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## Laser-assisted zona pellucida hatching of frozen-thawed in vivo-produced bovine embryos

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**LASER-ASSISTED ZONA PELLUCIDA HATCHING OF FROZEN-THAWED  
IN VIVO-PRODUCED BOVINE EMBRYOS**

**A Thesis**

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

**in**

**The Interdepartmental Program in  
the School of Animal Sciences**

**by  
Mindy K. Chiasson  
B.S., Louisiana State University, 2006  
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## ABSTRACT

Incomplete zona hatching or failure of the zona to rupture compromises post-transfer embryo viability and conceptus development. Assisted hatching prior to the transfer of frozen-thawed bovine embryos has been proposed as a means to increase recipient pregnancy rates. The objective of this study was to determine if laser-assisted hatching would improve in vivo-produced frozen-thawed bovine embryo hatching and pregnancy rates. In Experiment I, II and III frozen direct-transfer embryos received either two or three symmetrical rents at either 40% or 80% through the outer zona surface using the Hamilton Thorne XYClone<sup>®</sup> (Hamilton Thorne Biosciences) diode laser at 90% power with a 600  $\mu$ second pulse (Treatment A) or no zona renting (Treatment B). Embryo hatching rates combined were 51% of 86 embryos for Treatment A and 54% of 86 embryos for Treatment B. In Experiment IV, in vivo-produced nonsurgically collected direct transfer frozen-thawed Hereford embryos (n = 64) were utilized. In Experiment V, in vivo-produced nonsurgically collected glycerol frozen-thawed Brangus embryos (n = 46) were utilized. In Experiments IV and V, embryos received three symmetrical rents ~40% through outer zona surface at 90% power with a 600  $\mu$ second pulse (Treatment A) or no zona renting (Treatment B). In Experiment IV, treatment did not affect pregnancy rates at 35 days or 60 days of gestation and were 41% and 28% for Treatment A and 44% and 41% for Treatment B, respectively. Likewise, there was no difference in calving rate for recipients confirmed pregnant at 60 days for Treatment A (89%) and Treatment B (77%). In Experiment V, pregnancy rates at 35 days and at 60 days of gestation were not affected by treatment and were 65% and 65% for Treatment A and 78% and 65% for Treatment B, respectively. Calving rates were not different for those recipients in Experiment V confirmed pregnant at 60 days for Treatment A (73%) and Treatment B (73%). In conclusion, laser-assisted hatching does not increase the number of in vivo-produced bovine embryos that hatch following in vitro culture or increase pregnancy rates of recipients receiving in vivo-produced frozen-thawed embryos.

## **CHAPTER I**

### **INTRODUCTION**

The application of assisted reproductive technologies on mammalian embryos has been increasing over the last 20 years. The use of assisted reproductive techniques includes the dissection of embryos, inner cell mass (ICM) transfer, nuclear transfer, gene targeting and intracytoplasmic sperm injection (ICSI) and more recently assisted hatching using a diode laser. It has also been demonstrated that with additional application of a diode laser will improve the efficiency of these techniques. For example, openings or rents are made in the zona pellucida using the laser to allow for the incursion of the sperm head when conducting ICSI (Rienzi et al., 2001) or the injection of embryonic stem cells into an embryo (Gridley et al., 2007). It has also been noted that ICSI human embryos that were laser-assisted and transferred have increased implantation rates compared with those with an intact zona pellucida (Wright et al., 1990). The application of laser-assisted hatching could be beneficial in the transfer of in vitro or in vivo-produced embryos by increasing blastocyst hatching rates, which may increase implantation rates and subsequently increase pregnancy rates.

The four methods of assisted hatching are mechanical, chemical, enzymatic or laser manipulation. Mechanical hatching is conducted by incomplete zona pellucida dissection of sheep (Trounson et al., 1974) and human embryos (Cohen et al., 1990). The problem associated with this method is the difficulty of creating a rent of a constant size. Tyrode solution is a chemical means that allows for larger and more consistent rent size. The application of acid is utilized for renting the zona pellucida (Cohen et al., 1992). However, this solution could potentially be toxic to the embryo. Pronase solution is used to enzymatically dissolve or thin the zona pellucida. The 1.48  $\mu\text{m}$  infrared diode laser is the most recent addition to the list of assisted hatching options. The application of laser-assisted hatching technique has been

proposed as a more consistent and easier than mechanical and chemical means. Balaban et al. (1992) conducted a study comparing these four different techniques on human embryos and showed similar implantation and pregnancy rates. Implantation and pregnancy rates were not different for mechanical (19% and 49%), chemical (17% and 46%), enzymatic (19% and 47%) and diode laser (19% and 48%) and when compared with the control (22% and 48%). However, problems are associated with the application of these different assisted hatching methods (embryo degeneration and the occurrence of monozygotic twinning) needs to be evaluated (Balaban et al., 2002).

In 1890, Walter Heape performed the first successful pregnancy following the transfer of a rabbit embryo. It was Wilmut and Rowson (1973) that were the first to produce a live calf from an in vivo-produced bovine embryo. Cryopreservation has not only allowed the transfer of embryos at a later date following collection, but has also allowed the handling of embryos at a much larger scale in commercial production. This permits the ability for exceptional genetics to be transferred around the world so indigenous and exotic breeds can be preserved in embryo cryopreservation banks. However, pregnancy rates following frozen-thawed embryo transfer are lower than those of fresh in vivo-produced embryos. Therefore, the effects of cryopreservation can be detrimental to the viability and developmental competence of embryos. Calving rates of frozen-thawed flushed in vivo-produced bovine embryos are lower than those of fresh in vivo-produced bovine embryos (Baguisi et al., 1998).

Structural changes to the zona pellucida due to cell damage has been shown to occur in bovine embryos during the freezing and/or thawing procedure (Mohr and Trounson et al., 1981). It has been shown that damage to the embryo may include membrane disruption, damage to junctions between trophectoderm cells, cytoskeletal defects, blastomere extrusion and possibly zona pellucida hardening (Willadsen et al., 1977; Mohr et al., 1981; Baguisi et al., 1998). The combination of zona pellucida hardening and the decrease in viable cells caused by

the exposure to cryopreservation could potentially impair the blastocyst's ability to hatch. A marked increase in injury to the bovine embryo has been shown to occur between day-5 and day-13 (Hyttel et al., 1986) and ~30% of frozen-thawed bovine embryos have exhibited damaged zonae pellucidae (Tervit et al., 1981).

The decrease in pregnancy rates can be approximately 10 to 13% between fresh and frozen-thawed bovine embryos of comparable quality grades (Hasler et al., 2001). Pregnancy rates with frozen-thawed bovine embryos are variable between 3 and 60% after transfer. It has been suggested that a rent made in the zona pellucida using laser-assisted hatching may improve pregnancy rates of frozen-thawed in vivo-produced bovine embryos (Baguisi et al., 1998; Taniyama et al. 2007).

Assisted hatching may promote hatching of the blastocyst from the zona pellucida, permitting interaction between the embryo and maternal endometrium resulting in an increase in pregnancy rates. However, introducing a rent in the zona, utilizing laser-assisted hatching, may cause hatching through a thickened zona pellucida leading to incomplete hatching of the blastocyst. The blastocyst hatches by a weakening of the zona pellucida through a combination of the secretion of lysin and increased pressure, which allows the embryo to escape through an opening in the zona pellucida (Gordon et al., 1993). The effects of assisted hatching on cryopreserved human embryos after thawing have shown both promising (Strohmer et al., 1992; Peterson et al., 2005) and dubious (Peterson et al., 2006) results based on implantation rates and improving pregnancy.

Lopatarova et al. (2001) conducted an experiment comparing frozen-thawed in vivo-produced bovine embryo pregnancy rates after zona pellucida manipulation. Rents were made in the embryo's zona pellucida by either a microsurgical knife or with a micro-blade. The length of the rent was between 30-50  $\mu\text{m}$ . After thawing and morphological evaluation, a rent was

made in the zona pellucida and embryos were then cultured in vitro or transferred. The results of in vitro experiments showed higher hatching rates for both, but there were no difference in implantation rates after assisted hatching. Pregnancy rates following transfer of in vivo grade 2 or 3 bovine embryos were 58.7% for the assisted hatched group and 47.9% for the control groups. The results from the transfer of frozen-thawed grade-1 bovine embryos demonstrated the use of assisted hatching contributed to better pregnancy rates (57.5% vs. 45.3%). However, it has been shown that zona manipulation of frozen-thawed grade 2 bovine embryos had no effect on the pregnancy rates (30% vs. 33.3%; Lopatarova et al., 2001).

Balaban et al. (2006) showed an increase in pregnancy rates of in vitro frozen-thawed human embryo transfers after applying laser-assisted hatching. The application of laser-assisted hatching increased implantation (20.1% vs. 9.9%) and pregnancy rates (40.9% vs. 27.3%) for laser-assisted and control, respectively. There have been other studies showing the successful clinical application of laser-assisted hatching in human in vitro fertilization program (Malter et al., 1989; Strohmer et al., 1992; Neev et al., 1995; Rink et al., 1996; Peterson et al., 2005).

Menges et al. (2008) conducted a study that evaluated laser-assisted hatching utilizing the XYClone<sup>®</sup> laser system (Hamilton Throne Biosciences, Beverly, MA) on in vitro-produced bovine embryos. The results demonstrate that laser-assisted hatching using the XYClone<sup>®</sup> does not have an effect on in vitro embryo viability and development. An increase in the 60-day pregnancy rate was noted for embryos that were laser-assisted compared with the controls. The data showed the diode laser could be utilized to improve 60-day pregnancy rates of in vitro-produced embryos (Menges et al., 2008).

Peterson et al. (2006) conducted a study to evaluate the effect of thinning one quarter of the zona pellucida of frozen-thawed human embryos using qLZT-AH laser system (Fertilase<sup>™</sup> system; Medical Technologies Montreux, Lausanne, Switzerland) on implantation and



pregnancy rates. A total of 350 laser-assisted cryopreserved human embryo zonas were cut to the depth of 50 to 80% of the original thickness and were compared with 352 zona intact embryos. No difference in implantation or pregnancy rate was found after using this technique, this supports similar findings of Ng et al. (2005). Therefore, it has been suggested that laser-assisted hatching should not be performed on all frozen-thawed human embryos (Peterson et al., 2006; Valojerdi et al., 2009).

The objective of this study was to evaluate the effects of laser-assisted zona pellucida hatching on frozen-thawed in vivo-produced bovine embryos after in vitro culture and transfer. Blastocyst hatching patterns and complete hatching was compared with rents made with the application of laser-assisted hatched group (Treatment A) to the intact zona pellucida sham control group (Treatment B) when exposed to in vitro culture. Pregnancy and calving rates of frozen-thawed in vivo-produced bovine embryos either laser-assisted hatched (Treatment A) or intact zona pellucida (Treatment B) were compared to determine if increased pregnancy rates and calving rates was associated with partial dissection of zona pellucida using the application of the XYClone<sup>®</sup> diode laser.

## CHAPTER II

### LITERATURE REVIEW

#### **Functions of the Zona Pellucida**

The mammalian growing oocytes, ovulated oocytes and early embryos are surrounded by an extracellular coat. This coat is referred to as the zona pellucida in mammals (Goudet et al., 2008). The zona pellucida, a name derived from Latin, means transparent (pellucida) belt or girdle (zona). In the ovarian primordial follicles the non-growing oocytes lack the zona pellucida (e.g. mouse). The zona is produced during the late stages of oogenesis. The growth stage initiates the increase in the thickening of the zona pellucida as oocytes increase in diameter. The thickness of the zona varies from 2 to 20  $\mu\text{m}$  depending on the species (Wassarman et al., 2004). The average reported thickness of the zona pellucida for the cow is between 12 and 15  $\mu\text{m}$  (Hartman et al., 1931).

The zona pellucida provides species-specific binding of sperm and blocking of multiple sperm from entering the ovum and referred to as polyspermy. It also maintains blastomere structure during preimplantation development, protects the ovum during oviduct transport and protects the oocyte and/or embryo during the early stages of development from infection (Vanroose et al., 2000).

Upon completion of the acrosome reaction, species-specific sperm binding occurs. The removal of the zona pellucida from unfertilized ova removes this sperm-specific barrier when exposed to in vitro culture. After fertilization, the structure of the zona pellucida is changed such that it no longer permits binding of sperm. An expanded blastocyst must hatch from the zona pellucida before implantation can occur in the uterus.

## **Structure of the Zona Pellucida**

The zona pellucida structure is primarily composed of glycoproteins, whereby water molecules are taken up by proteins and carbohydrates (Nara et al., 2006). The zona is an elastic structure that is porous and aids in the transportation of large macromolecules (antibodies, small viruses and nutrients). The zona pellucida can enhance or restrict the embryo's growth and developmental competence (Sinowatz et al., 2001). The zona pellucida can be dissolved by exposing the zona pellucida to in vitro culture conditions with a pH of 2.5 to 3.5, high temperatures or low ionic strength buffers (Wassarman et al., 2004).

The zona structure consists of long, interconnected filaments. The filaments that make up the inner layer of the zona pellucida are oriented radially. The filaments that collect the outer zona pellucida layer are oriented tangentially. The middle layer of the zona pellucida is constructed as a double refraction (Sifer et al., 2006).

### Zona Pellucida Glycoproteins

In cattle, the zona is composed of three zona pellucida glycoproteins (ZP1, ZP2 and ZP3) (Sinowatz et al., 2001). These three glycoproteins make up 95% of the total zona protein and were named according to the cDNA size (Harris et al., 1994; Green et al., 1997).

The glycoproteins are synthesized by the oocyte, follicle cells or both depending upon the species. In the cow, it has been shown that both the oocyte and follicle cells contribute to the synthesis of the zona pellucida glycoproteins and synthesis occurs during all stages of follicular development (Sinowatz et al., 2001). A surplus of glycoproteins is found in the inner surface of the zona or close to the plasma membrane of the oocyte. In the mouse, the zona pellucida thickens from the region closest to the plasma membrane and outermost layer of the zona pellucida (Wassarman et al., 2004).

The glycoproteins are arranged in a specific order in the zona pellucida. ZP2 and ZP3 form long filaments that make up the extracellular coat and ZP1 serves as the connection between these filaments (Wassarman et al., 1987; Sinowatz et al., 2001; Wassarman et al., 2004). These three glycoproteins are acidic with a pH between 4.1 and 5.2. Their acidity is associated with asparagine- (N-) and serine/threonine- (O-) linked oligosaccharides. In cattle oocytes, the percentage of carbohydrate chains that form the N-linked oligosaccharides are neutral (23%) and acidic (77%) (Katsumata et al., 1996). The oligosaccharides attached to the zona pellucida glycoproteins are sialylated and sulfated (Wassarman et al., 2004). All acidic chains are neutralized by sialidase digestion. It has been shown that the acidity of the proteins from fertilized ova is weak when compared with unfertilized ova. Sialidase released from the oocyte at fertilization causes the change in acidity of the associated glycoproteins (Katsumata et al., 1996).

These oligosaccharides are responsible for sperm binding, initiation of the acrosome reaction and penetration of the zona pellucida (Braden et al., 1954; Rath et al., 2005). ZP3 is the primary species-specific sperm receptor that induces the acrosome reaction following binding of the sperm to the zona pellucida. Binding of the sperm to the zona pellucida occurs at the O-linked oligosaccharides that are present on ZP3 (Bleil et al., 1980; Sinowatz et al., 2001; Sun et al., 2003; Wassarman et al., 2004). In the mouse, binding of sperm to the ovum is mediated by adhesion molecules  $\beta$ -1,4-galactosyltransferase (GalTase) that are located on the surface of the sperm and connect to the zona glycoprotein ZP3 (Miller et al., 1992). The factors that are involved in the acrosomal signal transduction pathway include G proteins, inositol-3,4,5-triphosphate ( $IP_3$ ),  $IP_3$  receptors, phospholipase C, and  $Ca^{2+}$  (Wassarman et al., 2008).

In mammalian ova, ZP2 is the secondary sperm receptor. The binding of sperm to ZP2 is mediated by the sperm proteases, which are enzymes that digest protein. Acrosin is released from the acrosome and acts like a protease. The  $Ca^{2+}$  influx during fertilization causes the

contents of the oocyte cortical granules to be released into the plasma membrane at the time of sperm-oocyte fusion, which alters ZP2 and ZP3 to become ZP2f and ZP3f (Sun et al., 2003; Wassarman et al., 2004). This change can be blocked by either newborn calf serum (NCS) or fetuin (Schroeder et al., 1990; Kalab et al., 1991).

### **Physical Properties of the Zona Pellucida**

The physical properties of the zona pellucida reflect their chemical structures. When the zona pellucida is stretched and released instantly, it returns to its original position. If the zona pellucida is stretched and held for an extensive period of time, upon release it will return to an intermediate position (Green et al., 1997). When the oocyte is exposed to vigorous pipetting, the zona pellucida may crack without losing its circular shape. The zona pellucida is an elastic solid that has the ability to recover from the temporary expansion caused by modest pipetting (Drobinis et al., 1988; Green et al., 1997).

Green et al. (1997) suggested that neutral or acidic carbohydrates account for virtually half the mass of the mouse zona pellucida. The zona structure is considered to have a low ionic strength. In the pig, the zona pellucida will separate when exposed low ionic strength at 37°C more quickly than at a high ionic strength. This separation caused by low ionic strength can be blocked by lowering the temperature.

Lower temperatures cause tension on the zona pellucida filaments, which originates from swelling without the separation of its components. The tension and exposure to proteolytic cleavage on the zona pellucida causes it to rupture. These observations demonstrate that low ionic strength results in permanent changes and swelling of the zona pellucida (Green et al., 1997).

## Cortical Reaction

Fertilization is marked by the release of intracellular  $\text{Ca}^{2+}$ , which is essential for ova activation and the onset of early embryonic development. Following sperm penetration or activation of ova, cortical granules release their contents into the perivitelline space, which is termed the cortical reaction. Cortical granules are located in the cortex of the meiotic metaphase II (MII) stage oocyte. The release of the cortical granule exocytosis following the cortical reaction will cause structural changes to the zona pellucida and perivitelline space.

The cortical granule exocytosis signal pathway involves phosphatidylinositol biphosphate ( $\text{PIP}_2$ ),  $\text{IP}_3$ , diacylglycerol (DAG), calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) and G proteins. Low  $\text{Ca}^{2+}$  levels will stimulate a short cortical reaction, while numerous pulsated levels are required for a complete cortical reaction.

The increase of intracellular  $\text{Ca}^{2+}$  in mammalian oocytes occurs within 1 to 4 minutes after the sperm binding to the plasma membrane. The cortical reaction takes place for 5 to 30 minutes following the first spike in  $\text{Ca}^{2+}$  increase. CaMKII is activated in response to the rise in intracellular  $\text{Ca}^{2+}$ . CaMKII is required for cortical granule exocytosis to occur. PKC aids in the sperm acrosomal exocytosis and is activated by DAG, which induces the cortical granule exocytosis. It has been shown in sea urchins that activation of the G protein will initiate the  $\text{IP}_3$  production causing an increase in  $\text{Ca}^{2+}$  and cortical granule exocytosis. In the mouse, it has been shown that cortical granule N-acetylglucosaminidase adhere to sperm GalTase-binding sites thus blocking polyspermy (Miller et al., 1993). Exocytosis of cortical granules is one of the earliest  $\text{Ca}^{2+}$  dependent events that result in the block to polyspermy (Abbott et al., 2001; Sun et al., 2003).

### Polyspermy Block

Polyspermy is the invading of the oocytes cytoplasm by more than one sperm. Polyspermy can take place in insects, reptiles, birds and mammals. In mammals, polyspermy will result in failure for the embryo to properly develop. The  $\text{Ca}^{2+}$  oscillations, after fertilization of ova, induce ovum activation (Vitullo et al., 1992). Thus activation process initiates embryonic development and blocks polyspermy.

The  $\text{Ca}^{2+}$  oscillations are believed to control the initiation of the polyspermy block. In the mouse, the initiation of the polyspermy block is slower in ova with modest or completely suppressed  $\text{Ca}^{2+}$  than ova that go through normal levels of  $\text{Ca}^{2+}$  oscillations (Gardner et al., 2007). The cause of polyspermic fertilization during in vitro is due to the delay or incomplete cortical granule exocytosis interrupting the zona pellucida reaction or zona hardening.

### Hardening of the Zona Pellucida

Braden et al. (1954) reported the occurrence of the mouse zona pellucida reaction or zona hardening observed under in vivo conditions. It was noted that only a small number of sperm were removed from fertilized mouse ova. It was proposed that sperm penetration generated the ovum reaction, which prevents any additional sperm from entering the zona pellucida. It was suggested that cumulus cells surrounding the oocyte may protect the zona pellucida against hardening. Once the ovum is removed from the cumulus cells zona pellucida hardening can occur. The hardening of the zona pellucida has a direct influence on the ability of the ovum to fertilize (Katska et al., 1989).

The proteolytic conversion of ZP2 to ZP2<sub>f</sub> is considered to be the cause of the hardening of the zona pellucida following fertilization (Wassarman et al., 2008). Zona pellucida hardening is caused by proteolysis of the ZP2 (Velásquez et al., 2007). Fetuin is a plasma glycoprotein

found in fetal and neonatal bovine calves and can prevent the conversion of ZP2 to ZP2<sub>f</sub> by inhibiting proteolytic activity when added to in vitro culture (Schroeder et al., 1990).

Zona hardening is associated with the modification of the physical properties of the zona, or the thickening of the zona pellucida. Zona pellucida hardening is marked by the increase to the resistance of protease. The increased resistance to proteolytic digestion is not always marked by an increase in thickness of the zona pellucida. The increase in thickness requires an increase in swelling pressure, which is caused by an increase in the number of inter-filament cross-links within the zona pellucida (Green et al., 1997). The increase in pressure is due to the interaction between the inter-filament cross-links and tyrosine residues in the mouse (Green et al., 1997) and between the disulfide bond formation and the zona pellucida protein components in the rat and cow (Iwamoto et al., 1999).

The resistance to proteolytic digestion is directly associated to contact with proteolytic enzymes, which conceal the sites of attack or by cross-linking the zona pellucida preventing proteins from unfolding (Green et al., 1997). Nara et al. (2006) showed the thickness of the bovine zona pellucida of oocytes isolated from the ovary and embryos using transmission electron microscopy. The results show that the thickness of the zona pellucida of ovarian oocytes was  $11 \pm 0.6 \mu\text{m}$  and was then decreased after fertilization to  $7 \pm 0.2 \mu\text{m}$ , which suggests that compaction of the zona pellucida structure occurs after fertilization.

The zona reaction is the basis for increased resistance to zona dissolution by various agents. The zona pellucida can be removed using proteases, disulfide-bond reduction, sodium periodate, low pH and high temperatures (Drobnis et al., 1988). Hardening of the zona pellucida in cattle can be initiated by insertion of cumulus-free oocytes in both homological and heterological oviducts exposed to in vitro or in vivo conditions (Katska et al., 1989). In the mouse, the zona pellucida of unfertilized oocytes were found to dissolve more readily than 2-cell embryos. However, no differences in solubility properties of zonae pellucidae have been noted



between in vivo-produced 2-cell mouse embryos and in vitro-produced embryos (Inoue et al., 1974). In the cow, the zona pellucida was shown to be insoluble in isotonic medium at pH 3.0, while the zona pellucida of the pig oocytes are solubilized within 20 seconds after being placed in lactic acid-PBS at pH 3.6. These results indicate that bovine zonae pellucidae have a more rigid structure than that of pigs.

Cleavage of hydrogen bonds and the decline of disulfide association are essential for solubilization of the zona pellucida to occur (Iwamoto et al., 1999). In a study using mouse oocytes, Drobin et al. (1988) illustrated that structural changes were not correlated with the resistance to dissolution. The hamster zona pellucida becomes more elastic over time rather than becoming more rigid. These findings suggest that not every species has spontaneous zona pellucida hardening.

Changes in the structure of the zona pellucida and plasma membrane of aged mouse oocytes are often associated with a decrease in fertility (Longo et al., 1981). Mouse (Larman et al., 2006), pig (Coy et al., 2008), sheep (Huneau et al., 1994), horse (Dell'Aquila et al., 1999) and cattle (Iwamoto et al., 1999) oocytes have been shown to undergo spontaneous zona pellucida hardening when exposed to in vitro culture conditions. Katska et al. (1989) showed that preovulatory bovine oocytes had very rapid zona pellucida digestion by pronase, which implies high sensitivity of the zona pellucida in small and medium size follicles of 2 to 6 nm in diameter. It was concluded that hardening of the zona pellucida does not occur at all stages of follicular development and oocyte maturation. These alterations may affect the viability of manipulated mammalian oocytes and embryos.

### **Hatching of Blastocyst from the Zona Pellucida in Mammals**

During early preimplantation development, most mammalian embryos shed the zona pellucida in a process known as hatching. Hatching of the blastocyst must occur within the time period that the uterus is receptive or pregnancy will not ensue. The two factors that facilitate

rupture of the zona pellucida and blastocyst escape are proteolytic sublysis of the zona pellucida either by the embryo or the endometrial lining of the dam (Perona and Wassarman, 1986) and/or the hydrostatic pressure that is generated by the fluid accumulation in the expanding blastocoel cavity (McLaren et al., 1970).

Morishita et al. (1993) suggested that protein synthesis in the hatched embryos is higher than in the embryos that failed to hatch. Furthermore, trophoblast cells secrete trypsin-like substances, plasmin, glutamine, insulin and epidermal growth factor, which are products of amino acids. The protein synthesis in embryos is considered a critical factor in blastocyst hatching. It was suggested that the culturing the mouse blastocyst with human placental cells enhances RNA synthesis and RNA synthesis has been found to promote blastocyst hatching (Morishita et al., 1993).

Hitoshi et al. (1990) suggested that trypsin-like protease secreted from mouse embryos does not completely dissolve the zona pellucida. Partially digested or weakening of the zona pellucida enables the mouse embryo to shed the zona pellucida. Due to the amino acid sequence of ZP3, which consist of arginine residue with about 26% threonine (or serine)-arginine-X sequence, the zona pellucida is susceptible to protease digestion (Hitoshi et al., 1990). This concept is supported by the culturing of mouse embryos in altered amino acid solutions and in the presence of serine protease.

Schiewe et al. (1995) conducted a study using the blastocyst anti-hatching mouse model to show that embryonic secretion of a zona lysin is the primary control of hatching. The trophoblast is responsible for secreting the zona lysin (e.g., trypsin) at the early blastocyst stage. The possible reduction of trypsin has been proposed as a factor that could inhibit hatching and prevent implantation of morphologically normal appearing embryos (Schiewe et al., 1995). The inability to hatch may be due to a decrease in lysin production below the level needed to thin the zona pellucida or the inability of some embryos to secrete lysin.

It has also been shown that bovine embryos produce a serine protease and plasminogen activator during blastocoelic expansion and hatching (Menino et al., 1986; Cannon et al., 1998). The two types of plasminogen activators are the tissue type (tPA) and urokinase-type (uPA). It has been suggested that tPA is directly correlated with the hardening of the zona pellucida in rats (Zhang et al., 1991), while uPA is involved in the hatching of sheep blastocyst (Menino et al., 1989). Menino et al. (1986) showed that the addition of uPA plasminogen to in vitro culture medium will increase bovine blastocyst hatching rates.

The mechanical pressure applied on the zona pellucida by the expanding blastocyst is regulated by the enzymatic system that pumps fluid into the developing blastocoele. The pressure exerted by the expanding blastocyst applied against the zona pellucida before hatching has little effect on the escape of the embryo from the zona pellucida. Schiewe et al. (1995) demonstrated that the mouse embryos exposed to in vitro culture conditions decreases hatching rates but does not have any effect on blastocyst expansion using a protein-free medium anti-hatching model. Results showed that expanded blastocyst cell numbers and zona pellucida digestion were not different between mouse embryos that were cultured in serum-free human tubal fluid medium compared with those cultured with serum. The primary mechanism of blastocyst hatching is now thought not due to physical expansion. This was shown, in another study, where human embryos inserted into oil droplets to increase pressure within the zona pellucida, blastocyst hatching did not occur (Gordon et al., 1993).

Schiewe et al. (1995) showed there was no difference in the enzymatic digestion of the zona pellucida, which suggests that abnormal zona pellucida hardening and physical expansion are not factors in the blastocyst inability to hatch completely. Gordon et al. (1993) reported that the ratio of lysin produced and zona pellucida thickness determines whether the embryo will be capable of hatching. Embryos with an abnormal zona pellucida or following trauma, such as freezing and/or thawing may benefit from the application of assisted hatching.

## **Effects of Cryopreservation on Embryos**

Embryo transfer (ET) technology was first reported in rabbits by Heape in 1891. In 1951 Willet et al. reported successful results of embryo transfer in cattle. Cryopreservation provides a means of transferring embryos at a later date, handling of embryos on a larger scale, allows genetic material to be moved around the world and indigenous and exotic breeds to be preserved for embryo banks.

In 1949, Polge et al. discovered that glycerol provided the cryoprotection needed for spermatozoa survive following freezing and thawing. Smith et al (1952) results showed that rabbit embryos survived cryopreservation in glycerol media. Freezing of mammalian oocytes first involved freezing of the whole ovary and grafting to another female (Parkes et al., 1953; Deanesly, 1954). The cryopreservation of mammalian embryos has been effectively applied to numerous species. Reports from the early 1970s showed that successful development of in vivo frozen-thawed mouse embryos resulted in pregnancy rates of 65% (Whittingham, 1971; Whittingham et al., 1972). In the 1970s frozen-thawed embryos from goats (Bilton et al., 1976b), sheep (Willadsen et al., 1974; Bilton et al., 1976a) and cattle (Wilmot et al., 1973; Bilton et al., 1977) produced viable offspring. Wilmot and Rowson (1973) were the first to report successful pregnancies in cattle from the transfer of frozen-thawed blastocysts.

As early as the 1950s and 1960s, studies indicated that a very low percentage of rabbit embryos developed after the freeze-thaw process (Chang, 1948; Smith, 1952). Bank and Maurer (1974) showed that rabbit embryos at the 8-cell stage did not survive after cryopreservation and it was noted that the injury was caused by osmotic changes during the thaw process. Results from other studies have shown a reduction in the pregnancy rates following the transfer of frozen-thawed compared with fresh bovine embryos. Pregnancy rates have been reported to be 10 to 13% higher in cattle for fresh compared with frozen-thawed

embryos of comparable grades (Hasler et al., 2001). Generally, pregnancy rates following frozen-thawed mouse embryo transfer are reduced (Whittingham et al., 1976; Rall et al., 1985).

Successful cryopreservation of bovine embryos reduces the need for suitable recipients at the time of embryo collection. The viability of the embryo is determined by the embryos ability to cleave, form the blastocoel cavity and hatch from the zona pellucida. Today embryos are assigned grades based on color, blastomere size, cytoplasmic granulation and degree of blastomere fragmentation. When transferring bovine embryos based on this grading system, pregnancy rates of 55 to 65% can be achieved when excellent quality in vivo-produced embryos are utilized (Overstrom, 1996). However, when cryoprotective agents (glycerol, propylene glycol and ethylene glycol) have been added to the embryos prior to freezing they are often embryo toxic (Dobrinsky, 1996).

Over the years, embryos have been frozen and thawed at different stages with varying results. For example, human embryos that are frozen at the pronuclear stage have higher pregnancy and live birth rates when compared with early cleaved embryos. The blastocyst stage is usually the stage of choice for freezing (Huang et al., 2007). The injury induced by cryopreservation is more pronounced in day-5 and day-13 bovine embryos (Hyttel et al., 1986).

Mohr and Trounson et al. (1981) demonstrated that the collapse of the blastocoele and damage to cell components occurred in the embryos during the freezing and/or thawing process supporting a previous study by Willadsen et al. (1977). Cells suffer extensive physical stress during cryopreservation procedures. Research shows that cryopreservation can cause damage to the microvilli on the plasma membrane, swelling of the mitochondria, accumulation of molecular debris and reduction in the junction contacts between the trophoblastic cells (Mohr et al., 1981; Fair et al., 2001; Moreira et al., 2005). Kaidi et al. (2000) showed that there is a decrease in total cell number and death of inner cell mass (ICM) cells of frozen-thawed bovine embryos. The trophoblastic cells are more often affected by the freezing and/or thawing

procedures (Kaidi et al., 2000). Those cells are directly associated with metabolic activity during blastocyst expansion and hatching.

Intact blastomere plasma membranes is critical to the function and viability of the embryo and provides a barrier to the extracellular environment. This blastomere plasma membrane establishes and maintains ionic gradients, intracellular pH and solute specific exchanges. Cryopreservation compromises membrane integrity, which can lead to cell death. Dobrinsky et al. (1996) showed that cryopreservation causes a decrease in cytoplasmic microfilament and microtubules, but the association of the cytoskeleton and cellular nuclei were unaltered.

Cryopreservation causes cell death to occur in mammalian embryos through apoptosis or necrosis. Necrosis may be due to solution effects, osmotic effects or physical injury by growing ice crystals. Apoptosis is the programming of cell failure as a response to subnecrotic events. Cells with more flexible membranes will usually have less membrane damage than those with more rigid membranes. Injury to the embryo during the freeze and/or thaw process includes membrane disruption, cytoskeletal destabilization and possibly zona pellucida hardening (Baguisi et al., 1998).

Calving rates in frozen-thawed in vivo-derived bovine embryos are lower than those achieved by transfer of fresh in vivo embryos (Baguisi et al., 1998). The zona pellucida has been shown to be damaged in approximately half of frozen-thawed bovine embryos (Hyttel et al., 1986). Damage caused by cryopreservation may involve changes on the zona pellucida and the loss of embryo mass/volume, which can affect the embryos ability to hatch and produce pregnancy (Bielanski et al., 1986; Morieira et al., 2005). It has been demonstrated in mouse oocytes that the duration of time it takes for the zona pellucida to be dissolved enzymatically was greater in frozen-thawed mouse oocytes than fresh oocytes. It was suggested that

cryopreservation may induce cortical granule exocytosis in these oocytes, which may cause zona pellucida hardening (Coticchio et al., 2007; Gardner et al., 2007).

Abnormal zona pellucida hardening, increased thickness and reduction in lysin have been proposed as factors that could inhibit hatching and prevent implantation of embryos that appear morphically normal. Tervit et al. (1981) reported that 30% of frozen-thawed bovine embryos had damaged zonae pellucidae. However, there have been conflicting reports on the effect of freeze-thaw process on the hardening of the zona pellucida of cryopreserved embryos. A study conducted on mouse frozen-thawed 2-cell embryos, showed there was no hardening of the zona pellucida or even a slight softening. Other reports indicate that there are no additional effects of the cryopreservation process other than modifications to the mouse zona pellucida after fertilization (Matson et al., 1997). However, it appears that bovine embryos are much more fragile than mouse embryos. Some studies have suggested that partial dissection assisted hatching may be beneficial in improving pregnancy rates of the transfer of frozen-thawed mouse (Gordon et al., 1993) and human embryos (Gabrielsen et al., 2004).

#### **Four Methods for Assisted Hatching**

The success of embryo transfer depends upon communication between the transferred embryo and the endometrium. In vivo hatching of the embryo at the blastocyst stage is an essential step in the events culminating in successful implantation. The thinning of the zona pellucida is required for in vivo hatching to occur. It has been shown that pregnancy rates range from 10% to 29% for embryos with a consistent zona pellucida thickness compared with a thin zona pellucida (Cohen et al., 1989). Later, Cohen et al. (1990) reported reduced implantation rates following in vitro fertilization (IVF) of human embryo may be due to the inability of the embryo to hatch from the zona pellucida, which is supported by other studies (Hasler et al., 1995; Chao et al., 1997). Also, extended culture may induce zona pellucida hardening, which can be evaluated on dissolution times of the zona pellucida using a protease solution. It has

been proposed that zona pellucida hardening may be due to cryopreservation, which could inhibit hatching. Furthermore, zona pellucida hardening has been shown to be increased by the frozen-thaw process (Gabrielsen et al., 2004).

Several techniques for assisted hatching have been introduced to breach or to thin the zona pellucida and promote the embryo hatching process. Assisted hatching can be performed by mechanical (Cieslak et al., 1999), chemical (Cohen et al., 1990), enzymatic (Fong et al., 1998) or laser manipulation (Neev, 1995). Assisted hatching was initially performed using mechanical means. Mechanical hatching is a partial zona pellucida dissection. The embryo is held in position by a holding pipette and the micro-needle is passed through the zona pellucida and a rent is made (Cieslak et al., 1999). One of the limitations of this technique is the difficulty of creating an opening of consistent size.

The second technique allowing for larger and more consistent rent size, is the use of the chemical method (acid Tyrode solution) for drilling of the zona pellucida. Embryos are stabilized with a holding pipette and a 30  $\mu\text{m}$  diameter rent in the zona pellucida is created by a Tyrode acid washed over the surface of the zona pellucida (Cohen et al., 1990). Embryos are then rinsed to remove excess Tyrode solution from the zona pellucida. However, inconsistency and possible embryo toxicity remain as potential problems.

Enzymatic methods to dissolve or thin the zona pellucida are performed by using pronase solution. Embryos are transferred to the pronase solution for 60 seconds for initial dissolving of the zona pellucida (Fong et al., 1998). The zona pellucida is thinned but not completely removed. If no change in the zona pellucida is noted the embryo is then incubated with pronase for an additional 30 to 60 seconds. The embryos are then washed twice to remove any remaining pronase.

The 1.48  $\mu\text{m}$  infrared diode laser is the most recent addition to the list of assisted hatching options (Balaban et al., 2002). The laser is computer controlled and does not require



contact with the embryo. The laser is directly attached to an inverted microscope. The laser has three preset energy intensities of low (35 mW), medium (45 mW) and high (55 mW). Rents can be made at a low power for a very thin zona (<10 µm), medium power (10 to 15 µm), and high power for a thick zona (>15 µm). Laser-assisted hatching is more controlled and consistent compared when with both mechanical and chemical assisted hatching.

A study comparing these four different techniques showed similar post-transfer implantation and pregnancy rates of IVF and ICSI human embryos. Balaban et al. (2002) reported implantation and pregnancy rates for partial zona dissection was 19% and 49%, acid Tyrode's was 17% and 45%, pronase zona thinning was 19% and 47% and laser-assisted was 19% and 48%, respectively. Studies have shown that assisted hatching could be associated with increased implantation rates after IVF, embryo transfer or frozen-thawed embryo transfers in humans (Stein et al., 1995; Sifer et al., 2006). Balaban et al. (2002) also reported twinning rates of 51% of partial zona dissection, 40% of embryos exposed to Tyrode's, 45% of pronase zona thinning and 43% of laser-assisted transferred human embryos. The potential complications (embryo degeneration and monozygotic twinning) using the different assisted hatching methods needs to be further evaluated (Balaban et al., 2002).

Recent studies have reported controversial issues on frozen-thawed embryos related to assisted hatching. Gabrielsen et al. (2004) reported that zona renting (opening) of human embryos using Tyrode's acid increased implantation rates in the assisted hatched group compared with the control group. Although, Sifer et al. (2006) showed that enzymatic zona pellucida thinning prior to human embryo transfer did not improve hatching ratio of cryopreserved embryos when compared with controls.

Cohen et al. (1991) reported that only 16% of 167 of partial zona dissection embryos escaped through narrow holes and hatched completely, while others were trapped in a typical figure-eight shape and 72% of 60 embryos escaped through larger openings. It was proposed

that the human zona pellucida needs to be rented completely through the zona pellucida to enhance implantation ability of embryos.

However, thinning of the human zona pellucida rather than a complete zona renting has been shown to increase blastocyst hatching and implantation rates (Sifer et al. 2006). Zona thinning with pronase advantages are the outer layer is dissolved and the inner layers are weakened by the enzyme. Pronase has been shown to be more effective than trypsin in the digestion of the porcine zona pellucida (Kolbe et al., 2005). However, it has also been suggested that thinning zona of mouse embryos increases the possibility of blastomere loss or the entire embryo during contractions of the female reproductive tract or the inhibition of natural expansion of blastocyst. To avoid these disadvantages, thinning of the mouse zona pellucida was attempted and improvement of blastocyst hatching was noted.

Primi et al. (2004) reported that laser-assisted hatching through complete zona pellucida dissection of human embryos could have a negative effect when not supported by an antibiotic treatment. The implantation rate was 1.6% for the laser-assisted hatched group compared with 6% for group treated with antibiotic, which is consistent with the results published by Cohen et al. (1990).

Jones et al. (2006) compared laser-assisted hatching and acidified Tyrode's hatching by evaluating human blastocyst development rates. Blastocyst development rates and blastocyst quality were similar in both the chemical zona drilling group (47.5%) and in the laser-assisted hatched group (46.8) again suggesting that laser-assisted hatching does not impair embryonic development at the blastocyst stage. These findings were also supported a similar laser assisted embryo hatching study (Lanzendorf et al., 2007). Lan et al. (2008) conducted a study comparing the effects of complete chemical removal of the zona pellucida of IVF human embryos by using acidic Tyrode's solution versus 1.48  $\mu\text{m}$  diode laser (Fertilase) and showed no difference.

Various types of lasers such as erbium-yttrium-aluminium-garnet (Er:YAG) laser (Antinori et al., 1994), non-contact 1.48  $\mu\text{m}$  diode laser (Germond et al., 1995), non-contact holmium-yttrium-scandium-gallium garnet (Ho:YSGG) laser (Neev et al., 1995), and non-contact PALM UV laser (Schiewe et al., 1995) have been used for precise opening of the zona pellucida of bovine embryos. Laser-assisted hatching focuses light through the zona pellucida using optics attached to micromanipulator. The advantage of the Zilos-tk laser (Hamilton Thorne Research, Beverly, MA), which was used in the study by Jones et al. (2005), is that no micromanipulator is required and adjustment of the microscope stage moves the embryo and zona into the path of the laser. The laser creates a lateral trough through the zona pellucida. The rent diameter is determined by the focus of the laser beam and pulse duration. The escape of the embryo from the zona pellucida often requires multiple rents. Consistency of the rent diameter is one advantage of this method over acid or enzymatic renting. It also can be used in other procedures such as intra cytoplasmic sperm injection (ICSI). Laser-assisted zona pellucida hatching rather than zona pellucida breaching may lead to better implantation rates.

### **Laser Effects in the Manipulation of Human Embryos**

Assisted hatching was proposed by Cohen et al. (1990) as a way of improving implantation rates. Studies using mouse (Chen et al., 1995), cow (Park et al., 1999), pig (Beebe et al., 2004) and human (Balaban et al., 2006) models have been shown that assisted hatching can increase rates of embryo hatching. Studies have also shown that assisted hatching increases pregnancy rates in women who have a history of previous IVF failures. It has been reported that older patients whose embryos have thick zonae and embryos that have been cryopreserved benefit from assisted hatching (Cohen et al, 1990; Elhelw et al., 2004).

Antinori et al. (1994) reported successful pregnancies using a contact ErYAG laser. The laser emits UV radiation and infrared (IR) and both can cause damage to the embryo. The diameter of the laser beam is normally 4.5 to 5  $\mu\text{m}$  and the embryo or oocyte zona pellucida is

14 to 18  $\mu\text{m}$  thick. A study conducted used IR light at wavelength around 1,480 nm from an InGaAsP laser diode, reported no embryo damage. The use of 1,480 nm laser for assisted hatching reduces contamination because embryo contact was not needed (Rink et al., 1996). The IR beam that reaches the embryo's edge is  $\sim 60 \mu\text{m}$  above the floor of the Petri dish. The zona pellucida is dissolved where the laser beam and zona pellucida meet. The application of laser-assisted hatching allows the embryo to hatch and provides access to the blastomeres for other techniques (Tadir et al., 2007).

### Laser Thermal Effects

Scientists believe that the zona pellucida has the same absorption and thermal properties as water (Rink et al., 1996). During a laser pulse, the medium in and near the beam is heated to temperatures above  $100^\circ\text{C}$ . The heat produced from the laser beam is quickly reduced by heat transfer after the pulse ends. The transferred heat increases the temperature of the area of the medium around the laser beam, dissolving the zona pellucida (Tadir et al., 2007).

The energy and heat is emitted in the medium as the IR (e.g., XYClone) laser beam passes upward from the floor through the medium and embryos that are located at or in close proximity to the floor of the sterile Petri dish. The laser beam is focused on the outer section of the zona pellucida at 50 to 75  $\mu\text{m}$  above the floor of the dish. The cooling rate is very fast once the pulse is complete. The length of time at which the temperature remains high is dependent on the pulse duration (Tadir et al., 2007).

### **Laser-Assisted Hatching to Improve Pregnancy Rates of In Vitro-Fertilized Embryos**

Implantation is a prerequisite for implantation and pregnancy of the embryo in the uterine lining is impossible in most mammals unless hatching occurs. It has been suggested that the impaired hatching of human embryos resulting from IVF may contribute to lower implantation rates and pregnancy rates of 53% (Sagoskin et al., 2007). It has been demonstrated that

implantation and pregnancy rates of in vitro fertilized mouse (Alikani et al., 1992) and human (Liu et al., 1993; Tucker et al., 1993; Wiemer et al., 1993; Schoolcraft et al., 1994; Stein et al., 1995; Hellebaut et al., 1996; Sifer et al., 2006) embryos may be enhanced by mechanical, chemical, enzymatic assisted hatching techniques. The types of lasers that have been used to rent the zona have included krypton fluoride excimer laser (Blanchet et al., 1992), erbium:yttrium aluminum garnet laser (Conia and Voelkel 1994), a noncontact holmium:yttrium scandium garnet laser (Schiewe et al., 1995; Neev, et al., 1995) and a nitrogen-pulsed laser (Godke et al., 1990, Beetem et al., 1990).The 1480 nm, Infrared Solid State Diode laser is also used for laser-assisted zona pellucida drilling of cow (Pryor et al., 2008; Menges, et al., 2008), mouse (Gao et al., 1998) and human embryos (Tadir et al., 2007).

#### Laser-Assisted Hatching of Human and Mouse Embryos

In humans, Balaban et al. (2006) showed a significant increase in frozen-thawed embryo transfer pregnancy rates when applying laser-assisted hatching. The laser was used to thin the zona pellucida by 75% of its original thickness. The application of laser-assisted hatching subsequently increased implantation (20.1% vs. 9.9%) and pregnancy (40.9% vs. 27.3%) rates. Other studies have also shown the successful application of laser-assisted hatching in an in vitro fertilization program of mouse (Neev et al., 1995; Rink et al., 1996) and human embryos (Strohmer et al., 1992; Peterson et al., 2005).

In a study conducted by Lanzendorf et al. (2007), showed no differences between clinical or multiple pregnancy rates with the application of laser-assisted hatching using ZILOS-tk laser and assisted hatching using acidified medium. Likewise, Sagoskin et al. (2007) showed that the application of ZILOS-tk laser did not improve clinical outcomes among patients. The results were determined for laser-assisted and control (no treatment) groups for fetal cardiac activity (54% vs. 53%), spontaneous pregnancy loss (16% vs. 13%) and live birth (47% vs. 46%) were similar, respectively.

However, Ali et al. (2003) showed the laser-assisted hatching in human increased pregnancy rates for women  $\geq 36$  years but not for women  $\geq 37$  years. The results also showed that embryos with thin zonae ( $\geq 16 \mu\text{m}$ ) had increased pregnancy rates from laser-assisted hatching while embryos with thick zonae ( $\geq 17 \mu\text{m}$ ) or those females with repeated IVF failures (37 to 50%) did not. These results agree with those reported by Elhelw et al. (2004) using similar laser-assisted hatching on human embryos.

Little has been documented regarding the efficacy of laser-assisted hatching of frozen-thawed embryos prior to transfer. Petersen et al. (2006) conducted a study to determine the effect on pregnancy rates compared with thinning one quarter of the zona pellucida of frozen-thawed human embryos to a depth of 50 to 80% of the total zona thickness using qLZT-AH laser system (Fertilase™ system; Medical Technologies Montreux, Lausanne, Switzerland). A total of 350 laser-assisted cryopreserved human embryos were compared with 352 intact zona embryos. No difference in implantation or pregnancy rates were found after using qLZT-AH for assisted hatching. These results support the finding of Ng et al. (2005) and Valojerdi et al. (2010), which showed that renting of the zona pellucida did not improve implantation and pregnancy rates and laser-assisted hatching should not be performed routinely on all frozen-thawed human embryos at cleavage stage.

#### Human Protocol Using the ZILOS-tk Laser

Assisted hatching is typically performed to enhance implantation in cases where patients that are greater than 36 years of age, following multiple failed IVF attempts and whenever embryos showed abnormal zona pellucida characteristics (Ali et al., 2003). Sapir and Fisch et al. (2006) recommend that laser-assisted hatching should be performed a few hours or immediately prior to embryo transfer.

Stevens et al. (2006) described a laser protocol in human IVF laboratory as follows. The embryo is placed in an oil droplet in a dish at the center of the stage. Focus the embryo using

the high power objective (20X). Rents should be made so that hatching occurs at the rent or at an area of fragmentation. The embryo should be positioned when using the laser so that the area of the rent will be between twelve and 6 o'clock. Once the embryo is in focus, the laser pulse should be set to 800  $\mu$ seconds and the embryo moved so that the part of the zona pellucida is aligned with the red circle. Rents are made on the outer edge of the zona pellucida using the fire button with the mouse cursor on the computer screen. The embryo is moved again so that the next pulse ruptures the inner membrane or until the rent is twice the width of the red circle (20 to 30  $\mu$ m) is made in the zona pellucida and the inner membrane is broken along this width. This usually takes four to six different laser rents.

#### Laser-Assisted Hatching in Bovine In Vitro-Produced Blastocyst

Assisted hatching by thinning of the zona pellucida has been shown to improve hatching rates of in vitro-produced mouse and bovine embryos (Gao et al., 1996; Gao et al., 1998). Schmoll et al. (1999) investigated effects of a single rent with 10 to 15  $\mu$ m length produced by a single laser pulse and the evaluated hatching rates of bovine in vitro-produced blastocysts. At 7 days after fertilization, embryos were evaluated and blastocysts were assigned to three groups. Zona pellucida thickness and zona pellucida diameter were measured. In Experiment I, zona pellucida was rented by a single laser pulse using the FERTILASE™ laser (MTM Medical Technologies Montreux SA, Switzerland) to create an opening 10 to 15  $\mu$ m in length. In Experiment II, zona pellucida was opened by series of 4 to 5 pulses to open the zona pellucida for at least a minimum of 40  $\mu$ m in length. The control group did not receive the laser treatment. Zona pellucida diameter and zona pellucida thickness, embryo development were evaluated 24 hours and 48 hours after laser treatment.

The results of this study showed that a single rent in the zona pellucida with a length of 10 to 15  $\mu$ m does not improve hatching rates of bovine in vitro-produced embryos (Schmoll et al., 1999). In Experiment I the percentage of hatched blastocyst at 24 hours in the laser group

was 7% compared with 6% in the control group. At 48 hours following laser treatment, the hatched blastocyst rate in the laser group was 15% compared with 20% for the control group. The single rent caused the blastocyst to form an 8-shaped figure where the two halves are connected by a thin cell bridge. Hatched blastocyst in Experiment II at 24 hours was 13% for the laser group compared with 6% in the control group. At 48 hours in Experiment II in the laser group the hatched blastocyst rate was 57% compared with 20% in the control group. Hatching rate was improved in Experiment II with rents larger than 40  $\mu\text{m}$ , which corresponds with the hatching results reported by Park et al. (1999) using the InGaAsP diode laser (MTM Medical Technologies Montreux, Lausanne, Switzerland).

#### Utilization of Laser-Assisted Hatching in In Vitro-Fertilized Bovine Embryos

In vitro fertilization and culture has been shown to decrease hatching rates following transfer, which results in lower pregnancy rates over that of the controls. Menges et al. (2008) conducted a study that evaluated the effects of laser-assisted hatching using the XYClone<sup>®</sup> laser system (Hamilton Thorne Biosciences, Beverly, MA) to rent the zona pellucida of in vitro-produced bovine embryos. The XYClone<sup>®</sup> laser is the same as the ZILOS-tk; however, the XYClone<sup>®</sup> laser was not approved for clinical use. The effect of treatment on embryo survival prior to transfer into an estrus synchronized recipients and pregnancy rates were evaluated.

In Experiment I, oocytes were in vitro matured, fertilized and cultured. On days 5, 6 and 7 of in vitro culture, embryos were randomly divided into three treatment groups (no treatment, sham zona pellucida rent, or zona pellucida rent). Control embryos were immediately returned to the incubator following selection, while sham treated embryos were exposed to all conditions as rent group, except laser-assisted hatching. Rents were made using the XYClone<sup>®</sup> laser with a power of 90% and pulse length of 600  $\mu\text{s}$ . Embryos were cultured until day-8 and evaluated on percentage of live cell culture.



In Experiment II, in vitro-produced embryos were randomly divided into two groups, control and rent. On day-7 (the day of transfer), rents were made in the zona pellucida with the laser using 80% power and pulse length of 500 µseconds, prior to transfer into estrus synchronized recipients. Pregnancy rates were determined using ultrasound at days 30 and 60 of gestation.

The mean proportion of live cells on day 8 for the control group was 93%, sham group was 90% and rented group was 93%. These results suggest that laser-assisted hatching using the XYClone<sup>®</sup> does not inhibit embryo viability and development. In Experiment II, pregnancy rate at day 30 of gestation was 54% in the rented group and 49% in the control group. Pregnancy rates at 60 days of gestation were greater in the rented group (58%) compared with the control group (46%). This difference suggests that 60-day pregnancy rates of in vitro-produced embryos may be improved with the application of laser-assisted hatching. However, calving rates need to be determined.

#### Effect of Lipid Segregation With or Without Laser Drilling Post-Thaw In Vitro Bovine Embryos

The cryopreservation of embryos with increased lipid content can increase cell damage, which may decrease hatching rates. Pryor et al. (2008) did a study to determine if lipid segregation with or without zona pellucida renting affected post-thaw in vitro-produced bovine embryos survivability and development. Embryos were randomly allotted into Treatment 1 (control), Treatment 2 (CB or 7.5µg/ml Cytochalasin B for 20 min), Treatment 3 (CBCF or CB with 20 min Centrifugation (CF) at 16,000 x g, for lipid segregation), Treatment 4 (CBCFLAH or CB with 20 min CF and freeze then laser-assisted hatching (LAH) upon thaw) or Treatment 5 (LAH only post-thaw).

Embryos in treatment groups 4 and 5 received a single rent with the power strength 90% power and pulse length 600 µseconds using XYClone<sup>®</sup> laser. Embryos were stained in holding medium containing 2.5 µg/ml Bisbenzimidazole Hoechst 33342 and 5 µg/ml Propidium

Iodide for 15 minutes. Embryo cell counts were obtained by exposing embryos to ultra-violet (UV) light.

The results showed no mean differences between treatments for survivability and embryo development. At the morula stage, no difference was noted between CBCFLAH (77%) compared with LAH (72%), CBCF (69%) and the control group (68%) but had a greater percentage of live cells than CB (65%) (Pryor et al., 2008). Overall, CBCFLAH and LAH had a higher number of both total and live cells than the control group.

### **Application of Assisted Hatching of In Vivo Fresh or Frozen-Thawed Bovine Embryos**

The embryo transfer industry started developing in the United States in the early 1970s, with fresh embryos were surgically recovered and transferred. The recovery and transfer of embryos today are nonsurgical and more than 50% of cattle embryos harvested are cryopreserved after collection. Embryo cryopreservation has been considered to be a successful part of assisted reproductive technologies, but there tends to be a reduction in implantation and pregnancy rates when compared with fresh embryos. It has been suggested that embryo exposure to cryoprotectants and abrupt temperature changes may cause cell damage and zona pellucida hardening, and consequently may inhibit blastocyst hatching.

Assisted hatching may facilitate hatching of blastocyst stage embryos and subsequent interaction between the embryo and the endometrium, which could subsequently increase implantation and pregnancy rates. It has been shown that introducing an opening or rent in the zona pellucida may lead increased instance of to hatching in an hourglass pattern and causing the embryo to become trapped within the zona when a thickened zona pellucid is present (Baguisi et al., 1998). However, the thinning of the zona pellucida has been shown to increase the complete hatching of the blastocyst through the zona pellucida (Gordon et al., 1993). Comparative studies of assisted hatching on cryopreserved human embryos after thawing have

been reported to be effective (Strohmer et al., 1992; Peterson et al., 2005) or not (Peterson et al., 2006) on improving implantation and pregnancy rates.

Baguisi et al. (1998) conducted a study to determine if hatching failure is a result of loss of viability due to cryopreservation and if the application of assisted hatching of the zona pellucida would improve bovine hatching rates. Embryos were collected at slaughter from superovulated crossbred heifers at day-6 or day-7 post-estrus. Embryos were frozen in 1.4 M glycerol and thawed using 1.0 M sucrose in phosphate-buffered saline supplemented with 15% fetal calf serum. Assisted hatching was conducted by puncture, slicing or pronase dissolution. Following zona treatment, grades 1, 2 and 3 were cultured separately in vitro culture medium CR1aa and supplemented with 15% FCS in 5% CO<sup>2</sup> at 39°C. Blastocyst hatching rates were recorded daily for 4 days. Results indicated that ~70% of embryos with grades 1 and 2 with intact zona pellucida were viable but 50% of those embryos failed to hatch following cryopreservation and exposure to culture in vitro. Furthermore, ~30% of embryos with a grade of 3 were viable and a third of those hatched. It was suggested that the exposure to enzymatic dissolution of the zona with pronase may have damaging effects causing the decrease in hatching rates. Using the single puncture method through the zona pellucida, the embryos hatched incompletely. Embryos were “pinched” and caused some to split in two. Slicing the zona pellucida with a large cut resulted in complete hatching. The results from this study suggest that mechanical assisted hatching may increase hatching rates of cryopreserved embryos (Baguisi et al. 1998).

Taniyama et al. (2007) conducted an experiment designed to improve pregnancy rates by applying the assisted hatching technique to poor quality embryos. Embryos were collected from superovulated Japanese Black cows on day-7 post-insemination. The assisted hatching was performed by renting through 20% of the zona pellucida circumference using a micromanipulator. After renting, one or two embryos were transferred fresh into the uterine horn

of recipient cows on day-7 of the estrous cycle. Pregnancy and calf production rates were compared.

Pregnancy rates were determined at day-45 of gestation and calving rates were determined by the number of calves born divided by the number of pregnancies. Pregnancy rates of poor quality embryos in the assisted hatched group where two embryos were transferred into one recipient (60.3%; 44/73) were higher than those in the single fresh embryo transfer intact zona group (25.0%; 6/24) and in the single fresh embryo transfer assisted hatched group (44.0%; 37/84). Calving rates were 67.3%, 45.5% and 35.6% for the double embryo transfer assisted hatched group, the double embryo transfer zona intact group and the single fresh embryo transfer assisted hatched group, respectively. Pregnancy rates of poor quality bovine embryos after double embryo transfer were improved by assisted hatching when compared to those of single fresh embryo transfer with non-assisted hatched embryos (Taniyama et al., 2007).

Lopatarova et al. (2001) conducted a study to determine the effect of assisted hatching on in vitro and in vivo survival of in vivo-produced, fresh or frozen-thawed bovine embryos of various qualities. Day-7 bovine embryos were collected from superovulated Holstein-Friesian cows washed with PBS + 10% FCS and evaluated. Morphologically intact compacted morulae and early blastocysts of grades 1, 2 and 3 were used either for randomly assigned to a treatment group and exposed to in vitro culture or transfer into a recipient. Embryos with a grade of 1 or 2 were cryopreserved. Expanded blastocysts were not assisted hatched due to a little or no perivitelline space. Rents were made in the zona pellucida by either a microsurgical knife or with a micro-blade. The length of the rent varied between 30 to 50  $\mu\text{m}$ .

The treated embryos were either cultured in vitro or immediately transferred. The embryos were frozen in 1.5 mol/L ethylene glycol with 0.1 mol/L sucrose. After thawing and evaluation, a rent was made in the zona pellucida and embryos were then cultured in vitro or

transferred. Fresh or frozen-thawed embryos were transferred into the ipisilaterally horn from the corpus luteum. Embryos that remained with intact zonae pellucidae were used as controls. Rectal palpation for pregnancy rates was conducted between days 35 and 50 of gestation. The results of in vitro and in vivo experiments showed higher hatching rates, but no difference was noted for implantation rates after assisted hatching. There was no difference in pregnancy rates observed after the transfer of fresh in vivo embryos with the grade of 1 of assisted hatched group (64.6%; 42/65) and control (63.9%; 39/41). It was suggested that there was no treatment effect and similar pregnancy rates were noted between assisted hatched and control groups. It was suggested that no difference noted for fresh grade-1 embryos was due to the cell number and the quality of the embryo. Pregnancy rates after the transfer of in vivo embryos with a grade of 2 or 3 were 58.7% (37/63) for the assisted hatched group and 47.9% (35/73) for the control group. The results of the transfer of frozen-thawed grade-1 embryos assisted group had higher pregnancy rates compared with the control group (57.5% vs. 45.3%), respectively. The application of assisted hatching of frozen-thawed grade-2 embryos did not affect pregnancy rates (30% vs. 33.3%). In summary, assisted hatching prior to transfer of fresh or frozen-thawed, morphologically normal embryos were associated with increased pregnancy rates (Lopatarova et al., 2001).

The application of assisted hatching on frozen-thawed in vivo-produced embryos requires more investigation to determine embryos hatching, pregnancy and calving rates following transfer.

## **CHAPTER III**

### **ASSISTED HATCHING OF IN VITRO CULTURED FROZEN-THAWED IN VIVO-PRODUCED BOVINE EMBRYOS USING INFRARED CLASS I LASER**

#### **Introduction**

The hatching of a mouse embryo occurs by an opening in the zona pellucida created by enzymes (Perona and Wassarman, 1986) and the expansion of the blastocoel by fluid accumulation (McLaren et al., 1970). Gordon et al. (1993) reported that the ratio of lysin produced and zona pellucida thickness determines whether the embryo will hatch.

Injury to the bovine embryo caused by cryopreservation may affect cleavage, compaction, blastocyst formation and hatching (Willadsen et al., 1977). Injury to the bovine embryo during the freeze and/or thawing process includes damage to the plasma membrane, organelles, cytoskeleton and possibly inducing zona pellucida hardening (Dobrinsky, 1996; Baguisi et al., 1998). Disruption of intracellular pathways is one cause that jeopardizes the embryos ability to develop and hatch from the zona pellucida. It has been shown that approximately 30% of frozen-thawed bovine embryos have damaged zonae pellucidae (Tervit et al., 1981). These cryopreserved embryos may benefit from assisted hatching by increasing blastocyst hatching rates.

Assisted hatching has been proposed as a means to promote the natural hatching process of the blastocyst. It has been demonstrated that implantation and pregnancy rates are increased by mechanical, chemical, enzymatic and laser-assisted hatching techniques of in vitro fertilized murine (Cohen et al., 1991; Alikani et al., 1992; Gordon et al., 1993; Chen et al., 1995; Gao, 1998), human (Tucker et al., 1993; Liu et al., 1993; Schoolcraft et al., 1994; Wiemer et al., 1994; Stein et al., 1995; Hellebaut et al., 1996; Sifer et al., 2006) and bovine embryos (Gao et al., 1996; Basovskii et al., 1999; Lopatarova et al., 2001). However, contradicting evidence has

been reported on the effect of laser-assisted hatching on hatching and pregnancy rates of frozen-thawed in vivo-produced embryos. Petersen et al. (2006) showed no difference in implantation or pregnancy rates of frozen-thawed human embryos when rents were introduced at a depth of 50 to 80% through the zona pellucida using the qLZT-AH laser system (Fertilase™ system; Medical Technologies Montreux, Lausanne, Switzerland). Baguisi et al. (1998) showed that improved viability and hatching rates of frozen-thawed in vivo-produced bovine embryos resulted after the application of mechanical, chemical and enzymatic assisted hatching techniques.

However, research has also shown that when a single rent was placed in the zona pellucida a percentage of bovine embryos hatch incompletely (Baguisi et al., 1998). In this study, a single rent in the zona pellucida caused constriction of the trophoblast and the inner cell mass in embryos. The trophoblast cells numbers may have been reduced as the blastocyst hatched through the narrow gap. The embryos became “pinched” as they escaped through a small opening in the zona, causing splitting of the embryo. Balaban et al. (2002) reported twinning rates of 51% of partial zona dissected and 43% of laser-assisted transferred human embryos. The instance of monozygotic twins resulting from the application of assisted hatching could increase fetal loss (Balaban et al., 2002).

Another possibility is the premature hatching of mouse embryos through rents made in the zona pellucida prior to expansion and growth of the blastocyst. It has been shown that the blastocyst will hatch through these rents, but complete hatching was dependent on the size of the rent (Gordon et al., 1993). The incidence of trapped embryos could be avoided if larger rents were induced using acidic Tyrode’s solution or the thinning of the zona pellucida by laser-assisted hatching. Human embryos with multiple rents in their zonae hatched through the largest opening available (Cohen, 1992). In most cases when the zona pellucida receives

multiple rents, complete hatching will occur. It has been hypothesized that assisted hatching may improve the success of compromised cryopreserved embryos (Baguisi et al., 1998).

The first objective of this experiment was to determine the number of rents and depth of the rents made in the outer section of the zona pellucida with the use of the infrared diode laser to increase hatching rates of frozen-thawed in vivo-produced bovine embryos. The second objective was to evaluate if incomplete hatched embryos after laser treatment would result in the production of bovine demi-embryos.

## **Materials and Methods**

### Experimental Embryos

Purebred Hereford (Grandview Herefords, WV), Angus and Charolais (Southern Cattle Company, FL) frozen-thawed in vivo-produced embryos were used for treatment and in vitro culture in this study. Embryos were flushed and frozen using protocols described in the Manual by International Embryo Transfer Society (Robertson et al., 1998).

In Experiment I, in vivo-produced Hereford embryos (n= 40) were produced from the same herd and were collected and processed by the same technicians. Embryos from 9 different donors were used across treatments in this experiment. Embryos were frozen in ethylene glycol medium and prepared for direct embryo transfer at a later date.

In Experiment II, the in vivo-produced Angus and Charolais embryos were collected from the same herd. The embryos were collected and processed by several technicians. Embryos from 11 different Angus embryo donors and eight Charolais donors were stratified across treatments in this experiment and embryos were frozen using a standard International Embryo Transfer Society protocol (Robertson et al., 1998). Embryos were frozen in ethylene glycol for direct transfer at a later date. Embryos from both breeds (n= 132) were used across treatments.



In both experiments, frozen bovine embryos were thawed according to methods described by Mapletoft (2006). Briefly, upon removal of embryos from liquid nitrogen, embryos were allowed to air thaw at room temperature for 15 seconds and then were submerged in a 30°C water bath for an additional 15 seconds. After thawing, straws were wiped of excess water and embryos were then expelled into a 35-mm sterile Petri dish. The straw was opened using scissors and contents were expelled using a 1 ml sterile syringe by removing the end of the straw without the cotton plug and placing the syringe on the unplugged end, next removing the cotton plugged end of the straw and lastly pushing the contents of the straw into the Petri dish. Contents from the first column and last column were expelled separately from the center column containing the embryo.

Embryos were removed from the cryoprotectant then washed and held at room temperature in 3 ml of TALP HEPES buffer for 7 minutes before being washed twice through 40 or 70 µl droplets of TALP HEPES covered with sterile mineral oil. While in TALP HEPES wash, the embryos were evaluated and subjected to the renting procedure. Embryos were held in TALP HEPES for 15 minutes and allowed to expand.

Embryos were evaluated under a 40X magnification using a Nikon inverted microscope. Evaluation was conducted according to methods described by Robertson et al. (1998). Embryos were assigned grades of 1 to 4 (1 = excellent to 4 = poor) and stage of development was recorded (4 = morula to 7 = expanded blastocyst).

#### Laser-Assisted Zona Pellucida Renting

The Hamilton Thorne XYClone® (Hamilton Throne Biosciences, Beverly, MA) class I 1,480 nm, infrared, solid state, diode laser was used in this study. This system includes a 40X objective, video camera, laser renting software and a laptop computer. The 40X objective is a

high transmission, visible infrared, long distance laser and was attached to a Nikon inverted microscope (Figure 3.1).

Embryo measurements were recorded by translating pixels into micrometers. Calibration was conducted using a Ronchi rule slide. Magnification calibration was conducted twice during this study (March 8, 2008 and August 7, 2009). Target alignment was tested by covering a 2 cm x 2 cm area with three or four lines of a black dry erase marker on a coverslip. The area of black ink served as a target. The pulse was set at 1,000  $\mu$ seconds and the power to 100%.

The positioning of the XYClone<sup>®</sup> laser objective module, adjustment of the microscope and focus, and alignment of the zona pellucida in the path of the laser beam were completed before firing the laser. The image was adjusted to the focal plane at the midsection of the embryo at its largest diameter. To determine correct focus, the border of the embryo was observed. A standard color digital camera with video capture connected to a Panasonic laptop computer was used to view the embryos. Images of embryos were captured before and after renting.

Measurements of zona thickness and embryo diameter were recorded. Five measurements of the zona thickness were taken and a mean and standard deviation were calculated. To reduce variability and lessen bias, the five measurements of the zona pellucida were recorded at the 3, 5, 7, 10 and 12 o'clock positions. Two measurements of embryo diameter at the 6 to 12 o'clock position and 3 to 9 o'clock position were recorded and a mean and standard deviation were calculated.

The isotherms appear on the laptop screen as a series of six concentric circles. Each circle specifies the max temperature reached at that radius of the circle when the laser is fired its present power. The interior ring identifies the laser beam diameter and the second ring is the approximate rent size. Firing of the laser was controlled through the software interface. Each

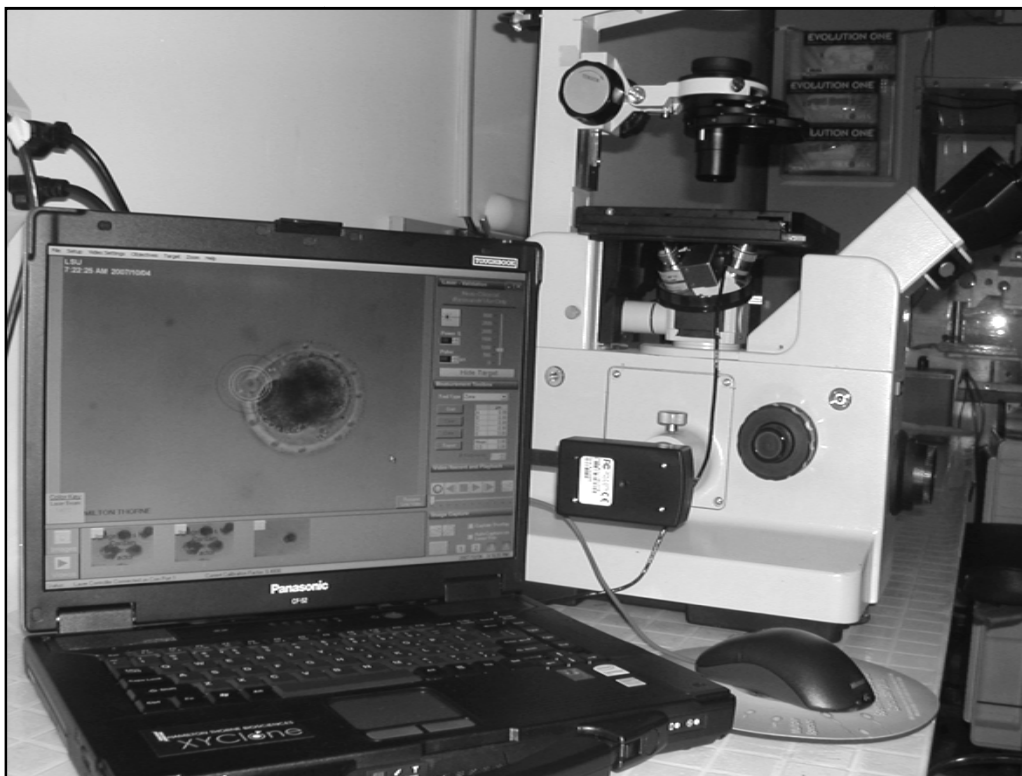


Figure 3.1. The XYClone<sup>®</sup> (Hamilton Thorne) diode laser was attached directly to a Nikon inverted microscope connected to a Panasonic laptop computer.

embryo received either two or three rents through the outer zona surface using the diode laser at 90% power with a 600 µsecond pulse.

#### In Vitro Culture of Embryos

After evaluation, measurements and treatment, embryos were washed twice through 40 or 70 µl droplets of CR1aa medium supplemented with 10% calf serum under sterile mineral oil. Embryos were then placed into 20 µl droplets of CR1aa medium supplemented with 10% calf serum with 10 embryos per droplet. The droplets containing the embryos were then placed in an air tight gas chamber, which was flushed with 5% CO<sub>2</sub>, 5% O<sub>2</sub> and N<sub>2</sub> gas mixture for 3 minutes. The gas chamber was then placed in an incubator set at 39°C in 5% CO<sub>2</sub> of humidified air. Embryos were cultured until day-11 of age (day-4 of culture).

#### Experimental Design

The objective of Experiments I and II was to determine the effects of number of rents and location using the XYClone<sup>®</sup> diode laser on the outer portion of the zona pellucid on hatching pattern and rate of frozen-thawed in vivo-produced bovine embryos exposed to in vitro culture conditions. The objective for Experiment III was to determine if laser-assisted hatching using the XYClone<sup>®</sup> diode laser would increase hatching rates of frozen-thawed in vivo-produced bovine embryos in an in vitro culture system. In both experiments, embryos were evaluated throughout culture and hatching rates were determined on embryo age day-11 (day-4 of culture).

In Experiments I and II, embryos with assorted grades, zona thickness and embryo diameter were randomly allotted to either laser-assisted (Treatment A) or embryos which remained with an intact zona (Treatment B). In Experiment I, embryos received two vertical rents that were 80% through the zona at three separate locations (Treatment A, n= 10)

(Figure 3.2). In Experiment II, embryos received two symmetrical rents 40% through the zona at three separate locations (Treatment A, n= 10) (Figure 3.3).

In Experiments I and II, rents were located at three separate positions on the outer zona surface. In both experiments, the XYClone<sup>®</sup> diode laser was set at 90% power with a 600 µsecond pulse and this setting was applied to embryos in Treatment A. In both experiments a sham control (Treatment B, n= 10) was utilized, where the laser was fired into the sterile mineral oil located on the outside of the medium droplet, where the embryo was located (Figure 3.5).

In Experiment III, embryos with assorted grades, zona thickness and embryo diameter were randomly allotted to either laser-assisted (Treatment A, n= 66) or embryos remained with an intact zona (Treatment B, n= 66). Embryos received three rents that were symmetrically 40% through the zona and located at one position on the outer zona surface using the XYClone<sup>®</sup> diode laser at 90% power with a 600 µsecond pulse (Treatment A) (Figure 3.4) or Treatment B as described for Experiments I and II (Figure 3.5).

In Experiments I, II and III, all embryo grades (1= excellent and 4= poor) and the stage of embryo development (4= morula to 7= expanded blastocyst) were recorded before in vitro culture. Means of embryo grade, zona thickness and embryo diameter were also recorded. After 4 days of in vitro culture, embryos were recorded as having failed to hatch or as hatched.

### Statistical Analysis

Mean embryo grade, zona thickness and embryo diameter across treatment groups was analyzed using Proc GLM. Hatching status at day-11 of age (4 days following exposure to in vitro culture) was analyzed using a Chi-Square (SAS, Cary, NC). Significant difference was set at the 0.05 level.

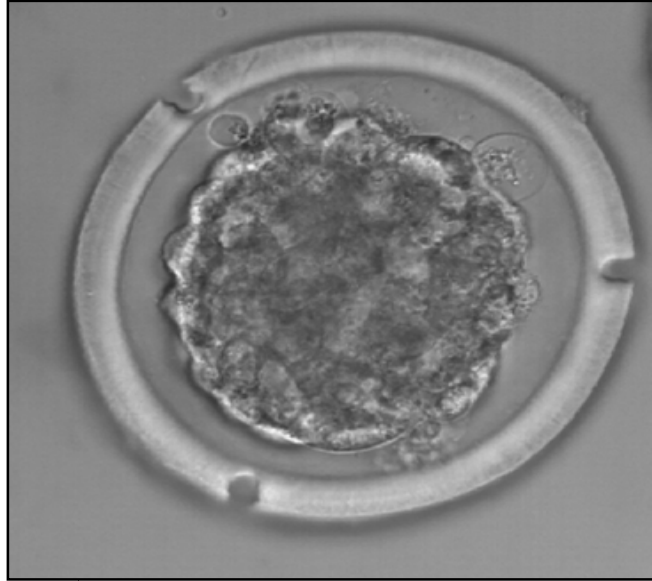


Figure 3.2 Experiment I, Treatment A in vivo-produced frozen-thawed bovine embryo with two vertical rents 80% through the zona pellucida in three different positions.

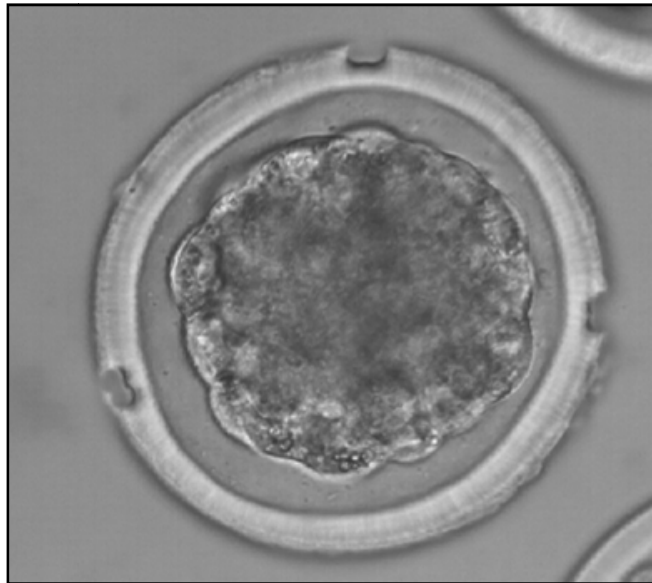


Figure 3.3 Experiment II, Treatment A in vivo-produced frozen-thawed bovine embryo with two symmetrical rents 40% through the zona pellucida in three different positions.

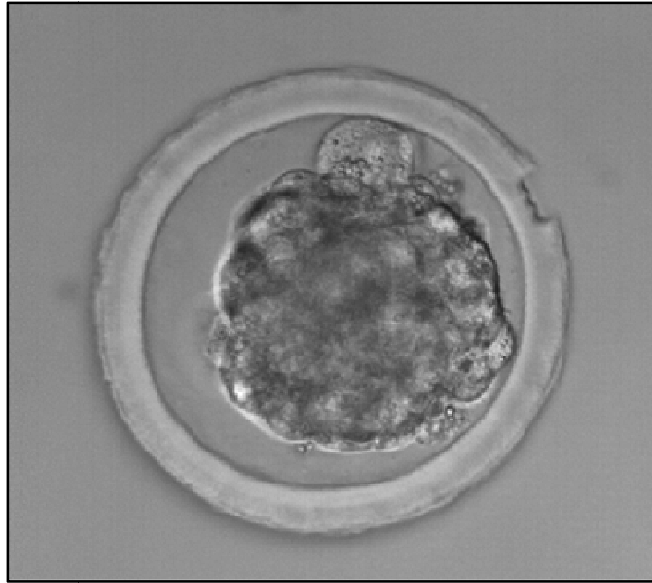


Figure 3.4 Experiment III, Treatment A in vivo-produced frozen-thawed bovine embryo with three symmetrical rents 40% through the zona pellucida.

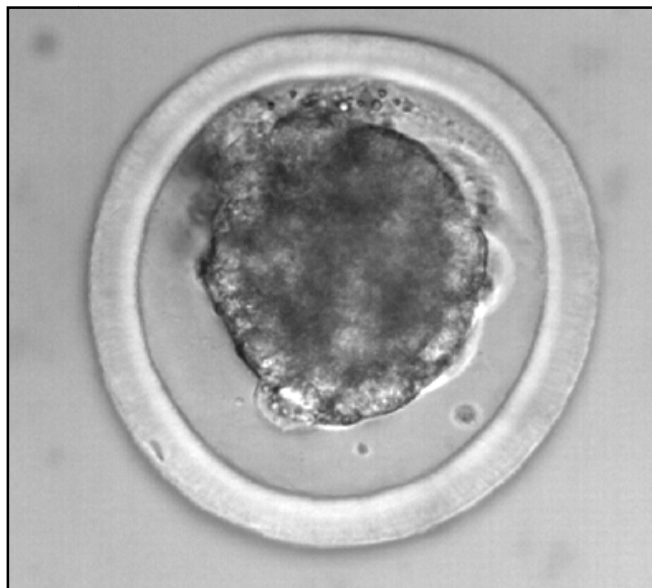


Figure 3.5 Experiments I, II and III, Treatment B in vivo-produced frozen-thawed bovine embryo with intact zona pellucida.

## Results

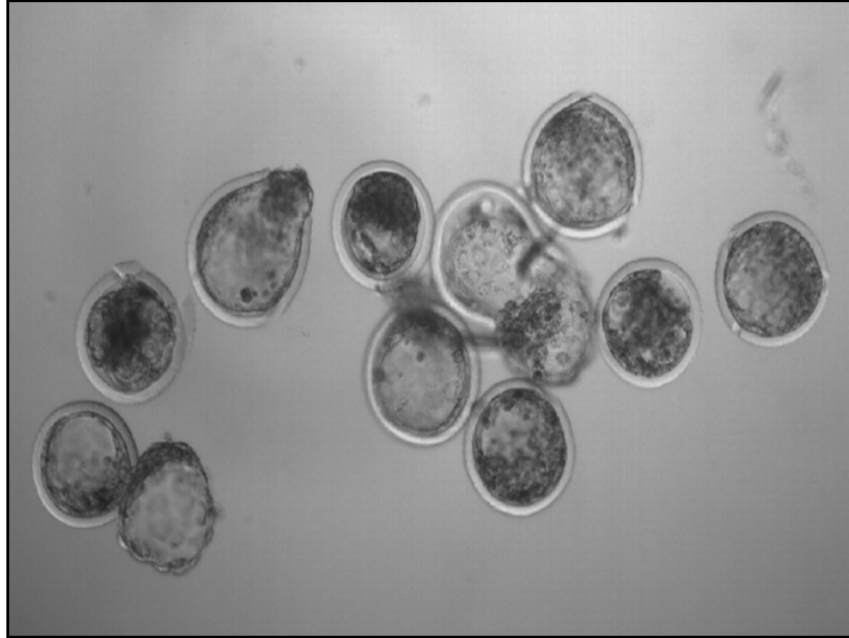
In Experiment I, it appeared that blastocysts (30%) in Treatment A (Figure 3.6) started the hatching process a day earlier than the blastocysts in Treatment B (Figure 3.7). On day-4 of culture cells of the embryos in Treatment A were partially protruding through the rents made using the XYClone<sup>®</sup> diode laser, which caused “pinching” of three embryos. During in vitro culture, one embryo in Treatment A was “pinched” during the hatching process, which caused the embryo to divide into two parts.

There was no difference in zona thickness between Treatment A ( $13.51 \pm 0.2 \mu\text{m}$ ) and Treatment B ( $12.55 \pm 0.3 \mu\text{m}$ ), respectively (Table 3.1). In both Treatment A and Treatment B, 90% of the embryos hatched in Experiment I. However, one embryo in Treatment A split after being exposed to treatment and in vitro culture. The embryo that split was counted as being two embryos. The number of embryos that were degenerating or those embryos that failed to hatch completely during in vitro culture were 2 (20%) for Treatment A and 1 (10%) for Treatment B. There was no difference between the percent age of embryos that were degenerating or those that failed to hatch and embryos which completely hatched in Treatments A and B (Table 3.2).

In Experiment II, there was no difference in mean embryo grade ( $1.5 \pm 0.2$  and  $1.6 \pm 0.2$ ), zona thickness ( $12.7 \pm 0.3 \mu\text{m}$  and  $13.9 \pm 0.5 \mu\text{m}$ ) or embryo diameter ( $149.4 \pm 0.7 \mu\text{m}$  and  $150.3 \pm 1.1 \mu\text{m}$ ), for Treatments A and B, respectively (Table 3.3). On day-4 of in vitro culture, all hatched blastocyst in Treatment A (Figure 3.8) appeared to be viable and expanded, while the embryos in Treatment B were beginning to shed embryonic cells (Figure 3.9). Three embryos in Treatment A were “pinching” but none of these split during in vitro culture (Figure 3.8).

In Experiment II, there was no difference in hatching rates between treatment groups, and were 90% for both Treatments A and B. The embryos that were degenerating and





Figures 3.6. Experiment I, in vivo-produced frozen-thawed bovine embryos in Treatment A on day-2 of in vitro culture.

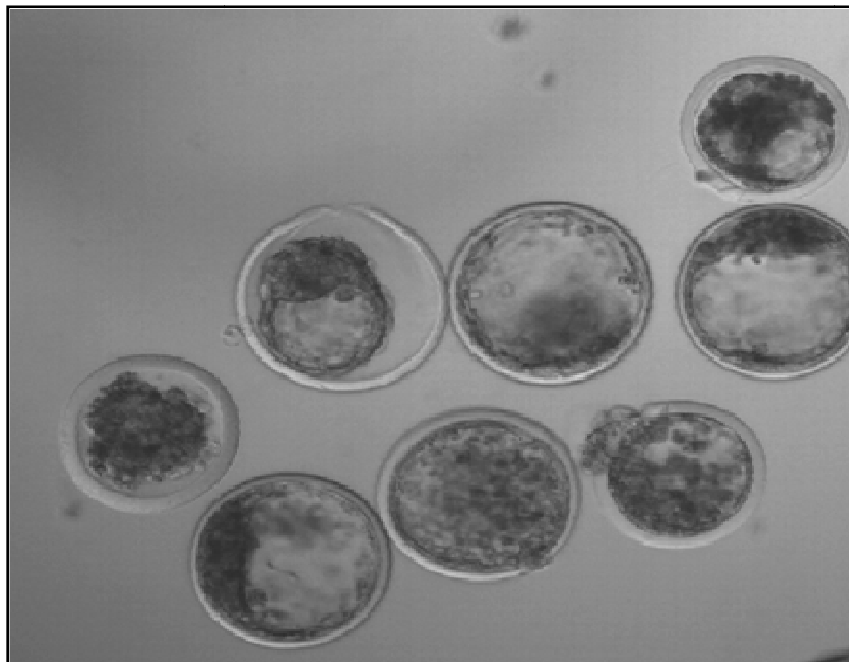


Figure 3.7. Experiment I, in vivo-produced frozen-thawed bovine embryos in Treatment B on day-2 of in vitro culture.

Table 3.1. Experiment I, mean embryo grade, zona thickness and embryo diameter of in vivo-produced frozen-thawed bovine embryos ( $\pm$ SE) before exposure to treatment.

Treatment	Mean Grade	Zona Thickness ( $\mu$ m)	Embryo Diameter ( $\mu$ m)
Treatment A (n= 10) LAH <sup>a</sup>	1.7 $\pm$ 0.1	13.51 $\pm$ 0.2	153.8 $\pm$ 2.0
Treatment B (n=10) Sham Control	2.1 $\pm$ 0.2	12.55 $\pm$ 0.3	144.3 $\pm$ 2.0

<sup>a</sup> Laser-assisted hatching.

Table 3.2. Experiment I, effects of laser-assisted hatching (LAH) of in vivo-produced frozen-thawed bovine embryos after 4 days of in vitro culture.

Treatment	Failed to Hatch (%)	Hatched Blastocyst (%)
Treatment A (n= 10) LAH	2 (20)	9(90) *
Treatment B (n=10) Sham Control	1(10)	9 (90)

\* One of the embryos split after treatment exposure during in vitro culture.

Table 3.3. Experiment II, mean embryo grade, zona thickness and embryo diameter of in vivo-produced frozen-thawed bovine embryos ( $\pm$ SE) before exposure to treatment.

Treatment Group	Mean Grade	Zona Thickness ( $\mu$ m)	Embryo Diameter ( $\mu$ m)
Treatment A (n= 10) LAH <sup>a</sup>	1.5 $\pm$ 0.2	12.7 $\pm$ 0.3	149.4 $\pm$ 0.7
Treatment B (n=10) Sham Control	1.6 $\pm$ 0.2	13.9 $\pm$ 0.5	150.3 $\pm$ 1.1

<sup>a</sup> Laser-assisted hatching.

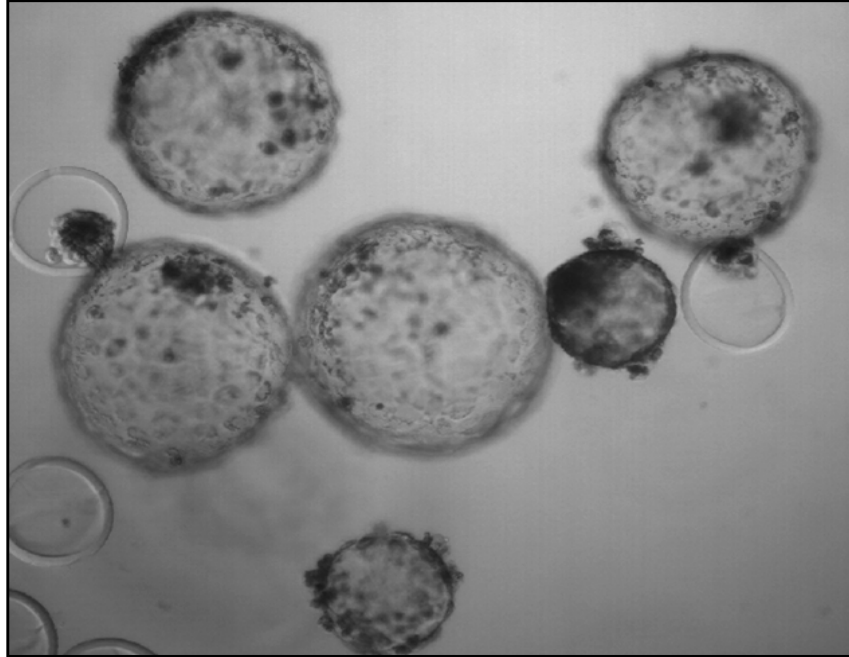


Figure 3.8. Experiment II, Treatment A in vivo-produced frozen-thawed bovine embryos on day-11 or 4 days after in vitro culture.

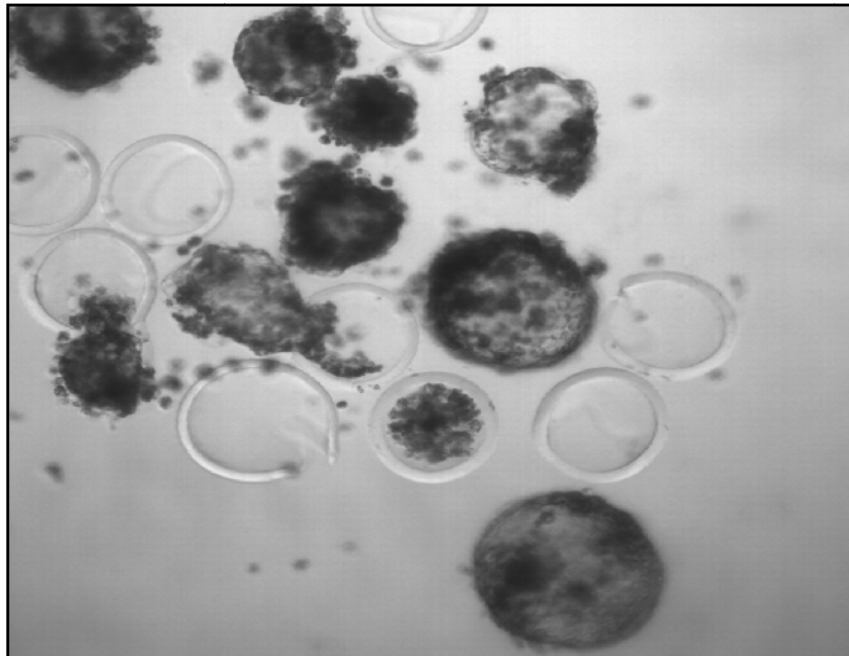


Figure 3.9. Experiment II, Treatment B in vivo-produced frozen-thawed bovine embryos on day-11 or 4 days after in vitro culture.

failed to hatch during in vitro culture were 1 (10%) for Treatment A and 1 (10%) for Treatment B (Table 3.4).

In Experiment III, there was no difference in the means for embryo grade ( $1.7 \pm 0.06$  and  $1.8 \pm 0.06$ ), zona thickness ( $13.4 \pm 0.3$  and  $13.7 \pm 0.2$ ) or embryo diameter ( $162.6 \pm 0.9$  and  $161.7 \pm 0.7$ ) for Treatments A and B, respectively (Table 3.5). Hatching rates were not affected by treatment and were 33/66 (50%) of embryos that hatched in Treatment A and 36/66 (55%) in Treatment B (Table 3.6).

The hatching data were combined from Experiments I, II and III and analyzed. The combined hatching rates were not different between Treatment A (n=86) and Treatment B (n=86) and were 59% and 63%, respectively (Table 3.7).

## **Discussion**

These experiments indicate that the renting of the zona pellucida using a diode laser did not increase the hatching rates of frozen-thawed in vivo-produced direct transfer bovine embryos when exposed to in vitro culture. Embryos in Experiment III appeared to be of lower in embryo quality after thawing than in Experiment I and Experiment II. The cause is unknown. Unfortunately, pre-freeze grades were not available. This lower embryo quality could have been caused by improper handling, freezing and/or storage of embryos.

Cryoprotectants often lead to chemical toxicity and osmotic injury to the cells (Rall et al., 1987). The abnormal zona hardening has been suggested as the cause for the inability of the blastocyst to hatch. It has been shown in multiple studies that the viability of bovine embryos following cryopreservation was decreased over that of similar fresh bovine embryos (Bank et al., 1974; Rall et al., 1985; Tervit et al., 1981; Hyttel et al., 1986; Baguisi et al., 1998). The injury is more pronounced in day-5 and day-13 embryos compared with fresh bovine embryos

Table 3.4. Experiment II, effects of laser-assisted hatching (LAH) of in vivo-produced frozen-thawed bovine embryos after 4 days of in vitro culture.

Treatment	Failed to Hatch (%)	Hatched Blastocyst <sup>a</sup> (%)
Treatment A (n= 10) LAH	1 (10)	9 (90)
Treatment B (n=10) Sham Control	1 (10)	9 (90)

<sup>a</sup>Embryos in laser-assisted treatment group appeared somewhat more viable than control group.

Table 3.5. Experiment III, mean embryo grade, zona thickness and embryo diameter of in vivo-produced frozen-thawed bovine embryos ( $\pm$ SE) before exposure to treatment.

Treatment	Mean Grade	Zona Thickness ( $\mu$ m)	Embryo Diameter ( $\mu$ m)
Treatment A (n= 66) LAH <sup>a</sup>	1.7 $\pm$ 0.06	13.4 $\pm$ 0.3	162.6 $\pm$ 0.9
Treatment B (n=66) Sham Control	1.8 $\pm$ 0.06	13.7 $\pm$ 0.2	161.7 $\pm$ 0.7

<sup>a</sup> Laser-assisted hatching.



Table 3.6. Experiment III, effects of laser-assisted hatching (LAH) of in vivo-produced frozen-thawed bovine embryos after 4 days of in vitro culture.

Treatment	Failed to Hatch (%)	Hatched Blastocyst (%)
Treatment A (n=66) LAH	33 (50)	33 (50)
Treatment B (n= 66) Sham Control	30 (45)	36 (55)

Table 3.7. Experiments I, II and III combined effects of laser-assisted hatching (LAH) of in vivo-produced frozen-thawed bovine embryos after 4 days of in vitro culture.

Treatment	Failed to Hatch (%)	Hatched Blastocyst (%)
Treatment A (n=86) LAH	36 (42)	51 (59)*
Treatment B (n= 86) Sham Control	32 (37)	54 (63)

\* One of the embryos split into two after treatment.

(Hyttel et al., 1986). Several techniques for assisted hatching have been introduced to thin the zona pellucida and promote the process of hatching. The overall objective of Experiments I, II and III was to determine if laser-assisted hatching using the XYClone<sup>®</sup> would improve hatching rates of frozen-thawed in vivo-produced bovine embryos when exposed to in vitro culture conditions.

Structural and ultrastructural changes that are caused by the freezing and/or the thawing process can decrease the post-thaw quality of bovine embryos (Mohr et al., 1981; Hyttel et al., 1986; Baguisi et al., 1998; Moreira et al., 2005). In our study, to reduce the bias, frozen-thawed in vivo-produced bovine embryos were randomly allotted to treatments before grading and embryo measurements were recorded in all three experiments.

The physical, chemical or osmotic damage to cells during cryopreservation procedures and could have been the causing necrotic cells and the decrease in quality noted in embryos in Experiment III before being frozen at the embryo transplantation unit. Damage caused to the embryo during cryopreservation may include membrane disruption, cytoskeletal destabilization, and possibly zona pellucida hardening (Mohr et al., 1981; Baguisi et al., 1998; Agarwal et al., 2009). It has been reported that cryopreservation can cause changes to the zona pellucida and when combined with the loss of embryo mass/volume may affect the embryo's ability to hatch in vivo (Whittingham et al., 1976; Tervit et al., 1981; Hyttel et al., 1986; Moreira et al., 2005).

Although not significant, our results may indicate a change in hatching pattern of the in vivo-produced bovine embryos with the position and the depth of the rents made using the diode laser. It has been shown that introducing an artificial opening in the human zona pellucida (i.e., laser-assisted hatching) can cause hatching of the embryo through a thicker zona pellucida in an hourglass pattern and with entrapment of the embryo (Cohen et al., 1991). This pattern occurred in Experiment I, indicating that introducing two vertical rents 80% through the zona pellucida at 600  $\mu$ second pulse and

90% power caused an irregular hatching pattern (Figure 3.10). The protruding of the embryo through the rent caused “pinching” and splitting of one embryo (Experiment I). Monozygotic twinning is the result of an embryo splitting, which in most cases is not desirable due to more retained placentas, increased dystocia, increased postpartum interval, lower conception rates, decreased survival at birth and lighter calves (Gregory et al., 1990).

Cohen et al. (1991) also showed that 16% of partial zona dissection embryos hatched completely and the remainder failed to hatch becoming trapped within the zona. However, with larger rents 72% of the embryos hatched completely. In Experiment II, thinning of the bovine zona pellucida was used in an effort to improve blastocyst hatching and to avoid loss of blastomeres. Thinning of the human zona pellucida rather than a complete zona renting has been previously shown to increase blastocyst hatching and implantation rates (Kolbe et al., 2005; Sifer et al. 2006).

Although some cell “pinching” was observed in Experiment II when two symmetrical zona rents were made 40% through the outer zona pellucida. This allowed for a more natural hatching process to take place. When the zona was thinned, as in Experiment III using three symmetrical rents using the same pulse and power removal of 40% of the zona pellucida, the embryo hatched through a large opening in the zona pellucida. These results are in agreement with those of previous studies conducted on in vivo-produced mouse embryos (Cohen et al., 1991; Gordon et al., 1993) (Figure 3.11). This suggests that allowing the embryo to exert internal force to hatch should prevent “pinching” and subsequently reduce monozygotic twinning.

Baguisi et al. (1998) showed that not only does creating an artificial opening by a renting the zona pellucida change hatching patterns, but embryos also hatched incompletely. These findings agree with other hatching studies using in vitro bovine embryos (Schmoll et al., 1999;

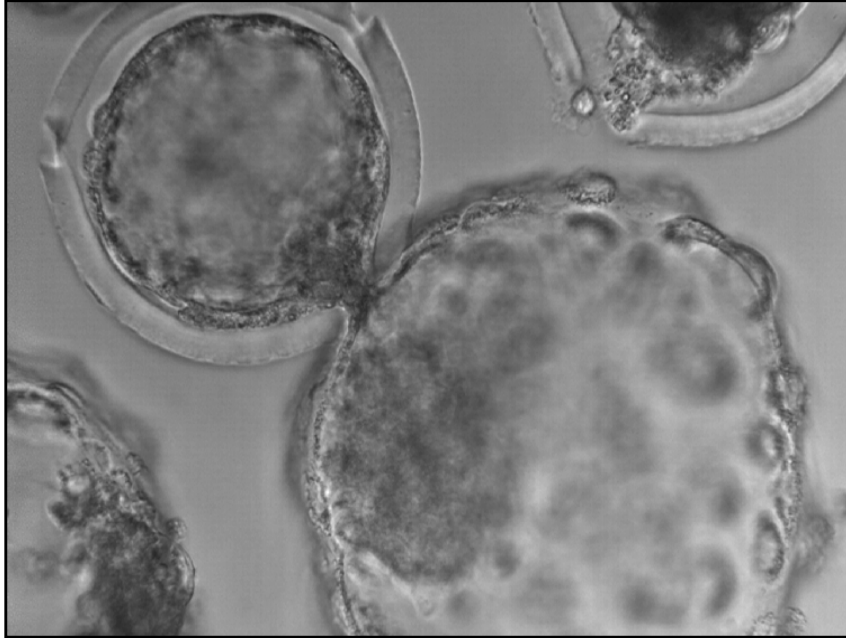


Figure 3.10. In vivo-produced frozen-thawed bovine embryo from Treatment A in Experiment I, seeping and pinching of blastocyst through two vertical rents made 80% through the outer zona pellucida.



Figure 3.11. In vivo-produced frozen-thawed bovine embryos from Treatment A in Experiment III, hatching naturally through three symmetrical rents made 40% through the outer zona pellucida using the XCYlone<sup>®</sup> diode laser.

Park et al., 1999). When comparing the two vertical rents made 80% (Experiment I) through the outer zona pellucida to the three symmetrical rents made through 40% (Experiment III) of the outer zona pellucida results showed incomplete hatching only occurred in Experiment I. In Experiment III, the embryos exhibited less variable hatched diameter and with no visible loss in embryo quality.

Cell “pinching” was observed in Experiment II but no embryo splitting occurred. However, differences in embryo quality noted on day-4 of culture between Treatments A and B may due to a delay in the hatching of embryos. An adjustment in the number of rents was made from two symmetrical rents (Experiment II) to three symmetrical rents (Experiment III) to embryos in Treatment A to circumvent this delay in hatching and reduce “pinching” of the embryo during the hatching process. Treated embryos in Experiment III received three symmetrical rents 40% through the outer zona pellucida and 17  $\mu\text{m}$  in length and no “pinching”, twinning or delay in embryo development was observed. Similar results, from a previous study, showed that a rent made using a laser in the zona pellucida with a diameter of 10 to 15  $\mu\text{m}$  does not improve hatching rates of in vitro-produced bovine embryos (Schmoll et al., 1999).

The trophoctoderm is responsible for secreting a serine protease at the early blastocyst stage, which aids in the thinning of the zona pellucida (Menino et al., 1986). The bovine embryos inability to hatch may be due to the damage caused by the cryopreservation process to the trophoctoderm cells, plasma membranes and zona pellucida (Willadsen et al., 1977; Mohr et al., 1981; Fair et al., 2001; Moreira et al., 2005). This may result in decreased total cell number, increased cellular debris and death of the inner cell mass.

It has been suggested that cryopreserved human embryos may pose damage to the zona pellucida or embryos with increased cellular trauma may benefit from the application of laser-assisted hatching (Kung et al., 2003; Ng et al., 2005; Balaban et al., 2006; Hiraoka et al.,

2008). However, conflicting results have been reported among studies evaluating the cause of hatching failure in cultured and cryopreserved human embryos (Cohen et al., 1990; Strohmer et al., 1992; Primi et al., 2004; Peterson et al., 2005; Balaban et al., 2006; Valojerdi et al., 2009).

The results from the present study show that the application of laser-assisted hatching using the XYClone<sup>®</sup> diode laser, at the settings used, did not affect blastocyst hatching rates of in vivo-produced frozen-thawed bovine embryos exposed to in vitro culture.

## **CHAPTER IV**

### **EFFECT OF LASER-ASSISTED HATCHING PRIOR TO THE TRANSFER OF FROZEN-THAWED IN VIVO-PRODUCED BOVINE EMBRYOS**

#### **Introduction**

Embryo cryopreservation has been a successful assisted reproductive technology, but field results generally indicate pregnancy rates are lower compared with similar fresh transferred embryos. Therefore, it appears as though the viability and developmental competence of the embryo may be reduced following cryopreservation. Similarly, calving rates of frozen-thawed in vivo-produced bovine embryos are generally lower than those achieved by the transfer of fresh bovine embryos (Bank et al., 1974; Tervit et al., 1981; Hyttel et al., 1986; Baguisi et al., 1998).

It has been suggested that lower pregnancy rates following the transfer of in vitro fertilized embryo transfers is due to the inability of the blastocyst to hatch from the zona pellucida (Cohen et al., 1990). It has been demonstrated that structural changes and damage to cell components of bovine embryos occur during the freezing and/or thawing process (Mohr and Trounson, 1981). Because cryopreservation may alter the zona pellucida, decreased in vivo hatching rates of those frozen-thawed embryos may occur. Fetal loss of 10 to 13% and pregnancy rates ranging from 3 and 60% for fresh and frozen-thawed bovine embryos of comparable quality have been reported (Hasler et al., 2001). Studies have suggested that the thinning of the zona pellucida through assisted hatching may be beneficial to pregnancy rates of transferred frozen-thawed murine (Montag et al., 1999) and human embryos (Kung et al., 2003; Gabrielsen et al., 2004; Ng et al., 2005; Balaban et al., 2006; Hiraoka et al., 2008).

Assisted hatching by thinning of the zona pellucida has been reported to improve hatching rates of in vitro-produced mouse (Gao et al., 1998) and bovine embryos (Menges et al., 2008). Similar results show an increase in pregnancy rates with the application of laser-



assisted hatching applied to frozen-thawed human embryo transfer (Kung et al., 2003; Balaban et al., 2006; Hiraka et al., 2008; Blessmann-Roset et al., 2009). Thinning of the zona pellucida of human embryos with the use of a 1480 nm diode laser has been reported to increase implantation (20.1% vs. 9.9%) and pregnancy rates (40.9% vs. 27.3%) when 25% of the zona pellucida is removed from the 3 o'clock to the 12 o'clock position (Balaban et al., 2006). However, Valojerdi et al. (2009) reported no difference in implantation or pregnancy rate was detected and these findings suggested that laser-assisted hatching may have adverse effects on the embryonic cells and should not be performed routinely on frozen-thawed human embryos.

It has been suggested that impaired zona pellucida hatching of mammalian embryos resulting from embryo freeze and thawing process can contribute to lower implantation rates in the rabbit (Chang, 1948; Smith, 1952; Bank and Maurer, 1974) and cow (Baguisi et al., 1998). It has been reported that implantation and pregnancy rates of in vitro and in vivo human embryos may be enhanced by mechanical, chemical, enzymatic assisted hatching techniques (Liu et al., 1993; Stein et al., 1995; Balaban et al., 2002).

The objective of Experiments IV and V was to determine if laser-assisted hatching would increase pregnancy and calving rates of in vivo-produced frozen-thawed bovine embryos. Embryo post-thaw parameters were evaluated and compared also with subsequent pregnancy rates.

## **Material and Methods**

### **Experimental Embryos**

In vivo-produced nonsurgically collected direct-transfer Hereford embryos (n = 64) were used in Experiment IV. In Experiment V, in vivo-produced nonsurgically collected glycerol frozen

Brangus embryos (n = 46) were utilized. In both experiments, in vivo bovine embryos were flushed and frozen using protocols described by Robertson et al. (1998).

In Experiments IV, the in vivo-produced Hereford embryos were donated from Grandview in Beaver, WV and were collected from the same herd and processed by the same technicians. Embryos from 9 different donors were stratified across treatments. No pre-freeze embryo quality grades were available for embryos used in this experiment. Embryos were frozen in ethylene glycol medium to be utilized for direct transfer at a later date.

In Experiment V, in vivo-produced Brangus embryos were donated by Dr. Charles Looney of OvaGenix (College Station, TX) and were collected from different herds and processed at OvaGenix using an OvaGenix standard protocol. In this experiment, embryos from eight donors were stratified across two treatments groups.

In Experiments IV and V, two embryos were randomly selected, identified and thawed at one time. After being removed from liquid nitrogen, embryos were allowed to air thaw for 15 seconds, before being placed in a 30°C water bath for an additional 15 seconds and then any excess water was removed from the straw before expelling its contents. The straw was opened using scissors and contents were expelled using a 1-ml sterile syringe. The end of the straw without the cotton was cut first and the syringe was placed on the unplugged end, then the cotton end of the straw was removed before pushing the contents of the straw into the 35-mm sterile Petri dish. Contents from the first column and last column were expelled separately from the center column.

Embryos were located, removed from the cryoprotectant and held in 3 ml of 1M sucrose (Bioniche, Athens, GA) for 7 minutes at room temperature in a 35-mm sterile Petri Dish. Embryos were then transferred to a 4-well sterile Petri dish containing 3 ml of Phosphate-Buffered Saline (Gibco, Carlsbad, CA) with 10% calf serum (Experiment IV) or in ViGRO

Holding Plus™ (Bioniche, Athens, GA; Experiment V). Embryos were then washed twice in the holding medium, evaluated for stage and grade then treatments were randomly applied.

Embryos were evaluated under a 40X magnification using a Nikon inverted microscope. Embryos were assigned grades of 1 to 4 (1 = excellent and 4 = poor) and stage of embryo development (4 = morula to 7 = expanded blastocyst) was recorded. Treatments A and B were then applied. Embryos were then reloaded in holding medium into a 0.25-ml sterile plastic straws. The embryo in Treatment A was identified with a mark made using a black permanent marker on the straw before being loaded in an IMV embryo transfer gun (Bioniche, Athens, GA). A sterile plastic blue embryo transfer sheath was placed over the gun and locked into place. A sterile plastic jacket sheath was then placed over the blue embryo transfer sheath (Reproductive Resources, Walworth, WI) to prevent vaginal contamination during transfer to recipient. Embryos were held at room temperature for no more than 15 minutes before transfer into a recipient cow on day-7 of her estrous cycle.

#### Laser-Assisted Zona Pellucida Renting

Measurements of zona thickness and embryo diameter were recorded from images using the XYClone® software. Five measurements of the zona thickness were taken and a mean and standard error ( $\pm$ SE) were calculated. The five measurements of the zona pellucida thickness were measured at the 3, 5, 7, 10 and 12 o'clock positions. Two measurements of embryo diameter were recorded and a mean and standard error ( $\pm$ SE) calculated on the embryo. These two measurements were taken from the 6 to 12 o'clock position and 3 to 9 o'clock position.

Based on results from Experiment I, II and III, embryos in Experiments IV and V received three symmetrical rents 40% through the total zona pellucida thickness located on the outer surface each 17  $\mu$ m in length using the diode laser at 90% power with a 600  $\mu$ second pulse.

### Recipients for Embryo Transfer

In Experiment IV and V, recipients used were crossed breed beef cows 2 years of age from the same herd. Recipients were selected based on being 50 days postpartum, having a body condition greater than 3 and prior reproductive soundness.

The estrous cycles of the recipient females were synchronized using an EAZI-BREED™ CIDR (progesterone controlled internal drug release) (Pfizer Animal Health, NY, NY) synchronization protocol utilized is described as follows: On day-0, cows received either a new or once-used CIDR vaginally inserted. On day-7, the CIDRs were removed and a single dosage of 25 mg (im) of prostaglandin F<sub>2</sub>α (Lutalyse®, Pfizer Animal Health, New York, NY) was administered to each female. Estroject™ (Estroject, Inc.) estrus detectors were applied to each recipient and were used to aid in visual observations between day-9 and 11 of the protocol. Recipients were selected based on observed visual estrus 7 days prior to embryo transfer and rectal ultrasonography was used in the confirmation of a corpus luteum 7 days after the onset of estrus.

Embryos were transferred nonsurgically by the same technician into these synchronized beef recipient females on day-7 of their estrous cycle. The recipient was prepped for embryo transfer by cleaning of the tailhead with sterile gauze and alcohol (70% ethonal) followed by an epidural injection with 100 mg of lidocaine. The vulva was then cleaned using a paper towel before insertion of the embryo transfer gun with sterile plastic cover. After insertion of the embryo transfer gun into the vagina, the sterile plastic cover was removed before entering the cervix. Embryos were deposited distally to the external uterine bifurcation into the horn ipsilateral to the corpus luteum. In both experiments, pregnancy rates were determined via rectal ultrasonography using Sonosite MicroMaxx® (Bothell, WA) at 35 days and 60 days of gestation. Live births and sex of calves were also recorded at the time of parturition.

## Experimental Design

In Experiments IV and V, the treatment groups were either laser-assisted (Treatment A) or zona left intact (Treatment B). Embryos were randomly allotted to either Treatment A (n = 32 and n = 23) or Treatment B (n = 32 and n = 23), respectively. Embryos in Treatment A received three symmetrical rents 40% through the total zona thickness located the outer portion of the zona pellucida and were 17  $\mu\text{m}$  in length using the XYClone<sup>®</sup> diode laser at 90% power with a 600  $\mu\text{second}$  pulse. Treatment B was a sham control, where the laser was fired into the sterile mineral oil located on the outside of the medium droplet where the embryo was located.

## Statistical Analysis

The effect of mean embryo grade, zona thickness and embryo diameter were analyzed by the number of pregnant and open females resulting from in vivo-produced frozen-thawed bovine embryos, was tested using Proc GLM and LSMEANS. The effect of treatment on embryo grades and pregnancy rates were analyzed using a Fisher's Exact test. Also, when analyzing treatment effect on pregnancy rates at 35 days and 60 days, a Chi-Square analysis was used and for data that had less than 5 observations per cell, a Fisher's Exact test was used. Chi-Square (SAS, Cary, NC) was performed to determine difference on calves that were born alive compare across treatments. Two images, one from Treatment A and another from Treatment B, were lost due to computer malfunction thus no zona thickness and embryo diameter measurements for these embryos were not included in the statistical analyses. The two embryos, however, were included in effect of treatment on pregnancy rates and live calves. Significance was determined at the  $P < 0.05$  level.

## **Results**

In Experiment IV, mean embryo grade, zona thickness and embryo diameter were not different across treatment groups (Table 4.1). Mean embryo quality grades ( $\pm\text{SE}$ ) were  $2.0 \pm 0.1$

Table 4.1. Mean embryo grade, zona thickness and embryo diameter ( $\pm$ SE) were compared across treatments applied to in vivo-produced frozen-thawed direct-transfer Hereford embryos.

Treatment	Mean Grade <sup>*</sup>	Zona Thickness ( $\mu$ m)	Embryo Diameter ( $\mu$ m)
Treatment A (n = 32) LAH <sup>a</sup>	2.0 $\pm$ 0.1	11.9 $\pm$ 0.3	149.8 $\pm$ 0.9
Treatment B (n = 32) Sham Control	2.1 $\pm$ 0.1	11.9 $\pm$ 0.2	150.7 $\pm$ 0.8

<sup>\*</sup> Grade was assigned from excellent (grade 1) to poor (grade 4) quality embryos.

<sup>a</sup> Laser-assisted hatching.

for Treatment A and  $2.1 \pm 0.1$  for Treatment B. Mean zona thickness was  $11.9 \pm 0.3$  for Treatment A and  $11.9 \pm 0.2$  for Treatment B. Mean embryo diameter was  $149.8 \pm 0.9$  for Treatment A and  $150.7 \pm 0.8$  for Treatment B. Also, embryo grade, zona thickness and embryo diameter were not different for pregnant or nonpregnant females across treatment groups.

In Experiment IV, embryo grade did not affect pregnancy rates at 35 days or 60 days of gestation for pregnant recipients (Figure 4.1 and 4.2). Zona thickness did not affect 35-day pregnancy rates for pregnant recipients from Treatment A ( $11.6 \pm 0.4$ ) and Treatment B ( $11.8 \pm 0.4$ ) when compared with open recipients from Treatment A ( $12.1 \pm 0.3$ ) and Treatment B ( $11.9 \pm 0.3$ ) (Figure 4.3). Zona thickness did not affect 60-day pregnancy rates for pregnant recipients from Treatment A ( $11.4 \pm 0.4$ ) and Treatment B ( $11.9 \pm 0.4$ ) when compared with open recipients from Treatment A ( $12.1 \pm 0.3$ ) and Treatment B ( $11.8 \pm 0.3$ ) (Figure 4.4). Embryo diameter did not affect 35-day pregnancy rates for pregnant recipients from Treatment A ( $151.3 \pm 1.4$ ) and Treatment B ( $151.3 \pm 1.4$ ) compared with open recipients from Treatment A ( $148.8 \pm 1.1$ ) and Treatment B ( $150.2 \pm 1.1$ ) (Figure 4.5). Embryo diameter did not affect at 60-day pregnancy rates for pregnant recipients from Treatment A ( $151.2 \pm 1.6$ ) and Treatment B ( $151.3 \pm 1.4$ ) and were not different when compared with open recipients from Treatment A ( $149.2 \pm 1.0$ ) and Treatment B ( $149.9 \pm 1.1$ ) (Figure 4.6).

In Experiment IV, 35-day pregnancy rates were not different for grades 1, 2 and 3 (Figure 4.7 and 4.8). Pregnancy rates for grade-1 embryo at both 35 days and 60 days of gestation for Treatment A were 33% (2/6) and Treatment B were 57% (4/7). Pregnancy rates for grade-2 at 35 days and 60 days of gestation for Treatment A were 45% (10/22) and 36% (8/22) compared with Treatment B were 37% (6/16) and 31% (5/16), respectively. Pregnancy rates for a grade-3 embryos at 35 days and 60 days of gestation for Treatment A were 25% (1/4) and 0% (0/4) compared with Treatment B were 44% (4/9) and 44% (4/9), respectively.

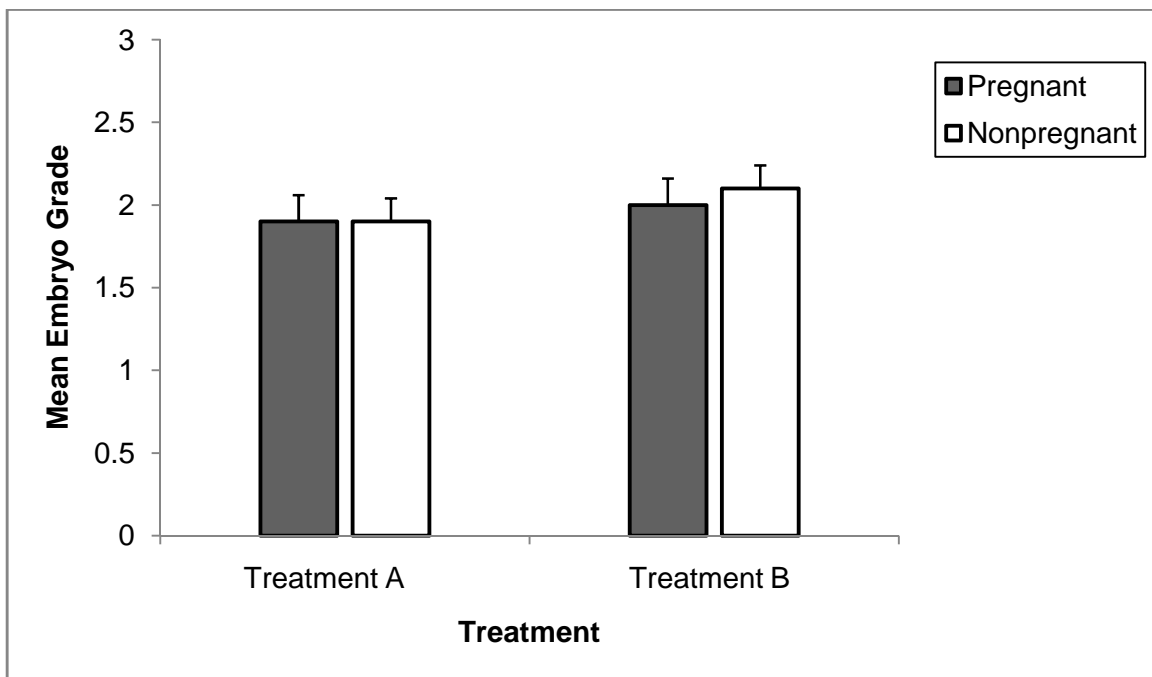


Figure 4.1. Experiment IV, effect of embryo grade on 35-day pregnancy rates of recipients receiving either LAH (n = 32) or non-LAH (n = 32) in vivo-produced frozen-thawed direct transfer Hereford embryos.



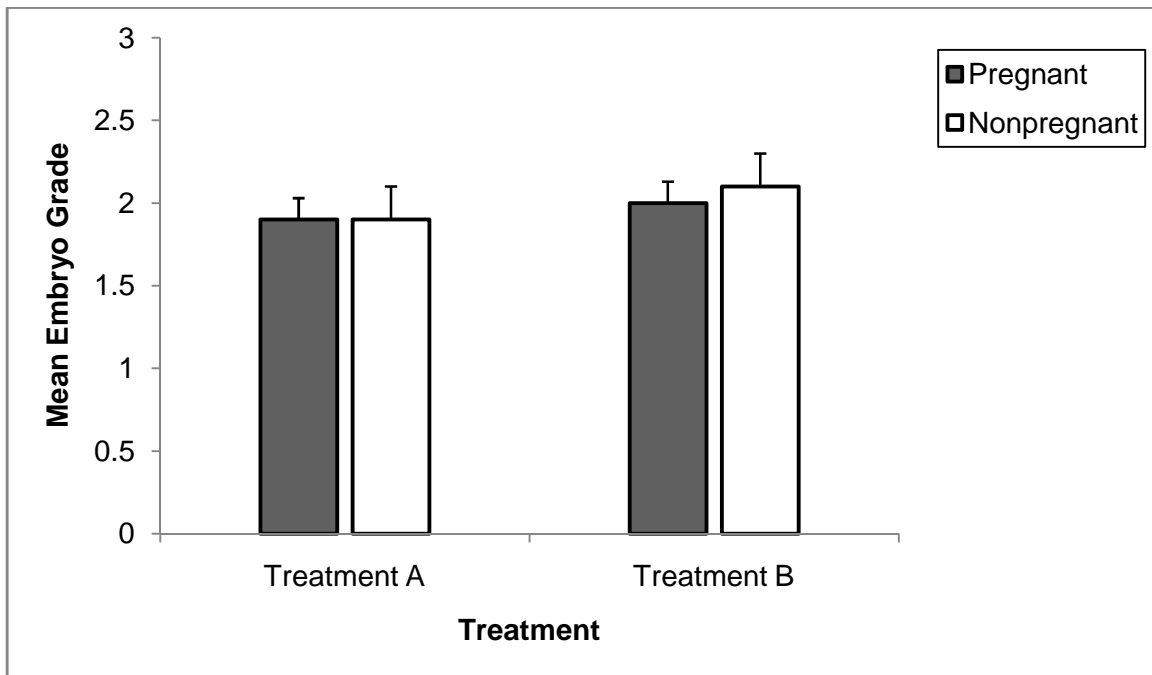


Figure 4.2. Experiment IV, effect of embryo grade on 60-day pregnancy rates of recipients receiving either LAH (n = 32) or non-LAH (n = 32) in vivo-produced frozen-thawed direct transfer Hereford embryos.

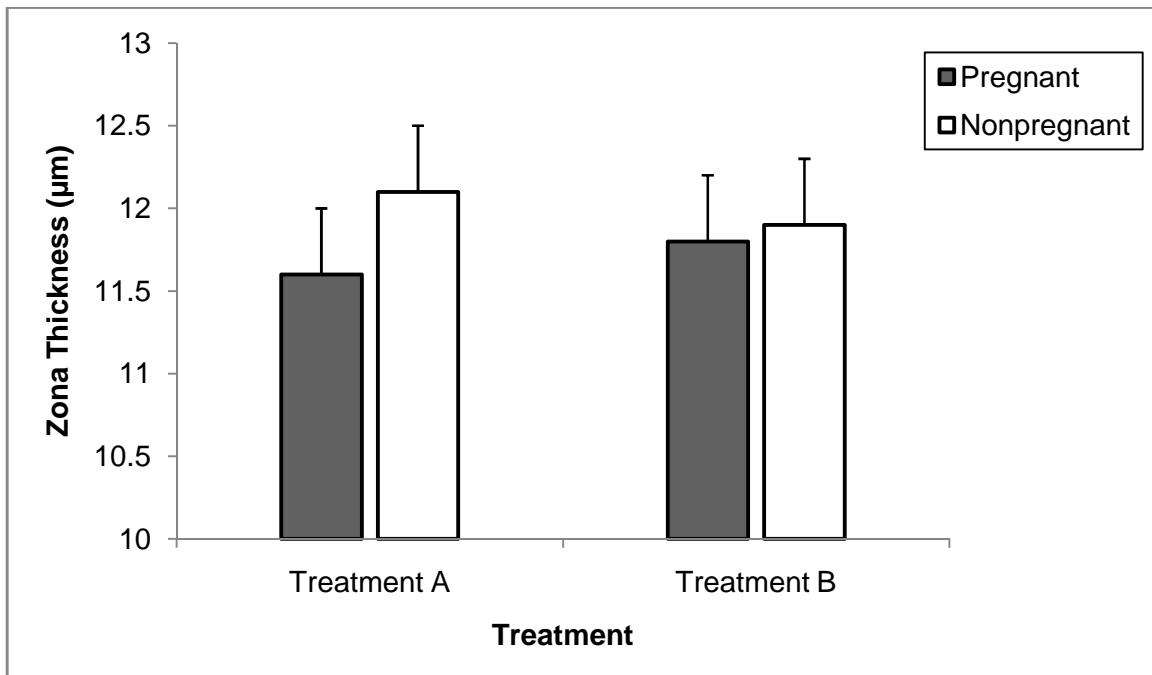


Figure 4.3. Experiment IV, effect of zona thickness on 35-day pregnancy rates of recipients receiving either LAH (n = 32) or non-LAH (n = 32) in vivo-produced frozen-thawed direct transfer Hereford embryos.

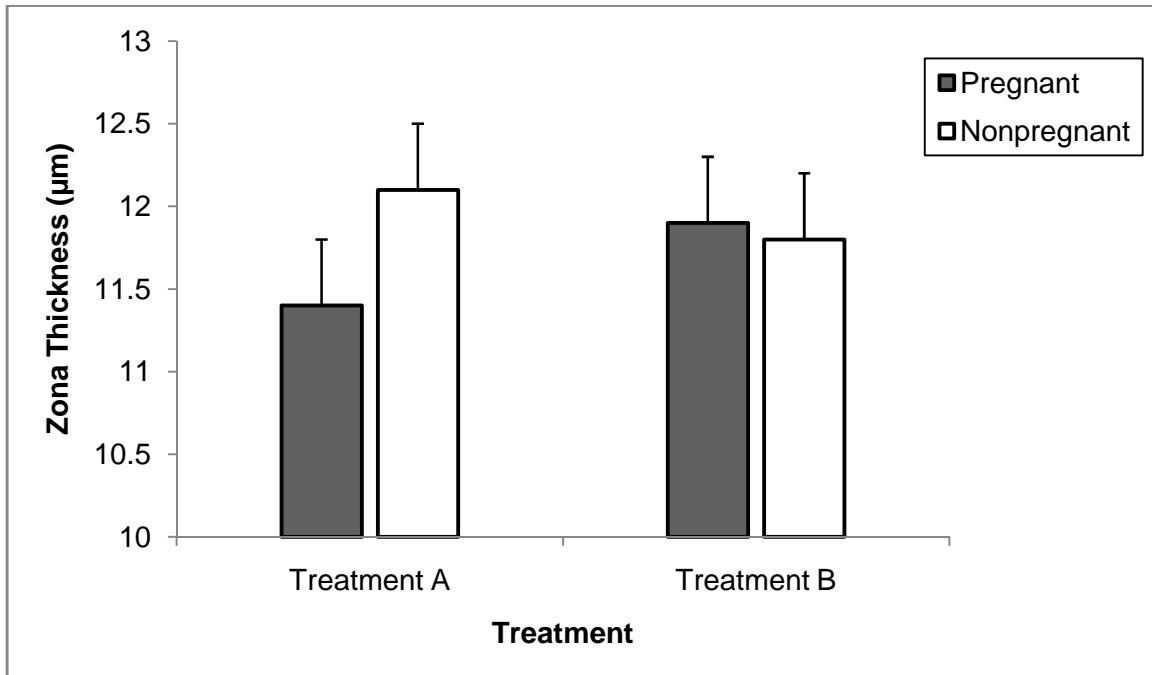


Figure 4.4. Experiment IV, effect of zona thickness on 60-day pregnancy rates of recipients receiving either LAH (n = 32) or non-LAH (n = 32) in vivo-produced frozen-thawed direct transfer Hereford embryos.

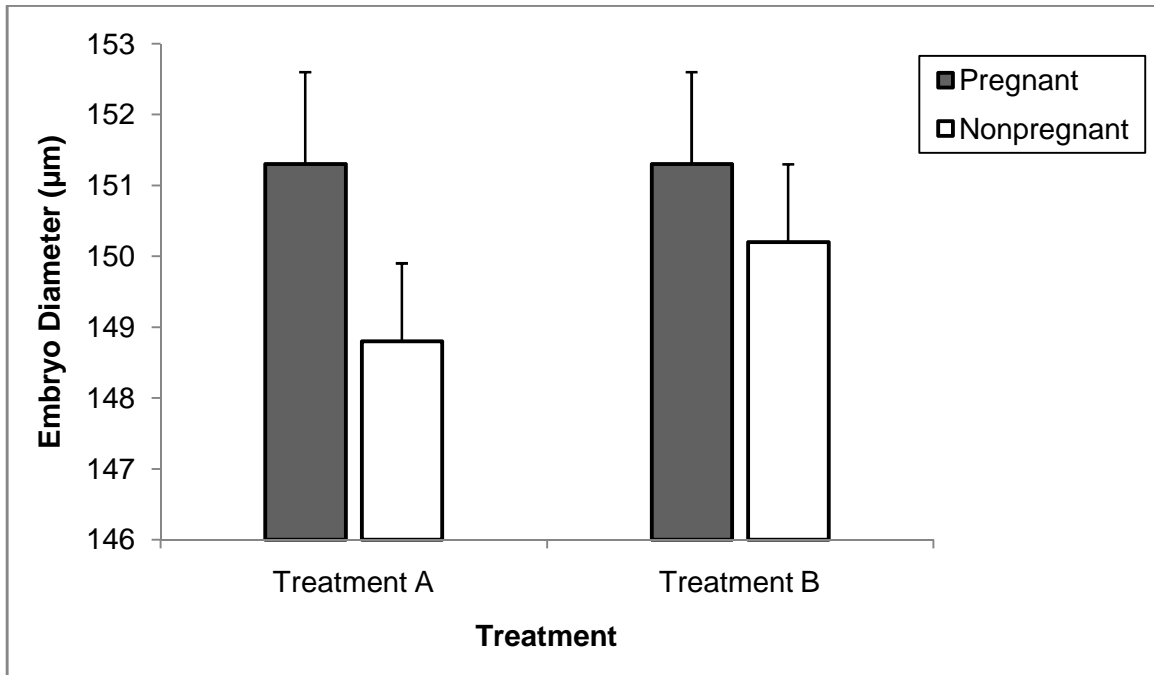


Figure 4.5. Experiment IV, effect of embryo diameter on 35-day pregnancy rates of recipients receiving either LAH (n = 32) or non-LAH (n = 32) in vivo-produced frozen-thawed direct transfer Hereford embryos.

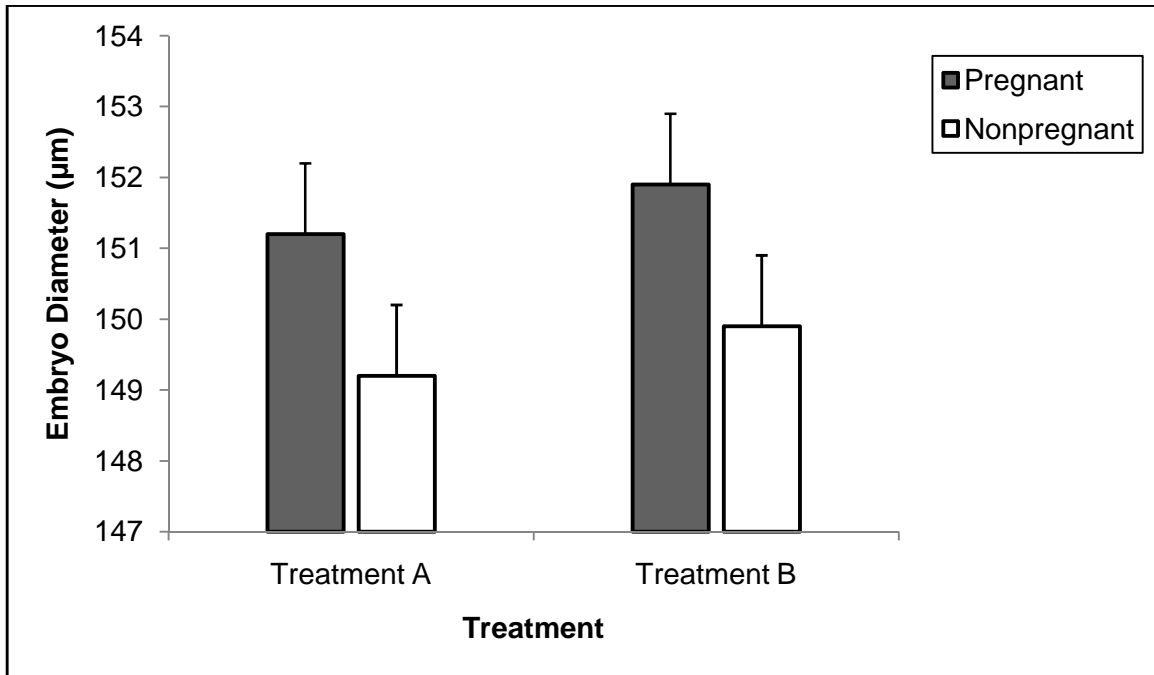


Figure 4.6. Experiment IV, effect of embryo diameter on 60-day pregnancy rates of recipients receiving either LAH (n = 32) or non-LAH (n = 32) in vivo-produced frozen-thawed direct transfer Hereford embryos.

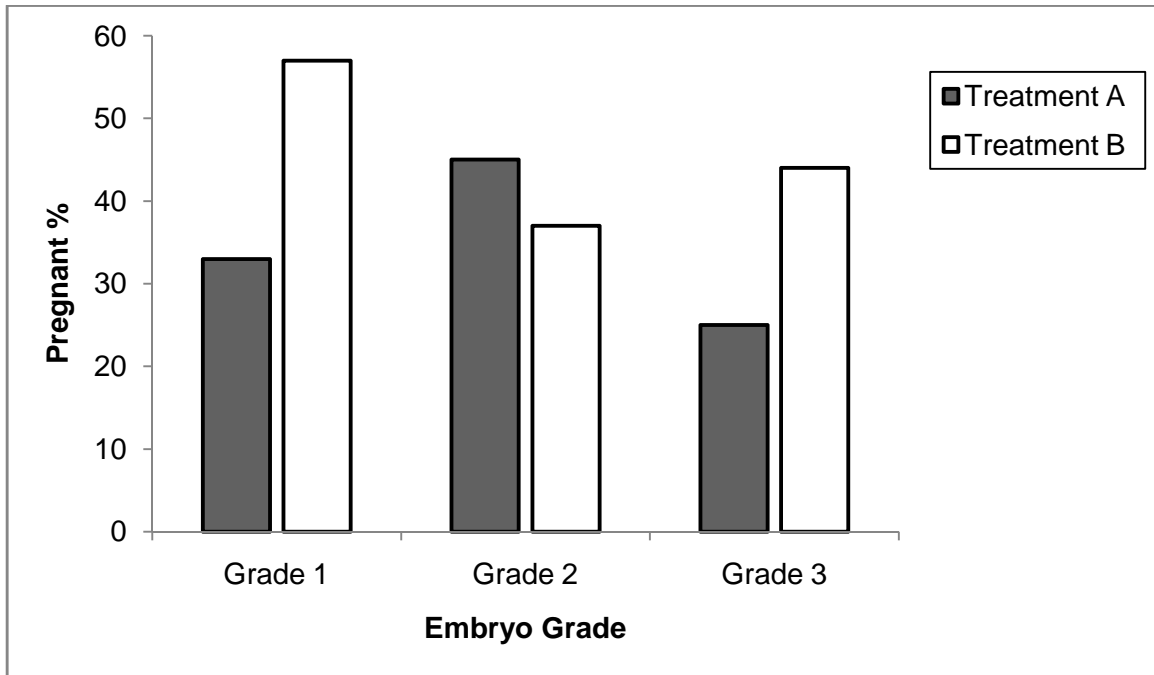


Figure 4.7. Experiment IV, 35-day pregnancy rates compared with grades of in vivo-produced frozen-thawed Hereford embryos. There were no difference between grades 1, 2 and 3 and pregnancy rates.

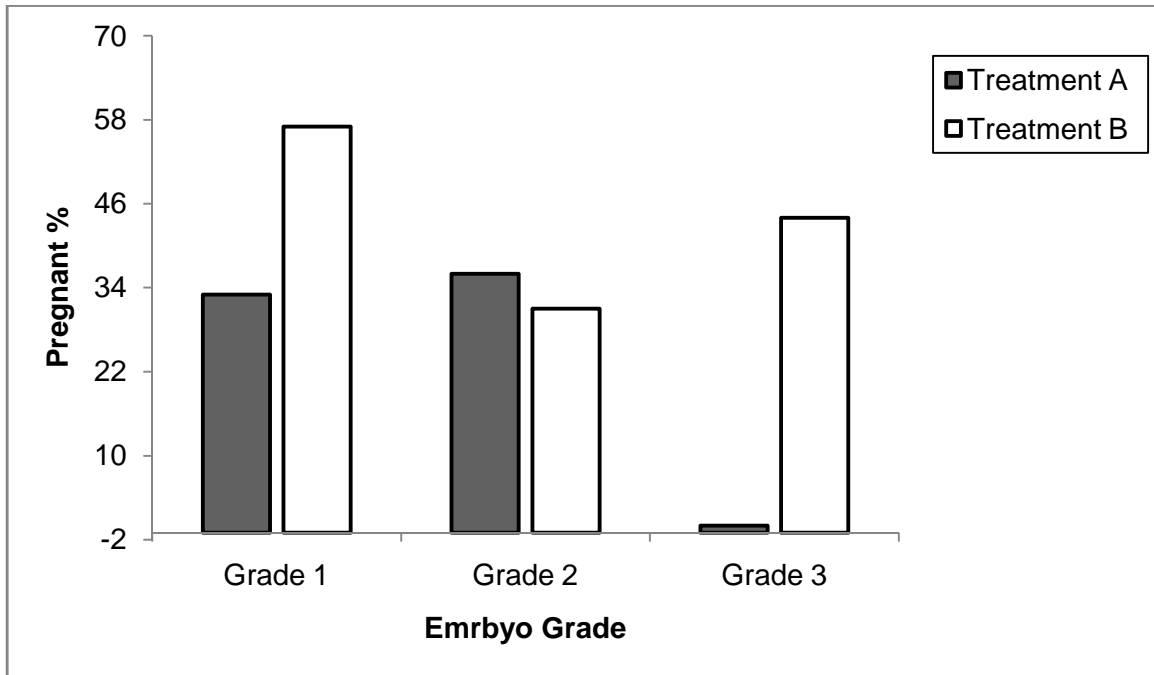


Figure 4.8. Experiment IV, 60-day pregnancy rates compared with embryo grade of in vivo-produced frozen-thawed Hereford embryos. No difference between grades 1, 2 and 3 and pregnancy rates.

In Experiment IV, treatment did not affect pregnancy rates at 35 days and 60 days of gestation. Pregnancy rates at 35 days and 60 days of gestation for Treatment A were 41% (13/32) and 28% (9/32) and for Treatment B were 44% (14/32) and 41% (13/32), respectively (Table 4.2). Likewise, there was no difference in calving rates for recipients confirmed pregnant at 60 days of gestation for Treatment A with 89% (8/9) and 77% (10/13) for Treatment B. Also, there was no twinning that occurred in either treatment group (Table 4.3).

In Experiment V, mean embryo grade, zona thickness and embryo diameter were not different across treatments (Table 4.4). Mean embryo quality grades were  $2.0 \pm 0.1$  for Treatment A and  $2.0 \pm 0.1$  for Treatment B. Mean zona thickness was  $13.3 \pm 0.3$  for Treatment A and  $13.6 \pm 0.3$  for Treatment B. Mean embryo diameter was  $162.9 \pm 1.3$  for Treatment A and  $162.9 \pm 1.3$  for Treatment B.

Also, embryo grade, zona thickness and embryo diameter were not different between pregnant and nonpregnant females. Mean embryo grades did not affect at 35 days of gestation for pregnant recipients from Treatment A ( $2.0 \pm 0.2$ ) and Treatment B ( $1.8 \pm 0.2$ ) were compared with open recipients from Treatment A ( $1.8 \pm 0.2$ ) and Treatment B ( $1.5 \pm 0.3$ ) (Figure 4.9). There was no difference in mean embryo grades at 60 days of gestation for pregnant recipients from Treatment A ( $1.9 \pm 0.2$ ) and Treatment B ( $1.8 \pm 0.2$ ) were compared with open recipients from Treatment A ( $1.9 \pm 0.2$ ) and Treatment B ( $1.7 \pm 0.2$ ) (Figure 4.10).

Zona thickness did not affect pregnancy rates at 35 days of gestation for pregnant recipients from Treatment A ( $13.2 \pm 0.4$ ) and Treatment B ( $13.8 \pm 0.4$ ) when compared with open recipients from Treatment A ( $13.6 \pm 0.6$ ) and Treatment B ( $12.9 \pm 0.8$ ) (Figure 4.11). Zona thickness did not affect pregnancy rates at 60 days of gestation for pregnant recipients from Treatment A ( $13.2 \pm 0.4$ ) and Treatment B ( $14.0 \pm 0.4$ ) when compared with open recipients from Treatment A ( $13.5 \pm 0.5$ ) and Treatment B ( $12.8 \pm 0.6$ ) (Figure 4.12). Embryo diameter did not



Table 4.2. Effect of treatment on pregnancy rates with the application of laser-assisted hatching on in vivo-produced frozen-thawed direct-transfer Hereford embryos on day-7 of the recipient's estrous cycle.

Treatment	35-Day Pregnancy (%)	60-Day Pregnancy (%)
Treatment A (n = 32) LAH <sup>a</sup>	13 (41)	9 (28)
Treatment B (n = 32) Sham Control	14 (44)	13 (41)

<sup>a</sup> Laser-assisted hatching.

Table 4.3. Effect of laser-assisted hatching on in vivo-produced frozen-thawed direct-transfer Hereford embryos on recipient calving rates.

Treatment	# Pregnancy at Day-60	# Born Alive (%)
Treatment A (n = 32) LAH <sup>a</sup>	9	8 (89)
Treatment B (n = 32) Sham Control	13	10 (77)

<sup>a</sup> Laser-assisted hatching.

Table 4.4. Mean embryo grade, zona thickness and embryo diameter ( $\pm$ SE) of in vivo-produced frozen-thawed glycerol Brangus embryos.

Treatment	Mean Grade <sup>*</sup>	Zona Thickness ( $\mu$ m)	Embryo Diameter ( $\mu$ m)
Treatment A (n = 23) LAH	2.0 $\pm$ 0.1	13.3 $\pm$ 0.3	162.9 $\pm$ 1.3
Treatment B (n = 23) Sham Control	2.0 $\pm$ 0.1	13.6 $\pm$ 0.3	162.9 $\pm$ 1.3

\* Embryo grade was assigned from excellent (grade 1) to poor (grade 4) quality embryos.

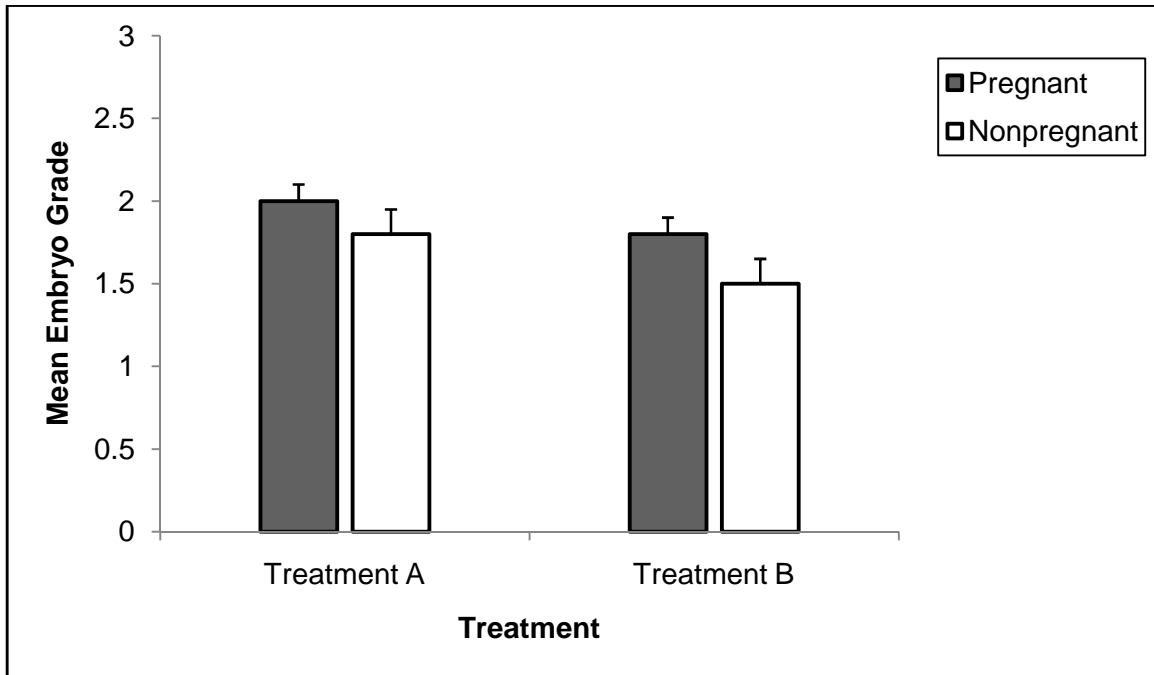


Figure 4.9. Experiment V, effect of mean embryo grade on 35-day pregnancy rates of recipients receiving either LAH (n = 23) or non-LAH (n = 23) in vivo-produced frozen-thawed glycerol Brangus embryos.

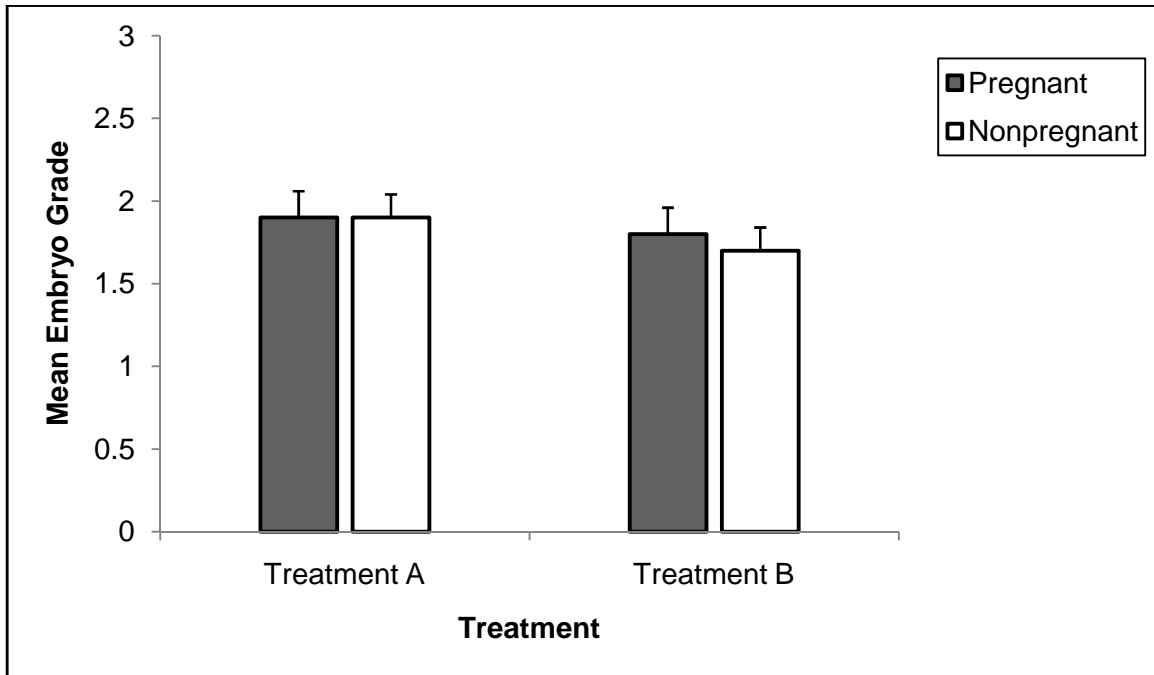


Figure 4.10. Experiment V, effect of mean embryo grade on 60-day pregnancy rates of recipients receiving either LAH (n = 23) or non-LAH (n = 23) in vivo-produced frozen-thawed glycerol Brangus embryos.

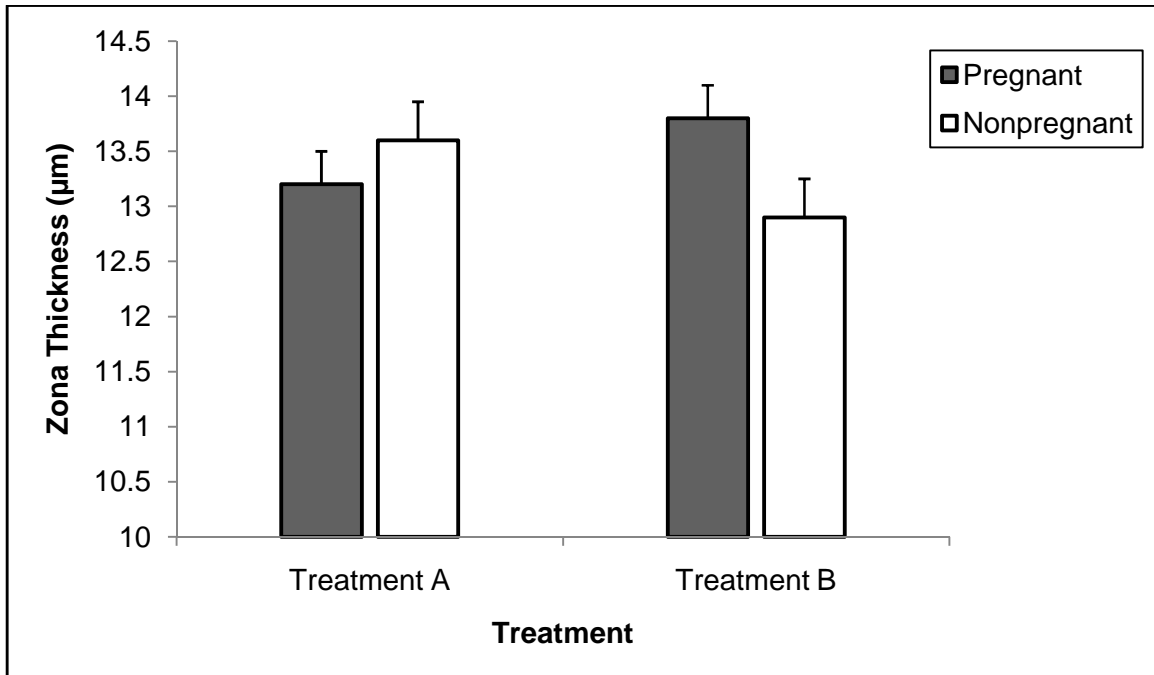


Figure 4.11. Experiment V, effect of zona thickness on 35-day pregnancy rates of recipients receiving either LAH (n = 23) or non-LAH (n = 23) in vivo-produced frozen-thawed glycerol Brangus embryos.

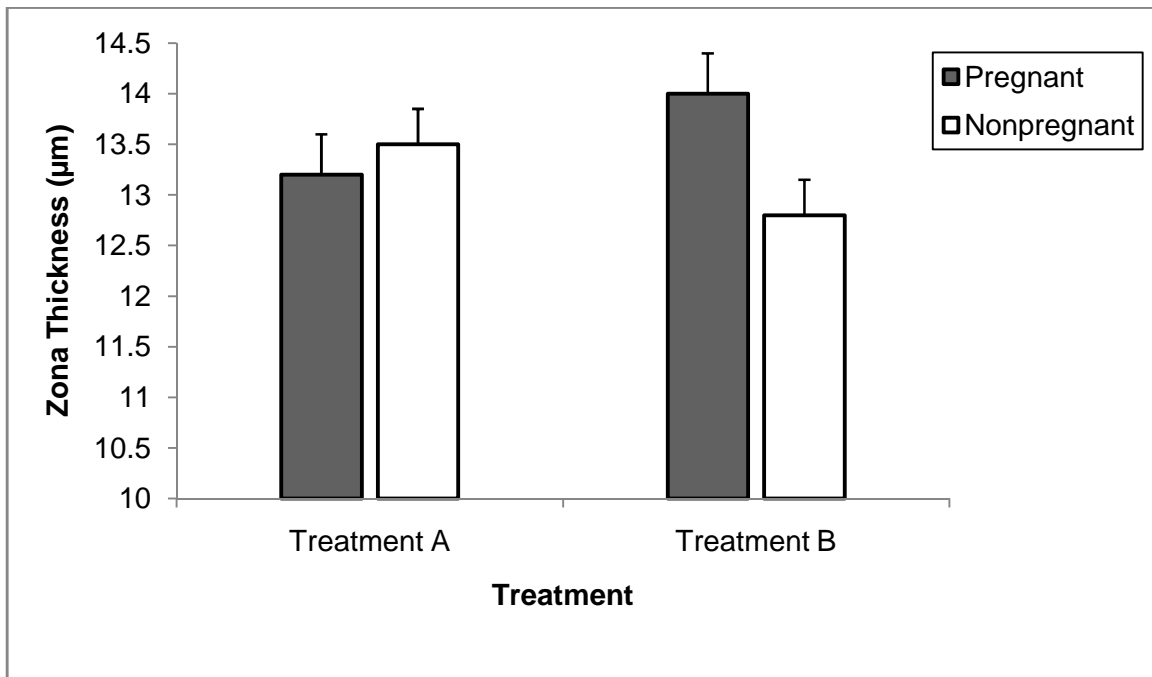


Figure 4.12. Experiment V, effect of zona thickness on 60-day pregnancy rates of recipients receiving either LAH (n = 23) or non-LAH (n = 23) in vivo-produced frozen-thawed glycerol Brangus embryos.

(161.4±1.6) and Treatment B (162.7±1.4) when compared with open recipients from Treatment A (165.7±2.1) and Treatment B (163.8±2.9) (Figure 4.13). Embryo diameter did not affect pregnancy rates at 60 days of gestation for pregnant recipients from Treatment A (161.1±1.6) and Treatment B (162.2±1.5) compared with open recipients from Treatment A (166.2±2.1) and Treatment B (164.3±2.2) (Figure 4.14).

Results from Experiment V, 35-day and 60-day pregnancy rates were not different for grades 1, 2 and 3 (Figures 4.15 and 4.16). Pregnancy rates for grade-1 embryo at both 35 days and 60 days of gestation for in Treatment A were 57% (4/7) compared with 71% (5/7) for Treatment B. The pregnancy rate for grade-2 embryos at 35 days of gestation was 83% (10/12) for Treatment A compared with 79% (11/14) for Treatment B. The pregnancy rate for grade-2 embryos at 60 days of gestation for Treatment A was 75% (9/12) compared with Treatment B which were 57% (8/14). Pregnancy rates for grade-3 embryos at both 35 days and 60 days of gestation for Treatment A were 50% (2/4) and 100% (2/2) for Treatment B.

In Experiment V, treatment did not affect day-35 or 60 pregnancy rates and were 69% (15/23) and 65% (15/23) for Treatment A and 78% (18/23) and 65% (15/23) for Treatment B, respectively (Table 4.5). Overall, treatment had no effect on calving rates after the transfer of in vivo-produced frozen-thawed Brangus embryos. In Treatment A 73% (11/15) of calves were born alive and for Treatment B was 73% (11/15) (Table 4.6). No twinning was occurred across treatment groups.

In Experiments IV and V, results were combined and analyzed. Pregnancy rates were not affected at either 35 days or 60 days by treatment and were 51% (28/55) and 44% (24/55) for Treatment A and 58% (32/55) and 51% (28/55) for Treatment B, respectively (Table 4.7). Overall, treatment did not affect calving rates for recipient receiving in vivo-produced frozen-thawed bovine embryos. In Treatment A 79% (19/24) of calves were born alive and for



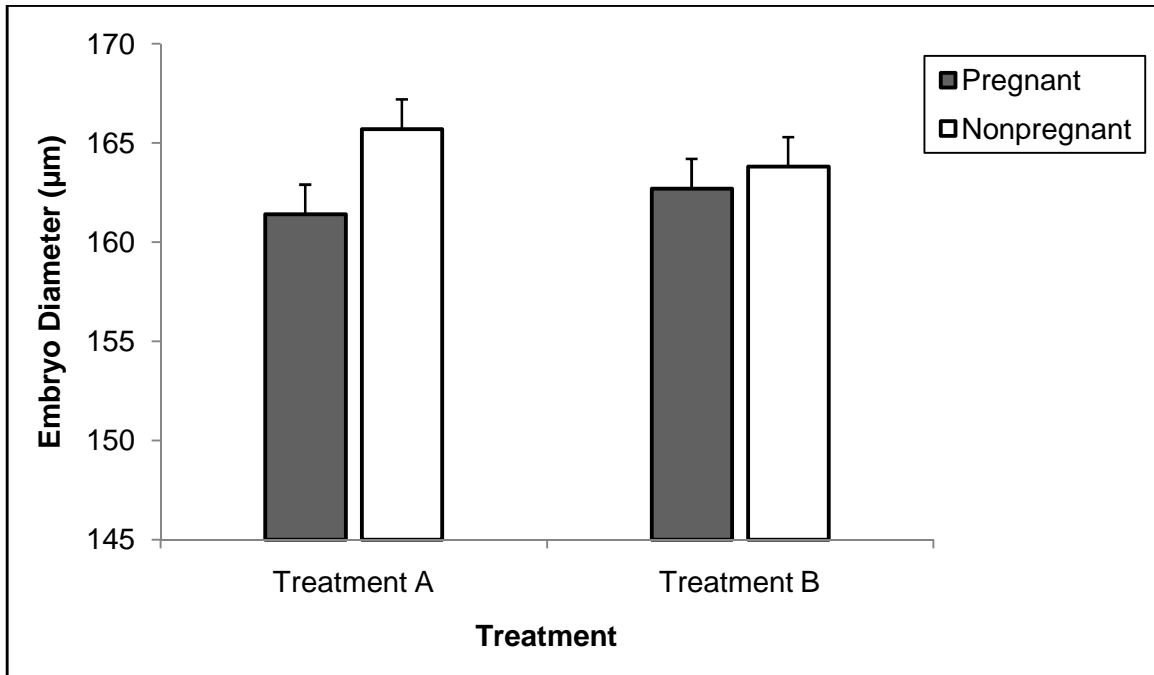


Figure 4.13. Experiment V, effect of embryo diameter on 35-day pregnancy rates of recipients receiving either LAH (n = 23) or non-LAH (n = 23) in vivo-produced frozen-thawed glycerol Brangus embryos.

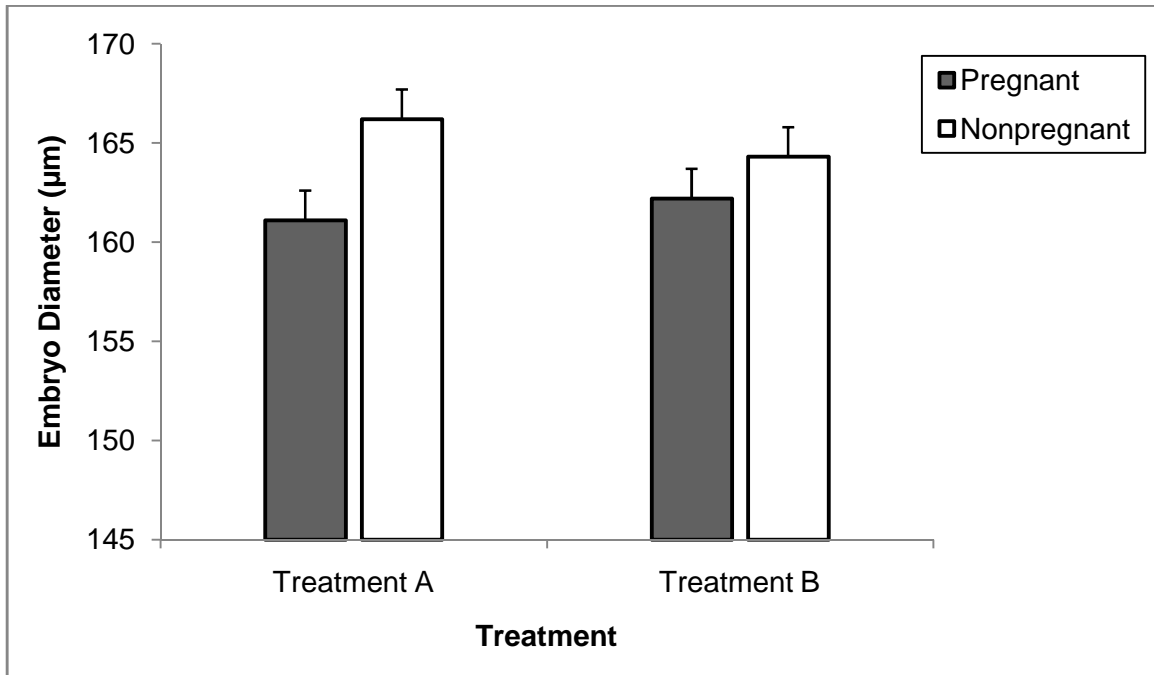


Figure 4.14. Experiment V, effect of embryo diameter on 60-day pregnancy rates of recipients receiving either LAH (n = 23) or non-LAH (n = 23) in vivo-produced frozen-thawed glycerol Brangus embryos.

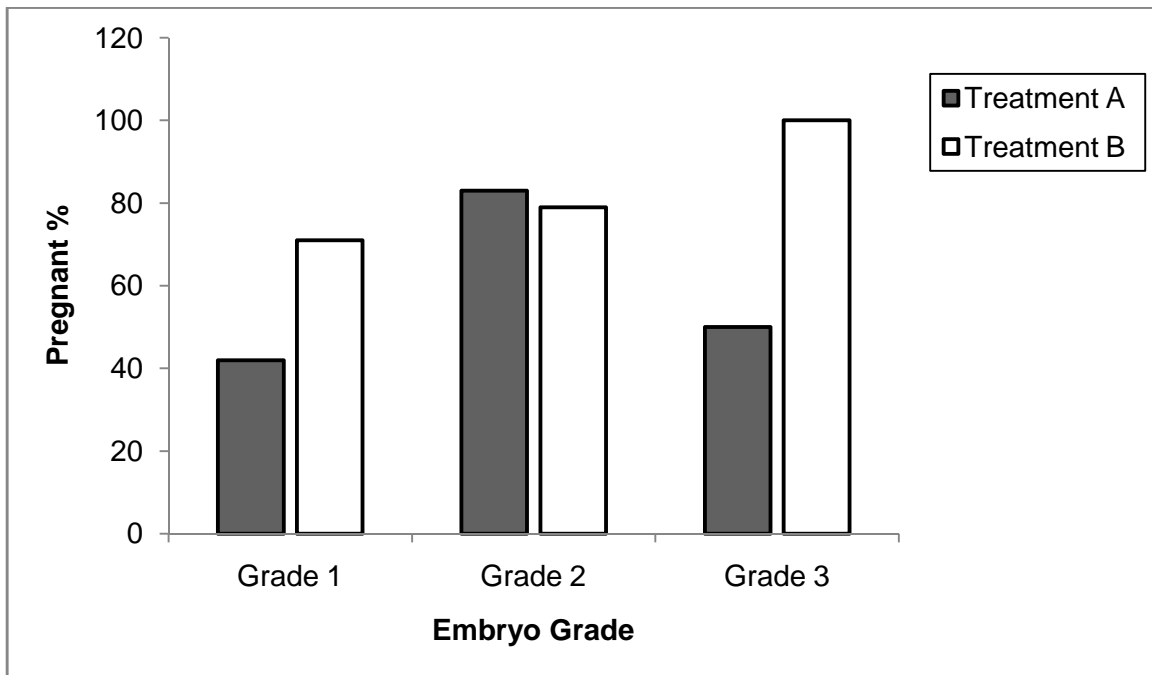


Figure 4.15. Experiment V, 35-day pregnancy rates compared with grades of in vivo-produced frozen-thawed glycerol Brangus embryos. There was no difference between grades 1, 2 and 3 and pregnancy rates.

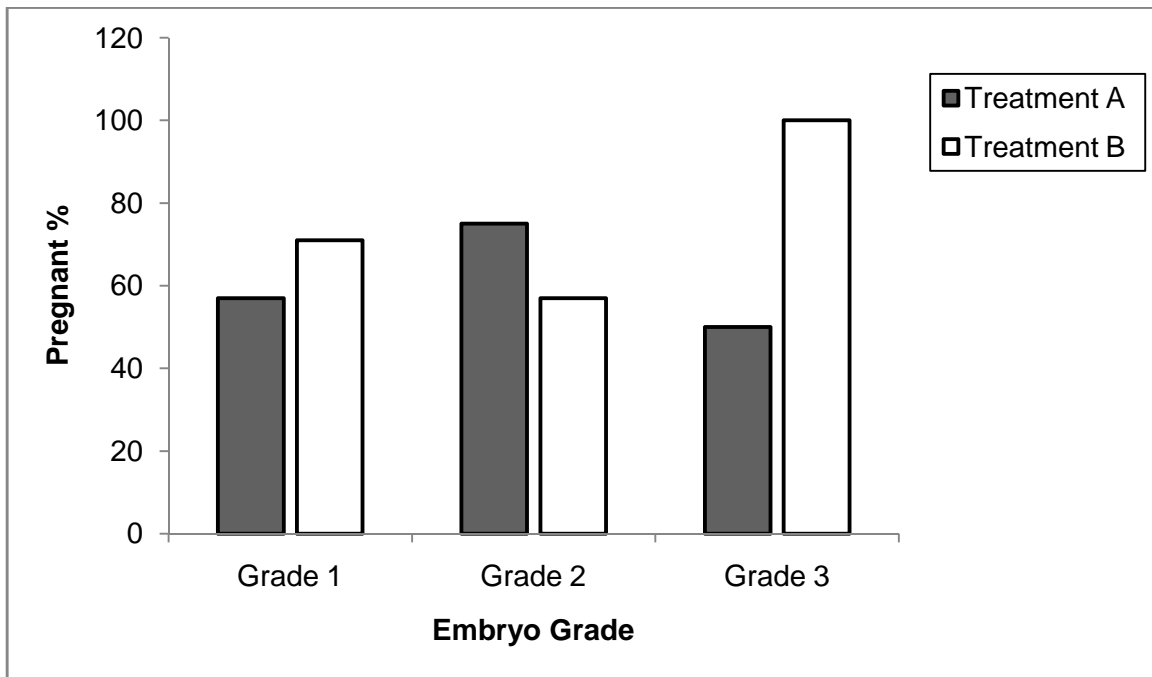


Figure 4.16. Experiment V, 60-day pregnancy rates compared with grades of in vivo-produced frozen-thawed glycerol Brangus embryos. There was no difference between grades 1, 2 and 3 and pregnancy rates.

Table 4.5. Effect of treatment on pregnancy rates with the application of laser-assisted hatching on in vivo-produced frozen-thawed glycerol Brangus embryos transfer on day-7 of the recipient's estrous cycle.

Treatment	35-Day Pregnancy (%)	60-Day Pregnancy (%)
Treatment A (n = 23) LAH <sup>a</sup>	16 (69)	15 (65)
Treatment B (n = 23) Sham Control	18 (78)	15 (65)

<sup>a</sup> Laser-assisted hatching.

Table 4.6. Effect of laser-assisted hatching on in vivo-produced frozen-thawed glycerol Brangus embryos on recipient calving rates.

Treatment	# Pregnancy at Day-60	# Born Alive (%)
Treatment A (n = 23) LAH <sup>a</sup>	15	11 (73)
Treatment B (n = 23) Sham Control	15	11 (73)

<sup>a</sup> Laser-assisted hatching.

Table 4.7. Experiments IV and V combined effects of treatment on pregnancy rates with the application of laser-assisted hatching on in vivo-produced frozen-thawed bovine embryos transfer on day-7 of the recipient's estrous cycle.

Treatment	35-Day Pregnancy (%)	60-Day Pregnancy (%)
Treatment A (n = 55) LAH <sup>a</sup>	29 (52)	24 (44)
Treatment B (n = 55) Sham Control	32 (58)	28 (51)

<sup>a</sup> Laser-assisted hatching

Treatment B 75% (21/28) of the calves were born alive (Table 4.8) (Figures 4.17 and 4.18). No twinning occurred across treatment groups.

## **Discussion**

The modifications of embryos caused by cryopreservation can be structural changes and/or damage to cell membranes (Willadsen et al., 1976; Mohr et al., 1981). Kaidi et al. (2000) reported a decrease in total cell number and an increase in apoptotic of trophoblastic and ICM cells in frozen-thawed bovine embryos compared with fresh embryos. The metabolic activity mediated by these cells is required for blastocyst expansion and subsequent hatching from the zona pellucida.

It has been suggested that metabolic activity may provide a means to assess the viability or developmental competence of embryos. In bovine in vivo-produced embryos, glucose and glutamine uptake and utilization are increased from the morula to blastocyst stage and both are required for expansion of blastocyst (Rieger et al., 1992). The increase in ATP is directly associated with embryo cavitation, blastocyst expansion and subsequent hatching (Rieger et al., 1992; Overstrom, 1996; Gardner, 1998; Khurana et al., 2000). It has been reported that embryos with increased glucose uptake will have a higher probability of developing when exposed to in vitro and in vivo conditions (Renard et al., 1980; Butler et al., 1989; Rieger et al., 1992). Glucose transporters are located on the trophoblastic cells and are responsible for the uptake of the glucose, thus providing glucose to the ICM (Gopichandran et al., 2003).

Embryos in Experiments IV and V were evaluated on embryo grade and diameter to determine viability and blastocyst expansion. It has been reported that when using this grading system pregnancy rates of 55 to 65% were achieved for excellent quality bovine embryos (Lindner et al., 1983; Overstrom, 1996). However, it has been shown that excellent quality embryos do not always display normal metabolic activity due to injury to the trophoblastic



Table 4.8. Experiments IV and V effect of treatment on calving results with the application of laser-assisted hatching on in vivo-produced frozen-thawed bovine embryos on day-7 of the recipient's estrous cycle.

Treatment	# Pregnancy at Day 60	# Born alive (%)
Treatment A (n = 55) LAH <sup>a</sup>	24	19 (79)
Treatment B (n = 55) Sham Control	28	21 (75)

<sup>a</sup> Laser-assisted hatching



Figure 4.17. Experiment IV, laser-assisted hatched in vivo-produced frozen-thawed Hereford heifer calf.



Figure 4.18. Experiment V, laser-assisted hatched frozen-thawed in vivo-produced Brangus heifer calf.

cells during cryopreservation. Therefore, embryo grading may be limited when attempting to determine embryo viability (Lindner et al., 1983). It has been proposed that laser-assisted hatching could promote hatching, which in turn will increase pregnancy rates of embryos that have suffered injury during the freezing and/or thawing process.

In Experiments IV and V, we showed that 35-day and 60-day pregnancy rates were not different for assisted and non-assisted embryo of grades 1, 2 and 3. These results suggest that the application of laser-assisted hatching does not rescue those in vivo-produced frozen-thawed bovine embryos that are of excellent/good or even poor quality but fail to hatch. Similar results have been reported in other studies designed to evaluate the efficacy of renting the zona pellucida of frozen-thawed human (Ng et al., 2005; Petersen et al., 2006; Valojerdi et al., 2009) and bovine (Schmoll et al., 1999) embryos on pregnancy rates. A factor that affects pregnancy rates of in vivo-produced bovine embryos that are transferred is the timing of recipient synchrony using embryo quality and stage (Hasler et al., 1987). Results from Experiments IV and V combined suggest that at the laser settings and rent sizes used in these experiments, treatment did not affect pregnancy and calving rates.

It has been shown that cryopreservation alters the zona pellucida or the hardening of zona pellucida, which inhibits hatching (Moreira et al., 2005). The ability of the embryo to escape from the zona pellucida is a prerequisite for implantation and pregnancy. Hatching of the blastocyst from the zona pellucida includes the secretion of proteolytic sublysis produced by the trophectoderm cells and/or pressure produced from fluid increase in the expanding blastocoel (Schiewe et al., 1995). Trophectoderm cells secrete trypsin-like substances, plasmin, glutamine, insulin and epidermal growth factors. The protein synthesis of embryos is considered to play an important role in thinning of the zona pellucida and hatching (Morishita et al., 1993). Results from the present experiments showed treatment did not affect pregnancy and calving rates of frozen-thawed in vivo-produced bovine embryos at the settings conducted in Experiments IV

and V when compared with zona pellucida thickness and is supported by previous studies on human (Schiewe et al., 1995) and bovine (Baguisi et al., 1998) embryos.

Emiliani et al. (2000) showed that the percent of re-expanded mouse blastocyst 24 hours after thawing was lower in embryos frozen in ethylene glycol than those frozen in glycerol. Although there was no difference across treatments in our study, the in vivo-produced bovine embryos cryopreserved in glycerol (Experiment V) had an overall higher pregnancy rates than those in vivo-produced bovine embryos cryopreserved in ethylene glycol (Experiment IV).

Assisted hatching has been proposed as a means of increasing hatching and pregnancy rates of otherwise viable human (Peterson et al., 2005; Balaban et al., 2006) and bovine (Schmoll et al., 1999) embryos. However, the overall results from the Experiments IV and V combined suggest that laser-assisted hatching using the XYClone<sup>®</sup> diode laser at the settings used in these experiments does not increase pregnancy rates or subsequent calving rates of frozen-thawed in vivo-produced bovine embryos.

## **CHAPTER V**

### **SUMMARY AND CONCLUSIONS**

The first objective of Experiments I, II and III was to determine the number of rents and depth of the rents made in the outer section of the zona pellucida using an infrared diode laser to avoid incomplete embryo hatching and reduce the possibility of twinning. The second objective was to establish the effect of laser-assisted hatching on blastocyst hatching rates of frozen-thawed in vivo-produced bovine embryos exposed to in vitro culture conditions. The objective of Experiments IV and V was to determine if laser-assisted hatching would increase pregnancy rates and calving rates of frozen-thawed in vivo-produced bovine embryos. Pregnancy rates were also compared with embryo quality across treatments (laser vs. no laser sham control).

The results from Experiments I, II and III indicate that a change in hatching pattern occurred in the in vivo-produced bovine embryos when the position and the depth of the rents made using the diode laser were changed. In Experiment I, three embryos in Treatment A were observed as being “pinched” during hatching, which resulted in one splitting into two parts (demi-embryos). In Experiment II, the quality of the hatched embryo was different on day-4 of culture across treatment groups. This may have been due to delayed hatching of embryos in the assisted hatched group. In Experiment II, three embryos were noted as being “pinched” but no twinning occurred. However, in Experiment III when three symmetrical rents were made on the outer zona pellucida a depth of 40% of the original thickness of zona pellucida, embryos hatched completely. Embryos were noted as hatching through a larger openings in the zona pellucida and supports results from previous studies on thinning of the zona pellucida of mouse (Cohen et al., 1991; Gordon et al., 1993), cow (Baguisi et al., 1998; Schmoll et al., 1999; Park et al., 1999), pig (Kolbe et al., 2005) and human( Sifer et al. 2006) embryos.

The decreased hatching rates of sheep (Willadsen et al., 1977) and bovine (Mohr et al., 1981; Fair et al., 2001; Moreira et al., 2005) embryos have been reported to be due to the damage caused during the freeze and thawing process to the trophectoderm cells, plasma membranes and zona pellucida. When Experiments I, II and III were combined, we showed that the application of laser-assisted hatching using the XYClone<sup>®</sup> diode laser at the settings stated here in did not increase hatching rates of frozen-thawed in vivo-produced bovine embryos when exposed to in vitro culture.

Based on the results from Experiments I, II, and III, the laser-assisted hatched embryos in Experiments IV and V received three symmetrical rents 40% through the zona pellucida thickness which were 17 µm in length and located on the outer surface. The diode laser settings were 90% power with a 600 µsecond pulse.

In Experiment IV and V, treatment did not affect pregnancy rates. These results suggest that the application of laser-assisted hatching (at the settings used in these experiments) does not salvage those frozen-thawed in vivo-produced bovine embryos that are of excellent/good or even poor quality that fail to hatch and implant.

Assisted hatching has been suggested as a means of increasing hatching and pregnancy rates of otherwise nonviable bovine (Schmoll et al., 1999) and human (Peterson et al., 2005; Balaban et al., 2006) embryos. However, results from the Experiment IV and V suggest that laser-assisted hatching using the XYClone<sup>®</sup> diode laser (at the settings used in these experiments) does not increase pregnancy rates or subsequent calving rates of in vivo-produced frozen-thawed bovine embryos over the control embryos when transferred to recipients on day-7 of their estrous cycle.

In conclusion, laser-assisted hatching using the XYClone<sup>®</sup> diode laser at the settings used in this study did not improve frozen-thawed in vivo-produced bovine embryos hatching rates when exposed to in vitro culture conditions or pregnancy and calving rates after transfer to recipients on day-7 of their estrous cycle. More research is needed on the effects of laser-assisted hatching of frozen-thawed in vivo-produced bovine embryos when exposed to in vitro culture and pregnancy rates following transfer.

## REFERENCES

- Abbott, A. L. and T. Ducibella. 2001. Calcium and the control of mammalian cortical granule exocytosis. *Front. Biosci.* 6: D792-D806.
- Ali, J., S. Rahbar, H. Burjar, A. M. Sultan, M. Al Flamerzi, and M. A. Shahata. 2003. Routine laser assisted hatching results in significantly increased clinical pregnancies. *J. Assist. Reprod. Genet.* 20: 177-181.
- Alikani, M. and J. Cohen. 1992. Micromanipulation of cleaved embryos cultured in protein-free medium: A mouse model for assisted hatching. *J. Exp. Zool.* 263: 458-463.
- Antinori, S., C. Versaci, P. Fuhrberg, C. Panci, B. Caffa, and G. H. Gholami. 1994. Seventeen live births after the use of an erbium-yttrium aluminum garnet laser in the treatment of male factor infertility. *Hum. Reprod.* 9: 1891-1896.
- Baguisi, A., J. F. Roche, and M. P. Boland. 1998. Assisted hatching of cryopreserved bovine embryos. *Theriogenology* 49: 161.
- Baguisi, A., P. Lonergan, E. W. Overstrom, and M. P. Boland. 1999. Vitrification of bovine embryos: Incidence of necrosis and apoptosis. *Theriogenology* 51: 162.
- Balaban, B. B. Urman, C. Alatas, R. Mercan, A. Mumcu, and A. Isiklar. 2002. A comparison of four different techniques of assisted hatching. *Hum. Reprod.* 17: 1239-1243.
- Balaban, B., B. Urman, K. Yakin, and A. Isiklar. 2006. Laser-assisted hatching increases pregnancy and implantation rates in cryopreserved embryos that were allowed to cleave in vitro after thawing: A prospective randomized study. *Hum. Reprod.* 21: 2136-2140.
- Bank, H. and R. R. Maurer. 1974. Survival of frozen rabbit embryos. *Exp. Cell Res.* 89: 188-196.
- Beebe, L. F. S., R. D. A. Cameron, A. W. Blackshaw, H. Keates, and M. B. Nottle. 2004. Assisted hatching improves post-warming in vitro viability of vitrified porcine blastocysts. *Reprod. Fertil. and Devel.* 16: 164-164.
- Beetem, D. D., D. Huffman, and R. A. Godke. 1990. Zona pellucida microsurgery using a nitrogen laser. *Theriogenology* 33: 194
- Bielanski, A., C. L. Lutze-Wallace, and S. Nadin-Davis. 2003. Adherence of bovine viral diarrhea virus to bovine oocytes and embryos with a hardened zona pellucida cultured in vitro. *Can. J. Vet. Res.* 67: 48-51.
- Bilton, R. J. and N. W. Moore. 1976a. Effects of ice seeding and of freezing and thawing rate on the development of sheep embryos stored at minus 196 celsius. *Theriogenology* 6: 635.
- Bilton, R. J. and N. W. Moore. 1976b. In vitro culture, storage and transfer of goat embryos. *Aust. J. Biol. Sci.* 20: 125-129.



- Bilton, R. J. and N. W. Moore. 1977. Successful transport of frozen cattle embryos from new zealand to australia. *J. Reprod. Fertil.* 50: 363-364.
- Blanchet, G. B., J. B. Russell, C. R. Fincher, Jr., and M. Portmann. 1992. Laser micromanipulation in the mouse embryo: A novel approach to zona drilling. *Fertil. Steril.* 57: 1337-1341.
- Bleil, J. D. and P. M. Wassarman. 1980. Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zona pellucidae possessing receptor activity for sperm. *Cell* 20: 873-882.
- Blessmann-Roset, J., N. Rives, B. Clavier, J. P. Milazzo, S. Mazurier, N. Mousset-Simeon, and B. Mace. 2009. Laser assisted hatching: Rouen university hospital outcomes. *Gynecol. Obstet. Fertil.* 37: 313-320.
- Braden, A. W., C. R. Austin, and H. A. David. 1954. The reaction of zona pellucida to sperm penetration. *Aust. J. Biol. Sci.* 7: 391-409.
- Brandao, D. O., P. Maddox-Hyttel, P. Lovendahl, R. Rumpf, D. Stringfellow, and H. Callesen. 2004. Post hatching development: A novel system for extended in vitro culture of bovine embryos. *Biol. Reprod.* 71: 2048-2055.
- Bronson, R. A. and A. McLaren. 1970. Transfer to the mouse oviduct of eggs with and without the zona pellucida. *J. Reprod. Fertil.* 22: 129-137.
- Butler, W. R. and R. D. Smith. 1989. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. *J. Dairy Sci.* 72: 767-783.
- Cannon, M. J. and A. R. Menino, Jr. 1998. Changes in the bovine zona pellucida induced by plasmin or embryonic plasminogen activator. *Mol. Reprod. Dev.* 51: 330-338.
- Chang, M. C. 1948. The effects of low temperature on fertilized rabbit ova in vitro, and the normal development of ova kept at low temperature for several days. *J. Gen. Physiol.* 31: 385-410.
- Chatot, C. L., C. A. Ziomek, B. D. Bavister, J. L. Lewis, and I. Torres. 1989. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J. Reprod. Fertil.* 86: 679-688.
- Chen, S. U., H. N. Ho, H. F. Chen, K. H. Chao, M. Y. Wu, S. C. Huang, T. Y. Lee, and Y. S. Yang. 1995. Effect of assisted hatching by partial zona pellucida dissection on mouse embryos in vitro. *J. Formos. Med. Assoc.* 94: 463-468.
- Chian, R. C., M. Kuwayama, L. Tan, J. Tan, O. Kato, and T. Nagai. 2004. High survival rate of bovine oocytes matured in vitro following vitrification. *J. Reprod. Dev.* 50: 685-696.
- Cieslak, J., V. Ivakhnenko, G. Wolf, S. Sheleg, and Y. Verlinsky. 1999. Three-dimensional partial zona dissection for preimplantation genetic diagnosis and assisted hatching. *Fertil. Steril.* 71: 308-313.

- Coates, A. A. and A. R. Menino, Jr. 1994. Effects of blastocoelic expansion and plasminogen activator activity on hatching and zona pellucida solubility in bovine embryos in vitro. *J. Anim. Sci.* 72: 2936-2942.
- Cohen, J. 1991. Assisted hatching of human embryos. *J. In Vitro Fertil. Embryo. Transf.* 8: 179-190.
- Cohen, J., M. Alikani, J. Trowbridge, and Z. Rosenwaks. 1992. Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. *Hum. Reprod.* 7: 685-691.
- Cohen, J., C. Elsner, H. Kort, H. Malter, J. Massey, M. P. Mayer, and K. Wiemer. 1990. Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. *Hum. Reprod.* 5: 7-13.
- Cohen, J. and D. Feldberg. 1991. Effects of the size and number of zona pellucida openings on hatching and trophoblast outgrowth in the mouse embryo. *Mol. Reprod. Dev.* 30: 70-78.
- Cohen, J., K. L. Inge, M. Suzman, S. R. Wiker, and G. Wright. 1989. Videocinematography of fresh and cryopreserved embryos: A retrospective analysis of embryonic morphology and implantation. *Fertil. Steril.* 51: 820-827.
- Cohen, J. H. Malter, C. Elsner, H. Kort, J. Massey, and M. P. Mayer. 1990. Immunosuppression supports implantation of zona pellucida dissected human embryos. *Fertil. Steril.* 53: 662-665.
- Conia, J. and S. Voelkel. 1994. Optical manipulations of human gametes. *Biotechniques* 17: 1162-1165.
- Coticchio, G., M. A. Bonu, R. Sciajno, E. Sereni, V. Bianchi, and A. Borini. 2007. Truths and myths of oocyte sensitivity to controlled rate freezing. *Reprod. Biomed. Online.* 15: 24-30.
- Coy, P., J. Gadea, R. Romar, C. Matas, and E. Garcia. 2002. Effect of in vitro fertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and in vitro development in pigs. *Reproduction* 124: 279-288.
- Coy, P. L. Grullon, S. Canovas, R. Romar, C. Matas, and M. Aviles. 2008. Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction* 135: 19-27.
- De Felici, M., S. Dolci, and G. Siracusa. 1991. An increase of intracellular free  $Ca^{2+}$  is essential for spontaneous meiotic resumption by mouse oocytes. *J. Exp. Zool.* 260: 401-405.
- De Vos, A. and A. Van Steirteghem. 2000. Zona hardening, zona drilling and assisted hatching: New achievements in assisted reproduction. *Cells Tissues Organs* 166: 220-227.
- Deanesly, R. 1954. Immature rat ovaries grafted after freezing and thawing. *J. Endocrinol.* 11: 197-200.

- Dell'Aquila, M. E., M. De Felici, S. Massari, F. Maritato, and P. Minoia. 1999. Effects of fetuin on zona pellucida hardening and fertilizability of equine oocytes matured in vitro. *Biol. Reprod.* 61: 533-540.
- Dobrinsky, J. R. 1996. Cellular approach to cryopreservation of embryos. *Theriogenology* 45: 17-26.
- Doklas, A., I. L. Sargent, and C. Ross. 1991. The human blastocyst: Its morphology and hcg secretion in vitro. *Human Reprod.* 6: 1143- 1151.
- Douglas-Hamilton, D. H. and J. Conia. 2001. Thermal effects in laser-assisted pre-embryo zona drilling. *J. Biomed. Opt.* 6: 205-213.
- Drobnis, E. Z., J. B. Andrew, and D. F. Katz. 1988. Biophysical properties of the zona pellucida measured by capillary suction: Is zona hardening a mechanical phenomenon? *J. Exp. Zool.* 245: 206-219.
- Ducibella, T., S. Kurasawa, P. Duffy, G. S. Kopf, and R. M. Schultz. 1993. Regulation of the polyspermy block in the mouse egg: Maturation-dependent differences in cortical granule exocytosis and zona pellucida modifications induced by inositol 1,4,5-trisphosphate and an activator of protein kinase C. *Biol. Reprod.* 48: 1251-1257.
- Echternkamp, S. E. and K. E. Gregory. 1999. Effects of twinning on postpartum reproductive performance in cattle selected for twin births. *J. Anim. Sci.* 77: 48-60.
- Edwards, R. G. 1964. Cleavage of one- and two-celled rabbit eggs in vitro after removal of the zona pellucida. *J. Reprod. Fertil.* 7: 413-415.
- El Mestrah, M. and F. W. Kan. 2001. Distribution of lectin-binding glycosidic residues in the hamster follicular oocytes and their modifications in the zona pellucida after ovulation. *Mol. Reprod. Dev.* 60: 517-534.
- el-Danasouri, I. L. M. Westphal, Y. Neev, J. Gebhardt, D. Louie, and M. W. Berns. 1993. Zona opening with 308 nm XeCl Excimer laser improves fertilization by spermatozoa from long-term vasectomized mice. *Hum. Reprod.* 8: 464-466.
- Elhelw, B. A., M. M. El Sadek, and K. M. El Nomrosy. 2004. Assisted hatching: Routine or selective application in IVF. *Middle East Fertil. Soc. J.* 9: 198-201.
- Emiliani, S., M. Van den Bergh, A. S. Vannin, J. Biramane, and Y. Englert. 2000. Comparison of ethylene glycol, 1,2-propanediol and glycerol for cryopreservation of slow-cooled mouse zygotes, 4-cell embryos and blastocysts. *Hum. Reprod.* 15: 905-910.
- Enright, B. P., P. Lonergan, A. Dinnyes, T. Fair, F. A. Ward, X. Yang, and M. P. Boland. 2000. Culture of in vitro produced bovine zygotes in vitro vs in vivo: Implications for early embryo development and quality. *Theriogenology* 54: 659-673.
- Fahning, M. L. and M. A. Garcia. 1992. Status of cryopreservation of embryos from domestic animals. *Cryobiology* 29: 1-18.

- Fair, T., P. Lonergan, A. Dinnyes, D. C. Cottell, P. Hyttel, F. A. Ward, and M. P. Boland. 2001. Ultrastructure of bovine blastocysts following cryopreservation: Effect of method of blastocyst production. *Mol. Reprod. Dev.* 58: 186-195.
- Fong, C. Y., A. Bongso, S. C. Ng, J. Kumar, A. Trounson, S. Ratnam. 1998. Blastocyst transfer after enzymatic treatment of the zona pellucida: Improving in-vitro fertilization and understanding implantation. *Hum. Reprod.* 13: 2926-2932.
- Gabrielsen, A. I. Agerholm, B. Toft, F. Hald, K. Petersen, J. Aagaard, B. Feldinger, S. Lindenberg, and J. Fedder. 2004. Assisted hatching improves implantation rates on cryopreserved-thawed embryos. A randomized prospective study. *Hum. Reprod.* 19: 2258-2262.
- Gao, C., B.E. Elts, Y. Han, J.A. Carter, and R.A. Godke. 1996. Using a nitrogen-pulse laser for assisted hatching of in vitro cultured bovine embryos. *Anim. Reprod.* 3: 22.
- Gao, C. 1998. Laser-assisted hatching of murine and bovine embryos, M.S. Thesis. Louisiana State University Baton Rouge.
- Gardner, A. J., C. J. Williams, and J. P. Evans. 2007. Establishment of the mammalian membrane block to polyspermy: Evidence for calcium-dependent and -independent regulation. *Reproduction* 133: 383-393.
- Gardner, D. K., C. B. Sheehan, L. Rienzi, M. Katz-Jaffe, and M. G. Larman. 2007. Analysis of oocyte physiology to improve cryopreservation procedures. *Theriogenology* 67: 64-72.
- Germond, M., D. Nocera, A. Senn, K. Rink, G. Delacretaz, and S. Fakan. 1995. Microdissection of mouse and human zona pellucida using a 1.48-microns diode laser beam: Efficacy and safety of the procedure. *Fertil. Steril.* 64: 604-611.
- Germond, M., D. Nocera, A. Senn, K. Rink, G. Delacretaz, T. Pedrazzini, J. P. Hornung. 1996. Improved fertilization and implantation rates after non-touch zona pellucida microdrilling of mouse oocytes with a 1.48 microm diode laser beam. *Hum. Reprod.* 11: 1043-1048.
- Germond, M., A. Senn, K. Rink, G. Delacretaz, and P. De Grandi. 1995. Is assisted hatching of frozen-thawed embryos enhancing pregnancy outcome in patients who several prious nidation failure? (Abstract). *J. of Fertil. and Reprod.* 3: 112
- Gopichandran, N. and H. J. Leese. 2006. The effect of paracrine/autocrine interactions on the in vitro culture of bovine preimplantation embryos. *Reproduction* 131: 269-277.
- Gordon, J. W. and U. Dapunt. 1993. A new mouse model for embryos with a hatching deficiency and its use to elucidate the mechanism of blastocyst hatching. *Fertil. Steril.* 59: 1296-1301.
- Goudet, G., S. Mugnier, I. Callebaut, and P. Monget. 2008. Phylogenetic analysis and identification of pseudogenes reveal a progressive loss of zona pellucida genes during evolution of vertebrates. *Biol. Reprod.* 78: 796-806.

- Green, D. P. 1997. Three-dimensional structure of the zona pellucida. *Rev. Reprod.* 2: 147-156.
- Gridley, T. and R. Woychik. 2007. Laser surgery for mouse geneticists. *Nat. Biotechnol.* 25: 59-60.
- Gulyas, B. J. 1980. Cortical granules of mammalian eggs. *International Rev. Cytol.* 63: 357-392.
- Harris, J. D., D. W. Hibler, G. K. Fontenot, K. T. Hsu, E. C. Yurewicz, and A. G. Sacco. 1994. Cloning and characterization of zona pellucida genes and cdnas from a variety of mammalian species: The ZPA, ZPB and ZPC gene families. *DNA Seq.* 4: 361-393.
- Hartman, C. G., W. H. Lewis, F. W. Miller, and W. W. Swett. 1931. First findings of tubal ova in the cow, together with notes on oestrous. *Anat. Rec.* 49: 265.
- Hartshorn, C., A. Anshelevich, and L. J. Wangh. 2005. Laser zona drilling does not induce hsp70i transcription in blastomeres of eight-cell mouse embryos. *Fertil. Steril.* 84: 1547-1550.
- Hasler, J. F. 2001. Factors affecting frozen and fresh embryo transfer pregnancy rates in cattle. *Theriogenology* 56: 1401-1415.
- Hasler, J. F., W. B. Henderson, P. J. Hurtgen, Z. Q. Jin, A. D. McCauley, S. A. Mower, B. Neely, L. S. Shuey, J. E. Stokes, and S. A. Trimmer. 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* 43: 141-152.
- Hasler, J. F., A.D. McCauley, W.F. Lathrop, and R.H. Foote. 1987. Effect of donor-embryo-recipient interactions on pregnancy rate in a large-scale bovine embryo transfer program. *Theriogenology* 27: 139-168.
- Hellebaut, S. P. De Sutter, D. Dozortsev, A. Onghena, C. Qian, and M. Dhont. 1996. Does assisted hatching improve implantation rates after in vitro fertilization or intracytoplasmic sperm injection in all patients? A prospective randomized study. *J. Assist. Reprod. Genet.* 13: 19-22.
- Herrler, A. and H. M. Beier. 2000. Early embryonic coats: Morphology, function, practical applications. An overview. *Cells Tissues Organs* 166: 233-246.
- Hiraoka, K., M. Fuchiwaki, T. Horiuchi, T. Murakami, M. Kinutani, and K. Kinutani. 2008. Effect of the size of zona pellucida opening by laser assisted hatching on clinical outcome of frozen cleaved embryos that were cultured to blastocyst after thawing in women with multiple implantation failures of embryo transfer: A retrospective study. *J. Assist. Reprod. Genet.* 25: 129-135.
- Hitoshi, Y. 1990. Clinical significance of interleukin-5 analysis. *Nippon Rinsho (Suppl)* 48: 822-825.
- Hoodbhoy, T., P. Dandekar, P. Calarco, and P. Talbot. 2001. P62/p56 are cortical granule proteins that contribute to formation of the cortical granule envelope and play a role in mammalian preimplantation development. *Mol. Reprod. Dev.* 59: 78-89.

- Huang, J. Y., J. T. Chung, S. L. Tan, and R. C. Chian. 2007. High survival and hatching rates following vitrification of embryos at blastocyst stage: A bovine model study. *Reprod. Biomed. Online* 14: 464-470.
- Huneau, D., N. Crozet, and M. Ahmed-Ali. 1994. Estrous sheep serum as a potent agent for ovine IVF: Effect on cholesterol efflux from spermatozoa and the acrosome reaction. *Theriogenology* 42: 1017-1028.
- Hyttel, P., H. Lehn-Jensen, and T. Greve. 1986. Ultrastructure of bovine embryos frozen and thawed by a two-step freezing method. *Acta Anat.* 125: 27-31.
- Inoue, M. and D. P. Wolf. 1974. Comparative solubility properties of the zonae pellucidae of unfertilized and fertilized mouse ova. *Biol. Reprod.* 11: 558-565.
- Iwamoto, K., K. Ikeda, N. Yonezawa, S. Noguchi, K. Kudo, S. Hamano, M. Kuwayama, and M. Nakano. 1999. Disulfide formation in bovine zona pellucida glycoproteins during fertilization: Evidence for the involvement of cystine cross-linkages in hardening of the zona pellucida. *J. Reprod. Fertil.* 117: 395-402.
- Jones, A. E., G. Wright, H. I. Kort, R. J. Straub, and Z. P. Nagy. 2006. Comparison of laser-assisted hatching and acidified tyrode's hatching by evaluation of blastocyst development rates in sibling embryos: A prospective randomized trial. *Fertil. Steril.* 85: 487-491.
- Kaidi, S., S. Bernard, P. Lambert, A. Massip, F. Dessy, and I. Donnay. 2001. Effect of conventional controlled-rate freezing and vitrification on morphology and metabolism of bovine blastocysts produced in vitro. *Biol. Reprod.* 65: 1127-1134.
- Kalab, P., G. S. Kopf, and R. M. Schultz. 1991. Modifications of the mouse zona pellucida during oocyte maturation and egg activation: Effects of newborn calf serum and fetuin. *Biol. Reprod.* 45: 783-787.
- Katska, L., G. Kania, Z. Smorag, E. Wayda, and G. Plucienniczak. 1999. Developmental capacity of bovine IVM/IVF oocytes with experimentally induced hardening of the zona pellucida. *Reprod. Domest. Anim.* 34: 255-259.
- Katska, L., P. Kauffold, Z. Smorag, U. Duschinski, H. Torner, and W. Kanitz. 1989. Influence of hardening of the zona pellucida on in vitro fertilization of bovine oocytes. *Theriogenology* 32: 767-777.
- Katsumata, T., S. Noguchi, N. Yonezawa, M. Tanokura, and M. Nakano. 1996. Structural characterization of the n-linked carbohydrate chains of the zona pellucida glycoproteins from bovine ovarian and fertilized eggs. *Eur. J. Biochem.* 240: 448-453.
- Khalifa, E.-A., M. J. Tucker, and P. P. Hunt. 1992. Cruciate thinning of the zona pellucida for more successful enhancement of blastocyst hatching in the mouse. *Hum. Reprod.* 7: 532-536.

- Kim, D. H., D. S. Ko, H. C. Lee, H. J. Lee, W. I. Park, S. S. Kim, J. K. Park, B. C. Yang, S. B. Park, W. K. Chang, and H.T. Lee. 2004. Comparison of maturation, fertilization, development, and gene expression of mouse oocytes grown in vitro and in vivo. *J. Assist. Reprod. Genet.* 21: 233-240.
- Ko, C. S., D. C. Ding, T. W. Chu, Y. N. Chu, I. C. Chen, W. H. Chen, and G. J. Wu. 2008. Changes to the meiotic spindle and zona pellucida of mature mouse oocytes following different cryopreservation methods. *Anim. Reprod. Sci.* 105: 272-282.
- Kolbe, T. and W. Holtz. 2005. Differences in proteinase digestibility of the zona pellucida of in vivo and in vitro derived porcine oocytes and embryos. *Theriogenology* 63: 1695-1705.
- Kung, F. T., Y. C. Lin, Y. J. Tseng, F. J. Huang, M. Y. Tsai, and S. Y. Chang. 2003. Transfer of frozen-thawed blastocysts that underwent quarter laser-assisted hatching at the day 3 cleaving stage before freezing. *Fertil. Steril.* 79: 893-899.
- Lan, K. C., F. J. Huang, Y. C. Lin, F. T. Kung, and S. Y. Chang. 2008. Zona-free versus laser zona-assisted hatching blastocyst transfer: A comparison of outcomes. *Fertil. Steril.* 91: 1959-1962.
- Landim-Alvarenga, F. C., S. E. Boyazoglu, L. R. Carvalho, Y. H. Choi, E. L. Squires, G. E. Seidel, Jr. 2002. Effects of fetuin on zona pellucida hardening, fertilization and embryo development in cattle. *Anim. Reprod. Sci.* 71: 181-191.
- Lanzendorf, S. E., R. R. Kazer, P. E. Patton, and D. P. Wolf. 1992. Cortical granule complements in human oocytes undergoing partial zona dissection. *Mol. Reprod. Dev.* 31: 131-134.
- Lanzendorf, S. E., F. Nehchiri, J. F. Mayer, S. Oehninger, and S. J. Muasher. 1998. A prospective, randomized, double-blind study for the evaluation of assisted hatching in patients with advanced maternal age. *Hum. Reprod.* 13: 409-413.
- Lanzendorf, S. E., V. S. Ratts, K. H. Moley, J. S. Goldstein, M. H. Dahan, and R. R. Odem. 2007. A randomized, prospective study comparing laser-assisted hatching and assisted hatching using acidified medium. *Fertil. Steril.* 87: 1450-1457.
- Larman, M. G., C. B. Sheehan, and D. K. Gardner. 2006. Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. *Reproduction* 131: 53-61.
- Lassalle, B., J. Testart, and J. P. Renard. 1985. Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. *Fertil. Steril.* 44: 645-651.
- Laufer, N., D. Palanker, Y. Shufaro, A. Safran, A. Simon, and A. Lewis. 1993. The efficacy and safety of zona pellucida drilling by a 193-nm Excimer laser. *Fertil. Steril.* 59: 889-895.
- Lee, D. R., J. E. Lee, H. S. Yoon, H. J. Lee, M. K. Kim, and S. I. Roh. 1997. The supplementation of culture medium with protease improves the hatching rate of mouse embryos. *Hum. Reprod.* 12: 2493-2498.

- Li, L., S. Munne, F. Licciardi, J. Neev, Y. Tadir, M. Berns, R. Godke, and J. Cohen. 1993. Microinjection of FITC-dextran into mouse blastomeres to assess topical effects of zona photoablation. *Zygote* 1: 43-48.
- Lindner, G. M. and R. W. Wright, Jr. 1983. Bovine embryo morphology and evaluation. *Theriogenology* 20: 407-416.
- Liu, H. C., J. Cohen, M. Alikani, N. Noyes, and Z. Rosenwaks. 1993. Assisted hatching facilitates earlier implantation. *Fertil. Steril.* 60: 871-875.
- Longo, F. J. 1980. Changes in the zona pellucidae and plasmalennae of aging mouse eggs: Evidence for enzyme's role in hardening of the zona pellucida. *Gamete Res.* 3: 279-290.
- Longo, F. J. 1981. Changes in the zones pellucidae and plasmalemma of aging mouse eggs. *Biol. Reprod.* 25: 399-411.
- Looney, C. R., B. R. Lindsey, C. L. Gonseth, and D. L. Johnson. 1994. Commercial aspects of oocyte retrieval and in vitro fertilization (IVF) for embryo production in problem cows. *Theriogenology* 41: 67-72.
- Lopatarova, M., L. Holy, and A. Vinkler. 2001. Effect on survival of micromanipulating the zona pellucida of bovine embryos. *Acta Anat. (Basel)* 70: 49-56.
- Malter, H. E. and J. Cohen. 1989. Blastocyst formation and hatching in vitro following zona drilling of mouse and human embryos. *Gamete Res.* 24: 67-80.
- Mandelbaum, J. 1996. The effects of assisted hatching on the hatching process and implantation. *Hum. Reprod.* 11 Suppl. 1: 43-50; discussion 51-45.
- Mapletoft, R. J. 2006. Bovine embryo transfer. *IVIS Reviews in Veteriary Medicine No. R0104.1106.* International Veterinary Information Service, Ithaca.
- Massip, A. 2001. Cryopreservation of embryos of farm animals. *Reprod. Domest. Anim* 36: 49-55.
- Matson, P. L., J. Graefling, S. M. Junk, J. L. Yovich, and W. R. Edirisinghe. 1997. Cryopreservation of oocytes and embryos: Use of a mouse model to investigate effects upon zona hardness and formulate treatment strategies in an in-vitro fertilization programme. *Hum. Reprod.* 12: 1550-1553.
- Maurer, R. R. 1978. Freezing mammalian embryos: A review of the techniques. *Theriogenology* 9: 45-68.
- Mavrides, A. and D. Morroll. 2002. Cryopreservation of bovine oocytes: Is cryoloop vitrification the future to preserving the female gamete? *Reprod. Nutr. Dev.* 42: 73-80.
- Mavrides, A. and D. Morroll. 2005. Bypassing the effect of zona pellucida changes on embryo formation following cryopreservation of bovine oocytes. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 118: 66-70.



- McLaren, A. 1968. A study of blastocysts during delay and subsequent implantation in lactating mice. *J. Endocrinol.* 42: 453-463.
- McLaren, A. 1970. The fate of the zona pellucida in mice. *J. Embryol. Exp. Morphol.* 23: 1-19.
- Menges, S., H. Wei, D. Faber, D. Kraemer, and C. Long. 2008. Utilization of laser assisted hatching to improve the pregnancy rate of in vitro fertilized bovine embryos. *Reprod. Fertil. Dev.* 20: 150.
- Menino, A. R., Jr. A. R. Dyk, C. S. Gardiner, M. A. Grobner, M. A. Kaaekuahiwi, and J. S. Williams. 1989. The effects of plasminogen on in vitro ovine embryo development. *Biol. Reprod.* 41: 899-905.
- Menino, A. R., Jr. and J. L. O'Claray. 1986. Enhancement of hatching and trophoblastic outgrowth by mouse embryos cultured in whitten's medium containing plasmin and plasminogen. *J. Reprod. Fertil.* 77: 159-167.
- Miller, D. J., X. Gong, G. Decker, and B. D. Shur. 1993. Egg cortical granule n-acetylglucosaminidase is required for the mouse zona block to polyspermy. *J. Cell Biol.* 123: 1431-1440.
- Miller, D. J., M. B. Macek, and B. D. Shur. 1992. Complementarity between sperm surface beta-1,4-galactosyltransferase and egg-coat ZP3 mediates sperm-egg binding. *Nature* 357: 589-593.
- Mintz, B. 1962. Experimental study of the developing mammalian egg: Removal of the zona pellucida. *Science* 138: 594-595.
- Modinski, J. A. 1970. The role of the zona pellucida in development of mouse eggs in vivo. *J. Embryol. Exp. Morphol.* 23: 539-547.
- Mohr, L. R. and A. O. Trounson. 1981. Structural changes associated with freezing of bovine embryos. *Biol. Reprod.* 25: 1009-1025.
- Montag, M., B. Koll, P. Holmes, and D. V. van. 2000. Significance of the number of embryonic cells and the state of the zona pellucida for hatching of mouse blastocysts in vitro versus in vivo. *Biol. Reprod.* 62: 1738-1744.
- Montag, M., B. Koll, and V. Van Der. 2000. Use of a laser to evaluate zona pellucida hardness at different stages of mouse embryonic development in vitro and in vivo. *J. Assist. Reprod. Genet.* 17: 178-181.
- Moreira, D. S. and R. Metelo. 2005. Relation between physical properties of the zona pellucida and viability of bovine embryos after slow-freezing and vitrification. *Reprod. Domest. Anim.* 40: 205-209.
- Morishita, T., M. Yokoyama, M. Nozaki, M. Sano, and H. Nakano. 1993. Improvement in blastocyst hatching of mouse embryos cocultured with human placental cells. *J. Assist. Reprod. Genet.* 10: 463-467.

- Nara, M., N. Yonezawa, T. Shimada, K. Takahashi, M. Tanokura, F. Yumoto, H. Nakagawa, K. Ohashi, S. Hamano, and M. Nakano. 2006. Fourier transform infrared spectroscopic analysis of the intact zona pellucida of the mammalian egg: Changes in the secondary structure of bovine zona pellucida proteins during fertilization. *Exp. Biol. Med.* (Maywood.) 231: 166-171.
- Neev, J., M. C. Schiewe, V. W. Sung, D. Kang, N. Hezeleger, M. W. Berns, and Y. Tadir. 1995. Assisted hatching in mouse embryos using a noncontact ho:Ysgg laser system. *J. Assist. Reprod. Genet.* 12: 288-293.
- Neev, J., Y. Tadir, P. Ho, M. W. Berns, R. H. Asch, and T. Ord. 1992. Microscope-delivered ultraviolet laser zona dissection: Principles and practices. *J. Assist. Reprod. Genet.* 9: 513-523.
- Ng, E. H., F. Naveed, E. Y. Lau, W. S. Yeung, C. C. Chan, O. S. Tang, and P. C. Ho. 2005. A randomized double-blind controlled study of the efficacy of laser-assisted hatching on implantation and pregnancy rates of frozen-thawed embryo transfer at the cleavage stage. *Hum. Reprod.* 20: 979-985.
- Nieder, G. L. and T. L. Caprio. 1990. Early embryo development in the Siberian hamster (*Phodopus sungorus*). *Mol. Reprod. Dev.* 27: 224-229.
- Nijs, M., P. Vanderzwalmen, G. Segal-Bertin, L. Geerts, E. Van Roosendaal, L. Segal, A. Schoysman-Deboeck, and R. Schoysman. 1993. A monozygotic twin pregnancy after application of zona rubbing on a frozen-thawed blastocyst. *Hum. Reprod.* 8: 127-129.
- Obruca, A., H. Strohmer, D. Sakkas, Y. Menezo, A. Kogosowski, Y. Barak, and W. Feichtinger. 1994. Assisted hatching and micro-fertilization. *Contracept. Fertil. Sex* 22: 303-305.
- Overstrom, E. W. 1996. In vitro assessment of embryo viability. *Theriogenology* 45: 3-16.
- Palermo, G., H. Joris, P. Devroey, and A. C. Van Steirteghem. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 340: 17-18.
- Park, S., E. Y. Kim, S. H. Yoon, K. S. Chung, and J. H. Lim. 1999. Enhanced hatching rate of bovine IVM/IVF/IVC blastocysts using a 1.48-micron diode laser beam. *J. Assist. Reprod. Genet.* 16: 97-101.
- Parkes, A. S. and A. U. Smith. 1953. Regeneration of rat ovarian tissue grafted after exposure to low temperatures. *Proc. R. Soc. Lond. B Biol. Sci.* 140: 455-470.
- Perona, R. M. and P. M. Wassarman. 1986. Mouse blastocysts hatch in vitro by using a trypsin-like proteinase associated with cells of mural trophoctoderm. *Dev. Biol.* 114: 42-52.
- Petersen, C. G., A. L. Mauri, R. L. Baruffi, J. B. Oliveira, V. Felipe, F. C. Massaro, and J. G. Franco, Jr. 2006. Laser-assisted hatching of cryopreserved-thawed embryos by thinning one quarter of the zona. *Reprod. Biomed. Online.* 13: 668-675.

- Petersen, C. G., A. L. Mauri, R. L. Baruffi, J. B. Oliveira, F. C. Massaro, K. Elder, and J. G. Franco Jr. 2005. Implantation failures: Success of assisted hatching with quarter-laser zona thinning. *Reprod. Biomed. Online* 10: 224-229.
- Polge, C., A. U. Smith, and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* 164: 666.
- Primi, M. P., A. Senn, M. Montag, V. Vander, J. Mandelbaum, A. Veiga, P. Barri, and M. Germond. 2004. A european multicentre prospective randomized study to assess the use of assisted hatching with a diode laser and the benefit of an immune-suppressive/antibiotic treatment in different patient populations. *Hum. Reprod.* 19: 2325-2333.
- Pryor, J. H., C. R. Looney, S. Romo, D. C. Kraemer, and C. R. Long. The effect of lipid segregation with or without zona pellucida laser drilling on post-thaw embryo development of in vitro-produced bovine embryos. *Reprod. Fertil. Dev.* 20: 125-126.
- Rall, W. F. and G. M. Fahy. 1985. Ice-free cryopreservation of mouse embryos at -196° C by vitrification. *Nature* 313: 573-575.
- Rath, D., E. Topfer-Petersen, H. W. Michelmann, P. Schwartz, and S. Ebeling. 2005. Zona pellucida characteristics and sperm-binding patterns of in vivo and in vitro produced porcine oocytes inseminated with differently prepared spermatozoa. *Theriogenology* 63: 352-362.
- Renard, J. P., A. Philippon, and Y. Menezo. 1980. In-vitro uptake of glucose by bovine blastocysts. *J. Reprod. Fertil.* 58: 161-164.
- Rieger, D., N. M. Loskutoff, and K. J. Betteridge. 1992. Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured in vitro. *J. Reprod. Fertil.* 95: 585-595.
- Rienzi, L., E. Greco, F. Ubaldi, M. Iacobelli, F. Martinez, and J. Tesarik. 2001. Laser-assisted intracytoplasmic sperm injection. *Fertil. Steril.* 76: 1045-1047.
- Rink, K., G. Delacretaz, R. P. Salathe, A. Senn, D. Nocera, M. Germond, P. De Grandi, and S. Fakan. 1996. Non-contact microdrilling of mouse zona pellucida with an objective-delivered 1.48-microns diode laser. *Lasers Surg. Med.* 18: 52-62.
- Rizos, D., F. Ward, P. Duffy, M. P. Boland, and P. Lonergan. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* 61: 234-248.
- Robertson, A. I. and R.E. Nelson. 1998. Certification and identification of the embryo. 3rd ed. International Embryo Transfer Society, Savoy, IL.
- Rosenkrans, C.F. and N.L. First. 1994. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. *J. Anim. Sci.* 72: 434-437.

- Sagoskin, A. W., M. J. Levy, M. J. Tucker, K. S. Richter, and E. A. Widra. 2007. Laser assisted hatching in good prognosis patients undergoing in vitro fertilization-embryo transfer: A randomized controlled trial. *Fertil. Steril.* 87: 283-287.
- Saunders, K. M. and J. E. Parks. 1999. Effects of cryopreservation procedures on the cytology and fertilization rate of in vitro-matured bovine oocytes. *Biol. Reprod.* 61: 178-187.
- Sawada, H., K. Yamazaki, and M. Hoshi. 1990. Trypsin-like hatching protease from mouse embryos: Evidence for the presence in culture medium and its enzymatic properties. *J. Exp. Zool.* 254: 83-87.
- Scarpeci, S. L., M. L. Sanchez, and M. O. Cabada. 2008. Cellular origin of the bufo arenarum sperm receptor gp75, a ZP2 family member: Its proteolysis after fertilization. *Biol. Cell* 100: 219-230.
- Schiewe, M. C. 1996. An effective, simplified, and practical approach to intracytoplasmic sperm injection at multiple IVF centers. *J. Assist. Reprod. Genet.* 13: 238-245.
- Schiewe, M. C., E. Araujo, Jr., R. H. Asch, and J. P. Balmaceda. 1995. Enzymatic characterization of zona pellucida hardening in human eggs and embryos. *J. Assist. Reprod. Genet.* 12: 2-7.
- Schiewe, M. C., N. L. Hazeleger, C. Scilimenti, and J. P. Balmaceda. 1995. Physiological characterization of blastocyst hatching mechanisms by use of a mouse antihatching model. *Fertil. Steril.* 63: 288-294.
- Schiewe, M. C., J. Neev, N. L. Hazeleger, J. P. Balmaceda, M. W. Berns, and Y. Tadir. 1995. Developmental competence of mouse embryos following zona drilling using a non-contact holmium:Yttrium scandium gallium garnet (ho:Ysgg) laser system. *Hum. Reprod.* 10: 1821-1824.
- Schmoll, F., H. Schneider, M. Montag, K. Rink, K. Wimmers, E. Tholen, S. Ponsuksili, H. van der Ven, and K. Schellander. 1999. Laser assisted hatching in bovine in vitro produced blastocysts. *Theriogenology* 51: 253-253.
- Schoolcraft, W. B., T. Schlenker, M. Gee, G. S. Jones, and H. W. Jones, Jr. 1994. Assisted hatching in the treatment of poor prognosis in vitro fertilization candidates. *Fertil. Steril.* 62: 551-554.
- Schroeder, A. C., R. M. Schultz, G. S. Kopf, F. R. Taylor, R. B. Becker, and J. J. Eppig. 1990. Fetuin inhibits zona pellucida hardening and conversion of ZP2 to ZP2f during spontaneous mouse oocyte maturation in vitro in the absence of serum. *Biol. Reprod.* 43: 891-897.
- Schutze, K., A. Clement-Sengewald, and A. Ashkin. 1994. Zona drilling and sperm insertion with combined laser microbeam and optical tweezers. *Fertil. Steril.* 61: 783-786.
- Seidel, G. E. Jr. 2006. Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology* 65: 228-235.

- Sifer, C., A. Sellami, C. Poncelet, P. Kulski, B. Martin-Pont, J. Bottero, R. Porcher, I. Cedrin-Durnerin, J. N. Hugues, and J. P. Wolf. 2006. A prospective randomized study to assess the benefit of partial zona pellucida digestion before frozen-thawed embryo transfers. *Hum. Reprod.* 21: 2384-2389.
- Sinowatz, F., E. Topfer-Petersen, S. Kolle, and G. Palma. 2001. Functional morphology of the zona pellucida. *Anat. Histol. Embryol.* 30: 257-263.
- Smith, A. U. 1952. Behavior of fertilized rabbit eggs exposed to glycerol and to low temperatures. *Nature* 170: 374-375.
- Stein, A., O. Rufas, S. Amit, O. Avrech, H. Pinkas, J. Ovadia, and B. Fisch. 1995. Assisted hatching by partial zona dissection of human pre-embryos in patients with recurrent implantation failure after in vitro fertilization. *Fertil. Steril.* 63: 838-841.
- Strohmer, H. and W. Feichtinger. 1992. Successful clinical application of laser for micromanipulation in an in vitro fertilization program. *Fertil. Steril.* 58: 212-214.
- Sun, Q. Y. 2003. Cellular and molecular mechanisms leading to cortical reaction and polyspermy block in mammalian eggs. *Microsc. Res. Tech.* 61: 342-348.
- Tadir, Y. and D. H. Douglas-Hamilton. 2007. Laser effects in the manipulation of human eggs and embryos for in vitro fertilization. *Methods Cell Biol.* 82: 409-431.
- Tadir, Y., J. Neev, P. Ho, and M. W. Berns. 1993. Lasers for gamete micromanipulation: Basic concepts. *J. Assist. Reprod. Genet.* 10: 121-125.
- Tadir, Y., W. H. Wright, O. Vafa, L. H. Liaw, R. Asch, and M. W. Berns. 1991. Micromanipulation of gametes using laser microbeams. *Hum. Reprod.* 6: 1011-1016.
- Takahashi, K., M. Takenaka, and B. Ishizuka. 1994. The effect of assisted hatching on patients repeatedly failed to conceive by in vitro fertilization. *Nippon Sanka Fujinka Gakkai Zasshi* 46: 1009-1012.
- Talbot, P. and P. Dandekar. 2003. Perivitelline space: Does it play a role in blocking polyspermy in mammals? *Microsc. Res. Tech.* 61: 349-357.
- Taniyama, A., Y. Watanabe, Y. Nisino, and T. Inoue. 2007. Increased pregnancy rates of poor quality bovine embryos by assisted hatching. *Reprod. Fertil. and Develop.* 19: 219-220.
- Tarkowski, A. K. 1959. Experiments on the development of isolated blastomers of mouse eggs. *Nature* 184: 1286-1287.
- Tervit, H. R. and R. P. Elsdon. 1981. Development and viability of frozen-thawed cattle embryos. *Theriogenology* 15: 395-403.
- Trounson, A. O. and N. W. Moore. 1974. The survival and development of sheep eggs following complete or partial removal of the zona pellucida. *J Reprod. Fertil.* 41: 97-105.
- Tsunoda, Y., T. Yasui, K. Nakamura, T. Uchida, and T. Sugie. 1986. Effect of cutting the zona pellucida on the pronuclear transplantation in the mouse. *J. Exp. Zool.* 240: 119-125.

- Tucker, M. J., N. M. Luecke, S. R. Wiker, and G. Wright. 1993. Chemical removal of the outside of the zona pellucida of day 3 human embryos has no impact on implantation rate. *J. Assist. Reprod. Genet.* 10: 187-191.
- Vajta, G., P. Holm, T. Greve, and H. Callesen. 1997. Survival and development of bovine blastocysts produced in vitro after assisted hatching, vitrification and in-straw direct rehydration. *J. Reprod. Fertil.* 111: 65-70.
- Valojerdi, M. R., P. Eftekhari-Yazdi, L. Karimian, F. Hassani, and B. Movaghar. 2009. Effect of laser zona thinning on vitrified-warmed embryo transfer at the cleavage stage: A prospective, randomized study. *Reprod. Biomed. Online* 20: 234-242.
- Van Steirteghem, A. C., Z. Nagy, H. Joris, J. Liu, C. Staessen, J. Smitz, A. Wisanto, and P. Devroey. 1993. High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod.* 8: 1061-1066.
- Van Wagtenonk-De Leeuw, A. M., J. H. Den Daas, T. A. Kruip, and W. F. Rall. 1995. Comparison of the efficacy of conventional slow freezing and rapid cryopreservation methods for bovine embryos. *Cryobiology* 32: 157-167.
- Vanroose, G., H. Nauwynck, A. V. Soom, M. T. Ysebaert, G. Charlier, P. V. Oostveldt, A. de Kruif,. 2000. Structural aspects of the zona pellucida of in vitro-produced bovine embryos: A scanning electron and confocal laser scanning microscopic study. *Biol. Reprod.* 62: 463-469.
- Vanroose, G., H. Nauwynck, A. Van Soom, E. Vanopdenbosch, and A. de Kruif. 1998. Replication of cytopathic and noncytopathic bovine viral diarrhea virus in zona-free and zona-intact in vitro-produced bovine embryos and the effect on embryo quality. *Biol. Reprod.* 58: 857-866.
- Velasquez, J. G., S. Canovas, P. Barajas, J. Marcos, M. Jimenez-Movilla, R. G. Gallego, J. Ballesta, M. Aviles, and P. Coy. 2007. Role of sialic acid in bovine sperm-zona pellucida binding. *Mol. Reprod. Dev.* 74: 617-628.
- Vitullo, A. D. and J. P. Ozil. 1992. Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. *Dev. Biol.* 151: 128-136.
- Voelkel, S. A. and Y. X. Hu. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. *Theriogenology* 37: 1117-1131.
- Wassarman, P., J. Chen, N. Cohen, E. Litscher, C. Liu, H. Qi, and Z. Williams. 1999. Structure and function of the mammalian egg zona pellucida. *J. Exp. Zool.* 285: 251-258.
- Wassarman, P. M. 1987. Early events in mammalian fertilization. *Annu. Rev. Cell Biol.* 3: 109-142.
- Wassarman, P. M., L. Jovine, and E. S. Litscher. 2004. Mouse zona pellucida genes and glycoproteins. *Cytogenet. Genome Res.* 105: 228-234.

- Wassarman, P. M. and E. S. Litscher. 2008. Mammalian fertilization: The eggs multifunctional zona pellucida. *International J. Dev. Biol.* 52: 665-676.
- Whittingham, D. G., S. P. Leibo, and P. Mazur. 1972. Survival of mouse embryos frozen to -196 degrees and -269° C. *Science* 178: 411-414.
- Wiemer, K. E., Y. Hu, M. Cuervo, P. Genetis, and D. Leibowitz. 1994. The combination of coculture and selective assisted hatching: Results from their clinical application. *Fertil. Steril.* 61: 105-110.
- Willadsen, S. M. 1977. Factors affecting the survival of sheep embryos during deep-freeze and thawing. *The freezing of mammalian embryos. Ciba Fbn. Symp.* 52: 175-189.
- Willadsen, S. M., C Polge, L. E. A. Rowson, and R. M. Moor. 1974. Preservation of sheep embryos in liquid nitrogen. *Cryobiology* 11: 560.
- Willett, E. L., W. G. Black, L. E. Casida, W. H. Stone, and P. J. Buckner. 1951. Successful transplantation of a fertilized bovine ovum. *Science* 113: 247.
- Wilmut, I. and L. E. Rowson. 1973. Experiments on the low-temperature preservation of cow embryos. *Vet. Rec.* 92: 686-690.
- Wright, G., S. Wiker, C. Elsner, H. Kort, J. Massey, D. Mitchell, A. Toledo, and J. Cohen. 1990. Observations on the morphology of pronuclei and nucleoli in human zygotes and implications for cryopreservation. *Hum. Reprod.* 5: 109-115.
- Zhang, X., J. Rutledge and D. T. Armstrong. 1991. Studies on zona hardening in rat oocytes that are matured in vitro in a serum-free medium. *Mol. Reprod. Dev.* 28: 292-296.

## APPENDIX A

### COMPOSITION OF TALP HEPES HOLDING MEDIUM USED TO WASH IN VIVO- PRODUCED BOVINE EMBRYOS CULTURED

Components	Catouloge no.	Amount
BSA, Fraction V	Sigma A-4503	6 mg/ml
HEPES- TL	Bioniche IVF022	20 ml/ml
Na Pyruvate	Sigma P-4562	0.2 ml/ml
Penicillin/Streptomycin	Gibco 15140-122	0.2 ml/ml



## APPENDIX B

### COMPOSITION OF CR1aa STOCK MEDIUM

Components	Catouloge no.	Amount
NaCl	Sigma S-5886	670 mg/ml
KCl	Sigma P-5405	23.1 mg/ml
NaHCO <sub>3</sub>	Sigma S-8875	220 mg/ml
Pyruvic Acid	Sigma P-4562	4.4 mg/ml
Lactic Acid	Sigma L-4388	54.6 mg/ml
Embryo Transfer Water	Sigma W-1503	100 ml/ml
Phenol Red	Sigma P-0290	1 ml/ml

Rosenkrans and First (1994).

## APPENDIX C

### COMPOSITION OF CR1aa CULTURE MEDIUM USED TO CULTURE IN VIVO-PRODUCED BOVINE EMBRYOS FROM DAY 7 TO 11

Components	Catouloge no.	Amount
CR1aa Stock	*	4.52 ml/ml
Basal Medium Eagle (BME) X50	Sigma B-6766	100 µl/ml
Modified Eagle's Medium (MEM) X100	Sigma M-7145	50 µl/ml
Calf Serum	HyClone 30073	250 µl/ml
Penicillin/Streptomycin	Gibco 15140-122	50 µl/ml
L-Glutamine (X100)	Sigma G-8790	0.195 mg/ml
Alanine (X100)	Sigma A-7469	0.215 mg/ml

\* Rosenkrans and First (1994).

## **VITA**

Mindy K. Chiasson, the daughter of Lisa and Robert Chiasson, was born September 5, in Abbeville, Louisiana. Upon graduation from Erath High School in 2002, she attended Louisiana State University, where she received her Bachelor of Science degree in Dairy Science in 2006.

Upon graduation she accepted the position as research technician at the LSU AgCenter's Dairy Research Farm in Baton Rouge, Louisiana. She then transferred later that year to the LSU AgCenter's Reproductive Biology Center in St. Gabriel, Louisiana, as a scientific research technician.

In May 2006, she began working on her master's degree in reproductive physiology under the guidance of Dr. Robert A. Godke at LSU. In January of 2010, she accepted the position as research associate at the LSU AgCenter's Iberia Research Station located in Jeanerette, Louisiana. She is now a candidate for the degree of Master of Science in Reproductive Physiology in the School of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.