Vitrification, warming in sucrose-free medium and transfer of goat and mouse embryos

DEPARTMENT OF ANIMAL SCIENCE

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Vitrification, warming in sucrose-free medium and transfer of goat and mouse embryos

Dissertation

to obtain the Ph. D. degree

in the International Ph. D. Program for Agricultural Sciences in Göttingen (IPAG) at the Faculty of Agricultural Sciences,

Georg-August-University Göttingen, Germany

presented by

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Göttingen, May 2020

D7

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Date of dissertation: 12th May, 2016

DEDICATION

To

God

My beloved Husband and Children

My Mom and Dad

ACKNOWLEDGEMENTS

First of all I would like to thank to the Deutscher Akademisher Austausch Dienst (DAAD) for providing me with the scholarship to study my PhD.

I would like to express my deepest gratitude to my supervisor Prof. Dr. Wolfgang Holtz for the invitation to initiate this journey, for all the knowledge he shared with me and the trust he had in me to make this project possible, his patience and friendship. I also would like to thank my supervisor Prof. Dr. Dr. Matthias Gauly for permitting me to join his work group, all his advices and support during this time. I would like to offer special thanks to Prof. Dr. Christoph Knorr for accepting being part of my disputation committee, although he is no longer with us, continues to inspire by his example and dedication.

Special thanks to the company Consartic, Schoellkrippen, Germany for facilitating us the CryoLogic Vitrification Method- CVM Ring fibreplug™ used in this study, especially to Mr. Tobias Gundelach for the support and enthusiasm he put in this work. My thanks also to the company Labotect, Göttingen, Germany for providing the embryo transfer catheters.

This work would not have been possible without the help of many people: first I want to express special thanks to Dr. Miguel Camacho for being my right hand, my support, my best friend and for always being there for me, thank you for the effort put in this work. Common destiny we were meant to be. I want to thank Dr. Manuela Ropeter and Dr. Moataz El-Gayar for the valuable training and support I got from them. This work would not have been possible without the help of the animal keepers: Dieter, Jürgen, Max, Jenny, Kristina, Ricarda and Louisa, thank you for always being able to help whenever it was needed. Special thanks to Dr. Gürbüz Das for helping on the statistics and Ms. Elli Stüwe for the samples analyzed, your advices and your friendship. I want to thank to my PhD fellows for being always there to help with the goats, mice, contributions, advices, friendship, shots and taking care of Mickey whenever we needed it: Dr. Pilasrak Panpraset, Dr. Kalyakorn Wongrak, Dr. Katrin Geburt, Dr. Kerstin Wagner, Dr. Sabrina Weber, Dr. Mazahr Shahin, Dr. Abdussamad Abdussamad, Dr. Mohammed Saleh, Dr. Stefanie Ammer, Dr. Sophie Meyer, Dr. Anna Stölzl, Dr. Daniela Marthold, Dr.

Shayan Rahimian, Dr. Vu Hieu, Dr. Johanna Trautmann, Dr. Daniel Gieseke, Rania Shamseldin, and Matthias Maier. Thanks to the bachelor and master students: Jennifer, Marie, Wasim, Eugenia, Elena, Jessica, Fernanda and Eric to make the work on the lab more fun. To my senior collegues Dr. Rogelio Ledezma, Dr. Morten Friedrich, Dr. Judith Friedrich, Dr. Gürbüz Das, Dr. Alexander Riek, Dr. Christian Lambertz, Dr. Uta König, Dr. Daniel Mörlein and the technical staff Dirk Teuteberg, Andrea Hartmann, Erwin Tönges, Birgit Sohnrey, Burchhard Möllers and Andrea Armbrecht for your friendship and support during these years.

Thanks to my Family in Göttingen: Daisy, Daniela, Daniel, Eugenia, Guadalupe, Ariana, Mariela, Ramon, Valeria, Jorge, Jose, Anika, Pablo, Monica, Juan-Ra, Ikal, Surem, Pablito y Sofí for being always there. Specially to my motivation and inspiration my children Miguel for permits me being mother and PhD student at the same time and Matías who came later to complete this family with his joy.

Special thanks for the long-distance support to my Parents, Sisters, Grand Parents, Nieces, Nephews, the entire family, my in-laws and friends in Mexico for encourage me to keep going during these years.

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List of Abbreviations

°C	Celsius degrees
Ch	Channel
CIDR	Control Internal Drug Release
cm	Centimeter
CO_2	Carbon dioxide
CVM	CVM Ring Fibreplug
ME_2SO	Dimethyl sulfoxide
E	East
eCG	Equine Chorionic Gonadotropin
e.g.	for example
EG	Ethylene Glycol
FDA	U.S. Food and Drug Administration
FSH	Follicle stimulating hormone
g	Grams
h	Hour (s)
hCG	Human Chorionic Gonadotropin
HCl	Hydrogen chloride
HM	Holding medium
H_2O	Water
ID	Internal diameter
i.m.	Intramuscular
IU	International Units
i.v.	Intravenous
Kg	Kilograms
LN_2	Liquid nitrogen
M	Molar
µm	Micrometers
µg	Micrograms
µl	Microliter
mg	Milligrams
mm	Millimeters

m*M*	Millimolar
ml	Milliliter
min	Minutes
mOsm	Milliosmole
N	North
n	Number of animals/ embryos
No	Number
NaOH	Sodium hydroxide
ng	Nanograms
NMRI	Naval Medical Research Institute
OPS	Open Pulled Straw
PBS	Dulbecco's phosphate buffered saline medium
pH	Potential Hydrogen
pLH	Porcine luteinizeiting hormone
PGF2α	Prostaglandin f 2 alpha
SAS	Statistical Analyses Software
s	Seconds
SE	Standard Error
TCM	Tissue Culture Medium
vs	Versus

SUMMARY

In farm animals, the usage of assisted reproduction programs, implying biotechnologies such as estrus synchronization, superovulation, cryopreservation of embryos and embryo transfer, to mention some, helps to improve productivity and genetic value of the flock. The general objective of this investigation was the establishment of suitable means for embryo vitrification and embryo transfer that are easier to employ in assisted reproduction programs under farm conditions of small ruminants than existing methods.

The objective was addressed in three separate trials:

1. Assessment of the survival rate of murine embryos after vitrification by an open or closed system and one-step warming in two different warming media.
2. Evaluation of the efficiency of two vitrification systems and one-step warming in sucrose free medium for the cryopreservation of goat embryos.
3. Comparison of the effectiveness of semi-laparoscopical versus transcervical embryo transfer in goats.

The first experiment compares two different vitrification systems and two different warming solutions in mouse embryos. Cryopreservation of embryos is of considerable relevance for the implementation of embryo transfer programs and the establishment of embryo banks in several mammalian species. Vitrification was performed using "Open Pulled Straw (OPS) or CVM RingFibre plug™ (CVM) devices. Warming was carried out either in a warming solution containing 0.33 M sucrose or in a solution devoid of sucrose. Differences between vitrification systems were not significant. Warming in sucrose-containing diluent resulted in an expansion rate of 64%, as compared to 86% in a solution devoid of sucrose; reported hatching rates were 45% vs. 9%, respectively ($p<0.05$). Upon transfer, implantation rates for OPS- and CVM were 50% and 27%, respectively, compared with 55% for freshly collected embryos. The implantation rate after warming was 43% for sucrose-containing and 33% for sucrose-free medium. In conclusion: a) both vitrification systems are suitable for vitrifying mouse blastocysts; b) warming in sucrose-free diluent yields better embryo survival rates than in diluent containing 0.33 M sucrose.

The second experiment outlines ways of non-surgical collection and semi-laparoscopic transfer of caprine embryos. Two different ways of embryo cryo-preservation by way of vitrification are described; the open pulled straw (OPS) procedure, known to be well suited and the solid surface procedure called for in situations where contact between embryos and non-sterile liquid nitrogen is to be avoided. Based on 13 transfers of OPS-vitrified and 9 transfers of solid surface-vitrified blastocysts (2 blastocysts/recipient) it was shown that either procedure is applicable (54% vs. 56% pregnancy- and 39% vs. 44% kidding rate). Furthermore the experiment showed that warming of vitrified embryos may be accomplished by one-step procedure (88% transferable post-warming embryos), opening up the possibility to transfer vitrified embryos under field conditions.

The third experiment consisted of an attempt to replace the semi- laparoscopic embryo transfer commonly practiced in our group by a noninvasive transcervical transfer technique. Pluriparous Boer goats (n=31) served as recipients. were submitted to an estrus synchronization protocol during the breeding season, consisting of insertion of a progestogen-containing CIDR for 7 days, followed, upon withdrawal 7 days later, by two doses of 5 mg dinoprost, applied at 12h interval. Does in estrus where considered suitable recipients and embryo transfer was carried out six days after the last day of standing estrus. For semi-laparoscopic embryo transfer does (n=22) were anesthetized and positioned in dorsal recumbency. Ovaries where laparoscopically inspected to localize the ovary with at least one corpus luteum. With the aid of a blunted uterine tenaculum forceps (Pozzi; Aesculap, Germany), 255mm long, introduce via a 20 to 30 mm incision along the linea alba cranial to the udder, the tip of the uterine horn ipsilateral to the ovary displaying a corpus luteum was grasped under laparoscopic control. A loop of 20 to 30 mm of uterine horn close to the utero-tubal junction was gently exteriorized. A puncture hole was made with a blunted 22 g hypodermic needle about 50 mm from the utero-tubal junction, through which a 20 µl unopette was introduced to deposit two embryos in the uterine lumen. Recipients received randomly selected embryos vitrified either with OPS (13 does) or CVM (9 does). For the non-surgical transcervical transfer nine pluriparous Boer goats served as recipients. To immobilize the does and prevent them from squatting, they were placed in a crate equipped with a hammock with holes for the front legs. A duck-bill speculum was introduced into

the vagina, the os cervix was located and the lip of the os cervix was grasped with the aid of sharp –pointed uterine tenaculum forceps (255mm long) and carefully pulled caudally until it almost reached the vulvar orifice. A transfer catheter set for human embryo transfer, consisting of an atraumatic outer curved guiding cannula was introduce through the cervical canal and directed to the desired uterine horn (ipsilateral to the corpus luteum identified ultrasonographically). Recipients were randomly divided up, so four does received embryos vitrified with OPS and five does embryos vitrified by the CVM method. Pregnancy was diagnosed by ultrasound 30 days and day 45 after transfer. Pregnancy rate for twenty-two does that received embryos semi-laparosocpically was 55% whereas, of nine does that received embryos via transcervical transfer only one remained pregnant. The semi- laparoscopic embryo transfer technique proved to be effective, however still being a surgical procedure, entails anesthesia, a surgeon and aseptic environment, which are aspects not easy to maintain on-farm. Based on these results and previous trials by our work group, transcervical transfer of embryos could result in healthy born kids, however the pregnancy rates are very low and in order to get better outcomes the technique must be substantially improved.

CHAPTER 1

General introduction and literature review

1. 1. General introduction

The assisted reproduction techniques include the use of synchronization protocols, artificial insemination, superovulation, embryo collection, in vitro fertilization, cryopreservation of gametes and embryo transfer. Apart of being important tools in controlled breeding, they may improve the genetic quality of the flock. In goats estrous synchronization, superovulation and artificial insemination are well documented. Embryo cryopreservation and transfer, however, are less developed. Embryo cryopreservation is of immeasurable value to the implementation of assisted reproduction programs. It has been applied to create embryo banks of various mammalian species including embryos of high genetic animals, species in danger of extinction and for transportation purposes.

There are two different methods of cryopreservation the Slow Freezing and Vitrification. Slow freezing is the method of cryopreservation most used for mammalian embryos around the world. Its protocols are well established; however, it is time consuming and requires sophisticated equipment. Vitrification constitutes direct conversion of water from a liquid to a glassified state, by direct exposition of the sample to liquid nitrogen. The relatively low cost and the speed in which its protocols are performed, makes vitrification a more desirable technique to cryopreserved embryos. Prior to vitrification embryos are exposed to extremely high concentration of cryoprotectants which after warming need to be diluted due to their cytotoxic effect. A disadvantage of vitrification is that, under field conditions, direct transfer of embryos is not possible due to the multiple steps required to remove the highly concentrated cryoprotectants by passaging the embryos through a sequence of dilution media. To overcome this, one-step warming protocols might be employed, allowing direct transfer under farm conditions. In the present investigation the effectiveness of two vitrification systems, the Open Pulled Straw and CVM Ring Fibreplug, to vitrify mice (experiment 1) and goat (experiment 2) embryos was compared, as well as the possibility of one step-warming instead of the conventional multiple-step protocol.

Embryo transfer allows the dissemination of high genetic traits among the flock. Because of the anatomical disposition of the reproductive tract on goats, earlier embryo transfer techniques in goats implied surgery, thus being difficult to apply under farm conditions. Methods to improve this technique and make the procedure less invasive include the use of laparoscopy, minimizing post-surgical adhesions. The development of a suitable

transcervical transfer will avoid the need of surgery, use of narcotic agents and post-surgery care and distress, making it a more practicable technique under farm conditions. The last experiment of this study was an attempt to perform a non-surgical transcervical embryo transfer and compare it with a semi-laparoscopical embryo transfer (experiment 3).

1.2. Literature review

1.2.1. Embryo cryopreservation

Cryopreservation comprises the exposition of embryos to extremely low temperatures which could compromise the following embryo development; consequently, an optimal cryopreservation method should allow the preservation with minimal cell damage. Until now two different methods have been established, slow freezing (Whittingham et al., 1972) and vitrification (Rall and Fahy, 1985). The most important factors for a method of cryopreservation to be successful are the cooling rates (°C/min), the viscosity of the medium and the warming process (Saragusty and Arav, 2011; Vajta and Kuwayama, 2006; Massip, 2001).

With the purpose of protecting the cells to avoid injuries during the chilling process, before cryopreservation, embryos should be exposed to an equilibration process in media containing cryoprotectants. The time of equilibration and the concentration of cryoprotectants vary depending on the method of cryopreservation (Shaw and Jones, 2003). With slow freezing, equilibration takes much longer than with vitrification (Mogas, 2014). Cryoprotectants act by stabilizing the lipid membranes by hydrogen bonding with polar head groups of membrane lipids (Crowe et al., 1984). There are two types of cryoprotectants; permeating, such as ethylene glycol (EG), glycerol, dimethyl-sulfoxide (DMSO) and propylene glycol, and non-permeating, such as sucrose, glucose or fructose (Saragusty and Arav, 2011; Chen et al., 2005; Luz et al., 2009; Barcelo-Fimbres and Seidel, 2007-b).

1.2.1.1. Slow freezing

The first successful cryopreservation of embryos by slow freezing was reported by Whittingham et al. (1972) in murine embryos. It constitutes the slow exchange of fluids, under subzero temperatures between intra- and extra- cellular space (Valojerdi et al., 2009). Nowadays, automatic freezers controlling the slow freezing process are available. The cryoprotectants most commonly used in these protocols are glycerol and sucrose. Due to their low toxicity and low concentration, it is possible to perform an in-straw dilution after thawing, making direct transfer of embryos possible. The possibility of direct transfer makes slow freezing an ideal method to be used under field conditions. Protocols are well established; however, they are time consuming and require special equipment.

1.2.1.2. Vitrification

Vitrification is defined as the instant conversion of a liquid into a glass-like form accomplished by extremely rapid cooling rates. Contrary to slow freezing embryos are exposed to high concentrations of cryoprotectants and immediate submersion in liquid nitrogen (-196°C), circumventing the processes of crystallization and cellular dehydration (Yuswiati and Holtz.,1990; Shaw and Jones., 2003). Because of the extreme velocity of cooling during vitrification, embryos need to be exposed to high concentrations of cryoprotectants to avoid rupture of the zona pellucida and to reduce chilling injuries. The first successful vitrification was accomplished in mouse embryos by Rall and Fahy reported in 1985.

There are open and closed vitrification systems. The open system implies direct contact with liquid nitrogen. Closed systems, on the other hand, have the advantage of avoiding direct contact of the embryo with liquid nitrogen, reducing the risk of sample contamination. In an attempt to achieve higher cooling rates and provide maximal biosecurity for the embryos more than 30 different vitrification devices have been developed. The best-known open system is the “open- pulled-straw” (OPS) developed by Vajta et al. (1999). It comprises a thinly drawn-out French straw that picks up the embryo in a tiny drop of vitrification medium by capillarity force, followed by immediate submersion in liquid nitrogen. However, because it is known that open systems do not fully satisfy the biosecurity requirements of the European Directive on Tissue Storage and the U.S. Food and Drug Administration (FDA), it is mostly used only for research purposes. Among the closed systems available on the market aspect such as total biosafety, easy handling and acceptable recovery rates have not yet been entirely accomplished.

Over the last 10 years vitrification has been improved. The main goal was to decrease the volume of the medium to 1µl per sample, suggesting that by reducing the sample volume, the volume of cryoprotectants could also be lowered, thus cytotoxicity will be lower. It has also been suggested that the smaller the volume of the sample, the higher the viscosity and the probability of vitrification (Yavin and Arav., 2007; Saragusty and Arav, 2011).

Vitrification has been described as the most efficient method to preserve embryos (Chang et al., 2008). However, the warming of vitrified embryos is based on various steps,

exposing embryos to media containing decreasing doses of non-permeable substances in order to remove the permeable cryoprotectants (Gibbons et al., 2011; El-Gayar et al., 2008). The latter forms a barrier for the transfer of vitrified embryos in most farm conditions due to the need of laboratory equipment and manipulation of the embryo during warming. Recent studies on mice (El-Gayar et al., 2008; Rodriguez et al., 2010) and cows (Trigal et al., 2013; Morato and Mogas., 2013a) embryos, demonstrated that after one-step warming in-vitro and in-vivo embryo cleavage is comparable with those thawed by three steps warming protocol.

It has been suggested that the major damages of vitrified embryos to the zona pellucida and consequently to the inner cell mass occur during warming rather than during cooling (Kasai et al., 1996). To prevent osmotic injuries during warming it is recommended to hold the devices containing the embryos at room temperature for some seconds before exposing them to the warming media. However, the time between liquid nitrogen exposure and warming media should not exceed 30 seconds, otherwise the sample may suffer crystallization causing damage to the zona and the inner cell mass (Rall and Meyer, 1989; Kasai et al., 1996).

1.2.2. Embryo transfer

Surgical transfer by laparotomy in goats has been well established in the past (Warwick et al., 1934). However, this techniques in invasive, requires post-surgical care and is known to cause adhesions thus, making it difficult to use an animal repeatedly. The surgical technique has been simplified by the use of an endoscope (McKelvy et al., 1984). That technique is less invasive, formation of adhesions are much lower, distress of the animal is also lower and recovery after the intervention is faster. Another variant of the laparoscopic transfer is the semi- laparoscopic transfer (Wallenhorst and Holtz, 2002; Al-Yacoub et al., 2010) which permits the direct visualization of the transfer site. It is know that laparoscopical methods are the most efficient (Abdullah et al., 1995; Ishwar and Memon, 1996; Rahman et al., 2008). However, still being a surgical procedure, it entails anesthesia, a surgeon, aseptic environment and special equipment, aspects not easy to maintain under on-farm conditions. Non-surgical transcervical transfer has also been attempted. Besides being poorly established, non-surgical transfer has, as yet, never produced satisfactory results

(Otsuki and Soma, 1964; Lin et al., 1979; Flores-Foxworth et al., 1992; Fonseca et al., 2014; Holtz and Sohnrey, unpublished data). The development of an efficient trancervical technique could avoid the aspects mentioned above and enable embryo transfer on farm (Fonseca et al., 2014).

1.2.3. References

AbdelHafez F., Xu J., Golderberg J. and Desai N., 2011. Vitrification in open and close carriers at different cell stages: Assesssment of embryo survival, development, DNA integrity and s ability during vapor phase storage for transport. BMC Biotechnology 11-29.

Abdullah R.B., Shamsul A., Malik A. and Wan Khadijah W.E., 1995. Production of kids through embryo transfer in goats assisted by a laparoscope. Proc 7th Malaysia Soc Anim Prod Ann Conf, (MSAP'95), Penang, Malaysia: 59-60.

Al-Yacoub A.N., Gauly M. and Holtz W., 2010. Open pulled straw vitrification of goat embryos at various stages of development. Theriogenology 73: 1018-1023.

Barcelo-fimbres M., and Seidel G.E., 2007b. Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during in vitro culture of bovine embryos on embryonic development after cryopreservation. Molecular reproduction and development 74: 1395- 1405.

Begin I., Bhatia B., Baldassarre, Dinnyes A. and Keefer C.L., 2003. Cryopreservation of goat oocytes and in vivo derived 2- to 4-cell embryos using cryoloop (CLV) and solid-surface vitrifcation (SV) methods. Theriogenology 59: 1839-1850.

Bielanski A., Davis N.S., Sapp T. and Lutze-Wallace C., 2000. Viral contamination of embryos cryopreserved in liquid nitrogen. Cryobiology 40:110–6.

Bielanski A., Bergeron H., Lau P.C. and Devenish J., 2003. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. Cryobiology 46: 146–52.

Chang C.C., Shapiro D.B., Bernal, Wright G., Kort H.I. and Nagy Z.P., 2008. Two successful pregnancies obtained following oocyte vitrification and embryo re-vitrification. Reprod Biomed Online 16: 346-349.

Chen S.U., Lien Y.L., Chen H.F., Tsai Y.Y., Chang L.J. and Yang Y.S., 2005. Observational clinical follow-up of oocyte cryopreservation using a slow-freezing method with 1, 2- propanediol plus sucrose followed by ICSI. Hum Reprod 20:1975–1980.

Crowe L.M., Mouradian R., Crowe J.H., Jackson S.A. and Womersley C., 1984. Effects of carbohydrates on membrane stability at low water activities. Biochim. Biophys. Acta: 769:141–50.

El-Gayar M., Gauly M. and Holtz W., 2008. One-step dilution of open-pulled-straw (OPS)-vitrified Mouse blastocysts in sucrose-free medium. Cryobiology 57: 191-194.

Flores-Foxworth G.A., McBride B.M. and Kraemer D.C., 1992. A comparison between laparoscopic and transcervical embryo collection and transfer in goats. Theriogenology 37:213.

Fonseca J.F., Esteves L.V., Zambrini F.N., Brandão F.Z., Peixoto M.G.C.D., Verneque R.S., Siqueira L.G.B. and Viana J.H.M., 2014. Viable offspring after successful non-surgical embryo transfer in goats. Arq. Bras. Med. Vet. Zootec. 66 no.2.

Gibbons A., Cueto M.I. and Pereyra B.F., 2011. A simple vitrification technique for sheep and goat embryo cryopreservation. Small Rumi Res 95: 61-64.

Ishwar A.K. and Memon M.A., 1996. Embryo transfer in sheep and goat: a review, Small Rumin Res 19: 35-43.

Kasai M., 1996. Simple and efficient methods for vitrification of mammalian embryos. Anim Reprod Sci 42, 67–75.

Lin A.Ch., 1979. Non-surgical embryo transfer in goats. Memoirs of the College of Agriculture (National Taiwan University)19:25.

Luz M.R., Holanda C.C., Pereira J.J., Teixeira N.S., Vantini R., Freitas P.M.C., Salgado A.E.P., Oliveira S.B., Guaitolini C.R.F. and Santos M.C., 2009. Survival rate and in vitro development of in vivo-produced and cryopreserved dog embryos. Reprod Fert Dev 22:208–209 (abstr).

Massip A., 2001. Cryopreservation of Embryos of Farm Animals. Reprod Dom Anim 36, 49-55.

McKelvey W.A. and Robinson J.J., 1984. Normal lambs born following transfer of embryos by laparoscopy. Vet Rec 115:230.

Mogas T., 2013. New insight on cryopreservation of oocytes and embryos. 30th Annual Meeting A.E.T.E proceedings, Dresden, Germany: 61-76.

Morato R. and Mogas T., 2013a. One step cryoprotectant dilution following vitrification of in vitro-produced bovine embryos. Reproduction, Fertility and Developent 25 (1): 182 Proceedings of the Annual Conference of the International Embryo Transfer Society, Hannover, Germany.

Otsuki K. and Soma T., 1964: Transfer of the fertilized ova through the cervix of goats. Bull Natl Inst Anim Ind 6: 27.

Panagiotidis Y.G., Papatheodour A., Prapas Y., Kasapi E., Goudakou M., Passadaki T., Nikelettos N., Veletza S., Prapas G. and Maroulis G., 2013. Assessment of open and close vitrification protocol for the cryopreservation of blastocysts from an elective non fresh embryo transfer oocyte donation program. Cryo congress Proceedings, Poster abstract: A-13.

Papatheodour A., Panagiotidis Y.G., Vanderzwalmen P., Kasapi L., Goudakou M., Papadaki T., Prapas N., Zikopoulos K., Georgiou I. and Prapas Y., 2013. Open versus close oocyte vitrification system in oocyte donation program: a prospective observational study. Cryo congress Proceedings, Poster abstract: A-13.

Rahman A.N.M.A., Abdullah R.B., Khadijah W.E.W., 2008. A Review of Reproductive biotechnologies and their application in goat. Biotechnology 7: 371-384.

Rall W.F. and Meyer T.K., 1989. Zona fracture damage and its avoidance during the cryopreservation of mammalian embryos. Theriogenology 31 (3): 683-692.

Rall W.F. and Fahy G.M., 1985. Ice free cryopreservation of mouse embryos at -196°C by vitrification. Nature 313: 573-575.

Rodriguez P.,Ongarratto F., Scherer D., de Avila-Rodriguez B. and Rodriguez J., 2010. Survival of vitrified Mouse blastocysts loaded into glass micro-capillaries. Rev colomb Cienc Pecu 23:28-34.

Saragusty J. and Arav A., 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. Socie Repro and Fert, 141: 1-19.

Shaw J.M. and Jones G.M., 2003. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. Human Repro Update 9: 583-605.

Trigal B., Gómez E., Caamaño J.N., Muño M., Correia E., Martín., Carrocera S. and Diez C., 2013. Improved survival to one-step rehydration of vitrified-warmed versus frozen- t awed in Vitro-produced bovine blastocysts. Reprod Fert Dev 25 (1), 189 Proceedings of the Annual Conference of the Germany International Embryo Transfer Society, Hannover.

Vajta G. and Kuwayama M., 2006. Improving cryopreservation systems. Theriogenology 65:236-244.

Valojerdi M.R, Eftekhari-Yazdi P., Karimian L., Hassani F. and Movaghar B., 2009.Vitrification versus slow freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos. J Ass Reprod Gene 26(6): 347-354.

Wallenhorst S. and Holtz W., 2002. Embryo collection in prepuertal gilts and attempts to develop an improved embryo transfer technique. Vet. Rec. 150, 749-751.

Warwick B.L., Berry R.O. and Horlachwer W.R., 1934. Results of mating rams to Angora female goats. Proceedings of the American Society of Animal Production 34: 225 (abstr).

Whittingham D.G., Leibo S.P. and Mazur P., 1972. Survival of mouse embryos frozen to -196 degrees and -269 degrees C. Science 178, 411–414.

Yavin S. and Arav A., 2007. Measurement of essential physical properties of vitrification solutions. Theriogenology 67: 81–89.

Yuswiati E. and Holtz W., 1990. Successful transfer of vitrified goat embryos. Theriogenology 34:629-632.

CHAPTER 2

Vitrification of mouse blastocysts by open or closed system and warming in sucrose-containing or sucrose-free diluent

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Published in CryoLetters 41 (3), 135-139 (2020)

Abstract

BACKGROUND: Cryopreservation of embryos is of considerable relevance for the implementation of embryo transfer programs and the establishment of embryo banks in several mammalian species. OBJECTIVE: The present investigation compares two different vitrification systems and two different warming solutions. MATERIALS AND METHODS: Vitrification was performed using "Open Pulled Straw (OPS) or CVM RingFibre plug™ (CVM) devices. Warming was carried out either in a warming solution containing 0.33 M sucrose or in a solution devoid of sucrose. RESULTS: Differences between vitrification systems were not significant. Warming in sucrose-containing diluent resulted in an expansion rate of 64%, as compared to 86% in a solution devoid of sucrose; reported hatching rates were 45% vs. 9%, respectively ($p<0.05$). Upon transfer, implantation rates for OPS- and CVM were 50% and 27%, respectively, compared with 55% for freshly collected embryos. The implantation rate after warming was 43% for sucrose-containing and 33% for sucrose-free medium. CONCLUSION: a) both vitrification systems are suitable for vitrifying mouse blastocysts; b) warming in sucrose-free diluent yields better embryo survival rates than in diluent containing 0.33 M sucrose.

Keywords: vitrification; mouse embryos; Open Pulled Straw vitrification; CVM RingFibre plug vitrification; sucrose-free reconstitution diluent.

2.1. Introduction

Cryopreservation of embryos is of considerable relevance for the implementation of embryo transfer programs and the establishment of embryo banks in several mammalian species. The first successful cryopreservation was accomplished in mouse embryos by Whittingham (15) using a slow freezing protocol. A decade later, vitrification was invented (11), using an ultra-rapid freezing protocol and extremely high concentrations of cryoprotectants. With this system liquids are converted to a glass-like solid state, circumventing potentially detrimental intra- and extracellular ice crystal formation. Vajta et al. (14) invented the "open-pulled-straw" (OPS) protocol using extremely thin and thin-walled receptacles that are not sealed, achieving cooling rates of 16,700°C min-1 (3). An objection to the method is the potential risk of contamination through non-sterile liquid nitrogen on

account of the unsealed straws. Therefore "solid surface vitrification" was invented, a technique that avoids contact of embryos with liquid nitrogen and achieves a cooling rate of 10,000°C min-1 (3).

The objectives of the present investigation were: a) to determine whether it is possible to achieve equally good results with a closed solid surface vitrification method as with the open-pulled-straw (OPS) method; and b) to warm embryos of either vitrification method in a single step in a sucrose-free diluent, which would enhance the practicability of the transfer of vitrified embryos to surrogate mothers under field conditions. The mouse served as a model for farm animals in which embryo transfer plays an increasingly important role.

2.2. Materials and methods

For the OPS procedure, ultra-thin straws are required which can be hand-fabricated by pulling 0.25 mL mini-straws (Minitube, Landshut, Germany) to half the original diameter over a hot plate at 200°C as described by El Gayar and Holtz (6). The commercially available equipment required for solid surface vitrification is depicted in Figure 2.1.

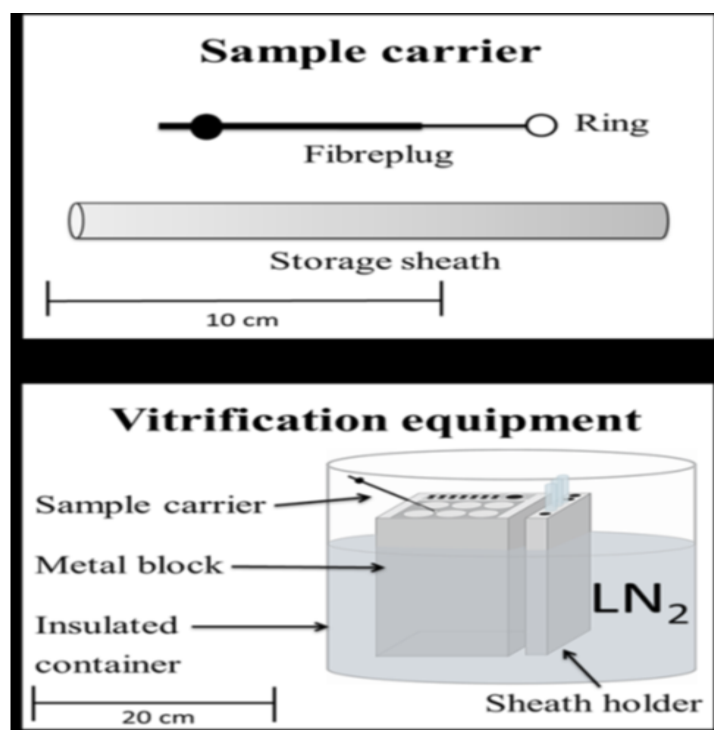

Figure 2.1. Solid surface vitrification carrier and equipment.

Blastocysts of grade 1 or 2, by the guidelines of the International Embryo Technology Society (IETS) (13), pooled from five donor mice at a time, were transferred to the aforementioned medium supplemented with 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (Me2SO). After 3 min, blastocysts were transferred to a 1 µL droplet of the culture medium supplemented with 16.5% EG, 16.5% Me2SO and 0.5 M sucrose. Within less than 40 s, vitrification was carried out by the OPS- and the solid surface-procedure. With the OPS protocol, slightly modified by El Gayar and Holtz (6), droplets were picked up by the narrow end of the OPS-straw owing to capillarity force, whereupon the straw was immediately submerged in liquid nitrogen, thin-end-first. With the solid surface-procedure droplets were placed on the ring of the carrier with the aid of an Eppendorf pipette where they adhered owing to surface tension. When lowering the ring onto the aluminum-nickel block cooled by liquid nitrogen (Fig. 1), the droplet was instantly transformed to a vitrified bead. The carrier with the adhering bead was sealed in a pre-cooled plastic sleeve and submerged in liquid nitrogen.

For warming of vitrified embryos, OPS straws were held in the air for 10 s before the thin end was dipped into holding medium at 37°C while the wide end was occluded with the tip of a finger so embryos were driven from the straw by the expanding air. With the solid surface system, the carrier was removed from the plastic sleeve and held in the air for 10 s. Then the ring-end was dipped into holding medium at 37°C. With either vitrification method six batches of five embryos were warmed in holding medium containing 0.33 M sucrose, another six batches of five embryos were warmed in medium devoid of sucrose. After 3 min, embryos were washed three times in 20 µL M16 medium (15) and cultured in 20 µL drops of M16-culture medium covered with embryo culture-tested paraffin oil (Sigma Aldrich M 8410, Steinheim, Germany) in an incubator at 37°C under humidified air with 5% CO2. Beforehand, culture dishes with medium had been preconditioned in the incubator for 2 h. Embryos were inspected at 20 - 40x magnification after 2 h and 24 h of incubation and classified from excellent [1] to degenerate [4] according to the IETS classification (13). Expansion and hatching rates were also recorded. As a control, six batches of five freshly collected grade 1 or 2 blastocysts, pooled from five donor mice each, were washed three times in M16 culture medium and cultured in the same manner as the vitrified-warmed embryos.

A small-scale in vivo trial was conducted to verify the meaningfulness of the in vitro findings. Twelve NMRI females, similar in age as the donors, served as surrogate mothers. They were caged with a vasectomized adult male overnight and, when a vaginal plug was detected, served as recipients. Two days after the vaginal plug was detected embryos were transferred as described by McLaren and Michie (9) and El Gayar et al. (5). Twelve recipient mice received five vitrified-warmed embryos transferred to one uterine horn and five freshly collected blastocysts to the contralateral uterine horn. Whereas six of the recipients received embryos that had been vitrified by the OPS procedure, the other six received embryos that been vitrified by the solid surface-procedure. Of the six recipients per group, three received embryos that had been warmed in medium containing 0.33 M sucrose, the other three received embryos that had been warmed in medium devoid of sucrose. Recipient mice were sacrificed 10 days after transfer and viable fetuses in each uterine horn were counted.

Data of the in vitro trial relating to embryo quality were analyzed with the non-parametric Kruskal-Wallis test. In case of significant effects, Dunn's multiple comparison post-hoc test ($P = 0.05$) was performed with the aid of SAS macro, and implemented in the NPAR1WAY procedure. Differences in expansion/hatching and pregnancy outcomes were determined by Chi-square test ($P = 0.05$).

2.3. Results

As shown in Table 2.1., there was no significant difference ($P>0.05$) between the OPS and the CVM RingFibre plug™ procedure with regard to post-warming recovery of embryos (83% vs. 77%), proportion of expanded blastocysts after 2 h in culture (82% vs. 67%) and proportion of hatched blastocysts after 24 h in culture (32% vs. 22%).

Expansion and hatching rates of vitrified embryos warmed in diluent devoid of sucrose (86% and 45%, respectively) were significantly higher ($P<0.05$) than of embryos warmed in diluent containing 0.33 M sucrose (64% and 9%, respectively) and did not differ from that of freshly collected control embryos (87% and 57%, respectively) ($P>0.05$). The morphological appearance of embryos warmed in sucrose-free diluent was superior to that of embryos warmed in sucrose containing medium (1.7 ± 0.1 vs 2.5 ± 0.1 on a scale of 1

to 4; P<0.05). Transfer of five OPS-vitrified blastocysts to each of six recipient mice resulted in an implantation rate across warming media of 50%, as compared to 27% for blastocysts vitrified by the solid surface-procedure (P>0.05). The implantation rate of freshly collected control blastocysts that were transferred to the contralateral uterine horn of 12recipients was 55%, which was not significantly different from the OPS-vitrified embryos (50%) but significantly higher than that of the solid surface vitrified embryos (27%, P<0.05). Warming of vitrified embryos in sucrose-free diluent across vitrification methods yielded an implantation rate of 33% as compared to 43% for embryos warmed in sucrose-containing diluent (P>0.05).

Table 2.1. Post-warming expansion rate after 2 h and hatching rate after 24 h of mouse blastocysts cultured in vitro after vitrification by the OPS or the solid surface CVM RingFibre plug™ procedure and warming in diluent containing 0 M or 0.33 M sucrose, as compared to freshly collected blastocysts (Control).

Variable	OPS			CVM			Sucrose conc. (M)			Control
	0.00	0.33	Total	0.00	0.33	Total	0.00	0.33	Total	
No. of vitrified blastocysts	30	30	60	30	30	60	60	60	120	30
Post-warming recovery (%)	90	77	83	73	80	77	82	78	80	-
Expansion rate (%)	89	74	82^{ab}	82	54	67^{b}	86^{x}	64^{y}	75	87^{ax}
Hatching rate (%)	48	13	32^{a}	41	4	22^{a}	45^{x}	9^{y}	27	57^{bx}

$^{ab, xy}$ **Means with different superscripts within rows differ (P<0.05)**

2.4. Discussion

The results indicate that vitrification of mouse blastocysts can be accomplished with both the OPS- and the solid surface-procedure, corroborating studies in humans (8, 10) and a study in mice (1). From a practical viewpoint the solid surface-procedure requires more skill and experience on the side of the technician, as it is pertinent to minimize the time required for placing the embryo on the ring of the carrier (under a stereoscope), lower the

carrier onto the metal block, insert it into the sheath and seal the sheath before submerging it in liquid nitrogen. The OPS procedure is easier to carry out because the droplet containing the embryos is readily sucked into the OPS straw which can then be directly submerged in liquid nitrogen. With either procedure occasionally an embryo was lost in the process, presumably due to a lack in dexterity on the part of the operator. With the CVM RingFibre plug™ procedure some of them could be retrieved from the sheath, without any loss in viability. The equipment required for the CVM RingFibre plug™ method is expensive in comparison to OPS straws which may be acquired commercially or self-manufactured at low expense. Which of the vitrification procedures to prefer may be individually decided on the basis of experience, availability of funds and considerations concerning biosafety, e.g., if embryos are to be exported.

In human assisted reproduction, vitrification has gained in popularity as it yields higher pregnancy- and live birth rates than conventional slow freezing (12). In farm animals, slow freezing is still standard. Permeating cryoprotectants, that are required to avoid chilling injury, are highly cytotoxic to unfrozen embryos and need to be removed immediately after liquefaction. This is accomplished either by stepwise dilution of the cryoprotectant or by a temporary sojourn of the embryo in a sucrose containing diluent. Both methods imply the availability of laboratory conditions and a stereoscope, rendering the procedure impractical for application in the field. For bovine embryos an “in-straw” system has been devised, consisting of straws containing the embryo in a small amount of diluent containing the cryoprotectant, separated by an air bubble from a large amount of sucrose-containing thawing diluent. After thawing, the fluid columns are mixed by shaking. With this system reasonable pregnancy rates are accomplished. The present study suggests that with vitrified embryos these efforts can be dispensed with because one-step warming with sucrose-free warming diluent provided better post-warming expansion- and hatching rates than warming in sucrose-containing diluent. Isachenko et al. (7), El-Gayar et al. (4) and Al Yacoub et al. (2), working with rat and mouse embryos, surmised that, due to the extremely brief exposure to vitrification solution before submersion in liquid nitrogen, only minimal amounts of cryoprotectants are able to enter the cells of the embryo. Therefore, the stress of de- and rehydration during passage through sucrose-containing medium appears to be

dispensable. Consequently, one-step warming of vitrified embryos in diluent devoid of osmotically active additives appears to be preferable. This would facilitate the transfer of vitrified embryos under field conditions.

The in vivo trial was conducted to verify the findings of the in vitro experiment. Although based on limited data, the approach of transferring cryopreserved embryos to one uterine horn and freshly collected control embryos to the contralateral horn of each recipient, improves the informative value. Transfer of both OPS- and solid surface-vitrified embryos resulted in the development of viable fetuses, though with a bias in favor of the OPS procedure (50% vs. 27%). More extensive in vivo studies would be required to substantiate a possible significant difference and attempts should be made to transfer the results to farm animal embryos.

In conclusion, for vitrification of mouse blastocysts the hygienically safe solid surface procedure appears to be a viable alternative to the established OPS procedure. More data will be required to substantiate this finding. Furthermore, one-step warming of vitrified mouse blastocysts in sucrose-free diluent is possible, facilitating application of the vitrification technique to serve as a practicable means of transferring embryos.

Acknowledgments

The authors thank Consartic, Schoellkrippen, Germany for making the CVM Ring-Fibreplug™ vitrification equipment available. G. Das helped with the statistical analysis. The senior author (G.D.) was supported by a fellowship of the German Academic Exchange Service (DAAD), and the second author (C.M.) by a fellowship of the Consejo Nacional de Ciencia y Tecnología (CONACYT) - German Academic Exchange Service (DAAD).

2.5. References

1. Abdelhafez F, Xu J, Golderberg J & Desai N (2011) BMC Biotech 30, 11-29.

2. Al Yacoub AN, Gauly M & Holtz W (2013) CryoLetters 34, 466-470.

3. Criado-Scholz E (2012) Current Frontiers in Cryopreservation, (ed) Katkov I, In Tech, Open Access Publisher, ISBN: 978-953-51-0302-08.

4. El-Gayar M, Gauly M & Holtz W (2008) Cryobiology 57, 191-194.

5. El-Gayar M, Gauly M & Holtz, W (2010) CryoLetters 31, 454-459.

6. El-Gayar M & Holtz W (2001) J Anim Sci 79, 2436-2438.

7. Isachenko V, Isachenko EF, Ostashko FI & Grishenko VI (1997) Cryobiology 34, 157-164. 8. Kuwayama M, Vajta G, Leda S & Kato O (2005) Repro Biomed Online 11, 608-614.

9. McLaren A & Michie D (1956) J Exp Biol 33, 394-416. 10. Panagiotidis YG, Vanderzwalmen P, Prapas, Y, Kasapi E, Goudakou M, Papatheodour A, Passadaki T, Petousis S, Nikelettos N, Veletza S, Prapas G & Maroulis G (2013) Reprod Bio Med Online 26, 470- 476.

11. Rall WF & Fahy GM (1985) Nature 313, 573–575.

12. Schiewe MC, Zozula S, Nugent N, Waggoner K, Borba J, Gamboa L & Whitney JB (2017) J Vis Exp 121, 54871.

13. Stringfellow DA & Givens MD (2010) Manual of the International Embryo Transfer Society (IETS), 4th edition, IET, Champaign, IL, USA.

14. Vajta G, Booth PJ, Holm P, Greve T & Callesen H (1997) CryoLetters 18, 191-195.

15. Whittingham DG (1971) Nature 233, 125-126.

CHAPTER 3

Technical Note: Transfer of caprine blastocysts vitrified by the open pulled straw (OPS) or the solid surface procedure and warmed in sucrose-free medium

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Small Ruminant Research 165 (2018) 111–114

Abstract

The present paper outlines ways of non-surgical collection and semi-laparoscopic transfer of caprine embryos. Two different ways of embryo cryo-preservation by way of vitrification are described; the open pulled straw (OPS) procedure, known to be well suited and the solid surface procedure called for in situations where contact between embryos and non-sterile liquid nitrogen is to be avoided. Based on 13 transfers of OPS-vitrified and 9 transfers of solid surface-vitrified blastocysts (2 blastocysts/recipient) it was shown that either procedure is applicable (54% vs. 56% pregnancy- and 39% vs. 44% kidding rate). Furthermore the experiment showed that warming of vitrified embryos may be accomplished by one-step procedure (88% transferable post-warming embryos), opening up the possibility to transfer vitrified embryos under field conditions.

Keywords: Goat, Embryo transfer, Cryopreservation, Vitrification, One-step-warming.

3.1. Introduction

Cryopreservation of embryos has become an established component of assisted reproduction in various mammalian species. In farm animals conventional slow freezing is most commonly applied. An extremely rapid cooling procedure, called vitrification, was introduced by Rall and Fahy in 1985. The procedure comprises brief exposure of embryos to highly concentrated permeating cryoprotectants, immediately followed by cooling at an ultra-rapid rate of 16,700 °C/min (Criado- Scholz, 2012) accomplished by direct submersion in liquid nitrogen. This approach circumvents ice crystal formation and dehydration of embryonic cells. A major breakthrough was achieved by the invention of the "open pulled straw" (OPS) procedure by Vajta et al. (1997). A point of criticism with this procedure is the potential risk of contamination resulting from direct contact of the embryos with non-sterile liquid nitrogen (Bielanski et al., 2000). The European Directive on Tissue Storage and the U.S. Food and Drug Administration call for hermetically closed aseptic systems precluding contact of embryos with liquid nitrogen (Abdelhafez et al., 2011). For that reason, the "solid surface" vitrification technique was invented, an approach by which the embryo, suspended in a tiny drop of vitrification medium, is brought in contact with the surface of a metal block cooled with liquid nitrogen. With this procedure the cooling rate approaches 10,000 °C/min (Dinnyes et al., 2000; Begin et al., 2003; Somfai et al., 2010;

Beebe et al., 2011). Once vitrified, embryos are sealed in a plastic sleeve and stored submerged in liquid nitrogen (Lindemans et al., 2004; Beebe et al., 2011).

The first successful vitrification of caprine embryos was reported by Yuswiati and Holtz (1990). When comparing transfer of caprine blastocysts cryopreserved by conventional freezing vs. vitrification by the OPS procedure, El-Gayar and Holtz (2001) and Al-Yacoub et al. (2010) achieved higher pregnancy rates with the latter. The objective of the present investigation was to determine whether it is possible to employ the solid surface vitrification procedure instead of the OPS procedure in goats. An additional aspect addressed in the present study was the warming of vitrified caprine blastocysts in a single step in sucrose-free holding medium as reconstitution diluent.

3.2. Materials and methods

3.2.1. Superovulation and embryo collection

Embryos were obtained from 31 pluriparous Boer goat does from the breeding flock of the Department of Animal Science at Goettingen University in Germany (51°46′N, 9°41′E). Does were, on average, 3.7 (2–7) yrs. of age and weighed 60 (46–79) kg. They were synchronized by providing them with progestogen releasing intravaginal pessaries that remained in place for 7d. Beginning 48 h before pessary removal porcine Follicle Stimulating Hormone (pFSH) supplemented with 40% porcine Luteinizing Hormone (pLH) (Nowshari et al., 1995) was administered by six i.m. injections at 12 h intervals of 4, 4, 2, 2, 2 and 2 Armour Units (1 AU corresponds to 10 µg purified FSH). Along with the last two FSH injections, 5 mg dinoprost (1 mL Dinolytic; Zoetis, Berlin, Germany) was administered. Does were tested for estrus with an aproned adult male at 8 h intervals and, when exhibiting standing estrus, mated once daily. Seven days after the last mating embryos were collected transcervically as described by Pereira et al. (1998) and Suyadi et al. (2000). Briefly, 16 h before collection 5 mg dinoprost was administered to induce luteolysis. With the aid of a duckbill speculum and pen light the lip of the external os cervix was grasped with a 255mm long sharp-pointed uterine tenaculum forceps (Possi; Aesculap, Tuttlingen, Germany) and gently pulled caudally almost to the vulvar orifice. A flushing catheter (Ruesch, Nelaton-Robinson, Art NR. 220500, Ch 12, Kernen, Germany) with a pliable steel stylet inserted was passed through the cervical canal. The stylet was removed,

and the catheter was further advanced into one uterine horn directed by a finger in the vaginal fornix. After infusing 20 mL Dulbecco's phosphate buffered saline (PBS), supplemented with 0.06% bovine serum albumin (BSA; A9647-50G, Sigma-Aldrich, Steinheim, Germany), 100 IU/mL penicillin and 100 µg/mL streptomycin (PAA P11-010, Darmstadt, Germany), the reflux was collected via embryo filter (75 µm Em Con Embryofilter, Albrecht, Aulendorf, Germany). To maintain an uninterrupted flow the catheter had to be gently moved to and fro. This procedure was repeated 8 times. Then the catheter was partially withdrawn and directed into the other uterine horn which was also flushed 8 times. Occasionally cellular debris was encountered in the first of a series of flushings. In these cases, the flushing was diluted by adding more diluent. Embryos that were recovered were assessed for developmental stage and morphological intactness under a stereoscope equipped with a warming stage at 20 to 40x. Blastocysts with intact zona pellucida and uniform blastomeres without visible cellular damage were vitrified.

3.2.2. Vitrification and warming of blastocysts

Within 30–60 min after flushing, embryos were transferred to 800 µL holding medium consisting of TCM 199 (M0650, Sigma, Steinheim, Germany) containing 2mM sodium bicarbonate, 2mM sodium pyruvate, 25mM Hepes-sodium salt and 1mM L-Glutamine, supplemented with 20% heat-inactivated male goat serum, at pH 7.4, osmotic pressure 280 mOsm and temperature 37 °C. Each embryo was washed twice in holding medium and, after 3 min equilibration, transferred to holding medium supplemented with 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (Me2SO) (vitrification solution No. 1). After 3 min, embryos were transferred to a 1 µL droplet of holding medium supplemented with 0.5M sucrose, 16.5% EG and 16.5% Me2SO (vitrification solution No. 2) with the aid of a 1 µL Eppendorf pipet and, within less than 40 s, they were vitrified either, at random, by the open pulled straw (OPS) or the solid surface (CVM Ring Fibreplug®) procedure.

3.2.2.1. OPS vitrification

Straws required for OPS vitrification were fabricated by softening French ministraws (0.25 mL, Minitueb, Landshut, Germany) over a hot plate at 200 °C, pulling them

to approximately half the original diameter and cutting them at the thinnest point. Vitrification was conducted according to the procedure of Vajta et al. (1997), slightly modified by El-Gayar and Holtz (2001). By touching the droplet of vitrification solution No. 2 containing the embryo with the narrow end of the drawn-out straw it was drawn into the straw by capillary force. Immediately afterwards the straw was submerged in liquid nitrogen, thin-end-first, where it was stored until transfer. For transfer, straws were removed from liquid nitrogen, held in the air for 10 s before dipping the thin end into 800 µL of holding medium at 37 °C serving as reconstitution diluent, while occluding the wide end with the tip of a finger. Embryos were expelled from the straw into the reconstitution diluent by the air warming inside the straw.

3.2.2.2. Solid surface vitrification

The equipment used for solid surface vitrification (CVM Ring Fibreplug®, manufactured by CVM™-CryoLogic, Australia) is commercially available. It consists of a metal (aluminum-nickel) block submerged in liquid nitrogen to 20mm from the top and an embryo carrier consisting of a plastic rod, 80mm long and 2mm in diameter, with a ring of 1.2mm inner diameter of 0.1mm nylon thread attached to the tip. With the aid of a 1 µL Eppendorf pipet the 1 µL-droplet of vitrification solution No. 2 containing the embryo is deposited on the ring of the carrier. There it adheres due to surface tension and, as soon as the tip of the carrier is lowered onto the metal block, it turns into a vitrified bead. The carrier with the bead is inserted into a plastic sleeve standing in liquid nitrogen. After sealing the top of the sleeve with a special welder, supplied by the manufacturer, it is completely submerged in liquid nitrogen. For warming, sleeves were partially removed from liquid nitrogen, cut open at the top and the carrier, after removal from the sleeve, was held in the air for 10 s before the ring end was dipped into the reconstitution diluent at 37 °C.

With either procedure, after warming embryo morphology was assessed under a stereoscope equipped with a warming stage. Embryos were classified from 1 (very good) to 5 (degenerate) according to the guidelines of the International Embryo Transfer Society (Stringfellow and Givens, 2010). Of 60 vitrified-warmed embryos recovered, 42 that were

classified as “very good” or “good” were transferred, two embryos at a time, to 21 recipients. Transfer was carried out within 5–10 min after removal of the embryos from liquid nitrogen.

3.2.3. Embryo transfer

Recipients were pluriparous Boer goat does of similar origin, weight and age as the donors. They were synchronized during the breeding season by using intravaginal progesterone-impregnated CIDRs. Upon CIDR removal after 7d, two i.m. injections of 5 mg dinoprost were administered at 12 h interval. Estrus detection was carried out at 8 h intervals with the aid of an aproned adult male. Six days after the last standing estrus semi-laparoscopic embryo transfer was performed. Beforehand, does were deprived of feed for 2 d and of water for 1 d. They were anesthetized by i.v. administration of 0.1 mL/10 kg Sedaxylan (20 mg xylazine; Eurovet Animal Health, Bladel, Netherlands) and 0.1 mL/10 kg Ursotamin (0.1 g ketamine; Serumwerke, Bernburg, Germany). After shaving, washing and disinfection of the area cranial to the udder, does were placed on a laparoscopy cradle in dorsal recumbence. The rear of the animal was elevated to an angle of 20°. The skin was nicked with the tip of a scalpel blade about 8 cm cranial to the udder on the mid-line and 8 cm lateral to the mid-line. At these points cannulae of 5mm diameter with a trocar inserted were punched through the abdominal wall. Trocars were removed and by introducing the endoscope (Panaview-Optic, 5mm diameter, 25° angle, Wolf, Knittlingen, Germany) through the lateral cannula the reproductive organs could be inspected, assisted by an exploratory probe (400mm long, 5mm in diameter; Storz, Tuttlingen, Germany) introduced through the mid-line cannula. To create sufficient intra-abdominal space the abdominal wall was manually lifted up by the lateral cannula. If a well-developed corpus luteum was identified, the punch hole on the mid-line was extended to a 20–30mm incision with a scalpel blade. Under endoscopic control the tip of the uterine horn ipsilateral to the ovary carrying a corpus luteum was grasped close to the utero-tubal junction with blunted 255mm tenaculum forceps (Possi; Aesculap, Tuttlingen, Germany) introduced through the small incision. Care was taken not to pinch the organ. A loop of 30–40mm of uterine horn was exteriorized and, while holding it gently between thumb and index finger, punctured with a blunted 22-gauge hypodermic needle. Through the puncture hole a unopette (20 µL; Becton Dickinson, Plymouth, UK) containing two embryos, suspended in 0.2 mL medium,

was introduced. Embryos were deposited in the uterine lumen 20–50mm from the utero-tubal junction. After repositioning the uterus, the skin incision was closed with a single suture. A prophylactic i.m. injection of 200,000 IU Procain Penicilin G and 200 mg Dihydrostreptomycin sulphate was administered. Thirteen recipients received embryos vitrified by the OPS procedure; nine by the solid surface procedure. Thirty days after transfer, pregnancy was diagnosed by transrectal ultrasonography as described by Padilla-Rivas et al. (2005).

Recovery and re-expansion of blastocysts as well as pregnancy rate were analyzed by Chi-square test; embryo quality by Kruskal-Wallis test (Steel and Torrie, 1960).

Table 3.1. Post-warming recovery and transfer of caprine blastocysts cryopreserved by open pulled straw (OPS, 13 recipients) and solid surface ("CVM Fibreplug™", 9 recipients) vitrification.

Vitrification system	Number of vitrified blastocysts	Post-warming recovery %	Transferable blastocysts %	Recipients pregnant (30d) %	Recipients kidding %	Mean litter size
OPS	36	78	93	54	38	1.0
CVM	24	92	82	56	44	1.5
Overall	60	83	88	55	41	1.2

Differences were not statistically significant.

3.3. Results and discussion

As shown in Table 3.1., post- warming recovery rates of OPS- and solid surface-vitrified embryos were 78% and 92%, respectively ($P > 0.05$). Mean embryo quality after warming was 1.60 (SEM 0.14) for OPS- and 1.54 (SEM 0.16) for solid surface-vitrified embryos; the proportion of transferable embryos (quality 1 and 2) being 93% and 82% respectively (both $P > 0.05$). Pregnancy rates 30 days after transfer were 54% for OPS- and 56% for solid surface-vitrified embryos ($P > 0.05$); corresponding kidding rates were 38% and 44% ($P > 0.05$), and litter size 1.0 and 1.5, respectively. These results indicate that for the vitrification of caprine blastocysts both the OPS and the solid surface procedure are suitable. This result agrees with findings by Begin et al. (2003) who vitrified caprine oocytes and two- to four-cell embryos, and with findings on human (Kuwayama et al., 2005) and murine embryos (Abdelhafez et al., 2011). The slightly higher post-warming recovery of embryos vitrified by the solid surface procedure may be explained by the fact that in some

cases embryos that got detached from the carrier could be recovered from the sleeve and picked up with a 10 µL Eppendorf pipette. With regard to the proportion of embryos that were of transferable quality there was no difference between OPS- and solid surface-vitrification (93% vs. 83%), indicating that the difference in cooling rate (16,700 vs. 10,000 °C/min) appears to be negligible.

From a technical point of view, the OPS-procedure is easier to carry out than the solid surface procedure investigated in the present study. Embryos are readily picked up owing to capillary force when touched with the tip of the OPS straw, which can then be instantly immersed in liquid nitrogen. The solid surface procedure requires considerable skill and experience to minimize the time needed for placing embryos on the carrier (under a stereoscope), insert the carrier with the vitrified bead into a sleeve and seal the sleeve before submerging it in liquid nitrogen. Warming and embryo recovery, on the other hand, is easier to accomplish with the solid surface system, although dexterity and routine are required to avoid detachment of vitrified beads from the ring at the tip of the carrier. On the whole, the OPS procedure is easier to implement and, due to the possibility to fabricate your own equipment (Vajta et al., 1997; El-Gayar and Holtz, 2001; Sun et al., 2008), less costly, whereas the solid surface system is more demanding with regard to skill and expense, but has the advantage of being hygienically sound, which is relevant in situations where export of embryos is intended. An accurate assessment which of the two is the more efficient vitrification technique would require more data generated under comparable conditions.

A principal reason for vitrification not being more commonly used for embryo cryopreservation in farm animals is the assumption that removal of the cytotoxic permeating cryoprotectants present in high concentration in vitrification medium after warming is essential. That applies to slow freezing where it is accomplished by stepwise dilution or by a temporary sojourn in sucrose-containing diluent, where sucrose has the function of maintaining high extracellular osmotic pressure while permitting the cryoprotectants to diffuse from the cells without them suffering osmotic damage by massive influx of water (Mazur and Schneider, 1986; Pedro et al., 1997). Insertion of this additional step would wield the method less suitable for application in the field, as it would require the availability of a stereoscope and laboratory equipment. For bovine embryos cryopreserved by slow freezing an in-straw dilution procedure has been devised that provides acceptable pregnancy rates, although success rates are not quite as good as with the more cumbersome laboratory procedure. It has been attempted to establish in-straw systems for vitrified embryos (Isachenko et al., 2003; Yang et al., 2007; Inaba et al., 2011; Caamano et al., 2014). Experience with

murine blastocysts of our own group (El-Gayar et al., 2008; Al- Yacoub et al., 2013) and others (Yang et al., 2007; Rodriguez et al., 2010), as well as a single report on caprine embryos by Guignot et al. (2006) on single step warming, gave rise to the notion that these efforts are dispensable. This assertion appears justified in view of 88% transferable blastocysts after warming in a single step in sucrose-free diluent achieved in the present study. It may be surmised that, due to the extremely short exposure of embryos to the vitrification solution with highly concentrated permeating cryoprotectants, merely small amounts of cryoprotectants are able to penetrate the blastomeres before they are plunged into liquid nitrogen As a consequence, there will not be much of a cytotoxic effect, nor will there be a substantial osmotic differential between the cytoplasm of the embryonic cells and the warming medium and, as a consequence, not much of an osmotic strain acting upon the cells.

It may be concluded that solid surface vitrification of caprine embryos is a viable alternative to the well-proven OPS procedure under circumstances where strict hygienic precautions are in demand. Furthermore, this study shows that vitrified blastocysts may be warmed in a single step in sucrose-free medium. Vitrification, having been shown to yield more favorable pregnancy rates in goats than conventional slow freezing, qualifies as a practicable option opening up the possibility to transfer vitrified embryos under field conditions. These findings encourages the employment of vitrification as an efficient means of cryopreserving caprine embryos and, with the availability of a non-surgical means of embryo collection and a low invasive procedure for embryo transfer may serve as encouragement to seriously consider utilizing the biotechnology of embryo transfer, perhaps combined with associated biotechnologies, for promoting progress in breeding, worldwide trade in genetic resources and, where populations are in danger of extinction, the establishment of embryo banks in goats.

Conflict of interest

None of the authors have any conflict of interest to declare.

Acknowledgments

The authors owe thanks to Consartic, Schoellkrippen, Germany for making the CVM Ring Fibreplug® vitrification equipment available. pFSH and pLH was donated by J.-F. Beckers (Faculty of Veterinary Medicine, State University of Liège, Belgium). G. Das

assisted with the statistical analysis. D. Daniel and the barn staff deserve thanks for assisting with the handling of the animals. The senior author (G.D.) was supported by a fellowship of the German Academic Exchange Service (DAAD); the second author (C.M.) by a fellowship of the Consejo Nacional de Ciencia y Tecnologia (CONACYT) – German Academic Exchange Service (DAAD) Mexico.

3.4. References

1. Abdelhafez, F., Xu, J., Golderberg, J., Desai, N., 2011. Vitrification in open and closed carriers at different cell stages: assessment of embryo survival, development, DNA integrity and stability during vapor phase storage for transport. BMC Biotechnol. 11, 29.

2. Al-Yacoub, A.N., Gauly, M., Holtz, W., 2010. Open pulled straw vitrification of goat embryos at various stages of development. Theriogenology 73, 1018–1023.

3. Al-Yacoub, A.N., Gauly, M., Holtz, W., 2013. Is sucrose required in open pulled straw (OPS) vitrification of mouse embryos? CryoLetters 34, 466–470.

4. Beebe, L.F., Bouwman, E.G., Mcllfatrick, S.M., Nottle, M.B., 2011. Piglets produced from in vivo blastocysts vitrified using the cryologic vitrification method (solid surface vitrification) and a sealed storage container. Theriogenology 75 (8), 1453–1458.

5. Begin, I., Bhatia, B., Baldassarre Dinnyes, A., Keefer, C.L., 2003. Cryopreservation of goat oocytes and in vivo derived 2- to 4-cell embryos using cryoloop (CLV) and solidsurface vitrifcation (SSV) methods. Theriogenology 59, 1839–1850.

6. Bielanski, A., Nadin-Davis, S., Saap, T., Lutze-Wallace, C., 2000. Viral contamination of embryos cryopreserved in liquid nitrogen. Cryobiology 40, 110–116.

7. Caamano, J.N., Gomez, E., Trigal, B., Munoz, M., Carrocera, S., Martin, D., Diez, C., 2014. Survival of vitrified in vitro-produced bovine embryos after one-step warming instraw cryoprotectant dilution procedure. Theriogenology 83, 881–890.

8. Criado-Scholz, E., 2012. The problem of contamination: open vs. closed vs. semi-closed vitrification systems. In: Katkov, Igor (Ed.), Current frontiers in cryopreservation. In Tech, open access publisher.

9. Dinnyes, A., Dai, Y., Jiang, S., Yang, X., 2000. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. Biol. Reprod. 63, 513–518.

10. El-Gayar, M., Holtz, W., 2001. Technical note: vitrification of goat embryos by the open pulled-straw method. J. Anim. Sci. 79, 2436–2438.

11. El-Gayar, M., Gauly, M., Holtz, W., 2008. One-step dilution of open-pulled-straw (OPS)- vitrified mouse blastocysts in sucrose-free medium. Cryobiology 57, 191–194.

12. Guignot, F., Bouttier, A., Baril, G., Salvetti, P., Pignon, P., Beckers, J.F., Touze, J.L., Cognie, J., Traldi, A.S., Cognie, Y., Mermillod, P., 2006. Improved vitrification method allowing direct transfer of goat embryos. Theriogenology 66, 1004–1011.

13. Inaba, Y., Aikawa, Y., Hirai, T., Hashiyada, Y., Yamanouchi, T., Misumi, K., Oshtake, M., Somfai, T., Kobayashi, S., 2011. In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. J. Reprod. Dev. 57, 437–443.

14. Isachenko, V., Folch, J., Isachenko, E., Nawroth, F., Krikokharchenko, A., Vajta, G., 2003. Double vitrification of rat embryos at different developmental stages using an identical protocol. Theriogenology 60, 445–452.

15. Kuwayama, M., Vajta, G., Leda, S., Kato, O., 2005. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. Reprod. Biomed. Online 11, 608–614.

16. Lindemans, W., Sangalli, L., Kick, A., Earl, C.R., Fry, R.C., 2004. Vitrification of bovine embryos using the CLV method. Reprod. Fertil. Dev. 16, 174 (abstr.).

17. Mazur, P., Schneider, U., 1986. Osmotic response of preimplantation mouse and bovine embryos and their cryobiological implications. Cell Biophys. 8, 259–285.

18. Nowshari, M.A., Beckers, J.F., Holtz, W., 1995. Superovulation of goats with purified pFSH supplemented with defined amounts of pLH. Theriogenology 43, 797–802.

19. Padilla-Rivas, G.R., Sohnrey, B., Holtz, W., 2005. Early pregnancy detection by real-time ultrasonography in Boer goats. Small Rumin. Res. 58, 87–92.

20. Pedro, P.B., Zhu, S.E., Makino, N., Edashige, K., Kasai, M., 1997. Effects of hypotonic stress on the survival of mouse oocytes and embryos at various stages. Cryobiology 35, 150–158.

21. Pereira, R.J.T.A., Sohnrey, B., Holtz, W., 1998. Nonsurgical embryo collection in goats treated with prostaglandin F2α and oxytocin. J. Anim. Sci. 76, 360–363.

22. Rall, W.F., Fahy, G.M., 1985. Ice-free cryopreservation of mouse embryos at −196 degrees Celsius by vitrification. Nature 313, 573–575.

23. Rodriguez, P., Ongarratto, F., Scherer, D., de Avila Rodriguez, B., Rodriguez, J., 2010.

24. Survival of vitrified mouse blastocysts loaded into glass micro-capillaries. Rev.

Colomb. Cienc. Pecu. 23, 28–34.

25. Somfai, T., Noguchi, J., Kaneko, H., Nakai, M., Ozawa, M., Kashiwazaki, N., Egerszegi, I., Rátky, J., Nagai, T., Kikuchi, K., 2010. Production of good-quality porcine blastocysts by in vitro fertilization of follicular oocytes vitrified at the germinal vesicle stage. Theriogenology 73, 147–156.

26. Steel, R.G.D., Torrie, J.H., 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co., New York.

27. Stringfellow, D.A., Givens, M.D., 2010. Manual of the International Embryo Transfer Society (IETS), 4th ed. IETS, Champaign, IL.

28. Sun, X., Li, Z., Yi, Y., Chen, J., Leno, G.H., Engelhardt, J.F., 2008. Efficient term development of vitrified ferret embryos using a novel pipette chamber technique. Biol. Reprod. 79, 832–840.

29. Suyadi, Sohnrey, B., Holtz, W., 2000. Transcervical embryo collection in Boer goats. Small Rumin. Res. 36, 195–200.

30. Vajta, G., Booth, P.J., Holm, P., Greve, T., Callesen, H., 1997. Successful vitrification of early stage bovine in vitro-produced embryos with the Open Pulled straw (OPS) method. CryoLetters 18, 191–195.

31. Yang, Q.-E., Hou, Y.-P., Zhou, G.-B., Yang, Z.-Q., Zhu, S.E., 2007. Stepwise in-straw dilution and direct transfer using open pulled straws (OPS) in the mouse: a potential model for field manipulation of vitrified embryos. J. Reprod. Dev. 53, 211–218.

32. Yuswiati, E., Holtz, W., 1990. Successful transfer of vitrified goat embryos. Theriogenology 34, 629–632.

CHAPTER 4

Semi-laparoscopic and transcervical transfer of goat embryos

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Abstract

Embryo transfer in goats is generally accomplished by laparoscopy. With the objective to establish a non-surgical technique, in the present study transcervical transfer of vitrified embryos to non-anesthetized does was attempted and compared to semi-laparoscopic transfer. Embryos vitrified either by the “Open Pulled Straw” (OPS) or the CryoLogic Vitrification Method-“CVM Ring Fibreplug™” method, were warmed in a single step in sucrose-free medium. Thirty-one Boer does served as recipients. In 22 does Semi- laparoscopic transfer was performed and in nine recipients non-surgical transcervical transfer using a human transfer catheter. Pregnancy was diagnosed by transrectal ultrasonography 30 days after transfer. Recovery rate of vitrified embryos after warming was 78% for OPS- and 88% for CVM-vitrified embryos (P>0.05). The corresponding proportions of transferable embryos (quality 1 or 2 on a scale of 1 to 3) were 87% and 80%, respectively (P>0.05). For OPS and CVM vitrified blastocysts pregnancy rates 30 days after semi-laparoscopic transfer were 54% and 56% and kidding rates, 39% and 44% (both P>0.05). Thirty days after semi-laparoscopic transfer 12 out of 22 does (55%) were found pregnant, nine of which (41%) kidded (average litter size 1.2), whereas, after transcervical transfer, only one out of 9 does (11%) were found pregnant giving birth to a single kid. These results permit the conclusion that transcervical transfer of embryos is possible but better means have to be established to warrant acceptable pregnancy rates. As of now semi-laparoscopic embryo transfer is the far more promising technique.

Keywords: goat; laparoscopy; embryo transfer; transcervical transfer; semi-endoscopic transfer

4.1. Introduction

The first embryo transfer in goats was reported by Warwick et al. (1934). During the early 1980s, ten years later than in cattle, it was considered an established technique (Thibier and Guerin, 2000). The successful outcome of embryo transfer depends on factors related to both embryo and the recipient. Quality and stage of the embryo, age, parity and condition of the recipient and the presence of corpora lutea at the time of transfer, are essential factors. Various studies showed that morphologically immaculate embryos have a greater chance to be carried to term than poorer grade embryos (Schneider et al., 1980; Hasler, 2001; Bari et al., 2003). They are more likely to adjust to or alter the uterine environment in their favor (Bari et al., 2003). However, no difference in embryo survival rate between embryos qualified as excellent or good (Alabart et al., 1995), or between Grade 1 and 2 embryos (Sreenan and Diskin, 1987), has been reported. Other factors that have been

reported to have an influence include the number of embryos transferred (Armstrong et al., 1983; Tervit et al., 1986), the site of transfer (Moore and Shelton, 1964; Rowson and Moor, 1966; Armstrong and Evans, 1983; Holtz et al., 2000) and synchrony of donor and recipient (Moore and Shelton, 1964; Hasler et al., 1987; Spell et al., 2001).

In cows and mares, embryo transfer techniques are well established. The reproductive organs may be manipulated per rectum which, on account of morphological conditions, is not possible in small ruminants (McMillan and Hall, 1994; Ishwar and Memon, 1996). However, it is possible to conduct artificial insemination as well as collection and transfer of embryos by laparoscopic means (Abdullah et al., 1995; Nowshari et al., 1995; Ishwar and Memon, 1996; El- Gayar et al., 2001; Guinot et al., 2006; Rahman et al., 2008; Lopez-Saucedo et al., 2013). One of two objectives of the present experiment was to attempt to transfer vitrified goat embryos non-surgically. In addition, the transferability of embryos cryopreserved by the OPS and the CryoLogic Vitrification Method -CVM vitrification technique was compared.

4.2. Material and methods

4.2.1. Superovulation and embryo collection

Embryos were collected from 60 pluriparous Boer goat does from our own breeding flock at Goettingen, Germany (51°46’N, 9°41’E), on average 3.7 (2 to 7) years old and weighing 60 (46 to 79) kg. Estrus was synchronized by providing does with progestagen releasing intravaginal pessaries, either “Controlled Internal Drug Releasing” devices (CIDR, Easy Breed, Zoetis, Berlin, Germany) containing 0.3g progesterone or Chronogest sponges (Intervet, Beaucouzé, France) containing 20mg flurogestone acetate. Pessaries were removed after 7 days and superovulation was induced by administering porcine Follicle Stimulating Hormone (pFSH), supplemented with 40% porcine Luteinizing Hormone (pLH) (Nowshari et al., 1995) at dosages of 4, 4, 2, 2, 2 and 2 Armour Units at 12h intervals, beginning 48 hours before pessary removal. Along with the last two FSH injections does received i.m. injections of 5 mg dinoprost (1ml Dinolytic; Zoetis, Berlin, Germany). Does were tested for estrus with an aproned adult male at 8-hour intervals and mated daily as long as they would posture. Seven days after the end of standing estrus, transcervical embryo collection was conducted as described by Suyadi et al. (2000).

Briefly, sixteen hours before embryo collection luteolysis was induced by i.m. administration of 5.0 mg dinoprost. With the aid of a duck-bill speculum and pen light a lip of the os cervix was grasped with a 255mm long sharp-pointed uterine tenaculum forceps (Possi; Aesculap, Tuttlingen, Germany) and gently pulled caudally almost to the vulvar orifice. A flushing catheter (Ruesch, Nelaton-Robinson, Art No. 220500, Ch 12, Kernen, Germany) with a pliable stylet inserted was passage through the cervical canal. After removal of the stylet the catheter was advanced further into one uterine horn directed by a finger located in the fornix. After flushing the horn eight times with 20ml Dulbecco's phosphate buffered saline (PBS) supplemented with 0.06% bovine serum albumin (BSA; A9647-50G, Sigma-Aldrich, Steinheim, Germany), 100 IU/ml penicillin and 100 µg/ml streptomycin (PAA P11-010, Darmstadt, Germany), the catheter was partially withdrawn and directed into the other uterine horn which was also flushed eight times. The reflux of each flushing was collected via embryo filter (75µm Em Con Embryofilter, Albrecht, Aulendorf, Germany). To maintain an uninterrupted flow, the catheter had to be gently moved to and fro. Embryos were recovered and evaluated for morphological intactness at 20 to 40x under a stereoscope equipped with a warming stage. Only blastocysts with no visible morphological defects were vitrified. Unless otherwise stated, all chemicals used were purchased from Sigma Aldrich (Steinheim, Germany).

4.2.2. Vitrification and warming of blastocysts

Within 30 to 60 minutes after collection, embryos (n=36) were randomly allocated to either the open pulled straw (OPS) or the CVM Ring Fibreplug™ (CVM) vitrification system. Vitrified embryos were warmed by removing the straw from liquid nitrogen, holding it in the air at room temperature for 10 seconds and submerging its tip in holding medium at 37°C. Within five minutes after warming the morphological appearance of the embryos was assessed at 20 to 40x under a stereoscope equipped with a warming stage. Embryos classified as morphologically very good or good, according to the guidelines of the International Embryo Technology Society (Stringfellow and Givens, 2010), were transferred.

4.2.3. Embryo transfer

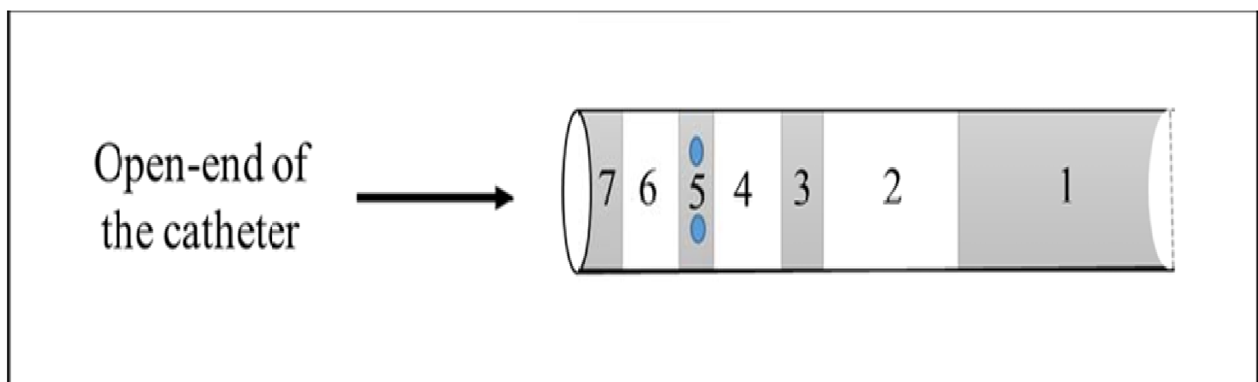
Thirty-one pluriparous Boer goat does from our own breeding flock of similar weight and age as the donors served as recipients. During the breeding season (September

to January) does were synchronized with CIDRs that remained in place for 7 days, followed by two i.m. injections of 5 mg dinoprost administered at 12h interval. Does were checked for estrus eight-hourly with an aproned adult buck. Six days after the end of standing estrus embryo transfer was performed. Before semi-laparoscopic transfer recipients were deprived of feed for 48h and of water for 24h.

Semi-laparoscopic embryo transfer was performed in 22 goats, as described by Al-Yacoub et al. (2010). Briefly, does were anesthetized by i.v. administration of 0.1ml /10kg Sedaxylan (20mg xylazine; Eurovet Animal Health, Bladel, Netherlands) and 0.1ml/10kg Ursotamin (0.1g ketamine; Serumwerke, Bernburg, Germany). The area cranial to the udder was clipped, washed and disinfected with iodine. Then does were placed on a laparoscopy cradle in dorsal recumbence. Approximately 8 cm cranial to the udder, on the mid-line and 8 to 10cm lateral to the mid-line, the skin was nicked with the tip of a scalpel blade. At these points two trocars of 5 mm diameter, enclosed in a cannula, were punched through the abdominal wall. After withdrawing the trocars, an endoscope (Panaview-Optic, 5 mm diameter, 25° angle, Wolf, Knittlingen, Germany), through the laterally located cannula and an exploratory probe (5mm diameter, 400mm in length, Storz, Tuttlingen, Germany); through the other cannula were introduced. By raising the abdominal wall with the laterally located cannula, enough intra-abdominal space was created to visualize the reproductive organs. After noting which ovary carried a well-developed corpus luteum, the punch hole on the mid-line was extended to a 20 mm incision with the aid of a scalpel. A blunted tenaculum forceps of 255mm length (Possi; Aesculap, Tuttlingen, Germany) was introduced and the tip of the ipsilateral uterine horn close to the utero-tubal junction, was grasped taking care not to pinch it. A loop of about 3 cm of uterine horn was exteriorized through the incision and, holding it gently between thumb and index finger, the wall was punctured with a blunted 22-gauge hypodermic needle 3 to 5 cm from the utero-tubal junction. Through the puncture hole a unopette (20µl; Becton Dickinson, Plymouth, UK) was introduced and two embryos, suspended in about 0.2 mL medium, were deposited in the uterine lumen about one cm from the insertion point. After repositioning the uterine horn, the mid-line incision was closed with a single skin suture. Does received a prophylactic i.m. injection of 2 mL penicillin/streptomycin (200000 UI Procain Penicilin G/200mg Dihydrostreptomycisulphate). Of the 22 recipients, 13 received embryos vitrified by the OPS method, and 9 embryos vitrified by the CVM method. Pregnancy was diagnosed by transrectal ultrasonography conducted 30 and 45 days after transfer.

Non-surgical transfer of embryos was carried out in nine recipients as follows. Does were immobilized and prevented from squatting by placing them in a crate equipped with a hammock with holes for the front legs (Suyadi et al., 2000). A sharp-pointed tenaculum forceps was used to grasp and gently pull the os cervix toward the vulvar orifice in the way described for embryo collection. A transvaginal embryo transfer catheter set for humans (No. 13369, Labotect, Goettingen, Germany), consisting of an outer round-tip guiding cannula, 230 mm in length and 1.5 mm outer diameter, enclosing an inner catheter of 0.7 mm outer and 0.5 mm inner diameter was used. The guiding cannula reinforced by a stainless-steelu stylet was passaged through the cervix. After removal of the stylet, the cannula was advanced and directed into the uterine horn ipsilateral to the corpus luteum (located by ultrasonography beforehand) with a finger located in the fornix. The inner catheter, loaded by successive aspiration (with the aid of a 1ml syringe) of 0.2 ml holding medium, 2 mm air, 0.02 ml holding medium, 1 mm air, a small quantity holding medium containing two embryos, 1 mm air and another 0.01 ml holding medium (Fig 4.1) was threaded into the guiding cannula. After expulsion of the embryos, inner and outer catheter were withdrawn. Pregnancy detection by transrectal ultrasonography was conducted 30 and 45 days after transfer. Four of nine recipients received embryos vitrified by the OPS technique, five by the CVM technique. Blood samples were taken by jugular venipuncture 14 and 21 days after transfer and analyzed for content of Pregnancy Associated Glycoprotein (PAG). Does received a prophylactic i.m. injection of 2 mL penicillin/streptomycin (200000 UI Procain Penicilin G- 200mg Dihydrostreptomycin sulphate).

Figure 4.1. Loading of embryo transfer inner catheter; 1: 0.2 ml medium; 2: 2 mm air; 3: 0.02 ml medium; 4:1 mm air; 5: a small quantity of medium containing the embryos; 6: 1 mm air and 7: 0.01 mm medium.



4.2.4. Statistical analysis

Recovery, re-expansion and pregnancy rates were analyzed using the Chi-square test (Steel and Torrie, 1960).

4.3. Results

As shown in Table 4.1, recovery rate after warming was 78% for OPS- and 88% for CVM-vitrified embryos (P>0.05). The proportions of transferable embryos (quality 1 and 2) were, correspondingly, 87% and 80% (P>0.05). Transrectal ultrasonography 30 days after transfer revealed that pregnancy rates for semi-laparoscopically transferred vitrified blastocysts were 54% for OPS and 56% for CVM (P>0.05) and kidding rates 39% and 44%, respectively (P>0.05). Thirty days after semi-laparoscopic transfer, 12 out of 22 does (55%) were found pregnant, nine of which (41%) kidded (average litter size 1.2), whereas, after transcervical transfer, only one out of 9 does (11%) were found pregnant giving birth to a single kid. In the doe that did eventually kid, serum PAG concentration on day 14 after transfer was 0.7ng/ml and on day 21, 1.7ng/ml. Three of the does returned to estrus with a delay and had serum PAG levels at 14 and 21 days after transfer of 0.6ng/ml. However, no fetal structures were discernible by ultrasound. In the remaining does serum PAG levels remained below 0.4ng/ml.

Table 4.1. Post-warming recovery and pregnancy rate of goat embryos transferred via semi-laparoscopic or by transcervical means.

Method of vitrification	Blastocysts vitrified	Post-warming recovery		Transferable embryos		Semi laparoscopic transfers				Transcervical transfers			
						Pregnant recipients day 30				Pregnant recipients day 30			
		n	%	n	%	n	%	Does kidding	Litter size	n	%	Does kidding	Litter size
OPS	50	39	78	34	87	7/13	54	5/13	1.0	0/4	0	0/4	-
CVM	40	35	88	28	80	5/9	56	4/9	1.5	1/5	20	1/5	1.0
Overall	90	74	82	62	84	12/22	55^{a}	9/22	1.2	1/9	11^{b}	1/9	1.0

^{ab} Within rows means with different superscripts differ ($P<0.05$)

4.4. Discussion

The prime intention of the present study was to establish a non-surgical technique for transfer ng embryos in goats. The semi-laparoscopic transfer technique was intended as control method and, also, to compare OPS-vitrified and CVM-vitrified blastocyst. The semi- laparoscopic technique originally established by Bari et al. (1999) for embryo recovery in sheep, later employed for embryo transfer in pigs (Wallenhorst and Holtz, 2002) and goats (Al-Yacoub et al., 2010) proved to be effective leading to a pregnancy rate of 55%. Most other authors reported similar results (Abdullah et al., 1995; Ishwar and Memon, 1996; Rahman et al., 2008) with the exception of El-Gayar et al. (2001) who reported a kidding rate of 93%. The semi- laparoscopic procedure has many advantages over a surgical approach: fewer post-surgical adhesions (McKelvey et al., 1984), the possibility to be conducted repeatedly, much faster and, of particular relevance, associated with considerably less post-surgical distress on the side of the animal. In the present study none of the goats showed signs of post-surgical pain or distress. Nevertheless, endoscopy remains a surgical intervention involving anesthesia, a reasonably aseptic environmental, sophisticated equipment and an experienced, skilled operating team. The advantages of the non-surgical transcervical technique attempted in the present study over the semi-laparoscopic procedure is that animals do not need to be of anesthetized, there is no need for pre-surgical starving and post-surgical care and substantial saving in time and expense. However, as yet non-surgical procedures have never produced satisfactory results (Otsuki and Soma, 1964; Lin et al., 1979; Flores-Foxworth et al., 1992; Fonseca et al., 2014; Holtz and Sohnrey, unpublished data). The present study is no exception: pregnancy rate was 11% and only one viable kid was born. At the present state the semi-laparoscopic approach is still preferable. Further research should be focused on ways of improving the transcervical technique. One concern might be irritation of the uterine epithelium as the tip of the catheter is advanced into the curved uterine horn. It might cause an inflammatory response at the transfer site. Bin Wu (2012) suggested that in humans it is critical to avoid the initiation of uterine contractility. Therefore, pliable catheters and gentle manipulation appear pertinent. Mansour and Aboulghar (2002), suggested that the adhesiveness of the cervical mucus picked up during the cannulation procedure might result in removal of the embryo from the site of deposition during catheter withdrawal. Experimenting with catheters of different lengths and pliability showed that the majority of catheters destined for humans required reinforcement to penetrate the caprine cervix. A suitable pliable stylet has to be available. The transvaginal embryo transfer catheter set (No. 13369, Labotect, Goettingen, Germany) used in the present study was selected for permitting passage of the cervical canal with the

least complication. Moreover, it had the smoothest tip. However, the inner catheter carrying the embryos to be transferred was partly reinforced by an integrated aluminum stylet which is not flexible enough to negotiate the convolutions of the uterine horn. Consequently the desired transfer site might not have been reached. Occasionally, the aluminum stylet had even damaged the outer catheter. Most other catheters were considered unsuitab e because the lumen was too sma l for the reinforcing stylet to be inserted. Use of a special endoscope, small enough to be threaded through the cervix, proposed by Colagross- Shoulten et al. (2014) might help to determine what it takes to reach a suitable site for embryo deposition without triggering a rejection mechanism.

Other feasible causes for the failure to establish a transcervical transfer might be the release of oxytocin induced by manipulation of the cervix. Otsuki and Soma (1964) suggested that oxytocin might cause the release of prostaglandin F2α. The contractions brought about by these hormones might result in expulsion of embryos. With the intention to block the release of oxytocin, Otsuki and Soma (1964) applied hyoscine-n-butylbromide or cocaine hydrochloride, yet without noticeable effect. Agrawal and Bhattacharyya (1982) reported a pregnancy rate of 39% after penetrating the cervix without fixation of the os cervix.

The present and previous unpublished results indicate that transcervical transfer of embryos in goats is possible but future studies will have to step by step lead to establishing a practicable solution.

Acknowledgments:

The authors owe thanks to J.F. Beckers (Faculty of Veterinary Medicine, State University of Liège, Belgium) for providing pFSH and pLH, Labotect, Göttingen, Germany for providing the embryo transfer set catheters, Consartic, Schoellkrippen, Germany for making the CVM Ring Fibreplug™ vitrification equipment available. The senior author (G.D.) was supported by a fellowship of the German Academic Exchange Service (DAAD); the second author (C.M.) by a fellowship of the Consejo Nacional de Ciencia y Tecnologia (CONACYT) - German Academic Exchange Service (DAAD).

4.5. References

1. Abdullah R.B., Shamsul A., Malik A. and Wan Khadijah W.E., 1995. Production of kids through embryo transfer in goats assisted by a laparoscope. Proc 7th Malaysia Soc Anim Prod Ann Conf, (MSAP'95), Penang, Malaysia: 59-60.

2. Agrawal K.P. and Bhattacharyya N.K., 1982. Non-surgical transplantation of embryos in goats. Proc Intern Congr on Goat Disease, Tucson, AZ: 340 (abstr).

3. Al-Yacoub A.N., Gauly M. and Holtz W., 2010. Open pulled straw vitrification of goat embryos at various stages of development. Theriogenology 73: 1018-1023.

4. Anon, 1994. Embryo transfer in small ruminant. Norsk Veterinaertidsskrift, 106: 750-753.

5. Armstrong D.T., Pfitzners A.P., Warnes G.M. and Seamark R.F., 1983. Superovulation treatment and embryo transfer in Angora goats. J Repro Fert 67: 403-410.

6. Armstrong D.T. and Evans G., 1983. Factors influencing success of embryo transfer in sheep and goats. Theriogenology19: 31-42.

7. Bari F., Khalidb M. and Haresign W., 2003. Factors affecting the survival of sheep embryos after transfer within a MOET program. Theriogenology 59: 1265-1275.

8. Bin Wu, 2012. Advances in Embryo Transfer, Advances in Embryo Transfer, InTech, Available from: http://www.intechopen.com/books/advances-in-embryo-transfer/advancesin- embryo-transfer.

9. Cognie Y., 1999. State of the art in sheep-goat embryo transfer. Theriogenology 51: 105-116.

10. Colagross-Schouten A., Allison D., Brent L. and Lissner E., 2014. Successful use of endoscopy for transcervical cannulation procedures in the goat. Repro Dom Anim 49: 909-912.

11. El-Gayar M. and Holtz W., 2001. Technical note: Vitrification of goat embryos by the open pulled-straw method. J Anim Sci 79: 2436-2438.

12. Flores-Foxworth G.A., McBride B.M. and Kraemer D.C., 1992. A comparison between laparoscopic and transcervical embryo collection and transfer in goats. Theriogenology 37:213.

13. Fonseca J.F., Esteves L.V., Zambrini F.N., Brandão F.Z., Peixoto M.G.C.D., Verneque R.S., Siqueira L.G.B. and Viana J.H.M., 2014. Viable offspring after successful non-surgical embryo transfer in goats. Arq. Bras. Med. Vet. Zootec. 66 no.2.

14. Guignot F., Bouttier A., Baril G., Salvetti P., Pignon P., Beckers J.F., Touze J.L., Cognie J., Traldi A. S., Cognie Y. and Mermillod P., (2006). Improved vitrification method allowing direct transfer of goat embryos. Theriogenology 66, 1004-1011.

15. Hasler J. F., McCauley A. D., Lathrop W. F. and Foote R. H., 1987. Effect of donor-embryo- recipient interactions on pregnancy rate in a large-scale bovine embryo transfer program. Theriogenology 27:139-168.

16. Hasler J. F., 2001. Factors affecting frozen and fresh embryo transfer pregnancy rates in cattle. Theriogenology 56:1401-1415.

17. Holtz W.,Nowshri M. and Sohnrey B., 2000. Unilateral or bilateral transfer of embryos in Boer goat. 7th Intern Conf Goats, Nouzilly, France, May 15-21, 494.

18. Holtz W., 2005. Recent developments in assisted reproduction in goats. Small Rumin Res 60: 95-110.

19. Ishwar A.K. and Memon M.A., 1996. Embryo transfer in sheep and goat: a review, Small Rumin Res 19: 35-43.

20. Lin A.Ch., 1979. Non-surgical embryo transfer in goats. Memoirs of the College of Agriculture (National Taiwan University)19:25.

21. Lopez J., Ramon J., Barroso J., Gutierrez A., Fierro R. and Piña R., 2013. Superovlation, in vivo embryo recovery and cryopreservation for Aoudad (ammotragus lervia) females using osmotic pumps and vitrification: a preliminary experience and its implication for conservation. Trop Conserv Sci 6(1): 149-157.

22. Mansour R.T. and Aboulghar M.A., 2002. Optimizing the embryo transfer technique. Hum. Reprod 17(5): 1149-1153.

23. McMillan W.H. and Hall D.R.H., 1994. Laparoscopic transfer of ovine and cervine embryos using the transpic technique. Theriogenology (42), 137–146.

24. McKelvey W.A. and Robinson J.J., 1984. Normal lambs born following transfer of embryos by laparoscopy. Vet Rec 115:230.

25. Moore N.W., Shelton J.N., 1964. Egg transfer in sheep. Effect of degree of synchronization between donor and recipient, age of egg, and site of transfer on the survival of transferred eggs. J Repro Fertil 7:145-152.

26. Nowshari M.A., Beckers J.F. and Holtz W., 1995. Superovulation of goats with purified pFSH supplemented. Theriogenology 43, 797-802.

27. Otsuki K. and Soma T., 1964: Transfer of the fertilized ova through the cervix of goats. Bull Natl Inst Anim Ind 6: 27.

28. Pereira R.J.T.A., So nrey B. and Holtz W., 1998. Nonsurgical embryo collection in goats treated with prostaglandin F2-alpha and oxytocin. J Anim Sci 76, pp. 360-363.

29. Rahman A.N.M.A., AbdullahR.B., Khadijah W.E.W., 2008. A Review of Reprodutive biotechnologies and their application in goat. Biotechnology 7: 371-384.

30. Rowson L.E.A. and Moor R.M., 1966. Embryo transfer in the sheep: the significance of synchronizing oestrus in the donor and recipient animal. J Repro and Fertil 11: 207-212.

31. Schneider H. J. Jr., Castelberry R. S. and Griffen J.L., 1980. Commercial aspects of bovine embryo transfer. Theriogenology13:73-85.

32. Sohnrey B., Holtz W., 2005. Technical Note: transcervical deep cornual insemination of goats. J Anim Sci 83:1543-1548.

33. Stringfellow D.A., Givens, M.D., 2010. Manual of the (IETS). 4th ed.Champaign, IL: IETS International Embryo Transfer Society.

34. Suyadi, Sohnrey B., Holtz W.,2000. Transcervical embryo collection in Boer goats. Small Rumin Res 36: 195–200.

35. Tervit H.R., 1986. Embryo transfer and artificial insemination in Conference, New Zealand. Angora goats. Proc Mohair

36. Thibier M. and Guérin B., 2000. Embryo transfer in small ruminants: the method of choice for health control in germ plasm exchanges. Journal of Livestock and Production Science. 62, 253-270.

37. Wallenhorst S. and Holtz W., 2002. Embryo collection in prepuertal gilts and attempts to develop an improved embryo transfer technique. Vet. Rec. 150, 749-751.

38. Warwick B.L., Berry R.O. and Horlachwer W.R., 1934. Results of mating rams to Angora female goats. Proceedings of the American Society of Animal Production 34: 225.

CHAPTER 5

General discussion

General discussion

This dissertation constitutes three studies concerned with the cryopreservation and transfer of embryos in the mouse as a model and the goat as farm animal.

In the first study the in vitro survival of mouse blastocysts cryopreserved by two different vitrification systems (Open Pulled Straw- [OPS] and CryoLogic Vitrification Method -CVM Ring Fibreplug™ [CVM]) and one-step warming in two different warming media were tested. In the “open” vitrification system chances are that the embryo gets into contact with liquid nitrogen which is avoided in the “closed” system. The objective of vitrification is to achieve extremely high cooling rates minimizing chilling injury and preserving biosecurity of the embryo. From a practical point of view the OPS method, representing an open system, was found to be easier to handle and, being handmade, is less costly. The CVM Ring Fibreplug® method, representing a closed system, has the advantage of providing a hygienically sound approach by avoiding direct contact with liquid nitrogen. This minimizes the risk of contamination. The commercially available equipment required is comparably high-priced and execution is more demanding with regard to experience and skill. The results achieved lead to the conclusion that both vitrification systems are equally suitable to cryopreserve mouse blastocysts. Another important finding forthcoming from the present study is that vitrified murine embryos may be warmed in a single step in medium devoid of sucrose. In fact, substantially better in vitro expansion and hatching rates were obtained than in embryos warmed in sucrose-containing medium as generally advised. A simple one-step warming procedure in sucrose-free medium provided highly satisfactory results. If applicable to domestic species, the latter finding will facilitate practicability of vitrification as means of embryo cryopreservation in the field.

In the second study, the ability of the Open Pulled Straw (OPS) and the CVM Ring-Fibreplug® (CVM) vitrification systems to be used for vitrifying goat embryos was investigated. With the experience obtained from the mouse-experiment the one-step warming approach in medium devoid of sucrose was applied. Regardless of the respective vitrification system healthy offspring was obtained from the transfer of vitrified goat blastocysts.

Based on the present findings it may be suggested that, since both systems are virtually equally effective, the selection of the vitrification system can be grounded on the experience of the technician, the availability of funds and considerations concerning biosafety if e.g. export of embryos is intended. One-step warming of vitrified embryos in warming medium devoid of sucrose is possible, enhancing practicability of the transfer of vitrified embryos in the field.

In the third study non-surgical transcervical transfer of vitrified embryos to non-anesthetized does was attempted and compared to semi-laparoscopic transfer. The main limiting issue of the practical use of embryo transfer in goats under farm conditions is the need of surgery. This study confirms earlier attempts showing that transcervical embryo transfer is not impossible. However, in order to get satisfactory pregnancy rates, the technique must be substantially improved. The principal suggestions for follow-up trials are to find more suitable catheter material and devise methodological modifications. The use of a special endoscope, small enough to be threaded through the cervix, which has recently become available, might help to determine what it takes to reach an appropriate site for embryo deposition without triggering a rejection mechanism.

Annex

Media Preparation

Holding Medium (HM)

Ingredients	For 100ml	Source
$NaHCO_3$	0.0168g	S-4019, Sigma
Na-pyruvate	0.0220g	P5280, Sigma
Hepes (Na-Salt)	0.650g	H3784, Sigma
L-Glutamine	0.0146g	G-5763, Sigma
Medium 199 (with Earle's Salts)	10ml	M-0650, Sigma
Sterile H_2O	90ml	Ampuwa™

*pH

After mixing the ingredients of HM, pH is adjusted with HCL or NaOH to 7.4

*Osmolarity

The osmolality of HM should be between 275-285 mosmoles. To adjust the osmolality you can use the next formula:

$$Q^* (a-b)/a = x$$

Q: quantity of medium made up (ml)

a: Osmolality measured

b: Osmolality desire

x: amount of medium to be discarded and replaced by sterile water (ml)

*Filtration

After pH and osmolality are measured, HM should be sterilized by filtering it through disposable 0,2µm filters (Sarstedt™, Germany), if serum will be added filtering should be made after serum supplementation.

*Serum supplementation

The addition of serum for embryo manipulation is 20% of Inactive Goat-Bock Serum, for example for 100ml of supplemented Holding medium: 20ml Inactive serum+ 80ml of holding medium. Sterilize HM+20% by filtering.

Reference: El-Gayar M., 2001. Vitrification and sexing of goat embryos and coating of foreign objects in the rabbit oviduct. Doctoral Dissertation. Animal Science Department, Georg August University, Goettingen, Germany.

Serum inactivation

1. Take male goat blood samples from jugular vein in a clean 50ml Falcon tube (as much as it will be need for about 6 months).
2. Storage of the blood for 24hrs at 4°C.
3. Centrifuge at 3000U/min for 10 min.
4. Separate the serum fraction in glass 5ml tubes.
5. In order to inactivate the serum the glass tubes are put into water bath at 56°C, once it has reached the 56°C temperature, it should stay in the bath for 25min , the temperature should be continuously measured during the 25 min, it should not pass over 57°C.
6. After inactivation; centrifuge the 5ml tubes containing the serum for 10min at 3000U/min.
7. Separate the serum (do not take the pellet in the end of the tube) and put it into tubes of the needed quantity. Mostly need for the media are 1ml, 2ml, 5ml and 10ml.

Stocks for M2 and M16 media

Stock A (x10 concentration)	Component	g/100ml	Source
	NaCl	5.534g	S-5886 Sigma
	KCl	0.356g	P-5405 Sigma
	KH_2PO_4	0.162g	P-5655 Sigma
	$MgSO_4$ x $7H_2O$	0.293g	M-9397 Sigma
	Sodium lactate 60% Syrup	4.349g (weight in a petri dish)	L1375 Sigma
	Glucose	1.000g	G6152 Sigma
	Pen-Strep 10000IU/ml (stored at -20°C)	1ml	PAA P11-010
	Sterile H_2O	Complete to 100ml	Ampuwa™
Stock B (x10 Concentration)	**Component**	**g/100ml**	**Source**
	$NaHCO_3$	2.101	S-4019 Sigma
	Phenol red	0.010[a]	Serva 32095
Stock C (x100 Concentration)	**Component**	**g/10ml**	**Source**
	Sodium pyruvate	0.036	P5280 Sigma
Stock D (x100 Concentration)	**Component**	**g/10ml**	**Source**
	$CaCl_2 \cdot 2H_2O$	0.252	C7902 Sigma
Stock E (x10 Concentration)	**Component**	**g/100ml**	**Source**
	HEPES	5.958	H3784 Sigma
	Phenol red	0.010[a]	Serva 32095

[a] The concentration of phenol red can be decrease to 0.0001-0.001g/liter, because it may be embryotoxic.

Procedure:

Preparation of Stocks A, B, C and D

1. Weigh the salts (except sodium lactate) into a designated volumetric flask and bring up to volume using 2x distilled water.
2. Weigh the sodium lactate into a designated 10-ml beaker.
3. Add the sodium lactate to the volumetric flask.
4. Rinse the baker several times with 2x distilled water. Add the washings to the volumetric flask and bring up to volume using 2x distilled water.
5. Filter all stocks through a Millipore filter. Convenient aliquots in sterile tubes can be made at this time according to the potential use (see table of M2 and M16 preparation).

Preparation of Stock E

1. Weigh the HEPES and phenol red into a designated beaker.
2. Add ~50ml of 2x distilled water and allow to dissolve.
3. Adjust the pH to 7.4 with 0.2M NaOH.
4. Pour into a 100-ml volumetric flask.
5. Rinse the beaker with 2x distilled water, add washings to the volumetric flask, and adjust to 100ml.
6. Filter all stocks through a Millipore filter. Convenient aliquots in sterile tubes can be made at this time according to the potential use (see table of M2 and M16 preparation).

Storage: If store in a refrigerator at 4°C, stocks A,D, and E can be kept for up to 3 months. Stocks B and C must be changed every other week. All stocks stored frozen at -20°C can be kept for longer periods.

Preparation of M2 and M16 Media from Concentrated Stocks

1. Measure 2x distilled water accurately into a designated conical flask. Aplastic 50-ml tube or a sterile culture bottle may be used instead of conical flask.
2. Measure stock solutions using plastic pipettes or tips. Leave the pipette in the conical flask.
3. Rinse the pipettes in the flask by sucking up water/ medium mixture two or three times.
4. Measure the osmolality of the medium (optional).
5. Gas the M16 medium by bubbling with 5% CO2, 95%air for ~15min to adjust the pH to 7.4 (optional).
6. Add the BSA to the medium to a final concentration of 4mg/ml; allow dissolving slowly and mixing gently. Do not shake the medium because it will froth and denature the protein.
7. If necessary, readjust the pH of M2 medium with 0.2N NaOH to pH 7.2-7.4, using color standards.
8. Filter the medium through a Millipore filter into sterile plastic tubes. Gas the space in the tubes with M16 medium with 5% CO2, 95% air for 30 seconds, and cap tightly to maintain a pH of 7.2-7.4.
9. Store at 4°C for 1-2 weeks.

Reference: Nagy A., Gertsenstein M., Vintersten K., Behringer R., 2003. Manipulating the mpuse embryo, a laboratory manual, third edition. Cold spring harbor laboratory press.

PBS Dulbecco's (Flushing and Culture of goat embryos)

Ingredients	Source	g/100ml	g/l	g/3l	g/5l
NaCL (Sodium Chloride)	S-5886 Sigma	0.800	8	24	40
KCL (Potassium Chloride)	P-5405 Sigma	0.020	0.200	0.600	1
$MgCL_2$ x $6H_2O$ (Magnesium Chloride Hexahydrate)	M-2393 Sigma	0.010	0.100	0.300	0.500
Na_2HPO_4 (Sodium phosphate)	S-5136 Sigma	0.100	1	3	2
NaH_2PO_4 (Sodium phosphate monobasic)	S-5011 Sigma	0.015	0.150	0.450	0.750
KH_2PO (Potassium Phosphate)	S-5655 Sigma	0.020	0.200	0.600	1
Na Pyruvate (Pyruvic acid/Sodium pyruvate)	S-5280 Sigma	0.036	0.360	0.108	0.180
D (+) Glucose (water free)	G-6152 Sigma	0.100	1	3	5
Penicillin/Streptomycin 10000	PAA P11-010 (-20°C)	1ml	10ml	30ml	50ml
H_2O (fill out till the desire amount is complete)	*Culture: Ampuwa™. *Flushing: 2x distill water	Up to 100ml	Up to 1lt	Up to 3lt	Up to 5lt
CaCl (Calcium Chloride [**Stock***]) if it WON'T be freeze before use, if the media should be frozen then add after thawing	C-7902 Sigma *Stock	1ml	10ml	30ml	50ml

***CaCL (C-7902 Sigma) stock 13.2mg /ml of water, filter through 2µl filter and store at 4°C.**

*Supplementation of BSA for goat embryos

Culture Medium	g/100ml	g/l
BSA 0.6%	0.600	6
Flushing Medium	**g/l**	**g/5l**
BSA 0.06%	0.600	3

- pH should be 7.2 and it can be fix with NaOH or HCL.
- Osmolality 265-295mmol (+/- 15).
- After pH and osmolality are measured, PBS should be sterilized by filtering it through disposable 0,2µm filters.
- Medium can be store at -20°C.

Reference: El-Gayar M., 2001. Vitrification and sexing of goat embryos and coating of foreign objects in the rabbit oviduct. Doctoral Dissertation. Animal Science Department, Georg August University, Goettingen, Germany.

Vitrification and Warming

Media and supplements

- Holding medium +20% Inactive serum
- Ethylene glycol (EG; Sigma Aldrich,St. Louis,MO, USA;E9129)
- Dimethylsulfoxide (DMSO; Sigma; D2650, 5ml ampoules)
- VitrSucrose

Storage

Basic medium can be stored as instructed by the producer.

EG can be stored at room temperature for years. For DMSO, use one ampoule after opening for no more than 3 weeks).

Vitr-Sucrose media

Put 5.13 g sucrose in a 50 ml tube, add HM to the 20 ml mark, dissolve (may require vortexing, slight warming and patience), complete again with HM to 21 ml. Filter, store at 4ºC.

Warm-Sucrose media

Put 17.1 g sucrose in a 50 ml tube, add HM to the 50 ml mark to each, dissolve (may require vortexing, slight warming and patience), complete again with HM to 50 ml in both tubes filter, store at 4ºC.

Protocols for 4 well dish preparation for vitrification and warming solutions, including time of exposition of embryos to the solutions:

Protocol of equilibration for vitrification

Vitrification	Media	EG µl	ME_2SO µl
Well 1	HM 800µl	-	-
Well 2	HM 800µl	-	-
Well 3 (3min)	HM 850µl	75µl	75µl
Well 4 (40-45 sec)*	Vitr-Sucrose 670µl	165µl	165µl

* The last step of the protocol should be carried out in a 1µl drop of medium of the well 4, after 40-45 s in that drop, embryos are then place in the vitrification device of choice and immediately after be submerge in LN_2 to achieved vitrification.

Reference: Gabor Vajta, 2012. http://www.gaborvajta.com/downloads/media_and_equilibration- dilution_parameters_of_cattle_blastocysts.pdf

Protocol of Warming in 1 Step Sucrose Free

Warming	HM
Well 1 (5min)	800µl

Protocol of Warming in 1 Step 0.3M Sucrose

Warming	HM	Warm-Sucrose
Well 1 (5min)	800µl	400 µl

Protocol of Warming in 3 Steps in sucrose containing media

Warming	HM	Warm-Sucrose
Well 1 (0.3M) Recovery	800µl	400 µl
Well 2 (0.3M) 5 min	800µl	400 µl
Well 3 (0.2M) 5min	800µl	200 µl
Well 4 5min	800µl	-

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Publications:

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- Camacho M., Garza D., Gauly M., Holtz W., 2019. Superovulation of Boer goats with different synchronization regimens at different times of the year in the northern temperate zone. Small ruminant research 177: 106-110.

- Garza D., Camacho M., Gauly M., Holtz W. 2018. Technical note: Transfer of caprine blastocysts vitrified by the open pulled Straw (OPS) or the solid Surface procedure and warmed in sucrose-freemedium. Small Ruminant Research 165: 111-114.

- Camacho M., Stiehl J., Garza D., Gauly M., Holtz W. 2017. Seasonality of Boer goats in nothern Europe and induction of estrous out of season. Small Rumi-nant Reseach 151: 23-31.

- Ledezma R., Garza D., Moreno G., Manzanares N., Picón F., Ramirez R., Sanchez F. 2015. Efecto del CIDR post inseminación sobre la tasa de preñez en vacas de carne. Revista Ciencia UANL 73: 62-68.

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