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MITIGATION OF HEAT STRESS EFFECT ON IN VITRO MATURED OOCYTES USING VITAMIN C IN GOAT

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SUMMARY

The current investigation aimed to assess the effect of induced heat stress (40 and 42 ºC) on the maturation of goat oocyte in vitro. In addition to study the effect of vitamin C supplementation during IVM as a way to mitigate the heat stress effects. A total of 438 of oocyteswere recovered from goat ovaries using slicing technique. The oocytes were cultured in prewarmed (38.5, 40, and 42 ºC, according to the experimental group) IVM medium with or without vitamin C (VC), under mineral oil in CO_2 incubator 5% CO_2, with 95% humidity. Six treatment groups were established: T1 (control, 38.5°C, no vitamin C), T2 (38.5°C, 50 µg/mL vitamin C), T3 (40°C, 50 µg/mL vitamin C), T4 (40°C, no vitamin C), T5 (42°C, 50 µg/mL vitamin C), and T6 (42°C, no vitamin C). The results of cumulus expansion that were obtained in this study were as follows; 81.67, 90, 87.22, 72.67, 71.69, and 55.59% for T1, T2, T3, T4, T5, and T6 groups, respectively. The data of the first polar body extrusion were recorded as 25.66, 41.33, 31.11, 17.56, 25.86, and 10.55% for T1, T2, T3, T4, T5, and T6 groups, respectively. In conclusion, heat stress (42 ºC) significantly reduced goat oocyte's maturation in vitro, however, vitamin C supplementation (50 µg/mL) to IVM medium may improveoocyte maturation rate and/or reduced the negative effects of heat stress.

Keywords: Goat, oocyte, IVM, heat-stress, vitamin C

INTRODUCTION

Heat stress affects all living creatures when the temperature rises above 25°C. Elevated temperatures (35-40ºC) significantly alter the body's physiological and metabolic processes (Asseng *et al.*, 2021). Heat stress is becoming a greater global health problem as heat waves occur more frequently, especially in moderate climate areas. In particular, heat stress seriously compromises the ability of both male and female animals and humans to reproduce (Boni, 2019).

Goats were domesticated about 10000 years ago (Monteiro *et al.*, 2018). Because of their resilience to varied environmental and climatic circumstances, more than thousand goat species occur in the globe, varies in size, form, and production category, presenting a significant importance by providing meat, milk, fiber, hide, and fertilizer (Lohani and Bhandari, 2021 and Naderi et al., 2008).

A mismatch between the amount of heat obtained from various sources, such as the body's metabolism and the surrounding environment, and the body's heat-dissipation mechanism causes heat stress, which raises the organism's internal temperature (Bernabucci *et al.*, 2014; Brown-Brandl, 2018; Collier *et al.*, 2017, and Lees *et al.*, 2019).

The disruption of oocyte functionality in heat-stressed bovine could be due to the modifications of oocyte membrane features such as structure of the fatty acids, physical attributes, or alterations in follicular fluid contents that surrounding the oocyte, for instance, insulin-like growth factor binding proteins or hormones (De Rensis *et al.*, 2017; Lopes *et al.*, 2012, and Roth, 2017).

The majority of *in vitro* models of heat stresshave confirmed a decrease in blastocyst formation when oocytes are exposed to maturation temperatures ranging from 39.5 to 43ºC, with 40 to 41.5ºC being the most commonly used range according to (Gómez-Guzmán *et al.*, 2024).

Exposure to the environment can have an impact on the quality of the embryo; it has been demonstrated that heat stress can disrupt the oocyte's RNA, produce heat shock proteins and other associated proteins, as well as antioxidants, endangering the final stages of development (Pavani *et al.*, 2015, and Sakatani, 2017). Rispoli *et al.* (2011) showed a decrease in the cleavage rate, embryo development, and the percentage of blastocyst coming from oocytes exposed to heat stress (12 hrs. at 41ºC).

Heat stress increases ROS production in bovine oocytes (Nabenishi *et al.*, 2012) and reduces the number of ovarian follicles that can properly develop (Roth and Hansen, 2004a). A few antioxidants might have a positive impact on this issue, e.g., retinol (Lawrence *et al.*, 2004), cysteine (Nabenishi *et al.*, 2012) or melatonin (Cebrian-Serrano *et al.*, 2013). The addition of melatonin to IVM media reduces the generation of ROS in maturing oocytes, and therefore, elevated oocytes' quality. An additional antioxidant that may help enhance oocytes' capacity for development is retinol (Eberhardt *et al.*, 1999). In addition, insulin-like growth factor 1 (IGF1) has been proposed as a potential support for HS-affected

bovine embryos (Bonilla *et al.*, 2015; Lima *et al.*, 2006; Nabenishi *et al.*, 2012, and Stamperna *et al.*, 2021).

Oral vitamin C (VC) supplementation to ewes increased their ability to with stand water restriction (Ghanem *et al.*, 2008) and improved the fertility and body weight of pregnant ewes and their new-born lambs (Halilo˘glu and Serpek, 2000). Recently,it was reported (Sammad *et al.*, 2024) that,by controlling the metabolism of amino acids in granulosa cells, VC has been shown to mitigate the detrimental effects of heat stress on reproductive activities. Therefore, the current study aimed to investigate the effect of supplementing goat oocyte with VC during in vitro incubation on the rate of maturation.

The current study composed of six experimental groups to investigate the effect of temperature increase on the *in vitro* maturation of goat oocytes. In addition to study the effect of vitamin C (ascorbic acid) addition to alleviate the potential negative effects caused by heat stress

MATERIALS AND METHODS

Work location:

The study has been conducted at the Embryo Manipulation Unit (EMU), Desert Research Center (DRC), Cairo, Egypt.

In vitro maturation protocol:

All media were obtained from Sigma-Aldrich (St. Louis, MO, USA) or otherwise noted. The goat IVM procedure was conducted according to AbdElkhalek *et al.*(2024). The goat ovaries were collected from local slaughter houses near Cairo, transported to the lab in thermos container filled with saline solution (35 ºC) fortified with gentamycin 50 µg/mL within two hours after slaughter.

Oocytes (n= 438) were recovered from goat ovaries using slicing technique. The oocytes were washed three times using wash medium (WM) consisted of TCM-199 with HEPES, 10% FBS (v/v) and 50 µg/mL gentamycin. Then followed by a final wash with IVM media which consisted of TCM-199, 10% FBS, 50 µg/mL gentamycin, 10 ng/mL epidermal growth factor (EGF), 1 µg/mL estradiol (E2), 0.25 mg/mL Na^+ pyruvate, 20 IU/mL equine Chorionic Gonadotropin (eCG, Gonaser®, 500 IU), and 20 IU/mL human Chorionic Gonadotropin (hCG, choriomon®, 5000 IU). The experimental groups differed in maturation media (with/without 50 µg/mL of vitamin C) and culture conditions according to the experimental design. Groups of 20-25 oocytes per group was cultured in 100 µL drop of the pre warmed (38.5, 40, and 42ºC, according to the experimental group) IVM medium each under mineral oil in CO_2 incubator 5% CO_2, with 95% humidity.

Experimental design:

The immature goat oocytes were randomly distributed into six experimental groups (three replicates each). The first group (T1) served as control; the oocytes were cultured at 38.5ºC with basic IVM medium without VC addition. T2: oocytes matured at 38.5ºC with VC 50 µg/mL. T3: oocytes matured at 40ºC with vitamin C50 µg/mL. T4: oocytes matured at 40ºC without vitamin C. T5: oocytes matured at 42ºC with vitamin C50 µg/mL. T6: oocytes matured at 42ºC without vitamin C.

Evaluation of oocyte maturation:

After 24 h of IVM, the rate of maturation was assessed based on the cumulus cells expansion (Fig. 1), and the first polar body extrusion. To examine the first polar body (Fig. 2), two minutes of moderate pipetting were applied to remove cumulus cells, and the oocytes were assessed under the inverted microscope (Leitz Fluovert FU Leica Microsystems, Wetzlar, Germany).

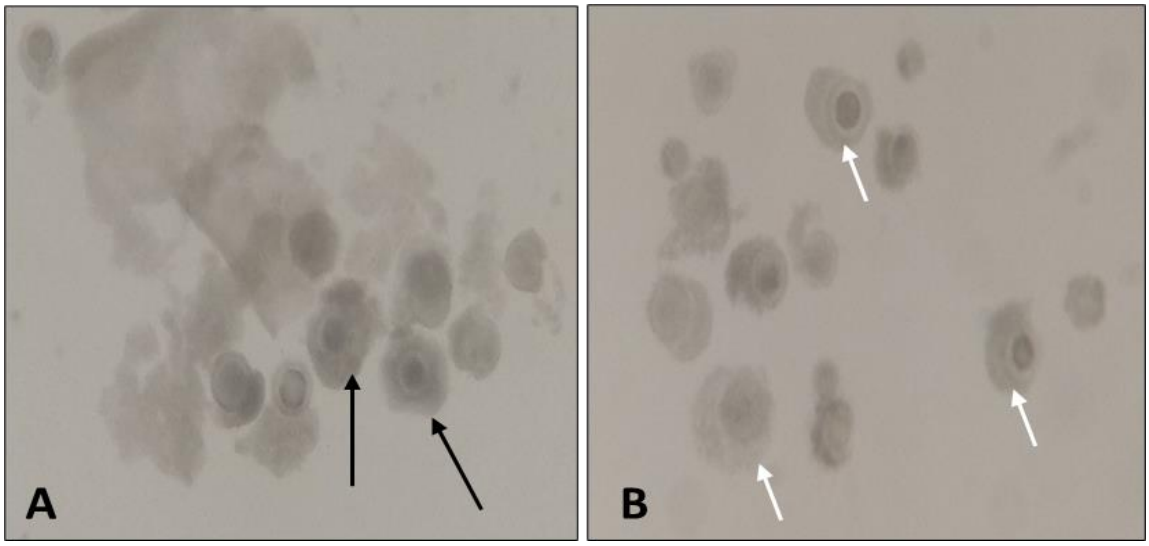

Fig. 1. Goat oocytes. (A) immature oocytes with compacted cumulus cells (black arrows). (B) mature oocytes with expanded cumulus cells (white arrows). Pics were captured under a stereo microscope at a magnification of 50X.

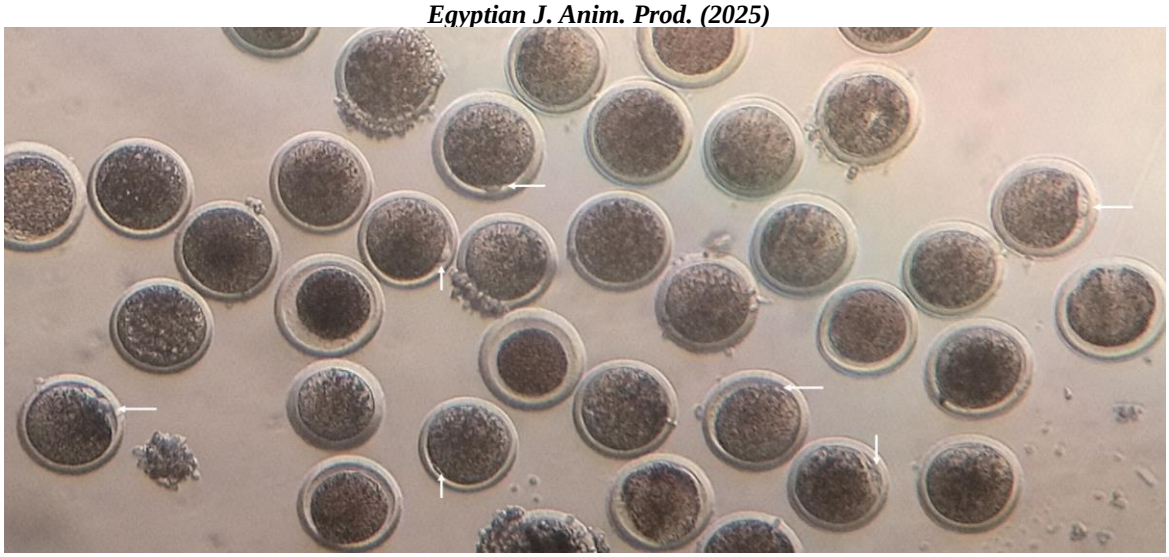

Fig. 2. First polar body extrusion in goat oocytes after *in vitro* maturation (white arrows). The picture was captured using an inverted microscope with a magnification of 400X.

Statistical analysis:

The data of oocyte maturation (due to expansion and first polar body rates) were subjected to angular transformation. All data were analyzed by a two-way analysis procedure (ANOVA) using the General Linear Models (GLM) procedure of SAS software (SAS, 2004) as follows:

$Y_{ijk} = \mu + Tr_i + Temp_j + (Tr*Temp)_{ij} + e_{ijk}$ Where,

Y_{ijk} = any observation of $_j$th temperature within $_i$th treatment.

μ = overall mean.

Tr_i = effect of $_i$th treatment (i: 1,2).

$Temp_j$ = effect of $_j$th temperature (j: 1,2,3).

$(Tr*Temp)_{ij}$ = effect of the interaction between treatment and temperature.

e_{ijk} = experimental error.

Differences in the mean values between the groups were assessed via Duncan's multiple range test (Duncan, 1955), the effects were considered significant if $P < 0.05$.

RESULTS

The results of cumulus expansion that were obtained in this study were as follows; 81.67, 90, 87.22, 72.67, 71.69, and 55.59% for T1, T2, T3, T4, T5, and T6 groups, respectively. Meanwhile, the data of the first polar body extrusion were recorded as 25.66, 41.33, 31.11, 17.56, 25.86, and 10.55% for T1, T2, T3, T4, T5, and T6 groups, respectively, as showed in Table1.

Table 1. Effect of vitamin C supplementation on cumulus cells expansion and first polar body extrusion during oocyte IVM under heat stress in goat

Items	Temp	Treatment		Overall	P-value Tr	P-value Temp	P-value Tr*Temp
		C	VC				
Expansion	38.5	81.67^{b}±2.84	90.00^{a}±1.15	85.83^{a}±2.31			
	40.0	72.67^{c}±1.76	87.22^{a}±1.46	79.95^{b}±2.41	0.0001	0.0001	0.051
	42.0	55.59^{d}±1.31	71.69^{c}±0.90	63.64^{c}±2.67			
Overall		69.97^{b}±3.96	82.97^{a}±2.91				
Polar body	38.5	25.66^{c}±1.20	41.33^{a}±1.33	33.50^{a}±3.59			
	40.0	17.56^{d}±1.23	31.11^{b}±1.11	24.33^{b}±3.12	0.0001	0.0001	0.052
	42.0	10.55^{e}±0.32	25.86^{c}±0.93	18.20^{c}±3.45			
Overall		17.92^{b}±2.24	32.76^{a}±3.59				

a,b,c,d means of Temp and treatment with different superscripts are significant differences ($P<0.05$), a-e means of different experimental subgroups with different superscripts are significant differences ($P<0.05$). Temp: stand for temperature level (38.5, 40, and 42 ºC), C: for groups without vitamin C supplementation, and VC: stand for treated groups with 50 µg/mL of vitamin C.

DISCUSSION

Heat stress substantially impacted oocyte's maturation in this study. This is in agreement with previous reports indicated that heat stress was negatively affected oocyte competence *in vivo* and *in vitro* in bovine (Payton *et al.*, 2018 and Roth and Hansen, 2004b). This negative effect could be due to the direct exposure of matured oocytes to heat stress, this could alter the functions of mitochondria, which consequently transferred to the early embryo.

The mechanism of mitochondrial function alteration includes alterations in transcripts involved in the electron transport chain and oxidative phosphorylation events related to protein

translocation into mitochondria and protein synthesis inside mitochondria. In conjunction, these heat-induced transcriptome alterations may reflect structural and functional abnormalities in oocyte mitochondria (Payton *et al.*, 2018).

The findings of the current investigation showed that the increased temperature significantly impaired the maturation process of oocyte in goat. Moreover, it was also found that the treatment with vitamin C significantly alleviated the negative effect of increased temperature on the maturation rate in comparison with the untreated groups.

The results of this study (according to the polar body extrusion) indicated that increased temperature to 40 and 42ºC, significantly reduced the maturation rate of goat oocytes *in vitro* (T4 and T6) compared to the control group (T1) which incubated at normal temperature (38.5ºC). However, it was also found that supplementing IVM medium with vitamin C significantly increased the maturation rate of the oocytes exposed to high temperature (40 and 42ºC) in T3 and T5 in comparison with T4 and T6 which were not supplemented with VC. Noteworthy to mention that vitamin C increased the maturation rate in T3 at 40 ºC in comparison to the control, and no significant difference was found between T5 at 42ºC compared to the control group.

Moreover, the data obtained from this investigation revealed that supplementing IVM of goat oocytes with 50 µg/mL of vitamin C in T2 at normal incubation temperature (38.5ºC) significantly enhanced the *in vitro* maturation compared to the control group (T1), and all other experimental groups. In the same trend, the cumulus expansion results showed that the highest significant rates were found in T2 and T3 followed by T1. And the lowest expansion rate was found in T6.

The improvement of VC supplement to oocyte IVM medium could be due to the protective role of vitamin C against the heat stress damage. Similarly, Yin *et al.* (2018) found that treating H9C2 cells with 20 µg/mL vitamin C for 16 h, stimulated the endogenous antioxidants and heat shock proteins (HSPs) production which reduced the damage to nuclei and mitochondria, leading to relieve the heat stress effects. In addition, it was found (Sun *et al.*, 2019) that pretreatment with vitamin C could effectively inhibit apoptosis, lipid peroxidation, and lactate dehydrogenase (LDH) activity in Sertoli cells.

Furthermore, the addition of vitamin C to heat-stressed granulosa cells considerably increased the proportion of PCNA protein in bovine (Sammad *et al.*, 2024). The PCNA protein found to be among the primary proteins of cell proliferation potential under natural conditions and mainly implicates in the S phase of the cell cycle during stress and apoptosis (Habibi *et al.*, 2022 and Scott *et al.*, 2001).

On the other hand, it was reported that Heat-stressed oocytes undergo germinal vesicle breakdown and progress to metaphase II sooner than non-stressed counterparts (Edwards *et al.*, 2005, and Hooper *et al.*, 2015). Which implies that heat stress accelerates the aging of oocytes, this was supported by the similarity in ATP levels and reduction of development between in vitro aged oocytes and heat stressed oocytes in bovine (Edwards *et al.*, 2005; Hooper *et al.*, 2015; Koyama *et al.*, 2014; Payton *et al.*, 2018; and, Takahashi *et al.*, 2013).

CONCLUSION

In conclusion, heat stress at 42ºC significantly reduced goat oocyte's maturation *in vitro*, however, vitamin C supplementation (50 µg/mL) to IVM medium may improve the oocyte maturation rate and reduced the negative effects of heat stress.

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التخفيف من تأثير الإجهاد الحراري على بويضات الماعز أثناء الإنضاج المعملي باستخدام فيتامين ج

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يهدف البحث إلى تقييم تأثير الإجهاد الحراري المحدث (٤٠، ٤٢ درجة مئوية) على نضج بويضات الماعز في المختبر. بالإضافة إلى دراسة تأثير إضافة فيتامين ج أثناء الإنضاج المعملي، كوسيلة للتخفيف من آثار الإجهاد الحراري. تم الحصول على البويضات (ن = ٤٣٨) من مبايض الماعز باستخدام تقنية التقطيع. وتم إستزراع البويضات في وسط IVM مُهيئ مسبقًا (٣٨,٥، ٤٠ و٤٢ درجة مئوية، وفقًا للمجموعة التجريبية) مع أو بدون فيتامين ج بتركيز ٥٠ ميكروجرام لكل مللي، تحت طبقة من الزيت المعدني في حاضنة ثاني أكسيد الكربون ٥٪ CO2، مع رطوبة ٩٥٪. المجموعة الأولى (T1) الضابطة؛ تم إستزراع البويضات عند ٣٨,٥ درجة مئوية باستخدام وسط IVM الأساسي دون إضافة فيتامين ج. المجموعة الثانية (T2): ٣٨,٥ درجة مئوية مع إضافة فيتامين ج. المجموعة الثالثة (T3): ٤٠ درجة مئوية مع فيتامين ج. المجموعة الرابعة (T4): ٤٠ درجة مئوية بدون إضافة فيتامين ج. المجموعة الخامسة (T5): ٤٢ درجة مئوية مع إضافة فيتامين ج. المجموعة السادسة (T6): ٤٢ درجة مئوية بدون إضافة فيتامين ج. وكانت نتائج تمدد الخلايا المحيطة بالبويضة في هذه الدراسة كما يلي؛ ٨١,٦٧، ٩٠، ٨٧,٢٢، ٧٢,٦٧، ٧١,٦٩، و٥٥,٥٩٪ للمجموعات T1، T2، T3، T4، T5، T6 على التوالي. كما تم تسجيل بيانات خروج الجسم القطبي الأول كما يلي ٢٥,٦٦، ٤١,٣٣، ٣١,١١، ١٧,٥٦، ٢٥,٨٦، و١٠,٥٥٪ للمجموعات T1، T2، T3، T4، T5، وT6، على التوالي. نستنتج من الدراسة أن زيادة الإجهاد الحراري الى ٤٢ درجة مئوية أدى إلى انخفاض كبير في نضج بويضات الماعز في المختبر. بالإضافة إلى ذلك، أيضًا يجب الاشارة إلى أن اضافة فيتامين ج (٥٠ ميكروجرام/مل) إلى بيئة الإنضاج المعملي تزيد بشكل كبير من معدل نضج البويضات وتقلل من الآثار السلبية للإجهاد الحراري.