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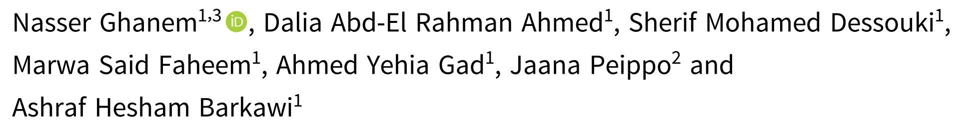
# Cellular and molecular alterations of buffalo oocytes cultured under two different levels of oxygen tension during *in vitro* maturation



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### Summary

This study was conducted to monitor the cellular and molecular changes of buffalo cumulus–oocytes complexes (COCs) cultured under high or low oxygen levels. Morphologically good quality COCs ($n = 1627$) were screened using brilliant cresyl blue (BCB) staining and placed into three groups (BCB+, BCB− and control). All groups of COCs were cultured under low (5%) or high (20%) oxygen tensions. Intracellular and molecular changes including oocyte ultrastructure, lipid contents, mitochondrial activity and transcript abundance of genes regulating different pathways were analyzed in the matured oocyte groups. The results revealed that oxygen tension did not affect cumulus expansion rates, however the BCB+ group had a higher ($P \leq 0.05$) expansion rate compared with the BCB− group. BCB− oocytes recorded the lowest meiotic progression rate ($P \leq 0.05$) under high oxygen levels that was linked with an increased level of reactive oxygen species (ROS) compared with the BCB+ oocytes. Ultrastructure examination indicated that BCB+ oocytes had a higher rate of cortical granules migration compared with BCB− under low oxygen tension. In parallel, our results indicated the upregulation of *NFE2L2* in groups of oocytes cultured under high oxygen tension that was coupled with reduced mitochondrial activity. In contrast, the expression levels of *MAPK14* and *CPT2* genes were increased ($P \leq 0.05$) in groups of oocytes cultured under low compared with high oxygen tension that was subsequently associated with increased mitochondrial activity. In conclusion, data from the present investigation indicated that low oxygen tension is a favourable condition for maintaining the mitochondrial activity required for nuclear maturation of buffalo oocytes. However, low-quality oocytes (BCB−) responded negatively to high oxygen tension by reducing the expression of gene-regulating metabolic activity (*CPT2*). This action was an attempt by BCB− oocytes to reduce the increased levels of endogenously produced ROS that was coupled with decreased expression of the gene controlling meiotic progression (*MAPK14*) in addition to nuclear maturation rate.

## Introduction

Buffaloes play a pivotal role in the Egyptian agricultural economy. This is attributed to their substantial contribution to meat and milk production. However, buffaloes reproduce at a very low rate due to silent ovulation, unordinary follicular dynamics, and low numbers of ovarian follicles (Barkawi *et al*., 2008). Therefore, assisted reproductive technologies (ARTs) have become an essential tool to enhance buffalo reproductive efficiency (Madan *et al*., 1996). The efficiency of *in vitro* embryo production is extremely important for producing viable embryos (Feugang *et al*., 2009). Despite the increased rates of *in vitro* embryo production (IVP) over the last decades, the quality of IVP embryos is still lower than that of their *in vivo* counterparts. This is mainly attributed to the composition of the culture medium, its pH, and the choice of oxygen tension used during IVP (Du Plessis *et al*., 2008).

Suboptimal conditions of *in vitro* culture ultimately compromise blastocyst quality due to increased accumulation of reactive oxygen species (ROS) (Oyamada and Fukui, 2004). In fact, ROS could be increased by high oxygen levels during any step of IVP (Goto *et al*., 1993; Bavister, 1995; Karagenc *et al*., 2004). The normal physiological concentration of oxygen in the reproductive tract of bovine females is approximately 5% (Bavister, 1995). High levels of oxygen (20%) are responsible for inducing oxidative stress during *in vitro* preimplantation development (Karagenc *et al*., 2004). Ovarian follicles are dynamic structures that undergo growth, ovulation, and luteinization processes during the reproductive cycle. Due to the lack of a direct blood supply, oxygen concentration decreases in follicular fluid during follicular maturation, reaching its lowest level in preovulatory follicles (Fischer *et al*., 1992). The dissolved oxygen in human

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ovarian follicular fluid is predicted to be 1.5–6.7% based on mathematical modelling (Redding *et al.*, 2008). Several studies have confirmed these values and the estimated level of dissolved oxygen ranges between 1% and 5% (Van Blerkom *et al.*, 1997; Huey *et al.*, 1999),

There is an equilibration system that keeps the generation of oxygen species in balance in any living cell (Whitaker, 2007). When this system is compromised and the available antioxidants cannot maintain oxygen concentrations within the appropriate level, an overabundance of ROS is generated and induced oxidative stress leads to programmed cell death (Fiers *et al.*, 1999). Various types of ROS, such as superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (OH) can trigger oxidative stress. These types of ROS originate from the environment, as well as from within oocytes and embryos as a by-product of energy metabolism, in particular from mitochondrial phosphorylation and glycolysis (Guerin *et al.*, 2001; Balaban *et al.*, 2005).

Oxidative stress has broad effects on the reproductive system and has resulted in impairment of the functionality of ovarian steroidogenesis, ovulation (Agarwal *et al.*, 2005), embryogenesis (Bedaiwy *et al.*, 2004), and maintenance of pregnancy (Myatt and Cui, 2004). It is noteworthy that oxidative stress has negatively affected the nuclear maturation of oocytes (Cetica *et al.*, 2001; Ali *et al.*, 2003). Accumulation of ROS increases the incidence of chromosomal errors, disorganization of the microtubule, disassembly of meiotic spindles, and finally has compromised the quality of mice oocytes during IVM (Tarin *et al.*, 1996; Choi *et al.*, 2007; Tamura *et al.*, 2008). Furthermore, oxidative stress has a harmful effect on subsequent preimplantation development by perturbation of various cellular organelles such as mitochondria in addition to biochemical composition such as lipids (Halliwell, 1989; Nasr-Esfahani *et al.*, 1990). Buffalo oocytes have a high lipid content, which makes them less resistant to oxidative stress caused by ROS during *in vitro* maturation (Boni *et al.*, 1992).

Therefore, the current study was designed to determine the cellular and molecular changes found in buffalo oocytes selected by BCB staining (good quality vs. bad quality) and matured *in vitro* under two levels of oxygen (20% vs. 5%).

## Materials and methods

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

### Experimental design

Cumulus–oocyte complexes (COCs) were distributed into three groups that were divided into two sub-groups based on oxygen tension, as follows: control (C5% $n = 224$, C20% $n = 348$); BCB+ (BCB+ 5% $n = 269$, BCB+ 20% $n = 358$) and BCB− (BCB− 5% $n = 181$ BCB− 20% $n = 247$). Different parameters were evaluated for oocytes following IVM under these two conditions.

### In vitro *assessment of oocytes maturation*

Buffalo ovaries from cyclic animals were collected from a local Egyptian slaughterhouse and kept at 34–37°C in a thermos container in 0.9% NaC1 supplemented with 50 μg/ml gentamycin. COCs were aspirated from 2–8 mm follicles using an 18-gauge needle attached to a 10-ml disposable syringe. The aspirated COCs were classified morphologically under a stereomicroscope based on the number of cumulus layers and ooplasm homogeneity (Faheem and Barkawi, 2012). After staining with BCB, COCs were washed twice with modified Dulbecco's phosphate-buffered saline (mDPBS). The stained COCs were examined under a stereomicroscope and divided into two groups based on their cytoplasm coloration; oocytes with any degree of blue coloration in the cytoplasm were considered BCB+, while oocytes without blue cytoplasm were considered BCB− (Manjunatha *et al.*, 2007). The control group was not exposed to BCB stain. All groups of COCs were washed twice in the maturation medium (Faheem *et al.*, 2015), which consisted of TCM-199 supplemented with 10% FBS, 5 μg/ml FSH, 1 μg/ml estradiol-17β, 0.15 mg/ml glutamine, 22 μg/ml Na-pyruvate and 50 μg/ml gentamycin. In total, 10–15 COCs from each group were seeded into drops of 100 μl maturation medium under mineral oil and incubated at 38.5°C in 5% $CO_2$, 90% $N_2$ and 5% $O_2$ (triple gas mix); or 5% $CO_2$ + $N_2$ 75% and 20% $O_2$, respectively. The phase of oocytes nuclear maturation was assessed after 22–24 h of IVM. Oocytes were denuded, then fixed in a Petri dish containing fixation solution (methanol:glacial acetic acid, 3:1) and left for 24 h. After the fixation period, oocytes were placed on glass slides and stained with 1% aceto orcein for 5 min, then covered with a glass coverslip before examination under an inverted microscope. Matured oocytes were evaluated in relation to their meiotic stage as: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) stages (Faheem and Barkawi, 2012).

### Preparation of mature oocytes for transmission electron microscopy (TEM)

After 22–24 h of IVM, the cumulus cells were removed by repeated pipetting of intact COCs into IVM medium supplemented with 0.25% of hyaluronidase. Denuded oocytes were fixed in 2.5% glutaraldehyde supplemented with 0.1 M sodium phosphate buffer (pH 7.4) at room temperature for 15 min, then washed three times in sodium phosphate buffer for 30 min each. Next, oocytes were treated with osmium tetroxide ($OsO_4$ 1%) and dehydrated in increasing concentrations of ethanol before treatment with 100% ethanol twice for 15 min each, according to Anguish and Coonrod (2013). Dehydrated oocytes were infiltrated with Spurr's epoxy resin, and then were sectioned (90 μm thick) using an ultramicrotome (Leica model EM-UC6, Japan) and finally mounted on copper grids (400 mesh). Stained sections were examined under a JEOL transmission electron microscope (JEM-1400 TEM, Japan). Images of mitochondria, lipid droplets, and cortical granules, were captured to evaluate their cellular structure. Total mitochondrial number per $\mu m^2$ was counted precisely ($n = 6$ oocytes × 4 fields/oocyte). The number of each type of peripheral and central mitochondrial distribution was counted per $\mu m^2$. In addition, the number and distribution of lipid droplets per $\mu m^2$ were determined ($n = 6$ oocytes × 2 fields/oocyte). Finally, the cortical granule migration pattern was calculated.

### Evaluation of oocyte cytoplasmic lipid content

Nile red stock solution was prepared by dissolving 1 mg/ml of the stain in DMSO and then stored in the dark. Briefly, matured oocytes ($n = 10$/group) were denuded from cumulus cells and washed twice in mDPBS. The washed oocytes were fixed in 4% paraformaldehyde in PVP-PBS for 30 min. The fixed oocytes were first stained in 500 ml of a 10 mg/ml Nile red solution adjusted in PVP-PBS solution (0.3% [wt/vol] PVP in 1 M PBS) for 3 h. Stained oocytes were washed three times in PVP-PBS (15 min each) as detailed in Ghanem *et al.* (2014).

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**Table 1.** List of primers used for quantitative real-time PCR analysis

| Gene name | GenBank accession number | Primer sequence | Fragment size (bp) |
|---|---|---|---|
| CPT2 | NM_001045889 | F: 5´-CCGAGTATAATGACCAGCTC-3´ | 152 |
| | | R: 5´-GCGTATGAATCTCTTGAAGG-3´ | |
| NFE2L2 | NM_001011678 | F: 5´-TAAAACAGCAGTGGCTACCT-3´ | 159 |
| | | R: 5´-GAGACATTCCCGTTTGTAGA-3´ | |
| SOD2 | NM_201527 | F: 5´-GTGATCAACTGGGAGAATGT-3´ | 163 |
| | | R: 5´-AAGCCACACTCAGAAACACT-3´ | |
| MAPK14 | XM_006050391 | F: 5´-GATTTCGGACTGGCTCGACA-3´ | 269 |
| | | R: 5´-TTTCTTGCCTCATGGCTTGG-3´ | |
| GAPDH | NM_001034034.2 | F: 5´-AGGTCGGAGTGAACGGATTC-3´ | 219 |
| | | R: 5´-GGAAGATGGTGATGGCCTTT-3´ | |

### Evaluation of oocyte mitochondrial activity

Mitochondrial activity was measured using a commercial kit (MitoTracker Green FM; Invitrogen, Carlsbad, CA, USA) based on Ghanem *et al.* (2014). Oocytes stained with Nile red were subsequently used for mitochondrial activity measurement. Oocytes were washed three times in 500 µl PVP-PBS (15 min each) and incubated for 5 min with 125 nM of MitoTracker dissolved in PVP-PBS. The stained oocytes were washed twice in PVP-PBS (10 min each). Next, oocytes were mounted in glycerol on clean slides. Finally, a coverslip was placed over the oocytes (without compressing). An epifluorescence microscope was used to excite both Nile red and MitoTracker Green FM lipophilic fluorescent dyes (580–596 nm).

### Measurement of ROS

For quantification of $H_2O_2$, matured oocytes were incubated in PVP-PBS supplemented with 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) for 20 min at 38.5°C (Fakruzzaman *et al.*, 2015). After incubation, oocytes were washed three times with PVP-PBS, then mounted onto glass slides, and visualized immediately under an epifluorescence microscope at 490 nm excitation and 525 nm emission. Images analysis was carried out using ImageJ software (https://imagej.nih.gov/ij/download.html).

### Gene expression

#### RNA extraction and cDNA synthesis

Three replicates from each experimental group were used for RNA isolation and cDNA synthesis ($n = 15$–20 matured oocytes/replicate). Following the manufacturer's instructions, total RNA was extracted using the PicoPure RNA isolation kit (MDS Analytical Technologies GmbH, Ismaning, Germany). Matured oocytes were mixed thoroughly with lysis buffer and incubated at 42°C for 30 min. The complete lysate was loaded into a preconditioned spin column and centrifuged shortly for 2 min at 3600 *g* to allow the RNA to bind to the spin column. DNA was removed using an on-column RNase-free DNase kit (Qiagen GmbH, Hilden, Germany). The column was washed with two different washing buffers. Finally, RNA was eluted with 12 µl RNase-free water.

For each sample, cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (Life Technologies Corporation, California, USA). Total RNA from all samples was adjusted by adding RNase-free water to achieve the same concentration of RNA. A 14.2 µ1 volume of RNA sample was added to 1 µ1 10× RT buffer, 0.8 µ1 25× dNTP, 2 µ1 random primers, 1 µ1 RNase inhibitor, and 1 µ1 Multiscribe reverse transcriptase. The mixture was incubated in a cycle of 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and holding at 4°C. The cDNA samples were stored at −20°C until real-time polymerase chain reaction (PCR) quantification.

#### Real-time PCR analysis

Real-time PCR primers (Table 1) were designed using Primer3 software (http://primer3.wi.mit.edu//). *GAPDH* was used as a reference gene for real-time PCR quantification. The PCR mixture was composed of 10 µl Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, California, USA), 0.2 µl forward primer, 0.2 µl reverse primer, 7.6 µl nuclease-free water, and 2 µl cDNA. Thermal cycling conditions were set as 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s and a final step at 60°C for 1 min. After the end of the last cycle, a dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements at 7-s intervals until the temperature reached 95°C. Relative expression was calculated using the relative quantification method ($2^{-\Delta\Delta CT}$ method) (Bermejo-Alvarez *et al.*, 2010).

### Statistical analysis

All data were analyzed using analysis of variance (ANOVA) and the general linear model procedure (SAS, 2004), followed by multiple pairwise comparisons using Tukey's test. The data were expressed as mean ± standard error. The effect of oxygen level (5% vs. 20%) and oocyte type (BCB+ vs. BCB− vs. control) and the interaction between these two factors were tested by applying the following model:

$$Y_{ijk1} = \mu + R_i + A_j + (R_i{*}A_j)_{ijk} + e_{ijk1}$$

where: $Y_{ijk1}$ = individual observation; µ = overall mean; $R_i$ = effect of BCB staining (BCB+ = 1 and BCB− = 2); $A_j$ = effect of oxidative stress (oxygen level) (5% = 1 and 20% = 2); $(R_i{*}A_j)_{ijk}$ = the interaction between BCB and oxidative stress; $e_{ijk1}$ = experimental error supposed to be randomly distributed (0, σ2). A probability value ($P$-value) ≤ 5% was considered to be statistically significant.

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**Table 2.** Cumulus expansion and nuclear maturation rate of buffalo oocytes cultured under 5% and 20% oxygen tensions during *in vitro* maturation

| Maturation rate | Oxygen level | | Quality | | |
|---|---|---|---|---|---|
| | 5% | 20% | Control | BCB(+) | BCB(−) |
| Cumulus expansion rate (%) | $92.45^a \pm 1.21$ | $93.63^a \pm 1.07$ | $94.26^a \pm 1.4$ | $95.49^a \pm 1.4$ | $89.73^b \pm 1.4$ |
| Nuclear maturation rate (%) | $77.26^a \pm 5.73$ | $56.23^b \pm 3.95$ | $69.07^a \pm 5.63$ | $70.67^a \pm 6.08$ | $60.51^a \pm 6.35$ |

Abbreviations: bp, base pair; PCR, polymerase chain reaction.
Values with different superscripts (a, b) within the same row and experimental category (oxygen level, quality) indicate significant difference at $P \leq 0.05$.

**Table 3.** Cortical granules distribution (migrated and non-migrated) and numbers *in vitro* matured buffalo oocytes cultured under low (5%) or high oxygen tensions (20%)

| Cortical granules number | Oxygen level | | BCB | | |
|---|---|---|---|---|---|
| | Oxygen level 5% | Oxygen level 20% | Control | (+) | (−) |
| Migrated | $3.55^a \pm 0.26$ | $2.44^b \pm 0.26$ | $2.75^a \pm 0.32$ | $3.58^a \pm 0.32$ | $2.66^a \pm 0.32$ |
| Non-migrated | $1.16^a \pm 0.26$ | $0.88^a \pm 0.26$ | $0.91^a \pm 0.32$ | $0.66^a \pm 0.32$ | $1.50^a \pm 0.32$ |
| Total number | $4.72^a \pm 0.11$ | $3.33^b \pm 0.11$ | $3.66^b \pm 0.13$ | $4.25^a \pm 0.13$ | $4.166^a \pm 0.13$ |

Values with different superscripts (a, b) within the same row and experimental category (oxygen level, quality) indicate significant difference at $P \leq 0.05$.

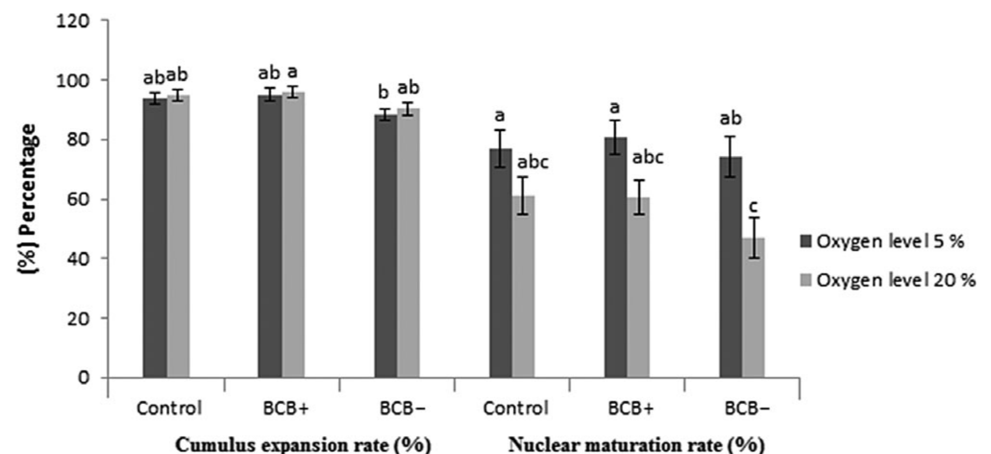


**Figure 1.** Cumulus expansion and nuclear maturation rate of buffalo oocytes cultured under 5% and 20% oxygen tension during *in vitro* maturation. Bars with different superscripts (a–c) indicate significant difference at $P \leq 0.05$. Cumulus expansion rate: numbers of oocyte that have completely or partially expanded in cumulus layers/total numbers of cultured oocytes. Nuclear maturation rate (%): oocytes that have reached telophase and MII stages.

## Results

### In vitro *maturation assessment*

The nuclear maturation of buffalo COCs was assessed to gain insight into the possible interaction of different levels of oxygen tension on the first crucial step of oocyte developmental competence when classified into different qualities using BCB staining. Oxygen tension had no significant effect on cumulus expansion rates (Table 2), however BCB+ had a higher ($P \leq 0.05$) expansion rate (95.49%) than BCB− (89.73%). Conversely, oxygen tension had a significant effect on nuclear maturation rate (oocytes that reached the MII stage). Regardless of oocyte quality, high oxygen level (20%) reduced ($P \leq 0.05$) the rate of oocyte maturation by about 20% (Table 2). Moreover, BCB− oocytes had a lower ($P \leq 0.05$) rate of MII stage under the high oxygen level (20%) compared with their counterparts cultured under low oxygen tension (Fig. 1).

### *Cortical granules migration*

The migration of cortical granules is considered as a marker of cytoplasmic maturation of COCs. Therefore, we examined the distribution of these cellular organelles in oocytes of different qualities and after *in vitro* maturation under the two oxygen levels. The total number of cortical granules (Table 3) was affected by the oxygen level, being significantly higher for oocytes matured under 5% (4.72 ± 0.11) than 20% (3.33 ± 0.11). Similarly, the number of migrated (peripheral distributed) cortical granules was increased ($P \leq 0.05$) under low (3.55 ± 0.26) compared with high (2.44 ± 0.26) oxygen tension. In addition, BCB+ and BCB− oocytes had higher cortical granule numbers compared with the control group. The number of migrated cortical granules was higher in BCB+ compared with the BCB− and control groups under low oxygen level (Fig. 2), however there were no differences among oocytes groups under high oxygen level. Moreover, there were no differences among all experimental groups concerning non-migrated cortical granules under high or low oxygen tensions. In general, oocytes cultured under high oxygen tension had lower ($P \leq 0.05$) cortical granule numbers compared with those cultured under low oxygen tension (Table 3 and Fig. 3).

### *Lipid droplet number, distribution, and fluorescence intensity*

Integration of the data on number and distribution of cellular organelles such as mitochondria with their activity measured by

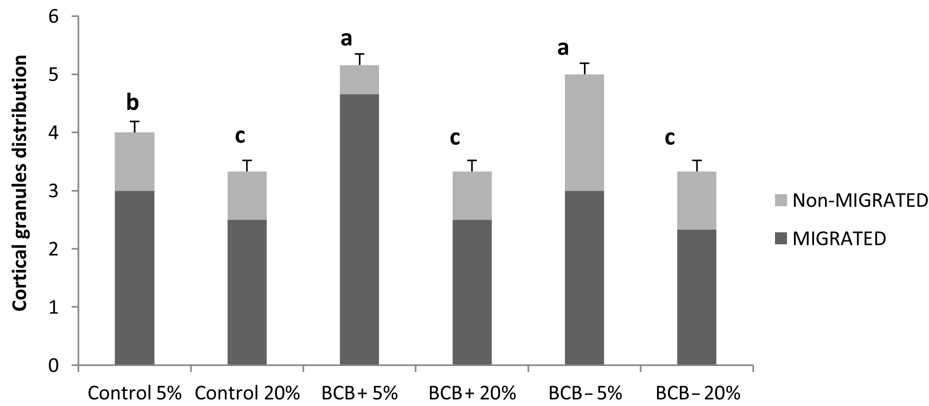


**Figure 2.** Percentage of cortical granules distribution (migrated and non-migrated) in buffalo matured oocytes cultured under low (5%) or high oxygen tension (20%) during *in vitro* maturation. Bars with different superscripts (a–c) indicate significant difference at $P \leq 0.05$. Migrated: moved peripherally beneath the plasma membrane. Non-migrated: diffused in the cytoplasm.

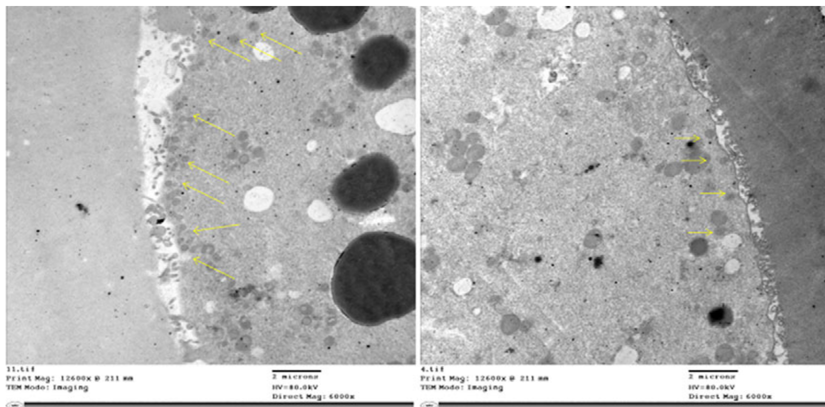

**Figure 3.** Representative photographs of the cortical granules of *in vitro* matured Egyptian buffalo oocytes taken with a transmission electron microscope (×6000).

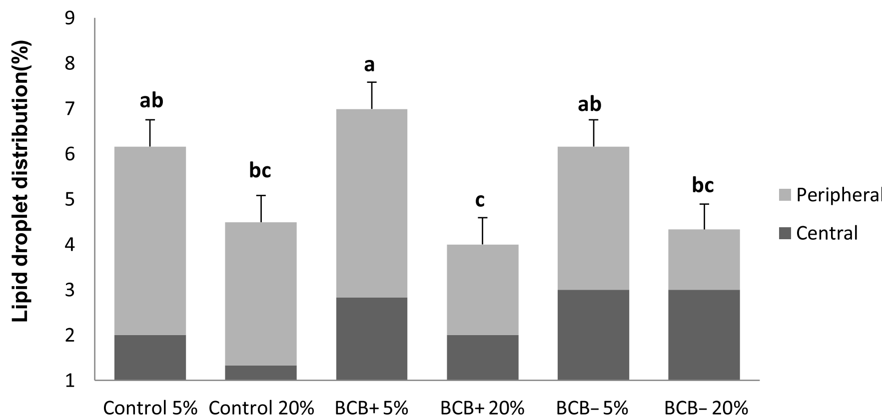


**Figure 4.** Percentage of lipid droplet distribution (peripheral and central) of buffalo mature oocyte cultured under low (5%) or high oxygen tensions (20%) during *in vitro* maturation. Bars with different superscripts (a–c) indicate significant difference at $P \leq 0.05$. Central: located in the centre of the cytoplasm. Peripheral: moved peripherally beneath the plasma membrane.

fluorescence intensity to understand their possible role during *in vitro* maturation of COCs cultured under two different levels of oxygen tension has not been described previously. As shown in Fig. 4, the intracellular lipid droplet number was increased in oocytes cultured under low compared with high oxygen tension. However, there were no significant differences in this parameter among control, BCB+, and BCB− groups.

The numbers of central intracellular lipid droplets were similar under the two oxygen levels and in all oocyte quality groups. However, the lowest number of peripheral lipid droplets was recorded in oocytes cultured under low oxygen tension and BCB− oocytes. Intracellular lipid fluorescence intensity was higher ($P \leq 0.05$) in the control and BCB− oocytes compared with BCB+ oocytes cultured under low oxygen tension. In addition, there were no differences in lipid fluorescence intensity among all groups of oocytes cultured under high oxygen tension (Fig. 5).

### *Mitochondrial number, distribution, and fluorescence intensity*

The total mitochondrial number was similar in all oocyte groups regardless of oxygen level and quality of oocytes (Figs 6 and 7). Moreover, the central mitochondrial distribution was more intense than the peripheral type in all oocyte groups under the two oxygen levels (Table 4).

As shown in Fig. 8, the fluorescence intensity of mitochondrial activity was similar in control, BCB+, and BCB− oocytes. In addition, the groups of oocytes cultured under low oxygen levels

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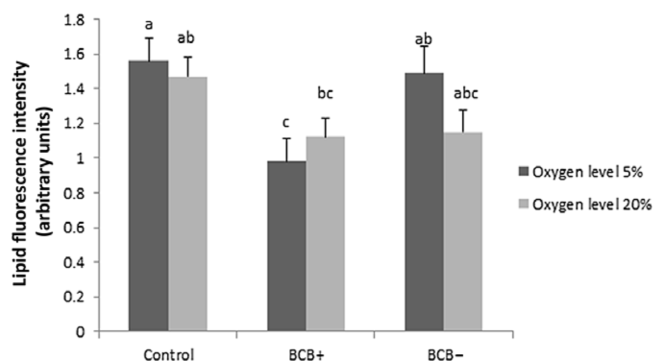


**Figure 5.** Fluorescence intensity of intracellular lipid content per buffalo mature oocytes cultured under low (5%) or high oxygen tensions (20%) during *in vitro* maturation. Bars with different superscripts (a–c) indicate significant difference at $P \leq 0.05$.

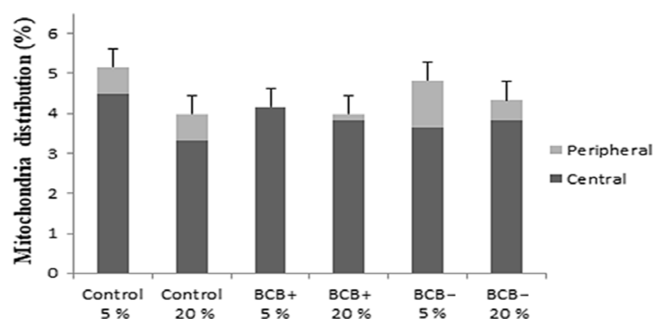


**Figure 6.** Percentage of mitochondria distribution (peripheral and central) of mature buffalo oocytes cultured under low (5%) or high oxygen tensions (20%) during *in vitro* maturation. Central: diffused in cytoplasm. Peripheral: moved peripherally beneath the plasma membrane.

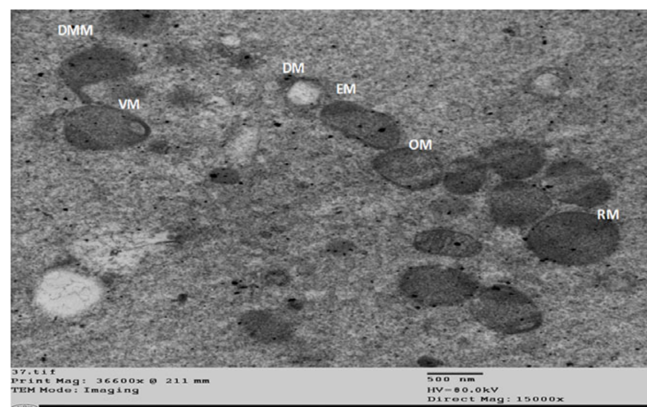


**Figure 7.** Electron micrograph of Egyptian buffalo oocytes showing oval (OM), round (RM), vacuolated (VM), distorted mitochondrial membrane (DMM) and deformed (DM) forms in matured oocytes (×15,000).

revealed an insignificant elevation in mitochondrial activity compared with their counterparts cultured under high oxygen levels, except for BCB+ oocytes.

### *Reactive oxygen species levels*

The level of ROS was measured to detect the effect of oxygen tension on the microenviroment of oocytes and subsequent effect on cytoplasmic and nuclear maturation of buffalo BCB+ and BCB− COCs. The ROS level in the cytoplasm of BCB− oocytes cultured under 20% oxygen was significantly ($P \leq 0.05$) higher than in BCB− group cultured under 5% oxygen (Fig. 9).

### *Gene expression*

Maturation of COCs is dependent on cytoplasmic, nuclear and molecular changes. Therefore, four genes were selected according to their biological roles and their profile link with subcellular, cytoplasmic and nuclear measurements performed during COC maturation. The *MAPK14* gene is involved in oocyte maturation (Fan and Sun, 2004; Villa-Diaz and Miyano, 2004), while *SOD2* plays a major role in cellular defence mechanisms against oxidative stress (Balasubramanian *et al.*, 2007; Ma *et al.*, 2017). Additionally, *CPT2* encodes a protein that oxidizes long-chain fatty acids (FAs) in mitochondria (Spikings *et al.*, 2007; Downs *et al.*, 2009) and *NFE2L2* induces the expression of a variety of antioxidant response element-dependent genes to regulate physiological and pathophysiological outcomes after exposure to different stressors (Ma *et al.*, 2018; Khadrawy *et al.*, 2019).

According to the oxygen level, the transcript abundance of *MAPK14* was lower in all groups of oocytes cultured under high oxygen tension than those cultured under low oxygen tension. The gene expression profile of *MAPK14* was increased ($P \leq 0.05$) in control and BCB+ oocytes compared with BCB−. BCB− oocytes recorded the lowest ($P \leq 0.05$) expression profile of *MAPK14* under high oxygen tension (Fig. 10).

There were no significant differences in the *SOD2* expression profile (Fig. 9) among all experimental groups, although oocytes cultured under low oxygen tension had a higher expression than those cultured under high oxygen tension.

The expression profile of the *CPT2* gene (Fig. 9) was increased in all groups of oocytes cultured under low compared with high oxygen tensions. The highest ($P \leq 0.05$) expression of *CPT2* was recorded in the BCB− group cultured under low oxygen levels. In contrast, BCB− oocytes cultured under high oxygen levels expressed the lowest transcript abundance of *CPT2* during *in vitro* maturation.

The expression profile of *NFE2L2* (Fig. 9) was upregulated ($P \leq 0.05$) in all groups of oocytes cultured under high oxygen tension compared with their counterparts cultured under low oxygen tension. The BCB+ oocytes cultured under high oxygen tension expressed the highest ($P \leq 0.05$) profile of the *NFE2L2* gene while BCB− oocytes cultured under low oxygen tension recorded the lowest expression of this gene.

## Discussion

Despite the progress in buffalo IVP (Madan *et al.*, 1994a) and embryo transfer (Madan *et al.*, 1994b), the efficiency of IVP is still very low, in terms of transferable embryos rate (TE) and development to term (Madan *et al.*, 1996). Seasonal variations in embryo development rate have been linked to alterations in oocyte quality in Italian buffaloes (Di Francesco *et al.*, 2011). The quality of the oocyte is one of the main factors that affects embryo yield, while culture conditions influence embryo quality (Rizos *et al.*, 2002). The results of the current study inferred no effect of oxygen tension level (20% vs. 5%) and oocyte quality (BCB+ vs. BCB−) on cumulus expansion rate. However, nuclear maturation rate was increased in all groups of oocytes cultured under low oxygen tension (5%) compared with groups cultured under high oxygen tension (20%). Similarly, Kumar *et al.* (2015) have reported the

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**Table 4.** Number and distribution of mitochondria of *in vitro* matured buffalo oocytes (in 1 μm$^2$ per oocyte) cultured under low (5%) or high oxygen tensions (20%)

| | Oxygen level | | BCB | | |
|---|---|---|---|---|---|
| Mitochondria number | Oxygen level 5% | Oxygen level 20% | Control | (+) | (−) |
| Central | 4.11$^a$ ± 0.30 | 3.66$^a$ ± 0.30 | 3.91$^a$ ± 0.37 | 4.00$^a$ ± 0.37 | 3.75$^a$ ± 0.37 |
| Peripheral | 0.61$^a$ ± 0.19 | 0.44$^a$ ± 0.19 | 0.66$^{ab}$ ± 0.23 | 0.08$^b$ ± 0.23 | 0.83$^a$ ± 0.23 |
| Total number | 4.72$^a$ ± 0.26 | 4.05$^a$ ± 0.26 | 4.58$^a$ ± 0.33 | 4.08$^a$ ± 0.33 | 4.50$^a$ ± 0.33 |

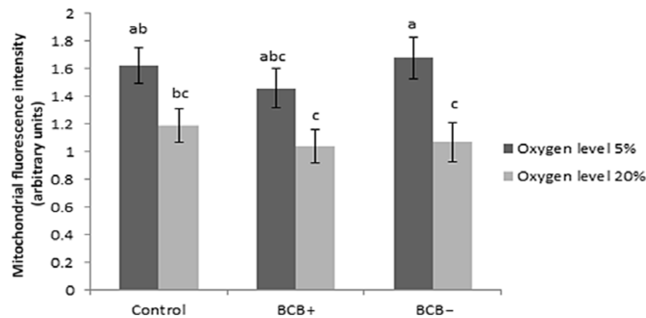


**Figure 8.** Fluorescence intensity of intracellular mitochondrial activity in mature buffalo oocytes cultured under low (5%) or high oxygen tensions (20%) during *in vitro* maturation. Bars with different superscripts (a–c) indicate significant difference at $P \leq 0.05$.

enhancement of maturation and blastocyst rates at low oxygen levels (5%). *In vitro* culture of preimplantation stages at low oxygen tension has improved the rate of nuclear maturation, embryo quality, and development to the blastocyst stage in different species (Farrell and Foote, 1995; Hashimoto *et al.*, 2000; Van Soom *et al.*, 2002; Ciray *et al.*, 2009).

In the present study, BCB− oocytes recorded the lowest maturation rate during *in vitro* maturation under high oxygen tension. It has been established that BCB− oocytes had lower developmental competence than BCB+ oocytes in buffalo (Manjunatha *et al.*, 2007), bovine (Alm *et al.*, 2005), camel (Fathi *et al.*, 2017), equine (Mohammadi-Sangcheshmeh *et al.*, 2011), sheep (Catalá *et al.*, 2011), goat (Rodríguez-González *et al.*, 2003), and mice (Wu *et al.*, 2007). The reduction of nuclear maturation rate of BCB− oocytes under high oxygen tension may be due to chromosomal defects that have occurred under oxidative stress (Tarin *et al.*, 1996; Choi *et al.*, 2007; Tamura *et al.*, 2008), but this needs to be confirmed. The higher rates of nuclear maturation and cortical granule migration in BCB+ oocyte support the idea for increased nuclear and cytoplasmic maturity of this group of oocytes. Conversely, delayed migration of cortical granules in BCB− could be due to cytoskeleton disorganization (Silva *et al.*, 2013; Mao *et al.*, 2014) that resulted in unsynchronized events of nuclear, as well as cytoplasmic, maturation in buffalo BCB− oocytes under high oxygen tension. The low maturity of BCB− was also linked to the reduced expression of *MAPK14* under high oxygen tension. In support of our results, MAPK activity was reduced in porcine oocytes cultured under thermal stress and this subsequently led to decreased viability (Yen *et al.*, 2014). The MAPK family has a crucial role in the meiotic progression of oocytes from different mammalian species (Fan and Sun, 2004; Villa-Diaz and Miyano, 2004; Baatout *et al.*, 2007; Baluch and Capco, 2008; Torner *et al.*, 2008; Uzbekova *et al.*, 2009), as indicated by its involvement in correct chromosome segregation and meiotic spindle stabilization (Baluch and Capco, 2008).

Surprisingly, downregulation of *MAPK14* was coupled with increased ROS levels in BCB− oocytes under high oxygen tension. Interestingly, ROS accumulation could be a key factor that causes low maturation rate in this group of oocytes. This result is supported by early studies that reported an increased incidence of chromosomal errors and disorganization of meiotic spindles in oocytes that had a high level of ROS (Tarin *et al.*, 1996; Choi *et al.*, 2007; Tamura *et al.*, 2008). However, studies on *MAPK14* have reported its upregulation under increased levels of ROS, which represents an essential trigger for oocyte maturation (Shkolnik *et al.*, 2011). These results supported our hypothesis that events controlling maturation (cortical granules migration, nuclear maturation, and genes regulating these events) are dysregulated in buffalo BCB− oocytes under high oxygen tension.

Nuclear erythroid 2-related factor 2 (NFE2L2, also know as NRF2) is an important transcriptional factor that regulates antioxidant defence mechanism, while its downregulation decreased mitochondrial activity and finally compromised cellular proliferation (Khadrawy *et al.*, 2019). Our results indicated the upregulation of *NFE2L2* in groups of oocytes cultured under high oxygen tension coupled with reduced mitochondrial activity, which reflected the enhanced molecular response of oocytes to increased oxygen level. The expression of *NFE2L2* and level of ROS were elevated in bovine granulosa cells cultured under heat shock (Alemu *et al.*, 2018). Increased expression of *NFE2L2* could be an attempt by the oocyte to protect itself from any possible deleterious effects of high levels of ROS on meiotic progression. This idea was confirmed in a previous study that demonstrated the reduction of meiotic defects in aged oocytes after overexpression of *NFE2L2* (Ma *et al.*, 2018). Interestingly, the expression of *NFE2L2* was higher in BCB+ than BCB− oocytes under high oxygen tension. This supported the idea that BCB+ oocytes were more competent than BCB− at the molecular level and that this ensured meiotic progression and subsequent embryonic development (Torner *et al.*, 2008). Moreover, this added a new explanation for the increased developmental competence of BCB+ by upregulating antioxidant defence genes in response to any change in their microenvironment.

The redox balance of mammalian cells is regulated by several antioxidant enzymes such as peroxiredoxin (PRDX), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) (Cetica *et al.*, 2001; Rizzo *et al.*, 2012). Surprisingly, the transcript abundance of *SOD2*, which is considered to be the downstream gene of *NFE2L2*, did not show clear changes under high oxygen tension. However, previous studies have reported that embryos developed under low $O_2$ levels had higher expression profiles for *MnSOD* and *PRDX5* compared with their counterparts developed under high $O_2$ levels to neutralize excess ROS generated as a by-product of preimplantation metabolic activity (Balasubramanian *et al.*, 2007; Ma *et al.*, 2017). One factor behind the observed molecular alteration in buffalo oocytes could be the species effect on gene expression profiles,

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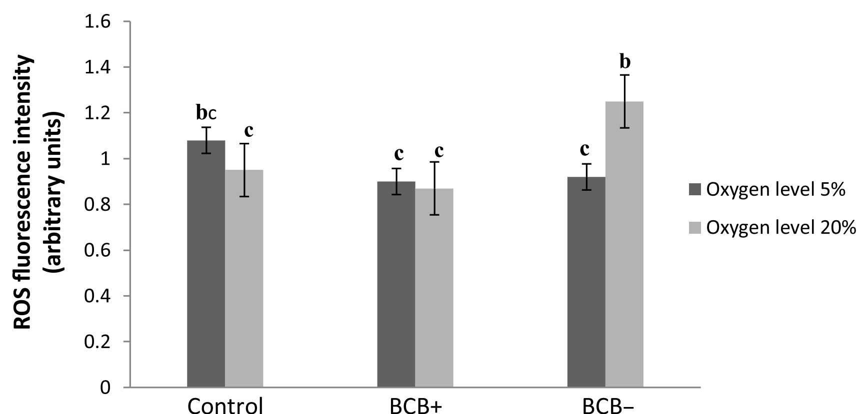


**Figure 9.** Fluorescence intensity of reactive oxygen species (ROS) of matured buffalo oocytes cultured under low (5%) or high oxygen tensions (20%) during *in vitro* maturation. Bars with different superscripts (b, c) indicate significant difference at $P \leq 0.05$.

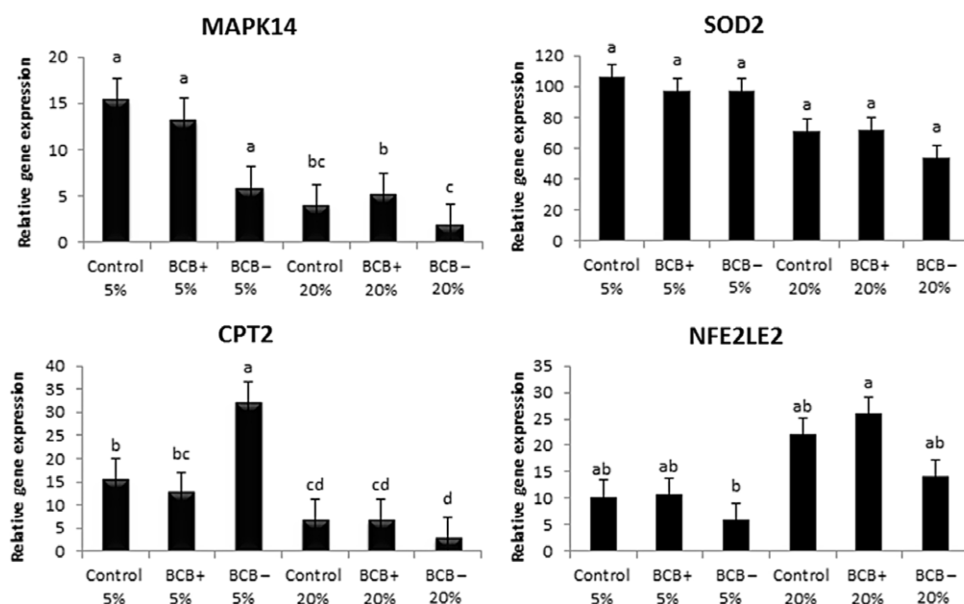


**Figure 10.** Gene expression profiles of MAPK14, SOD2, CPT2 and NFE2L2 in matured buffalo oocytes cultured under low (5%) or high oxygen tensions (20%) during *in vitro* maturation. Bars with different superscripts (a–d) indicate significant difference at $P \leq 0.05$.

as indicated in previous studies (Combelles *et al.*, 2009; Kandil *et al.*, 2010; Ghanem and El-Sayed, 2012). For example, the transcripts of the cell cycle namely *MPHOSPH9* (Kandil *et al.*, 2010) and calcium ion binding such as *ANXA2* (Ghanem and El-Sayed, 2012) were expressed in buffalo oocytes on an opposite pattern compared with that of their bovine counterparts during *in vitro* maturation.

Enhanced nuclear maturation of buffalo BCB+ was coupled with an increased rate of centrally located mitochondria and this was in accordance with data reported in bovine (Silva *et al.*, 2013). Recently, porcine BCB+ oocytes recorded a higher rate of diffuse distribution of mitochondria compared with BCB– oocytes after IVM as an indicator of enhanced cytoplasmic maturation (Fu *et al.*, 2015). The change in mitochondrial distribution linked with enhanced nuclear maturation of BCB+ oocytes could either reflect normal cytoplasmic changes that orchestrate progression to maturation or the enhanced metabolic activity required for the maturation process. However, additional investigations are needed to confirm this idea. Our results indicated the enhancement of mitochondrial fluorescence intensity in groups of oocytes cultured under low oxygen tension compared with their counterparts that were cultured under high oxygen tension, however there was no difference between BCB+ and BCB– oocytes. Mitochondria are maternally inherited and represent the primary source of ATP in the oocyte (Van Blerkom, 2011). Moreover, mitochondrial activity reflects the process of energy production during preimplantation and is relevant to IVP success (Krisher *et al.*, 2007; Van Blerkom, 2011; Ma *et al.*, 2017; García-Martínez *et al.*, 2018; Belli *et al.*, 2019).

In parallel, the number of mitochondria and lipid levels tend to increase in oocytes cultured under low, compared with high, oxygen tension, which indicated the ability of these oocytes to maintain the sufficient energy production required for nuclear progression (Van Blerkom, 2011; Ma *et al.*, 2017; Belli *et al.*, 2019). Recently, it has been demonstrated that mice embryos when developed under 20% $O_2$ had fewer mitochondria and abnormal mitochondria shapes compared with those under 5% $O_2$ (Ma *et al.*, 2017; Belli *et al.*, 2019). However, the central distribution and fluorescence intensity of oocyte lipid droplets were, to some extent, higher in BCB– oocytes under low or high oxygen tension compared with the other experimental groups. In another study conducted in bovine, lipid droplets were more localized in the inner cytoplasm than the periphery of the oocytes without changing lipid droplet density after treatment with L-carnitine (Chankitisakul *et al.*, 2013). The ability of BCB– oocytes to modulate intracellular localization and activity of mitochondria, as well as lipid, could be associated with their low developmental competence. In support of this idea, our data demonstrated

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increased transcript abundance of the *CPT2* gene in all groups of oocytes cultured under low oxygen tension, and the highest profile was observed in the BCB− group linked with increased mitochondrial activity. The increased activity of mitochondrial and expression of mitochondrial replication factor during IVM of BCB− oocytes were reported by Torner *et al*., (2008). While, Spikings *et al*. (2007) have demonstrated lower copies of mtDNA and a delay in its replication in BCB− because of delayed onset of expression of their nuclear-encoded replication factors during IVM. The induction of meiotic progression was enhanced with the increased mitochondrial metabolic activity of FAs through β-oxidation (Downs *et al*., 2009). Therefore, it seems that low oxygen tension provides a favourable condition for oocytes to increase β-oxidation and subsequently increased mitochondrial activity by upregulation of *CPT2*. However, high oxygen tension increased the level of ROS that downregulated genes orchestrating mitochondrial activity and finally compromised nuclear maturation in BCB− oocytes.

In conclusion, the results of the current investigation have demonstrated an increase in the maturation rate of buffalo oocytes cultured under low oxygen tension. Conversely, nuclear maturation (progression to meiotic stages) of buffalo BCB− oocytes was reduced and this was coupled with cytoplasmic (cortical granules migration, distribution of mitochondria and lipid) and molecular alterations (reduced expression of *MAPK14* and *CPT2* genes) under high oxygen tension.

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**Ethical standards.** This study did not require any ethical approval.

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