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Effect of antioxidant ascorbic acid on *in vitro* maturation of Caprine oocytes under normal and elevated temperatures

S.B. Khanday, J.A. Ahmed*, N. Nashiruddullah[1], U. Sharma[2] and D. Chakraborty[3]

Division of Veterinary Physiology and Biochemistry Faculty of Veterinary Science and Animal Husbandry,
Sher-e-Kashmir University of Animal Sciences and Technology. R.S. Pura-181 102, Jammu and Kashmir, India.
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ABSTRACT

The aim of the present study was to assess the effect of ascorbic acid on *in vitro* maturation of caprine oocytes under normal and elevated temperatures. Goat ovaries were collected at slaughter and both A and B grade cumulus-oocyte-complexes (COCs) were aspirated out and were matured *in vitro* under normal (38.5°C) and elevated temperatures (41°C). On the basis of cumulus expansion and nuclear maturation, the maturation competencewere compared with and without ascorbic acid supplementation (100 µM). Heat stress significantly (P≤ 0.01) reduced cumulus expansion, maturation rate and lowered metaphase stage II of nuclear maturation. Ascorbic acid improved developmental competence of oocytes during heat stress (41°C) and ascorbic acid supplemented COCs demonstrated significantly (P≤ 0.05) higher maturation rates when compared to non-supplemented groups.

Key words: Ascorbic acid, Goat oocytes, Heat stress, *In vitro* maturation.

INTRODUCTION

Heat stress is one of the major factors responsible for reduced fertility in farm animals. Goats keep normothermia at 38.5-39.7°C (Robertshaw, 2004) and are quite hardy and relatively resistant to thermal stress. Yet goats suffer from heat stress when the environmental temperature exceeds beyond their comfort zone, i.e. 13-27 °C for Indian goats (Jyotiranjan *et al*., 2017). Elevated temperature negatively affects the reproductive functions of the animals (Ealy *et al*., 1993). Generation of Reactive Oxygen Species (ROS) has been suspected during heat stress (Nabenishi *et al*., 2012a; 2012b). On the other hand, supplementation of antioxidants has been reported to reduce the deleterious effects of heat stress on oocytes (Ali *et al*., 2003).

Ascorbic acid is a water soluble antioxidant present in ovaries, corpus luteum, follicular fluid (Padh, 1991; Das *et al*., 1993; Lutwak-Mann, 1954). It inhibited the apoptosis in bovine granulosa cells and murine cumulus oocyte complexes (Tilly and Tilly, 1995; Eppig *et al.*, 2000; Murray *et al*., 2001), reduced intracellular levels of reactive oxygen species (Castillo-Martín *et al.*, 2014b) and heat shock protein 70 (HSPA1A) (Castillo-Martín *et al*., 2014a). As antioxidant supplement, ascorbic acid has many beneficial effects including improvement of nuclear maturation and developmental competence of oocytes matured *in vitro* (Pernes *et al*., 2016). The role of ascorbic acid as antioxidant on *in vitro* maturation of oocytes is documented by various authors in literature; however the studies on the effect of ascorbic acid on *in vitro* maturation of oocytes under heat stress are very less. The present study was designed to study the developmental competence of *in vitro* matured caprine oocytes with ascorbic acid supplementation and to study the effect of ascorbic acid intervention on *in vitro* maturation of caprine oocytes under heat stress. It is envisaged that the findings will allow to devise strategies in using suitable antioxidants such as ascorbic acid in overcoming both *in vivo* as well as *in vitro* reproductive failures associated with heat stress.

MATERIALS AND METHODS

Aspiration media: HEPES buffered TCM-199, BSA, gentamicin (50 µg/ml), L-glutamine.

Washing media: HEPES buffered TCM-199, FBS, gentamicin (50µg/ml), L-glutamine, sodium pyruvate, cysteamine (50 µM/ml).

Oocyte maturation media (OMM): FSH (5 µg/ml), follicular fluid, estradiol 17-β (1µg/ml), cysteamine (100 µM/ml).

Oocyte recovery and selection: The ovaries were collected from a local slaughter house and were transported to the laboratory suspended in sterile pre-warmed (37°C) 0.9% normal saline solution supplemented with 50 µg/ml gentamicin in insulated containers within 2 hrs of slaughter. The ovaries were washed 3-4 times with normal saline containing antibiotics and trimmed free of extraneous tissue and rinsed in normal saline. By using a sterile 18 gauge needle attached to a 5 ml syringe containing aspiration medium, the cumulus oocyte complexes were collected by aspiration of surface follicles. Under the stereozoom microscope, the

*Corresponding author's e-mail: jafrinahmed@rediffmail.com
[1]Division of Veterinary Pathology, R.S. Pura-181 102, Jammu and Kashmir, India.
[2]Division of Veterinary Gynaecology and Obstetrics, R.S. Pura-181 102, Jammu and Kashmir, India.
[3]Division of Animal Genetics and Breeding, R.S. Pura-181 102, Jammu and Kashmir, India.

COCs were separated from the debris, picked out individually with the help of lifter, placed into another petri dish with washing medium, and graded according to Jainudeen *et al*., (2000). Only grade A and grade B COCs were selected for *in vitro* maturation.

IVM droplets were prepared by taking 50 µL of OMM into a 35-mm petri dish. Selected COCs (A and B grade) were washed 6 times in washing medium and 2 times in OMM medium and then transferred to each OMM droplet and covered with 0.2 µm of sterile, filtered mineral oil. The petri dish containing COCs was incubated at 38.5°C with 5% CO_2 and humidified air for 24 hrs. For inducing heat stress, COCs were exposed to a temperature of 41°C during the first 12 hr of IVM and again 38.5°C for remaining 12 hrs with 5% CO_2 and humidified air as described by Roth and Hansen, (2004b).

Fixation and staining of oocytes: After completion of IVM, the oocytes were prepared for fixation and staining. *In vitro* matured COCs were exposed to 0.2% hyaluronidase for 1 minute to loosen the cumulus cells and thenvortexed for 2-3 minutes so that they become free of cumulus cells. Cumulus free oocytes were washed 3 times in OMM and then fixed with acetic alcohol (acetic acid and ethyl alcohol, 1:3 parts) and stained with aceto-orcein stain.

Experimental design: *In vitro* maturation of caprine oocytes was determined microscopically under Inverted microscope (Trinocular Stereozoom MS2-TR Mac Olympus) after 24 h of incubation in OMM medium through experiments 1-2 and repeated many times.

Experiment 1: Effect of ascorbic acid on cumulus expansion of COCs: After 24 hours of incubation in OMM media, the degree of cumulus expansion of oocytes was qualitatively determined under a microscope for caprine oocytes with ascorbic acid supplementation and under heat stress.

Experiment 2: Effect of ascorbic acid on nuclear maturation: *In vitro* maturation of oocytes was also confirmed by demonstration of nuclear stage of maturation by staining of oocytes. Metaphase II was taken for comparative performance of oocytes under different maturation media (i.e. normal maturation media and ascorbic acid supplemented media) and temperatures of incubation (i.e. normal temperature at 38.5 °C and under heat stress at 41°C).

RESULTS AND DISCUSSION

Experiment 1: Effect of antioxidant on cumulus expansion of COCs: The results of percentage of caprine COCs undergoing cumulus expansion during IVM in non supplemented and ascorbic acid supplemented media and different temperatures of incubation are presented in Fig 1 and the comparative pairwise chi square values are represented in Table 1.

When the maturation performance of COCs in the non supplemented group without heat stress (75.23%) is compared with heat stress group (42.98%), it becomes evident that heat stress drastically reduces the maturation percentage of COCs by 32.25% and also has been shown statistically significant (P≤0.01). Also the expansion of cumulus cells in heat stressed, non supplemented oocytes

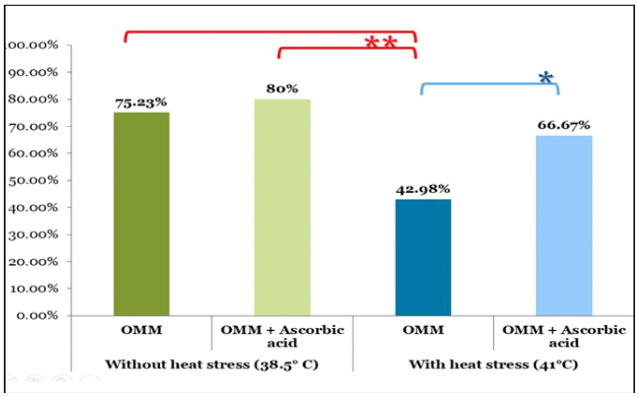

Fig 1: Maturation percent of caprine oocytes based on cumulus cell expansion in normal maturation media and ascorbic acid supplemented maturation media, with and without heat stress.

Table 1: Pairwise Chi-square values of *in vitro* maturation of caprine oocytes based on cumulus cell expansion in ascorbic acid supplemented oocyte maturation media (OMM), with and without heat stress.

		Without heat stress (38.5°C)		With heat stress(41°C)	
		OMM	**OMM + Ascorbic acid**	**OMM**	**OMM + Ascorbic acid**
Without heat stress (38.5°C)	**OMM**	-	0.08	5.86**	0.25
	OMM+ Ascorbic acid	-	-	7.01**	0.68
With heat stress(41°C)	**OMM**	-	-	-	4.01*
	OMM + Ascorbic acid	-	-	-	-

*Significant (P≤0.05) and **Significant (P≤0.01).

was significantly poorer than those of non heat stressed ones (both non-supplemented and ascorbic acid supplemented group). When ascorbic acid was added to OMM in heat stressed oocytes, the cumulus expansion was increased by 23.69% from non supplemented heat stressed oocytes. Ascorbic acid addition to the heat stressed group could bring a significant (P≤0.05) increase in oocyte maturation percentage than non-supplemented OMM.

In this study ascorbic acid (100 µM) was found to cause increased expansion of cumulus cells and increase in MII nuclear maturation which is similar to the reports of Miclea *et al*., (2008) and Elsayed *et al*., (2015) who found an increased cumulus cell expansion and significant increase in maturation rates in porcine and camel oocytes, respectively. Similar results were reported in porcine oocytes (Hossein *et al*., 2007) and caprine oocytes (Hammami *et al*., 2013; Hammami (2014) where ascorbic acid was found to improve cytoplasmic maturation and embryo development. Although the results could slightly vary with different concentrations used, in general, ascorbic acid increased expansion of cumulus cells.

Experiment 2: Effect of ascorbic acid on nuclear maturation: A total of 230 oocytes were observed for nuclear maturation (Metaphase II stage) with and without ascorbic acid addition in OMM, in non heat stress and heat stress conditions as presented in Fig 2 and the pairwise Chi-square values observed with ascorbic acid supplemented and non supplemented OMM, with and without heat stress is presented in Table 2. Extrusion of first polar body of metaphase II was observed (Fig 3) after 24 hours of maturation after aceto-orcein staining.

The percentage of oocytes in MII stage is more in group without heat stress (72.86%) than the group exposed to higher temperature (40%). There was a dramatic and significant difference (P≤0.05) of MII nuclear maturation stage of oocytes between the heat and non-heat stressed, non supplemented group. In ascorbic acid supplemented media, MII stage of nuclear maturation of heat stressed group increases (64%) but there was no significant difference when compared to non-supplemented heat stressed group (40%).

Nuclear maturation status achieved with addition of ascorbic acid is comparable to findings of Pernes *et al*., (2016) who found that ascorbic acid increased the meiotic resumption of canine oocytes and higher proportions of oocytes reached MI and MII stage. Significant improvement

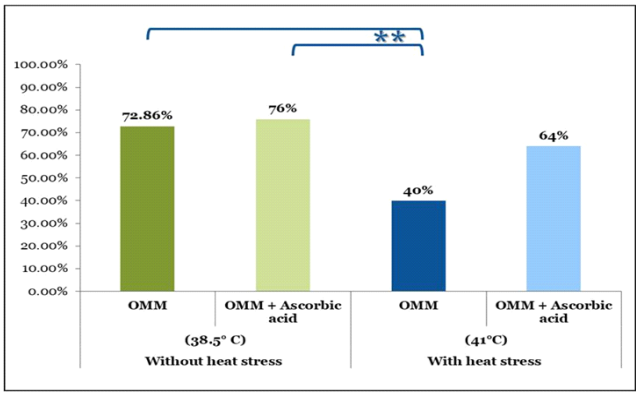

Fig 2: Expression percentage of nuclear maturation stae MII observed during *in vitro* maturation of caprine oocytes in normal maturation media and ascorbic acid supplemented maturation media, with and without heat stress.

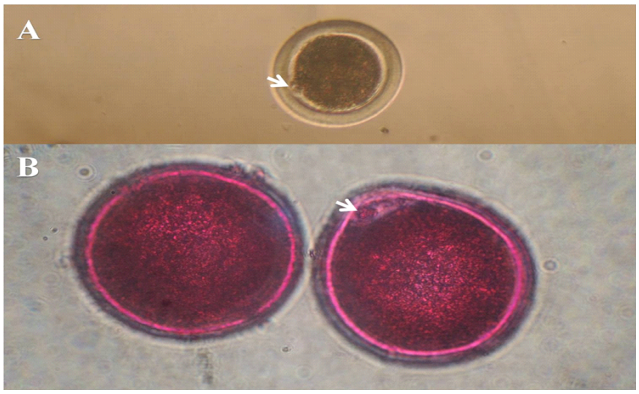

Fig 3: Caprine oocyte showing extrusion of first polar body (white arrow) after *in vitro* maturation. (A) Before staining and (B) After aceto-orcein staining.

Table 2: Pairwise Chi-square values of nuclear maturation stage MII observed after *in vitro* maturation of caprine oocytes in ascorbic acid supplemented oocyte maturation media (OMM), with and without heat stress.

		Without heat stress (38.5°C)		With Heat stress (41°C)	
		OMM	**OMM+ Ascorbic acid**	**OMM**	**OMM + Ascorbic acid**
Without Heat stress (38.5°C)	**OMM**	_	0.02	3.94*	0.20
	OMM + Ascorbic acid	_	_	3.98*	0.30
With Heat Stress (41°C)	**OMM**	_	_	_	2.03
	OMM + Ascorbic acid	_	_	_	_

*Significant (P≤0.05) and **Significant (P≤0.01).

in maturation rate of MII oocytes with ascorbic acid supplemented media were reported by Dorra *et al.*, (2012) and Nadri *et al.*, (2009) in rabbit and mice oocytes, respectively.

Exposure of COCs to elevated temperature during first 12 h of maturation caused reduction in cumulus expansion and nuclear maturation, which may be due to oxidative stress caused by increased concentration of ROS (Nabenishi *et al.*, 2012a; 2012b). Addition of ascorbic acid could bring significant improvement in maturation rates in heat stressed COCs. The role of ascorbic acid in preventing free radical initiated peroxidative tissue damage is well documented (Rose and Bode, 1993; Tilly and Tilly, 1995). It could protect the oocytes from oxidative stress by increasing the intracellular glutathione (GSH) content (Tatemoto *et al.*, 2000) which protected cell membranes from circulating oxidants (Kosower and Kosower, 1973) and decreased ROS accumulation in MII oocytes which allowed a better ooplasmic maturation and subsequent embryo development in porcine (Kere *et al.*, 2012). It can be, therefore, assumed that supply of ascorbic acid in oocyte maturation media (OMM) increases the GSH content, which protects the cumulus cell membranes from ROS and increases the levels of MPF activities of oocytes, thereby, prevents the apoptosis of cumulus cells as well as oocytes.

CONCLUSION

In conclusion, supplementation of 100µM L-Ascorbic acid in oocyte maturation media (OMM) has been demonstrated to improve developmental competence of oocytes and increased maturation rates during heat stress.

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