by the electron transport chain. *C*. Cerulenin is as a fatty acid synthase inhibitor).



3.3. Objective.

The aims of this study were to determine whether glucose or fructose is the best hexose alternative for supporting embryo development, and interacted with metabolic regulators to decrease cytoplasmic lipid accumulation in embryos.

3.4. Materials and Methods.

3.4.1. Experimental Design.

For the first experiment, 2400 oocytes were used to produce embryos that were subjected to a 2 x 5 x 4 factorial experimental design with 2 energy substrates (glucose or fructose), a control plus 4 additives (0.3 μM phenazine ethosulfate (PES); 30 μM 2,4-dinitrophenol (DNP); 0.3 μM PES+ 30 μM DNP; and fetal calf serum 10% (FCS)), and semen from 4 bulls (A, B, C, and D) replicated on two days for each bull.

For the second experiment 1800 oocytes were used to produce embryos that were subjected to a 2 x 5 x 6 factorial experimental design with 2 energy substrates (glucose or fructose), a control plus 4 additives (0.3 μM PES; 0.3 μM PES+ 30 μM DNP; cerulenin 1 μg/ml (C1); and cerulenin 3 μg/ml (C3)), and semen from 6 bulls (A, B, C, D, E, and F) one batch of oocytes (ovaries) per bull.

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St Louis, MO).

3.4.2. In vitro production of embryos

3.4.2.1 Oocyte Collection and In Vitro Maturation

Bovine ovaries from mature cows were obtained from abattoirs and transported to the laboratory in 3 h in 0.15 M NaCl saline at ambient temperature, approximately 22 to

25°C. After arriving at the laboratory, ovaries were trimmed of extraneous tissue, and rinsed once again in 0.15 M NaCl. Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm antral follicles with an 18-gauge needle attached to a tubing system connected to a vacuum aspiration pump with ~50 mm Hg of pressure. Oocytes with more than 3 layers of unexpanded cumulus cells and morphologically bright and evenly granulated cytoplasm were selected for IVM. COCs were washed twice in chemically defined medium (CDM) with hepes buffer for handling oocytes and once with maturation medium (De La Torre-Sanchez et al., 2006b). Fifty COCs were matured per well of 4-well plates (Nunclon, Roskilde, Denmark), containing 1 ml of maturation medium CDM with 0.5% fatty acid-free BSA (Sigma A-6003, Lot # 063K7525) and hormones (15 ng/ml of FSH (NIH-FSH-S17; Bethesda, MD), 1μg/ml of LH (USDA-LH-B-5; Beltsville, MD), 1.0 μg/ml of estradiol-17β (Sigma E-2257), 50 ng/ml EGF (Sigma E-9644) and 0.1mM cysteamine (Sigma M-6500). Oocytes were incubated at 38.5°C with humidified 5% CO_2 in air for 23 h as described by De La Torre-Sanchez et al. (2006b).

3.4.2.2 Sperm Preparation.

Frozen semen was from bulls of proven fertility and contained at least 40 % progressive motile sperm after thawing. Straws of semen were thawed in water at 35°C for 30 sec. Semen from one straw was gently expelled into a 15-ml centrifuge tube and centrifuged 20 min at ~400 x g at 23°C through a Percoll gradient (Sigma P-1644) with 2 ml 90%: 2 ml 45% Percoll in sperm (sp)-TALP medium (modified Tyrode's) as described by Parrish et al. (1989). The supernatant was discarded, and the sperm pellet (approximately 100 μl) was washed with 5 ml of chemically defined fertilization medium

(Fert-CDM; De La Torre-Sanchez et al., 2006b), supplemented with 0.5% BSA, 5 mM caffeine (Sigma C-0750) and 2 μg/ml heparin (Sigma H-3125). The sample was centrifuged again for 5 min at 400 x g at 23°C, and the supernatant was discarded. Approximately 90 to 100 μl remained, and a 5-μl aliquot was taken to determine the sperm concentration with a hemacytometer. The sperm concentration was adjusted to 5 x 10^6 spermatozoa per ml to give final concentrations of 0.5 x 10^6 spermatozoa per ml in Fert-CDM.

3.4.2.3 In Vitro Fertilization.

Following in vitro maturation, 50 oocytes were randomly assigned in 450 μl of Fert-CDM medium per well of 4-well dishes, and 50 μl of sperm suspension were added to give final volume of 500 μl per well. Gametes were incubated with the semen for 18 h at 38.5°C in an atmosphere of humidified 5% CO_2 in air.

3.4.2.4 Culture of Embryos.

Following in vitro fertilization, presumptive zygotes were removed from wells and transferred to 1.0-ml microcentrifuge tubes with approximately 100 μl of Fert-CDM (De La Torre-Sanchez et al., 2006b), and then vortexed for 50 to 60 sec to remove cumulus cells. These procedures were carried out in a room at 28 °C. Embryos then were rinsed three times in Hepes CDM-1 (H-CDM-1). Early culture (day 0 to 2.5 post fertilization) was done in a new 4-well dish, containing 500 μl of CDM supplemented with 0.5% fatty acid-free BSA, non-essential amino acids (NEAA), and 10 μM EDTA (CDM-1) as described by De La Torre-Sanchez et al. (2006b), and incubated at 39°C

under 90% N_2, 5% O_2, and 5% CO_2. After 60 h of culture, embryos were examined with a stereomicroscope (15 to 20×) for cleavage rate, and all uncleaved ova and embryos less than 8 cells were discarded. The rest were cultured in new dishes with CDM-2 (CDM supplemented with 0.5% fatty acid-free BSA, NEAA and essential amino acids and 2 mM of energy substrate) from day 2.5 to day 7.5 post fertilization.

3.4.2.5. Addition of Metabolic Regulators.

A 2000x stock of each chemical was prepared in nanopure water for PES (Sigma, P-4544), and DMSO for DNP (Sigma D-7004); then a 100x stock was made for each compound in HCDM-2. A stock solution of cerulenin was prepared with dimethyl formamide (DMF) (Sigma D-4551) at 50 mg/ml and then diluted to the desired concentration in HCDM-2. These stocks were stored at 5°C and added to CDM-2 at the time of equilibration. The final concentrations of the metabolic regulators for each treatment for the first experiment were: PES, 0.3 μM; DNP, 30 μM; mix of PES, 0.3 μM + DNP, 30 μM; and 10% FCS (Gemini Bio-Products Inc., West Sacramento, CA, lot# A20206P) as a negative control. For the second experiment treatments were: PES, 0.3 μM; mix of PES, 0.3 μM + DNP, 30μM; cerulenin, 1 μg/ml; and cerulenin, 3 μg/ml. These concentrations were based on the dose-response studies of De La Torre-Sanchez et al. (2006b), except for cerulenin.

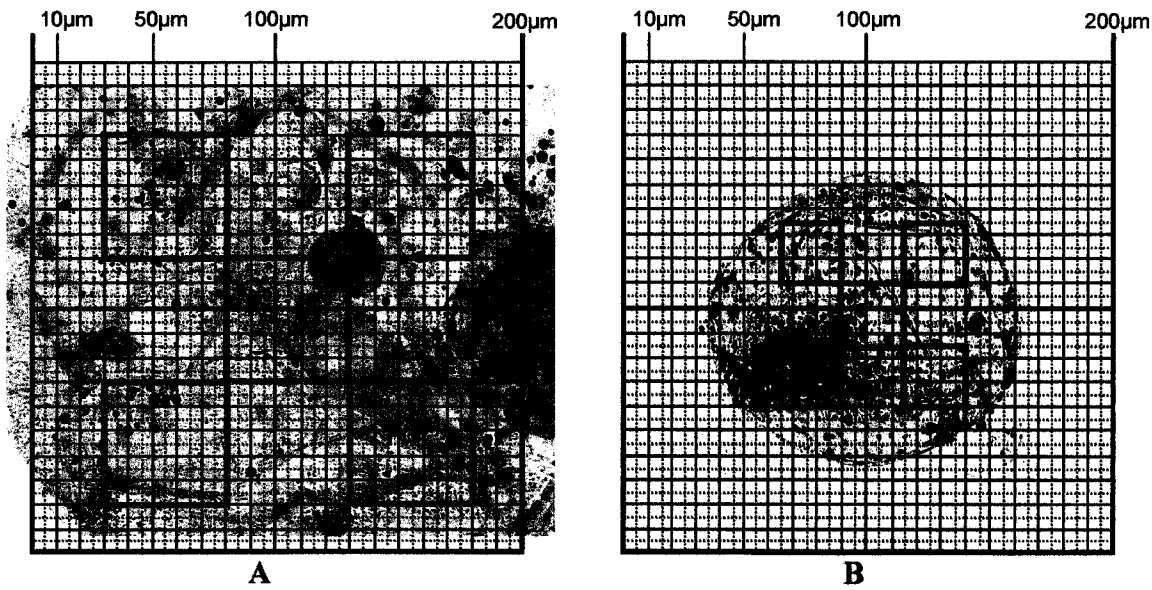
3.4.2.6. Evaluation of Embryos.

The accumulation of the lipids in the cytoplasm of embryonic cells was quantified by staining embryos with Sudan Black B, a lipophilic dye (Sigma S-0395), using a

modification of the technique described by Abe et al. (2002). Briefly, the embryos were fixed in 10% formalin in modified phosphate-buffered saline (mPBS; Elsden and Seidel, 1995) at pH 7.4 for 2 h at room temperature, rinsed in distilled water plus 0.05% PVA, and then transferred to 50% ethanol in water for 2 min. Then, they were stained in about 4 drops of 1% (w/v) Sudan Black B in 70% ethanol for 1 min in a round-bottom glass well. After a 3x wash in 50% ethanol, 5 min each, stained embryos were rinsed in distilled water plus 0.05% PVA for 5 min and then mounted in glycerol on glass slides. The numbers of Sudan Black granules were counted as follows. Embryos were visualized at 600x and the equatorial part of the embryo was focused (which generally is the part with the largest amount of lipid droplet accumulation). A digital photograph was taken and the image was digitally enhanced to equalize the dark tones and get a better visualization of the Sudan Black B granules.

A grid of 40,000 μm^2 (200 by 200 μm^2) was constructed using digital tools with a Neubauer chamber as a reference with intergrids of 25 μm^2 (5 by 5 μm^2) (Figure. 3.2 A), and 5 squares of 2500 μm^2 (50 by 50 μm^2) were randomly placed over the embryo image. For small blastocysts a small size grid was constructed of 5 squares of 625 μm^2 (25 by 25 μm^2) (Fig. 3.2 B) and multiplied by four to equalize the areas relative to data for large embryos. The numbers of granules in focus were counted and classified by diameter as small (<2 μm), medium (2 to 6 μm), and large (>6 μm) (Abe et al., 2002). Results are reported as numbers of small, medium, large, and medium + large granules, per $1x10^3$ μm^2.

Figure. 3.2. Photomicrograph at 600x of 40,000 μm² grid. (**A.** Grid over a large Blastocyst stained for Sudan back B with 5 squares 50 by 50 μm², **B.** Grid over a small blastocyst stained with Sudan back B with 5 squares of 25 by 25 μm²).



For the last response, large droplets were converted to an equivalent number of medium droplets; considering an average size of 4 μm for a medium droplet and 8 μm for a large droplet, and taking into account that the comparison has to be made in terms of volume, one large droplet was equivalent to 8 medium droplets. Note that this is a method to determine relative lipid granule volume in the embryo, and served to compare lipid accumulation in embryos from different treatments. Calculating the absolute amount of lipids in an embryo requires different methodology.

Cleavage rates were assessed 2.5 days after being placed in CDM-1, and embryos at the 8-cell stage were placed into culture into CDM-2; 2- to 6-cell embryos were considered cleaved, but were not cultured further. The percentage development to blastocysts was evaluated 7.5 days after fertilization. Percentages were transformed using

arcsin square root, and the transformed data were then analyzed by ANOVA using the SAS statistical software package general linear model (GLM). Sources of variation in the model included all main effects (all considered as fixed effects), all possible 2 way interactions, and residual error. If the ANOVA was significant ($P < 0.05$), means were separated by Tukey's w procedure. Data are reported as untransformed least-squares means.

3.5. Results

3.5.1. Embryo Production for Experiment 1.

For the hexose main effect (Table 3.1), the rate of 8 cell production per oocyte was not different between glucose and fructose, but the blastocyst rates per oocyte matured and per 8- cell embryo were higher ($P < 0.05$) for fructose than glucose. Numbers of cells per blastocyst did not differ between glucose and fructose treatments, 92.6 ± 2.7 (N=84) and 93.0 ± 2.8 (N=79), respectively. As shown in Table 3.2, bull B was lower than the others bulls ($P < 0.05$) for 8- cell embryo production and cleavage rate. For blastocyst production, bull D was higher than the other bulls, for both blastocysts per 8- cell embryo, and per oocyte. For effects of metabolic regulators (Table 3.3), DNP and DNP+PES were lower for blastocyst rate per oocyte matured and per 8-cell embryo ($P < 0.05$) than the other treatments. Numbers of cells per blastocyst was higher for FCS than DNP and DNP+PES, but not different from control or PES (Table 3.3).

Table 3.1. Main effect hexose treatment means for development of bovine embryos produced in vitro (±SE).

Hexose	No. zygotes in CDM-1 medium	8 cell embryos per oocyte (%)	No. 8 cell embryos in CDM-2 medium	Blastocysts per oocyte (%)	Blastocysts per 8 cell embryo (%)
Experiment 1					
Fructose	1200	69.6 ± 1.3	835	23.0±0.9 [a]	32.6±1.2 [a]
Glucose	1200	68.1 ± 1.3	817	18.9±0.9 [b]	26.4±1.2 [b]
Experiment 2					
Fructose	0	Not done	522	30.4 ± 1.3 [c]	52.8 ± 1.6 [c]
Glucose	1800	58.0± 0.8	522	26.5 ± 0.8 [d]	47.6 ± 1.0 [d]

[a,b,c,d] Values without common superscripts in the same column within experiments differ ([a,b] P<0.05; [c,d] P<0.01).

Table 3.2. Main effect treatment means for bulls for development of bovine embryos in vitro (±SE).

Bull	No. oocytes	Cleaved (%)	8 cell embryos per oocyte (%)	Blastocysts per oocyte (%)	Blastocysts per 8 cell embryo (%)
Experiment 1					
A	600	82.5±1.6 [a]	70.4±1.8 [a]	19.0±1.4 [a]	26.8±1.7 [a]
B	600	76.4±1.6 [b]	60.3±1.8 [b]	17.5±1.4 [a]	25.7±1.7 [a]
C	600	82.0±1.6 [a]	72.5±1.8 [a]	19.3±1.4 [a]	26.6±1.7 [a]
D	600	85.3±1.6 [a]	72.1±1.8 [a]	27.8±1.4 [b]	38.9±1.7 [b]
Experiment 2					
A	300	74.0±1.4 [a]	56.6±1.9 [a]	37.9±2.0 [c]	55.2±2.4 [c]
B	300	68.3±1.4 [a]	54.5±1.9 [a]	33.8±2.0 [c]	51.0±2.4 [c]
C	300	65.7±1.4 [a]	57.0±1.9 [a]	31.9±2.0 [c]	50.0±2.4 [c]
D	300	81.7±1.4 [b]	64.0±1.9 [b]	28.5±2.0 [c]	50.0±2.4 [c]
E	300	78.1±1.4 [a]	57.0±1.9 [a]	25.2±2.0 [c,d]	48.8±2.4 [c,d]
F	300	58.0±1.4 [a]	56.2±1.9 [a]	21.0± 2.0 [d]	44.4±2.4 [d]

[a,b,c,d] Values without common superscripts in the same column within experiments differ (P<0.05).

3.5.2. Lipid Droplets for Experiment 1.

Numbers of small droplets (Figure 3.3) were lower for PES than for the control and the rest of the metabolic regulators ($P < 0.05$), with no differences among DNP, DNP+PES and control ($P > 0.05$); FCS was higher ($P < 0.05$) than any other groups.

Numbers of medium size droplets were significantly lower ($P < 0.05$) for PES, DNP, and DNP+PES than the control and FCS. Numbers of large size droplets were similar for PES, as DNP, and DNP+PES, but different ($P < 0.05$) from the control and FCS; however DNP did not differ ($P > 0.05$) from the control. Numbers of medium + large size lipid droplets (Figure 3.4) were similar for PES, DNP, and DNP+PES, but all of these were lower ($P < 0.05$) than the control and FCS. There was no effect of hexose (glucose or fructose) on lipid droplet sizes (Table 3.4). Photomicrographs of typical embryos for each treatment are in Figure 3.5.

Table 3.3. Main effect means of additives for development of bovine embryos *in vitro* (±SE).

Additive	No. oocytes	Blastocysts per oocyte (%)	Blastocysts per 8 cell embryo (%)	No. cells per blastocyst *
Experiment 1				
Control	480	21.8±1.4 [a]	30.5±1.9 [a]	97.8 ± 3.9 [a,b]
FCS 10%	480	24.5±1.4 [a]	34.1±1.9 [a]	98.6 ± 4.0 [a]
PES	480	24.0±1.4 [a]	34.1±1.9 [a]	94.0 ± 4.9 [a,b]
DNP	480	17.0±1.4 [b]	24.2±1.9 [b]	87.3 ± 4.2 [b]
DNP+PES	480	17.0±1.4 [b]	24.2±1.9 [b]	86.3 ± 4.7 [b]
Experiment 2				
PES	360	35.8±1.6 [c]	59.3±2.0 [c]	Not done
DNP+PES	360	31.9±1.6 [c]	55.5±2.0 [c]	
Control	360	32.8±1.6 [c]	56.6±2.0 [c]	
Cerulenin 1	360	32.8±1.6 [c]	55.1±2.0 [c]	
Cerulenin 3	360	13.6±1.6 [d]	24.4±2.0 [d]	

[a,b,c,d] Values without common superscripts in the same column within experiments differ ([a,b] $P<0.05$; [c,d] $P<0.01$). * N=25 to 39 per group.

3.5.3. Embryo Production for Experiment 2.

The blastocyst rate per oocyte matured and per 8 cell embryo was higher ($P < 0.05$) for fructose than glucose (Table 3.1). Semen from bulls A, B, C, and D, (Table 3.2) resulted in more blastocysts per oocyte than bull E ($P < 0.05$). The only significant effect

of metabolic regulators was on blastocyst rate per 8-cell embryo ($P < 0.05$); the highest concentration of cerulenin was toxic relative to the other treatments (Table 3.3).

Figure 3.3. Means ± SEM of small, medium and large lipid droplets stained with Sudan black B with metabolic regulators for the first experiment.

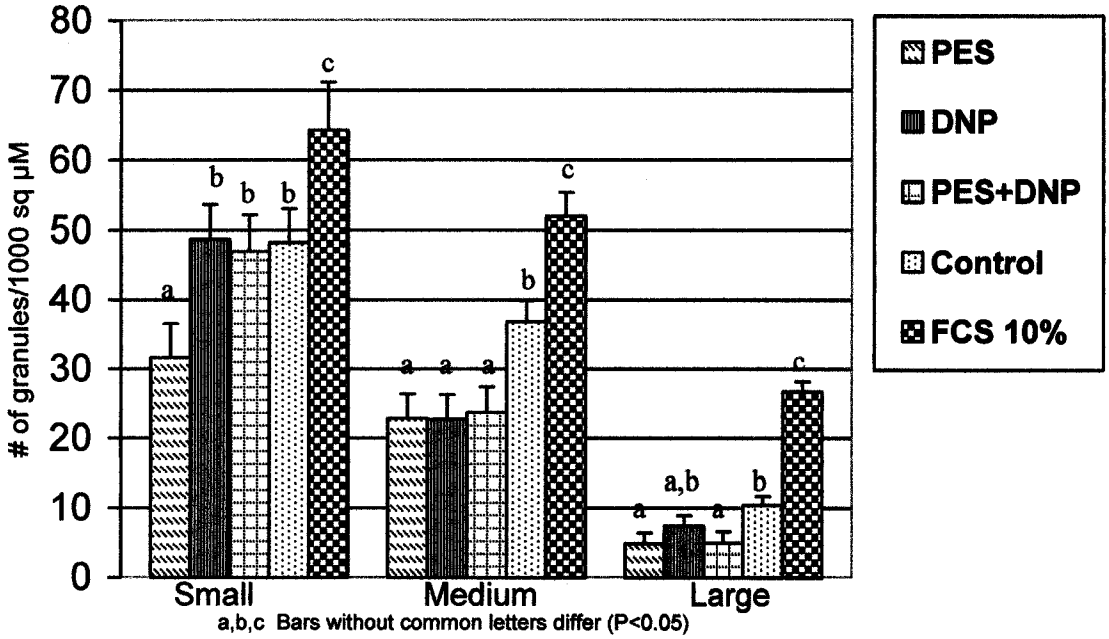


Figure. 3.4. Means ± SEM for metabolic regulators for the first experiment for medium plus large lipid droplets.

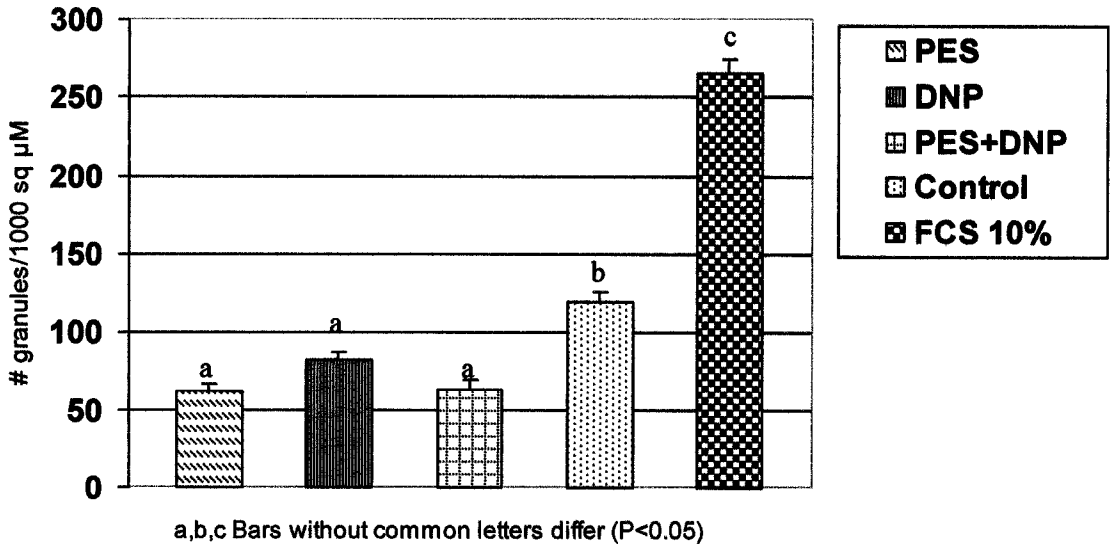


Figure 3.5. In vitro produced embryos from experiment 1 stained with Sudan black B (Photographed with a 60X objective).

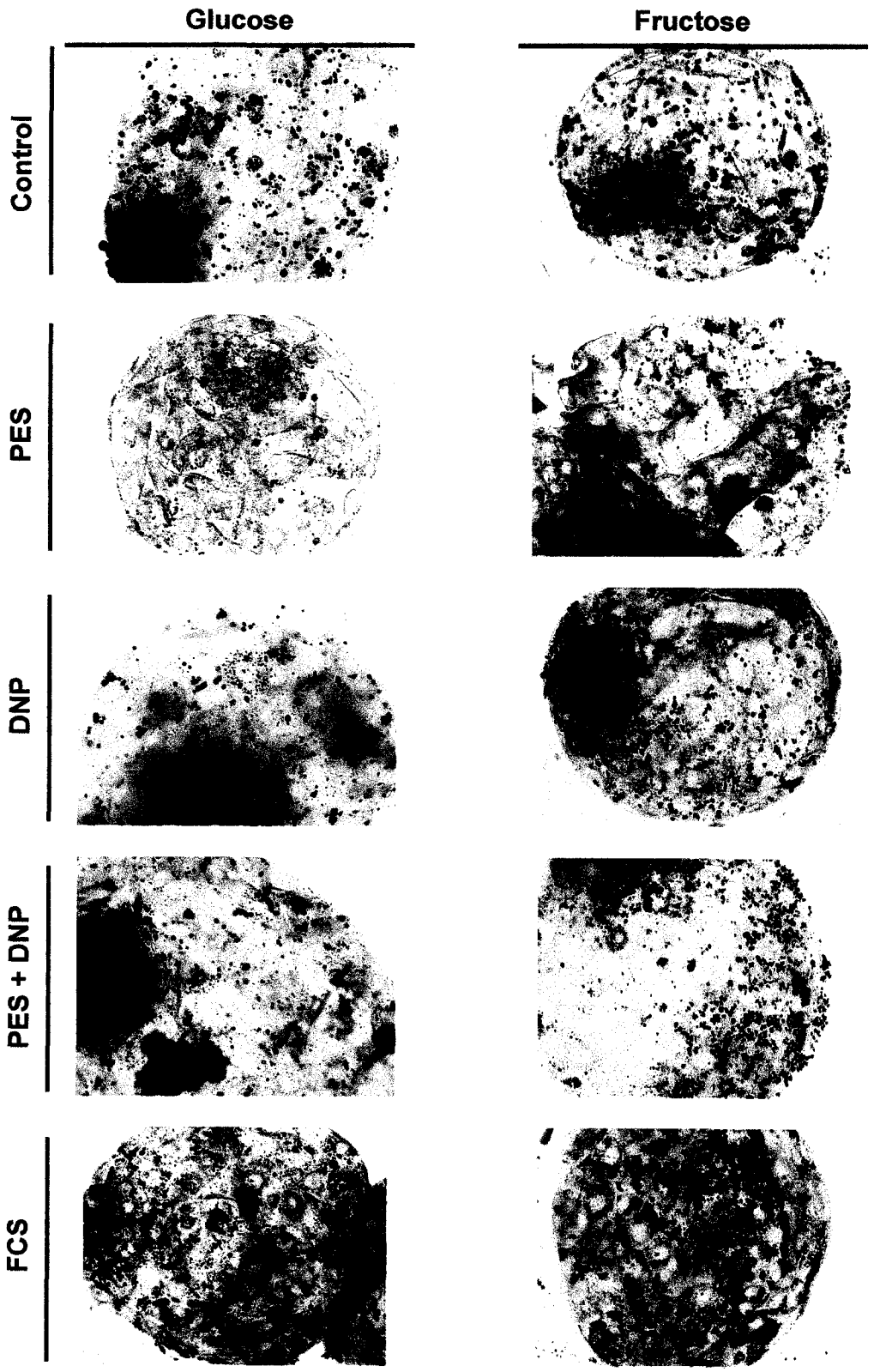


Table 3.4. Means ± SEM of hexose effects on lipid droplet production of bovine blastocysts produced in vitro.

Hexose	Small size	Medium size	Large size	Medium+Large size
Experiment 1				
Glucose	47.3 ± 3.0	32.0 ± 2.2	11.6 ± 0.9	124.2 ± 8.0
Fructose	48.5 ± 3.0	31.2 ± 2.2	10.1 ± 0.9	113.2 ± 8.0
Experiment 2				
Glucose	38.7 ± 2.7	30.4 ± 1.5[a]	3.7 ± 0.3	74.0 ± 6.7
Fructose	42.2 ± 3.9	23.6 ± 2.2[b]	4.5 ± 0.4	87.6 ± 8.7

[a,b] Values with different superscripts in the same column differ (P<0.05).

3.5.4. Lipid Droplets for Experiment 2.

The number of small droplets did not differ among treatments (P > 0.05) (Figure 3.6) except that cerulenin 3 had more small droplets than all treatments except the control (P < 0.05). Numbers of medium size droplets were lower (P < 0.05) for PES and DNP+PES than cerulenin 1, cerulenin 3 and the control. Numbers of large lipid droplets were similar for PES, and DNP+PES; PES was lower (P < 0.05) than the control, but not different between control and DNP+PES; cerulenin 3 was not different from the control, but was significantly higher than the rest of the treatments (P < 0.05). Numbers of medium + large (Figure 3.7) size droplets were similar for PES and DNP+PES, but different from the control, cerulenin 1, and cerulenin 3 (P < 0.05). There was no effect of hexose (glucose or fructose) for lipid droplets (Table 3.4), except for medium size droplets, for which fructose resulted in fewer droplets than glucose (23.6 vs. 30.4 droplets, respectively), (P < 0.05). Photomicrographs of typical embryos for each treatment are in Figure 3.8.

Figure 3.6. Means ± SEM for metabolic regulators for experiment 2 of small, medium and large lipid droplets.

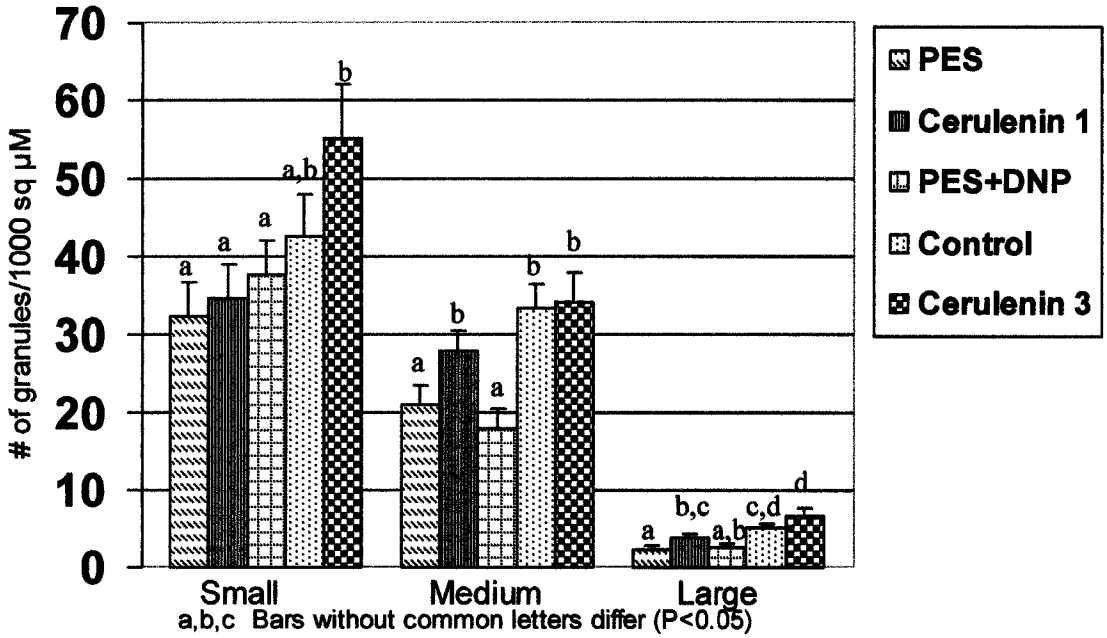


Figure 3.7. Means ± SEM for metabolic regulators for experiment 2 of medium plus large lipid droplets.

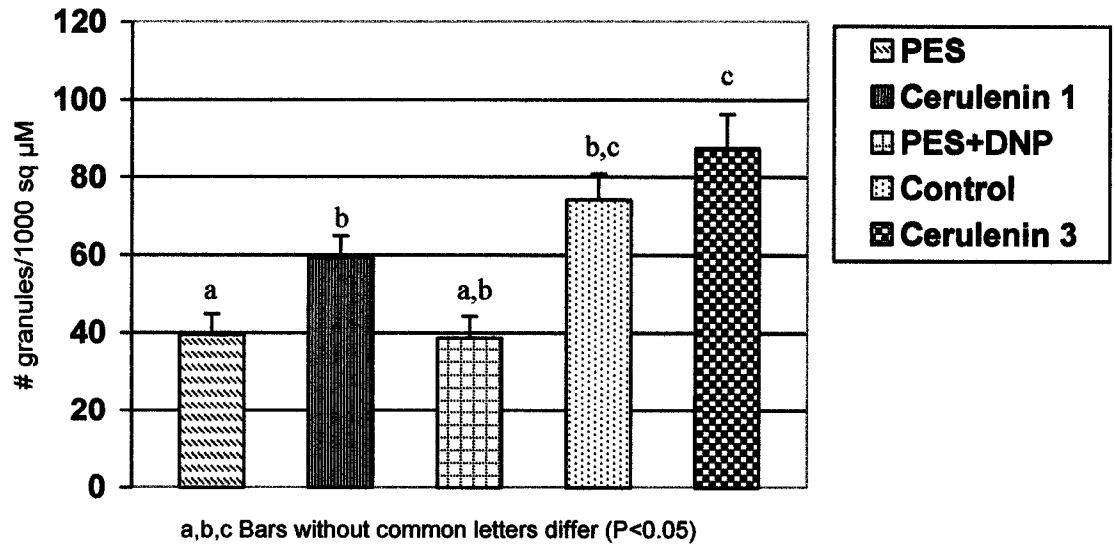
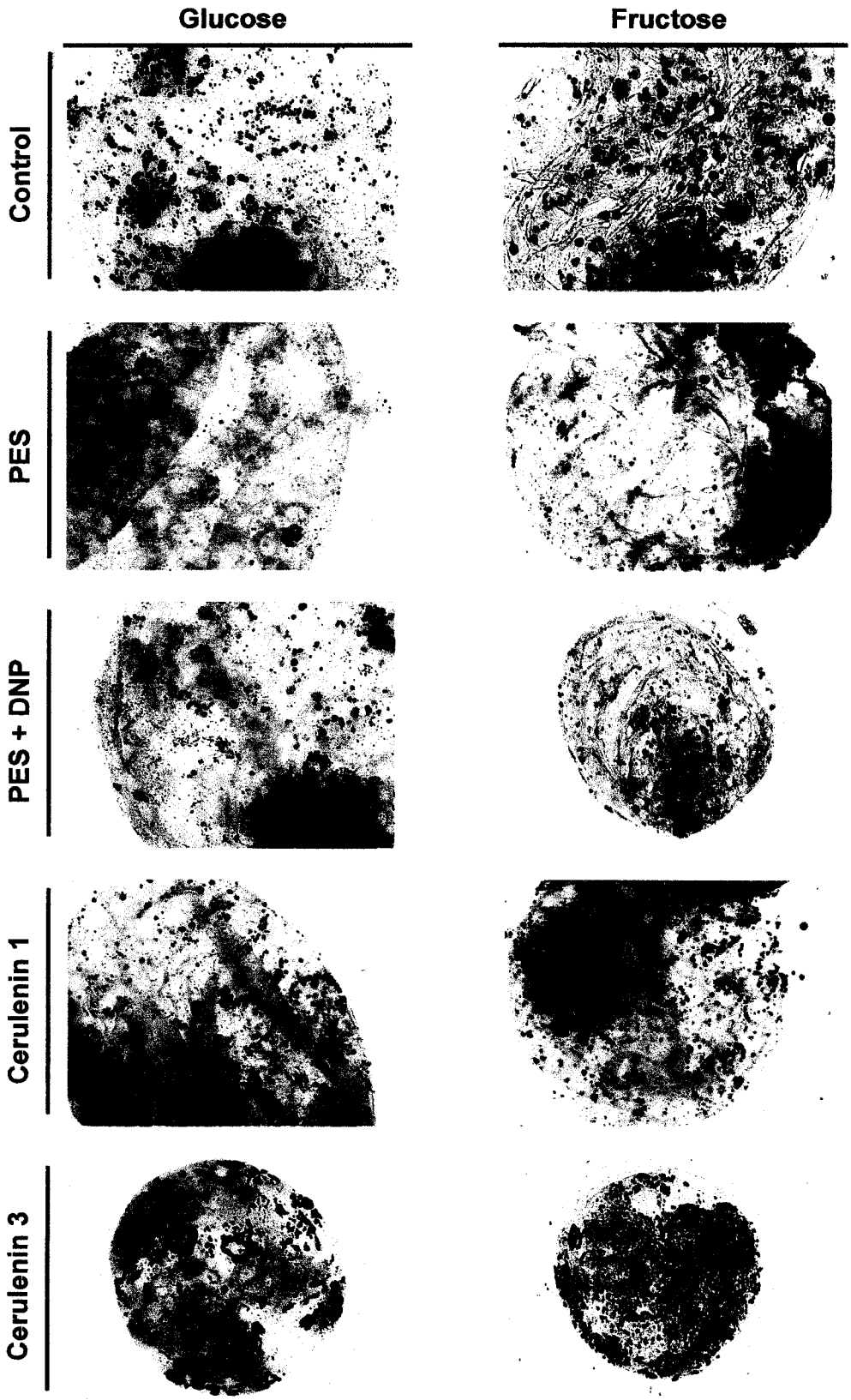


Figure 3.8. In vitro produced embryos from experiment 2 stained with Sudan black B (Photographed with a 60X objective).



3.6. Discussion

3.6.1. Hexoses.

In the first experiment there was no significant difference between the use of glucose or fructose as energy substrate at low concentration (0.5 mM) in the rate of 8-cell production per oocyte (68.1 vs. 69.6%, respectively). These results might be expected, first because from zygote to the 8- to 16-cell stage, pyruvate and lactate are the preferred energy sources (Leese and Barton, 1984). Also, hexose metabolism via glycolysis appears to have minimal importance in early embryos (Gardner et al., 1993). Furthermore, if high levels of glycolysis are induced in mice (Scott and Whittingham, 2002), rats (Matsumoto and Sugawara, 1998), and hamsters (Seshagiri and Bavister, 1991) by use of high glucose, phosphate, or no amino acids in the medium, this leads to the "Crabtree effect" (Koobs, 1972), and a 2-cell developmental block. Also, glucose-6-phosphate fails to inhibit the enzyme hexokinase allosterically in the early embryo, which leads to reduction of phosphate pools in the mitochondria because of the continued phosphorylation of glucose by the kinase and ADP generated in this reaction in the mitochondria (Gardner et al., 2000). The use of EDTA during this time improves blastocyst production (Olson and Seidel, 2000), possibly because EDTA inhibits glycolysis by chelating Mg^{++} ions, which are needed for the kinase enzymes (Lane and Gardner, 1997). However, EDTA may have some other effect because EDTA is added at 10-μM concentration and the media contain 500 μM magnesium.

During this stage, glucose transporters 1 (Glut-1), 3 (Glut-3), and 8 (Glut-8) are found in the embryos; however, Glut-1 is in the lateral membrane of the cells (Augustin et al., 2001), so its role could be intracellular rather than for glucose uptake from the outside environment. Glut-8 is an insulin-regulated transporter, so Glut-3 is the main transporter for moving glucose from the external environment at this stage, as well as at the blastocyst stage (Pantaleon et al., 1997). The Glut-5 transporter has a higher affinity for fructose than glucose, but is not present during this period of time because transcription starts at the 8- to 16-cell stage in cattle, which coincides with the time of embryonic genome activation (Augustin et al., 2001). Probably this explains why glucose, but not fructose, can be toxic at high doses (5.4 mM) in early stages as shown by Kwun et al. (2003).

The use of high glucose concentration in media for culturing precompaction stage embryos is detrimental in mice (Schini and Bavister, 1988; Menezo and Khatchadourian, 1990; Downs and Dow, 1991), cattle (Kim et al., 1993), hamsters (Schini and Bavister, 1988), and human embryos (Quinn, 1995); similar effects are seen with embryonic death in diabetic mice (Moley et al., 1996). Some research suggest that this toxic effect is also related to disruption of mitochondrial organization (Barnett et al., 1997) and reduction of respiration (Seshagiri and Bavister, 1991), resulting in arrest of embryonic development (Seshagiri and Bavister, 1989).

In both experiments the blastocyst rates per oocyte matured and per 8-cell embryo were higher for fructose than glucose. Using fructose between fertilization and the

blastocyst stage increased blastocysts per oocyte 22% (4.1 percentage points) and blastocysts per 8-cell embryo 24% (6.2 percentage points) in the first experiment. Meanwhile in the second experiment the use of fructose at the time of the genome activation (8-cell embryo) to blastulation, increased blastocysts per oocyte 15% (3.9 percentage points) and the blastocyst per 8-cell embryo 11% (5.2 percentage points). Though the blastocyst rate differed between experiments for unknown reasons, the fructose effect was similar. These results are consistent with other experiments in which fructose showed advantages over glucose, particularly less toxicity at a high dose (5.4 mM) (Kwun et al., 2003), including higher cleavage (74% vs. 66%) and more morulae (27% vs. 21%) in bovine embryos (Chung et al., 1996). In bovine embryos, fructose at a low dose (0.25 mM) from the zygote stage to the precompaction stage resulted in slightly more morulae and blastocysts (48 and 35%) than glucose (36 and 27%) (Chung et al., 2000); Fructose also improved the developmental competence of bovine SCNT embryos relative to glucose (Kwun et al., 2003); increased total cell number in hamster (Ludwig et al., 2001), and bovine embryos (Kwun et al., 2003); and reduced the index of DNA-fragmented nuclei in porcine blastocysts as well as enhanced embryo quality (Wongsrikeao et al., 2005).

The effects of different hexoses on embryo development may be species-specific; fructose does not appear to have an advantage over glucose on percent embryo development for in vitro-produced porcine embryos, but it did improve embryo quality (Wongsrikeao et al., 2005). In some strains of mice (OF1 and C57B1) fructose alone was not the best energy source, (Sakkas et al., 1993), but in the outbred Swiss strain, fructose

eliminated the 2-cell block and resulted in higher blastocyst rates than glucose (Menezo and Khatchadourian, 1990). Analogous beneficial effects have been obtained by the use of fructose during embryo development in hamsters as determined by embryo implantation rate, and fetal development and weight (Ludwig et al., 2001).

Glucose is the most used energy source for culture of tissues and embryos, usually at the concentration (5.6 mM; Biggers, 1998) found in blood of monogastric species like pigs (Nichol et al., 1992), mice and humans (Lee et al., 1978; McDonald, 1980). However, the concentration of glucose in ruminant blood is lower: cattle 3.0 to 3.5 mM (Meier et al., 2003), and sheep 3.6 to 4.8 mM (Maas et al., 2001). Glucose concentration in the reproductive tract of cattle is very low 2 to 2.85 mM in follicular fluid (Leroy et al., 2004), and 0.04 to 0.2 mM in oviductal fluid (Carlson et al; 1970; Parrish et al., 1989). Therefore, early embryos cultured in a medium at 5.6 mM glucose are exposed at least 28 times more glucose than the in vivo concentration in the oviduct. The use of glucose at 5.6 mM is not appropriate for development of early embryos of most monogastric mammals including: hamsters (Schini and Bavister, 1988), mice (Lawitts and Biggers, 1991; Scott and Whittingham, 2002), rats (Kishi et al., 1991; Miyoshi et al., 1994), and humans (Conaghan et al., 1993; Quinn, 1995), and especially polygastrics such as cattle (Peippo et al., 2001) and sheep (Thompson et al., 1992). Use of high levels of glucose post-compaction produces further detrimental effects in blastocysts (Colton et al., 2002), and in mouse embryos leads to a significant decrease in postimplantation fetal development compared with a low dose of glucose (Ludwig et al., 2001). Also the use of glucose during in vitro culture increases glycogen accumulation relative to no glucose

(Ozias and Stern, 1973). Kimura et al. (2005) proposed that the effect of the high glucose concentration is one of the main determinants of sex ratio distortion in vitro. The use of glucose at blood serum concentrations was detrimental for female embryos, which arrested in the transition of post-compaction stage of morulae to blastocysts (Gutiérrez-Adán et al., 2001; Larson et al., 2001; Peippo et al., 2001); this leads to a change in the sex ratio of blastocysts, favoring the production of male embryos. The use of medium lacking glucose or with fructose normalized the sex ratio (Kimura al., 2005).

3.6.2. Bull Effects.

In these experiments, a bull effect was found, corroborating that different sires manifest differences in cleavage and blastocyst rates when sperm are used for identical co-incubation times and sperm concentrations during in vitro fertilization. In experiment 1, bull B was lower than the others bulls for cleavage rate, but not for blastocyst production, and bull D was not different from bulls A and C for 8-cell embryo production, but was higher for blastocyst production. However, in the experiment 2 bulls A, B, C, and D, resulted in more blastocysts than bull E. There are no clear explanations for these bull effects. It is been suggested by Ward et al. (2003) that the fertilizing capacity of bulls depends on differences in the kinetics of sperm penetration. They explained that sperm from some bulls penetrate oocytes faster than other bulls, resulting in earlier cleavage and blastocysts of better quality. Also the heparin concentration during IVF is an important factor to consider, because different bulls may require different concentrations to achieve acceptable rates of fertilization and embryo development (Fukui et al., 1990; Hillery et al., 1990; Lu and Seidel, 2004).

3.6.3. Metabolic Regulators.

Pharmacological regulation of embryo metabolism has been evaluated in many studies. The use of 0.1 to 0.3 μM PES during in vitro production of cattle embryos had no effect on blastocyst rate, inner cell mass or embryo quality, or cell number compared to controls; however, 0.9 μM PES was detrimental (De La Torre-Sanchez et al., 2006a). Embryos cultured with 0.3 μM PES had higher glycolysis and PPP rates but lower glucose uptake, compared with DNP, sodium azide and the control (De La Torre-Sanchez et al., 2006b). Several authors have reported an increase in glucose metabolism via the PPP by PES for embryos produced in vitro in rats (Dufrasnes et al., 1993), mice (O'Fallon and Wright, 1986), and cattle (Rieger and Guay, 1988; De La Torre-Sanchez et al., 2006a). Dufrasnes et al. (1993) reported 10-fold increases in PPP, and glycolysis was reduced by 50%, when using a toxic dose of PES for rat embryos. This is probably because $NADPH^{+}$ is necessary for some important pathways; for example, besides being involved in lipid biosynthesis (Wales and Du, 1993), it is also required for inhibiting peroxidation by reducing intracellular glutathione (Harvey et al., 2002).

The mechanism by which DNP has beneficial effects is not totally understood. Partial inhibition of mitochondrial ATP production may lead to a more suitable redox state in the embryo that favors metabolism via glycolysis (Macháty et al., 2001), so glucose is used in other ways than lactate production (Thompson and Peterson, 2000). There are reports of beneficial effects of DNP during in vitro culture in different species at various concentrations; in bovine embryos 10 to 30 μM DNP increased glucose uptake (Thompson et al., 2000; De La Torre-Sanchez et al., 2006a), increased numbers of cells

in embryos (Thompson et al., 2000; Harvey et al., 2004) increased blastocyst rate (Thompson et al., 2000; De La Torre-Sanchez et al., 2006b); and increased expression of hypoxia factors 1 and 2 α (Harvey et al., 2004). However, bovine embryos cultured with 90 μM DNP developed more slowly and had darker appearance than lower doses (10 and 30 μM) (De La Torre-Sanchez et al., 2006b), and 100 μM DNP reduced embryo development (Thompson et al., 2000); a dose of 1000 μM disrupted bovine embryo development (Donnay and Leese, 1999; Thompson et al., 2000). Embryos of rats, however, are very resistant to DNP and can tolerate and develop normally at 1000 μM (Thompson et al., 2000). For pig embryos, DNP at 100 μM was superior to 10 and 50 μM for stimulating blastocyst development and cell number (Macháty et al., 2001); in mice, 50μM DNP increased metabolism via glycolysis and oxidation of glucose (O'Fallon and Wright, 1986), and in sheep 50μM DNP increased glucose metabolism when pyruvate and lactate were not present (Thompson et al., 1991). Note that in all of these studies, dose effects of DNP may depend on the composition of the media used.

In this study, DNP and the combination of DNP with PES, but not PES alone decreased blastocyst production 40% (10 percentage points) in the first experiment, but not in the second experiment. A possible explanation is that in the first experiment, a single dilution step of DNP in DMSO was done from the 2000x stock to the media, without intermediate dilution to a 100x stock; the 1:2000 DMSO could have had a toxic effect, as suggested a separate experiment where a single dilution step of DMSO to the media without DNP was done, resulting in small detrimental effects for embryo production compared to two step dilution and the control (data not shown).

Previous work has shown that cerulenin decreased ATP and lipid production in neurons (Landree et al., 2004), led to apoptosis in cancer cell lines by inhibiting fatty acid synthase (Kuhajda et al., 2000), and caused loss of weight and reduced feed intake in wild type and leptin deficient ob/ob mice (Loftus et al., 2000). Cerulenin in neurons at 5 to 20 μg/ml was not toxic (Landree et al., 2004). To our knowledge there are no previous reports of use of cerulenin in embryos. Bovine embryos were very sensitive to this mycotoxin; in a preliminary experiment we compared control, 10 μg/ml and 50 μg/ml of cerulenin, added at the 8-cell stage; these concentrations killed all embryos (data not shown). In the second experiment 1 μg/ml of cerulenin did not affect the embryo development, but 3 μg/ml reduced the blastocyst rate 32%, and resulted in blastocysts of poor quality (Fig. 8). Therefore, 1 μg/ml was very close to the minimum dose of cerulenin for embryos without negative effects.

In the first experiment, lipid accumulation was less for PES, DNP, and DNP+PES, than the control and FCS (62, 82, 64 vs. 119 and 266 lipid droplets per $1x10^3$ μm^2, respectively). FCS-treated embryos accumulated 4.3 times and the control 2 times more lipid droplets than PES-treated embryos. Some authors have postulated that lipid accumulation can be either uptake from the culture environment (McEvoy et al., 2001) or be produced by the embryo as result of impaired β-oxidation metabolism, due to abnormal mitochondrial activity (Dorland et al., 1994). In this experiment the medium was chemically defined, with fatty acid free BSA as the only undefined compound; therefore, lipid accumulation in the control group had to result from impaired metabolism of the embryo; lipid accumulation with FCS could have been due to both mechanisms.

Previous reports also have shown that FCS increased the number and size of lipid droplets in morulae and blastocysts (Gardner et al., 1994; Thompson et al., 1995; Crosier, et al., 2000; Abe et al., 2002), and produced organelle abnormalities (Abe et al., 1999) including mitochondrial degeneration (Dorland et al., 1994). In our culture system there was no benefit of using FCS on embryonic development. This is consistent with other reports in which there was no advantage of using FCS instead of albumin in culture media for human embryos (Menezo et al., 1984) or bovine embryos at day 7 of culture (Rizos et al., 2003). In addition international regulations are evolving to eliminate animal products in media; FCS can be source of pathogenic viruses (Blake et al., 2002).

In the second experiment, lipid accumulation was less for PES and DNP+PES, than for the control and cerulenin. There was no advantage of the combination of DNP+PES on lipid accumulation and embryo development in either experiment. Cerulenin was ineffective in reducing lipid accumulation; one explanation could be that cerulenin is simply toxic to bovine embryos, and the non-toxic dose had no inhibitory effect on fatty acid synthase. The high dose of cerulenin accumulated more lipid than the low dose, but neither was different from the control ($P > 0.1$).

In a previous experiment (De La Torre-Sanchez et al., 2006a), small lipid droplet accumulation was higher in in vivo-produced embryos than in vitro-produced embryos treated with PES, DNP or the in vitro control; medium size lipid droplets for PES and DNP were higher than for in vivo-produced embryos, but were lower than the in vitro control, and the large lipid droplets were lower for in vivo-produced embryos and PES, with no differences between DNP and the control. Thus embryos treated with PES

accumulated more lipids than in vivo-produced embryos, but less than the in vitro control or the DNP-treatment.

The effect of hexose on lipid accumulation was minor in these experiments; in the first experiment, there was no effect of glucose or fructose on numbers of lipid droplet, although in the second experiment, fructose resulted in fewer medium size droplets than glucose. A possible explanation is that the method used to measure lipid accumulation was not sufficiently sensitive to detect small changes in lipid accumulation in experiment

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CHAPTER IV

EFFECTS OF FETAL CALF SERUM, PHENAZINE ETHOSULPHATE AND EITHER GLUCOSE OR FRUCTOSE DURING IN VITRO CULTURE OF BOVINE EMBRYOS ON EMBRYONIC DEVELOPMENT AFTER CRYOPRESERVATION

4.1 Summary.

This study investigated effects of hexoses, FCS, and PES during the culture of bovine embryos on blastocyst development and survival after cryopreservation by slow freezing or vitrification. In vitro-produced bovine zygotes were cultured in CDM-1 with 0.5 mM glucose; after 60 h, 8-cell embryos were cultured 4.5 d in CDM-2. The eight-cell embryos were randomly allocated to a 2 x 3 x 2 x 3 factorial experimental design with two energy substrates (2 mM glucose or fructose); three additives (0.3 μM PES, 10% FCS, and control); two cryopreservation methods using no animal products (conventional slow freezing or vitrification); and semen from three bulls with two replicates for each bull. A total of 1107 blastocyst were produced.

Fructose resulted in 13% more blastocysts per oocyte than glucose (37.2 vs. 32.9%), and per 8- cell embryo (51.3 vs. 45.3%) ($P < 0.01$). No differences were found for additives ($P > 0.1$) control, FCS, or PES for blastocysts per oocyte or per 8-cell embryo. There was a significant interaction ($P < 0.05$) between additives and hexoses for blastocyst production; although trends were similar, the benefit of fructose compared to glucose was greater for controls than for FCS or PES. Culture of embryos with PES, which reduces cytoplasmic lipid content, improved cryotolerance of bovine embryos; post cryopreservation survival of blastocysts averaged over vitrification and slow freezing (between which there was no difference) was 91.9, 84.9, and 60.2% of unfrozen controls ($P < 0.01$) for PES, control and FCS groups, respectively.

4.2. Introduction.

Embryo transfer in cattle has become widespread (Hasler, 2003), partly because of cryopreservation with the conventional slow freezing technique using ethylene glycol (EG) for direct transfer (Leibo, 1984; Voelkel and Hu, 1992). It has been more than three decades since the report of the first successfully pregnancy from a cryopreserved bovine embryo (Wilmut and Rowson, 1973). However, new methods for cryopreservation such as vitrification (Sommerfeld and Niemann, 1999; Campos-Chillon et al., 2006), could simplify embryo transfer even more. Vitrification is a rapid and economical alternative to cryopreservation by conventional freezing (Vajta and Nagy, 2006), and we have developed a simple and efficacious vitrification technique for direct embryo transfer (Campos-Chillon et al., 2006).

Since the first calf produced by in vitro fertilization was reported by Brackett et al. in 1982, in vitro production of bovine embryos has increased significantly. In 2004 more than 239,000 of such embryos were transferred, and less than 46% were frozen embryos (Thibier, 2005). The production of embryos in vitro so far has had little commercial use in most countries; the main use in the United States is for research purposes. Some of the main limitations are related to abnormal morphology, metabolism (Khurana and Niemann, 2000), and large offspring syndrome (LOS) (Lazzari et al., 2002).

Several studies indicate that in vitro-produced ruminant embryos do not survive cryopreservation as well as those produced in vivo. For some reason, the chilling and freezing sensitivity and post transfer developmental competence are lower than for in vivo-derived embryos (Pollard and Leibo, 1993; Rizos et al., 2001; Rizos et al., 2002; Stojkovic et al., 2002; Mucci et al., 2006), which leads to poor embryo transfer results, with pregnancy rates as low as 30% (Hasler, 2003). The higher lipid accumulation in in vitro-produced embryos has been correlated with reduced cryotolerance (Abe et al., 2002). This concept was first demonstrated in pig embryos; cryotolerance was increased by delipidation by microsurgically removing cytoplasmic lipid droplets in early cleavage stage embryos (Nagashima et al., 1994; Nagashima et al., 1995). Changes in zona pellucida properties (Pollard and Leibo, 1993) and buoyant density (Leibo and Loskutoff, 1993; Massip et al., 1995), likely due to increased lipid content, have been reported for in vitro-produced embryos.

Some research suggests that embryo survival after cryopreservation is directly related to procedures for culturing embryos (Rizos et al., 2001), including co-culture (Leibo and Loskutoff, 1993) or adding fetal calf serum (FCS) (Abe et al., 2002; Mucci et al., 2006). Although there are beneficial properties of FCS as a source of nutrients and anti-oxidants (Catt, 1994), use of FCS has been correlated with LOS (Young et al., 1998), altering metabolism (Gardner et al.,1994), altered gene expression (Lazzari et. al., 2002), excess lipid accumulation (Abe et al., 2002; Barcelo-Fimbres and Seidel, 2007), abnormalities of organelles (Abe et al., 1999), and premature blastulation (Holm et al., 2002) of embryos. In addition, FCS can be a source of pathogenic viruses (Blake et al., 2002), and international regulations are evolving to eliminate animal products in media for embryos.

Chemicals that regulate metabolism can be used to improve embryonic development; for example, phenazine ethosulphate (PES) an electron acceptor for NADPH, results in NADP and has been used for mouse oocytes (Downs et al., 1998) and bovine embryos (De La Torre-Sanchez et al., 2006a, b). NADP promotes conversion of glucose-6-phosphate to 6- phosphogluconate, stimulating the pentose phosphate pathway (PPP), and decreasing lipid production (De La Torre-Sanchez et al., 2006a; Barcelo-Fimbres and Seidel, 2007). We postulated that PES treatment might increase survival rates after cryopreservation of in vitro produced embryos due to less lipid accumulation.

Most systems for producing mammalian embryos in vitro use glucose as an energy source in media despite putative toxic effects as reviewed in the companion paper

to this one (Barcelo-Fimbres and Seidel, 2007). In previous reports, use of fructose during in vitro culture increased total cell number in hamster embryos (Ludwig et al., 2001), reduced the index of DNA-fragmented nuclei in porcine blastocysts and enhanced embryo quality (Wongsrikeao et al., 2005), and showed a tendency to reduce lipid granule accumulation in bovine embryos (Barcelo-Fimbres and Seidel, 2007). This might lead to differences in embryo cryosurvival.

4.3. Objective.

The aims of this experiment were to determine the effects of glucose vs. fructose and use of metabolic regulators on development of bovine blastocysts produced in vitro, and success of their cryopreservation by both vitrification and conventional procedures.

4.4. Materials and methods

4.4.1. Experimental Design.

Eight cell embryos resulting from 3822 oocytes were subjected to a 2 x 3 x 2 x 3 factorial experimental design with 2 energy substrates (glucose or fructose); 3 additives (0.3 μM phenazine ethosulfate (PES), 10% of fetal calf serum (FCS), and control); 2 cryopreservation methods (conventional freezing or vitrification); and semen from 3 bulls (A, B, and C), two replicates for each bull.

4.4.2. In Vitro-production of embryos

4.4.2.1. Oocyte Collection and In Vitro Maturation

Bovine ovaries from mature cows were obtained from an abattoir (Gering, Nebraska) and transported to the laboratory within 5-6 h of slaughter in 0.15 M NaCl at ambient temperature, approximately 22 to 25°C. After arriving at the laboratory, ovaries were trimmed of extraneous tissue, and rinsed once again in 0.15 M NaCl. Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm antral follicles with an 18-gauge needle attached to a vacuum aspiration pump with 40-50 mmHg of pressure. Oocytes with more than 3 layers of unexpanded cumulus cells and morphologically bright and evenly granulated cytoplasm were selected for in vitro maturation (IVM). COCs were washed twice in chemically defined medium (CDM) (De La Torre-Sanchez et al., 2006b) with Hepes buffer for handling oocytes (HCDM-1), and once with maturation medium. Fifty COCs were matured in a well of 4-well plates (Nunclon, Roskilde, Denmark), containing 1 ml of CDM maturation medium with 0.5% fatty acid-free BSA (Sigma A6003) and hormones (15 ng/ml of FSH (NIH-FSH-S17; Bethesda, MD), 1μg/ml of LH (USDA-LH-B-5; Beltsville, MD)), 1.0 μg/ml of estradiol-17β(Sigma E2257), 50 ng/ml EGF (Sigma E9644) and 0.1mM cysteamine (Sigma M6500). Oocytes were incubated at 38.5°C with 5% CO_2 in humidified air for 23 h as described by De La Torre-Sanchez et al. (2006b).

4.4.2.2. Sperm Preparation

Frozen semen from three bulls of proven fertility, having at least 40% progressive motile sperm after thawing, were thawed in water at 35°C for 30 sec. Semen was gently expelled on top of a Percoll gradient (Sigma P1644) (2 ml 90%: 2 ml 45% Percoll in sperm (sp)-TALP medium (modified Tyrode's) as described by Parrish et al. (1989)). The

Percoll was in a 15-ml centrifuge tube that was centrifuged 20 min at ~400 x g at 21-24°C. The supernatant was discarded, and the sperm pellet (approximately 100 µl) was washed with 5 ml of chemically defined fertilization medium (Fert-CDM) (De La Torre-Sanchez et al., 2006b) supplemented with 0.5% BSA (fatty acid free; Sigma A6003), 5 mM caffeine (Sigma C0750), and 2 µg/ml heparin (Sigma H9399).

The sample was centrifuged again for 5 min at 400 x g at 21-24°C. After discarding the supernatant, approximately 90 to 100 µl remained and a 5 µl aliquot was used to determine sperm concentration with a hemacytometer. The sperm concentration was adjusted to 5 x 10^6 spermatozoa per ml in Fert-CDM.

4.4.2.3. In Vitro Fertilization

Following in vitro maturation, groups of 50 oocytes were placed in 450 µl of Fert-CDM medium per well of 4-wells dishes (Nunclon, Roskilde, Denmark); 50 µl of sperm suspension at 5 x 10^6 spermatozoa per ml were added, resulting in a final volume of 500 µl per well and a sperm concentration of 0.5 x 10^6/ml. Gametes were co-incubated for 18 h at 38.5°C in an atmosphere of humidified air and 5% CO_2.

4.4.2.4. Culture of Embryos

Following in vitro fertilization, presumptive zygotes were removed from wells, and transferred to 1.0-ml microcentrifuge tubes with approximately 100 µl of Fert-CDM, and then vortexed for 50 to 60 sec in a room at 28°C to remove cumulus cells. They were rinsed three times in handling medium (H-CDM-1). Early culture (day 0 to 2.5 post

fertilization) was done in 500 µl of fresh CDM with 0.5 mM glucose supplemented with non-essential amino acids (NEAA) (Sigma M7145), and 10 µM EDTA (CDM-1) as described by De La Torre-Sanchez et al. (2006b), and incubated at 39°C under 90% N_2, 5% O_2, and 5% CO_2. After 60 h of culture, embryos were examined with a stereomicroscope (15 to 20×) for cleavage rate, and all uncleaved ova and embryos less than 8 cells were discarded; the rest were transferred to a new culture dish with 500 µl CDM-2 (CDM supplemented with NEAA and essential amino acids (Sigma 6766) and 2 mM of hexose) and cultured from day 2.5 to 7.0 post fertilization.

Half of the 8-cell embryos were placed with CDM-2 plus 2 mM fructose, and half, with CDM-2 plus 2mM glucose, factorially with the additives (PES, FCS and Control). Embryos were evaluated on day 7 of culture to determine the final stage of development.

4.4.2.5. Addition of Metabolic Regulators

A 2000x stock of PES (Sigma, P4544) was prepared in nanopure water, and then a 100x stock was made in HCDM-2. This stock was stored at 5°C and added to CDM-2 at the time of equilibration. The final concentrations were: PES 0.3 µM, FCS 10%, and a control without additives.

4.4.2.6. Cryopreservation of Embryos

A total of 1107 blastocysts were produced in 6 replicates (semen of 3 bulls twice each) for the 6 treatments (2 hexoses and 3 additives). Twenty blastocysts produced in

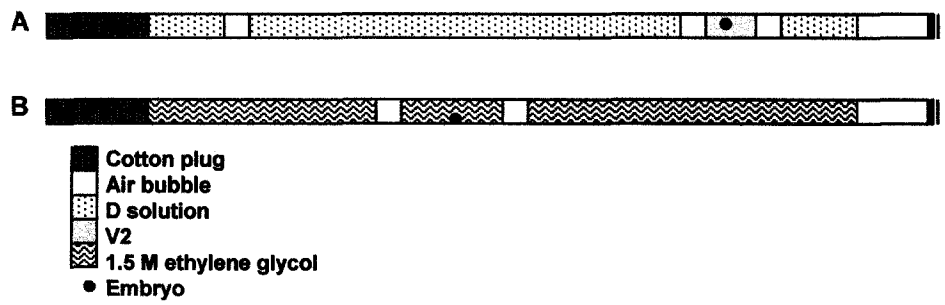
each treatment replicate combination were cryopreserved; 10 of them were frozen conventionally and 10 were vitrified. An average of 10 blastocysts of each subclass were not frozen and kept as controls. Two straws with 5 embryos each were used for each treatment x replicate. The base medium used for cryopreservation in this experiment was Syngro holding medium (SH) (Bioniche, Pullman, WA), with 0.1% w/v sodium pluronate and 0.2% w/v polyvinyl alcohol (PVA) (Sigma P8183), but no BSA or other animal products.

Vitrification: Embryos were placed into SH and held briefly at room temperature (20-24°C) until vitrification; they were transferred using a P10 Pipetman in 1 µl or less into 500 µl of vitrification solution one (V1: 5M (28% v/v) EG in SH) for 3 min to equilibrate, and then into a 6-µl drop of vitrification solution two (V2: 7M EG (39% v/v)) in SH + 0.5 M galactose (9% w/v, Sigma G5388) and Ficoll 70 (18% w/v, Sigma F2778) for 45±5 sec. Immediately after placing the embryo into V2, D solution (SH plus 0.5 M galactose (9% w/v)), then a column of V2 containing the embryos, and finally another column of D medium were aspirated into a 0.25 ml straw (Figure 4.1A). Then the straw was sealed with heat. Once 45±5 sec had elapsed, the straw was plunged embryo end first into liquid nitrogen and then the entire straw was slowly submerged.

Conventional Freezing: Embryos were placed into SH, held briefly at room temperature until cryopreservation, and then transferred to 500 µl of SS medium (SH plus 1.5 M EG plus 0.1 M sucrose) for 10-15 min at room temperature. Straws of 0.25 ml were loaded in 3 columns of fluid with 5-mm air columns between them. Straws were

loaded so that embryos were placed in the middle column, and enough medium was drawn into the straw in the third column to thoroughly wet the cotton plug, leaving 1 cm of air on the end opposite the cotton plug (Figure 4.1B). The end of the straw was heat-sealed before straws were placed into a programmable freezing machine. Straws were seeded at -7°C and held for 10 min. Seeding was done by touching the ends of the column of fluid with the embryos with forceps dipped in liquid nitrogen, and returning the straw to the freezing machine quickly. Embryos were cooled to -32°C at 0.5°C per min; when the straws reached -32°C, they were plunged into liquid nitrogen.

Figure 4.1. Straws loaded for embryo cryopreservation, A) embryo vitrification, and B) embryo freezing.



4.4.2.7. Thawing and Subsequent Culture of Embryos

Straws from both procedures were removed from liquid nitrogen, held in air for 10 sec, and then placed into a semen thaw box (35°C) for 20 sec. For vitrification, straws were removed from water and thoroughly shaken 4 times to mix columns. For both methods the embryos were held at room temperature (20-24°C) for 4 min, and the heat-sealed end of straw was cut off and the embryos expelled into SH at room temperature (20-24°C). Thawed embryos were rinsed through five 50 to 60 µl drops of SH before being cultured in CDM-2 for 48 h.

4.4.2.8. Evaluation of Embryos

In this experiment survival was defined as re-expansion of the blastocoele and its maintenance at 24 and 48 h post-thaw. Survival was adjusted relative to controls using the same number of treated blastocysts that were not cryopreserved, but were held at room temperature briefly and then cultured for an additional 48 h. Blastocysts were scored at 24 and 48 h for stage of development (5-early, 6-full, 7-expanded, 8-hatched), quality (1-excellent, 2-good/fair, 3-poor, and 4-dead), lightness (1-lighter, ...4-darker) and inner cell mass (ICM) (1-large, compacted ICM; 2- large, less compacted ICM; 3-medium, loose ICM; 4-scarce, loose ICM). The percentage development to blastocysts before cryopreservation was evaluated 7.5 days after the onset of fertilization.

Data were analyzed by ANOVA using the GLM procedure of the SAS statistical software package. Sources of variation in the model included energy source (glucose and fructose), additives (PES, FCS and control) method of cryopreservation (conventional freezing and vitrification), bulls, and all possible first order interactions; all factors were considered as fixed effects. The arc sin transformation was applied to percentage data. If the ANOVA was significant ($P < 0.05$), means were separated by the LSD procedure. Data are reported as untransformed least-squares means. Main effect means are presented in the absence of significant interactions.

4.5 RESULTS

4.5.1 Blastocyst Production of IVF Embryos

The blastocyst rates per oocyte matured and per 8-cell embryo produced were different between glucose and fructose ($P < 0.001$) (Table 4.1) (32.9 vs. 37.2% and 45.3 vs. 51.3%, respectively). No differences ($P > 0.1$) were found for the additives FCS, PES and the control for blastocyst rate per oocyte matured or blastocyst rate per 8-cell embryo produced. Frozen semen of bull A resulted in more blastocysts per 8-cell embryo and per oocyte than semen from bulls B and C ($P < 0.01$).

There was a significant interaction ($P < 0.05$) between additives and hexoses for blastocyst production (Table 4.2; Figure 4.2 and 4.3). Fructose was superior to glucose for control and FCS treatment for blastocysts per oocyte, and the control group for blastocysts per 8-cell embryo; however, fructose was not superior to glucose for the PES treatment.

Table 4.1. Main effect treatment means of hexoses, additives, and bulls for development, stage score and hatching of bovine embryos in vitro (±SE).

Responses	No. 8-cell embryos	Blastocysts per oocyte (%)	Blastocysts per 8-cell embryo (%)	Blastocyst stage (5=early blastocyst ... 8=hatching)	Blastocysts hatching (%)
Hexose					
Fructose	1162	37.2 ± 0.9 [a]	51.3 ± 1.2 [a]	6.9 ± 0.02	12.2 ± 1.3
Glucose	1185	32.9 ± 0.9 [b]	45.3 ± 1.2 [b]	6.8 ± 0.02	11.6 ± 1.3
Additives					
Control	789	34.7 ± 1.0	47.9 ± 1.5	6.9 ± 0.03	13.6 ± 1.6
FCS 10%	792	35.0 ± 1.0	48.3 ± 1.5	6.8 ± 0.03	11.9 ± 1.6
PES	766	35.4 ± 1.0	48.8 ± 1.5	6.8 ± 0.03	10.5 ± 1.6
Bull					
A	791	40.6±1.0 [a]	56.3±1.5 [a]	6.9±0.03	12.6±1.6
B	834	32.3±1.0 [b]	45.7±1.5 [b]	6.9±0.03	9.9±1.6
C	722	32.2±1.0 [b]	42.9±1.5 [b]	6.9±0.03	13.3±1.6

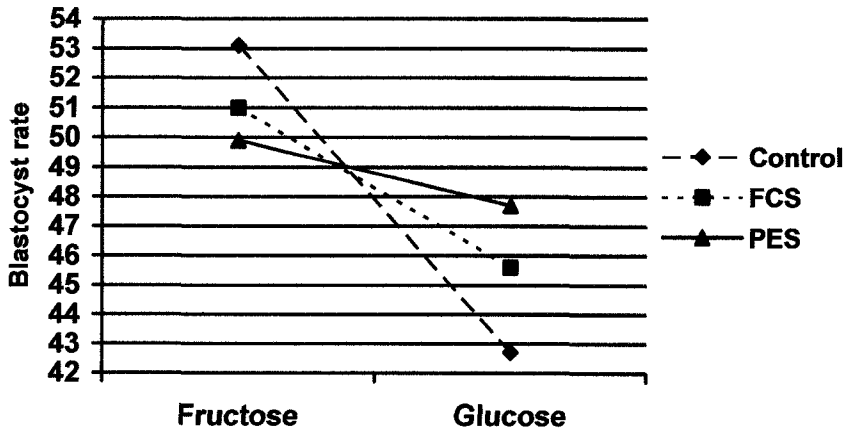
[a,b] Means with different superscripts in the same column within main effects differ ($P < 0.01$).

Table 4.2. Interaction means of additives and hexoses for development of bovine embryos in vitro (±SE).

Treatments		No. 8-cell	Blastocysts per	Blastocysts per 8-cell
Hexoses	Additives	embryos	oocyte (%)	embryo (%)
Fructose	Control	395	38.4 ± 1.5^{a}	53.1 ± 2.1^{a}
Fructose	FCS	389	36.9 ± 1.5^{a}	51.0 ± 2.1^{a}
Fructose	PES	378	$36.1 \pm 1.5^{a,c}$	49.9 ± 2.1^{a}
Glucose	Control	394	31.0 ± 1.5^{b}	$42.7 \pm 2.1^{b,c}$
Glucose	FCS	403	$33.1 \pm 1.5^{b,c}$	$45.6 \pm 2.1^{a,c}$
Glucose	PES	388	$34.6 \pm 1.5^{a,b}$	$47.7 \pm 2.1^{a,c}$

[a,b,c] Values without common superscripts in the same column differ ($P < 0.05$).

Figure 4.2. Interaction means of hexoses and additives for blastocyst rate per eight cell embryo produced in vitro ($P < 0.05$).



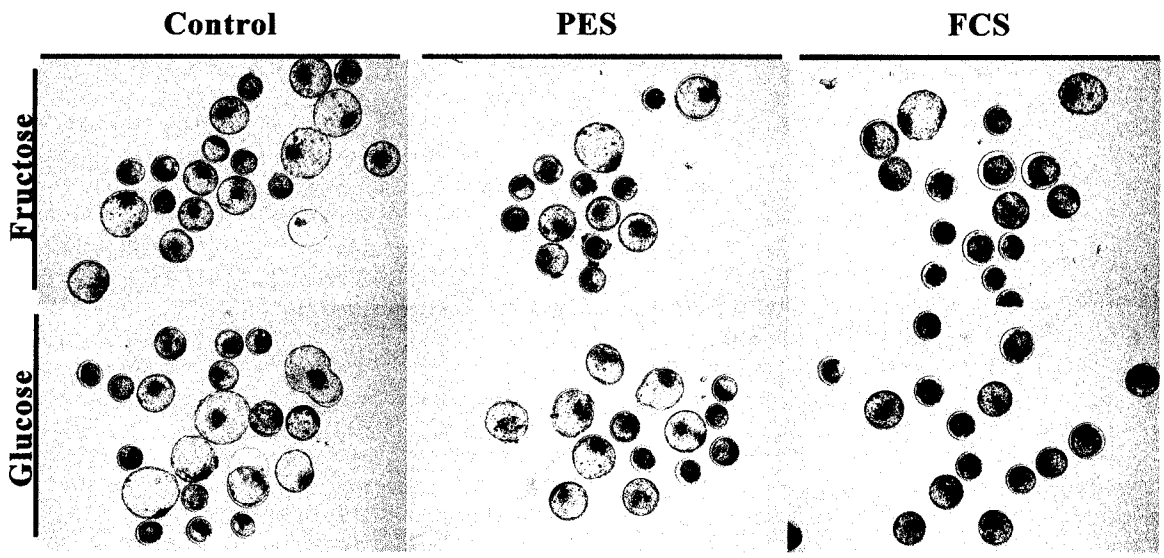
4.5.2 Blastocyst Stage of IVF Embryos

The embryonic stage at the end of the culture period was not different between glucose and fructose ($P > 0.1$) (Table 4.1) (6.8 vs. 6.9, respectively). No differences ($P > 0.1$) were found for the additives FCS, PES, or the control for stage of blastocysts developed in vitro. No effect for blastocyst stage was found by the use of semen from different bulls.

4.5.3 Hatching of IVF Embryos

The blastocyst hatching rates of the IVF embryos produced at 8 days of culture, were not different between glucose and fructose ($P > 0.1$) (Table 4.1) (12.2 vs. 11.6%, respectively). No differences ($P > 0.1$) were found for the additives FCS, PES, or the control for hatching, nor was a bull effect found.

Figure 4.3. In vitro-produced blastocysts from treatments with different energy substrates and additives (Photographed with a 10X objective).



4.5.4 Survival of Embryos after Cryopreservation

After cryopreservation, survival of blastocysts produced with glucose and fructose (Table 4.3; Figure 4.4) did not differ at 24 or 48 h. The effect of additives after cryopreservation (Table 4.4; Figure 4.4) was that PES was superior to the control and FCS at 24 and 48 h ($P < 0.001$). The comparison of FCS vs. control was also significant for survival ($P < 0.001$); however, embryo survival rates did not differ between methods

of cryopreservation (Table 4.5; Figure 4.4), at 24 or 48 h ($P > 0.1$). There were no significant interactions among treatments ($P > 0.1$) for any survival response.

Table 4.3. Main effect means of hexoses during culture in vitro for survival rate and embryo characteristics after cryopreservation of bovine embryos (±SE).

Responses	Fructose[a]		Glucose[a]	
	24 h	**48 h**	**24 h**	**48 h**
Survival rate, % (unfrozen control)	90±1.6	78±2.5	91±1.6	80±2.5
No. Blastocysts (unfrozen control)	180	180	180	180
Survival rate, % of unfrozen control survival rate	80.5±2.2	75.8±2.5	77.5±2.2	72.1±2.5
No. Blastocysts Cryopreserved	360	360	360	360
Embryo quality (1=excellent . . . 4=degenerate)	2.7±0.1	2.9±0.1	2.7±0.1	3.0±0.1
Lightness(1=light . . . 4=dark)	2.2±0.1	2.2±0.1	2.3±0.1	2.3±0.1
ICM score (1=large, compact . . . 4=few scattered cells)	2.3±0.1	2.3±0.1	2.2±0.1	2.4±0.1
Embryo stage (5=early blastocyst . . . 8=hatched)	6.7±0.1	6.8±0.1	6.5±0.1	6.7±0.1
Hatching/hatched, %	14.7±1.3	19.1±1.7	13.7±1.3	15.5±1.7

[a] Treatment means did not differ for any response ($P > 0.1$).

Figure. 4.4. In vitro-produced blastocysts for treatments with different energy substrates, additives and cryopreservation methods 24 hrs after thawing (Photographed with a 10X objective).

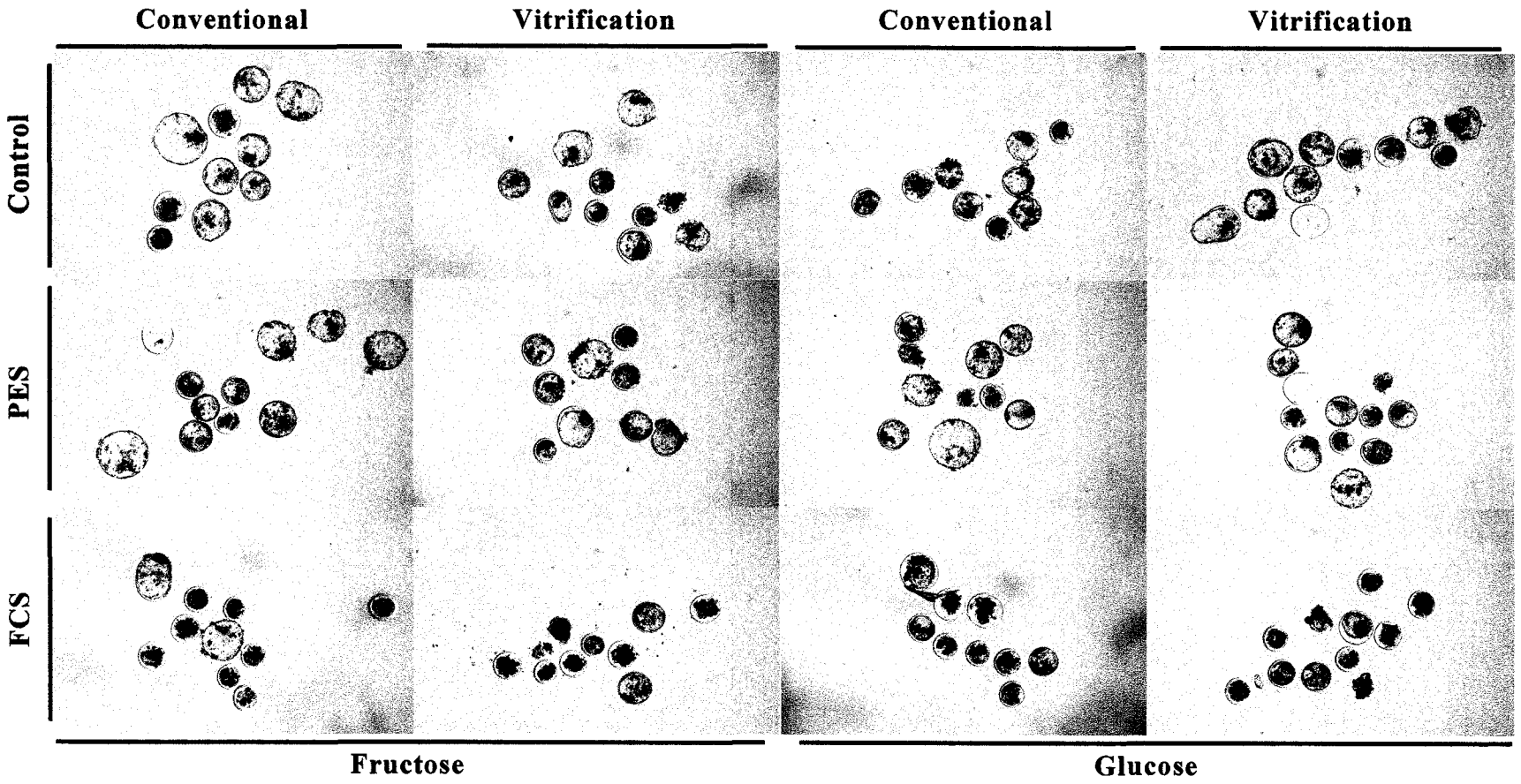


Table 4.4. Main effect means of additives during culture in vitro for survival rate and embryo characteristics after cryopreservation of bovine embryos (±SE)

Responses	Control		FCS		PES	
	24 h	48 h	24 h	48 h	24 h	48 h
Survival rate, % (unfrozen control)	92±2.0	84±2.1	88±2.0	77±2.1	90±2.0	79±2.1
No. Blastocysts (unfrozen control)	120	120	120	120	120	120
Survival rate, % of unfrozen control survival rate	84.9±2.7 [a]	77.0±3.0 [a]	60.2±2.7 [b]	60.3±3.0 [b]	91.9±2.7 [c]	84.6±3.0 [c]
No. Blastocysts Cryopreserved	240	240	240	240	240	240
Embryo quality (1=excellent . . . 4=degenerate)	2.5±2.7 [a]	2.8±2.7 [a]	3.2±2.7 [b]	3.3±2.7 [b]	2.4±2.7 [a]	2.7±2.7 [a]
Lightness(1=light . . . 4=dark)	2.2±0.1 [a]	2.2±0.1 [a]	2.8±0.1 [b]	2.8±0.1 [b]	1.9±0.1 [c]	1.8±0.1 [c]
ICM score (1=large, compact . . . 4=few scattered cells)	2.1±0.1 [a]	2.3±0.1 [a]	2.5±0.1 [b]	2.6±0.1 [b]	2.0±0.1 [a]	2.2±0.1 [a]
Embryo stage (5=early blastocyst . . . 8=hatched)	6.6±0.1	6.8±0.1	6.5±0.1	6.7±0.1	6.7±0.1	6.9±0.1
Hatching/hatched, %	16.0±2 [a]	20.5±2 [a]	10.6±2 [b]	12.1±2 [b]	15.9±2 [a]	19.3±2 [a]

[a,b,c] Means with different superscripts in the same row within times differ ($P < 0.01$).

Table 4.5. Main effect treatment means of method of cryopreservation for embryo survival rate and embryo characteristics after cryopreservation of bovine embryos (±SE).

Responses	Freezing		Vitrification	
	24 h	48 h	24 h	48 h
No. Blastocysts	360	360	360	360
Survival rate, % of unfrozen control survival rate	80.4±2.2	75.9±2.5	77.7±2.2	72.0±2.5
Embryo quality (1=excellent . . . 4=degenerate)	2.6±0.1	2.9 ± 0.1	2.7 ± 0.1	3.0±0.1
Lightness(1=light . . . 4=dark)	2.2±0.1	2.2±0.1	2.3±0.1	2.3±0.1
ICM score (1=large, compact . . . 4=few scattered cells)	2.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.4 ± 0.1
Embryo stage (5=early blastocyst . . . 8=hatched)	6.8± 0.1[a]	6.9± 0.1	6.5± 0.1[b]	6.7±0.1
Hatching/hatched, %	15.8±1.4	19.1±1.7	13.0±1.4	16.0± .7

[a,b] Values with different superscripts differ ($P < 0.01$).

4.5.5 Embryo Recovery after Cryopreservation

The embryo recovery rate from straws for freezing and vitrification were not different ($P > 0.1$), (99.2 vs. 98.6%, respectively). The shaking method with vitrification resulted in similar recovery of embryos from straws to the conventional method.

4.5.6 Embryo Quality after Cryopreservation

After cryopreservation, the hexose effect for quality of blastocysts produced with glucose and fructose (Table 4.3) did not differ at 24 and 48 h. The effect of additive on the embryo quality score after cryopreservation (Table 4.4) resulted in higher quality for PES and controls than FCS at 24 and 48 h ($P < 0.001$). The quality scores for method of cryopreservation (Table 4.5), were not different at 24 and 48 h ($P > 0.1$).

4.5.7 Embryo Lightness after Cryopreservation

The lightness of blastocysts produced with glucose and fructose (Table 4.3) did not differ at 24 and 48 h. Blastocysts after cryopreservation (Table 4.4) were lighter for PES than the control and FCS at 24 and 48 h ($P < 0.001$). The lightness of blastocysts did not differ between methods of cryopreservation (Table 4.5).

4.5.8 Embryo Inner Cell Mass (ICM) after Cryopreservation

The ICM quality of blastocysts produced with glucose and fructose (Table 4.3) did not differ at 24 or 48 h after cryopreservation. The additive effect (Table 4.4), resulted in higher quality scores for PES and the control than FCS at 24 and 48 h ($P < 0.001$); the method of cryopreservation (Table 4.5), had no effect on ICM quality ($P > 0.1$).

4.5.9 Embryo Stage after Cryopreservation

The embryo stage after thawing was not affected by previous culture in glucose vs. fructose (Table 4.3). There was no additive effect on embryo stage after cryopreservation (Table 4.4) ($P > 0.1$). Vitrification resulted in less advanced blastocysts than freezing at 24 h ($P < 0.01$) but not 48 h ($P>0.1$) (Table 4.5).

The hatching rate per embryo thawed was not affected by previous culture with glucose or fructose (Table 4.3). Fewer embryos hatched in the FCS group than in PES or control groups (Table 4.4) at 24 and 48 h ($P < 0.001$). Hatching scores (Table 4.5) were not different between vitrification and freezing methods at 24 or 48 h ($P > 0.1$).

4.6 DISCUSSION

4.6.1 Hexose Effects

In this experiment, blastocyst rates per oocyte matured and per 8-cell embryo were higher for fructose than glucose. Using fructose after embryonic genome activation (8-cell embryo) to blastulation increased both blastocyst rates per oocyte and per 8-cell embryo by 13% (4.3 and 6 percentage points, respectively). These results are consistent with publications in mice (Menezo and Khatchadourian, 1990), hamsters (Ludwig et al., 2001), and for cattle IVF and SCNT embryos (Kwun et al., 2003; Kimura et al., 2005; Barcelo-Fimbres and Seidel, 2007). The improvement in embryo development of fructose versus glucose has been demonstrated during in vitro culture of bovine embryos using a single culture medium (Kimura et al., 2005), and a sequential media system (Chung et al., 2000; Barcelo-Fimbres and Seidel, 2007); however, the beneficial effect of the fructose appears to be after the 8-cell stage (Barcelo-Fimbres and Seidel, 2007). We found no effect of hexose on blastocyst stage or hatching ($P < 0.01$).

4.6.2 Cryopreservation Method

There are important differences in cryopreservation techniques. Conventional cryopreservation requires removing water from cells by osmosis to prevent damaging ice crystal formation; this requires slow cooling rates and cryoprotectants. However, vitrification implies formation of glass without ice crystal formation; high cooling rates and high concentrations of cryoprotectants are used, which increase the risk of osmotic and toxic damage (Kuwayama et al., 1994).

The conventional freezing technique for cryopreservation of embryos is a well established, basic protocol with only minor variations among studies (Voelkel and Hu, 1992; Hasler et al., 1995). Pregnancy rates with this technique have exceeded 55% in large scale trials (Hasler et al., 1995; Nibart and Humblot, 1997). However, for vitrification there are many variations in protocols (Ishimori et al., 1993; Vajta et al., 1999; Park et al., 1999; Kim at al., 2004; Vajta and Nagy, 2006). Vitrification of in vitro-derived embryos results in highly variable pregnancy rates after transfer; the reported range is 23 to 50% of transferred embryos, but most of studies involved small numbers of embryos (Kuwayama et al., 1992; Tachikawa et al., 1993; Wurth et al., 1994; Agca et al., 1994; Delval et al., 1996; Holm et al., 1996). There was one experiment with large numbers of embryos transferred that compared slow freezing versus vitrification, and both procedures resulted in similar pregnancies rates (Van Wagtendonk-de Leeuw et al., 1997).

In vitro-produced embryos do not survive as well as in vivo-produced embryos using slow freezing (Leibo and Loskutoff, 1993; Hasler et al., 1995). Therefore, improvement of cryotolerance of in vitro-derived embryos is an important goal; some data indicate that vitrification, rather than slow freezing, may be better suited for their survival (Leibo and Loskutoff, 1993; Mahmoudzadeh et al., 1994; Pollard and Leibo, 1994; Mucci et al., 2006; Vajta and Nagy, 2006). In the current experiment, no differences in survival rates were detected between vitrification and slow freezing. Of the many responses evaluated, the only significant difference between vitrification and

conventional freezing was observed at 24 h after thawing; vitrified embryos had delayed development compared to those that were frozen; however, by 48 h after thawing this difference was no longer significant.

4.6.3 Additive Effects

In the current experiment, we found no effect of additive on blastocyst stage or hatching prior cryopreservation ($P < 0.01$). PES or FCS as main effects resulted in similar blastocyst production as the control, but there were interaction effects; fructose was superior to glucose for the control and FCS for blastocysts per oocyte, and the control for blastocysts per 8-cell embryo. We found that it is possible to produce bovine embryos in vitro in FCS-free medium without affecting blastocyst yield or quality (Barcelo-Fimbres and Seidel, 2007) as did Mucci et al. (2006); furthermore, elimination of biological products may facilitate meeting international regulations on embryo transport, eliminating some biosecurity issues. The holding media used for vitrification and slow freezing had no animal products, as was the case with a previous vitrification experiment (Walker and Seidel, 2005).

Blastocysts cultured with FCS had 41.0 and 52.7% (24.7 and 31.7 percentage points) lower survival rates at 24 h post-thawing than control and PES-treated embryos, respectively. Results were similar at 48 h post-thawing. Mucci et al. (2006) reported a similar reduction in survival rates for bovine embryos cultured with estrous cow serum versus the control at 24 and 48 h posthawing (26.9 and 23.6 percentage points decreases).

The lightness of embryos cryopreserved and thawed was evaluated subjectively. Lightness appears to be correlated with lipid content. Embryos treated with PES during in vitro culture were clearer than controls and FCS. FCS-treated embryos were darker than controls. Similarly, cryopreserved FCS-treated embryos had lower embryo quality, hatching rate and ICM quality than PES-treated and control blastocysts at 24 and 48 h after thawing. However, no effect of additives was found for embryonic stage of blastocysts that survived.

Bovine zygotes fertilized in vitro but cultured in vivo had similar cryotolerance as those produced entirely in vivo (Enright et al., 2000; Rizos et al., 2002, Lonergan et al., 2003); however, those embryos produced entirely in vitro had the lowest cryotolerance (Rizos et al., 2002; Lonergan et al., 2003). This reduction in cryotolerance appears to be altered by embryo culture conditions (Rizos et al., 2001; Rizos et al., 2003; Mtango et al., 2003).

Culture conditions can modify embryonic characteristics, particularly the use of serum, which can lead to LOS in cattle and lambs (Thompson et al., 1995; Young et al., 1998); its absence results in less lipid accumulation in embryos (Thompson et al., 1995; Abe et al., 2002). The presence of serum in culture media compromised embryo survival compared with embryos produced in media without serum (Rizos et al., 2002, 2003; Mtango et al., 2003; Lane at al., 2003). Bovine embryos produced in vitro tend to have darker cytoplasm and lower buoyant density than in vivo–produced embryos (Pollard and Leibo, 1994), as a consequence of higher lipid content and different lipid composition

(Abd El Razek et al., 2000). The success rates of cryopreservation of in vitro-produced embryos appear to be highly correlated with cytoplasmic lipid content (Martino et al., 1996). One of the clearest demonstrations was that microsurgical removal of cytoplasmic lipid droplets in embryos increased cryotolerance, not just in early embryonic stages of pig (Nagashima et al., 1994, 1995) and cattle (Murakami et al., 1998; Diez et al., 2001), but also in resulting morulae and blastocysts in pigs (Ushijima et al., 2004) and cattle (Ushijima et al., 1999). Nagashima et al. (1994, 1995) showed that more than half of delipidated pig embryos survived compared to none in the control group. Use of centrifugation to isolate the lipids but without removal in bovine embryos also increased cryosurvival (Murakami et al., 1998).

The specific mechanism by which lipid accumulation in in vitro-derived embryos modifies cryotolerance is unknown, and may only be indirectly related to cytoplasmic lipids (Seidel, 2006). One possible mechanism is lipid peroxidation, which is amplified by cryopreservation due to an increase in levels of oxygen radicals, so the high lipid content in the embryo could produce more oxygen radicals that lead to embryonic death. In in vitro produced mouse embryos, the use of antioxidants such as ascorbate to inhibit lipid peroxidation increased survival after cryopreservation (Tarin and Trounson, 1993; Lane et al., 2002). However the beneficial effect of ascorbate was more evident for slow freezing than vitrification (Lane et al., 2002).

Unsaturated fatty acids such as linoleic acid bound to albumin have been added during culture; even when this did not affect embryo development, it improved survivals

rates using slow freezing of bovine morula and blastocysts (Imai et al., 1997; Hochi et al., 1999). Hochi et al. (1999) suggest that incorporation of unsaturated fatty acids into the lipid bilayer, or removal of cholesterol by binding to albumin can produce an increase in membrane fluidity. Bovine embryos supplemented with FCS during culture had higher levels of saturated fatty acids such as palmitic, stearic and palmitoleic (13.5, 8.1, and 12.6%, respectively) than the control; for unsaturated fatty acids oleic acid increased (8.1% of fatty acids), but linoleic and linolenic were not different from the non serum-supplemented control (Sata et al., 1999).

Culturing in vitro-produced blastocysts with 10% FCS resulted high levels of palmitic, stearic and oleic acids; saturated fatty acids were higher (72%), than monounsaturated and polyunsaturated fatty acids (14.7 and 13.3%, respectively) (Lapa et al., 2005). A possible explanation is that the use of serum could promote incorporation of saturated fatty acids and cholesterol into the membranes, making them less permeable, and this could explain the higher susceptibility to cryopreservation of embryos cultured with serum.

In previous experiments, there was a decrease of cytoplasmic lipid droplet accumulation by removing FCS from culture media for bovine embryos, compared to controls using fatty acid free BSA (Barcelo-Fimbres and Seidel, 2007), or using recombinant human serum albumin (De La Torre-Sanchez et al., 2006a). This reduction of cytoplasmic lipid droplets in bovine embryos is consistent after use of PES, compared with controls (Barcelo-Fimbres and Seidel, 2007). The explanation of this lipid reduction

is that PES oxidizes NADPH, which is required for synthesizing numerous lipids, particularly long-chain fatty acids. Nevertheless these PES-treated embryos still have more cytoplasmic lipid accumulation than in vivo-produced embryos (De La Torre-Sanchez et al., 2006a).

In the current experiment, we have demonstrated that bovine blastocysts produced by culture with PES survived conventional cryopreservation and vitrification better than no additive or 10% fetal calf serum. Averaged over both cryopreservation procedures, PES compared with the control improved survival rates 8.3 and 9.9% (7 and 7.6 percentage points) at 24 and 48 h after thawing. Thus, it was possible to improve the success of cryopreservation of in vitro-produced embryos by manipulating embryo metabolism and eliminating FCS from culture medium. Seidel (2006) mentioned that the most frequent approach to deal with the lower success rates of cryopreservation of in vitro-derived embryos is modifying the cryopreservation procedures, but this results in limited improvements. However, another alternative is modifying the embryos themselves to make them more cryopreservable. This could be more attractive under some circumstances. Future research should focus on embryo transfer and production of live offspring to evaluate cryopreservation systems and improvements in embryo cryopreservation.

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CHAPTER V

EFFECTS OF PHENAZINE ETHOSULPHATE (PES) DURING CULTURE OF EMBRYOS ON PREGNANCY RATE AND PRE AND POSTNATAL CALF DEVELOPMENT AFTER EMBRYO TRANSFER

5.1 Summary.

Oocytes collected from slaughterhouse ovaries were matured, fertilized, and resulting embryos cultured in vitro by standard procedures in a chemically defined medium. Day 0 of culture was 18 ± 2 h after the onset of IVF. From 2.5 to 6.5 d of culture, half of the eight cell embryos served as controls, and the rest were exposed to 0.3 μM PES, which decreases lipid content of embryos. PES or control blastocysts of good quality were transferred nonsurgically to synchronized recipients in estrus 6 to 7.5 d earlier, resulting in 9 calves in each group. These in vitro- produced pregnancies were

evaluated weekly between 35 and 98 d post estrus by ultrasonography, and post-natal development of the calves was monitored for 1 month. Use of PES during in vitro culture had no effect on pregnancy rates compared to control at 35 or 98 d ($P > 0.1$). Recipients at 7 to 7.5 d post estrus had higher pregnancy rates than at 6 to 6.5 d ($P < 0.05$). In vitro-derived fetuses that aborted showed retarded fetal and placental development compared to those that went to term, but there was no difference in fetal loss between the PES treatment and controls. One calf in the PES group weighing 36 Kg was born dead at 252 d of gestation, and another calf in this group was dead and weighing 22.2 kg when parturition was induced at 310 d of gestation. It is unclear whether these two abnormal calves were caused by the PES treatment, or were due to in vitro procedures in general. In conclusion, the use of PES during in vitro culture did not affect pregnancy rates, conceptus losses between d 35 and 98 of pregnancy, nor fetal post-natal development in calves born normally.

5.2 Introduction

In vitro-derived bovine embryos have many potential uses in research and in production agriculture; however, considerable gestational and postnatal differences between in vitro-produced and in vivo-derived calves have been reported for pregnancy rates (Hasler, 2000a; Bertolini et al., 2004), gestation length (Kruip and den Daas, 1997; Jacobsen et al., 2000a; Rerat et al., 2005), placentation (Farin and Farin, 1995; Bertolini et al., 2002; Bertolini et al., 2004), dystocia (Szenci, et al., 1988; Hasler, 2000b; Jacobsen et al., 2000a), rate of hydrallantois (Hasler et al., 1995, Cibelli et al., 1998, Van Wagtedonk-de Leew et al., 2000), abortions and postnatal mortality (Hill et al., 2000;

Van Wagtedonk-de Leew et al. 2000; Bertolini et al., 2004), birth weight and growth rates (Rerat et al., 2005; Jacobsen et al., 2000a), postnatal clinical traits (Sangild et al., 2000; Rerat et al., 2005), and abnormal offspring syndrome (AOS) (McEvoy et al., 1997; Farin et al., 2006). These issues have contributed to delayed commercial application of this technology. These abnormalities vary in incidence, likely due to different in vitro production systems used, in different laboratories.

In vitro–produced embryos (IVP) usually have markedly more lipid content than in vivo controls (De la Torre-Sánchez et al., 2006a,b), resulting in unusually high rates of embryonic death, and lower survival after cryopreservation. We have found that culture of embryos in the presence of phenazine ethosulfate (PES) markedly decreases their lipid content. PES is an electron acceptor that oxidizes NADPH to NADP, which increases flux of glucose through the pentose phosphate pathway and glycolysis (De la Torre-Sanchez et al., 2006a,b), decreases lipid accumulation in in vitro-produced bovine embryos (De la Torre-Sanchez et al., 2006a,b; Barcelo-Fimbres and Seidel, 2007a) and increases embryo survival after cryopreservation relative to controls (Barcelo-Fimbres and Seidel, 2007b). De la Torre-Sánchez et al. (2006a,b) found that IVP embryos cultured with PES, transferred at day 7, and recovered back on day 14, had similar surface area and development rates as control embryos and those cultured with other metabolic regulators. However, it is important to determine the effects of PES as additive to culture media on pregnancy rates, embryonic and fetal losses, fetal size, and neonatal survival of calves.

Ultrasonography was used to monitor pregnancies in this study during the first 3 months of gestation. This noninvasive technology has been used successfully to monitor development of the fetus and placental membranes for in vivo, in vitro and nuclear transfer bovine pregnancies (Curran et al., 1986; Hugues and Davies, 1989; Kahn, 1989; Bertolini et al., 2002; and Breukelman et al., 2004, 2005).

Therefore, the aims of this study were to determine if use of PES is detrimental for embryonic and post natal development compared with in vitro-produced controls cultures without PES and cryopreserved in vivo produced embryos. We also studied effects of days post estrus of the recipient at embryo transfer (synchrony) and problems during embryo transfer (ETgrade) on pregnancy rates.

5.3 MATERIALS AND METHODS

5.3.1 Production of IVP embryos

5.3.1.1 Oocyte Collection and In Vitro Maturation

Chemicals were purchased from Sigma Chemical Company, St Louis, Missouri unless specified otherwise.

Bovine ovaries from mature cows of various beef breeds were obtained from an abattoir and transported to the laboratory within 5-6 h of slaughter in 0.15 M NaCl at ambient temperature, approximately 22 to 25°C. Ovaries were then rinsed again in 0.15 M NaCl, and cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm antral follicles with an 18-gauge needle attached to a vacuum aspiration pump with less than 50 mmHg of pressure. Oocytes with more than 3 layers of unexpanded cumulus cells and morphologically bright and evenly granulated cytoplasm were selected for in vitro maturation (IVM). COCs were matured and embryos were cultured as described by De La Torre-Sanchez et al. (2006b). Briefly, COCs were washed twice in chemically defined medium (CDM) with 20 mM Hepes buffer for handling oocytes (HCDM-M), and once with maturation medium. All CDM used in this study for maturation, fertilization, and culture of embryos were supplemented with 0.5% fatty acid-free BSA (Sigma A6003). Fifty COCs were matured per well in 4-well plates (Nunclon, Roskilde, Denmark), containing 1 ml of CDM maturation medium supplemented with 15 ng/ml of FSH (NIH-FSH-S17), 1μg/ml of LH (USDA-LH-B-5) (FSH and LH from National Hormone and Peptide Programs Torrance, California), 1.0 μg/ml of estradiol-17β (Sigma E2257), 50 ng/ml EGF (Sigma E9644) and 0.1mM cysteamine (Sigma M6500). Oocytes were incubated at 38.5°C with 5% CO_2 in humidified air for 23 h.

5.3.1.2 Sperm Preparation

Frozen semen from one of three bulls of proven fertility, having at least 40% progressive motile sperm after thawing, was thawed on a given day in water at 37°C for 30 sec. Semen was gently expelled on top of a Percoll gradient (Sigma P1644) (2 ml

90%: 2 ml 45% Percoll in sperm TALP medium (modified Tyrode's) as described by Parrish et al. (1989). The Percoll was in a 15-ml conical tube that was centrifuged 20 min at ~400 x g at 21-24°C. The supernatant was discarded, and the sperm pellet (approximately 100 µl) was washed with 5 ml of chemically defined fertilization medium (Fert-CDM) (De La Torre-Sanchez et al., 2006b) supplemented with 5 mM caffeine (Sigma C0750) and 2 µg/ml heparin (Sigma H9399).

The sample was centrifuged again for 5 min at 400 x g at 21-24°C. After discarding the supernatant, approximately 90 to 100 µl remained, and a 5 µl aliquot was used to determine sperm concentration with a hemacytometer. The sperm concentration was adjusted to 5 x 10^6 spermatozoa per ml in Fert-CDM.

5.3.1.3 In Vitro Fertilization

Following in vitro maturation, groups of 50 oocytes were placed in 450 µl of Fert-CDM medium per well of 4-wells dishes (Nunclon, Roskilde, Denmark); 50 µl of sperm suspension at 5 x 10^6 spermatozoa per ml were added, resulting in a final volume of 500 µl per well and a sperm concentration of 0.5 x 10^6/ml. Gametes were co-incubated for 18 h at 38.5°C in an atmosphere of humidified air and 5% CO_2.

5.3.1.4 Culture of Embryos

Following in vitro fertilization, presumptive zygotes were removed from wells, and transferred to 1.0-ml microcentrifuge tubes with approximately 100 µl of Fert-CDM, and then vortexed for 50 to 60 sec in a room at 28°C to remove cumulus cells. They were

rinsed three times in handling medium (H-CDM-1). Early culture (day 0 to 2.5 post fertilization) was done in 500 µl of fresh CDM with 0.5 mM glucose supplemented with non-essential amino acids (NEAA) (Sigma M7145), and 10 µM EDTA (CDM-1) as described by De La Torre-Sanchez et al. (2006b), and incubated at 39°C under 90% N_2, 5% O_2, and 5% CO_2. After 60 h of culture, embryos were examined with a stereomicroscope (15 to 20×) for cleavage rate, and all uncleaved ova and embryos less than 8 cells were discarded; the rest were transferred to a new culture dish with 500 µl CDM-2 (CDM supplemented with NEAA and essential amino acids (Sigma 6766) and 2 mM of glucose) and cultured from day 2.5 to 7.0 post fertilization. Half of the 8-cell embryos were placed with CDM-2 with PES (Sigma, P4544) at 0.3 µM; the rest were controls. Embryos were evaluated on day 6.5 of culture and selected for embryo transfer.

5.3.2 Embryo transfer

For transfer of IVP embryos, recipients were synchronized in two ways: first, 25 mg of prostaglandin $F_{2\alpha}$ (Lutalyse®, Pfizer, New York, New York) was injected i.m. (day 0), estrus was detected for 3 d, and embryo transfer was performed 6 to 7.5 d after estrus. To resynchronize non pregnant recipients, immediately after embryo transfer a progesterone-releasing intravaginal device (CIDR®, InterAg, Hamilton, New Zealand) was placed (day 0) into each recipient and removed 12 d later; cows were observed for estrus for 3 d, and embryos were transferred 6 to 7 d after estrus. Cows with a detectable corpus luteum were used.

Fresh day 7 blastocysts of good quality with a compact inner cell mass were loaded individually into a 0.25 ml straw and transferred nonsurgically under epidural anesthesia into the uterine horn ipsilateral to the ovary with a corpus luteum as described by Elsden and Seidel (1995). Thirty eight PES-treated embryos and thirty five control embryos were transferred.

As a control for embryo development, in vivo-derived bovine embryos were obtained after superovulation of Angus-cross cows with declining doses of FSH (Folltropin®, Bioniche, Belleville, Ontario, Canada) and artificial insemination (AI) as previously described (De la Torre-Sanchez et al., 2006a). The donor cows were inseminated with semen from the same Angus bulls used for the IVP procedure. Seven days after donors were observed in estrus embryos were recovered nonsurgically and cryopreserved by conventional freezing. Embryos were transferred to recipients 6 to 8 d post estrus. Because of high costs of keeping pregnant cows, at d 49 of pregnancy those recipients receiving the in vivo-derived embryos were given 25 mg of $PGF_{2\alpha}$ i.m. to cause abortion so that they could be reused.

5.3.3 Evaluation of pregnancy rates of IVP embryos

When IVP embryos were transferred, these data were recorded: embryo treatment group, corpus luteum side, corpus luteum structure (solid vs. clear lumen), ET grades (grade 1, good; grade 2, some cow movement at the moment of transfer; and grade 3, problematic (much cow movement and/or bloody transfer gun)), days post estrus of the recipient (synchrony), and protocol used for recipients synchronization ($PGF_{2\alpha}$ vs.

CIDR). Pregnancies were evaluated weekly between 35 and 98 d post estrus by transrectal ultrasonography.

5.3.4 Evaluation of Fetal development during gestation

The weekly evaluations of pregnant cows receiving in vitro-derived embryos using transrectal ultrasound were discontinued if the fetus was no longer accessible for ultrasonography because it had dropped over the pelvic rim; this usually did not occur until after d 98 of gestation. The ultrasound evaluations were done using an Aloka 500 ultrasound machine with a 5.0 MHz linear rectal probe. Fetal and placental development were monitored using the ultrasound machine tools for length and area estimation, but in addition, ultrasound images were recorded on video tape and subsequently analyzed by a single person. The fetal heart rate was determined from the video (10 to 15 sec video clip) with a stopwatch. The fetal and placental structures measured were: crown-rump length measuring the distance from the crown to tail in a sagittal view at 35 to 70 d (Figure 5.1), cross-section of the fetal abdomen at the insertion of the umbilical cord (CAU) at 49 to 98 d (Figure 5.1), head length from the occipital bone to the nose tip at 49 to 98 d (Figure 5.1), biparietal diameter of the cranium (BPD) by a perpendicular line to head length at 49 to 98 d (Figure 5.1), amniotic sac area from a ventral-dorsal fetus view at 35 to 56 d (Figure 5.2), and placentome area; two placentomes were measured per fetus (one close and the other distant from the fetus) at 42 to 98 d (Figure 5.2).

Fetal and placental growth parameters were used to detect potential differences including abnormalities between treatments (control vs. PES-treated embryos). Based on

the outcome of the pregnancies, the fetal and placental structures were classified into three groups: group 1 (n=16, data of treatments (PES vs. control) were pooled because no significant differences were found) were fetuses that resulted in normal term calves (term), group 2 (n=2) were calves that died just before or after birth (abnormal), and group 3 (n=10) were fetuses aborted before 70 d of pregnancy (aborted). Fetal death was defined as cessation of heart beating, detected from the sonogram.

The pregnant cows with in vivo-derived embryos underwent transrectal ultrasound evaluations weekly on days 35, 42, and 49 after estrus. Crown-rump length, fetal heart rate and amniotic sac area were evaluated from 35 to 49 d; placentome area was evaluated at 42 and 49 d; and head length, BDP and CAU were evaluated at d 49.

The IVP embryos (n=18), were compared with in vivo produced embryos (n=34), on days 35, 42 and 49 after estrus.

Figure 5.1. Ultrasound images of in vitro-produced fetuses illustrating sites of measurements of crown-rump length, cross-section of the fetal abdomen at the insertion of the umbilical cord (CAU), head length and biparietal diameter of the cranium (BPD) at different times from 35 to 98 d post estrus.

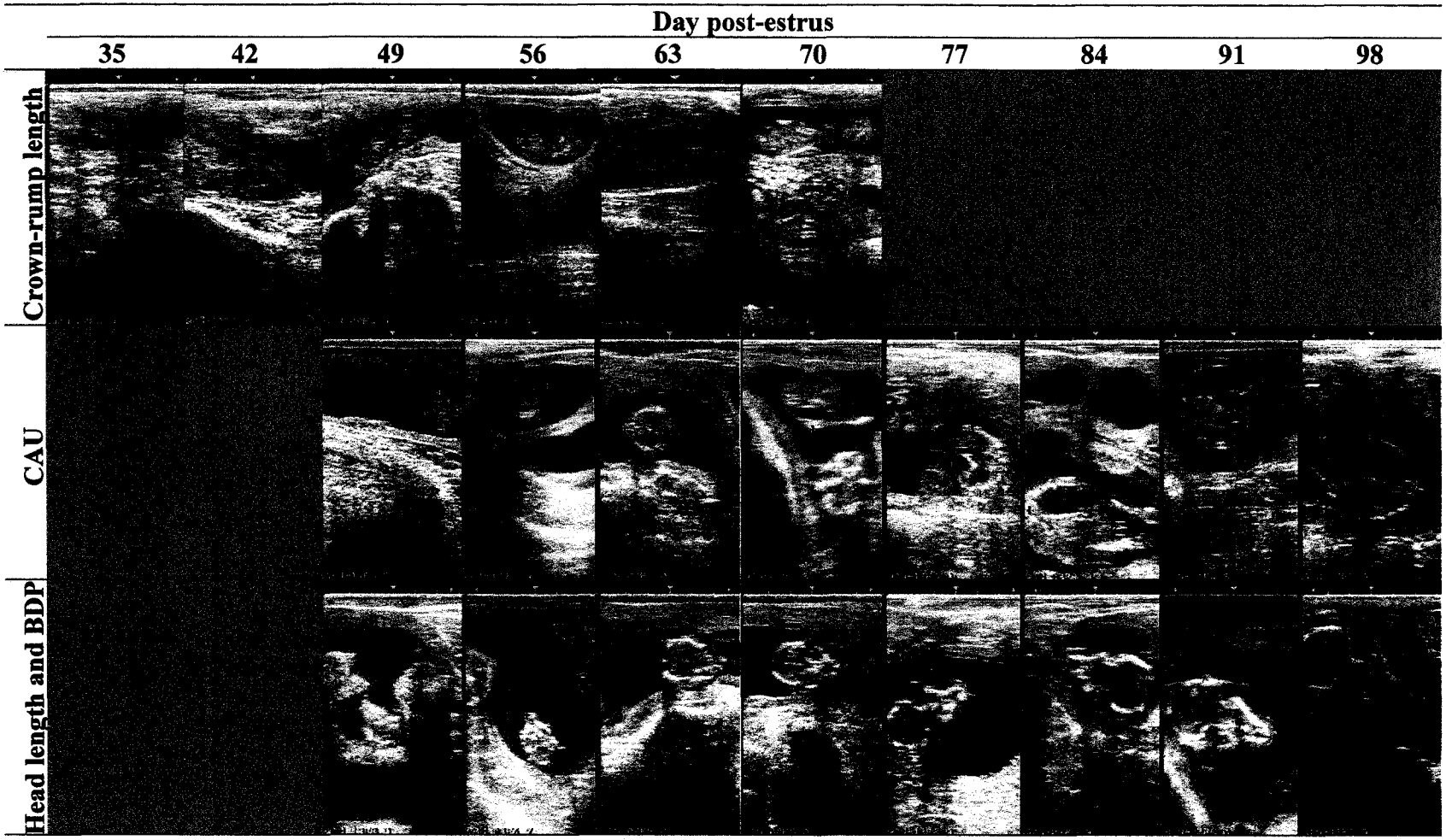
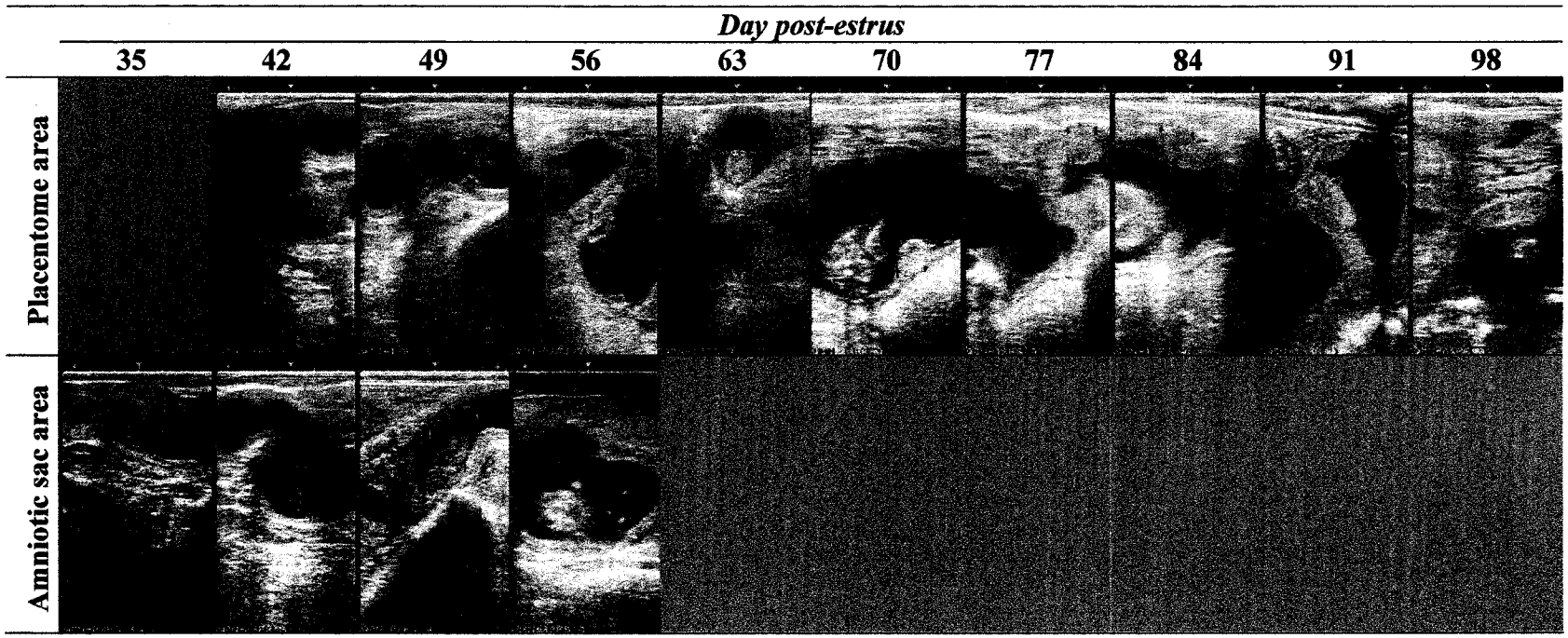


Figure 5.2. Ultrasound images of in vitro-produced fetal structures: placentome area and amniotic sac area at different times from 35 to 98 d post estrus.



5.3.5 Calving and calf evaluations

About 3 weeks before expected parturition cows were monitored twice daily. Close to parturition they were checked at 6 h intervals. At calving we recorded the dystocia score (1= no assistance, 2=minor assistance, 3= heavy extraction, 4=cesarean). For the first 90 min after calving, the calf was under observation to record calf vigor score (1=Aggressive ... 4=lazy). Two h after calving, calves were weighed, and sex, abnormalities if any, clinical traits (respiration rate (breaths/min), and heart rate (beats/min), and temperature (°C)) were recorded; a blood sample was taken by venipucture from jugular vein (~5 ml). After centrifugation of the blood, plasma protein levels (using a refractometer, mg/dl) and hematocrit were evaluated. One month after birth calves were weighed, and average daily gain (ADG) was calculated.

When natural calving had not occurred by day 310 (1 case), an i.m. injection of 30 mg of dexamethasone was given, to induce parturition.

5.3.6 Statistical analysis

Data were analyzed using the SAS statistical software package (SAS Institute, Cary, North Carolina). Gestation length, and birth weight were analyzed with a mixed linear model (proc mixed), where sex of the calf, and the treatment were considered fixed effects, and the sire was considered as a random effect. Fetal and placental development were evaluated over time, 35, 42, 49, 56, 63, 70, 77, 84, 91 and 98 d for in vitro and 35, 42, and 49 d for in vivo-derived embryos. Crown-rump length, fetal heart rate, amniotic sac area, placentome area, head length, and CAU were evaluated using a regression

model (proc REC), analyzing linear and quadratic terms. Dummy variables were used for categorical variables: treatments (control and PES), pregnancy outcome (term, abnormal and aborted) and embryo origin (in vitro and in vivo). Differences between and within treatments in sex ratio, pregnancy rate, abortions and the number of calves dead at birth were determined by Fisher's Exact Test. The statistical power of the design for detecting differences in fetal development during gestation and birth weight of calves was done using Java Applets computer software (Lenth, 2006).

5.4 RESULTS

5.4.1 Pregnancies from *in vitro*-produced embryos after embryo transfer

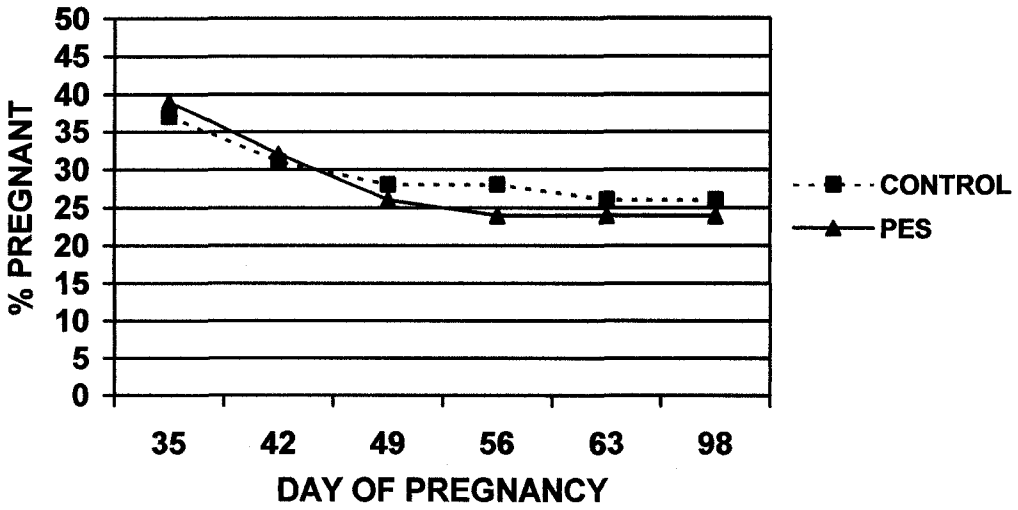
Use of PES during in vitro culture had no effect on pregnancy losses (Figure 5.1) nor pregnancy rates at 35 or 98 d compared to controls nor did synchronization protocol, or corpus luteum side or structure ($P > 0.1$) (Table 1). There was a significant effect of synchrony on pregnancy rates at 35 d ($P < 0.06$) and 98 d ($P < 0.05$) (Table 1); the 98d pregnancy rate was higher for recipients in estrus 7-7.5 d (49%) than 6-6.5 d (29%) earlier. Pregnancies from ET grade 1 were higher than 2 and 3 at 35 and 98 d ($P < 0.05$) (Table 1). There was no interaction among treatments ($P > 0.1$).

5.4.2 Development of fetal and placental structures

Embryos treated with PES were not significantly different ($P > 0.1$) from controls for fetal development responses CAU, head length and BPD (Figure 5.4); fetal heart rate, and crown-rump length (Figure 5.5); amniotic sac area, and placentome area (Figure 5.6). However, a fetal age effect representing normal growth was found for all of these

responses ($P < 0.05$) (Figures 5.4, 5.5, 5.6). There was no age x treatment interaction for any response ($P > 0.1$). The analysis of the time effect by regression was statistically significant for the quadratic term for amniotic sac area, crown-rump length, placentome area, CAU and fetal heart rate ($P < 0.01$) in addition to a significant linear effect.

Figure 5.3. Pregnancy losses of controls and embryos cultured with PES.



For in vitro produced embryos, a significant effect of pregnancy status was found: group I (normal term) and group II (abnormal term) were significantly different from group III (aborted) ($P < 0.05$) (Figures 5.4, 5.5) for all the fetal structures (fetal heart rate, crown-rump length, CAU, head length and BPD) and fetal membranes components: (amniotic sac area and placentome area) (Figure 5.6). There was a significant interaction of pregnancy status by days of pregnancy ($P < 0.05$). The aborted fetuses (group III) had a retarded development for all the fetal and placental structures measured after 56 d of pregnancy.

TABLE 5.1. Pregnancy rates (%) ± SEM at 35 and 98d for in vitro-produced embryos according to treatment, synchronization protocol, corpus luteum side and structure, and ET grade at the time of embryo transfer.

		DAYS POST-ESTRUS	
RESPONSES	**No. recipients**	**35**	**98**
Treatment			
Control	35	37±8	26±7
PES	38	40±8	24±7
Synch protocol			
$PGF_{2\alpha}$	40	43±8	25±7
CIDR	33	33±9	24±8
CL side			
Left	36	44±8	31±7
Right	37	32±8	19±7
CL structure			
Crown	47	34±7	19±6
Lumen	26	46±9	35±8
Synchrony			
6-6.5 d	38	29±7 [c]	15±7 [a]
7-7.5 d	35	49±7 [d]	34 ±7 [b]
ET grade			
1	40	53±7 [a]	38±7 [a]
2	20	30±10 [b]	15±9 [b]
3	13	8±12 [b]	0±8 [b]

[a,b,c,d] Values with different superscripts within responses in the same column differ ([a,b] $P < 0.05$; [c,d] $P < 0.06$).

The fetuses derived from in vitro produced embryos were not significantly different from the in vivo-derived fetuses ($P > 0.1$) for fetal and placental development, crown-rump length amniotic sac area, and fetal heart rate 35 to 49d, placentome area 42 to 49 d, and CAU, head length, BPD 49 d post-estrus (Table 2, Figures. 5.5, 5.6). A fetal age effect was found for in vivo and in vitro-produced embryos for crown-rump length, amniotic sac area, and placentome area ($P < 0.05$), but not for fetal heart rate ($P > 0.1$),

but this analysis only concerned d 35 to 49 of gestation. The analysis of the age effect by regression also was statistically significant for the quadratic term for crown-rump length and amniotic sac area ($P < 0.01$).

TABLE 2. Means ± SEM of fetal development (CAU, head length and BDP) of bovine fetuses produced *in vivo* vs. *in vitro*.

Measurement (unit) [a]	FETUS AGE (d)	TREATMENTS [b] N	*In vivo*	N	*In vitro*
CAU (cm^2)					
	49	34	1.17±0.03	18	1.23±0.04
Head length (cm)					
	49	34	1.52±0.08	18	1.54±0.10
BDP (cm)					
	49	34	0.89±0.02	18	0.90±0.03

[a] CAU=cross-section of the fetal abdomen at the insertion of the umbilical cord, and BDP=biparietal diameter of the cranium.
[b] No treatment differences ($P > 0.1$).

5.4.3 Post-natal development of calves from *in vitro*-produced embryos

At birth, live calves resulting from pregnancies from PES-treated embryos and the control group were not different in the dystocia or calf vigor scores ($P > 0.1$), because no assistance was required for parturition for any of the calves, and the vigor was aggressive for all calves. The sex ratio was not different between the control and the PES-treated embryos (0.78 vs. 0.89 respectively) ($P > 0.1$), but the pooled data (0.83 males) was different ($P < 0.05$) from the 0.51 ratio normally found in cattle (Foote, 1977).

No significant differences of treatments (PES-treated embryos vs. control), sires or sex of the calves were found in mean gestation length, birth weight, blood plasma protein and hematocrit, respiration rate, heart rate, body temperature at birth, or the average daily gain and body weight of calves at 30 d (Tables 5.3, 5.4, 5.5).

Fig 5.4. Fetal development (CAU=cross-section of the fetal abdomen at the insertion of the umbilical cord, head length, and BDP=biparietal diameter of the cranium) for treatment (control vs. PES) and pregnancy status (abortion, abnormal term development and normal term) of bovine fetuses produced in vitro.

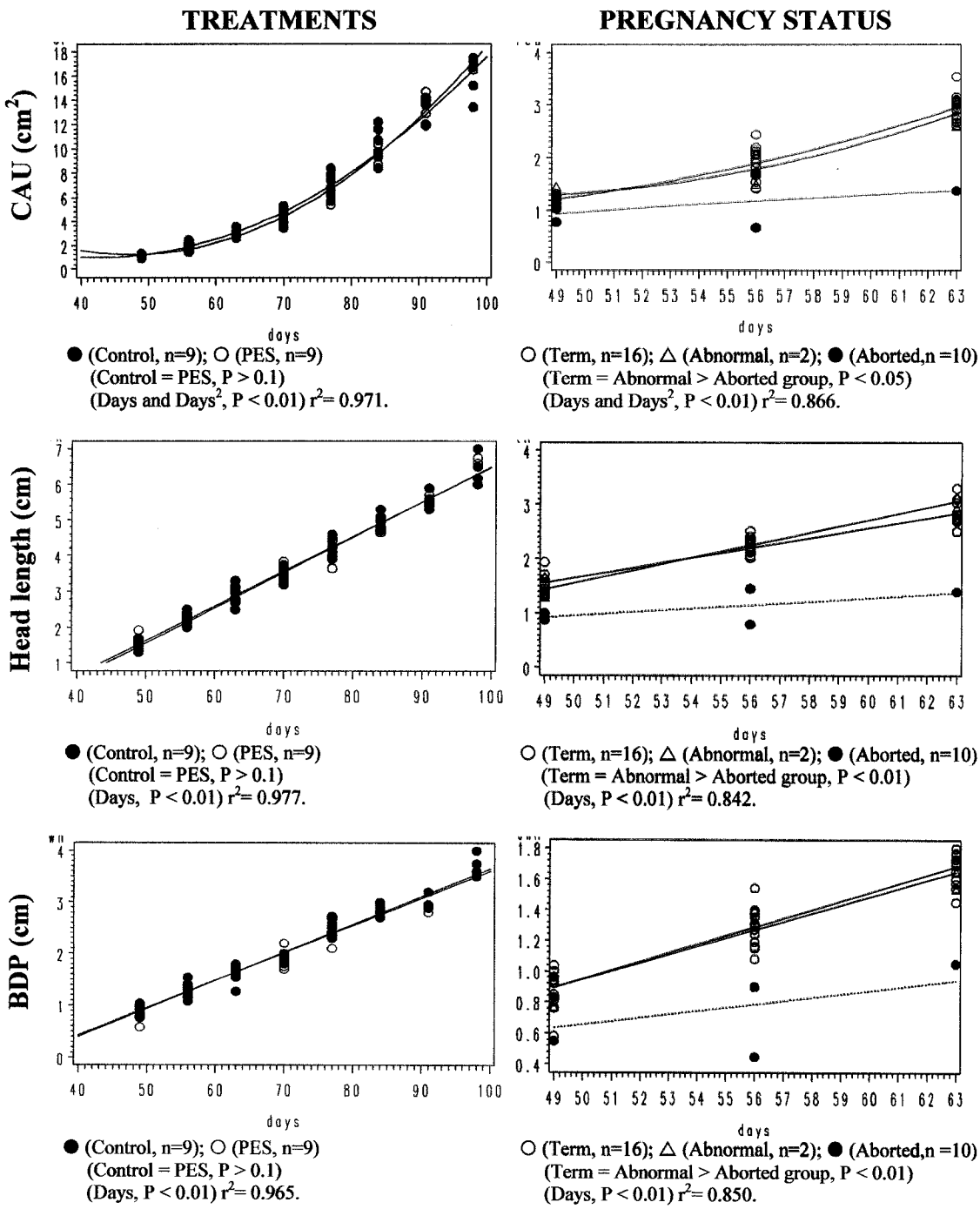


Figure 5.5. Fetal development (crown-rump length, and fetal heart rate) for treatment (control vs. PES), pregnancy status (abortion, abnormal development and normal term) and fetuses of different origin (In vitro vs. In vivo).

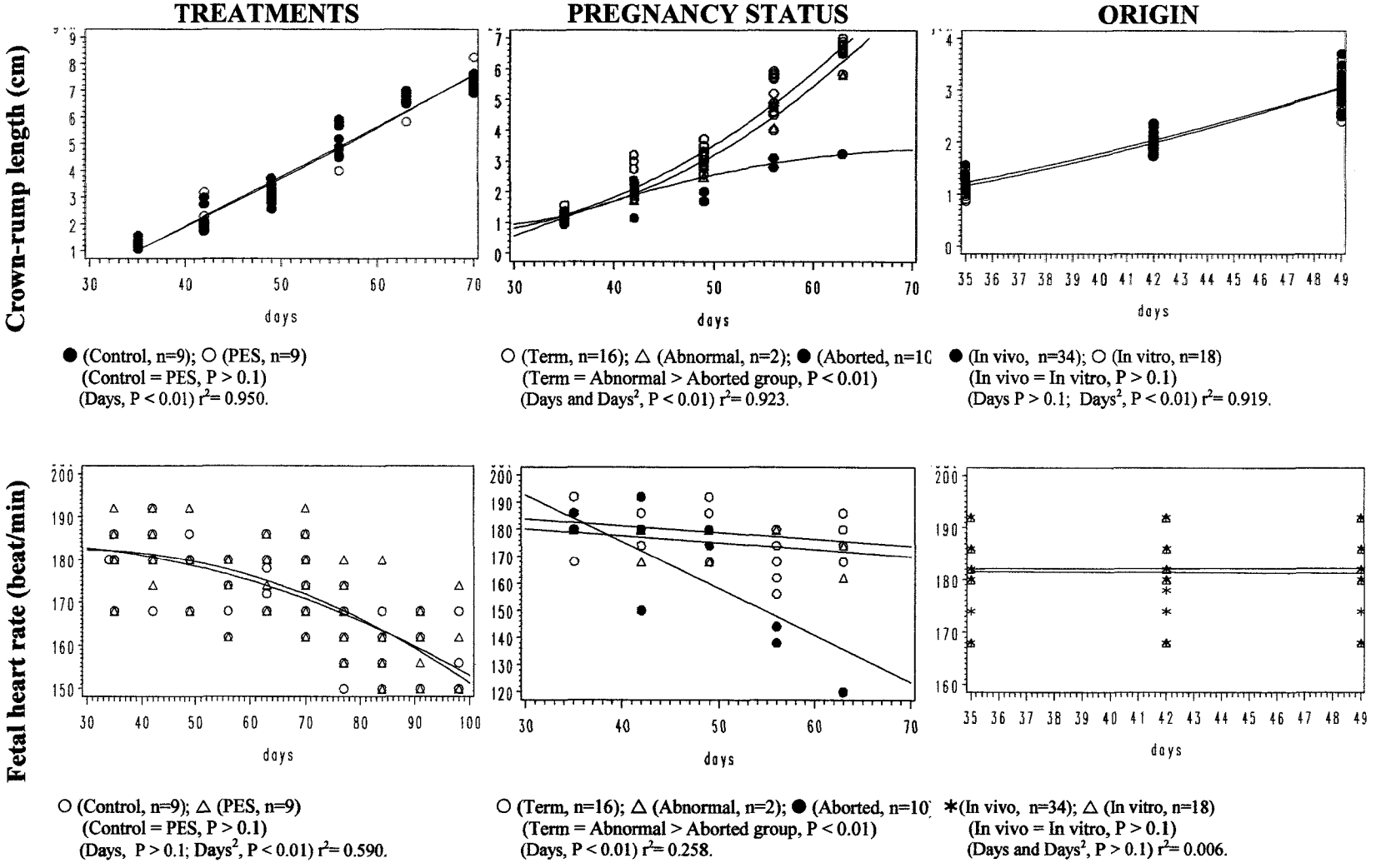
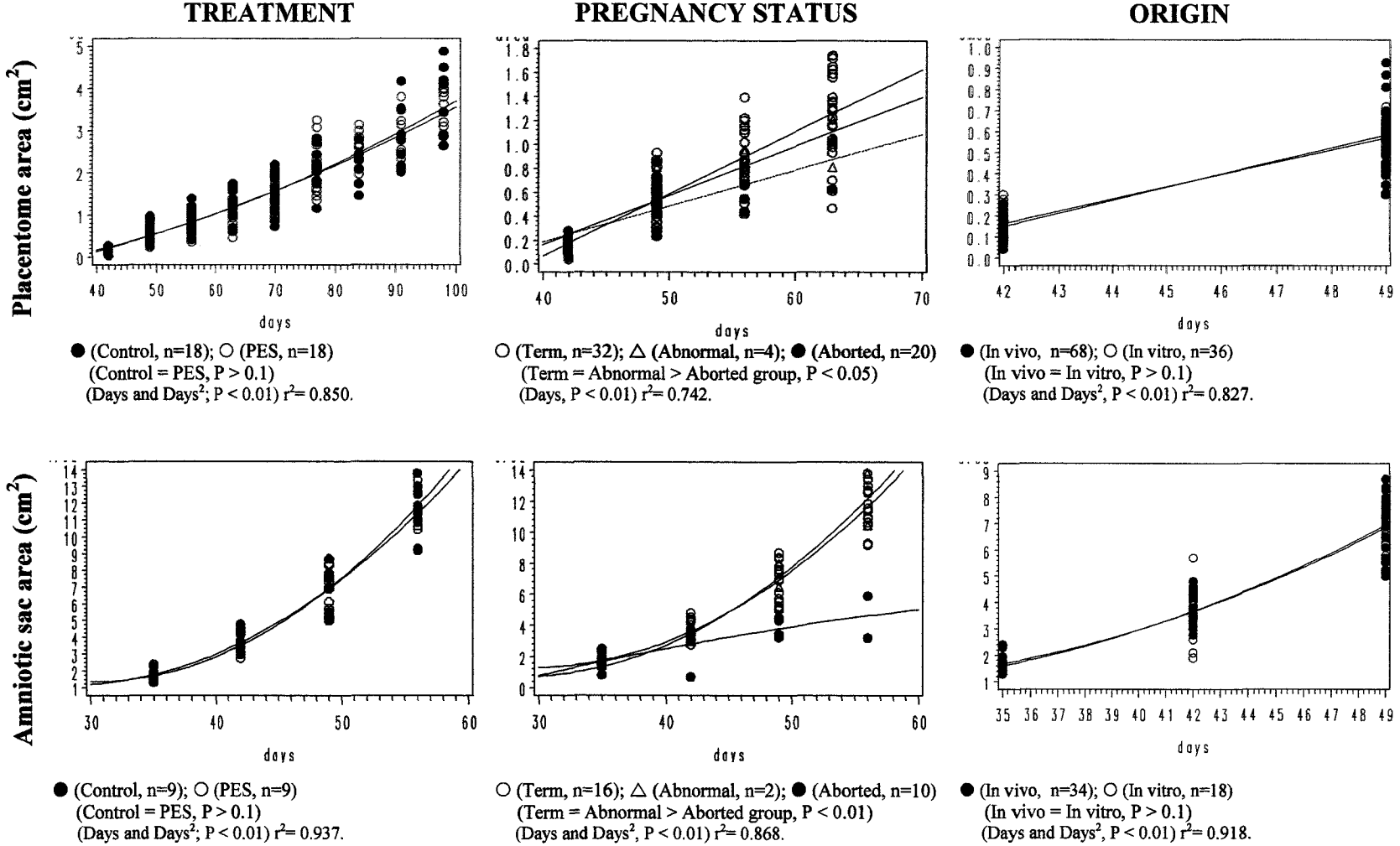


Figure 5.6. Fetal membrane development (placentome area and amniotic sac area) for treatment (control vs. PES), pregnancy status (abortion, abnormal term development and normal term) and fetuses of different origin (In vitro vs. In vivo).



No phenotypic abnormalities were observed at birth (Figures 5.7, 5.8); however, 2 calves died (previously referred as group 2), one at premature birth (252 d of gestation) and the other some hours after birth. Both calves belonged to the PES-treated group, and this treatment could have resulted in the deaths; however these abnormalities probably were done to the general effect of IVP rather than a treatment effect for several reasons: first, these calves showed normal development as fetuses and their placentas compared to the normally viable fetuses during the first trimester of gestation. Second, necropsy did not reveal any macroscopic abnormalities other than small size for one calf; however, no microbiological analyses were done to rule out infection. Third, these calves had very different characteristics; the premature was of normal weight, 36 kg, and the other was very small after birth (22.2 kg), suggesting completely different causes of the abnormalities.

TABLE 5.3. Means ± SEM of treatments effects on fetal responses during gestation, at birth and 30d after birth of calves produced *in vitro*.

	TREATMENTS [a]	
RESPONSES	**Control (n=9)**	**PES (n=7)**
Gestation		
Gestation length (d)	284.9±1.5	286.9±1.7
At birth		
Birth weight (kg)	43.9±1.5	44.2±1.7
Plasma protein (g/dl)	5.8±0.6	6.2±0.7
Hematocrit (%)	37.9±1.1	36.9±1.3
Respiration rate (breaths/min)	57.4±3.5	53.5±4.0
Heart rate (beats/min)	130±7.3	129.5±8.4
Temperature (°C)	39.2±0.2	38.8±0.2
30 d After birth		
Weight at 30 d (kg)	94.2±4.2	91.6±4.8
Average daily gain (kg)	1.68±0.12	1.58±0.13

[a] No treatment differences (P > 0.1).

TABLE 5.4. Means ± SEM of sire effects on fetal responses during gestation, at birth and 30d after birth of calves produced *in vitro*.

	BULLS [a]		
RESPONSES	**A (n=9)**	**B (n=5)**	**C (n=2)**
Gestation			
Gestation length (d)	285.3±1.4	285.0±2.1	287.5±2.5
At birth			
Birth weight (kg)	42.0±1.4	42.5±2.1	47.6±2.5
Plasma protein (g/dl)	5.9±0.6	7.2±0.9	4.9±1.1
Hematocrit (%)	36.7±1.1	37.8±1.6	37.7±1.9
Respiration rate (breaths/min)	55.2±3.4	60.2±4.9	51.0±6.1
Heart rate (beats/min)	131.2±7.0	143.1±10	125.0±12
Temperature (°C)	38.9±0.2	39.0±0.2	39.1±0.3
30 d After birth			
Weight at 30 d (kg)	89.9±4.0	89.0±5.9	99.7±7.3
Average daily gain (kg)	1.59±0.11	1.55±0.17	1.74±0.20

[a] No bull differences (P > 0.1)

TABLE 5.5. Means ± SEM of sex effects on fetal responses during gestation, at birth and 30d after birth of calves produced *in vitro*.

	SEX [a]	
RESPONSES	**Male (n=13)**	**Female (n=3)**
Gestation		
Gestation length (d)	285.3±1.2	286.6±2.2
At birth		
Birth weight (kg)	41.9±1.3	46.2±2.3
Plasma protein (g/dl)	5.9±0.5	6.1±0.9
Hematocrit (%)	39.1±1.0	35.7±1.7
Respiration rate (breaths/min)	55.9±3.0	55.0±5.4
Heart rate (beats/min)	123.0±6.2	136.0±11
Temperature (°C)	39.1±0.1	38.9±0.2
30 d After birth		
Weight at 30 d (kg)	91.1±3.6	94.6±6.4
Average daily gain (kg)	1.64±0.10	1.62±0.18

[a] No sex differences (P > 0.1).

Figure 5.7. Photographs from calves of control embryos produced in vitro, at birth and 30d later.

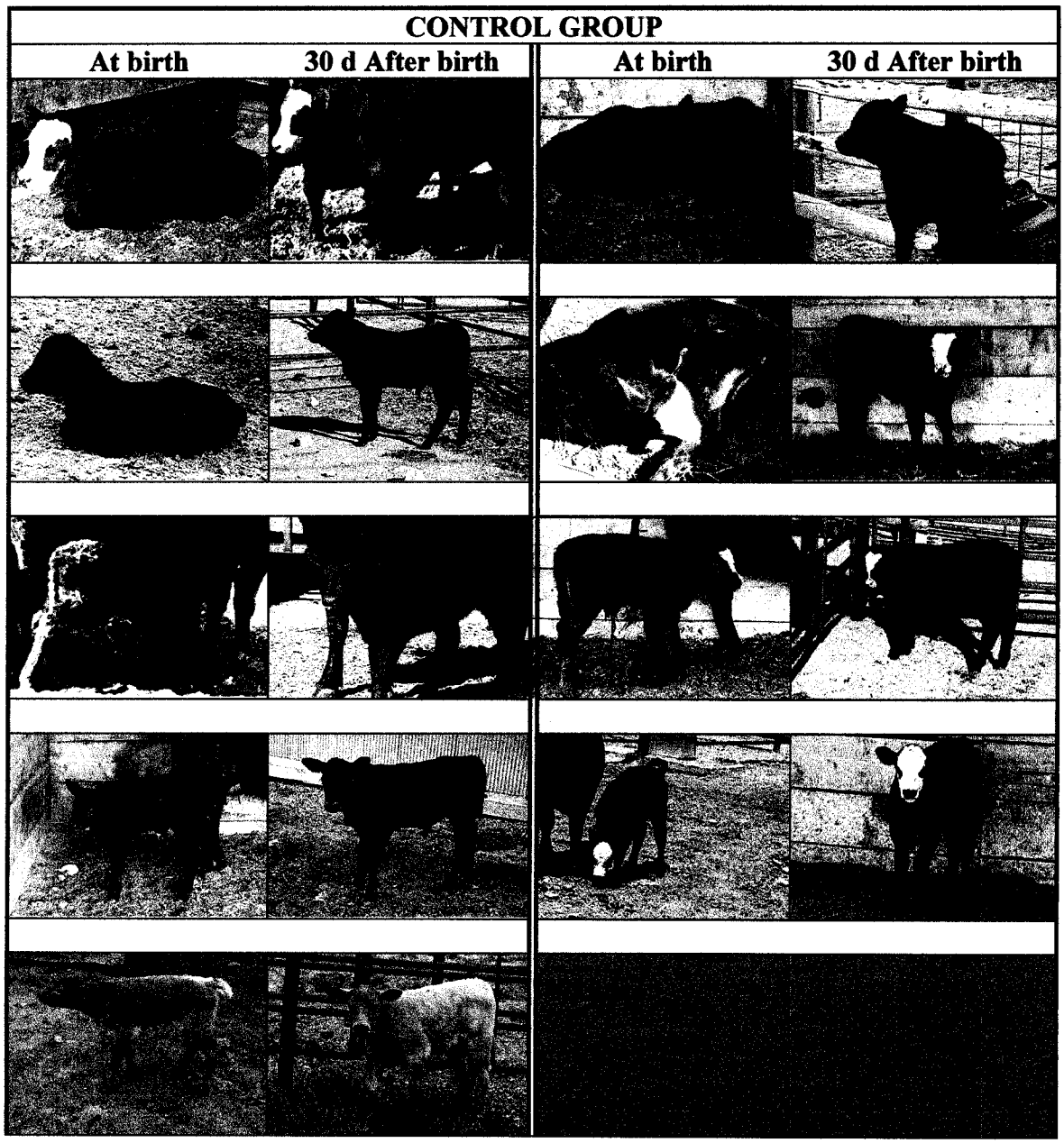
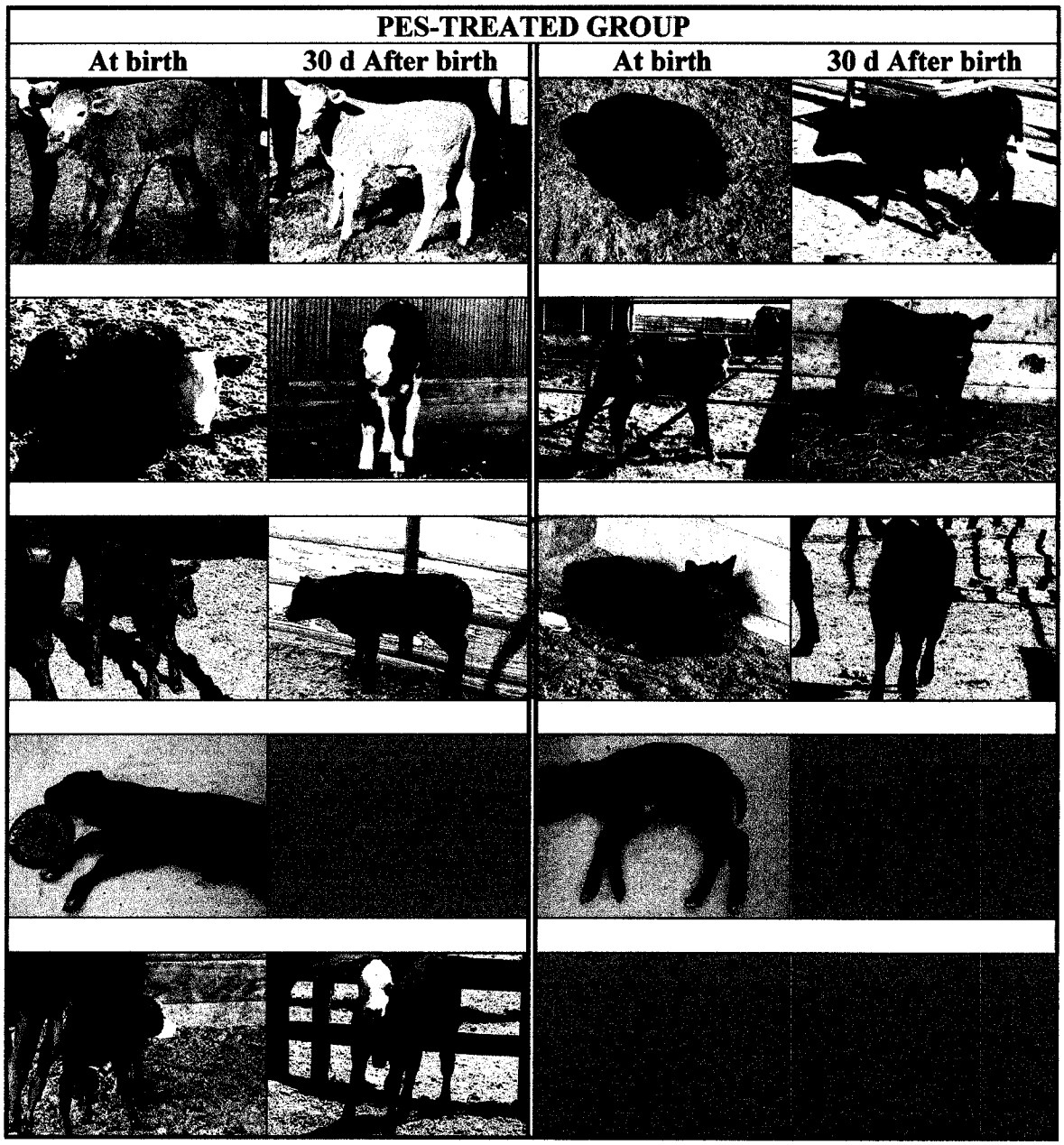


Figure 5.8. Photographs of calves from PES-treated embryos produced in vitro, at birth and 30d later.



5.5 DISCUSSION

We examined the statistical power of this design for detecting differences in fetal development during gestation and birth weight of calves. With the observed standard deviation of 3 kg (Table 5.6) for body weight, α= 0.05, β=0.2, 1-tail test and an average of 8 calves per group, we could have detected a 3.9 kg difference between treatments and control groups. Since excess size of AOS calves is defined as more than two standard deviations higher in birth weight than normal (Heyman et al., 2002), this experiment was capable of detecting differences in AOS between treatments. Parameters for fetal ultrasound evaluations are also presented in Table 5.6. These illustrate that for continuous responses, this experiment had reasonable statistical power to detect differences if they existed. Table 5.7 had similar information about the power to detect substantive differences between in vitro- and in vivo-produced calves.

TABLE 6. Statistical power of continuous responses (assumes α=0.05, β=0.2, 1-tail test) of in vitro-produced embryos.

RESPONSE	Units	n1/n2[a]	Mean	SD from ANOVA	Difference detectable	% Difference detectable
Fetal development						
Crown-Rump length	cm	9/9	4.4	0.4	0.49	11.4
CAU	cm^2	9/9	5.7	0.7	0.86	15.0
Head length	cm	9/9	3.5	0.2	0.25	7.1
BDP	cm	9/9	2.0	0.1	0.12	6.0
Placentome area	cm^2	18/18	1.5	0.3	0.25	16.6
Amniotic sac area	cm^2	9/9	6.3	1.0	1.20	19.0
Fetal heart rate	beats/min	9/9	172.0	7.9	9.70	5.6
Gestation length	days	9/9	285.0	3.4	4.20	1.4
Postnatal development						
Birth weight	kg	7/9	41.5	3.0	3.90	9.4
Temperature	°C	7/9	39.1	0.4	0.60	1.5
Respiration rate	breaths/min	7/9	56.8	8.0	12.20	21.5
Heart rate	beats/min	7/9	132.0	17.0	25.90	19.6
Weight at 30 d	kg	7/9	89.9	10.0	11.70	13.0
Average daily gain	kg	7/9	1.6	0.3	0.46	28.8

[a] n1=PES; n2=control.

TABLE 7. Statistical power of continuous responses (assumes α=0.05, β=0.2, 1-tail test) of in vivo vs. in vitro-produced embryos.

RESPONSE	Units	n1/n2[a]	Mean	SD from ANOVA	Difference detectable	% Difference detectable
Fetal Development						
Crown-Rump length	cm	34/18	2.1	0.23	0.20	9.5
CAU	cm^2	34/18	1.2	0.20	0.17	14.2
Head length	cm	34/18	1.5	0.50	0.42	28.0
BDP	cm	34/18	0.9	0.13	0.11	12.2
Placentome area	cm^2	62/36	0.4	0.10	0.06	15.0
Amniotic sac area	cm^2	34/18	4.2	0.68	0.57	13.6
Fetal heart rate	beats/min	34/18	181.6	6.80	5.70	3.1

[a] n1=in vivo; n2=in vitro produced embryos.

5.5.1 Pregnancy rates and losses with in vitro produced embryos

In this experiment there were considerable pregnancy loses (13.5 percentage points of cumulative fetal losses between d 35 and the end of gestation); however, no significant differences were found between treatments (control vs. PES) (Figure 5.3), corpus luteum side or corpus luteum structure. Others have found that pregnancy rates are substantially lower for IVP than AI or routine embryo transfer (Farin and Farin, 1995, Hasler et al., 1995; van Wagtendonk-de Leeuw et al., 2000). Cumulative incidence of fetal loss between 31 to 260 d of gestation of in vivo-derived pregnancies varies from 3.6% to 10.8% (Paisley et al., 1978; Forar et al., 1996). Even higher embryonic losses than in vivo-derived were reported by Bertolini et al. (2004); they found 32% pregnant on d 30 and 14% pregnant on d 60 for in vitro-derived pregnancies (18 percentage points of cumulative fetal loss), and 38% pregnant on d 30 and 26 % pregnant on d 60 for in vivo-derived pregnancies (12 percentage points of cumulative fetal loss). Hasler et al. (1995) reported 11% of 546 IVP pregnancies aborted between the 2 mo pregnancy determination

and term. In somatic cell nuclear transfer clones, losses during the first trimester are up to 37 percentage points (45% pregnant on d 30 and 8% pregnant on d 60) (Hill et al., 2000).

Fetal abnormalities are a major reason for pregnancy losses of natural pregnancies (Wilmut et al., 1986). Research done with somatic cell clones has shown that embryonic losses during the first 3 months are related to defective placental formation with reduced placentome numbers (Farin and Farin, 1995; Hill et al., 2002; Lee et al., 2004), and deficient angiogenesis and chorioallantoic development (Hill et al., 2000). Pregnancy losses in the last 6 months of gestation are associated with abnormal offspring syndrome, which includes abnormalities in placentas (edema), fetal size and vital organ hypertrophy (heart and liver) (Wilson et al., 1995; Young et al., 1998; Heyman et al., 2002). Some of the losses from cloned pregnancies almost certainly are due to the in vitro procedures involved other than cloning per se.

The presence of corpora lutea with a large cavity, often termed cystic, has been studied by Kahn (1989); in his study, a high incidence of such cavities was found in cows (36 to 41%), but this decreased after day 9. However, no pathological implications of cavities were found in early pregnancies, which is consistent with our findings.

The administration of exogenous progesterone by a progesterone releasing device after embryo transfer has been reported by Looney et al. (2006); a group of cows received supplemental progesterone (CIDR) on the day of transfer for a total of 14 days. There was no effect on pregnancy rates, so this procedure appears not to have detrimental

effects on the fetus. This is relevant in terms of resynchronization, because according to our results, resynchronization using a progesterone device for 12 d after ET resulted in similar pregnancy rates as synchronization with prostaglandin.

5.5.2 Ultrasound evaluations

In the IVP fetuses, there was no difference in fetal and conceptus ultrasound measurements between treatments (control vs. PES-treated embryos), nor between IVP vs. in vivo-derived embryos. Chavatte-Palmer et al. (2006) reported no differences in fetal and placental measurements mode via ultrasound between AI and IVP-produced fetuses.

In the present study the fetal heart rate was 181.5 for both the control and PES-treated group. The fetal heart rate decreased significantly for both groups as pregnancy progressed: d 77, 166 to 162 bpm, respectively and d 98, 156 to 158 bpm, respectively. Kahn (1989) reported similar fetal heart rate at d 60 and d 100, 163 and 160 bpm, and Bruekelman et al. (2004) reported a decline to 160 bpm by d 100.

The crown-rump length, head length, and biparietal diameter measurements of fetuses that went to term were consistent with measurements done in IVP calves (Kahn, 1989; Chavatte-Palmer et al., 2006). Kastelic et al. (1988) reported similar measurements to the current study for crown-rump length of in vivo conceived pregnancies at 41 and 57 d of gestation. One report indicated no differences in growth between AI vs. IVP fetuses for crown-rump length (Pace et al., 2004). However, Hill et al. (2000) reported that those

SCNT embryos that failed to survive had significantly smaller crown-rump lengths, similar to our findings for in the in vitro-derived aborted group.

In our study of in vitro-produced pregnancies, we found that fetuses that died during the first 98 d of the study had significantly different amniotic sac area, placentome area, fetal heart rate, crown-rump length, CAU, head length and BDP from the viable fetuses. Therefore, measurement of fetal and placental structures can be used to predict fetus viability. Previous studies have shown that fetal death can be predicted accurately using fetal heart rate in bovine (Kastelic et al., 1988; Bruekelman et al., 2004) and human fetuses (Achiron et al., 1991; Doubilet and Benson, 1995). Bruekelman et al. (2005) found a reduction of fetal heart rate, decreased volume of allantoic fluid, and segregation of chorioallantoic membranes as fetuses died at various stages after induced abortions.

No differences in placentome area were found between control vs. PES-treated embryos in the IVP group, or when compared to the placentomes of in vivo-derived fetuses. Consistent with our findings, previous authors did not find differences between in vivo vs. in vitro-derived embryos at 90 or 180 d of gestation (Jacobsen et al., 2000b, Bertolini et al., 2004). However fetuses that aborted before the day 98 of gestation had retarded placentome development after day 49 of gestation. Alterations in placentomes of in vitro-produced embryos during gestation have been reported previously, including reduced numbers of placentomes, (Farin and Farin, 1995); giant placentomes with 33% heavier calves than controls (Bertolini et al., 2002); and placentomes with dissimilar surface (longer, wider and thinner than in vivo pregnancies), (Bertolini et al., 2004).

5.5.3 Gestation length, calving and birth weight

Effects of in vitro culture on gestation length vary among studies. Jacobsen et al. (2000a) reported no differences in gestation length for calves produced by AI, IVP-defined, and IVP-serum supplemented media, (277, 280, and 281d, respectively). In another report gestation length was longer for IVP (298d) than AI (287) calves (Rerat et al., 2005). One large study showed that IVP-derived embryos had significantly longer gestations than AI calves (284.2 vs. 281.4) (van Wagtendonk-de Leeuw et al., 1998). The delayed calving is typically related with AOS (Kruip and den Daas, 1997). In the present experiment no differences in gestation length were found between treatments, probably because no AOS was observed.

Birth weight (BW) is an important determinant of perinatal outcome because excessive birth weight of calves is a major cause of perinatal mortality in calves (Bellows et al., 1971; Nix et al., 1988; Ferrell, 1989). High birth weight of calves can lead to dystocia which alters the acid-base balance in the new born calf by decreasing the pH and increasing the pCO_2 and lactate concentrations in blood (Szenci et al., 1988). In another study 75% of IVP calves from embryos cultured in serum-supplemented medium required assistance, compared with 33% of IVP embryos cultured in defined medium, and 40 % for AI calves (Jacobsen et al., 2000a). IVP and cloned calves tend to be larger than normal (Jacobsen et al., 2000a), but the higher BW can be partially explained by longer gestation and more male calves. The use of co-culture or serum is a likely cause of the increased BW (Jacobsen et al., 2000b; Sinclair, 1999). Van Wagtendonk-de Leeuw et

al. (1998) found an increase of 10% in BW (44.1 vs. 48.3 kg) from IVP calves compared to AI calves. Jacobsen et al. (2000a) reported BW for calves produced by AI, IVP-defined and IVP serum-supplemented of 39.0, 41.9, vs. 45.2 kg, respectively. However in a study where no co-culture or FCS was used, no difference in BW between IVP vs. AI produced calves were found (49.7 vs. 46.0 kg respectively) (Rerat et al., 2005).

Rerat et al. (2005) reported faster growth rates with a higher gain to feed ratio for the first month after birth, and larger body weight after 8 wks for IVP than AI calves; this faster growth rate after birth was also seen in lambs (Walker et al., 1996), but not in IVP humans (Wennerholm et al., 1998). In this experiment, the average daily gain (ADG) was studied from birth to one month of age; we found no effect on ADG of treatment, sex or sire; the ADG was between 1.59 to 1.74 kg per day. Rerat et al. (2005) also reported that in vitro–derived calves have higher growth performance with identical nutrient intake per kg of BW, than AI calves.

AOS often occurs due to IVP and nuclear transfer manipulation, possibly due to epigenetic modification of imprinted genes, which affects gene expression later in development (Young et al., 1998; Young and Fairburn, 2000; Young et al., 2001). Previously, AOS have been referred to as 'large offspring syndrome' or 'large calf syndrome'; however, these referred terms are misnomers (Farin et al., 2006), because although some offspring with this syndrome are oversize at birth, most are of normal size. AOS is a broad term with various abnormal phenotypes, characterized by offspring with aberrant fetal and placental development, increase fetal myogenesis, dystocia,

dysfunctional perinatal pulmonary activity, organomegaly, and increased early mortality (Thompson and Peterson, 2000; Walker at al., 2000; Sinclair et al., 2000, Bertolini and Anderson, 2002; Farin et al., 2006); those that were oversize at birth and remain alive maintain abnormal organ size during later life (McEvoy et al., 1997).

Various conditions seem to cause AOS, including culture under less than optimal conditions (Lonergan et al., 2003a); FCS supplementation (Young *et al.,* 1998); high ammonia, which enhances activity of growth factors present in serum (McEvoy et al., 1997); complex media (Thompson et al., 1995, Sinclair et al., 1999), and co-culture. Heyman et al. (2002) defined oversize calves as birth weight higher than the mean birth weight plus two standard deviations. In the current experiment (mean birth weight 41.5 ± 3.9 kg), calves weighing more than 49 kg at birth would be considered large calves. However, none of the calves was in this category, and there was little evidence of other manifestation of AOS with the procedures used other than the high rates of early pregnancy loss (Figure 5.3).

An increase in hydrallantois has been described by various authors, with unknown cause and variable progression during gestation (Hasler et al., 1995, Cibelli et al., 1998, Van Wagtendonk-de Leeuw et al., 2000). Hasler et al. (1995) reported that 1% of IVP recipients had hydrallantois, which is higher than the normal rate (1 in 7500 pregnancies) (Hasler, 1998). No hydrallantois was observed in the present experiment.

5.5.4 Postnatal clinical traits

Several clinical traits for IVP calves were studied; no treatment differences were found due to sex, sire or in vitro vs. in vivo origin on respiratory frequency. Lower respiratory frequency rates than in the present study were reported by Rerat et al. (2005), who found there were no differences between IVP vs. AI produced calves (34 vs. 31 breaths/min, respectively). However, Sangild et al. (2000), reported higher respiratory frequency for IVP calves than controls (66 vs. 56 breaths/min). For heart rate, similar results to the present experiment were published for Sangild et al. (2000). Rerat et al. (2005) reported higher means of heart rates for IVP vs. AI produced calves (167 vs. 154 beats/m respectively). Lower heart rates have been reported when a C-section was done (van Wagtendonk-de Leeuw et al., 2000). The rectal temperature was as previously reported by Rerat et al. (2005) for IVP calves at birth, who found no differences between IVP and AI produced calves (38.6 vs. 38.3 °C). However, another report showed elevated temperature for IVP calves than controls (38.9 vs. 37.4 °C, respectively) (Sangild et al., 2000). Reduced viability of IVP calves has been reported (Sangild et al., 2000), but this was not seen in our study.

Jacobsen et al. (2000a) reported neonatal changes in clinical and hematological values for IVP calves; however, most of these stabilized by 6 h after birth. Changes in blood chemistry appear to be influenced by calving difficulty and BW (Jacobsen et al., 2000a; Sangild et al., 2000). The results obtained for hematocrits in the present study are comparable to those found by Rerat et al. (2005), who detected no differences at birth between IVP and AI-derived calves (0.37 vs. 0.38 L/L, respectively). Hadorn et al.

(1997) have noted that all calves have lower hematocrit values due to hemodilution after colostrum intake.

In the present experiment the sex ratio of calves that were born was skewed toward males ($P < 0.05$), as found in previous experiments (Massip et al., 1996; van Wagtendonk-de Leeuw et al., 1998, 2000; Hoelker et al., 2006). This effect has been addressed by several authors; some possible explanations are glucose toxicity to female embryos during culture (Kimura et al., 2005), faster development of male embryos to the blastocyst stage during the in vitro culture (Hasler et al., 1995; Van Soom et al., 1997; Lonergan et al., 1999; Gutierrez-Adan et al., 2001) and superior viability of IVP-derived male embryos after transfer to recipients (Hoelker et al., 2006).

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CHAPTER VI

EFFECTS OF EMBRYO SEX (BY UTILIZATION OF SEXED SEMEN), GLUCOSE OR FRUCTOSE ON EMBRYO DEVELOPMENT

6.1. Summary

The objective of this study was to determine if glucose and/or fructose affects female embryos differently from male embryos using a sexed semen approach. Blastocysts were produced in a factorial design with sperm sex (non-sexed, X-bearing and Y-bearing); 4 energy substrates (no hexose control, glucose, fructose, and glucose + fructose); and semen from 3 bulls (A, B, and C), two replicates for each bull. Data were analyzed by ANOVA. There was no effect on cleavage and eight cell rates for the hexose treatment ($P > 0.1$). However, blastocyst rates per oocyte and per eight cell embryo were higher for fructose than control, glucose, or glucose + fructose ($P < 0.05$). Glucose treatment resulted in retarded development compared to the other hexose treatments ($P < 0.05$). We found an interaction of hexose (glucose and fructose) and sexed of sperm (X-bearing and Y-bearing) ($P < 0.05$), where glucose was detrimental to female embryos and fructose improved development to 8-cell and blastocyst stages. Lower cleavage rates were found when fertilizing with X-bearing sperm; however, eight cell rates, blastocyst

rates per oocyte and per eight cell embryo were not different fertilizing with X-, Y-bearing and non-sexed semen ($P > 0.1$). There was no bull effect on rates of cleavage, eight cell production, or blastocysts per oocyte or per eight cell embryo ($P > 0.1$). In conclusion, fructose improved embryonic development; and glucose retarded embryonic development and was toxic for female embryos. Sexed sperm had similar results to non-sexed sperm, and female embryos had retarded embryonic development.

6.2. Introduction

In a dairy cattle the sex ratio of males-females born is very close to 0.5, either by natural service or artificial insemination (Foote, 1977), but this sex ratio is skewed toward males with in vitro produced bovine embryos (Avery et al., 1991, 1992; Xu et al., 1992; Massip et al., 1996; van Wagtendonk-de Leeuw et al., 1998, 2000; Gutiérrez-Adán et al., 2001; Larson et al., 2001; Peippo et al., 2001; Kimura et al., 2005; Hoelker et al., 2006). This change in the male-female ratio is not entirely understood, but there are several hypotheses about how this occurs, and it is affected by the kind and concentration of hexose used as an energy substrate in media (Kimura et al., 2005). Male embryos develop develop more rapidly to the blastocyst stage during in vitro culture or development of female embryos is delayed by metabolic differences (Hasler et al., 1995; Van Soom et al., 1997; Lonergan et al., 1999; Gutierrez-Adan et al., 2001); IVP-derived male embryos appear to have superior viability after transfer to recipients (Hoelker et al., 2006).

In previous experiments we found an increase in blastocyst production using fructose as energy substrate, especially at postcompaction stage (Barcelo-Fimbres-Seidel, 2007a,b); however, this increase in blastocysts could be from an increase in blastocyst production or due to rescuing female embryos, due to the putative glucose toxicity effect during in vitro culture (Kimura et al., 2005). Metabolic and redox differences have been reported previously due to the existence of two transcriptionally active X-chromosomes in female embryos (Epstein et al., 1978; Mak et al., 2004; Okamoto et al., 2004).

The utilization of sexed semen allows determining the effect of sex on embryo development, and whether if there is a sex preference for hexose, which could explain the higher blastocyst rates obtained by the use of fructose in previous experiments (Barceló-Fimbres and Seidel, 2006a,b). We hypothesized that female embryos will have delayed development compared to male embryos, with glucose but not fructose. We also considered that combination of glucose and fructose might be superior to glucose or fructose alone on embryo development, because both hexoses are present in the reproductive tract fluids, and the combination could result in a more balanced metabolism. According to some results, the cell number and differentiation of the blastomeres is affected differently depending on the hexoses used. Possibly fructose stimulates blastomere differentiation into trophectoderm cells, increasing the total cell number per embryo (Kwun et al., 2003).

The aim of this study was to determine if glucose and/or fructose or the combination affects female embryos differently from male embryos.

6.3. Materials and methods

6.3.1. Experimental Design

Zygotes resulting from 1851 oocytes were subjected to a 3 x 4 x 3 x 2 factorial experimental design with 3 sperm sexes (non-sexed, X-bearing and Y-bearing); 4 energy substrates (no hexose control, glucose, fructose and glucose + fructose); and semen from 3 bulls (A, B, and C) two replicates for each bull.

6.3.2. Procedures for producing embryos

6.3.2.1. Oocyte Collection and In Vitro Maturation. Bovine ovaries from mature cows were obtained from local abattoirs and transported to the laboratory in 1-2 h in 0.15 M NaCl at ambient temperature, approximately 22 to 25°C. After arriving at the laboratory, ovaries were trimmed of extraneous tissue, and rinsed once again in 0.15 M NaCl. Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm antral follicles with an 18-gauge needle attached to a tubing system connected to a vacuum aspiration pump with 40-50 mm Hg of pressure. Oocytes with at least 3 layers of unexpanded cumulus cells and morphologically bright and evenly granulated cytoplasm were selected for IVM. COCs were washed twice in chemically defined medium (CDM) with Hepes buffer for handling oocytes, and once with maturation medium (De La Torre-Sanchez et al., 2006b). Fifty COCs were matured per well of 4-well plates (Nunclon, Roskilde, Denmark), containing 1 ml of maturation medium CDM with 0.5% fatty acid-free BSA (Sigma A-6003, Lot # 063K7525) and gonadotropin hormones (National Protein and Peptide

Program, Torrance, CA) (15 ng/ml of FSH (NIH-FSH-S17), 1μg/ml of LH (USDA-LH-B-5), 1.0 μg/ml of estradiol-17β (Sigma E-2257), 50 ng/ml EGF (Sigma E-9644) and 0.1mM cysteamine (Sigma M-6500). Oocytes were incubated at 38.5°C with humidified 5% CO2 in air for 23 h.

6.3.2.2. Sperm preparation

Sexed and non sexed sperm frozen after sorting at 40 psi with $5x10^6$ cells per 0.25 mL straw from each bull were used in these experiments. Accuracy of sorting was ~ 95% X or Y sperm. Straws of semen were thawed in water at 35°C for 30 sec. Semen from one straw was gently expelled on top of a Percoll gradient (Sigma P-1644) using the mini-percoll system, with 0.45 mL 90%: 0.5 mL 45% Percoll in sperm (sp)-TALP medium (modified Tryode's), and centrifuged 20 min at ~400 x g at 23°C in a 1.5 mL eppendorff tube. The supernatant was discarded, and the sperm pellet (50 μL) was washed with 1 mL of chemically defined fertilization medium (Fert-CDM) (De la Torre-Sanchez et al., 2006b), supplemented with 0.5% BSA and 5 mM caffeine (Sigma C-0750) and 2 μg/mL heparin (Sigma H-3125). The sample was centrifuged again for 5 min at ~400 x g at 23°C, and the supernatant was discarded again. Approximately 40-50 μL for sexed and non-sexed semen remained, and a 2 μL aliquot was taken to determine sperm concentration with a hemacytometer. In each experiment three sperm stocks were prepared (X-bearing, Y-bearing and non sexed), at 4 x 10^6 spermatozoa per mL, giving a final concentration of 1 x 10^6 spermatozoa/mL in Fert-CDM in each treatment (Campos-Chillon et al., 2003).

6.3.2.3. In Vitro Fertilization

Fertilization was done in micro-droplets under oil. The matured oocytes were removed from maturation medium and washed once in 1 mL of Fert-CDM and transferred in groups of 30 in 10 μL using a Pipetman (P-10) into 50 μL drops of F-CDM under mineral oil in 60 x 15 petri dishes (Beckton Dickinson, NJ, USA). Fertilization took place by adding 20 μL of sperm suspension per drop; co-incubation of gametes was for 18 h at 38.5°C, 5% CO_2 in air with maximum humidity.

6.3.2.4. Culture of Embryos

Following in vitro fertilization, presumptive zygotes were removed from wells and transferred to 1.0-ml microcentrifuge tubes with approximately 100 μl of Fert-CDM (De La Torre-Sanchez et al., 2006b), and then vortexed for 50 to 60 sec to remove cumulus cells. These procedures were carried out in a room at 28°C. Embryos then were rinsed three times in Hepes CDM-1 (H-CDM-1). Early culture (day 0 to 2.5 post fertilization) was done in a new 4-well dish, containing 500 μl of CDM supplemented with 0.5% fatty acid-free BSA, 0.5 mM hexose (control 0 mM; 0.5 mM glucose; 0.5 mM, fructose; and 0.25 glucose + 0.25 fructose), non-essential amino acids (NEAA), and 10 μM EDTA (CDM-1) as described by De La Torre-Sanchez et al. (2006b), and incubated at 39°C under 90% N2, 5% O2, and 5% CO2. After 60 h of culture, embryos were examined with a stereomicroscope (15 to 20×) for cleavage, and all uncleaved ova and embryos less than 8 cells were discarded. The rest were cultured in new dishes with CDM-2 (CDM supplemented with 0.5% fatty acid-free BSA, NEAA and essential amino acids and 2 mM of hexose (control 0 mM; 2.0 mM glucose; 2.0 mM, fructose; and 1.0

glucose + 1.0 fructose)) from day 2.5 to day 7 post fertilization. Embryos were evaluated 168 h after removal from fertilization medium to determine the final stage of development.

6.3.3. Evaluating embryos

Embryo development was evaluated at 2.5 and 8 d after fertilization to determine the final stage of development. Cleavage and 8-cell embryos rates were evaluated 2.5 d after fertilization. Blastocysts at day 7 were scored for stage of development (5=early ...8=hatched), and the percentage development to blastocysts was evaluated 7 d after fertilization.

6.3.4. Statistical analysis

Data were analyzed by ANOVA using the SAS statistical software package. Sources of variation in the model included sex of sperm (non-sexed, X-bearing and Y-bearing), energy source (glucose, fructose, combination and no hexose), and bulls (A, B, and C), and all possible first order interactions; all factors were considered as fixed effects. The subset of the effect of glucose or fructose and male vs. female embryos was also analyzed separately. The arc sin transformation was applied to percentage data. If the ANOVA was significant ($P < 0.05$), means were separated by the LSD procedure. Data are reported as untransformed least-squares means.

6.4. RESULTS

6.4.1 Hexose effect

No differences in cleavage and eight cell rates were observed using control and the hexoses treatments (glucose, fructose or the combination) ($P > 0.1$) (Table 6.1). However, blastocyst rates per oocyte and per eight cell embryo were higher for fructose than control, glucose and the combination ($P < 0.5$) (Table 6.2). For blastocyst stage, glucose resulted in retarded development relative to fructose and the control group ($P < 0.05$). No interactions were found between treatments using the full model ($P > 0.1$).

Table 6.1. Main effect least-squares means for development of bovine embryos (±SE).

FACTORS	No. oocytes	Cleaved (%)	Eight cell rate (%)
Hexose			
Control (no-hexose)	469	72.6 ± 2.4	44.8 ± 3.3
Fructose	457	71.3 ± 2.4	49.9 ± 3.3
Glucose	465	72.7 ± 2.4	44.7 ± 3.3
Glucose+Fructose	460	71.7 ± 2.4	47.2 ± 3.3
Sperm Sex			
Non-sexed	631	76.3 ± 2.1 [a]	48.3 ± 2.9
X-Bearing	597	68.5 ± 2.1 [b]	45.2 ± 2.9
Y-Bearing	623	71.4 ± 2.1 [ab]	46.3 ± 2.9
Bull			
A	651	72.3 ± 2.1	45.5 ± 2.9
B	618	74.8 ± 2.1	49.8 ± 2.9
C	582	69.2 ± 2.1	44.6 ± 2.9

[a,b] Values without common superscripts within factors in the same column differ ($P < 0.05$).

The subanalysis of sexed sperm (X-bearing and Y-bearing) and individual hexoses (glucose vs. fructose) resulted in a significant interaction for blastocysts per oocyte and per eight cell embryo ($P < 0.05$) (Table 5.3; Figures 6.1, 6.2). Glucose was

detrimental for the X-bearing fertilized group, and/or fructose was beneficial for this group.

Table 6.2. Main effect least-squares means for development of bovine embryos (±SE).

FACTORS	No. oocytes	Blastocysts per oocyte (%)	Blastocysts per eight cell (%)	Blastocyst Stage (5=early ... 8=hatched)
Hexose				
Control (no-hexose)	469	14.9 ± 1.4 [b]	33.4 ± 3.0 [a]	6.1 ± 0.1 [a]
Fructose	457	19.4 ± 1.4 [a]	39.8 ± 3.0 [b]	6.2 ± 0.1 [a]
Glucose	465	15.0 ± 1.4 [b]	32.7 ± 3.0 [a]	5.7 ± 0.1 [b]
Glucose+Fructose	460	13.1 ± 1.4 [b]	31.5 ± 3.0 [a]	5.9 ± 0.1 [a,b]
Sperm Sex				
Non-sexed	631	16.4 ± 1.2	35.6 ± 2.7	6.1 ± 0.1 [a]
X-Bearing	597	15.1 ± 1.2	33.2 ± 2.7	5.7 ± 0.1 [b]
Y-Bearing	623	15.4 ± 1.2	34.1 ± 2.7	6.1 ± 0.1 [a]
Bull				
A	651	15.1 ± 1.2	34.2 ± 2.7	6.1 ± 0.1
B	618	16.4 ± 1.2	34.5 ± 2.7	5.9 ± 0.1
C	582	15.2 ± 1.2	34.3 ± 2.7	6.0 ± 0.1

[a,b] Values without common superscripts within factors in the same column differ ($P < 0.05$).

Table 6.3. 2-way least-squares means of hexose and sperm sex for blastocyst production in vitro (±SE).

FACTORS		No. oocytes	Blastocyst per oocyte (%)	Blastocyst per eight cell (%)
Hexose	**Sperm sex**			
Fructose	X-Bearing	150	20.4 ± 1.9 [a]	43.2 ± 3.9 [a]
Fructose	Y-Bearing	156	17.7 ± 1.9 [a,b]	38.5 ± 3.9 [a,b]
Glucose	X-Bearing	151	10.6 ± 1.9 [b,c]	24.2 ± 3.9 [b,c]
Glucose	Y-Bearing	156	14.7 ± 1.9 [c]	33.9 ± 3.9 [c]

[a,b,c] Values without common superscripts in the same column differ ($P < 0.05$).

Figure 6.1. Interaction least-squares means (±SE) of hexose (glucose and fructose) and sperm sex (X- bearing and Y-bearing) for blastocyst per oocyte in vitro.

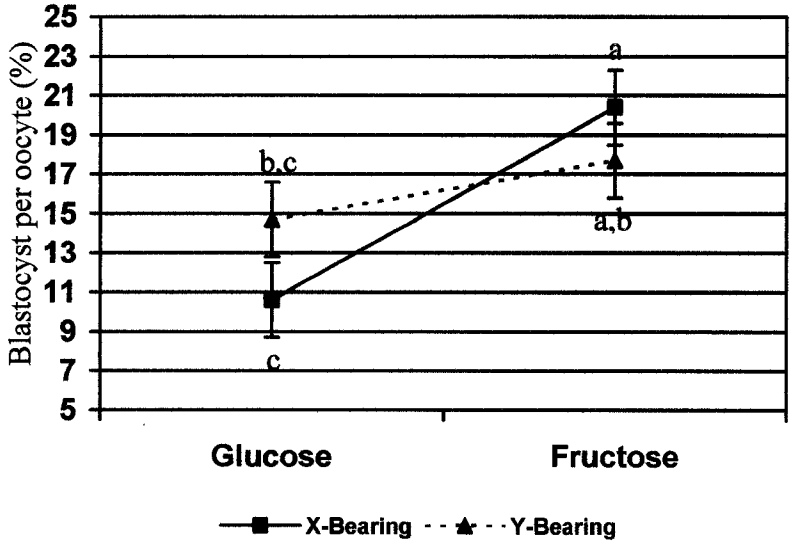
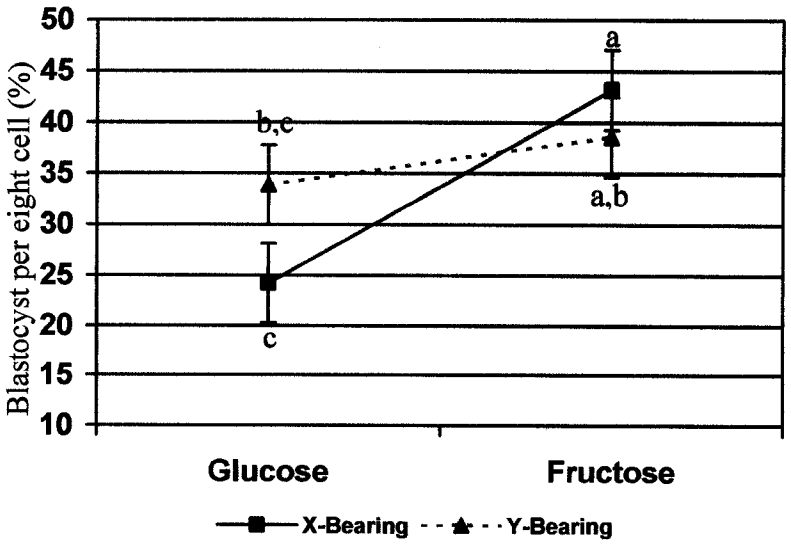


Figure 6.2. Interaction least-squares means (±SE) of hexose (glucose and fructose) and sperm sex (X- bearing and Y-bearing) for blastocysts per eight cell.



6.4.2. Sperm sex effect

X sperm resulted in lower cleavage rates than non-sexed ($P < 0.05$); however, the cleavage rate was not different between hexose treatments ($P > 0.1$) (Table 6.1). Also, blastocyst rates per oocyte and per eight cell embryo were not different due to the sex of sperm ($P > 0.1$) (Table 6.2). Fructose resulted in 30% more blastocyst per oocyte (4.4 percentage points) than the control and glucose groups. X-bearing sperm resulted in retarded development at the blastocyst stage compared to Y-bearing and non-sexed sperm ($P < 0.5$). No interactions were found between treatments ($P > 0.1$).

6.4.3. Bull effect

No differences in cleavage and eight cell rates were observed using three different bulls (A, B and C) ($P > 0.1$) (Table 6.1). Likewise blastocyst per oocyte and per eight cell embryo and stage of blastocyst development were not different between bulls ($P > 0.1$) (Table 6.2). No interactions were found between treatments ($P > 0.1$).

6.5. DISCUSSION

We found a detrimental effect of glucose during in vitro culture. Even when glucose was not different to the control group in percent blastocysts per oocyte, the blastocyst produced were retarded in development. It is proposed that the effect of the high glucose concentration is one of the main determinants (Kimura et al., 2005) of sex

ratio distortion. The use of glucose at blood serum concentrations is detrimental for the female embryo, which arrests at the transition of the post-compaction stage of morulae to blastocyst (Gutiérrez-Adán et al., 2001; Larson et al., 2001; Peippo et al., 2001); this leads to changes the sex ratio of the blastocysts favoring the production of male embryos. The use of medium lacking glucose or with fructose normalizes the sex ratio (Kimura et al., 2005). However, in our study the control group with no hexose was inferior in blastocyst production to the fructose group. The use of 1μM 6-aminonicotiamide (6-AN) and inhibitor of PP pathway by inhibition of G6PD reversed the male ratio (0.46) distortion caused by the effect of high glucose concentration (0.67), but also reduced the blastocyst rate (Kimura et al., 2005). The same effects of reversal ratio and compromised embryo development were observed with another G6PD inhibitor dehydroepiandrosterone (DHEA) (Kimura et al., 2005).

According to some results, the cell number and differentiation of the blastomeres is affected differently depending of the hexose that is used. Kimura et al. (2003) found that fructose stimulated blastomere differentiation into trophectoderm cells; this was also seen in hamsters (Lugwig et al., 2001). On the other hand, glucose stimulated blastomere differentiation into inner cell mass without increasing the total cell number (Kwun et al., 2003); also, in the mouse glucose is the preferred energy substrate for the ICM (Hewitson and Leese, 1993). This can be important because cell numbers in blastocysts can affect the percentage of live offspring after transfer (Van Soom et al., 1997), and the oversize ICM could result in abnormal fetal development including abnormal offspring syndrome (Thompson et al., 1995). A confounding effect is that apoptosis in blastocysts is more

dominant in the ICM than the trophoblast cells (Brison and Schultz, 1997; Byrne et al., 1999). However, the combination of hexoses in this experiment was not superior to the fructose group.

One surprising result was the similar results with no hexose to glucose. Apparently many embryos developed just fine for a week in vitro in the absence of any hexose. De la Torre et al. (2006b) similarly found no differences in blastocyst production using 0, 0.5, 2.0, or 8.0 mM glucose ($P > 0.1$).

6.5.1. Delayed development of female embryos by metabolic differences

Female embryos had retarded development relative to males ($P < 0.05$). In vitro-produced male embryos during the preimplantation period have a higher metabolic rate and faster growth than female embryos; in a study in mice, the faster developing embryos (expanded blastocysts) were mostly males (75%), and the slower developing embryos (early blastocyst) were mostly females (62%) (Pérez-Crespo et al., 2005); this also occurred in bovine female embryos delayed from the morula to blastocyst transition (Kimura et al., 2001). These differences in growth and metabolisms can be due to the existence of two transcriptionally active X-chromosomes (Epstein et al., 1978; Mak et al., 2004; Okamoto et al., 2004); for example, this double expression leads to higher activity of the enzyme G6PD in the female embryo (Iwata et al., 2002), which increased flux through the PPP pathway 4-fold greater than male embryos (Tiffin et al., 1991); however, males had twice the glucose metabolism as female embryos (Tiffin et al., 1991). In the present experiment, retardation in female embryos was also demonstrated using fertilization with sexed semen.

6.5.2. Dimorphic generation and tolerance to free radicals

Some authors proposed that the change in sex ratio occurs due to generation of oxygen free radicals (Iwata et al., 1998). However, increasing the oxygen levels to 20% decreased blastocyst development but did not affect the sex ratio (Kimura et al., 2004). In other words, oxygen free radicals affected both sexes equally; another possibility is that a high glucose concentration leads to a grater flux through PP pathway, which causes an imbalance in metabolism, generating more free radicals.

Three important components that control the amount of free radicals are related to double expression of X-linked genes: the enzymes G6PD, hypoxanthine phosphoribosyl transferase (HPRP), and X-linked inhibitor of apoptosis protein (XRAP) a protein that controls apoptosis through modulating caspase activation and activity. These enzymes are expressed at higher levels in female than male embryos in cattle (Gutiérrez-Adán et al., 2000; Jiménez et al., 2003), and humans (Taylor et al., 2001; Wrenzycki et al., 2002).

6.5.3. NADP and G6PD

Reduced NADP (NADPH) is a defense against oxidative stress, maintaining glutathione in its reduced form, which is essential for detoxification of reactive free radicals and lipid hydroperoxides (Nicol et al., 2000). NADPH is produced by several reactions, but mainly in the first step of the PP pathway, in the conversion of glucose 6-phosphate to ribose5phosphate by G6PD, which is the unique enzyme activated in response to oxidative stress, causing reduced NADP. This is why G6PD is considered

guardian of the cell redox potential (Filosa et al., 2003); high G6PD activity is related with high embryonic competence (Iwata et al., 2002). As demonstrated by Filosa et al (2003), embryonic cells increase PPP activity with oxidative stress. Also it is hypothesized that G6PD is a key enzyme that controls sex ratio in wild animal populations, by favoring the survival of female embryos under oxidative stress conditions (Gutiérrez-Adán et al., 2000). Recent studies suggest that the female embryos produce less H_2O_2, and survive better and resist heat stress better than male embryos under conditions that produce ROS in vivo or in vitro. This is due to the presence of the X-related genes, because when inhibiting G6PD with DHEA, all the differences between females and males disappeared (Perez-Crespo et al., 2005). This leads us to think that some transcription factors can be redox regulated, and more active when NADPH is high.

The level of transcription of G6PD is higher in the female embryo, but BAX (gene implicated in resistance to apoptosis), SOX (sarcosine oxidase, involved in the response to stress), and Oct-4 (related to pluripotency) were higher in the male; however, with inhibition of G6PD by DHEA no differences were observed between sexes in gene expression (Perez-Crespo et al., 2005). The higher expression of G6PD and resultant increase in NADPH is the most likely explanation of female embryo resistance for oxidative stress, and the higher expression of BAX, SOX may be a compensatory mechanism for protection for male embryos (Perez-Crespo et al., 2005).

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CHAPTER VII

VALIDATION OF QUANTITATIVE FLUORESCENCE FOR DETERMINING LIPID CONTENT OF BOVINE OOCYTES AND BLASTOCYSTS STAINED WITH NILE RED DYE

7.1. Summary

The main aim of this work was to test and validate a fluorescence approach to quantify lipid accumulation in bovine oocytes and blastocysts. For experiment 1, denuded oocytes from 2 ovary sources (cows vs. postpubertal heifers) were evaluated as well as blastocysts, in a factorial design with 2 ovary sources (cows vs. heifers), 3 additives in CDM-2 (Control; 10% FCS; and 0.3μM PES, an electron acceptor that oxidizes NADPH) and 2 blastocyst stages (early vs. expanded); all blastocysts were graded for darkness (1=clear ... 4=dark). In experiment 2, denuded oocytes were used to measure lipid content in a factorial design with 2 ovary sources (cows vs. heifers), and 4 darkness grades (1=clear ... 4=dark). To quantify lipid accumulation, oocytes and 7.5 d blastocysts were fixed and stained with 1 μg/mL Nile red dye in mPBS overnight, rinsed twice, and then mounted on a slide. A digital photograph of the equatorial part of the oocyte and

embryo (including inner cell mass) was taken at 200x, and fluorescence intensity (Arbitrary Fluorescence Units, AFU) was measured with Image Pro software from 0 to 255 shades for each pixel (0=no lipids; 255=greatest lipid accumulation); all units were adjusted to a 15,000 μm^2 basis. Reverse images of the same photographs were used to count numbers of cytoplasmic lipid droplets, classified by diameters as small (S, <2 μm), medium (M, 2 to 6 μm), or large (L, >6 μm); the averages of 1, 4 and 6 μm diameters for S, M and L were used to calculate the volume of lipid droplets per 15,000 μm^2 (SML). Data were analyzed by regression and ANOVA. The linear regression of SML with AFU in oocytes had an r^2=0.84, and blastocysts r^2=0.910. SML and AFU were highly correlated, and had similar coefficients of variation from ANOVA (38 and 44%, respectively). Treatment differences were of similar magnitude with both techniques. Lipid content of oocytes and blastocysts from heifers and cows was similar ($P > 0.1$); PES reduced lipid accumulation, and FCS increased it relative to the control for AFU (18.6 vs. 46.6 vs. 36.9 units, respectively), and SML (1763 vs. 4081 vs. 3310, respectively) (all, $P < 0.01$). Early blastocysts resulted in more lipid accumulation than expanded ones for AFU (41.5 vs. 26.6, respectively) and SML (3519 vs. 2583, respectively) (both, $P < 0.01$). We found a strong relationship between oocyte and blastocyst darkness and lipid content ($P < 0.01$). In conclusion fluorescence after staining is a reliable technique for quantifying lipid droplets in bovine oocytes and blastocysts; using AFU adjusted for area resulted in similar discrimination to SML, but is faster and more objective than the SML approach.

7.2. INTRODUCTION

Lipid accumulation in oocytes and embryos has been assessed with quantitative methods such as thin-layer and gas chromatography (Menezo et al., 1982; Waterman and Wall, 1988; Coull et al., 1998; McEvoy et al., 2000; Kim et al., 2001; Sinclair et al., 2002; Reis et al., 2003) and neosynthesis of lipids coupled with HPLC (Abd El Razek et al., 2000). These quantification techniques, however, require special, expensive equipment and pools of 4 to 1000 oocytes. Enzymatic-microfluorescence assays require at least 1-3 embryos (Ferguson and Leese, 1999; Majerus et al., 2000; Sturmey and Leese, 2003); assays are easier to use but required 100 oocytes (Kim et al., 2001).

Simpler semi-quantitative techniques using a single oocyte or embryo have been reported, where quantification of number and density of lipid droplets has been evaluated with electron microscopy (Crosier et al., 2000; Crosier et al., 2001; Kikuchi et al., 2002; Rizos et al., 2002) or light microscopy using Sudan Black B dye based on size of lipid droplets (Abe et al., 2002; De la Torre-Sanchez et al., 2006b, Barcelo-Fimbres and Seidel, 2007a). Recently an easy, repeatable and sensitive technique has been reported for amounts of lipids by quantification of fluorescence of Nile Red using microscopy with single oocytes or embryos (Genicot et al., 2005; Leroy et al., 2005).

Nile red is a lipophilic dye that fluoresces yellow at 580-596 nm with apolar lipids (neutral) like triglycerides, and orange 597-620 nm with polar lipids such as the phospholipid bilayer (Greenspan and Fowler, 1985). The amount of fluorescence emitted by the dye is correlated with lipid accumulation, so higher fluorescence equates with

higher the lipid content. With this approach murine oocytes emitted 2.8-fold lower fluorescence than bovine embryos, and the latter, 2.4 times less than porcine oocytes (Genicot et al., 2005).

In previous experiments, we found addition of phenazine ethosulfate (PES) during culture decreases lipid content in bovine blastocysts (De la Torre-Sanchez et al., 2006b, Barcelo-Fimbres and Seidel, 2007a); however, the addition of 10% fetal calf serum (FCS) increases it (Barcelo-Fimbres and Seidel, 2007a). Lipid content in these experiments was measured using Sudan Black B dye based on size of lipid drops, so the use of these additives could serve as reference to test a new, faster and more objective procedure using Nile red dye.

The objective of this experiment was to test and validate a fluorescence technique to quantify lipid accumulation in bovine oocytes and blastocysts, using ovaries from heifers and mature cows.

7.3 Materials and Methods

7.3.1. Experimental Designs

Experiment 1: Validation and test of fluorescence of Nile Red dye to quantify lipid accumulation in oocytes and blastocysts. In this experiment, lipid accumulation in oocytes was validated with 50 oocytes obtained after aspiration from two ovary sources (cow vs. heifer-derived ovaries). Validation of lipid accumulation in blastocysts was done

with 60 blastocysts (5 per subclass) produced from a factorial design with two ovary sources (cow vs. heifer-derived ovaries), three additives in CDM-2 (Control; 10% FCS; and 0.3 μM PES) and blastocyst stage (early (small blastocoele to early expanded) vs. late (fully-expanded)). Also, all blastocysts were graded for darkness (1=clear ... 4=dark).

Experiment 2: Evaluation of the lipid accumulation in bovine oocytes. For the second experiment, immature denuded oocytes were used to measure lipid accumulation. Denuded oocytes (n= 256) were obtained from two ovary sources (cows vs. heifers), and classified subjectively in four darkness grades (1=clear ... 4=dark).

7.3.2. Embryo production

7.3.2.1. Oocyte Collection and In Vitro Maturation. Bovine ovaries from mature cows and heifers were obtained from local abattoirs and transported to the laboratory in 1-2 h in 0.15 M NaCl saline at ambient temperature, approximately 22 to 25°C. After arriving at the laboratory, ovaries were trimmed of extraneous tissue, and rinsed once again in 0.15 M NaCl. Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm antral follicles with an 18-gauge needle attached to a tubing system connected to a vacuum aspiration pump with 40-50 mm Hg pressure. Oocytes with at least 3 layers of unexpanded cumulus cells and morphologically bright and evenly granulated cytoplasm were selected for IVM. COCs were washed twice in chemically defined medium (CDM) with Hepes buffer for handling oocytes and once with maturation medium (De La Torre-Sanchez et al., 2006b). Fifty COCs were matured per well of 4-well plates (Nunclon,

Roskilde, Denmark), containing 1 ml of maturation medium CDM with 0.5% fatty acid-free BSA (Sigma A-6003, Lot # 063K7525) and gonadotropin hormones (National Protein and Peptide Program, Torrance, CA) (15 ng/ml of FSH (NIH-FSH-S17), 1μg/ml of LH (USDA-LH-B-5), 1.0 μg/ml of estradiol-17β (Sigma E-2257), 50 ng/ml EGF (Sigma E-9644) and 0.1mM cysteamine (Sigma M-6500). Oocytes were incubated at 38.5°C with humidified 5% CO_2 in air for 23 h.

7.3.2.2. Sperm Preparation. Frozen bovine semen contained at least 35 % progressive motile sperm after thawing. Straws of semen were thawed in water at 35°C for 30 sec. Semen from one straw was gently expelled into a 15-ml centrifuge tube and centrifuged 20 min at ~400 x g at 23°C through a Percoll gradient (Sigma P-1644) with 2 ml 90%: 2 ml 45% Percoll in sperm (sp)-TALP medium (modified Tyrode's) as described by Parrish et al. (1989). The supernatant was discarded, and the sperm pellet (approximately 100 μL) was washed with 5 ml of chemically defined fertilization medium (Fert-CDM; De La Torre-Sanchez et al., 2006b), supplemented with 0.5% BSA, 5 mM caffeine (Sigma C-0750) and 2 μg/ml heparin (Sigma H-3125). The sample was centrifuged again for 5 min at 400 x g at 23°C, and the supernatant was discarded. Approximately 90 to 100 μl remained, and a 5-μl aliquot was taken to determine the sperm concentration with a hemacytometer. The sperm concentration was adjusted to 5 x 10^6 spermatozoa per ml to give final concentrations of 0.5 x 10^6 spermatozoa per ml in Fert-CDM (see below).

7.3.2.3. In Vitro Fertilization. Following in vitro maturation, ~50 oocytes were placed in 450 μl of Fert-CDM medium per well of 4-well dishes, and 50 μl of sperm suspension

were added to give final volume of 500 µl per well. Gametes were co-incubated for 18 h at 38.5°C in an atmosphere of humidified 5% CO_2 in air.

7.3.2.4. Culture of Embryos. Following in vitro fertilization, presumptive zygotes were removed from wells and transferred to 1.0-ml microcentrifuge tubes with approximately 100 µl of Fert-CDM (De La Torre-Sanchez et al., 2006b), and then vortexed for 50 to 60 sec to remove cumulus cells. These procedures were carried out in a room at 28°C. Embryos then were rinsed three times in Hepes CDM-1 (H-CDM-1). Early culture (day 0 to 2.5 post fertilization) was done in a new 4-well dish, containing 500 µl of CDM supplemented with 0.5% fatty acid-free BSA, 0.5 mM fructose, non-essential amino acids (NEAA), and 10 µM EDTA (CDM-1) as described by De La Torre-Sanchez et al. (2006b), and incubated at 39°C under 90% N_2, 5% O_2, and 5% CO_2. After 60 h of culture, embryos were examined with a stereomicroscope (15 to 20×) for cleavage, and all uncleaved ova and embryos less than 8 cells were discarded. The rest were cultured in new dishes with CDM-2 (CDM supplemented with 0.5% fatty acid-free BSA, NEAA and essential amino acids and 2 mM of fructose) from day 2.5 to day 7.5 post fertilization. Embryos were evaluated 180 h after the end of fertilization to determine the final stage of development.

7.3.2.5. Addition of Metabolic Regulators. A 2000x stock of PES (Sigma, P-4544) was prepared in nanopure water; then a 100x stock was made in HCDM-2. This stock was stored at 5°C and added to CDM-2 at the time of medium equilibration. The final concentrations of the metabolic regulators were: PES, 0.3 µM; and 10% FCS (Gemini

Bio-Products Inc., West Sacramento, CA, lot# A20206P) which was considered as a negative control. PES concentration was based on dose-response studies of De La Torre-Sanchez et al. (2006b).

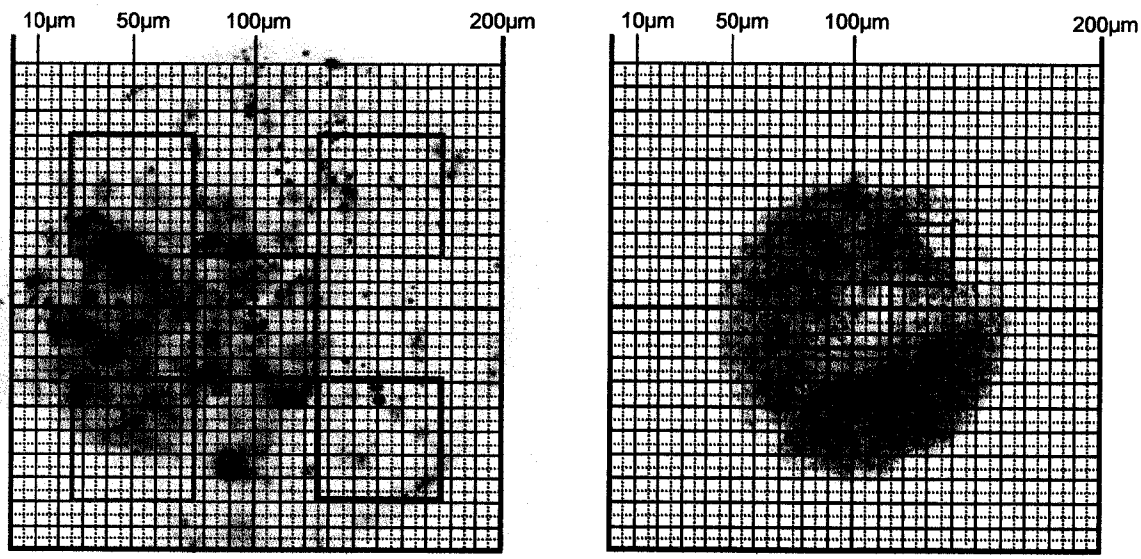
7.3.3. Lipid quantification

To quantify lipid content, oocytes and blastocysts were fixed in 10% formalin in modified phosphate-buffered saline (mPBS; Elsden and Seidel, 1995) plus 0.01% PVA at pH 7.4 for one day, and then stained overnight in 500 μL of 1 μg/mL Nile red dye (Sigma, N3013) dissolved in mPBS (dose based on work done by Genicot et al., 2005), then rinsed twice in mPBS and mounted on a slide with glycerol; finally, a cover slide with paraffin-vaseline was placed without pressing the oocyte or embryo. A stock solution of 100 μg/mL Nile red was made in DMSO and stored at room temperature; the stock was diluted in mPBS plus 0.01% PVA to get a final concentration of 1 μg/mL. This solution and slides were stored in the dark.

An epifluorescence microscope (Nikon E-800) was used to excite the lipophilic fluorescent dye Nile red (590 nm), capturing a digital photograph of the equatorial part of the oocyte or blastocyst (including the inner cell mass, ICM) using a 20 X objective. Fluorescence intensity (Arbitrary fluorescence units, AFU) was measured with computer software Image Pro from 0 to 255 shades for each pixel (0=no lipids; 255=greatest lipid accumulation). The AFU were adjusted by background to compensate for variation of the UV lamp intensity. The area from all oocytes and blastocysts was measured, and the fluorescence was adjusted to a 15,000 μm^2 basis (which is equivalent to an embryo of

~138 μm of diameter). For experiment 1, immature oocytes and 7.5 d blastocysts were used, and the fluorescence was measured as explained above. Then reverse images of the same photographs were used to count numbers of cytoplasmic lipid droplets. The numbers of granules in focus were counted and classified by diameter as small (S, <2 μm), medium (M, 2 to 6 μm), or large (L, >6 μm) (Abe et al., 2002). Lipid droplets were counted as previously described Barcelo-Fimbres and Seidel, 2007a. Briefly, a grid of 40,000 μm^2 (200 by 200 μm^2) was constructed using digital tools with a Neubauer chamber as a reference with intergrids of 25 μm^2 (5 by 5 μm^2) and 5 squares of 2500 μm^2 (50 by 50 μm^2) randomly placed over the embryo image. For oocytes and small blastocysts, a small size grid was constructed of 5 squares of 625 μm^2 (25 by 25 μm^2) and multiplied by four to equalize the areas relative to data for large embryos (Figure 7.1).

Figure 7.1. Negative photomicrographs visualizing Nile red dye of large blastocyst with a grid of 40,000 μm^2 with 5 squares 50 by 50 μm^2 outlined **(A)** and oocyte with a grid of 40,000 μm^2 showing 5 squares of 25 by 25 μm^2 **(B).**



To validate the fluorescence approach relative to our previous technique (Barcelo-Fimbres and Seidel, 2007a), a relative lipid granule volume in the embryo was calculated (to calculate absolute amount of lipids in an embryo requires different methodology). First, large droplets were converted to an equivalent number of medium droplets (medium + large, ML); considering an average size of 4 μm for a medium droplet and 8 μm for a large droplet, and taking into account that the comparison has to be made in terms of volume, one large droplet was equivalent to 8 medium droplets. Second, large and medium droplets were converted to an equivalent number of small droplets (small + medium + large, SML); considering an average size of 1 μm for a small, 4 μm for a medium and 8 μm for a large droplet; in terms of volume, one large droplet was equivalent to 8 medium and 64 small droplets. ML and SML were used to calculate the relative volume of lipid droplets per 15,000 μm^2.

7.3.4. Evaluations of embryos

For experiment 1, 7.5 d blastocysts were scored by stage (early and expanded) and lightness (1= lighter, ... 4= darker). Denuded oocytes from ovaries derived from cows and heifers were used in experiment 2, and they were graded subjectively according to the color (1= lighter, ... 4= darker).

7.4. Statistical procedures

For comparison of lipid measurement techniques a regression model (proc REC) was used for evaluating the linear and quadratic terms. Lipid droplets and fluorescence evaluations were then analyzed in a factorial design by ANOVA, and blastocyst darkness

was evaluated using one way-ANOVA, using the SAS statistical software package general linear model (GLM). Sources of variation in the model included all main effects (all considered as fixed effects), all possible 2 way interactions, and residual error. If the ANOVA was significant ($P < 0.05$), means were separated by Tukey's w procedure.

7.5 RESULTS

Experiment 1 was done to validate and test a fluorescence technique to quantify lipid accumulation in oocytes and blastocysts treated in various ways (Table 7.1). Regression analysis of lipid quantification by fluorescence intensity (arbitrary florescence units) with the volume of lipid droplets (Medium + Large (ML); and Small + Medium + Large (SML)) was done in oocytes and blastocysts (Figure 7.2). In oocytes fluorescence intensity was only modestly correlated with ML, $r^2=0.50$, but highly correlated with SML, $r^2=0.84$. For blastocysts, fluorescence intensity was highly correlated with both ML, $r^2=0.910$, and SML, $r^2=0.912$. These relationships were linear for both oocytes and blastocysts; the quadratic terms were not significant for any response ($P > 0.1$).

Lipid accumulation in oocytes and blastocysts measured by fluorescence intensity and lipid droplet quantification (relative lipid granule volume of S+M+L) were similar whether derived from heifers or cows ($P > 0.1$) (Table 7.1). Differences in lipid content of blastocysts of different stages (early vs. late blastocysts), blastocysts treated with additives (Control; 10% FCS; and 0.3 μM PES), and stage (early vs. late) were of similar magnitude with both techniques used. There was more lipid in early than late blastocysts

($P < 0.01$), and FCS had more lipids, and PES-treated blastocysts less lipids than controls ($P < 0.01$) (Table 7.1).

Table 7.1. Main effect treatment least-squares means (±SE) of ovary source, stage, and treatment on lipid accumulation using Nile red (arbitrary fluorescence units, AFU) and SML (small + medium + large lipid droplets per 15,000 μm^2) of bovine embryos in vitro -Experiment 1.

RESPONSES	No.	AFU	SML
OOCYTES			
Source			
Heifers	25	48.3 ± 3.5^{a}	63.2 ± 3.3^{a}
Cows	25	50.5 ± 3.5^{a}	67.6 ± 3.3^{a}
BLASTOCYSTS			
Source			
Heifers	30	34.5 ± 2.8^{a}	3038 ± 142^{a}
Cows	30	33.6 ± 2.8^{a}	3065 ± 142^{a}
Stage			
Early blastocysts	30	41.5 ± 2.8^{a}	3519 ± 142^{a}
Late blastocysts	30	26.6 ± 2.8^{b}	2583 ± 142^{b}
Treatment			
PES	20	18.6 ± 3.4^{a}	1763 ± 173^{a}
Control	20	36.9 ± 3.4^{b}	3310 ± 173^{b}
FCS	20	46.6 ± 3.4^{c}	4081 ± 173^{c}

Values without common superscripts within factors in the same column differ (^{a,b,c} $P < 0.01$).

We found a strong relationship between blastocyst darkness grade and lipid content. The highest to the least lipid accumulation was 4 (dark), 3, 2, and 1 (clear); using the fluorescence approach these categories measured 90.2, 53.7, 38.7 and 17 AFU, respectively, and for SML, 4638, 3180 2111, and 813 lipid droplets per 15,000 μm^2, respectively, so these differences were detected similarly with both techniques (Table 7.2).

Figure 7.2. Linear regression of Arbitrary Florescence Units (AFU) with Medium + Large (ML) and Small + Medium + Large (SML) lipid droplet volumes of oocytes and blastocysts.

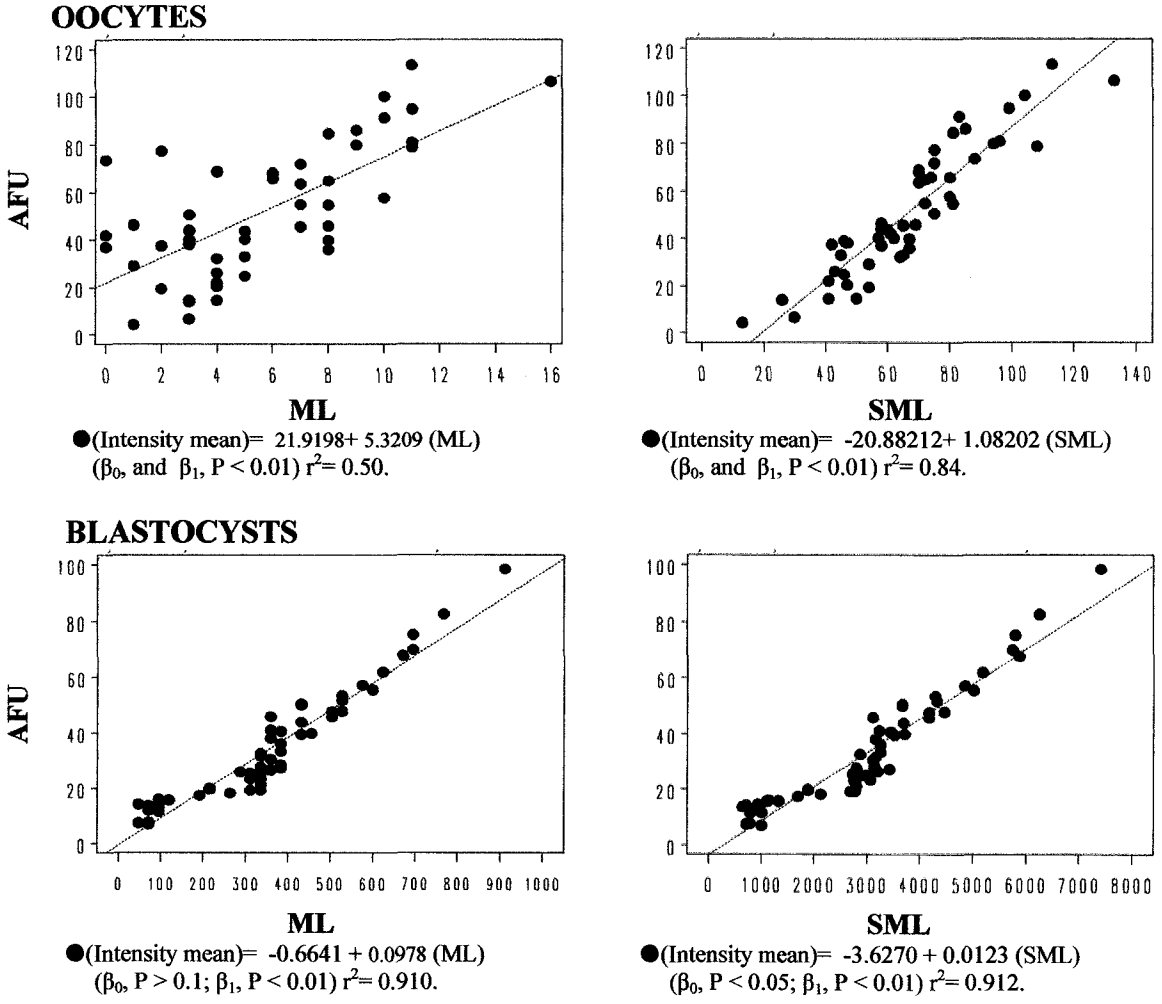


Table 7.2. Least-squares means (±SE) of blastocyst darkness grade on lipid accumulation using Nile red (arbitrary fluorescence units, AFU) and SML (small + medium + large lipid droplets per 15,000 μm^2) of bovine embryos *in vitro* -Experiment 1.

Darkness grade	No.	AFU	SML
1 (clear)	8	17.0 ± 7.0^{a}	813 ± 315^{a}
2	18	38.7 ± 5.2^{b}	2111 ± 192^{b}
3	14	53.7 ± 6.0^{c}	3180 ± 223^{c}
4 (dark)	20	90.2 ± 5.1^{d}	4638 ± 187^{d}

Values without common superscripts within factors in the same column differ (^{a,b,c,d} P < 0.01).

In experiment 2, immature oocytes were used to determine lipid content measured using fluorescence with Nile red. No differences were found in lipid accumulation from heifer vs. cow-derived immature oocytes (39.6 vs 42.6 AFU, respectively). However, there was a significant effect for oocyte grade ($P < 0.01$); color (darkness) was related to lipid amount. The highest to the least lipid accumulation was 4 (dark), 3, 2, and 1 (clear) (63.6, 45.6, 36.3 and 18.9 AFU, respectively) (Table 7.3).

Table 7.3. Main effect treatment least-squares means (±SE) of ovary source, and color grade (darkness) of immature bovine oocytes stained with Nile red -Experiment 2.

RESPONSES	No. oocytes	Arbitrary Fluorescence Units
Source		
Heifers	177	39.6 ± 1.7^{a}
Cows	79	42.6 ± 2.2^{a}
Oocyte grade		
1 (clear)	15	18.9 ± 4.5^{a}
2	50	36.3 ± 2.5^{b}
3	81	45.6 ± 1.9^{c}
4 (dark)	110	63.6 ± 1.8^{d}

Values without common superscripts in the same column differ (^{a,b,c,d} $P < 0.01$).

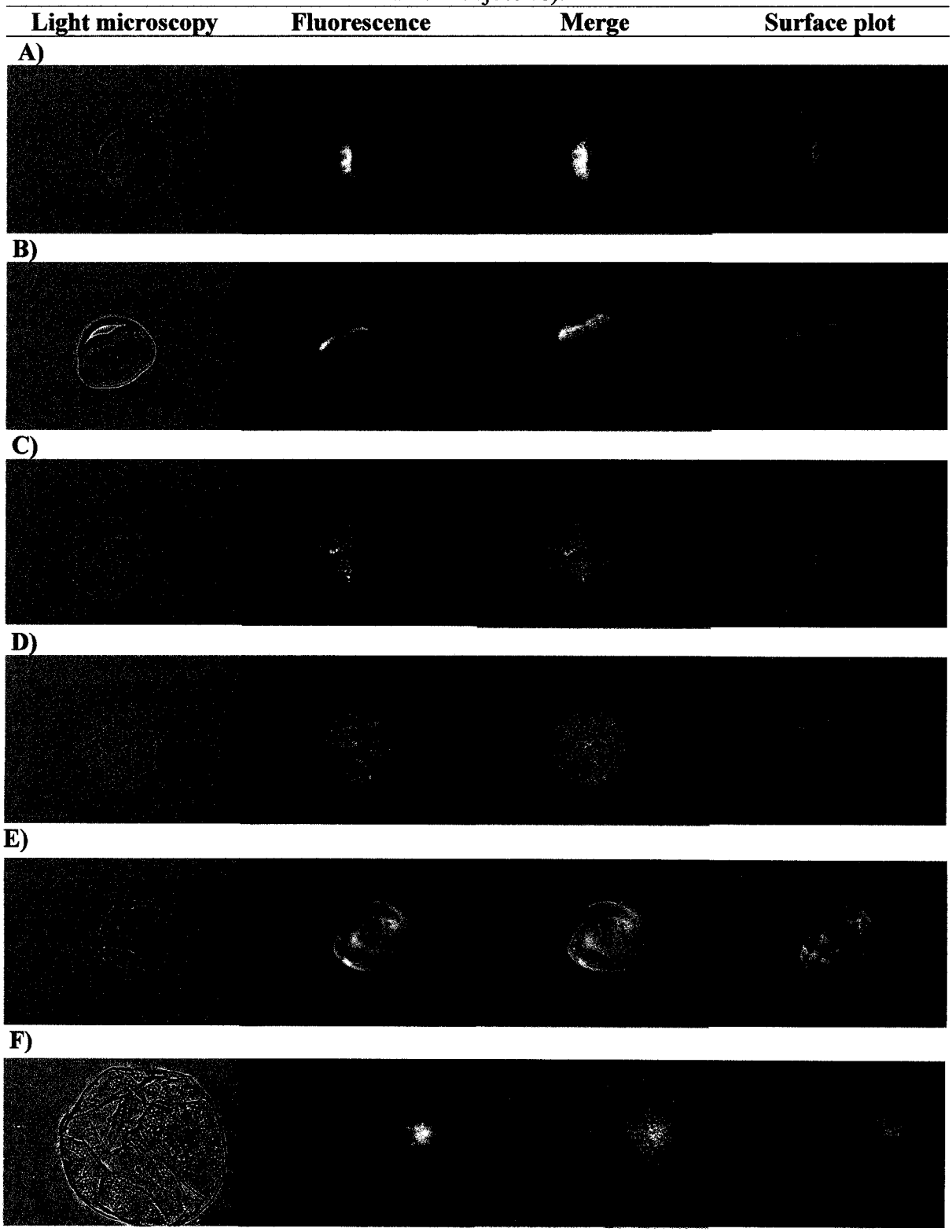
7.6. DISCUSSION

Bovine oocytes contain abundant stores of lipids, with triglycerides the most abundant lipids in both immature and in vitro-matured oocytes (Kim et al., 2001; Sinclair et al., 2003; McEvoy et al., 2000). Lipid accumulation is associated with embryo darkness (Sata et al., 1999; Abe and Hoshi, 2003; Leroy et al 2005), and impaired embryonic quality (Hill and Kuehner, 1998; Reis et al., 2003; Rizos et al., 2003).

Genicot et al. (2005) demonstrated that the dark clusters in oocyte cytoplasm corresponded to lipid droplets stained with Nile red dye. We also confirm that Nile red is very specific to lipids; after centrifuging, staining and merging fluorescence and light microscopy photographs of oocytes, the stain was strictly localized in the lipid cluster, and not in zona pellucida or other cellular compartments (Figure 7.3 A, B). We found different patterns of lipid droplet distribution in the cytoplasm of oocytes; some had small size lipid droplets, medium size or lipid clusters, but mostly in a homogeneous distribution pattern (Figure 7.3 C, D). This homogeneous pattern was corroborated since the position of the microscopic focal plane at intervals of 20 μm within oocytes had no influence in the amount of fluorescence (Genicot et al., 2005). The lipid droplets are associated with endoplasmic reticulum and mitochondria, so that their distribution during maturation may be related to possible metabolic changes (Kruip et al., 1983).

When classifying lipid droplets by size, as previously described (S, <2; M, 2 to 6 and L, >6 μm), the average percentages of lipid droplets per oocyte were 81.3, 18.5 and 0.2 % for S, M and L, respectively (data not showen). Nevertheless, in terms of volume, the percentages of the total lipid counted were 34, 60 and 6% for S, M and L respectively (data not shown). Thus, in frequency most of the lipid droplets were less than 2 μM; however, in volume this represented 34% of lipid, which could explain the lower coefficients of regression comparing Nile red fluorescence intensity vs. lipid droplets volume using ML than SML (Figure. 7.2).

Figure 7.3. Photomicrographs by light microscopy, fluorescence, merge and surface plot of immature oocytes (A and B: centrifuged at 10,000 x G for 5min; C and D: patterns of lipid granule distribution; E: early blastocyst; F: expanded blastocyst. (Photographed with a 20 x objective).



For blastocysts, the average percentage of lipid droplets per oocyte was 56, 38 and 6% for S, M and L, respectively. However, in terms of volume, the percentages of total lipid were 7, 43 and 49% for S, M and L respectively. This may explain very similar coefficients of regression comparing Nile red fluorescence intensity vs. lipid droplet volume using ML or SML (Figure. 7.2). Thus, the bulk of lipid droplet accumulations in IVP blastocysts is in medium and large droplets, as we have previously reported (De la Torre-Sanchez et al., 2006b; Barcelo-Fimbres and Seidel, 2007a).

In the present study we used black and white instead of color photomicrographs, because we found that black and white gave better resolution in terms of intensity than those in color (Figure 7.4 A, B, C, D).

We found a significant relationship between ooplasm darkness of immature oocytes (Figure. 7.5 F) and blastocysts and lipid accumulation by classifying them in 4 categories (1=clear...4=dark), so darker the ooplasm means higher lipid accumulation. Similar significant results for oocytes were reported by Leroy et al. (2005), classifying the ooplasm in three categories (1=uniform dark, 2=gray or granulated, 3=very pale). We did not evaluate oocyte competence by category; however, there are reports that clear or granulated ooplasm resulted in lower blastocyst rates in vitro (Hawk and Wall, 1994; Bilodeau-Goeseels and Panich, 2002).

Figure 7.4. Black and white fluorescence photomicrograph **(A),** and its intensity surface plot **(B)** and color photomicrograph **(C)** and its intensity surface plot **(D).** (Photographed with a 20x objective).

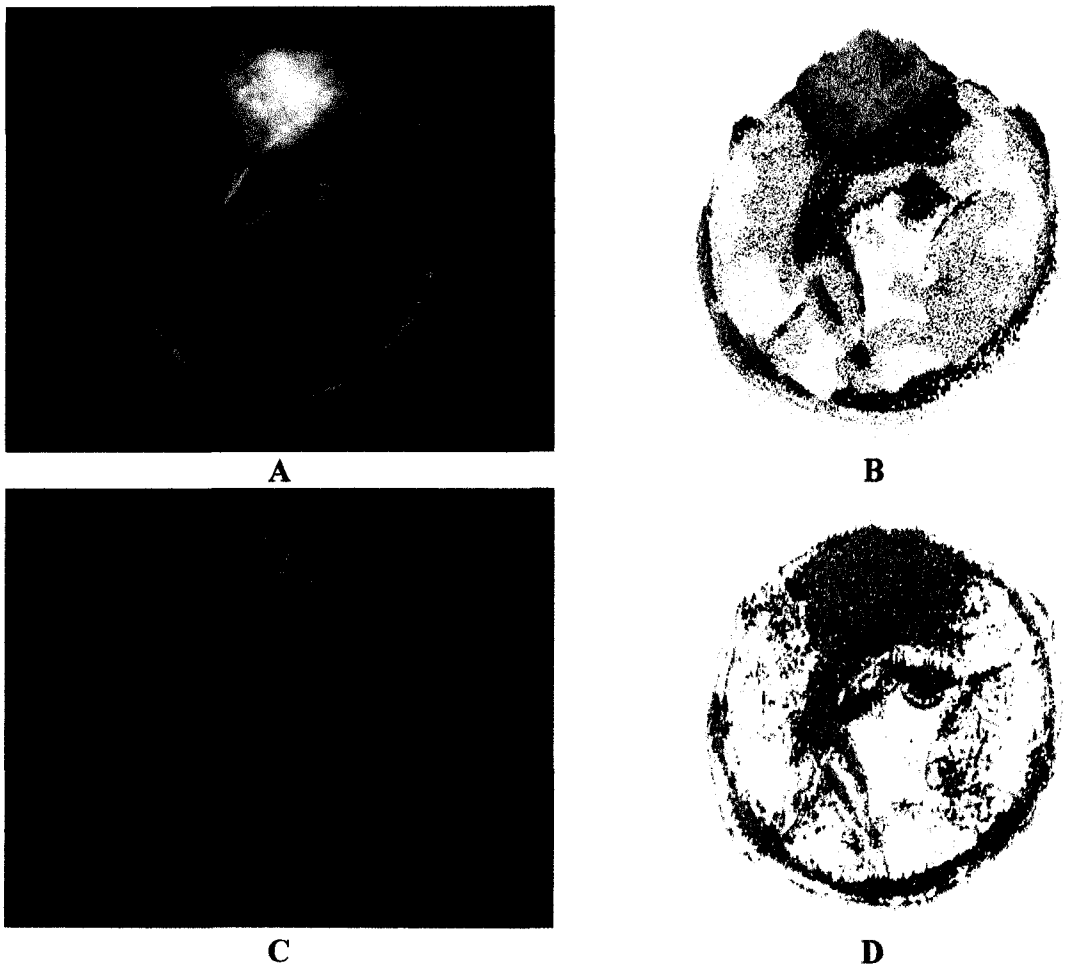
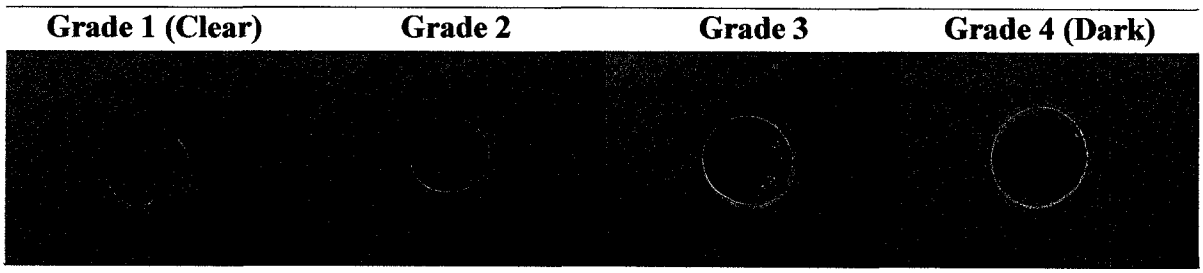


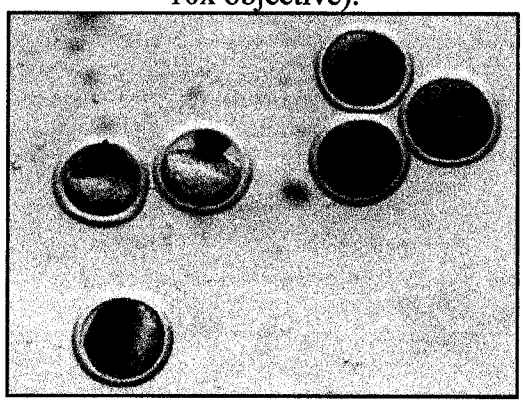
Figure 7.5. Photomicrograph by light microscopy of color grade from left to right: (1=clear ... 4=dark). (Photographed with a 10x objective).



The oocytes and blastocysts are colorless, so filters used in microscopes will determine the color observed. For objective oocyte or blastocyst evaluation, it is important that the same microscope parameters are used. We related the darkness of oocytes and blastocysts to lipid content, which is perceptible in the microscope because lipids limit the amount of light that passes through the oocyte or embryo; redistributing the lipid content by centrifugation of ooplasm of bovine oocytes gives a clear appearance (Figure 7.6). This may explain why there are differences in darkness between species, for example murine oocytes are clearer than cattle, and cattle clearer than pigs; the lipid content is least in murine and highest pig oocytes (Genicot et al., 2005).

We did not find differences in lipid content in oocytes and blastocysts derived from heifers and cows using Nile red or the lipid droplets counting approach. Similarly, Majerus et al. (2000) reported similar results for calf oocyte-derived 7 d blastocysts vs. adults oocyte-derived embryos (64±15 vs. 65±6 ng per embryo) using an enzymatic-microfluorescence essay to measure lipid accumulation.

Figure 7.6. Photomicrograph of denuded bovine oocytes, the three oocytes on the right are normal oocytes, and the three on the left were centrifuged (~10,000 x g) notice that the ooplasm gets clear after the lipid content moves to one side. (Photographed with a 10x objective).



Previous reports have indicated that lipid content in the bovine oocyte increases with increasing size of oocytes (Fair et al., 1995). However we did not find differences in oocyte diameter in experiment-2 ($P > 0.1$), where post-pubertal heifer-derived oocytes were 125 ± 0.8 (n=177) and those from cows 126 ± 1.2 (n=79) μm in diameter.

In the present experiment, we found that both techniques for measuring lipid content in blastocysts treated with additives and blastocysts stages (early vs. expanded) gave results of similar magnitude. Early blastocysts (early blastocysts to early expanded) had more lipid content than late stage (expanded and hatched) (Figure 7.3 E, F). We found that PES during culture decreases lipid content, and FCS increased it relative to controls, as previously reported (De la Torre-Sanchez et al., 2006b, Barcelo-Fimbres and Seidel, 2007a). The addition of PES decreased lipid content of embryos 50% and 53%, and FCS increased it 26 and 23% relative to controls using Nile red and lipid droplet counting respectively. SML and AFU were highly correlated and had similar coefficients of variation from ANOVA (38 vs. 44%, respectively).

We adjust fluorescence intensity to the size of oocytes and blastocysts, even through the adjusted and non adjusted gave similar significant differences. When the treatments were analyzed statistically, the AFU adjusted explained the variances a little better than without adjusted (r^2=0.52 vs 0.48). We also compared lipid droplet counting with fluorescence intensity (AFU) of blastocysts used in experiment 1 using the free software Image J (NIH Image, Bethesda, Maryland, Available at http://rsb.info.nih.gov/ij/download.html), and we found that this system also resulted in a

high correlation fluorescence for both ML, r^2=0.71, and SML, r^2=0.72, although not as high as the system that we used.

The technique reported to quantify lipid accumulation in the present study using Nile red to stain oocytes and blastocysts detected treatment differences in similar magnitudes, but is more objective and less time consuming than the lipid drop counting technique that we have been previously reported (Abe et al., 2002; De la Torre-Sanchez et al., 2006b; Barceló-Fimbres et al., 2007a). The procedure that we used to analyze lipids is simpler than that reported by Genicot et al. (2005), who amplified and quantified the fluorescence using photomultiplier and a photometer respectively.

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CHAPTER VIII

EFFECTS OF METABOLIC REGULATORS ON EMBRYONIC DEVELOPMENT AND LIPID CONTENT OF EMBRYOS DERIVED FROM OOCYTES OF HEIFERS AND COWS

8.1. Summary

The objective of these experiments was to evaluate lipid accumulation and embryonic development of bovine morulae and blastocyst treated with different chemicals and derived from oocytes from ovaries from heifers and cows. For the experiment 1, blastocysts were produced in a factorial design with 2 ovary sources (cows vs. heifers), 3 additives in CDM-2 (Control; 10% FCS; and 0.3 μM PES), and 4 bulls (2 days each); in experiment 2, morulae were used in a factorial design with 2 ovary sources (cows vs. heifers), and 7 treatments (Control and 2 and 8 mM caffeine, 1 and 4 μM epinephrine, and 10 and 40 μM forskolin). To quantify lipid accumulation, oocytes and d 7 blastocysts were fixed and stained with 1 μg/mL Nile red dye. A digital photograph of the equatorial part of the morula or blastocyst (including inner cell mass) was taken at 200x, and fluorescence intensity was measured with Image Pro software from 0 to 255 shades for each pixel (0=no lipids; 255=greatest lipid accumulation); all units were adjusted to a 15,000 μm^2 basis.

No differences in cleavage or eight cell rates were found in either experiment, or morula production in experiment 2 ($P > 0.1$). However, blastocyst rates per oocyte and eight cell in both experiments or per morula in experiment 2 were higher for cows than

heifers ($P < 0.05$). No differences in blastocyst stage or hatching rates were found in experiment 1 ($P > 0.1$); however in experiment 2 cow blastocysts were more advanced in development ($P < 0.01$). No effect of additives was found in experiment 1 (Control vs. PES vs. FCS) for blastocyst production; however in experiment 2, and 8 mM caffeine was detrimental for blastocyst development and stage ($P < 0.01$), and epinephrine and forskolin were similar to the control ($P > 0.1$). In experiment 1 no difference in lipid content was found measuring the whole embryo-derived from cows vs. heifers; however cow blastocysts had more lipids in the embryonic mass ($P < 0.05$). In experiment 2, more lipid content was found in whole embryos and trophoblast in heifer-derived than cow blastocysts. Early blastocysts had more lipid accumulation per unit area than expanded blastocysts in both experiments. PES-treated embryos resulted in less lipid accumulation compared to the control, and FCS increased lipid ($P < 0.05$) in experiment 1, and forskolin resulted in less lipid content than control, caffeine and epinephrine-treated embryos in experiment 2 ($P < 0.05$).

In conclusion, mature cows were a better source of oocytes than feedlot heifers for embryo development; however, they had more lipid content in the embryonic mass. Progression of the early blastocyst to expanded blastocyst resulted in decreased lipid content. PES decreased, and FCS increased lipid content relative to control, but neither affected embryonic development. Forskolin reduced lipid content relative to control, caffeine and epinephrine-treated embryos. High doses of caffeine were detrimental to embryos.

8.2. Introduction

Cattle oocytes and embryos contain considerable lipid in the form of triglycerides (Ferguson and Leese, 1999; Sinclair et al., 2002), which are an important source of energy during oocyte maturation, fertilization, and early embryo cleavage (Ferguson and Leese al., 1999; Kim et al., 2001; Sinclair et al., 2002; Ferguson and Leese, 2006); phospholipid and cholesterol also are important for function and structure of biological membranes (Cribier et al., 1993). In vitro-produced (IVP) bovine blastocysts have darker cytoplasm and lower buoyant density (Pollard and Leibo, 1994) as a consequence of higher lipid content than their in vivo counterparts (Abd El Razek et al., 2000). Lipid accumulation is associated with embryo darkness (Sata et al., 1999; Abe and Hoshi, 2003; Leroy et al 2005c), impaired embryonic quality (Hill and Kuehner, 1998; Reis et al., 2003; Rizos et al., 2003), and reduced cryotolerance (Pollard and Leibo, 1993; Hill and Kuehner, 1998; Abe et al., 2002).

Cryotolerance of embryos can be improved by reduction of lipid droplet accumulation by microsurgery (Nagashima et al., 1994; Nagashima et al., 1995). We have previously reported increased embryonic cryosurvival (Barcelo-Fimbres and Seidel, 2007b) by partial reduction in lipid droplet accumulation by addition of the metabolic regulator phenazine ethosulphate during culture (De La Torre-Sanchez et al., 2006a; Barcelo-Fimbres and Seidel, 2007a); this indirect of PES occurs because it oxidizes NADPH, which is essential for fatty acid elongation. However, embryos treated with PES accumulated more lipids than in vivo-produced embryos (De La Torre-Sanchez et al., 2006a). Chemical stimulation of lipolysis using forskolin during culture has been

assessed in porcine embryos, improving embryonic cryotolerance (Men et al., 2006). However, they assessed lipolysis indirectly by glycerol release to the medium, but not by lipid reduction directly.

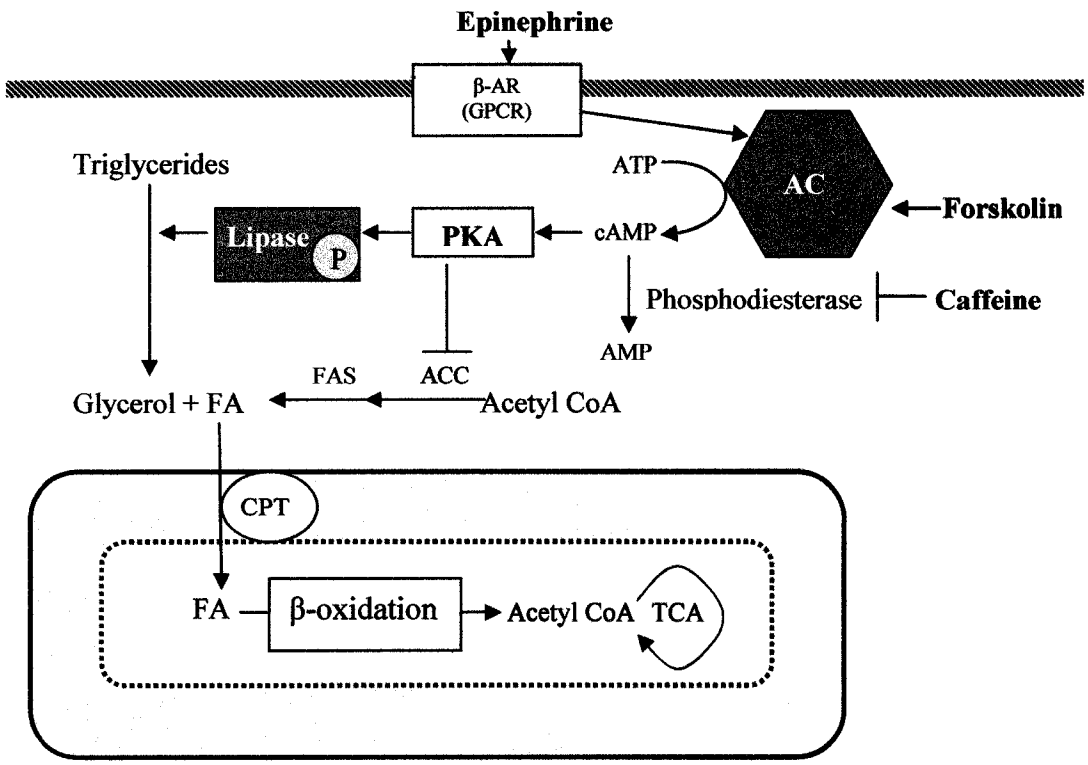
In mammalian cells a number of hormones regulate metabolism of lipids, including lipolytic agents such as catecholamines (epinephrine and norepinephrine), ACTH, and glucagon, and anti-lipolytic agents such as melatonin and insulin (Vaughan et al., 1964; Holm, 2003). Some chemicals are able to stimulate intracellular lipolysis of triacylglycerols by acting on different components of the lipolytic pathway: catecholamines and isoproterenol bind β-adrenergic receptors (β-AR) present in preimplantation embryos (Čikoš et al., 2005), coupled to adenylate cyclase (AC) via G-protein coupled receptor (GPCR) and increase production of cAMP and activation of PKA (Londos et al, 1999); forskolin activates AC directly (Seamon et al., 1981), and Xanthines (theophyline and caffeine) inhibit phosphodiesterases preventing cAMP degradation. So, the addition of epinephrine, forskolin or caffeine during in vitro culture may stimulate lipolysis and decreased lipid accumulation in embryos (Figure 8.1).

Any female is a potential donor of oocytes if antral follicles are present in the ovary. However, oocytes derived from prepubertal animals have poorer developmental competence in vitro than from mature cows (Majerus et al., 2000; Galli et al., 2003; Rizos et al., 2005). Heifers often are fattened in feedlots with high energy diets, frequently supplemented with the progestagen melengestrol acetate (MGA) to promote growth, with the additional benefit of artificial diestrus, which eliminates estrus

(Zimbelman and Smith, 1966; DeBois and Bierschwal, 1970; Imwalle et al., 1998). This could lead to poorer quality oocytes and subsequent development of embryos including more lipid accumulation in the oocytes, which remains as embryos develop in vitro. However, studies are required to study the effects of high energy diets and/or MGA feeding on oocytes, and ways to optimize the use of such ovaries.

The objective of these experiments was to evaluate lipid accumulation and embryonic development of bovine morulae and blastocysts treated with different chemicals, using ovaries from heifers and mature cows.

Figure 8.1. Lipolysis induced by epinephrine and forskolin activating adenylate cyclase (AC) and AMP-activated protein kinase (PKA), and caffeine inhibiting phosphodiesterases, maintaining high levels of cAMP, with further activation lipase and transport of fatty acids (FA) to the mitochondria by carnitine palmitoyltransferase (CPT) for oxidation. Lypogenesis is produced by FA synthase (FAS) and acetyl-CoA carboxylase (ACC).



8.3 Materials and Methods

8.3.1. Experimental Designs

Experiment 1: Evaluation of embryonic development and lipid accumulation in bovine blastocysts. A total of 2,943 oocytes were subjected to a two by three by four factorial experimental design with two ovary sources (cows vs. heifers), three additives (none (control), PES and 10%FCS) and semen from four bulls with oocytes from 2 days for each bull (A, B, C, and D). This experiment was thus replicated using 8 batches of ovaries (4 bulls, 2 days each).

Experiment 2: Evaluation of lipolytic agents on embryonic development and lipid accumulation in bovine blastocysts. A total of 2,619 oocytes were used to produce morulae and early blastocysts for this experiment. The experiment was replicated 6 times using semen from 3 bulls, twice for each bull. (Half of the batches of ovaries were from cows and the rest from heifers). At d 6 embryos were evaluated, and 755 morulae were randomly selected to be controls or exposed to different lipolytic agents (2 and 8 mM caffeine, 1 and 4 μM epinephrine, and 10 and 40 μM forskolin).

8.3.2. Embryo production

8.3.2.1 Oocyte Collection and In Vitro Maturation. Bovine ovaries from postpubertal heifers and mature cows were obtained from local abattoirs and transported to the laboratory within 3-4 h in 0.15 M NaCl at ambient temperature, approximately 22 to 25°C. After arriving at the laboratory, ovaries were trimmed of extraneous tissue, and

rinsed once again in 0.15 M NaCl. Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm antral follicles with an 18-gauge needle attached to a tubing system connected to a vacuum aspiration pump with 40 to 50 mm Hg pressure. Oocytes with at least 3 layers of unexpanded cumulus cells and morphologically bright and evenly granulated cytoplasm were selected for IVM. COCs were washed twice in chemically defined medium (CDM) with Hepes buffer for handling oocytes, and once with maturation medium (De La Torre-Sanchez et al., 2006b). Fifty COCs were matured per well of 4-well plates (Nunclon, Roskilde, Denmark) containing 1 ml of maturation medium CDM with 0.5% fatty acid-free BSA (Sigma A-6003, Lot # 063K7525) and gonadotropin hormones (National Protein and Peptide Program, Torrance, CA) (15 ng/ml of FSH (NIH-FSH-S17), 1μg/ml of LH (USDA-LH-B-5), 1.0 μg/ml of estradiol-17β (Sigma E-2257), 50 ng/ml EGF (Sigma E-9644), and 0.1mM cysteamine (Sigma M-6500). Oocytes were incubated at 38.5°C with humidified 5% CO_2 in air for 23 h. In the experiment 2, oocytes from mature cows were obtained from Bomed (Madison, WI) transported over night in the maturation medium described above at 38°C; maturation was completed in an incubator upon arrival.

8.3.2.2 Sperm Preparation. Frozen semen was from bulls of proven fertility, and at least 40 % progressive motile sperm after thawing. Straws of semen were thawed in water at 35°C for 30 sec. Semen from one straw was gently expelled into a 15-ml centrifuge tube and centrifuged 20 min at ~400 x g at 23°C through a Percoll gradient (Sigma P-1644) of 2 ml 90%: 2 ml 45% Percoll in sperm (sp)-TALP medium (modified Tyrode's) as described by Parrish et al. (1989). The supernatant was discarded, and the sperm pellet

(approximately 100 μL) was washed with 5 ml of chemically defined fertilization medium (Fert-CDM; De La Torre-Sanchez et al., 2006b), supplemented with 0.5% BSA, 5 mM caffeine (Sigma C-0750) and 2 μg/ml heparin (Sigma H-3125). The sample was centrifuged again for 5 min at 400 x g at 23°C, and the supernatant was discarded. Approximately 90 to 100 μl remained, and a 5-μl aliquot was taken to determine the sperm concentration with a hemacytometer. The sperm concentration was adjusted to 5 x 10^6 spermatozoa per ml to give a final concentration of 0.5 x 10^6 spermatozoa per ml in Fert-CDM.

8.3.2.3. In Vitro Fertilization. Following in vitro maturation, ~50 oocytes were placed in 450 μl of Fert-CDM medium per well of 4-well dishes, and 50 μl of sperm suspension were added to give final volume of 500 μl per well. Gametes were co-incubated for 18 h at 38.5°C in an atmosphere of humidified 5% CO_2 in air.

8.3.2.4. Culture of Embryos. Following in vitro fertilization, presumptive zygotes were removed from wells and transferred to 1.0-ml microcentrifuge tubes with approximately 100 μl of Fert-CDM (De La Torre-Sanchez et al., 2006b), and then vortexed for 50 to 60 sec to remove cumulus cells. These procedures were carried out in a room at 28°C. Embryos then were rinsed three times in Hepes CDM-1 (H-CDM-1). Early culture (day 0 to 2.5 post fertilization) was done in a new 4-well dish, containing 500 μl of CDM supplemented with 0.5% fatty acid-free BSA, 0.5 mM fructose, non-essential amino acids (NEAA), and 10 μM EDTA (CDM-1) as described by De La Torre-Sanchez et al. (2006b), and incubated at 39°C under 90% N_2, 5% O_2, and 5% CO_2. After 60 h of

culture, embryos were examined with a stereomicroscope (15 to 20×) for cleavage, and all uncleaved ova and embryos less than 8 cells were discarded. The rest were cultured in new dishes with CDM-2 (CDM supplemented with 0.5% fatty acid-free BSA, NEAA and essential amino acids and 2 mM of fructose) from day 2.5 to day 7 post fertilization. For experiment 2, after 3 days in CDM-2 morulae were placed randomly in the different treatments. Embryos were evaluated 168 h after the end of fertilization to determine the final stage of development.

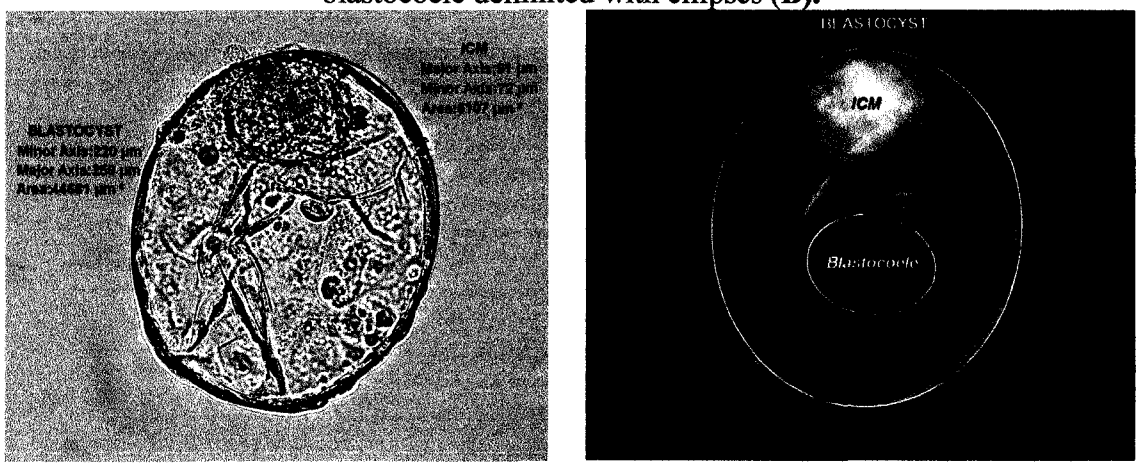
8.3.2.5. Addition of Metabolic Regulators. For experiment 1, a 2000x stock of PES (Sigma, P-4544) was prepared in nanopure water; then a 100x stock was made in HCDM-2. This stock was stored at 4°C and added to CDM-2 at the time of medium equilibration. The final concentration of PES was 0.3 μM; 10% FCS (Gemini Bio-Products Inc., West Sacramento, CA, lot# A20206P) was considered as a negative control. PES concentration was based on the dose-response studies of De La Torre-Sanchez et al. (2006b). For experiment 2, a 200 mM stock of caffeine (Sigma C-0750) and 100 mM epinephrine (Sigma E-4642) were prepared in nanopure water; a 100 mM stock of forskolin (Biosciences, CA, Cat No. 34420) was prepared in DMSO. These stocks were stored at -20°C and added to CDM-2 at the time of medium equilibration. Doses of chemicals to regulate lipolysis were chosen based on previous work in porcine embryos (Men et al., 2006); 10 μM forskolin, 2 mM caffeine and 1 μM epinephrine have been used during in vitro fertilization procedures; we also studied 4X the base dose. The final concentrations of the lipolytic agents thus were: 2 and 8 mM caffeine; 1 and 4 μM epinephrine; and 10 and 40 μM forskolin.

8.3.2.6. Quantification of lipid accumulation

To quantify lipid accumulation, morulae and blastocysts were fixed in 10% formalin in modified phosphate-buffered saline (mPBS; Elsden and Seidel, 1995) plus 0.01% PVA at pH 7.4 for one day, and then stained overnight in 500 μL of a 1 μg/mL Nile red dye (Sigma, N3013) solution dissolved in mPBS (dose based on work done by Genicot et al., 2005). Slides then were rinsed twice in mPBS and mounted on a slide with glycerol. Finally, a cover slip with paraffin-vaseline was placed on the slide without pressing the embryo. A stock solution of 100 μg/mL Nile red was made in DMSO and stored at room temperature; the stock was diluted in mPBS plus 0.01% PVA to get a final concentration of 1 μg/mL. This solutions and slides were stored in the dark.

An epifluorescence microscope (Nikon E-800) was used to excite the lipophilic fluorescent dye Nile red (590 nm), capturing a digital photograph of the equatorial part of the morulae or blastocyst (including the inner cell mass, ICM) using a 20 X objective. Fluorescence intensity (arbitrary fluorescence units, AFU) was measured with computer software Image Pro from 0 to 255 shades for each pixel (0=no lipids; 255=greatest lipid accumulation). The AFU were adjusted by background to compensate for variation of the UV lamp intensity. The area of all morulae and blastocysts was measured, and the fluorescence was adjusted to a 15,000 μm^2 basis (which is equivalent to an embryo of ~138 μm of diameter). Lipid content of the embryonic mass (EM) and blastocoele was obtained measuring AFU in equal size areas in the embryo (Figure 8.2 A, B). Lipid accumulation for the blastocoel and embryonic mass, as well as their difference were evaluated.

Figure 8.2. Light photomicrograph showing embryo and inner cell mass (ICM) areas. (**A**); Fluorescence photomicrograph measuring intensity of whole embryo, ICM and blastocoele delimited with ellipses (**B**).



A B

8.3.2.7. Evaluation of Embryos

Embryonic development was evaluated in both experiments; cleavage rates were assessed 2.5 days after being placed in CDM-1, after which embryos at the 8-cell stage were placed into culture into CDM-2; 2- to 6-cell embryos were considered cleaved, but were not cultured further. Morulae rates were evaluated at day 6 in experiment 2. The percentages of development to blastocysts and hatched state were evaluated 7 d after fertilization. For experiment 1, 7 d blastocysts were classified by stage: early (< 50 % blastocoele), blastocyst (>50 % blastocoele to early expanded), and expanded (expanded and hatched blastocysts). In both experiments, blastocyst stage development was also scored (5=early, 6=full, 7=expanded, 8=hatched).

8.3.3. Statistical procedures

Percentages of embryonic development during in vitro production (cleavage, morula, blastocyst and hatched rates) were transformed using arcsin square root. Transformed

data, fluorescence evaluations and embryo stage were then analyzed by ANOVA using the SAS statistical software package general linear model (GLM). Homogeneity of variance and normality were checked by Levene's test and plotting studentized residuals vs. predicted values. Sources of variation in the model included all main effects (all considered as fixed effects), all possible 2 way interactions, and residual error. If the ANOVA was significant ($P < 0.05$), means were separated by Tukey's w procedure. Data are reported as untransformed least-squares means.

8.4. Results

8.4.1. Embryonic development

In experiment 1, oocytes derived from heifers did not differ in cleavage and eight cell rate from those derived from mature cows ($P > 0.1$). However, 19% more blastocysts were produced per cow oocyte (4.2 percentage points) and 16% more per cow eight cell embryo (5.9 percentage points) than heifers ($P < 0.01$). No differences in stage and hatched rate were found due to ovary source ($P > 0.1$) (Table 8.1).

In experiment 2, oocytes derived from heifers did not differ in cleavage, eight cell, morulae per oocyte and morulae per eight cell rates from those derived from mature cows ($P > 0.1$) (Table 8.2). However, 101% more blastocysts were produced per cow oocyte (10.3 percentage points) and 95% more per cow eight cell embryo (33 percentage points) than heifer ova ($P < 0.01$) (Table 8.2). Differences in blastocyst stage were found ($P < 0.05$), where cow-derived blastocysts were more advanced than heifer blastocysts (Table 8.3).

There was no bull effect for cleavage and eight cell rate in experiment 1 ($P > 0.1$). However bull B had highest and D the lowest blastocysts per oocyte ($P < 0.05$), but bull A and C were not different from other bulls ($P > 0.1$). Bull B produced more blastocysts per eight cell embryo than D ($P < 0.05$), but both were not different from bulls A and C ($P > 0.1$). No differences in stage and hatched rate were found for sires ($P > 0.1$) (Table 8.1). In experiment 2, there was no bull effect for cleavage, eight cell, morulae per oocyte and per eight cell rates ($P > 0.1$). However bull C had higher blastocysts per oocyte and per eight cell than B ($P < 0.05$), but bull A was not different from bulls B and C ($P > 0.1$) (Table 8.2). Note that bull A in experiment 1 is not true the same as bull A in experiment 2.

For culture treatments in experiment 1, no differences in blastocysts per oocyte and eight cell were found ($P > 0.1$). However, PES-treated blastocysts and controls were more advanced in development than FCS-treated embryos ($P < 0.1$) (Table 8.1). No differences in hatched rate were found for treatments ($P > 0.1$) (Table 8.1). No significant interaction was found for any response ($P > 0.1$). The addition of lipolytic agents at the morula stage in experiment 2, was not detrimental for blastocyst development, with exception of caffeine, for which the low dose had a numerical but not significant ($P > 0.1$) decrease in development; However, the higher dose was very detrimental for embryos ($P < 0.01$); those blastocysts that survived the treatment had retarded development ($P < 0.01$); however, the lower dose of caffeine was not different to the control ($P > 0.1$) (Table 8.3).

Table 8.1. Main effect treatment least-squares means of ovary source, bull, and treatment on embryonic development of bovine embryos *in vitro* (±SE)-Experiment 1.

RESPONSES	No. oocytes	Cleaved (%)	Eight cell rate (%)	Blastocysts per oocyte (%)	Blastocysts per eight cell (%)	Blastocyst Stage (5:early... 8:expanded)	Hatched rate (%)
Ovary source							
Heifer	1914	72.5 ± 2.9	58.2 ± 2.5	21.8 ± 2.0 [a]	37.4 ± 2.1 [a]	6.4 ± 0.1	14.1 ± 2.4
Cow	1029	78.2 ± 2.9	59.5 ± 2.5	26.0 ± 2.0 [b]	43.3 ± 2.1 [b]	6.4 ± 0.1	11.9 ± 2.4
Bull							
A	605	71.4 ± 1.4	60.0 ± 3.6	24.5 ± 1.4 [b]	41.2 ± 2.5 [ab]	6.4 ± 0.1	9.6 ± 3.3
B	997	78.5 ± 1.7	64.9 ± 3.6	30.0 ± 1.4 [a]	46.7 ± 2.5 [a]	6.3 ± 0.1	10.1 ± 3.3
C	604	75.4 ± 1.8	59.8 ± 3.6	23.0 ± 1.4 [b]	38.3 ± 2.5 [ab]	6.4 ± 0.1	17.6 ± 3.3
D	737	76.2 ± 2.8	50.6 ± 3.6	17.8 ± 1.4 [c]	35.1 ± 2.5 [b]	6.4 ± 0.1	15.0 ± 3.3
Treatment							
Control	984	--	--	23.2 ± 1.3	39.3 ± 2.5	6.4 ± 0.1 [d]	13.3 ± 2.9
PES	973	--	--	25.0 ± 1.3	42.3 ± 2.5	6.5 ± 0.1 [d]	15.9 ± 2.9
FCS	986	--	--	23.2 ± 1.3	39.4 ± 2.5	6.2 ± 0.1 [e]	10.0 ± 2.9

Values without common superscripts in the same column differ ([a,b,c] $P < 0.05$; [d,e] $P < 0.01$).
(--) No data because treatments were added at the 8-cell embryo stage.

Table 8.2. Main effect least-squares means of embryonic development of bovine embryos *in vitro* (±SE)-Experiment 2.

FACTORS	No. oocytes	Cleaved (%)	Eight cell rate (%)	Morulae per oocyte (%)	Morulae per eight cell (%)	Blastocysts per oocyte (%)	Blastocysts per eight cell (%)
Ovary source							
Heifer	1581	74.7 ± 3.6	60.5 ± 2.8	28.2 ± 0.8	46.5 ± 3.0	10.1 ± 2.1 [a]	34.7 ± 3.8 [a]
Cow	1038	67.8 ± 3.6	56.6 ± 2.8	30.3 ± 0.8	53.8 ± 3.0	20.4 ± 2.1 [b]	67.7 ± 3.8 [b]
Bull							
A	854	68.1 ± 4.4	53.4 ± 3.5	28.2± 1.0	53.3 ± 3.8	15.0 ± 1.4 [a,b]	51.3 ± 4.5 [a,b]
B	901	65.8 ± 4.4	54.6 ± 3.5	26.6 ± 1.0	48.8 ± 3.8	11.0 ± 1.4 [a]	40.0 ± 4.5 [a]
C	864	79.9 ± 4.4	67.8 ± 3.5	33.1 ± 1.0	48.9 ± 3.8	20.4 ± 1.4 [b]	62.4 ± 4.5 [b]

Values without common superscripts in the same column differ ([a,b] $P < 0.05$).

Table 8.3. Main effect least-squares means of embryonic development of bovine embryos *in vitro* (±SE)-Experiment 2.

FACTORS	No. Morulae	Blastocysts per morula (%)	Blastocyst Stage (5:early...8:expanded)
Ovary source			
Heifer	444	30.9 ± 3.1^{a}	5.6 ± 0.1^{a}
Cow	311	55.0 ± 3.1^{b}	6.0 ± 0.1^{b}
Treatments			
Control	106	51.6 ± 5.8^{a}	$5.8 \pm 0.1^{a,b}$
Caffeine 2 mM	108	38.8 ± 5.8^{a}	5.6 ± 0.1^{b}
Caffeine 8 mM	109	4.5 ± 5.8^{b}	5.0 ± 0.4^{c}
Epinephrine 1 μM	107	53.7 ± 5.8^{a}	6.0 ± 0.1^{a}
Epinephrine 4 μM	106	49.4 ± 5.8^{a}	6.0 ± 0.1^{a}
Forskolin 10 μM	109	48.2 ± 5.8^{a}	5.9 ± 0.1^{a}
Forskolin 40 μM	110	54.2 ± 5.8^{a}	6.1 ± 0.1^{a}

Values without common superscripts in the same column differ (^{a,b,c} $P < 0.01$).

8.4.2. Lipid content

In experiment 1, no differences were found in lipid accumulation between cow and heifer-derived blastocysts for whole embryo or trophoblast ($P > 0.1$); however, a higher lipid accumulation in the embryonic mass was found in cow than heifer-derived blastocysts ($P < 0.01$), which was reflected in a higher embryonic mass-trophoblast difference ($P < 0.01$) Table 8.4. For culture treatments, FCS had more lipid content, and PES-treated blastocysts had less lipid than controls ($P < 0.01$) (Table 8.4, Figure 8.5). Early blastocysts resulted in higher lipid content, and expanded had less than the middle blastocyst stage ($P < 0.01$) (Table 8.4).

There were significant interactions ($P < 0.01$) between treatments and blastocyst stages for whole embryo and embryonic mass; FCS-treated embryos had higher lipid content than PES and control groups for early blastocyst, but this difference was less at true expanded blastocyst satge for whole embryos (Table 8.5, Figure 8.3). Also, there

were significant interactions ($P < 0.01$) between ovary source and blastocyst stages for embryonic mass and embryonic mass-trophoblast difference, because cow blastocysts had higher lipid content than heifers for early blastocyst and blastocyst stages, a difference that diminished greatly by the expanded blastocysyt stage (Table 8.6, Figure 8.4).

Table 8.4. Main effect treatment least-squares means (±SE) of lipid accumulation (arbitrary fluorescence units) of blastocysts produced *in vitro* according to ovary source, treatment and stage.-Experiment 1.

RESPONSES	No. blastocysts	Whole Embryo	Embryonic Mass	Trophoblast	Difference
Ovary source					
Cow	192	46.1± 1.2	94.6 ± 2.1 [a]	35.0 ± 1.3	59.7 ± 1.7 [a]
Heifer	390	44.3± 1.8	72.7 ± 1.4 [b]	37.7 ± 0.9	35.0 ± 1.2 [b]
Treatment					
Control	203	44.5 ± 1.8 [a]	85.2 ± 2.1 [a]	36.5 ± 1.3 [a]	48.7 ± 1.7 [a]
FCS	201	64.4 ± 1.9 [b]	107.4 ± 2.2 [b]	52.5 ± 1.3 [b]	54.9 ± 1.8 [b]
PES	178	23.8 ± 2.0 [c]	58.4 ± 2.3 [c]	19.9 ± 1.4 [c]	38.5 ± 1.9 [c]
Stage					
Early blastocyst	159	58.4 ± 2.0 [a]	92.1 ± 2.4 [c]	39.7 ± 1.4 [c]	52.4 ± 1.9 [a]
Blastocyst	139	47.7 ± 2.2 [b]	84.7 ± 2.6 [d]	36.4 ± 1.6 [c]	48.3 ± 2.1 [a]
Expanded blastocyst	284	26.5 ± 1.4 [c]	74.2 ± 1.7 [e]	32.7 ± 1.0 [d]	41.4 ± 1.3 [b]

Values without common superscripts in the same column differ ([a,b,c] $P < 0.01$; [c,d,e] $P < 0.05$).

Table 8.5. 2-way least-squares means (±SE) of treatment and blastocyst stage for lipid accumulation (arbitrary fluorescence units) of bovine embryos in vitro -Experiment 1.

FACTORS		No.		
Treatment	**Stage**	**Blastocysts**	**Whole embryo**	**Embryonic mass**
Control	Early blastocyst	48	61.5 ± 3.4 [b]	96.3 ± 3.9 [b,c]
Control	Blastocyst	47	47.1 ± 3.6 [c]	87.8 ± 4.2 [c]
Control	Expanded blastocyst	108	24.9 ± 2.3 [d]	71.5 ± 2.7 [d]
FCS	Early blastocyst	74	82.3 ± 2.9 [a]	117.1 ± 3.4 [a]
FCS	Blastocyst	39	66.9 ± 3.9 [b]	102.1 ± 4.6 [b]
FCS	Expanded blastocyst	88	44.0 ± 2.5 [c]	103.1 ± 3.0 [b]
PES	Early blastocyst	37	31.6 ± 2.5 [d]	63.0 ± 4.7 [d]
PES	Blastocyst	53	29.2 ± 3.4 [d]	64.2 ± 3.9 [d]
PES	Expanded blastocyst	88	10.6 ± 4.0 [e]	48.1 ± 2.9 [e]

[a,b,c,d,e] Values without common superscripts in the same column differ ($P < 0.05$).

Figure 8.3. Interaction least-squares means of treatment and stage (1=early blastocyst, 2=blastocyst, 3=expanded blastocyst) for lipid accumulation of bovine embryos in vitro (±SE) Experiment 1.

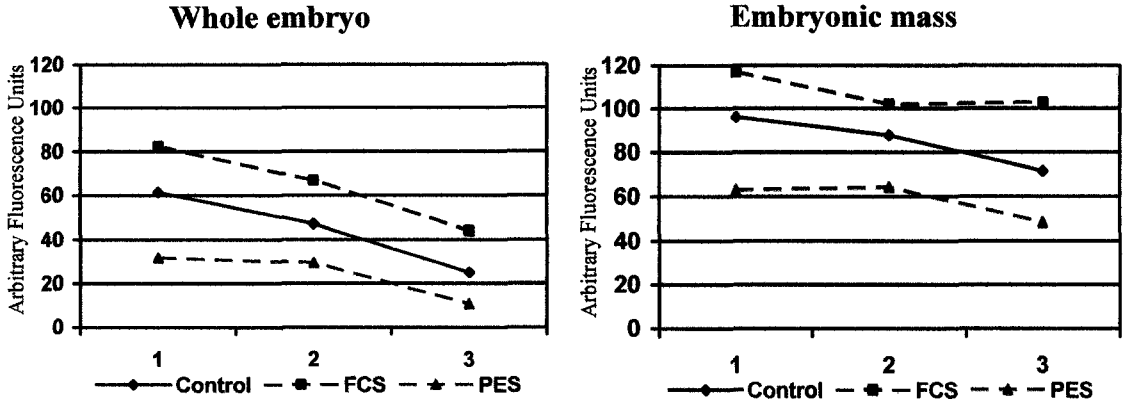


Table 8.6.2-way least-squares means (±SE) of ovary source and blastocyst stage for lipid accumulation (arbitrary fluorescence units) of bovine embryos in vitro. Experiment 1.

FACTORS		No.		
Source	**Stage**	**Blastocysts**	**Embryonic mass**	**ET difference**
Cow	Early blastocyst	53	106.9 ± 3.8 [a]	68.7 ± 3.1 [a]
Cow	Blastocyst	37	98.8 ± 4.5 [a]	63.2 ± 3.6 [a]
Cow	Expanded blastocyst	102	78.2 ± 2.7 [b]	47.1 ± 2.2 [b]
Heifer	Early blastocyst	106	77.3 ± 2.7 [b]	36.1 ± 2.2 [c]
Heifer	Blastocyst	102	70.6 ± 2.7 [c]	33.3 ± 2.2 [c]
Heifer	Expanded blastocyst	182	70.2 ± 2.0 [c]	35.7 ± 1.6 [c]

[a,b,c] Values without common superscripts in the same column differ (P <0.05).

Figure 8.4. Interaction least-squares means of ovary source and stage (1=early blastocyst, 2=blastocyst, 3=expanded blastocyst) for lipid accumulation of bovine embryos in vitro (±SE) Experiment 1.

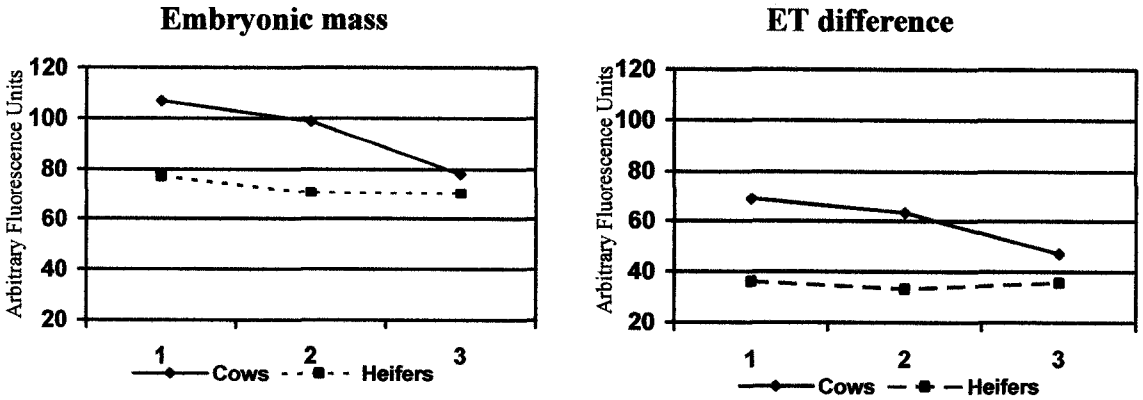
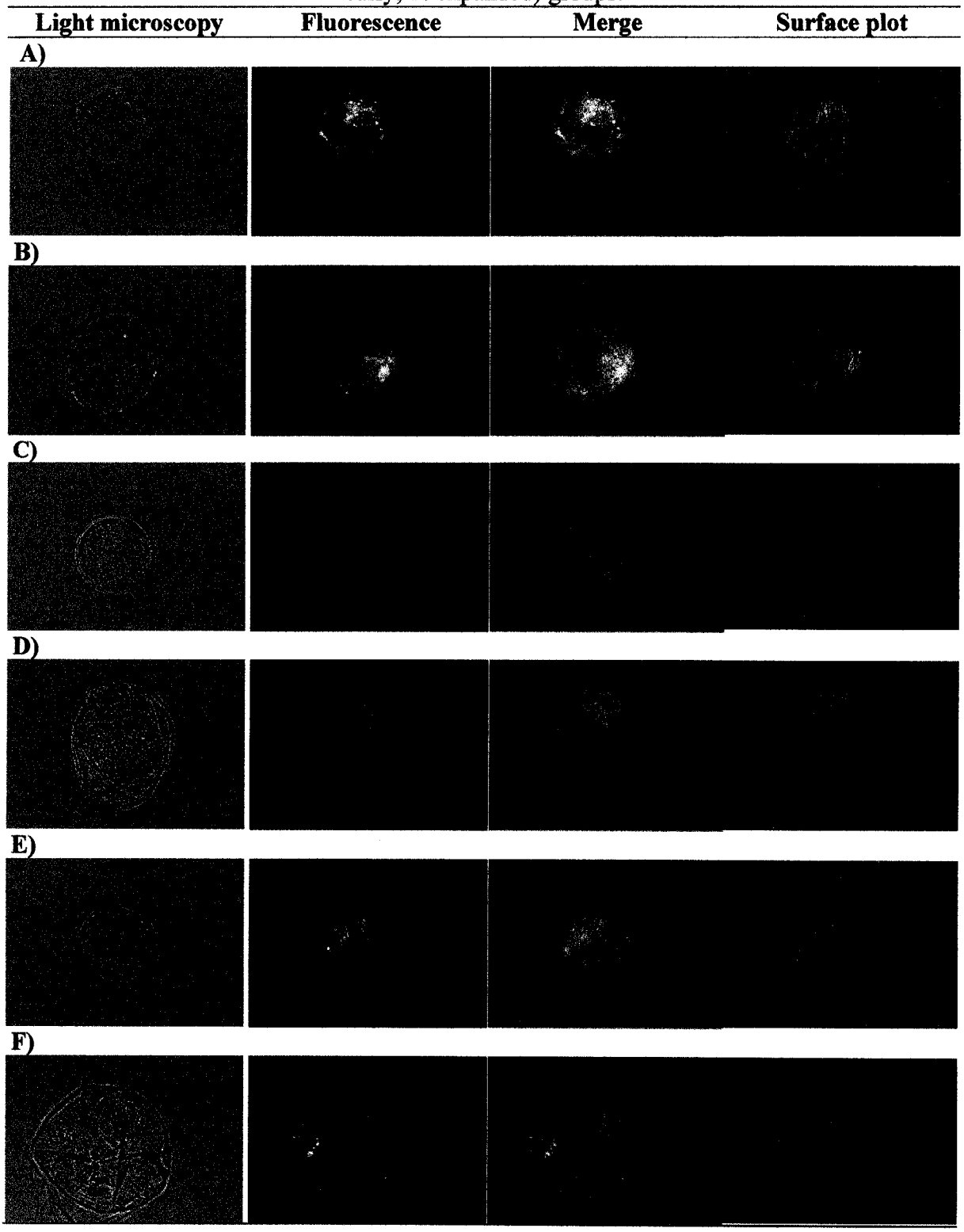


Figure 8.5. Photographs of blastocysts by light microscopy, fluorescence, merge and surface plot of control (A: early; B: expanded), PES (C: early; D: expanded), and FCS (E: early; F: expanded) groups.



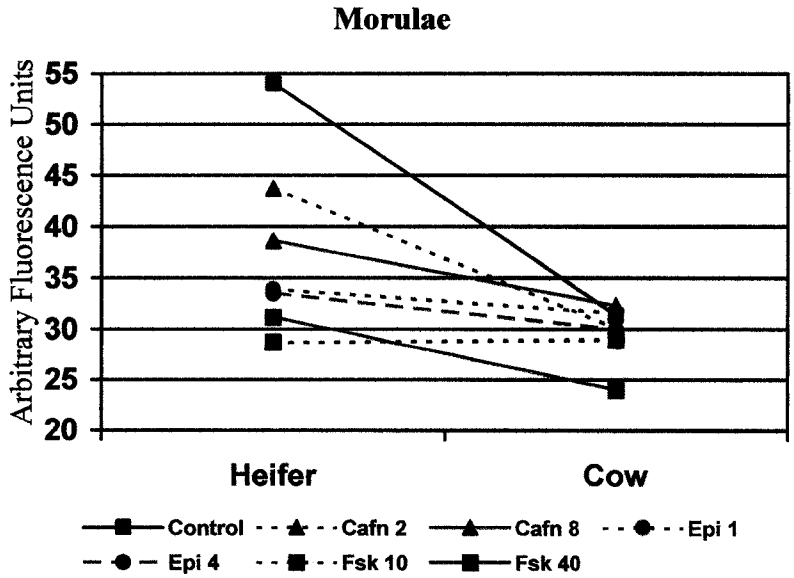
In experiment 2, there was a significant interaction ($P < 0.01$) between treatments and ovary source of morulae; heifer-derived morulae had higher lipid content for the control, and cow derived morulae had the least lipid content for forskolin 40 μM (8.7, Figure 8.6).

Table 8.7. 2-way least-squares means of ovary source and blastocyst stage for lipid accumulation of bovine morulae in vitro (±SE)-Experiment 2.

FACTORS			
Source	**Treatment**	**No. Morulae**	**Arbitrary fluorescence units**
Cow	Control	15	$31.4 \pm 2.8^{d,e}$
Cow	Caffeine 2 mM	20	$30.8 \pm 2.4^{d,e,f}$
Cow	Caffeine 8 mM	45	$32.3 \pm 2.6^{c,e}$
Cow	Epinephrine 1 μM	13	$31.9 \pm 3.0^{d,e}$
Cow	Epinephrine 4 μM	13	$30.2 \pm 3.0^{d,e,f}$
Cow	Forskolin 10 μM	12	$30.0 \pm 3.0^{d,e,f}$
Cow	Forskolin 40 μM	12	24.0 ± 3.1^{f}
Heifer	Control	29	54.8 ± 2.0^{a}
Heifer	Caffeine 2 mM	40	43.7 ± 1.7^{b}
Heifer	Caffeine 8 mM	58	38.5 ± 1.4^{c}
Heifer	Epinephrine 1 μM	36	33.9 ± 1.8^{d}
Heifer	Epinephrine 4 μM	38	33.5 ± 1.8^{d}
Heifer	Forskolin 10 μM	35	$28.7 \pm 1.8^{e,f}$
Heifer	Forskolin 40 μM	36	$31.2 \pm 1.8^{d,e}$

^{a,b,c,d,e,f} Values without common superscripts in the same column differ ($P < 0.05$).

Figure 8.6. Interaction least-squares means (±SE) of ovary source (heifer vs. cow) and treatments (control; Caffeine 2 mM, Cafn 2; Caffeine 8 mM, Cafn 8; Epinephrine 1 μM, Epi 1; Epinephrine 4 μM, Epi 4; Forskolin 10 μM, Fsk 10; Forskolin 10 μM, Fsk 10;) for lipid accumulation of bovine morulae in vitro.-Experiment 2.



In experiment 2, differences were found in lipid accumulation between cow and heifer-derived blastocysts for whole embryo and trophoblast ($P < 0.01$); however, embryonic mass and the embryonic mass-trophoblast difference did not differ ($P > 0.1$) (Table 8.8). Early blastocysts resulted in higher lipid content, and expanded had less than the middle blastocyst stage for trophoblast ($P < 0.01$), but were not different for whole embryos or embryonic mass ($P > 0.1$) (Table 8.8). There was no effect of blastocyst stage on the embryonic mass-trophoblast difference ($P > 0.1$).

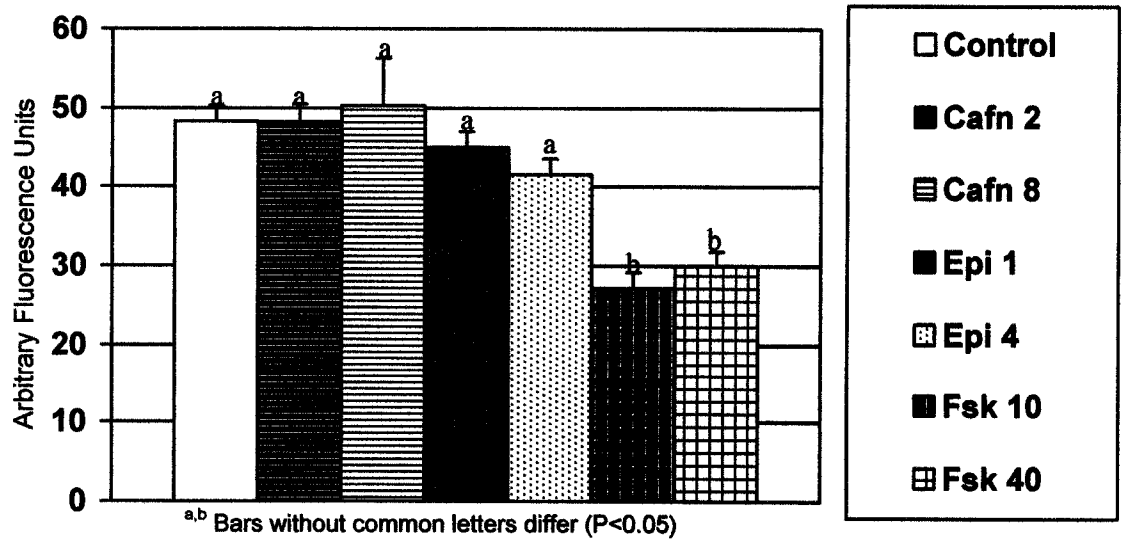
For lipolytic culture treatments, forskolin resulted in less lipid content than control, epinephrine and caffeine treatments for whole embryo, embryonic mass and trophoblast ($P < 0.01$) (Table 8.8, Figure 8.7). However, embryonic mass-trophoblast difference was not different between treatments ($P > 0.1$) (Table 8.8).

Table 8.8. Main effect treatment least-squares means (±SE) of lipid accumulation (arbitrary fluorescence units) of morulae produced *in vitro* according to ovary source and treatments.-Experiment 2.

RESPONSES	No. blastocysts	Whole Embryo	Embryonic Mass	Trophoblast	Difference
Ovary source					
Cow	140	36.7± 1.4 [a]	94.3 ± 2.0	35.9 ± 1.1 [a]	58.4 ± 1.8
Heifer	173	46.5 ± 1.5 [b]	94.8 ± 2.2	39.6 ± 1.2 [b]	55.2 ± 2.0
Stage					
Early blastocyst	150	48.6 ± 1.4 [c]	98.7 ± 2.0 [a]	39.9 ± 1.1 [a]	59.8 ± 1.8
Blastocyst	86	45.8± 1.7 [c,d]	94.2 ± 2.5 [a,b]	38.8 ± 1.3 [a]	54.3 ± 2.2
Expanded blastocyst	77	30.5 ± 2.0 [d]	90.7 ± 2.8 [b]	34.4 ± 1.5 [b]	56.3 ± 2.5
Treatment					
Control	53	48.3 ± 2.0 [a]	97.7 ± 2.9 [c]	41.8 ± 1.5 [c]	55.9 ± 2.6
Caffeine 2 mM	41	48.2 ± 2.3 [a]	99.0 ± 3.3 [c]	41.3 ± 1.8 [c]	57.7 ± 3.0
Caffeine 8 mM	5	50.3 ± 6.4 [a]	102.0 ± 6.0 [c]	39.9 ± 4.7 [c]	62.5 ± 8.3
Epinephrine 1 mM	54	45.0 ± 2.0 [a]	104.0± 2.8 [c]	42.2 ± 1.5 [c]	61.9 ± 2.5
Epinephrine 4 mM	49	41.5 ± 2.0 [a]	94.9 ± 3.0 [c]	40.6 ± 1.6 [c]	54.3 ± 2.7
Forskolin 10 µM	51	27.2 ± 2.0 [b]	77.9 ± 2.0 [d]	28.0 ± 1.6 [d]	50.0 ± 2.6
Forskolin 40 µM	60	30.0 ± 1.8 [b]	85.8 ± 2.7 [d]	30.3 ± 1.4 [d]	55.5 ± 2.4

Values without common superscripts in the same column differ ([a,b,c] $P < 0.01$; [c,d] $P < 0.05$).

Figure 8.7. Least-squares means ± SEM for lipolytic medium treatments (control; Caffeine 2 mM, Cafn 2; Caffeine 8 mM, Cafn 8; Epinephrine 1 µM, Epi 1; Epinephrine 4 µM, Epi 4; Forskolin 10 µM, Fsk 10; Forskolin 10 µgM, Fsk 10;) for lipid content of whole bovine blastocyst in vitro.-Experiment 2.



8.5. DISCUSSION

8.5.1. Developmental competence of oocytes derived from cows and heifers

A variety of studies comparing calf vs. cow oocytes for in vitro development, have found that oocytes of calves are less developmentally competent than those from cows, but few studies are done to relate differences between older heifers and cow-derived oocytes. However, these studies in calves could related to differences between heifer and cow-derived oocytes.

Oocytes derived from calves have structural and metabolic differences including smaller diameter (Duby et al., 1996; Gandolfi et al., 1998; Steeves and Gardner, 1999); more microvilli and endocytic vesicles and fewer mitochondria (de Paz et al., 2001); differences in protein synthesis (Gandolfi et al., 1998; Levesque and Sirard, 1994; Salamone et al., 2001); and lower metabolic rates (Gandolfi et al., 1998). Also oocytes derived from calves show differences in IVP: slower rates of nuclear maturation (Khatir et al., 1998); incomplete cytoplasmic maturation with defects in cortical granule distribution and calcium profile after IVM (Duby et al., 1996); slower development in vitro (Majerus et al., 2000); less development to blastocyst from parthenotes, IVF and nuclear transfer techniques (Levesque and Sirard, 1994; Damiani et al., 1996; Revel et al., 1995; Khatir et al., 1996; Majerus et al., 2000; and Salamone et al., 2001), and lower ability to maintain pregnancy after transfer (Revel et al., 1995; Fry et al., 1998; Brogliatti et al., 1999).

In the present work, we found no differences in fertilization, cleavage, and eight cell rates in both experiments, and morula production in experiment 2 between cow and heifer-derived oocytes; however, although the blastocyst yield was higher for cow-derived oocytes. No differences in fertilization suggest there are differences in of oocyte structures like zona pellucida and cortical granules for capacity of fertilization, although such defects were reported in calves (Duby et al., 1996).

Zhang et al. (1991), also reported no differences in maturation, fertilization, and cleavage rates (93, 78.3, and 60.6% vs. 94.3, 77.3, and 54.7%, respectively) using cows or heifer-derived oocytes from mixed dairy and beef animals; however, more cow than heifer-derived oocytes development to blastocysts (27.5 and 16.4 %, respectively). Similarly, another report found similar cleavage rates (80.8 vs. 79.9%), and higher blastocyst rates at day 7 (42.6 vs. 29%) and 8 (46.5 vs. 33.4%) ($P < 0.01$), in cow than heifer-derived oocytes using crossbred beef animals (Rizos et al., 2005).

Using slaughterhouse derived oocytes, Checura et al. (2002) found that fertilization, cleavage, eight cell and blastocyst rates were all higher for cow than heifer-derived oocytes, which differs from our findings. In the same way, Galli et al. (2003), using ovaries from a BSE-infected cow herd, found higher cleavage and blastocyst rates from cow than heifer-derived oocytes (68.6 and 21.8% vs. 62.7 and 15.6%, respectively). Rizos et al. (2005) reported no differences in developmental ability in oocytes from dairy heifers vs. postpartum lactating cows. However, low number of oocytes were used and low blastocyst rates were found in both groups at d 7 (12.4 vs. 8.1%, respectively); also,

postpartum lactating cows may have negative energy balance, which could affect oocyte quality (Wilbank et al., 2002), oocyte competence, and reduction of class 1 follicles of 3-5 mm (Lucy et al., 1991).

With oocytes obtained by oocyte pick up, no significant differences in the percentage of oocytes cleaving after fertilization, or in the percentage reaching the blastocyst stage were found between heifers and cows (Rizos et al., 2005). Howerver, Galli et al. (2001) using cows, heifers (9 mo) and calves (2-8 mo) found higher transferable and freezable embryos per cleaved ovum in cows than in heifers and calves. In another report, cow-derived oocytes produced higher cleavage rates and more transferable embryos per aspiration than those from heifers (71%, and 2.8 vs. 67%, and 1.5, respectively) (Galli et al., 2003).

From reviewing research done in bovine nuclear transfer (NT), there are two reports using calf-derived oocytes, where lower 2-cell and blastocyst rates of NT embryos were obtained compared to cow-derived oocytes (Mermillod et al., 1998; Salamone et al., 2001). Similarly, oocytes derived from cows had greater capacity to reprogram donor cell DNA following NT compared to heifer oocytes based on in vitro development from the 2-cell stage to morula/blastocyst stages, and pregnancy rates (Aston et al., 2006).

We did not find differences in stage of development of blastocysts originating from heifer vs. cow-derived oocytes. Likewise, there is a report where grade-one

blastocysts developed at a similar rate (14.6 and 10.2 %) from cow as heifer-derived oocytes (Galli et al., 2003). We observed a numerical increased in hatching for cow derived oocytes and PES treatment, although this was not significant ($P > 0.1$). Nevertheless, there is a report where more blastocysts hatched from cow than heifer-derived oocytes (22 vs. 8.6%) (Zhang et al., 1991).

8.5.2. Molecular differences of competence of oocytes derived from cows and heifers

It takes approximately 180 d for a primordial, and 80 d for an antral follicle to reach the preovulatory stage (Campbell et al., 1995). However, oocyte competence is acquired during oocyte growth at a diameter of about 110 μm, and concomitant with follicle development (Hyttel et al 1997); competence consists in acquiring the ability to be fertilized and develop to the blastocyst stage (Eppig et al., 2002, Matzuk et al., 2002). It is hypothesized that prepubertal calf oocytes are defective in oocyte capacitation, because previous studied have shown that aspirating 2-5 mm follicles from ovaries leads to a lower oocyte diameter in calves (112-119 μm) than cows (117-123 μm), and oocyte diameter may be related in the capacity of oocytes to reach the metaphase II stage and subsequent development (Fair et al. 1995; Otoi et al., 1997). Rizos et al. (2005) tested the influence of the postfertilization environment of heifer and cow presumptive zygotes in vivo using the ewe oviduct; interestingly, more cow than heifer-derived oocytes reached the blastocyst stage (53.1 vs. 25.2%, respectively), so this supports that the theory of oocyte competence due to intrinsic oocyte quality. Gonadotropin-stimulated prepubertal animals exhibit higher cleavage and blastocyst development in dairy (Presicce et al., 1997) and beef heifers (Lu et al., 1991) than unstimulated heifers. Also, Galli et al.

(2001) reported an increase in transferable and freezable embryos using OPU oocytes from calves (2-3 mo) stimulated with eCG in vs. non-treated control. This could be a good approach for OPU, but is not relevant for slaughter-derived oocytes. Nevertheless, oocyte competence after gonadotropin priming is still lower than for mature cows (Presicce et al., 1997).

Looney et al. (1995) reported that stimulated heifers under 240 d of age produced 0% blastocysts vs. 19% blastocysts from similarly stimulated heifers over 240 d of age. There is evidence of gradual age-dependent oocyte competence acquisition in cattle oocytes. Gradual increase of cleavage and blastocyst rates was observed in OPU-derived oocytes of prepubertal dairy cattle from 5 to 11 mo of age (Presicce et al., 1997). However in another report with slaughterhouse beef heifer ovaries, separated by heifer ages (12-18, 19-24, and 25-30 months), no differences in cleavage or blastocyst rates were observed between the three age groups, but cow-derived oocytes produced more blastocysts at d 8 (Rizos et al., 2005).

In terms of gene expression, from a microarray experiment using mRNA from germinal vesicle stage oocytes from adult vs. 24 week old calves (model of poor development), 193 genes displayed greater RNA abundance in adults and 223 genes were greater in prepubertal oocytes (Patel et al., 2007). Interestingly they reported an increase in mRNA abundance for follistatin, and the βB subunit of inhibin/activin in the oocyte compartment of COCs collected from adult vs. prepubertal animals. The βB subunit of

inhibin/activin is a subset of the TGF-β family, and follistatin binds to it; these molecules are associated with folliculogenesis and oocyte maturation (Knight and Glister, 2001).

The main role of follistatin is to regulate activin activity (Knight and Glister, 2001), but follistatin also binds bone morphogenic proteins (BMP) (Fainsod et al., 1997; Iemura et al., 1998), important for oocyte and embryo development. The addition of low doses to COCs in vitro increased embryonic development and did not antagonize the effects of activin A (Silva and Knight. 1998). Patel et al. (2007) suggested that follistatin could have a stimulatory role in early development. Lee et al. (2007) tested this hypothesis by adding follistatin in vitro at the zygote stage, which enhanced the proportion of early cleaving embryos, blastocyst rates and number of trophectoderm cells. Those embryos that cleave faster have more chance to reach the blastocyst stage (Plante and King, 1992; Grisart et al., 1994; Holm et al., 1998).

8.5.3. Lipid accumulation

Lipids appear to be an important source of energy during bovine oocyte maturation, fertilization, and early embryo cleavage (Ferguson and Leese, 1999; Kim et al., 2001; Sinclair et al., 2002; Ferguson and Leese, 2006). An increase in lipolytic activity during maturation has been reported (Cetica et al., 2002); also, bovine oocytes and zygotes exposed to methyl palmoxirate to block FA transport across the inner mitochondrial membrane prior to oxidation showed a reduced capacity to form blastocysts post fertilization (Ferguson and Leese, 2006). This indicates that high levels of ATP may needed by the oocyte during cytoplasmic and nuclear maturation and early

cleavage. Triglycerides are the main class of lipid synthesized in oocytes and embryos of mice (Flynn and Hillman, 1978), sheep (Coull et al., 1998; Ferguson and Leese, 2000) cattle (Ferguson and Leese, 1999, McEvoy et al., 2000; Sinclair et al., 2002) and pigs (McEvoy et al., 2000; Sturmey and Leese, 2003). Bovine oocytes contain abundant stores of lipids, with triglycerides the most abundant lipids in immature and in vitro-matured oocytes (Kim et al., 2001; Sinclair et al., 2003; McEvoy et al., 2000). Various studies found that palmitic, oleic, and stearic acids are the most abundant FA in descending order of abundance (Kim et al., 2001; McEvoy et al., 2000; Zeron et al., 1999); however, other reports found oleic as the most abundant and palmitic and stearic as the second and third in order of abundance (Yao et al., 1980; Khandoker et al., 1997). Interestingly these three long chain FA are also the most abundant of the total fatty acids (61-74%) in follicular, oviductal and uterine fluids of cow and pig (Khandoker et al., 1997) and rabbit oviduct (Waterman and Wall, 1988).

Analysis of genes involved in lipid metabolism in in vivo-derived, matured oocytes and blastocysts suggests that relative abundance of mRNA for FA transport (FA translocase, FAT/CD36; FA transport protein, FATP1) and catabolism (Carnitine palmitoyltransferase I, CPT-I; AMP-activated protein kinase gamma 1, AMPK) was >10 times higher during oocyte maturation than at the blastocyst stage; in contrast transcripts for fatty acid synthesis (acetyl-CoA carboxylase, ACC; FA synthase, FAS) increased > 5 times during blastocyst stage. Interestingly no transcripts for CPT-I were detected at the blastocyst stage (Algriany et al., 2007). This should be interpreted carefully; even when the switch in gene expression is evident from a catabolic to a anabolic phase from oocyte

to blastocyst stage, some of this protein may be present even if the transcript is not. For example, Haggarty et al. (2006) reported FA oxidation in human blastocysts, so catabolism of FA may occur even when the transcript for the mitochondrial transported CPT-I does not. Thus, protein analysis is required to dissect these findings. In measuring lipid accumulation in in vivo-derived embryos by enzymatic-microfluorescence assay, the triglyceride concentration remained stable from the two-cell to blastocyst stage (Ferguson and Leese, 1999). This may indicate that triglyceride catabolism in vivo is not an obligatory event.

In the first experiment, we found that the FCS group had more lipid content, and PES-treated blastocysts had less lipid than control embryos, as previously reported in the whole embryo using Sudan black B dye and counting lipid droplets (De la Torre et al., 2006b; Barcelo-Fimbres and Seidel, 2007a). In the present experiment, FCS increased lipid content more than 40% in the whole embryo and trophoblast, and 27% in the embryonic mass compared with controls; meanwhile PES decreased lipid more than 50% in the whole embryo and trophoblast, and 69% in the embryonic mass compared with controls. Adding FCS at the 4-cell stage to IVP embryos resulted in doubling of triglyceride reserve by the blastocyst stage (Ferguson and Leese, 1999). Morulae cultured in presence of serum had higher fluorescence emission of Nile red than those cultured without serum, increasing lipid content by 30% of in d 6 morulae (Leroy et al., 2005).

No differences in lipid content of the whole embryo were found between cow and heifer-derived blastocysts. Similarly, Majerus et al. (2000) reported similar results for

calf oocyte-derived 7 d blastocysts vs. adults oocyte-derived embryos (64±15 vs. 65±6 ng per embryo) using an enzymatic-microfluorescence assay to measure lipid accumulation. Interestingly a 30% higher lipid accumulation in the embryonic mass was found in cow than heifer blastocysts ($P < 0.01$). This could be simply due to more cells in the embryonic mass for the cow-derived embryos. We did not find previous reports about lipid contents in the embryonic compartments.

We found that early blastocysts had a higher lipid content in the whole embryo, embryonic mass, and trophoblast than expanded blastocysts in both experiments. The difference in lipid content between embryonic mass and trophoblast was evident by the reduction on lipid content in the trophoblast. This decrease in lipid content could be attributed to fatty acid utilization through β-oxidation for generation of energy by mitochondria for blastocoele fluid production (Wiley, 1987). Blastocoele formation requires function of Na^+/K^+ ATPases, whose fuel is ATP (Watson et al., 1999).

In the experiment 2, we found less lipid accumulation in cow than heifer morulae; however the interaction with treatments was significant ($P < 0.01$); the heifer control group was the highest in lipid content ($P < 0.05$). In experiment 2, the cow ovary source was different than experiment 1. Also, the blastocyst lipid content was higher for whole embryos, but not for the embryonic mass for heifer blastocysts. This difference can be attributed to higher lipid content in the trophoblast. Differences in lipid contents between heifer and cow embryos may be attributed to breed (Adamiak et al., 2004; Leroy et al.,

2004; Visintin et al., 2002), lactational stage in dairy cattle (Leroy et al., 2006) or nutritional management (Fouladi-Nashta et al., 2007; Adamiak et al., 2004).

For lipolytic culture treatments, forskolin resulted in less lipid content than control, epinephrine and caffeine treatments for whole embryo, embryonic mass and trophoblast ($P < 0.1$) (Table 8.8, Figure 8.7). However, embryonic mass-trophoblast difference was not different between treatments ($P > 0.1$) (Table 8.8).

We found that embryos treated with forskolin after the morula stage had less lipid content than the other treatments and control. This experiment confirms the lipolytic action previously reported by Men et al. (2006), which assessed lipolysis indirectly by glycerol release to the medium. Forskolin, a diterpene from the roots of *Coleus forskohlii*, is a potent activator of adenylate cyclase (Metzger and Lindner, 1981; Seamon et al., 1981) and and increases levels of cAMP in a rapid and reversible manner (Seamon et al., 1981). It also, activates lipase (Honnor et al., 1985; Stralfors and Belfrage, 1983) without affecting phosphodiesterases activity (Lindner et al., 1978). Even when there was 56% reduction in lipid content by forskolin, there was no benefit of using a higher dose (40 μM) than 10 mM. Similarly, Litosch et al. (1982) reported on higher cyclic AMP levels in adiposities cells using 100 μM vs. 10 mM; no differences between doses were found in lipolysis measured by glycerol release.

Recent works suggests that catecholamines in CHO cells activate not only PKA, but also ERK (extracellular-signal-regulated-kinase) and phosphorylating lipase, thus

acting in a dual way to stimulate lipolysis (Soeder et al., 1999; Greenberg et al., 2001). However, addition of epinephrine and caffeine during culture did not affect lipid content in embryos at the doses used during in vitro culture; however, an initial decrease was detected in morulae with high lipid content (heifer morulae). Thus, higher dose of epinephrine may be required to get an important lipolytic effect. However, caffeine inhibiting phosphodiesterases compromised embryonic development at the morula stage at 8 mM. Caffeine is mostly used at 2 mM concentration to stimulate fertilization (De la Torre et al., 2006b); however, there is one report of successful using a 10 mM dose during in vitro fertilization (Xu et al., 2006).

8.6. CONCLUSION

These results suggest that higher a percentage of oocytes derived from cows than heifers can develop into blastocyst in vitro. Lipid content in cow vs. heifer-derived blastocysts was not consistent in both experiments. Progression of the early blastocyst to expanded resulted in a decreased lipid content. PES decreased and FCS increased lipid content relative to controls, but neither affected embryonic development. Forskolin reduced lipid content relative to control, caffeine and epinephrine-treated embryos. High doses of caffeine were detrimental to embryos.

8.7. REFERENCES

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CHAPTER IX

RETROSPECTIVE STUDY OF EMBRYIONIC DEVELOPMENT OF OOCYTES DERIVED FROM MATURE COWS AND HEIFERS

9.1 Summary

The objective of this experiment was to evaluate oocyte collection, development of embryos from oocytes from heifers and mature cows, and the influence of corpora lutea rate per batch of ovaries on blastocysts rates. We analyzed 72 in vitro production cycles (33,982 oocytes) during one year, in a factorial design with 2 ovary sources (heifers (53 IVP cycles), and mature cows (19 IVP cycles)) and semen from 4 bulls (A, B, C, and D). Percentages of embryonic development during in vitro production (cleavage, eight cell and blastocyst rate) were analyzed by ANOVA. For embryonic development according to the percentage of ovaries with corpora lutea per batch of ovaries, a regression model was used. No differences in cleavage (74.0 vs. 73.3%), or eight cell rates (49.5 vs. 54.6%) were found between heifer and cow derived embryos,

respectively ($P > 0.1$). However, more blastocysts per oocyte (25.2 vs. 13.2%) and per eight cell embryo (45.5 vs. 25.5%) were obtain from cow than heifer-derived ovaries ($P < 0.01$). More grade one oocytes were obtained per ovary of heifers than those from mature cows (10.0. vs. 8.0, $P < 0.01$). The proportion of corpora lutea per batch of ovaries was higher from cow than heifer ovaries (25.8 ± 2.7 vs. 11.4 ± 1.6 %, respectively, $P < 0.01$). Also, we found a correlation between the corpora lutea rate present per batch of ovary derived from heifers but not from mature cows ($P < 0.01$). In conclusion, we confirmed that oocytes from mature cows are more developmental competent than heifer-derived oocytes; however, more good quality oocytes were recovered per heifer ovary. Higher proportion of corpora lutea per batch of ovaries in heifers is related to better embryonic development, likely because batches of ovaries with few corpora lutea were from heifers fed an orally active progestin.

9.2 Introduccion

In vitro production of bovine embryos for commercial purposes may allow production of inexpensive embryos using ovaries from slaughterhouses. However, these ovaries are from animals of different backgrounds (e.g. age, breed, health, and reproductive and physiological status), so the source of ovaries could be an important factor in terms success of in vitro production of embryos. Previous reports have showed that oocyte quality is one of the main factors to consider in the variability of success of blastocyst production during in vitro production (IVP) of embryos (Rizos et al., 2002, 2003).

In the United States some abattoirs slaughter hundreds of postpubertal heifers per day, so a considerable number of ovaries are discarded which could be used for in vitro production of embryos. Oocytes derived from prepubertal animals seem to have poorer developmental competence in vitro than mature cows (Majerus et al., 2000; Galli et al., 2003; Rizos et al., 2005). The reasons for these differences in blastocyst production are mostly attributed to a prepubertal effect; nevertheless the bases of this problem are unknown. Besides age effects there are more confounding factors, as nutritional and hormonal supplementation. Heifers often are fattened in feedlots with high energy diets, usually supplemented with the progestagen melengestrol acetate (MGA) to promote growth with the additional benefit of artificial diestrus, which eliminates estrus (Zimbelman and Smith, 1966; DeBois and Bierschwal, 1970; Imwalle et al., 2002). This may affect oocyte quality; however, more studies are necessary to find ways to optimize the use of such oocytes.

We have observe in our laboratory that most of the ovaries acquired for in vitro production of embryos from some batches of heifers contain many medium size follicles, and few of the ovaries have corpora lutea, compared to mature cows batches. The incidence of ovaries with these persistent follicles or corpora lutea and the resultant embryonic development in heifers-derived batches of oocytes are very variable. Beside this, is has been reported that oocytes collected in presence of a dominant follicle are of lesser quality than those collected during follicular growth (Garcia and Salaheddine, 1998; Hagemann et al., 1999).

The objective of this experiment was to evaluate oocyte collection, and embryonic development of oocytes from heifers and mature cows, and the influence of corpora lutea rate per batch of ovaries through a retrospective study.

9.3. Materials and Methods

9.3.1. Experimetal design

Embryonic development of oocytes derived from heifers and cows during one year. For this experiment, seventy-two IVP cycles were evaluated during one year, with oocytes (n=33,982) derived from heifers (53 IVP cycles) and mature cows (19 IVP cycles), and semen from four bulls (A, B, C, and D).

9.3.2 Embryo production

9.3.2.1. Oocyte Collection and In Vitro Maturation. Bovine ovaries from heifers and mature cows were obtained from local abattoirs and transported to the laboratory in 1-2 h in 0.15 M NaCl saline at ambient temperature, approximately 22 to 25°C. After arriving at the laboratory, ovaries were trimmed of extraneous tissue, and rinsed once again in 0.15 M NaCl. Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm antral follicles with an 18-gauge needle attached to a tubing system connected to a vacuum aspiration pump with less than 50 mm Hg of pressure. Oocytes with at least 3 layers of unexpanded cumulus cells and morphologically bright and evenly granulated cytoplasm were selected for in vitro maturation. COCs were washed twice in chemically defined medium (CDM) with Hepes buffer for handling oocytes, and once with maturation

medium (De La Torre-Sanchez et al., 2006b). Fifty COCs were matured per well of 4-well plates (Nunclon, Roskilde, Denmark), containing 1 ml of maturation medium CDM with 0.5% fatty acid-free BSA (Sigma A-6003, Lot # 063K7525) and gonadotropin hormones (National Protein and Peptide Program, Torrance, CA) (15 ng/ml of FSH (NIH-FSH-S17), 1μg/ml of LH (USDA-LH-B-5), 1.0 μg/ml of estradiol-17β (Sigma E-2257), 50 ng/ml EGF (Sigma E-9644) and 0.1mM cysteamine (Sigma M-6500). Oocytes were incubated at 38.5°C with humidified 5% CO_2 in air for 23 h.

9.3.2.2. Sperm Preparation. Frozen semen was from four bulls and contained at least 35 % progressive motile sperm after thawing. Straws of semen were thawed in water at 35°C for 30 sec. Semen from one straw was gently expelled into a 15-ml centrifuge tube and centrifuged 20 min at ~400 x g at ~23°C through a Percoll gradient (Sigma P-1644) of 2 ml 90%: 2 ml 45% Percoll in sperm (sp)-TALP medium (modified Tyrode's) as described by Parrish et al. (1989). The supernatant was discarded, and the sperm pellet (approximately 100 μL) was washed with 5 ml of chemically defined fertilization medium (Fert-CDM; De La Torre-Sanchez et al., 2006b), supplemented with 0.5% BSA, 5 mM caffeine (Sigma C-0750) and 2 μg/ml heparin (Sigma H-3125). The sample was centrifuged again for 5 min at 400 x g at 23°C, and the supernatant was discarded. Approximately 90 to 100 μl remained, and a 5-μl aliquot was taken to determine the sperm concentration with a hemacytometer. The sperm concentration was adjusted to 5 x 10^6 spermatozoa per ml to give final concentrations of 0.5 x 10^6 spermatozoa per ml in Fert-CDM (See below).

9.3.2.3. In Vitro Fertilization. Following in vitro maturation, ~50 oocytes were placed in 450 µl of Fert-CDM medium per well of 4-well dishes, and 50 µl of sperm suspension were added to give final volume of 500 µl per well. Gametes were co-incubated for 18 h at 38.5°C in an atmosphere of humidified 5% CO_2 in air.

9.3.2.4. Culture of Embryos. Following in vitro fertilization, presumptive zygotes were removed from wells and transferred to 1.0-ml microcentrifuge tubes with approximately 100 µl of Fert-CDM (De La Torre-Sanchez et al., 2006b), and then vortexed for 50 to 60 sec to remove cumulus cells. These procedures were carried out in a room at 28 °C. Embryos then were rinsed three times in Hepes CDM-1 (H-CDM-1). Early culture (day 0 to 2.5 post fertilization) was done in a new 4-well dish, containing 500 µl of CDM supplemented with 0.5% fatty acid-free BSA, 0.5 mM fructose, non-essential amino acids (NEAA), and 10 µM EDTA (CDM-1) as described by De La Torre-Sanchez et al. (2006b), and incubated at 39°C under 90% N_2, 5% O_2, and 5% CO_2. After 60 h of culture, embryos were examined with a stereomicroscope (15 to 20×) for cleavage, and all uncleaved ova and embryos less than 8 cells were discarded. The rest were cultured in new dishes with CDM-2 (CDM supplemented with 0.5% fatty acid-free BSA, NEAA and essential amino acids and 2 mM of fructose) from day 2.5 to day 7 post fertilization. Embryos were evaluated 168 h after fertilization to determine the final stage of development.

9.3.3. Evaluation of embryos

Cleavage rates were assessed 2.5 days after being placed in CDM-1, after which embryos at the 8-cell stage were placed into culture into CDM-2; 2- to 6-cell embryos were considered cleaved, but were not cultured further. The percentage development to blastocysts and hatched rate were evaluated 7 days after fertilization.

9.3.4. Statistical evaluations

Percentages of embryonic development during in vitro production (cleavage, eight cell and blastocyst rate) were transformed using arcsin square root. Transformed data were then analyzed by ANOVA. For evaluating embryonic development according to the rate of corpora lutea rate per batch of ovaries, a regression model (proc REC) was used including the linear and quadratic terms, using the SAS statistical software package general linear model (GLM). Sources of variation in the model included all main effects (all considered as fixed effects), all possible 2 way interactions, and residual error. If the ANOVA was significant ($P < 0.05$), means were separated by Tukey's w procedure. Data are reported as untransformed least-squares means.

9.4. RESULTS

In this study, ovaries derived from heifers resulted in more oocytes per ovary than mature cows (10.0 ± 0.3 vs. 8.0 ± 0.5) ($P < 0.01$). No significant effect was found for cleavage and eight cell rate ($P > 0.1$). However, blastocysts rates were 90%higher per oocyte (12 percentage points) and 78% higher per eight cell embryo (20 percentage points) for oocytes derived from mature cows vs. heifers ($P < 0.01$) (Table 9.1).

There was a bull effect for cleavage where the bull C was higher than A, B and D ($P < 0.01$). No effect for bull for eight cell rate was found ($P > 0.1$), but blastocysts per oocyte was higher for bulls A and B than C and D ($P < 0.01$). Blastocysts per eight cell was higher for bull A than bulls C and D ($P < 0.01$), but bull B was not different to A and D. No interaction of bull by ovary source for cleavage, eight cell, blastocysts per oocyte and blastocyst per eight cell rates was found (Table 9.1).

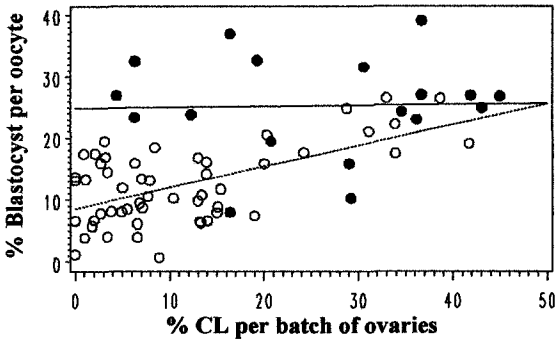
The proportion of ovaries with corpora lutea per batch of ovaries was higher from cow than heifer ovaries (25.8 ± 2.7 vs. 11.4 ± 1.6 %, respectively, $P < 0.01$). There was a highly significant ($P < 0.01$) correlation of blastocyst development per oocyte with the rate of corpora lutea present per batch of ovaries (Fig 1). Blastocyst development per oocyte and per eight cell embryo were lower with low corpora lutea rates per batch of ovaries from oocytes derived from heifers but not for mature cows. The quadratic term was not significant ($P > 0.1$).

Table 9.1. Main effect treatment least-squares means of ovary source and bull on embryonic development of bovine embryos *in vitro* (±SE).

RESPONSES	No. oocytes	Cleaved (%)	Eight cell rate (%)	Blastocysts per oocyte (%)	Blastocysts per eight cell (%)
Ovary source					
Heifers	28815	74.0 ± 1.1	49.5 ± 2.0	13.2 ± 1.2 [a]	25.5 ± 1.6 [a]
Cows	5167	73.3 ± 1.8	54.6 ± 3.3	25.2 ± 1.9 [b]	45.5 ± 2.7 [b]
Bull					
A	11018	70.3 ± 1.4 [b]	51.4 ± 2.6	21.1 ± 1.4 [a]	39.5 ± 2.1 [a]
B	11287	72.0 ± 1.7 [b]	54.2 ± 3.2	21.3 ± 1.7 [a]	37.5 ± 2.6 [ab]
C	8228	79.9 ± 1.8 [a]	52.8 ± 3.2	15.7 ± 1.7 [b]	28.9 ± 2.6 [c]
D	3449	72.4 ± 2.8 [b]	47.7 ± 5.1	14.1 ± 2.6 [b]	30.4 ± 4.1 [bc]

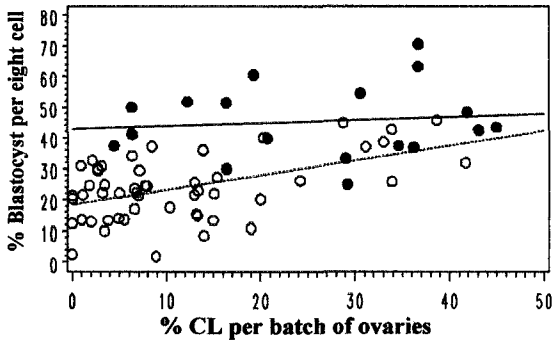
[a,b] Values without common superscripts in the same column differ ($P < 0.01$).

Figure 9.1. In vitro development of oocytes of different sources (heifers vs. cows) according to corpora lutea per batch of ovaries



●—(Cows)=24.758 + 0.016 (CL rate); r^2=0.001
○- -(Heifers)= 8.518 + 0.340 (CL rate); r^2=0.354

Ovary Source effect (Cows vs. Heifers) (P < 0.01).



●— (Cows)= 42.898 + 0.099 (CL rate); r^2=0.012
○- - (Heifers)= 18.394 + 0.477 (CL rate) ; r^2=0.255

Ovary Source effect (Cows vs. Heifers) (P < 0.01).

9.5. DISCUSSION

9.5.1 Oocyte recovery of oocytes derived from cows and heifers

In this study of 72 batches of ovaries, we recovered more grade one oocytes from ovaries of heifers per collection than those from mature cows (10.0 ± 0.3 vs. 8.0 ± 0.5, P < 0.01). An early study shows that calf ovaries exhibit larger pools of antral follicles visible on their surface than adult ovaries (Erickson, 1966), thus offering the potential of recovering more oocytes from calves.

Moreno et al. (1992) recovered more total oocytes per ovary of heifers than cows slaughterhouse ovaries (13.76 vs. 5.67) (P < 0.05). The percentages of grade one oocytes were 28.9 vs. 21.5% from heifers vs. cows respectively. Interestingly they obtained a higher percentage of grade 1 oocytes (compacted and 3 or more layers of cumulus cells)

from heifers with no CL on ovaries than with CL. Nevertheless they recovered a high number of denuded oocytes (ranging from 23 to 42% of total oocytes aspirated in the different groups); this may be explained by high pressure used to aspirate oocytes (120 mm Hg). In contrast there is another report where no significant difference was observed in terms of number of follicles aspirated per ovary (14.5 vs. 16.4), or number of oocytes grade 1–2 recovered (3.6 vs. 3.8) for cows and heifers under 30 months of age (Rizos et al., 2005).

Rizos et al. (2005), using ovum-pick up found significantly more follicles (10.4 versus 7.8), higher numbers of total oocytes recovered (4.7 versus 2.8), with more grade 1-2 oocytes recovered/animal (3.0 versus 1.8) from ovaries of heifers than cows. However, there is a large retrospective analysis where cows yielded more oocytes per session than heifers (10.0 vs. 7.8, respectively) (Galli et al., 2003).

9.5.2. Developmental differences of oocytes derived from cows and heifers

We found a correlation between blastocyst yield and the proportion of CL per batch of ovaries from heifers. Blastocysts rates per oocyte were 90% higher (12 percentage points) from mature cows vs. heifers. This experiment is a retrospective study where the proportion of CL per batch of ovaries was higher in cows than heifers (25.8 ± 2.7 vs. 11.4 ± 1.6, $P < 0.01$). To validate these data, we compared this information with a prospective experiment where a higher blastocyst yield was observed from heifer-derived oocytes than in the present retrospective analysis. There was a higher CL proportion too; corpora lutea per batch of ovaries was not different for cows and heifers (32.6 ± 8 vs.

29.8 ± 8, respectively) ($P > 0.1$); however, mean blastocyst rates per oocyte matured in the prospective experiment in cows and heifers (26.0 vs. 21.8, respectively, $P<0.05$) from 8 replicates (4 each) corresponded closely to the regression line for each category with the proportion of CL per batch of ovaries (Figure 9.1). For cows 32.6% of CL per batch corresponds to 25.8% of blastocyst rates per oocyte, and for heifers 29.8% corresponded to 19.0% blastocyst rate per oocyte, respectively. This is less than 2% difference between the calculated rate and the actual rates of the prospective study.

The relation of the CL rate per batch of ovaries with blastocyst yield is not likely related to the diestrus phase; as previously reported by de Wit et al. (2000), there were no differences in oocyte quality and blastocyst production for oocytes collected from ovaries in early-luteal, late-luteal, and follicular phases, or from non-cyclic ovaries. Also, this was confirmed by Chian et al. (2002), who found no differences in blastocyst production using oocytes collected from ovaries at different stages. Rather this effect could be masked by hormonal supplementation or a nutritional effect.

Mature cows are usually slaughtered because of age, physical or reproductive problems, frequently without a feedlot preparation; on the other hand, heifers are fed in feedlots with high energy diets and usually supplemented with MGA, which may be the reason for lower blastocyst production per oocyte for heifers.

Melengestrol acetate is an inexpensive, orally administrated progestagen, approved in 1968 for growth promotion by FDA, and recently granted zero-withdrawal status period to slaughter. MGA suppresses estrous behavior in feedlot heifers

(Zimbelman and Smith, 1966; DeBois and Bierschwal, 1970; Imwalle et al., 2002), inhibits ovulatory LH surge (Zimbelman and Smith, 1966; Imwalle et al., 2002) blocking ovulation (Custer at al 1994; Yelich et al., 1997), and increases the diameter of the largest follicle (Imwalle et al., 1998), leading to development of persistent dominant follicles (PDF) in up to 90% of MGA-treated cows by d 10 of treatment (Yelich et al., 1997). The MGA dose typically used to suppress estrus do not inhibit pulsatile release of LH; rather it is stimulated (Kojima et al., 1995; Imwalle et al., 1998), increasing growth of multiple follicles and estrogen biosynthesis. As these follicles regress, they are replaced by new follicles. These may explain the higher harvest of follicles and oocytes from heifer ovaries. The negative effect of MGA fed heifers on IVP is unclear; however, if heifers that do not consume adequate amounts of the MGA, the estrus suppression effect can be overcome, and they ovulate (Yelich et al., 1997). Large, persistent follicles yield developmentally less-competent oocytes (Mihm et al., 1999); also those collected in presence of a dominant follicle are of leaser quality than those collected during follicular growth (Garcia and Salaheddine, 1998; Hagemann et al., 1999). Therefore, although oocytes from PDF can be fertilized, their subsequent development is compromised (Ahmad et al., 1995). These MGA effects are seen in vivo during estrus synchronization and AI, where fertility is reduced using long-term MGA treatment (DeBois and Bierschwal, 1970); however shorter terms (<10 d) did not reduce conception rates (Larson, 1998; Funston et al., 2002).

9.6. CONCLUSIONS

In this retrospective analysis, we confirmed that oocytes from mature cows are more developmental competent than heifer-derived oocytes. However, more good quality oocytes are recovered per heifer ovary. Also, we found a correlation between the corpora lutea rate per ovary per batch derived from heifers but not from mature cows.

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CHAPTER X

CONCLUSIONS

For experiments performed in chapter 3, the objective was to determine if replacing glucose with fructose would decrease cytoplasmic lipid accumulation during culture of embryos with or without regulators of metabolism; we found that fructose produced more blastocysts than glucose, PES reduced lipid accumulation, and FCS and Cerulenin 3 increased lipid accumulation relative to controls.

In chapter 4, we investigated effects of hexoses, FCS, and PES during the culture of bovine embryos on blastocyst development and survival after cryopreservation by slow freezing or vitrification; we found that replacement of glucose with fructose resulted in higher in vitro blastocyst production, but no differences were found for this response between PES, FCS and control groups. Cryopreservation by vitrification and conventional freezing resulted in similar survival rates of blastocysts. Reduction of cytoplasmic lipid content of bovine embryos by culturing them with PES improved cryotolerance relative to controls cultured without serum. Embryos cultured in the presence of FCS had poorer survival post cryopreservation than controls or the PES treatment.

The objectives of the research in chapter 5, were to determine if use of PES is detrimental for embryonic and post natal development compared with in vitro-produced

controls cultured without PES, and cryopreserved in vivo-produced embryos. We also studied effects of days post estrus of the recipient at embryo transfer (synchrony) and problems during embryo transfer on pregnancy rates. Use of PES during in vitro culture did not affect pregnancy rates nor conceptus losses between d 35 and 98 of pregnancy, and had no effect on post-natal fetal development. There was no effect of synchronization protocol, CL side or CL structure on pregnancy rates. However, recipients 7 to 7.5 d post estrus at the time of ET had higher pregnancy rates than those 6 to 6.5 d post estrus, and non problematic ET pregnancy rates were superior to those when problems occurred during embryo transfer. The pregnancies for in vitro-produced embryos resulting in abortion showed retarded fetal and placental development well before abortion occurred compared to those that developed to term. In vitro-produced fetuses were similar to in vivo controls during early pregnancy.

The reseach in chapter 6, was designed to determine if glucose and/or fructose or in culture media affects female embryos differently from male embryos. Sexed semen was used to produce embryos. Fructose improved embryonic development of both sexes of embryos; glucose retarded development of female embryos and was toxic for female embryos. Sexed semen resulted in similar cleavage and blastocysyt production as non-sexed semen, and female embryos had retarded embryonic development relative to male embryos.

The objective of the experiment in chapter 7 was to test and validate a fluorescence approach to quantify lipid accumulation in bovine oocytes and blastocysts; we found that fluorescence after staining is a reliable technique for quantifying lipid

droplets in bovine oocytes and blastocysts; measuring flourescence adjusted for area of the embryos resulted in similar discrimination to measuring and counting lipid droplets, but is faster and more objective.

In chapter 8, the objective was to evaluate lipid accumulation and embryonic development of bovine morulae and blastocysts treated with different chemicals using ovaries from post pubertal heifers and mature cows; we found that higher percentage of oocytes derived from cows than heifers developed into blastocysts in vitro. Progression of early to expanded blastocysts resulted in progressively decreased lipid content. PES decreased and FCS increased lipid content relative to controls, but neither affected rates of embryonic development. Forskolin reduced lipid content relative to control, caffeine and epinephrine-treated embryos. High doses of caffeine were detrimental to embryos.

The objective of reseach in chapter 9 was to evaluate oocyte collection success and development of embryos from oocytes from heifers and mature cows, and the influence of corpora lutea rate per batch of ovaries on blastocysts rates, with a retrospective analysis; we confirmed that oocytes from mature cows are more developmental competent than heifer-derived oocytes; however, more good quality oocytes were recovered per heifer ovary. Higher proportions of ovaries with corpora lutea per batch of ovaries in heifers is related to better embryonic development, likely because batches of ovaries with few corpora lutea were from heifers fed an orally active progestin.

CHAPTER XI

APPENDICES

Appendix 1: Hepes-buffered chemically defined medium-maturation (HCDM-M)

NaCl	93.5	mM
KCl	6.0	mM
KH_2PO_4	1.0	mM
Na-Citrate.$2H_2O$	0.5	mM
$NaHCO_3$	5.0	mM
$CaCl_2.2H_2O$	2.0	mM
D-Glucose	0.5	mM
Glycine	4.9	mM
Alanine-Glutamine	1.0	mM
Hepes 1:1 (Hepes acid [Sigma H 4034]: Hepes Na salt [Sigma H 3784])	20.0	mM
L-Lactate	10.0	mM
Na-Pyruvate	0.5	mM
$MgSO_4$	0.5	mM
Nonessential amino-acids[a]	10.0	µl/ml
BSA (fatty acid free) (Sigma A 6003)	2.5	mg/ml
pH was adjusted to 7.3 – 7.4 by addition of NaOH or HCl		
Sterilization was by filtration through a 0.22 µm filter		
Heparin (Sigma H 3149)	20.0	µg/ml
Gentamycin sulfate	25.0	µg/ml

[a] Final concentration equivalent to Minimal Essential Medium

Appendix 2: Chemically defined medium-maturation (CDM-M)

NaCl	71.0	mM
KCl	6.0	mM
KH_2PO_4	1.0	mM
Na-Citrate.$2H_2O$	0.5	mM
$NaHCO_3$	25.0	mM
$CaCl_2.2H_2O$	2.0	mM
D-Glucose	0.5	mM
Glycine	4.9	mM
Alanine-Glutamine	1.0	mM
Hepes 1:1 (Hepes acid [Sigma H 4034]: Hepes Na salt [Sigma H 3784])	20.0	mM
L-Lactate	10.0	mM
Na-Pyruvate	0.5	mM
$MgSO_4$	0.5	mM
Myo-inositol	2.77	mM
Nonessential amino-acids[aa]	10.0	µl/ml
Taurine	0.1	mM
BSA (fatty acid free) (Sigma A 6003)	5.0	mg/ml
pH was adjusted to 7.3 – 7.4 by addition of NaOH or HCl		
Sterilization was by filtration through a 0.22 µm filter		
FSH (NIDDK-oFSH-20)	15.0	ng/ml
LH (USDA-LH-B-5)	1.0	µg/ml
Epidermal Growth Factor	50.0	ng/ml
Estradiol 17ß	0.1	µg/ml
Cysteamine	0.1	mM
Gentamycin sulfate	25.0	µg/ml

[a] Final concentration equivalent to Minimal Essential Medium

Appendix 3: Chemically defined medium-fertilization (F-CDM)

NaCl	85.0	mM
KCl	6.0	mM
KH_2PO_4	1.0	mM
Na-Citrate.$2H_2O$	0.5	mM
$NaHCO_3$	25.0	mM
$CaCl_2.2H_2O$	2.0	mM
D-Glucose	0.5	mM
Glycine	4.9	mM
Alanine-Glutamine	1.0	mM
Hepes 1:1 (Hepes acid [Sigma H 4034]: Hepes Na salt [Sigma H 3784])	20.0	mM
L-Lactate	10.0	mM
Na-Pyruvate	0.5	mM
$MgSO_4$	0.5	mM
Nonessential amino-acids[a]	10.0	µl/ml
Caffeine	2.0	mM
BSA (fatty acid free) (Sigma A 6003)	5.0	mg/ml
pH was adjusted to 7.5 – 7.6 by addition of NaOH or HCl		
Sterilization was by filtration through a 0.22 µm filter		
Heparin (Sigma H 3149)	2.0	µg/ml
Gentamycin sulfate	25.0	µg/ml

[a] Final concentration equivalent to Minimal Essential Medium

Appendix 4: Percoll gradient

90% Percoll

Percoll		4.5	ml
10xSPTL		0.5	ml
100xSPAD		0.05	ml
$NaHCO_3$ (25 mM)		0.05	ml

45% Percoll

90% Percoll		2.0	ml
1xSPTL		2.0	ml

10x Sperm talp stock solution (SPTL)

NaCl		4.675	g
KCl		0.23	g
NaH_2PO_4		0.036	g
Hepes (acid)		1.192	g
Hepes (salt)		1.302	g
D-L Lactate (60% Syrup)		3.68	ml
H_20	up to	100.0	ml

100xSPAD stock solution

$CaCl_2.2H_20$		0.308	g
MgCl		0.0602	g
H_20		10.0	ml

1x SPTL

10xSPTL		2.5	ml
100xSPAD		0.2	ml
H_20		17.3	ml

Appendix 5: Hepes-buffered chemically defined medium-1 (HCDM-1)

NaCl	93.5	mM
KCl	6.0	mM
KH_2PO_4	1.0	mM
Na-Citrate.$2H_2O$	0.5	mM
$NaHCO_3$	5.0	mM
$CaCl_2.2H_2O$	2.0	mM
Hexose (D-Glucose or D-Fructose)	0.5	mM
Glycine	4.9	mM
Alanine-Glutamine	1.0	mM
Hepes 1:1 (Hepes acid [Sigma H 4034]: Hepes Na salt [Sigma H 3784])	20.0	mM
L-Lactate	10.0	mM
Na-Pyruvate	0.5	mM
$MgSO_4$	0.5	mM
Nonessential amino-acids[a]	10.0	µl/ml
BSA (fatty acid free) (Sigma A 6003)	2.5	mg/ml
pH was adjusted to 7.3 – 7.4 by addition of NaOH or HCl		
Sterilization was by filtration through a 0.22 µm filter		
Gentamycin sulfate	25.0	µg/ml

[a] Final concentration equivalent to Minimal Essential Medium

Appendix 6: Chemically defined medium-1 (CDM-1)

NaCl	72.0	mM
KCl	6.0	mM
KH_2PO_4	1.0	mM
Na-Citrate.$2H_2O$	0.5	mM
$NaHCO_3$	25.0	mM
$CaCl_2.2H_2O$	2.0	mM
Hexose (D-Glucose or D-Fructose)	0.50	mM
Glycine	4.9	mM
Alanine-Glutamine	1.0	mM
Hepes 1:1 (Hepes acid [Sigma H 4034]: Hepes Na salt [Sigma H 3784])	20.0	mM
L-Lactate	10.0	mM
Na-Pyruvate	0.5	mM
$MgSO_4$	0.5	mM
Myo-Inositol	2.77	mM
EDTA	0.01	mM
Nonessential amino-acids[a]	10.0	µl/ml
Taurine	0.1	mM
BSA (fatty acid free) (Sigma A 6003)	5.0	mg/ml
pH was adjusted to 7.3 – 7.4 by addition of NaOH or HCl		
Sterilization by filtration through 0.22 µm filter		
Gentamycin sulfate	25.0	µg/ml

[a] Final concentration equivalent to Minimal Essential Medium

Appendix 7: Chemically defined medium-2 (CDM-2)

NaCl	76.0	mM
KCl	6.0	mM
KH_2PO_4	1.0	mM
Na-Citrate.$2H_2O$	0.5	mM
$NaHCO_3$	25.0	mM
$CaCl_2.2H_2O$	2.0	mM
Hexose (D-Glucose or D-Fructose)	2.0	mM
Glycine	4.9	mM
Alanine-Glutamine	1.0	mM
Hepes 1:1 (Hepes acid [Sigma H 4034]: Hepes Na salt [Sigma H 3784])	20.0	mM
L-Lactate	5.0	mM
Na-Pyruvate	0.2	mM
$MgSO_4$	0.5	mM
Myo-Inositol	2.77	mM
Essential amino-acids[a]	20.0	µl/ml
Nonessential amino-acids[a]	10.0	µl/ml
BSA (fatty acid free) (Sigma A 6003)	5.0	mg/ml
pH was adjusted to 7.3 – 7.4 by addition of NaOH or HCl		
Sterilization was by filtration through a 0.22 µm filter		
Gentamycin sulfate	25.0	µg/ml

[a] Final concentration equivalent to Minimal Essential Medium

Appendix 8: Hepes-buffered chemically defined medium-2 (HCDM-2)

NaCl	97.5	mM
KCl	6.0	mM
KH_2PO_4	1.0	mM
Na-Citrate.$2H_2O$	0.5	mM
$NaHCO_3$	5.0	mM
$CaCl_2.2H_2O$	2.0	mM
Hexose (D-Glucose or D-Fructose)	2.0	mM
Glycine	4.9	mM
Alanine-Glutamine	1.0	mM
Hepes 1:1 (Hepes acid [Sigma H 4034]: Hepes Na salt [Sigma H 3784])	20.0	mM
L-Lactate	5.0	mM
Na-Pyruvate	0.2	mM
$MgSO_4$	0.5	mM
Essential amino-acids[a]	20.0	µl/ml
Nonessential amino-acids[a]	10.0	µl/ml
BSA (fatty acid free) (Sigma A 6003)	2.5	mg/ml
pH was adjusted to 7.3 – 7.4 by addition of NaOH or HCl		
Sterilization was by filtration through a 0.22 µm filter		
Gentamycin sulfate	25.0	µg/ml

[a] Final concentration equivalent to Minimal Essential Medium

Appendix 9: Syngro Holding medium (SH)

Polyvinyl alcohol (PVA) (Sigma P8183)	20.0	mg
Syngro holding medium 0.1% w/v sodium pluronate (AB technology)	10.0	ml

Appendix 10: 5 M vitrification medium-1 (V1)

Ethylene glycol	1.4	ml
SH	3.6	ml

Appendix 11: 7 M vitrification medium-2 (V2)

Ethylene glycol	1.95	ml
SH	3.05	ml
0. 5 M Galactose	0.45	g/5ml
18% Ficoll 70	0.9	g/5ml

Appendix 12: Dilution medium (D-Solution)

SH	5.0	ml
0.5 M Galactose	0.45	g/5ml

Appendix 13: Freezing medium (SS medium)

SH	4.58	ml
1.5 M Ethylene glycol	0.42	ml
0.1 M Sucrose	0.17	g/5ml