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Short communication

Low oxygen tension improves developmental competence and reduces apoptosis in hand-made cloned buffalo (*Bubalus bubalis*) embryos

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ABSTRACT

This study compared the effects of oxygen tension on the developmental competence, quality and expression level of some important genes in somatic cell nuclear transfer (SCNT) buffalo embryos produced through hand-made cloning. Following in vitro culture (IVC) of reconstructed embryos under 5% and 20% oxygen tension, the blastocyst rate (72.0 ± 4.78 vs. $58.0 \pm 4.61\%$) and total cell number (413.5 ± 66.7 vs. 265.4 ± 29.8) were higher ($P < 0.01$), and the apoptotic index (2.46 ± 0.71 vs. 11.13 ± 1.52) was lower ($P < 0.01$), respectively, although there was no effect on the cleavage rate. The relative mRNA abundance of hypoxia inducible factors (*HIF1α* and *HIF2α*), was higher ($P < 0.01$) whereas that of oxidative stress – (*SOD-2*, *PRDX1* and *GPX-1*) and apoptosis-related genes (*CASPASE3* and *P53*) was lower ($P < 0.05$) in reconstructed embryos cultured under 5% oxygen than in those cultured under 20% oxygen tension. In conclusion, these results indicate that lowering the oxygen tension during IVC of SCNT embryos from 20 to 5% improves their developmental competence and quality, reduces the apoptosis level and that many of these effects may be elicited through altering the expression of transcription factors.

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1. Introduction

Although somatic cell nuclear transfer (SCNT) has been successfully used to produce cloned animals in several mammalian species, the cloning efficiency is very low; typically, less than 10% of cattle and 6% of pig reconstructed embryos give rise to live-born animals (EFSA, 2010). The in vitro developmental competence of SCNT embryos is not lower than that of embryos produced through in vitro fertilization (IVF) as indicated by similar or higher blastocyst rate (Anand et al., 2008; Selokar et al., 2012, 2013). However, the in vivo developmental competence of SCNT embryos is severely compromised since, following their transfer to recipients, an abrupt fetal loss is observed within the first trimester followed by an abortion rate higher than that observed in IVF embryos (Heyman et al., 2002).

Among many other factors, abnormal culture conditions, resulting in compromised embryo quality, could be an important one leading to low live-birth rate obtained with SCNT embryos. Oxidative stress, which arises due to high oxygen tension, is a major factor that adversely affects embryo yield and quality when in vitro culture (IVC) is performed under 5% CO_2 in air, resulting in oxygen tension of over 20%, compared to that of 3.5–8% encountered by the embryos in the reproductive tract of most mammals (Fischer and Bavister, 1993). Several reports on production of IVF embryos suggest that IVC under low oxygen improves blastocyst development and quality across many species (reviewed by Harvey, 2007). To our

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knowledge, there is only one report on the effects of oxygen tension during IVC on SCNT embryos (Xiong et al., 2014). The present study was undertaken to compare the yield, quality and expression level of some important genes in SCNT embryos produced under 5% and 20% oxygen tension.

2. Materials and methods

Chemicals were obtained from the Sigma Chemical Company (St. Louis, MO, USA), media were purchased from GIBCO (Grand Island, NY, USA) and the disposable plastic ware was from Nunc (Roskilde, Denmark) unless otherwise mentioned.

2.1. Production of hand-made cloned embryos

Somatic cell preparation was performed as described previously to establish the culture of donor karyoplasts (Shah et al., 2008). Preparation of recipient oocytes (maturation, cumulus/zona removal and manual enucleation), fusion, activation and culture of NT embryos were performed as described previously (Selokar et al., 2012). The fused embryos were divided randomly into two groups and were cultured under 5% or 20% oxygen tension for 8 days. For examining the quality of embryos, total cell number (TCN) and the level of apoptosis in day 8 blastocysts were determined by TUNEL staining (Supplementary Fig. 1) as described earlier (Selokar et al., 2013).

2.2. Gene expression analysis

For gene expression analysis, RNA was isolated from group of 3–4 blastocysts using the RNAqueous micro kit (Ambion, Austin, TX) according to the manufacturer's instructions. The genomic DNA contamination was removed by DNase treatment at 37 °C for 20 min. The RT reaction was achieved using the M-MLV RT provided in superscript reverse transcriptase II kit (Invitrogen Co., Grand Island, NY). Real-time RT-PCR was performed using the optimized primer sets shown in Table 1 on CFX96 real time system (Bio-Rad, Hercules, CA, USA) with maxima@SYBR Green master mix (Fermentas, St. Leon-Rot, Germany) at the following thermal cycling conditions: 95 °C for 5 min, followed by 40 PCR cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s as described earlier (Sharma et al., 2011) using β-actin as the housekeeping gene. Melting peaks were determined using melting curve analysis in order to ensure the specific amplification. For comparison, the average expression level of each gene from 20% atmospheric embryo group was set as 1 and performed three separate experiments with three replicates for each gene.

2.3. Statistical analysis

The percentage data was analyzed using SYSTAT 12.0 (SPSS Inc., Chicago, IL, USA) after arcsine transformation. Differences between means were analyzed by Student's '*t*' test for blastocyst rate, apoptotic index and gene expression. Differences were regarded as significant at $P < 0.05$ or $P < 0.01$.

3. Results and discussion

The blastocyst rate and TCN were higher ($P < 0.01$) and the apoptotic index was lower ($P < 0.01$) for reconstructed embryos cultured under 5% oxygen than for those cultured under 20% oxygen tension although there was no effect on the cleavage rate (Table 2). Our results on SCNT embryos agree with those of earlier studies on IVF embryos across many species such as cattle, goat, sheep, pig, rabbit, mouse and human (reviewed by Harvey, 2007), with our earlier study in buffalo (Elamaran et al., 2012) and with the only report available on SCNT embryos (Xiong et al., 2014) in terms of improved blastocyst rate, higher TCN and lower apoptotic index following IVC in 5% than in 20% oxygen tension. This indicates that there is commonality among the mechanisms through which high oxygen tension exerts its adverse effects during IVC of embryos, irrespective of the nature of method through which they are produced. Whereas increased oxidative stress, as a consequence of higher production of reactive oxygen species (ROS) may play an important role in eliciting

Table 1
Real-time PCR primers for each target gene.

Gene	Sequence	Product size	Acc. no
HIF1α	F-TCTCATCCAAGAAGCCCTAA R-AATAATGTTCCAATTCCTACTGCT	166	NM_174339.3
HIF2α	F-CCTCGCATTTGATGTGGAAA R-TGAACTCATCATTAGGGACATTT	104	AB018399.1
SOD2	F-TGGAGAAGGGTGATGTTACAG R-TTAGGGCTCAGATTTGTCCA	105	NM_201527.2
PRDX5	F-CCCGATTAAGGTTGGAGATG R-CAAACAGCACTCCCTTCTTG	109	NM_174749.2
GPX1	F-AACGTAGCATCGCTCTGAGG R-AGCATGAAGTTGGGCTCGAA	203	JQ031269.1
P53	F-GGAAGAATCACAGGCAGAACTC R-ACTTCATTCGGACATTCATCCA	176	AB571118.1
CASPASE3	F-TGGTATTGAGACAGACAGTGG R-AGCATCTCACAAAGAAGCCTG	158	NM_001077840.1
β-ACTIN	F-ACCACACCTTCTACAACGAG R-GAACATGATCTGGGTCATCTTC	112	NM_001206502.1

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