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DETERMINATION OF 70 KDA PROTEINS FOR DIFFERENT TEMPERATURES IN CATTLE OOCYTES MATURED *IN VİTRO*

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Abstract

This study was conducted to determine the effect of 36.5 (low) or 38.5 °C (conventional) culture temperatures during *in vitro* maturation (IVM) on 70 kDa proteins in cattle oocytes. In the study, cattle cumulus-oocyte complexes (COC) were subjected to IVM in bicarbonate-buffered TCM–199 supplemented with 10% FCS for 22 hours with a humidified 5% CO2 in air at either 36.5 or 38.5 °C culture temperatures. Maturation of COCs was determined according to fully cumulus expansion at the end of IVM. The cumulus cells of matured COCs were removed by vortexing in TCM-199 with HEPES buffered TCM-199 containing 0.1% hyaluronidase enzyme. Following, denuded oocytes were washed in 9.6 % phosphate-buffered saline (PBS) solution for protein isolation. Denuded oocytes (n = 100 for both groups) were centrifuged for 5 min at 300 g at 4 °C for PBS removed than the lysis buffer (100 µl) was added and shake 15 min on ice for 30 minutes. For protein isolation, the protein was centrifuged again at 13,000 g for 5 minutes and the protein samples were stored at -20 ° C. To increase the concentration of isolated protein samples, the samples were frozen at -80 °C for 24 hours and lyophilized at 0.140 hPa in a lyophilizer. Lyophilized protein samples were stored at -20 °C until SDS-PAGE analysis. The level of 70 kDa proteins in oocytes was determined by SDS-PAGE method. There were no significant differences between low (81.11%) or conventional (84.41%) incubation temperatures in IVM in terms of full cumulus expansion. The 70 kDa proteins band size in SDS-PAGE polyacrylamide gel image of cattle oocytes maturated at low culture temperature was weak compared to cattle oocytes maturated at conventional culture temperature. The results of this study may show that low incubation temperature during IVM decreases the amount of 70 kDa proteins in cattle oocytes.

Keywords: Oocyte, Maturation, Culture temperature, 70 kDa Proteins, Cattle

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1. Introduction

It has been studied for a long time to understand effect heat stress on mature and immature cattles oocytes and also it caused to infertility (Ispada et al., 2018). Cattle oocytes are susceptible to environmental condition factors such as heat stress, temperature and humidity

(Badinga et al., 1993; Al-Katanani et al., 2001). *In vitro* embryo production experiments have been carried out in cattle, but this reproductive biotechnology technique is not yet satisfactory to obtain sufficient quantity of good quality transferable embryos (Pfeifer et al., 2008; Koçyiğit et al., 2015). *İn vitro* production of the cattle embryo is affected by many factors (Camargo et al., 2006). One of the basic factors is essential culture conditions of the oocyte during *in vitro* maturation (IVM) for normal maturation (Cetica et al., 2001). Maturating oocytes (Roth and Hansen 2004; Sen, 2014) in *in vitro* at high temperature (Lánská et al., 2006) (41 °C) were initiated cellular and molecular changes in the oocyte that causing to the decreased potential of development (Ispada et al., 2018). During maturation 12–14 h heat shock interrupted oocyte microtubule and microfilament texture (Roth and Hansen 2005) and spindle positioning (Ju et al. 2005), diminished nuclear maturation (Roth and Hansen 2005; Rodrigues et al. 2016), affection distribution of cortical granule (Maya-Soriano et al. 2013) and reducing mitochondrial function in oocyte (Nabenishi et al. 2012). Various approaches have been employed to improve maturation and developmental competence of cattle oocytes following *in vitro* fertilization (Katska-Ksiazkiewicz and Alm, 2005; Cevik et al., 2014). *İn vitro* maturation of cattle oocytes has been studied with different supplementation to culture media such as follicular fluid, co-culture (Moulavi et al., 2006), growth factors or gonadotropic hormones (Cevik et al., 2011). However, developmental competence of cattle oocytes not only depends on the composition of the culture media but also incubation conditions such as atmospheric condition, humidity and temperature (Leese et al., 2008).

IVM of cattle oocytes are performed at 38°C to 39°C, as this temperature is close to the rectal temperature in cattle (Shi et al., 1998). However, previous studies demonstrated that the temperature in preovulatory follicles is 1.5 to 2°C cooler than their adjacent stroma in cattle (Hunter, 2005). The existence of follicular cooling raises the question of whether oocytes develop advantageously at lower temperatures. The reduced temperature might be required for successful oogenesis or oocyte maturation, for subsequent embryonic or fetal development (Hunter et al. 2006).

Heat shock proteins (HSPs), are an protein group that is express when cells are exposed to high temperatures (Aufricht, 2005). The phenomenon heat shock response that leads to a dramatic increase HSPs, are often regulated by the heat shock factor (Morimoto and Santoro, 1998; Sarge et al., 2009). HSP70 is the most important and well-studied protein of all HSP family/families that protect cells, tissues and whole organisms from severe thermal stress (Gao et al., 2014). HSP70 can be expressed in the cell by many stress such as infections other than heat, dehydration, treatment with toxic agents, and ultraviolet light (Morimoto and Santoro, 1998, Aufricht, 2005, Petrof et al., 2005). However, high levels of HSP70 expression probably impose costs on individuals exposed to thermal stress by influencing their fecundity, growth or survival (Sørensen et al., 2003; Hamdoun et al., 2003; Karl et al., 2009). Most of these studies infer links between the high expression of HSP70 and thermal adaptation by comparing HSP70 and heat tolerance of animals from different seasons or from different thermal environments (Gao et al., 2014).

The best protected and most studied group in organisms is the 70 kDa heat shock protein family (Morimoto et al., 1986; Feige et al., 1996). The 70-kD heat shock proteins have been studied in different animal models, including (Bernardini et al., 2003) cattle oocytes (Camargo et al., 2006) and embryos (Tavares et al., 2005).

The aim of the present study was, therefore, to determine the effect of 36.5 (low) or 38.5 °C (conventional) culture temperatures during *in vitro* maturation (IVM) on 70 kDa proteins in cattle oocytes.

2. Material and Method

2.1. Oocyte Collection

All chemicals and media used in this study were purchased from Sigma-Aldrich, Turkey. In the present study, cattle ovaries were collected from a local slaughterhouse and transported to the laboratory in a thermos filled with physiological saline solution (0.9% w/v NaCl) containing 0.1 µl/mL antibiotic-antimycotic solution (10.000 IU penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml) at 34.0±2.0°C within 3 h after slaughter. Cumulus-oocyte complexes were recovered from follicles 2–8 mm in diameter by aspiration, using an eighteen-gauge needle fixed to ten ml syringe and then cumulus-oocyte complexes were washed two times with Hepes-buffered medium-199. Cumulus-oocyte complexes were assessed morphologically and only oocytes with compact, intact cumulus cells around and homogeneous cytoplasm were selected for maturation (Sen, 2015).

2.2. Preparation Culture Medium and Heat Treatments

A total of 400 cumulus-oocyte complexes were subjected to *in vitro* maturation. Maturation medium was prepared according to Cevik et al. (2011). Cumulus-oocyte complexes were separately placed in 500 µl of maturation medium covered with 300-µl mineral oil in four-well dishes approximately thirty cumulus-oocyte complexes per well. Maturation medium was sodium bicarbonate-buffered Medium 199 (M4530; Sigma, Turkey) containing Earle's salts and L-glutamine supplemented with 5.5 µg/mL sodium pyruvate, 1% v/v antibiotic-antimycotic solution and 10% v/v (growth facrors) heat-inactivated fetal bovine serum.

Selected cumulus-oocyte complexes were matured for 22 hours in a humidified atmosphere of 5% CO2 in the air at 36.5 °C (low) or 38.5 °C (conventional). Cumulus cell expansion of cumulus-oocyte complexes at both culture temperatures were evaluated at the end of maturation period under a stereomicroscope. Cumulus-oocyte complexes with fully expanded cumulus cell layer

considered as maturated oocytes.

The cumulus cells of matured COCs were removed by vortexing in TCM-199 with hepes buffered TCM-199 containing 0.1% hyaluronidase enzyme. Following, denuded oocytes were washed three times in 9.6 % phosphate-buffered saline (PBS) solution for protein isolation. Denuded oocytes (n= 100 for both groups) were centrifuged for 5 min at 300 g at 4 °C to remove PBS. The lysis buffer (100 µl) was then added and shake 15 min on ice for 30 minutes.

2.3. Isolation Protein and Lyophilization

For protein isolation, the protein was centrifuged again at 13,000 g for 5 minutes, and the protein samples were stored at -20 untill SDS-PAGE gel electrpphoresis. To increase the concentration of isolated protein samples, firstly the samples were frozen at -80 °C for 24 hours and secondly lyophilized at 0.140 hPa in a lyophilizer. Lyophilized protein samples were stored at -20 °C until SDS-PAGE analysis. The level of 70 kDa proteins in oocytes was determined by SDS-PAGE method.

2.4. Statistical Analysis

Treatment effects (temperature) on cumulus cell expansion of cattle oocytes were analyzed by chi-square ($\chi 2$) test. Statistical analyses were performed by the SPSS 17.0 package program (SPSS, Chicago, IL, USA).

3. Results and Discussion

Maturation ratio according to cumulus expansion of cattle oocytes matured *in vitro* either at 36.5°C or 38.5°C maturation temperatures were presented in Table 1. There were no significant differences between low (81.11%) or conventional (84.41%) incubation temperatures in IVM in terms of whole cumulus expansion. The results of the present study showed that culture temperature during *in vitro* maturation had no effect on cumulus cell expansions of cattle oocytes.

Ye et al. (2007) suggested that lower follicular ambient temperature is advantageous to complete oocyte maturation or development, within the follicular microenvironment. The results of the present study show that culture temperature during *in vitro* maturation had no effect on cumulus cell expansions of cattle oocytes. Similar to the results of our study, previous studies have shown that low incubation temperature during IVM did not affect the rate of cumulus expansion (Shi et al., 1998; Lenz et al., 1983; Ravindranatha et al., 2003; Sen and Kuran, 2018).

Table 1. Maturation ratio according to cumulus expansion of cattle oocytes matured *in vitro* either at 36.5°C or 38.5°C maturation temperatures.

CT (°C)	Total oocytes	Matured oocytes	Matured oocytes ratio (%)	Std Error	P
36.5 C°	1393	1121	81.11	3.41	0.554
38.5 C°	1501	1325	84.41	2.26	

CT= culture temperatures

SDS-PAGE polyacrylamide gel image of cattle oocytes maturated at 36.5 °C (low) or 38.5 °C (conventional) culture temperatures is present Figure 1.

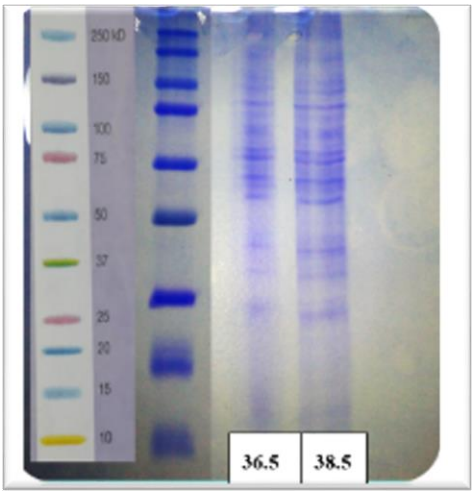

Figure 1. SDS-PAGE polyacrylamide gel image of cattle oocytes maturated at low or conventional culture temperatures.

Among the major heat shock proteins produced by most cells are those that belong to 70 kDa heat shock proteins family (Parsell and Lindquist, 1993). 70 kDa heat shock proteins protect cells against adverse effects of stress (Hendry and Kola, 1991), and function in the absence of stress as a molecular chaperone (Becker and Craig, 1994). In mammals, cytosolic members of the 70 kDa heat shock proteins family appear in two isoforms: a constitutively synthesised variant and a highly heat-inducible form. These two proteins exhibit a high degree of relatedness, although encoded by distinct genes (Welch and Feramisco, 1982). Experiments utilising metabolic labelling with 35S-amino acids and two-dimensional gel electrophoresis have indicated that maturing cattle oocytes synthesise 70 kDa heat shock proteins but the magnitude of synthesis is not increased upon heat shock (Edwards and Hansen, 1997). By the 2-cell stage, however, heat shock increases the synthesis of 70 kDa heat shock proteins. The heat-inducibility of 70 kDa heat shock protein genes at the 2-cell stage occurs even in the presence of α-amanitin (Edwards et al., 1997), suggesting either that the increase in 70 kDa heat

shock protein synthesis is regulated post-transcriptionally or, as with Drosophila (Gilmour and Lis, 1986), that RNA polymerase II is bound to the promoter before heat shock and thereby prevents the inhibitory effect of α-amanitin on transcription.

In previous studies, Kawarsky and King, (2001) determined that heat stress of HSP70 mRNA can be transcribing under control conditions in early embryonic period during *in vitro* maturation of cattle oocytes. As a result of confocal laser scans, it was reported that hsp70 protein was not affected by high temperature exposure in the mature and immature oocyte ooplasm of oocytes and embryos and this protein may be related to meiotic spindle (Kawarsky and King, 2001). Therefore, in our study maturation cattle oocyte at low incubation temperature (36.5 °C) and conventional culture temperature (38.5 °C) in *in vitro* may be a reason for the low amount of protein in 70kDa.

In the present study, the 70 kDa proteins band size in SDS-PAGE polyacrylamide gel image of cattle oocytes maturated at low culture temperature was weak compared to cattle oocytes maturated at conventional culture temperature. The results of this study may show that low incubation temperature (36.5 °C) during IVM did not have any effects on cumulus expansion and low incubation temperature during IVM decrease the amount of 70 kDa proteins in cattle oocytes. In the future, expression studies can be to describe during the heat stress in the cells. Specificity of the stress response to the cell type can be studied. The effect of nutrient content on oocytes and the synthesis of hsp70 can be investigated.

Conflict of interest

The authors declare that there is no conflict of interest.

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