RESEARCH ARTICLE

Effect of Oxygen Tension on *In Vitro* Maturation of Buffalo Cumulus Oocyte Complexes

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Abstract

This study investigated the effect of oxygen (O_2) tension during *in vitro* maturation (IVM) of buffalo cumulus oocyte complexes (COCs). Buffalo ovaries (n=337) were collected from a slaughterhouse of Ahmedabad Municipal Corporation, Ahmedabad. A total of 801 COCs were retrieved using aspiration method using 18 G 1.5′ needle and 5 mL syringe. The COCs were graded into four groups based on cumulus layers. Two IVM conditions were compared: a benchtop incubator with 5% O_2 and a conventional incubator with ambient O_2 (20%). The COCs of the first three grades were randomly allocated to two groups and the effect on IVM was evaluated using chi square test. The 5% O_2 group exhibited a higher maturation rate (89.00%, 365/410) compared to the 20% O_2 group (73.66%, 288/391), though the difference was not statistically significant (p=0.21). The findings suggest that lower O_2 tension during IVM may enhance COCs maturation, potentially improving embryo development outcomes.

Key words: Benchtop incubator, Buffalo oocytes, Conventional incubator, IVM, O_2 tension.

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Introduction

The Indian subcontinent holds the world's best buffalo germplasm in terms of milk production and resilience to challenging climates. Globally, efforts to genetically improve buffaloes have only recently gained momentum. Buffaloes face challenges with reproductive efficiency, including delayed puberty, weak estrus expression, low embryonic survival rates, extended inter-calving intervals, prolonged postpartum ovarian inactivity, seasonal fertility, and reduced conception rates, especially with artificial insemination (Singh *et al.,* 2009; Suthar and Dhami, 2010). To address these physiological issues, assisted reproductive technologies (ART), such as timed artificial insemination (TAI), super-stimulation, ovum pick-up (OPU), *in vitro* embryo production (IVEP), and embryo transfer (ET) have been introduced to enhance the offspring numbers from genetically superior buffaloes (Kumar *et al.,* 2023). The IVEP, utilizing superior germplasm from both male and female animals simultaneously, is gaining prominence over other reproductive techniques like, multiple ovulation and embryo transfer-MOET (Madan *et al.,* 1996; Suthar and Shah, 2009). The IVEP process consists of three key stages: *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* embryo culture (IVC) (Gasparrini, 2002; Suthar and Shah, 2009).

In many mammalian species, the oxygen concentration within the reproductive tract ranges from 1.5 to 8.7% (Fischer and Bavister, 1993), while Van Blerkom (1998) had reported dissolved oxygen levels in follicular fluid between 1% and 5%. In contrast, the oxygen concentration commonly used in standard *in vitro* maturation (IVM) procedures in clinical and research laboratories, as well as in the livestock industry, is 20-21 %, which corresponds to atmospheric levels unless at high altitudes. Elevated oxygen levels beyond physiological norms increase the risk of ROS formation (Johnson and Nasresfahani, 1994), potentially disrupting the balance between pro-oxidants and antioxidants and resulting in cellular damage (Alonso-Alvarez *et al.,* 2004). Reactive oxygen species (ROS) are oxygen free radicals that can harm biological systems (Prescott and Bottle, 2017). These radicals have been shown to reduce the success rate of *in vitro* maturation and embryo production in cattle, with

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estimates indicating that only 30-40% of oocytes develop into blastocysts. These highly toxic ROS can lead to significant cellular damage, including membrane lipid peroxidation, enzyme inactivation, and DNA damage in various cell types, such as oocytes (Umaoka *et al.,* 1992) and spermatozoa (Alvarez and Storey, 1983).

Despite some studies investigating the effect of oxygen tension during IVM on oocyte developmental competence, the findings have been inconsistent. For instance, in mice, 5% O_2 during IVM has been reported to be either beneficial (Haidri *et al.,* 1971) or harmful (Hu *et al.,* 2001) to nuclear maturation, with some studies indicating it has no effect on subsequent oocyte development (McNatty, 1978). In cattle, IVM at 20% O_2 was found to be more effective for nuclear maturation in a medium containing 5.6 mM glucose (Pinyopummintr and Bavister, 1995). Conversely, another study showed that IVM at 5% O_2 resulted in higher cleavage rates and better development into blastocysts compared to 20% O_2 in a medium with 20 mM glucose (Hashimoto *et al.,* 2000). These conflicting results highlight the need to consider how varying oxygen conditions during IVM may influence outcomes when assessing the effects of low oxygen environments on IVM success. Hence, this study was aimed to see the effect of two levels of oxygen tension on *in vitro* maturation of buffalo cumulus oocyte complexes.

Materials and Methods

The present study was carried out at IVF lab of Gujarat Biotechnology Research Centre (GBRC), Gandhinagar, Gujarat (India). Buffalo ovaries (n=337) were collected from the slaughterhouse facility of Ahmedabad Municipal Corporation (AMC), Ahmedabad, Gujarat and rinsed with normal saline (NS) containing 1% (w/v) antibiotics (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B; Gibco, USA; Vassena *et al.,* 2003). They were then stored in an isothermal container at 34-36°C with NS and transported to the GBRC within 3-4 h post-slaughter.

Aspiration and Grading of Cumulus Oocyte Complexes

Ovaries were processed for COCs aspiration under laminar air flow using sterile 18 G needle and 5 mL syringe with 0.5 mL of OPU media (IMV, France). In all 16 aspiration sessions were conducted. COCs were collected in TCM wash media (BO-IVM: IVF Bioscience) and washed thrice before transferring them in IVM media. The COCs were classified according to the standard classification criteria (Das *et al.,* 1996). Grade I: COCs with five layers and above cumulus mass; Grade II: COCs with 3 to 4 layers of cumulus cells, uniform granulation of ooplasm and no nuclear irregularities of the COCs; Grade III: COCs with 1 or 2 layers of cumulus cells with uniform granulation of ooplasm and fragmented oocyte nucleus; and Grade IV: COCs with no layers of cumulus cells or denuded oocytes or/ and uneven cytoplasm and misshapen with partially absent or vacuolated cytoplasm and nucleus.

Equilibration of *In Vitro* Maturation Media

Twelve h prior to COCs aspiration from the ovaries, the *in vitro* maturation (IVM) media (BO-IVM; IVF Bioscience) was equilibrated in two different gases environment: group-I, in CO_2 incubator (5% CO_2 in air, *i.e.* 20% O_2) and group-II, in Benchtop incubator (Cooper Surgical, USA, with 5% CO_2, 5% O_2, balanced with N_2, and relative humidity >90%). The IVM procedure was conducted in 90 µL microdrops covered with mineral oil overlay. The IVM media was drawn into 1.5 mL microcentrifuge tubes using a sterile syringe and needle, and drops of 90 µL of IVM media were prepared in tissue culture tested 35 mm petri dishes with 4 mL of mineral oil overlay depending on the number of aspirations. The petri dishes were then covered with lids and placed in the two different incubators for media equilibration.

In Vitro Maturation Procedure

The aspirated COCs, along with ovarian tissues, were placed in a 100 mm petri dish. COCs were located using a stereo-zoom microscope. After examining the 100 mm petri dish twice, the COCs were transferred to pre-warmed wash media (90 µL) and washed three times. They were then classified based on their morphology. Then, the COCs were washed in three drops of pre-equilibrated IVM media in a 35 mm petri dish and finally transferred into drops of pre-equilibrated IVM media randomly in group-I, in CO_2 incubator (5% CO_2 in air), and group-II, in Benchtop incubator (Cooper Surgical, USA, with 5% CO_2, 5% O_2, balanced with N_2), set at 38.5°C for 18-22 h (a maximum of 20 COCs in each 90 µL drop) in an IVM petri dish. The IVM study of COCs was conducted across 16 sessions, eight sessions each under 5% (410 COCs) and 20% (391 COCs) O_2 tension.

Maturation was assessed based on the expansion of cumulus cells after 18-22 h of IVM. The oocyte was quickly evaluated for maturation under a stereo-zoom microscope at 5X magnification and recorded for further analysis.

Statistical Analysis

The statistical analysis was performed using SPSS26 (IBM private limited, Bengaluru, India). The maturation rates between the two groups (5% O_2 & 20% O_2) were compared using the chi square test.

Results and Discussion

COCs Maturation Rate

For this study, total 337 ovaries were collected, and 1192 (74.5±5.34/session) follicles were observed for aspiration of COCs. Total 801 (50.06±4.50) COCs were aspirated with recovery rate of 66.87%. The details on maturation rate in 5% and 20% O_2 tension are depicted in Table 1. For the 5% and 20% O_2 tension conditions, total 410 (51.25±7.32/session) and

391 (48.88±5.73) COCs were used and kept for IVM. After IVM under 5% and 20% O_2 tension conditions 365 (45.63±7.09/session) COCs with 88.00±1.46% maturation rate and 288 (36.00±4.48/session) COCs with 73.05±1.60% maturation rate was achieved, respectively. No significant effect was observed between two O_2 tension conditions on maturation rate, however, under 5% O_2 tension condition 3.25±2.56 more COCs per session were matured than the 20% O_2 tension conditions (p=0.21; Fig. 1, 2).

Table 1: Effect of two oxygen tension (5% & 20%) on maturation rate of COCs (per session) aspirated from buffalo ovaries of slaughter origin

Oxygen tension (%)	Total number of COCs kept for IVM	Number of COCs Matured	Maturation rate (%)
5	51.25 ± 7.32 [a]	45.63 ± 7.09 [a]	88.00 ± 1.46 [a]
20	48.88 ± 5.73 [a]	36.00 ± 4.48 [a]	73.05 ± 1.60 [a]

Meas with same superscript within coloumn do not differ significantly (p>0.05).

Our results are in consonance with previous study conducted on buffalo oocytes, where El-Sanea *et al.* (2021) reported significantly higher maturation rates under low oxygen tension (5% O_2; 85.00±1.30 %) compared to high oxygen tension (20% O_2; 72.40±1.00 %), suggesting that low oxygen conditions reduce oxidative stress and better mimic physiological environments. Contrary to the present finding, Pereira *et al.* (2010) found no significant difference in maturation rates between 5% O_2 (72.39%) and 20% O_2 (72.53%) tension during IVM. These differences underscore the complexity of oxygen tension during *in vitro* conditions. The oxygen tension during *in vitro* maturation (IVM), fertilization (IVF), and culture (IVC) significantly influences buffalo oocyte developmental competence (Pereira *et al.,* 2010; El-Sanea *et al.,* 2021).

The current study found that while the 5% O_2 tension resulted in a slightly higher number of matured COCs (3.25 ± 2.56/session), the difference was not statistically significant (p=0.21) from 20% O_2 tension. Thus, it might be concluded that both 5% and 20% oxygen tensions support COCs maturation *in vitro*, however further studies are required including a greater number of COCs and with looking at cleavage, blastocyst and hatching rate.

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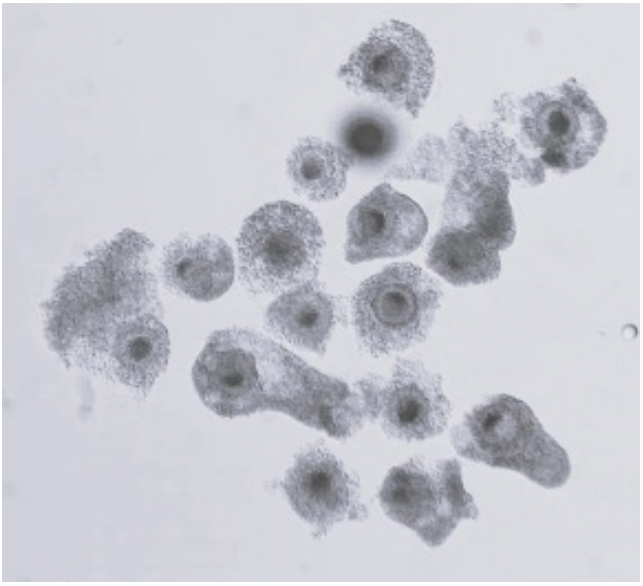

Fig. 1: Immature COCs after aspiration

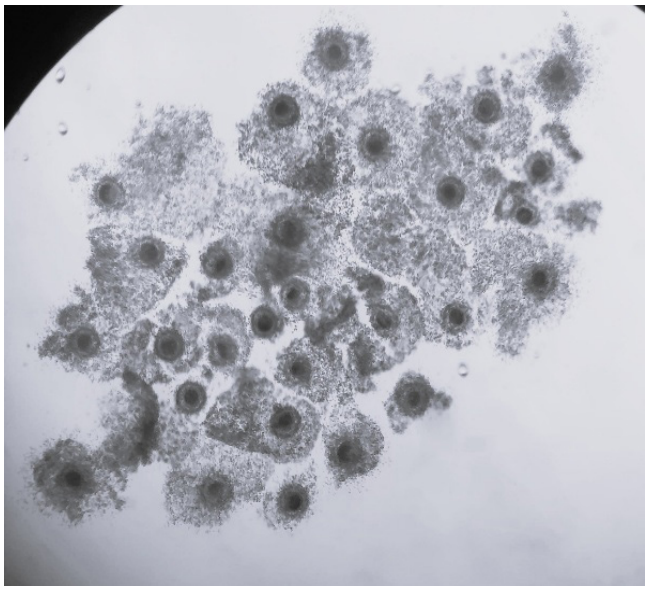

Fig. 2: Mature COCs after 24 h of *in vitro* maturation

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