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Developmental Competence of Buffalo Oocytes Cultured Under Different Oxygen Tensions after Selection with Brilliant Cresyl Blue

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ABSTRACT

The aim of this investigation was to follow up *in vitro* preimplantation development of buffalo cumulus-oocyte complexes (COCs) after BCB test and followed by *in vitro* maturation under two different levels of oxygen tension. Cumulus-oocyte complexes (n=1045) were selected with BCB staining (oocytes with any degree of blue color in cytoplasm was defined as BCB+, oocytes without any degree of blue color in cytoplasm was defined as BCB-) in addition to a third control group. The previous experimental groups (BCB+, BCB-, control) were matured *in vitro* under low (5%) and high oxygen tension (20%), followed by *in vitro* fertilization and *in vitro* culture of presumptive zygotes. There were no differences ($P \leq 0.05$) in cleavage, morula and transferable embryos rates among BCB+, BCB- and control group. However, blastocyst rate was greater significantly in control group (14.4 ± 2.0) than BCB- COCs (8.4 ± 1.9). According to the oxygen tension effect, the rate of morula and transferable embryos was increased ($P \leq 0.05$) in buffalo COCs developed under low oxygen tension (11.6 ± 1.4 and 23.8 ± 1.9) compared to high oxygen tension group (7.4 ± 1.4 and 17.9 ± 2.1). In addition, cleavage, morula, blastocyst and transferable embryos rates were greater in BCB+ under low (43.6 ± 3.9, 14.9 ± 2.5, 14.1 ± 2.9 and 28.4 ± 3.6) than high oxygen tension group (33.5 ± 3.9, 7.1 ± 2.5, 11.6 ± 2.9 and 18.8 ± 3.6) which may reflect enhanced biological processes controlling early development. Moreover, blastocyst rate was significantly higher in control group cultured under high (12.0 ± 2.9) and low (16.9 ± 2.8) oxygen level than their counterparts of BCB- group (9.3 ± 2.9 and 7.6 ± 2.6, respectively). In conclusion, there was no differences in embryo development between BCB+ and BCB-, COCs; therefore, oocyte selection based on BCB staining is not an effective tool to select developmental competent buffalo COCs. Buffalo morula and transferable embryos prefer low oxygen tension for early development, which should be applied during *in vitro* embryo production of this species.

Keywords: Brilliant cresyl-blue staining, Cumulus-oocyte complex, Morula, Preimplantation.

INTRODUCTION

Egyptian buffaloes represent an important livestock resource for producing meat and milk production in agriculture national economy. Reproductive efficiency is a key factor affecting female buffalo productivity; however, buffaloes are characterized by late of puberty and sexual maturity, long postpartum, poor oestrous signs, low pregnancy rate and long calving intervals (Barile, 2005). Noteworthy, several Assisted Reproductive Technologies (ART's) are becoming important tools that applied for accelerating the genetic gain of milk production and enhancing efficacy of dairy animal's reproductive performance (Hafez, 2015).

In this regard, *in vitro* production of buffalo embryos (IVEP) technique has improved during the last decades. However, the quality and rate of transferable embryos produced still low than that required for breeding programs (Gasparrini, 2007; Goszczynsk et al., 2018). It is well established that the development of embryos depends on various factors like culture conditions (*in vivo* versus *in vitro* conditions), constituents of culture media, pH and oxygen tension (Simopoulou et al., 2018).

Suboptimal conditions for *in vitro* culture eventually reduced the development rate and quality of blastocysts because of reactive oxygen species (ROS) accumulated during pre-implantation development due to insufficient oxygen level (Bavister, 1995; Karagenc et al., 2004; Oyamada and Fukui, 2004; Torres-Osorio et al., 2019). The primary gas phases used in IVF laboratories are 5% carbon dioxide (CO_2) in air (20% O_2), and 90% Nitrogen (N_2). The oxygen level of most mammalian species in the oviduct and uterus ranged from 2 to 8 % (Fischer et al., 1992). There is a balanced system in any living cell, which keeps the oxygen species generation and scavenging in balance (He et al., 2017). An imbalance between the Reactive Oxygen Species (ROS) produced and the intracellular antioxidants causes oxidative stress (Fiers et al., 1999).

Various types of ROS cause oxidative stress, which either produced intracellularly or derived from extracellular environment. Indeed, the main sources of ROS are free radicals such as superoxide anion, hydrogen peroxide, hydroxyl

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radical, hydroxyl ion, and nitric oxide produced during oxygen reduction reactions (Balaban et al., 2005; Agarwal et al., 2006). The effects of ROS in mammalian reproductive tract could be negative or positive depends on the duration of exposure and level of ROS (Agarwal et al., 2005). Indeed, an adequate amount of ROS is required for ovarian steroid biosynthesis and induction of ovulation events.

Physiological levels of ROS are required during *in vitro* maturation (IVM) to reinitiate meiosis of the oocytes (Shkolnik et al., 2011). However, an elevated level of ROS during *in vitro* maturation can cause chromosomal errors and finally reduced developmental competence of the oocyte (Sasaki et al., 2019). Increased ROS generation has been shown to cause alterations in the organization of microtubules and chromosomal alignment of metaphase II meiotic spindles in mouse oocytes (Cheung et al., 2016; Kala et al., 2016). Moreover, minimal levels of oxidative stress during embryonic growth may be beneficial for early development (Combelles et al., 2009; Shkolnik et al., 2011). In contrast, embryos exposed to high levels of ROS are morphologically poor quality and at risk of early embryonic development blockage. An increase in ROS production has been found to result in embryonic arrest at two-cell stage of development (Du plessis et al., 2008; Ashraf et al., 2016). Therefore, the current study was designed to assess the developmental competence of buffalo oocyte after *in vitro* maturation under two levels of oxygen tension followed by *in vitro* fertilization and early embryonic development up to the blastocyst stage.

MATERIALS AND METHODS

Experimental design

Good quality COCs of grade A and B were used in the current investigation. In this experiment, good quality cumulus-oocyte complexes (COCs) were distributed in three groups (BCB+,BCB- and control) and according to oxygen tension divided into two subgroups as follows: Control 5% n=160, Control 20% n=174, BCB+5% n=160, BCB+20% n=197, and BCB-5% n=169, BCB-20% n=185).

Collection of ovaries and oocyte retrieval

Buffalo ovaries (n=1045) were collected from local slaughterhouses (El-Monieb and El-Warak) during Winter season, from October 2019 to March 2020. Within 3 hrs after slaughtering, ovaries were transported to the laboratory (Embryology Lab, Research Park, Faculty of Agriculture, Cairo University) in physiological saline (0.9% NaC1 supplemented with 50 µg / ml gentamycin) and maintained at 34-37°C. Ovaries were washed once in 70% ethanol and three times in warm sterile physiological saline. Cumulus-oocyte complexes (COCs) were collected by aspiration of antral follicles using 18-gauge needle attached to a 10-ml syringe. Oocytes were washed twice in washing medium TCM-199-Hepes medium supplemented with 2% fetal bovine serum (FBS), 0.3 mg/ml glutamine and 50 µg/ml gentamycin. The evaluation of recovered COCs was done under a stereo microscope, according to their cumulus layers and ooplasm homogeneity as stated by (Chauhan et al., 1998). Compact COCs that had a mass of ≥ 5 layers of cumulus cells and homogeneous ooplasm (Grade-A) and those had 3-4 layers of cumulus cells and homogeneous ooplasm (Grade-B) were used in the current study.

Brilliant cresyl blue staining test

Cumulus-oocyte complexes were washed three times in Dulbecco's PBS (DPBS) and adjusted by adding 0.4 % BSA (Fraction V), then COCs were exposed to 26 mM of BCB (B-5388, Sigma) and diluted in mDPBS at 38.5 ° C for 90 min in a humidified air atmosphere without CO_2. Directly after washing twice in mDPBS, COCs were examined under a stereomicroscope and according to their cytoplasm coloration were divided into two classes (Figure 1); oocytes with any degree of blue cytoplasm coloration (BCB+) and oocytes with no blue cytoplasm coloration (BCB-), While control group was incubated immediately without BCB exposure.

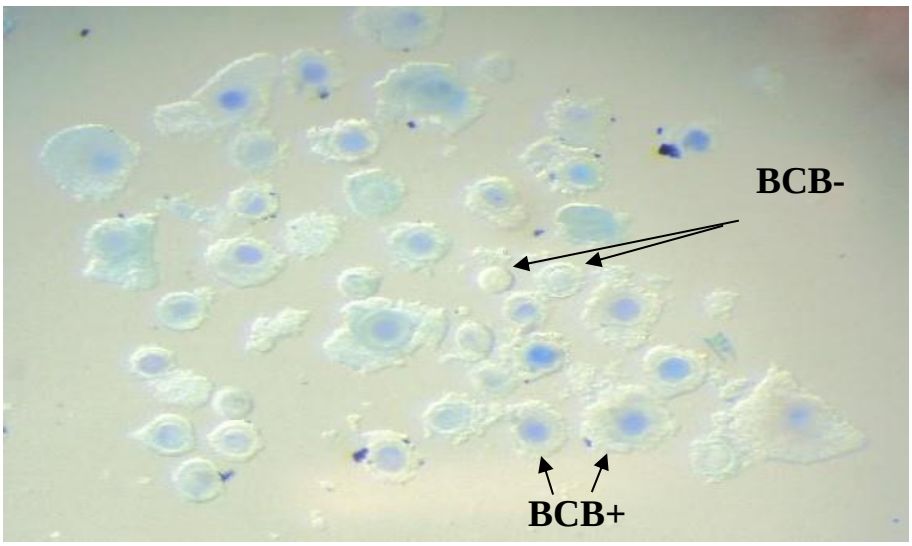

Figure 1. Brilliant cresyl blue stained of Egyptian buffalo oocytes. Blue stained oocytes are designated as brilliant cresyl blue (BCB +) and colorless oocytes are designated as brilliant cresyl blue (BCB-).

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***In vitro* oocytes maturation**

Before commencing the maturation process, the maturation medium was prepared in advance. The medium was held for equilibrium at 38.5° C and 5 % CO_2 and incubated for 2 hrs. All COC groups were washed twice in the ripening medium consisting of TCM-199 combined with 10 % FBS (F7524, Sigma, Germany), 1 µg / ml estradiol-17β (E2758, Sigma, USA), 0.15 mg / ml glutamine, 22 µg / ml, Na-pyruvate (P-4562;Sigma, USA), 5 µg / ml FSH (F2293, Sigma, USA), and 50 µg / ml gentamycin. A total of 10-15 COCs were seeded from each category (Control, BCB+, BCB-) in drops of 100 µl of maturation medium under mineral oil (M8410, Sigma, USA) in a 3.5 cm Petri dish. The cumulus-oocyte complexes were incubated at 38.5° C for 24 hrs in a humidified atmosphere with 5 % CO_2 under 5% oxygen level or 20% oxygen level.

***In vitro* fertilization**

Frozen straw of Egyptian buffalo bull semen (0.25 ml) was thawed in warmed water at 38°C for 30 seconds and opened after wiped with 70% ethanol alcohol. Spermatozoa were washed twice in the sperm washing medium namely Tyrode Albumin Lactate Pyruvate (TALP) medium (modified Ca2+ free TALP medium) by centrifugation at 1800 rpm for 5min and one time in fertilization medium (modified TALP medium) according to Stinshoff et al. (2011). Spermatozoa pellet was re-suspended in the fertilization medium. The concentration of sperm was adjusted to be 2×106 (Gasparrini, 2002). Matured oocytes (10-15 oocytes per drop) were placed in a small petri dish containing 50 µl droplets of fertilization medium. Aliquots of the sperm suspension were added to each droplet containing mature oocytes. The oocytes and sperm were co-cultured in an incubator at 38.5˚C in 5% CO_2 in air, with maximal humidity for 18 hrs.

***In vitro* culture**

The received zygotes were washed four times after 18 hrs of fertilization by repeated pipetting to denude cumulus cells. The presumptive zygotes of all groups (control, BCB+ and BCB-) were placed into 50 µl droplets of *in vitro* culture medium in 4 well petri dish and covered with paraffin oil. The culture medium composed of TCM-199 supplemented with 3 mg/ml BSA, 22 µg/ml Na-pyruvate, 10 µl/ml NEAA (100 X), 20 µl/ml EAA (50 X) and 50 µg/ml gentamycin sulphate. During the culture period, presumptive zygotes were kept at 38.5˚C in a humidified atmosphere of 5% CO_2 in air. Half of the medium was changed every 48 hrs, the cleavage rate was checked after 48 hrs post insemination (Figure 2) and blastocyst rate was checked on day 7 (Figure 3).

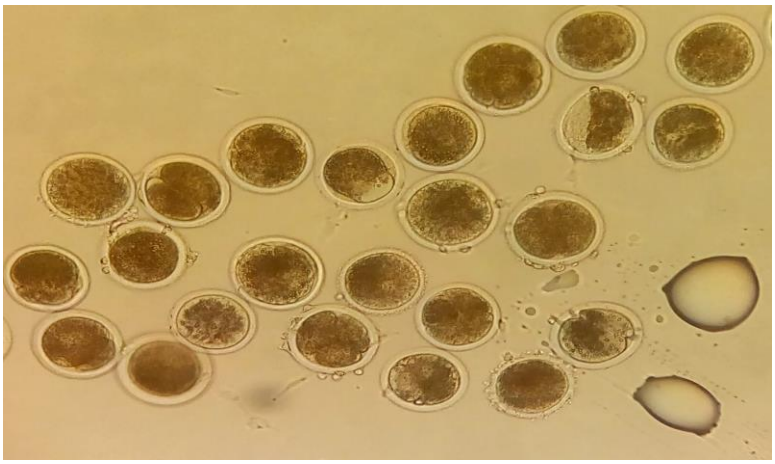

Figure 2. Embryos at 2-Cell, 4-Cell, 8-Cell and degenerated embryos after 48 hours of culture under oxidative stress (20%O_2) in Egyptian buffalo oocytes

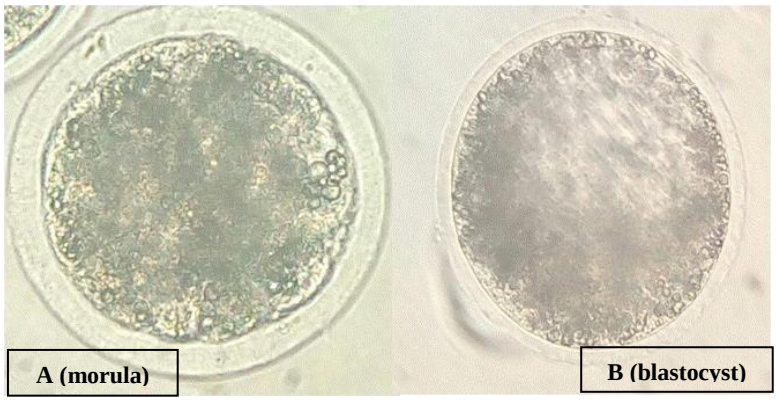

Figure 3. Embryos at morula and blastocyst stages under 5% O_2 level in Egyptian buffalo oocytes

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Statistical analysis

Statistical analysis of current data was done using the SPSS program package (SPSS Inc., Chicago, 183 Illinois, USA). The effect of oxygen level (5% versus 20%) and oocyte type (BCB+ versus BCB- versus control) on the previous traits were tested by applying the following model:

$Yijk1=\mu+Ri+Aj+(Ri*Aj)ijk+eijk1$

Where:

Y ijk1= individual observation; μ= Overall mean; Ri= Effect of BCB staining (BCB+ =1 and BCB-=2); Aj= Effect of oxygen level (5%=1 and 20%=2); (Ri*Aj) ijk = The interaction between BCB and oxygen level; e ijk1= Experimental error supposed to be randomly distributed $(0, \sigma 2)$. The data were expressed as mean ± standard error. Probability values up to ≤ 5% was considered significant.

Ethical approval

The present work has been conducted in accordance with the guidelines of the ethical committee of Faculty of Agriculture, Cairo University. The experimental lab work was executed at Cairo University Research Park, Faculty of Agriculture, Cairo University, Egypt.

RESULTS AND DISCUSSION

Although the efficiency of *In vitro* embryo production has improved in Buffalo, the rate of transferable embryos (TE) and the rate of development to term is still very low (Gasparrini, 2002). The outcome and quality of *in vitro* produced embryos are generally still lower than *in vivo* counterparts in other mammalian species (Marsico et al., 2019). Different factors play a key role in assessing the quality of *in vitro* production (IVP). The competence of COCs to reach transferable embryos stages is considered one of the main factors influencing the yield of the pre-implantation embryos (Reader et al., 2017), while the conditions of culture affect the quality of the blastocysts (Rizos et al., 2002). In the current study, we investigated the interaction of oxygen tension levels with the quality of buffalo oocytes (using BCB staining model) after *in vitro* maturation, fertilization and early embryonic development up to the blastocyst stage.

Embryonic development of buffalo oocytes under two levels of oxygen tension

The results indicated that there were no significant differences between experimental groups (BCB+, BCB- , and control) neither in cleavage rate nor in the rates of morula and transferable embryos developed under the two oxygen levels. However, the cleavage, morula, blastocyst and transferable embryos rates were greater in BCB+ under low than high oxygen level (Table 1). Moreover, blastocyst rate was significantly increased in the control group compared to BCB- oocyte group when cultured under low and high oxygen tension (Table 1 and figure 4). The improvement of development rates of preimplantation embryos of BCB+ group under low compared to high oxygen tension may reflect enhanced biological processes controlling early development. Indeed, embryo development rate was enhanced when the intracellular mitochondrial activity was increased under low level of oxygen (Ma et al., 2017; Belli et al., 2019). Noteworthy, High oxygen tension could cause oxidative stress which directly compromises early embryonic development by disturbing mitochondria (Ma et al., 2017).

The increased blastocyst rate of control group compared to BCB- is due to lower total number of embryo cells and mitochondria content of BCB- group (Fakruzzaman et al., 2013). Selection of COCs based on BCB staining during IVP of bovine indicated improvement of blastocyst development rate, quality, activity of mitochondria and increased expression of pregnancy associated candidate genes in BCB+ compared to BCB- group (Fakruzzaman et al., 2013). This indicates that the increased development rate of BCB+ embryos under low oxygen tension is due to its inherent intracellular potential.

Table 1. Cleavage rate, morula rate, blastocyst rate and transferable embryos rate of buffalo COCs either selected with BCB staining or cultured under low and high oxygen tension

Groups	Oxygen level (5%)				Oxygen level (20%)			
	Cleavage* rate (%)	Morula rate (%)	Blastocyst rate (%)	Transferable embryos ** (%)	Cleavage* rate (%)	Morula rate (%)	Blastocyst rate (%)	Transferable embryos ** (%)
Control	35.5 ± 3.7 ^{a***}	7.7 ± 2.3 ^{a}	16.9 ± 2.8^{a}	24.3 ± 3.4 ^{a}	31.6 ± 3.9 ^{a}	6.1 ± 2.5 ^{a}	12.0 ± 2.9^{a}	17.9 ± 3.6 ^{a}
BCB+	43.6 ± 3.9 ^{a}	14.9 ± 2.5 ^{a}	14.1 ± 2.9^{ab}	28.4 ± 3.6 ^{a}	33.5 ± 3.9 ^{a}	7.1 ± 2.5 ^{a}	11.6 ± 2.9^{ab}	18.8 ± 3.6 ^{a}
BCB-	30.3 ± 3.6 ^{a}	12.1 ± 2.2 ^{a}	7.6 ± 2.6^{b}	18.9 ± 3.2 ^{a}	34.5 ± 3.9 ^{a}	9.0 ± 2.5 ^{a}	9.3 ± 2.9^{b}	17.1 ± 3.6 ^{a}

Values with different superscripts across treatments within the same column indicate significant differences (P ≤ 0.05). *Cleavage rate= Numbers of oocytes cleaved/Numbers of oocytes inseminated; **Transferable embryos (Day 7): numbers of morula and blastocyst embryos; ***Least Square Means± Standard Error; BCB+: oocytes with any degree of blue color in cytoplasm; BCB-: oocytes without any degree of blue color in cytoplasm

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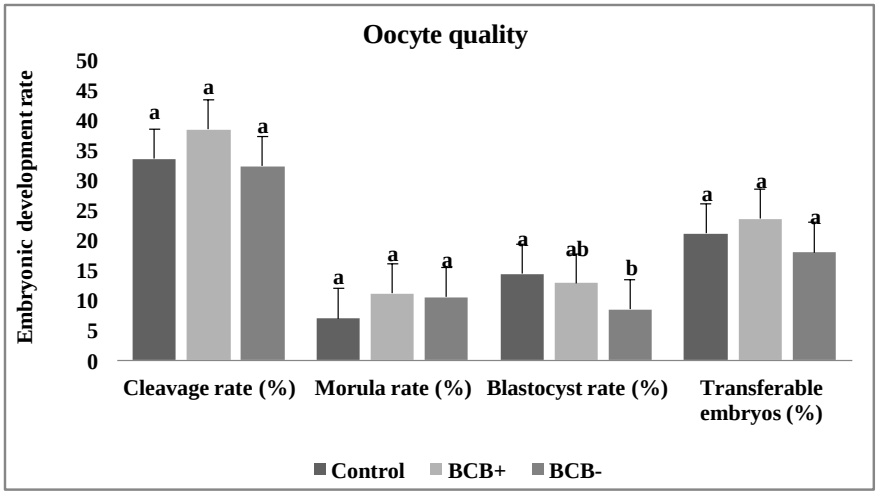

Figure 4. Effect of oocytes brilliant cresyl blue staining on *in vitro* embryonic development of Egyptian buffalo oocytes. Bars with different superscripts (a, b) indicate significant differences (P ≤0.05).

Effect of brilliant cresyl blue staining

The inefficiency of *in vitro* embryo production was associated with oocyte quality at the beginning of maturation (Gasparrini, 2002). Identifying and selecting oocytes with high developmental competence is a critical step towards successful embryo technology (Egerszegi et al., 2010). Many studies used brilliant cresyl blue (BCB) staining tests to monitor and identify oocyte quality for different species such as buffalo and ovine (Manjunatha et al., 2007; Wang et al., 2012).

Previous work has shown that BCB+ oocytes are more competent and yield more blastocysts compared to BCB− oocytes. BCB testing has been shown to enable the selection of larger oocytes with higher levels of *in vitro* maturation and fertilization and an increased percentage of normal fertilized oocytes (Wang et al., 2012). The ability of BCB+ derived oocyte to be fertilized and developed to morula and blastocyst stages was significantly higher than morphologically selected oocytes only (Rodriguez-Gonzalez et al., 2003; Alm et al., 2005; Manjunatha et al., 2007; Silva et al., 2013). Indeed, BCB selects larger oocytes with more mitochondria and activation of the maturation-promoting factor (MPF) (Catalá et al., 2011). On the other side, less competent oocytes (BCB-) displayed fertilization failure or developmental arrest due to delayed mitochondrial DNA (mtDNA) replication attributed to the delayed expression of their nuclear replication factors.

Our results indicated a lower blastocyst rate in BCB- oocytes than control group (Table 2 and figure 4). In agreement with our findings, Wongsrikeao et al. (2006) reported that BCB staining has a negative impact on the cleavage and development of porcine embryos after *in vitro* maturation. Opiela et al. (2008) consider the BCB staining test as questionable because of the lack of significant differences in blastocyst formation between BCB+ and control group. Pawlak et al. (2014) stated *that* BCB staining test was less successful selection method with high apoptosis incidence and major variations in the diameter of the BCB+ oocyte. In contrast, Manjunatha et al. (2007) stated that before *in vitro* maturation, staining of buffalo oocytes with BCB stain had identified developmentally competent oocytes for IVP. Relative to conventional oocyte selection based solely on morphology, the yield of blastocyst has been significantly improved in BCB+ selected oocytes. Fathi et al. (2017) found that in BCB+ groups, indices of early embryonic development; cleavage, morula and blastocyst stage growth were substantially improved compared to the BCB− and control groups.

Table 2. Effect of oocytes quality on *in vitro* embryonic development of Egyptian buffalo oocytes

Treatments	Cleavage rate* (%)	Morula rate (%)	Blastocyst rate (%)	Transferable embryos** (%)
Control	33.6 ± 2.7 ^{a***}	6.9 ± 1.7 ^{a}	14.4 ± 2.0^{a}	21.1 ± 2.5 ^{a}
BCB+	38.5 ± 2.8 ^{a}	11.1 ± 1.8 ^{a}	12.8 ± 2.1^{ab}	23.6 ± 2.5 ^{a}
BCB-	32.4 ± 2.7 ^{a}	10.5 ± 1.7 ^{a}	8.4 ± 1.9 ^{b}	18.0 ± 2.4 ^{a}

Values with different superscripts (a, b) across treatments within one column indicate significant differences (P ≤0.05). *Cleavage rate= Numbers of oocytes cleaved/Numbers of oocytes inseminated; **Transferable embryos (Day 7): numbers of morula and blastocyst embryos; ***Least Square Means± Standard Error; BCB+: oocytes with any degree of blue color in cytoplasm; BCB-: oocytes without any degree of blue color in cytoplasm

Oxygen concentration

Karagenc et al. (2004) found that high oxygen concentrations (20% in air) decreased the percentage of embryonic development due to increased level of ROS in rodents, swine (Kitagawa et al., 2004; Booth et al., 2005), goats (Batt et al., 1991) and bovine (Takahashi et al., 2000). According to the oxygen tension effect, the rate of morula and transferable

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embryos was increased (P≤0.05) in buffalo COCs developed under low oxygen tension compared to high oxygen tension group (Table 3 and figure 5).

Table 3. Effect of oxygen levels on *in vitro* embryonic development in Egyptian buffalo oocytes

Oxygen level	Cleavage rate* (%)	Morula rate (%)	Blastocyst rate (%)	Transferable embryos** (%)
5%	36.5 ± 2.2 [a***]	11.6 ± 1.4[a]	12.8 ± 1.6 [a]	23.8 ± 1.9[a]
20%	33.2 ± 2.3 [a]	7.4 ± 1.4 [b]	10.9 ± 1.7 [a]	17.9 ± 2.1[b]

Values with different superscripts (a, b) across treatments within one column indicate significant differences (P ≤0.05). *Cleavage rate= Numbers of oocytes cleaved/Numbers of oocytes inseminated; **Transferable embryos (Day 7): numbers of morula and blastocyst embryos; ***Least Square Means± Standard Error; BCB+: oocytes with any degree of blue color in cytoplasm; BCB-: oocytes without any degree of blue color in cytoplasm

However, there were no significant differences in cleavage and blastocyst rates under two oxygen levels. In terms of blastocyst formation, embryo developmental competence was significantly higher in embryos grown under low concentration of oxygen (5 % O_2 + 5 % CO_2 + 90 %N_2) than those developed in atmospheric oxygen (air + 5 % CO_2) in bovine (Thompson et al., 1990; Liu and Foote, 1995), mouse (Orsi and Leese, 2001) human (Dumoulin et al., 1999) and porcine (Booth et al., 2005). Similarly, for the development of high-quality ovine blastocysts, high oxygen concentration (20 versus 5% O_2) during IVF was found to be unfavorable (Leoni et al., 2007). Amin et al. (2014) found that there was no significant difference in the cleavage rate between groups. On the other hand, cultivating embryos below 5 % oxygen resulted in blastocyst levels significantly higher (P ≤0.05) than those cultivated at 20% oxygen.

The high concentration of O_2 in the culture media can increase certain enzymatic reactions, such as xanthine oxidase and NADPH oxidase activity, leading to increased levels of ROS in embryonic cells (Goto et al., 1993; Lopes et al., 2010). The high level of intracellular ROS increased incidence of DNA damage, lipid peroxidation, and apoptosis, which subsequently reduced embryo development (Takahashi, 2012). The physiological level of oxygen has enhanced embryo development through regulation of development related genes (Rinaudo et al., 2006; Bermejo-Álvarez et al., 2010).

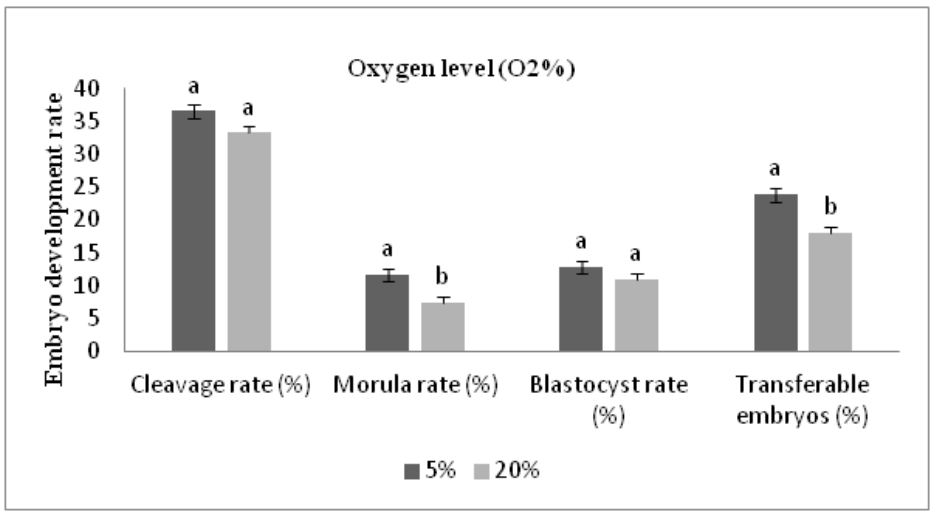

Figure 5. *In vitro* embryonic development of Egyptian buffalo oocytes under two oxygen levels**.** Bars with different superscripts (a, b) indicate significant differences (P ≤0.05).

CONCLUSION

The results of the present investigation have clearly indicated that preimplantation embryo development is similar in BCB+ and BCB- COCs, which discourages the use of this test in the selection of buffalo COCs. Moreover, transferable buffalo embryos (morula and blastocyst) developed at a higher rate under low oxygen tension (5% O_2). Therefore, it is recommended to culture buffalo pre-implantation embryos at 5% of oxygen during *in vitro* development.

DECLARATIONS

Competing interests

The authors have no competing interests, and we are with respect to this search and in agreement with each other. In addition, we have no conflict with authorship or article publication.

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Author`s contribution

All authors have contributed to design of the study, writing and revision of the manuscript. In addition, Dalia Abdel Rahman Ahmed who conducted the laboratory work, analyzed the data wrote the research article. All the authors approved and agreed to publish the manuscript.

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