Open Access
Asian-Australas J Anim Sci
Vol. 31, No. 6:827-834 June 2018
https://doi.org/10.5713/ajas.17.0569
pISSN 1011-2367 eISSN 1976-5517

AJAS
Asian-Australasian Journal of Animal Sciences

Low incubation temperature successfully supports the *in vitro* bovine oocyte maturation and subsequent development of embryos

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Submitted Jul 31, 2017; Revised Nov 19, 2017; Accepted Nov 23, 2017

Objective: The aim of this study was to compare the effects of 36.5°C and 38.5°C incubation temperatures on the maturation of bovine oocytes and developmental competence of embryos.
Methods: In experiment 1, oocytes were maturated in bicarbonate-buffered TCM–199 for 22 hours in a humidified atmosphere of 5% CO_2 in the air at either 36.5°C or 38.5°C and nuclear maturation status were determined. In experiment 2, *in vitro* fertilized oocytes were allocated randomly into synthetic oviductal fluid medium with or without a mixture of 1 mM L-glutathione reduced and 1,500 IU superoxide dismutase and cultured in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 in the air at 38.5°C for 8 days.
Results: There were no significant differences between incubation temperatures in terms of oocyte maturation parameters such as cumulus expansion, first polar body extrusion and nuclear maturation. Incubation temperatures during *in vitro* maturation had no effects on developmental competence of embryos, but supplementation of antioxidants increased ($p<0.05$) developmental competence of the embryos. Blastocysts from oocytes matured at 38.5°C had comparatively higher inner cell mass, but low overall and trophectoderm cell numbers ($p<0.05$).
Conclusion: The results of present study showed that maturation of bovine oocytes at 36.5°C may provide a suitable thermal environment for nuclear maturation and subsequent embryo development.

Keywords: Bovine; *In vitro* Maturation; Culture Temperatures; Antioxidants; Embryo Development

INTRODUCTION

Many researchers have investigated conditions required to improve *in vitro* maturation (IVM) and subsequent development of cattle oocytes [1,2]. In this respect various supplements to culture media have been examined including serum [3], follicular fluid, co-culture [4], growth factors and/or gonadotropic hormones [2]. However, IVM of bovine oocytes is not only related to the constituents of the culture medium but also to the incubation temperature [5]. A basic variable in the cellular environment of *in vitro* cultures is temperature [4]. Conventional *in vitro* bovine embryo production technology including oocyte maturation, fertilization and embryo culture is performed at 38.5°C or 39°C which represents core body temperature of cattle [6]. However, Leese et al [5] reported that preovulatory follicles have a temperature of approximately 1.5°C to 2°C less than that of the ovarian stroma or core body temperature in cattle, but this fact is rarely or never taken into account in *in vitro* embryo production. Moreover, McEvoy et al [7] suggested that present *in vitro* oocyte maturation technology is far from the *in vivo* reality due to incubation temperature employed. Also, Hunter et al [8] suggested that *in vivo* formation of meiotic and cytoplasmic maturation of oocyte, which occur at low temperature, should be considered in *in-vitro* studies. These phenomena lead

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to the suggestion that a temperature lower than core body temperature of cattle may provide better oogenesis or maturation of the bovine oocytes when cultured *in vitro*, and therefore, may result in an improvement in the subsequent development after fertilization.

Intra-cellular ATP production is realized as a result of oxidative metabolism, but also reactive oxygen species (ROS) are produced as undesired by-products of the process [9]. The amount of ROS production can vary depending on the rate of oxidative metabolism, and higher metabolic activity is associated with increased amounts of ROS production [10] that may cause cellular damage such as cellular enzymes inactivation, DNA damage and cell membrane lipid peroxidation in embryonic cells [5]. Generation of ROS is an unavoidable result of oxidative reactions, but various preservative scavengers such as superoxide dismutase, catalase and glutathione remove radical species before they can cause significant damage in *in vitro* embryo production systems. However, it would be possible to achieve a lower production of ROS with reduced metabolic activity and/or oxidative metabolism rate. In that case, culture media may require comparatively less or no supplementation with an antioxidant system against the ROS. Therefore, if environmental conditions of the *in vitro* culture of oocytes do not allow an increase in the rate of embryo metabolism, it may be possible to eliminate or reduce supplementations of the culture media with antioxidants. To this effect Leese [11] has put forward the 'Quiet Embryo Hypothesis', which proposed that low (quiet) embryo metabolism would better serve the embryo viability than active metabolism. This leads to the notion that the oocyte and the early embryo may function better at a lower temperature which is also representative of the *in vivo* preovulatory follicle environment [5]. Therefore, in this study we tested the hypothesis that exposure to lower temperature (36.5°C) representative of the *in vivo* thermal conditions of the bovine preovulatory follicles, improves the maturation and subsequent post-fertilization development of the bovine oocytes. The main aim was to compare the effects of low (36.5°C) and conventional (38.5°C) culture temperatures on the oocyte maturation parameters and the embryonic development including the cell numbers, diameter and blastocyst quality.

MATERIALS AND METHODS

All chemicals and media used in this study were purchased from Sigma-Aldrich, Turkey, except where otherwise indicated.

Collection and *in vitro* maturation of oocytes

Within 3 h after slaughter bovine ovaries, which were collected from a local slaughterhouse, were transported to the laboratory in 0.9% NaCl (w/v) containing 0.1% v/v antibiotic solution (10,000 IU penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL) at 33.2°C±2.0°C. Cumulus-oocyte complexes (COCs) were aspirated from follicles (2 to 8 mm in diameter) with an 18-gauge needle attached to a 10-mL syringe. The COCs were collected in 3 to 4 mL Hepes-buffered TCM–199 (M7528; Sigma, Samsun, Turkey) containing Earle's salts and supplemented with 1% v/v antibiotic-antimycotic solution, 100 μg/mL L–glutamine and 5% v/v heat-inactivated fetal calf serum (FCS). COCs were then classified as Grade A and Grade B according to previously described criteria [2]. Grade A COCs had homogeneous cytoplasm with an intact cumulus cells around oocytes and Grade B COCs had homogenous cytoplasm but an unevenly surrounding cumulus investment around oocytes. After classification, COCs were washed three times in Hepes-buffered TCM–199 and then twice in maturation medium. Maturation medium was sodium bicarbonate-buffered TCM–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, 10% v/v heat-inactivated FCS, 5.0 μg/mL luteinizing hormone, 0.5 μg/mL follicle stimulating hormone and 10 ng/mL epidermal growth factor. Grade A and Grade B COCs were separately placed in 500 μL of maturation medium (approximately 25 to 35 COCs per well) covered with 300 μL mineral oil in four-well culture dishes (Nunc, Roskilde, Denmark) and matured for 22 hours in a humidified atmosphere of 5% CO_2 in the air.

Assessment of maturation parameters of oocytes

Cumulus cell expansions of COCs were evaluated at the end of maturation period under a stereomicroscope. COCs with fully expanded cumulus cell layer were considered as maturated oocytes. After evaluation, COCs were washed three times in Hepes-buffered TCM–199. Subsequently, COCs were completely denuded of cumulus cells by vortexing and first polar body of oocytes was evaluated. Following, oocytes were fixed with a mixture of acetic acid: ethanol (1:3, v/v) for 24 h at 4°C for evaluation nuclear status. The fixed oocytes were mounted on glass slides with 90% glycerol in Dtulbecco's phosphate-buffered saline (pH 7.4) containing 10% (w/v) Hoechst 33342 (H-6024) at room temperature for 15 min and examined under fluorescent microscope. Excitation was induced at 365 nm and the emission was viewed through a 420-nm barrier filter. Nuclear maturation was evaluated without knowing the treatment groups by an independent evaluator and classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (M I) (including anaphase I and telophase I), metaphase II (M II) as described by Cevik et al [2].

Spermatozoa preparation and *in vitro* fertilization

Following maturation period, COCs were washed twice in Hepes-buffered TCM–199 and then twice in *in vitro* fertil-

ization (IVF) medium. The IVF medium was glucose-free modified Tyrode's albumin lactate pyruvate (TALP; adjusted pH 7.4 and 280 to 300 mOsm/kg) supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free bovine serum albumin (BSA), 10 mg/mL heparin-sodium salt and 0.5 μL/mL antibiotic-antimycotic solution. COCs were transferred into 46 μL IVF drops (approximately 15 COCs per drop) covered with mineral oil. Single bull frozen semen was used for IVF after thawed. Separation of motile sperm cells in the frozen–thawed semen was carried out by Percoll density gradient method. The sperm density was assessed by a hemocytometer using a phase contrast microscope at a magnification of 400× and adjusted to 50×10^6 spermatozoa/mL with IVF medium. The sperm motility was visually checked for acceptable motility (at least 80% progressively motile). The oocytes were fertilized with 2 μL diluted semen and 2 μL of penicillamine, hypotaurine and epinephrine cocktail (20 μM penicillamine, 10 μM hypotaurine, and 1 μM epinephrine in final concentration) per IVF drop for 22 hours in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 in air at 38.5°C.

In vitro culture

After IVF, the putative zygotes were washed three times in Hepes-buffered TCM–199 and vortexed for 5 min to remove cumulus cells layer. The naked zygotes were washed twice in Hepes-buffered TCM–199 and then twice in synthetic oviductal fluid (SOFaa) embryo culture media. The SOFaa embryo culture media was supplemented with 40 μg/mL sodium pyruvate, 8 mg/mL fatty acid-free BSA, 20 μL/mL non-essential amino acids solution (100×), 10 μL/mL amino acids solution (50×) and 0.5 μL/mL antibiotic-antimycotic solution on the day of use. The zygotes were placed in 50 μL drops (approximately 15 zygotes per drop) of SOFaa embryo culture media under mineral oil and cultured in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 in air at 38.5°C. The IVF day was accepted as day 0 of embryonic development. Zygotes cleaved were determined on day 3 of development. Percentages of zygotes developed to morula and blastocyst stages were evaluated on days 5 and 8, respectively.

Determination of diameter and quality of blastocyst

The diameter of each blastocyst was measured using a lens micrometer attached to an inverted light microscope (×320). The quality of the blastocysts was determined according to the morphological criteria of International Embryo Transfer Society [12]. Blastocysts were categorized as Grade I; excellent or good quality blastocysts with blastocoele filling the entire blastocyst, compact inner cell mass (ICM), multicellular cohesive trophectoderm (TE) and oval shaped, Grade II; moderate quality blastocysts with normal ICM, but no optimal (fragmented or necrotic) TE, and Grade III; poor quality blastocysts with very few cells or without ICM and with large vacuole instead of blastocoel.

Differential staining of inner cell mass and trophectoderm cells

Differential staining was conducted as defined by Van Soom et al [13] and Sen [14]. A fluorescence microscope was used to view stained blastocysts (Nikon Invert Microscope Eclipse Ti-FL, 340 to 380 nm excitation and 430 nm suppression). ICM nuclei labelled with bisbenzimide appeared blue and TE nuclei labelled with both bisbenzimide and propidium iodide appeared pink to red. The numbers of ICM and TE nuclei were counted directly under the fluorescence microscope using a 345-nm ultraviolet light filter.

Experimental design

Experiment 1: Bovine oocytes were matured either at 36.5°C or 38.5°C on the same day and in different incubators for each replication and then the maturation parameters were examined as described above. Temperature of incubators was attentively set at the beginning of each experiment and the sample location was monitored continuously by means of thermometer in the incubator. The experiment was repeated 5 times.

Experiment 2: Bovine oocytes, matured either at 36.5°C or 38.5°C, were subjected to IVF procedure. Following IVF, presumptive zygotes were cultured in SOFaa embryo culture medium supplemented with or without antioxidants (a mixture of 1 mM L-glutathione reduced and 1,500 IU superoxide dismutase). The experiment was repeated 7 times.

Statistical analysis

Treatment effects (temperature) on maturation parameters of bovine oocytes were analyzed by chi-square (χ^2) test. Effects of temperature during IVM and antioxidants in embryo culture on developmental competence of embryos and cell numbers of blastocyst were analyzed by the general linear model of the SPSS 17.0 package program (SPSS, Chicago, IL, USA) after than arcsine-transformation and logl0 transformation (cleaved embryos, morula and blastocyst yields were done arcsine-transformation; cell numbers of blastocyst were $\log_{10}$ transformation).

Quality of blastocysts was analyzed by chi-square (χ^2) test. Pearson correlation analysis were used for determine correlation between blastocyst diameter and cell numbers. Additionally, Kendall's Tau correlation analysis was applied for determine correlation between blastocyst diameter, development stages and quality. Duncan's test was used to determine differences between means, using a p value of 0.05 and untransformed mean±standard error of the mean values were presented in Tables. Data on proportion of morula or blastocyst stages are presented as ratio of the numbers of morula or blastocysts to the number of cleaved zygotes.

RESULTS

Effects of low incubation temperature during IVM on oocyte maturation parameters

The data on percentage of oocytes with expanded cumulus and first polar body extrusion (FPBE) are presented in Table 1. Low and conventional incubation temperatures in IVM had a similar effect on percentages of oocytes with expanded cumulus and FPBE in Grade A bovine oocytes. However, low incubation temperature during IVM decreased ($\chi^2 = 9.52$; $p< 0.05$) percentage of FPBE in Grade B oocytes. Various nuclear stages of oocytes are presented in Table 2. Although there were no significant differences between the incubation temperatures during IVM in terms of nuclear maturation status of Grade A bovine oocytes, a lower percentage of Grade B oocytes ($\chi^2 = 12.14$; $p<0.05$) reached to M II stage at low incubation temperature during IVM.

Effects of low incubation temperature during IVM on zygote development

Development rates of bovine zygotes are presented in Table 3. The incubation temperature during IVM did not influence cleavage rate and proportion of embryos developed to blastocyst stage following IVF. However, percentage of zygotes developed to morula stage was significantly higher ($p<0.05$) in zygotes from oocytes matured at conventional temperature than those matured at low temperature. In experimental groups, developmental competence of zygotes was lower ($p< 0.05$) in zygotes from Grade B oocytes than those of Grade A oocytes. Additionally, supplementation of antioxidants into embryo culture media improved blastocyst yield of embryos from Grade A oocytes ($p<0.05$).

Table 1. Percentage of bovine oocytes with cumulus expansion and first polar body extrusion, matured *in vitro* either at 36.5°C or 38.5°C maturation temperatures

Culture temperature (°C)	Oocyte quality	Cumulus expansion		First polar body	
		n	%	n	%
36.5	Grade A	623	96.2	78	79.5[a]
	Grade B	687	89.8	108	63.0[b]
38.5	Grade A	622	95.8	82	85.4[a]
	Grade B	751	90.2	112	74.1[a]

[a,b] Different letters in the same column indicate significant difference ($p<0.05$).

Effects of low incubation temperature during IVM on blastocyst formation

Percentages of blastocyst quality are presented in Table 4. The incubation temperature during IVM had no effect on blastocyst quality, but the antioxidant supplementation into embryo culture media improved blastocyst quality ($p<0.05$) only in zygotes from oocytes matured at conventional temperature. Percentage of Grade I quality blastocyst from Grade A oocytes

Table 2. Nuclear maturation status of bovine oocytes, matured *in vitro* either at 36.5°C or 38.5°C maturation temperatures per groups for 5 replicates

Culture temperature (°C)	Oocyte quality	Oocytes (n)	Nuclear maturation stages (%)			
			GV	GVBD	M I	M II
36.5	Grade A	82	0 (0)	5 (6.1)	10 (12.2)	67 (81.7)[a]
	Grade B	72	3 (4.2)	9 (12.5)	18 (25)	42 (58.3)[b]
38.5	Grade A	88	1 (1.1)	3 (3.4)	18 (20.5)	66 (75.0)[a]
	Grade B	85	5 (5.9)	8 (9.4)	19 (22.4)	53 (62.4)[ab]

GV, germinal vesicle; GVBD, germinal vesicle break down; M I, metaphase I (including anaphase I and telophase I); M II, metaphase II.
[a,b] Different letters in the same column indicate significant difference ($p<0.05$).

Table 3. Effect of incubation temperatures during *in vitro* maturation and antioxidants in embryo culture media on developmental competence of embryos[1)]

IVM condition (°C)	Oocyte quality	Antioxidants[2)]	Putative zygotes	Developmental competence of bovine embryos (%)		
				Cleaved	Morula	Blastocyst
36.5	Grade A	–	215	68.4±2.42[a]	45.0±2.60[b]	32.6±4.13[b]
		+	217	70.3±4.06[a]	46.3±3.26[b]	37.8±5.52[a]
	Grade B	–	215	56.0±3.34[b]	34.2±2.52[c]	21.7±3.95[c]
		+	219	49.8±5.62[b]	38.7±5.05[c]	18.5±2.66[c]
38.5	Grade A	–	194	71.7±2.46[a]	57.4±5.57[a]	29.8±2.57[b]
		+	205	78.0±2.05[a]	58.3±4.64[a]	42.7±5.27[a]
	Grade B	–	244	52.8±2.35[b]	39.6±3.68[c]	15.6±2.50[c]
		+	242	62.1±2.24[b]	34.4±2.93[c]	23.8±2.84[c]

IVM, *in vitro* maturation.
[1)] Results are presented as untransformed mean±standard error of the mean values per groups for 7 replicates.
[2)] A mixture of 1 mM L-glutathione reduced and 1,500 IU superoxide dismutase.
[a-c] Different letters in the same column indicate significant difference ($p<0.05$).

Table 4. Effect of incubation temperatures during in vitro maturation and antioxidants in embryo culture on blastocyst quality[1)]

IVM condition (°C)	Oocyte quality	Antioxidants[2)]	Blastocyst quality (%)		
			Grade I	Grade II	Grade III
36.5	Grade A	–	18.87[a]	73.58	7.55[b]
		+	19.78[a]	69.89	10.33[b]
	Grade B	–	0.00[c]	73.08	26.92[a]
		+	5.00[c]	65.00	30.00[a]
38.5	Grade A	–	14.00[b]	76.00	10.00[b]
		+	21.14[a]	71.00	7.86[b]
	Grade B	–	4.17[c]	66.66	29.17[a]
		+	0.00[c]	72.97	27.03[a]

IVM, *in vitro* maturation.
[1)] Results are cumulative data from 7 replicates.
[2)] A mixture of 1 mM L-glutathione reduced and 1,500 IU superoxide dismutase.
[a-c] Different letters in the same column indicate significant difference ($p<0.05$).

Table 6. Kendall's Tau correlation coefficients (95% confidence intervals) between diameter, development stages (early, mid, and expanded) and quality (grade I, II, and III) of blastocysts

IVM condition (°C)	Oocyte quality	Antioxidants[1)]	Traits		
			BD – BDS	BD – BQ	BDS – BQ
36.5	Grade A	-	0.685**	–0.419	0.458
		+	0.733**	–0.498	0.650*
	Grade B	–	0.501	–0.501	0.375
		+	0.521	–0.363	0.359
38.5	Grade A	–	0.706**	–0.417	0.452
		+	0.713**	–0.455	0.453
	Grade B	–	0.581	–0.509	0.385
		+	0.582	–0.266	0.307

IVM, *in vitro* maturation; BD, blastocyst diameter; BDS, blastocyst development stages; BQ, blastocyst quality.
[1)] A mixture of 1 mM L-glutathione reduced and 1,500 IU superoxide dismutase.
* $p<0.05$; ** $p<0.01$.

were higher ($p<0.05$) than those of Grade B oocytes.

Correlations between diameter and cell numbers, development stages and quality of bovine blastocysts

Pearson correlation coefficients between diameter and various cell numbers of bovine blastocysts are shown in Table 5. The relationship between diameter and cell numbers of ICM were insignificant, but the positive correlations were detected between diameter and TE cell numbers (0.710; $p<0.01$), diameter and overall cell numbers of blastocysts (0.798; $p<0.01$), except for blastocysts from Grade A oocytes. Although there were no significant correlations between ICM and TE cell numbers of blastocysts, positive correlations were detected between overall cell numbers and TE cell numbers (0.670; $p<0.05$), and overall cell number and ICM cell numbers of blastocysts (0.873; $p<0.01$). Kendall's Tau correlation coefficients between diameter, development stages and quality of bovine blastocysts are presented in Table 6. There was insignificant correlation between diameter and quality of blastocysts. The positive correlations were detected between diameter and blastocysts stages obtained from Grade A oocytes (0.709; $p<0.01$). Moreover, when blastocyst development level advances from early to expanded or hatched blastocysts the diameter of blastocysts increases in all experimental groups ($p<0.05$).

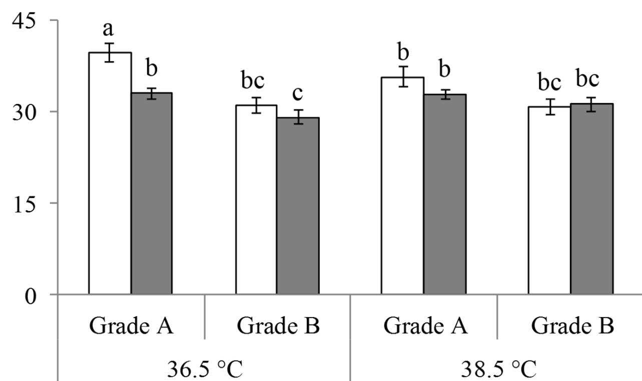
Effects of low incubation temperature during IVM on ICM, TE, and overall cell numbers

ICM, TE, and overall cell numbers of bovine blastocysts are presented in Figure 1. The incubation temperatures during IVM did not affect ICM, TE, and overall cell numbers of blastocysts. However, antioxidant supplementation increased ($p<0.05$) cell number of ICM in blastocysts from Grade A oocytes maturated at low incubation temperature. Also, TE and overall cell numbers were increased ($p<0.05$) by antioxidant supplementation in blastocysts from Grade A oocytes maturated at conventional incubation temperature. In all experimental groups, blastocysts produced from Grade B oocytes had lower ($p<0.05$) overall cell numbers than those of Grade A oocytes.

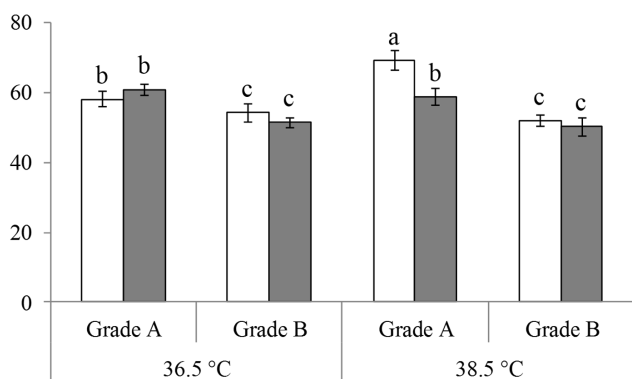
Table 5. Pearson correlation coefficients (95% confidence intervals) between diameter and cell numbers of various parts of blastocysts

IVM condition (°C)	Oocyte quality	Antioxidants[1)]	Traits					
			BD – ICM	BD – TE	BD – OCN	ICM – TE	ICM – OCN	TE – OCN
36.5	Grade A	-	0.245	0.510	0.504	0.373	0.720**	0.909**
		+	0.528	0.883**	0.891**	0.069	0.629*	0.819**
	Grade B	–	0.482	0.644*	0.676*	0.161	0.671*	0.840**
		+	0.507	0.781**	0.843**	0.080	0.651*	0.876**
38.5	Grade A	–	0.504	0.730**	0.815**	0.259	0.614*	0.920**
		+	0.433	0.777**	0.712**	0.395	0.728**	0.924**
	Grade B	–	0.467	0.768**	0.781**	0.160	0.694*	0.889**
		+	0.518	0.749**	0.866**	0.026	0.659*	0.814**

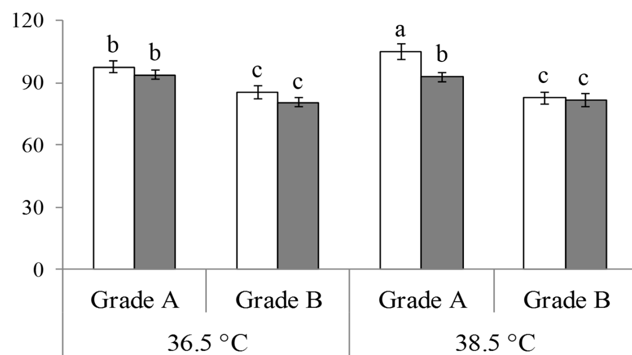
IVM, *in vitro* maturation; BD, blastocyst diameter; ICM, inner cell mass cell numbers; TE, trophectoderm cell numbers; OCN, overall cell numbers.
[1)] A mixture of 1 mM L-glutathione reduced and 1,500 IU superoxide dismutase.
* $p<0.05$; ** $p<0.01$.



(a)



(b)



(c)

Figure 1. Effect of temperature during *in vitro* maturation and antioxidants in embryo culture on inner cell mass (a), trophectoderm (b), and overall (c) cell numbers of blastocysts from Grade A or Grade B oocytes matured at either 36.5°C (n = 152) or 38.5°C (n = 165). The error bars represent the standard error of means and bars with different letters are significantly different at p<0.05. The white bars represent the embryo culture media with antioxidants, whereas the gray bars represent the antioxidants free media.

Effects of low incubation temperature during IVM on blastocyst diameter

Diameter of bovine blastocysts is presented Figure 2. The incubation temperatures during IVM had no significant effect on blastocyst diameter at the end of embryo culture period, but supplementation of antioxidants into embryo culture media increased (p<0.05) diameter of blastocysts. Oocyte quality had significant effect on blastocyst diameter. The blastocysts obtained from Grade A oocytes had higher diameter than that of Grade B oocytes (p<0.05).

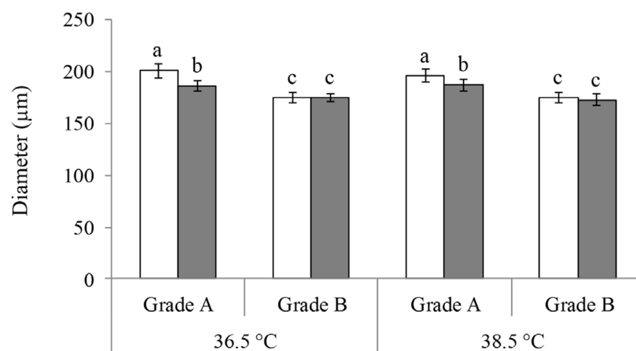


Figure 2. Effect of temperature during *in vitro* maturation and antioxidants in embryo culture on diameter of blastocysts from Grade A or Grade B oocytes matured at either 36.5°C (n = 152) or 38.5°C (n = 165). The error bars represent the standard error of means and bars with different letters are significantly different at p<0.05. The white bars represent the embryo culture media with antioxidants, whereas the gray bars represent the antioxidants free media.

DISCUSSION

In the present study an attempt was made to carry out IVM of bovine oocytes at low temperature of 36.5°C, which is considered to be the temperature of preovulatory follicles and at which supposedly *in vivo* oocyte maturation occurs. The results for the Grade A oocytes showed that cumulus expansion rate, first polar body formation and the proportion of oocytes reaching the M II stage were similar between the tested two maturation temperatures (36.5°C or 38.5°C). This suggests that low incubation temperature of 36.5°C during the maturation process does not alter the progress of bovine nuclear maturation. Similarly, previous studies have shown that low incubation temperature during IVM did not affect the rate of cumulus expansion [15,16], first polar body formation [15-17] and proportion of oocytes that reached to M II stage [4,6,15, 16]. Ye et al [4] have reported that addition of follicular fluid to the maturation media did not alter nuclear maturation level of porcine oocytes matured at 35.5°C, compared to counterparts matured at 39.0°C and 37.0°C. Moreover, supplementation of estrous cow serum into the maturation media did not affect nuclear maturation level of bovine oocytes cultured at 37°C and 39°C [18]. Overall, these studies suggest that serum may protect oocytes from possible negative effects, if any, of low incubation temperatures. It has been suggested previously [4] that, lower follicular ambient temperature is advantageous to complete oocyte maturation or development, within the follicular microenvironment. However, our results for Grade B oocytes have shown that low incubation temperature decreased the proportion of oocytes that reached M II or first polar body formed. These results suggest that lower

temperatures do have negative impact on the maturation process which in our study was not obvious in the case of Grade A oocytes but was present in the case of comparatively vulnerable Grade B oocytes probably due to less cumulus cell layers which are known to assist in the nuclear maturation of mammalian oocytes [19].

As expected, embryos from Grade A oocytes had better developmental competence following IVF. Although cleavage rates of embryos obtained from oocytes matured at 36.5°C were similar to those from matured oocytes at 38.5°C, the proportion of embryos in the morula stage were higher for Grade A oocytes matured at 38.5°C. However, this difference disappeared at the blastocyst stage. Shi et al [6] have similarly shown that neither 37.0°C nor 38.5°C incubation temperatures during lVM had any significant effect on embryonic development until development to the blastocyst stage. Ravindranatha et al [16] reported that embryos from buffalo oocytes maturated at four different temperatures had similar development to 8 to 16 cells and blastocysts. Antioxidant supplementation, as expected, increased the ratio of embryos in the blastocyst stage, irrespective of maturation temperature whether the oocytes were maturated at 36.5°C or at 38.5°C. Similar results on the effect of antioxidant supplementation have been informed by Luvoni et al [9] and Uysal et al [20].

The viability of the pre-implant embryo depends on a well-organized formation of ICM cells [21]. Ganeshan et al [21] reported that *in vitro* environmental or culture conditions have primarily major effects at the developmental potential of the ICM cell lineage rather than the TE cell lineage. Previous studies reported that *in vitro* produced blastocysts had less ICM cells than *in vivo* counterparts [22,23]. Van Soom et al [13] and Kuran et al [24] reported that lower ICM or overall cell numbers of *in vitro* derived blastocysts are related to poor embryo quality. In the present study, incubation temperatures during IVM did not influence overall, ICM and TE cell numbers, but blastocysts obtained from Grade A oocytes matured at 36.5°C and cultured in antioxidants supplemented media, had more ICM cells. This could be a sign of an improvement in antioxidant defense system and low culture temperature during maturation had strengthened the antioxidant defense system in the morula and blastocyst stage of embryo development and may increase the quality of embryos. Additionally, this improvement may be caused by mimicking of the *in vivo* Graaf follicle environmental conditions. On the contrary, blastocysts, which were obtained from good quality oocytes matured at 38.5°C and cultured in antioxidant supplemented media, had more TE cells. These results indicate that bovine oocytes maturation in conventional culture temperature with antioxidant supplementations in embryo culture have improved subsequent development of embryonic TE cells from balancing the metabolic activity. Because, Hewitson and Leese [25] showed that TE cells consume more oxygen and nutrients due to having more mitochondria than the ICM cells. Also, ICM cells have more glycolytic metabolism than those of the TE in mouse embryo [25]. This low metabolic activity may reflect pluripotency of ICM cells [26]. Unfortunately, observations regarding to the embryo transfer and metabolic activity were not recorded in the present study. However future studies may help to interpret whether low maturation temperature had any effect on ICM cell metabolic activity and success rate of embryo transfer in cattle.

In the present study, we have observed that the incubation temperatures during maturation did not influence blastocyst diameter, but supplementation of antioxidants during embryo culture increased both overall cell number and diameter of blastocysts. *In vitro* produced blastocysts with a smaller diameter are known to have some anomalies in mitotic, osmotic or secretory activity of blastomeres [27]. Thus, these results of the present study may indicate that supplementation of antioxidants in embryo culture media may improve cell proliferation or the osmotic/secretory activity of the blastomeres in blastocyst rather than reflecting the culture temperatures during maturation stage.

In conclusion, the results of the present study demonstrated that bovine oocytes can complete maturation process at 36.5°C culture temperature and they exhibit similar embryonic development following IVF as the oocytes matured at conventional (38.5°C) culture temperature. Low culture temperature during IVM could be useful to bovine *in vitro* embryo production programs. Future studies are required to investigate the effect of low incubation temperature during maturation on the metabolic activity, gene expression, DNA methylation and antioxidant capacity of both oocytes and embryos.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

The authors acknowledge the financial support by the Ondokuz Mayis University Scientific Research Projects Coordination Unit (Project no: ZRT.1904.11.012) to carry out this study. We also thank Dr. Muhammad Khalid, Royal Veterinary College, and University of London, UK for his valuable critical editing of the manuscript.

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