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Cumulus cell expansion and ultrastructural changes in *in vitro* matured bovine oocytes under heat stress

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Summary

Cumulus-oocyte complexes (COCs) from cows were matured under normal (38.5°C) and elevated temperatures (41°C) simulating heat stress and their maturation was assessed based on measurement of cumulus expansion in both groups. There was a significant reduction (P<0.01) in maturation rate in the heat stressed oocytes. The ultrastructural events associated with *in vitro* oocyte maturation and changes associated with elevated temperature were also studied by transmission electron microscopy (TEM). Normal maturation cellular events were marked by migration of Golgi and mitochondria from the cortical regions, and conversely by a migration of cortical granules from the inner regions to a sub-perivitelline zone. Heat stressed oocytes (41°C) were not only marked by a reduction in rate and less cumulus cell expansion, but also by a reduction in cortical granule migration. The mitochondria appeared swollen with cristolysis. Ribosomal disruption and an abundance of free ribosomes were also seen. Changes in the cumulus cells include nuclear chromatin margination, condensation and karyolysis, formation of nuclear and cell membrane blebs, and typical membrane bound vesicles enclosing cell fragments indistinguishable from apoptosis. Evidently, heat stress can be associated with reduced cytoplasmic events of oocyte maturation, thereby decreasing the oocyte competence and can be associated with apoptosis of the cumulus cells and therefore compromise the survival of the oocyte itself.

Key words: Bovine, Heat stress, IVM, Oocyte, TEM

Introduction

The oocyte remains sensitive to heat stress through the period of oocyte maturation (Hansen, 2013). Exposure to heat stress can occur due to elevated ambient temperature, often recognized as a major factor responsible for reduced fertility in farm animals (Ealy *et al*., 1995). The negative effects on the oocyte may include alterations in the cytoplasmic and nuclear maturation, protein expression, membrane alterations, induction of apoptosis, pre-mature aging and overall developmental competence (Maya-Soriano, 2012).

The present study was proposed to record visual ultrastructural changes associated with heat stressed oocytes during their maturation.

Materials and Methods

The current experiment was approved by the Institutional Animal Ethics Committee, College of Veterinary Science, Assam Agricultural University.

Recovery of cumulus-oocyte complexes

Ovaries from cows and heifers were obtained from a local abattoir located at Killing, Baridua, Meghalaya. The samples were transported to the laboratory and the cumulus-oocyte complexes (COCs) were processed for *in vitro* maturation (IVM) as described before (Ahmed *et al*., 2016). Only grade 'A' and 'B' COCs (Jainudeen *et al*., 2008) with more than 3-5 layers of compact cumulus cells surrounding the zonapellucida were selected for IVM.

In vitro oocyte maturation

In vitro maturation of bovine COCs was carried out as described before (Ahmed *et al*., 2016). Extent of cumulus expansion was assessed by measuring the oocyte diameter with cumulus cells before and after 24 h of incubation under a phase contrast microscope (Leica, Inverted Laboratory Microscope, DM IL LED) with LAS-EZ (Leica Application Suit) software under normal (38.5°C) and elevated temperature (41.0°C) as described before (Ahmed *et al*., 2016).

For inducing heat stress, the oocytes were exposed to

a physiologically relevant temperature of 41°C during the first 12 h of IVM and reverted to 38.5°C for the next subsequent 12 h as described by Roth and Hansen (2004), with other conditions remaining unaltered.

Electron microscope sample preparation

After 24 h culture in the maturation medium, five oocytes each from heat stressed group and control group were randomly selected and primary fixed in 1% Karnovsky's fixative prepared in 0.1 M sodium cacodylate buffer (pH = 7.2) for 12 h at 4°C. The specimens were then washed in 0.1 M sodium cacodylate buffer thrice for 15 min each and stored in the same buffer at 4°C till further use.

The oocytes were then washed, secondary fixed in osmium tetroxide and dehydrated in ascending grades of acetone, resin embedded in araldite and ultrathin sections prepared for the whole COC. Sections were mounted on 75-mesh copper grids and stained with uranyl acetate and lead citrate. These sections were observed and photographed with a Transmission Electron Microscope (Jeol, JEM-2100, 200 kV) at 80 KV.

Statistical analysis

Pairwise Chi-square test was performed to find differences in development competence during maturation between treatments.

Results

Assessment of oocyte maturation by visualization of cumulus expansion of COCs revealed a highly significant decrease ($P \leq 0.01$) in expansion rate from 80.30% under normal temperature (38.5°C) to 60.59% in heat stressed COCs (Table 1).

Table 1: Development competence of *in vitro* matured bovine oocytes under normal (38.5°C) and elevated temperature (41°C) based on cumulus cell expansion

Cumulus-oocyte complexes (COCs)	Without heat stress (38.5°C)	With heat stress (41°C)
No. of COCs	168	170
No. of COCs expanded	135	103
Expansion percentage	80.36	60.59*

* P<0.01

The various cellular changes in COCs from both groups observed under the transmission electron microscope are as follows:

In vitro matured oocytes (38.5°C)

The cytoplasmic granules were seen to migrate towards the cortex and occupy the area just beneath the oolemma. There is also a redistribution of mitochondria which become oriented in association with the endoplasmic reticulum and vacuoles. Most microvilli detach to form the perivitelline space. The number of lipid vacuoles also decreased inside the maturing oocyte. The ultrastructural changes associated with the non heat stressed COCs are represented in Figs. 1A-F. The cumulus cells also show marked expansion and appear elongated.

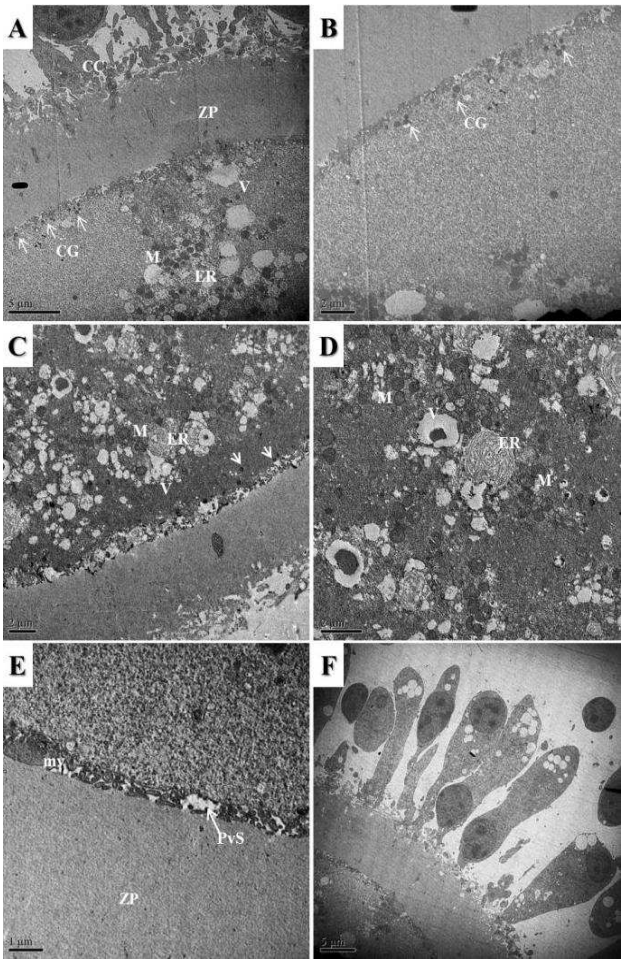

Fig. 1: Transmission electron micrographs showing ultra-structural changes associated with non heat stressed *in vitro* matured cumulus-oocyte complexes. **A-B**: Oocytes showing migration of cortical granules (CG) marked by white arrows, beneath the zona pellucida bearing cumulus cells (CC), **C-D**: Oocytes showing association of mitochondria (M) with endoplasmic reticulum (ER) and vacuoles (V). Cortical granules are marked with white arrows, **E**: Oocyte showing microvilli (mv) and perivitelline space (PvS), and **F**: Cumulus cells undergoing expansion and appear elongated

In vitro heat stressed matured oocytes (41°C)

The changes observed for heat stressed oocytes are presented in Figs. 2A-D. The cortical granules were scarce or absent and no migration was evident towards the cortex. The mitochondria are also found to be associated with the endoplasmic reticulum and vacuoles. In most cases the mitochondria appear swollen with cristolysis. Ribosomal disruption and an abundance of free ribosomes were marked.

The cumulus vestment appeared compact, degenerated and were sometimes indistinguishable from apoptosis (Figs. 3A-F). Changes in the nucleus include chromatin margination, condensation, nuclear membrane blebs and nuclear fragmentation. Cytoplasmic changes

included a generalized swelling of all organelles including the mitochondria, endoplasmic reticulum and Golgi apparatus. There was an abundance of lysosomes and free ribosomes. There was also prominent cytoplasmic vacuolation, cell membrane blebs and characteristic membrane bound cellular fragments indistinguishable from apoptotic bodies.

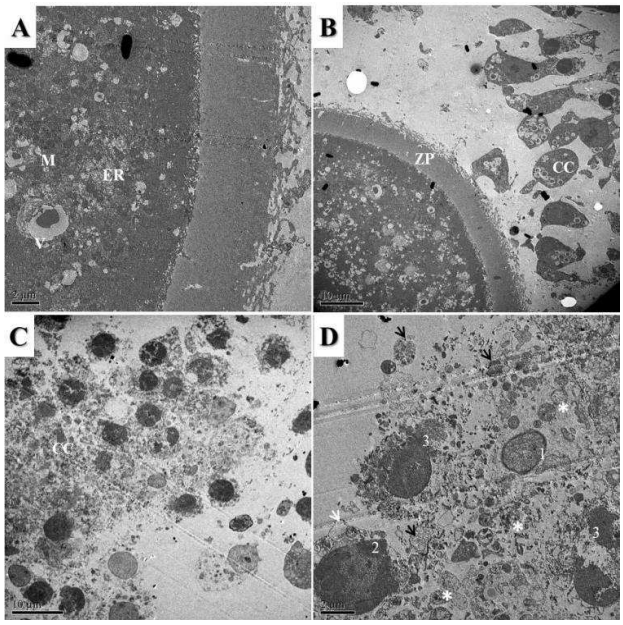

Fig. 2: Transmission electron micrographs showing ultra-structural changes associated with heat stressed and *in vitro* matured cumulus-oocyte complexes. **A**: Heat stressed, *in vitro* matured oocyte showing absence of cortical granules but association of mitochondria (M) with endoplasmic reticulum (ER) and vacuoles (V), **B**: COC showing compact cumulus cells (CC) with less expansion and detachment from zonapellucida (ZP), **C**: Compact cumulus cells undergoing degeneration, **D**: Numerous cumulus cells undergoing changes indistinguishable from apoptosis-nuclear chromatin margination (1), condensation (2) and fragmentation (3), mitochondrial swelling and rounding (*), membrane blebs (white arrow) and membrane bound cellular fragments (black arrows)

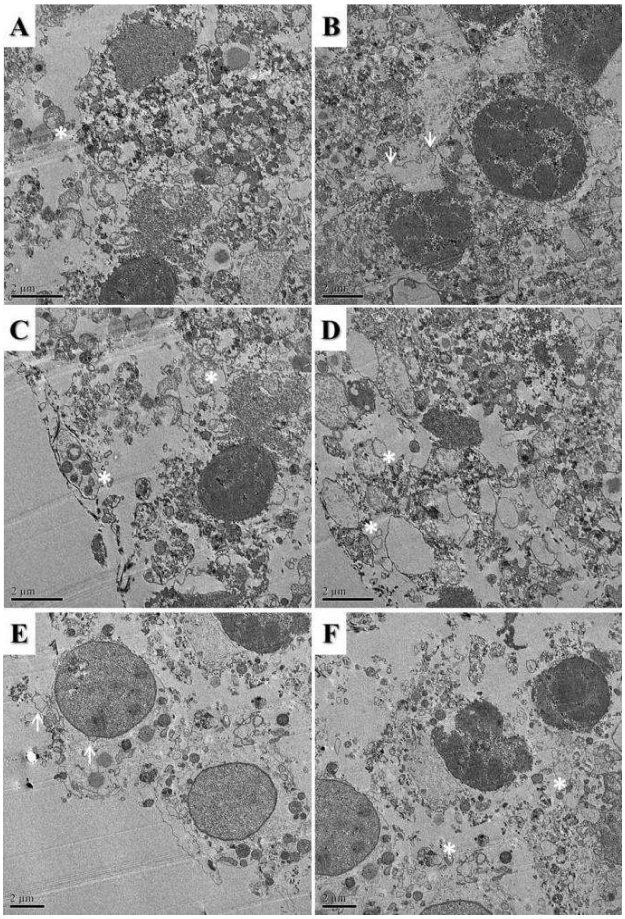

Fig. 3: Transmission electron micrographs showing ultra-structural changes indistinguishable from apoptosis in cumulus cells of heat stressed, *in vitro* matured cumulus-oocyte complexes. **A-F:** Numerous cumulus cells undergoing changes indistinguishable from apoptosis including nuclear chromatin margination, condensation and fragmentation, nuclear and cell membrane blebs (arrows) and membrane bound cellular fragments (*)

Discussion

It was evident that heat stress was associated with a significant decrease in the development competence of oocytes during maturation events. The ultrastructural changes observed in control (non heat stressed) cumulus-oocyte complexes (COC's) were consistent with the descriptive cytoplasmic changes associated with oocyte maturation (Hosoe and Shioya, 1997; Cetica *et al*., 2001). The migration of a majority of organelles from the inner regions of the oocyte to a cortical position is primarily mediated by the cytoskeletal network (Hyttel *et al*., 1997; Ferreira *et al*., 2009). This clustering, particularly of the mitochondria near other organelles and nuclear material is believed to be necessary for protein synthesis (Krisher and Bavister, 1998) as a source of ATP production (Stojkovic *et al*., 2001).

The maturation changes associated with induced heat stress were similar to those described by earlier workers (Ju *et al*., 2005; Roth and Hansen, 2005; Soto and Smith, 2009). Within the oocyte, heat stress evidently brought about a collapse of the cytoskeletal organization. Cytoskeletal modifications have been attributed to disruption in meiosis resumption during heat shock and associated with oocyte apoptosis (Roth and Hansen, 2005). The decrease in migration of cortical granules during heat stress have either been attributed to structural and organizational loss of microfilaments (Ju and Tseng, 2004; Rivera *et al*., 2004), or a pre-mature translocation of cortical granules possibly due to induced pre-mature aging of oocytes (Lawrence *et al*., 2004; Edwards *et al*., 2005; Schrock *et al*., 2007). Roth and Hansen (2005) argued that alterations in the cytoskeleton caused by heat shock may serve as a trigger for apoptosis; while conversely, disruption of cytoskeletal architecture may also be the result of activation of execution caspases associated with apoptosis.

The other salient feature of heat stress induced change was that of severe organellar disruption. In a study on heat stressed mouse early blastocysts, Qu *et al*.

(2009) similarly observed severe mitochondrial swelling, presence of phagolysosomes, multi-vesicular body and ribosomal detachments at 41°C. This organellar disruption, to an irreversible degree may be lethal to the survival of the oocytes. Evidently, mitochondrial dissociation during heat stress could also be associated with a decrease in ATP generation within the oocyte. A certain degree of reversible changes of mitochondrial swelling has been observed by Qu *et al*. (2009) at 39°C in mouse blastocysts when reverted back to 37°C, but repair was not easily achieved when pre-exposed to 41°C. Soto and Smith (2009) suggested that mitochondrial integrity could be one probable factor that determines progression to apoptosis, and emphasized its role in the integration and transmission of cell death signals. Nabenishi *et al*. (2012b) further observed that oocytes treated with cyclosporine-A which inhibits mitochondrial permeability transition pore opening, could decrease cumulus cell apoptosis under heat shock. An underlying mitochondrial dysfunction thus becomes evident, and when their integrity is compromised, the intrinsic mechanisms leading to apoptosis are probably triggered.

It may be possible that ribosomal detachments could also be associated with decreased protein synthesis. A similar notion has also been forwarded by Qu *et al*. (2009) in heat stressed mice blastocysts. This can be corroborated with the findings of Edwards and Hansen (1996) that suggested a decrease in protein synthesis by as much as 30-50 per cent during heat stress.

Under heat stress, a decrease in expansion rate was evident, apparently from the association of degenerate and apoptosis-like changes. It may be interesting to note, that the degenerate changes were particularly evident in the cumulus cells. Evidence of apoptosis of the cumulus cells in bovine oocytes under heat shock have been similarly reported by Nabenishi *et al*. (2012a). Since the role of the cumulus cell is vital to nurture the oocyte during maturation up to the MII stage (Tanghe *et al*., 2002) and facilitate exchange of nutrient and chemical signals (Warnes *et al*., 1977), their degeneration would invariably compromise the oocyte.

Heat stress induced by adverse climates can bring about failures in reproduction and development competence of oocytes as is obvious from *in vitro* heat stress (41°C) simulation. It can be associated with reduced cytoplasmic events of oocyte maturation thereby decreasing the oocyte competence and can be associated with apoptosis of the cumulus cells and therefore compromise the survival of the oocyte itself.

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Conflict of interest

The authors declare no conflicts of interest.

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