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Araştırma Makalesi ***Research Article***

Effect of Different Cooling Rates on Embryo Survivability and Pregnancy Rates in Freezing Sheep Embryos#

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Abstract

In the first stage of the study, oocytes obtained from ovaries from abbatoir (n=2990) were incubated in maturation medium for 24 h. The maturated oocytes were left for *in vitro* fertilization (IVF) for 20 hours. The cleavaged embryos (n=1305) were left for *in vitro* culture (IVC) for six days. Then morula-blastocyst stage embryos divided randomly in three groups (Grup I: 0.5°C/min, Grup II: 0.8°C/min, Grup III: 1°C/min). Embryos in each group (n=50) were frozen at different cooling rates in 1.5 M ethylene glycol containing freezing medium. As a result, 0.5°C/min cooling rate group was found as the most successful group (P<0.05). In the second stage of the study, *in vivo* embryos (morula-blastocyst) derived from donor sheep were frozen at 0.5 °C/min. Frozen-thawed nineteen embryos were transferred to hormonally pretreated seventeen recipients. At the 60th day of the transfer, three recipients were diagnosed as pregnant and one of them had twins. One of the three recipients gave birth and two sheeps had early embryonic loss are observed in the later ultrasound scanning. In the study, 0.5°C/min cooling rate in the slow freezing of sheep embryos was found out to be more successful cooling rates and pregnancy following a healthy lambing is achieved.

Özet

Koyun Embriyolarının Dondurulmasında Farklı Soğutma Hızlarının Embriyoların Canlılığı ve Gebelik Oranları Üzerindeki Etkisi

Çalışmanın ilk bölümünde, mezbahadan sağlanan ovaryumlardan kazanılan oositler (n=2990) olgunlaştırma medyumu içerisinde 24 saat süreyle olgunlaştırıldı. Ardından, 20 saat süreyle *İn Vitro* Fertilizasyona (İVF) bırakıldılar. Yarıklanma gösteren embriyolar (n=1305), Sentetik Ovidukt Fluid (SOF) medyumu içerisine alınarak altı gün süresince *İn Vitro* Kültüre (İVK) bırakıldılar. İVK sonrası elde edilen morula-blastosist aşamasındaki embriyolar rastlantısal şekilde üç farklı dondurma hızı grubuna eşit olarak ayrıldılar (Grup I: 0,5 °C /dk, Grup II: 0,8 °C /dk, Grup III: 1 °C /dk). Her bir gruptaki embriyolar (n=50), 1,5 M etilen glikol bulunan dondurma medyumu içerisinde farklı soğutma hızlarında donduruldu. Sonuçta 0,5 °C/dk soğutma hızının en başarılı grup olduğu belirlendi (P<0,05). Çalışmanın ikinci bölümünde, verici koyunlardan elde edilen *in vivo* embriyolar (Morula-Blastosist) çalışmanın birinci bölümünde bulunan en başarılı soğutma hızı (0,5 °C/dk) ile soğutularak donduruldu. Dondurulan 19 adet embriyo hormonel olarak hazırlanmış 17 alıcı koyuna transfer edildi. Transfer sonrası 60. günde yapılan ultrason muayenesinde üç adet koyunda gebeliklere ait embriyonik keseler gözlendi ve bu koyunlardan birinde de ikiz gebelik saptandı. Gebe koyunlardan bir tanesinde doğum gerçekleşti; diğer iki koyunda ise ileriki dönemde yapılan ultrason muayenesinde gebeliklerin sonlandığı gözlendi. Çalışma sonucunda, koyun embriyolarının dondurulması sırasında 0,5°C/dk soğutma hızının en başarılı hız olduğu ve bu yöntemle dondurulan embriyoların transferinden de gebelik elde edilebildiği saptandı.

Introduction

Assisted reproductive technologies such as oestrus synchronization, artificial insemination, multiple ovulation embryo transfer (MOET), gamete sexing, *in vitro* and transgenic animal production, nuclear transfer studies have been introduced to increase the amount of animal products obtained (Atalla, 2002; Ataman, 1998;

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Bari et al., 2003; Birler et al., 2010). However, the success of these technologies are largely related to the success of oocyte maturation, sperm DNA integrity, fertilization, embryo culture and cryopreservation of the oocyte and embryos *in vitro* (Akal and Selçuk, 2013; Boland et al., 2001). In order to perform the studies on reproductive biotechnology plurality of oocytes and embryos is needed (Dattena et al., 2000; Dattena et al., 2004). *In vitro* embryo production consists from four steps such as; oocyte collection, *in vitro* maturation, *in vitro* fertilization and *in vitro* culture. Cryopreservation of embryos have many benefits such as allowing to select the recipient, determine the transfer time, avoid the necessity of synchronous the donor and the recipients at same time, transfer the embryos intercontinentally, help saving endangered species, and save the herds from epidemic diseases (Fowler and Toner, 2005). *In vivo* sheep embryo production consists from these steps: Oestrus synchronization and superovulation of the donors, artificial insemination or natural breeding, collecting embryos from the uterus by surgical flushing technique and finding the embryos under microscopic observation (Garcia et al., 2006). Although, there are many studies on cryopreservation of cattle embryos (Gasparrini, 2002; Hasler et al., 1987; Kaidi et al., 1999), studies on sheep embryos are still rare (Fowler and Toner, 2005; Grazul-Bilska et al., 2006; Isachencko et al., 2003), little studies had been done about sheep embryos and a common cryopreservation method for sheep has not yet been developed (Fowler and Toner, 2005). The survivability rates of cryopreserved embryos show differences between researchers. The reasons can be sorted as; cooling, freezing and thawing procedures, developmental stages of the embryos, cell permeability, osmotic pressure and toxicity of the cryoprotectants used (Martinez et al., 2002). The aim of the study is to determine the effect of different cooling rates (0.5 ^{o}C/min, 0.8 ^{o}C/min and 1° C/min) on sheep embryo survivability and pregnancy rates using slow freezing methods.

Materials and Methods

Oocyte recovery

The present study was performed in accordance with guidelines for animal research from Istanbul University Ethics Committee on Animal Research (2010/166).

In the first step of the study it was aimed to produce embryo in vitro. The ovaries were placed in Dulbecco's phosphate buffered saline (PBS) at 38.5 °C and brought to the laboratory within 2-3 h. The ovaries were minced and rinsed with a washing medium (10 µg/mL heparin supplemented HEPES modified TCM 199) at 38.5 °C in order to obtain cumulus oocyte complexes (COCs). The oocytes with dark ooplasm and completely surrounded by at least four layers of cumulus cells were classified as Grade I and selected for *in vitro* maturation (IVM).

In vitro maturation (IVM)

The COCs were transferred into different four-well petri dishes containing 500 µL maturation medium under mineral oil in each well. Thirty to Forty COCs were placed in each well. Oocytes were maturated in 10 % fetal bovine serum (FBS), 10 µg/mL of FSH, 10 µg/mL of LH and 0.1 mg/mL sodium pyruvate bicarbonate buffered TCM 199 in humidified atmosphere containing 5% CO_2 at 38 °C for 24 h.

In vitro fertilization (IVF)

Healthy 2-3 years aged Kivircik rams were used as semen donor. Semen was collected by electro-ejaculation. Then, it was transferred onto a two layer discontinuous gradient formed by layering 1mL of the 45% Percoll solution on top of 1ml of 90% in a 15 mL conical tube, and centrifuged at 1500 g for 15 min at room temperature. The supernatant was removed and the sperm pellet was washed with 3 ml of HEPES supplemented SOF medium (hSOF) by centrifugation at 600 g for an additional 5 min. The pellet was then recovered after aspiration of the supernatant, and the spermatozoa were re-suspended in hSOF medium supplemented with 0.56 mg/ml heparin to induce capacitation. For each 30-40 maturated oocytes were co-incubated with the sperm suspension (800.000-1.000.000 spermatozoa/mL) at 38.5°C humidified atmosphere containing 5% CO_2, for 20 h. Fertilization day was considered as day 0.

In vitro culture (IVC)

The presumptive zygotes were pipetted and transferred into four-well petri dishes containing 500 µL SOF culture medium under mineral oil for IVC at 38.5°C for 8-9 days 5% CO_2, 5% O_2, and 90% N atmosphere. 1.5 mM Glucose was added to the culture media at 4th day of the culture period. Thereafter, the non-cleaved oocytes discarded and cleavage rates were determined. After the entire culture period, the embryos reached to morula–blastocyst stage were selected for freezing.

Embryo freezing and thawing

The study was performed at three different cooling rates (0.5 °C/min, 0.8 °C/min and 1°C/min) with 1.5 M ethylene glycol (EG) by automatic freezer (Cryologic CL 5500). Each embryo was loaded into 0.25 mL straws with a small amount (10 µL) of EG and separated by two air bubbles from two surrounding drops of 0.5 M sucrose solution (90 µL). After closing with PVP, the straws were placed in a controlled-rate freezer. The slow freezing program was carried out. Freezing was

accomplished by cooling from 18 °C to - 6 °C at three different rates (0.5°C/min, 0.8 °C/min and 1°C/min) for three different groups and then seeding was started by touched the metal stick on the upper side of the straws. At the end of the freezing procedure, the straws were directly plunged into the liquid nitrogen (LN_2). Fifty *in vitro* produced embryos (morula-blastocyst stage) were frozen for each different cooling rate group. Embryo thawing was performed at room temperature for 10 s and then in a water bath at 38 °C for 30 s. The cryoprotectant was removed in 2 steps with decreasing concentrations (0.5 and 0.25 M) of sucrose. Then, the embryos were washed three times with hSOF medium for eliminating the cryoprotectant traces.

Post thawing embryo evaluation

Evaluation of the survival rate after cryoprotectant removal was performed by culturing the embryos in 50 µL drops of SOF medium, under mineral oil at 38.5 °C in a controlled atmosphere (5% CO_2, 5% O_2, 90% N). The development (as assessed by blastocele re-expansion) and hatching stages were monitored at 1st, 24th and 48th h for determining the viability of embryos.

Superovulation and oestrus synchronization of the donor ewes

In the second stage of the study; 40 ewes and five rams were used. The ewes were treated with intravaginal progestogen-impregnated sponge containing 30 mg flurogestone acetate (FGA), maintained *in situ* for 14 days. Superovulatory treatment was performed using 1.5 mL FSH (Pluset Calier) by administered i.m. with 12-h intervals in decreasing doses to 1 mL over 96 h, starting 48 h before sponge removal. Starting from 16 h after sponge removal ewes were treated with GnRH (37,5 µg lesirelin asetat) and were observed every 6 h to establish the time of onset of estrus with the aid of teaser rams. Ewes were handmated and inseminated artificially at the beginning of estrus and subsequently every 6-8 h until the end of oestrus.

Embryo recovery

On the seventh day following insemination, laparotomy was performed under general anesthesia and the ovarian response was estimated by counting the numbers of corpora lutea. Embryos were recovered from genital tracts by retrograde flushing of the uterine horns with 20 mL modified PBS supplemented with 0.4 % bovine serum albumin (BSA) into glass recovery vessels and then transferred in modified PBS containing 20 % FCS. Collected embryos were harvested under a stereomicroscope at magnification of 40X. Obtained embryos were then observed under phase contrast inverted microscopy. The cleavage was considered as evidence of fertilization. Embryos were classified according to development stage and considered.

Embryo freezing and thawing

The recovered *in vivo* produced embryos were frozen according to the best group (cooling rate at 0.5°C/min) in the *in vitro* produced embryos at the first stage of the study.

Oestrus synchronization of the recipients

Twenty ewes were treated with intravaginal progestagen-impregnated sponge containing 30 mg FGA, maintained in situ for 14 days. Beginning sponge removal ewes were administered i.m. 500 IU Pregnant Mare Serum Gonadotropin (PMSG).

Embryo transfer to the recipients

Eight days after removal the sponges, the embryos were transferred to the recipients by laparotomy under anaesthesia. For anaesthesia, 2 mg/kg Lidocaine by epidural way and 0.05 mg/kg xylazine by intramuscular way were given. One or two embryos were transferred into a cornu uteri ipsilateral to an ovary containing at least one corpus luteum.

Statistical Analysis

Differences in viability, developmental stages, hatching rates of freeze - thawed embryos were analysed by Kruskal-Wallis Test. Confidence level of $P<0.05$ was considered significant.

Results

In the first step of the study, the *in vitro* produced embryos at different developmental stages were frozen according to three different cooling rates (Table 1). After thawing, the embryos cultured *in vitro* and checked for their survival abilities. The embryo survival rates are given in Table 2. Finally, 0.5 °C/min was the most successful (36%) cooling rate compared 0.8 and 0.5°C/min groups (8% and 6%) ($P<0.05$). In the second stage of the study, twelve sheeps superovulated and 19 morula stage healthy embryos were collected from three of them. After cultured *in vitro* at 24 h the embryos were developed and then frozen with 0.5°C/min cooling rate. After thawing, the 19 *in vivo* collected embryos were transferred to 17 superovulated ewes. Three pregnancies were detected by ultrasonographic examination at the 60th day of the transfer. Moreover, one of the sheep had twins (Figure 1). Including the twins, two sheep pregnancies were detected terminated at the second ultra-sonographic examination at 90th day. Finally, one pregnancy was resulted with a healthy birth (Figure 2).

Table 1. Developmental stages of in vitro embryos frozen with different cooling rates.

Tablo1. Farklı soğutma hızları ile dondurulan in vitro embriyoların gelişim basamakları.

Cooling Groups	Embryonic Developmental Stages							
	Morula (%)	Compacted Morula (%)	Early Blastocyst (%)	Blastocyst (%)	Expanded Blastocyst (%)	Hatching Blastocyst (%)	Hatched Blastocyst (%)	Total (n)
0.5 °C/min	4 (8)	11 (22)	16 (32)	14 (28)	2 (4)	2 (4)	1 (2)	50
0.8 °C/min	1 (2)	12 (24)	19 (38)	16 (32)	1 (2)	1 (2)	0 (0)	50
1 °C/min	1 (2)	15 (30)	15 (30)	17 (34)	2 (4)	0 (0)	0 (0)	50

Table 2. The viability of frozen-thawed in vitro produced embryos.

Tablo 2. İn vitro embriyoların donma-eritme sonrası canlılıkları.

Cooling Groups	Checking Time								
	1st			24th			48th		
	Normal	Z.P. defect	Deg.	Normal	Z.P. defect	Deg.	Normal	Z.P. defect	Deg.
0.5 °C/min	48 (96.00)^{a}	2 (4.00)^{A}	0 (0.00)*	18 (36.00)^{a}	2 (4.00)^{A}	30 (60.00)**	18 (36.00)^{a}	2 (4.00)^{A}	30 (60.00)*
0.8 °C/min	24 (48.00)^{b}	4 (8.00)^{A}	22 (44.00)***	9 (18.00)^{b}	4 (8.00)^{A}	37 (74.00)**	4 (8.00)^{b}	4 (8.00)^{A}	42 (84.00)**
1 °C/min	43 (86.00)^{a}	2 (4.00)^{A}	5 (10.00)**	3 (6.00)^{a}	2 (4.00)^{A}	45 (90.00)***	3 (6.00)^{b}	2 (4.00)^{A}	45 (90.00)***

Z.P.: Zona Pellucida; Deg.: Degenerated

a,b,A, *,**,***: Values with different superscripts in the same column are significantly different; ($P<0.05$).

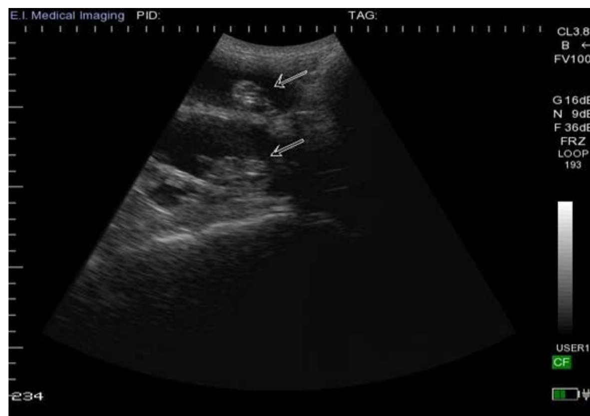

Figure 1. Twin pregnancies at the 60th day of ultrasonographic examination.

Şekil 1. Altmışıncı gün ultrason muayenesinde ikiz gebelik.

Figure 2. A healthy lamb born after transfer of cryopreserved embryo.

Şekil 2. Donmuş embriyonun transferi ile doğan sağlıklı bir kuzu.

Discussion

In the first stage of the study, *in vitro* maturation rate was 51.53%. Wani et al. (2012) added Epidermal Growth Factor (EGF) and cysteamine in maturation medium in their study and they found higher IVM rates (84.79% and 69.6%). This result shows that adding EGF and cysteamine to medium could increase the IVM rates. The presence of the EGF and IGF receptors on granulosa cells are the evidence that they are effective on IVM. It is stated that IGF shows synergetic action with

FSH and they stimulates the steroid production and oocyte maturation (O'Brien et al., 1997). Grazul-Bilska et al. (2006) found that adding EGF to medium could increase the cleavage rates of sheep embryos than the control group (59% vs. 78%). The cleavage rate of our study was 69.69%. Although we have not added EGF to IVM medium our results were similar to these researchers. O'Brien et al. (1997) found that the blastocyst rates of the oocyte collected from pubertal sheeps were significantly higher than those collected from prepubertal sheeps (34%-15%). However, in the same study pregnancy rates were not significantly different between two groups (30% vs. 40%). It is known that sheep shows seasonal oestrus and the oocytes collected in breeding season have higher maturation, fertilization and embryo production abilities than collected in anestrus period (Emsen and Koşum, 2009). The blastocyst rate of the present study (9.86%) was lower than the researchers' results. The difference could be related to the seheeps' age and season. Since the lambs' meat is preferred most in Turkey, the sheeps generally slaughtered at younger ages. The most of our materials (ovaries) were collected in anestrus season from the 6-10 months old (prepubertal) sheeps. In the present study, 150 morula-blastocyst stage embryos produced *in vitro* were frozen with three different cooling rates. After thawing and *in vitro* culture, the embryos were checked for their viability. At the end of the culture period (48 h), significantly highest viability rates were in 0.5 C °/min cooling group (36% vs. 8% and 6%). Dattena et al. (2000), found 28% viability rates after thawing from frozen embryos produced from prepubertal sheep. These results are similar to our findings. The similarity may due to using of prepubertal sheep as oocyte sources in both studies. Moreover, it is stated that the *in vitro* produced ruminant embryos have darker cytoplasm and more sensitive to freezing than the collected *in vivo* (Sungur and Yurdaydın, 1991). When the ambient temperature decreases, the number and the size of extracellular harmful ice crystals increase. When the freezing rate is too slow the cells lead to death since the osmotic shock and solid effects. Contrarily, when the freezing rate is too fast the cells lead to death since the intracellular ice crystal formation (Thibier and Guerin, 2000; Tominga et al., 2007). According to the 48 h culture results, the survival rates of the frozen thawed embryos were inversely proportional (18%, 4% and 3%) with cooling rates (0.5, 0.8 and 1 °C/min). Therefore, these results are supported the idea that when the ambient temperature decreases, the number and the size of extracellular harmful ice crystal increase and when the cells freeze too fast they lead to death due to the intracellular ice crystals formation (Thibier and Guerin, 2000). Moreover, researchers stated that the little sized ice crystals are reorganised and transformed to larger and harmful shapes during thawing procedure (Thibier and Guerin, 2000; Vajta et al., 1998). In the second stage of this study, 19 morula stage embryos were collected at the 7th day of the oestrus cycle from the three of superovulated 12 sheep. Before freezing, these embryos were cultured *in vitro* for 24 - 48 h additionally. 16 of them were developed to blastocyst and two of them remained at morula stage. After freezing and thawing they were transferred to the uterus of 17 recipients. At 60th day of gestation we found three pregnancies by ultra-sonographic examination (17.63%). It is well known that the pregnancy rates could be influenced by the quality and developmental stages of transferred embryos (Sağırkaya and Bağış, 2003; Vajta et al., 1998). Bari et al. (2003) compared viability of frozen-thawed sheep embryos collected at 5th and 6th days of oestrus. They found that the blastocyst stage embryos were collected at 6th days had higher viability rates than the morula stage embryos collected at 5th day. In the present study the *in vivo* developed embryos collected at 6th days of the oestrus were at morula stages and this may have influenced the viability of frozen embryos after thawing. The success of MOET program is related to many factors such as; embryo quality, developmental stage, the age of recipient and donors, the corpus luteums' form and numbers (Vajta et al., 1998; Vieria et al., 2007). Nutrition is one of the most important factors which affects the *in vitro* and *in vivo* embryo development (Robinson et al., 2006). It is known that energy need is the major factor that affects the reproductive processes. The synthesis and secretion of gonadotropins are affected from the energy intake (Wani et al., 2012). In the present study, 35 non-fertilised oocyte and nine zona pellucida were obtained from the sheeps' uterus flushing. Moreover, at the 6th day of oestrus we were expected to collect blastocyst stage embryos but they were developed to just morula stages. The materials of the study were kept at the faculty farm. The feeding program which was applied in the farm was not suitable for embryo recovery. The insufficient energy and protein intake might be the cause the failure of IVF and further embryonic developments.

In conclusion, 0.5 °C/min cooling rate in the slow freezing of sheep embryos was found more successful than the other cooling rates and despite the adverse conditions, pregnancies and a healthy lambing was achieved with the embryos frozen with this method. It can be stated that 0.5°C/min cooling rate is more suitable than 0.8 and 1°C/min for freezing the *in vivo* and *in vitro* produced sheep embryos.

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