İstanbul Üniversitesi
Veteriner Fakültesi Dergisi

Journal of the Faculty of
Veterinary Medicine Istanbul University

İstanbul Üniv. Vet. Fak. Derg. / J. Fac. Vet. Med. Istanbul Univ., 41 (1), 37-42, 2015
doi: 10.16988/iuvfd.2015.39408

Araştırma Makalesi *Research Article*

Effects of Cysteamine on Sheep Embryo Cleavage Rates

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Geliş Tarihi / Received:
28 February 2014

Kabul Tarihi / Accepted:
26 March 2014

Anahtar Kelimeler:
Koyun, sisteamin, olgunlaşma, bölünme oranı, embriyo

Key Words:
Sheep, cysteamine, maturation, cleavage rate, embryo

Abstract

Oxidative stress during in vitro culture leads to defects in development of gametes and embryos. Several antioxidants such as cysteamine, L-ascorbic acid, beta mercaptoethanol, cysteine, glutathione, proteins, vitamins have been used to supplement culture media to counter the oxidative stress. This study was conducted to detect the effect of adding cysteamine to the maturation medium to subsequent cleavage rates of sheep embryos. Totally 604 ovaries were obtained by ten replica and 2060 oocytes were collected. The cumulus oocyte complexes were recovered by the slicing method. A total of 1818 selected oocytes were divided into two groups and used for maturation (88.25%). The first group was created as supplemented with cysteamine (Group A) and second group (Group B, control) without cysteamine in TCM-199. The two groups were incubated for 24 h at 38.8 °C in an atmosphere of 5% CO_2 in humidified air for in vitro maturation (IVM). After IVM, oocytes were fertilized with 50 x 10^7 / mL fresh ram semen in BSOF medium for 18 h. After fertilization, maturation groups were divided into two subgroups with different culture media: Group AI-SOF (Synthetic Oviduct Fluid medium), Group AII-CR1aa (Charles Rosencrans medium), Group BI-SOF and Group BII-CR1aa were achieved. Cleavage rates were evaluated at day 2. post insemination. The rates of cleavage were detected as 59.54% (184/309), 55.44% (173/312), 65.34% (215/329), 59.34% (200/337) respectively, with showing no statistically significant difference between the groups at the level of P>0.05. In conclusion, supplementing cysteamine to maturation media in TCM-199 did not affect the cleavage rates of sheep embryos in SOF and CR1aa culture media.

Özet

Sisteaminin Koyun Embriyo Bölünme Oranlarına Etkileri

In vitro kültür esnasında oksidatif stres gametlerin ve embriyoların gelişiminde hasarlara yol açar. Sisteamin, L-askorbik asit, beta merkaptoetanol, sistein, glutatyon gibi birçok antioksidanlar, proteinler, vitaminler oksidatif stresi önlemek için kültür medyumlarına eklenmiştir. Bu çalışma olgunlaşma medyumuna sisteamin ilavesinin koyun embriyonik bölünme oranları üzerine etkisinin saptanması için tasarlandı. Toplam 604 ovaryum 10 kerede toplandı ve 2060 koyun oositi elde edildi, doğrama metodu ile 1818 adet oosit kumulus kompleksleri seçilerek olgunlaşma amacıyla iki gruba ayrıldı (%88,25). İlk grup (Grup A) TCM-199 medyumuna sisteamin eklenen oositler, diğer grup (Grup B, kontrol) TCM-199 medyumuna sisteamin eklenmeyen oositler olarak oluşturuldu. İki grupta 24 saat boyunca 38,8 °C'de %5 CO_2 altında, nemli havada in vitro maturasyon (IVM) için inkübe edildi. Oositler, IVM sonrası 50 x 10^7 / mL taze koç sperması ile BSOF medyumunda 18 saat süreyle döllendi. Döllenme sonrası, olgunlaşma grupları değişik kültür medyumları ile iki alt gruba ayrıldı ve Grup AI-SOF (Sentetik Ovidukt Sıvısı Medyumu), Grup AII-CR1aa (Charles Rosencrans Medyumu), Grup BI-SOF ve Grup BII-CR1aa elde edildi. Bölünme oranları döllenme sonrası 2. günde değerlendirildi. Bölünme oranları gruplar arasında istatistik açıdan önemli fark olmadan (P>0,05) sırasıyla, %59,54 (184/309), %55,44 (173/312), %65,34 (215/329), %59,34 (200/337) olarak saptandı. Sonuç olarak, TCM-199 olgunlaşma medyumuna sisteamin eklenmesinin SOF ve CR1aa kültür medyumlarındaki koyun embriyo bölünme oranları üzerine bir etkisinin olmadığı saptandı.

Introduction

The sheep is a valuable livestock around the world especially in less developed countries having limited land and natural sources. They are suitable to small and low-capital input farms in the rural areas of developing countries as they are the main source of wool, milk and meat. Thus, assisted reproduction technologies have gained importance in recent years to produce high-yielding lambs in large numbers. Hence, in vitro techniques are considered as a key technology in sheep reproduction and the biomedical field for valuable genetic transfer (Farag et al., 2009). Despite using different type of culture media such as SOF, CR1, TCM-199 etc in different laboratories for in vitro culture, the same results could not be able to achieved and there are still some problems and failures in in vitro embryo production (Camargo et al., 2006). The most important problem is oxidative stress in development of embryos during in vitro culture, and this stress leads to genetic defects in embryos. In their natural environment, oocytes and embryos are protected from oxidative stress by free radical scavengers which are present in oviductal and follicular fluids, and also by antioxidant enzyme systems such as glutathione peroxidase, superoxide dismutase etc (Lapointe et al., 1998; Oyawoye et al., 2003). However, the oocytes and embryos are deprived of this kind of a protection during in vitro production (Natarajan et al., 2010b). Antioxidant metabolites (cysteine, glutathione, taurine and hypotaurine) and enzymes (superoxide dismutase, catalase, glutathione peroxides) were used in biological systems to protect the cellular components such as DNA, proteins and lipids against oxidative damage (Gupta et al., 2009). Glutathione (GSH) is a tripeptide thiol compound that has many functions in intracellular physiology and metabolism (Shabankareh and Zandi, 2010). Glutathione content increases during development and oocyte maturation in the ovary as the oocyte approaches to ovulation (Perreault et al., 1998) and protects the oocyte in later stages of fertilization (Teleford et al., 1990). Thiol compounds such as cysteamine or β-mercaptoethanol can stimulate GSH synthesis and can decrease hydrogen peroxide levels thus improve embryonic development (de Matos et al., 1996; de Matos et al., 1999; de Matos and Furnus, 2000). Many researchers used various antioxidant substances such as retinol, L-ascorbic acid, cysteamine, α-tocopherol for in vitro sheep oocytes culture media (Livingston et al., 2009; Natarajan et al., 2010a; Natarajan et al., 2010b; Shabankareh and Zandi, 2010). According to our knowledge, there are few literatures that indicate the effect of cysteamine as an antioxidant on in vitro maturation and cleavage rate of sheep embryos (Freitas and Melo, 2010; Shabankareh and Zandi, 2010), so in the current study we aimed to determine the effect of adding cyteamine to maturation medium and subsequent cleavage rates of embryos in SOF or CR1aa media.

Materials and Methods

Oocyte recovery

Ten replica 604 ovaries were obtained and 2060 ovine oocytes were collected, from these oocytes 1818 were used for maturation (88.25%). Ovine ovaries were obtained from a local abattoir and transported to the laboratory in PBS supplemented with antibiotic combination at 30-35 °C within 2-3 h of slaughter. The cumulus oocyte complexes (COCs) were recovered by slicing method of antral follicles of 2-8 mm in diameter.

In vitro maturation (IVM)

Selected oocytes were washed three times in H-SOF and once in Oocyte Maturation Medium (OMM) and placed in petri dishes containing 500 µL of the same medium. Oocytes were divided into two groups. TCM-199 supplemented with 10% FCS (v/v), 1 µg/mL FSH, 10 µg/mL LH, 0.3 mM Na pyruvate and 100 µM cysteamine. Osmotic pressure of TCM-199 medium was 283±10 mOsm/kg and pH was 7.2-7.4. Cysteamine was added to the first group oocytes in OMM medium (Group A), in the second group it did not contain cysteamine in OMM medium (Group B, control), and both of them were incubated for 24 h at 38.8 °C in an atmosphere of 5% CO_2 in humidified air.

In vitro fertilization (IVF)

After maturation the oocytes that had expanded cumulus oophorus cells were accepted as maturated (Figure 1, 2) and they were transferred in B-SOF medium that was recently incubated in a gas environment as Group A and B. BSOF medium was supplemented with 0.1 mM Na pyruvate, 1 mM glutamine, 2% oestrus sheep serum (v/v), 0.07 mM streptomycine, 0.14 mM kanamycine and 0.2 mM penicillin combination. Osmotic pressure of BSOF medium was 283±10 mOsm/kg and pH; 7.9. Fresh ram semen was used for fertilization. Spermatozoa were incubated for 20 min for swim-up process in H-SOF medium, counted in Thoma and calculated as 50 x 10^7 / mL for fertilization. HSOF medium was supplemented with 0.7 mM Na pyruvate, 2 mM glutamine, 3 mg/mL BSA, 0.14 mM streptomycine, 0.28 mM kanamycine and 0.4 mM penicillin combination. Then they were

incubated for 18 h fertilization at 38.5 °C in 5% CO_2. Osmotic pressure of HSOF medium was 283±10 mOsm/kg and pH was 7.2-7.4.

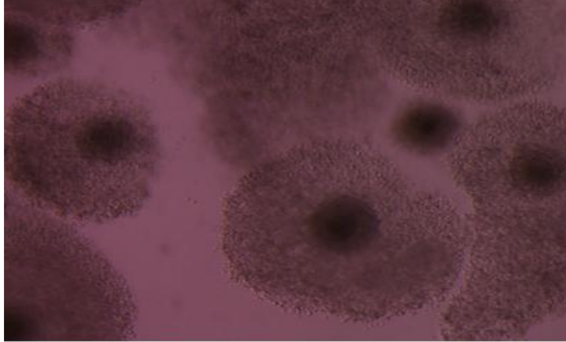

Figure 1. Expanded cumulus oophorus cells of sheep oocytes after in vitro maturation.

Şekil 1. In vitro olgunlaşmadan sonra koyun oositlerinin genişlemiş kumulus ooforus hücreleri.

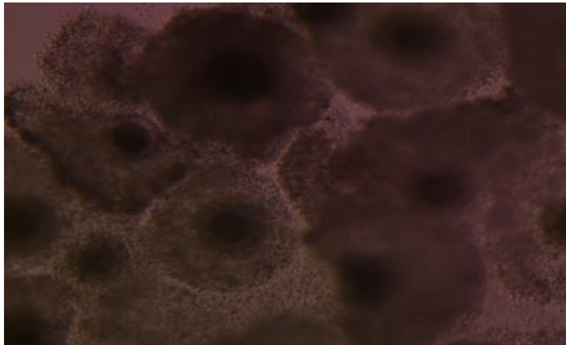

Figure 2. In vitro maturated sheep oocytes.

Şekil 2. İn vitro olgunlaştırılmış koyun oositleri.

In vitro culture (IVC) and cleavage controls

Maturated ovine oocytes in OMM medium with or without cysteamine were tested in SOF and CR1aa media. For this reason the two groups were divided into two sub-groups and totally 4 culture groups were formed. Group AI-SOF, Group AII-CR1aa, Group BI-SOF, Group BII-CR1aa. SOF medium supplemented with 0.3 mM Glutamine, 4 mg/mL BSA, 0.35 mM Na pyruvate, 0.07 mM streptomycine, 0.14 mM kanamycine and 0.2 mM penicillin combination. Osmotic pressure of SOF medium was 283 mOsm/kg and pH; 7.2-7.4. CR1aa medium supplemented with 0.2 mM glutamine, 3 mg/mL BSA, 5% FCS (v/v), 0.2 mM penicillin, 0.14 mM streptomycine. Osmotic pressure of CR1aa medium was 283 mOsm/kg and pH; 7.2-7.4. The presumptive zygotes were vortexed in tubes containing H-SOF to remove the cumulus cells and spermatozoa and they were cultured in 100 µl droplets SOF and CR1aa medium at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2 for 48 h. Cleavage was assessed on day 2 of IVC.

Statistical Analysis

At the end of 48 h cleavage rates were evaluated with SPSS 13.0 packet programme according to Chi-square test. Level of significance was P<0.05.

Results and Discussion

The aim of the present study was to determine the effect of addition of cysteamine to maturation medium on subsequent cleavage rates of sheep embryos in SOF or CR1aa media. Current study results are indicated in Table 1.

Table 1. Mean cleavage rate (%) of embryos in the culture groups that have been maturated in TCM-199 with/without cysteamine of this study.

Tablo 1. Sisteaminli veya sisteaminsiz TCM-199 medyumunda olgunlaştırılmış embriyoların kültür gruplarında ortalama bölünme oranları (%).

Parameter (%)	Group I	Group II	Group III	Group IV	Significance
	(AI-SOF)	(AII-CR1aa)	(BI-SOF)	(BII-CR1aa)	
	59.5	55.4	65.3	59.3	**NS**

NS: Non-significant

According to the results there was no statistically significant (P>0.05) difference detected between the groups for cleavage rates (Figure 3) in this study. The enzymatic and chemical reactions occur during oocyte maturation can affect the development of embryos. Successful in vitro maturation depends on nuclear and cytoplasmic maturation of oocytes (Freitas and Melo, 2010). Factors facilitating oocyte maturation are in protein structure (maturation-promoting factor, cell division cycle kinase, and mitogen-activated protein

kinase), mitochondria, endoplasmic reticulum and fat (Arlotto et al., 1996; Fair et al., 1995; Gardner et al., 2004; Rizos et al., 2002). Reactive oxygen species affect processes like oocyte maturation, fertilization and embryo development but also pregnancy (Lagod et al., 2001). The addition of thiol compounds such as cysteamine can be effective on embryo development and it increases intracytoplasmic glutathione concentration and leads to protection of cell from culture oxidative stress (Cognie et al., 2003). Glutathione content improves the maturation of oocytes when the oocyte approaches to ovulation (Lafleur et al., 1994; Perreault et al., 1988; Teleford et al., 1990), besides addition of cysteamine increases glutathione synthesis and decrease the hydrogen peroxide levels (de Matos et al., 1996; de Matos et al., 1999; de Matos et al., 2000). Because of the chemical composition of culture media oocytes and embryos can be affected in different ways during their development stages. Shabankareh and Zandi (2010) reported that when cysteamine supplemented with EGF and IGF-I, they have failed to improve the cleavage rate but improved the blastocyst rate. Gasparrini et al. (2003) and Gasparrini et al. (2006) have reported that supplementation with cysteamine during in vitro maturation did not improve the rate of cleavage in buffalo embryos. This study's results are in line with them. Although their results have indicated us antioxidant substances did not affect on cytoplasmic maturation of oocytes positively, these substances have facilitated the embryos to reach blastocyst stage. The formation and the amount of reactive oxygen species occur insufficiently at the initial cleavage stages, subsequently reactive oxygen species amounts may attend to increase when the embryo reaches to blastocyst stage because of the metabolic products and apoptotic cells. At the begining of this study, the effect of cysteamine on maturation of sheep oocytes was investigated because there are few researches about cysteamine on sheep oocyte maturation. Sandal and Özdaş (2012) reported that no statistically significant difference was found between bovine oocytes maturation supplemented with or without cysteamine. SOF (Gardner et al., 1994; Gomez et al., 1998; Walker et al., 1996) and CR1aa (Rosenkrans et al., 1993; Sreenivas et al., 2013) are prefferable media for sheep embryo development (Freitas and Melo, 2010), in current study, the sheep oocytes that were supplemented with cysteamine or not in those two media were searched. Wani et al. (2012) have reported that cysteamine supplementation did not affect the maturation rate when compared to control medium. Furthermore, they used TCM-199 for culture medium different from current study, they found the cleavage rate in TCM-199 with cysteamine as 52.7%, which is lower than this study when compared to either SOF (59.5%) or CR1aa (55.4%) with cysteamine addition. As chemical substances act on the development of embryos in different ways in different culture environments, the results obtained from this study are in line with Wani et al. (2012).

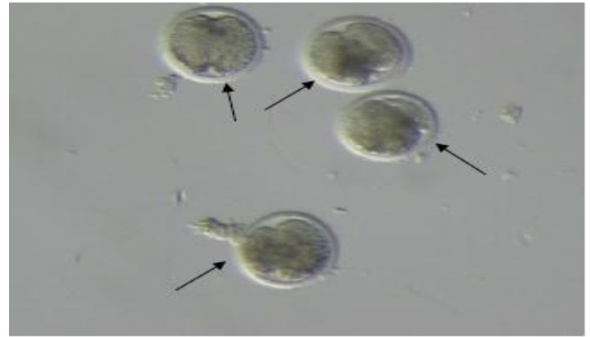

Figure 3. Two-cell cleavaged embryos (arrows).
Şekil 3. İki hücreli bölünmüş embriyolar (oklar).

Conclusion

Cysteamine supplementation to maturation medium showed no positive effect on cleavage rate of sheep embryos cultured in SOF or CR1aa media in this study, it is thought that the positive effects of antioxidant substances may occur by decreasing the metabolic wastes in culture medium in embryos that were incubated for long period and future researches may be required to clarify this hypothesis about the effect of antioxidants on embryos in different culture environments.

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