Open pulled straw vitrification of murine and caprine embryos and timed deep uterine insemination of goats

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DEDICATION

To

My most beloved and respectful parents

and

My beloved wife and child

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General Introduction:

The continuous expansion of the world population raises many questions related to food resources in terms of costs and quality of production. These inquiries draw attention to livestock production and in many parts of the world especially, to goats as an important domestic species supplying milk, meat and fiber. Enhancing the genetic progress in the breeds of this species, to cover the food and industry demands, calls for progress in reproduction management and biotechnology. This thesis describes recent efforts generated in this field directed at an addition or improvement in this area.

Cryopreservation of gametes and embryos helps preserving genetic variability, propagate superior breeding stock and conserve populations threatened by extinction. The ability of cryopreserving, thawing and establishing pregnancies with supernumerary preimplantation embryos has become an important tool in reproduction management in cattle. Although success rates with the transfer of cryopreserved embryos have increased over time, there is still room for improvement. There are two major approaches for embryo cryopreservation, slow-rate freezing (conventional) and vitrification. In goats, few results of success are reported after transfer of vitrified embryos. Previous successful transfer of goat blastocysts vitrified with the open pulled straw (OPS) method, with high embryo survival rate, was reported in our group (El-Gayar and Holtz 2001). This success drew attention to the applicability of this method to the other developmental stages of the caprine embryos. This question is covered in the first experiment of the first section of this thesis.

The second experiment in the first section of this thesis describes an attempt to study the effect of sucrose in the vitrification and warming media on post-warming in vitro survival of OPS-vitrified mouse blastocysts. Addition of moderate concentrations of sucrose to the vitrification media helps decreasing the toxicity of the high concentrations of permeating cryoprotectants required in the OPS vitrification media. Warming of vitrified embryos is usually practiced in sucrose solutions to dilute the toxic cryoprotectants. Performing this step in a sucrose-free medium would enable microscope-free embryo transfers and help in establishing a more practical application of OPS vitrification in the field.

The investigation covered in the second section of the thesis addressed the question whether it is possible to replace Gonadotropin Releasing Hormone (GnRH) as ovulation inducing agent

in prostaglandin $F_2\alpha$ ($PGF_{2\alpha}$)-synchronized goats by human Chorionic Gonadotropin (hCG) as part of a protocol leading to fixed-time insemination. The underlying intention was to reduce the incidence of short cycles by providing a more sustained stimulation of the corpus luteum. Timed insemination took place by way of deep uterine insemination, a technique developed in our group (Sohnrey and Holtz 2005) with slight modifications to diminish stress on animals and operators.

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SECTION I

Open pulled straw vitrification of murine and caprine embryos

1 Open pulled straw (OPS) vitrification of various stages of caprine embryos

1.1 Introduction:

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues (Pegg, 2007). The technology of cryopreserving gametes and embryos, coupled with the embryo transfer, is used for preserving genetic strains in laboratory animals, for breeding of superior animals and rare breeds in farm animals, and for solving the infertility problems in humans. Furthermore, it could be used as a means of conservation of endangered animal species (Kasai 2002). The ability of cryopreserving, thawing, and establishing pregnancies with supernumerary preimplantation embryos has become an important tool in reproduction management in farm animals (Smith and Silva, 2004). Although success rates with transfer of cryopreserved embryos have increased over time, there is still room for improvement.

There are two major approaches for embryo cryopreservation in farm animals. The first one is the slow-rate freezing method (conventional), which can be interpreted as an attempt to create a delicate balance between various damage-causing factors surrounding the embryo during freezing including ice crystals, fracture, toxic and osmotic damage (Vajta and Kuwayama, 2006). In 1972 conventional slow-freezing proved to be a successful method for cryopreservation of mammalian embryos, as offspring was obtained after applying the method on mouse embryos (Whittingham et al., 1972). This success opened the door for more pioneering achievements in the field of embryology. In 1976 the first successful cryopreservation of goat embryos was reported by Bilton and Moore.

The second approach of embryo cryopreservation is vitrification, which consists of a glass-like solidification without formation of ice crystals involving high concentrations of cryoprotectants parallel with very high cooling (Vajta, 2000). The first successful vitrification of a mammalian embryo was reported in mouse embryos by Rall and Fahy (1985). Five years later, the first successful vitrification of goat embryos was reported (Yuswiayti and Holtz, 1990), though with limited success. Applying the open pulled straw (OPS) vitrification method, developed by Vajta et al. (1997), to goat blastocysts resulted in high embryo survival, (64%) comparable to the best results achieved with conventional slow-freezing in goat embryos (El Gayar and Holtz, 2001).

1.2 Review of literature:

1.2.1. Cryopreservation of embryos:

Cryopreservation of embryos as a practice could be divided into two major methods: conventional cryopreservation (slow freezing) and vitrification. The most widely used technology is the conventional method (slow freezing) which depends on maintaining a delicate balance between cryoprotectants at low concentration (1–1.5 M) and the aqueous embryo compartment (Guignot et al., 2006). This method of cryopreservation requires an accurate control of the quantity of ice formed during cooling and warming. In this slow freezing method, it is necessary to use programmed freezing unit and the freezing process itself takes a long time.

Generally, in slow-freezing of mammalian embryos, the embryo is suspended in a physiological solution containing 1-2 M cryoprotectant, then ice seeded and cooled very slowly (0.3-0.5° C/min) to allow the cellular contents to become concentrated, due to the gradual dehydration, in response to the concentration of the extracellular unfrozen fraction during the growth of extracellular ice. After obtaining sufficient concentration (usually at -30° C), the embryos, together with the extracellular unfrozen fraction, would be vitrified in liquid nitrogen (Kasai, 2002).

On the other hand, the strategy of the vitrification method depends on the solidification of liquid (avoiding ice crystal formation) using high concentrations of cryoprotectants (6–7.5 M) parallel with very high cooling rates (Guignot et al., 2006). Conventional slow freezing is the mainly practiced cryopreservation method in farm animals, as the method was established and standardized in many farm animal species since a long time with satisfactory results. In terms of experimental practices, both of the conventional freezing and vitrification methods (Table 1) have been used widely for the cryopreservation of embryos in domestic animals with variable success.

1.2.2 Disadvantages and injuries:

Conventional slow-freezing is a time consuming method as 2-3 hr is to be expected for preparation, equilibration and freezing of embryos. The programmed freezing unit is costly

equipment consisting of an alcohol path and programmed cooling unit attached to a computer with a special freezing program.

The strategy of conventional slow cooling depends on creating a delicate balance between the various factors engaged in the freezing scheme (Vajta, 2000), which is not easily maintained and could result in a damage such as formation of ice crystals, osmotic injury, toxic effect of cryoprotectants, concentration of intracellular electrolytes, chilling injury, osmotic swelling or shrinkage, fracture of the zona pellucida and change in the intracellular organelles, cytoskeleton and cell-to-cell contacts (Massip et al., 1995; Dobrinsky, 1996; Kasai et al., 1996; Saha et al., 1996; Kasai, 2002).

Table 1 Embryo cryopreservation methods (Pereira and Marques 2008)

Attributes	Conventional slow freezing	Vitrification
Container	Standard 0.25 ml straws	Several devices for loading embryos 0.25 ml straws, OPS, cryoloop, ..etc
Cryoprotectant	Low concentrations (1–1.5 M)	High conc./reduced volume and time
Cooling rate	Seeding at -5 to -7, controlled slow cooling (0.1 to 0.3° C/min)	Ultrarapid cooling (20000° C/ min using OPS or cryoloop)
Preservation	Plunging into LN_2 at -30 to -70°C, storage (-196° C)	Direct plunging and storage in LN_2 (-196° C)

On the other hand, the strategy of vitrification is based on total elimination of ice crystal formation, depending on high concentrations of toxic cryoprotectants. Unfortunately, most of the possible injuries affecting embryos and oocytes during cryopreservation including cryoprotectant toxicity, glass-fractures, de-vitrification and re-crystallization and chilling injury are possible sources of damage that are to be considered and avoided during vitrification (Vajta, 2000; Yavin and Arav, 2007). Recent in vivo and in vitro results on survival of vitrified embryos provide convincing evidence that the toxic, osmotic and other harmful effects caused by the vitrification process are not exceeding but are rather below that caused by conventional slow freezing (Kuwayama, 2007).

Despite the efforts to achieve successful cryopreservation, characterized by acceptable post-thawing in vitro or in vivo survival rates, there are fundamental characteristics of embryos (Table 2) of some species (e.g. the pig), or of embryos of some stages (e.g. early cleavage

stages in cattle), or of oocytes of most species (excepting mice and humans) that complicate solutions (Leibo et al., 1996). These cases drive the cryobiological investigations to try to find modified cryopreservation variables to allow successful cryopreservation under these restrictions. Modifying the cryopreservation methods to satisfy specific characteristics of an embryo (species) or a stage of development, may involve using new cryoprotectants that are less toxic, various concentrations and mixtures of various cryoprotectants, studying the interactions of cooling and warming rates regarding the variable cryoprotectant mixtures used, and trying simpler new containers for the embryos during preservation (Leibo et al., 1996).

Table 2 Differences in cryopreservation tolerance of oocytes and embryos from various species, stages of development and derivations (Pereira and Marques, 2008)

Criteria	More tolerant	Less tolerant
Species	Bovine, ovine	Porcine, equine
Developmental stage	Morula,	Oocytes
	Early Blastocyst	Hatched Bla
	Blastocyst	
Origin	In vivo derived embryos	In vitro derived embryos
		Micromanipulated embryos

1.2.3 Zona pellucida:

The zona pellucida (ZP) is a relatively thick extracellular coat that surrounds the plasma membrane of the oocyte of eutherian mammals (Green, 1997; Wassarman and Litscher, 2008). The name of this coat is derived from Latin, meaning transparent (pellucida) belt or girdle (zona). It is a spherical shell of remarkably uniform thickness (5-10 μm in eutherian mammals; Green, 1997). The ZP is usually present throughout the preimplantation stages of the embryonic development until it is shed as the blastocyst has reached maturity and is ready for implantation. Generally, the ZP consists of long interconnected filaments that are organized into long cross-linked fibrils that constitute the extracellular coat (Green, 1997; Wassarman and Litscher, 2008).

It is composed of only 3 glycoproteins (ZP 1-3) that are synthesized and secreted exclusively by growing oocytes (Wassarman and Litscher, 2008), two of them (ZP2 and ZP3) are

organized into long filaments exhibiting a cyclic repetition and the third glycoprotein, ZP1 serves as a cross-linker of the filaments (Wassarman et al., 1999). The presence of both ZP2 and ZP3 is absolutely required for the assembly of the ZP during oocyte growth. The third glycoprotein ZP3 functions as a primary sperm receptor as it has oligosaccharides that are recognized by the spermatozoon, and it has the function to induce the acrosome reaction during fertilization (Wassarman et al., 1999).

The zona pellucida has several important functions before, during, and after fertilization. As mentioned before, the ZP has a specific glycoprtein (ZP3) that acts in regulating interactions between ovulated eggs and the free swimming spermatozoa during and following fertilization. The zona pellucida acts as a barrier against the fertilization of eggs from one species by spermatozoa of another species (Yanagimachi, 1994; Wassarman, 1999) as it contains species-specific sperm receptors that are recognized primarily by spermatozoa of the same species (Wassarman et al., 1999). It also plays an important role during fertilization in that it serves as a barrier to polyspermic fertilization that might occur after the fusion of the first ZP-penetrating spermatozoon with the vitelline membrane of the oocyte, which, in turn, inactivates the ZP sperm-receptors and disallows any further spermatozoa to bind to the ZP (Bearden and Fuquay 1997; Wassarman et al., 1999).

The zona pellucida is believed to play a role in permitting the communication between oocytes and follicle cells during oogenesis, and serves as a protective shell for oocytes, zygotes and embryos during development (Wassarman et al., 1999). More precisely, for preimplantation embryos, the ZP enables the cleaving embryos to be transferred freely and more easily through the oviduct until reaching the uterus and its presence has been considered as a necessity for normal early development in vivo (Modlinski, 1970). The role of keeping the blastomeres of an embryo together is also attributed to the ZP, which, besides, protects different embryos from fusion during cleavage and development (Nowshari, 1990). Trounson and Moore (1974) attributed the protection of the inner cell mass of an embryo from the uterine environment to the intact ZP.

1.2.3.1 Zona pellucida and cryopreservation:

The presence of an intact ZP surrounding the preimplantation embryo seems to play a critical role in successful cryopreservation and transfer of mammalian embryos. The contribution of the ZP during the slow freezing appears to have two major functions. Firstly, it creates a physical barrier against extracellular ice crystals, while the second major function was found

to be the creation of an osmotic buffer around the embryo (Lehn-Jensen and Rall, 1983; Lehn-Jensen and Willadsen, 1983). Investigating the role of the ZP during freezing and thawing of bovine embryos, assumed that the ZP could be judged as a limiting factor or "cell membrane" when considering the dynamics of water permeability and shrinkage of the system, since the reduced loss of water during the freezing of embryos in the presence of the ZP results in intracellular ice formation, which, in turn, bears upon the further development of the embryos (Kanagawa et al., 1979).

1.2.4 Developmental stage of Embryo:

The developmental stage of the embryo is one of the most important determinants for success of embryo cryopreservation. In mammals, the newly ovulated female gamete is referred to as oocyte. Upon fertilization, the oocyte becomes a one-cell embryo, which is usually called zygote. Division of the zygote will create 2-cell, 4-cell, etc. stages (each cell is called a blastomere). At the 16-cell stage, the embryo is referred to as morula (Latin for mulberry). Further development of the morula will give a compacted morula that is characterized by a change in shape of embryo cells from spherical to polygonal. When a fluid-filled central cavity (blastocoele) forms, the embryo is referred to as a blastocyst. Until this stage, the ZP is still enveloping the embryo and expansion of the blasocoele will produce an expanded blastocyst with a thinner ZP. When the expansion of the blasocyst reaches a certain stage, the embryo hatches from the ZP and is called hatched blastocyst. Blastocyst formation is one of the important criteria of in vitro viability of early embryonal stages before morula, while hatching of the embryo is an indicator usually used to test viability of morulae and above stages.

In the caprine, the blastocyst seems to be the most suitable stage for cryopreservation with pregnancy rates ranging between 42 and more than 85% (Li et al., 1990; Puls-Kleingeld et al., 1992; Nowshari and Holtz 1995; El Gayar and Holtz, 2001). Blastocyst appears to be more suitable for conventional cryopreservation than morula (Nowshari, 1990; Puls-Kleingeld et al., 1992) Culturing of the goat morulae to the blastocyst stage before cryopreservation is advised as it improved success rates (Nowshari and Holtz, 1995). Cell association in caprine morulae and compacted morulae was found to be weaker when compared with that in bovine and ovine morule resulting in a weaker chance of development after cryopreservation (Udy, 1987). Chances of erroneously classifying embryos as morphologically sound are higher in the morula stage and might result in the transfer of embryos that lack the capability of

development (Nowshari and Holtz, 1993). By contrast, Le Gall et al. (1993) claim that, in the goat, morulae are more suitable for cryopreservation than blastocysts.

Despite all the claims of the importance of the presence of an intact ZP during cryopreservation of embryos (Lehn-Jensen and Rall, 1983; Niemann et al., 1986; Nowshari and Holtz, 1993), high kidding rates (50%) were achieved when transferring frozen-thawed ZP-free or ZP-damaged caprine embryos (Chemineau et al., 1986). Four years later, Li et al. (1990) reported a pregnancy rate of 67% attained when transferring frozen-thawed hatching and hatched blastocysts of goats.

The pioneering efforts of vitrification of goat embryos resulted in relatively limited success (Yuswiati and Holtz, 1990). More acceptable results were attained later with in vivo and in vitro-derived goat embryos (Traldi et al., 1999). Low in vitro survival (13%) was reported after "cryoloop vitrification" of caprine in vivo-derived 2- to 4-cell caprine embryos (Begin et al., 2003). With the Open-pulled-straw (OPS) vitrification of caprine blastocysts spectacular success rates were reported. Overall embryo survival amounted to 64% for OPS-vitrified blastocysts as compared to 42% in conventionally frozen ones, the statistical difference being significant (El-Gayar and Holtz, 2001). Morulae and early blastocysts collected 7 d after the first insemination were successfully vitrified with the OPS method using ficoll and sucrose in addition to EG and ME_2SO in the vitrification medium, achieving a kidding rate ranging from 43 to 51% (Hong et al., 2007). Low embryo survival (14%) was achieved when goat morulae and blastocysts were vitrified with the OPS method, using 0.4 M sucrose added to 18 % EG and 18 % Me_2SO as a vitrification medium (Guignot et al., 2006). Hong et al. (2007) and Guignot (2006) did not differentiate between morulae and blastocysts, therefore an effect of stage of embryonal development on success rates with cryopreservation can not be assessed. As yet, no experience exists with the cryopreservation of morulae and hatched blastocysts by the OPS vitrification method devised by Vajta et al. (1998).

1.2.5 Vitrification:

Vitrification can be defined as the process by which a liquid is solidified into a non-crystalline (glass-like) phase by greatly increasing the viscosity and highly lowering the temperature (Luyet and Hoddap, 1938). Avoiding crystallization can be achieved by lowering the freezing point through addition of cryoprotectants at high concentration. With lowering of the temperature the liquid solution becomes more viscous and, at high enough solute concentration and low enough temperature, it becomes glassy solid (vitrified). As nucleation

rate is minimized at very low temperatures, vitrification can be theoretically achieved with very high cooling rate without using cryoprotectants (Meryman, 2007).

In order to avoid the lethal effect of ice crystal formation during cryopreservation of cells, Luyet (1937) drew attention to the feasibility of vitrification as an inexpensive and simple method. Rall and Fahy (1985) were pioneers in applying this method for cryopreservation of mammalian (mouse) embryos.

Successful vitrification depends on three major factors; viscosity of the sample, cooling rate and sample volume. The relation between these three factors is shown in the following equation:

$$\text{Probability of vitrification} = \frac{\text{Cooling rate} \times \text{Viscosity}}{\text{Volume}}$$ (Yavin and Arav, 2007)

The method involves the addition of high concentrations of cryoprotectants which, at extremely low temperatures, are in an amorphous state without crystals. At the concentration required for vitrification these solutions are toxic at room temperature. The toxic effect is eliminated by exposing the embryos to low temperatures or reducing the exposure to very short periods of time (Ishida et al., 1997).

High cooling and warming rates are required parallel with the high concentrations of cryoprotectants to accomplish a successful vitrification (Kong et al., 2000). Accelerating the speed of temperature change offers two important advantages: first, it may permit the use of lower concentrations of cryoprotectants with consequent reduction in toxicity and, secondly it may result in less severe chilling injury due to the rapid passage through the dangerous temperature zone (Vajta et al., 1998).

Generally, nowadays there are two main types of vitrification: firstly, conventional vitrification in which embryos, suspended in a highly concentrated solution of cryoprotectants (5-8 mol/L), are loaded into a straw and directly plunged into liquid nitrogen. Despite the high levels of embryo viability achieved with this method, the toxicity obstacle has to be considered. The second type is the ultrarapid vitrification, which depends on vitrifying the samples containing the embryos with very high cooling rates (20000 – 24000° C/min) through direct contact between the samples containing the embryos and liquid nitrogen. This trend is achieved by using special containers (eg. electron microscope grids, OPS and cryoloops; Martino et al., 1996; Vajta et al., 1997; Lane et al., 1999) or with a completely container-less method (micrdrops), by directly plunging a microdropdrop of 4-8 µL into liquid nitrogen without any thermal insulation layer (Landa and Tepla, 1990; Papis et al., 1999). Using

partially solidified liquid nitrogen (nitrogen slush) instead of liquid nitrogen is another trend used to increase the cooling rate during vitrification, as nitrogen slush has a temperature of about -210° C (Martino et al., 1996).

1.2.6 Cryoprotectants:

Cryoprotectants are additional chemicals used in cryopreservation to decrease the chances of intracellular ice formation. They are usually separated into two broad classes based on their ability to diffuse across cell membranes: Permeating cryoprotectants are able to pass across cell membranes; non-permeating agents can not.

1.2.6.1 Permeating cryoprotectants:

Permeating cryoprotectants are small molecules that readily permeate the membranes of cells, form hydrogen bonds with intracellular water molecules and lower the freezing temperature of the resulting mixture, preventing ice crystallization (Pereira and Marques, 2008). Examples of permeating cryoprotectants are: Propylene glycol, glycerol, ethylene glycol (EG) and dimethyl sulfoxide (Me_2SO). Cryopreservation is generally based on the avoidance or minimization of the intracellular ice formation, parallel with minimizing the possible damage that might strike the cells originating from the environment of the concentrated solutes during cooling (McGann 1978). As the permeating cryoprotectants are extremely toxic at high concentrations, considerable exertions were applied to decrease this toxicity by applying less toxic and more permeable chemicals (first of all EG), using a mixture of two or three cryoprotectants to reduce the specific toxicity of each of them, to make stepwise addition of cryoprotectants, to minimize the volume of the solution and the container, and to cool the temperature of the solution to around 0º C when adding the targeted high concentration of cryoprotectants (Vajta and Kuwayama 2006). Propylene glycol (1,2-propanediol; PROH) is one of the most commonly used permeating cryoprotectant which is usually added at a concentration of 1.5 M in cases of slow-freezing, where the toxicity is low, compared with the danger of ice crystal formation which is prohibited in PROH case with 6 M concentration (Pereira and Marques, 2008).

For caprine embryos, EG is considered the most suitable cryoprotectant and is usually added in a concentration of 1.5 M (Le Gal et al., 1993; Fieni et al., 1995). Glycerol, which is usually added at 1 to 1.4 M concentration, also proved to be a successfully used cryoprotectant for goat embryos (Rong et al., 1989; Puls-Kleingeld et al., 1992; Nowshari and Holtz, 1995). Using of a highly toxic cryoprotectnt (Me_2SO) resulted in excellent results when it was used alone in slow-freezing (Li et al., 1990) or mixed at high concentrations with another cryoprotectnt (EG) in case of OPS vitrification (El Gayar and Holtz, 2001).

1.2.6.2 Non-permeating cryoprotectants:

Nonpermeating cryoprotectants remain extracellularly, drawing free water from within the cells and causing dehydration of the intracellular space. They are usually used in combination with permeating cryoprotectants to increase the net concentration of the permeating cryoprotectant inside the cell and also preventing ice-crystal formation (Pereira and Marques, 2008). Large-molecular-weight polymers (macromolecules) are frequently used as extracellular cryoprotectants. They can help in reducing the amount of intracellular cryoprotectants necessary for vitrification and, as a result, reducing the toxicity of the solution. Polymers are also known to protect against zona pellucida cracking (Pereira and Marques, 2008). Several macromolecules are commonly used in cryopreservation such as polyethylene glycol 8000, polyvinylpyrrolidone 360,000, Ficoll 70,000 or 400,000, polyvinyl alcohol and dextran. Ficoll was successfully used for cryopreservation of bovine (Lane et al. 1999b) and human (Zech et al., 2005) embryos, but Laowtamathron et al. (2005) proved that this polymer has no beneficial effect on bovine embryo survival. These contradictions confirm the emerging idea that macromolecules can alter the vitrification properties of solutions leading to variability (Pereira and Marques, 2008). Several hypotheses have been proposed clarifying the mechanism of cryoprotection by non-permeating cryoprotectants (Takahashi et al., 1988). Nash (1966) suggested that they might act in a similar way as the permeating cryoprotectants, by forming numerous and strong bonds. Farrant (1969) suggested that a polymer such as polyvinyl pyrrolidone (PVP) may show enhanced colligative properties at higher concentration and may protect the cells by lowering the external salt concentration at a given subfreezing temperature in a way of action similar to that of the low molecular weight cryoprotectants. A kind of direct interaction between the polymers and the cell membranes was also proposed to elucidate the mechanism of action of these non-permeating

cryoprotectants (Meryman, 1966). McGann (1978, 1979) presented a hypothesis called "squeeze out" in which he hinted that the non-permeating cryoprotectants osmotically "squeeze" water from the cells primarily during the initial phase of freezing at temperatures between -10 and -20ºC, when these cryoprotectants become concentrated in the extracellular part. Williams (1983) reported that the cryoprotective properties of polymers depend mainly on their ability to alter the physical properties of the solutions during the freezing process. Based on thermal analysis, the capability of a certain non-permeating cryoprotectant (hydroxyethyl starch, HES) to absorb a certain portion of the water and keeping it away from freezing was hypothesized to explain the protective action of HES during cryopreservation (Körber and Scheiwe, 1980). Thermodynamic properties (limiting glass transition temperature T'_g) of the polymers used as cryoprotectants were found to have a very critical effect on their cryoprotective properties (Takahashi et al., 1988). A limiting glass transition is defined as a glass transition in an aqueous solution which is in equilibrium with ice at the glass transition temperature, having been formed by the removal of H_2O from the solution by the growth of ice crystals alone. Because of the viscosity of the polymer solutions getting very high as their temperature approaches the T'_g, the polymers allow the cells to supercool a moderate amount between 0º C and the T'_g of the surrounding solution (Takahashi et al. 1988). Ice formation in the extracellular space concentrates the extracellular solution, which, in turn, extracts water from cells, concentrating cellular contents and subjecting the cells to osmotic stress. It also increases the extracellular viscosity, reducing the rate of water withdrawal from cells toward the extracellular ice crystals. Below - 70º C, high intracellular viscosity inhibits the growth of intracellular ice crystals (Takahashi et al. 1988). Currently, most solutions used for bovine oocyte and embryo vitrification contain fetal calf serum (FCS) or bovine serum albumin (BSA) as the macromolecular component (Pereira and Marques, 2008). Partial inclusion of macromolecules (PVP, ficoll) and sugars into the vitrification solutions to substitute part of the permeating cryoprotectants and to increase the viscosity of the vitrification medium have been suggested to reduce the toxicity of the media (Kuleshova et al., 1999; Shaw et al., 1997). However, the composition of the vitrification solution seems to be the most critical determinant affecting the vitrification success (Guignot et al., 2006). A positive effect has been noticed when sugars (sucrose or trehalose) were added to the vitrification solution (Kuleshova et al., 1999) and also polymers (ficoll and dextran) were found to be effective when used to design polymer-based vitrification solutions that help in minimizing the inclusion of the toxic permeating cryoprotectants in the vitrification solutions (Kuleshova et al., 2001) In cattle, the addition of 0.1 or 0.3 M sucrose in the last step of equilibration has

significantly enhanced the hatching rate of Day 6 and Day 7 embryos as compared to 0.0 and 0.5 M sucrose (Martinez et al., 2002). Addition of sucrose (0.4 M) into the vitrification solution for conventional and OPS vitrification, resulted in acceptable embryo survival of goat morulae, blastocysts and expanded blastocysts (Guignot et al., 2006).

As dehydration of embryos during conventional slow freezing is believed to be helpful in performing a successful cryopreservation, it was also suggested that dehydration of oocytes or embryos might be more important for achieving successful vitrification than the absolute amount of cryoprotectant present in the cells (Martino et al., 1996; Kuleshova et al., 2001). The addition of the carbohydrates to the vitrification solution parallel with the permeating cryoprotectants aids in accomplishing the desired dehydration of embryos (Ali and Shelton, 1993a; Kasai et al., 1992). The inclusion of sucrose or trehalose into vitrification solutions is widely used for cryopreservation of murine, bovine, and ovine embryos (Kasai et al., 1990; Ali and Shelton, 1993a; Saha et al., 1996). The vitrification physical properties of a solution were not highly altered when penetrating cryoprotectants were partially replaced by polymers (e.g. PVP, ficoll and dextran; Shaw et al., 1997).

1.2.6.3 Removal of cryoprotectants:

Removal of permeating cryoprotectants from the cryopreserved embryos is a very important step directly associated with thawing to prevent excessive osmotic swelling of the thawed embryos caused by the rapid influx of water in response to the accumulation of high concentrations of cryoprotectants within the embryos (Leibo, 1989). The dangerous swelling and damage of the embryo after thawing is usually prevented by gradual passing the thawed embryos through solutions of decreasing osmotic pressure, composed of decreasing concentrations of permeating cryoprotectants with sucrose (Puls-Kleingeld et al., 1992; Nowshari and Holtz, 1995) or even without sucrose (Li et al., 1990; Le Gall et al., 1993). Sucrose solutions alone are also a common way of removing cryoprotectants after thawing in conventionally cryopreserved (Le Gall et al., 1993) or vitrified (El Gayar and Holtz, 2001) embryos. Presently, only disaccharides, mainly sucrose, are widely employed in the media used to remove cryoprotectants from vitrified-warmed embryos (Kuleshova et al. 1999).

1.2.7 Cooling rate:

The rate of cooling is a very important factor that has a direct influence on embryo survival during cryopreservation. In case of conventional slow freezing, the optimal freezing rate usually, considers the relative tolerance of the embryo (species, stage, origin) to the toxicity of the cryoprotectants used and the possible damage caused by the ice crystals. In conventionally cryopreserved embryos intracellular ice crystal formation is affected by the cooling rate, which depends on surface-to-volume ratio of the embryos, water permeability coefficient and temperature (Leibo, 1989).

Sometimes, vitrification is described as the result of extremely high cryoprotectant concentrations parallel with extremely high cooling rates (Kuwayama et al., 2007). However, vitrification does not necessarily require high cryoprotectant concentrations, because even pure water can be vitrified if the cooling rate is high enough (-10^7 ºC/s, Rall, 1987). High cooling rate, when used in vitrification, have the advantage of decreasing the concentration of the cryoprotectants used and preventing passing of the embryos, during cooling, through the dangerous temperature zone (+15 to –5ºC) leading to a reduction in chilling injury (Kuwayama, 2007). Direct plunging of traditional 0.25 ml insemination straws into liquid nitrogen leads to a maximum cooling and warming rate of < 2000º C/min (Vajta et al., 1998). In the last 20 years different approaches have been suggested and applied to maximize the cooling rate of the embryos during vitrification. However, nearly all the suggestions and applications tended toward the direct contact between the cryoprotectant solution and liquid nitrogen. Direct immersion of the embryos in liquid nitrogen was the simplest and most direct way to achieve this goal (Landa and Tepla, 1990). This procedure, originally developed for mouse embryos, was later successfully applied to bovine embryos (Riha et al., 1991). Minimizing the volume of the vitrification solution holding the embryo during vitrification provides a golden chance for maximizing both cooling and warming rates, and also minimizes the chance of ice crystal formation in the small sample giving suitable circumstance for successful vitrification (Rall, 1987; Kuwayama, 2007). Reduction of the sample volume, combined with accelerated cooling, enabled reduction of the cryoprotectant concentration (Yavin and Arav, 2007). In few systems, where it is possible to measure the cooling rate, the critical rate required to avoid chilling injury and to benefit from minimizing the cryoprotectant concentration needed was found to be around 20,000º C/min in case of the OPS method (Vajta et al., 1998).

1.2.8 Warming rate:

Warming rate is also a very important determinant for achieving successful cryopreservation of embryos. The optimum warming rate for a given type of cell is highly dependent on the optimum cooling rate that preceded it. Early investigators assumed that rapid warming of mammalian cells after cryopreservation was always better because there was less time for intracellular re-crystallization to occur and for exposure to cryoprotectants. However, the first investigations of mouse embryo freezing by Whittingham et al. (1972) proved that there are exceptions to this rule. Their study showed that embryos cryopreserved by slow-cooling had greater post-thaw survival chances when warmed slowly. In fact, they reported that embryo survival was dependent on a slow warming rate. They concluded that poor survival using faster warming rates is most likely due to osmotic stress. Many factors are believed to play a role in determining the rate of thawing, including size of cryopreserved embryo, volume reduction during cryopreservation and permeability of the embryonal cells to water and cryoprotectants used (Whittingham et al. 1972; Leibo et al., 1989).
In case of vitrified embryos, the high warming rate is believed to improve chances of post-thaw survival as they give no chance for re-crystallization due to rapid passing through the dangerous temperature zones where ice crystals begin to form (Yavin and Arav, 2007).

1.2.9 Open pulled straw (OPS):

In 1997, Vajta et al. invented a capillary system called open-pulled straw (OPS). They heat-softened 0.25 ml insemination straws over a hot plate and pulled them manually until decreasing the inner diameter and wall thickness of the central part from 1.7 to about 0.8 mm and from 0.15 to about 0.07 mm, respectively. Then the pulled straw was cut with a razor blade at the narrowest point. Loading of embryos into the OPS happened by capillary action by touching a minute drop (2.0 μL) of vitrification medium containing the embryos, by the narrow end of the pulled straw. The vitrification solution containing the embryos forms a 2-3 cm column (1-1.5 μL) at the tip of the narrow end of the open straw. Open straws loaded with embryos are directly plunged into liquid nitrogen thin-end-first to achieve a cooling rate of about 22 500° C/min (Vajta et al., 1998).

1.2.10 Cryopreservation of goat embryos:

Bilton and Moore (1976) reported the first successful cryopreservation of goat embryos, whereas, the first successful transfer of vitrified goat embryos was reported by Yuswiati and Holtz (1990). Both, conventional slow freezing and vitrification are used for embryo cryopreservation nowadays, with a trend, during the last decade, of preferring vitrification, providing a cheaper and more practical substitute for the time consuming and more expensive traditional method. During the last three decades, variable developmental stages of goat embryos between 16-cell stage embryos and hatched blastocysts were cryopreserved and transferred (Table 3). Morulae have been cryopreserved with unsatisfactory success rates (Puls-Kleingeld et al., 1992). On the other hand, blastocysts proved to be more suitable (Li et al., 1990; Puls-Kleingeld et al., 1992) for conventional cryopreservation. High pregnancy rates (67%) were also reported when transferring frozen-thawed hatching and hatched blastocysts (Li et al., 1990). The Open-pulled-straw (OPS) vitrification technique for blastocyst stage goat embryos produced rather spectacular success rates. Overall embryo survival amounted to 64% for OPS-vitrified as compared to 42% for conventionally frozen embryos, with statistically significant difference (El-Gayar and Holtz, 2001). Successful vitrification of morulae and early blastocysts was reported, applying the OPS method, using ficoll and sucrose in addition to EG and Me_2SO in the vitrification medium. Kidding rates ranged from 43 to 51% (Hong et al., 2007). Low embryo survival (14%) was achieved when goat morulae and blastocysts were vitrified with the OPS method, using 0.4 M sucrose added to 18 % EG and 18 % Me_2SO as a vitrification medium (Guignot et al., 2006). In both the latter investigations no differentiation between morulae and blastocysts took place, compromising possible interpretation.

No experience with the vitrification approach of Vajta et al. (1998) exists with regard to other developmental stages (e.g. hatched blastocyst) using OPS method or even with morulae using.

Table 3 Results of the transfer of vitrified-warmed goat embryos as reported in the literature

Reference	Stage	Cryoprotectant	Method	Success rate
Yuswiati and Holtz (1990)	Morula + Blastocyst	10% Gly + 20% Propanediol (equilibration) 25% Gly + 25% Propanediol	0.25 ml Straw	22% kidding
Traldi et al.(1999)	Expanded blastocyst	Gly + EG	Straw	45% kidding
El-Gayar and Holtz (2001)	Blastocyst	10% EG + 10% Me_2SO 20% EG + 20% Me_2SO	OPS	64% embryo survival
Begin et al.(2003)	2-4 cells	10% EG + 10% Me_2SO (equilibration) 20% EG + 20% Me_2SO + 10mg\ml Ficoll + 0.65 M Suc	Cryoloop	13% blastocyst formation
Huang et al. (2006)	Expanded blastocyst	16.5% EG +16.5% Me_2SO 20% EG +16.5% Me_2SO	Microdrop	64% 53% embryo survival
Guignot et al. (2006)	Morula + Blastocyst	10% Gly (equilibration) conventional dilution 10% Gly + 20% EG (equilibration) direct transfer 25% Gly + 25% EG	0.25 ml Straw	35% 28%
		7.5% EG + 7.5% Me_2SO (equilibration) 18%% EG + 18% Me_2SO + 0.4 M Suc	OPS	14% Embryo survival
Hong et al. (2007)	Morula + Blastocyst	10% EG (equilibration) 30% EG + 30% Ficoll + 0.5 M Suc 40% EG + 30% Ficoll + 0.5 M Suc	OPS	33% 46%
		10% EG + 10% Me_2SO (equilibration) 15% EG + 15% Me_2SO + 30% Ficoll + 0.5 M Suc 20% EG + 20% Me_2SO + 30% Ficoll + 0.5 M Suc		51% 43% kidding

EG = Ethylene glycol Gly = Glycerol Me_2SO = Dimethylsulfoxide
OPS = Open pulled straw Suc = Sucrose

1.3 Materials and Methods:

1.3.1 Animals:

The experiment was conducted on Boer goats from our own breeding flock at Goettingen, Germany (9º 41' E, 51º 46' N) during the breeding season (October to January). The goats were group-housed in an open barn with straw-bedding and an outdoor concrete run. They were fed a daily ration of 600 g concentrate, consisting of equal parts of a pelleted diet for breeding ewes (16% crude protein, 10.2 MJ ME/ kg, supplemented with 43 mg/kg Se, 12 mg/kg I and 5000 mg/kg Zn), oats and dried sugar beet pulp and had free access to wheat or barley straw, salt lick and water. Once daily the complete flock was routinely tested for estrus with an aproned male.

1.3.2 Donors preparation and superovulation:

The estrous cycles of the donors were synchronized as follows. Does with serum progesterone concentrations in excess of 5 ng/mL - assessed by ELISA according to van de Wiel and Koops (1986, modified by Moeller 1991) - were treated with an i.m. injection of $PGF_{2\alpha}$ (5.0 mg Dinoprost, Dinolytic®, Pfizer, Karlsruhe, Germany) followed, seven days later, by i.m. administration of 0.004 mg of the GnRH analog Buserelin (1 ml Receptal®, Intervet, Unterschleissheim, Germany). Superovulation was induced by administering 6 s.c. injections of 4, 4, 2, 2, 2, and 2 A.U. of pFSH supplemented with 40% LH (Nowshari et al., 1995) at 12-h intervals, beginning 5 days after the Dinoprost injection. Along with the last 2 FSH injections the does received 2 i.m. doses of 5.0 mg Dinoprost. Eighteen h after the last $PGF_{2\alpha}$ injection the does were randomly assigned to three treatment groups. The control group received an i.m. injection of 1 mL of sterile physiological saline solution, the GnRH group an i.m. injection of 0.004 mg of the GnRH analog Buserelin (1 ml Receptal®) and the hCG group an i.m. injection of 500 I.U. human chorionic gonadotropin (hCG, 1.0 mL Chorulon®, Intervet, Beaucouzé, France). After the end of the hormone treatment does were tested for estrus with an aproned male at 6 h intervals (6 am, 12 am, 6 pm, and 12 pm). The does showing signs of estrus were hand mated twice daily as long as they allowed the male to mount. To counteract the effect of premature CL regression, donors were provided with

progestogen containing ear implants (Crestar®, 3.3 mg Norgestomet, Intervet, Intervet, Beaucouzé, France) 12 h after the last mating. The implants were removed 20 h before embryo collection, simultaneously with an i.m. administration of 5.0 mg Dinoprost.

1.3.3 Collection of embryos:

In order to obtain morulae, blastocysts and hatched blastocysts, does were flushed on days 6, 7-8 and 8.5-9 after the last mating, respectively, applying the transcervical flushing technique described in Suyadi et al. (2000).

The flushing medium (Dulbecco's Phosphate Buffered Saline, PBS) was supplemented with 0.06 % BSA (Sigma A 9647, Steinheim, Germany) and had a temperature of 39°C (Table 4). Embryos were recovered from the flushings under a stereo microscope (M8, Wild Heerbrugg, Switzerland) at X20-50 and classified based on morphological characters according to Lindner and Wright (1983).

Table 4 Composition of (PBS) flushing medium used for collection of goat embryos

Component	Concentration [a]	Source
NaCl	8000 mg/L	Sigma, Steinheim, Germany
KCl	200 mg/L	Sigma, Steinheim, Germany
$MgCl_2.6H_2O$	100 mg/L	Sigma, Steinheim, Germany
Na_2HPO_4	1000 mg/L	Sigma, Steinheim, Germany
NaH_2PO_4	150 mg/L	Sigma, Steinheim, Germany
KH_2PO_4	200 mg/L	Sigma, Steinheim, Germany
Glucose	1000 mg/L	Sigma, Steinheim, Germany
Na-Pyruvate	36 mg/L	Sigma, Steinheim, Germany
BSA	600 mg/L	Sigma, Steinheim, Germany
$CaCl_2$	100 mg/L	Sigma, Steinheim, Germany
Penicillin-Streptomycin 10 000 IU/ml	10 ml	Gibco, Paisley, Scotland

[a] In sterile water (Ampuwa®, Fresinius, Germany) (PH 7.2)

1.3.4 Cryopreservation of embryos:

One-2 h after collection, morphologically intact embryos classified as “very good” or ”good” at the various stages of development from each recipient were divided up into those to be cryopreserved conventionally and those to be vitrified by the OPS method described by El-Gayar and Holtz (2001). For conventional freezing only blastocyst and hatched blastocysts were used, for vitrification morulae as well.

1.3.4.1 Conventional cryopreservation method:

For conventional cryopreservation embryos were washed 3 times for 3 min at a time in M2 medium (Hogan et al., 1986). They were then transferred to M2 medium containing 0.5, 1.0 and 1.5 M ethylene glycol (EG, Sigma E 9129, Steinheim, Germany) for 10, 10 and 20 min, respectively. Two embryos at a time were loaded into 0.25 mL straws (Minitüb, Tiefenbach, Germany), labeled with the donor number, stage and quality of the embryos and date. Up to this point all operations were conducted at room temperature. As a next step the straws were transferred to an alcohol bath (Haake, Karlsruhe, Germany) pre-cooled to -6° C, seeded after 10 min and, after another 10 min, cooled to –35° C at a rate of 0.5° C/min. After 15 min at -35° C, straws were immersed in liquid nitrogen.

1.3.4.1.1 Thawing of conventionally frozen embryos:

For thawing, straws were removed from liquid nitrogen, waved in the air at room temperature for 7 to 8 sec, and immersed in a water bath at 35° C for 8 to 10 sec. The contents of the straws were emptied into a tissue culture dish from where the embryos were recovered and passed, at room temperature, through 3 dishes containing 1.0 mL M2 medium with 0.5 M sucrose and 0.75 M EG (3 min); M2 medium with 0.5 M sucrose (5 min) and M2 medium with no additions (10 min). They were kept in an incubator at 39° C under a humidified atmosphere of air until transfer.

1.3.4.2 Open pulled straw (OPS) vitrification:

For OPS vitrification, 0.25 mL straws (Minitüb, Tiefenbach, Germany) were softened over a hot plate at 200° C and pulled until the thinnest point had approximately half the original diameter and wall thickness. By cutting at the thinnest point, two OPS-straws were obtained. One embryo at a time was equilibrated for 10 min in 1.0 mL holding medium consisting of Medium 199 (PAA Laboratories, Pasching, Germany) supplemented with 0.022 g/100 mL pyruvic acid (Sigma P 5280, Steinheim, Germany), 0.0146 g/100 mL L-glutamin (Sigma G 5763, Steinheim, Germany) and 20% heat-inactivated goat serum. Thereafter it was transferred to 1.0 ml of holding medium supplemented with 10% ethylene glycol (EG) (Sigma E 9129, Steinheim, Germany) and 10% dimethyl sulfoxide (Me_2SO) (Sigma D2650, Steinheim, Germany), and, after 1 min, to a 20 µL droplet containing 20% EG and 20% DMSO. Within less than 25 sec, the embryo, suspended in 1-2 µL medium, was aspirated into the thin end of a labeled drawn-out straw by capillary force. Immediately the straws were plunged into liquid nitrogen (-196° C) in a vertical position with the thin end first.

1.3.4.2.1 Warming of vitrified embryos:

For warming, the straws were removed from liquid nitrogen and the thin end was immediately dipped into warming medium at 39° C while the opposite end was occluded with the tip of a finger. As the contents of the straw liquefied and the air expanded, the embryos slid out into the medium (holding medium containing 0.33 M sucrose). After 1 min, the embryo was passaged, for 1 min at a time, through 2 sequential dishes of holding medium containing 0.33 and 0.20 M sucrose, respectively, and ended up in holding medium devoid of sucrose. Within 5 min thereafter, the embryos were transferred to the uterine horn of a recipient.

1.3.5 Embryo transfer:

To assess the difference in vivo survival of OPS-vitrified embryos and embryos cryopreserved by the conventional slow freezing procedure, cryopreserved-thawed embryos were transferred to synchronized recipients, and the does were allowed to carry to term.

1.3.5.1 Preparation of recipients for embryo transfer:

Embryos were transferred within 15 minutes after thawing. Recipients were pluriparous does from our own breeding flock, estrus induced by i.m. injection of 0.004 mg of Buserelin, followed, 7 days later, by i.m. injection of 5.0 mg Dinoprost. Estrus detection with an aproned buck was conducted twice daily and does in estrus, within 48-72 h after prostaglandin treatment, were considered suitable recipients. Morulae, blastocysts and hatched blastocysts were transferred 5, 6-7 or 7.5-8 days after the end of estrus, respectively. The does had been deprived of feed for 2 days and of water for 1 day. To be sure of a functional corpus luteum present, a blood sample was analyzed for progesterone is just on the morning of the intended transfer.

1.3.5.2 Laparoscopic embryo transfer:

The does were anesthetized by i.v. injection of 1.0 mL Seaxylan® (20 mg xylazin, WDT, Garbsen, Germany) and 1.0 mL Ursotamin® (0.1 g ketamin, Serumwerke Bernburg, Germany) and placed on a laparoscopy cradle in dorsal recumbence. Following the technique described in Sohnrey and Holtz (2005) the ovaries were inspected laparoscopically. Through a 3 cm incision the uterine horn ipsilateral to the ovary displaying at least one corpus luteum was very gently grasped with the aid of a blunted tenaculum forceps and a loop of 3-4 cm was exteriorized. With the aid of a unopette (20 µL, Beckton Dickinson, Plymouth, UK) usually 2, on rare occasions 1 or 3 embryos were deposited in the uterine lumen through a puncture hole in the uterine wall about 5 cm from the utero-tubal junction Figure 1. After repositioning of the uterus the incision was closed with a single suture. Four weeks after transfer the does were subjected to real-time ultrasonography (Aloka SSD 500) with a rectal 7.5-MHz linear-array transducer, as described elsewhere (Padilla Rivas et al., 2005). At parturition, kidding rate and litter size were recorded.

1.3.6 Statistical analysis:

Differences between five groups for pregnancy, kidding and embryo survival were analyzed with the Chi Square test by performing all pair-wise comparisons. The differences were considered to be significant when corresponding P value was less or equal to 0.05 ($P \leq 0.05$). All the statistical tests were analyzed by SAS program.

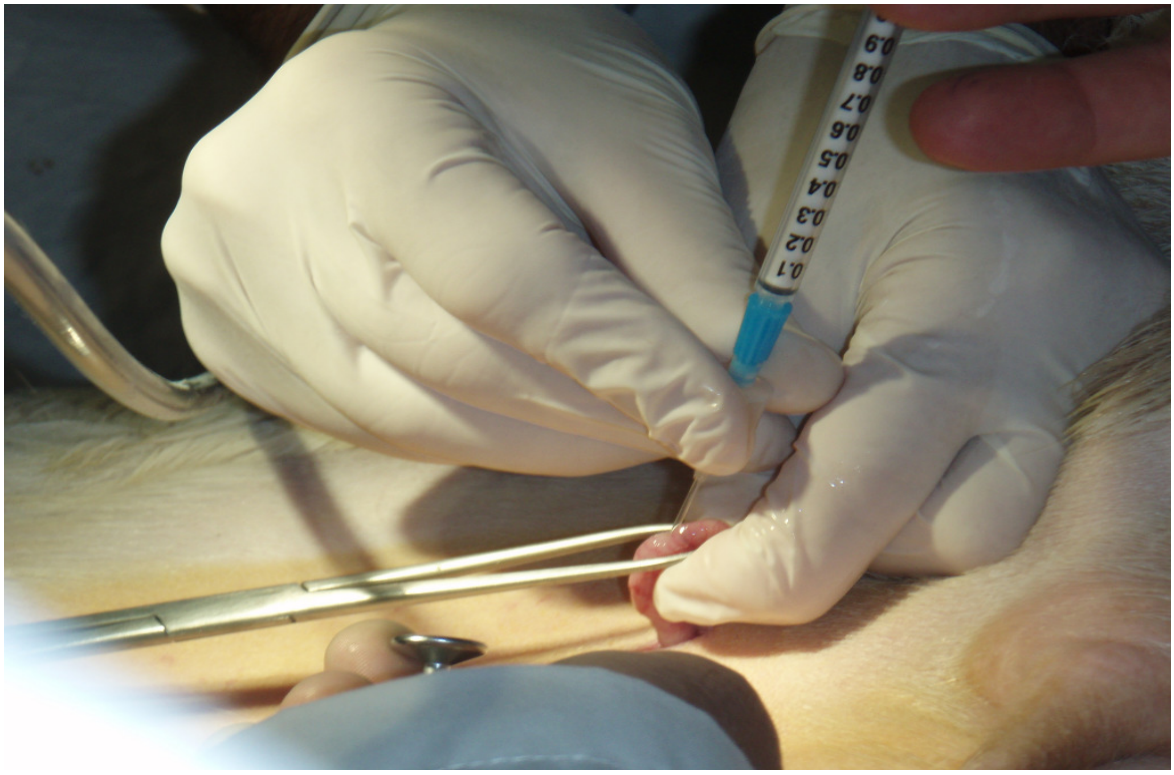

Figure 1 Laparoscopic transfer of embryos with a unopette

1.4 Results:

The results of the experiment are summarized in Table 1. In 9 out of 48 recipients the intended transfer of 2 embryos/recipient was not implemented because, in order to be able to trace back each kid to its genetic parent, mixing of embryos from different donors was not permissible. As indicated in Table 1, 7 does received a single embryo and 2 does received three embryos.

Conventional cryopreservation of morulae was not attempted as it was known from earlier experience that chances for survival are minimal. As shown in Table 1, cryopreservation of morulae by OPS vitrification was equally ineffectual.

Blastocysts cryopreserved by conventional means gave rise to pregnancies in 50% (5/10) of the recipients. Due to one abortion, the kidding rate was 40%. Litter size averaged 2.0 and total survival of transferred blastocysts was 42%. With the OPS vitrification method, 82% (9/11) of the recipients got pregnant and went to term, average litter size was 1.8 and overall embryo survival 70%. The difference in kidding rate between conventionally frozen and OPS-vitrified blastocysts (40 vs. 82%) was statistically significant ($P<0.05$).

The cryopreservation of hatched blastocysts was less effectual. Transfer of conventionally frozen and OPS-vitrified embryos both resulted in 3 out of 9 recipients (33%) pregnant. Due to 1 abortion in the group that received vitrified embryos the kidding rate was only 22%, as compared to 33% in the group that received conventionally frozen embryos. In every case a singlet was born, leading to embryo survival rates of 19% in the group receiving conventionally frozen embryos and 13% in the group receiving OPS vitrified embryos. Regardless of the cryopreservation technique, pregnancy, kidding and embryo survival rates achieved by transferring cryopreserved hatched blastocysts were significantly lower than what was recorded for non-hatched blastocysts ($P<0.05$).

Only 3 pregnancies were reported in case of hatched blastocysts cryopreserved with the conventional method out of the 9 recipients that received embryos, resulting in a pregnancy rate of 33%, and an equal kidding rate as all of the 3 goats kidded. Similar pregnancy rate (33%) was obtained in the conventionally frozen hatched blastocysts group, as 3 out of the 9 recipients were found to be pregnant by d 30. kidding rate was decreased to 22% as one goat aborted. Embryo survival rates were 19 and 13% in conventional-hatched blastocysts and OPS-hatched blastocysts groups respectively. No significant differences were found between the two hatched blastocysts groups. But both of the groups had significantly lower ($P<0.05$)

pregnancy, kidding and embryo survival rates when compared with the OPS-blastocyst group. Unfortunately, none of the recipients that received OPS-vitrified goat morulae were found to be pregnant by d 30.

Table 5 Transfer of caprine embryos of various developmental stages cryopreserved either conventionally or by OPS vitrification. Unless otherwise stated, recipient does received 2 embryos

Stage of development	Method of cryopreservation	Number of recipients	Recipients pregnant		Recipients kidding		Average litter size	Embryo survival
			n	%	n	%		%
Morula	Coventional [a]	-	-	-	-	-	-	-
	OPS	9	0	0 [z]	0	0 [z]	-	0 [z] (0/18)
Blastocyst	Conventional	10 [b]	5	50 [xy]	4	40 [f x]	2.0	42 [xy] (8/19)
	OPS	11 [c]	9	82 [x]	9	82 [y]	1.8	70 [x] (16/23)
Hatched blastocyst	Conventional	9 [d]	3	33 [yz]	3	33 [xz]	1.0	19 [yz] (3/16)
	OPS	9 [e]	3	33 [yz]	2	22 [fxz]	1.0	13 [yz] (2/15)

[x,y,z] Within columns, values with different superscripts are different ($P<0.05$, χ^2 test)
[a] Not included in the experiment
[b] One recipient received 1 embryo only
[c] One recipient received 1, 2 recipients 3 embryos
[d] Two recipients received 1 embryo only
[e] Three recipients received 1 embryo only
[f] One recipient aborted

1.5 Discussion

The present investigation constitutes an attempt to extend the favorable results achieved with OPS vitrification of caprine blastocysts (El Gayar and Holtz, 2001) to other embryonal stages (morulae and hatched blastocysts).

From earlier studies (Li et al., 1990; Puls-Kleingeld et al., 1992) it is known that, in most cases, caprine morulae do not survive conventional freezing. Therefore, conventional freezing of morulae was not included in the present investigation. In an earlier study involving vitrification of goat embryos - though not by the OPS method (Yuswiati and Holtz, 1990) – one of the two kids born originated from a morula. Two recent investigations report pregnancy rates of 14% (Guignot et al., 2006) and – on account of various methodological variations - between 33-51% (Hong et al., 2007) upon transfer of vitrified caprine morulae and blastocysts. It was not distinguished between embryonal stages; however, therefore there is no way of telling whether morulae had survived. In the present study not a single recipient provided with OPS vitrified morulae remained pregnant, indicating that the vitrification procedure established and applied by Vajta et al. (1998) and El Gayar and Holtz (2001), is not suitable for the cryopreservation of caprine morulae.

Blastocysts have repeatedly been shown to be a suitable stage for conventional cryopreservation (Puls-Kleingeld et al., 1992; Nowshari and Holtz, 1995). In fact, embryos recovered as morulae were successfully cryopreserved after in vitro culture to the blastocyst stage (Nowshari and Holtz, 1995). In the present investigation, the results arrived at with the cryopreservation of blastocysts were in almost perfect agreement with those of an earlier study (El-Gayar and Holtz, 2001): after conventional freezing, in either study 42% of the transferred blastocysts were carried to term; after OPS vitrification embryo survival was slightly improved over the previous study (70 vs. 64%), whereas the proportion of does kidding was a bit lower (82% vs. 93%).

To our knowledge this is the first report of successful OPS vitrification of hatched goat blastocysts. The transfer results, however, were discouragingly low, regardless whether conventional freezing or OPS vitrification protocols were involved. This is disappointing, because it implies that there is still an efficient means of cryopreserving embryos without an intact zona pellucida lacking. This includes bisected (Nowshari and Holtz, 1993; Oppenheim et al., 2000) or biopsied embryos (El-Gayar and Holtz, 2005). The zona pellucida as a protective coat is known to control osmotic pressure (Bronson and McLaren, 1970), transport and diffusion of nutrients and metabolites (Leoni, et al., 2002) and, in all likelihood, also

foreign substances including cryoprotectants. It is, by no means, a static structure, changing its character during tubal passage (Kolbe and Holtz, 2005), fertilization (Yanagimachi, 1988; Suzuki et al., 1996) or cryopreservation (Moreira da Silva and Metelo, 2005). The exact mechanism by which the zona pellucida provides protection to the embryo is not fully understood. Current methods for cryopreserving embryos, being devised empirically, are tailored to embryos with an intact zona. It might be advisable to devise specific protocols for zona-free embryos. This might involve modification of concentrations and exposure times of cryoprotectants, possibly employment of alternative cryoprotectants that are more effective or less toxic (e.g. glycerol, Chemineau et al., 1986; Fiẻni et al., 1995), etc.

By way of conclusion, the outcome of the present experiment convincingly confirms the earlier finding that OPS vitrification of caprine blastocysts provides for higher pregnancy and embryo survival rates than conventional freezing. Attempts to apply the OPS technique to caprine morulae were of no avail since no pregnancies were accomplished. For the cryopreservation of hatched blastocysts the OPS vitrification method was no more effective than conventional cryopreservation, yet, the technique is less elaborate, less time consuming and less costly.

2 Effect of sucrose in vitrification and warming media on the in vitro survival of OPS vitrified mouse blastocysts

2.1 Introduction:

Conventional cryopreservation of mammalian embryos comprises a controlled crystallization process and leads to partial dehydration of the blastomeres. It requires a programmable freezing unit, 1-2 hours of time and the seeding of straws once they have reached a certain temperature. Vitrification, on the other hand, comprises the solidification of liquids containing high concentrations of penetrating cryoprotectants at extremely high cooling rates to reach a glass-like state. It requires less equipment and time and circumvents the detrimental effect of ice crystal formation. Luyet (1937) was the first to draw attention to the feasibility of vitrifying biological material and Rall and Fahy (1985) were the first to successfully vitrify mammalian embryos. At a cooling rate of 20000° C/min, accomplished with the OPS method, the critical temperature encompassing the phases of homogenous nucleation and transition to the glass phase is passed fast enough to minimize chilling injury and damage inflicted by rapid transmembranous water passage (Vajta et al., 1998). The penetrating cryoprotectants present in vitrification media at extremely high concentrations are highly cytotoxic to unfrozen cells. Therefore it is critical to bring the temperature down as fast as possible after exposure of the embryos to the cryoprotectants and have the cryoprotectants removed from the cells as a soon as the medium has liquefied upon warming (Ishida et al., 1997; Kong et al. 2000). With conventional freezing, sucrose is used to reduce physical and chemical damage to the cells, especially after thawing (Martino et al., 1996; Kuleshova et al., 2001). That might be the reason why sugars are included with vitrification and warming media. According to Shaw et al. (1997) the properties of vitrification media remain unaltered after the addition of disaccharides or polymers such as PVP, ficoll and dextran. Sucrose and trehalose have been added to vitrification media in murine, bovine and ovine embryos (Kasai et al., 1990; Ali and Shelton, 1992; Saha et al., 1996). Whereas disaccharides are commonly used to alleviate osmotic stress during the removal of penetrating cryoprotectants from blastomeres (Kuleshova et al. 1999), recent findings (El-Gayar et al., 2008) suggests that, in that context, this is not a necessity. The present investigation addresses the question whether, with the vitrification of murine blastocysts, sucrose as a component of vitrification or warming media is a requirement.

2.2 Review of literature:

2.2.1 Vitrification of murine embryos:

2.2.1.1 Developmental stage of embryos:

The developmental stage of the embryo is one of the most important determinants for success of embryo cryopreservation. In case of murine embryos, all embryo stages (1-cell to hatched blastocyst) appear to have a good chance of survival after vitrification, depending on the cryoprotectants used in the vitrification solution, the container and the dilution method (Table 6).

For 1-cell stage embryos the post-warming development of the vitrified embryos ranged between 0% in vitro development (Kono and Tsunoda 1987) to higher acceptable rates of development in vitro (67%, Zhou et al., 2005) and in vivo (9%, Yan et al., 2008) when using the OPS method. Two-cell stage embryos appear to be more suitable for vitrification than 1-cell stage embryos with in vitro development rates ranging from 22% (Uechi et al., 1999) to 100% (Kuleshova and Shaw 2000) when straw in straw dilution was applied. An in vivo survival rate of 76% was achieved when 2-cell vitrified-warmed embryos were transferred.

After vitrification of 4-cell murine embryos, in vitro development ranged from 70% (Ali and Shelton, 1993b) to 88% (Bernart et al., 1994). When the OPS method was used to vitrify this stage, the in vitro development reached 88% (Zhou et al., 2005).

The pioneering efforts of vitrification of murine embryos were actually accomplished with 8-cell embryos (Takeda et al., 1984; Rall and Fahy 1985). The development of vitrified-warmed murine 8-cell embryos ranged from 8% (Hredzák et al., 2006) to 100% (Kuleshova and Shaw 2000) in vitro development. The morula is one of the most studied embryo stages in the murine for the employment of vitrification resulting in high in vitro survival rates (usually >70%; Table 6). In contrast to most of the reports, low in vitro survival rates (29%) were also reported (Takahashi and Kanagawa 1985). High in vitro (97%) and in vivo (74%) development was achieved by Kasai et al. (1992), and when the OPS method was applied to vitrify morulae, 100% in vitro and 33% in vivo development was reported (Zhou et al., 2005).

Murine blastocysts seem to be more sensitive than morulae as results of in vitro and in vivo development were variable depending on the respective cryoprotectants used (Table 6). When glycerol and polyethylene glycol were used as cryoprotectants for vitrifying mouse blastocysts, 5 and 6% in vitro development was recorded for early and expanded blastocysts,

respectively (Cseh et al., 1999). On the other hand, 96% in vitro development and 55% fetal developments in vivo were recorded when a cryoloop was employed to vitrify mouse blastocysts (Lane et al., 1999). The few reports regarding vitrification of murine hatched blastocysts showed that hatching of the embryo has no detrimental effect on post-warming survival of vitrified embryos. The success of vitrification of hatched murine blastocysts ranged from 65% (Zhu et al., 1996) to 99% (Zhou et al., 2007) for in vitro development, while the corresponding in vivo development rates reported in these two reports were, 31% and 42%, respectively.

2.2.1.2 Cryoprotectants:

All of the permeating cryoprotectants used for cryopreservation of embryos in farm animals or humans, had actually proved to be successful with mouse embryos, the mouse being a favorite model animal for experimental medicine and biotechnology. Variable permeating cryoprotectants (e.g. glycerol, propylene glycol, propandiol, 1, 3 butanediol, ethylene glycol and Me_2SO) were used at different concentrations (Table 6) ranging from 1.0 M (acetamide, Nakao et al., 1997) to 20.5 M (Me_2SO, Kono and Tsunoda 1987). Incorporation of permeating cryoprotectants at concentrations calculated as a percentage of the vitrification solution were also reported and ranged from 10% (butanediol, Valdez et al., 1992) to 40% (EG, Zhu et al., 1996; Ishida et al., 1997) for each of the permeating cryoprotectants used in the vitrification solution.

Non-permeating cryoprotectants were also included in many of the vitrification solutions reported in Table 6. (e.g. sucrose, trehalose, polyethylene glycol, bovine serum albumin, dextran and ficoll), with molar concentrations ranging from 0.25 M (Bernart et al., 1994) to 1.1 M (Krag et al., 1985). In gene Percentage-basis addition of non-permeating cryoprotectants to the vitrification solutions ranged between 6% (Cseh et al., 1999) and 35% (Kuleshova and Shaw 2000). The combination of different permeating and non-permeating cryoprotectants to design new vitrification solutions depends on creating a balance between the cryoprotectants considering their permeability, toxicity and glass-forming ability.

2.2.1.3 Vitrification method:

Vitrification of murine embryos has been practiced by loading the embryos with the high concentrated vitrification solution into a 0.25 ml French insemination straw and plunging the straw directly into liquid nitrogen to get a cooling rate of 2500°C/min (Kasai et al., 1990; Zhu et al., 1996). While in the new containers (OPS, cryoloop, Vit-Master) or container-less vitrification (microdrops) that enable direct contact between micro-drop (1 to 2 μL) of vitrification solution carrying the embryos and liquid nitrogen to create a cooling rate of about 20-22000°C/min (Vajta et al., 1998; Lane et al., 1999). Vitrification of murine embryos by plunging the OPS straws into slush nitrogen (SN_2, -210°C) to achieve a higher cooling rate, resulted in increased survival and development of vitrified-warmed biopsied or zona-opened embryos as compared to those vitrified by plunging into liquid nitrogen (Lee et al., 2007).

2.2.1.4 Removal of cryoprotectants:

Removal of permeating cryoprotectants from the cryopreserved embryos is an important step to dilute the toxic cryoprotectants out and to avoid excessive osmotic swelling in the process (Leibo, 1989). This step usually achieved by gradual passaging of the warmed murine embryos through solutions of decreasing osmotic pressure, composed of decreasing concentrations of sucrose (Ali and Shelton, 1993b; Lane et al., 1999) or trehalose (Ishida et al., 1997). This step-wise dilution might also be performed in-straw to enable the direct transfer of warmed embryos using the vitrification straw (OPS) as a transfer catheter (Yang Qe et al., 2007). One-step dilution is also practiced by diluting the embryos in one sucrose solution (Cseh et al., 1999) or by introducing the sucrose solution into the vitrification straw to enable one-step in straw dilution (Rall and Wood 1994; Kasai et al., 2002). Recently, OPS vitrified murine blastocysts were successfully diluted in a sucrose-free medium in one step (El-Gayar et al., 2008)opening the door for conducting direct transfer of embryos and facilitating the applicability of OPS-vitrification in the field.

Table 6 Results of in vitro and in vivo development of mouse embryos cryopreserved by vitrification as reported in the literature

Reference	Embryo stage (Container)	Cryoprotectants	Development	
			In vitro	In vivo
Takeda et al. (1984)	8-cell	3.0 M Gly + 0.25 M Suc	65%	-
Williams and Johnson (1985)	Morula	2.0 M Gly + 0.5 M Suc	84%	-
Rall and Fahy (1985)	8-cell	20.5 M Me_2SO + 15.5 M Acetamide + 10% Propyleneglycol + 6% Polyaethyleneglycol	88%	-
Krag et al. (1985)	8-16 cell	1.4 M Gly (equilibration) 1.4 M Gly + 1.1 M Suc	67%	-
		1.4 M Gly (equilibration) 1.4 M Gly + 1.1 M Trehalose	57%	-
Takahashi and Kanagawa (1985)	Morula	3.0 M Gly + 0.25 M Suc	72%	-
		4.0 M Gly + 0.25 M Suc	29%	-
Biery et al. (1986)	Morula	2.5 M Gly	63%	-
		3.0 M Gly	59%	-
Szell and Shelton (1986b)	8-cell	3.0 M Gly	70%	-
Williams and Johnson (1986)	Morula	2.0 M Gly + 0.5 M Suc	67%	-

Reference	Embryo stage (Container)	Cryoprotectants	Development	
			In vitro	In vivo
Scheffen et al. (1986)	Morula	10% Gly + 20% Propandiol (equilibration)	80%	-
	E.blastocyst	25% Gly + 25% Propandiol	82%	-
	Blastocyst		39%	-
Kono and Tsunoda (1987)	1-cell	20.5 M Me_2SO + 15.5 M Acetamide + 10% Propyleneglycol + 6%	0%	0%
	2-cell	Polyaethyleneglycol (VS1)	46%	22%
	8-cell		86%	44%
Kono and Tsunoda (1987)	Morula	20.5 M Me_2SO + 15.5 M Acetamide + 10% Propyleneglycol + 6%	77%	31%
	Blastocyst	Polyaethyleneglycol (VS1)	29%	43%
	1-cell	12.5% VS1 + 25% VS1	19%	20%
	Blastocyst	50% VS1 + 100% VS1	44%	70%
Van der Zwalmen et al. (1988)	Blastocyst	10% Gly + 20% Propandiol	> 40%	-
		25% Gly + 25% Propandiol (at 4°C)	> 80%	-
Lopes et al. (1989)	Morula	10% Gly + 20% Propandiol (equilibration)	68	28
	E.blastocyst	25% Gly + 25% Propandiol (at 4°C)	46	14
	Blastocyst		26	5
Kasai et al. (1990)	Morula	40% EG + 30%Ficoll + 0.5 M Suc	98%	51%
Kasai et al. (1992)	Morula	40% EG + 18%Ficoll + 0.3 M Suc	97%	74%
Valdez et al. (1992)	Blastocyst	20% EG + 20% Me_2SO + 10% 1,3 Butanediol	95%	45%
		10% EG + 10% Me_2SO + 5% 1,3 Butanediol (equilibration)		
		20% EG + 20% Me_2SO + 10% 1,3 Butanediol	96%	54.2%

Reference	Embryo stage (Container)	Cryoprotectants	Development	
			In vitro	In vivo
Zhu et al. (1993)	Ex.blastocyst	20% EG (5 min at 25°C, equilibration) 40% EG + 30%Ficoll + 0.5 M Suc. (0.5 min)	94%	58%
Ali and Shelton (1993b)	2-cell	5.5 M EG + 1.0 M Suc	71%	-
	4-cell		70%	-
	8-cell		87%	-
	Morula		98%	33.3%
	E. blastocyst		97%	25%
	Blastocyst		97%	5.4%
Bernart et al. (1994)	1-cell	3.5 M Me_2SO + 0.25 M Suc	32%	-
	2-cell		73-76%	-
	4-cell		87-88%	-
	8-cell		81%	-
Rall and Wood (1994)	8-12 cell	1.6 M Gly + 6% BSA (equilibration) 4.2 M Gly + 6% BSA (equilibration) 6.5 M Gly + 6% BSA	97%	41%
Zhu et al. (1996)	H. blastocyst	40% EG + 30% Ficoll + 0.5 M Suc (one step-directly in 40%EG)	65%	31%
		10 or 20% Gly (5 or 10 min. equilibration) 40% Gly + 30% Ficoll + 0.5 M Suc	89-94%	54%
Nakao et al. (1997)	2-cell	1 M Me_2SO (equilibration)		
	(cryotube)	1.95 M Me_2SO + 1 M acetamide + 2.85 M propylene glycol	92%	56.5%
Ishida et al. (1997)	8-cell	40% EG + 18%Ficoll + 0.3 M Suc (1min)	45%	-
		(5 min)	62%	-
		(10 min)	60 %	-

Reference	Embryo stage (Container)	Cryoprotectants		Development	
				In vitro	In vivo
Lane et al. (1999)	Blastocyst	10% EG + 10% Me_2SO (equilibration)			
	(Cryoloop)	20% EG + 20% Me_2SO + 10mg/mL Ficoll + 0.65 M Suc		96%	55%
Uechi et al. (1999)	2-cell	40% EG + 18%Ficoll + 0.3 M Suc		22%	-
Cseh et al. (1999)	Morula	10% Gly + 20% propylene glycol (equilibration)	(RT)	66%	
		25% Gly + 25% propylene glycol	(4°C)	70%	-
	E.blastocyst		(RT)	33%	-
			(4°C)	38%	-
	Ex.blastocyst		(RT)	5%	-
			(4°C)	57%	-
	Morula	1.625 M Gly + 1.5% polyethyleneglycol (equilibration)	(RT)	93%	-
		6.5 M Gly + 6.0 % polyethyleneglycol	(4°C)	83%	-
	E.blastocyst		(RT)	36%	-
			(4°C)	5%	-
	Ex.blastocyst		(RT)	6%	-
			(4°C)	57%	-
					-
Kong et al. (2000)	Blastocysts	10% EG + 10% Me_2SO (equilibration)	OPS	89%	-
	(OPS or GMP)	16.5% EG + 16.5% Me_2SO	GMP	90%	-
Kuleshova and Shaw (2000)	2-cell	25% EG + 35% Dextran	(single straw)	100%	-
			(straw in straw)	100%	-
		25% EG + 35% Ficoll	(single straw)	100%	76%
			(straw in straw)	100%	76%
	8-cell	25% EG + 35% Ficoll	(single straw)	98%	-
			(straw in straw)	100%	-
Zheng et al. (2004)	Morula	40% EG + 18% Dextran + 0.5 M Suc		92%	15%
		40% EG + 18% Ficoll + 0.5 M Suc		84%	16%
Huang et al. (2005)	Blastocysts	10% EG+ 10% Me_2SO (equilibration)			
	(Vit-Master)	20% EG + 20% Me_2SO + 0.5 M Suc		50%	-

Reference	Embryo stage (Container)	Cryoprotectants		Development	
				In vitro	In vivo
Hredzàk et al. (2005)	2-cell	20% EG +18% Ficoll + 0.5 M Suc (equilibration)		22%	11%
		40% EG +18% Ficoll + 0.5 M Suc			
Zhou et al. (2005)	1-cell	10% EG + 10% Me_2SO (equilibration)		53-67%	-
	2-cell	15% EG + 15% Me_2SO + 30% Ficoll + 0.5 M Suc		63-69%	-
	4-cell			82-86%	-
	8-cell			90-93%	-
	Morula			97-100%	33%
	E.blastocyst (OPS)			89-100%	40%
Hredzàk et al. (2006)	8-cell	EG	0.25 ml straws	8%	-
			pipetting tip	38%	-
			micro-drops	60%	-
Lee et al. (2007)	4-cell	1.5 M EG (equilibration)	OPS	84%	-
	(OPS) +	5.5 M EG + 1.0 M Suc	EM grid	81%	-
	(EM Grid)		OPS: In liquid nitrogen	78%	-
			In slush nitrogen	82%	-
Yang et al. (2007)	Morula	30% EG + 30% Ficoll + 0.5 M Suc			
		(1-step in straw dilution for 3, 5, 8, 12, and 16 min)		64-76%	59%
		(in straw dilution for 20 min)		52%	(5min)
Yang Qe et al. (2007)	Blastocyst (OPS)	10% EG + 10% Me_2SO (equilibration)			
		15% EG + 15% Me_2SO + 30% Ficoll + 0.5 M Suc Or		81%	
		20% EG + 20% Me_2SO + 30% Ficoll + 0.5 M Suc		71%	
			(Stepwise in-OPS dilution)		42%
		10% EG (equilibration)			
		30% EG + 30%Ficoll + 0.5 M Suc Or		55%	
		40% EG + 30%Ficoll + 0.5 M Suc		79%	

Reference	Embryo stage	Cryoprotectants		Development	
				In vitro	In vivo
Zhou et al. (2007)	H.blastocyst (OPS)	10% EG (equilibration) 30% EG + 30% Ficoll + 0.5 M Suc		88%	-
	H.blastocyst (OPS)	10% EG + 10% Me_2SO (equilibration) 30% EG + 30% Ficoll + 0.5 M Suc		99%	36-42%
El-Gayar et al. (2008)	Blastocyst (OPS)	10% EG + 10% Me_2SO (equilibration)	3-step dilution	83%	42%
		20% EG + 20% Me_2SO	1-step dilution in 0.66 M Suc.	76%	-
			1-step dilution in 0.33 M Suc.	73%	-
			1-step dilution in 0.0 M Suc.	79%	40%
Jin et al. (2008)	Morula	30% EG + 30% Ficoll + 0.5 M Suc		93%	-
		40% EG + 30% Ficoll + 0.5 M Suc		99%	-
Liu et al. (2008)	8-cell (Vit-Master)	10% VS			
		50% VS			
		100% VS: 38% EG + 0.5 M Suc		48%	-
		38% Gly + 0.5 M Suc		27%	-
		38% Me_2SO + 0.5 M Suc		29%	-
		38% propylene glycol + 0.5 M Suc		28%	-
Yan et al. (2008)	1-cell	-		33%	9%
	2-cell (OPS)			61-70%	50%
Graves-Herring et al. (2009)	2-cell	7.5% EG + 7.5% Me_2SO (equilibration)		52%	31%
		15% EG + 15% Me_2SO			

BSA = Bovine serum albumin
E.blastocyst = Early blastocyst
EG = Ethylene glycol
EM grid = Electron microscope grid
Ex.blastocyst = Expanded blastocyst
Gly = Glycerol
GMP = Glass micropipette
Me_2SO = Dimethylsulfoxide
OPS = Open pulled straw
Suc = Sucrose

2.3 Materials and Methods:

2.3.1 Animals

Embryos were obtained from NMRI mice housed under standard rearing conditions (temperature 22 ± 2 ° C; relative humidity 55 ± 5%) with a photoperiod of 12 h dark and 12 h light and fed a commercial pelleted maintenance diet (ssniff M-Z, 10 mm, Soest, Germany).

2.3.2 Embryo donors

Virgin 6-8 week old females were superovulated by intraperitoneal injection of 7.5 IU eCG (Intergonan®, Intervet, Unterschleissheim, Germany) followed, 48 h later, by 5.0 IU hCG (Chorulon®, Intervet, Boxmeer, Holland). Immediately after hCG administration, females were individually caged overnight with a fertile male. Females that had a vaginal plug on the next morning served as embryo donors.

2.3.3 Collection of embryos

Ninety six hours after the hCG injection, donors were sacrificed by cervical dislocation. The uteri were removed, washed 3 times in M2 medium at 37º C (Hogan et al., 1986) and flushed toward the cervix with 0.8-1.0mL of M2 medium. Recovered embryos were washed 3 times (5 min each) in fresh M2 medium and inspected under the microscope at X40. Morphologically intact blastocysts, pooled from 3-5 donors, were transferred to culture dishes (35×10mm, Greiner, Frickenhausen, Germany) containing 3.0 ml fresh M2 medium. All operations were conducted at a constant 37°C. The culture dishes were kept in an incubator (Type 85, Melag, Berlin, Germany) to be cultured at 37ºC under a moisture-saturated atmosphere of air.

2.3.4 Vitrification of embryos

The embryos were vitrified within 1-2 h after flushing by the open pulled straw (OPS) method of Vajta et al. (1998) as modified by El Gayar and Holtz (2001). Briefly, 0.25 ml semen straws (Minitüb, Germany) with an external diameter of 1.7mm were drawn out over a hot

plate at 200° C to reach half the original diameter and wall thickness at the thinnest point. By cutting the straws at the thinnest point, two OPS-straws were derived. The holding medium consisted of Medium 199 (PAA Laboratories, Pasching, Germany) supplemented with 0.022g/100mL pyruvic acid (Sigma P 5280, Steinheim, Germany), 0.0146g/100mL L-glutamin (Sigma G 5763, Steinheim, Germany) and 20% heat-inactivated goat serum and was hepes-buffered. Two or 3 embryos at a time were washed twice in holding medium for 5 min at a time before being transferred to vitrification solution no. 1, which consists of holding medium supplemented with 10% ethylene glycol (EG) (Sigma E9129, Steinheim, Germany) and 10% dimethyl sulfoxide (Me_2SO) (Sigma D2650, Steinheim, Germany). After 1 min embryos suspended in 1-2 µL medium were transferred to a 20 µL droplet of vitrification medium no. 2 consisting of holding medium with 20% EG and 20% Me_2SO. To examine the effect of sucrose in the vitrification medium, the medium no. 2 was supplemented with 0.0, 0.4 or 0.8 M sucrose (Sigma S 9378, Steinheim, Germany). Within less than 30 sec the embryos were aspirated into the thin end of the drawn-out straw by capillary force, and the straw was immediately plunged into liquid nitrogen thin-end-first.

2.3.5 Warming of embryos

After the storage period the straws were removed from liquid nitrogen and the thin end was immediately dipped into holding medium at room temperature, while the wide end was occluded with the tip of a finger. As the medium in the straw liquefied and the air expanded, the embryos slid out into the holding medium. To examine the effect of sucrose in the dilution medium, the holding medium was supplemented with 0.00, 0.25 or 0.50 M sucrose. After 3 min in that medium the embryos were transferred to holding medium devoid of sucrose and, after another 40 sec, passaged, for 1 min at a time, through two sequential dishes of potassium simplex optimized medium (KSOM, GM 501, Gynamed, Germany) at 37°C.

2.3.6 In vitro culturing

Eventually the embryos were cultured in KSOM drops under oil. The culture dishes (85×15mm, Greiner, Frickenhausen, Germany) were prepared ahead of time by placing three 50 µL-drops of KSOM on their bottom and cover these with a thin layer of embryo culture-tested light paraffin oil (Sigma M 8410, Steinheim, Germany). The dishes were kept in an incubator (C60, Labotect, Göttingen, Germany) at 37° C under a humidified atmosphere of

5% CO_2, 5% O_2 and 90% N_2 for 24 h. The paraffin oil had been preconditioned by mixing it thoroughly with an equal volume of KSOM and keeping it in an incubator for 24 h under the above mentioned culture conditions.

Every time embryos were to be thawed, 3 dishes were prepared. The drops in dish 1 received embryos that had been warmed in medium devoid of sucrose, those in dish 2 in medium containing 0.25 and those in dish 3 in medium containing 0.50 M sucrose. The three drops within a dish received embryos vitrified in media containing 0.0, 0.4 and 0.8 M sucrose, respectively. Between 5 and 6 embryos (pooled from 2-3 straws) were placed in each drop. The experimental design was thus a 3x3 factorial, the factors being "sucrose concentration in vitrification medium" and "sucrose concentration in dilution medium". The experiment was replicated six times. The proportion of embryos reaching the expanded hatched blastocyst stage, respectively, was assessed under a phase microscope (SZ 11, Olympus, Hamburg) at X 50-100 after 24 and 48 h of culture, respectively.

2.3.7 Statistical analysis

The data were analyzed with Proc GLM of SAS considering the concentrations of sucrose in the vitrification and thawing media as well as the interaction between the two as factors. Post-hoc comparisons were performed with t- test when there was a significant main effect or interaction ($P<0.01$).

2.4 Results:

The in vitro expansion and hatching rates recorded subsequent to vitrification (Table 7) indicated that sucrose as a component of the post-thaw dilution medium was ineffective ($P>0.05$). To the contrary, differences in sucrose content of vitrification media proved to be effective: at 0.4 M sucrose, expansion rates (84-87%) were higher than at 0.0 (76-82%) or 0.8 M (40-54%). Whereas the difference between 0.0 and 0.4 M was non-significant ($P>0.05$), that to 0.8 M was statistically significant ($P<0.01$). The hatching rates followed a similar tendency, although only about half the embryos that had reached the expanded blastocyst stage continued to hatching: 45 and 47%, respectively, in vitrification media containing 0.0 and 0.8 M sucrose, respectively as compared to almost 60% if the sucrose concentration was 0.4 M ($P<0.01$).

Table 7 In vitro expansion and hatching rates, of OPS vitrified mouse blastocysts exposed to various concentrations of sucrose before (vitrification medium) and after (dilution medium) vitrification, recorded 24 and 48 h after warming, respectively.

Sucrose concentration in vitrification medium (M)	Sucrose concentration in dilution medium (M)							
	0.0		0.25		0.50		Total	
	Exp	Hatch	Exp	Hatch	Exp	Hatch	Exp	Hatch
0.0	81^{a}	37^{A}	76^{a}	29^{A}	82^{a}	42^{A}	80	36
0.4	87^{a}	50^{B}	85^{a}	44^{B}	84^{a}	57^{B}	85	50
0.8	41^{b}	22^{C}	54^{b}	19^{C}	40^{b}	23^{C}	45	21
Total	70	36	72	31	69	41	70	36

Within columns values with different superscripts differ ($P<0.01$, t-test).

2.5 Discussion:

The finding that the addition of a disaccharide to the vitrification medium favors post-thaw vitality of vitrified mouse embryos is in agreement with the observations of Kasai et al. (1990) and Kuleshova et al. (2001). Presumably sucrose is instrumental in alleviating the toxicity of penetrating cryoprotectants by extracting intracellular water and, as a consequence, limiting the amount of hydrophilic substances from entering the cells (Kasai et al., 1990). Apparently a concentration of 0.4 M sucrose in vitrification medium was capable of accomplishing this objective, whereas sucrose at a concentration of 0.8 M was excessive and, therefore, deleterious to cells. Kuleshova et al. (1999) showed that mouse oocytes placed into medium containing 1.0 M sucrose were irreversibly damaged.

Generally disaccharides, mostly sucrose, are incorporated in post-thaw media (Kuleshova et al., 1999), the objective being to permit penetrating cryoprotectants to diffuse out of the cells without water rushing in and straining cell membranes (Massip et al., 1987). This can occur gradually by decreasing concentrations (Vajta et al., 1998; El-Gayar and Holtz, 2001) or in a single step (Kasai et al., 2002). Moreover, non-permeating carbohydrates are assumed to exert a protective action on the structural and functional integrity of cells (Crowe et al., 1984).

One-step warming (dilution) has been successfully applied in vitrified bovine (Saha et al., 1996), caprine (Guignot et al., 2006) and murine (Kasai et al., 2002) embryos. The lack of an effect of sucrose in warming media on expansion and hatching of embryos, as recorded in the present investigation, agrees with recent findings by El-Gayar et al. (2008). These authors did, however, attain better hatching rates than in this experiment. This might be due to the difference in culture media used for in vitro culture. KSOM, the medium used for in vitro culture in this experiment, contains comparatively low concentrations of NaCl, KCl, KH_2PO_4, lactate and glucose (Lawitts and Biggers, 1993). The medium was developed for allowing zygotes of outbred mouse strains to overcome the two-cell culture block (Erbach et al., 1994). El-Gayar et al. (2008) cultured in M16 culture medium supplemented with BSA.

It has been shown by Paria and Dey (1990) and Lane and Gardner (1992) that mouse embryos, cultured in small volumes of medium and/or in groups had more blastomeres, developed better and, most importantly, displayed superior post-transfer viability. In mouse

embryos cultured in the absence of exogenous protein, development to the hatched blastocyst stage was observed when the density was 10 embryos10/µL of medium but not so if it was 1 embryo/10µL (Quinn et al., 1993). Autocrine and/or paracrine factors are believed to be responsible and are thought to be diluted out in large volumes. Our density of 5-6 embryos per 50 µL drop might have been too low.

It may be concluded that incorporation of sucrose into vitrification medium at a concentration of 0.4 M will enhance post-thaw development of OPS-vitrified mouse blastocysts. At a concentration as high as 1.0 M, however, sucrose has a detrimental effect. In the medium the embryos are suspended in after warming, sucrose is not required. This opens up the possibility to transfer vitrified embryos to recipients directly after warming without the necessity of a microscope or other laboratory equipment.

SECTION II

Fixed-time deep uterine insemination in synchronized goats after ovulation induction with GnRH or hCG

3 Fixed-time deep uterine insemination in synchronized goats after ovulation induction with GnRH or hCG

3.1 Introduction:

No reproduction technology has contributed more towards the genetic improvement of farm animals than artificial insemination (AI), and in certain regions AI in goats has become an established biotechnology; e.g. in France about 10% of the goat population is artificially inseminated. As a rule, goats to be inseminated are estrus synchronized, most commonly by means of progestogen-impregnated vaginal pessaries (sponges or CIDR), combined by an injection of equine Chorionic Gonadotropin (eCG) (Corteel et al., 1988; Leboeuf et al., 2003; Holtz, 2005). Repeated use of eCG may result in antibody formation (Roy et al., 1999; Drion et al., 2001), leading to delays in estrus, preovulatory LH surge and ovulation. Under those conditions fixed-time AI protocols will not yield the desired result unless they are modified accordingly (Baldassarre and Karatzas, 2004).

In cattle, injection of a Gonadotropin Releasing Hormone (GnRH)-agonist followed, 7 days later by prostaglandin F2α ($PGF_{2\alpha}$), has proven to be effective in synchronizing estrus (Thatcher et al., 1989; Twagiramungu et al., 1992; Wolfenson et al., 1994). The intention of this treatment is to bring about corpus luteum (CL) regression and control of follicular waves. Ovulation may be controlled by following the prostaglandin treatment up, 48 h later, with another injection of GnRH. This protocol, originally established by Pursley et al. (1995), became to be known as "Ovsynch" and has become an accepted way of administering fixed-time AI in cattle. Cows are usually inseminated 16-20 h after the ovulation inducing GnRH injection (Pursley et al., 1995; Yamada, 2005).

Recently a corresponding protocol was compared to the conventional sponge-eCG treatment in goats (Holtz et al., 2008). On the whole the results were encouraging; the only disadvantage being that in 29% of the cases premature return to estrus was encountered. The objective of the present experiment, therefore was to replace GnRH as the ovulation inducing agent by human Chorionic Gonadotropin (hCG), conjecturing that the long half-life of hCG

(De Rensis and Peters, 1999) will provide a more sustained stimulation of the CLs thus rendering them less prone to premature regression.

As part of this study the insemination technique described by Sohnrey and Holtz (2005) was in some animals slightly modified with the intention to reduce strain to animals and operators.

3.2 Review of Literature:

3.2.1 Estrous cycle in goats:

Estrus is defined as the time at which the female has receptivity to the male and stands for mating, and the period of time between two periods of estrus is defined as the estrous cycle (Bearden and Fuquay, 1997). The length of the estrous cycle in goats ranges from 19 to 21 days in most of the breeds (Shelton, 1978). Whereas breeds such as the Venezuelan tropical goat have an estrous cycle within the range stated above (21 d, Gonzalez and Bury, 1982) or the Boer goat (20.7 d, Greyling, 2000) there are breeds having an extreme duration such as the pygmy goats (24 d, Jarosz et al., 1971). Behavioral duration of estrus is also influenced by the breed, ranging between 22 h of sexual receptivity in Angora goats (Van Rensburg, 1971) and 96 h in pygmy goats (Jarosz et al., 1971). The most commonly stated estrus length is 36 to 37 h (Shelton, 1978; Greyling, 2000). When the follicular dynamics were studied in goats, previous reports revealed that the goats have one to four ovarian follicular waves during the estrous cycle and four waves case seems to be the most common (Ginther and Kot, 1994; Castro et al., 1999). Only the first and the fourth waves have evident follicular dominance and follicles from the first wave and the ovulatory (fourth) wave reach larger diameter and have greater tenacity compared to the second and third waves (Ginther and Kot, 1994; Castro et al., 1999). A follicular wave was defined by sequential days of entry of follicles ≥6 mm into the wave, and the emergence day was defined as the first day that the ≥6 mm follicles were 3 mm (Ginther and Kot, 1994). Ovarian follicular dynamics could be involved in affecting the interval of time between the end of estrus synchronization treatment (removal of sponge) and onset of estrus (Fonseca et al., 2005). Ginther and Kot (1994) reported that the fourth wave emerged, on average, on day 13 of the cycle and the inter-wave intervals (days) were longer for waves 1 and 4 compared to those for waves 2 and 3. The time of ovulation in goats was reported differently in different sources. It was reported to occur generally a few hours after the termination of standing estrus (Shelton, 1978) or between 32 and 48 h after the onset of estrus (Rao and Bhattacharyya 1980). A value of 38 h after the onset of estrus (Greyling and van Niekerk, 1990) or even more precisely 36.8 h (Greyling, 2000) was reported in Boer goat does. Although a wide range between 24 to 103 h has been reported (Riera, 1982). The Preovulatory luteinizing hormone (LH) peak, which is an indicator of ovulation, was found to occur 8 h (Greyling, 2000) to 11.6 h (Greyling and van Niekerk, 1990) after the onset of

estrus. The corpus luteum was found to reach its maximum diameter between days 8 and 14 of the cycle (Castro et al., 1999). When the follicular dynamics of goats were monitored by ultrasonography, it has been noticed that the early lysis of the CL, within the first 5 to 6 days of the estrous cycle, created a tight and synchronized period of ovulations with a shorter interval between lysis and ovulation when compared with the lysis occurring at a more advanced luteal stage (Days 11 to 16; Gonzalez-Bulnes et al., 2005). Progesterone concentrations begin to rise 2 d after estrus to reach the peak on day 12 to 15 of the cycle (Braun et al., 1988). Progesterone level variability is related to breed (Fonseca and Torres, 2005) and number of corpora lutea (Jarrell and Dziuk, 1991; Appavu and Holtz, 1992; Boscos et al., 2003).

3.2.2 Hormonal regulation of the estrous cycle:

The estrous cycle in the doe, similar to other mammals, is controlled through the endocrine system. Most of the hormonal regulation of reproductive processes comprises the hypothalamic-anterior pituitary-gonadal axis (
Table 8). Releasing hormones from the hypothalamus (GnRH) control the function of the anterior pituitary. Gonadotropic hormones from the anterior pituitary (FSH and LH) control the functions of the ovary involving the production of ova and hormones. In turn, through feed back mechanism involving the hypothalamus, the steroid and protein hormones of the ovary regulate the release of the gonadotropins. The gonadal steroids (estradiol and progesterone) maintain optimum conditions for reproduction through their effects on mating behavior and the female reproductive tract. Prostaglandin $F_{2\alpha}$, produced in the uterus, is an important determinant for continuation of pregnancy or beginning of a new estrous cycle in the doe, as it is responsible for CL regression. Other hormones produced by the placenta in some species (hCG in humans) and (eCG in mares) have gonadotropin-like effects and can be applied in biotechnological control of reproduction in goats. All hormones regulating the reproductive cycle in females are summarized in
Table 8; their interactions are summarized in Figure 2

Table 8 Hormones that regulate the reproductive cycle in the female (Bearden and Fuquay, 1997)

Gland	Hormone	Chemical class	Principal function
Hypothalamus	Gonadotropin- releasing hormone (GnRH)	Peptide	1) FSH and LH release
Anterior pituitary	Follicle stimulating hormone (FSH)	Protein	1) Follicle growth 2) Estrogen release
	Luteinizing hormone (LH)	Protein	1) Ovulation 2) Corpus luteum (CL) formation and function
Ovary	Estrogens (Estradiol)	Steroid	1) Mating behavior 2) Secondary sex characteristics 3) Maintenance of female duct system 4) Mammary growth
	Progestins (Progesterone)	Steroid	1) Maintenance of pregnancy 2) Mammary growth
	Inhibin	Protein	1) prevents FSH release
Placenta	Human chorionic gonadotropin (hCG)	Protein	1) LH-like
	Equine chorionic gonadotropin (eCG)	Protein	1) FSH-like (Supplementary CL in mare
Uterus	Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)	Lipid	1) CL regression 2) Parturition

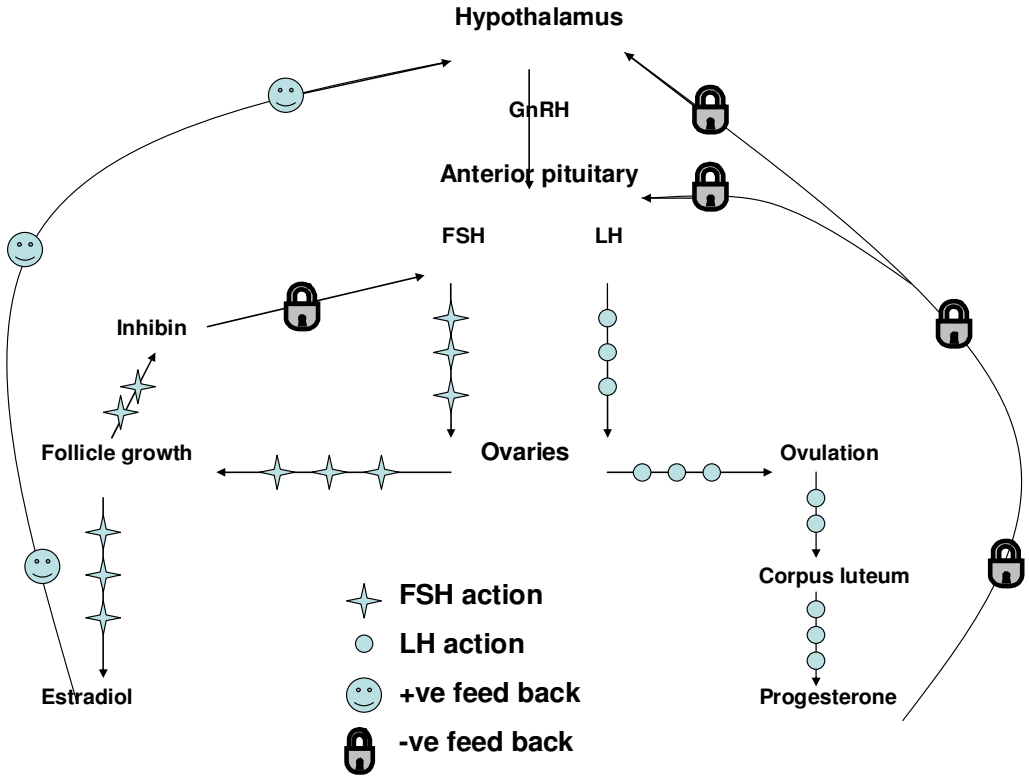

Figure 2 Hormonal regulation of the reproductive cycle in females.

The interactions are summarized as follows:

a. GnRH from the hypothalamus stimulates the release of FSH and LH from the anterior pituitary.
b. FSH stimulates production of estradiol and inhibin by granulosa cells in the ovarian follicle.
c. Inhibin selectively inhibits release of FSH and exerts paracrine effect on other follicles.
d. When progesterone is low, high concentrations of estradiol stimulate a greater surge of GnRH, FSH, and LH by positive feedback control.
e. LH surge stimulates ovulation of dominant follicles.
f. LH stimulates production and release of progesterone by granulosa cells in the corpus luteum.
g. High concentrations of progesterone inhibit the release of GnRH, FSH, and LH by negative feedback control.

3.2.3 Estrus synchronization:

Estrus synchronization in farm animals focuses on manipulating either the luteal or the follicular phase of the estrous cycle. In does, the opportunity for controlling the estrous cycle is greater during the luteal phase, which has a longer duration and is more responsive to manipulation (Wildeus, 2000). Strategies can be employed to extend the luteal phase by supplying exogenous progesterone (Corteel et al., 1988) or to shorten this phase by prematurely regressing existing corpora lutea (CL) (Nuti et al., 1992). Other synchronization strategies like artificial light treatment (with- or without melatonin administration; Wuliji et al., 2003) or utilizing the male effect by exposing the does to males after a period of isolation (Fletcher et al., 2002) or an ovsynch protocol (Holtz et al., 2008) were found to be successful synchronization strategies in goat. Successful synchronization protocols must not only establish tight synchrony, but are also expected to provide acceptable levels of fertility upon natural mating or artificial insemination (Whitley and Jackson 2004).

3.2.3.1 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-based synchronization:

Prostaglandin $F_{2\alpha}$-based estrus synchronization depends on termination of the luteal phase through regression of the CL to control the estrous cycle. This system is restricted to breeding season and is applicable to cycling females in which the presence of functional CL is ensured. The two commonly used products in $PGF_{2\alpha}$-based systems are $PGF_{2\alpha}$ (Lutalyse® or Dinolytic®; Pfizer) and the prostaglandin analog cloprostenol (Estrumate®; Bayer, Shawnee Mission, KS; Wildeus, 2000). A single injection of a dose of Prostaglandin $F_{2\alpha}$ or one of its analogs, injected in the presence of a functional CL, is effective in inducing luteolysis (Greyling and Van Niekerk, 1986; Corteel and Leboeuf, 1990). This regimen is appropriate to be applied in cycling animals between the 4^{th} and 16^{th} day of the estrous cycle (Holtz, 2005). This fact was confirmed by Nuti et al. (1992) when dairy goats injected with cloprostenol (125 µg) on d 6 or 12 of the estrous cycle, showed no difference in estrous response and timing of estrus and LH surge. To be sure that all the does in the flock have a functional CL and are ready to respond, two $PGF_{2\alpha}$ injections administered 10 or 11 days apart are advocated (Nandy et al., 1990; Romano, 1998). In Boer goats the double $PGF_{2\alpha}$ injection system, proved to lack efficacy during the anestrous and transitional seasons (Greyling and Van Niekerk, 1991). Out of season and even during the periods of the season, in which the

does have no functional corpora lutea, prostaglandin-based treatments are of no avail (Holtz, 2005).

3.2.3.2 Progestogen based synchronization:

Variable forms of progestogens accompanied also with different administration methods have been employed to extend the lifespan of the corpus luteum for estrus synchronization in goats. Estrus synchronization or even induction using these protocols have been used in cycling does, as well as in seasonally anestrus does (Amoah and Gelaye 1997; Wildeus, 2000; Whitley and Jackson 2004; Holtz, 2005). The most common administration route in this synchronization strategy is the progestagen-containing vaginal pessaries. These pessaries are polyurethane sponges impregnated with fluorogestone acetate (FGA) or medroxyprogesterone acetate (MAP) or they are Y-shaped silicon-coated devices ("controlled internal drug release", CIDR) impregnated with progesterone (Holtz, 2005). Implants impregnated with the highly potent synthetic progestagen norgestomet may be inserted under the skin of the upper side of the ear (Bretzlaff and Madrid, 1985; Holtz and Sohnrey, 1992; Yuswiati and Holtz, 1996; Mellado et al., 2000; Medan et al., 2003) or on the under side of the tail (East and Rowe, 1989). The subcutaneous implants devised to be used in bovines ("Synchromate B" or "Crestar" Intervet) were cut in half to be used in goats. These manipulated implants proved to be as efficient as the sponges in synchronizing estrus in goats (Holtz and Sohnrey, 1992).

The traditional protocol used for synchronization of estrus in goats for the purpose of performing timed AI, was developed more that 20 years ago and is still recommended by many breeders and research groups. This traditional protocol consists mainly of an extended period (11-17 days) of exposure to progestagen (progesterone or a synthetic analog) in association with an injection of eCG, administered i.m. at the end of the treatment to induce ovulation (Corteel et al., 1988). The administration of eCG was found to be necessary for stimulating a satisfactory ovulatory response in lactating and non-lactating Angora goats, and increasing the dose of eCG, increased the ovulation rate in non-lactating goats and advanced the time of ovulation in both lactating and non-lactating does (Ritar et al., 1984). Induction of estrus during the anestrous season was successfully achieved in colored mohair goats by applying multiple injections of eCG (total of 950 IU) without any progestagen pretreatment (Karaca et al., 2008). Fertility rates exceeding 60% were achieved when the traditional FGA vaginal sponge-eCG protocol was used in combination with single cervical AI using frozen-thawed semen at a predetermined time after the end of treatment (Corteel et al., 1988). FGA

sponges, combined with eCG for estrus synchronization in Damascus goats, resulted in a high kidding rate of 80%, compared to 52% resulting from synchronizing of estrus with two $PGF_{2\alpha}$ injections 11 days apart (Al-Merestani et al., 2003). On the other hand, poor fertility rates were achieved with the sponge-eCG protocol due to a delay in the onset of estrus (Baril et al., 1993). This delay is believed to be associated with the presence of anti-eCG antibodies subsequent to repeated treatment with this agent (Baril et al., 1996). However, animals treated with the same protocol for the first time, tended to show a large variation in the time of onset of estrus (Baril et al., 1993). The synchronization of estrus in goats with a norgestomet implant or half-implant did not reduce the variability in estrus occurrence and preovulatory LH peak, when compared with the results attained when the FGA vaginal sponges were used (Freitas et al., 1997a). These facts suggest that factors other than eCG antibodies could play a rule in affecting the response of goats to an estrus synchronization treatment. When AI was conducted at observed estrus occurrence, kidding rate tended to be lower in animals synchronized with a whole norgestomet implant compared with those synchronized with vaginal FGA sponges and significantly lower in the goats that received a halved ear-implant (Freitas et al., 1997a). Trying to increase the progestagen level at the end of a synchronization treatment by insertion of an additional vaginal sponge 2 or 4 days before the end of the treatment, was found not to be efficient in reducing variability among animals in the onset of estrus. The kidding rate of the goats that received an extra sponge significantly decreased (Freitas et al., 1996). The use of intravaginal hormone-releasing devices could cause some problems related to the vaginal irritation such as vaginitis, and could create a kind of negative implication regarding the legal limits of progestogen residues allowed in milk of dairy goats (López-Sebastian et al., 2007). Washing the vagina of the goat with a physiological solution after sponge withdrawal, increased the kidding rate, especially those resulting from the first mating after synchronization (Al-Merestani et al., 2003). These results are supported by a report suggesting that the vaginal sponge creates an unsuitable medium for fertilization in the first stimulated estrus resulting after the synchronization (Cognie and Mauleon, 1983). The goats tend to have variability in the occurrence of estrus after removal of progestagen, which leads to decreased fertility when the goats are inseminated at a predetermined time after progesterone withdrawal (Freitas et al., 1997b). The timing of estrus and LH surge were found to be less variable among goats synchronized by a progestagen treatment when compared with goats having natural estrous cycle, which suggests that physiological limits of the goat could restrict the possible improvements in the estrous synchronization based on progestagen (Freitas et al., 1997b).

3.2.3.3 Short term protocol:

A practical estrus synchronization protocol, called Short-Term Protocol, was developed for small ruminants to avoid the traditional trend of long time exposure to progestagen (Menchaca and Rubianes, 2004). This protocol involves a short period of exposure to progestin (i.e. 5-7 days) associated with a $PGF_{2\alpha}$ injection applied at the beginning of the treatment and a small dose of eCG (200-350 IU) at the end of the progestin phase. Pregnancy rates associated with this short-term protocol in goats jumped up to 74 % when a single AI was performed after detection of estrus, or up to 75 % when a fixed-time AI was applied 54 h after the end of the treatment (Rubianes et al., 2001).

When estradiol benzoate (EB) was administered 24 h after withdrawal of the device LH surge and ovulation were synchronized in a similar way as in the eCG case (Menchaca et al., 2007). But a higher pregnancy rate was achieved when eCG was used in the short-term protocol to induce ovulation, compared to that achieved with EB (Menchca and Rubianes 2007). In the short-term protocol, the LH peak is induced 40 h after the end of progesterone treatment, while ovulation comes to pass 20 h later (60 h after the end of progesterone treatment, (Menchaca et al., 2007). The reduction in duration of progestagen treatment for 6 or 9 days was found to be practicable, as it causes no reduction in estrous response with acceptable fertility and kidding rate in the 9-day protocol, but not for the 6-day protocol (Fonseca et al., 2005). A pregnancy rate of 64% was achieved when the goats were synchronized for timed AI using the short-term protocol and inseminated with liquid semen 54 h after the end of the progesterone treatment. While the rate diminished to 49% when insemination was performed 48 h after the end of the treatment (Menchaca and Rubianes 2007).

3.2.3.4 Male effect:

In "photoperiodic rigid breeds", manipulating the sexual activity season is possible by applying alterations in the photoperiod only (Delgadillo et al., 2004). Artificial long days, whether or not accompanied with melatonin administration, were successfully applied to stimulate the sexual activity of goat males out of season. These bucks were able to induce the sexual activity of anestrous females through the male effect (Delgadillo et al., 2004). Exposing seasonally anovulatory females to a male after an isolation period can stimulate ovulation in goat herds (Shelton, 1960). This strategy, known as the male or buck effect was found to be successful in initiating short ovarian cycles (5 to 7 days) in some female goats. In

this case, estrus and ovulation occur about 7 to 9 days after the induction by the buck effect, and the CL formed after ovulation is usually with a normal life-span (Chemineau, 1983). So, a new postovulatory wave at the beginning of the cycle, simultaneously with the presence of a large growing follicle accompanied with low progesterone levels, seems to be the most suitable condition to promote estrus synchronization and its early occurrence after treatment.

3.2.3.5. (IMA.PRO2®):

IMA.PRO2® is a new protocol that was designed to induce and synchronize ovulation in goats during the non-breeding season, allowing for timed AI without any need for prior estrus detection, and depends on "male effect" to induce ovulation and a single 25 mg dose of progesterone administered at the time of buck exposure, with the early lysis of the induced CL by administering 75µg of cloprostenol 9 days later (López-Sebastian et al., 2007). This protocol resulted in a high degree of estrus and ovulation synchronization and a high fertility after performing a single timed AI. The method has additional benefits of reducing costs (treatment and animal handling costs) parallel with lowering the detrimental effects on animal health (reduced vaginal irritations and related diseases) (López-Sebastian et al., 2007).

Induction of estrous cycle and ovulation in goats, performed by the male-effect and progesterone, does not warrant sufficiently accurate timing to allow for performing a successful timed AI. In order to create a suitable synchronization for this purpose, a single dose of cloprostenol ($PGF_{2\alpha}$) has been administered (López-Sebastian et al., 2007). Administration of a luteolytic agent during the early luteal phase (as in **IMA.PRO2®**) results in a tighter synchronization of estrus (Gonzalez-Bulnes et al., 2005). This is a critical point when timed AI is practiced, since low fertility of goats involved in these programs might be related to the variability of timing of estrus (Freitas et al., 1997b). In other words, it could be supposed that the delayed ovulation of females managed under AI programs could easily decrease the fertility of the herd (López-Sebastian et al., 2007).

3.2.3.6. Ovsynch:

Injection of a gonadotropin releasing hormone (GnRH)-agonist followed, 7 d later, by a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection has proved to be effective in synchronizing estrus in cattle

(Thatcher et al., 1989; Twagiramungu et al., 1992; Wolfenson et al., 1994). This regimen brings about regression of corpora lutea and controls the wave-like growth of ovarian follicles. If the prostaglandin treatment is followed up, 48 h later, by another GnRH-injection, the time of ovulation is more precisely controlled. This protocol, originally established by Pursley et al. (1995), became to be known as "Ovsynch" and has become a useful means of applying timed AI in cows. In this system, the most suitable time for insemination is 16-20 h after the second GnRH injection (Pursley et al., 1995; Yamada, 2005). A drawback associated with the Ovsynch protocol in cattle is the occasional occurrence of premature luteal regression (Schmitt et al., 1996). This problem is much more prominent in goats (Holtz et al., 2008). To test the hypothesis that the LH surge induced by injection of a GnRH-agonist might be too short-lived to permit full-fledged corpus luteum (CL) formation, in the present experiment the GnRH-agonist was substituted by human chorionic gonadotropin (hCG). This substance, extracted from urine of pregnant women, has been shown to have LH activity. When administered in cattle, the plasma concentrations of hCG increased clearly for up to 30 h after administration and returned to the baseline by about 60 h (De Rensis and Peters, 1999).

3.2.4 Advantages of substituting the traditional protocol:

The advantages of using substitute programs for estrus synchronization other than the traditional Progestagen-eCG protocol are summarized as follows:

The first benefit is the avoidance of using progestagens. The European Union and many other countries control the usage of progestagens strictly through legislation (Maximum Residue Limits, RD 2178/2004) prohibiting the presence of residues in milk and meat (López-Sebastian et al., 2007). Intravaginal progestagens are really recognized to have negative effects on fertility, through affecting sperm transport and viability (Hawk and Conley, 1975).

The second advantage is avoiding the use of eCG, which is a hormone of animal origin, that might carry some health concerns considering its inter-species use, but formation of eCG antibodies in goats after the repetitive use of eCG is a major drawback (López-Sebastian et al., 2007; Hervé et al., 2004). The creation of these antibodies has detrimental effects on the fertility yields of the AI programs in goats as a result of the delay in estrus and/or ovulation or even the prevention of the ovulatory response (Drion e al., 2001).

3.2.5 Short estrous cycles:

Goats require a functional CL during the whole period of gestation. Irrespective the season, some goats tend to show normal or short estrous cycles. This might be related to the endogenous progesterone capacity of the goats (Lu Meng Chao et al., 2008). The proportion of cycles of abnormal length (too short or too long) was higher at the beginning and at the end of the breeding season (Rivera et al., 2003). At least one abnormally short estrous cycle is usually shown by does at the beginning of the mating season (Camp et al., 1983). The short estrous cycles are not only limited to the beginning of the breeding season, shortened cycles also have been observed in does after the postpartum period (Riera et al., 1982) or after $PGF_{2\alpha}$-induced abortions (Brettzlaf et al., 1982) and in goats superovulated with eCG (Armstrong et al., 1982; 1987). Superovulation by eCG treatment resulted in both normal and abnormal estrous cycles. Other reports of short cycles in goats were identified after eCG treatment for ovulation induction and/or estrus synchronization (Armstrong et al., 1983). The lifespan of the CL and progesterone concentrations are usually used to distinguish between the normal and short estrous cycles (Lu Meng Chao et al., 2008). Abnormal CL leads to an early luteal regression and shorter estrous cycles (Saharrea et al., 1998). An abnormal CL of less than 2 mm diameter and pale color began to regress 3 days after ovulation (Gilbert et al., 1990). In a later experiment, larger corpora lutea (about 7 mm in diameter) were detected to regress on day 1 after estrus (Lu Meng Chao et al., 2008). Functionally poor CL that produces low progesterone was believed to be the major cause of high embryo loss after estrus synchronization and mating of Korean native goats (Lu Meng Chao et al., 2008). Recovery rates (as % of ovulations) of embryos flushed from goats with regressed CL at the time of flushing were below 25% (Armstrong et al., 1987).

Superovulated nanny goats were treated with a prostaglandin synthetase-inhibitor (flunixin meglumin) to examine the involvement of $PGF_{2\alpha}$ in the early luteal regression in superovulated goats. Intermittent surges in plasma concentrations of a prostaglandin metabolite (PGFM) in the untreated goats, accompanied with evidence of premature luteal regression, suggests that the premature release of $PGF_{2\alpha}$ may drive the premature regression of the CL in superovulated does (Battye et al., 1988). The problem of premature luteal regression in superovulated goats was overcome using a prostaglandin synthetase-inhibitor (Gilbert et al., 1990), or by administering extra exogenous progesterone to replace the decrease in progesterone concentration associated with spontaneous premature luteal regression (Gilbert et al., 1990).

3.2.6 Semen and insemination:

Semen from caprine bucks may be utilized for insemination as fresh, refrigerated or frozen. Techniques of insemination in goats are classified depending on the site of semen deposition during insemination into vaginal, cervical and intrauterine (Evans and Maxwell, 1987; Chemineau and Cognié, 1991; Leboeuf et al., 2000). Employing frozen semen in AI programs facilitates the distribution and exchange of superior genetic material and allows the use of semen in and out of the breeding season. It also overcomes the problem of farm dispersion, and provides a chance for longer utilization of gametes from superior males after accurate genetic evaluation (Baldassare and Karatzas, 2004; Viudes-de-Castro et al., 2007).

Cryopreservation of semen from bucks is considered a challenge, as some workers prefer to remove the seminal plasma by centrifugation to protect the spermatozoa from the toxic effect of lysolecithin. Lysolecithin is a by-product of the reaction between an enzyme having a triglyceride lipase activity that originates in the bulbourethral gland (Pellicer-Rubio et al., 1997) and the lecithin of the egg yolk or skim milk contained in the semen diluents used (Chemineau et al., 1999). Centrifugation of semen is detrimental to the plasma membrane of the spermatozoa; possibly more so than the alleged effect of the enzyme. This fact guided some investigators to process caprine semen much in the way as bovine semen (Evans and Maxwell, 1987; Tuli and Holtz, 1994, 1995). AI performed with frozen-thawed semen gives lower success rates than with cooled semen (Viudes-de-Castro et al., 2007). It is, of course, not to be compared with that performed with fresh semen, as the latter has success rates comparable to natural service (Holtz, 2005). High kidding rates of 78% and 74% were achieved with liquid buck semen when conducting cervical and vaginal insemination, respectively, in Norwegian dairy goats (Paulenz et al., 2005). Factors such as farm and depth of semen deposition in the female reproductive tract have a direct effect on the success rates of AI with frozen-thawed semen. Pregnancy rates range from 40% to 65% (Ritar et al., 1990; Leboeuf et al., 1998; Salvador et al., 2005).

The conventional procedure of AI in goats involves lifting up of the rear quarters of the doe with its forelegs remaining on the ground. Using a duckbill speculum and a penlight, the cervical os is located and, under visual control, the insemination pipette is passed through the cervix to deposit the semen in the uterine body (Holtz, 2005). Deposition of the frozen-thawed semen during traditional AI in a deeper position toward the uterus will increase the pregnancy rate (Ritar et al., 1990; Salvador et al., 2005). But, the passage of the traditional insemination catheter into the goat uterus is sometimes difficult due to the anatomical barrier

caused by cervical rings or folds (Viudes-de-Castro et al., 2007). Passage was attained in only 17.5% of Murciano-Grandian goats inseminated using the traditional method (Salvador et al., 2005). In such a case, semen has to be deposited intracervical or even caudal to the external cervical os. When high pregnancy rates (>70%) are desired and intrauterine deposition of semen is a problem, e.g. in nulliparous, small breeds (e.g. Nigerian dwarf) or individuals, laparascopic AI can help solve the problem. With laparascopic AI higher and more consistent pregnancy rates are usually accomplished (Ritar et al., 1990; Vallet et al., 1992). This technique has the advantage of requiring only one tenth of the number of spermatozoa usually used. It is however, a procedure posing technical difficulties (Evans and Maxwell, 1987; Chemineau and Cognié, 1991). Animals have to be fixed on a special cradle in dorsal recumbancy in a head-down position at an angle of about 45°. The abdominal cavity is insufflated with air or an inert gas, and the abdominal wall is punctured in two positions using a trocar to insert the cannulae that permit the introduction of a laparascope and an insemination instrument. Using a special insemination pipette ("aspic", Cassou, IMV, L'Aigle, France) the uterine wall is punctured about 5 cm from the uterine bifuraction to deposit the semen in the uterine lumen. The operation is not really painful for the animals, but according to (Holtz, 2005) they do not exactly relish the experience.

An insemination technique described by Sohnrey and Holtz (2005), provides for deep uterine deposition of semen via transcervical route, and was found to result in a kidding rate of 71% vs. 53% in does inseminated laparascopically. This method comprises insertion of a catheter into the uterine cavity with the purpose of depositing the semen as deep as possible in the uterine horns (see Materials and Methods).

3.3 Materials and Methods:

3.3.1 Animals, housing and feeding

The experiment was conducted in the middle of the breeding season (October to January) on Boer goats from our own breeding flock at Goettingen, Germany (9º 41' E, 51º 46' N). The goats, group-housed in open barns with straw-covered floor and outdoor concrete runs, were fed a daily ration of 600 g concentrate, consisting of equal parts of a pelleted diet for breeding ewes (16% crude protein, 10.2 MJ ME/kg, supplemented with 43 mg/kg Se, 12 mg/kg I and 5000 mg/kg Zn), oats and dried sugar beet pulp and had free access to wheat or barley straw, salt lick and water. A total of 60 does, 2–7 years of age and weighing 45–68 kg, that had kidded at least once, were randomly assigned to one of three treatment groups. Estrus was detected with an aproned male at 8 h intervals (8:00, 16:00, and 24:00 h). All the animals were routinely tested for estrus with an aproned male.

3.3.2 Ovarian monitoring

Echographic inspection of the ovaries was conducted by way of a real-time scanner (ALOKA SSD 500) equipped with a transrectal 7.5 MHz linear-array transducer. To estimate the ovulation rate, the number of large follicles was recorded just before and 20 h after insemination. With this approach, ovulation rate could be closely approximated as indicated by a correlation coefficient of r = 0.82, assessed between ultrasonic and laparoscopic inspection on 50 goats by Suyadi and Holtz (unpublished data).

3.3.3 Estrus synchronization

Blood samples were drawn from the jugular vein between days 5 to 12 of the estrous cycle (day 1 = first day of standing estrus) and analyzed for progesterone content by ELISA according to Van de Wiel and Koops (1986, modified by Moeller 1991).
Treatment commenced if the serum progesterone concentration exceeded 5 ng/mol. The does of Group 1 (n=20) served as controls. They received an i.m. injection of $PGF_{2\alpha}$ (5.0 mg Dinoprost, Dinolytic®, Pfizer Pharmacia, Karlsruhe, Germany). The goats of Group 2 (n = 20) received 5.0 mg $PGF_{2\alpha}$ followed, 48 h later, by an i.m. injection of 0.004 mg of the GnRH-

analog Buserelin (1 ml Receptal®, Intervet, Unterschleissheim, Germany). The treatment of the does in Group 3 (n = 21) resembled that of Group 2, except that, instead of GnRH, they received an i.m. injection of 500 I.U. hCG (Chorulon®, Intervet, Unterschleissheim, Germany). Whereas the does of Group 1 were inseminated 12 to 14 h after the onset of standing estrus, the does of Groups 2 and 3 were inseminated at a fixed time, 16 h after the ovulation-inducing injection of GnRH, respectively hCG.

3.3.4 Estrus detection

Beginning 24 h after the $PGF_{2\alpha}$ injection, the treated does were individually tested for estrous symptoms with an aproned buck at 8 h intervals (8:00, 16:00, 24:00 h) for up to 5 min at a time. Estrous behavior, evidenced by immobility reflex or tail flagging (Holtz et al., 2008) was recorded.

3.3.5 Deep uterine insemination

The does were inseminated with pellet-frozen semen from eight different males that were allocated to treatment groups at random (200×10^6 spermatozoa in Tris-glucose-egg yolk extender containing glycerol as cryoprotectant) (Nagase et al., 1964). The procedure of deep uterine insemination has been described in Sohnrey and Holtz (2005) and all the equipments required for this insemination are shown in Figure 3. Briefly, the hind quarters of the goat were elevated, the external cervical os was located with the aid of a duckbill speculum (UA045, IMV) and penlight and grasped, from the side (Figure 4), with a 255 mm long sharp-pointed forceps ("Possi," Aesculap, Tuttlingen, Germany). After removing the speculum the animal was set back on its feet. In some of the animals the insemination procedure described by Sohnrey and Holtz (2005) was slightly modified. Instead of elevating their hind quarters to localize the cervical os, they were placed in a restraining device that prevented them from moving about or squatting during the insemination process. The device consists of a hammock with holes for the front limbs and has been described in Suyadi et al. (2000). The cervix was gently drawn toward the vulva and a catheter with 3.2 mm o.d. braced with a stainless steel stylet was introduced. As soon as the cervix had been passaged, the stylet was removed and the catheter was advanced another 10-12 cm, directing it toward one of the uterine horns with a finger located in the vaginal fornix (Figure 5). A polyethylene (PE)

tube (Portex, Hythe, Kent, U.K.), 40 cm in length with an o.d. of 1.52 mm, into which the thawed inseminate had been aspirated, was threaded all the way through the catheter. After drawing the catheter back about 2 cm so the PE tube protruded from its tip, half of the inseminate was expelled by pushing the plunger of the 1 ml-syringe attached to the other end of the PE tube. The protruding PE tube was then pulled back and the catheter was withdrawn far enough to be able to advance it into the other uterine horn where the remaining half of the inseminate was deposited in a similar fashion.

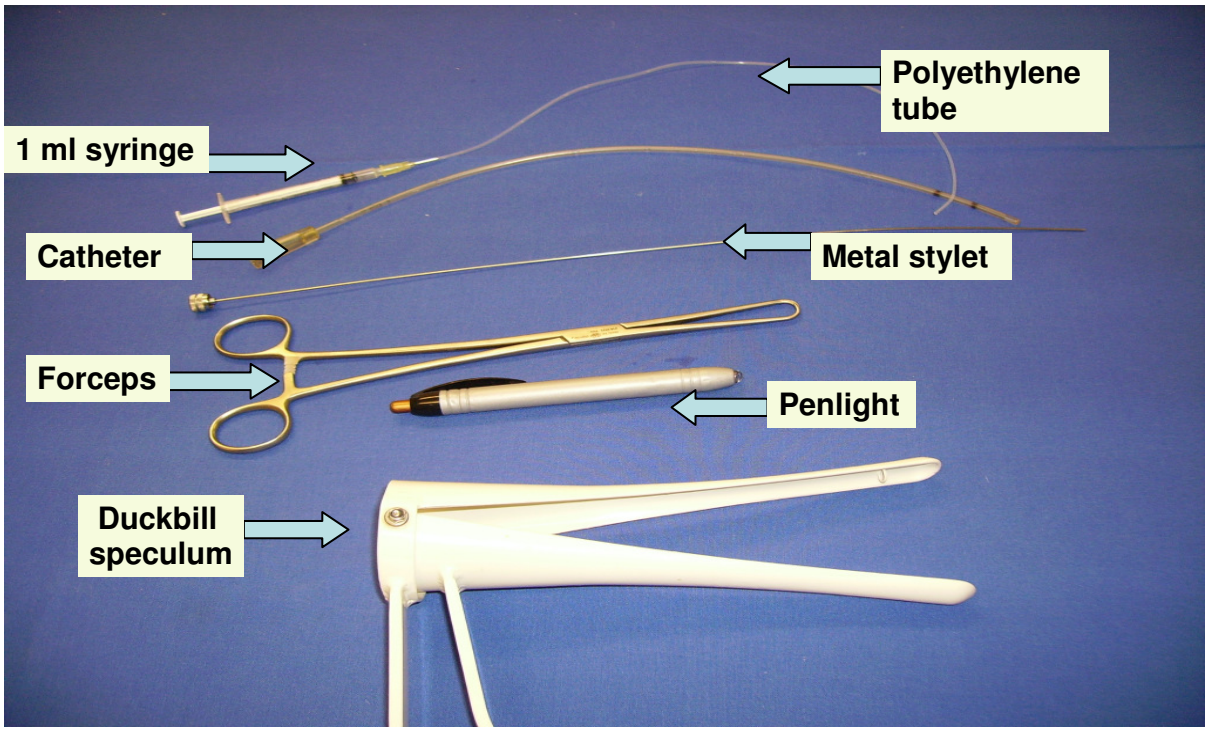

Figure 3 The equipments required for the deep uterine insemination according to Sohnrey and Holtz (2005)

3.3.6 Premature luteal regression monitoring

Blood samples were collected via jugular venipuncture on days 2, 4, 6 and 8 after insemination to monitor the progesterone profile in the blood serum during this period. After 20 to 24 h at 4° C, the blood was centrifuged at 3000 x g for 10 min and in the serum the progesterone concentration was assessed by ELISA method as described before. A concentration of >1.5 ng/ml was considered as an indication of an active CL (Nowshari and Holtz, 1993). If the progesterone concentration dropped to a value lower than 1.5 ng/ml

during the first 8 days after insemination, the goat was considered as having a premature luteal regression (short cycle).

3.3.7 Pregnancy detection

To monitor the success of insemination, serum progesterone of inseminated goats were measured on d 21 and 28 after insemination. Pregnancy diagnosis was confirmed by rectal ultrasonography (Aloka SSD 500 with 7.5 MHz linear-array transducer) as described by Padilla Rivas et al. (2005) four weeks after insemination.

3.3.8 Statistical analysis

The effect of treatment on the interval between prostaglandin injection and onset of estrus, duration of estrus and number of ovulations was evaluated by analysis of variance using the program Proc GLM of SAS. The differences among groups regarding premature CL regression and pregnancy were tested for significance by Chi square test.

Figure 4 Grasping of the external cervical os with the foreceps aided by a duckbill speculum and a penlight

Figure 5 Deep introduction of the insemination catheter into the uterus horn directed with a finger located in the vaginal fornix

3.4 Results:

As indicated in Table 9, all of the 60 goats showed signs of estrus, though 3 (15%) of the GnRH-group and 2 (10%) of the hCG-group did not posture, but exhibited tail flagging only. The time passing between $PGF_{2\alpha}$ treatment and the onset of estrus was 44.5, 46.6 and 41.6 h for control, GnRH- and hCG treated groups, respectively. The duration of estrus was, on average, 37.1 h in the GnRH-group, which was significantly shorter ($P<0.05$) than in the control and the hCG group (46.4 and 48.4 h, respectively). Ovulations were recorded in 19 of the 20 control does (95%) as compared to 17 of the 20 does in the GnRH group (85%) and 16 of the 20 does in the hCG group (80%). The number of ovulations per doe ovulating was similar for the three groups (2.5, 2.4 and 2.1, respectively, $P>0.05$).

Abnormally short cycles were recorded in a single goat of the control group (5%), in 8 of 20 does (40%) of the GnRH- and 7 of 20 does (35%) in the hCG-group, the difference between the control and the treatment groups being significant ($P<0.05$). The progesterone profiles depicted in Figure 6 indicate that the early return to estrus is a consequence of premature CL regression. The duration of the short cycles (from onset of the preceding to onset of the new estrus) was 5.5 days (range 5-6) for the GnRH group (n = 4) and 4.75 days (range 4-5) for the hCG group (n = 4). The remaining animals (one in the control-, 4 in the GnRH- and 3 in the hCG group) were not observed in estrus but were recognized to have short cycles by serum progesterone values.

Four weeks after insemination 12 of 20 does (60%) of the control group, 10 of 20 does (50%) of the GnRH group and 7 of 20 does (35%) of the hCG group were diagnosed pregnant. Of the 5 does failing to display posturing behaviour, 1 got pregnant and carried twins to term; 2 had short and 2 had normal length cycles. Of the 8 does with no ovulation recorded, 3 did get pregnant. When disregarding does with premature CL-regression, pregnancy rates for control-, GnRH- and hCG- treated groups, respectively, were 63, 83 and 54%, the differences being not statistically significant. All does that had been diagnosed pregnant, with the exception of 2 from the GnRH group that aborted, went to term, average litter size being 1.83, 1.88 and 1.71 for the control-, GnRH- and hCG-groups, respectively ($P>0.05$).

Table 9 Results of ovulation control with GnRH or hCG in synchronized does subjected to a single fixed-time insemination with frozen-thawed semen, as compared to does inseminated 12 to14 h after the onset of estrus (Control).

Parameter	Control	GnRH	hCG	P
Number of does treated	20	20	20	
Does posturing (%)	100	85	90	
Does tail flagging only (%)	0	15	10	
Onset of estrus (h)[c]				
Mean	44.5	46.6	41.6	0.45
S.E.M.	2.8	2.9	2.7	
Range	26-74	26-66	26-58	
Duration of estrus (h)				
Mean	46.4[a]	37.1[b]	48.4[a]	0.04
S.E.M.	3.3	3.3	3.2	
Range	24-72	16-56	24-80	
Does ovulating (%)	95	85	80	
Number of ovulations (mean ± S.E.M.)[d]	2.5 ± 0.22	2.4 ± 0.23	2.1 ± 0.24	0.45
Incidence of short cycles (%)	5[a]	40 [b]	35 [b]	0.03
Does pregnant (%)[e]	60 (63)	50 (83)	35 (54)	0.21
Does kidding (%)[e]	60 (63)	40 (67)	35 (54)	0.64
Litter size (mean ± S.E.M.)[f]	1.83 ± 0.17	1.88 ± 0.13	1.71 ± 0.29	0.48

[a,b] Values with different superscripts within rows differ ($P < 0.05$)
[c] After injection of $PGF_{2\alpha}$.
[d] Taking only goats that ovulated into consideration.
[e] The values in parentheses indicate results when goats with short cycles are excluded.
[f] Taking only goats that kidded into consideration.

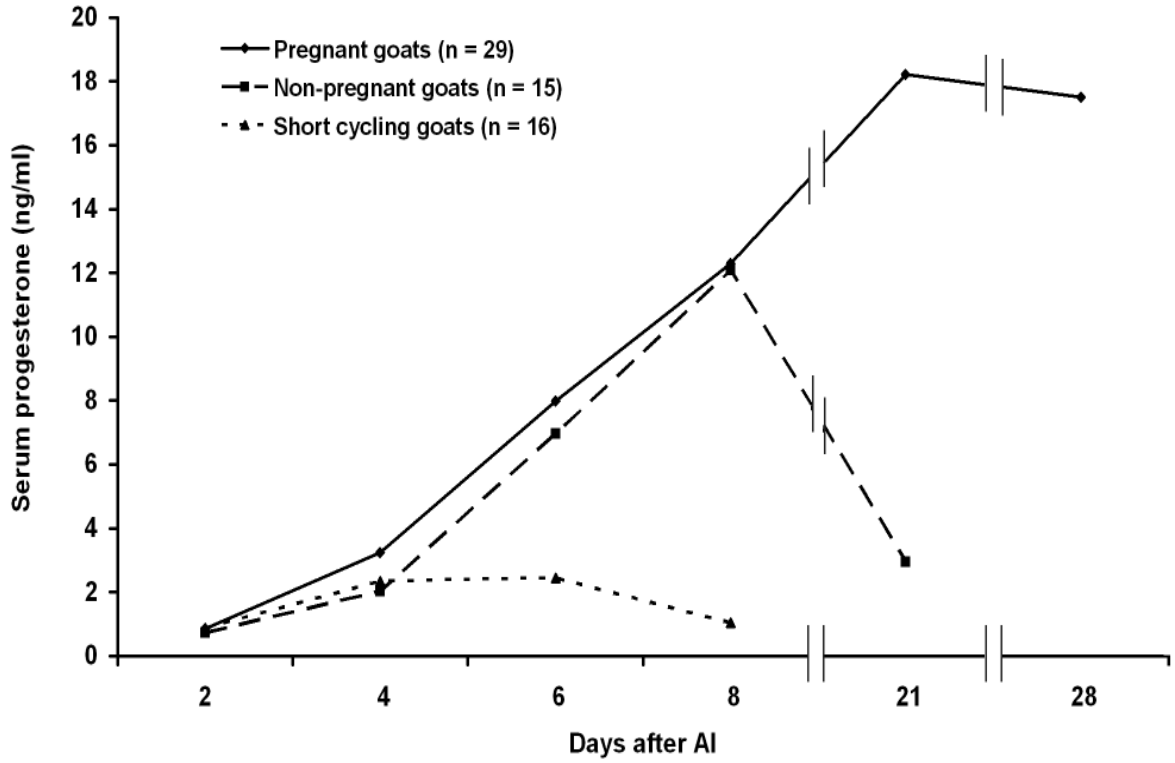

Figure 6 Progesterone profiles in pregnant goats, non pregnant goats and goats with short estrous cycles.

3.5 Discussion:

One objective of the present investigation was to verify an earlier finding (Holtz et al., 2008) indicating that estrus synchronization by means of $PGF_{2\alpha}$-GnRH treatment is a suitable means of permitting fixed-time insemination in goats. The outcome was encouraging in so far as estrus synchronization was accomplished in all does irrespective of the ovulation inducing agent. The slight superiority of GnRH- over hCG treated does might be coincidental and would require verification on a larger scale. The only significant difference between GnRH- and hCG treated groups was the shorter estrus period observed in the GnRH group. Presumably the immediate release of LH known to occur in response to GnRH-treatment (Holtz et al., 2008) induced ovulation and, as a sequel, decline in plasma estrogen concentration sooner than in the other groups. Variability in the onset and duration of estrus was unaffected by treatment.

The main objective of the investigation was to reduce the incidence of short cycles. To that end GnRH, serving as ovulation inducing agent in the ovsynch protocol, was replaced by hCG. Short cycles as a sequel to synchronization (Armstrong et al., 1983; Chemineau et al., 1986, 1999; Cognié et al., 2003) and superovulation treatment (Holtz, 2005) are commonly encountered in goats both with the conventional sponge-eCG protocol and, as indicated in this and a preceding study (Holtz et al., 2008), to an even higher extent with $PGF_{2\alpha}$-GnRH synchronized goats. In cows, short cycles were recorded in 16% (Schmitt et al., 1996) and 11% (Geary et al., 2001) of animals subjected to an ovsynch-protocol. The reason for the high frequency of animals with premature corpus luteum regression is not clear (Cognié et al., 2003). The inadequate LH surge made responsible for short cycles in cattle (Taponen et al., 2002) can, according to a recent study (Holtz et al., 2008), not serve as a reason in goats; the LH surge induced by buserelin in the course of the ovsynch treatment was not visibly different from that following the sponge-eCG treatment.

If it were not for the high incidence of premature luteal regression, pregnancy rates to fixed-time insemination in $PGF_{2\alpha}$-GnRH synchronized goats, would have been exceptionally good (83%). Whether the two abortions that happened to occur in that particular group were associated with the treatment, is open to conjecture. Spontaneous abortions with no obvious pathogenic reason are an occasional occurrence in our flock. The objective to decrease the

incidence of premature luteal regression by replacing the GnRH agonist by hCG was not achieved. Apparently hCG failed to exert the anticipated sustained lutealtropic effect.

Ever since the availability of a practicable technique for deep uterine insemination of goats by transcervical route (Sohnrey and Holtz, 2005) it has become the method of choice in our breeding flock. In the present experiment the technique was slightly modified. By moving the does into an immobilization crate instead of lifting their hind quarters up, the physical strain to animal and operators was substantially decreased. This was found to be particularly convenient with heavy or excitable animals.

As a conclusion, it may be stated that the result of the present study corroborates the findings of the previous investigation showing that synchronization of goats by way of prostaglandin F2 alpha-GnRH treatment would yield excellent pregnancy rates if not affected by a high incidence of premature luteal regression. Substitution of the GnRH agonist by hCG did not solve the problem. In the interest of the AI industry and effective embryo transfer programs in goats the problem of premature luteal regression calls for more scientific attention.

4 General Discussion

The finding that the addition of a disaccharide to the vitrification medium favors post-thaw vitality of vitrified mouse embryos is in agreement with the observations of Kasai et al. (1990) and Kuleshova et al. (2001). Presumably sucrose is instrumental in alleviating the toxicity of penetrating cryoprotectants by extracting intracellular water and, as a consequence, limiting the amount of hydrophilic substances from entering the cells (Kasai et al., 1990). Apparently a concentration of 0.4 M sucrose in vitrification medium was capable of accomplishing this objective, whereas sucrose at a concentration of 0.8 M was excessive and, therefore, deleterious to cells. Kuleshova et al. (1999) showed that mouse oocytes placed into medium containing 1.0 M sucrose were irreversibly damaged.

Generally disaccharides, mostly sucrose, are incorporated in post-thaw media (Kuleshova et al., 1999), the objective being to permit penetrating cryoprotectants to diffuse out of the cells without water rushing in and straining cell membranes (Massip et al., 1987). This can occur gradually by decreasing concentrations (Vajta et al., 1998; El-Gayar and Holtz, 2001) or in a single step (Kasai et al., 2002). Moreover, non-permeating carbohydrates are assumed to exert a protective action on the structural and functional integrity of cells (Crowe et al., 1984).

The lack of an effect of sucrose in warming media on expansion and hatching of embryos, as recorded in the present investigation, agrees with recent findings by El-Gayar et al. (2008). These authors did, however, attain better hatching rates than in this experiment. This might be due to the difference in culture media used for in vitro culture. KSOM, the medium used for in vitro culture in this experiment, contains comparatively low concentrations of NaCl, KCl, KH_2PO_4, lactate and glucose (Lawitts and Biggers, 1993). The medium was developed for allowing zygotes of outbred mouse strains to overcome the two-cell culture block (Erbach et al., 1994). El-Gayar et al. (2008) cultured in M16 culture medium supplemented with BSA.

It has been shown by Paria and Dey (1990) and Lane and Gardner (1992) that mouse embryos, cultured in small volumes of medium and/or in groups had more blastomeres, developed better and, most importantly, displayed superior post-transfer viability. In mouse embryos cultured in the absence of exogenous protein, development to the hatched blastocyst stage was observed when the density was 10 embryos10/µL of medium but not so if it was 1

embryo/10µL (Quinn et al., 1993). Autocrine and/or paracrine factors are believed to be responsible and are thought to be diluted out in large volumes. Our density of 5-6 embryos per 50 µL drop might have been too low.

It may be concluded that incorporation of sucrose into vitrification medium at a concentration of 0.4 M will enhance post-thaw development of OPS-vitrified mouse blastocysts. At a concentration as high as 1.0 M, however, sucrose has a detrimental effect. In the medium the embryos are suspended in after warming, sucrose is not required. This opens up the possibility to transfer vitrified embryos to recipients directly after warming without the necessity of a microscope or other laboratory equipment.

The second investigation constitutes an attempt to extend the favorable results achieved with OPS vitrification of caprine blastocysts (El Gayar and Holtz, 2001) to other embryonal stages (morulae and hatched blastocysts).
Conventional freezing of morulae was not included in the present investigation as we know from earlier studies (Li et al., 1990; Puls-Kleingeld et al., 1992) that, in most cases, caprine morulae do not survive conventional freezing. Two recent investigations report pregnancy rates of 14% (Guignot et al., 2006) and – on account of various methodological variations - between 33-51% (Hong et al., 2007) upon transfer of vitrified caprine morulae and blastocysts. It was not distinguished between embryonal stages; however, therefore there is no way of telling whether morulae had survived. In the present study not a single recipient provided with OPS vitrified morulae remained pregnant, indicating that the vitrification procedure established and applied by Vajta et al. (1998) and El Gayar and Holtz (2001), is not suitable for the cryopreservation of caprine morulae.

Blastocysts have repeatedly been shown to be a suitable stage for conventional cryopreservation (Puls-Kleingeld et al., 1992; Nowshari and Holtz, 1995). In the present investigation, the results arrived at with the cryopreservation of blastocysts were in almost perfect agreement with those of an earlier study (El-Gayar and Holtz, 2001): after conventional freezing, in either study 42% of the transferred blastocysts were carried to term; after OPS vitrification embryo survival was slightly improved over the previous study (70 vs. 64%), whereas the proportion of does kidding was a bit lower (82% vs. 93%).
To our knowledge this is the first report of successful OPS vitrification of hatched goat blastocysts. The transfer results, however, were discouragingly low, regardless whether

conventional freezing or OPS vitrification protocols were involved. This is disappointing, because it implies that there is still an efficient means of cryopreserving embryos without an intact zona pellucida lacking. This includes bisected (Nowshari and Holtz, 1993; Oppenheim et al., 2000) or biopsied embryos (El-Gayar and Holtz, 2005). The zona pellucida as a protective coat is known to control osmotic pressure (Bronson and McLaren, 1970), transport and diffusion of nutrients and metabolites (Leoni, et al., 2002) and, in all likelihood, also foreign substances including cryoprotectants. It is, by no means, a static structure, changing its character during tubal passage (Kolbe and Holtz, 2005), fertilization (Yanagimachi, 1988; Suzuki et al., 1996) or cryopreservation (Moreira da Silva and Metelo, 2005). The exact mechanism by which the zona pellucida provides protection to the embryo is not fully understood. Modification of the media used and the cryopreservation methods applied might be necessary to be suitable for the hatched embryos.

By way of conclusion, the outcome of the present experiment convincingly confirms the earlier finding that OPS vitrification of caprine blastocysts provides for higher pregnancy and embryo survival rates than conventional freezing. Attempts to apply the OPS technique to caprine morulae were of no avail since no pregnancies were accomplished. For the cryopreservation of hatched blastocysts the OPS vitrification method was no more effective than conventional cryopreservation, yet, the technique is less elaborate, less time consuming and less costly.

One objective of the third investigation was to verify an earlier finding (Holtz et al., 2008) indicating that estrus synchronization by means of $PGF_{2\alpha}$-GnRH treatment is a suitable means of permitting fixed-time insemination in goats. The outcome was encouraging in so far as estrus synchronization was accomplished in all does irrespective of the ovulation inducing agent. The slight superiority of GnRH- over hCG treated does might be coincidental and would require verification on a larger scale. The only significant difference between GnRH- and hCG treated groups was the shorter estrus period observed in the GnRH group. Presumably the immediate release of LH known to occur in response to GnRH-treatment (Holtz et al., 2008) induced ovulation and, as a sequel, decline in plasma estrogen concentration sooner than in the other groups.

The main objective of the investigation was to reduce the incidence of short cycles. To that end GnRH, serving as ovulation inducing agent in the ovsynch protocol, was replaced by

hCG. Short cycles as a sequel to synchronization (Armstrong et al., 1983; Chemineau et al., 1986, 1999; Cognié et al., 2003) and superovulation treatment (Holtz, 2005) are commonly encountered in goats both with the conventional sponge-eCG protocol and, as indicated in this and a preceding study (Holtz et al., 2008), to an even higher extent with $PGF_{2\alpha}$-GnRH synchronized goats. The reason for the premature corpus luteum regression is not clear (Cognié et al., 2003). The inadequate LH surge made responsible for short cycles in cattle (Taponen et al., 2002) can, according to a recent study (Holtz et al., 2008), not serve as a reason in goats.

If it were not for the high incidence of premature luteal regression, pregnancy rates to fixed-time insemination in $PGF_{2\alpha}$-GnRH synchronized goats, would have been exceptionally good (83%). Whether the two abortions that happened to occur in that particular group were associated with the treatment, is open to conjecture. Spontaneous abortions with no obvious pathogenic reason are an occasional occurrence in our flock. The objective to decrease the incidence of premature luteal regression by replacing the GnRH agonist by hCG was not achieved. Apparently hCG failed to exert the anticipated sustained lutealtropic effect.

Ever since the availability of a practicable technique for deep uterine insemination of goats by transcervical route (Sohnrey and Holtz, 2005) it has become the method of choice in our breeding flock. In the present experiment the technique was slightly modified. By moving the does into an immobilization crate instead of lifting their hind quarters up, the physical strain to animal and operators was substantially decreased. This was found to be particularly convenient with heavy or excitable animals.

As a conclusion, it may be stated that the result of the present study corroborates the findings of the previous investigation showing that synchronization of goats by way of prostaglandin F2 alpha-GnRH treatment would yield excellent pregnancy rates if not affected by a high incidence of premature luteal regression. Substitution of the GnRH agonist by hCG did not solve the problem. In the interest of the AI industry and effective embryo transfer programs in goats the problem of premature luteal regression calls for more scientific attention.

5 Summary

The thesis consists of two sections, the first of which is comprised of two experiments involving the open-pulled-straw (OPS) vitrification method for cryopreserving caprine and murine embryos.

In the first experiment the applicability of the OPS vitrification method, found to be effective in cryopreserving caprine blastocysts (El-Gayar et al., 2001), to other embryonal stages was investigated. Morphologically intact morulae, blastocysts, hatching and hatched blastocysts, collected from superovulated does between day 6 and day 9 after the onset of estrus, were vitrified by the OPS method. French mini-straws were heat-softened, pulled and cut at the narrowest point to get a modified straw with a diameter of approximately half the original one. Embryos were equilibrated in medium 199 supplemented with 20% goat serum, containing 10% ethylene glycol (EG) and 10% dimethyl sulfoxide (Me_2SO), at 39°C for 1 min and then in 20% EG and 20% Me_2SO for 20 seconds. The embryos were loaded into the narrow end of the modified straw by capillary force and the straws were plunged directly into liquid nitrogen. After step-wise thawing in sucrose solution at 39°C, the embryos were transferred to synchronized recipients (-24 h) by endoscopic means. As a control, conventional freezing (embryos equilibrated in M2 medium containing 1.5 m EG, loaded into 0.25 ml straws and cooled at a rate of 0.5°C/min) was employed. Of 11 recipients receiving OPS-vitrified blastocysts, 9 (82%) became pregnant and all of them kidded. The corresponding values for conventional freezing were 50% pregnant and 40% (4/10) kidding. Overall embryo survival amounted to 70% (16/23) for OPS vitrified and 42% (8/19) for conventionally frozen embryos. Of 9 recipients receiving OPS-vitrified hatched blastocysts, only 3 (33%) get pregnant and 2 (22%) kidded, resulting in an embryo survival of 13% (2/15). When hatched blastocysts were frozen by the conventional method, a similar pregnancy rate was achieved 33% (3/9) and all of the pregnant goats went to term, resulting in an embryo survival rate of 19% (3/16). In case of hatched blastocysts, there was no significant difference between cryopreservation techniques ($P<0.05$). Morulae were only cryopreserved by the OPS method, as experience tells that conventional freezing is of no avail. It turned out that vitrification of morulae was equally futile. In conclusion, the OPS method did not only prove to be particularly practical and cheap but also more successful than the conventional method of cryopreservation of caprine blastocysts and hatched blastocysts.

The second experiment was performed to investigate the effect of sucrose as a component of vitrification medium and post-warming dilution medium when cryopreserving murine blastocysts. Morphologically intact mouse blastocysts collected from superovulated 6- to 8-week-old virgin female NMRI mice were vitrified by the OPS vitrification method as described above. However, in this experiment the blastocysts were exposed, at the second step of the vitrification procedure (medium containing 20% Me_2SO + 20% EG) to 0.0, 0.4 or 0.8 M sucrose. Embryos from each of the 3 vitrification treatments were warmed and diluted at room temperature in medium containing 0.00, 0.25 or 0.50 M sucrose. Eventually, the embryos were cultured in potassium simple optimized medium (KSOM) microdrops under oil to monitor in vitro development. It turned out that sucrose as a component of the post-thaw dilution medium had no effect on expansion or hatching rates regardless of the respective concentration ($P>0.05$). To the contrary sucrose in the vitrification medium exerted a significant ($P<0.001$) effect on both expansion and hatching rate. Expansion rates were highest (84-87%) in vitrification medium containing 0.4 M sucrose, the difference between this group and that devoid of sucrose (76-82%) being non-significant ($P>0.05$). Medium containing 0.8 M sucrose however, was significantly inferior to media containing 0.0 or 0.4 M sucrose (40-54%, $P<0.001$). Hatching rates followed a similar trend. At 0.4 M sucrose it amounted to 44-57% at 0.0 M to 29-42% and at 0.8 M to 19-23. As with the expansion rate; the high concentration was significantly inferior to zero or a low concentration of sucrose ($P<0.001$). Only about half the embryos that had reached the expanded blastocyst stage continued to hatching. In the 0.0 and 0.8 M sucrose groups, it amounted to 45 and 47%, respectively, compared to almost 60% in the 0.4 M sucrose group. It may be concluded that the incorporation of sucrose into vitrification medium at low concentrations is beneficial to post-warming survival of mouse blastocysts. Warming of vitrified blastocysts can be performed in a single-step omitting the incorporation of sucrose in the dilution medium.

The second section of the thesis consists of an attempt to apply the technique of fixed-time deep uterine insemination in synchronized goats. Ovulation induction was accomplished with the commonly used Gonadotropin Releasing Hormone (GnRH) or human Chorionic Gonadotropin (hCG). The question was whether hCG may be substituted for GnRH as ovulation inducing agent in prostaglandin F2α ($PGF_{2\alpha}$)-synchronized goats. The underlying intention was to reduce the incidence of short cycles by providing a more sustained stimulation of the corpus luteum (CL), conjecturing that this will render the CL a less prone to

premature regression. Sixty pluriparous Boer goat does were randomly assigned to 3 treatment groups. All does were subjected to an i.m. injection of 5 mg Dinoprost (Dinolytic®, Pfizer) during the luteal phase of the estrous cycle. The 20 does constituting the control group were inseminated 12-14 h after the onset of detected estrus. In another 20 does the Dinoprost treatment was followed up, 48 hours later, by an i.m. injection of GnRH (0.004 mg Buserelin, Receptal®, Intervet); the remaining 20 does by an i.m. injection of 500 I.U. hCG (Chorulon®, Intervet). The does of the latter two groups were artificially inseminated 16 h later. Ovarian activity was monitored by ultrasonography and estrus detection was performed, at 8 h intervals, with an aproned male. Artificial insemination was conducted by the deep uterine method described in Sohnrey and Holtz (2005). In some of the goats, the procedure was slightly modified in that, instead of lifting up the hind quarters of the animal in order to introduce the insemination catheter, the does stood in a restraining crate equipped with a hammock-like sling with holes for the forelimbs (Suyadi et al., 2000). The sling prevented the animal from crouching or moving about while being inseminated. Three of the goats (15%) of the GnRH group and 2 (10%) of the hCG group exhibited tail flagging only, all others displayed standing estrus. There were no differences among groups with regard to the time between $PGF_{2\alpha}$ treatment and onset of estrus (44.5, 46.6 and 41.6 h for controls, GnRH- and hCG groups, respectively). Duration of estrus was, on average, 10 hours shorter in the GnRH group than in the other groups (37.1 versus 46.4 and 48.4 h, $P<0.05$). Ovulation numbers were virtually similar (2.5, 2.4 and 2.1). The incidence of premature luteal regression was significantly lower in the control group compared with the GnRH- and hCG groups (5 vs. 40 and 35%, $P<0.05$). Pregnancy rate in the control group was 60%, in the GnRH group 50% and in the hCG group 35%. Corresponding kidding rates were 60, 40 and 35%. When disregarding does with premature CL regression, the corresponding pregnancy rates were 63, 83 and 54% and kidding rates were 63, 67 and 54%. The numbers of kids born to control-, GnRH- and hCG groups were 1.83, 1.88 and 1.71, respectively. In conclusion, estrus synchronization is accomplishable by injection of $PGF_{2\alpha}$ during the luteal phase; ovulation may be terminated by injection of either GnRH or hCG. The hoped-for reduction in the incidence of premature luteal regression by using hCG instead of GnRH was not achieved.

6 References

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