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COMPARISON OF TWO SYSTEMS OF *IN VITRO* CATTLE EMBRYO PRODUCTION: REDUCED OXYGEN VERSUS ATMOSPHERIC OXYGEN CONTENT

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ABSTRACT

Currently, two culture systems for *in vitro* embryo production (IVP) of mammals are used. Each of these systems has its own pluses and minuses. Culture at atmospheric oxygen level (20 %) requires conventional CO_2 incubator and co-culture with a feeder cells, which involves procedures for maintaining these cells in culture. Culture at reduced oxygen level (5 %) requires special incubating system making IVP procedures more expensive than using conventional technique. The aim of this study was to examine, which of these culture systems can provide higher blastocyst yield after *in vitro* fertilization of fresh or vitrified oocytes. Bovine oocytes derived from the ovaries of slaughtered cows were matured *in vitro*, then part of them were vitrified by an ultra-rapid vitrification technique. After warming, vitrified (n = 482) and fresh oocytes (n = 558; control) were fertilized *in vitro* using frozen bull semen. Following 20 hours, presumptive zygotes, developed from both vitrified and fresh oocytes, were divided into two groups and incubated either under atmospheric oxygen content (AOC; 20 % O_2) in a conventional SANYO CO_2 incubator at 38.5 °C and 5 % CO_2, or under reduced oxygen content (ROC; 5 % O_2) in a MIRI multiroom incubator at 38.5 °C and 6.1 % CO_2 until day 8. Experiments were performed in 5 replicates. Zygotes, obtained from fresh oocytes, reached higher cleavage (76.72 %) and blastocyst (39.34 %) rates, when cultured in ROC compared to AOC (65.6 % and 29.25 %, resp.). Similarly, in vitrified oocytes, cleavage (71.9 %) and blastocyst (22.8 %) rates were significantly higher ($p < 0.05$) in ROC than in AOC (56.2 % and 14.0 %, resp.). Embryo cell number was also significantly higher in blastocysts of both fresh (165.5 ± 8.9) and vitrified (154.54 ± 7.8) groups cultured under ROC, when compared to the AOC level (108.67 ± 3.6 and 98.04 ± 5.82, resp.). In conclusion, the culture system with a reduced oxygen showed to be more efficient for bovine IVP than the system with an atmospheric oxygen in a conventional CO_2 incubator. This finding is important especially when *in vitro* produced embryos are derived from cryopreserved oocytes.

Key words: bovine; oocyte; embryo; blastocyst; oxygen tension; culture

INTRODUCTION

The developmental potential of embryos produced *in vitro* is still less than those recovered *in vivo* (Correa *et al.*, 2008). One of the differences between *in vivo* and *in vitro* environments is the oxygen tension. For bovine *in vitro* embryo production, two culture systems are currently used: culture at atmospheric oxygen content (20 %) or culture at reduced oxygen content (5 %). Embryo culture is most commonly carried out at atmospheric O_2 concentration, which is about 20 %. However, the physiological oxygen content in the oviduct and uterus of most mammals is lower (Fischer and Bavister, 1993) and fluctuating in the range of 2 – 6 % in the air (Ottosen *et al.*, 2006). Greater O_2 tension during culture is detrimental for embryo development, probably due to the

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accumulation of reactive oxygen species (ROS), whose levels in embryos developed under high O_2 tension were greater than those embryos developed under low O_2 (Belli *et al.*, 2020).

First *in vitro* culture under low oxygen content has been performed on human embryos in the early 1970s (Edwards *et al.*, 1970). Later, the effects of different oxygen concentrations, combined with other interventions, were examined on animal embryo cultures (mouse: Brinster and Troike, 1979; sheep: Betterbred and Wright, 1985; Thompson *et al.*, 1990; cattle: Nakao and Nakatsuji, 1990; Thompson *et al.*, 1990; goat: Batt *et al.*, 1991) but with conflicting results. Though, further studies have suggested that high O_2 concentration during *in vitro* culture can increase oxidative stress and lead to the developmental blockage of embryos (Goto *et al.*, 1993; Johnson and Nasr-Esfahani, 1994). However, the mechanism, by which reduced oxygen tension could improve embryo development *in vitro*, is still unclear. More recent studies examining effect of ultra-low oxygen level (2 %) on human embryo culture suggested that the best oxygen level may depend on the embryo stage (Morin, 2017; Crawford and Ledger, 2019). Wale and Gardner (2010) in their study on mice embryos demonstrated that oxidative stress might have an adverse impact on embryos only at cleavage stages, while further culture of embryos from Days 2 – 3 under 20 % of oxygen might not influence embryo development thereafter. They concluded that oxidative stress might be detrimental exclusively before embryonic genome activation.

These findings suggest that embryo culture using tri-gas incubators (5 % O_2, 6 % CO_2 and 89 % N_2) should be preferred. The latest guidelines published by the European Society of Human Reproduction and Embryology (ESHRE) recommended low O_2 tension for embryo culture (ESHRE, 2016). However, tri-gas culture systems require special incubating devices that allow the use of nitrogen gas to purge oxygen out of the incubator, thereby increasing the costs of embryo culture pro-cedures and making it more expensive than standard incubation conditions (Agarwal *et al.*, 2022). Some laboratories around the world still perform embryo culture under atmospheric O_2 content around 20 % (Herbemont *et al.*, 2021). Moreover, embryo culture under hypoxia (5 %) is not standardized yet, as human embryos are cultured under atmospheric O_2 tension in up to 25 % of laboratories performing assisted reproductive technology (ART) procedures (Gardner and Kelley, 2017).

The aim of this study was to examine, which system can provide higher blastocyst yield after IVF of fresh or vitrified oocytes: embryo culture at atmospheric oxygen content (AOC; 20 % O_2) or culture at reduced oxygen content (ROC; 5 % O_2).

MATERIAL AND METHODS

Oocyte collection and *in vitro* maturation

The oocytes were recovered from antral follicles (2 – 8 mm) of bovine ovaries collected from undefined cows at a local slaughterhouse. Using visual inspection under a stereomicroscope, only the oocytes with several layers of cumulus cells and homogeneous ooplasm were selected for *in vitro* maturation (IVM). The oocytes (n = 1040) were matured *in vitro* using incubation in an *in vitro* maturation medium containing M 199 (Gibco), sodium pyruvate (0.25 mmol/L, Sigma), gentamycin (50 mg/mL, Sigma), 10 % foetal bovine serum (FBS, Gibco) and FSH/LH (1/1 IU, Pluset, Minitube) at 38.5 °C and 5 % CO_2. The length of maturation lasted 21 hour s in the case of oocytes intended for vitrification (n = 482) and 24 hours in the case of fresh control oocytes (n = 558).

Oocyte vitrification and warming

Mature oocytes were vitrified using ultra-rapid cooling technique in minimum volume on the electron microscopy grids (EMGs), as described previously (Olexikova *et al.*, 2020). Briefly, the oocytes were incubated for 12 min in the equilibration medium (M199, 3 % ethylene glycol, 10 % FBS, 50 µg/mL gentamicin). Then, the oocytes were washed in the vitrification medium (M199, 10 % FBS, 30 % ethylene glycol, 1 M sucrose (Sigma)) for 25 sec, placed onto EMGs and immediately plunged into a container with liquid nitrogen for storage for at least one week. Afterwards, oocytes were thawed in a warming solution (M199, 10 % FBS, 0.5 M sucrose, 50 µg/mL gentamicin) for 1 min at 37 °C, washed in a set of media with decreasing sucrose concentrations (0.25, 0.125 and 0.0625 M) and finally washed in a medium without cryoprotectant (M199, 10 % of FBS, 50 µg/ml of gentamicin).

Oocyte IVF and embryo culture

Warmed oocytes were evaluated for their survival using light microscopy inspection based on the integrity of the ooplasm and the *zona pellucida*. Selected oocytes were incubated 3 hours for post-warming recovery in

the IVM medium. Fresh IVM oocytes (n = 558) served as a control. Afterwards, oocytes were fertilized *in vitro* (IVF) using frozen-thawed bull semen as described in Olexikova *et al.* (2020). Following IVF (20 hours), presumptive zygotes were divided into two groups based on the culture system to be used.

In a culture system under an atmospheric oxygen content (AOC), presumptive zygotes were placed on a monolayer of BRL-1 cells (Buffalo Rat Liver, ECACC), which were prepared just before the IVF step, and cultured in a B2 Menezo medium in a conventional CO_2 incubator – Sanyo, MCO 15AC (Figure 1) at 38.5 °C, 5 % CO_2 and 20 % O_2 (atmospheric oxygen tension).

In a culture system under a reduced oxygen content (ROC), presumptive zygotes were placed to an *in vitro* culture medium (IVF bioscience) and cultured in a MIRI multiroom incubator (ESCO Medical; Figure 2) at 38.5 °C, 6.1 % CO_2 and 5 % O_2 (reduced oxygen tension).

The embryo cleavage rate was determined on the day 2 of *in vitro* culture. Development to the blastocyst stage was monitored on the day 8 of *in vitro* culture.

Figure 1. CO_2 incubator – Sanyo, MCO 15AC

Figure 2. MIRI multiroom incubator (ESCO Medical)

Cell number evaluation

Blastocyst total cell number was evaluated by labelling the blastocyst nuclei using a Vectashield anti-fade medium containing DAPI fluorochrome (Vector laboratories). Blastocysts were placed onto coverslips, covered with a 2 µl of Vectashield with DAPI, attached to microslides to make a sandwich and analysed under a Leica fluorescence microscope.

Statistical analysis

The differences in the mean value of tested groups versus control group were evaluated by t-test. Embryo culture experiments were performed in 6 replicates. Development (cleavage and blastocyst rates) and total cell numbers were analysed by Pearson's Chi-square test. Values were considered as statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The embryos cultured in both groups showed different quality, which was evident even based on visual evaluation under a light stereomicroscope. In particular, the embryos cultured in AOC system showed darker appearance (Figure 3A), when compared with clearer and more translucent appearance in the embryos developed under ROC conditions (Figure 3B). Moreover, the embryos cultured in a reduced oxygen system were visually larger in size than those cultured under atmospheric oxygen level.

Zygotes, obtained from fresh oocytes (Table 1), reached higher cleavage and blastocyst rates, when cultured in presence of reduced oxygen (ROC), compared to those cultured under atmospheric oxygen tension (AOC). In the zygotes derived from vitrified oocytes the similar

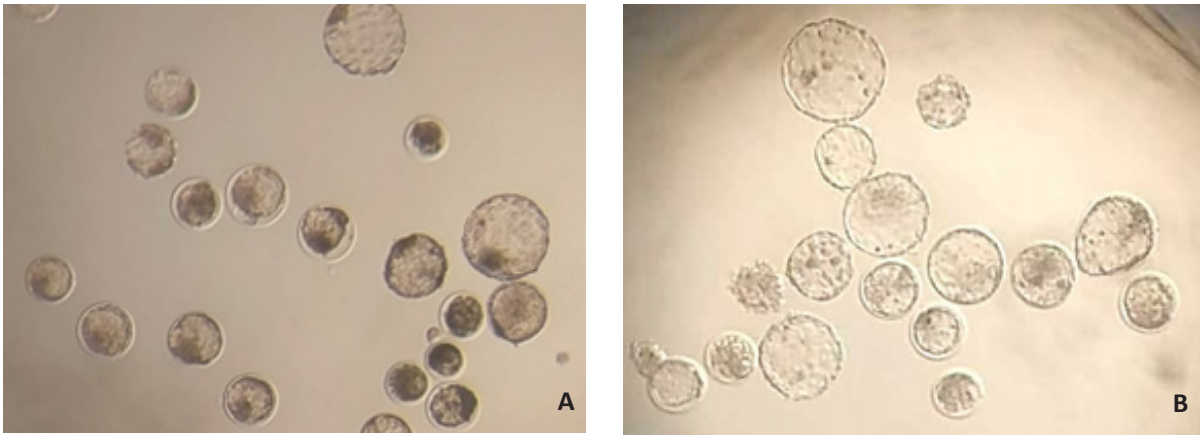

Figure 3. A – bovine embryos cultured under atmospheric oxygen content (AOC); B – bovine embryos cultured under reduced oxygen content (ROC)

Table 1. The effect of the culture system on the development of embryos derived from fresh oocytes

Groups	IVF oocytes, n	Cleavage rate, n (%)	Blastocyst rate, n (%)
Fresh-AOC	253	166 (65.61)[a]	74 (29.25)[a]
Fresh-ROC	305	234 (76.72)[b]	120 (39.34)[b]

[a]versus[b] – differences are significant at $p < 0.05$. AOC – atmospheric oxygen content (20 %); ROC – reduced oxygen content (5 %).

Table 2. The effect of the culture system on the development of embryos derived from vitrified oocytes

Groups	IVF oocytes, n	Cleavage rate, n (%)	Blastocyst rate, n (%)
Vitrified-AOC	315	177 (56.19)[a]	44 (13.97)[a]
Vitrified-ROC	167	120 (71.86)[b]	38 (22.75)[b]

[a]versus[b] – differences are significant at $p < 0.05$. AOC – atmospheric oxygen content (20 %); ROC – reduced oxygen content (5 %).

trend was observed (Table 2), where cleavage and blastocyst rates were significantly higher under reduced oxygen compared to the atmospheric oxygen content.

One of the essential quality traits of IVP embryos is the number of cells at the blastocyst stage. Our results of cell counting (Figure 4) show that the total cell number of embryos was significantly higher in blastocysts of both fresh (165.5 ± 8.9) and vitrified (154.54 ± 7.8) groups cultured under reduced oxygen compared to those cultured under an atmospheric oxygen level (108.67 ± 3.6 and 98.04 ± 5.82, resp.).

All these data indicate that the culture system with reduced oxygen content is more beneficial for *in vitro* production of bovine blastocysts both from fresh and vitrified oocytes. Our findings agree with the study of Herbemont *et al.* (2021), who revealed a significant improvement in blastocyst quality at Day 5 and Day 6, when embryo culture was performed under reduced oxygen (5 % O_2) compared to 20 % O_2. They suggested that oxidative stress might be less harmful on embryo development after compaction.

Several earlier studies on animal embryos reported a deleterious effect of the greater O_2 level.

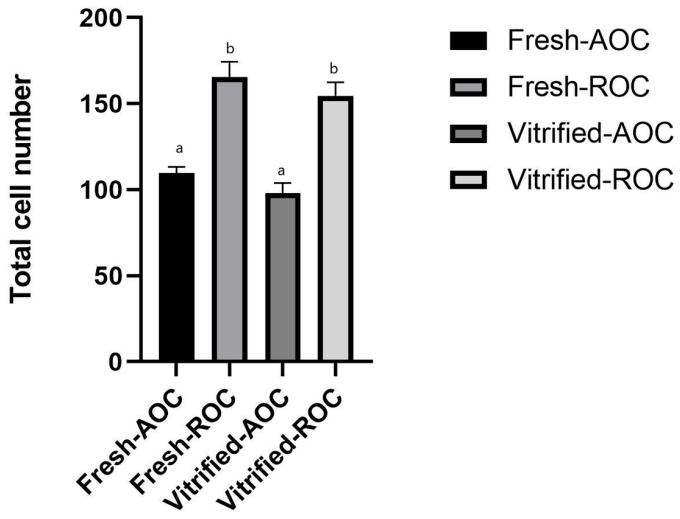

Figure 4. The effect of a culture system on the embryo cell number in fresh and vitrified embryos; [a] versus [b] – differences are significant at p ≤ 0.001; AOC – atmospheric oxygen content (20 %); ROC – reduced oxygen content (5 %)

Oppositely, embryo development was improved, when culture was performed at 5 % O_2 (Takahashi *et al.*, 2000; Yuan *et al.*, 2003; Kitagawa *et al.*, 2004). These authors concluded that a lesser O_2 tension would decrease formation of ROS and diminish oxidative stress leading to better embryo development. However, when oxygen concentration was decreased to 2 % during the post-compaction period, development of bovine embryos to the blastocyst stage was unaffected (Harvey *et al.*, 2004; 2007). It seems different in human embryos. Particularly, Kaser *et al.* (2018) reported that human embryos cultured at ultra-low oxygen concentrations (2 %) are more likely to develop into good blastocysts than the respective sibling embryos cultured under 5 % oxygen. Gelo *et al.* (2019) demonstrated, that culturing of human embryos in benchtop incubators under 5 of oxygen level produced more blastocysts, which resulted in higher pregnancy rates and, therefore, was a better alternative for embryo selection. On the other hand, De Munck *et al.* (2019) showed that lowering oxygen tension from 5 to 2 % since day 3 onwards seems not to further improve human embryo development during extended culture.

According to Herbemont *et al.* (2021), if hypoxia (reduced oxygen level) is not a tool to produce more human embryos at all but rather to produce top-quality blastocysts. This conclusion corresponds to our results on bovine embryos, where the blastocysts developed under reduced oxygen content appeared to be larger and contained more embryo cells than the blastocysts developed in a conventional incubator with atmospheric oxygen level. Some of the studies evaluating the impact of oxygen on embryo development either describe the use of different kind of incubators, e.g. large-box incubators in atmospheric conditions and benchtop incubators in hypoxia (Guo *et al.*, 2014; Peng *et al.*, 2015; Gelo *et al.*, 2019), or do not specify the type of incubators used (Kea *et al.*, 2007). Large-box incubators are less stable than benchtop incubators, causing additional stress to embryos because of temperature and pH variations. This note indicates that not only oxygen level but also volume of an incubator may affect the blastocyst outcome. It thus can explain the fact, that in our study, more quality blastocysts were harvested from zygotes cultured in a benchtop MIRI multiroom incubator when compared to classical MCO 15AC incubator with a substantially higher air volume.

Different antioxidant mixtures in the culture media during *in vitro* culture in order to reduce oxidative stress could be used to improve embryo development (Silva *et al.*, 2015; Belli *et al.*, 2019; Agarwal *et al.*, 2022;

Zarbakhsh, 2021). It was demonstrated that the addition of some antioxidants to the culture media (Von Mengden *et al.*, 2020), such as 7,8-dihydroxyflavone (Choi *et al.*, 2013), quercetin and taxifolin (Kang *et al.*, 2016), glutathione (Olexiková *et al.*, 2023) and astaxanthin (Dujičková *et al.*, 2024) during *in vitro* culture reduced ROS levels in oocytes and improved the developmental competence of the embryos.

Other factors, which may affect a blastocyst outcome, are the gamete or embryo cryopreservation with vitrification (Khalili *et al.*, 2012; 2017). In most published studies, only transfers with fresh embryos were taken into account, which might underestimate the effect of oxygen level on embryos derived from cryopreserved oocytes (Van Montfoort *et al.*, 2020). These authors demonstrate that human embryo culture under 5 % of oxygen level, as compared to 20 % for as little as 2 days, already resulted in a higher number of good quality embryos, which were more suitable for cryopreservation. Consequently, the cumulative ongoing pregnancy and live birth rate after transfer of fresh and frozen embryos from one cycle was significantly higher in the 5 % oxygen group as compared to the 20 % oxygen group (Van Montfoort *et al.*, 2020). In our study we used both fresh and vitrified oocytes as sources of IVP embryos. Our data show that cleavage rate of embryos, derived from vitrified oocytes and developed under reduced O_2, was higher (71.86 %) than those of fresh-derived embryos cultured in 20 % of O_2 (65.61 %). Although, blastocyst rate of vitrification-derived embryos cultured under reduced O_2 was lower (22.75 %) than the blastocyst rate in fresh-derived embryos cultured in 20 % of O_2 (29.25 %), this value was close to those in fresh-derived embryos, as the difference among these values was not substantial.

CONCLUSION

Our experiments indicate that the culture of bovine zygotes under reduced O_2 tension in a multiroom incubator can provide higher blastocyst yield than the co-culture on a feeder cell monolayer under atmospheric oxygen level in a conventional CO_2 incubator. This finding is important mainly for *in vitro* production of embryos derived from cryopreserved oocytes, which are most sensitive to culture conditions.

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AUTHOR'S CONTRIBUTIONS

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Investigation: OLEXIKOVÁ, L., DUJÍČKOVÁ, L., NESVADBOVÁ, A.

Data curation and supervision: MAKAREVICH, A., OLEXIKOVÁ, L.

Writing-original draft preparation: MAKAREVICH, A., OLEXIKOVÁ, L.

Writing-review and editing: MAKAREVICH, A., OLEXIKOVÁ, L., BEZDÍČEK, J.

Project administration: MAKAREVICH, A., BEZDÍČEK, J.

All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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