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EVALUATION OF SHEEP OOCYTES SUBMITTED TO HEAT STRESS INDUCED DURING IN VITRO MATURATION

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ABSTRACT

The aim of this work was to evaluate the effect of heat stress during oocyte maturation on ovine in vitro embryo production. Ovaries were collected at abattoirs and oocytes retrieved from follicles ranging from 2 and 6 mm in diameter. After selection, all oocytes, in 10 replicates, were placed in in vitro maturation (IVM) during 24 hours. The oocytes were submitted to heat stress of 41 ºC during 3, 6, 12, 18 and 24 hours and were further transferred to 39 ºC in order to complete IVM, which was the temperature of maturation of control oocytes. Embryonic development was determined on days 3, 4, 5, and 8 post-fertilization. Embryo evaluation was performed as total cell count by DAPI staining and determination of positive blastomere for apoptosis by the TUNEL assay. We observed that heat stress diminishes ($P < 0.05$) oocyte maturation capacity in accordance with exposure time of 41 ºC. In the group of oocytes incubated at 39 ºC, 70.70% matured, while in the groups exposed to heat stress of 41º C, only 45.28%, 35.17%, 12.30%, 9.74% and 4.60% matured, respectively, after 3, 6, 12, 18 and 24 hours of incubation. The duration of exposure to heat stress is inversely proportional ($P < 0.05$) to embryonic development capacity and directly proportional ($P < 0.05$) to the number of blastocysts positive to apoptosis. However, the cleavage rate and embryonic development from 8 to 16 cells and morulae stages were affected by heat stress ($P > 0.05$) only up to 18 hours of incubation. The results allow the conclusion that heat stress during oocyte in vitro maturation reduces the quantity and quality of ovine embryos produced in vitro determined by the high incidence of apoptosis.

KEYWORDS: blastocyst; IVF; IVM; IVP.

AVALIAÇÃO DE EMBRIÕES OVINOS PROVENIENTES DE OÓCITOS SUBMETIDOS A ESTRESSE CALÓRICO DURANTE A MATURAÇÃO IN VITRO

RESUMO

Neste trabalho foi avaliado o efeito do estresse calórico durante a maturação de oócitos sobre a produção *in vitro* de embriões ovinos. Os ovários foram obtidos em abatedouro e os oócitos colhidos de folículos de 2 a 6 mm de diâmetro. Após seleção, os oócitos, em 10 replicações, foram colocados para maturação *in vitro* (MIV) durante

24 horas. Os oócitos submetidos ao estresse térmico de 41º C durante 3, 6, 12, 18 e 24 horas foram posteriormente transferidos para completar a MIV a 39 ºC, mesma temperatura utilizada para maturação dos oócitos do grupo controle. O desenvolvimento dos embriões foi determinado nos dias 3, 4, 5 e 8 pós-fecundação. A avaliação da qualidade dos embriões foi efetuada através da contagem total de células coradas pelo DAPI e da determinação do número de blastômeros positivos para apoptose através do teste de TUNEL. Observou-se que o estresse térmico diminuiu (P < 0,05) a capacidade de maturação dos oócitos de acordo com o tempo de exposição à temperatura de 41º C. No grupo de oócitos incubados a 39° C, 70,70% maturou, enquanto que nos grupos expostos ao estresse térmico, apenas 45,28%, 35,17%, 12,30%, 9,74% e 4,60% maturaram, respectivamente, após 3, 6, 12, 18 e 24 horas de incubação. A duração de exposição dos oócitos ao estresse calórico é inversamente proporcional (P < 0,05) à capacidade de desenvolvimento embrionário e diretamente proporcional (P < 0,05) ao número de blastocistos positivos para apoptose. Todavia, o efeito deletério do estresse térmico sobre a clivagem e os embriões nos estádios de 8 a 16 células e de mórula foi crescente (P > 0,05) somente até 18 horas de incubação. Os resultados permitem concluir que o estresse calórico durante a maturação in vitro de oócitos reduz a quantidade e a qualidade dos embriões ovinos produzidos in vitro determinadas pela alta incidência de apoptose.

PALAVRAS-CHAVE: blastocisto; FIV; MIV; PIC.

INTRODUCTION

The productive chain of the sheep industry in Northeast Brazil has shown its potential as an activity that fits well to the adverse climatic conditions of the region, recognized by the potential for the production of meat and sheep skin (SIMPLÍCIO et al., 2007). The high environment temperature is one of the major responsible factors for the reduced fertility of animals raised on farms (HANSEN, 2009). It has been reported that the viability of bovine oocytes and embryos is lower during the warm seasons than in the cold seasons (ROTH, 2008; EDWARDS et al., 2009). This seasonal depression of the reproductive performance can be determined by several factors, including management, inappropriate environment, age and species-specific sensitivity to these factors (BADINGA et al. 1985).

In vitro studies have also demonstrated that heat stress has a negative effect on the viability and development capacity of mammalian embryos (JU et al., 1999; PAULA-LOPES & HANSEN, 2002ab; TSENG et al., 2004; ROTH, 2008; HANSEN, 2009).

We evaluated the effect of heat stress during oocyte maturation on in vitro production of sheep embryos, aiming to provide data related to the mechanisms of cell death and reduced competence of oocytes from animals naturally exposed to high temperature.

MATERIAL AND METHODS

Ovaries were obtained from ewes at a slaughterhouse located in the Metropolitan Region of Recife - PE (latitude 08° 03' 14'' S and longitude 34° 52' 52'' W). In the dry season, from October 2009 to March 2010, the temperature ranged from 23 to 33 °C and the relative humidity was 71%. In the rainy season, from April to September 2010, the temperature ranged from 18 to 31 °C and the relative humidity was 85% in 2009 (INMET, 2009).

Within a maximum period of one hour after slaughter, the ovaries were transported to the laboratory in thermos containing saline solution heated at 30 °C, and 30 g mL^{-1} of gentamicin sulfate. The oocytes harvested from ovarian follicles measuring from 2 to 6 mm in diameter were deposited on Petri dishes containing collection medium constituted of 8.0 mg of sodium bicarbonate solution, 45.0 mg of glucose, 5.6 mg of sodium pyruvate, 11.9 mg of HEPES, 2.5 mg of gentamicin sulfate and 20.0 mg of polyvinyl alcohol in 50 ml of TALP.

Immediately after the selection and based on morphological classification described by CHIAMENTI et al. (2013), we washed the oocytes, in 10 replicates, three times in collection medium and distributed them into 100 µL drops of the basic medium for in vitro maturation constituted by TCM 199 supplemented with 50 g mL^{-1} of sodium pyruvate, 2.6 mg mL^{-1} of sodium bicarbonate, 10% fetal bovine serum (FBS), 50 g mL^{-1} of gentamicin sulfate, 20 µg mL^{-1} of FSH / LH (Pluset ®) and 1 mg mL^{-1} Polyvinyl alcohol.

The in vitro maturation was performed for 24 hours in a humidified atmosphere containing 5% CO_2. The oocytes in control group were incubated at 39 °C and submitted to heat stress at

41 °C for 3, 6, 12, 18 and 24 hours of incubation, except for the 24 hour group, which was subsequently transferred to another greenhouse at 39 ° C to complete in vitro maturation.

At the end of the in vitro maturation period, we selected the oocytes, based on the expansion of cumulus cells (CHIAMENTI et al., 2013), to expose them to sperm treated with modified defined medium (mDM), according to KESKINTEPE et al.(1998). This medium was composed of 0.1250 g of glucose, 0.1552 g of sodium bicarbonate, 0.0069 g of sodium pyruvate, 0.0500 g of polyvinyl alcohol, 0.0500 g of caffeine and 50 µg mL^{-1} of gentamicin in 50 mL mDM. In vitro fertilization was performed with fresh semen at 2 X 10^6/mL sperm concentration.

After 18 hours of incubation, the potential zygotes were denuded and transferred to drops of 100 mL synthetic fluid of modified oviduct, supplemented with 10% fetal bovine serum. The potential zygotes were incubated at 39 °C in a humidified atmosphere containing 5% CO_2, 5% O_2 and 90% N_2 for eight days, depending on the experimental group. We emphasize that the assessments were made on the third day (D-3) as for cleavage, on the fourth day (D-4) for embryos with 8-16 cells, on the fifth day (D-5) for the morula stage, and on the eighth day (D-8) for the blastocysts.

After the period of embryo cultivation, we assessed blastocysts quality by total cell count with DAPI staining and carried out DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling test (TUNEL) as recommended by PAULA-LOPES & HANSEN (2002a) and by ROTH & HANSEN (2004). The aforementioned evaluations were performed using a fluorescence microscope at 1000x magnification.

Statistical analysis was performed by analysis of variance by the least squares method. Results were expressed as mean and standard deviation (analysis of variance between groups). Data in percentages were arcsine-square root of x/100 transformed and submitted to analysis of variance and F test for variances at 5% significance. The data were transformed but not analyzed by a nonparametric method to preserve the experimental model. Then a Student t test for comparison of means was conducted, at 5% significance, for equivalent or different variances, as it was observed at the F test for variance.

RESULTS

Heat stress decreases (P <0.05) the ability of oocyte maturation in relation to the time of exposure to a temperature of 41 °C (Table 1). In the group incubated at 39 ° C, 70.70% of oocytes matured, while in incubated groups exposed to thermal stress at 41 °C for 3, 6, 12, 18 and 24 hours, only 45.28%, 35.17%, 12.30%, 9.74% and 4.60% matured, respectively.

The data in Table 2 show that the duration of exposure of oocytes to heat stress is inversely proportional (P <0.05) to the ability of embryo development until the blastocyst stage and directly proportional (P <0.05) to the number of blastocysts positive for apoptosis (P <0.05). However, the deleterious effect of heat stress on cleavage and embryos at stages of 8 to 16 cells and morula stage was similar (P <0.05) only until 18 hours of incubation.

TABLE 1: Mean values ($\bar{x}$ ± s) of selected ovine oocytes submitted to in vitro maturation (IVM) at 39 °C for 24 hours and at 41 °C for 3, 6, 12, 18 and 24 hours of heat stress and selected for in vitro fertilization (IVF)

	IVM 39ºC	IVM 41° C				
Oocytes	24 hours ($\bar{x}$ ± s)	3 hours ($\bar{x}$ ± s)	6 hours ($\bar{x}$ ± s)	12 hours ($\bar{x}$ ± s)	18 hours ($\bar{x}$ ± s)	24 hours ($\bar{x}$ ± s)
Selected for IVM	107.2±5.22	110.4±5.66	106.7±4.92	100.8±4.28	101.6±4.16	99.8±5.73
Matured	75.8±4.54^a	50.0±3.74^b	35.4±3.77^c	12.4±1.71^d	9.9±1.59^e	4.6±.96^f

Values with different superscript letters in the same row are different (P <0.05) by Student's t test.

TABLE 2: Mean values ($\bar{x}$ ± s) of cleavage (D-3), of embryos with 8 to 16 cells (D-4), of morulas (D-5) and of blastocysts (D-8), as well as of blastocysts positive for apoptosis after in vitro maturation (IVM) of ovine oocytes at 39 ºC for 24 hours and at 41 ºC for 3, 6, 12, 18 and 24 hours of heat stress

	IVM 39º C	IVM 41º C				
	24 hours	3 horas	6 hours	12 hours	18 hours	24 hours
Embryos	($\bar{x}$ ± s)	($\bar{x}$ ± s)	($\bar{x}$ ± s)	($\bar{x}$ ± s)	($\bar{x}$ ± s)	($\bar{x}$ ± s)
Cleaved D-3	32.5±3.97[a]	20.0±2.49[b]	11.5±1.08[c]	3.4±0.84[d]	2.4±0.51[e]	2.7±0.82[e]
8 to 16 Cells D-4	24.3±2.62[a]	14.5±2.06[b]	6.9±0.73[c]	2.6±0.51[d]	1.4±0.69[e]	1.9±0.73[e]
Morula D-5	19.9±2.68[a]	11.0±2.21[b]	3.8±0.63[c]	2.4±0.51[d]	1.0±0.81[e]	0.7±0.67[e]
Blastocysts D-8	9.14±2.44[a]	4.16±0.96[b]	2.53±1.25[c]	1.58±0.51[d]	0.68±0.67[e]	0.20±0.42[f]
Blastocysts (Apoptosis)	9.18±0.56[a]	13.04±0.51[b]	18.51±0.52[c]	31.25±0.52[d]	71.42±0.52[e]	100±0.42[f]

Values with different superscript letters in the same row are different ($P < 0.05$) by Student's t test.

DISCUSSION

EDWARDS & HANSEN (1997), JU et al. (1999), ROTH & HANSEN (2004), TSENG et al. (2004) and ROTH (2008) proved that in vivo and in vitro changes in the microenvironment of bovine oocytes, caused by heat stress, affect the viability and the kinetics of embryonic development.

The effect of heat stress on in vitro mammalian embryos and oocytes depends on the temperature and duration of hyperthermia. JU et al. (1999) reported that the temperature at 43 °C reduces both the competence of oocytes as the embryo development in bovine, even with an exposure time of only 45 minutes. In this work, we based the choice of time of exposure to heat stress on previous studies of JU & TSENG (2004) and PAYTON et al. (2011), who demonstrated the oocyte susceptibility to thermal stress at 41 °C at different time intervals. In addition, temperature of 41 °C reflects the average value of body temperature in animals exposed to heat stress in vivo (RIVERA & HANSEN, 2001). The data corroborate the observations that gametes maturation was reduced proportionally to the duration of exposure to heat stress at 41 °C.

The interval between oocyte maturation and the first cleavage is, according to EALY et al. (1995), the most susceptible period to the effects of heat stress on embryonic development and after genome activation, the embryo becomes more resistant to stress (EDWARDS & HANSEN, 1997; JU et al., 1999). In this work, heat stress during oocyte maturation, even after shorter incubation periods, showed marked effect on embryo development. The reduction in the in vitro production of embryos up to the morula stage was proportional to the duration of heat stress, except between 18 and 24 hours of incubation, which had similar rates from cleavage to the morula stage. This fact suggests that the period of oocytes nuclear maturation is critical to gamete's susceptibility to heat stress (TSENG et al., 2004). However, the groups 18 and 24 hours of heat stress differed in blastocysts production, possibly due to the quality of these embryos.

Heat stress induces apoptosis of oocytes and embryos (EDWARDS & HANSEN, 1997; ROTH & HANSEN, 2004). Apoptosis has a direct effect on embryo viability, because by inhibiting the activity of type-II caspases, cleavage rate is recovered after heat stress in bovine (ROTH & HANSEN, 2004). As for the embryos quality in this study, the percentage of apoptotic blastomeres, produced from oocytes exposed to heat stress with exposure times of 6 and 12 hours, is similar to the results found by PAULA-LOPES & HANSEN (2002a) for bovines after exposure at 41 °C for 9 hours. These authors stated that it is possible to observe a higher apoptosis incidence after severe stress, affecting embryonic development, as observed in this study during the incubation periods of 18 and 24 hours, where apoptosis in blastocysts reached 71.42 and 100%, respectively.

We observed apoptosis induction after heat shock in vitro sheep embryos, as it occurs in bovines and other mammals, suggesting that the low viability of embryos exposed to hyperthermia in the female reproductive tract may be a cause of pregnancy loss

during the preimplantation in sheep. A better understanding of the physiological responses of cells, gametes and embryos submitted to heat stress may allow the development of protocols for the production of embryos that better resist heat stress in vitro and in vivo (WANG et al., 2009; ANDREU-VAZQUEZ et al., 2010; SHEHAB-EL-DEEN et al., 2010; PAYTON et al., 2011). For example, the addition of retinoids enhances in vitro maturation of oocytes exposed to heat stress (LAWRENCE et al., 2004; MAYA-SORIANO et al., 2012).

CONCLUSION

The duration of heat stress exposure during ovine oocyte in vitro maturation is inversally proportional to the quantity and quality of embryos produced in vitro.

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