Global gene expression analysis of *in vitro* produced cryopreserved bovine embryos using vitrification and slow freezing techniques

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By

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TITLE OF THESIS: Global gene expression analysis of cryopreserved *in vitro* produced bovine embryos using vitrification and slow freezing techniques

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ABSTRACT

The overall objective of this thesis was to analyze developmental and gene expression changes in cryopreserved *in vitro* produced (IVP) bovine embryos using two techniques; vitrification and slow freezing.

Specifically, the first objective was to study and compare the blastocyst development of IVP bovine day 6 morulae [day 0 = *in vitro* fertilization (IVF)] after cryopreservation using vitrification and slow freezing (end point = development to expanded blastocyst stage on day 7-8). The blastocyst development rate for the vitrification group (52±4.6%) was higher ($p<0.001$) than that of the slow freezing group (35±4.2%). Blastocyst development rate was the highest in unfrozen control group (78 ± 3.6%). Re-expansion of vitrified morulae upon warming and correlation with subsequent blastocyst conversion rate was studied. No significant correlation was found between the vitrified morula re-expansion upon warming and blastocyst rate (Pearson's correlation = -0.048; $p>0.05$).

Data obtained from above study of cryopreserved embryos was also utilized for comparing the effect of season (cleavage rate and morula rate) and season x cryopreservation interaction on blastocyst conversion rate. Fall season had lower cleavage (67±1.6%; day 2) and morula (22±1.4%; day 6) rates than other seasons (74±1.1% and 30±1.2%, respectively; $p<0.05$). Spring, summer and winter seasons did not differ ($p>0.05$). Blastocyst conversion rate differed ($p<0.05$) between summer (63±4.5%) and spring (85±3.3%) seasons in unfrozen groups, between summer (20±4.1%) and all other seasons (43±8.3%) in slow freezing groups and did not differ among the seasons in the vitrification group ($p=0.19$).

Another aim of the study was to determine the effect of different cryoprotectants (glycerol and ethylene glycol) on the blastocyst conversion rates from slow frozen morulae. The blastocyst conversion rate had a tendency ($p=0.065$) to be higher in the ethylene glycol group (31±5.3%) than the glycerol group (18±4.3%).

A final set of experiments was designed to compare the global gene expression analysis of vitrified and slow frozen IVP embryos to control IVP embryos. Day 6 IVP bovine morulae were randomly distributed across three groups: unfrozen control, vitrified

and slow frozen. These embryos were allowed to grow to expanded blastocyst stage in culture for 24-48 h without treatment (control) or after warming (vitrification and slow freezing groups). Four successful replicates were conducted for each group on separate dates (6-7 embryos per group per replicate). Total RNA was extracted, RNA quality was tested using bio-analyzer and samples were hybridized on microarray slides. Images acquired from microarray slides were analyzed using ArrayPro™, ELMA, FlexArray and Ingenuity pathway analysis softwares. The vitrified group had 64 differentially regulated (up and down regulated) genes as compared with control group, while a total of 162 genes were differentially expressed in slow frozen group as compared with control group. Upon *in silico* analysis differential gene expression showed 76 genes up and downregulated between slow freezing and vitrification groups. Interestingly, vitrification embryos showed highest gene expression changes in lipid metabolism, cellular movement, cell-cell signaling, molecular transport and vitamin and mineral metabolism. Detailed pathway analysis showed the affected mechanisms of lipid peroxidation and steroid biosynthesis pointing towards the impairment of further embryo development. In addition, pathways involved in blastocyst hatching and apoptosis were affected. In slow frozen embryos, only three genes were affected. The pathways suggest involvement in cell structure and cell adhesion/MAPK pathways.

The study suggests that even if the survival of vitrified embryos is significantly higher than the slow frozen embryos, the viability, innate developmental capacity and future uterine implantation capability of vitrified embryos may be lower than that of slow frozen survivor embryos.

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“What you seek is seeking you”

Rumi

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A journey comes to an end when travelled with the constant loving, free-spirited yet dedicated efforts. I believe my journey has started not ended.

"I dedicate and submit all my thesis work to my Parents and My Family"

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LIST OF ABBREVIATIONS

µl: Microliter
A.a.: Amino acids
ABC/ABCC2: ATP Binding Cassette subfamily C
ACTG2: Actin-gamma 2
AETS: American Embryo Transfer Society
AI: Artificial insemination
AKR1B1: Aldo-keto reductase 1 B1
ANKRD1 Ankyrin repeat domain-containing protein 1
ANXA3: Annexin A 3
APOA1: Apolipoprotein A 1
AQP: Aquaporin
ART: Assisted reproductive technologies
aRNA: Amplified Ribonucleic acid
ATP: Adenosine Triphosphate
ATP5A1: ATP synthase-5 alpha subunit-1
ATRA: All-Trans-Retinoic Acid
Bax: BCL-2 associated protein
BCL-XL: B-cell lymphoma-extra large
BME: Basal Medium Eagle
BO: Brackett-Oliphant
BSA: Bovine serum albumin
CAGR: Canadian Animal Genetic Resources
CALB1: Calbindin 1
CAM: Cell adhesion molecules
cAMP: Cyclic adenosine monophosphate
CCL17: Chemokine C-C ligand 17
CEBPB: CCAAT/enhancer-binding protein beta
CHUK: Conserved helix-loop- helix ubiquitous kinase
CLA: Trans-10, cis-12 conjugated linoleic acid
CLDN23: Claudin 23
CMCF: Canadian Macromolecular Crystallography Facility
CR1: Charles Rosenkrans 1
CS: Newborn calf serum
CO2: Carbon dioxide
COC: Cumulus-oocyte complexes
COL4A1: Collagen 4 subunit A type 1
COL9A1: Collagen 9 subunit A type 1
CYP11A1: Cytochrome-P-450 subunit 11 Type 1
D/d= Day
DSC2: Desmocollin 2
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DPBS: Dulbecco’s phosphate buffered saline
ECM: Embryo culture medium
EEM: Extra embryonic membrane

EG: Ethylene glycol
EGF: Epidermal growth factor
EGFLAM: EGF-like, fibronectin Type III and laminin G domains
ELMA: EmbryoGene LIMS Microarray and Analysis
ERFP: European regional focal point for animal genetic resources
ERK: Extracellular signal regulated kinase
ET: Embryo transfer
FAO: Food and Agriculture Organization
FBC: Forced blastocoele collapse
FCS: Fetal calf serum
FGF/FGF2: Fibroblast growth factor
FSH: Follicle stimulating hormone
FXR: Farsenoid X receptors
GC: Guanine Cytosine
GnRH: Gonadotropin-releasing hormone
GPR3: G-protein coupled receptor 3
GV: Germinal vesicle
GVBD: Germinal vesicle breakdown
h: Hour
HDL: High density lipoprotein
hESC: Human embryonic stem cells
HNF4α- Hepatocyte nuclear factor-4α
HS: Holding Solution
HSD3β1: Hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 1
HSPA5: Heat Shock Protein 70kDa protein 5
HSP10A1-Heat shock protein 10 kDa protein 1
ICM: Inner cell mass
ICSI: Intracytoplasmic sperm injection
IETS: International Embryo Transfer Society
IFNγ- Interferon-gamma
IFNτ2: Interferon Tau 2
IGFBP: Insulin like growth factor binding protein
IIF: Intracellular ice formation
IL1β: Interleukin 1β
IVC: *In vitro* culture
IVD: *In vivo* derived
IVF: *In vitro* fertilization
IVM: *In vitro* maturation
IVP: *In vitro* production
KRT 7, 8, 18, 19: Keratin 7, 8, 18, 19
kCal: Kilo Calories
LH: Luteinizing hormone
LIF: Leukemia inhibitory factor
LN_2: Liquid nitrogen
LPS: Lipopolysaccharide
min: Minute

mL: Milliliter
mol^{-1}: per mole
MAPK-Mitogen activated protein kinase
MEM: Minimal essential medium
MIAME: Minimum information about a microarray experiment
mRNA: Messenger ribonucleic acid
MMP: Matrix-metalloproteinase
MTOCS: Microtubule organizing center
MZT: Maternal-zygote transition
n: Number
N_2: Nitrogen
NAD^+: Nicotinamide adenine dinucleotide
NADH: Nicotinamide adenine dinucleotide hydrogenase
NCBI: National center for biotechnology information
NFκβ: Nuclear factor- kappa beta
NMR: Nuclear magnetic resonance
NSAID: Non-steroid anti-inflammatory drug
O_2: Oxygen
OPS: Open pulled straw
OPU: Ovum pick up
PAF: Platelet-activating factor
PAG2: P-antigen family 2 (prostrate associated)
PCOS: Polycystic ovarian syndrome
PDGFβ- Platelet derived growth factor-β
PES: Phenazine ethosulphate
PG: Prostaglandin
PGE2: Prostaglandin E 2
PGF2α: Prostaglandin F2α
PGS: Primordial germ cells
PLA2R1: Phospholipase 2 receptor 1
PLAC8: Placenta specific gene 8 protein
PLAU/uPA: Urokinase type plasminogen
PRDX2: Peroxiredoxin 2
PTGS2/COX2: Prostaglandin synthase 2/ Cyclooxygenase2
PvS: Perivitelline space
qRT-PCR: Quantitative real time-Polymerase Chain Reaction
RBC: Red blood cells
RNA: Ribo nucleic acid
ROS: Reactive oxygen species
RXR: Retinoid X receptor
s: Second
SER: Smooth endoplasmic reticulum
SNP: Single nucleotide polymorphism
SPP1: Secreted phosphoprotein 1, Osteopontin
StARD1 and 3: Steroidogenic acute regulatory protein 1 and 3
TE: Trophoectoderm/ Trophoectodermal
TGC: Trophoblastic giant cells
TGFβ: Transfromation growth factor β
TJP1: Tight junction proetin 1

TKTL1: Transketolase 1
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF: Vascular endothelial growth factor
VIM: Vimentin
vs: Versus
VS1: Vitrification solution 1
VS2: Vitrification solution 2
WOW: Well of wells
WNT: Wnt signalling proteins
ZP3: Zona protein 3

CHAPTER 1 **INTRODUCTION**

In both developing and developed countries, local and culturally important animal breeds and their gene bank is endangered because of increasing food market pressure. Today's consumer requires a high variety and quality of food leading to high selection pressure on the livestock animals (Canadian Animal Genetic Resources, CAGR). This intense selection pressure however, challenges the population of naturally or traditionally occurring animals breeds. Moreover, there is a worldwide awareness to save and rejuvenate the endangered breeds, to conserve allelic variation and genetic diversity (FAO 2007).

Along with this, to enhance food security, new methods are being explored to propagate the high producing genetic resources (FAO 2007). Cryopreservation of biological material is an important aspect of agriculture, animal food industry, human reproductive medicine and beyond (Mazur 1970). For instance, cryopreservation of gametes and embryos is important in farm animal genetics, breeding and conservation. It is an insurance against any unforeseen natural calamities or disease threats (Prentice and Anzar 2011). It also provides means to transport these gametes and embryos across the world, thus, allows introduction of high quality genome in herds. Also, it replaces and supports the highly expensive *in situ* and *ex situ-in vivo* live animal conservation strategy for gene banking (FAO 2007; Woelders *et al.* 2012).

Cryopreservation technique has been derived from naturally occurring biological beings, able to survive at below zero temperatures (Mazur 1970). Cryopreservation technique was first applied successfully in mouse embryos (Whittingham *et al.* 1972) and subsequently transferred in bovines and other farm animals.

Cryopreservation of animal gametes *viz. sperm and oocytes* provides a chance to preserve the male and female complement of genes separately, for a longer period and for selection of higher genetic merit herd at later stages. Also, due to its high availability semen is successfully cryopreserved and provides a chance of introduction of heterozygosity into any animal population easily (Prentice and Anzar 2011; Woelders *et al.* 2012). Storing oocytes to create ova banks helps in avoiding backcrossing to create healthy herds, when used with semen for *in vitro* fertilization (IVF). However, oocytes are highly susceptible to cryopreservation (Arav 2014) and reliable methods for oocyte cryopreservation are still under development for many livestock species (Prentice and Anzar 2011). Embryo cryopreservation is a dependable method for establishing gene banks and can be utilized to create a healthy herd of required genetic makeup without any need for complement of other gender. It is especially helpful in rejuvenating a lost or endangered breed population in a shorter period of time (Woelders *et al.* 2012). Therefore, embryo cryopreservation has been used as well as explored to a greater extent and is successful (Arav 2014).

Various events viz. cryoprotectant equilibration, cooling, extracellular ice formation cellular dehydration and subsequently storage at vitrified state at -196º C; ideally define embryo cryopreservation (Mazur 1963). Thawing or warming of embryos upon recovery is the final step. Based upon these, several different cryopreservation techniques like slow freezing or programmed freezing and rapid freezing, ultra-rapid freezing or vitrification were tried overtime. However, these techniques are merely the derivation or advancements of one another.

With time two major techniques; slow freezing and vitrification have been developed and widely applied in research and field conditions. Slow freezing, as the name suggests is the freezing of embryos at a slow/programmed cooling rates and in a moderate concentration of cryoprotectants (Mazur 1970). It typically involves the conventional steps of cryoprotectant equilibration, cooling the embryos at a programmed rate of 0.3-0.5ºC per minute to -35ºC and plunging them into liquid nitrogen (N_2) for storage (Carvalho 1995). In vitrification, on the other hand, a high concentration of cryoprotectants and exponential rates of cooling are used. Vitrification of embryos usually involves cryoprotectant equilibration in a less concentrated followed by short exposure to highly concentrated cryoprotectant and plunging the specimen directly into liquid N_2.

Vitrification is although a simple to accomplish and inexpensive method to cryopreserve compared with slow freezing. Also, it can be frequently used in field conditions and less equipped laboratories. However, it requires high technical expertise to use the vitrification technique.

Slow freezing ideally does not involve intra-cellular ice formation and the survivability of embryos during slow freezing depends on extracellular ice formation as well as proper cryoprotectant equilibration to move the water out of cells, before freezing. However, there are chances of ice formation intracellularly, which is the major reason for cell injury during slow freezing (Mazur 1963). Vitrification involves the formation of glass like structure with the use of highly viscous cryoprotectants and exponential cooling rates for embryos. This, however, is associated with other possible cellular hazards due to use of high cryoprotectant concentration that prove toxic if used

for longer durations. Also, accelerated rates of cryopreservation cause intracellular fractures, sometimes. All these reasons have driven all efforts to establish better and customized protocols for both techniques in the field. Therefore, we wanted to study the impact of vitrification and slow freezing technique on post-warming development of *in vitro* produced (IVP) bovine morulae.

Although, vitrification is a better technique in embryo survival than slow freezing; several studies have been conducted to study the damage caused by them at cellular (Sommerfeld and Niemann 1999), ultra-structural and physiological level. However, not many studies have been conducted on the global gene expression of embryos surviving post-cryopreservation using vitrification and slow freezing. Therefore, we wanted to study the same, as it will provide a deeper insight into the effects of cryopreservation on bovine embryo development.

Embryo cryopreservation is species, stage and origin (*in vitro* or *in vivo*) dependent (Massip 2001; Vajta and Nagy 2006). For example, embryos of sheep and bovine are more tolerant to freezing than pig and horses due to biochemical and anatomical differences. Also, *in vivo* derived (IVD) embryos are more resistant to freezing injury than *in vitro* produced ones. One of the reasons for this difference is mainly the cytoplasmic lipid content. Higher cellular lipid content contributes to the fragility of embryonic health upon cryopreservation (Massip *et al.* 1995). Also, cell membrane and cytoskeletal structure damage is also determined by the origin of embryos. Apart from this, different stages of embryos also determine the cryo-survivability. For example, early cleaved embryos (2-16 celled) are more sensitive to cryopreservation and morula-blastocyst stages are the most resistant ones. Therefore, the packaging material and

protocols for both techniques varies according to the type of specimen and species involved (Saragusty and Arav 2011).

Bovine embryos are resistant to cryopreservation, but it is dependent on origin of embryos (*in vitro* vs *in vivo*) (Massip *et al.* 1995; Massip 2001). However, embryo production is dependent on season in certain cattle breeds. Heat stress associated with summer season has an impact on the quality, developmental competence as well as gene expression of oocytes (Gendelman and Roth 2012). Also, the follicular development and hormonal balance gets affected due to delayed effects of summer (Mazur 1970; Sartori *et al.* 2002). Thus, we wanted to study the impact of season on IVP of embryos and their survival after using different cryopreservation techniques.

Cryoprotectant equilibration is an important step in both the techniques of cryopreservation. Proper equilibration ensures better survival of embryos. Also, permeability of cryoprotectant determines the type and toxicity of cryoprotectant (Mazur 1970). Ethylene glycol (EG) and glycerol are being used for long periods in slow freezing of embryos. Ethylene glycol however, is a better cryoprotectant and allows direct transfer of embryos; without involving the step of cryoprotectant dilution upon warming as opposed to glycerol which is not as easily removed from embryos. In our study, we wanted to test the efficacy of both the cryoprotectants in slow freezing of IVP bovine morulae.

CHAPTER 2 **LITERATURE REVIEW**

2.1 Conservation of animal genetic resources

High market demands of superior commercial livestock breeds for increased productivity puts high selection pressure on animal population. Livestock animal breeding strategy adopted by companies worldwide, is leading to extinction of local and rare breeds. According to Food and Agriculture Organization (FAO), this extinction rate is accelerated with at least one livestock breed being lost per month (Pilling and Rischkowsky 2007; Buerkle and Rieseberg 2008). Domestic livestock breeds are considered social and cultural heritage, because of phenotypic characteristics adopted by such breeds relative to different environments over the period of time (FAO 2007). Decline in population of such breeds is considered damage to communities and their cultural heritage, especially in developing countries. To ensure food security, along with production of high yielding superior germplasm breeds, sustenance of regionally adapted breeds is equally important to keep genetic diversity intact. Degrading biological diversity leads to ecological imbalance and poses increased threat during large scale culling of animal population during epidemic disease and natural calamities. Thus, commercial breeds are most prevalent, yet their genetic diversity is low as only few selected sires are used for breeding (Stachowicz *et al.* 2011). On the other hand, commercially less important breeds are low in population number and have chances of extinction and genetic drift. Thus, conservation of animal genetics is important to re-establish declining animal population, to overcome genetic drifts and inbreeding, to apply breed selection or for research.

In situ conservation or conservation by utilization, is one of the ways of preserving livestock genome in the form of live animals. This can be done by funding local farmers

to cultivate and preserve local and rare breeds or utilizing the genetic diversity during breeding the small animal populations. However, the genetic information is lost as soon as the rare individuals in that population die. Apart from this *ex situ in vivo* germplasm conservation involves keeping live animals in a controlled monitored environment, which however, incurs huge expenses for long-term maintenance.

Creating a germplasm gene bank serves as insurance for conservation and recovery of lost breeds in future as well as to strategize breeding within small populations to minimize inbreeding. Maintenance of animal diversity is therefore important for changing market demands and associated regulations. To establish gene banks a diverse genome for different breeds needs to be preserved. Molecular markers are now a day utilized for characterization of pedigree and genetic diversity. Single nucleotide polymorphism (SNP) markers are the method of choice for analyzing genetic diversity and are more successful in targeting conserved populations rather than random or pedigree based animal selection. Commercial breeds are generally highly inbred therefore require small gene pool banking to cover the whole genome scenario.

For some commercial cattle breeds like Holstein, SNP chips have been used to compare the highly related breeds and to detect the frequent polymorphisms across the genome (Engelsma *et al.* 2012).

A global effort wave has already started which includes use of assisted reproductive technologies for propagating and preserving germplasm (FAO 2007). Interestingly, FAO has come up with guidelines to create and manage *in vivo* animal populations as well as *in vitro* genetic resources (FAO, 2010). In the light of declining biodiversity, several other organizations and conferences have been launched for example Rare breeds

Canada, programs like Canadian Animal Genetic Resources (CAGR), European Regional Focal Point for Animal Genetic Resources (ERFP) and Convention on biological diversity. CAGR and ERFP programs for example, work in coordination with international organizations like FAO to implement genetic resource acquirement and conservation in the form of cells or tissues. The main agenda of these organizations is to acquire, evaluate, characterize and maintain animal genetic resources. Animal gene bank development by germplasm cryopreservation however, needs intensive technical support pertaining reproductive physiology, genetics, cryobiology and data management. Also, long-term sustainability, establishment and operation of such gene banks require allocation of stakeholders (FAO 2007). In addition, constant monitoring of breed statistics enlisting breeding population size, male and female ratio and number of subpopulations to maintain tracking records is necessary. Such registers may be helpful is assessing phenotype and genotype of the breeding populations (Patterson and Silversides 2003; Prentice and Anzar 2011). Cryoconservation using oocytes, sperm, embryos, reproductive tissues and/or somatic cells, is an important component of such ART based approach. Gamete cryopreservation is a highly tested and an established method of genome banking in several domestic livestock species.

Semen cryopreservation is well established for various species and protocols have been developed for its use in artificial insemination (AI) and *in vitro* fertilization (IVF). Semen is a good instrument for recovery of lost breed, which however, is done through "backcrossing" and is a long and expensive method (Prentice and Anzar 2011). Semen storage can be done after sperm sexing, where the facility is available. Also, due to its easy availability semen cryopreservation makes it a widespread option for genome

banking. Semen is therefore, frozen and easily transported across the world. However, semen storage covers only half of genetic complement and poses a disadvantage that sperm mitochondrial genome is not passed to next generation and is therefore not conserved. Also, not enough rare breed bulls are available at AI stations and have to be accessed in farm conditions, which might increase the collection and cryopreservation costs. Epididymal spermatozoa are generally the most reliable source for cryopreservation with better post thaw quality than ejaculated semen (Woelders *et al.* 2012).

Oocyte cryo-storage along with semen, thus, provides another option of restoration of lost breeds (Woelders *et al.* 2012). Oocyte cryopreservation is however, still questionable due to its large surface-volume ratio and low permeability to cryoprotectants. Oocyte cryopreservation is developmental stage, origin (*in vitro* or *in vivo*) and species dependent. For example, GV stage oocytes have lower susceptibility to cryopreservation, therefore, oocytes are mostly cryopreserved at immature GV stage and MII stage (Ledda *et al.* 2001). For post-cryopreservation survival of oocytes, sustenance of cellular structure and mostly the mitotic spindle in MII stage oocytes is important. Oocyte plasma membrane, cortical granules and intercellular projections into cumulus cells are important determining factors for oocyte survival (Chen *et al.* 2003). However, oocytes suffer from cryoinjuries at lower temperatures leading to meiotic spindle, chromosomal, cell membrane alterations, leading to misdistribution of cortical granules and impaired fertilization (Massip 2003). Less reliable protocols are however, available for oocyte cryopreservation, but general success in oocyte cryopreservation is low

(Prentice and Anzar 2011). Membrane stability of unfertilized oocytes differs from embryos; due to this embryos are better and more successful option for genome banking.

Embryos provide the full genetic complement to conserve animal genome and are the fastest way to recover and establish a lost animal breed in single generation; however, a larger number of embryos would be required for the same. Over the years, embryo transfer (ET) has turned into an international industry with more than 3,00,000 fresh or frozen ET, which is an expensive procedure (Hasler 2001). Embryos have been successfully cryopreserved in almost all livestock species. Like oocytes, cryopreservation susceptibility of embryos varies with species, stage and origin. Horse and pig embryos are highly susceptible to cryopreservation than bovine and ovine embryos. ET and collection technique has been developed for only few species therefore, not used routinely. Embryo cryopreservation and transfer is successfully used in cattle and sheep, but not in horse and pig.

Ovaries cryopreservation is an emerging concept in germplasm preservation. Techniques are being developed for ovarian tissue banking for oocyte recovery and IVF at later stages (Terazono *et al.* 2012; Woelders *et al.* 2012). This can be achieved by allografting the cryopreserved ovarian tissue and is a method of choice for reassuring fertility in cancer-affected women.

Another possibility could be cryopreserving primordial germ, stem and somatic cells, which are easier to freeze, but is not a cost-effective method of germplasm preservation. In many farm animal species, pigs, cattle, buffalo, sheep and goats, live offspring have been obtained using somatic cells (Campbell *et al.* 2007). Cryopreservation of human embryonic stem cells (hESCs) is a viable approach for

regenerative medicine. In this technique, pluripotent cells from inner cell mass (ICM) are extracted and can be cryopreserved and stored for future use. These cells maintain their pluripotency and can differentiate into any of the germ cells and can be propagated *in vitro* indefinitely. However, cryopreservation protocols for these cells are still under construction and optimization, as previous protocols have been found to induce gene expression changes related to embryo morphogenesis after thawing and culture (Wagh *et al.* 2011).

Avian germplasm preservation is another important facet for wild and rare breed line maintenance and propagation. Semen cryopreservation is possible in avian species with no possibility of ova and embryo cryopreservation. However, ovary cryopreservation is viable and utilizable in birds. In a study on quail ovaries, vitrification yielded better results than slow freezing (Liu *et al.* 2010). Use of cryopreserved semen along with ovaries allografting could be a better option to recover a whole breed in this scenario. Also, primordial germ cell (PGC) retrieved from blood has been used to cryopreserve after propagation as clonal cell lines. This type of gene banking has an advantage of escaping backcrossing otherwise needed with other cryopreservation methods (semen or ova) for lost breeds re-establishment (Macdonald *et al.* 2010).

2.2 Biophysical events during cryopreservation

Scientists have extensively studied events occurring during cellular cryopreservation, in early periods of the technique development (Mazur 1963; Fuller and Paynter 2004; Leibo and Pool 2011). Several parameters were described that change along the course of cryopreservation and determine cellular/embryo survivability.

Liquid water plays an essential part in physiological and structural function of living systems. It is an essential component of cryobiology as well. Freezing removes cellular water by turning into ice and 90% of this water freezes either inside or after flowing outside the cells, 10% however, remains unfrozen. Low temperature exposure is not lethal to all type of cells and some of them survive cooling. Due to freezing of liquid water, concentration reduces and cells lose osmotic equilibrium. Cell injury, during cooling and freezing, is mainly attributed to intracellular ice formation (Mazur 1965). Plasma membrane permeability is an important factor in cell death. Factors determining cell survival and ice formation during cryopreservation are discussed below.

2.2.1 *Osmotic changes*

During the process of freezing and thawing cells undergo major osmotic changes, as they are exposed to highly anisotropic solutions and extreme temperature changes (Mazur and Schneider 1986). When the cells are cooled at slow cooling rates in the presence of cryoprotectants, the external aqueous medium freezes and protoplast gets supercooled which creates high vapor pressure inside the cells than external environment. This creates an osmotic gradient between extra and intracellular environment, thus leading to movement of water outside the cells. A slow cooling rate is required to maintain osmotic equilibrium with the outside medium, by dehydration. The fundamental principle of osmotic process is that it does not involve any cellular metabolic processes and cell maintains equilibrium of solutes according to its plasma membrane permeability. The following equation describes the same:

$$dV/dt=L_pA(\pi^i-\pi^e)$$

Where L_p is hydraulic conductivity with units μ^3/μ^2/min-atm and A is the area of the cell surface. π^e is the external osmotic pressure (Fuller and Paynter 2004). This vapor pressure difference forces the water out of cells and protoplast gets concentrated. The movement of water is defined by two factors; plasma membrane permeability of cells and surface area-volume ratio (Mazur 1963). Equilibrium cooling maintains intra and extra cellular osmotic pressure. This means that higher cooling rate sets an osmotic disequilibrium between a cell and its environment, which leads to slow movement of water from the cell. Thus, protoplast does not maintain at freezing point and super-cooling increases. Cells having higher membrane permeability and lower cellular surface area-volume ratio, have increased water flow out of cells in a given interval of time. Larger cells therefore retain higher percentage of super-cooled water at a given temperature. Permeability of cells has been found to be very much dependent on a class of cell trans-membrane protein called aquaporins (AQPs) (Jin *et al.* 2011).

In a study, mouse and bovine embryos were proved to behave as osmometers in such solutions, across the wide range of tonicity (4 times to one-third). However, the embryos become damaged and leaky outside this range (Mazur and Schneider 1986). During cellular cooling, as the cell protoplasm begins to cool and temperature drops below freezing point, super-cooling occurs. This leads to building up of high intracellular osmotic pressure that keeps forcing the supercooled water out of cell through permeable membrane until equilibrium is reached thus, concentrating the protoplast. It leads to further depression in freezing point and hence, glass transformation occurs, leading to vitrification (Jones, 1965). There is however, a possibility of ice formation before attaining equilibrium, which removes osmotic pressure between intra and extra-cellular

environment. The equations describing interaction between cellular permeability to water, relevant temperature co-efficient and surface area-volume ratio, have been elaborated by Mazur (1965). This phenomenon is discussed below.

2.2.2 ***Solution effect***

Slow cooling of cells causes solute thermodynamics to come into action. Ice formation in extracellular solution induces chemical potential differential. At equilibration point, this chemical potential of ice and solution in contact are equal. Chemical potential of a solution however, is a function of pressure, temperature and concentration. At slower cooling rates, extracellular ice formation takes place which induces movement of intracellular water out of cells, depending on the permeability of cellular membrane. This results in cell dehydration and shrinkage known as a "solution effect" (Fahy 1980) When the cooling rate is too rapid for water to move out of the cell at a rate sufficient to reach equilibrium with the extracellular solution, supercooling will take place and eventually, equilibrium will be attained by intracellular freezing, resulting in cell death (Mazur 1963)

2.2.3 ***Intracellular ice formation***

Successful cryopreservation is related with no or little ice formation. Although, procedure like equilibrium slow freezing avoids ice formation, its possibility is not completely eliminated. Ice formation causes damage to the cells or tissues to be frozen. To completely eliminate ice formation, initially, several slow/equilibrium cooling rates (1000ºC/min, 100ºC/min, 1ºC/min etc.) upon various type cells like RBCs, yeast (Mazur 1963)(Mazur, 1963), oocytes and embryos were tested. Only at slower cooling rates (less

than 1º C/min), successful cryopreservation with least ice formation has been noted in most of the cells (Mazur 1963; Leibo and Pool 2011). Ice nucleation is necessary to induce ice formation in a supercooled solution. Nucleation is in turn dependent on the cooling rate and osmolality of the solutions (Rall and Fahy 1985) Therefore, different type and concentration of highly anisotropic solutions have been used to notice the drop in the ice nucleation temperature (Shabana and McGrath 1988; Ruffing *et al.* 1993). Nucleation is also dependent on the state of cellular plasma membrane and cell protection during freezing. For intracellular ice formation (IIF) the presence of extracellular ice in close contact with the cell is necessary. Induction of nucleation is however necessary, which is otherwise predicted to occur at -33º C in embryos immersed in 1 M DMSO. External medium containing ice can also induce intracellular ice nucleation. Therefore, ice and supercooled water can coexist only if there is a barrier between them, and this barrier must be the cell wall or the plasma membrane. Ice has an open structure as ice has four water molecules hydrogen bonded around a central water molecule. This is responsible for lower density of ice and extensive hydrogen bonding gives it a high heat of fusion and sublimation. The cellular membrane is more permeable to water (Davson and Danielli 1943) than even other solutes. Although, intracellular ice formation after supercooling can only occur upon seeding, the high permeability puts doubt on the barrier function of cellular membrane. The cell in which water has reached its equilibrium value or around 10% of initial volume cannot undergo ice formation. This 10% residual water is incapable of freezing (Wood and Rosenberg 1957). Mazur (1965), as discussed the dynamics of extracellular ice formation and its role in intracellular ice seeding, in details. Plasma membrane acts as a barrier for the nucleation of ice crystals in the supercooled

water inside the cells. This creates hyperosmotic pressure inside cells causing water to flow out of cells. Rapid movement of water out of the cells reduces chemical potential of remaining protoplast water and precludes intracellular ice formation (Mazur 1965). However, plasma membrane also helps in supercooling of cytoplasm by acting as a barrier for nucleation.

On the other hand, below certain temperatures (-5º to -15ºC) membranes are noted to lose their barrier property. The reason for this loss of barrier property might be the alteration in cell membrane structures such as membrane pores lose their confirmations and become large.

Large and sensitive cells like oocytes are not suitable for slow cooling procedure and vitrification has been tried to cryopreserve them.

2.3 Embryo development

The early development of embryos is a series of events involving ovulation, oocyte maturation, fertilization with spermatozoa, cleavage of blastomeres and implantation of hatched blastocysts to finally develop into a fetus.

Oocyte undergoes various degrees of changes to become fully mature or capable for fertilization. *In vivo* and *in vitro* matured oocytes however, have significantly different quality. Oocyte quality depends on the size of antral follicle taken from, stage of follicular wave and site of maturation (Lonergan and Fair 2008). Oocyte quality further affects the embryo development (Hyttel *et al.* 1989; Rizos *et al.* 2002f; Lonergan and Fair 2008).

In vitro bovine embryo production is considered in-efficient system, but these

embryos serve as good model systems for *in vivo* produced embryos (Lonergan and Fair 2008). Many studies have been conducted to compare the *in vitro* and *in vivo* oocyte maturation and embryo development. Both oocytes and embryos differ at morphological, ultra-structural, biochemical and gene expression level (Hyttel and Niemann 1990; Badr *et al.* 2007). For example, these embryos differed in cell size, shape and number, peri-vitelline space, morula compaction and blastocyst quality (Lindner and Wright 1978; Massip *et al.* 1995). Also, these *in vivo* and *in vitro* produced embryos differed in development rates and cryo-survival (Sakkas *et al.* 1989; Papadopoulos *et al.* 2002; Rizos *et al.* 2002f).

The IVC provides deficient environment, thermal shocks, light exposure, direct exposure to atmospheric gas and static culture conditions with shortage of metabolites, to the embryos. On the other hand, *in vivo* derived (IVD) embryos develop in constant body temperature, gaseous tension and darkness with permanent dynamic exchanges between mother and embryos (Rieger and Betteridge 1989; Massip *et al.* 1995).

Biochemically, *in vivo* produced embryos differ than IVP embryos. *In vitro* produced bovine embryos get their embryonic genome activated earlier than *in vivo* ones, which is a rather important phase of early embryo development (Holm *et al.* 2002; Lonergan *et al.* 2003a). Similarly, IVC systems impact the metabolism of embryos; for example, *in vitro* produced embryos differ in oxygen consumption and glucose metabolism than *in vivo* ones (Rieger *et al.* 1995; Lopes *et al.* 2007; Lonergan and Fair 2008). Also, more chromosomal abnormalities exist in IVP embryos than *in vivo* ones. In embryos cultured in SOF+FCS only 8.8% of blastocysts were normal diploid and rest were mixoploids. *In vivo* culture reduced the mixoploidy, significantly ($p<0.01$)

(Lonergan *et al.* 2003b; Lonergan and Fair 2008).

2.3.1 *In vivo development*

Oocytes are the female gamete, nurtured and developed in the functional units of ovaries i.e. follicles. Oocyte maturation is an important aspect of embryo formation and is relevant to study for developing any infertility treatments. Many ultra-structural, biochemical and molecular changes occur during oocyte maturation that further determines its developmental competence.

At birth, oocytes reach a stage of prophase-I of meiosis and are arrested for a defined period of time. High level of cAMP produced in MAPK pathway is released through cumulus cells into oocytes, which maintain this meiotic arrest (Richard 2007; Li and Albertini 2013). The development period before maturation involves accumulation of transcriptome necessary for subsequent developmental stages *viz.* meiotic resumption, fertilization and early cleavage of embryos (Sirard *et al.* 2007). During this period, up to adolescence, oocytes grow in size and acquire the protective glycoprotein layer, zona pellucida (ZP) (Sirard *et al.* 2007). At the same time, follicular growth and differentiation takes place. Oocytes undergo meiosis and mature in such developing follicles, out of which one is destined to become the dominant follicle. Several signalling events stimulate follicular differentiation *viz.* variation in FSH levels; for ovulation and oocyte developmental competence (Sirard *et al.* 2007). Selection pressure on developing follicles also determines the developmental competence of oocytes. Follicles that fail to ovulate die and become atretic.

Oocytes resume meiosis during late pre-ovulatory stages, upon LH surge via mural LH receptors on mural granulosa cells (Mehlmann 2005b). A decrease in cAMP is necessary for meiotic resumption, which occurs after gonadotropin-LH surge (Sun *et al.* 2009). Meiotic resumption thus, corresponds with breakdown of nuclear membrane, chromosomal condensation and meiotic spindle organization. For both nuclear and cytoplasmic maturation, translation activation of oocytic mRNA content is required. This occurs usually by poly-A tail extension or mRNA release from translation inhibitors (Stitzel and Seydoux 2007). Thus, oocyte maturation process removes meiotic inhibition of oocytes arrested at prophase I and is accompanied by mRNA and protein degradation. Mostly these are the proteins involved in meiotic arrest and maturation.

cAMP is known to play major role in meiotic arrest and resumption. Other molecules like G-proteins, epidermal growth factors, adenylyl cyclase and phosphodiestrase provide supporting role in regulating cAMP concentrations (Mehlmann 2005a; Richard 2007). Intracellular cAMP levels is maintained by G-proteins signaling molecules and oocytes in female mice with null mutation for G-protein coupled receptors 3 (GPR3) show meiotic resumption in antral follicles (Mehlmann *et al.* 2004). Gonadotrophic stimulation induces MAPK cascade activation in granulosa cells, which leads to meiotic resumption and spindle formation in oocytes (Sun *et al.* 2009). FSH/LH addition in IVC expedites the MAPK phosphorylation. *In vitro* maturation however, alter the gene expression of oocytes as compared with *in vivo* ones (Yuan *et al.* 2011).

After completion of meiosis-I, oocytes again get arrested in metaphase-II stage of meiosis, which is released only after fertilization. This phenomenon is known as oocyte activation. For all these changes to take place an intercellular communication is

established between oocyte and granulosa cells via bi-directional transzonal projections (Li and Albertini 2013). Similarly, cumulus cells surrounding the oocyte play important role in maturation. FSH exerts the stimulatory effects on oocytes via cumulus cells, induce LH receptors and prevent pre-antral follicular atresia. Cumulus cells expansion occurs via proteoglycans network and are in connection with the oocyte (Eppig *et al.* 1997; Sirard *et al.* 2007). Cumulus cells are involved in bi-directional communication with oocyte regulating metabolism and developmental competence (Krisher 2014).

Fully developed oocytes undergo both nuclear and cytoplasmic maturation process. However, studies suggest that oocyte might acquire its competence before GVBD stage (Sirard *et al.* 2007). The developmental competence of the oocytes obtained from dominant follicles is depicted by several morphological changes (Assey *et al.* 1994; Rizos *et al.* 2002f). For example, mitochondria, smooth endoplasmic reticulum (SER) and cortical granules' shape and cytoplasmic location are good indicators of cytoplasmic maturation of oocytes (Leoni *et al.* 2015). This cytoplasmic maturation is essential for blockage of polyspermy at fertilization and early embryonic development. Some subtler changes include molecular maturation of oocytes and emergence of Ca^{2+} waves upon fertilization, which greatly define the developmental competence of oocytes into future embryos (Sirard *et al.* 2006; Stitzel and Seydoux 2007). This includes accumulation of RNA and proteins essential for development up to maternal-zygote transition and beyond (Brevini *et al.* 2007).

Thus, oocyte maturation is an event for preparation of oocyte to acquire necessary information for future fertilization and early embryonic development.

Fertilization is the union of oocyte (female gamete) and spermatozoa (male gamete) to produce a new organism. For this event to take place, oocyte and spermatozoa travel to ampulla region of oviduct in mammals (Brevini and Pennarossa 2013). Oocyte-spermatozoa fertilization event is the critical stimulus for second meiotic resumption.

Before fertilization, capacitation of spermatozoa is an essential step. In cattle, oviductal membrane secretions play great role in sperm capacitation. Sperm loose their de-capacitation factors after entering oviductal fluid. Exposure to high-density lipoproteins in these secretions removes bovine seminal plasma protein attached to sperm membrane and promotes formation of zona pellucida receptors on it. Interestingly, both proteins and carbohydrate receptors play a role in recognition during capacitation and entry of sperm into the oocyte (Lefebvre *et al.* 1997; Gabler *et al.* 1999). For example, a cell-adhesion glycoprotein "osteopontin" secreted by oviductal epithelial layer fluctuates between ovarian cycles and may have potential impacts in gamete interactions (Gabler *et al.* 2003). The cap of sperm is transformed in an acrosome over the elongated nucleus in a mature sperm. The acrosomal reaction and removal of glycoprotein from plasma membrane are part of the process and are activated after contact with ZP. This reaction allows fusion of spermatozoa membrane and outer ZP. The "chosen sperm" thus, enters the oocyte. Cytosolic wave of Ca^{2+} after sperm penetration defines the resumption of meiosis in oocyte and oocyte cortical reaction blocks further polyspermy of oocytes (Williams 2002). Cortical granules are made up of proteases, peroxidases, mucopolysacharides, plasminogen activator and acid phosphatases (Brevini and Pennarossa 2013). Fertilized zygote has two pronuclei, female and male; which merge to form the embryo. The syngamy of pronuclei is facilitated by spermatozoa centrioles,

which brings them closer. Other growth factors such as fibroblast growth factor-1, 2, vascular endothelial growth factor (VEGF), insulin growth factor and platelet-derived growth factor (PDGF-B) support maturation of oocyte and early embryonic growth (Viuff *et al.* 1995; Einspanier *et al.* 1999).

After fertilization embryos are capable of supporting early cellular divisions and differentiation without much external support (Hoelker *et al.* 2009). Microtubule organizing centres (MTOCs) of oocytes contribute to early mitotic divisions. After fertilization, oocytes undergo maternal-zygotic transition (MZT) and embryo starts depending on its own machinery for the production of mRNA and proteins supporting further development (Camous *et al.* 1986; Schier 2007). MZT takes place at different embryonic stages in different species. In bovine embryos, a minor and a major embryonic genome activation takes place (Badr *et al.* 2007). Minor genome activation takes place at 1-4 celled stages and major genome activation happens at 8-16 celled stage for bovine embryos (Vigneault *et al.* 2009). Interestingly, ribosomal genes start expressing at 4-celled stage, which in turn are essential machinery for protein synthesis (Hyttel *et al.* 2000). Embryonic arrest at 8-16 celled stage is attributed to poor transcription and cytoplasmic factors (Meirelles *et al.* 2004). Rapid cell cycles and absence of transcriptional machinery are proposed to suppress the zygotic genome activation during early cleavage (Schier 2007; Stitzel and Seydoux 2007). Histones and DNA methylation are supposed to play a key role in this until nucleo-cytoplasmic ratio and DNA quantity reaches a certain quantity (Kikyo and Wolffe 2000; Ruzov *et al.* 2004; Schier 2007). Maternal RNAs are then removed or repressed by the microRNAs targeting the 3'UTR

regions, which may otherwise block the embryonic transcriptional activities (Tadros and Lipshitz 2005; Schier 2007).

The pre-implantation embryos develop to morula and blastocyst. Morula compaction occurs with the help of inter-cellular junctions and adhesive molecules. These molecules define the success of compaction, as proposed by a transcriptional study (Boni *et al.* 1999). This is an essential step leading to cavitation and blastocyst formation.

2.3.2 *In vitro development*

In vitro fertilization (IVF) is an established assisted reproductive technology, both in domestic animals and humans (Badr *et al.* 2007). IVF of mammalian oocytes successfully started in rabbits (Chang 1959), followed by reports in other species (Whittingham *et al.* 1979; Brackett *et al.* 1982). However, *in vitro* development of embryos is limited and is dependent on oocyte quality as well as culture conditions (Rizos *et al.* 2002f). The procedure has undergone numerous modifications for standardization of protocols and involves several critical points (Oocyte maturation, sperm capacitation, fertilization, cleavage and blastocyst formation). Interestingly, developmental potential of *in vitro* pre-implantation embryos, can also be measured on the stage specific molecular markers (Rekik *et al.* 2011).

The source of matured oocytes (*in vivo* or *in vitro*) also affects the embryo developmental potential (Farin *et al.* 2001). Oocytes acquired from 2-8 mm sized follicles of slaughterhouse ovaries for *in vitro* production (IVP) of embryos may acquire nuclear maturation but do not express full developmental competence due to poor cytoplasmic maturation (Lonergan and Fair 2008). Also, these oocytes are being removed from a

“negative” follicular fluid environment without getting the usual “positive” post-ovulation LH surge stimulus. However, such oocytes gain the ability to resume meiosis spontaneously in the absence of follicular inhibition (Sirard and Coenen 2006). *In vitro* matured (IVM) oocytes and IVP embryos however, serve as good study models for *in vivo* counterparts. For example, IVM of oocytes has revealed many metabolic aspects of different maturation stages. In spite of energy metabolism; amino acids (a.a.) and fatty acids are extensively taken up by these oocytes. Several of them are species and stage specific, for example, glutamine enhances MII stage maturation and alanine and glycine content of follicular fluid indicate future COC competence (Krisher 2014).

In vitro maturation of oocytes is marked morphologically by cumulus cells expansion. Mature oocytes are evaluated microscopically by different staining methods to observe first polar body expulsion, mitochondrial distribution and relocation as well as mitotic spindle organization. Oocyte quality and competence is also defined by the composition and appearance of cytoplasm. Like oocytes with dark cytoplasm and more mitochondria have more developmental competence (Jeong *et al.* 2009).

In vitro sperm capacitation was successfully implied in 1982, with successful calf birth (Brackett *et al.* 1982). In cattle, pH 7.4 was found to be optimal for *in vitro* capacitation (Lu *et al.* 1987). Simultaneously, capacitation was found to convert the net negative charge on the sperm membrane (Gordon, 2003).

However, initial studies reveal that retaining of cumulus cells during IVF does not have any marked effect of sperm capacitation but affects the pro-nucleus formation rate.

Hypotaurin emerged as a good agent for increased capacitation of spermatozoa in IVC (Guerin *et al.* 1995).

In vitro culture and production of embryos are affected by external factors such as atmosphere, oocyte and sperm quality (Farin *et al.* 2001). *In vitro* developed embryos are nutritionally deprived and show anomalies as compared to *in vivo* counter-parts. These embryos show morphological, biochemical and molecular *viz.* gene expression differences, than the later embryos (Badr *et al.* 2007). Morphologically, IVP embryos showed changes in cytoplasm, shape and number of blastomere and organelle distribution within them (Massip *et al.* 1995). Cellular anomalies like uneven shape of mitochondria, distribution of cortical granules and low cell-cell coupling was found in IVP bovine embryos (Hyttel *et al.* 1989). This might be the reason for reported *in vitro* non-compacted morulae and darker blastocysts (Farin *et al.* 2001). Also, IVP embryos had lower peri-vitelline space, number of blastomeres and inner cell mass ratio (Van Soom and de Kruif 1992). Interestingly, zona structure of IVP embryos is debatable (Massip *et al.* 1995). Ultrastructure of IVP embryos also point towards low viability of these embryos with altered nucleolus and mitochondrial cristae as well as low heterochromatin and ribosomes (McReynolds and Hadek 1972). *In vitro* culture conditions alter the physiology and biochemistry of embryos as well. For example, lipid content of IVP embryos is higher than the *in vivo* produced embryos, which makes the former embryos more buoyant (Leibo 1993). Glucose and amino acid like glutamine are metabolized differently in these embryos and at different stages. This is further useful for improving the culture conditions for the IVP embryos (Rieger *et al.* 1995). Cattle embryos developed *in vitro* get the maternal-embryo transition of gene expression at later stages

than *in vivo* produced embryos (Holm *et al.* 2002). This phenomenon is important and stage dependent as well as determines the embryo kinetics and development. Therefore, embryo development and embryonic differentiation is delayed in IVC (Farin *et al.* 2001). An example of this is presence of vacuoles in blastomere cytoplasm of morulae produced under serum containing culture media (Enders *et al.* 1987; Farin *et al.* 2001). This is not the case with the *in vivo* produced embryos. Also, IVP morulae appeared to loose these vacuoles upon blastocyst development (Farin *et al.* 2001).

In vitro embryo culture conditions are critical and smallest factors like embryo density per microliter of media has dramatic effect on their future development (Hoelker *et al.* 2009).

So far, numerous studies have been conducted on IVP embryos in cattle varying in pregnancy rate, which is associated with the abnormal placenta development as well as cryodamage. Some studies therefore, reported up to 20% of abortions using IVP embryos. In the bovine, developmental rates differ than *in vivo* embryos. Culture conditions affect the timing of compaction and blastulation. The inner cell mass (ICM) in IVP embryos is also lower than IVD embryos and trophoblast differentiation occurs prematurely.

2.4 Embryo cryopreservation in assisted reproductive technologies (ART)

2.4.1 *Scale of embryo transfer in livestock industry*

The concept of embryo transfer (ET) to produce live offspring was introduced successfully in rabbits by Heape, in the year 1890. Embryo transfer is a great method to propagate and transfer superior genetic material and along with cryopreservation provides extended opportunities to move it around the world. Assisted reproductive techniques (ART) are of great importance in human and domestic animals to overcome reproductive failures, to increase number of offspring from selected females and to reduce genetic intervals in livestock in farm animals (Boerjan *et al.* 2000). In dairy cattle management, there are several diseases like repeat breeding, which affects the breeding population and poses challenge in economic success in animal production (Tanabe *et al.* 1985). This problem can be countered with the use of *in vitro* fertilization (IVF) and ET to improve the conception rate in repeat breeder cattle. ET can also help to circumvent infertility because of fertilization failure or embryonic loss due to heat stress. A number of techniques have been developed and applied in the ET industry starting from animal synchronization, superovulation, ovum pick up (OPU), artificial insemination (AI), uterine *in vivo* derived embryo flushing, IVF, intracytoplasmic sperm injection (ICSI), animal cloning and cryopreservation of IVD or IVF embryos using various techniques such as vitrification and slow freezing. Over the years collection of vital data for the growing ET industry in cattle and other domestic species became difficult to retrieve. Organizations such as International embryo transfer society (IETA), American Embryo

transfer society (AETA), Brazilian Society for Embryo Technology (SBTE) and many such organizations stand today for reporting the data across the world, with IETS being the only international one.

In cattle, across the years IETS has recorded increasing ET data for Holstein and Angus breeds with less number of registrations for Hereford breeds (Wheeler and George 2014). Gradually, protocols for superovulation, AI and embryo flushing have improved overtime leading to increased procurement of IVD embryos. *In vitro* production of embryos in cattle also slowly became part of small commercial ET service providers. In contrast to humans, in cattle oocytes are collected from the slaughterhouse ovaries and or through ultrasound guided OPU. In 1992 (Lu and Polge), a huge scale program reported production of 2000,000 blastocysts from 7,00,000 oocytes obtained from slaughterhouse ovaries. ET in cattle has been tried with the use of IVF embryos, frozen thawed embryos and with or without concurrent artificial insemination (AI) to establish successful pregnancy. The authors found 49% pregnancy rate in heifers and 41% pregnancy rates in repeat breeder cattle with the transfer of frozen thawed IVF embryos alongwith concurrent AI (Dochi *et al.* 2008). With the use of IVD embryos however, a pregnancy rate of 70% was obtained in repeat breeder cows. In general, scientists have observed lower pregnancy rates through frozen thawed IVP embryos rather than *in vivo* derived (IVD) embryos (Pollard and Leibo 1994; Hasler *et al.* 1997; Hasler 1998). *In vitro* produced embryos pose problems in pregnancy such as large calves, increased gestational length, decreased intensity of labor, increased abortions, congenital malformations, increased perinatal mortality and increased hydroallantois (Hasler 2003). Some authors have directly questioned the success of IVF-ET in comparison with the IVD embryos

(Sinclair *et al.* 1995; Holm and Callesen 1998) with few studies of success with IVF embryos to derive modest pregnancy rates (Duby *et al.* 1996; Taneja *et al.* 2000). *In vitro* produced ET provides opportunity to increase genetic gain by decreasing intergenerational intervals (Hasler 2003). This has been supported with the use of IVF in combination with OPU technique and using for collecting oocytes in early pregnancy from a high demand superior genetic merit donor animal (Eikelmann *et al.* 2000). At a commercial service provider (Boviteq®) the scientists directly compared the results of IVP and conventional *in vivo* ET programs. IVP was found to produce 3.4 times more embryos and 3.2 times more pregnancies in a 60-day period, with only one superovulation per donor (Bousquet *et al.* 1998).

The ET industry was setup with the efforts of two pioneer groups; researchers who developed the technique and commercial practitioners who developed the setup to reach the cattle industry. In the year 2013, bovine ET industry recorded the highest ever number of collections and transfers globally (IETS, 2013). For the first time over 5,00,000 IVF, OPU embryos were collected and over 4,00,000 were transferred. The commercial industry of cattle ET started establishing in North America in the year 1970s (Hasler 2003). There has been a constant activity of ET in Holstein cattle in North America (Hasler 2003). During these years both the numbers of ET practitioners and donors increased rapidly. In the year 2013, North America accounted for half of global activity of IVD embryos. A commercial IVF program by *Em Tran*® was done for the purpose of producing pregnancy in infertile cows in the year 1992 in USA and Denmark (Hasler *et al.* 1995). However, in the earlier years lack of adequate freeze thaw protocols made it necessary to maintain enough viable estrous synchronized animals. A transfer of

2268 fresh IVP bovine embryos over the period of two and a half years resulted in 1220 pregnancies (53.8%). Transfer of fresh and frozen IVF embryos were compared to obtain pregnancy rates of 59% and 42%, respectively. *In vivo* derived fresh and frozen embryos, however, resulted in 76% and 67% pregnancy rates (Hasler *et al.* 1995). On the contrary, OPU/IVP are more expensive than conventional ET to produce pregnancy (Schatten and Constantinescu 2008) This can only be applied for extremely valuable cattle and is dependent on the efficiency of IVP of embryos.

The widespread adoptions of ET of frozen thawed embryos lead to application of more practical approaches like direct embryo transfer of embryos frozen using cryoprotectants like ethylene glycol and hence increased possibility to spread it variety of practical field conditions (Voelkel and Hu 1992b). These direct ET of frozen thawed embryos can be done economically with least infrastructure and equipment. In another study, viability of cryopreservation and frozen thawed embryo transfer of *in vivo* embryos using DMSO or glycerol as the dehydrating agents and frozen using rapid freezing and DMSO resulted in better embryo survival (Prather *et al.* 1987). However, standardization of embryo freezing and transfer protocols and equipment are still under development.

2.5 Factors affecting embryo cryopreservation

Ability to survive cryopreservation is a good indicator of embryo quality (George *et al.* 2008; Sudano *et al.* 2012). Several factors regulate the outcome of embryo cryopreservation apart from the basic cryopreservation principles discussed above. Authors have described several key points for improving the post-cryopreservation outcome such as choice of embryo culture media (ECM) (Rizos *et al.* 2002f; Rizos *et al.* 2008; Sudano *et al.* 2011), cryoprotectants, cryopreservation method (Nicacio *et al.* 2012); embryonic stage (Sanchez-Osorio *et al.* 2008) and embryonic origin (*in vitro* vs *in vivo*) (Mucci *et al.* 2006; Aksu *et al.* 2012). Modifications of these parameters can lead the way to improve the IVP embryo production system hence, enhancing the quality of embryos produced and post-cryopreservation survival (Seidel 2006).

2.5.1 *Culture media*

Mammalian embryo nutritional usage and status are important determinants of its health and developmental potential. Post-compaction embryonic cells act like somatic cells and embryonic genome activation allows wide range of nutrient utilization (Gardner *et al.* 2000). For instance, the uptake of glucose in peri-compaction period increases in certain species like mouse (Leese and Barton 1984). Thus, culture system has an impact on the quality of embryos and effect on embryo metabolism (Rizos *et al.* 2002f). However, mammalian pre-implantation embryos exhibit a certain level of plasticity (Gardner and Leese 1988). Embryo on the other hand, compromises in its viability in return of any abuse. Therefore, ECM that are less stressful and do not provide sub-optimal conditions to the developing embryos, are required.

Several studies have been conducted to understand the different aspects, requirements and utilization of nutrients by the embryos in the culture systems. For example, nutrient availability, transport mechanism on plasma membrane, enzyme activity (tested *in vitro*) and control of enzyme activity through specific regulators were investigated overtime. Components of culture media have been defined, accordingly (Gardner *et al.* 2000).

Carbohydrates are the first basic requirement for energy production and utilization by the oocytes and embryos. Glucose is utilized by oocytes and pre-implantation embryos and is present in oviductal fluid. The oocytes themselves lack the capacity of utilizing glucose and cumulus cells provide the metabolic intermediates for metabolic use. Cumulus-oocyte cells surrounding the oocytes utilize glucose via several pathways like glycolysis and play a pivotal role in oocyte maturation (Huang and Wells 2010; Sutton-McDowall *et al.* 2010). Embryos however, utilize glucose especially during implantation period. Glucose metabolism through pentose phosphate pathway (PPP) produces NADPH, which is further required for antioxidant activity of glutathione and in nucleic acids and lipid biosynthesis of embryos (Krisher and Prather 2012). However, bovine blastocysts have lower ability to utilize glucose or pyruvate via TCA cycle (Krisher and Prather 2012). Also, higher glucose concentration could be inhibitory in some species. For example, the presence of 1.0 mM glucose in IVC media caused reduction in human blastocyst development (Conaghan *et al.* 1993). In *in vitro* conditions, the glucose utilization is dependent upon substrate availability rather than plasma membrane transport or enzyme activity dependent (Biggers *et al.* 1988; Gardner *et al.* 2000). Importance of glucose in embryo development varies with species. In mouse and hamster

embryos, lack of glucose in IVC media from eight celled to blastocyst stage causes loss of viability (Gardner and Lane 1996). In the early development however, oocyte and embryos do not encounter glucose as surrounding cumulus cells take up any glucose in the culture media and convert it into pyruvate and lactate (Leese and Barton 1985). Pyruvate enters into embryos readily by means of a facilitated carrier. Pyruvate is an anti-oxidant and reduces hydrogen peroxide levels in embryos (O'Fallon and Wright 1986). Pyruvate and lactate are the important for production of NAD^+ and NADH, which are important for the blastocyst stage (Krisher and Prather 2012). Studies show change in media components can change intracellular pyruvate and lactate ratio as well as NAD^+ and NADH ratio. Also, lactate concentration in culture medium impacts the mouse embryo viability (Gardner and Sakkas 1993).

Amino acids are abundantly present in oviductal fluid as well as are pooled in embryonic blastomeres (Devreker *et al.* 2001). Absence of amino acids in culture media causes stunted growth and affects viability of embryos (Camargo *et al.* 2006). These amino acids are classified in two groups; essential and non-essential amino acids and are required during different phases of embryonic development. Amino acids play a lot of roles in embryonic physiology. These amino acids act as energy sources, pH buffer, antioxidants and biosynthetic precursors and chelators. Non-essential amino acids play an important role in zygote and cleavage function (Van Winkle 2001).. Taurine and glutamine play an essential role in embryo development and are added to ECM (Van Winkle 2001). Absence of non-essential a.a. affects initial cleavage in embryos of mouse and cow. Also, they impact glucose metabolism and pH buffering in the culture media (Van Winkle 2001).

Enzyme kinetics studies, although low in number, have also revealed the importance of different isoforms of enzymes and specific regulators of enzymes in different embryo developmental phases. For example, two enzymes; hexokinase and 6-phosphofructokinase however, act as rate limiting for glycolytic pathway in 2-cell to morula staged embryos (Houghton *et al.* 2003). Hexokinase isozyme analysis, for example, reveals the presence of two different isozymes at different stages like zygote and blastocyst stage. This determined the important role of amino acid composition of culture media, in the ability to regulate metabolism at different embryonic stages. Similarly, after embryo-genome activation starts the metabolic machinery of embryos, it results in more production of proteins and increased ATP utilization. This ATP utilization is maximized during blastocoel formation and embryo expansion (Houghton *et al.* 2003).

Interestingly, spent culture media is an important way of determining embryo viability and future developmental potential, prior to embryo transfer. Nutrient utilization and metabolism could provide a good picture to design the embryo culture media. For example, amount of glucose utilization had been found to be related to better quality blastocysts leading to pregnancy. Mouse blastocysts with increased glucose utilization in culture lead to pregnancy as compared to others (Gardner and Leese 1987). Post-thawing cryopreserved bovine embryo re-expansion could also be predicted with relation to their metabolic activity of glucose and lactate production (Gardner and Lane 1996). According to "quiet embryo hypothesis", the more viable embryos exhibit "quieter metabolism" than less viable ones (Leese, 2002). For example, *in vitro* produced mouse blastocysts with higher glycolytic rates might have impaired implantation after embryo transfer (Lane and Gardner, 1996).

Post-fertilization culture media has an impact on major events like cleavage, embryo genome activation, morula compaction and first cellular differentiation into trophoectoderm (TE) and inner cell mass (ICM) (Marikawa and Alarcon 2009). Fetal calf serum (FCS) is an important component in embryo culture media but is known to be associated with large offspring syndrome (Lazzari *et al.* 2002; Rizos *et al.* 2003; Sudano *et al.* 2012). FCS is thus, an excellent source of nutrients like vitamins, amino acids and fatty acids and has been found to accelerate the embryonic growth; it also presents threat of pathogen transmission (Gomez *et al.* 2008c). Culture media with more serum affects the embryo structure. Thus, the presence of FCS in culture media has been found to be responsible for lipid accumulation in IVP embryonic blastomeres (Farin *et al.* 2001). An imbalance in cellular-mitochondrial redox mechanism on mitochondrial membrane, can affect lipid metabolism by impairing β-oxidation thus, increase lipid droplets in embryonic cells (Abe *et al.* 2002b; Barcelo-Fimbres and Seidel 2007a). Thus, low mitochondrial metabolism of lipids by such embryos leads to more accumulation of lipid droplets (Farin *et al.* 2001). Other authors have different opinions for the increased lipid concentration in FCS cultured IVP embryos. Some studies believe that embryos take up lipoproteins from culture media containing FCS (Sata *et al.* 1999) or embryos start synthesizing new tri-glycerides (Razek *et al.* 2000). Lipid accumulation in cells can also be attributed to impaired energy metabolism. Glucose metabolism in pre and post-compaction IVP embryos is referred to occur as “crabtree” effect (Gardner *et al.* 2000). Excess glycolysis leads to inhibition of oxidative phosphorylation and increase in concentration of lipid synthesis precursors (Rieger *et al.* 1992; Sudano *et al.* 2011). Experiments conducted to correlate the presence of serum at different concentrations in

culture media and formation of lipid droplets in IVP bovine embryonic blastomeres was approving (Sudano *et al.* 2011). Fetal calf serum not only affected the quality (blastomere cell number and apoptosis) of bovine embryos but higher FCS concentrations (5 and 10%) also resulted in lower embryo survival after vitrification, due to impaired blastocyst re-expansion (Sudano *et al.* 2011). Several culture conditions have been tried to improve the survival of IVP embryos after cryopreservation. Interestingly, use of a fatty acid synthesis inhibitor, phenazine ethosulphate (PES) along with lower FCS concentrations (2.5%) in the media, improved the post-vitrification embryo survival ($p<0.05$). Neutral lipid accumulation in the embryos affects the equilibrium between dehydration and rehydration during freezing and thawing and increases chances of ice crystallization (Vajta *et al.* 1999). Trans-10, cis-12 conjugated linoleic acid (CLA) addition to bovine embryo culture reduces neutral lipid accumulation and has been found to improve post-cryopreservation re-expansion (Batista *et al.* 2014). However, the hatching rate of these embryos was not improved. In another similar study, use of cis and trans CLA in serum containing embryo culture media resulted in 83.8% blastocyst rate post-cryopreservation of bovine embryos as compared to 69.3% in control group (without CLA). Also, authors suggested change in lipid profile of embryonic cell membrane with an increase in levels of highly unsaturated phosphotidyl choline (36-38 carbon structures) when the CLA supplement was added for both IVM and IVC (Batista *et al.* 2014). This suggests that improved cryopreservation in the treated group is related with changes in molecular mechanisms involved with the structural lipids of membranes and is dependent on the length of treatment as well (Leao *et al.* 2015). Culture conditions can also impact the gene expression of IVP embryos, for example relative gene expression found in post-

compaction embryos due to changing oxygen availability (Rizos *et al.* 2003; Lonergan and Fair 2008). In another study, reduced blastocyst development after cryopreservation of embryos cultured in FCS containing media was accompanied by detrimental gene expression changes (Rizos *et al.* 2003). Culture media is now available commercially and most companies have replaced the serum in the media with bovine serum albumin or synthetic components such as polyvinyl alcohol (Lim *et al.* 2007; Hasler 2014). Several studies indicate either global or individual gene expression changes due to change in culture medium as well. Culture media need to be optimized based on reducing any metabolic stress to the embryos. The mechanisms responsible for increased embryonic death after cryopreservation may be excessive lipid peroxidation. ROS production has a detrimental effect on IVP embryos (Massip *et al.* 1995).

Several studies have been conducted to improve and optimize the basic culture media, after initial reports on generation of IVM and IVF embryos (Hernandez-Ledezma *et al.* 1992; Hernandez-Ledezma *et al.* 1996). Different additions in basal culture media have been made through cellular co-culture media as well (Goto *et al.* 1988; Eyestone and First 1989). Use of cells in co-culture system provides benefits such as endogenous source of embryotrophic factors or protection against damaging chemicals or radicals by the supporting cells. In a study, different types of co-culture cells [oviductal cells (BOEC) or buffalo rat liver (BRL)] in the basic TCM-199 media were used for bovine embryo culture. Around 58% blastocyst rate was obtained in both the co-culture system (Hernandez-Ledezma *et al.* 1996). The co-culture of cells do not provide any advantage over just conditioning the culture medium with BOEC and BRL cells (Hernandez-Ledezma *et al.* 1996).

Embryo culture system is a sensitive microenvironment controlled system, which is expected to somewhat simulate *in vivo* conditions and provide constant support to developing embryos in terms of nutrients and gaseous components. Embryo culture density also has an impact on the gene expression and developmental capacity (Hoelker *et al.* 2009). Low embryo density (embryo number: media volume ratio) or single embryo culture had been found to be associated with low blastocyst development rates and decreased production of interferon-τ (Khurana and Niemann 2000). Well of wells (WOW) system has been developed to track the development of single embryos. In a study, effect of microenvironment and embryo density (WOW, 16 embryos/well and 50 embryos per well) was tested on embryo developmental capacity, embryo quality and gene expression profiles of IVP embryos. Zygotes stacked in a group of 16 (22.2%) developed to blastocysts lower than embryos grouped in 50 embryos per well (30.3%). Interestingly, embryos cultured in WOW system developed comparably to 50-embryo group (31.3 vs 30.3%). The quality of embryos (apoptotic cell index and differential cell counts) developed in WOW was not significantly different from control embryos. Interestingly, a considerable gene expression changes were found in embryos cultured in WOW as compared to control groups (16-embryo and 50-embryo group) as well as *in vivo* derived embryos and genes such as ATP5A1, PLAC8, KRT8, S100A10 and ZP3 related to cell structure and metabolism were differentially expressed (Hoelker *et al.* 2009).

2.5.2 *Cryoprotectants*

Exposing the biological material to low temperature may cause an imbalance in the living process, structure and physiology. To protect the cells from damaging effects of

subzero temperature and sudden thermodynamic changes during the process of cryopreservation, molecules called cryoprotectants were discovered. Cryoprotectants are simple, low molecular weight solvents like ethanol etc., which are utilized with the purpose to reduce cryoinjuries by reducing the formation of intracellular ice. In 1949, Polge discovered the cryoprotection ability of glycerol for the rooster spermatozoa, serendipitously (Polge *et al.* 1949).

These may be of two types; those that enter the cells (permeable) and those that do not enter the cells (non-permeable) (Vajta and Nagy 2006). The permeable cryoprotectants minimize the intracellular formation by penetrating in the cells and lowering freezing point. While non-permeable cryoprotectants play their role by osmotic dehydration of cells, the high concentration of non-permeable solutes exerts osmotic stress on the cells (FAO 2012). These when used in combination with permeable cryoprotectants also have additive protective effects by stabilizing intracellular structures and cellular membranes. The permeating cryoprotectants entering the cells like glycerol, EG and DMSO provide cryoprotection by stabilizing the cell membrane lipids by hydrogen bonding with the polar heads of membrane lipids which is an important phenomenon especially for severely dehydrated states (Crowe *et al.* 1984). Several protocols of cryopreservation and combinations of cryoprotectants have been tried over the years to derive successful results in various species. These combinations have been derived based on type and permeability of cryoprotectants into the cells (Leibo and Pool 2011).

The cryoprotectants and water move across the cellular membrane by two pathways; simple diffusion through lipid bilayer and facilitated diffusion through

channels. Simple diffusion is a temperature dependent phenomenon where the permeability of water/cryoprotectant is low while activation energy (E_a= kCal mol^{-1}) is high. This needs a longer exposure of cells to cryoprotectant solutions to dehydrate the cells and entry of cryoprotectants. On the contrary, facilitated diffusion provides high permeability and low E_a (Jin *et al.* 2011). In facilitated diffusion, time period of cryoprotectant exposure needs to be short. Removal of cryoprotectant upon warming/thawing is equally important and is temperature-dependent; however, facilitated diffusion also removes this obstacle. Some authors suggest movement of molecules such as water, DMSO, glycerol and ethylene glycol via channels using facilitated diffusion in morula stage embryos. Aquaporin 3 (AQP3) plays a principle role in the rapid movement of glycerol and ethylene glycol but not DMSO (Edashige *et al.* 2003). Therefore, depending upon permeability and pathway adopted by various cryoprotectants, vitrification protocols suitable for different embryonic stages need to be defined. For example, ethylene glycol can be used in a one-step method for mouse morulae while requires a pre-treatment with mouse early staged embryos. Jin and co-workers found the maximum permeability of ethylene glycol in bovine morulae (P_s= 12.34±4.76 X 10^{-3} cm min^{-1}) at 25° C with least activation energy (E_a=1.8 kCal mol^{-1}). However, it did not differ significantly for blastocyst (E_a=10.85±2.74; P_s= 3.9) staged embryos rather than 16-cell staged embryos (E_a= 2.04±1.3; P_s= 10.3). To confirm the role of AQP3 in diffusion of cryoprotectants across the cell membrane, injection of double stranded (ds) AQP3 in bovine morulae suppressed the expression of AQP3. These embryos changed volume in a glycerol solution much slowly than non-injected intact bovine morulae (Jin *et al.* 2011).

Earlier attempts of cryopreservation utilized glycerol as cryoprotectant, in field conditions especially for commercial setup in cattle embryo cryopreservation. However, over the years Ethylene glycol (EG) proved to be least toxic because of its higher permeability and helpful in direct transfer of embryos (Vajta and Nagy 2006). Thus, it was more adaptable in field conditions but gave comparable pregnancy rates as glycerol in cattle (Voelkel and Hu 1992b; McIntosh and Hazeleger 1994). The bovine embryos frozen with 1.5 M EG, 1.5M propylene glycol (PG), 1.5 M DMSO and 1.4 M Glycerol gave embryo survival rates such as 80%, 16%, 35% and 60% (Voelkel and Hu 1992b). In ovine embryos, cryopreservation (slow freezing) of embryos (morulae and blastocysts) was done using two different cryoprotectants; glycerol and ethylene glycol. The pregnancy rates obtained from cryopreserved blastocysts did not differ significantly between ethylene glycol and glycerol (40.2 vs 51.3%) (Martinez and Matkovic 1998). However, for morulae, pregnancy rates from ethylene glycol group was better than glycerol (59.4% vs 29.7%) (Martinez and Matkovic 1998). Comparison of ethylene glycol-Ficol-sucrose and propylene glycol-glycerol was done in ovine embryo vitrification, which resulted in pregnancy rates of 40 and 28.1%, respectively (Martinez and Matkovic 1998).

2.5.3 *Cryopreservation methods*

Supercooled water was first identified 200 years ago by Gey-Lussac. Since then cryopreservation technology has advanced a lot, with high survival rates of oocytes and embryos using slow freezing as well as vitrification. These techniques are dependent upon rate of cooling and concentration of cryoprotectants used. The velocity of cooling is dependent upon thermal mass of the sample and its surface area. Thus rapid cooling can

be achieved with the lowest thermal mass and high surface area: volume ratio of the sample. The development and use of the two most commonly used techniques like slow freezing and vitrification is described below.

2.5.3.1 Slow freezing

In 1949, Polge and coworkers in an attempt to repeat Luyet's work discovered the cryoprotection ability of glycerol and hence paved the way for development of slow freezing (Polge *et al.* 1949). In slow freezing a delicate balance between various damaging factors including ice crystal formation, fracture, toxic and osmotic damage is created. As the name suggests, this technique utilizes the slow and controlled cooling rates as well as low cryoprotectant concentrations to freeze the samples (cells, oocytes or embryos). In the technique, embryos are equilibrated in the cryoprotectants, depending upon the surface area and permeability of embryos and oocytes. Embryos are then transferred to sub-zero temperatures like -6ºC to -7ºC. Ice seeding is done after equilibration at this temperature for 5-10 mins. These embryos are then cooled to -35ºC at the rate of 0.3ºC to 0.6ºC per min and then directly plunged into liquid nitrogen. The cryoprotectant equilibration and slow cooling of embryos derives water out of cells. This leads to ice formation in the extracellular water further dehydrating the cells and vitrification at -196ºC in liquid nitrogen.

In a study, on IVP human cleavage staged embryos were slow frozen and vitrified. Only 61.8% and 85.5% embryos survived, with slow freezing embryos resulting in no pregnancy, while vitrified embryos produced 25% pregnancies and 15% implantation (Son *et al.* 2009). In another study mouse and human blastocysts were cryopreserved with slow freezing and embryo survival rates and apoptosis rates were observed. Only

82.5% mouse and 83.3% human blastocysts survived slow freezing and DNA integrity index of these embryos were 75.8% in mouse and 76.5% in humans as compared to fresh embryos (93.6% and 92.4%, respectively) (Li *et al.* 2012). In freezing rabbit morulae, using slow freezing and open pulled straw (OPS) vitrification resulted in 55% and 71% blastocysts rate (Naik *et al.* 2005). Live offspring rate in both groups was 19% and 29%, respectively. In another report, higher survival and implantation rates were found after slow freezing than vitrification of human embryos (Mauri *et al.* 2001). In another randomized control study on day 3 human embryos (Balaban *et al.* 2008), vitrification yielded superior embryo survival (94.8% vs 88.7%) and blastocyst development rates (60.3 vs 49.5%) than slow freezing (Balaban *et al.* 2008).

In slow freezing, low cryoprotectant concentration prevents serious toxic and osmotic damage. Although, all precautions have been taken and protocols optimized for embryo cryopreservation using slow freezing, the technique is still associated with intracellular ice formation. Importantly, in slow freezing, during cooling at lower temperatures, the extracellular ice formation leads to dangerously high concentration of intracellular solutes. Use of rapid cooling rates can reduce the exposure period of embryos to cryoprotectants and can eliminate chances of such toxic and osmotic effects. This speculation has lead to the development of vitrification technique and it may prove to be a method of choice for embryo cryopreservation in humans and many domestic species.

2.5.3.2 Vitrification

Vitrification i.e. achievement of a “glass-like state”, is a radical non-equilibrium cryopreservation approach to preserve embryos using high concentration of

cryoprotectants and very high cooling rates (20,000ºC per min or higher) at -196ºC. The higher the cooling rate the lower the concentration and amount of cryoprotectant required. For vitrification protocol to develop and be applied successfully, a balance is required between a safe device to provide maximum cooling and warming rate and escaping toxic effects of high cryoprotectant concentrations. Thus, several protocols and devices have been tried for making the vitrification protocols quicker and more effective (Saragusty and Arav 2011). The smaller the volume of sample containing vitrification solutions, higher is the probability of vitrification without possibility of ice formation (Arav *et al.* 2002). To reduce the volume, many surface vitrification techniques like EM grids (Martino *et al.* 1996), minimum drop size (Arav and Zeron 1997), cryoleaf (Chian *et al.* 2005), cryotop (Kuwayama and Kato 2000) and tubing techniques such as plastic straw (Rall and Fahy 1985), open pulled straw (OPS) (Vajta *et al.* 1997; Vajta *et al.* 1998) etc. were developed. Also, decrease in volume of sample provides acceleration to the cooling rates, while vitrification. The other factor that contributes in the success of the procedure is optimally shortening the exposure of vitrification solution to avoid the toxicity of high concentration of cryoprotectants (Kasai *et al.* 1996).

Open pulled straw method has been used successfully in bovine, rabbit (Naik *et al.* 2005; Vicente *et al.* 2013) and pig embryos (Berthelot *et al.* 2001) as well as bovine oocytes (Vieira *et al.* 2002). In humans, vitrification of cleavage staged embryos yielded 79% embryo survival rates and 14% of implantation from ET (Mukaida *et al.* 1998). Vitrification of human blastocyst staged embryos provided 79% blastocyst survival rates, with 23% implantation rates (Vanderzwalmen *et al.* 2003). In another study, 82% blastocysts and 73% of morulae survived (Cremades *et al.* 2004). A blastocyst survival

rate of 97% and an implantation rate of 47% of human vitrified blastocysts has been reported after induced hatching of warmed blastocysts before ET (Mukaida *et al.* 2006). Therefore, vitrification is a method of choice in most human embryo cryopreservation setups. Rabbit morula vitrification however, had been found to be responsible for reduction in early fetal growth and losses throughout gestation (Vicente *et al.* 2013).

Evaluation of vitrified bovine embryos post-warming, for apoptosis and DNA fragmentation as well as gene expression related to apoptosis (survivin, Fas, caspase-3 and Hsp 70) after vitrification, was done. The blastocysts showed a significantly higher apoptotic index (11.9%) than non-frozen groups (3.34%) (Park *et al.* 2006). The relative abundance of survivin, Fas, caspase-3 and Hsp 70 expression increased in frozen-thawed embryos in comparison with the non-frozen controls. This indicated triggering of stress response and poor quality of pre-implantation embryos.

Ovine embryos were cryopreserved using both controlled slow freezing and open pulled straw vitrification yielded equivalent embryo survival (36-45%) and lambing rates (55-68%) (Bettencourt *et al.* 2009). Authors suggest that pre and post cryopreservation embryo survival rates were similar, suggesting no effect significant effect on embryo viability.

Vitrification protocol has two glitches 1) embryos cannot be transferred directly and embryo warming requires microscopic examination, 2) biohazard of pathogen transmission mediated by liquid N_2 (Vajta and Nagy 2006).

To summarize both the techniques; during slow freezing extracellular water crystallizes, resulting in an osmotic gradient that draws water from the intracellular environment till vitrification occurs. In cryopreservation by vitrification, both intra and

extracellular compartments apparently vitrify after cellular dehydration has already occurred. (Saragusty and Arav 2011).

2.5.4 *Origin of embryos*

Many studies have compared the freeze-thaw survivability and further development of *in vitro* and *in vivo* produced embryos (Massip *et al.* 1995).

2.5.4.1 In vivo **derived**

Although *in vivo* produced embryos are more cryotolerant, their survivability is dependent upon breeds and species of origin (Steel and Hasler 2004). A study was conducted to compare the ultra-structure of IVD and IVP embryos after cryopreservation (Fair *et al.* 2001). The study proved that ultrastructure of both IVD and IVP embryos after cryopreservation showed changes as compared to the non-cryopreserved embryos. Overall scale of damage was, however, less in IVD embryos as compared to IVP embryos. The changes suggested in IVP embryos after cryopreservation are already preceeded by poor morphology as suggested by many studies (Prather and First 1993).

2.5.4.2 In vitro **produced**

Cryopreservation of IVP embryos can be utilized to redeem genetic material from superior cows that have been culled inevitably due to health problems such as repeat breeding, lameness, udder problems and chronic endometritis (Hosseini *et al.* 2013). During past few decades improved knowledge of embryo culture media, embryo structure, morphology and ultrastructure has lead to increased production of IVP embryos in domestic animals (Sudano *et al.* 2012). *In vitro* produced embryos are however, highly

sensitive to cryopreservation, which is a major drawback for dissemination of IVP embryo cryopreservation technology (Abe *et al.* 2002b; Rizos *et al.* 2002a; Rizos *et al.* 2003; George *et al.* 2008). As discussed earlier, IVP embryos exhibit characteristics such as detached blastomeres, highly electron-dense cytoplasm, retarded growth, high sugar metabolism, swollen blastomeres (Van Soom *et al.* 1992), a fragile zona pellucida (Duby *et al.* 1996), differential intercellular communication (Boni *et al.* 1999), higher chromosomal abnormalities and high thermal sensitivity (Gardner *et al.* 2000; Abe *et al.* 2002a; Sudano *et al.* 2012). Variations in cryotolerance of IVD and IVP embryos can be predicted at ultrastructural levels as well (Fair *et al.* 2001). The IVP embryos with reduced cryotolerance showed wider peri-vitelline space, vacuoles in trophoblast and a sparse population of microvilli (Abe *et al.* 1999; Crosier *et al.* 2001; Papadopoulos *et al.* 2002). Also, IVP of embryos in cattle have been related with atleast 3.7% of calves having congenital malformations (Boerjan *et al.* 2000).

These indicate that IVP embryos are more sensitive to cryopreservation, which reduces conception rates relative to *in vivo*-produced embryos (Farin and Farin 1995; Sudano *et al.* 2011). Also, damage to mitochondria, RER as well as desmosomes and cell-cell tight junctions alterations were found to be more in *in vitro* produced post-thawed embryos than *in vivo* embryos (Vajta *et al.* 1997; Fair *et al.* 2001). In addition, IVP embryos survive less than IVD embryos (Hasler *et al.* 1995; Fair *et al.* 2001).

High lipid accumulation is considered one of the main reasons for the increased cryopreservation sensitivity of IVP embryos than IVD ones (Abe *et al.* 2002b; Mucci *et al.* 2006; Sudano *et al.* 2012). As discussed earlier (section 2.5.1), the reasons for higher lipid accumulation could be use of FCS in culture media and/or abnormal glucose

metabolism (Barcelo-Fimbres and Seidel 2007b; Sudano *et al.* 2011). A research shows that post vitrification bovine embryo survival was significantly correlated with apoptosis rates and lipid droplet in fresh embryos (Sudano *et al.* 2012). On the other hand, in the same study, embryo cleavage rate, total cell number and quality degree score did not impact the cryosurvival outcome.

Surprisingly, in a comparative study of pregnancy rates from fresh and vitrified IVD and IVP ovine embryos revealed consistently low results from IVP embryos in both the treatments (fresh and vitrified) (54% versus 5%, respectively) with respect to IVD group (90% versus 50%) (Papadopoulos *et al.* 2002). It has been observed that culture of IVD zygotes *in vitro* uptil blastocyst stage results in low cryotolerance of these embryos. Contrarily, culture of IVP zygotes in ewe oviduct *in vivo* to blastocyst stage, increased their cryotolerance significantly (Papadopoulos *et al.* 2002; Rizos *et al.* 2002a).

Embryonic cell death induced due to apoptosis is considered important criteria to determine the embryo quality (Matwee *et al.* 2000). Furthermore, apoptosis induced to sub-optimal *in vitro* conditions can also indicate the embryonic health (Betts and King 2001). Several studies have indicated altered apoptotic gene expression in such culture conditions, suggesting lower embryo quality (Rizos *et al.* 2002e; Kuzmany *et al.* 2011).

2.5.5 *Embryonic stages*

Embryonic stages contribute highly to the outcome of cryopreservation (Shirazi *et al.* 2010b). The early staged embryos are difficult to retrieve *in vivo,* but extensive data is available for *in vitro* produced early embryo cryopresevation (Massip 2001). Unfortunately, IVP pre-compaction embryos are suggested to be more prone to chilling

injury in cattle, pigs and sheep (Pollard and Leibo 1994; Cuello *et al.* 2004a). Some success had been observed with the use of small volume for freezing for example with the use of open pulled straw technique for vitrification of early IVP bovine embryos. Advanced stages like morula, early, middle and expanded blastocysts are more suitable and resilient option for the process of cryopreservation using both slow freezing and vitrification (Massip 2001; Prentice and Anzar 2011). As discussed in cryoprotectant section; post compaction stages are well equipped with the machinery (e.g. AQP3) to facilitate diffusion of cryoprotectants across the cellular membranes (Jin *et al.* 2011). Also, in morula and blastocyst stage, the intercellular connections including water channels aquaporins (AQP3) are well developed allowing proper diffusion of cryoprotectants and hence, less exposure time (Jin *et al.* 2011).

Vitrification of different stages (morula, early blastocysts or expanded blastocysts) of pig embryos was done using three techniques; open pulled straw: OPS; superfine open pulled straw: SOPS; and Vit-Master1 technology using SOPS: Vit-Master-SOPS. Although, vitrification system did not affect the embryo survival; expanded blastocyst staged embryos showed the best survival outcome *in vitro* with similar hatching rates as those of fresh *in vitro* counterparts (Cuello *et al.* 2004a). The hatching rate of fresh morula or early blastocyst stage embryos was higher than their vitrified counterparts. Another study conducted on the vitrification of different stages of sheep embryos (Shirazi *et al.* 2010b). Like pigs, sheep early embryos (4-cell, 8-cell and 16-cell embryos) were prone to freezing damage and showed lower re-expansion rates and blastocyst formation rates than morula and blastocyst staged embryos. Another study on sheep embryos revealed lower viability of frozen-thawed cleavage stage embryos as compared with the

blastocyst staged embryos and freeze-thaw survivability improved as embryo developmental stage progressed (Garcia-Garcia *et al.* 2006). Similar results were shown by the mouse embryos where blastocyst and expanded blastocyst rates after vitrification of one and two celled stage embryos were lower than later stages and increased progressively from 4-celled stage through morula and early blastocyst stage embryos (Zhou *et al.* 2005). For bovine embryos, a cryopreservation study using vitrified embryos at cleavage, morula and blastocysts was conducted. No significant difference was found among survival rates of different staged embryos; however, there was a trend of lower survival of blastocysts as compared with compacted morula (81.4% vs 92%) (Zhang *et al.* 2009). This can be attributed to the blastocoel fluid present in the blastocoel. The fluid present in the blastocoel cavity of early and expanded blastocysts turns into ice-crystals upon freezing and causes injury (Mukaida *et al.* 2006; Feng *et al.* 2010). Reduction of blastocoel fluid by forced blastocoele rupture (FBC) before cryopreservation had been related to higher survival rates due to low possibility of ice-crystallization.(Mukaida *et al.* 2006) FBC in expanded blastocyst stage bovine embryos improved their development and pregnancy rates, post-cryopreservation using slow freezing and vitrification techniques as compared with intact cryopreserved blastocysts (non-FBC) group (Min *et al.* 2014). The frozen thawed FBC blastocysts exhibited higher re-expansion and hatching rates than non-FBC group. In another study, vitrified blastocoele collapsed blastocysts yielded higher survival rates (100%) than blastocoele intact ones (89%) (Li *et al.* 2012). Cryopreservation damage can induce apoptosis due to physical and thermal stress in embryonic cells (Sudano *et al.* 2011). The rate of apoptosis was decreased in frozen thawed FBC blastocysts in the former study. Also, gene expression (Bcl-XL and Bax)

related to apoptosis was analyzed and pro-apoptotic gene like Bax increased while that of anti-apoptotic gene like Bcl-XL decreased in frozen thawed-FBC *in vitro* bovine blastocysts. (Li *et al.* 2012) This indicates improved quality of frozen thawed FBC blastocysts as compared with frozen thawed- non-FBC blastocysts. The mechanism of repair of ruptured TE of blastocoele cavity is however, still under under elucidation.

2.5.6 *Species of origin*

Although main emphasis of embryo cryopreservation has been on the cattle embryos, success has been reported in other domestic species such as sheep, goat, horse and pigs. Sheep, goat and cattle embryos are however, more resistant to cryopreservation damages and give more survival rates as compared to horse and pig embryos which are known for their sensitivity to chilling. Horse blastocyst stage embryos are prone to freezing injury due to their large size (Squires *et al.* 1999) and reasonable pregnancy rates had been obtained for smaller equine embryos (Hochi *et al.* 1994; Massip 2001). While pig embryos are suspected to be cryosensitive due to their high lipid content (Dobrinsky 1997), cryopreservation of hatched embryos have shown success in sparse studies while morulae and early blastocysts do not survive freezing, well (Hayashi *et al.* 1989; Dobrinsky 1997; Cuello *et al.* 2004c). This is suspected due to smaller size of lipid droplets in these stages. Pre-treatment with delipidation and cytoskeleton stability and centrifugation was found to improve survival of pig morulae and early blastocysts (Dobrinsky *et al.* 2000).

Sheep embryos were initially used as models for optimizing protocols for bovine embryo freezing by conventional methods with the use of DMSO as cryoprotectants

(Willadsen *et al.* 1978; Massip 2001). Ovine embryos improve in their freeze-thaw survivability with the advancing embryonic stages. They have been successfully vitrified using 3.5 M glycerol and 3.5 M propylene glycol as cryoprotectants, with lambing rates of around 52% (Szell and Windsor 1994). Vitrification of IVP ovine embryos in another study however, resulted in only 5% pregnancy rates as compared to IVD embryos where pregnancy rates were 50%, similar to previous studies (Papadopoulos *et al.* 2002). Interestingly, the embryo survival rates of IVP sheep embryos had been observed to be higher than IVP cattle embryos in the same culture conditions and cryopreserved using same technique and protocol (Rizos *et al.* 2002a) suggesting innate strength and quality of sheep embryos.

2.6 Effects of cryopreservation on embryo development

2.6.1 *Morphological and ultrastructural changes*

Post cryopreservation morphological and ultrastructural changes have been studied and discussed in both IVD and IVP embryos (Prather and First 1993; Pollard and Leibo 1994; Fair *et al.* 2001; Saragusty and Arav 2011). Cryoinjuries can occur due to cryoprotectant toxicity, subsequent exposure to low temperatures and during warming. Scientists have therefore, concentrated their efforts to understand and minimize these damages to cell and embryonic structure. For this several combinations and modifications in cryopreservation protocols have been studied to evaluate the post-cryopreservation damage (Saragusty and Arav 2011).

During cryopreservation, the cellular membranes and cell structure need to endure volumetric changes and membrane shrinkage in response to hyperosmotic cryoprotectant solutions. Cells must escape cryoprotectant toxicity, thermal phase changes in membrane

phospholipids and changes in membrane lipid composition, freeze induced dehydration, ice formation induced cell-cell intercellular damage (Mazur and Cole 1985), cryopreservation induced lipid peroxidation and loss of superoxide dismutase activity and recrystallization during warming (Mazur 1970; Arav *et al.* 1993). Another structure commonly damaged during cryopreservation is cytoskeleton. According to some studies, the stability of cytoskeleton prior to cryopreservation, can be preserved by using cytochalasin B or colchicine in culture media prior to cryopreservation (Dobrinsky *et al.* 2000; Saragusty and Arav 2011).

A study conducted to compare pre and post-thaw morphology of IVD and IVP bovine embryos was done. The non-cryopreserved IVD embryos' plasma membrane was apposed closely to zona pellucida, microvilli were stacked, trophoblasts were connected with tight junctions and desmosomes. In cryopreserved embryos however, plasma membrane was not closely apposed to zona pellucida, microvilli were broken and extended. These blastocysts showed perivitelline space (PvS) debris. Fresh IVM/IVF embryos on the other hand, had wider PvS. Blastocysts had small and large cavities surrounding ICM cells. Post-thaw blastocyst morphology was inferior, cavities surrounding ICM cells were large, wider PvS and there were lipid droplets in PvS. The number of degenerated nuclei and apoptotic bodies were 3-7 and 6 in IVP embryos, as compared to 4-7 and 2-6, respectively in IVD embryos (Fair *et al.* 2001).

Three phases of cryopreservation, incur different types of damage according to temperature ranges the cells pass through. Between +15ºC to -5ºC, chilling injury is the major factor, damaging cytoplasmic lipid droplets and microtubules (Leibo 1981). More rapid cooling and warming rates during vitrification, however, reduces chilling injury

such as lateral segregation of proteins and phospholipids within membranes by passing the critical temperature zone (+15ºC to -5ºC). During -5 to -80ºC phase, injury occurs mainly because of extracellular and intracellular ice formation. From -50 to -150ºC however, zona or cytoplasm fractures are the main cause of embryo death (Rall and Meyer 1989). Damage to intracellular junctions, organelles and cytoskeleton after cryopreservation of embryos had been reported in many studies (Vicente and Johnson 1992; Dobrinsky 1997; Vajta and Nagy 2006).

Several modifications in cryopreservation procedures have been tried to reduce cryoinjuries. For example, addition of PES and cytochalasin in embryo culture medium before subjecting them to cryopreservation has been tried to minimize and stabilize the cytoplasmic lipid droplets and microtubules, respectively (Seidel 2006). Studies suggest that cryoprotectant exposure have an impact on cytoskeletal dynamics and cellular organelles. The reports of loss of microvilli, intercellular junctions and cytoplasmic membrane stability have disruptive impact on cellular organelles (Bettencourt *et al.* 2009). Mitochondrial structure had been reported to be damaged due to cryopreservation in many studies thus, affecting the energy supply to cell boundaries (Vajta *et al.* 1997; Cuello *et al.* 2007).

2.6.2 *Biochemical changes*

The challenge in cryopreserving cells from homeotherms is in cooling the cells below body temperature that leads to cellular damage. The chilling injury may extend upto behavior and function of membrane lipids and proteins. Also, membrane lipids, which are in liquid crystalline state may solidify at lower temperatures. This can change their function, can cause protein denaturation and uncoupling of ATP synthesis from

electron transport chain mitochondrial enzymes (Shirazi *et al.* 2010b). This phenomenon further leads to production of reactive oxygen species (ROS) that damage to membranes and cryocapacitation like changes in boar sperm (Radomil *et al.* 2011). Cholesterol is present in cells and in plasma membranes. The ratio of cholesterol and PM phospholipids define the chilling sensitivity of PM, which is often damaged during cryopreservation (Seidel 2006; Saragusty and Arav 2011).

Gene expression changes can depict the effect of cryopreservation on embryos at subtler levels apart from ultra-structural changes. In a study by Saenz-de-Juano and coworkers (2014), gene expression changes were compared in cryopreserved rabbit embryos using vitrification with fresh embryos. Upon microarray analysis and quantitative gene expression analysis, the gene expression of vitrified group was found similar to that of fresh embryos (Saenz-de-Juano *et al.* 2014b). Also, the implantation and birth rates in vitrified group were higher than another treatment group (slow freezing; not compared for gene expression changes) (Saenz-de-Juano *et al.* 2014b). In another study, vitrification technique caused fetal losses during implantation periods in rabbit morula vitrification (Vicente *et al.* 2013). The gene expression tested using quantitative real time pointed towards possible impairment of foetal development during peri-implantation periods (Vicente *et al.* 2013). In cryopreservation studies on mouse embryos (8-cell and blastocysts), most transcript changes related to development, metabolism oxidative stress and apoptosis were established (Mamo *et al.* 2006; Dhali *et al.* 2007). Vitrified IVP bovine embryos were compared with control untreated group using microarray-based technology. A total of 383 genes were differentially expressed in vitrified embryos. Genes involved in apoptosis (PRDX2), heat shock (HSPA5), maternal recognition of

pregnancy (IFNT2 and PAG2), and cell differentiation and placenta formation (KRT18) were downregulated in vitrified embryos (Sudano *et al.* 2012). In another study, however, the molecular effects of cryopreservation treatment were found to be dependent upon origin of bovine embryos (*in vitro* vs *in vivo*) (Aksu *et al.* 2012). A total of 854 genes were found differentially expressed using microarray technology, in IVP vitrified embryos as compared to fresh counterparts. However, only 162 genes were differentially expressed among vitrified IVP and IVD embryos. Vitrification was found to be associated with upregulation of oxidative phosphorylation and stress responses in IVP embryos. Comparison of gene expression of IVP and IVD embryos have been reported in several studies (Rizos *et al.* 2002a; Lonergan *et al.* 2003b; Wrenzycki *et al.* 2004). These altered genes identified in IVF embryos were associated with a number of biological processes such as metabolism, cytokine signaling, and stress adaptation (Wrenzycki *et al.* 2004). Thus, oocytes and embryos when exposed to environmental deviations such as heat stress (Gendelman *et al.* 2010; Gendelman and Roth 2012), cryopreservation and IVC culture conditions lead to detrimental gene expression changes.

CHAPTER 3 **General objectives and Hypotheses**

Overall objective of the study was to determine the superiority of any one of the most commonly used cryopreservation techniques; vitrification or slow freezing for freezing bovine embryos. As discussed above, vitrification has been trying to replace slow freezing in the field of embryo cryopreservation for over a decade (Vajta and Nagy 2006). However, the ease of performing slow freezing technique without any prior technical expertise outweighs the option of doing vitrification. The developmental outcome of both techniques in bovine embryos is still debatable and scientists have long tried to adapt the techniques to the cellular properties of the embryonic blastomeres (Massip *et al.* 1995). Although, vitrification tends to yield better embryo survival rates than slow freezing, several studies pinpoint possible drawbacks of the former technique at morphological (Moreira da Silva and Metelo 2005; Coutinho *et al.* 2007; Dalcin *et al.* 2013) and biochemical (Dalcin *et al.* 2013) levels. Based on the previous observations the overall hypothesis of this thesis is that cryopreservation of embryos with vitrification and slow freezing causes significant changes in global gene expression which impact the quality of post-warmed embryos.

3.1 Objectives

A. To study the effects of vitrification and slow freezing on the post-warming development of *in vitro* produced bovine embryos

B. To compare the effects of ethylene glycol and glycerol as cryoprotectants in the development of slow frozen *in vitro* produced bovine embryos

C. To study the effect of vitrification and slow freezing techniques on global gene expression changes in *in vitro* produced bovine embryos.

D. To confirm the differential gene expression changes in the vitrified and slow frozen embryos using quantitative real time PCR.

CHAPTER 4 **Effect of cryopreservation techniques, season and cryoprotectants on the survival of *in vitro* produced bovine embryos**

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4.1 Abstract

Embryo cryopreservation is a major tool for conservation and propagation of animals possessing high genetic value. However, it adversely affects the survival of embryos. The objective of this study was to determine the effect of two cryopreservation techniques (vitrification vs. slow freezing) during different seasons of the year and the effect of two cryoprotectants (glycerol vs. ethylene glycol) used for slow freezing on the post-warming survival of *in vitro* produced (IVP) bovine morulae. In experiment 1, morulae [day 6 post-*in vitro* fertilization (d6 post-IVF)] were subjected to either vitrification (n=271 morulae) or slow freezing (n=281) or no freezing (control; n =249) in spring, summer, fall and winter seasons over a period of 3.5 years. After warming, the morulae were cultured for 1 to 2 days (d7-8 post-IVF) to expanded blastocyst stage for assessment of their survival. Data were compared using Glimmix procedure in SAS®. Blastocyst conversion rate (blastocysts obtained / number of morulae used; %) was higher ($p<0.05$) in vitrified (52±4.6) than slow freezing (35±4.2) group. Blastocyst conversion rate was higher ($p<0.05$) in the control group (78±3.6%) as compared to frozen groups (combined vitrification and slow freezing groups; 43.5±4.4%). Re-expansion upon warming of vitrified morulae and subsequent blastocyst conversion rate were not related (Pearson's correlation coefficient = 0.092; $p>0.05$). Fall season had lower cleavage (67±1.6%; d2 post-IVF) and morulae (22±1.4%; d6 post IVF) rates than

other seasons (74±1.1 and 30±1.2%, respectively; $p<0.05$). Spring, summer and winter season did not differ significantly among themselves ($p>0.05$). Blastocyst conversion rate differed ($p<0.05$) between summer (63±4.5%) and spring (85±3.3%) seasons in control group, between summer (20±4.1%) and all other seasons (43±8.3%) in slow freezing group, and did not differ among seasons ($p=0.19$) in vitrification group. In experiment 2, glycerol (n=86 morulae) and ethylene glycol (n=75) were used to cryopreserve bovine morulae using slow freezing method. Blastocyst conversion rate had a tendency ($p=0.07$) to be higher in ethylene glycol (31±5.3) than in glycerol (18±4.3) group. In conclusion, vitrification is a better technique than slow freezing for cryopreservation of IVP bovine morulae. Winter and spring are better seasons to conduct IVF in cattle. Ethylene glycol may be a better cryoprotectant than glycerol for slow freezing of IVP bovine morulae.

Keywords: vitrification; slow freezing; IVF, bovine morula; cryopreservation; genetics

4.2 Introduction

Market demand for high quality and quantity of food puts intense selection pressure on livestock species to propagate the individuals and breeds with "desirable" traits. Such efforts lead to loss of bio-diversity within a breed and loss of rare livestock breeds (Hiemstra 2011; Prentice and Anzar 2011; Woelders *et al.* 2012). Therefore, global efforts for conservation and rejuvenation of endangered breeds and lost gene pools are in progress (FAO 2007). Embryo cryopreservation and transfer provides a way for rapid genetic improvement and re-establishment of herds.

Embryo cryopreservation is an important part of the growing embryo transfer industry. It has a major role in transportation of high quality animal genome across the world. Its potential is highlighted in survey data from the *American Embryo Transfer*

Association (AETA 2013) , documenting the transport of 10,993 dairy and 4,903 beef cattle frozen embryos around the globe from USA. Total number of frozen embryos (dairy and beef) transferred across USA was around 129,100 during the year 2013 (AETA 2013). Similar statistics presented by *International Embryo Transfer Society* show transfer of 344,149 frozen *in vivo* produced and 42,004 frozen *in vitro* produced (IVP) bovine embryos in the year 2013 (Wheeler and George 2014).

Mammalian cryopreservation techniques store cells in liquid nitrogen at -196°C and aim to minimize the damaging effects of ice crystal formation during freezing and thawing cycle. Such intracellular ice formation is dependent on cooling and warming rates, and surface area/volume of cells to be frozen (Mazur 1963; Mazur 1970; Seki *et al.* 2014). Initially, cells, oocytes and embryos were frozen with programmed cooling rates but simpler and faster freezing techniques such as vitrification have been recently introduced recently (Vajta and Nagy 2006). These techniques are differentiated by two factors: cryoprotectant concentration and rate of cooling (Leibo 1993; Massip 2001). Before programmed slow freezing of embryos, a non-permeating cryoprotectant solution is used to create differential osmotic pressure that drives water out of cells with concomitant increase in cytoplasmic solute concentration. This depresses the freezing temperature of the cytoplasm and lowers intracellular ice crystal formation (Mazur 1963; Mazur 1970; Seki *et al.* 2014). Vitrification, on the other hand, is the preservation of biological cells in highly viscous permeating cryoprotectant solutions to achieve a glass like state with ultra-rapid cooling rates (>1000°C/ min) thus avoiding intracellular ice crystal formation (Rall and Fahy 1985; Rall 1987; Kuwayama 2007).

Recent studies however, point out that warming rate might be more important than

viscosity of cryoprotectant solution and cooling rate in survival of vitrified embryos (Seki *et al.* 2014). Warming of vitrified embryos is accompanied with a phenomenon of embryonic rehydration and removal of cryoprotectants, followed by regaining of embryonic shape. This "embryo re-expansion" is proposed as an indicator of embryonic vitality and developmental potential in many studies (Tachikawa *et al.* 1993; Kaidi *et al.* 2000; Aksu *et al.* 2012). Therefore, we wanted to confirm the validity of the relationship between warming re-expansion of vitrified IVP bovine morulae and subsequent blastocyst formation.

Embryo survival post-vitrification and slow freezing have been studied in various species (Liebermann and Tucker 2004; Lin *et al.* 2010; Li *et al.* 2012; Zander-Fox *et al.* 2013). Some of the studies have also tested the effect of vitrification and/or slow freezing on embryo morphology, i.e. live-dead cell ratio and apoptosis (Kader *et al.* 2010; Morato *et al.* 2010; Li *et al.* 2012).

One of the important steps during embryo cryopreservation is "cryoprotectant equilibration" (Mazur 1970). Various concentration and composition of cryoprotectants have been tested to develop a safer combination that gives optimum embryo survival as well as is embryo transfer-friendly. Ethylene glycol (EG) is a lower molecular weight cryoprotectant than glycerol and allows the direct transfer of embryos into recipient animals without its removal during warming (Voelkel and Hu 1992b; Massip 2001). In the present study, we test the impact of cryoprotectants, drawing the comparison between ethylene glycol and glycerol on the development of IVP bovine embryos.

Oocyte quality and embryo survival is affected in dairy cattle breeds due to summer

heat (Gendelman *et al.* 2010; Braga *et al.* 2012; Gendelman and Roth 2012). IVF has been tried as an alternative to circumvent such kind of stresses (Rutledge 2001; Al-Katanani *et al.* 2002). However, the impact of different seasons on *in vitro* bovine embryo production and their post-cryopreservation survival has not been explored extensively. Our data were gathered over a 3.5-year period and therefore, the experimental design allowed us to examine the role of different seasons in embryo production and their cryo-survival.

The objective of this study was to determine the effects of common cryopreservation techniques (slow freezing *vs* vitrification), season (spring, summer, fall and winter) and cryoprotectants (Ethylene glycol *vs* glycerol for slow freezing) on *in vitro* bovine embryo development. Also, the relationship between re-expansion of vitrified-warmed morulae and subsequent development to blastocyst stage was examined.

4.3 Material and methods

4.3.1 *Chemical and supplies*

Dulbecco's phosphate buffer saline (DPBS-1X Ca^{2+}-Mg^{2+} plus- Cat# 21300-025), newborn calf serum (CS), TCM-199 (Cat# 12340-030) and MEM essential amino acids (Cat# 11140-050) were purchased from Invitrogen® Inc. (Burlington, ON, Canada). Lutropin-V (LH), Folltropin-V (FSH; Cat # PHD075) and Vigro™ Ethylene Glycol Freeze Plus media (Cat#624034) were obtained from Bioniche® Animal Health, Inc. (Belleville, ON, Canada). All other chemicals and reagents were purchased from Sigma-Aldrich® (Oakville, ON, Canada). For vitrification, cryotops (Cat# 81111) were purchased from Kitazato® Co. (Fuzi, Shizuoka, Japan) and for slow freezing 0.25 ml

Mini straw were purchased from IMV® Tech., (Woodstock, ON, Canada).

4.3.2 *In vitro maturation and fertilization*

Bovine ovaries were procured from the commercial Cargill® abattoir in Calgary and transported in controlled temperature coolers at 22ºC within 12 h. Extra tissues were removed and ovaries were washed with normal saline. *In vitro* maturation (IVM), *in vitro* fertilization (IVF) and embryo culture were conducted as described earlier (Prentice *et al.* 2011). Briefly, cumulus oocyte complexes (COC) were aspirated from 2-8 mm follicles using 18-gauge needle containing 1 ml holding solution [HS; 5% (v/v) new born calf serum (CS) in DPBS]. The COCS from pooled aspirated fluid were searched under a stereomicroscope, washed (3X) in HS and were graded (de Loos *et al.* 1989). The COCS (first and second grade), with more than 3 layers of cumulus cells, uniform cytoplasm and no visual signs of degeneration, were selected for further processing. The COCS were washed (3X) in maturation medium [TCM-199 + 5% CS + LH (5µg/ml) + FSH (0.5µg/ml) + gentamicin (0.05 µg/ml)] at 37˚C. The groups of 20 COCS were placed in 100 µl droplets of maturation medium under mineral oil at 37˚C in 5% CO_2 and high humidity, for 22-24 h.

Frozen semen straws from Holstein dairy bulls were thawed in a water bath at 37˚C, for at least 1 min. Sperm were washed and live sperm were isolated with Percoll density gradient method (Parrish *et al.* 1995). The isolated sperm were diluted to a concentration of 3 million cells per ml using Brackett-Oliphant (BO) (Brackett and Oliphant 1975) fertilization media [BO stock A + BO stock B + sodium pyruvate (1.3% w/v) + gentamicin (0.05 µg/ml)]. Eight 100-µl diluted sperm droplets were made under

mineral oil and approximately 20-25 oocytes per droplet were co-incubated (IVF= day (d) 0) at 38.5˚C, 5% CO_2 and high humidity for 18-22 h.

4.3.3 *Embryo culture*

After IVF, fertilized oocytes were washed (3X) in Charles Rosenkrans 1 (CR1aa) embryo culture media (5% calf serum (v/v), 2% BME essential amino acids (v/v), 1% MEM essential amino acids (v/v), 1% L- glutamic acid (v/v), 0.3% BSA (w/v) and 0.05 µg/ml gentamicin in CR1 medium; (Rosenkrans *et al.* 1993)). The presumptive zygotes were transferred to a petri dish containing 100 µl droplets of CR1aa media and incubated at 38.5˚C in 5% CO_2, 5% O_2 and 90% N_2 for 6 days. Cleavage rate was recorded d2 post-IVF (number of cleaved zygotes/number of oocytes incubated on d0*100).

Morulae were collected, washed (3X) and graded on d6 post-IVF. Percent morulae rates were recorded using two criteria (number of morulae/number of oocytes incubated on d0*100; number of morulae/number of cleaved zygotes on d2*100). First and second grade compact morulae with 50-85% uniform blastomeres and low peri-vitelline cellular debris (Stringfellow and Siedel 1998), were selected for experiment and were divided into 3 treatment groups: control (unfrozen), slow freezing and vitrification (detailed description in Experimental design section).

4.3.4 *Vitrification and slow freezing procedures*

The morulae morulae were washed (3X) with HS. Morulae/early blastocysts in control (unfrozen) group were then continued to culture in CR1aa up till d7-8 post-IVF.

Vitrification of embryos was done as described earlier (Prentice *et al.* 2011).

Briefly, the morulae were incubated in vitrification solution 1 [VS1; 7.5% ethylene glycol (EG, v/v) + 7.5% dimethyl sulfoxide (DMSO, v/v) + 20% CS (v/v) in 1X DPBS] at 37˚ C for 5 min. Approximately, 3-4 morulae were transferred through three 20 µl drops of vitrification solution 2 [VS2; 15% EG + 15% DMSO + 20% CS + 17.1% sucrose (w/v) in 1X DPBS] at 37˚ C within 1 min. Morulae were then placed on a cryotop; extra VS2 was aspirated carefully to minimize the droplet and embryos were immediately plunged in liquid nitrogen (N_2). After at least 24 h, morulae were warmed in warming solution [0.5 M sucrose (w/v) + 20% CS (v/v) in 1X DPBS] for 5 min. and washed with HS (3X). Morula re-expansion was noted down at the end of 5 min incubation in warming solution. All morulae were cultured up to d8-9 post-IVF in freshly prepared CR1aa to record the number of expanded blastocysts. Blastocyst conversion rate was calculated as number of blastocysts / number of morulae used in each group*100.

Slow freezing was conducted as described earlier (Carvalho *et al.* 1996). Morulae were incubated in glycerol based cryoprotectant solution for freezing [CPF; 1.5 M glycerol + 5 % CS in 1X DPBS] for 10 min at room temperature (RT). Three to four morulae were transferred to 0.25 ml straws and frozen in a methanol bath using a controlled rate Bio-Cool® III-80 freezer, FTS systems, SP Industries, Inc. (StoneRidge, NY, USA), as follows. Embryo containing straws were transferred directly from RT to -7°C and held for 5 min. Ice seeding was done by touching the straws with liquid N_2 dipped Q-tip™. Embryos were held at -7ºC for additional 10 min. Embryos were then frozen at a rate of 0.3-0.5°C/ min to -35ºC, held at -35°C for 5-10 min and straws were directly plunged into liquid N_2. Embryos were warmed after 24 h storage by incubating in 0.7 M sucrose in HS at 37ºC for 5 min. These were washed with HS and transferred to

100 µl-droplets of embryo culture media at 37ºC to d8-9 post-IVF.

In experiment 2, morulae were either frozen using glycerol based CPF (as described above in slow freezing) or commercial ethylene glycol (EG) based medium [Vigro Freeze plus™, Bioniche®; 1.5M ethylene glycol, 0.4% BSA and 0.1M sucrose in DPBS]. Slow freezing protocol described above was followed for both the cryoprotectants. Glycerol group morulae were warmed in 0.7M sucrose + 5% CS in 1X DPBS for 5 min. while EG group morulae were warmed in 5% CS (v/v) in 1X DPBS, for 10 min. Morulae were then cultured to d8-9 post-IVF.

4.3.5 *Experimental design*

A total 22 replicates of bovine IVF (i.e., batches of ovaries on different dates) using 5100 oocytes were conducted from October, 2011 to March, 2014 including all four seasons (spring, summer, fall and winter). Data from 13 replicates was used to study blastocyst development from IVP bovine morulae (d6 post-IVF) in control, vitrification and slow freezing groups. For seasonal-impact analysis, data from all 22 replicates were used to study effects on embryo culture (Cleavage rate and Morula rate) and survival after freezing [19 replicates for unfrozen control group, 13 replicates for cryopreserved groups (common with control group replicates)]. Data from 3 replicates were used to compare the effects of glycerol and ethylene glycol as cryoprotectants during slow freezing of bovine morulae.

4.3.5.1 Experiment 1: Development of in vitro produced bovine morulae to blastocyst stage following vitrification and slow freezing in different seasons.

The objective of this experiment was to compare the development of IVP bovine morulae after cryopreservation using vitrification and slow freezing techniques. A total of 801 embryos from 13 replicates were randomly distributed to one of three treatment groups; control (unfrozen), vitrification, or slow freezing. In control group, morulae (n=249) were directly transferred to embryo culture for blastocyst development. In other treatment groups, morulae were cryopreserved by vitrification (n=271) or slow freezing (n=281) and stored in liquid N_2 for at least 24 h. Morulae were warmed and transferred to CR1aa and incubated upto d8-9 post-IVF to record their development in blastocyst stage (n=13 replicates). The re-expansion of vitrified morulae was noted in warming solution to correlate with subsequent blastocyst development from a subset of replicates (n=9).

During this experiment, data on embryo development (cleavage rate on d2 post-IVF, morulae rate on d6 post-IVF and blastocyst conversion rate (unfrozen control, vitrification and slow freezing on d8-9 post-IVF) were recorded for four seasons over the period of 3.5 years. Seasons (spring, summer, fall and winter) were defined based on dates for equinoxes and solstices in northern hemisphere. A statistical analysis of accumulated data was done for the effect of season, cryopreservation technique and their interaction on fertilization and blastocyst development. Separate analyses were done for seasonal effect on blastocyst development in control, vitrification and slow freezing groups.

4.3.5.2 Experiment 2: Effect of cryoprotectant (ethylene glycol and glycerol) on blastocyst development from frozen morulae using slow freezing

The objective of this experiment was to compare the effect of two cryoprotectants (ethylene glycol and glycerol) used in slow freezing of bovine embryos. *In vitro* produced bovine morulae (n=161; 3 replicates) were collected as described above and subjected to two different cryoprotectants; ethylene glycol (EG; n=75) and glycerol (Gly; n=86) in slow freezing method. This study was repeated 3 times on different dates.

4.3.6 *Statistical analysis*

The data were analyzed using Proc Glimmix in SAS® enterprise guide 4.3 (SAS). Analyses were performed using link-function for binary distribution (yes/no response variable). Technique (3 levels; control, vitrification, slow-freezing), season (4 levels, spring, summer, fall and winter) and cryoprotectant (2 levels; glycerol vs. EG) were considered categorical fixed-effect explanatory variables and replicate was considered as a random factor. In one analysis, blastocyst conversion rate in control (unfrozen), vitrification and slow freezing groups were compared.

In a separate analysis, embryo development (cleavage rate and morulae rate) in four different seasons was compared. In the third set of analyses, effect of season was examined (separate analysis for control, slow-freezing and vitrification groups). Relationship between expansion of vitrified morulae and blastocyst development was analyzed by calculating Pearson's correlation co-efficient.

Last analysis was performed for Experiment 2 and compared the effect of different

cryoprotectants (EG versus Gly) on blastocyst conversion rate. The level of significant difference for main effects and interaction was set at $p \leq 0.05$ and post-hoc group comparisons of LSmeans were performed using Tukey's method.

Data were arranged in columns for Replicate, Treatment, Season, Outcome (each embryo was coded as 1=successful development, 0=no development) and following common SAS syntax model was followed with little modifications: Proc glimmix method=quad; class Replicate Treatment Season; model Outcome (event="1")=Treatment Season Treatment*Season/ dist=bin link=logit; random intercept/subject=Replicate; run. If the p value for Treatment or Treatment*Season was ≤0.05, then least square means were compared by adding lsmeans Treatment/diff lines ilink or adjust=tukey; or lsmeans Treatment*Season/diff lines ilink or adjust=tukey; respectively to the syntax.

4.4 Results

4.4.1 *Experiment 1: Development of in vitro produced bovine morulae to blastocyst stage following vitrification and slow freezing in different seasons.*

Blastocyst conversion rates for control (unfrozen), vitrification and slow freezing group are presented in Fig. 1. Blastocyst rate was the highest in control (78±3.6%) followed by vitrification (52±4.6%) and slow freezing (35±4.2%) groups ($p<0.001$). Only 72.7% of morulae re-expanded upon warming and out of these only 70.8% converted into blastocysts. No significant relationship was found between re-expansion upon warming of vitrified morulae and subsequent blastocyst rate (Pearson's correlation coefficient = -

0.048; $P > 0.05$).

The cleavage and morulae rates in different seasons are presented in Table 1. The cleavage (Day 2 post-IVF) and morulae (Day 6 post-IVF) rates were the lowest in the fall (67±1.6% and 22±1.4%; $p < 0.05$) season than other seasons (combined rates among 3 seasons: 73.7±1.1 and 30±1.2%, respectively). Blastocyst rate (%) in unfrozen control group was the highest in spring (85±3.3) as compared with summer (63±4.5) being the lowest ($p < 0.05$; Table 2). In fall and winter, the corresponding blastocyst rates were 67±6.7 and 67±5.2, respectively ($p > 0.05$). Blastocyst rate from vitrified embryos did not differ due to seasons ($p > 0.05$; Table 2). But, slow frozen embryos yielded the lowest blastocyst rate (Table 2) in summer (20±4.1) as compared with other seasons (43±8.3; $p < 0.05$).

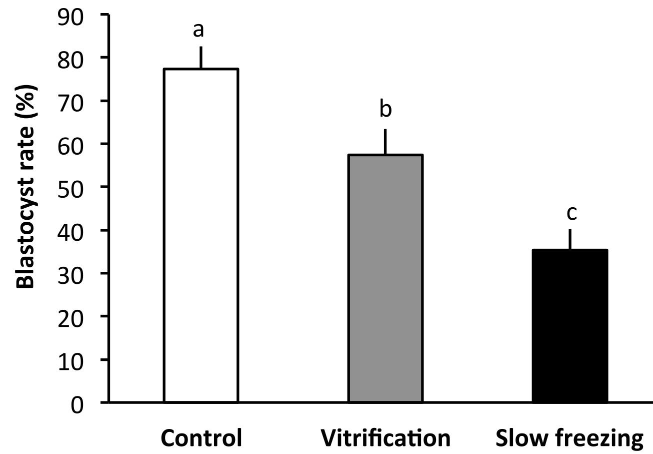

Figure 4.1 Effect of cryopreservation technique (vitrification vs. slow freezing) of bovine morulae (Day 2 post-IVF) on subsequent blastocyst formation (Day 8 post-IVF). Each bar represents mean±SEM.

a-cDifferent letters on the bars represent statistical difference ($p<0.05$).

Table 4.1. Effect of season on cleavage rate (Day 2 post-IVF) and morulae rate (Day 6 post-IVF) in *in vitro* produced bovine embryos (mean±SEM).

End point	Spring (1798/6)*	Summer (1602/8)	Fall (919/4)	Winter (783/4)	*p-value*
Cleavage rate**	$74±1.0^{a}$	$74±1.1^{a}$	$67±1.6^{b}$	$73±1.6^{a}$	*0.003*
Morulae rate***	$41±1.4^{a}$	$41±1.4^{a}$	$32±1.9^{b}$	$40±2.0^{ab}$	*0.011*
Morulae rate****	$30±1.1^{a}$	$30±1.1^{a}$	$22±1.4^{b}$	$30±1.6^{a}$	*<0.001*

*Value in parenthesis under each season represents the number of oocytes used/number of replicates

**Cleavage rate based on number of oocytes subjected to IVF

*** Morulae rate based on number of cleaved embryos

****Morulae rate based on number of oocytes subjected to IVF

^{a,b}Different superscripts within a row represent statistical difference ($p<0.05$).

Table 4.2. Effect of season on blastocyst rate (Day 8 post-IVF) in control, vitrified and slow frozen *in vitro* produced embryos (mean±SEM).

Treatment	Spring	Summer	Fall	Winter	*p-value*
Control	85±3.3^{a}* (102)**	63±4.5^{b} (56)	67±6.7^{ab} (54)	67±5.2^{ab} (37)	*0.015*
Vitrification	66±8.9^{a} (87)	39±10.6^{a} (92)	52±1.5^{a} (50)	71±13.4^{a} (42)	*0.187*
Slow freezing	49±6.8^{a} (60)	20±4.1^{b} (126)	42±8.5^{a} (53)	37±9.6^{a} (50)	*0.002*

*Blastocyst rate based on the number of morulae used

**Value in parenthesis represents the number of morulae used in 13 replicates

^{a,b}Different superscripts within a row represent statistical difference.

4.4.2 *Experiment 2: Effect of cryoprotectant (ethylene glycol and glycerol) on blastocyst development from frozen morulae using slow freezing*

Slow freezing of bovine morulae was tested using two different cryoprotectants; ethylene glycol and glycerol. Blastocyst conversion rate from morulae frozen in ethylene glycol (31±5.3) was numerically higher (Fig. 4.2) and had a tendency for a difference than glycerol group (18±4.3; p= 0.065).

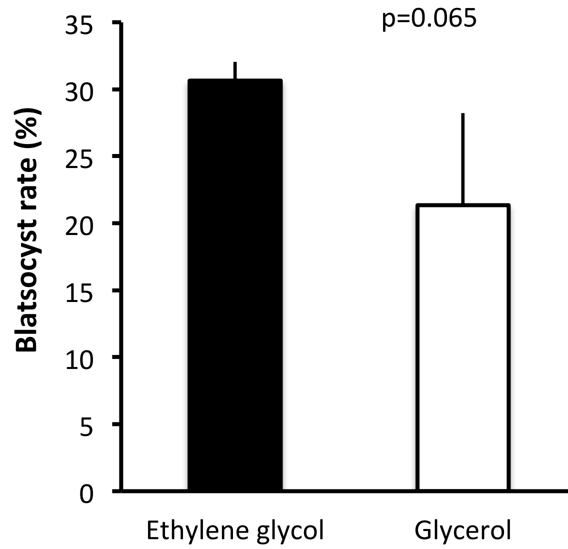

Figure 4.2. Effect of cryoprotectant in slow freezing of bovine morulae on subsequent blastocyst development. Each bar represents mean±SEM.

4.5 Discussion

In the present study, effects of two different techniques of cryopreservation and four seasons on survival and development of *in vitro* produced bovine morulae were tested. Results obtained in 13 replicates over a 3.5 years period using more than 500 embryos document that 55% of morulae developed to blastocysts after vitrification as compared to 35% after programmed slow freezing. However, cryopreserved embryos are more vulnerable and had less developmental potential than unfrozen control ones (78%). Results of our study also suggested that season of conducting IVF affected the developmental potential of bovine oocytes and embryos. The impact of season does not extend to the outcome of vitrified embryos; however, embryos produced from oocytes obtained in summer were the most sensitive to slow freezing. Also, we found ethylene glycol a better cryoprotectant than glycerol for slow freezing of IVP bovine morulae.

Vitrification is a hands-on technique and does not require a sophisticated programmable cell freezer. Therefore, it is easy to perform yet it requires technical skills. Slow freezing, although can be performed with less technical skill and training, is more expensive as it requires programmable cell freezer, takes longer time to perform and is less convenient for on-sight farm use. Ideally, slow freezing includes establishment of delicate balance between cryoprotectant equilibration and cooling of embryos, to avoid intracellular freezing (Vajta and Kuwayama 2006). Intracellular ice formation is immensely dependent on the rate of cooling applied, as it determines the speed of cell shrinkage (Mazur 1963). Hence, freezability of cells is reliant on permeability of cell membrane and cell-surface volume ratio (Mazur 1963; Mazur 1970). Despite all efforts, slow freezing is known to cause intra-cellular ice crystallization (Vajta and Nagy 2006) that eventually leads to cell damage and embryonic death. Contrarily, vitrification

involves complete elimination of cellular freezing by cell volume shrinkage with high cryoprotectant concentration, ulta-rapid cooling and glass-phase formation at -196°C instead of ice formation. Also, vitrification appears to give better embryo survival rates than the slow freezing (Liebermann and Tucker 2004; Lin *et al.* 2010; Li *et al.* 2012; Zander-Fox *et al.* 2013). Mouse (Vutyavanich *et al.* 2009; Zander-Fox *et al.* 2013), rabbit (Naik *et al.* 2005) and humans (Son and Tan 2009), embryos frozen with vitrification yielded higher blastocyst and pregnancy rate than slow freezing. Despite all advantages, vitrification exposes embryos to toxic concentration of cryoprotectants and may also damage cell organelles resulting in poor quality embryos in horses (Hendriks *et al.* 2014). Conversely, other reports suggested slow freezing resulted in a higher proportion of fragmented equine embryos than vitrification (Hendriks *et al.* 2014). DNA integrity of slow-frozen mouse embryos was however, affected in one (Li *et al.* 2012) but not in another (Kader *et al.* 2010) study. A combination of these factors likely resulted in better embryo survival rates after vitrification than slow freezing of in vitro produced bovine embryos in our study and other species (Kuleshova and Lopata 2002; Balaban *et al.* 2008; Saragusty and Arav 2011). In contrast to morulae vitrification in our study, one report suggested lower blastocyst-hatching rates after vitrification of expanded bovine blastocysts than slow frozen ones (Nicacio *et al.* 2012). It is apparent from above discussion that freezability and survival of embryos using any technique is also, species, embryo stage and origin (*in vivo* or *in vitro*) specific (Massip 2001; Vajta and Nagy 2006).

Exposure of embryos to high cryoprotectant concentration in vitrification leads to shrinkage of cell volume, due to loss of water. Upon warming, embryos are exposed to

diluting solution that results in removal of cryoprotectants and re-expansion of embryos to their original size. Re-expansion of vitrified bovine blastocysts to attain their original size is utilized as a criterion for their survivability and developmental potential (Kaidi *et al.* 2000; Aksu *et al.* 2012). Our study, on the other hand, showed that re-expansion of vitrified morulae upon warming is not a good indicator of embryo viability and subsequent blastocyst formation. Such contradictory results might be due to embryonic stage vitrified in previous studies, i.e. blastocyst vs that used in our study, i.e. morulae.

Thermal and nutritional stress during summer season contributes to seasonal infertility in many cattle breeds (Roth *et al.* 2000; De Rensis and Scaramuzzi 2003). However, to the best of our knowledge, no study has been conducted to test the cryo-survivability of IVP embryos in different seasons in cattle. Oocytes obtained from heat stressed cows had reduced developmental competence as well as altered gene expression patterns during oocyte maturation and early embryonic development (De Rensis and Scaramuzzi 2003; Gendelman and Roth 2012). A seasonal study suggested that heat damage extended to a subset of ovarian oocyte pool and recovery of oocyte competence took a period of up to 2-3 estrous cycles (Roth *et al.* 2001). Some reports suggested that summer heat stress might impair pre-ovulatory follicular function as well as oocyte quality and early embryo development in the fall season due to carry-over effects from summer (Roth *et al.* 2000; Roth *et al.* 2001; Gendelman *et al.* 2010). Delayed cleavage of fertilized oocytes obtained from cows during hot season (May-November) have been reported (Gendelman *et al.* 2010) and embryo transfer from non-heat stressed super-ovulated cows appear to improve the conception rates in lactating heat-stressed dairy cows (Drost *et al.* 1999). Our results indicated season has a strong impact on early

embryo development after IVF, i.e. cleavage rate and morula rate. Fall season had the lowest cleavage and morula rate than any other season. Also, blastocyst conversion rates from unfrozen control and slow frozen morulae were affected by the season of their production. However, it is noteworthy that our study was conducted in Canada where milder summer temperatures are less likely to cause “summer heat stress”; rather, summer is associated with postpartum period, calf rearing, pasture grazing and breeding in beef cows. Remarkably, similar seasonal trends have been found in human IVF, with highest cleavage rate and morphological embryo quality in spring, average in winter and summer and lowest in autumn (Rojansky *et al.* 2000).

Summer stress not only affects the maturation of oocytes but also the maternal pool of transcripts in early embryos. For example, bovine embryos produced in hot season have decreased expression of *POU5F1* gene (Gendelman *et al.* 2010), which is essential for determining the cell lineages during blastocyst formation (Goissis and Cibelli 2014). This might be the reason for less potential of embryos to convert to blastocysts. Our results are in conjunction with the above-mentioned study as blastocyst conversion rate in unfrozen IVP embryos was the lowest in summer season as compared to other seasons. In support of this, in a seasonal study blastocyst formation was lower in a hot season (May-November) than a cold (December to April) one (Gendelman *et al.* 2010). Interestingly, season of conducting IVF appears to have a significant impact on blastocyst rates in seasonal breeders like sheep as well (Mara *et al.* 2014). However, not many studies have been conducted over the impact of season of embryo production on their post-cryopreservation survival. In our study, morulae frozen in summer using slow freezing yielded lowest blastocyst conversion rate than other seasons.

Our findings in slow freezing group are contrary to a report, in which season of embryo freezing did not affect pregnancy rates in cattle (Hasler 2001). Similar to this later study, seasonal analysis in our study shows vitrification technique is independent of the source and quality of embryos and reaffirms its uniformity and superiority to slow freezing. More importantly, our study provides useful insight for "out-of-season" or "non-breeding" season *in vitro* embryo production and cryopreservation in other species.

Lastly, our study suggested a trend towards better survivability of bovine morulae after slow freezing using ethylene glycol as a permeating cryoprotectant than glycerol. Use of glycerol utilizes either a 3-step, 2-step or a single step cryoprotectant dilution procedure before embryos can be transferred into animals (Prather *et al.* 1987). Ethylene glycol is a more permeable and less toxic cryoprotectant (Kasai 1994; Massip 2001) than glycerol (Jin *et al.* 2011) and allows direct transfer of bovine embryos into animals (Voelkel and Hu 1992a). Also, direct transfer of embryos eliminates the need for on-site technicians. The outcome of embryo freezing using these agents is controversial. Post-warming viability of embryos frozen in EG was significantly higher than glycerol (Voelkel and Hu 1992b). In another study, blastocyst survival rates were better with glycerol (Hasler *et al.* 1997). Similar to our study, glycerol yielded lower embryo development rates in pigs than ethylene glycol especially in higher concentrations (Weber and Youngs 1994). *In vitro* produced (*in vitro* matured, fertilized and cultured) embryos are more sensitive to freezing than *in vivo* produced embryos (Leibo 1993). This could be a possible reason of a low embryo survival following cryopreservation in our study.

In conclusion, the study demonstrated the superiority of vitrification procedure over

programmed slow freezing for cryopreservation of in vitro produced bovine morulae. In contrast to results of other studies on bovine blastocysts, our study showed that re-expansion of vitrified in vitro produced bovine morulae upon warming is not a good indicator of embryo viability and subsequent blastocyst formation. In future, it will be interesting to test the viability of both freezing techniques on organelle (electron microscopy), cellular (immunohistochemistry) and molecular (microarrays, next-generation sequencing, qPCR) levels. Winter and spring were better seasons to conduct IVF in cattle. Season of conducting IVF for bovine embryo production should be considered in experimental designs. Furthermore, impact of season of embryo cryopreservation in seasonal breeders needs further attention and can be investigated. Finally, slow freezing using ethylene glycol tended to provide marginally better morulae to blastocyst conversion rate than glycerol.

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CHAPTER 5 **Cryopreservation affects global gene expression of *in vitro* produced bovine embryos**

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5.1 Abstract

An experiment was planned to compare the transcriptomic changes in bovine embryos cryopreserved using vitrification and slow freezing compared to unfrozen control group. Compact morula-stage *in vitro* produced bovine embryos were cryopreserved using the two techniques and allowed to develop to expanded blastocyst stage post-warming (n=7 replicates). Morula to blastocysts was higher (P<0.05) in control (72%) and vitrified (77%) embryos compared to slow frozen (34%) embryos. RNA was extracted from expanded blastocyst staged embryos and microarray based analysis of differential gene expression was done using dye-swap method (n=4 biological replicates) to compare vitrification and slow freezing with unfrozen embryos (reference group). An *in silico* gene expression comparison was conducted among vitrification and slow freezing (reference) groups. A total of 20 genes were upregulated and 44 genes were downregulated in vitrified embryos as compared to unfrozen embryos (Flexarray analysis; fold change ≥ ±2, P<0.05). In slow freezing treated embryos no genes were differentially expressed at fold change ≥ ±2, but 102 genes were upregulated and 63 genes were downregulated at a at fold change ≥ ±1.5 (P<0.05) in comparison with unfrozen embryos. Six differentially expressed genes (CYP11A1, TKT, PLAU, KRT19,

CLDN23 and AKR1B1) in vitrified embryos and one gene (SPP1) in slow freezing treated embryos were used by real-time PCR to confirm the microarray results. Vitrified embryos exhibit gene expression changes mainly involved in embryo implantation (PTGS2, CALB1), lipid peroxidation and ROS generation (HSD3β1, AKR1B1, APOA1) and blastocyst development especially cell differentiation (KRT19, CLDN23). The slow frozen embryos, however, showed lower but significant changes in the expression of genes related to cell signaling (SPP1), cell structure and differentiation (DCLK2, JAM2 and VIM) and lipid metabolism (PLA2R1 and SMPD3). Differential gene expression changes in the vitrified embryos as compared to slow freezing treated embryos were similar to those found in comparison with unfrozen (reference) embryos. In conclusion, although the vitrified embryos provide better embryo survival rates post-warming than slow freezing treated bovine embryos, their further development beyond blastocyst stage might be affected, based on changes in gene expression related to lipid metabolism, steroidogenesis (CYP11A1, HSD3β1), cell differentiation (CLDN23, ACTG2) and placentation (PTGS2). According to our study, although slow frozen embryos yielded lower embryo survival rates, they have the better quality to grow better at later stages.

Keywords

Bovine embryo, cryopreservation, CYP11A1, global gene expression, implantation, *in vitro* embryo culture, microarray, morula, slow freezing, vitrification.

5.2 Introduction

The International Embryo Transfer Society (IETS) reported the production of 517,857 embryos by superovulation and 29,041 *in vitro* produced (IVP) bovine embryos in 2014 (IETS, 2014). Cryopreservation of bovine embryos is a widely used method for

the trade of genetically superior animals and conservation of genetic diversity in beef and dairy cattle. According to the American Embryo Transfer Association report (AETA 2013), 13,409 dairy and 5,111 beef cattle embryos were transported across the world. Likewise, 32,274 pregnancies were established using frozen-thawed bovine embryos across Canada (Canadian Embryo Transfer Association, 2013).

Two most commonly used embryo cryopreservation techniques are 1) equilibrium, slow or programmed freezing, and 2) non-equilibrium freezing or vitrification (Leibo 2008). These techniques are based on thermodynamic and osmotic principles and the cell freezing is achieved by using a high concentration of cell permeating or non-permeating cryoprotectants and a certain cooling rate (Leibo and Pool 2011). Cryopreservation is a radical procedure and has deleterious developmental, morphological and biochemical after-effects on oocytes and embryos (Wilmut 1972; Wilmut and Rowson 1973; Pollard and Leibo 1994). Nevertheless, both techniques aim to avoid damage to cellular health of morulae/blastocysts caused by intracellular ice formation (Leibo 2008; Leibo and Pool 2011); however, it is generally believed that cells undergoing vitrification are less prone to ice crystal formation as compared to slow freezing (Vajta and Nagy 2006; Anzar *et al.* 2014). Most of the post-cryopreservation developmental studies have documented improved or equivalent bovine embryo survival (Nedambale *et al.* 2004a), blastocyst hatching (Nedambale *et al.* 2004b) and pregnancy rates (van Wagtendonk-de Leeuw *et al.* 1997; Vajta and Nagy 2006) after vitrification than slow freezing. Morphologically, however, both vitrification and slow freezing can cause intracellular/extracellular fractures in freezing planes (Leibo *et al.* 1978; Lehn-Jensen and Rall 1983), acute shrinkage in cell volume in bovine embryos (Lehn-Jensen and Rall 1983; Leibo 2008)

and organelle damage and dislocation (Popelkova *et al.* 2005) in rabbit embryos and oocytes. TUNEL-based assays on embryos showed low DNA-integrity indices after slow freezing and vitrification in mouse, human and bovine species (Kader *et al.* 2010; Morato *et al.* 2010; Li *et al.* 2012). Other studies reported skewed inner cell mass to trophoblast ratio and localization of reactive oxygen species in mitochondria after vitrification (Gomez *et al.* 2008a; Martino *et al.* 2013).

Many studies have been conducted to identify gene expression related to different developmental stages of bovine oocytes and pre-implantation embryos and their cryopreservation (Vigneault *et al.* 2004; Yao *et al.* 2004; Rekik *et al.* 2011; Robert *et al.* 2011); (Aksu *et al.* 2012). These and similar studies provide an overview of changes occurring in oocytes, granulosa cells and pre-implantation embryos during extreme stress conditions such as heat shock (Gendelman *et al.* 2010; Gendelman and Roth 2012), hormonal changes (Carter *et al.* 2010; Gilbert *et al.* 2012; Dias *et al.* 2013; Dias *et al.* 2014) and laboratory handling (Plourde *et al.* 2012). For example, vitrification of mouse embryos (Dhali *et al.* 2007) exhibited differential apoptotic and DNA methylation gene expression using quantitative-real time PCR (qRT-PCR). In a TUNEL based analysis, mouse embryos showed significant decrease in DNA integrity index after vitrification, but slow frozen embryos did not show any damage (Kader *et al.* 2010). On the contrary, bovine embryos cryopreserved by slow freezing revealed higher pro-apoptotic gene expression by real time PCR as compared to vitrification (Stinshoff *et al.* 2011). These studies have examined the individual genes in isolation. In order to fully understand the effects of vitrification and controlled slow freezing on cellular and molecular pathways, there is a need to compare the global gene expression in *in vitro* produced bovine

embryos. Such subtle but cumulative changes may influence the embryo development at a morphological level and may have long-term effects. Therefore, our study aimed to study the effect of cryopreservation on *in vitro* produced (IVP) bovine morulae using a microarray- based comparison of global gene expression changes. We compared the gene expression in unfrozen (control) IVP embryos with two cryopreservation techniques; vitrification and slow freezing as well as between the two freezing protocols. The study allowed to examine if similar or diverse cellular pathways and mechanisms are involved in embryo survival or failure after these two cryopreservation treatments and will provide insights to overcome such alterations by modifying the culture conditions, freezing procedure or thawing/post-thawing environment.

5.3 Material and Methods

5.3.1 *Cumulus oocyte complex (COCs) collection*

Beef cattle ovaries were collected at a commercial abattoir (Cargill®, Calgary) and were transported at 20-25°C to Saskatoon within 12-18 h. Ovaries were trimmed of any extra tissue and washed with normal saline at room temperature. Follicular fluid containing cumulus oocyte complexes (COC) was aspirated from <4mm ovarian follicles using an 18-gauge needle attached to a 5 ml syringe and pooled among ovaries for further processing.

5.3.2 *In vitro embryo (morulae) production*

The follicular fluid was subjected to COC searching under a stereomicroscope. COC were placed and washed in holding solution (HS; 5% CS in 1X DPBS). COC grading was done as described (de Loos *et al.* 1989) and only first and second grade

oocytes were used for the study. COC were washed 3X in maturation media [TCM-199 supplemented with 5% CS, LH (5µg/ml), FSH (0.5µg/ml) and gentamicin (0.05 µg/ml)] and matured *in vitro* for 22-24 h at 37°C in high humidity and 5% CO_2 (~20 oocytes/100µl drop under mineral oil).

For *in vitro* fertilization (IVF) [Day 0 (d0)= IVF], *in vitro* matured (IVM) COC were incubated in Brackett-Oliphant (BO) fertilization medium (Brackett and Oliphant 1975) [BO stock A + BO stock B + sodium pyruvate (1.3% w/v) + gentamicin (0.05 µg/ml)] with frozen-thawed semen from a fertile healthy dairy bull ($3X10^6$/ml final concentration) for 18-22 h at 37°C in 5% CO_2. The zygotes were washed and transferred to *in vitro* culture (IVC) medium (Rosenkrans *et al.* 1993) [CR1+ essential amino acids+ 5% CS] at 37°C in humidified 5% CO_2, 5% O_2 and 98.5% N_2 for further development to morula stage embryos. On d7 post-IVF, compacted morulae were collected, washed in HS, graded (as per IETS, 1998) and divided into three treatment groups; control, vitrification and slow freezing.

5.3.3 *Cryopreservation of morula-*

5.3.3.1 Control

Morulae assigned to control group were transferred back to IVC medium and cultured for 24-48 h.

5.3.3.2 Vitrification

Vitrification was done as described earlier (Prentice *et al.* 2011). Briefly, morulae were washed in HS and were equilibrated in vitrification solution 1 [VS1; 7.5% Ethylene

glycol (EG, v/v) + 7.5% dimethyl sulfoxide (DMSO, v/v) + 20% CS (v/v) in 1X DPBS] for 5 min at room temperature. Morulae (n=3 to 4 in a given batch) were then transferred through three 20 μl droplets of vitrification solution 2 [VS2; 15% EG + 15% DMSO + 20% CS + 17.1% sucrose (w/v) in 1X DPBS] at 37°C within 1 min., placed on cryotop (Kitazato® Co., Fuzi, Shizuoka, Japan) with minimal amount of medium and immediately plunged in liquid N_2. Morulae were stored in liquid N_2 for at least 24 h before warming.

5.3.3.3 Slow freezing

Slow freezing was done as described (Carvalho *et al.* 1996). Briefly, morulae were washed and incubated in cryoprotectant freezing solution [CPF (constituted in lab); 1.5 M glycerol + 5 % CS (v/v) in 1X DPBS] for 10 min at room temperature. Embryos were transferred to 0.25 ml plastic straws (IMV® Tech., Woodstock, ON, Canada), sealed and kept for 5 min in the methanol bath of the controlled rate freezer (Bio-Cool® III-80, FTS systems, SP Industries, Inc., StoneRidge, NY, USA) already set at -7°C. Ice seeding was done using a liquid N_2 dipped Q-tip™ and embryos were again incubated for 10 min at -7°C. They were cooled to -35°C at a rate of 0.5°C/min and straws were plunged directly into liquid N_2. Morulae were stored for 24 h in liquid N_2 before thawing.

5.3.3.4 Warming/thawing

Vitrified morulae were transferred from cryotop to warming solution [0.5M sucrose (w/v) + 20% CS (v/v) in 1X DPBS] and incubated for 5 min at 37°C. Embryos were washed with HS and were incubated in IVC medium for 24-48 h. Similarly, 0.25 ml plastic straws containing slow-frozen embryos were thawed in air for 10s and in water bath at 37°C for 1 min. The embryos were transferred to thawing solution [0.7 M

sucrose+5% CS (v/v) in 1X DPBS] and incubated for 5 min at 37°C. Embryos were then washed with HS and cultured for 24-48 h.

5.3.3.5 Blastocyst collection

Recovered morulae from each treatment group were cultured for 24-48 h to expanded blastocyst stage and examined for quality. Blastocyst conversion rates were calculated for each treatment group (control, vitrification and slow freezing). The statistical analyses were performed using link-function for binary distribution (yes/no response variable). Three treatments (unfrozen control, vitrification, slow-freezing) were considered categorical fixed-effect explanatory variables and replicate was considered as a random factor. In one analysis, the blastocyst rate in unfrozen control, vitrification and slow freezing groups was compared. The data were analyzed using Proc Glimmix in SAS® Enterprise Guide 4.3 (SAS). Data were arranged in columns for replicate, treatment, outcome (each embryo was coded as 1=successful development, 0=no development). Following common SAS syntax model was used: Proc glimmix method=quad; class= replicate treatment; model outcome (event="1")=treatment/ dist=bin link=logit; random intercept/subject=Replicate; run. If the p-value for treatment was ≤0.05, then least square means were compared using Tukey's test.

Blastocysts from each treatment group (Control, vitrification and slow freezing, as described above), per replicate, were collected in groups of 5 to 7 blastocysts in 50-100 µl RNAse-free water in cryo-tubes (RNase-free; Neptune® cryovials, 0.5 ml tubes). Only those embryos were used that were successful to transition from morula to expanded blastocyst (Figure. Supplementary 1). These tubes were flash frozen by plunging into

liquid N_2 and were shipped to Dép. des Sciences Animales, Université Laval, Quebec city, QC for further processing. A total of five IVF/IVC/cryopreservation cycles (i.e., biological replicates) were conducted on separate dates. Four (of the 5) biological replicates were used for Microarray experiment (i.e., one cryo-tube per group per IVC cycle). Three biological replicates were used for qRT-PCR analysis. Two IVC cycles were common between microarray analysis and qRT-PCR, however, separate tubes were created (i.e., separate set of blastocysts were used) for the two analyses.

5.3.3.6 RNA extraction, quality analysis and amplification

All procedures for microarray experiment were conducted according to protocols described previously (Cagnone *et al.* 2012; Dias *et al.* 2013) with little modifications.

Total RNA was extracted from embryos using Arcturus Picopure® RNA isolation kit (Cat#KIT0204, Life Technologies, Burlington, ON, Canada), with all the setup and protocol for safe RNase free extraction. Briefly, embryos were subjected to DNAse I (Cat#79254, Qiagen® Inc., Toronto, ON, Canada) digestion, samples were mixed with extraction buffer and incubated at 42°C for 30 min. Samples were mixed with 70% ethanol and added to pre-conditioned columns. Total RNA was eluted in 13µl of elution buffer, 1.5µl of eluted RNA was used to analyze the quality using Agilent 2100 Bioanalyzer™ and Agilent RNA 6000® pico kit (Cat# 5067-1513, Agilent technologies, Santa Clara, CA) and the remaining was stored in -80°C freezer until further processing. High quality RNA samples with RNA integrity number (RIN) more than 7.0 were amplified using T7 RNA amplification procedure using RiboAmp® HS^{Plus} RNA

Amplification Kit (Cat# KIT0525, Life™ technologies, Burlington, ON, Canada) and used for microarray hybridization.

5.3.3.7 Microarray hybridization and scanning

Amplified antisense RNA (aRNA) samples from four biological replicates per treatment group (3 treatment groups: control, slow freezing and vitrification; n=12 samples) were labeled with ULS™ system based fluorescent labeling kit (Cat# EA-021, Kreatech® Diagnostics, Amsterdam, The Netherlands), according to manufacturer's protocol. Control group was used as the reference for other treatment groups, therefore, 4µg amplified RNA from each control group sample was labeled while for slow freezing and vitrification groups, 2.5µg amplified RNA was labeled with Cy-3 or Cy-5 dye (ULS™ Fluorescent Labeling Kit for Agilent® arrays; Kreatech® #EA-021). Labeling efficiency for both dyes was recorded with ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) with a minimum of 30 pmol/µg (dye concentration/aRNA concentration) for each sample and the non-reacted residual labeling dye was filtered out using another round of RNA isolation kit (Cat#KIT0204, Life™ Technologies, Burlington, ON, Canada) without DNAse treatment.

Two Bovine embryo and splice transcriptome Agilent® microarray slides (BESTv1-4X44K format, GEO Accession #: GPL13226, #Agilent-028298, Agilent® technologies; (Robert *et al.* 2011) were used. Each slide had 4 arrays and each array contained 43, 671 (genes and isoforms excluding control) probes. One slide was used to perform simple contrast design in full dye swap to compare control (reference) versus vitrification groups and the second one to compare control (reference) versus slow-freezing group. On

each array, 825 ng of Cy-3 or Cy-5 labeled aRNA from control group was hybridized with 825 ng of oppositely (i.e., Cy-5 or Cy-3, respectively) labeled aRNA from treatment group (vitrification or slow freezing) using dye-swap design (n=2 such dye-swap comparisons for control vs treatment group; four biological replicates for each control vs treatment comparison). Slides were incubated for 17 h at 65°C in a pre-heated hybridization oven, washed with wash buffers, dried and scanned using Tecan PowerScanner™ (Tecan Group Ltd., Mannedorf, Switzerland) (Robert *et al.* 2011). Comparison of vitrification and slow freezing group was done *in silico* by extracting the median background and spot intensities from the Slide 1 or 2 (Supplementary figure 2).

5.3.3.8 Data normalization and statistical analysis

Data normalization and analysis was conducted as described (Dias *et al.* 2013). After laser scanning the slides, image files and median signal intensities from each spot were obtained using Array-Pro™ software (Media Cybernetics Inc., Rockville, MD, USA). The gene-spot intensity file was uploaded in MIAME-compliant ELMA (EmbryoGENE Laboratory Information Management System and Microarray analysis) (Grant *et al.* 2014) portal and validation analyses of linearity, specificity and variability of microarray hybridization were done using in-built Gydle™ software (http://www.gydle.com). The background and spot median intensities were uploaded and analyzed in Flexarray® software Version 1.6.3 (Blazejczyk, 2007). For background normalization, background signal intensity was subtracted from median grayscale signal intensity of spots to obtain required correct signal intensity. In case of higher background intensity for a spot than the signal intensity, negative value was replaced with 0.5 as a default (false spots). The median value for each target was transformed to the $\log_2$ value

and normalized “within array” for dye bias using non-parametric regression (locally weighted scatter plot smoothing “lowess”), and subjected to between array normalization to unify intensities across the arrays using Quantile normalization methodology (GEO #: GSE45381), respectively (Bolstad *et al.* 2003). Linear Models for Microarray and RNA-Seq Data (LIMMA) simple statistical analyses were done in Flexarray® and lists of up-regulated and down-regulated genes were obtained for each comparison (Wettenhall and Smyth 2004) (*i.e.* control (reference group) vs vitrification; control (reference group) vs slow freezing). Statistically adjusted signal intensity data for vitrification and slow frozen was derived from the previously acquired data for control-vitrification and control-slow freezing groups and an *in silico* Flexarray analysis was then conducted between vitrification and slow freezing (reference) group. A gene was considered differentially expressed if the fold change was ≥ ±2 with a p-value of <0.05. As no genes were detected as differentially expressed at a fold change of ±2 in the control vs slow freezing comparisons, additional analysis was performed by lowering the fold change threshold to ≥ ±1.5.

5.3.3.9 Ingenuity based functional analysis

The normalized and differential expression data from FlexArray® were uploaded and analyzed in Ingenuity Pathway analysis™ software. Differential gene lists from control vs vitrification (≥ ±2-fold change and p-value<0.05); control vs slow freezing (≥ ±1.5 fold change, p-value<0.05) and vitrification vs slow freezing (≥ ±2 fold change, p-value<0.05) were compared for functional analysis to obtain molecular, cellular and functional correlations.

5.3.3.10 Quantitative real time PCR (qPCR) analysis-

Blastocyst, total RNA and cDNA production

Total RNA extraction from the three biological replicates (each biological replicate included control, slow freezing and vitrification group; 5-7 expanded blastocysts per sample) was done using Arcturus Picopure® RNA isolation kit (Cat#KIT0204, Life Technologies, Burlington, ON, Canada) and quality was tested using Agilent 2100 Bioanalyzer™ (Agilent technologies, Santa Clara, CA) as described above. No amplification was performed and the extracted RNA was reverse transcribed to cDNA using qScript™ cDNA supermix (Cat#95048-100, Quanta biosciences, Inc., MD, USA) following kit instructions. Total cDNA quantity was measured using Nanodrop and stored in -80º C until further use.

In addition, total RNA and cDNA were obtained from extra samples of pooled IVP bovine expanded blastocysts for primer optimization and standard curve generation.

Gene selection, primer designing and optimization

A total of 7 genes (CYP11A1, KRT19, PLAU, CLDN23, AKR1B1, TKTL1 and SPP1) and one housekeeping gene (CHUK) (Cagnone *et al.*, 2012) were selected for performing qPCR analysis. Primers were designed 29-September, 2014 and 27-November, 2014 using Primer 3® (http://frodo.wi.mit.edu/primer3/) (Bos Taurus, genome sequence 4.6.1) and Clone Manager® basic version 9.4 (http://www.scied.com/pr_cmbas.htm) softwares. Primer analysis was done using IDT PrimeQuest Oligo Analyzer website (http://scitools.idtdna.com/analyzer/Applications/OligoAnalyzer/) and NCBI blast

(http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to confirm gene and species specificity of primers. A selected primer met these essential criteria: 40-60% GC content and no hairpin, self-dimer or heterodimer formation. Primer testing and optimization was done using end-point PCR implying *Taq* DNA polymerase (Cat#201203, Qiagen, Inc., Toronto, ON, Canada) kit. The list of selected genes and primers is presented in Table 5.1.

Bovine expanded blastocyst cDNA samples (other than those required for the study) were used to amplify the PCR products. These specific products were visualized using 1% agarose gel electrophoresis. The product size of each product and any undue amplification were confirmed. The bands of gel containing products were cut and eluted by using QIAquick Gel Extraction kit (Cat# 28704, Qiagen, Toronto, ON, Canada) and cDNA content was measured using Nanodrop, ND-100. DNA sequencing of each amplified gene product was done to further test gene specificity. The amplified cDNA of each gene was diluted (10^{-2} to 10^{-11}) to develop standard curve for qPCR measurements. To optimize the use of cDNA in real time PCR, cDNA equivalent to 0.02 embryos was used from each treatment group per replicate.

Quantitative real time PCR was done on Stratagene® Mx3005P fast thermal cycler (Agilent technologies, Santa Clara, CA) and QuantiFast® SYBR® green PCR kit (Qiagen®, Toronto, ON, Canada).

Statistical Analyses

Cycle threshold (C_T) values were recorded for each selected gene for every treatment group. These C_T values were used to calculate differential expression in treated embryos in comparison with the control group. At first, PCR efficiency was calculated

using standard curve data for each gene and software used pair-wise fixed reallocation randomization test using Relative Expression Software Tool (REST® 2009, Qiagen®) (Pfaffl *et al.* 2002). PCR efficiency equal to 1 was considered optimum for each gene.

The relative levels of a transcript for each treatment group were calculated as Cycle threshold (C_T) normalized separately (ΔC_T) for levels of transcripts for one "house-keeping" gene – Conserved Helix-Loop-Helix Ubiquitous Kinase (CHUK). A lower C_T (or Δ C_T) of "1" indicates approximately a two-fold higher concentration of RNA. The significance of differences of mean values of ΔC_T between treated and control embryos were determined using a student T-test in Microsoft® excel.

Table 5.1 List of genes for qPCR analysis and their respective primers sequences.

Gene	*Strand (5'-3')*	*Primer sequence*	*Tm (ºC)*
AKR1B1	Forward	CCAACCACATCGTGCTCTAC	55
	Reverse	CCCACCTCGTTCTCATTCTG	55
CLDN23	Forward	AAACACCTGGCTCGGAGTC	55
	Reverse	AGGGCCTTGATTCCTCTGG	55
CYP11A1	Forward	ATCCAGTGTCTCAGGACTTCGT	61
	Reverse	GAACATCTTGTAGACGGCATCA	61
KRT19	Forward	GAGGAGCTGAACAGGGAGGT	61
	Reverse	CTGGGCTTCGATACCACTGA	61
PLAU	Forward	GCTGGTGTTCTGTGTCTG	55
	Reverse	GGTCGGAAGGGATAACTG	55
SPP1	Forward	ATT GTG GCT TAC GGA CTG	54
	Reverse	TTG GCG TGA GTT CTT TGG	54
TKT	Forward	ACAAGCCAAGGTGGTCCTGAAGAA	62
	Reverse	TAGCACGGGCACTGTCAAGAATGA	62
CHUK	Forward	TGATGGAATCTCTGGAACAGCG	56
	Reverse	TGCTTACAGCCCAACAACTTGC	56

5.4 Results

5.4.1 *Morula to blastocyst development rate*

The blastocyst development rate did not differ between vitrification (77.3±4.5) and control unfrozen (72.4±9.8) groups ($p>0.05$). The slow freezing (34.4±9.2) group had the lowest embryo survival rates among the three treatment groups ($p<0.05$) (Figure 5.1).

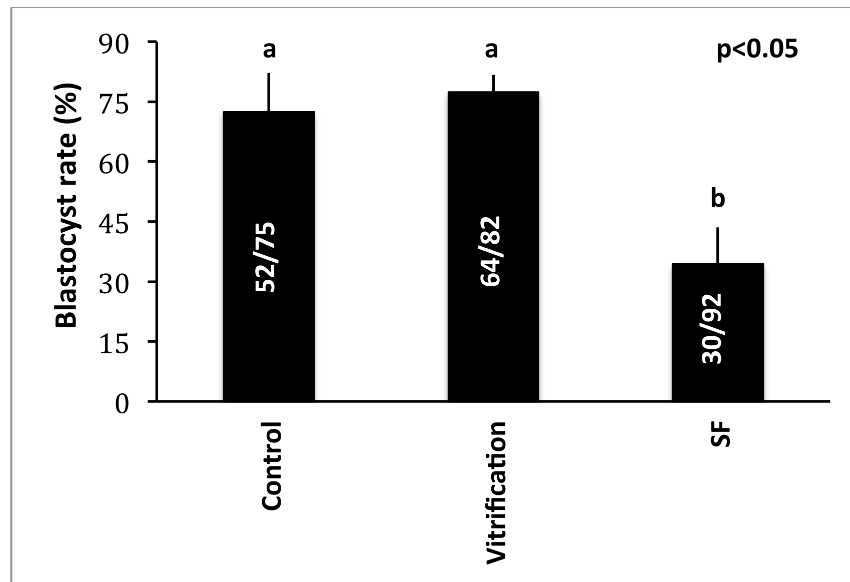

Fig. 5.1 Blastocyst development rate of vitrified and slow frozen cryopreserved IVP bovine morulae as compared to unfrozen control group. Bars represents mean±SEM (Percent number of expanded blastocyst/number of morulae used per group) from five replicates. Different superscripts[a,b] represent statistical difference ($p<0.05$) calculated using Tukey-Kramer test.

5.4.2 ***Differential gene expression profile***

Using Flexarray® software, a total of 64 differentially expressed genes were found between the control and vitrified embryos (fold change ≥ 2; p-value< 0.05) (Table 5.2). A total of 1 and 165 genes were differentially expressed between control and slow frozen embryos (Fold change ≥ 2 and ≥ 1.5, respectively; p-value<0.05) (Table 5.2). An *in silico* Flexarray® between vitrification and slow freezing (reference) group, detected that 75 genes were differentially regulated (Table 5.2) at fold change of ≥ 2. The list of top 5 up and down-regulated genes from Flexarray® software analysis is presented in Table 5.3.

Table 5.2. Upregulated and downregulated (p<0.05) trasncripts in different embryo treatment groups versus (vs) reference groups. Transcripts with known functions and novel transcripts are listed separately.

Treatments	Fold change	Upregulated genes		Downregulated genes	
		Known	Novel	Known	Novel
Vitrified vs control	≥±2	7	13	33	11
Slow frozen vs control	≥±2	0	0	1	0
Slow frozen vs control	≥±1.5	35	67	49	14
Vitrified vs slow frozen	≥±2	10	15	30	20

Table 5.3 Top five up and downregulated gene transcripts detected by Flexarray® analysis between Vitrified versus unfrozen (Reference group) embryos (A); Slow frozen versus unfrozen (Reference group) embryos (B); and Vitrified versus slow frozen(Reference group) embryos (C).

Genes	*Description*	*Fold change*	*p-value*
A. Vitrification versus control			
WBP5	WW binding protein 5	2.148	$1.49 X10^{-05}$
TKTL1	Transketolase 1	2.119	$8.63 X10^{-04}$
HS3ST5	Heparan sulpfate (glucosamine)-O-sulphotransferase 5	2.113	$6.68 X10^{-04}$
TRIM64/TRIM64B	Tripartite motif 64-B	2.100	$1.33 X10^{-05}$
COL9A2	Collagen, type IX alpha 2	2.078	$5.82 X10^{-05}$
CYP11A1	Cytochrome P450 family 11 subfamily A polypeptide 1	-3.971	$3.43X10^{-04}$
CCL17	Chemokine (C-C motif) ligand 1	-2.946	$4.39 X10^{-04}$
FADS2	Fatty acid desaturase 2	-2.897	$1.54 X10^{-04}$
HEBP2	Heme binding protein 2	-2.817	$5.48 X10^{-05}$
KRT19	Keratin 19	-2.784	$2.36 X10^{-03}$
B. Slow freezing versus control			
PLA2R1	Phospholipase A 2 recepetor-1, 180 kDa	1.983	$3.99 X10^{-03}$
DCLK2	Doublecortin-like kinase-2	1.829	$2.56 X10^{-02}$
FBXO32	F-box protein 32	1.774	$8.90 X10^{-04}$
SMPD3	Sphingomyelin phosphodiestrase 3, neutral membrane	1.772	$7.48 X10^{-03}$
ZMYM6	Zinc finger, MYM-type 6	1.772	$2.48 X10^{-02}$
SPP1	Secreted Phosphoprotein 1	-2.197	$1.14X10^{-03}$
VIM	Vimentin	-1.959	$6.0 X10^{-03}$
PAH	Phenylalanine hydroxylase	-1.928	$9.33 X10^{-03}$
TBX18	T-box 18	-1.886	$1.07 X10^{-02}$
TXNL4	Thioredoxin like 4B	-1.857	$4.10X10^{-03}$
C. Vitrification versus slow freezing			
SDS	Serine dehydratase	2.482	$1.24 X10^{-02}$
SERPINA5	Serpin peptidase inhibitor, clade A (alpha 1, antiproteinase, antitrypsin), member 5	2.469	$5.71 X10^{-04}$
AGXT2L1	Alanine-glyoxylate aminotransferase 2-like 1	2.326	$2.48 X10^{-03}$
LYZ3	Lysozyme 3	2.284	$2.10 X10^{-03}$
GLDC	Glycine dehydrogenase (decarboxylating)	2.161	$2.64 X10^{-03}$
NAGK	N-acetylglucosamine kinase	-3.861	$4.83X10^{-02}$
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	-3.326	$7.89 X10^{-04}$
CCL17	Chemokine (C-C motif) ligand 17	-2.738	$1.92 X10^{-03}$
HEBP2	Heme binding protein 2	-2.631	$1.95 X10^{-03}$
CLDN23	Claudin 23	-2.455	$9.85X10^{-04}$

5.4.3 ***Upstream regulators***

To understand broader implications of gene expression changes in the treated embryos, a list of potentially "inhibited" or "activated" upstream regulators was generated using IPA analysis. The analysis was based on the activation (z-) score ≥ ± 2 and p-value <0.05. Activation (z-) score represent the direction of change for the function. Compared to the unfrozen embryos, vitrified group embryos had two "inhibited" upstream regulators (Table 5.4) i.e. NFκβ and Tretinoin. To provide a better picture, other upstream regulators with z-scores < ± 2 are also included in Table 5.4. Potential upstream regulators in vitrified embryos were included in a network diagram as core molecules to explain downstream changes in gene expression (Figure 5.4). No upstream regulator was predicted to be affected in slow frozen embryos (slow freezing vs control embryos).

Table 5.4 Upstream regulators predicted by IPA software to be inhibited/downregulated (decreased influence upon downstream targets) based on the differential expression of target molecules identified in vitrified expanded blastocysts versus unfrozen embryos (reference group). Upward and downward arrows indicate up-regulation and down-regulation of the transcripts in the dataset.

Upstream regulator	*Molecule type*	*Predicted activation state*	*Activation score*	*p-value*	*Target molecules in dataset (Gene symbols)*
NFκβ	*Complex*	*Inhibited*	*-2.165*	*1.55E-04*	*↓PTGS2, ↓PLAU, ↓KRT19, ↓CCL17, ↓CALB1, ↓ANKRD1*
Tretinoin	*Chemical endogenous mammalian*	*Inhibited*	*-2.124*	*1.68E-02*	*↓PTGS2, ↓PLAU, ↓KRT19, ↓CCL17, ↓CALB1, ↓CYP11A1, ↑COL9A2*
CEBPβ	*Transcription regulator*		*-1.972*	*2.57E-03*	*↓FHL2, ↓SLC10A1, ↓PTGS2, ↓CYP11A1*
IGF1R	*Transmembrane receptor*		*-1.969*	*2.08E-05*	*↓ANKRD1, ↓PTGS2, ↓PLAU, ↓KRT19, ↓CYP11A1*
EGF	*Growth factor*		*-1.831*	*8.30E-05*	*↓PTGS2, ↓PLAU, ↓KRT19, ↓CYP11A1, ↓APOA1,*

				↓CCL17
IFNγ	*Cytokine*	*-1.731*	*2.92E-04*	*↓FHL2, ↓CALB1, ↓PTGS2, ↓PLAU, ↓KRT19, ↓CYP11A1, ↓IFI30*
TGFβ1	*Growth factor*	*-1.546*	*2.88E-03*	*↓PLAU, ↓KRT19, ↓CYP11A1, ↓IFI30, ↓ABCC2, ↓ANKRD1, ↓ACTG2, ↓PTGS2*

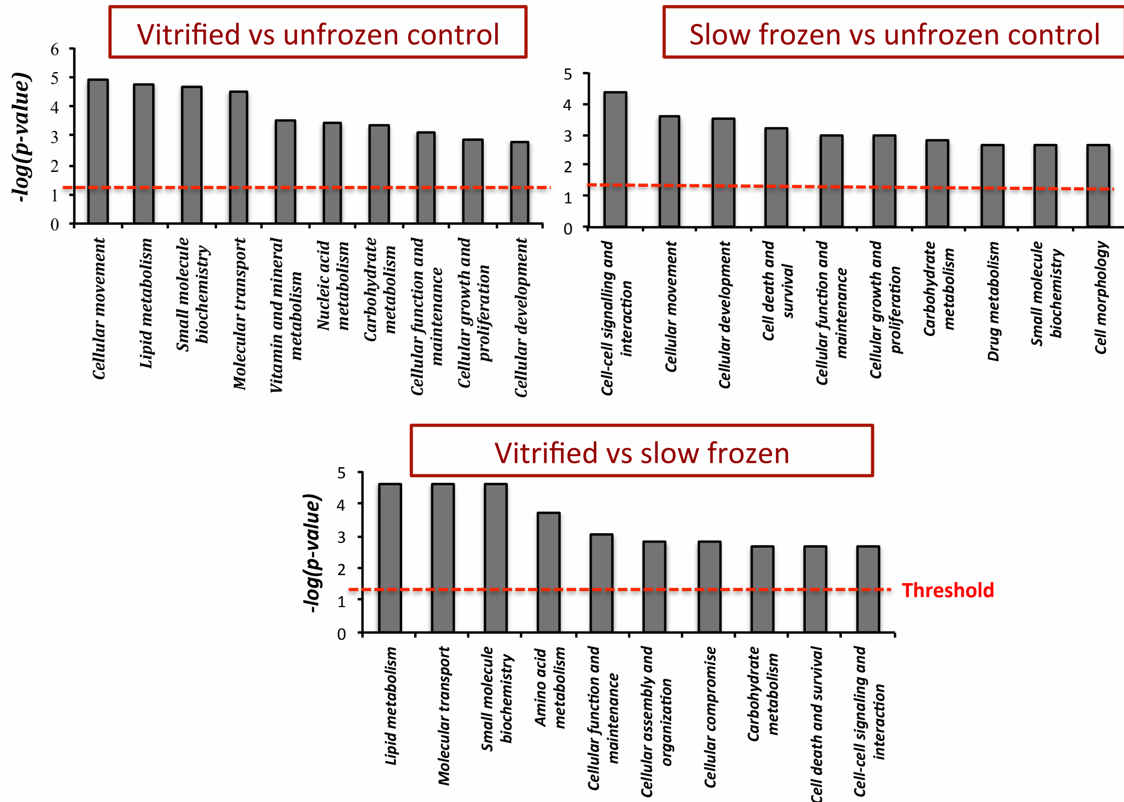

Figure 5.2 Functional analysis of differential gene expression in IVP bovine embryos based on –log (p-value) obtained with IPA® software. Higher log values relate to higher significance of the functions. Top 10 cellular and molecular functions in each comparison are illustrated. Taller bars are more significant than shorter bars and red line represents the cut-off value for p-value <0.05, -log-value= 1.3.

5.4.4 *Pathway and network analysis:*

The differential gene list from the Flexarray® software analyses for each treatment comparison were uploaded in Ingenuity® Pathway Analysis (IPA) software and analyzed for the most possibly affected cellular, molecular, physiological and disease-related pathways. Top 10 affected cellular-molecular pathways in vitrified and slow frozen embryos as compared with unfrozen embryos (control; reference group) as well as vitrification versus slow freezing (reference) groups are presented (Figure 5.2). Genes involved in the well-known canonical pathways were also examined using the IPA software and top 10 affected canonical pathways are presented (Figure 5.3). A network of top differentially expressed genes (p-value < 0.05; fold change $\geq \pm 2$) in vitrified embryos as compared to untreated control embryos was developed in IPA® (Figure 5.4).

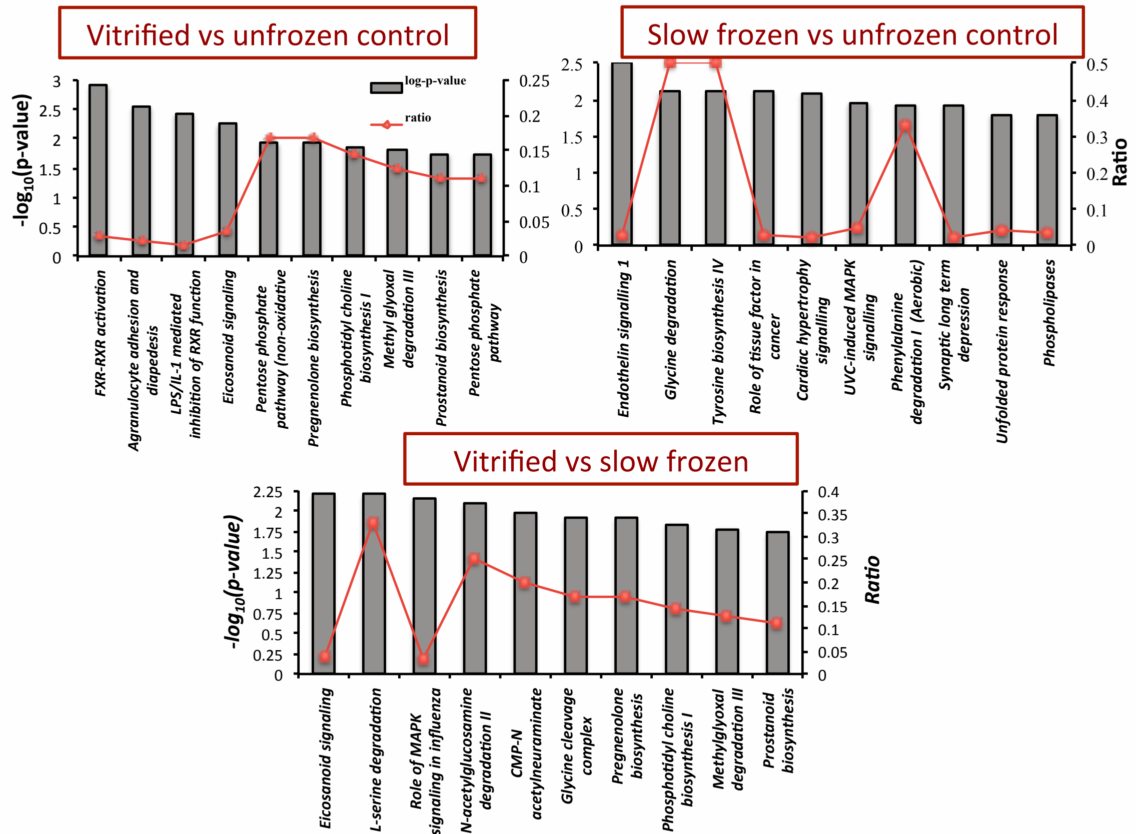

Figure 5.3 Canonical pathway analysis of gene expression of treated IVP bovine embryos as compared to reference groups. Score ratio (red line graphs) depicts the number of genes affected in the treatment versus the total number of genes involved in the pathway (y-axis on right side of each figure). Taller bars are more significant than shorter bars (-log-value= 1.3 is equivalent to p-value <0.05). Data analysis and graphs generated with IPA® software.

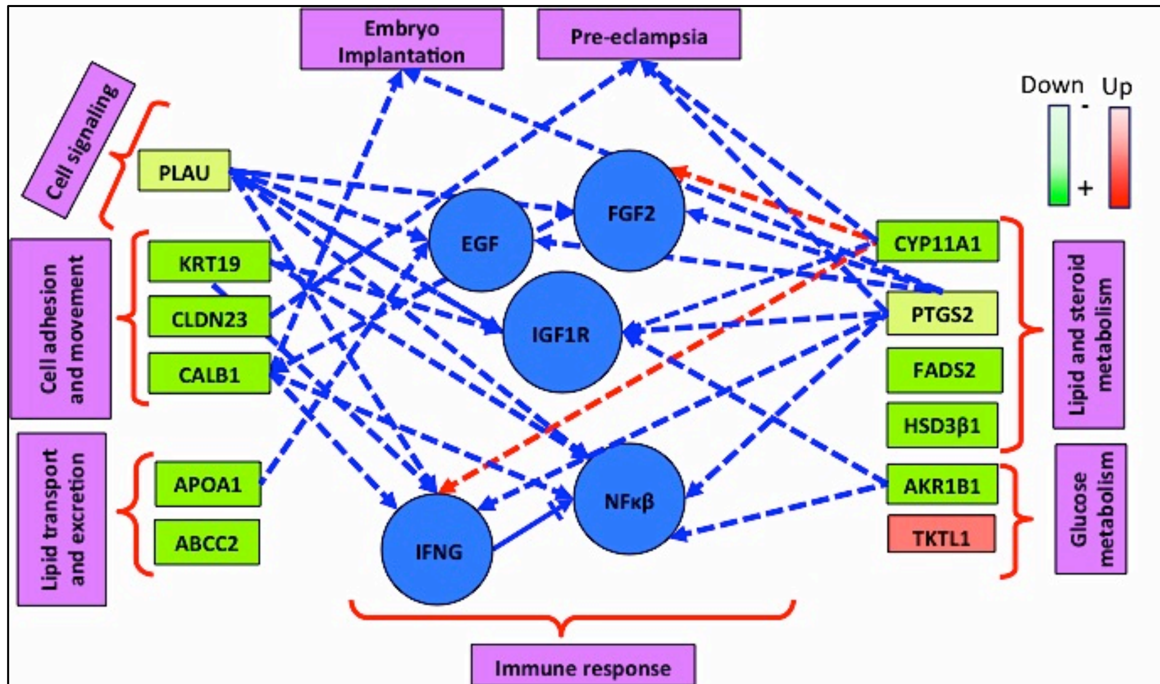

Figure 5.4 A functional network of differentially expressed genes (rectangular boxes; green=downregulated, red=upregulated) in vitrified compared with control embryos. The circular nodes represent the affected upstream regulators determined by IPA® analysis. The lines (Blue line = possible inhibition, red line = possible activation of upstream regulator) represent the relationship among the molecules. Relationship lines are supported by at least one reference derived from the literature, textbooks, and/or canonical pathways stored in Ingenuity® Knowledge Base. Based upon the state (downregulated and upregulated) of the genes, all upstream regulators in this network are predicted inhibited or downregulated (blue circles).

5.4.5 ***Quantitative real time PCR:***

Based on microarray data and function analysis, 6 genes were selected from differential genes of vitrification and control untreated group and 1 gene was selected from slow freezing and control untreated group for validation with quantitative real time PCR (Vitrification-CYP11A1, PLAU, CLDN23, KRT19, AKR1B1, TKTL1 and Slow freezing- SPP1). After quantification in three independent biological replicates from treatment (vitrification and slow freezing) and control groups, differential expression was validated for 4 of the 7 genes (90% confidence level; $P \leq 0.01$; Figure 5.5).

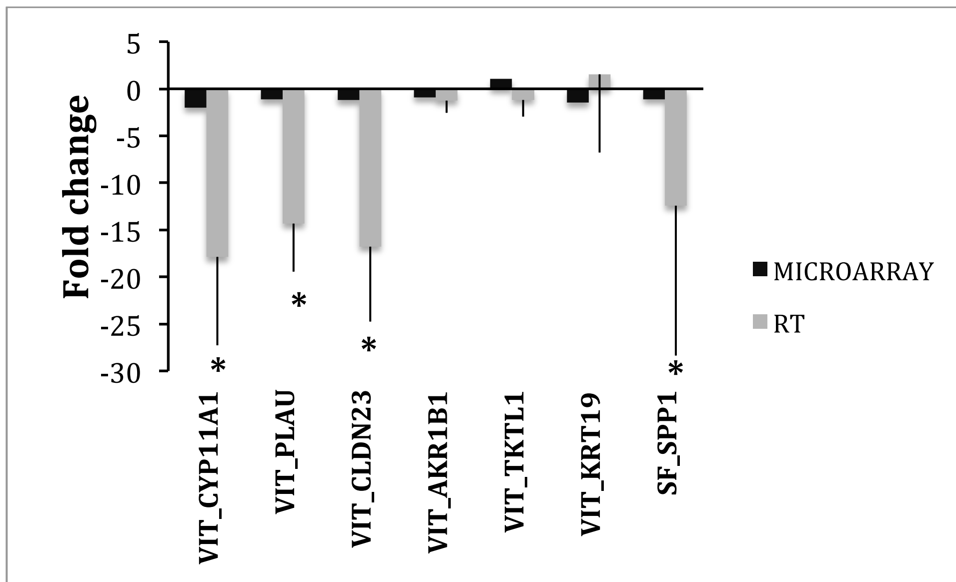

Figure 5.5 Quantification (fold-change; mean ±SEM) of the mRNA profile of *in vitro* produced bovine blastocysts after cryopreservation treatment [Vitrification (VIT) vs control and slow freezing (SF) vs control] using real-time (RT) PCR (n=3 replicates per group). Black bars represent the differential level of expression of transcripts detected in the microarray experiment, while light grey bars represent the differential level of expression of the same transcripts obtained by real-time PCR. Bars with superscript* are downregulated in the treatment group than in the untreated control group (P≤0.01).

5.5 Discussion

Microarray-based analysis of vitrified and slow frozen embryos point towards the two postulates; 1) the quality of surviving vitrified embryos is degraded as compared with control IVP and/or surviving slow frozen embryos and 2) embryos obtained after vitrification may be impaired for future implantation in the uterus. Vitrification technique is known to provide better embryo survival rate in both bovine and humans than slow freezing as was the case in our experiment as well (77% versus 34% morula to blastocyst conversion rates, respectively). Although 2/3rd did not survive the slow freezing, interesting to note that transcriptome of the remaining surviving slow frozen embryos was very similar to the unfrozen control embryos.

In the present study, the most profound impact of vitrification on *in vitro* produced bovine embryos was observed on genes regulating lipid metabolism and excretion. Abrupt changes in cellular volume, a high amount of physical/osmotic pressure on the embryonic cells and vitrification turns the fluidic lipid portion of cell membrane to gel phase, known as lipid phase transition (Leibo 1981) thus causing irreversible damage to certain portion of cell membranes (Leao *et al.* 2014). The substrates for steroid biosynthesis *i.e.* arachidonic acid and polyunsaturated fatty acids (PUFA) are derived from membrane phospholipids. In the present study, the genes involved in steroid biosynthesis, pregnenolone biosynthesis and eicosanoid signaling (cytochrome P450 subunit 11 type A 1 (CYP11A1; -3.971 fold downregulation), 3-beta hydroxy steroid dehydrogenase, delta-isomerase type 1 (HSD3β1; -2.37), ATP-binding cassette subfamily C-2 (ABCC2; -2.02) and prostaglandin synthase 2 (PTGS2; -2.322) / cyclooxygenase 2 (COX2) were significantly downregulated in vitrified embryos. Interestingly, the genes

involved in purine metabolism and sphingolipid metabolism were upregulated in IVP vitrified blastocysts (Aksu *et al.* 2012) as opposed to downregulation in morulae in the present study. The lipid metabolism genes (CYP11A1; -3.971, HSD3β1; -2.37 and APOA1; -2.02) are also involved in retinoids and their receptor (FXR/RXR) pathway. It is interesting to record that FXR/RXR are expressed in inner cell mass (ICM) and trophoectodermal (TE) cells and enhance blastocyst development and hatching in sheep and cattle (Eberhardt *et al.* 1999; Mohan *et al.* 2001).

The steroid metabolite (PGF2α, PAF, progesterone and β-estradiol) catalyzed by these (CYP11A1; -3.971, PTGS2; -2.322 and HSD3β1; -2.37) enzymes are critical for pre-implantation embryo development (Bazer *et al.* 2015) like transformation of morula to blastocysts, shedding and dissolution of zona pellucida and embryo elongation (Dickmann and Dey 1974; Dickmann *et al.* 1975). Furthermore, detected downregulated genes involved in steroid metabolism (CYP11A1; -3.971, HSD3β1; -2.37, PTGS2; -2.322 and AKR1B1; -2.566) are also implicated in placentation and implantation of embryos at later stage (Shemesh *et al.* 1992; Hoffmann and Schuler 2002; Breuiller-Fouche *et al.* 2010). This study therefore, suggests the possibility of impaired implantation in vitrified embryos. Detected impaired gene expression of CYP11A1, PTGS2, disturbed lipid metabolism and predicted defective implantation are all hallmarks of transcriptional deregulations in pre-eclamptic conditions (Vaiman *et al.* 2013). This hypothesis will be further discussed in subsequent sections alongwith other genes and pathways.

Two genes associated with cellular uptake and efflux of cholesterol and fatty acids (apolipoprotein type A1 (APOA1; -2.02) and ABCC2; -2.02) from the external media (Tall 1998; Boisvert *et al.* 1999; Rigotti and Krieger 1999; Abumrad *et al.* 2000) are also

involved in waste disposal/detoxification (Jonker *et al.* 2009), e.g., ABC transporters such as ABCC2 actively detoxify the cells by efflux of xenobiotics during oxidative stress under hypoxic conditions (Aye and Keelan 2013). This may be an important mechanism for the survival of a semi-autonomous preimplantation stage embryos. Enzymes such as Aldo-keto reductases (AKR1B1) also perform cellular detoxification that protects against toxic carbonyl-compounds (*e.g.* oxalic acid *i.e.* major metabolite of ethylene glycol metabolism). Therefore, the down-regulation of these molecules may be an indicator of accumulated chemical stress at cellular level. In another study, exposure of IVP embryos to cryopreservation increase the expression of stress related proteins like HSPA1A (Stinshoff *et al.* 2011).

In the present study, the above-mentioned down-regulation in lipid and glucose metabolism as well as nutrient (lipids and organic anion) transporter genes in vitrified – survived embryo correspond to the "quiet embryo hypothesis" (Leese 2002). According to this hypothesis, the survival of pre-implantation embryos under stressful conditions is associated with their low metabolic activity, i.e. reduced exogenous nutrient supply and increased utilization of endogenous resources. Morulae and blastocysts however, have higher metabolic rates than preceding embryonic stages and downregulation of embryonic metabolism is sometimes associated with embryonic diapause (Leese *et al.* 2008). The relationship between low metabolic activity and higher embryo viability was supported in our study as well, as more viable embryos showed low lipids and fatty acid metabolism, indicating acceptable quality of vitrified-survived embryos. The use of serum and/or BSA in IVP embryos render them susceptible to cryopreservation induced damage (Abe *et al.* 2002b; Mucci *et al.* 2006; Gardner 2008). Fewer studies had been

conducted to change the culture media to improve cryopreservation outcome (Sudano *et al.* 2011). The addition of phenazine ethosulfate, a metabolic regulator that inhibits fatty acid synthesis, reduced lipid accumulation and increased blastocyst re-expansion after vitrification (Sudano *et al.* 2011). Thus, the down-regulation of lipids' metabolism and accumulation genes in vitrified embryos may be supportive of their survival.

An important aspect of bovine embryo development is the formation of blastocyst from morula stage and involves differentiation of blastomeres into ICM and TE cells. During morula compaction and formation of blastocoel, adhesion plaques lead to formation of polarized transporting trophectoderm (TE) epithelium and non-polarized inner cell mass (ICM) cells (Rodriguez-Boulan and Nelson 1989; Wiley *et al.* 1990). Genes related to cell adhesion (Claudin 23 (CLDN23); -2.318) and actin-cytoskeletal component (Actinγ2 (ACTG2); -2.010) were downregulated in vitrified embryos in the current study. These molecules prevent leakage of fluid during blastocoel formation and support blastocyst expansion and hatching process (Biggers *et al.* 1988; Watson and Barcroft 2001). Taken together with results of another report that revealed downregulation of tight junction and cell adhesion proteins (Tight junction protein and Desmocollin 2) in vitrified bovine blastocysts (Stinshoff *et al.* 2011), our results support the notion that vitrification may adversely affect or delay hatching.

Under *in utero* conditions, cytokines such as (IFNτ and IFNγ) and growth factors (EGF, FGF, TGFβ and IGFBPB), which play a major role in cell growth, proliferation and differentiation, are important for transformation of morula to blastocyst and for further hatching. Some of them like IFNτ and IFNγ play a major role in embryo signaling, maternal recognition of pregnancy, immune regulation and establishment of

pregnancy (Charpigny *et al.* 1988; Sharkey 1998; Godornes *et al.* 2007). IFN-τ secreted by implanting embryos, inhibits the production of prostaglandin synthase (PGS) in ruminants (Godkin *et al.* 1997). Interestingly, expression of upstream regulator IFN-γ gene was predicted to be downregulated by IPA analysis (based on downregulation of FHL2; -2.145, CALB1; -2.165, PTGS2; -2.322, PLAU; -2.205, KRT19; -2.783, CYP11A1; -3.971, IFI30; -2.276) in vitrified bovine embryos in this study. Interestingly, one earlier study documented the downregulation of IFN-τ gene expression in bovine vitrified embryos (Sudano *et al.* 2012) but another study failed to detect change in IFN expression levels (Stinshoff *et al.* 2011). In this study urokinase-based plasminogen activator (PLAU also known as uPA), with a suggested role in extra-embryonic matrix (EEM) digestion, was downregulated (-2.205) in vitrified embryos as compared to unfrozen embryos. It is important to consider that bovine embryos showing downregulation of PLAU were unable to implant (El-Sayed *et al.* 2006). Similar to KRT18 (Sudano *et al.* 2012), in the current study, the keratin family gene (KRT19; -2.783) was also downregulated in the vitrified embryos. Taken together, downregulation of PLAU and keratin family along with predicted decrease in upstream regulator IFNγ suggest the possible impairment in early embryo recognition and implantation process in the vitrified embryos.

In the current study, IPA analysis of differentially expressed genes indicated apoptosis (NFκβ, CYP11A1; -3.971, AKR1B1; -2.566, CALB1; -2.165 and PLAU; -2.205) and necrosis (TGFβ, IFNγ, PLAU; -2.205, KRT19; -2.783, CYP11A1; -3.971, IFI30; -2.276, ABCC2; -2.02, ACTG2; -2.010, CCL17; -2.946) pathways in vitrified embryos. Necrosis is a large scale cellular damage associated with membrane damage,

nuclear disintegration and cellular swelling, thus may affect whole embryo survival (Proskuryakov *et al.* 2003). Apoptosis is usually associated with single cell damage with cytoplasm shrinkage, chromatin condensation and DNA damage leaving adjacent cells intact (Bredensen 1996). Early cleaved blastomeres are more resistant to apoptosis rather than inner cell mass and trophoblast (Weil *et al.* 1996; Matwee *et al.* 2000). Apoptosis is a normal process in early embryos for blastomere selection (Plante and King 1994) and is a strategy of embryo survival under stressful circumstances (Antunes *et al.* 2010). This seems to be true for survived vitrified embryos in this study.

Interestingly, survivor slow frozen embryos showed fewer gene expression changes as compared to control unfrozen but their transcriptome differed significantly from that od the vitrified embryos. These results point towards the surviving efforts of slow frozen embryos. For example, up-regulation of cell structure and morphology maintenance genes such as microtubule polymerization genes like double-cortin kinase 2 (DCLK2; 1.829 fold upregulated), Zinc finger, MYM 6 (ZMYM6; 1.772) and proteolytic inhibitor such as serine peptidase inhibitor kunitz type 1 (SPINT1; 1.691) compared to connrol group may be the compensatory mechanisms for the cell structure damage. Other genes like Junctional adhesion molecule 2 (JAM2; -1.664) and Vimentin (VIM; -1.959) were however, downregulated in slow frozen embryos. VIM encodes a protein member of cellular intermediate filaments known to enhance cell elasticity, capacity to adapt to stress and is important for normal bovine embryo development (Maddox-Hyttel *et al.* 2003). Other upregulated genes in slow frozen embryos related to membrane lipid metabolizing enzymes; Phospholipase A 2 recepetor-1, 180 kDa (PLA2R1; 1.983) and Sphingomyelin phosphodiestrase 3, neutral membrane (SMPD3; 1.772) depict membrane

damage but also utilization of embryo's internal resources for metabolism. These changes point towards the viability and better quality of slow frozen embryos that survived the transition from morula to blastocyst after cryodamage.

During recent years, vitrification is replacing slow freezing in field conditions due to high embryo survival rates as well as ease of conducting the former technique (Vajta and Nagy, 2006). Our study documented a similar morula to blastocyst transition rates in vitrified embryos (77%) compared to unfrozen control (72%) while many slow frozen embryos failed during this transition process (34% survival). Similar results have been reported in earlier studies on bovine embryos (Nedambale *et al.* 2004b; Vajta and Nagy 2006). Interestingly, upon *in silico* comparison between slow freezing and vitrification group, gene expression changes similar to vitrified versus control group were recorded. Similar pathways like lipid metabolism and cell movement and adhesion were affected in vitrified embryos as compared to slow frozen embryos. It is noteworthy that the transcriptome of the surviving slow frozen bovine embryos in the present study was very similar to the unfrozen control (no gene transcripts were detected at ≥ 2 fold change) thereby perhaps pointing to higher developmental potential of these embryos in future as compared to vitrified embryos. Based on historical data, equivalent embryo implantation and pregnancy rates are expected after transfer of vitrified (45.1%) and slow freezing (44.5%) bovine embryos (van Wagtendonk-de Leeuw *et al.* 1997). Studies conducted on rabbit morulae following vitrification demonstrated impaired trophoblast proliferation and differentiation, retarded fetal development and an altered gene expression (ANXA3, EGFLAM and TNAIP6) compared to slow freezing embryos (Vicente *et al.* 2013; Saenz-de-Juano *et al.* 2014a). Based on discussion in previous sections and considering the

pregnancy rate data in cattle, we postulate that higher failure rates in vitrified embryos compared to slow frozen embryos during the pre-implantation period, during maternal recognition of pregnancy and perhaps during peri-implantation period, thereby resulting in similar pregnancy rates by 30 days of gestation. Conversely, some scientists consider that development and implantation are better in vitrified embryos than slow freezing (Leibo 2012). On the other hand, our hypothesis is supported by rabbit studies wherein slow-frozen embryos were lost during two development phases (before implantation and around placentation time) while the vitrified embryos reaching the late blastocyst stage were implanted successfully but had a higher mortality rate post-implantation (Saenz-de-Juano *et al.* 2014b).

To conclude, this study revealed the superiority of survived slow frozen embryos as compared to vitrified embryos in terms of changes in their gene expression. Though vitrification provided a higher embryo survival than slow freezing, the quality and viability of vitrified embryos might be questionable in terms of further embryonic development. The vitrified embryos showed downregulation of genes involved in lipid metabolism, cell differentiation and cell adhesion. However, the mechanisms affected due to these gene expression changes might lead to impaired implantation of these embryos. Although the survival rate of slow frozen embryos was poor, the level of gene expression changes was low and comparable to control unfrozen embryos. Also, the gene expression changes in vitrified embryos when compared to slow frozen embryos was similar to when compared with control embryos. This suggests the need for subsequent experiments to be performed to study the differential implantation and pregnancy rates in vitrified and slow frozen embryos. Also, metabolomics study should be performed on the

pre and post-thawing embryo culture media from vitrified and slow frozen embryos to confirm upon these changes.

CHAPTER 6 **General discussion**

Cryopreservation of mammalian oocytes and embryos is an important application of assisted reproductive technology (ART). For recovering lost breeds, global transport of genetic material, genetic improvement, food security, long term preservation and maintenance of genetic diversity requires development of cryostorage gamete and embryo banks. Also, with the growing food and animal industry the increasing demand of oocyte and embryos can only be met by storing them in a frozen state. The first successful pregnancy was achieved in bovines in the year 1973 (Wilmut and Rowson 1973; Leibo and Pool 2011). This success lead to the development of two commonly used cryopreservation techniques *viz.* vitrification and slow freezing in animal industry. Apart from animal industry, cryopreservation has an important implication in human medicine of producing live offspring and chances of rescuing the gametes and embryos in infertility and cancer patients. Therefore, keeping in mind the importance of gamete and embryo cryopreservation various techniques and protocols have evolved overtime.

In this thesis, four studies were conducted to compare the outcome of cryopreservation of day 6 IVP bovine morulae by using vitrification and slow freezing. The embryo development to blastocyst stage was studied to compare the efficiency of the techniques, in the first study. The data obtained from this study was analyzed to study the impact of season of conducting *in vitro* fertilization and embryo cryopreservation on the development of embryos. In the second study, the embryos were assigned slow frozen using two cryoprotectants; ethylene glycol and glycerol. Subsets of replicates of embryos obtained from the first study were assigned for microarray based analysis of unfrozen control embryos and treated embryos (vitrification and slow freezing). Several

differentially expressed genes were selected for both treatment groups and were analyzed using quantitative real time PCR.

Cryopreservation of embryos affects embryonic health and their further development. Outcome of cryopreservation is dependent upon the type of techniques used; vitrification or slow freezing. In the first study, our results indicated significant difference between morula to blastocyst development after vitrification and slow freezing. The blastocyst development was higher in control unfrozen embryos than other treatment groups. Commercial embryo transfer requires a higher survival and development outcome of embryos. Formation of blastocyst from morula is a critical stage of embryo development and is a determinant of bovine pregnancy outcome. The production cost of embryo transfer and cryopreservation is high for superior genetic breeds. Slow freezing of embryo involves the use of specialized expensive equipment but less technical expertise and allows freezing of bulk of embryos in less time. However, vitrification of embryos is easily adaptable but requires high technical expertise and more time for performing the technique. The embryo survival outcomes of both are controversial and are highly variable with the species involved (Palasz and Mapletoft 1996). For a few decades, the vitrification technique has been attempted as a replacement ofslow freezing for bovine and other domestic animals' embryo freezing because of the ease for doing the technique and better outcome. Vitrification has yielded higher embryo survival outcomes in many species including humans (Levron *et al.* 2014), mouse (Li *et al.* 2012) and rabbit (Naik *et al.* 2005). Live offspring have been born from vitrified-warmed embryos in domestic animals (Vajta and Nagy 2006; Somfai *et al.* 2014) and human (Desai *et al.* 2010). The extensive data collection in this study confirms the

superiority of vitrification technique than slow freezing for cryopreservation of bovine embryos.

Heat stress is a major management issue in commercial dairy animal production systems, as it alters the reproductive physiology especially follicular dynamics in lactating cattle (Roth *et al.* 2000; Wolfenson *et al.* 2000). A study has already shown that a period of 2-3 estrous cycles is required for recovery from damage due to heat stress (Gendelman and Roth 2012). In this perspective, the data obtained in study one was analyzed for the impact of external environmental factors such as season on the development on IVF and IVP of embryos. Also, we wanted to analyze the impact of season and cryopreservation treatment on the post-warming survival of cryopreserved embryos. Our study revealed that season had impact on the outcome of IVF and fall season had lowest cleavage and morula rate than other seasons. Blastocyst rate in unfrozen control group was highest in spring and lowest in summer season. The effect of season on bovine oocyte quality extends not only to embryo development and quality but also affects the transcriptional gene expression during early embryo development (Gendelman and Roth 2012). The study found that gene expression of POU5F1 was lowered in hot season. Its upregulation had been shown to be associated with high quality blastocysts (Schultz 2002). Thus, embryos produced during low production season may be of low quality. The season of embryo cryopreservation also showed an impact on the post-thaw development as well. In the slow frozen group, blastocyst rate was lowest in summer season than other seasons. Outcome of vitrification, on the other hand, was not affected by the seasonal variation of oocyte collection. This is in conjunction with the previous observation made, where no significant difference was found in pregnancy rates

after transfer of fresh and vitrified IVP bovine embryos (Stewart *et al.* 2011). Thus, this study provides an insight for the livestock owners and dairy and beef production companies to manage and manipulate their oocyte collection, embryo production and cryopreservation strategy according to season, to minimize losses. High yielding cattle usually have difficulty in maintaining normothermia. Also, a delayed effect of heat stress is known to persist in autumn season, which affects the follicular steroidogenic capacity and indicated to induce variation in follicular dynamics (Wolfenson *et al.* 1997). It is interesting to note that the ovary collection, IVF and cryopreservation of embryos were done in a temperate climate of central Canada and the impact of heat stress in not extreme in this climatic zone. However, as mentioned above stress of lactation in high yielding cattle affects reproductive physiology. The nutritional stress during hot season also, continues its effect in autumn season, which leads to low quality of oocytes and embryos in cattle (Gendelman and Roth 2012). Also, this seasonality has been found to be related to light intensity and photoperiod (Cummings 2010). This is transduced into neuroendocrine changes through variations in melatonin secretion from the pineal gland. Melatonin further changes the secretory activities of gonadotrophin releasing hormone (GnRH) and modulates the discrete areas in hypothalamus. Interestingly, season was found to have significant impact in IVF rate in humans and it was found to be highest in spring season (Braga *et al.* 2012). Therefore, this study can also help in human medicine to provide better pregnancy outcomes by performing procedures in favorable seasons. Also, it stimulates us towards the exploration of embryo cryosensitivity in seasonal breeder animals, in different seasons.

Cryoprotectant type and concentration used in slow freezing procedure greatly determine the outcome of cryopreservation. There have been efforts to determine the best cryoprotectant for embryo cryopreservation in terms of their toxicity and their ability to minimize the cryoinjury (Palasz and Mapletoft 1996). There are mainly two types of cryoprotectants; permeable and non-permeable. Both type of cryoprotectants have a mechanism to stabilize intracellular structures and the cell membranes. Permeable cryoprotectants enter the cells, cause dehydration and hence, minimize ice formation. Slow freezing usually utilize a single cryoprotectant while vitrification involves the use of mixture of cryoprotectants (Palasz and Mapletoft 1996). Two cryoprotectants ethylene glycol and glycerol have been long used in bovine embryo cryopreservation. Polyols like glycerol regulate dehydration and protect the protein structure. Glycerol is however, a poor stabilizer of membrane as well as induces membrane fusion at high concentrations and temperatures (Womersley *et al.* 1986). Only rapid thawing can prevent this. An approach to transfer cryopreserved embryos directly to animals without thawing has been adopted in field conditions. Low molecular weight cryoprotectants such as ethylene glycol or 1,2- propylene glycol are highly permeating and therefore, avoid osmotic shock to embryos when directly transferred to the isotonic uterine environment. High molecular weight glycerol based cryoprotectant, however, causes osmotic shock under such conditions. Depending upon previous observations, we hypothesized that ethylene glycol is a better cryoprotectant than glycerol for success of slow freezing bovine embryos. Two cryoprotectants ethylene glycol and glycerol, were used to slow freeze day6 IVP bovine morula. These embryos were allowed to develop to expanded blastocyst stage. Our study revealed better embryo survival and blastocyst development rates using ethylene glycol

than glycerol. Therefore, it is recommended to utilize ethylene glycol for freezing the IVP bovine embryos, which is otherwise also the cryoprotectant of choice for direct transfer of embryos.

Since the technique for cryopreservation had its advent, a significant amount of research had been conducted in cryobiology to determine the causes and consequences of freezing injury. Many studies have reported morphological and ultrastructural changes in oocytes and embryos post-cryopreservation using vitrification and slow freezing (Mucci *et al.* 2006; Shirazi *et al.* 2010a; Zander-Fox *et al.* 2013). Some studies have reported a decrease in a total cell number of cryopreserved blastocyst after slow freezing than vitrification (Gomez *et al.* 2009; Zander-Fox *et al.* 2013). Similarly, isolated reports have been made on the biochemical and gene expression changes induced due to cryopreservation (Kaidi *et al.* 2001; Park *et al.* 2006; Stinshoff *et al.* 2011; Aksu *et al.* 2012). Most developmental, morphological and biochemical studies point towards the better embryo survival and quality after vitrification than slow freezing. However, no significant study has been made to analyze the global gene expression changes in cryopreserved bovine embryos. Therefore, we designed a microarray based gene expression analysis study to inspect changes in bovine early embryos after cryopreservation using vitrification and slow freezing.

In the microarray analysis, a coherent picture of subtle gene expression changes occurring in the two groups; vitrification and slow freezing in comparison with the untreated IVP embryos can be established. Four biological replicates of each treatment group were compared on different microarray slides with the same untreated control group. The data obtained was background corrected (ArrayPro and ELMA) and

statistically analyzed for differentially expressed genes (Flexarray™). This extracted information was further processed using Ingenuity™ pathway analysis software to determine most affected gene expression as well as biological pathways.

A total of 64 genes were differentially regulated in control and vitrified embryos. The major pathways and genes affected in vitrified embryos were of lipid metabolism (CYP11A1, PTGS2, HSD3β1 and AKR1B1), cell-to-cell signaling and transport (ABCC2, APOA1), cell junction, adhesion (CLDN23) and movement (PLAU) and vitamin and mineral metabolism (FXR/RXR pathway). These changes point towards defective development of blastocyst from morula stage. Most of the genes downregulated in vitrified embryos have been shown to be vital to morula compaction, blastocyst development and hatching (Cui *et al.* 2007). Therefore, the vitality and functionality of vitrified embryos after warming maybe questionable.

As shown in a recent study, vitrification procedure used in our experiments, rules out the chances of ice formation in embryos (Anzar *et al.* 2014). By avoiding ice formation, vitrification inflicts lower morphological damage to the embryonic blastomeres. Therefore, the most apparent functional damages to the vitrified embryos shown in our study may be mainly due to extreme cryoprotectant concentrations used in the procedure. *In vitro* produced embryos are known to be more cryosensitive and more buoyant than IVD embryos, mainly due to their high lipid content (Leibo 1993; Seidel 2006). Changes in pre-vitrification culture media therefore, might have a great impact on the embryo cryosenstivity and quality upon survival. Thus, several changes in culture media have been tried to improve post-vitrification IVP bovine embryo survival. To remove lipid droplets a variety of experimental procedures had been tried, which includes

serum-free culture medium and de-lipidation using centrifugation and micromanipulation (Pryor *et al.* 2011). Also, other modifications in culture media and addition of factors such as retinoids (Gomez *et al.* 2008a), beta-mercaptoethanol (Nedambale *et al.* 2006), neutral lipids such as trans-10, cis-12 conjugated linoleic acid (Batista *et al.* 2014) and use of serum-free media (Rizos *et al.* 2003) have been shown to affect the cryosurvivability and post-vitrification quality of bovine embryos. Thus, changes in embryo production and cryopreservation strategies may impart better survival to them.

The vitrification survivor embryos showed the gene expression changes, which might affect the future developmental potential of the embryos. The possible major affected mechanism predicted was implantation of embryos.

The slow frozen embryos in our study had significantly reduced gene expression only for three genes VIM, SPP1 and PLA2R1 as compared to control untreated bovine embryos. Vimentin is an intermediate filament that has an important role in induction of cellular migration and is a marker for epithelial to mesenchymal transition (Hyder *et al.* 2015). Another molecule such as SPP1 is an important molecule of ECM and has a marked role in cell-cell adhesion, cell-ECM communication, migration of immune cells and decrease cell death by reducing ROS species. It also has a marked role in embryo implantation and placentation (Johnson *et al.* 2003). The differential gene expression changes in slow freezing survivor embryos were however, not significant.

Our study is a comprehensive overview of impact of the two most commonly used cryopreservation techniques; vitrification and slow freezing on the development and global gene expression of bovine embryos. The embryo development study showed a higher blastocyst development in control untreated embryo than vitrified and slow frozen

embryos. Vitrified embryos showed higher development than slow frozen embryos. However, the differential gene expression changes show a lower embryo quality post-warming of vitrified embryos than slow frozen embryos. Our study is a good insight for commercial embryo production technologists to choose their procedures, wisely for better outcomes. However, combining this study with the microscopic and/or TUNEL evaluation of embryo quality could have been an asset. As expected from previous observations, the gene expression changes in slow frozen embryos were related with structural damage to the embryos. The development of morulae to blastocyst stage after cryopreservation provided us a chance to study the gene expression changes affected during the process.

CHAPTER 7 **Conclusions and future directions**

This study provides a chance to imply the effect of most commonly used embryo culture environment and cryopreservation techniques upon defining the improvements in future protocols of embryo cryo-storage. More studies can be directed to make the *in vitro* produced embryos resistant to cryopreservation-induced damages. Certain additions to media like use of anti-oxidants and reducing the exposure time to serum based culture media can be helpful. Also, changes in the cryopreservation media such as using serum free culture media or composition to suit the post-thaw embryo requirements *e.g.* use of anti-oxidants, can be derived from our study. Moreover, this study provides an overview of pre-implantation embryonic physiology, behavior and responses under extreme stressful conditions in comparison with the normal embryos. Interestingly, the gene expression changes studied after microarray analysis in our study was done on human and mouse genomic data-based software called Ingenuity pathway analysis. Therefore, this study provides a great insight for inducing changes in embryo production protocols in human medicine.

Embryos are the functional germinal units of a new life. The exposure of embryos to extreme cryopreservation temperatures requires a full armor of possible protection to ensure their survival and development in real time, post-warming.

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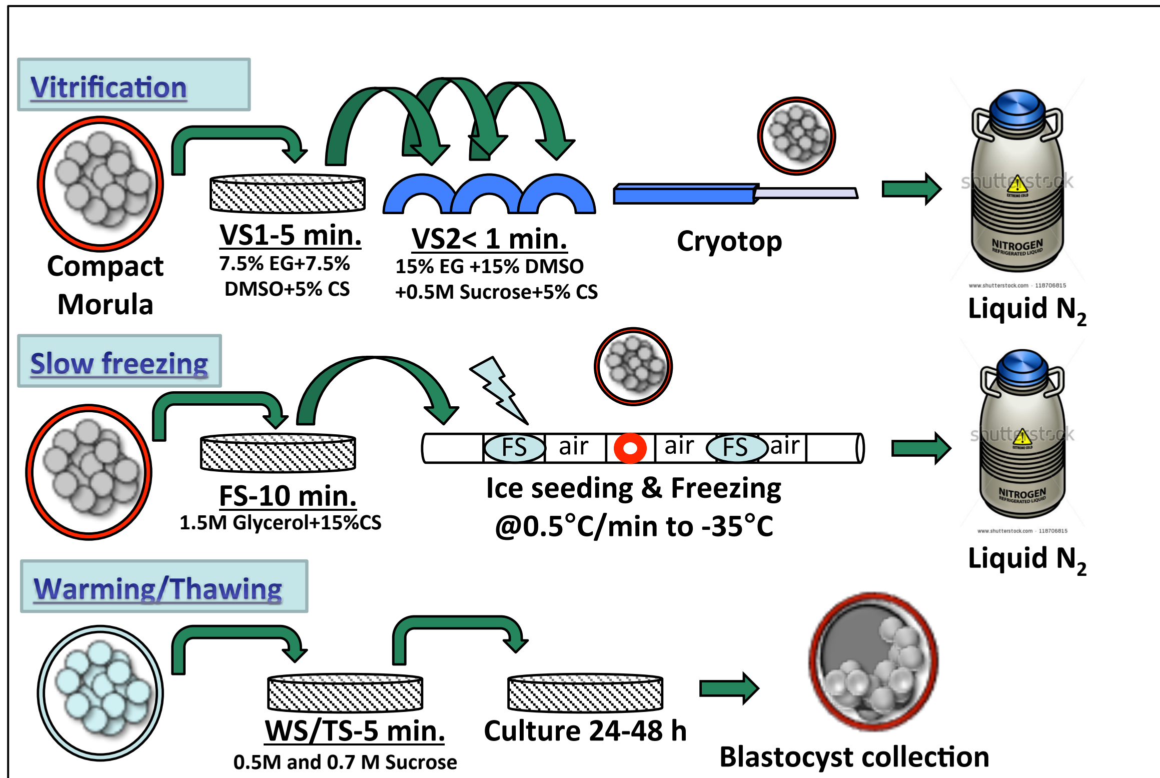
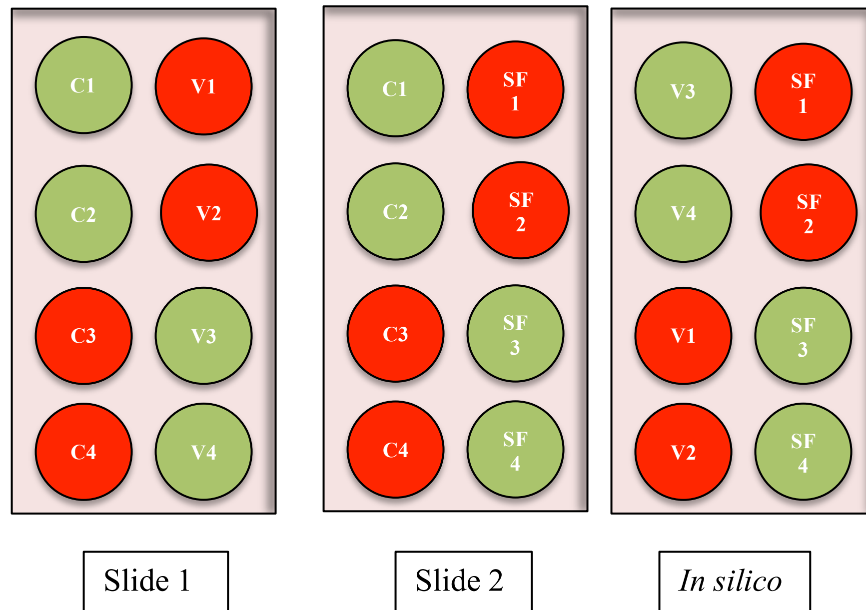
Appendix-I



Supplementary Figure 1. Cryopreservation protocol overview (vitrification and slow freezing) for IVP bovine embryos. VS1- Vitrification solution 1; VS2- Vitrification solution 2; FS- Freezing solution; EG- Ethylene Glycol; DMSO- Dimethyl Sulfoxide; CS- Calf serum; WS-Warming solution; TS- Thawing solution. (Liquid Nitrogen tank photo from Shutterstock®).



Supplementary Figure 2. Microarray hybridization scheme; Slide 1: Control (reference) vs Vitrification; Slide 2: Control (reference) vs Slow freezing; Sample from same control (reference) group of each biological replicate was used to compare with two treatment groups on separate slides; *In silico* analysis-Vitrification vs Slow freezing (reference). Green color refers to Cy3 labeled samples and red represents Cy5 labeled samples. A dye swap technique (as shown) was utilized to compare among the treatment groups using 4 different biological replicates. C1-4- Control reference group (4 biological replicates); V1-4- Vitrification group; SF1-4- Slow freezing group. *In silico* analysis for vitrification vs SF group was done virtually only.