DISSERTATION

REGULATION OF GLUCOSE METABOLISM IN BOVINE EMBRYOS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOSE FERNANDO DE LA TORRE SANCHEZ ENTITLED REGULATION OF GLUCOSE METABOLISM IN BOVINE EMBRYOS BE ACCEPTED AS FULLFILING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR IN PHILOSOPHY.

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ABSTRACT OF DISSERTATION

REGULATION OF GLUCOSE METABOLISM IN BOVINE EMBRYOS

The objective of this research was to evaluate glucose metabolism and other characteristics of bovine embryos treated with molecules that regulate carbohydrate metabolism to improve normality of embryos produced in vitro. A preliminary experiment compared in vitro development of embryos exposed to four levels of phosphate (1250, 250, 50 and 0 μM) and three levels of citrate (500, 50 and 0 μM) in a chemically defined media system (CDM1/CDM2) plus fatty acid-free BSA. A phosphate x citrate interaction for lightness-darkness (dark= more lipids) of embryos at 36 h ($P<0.04$) was found. Citrate decreased embryo lipid accumulation (darkness) in the presence of the highest concentration of phosphate compared to other treatments.

Four experiments were then done to evaluate the toxicity/beneficial effects of four regulators of glucose metabolism on development of embryos. Phenazine ethosulfate (PES), phloretin (PL), pyrroline-5-carboxylate (P5C), and sodium azide (NaN_3) were included at four doses (0, 0.1, 0.3, and 0.9 μM; 0, 30, 90, and 270 μM; 0, 9, 27, and 81 μM; and 0, 3, 9, and 27 μM, respectively) in factorial combination with glucose concentrations of 0, 0.5, 2 and 8 mM. Compact morulae produced in vitro using the CDM1/CDM2 culture system were allocated to treatments (~5760 total morulae), and evaluated blindly after 36 and 72 h for stage of development, morphological quality,

lightness-darkness, inner cell mass quality, percentage of blastocysts, and number of cells. The highest PES dose evaluated (0.9 μM) resulted in poorer development than treatments with lower doses ($P<0.05$). Presence of PL was detrimental for many of the responses studied, especially for the two highest doses. P5C had little effect on post-compaction embryos at any dose. Inclusion of 27 μM NaN_3 in culture medium resulted in lighter embryos at 72 h compared to lower or no NaN_3 ($P<0.01$). There was little effect of NaN_3 on quality of embryos except the higher NaN_3 levels resulted in more advanced and better quality embryos at 36 h of culture, when cultured in 2 mM glucose, but not at other concentrations. The dose-response information obtained for these four regulatory molecules was used to choose effective but non toxic doses for later experiments.

Two more screening experiments were completed with 2, 4-dinitrophenol (DNP). First, embryos were produced with the CDM1/CDM2 system, and allocated to treatments at the 8- to 16-cell stages. Three doses of DNP (10, 30, and 90 μM), and three times of exposure (early, late and both) were evaluated. A tenth treatment was no DNP (control). Embryos cultured in 90 μM DNP developed slower and were darker than embryos cultured at lower doses. When compared to the control, 30 μM DNP-treated embryos had a higher blastocysts rate. DNP in early culture resulted in more advanced and lighter embryos than in late culture. The second experiment was done in the G1/G2 chemically defined system, using 30 μM DNP at different developmental stages. With this system DNP was detrimental when embryos were exposed at early cleavage stages, but had little effect on embryo development with exposure at later stages.

Two experiments were done to compare in vivo versus in vitro-produced embryos at two developmental stages, and to evaluate how changing development conditions (vivo to vitro and vice versa) at the compaction stage affects blastocyst development. Day 5 and day 7 embryos were produced in vitro, and obtained in vivo with the G1/G2 system. In the second experiment, day-7.5 embryos were produced in four different ways: vitro-vivo (VT-VI); embryos were produced in vitro with the G1/G2 system; on day five embryos were transferred to recipient cows, and recovered 2½ days later. Vivo vitro (VI-VT): In vivo embryos were collected at day 5, and then placed in culture in G-2 for 2½ days. Vitro-Vitro (VT-VT): Day-7.5 embryos were produced completely in vitro in G1/G2. Vivo-Vivo (VI-VI): day-5 in vivo embryos were recovered, transferred to a recipient cow, and 2½ days later embryos were recovered from recipients. In the first experiment Day-7 vitro (D7VT) embryos metabolized more glucose than Day-5 vitro embryos (D5VT) ($P<0.06$); also, the two groups of day-7 embryos had more cells than their day-5 counterparts ($P<0.01$). The D7VT group had more medium and large lipid granules, when compared to D7VI ($P<0.01$), and to D5VT ($P<0.01$). In experiment 2, VT-VI and VI-VT embryos metabolized less glucose than VI-VI and VT-VT embryos (10.7±1.6 and 9.0±4.5 vs 15.4±1.7 and 19.3±2.0, respectively, $P<0.01$). Embryos produced completely in vitro (VT-VT) had higher lactate production than embryos produced in vivo (VI-VI) ($P<0.06$). No other differences were detected in metabolic parameters and numbers of cells per embryo among the treatments. Embryos graded as good metabolized more glucose (16.6±1.18 pMol/embryo/h) than those graded fair (10.6±2.48 pMol/embryo/h) ($P<0.05$). This effect was most pronounced on VT-VT group ($P<0.05$ for quality x group interaction). Amounts of glucose metabolized were similar

among embryos produced completely in vivo versus in vitro, but were lower in VI-VT and VT-VI groups ($P<0.05$). In vitro-produced embryos had higher lactate production than in vivo embryos.

In the last experiment, we compared effects of three metabolic regulators added to G2 medium during compaction and blastulation stages of in vitro-produced bovine embryos. Eight- and 16-cell embryos were randomly allocated to the following treatments: PES, 0.3 μM; NaN_3, 27 μM; DNP, 30 μM; and control, no metabolic regulator. Forty to 50 8- to 16-cell embryos were allocated per treatment per replicate, resulting in 10-15 normal seven-day embryos for evaluation. Radiolabeled glucose was used to study glucose metabolism and pentose phosphate pathway (PPP) rates. Glucose uptake and lactate production was determined by microfluorometry. For lipid quantification, embryos were fixed and stained with Sudan Black B, and lipid granules were quantified microscopically. In four replicates, embryos were transferred to synchronized recipients, and recovered seven days later for evaluation. No differences were found among treatments in blastocyst rate, stage, quality and lightness; average blastocyst production from 8- to 16-cell embryos was 40.3%. PES-treated embryos had the highest glucose metabolism, 18.5 pMol/embryo/h ($P<0.05$ compared to NaN_3) and tended to have a higher PPP rate ($P<0.12$) than controls (50.5% vs 22.1); however, glucose uptake was lower (13.1 pMol/embryo/h) and glycolysis was higher with PES than other treatments (187.3%) ($P<0.01$). Lipid accumulation of embryos from PES was markedly lower than any other in vitro treatment ($P<0.01$), but still higher than in vivo embryos from the previous experiment ($P<0.01$). NaN_3-treated embryos metabolized the

least glucose (11.1 pMol/embryo/h, $P<0.05$), but had highest glucose uptake (38.9 pMol/embryo/h). Lipid accumulation was high in this treatment. DNP-derived embryos had intermediate values in all the metabolic parameters studied, and also were characterized by accumulation of lipids similar to controls. No treatment differences were found in developmental competence when day-7 embryos were transferred to recipients and recovered 1 week later (average 48.6% of recovery of live embryos). The most important conclusion from this research is that 0.3 μM PES resulted in in vitro production of embryos that were more similar to in vivo-produced embryos than controls with respect to amounts of cytoplasmic lipid.

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DEDICATION

To my beloved wife Mercedes.

"Puerto seguro en mares tempestuosos, piedra angular, fuente inagotable de amor, alegría que embellece lo que toca"

To Fernando Emmanuel, Jose Bernardo and Miriam Mercedes, my everlasting source of inspiration.

To My Parents, Vicente de Jesus†, Maria Josefina†, Yolanda† and Gustavo.

To my brothers and sisters.

To my friends.

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CHAPTER 1

INTRODUCTION

Eighteen years ago, Hansel[1] predicted that by the turn of the twenty first century, farm animal breeding systems would start a change from the age of artificial insemination to a new age of artificial inembryonation, in which trained technicians would put in the reproductive tract of several domestic species, age-matched, genetically superior/engineered embryos, produced either in vivo or in vitro, with higher success rates resulting from development of methods to reduce early embryonic death. Except for reduction of early embryonic death, these technologies have been developed, but not to the extent of practical application in the animal industry.[2] Moreover, although some biotechnologies have been achieved "technically", they have resulted in very modest practical application. A pertinent example is the production of transgenic animals whose genetic modification would result in better productive characteristics or pharmaceutical applications. Seidel[3] mentions that hundreds of millions of dollars were invested in transgenic farm-animal research between 1983 and 1997, without any specific product released to the animal industry. He predicts, however, the advent of a "golden age" for this technology in the upcoming years, since an extensive foundation of technology has accumulated, providing an opportunity to get a pay back for all the economic and human resources already spent.

In vitro production of embryos (IVP) is one of the technologies, well established technically, that is without the expected widespread commercial application. Transfer of in vitro-produced embryos as a way to produce genetically superior animals is still way behind the volume of transfers done with embryos produced in vivo. Data retrieved by the International Embryo Transfer Society in 2002[4] show that more than half a million in vivo-produced bovine embryos were transferred worldwide, whereas transfer of in vitro-produced embryos in the same species accounted for only about 83,000. Moreover, almost half of these transfers were done in Brazil, where there was an explosive increment of commercial IVP in the previous year. It is uncertain if such volume will be maintained in the future in that country. Production of embryos in vitro for research purposes (both, research on the IVP system itself to generate new data on regulation of embryo development, and the use of in vitro-produced embryos as a research tool for other technologies) has a very different history. It is very difficult to determine the precise volume of IVP embryos produced in research laboratories, but it is known that more than 100 facilities produce several tens of thousands of embryos per year, leading to the inference that the annual volume of production would be in the range of millions, mostly in cattle.

Despite the many improvements made to IVP embryo systems, the in vitro-produced embryo clearly is not an attractive product for animal producers. The reason is that this type of embryo is still abnormal, compared with its in vivo counterpart. Researchers have found it very difficult to recreate the physiological conditions that occur in the closely regulated environment of the oviduct and uterus, so it is not

surprising that there are still technical issues complicating the usefulness of this technology for animal production systems.[5]

IVP embryos have several inadequacies, including altered metabolism,[6, 7] morphological abnormalities such as impaired compaction and premature blastulation,[8] ultrastructural alterations such as mitochondrial abnormalities,[9] alterations on karyotype,[10] and cytoplasmic lipid accumulation.[11, 12] IVP embryos also are characterized by reduced cryotolerance,[13] altered gene expression,[14] and post-transfer developmental effects, such as reduced viability,[15] increased rates of abortion, and the so called large offspring syndrome.[16, 17]

Much of the problem of abnormal development of IVP embryos may be because in vitro environments for embryos frequently have been designed based on empirical assumptions derived from knowledge in culture of somatic cells, rather than precise requirements of embryos.[18] During the last 20 years, however, much of the research on in vitro production of mammalian embryos has been focused on determining the requirements of embryos and designing culture systems to fit those needs. Amongst the most remarkable achievements have been the development of chemically defined and semi-defined media,[19, 20] and the introduction of sequential media systems to fit the changing requirements of embryos as they go through developmental stages.[21] Some groups have reported production of much less abnormal embryos using the already mentioned recent advances in culture systems.[19, 22] However, with current knowledge in

embryo production systems, we still can not reliably produce embryos with the characteristics of those routinely produced in vivo[23]

One recent approach to improving embryo culture systems concerns metabolic regulation. This involves addition of pharmacological agents to culture media, with the aim of correcting aberrations in metabolic profiles of embryos. A well known example is addition of EDTA to early cleavage stage embryos to down-regulate the glycolytic rate, hence avoiding detrimental effects of excessive glycolysis in early embryos of several species.[24] Other approaches to manipulating glucose utilization of embryos at the post-compaction stages have been investigated recently, based on the finding that post-compaction, IVP embryos use glucose inappropriately, with consequent effects on developmental competence of these embryos.[25]

The aim of this research is to explore glucose metabolism and metabolic regulation of post-compaction bovine IVP embryos. We hypothesize that post-compaction embryos cultured in vitro in chemically defined media will show elevated glycolytic rates and decreased developmental capacity compared to in vivo counterparts, and that addition of metabolic regulators to IVP embryos will induce more physiological glucose metabolism, with resultant improved developmental capacity.

CHAPTER 2

LITERATURE REVIEW

In Vitro production of embryos is a well-established technology in humans and various domestic and laboratory species, with broad applications in basic and applied sciences.[26] In vitro manipulation and culture of mammalian embryos at different stages of early development are required for many medical, scientific and commercial processes, including production of transgenic animals, gene targeting and assisted reproduction technologies. Cloning from embryonic and somatic adult cells also requires similar procedures.[5, 27]

IVP from trans-vaginally collected immature oocytes in cows is a valuable alternative to conventional embryo production to produce large numbers of embryos and pregnancies.[28, 29] Embryo production using oocytes derived from slaughterhouse ovaries is an alternative with commercial applications under certain circumstances.[29] In the same way, human IVP to the blastocyst stage in defined culture media has been used routinely to provide high pregnancy rates.[30] Despite these encouraging facts, IVP in cattle is not used widely, mainly due to the adverse effects reported in such procedures on post-implantation growth and development plus the expenses associated with lowered

pregnancy rates.[31] As previously mentioned, embryos produced in vitro show a series of morphological and developmental abnormalities that merit analysis.

2.1. Differences between in vitro-produced and in vivo-derived embryos.

2.1.1. Altered metabolism.

The main observed feature of mammalian embryos produced in vitro is high levels of glucose metabolism, which in early cleavage stages can result in depletion of cytoplasmic phosphate pools and disruption of embryo functions (crabtree-like effect).[32] In post compaction development, this elevated glucose metabolism is accompanied by excessive lactate production in a phenomenon known as aerobic glycolysis.[7, 33] This event, along with the rationale of glucose utilization in culture media will be discussed in more detail later. Excessive aerobic glycolysis has a negative effect on post-transfer developmental competence of murine embryos.[34]

2.1.2. Morphological abnormalities.

Holm et al.[8] found that in vitro-produced bovine blastocysts cultured in presence of serum had a higher incidence of poor compaction and premature blastulation. They found that serum resulted in premature blastocoele formation, without cell proliferation. Similarly, Miller et al.[35] found that in vitro culture conditions can significantly reduce the time in which embryo blastomeres form tight junctions, resulting in defective compaction and blastulation. This effect was more pronounced when embryos were

cultured in more complex systems (co-culture, serum supplementation), than under simpler culture conditions (SOF-aa + 16 mg/ml FAF-BSA).

2.1.3. Ultrastructural abnormalities.

Abnormalities can also be found at the sub-cellular level. Crosier et al., using transmission electron microscopy, performed a detailed morphometric analysis of ultrastructural characteristics of in vivo-derived and in vitro-produced, compact morulae[36] and blastocysts.[37] In both papers, they used three in vitro systems: embryos produced in vitro in the presence of 10% estrous cow serum (IVPS); embryos produced in vitro with serum restriction at the early cleavage stages (IVPSR) and embryos produced entirely in modified SOF + 6 mg/ml FAF-BSA (mSOF). In vivo embryos were obtained from superovulated cows. The most striking characteristic found in morulae, was an eight-fold reduction in the volume density of mature mitochondria in all in vitro treatments, compared to in vivo-derived embryos; all in vitro-produced embryos also had a higher proportional volume of cytoplasm to all cellular components when contrasted to in vivo-derived embryos, and a reduced proportional volume density of the nucleus to cytoplasm and other cellular components was observed in IVPS embryos compared to in vivo embryos. Analysis of blastocysts revealed similar tendencies, although trends were different when different degrees of blastocyst development (early, full or expanded blastocyst) were analyzed separately. Overall, IVPSR embryos had a lower proportional cytoplasm volume to all cellular components than in vivo and mSOF embryos; an effect on mitochondrial profiles was also found, but not as marked as with morulae. In general, the total mitochondria (including mature, immature and vacuolated) volume density was

decreased in all in vitro treatments, compared to in vivo embryos. Finally, a reduced nucleus volume density was observed in IVPS and IVPSR embryos, compared to in vivo-generated embryos. Others have reported abnormal mitochondrial profiles due to in vitro conditions. Barnett and Bavister[9] reviewed effects of in vitro conditions on mitochondrial patterns in several species, revealing an overall increment on abnormalities, such as reduced number, lack of elongation, reduced size, swollen, absence of cristae and matrix, and abnormal cytoplasmic distribution.

Karyotype alteration is another ultrastructural aberration induced by in vitro-production systems. Viuff et al.,[10] using fluorescence in situ hybridization-based analysis, determined the chromosomal number on cells of in vitro-produced and in vivo-obtained bovine embryos, finding a higher incidence of mixoploid embryos (embryos with a mixture of normal diploid cells and presumptive polyploid cells) in in vitro- than the in vivo-derived embryos (72 vs 25%, $P<0.0001$). In both cases, most embryos had less than 10% of polyploid cells. Studies performed in mice demonstrated that diploid-tetraploid chimeras exhibited developmental retardation and abnormal placental growth;[38] however, it is not known if an increase of mixoploidy induced by culture conditions could have effects on post-transfer development of mammalian embryos.[18]

One of the most evident features of IVP embryos observed under the light microscope is the darkness of their cells. This feature appears to be related to abnormal accumulation of cytoplasmic lipid droplets. Abe et al.[11] found lightness differences after 8-cell stage between IVP embryos produced either in presence or absence of serum.

Embryos produced in presence of serum, appeared darker, and when lipid contend was quantified using Sudan black stain, darkness was correlated with lipid content. Crosier et al.,[36, 37] compared lipid content on in vitro versus in vivo embryos, using EM micrographs and point-count methods for quantification, and found higher volume density of lipids for in vitro-produced morulae and blastocysts than in their respective in vivo counterparts. Lipid accumulation is suggested to result from two different processes. First, the embryo can uptake large amounts of lipids from the culture environment, especially when good sources of lipids are present (serum).[18] Second, it is postulated that lipid accumulation results from the culture-induced impaired activity of mitochondria, and their inability to metabolize complex lipids through β-oxidation.[39] Which ever is the cause of lipid accumulation, it represents a threat for embryo survival as lipid-loaded embryos are more susceptible to oxidative damage[18] and have reduced chilling and freezing tolerance when subjected to cryopreservation procedures.[13, 40, 41]

2.1.4. Altered gene expression.

One of the most studied features of IVP systems is patterns of gene expression of pre-implantation mammalian embryos. Many of the characteristics already explained and to be explained in the present section are consequences of differential gene expression by in vitro-produced embryos. In vitro conditions can cause deviations from the normal mRNA expression of imprinted genes, by causing changes in the normal patterns of DNA methylation.[42, 43] Lane and Gardner[44] give an example of aberrant expression of imprinted genes; 300 μM ammonium in the culture medium significantly altered expression of the imprinted gene H-19 in mouse embryos. They mention that culture of

embryos in medium containing amino acids can easily produce ammonium that is detrimental to embryo physiology and gene expression.

It is important to consider the timing during in vitro embryo development when gene expression is most affected. During maturation and early cleavage, bovine oocyte/embryo development relies mostly on mRNA's that were specified by the maternal genome, mostly before oocyte maturation.[45] For that reason, in vitro conditions during maturation and fertilization might not have a significant effect on gene expression.[46] However, after the embryo genome starts its activation process (which occurs around the 4-8 cell stage in cattle[47]), it becomes more vulnerable to culture-induced transcriptional deviations. Rizos et al.,[48] found that the intrinsic quality of the oocyte, determined by maturation conditions (in vivo or in vitro), will affect the proportion of oocytes that will become blastocysts, whereas conditions of embryo culture will affect blastocyst quality, regardless of maturation conditions. They conclude that differences on blastocyst quality observed on embryos cultured in vivo or in vitro are consequence of differences in gene expression induced by the culture environment.

Several specific gene-induced abnormalities on embryo development have been documented. Rizos et al.,[49] analyzed the relative abundance of mRNA transcripts of seven genes related to apoptosis, oxidative stress, communication through gap junctions, and differentiation and implantation, in bovine blastocysts developed in three in vitro systems (IVP in SOF, co-culture of IVM/IVF zygotes, and sheep oviduct culture of IVM/IVF zygotes) and in vivo-derived blastocysts. Several interesting differential mRNA

expression features were found. An exacerbated expression of Bax, a transcript related to apoptosis was found in embryos cultured in SOF. MnSOD, a transcript that has been related to mitochondrial activity, was more abundant for in vivo-derived embryos than embryos produced in SOF and co-culture, suggesting a transcriptionally-induced mitochondrial malfunction of embryos produced in vitro. SOX transcript was lower on vivo and oviduct-cultured embryos than SOF and co-culture embryos; SOX enzyme is responsible for lipid peroxidation, and therefore, associated with higher production of H_2O_2 and hence reactive oxygen species (ROS). LIF and LR-β, are two transcripts associated to early differentiation and implantation. They were over-expressed in all in vitro systems, at the blastocyst stage, indicating possible abnormal development of ICM and throphectoderm.

Knijn et al.,[46] compared in vivo-derived embryos with in vitro-produced embryos in three different maturation systems. They found reduced expression of several developmentally important genes due to in vitro conditions. Desmocolin 2 and plakophilin had lower expression at the blastocyst stage, with a possible relation with impaired compaction. The reduced expression of the glucose transporter 1 found in IVP embryos, may be related to the abnormal glucose metabolism mentioned earlier (aerobic glycolysis).

Miller et al[35] found that embryos that start compaction at day 6 with blastocyst formation at day 7 (short compaction length) had reduced expression of a set of 6 transcripts involved with formation of tight junctions, when compared to embryos that

start compaction at day 5 with blastocyst formation at day 7 (long compaction length), irrespective of culture system. IVP embryos tend to have short compaction length.. These transcripts are expressed primarly by the embryo genome, at the 8- to 16-cell stage in the bovine embryo, just before the compaction process starts. The short compaction lenght may not allow enough time for the full expression of mRNA's concerned with tight junctions, resulting in defective formation of the intercellular seal, resulting in failure of the cavitation process. Use of serum on in vitro culture systems can exacerbate the abnormal gene expression of IVP embryos, already explained.[40, 50]

Several post transfer consequences due to altered in vitro-induced gene expression have been documented, including higher incidence of early embryonic and fetal abnormalities[15, 51] and the occurrence of the so called "large offspring syndrome".[16, 17, 27]

2.2. In vitro culture of mammalian embryos.

The problem of non-normality of in vitro-produced embryos initiated a relevant change goals, to not only to produce quality blastocysts, but also to be sure that those blastocysts are developmentally competent and result in healthy offspring. Therefore, metabolic study of pre-elongation embryos has became a priority, resulting in considerable research including how embryos take up and metabolize available nutrients under in vitro culture conditions. The next section reviews development of in vitro culture systems, with emphasis on bovine embryos.

2.2.1. Evolution of bovine in vitro culture systems.

2.2.1.1. Early endeavors to culture bovine embryos in vitro.

The era of culturing bovine embryos for more than a day or two began in the early 1970s when several groups reported successful in vitro development of embryos to further stages.[52-55] In these experiments, embryos were produced in vivo, collected from the female reproductive tract, and then cultured in vitro, since techniques for in vitro maturation of oocytes, in vitro sperm capacitation and in vitro fertilization were not well developed. Since then, embryos have been cultured in vitro under a variety of conditions, including simple salt solutions, co-culture with somatic cells, and semi-defined and defined media; more recent developments include sequential and microfluidic systems.

In vivo-produced bovine embryos were successfully cultured in vitro from the 1-cell to the blastocyst stage for the first time in 1972[55] in a medium called "synthetic oviduct fluid" (SOF), which was based on the biochemical composition of sheep oviduct fluid. Further results with SOF and other media originally developed to culture somatic cells were disappointing, however, particularly for in vitro-produced embryos. Retarded development, a developmental 8-cell block, and poor viability were the main reasons that co-culture systems and more complex media evolved, leading to improvements in in vitro culture of embryos by the late 1980s.[56]

2.2.1.2. Co-culture systems.

When appropriately formulated and supplemented with a protein source, co-culture systems result in good developmental rates of morphologically sound blastocysts, with good pregnancy rates after embryo transfer[57, 58]. Until recently, most commercial production of IVP embryos was done with co-culture systems or in vivo culture in heterologous species (e. g. culture of bovine embryos in the sheep oviduct). The reasons for good results with co-culture are due to several beneficial actions. Feeder cells appear to modify the composition of medium making it more appropriate for embryo culture,[59] provide embryotrophic factors,[60] remove inhibitory or toxic components, and protect the embryo against the action of reactive oxygen species (ROS).[61] There is a trend, however, among those doing IVP for research, to use chemically defined and semi-defined media, without the help of somatic cells. Reasons for this will be explained further on.

Most media used for co-culture are commercially available formulations designed to support the growth of somatic cells (e. g. TCM-199, Ham's F10). Embryos, however, have different nutrient requirements as deduced from the composition of tubal and uterine fluids and by experiments in which the metabolic profiles of individual embryos have been assessed. For example, the glucose concentration in many commercially available media has been set at human blood levels (5.55 mM), but embryos develop best at lower concentrations; in fact, this concentration of glucose can even be detrimental, particularly for early cleavage bovine embryos. With co-culture, however, embryos, develop reasonably well in these media. This apparently contradictory situation is explained by changes that feeder cells cause in the medium (e. g. lowering the concentrations of ions

and glucose and increasing lactate concentrations).[62, 63] A variation on co-culture is to use conditioned medium, which is made by culturing cells for several days and then collecting the medium without the cells.[63]

The cell types used for co-culture most frequently are primary cells (granulosa, cumulus, oviductal epithelia, uterine epithelia, and fibroblast cells) or partially transformed cell lines (e.g. buffalo rat liver and Vero cells). Granulosa cells have been used extensively because they are readily available in follicular aspirates and are relatively easy to plate in culture dishes. The problem is that they are usually collected from a random population of follicles, giving a very heterogeneous population, and hence have high variability among batches.[61] Medium components are more physiological for embryos when co-culture is established using oviductal cells,[59] so they may be the most appropriate material for embryo co-culture; however, it is not possible to establish a long-term culture with these cells types, since epithelial cells revert to fibroblast type morphology after few passages. Although not very popular, fibroblasts harvested from embryos, smooth muscle or uterus also have been used to co-culture bovine embryos.[64]

Good quality embryos can also be produced using BRL and Vero cells.[65, 66] However, cell types unrelated to reproduction do not produce specific embryotrophic factors. There also may be, depending of the source, a higher risk of contamination with mycoplasms or viruses.[61]

2.2.1.3. Use of blood serum in culture media.

Serum is usually present in co-culture systems as a source of nutrients, vitamins, growth factors, hormones and anti-oxidants, all of which are beneficial for embryo development.[67] The presence of serum, however, represents an important source of variation,[68] which diminishes the validity of results in experimental data; it is also responsible for abnormal development in embryos, including altered metabolism,[69] accumulation of cytoplasmic lipids,[11] ultrastructural organelle abnormalities,[12] impaired compaction, premature blastulation,[8] and changes in the quantitative expression of developmentally important genes.[16] Serum also is a potential source of viral and prion contamination.[70] Serum putatively is responsible for increased abnormalities observed in calves derived from IVP embryos, generically known as large offspring syndrome.[17]

A major disadvantage of co-culture systems is that most feeder cells require serum components. Many of the beneficial factors present in serum have been identified and isolated, and can be added separately in defined media; however, there are still undefined components required for most co-culture systems for optimal development of embryos.

Even though feeder cells are able to protect embryos from most detrimental effects of serum, co-culture systems are still largely undefined and variable, making this inappropriate for some kinds of research, particularly when effects of certain components in the culture medium are tested. Co-culture is, therefore, restricted to certain types of experiments, and for commercial use.

Serum has been tested in culture systems without feeder cells, with good results in terms of blastocyst rates, but with major negative effects on embryo morphology and developmental competence, as well as a higher incidence of large offspring syndrome. For that reason, its use has been almost discontinued in embryo culture systems, other than for oocyte maturation.

Because of the constraints of co-culture and serum, particularly when studying specific requirements of embryos in vitro, the need for more defined culture systems is evident. Achieving good results without the help of feeder cells and serum, however, has been challenging.

2.2.1.4. Defined and semi-defined culture media.

As mentioned previously, in co-culture systems feeder cells and serum provide embryos with growth factors and protection against toxic elements. Therefore, when embryos are cultured in a completely defined medium, the formulation ideally should contain all the nutrients and growth factors required by the embryo, as well as antioxidants, chelators and other protective molecules. Defined media, initially developed in the 70s and early 80s, were unable to meet these requirements, and results were poor in terms of embryo production and quality, particularly with in vitro-produced embryos.[56, 71]

Addition of bovine serum albumin (BSA) provided embryos with some of the protective features lacking in the defined system, plus some important adsorbed nutrients such as amino acids, fatty acids, and citrate.[70, 72] BSA improves the capacity of defined media to support embryo development substantially.[25] However, this protein can not be completely purified and is still somewhat undefined,[56] with the variability implications already discussed. BSA can be subjected to organic solvent extraction of fatty acids and other lipophylic compounds, making it essentially fatty acid-free (FAF). FAF-BSA has been used extensively in culture media, with acceptable results. Although still undefined, it is much less variable than serum. Media containing FAF-BSA are generically known as semi-defined media, and are used by many for research purposes.

A landmark in development of defined and semi-defined media, was inclusion of amino acids, which resulted in major benefits as explained in the next section. For a completely defined medium, some have substituted BSA with synthetic macromolecules such as polyvinyl pyrrolidone (PVP) or polyvinyl alcohol (PVA)[57]. Embryos, however, develop more poorly than when cultured in presence of serum albumin.[19]

A recent approach is to use human serum albumin produced with recombinant technology, in combination with hyaluronan, a macromolecule produced by yeast fermentation and present in reproductive fluids. Therefore, both molecules are completely defined. Completely defined media containing these molecules and supplemented with citrate (which is usually bound to BSA) have been successfully used for production of bovine blastocysts.[20]

Culturing embryos in completely defined media assures higher repeatability over replicates, giving an advantage for reliability in experiments involving responses of embryos to modifications in culture conditions. Embryos cultured in completely defined medium, however, are more sensitive to non-physiological factors such as contaminants present in the medium or that accumulate after the embryos interact with the culture medium for a certain time, as they do not have the protective effects of feeder cells, serum or BSA.

As embryos are more exposed to toxic factors in defined systems, amino acids, chelators, antioxidants and use of superoxide dismutase (SOD), as well as low oxygen concentrations during culture should be considered.[56] Toxic products are generated in culture by embryo metabolism or by spontaneous breakdown of molecules; for example, glutamine is unstable at culture temperatures and generates ammonia which is toxic for embryos if it accumulates in medium.[21, 44] To avoid ammonia toxicity, several strategies can be used: changing embryos to fresh medium after 2-3 d, culturing embryos in large volumes (500 μl wells) without an oil layer to allow dissipation of ammonia out of the medium, and finally, use of more stable sources of glutamine such as the dipeptide alanyl-glutamine.[73]

Growth factors often are added to chemically defined media to promote better development. However, embryos produce some of these factors, and culturing embryos in groups allows autocrine/paracrine growth factor transfer between embryos.[69]

Inappropriate use of growth factors, however, may lead to the same problems caused by serum.[74]

2.2.1.5. Sequential culture systems.

The rationale for sequential culture is based on metabolic studies in which important differences have been found between embryos at early and advanced stages of development (explained in next section). This "duality" of metabolic behavior has led to evolution of sequential media systems, in which embryos are cultured in media of different composition, according to their stage-specific requirements.[21] Overall, bovine embryos are cultured from the zygote to the 8- to 16-cell stage in a medium with low glucose and non-essential amino acids, and then transferred to a second medium where they will remain through the blastocyst stage. This second environment will contain higher glucose and the complete set of amino acids. The rationale for this media composition will be explained in the next section.

2.2.2. Metabolism of bovine embryos cultured in vitro.

Early bovine embryos, from zygote to 8- to 16-cell stage, have metabolic activity that resembles the paucity that characterizes the oocyte. Overall, early embryos rely on pyruvate and lactate as energy sources, pyruvate being the most important and obligatory substrate for the first cleavage division. Although little glucose is metabolized, a striking feature observed in early embryos cultured in presence of high glucose in the absence of amino acids is a block of development (8- to 16-cell developmental block).[71]

Under these conditions, the embryo will show an exacerbated rate of glycolysis, similarly to tumor cells. This phenomenon, known as the "Crabtree effect",[75] results from failure of the glycolysis intermediate product glucose-6-phosphate to allosterically inhibit the isoform of hexokinase present in the early embryo, causing depletion of mitochondrial phosphate pools due to the continued phosphorylation of glucose and the subsequent re-phosphorylation of the ADP generated in this reaction in the mitochondrion.[32] Mitochondrial depletion of phosphate ultimately causes a disruption in mitochondrial activity, which is often manifested as lipid accumulation in blastomere cytoplasm.[39] Several modifications have been made to early embryo culture media to prevent this detrimental effect. First, the glucose concentration has been reduced, as glucose availability rather than glucose transport or enzyme activity is the main factor determining the glycolytic rate[32]. Less than 1 mM glucose is advised for cleavage stage embryos, 0.5 mM being the most common level used. Amino acids in the medium also help to control the glycolytic rate, as they are alternate energy sources as well as regulators of energy metabolism.[76] Finally, glycolysis can also be regulated using pharmacological agents such as EDTA, which inhibits glycolysis by chelating Mg++ ions, which are necessary for completion of glycolytic reactions in which kinase enzymes participate.[24] EDTA is added to early development medium at concentrations around 10 μM, but can be beneficial at concentrations as low as 3 μM.[77, 78] Higher concentrations can be detrimental as pyruvate and glutamine oxidation can be also inhibited; however, in one report, addition of 100 μM EDTA in early culture improved quality of embryos.[79] EDTA may also exert a beneficial effect by chelating heavy metals in the culture medium, particularly if less pure chemicals are used.[78]

Mg++ chelation by EDTA is difficult to explain in terms of molar concentrations, since the amount of Mg++ present in the culture medium is about 50 times higher than EDTA molecules (0.5 vs 0.01 mM); this is without considering that the available EDTA molecules can also chelate other divalent anions and heavy metals. However, in a recent report, EDTA reduced the glycolytic rate in early cleavage mouse embryos, reducing the availability of Mg++ ions, and reducing the activity of at least one kinase enzyme in the glycolytic pathway. The glycolytic rate was restored when additional Mg++ ions were added to the medium, thus demonstrating the role of EDTA as glycolytic inhibitor.[80] Perhaps the EDTA acts within a cellular compartment in which Mg++ concentrations are very low, or acts via a mechanism other than lowering free Mg++ concentrations.

After the 8- to 16-cell stage, and coincident with the activation of embryonic genome, the embryo experiences a switch in the carbohydrate preference, from pyruvate and lactate to glucose; this sugar is the main source of energy for the compaction and blastulation stages.[81-83] Amino acids are also a significant source of energy,[30] especially glutamine, which is utilized in the first two cleavage divisions and after compaction.[84]

Glucose, probably the most controversial energy substrate used in culture of mammalian embryos, raised early concerns about putative toxicity,[85-87] leading to the decision of some investigators to remove this from the culture medium.[88, 89] However, ample evidence supports the importance of this molecule for pre-elongation development of mammalian embryos[21]. This is explained by several important functions of glucose

other than as energy substrate, that will be mentioned later on. Further improvements made to culture media, particularly addition of amino acids, along with a better understanding of embryo physiology at different stages of development, allowed inclusion of glucose without adverse effects when culturing embryos of various mammalian species.[69, 76, 90]

As the embryo progresses to the morula stage, the metabolic profiles change, in that glucose becomes the most important energy substrate, with lesser dependence on pyruvate and lactate. The embryo is now able to take up larger amounts of glucose, and the glycolytic and tricarboxylic acid cycle pathways become necessary.[30, 91, 92] This switch responds to the increased energy requirement of embryos at this stage, which is associated with two main events. First, is the activation of embryonic genome, which is accompanied with higher biosynthetic rates, and second is blastocoele formation, which requires functioning of the active transporters, Na+/K+ ATPases, whose fuel is ATP. [45, 93]

A feature observed only in embryos developed in vitro, is excessive production of lactate from the uptake of glucose. Whereas in vivo embryos utilize about 40 to 50% of the glucose for producing lactate, their in vitro counterparts convert almost 100% of glucose into lactate.[7] This paradoxical characteristic (metabolism of glucose through the less efficient pathway even though more energy is required) has been called "aerobic glycolysis",[94] since this is the normal fate of the glycolytic pathway under anaerobic conditions; however, in this case, it occurs in the presence of oxygen. This aberrant

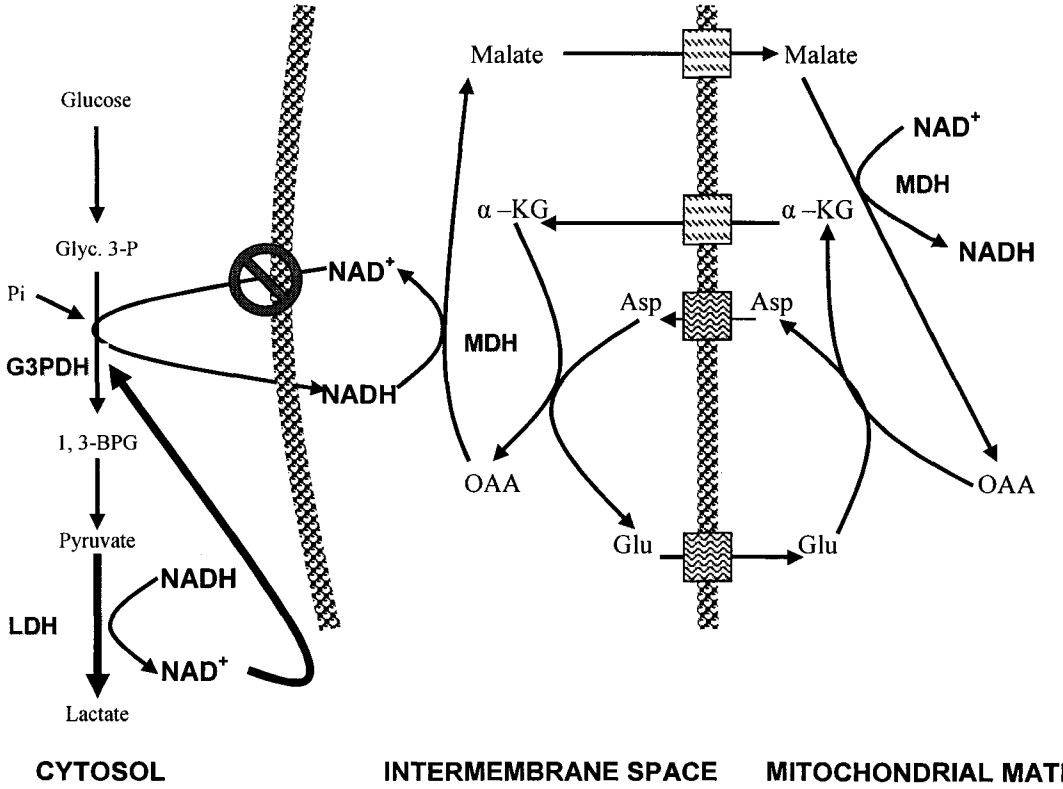
metabolic condition has been linked with embryo viability, since embryos converting most of the glucose taken up into lactate are less developmentally competent after transfer than embryos with lower percentages of lactate conversion.[19, 76]

The most plausible explanation for this phenomenon resides in a putative failure of the malate/aspartate shuttle, which normally moves the NAD^+ generated by the respiratory chain in the mitochondria to the cytoplasm, to be used in the glycolytic pathway.[95] As the NAD^+ can not be transported to the cytoplasm, the embryo needs to generate this from other sources to maintain the glycolytic pathway; therefore, the pyruvate generated is reduced to lactate, in order to oxidize NADH and generate the needed NAD^+.[96] Additionally, exogenous pyruvate seems to be readily oxidized in the tricarboxylic acid cycle, presumably due to cell compartmentalization in terms of substrate metabolism; thus, the glucose-derived pyruvate is channeled to a different compartment than exogenous pyruvate, and is almost entirely converted to lactate, whereas the exogenous pyruvate can be oxidized.[23] Figure 2.1 illustrates the putative dynamics of NAD^+/NADH in the IVP embryo.

Because glucose could potentially cause all of these metabolic disturbances, many people were tempted to take it out of culture media; however, there are two reasons for not doing this. First, glucose is not only a source of energy currencies, but also has an important biosynthetic role; if glucose is shifted to the PPP, it will function as a substrate for the generation of biosynthetic molecules like triacylglycerols, glycoproteins;[97] ribose-5-phosphate (required for nucleic acid synthesis),[98] and NADPH, which is

required in biosynthesis of lipids and other complex molecules,[99] as well for reduction of intracellular glutathione.[100]

Figure 2.1. Malate-Aspartate shuttle in the IVP embryo.



Glyc. 3-P= Glyceraldehyde 3-phosphate; Pi= inorganic phosphate; 1, 3-DPG= 1, 3-diphosphoglycerate; G3PDH= glucose 3-phosphate dehydrogenase; LDH= lactate dehydrogenase; α-KG= α-ketoglutarate; Asp= aspartate; Glu= glutamate; OAA= oxaloacetate; MDH= malate dehydrogenase; = putative in vitro-derived inhibition of NAD^+ availability in the cytoplasm from the mitochondrion; = Malate-α-ketoglutarate carrier; = Glutamate-aspartate carrier; bold arrows= enhanced reactions due to malate-aspartate shuttle inhibition.

Second, glucose can also be stored in the form of glycogen,[92] and it is proposed that the embryo needs this form of energy reserve during implantation, when embryos of some species will spend a period of time until the vasculature is established, without availability of nutrients in the surrounding fluids. If the embryo is cultured in medium lacking glucose, it will not be able to increase glycogen stores; also it will have to utilize

part of the energy storage originating in the oocyte to cover energy demands, with subsequent viability compromised at a later time.[21, 101]

With this background, the decisions towards development of culture media able to generate blastocysts with adequate developmental potential must be directed not to elimination of glucose from the culture media, but to provide adequate conditions that will permit taking advantage of glucose benefits, without its possible adverse effects.

2.3. Metabolic regulation.

A relatively new concept in embryo culture is metabolic regulation, which consists in the addition of non-physiological compounds to regulate the metabolic pathways of embryo's cells to modify metabolism in favor of embryonic development. The best-known example of metabolic regulation may be addition of EDTA to reduce glycolysis in early cleavage embryos.[77-79]

Recently Thompson's group[102-104] improved in vitro development of bovine and porcine embryos, by using non-toxic concentrations of sodium azide (NaN3), an inhibitor of cytochrome oxidase a3 in the electron chain transport cascade, and 2, 4-dinitrophenol (DNP) an uncoupler of oxidative phosphorylation from electron transport, to sub-acutely down regulate mitochondrial ATP production. The mechanism for this remains to be elucidated, but they propose that partial inhibition of mitochondrial ATP production regulates the relative contribution of glycolytic ATP vs mitochondrial ATP production, establishing an appropriate redox state which favors adequate glucose metabolism.

Other studies on metabolic regulation have been performed by Downs et al.[105, 106] in mouse oocytes using Pyrrolyne-5-carboxylate (P5C) and phenazine ethosulfate (PES), which are electron aceptors for NADPH. This produces NADP, which is necessary for the conversion of glucose-6-phosphate into 6-phosphogluconate, in this way stimulating the PPP. Whether these compounds can be used to increase the rate of PPP in post-compaction, in vitro-produced bovine embryos, thereby improving the glucose metabolism, remains to be investigated.

Phloretin, a bioflavonoid, acts as an inhibitor of GLUT transporters.[107] Phloretin reduces glucose utilization in mammalian embryos,[108, 109] and partial inhibition of glucose uptake by phloretin overcomes the developmental 2-cell block in CF-1 mouse embryos, shown when cultured in a medium containing glucose and phosphate.[110] Phloretin has also proven effective to increase the relative PPP rate in sperm, by reducing significantly the glycolytic rate, but maintaining the PPP rate at the same level.[111] Although the mechanism for this shift in glucose metabolism is not well understood, the question arising is whether phloretin could have the same effect on post-compaction bovine embryos, and hence improve bovine embryo development.

Metabolic regulation research warrants further investigation, since we are far from mimicking in vitro, the conditions and dynamics that the embryo normally faces when it develops in the tubal and uterine environments. The oviduct is one of the most dynamic and less studied epithelia.[112] One of the main concerns about glucose utilization in post-compaction development in vitro is excessive production of lactic acid,

which causes insufficient partitioning of glucose to the PPP, and results in the metabolic consequences already explained. The metabolic regulation approach proposed in the present document, could give a window of opportunity to achieve more physiological utilization of glucose during post-compaction development.

The use of completely chemically defined media, is central for obtaining reliable data on any kind of embryo evaluation, including metabolic assessments. Wrenzycki et al. [42] found more laboratory-to-laboratory consistency in transcriptional activity of in vitro-cultured embryos when a chemically defined media system was used. Most commercially available BSA is heterogeneous and has various amounts of bound molecules, which could introduce uncontrolled variability to these evaluations.[77, 113] Unfortunately, substitution of BSA with other defined synthetic macromolecules (PVP, PVA) is not entirely successful, since the embryo production is lower in such systems, and embryos are of poorer quality.[19, 77, 114] Other recent approaches to effectively substitute BSA, have been the use of completely defined serum albumin, produced with recombinant technology (rSA),[70] plus the macromolecule hyaluronan, produced with yeast fermentation technology or extracted from rooster comb.[113, 115, 116] Combined, rSA and hyaluronan, have demonstrated synergistic benefits for embryos cultured in completely defined medium.[117] With these considerations, it was decided to use a chemically defined media system, containing human rSA and hyaluronan for culturing embryos subjected to metabolic evaluations.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1. In vitro production of embryos using G1/G2 and CDM1/CDM2 sequential media.

Two sequential media formulations were used for producing in vitro embryos, the G1/G2 system, developed by Gardner's laboratory,[21] and CDM1/CDM2, developed by Seidel's laboratory at Colorado State University.[77] Both systems evolved in parallel.

3.1.1. G1/G2 system.

The formulation of G1/G2 sequential media, as previously published[21] is presented in the Table 3.1. All media required for all the in vitro procedures, from maturation to culture were based on G1 and G2 media. Maturation medium consisted of G2 supplemented with 0.1 mM cysteamine, 15 ng/ml oFSH (NIDDK-oFSH-20), 1 μg/ml bLH (NIH-LH-S1), 1 μg/ml 17 β estradiol (Sigma Cat # E2257) and 50 ng/ml hrEGF (Sigma Cat # E9644) (G-Mat).

Fertilization medium was prepared using G1 without EDTA, rHSA and hyaluronan, but supplemented with 10 mM more NaCl, 2 mM caffeine, 2.5 mg/ml fatty

acid-free, crystalline bovine serum albumin (BSA), and 2 μg/ml heparin (G-Fert). Embryos were cultured in G1 for 3 days and G-2 for 3½ days. Procedures for embryo production are described next. G1 and G2 media were prepared in a different laboratory, therefore, upon arrival, a sample of each batch was placed on incubation and pH measured after equilibration. pH was adjusted to 7.2-7.3 in the sample and the correspondent amount of NaOH or HCl added to the total amount of medium. Another sample was taken and equilibrated to corroborate that the pH was in the desired range.

Table 3.1. Composition of G1/G2 sequential media.

Component (mM)	**G1**	**G2**
NaCl	90.1	90.1
KCl	5.5	5.5
Na_2HPO_4	0.25	0.25
Na-Citrate $2H_2O$	0.5	0.5
$NaHCO_3$	25.0	25.0
$CaCl_2$ $2H_2O$	1.8	1.8
D-Glucose	0.5	3.15
Alanyl-Glutamine	0.5	0.5
L-Lactate	10.5	10.5
Na-Pyruvate	0.32	0.1
$MgSO_4$	1.0	1.0
MEM aa sol. 100x (%)	1.0	1.0
BME aa sol. 50x (%)	0.0	2.0
rHuman SerumAlbumin (mg/ml)	2.5	2.5
Hyaluronan (mg/ml)	0.25	0.25
BME vit sol. 100x (%)	0.0	1.0
EDTA	0.01	0.0
Taurine	0.1	0.0
Gentamicin (μg/ml)	25	25
Osmolarity by formula	**273.2**	**278.1**

Bovine ovaries were collected post-mortem, trimmed, and rinsed twice in 0.15 M sterile NaCl solution (saline), and transported to the laboratory in saline at 23-25°C. Time lapse from collection to starting aspiration ranged from 3 to 5 h. The above temperature for transporting ovaries was selected as best for ovaries subjected to prolonged transportation times [118]. Oocytes were aspirated at room temperature from 2-8 mm follicles using a vacuum aspiration pump (GenX International) with a negative pressure ~80 mmHg. The suction system was attached to a two-way rubber stopper connected to a 50 ml high-clarity polypropylene conical centrifuge tube (Falcon Cat. # 352070). The other stopper opening had an 18ga x 1" needle (Monoject Cat # 8881201530) attached, so that when the follicle is pierced, the follicular fluid along with the oocyte is aspirated directly to the conical tube.

After collection, follicular fluid was allowed to settle for about 10 min to allow a pellet of oocytes and debris to accumulate in the bottom of the tube. The pellets were then aspirated with a 5 ml serological pipet, (Falcon Cat. # 357543) and placed in 60x15 mm standard cell culture dishes (Falcon Cat. # 353002) where G-Mops (20 mM Mops-buffered G medium for handling oocytes and embryos) was added in an approximate ratio of 1:3 (pellet:medium). Dishes were then kept at 38°C on a warming plate. Oocytes were searched using a stereomicroscope at 15-20x and transferred to 30x10 mm standard cell culture dishes (Falcon Cat. # 353001) containing warm G-Mops. After three rinses in G-Mops, oocytes were rinsed in G-Mat and transferred to G-Mat for maturation. Wells containing G-Mat were set up the morning of oocyte collection (at least 6 h before

transferring oocytes to wells) as follows: once the hormones and cysteamine were added to medium, polystyrene four-well multidishes (Nunc Cat. # 176740) were filled with 700 µl medium in each well and placed in the incubator (6% CO_2 in air, 38.5 °C, 100% humidity) for equilibration. When oocytes were ready, ~33 occytes were placed in each well, layering the wells with 250 µl of previously equilibrated-, embryo tested-light mineral oil (Fisher Cat. # 0121-4) immediately after oocytes were placed. Dishes were then placed back in the incubator for in vitro maturation for 23 h under the incubation conditions already mentioned.

Frozen straws of semen were thawed in a 35-37°C-water bath for 30 sec. The contents of the straw was placed over a 45-90% gradient of percoll (Sigma Cat. # P1644) in sperm-Talp (2 ml each) in a 15 ml high-clarity polypropylene conical centrifuge tube (Falcon Cat. # 352096). For 90% percoll preparation, a 10x solution of sperm-Talp without Ca^{++} and Mg^{++} salts was mixed in a 1:9 ratio with percoll; then 5 mM $NaHCO_3^-$ and Ca^{++} and Mg^{++} salts were added. Fourty five % percoll was prepared mixing equal amounts of 90% percoll and sperm-Talp whit Ca^{++} and Mg^{++} salts. The tube was then centrifuged 20 min at 400g; normal, motile sperm preferentially pass through the two percoll layers leaving most of the abnormal and non motile sperm behind as well as semen extender and seminal plasma. The supernatant was removed and 5 ml of G-Mops were added to re-suspend the semen pellet and centrifuge it again at 400g for 5 min to wash the pellet. After that, the supernatant was removed again and the pellet of semen left in an approximate volume of 100 µl. A sample of semen was taken from the pellet and diluted 20 times in distilled water and counted in two hemacytometer chambers to

determine the sperm concentration. Finally, the sperm concentration was adjusted to $5x10^6$ sperm/ml by adding the pertinent amount of G-Fert. During the first semen centrifugation, the oocytes were transferred from G-Mat to G-Fert, with rinses in G-Mops and G-Fert, once each. 430 µl G-Fert wells were prepared in advance to allow equilibration; this time, the oil was added at the time of preparation of dishes. 50 oocytes were added per well, in as little medium as possible (~ 20 µl); thus when 50 µl of semen was added, a final volume of ~ 500 µl was achieved with a final sperm concentration of $5x10^5$ sperm/ml. After addition of semen, heparin was added directly to the wells, from frozen stock, final concentration of heparin in G-Fert was 2 µg/ml. Sperm and oocytes were co-cultured 18 h to allow in vitro fertilization to occur, at the same incubation conditions as for maturation.

Before transferring presumptive zygotes from fertilization wells to culture conditions, cumulus cells were removed by vortexing as follows. Groups of 150 or fewer zygotes were transferred to G-Mops and then to a 0.6 ml siliconized microcentrifuge tube (Fisher Cat. # 02-681-330) in a small volume (~ 100 µl). The tube was vortexed at high speed for 60 s and rinsed with ~500 µl G-Mops, allowing 2-3 min for zygotes to sink to the bottom before taking them out with a glass pipette. Zygotes were cleaned up by passing them through a series of 5-6 G-mops drops to remove all cumulus cells from the zygotes. Zygotes then were transferred to four-well dishes (50 zygotes/500 µl well) of previously equilibrated, oil-layered G-1 where they remained 3 days under 6% CO_2, 5% O_2, 89% N_2 atmosphere, at 38.5 °C, 100% humidity.

At the end of culture in G-1, embryos were transferred to G-Mops, and 8- to 16-cell stage embryos were selected, discarding the unfertilized ova, and 2- and 4-cell stages. At this point the cleavage, and 8- to 16 cell percentages, were recorded. Embryos had to be rinsed thoroughly in G-Mops before being transferred to G-2 to avoid carry over of G-1 components, especially those which might be detrimental in late culture such as the EDTA. Embryos remained in G-2 3 ½ days up to the blastocysts stage under the same incubation conditions as for G-1.

3.1.2. CDM1/CDM2 system.

Table 3.2 presents the formulas for CDM1/CDM2 used in the experiments in which this system was selected, and which are a modification of previously published formulas [77]. Maturation, fertilization and handling media were based on CDM1 and CDM2 media, except for maturation, for which some experiments were done using TCM-199-based medium, whereas for other experiments maturation medium was based on CDM. TCM-199 maturation medium was prepared using 199 medium, HEPES modification (Sigma Cat # M-7528) supplemented with 1.0 mM glutamine and 0.2 mM Na-pyruvate. CDM-based maturation medium was CDM-1, but with 2 mM Glucose, and without EDTA (CDM-M). Both maturation media were supplemented with cysteamine and hormones (FSH, LH, 17 β estradiol, and hrEGF) as used in G-Mat.

Fertilization medium was prepared using CDM1 without EDTA, and supplemented with 14 mM NaCl, 2 mM caffeine, and 2 μg/ml heparin (CDM-F). Culture was performed in CDM1 (2½ or 3 days) and CDM2 (3½ or 4 days) formulated as in table

3.1.2. Appropriate versions of Hepes-buffered CDM's were used for handling steps; thus, Hepes-buffered CDM1 plus 20 μg/ml heparin (HCDM-M) was used for handling oocytes, and Hepes-buffered CDM1 and CDM2 (HCDM-1, HCDM-2) were used for handling of early and advanced embryos, respectively. Note that the Hepes concentration in Hepes-buffered media is the same as culture media. The lower concentration of sodium bicarbonate (5 mM) allows Hepes-buffered media to maintain pH for longer periods of time.

Table 3.2. Composition of CDM1/CDM2 sequential media.

Component (mM)	**CDM1**	**CDM2**
NaCl	72	76
KCl	6	6
KH_2PO_4	1	1
Na-Citrate $2H_2O$	0.5	0.5
$NaHCO_3$	25.0	25.0
HEPES (50% free acid, 50% Na salt)	20	20
$CaCl_2$ $2H_2O$	2	2
D-Glucose	0.5	2
Alanyl-Glutamine	1	1
Glycin	4.9	4.9
Myo-Inositol	2.77	2.77
L-Lactate	10	5
Na-Pyruvate	0.5	0.2
$MgSO_4$	0.5	0.5
MEM aa sol. 100x (%)	1.0	1.0
BME aa sol. 50x (%)	0.0	2.0
Bovine SerumAlbumin (mg/ml)	5.0	5.0
EDTA	0.01	0.0
Taurine	0.1	0.0
Gentamicin (μg/ml)	25	25
Osmolarity by formula	**274.9**	**275.2**

Ovary handling and oocyte aspiration were performed as described previously in this section. Oocyte searching and rinsing was performed using HCDM-M as a handling medium, as described previously. Maturation set up was done in the same way as for G1/G2 system, but for CDM-M, 50 oocytes were matured in 1 ml of medium, and no oil was used to overlay the wells. Oocytes were incubated 23 h at 5% CO_2 in air, 38.5 °C, 100% humidity.

The semen preparation protocol was the same as for G1/G2, but HCDM-1 was used for washing semen instead of G-Mops, and CDM-F instead of G-Fert was used to dilute the semen and for the fertilization per se. Oil was not used to overlay wells, and a small amount of nanopure water (~ 3 ml) was placed in the middle of the four well dish to avoid dehydration of medium when wells were outside of the incubator. Co-culture time was also 18 h., at the same incubation conditions as for maturation in CDM-M.

Removing cumulus cells was performed as for G1/G2, but using HCDM-1. Once cleaned, presumptive zygotes were transferred to four-well dishes (50 zygotes/500 µl well) of previously equilibrated CDM1 where they remained 2 days under 5% CO_2, 5% O_2, 90% N_2 atmosphere, at 38.5 °C, 100% humidity.

Before being transferred to CDM2, 8- to 16-cell embryos were selected, discarding the unfertilized ova, and 2- and 4-cell stages. After rinsing thoroughly in HCDM-1, embryos were transferred to CDM2, where they remained 5 days (embryos

were transferred to fresh CDM-2 after 48 h culture) under the same incubation conditions as for CDM1.

3.2. Superovulation procedures, embryo recovery and embryo transfer.

To optimize use of cows per unit time, a program named 'donor quick turn around' was used [119], consisting in the use of controlled internal drug releasing device EAZI-BREED™-CIDR® (Pfizer Cat # NDC 0009-5207-01) which is a Y-shaped silicone device that contains progesterone and is designed to be implanted in the vagina. With this method, cows can be superovulated at shorter intervals than normal (60-70 days), achieving an average interval between superovulations of 40 days, with good results. The whole superovulation scheme was performed following recommendations of Seidel et al. [120], and it is described next.

All procedures done with experimental animals were approved by the CSU animal care and use committee (Approval # 02-165A). Angus heifers and cows aged 2 to 9 years (n=23) were used as donors. Physical and reproductive soundness evaluations were performed to insure that they met minimal characteristics of health and reproductive soundness. Animals were confined in pens, and fed 10 Kg alfalfa hay daily with mineral blocks and water 'ad libitum'. Cows were observed twice a day for estrus. Estrus was synchronized using serial injections of 25 mg $PGF_2\alpha$ (Lutalyse® Pfizer Cat. # NDC 0009-0327-10) to form three groups of cows (8, 8, and 7) for superovulation purposes. Superovulation wase initiated at the middle of the estrous cycle (days 9 to 13 after estrus)

using a 4-day treatment period of FSH (Folltropin®-V Bioniche Cat. # DIN 00867357) combined with $PGF_2\alpha$, as shown in table 3.3.

Table 3.3. Superovulatory schedule using FSH and $PGF_2\alpha$

Day of treatment	Time[1]	Dose (mg)	Hormone
Day 1	a.m.	50	FSH
	p.m.	50	FSH
Day 2	a.m.	40	FSH
	p.m.	40	FSH
Day 3	a.m.	20	FSH
		25	$PGF_2\alpha$
	p.m.	20	FSH
		12.5	$PGF_2\alpha$
Day 4	a.m.	20	FSH
	p.m.	20	FSH
Day-5	a.m.		**Estrus**

[1]All the hormones were given i.m. at approximately 12 h intervals.

Artificial Insemination was performed 12 and 24 h after detected estrus using frozen-thawed semen at 2 x 10^7 sperm/dose. Depending on the experiment, embryos were collected either at 5, 7 or 7.5 days after estrus. Embryos were collected nonsurgically, using modified Dulbecco's phosphate-buffered saline (mPBS) [121], supplemented with 0.05% PVA as a surfactant. mPBS+PVA formula is presented in table 3.4. Uterine horns were flushed using a two-way Foley catheter (AB Technology Cat # ECE011) with an inflatable balloon behind the tip opening to fix the catheter in the uterine body to avoid medium reflux to the vagina. Flushing medium was collected in an embryo collection filter (AB Technology Cat. # ECE050) with an 80 μM pore size screen, which allows most of the flush debris to pass through the filter, but retains the embryos. Filter content was poured in a 100mm-square grid dish (Falcon Cat # 351012) and the filter jet-washed

with mPBS using an all-plastic 50 ml syringe (Henke Cat. # 4-500.000DO) with a 22GAx1½" needle (Monoject Cat # 8881250206) attached. Embryos were searched under stereomicroscope at 10-15X . As embryos were found, they were picked up using a fire-pulled and polished, sterilized 4 mm glass pipette (VWR Cat. # 80200-4) and rinsed in G-Mops. Finally, embryos were placed in G-2 drops under oil to wait under incubation conditions (6% CO_2-5% O_2,-89% N_2, 38.5 °C, 100% humidity) for further procedures.

Table 3.4. Composition of Dulbecco's phosphate-buffered saline (mPBS) supplemented with PVA.

Component	**mM**
NaCl	136.89
KCl	2.68
$Na_2HPO_4 - 7H_2O$	8.09
KH_2PO_4	1.47
D-Glucose	5.55
Na-Pyruvate	0.33
$CaCl_2 - 2H_2O$	0.9
$MgSO_4 - 7H_2O$	0.49
Streptomycin-sulfate (mg/ml)	50
Na-penicillin G (mg/ml)	67.7
PVA (mg/ml)	0.5
Osmolarity by formula	**307.68**

Management of cows for the "donor quick turn around" program was as follows:

1. On the day of embryo collection, cows were injected with 25 mg PGF2α and a CIDR® was inserted in the vagina.

2. 10-12 days later (timing depends on when to aim the next superovulation), the CIDR® was removed and 25 mg PGF2α injected again. The donor was expected in estrus 2-3 days later.

3. Donors were started on the next FSH treatment 9-13 days after estrus.

4. If the donor did not show estrus 3½ days after removing the CIDR®, another CIDR® was inserted at that time. The donor was started on FSH 5 to 7 days later, with removal of the CIDR® at the time of first PGF2α injection during the superovulation treatment.

Embryo transfer was performed by the nonsurgical, trans-vaginal method, as described by Seidel et al.[120] Briefly, embryos were rinsed twice in G-Mops and loaded in a previously G-Mops-rinsed, 0.25 cc, γ-radiated plastic straw (IMV Cat. # 005592), in a 4-cm medium column, separated with 1-cm air columns to medium columns in both ends of the straw. Once embryos were loaded, the straw was placed in a miniaturized transfer syringe (IMV Cat # ZA035) with coupled axial delivery sheath (IVM Cat # ZA133). A sanitary sleeve (IVM Cat. # ZA460) was placed over the transfer syringe to prevent contamination from the vaginal environment.

Recipient cows were selected based on synchrony (always within one day) with embryos (for day-5 transfers, cows with exact synchrony or older than embryos were preferred), presence of a corpus luteum showing appropriate parenchyma volume under ultrasound examination, and general healthy appearance at the time of transfer. After appropriate restraint and epidural anesthesia with 5 ml of 2% procaine, embryos were deposited as deeply as possible without undue manipulation in the uterine horn ipsilateral to the corpus luteum. The side of corpus luteum and number of embryos transferred were recorded as a reference for later embryo recovery.

3.3. Measurement of metabolic pathway activity.

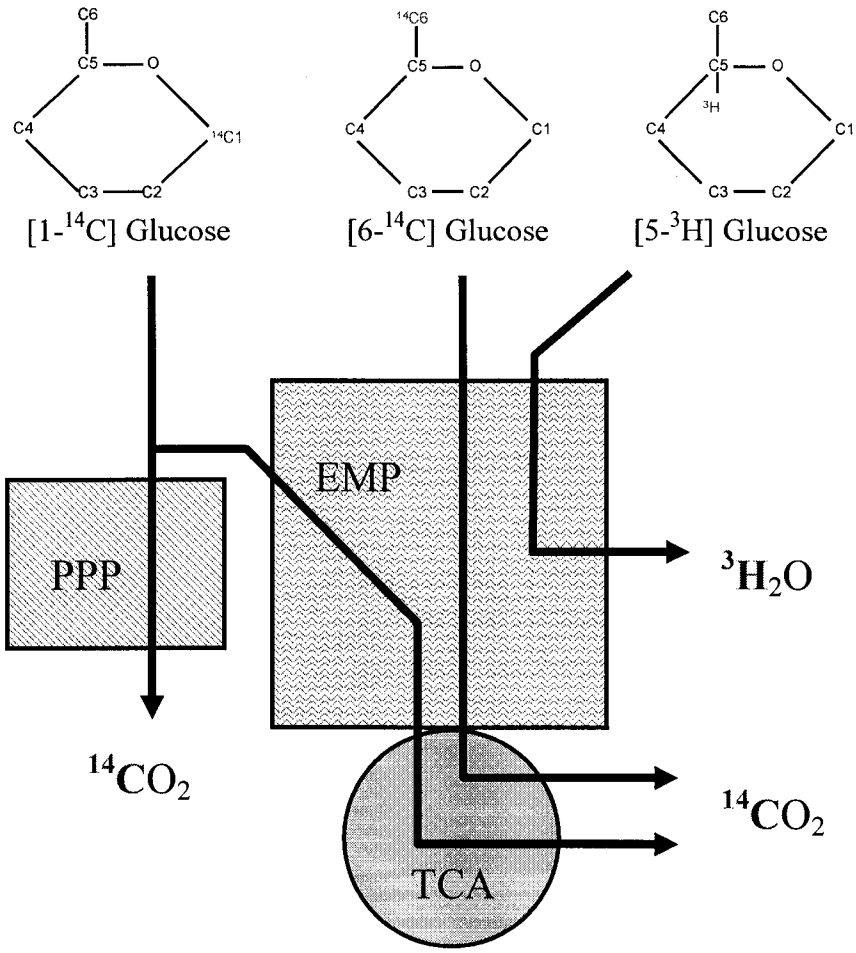
3.3.1. Radio-labeled substrates technique.

Flux of glucose through Embden-Meyerhof pathway (EMP), the tricarboxylic acid cycle (TCA), and the pentose phosphate pathway (PPP) was determined using radio-labeled glucose as initially described by O'Fallon and Wright,[122] with further modifications by Rieger et al.[84] Except for some slight modifications, procedures were are as described by Rieger. [123]

The approach to measure the fate of glucose through the metabolic pathways in embryos is to add to the culture medium, glucose labeled with ^{14}C at different carbon positions, and with ^{3}H at the hydrogen position 5, and recover the label in the CO_2 and H_2O molecules released by the embryo as metabolic end products. Glucose labeled at carbon 1 releases the label as CO_2 either if glucose is metabolized in the PPP or if it passes through the EMP and TCA, whereas label in carbon 6 is released in the EMP + TCA but not PPP. Glucose labeled in position 5 with tritium, will release the label in a H_20 molecule in the second to last step of EMP. Therefore, depending on the type of labeled glucose added, the label recovery will be indicative of specific pathway activity. Thus, EMP will be measured by collecting $^{3}H_2O$ released from [5-^{3}H] glucose, whereas PPP will be measured by collecting $^{14}CO_2$ produced from [1-^{14}C] glucose after subtracting the amount of flux through the TCA cycle using 6-^{14}C labeled glucose on a

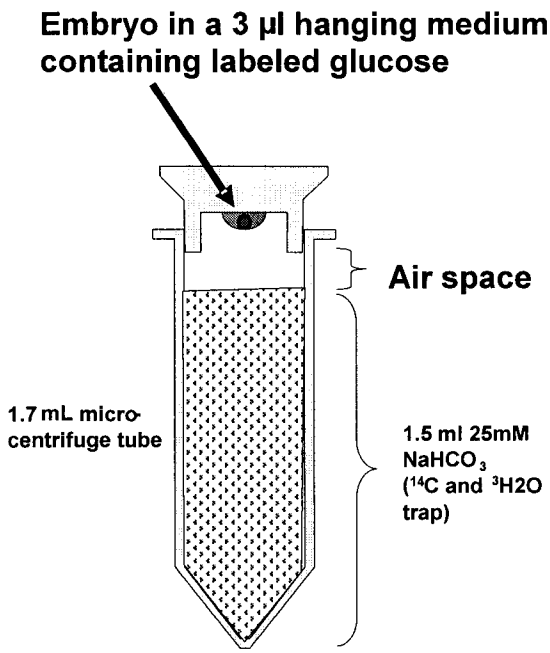
different subset of embryos from the same cohort, and adjusting for total glucose metabolized (EMP). Figure 3.1 illustrates the fate of the different labels used.

Figure 3.1. Fate of labeled molecules through embryo metabolism.



Embryos were incubated individually in a 3 µl drop of culture medium containing a known concentration of glucose, plus the labeled glucose, in a closed incubation chamber, as it is shown in figure 3.2.

Figure 3.2. Closed incubation chamber used to measure glucose metabolism in individual bovine embryos.



METABOLIC INCUBATION CHAMBER

Embryos to be evaluated were transferred to culture medium containing the radio label, with as little medium as possible, to avoid dilution of the label concentration. With a positive displacement microdispensor calibrated at 3 µl (Drummond Cat. # 3-000-105), each embryo was picked up along with 3 µl of medium and placed in the lid of a micro-centrifuge tube (VWR Cat. # 20170-355); the attachment band that joins the micro-centrifuge tube with the lid was cut out to facilitate loading embryos under the microscope. The tube was filled with 1.5 ml of 25 mM $NaHCO_3$ previously equilibrated at 6% CO_2, 5% O_2, 89% N_2. The lid was aligned to the tube, and a needle connected to a 6% CO_2, 5% O_2, 89% N_2 gas mixture tank was placed between the tube and the lid, gassing the air space for about 5 to 7 seconds to saturate the free space in the tube. The

lid was then quickly closed and the tube placed at 38.5 °C for 3 h. As the chamber is incubated, the $NaHCO_3$ traps the ^3H_2O and $^{14}CO_2$ released from the respective labeled glucose molecules. Previous experiments have demonstrated that with this technique, there is an adequate exchange between the labeled H_2O from the hanging drop and the normal H_2O from the trap;[122] also the CO_2 is adequately trapped by the $NaHCO_3$ solution in this closed system.[124] In this way, it is possible to measure two labels ([5-^{3}H] Glucose and [1-^{14}C] or [6-^{14}C] glucose) at the same time.

At the end of the incubation period, the tube was opened and the lid containing the embryo properly disposed. One ml of 25 mM $NaHCO_3$ solution was recovered from the tube and placed in a 10 ml fast turn cap, poly-Q vial (Beckman, Cat. # 566740) previously loaded with 200 µl 0.1 N NaOH to convert the bicarbonate into carbonate for counting.

Control preparations (sham) containing radio-labeled glucose but no embryos were included in each replicate to determine levels of passive diffusion and possible spontaneous breakdown of the labeled glucose, as well as the background of the liquid scintillation counter. Total radioactivity (total count) of glucose was determined in other control tubes by placing a droplet of identical size directly into the $NaHCO_3$ trap. One ml of bicarbonate solution was collected in these tubes after incubation and placed in scintillation vials with NaOH as with the embryo samples. The samples were stored at 4 °C until counting. For counting, 10 ml of Ecolite® liquid scintillation cocktail for aqueous samples (ICN, Cat. # 882475) were added to each scintillation vial. The samples

were then counted in a liquid scintillation β counter (Beckman, Model LS 5000 CE) for 5 min each. The raw counts per minute (c.p.m.) were automatically corrected for quenching and converted to disintegrations per minute (d.p.m.) by the machine. As two labeled substrates were used in the same sample (^{3}H and ^{14}C), samples were counted in two counting windows and corrected to obtain the d.p.m. for each label. The liquid scintillation counter used was programmed to do these determinations automatically.

At this point, the methodology for radiolabeled techniques has been described. In the following paragraphs the preparation of the medium with the label and the calculations to convert the d.p.m. into glucose concentrations will be explained.

The culture medium was the same one used for culturing the embryos subjected to evaluation (G-2), except unlabeled ("cold") glucose concentration was set at a lower level to allow an optimum ratio of cold and labeled glucose. Total glucose in medium was as follows: cold glucose: 0.2 mM; ^{3}H-glucose: 0.014 mM; ^{14}C-glucose: 0.5 mM, making a total 0.714 mM glucose concentration (explained later) .

Labeled glucose molecules used were: D-[1-^{14}C]glucose (specific activity 319 μCi/mg, radioactive concentration 200 μCi/ml, batch # 81); D-[6-^{14}C]glucose (specific activity 319 μCi/mg, radioactive concentration 200 μCi/ml, batch # 66); and D-[5-^{3}H]glucose (specific activity 110 mCi/ml, radioactive concentration 1 mCi/ml, batch # 57) (Amersham Pharmacia Biotech).

Ideally, it is recommended to add labeled compounds to medium at a specific activity of 0.25 μCi/μl. However, the specific activity of ^{14}C labels was not high enough to achieve that activity without having too much glucose in the medium (at a specific activity of 319 μCi/mg, 0.25 μCi/μl in the medium would be equivalent to 4.3 mM glucose). It was decided therefore to set the specific activity in 0.029 μCi/μl, which is equivalent to 0.5 mM glucose. To calculate the amount of label to dry (the solution in which the label is dissolved should not be present in the culture medium; therefore, it was necessary to dry the label by streaming the liquid with N_2 in a snap-cap tube [Falcon, Cat. # 35-2058] until it was dry), the amount of medium to be prepared and the radioactive concentration of the sample (200 μCi/ml) have to be considered. For example, to work with 30 embryos, it is necessary to prepare 3 x 30 = 90 μl, plus 30 μl for sham and total count samples and some extra medium, giving a total of 120 μl. For this we need 0.029 x 120 = 3.48 μCi. As the radioactivity of the sample is 200 μCi/ml, 17.4 μl of label will be dried and re-suspended in 120 μl of medium, giving a specific activity of 0.029 μCi/μl and a glucose concentration of 0.5 mM.

As ^{3}H label specific activity was much higher (110 mCi/mg), it was possible to add the label at the desired concentration of 0.25 μCi/μl, giving a glucose concentration of 0.014 mM. To calculate the amount to dry, and taking the same example of 120 μl of medium, the radioactive concentration of this label was 1.0 mCi/ml, and as we need 0.25 x 120 = 30 μCi, therefore, 30 μl of label was dried and re-suspended into 120 μl of medium to have the desired specific activity of 0.25 μCi/μl and a glucose concentration of 0.014 mM.

As previously mentioned, it is possible to add both the ^{14}C and the ^{3}H labels in the same medium to obtain separate data of the activity of each label in the same procedure. However, it is not possible to measure the two ^{14}C labels (1-^{14}C and 6-^{14}C) in the same sample; therefore, embryos in each replicate and each treatment were split into two sub-sets to measure each ^{14}C label in each group. ^{3}H label was included in all samples. For this reason, calculations for PPP which require data from 1-^{14}C and 6-^{14}C labels, are estimations, since the data are not obtained from the same embryos. Sub-sets of embryos were blocked to be similar in terms of stage and quality, and data were averaged per replicate, to give more objectivity to the calculations.

Calculations:

For ^{3}H label:

- Embryo activity (EA) = Embryo dpm – Sham dpm
- Labeled glucose concentration (LGC) in 3 µl drop: (0.014 mM x 3)/1x10^{6} = 4.2x10^{-8} mM = 4.2x10^{-11} M.
- As total counts ~ labeled glucose concentration, therefore we can calculate the embryo labeled glucose metabolism (ELGM) as the proportion embryo activity to the total count activity.
- ELGM = (EA x LGC)/total counts.

To obtain the absolute amount of glucose metabolized by the embryo (ETGM), we consider the total amount present in the medium (0.714 mM) and get the value, according with the data obtained with labeled glucose as follows:

- ETGM= (ELGM x 0.714)/0.014

Next, we correct the amount of glucose metabolized for the efficiency of product recovery of the system. To obtain this datum, an efficiency curve was obtained before the experiment. The methodology and results of this procedure are described at the end of this section. For this label, the product recovery efficiency was 71%, therefore:

- ETCGM= ETGM x .71

Finally, as the embryos were incubated for 3 h, the glucose metabolism value per embryo, per hour, was obtained by dividing the value by 3.

- ETCGM/h= ETCGM / 3

Embryo metabolism of glucose/h usually lies in the range of pico molar values; therefore it is convenient to convert molar concentration into pMol. This is achieved multiplying the value obtained times $1x10^{12}$.

- Glucose metabolism pMol/embryo/h = ETCGM/h x $1x10^{12}$.

For ^{14}C label:

Calculations were the same as with ^{3}H label, with the following modifications:

- LGC were: (0.5 mM x 3)/$1x10^{6}$ = $1.5x10^{-6}$ mM = $1.5x10^{-9}$ M.
- ETGM= (ELGM x 0.714)/0.5

For ^{14}C-Glucose the product recovery efficiency was 59%, therefore:

- ETCGM= ETGM x .59

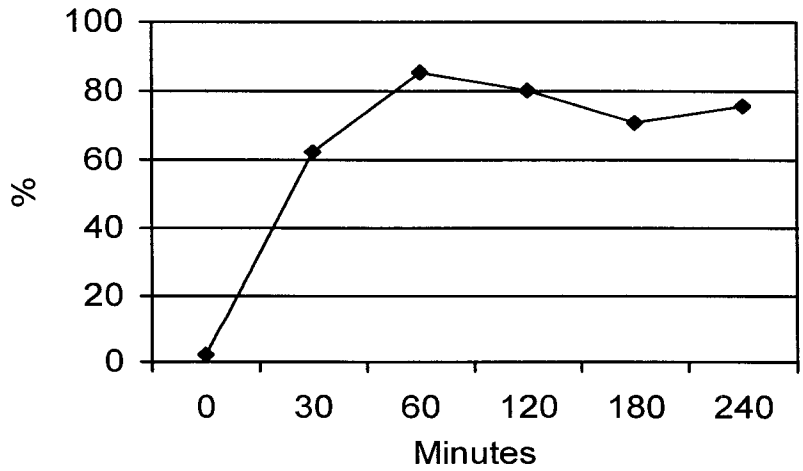
All the calculations above explained were handled in a spread sheet program.

Efficiency curve for product recovery:

This procedure was done only once to determine the proportions of $^{14}CO_2$ and $^{3}H_2O$ produced recovered in the bicarbonate trap. Figures 3.3 and 3.4 show data and efficiency curves for $^{14}CO_2$ and $^{3}H_2O$ labels, respectively.

$NaH^{14}CO_3$ and $^{3}H_2O$ were used as labels in known amounts in the 3 µl hanging drop. Calculations were done, considering the activity in µCi/µl, to add similar specific activity of labeled substrates as ^{3}H-Glucose and ^{14}C-Glucose (0.25 µCi/µl and 0.029 µCi/µl, respectively).

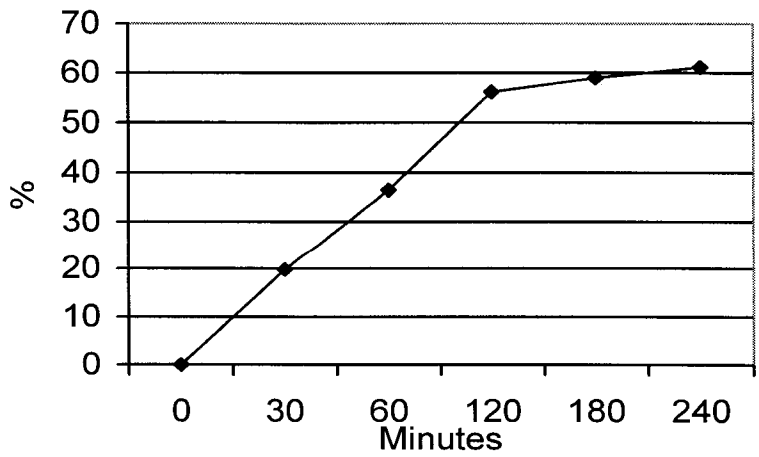
Figure 3.3. Product recovery and efficiency curve for label $^{3}H_2O$



^3H_2O had specific activity of 5 mCi/ml; to get a specific activity of 0.25 μCi/μl, we need to dilute out the sample 20 times. For 28-3 μl drops, a volume of 84 μl. Is needed. A total volume of 100 μl was prepared mixing 5 μl of ^3H_2O with 95 μl of H_2O. With $NaH^{14}CO_3$ specific activity of 50 μCi/ml, to get a specific activity of 0.029 μCi/μl in 100 μl 58 μl of $NaH^{14}CO_3$ were diluted with 42 μl of H_2O. $NaH^{14}CO_3$ and ^3H_2O will be exchanged with unlabeled molecules in the trap, when the closed system is placed in culture.

A series of hanging drop tubes were prepared and placed in culture following the same procedures described before (except that no embryo was placed in the hanging drop). Samples were taken at different time intervals for scintillation counting. The times of sampling were: 0, 30, 60, 120 and 180 and 240 min after the start of culture; four samples were taken for each time. Also four total count tubes were placed in culture. Samples for total counts were taken at time 240. This makes a total of 28 samples per label.

Figure 3.4. Product recovery and efficiency curve for label $NaH^{14}CO_3$



Calculations:

- Total counts = total recovery = 100% in the curve
- x-time recovery % = (x-time recovery value/total count recovery value) x 100.

Data from 4 replicates were averaged and a curve was plotted.

% of recovery was determined for the time we set for embryo evaluations (3 h) and that value was used as correction factor for metabolism calculations.

3.3.2. Quantitative microfluorometry.

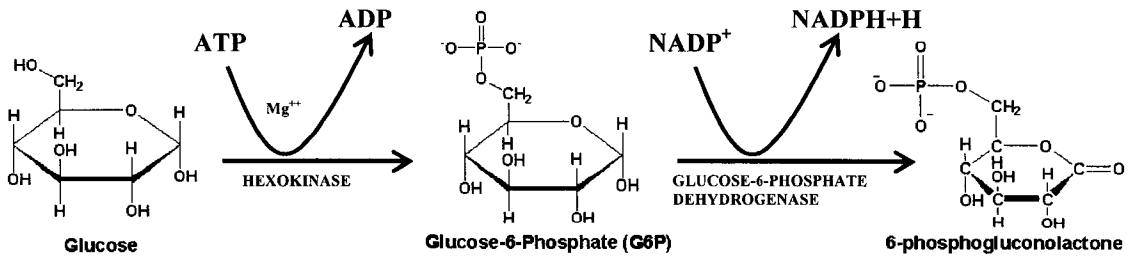
This technique was used to assess the glucose uptake and lactate production by individual embryos, and performed as described by Gardner et al.[82, 125] Individual embryos were placed in nanoliter droplets (200 nl for day-5 embryos, and 500 nl for day-7 and day-7.5 embryos) of medium, containing 0.5 mM glucose and no lactate, and incubated for 3 h in 60x15 mm cell culture dishes (day-5 embryos) or 0.6 ml microcentrifuge tubes (VWR Cat. # 20170-293) (day-7+ embryos), layered with light mineral oil under 6% CO_2, 5% O_2, 89% N_2, 38.5 °C, and 100% humidity incubation conditions. After culture, embryos were withdrawn and media samples kept frozen until analyzed.

Quantification of glucose and lactate were achieved using methods of enzymatic analysis employing the pyridine nucleotides NAD and NADP, which are oxidized or reduced in coupled reactions. When exposed to UV light (340 nm) only the reduced form of the nucleotides (NADH, NADPH) fluoresce. The intensity of fluorescence was quantified with a fluorescence microscope with photomultiplier and photometer

attachments (Leica, Fluorovert). Ten nl of an assay cocktail, containing specific substrates and co-factors required to optimize the specific reactions of interest, depending on the substrate to be determined (glucose or lactate) were pipeted on a siliconised (Sigmacote® Sigma Cat # SL-2)glass slide (Fisher Cat# 12-550-13) under heavy mineral oil (Sigma Cat. # M-8691). The initial fluorescence of the cocktail was determined and recorded. A 1 nl sample was then added to the cocktail drop, allowing time for the reaction to reach completion (3 min.). Fluorescence was measured again and the change in fluorescence quantified by a series of standard curves with linear relationship between known substrate concentration and fluorescence. Standard curves were generated each time samples were evaluated, using fresh substrates. The following cocktail components and reactions were used to determine glucose and lactate concentrations in samples.

Glucose. Assessment of this substrate requires a two-step reaction. First, glucose is phosphorylated to glucose-6-phosphate. Secondly, glucose is oxidized to 6-phosphogluconolactone. In this last reaction, NADP is reduced to NADPH. Figure 3.5 shows the enzymes and co-factors participating in these reactions.

Figure 3.5. Biochemical reactions involved in glucose concentration determination.



Glucose cocktail preparation:

Buffer:

- 2.52 g N-(2-Hydroxyethyl)piperazine-N'-3-propanesulfonic acid (EPPS)
- 10 mg Penicillin
- 10 mg Streptomycin
- pH was adjusted to 8.0 with 1M NaOH

Reagent cocktail:

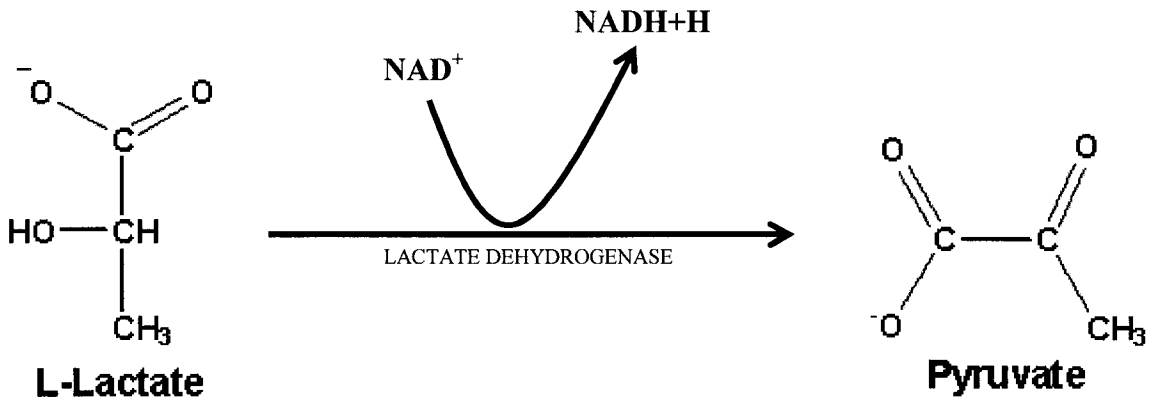
- 15 ml buffer
- 2 ml 5mM DL-Dithiothreitol (DTT)
- 2 ml 37 mM $MgSO_4$
- 1 ml 10 mM ATP
- 3 ml 10 mM NADP
- 1 ml hexokinase(EC 2.7.1.1, 12 U/ml) plus glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49, 6 U/ml)

  Reagent cocktail was frozen in aliquots and thawed as required.

As mentioned above, the reduced form of the nucleotide (NADPH) will fluoresce; therefore, the more glucose in the sample, the more fluorescence will occur. More fluorescence means less glucose was taken up by the embryo, and vice versa.

Lactate. This compound was assessed by a single reaction in which lactate was converted into pyruvate. In this case, NAD was reduced to NADH, as shown in Figure 3.6.

Figure 3.6. Biochemical reactions involved in lactate concentration determination.



Lactate cocktail preparation:

Buffer:

- 7.5 g glycine
- 5.2 g hydrazine
- 0.2 g EDTA
- Dissolve components in 49 ml of water; add 51 ml 2 M NaOH. (pH ~9.4).

Reagent cocktail:

- 0.45 ml buffer
- 0.40 ml water
- 25 µl lactate dehydrogenase (EC 1.1.1.27, 100 U/ml)
- 75 µl NAD^+ solution (40 mg/ml, from frozen aliquot)

Reagent cocktail must be prepared the day of assay.

The amount of lactate in the sample is directly correlated with the amount of fluorescence because of the higher concentration of NADH produced.

The percentage of glucose taken up and converted into lactate, was determined by assuming that one molecule of glucose produced 2 molecules of lactate. The noninvasive nature of the microfluorometry allowed later use of the same embryos for glucose flux determinations using radiolabeled substrates (Spindler et al., 1998).[126]

3.4. Short-term in vivo-culture of blastocyst-stage embryos to assess developmental competence.

Procedures were done following the methodology explained by Olson and Seidel.[127] Day-7 blastocysts were produced using the G1/G2 system, following procedures described in previous sections. Recipient cows were synchronized using 25 mg $PGF_2\alpha$ i.m. to be in estrus around the morning of the in vitro fertilization day. On day of transfer, cows were checked by trans-rectal ultrasound for the presence of a corpus luteum. Embryos were transferred to recipients (8-12 per cow) following the procedures explained in previous sections. Seven days later cows were flushed as previously described, using 2 l of saline + 0.05% PVA to recover day-14 embryos. Embryos at this stage are elongated or ovoid, ranging 0.1 to 3 cm length, so manipulation of uterus and handling of recovered medium has to be done using care to avoid breaking embryos in pieces, which can make it difficult or impossible to measure surface area. Once collected and isolated, embryos were measured using a calibrated eyepiece micrometer on a

stereomicroscope at 15 or 30x magnification. Visual evaluation was also performed, recording color (1= white, ... 4= tan), shape and if they were collapsed (1= not collapsed,... 4= completely collapsed).

3.5. Staining protocols for cell counting and lipid quantification

3.5.1. Staining protocols for cell counting.

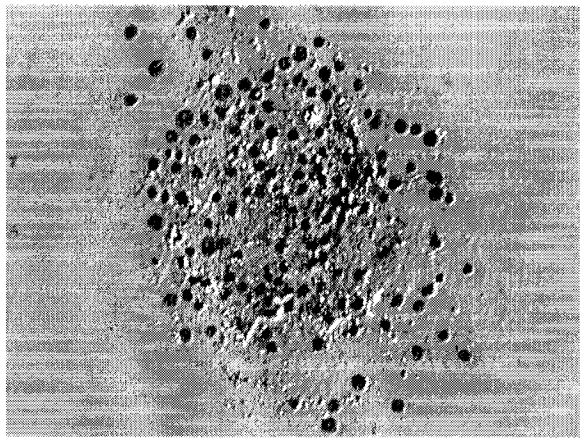
Three protocols were used for counting cells of embryos in the experiments described. The first two protocols allow simple counting of total cells, and the last allows differential counting of inner cell mass (ICM) and trophectoderm (TP) cells.

3.5.1.1. Giemsa staining.

For total cell counting, a previously reported[128] technique involving staining nuclei with giemsa stain was used. Embryos to be stained were placed in a warmed hypotonic solution of sodium citrate (0.9% w/v) in a glass multi-well dish for 10 to 15 min. Embryos were then transferred to a fixation solution prepared with absolute methanol, acetic acid and water in a 3:2:1 ratio, in a 35 mm plastic petri dish for about 1 min. The zona pellucida will start disintegrating, so embryos were monitored and removed as soon as the zona pellucida disappeared (indicated when the embryo detached from the bottom of the dish and started rolling around). Embryos were then transferred with as little as possible of fixation solution onto a slide previously cleaned with methanol. As the embryo plus fixation solution make contact with the slide, the embryo's cells will spread out on the surface; immediately, air was blown by mouth to dry the

solution as quickly as possible and have the embryo properly fixed in the slide. After fixation, slides were stained with a 5% Giemsa stain (Sigma Cat. # GS-500) solution in mPBS.[120] Slides were placed over paper towels with the embryos side up and staining solution was poured over the slides, covering the surface with the embryos, leaving the stain for at least 10 min and rinsing gently with deionized water. Slides were air-dried and counted under compound microscope at x100 to x 200 magnification. Nuclei of the cells of the embryo, plus a few metaphase plates were then counted. Figure 3.7 shows a stained embryo with nuclei that can be easily counted.

Figure 3.7. Bovine embryo fixed and giemsa-stained.



3.5.1.2. Hoechst stain.

Cells of embryos can be counted using Hoechst 33342 stain, without compromising structural integrity of embryos. This technique was, therefore, used when embryos were subjected to further staining procedures (lipid stain).

Embryos were incubated in 20 μg/ml Hoechst 33342 (Sigma Cat. # B-2261) in Hepes-buffered CDM for 20 min at 38 °C, and then fixed in 10% formalin in mPBS[120] at pH 7.4 for 2 h at room temperature. After washing in mPBS, they were stored at 4 °C, until cell counting. Cell numbers were determined by counting nuclei under a fluorescence microscope with a UV filter for Hoechst 33342 using a 20x objective. Note that autofluorescence from formalin will not interfere with the wavelength of Hoechst 33342. After counting, embryos were processed for lipid determination.

3.5.1.3. Differential staining.

A very simple technique described by Thouas et al.[129] was used. First, embryos were incubated in 500 μl of protein-free, Hepes-buffered CDM medium with 1% Triton X-100 (Sigma Cat. # T-9284) and 100 μg/ml propidium iodide (Sigma Cat. # P-4170), for 30 sec. Embryos then were transferred to 500 μl of a fixative solution 100% ethanol plus 25 μg/ml Hoechst 33258 (Sigma Cat. # B-2883), and stored at 4 °C overnight. The next day, embryos were transferred with as little fixation solution as possible to a 5 μl drop of glycerol on a siliconised glass slide. The glycerol drop plus embryo, was gently flattened with a coverslip until the embryo was moderately flattened to facilitate cell counting. Embryos were observed with a fluorescence microscope with excitation filters for red and blue fluorescence (460 nm) and red fluorescence only (560 nm). Hoechst 33258 stains all cells in the embryo, whereas propidium iodide stains only cells in the outer layer (presumably trophectoderm cells) which were permeabilized by the Triton

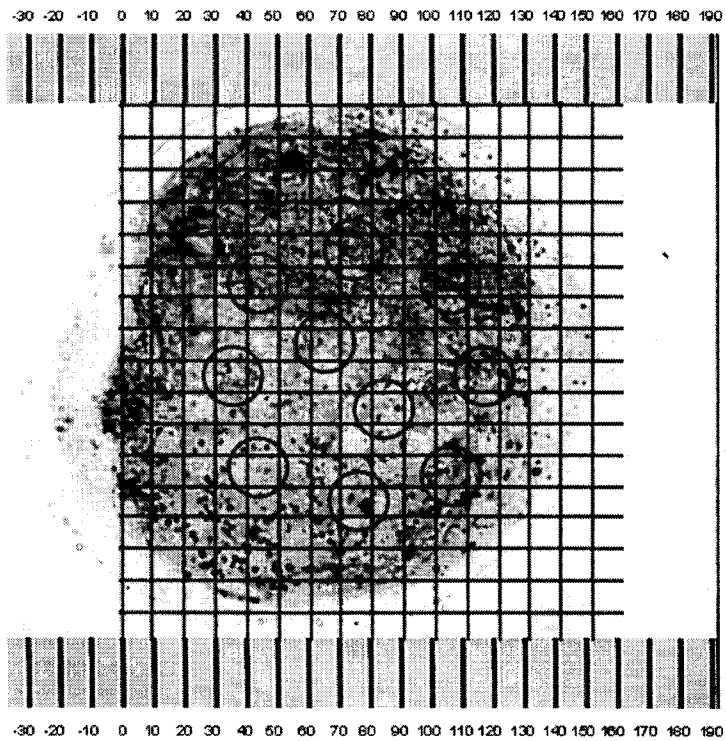
X-100. ICM number was obtained substracting propidium iodide stained cells (red) from the Hoechst stained cells (blue).

3.5.2. Staining procedure for lipid quantification.

Accumulation of lipid in the cytoplasm of embryonic cells was quantified by staining embryos with Sudan Black B (Sigma Cat. # S-2380), using a modification of the technique described by Abe et al. (2002).[11] Briefly, fixed embryos which were previously evaluated for total cell number by Hoechst staining, were rinsed in distilled water and then transferred to 50% ethanol/50% water for 2 min. Then, they were stained in about 4 drops of 1% (w/v) Sudan Black B in 70% ethanol for 0.5 to 1 min, in a rounded-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. Numbers of Sudan Black granules (lipids) were counted as follows. Embryos were visualized at 600x and the equatorial part of the embryo was set in focus (which generally was the part with the larger amount of lipid droplets in focus). A digital picture was taken and the image was digitally enhanced to equalize the dark tones and get a better appreciation of the Sudan Black B granules. Using digital tools, a grid was placed over the embryo image, and 10, 100 μM^2-squares were randomly selected (Figure 3.8.), counting the number of granules in focus, classified by diameter as small (<2 μM), medium (2 to 6 μM), and large (>6 μM) (Abe et al., 2002).[11] The results were reported as in number of small, medium, large, and medium+large granules, per $1X10^3$ μM^2. 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, it was calculated that 1 large droplet was equivalent to 8 medium droplets.. Note that this is a method to determine relative lipid granule concentration in the embryo, and served as a reference point to compare lipid accumulation in embryos from different treatments. There is no way to calculate the absolute amount of lipids in an embryo by using this methodology.

Figure 3.8. Measurement of lipids in an embryo stained with Sudan Black B.



CHAPTER 4

EFFECT OF PHOSPHATE AND CITRATE ON POST-COMPACTION DEVELOPMENT OF IN VITRO-PRODUCED BOVINE EMBRYOS

4.1. Summary.

In vitro-produced compact morulae were exposed to four levels of phosphate (1250, 250, 50 and 0 μM) and three levels of citrate (500, 50 and 0 μM) in a factorial arrangement. The experiment was replicated seven times using semen from three bulls. Embryos were evaluated blindly for % blastocysts, stage of development, morphological quality and lightness-darkness as a measure of lipid content. Main effects were not significantly different for any of the responses evaluated ($P>0.05$). However, there was a phosphate x citrate interaction for lightness-darkness after 36 h of culture ($P<0.04$). Citrate appeared to decrease lipid accumulation in the presence of phosphate. In the absence of phosphate, high concentrations of citrate increased lipid accumulation. This effect was no longer significant ($P>0.05$) at the 72 h evaluation. Higher concentrations of citrate and phosphate may delay lipid accumulation in bovine embryos in vitro.

4.2. Introduction.

This preliminary experiment was designed to find the best phosphate and citrate concentrations in the culture medium to be used in subsequent experiments done with the

CDM1/CDM2 system, in which a base concentration of 2 mM glucose was used in CDM2.

Glucose has been reported to be detrimental for mammalian embryo development when present in high or even moderate concentrations. [71, 86, 87] Developmental responses of embryos to glucose in the culture medium depend on stage and on interactions with other compounds.[9] The presence of the inorganic ion phosphate has been linked to the detrimental effect of glucose, at least in rodent embryos.[130] It is postulated that phosphate could disrupt mitochondrial activity of embryonic cells by excessively exacerbating the glycolytic pathway.[9] Some have postulated that the putative toxic effect of glucose is actually a toxic effect of phosphate, masked by the presence of the two compounds in the medium.[131] Recent improvements to culture media, like the addition of amino acids have overcome toxic effects of glucose, even in very sensitive species like the hamster.[131] However, there is no consensus on whether phosphate should be included in the medium or what is the best concentration for different species of embryos.

Lack of citrate in culture medium is another postulated cause of exacerbated glycolysis, since this tricarboxylic acid controls glycolytic flux allosterically.[132] Intracellular citrate could be lost by efflux if there is insufficient citrate in the surrounding medium, thus requiring extracellular availability to avoid this effect.

Inorganic phosphate is required for a wide array of enzymatic reactions and cellular signaling pathways. It is also essential for synthesis of nucleic acids and adenosine

phosphate compounds, among many others.[132] It is therefore essential for culture media. Citrate, an intermediate product of the tricarboxylic acid cycle, allosterically inhibits the enzyme phosphofructokinase (PFK); phosphate has opposite effect, since stimulates the activity of PFK.[132] As citrate has the capacity to control the glycolytic rate, it is postulated that citrate could overcome the putative toxic effects of phosphate in culture media containing glucose and amino acids.

4.3. Objective.

The objective was to evaluate effects of different levels of phosphate and citrate on post-compaction development of bovine embryos cultured with 2 mM glucose.

4.4. Materials and Methods.

Slaughterhouse oocytes were matured in TCM-199, and fertilized and cultured in the CDM1/CDM2 culture system as described in General Materials and Methods. At 2.5 days of post-fertilization culture in CDM1, plus 1.5 days culture in CDM2, compact morulae were selected and randomly allocated to treatments (8-12 morulae per subclass) for an additional 72 h of culture in CDM2. Phosphate and citrate were studied in a factorial design, with four levels of phosphate (1250, 250, 50 and 0 μM) and three levels of citrate (500, 50 and 0 μM). The experiment was replicated seven times, using three different bulls. Embryos were visually evaluated under a stereomicroscope at 50x. Evaluations were performed at 36 and 72 h of treatment culture. Each of the ~ 840 embryos was evaluated individually, but data within each well were averaged to provide appropriate experimental units. Wells were coded, and responses were evaluated

"blindly". Responses evaluated were % blastocysts at the end of culture period; stage of development at 36 and 72 h (5= early blastocyst,...8= hatched blastocysts); morphological quality at 36 and 72 h (1= excellent, ...3= poor); a subjective evaluation on a scale of 1-4 of the degree of lightness-darkness at 36 and 72 h (probably related to lipid accumulation in the embryo) (1= lighter,...4= darker); and quality of the inner cell mass at 36 and 72 h (1= large, compacted inner cell mass,...4= scarce, loose inner cell mass). Embryos then were fixed, giemsa-stained as described in General Materials and Methods, and cells counted. Data were analyzed by ANOVA, with the general linear models (GLM) procedure of the Statistical Analysis System (SAS),[133] using a factorial design, with factors phosphate (4), citrate (3) and bulls (3). Normality was tested on percentage responses, from plots of studentized residuals versus predicted values; arcsin $\sqrt{y}$ transformation was done, when necessary, to achieve normality. Means were separated by Tukey's HSD method.

4.5. Results and discussion.

Inspection of studentized residuals versus predicted values plots of percentage responses, revealed homogeneous distribution of variances. Therefore, it was decided to analyze data without transformation.

A bull effect was observed ($P<0.05$) for stage at 36 and 72 h (Table 4.1). Embryos produced using sperm of bull # 2 developed faster than those produced with sperm of bulls # 1 and 3. These bulls were chosen as a sample of the population of bulls rather than any interest in assessing the specific bulls used.

There were no significant main effects for phosphate or citrate for any response studied, at either 36 or 72 h evaluations (P>0.05) (tables 4.2 and 4.3); however, there was a phosphate x citrate interaction for darkness at 36 h (P=0.039)(Figure 4.1). Citrate appeared to decrease lipid accumulation in the presence of phosphate. In the absence of phosphate, high concentrations of citrate increased lipid accumulation. This effect was no longer significant (P>0.1) by 72 h.

Table 4.1. Least-squares means±SEM for main effects of the three bulls evaluated.

Response	Bull # 1	Bull # 2	Bull # 3
% Blastocysts	44.3±2.9	48.3±2.8	45.6±4.5
Stage 36 h*	5.7±0.14[a]	6.3±0.17[b]	5.7±0.11[a]
Stage 72 h	6.5±0.11[c]	6.9±0.08[d]	6.7±0.10[cd]
Quality 36 h#	1.37±0.05	1.29±0.05	1.29±0.07
Quality 72 h	1.84±0.12	1.60±0.08	2.01±0.16
Lightness 36 h^	2.00±0.08	2.00±0.08	1.91±0.08
Lightness 72 h	2.22±0.09	2.17±0.07	2.29±0.08
ICM 36 h@	1.69±0.10	1.68±0.10	1.47±0.09
ICM 72 h	1.88±0.10	1.78±0.07	2.04±0.16
Number of cells	95.8±6.2	89.3±5.0	113.5±8.8

a, b. Values within rows without common superscripts differ (P<0.05).
c, d. Values within rows without common superscripts differ (P<0.01).
* Early blastocyst= 5,... hatched blastocyst= 8.
# Excellent= 1, ... poor= 3.
^ Lighter=1, ...darker= 4.
@ Big, compact ICM= 1, ...scarce, loose ICM= 4.

Table 4.2. Least-squares means ±SEM for main effects of phosphate concentrations[a].

	Phosphate			
Response	**0 µM**	**50 µM**	**250 µM**	**1250 µM**
% Blastocysts	47.6±3.8	46.4±3.5	49.5±3.6	42.2±4.5
Stage 36 h*	6.3±0.30	5.8±0.12	5.9±0.10	5.8±0.18
Stage 72 h	6.6±0.11	6.8±0.09	6.8±0.10	6.7±0.15
Quality 36 h#	1.29±0.07	1.29±0.08	1.25±0.05	1.36±0.08
Quality 72 h	1.81±0.11	1.69±0.14	1.77±0.13	1.85±0.18
Lightness 36 h^	2.01±0.11	1.99±0.08	1.95±0.08	1.96±0.10
Lightness 72 h	2.14±0.10	2.17±0.09	2.23±0.07	2.32±0.11
ICM 36 h@	1.66±0.07	1.50±0.10	1.69±0.16	1.64±0.12
ICM 72 h	1.91±0.08	1.83±0.13	2.00±0.13	1.76±0.15
Number of cells	97.1±7.0	98.9±9.5	101.9±6.9	93.7±7.6

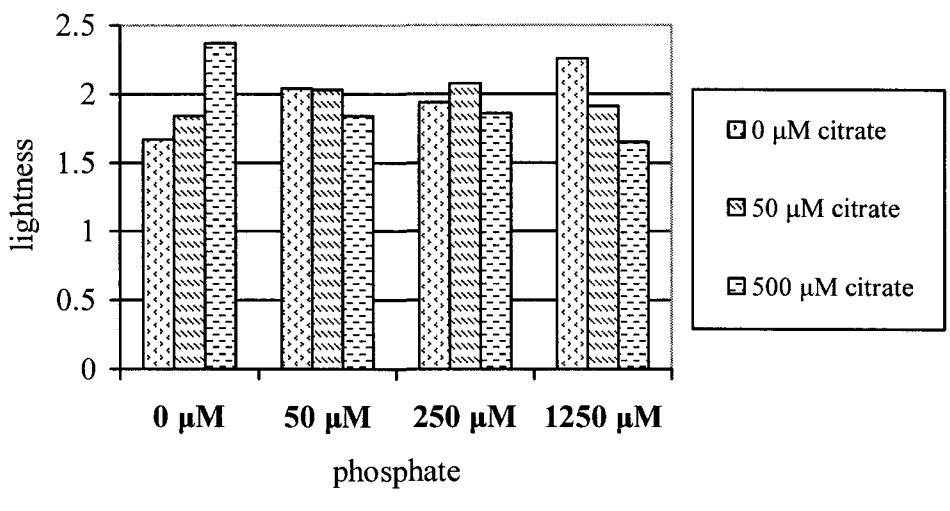
[a]No significant differences (P>0.1).
* Early blastocyst= 5,... hatched blastocyst= 8.
# Excellent= 1, ... poor= 3.
^ Lighter=1, ...darker= 4.
@ Big, compact ICM= 1, ...scarce, loose ICM= 4.

Table 4.3. Least-squares means ±SEM for main effects of citrate concentrations[a].

	citrate		
Variable	**0 µM**	**50 µM**	**500 µM**
% Blastocysts	47.0±3.8	45.5±2.9	46.9±3.3
Stage 36 h*	6.1±0.19	5.9±0.14	6.0±0.17
Stage 72 h	6.8±0.09	6.7±0.13	6.6±0.07
Quality 36 h#	1.35±0.07	1.34±0.05	1.19±0.05
Quality 72 h	1.63±0.09	1.87±0.12	1.83±0.14
Lightness 36 h^	2.03±0.08	1.96±0.08	1.95±0.08
Lightness 72 h	2.13±0.08	2.24±0.08	2.27±0.08
ICM 36 h@	1.66±0.11	1.68±0.13	1.54±0.07
ICM 72 h	1.90±0.09	1.76±0.10	1.97±0.13
Number of cells	101.5±6.8	93.9±8.1	98.7±4.7

[a]No significant differences (P>0.1).
* Early blastocyst= 5,... hatched blastocyst= 8.
# Excellent= 1, ... poor= 3.
^ Lighter=1, ...darker= 4.
@ Big, compact ICM= 1, ...scarce, loose ICM= 4.

Figure 4.1. Least-squares means+/- SEM for lightness^ at 36 h for the interaction phosphate x citrate



	phosphate			
citrate	0 µM	50 µM	250 µM	1250 µM
0 µM	1.67±0.20	2.04±0.17	1.94±0.17	2.26±0.17
50 µM	1.84±0.17	2.03±0.20	2.08±0.17	1.91±0.17
500 µM	2.37±0.17	1.84±0.17	1.86±0.17	1.65±0.20

Interaction significance, P= 0.039.
^1= lighter, ... 4= darker.

Except for lightness after 36 h of culture, in vitro-produced bovine embryos were not affected during the post-compaction period by changes in the concentrations of phosphate and citrate studied, as measured by development of blastocysts, cell numbers/embryo and several other responses. Overall, 46% of the morulae developed into blastocysts with an average of 98 cells each. This remarkable metabolic flexibility of mammalian embryos has also been noted numerous times by others.[50, 134] It does appear, however, that the higher concentrations of citrate and phosphate studied delay lipid accumulation in bovine embryos in vitro. As phosphate is required for a variety of functions in embryos, and since it was demonstrated to be non-toxic when citrate is present, and as lighter embryos where produced with 1.25 mM phosphate and 0.5 mM citrate, it is advisable to include phosphate at the highest concentration studied, along with the highest citrate concentration evaluated, and this was done in subsequent experiments using the CDM1/CDM2 culture system.

CHAPTER 5

EFFECT OF FOUR METABOLIC REGULATORS ON POST-COMPACTION DEVELOPMENT OF IN VITRO-PRODUCED BOVINE EMBRYOS, CULTURED AT DIFFERENT GLUCOSE CONCENTRATIONS

5.1. Summary.

Four experiments were done to evaluate the toxicity/beneficial effects of four metabolic regulators on embryo development at different glucose concentrations. Phenazine ethosulfate (PES), phloretin (PL), pyrroline-5-carboxylate (P5C), and sodium azide (NaN_3) were included at four doses (0, 0.1, 0.3, and 0.9 μM; 0, 30, 90, and 270 μM; 0, 9, 27, and 81 μM; and 0, 3, 9, and 27 μM, respectively). For all experiments, four glucose concentrations (0, 0.5, 2 and 8 mM) were evaluated in a factorial arrangement with four treatment concentrations. Experiments were replicated several times with semen from each of two or three bulls. Compact morulae produced in vitro using the CDM1/CDM2 culture system were allocated to treatments (the experimental unit was the average response of 8-12 embryos in a 500 μl well), and evaluated blindly at 36 and 72 h for stage of development, morphological quality, lightness-darkness (putative indicator of lipid content), and inner cell mass quality. Percentage of blastocysts and number of cells were evaluated at the end of culture period. The total number of morulae used was around

1440/experiment (~5760 morulae grand total). Bull main effects and interactions were found for several responses among the experiments.

The higher PES dose evaluated (0.9 μM) resulted in poorer development than treatments with lower or no PES included ($P<0.05$). A bull x PES interaction effect was found for lightness at 36 h; one bull produced darker embryos when cultured in 0.3 μM PES, whereas the other two bulls produced darker embryos with 0.9 μM PES ($P<0.03$). Presence of PL was detrimental for many of the responses studied, especially at the two highest doses. 30 μM PL resulted in a blastocyst rate 8% lower than the control ($P<0.1$); however this treatment was superior in other qualitative characteristics evaluated. Culture with 270 μM PL was detrimental to embryos for many responses, but embryos produced in this treatment were much lighter than those in other groups ($P<0.01$). This feature was not found in embryos cultured with high glucose (8 mM) ($P<0.04$ for the interaction glucose x PL). Except for a possible protective effect of P-5-C when embryos were cultured with 8 mM glucose ($P<0.08$ interaction P5C x glucose for quality at 36 h), P5C had little effect on post-compaction embryos. Inclusion of 27 μM NaN_3 in culture medium resulted in lighter embryos at 72 h compared to lower or no NaN_3 levels ($P<0.01$). Except for embryos cultured in 3 μM NaN_3, all NaN_3 levels resulted in more advanced and better quality embryos at 36 h of culture, when cultured in 2 mM glucose, but not other levels of glucose (interaction effects of $P<0.05$ for both responses). Several bull effects were found in these experiments, indicating that the bull used for semen for IVF can affect the quantity and quality of resultant blastocysts in addition to fertilization rates. The dose-response information obtained for these four molecules regulating

glucose metabolism should be useful for discriminating between effective and toxic doses for future experiments.

5.2. Introduction.

Phenazine ethosulfate and pyrroline-5-carboxylate are electron acceptors for reduced nicotinamide adenine dinucleotide phosphate (NADPH). By oxidizing NADPH to NADP, these compounds activate the NADP-requiring enzymes, including the first two enzymatic reactions in the oxidative phase of the PPP, consequently stimulating this pathway.[105] These compounds increase PPP activity in mammalian pre-implantation embryos. [122, 124] Glucose metabolism is abnormal in in vitro-produced embryos at pre- and post-compaction stages (e.g. the "Crabtree" effect).[32] Under in vitro conditions, post-compaction embryos show an exacerbated activity of the glycolytic pathway (glucose to lactate), even in presence of ample oxygen, a phenomenon known as "aerobic glycolysis".[7] Embryos that metabolize a high percentage of glucose uptake through glycolysis can compromise their developmental capacity.[19] A possible explanation is that under these conditions, too little glucose is partitioned to the PPP, which is important for its biosynthetic role in the cell.[97, 98] It is proposed that addition of PES and P5C at the post-compaction period could induce more balanced glucose utilization by stimulating the PPP, and consequently improve the production and quality of in vitro-produced bovine embryos.

Sodium azide, an inhibitor of cytochrome oxidase a3 in the electron chain transport cascade, improves in vitro development of bovine and porcine embryos[102, 104] when used in low concentrations by sub-acutely down-regulating mitochondrial ATP production. It was suggested that partial inhibition of mitochondrial ATP production by NaN_3 increases the relative contribution of glycolytic ATP, establishing an appropriate redox state which favors appropriate glucose metabolism.[102]

Glucose can gain entry into the embryo by three possible mechanisms: passive transport down a concentration gradient through water channels; facilitated transport using members of the Na^+ -independent glucose transporter glycoproteins (GLUT's); and active transport using a Na^+ -coupled carrier system.[110, 135] As the participation of active glucose transport systems in the pre-implantation embryo remains uncertain, the main mechanisms driving glucose into the embryo could be via concentration gradient and facilitated transport through GLUT's, particularly GLUT1, GLUT2 and GLUT3.[136] Phloretin (PL), a member of bioflavonoid family, which are compounds that occur naturally in both flowering and non-flowering plants, is a molecule with a flexible structure that mimics glucose and binds with high affinity to the outward face of the GLUT transporters, hence inhibiting their activity.[107] PL can drastically reduce glucose utilization in mammalian embryos.[108, 109] Partial inhibition of glucose uptake by PL overcame the developmental block of CF-1 mouse embryos (a random-bred mouse strain whose embryos have a developmental block at the 2-cell stage in many embryo culture media) cultured in a medium containing glucose and phosphate.[110] In another study, in which glucose metabolism of capacitated sperm was examined, addition of 0.5 mM PL

increased the proportion of glucose metabolized via the PPP by reducing the glycolytic rate significantly, but maintaining the PPP rate at the same level as controls.[111] The mechanism by which PL caused that shift in glucose metabolism, rather than merely inhibiting glucose uptake is not understood. It is unknown if PL could have the same effect on post-compaction bovine embryos, and hence improve bovine embryo development.

5.3. Objective.

The objective was to evaluate the toxicity/beneficial effect of four metabolic regulators on embryo development, in media with different glucose concentrations.

5.4. Materials and Methods.

Abattoir-derived oocytes were matured, fertilized and cultured in the CDM1/CDM2 system as described in General Materials and Methods. After 2½ days post-fertilization culture in CDM1, plus 1½ days culture in CDM2, compact morulae were selected and randomly allocated to treatments (8-12 morulae per subclass; total ~ 5760 morulae in the four experiments) for an additional 72 h of culture in CDM2. Four doses of each of the metabolic regulators and four levels (0, 0.5, 2, and 8 mM) of glucose were studied in a factorial design. Two to four bulls (A, B, C, and D) were used within each experiment. Metabolic regulator doses and preparation, as well as specific bulls used and replication structure for each experiment were as follows.

Phenazine ethosulfate. PES (Sigma, cat. # P-4544) was diluted in nanopure water in a 2000x stock and added to CDM2 at the corresponding concentration. Initially, PES was used at concentrations 0, 10, 30, and 90 μM, similar to those reported in other experiments.[122, 124] These doses, however, were lethal for embryos and were replaced by lower doses 0, 0.1, 0.3, and 0.9 μM. This experiment was replicated 10 times, three times each with bulls A and C, and 4 times with bull B.

Phloretin. A 2000x stock solution for each dose of PL (Sigma, cat. # P-7912) was prepared in 95% ethanol to have the same ethanol concentration (1/2000) in CDM2 for each treatment. Control groups were prepared with 1/2000 ethanol, to ensure equal culture conditions for all treatments. PL doses used were 0, 30, 90, and 270 μM. This experiment was replicated three times each with bulls A and B, and twice using bull C.

Pyrroline-5-carboxylate. P5C (Sigma, cat. # P-3545) was included at 0, 9, 27 and 81 μM in CDM2 from a 2000x stock prepared in DMSO. Treatments were adjusted to have the same DMSO concentration. The P5C experiment was replicated five times with bull B and four times using bull D.

Sodium azide. NaN_3 (Sigma, cat. # S-8032) was diluted with nanopure water and added to treatments at final concentrations of 0, 3, 9 and 27 μM. Bulls A, B, and D were used three times each, for a total of nine replicates.

Embryos were evaluated individually at 36 and 72 h of treatment culture, but data were averaged within each well to provide appropriate experimental units. Wells were coded and responses were evaluated "blindly". Percentages of blastocysts and cell numbers were evaluated at the end of culture period. The following responses were evaluated at 36 and 72 h of culture: stage of development (5= early blastocyst, 6=full blastocysts, 7= expanded blastocyst, 8= hatched blastocysts); morphological quality (1= excellent, 2= fair, 3= poor); a subjective evaluation on a scale of 1-4 of the degree of lightness-darkness (probably related to lipid accumulation in the embryo) (1= lighter,...4= darker); and quality of the inner cell mass (1= large, compacted inner cell mass, 2= large, less compact inner cell mass, 3= medium, loose inner cell mass, and 4= scarce, loose inner cell mass).

After the last evaluation, embryos were fixed, and stained with giemsa as described in General Materials and Methods, and cells were counted. Data were analyzed by ANOVA, with the general linear models (GLM) procedure of the Statistical Analysis System (SAS),[133] using a factorial design, with factors metabolic regulator (4), glucose (4) and bulls (2 or 3, depending on the experiment). Plots of studentized ranges versus predicted values were done for each percentage response, to check for normality. If necessary, arcsin $\sqrt{y}$ transformation was done to achieve normality. ANOVA was done on transformed data to obtain P-values, but least-squares means and standard errors were obtained from non-transformed data. Tukey's HSD procedure was used as multiple range test.

5.5. Results.

Least-squares means and standard errors of the means for all the responses, for main effects of metabolic regulators and glucose, and for the metabolic regulator x glucose significant interactions are presented in Appendix 1. After inspection of studentized residuals versus predicted values, it was determined that the % blastocysts response for the P5C experiment required the arcsin $\sqrt{y}$ transformation. ANOVA of transformed data was used to determine P-values; least squares means±SEM were computed from non-transformed data.

Phenazine ethosulfate. There were differences among the three bulls evaluated (A, B, and C) for six of the ten responses evaluated (Table 5.1). Semen from bull A produced a higher percentage of blastocysts and more advanced embryos at 36 h than bulls B and C ($P<0.02$ and $P<0.01$, respectively). At 72 h of culture, bulls A and B produced more advanced embryos than bull C ($P<0.01$). Bull B produced lighter embryos than bull C at 36 and 72 h of culture, with bull A intermediate in value ($P<0.05$). Finally, semen from bulls A and B resulted in embryos with more cells than bull C semen ($P<0.01$).

Table 5.1. Least-squares means±SEM for significant main effects of the three bulls evaluated.

	BULLS		
RESPONSE	**A**	**B**	**C**
Stage 36 h*	6.0±0.10^{ab}	6.3±0.07^{a}	5.8±0.07^{b}
Lightness 36 h^	1.92±0.09^{a}	2.09±0.06^{ab}	2.20±0.06^{b}
Stage 72 h	6.82±0.07^{a}	6.86±0.05^{a}	6.54±0.05^{b}
Lightness 72 h	2.06±0.08^{a}	2.25±0.06^{ab}	2.33±0.06^{b}
% blastocysts	32.5±2.9^{a}	42.5±2.1^{b}	36.8±2.1^{ab}
Number of cells	109±5.2^{a}	110±3.7^{a}	94±3.7^{b}

a, b. Values within rows without common superscripts differ, ($P<0.05$).
* Early blastocyst= 5,... hatched blastocyst= 8.
^ Lighter=1, ...darker= 4.

Embryos cultured in 0.9 μM PES produced fewer blastocysts (P<0.04) and tended to have inferior ICM quality at 36 h evaluation (P<0.06) than embryos cultured with no PES. Treatments with 0.1 and 0.3 μM did not differ from the control. Embryos cultured with 0.9 μM PES had also fewer cells than the other treatments (P<0.01). (Table 5.2).

Main effects for lightness score at 72 h were 2.06±0.08, 2.23±0.07, 2.38±0.08, and 2.19±0.07 for 0, 0.5, 2, and 8 mM glucose, respectively. Except for this significant effect (P<0.04), different levels of glucose did not affect embryo responses. Embryos cultured with no glucose were lighter than embryos cultured with 2 mM glucose (P<0.05). Lightness of embryos in 0.5 and 8 mM glucose treatments was not different from the other glucose treatments.

Table 5.2. Least-squares means±SEM for significant main effects of the four PES doses evaluated.

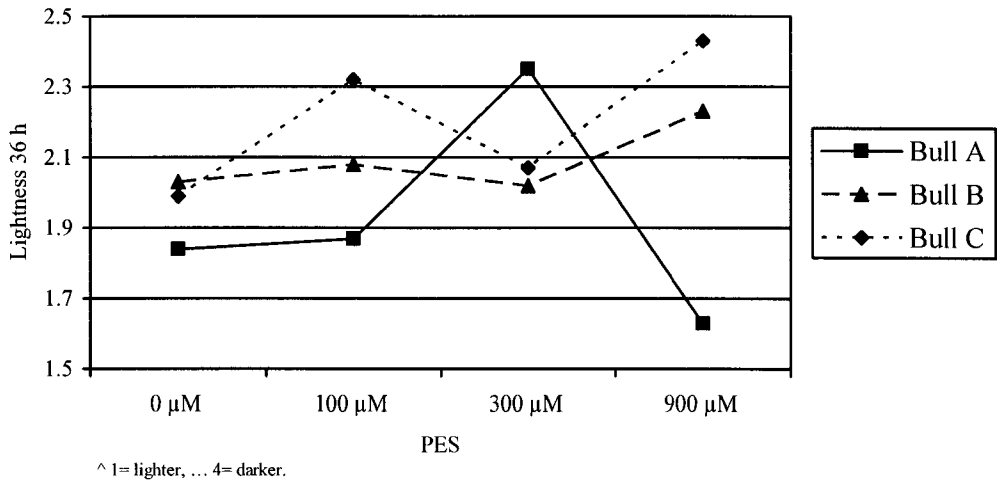
	PES (μM)			
RESPONSE	**0**	**0.1**	**0.3**	**0.9**
ICM 36 h@	1.24±0.07[a]	1.42±0.08[ab]	1.25±0.08[ab]	1.51±0.09[b]
% blastocysts	40.6±2.8[c]	39.2±2.8[cd]	39.1±2.8[cd]	30.3±2.8[d]
Cell number	116±4.9[e]	110±4.8[ef]	110±4.9[ef]	82±5.1[f]

a, b. Values within rows without common superscripts differ, (P<0.06).
c, d. Values within rows without common superscripts differ, (P<0.04).
e, f. Values within rows without common superscripts differ, (P<0.01).
@ Big, compact ICM= 1, ...scarce, loose ICM= 4.

There was a bull x PES interaction effect for lightness at 36 h (P<0.03). Bull A produced darker embryos when cultured in 0.3 μM PES, whereas the other two bulls produced darker embryos with 0.9 μM PES. (Figure 5.1).

Phloretin. Embryos fertilized with sperm from bull C had higher average cell numbers (137±6) than embryos from bull A (112±5), with embryos from bull B at an intermediate value (126±5) (P<0.01).

Figure 5.1. Least-squares means for lightness^ at 36 h for the interaction PES x Bull.



^ 1= lighter, ... 4= darker.

Embryos cultured with no PL produced more blastocysts than 270 µM PL ($P<0.01$). The blastocysts rate for 30 and 90 µM PL was 8% lower than the control ($P= 0.1$ and 0.08, respectively). 90 µM PL resulted in lower quality embryos than the control at 36 h of culture ($P<0.02$), and 0 and 30 µM PL yielded better quality embryos than 90 and 270 µM PL at 72 h ($P<0.01$). Embryos cultured with 0 or 30 µM PL were more advanced and had better ICM quality at 72 h of culture than embryos cultured with 90 or 270 µM PL ($P<0.01$). The average number of cells per embryo was highest for embryos cultured with 30 µM PL, although not significantly different from the control. Ninety and 270 µM PL resulted in the lowest cell numbers ($P<0.01$). Interestingly, embryos cultured with 270 µM PL were lighter than the other treatments at 36 and 72 h evaluations ($P<0.01$).Table 5.3 summarizes the results obtained for all significant responses for the main effect PL.

There was no significant main effect of glucose concentrations for any of the responses studied; however, there was a glucose x PL interaction effect for lightness at 36

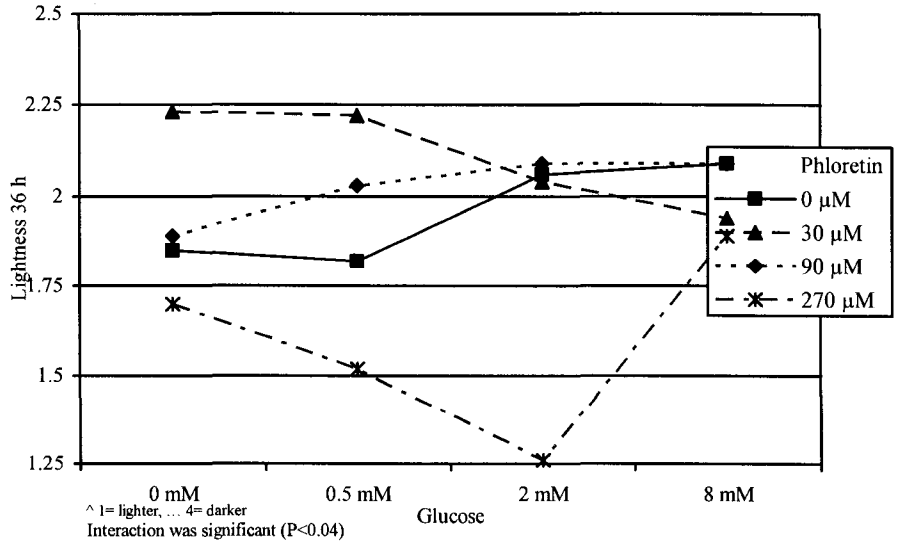
h (P<0.04). Although embryos cultured with 270 μM PL were consistently lighter than the other treatments, in presence of 8 mM glucose, they were almost as dark as the embryos from the other treatments (Figure 5.2).

Table 5.3. Least-squares means for significant main effects of the four PL doses evaluated

	PL (μM)			
RESPONSE	**0**	**30**	**90**	**270**
Quality 36 h[#]	1.06±0.03[c]	1.07±0.03[cd]	1.17±0.03[c]	1.06±0.03[cd]
Lightness 36 h[^]	1.96±0.06[a]	2.11±0.06[a]	2.02±0.06[a]	1.59±0.07[b]
Stage 72 h[*]	6.8±0.07[a]	6.9±0.07[a]	6.7±0.07[ab]	6.4±0.07[b]
Quality 72 h	1.49±0.11[a]	1.43±0.11[a]	1.97±0.11[b]	2.13±0.11[b]
Lightness 72 h	2.14±0.07[ab]	2.03±0.07[ab]	2.30±0.07[a]	1.89±0.07[b]
ICM 72 h[@]	1.51±0.12[a]	1.48±0.12[a]	1.95±0.13[b]	2.24±0.13[b]
% blastocysts	55.6±3.4[a]	47.6±3.4[a]	47.1±3.4[a]	34.1±3.4[b]
Cell number	141±6[ab]	149±6[a]	123±6[b]	87±6[c]

a, b. Values within rows without common superscripts differ, (P<0.01).
c, d. Values within rows without common superscripts differ, (P<0.02).
* Early blastocyst= 5,... hatched blastocyst= 8.
# Excellent= 1, ... poor= 3.
^ Lighter=1, ...darker= 4.
@ Big, compact ICM= 1, ...scarce, loose ICM= 4.

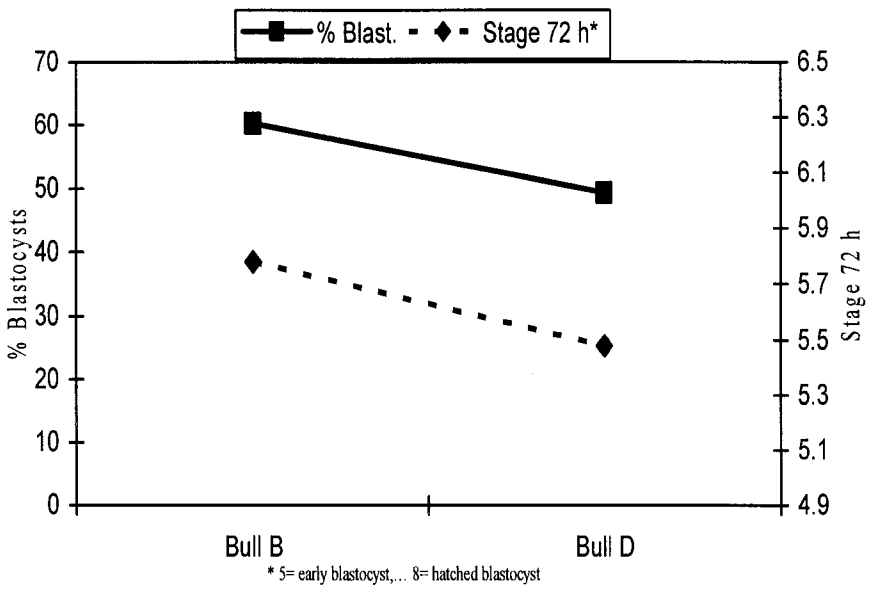
Figure 5.2. Least-squares means for lightness[^] at 36 h for the interaction PL x Glucose.



Pyrroline-5-carboxylate. Sperm from bull B produced more blastocysts and at more advanced stages (60.3±2.6 and 5.78±0.08, respectively) than embryos derived from bull D (49.3±2.3 and 5.48±0.07, respectively) ($P<0.01$) (Figure 5.3).

Neither P5C nor glucose induced any change, either negative or positive in the responses evaluated. There was, however, a tendency to improve the embryo quality at 36 h when any of the P5C doses were included for embryos challenged with high glucose concentrations (8 mM) ($P<0.08$ for the interaction P5C x glucose) (Figure 5.4). This effect was not observed at 72 h of culture.

Figure 5.3. % Blastocysts and Stage at 72 h for embryos produced with bulls B and D.



Sodium Azide. Sperm from Bull B resulted in more blastocysts per oocyte than bulls A and D in this experiment ($P<0.05$) (Figure 5.5). Embryos derived from the three bulls did not differ in any of the other responses evaluated.

Figure 5.4. Quality at 36 h for the interaction P5C x Glucose.

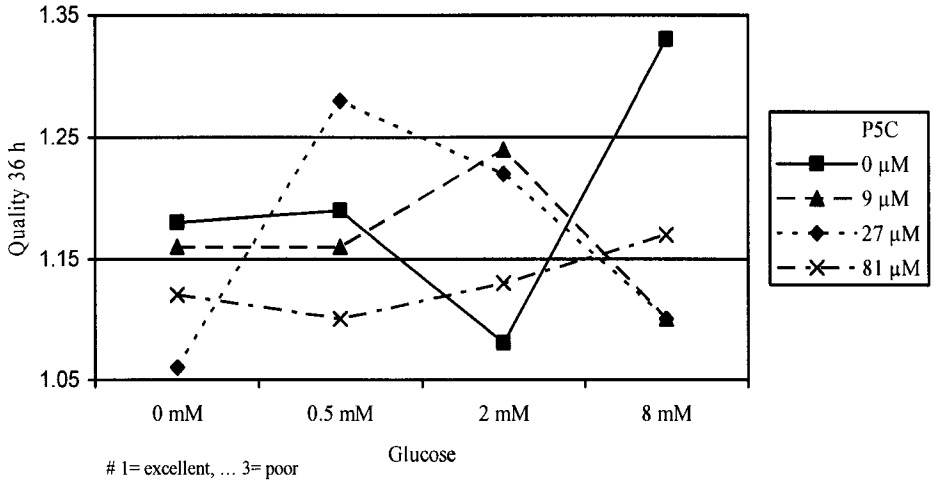
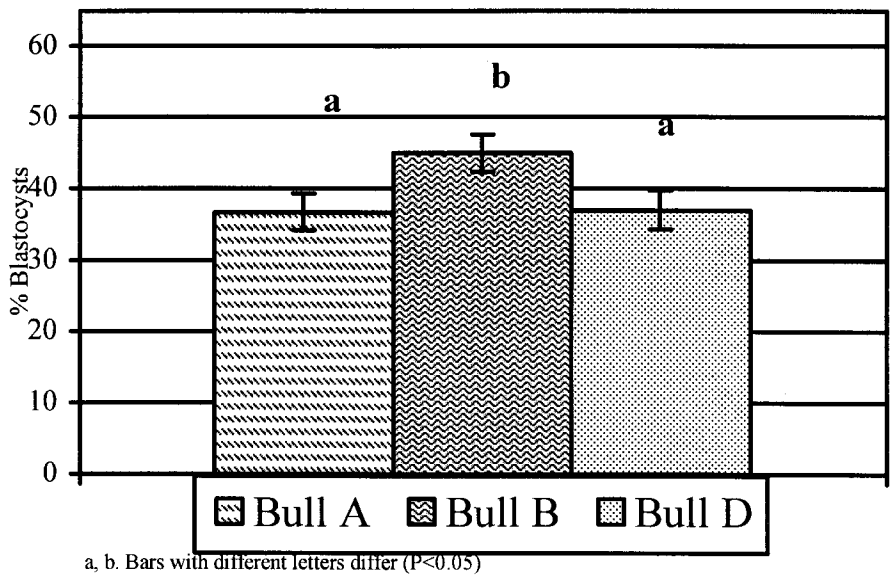


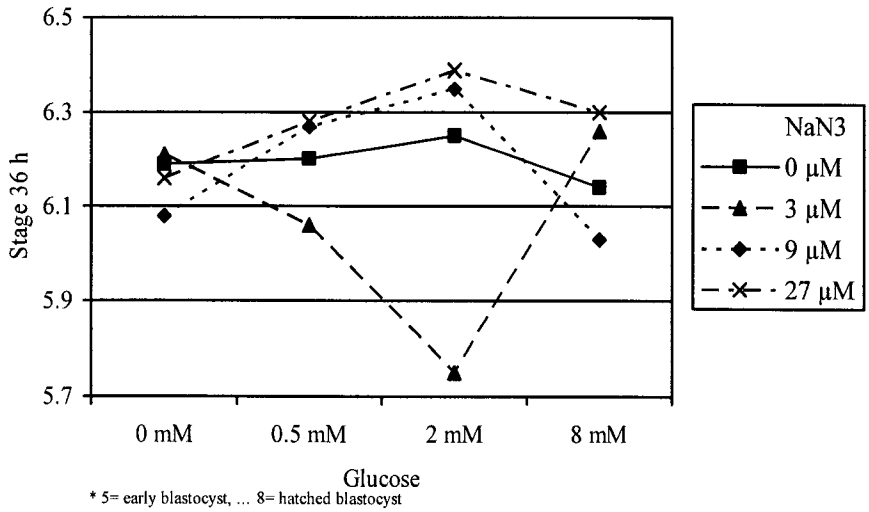
Figure 5.5. % blastocysts produced by different bulls.



Embryos cultured with the highest dose of sodium azide (27 µM NaN_3) were lighter at 72 h than embryos exposed to 9 µM NaN_3 (2.24±0.06 vs 2.00±0.06, for 9 and 27 µM, respectively; $P<0.01$). Control and 3 µM treatments were intermediate in values. There were no other significant effects for the remaining responses for NaN_3. Also, no significant effects were found when level of glucose was analyzed as a main effect. However, significant effects were found for the NaN_3 x glucose interaction. Embryos

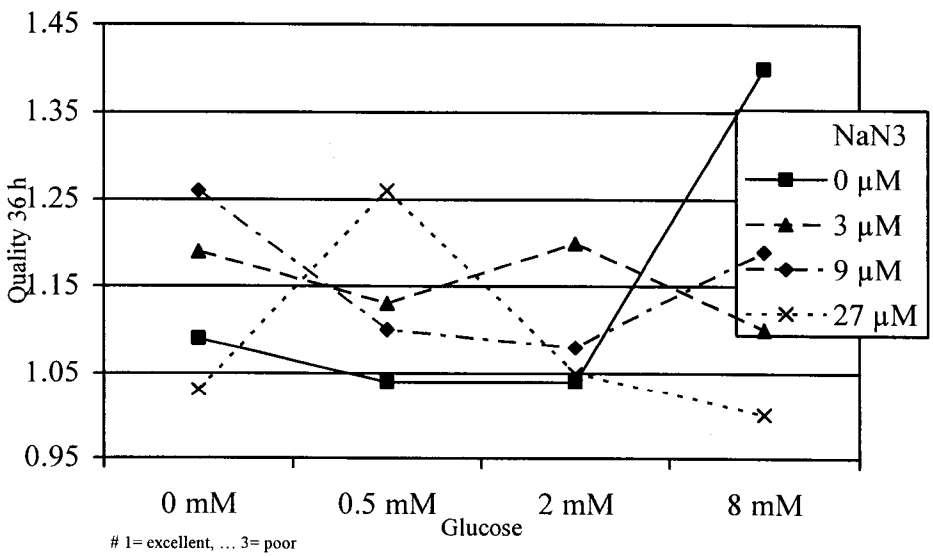
were retarded when cultured in 3 μM NaN_3, but not other levels of NaN_3 at 36 h of culture when cultured in 2 mM glucose, but not other levels of glucose ($P<0.05$) (Figure 5.6). Embryo quality was lower when no NaN_3 was added, compared with all NaN_3 doses at the highest glucose level ($P<0.04$) (Figure 5.7). These effects were no longer present at 72 h of culture.

Figure 5.6. Stage* at 36 h for the interaction NaN3 x glucose.



* 5= early blastocyst, ... 8= hatched blastocyst

Figure 5.7. Quality# at 36 h for the interaction NaN3 x glucose.



1= excellent, ... 3= poor

5.6. Discussion.

Although the characteristics of embryos derived from sperm of the four bulls evaluated was not completely consistent throughout the four experiments, Bull B was superior in several of the responses evaluated in at least three out of the four experiments. Bull C showed lower performance in some cases, and bulls A and D were considered average. Sperm from different bulls can result in marked differences in fertilization rates, with the same in vitro fertilization procedures, [137] but other responses also are affected[138, 139] by bull effects. In these experiments, we used compact morulae; unfertilized oocytes and embryos not going past the early cleavage stages were discarded. Bulls effects still contributed to differences in rates of blastocyst production and other responses related to embryo quality, even with morulae as the starting material. This confirms previously reported data concerning bull effects both on blastocyst rate and embryo quality, [138] regardless of fertilization capacity of bulls. This effect has been explained by Ward et al.[139] as the differential capacity of sperm from different bulls to penetrate the oocyte faster, leading to embryos having their first cleavage division sooner, which results in more and better quality blastocysts. The genome of sperm also can account for different blastocyst development, since morphogenesis resulting in blastocyst formation depends upon expression of the embryonic genome.[45]

The finding of differential performance of the sperm from individual bulls in these experiments might only be relevant for semen of these bulls. However, the importance of using semen of different bulls with a range of characteristics is that the experimental treatments are tested under a variety of conditions, and are even challenged

with sub-optimal conditions. Also, results can be validly applied to the populations of bulls used. Furthermore, interactions between bulls and the factors to be evaluated can be detected. For example, the highest dose of PES produced darker embryos at 36 h when bulls B and C were used, but when bull A was used, embryos were lighter at 36 h than for other bull-dose combinations.

As a main effect, glucose was of little overall significance among the experiments. Glucose is a necessary substrate for bovine embryos during the post-compaction stages, and is required to fuel the ATP-ases responsible for blastocoele formation.[93] This hexose is also an essential substrate for important biosynthetic pathways.[97-99] On the other hand, glucose was detrimental during in vitro culture of embryos, when present at blood concentrations or higher.[86, 87] According to these reports, detrimental effects would be expected with treatments without glucose or when higher than blood concentrations were included (8 mM). The few significant effects found did not correspond with the expected outcomes, making it difficult to correlate results with dose-related beneficial or detrimental effects (e.g. in PES experiment, embryos cultured with no glucose were lighter than 2 mM glucose, with intermediate values for 0.5 and 8 mM glucose). When interactions of glucose with metabolic regulators were analyzed, some interesting findings occurred, which will be discussed later. The lack of response of embryos to different glucose concentrations in the medium could be explained by the capacity of embryos to adapt to sub-optimal but non detrimental conditions (embryo plasticity), already mentioned by others,[50, 134] and by the fact that embryos cultured in chemically defined media containing amino acids are less

sensitive to suboptimal glucose concentrations in the medium.[83] Energy available via lactate, pyruvate, and glutamine in these media likely was sufficient for embryos when no glucose was present.

Phenazine ethosulfate affected important indicators of embryo development (percentage of blastocysts and cell number) negatively when used at 0.9 μM. However, lower doses did not differ from the values obtained for control embryos. Several authors have reported PES effects on mammalian embryos, specifically on the PPP rate.[109, 122, 124] However, none of them reported effects on embryo development and quality. Of particular significance is the report by Dufranses et al.[109] who mention that despite a ten-fold increase in the PPP rate of glucose utilization induced by PES, glycolysis was reduced by 50%. They infer that this reduction in glycolytic rate might be caused by a non-specific toxic effect of PES on embryos. This could be the reason for the detrimental effect observed when the highest PES dose was used. Importantly, the PES doses used in the papers previously mentioned,[109, 122, 124] were in a much higher range (10 to 25 μM) than the doses used in this experiment. As mentioned in the Materials and Methods section, a preliminary experiment was conducted, using doses similar to those reported elsewhere, resulting in lethal effects on embryos. One of the papers mentioned[124] was done using bovine embryos at high doses of PES; this discrepancy remains unexplained and may be of considerable importance.

Phloretin caused a negative effect on embryo development in most of the responses studied, some in a dose-response manner. Apparently there was a non-specific

detrimental effect of PL on embryos in addition to inhibition of GLUT transporters, since embryos cultured with no glucose did not show additional negative effects. The fact that embryos cultured with 270 μM PL were lighter at 36 and 72 h, does not necessarily mean that these embryos were of better quality, since they were worse for other responses. Likely, the clarity of these embryos is a result of abnormal metabolism caused by PL effects. The glucose x PL interaction was interesting. As seen in figure 5.2, the feature of lightness seen on embryos cultured in 270 μM disappears at the highest glucose level. This might indicate that despite the inhibition of glucose transport by PL, glucose could be driven into the embryo by the gradient formed by the high extracellular concentration,[140] somehow modifying embryo metabolism, and then lightness. None of the possible beneficial effects of PL proposed based on findings of Urner and Sakkas[111] was observed. Our results indicate that phloretin does not have the capacity to improve bovine embryo development during the post-compaction period.

Except for the bull effect found in the P5C experiment, embryos were not affected either by glucose levels nor by P5C doses. The tendency found in the glucose x P5C interaction for quality at 36 h, suggests a possible protective effect of P5C when embryos are challenged with high glucose (8 mM), since embryos exposed to high glucose had better quality when P5C was present at any dose, than when embryos were cultured without P5C. Interestingly, the overall percentage of blastocysts produced from morulae was higher in this experiment (~55%) than the percentage obtained in the other three experiments (~40-45%). This could indicate better oocyte quality during the time the experiment was done, or for random reasons the culture conditions were better, or both.

In any case, when embryos are subjected to more favorable conditions, it may be more difficult to detect favorable effects of regulators being tested.

Twenty seven μM NaN_3 produced lighter embryos at 72 h. There was also a positive effect of NaN_3 for stage at 36 h, when embryos were exposed to 2 mM glucose (except for embryos in 3 μM NaN_3). NaN_3 had the same protective effect on quality at 36 h as P5C (better quality embryos when exposed to 8 mM glucose at any NaN_3 dose). Though not definitive, this evidence might account for a positive effect of NaN_3 on post-compaction development of bovine embryos. Others have found solid evidence of a beneficial effect of NaN_3 on embryo development. Machaty et al.,[102] using porcine embryos found better blastocyst development when embryos were exposed to 20 μM NaN_3 than at lower or no NaN_3 doses. Thompson et al.[104] also found a positive effect of NaN_3 in bovine embryos, but disagreed regarding the optimal dose. They found that 5 μM NaN_3 were slightly better than 10 or 20 μM in terms of blastocyst rate. Partial inhibition of oxidative phosphorylation with sodium azide appears to be a promissing approach to improve embryo quality. The effect of this metabolic regulator on developmental competence of in vitro-produced mammalian embryos remains to be tested, however.

When studying many responses for many factorial treatment combinations, there are bound to be some Type I statistical errors, particularly when P~ 0.05. Therefore, a few of the statistically significant findings likely are not true, and those differing from expectations based on theory need to be verified by additional experiments.

CHAPTER 6

DOSE AND TIMING OF DINITROPHENOL TREATMENT DURING IN VITRO CULTURE OF BOVINE EMBRYOS

6.1. Summary.

Two experiments were completed to determine toxicity and possible beneficial effects of timing and doses of 2, 4-dinitrophenol (DNP) on embryo development. For the first experiment, 8- to 16-cell stage embryos were produced with the CDM1/CDM2 system. Three doses of DNP (10, 30, and 90 μM), and three times of exposure (EA, DNP for 2½ days, and no DNP for additional 2½ days; LA, no DNP for 2½ days, and DNP 2½ days; ALL, culture in DNP for the 5 day period) were evaluated in a factorial arrangement. A tenth treatment with no DNP exposure was used as a control. Semen from three bulls (A, B, and C) was used three times each. At the end of culture, percentage of blastocysts per 8- 16-cell embryo, stage of development, morphological quality, lightness-darkness, quality of the inner cell mass, and number of cells were evaluated. The second experiment was done in the G1/G2 system, using 30 μM DNP at different developmental stages, from the zygote stage to the blastocyst stage. The percentages of embryos reaching the expected stage at different times were recorded, and the embryos were fixed and stained to differentially count ICM and trophectoderm cells. Embryos cultured in 90 μM DNP in CDM developed slower and were darker than

embryos cultured at lower doses. When compared with controls, embryos cultured in 30 μM DNP had a higher blastocysts rate. DNP in EA produced more advanced and lighter embryos than in LA. When cultured in G1/G2, DNP did not improve embryo development or quality, but resulted in marked detrimental effects when embryos were exposed to DNP at the zygote to 8- to 16-cell stages. In conclusion DNP improved embryo development and quality from the 8- to 16-cell stage to the blastocyst stage with the CDM1/CDM2 embryo production system.

6.2. Introduction.

The two experiments in this Chapter are a continuation of the screening of metabolic regulators that was done with several compounds as reported in the previous chapter. Dinitrophenol (DNP) evaluations were performed differently from the other regulators, since glucose was not set at different levels, and the effects of the pharmacological agent were assessed at different times during embryo development. For that reason, the DNP information is presented in a separate Chapter.

For several years, DNP has been used to block oxidative phosphorylation in experiments designed to characterize the metabolic profiles of energy substrates in mammalian preimplatation embryos.[93, 122, 124, 141] More recently, it was observed that sub-acute inhibition of oxidative phosphorylation by low doses of DNP was beneficial by regulating how in vitro-produced, post-compaction embryos metabolize glucose, providing the possibility to use it as a metabolic regulator. Several papers reported beneficial effects of low DNP doses on in vitro development of porcine and bovine

embryos.[102-104] The mechanism by which DNP produces these beneficial effects is not fully understood. It is proposed that by inhibiting mitochondrial production of ATP, there is a better balance between cytoplasmic and mitochondrial ATP production, which favors glucose utilization in ways other than lactate production.[23, 25]

The aims of the experiments reported in this chapter were to test the effect of a range of doses of DNP at different times of embryo development. Also, DNP effects were tested on embryos produced in a completely defined media system in the second experiment.

6.3. Objectives.

- Determine the DNP dose that has no negative effects and possible beneficial effects on embryo development.
- Determine the best timing for DNP use during embryo development, and possible interactions with doses.
- Examine DNP effects when using chemically semi-defined and defined media systems.

6.4. Materials and Methods.

6.4.1. Experiment 1.

Maturation and fertilization of oocytes and culture of embryos were performed using the CDM1/CDM2 system described in General Materials and Methods. Presumptive zygotes were cultured 2½ days post-fertilization in CDM1. At that time, 8-

and 16-cell embryos were selected and allocated to experimental groups (25-30 embryos per subclass; total ~ 2500 embryos). Three doses of DNP (Sigma, cat # D-7004) (10, 30, and 90 μM) and three times of exposure (EA, culture in CDM2 plus DNP for 2½ days, then culture in CDM2 alone for additional 2½ days; LA, culture in CDM2 for 2½ days and then culture in CDM2 plus DNP 2½ days more; ALL, culture in CDM2 plus DNP the whole 5 day period,) were evaluated in factorial arrangement. A tenth treatment with no DNP exposure was used as a control. Semen from three bulls (A, B, and C) was used three times each. For ALL and control treatments, medium was refreshed after 2½ days in culture. Treatments were placed in 500 μL medium with no oil overlay in four well dishes, under 5% CO_2, 5% O_2, 90% N_2, 38.5 °C, 100% humidity incubation conditions. As DNP is a very lipophilic molecule, it was necessary to use DMSO to get it incorporated into the culture medium. Three 2000x stocks were prepared in DMSO, one per dose evaluated, and added to culture medium to get the required concentrations. 1/2000 DMSO was added to CDM2 to make it an appropriate control.

At the end of culture, embryos were evaluated individually, but data were averaged within each well to provide appropriate experimental units. Blind evaluation was not possible, since DNP changes the color of medium, and it was easy to determine which treatments were in the different wells. The following responses were evaluated: percentage of blastocysts per 8- 16-cell embryo; stage of development (5= early blastocyst, 6=full blastocysts, 7= expanded blastocyst, 8= hatched blastocysts); morphological quality (1= excellent, 2= fair, 3= poor); a subjective evaluation on a scale of 1-4 of the degree of lightness-darkness (probably related to lipid accumulation in the

embryo) (1= lighter,...4= darker); and quality of the inner cell mass (1= large, compacted inner cell mass, 2= large, less compact inner cell mass, 3= medium, loose inner cell mass, and 4= scarce, loose inner cell mass). After evaluation, embryos were fixed and stained with giemsa according to procedures in General Materials and Methods, and nuclei were counted to determine the number of cells per embryo. Data were analyzed by ANOVA, with the general linear models (GLM) procedure of the Statistical Analysis System (SAS).[133] Three different analysis were performed; the latter two to accommodate the control: a factorial design, with factors DNP dose (3), timing (3) and bulls (3); a factorial design 3x4, with factors bulls (3) and DNP doses (90 μM, 30 μM, 10 μM, and control; and a factorial design 3x4, with factors bulls (3) and timing (EA, LA, ALL, and control). Percentage data were tested for normality by plotting Studentized residuals versus predicted values to check for homogeneity of variances. If required, data were transformed to achieve normality, using the arcsin $\sqrt{y}/100$ transformation. Means were separated using Tukey's HSD test.

6.4.2. Experiment 2.

Embryos were produced from abattoir-derived oocytes, and matured, fertilized, and cultured on the G1/G2 system, as described in General Materials and Methods. Cumulus cells were removed after 18 h of fertilization, and presumptive zygotes were allocated to treatments (25-30 presumptive zygotes per subclass; total ~1300 embryos). The dose of DNP used for this experiment was the one with best results in experiment 1 (30 μM). Eight treatments were designed, with the aim to cover DNP exposure at different times. Culture was divided in three periods: in period 1, zygotes were placed in

G1 for 2.5 days; 8- to 16-cell embryos were placed in G2 for 2 days, making period 2; and morulae were placed in G2 for 2 days, as period 3. Table 6.1 illustrates DNP exposure during the three periods, for each treatment. This experiment was replicated three times each with semen of two bulls. Treatments were placed in 500 μL of medium in four well dishes, under 5% CO_2, 5% O_2, 90% N_2, 38.5 °C, 100% humidity incubation conditions. DNP stock solution was prepared and added to media as described for experiment 1.

Table 6.1. Timing and length of DNP exposure for the treatments evaluated

	Developmental stage		
Treatment #	**Zygote**	**8- to 16-cells**	**Morula**
1	---	---	---
2	DNP	---	---
3	DNP	DNP	---
4	DNP	DNP	DNP
5	---	DNP	DNP
6	---	---	DNP
7	DNP	---	DNP
8	---	DNP	---

At the end of each culture period, the percentage of embryos reaching the expected stage (8- to 16-cell embryo, compact morula, and blastocyst, respectively) was recorded. Blastocyst rate was evaluated twice, at 48 and 60 h of culture in 3rd period, which corresponds to 7 and 7½ day embryos. Embryos were then fixed and stained using the differential staining technique described in General Materials and Methods to obtain total numbers of cells, number of ICM cells, number of trophectoderm cells and percentage of cells in the ICM. Data were analyzed by ANOVA, with the general linear models (GLM) procedure of the Statistical Analysis System (SAS),[133] using a factorial design, with factors treatment (8) and bulls (2). Normality, was tested for all responses,

by plotting Studentized residuals versus predicted values to check for homogeneity of variances. If required, data were transformed to achieve normality, using arcsin $\sqrt{y/100}$ transformation. Means were separated using Tukey's HSD test.

6.5. Results.

6.5.1. Experiment 1.

Transformations of data were not required for analyses. Appendix 2 shows the ANOVA tables for the three analyses performed, for each of the responses. Table 6.2 shows the bull main effect means for the six responses studied. Embryos produced using semen from bull A achieved the best blastocyst rate, but tended to have an intermediate speed of development and tended to score lowest in quality. Embryos produced with sperm of bull B tended to attain the highest developmental stage average, but were intermediate in the other two responses. Embryos produced using bull C tended to develop faster than the other two groups, with tendency to achieve the best quality, although blastocyst production was the lowest.

Table 6.2. Blastocyst rate (%), stage, quality, lightness, ICM quality, and number of cells for the main effect bull.

Response	Bull A	Bull B	Bull C
Blastocyst rate[1]	50.3±2.5[a]	43.4±2.6[ab]	41.8±2.5[b]
Stage of development[*]	6.75±0.04[cd]	6.68±0.04[c]	6.80±0.04[d]
Quality[#]	1.42±0.04[e]	1.33±0.04[ef]	1.28±0.04[f]
Lightness[^]	1.89±0.05	1.94±0.05	1.8±0.05
ICM quality[@]	1.30±0.03	1.26±0.03	1.22±0.03
Number of cells	100.7±3.1	100.4±3.2	107.0±3.1

1. Calculated as percentage of blastocysts from 8- to 16-cell embryos.
* 1= early blastocyst, ... 8= hatched blastocyst.
# 1= excellent, ... 3= poor.
^ 1= lighter, ... 4= darker.
@ 1= big, compact ICM, ... 4= scarce, loose ICM.
a, b. Values within rows without common superscripts differ (P<0.05).
c, d. Values within rows without common superscripts differ (P<0.06).
e, f. Values within rows without common superscripts differ (P<0.08).

There were no significant (P>0.09) interactions between dose and time of exposure for any response; therefore, only main effects will be described. Table 6.3 describes the responses for the main effect dose. Exposure of embryos to 90 μM DNP averaged over times was detrimental, reflected in slower development and darker embryos.

Table 6.3. Blastocyst rate (%), stage, quality, lightness, ICM quality, and number of cells for the main effect dose.

Response	10 μM DNP	30 μM DNP	90 μM DNP
Blastocyst rate[1]	44.5±2.6	48.3±2.5	42.8±2.5
Stage of development*	6.84±0.04[a]	6.80±0.04[a]	6.60±0.04[b]
Quality#	1.31±0.04	1.31±0.04	1.41±0.04
Lightness^	1.83±0.05[c]	1.84±0.05[c]	1.97±0.05[d]
ICM quality@	1.24±0.03	1.24±0.03	1.29±0.03
Number of cells	102.6±3.2	105.9±3.1	99.7±3.1

1. Calculated as percentage of blastocysts from 8- to 16-cells embryos.
* 1= early blastocyst, ... 8= hatched blastocyst.
# 1= excellent, ... 3= poor.
^ 1= lighter, ... 4= darker.
@ 1= big, compact ICM, ... 4= scarce, loose ICM.
a, b. Values within rows without common superscripts differ (P<0.01).
c, d. Values within rows without common superscripts differ (P<0.06).

Table 6.4 illustrates the responses of embryos exposed to DNP at different times during culture. Embryos exposed to DNP between the 8- to 16-cell and morula stages were more advanced and lighter than embryos cultured with the inhibitor at post-compaction stages. Embryos exposed to DNP for the complete period, had intermediate values.

To compare DNP in terms of dose and timing with embryos not exposed to this compound, the three doses and the three timings were compared to control embryos which were not exposed to DNP. Bull effect was included as a factor in these evaluations, making the design a factorial 4x3.

Table 6.4. Blastocyst rate (%), stage, quality, lightness, ICM quality, and number of cells for the main effect timing.

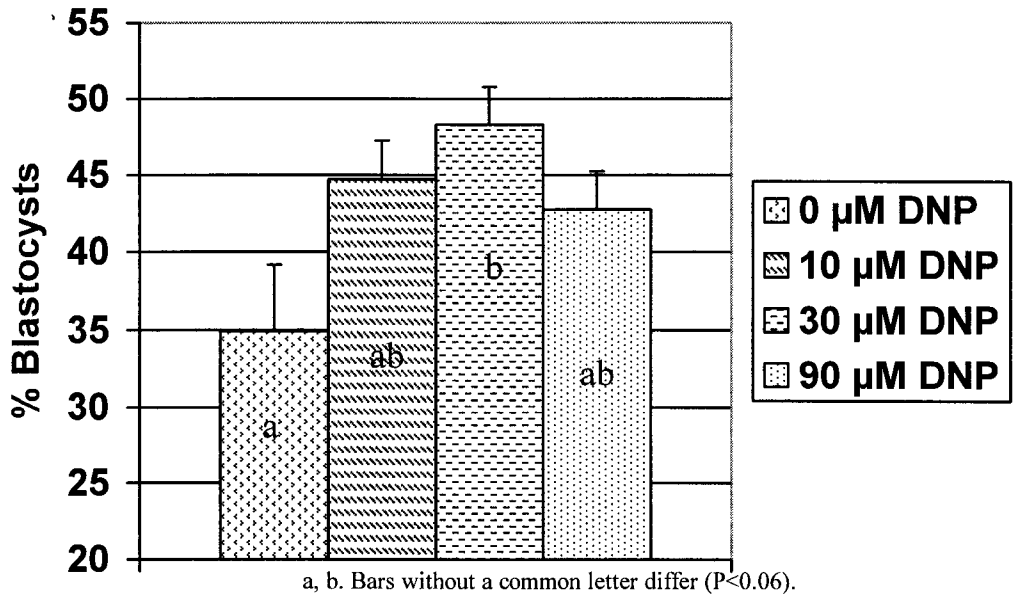
Response	EA	LA	ALL
Blastocyst rate[1]	43.8±2.6	48.0±2.5	43.8±2.5
Stage of development*	6.84±0.04[a]	6.68±0.04[b]	6.73±0.04[ab]
Quality#	1.28±0.04	1.38±0.04	1.37±0.04
Lightness^	1.79±0.05[c]	1.96±0.05[d]	1.88±0.05[cd]
ICM quality@	1.23±0.03	1.30±0.03	1.25±0.03
Number of cells	106.3±3.2	101.4±3.1	100.5±3.1

1. Calculated as percentage of blastocysts from 8- to 16-cells embryos.
* 1= early blastocyst, ... 8= hatched blastocyst.
# 1= excellent, ... 3= poor.
^ 1= lighter, ... 4= darker.
@ 1= big, compact ICM, ... 4= scarce, loose ICM.
a, b. Values within rows without common superscripts differ (P<0.01).
c, d. Values within rows without common superscripts differ (P<0.05).

Bull effects were similar to those obtained when data were analyzed in 3x3x3 arrangement; therefore, bull responses will not be presented. Embryos cultured with 30 μM DNP produced more blastocysts than the control group, with 10 and 90 μM resulting in intermediate values (34.9±4.3, 44.7±2.6, 48.3±2.5, and 42.8±2.5%, respectively for 0, 10, 30, and 90 μM DNP; P<0.06. Figure 6.1). Embryos cultured in 10 and 30 μM DNP were more advanced in development (6.83±0.04 and 6.80±0.04, respectively) than embryos in 90 μM DNP (6.60±0.04) (P<0.01). Control embryos were intermediate (6.70±0.07).

Exposure to DNP at post-compaction stages tended to result in higher numbers of blastocysts than the control (34.8±4.4, 43.9±2.6, 48.0±2.5, and 43.8±2.5%, for Control, EA, LA, and ALL groups, respectively; P<0.09); however, EA embryos tended to develop faster and be lighter (6.84±0.04, and 1.79±0.05, for stage of development and lightness, respectively) than LA group (6.68±0.04, and 1.96±0.05, for stage of development and lightness, respectively) (P<0.09, and P<0.07, respectively). Control embryos and those exposed to DNP for the complete period had intermediate responses.

Figure 6.1. Blastocyst production with different doses of DNP



a, b. Bars without a common letter differ (P<0.06).

6.5.2. Experiment 2.

Percentage responses did not require transformation for analysis. There were no differences attributable to bulls for any of the responses studied in this experiment (P>0.1).

Table 6.5 shows the developmental rate at 60, 108, 156, and 168 h, of embryos exposed to DNP at different times. Note that some treatments include exposure of DNP only at later stages; therefore, for early evaluations, those treatments can be considered as controls. Overall, a striking detrimental effect of DNP was observed when embryos were exposed at early stages, from the zygote stage to the 8- to 16-cells stage. None of the treatments was superior to the control.

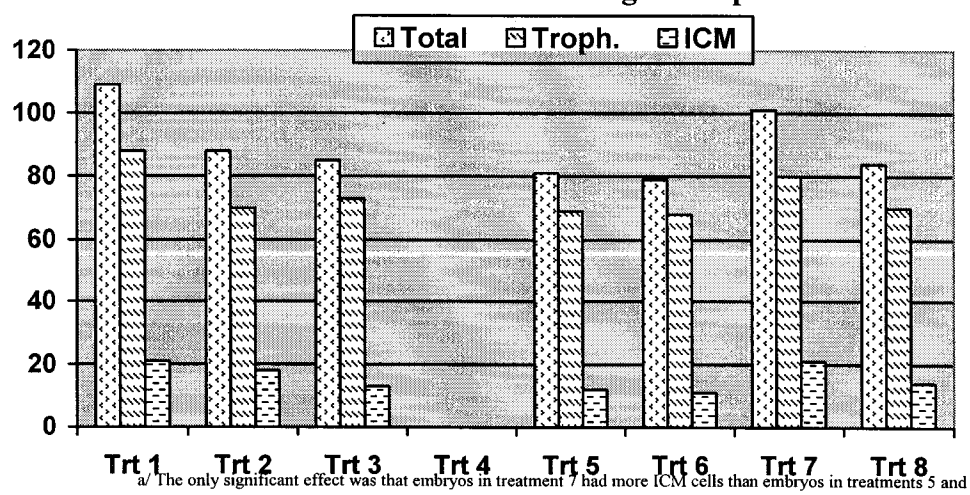
Table 6.5. Development responses[$] of embryos exposed to 30 µM DNP at different stages of development.

	Time of exposition to DNP			60 h eval	108 h eval	156 h eval	168 h eval
Trt #	Zygote	8- 16-C	Morula	8- 16-cell	Morula	Blast 7 d	Blast 7.5 d
1	-o-	-o-	-o-	47.3±5.5^{ab}	46.7±5.3^{c}	13.7±2.3^{c}	23.0±3.5^{c}
2	DNP	-o-	-o-	31.5±5.5^{b}	27.3±5.3^{cd}	8.5±2.3^{cd}	12.2±3.5^{cd}
3	DNP	DNP	-o-	37.5±5.5^{ab}	24.0±5.3^{d}	5.7±2.3^{cd}	9.4±3.5^{cd}
4	DNP	DNP	DNP	35.5±5.5^{ab}	26.8±5.3^{cd}	3.2±2.3^{d}	8.6±3.5^{d}
5	-o-	DNP	DNP	57.0±5.5^{a}	40.3±5.3^{c}	11.8±2.3^{cd}	19.4±3.5^{cd}
6	-o-	-o-	DNP	58.3±5.5^{a}	48.0±5.3^{c}	13.2±2.3^{cd}	21.0±3.5^{cd}
7	DNP	-o-	DNP	39.5±5.5^{ab}	38.2±5.3^{cd}	5.0±2.3^{cd}	12.7±3.5^{cd}
8	-o-	DNP	-o-	48.8±5.5^{ab}	36.8±5.3^{cd}	10.8±2.3^{cd}	18.4±3.5^{cd}

a, b. Values within columns without common superscripts differ (P<0.01).
c, d. Values within columns without common superscripts differ (P<0.05).
[$] Percentage of embryos reaching the stage correspondent to time of evaluation, from presumptive zygotes.

Figure 6.2 presents the differential cell count data; note that for this response, only blastocysts surviving to the end of culture were stained and counted. Therefore, there were fewer surviving embryos evaluated for treatments with DNP in early stages. Data for treatment 4 could not be estimated, because there were no blastocysts to stain in any of the replicates of one of the bulls. Neither the total number of cells nor the number of trophectoderm cells was different among the treatments; however, embryos in treatment 7 had more ICM cells (20.8±2.9) than embryos in treatments 5 and 6 (11.7±1.93, and 11.4±1.93, respectively). All the other treatments were intermediate.

Figure 6.2. Total number and allocation of cells on embryos exposed to 30 µM DNP at different times during development.[a]



a/ The only significant effect was that embryos in treatment 7 had more ICM cells than embryos in treatments 5 and 6.

6.6. Discussion.

The array of bulls used for experiment 1 showed differences in 3 of the 5 responses evaluated; none of the bulls, however, was outstanding in all characteristics. This proves the previously discussed effect of bulls, in other than fertilization characteristics, such as blastocyst rates, rate of development, and quality. This also highlights the already mentioned importance of using several bulls in IVP experiments.

Embryos cultured in the higher DNP dose (90 μM) had slower development and were darker than embryos cultured in lower doses. Bovine embryos at post-compaction stages rely mainly on glycolysis for energy production; oxidative phosphorylation is much less active.[81, 83, 142] Total inhibition of oxidative phosphorylation by using high doses of DNP (1000 μM), however, disrupts embryo development, as observed by several authors.[93, 104] The maximal dose with no toxic effects seems to be highly dependent on embryo stage (discussed later) and species. One hundred μM DNP reduced the percentage of grade 1 and 2 transferable bovine embryos, compared to embryos exposed to 10 μM DNP;[104] similarly, 100 μM DNP reduced the percentage of bovine compact morulae plus blastocysts developed, compared to 10 μM DNP.[103] However, the 100 μM dose improved blastocyst rates and cell numbers when compared to 50, 10 and 0 μM DNP in porcine embryos.[102] Rat embryos, which are much less reliant on oxidative phosphorylation are even more resistant to high doses, since they can still develop in presence of 1000 μM DNP.[143] Thompson[23] proposes the use of 5 to 10 μM DNP to partially inhibit oxidative phosphorylation in post-compaction bovine embryos, and improve embryo development and quality. There are no reports of DNP effects using

doses intermediates between 10 and 100 μM; however, we found that embryos exposed to 30 μM produced more blastocysts than embryos not exposed to DNP, with 10 and 90 μM DNP resulting in intermediate percentages.

Embryos exposed to DNP between the 8- to 16-cell and morula stages, but not at post-compaction stages, developed faster and were lighter than embryos exposed to DNP only at post-compaction stages; however, in this last group, there was a tendency to produce higher numbers of blastocysts than the control treatment, with embryos exposed at early stages in between. Early cleavage embryos are more dependent than advanced stages on metabolism of tricarboxylic acids via oxidative phosphorylation for energy production.[81] They are, therefore more susceptible to inhibition of this metabolic pathway. Mouse embryos at early cleavage stages (2- to 8-cell stages) were more susceptible to inhibition of oxidative phosphorylation than embryos at later stages.[144] The same effect was observed in rat embryos.[143] In all experiments involving use of DNP in bovine embryos reported by others, embryos were exposed at compaction and post-compaction stages. The metabolic shift from oxidative phosphorylation to glycolysis in the bovine embryo occurs by the time of compaction;[83] however, bovine embryos start taking up much more glucose from the 8- and 16-cell stages, with a two-fold increase in glucose metabolism at the 16-cell stage.[145] The data presented in that paper suggested that this transition process in the embryo is gradual and could start as early as in the 8-cell stage. In the present experiment, exposure to DNP at pre-compaction stages, but after 8-cell stage, was not detrimental for embryo development, with some advantages in terms of speed of development and possibly less lipid accumulation.

Results of dose and timing of exposure led to a second experiment, in which we studied the dose with better results (30 μM) by examining the timing of exposure, from the zygote to the blastocyst stage. We also tested DNP under chemically defined conditions, using a media system supplemented with human recombinant albumin and hyaluronan, instead of BSA (G1/G2). As mentioned for mouse and rat embryos,[143, 144] DNP was detrimental to bovine embryos at early cleavage stages (from zygote to 8- to 16-cell stages). Overall, exposure to 30 μM DNP at early cleavage stages led to lower morula and blastocysts rates, as well as fewer total and ICM cells. No beneficial effect was found for any of the treatments compared to control embryos with no DNP exposure. Embryos with DNP exposure after 8- to 16-cell stages, however, were similar to the control. To our knowledge, this is the first experiment in which DNP effects were tested using a chemically defined medium using recombinant albumin. Whether the beneficial effects of DNP are related to the use of media containing BSA, remains to be investigated.

CHAPTER 7

COMPARISION OF GLUCOSE METABOLISM AND LIPID ACCUMULATION OF IN VIVO-DERIVED VERSUS IN VITRO-PRODUCED EMBRYOS AT DIFFERENT STAGES OF EMBRYONIC DEVELOPMENT

7.1. Summary.

Two experiments were done to compare in vivo versus in vitro-produced embryos at two developmental stages, and to evaluate how a change in development conditions (vivo to vitro and vice versa) at the compaction stage affects blastocyst development. In experiment 1, day 5 and day 7 embryos were obtained in vivo and produced in vitro with the G1/G2 system. 16-24 embryos per group (~80/replicate) were obtained in four replicates; each replicate involved two collection days 2 days apart (days 5 and 7). Two-thirds of the embryos in each treatment were used for metabolic evaluations, and the other third were fixed and stained for cell counting and estimating lipid content using Sudan Black B stain. In experiment 2, day-7.5 embryos were produced in four different ways, as follows: vitro-vivo (VT-VI); embryos were produced in vitro in G1/G2 system, and selected at day five to be transferred to synchronized recipient cows. Two and one half days later, cows were flushed to recover the previously transferred embryos. Vivo vitro (VI-VT): Superovulated and artificially inseminated cows were collected at day 5. Embryos were then placed in culture in G-2 for 2.5 days. Vitro-Vitro (VT-VT): Day-7.5

embryos were produced completely in vitro in the G1/G2 system. Vivo-Vivo (VI-VI): Superovulated, artificially inseminated cows were flushed at day 5; embryos were transferred to a synchronized recipient cow, and 2.5 days later embryos were recovered from recipients. Metabolism assays, and staining for cell counting and lipid evaluation were done as in experiment 1.

In experiment 1, Day-7 vitro (D7VT) embryos metabolized more glucose than Day-5 vitro (D5VT) embryos ($P<0.06$); also, the two groups of day-7 embryos had more cells than their day-5 counterparts ($P<0.01$). D5VT embryos had more small lipid droplets than D7VT embryos ($P<0.05$). Day-7 vivo (D7VI) embryos had fewer medium lipid granules ($P<0.05$) and fewer large lipid granules ($P<0.01$) than D7VT. Also, D7VT had more lipids than D5VT ($P<0.01$). Overall, day-7 embryos had more lipid than day-5 embryos ($P<0.05$).

In experiment 2, VT-VI and VI-VT embryos metabolized less glucose than VI-VI and VT-VT embryos (10.7±1.6 and 9.0±4.5 vs 15.4±1.7 and 19.3±2.0, respectively $P<0.01$). Embryos produced completely in vitro (VT-VT) had higher lactate production than embryos produced on in vivo conditions (VI-VI) ($P<0.06$). No other differences were detected in metabolic parameters and number of cells per embryo among the treatments. Embryos graded as good metabolized more glucose (16.6±1.18 pMol/embryo/h) than embryos graded as fair (10.6±2.48 pMol/embryo/h) ($P<0.05$). This effect was most pronounced in the VT-VT group ($P<0.05$ for quality x group interaction). VT-VI, VI-VT, and VT-VT groups had very similar lipid accumulation ($P>0.1$). In

summary, change in culture conditions affected glucose metabolism. Glucose metabolism was similar among embryos produced completely in vivo versus in vitro; however, in vitro-produced embryos had higher lactate production.

7.2. Introduction.

Embryo development, from the zygote to blastocyst stages, can be divided into two well defined periods. From zygote to early cleavage stages (8- to 16-cell embryos), embryos resemble the latent state that characterizes the oocyte; the embryo relies on tricarboxylic acids for energy; glucose metabolism is almost nonexistent, and biosynthetic and transcriptional activities are low. [83, 91] At later phases (compaction and blastocyst stages), and upon activation of embryonic genome, embryos become much more active, with enhanced energy metabolism. Glucose becomes the main substrate for energy production, and biosynthetic and transcriptional activity increase.[93] Another issue, as reviewed in the previous section, is that in vitro-produced embryos are abnormal compared to in vivo-derived embryos in many respects, including morphological characteristics, ultrastructural features, metabolic profiles, gene expression, chilling and freezing sensitivity, and post-transfer developmental competence. [6-11, 13-17] It is not known to what extent IVP embryos show deviated characteristics from in vivo counterparts at the time of transition of the two mentioned phases, in contrast to the documented differences between IVP and in vivo-derived embryos at the blastocyst stage.

Embryos show temporal sensitivity to in vitro conditions, as described by Rizos et al.[48] They evaluated different combinations of in vivo or in vitro maturation, fertilization and culture of bovine oocytes/embryos. They found that maturation and fertilization in vivo increased blastocysts yield compared to in vitro-matured/fertilized oocytes, irrespective of the culture conditions. In vivo culture in sheep oviduct, however, improved the quality of blastocysts produced, compared to in vitro-cultured embryos, without affecting the blastocyst yield. In a later report,[13] they analyzed temporal effects of embryo culture, finding that in vitro matured/fertilized zygotes cultured in SOF for 2 or 4 days before transfer to sheep oviduct were more sensitive to a change in the environment than zygotes cultured in sheep oviduct for the first 2 or 4 days prior to development to the blastocyst stage in vitro. Embryos in these experiments were evaluated for quality and survivability after freezing/thawing procedures. Even though culture in the sheep oviduct is considered to closely mimic in vivo conditions in the reproductive tract of the cow,[29] it would be risky to assume that bovine embryos will have the same behavior if in vitro conditions are combined with in vivo development in the cow. Besides being a heterologous in vivo culture system, the main concern is that with this design, early and post-compaction embryos both are cultured in oviduct, while under normal conditions they develop in oviduct and uterus, respectively. To our knowledge, there has been no evaluation of temporal effects of culture conditions on bovine embryo development using the cow oviduct and uterus for culturing early and late embryos in vivo, respectively.

7.3. Objectives.

- To compare in vivo-derived versus in vitro-produced bovine embryos at compaction and blastocyst stages.
- To evaluate how a change on development conditions (in vivo to in vitro and vice versa) at the compaction stage affects blastocyst development.

7.4. Materials and Methods.

7.4.1. Experiment 1.

Four groups of embryos were studied. Day 5 and day 7 embryos produced in vitro, and embryos of the same two ages obtained in vivo. For defining the age of in vitro embryos, the time of starting fertilization was considered day 0, whereas for the in vivo counterparts, day 0 was the onset of estrus in the donor cow. With these parameters, in vivo and in vitro-produced embryos were at comparable stages on day 5 (Table 7.1). In vivo-derived embryos were obtained nonsurgically from superovulated, artificially inseminated cows, as described in General Materials and Methods. IVP embryos were produced from abattoir ovaries, using the G1/G2 IVP system, as described in General Materials and Methods. Superovulations and oocyte collections were scheduled to synchronize ages of in vivo and in vitro embryos. The experiment was replicated four times at 2- to 3-week intervals; each replicate involved two collection days 2 days apart (days 5 and 7). About 16-20 in vivo embryos per group (32-40/replicate) were studied, and similarly, ~24 in vitro embryos per group were studied (~48/replicate) (these are likely to be more variable in response, so more are required to get robust data). To ensure sufficient in vivo embryos for days 5 and 7, eight cows were superovulated per replicate,

collecting four each day. Ovarian ultrasound was performed in all eight cows before the 5 day collection to strategically allocate the donors to the two times, according to the superovulatory response. We tried to put more cows with better responses in the day-5 collection subclass, calculating to have ~20% more possible embryos to recover, given that we expected a lower recovery rate on day 5 because some embryos might still be in the oviduct at that time and thus could not be recovered.

Two thirds of the embryos in each treatment were used for metabolic evaluations, assessing each embryo individually. The other third were fixed and stained for cell counting and lipid content estimation. Embryos for metabolic evaluations were placed in a round bottom 5 ml snap-cap tube, in 1.5 ml of previously equilibrated G2 medium overlaid with 250 μl light mineral oil. Before closing the tube, the air space was gassed with a 6% CO_2, 5% O_2, 89% N_2 gas mixture. Embryos were transported at 38-39 °C, in a portable incubator (Minitube, Cat. # 19180/0000) to the laboratory where metabolic evaluations were performed in < 3 h. Upon arrival, embryos were immediately recovered from the transporting tube, rinsed in G-Mops, and transferred to individual G-2 drops. At this point, stage and quality of embryos were recorded. Each embryo was then transferred to a closed incubation chamber containing radiolabeled glucose to perform glucose metabolism evaluations as explained in General Materials and Methods. After incubation, embryos were recovered and transferred to a 200 nl drop (500 nl for day-7 embryos) of G-2 with 0.5 mM glucose, no pyruvate and no lactate, and incubated for microfluorometric analysis, as explained in General Materials and Methods. After this

second incubation, day-7 embryos were discarded, and day-5 embryos were placed back in culture in G-2, and evaluated for survival 2 days later.

Embryos for cell counting and lipid evaluation were stained with Hoechst 33342, as indicated in General Materials and methods, and nuclei were counted. After that, embryos were fixed with 10% formalin in PBS and stored at 4°C. Next day, the embryos were stained with Sudan Black B for lipid quantification, as indicated in General Materials and Methods.

7.4.2. Experiment 2.

Day-7.5 embryos were produced in four different ways, as follows:

Vitro-Vivo (VT-VI): Embryos were produced in vitro in G1/G2 system, from abattoir ovaries; at day five (in vitro-fertilization day, was day 0) ~ 32 embryos were selected for stage (16- to 32-cell) and quality (homogeneous blastomeres and starting compaction), and transferred to two previously synchronized recipient cows, ~16 embryos per cow in the uterine horn ipsilateral to the corpus luteum. At the beginning of the experiment, viable embryo recovery rates were low when estrus in recipients was delayed 0.5 or 1 day with respect to the embryo's age; therefore, for subsequent replicates, only cows with exact synchrony or up to ½ day earlier in estrus were used. Two and one half days later, cows were flushed to recover the previously transferred embryos. In vitro production of embryos, embryo transfer, and embryo collection procedures were done as described in General Materials and Methods.

Vivo vitro (VI-VT): Superovulated and artificially inseminated cows were collected at day 5 (First estrus detection was day 0). Selected embryos (same criteria as with VT-VI) were then placed in culture in G-2 for 2.5 days. In vivo generation of embryos and culture in G-2 were done according to procedures mentioned in General Materials and Methods.

Vitro-Vitro (VT-VT): Day-7.5 embryos were produced completely in vitro in the G1/G2 system, as described in General Materials and Methods. At day five, embryos were taken out of the incubator, placed in G-mops for ~30-60 min, and placed back in culture, to make an appropriate control.

Vivo-Vivo (VI-VI): Superovulated and artificially inseminated cows were flushed at day 5; embryos were transferred to a synchronized recipient cow, using one recipient for the production of each donor cow. Two and a half days later embryos were recovered from recipients. This system was used, instead of just collecting superovulated donors at day 7.5, to assure an appropriate control. A set of embryos collected at day 7.5 from a donor cow was evaluated to validate the VI-VI treatment as equivalent to in vivo-derived, day-7.5 embryos. Again, all procedures were done according to the General Materials and Methods section.

Embryo numbers and replicates were as described for experiment 1. For the treatments involving transferring embryos at day 5 and recovering them back at day 7.5,

20 to 30% additional embryos were transferred to compensate for incomplete recovery of embryos on day 7.5. For this experiment, culture procedures for microfluorometry assays were performed first, during transport of embryos. Embryos were evaluated individually and placed in a 500 nl drop of G-2 with 0.5 mM glucose, no pyruvate, and no lactate, in the bottom of a 0.6 ml microcentrifuge tube, covered with 50 µl light mineral oil and gassed with the previously mentioned gas mixture. Embryos were then transported in a portable incubator, and after 3 h, taken from the microcentrifuge tube to start incubation with radiolabeled glucose. The remaining handling and evaluation procedures were done as indicated in experiment 1 and General Materials and Methods. Embryo staining for cell counting and lipid evaluation were done as in experiment 1.

ANOVA was used to analyze metabolism values; total glucose metabolism, PPP rate (%), glucose uptake, glucose uptake/lactate production molar ratio (glycolytic rate), numbers of cells, and lipid content among the treatments. Embryo quality was also used as an independent variable for some metabolic responses. The four replicates in each experiment were done using semen from a different bull each time, so bull effects are confounded with replicate effects. A randomized complete block design was used, blocking for replicate/bull effects. For percentage responses, a residual plot of studentized residuals versus predicted values was done to check for homogeneity of variances and therefore, normality. If data were not normally distributed, responses were transformed to achieve normality, usually by using arcsin $\sqrt{y}$ transformation. Means were separated by Tukey's lsd method for experiment 1, since we were looking for specific pre-planned comparisons (day 5 vivo vs day 5 vitro, etc). Tukey's HSD method, was

used for experiment 2. Data was analyzed using the SAS statistical package, with the GLM procedure.[133]

7.5. Results.

7.5.1. Experiment 1.

There were insufficient numbers of experimental units for the PPP rate for some subclasses, and data were not normal, even after transformation. The statistical analysis was done anyway, and least squares means ±SEM are presented, with the warning that analysis might not be valid. Due to laboratory problems out of our control, valid microfluorometric data were not obtained; therefore, glucose uptake and glycolytic rate were not estimated. Table 7.1 shows glucose metabolism, PPP rate and numbers of cells for the four treatments. D7VT embryos metabolized more glucose than D5VT ($P<0.06$); also, the two groups of day-7 embryos had more cells than their day-5 counterparts ($P<0.01$). Orthogonal contrasts were also performed to compare all day 5 embryos versus all day 7 embryos and all in vivo versus all in vitro. Day seven embryos had larger cell numbers ($P<.001$) and metabolized more glucose($P<0.05$) than day five embryos.

Numbers of Sudan Black B positive granules (lipid droplets) are presented in four categories: small, medium, large, and medium+large granules. Figure 7.1 illustrates numbers of small lipid granules in embryos of the four treatments. D5VT embryos had more small lipid droplets, than D7VT ($P<0.05$). Overall, day 5 embryos had more small lipid granues than day 7 embryos ($P<0.05$). No other difference was detected.

Table 7.1. Least squares means± SEM for glucose metabolism, PPP rate (%), and cell numbers for embryos of two ages and two origins.

Variable	Gluc. Metab*.	PPP rate (%)^	# of cells
D5VI	6.7±1.1	32.1±14.2	17.2±2.8[c]
D5VT	7.6±1.3[a]	19.8±17.4	13.3±2.4[e]
D7VI	8.7±1.5	10.3±17.4	51.5±2.7[d]
D7VT	12.3±2.0[b]	0.0±24.6	47.9±2.9[f]

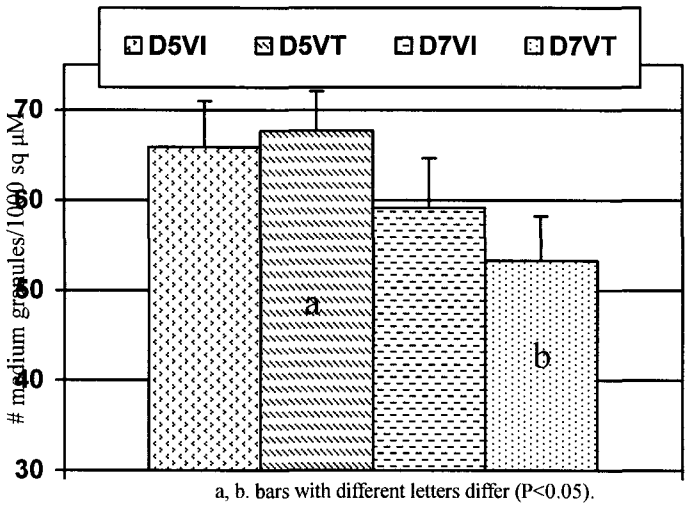
*Glucose metabolism and uptake is in picomol/embryo/h

^ PPP could be estimated from only two replicates, so SEM are large.

a, b. lsd planned comparison D5VT vs D7VT differ (P<0.06)

c, d; e, f. lsd planned comparisons D5VI vs D7VI, and D5VT vs D7VT differ (P<0.01).

Figure 7.1. Least squares means±SEM of small (<2 μM) Sudan Black B positive granules per $1x10^3$ μM^2 in embryos of two ages and two origins.



a, b. bars with different letters differ (P<0.05).

D7VI embryos had fewer medium size lipid granules than D7VT (P<0.05) (Figure 7.2). Also, overall, in vivo embryos had fewer medium lipid granules than in vitro embryos (P<0.01). For the large lipid granule response (Figure 7.3), D7VI had fewer granules than D7VT (P<0.01). Also, D7VT had more lipid accumulation than D5VT (P<0.01).

Figure 7.2. Least squares means±SEM of medium (2-6 µM) Sudan Black B positive granules per $1x10^3$ μM^2 in embryos of two ages and two origins.

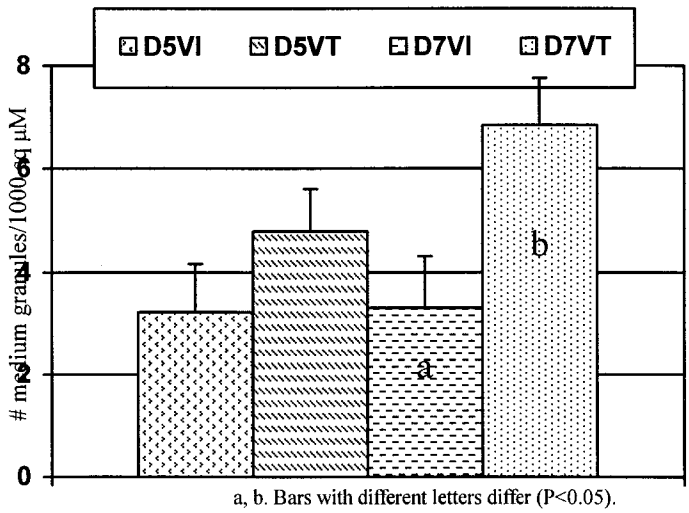


Figure 7.3. Least squares means±SEM of large (>6 µM) Sudan Black B positive granules per $1x10^3$ μM^2 in embryos of two ages and two origins.

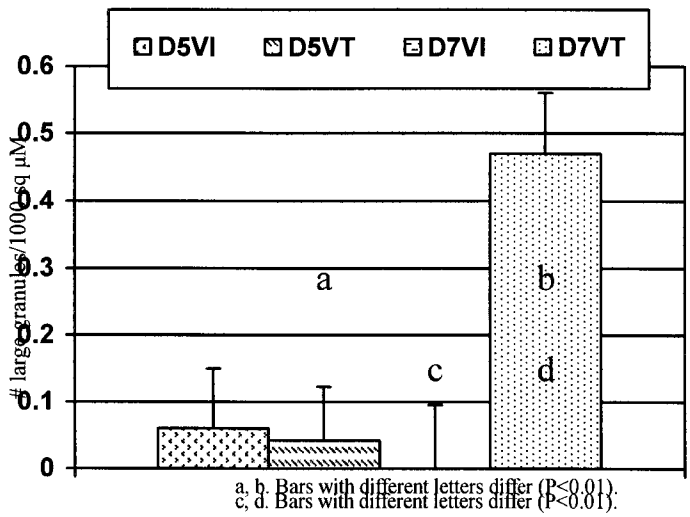
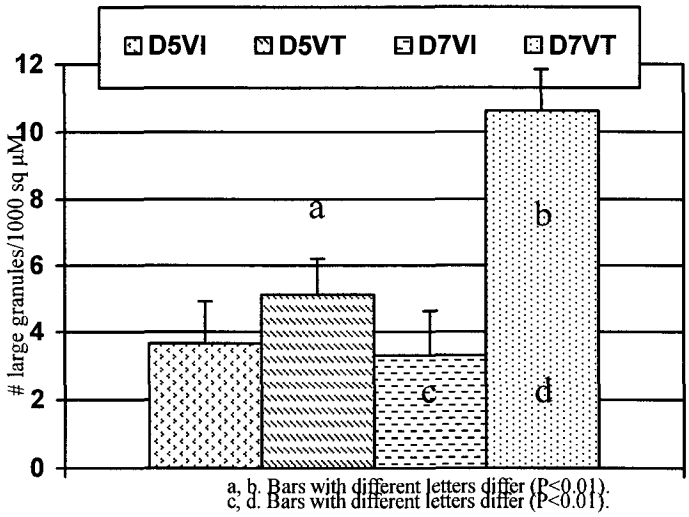


Figure 7.4 shows the data of medium and large drops combined, expressed in medium drops equivalency (see General Materials and Methods for calculations). Again, D7VT accumulated more lipids than D7VI, and than D5VT. (P<0.01).

Figure 7.4. Least squares means±SEM of medium+large Sudan Black B positive granules per $1x10^3$ μM^2 in embryos of two ages and two origins.



7.5.2. Experiment 2.

We found the same constraints for PPP analysis, as mentioned for experiment 1. To validate the VI-VI treatment as an in vivo control, we measured glucose metabolism in a group of 15 day-7.5 embryos, generated in vivo without the collection and re-transfer steps. Glucose metabolism of these embryos was similar to the value obtained in the VI-VI group (14.1±5.9 pmol/embryo/h). In some replicates, we did not recover adequate numbers of embryos that were transferred on day five. In addition, we did not get enough day-5 in vivo embryos for some replicates. These two factors combined, resulted in low availability of embryos for the VI-VI group, so we decided to use all the VI-VI embryos produced for metabolic determinations. For that reason, we do not have information of cell numbers and lipid accumulation for that group, but they presumably would be similar to the day 7 in vivo embryos from Experiment 1. There was also a problem in determining valid lactate production for the VT-VI group; therefore information for lactate production and glycolysis is not available for that group. PPP was estimated from

only two replicates, since sufficient data were not available from the other two replicates. Table 7.2 presents information on glucose metabolism, PPP rate, glucose uptake, lactate production, glycolysis, and cell numbers for treatments.

Table 7.2 Glucose metabolism, PPP rate (%), glucose uptake, glycolytic rate, and cell numbers of embryos produced in different culture conditions.

Culture conditions	Glucose Metabolism*.	PPP rate (%)	Glucose Uptake*	Lactate Production*	Glycolytic Rate (%)	# of cells
VT-VI	10.7±1.6[a]	41.5±21.4	60.7±12.2	n.a.	n.a.	52.7±12.6
VI-VT	9.0±4.5[a]	45.6±21.4	45.71±7.6	99.9±29.7[c]	192±39.7	73.4±5.5
VT-VT	19.3±2.0[b]	35.0±21.4	49.95±13.6	222±51.5[d]	234±68.8	61.9±5.7
VI-VI	15.4±1.7[b]	25.0±21.4	44.05±11.1	107±42.0[cd]	140±56.2	n.a.

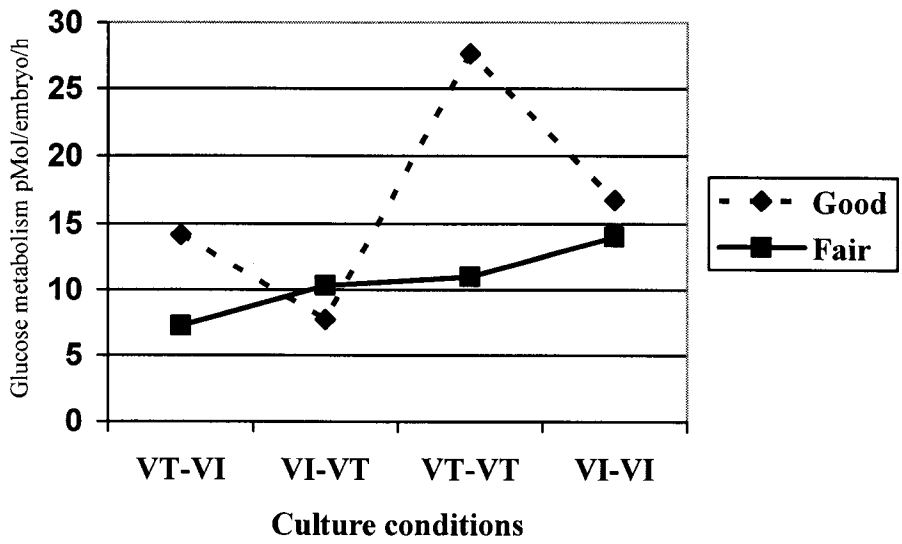
*in picomol/embryo/h
a, b. Values within columns without common superscripts differ, (P<0.01).
c, d. Values within columns without common superscripts differ, (P<0.06).
n.a. Data not available.

Embryos with a change in culture conditions (in vitro to in vivo or in vivo to in vitro) metabolized less glucose than embryos cultured in the same system all the time (P<0.01). Also, embryos produced completely in vitro (VT-VT) tended to have higher lactate production than embryos produced in vivo (VI-VI) (P<0.06). No other significant differences in metabolic parameters and number of cells among the treatments were detected.

Before glucose metabolism determination, embryos were visually evaluated and graded as good or fair (1 and 2). This quality evaluation was used as independent variable and glucose metabolism was evaluated among treatments with the two quality grades in a factorial arrangement. Grade 1 embryos metabolized more glucose (16.6±1.18 pMol/embryo/h) than grade 2 embryos (10.6±2.48 pMol/embryo/h) (P<0.05). There was

also an interaction effect for quality x treatment (Figure 7.5) ($P<0.05$). Grade 1 VT-VT embryos metabolized over twice as much glucose as grade 2 VT-VT embryos, and more than any other group.

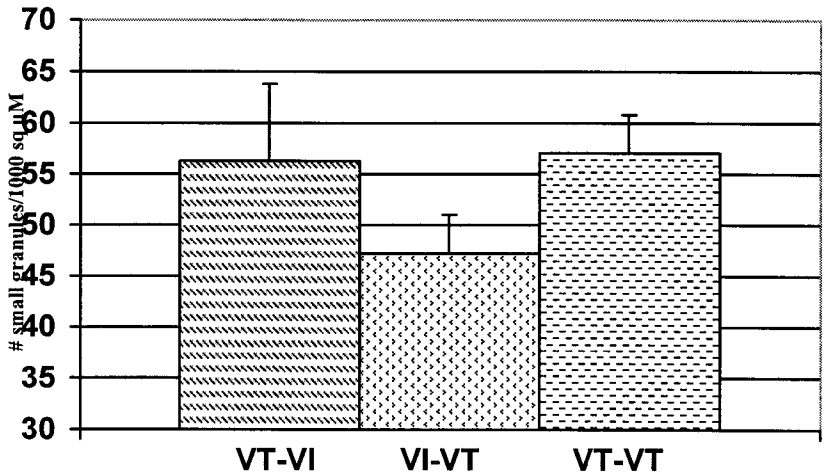
Figure 7.5. Glucose metabolism of embryos produced in four different culture conditions, and graded as good and fair quality at the time of assay.



Interaction was significant ($P<0.05$).

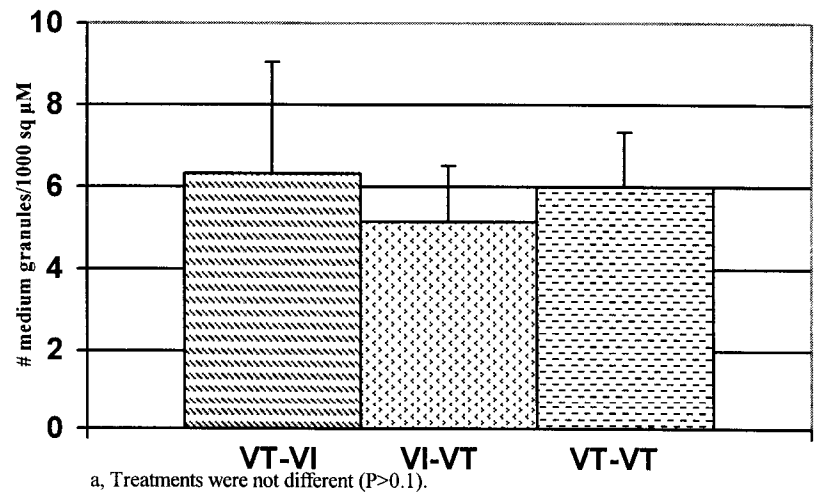
The three treatments evaluated (Figures 7.6 to 7.9) had very similar lipid accumulation, resulting in no differences in the four responses related to lipid granules density.

Figure 7.6. Least squares means±SEM of small (<.2 μM) Sudan Black positive granules per $1x10^3$ μM^2 in embryos under different culture conditions.[a]



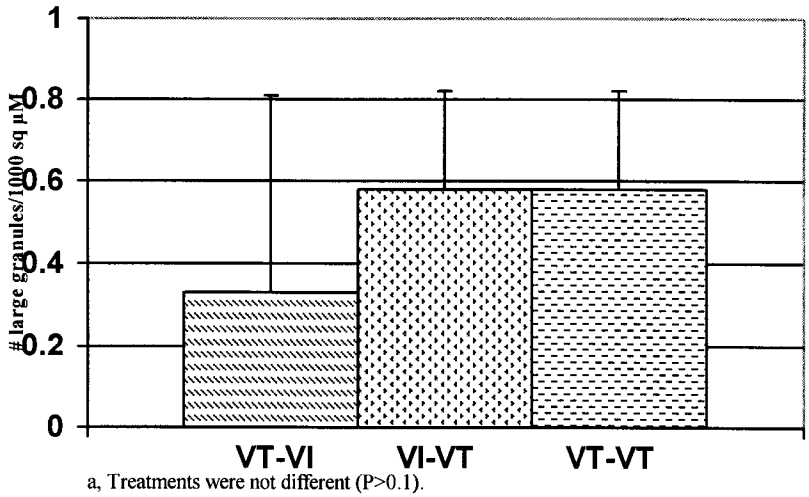
a, Treatments were not different (P>0.1).

Figure 7.7. Least squares means±SEM of medium (2-6 μM) Sudan Black positive granules per $1x10^3$ μM^2 in embryos under different culture conditions.[a]



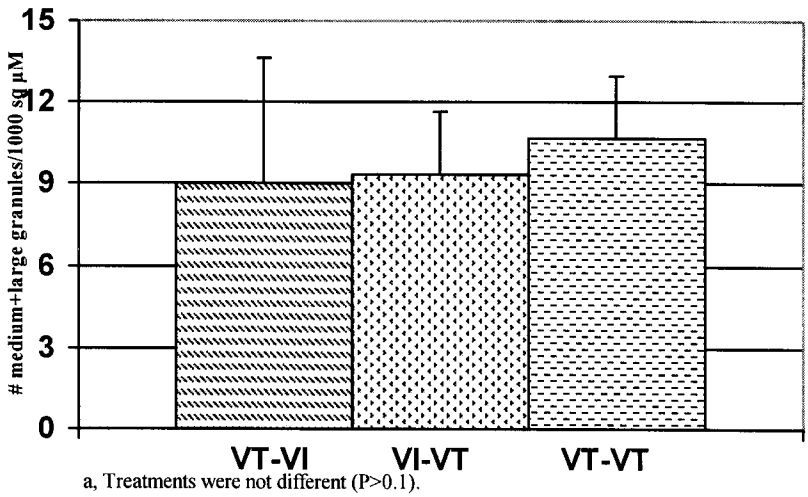
a, Treatments were not different (P>0.1).

Figure 7.8. Least squares means±SEM of large (>6 μM) Sudan Black positive granules per $1x10^3$ μM^2 in embryos under different culture conditions.[a]



a, Treatments were not different (P>0.1).

Figure 7.9. Least squares means±SEM of medium+large Sudan Black positive granules per $1x10^3$ μM^2 in embryos under different culture conditions.[a]



a, Treatments were not different (P>0.1).

7.6. Discussion.

Day-7 embryos obviously had more cells than day-5 embryos, both in vitro and in vivo. That 3-fold increment, however, does not correspond to the increments in glucose

metabolism observed in day-7 groups, compared to day-5 embryos. This can be explained by smaller size cells in blastocysts, compared to early morulae. Also, at the blastocysts stage, about 70% of the cells are allocated to trophoblast, and it has been reported that trophoblast cells are much less active in glucose uptake than the ICM.[146] The glucose metabolism values obtained for embryos produced in vitro (7.6 and 12.3 pMol/embryo/h, for day-5 and day-7 embryos, respectively), are similar to values obtained by Rieger et al.[145] They found glucose metabolism values of 1.68 and 6.59 pMol/embryo/h for 16-cell and compact morula (stages found with our day-5 embryos), whereas blastocysts and expanded blastocysts (corresponding to our day-7 embryos) metabolized 9.65 and 15.1 pMol/embryo/h, respectively. We did not find differences between in vivo and in vitro embryos at either developmental stage. These data correspond to Findings of Khurana and Niemann[7] who report very similar values of glucose metabolism for in vivo-derived and in vitro-produced embryos. (Early morula: vivo= 4.4, vitro= 3.8; Blastocyst: vivo= 8.7, vitro= 9.9, pMol/embryo/h).

Lipid accumulation responses can be summarized by two statements: Overall, in vivo-derived embryos were less lipid loaded than in vitro counterparts; and day-7 embryos accumulated more medium and large lipid granules than day-5 embryos. Day-7 in vitro embryos had much greater lipid content than day-5 in vivo embryos (see figures 7.2 - 7.4). Lipid accumulation has been regarded as a distinctive characteristic of in vitro-produced embryos,[36, 37] and has been commonly related to presence of serum in medium, arguing that embryos take up lipids from the medium.[11, 147, 148] The embryos produced in vitro in this experiment, however, were developed in a completely

chemically defined media system, with no lipids to be taken up. It is therefore concluded that embryos in the present study produced the lipids from endogenous substrates as a result of impaired metabolism induced by the in vitro conditions.

The most striking effect found in the second experiment was that glucose metabolism declined in both VI-VT and VT-VI groups but remained high when culture conditions did not change (VI-VI and VT-VT). Thus, changing conditions had more impact on glucose metabolism than the culture condition itself. Several reports have documented differences on quality and gene expression in embryos produced in combined systems.[13, 48, 49] Rizos et al., found that the 6-day period that follows fertilization determines developmental competence of embryos, since several major developmental events take place during that time, including first cleavage, embryonic genome activation, compaction and blastulation. They suggest that any modification of the culture environment which could affect these processes might have an effect on the quality of the resultant embryo. In a study similar to the present experiment, Lonergan et al.,[13] found that embryos cultured in vitro on early stages and then transferred to in vivo conditions (sheep ligated oviduct), were worse than embryos cultured in vivo in early stages and then transferred to in vitro conditions. In the current experiment, embryos produced in vitro during the entire culture period had doubled lactate production compared to VI-VT ($P<0.06$) and VI-VI groups. One of the characteristics previously reported for embryos produced in vitro, is increased lactate production, compared to in vivo counterparts. Lane and Gardner[76] found that mouse embryos cultured in vitro produced significantly more lactate, compared to in vivo-derived embryos (7.37 vs 2.94

pMol/embryo/h, $P<0.01$) They previously mentioned that excessive lactate production may have a direct effect on developmental competence of embryos.[90] Khurana and Niemann[7] also found increased lactate production for bovine embryos produced in vitro, compared to in vivo counterparts, at different developmental stages.

Good quality embryos metabolized more glucose than embryos graded as fair. In some other studies, no correlation was found between morphological evaluation and developmental competence,[90] but other studies do show a correlation.[31, 149] This experiment provides insights why qualitative evaluation of embryos might be related to the developmental capacity; others have shown that higher glucose metabolism and uptake are related to higher developmental competence.[150, 151] This difference was even more evident for the VT-VT group ($P<0.05$ for the quality x group interaction), emphasizing the value of qualitative evaluation of in vitro-produced embryos.

There was no treatment effect on lipid accumulation profiles in the second experiment; all lipid granule evaluations resulted in similar amount of lipids for all the treatments. It is important to recall that the VI-VI treatment was not included; therefore, we can not compare the amount of lipids found to in vivo conditions, although in experiment 1, the in vivo groups had much less lipid than the in vitro groups. We also note that the VT-VT group corresponds to the D7VT group of Experiment 1. The numbers of small, medium, and large lipid granules were virtually identical between these groups in Experiments 1 and 2, so this appears to be a robust method of measuring lipids.

CHAPTER 8

EFFECTS OF THREE METABOLIC REGULATORS DURING POST-COMPACTION DEVELOPMENT OF IN VITRO-PRODUCED BOVINE EMBRYOS

8.1. Summary.

The objective of this study was to compare three metabolic regulators, added during compaction and blastulation stages of bovine embryos in a chemically defined medium, on blastocyst rate and quality, glucose metabolism, lipid accumulation, and post-tranfer developmental competence. Slaughterhouse oocytes were matured and fertilized in vitro, and resulting embryos cultured in G1/G2 media. After 72 h culture in G1, 8- and 16-cell embryos were selected and randomly allocated to experimental treatments for 72 h as follows: PES, 0.3 μM; NaN_3, 27 μM; DNP, 30 μM; control, no metabolic regulator. Forty to 50 embryos at the 8- to 16-cell stages were allocated per treatment per replicate, resulting in 10-15 normal seven-day embryos for evaluation. Each of the four treatments was replicated three or four times, depending on the responses evaluated, using semen from a different bull each time. Radiolabeled glucose was used to study glucose metabolism and PPP rates. To determine glucose uptake and lactate production, embryos were incubated in G2 with 0.5 mM glucose with no lactate and no pyruvate. For lipid quantification, embryos were fixed and stained with Sudan

Black B, and lipid granules were quantified microscopically for this response; a fifth treatment (D7VI from experiment 1, chapter 7) was included as in vivo control. In four replicates, embryos were transferred to synchronized recipients, and embryos recovered seven days later were evaluated.

No differences were found among treatments in blastocyst rate, stage, quality and lightness; average blastocyst production from 8- to 16-cell embryos was 40.3%. PES-treated embryos had the highest glucose metabolism ($P<0.05$ compared to NaN_3) and tended to have a higher PPP rate ($P<0.12$) than controls (18.5 pMol/embryo/h, and 50.5%, respectively); however, glucose uptake was the lowest (13.1 pMol/embryo/h) and glycolysis the highest among treatments (187.%) ($P<0.01$). Lipid accumulation of in vivo-derived embryos was the lowest ($P<0.01$). Embryos from PES accumulated more lipids than in vivo embryos, but had less lipid than the other treatments and in vitro controls ($P<0.01$). NaN_3-derived embryos metabolized the least glucose (11.1 pMol/embryo/h, $P<0.05$), but had highest glucose uptake (38.9 pMol/embryo/h). Lipid accumulation was high in this treatment. DNP-derived embryos had intermediate values in all the metabolic parameters studied, and also were characterized by accumulation of lipids. No differences were found among treatments in developmental competence of day-14 embryos, with an average 48.6% recovery of live embryos from day-7 embryos transferred. Inhibitors (NaN_3) and uncouplers (DNP) of oxidative phosphorylation induced higher uptake of glucose, but did not cause increased glucose metabolism; these treatments also did not decrease lipid accumulation compared to controls. PES treatment increased glucose metabolism and tended to increase the relative amount of glucose

metabolized through the PPP, and clearly reduced the accumulation of lipids in embryos produced in a chemically-defined media system.

8.2. Introduction.

During the past 50 years, the formulation of media for culturing mammalian embryos in vitro has evolved from simple salt solutions, to complex and largely undefined co-culture and serum-supplemented systems, to more wisely designed, chemically defined formulations, intended to meet metabolic requirements of embryos (see Literature Review section). The extensive research foundation built over the years, mainly in rodent species, culminated in current defined or semi-defined systems aimed to resemble physiological conditions that are repeatable among experiments and laboratories.[71] Successful development of embryos of several species, including cattle, has been reported using media formulated with only chemically defined compounds.[56, 77, 85, 115, 152] In vitro-produced embryos, however, are still abnormal in many aspects, including metabolic profiles (described in the Literature Review section). Several approaches have been used to resolve the problem of metabolic aberrations of in vitro-produced embryos. Inclusion of amino acids has been one advance with important positive effects.[69, 76, 90, 153, 154] Inclusion of pharmacological compounds that affect metabolism, the so called metabolic regulators, has been another recent approach, with proven and promising results. Metabolic regulation refers to the inclusion in media formulations of compounds not present physiologically, with the capability to change metabolism of embryos towards better development. The best known example of

metabolic regulation is addition of EDTA to early cleavage stage embryos to down-regulate the exacerbated glycolysis observed when embryos are cultured in vitro.[77, 79, 80]

In chapters five and six, the possible positive and negative effects of five pharmacological compounds that affect carbohydrate metabolism of embryos were screened for effects on embryo morphology and development. Based on these preliminary results, three of these compounds, phenazine ethosulfate (PES), sodium azide (NaN3), and dinitrophenol (DNP), were selected for more comprehensive evaluation, including embryo development, metabolic profiles, accumulation of lipids and developmental competence post-transfer.

As previously mentioned, PES oxidizes NADPH to NADP, activating the enzymatic reactions for which NADP is required. This includes the first two enzymatic reactions in the oxidative phase of the PPP. PES is therefore a PPP stimulator,[105] that increases PPP activity in mammalian pre-implantation embryos. [122, 124] Addition of PES during post-compaction of in vitro-produced bovine embryos could induce more balanced glucose utilization by stimulating the PPP, and therefore reduce the glycolytic rate (conversion of glucose into lactate), with consequent improvement of the production and quality of IVP bovine embryos.

NaN3 and DNP are inhibitors of oxidative phosphorylation in the mitochondrion. NaN_3 inhibits cytochrome oxidase a3 in the electron chain transport cascade, and DNP uncouples oxidative phosphorylation from the respiratory chain.[132] At low

concentrations, these compounds sub-acutely down-regulate mitochondrial ATP production, increasing the relative contribution of glycolytic ATP, and establishing a more appropriate redox state which favors adequate glucose metabolism.[23, 25] Low doses of NaN3 and DNP improve in vitro development of bovine and porcine embryos.[102-104]

8.3. Objective.

The objective was to compare effects of addition of three metabolic regulators during compaction and blastulation stages of bovine embryos produced in a chemically defined media system, in terms of: blastocyst rate and quality, glucose metabolism, lipid accumulation, and post-transfer developmental competence.

8.4. Materials and Methods.

Slaughterhouse ovary collection and oocyte retrieval were performed as indicated in General Materials and Methods. G1/G2 media were used for maturation, fertilization and culture, following the procedures described in chapter 3. After fertilization, embryos were cultured in G1 for 72 h. Eight- and 16-cells embryos were selected and allocated to experimental treatments as follows:

A 2000x stock of each chemical was prepared in nanopure water for PES and NaN_3, and DMSO for DNP; then a 100x stock was made for each compound in G2. This last stock was stored refrigerated and added to G2 at the time of equilibration. The final concentration for each treatment was: PES, 0.3 μM; NaN_3, 27 μM; DNP, 30 μM. A G2 control treatment was also included. Eight- to 16-cell embryos were randomly allocated

to treatment dishes, 40-50 embryos per 500 μL medium in four-well dishes. Embryos remained in treatment wells for 72 h under 6% CO_2, 5% O_2, 89% N_2, 38.5 °C, 100% humidity incubation conditions. The experiment was replicated three or four times, depending on the set of embryos (explained below) using a different bull for each replicate of the four treatments. Three sets of embryos, were evaluated in three or four replicates follows:

1st set: glucose metabolism. Day-7 embryos were incubated with radiolabeled glucose, using the hanging drop incubation chamber previously described. Glucose metabolism and PPP rates were obtained. Four replicates were done for this set.

2nd set: blastocyst rate and quality, glucose uptake and lactate production, and lipid quantification. Day-7 embryos were visually evaluated for stage, quality and lightness, and then were incubated 3 h in 500 nl drops of G2 with 0.5 mM glucose with no lactate and no pyruvate; after embryos were withdrawn, drops were frozen and glucose and lactate concentration measured later, using the microfluorometric technique. Embryos were then fixed and stained with Sudan Black B for lipid quantification. Lipid quantification data obtained from D7VI group in experiment 1, chapter 7 were used to add a fifth group as in vivo control for lipid evaluations. The experiment was replicated three times for this set.

3rd set: blastocyst rate and quality, and developmental competence post-transfer. Day-7 embryos were evaluated with a stereomicroscope for stage, quality, and lightness.

After that, 8-12 embryos were loaded per treatment in a 0.25 ml straw in G-Mops, and transferred to synchronized recipients. Seven days later, Day-14 embryos were recovered nonsurgically. Surface area and percentage recovery of live embryos were determined. Four replicates were completed for this set.

All procedures mentioned above were done following methods described in the General Materials and Methods chapter. For blastocyst rate and quality, embryos were visually assessed using a stereomicroscope at 50x magnification. For each embryo or experimental unit, the following information was recorded: percentage of embryos that reached blastocyst stage by day 7.5, from the 8- and 16-cells embryos placed in treatment; stage of development (4= compact morulae, 5= early blastocysts, 6= full blastocysts, 7= expanded blastocysts, 8= hatched blastocysts); morphological quality (1= good, 2= fair, 3= poor); and lightness (1= lighter,... 4= darker). Responses were analyzed by ANOVA; arcsin $\sqrt{y}$ transformation was done on percentage responses to achieve normality if necessary from evaluating residual plots. A randomized complete block design was used, blocking for replicate/bull effects. Four replicates were done for the radiolabeled glucose experiment, using two bulls, twice each; therefore, a factorial 2x4 analysis was done, with factors bulls (2) and treatments (4). Means were separated by Tukey's LSD method, when applicable. Data were analyzed using SAS statistical package, with the GLM procedure.[133]

8.5. Results.

Percentage responses were examined for normality; none of these required transformation. Table 8.1 shows blastocyst rate, stage of development, quality, and

lightness of embryos from the four treatments evaluated. Data were compiled from seven replicates, and 1065 eight- to 16-cells total embryos were used. Embryos produced with NaN_3 tended to be more retarded (P=0.11) than control embryos. No differences among groups were found for any other response (P>0.1).

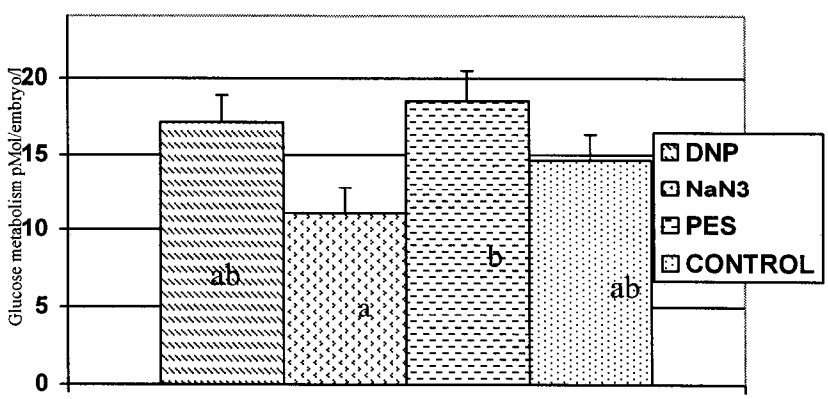
Table 8.1. Least squares means ±SEM for blastocyst rate, stage of development, quality, and lightness, of embryos cultured in chemically defined medium, with 3 metabolic regulators.[a]

Treatment	n	% Blastocysts[1]	Stage*	Quality#	Lightness^
DNP 30 μM	70	40.5±1.86	5.06±0.11	1.48±0.05	2.09±0.06
NaN_3 27 μM	70	41.1±1.86	4.92±0.11	1.38±0.05	2.09±0.06
PES 0.3 μM	70	37.7±1.86	5.24±0.11	1.42±0.05	2.00±0.06
Control	70	41.8±1.86	5.28±0.11	1.39±0.05	1.98±0.06

1. Percentage of blastocysts by day 7.5 from IVF, out of 8- and 16-cells embryos.
* 5= early blastocyst, ... 8= hatched blastocyst.
# 1= excellent, ... 3= poor.
^ 1= lighter, ... 4= darker.
a. No significant differences (P>0.1).

There was no bull effect on glucose metabolism (P>0.1); nor was a bull x treatment interaction effect observed (P>0.1). Embryos cultured in presence of PES at compaction and post-compaction stages metabolized more glucose than embryos exposed to NaN_3 during the same period (P<0.05) (Figure 8.1). Control and DNP embryos had intermediate glucose metabolism values.

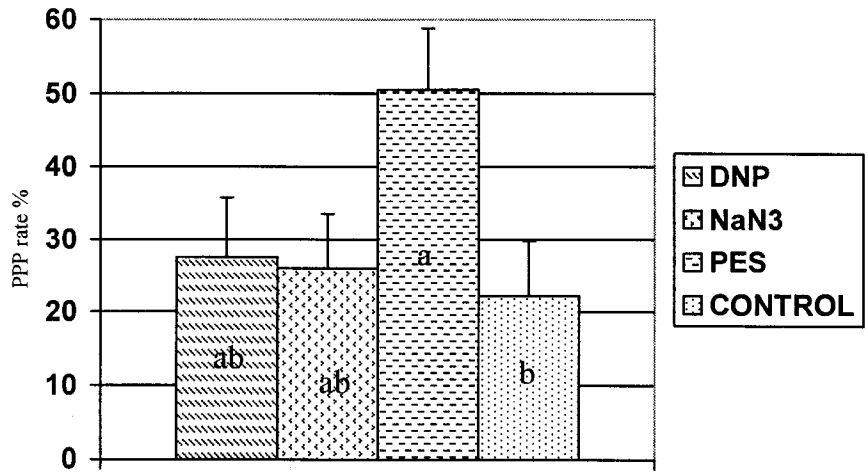
Figure 8.1. Glucose metabolism for embryos cultured with different metabolic regulators.



a, b. Bars without common letters differ (P<0.05).

For the PPP rate, embryos produced using bull 1 metabolized a higher percentage of glucose via the PPP pathway (49.3%) than embryos produced from bull 2 (13.8%) ($P<0.001$). There was no bull x treatment interaction effect ($P>0.1$). Embryos cultured in PES metabolized a much higher percentage of glucose via the PPP pathway than control embryos (50.5 vs 22.1%, respectively Figure 8.2); the difference was not significant however ($P=0.11$). DNP and NaN_3 groups used 27.5 and 26% of metabolized glucose in the PPP, respectively.

Figure 8.2. PPP rate (%) for embryos cultured with different metabolic regulators.

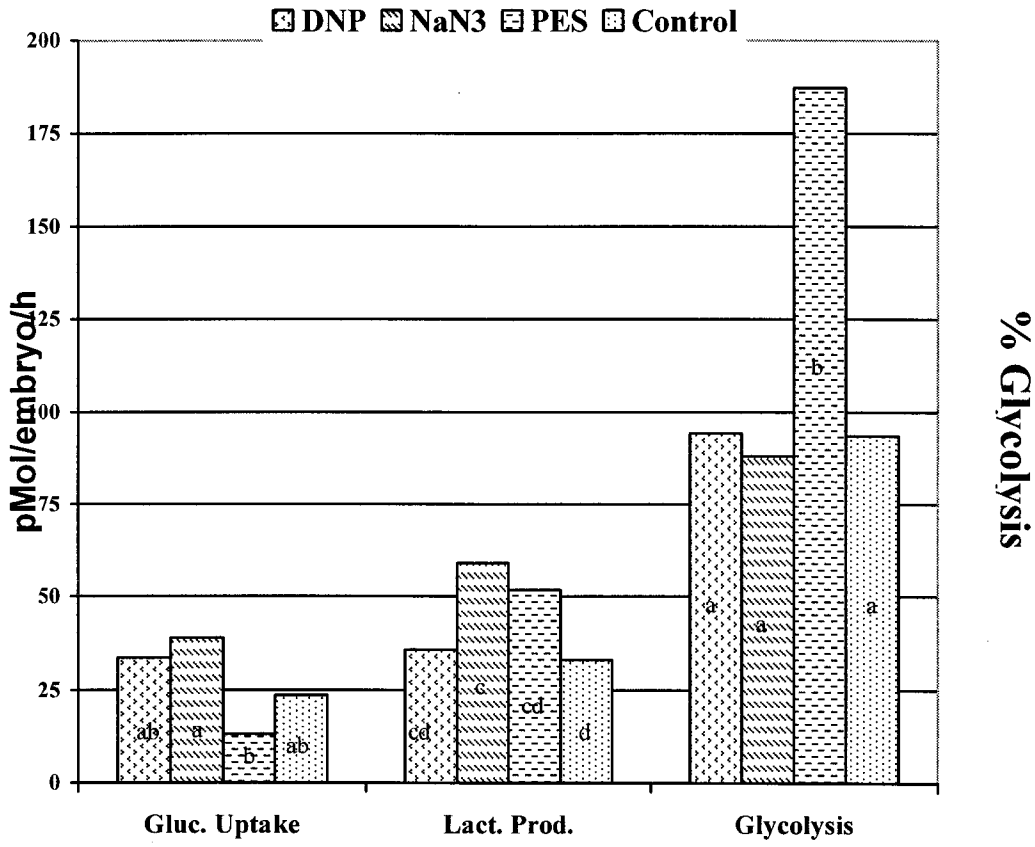


a, b. Bars without common letters differ ($P<0.11$).

Glucose uptake, lactate production and glycolysis for the treatments evaluated are presented in Figure 8.3. Embryos cultured with NaN_3 had higher glucose uptake than those produced in PES (38.9±4.2 vs 13.1±6.9 pMol/embryo/h; $P<0.01$). DNP and control embryos had intermediate values. NaN_3 treatment also tended to show higher lactate production than control embryos (58.91±7.2 vs 33.0±8.2; $P<0.08$). Finally, when the glycolytic rate was calculated (percentage of lactate production from glucose uptake on a

Molar basis), PES had a higher percentage (187.±21.09%), than DNP, NaN_3, and control treatments (94.2±14.2, 88.1±12.8, and 93.5±14.6, respectively) ($P<0.01$).

Figure 8.3. Glucose uptake, lactate production (pMol/embryo/h) and glyclolysis (%) for embryos exposed to three metabolic regulators at post-compaction stages



a, b. Bars without common letters within responses differ ($P<0.01$).
c, d. Bars without common letters within responses differ ($P<0.08$).

Lipid content was evaluated in ~ 100 embryos from the four in vitro groups and the in vivo control. As number of replicates of in vitro groups and in vivo embryos did not match, it was not possible to block per replicate, therefore, the five treatments were analyzed in a one way ANOVA. Figures 8.4, 8.5, 8.6, and 8.7 show numbers of small, medium, large and medium+large lipid granules per $1x10^3$ μM^2, respectively, for the four treatments evaluated. For last response, one large granule was considered equivalent to 8

medium granules (double diameter = 8-fold increase in volume), as explained in General Materials and Methods. Figure 8.8 shows microphotographs of in vitro-produced embryos from the different groups, stained with Sudan Black B.

Figure 8.4. LS means±SEM of small Sudan Black positive granules in embryos cultured with metabolic regulators.

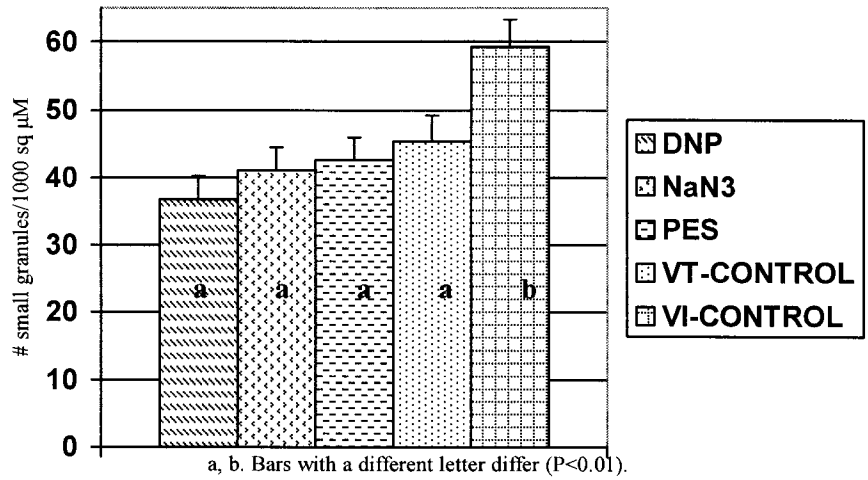


Figure 8.5. LS means±SEM of medium Sudan Black positive granules in embryos cultured with metabolic regulators.

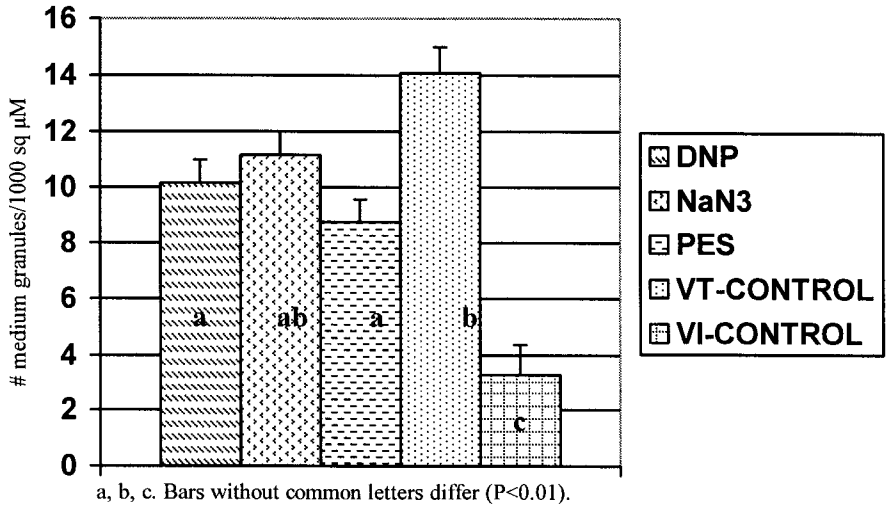
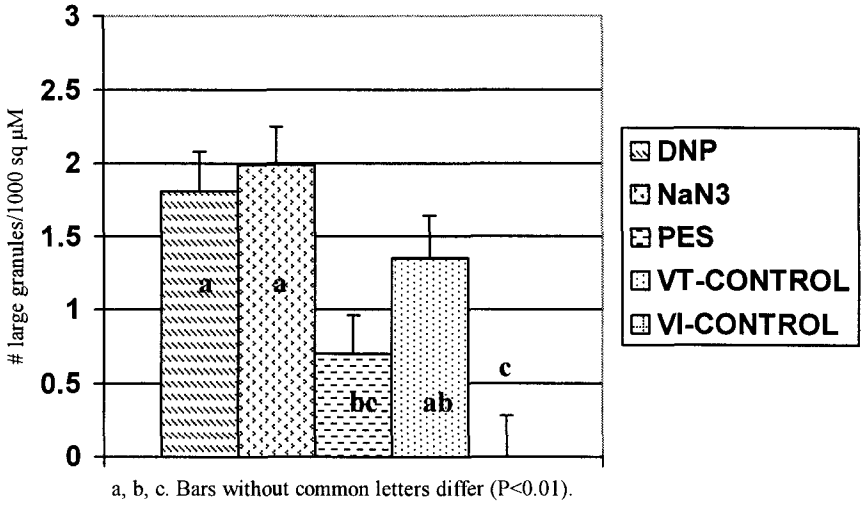
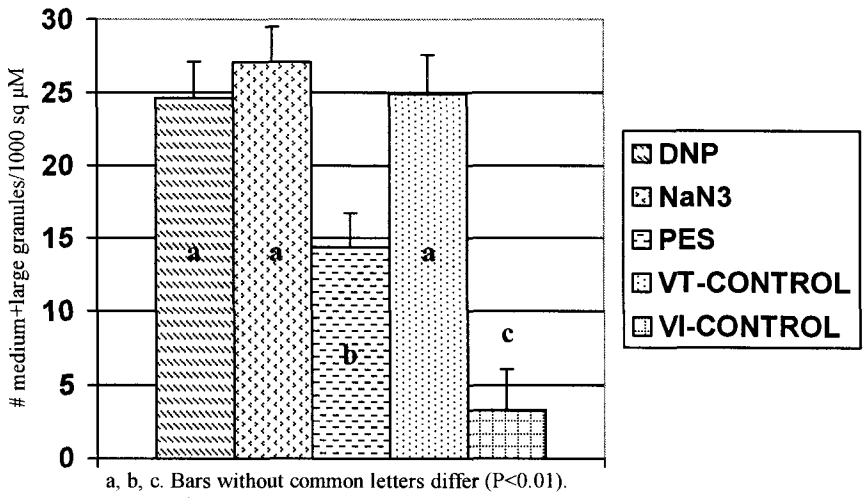


Figure 8.6. LS means±SEM of large Sudan Black positive granules in embryos cultured with metabolic regulators.



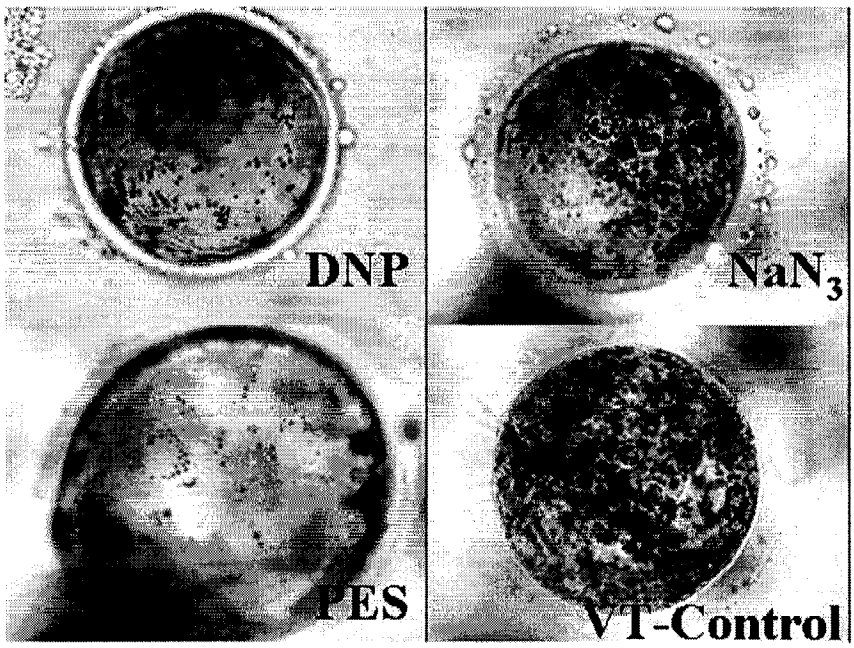
a, b, c. Bars without common letters differ (P<0.01).

Figure 8.7. LSmeans±SEM of medium+large Sudan Black positive granules in embryos cultured with metabolic regulators.



a, b, c. Bars without common letters differ (P<0.01).

Figure 8.8. In vitro-produced embryos from different groups, stained with Sudan Black B



There were no differences in surface area or development rate of day-14 embryos among the treatments studied (Table 8.2.).

Table 8.2. Least squares means ± SEM for surface area (mm^2) and development rate, of embryos cultured in chemically defined medium with 3 metabolic regulators.[a]

Treatment	n	Surface area (mm^2)	Development rate[1]
DNP	29	7.0±3.15	47.8
NaN_3	21	5.75±3.45	43.0
PES	19	1.12±3.86	49.7
CONTROL	27	5.02±3.45	54.0

[1] Percentage of live embryos recovered, out of embryos transferred.
[a] There were no significant differences (P>0.1)

8.6. Discussion.

Table 8.3 summarizes the responses of embryos for the four groups evaluated, as a basis for discussion, and Figure 8.8 depicts the mechanisms of action of the metabolic regulators used in this experiment.

Table 8.3. Summary of responses of embryos exposed to three metabolic regulators

Response	P-value	DNP	NaN_3	PES	Control
Glucose metabolism	P<0.05	↔	↓	↑	↔
PPP	P<0.12	↔	↔	↑	↓
Glucose uptake	P<0.01	↔	↑	↓	↔
Lactate production	P<0.08	↔	↑	↔	↓
Glycolysis	P<0.01	↓	↓	↑	↓
Medium lipid granules	P<0.001	↓	↔	↓	↑
Large lipid granules	P<0.01	↑	↑	↓	↔
Medium+large lipid granules	P<0.01	↑	↑	↓	↑

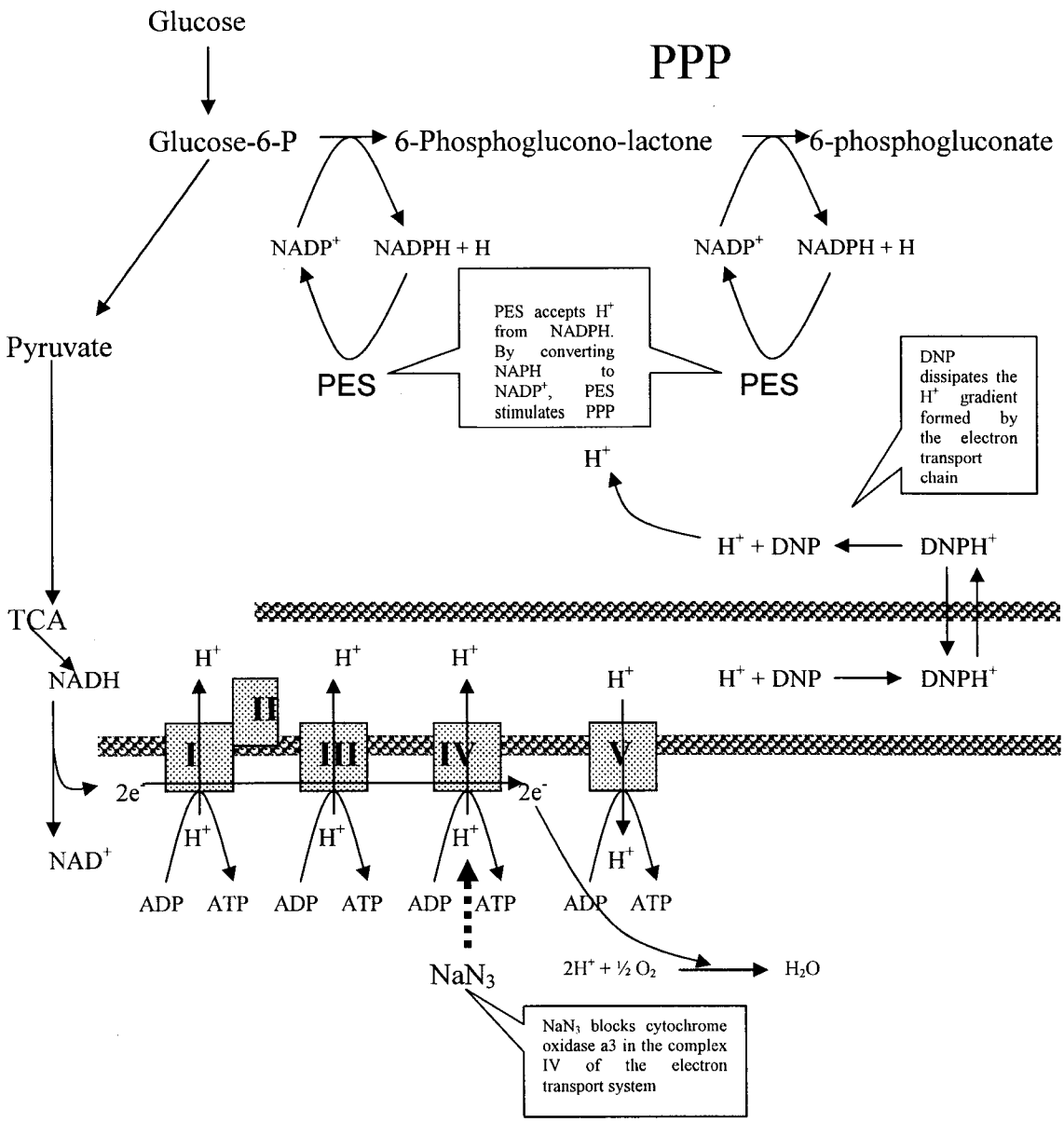
↑ Indicates significant higher response than ↓ in the same row.

↓ Indicates significant lower response than ↑ in the same row.

↔ Indicates intermediate response in the same row.

There were no differences among treatments in % blastocyst production or embryo quality (Table 8.1). This is not surprising because doses of respective metabolic regulator were chosen that were not toxic and similar to controls for embryo quality in the experiments discussed in chapters 5 and 6.

Figure 8.9. Mechanism of action of PES, DNP and NaN_3.



Embryos cultured in presence of NaN_3, had the highest glucose uptake, and released most lactate; however, the relative amount of glucose taken up and converted into lactate on a molar basis was lower than the other groups. Additionally, glucose metabolism, calculated as the amount glucose entering the EMP, was lower than the other

groups. NaN_3 thus promoted high glucose uptake, but restricted glucose utilization by the glycolytic pathway. Thompson et al.,[104] found significant increases ($P<0.05$) of glucose uptake when bovine embryos were exposed to 10 and 100 μM NaN_3. These increments, however, were not accompanied with corresponding increments in lactate production. They suggested that NaN_3 might be inducing glucose utilization by mechanisms other than aerobic glycolysis; however, they did not perform glucose metabolism evaluations. In the present study, the reduction in glucose metabolism was not accompanied by an increment in the PPP rate; therefore, it is more probable that the excess glucose was directed to anabolic processes. Sodium azide also had high lipid accumulation. Thompson [23] mentions that doses over 10 μM are detrimental for bovine embryo development. We did not find a detrimental effects of the dose used (27 μM) during the screening experiments, nor were any of the responses in this experiment different from controls.

Thompson et al. [104] mention that another possible way in which NaN_3 could be beneficial for embryos is by favoring a more appropriate REDOX state. Harvey et al., [100] mention that a perturbed REDOX state originated by sub-optimal culture conditions, not only alters ATP production, but also can have effects on gene expression, with post-developmental consequences. High doses of Mitochondrial transport inhibitors, however, could also result in oxidative damage. Ishiguro et al,[155] found oxygen hypersensitivity and oxidative damage of cultured somatic cells, and in *C. elegans* upon exposure to 125 to 500 μM NaN_3. They mention that defective electron transport in the mitochondria causes oxidative stress, mainly by the generation of reactive oxygen species. They also

mention that sensitivity to NaN_3 may vary among cell types. It could be possible, therefore, that the NaN_3 dose used in this experiment was excessive under the specific circumstances in which the study was done, causing an imbalance in the REDOX state and disturbing mitochondrial function, which has been mentioned as a cause of lipid accumulation with in vitro-produced embryos.[39]

Phenazine ethosulfate had opposite effects to NaN_3. With this compound, embryos decreased their glucose uptake, but glucose metabolism, and PPP activity increased. The stimulatory effect of PES on glucose metabolism through the PPP has been reported by others.[122, 124] Glycolysis of embryos treated with PES was higher than the other groups. It is important to note that this is in part an artifact caused by the low values obtained for glucose uptake, since glycolysis is calculated as the proportion of moles of lactate produced from glucose taken up. The absolute amount of lactate produced was not different from the control group, and was even numerically lower than NaN_3 group. Downs et al. [105] found that cumulus cell-enclosed murine oocytes responded to 10 μM PES with increased glucose uptake, but an even higher increase in lactate production. Lactate production of oocytes cultured with no PES accounted for 67% of glucose uptake, whereas with oocytes exposed to 10 μM PES this value increased to 90%. Given the glucose uptake and glucose metabolism values obtained for this group, it is probable that embryos used endogenous glucose for their metabolic requirements. Utilization of endogenous glucose for metabolic functions is normal for preimplantation embryos.[92]

One striking characteristic of embryos produced in the presence of PES was the significant lower lipid accumulation, compared to the other two metabolic regulators and in vitro control. As mentioned in Literature Review, lipids can accumulate in the embryo from uptake from the culture environment,[18] or as a result of culture-induced impaired activity of mitochondria, and their inability to metabolize complex lipids through β-oxidation.[39] The nature of the completely defined medium used in this experiment removes any possibility of exogenous uptake of lipids, making the second cause the most probable. As PES putatively enhances PPP, and given that this is a biosynthetic pathway involved in fatty acid synthesis, we might be tempted to deduce that PES caused an increase in lipids in the embryo, rather that a decrease. However, the specific mechanism of action of PES (Figure 8.8), is to stimulate the PPP by converting NADPH into $NADP^+$, and as the reduced form of this nucleotide is required for fatty acid synthesis, PES can not stimulate this biosynthetic pathway, since the net amount of NADPH does not increase with this mechanism of stimulating the PPP. In any case, lipid accumulation represents a threat for embryo survival, as lipid-filled embryos are more susceptible to oxidative damage,[18] and have reduced chilling and freezing tolerance when subjected to cryopreservation procedures.[13, 40, 41]

We included an in vivo control, using lipid accumulation data obtained from in vivo-derived embryos of the same age, produced in a previous experiment. Embryos produced in the PES group had less lipids than any other treatment, including the in vitro control; however, exposure of embryos to PES was unable to reduce lipid accumulation to the levels seen on in vivo controls. Considering the increased contribution of the PPP

to glucose metabolism by embryos in PES, along with less accumulation of lipids, culture of embryos with 0.3 μM of PES may be a promising approach for improving the quality of in vitro-produced embryos.

Embryos cultured in presence of DNP responded similarly to controls in terms of the glucose metabolism parameters evaluated. However, even though DNP was statistically similar to the control and to the highest group in terms of glucose metabolism and glucose uptake, the numerical values are closer to treatments with the highest numbers than the controls (glucose metabolism: PES = 18.5, DNP = 17.1, control = 14.7; glucose uptake: NaN_3 = 38.9, DNP = 33.6, control = 23.6). Lipid accumulation was also significant in DNP embryos, suggesting possible excessive inhibition of oxidative phosphorylation and disruption of mitochondrial function. In the only similar experiment found in the literature reporting metabolic parameters of embryos treated with sub-acute doses of DNP, 10 μM DNP increased ($P<0.05$) glucose metabolism of day-7 embryos, from 23.1±2.45 pMol/embryo/3h for the control, to 33.3±2.49 pmol/embryo/3h in DNP-treated embryos. Although there was no evidence of a beneficial effect of DNP in this experiment, the increase in blastocyst rate that 30 μM DNP generated with the CDM1/CDM2 system (Chapter 6), along with positive results reported in the literature,[102-104] suggest that we should not completely discard this metabolic regulator. Perhaps it should be included in future experiments, with more study of doses in the range of 10 to 30 μM, and in combination with PES. Regarding this last possibility, as PES and DNP have different mechanisms of action (Figure 8.8), and cause different responses in terms of glucose uptake and metabolism and lipid accumulation, a

combination of PES and DNP might result in embryos with adequate levels of glucose uptake and metabolism, with less lipid accumulation.

We did not find any significant difference in size, expressed as surface area, of day 14 embryos produced with the metabolic regulators, and maintained in vivo for seven days. PES-treated embryos were the smallest (at least 5 times smaller in surface area that the other groups); however, as the range of surface areas among the embryos was very large (from 0.03 to 56.12 mm^2; See raw data in Appendix 3), the variability of the data was very large, and very little was explained by the treatments in the statistical analysis (r^2=0.08, coefficient of variation= 154%).Median surface areas (Appendix 3) are much more similar among treatments than means, 1.24 mm^2 for controls vs 0.99 mm^2 for PES (medians of median replicates).

There was no difference in developmental competence rates among the treatments. Percentages of recovered live embryos, compared to total transferred embryos were very similar; the overall mean was 48.6%. Considering that some live embryos likely were not be recovered, this percentage is an underestimate. These data indicate that at least half of the embryos produced with the IVP system used in this experiment were developing normally one week after transfer to recipients. This provides validation of the metabolic data obtained.

CHAPTER 9

CONCLUSIONS

From experiments performed in chapters 4, 5, and 6, we demonstrated that semen from different bulls affected development responses of morulae, including percentage of blastocysts, morphological quality, degree of lightness/darkness and speed of development.

Morulae developed on CDM1/CDM2 sequential media system exhibited great flexibility during post-compaction stages in adapting to changing glucose concentrations in the range of 0 to 8 mM.

The screening experiments performed in chapters 5 and 6 were useful to identify toxic compounds, and to determine doses of metabolic regulators with no detrimental effects, as determined by morphological evaluation of embryos.

Characteristics previously reported for in vitro-produced embryos (lipid accumulation, and higher lactate production), compared to in vivo-derived embryos, were confirmed. Furthermore, embryos produced in combined in vitro/in vivo systems, were less metabolically active than embryos cultured in the same system, either in vitro or in

vivo. Additionally, embryos with good morphology metabolized more glucose than morphologically fair quality embryos.

At least 50% of the embryos produced with the G1/G2 system, with the selected doses of metabolic regulators were developmentally competent after transfer to recipient cows.

Embryos exposed to PES at the compaction and post-compaction stages had increased glucose metabolism and accumulated much less lipid, than in vitro-produced embryos exposed to other metabolic regulators, and in vitro controls, making this treatment a promising approach to producing better embryos in vitro. DNP might also be a useful metabolic regulator, and further evaluation using slightly lower doses, possibly combined with PES, is advised.

Evaluation of offspring generated from in vitro-produced embryos exposed to metabolic regulators is the next logical step in research on metabolic regulation during post-compaction development of bovine embryos.

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APPENDIX 1

LEAST-SQUARES MEANS± STANDARD ERRORS (LSM±SEM) FOR METABOLIC REGULATOR AND GLUCOSE MAIN EFFECTS, AND FOR METABOLIC REGULATOR X GLUCOSE SIGNIFICANT INTERACTIONS FOR EXPERIMENTS IN CHAPTER 5.

Phenazine ethosulfate.

Response	Effect	LSM	SEM	Diff	Significance
% Blastocysts	**Dose**				
	0 μM	40.6	2.76	a	
	0.1 μM	39.2	2.76	ab	
	0.3 μM	39.1	2.76	ab	
	0.9 μM	30.3	2.76	b	P=0.038
	Glucose				
	0.0 mM	41.2	2.8	a	
	0.5 mM	34.8	2.8	ab	
	2.0 mM	32.3	2.8	b	
	8.0 mM	40.8	2.8	a	P=0.059

Response	Effect	LSM	SEM	Diff	Significance
Stage 36 h	**Dose**				
	0 μM	6.0	0.09	a	
	0.1 μM	5.9	0.09	a	
	0.3 μM	6.1	0.09	a	
	0.9 μM	6.2	0.10	a	P>0.1
	Glucose				
	0.0 mM	6.1	0.09	ab	
	0.5 mM	6.0	0.09	ab	
	2.0 mM	6.2	0.10	a	
	8.0 mM	5.8	0.09	b	P=0.084

Response	Effect	LSM	SEM	Diff	Significance
Quality 36 h	**Dose**				
	0 μM	1.15	0.05	a	
	0.1 μM	1.24	0.04	a	
	0.3 μM	1.15	0.04	a	
	0.9 μM	1.23	0.05	a	P>0.1
	Glucose				
	0.0 mM	1	0.04	a	
	0.5 mM	1.20	0.05	a	
	2.0 mM	1.17	0.05	a	
	8.0 mM	1.20	0.05	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Lightness 36 h	**Dose**				
	0 μM	1.95	0.08	a	
	0.1 μM	2.09	0.08	a	
	0.3 μM	2.15	0.08	a	
	0.9 μM	2.10	0.09	a	P>0.1
	Glucose				
	0.0 mM	1.96	0.08	a	
	0.5 mM	2.11	0.08	a	
	2.0 mM	2.16	0.09	a	
	8.0 mM	2.05	0.08	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
ICM 36 h	**Dose**				
	0 μM	1.24	0.07	a	
	0.1 μM	1.42	0.08	ab	
	0.3 μM	1.25	0.08	ab	
	0.9 μM	1.5	0.09	b	P=0.053
	Glucose				
	0.0 mM	1.27	0.08	a	
	0.5 mM	1.42	0.08	a	
	2.0 mM	1.41	0.07	a	
	8.0 mM	1.33	0.09	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Stage 72 h	**Dose**				
	0 μM	6.74	0.07	a	
	0.1 μM	6.64	0.07	a	
	0.3 μM	6.70	0.07	a	
	0.9 μM	6.87	0.07	a	P>0.1
	Glucose				
	0.0 mM	6.86	0.07	a	
	0.5 mM	6.68	0.07	a	
	2.0 mM	6.68	0.07	a	
	8.0 mM	6.73	0.07	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Quality 72 h	**Dose**				
	0 μM	1.63	0.08	a	
	0.1 μM	1.65	0.08	a	
	0.3 μM	1.65	0.09	a	
	0.9 μM	1.83	0.09	a	P>0.1
	Glucose				
	0.0 mM	1.59	0.09	a	
	0.5 mM	1.67	0.08	a	
	2.0 mM	1.84	0.09	a	
	8.0 mM	1.65	0.08	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Lightness 72 h	**Dose**				
	0 μM	2.09	0.07	a	
	0.1 μM	2.16	0.07	a	
	0.3 μM	2.28	0.08	a	
	0.9 μM	2.32	0.08	a	P>0.1
	Glucose				
	0.0 mM	2.06	0.08	a	
	0.5 mM	2.23	0.07	ab	
	2.0 mM	2.38	0.08	b	
	8.0 mM	2.19	0.07	ab	P=0.032

Response	Effect	LSM	SEM	Diff	Significance
ICM 72 h	**Dose**				
	0 μM	1.76	0.09	a	
	0.1 μM	1.64	0.09	a	
	0.3 μM	1.72	0.09	a	
	0.9 μM	1.92	0.09	a	P>0.1
	Glucose				
	0.0 mM	1.62	0.09	a	
	0.5 mM	1.79	0.09	a	
	2.0 mM	1.87	0.09	a	
	8.0 mM	1.76	0.09	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
# of cells	**Dose**				
	0 μM	116.2	4.88	a	
	0.1 μM	109.9	4.83	a	
	0.3 μM	110.4	4.87	a	
	0.9 μM	82.3	5.07	b	P<0.001
	Glucose				
	0.0 mM	107.7	4.88	a	
	0.5 mM	110.0	4.88	a	
	2.0 mM	103.7	5.08	a	
	8.0 mM	97.3	4.83	a	P>0.1

Phloretin.

Response	Effect	LSM	SEM	Diff	Significance
% Blastocysts	**Dose**				
	0 μM	55.6	3.40	a	
	30 μM	47.6	3.35	a	
	90 μM	47.1	3.35	a	
	270 μM	34.1	3.40	b	P<0.001
	Glucose				
	0.0 mM	50.0	3.35	a	
	0.5 mM	45.6	3.35	a	
	2.0 mM	44.3	3.40	a	
	8.0 mM	44.5	3.40	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Stage 36 h	**Dose**				
	0 μM	6.15	0.10	a	
	30 μM	6.18	0.10	a	
	90 μM	6.03	0.10	a	
	270 μM	6.06	0.10	a	P>0.1
	Glucose				
	0.0 mM	6.07	0.10	a	
	0.5 mM	6.07	0.10	a	
	2.0 mM	6.23	0.10	a	
	8.0 mM	6.05	0.10	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Quality 36 h	**Dose**				
	0 μM	1.06	0.03	a	
	30 μM	1.07	0.03	ab	
	90 μM	1.17	0.03	b	
	270 μM	1.06	0.03	ab	P=0.015
	Glucose				
	0.0 mM	1.09	0.03	a	
	0.5 mM	1.04	0.03	a	
	2.0 mM	1.14	0.03	a	
	8.0 mM	1.08	0.03	a	P>0.1

Response	Effect		LSM	SEM	Diff	Significance
Lightness 36 h	**Dose**					
	0 μM		1.96	0.06	a	
	30 μM		2.10	0.06	a	
	90 μM		2.02	0.06	a	
	270 μM		1.59	0.07	b	P<0.001
	Glucose					
	0.0 mM		1.92	0.06	a	
	0.5 mM		1.90	0.06	a	
	2.0 mM		1.86	0.07	a	
	8.0 mM		2.00	0.07	a	P>0.1
	Dose x Glucose					
	0 μM	0.0 mM	1.85	0.12		
	30 μM	0.0 mM	2.23	0.12		
	90 μM	0.0 mM	1.90	0.12		
	270 μM	0.0 mM	1.70	0.12		
	0 μM	0.5 mM	1.82	0.12		
	30 μM	0.5 mM	2.22	0.12		
	90 μM	0.5 mM	2.03	0.12		
	270 μM	0.5 mM	1.52	0.14		
	0 μM	2.0 mM	2.06	0.12		
	30 μM	2.0 mM	2.04	0.14		
	90 μM	2.0 mM	2.09	0.12		
	270 μM	2.0 mM	1.27	0.14		
	0 μM	8.0 mM	2.09	0.14		
	30 μM	8.0 mM	1.93	0.12		
	90 μM	8.0 mM	2.09	0.12		
	270 μM	8.0 mM	1.89	0.13		P=0.034

Response	Effect	LSM	SEM	Diff	Significance
ICM 36 h	**Dose**				
	0 μM	1.13	0.06	a	
	30 μM	1.06	0.06	a	
	90 μM	1.18	0.07	a	
	270 μM	1.21	0.07	a	P>0.1
	Glucose				
	0.0 mM	1.16	0.07	ab	
	0.5 mM	1.04	0.07	a	
	2.0 mM	1.28	0.07	b	
	8.0 mM	1.11	0.07	ab	P=0.091

Response	Effect	LSM	SEM	Diff	Significance
Stage 72 h	**Dose**				
	0 μM	6.80	0.07	a	
	30 μM	6.91	0.07	a	
	90 μM	6.65	0.07	ab	
	270 μM	6.39	0.07	b	P<0.001
	Glucose				
	0.0 mM	6.70	0.07	a	
	0.5 mM	6.64	0.07	a	
	2.0 mM	6.80	0.07	a	
	8.0 mM	6.60	0.07	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Quality 72 h	**Dose**				
	0 μM	1.49	0.11	a	
	30 μM	1.44	0.11	a	
	90 μM	1.97	0.11	b	
	270 μM	2.13	0.11	b	P<0.001
	Glucose				
	0.0 mM	1.59	0.10	a	
	0.5 mM	1.88	0.10	a	
	2.0 mM	1.70	0.11	a	
	8.0 mM	1.87	0.11	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Lightness 72 h	**Dose**				
	0 μM	2.14	0.07	ab	
	30 μM	2.03	0.07	ab	
	90 μM	2.30	0.07	a	
	270 μM	1.90	0.07	b	P<0.01
	Glucose				
	0.0 mM	1.99	0.07	a	
	0.5 mM	2.15	0.07	a	
	2.0 mM	2.14	0.07	a	
	8.0 mM	2.09	0.07	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
ICM 72 h	**Dose**				
	0 μM	1.51	0.12	a	
	30 μM	1.48	0.12	a	
	90 μM	1.95	0.13	b	
	270 μM	2.24	0.13	b	P<0.001
	Glucose				
	0.0 mM	1.71	0.12	a	
	0.5 mM	1.95	0.12	a	
	2.0 mM	1.76	0.12	a	
	8.0 mM	1.76	0.13	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
# of cells	**Dose**				
	0 μM	140.8	6.02	ab	
	30 μM	149.5	5.93	a	
	90 μM	122.7	5.93	b	
	270 μM	87.3	6.10	c	P<0.001
	Glucose				
	0.0 mM	117.1	5.93	a	
	0.5 mM	127.2	5.93	a	
	2.0 mM	128.2	6.02	a	
	8.0 mM	127.8	6.10	a	P>0.1

Pyrroline-5-carboxylate.

Response	Effect	LSM	SEM	Diff	Significance
% Blastocysts	**Dose**				
	0 μM	54.7	3.49	a	
	9μM	53.2	3.49	a	
	27 μM	56.0	3.49	a	
	81 μM	55.2	3.49	a	P>0.1
	Glucose				
	0.0 mM	51.7	3.49	a	
	0.5 mM	56.9	3.49	a	
	2.0 mM	56.8	3.49	a	
	8.0 mM	53.7	3.49	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Stage 36 h	**Dose**				
	0 μM	4.69	0.06	a	
	9μM	4.63	0.06	a	
	27 μM	4.70	0.06	a	
	81 μM	4.64	0.06	a	P>0.1
	Glucose				
	0.0 mM	4.56	0.06	a	
	0.5 mM	4.66	0.06	a	
	2.0 mM	4.70	0.06	a	
	8.0 mM	4.73	0.06	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Quality 36 h	**Dose**				
	0 μM	1.19	0.03	a	
	9μM	1.17	0.03	a	
	27 μM	1.17	0.03	a	
	81 μM	1.13	0.03	a	P>0.1
	Glucose				
	0.0 mM	1.13	0.03	a	
	0.5 mM	1.18	0.03	a	
	2.0 mM	1.17	0.03	a	
	8.0 mM	1.18	0.03	a	P>0.1
	Dose x Glucose				
	0 μM 0.0 mM	1.18	0.07		
	9 μM 0.0 mM	1.16	0.06		
	27 μM 0.0 mM	1.06	0.07		
	81 μM 0.0 mM	1.12	0.07		
	0 μM 0.5 mM	1.19	0.06		
	9 μM 0.5 mM	1.16	0.06		
	27 μM 0.5 mM	1.28	0.07		
	81 μM 0.5 mM	1.10	0.07		
	0 μM 2.0 mM	1.08	0.06		
	9 μM 2.0 mM	1.24	0.06		
	27 μM 2.0 mM	1.22	0.06		
	81 μM 2.0 mM	1.13	0.06		
	0 μM 8.0 mM	1.33	0.06		
	9 μM 8.0 mM	1.11	0.06		
	27 μM 8.0 mM	1.07	0.06		
	81 μM 8.0 mM	1.17	0.07		P=0.077

Response	Effect	LSM	SEM	Diff	Significance
Lightness 36 h	**Dose**				
	0 μM	2.06	0.05	a	
	9μM	1.98	0.05	a	
	27 μM	2.00	0.05	a	
	81 μM	1.95	0.05	a	P>0.1
	Glucose				
	0.0 mM	1.13	0.03	a	
	0.5 mM	1.18	0.03	a	
	2.0 mM	1.17	0.03	a	
	8.0 mM	1.18	0.03	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
ICM 36 h	**Dose**				
	0 μM	1.27	0.04	a	
	9μM	1.15	0.04	a	
	27 μM	1.20	0.04	a	
	81 μM	1.17	0.04	a	P>0.1
	Glucose				
	0.0 mM	1.18	0.04	a	
	0.5 mM	1.18	0.04	a	
	2.0 mM	1.18	0.04	a	
	8.0 mM	1.25	0.04	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Stage 72 h	**Dose**				
	0 μM	5.62	0.11	a	
	9μM	5.54	0.11	a	
	27 μM	5.69	0.11	a	
	81 μM	5.69	0.11	a	P>0.1
	Glucose				
	0.0 mM	5.53	0.11	a	
	0.5 mM	5.71	0.11	a	
	2.0 mM	5.67	0.11	a	
	8.0 mM	5.63	0.11	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Quality 72 h	**Dose**				
	0 μM	1.52	0.06	a	
	9μM	1.62	0.06	a	
	27 μM	1.41	0.07	a	
	81 μM	1.56	0.06	a	P>0.1
	Glucose				
	0.0 mM	1.40	0.07	a	
	0.5 mM	1.56	0.06	a	
	2.0 mM	1.58	0.06	a	
	8.0 mM	1.58	0.06	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Lightness 72 h	**Dose**				
	0 μM	1.96	0.06	a	
	9μM	2.00	0.06	a	
	27 μM	1.92	0.06	a	
	81 μM	2.03	0.06	a	P>0.1
	Glucose				
	0.0 mM	1.96	0.06	a	
	0.5 mM	1.98	0.06	a	
	2.0 mM	1.96	0.06	a	
	8.0 mM	2.01	0.06	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
ICM 72 h	**Dose**				
	0 μM	1.38	0.05	ab	
	9μM	1.48	0.05	a	
	27 μM	1.33	0.05	b	
	81 μM	1.46	0.05	ab	P=0.078
	Glucose				
	0.0 mM	1.40	0.07	a	
	0.5 mM	1.56	0.06	a	
	2.0 mM	1.58	0.06	a	
	8.0 mM	1.58	0.06	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
# of cells	**Dose**				
	0 μM	96.4	4.00	a	
	9μM	100.7	3.83	a	
	27 μM	108.9	4.14	a	
	81 μM	100.7	3.83	a	P>0.1
	Glucose				
	0.0 mM	102.7	3.95	a	
	0.5 mM	101.1	4.00	a	
	2.0 mM	104.6	3.88	a	
	8.0 mM	98.4	3.95	a	P>0.1

Sodium azide.

Response	Effect	LSM	SEM	Diff	Significance
% Blastocysts	**Dose**				
	0 μM	41.9	3.00	a	
	3 μM	39.7	3.05	a	
	9 μM	36.0	3.00	a	
	27 μM	40.6	3.00	a	P>0.1
	Glucose				
	0.0 mM	41.7	3.05	a	
	0.5 mM	37.3	3.00	a	
	2.0 mM	37.6	3.00	a	
	8.0 mM	41.6	3.00	a	P>0.1

Response	Effect		LSM	SEM	Diff	Significance
Stage 36 h	**Dose**					
	0 μM		6.19	0.06	ab	
	3 μM		6.07	0.06	a	
	9 μM		6.18	0.06	ab	
	27 μM		6.29	0.06	b	P=0.096
	Glucose					
	0.0 mM		6.16	0.06	a	
	0.5 mM		6.20	0.06	a	
	2.0 mM		6.19	0.06	a	
	8.0 mM		6.18	0.06	a	P>0.1
	Dose x Glucose					
	0 μM	0.0 mM	6.19	0.13		
	3 μM	0.0 mM	6.21	0.12		
	9 μM	0.0 mM	6.08	0.12		
	27 μM	0.0 mM	6.16	0.12		
	0 μM	0.5 mM	6.20	0.11		
	3 μM	0.5 mM	6.07	0.12		
	9 μM	0.5 mM	6.27	0.12		
	27 μM	0.5 mM	6.28	0.11		
	0 μM	2.0 mM	6.25	0.11		
	3 μM	2.0 mM	5.75	0.12		
	9 μM	2.0 mM	6.35	0.12		
	27 μM	2.0 mM	6.40	0.12		
	0 μM	8.0 mM	6.13	0.11		
	3 μM	8.0 mM	6.26	0.12		
	9 μM	8.0 mM	6.03	0.14		
	27 μM	8.0 mM	6.30	0.12		P=0.049

Response	Effect		LSM	SEM	Diff	Significance
Quality 36 h	**Dose**					
	0 μM		1.14	0.04	a	
	3 μM		1.16	0.04	a	
	9 μM		1.16	0.04	a	
	27 μM		1.08	0.04	a	P>0.1
	Glucose					
	0.0 mM		1.14	0.05	a	
	0.5 mM		1.13	0.04	a	
	2.0 mM		1.10	0.04	a	
	8.0 mM		1.17	0.05	a	P>0.1
	Dose x Glucose					
	0 μM	0.0 mM	1.09	0.10		
	3 μM	0.0 mM	1.19	0.09		
	9 μM	0.0 mM	1.26	0.09		
	27 μM	0.0 mM	1.03	0.09		
	0 μM	0.5 mM	1.04	0.08		
	3 μM	0.5 mM	1.19	0.09		
	9 μM	0.5 mM	1.11	0.09		
	27 μM	0.5 mM	1.26	0.08		
	0 μM	2.0 mM	1.04	0.08		
	3 μM	2.0 mM	1.21	0.09		
	9 μM	2.0 mM	1.08	0.09		
	27 μM	2.0 mM	1.05	0.09		
	0 μM	8.0 mM	1.40	0.08		
	3 μM	8.0 mM	1.10	0.09		
	9 μM	8.0 mM	1.19	0.11		
	27 μM	8.0 mM	1.00	0.09		P=0.032

Response	Effect	LSM	SEM	Diff	Significance
Lightness 36 h	**Dose**				
	0 μM	2.09	0.06	a	
	3 μM	2.10	0.06	a	
	9 μM	2.11	0.07	a	
	27 μM	2.05	0.06	a	P>0.1
	Glucose				
	0.0 mM	2.03	0.06	ab	
	0.5 mM	2.18	0.06	a	
	2.0 mM	2.17	0.06	ab	
	8.0 mM	1.98	0.06	b	P=0.067

Response	Effect	LSM	SEM	Diff	Significance
ICM 36 h	**Dose**				
	0 μM	1.12	0.04	a	
	3 μM	1.14	0.05	a	
	9 μM	1.19	0.04	a	
	27 μM	1.06	0.04	a	P>0.1
	Glucose				
	0.0 mM	1.17	0.04	a	
	0.5 mM	1.12	0.04	a	
	2.0 mM	1.09	0.04	a	
	8.0 mM	1.14	0.04	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Stage 72 h	**Dose**				
	0 μM	6.89	0.06	a	
	3 μM	6.82	0.06	a	
	9 μM	6.76	0.06	a	
	27 μM	6.84	0.06	a	P>0.1
	Glucose				
	0.0 mM	6.75	0.06	a	
	0.5 mM	6.90	0.06	a	
	2.0 mM	6.88	0.06	a	
	8.0 mM	6.78	0.06	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Quality 72 h	**Dose**				
	0 μM	1.42	0.08	a	
	3 μM	1.47	0.08	a	
	9 μM	1.60	0.08	a	
	27 μM	1.43	0.08	a	P>0.1
	Glucose				
	0.0 mM	1.52	0.08	a	
	0.5 mM	1.50	0.08	a	
	2.0 mM	1.40	0.08	a	
	8.0 mM	1.49	0.08	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
Lightness 72 h	**Dose**				
	0 μM	2.22	0.06	ab	
	3 μM	2.05	0.06	ab	
	9 μM	2.24	0.06	a	
	27 μM	2.00	0.06	b	P<0.01
	Glucose				
	0.0 mM	2.11	0.06	a	
	0.5 mM	2.18	0.06	a	
	2.0 mM	2.09	0.06	a	
	8.0 mM	2.14	0.06	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
ICM 72 h	**Dose**				
	0 μM	1.30	0.06	a	
	3 μM	1.30	0.06	a	
	9 μM	1.47	0.06	a	
	27 μM	1.33	0.06	a	P>0.1
	Glucose				
	0.0 mM	1.37	0.06	a	
	0.5 mM	1.41	0.06	a	
	2.0 mM	1.30	0.06	a	
	8.0 mM	1.31	0.06	a	P>0.1

Response	Effect	LSM	SEM	Diff	Significance
# of cells	**Dose**				
	0 μM	132.3	5.05	a	
	3 μM	130.0	5.14	a	
	9 μM	132.4	5.05	a	
	27 μM	127.9	5.14	a	P>0.1
	Glucose				
	0.0 mM	130.6	5.14	a	
	0.5 mM	125.6	5.05	a	
	2.0 mM	137.3	5.05	a	
	8.0 mM	129.1	5.14	a	P>0.1

APPENDIX 2

ANALYSIS OF VARIANCE TABLES FOR THE THREE ANALYSES PERFORMED ON THE SIX RESPONSES, OF EXPERIMENT ONE IN CHAPTER 6.

Analysis 1. Factorial 3x3x3.

Response:Stage

Source of variation	d. f.	Mean square	P-value
Bull	2	0.1076	0.0553
Dose	2	0.4091	<0.0001
Bullxdose	4	0.0689	0.1143
Time	2	0.1861	0.0079
Bullxtime	4	0.0108	0.8726
Dosextime	4	0.0055	0.09595
Error	61	0.0354	
Corrected total	79		

r^2= 0.4489

Response:Quality

Source of variation	d. f.	Mean square	P-value
Bull	2	0.1332	0.0783
Dose	2	0.0980	0.1503
Bullxdose	4	0.0400	0.5304
Time	2	0.0742	0.2356
Bullxtime	4	0.0073	0.9639
Dosextime	4	0.0165	0.8568
Error	61	0.0501	
Corrected total	79		

r^2= 0.2226

Response:Lightness

Source of variation	d. f.	Mean square	P-value
Bull	2	0.1227	0.1190
Dose	2	0.1724	0.0524
Bullxdose	4	0.0044	0.9881
Time	2	0.1899	0.0394
Bullxtime	4	0.0920	0.1725
Dosextime	4	0.0173	0.8688
Error	61	0.0556	
Corrected total	79		

r^2= 0.2967

Response:% blastocysts

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0550	0.0497
Dose	2	0.0214	0.2993
Bullxdose	4	0.0137	0.5371
Time	2	0.0158	0.4095
Bullxtime	4	0.0071	0.8015
Dosextime	4	0.0136	0.5400
Error	61	0.0174	
Corrected total	79		

r^2= 0.2319

Response:ICM

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0449	0.1241
Dose	2	0.0176	0.4340
Bullxdose	4	0.0349	0.1662
Time	2	0.0279	0.2689
Bullxtime	4	0.0214	0.3983
Dosextime	4	0.0283	0.2579
Error	60	0.0208	
Corrected total	78		

r^2= 0.2970

Response:Number of cells

Source of variation	d. f.	Mean square	P-value
Bull	2	371.3506	0.2480
Dose	2	265.1006	0.3672
Bullxdose	4	98.0061	0.8245
Time	2	254.9386	0.3813
Bullxtime	4	94.0759	0.8351
Dosextime	4	227.6341	0.4845
Error	61	260.2892	
Corrected total	79		

$r^2 = 0.1798$

Analysis 2. Factorial 3 (bulls)x4 (doses).

Response: Stage

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0379	0.3912
Dose	3	0.2701	0.0004
Bullxdose	6	0.0615	0.1768
Error	77	0.0399	
Corrected total	88		

$r^2 = 0.3080$

Response: Quality

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0440	0.4185
Dose	3	0.0789	0.2011
Bullxdose	6	0.0430	0.5272
Error	77	0.0500	
Corrected total	88		

$r^2 = 0.1551$

Response: Lightness

Source of variation	d. f.	Mean square	P-value
Bull	2	0.2006	0.0539
Dose	3	0.1217	0.1468
Bullxdose	6	0.0116	0.9826
Error	77	0.0661	
Corrected total	88		

$r^2 = 0.1379$

Response: % blastocysts

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0846	0.0086
Dose	3	0.0431	0.0598
Bullxdose	6	0.0127	0.6046
Error	77	0.0167	
Corrected total	88		
r^2= 0.2181			

Response: ICM

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0056	0.7719
Dose	3	0.0101	0.7036
Bullxdose	6	0.0398	0.1022
Error	76	0.0216	
Corrected total	87		
r^2= 0.1661			

Response: Number of cells

Source of variation	d. f.	Mean square	P-value
Bull	2	1649.9847	0.0038
Dose	3	510.5892	0.1449
Bullxdose	6	449.5138	0.1503
Error	77	275.8006	
Corrected total	88		
r^2= 0.2203			

Analysis 3. Factorial 3 (bulls)x4 (times).

Response: Stage

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0363	0.4788
Timing	3	0.1246	0.0617
Bullxtiming	6	0.0216	0.8473
Error	77	0.0488	
Corrected total	88		
r^2= 0.1540			

Response: Quality

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0436	0.4383
Timing	3	0.0653	0.2985
Bullxtiming	6	0.0196	0.8934
Error	77	0.0524	
Corrected total	88		
r^2= 0.1144			

Response: Lightness

Source of variation	d. f.	Mean square	P-value
Bull	2	0.1913	0.0499
Timing	3	0.1390	0.0876
Bullxtiming	6	0.0676	0.3689
Error	77	0.0613	
Corrected total	88		
r^2= 0.2000			

Response: % blastocysts

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0848	0.0098
Timing	3	0.0392	0.0868
Bullxtiming	6	0.0081	0.8270
Error	77	0.0172	
Corrected total	88		
r^2= 0.1944			

Response: ICM

Source of variation	d. f.	Mean square	P-value
Bull	2	0.0066	0.7400
Timing	3	0.0260	0.4287
Bullxtiming	6	0.0295	0.2496
Error	76	0.0220	
Corrected total	87		
r^2= 0.1496			

Response: Number of cells

Source of variation	d. f.	Mean square	P-value
Bull	2	1646.2301	0.0039
Timing	3	496.8033	0.1549
Bullxtiming	6	445.3340	0.1557
Error	77	276.5753	
Corrected total	88		
r^2= 0.2181			

APPENDIX 3

INDIVIDUAL EMBRYO SURFACE AREA IN mm^2 PER TREATMENT, PER REPLICATE

Treatment	CONTROL				DNP				NaN_3				PES			
Replicate	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	0.46	0.03	3.17	0.41	2.10	0.04	0.13	0.43	0.48	0.79	0.19	0.07	0.05	0.53	0.11	0.62
2	0.77	0.25	4.23	0.43	6.38	0.05	1.59	0.50	3.06	2.12	0.24	0.21	0.11	2.31	0.28	1.39
3	1.53	0.25	6.35	0.78	11.48	0.20	2.08	0.50	5.10	3.31	0.64	0.93	0.20	3.70	0.66	1.66
4	2.30	0.31	18.18	0.95	17.35	0.20	2.64	0.66	7.78	3.31	1.42	1.11	0.20			1.73
5	10.2	0.31	22.48	0.99	17.35	0.44	4.46	0.76	13.27			1.40	0.44			2.49
6		0.44	29.75	1.13	56.12	0.56		0.83	15.31				0.44			
7		0.60	33.32	2.48				0.85	25.93				0.78			
8		1.00						0.96	41.48				0.79			
9								1.39								
10								1.66								
11								2.91								
12								3.12								