pISSN 1225-4991, eISSN 2288-0178
J. Emb. Trans. (2014) Vol. 29, No. 3, pp. 257～264
http://dx.doi.org/10.12750/JET.2014.29.3.257

Influence of Oxygen Consumption on Pregnancy Rates of Hanwoo Calves following Embryo Transfer

Hyun Kim[1], Nan-Hee Bok[1,2], Sung-Woo Kim[1], Yoon-Jung Do[1], Hwan-Hoo Seong[1], Jae-Hwan Kim[1], Dong Hun Kim[1], Min-Kyu Kim[2] and Yeoung-Gyu Ko[1†]

[1]*Animal Genetic Resources Station, National Institute of Animal Science, RDA, Namwon 590-832, Korea*
[2]*Department of Animal Science Graduate School, Chungnam National University, Daejeon 305-764, Korea*

ABSTRACT

Recently, several approaches have been used to measure the oxygen consumption rates of individual embryos, but relationship between oxygen consumption and pregnancy rates of Hanwoo following embryo transfer has not yet been reported. In this study, we investigated the correlation between oxygen consumption rate and pregnancy rates of Hanwoo embryo using a SECM. In addition to, the expression of apoptosis-related genes was determined using real-time PCR by extracting RNA according to the oxygen consumption of *in vivo* embryo. First, we found that the oxygen consumption significantly increased in blastocyst-stage embryos (blastocyst) compared to early blastocyst stage embryos, indicating that oxygen consumption reflects the embryo quality (Grade I). The oxygen consumption or GI blastocysts were significantly higher than those of GⅡ blastocysts (10.2×10^{14}/mol s^{-1} versus 6.4×10^{14}/mol s^{-1}, $p<0.05$). Pregnant rate in recipient cow was 0, 60 and 80% in the transplantation of embryo with the oxygen consumption of below 10.0, 10.0～12.0 and over 12.0 × 10^{14}/mol s^{-1}, respectively. Apoptosis regulatory genes, *Hsp-70.1* were significantly increased in over-10.0 group than below 10.0 group but in *Caspase-3*, *Bax* and *P53* gene, there was no significant difference. In conclusion, These results suggest that measurement of oxygen consumption maybe help increase the pregnant rate of Hanwoo embryos.

(Key words : oxygen consumption, Korean native cattle, pregnancy, apoptosis-related genes)

INTRODUCTION

To date, several parameters have been investigated as potentially predictive indicators of embryo quality and subsequent viability *in vitro*. Morphological evaluation (using the International Embryo Transfer Society (IETS) evaluation system) using a standard stereomicroscope is still the most widely used as a non-invasive technique for rapid routine assessment of bovine embryo quality and subsequent viability before transfer (Overstöm 1992, 1996; Boiso *et al.*, 2002; Farin *et al.*, 1995). However, this type of quality evaluation is subjective and requires specialized training (Merton, 2002; Overstöm, 1996).

With the exception of morphological evaluation, most viability assays that have been developed target a metabolic parameter that may be predictive with respect to bovine embryo survival *in vivo*. Unfortunately, these techniques are generally invasive and variably perturb the viability of embryos because embryos are exposed to cell permeating fluorescent dyes, UV illumination or radioactive probes (Overstöm, 1996). Actually, Oxygen consumption is a potential quality parameter in itself, as it provides a worthful indication of the overall metabolic activity of a single embryo (Leese, 2003) and a valuable parameter for evaluating embryo quality (Barnett *et al.*, 1996; Houghton, 1996). Embryonic oxygen consumption has earlier been estimated with a cartesian diver technique, by microspectophotometry and ultramicrofluorescence, using electrochemical methods, by an automatic scanning electrode and more recently by scanning electrochemical microscopy (SCEM) (Houghton, 1996; Trimarchi *et al.*, 2000a, b; Magnusson *et al.*, 1986; Overström *et al.*, 1992; Thompson *et al.*, 1995, 1996; Shiku *et al.*, 2001). Therefore, measurement of individual embryonic oxygen consumptive rates is an alternative and a promising

* This work was carried out with the support from the Agenda Program (No. PJ008632)" Rural Development Administration, Republic of Korea.
* This work was supported by 2013 Post Doctoral Fellowship Program of National Institute of Animal Science, Rural Development Administration, Republic of Korea.
† Correspondence : kog4556@korea.kr

approach for assessment of embryo quality and viability.

More recently, oxygen consumption by individual bovine embryos has been shown to be non-invasively quantified by SECM (Shiku *et al.*, 2001). It has been suggested that the oxygen consumption of an individual bovine IVF embryo was highly related to its morphological quality (Shiku *et al.* 2001). Trimarchi *et al.* (2000) reported that the oxygen consumption reflects the total cell number or the number of mitochondria in the embryo. Consequently, the quantification of oxygen consumption by single embryos combined with a morphological assessment is likely to improve the selection of embryos by facilitating the evaluation of their developmental competence.

The incidence of apoptosis has been suggested as an additional criterion to morphological evaluation of embryo to assess embryo quality and effectively predict embryo viability (Pomar *et al.,* 2005; Betts and King, 2001). During pre-implantation development, apoptosis is regulated by the activity of pro- and anti-apoptotic genes (Bergeron *et al.,* 1998). However, the relation between oxygen consumption of bovine pre-implantation embryos and expression of apoptosis regulatory genes has not yet been established.

Previous studies have provided evidence that embryonic oxygen consumptive rates are directly correlated with viability following embryo transfer. Therefore, if there is correlation between the oxygen consumption and the pregnancy rates of bovine following embryo transfer, the non-invasive assessment of oxygen consumption of each embryo may potentially be used as a predictive indicator of pregnancy rates. In the present study, we quantified the oxygen consumption of single Korean native cattle (Hanwoo) embryos by SECM technology for their pregnancy diagnosis.

MATERIALS AND METHODS

1. Animal Care and Use

The present study was approved by the Ethics Committee for the Care and Use of Experimental Animals, Animal Genetic Resources Station, National Institute of Animal Science, Korea.

2. *In Vivo* Embryo Production

In vivo-derived embryos from superovulated Korean native cows (Hanwoo) were collected as described. Superovulation was performed by injecting 20 armor units of follicle-stimulating hormone (SY Esrone, Samyang, Seoul, Korea) and 2 ml prostaglandin $F_{2\alpha}$ (SY Esrone, Samyang, Seoul, Korea) followed by artificial insemination (AI) of ejaculated sperm samples from a Hanwoo that were frozen in 0.5 ml straws and thawed in a 37℃ water bath for 30 sec. On Day 8 post-AI, embryos were recovered by uterine flashing. Blastocysts and expanded blastocyst stage embryos were selected and stored in Dubelco phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) containing 20% calf serum until measurement of oxygen consumption.

3. Synchronization and Embryo Transfer

Korean native cows (Hanwoo) crossbred recipients were synchronized with 3 ml prostaglandin $F_{2\alpha}$. Transferable embryos identified according to the International Embryo Transfer Society manual as either code 1 or 2 single blastocysts at Day 7 after insemination were then transferred into the ipsilateral uterine horn of each synchronized recipient on Days 7～8 after estrus. The recipients were fed and managed in the same manner, and estrous behavior was observed at least twice daily, in the morning and evening.

4. Measurement of Oxygen Consumption

All chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise stated.

Oxygen consumption by individual bovine embryos was non-invasively quantified by the recently developed SECM measuring system (FHK, HV-405, Tokyo, Japan) (Abe *et al.* 2004). A single blastocyst was transferred into a plate filled with 5 ml of embryo respiration assay medium-2 (ERAM-2; Research Institute for the Functional Peptides, Yonezawa, Japan) and the embryos dropped individually to the bottom of the microwell. The medium temperature was maintained at 37℃ on a warming plate (MATS502NLR; Tokai Hit, Shizuoka, Japan) on the microscope stage. The measurement instruments were covered with a plastic sheet and water saturated 5% CO_2 and 95% air was allowed to flow. The measurement of oxygen consumption was carried out according to the procedu repreviously described by Shiku *et al.* (2001). Briefly, Pt-microdisc electrodes, sealed in a tapered soft-glass capillary (PG10165-4: World Precision Instruments, Sarasota, FL, USA), were fabricated according to the literature (Matsue *et al.* 1993). A tip potential was held at −0.6 V vs Ag/AgCl with a potentiostat (RAP-1, IFP, Tokyo, Japan) to monitor the local oxygen concentration in the solution. The tip scanning rate was 19.1 μm/s.

A microelectrode with a Pt-disc radius <1.4 μm was selected so that the oxygen reduction current of the electrode was <1.0 nA. The accurate tip radius was determined by cyclic voltammetry in a 5.0-mM $K_4Fe(CN)_6$, 0.1 M KCl solution before the experiments. The XYZ-stage and the potentiostat were controlled by a notebook computer (FMV-BIBLO NE7/800; Fujitsu, Tokyo, Japan). Voltammetry of the Pt-microdisc electrode in ERAM-1 solution showed a steady-state oxygen reduction wave. No response from other electrochemically active species was observed near the embryo surface. The oxygen consumption rate of embryos was calculated by software, in which the oxygen concentration difference between the bulk solution and sample surface (ΔC), and the oxygen consumption rate (F) of a single sample was estimated according to the spherical diffusion theories (Shiku *et al.* 2001). After measurement of oxygen consumption, each embryo was frozen in a tube containing phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) supplemented with 0.3% polyvinylpyrrolidone (PVP) and stored at −80℃ until real time PCR.

5. Pregnancy Diagnoses

Pregnancy diagnoses were performed on Days 30 and 60 after embryo transfer using ultrasonography (Sonovet-600, Medison, Korea). Pregnancy was confirmed by observation of a fetus with a detected heartbeat in the intraluminal uterine fluid and embryonic membrane.

6. Relative Transcript Abundance (RA) of *Caspase-3*, *Bax*, *P53* and *Hsp-70.1* Genes

Optimized real-time quantitative polymerase chain reaction (qPCR) assays were used to detect transcripts for apoptosis-related genes, *Caspase-3*, *Bax*, *P53* and *Hsp-70.1* genes in bovine Day 8 blastocysts. Primer sequences, annealing temperature, and approximate sizes of the amplified fragments are listed (Table 1). Two pools of embryos for each treatment (n = 3～24 blastocysts/pool) were lysed in 20 ml of extraction buffer Dynabead mRNA DIRECT^{TM}Kit (DYNAL BIOTECH, #61012) by incubation at 42℃ for 30 min followed by centrifugation at 3,000 g for 2 min. RNA was kept frozen at −80℃ in the kit's extraction buffer until all samples were collected for analysis. The cells were washed with PBS and immediately used for RNA isolation.

Total RNA was isolated using Dynabead mRNA DIRECT^{TM} Kit (DYNAL BIOTECH, #61012), and cDNA was synthesized by Super Script II (200 U/ml) with oilgo-dT 16 primer. qPCR was done using with LightCycler (Roche Diagonostics GmbH, Mannheim, Germany) and LightCycler FastStart Thunderbird Rotor-Gene^{TM}SYBR$^{®}$Green (QIAGEN, Netherlands) according to the manufacturer' instructions. Primer sequences and the sizes of the amplified fragments of all transcripts are shown in Table 1. Bovine-specific primers were designed using PRIMER3 software (available at http://fokker.wi.mit.edu/primer3/), and the specificity for each primer set was confirmed by both electrophoresis of the PCR products and analyzing the melting (dissociation) curve after each qPCR. Twenty microlitter of the reaction solution consisted of 2 μl of the template (appropriate dilution was determined by gene), 10 μl of LightCycler FastStart Thunderbird SYBR qPCR Mix, 1 μl of 10 μM of

Table. 1. Description of forward (For) and reverse (Rev) primers used to assess expression of apoptosis regulatory genes

Gene	Primer sequence (5'-3' orientation)	Annealing temp (℃)	GenBank accession number
Caspase-3	For: tgtgcttctaagccatggtg Rev: ggaatccctcgatcactgaa	60	BC123503.1
Bax	For: ttttgcttcagggtttcatccagga Rev: cagctgcgatcatcctctgcag	62	NM173894
P53	For: atttacgcgcggagtatttg Rev: ccagtgtgatgatggtgagg	60	BC102440.1
Hsp-70.1	For: aacaagatcaccatcaccaaacg Rev: tccttctccgccaaggtgttg	60	NM174550
β-Actin	For: atgaggctcagagcaagaga Rev: atctgggtcatcttctcacg	60	AY141970

each primer. PCR amplification was performed as follows: pre-denature for one cycle at 95℃ for 15 min and 45 cycles at 95℃ for 15 sec, 60℃ for 20 sec and 72℃ for 30 sec. Melting curve analysis was performed at 65～95℃ with 0.1 C/sec temperature transition.

7. Statistical Analysis

Differences among groups were analyzed using oneway analysis of variance (ANOVA) by SPSS after arcsine transformation of proportional data. Data were expressed as mean + SEM. Comparisons of mean values among treatments were performed using Duncan's and Tukey's multiple comparisons test. Differences were considered to be significant when $P<0.05$.

RESULTS

1. Comparison of Oxygen Consumptive Rates of *In Vivo*- and *In Vitro*- Produced Embryos

The overall mean oxygen consumptive rate of *in vivo*-produced embryos was 4.0 ± 0.3^{a} (n = 2), which was significantly higher than the 2.4 ± 0.3^{b} (n = 8) observed for morulae *in vitro*-produced embryos (Table 2). Regardless of the apparent difference in mean oxygen consumptive rates between *in vivo*- and *in vitro*-produced embryos, embryo type was found not to influence embryonic oxygen consumption (Table 2). Instead, the stage of embryonic development and morphological quality within type significantly affected oxygen consumptive rates, with embryos of better morphological quality and at more advanced stages of development showing higher oxygen consumptive rates (Table 2).

Table 2. Oxygen consumption rates of *in vivo*- and *in vitro*-produced embryos in Hanwo

Stage of embryonic development		No. of embryos measured	Oxygen consumption rate ($F\times10^{14}$ mol s^{-1})*
Morulae	*In vivo*	2	4.0 ± 0.3^{a}
	In vitro	8	2.4 ± 0.3^{b}
Blastocyst	*In vivo*	13	4.2 ± 0.6
	In vitro	10	5.3 ± 1.5
Expended blastocyst	*In vivo*	5	5.5 ± 0.5
	In vitro	8	5.2 ± 0.7

Data are represented as mean ± S.D.(n=3-4)

*,a,b Different super scripts indicate significant difference ($p<0.05$).

2. Relationship between Each Morphological Stage (Early Blastocyst and Blastocyst) of Embryos and Oxygen Consumption

Initially, the data for each morphological stage (early blastocyst and blastocyst) of embryos of grade I and II were analysed to investigate any effects of the morphological stage on the oxygen consumptive rates. As shown in Table 3, there were no significant differences in oxygen consumptive rates between grade I and II embryos of early blastocyst. However, as shown in Table 3, the mean oxygen consumptive rate significantly increased with increasing morphological quality, being 10.2 ± 1.6^{b} (n = 24) and 6.4 ± 0.6^{a} (n = 3) for grade I and II embryos in blastocyst, respectively (^{a,b} $P<0.05$; values with different superscripts differ significantly).

3. Embryo Viability Following Transfer of *In Vivo*-Produced Embryos

Pregnancy of recipient cow was confirmed with rectal palpation after 60 days of embryo transfer. The associated pregnancy rates in recipient cow was 0% (5/0), 60% (6/10) and 80% (8/10) in the transplantation of embryos with the oxygen consumption of below 10.0, 10.0～12.0 and over 12.0 $\times10^{14}$/mol s^{-1}, respectively. The mean oxygen consumptive rate for embryos producing a pregnancy was significantly higher different from the mean oxygen consumptive rate of embryos that did not produce a pregnancy (High (Over 12.0) versus Low (Below 10.0); $P>0.05$).

Table 3. Oxygen consumption rates of *in vitro* derived bovine embryos according to the morphological quality.

Quality of embryos		No. of embryos examined	Oxygen consumption rate ($F\times10^{14}$ mol s^{-1})
Early blastocyst	Grade I	15	8.2 ± 2.8^{a}
	Grade II	4	7.2 ± 2.5^{a}
blastocyst	Grade I	24	10.2 ± 1.6^{b}
	Grade II	3	6.4 ± 0.6^{a}

Data are represented as mean ± S.D.(n=3-4)

a,b Different super scripts indicate significant difference ($p<0.05$).

4. Expression of Apoptosis-related Genes in *In Vivo*-Produced Bovine Embryos.

The expression of apoptosis-related genes *Caspase-3*, *Bax* (*bos taurus apoptosis regulator box-a*), *P53* and *Hsp-70.1* (*heat-shock protein 70.1*) were determined using real-time PCR by extracting RNA according to the oxygen consumption of *in vivo* embryo. As shown in Fig. 1, the level of expression of apoptosis-related genes such as *Caspase-3*, *Bax* and *P53*, there was no significant difference between over 10.0 group and below 10.0 group. However, in over 10.0 group, the *Hsp-70.1* transcript level was significantly higher ($P<0.05$) in comparison to below 10.0 group *in vivo*-derived blastocyst.

DISCUSSION

The respiration rates of individual day 7 *in vivo*-produced embryos were accurately measured with the SECM and the measured embryos subsequently individually transferred fresh to synchronized recipients. Hence, this study allowed us to compare the oxygen consumptive rates and viability of a single embryo following transfer. The SECM was accurate and consistent in measuring individual embryonic oxygen consumptive rates, as demonstrated by the high correlation between the first and the second measurement of the same embryo as well as by the negligible background noise of the empty control capillaries (which is likely attributed to the extremely small oxygen consumption by the electrochemical microsensor during the measurements). In addition, this technology did not interfere with embryo viability, as pregnancy rates resulting from the transfer of embryos after measurements of oxygen consumptive rate were in agreement with those published by others working with similar systems (Farin *et al.*, 1995; Hasler, 1998) and with the results routinely achieved following embryo transfer in the herd where this study was carried out. In fact, the pregnancy rates obtained in this study following measurements of embryo oxygen consumptive rates were slightly higher than the rates normally observed for each morphological quality.

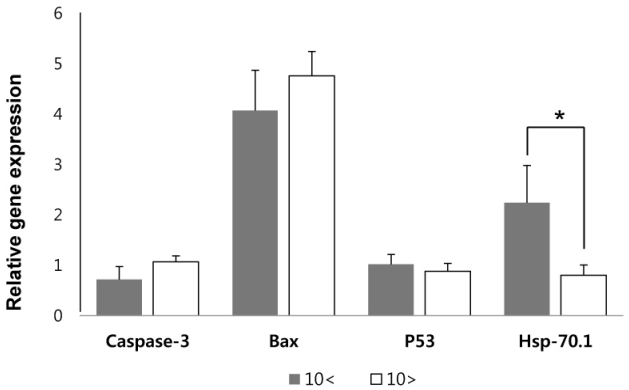

Fig. 1. Relative abundance of apoptosis-related genes in oxygen consumption rate over 10.0 group (black bars) and below 10.0 group (white bars) of *in vivo*-produced bovine embryos. Data are represented as mean ± S.D(n=3-4). * Different super scripts indicate significant difference ($p<0.05$).

The oxygen consumptive rates obtained in this study for *in vivo*-produced embryos are relatively different from those documented by Thompson *et al.* (1995) and Overstrom (1996), as they were 0.7 ± 0.1 and 1.47 ± 0.44 ($F \times 10^{14}$ mol s^{-1}), respectively. The observed difference might be attributed to differences in methodology, techniques and approaches, and species specificity, direct comparisons may not be possible. Nevertheless, the oxygen consumptive rates of grade I and II embryos were not significantly different in early blastocyst. The oxygen consumptive rates of *in vivo*-produced embryos decreased with decreasing morphological quality, indicating that morphological differences are at least partly reflected at the level of oxygen consumption. A similar result was found by Overstrom (1992), who did not observe any differences in oxygen consumption among embryos of different morphological qualities. The significant but limited correlation between embryo morphological quality and oxygen consumptive rates observed in this study and in a previous study using *in vitro*-produced embryos (Lopes *et al.*, 2005) suggests that combining measurements of oxygen consumption with assessment of morphological quality might improve embryo selection.

Correlation between embryo diameter and oxygen consumption has been reported earlier (Shiku *et al.*, 2001, 2004). The lack of correlation between oxygen consumptive rates and diameter observed in this study could be related to the fact that *in vivo*-produced embryos are a more homogeneous population with a more even size distribution as compared with *in vitro*-produced embryos (Holm *et al.*, 1998, 1999). Therefore, the issue regarding the correlation between oxygen consumptive rates and embryo diameter for *in vivo*-produced embryos awaits for further investigation.

In our study, we attempt to analyse the expression of apoptosis regulatory genes with respect to oxygen consumptive rates of *in vivo*-derived bovine embryos. The pro-apoptotic *Hsp-70.1* gene was expressed in all types of bovine oocytes and embryos,

with the highest expression in denuded oocytes (Yang and Rajamahendran, 2002). Similarly, the relative abundance of *Hsp-70.1* was found to be significantly higher ($P<0.05$) in oxygen consumptive rates high bovine embryos compared with that of low embryos. This signifies the role of *Hsp-70.1* in inducing apoptosis. However, the expressions of the other genes of cell death inducers (*Caspase-3*, *Bax* and *p53*) appear to have no correlation with oxygen consumptive rates of *in vivo*-derived embryos. Interestingly, our result demonstrates a significantly higher expression of apoptosis regulatory genes, *Hsp-70.1* mRNA in high oxygen consumptive rates, which could possibly pave the way towards DNA fragmentation detected at the blastocyst stage. Therefore, the pro-apoptosis *Hsp-70.1* gene can be used as potential markers for evaluation of *in vivo*-produced bovine preimplantation embryos. Further study is recommended to develop additional molecular signatures in *in vitro*-produced bovine pre-implantation embryos.

The pregnancy rates according to oxygen consumptive rate in Table 4 (and notably the observation that five embryos with the lowest oxygen consumption were associated with non-pregnancy) led us to speculate that very low levels of embryonic oxygen consumptive rates could be associated with non-pregnancy, whereas the best chance for pregnancy would be found among embryos with high values of oxygen consumption. In this case, very low oxygen consumptive rate would correlate with adverse alterations in oxidative phosphorylation and in other oxygen-related processes (Ziebe *et al.*, 1997), metabolic stress and consequently with reduced embryo viability and increased mortality. Furthermore, it could be expected that the culture conditions and media components such as glucose, amino acids, pyruvate and serum, which are known to affect embryo metabolism (Eckert *et al.*, 1998; Lane and Gardner, 2000; Donnay, 2002; Abe *et al.*, 2005), would have had quantifiable effects on the resulting embryos, which would have been detected during the oxygen consumption measurements.

Table 4. Pregnant rates according to oxygen consumption (high versus medium versus low) of bovine *in vivo*-produced embryos

Oxygen consumption category ($F\times10^{14}$ mol s^{-1})	No. of	
	Recipient	Pregnant (%)
Low (Below 10.0)	5	0 (0.0)
Medium (10.0~12.0)	10	6 (60.0)
High (over 12.0)	10	8 (80.0)

The presented experiment allows us to conclude that measuring the oxygen consumptive rates of individual Korean native cattle (Hanwoo) embryos using the SCEM influence their subsequent viability, as embryos developed into viable fetuses at rates considered normal under farm conditions and all calves born were normal and viable. As no statistically validated correlation between oxygen consumptive rate and pregnancy status could be demonstrated, it is possible that the use of this equipment will help in identifying the most viable *in vivo*-produced embryos just by measuring the oxygen consumptive rates, both because *in vivo*-produced embryos are known to be a more homogeneous population and because confounding factors such as the quality of the recipients seem to play a major role in the pregnancy outcome. On the contrary, it is possible that the use of this technology can bring new possibilities and prove beneficial in improving selection of *in vitro*-produced embryos.

REFERENCES

Abe H, Shiku H, Yokoo M, Aoyagi S, Moriyasu S, Minami hashi A, Matsue T and Hoshi H. 2006. Evaluting the quality of individual embryos with a non-invasive and highly sensitive measurement of oxygen consumption and by scanning electrochemical microscopy. J. Reprod. Dev. 52:S55-S64.

Barnett DK, Bavister BD. 1996. What is the relationship between the metabolism of preimplantation embryos and their developmental competence? Mol. Reprod. Dev. 43:105-33.

Bergeron L, Perez GI, Macdonald G, Shi L, Sun Y, Jurisicova A, Varmuza S, Latham KE, Flaws JA, Salter JC, Hara H, Moskowitz MA, Li E, Greenberg A, Tilly JL and Yuan J. 1998. Defects in regulation of apoptosis in caspase-2 deficient mice. Genes Dev. 12:1304-1314.

Betts DH, King WA. 2001. Genetic regulation of embryo death and senescence. Theriogenology 55; 171-191.

Boiso I, Veiga A and Edwards RG. 2002. Fundamentals of human embryonic growth *in vitro* and the selection of high-quality embryos for transfer. Reproductive Biomedicine OnLine 5:328-350.

Donnay I. 2002. Assessment of Mammalian Embryo Quality: Invasive and Non-invasive Techniques. Dordrecht, The Ne-

therlands: Kluwer Academic Publishers. 57-94.

Eckert J, Pugh PA, Thompson JG, Niemann H and Tervit HR. 1998. Exogenous protein affects development competence and metabolic activity of bovine preimplantation embryos *in vitro*. Reproduction Fertility and Development 10:327-332.

Farin PW, Britt JH, Shaw DW and Slenning BD. 1995. Agreement among evaluators of bovine embryos produced *in vivo* or *in vitro*. Theriogenology 44:339-349.

Gjørret JO, Hiemke MK, Steph JD, Birthe A, Larsson L and Maddox-Hyttel P. 2003. Chronology of apoptosis in bovine embryos produced *in vivo* and *in vitro*. Biol. Reprod. 69: 1193-1200.

Hasler JF. 1998. The current status of oocyte recovery, *in vitro* embryo production, and embryo transfer in domestic animals, with an emphasis on the bovine. Journal of Animal Science 76:52-74.

Holm P, Shukri NN, Vajta G, Booth P, Bendixen C and Callesen H. 1998. Developmental kinetics of the first cell cycles of bovine *in vitro* produced embryos in relation to their *in vitro* viability and sex. Theriogenology 50:1285-1299.

Holm P, Booth PJ, Schmidt MH, Greve T and Callesen H. 1999. High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology 52:683-700.

Houghton FD, Thompson JG, Kennedy CJ and Leese HJ. 1996. Oxygen consumption and energy metabolism of the early mouse embryo. Mol. Reprod. Dev. 44:476-485.

Lane M and Gardner DK. 1996. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. Hum. Reprod. 11:1975-8.

Leese HJ. 2003. What does an embryo need? Human Fertility. 6:180-185.

Lopez AS, Larsen LH, Ramsing N, Lovendahl P, Raty M, Peippo J, Greve T and Callesen H. 2005. Respiration rates of individual bovine *in vitro*-produced embryos measured with a novel, non-invasive and highly sensitive microsensor system. Reproduction 130:669-679.

Magnusson C, Hillensjo T, Hamberger L and Nilsson L. 1986. Oxygen consumption by human oocytes and blastocysts grown *in vitro*. Human Reproduction 1:183-184.

Matsue T, Koike S and Uchida I. 1993. Microamperometric estimation of photosynthesis inhibition in a single algal protoplast. Biochem. Biophys. Res. Commun. 197:1283-1287.

Merton S. 2002. Morphological Evaluation of Embryos in Domestic Animals. Dordrecht, The Netherlands: Kluwer Academic Publishers. 31-55.

Overstrom EW, Burke PA, Hagopian SS and Selgraph JP. 1992. Blastocyst oxidative metabolism and embryo viability. J. Cell Biol. 107:607.

Overstrom EW, Duby RT, Dobrinsky J, Roche JF and Boland MP. 1996. Viablity and oxydative metabolism of the bovine blastocyst. Theriogenology 37:269.

Pomar FJ, Teerds KJ, Kidson A, Colenbrander B, Tharasanit T, Aguilar B and Roelen BA. 2005. Differences in the incidence of apoptosis between *in vivo* and *in vitro* produced blastocysts of farm animal species: a comparative study. Theriogenology 63:2254-2268.

Shiku H, Shiraishi T, Ohya H, Matsue T, Abe H, Hoshi H and Kobayashi M. 2001. Oxygen consumption of single bovine embryos probed with scanning electrochemical microscopy. Anal. Chem. 73:3751-3758.

Shiku H, Shiraishi T, Aoyagi S, Utsumi Y, Matsudaira M, Abe H, Hoshi H, Kasai S, Ohya H and Matsue T. 2004. Respiration activity of single bovine embryos entrapped in a cone-shaped microwell monitored by scanning electrochemical microscopy. Analytica Chimica Acta 522:51-58.

Thompson JG, Partridge RJ, Houghton FD, Kennedy CJ, Pullar D, Wrathall AE and Leese HJ. 1995. Preliminary observations of the uptake of oxygen by day 7 bovine blastocysts. Theriogenology 43:3-37.

Trimarchi JR, Liu L, Porterfield DM, Smith PJ and Keefe DL. 2000a. Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. Biology of Reproduction 62:1866-1874.

Trimarchi JR, Liu L, Porterfield DM, Smith PJ and Keefe DL. 2000b. A non-invasive method for measuring preimplantation embryo physiology. Zygote 8:15-24.

Thrimarchi JR, Liu L, Smith PJS and Keefe DL. 2000. Noninvasive measurement of potassium efflux as an early indicator of cell death in mouse embryos. Biol. Reprod. 63:851-857.

Yang M, Rajamahendran R. 2002. Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced *in vitro*. Anim. Reprod. Sci. 15:159-169.

Ziebe S, Petersen K, Lindenberg S, Andersen AG, Gabrielsen A and Andersen AN. 1997. Embryo morphology or cleavage

stage: how to select the embryos for transfer after *in-vitro* fertilization. Hum. Reprod. 12:1545-9.

Received May 8, 2014, Revised May 18, 2014, Accepted September 22, 2014