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Research Article

The Effect of Cysteamine and Oviductal Cells in Different Culture Media on the Development of Sheep Embryos [1]

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Abstract

Sheep is a very important source of wool, meat and milk all over the world. 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 are used to supplement culture media to counter the oxidative stress. This study was aimed to detect the effect of cysteamine supplementation to the maturation medium and oviductal cell supplementation to culture medium on the subsequent development rates of sheep embryos with the control group. Oocytes were obtained from slaughtered sheep ovaries. Selected oocytes were incubated with or without 100 µM cysteamine in TCM-199 medium under 38.5-38.8ºC 5% CO_2 for 23 h. During IVF fresh semen was collected from ram by electroejaculation, they were washed in H-SOF medium and were fertilized in B-SOF medium with oocytes incubated for 18 hours under 38.5-38.8ºC 5% CO_2, 5% O_2 and 90% N_2. The oocytes were obtained from maturation medium with/without cysteamine (C+,-) and were cultured in SOF or CR1aa media with/without oviductal cells (Ov+,-) and were grouped as; Group Ia: SOF+(C+ Ov-), Group Ib: SOF+(C+Ov+), Group Ic: SOF+(C- Ov-) Group Id: SOF+(C- Ov+); Group IIa: CR1aa+(C+ Ov-), Group IIb: CR1aa+(C+ Ov+), Group IIc: CR1aa+(C- Ov-), Group IId: CR1aa+(C- Ov+). Embryos were incubated under 38.5-38.8ºC 5% CO_2, 5% O_2, 90% N_2 in culture medium for 7 days. Embryo developments were observed and recorded daily. GLM procedure found in SPSS packet program was used for statistical analysis in this study. In conclusion, the addition of cysteamine or oviductal cells in vitro culture media found to have any effect in terms of the capacity of reaching to blastocyst stage in SOF or CR1aa media and no statistical difference is detected between groups.

Keywords: *Cysteamine, Embryo, Oviductal cell, Medium, Sheep*

Kültür Medyumlarında Sisteamin ve Ovidukt Hücrelerinin Koyun Embriyo Gelişimi Üzerine Etkisi

Özet

Koyun yün, et ve süt kaynağı olarak dünya çapında önemli bir çiftlik hayvanıdır. İn vitro kültür esnasında oksidatif stres embriyo ve gametlerin gelişiminde defektlere neden olur. Sisteamin, L-askorbik asit, beta merkaptoetanol, sistein, glutatyon, proteinler, vitaminler gibi birçok antioksidanlar oksidatif stresi engellemek için kültür medyumunda kullanılır. Bu çalışmada maturasyon medyumuna sisteamin ve kültür medyumuna ovidukt hücreleri eklenen oositlerin kontrol grubu oluşturularak kültürleri sonucundaki gelişim aşamalarının değerlendirilmesi hedeflendi. Oositler mezbahada kesilen koyunların ovaryumlarından elde edildi. Seçilen oositler 100 µM sisteamin ilaveli ve ilavesiz TCM-199 medyumunda 38.5-38.8ºC'de %5 CO_2 altında 23 saat boyunca inkübe edildi. IVF aşamasında koçlardan elektroejakülasyon yöntemiyle elde edilen taze sperma H-SOF medyumunda yıkandıktan sonra fertilizasyon amacıyla B-SOF medyumunda bulunan oositler ile 18 saat 38.5-38.8ºC'de %5 CO_2, %5 O_2 ve %90 N_2'de inkübe edildi. Sisteaminli/sisteaminsiz (S +, -) maturasyon medyumundan çıkan oositler, SOF veya CR1aa medyumlarında ovidukt hücreli/ovidukt hücresiz (Ov +, -) şu şekilde gruplara bölündü; Grup Ia: SOF+(S+ Ov-), Grup Ib: SOF+(S+Ov+), Grup Ic: SOF+(S- Ov-) Grup Id: SOF+(S- Ov+); Grup IIa: CR1aa+(S+ Ov-), Grup IIb: CR1aa+(S+ Ov+), Grup IIc: CR1aa+(S- Ov-), Grup IId: CR1aa+(S- Ov+). Embriyolar 38.5-38.8ºC'de %5 CO_2, %5 O_2, %90 N_2 içeren inkubatörde 7 gün boyunca kültüre edildi. Kültür sonucunda embriyoların blastosit aşamasına kadar olan gelişimleri günlük olarak izlenerek kaydedildi. Bu çalışmada istatistik analiz için SPSS paket programında bulunan GLM prosedürü kullanıldı. Sonuç olarak, SOF ve CR1aa medyumlarında kültüre edilen koyun embriyolarının blastosiste ulaşma kapasitesi açısından in vitro üretim medyumlarında sisteamin veya ovidukt hücresi ilavesinin gelişimleri üzerine bir etkisinin olmadığı ve gruplar arasında istatistiksel bir önemin olmadığı tespit edildi.

Anahtar sözcükler: *Sisteamin, Embriyo, Ovidukt hücresi, Medyum, Koyun*

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INTRODUCTION

Sheep is a very important source of wool, meat and milk all over the world. In recent years, sheep population is significantly decreased in our country, the increase in the population of sheep can be achieved by the transfer of embryos obtained from *in vivo* and *in vitro* biotechnological methods. *In vitro* embryo production technologies in sheep and goats provide so many advantages; i) maintain to obtain plenty of embryo from the animals that have high genetic values as the oocytes can be harvested from the prepubertal, pregnant and even slaugtered sheep and goats ii) maintain a wonderful source for basic embryonic researches by its lower costs iii) provide a strategy for embryo transfer of some endangered animal species by interspecific embryo transfer [1,2]. The environment that supports the embryo development can be achieved by different culture conditions, oviduct cells and medium supplementation and gas atmospheres [3]. Embryo recovery and viability can be usually affected by different culture media and systems [4,5]. Culture media are species specific and there are many systems in the culture of *in vitro* fertilized oocytes. SOF medium is usually used for the development of sheep embryos *in vitro* when strengthen with amino acids supports the embryo development of sheep and goats [6], besides Hams-F-10, Thyrodes medium and CR1 media are used in *in vitro* sheep culture experiments [5-10]. TCM-199, Minimum essential medium (MEM) can be used for *in vitro* maturation of sheep oocytes and supplementing FSH and LH to maturation medium is essential for subsequent *in vitro* fertilization and culture [11,12]. Bovine serum albumin (BSA) can be used for fertilization medium to support the sperm capacitation [13]. Ewe serum, foetal calf serum, human serum and BSA can be used to support the culture media of sheep [10]. Aerobic metabolism causes the production of reactive oxygen species even under basal conditions [14]. The physiologic level of ROS; H_2O_2 and superoxide is produced especially during normal metabolism of mammal embryos [14]. During *in vitro* culture, embryos are exposed to inevitable suboptimal culture conditions and the production of ROS molecules exceeds the level of its *in vivo* production [14]. Oocytes and embryos are prevented against oxidative stress by oxygen scavengers present both in follicular and oviductal fluid. As the oocytes and embryos are moved away from their normal enviroment in *in vitro* production system, their defense system is lost [15]. Reactive oxygen species (ROS) has got so many harmful effects on the cells, these are DNA damage, lipid peroxidation, and oxidative changes of proteins [16]. Oxidative stress leads to mitochondrial damage, DNA, RNA and protein damage, reduction in sperm oocyte junction, especially embryo cell bloque during blastocyst period and cell death [17]. So many researchers have added various antioxidants to the culture medium in order to reduce the harmful effects of ROS on embryo development. Low molecular weight thiol compounds such as β-mercaptoethanol, cysteine, cystine and cysteamine are used to supplement oocyte maturation media and embryo culture media [18-24]. To develop *in vitro* embryo production oxidative stress during *in vitro* culture must be taken under control [25]. Glutathione is a natural antioxidant that is present in gametes and its level changes [26-29]. Cysteamin is a low molecular weight thiol compound which reduces cystine to cysteine and enhances oocyte glutathione synthesis [30]. de Matos *et al.*[31] and de Matos *et al.*[32] have reported the addition of cysteamine to the *in vitro* maturation media improves the rate of embryo development by increasing glutathione synthesis in cow and sheep. It was reported previously that oviduct cells in the oviduct play a major role in the embryo development [33-37]. Therefore, oviduct cell cultures are widely used during *in vitro* culture studies [6,38]. Usage of a co-culture passively influences the development of embryos at an early stage of development by reducing the negative effects of toxic substances within the culture media [39,40].

The aim of the present study is to detect the development competence of the embryos in which they were supplemented with cysteamine in maturation medium and oviductal cells in different culture media (Synthetic Oviduct Fluid-SOF and Charles&Rosencrans-CR1aa) by comparing them with controls.

MATERIAL and METHODS

Oocyte Recovery

Eight replica 604 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 and total 2060 oocytes were collected out of the season. The cumulus oocyte complexes (COCs) were recovered by slicing method of antral follicles of 2-8 mm in diameter.

Preparation of Oviductal Cells

The oviducts that were brought to laboratory at the same time with ovaries were freed from surrounding fat and connective tissue without compromising the integrity of the channel and washed with PBS 3 times. The epithel cells in the canal squeezed from the begining of uterotubal junction with the help of forceps were transferred into the TCM-199 medium. The epithel cells were centrifuged 3.000 rpm for 5 min and the supernatant was throwed away and the fresh medium was added over the pellet at the bottom and centrifugation was repeated for 3 times and the remaining pellet at the bottom of the tube was transferred to the 80 cm^2 tissue culture flask and incubated for 48 hours under 5% CO_2. At the end of this process two different sample obtained from the culture suspension that contain oviductal cells were transferred to different tubes and centrifuged at 3.000 g for 2 min. The same process repeated again. After the supernatant was threw away 2 ml medium was added up the remaining oviduct

cell pellet at the bottom of the tube and were transferred at an appropriate proportion to the culture dishes to be used.

In vitro Maturation (IVM)

Selected oocytes were washed 3 times in TCM-199 supplemented with 10% FCS (v/v), 1 µg/mL FSH, 10 µg/mL LH, 0.3 mM Na +L-glutamine + 50 µg/ml gentamycine sulphate±100 µM cysteamine and without cysteamine then were transferred to the 4 well dishes that contains the same medium and were incubated 38.5-38.8°C under 5% CO_2 in humidified air for 23 h.

In vitro Fertilization (IVF)

After maturation the oocytes that had expanded cumulus oophorus cells were accepted as maturated and they were transferred in B-SOF medium that was recently incubated in a gas environment. 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 collected from ram by electroejaculation and used for fertilization. Spermatozoa were incubated for 20 min for swim-up process in H-SOF medium, counted in Thoma slide and calculated as $4x10^5$-$1x10^6$/ml per oocyte 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 38.5-38.8°C in 5% CO_2, 5% O_2 and 90% N_2. Osmotic pressure of HSOF medium was 283±10 mOsm/kg and pH was 7.2-7.4.

In vitro Culture (IVC) and Blastocyst Controls

Maturated ovine oocytes in oocyte maturation medium (OMM) with or without cysteamine were tested in SOF and CR1aa culture media by addition of oviductal cells or not. Totally 8 culture groups were formed.

Group Ia: SOF+(C+ Ov-), **Group Ib:** SOF+(C+Ov+), **Group Ic:** SOF+(C- Ov-), **Group Id:** SOF+(C- Ov+);

Group IIa: CR1aa+(C+ Ov-), **Group IIb:** CR1aa+(C+ Ov+), **Group IIc:** CR1aa+(C- Ov), **Group IId:** CR1aa+(C- Ov+).

SOF medium supplemented with 5% FCS (v/v), 2% BME, 1% MEM, 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 2% BME, 1% MEM, 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 38.5-38.8°C in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 for 6-7 days. The development of embryos till blastocyst stage was evaluated and recorded daily. GLM procedure from SPSS packet program was used for this study.

RESULTS

Eight replica 604 ovaries were obtained and 2060 ovine oocytes were collected, from these oocytes 1626 were used for maturation, out of them 1056 oocytes were incubated in maturation medium with cysteamine whereas 570 oocytes were incubated in maturation medium without cysteamine. Total cleaved oocytes that were incubated in culture medium were 1034 and out of them 619 oocytes were obtained from maturation medium with cysteamine and 415 oocytes from maturation medium without cysteamine. At the end of the culture period totally 315 early blastocysts and blastocysts were obtained. In the used statistical model the main effects of SOF/CR1aa, cysteamine and oviductal cell status were taken part. These main effects bilateral interactions were found insignificant so these effects were not included in the statistical model. Average early blastocyst/blastocyst rate was detected as 376/1163=32.3% in all groups.

Embryo development cultured in SOF/CR1aa media with/without oviductal cells which were previously maturated with/without cysteamine is given in *Table 1* and *Table 2*.

DISCUSSION

In present study, development competence of the embryos were evaluated at the end of the culture period in which they were supplemented with cysteamine in maturation medium and oviductal cells in culture medium by comparing them with their controls.

It is well known that the addition of antioxidant substances to maturation medium is a critical point for to ensure the desired effect on the first phase of embryo development [41]. Gasparrini *et al.*[42] reported that the addition of cysteamine to the medium during buffalo oocyte maturation has increased the cytoplasmic glutathione content and subsequent embryo development. Cysteamine was added to medium during maturation period but the effect of addition of cysteamine on cytoplasmic glutathione content was not evaluated in current study.

Shabankareh and Zondhi [43] detected the blastocyst rate of bovine embryos 30.8% without cysteamine in maturation medium, wheras they found the same ratio as 35% when added cysteamine to maturation medium. In current study, the blastocyst rate (Group Ia) was found as 33.3% in the embryos that were maturated in medium

Table 1. *The effect of cysteamine and oviductal cells supplementation in different culture media on the development of sheep embryos*
Tablo 1. *Değişik kültür medyumlarında sisteamin ve ovidukt hücrelerinin eklenmesinin koyun embriyo gelişimi üzerine etkisi*

Groups	Group Ia %			Group Ib %			Group IIa %			Group IIb %			Total %		
	Oocyte Number	Clevaged Oocytes	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*
n1	35	15	4	41	18	3	36	12	2	41	12	2	153	57	11
n2	25	17	8	18	14	6	25	16	5	18	12	4	86	59	23
n3	29	16	3	24	16	4	29	16	4	24	7	1	106	55	12
n4	28	20	10	42	27	8	29	10	4	42	28	5	141	118	33
n5	52	30	7	60	42	10	60	30	5	60	38	7	232	140	29
n6	30	13	4	36	19	6	28	10	2	34	20	4	128	62	16
n7	25	19	12	19	15	6	20	16	6	24	19	13	88	69	37
n8	60	35	7	22	14	5	15	12	5	25	16	4	122	77	21
TOTAL	284 58.0%	165	55 33.3%	262 68.7%	180	48 26.6%	242 50.4%	122	33 27.0%	268 56.7%	152	40 26.3%	1056 73.1%	772	227 29.4%

Table 2. *The effect of cysteamine and oviductal cells supplementation in different culture media on the development of sheep embryos (continued)*
Tablo 2. *Değişik kültür medyumlarında sisteamin ve ovidukt hücrelerinin eklenmesinin koyun embriyo gelişimi üzerine etkisi (devam ediyor)*

Groups	Group Ic %			Group Id %			Group IIc %			Group IId %			Total %		
	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*	Oocyte Number	Clevaged Oocyte	Eb-bls*
n1	6	4	2	5	3	1	6	3	2	5	3	1	22	13	6
n2	22	17	8	21	16	7	15	13	7	26	18	5	84	64	27
n3	26	18	7	20	16	6	20	17	6	23	17	5	89	68	24
n4	10	7	3	9	7	2	10	6	2	10	6	3	39	26	10
n5	9	6	2	55	42	18	51	18	9	64	43	15	179	109	44
n6	15	11	3	9	5	2	12	9	4	10	7	3	46	32	12
n7	17	12	4	14	11	3	11	9	3	12	9	4	54	41	14
n8	8	3	2	22	18	4	20	13	5	7	4	1	57	38	12
TOTAL	113 73.4%	83	31 37.34%	155 76.12%	118	33 27.9%	145 74.4%	108	38 35.1%	157 67.5%	106	37 34.9%	570 68.5%	391	149 38.1%

** Early blastocyst/blastocyst; % Clevaged oocyte/Oocyte number*

with cysteamine, whereas the same ratio was found similar as 37.34% in Group Ic and 35.1% in Group IIc without cysteamine supplementation during maturation. In this study addition of cysteamine to maturation medium and later addition of oviductal cells to culture medium was found to have no effect on embryo development. We think that this difference can be originated because of the marked variations in terms of the material and methods variations of these two studies and could be attributed to different animal species. Besides Shabankareh and Zondhi [43] was added EGF and IGF-I to maturation medium suggests us more effective for embryo development. Shabankareh and Zondhi [43] preferred SOF medium to fertilize the maturated oocytes in their study, in this study BSOF medium was used and indeed in terms of the culture periods there are important differences in these two studies. In present study, SOF and CR1aa media were compared for culture and higher proportion of embryo development was determined in SOF medium.

Balasubramanian and Rho [24] added cysteamine to the maturation medium of bovine oocytes and were assessed significantly higher blastocyst rate on day 7 and 8 than control group. Cysteamine addition showed no different blastocyst yield when compared to control group in this study indicates us to think this difference could have been arisen because of the different animal species.

The addition of thiol compounds like cysteamine or beta-mercaptoethanol in the medium during sheep and bovine oocyte maturation was reported to facilitate the induction of glutathione synthesis and reduces the hydrogen peroxide levels and increase the embryo development [31,44,45]. We think their higher amount of cysteamine addition to maturation medium in their studies could have been lead to higher rate of embryo development than this study. Wani *et al.*[46] added cysteamine to maturation medium of sheep oocytes and was aimed to detect the effect of cysteamine on the development competence of sheep embryos after culture period and they used TCM-199 medium in their study for maturation as we did. They added 200 µM/ml cysteamine to maturation medium and after culture with TCM-199 they found 36.7% embryo rate that reached to morula stage. In current study 100 µM cysteamine was added to maturation medium, and at the end of the culture period in SOF or CR1aa media early blastocyst/blastocyst rates were detected as 33.3% and 27%, respectively and this result was consistent with the findings of Wani *et al.*[46] study.

Rodriquez-Dorta *et al.*[47] investigated the effect of goat oviductal cells on *in vitro* goat embryo development in SOF medium supplemented with 3 mg/ml BSA and they detected the cleavage rate as 83%, this rate was detected as 76.12% in present study.

The usage of somatic cell was encouraged to support the *in vitro* mammal' embryo development in the past. Firstly mouse embryo culture was used in mouse ovary organ culture. Till than, embryo somatic co-culture was used in a large scale of animal specie including buffalo. Somatic cells have highly effective embryotrophic impact. These are faster cleavage, less fragmentation, increased blastocyst cell numbers, advanced morphologic image, low apoptosis rate, advanced prenancy rates and live births [48]. During *in vivo* development through somatic cells like oviductal epitel cells embryonic environment can be regulated metabolically. These cells take part in several tasks like reducing of oxygen pressure partially and provision of useful metabolites for embryos. So the usage of oviductal epitel cells can be helpful during *in vitro* embryo development [49].

Özdaş *et al.*[49] were used SOF and B2 media for culturing bovine embryos and were added oviductal cells to these culture media and they found a positive effect of oviductal cells on development competence of embryos besides they were detected this effect as statistically significant in their study. In current study, conversely the addition of oviductal cells to culture media of sheep embryos were not found statistically significant. The difference organized from the addition of oviductal cells suggests us that can be arisen because of the media types and conditions used in laboratory can affect in different ways on embryo development. As it is determined that There is no difference with about the addition of oviductal cells on subsequent embryo development during culture period of fertilized oocytes when compared to control group in this study. We think this difference could have been arisen because of the planning of two studies and during culture periods two culture media (SOF and CR1aa) were used in this study but only SOF medium was used as common in the other study.

Sandal and Özdaş [23] supports our hypothesis that they did not find any effect of cysteamine supplementation to maturation medium on subsuquent embryo development of bovine embryos.

In conclusion, it was found to have any impact effect on sheep embryo development supplemented with cysteamine or oviductal cells in terms of the capacity of reaching to blastocyst stage cultured either in SOF nor CR1aa media and no statistical significance was detected between the groups in present study. We tried to emphasize the importance of different systems on embryonic development capability with this study. Still today the addition of antioxidants such as cysteamine to maturation medium and the effect of it on development of embryos during culture period is a matter under investigation.

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