Oxygen consumption and ROS production are increased at the time of fertilization and cell cleavage in bovine zygotes

A.S. Lopes¹,2*, M. Lane¹, and J.G. Thompson¹

¹Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, The University of Adelaide, Adelaide 5005, Australia
²Present address: Leuven Institute for Fertility and Embryology (LIFE), Heilig Hart Hospital, Leuven 3000, Belgium

*Correspondence address. Tel: +32-488249057; E-mail: ana.lopes@lifeleuven.be

Submitted on February 21, 2010; resubmitted on May 23, 2010; accepted on July 6, 2010

BACKGROUND: Oxygen consumption is a key indicator of metabolic activity within embryos. Increased oxidative activity and REDOX changes at the time of fertilization have been suggested to signal Ca²⁺ oscillations after sperm penetration. The objective of the present study was to determine the oxygen consumption and the REDOX status of zygotes and early embryos at the time of sperm penetration and cell cleavage and to investigate how metabolism relates to key temporal events and developmental competence.

METHODS: Individual oxygen-consumption rates of bovine in vitro matured oocytes and presumptive zygotes (n = 101) were measured using the Nanorespirometer at 0, 7, 12, 17 and 24 h after IVF. Using the Embryoscope, oxygen-consumption profiles of individual oocytes and embryos (n = 75) were recorded repeatedly from 6 h until 30 h after IVF and time-lapse images were acquired, at intervals of ~36 min. Oocytes and embryos were stained with Hoechst 33342 and visualization of nuclear stage was performed by fluorescence microscopy. To determine the REDOX status, cohorts of oocytes and zygotes (n = 55) were individually stained with REDOX-Sensor Red CC-1 and Hoechst 33342 at 0, 7, 12, 17 and 24 h after IVF and subsequently imaged by confocal microscopy.

RESULTS: A peak of oxygen consumption was observed at the time of fertilization and a smaller rise and fall in oxygen consumption could be detected prior to the first cell cleavage. Increased reactive oxygen species production was also observed at 7 h and then at 24 h after IVF, just preceding the first embryonic cleavage.

CONCLUSIONS: There are specific events during embryo development that appear to be associated with a change in oxygen consumption and REDOX state, indicating that both have a role in sperm-mediated oocyte activation and cell cleavage in bovine embryos.

Key words: oxygen / ROS / H₂O₂ / fertilization / cell cycle

Introduction

Oocytes and embryos rely on the production of energy to support continued development during preimplantation stages. The generation of cellular energy (ATP) in the mitochondria of oocytes and early embryos is mostly dependent on oxidative phosphorylation, a process that utilizes oxygen as a key energy substrate. Thus, oxygen is a key indicator of overall metabolic activity of single oocytes and embryos (Leese, 2003). In the oocyte, ATP production depends mostly on the number and activity of mitochondria present in the ooplasm, which seem to be strongly related to developmental competence, with lower mitochondrial activity and number associated with premature arrest of the oocyte, fertilization failure, and reduced embryo development (Van Blerkom 2004, 2008; Dumollard et al., 2007). Mitochondrial respiratory activity accounts for 30% of all the oxygen consumed by the embryo at early cleavage stages, a figure that increases to 60–70% at the blastocyst stage (Trimarchi et al., 2000). Since oxygen uptake is directly influenced by utilization rates, as there are no known facilitated transport systems for oxygen in embryos, changes in mitochondrial activity should be directly reflected by the oxygen consumption of the developing embryo.

Albeit an evolutionary breakthrough, oxidative phosphorylation came at a cost with the production of reactive oxygen species (ROS), in particular the superoxide anion (O₂⁻) and the hydroxy radical (OH⁻). Hydrogen peroxide (H₂O₂) is not a free radical per se, but usually a product of O₂⁻ dismutation, catalyzed by the superoxide dismutase. However, in the presence of a transition metal, H₂O₂ can form the extremely reactive and damaging OH⁻. ROS
can induce oxidative modifications of the cell components, thus indirectly causing DNA fragmentation, protein oxidation, lipid per oxidation and mitochondrial damage (reviewed by Guérin et al., 2001). Using in vitro culture models, it was shown that overproduction of intracellular ROS during early development of mammalian embryos affects metabolism and is detrimental for embryo survival (reviewed by Johnson and Nasr-Esfahani, 1994; Guérin et al., 2001). Sub-optimal in vitro culture conditions, especially high glucose concentrations, exposure to light and higher oxygen concentrations, when compared with in vivo conditions, have also been associated with increased ROS generation and perturbed developmental ability (Luvoni et al., 1996; Hashimoto et al., 2000; Guérin et al., 2001; Kitagawa et al., 2004). Nevertheless, the assumption that improved embryo development under low oxygen concentrations could be due to reduced ROS production is yet to be conclusively demonstrated (Harvey et al., 2002; Harvey, 2007).

Controversially, it is currently accepted that physiological concentrations of ROS participate in normal cell processes as major factors in growth and development regulation (reviewed by Lander, 1997; tractions of ROS participate in normal cell processes as major factors in growth and development regulation (reviewed by Lander, 1997; Harvey et al., 2002). Earlier studies have reported a rise in H2O2 production coincident with the time of sperm penetration in murine and bovine zygotes (Nasr-Esfahani et al., 1991; Dalvit and Thompson, unpublished data), and oocytes of marine invertebrates are also known to undergo a ‘respiratory burst’ at fertilization (Heinecke and Shapiro, 1992; Schomer and Epel, 1998), leading to the suggestion that mitochondrial activity is stimulated by Ca2+ waves around the time of sperm penetration. A brief pulse of H2O2 in bovine embryos at the 8-cell stage (embryonic genome activation) has also been positively correlated with subsequent development in vitro (Morales et al., 1999). Recently, assessment of redox metabolism in murine and bovine oocytes and embryos at different developmental stages confirmed that ROS have a key role with regard to in vitro maturation (IVM), fertilization and embryo development (Tanghe et al., 2003; Dumollard et al., 2007; Morado et al., 2009). Moreover, short-term exposure to H2O2 at the end of maturation seems to have a beneficial effect on subsequent embryo development (Vandaele et al., 2010).

Oxygen consumption has been determined by several techniques but recently, a non-invasive and highly sensitive device, capable of measuring the oxygen consumption of individual oocytes or embryos at a specific time and stage of development has been developed (The Nanorespirometer; Lopes et al., 2005). Using the Nanorespirometer, the relationship between the oxygen consumption of bovine embryos and their subsequent viability has been earlier demonstrated (Lopes et al., 2007). Based on the same principle, the Embryoscope (Unisense Fertilitech A/S, Aarhus, Denmark) is built inside a standard CO2 incubator (5% CO2 in air, at 38.5°C) and combines a time-lapse videomicrography with an oxygen microsensor (with a laser positioning system), which is able to automatically and repeatedly measure the oxygen consumption of oocytes and embryos. Correlations between the oxygen consumption of bovine and murine embryos and their subsequent developmental competence have been established using the Embryoscope (Callesen et al., 2005; Ottosen et al., 2007). Furthermore, Callesen et al. (2005) have briefly reported the observation of small pulses of oxygen consumption (increases of 3–10%) preceding cell division and lasting ~2 h. Detection of the timing of the first cleavage is critical for subsequent evaluation of embryo quality and viability, as early cleavage is considered a significant predictor of developmental potential, and has been associated with higher pregnancy and implantation rates (Sakkas et al., 2001; Salumets et al., 2003; Fancovits et al., 2005).

In the present study, we determined the individual oxygen consumption of bovine oocytes, fertilized zygotes and cleaving embryos by repeated measurements of oxygen consumption performed at short intervals during development. The bovine model was chosen as early bovine embryo kinetics and metabolism are similar to that of the human. Based on that assumption, we investigated whether oxygen consumption can be used as a non-invasive tool for signalling key temporal events occurring within fertilizing oocytes and early embryos, such as sperm penetration and cell cleavage. Moreover, we wished to relate this temporal activity with metabolic activity to develop an understanding of how metabolism relates to competence.

This was accomplished by evaluating the relationship between the REDOX flux and oxygen consumption within the fertilized zygotes and cleaving embryos, and correlating it to cell cycle events. Preliminary results of this work were presented earlier (Lopes et al., 2008).

Materials and Methods

Oocyte collection and IVM

Unless otherwise specified, all chemicals and reagents were purchased from Sigma (St Louis, MO, USA). The method used for in vitro embryo production has been reported elsewhere (Hussein et al., 2006). Briefly, bovine ovaries were collected from local abattoirs and transported to the laboratory in warm saline (30–35°C). Bovine immature cumulus—oocyte complexes (COCs) were aspirated from antral follicles (3–8 mm in diameter) using a 18-gauge needle and a 10-ml syringe containing ~2 ml HEPES-buffered tissue culture medium-199 (TCM-199; ICN Biochemicals, Irvine, CA, USA) supplemented with 50 µg/ml kanamycin, 0.5 mM sodium pyruvate, 50 µg/ml heparin and 4 mg/ml fatty-acid-free bovine serum albumin (FAF—BSA; ICPbio Ltd., Auckland, New Zealand). Intact COCs with compact cumulus vestments larger than 5-cell layers and evenly pigmented cytoplasm were selected under a stereomicroscope and washed twice in HEPES-buffered TCM-199 supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) and once in oocyte maturation medium. The medium used for oocyte maturation was Bovine VitroMat (Cook Australia, Eight Mile Plains, QLD, Australia), which is based on the ionic composition of bovine follicular fluid (Sutton-McDowall et al., 2005), supplemented with 0.1 IU/ml recombinant human FSH (Puregenon, Organon, Oss, Netherlands). COCs were cultured in pre-equilibrated 300 µl drops overlaid with mineral oil and incubated at 39°C with 5% CO2 in humidified air for 24 h.

In vitro fertilization

Fertilization in all experiments was performed using frozen-thawed semen from a single bull of proven fertility. In brief, thawed semen was layered over a discontinuous (45%; 90%) Percoll gradient (Amersham Biosciences, Uppsala, Sweden) and centrifuged at room temperature for 20–25 min at 700g. Following removal of the supernatant, the sperm pellet was washed with 500 µl Bovine VitroWash (Cook Australia) and centrifuged for 5 min at 200g. Spermatozoa were resuspended with IVF medium [Bovine VitroFert (Cook Australia) supplemented with 0.01 mM heparin, 0.2 mM penicillamine and 0.1 mM hypotaurine] and then added to the IVF medium drops at a final concentration of 1 × 104 spermatozoa/ml. COCs were inseminated in the IVF medium drops at a density of 10 µl of IVF medium per COC and incubated at 39°C with 5% CO2 in humidified
air for 24 h. In our laboratory, mean cleavage (evaluated on Day 2) and blastocyst rates (evaluated on Day 8) of in vitro produced bovine embryos were 87 and 33%, respectively.

Denudation, selection and evaluation of in vitro matured oocytes and zygotes

Cumulus cells surrounding the mature oocytes (non-fertilized group—control group) were removed by gently pipetting these up and down with a plastic pipette (130 μm inner diameter, Cook Australia) in 400 μl of Bovine VitroWash (Cook Australia), at 24 h after IVM. Similarly, presumptive zygotes were denuded at 6, 11, 16 or 23 h after IVF, according to the allocated group.

Oocytes and presumptive zygotes were visually inspected under a stereomicroscope with ×50 magnification and selected based on the morphologic characteristics and stage of development. In vitro matured oocytes were selected based on the presence of the first polar body. Presumptive zygotes at 6 and 11 h post-fertilization were randomly selected from the pool of in vitro matured oocytes displaying the first polar body and occasionally from the observation of the sperm cell inside the oocyte. Zygotes at 16 and 23 h post-fertilization were selected based on the presence of the second polar body.

Measurement of oxygen consumption

Using the Nanorespirometer

The Nanorespirometer (Unisense Fertilitech A/S) was used to measure the oxygen consumption of single oocytes or zygotes, as previously described (Lopes et al., 2005). Briefly, using a thin glass capillary, the selected oocytes or zygotes were loaded individually into the glass capillaries of the rosette. One empty glass capillary served as reference measurement without respiratory activity (= blank). The rosette with the embryos was submerged into a beaker (80 ml) containing HEPES-buffered TCM-199 supplemented with 4 mg/ml FAF–BSA, which was maintained at 38.5 °C in a semi-closed system, under a constant flow of humidified 5% CO₂ in 19.5% O₂. The system was left undisturbed for 1 h, until a steady state linear oxygen gradient was established inside the glass capillaries of the rosette. Subsequently, oxygen consumption gradients generated by the oxygen consumption of the oocyte or zygote were determined by measuring the oxygen concentration at consecutive measurement points inside the capillary with an oxygen microsensor (Clark-type oxygen microelectrode). Both oocyte or zygote-containing capillaries and empty capillaries were measured in duplicate. Calculation of the mean oxygen-consumption rate (nl/h) was performed as earlier described (Lopes et al., 2005) and based on two oxygen concentration profiles obtained from each oocyte or zygote, after subtraction of the blank values measured in the empty reference capillaries during each measurement session.

Using the Embryoscope

The oxygen consumption of presumptive zygotes was measured individually using the Embryoscope™ (Unisense Fertilitech A/S). The Embryoscope was built inside a standard IVF CO₂ incubator (5% CO₂, 19% O₂, at 38.5 °C) and combined a high-resolution microscope system (Leica 20 × LMPLFL objective) with a system for the measurement of oxygen consumption. It was capable of recording linear oxygen concentration gradients over a single oocyte or zygote placed in a microwell and although based on the same principle, this device was technically different from the Nanorespirometer.

The Embryoscope measured automatically and repeatedly the individual oxygen consumption by means of an oxygen microsensor (with a laser positioning system) guided by microcontrollers that permitted the alignment of the tip of the microsensor with the microwells of the EmbryoSlide™ (Unisense Fertilitech A/S). The EmbryoSlide™ was an embryo-tested polymer slide with 12 gas permeable microwells (depth 0.3 mm, diameter 0.7 mm), which were filled with 20 μl IVF medium, overlaid with mineral oil and equilibrated at 38.5 °C with 5% CO₂ in humidified air for 18 h.

Following equilibration, a plastic denudation pipette (130 μm diameter, Cook Australia) was used to load the embryos into the bottom of each microwell of the EmbryoSlide™ and the oxygen supply to the zygote was maintained through molecular diffusion from the overlaying culture medium down through the well. Two or three microwells of the EmbryoSlide™ contained only IVF medium, in order to serve as reference measurement without respiratory activity (= blank). The EmbryoSlide™ was inserted in the incubator 1 h before measurements were performed and an oxygen concentration gradient was established.

For calibration purposes, a Nunc 4-well slide (calibration dish) was prepared as follows: well 1—HCl (0.1 HCl, pH 1) and detergent (2% ES 7X-PF, MP Biomedicals, Irvine, CA, USA), for washing; well 2—Alkaline Sodium Ascorbate (0.1 M Na Ascorbate and 0.1 M NaOH), for the 0% O₂ calibration; well 3—Phosphate buffered saline (PBS) pH 7.2, for rinsing the microsensor; well 4—IVF media, the same as that used in the EmbryoSlide™, for the 19% O₂ calibration. The calibration dish containing the calibration and washing solutions was positioned parallel to the EmbryoSlide™, inside the incubator, ~1 h before each set of measurements. Calibration of the device was performed by sequentially moving the oxygen microsensor down into wells 1—4 of the calibration dish for acquisition of the reference values corresponding to 0 and 19% O₂.

Oxygen concentration gradients of individual zygotes were measured by the oxygen microsensor in all microwells of the EmbryoSlide™ and individual oxygen-consumption rates were calculated from the slope of the linear steady-state oxygen gradients measured. The estimated mean oxygen consumption (95% coefficient interval) in microwells filled with IVF medium but without zygotes was 0.08 (0.04—0.12) nl/h. Oxygen-consumption rates of individual embryos were obtained by subtracting the mean value of the blanks in each measurement session from the calculated oxygen-consumption rates.

During embryo development inside the Embryoscope, digital images were acquired between oxygen measurements at intervals of ~36 min. Subsequent analysis of the images allowed monitoring the individual embryo development and identifying the time of cell division on the embryos that developed beyond the 1-cell stage. From analysis of the time-lapse images, an activity pattern was generated for each individual embryo.

Processing of oocytes and zygotes after the oxygen measurements

After the oxygen measurements, the oocytes or zygotes were individually transferred to individual wells of a 4-well dish (Nunc, Roskilde, Denmark) containing 500 μl Bovine VitroWash (Cook Australia) and stained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA). The oocytes or zygotes were subsequently washed in Dulbecco’s-PBS and individually loaded into a 10 μl drop of 4-morpholinopropanesulfonic acid (MOPS)-buffered medium (G-MOPS without phenol-red; Lane and Gardner, 2004) on a clean microscope slide. A cover slip was fixed to the slide using Vaseline as a spacer and the oocytes or zygotes were imaged immediately using fluorescence microscopy, to determine the nuclear stage. Oocytes were classified as in vitro matured when a metaphase II chromosome configuration was present. Oocytes were considered fertilized when the sperm cell was visible, the two polar bodies were present and/or the zygote had initiated pronuclei formation.
Staining of oocytes and zygotes and determination of ROS levels by the Redox sensor red assay

To determine the REDOX state of cohorts of oocytes and zygotes at different stages of the cell cycle, oocytes and zygotes were allocated in groups according to stage of development, as previously described. In vitro matured oocytes and presumptive zygotes were co-incubated with 1 μM of RedoxSensor Red CC-1 (Molecular Probes) stock solution at 1 mM (the content of one vial dissolved in 11.5 μl of dimethyl sulfoxide) and 1 μM of Hoechst 33342 for 10 min in the dark, at 38.5°C.

Redox-Sensor Red CC-1 passively enters the embryo where it is oxidized in the cytosol, producing a red-fluorescent product. The REDOX potential of the cytosol determines differential distribution of the red-fluorescent product and the subsequent staining pattern. The Hoechst 33342 stain was used to visualize nuclei, polar bodies and pronuclei as this fluorescent stain labels DNA in fluorescence microscopy.

Oocytes and zygotes were subsequently washed in PBS and individually loaded into a 10 μl drop of G-MOPS, as previously described. Oocytes and zygotes were imaged immediately using a laser-scanning confocal microscope to determine the distribution pattern of the red-fluorescent product and the nuclear stage.

Images of fluorescently labelled oocytes and zygotes were acquired using a confocal microscope (Nikon TE2000E with a Nikon C1 Confocal Scanning Head, Nikon Corporation, Tokyo, Japan). Image analysis was performed using the Adobe Photoshop Pro software package (version 13, Adobe Systems Inc.) and a method adapted from Barnett et al. (1997). The mean red-staining intensity was determined in four different regions (1–4) within three areas of the oocyte or zygote (areas 1–3), as illustrated in Fig. 1. The mean pixel intensity was determined for each of the three areas in both oocytes and zygotes.

Experimental design

A total of 416 in vitro matured oocytes and presumptive zygotes were measured in this study during 54 measurement sessions. In vitro matured oocytes and zygotes were used in the assessment of individual oxygen consumption or in the determination of ROS levels, as follows:

Measurement of the oxygen consumption of individual oocytes and zygotes to determine differences according to cell cycle

Experiment 1: oxygen measurements with the Nanorespirometer. To determine the oxygen consumption of cohorts of oocytes or zygotes at different stages of the cell cycle, oocytes and zygotes were allocated in six groups, according to the time of fertilization: Group A—oocytes in vitro matured for 24 h, not fertilized, measured at 0 h after IVF (n = 15); Group B—presumptive zygotes, measured 7 h after IVF (time of sperm penetration; n = 25); Group C—presumptive zygotes, measured 12 h after IVF (time between sperm penetration and pronuclei formation; n = 15); Group D—zygotes, measured 17 h after IVF (time of pronuclei formation, n = 14); Group E—zygotes, measured 24 h after IVF (time before the first cleavage; n = 23); Group F—unfertilized in vitro matured oocytes, measured 7 h after transfer to IVF medium (negative control; n = 13). The oxygen consumption of these embryos was assessed in duplicate in independent measurement sessions (n = 20). Oocytes and zygotes were then immediately stained with Hoechst 33342 and classified according to cell stage by fluorescence microscopy.

Following visualization of the nuclear stage, oxygen-consumption rates of oocytes that were unfertilized but initially included in Group B (n = 4), were excluded from the final data analysis (thus Group B = 21). Furthermore, rates of zygotes from Groups C and D were recombined according to the presence or absence of pronuclei. Thus, the new Group C included oxygen-consumption rates of zygotes that did not show pronuclei and were measured at 12 and 17 h after IVF (n = 16), and the new Group D included oxygen-consumption rates of zygotes that showed pronuclei formation and were measured at 12 and 17 h after IVF (n = 13). Statistical analyses were performed before and after merging data from Groups C and D.

Experiment 2: oxygen measurements with the Embryoscope. A total of 75 oocytes and zygotes were measured in 8 measurement sessions. In six of the measurement sessions, the EmbryoSlide® was loaded with nine or ten presumptive zygotes, 6 h after IVF (n = 57). To investigate differences in the respiratory patterns associated with fertilization, the Embryoscope® was loaded in two of the measurement sessions with nine unfertilized in vitro matured oocytes that had been transferred to IVF medium at the supposed time of IVF (n = 18). The apparent oxygen consumption of the blanks (n = 21) was determined in two or three empty microwells, in all measurement sessions.

Measurements of individual embryonic oxygen consumption and time-lapse images were acquired until 30 h after IVF at intervals of ~36 min. Images were used for evaluation of developmental progress. It was not possible to detect the precise time of pronuclei formation by analysis of the images acquired by the Embryoscope, due to the high lipid content of the cytoplasm of bovine embryos and to the lack of resolution of the respective images. Thus, the correlation between time of pronuclei formation and corresponding oxygen consumption could not be evaluated. In case the first cell division was not detected, oocytes or zygotes were subsequently unloaded and stained with Hoechst 33342 for accurate cell-stage classification by fluorescence microscopy. Oxygen-consumption rates of presumptive zygotes that were found to be unfertilized and arrested at the MII stage were not used in the data analysis.

Furthermore, profiles of four embryos and six in vitro matured oocytes were regarded as non-consistent (linearity with a coefficient of determination <0.999) due to the presence of bubbles in the microwells and thus excluded from the analysis. Overall, 34 embryos and 12 unfertilized in vitro matured oocytes were included in the full statistical analysis.

Figure 1 Schematic of the template used to analyse the red-pixel intensity of confocal images from fluorescently stained oocytes and zygotes, showing four regions (1–4) within three areas (1–3).
Measurement of the REDOX state of oocytes and zygotes to determine differences according to cell cycle

Experiment 3: REDOX sensing with REDOX-Sensor RED CC-1 assay.

In vitro matured oocytes (Group A, 0 h after IVF; n = 16) and presumptive zygotes (Group B, 7 h after IVF, n = 7; Group C, 12 h after IVF, n = 10; Group D, 17 h after IVF, n = 13; Group E, 24 h after IVF, n = 11) were individually stained with REDOX-Sensor Red CC-1 and Hoechst 33342, and imaged by confocal microscopy to determine the REDOX potential and the distribution pattern of the red-fluorescent product as well as...

Figure 2. Confocal (a–e) and corresponding bright-field (f–j) images of oocytes and zygotes stained with REDOX-Sensor Red CC-1 and Hoechst 33342 at 0, 7, 12, 17 and 24 h after IVF.
the nuclear stage (Fig. 2). The REDOX state of Group F, corresponding to unfertilized in vitro matured oocytes measured 7 h after transfer to IVF medium, was not assessed in this experiment.

Following visualization of the nuclear stage, values obtained for zygotes of Groups C and D were pooled according to the presence or absence of pronuclei, as earlier described for the oxygen-consumption results. Thus, Groups C and D included in the data analysis comprised 12 and 9 zygotes, respectively.

Statistical analysis

Oxygen-consumption rates of individual oocytes and zygotes measured by the Nanorespirometer and the Embryoscope were tested for the distributional properties, and deviation from normal distribution was not significant (Univariate Procedure, SAS).

Oxygen-consumption rates of oocytes and zygotes measured by the Nanorespirometer were analysed using a linear mixed model (Mixed Procedure, SAS), including group as a fixed effect and time of measurement as a random effect. Least-squares means (LSMs) and associated SEM for the treatment factor were produced by this model. The random effect of time of measurement was tested by chi-square analysis, before being introduced in the random part of the model.

Oxygen-consumption rates of individual oocytes and zygotes assessed by the Embryoscope were analysed using a linear mixed model (Mixed Procedure, SAS), including group and time as fixed effects and embryo number as a random effect, due to repeated observations on each embryo. LSM ± SEM were produced by the previous model. The random effect of embryo number was tested by chi-square analysis, before being introduced in the random part of the model.

Changes in oxygen-consumption rates of zygotes at the time of cleavage (measured with the Embryoscope) were analyzed independently using a linear mixed model (Mixed Procedure, SAS). The model included time as a fixed effect and embryo number as a random effect. LSM ± SEM were produced by the previous model.

The red-pixel intensity measured following staining of oocytes and zygotes with REDOX-Sensor Red was tested for the distributional properties, and deviation from normal distribution was not significant (Univariate Procedure, SAS). Data were subsequently transformed using a square-root transformation, as this was effective in stabilizing variance and obtaining normally distributed data and residuals.

Following transformation, the red-pixel intensity measured in individual oocytes and zygotes was evaluated by a linear mixed model (Mixed Procedure, SAS). The model included the following fixed factors: an interaction of group and area measured. Pixel-intensity measurements were loaded in the EmbryoSlide following IVF. The mean oxygen-consumption rate of individual presumptive zygotes was affected by time of measurement ($P < 0.05$) and we were able to identify a peak of oxygen consumption (over and above baseline O$_2$ consumption) between 7 and 11 h after IVF (Fig. 4, red profile). Following an abrupt drop, a smaller rise and fall was observed between 22 and 25 h after IVF, at the time of the first cell cleavage. The oxygen-consumption profile of the control group, i.e. in vitro matured oocytes that did not undergo IVF, was significantly different (Fig. 4, blue profile; $P < 0.05$), with no peak at 7 h and decreasing gradually with time. Overall, oxygen-consumption rates of unfertilized in vitro matured oocytes were significantly higher than those of zygotes ($P < 0.05$), but this difference was not significant for all time points. In particular, mean oxygen-consumption rates acquired 7 h after IVF were not statistically different among both groups (0.52 nl/h for presumptive zygotes versus 0.55 nl/h for unfertilized in vitro matured oocytes; $P > 0.05$).

As mentioned earlier, we confirmed that a small oxygen-consumption peak was consistently present immediately before or at the exact time of cleavage for every presumptive zygote. Mean oxygen-consumption rates were subsequently grouped according to time of cleavage and Fig. 5 shows the mean oxygen-consumption profiles for embryos with the first cleavage at 22 h ($n = 5$), 23 h ($n = 2$), 24 h ($n = 6$) and 25 h ($n = 9$) after IVF. For embryos dividing at 22 h after IVF (Fig. 5a), we observed a double peak with the cytoplasmic cell division occurring at the lag phase between the two peaks. When considering embryos dividing at 23 and 25 h (Fig. 5b and d), division was seen at the onset of the oxygen-consumption peak (i.e. cleavage at 22.41 and 25.17 min and oxygen-consumption peaks at 24.30 and 25.52 min, respectively). For embryos showing the first division at 24 h after IVF (Fig. 5c), the time of division was consistent with the highest value of the oxygen consumption peak. It is important to notice that there is a discrepancy of 7–9 min between the image acquisition and oxygen measurement for each data point, with the image being obtained before the oxygen measurement.

Mean oxygen-consumption rates of zygotes that have undergone the first cleavage are shown in Table 1. Mean oxygen-consumption rates at the time of cleavage were affected by the time of measurement ($P < 0.05$). Considering only five points of measurement, there was a significant drop in oxygen consumption from ~72 min before cleavage, which indicated that the small peak of oxygen consumption occurred ~72 min before cleavage.

**Results**

**Oxygen consumption of in vitro matured oocytes and presumptive zygotes**

**Measurements using the Nanorespirometer**

The mean oxygen-consumption rates of in vitro matured oocytes or zygotes measured at 0, 7, 12, 17 and 24 h after IVF as well as the mean oxygen-consumption rate of the control group (where IVF was not performed) are shown in Fig. 3a. The values obtained from combining the profiles of zygotes measured at 12 and 17 h according to the presence or absence of pronuclei are presented in Fig. 3b.

**Measurements using the Embryoscope**

The first division was observed in 69% of the presumptive zygotes loaded in the EmbryoSlide following IVF. The mean oxygen-consumption rate of individual presumptive zygotes was affected by time of measurement ($P < 0.05$) and we were able to identify a peak of oxygen consumption (over and above baseline O$_2$ consumption) between 7 and 11 h after IVF (Fig. 4, red profile). Following an abrupt drop, a smaller rise and fall was observed between 22 and 25 h after IVF, at the time of the first cell cleavage. The oxygen-consumption profile of the control group, i.e. in vitro matured oocytes that did not undergo IVF, was significantly different (Fig. 4, blue profile; $P < 0.05$), with no peak at 7 h and decreasing gradually with time. Overall, oxygen-consumption rates of unfertilized in vitro matured oocytes were significantly higher than those of zygotes ($P < 0.05$), but this difference was not significant for all time points. In particular, mean oxygen-consumption rates acquired 7 h after IVF were not statistically different among both groups (0.52 nl/h for presumptive zygotes versus 0.55 nl/h for unfertilized in vitro matured oocytes; $P > 0.05$).

As mentioned earlier, we confirmed that a small oxygen-consumption peak was consistently present immediately before or at the exact time of cleavage for every presumptive zygote. Mean oxygen-consumption rates were subsequently grouped according to time of cleavage and Fig. 5 shows the mean oxygen-consumption profiles for embryos with the first cleavage at 22 h ($n = 5$), 23 h ($n = 2$), 24 h ($n = 6$) and 25 h ($n = 9$) after IVF. For embryos dividing at 22 h after IVF (Fig. 5a), we observed a double peak with the cytoplasmic cell division occurring at the lag phase between the two peaks. When considering embryos dividing at 23 and 25 h (Fig. 5b and d), division was seen at the onset of the oxygen-consumption peak (i.e. cleavage at 22.41 and 25.17 min and oxygen-consumption peaks at 24.30 and 25.52 min, respectively). For embryos showing the first division at 24 h after IVF (Fig. 5c), the time of division was consistent with the highest value of the oxygen consumption peak. It is important to notice that there is a discrepancy of 7–9 min between the image acquisition and oxygen measurement for each data point, with the image being obtained before the oxygen measurement.

Mean oxygen-consumption rates of zygotes that have undergone the first cleavage are shown in Table 1. Mean oxygen-consumption rates at the time of cleavage were affected by the time of measurement ($P < 0.05$). Considering only five points of measurement, there was a significant drop in oxygen consumption from ~72 min before cleavage, which indicated that the small peak of oxygen consumption occurred ~72 min before cleavage.

**ROS levels within in vitro matured oocytes and presumptive zygotes**

Pixel intensity determined following incubation of oocytes and zygotes with REDOX-Sensor Red CC-1 ($P < 0.05$) was influenced by the interaction of group and area measured. Pixel-intensity measurements can be observed in Fig. 6, but for simplification purposes only the
temporal measurements (groups) within each area are statistically compared in the graphic. A clear temporal effect in REDOX state was observed, with zygotes measured at 7 h and at 24 h after IVF showing a significantly higher mean pixel intensity compared with oocytes and zygotes evaluated at 0, 12 and 17 h after IVF (Fig. 6).

Furthermore, three significantly different patterns of red-fluorescent dye penetration emerged: (i) For unfertilized oocytes (Group A) measured at 0 h, the inner area of the oocytes had the lowest pixel intensity and the outer area showed the highest pixel intensity (Fig. 7a); (ii) For zygotes of Groups B–D, measured at 7, 12 or 17 h, it was revealed that the inner area had the lowest pixel intensity of the three areas but significant differences were not observed between the intermediate and the outer area (Fig. 7b); (iii) For zygotes of Group E measured at 24 h, an even distribution of the red-fluorescent product over the outer, intermediate and inner areas could be demonstrated (Fig. 7c).

The regions (1–4) within each area of the oocyte or zygote also affected mean pixel intensity, with region 2 showing the highest pixel intensity ($101.50 \pm 8.31^a$, $113.67 \pm 8.79^b$, $111.92 \pm 8.72^b, c$).
Discussion

In the present study, the individual oxygen consumption of bovine oocytes, fertilized zygotes and cleaving embryos were assessed at short intervals after IVF, without perturbing incubation conditions. Digital images of individual bovine embryos were acquired before the oxygen measurements and allowed a direct correlation between oxygen consumption and developmental stage. Bovine embryos follow similar patterns of metabolism and kinetics (specifically timing of pronuclear formation and first division), when compared with human embryos. Embryos of both species are also of similar size, being the higher lipid content of the cytoplasm the major difference between them. Consequently, the bovine model was considered appropriate for evaluating the oxygen consumption at key temporal events, and results might give an indication of the oxygen consumption changes during human early embryo development.

The data provided evidence of the existence of one major peak of oxygen consumption observed at the time of fertilization. Our hypothesis could be confirmed by the observation that unfertilized mature oocytes had a significantly lower oxygen consumption at 7 h (measured with the Nanorespirometer; Fig. 3) or a different oxygen-consumption pattern, with no detectable peaks (measured with the Embryoscope; Fig. 4). Coincident with the oxygen peaks, higher levels of ROS were identified at 7 and 24 h after IVF (Fig. 6). Similar findings were described by Heinecke and Shapiro (1992), who monitored the oxygen consumption of sea urchin eggs following fertilization, using a Clark-type oxygen electrode. At the time of fertilization, they observed a respiratory burst which was quantitatively

and 100.75 ± 9.06a for regions 1–4, respectively; a,b,c P < 0.05; values with different superscripts differ significantly).

Figure 5 Mean oxygen-consumption rates of embryos with the first cleavage at (a) 22 h, (b) 23 h, (c) 24 h and (d) 25 h after IVF. Time-lapse images were acquired during the measurements with the Embryoscope at intervals of ~36 min and used for evaluation of developmental progress (the time of the first cleavage is indicated by the red symbol).

Table 1 Mean oxygen-consumption rate of bovine zygotes (n = 34) in relation to the time of first cleavage.

<table>
<thead>
<tr>
<th>Time in relation to first cleavage (min)</th>
<th>Mean oxygen-consumption rate (nl/h)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>0.4988a</td>
<td>0.0099</td>
</tr>
<tr>
<td>36</td>
<td>0.4911b,c</td>
<td>0.0107</td>
</tr>
<tr>
<td>0</td>
<td>0.4920b,d</td>
<td>0.0100</td>
</tr>
<tr>
<td>36</td>
<td>0.4925b</td>
<td>0.010</td>
</tr>
<tr>
<td>72</td>
<td>0.4873c,d</td>
<td>0.0099</td>
</tr>
</tbody>
</table>

a,b,c, P < 0.05.
accounted for by H$_2$O$_2$ production. Nasr-Esfahani et al. (1991) have also shown a rise in ROS production at the time of fertilization in mice embryos.

It is now well established that ROS, in particular H$_2$O$_2$, are key signalling molecules that can influence cell proliferation, cell death, gene expression and are involved in the activation of several signalling pathways (Hancock et al., 2001). ROS are produced in the cytoplasm by oxidases and by the mitochondria (as a consequence of electron transport required for oxidative phosphorylation) (Nohl, 1994). The increase in oxygen consumption and ROS observed in our study around the time of fertilization suggests this may involve mitochondrial activity stimulated by sperm entry. In fact, several studies using ascidia eggs and mice embryos have established a correlation between increased oxygen consumption and Ca$^{2+}$ pulses (Dumollard et al., 2003, 2004, 2007; Van Blerkom et al., 2003). These authors suggested that the repetitive sperm-triggered Ca$^{2+}$ waves appear to activate mitochondrial activity and thereby increase oxygen consumption, which was also corroborated in our study. Thus, we could document and confirm the relationship between the REDOX flux and oxygen consumption around the time of sperm penetration. An alternative hypothesis is that sperm-derived NADPH oxidase initiates a wave of REDOX activity. Oocyte developmental competence is related indirectly to the levels of NADPH, as a function of the role of glutathione as a reducing agent (e.g. de Matos et al., 2002). In both scenarios, it would appear that the probable function of a REDOX pulse is to attenuate Ca$^{2+}$ signalling.

We observed that the mean oxygen-consumption rate acquired 7 h after IVF by the Nanorespirometer was significantly lower for unfertilized in vitro matured oocytes compared with zygotes and that similar findings could not be observed when using the Embryoscope. That could possibly be explained by the different status of the embryos in the two different settings (stressed or dormant) or by the different incubation conditions experienced by the embryos in the two distinct devices.

Following the first peak (which lasted from ~7–11 h after IVF), oxygen consumption decreased. Using the Nanorespirometer, we
were able to confirm that oxygen consumption was associated with pronuclei formation (oxygen consumption of cohorts of embryos measured at 12 or 17 h after IVF when pronuclei formation was subsequently observed; Fig. 3b). Similarly, Pantaleon et al. (2001), demonstrated that pronuclei formation after fertilization in mice was accompanied by increased glycolysis and glucose oxidation through the pentose phosphate cycle. These authors argued that this was a sperm-mediated effect, as parthenogenesis did not affect metabolism.

On the contrary, in the data acquired with the Embryoscope we were not able to identify a peak of oxygen consumption which was consistent with pronuclei formation. From observation of Fig. 5, one could possibly speculate about the existence of a minor peak around 15–17 after IVF. However, it could not be confirmed whether that increase in oxygen consumption corresponded in fact to the event of pronuclei formation, as accumulation of lipid droplets in the cytoplasm of bovine embryos obscures subcellular structures, and images acquired by the camera could not detect their presence. Despite the fact that pronuclei formation occurs most likely between 12 and 17 h after IVF, as previously described for bovine embryos (Comizzoli et al., 2000), the measurements continued uninterrupted until 30 h after IVF and thus it was not possible to identify pronuclei by staining. Considering the data retrieved by the Nanorespirometer irrespectively of pronuclei formation (Fig. 3a), it is clear that there is a trend (dashed line from Fig. 3a showing the trend) for an oxygen consumption profile which has strong similarities with the profile obtained from the repeated measurements using the Embryoscope. A smaller rise and fall in oxygen consumption was observed at the time of the first embryonic cleavage (between 22 and 25 h after IVF; Figs. 4 and 5). Similarly, oxygen consumption peaks were also shown to be coincident with the first cell division in a pilot study using an earlier version of the Embryoscope, which performed measurements of oxygen consumption at 20-min intervals (Callesen, unpublished observations). Both studies showed similar oxygen-consumption rates for embryos at similar developmental stages. This rise in oxygen consumption was not observed in the data retrieved with the Nanorespirometer, but in the cohort of embryos measured at 24 h after IVF using that device, the first cleavage had not yet occurred.

As earlier pointed out, the difference of ~0.1 nl/h observed between the rates acquired with the two instruments can possibly be due to the different incubation conditions, as fluctuations in temperature inside the Embryoscope were ~0.1°C, whereas in the Nanorespirometer these were of ~0.3°C. Furthermore, the gas composition was more accurately defined in the Embryoscope as embryos in the Nanorespirometer were held in a semi-closed system, under sub-optimal culture conditions. Moreover, the type of media and material used for the measuring unit could have interfered with the final results: embryos were loaded in glass structures in the Nanorespirometer and in plastic slides in the Embryoscope.

In our study, we were unable to detect any change in the REDOX state as a consequence of pronuclei formation. The use of another REDOX probe, such as 2',7'-dichlorodihydrofluorescein diacetate (DCDHF DA) fluorescent assay (Nasr-Esfahani et al., 1990), may provide a clearer picture of changes in ROS levels during these early stages of development.

Finally, we have described a significant increase in ROS levels at 24 h after IVF. The cohort of zygotes measured at 24 h after IVF with the Nanorespirometer was still at the 1-cell stage but the ROS levels were considerably higher than in other groups (Fig. 6). It is possible that this cohort of zygotes was on the verge of dividing, but that could not be demonstrated. This increase in ROS at 24 h after IVF is coincident with a respiratory peak of lower intensity observed in a cohort of embryos measured with the Embryoscope prior to the first cell division, between 22–25 h after IVF (Fig. 4, Table I). Morado et al. (2009) also demonstrated that ROS levels were significantly higher in 2-cell embryos than in in vitro matured bovine oocytes. The increase in oxygen consumption observed at the time of first cleavage is associated with a higher energy demand for the initiation of the process of cell division. This increase in metabolic activity triggered by the first cleavage is likely to be a consequence of increased mitochondrial activity. And since mitochondrial oxidative phosphorylation is the major source of ATP production at this stage (Leese, 1995; Thompson et al., 1996), oxygen consumption and ROS production would be increased. Increases in oxygen consumption and ROS can also be associated with other REDOX processes, for example xanthine oxidase and NADPH oxidase activity.

In conclusion, there are some specific events during embryo development that appear to be associated with a change in oxygen consumption and REDOX state, indicating that both have a role in sperm-mediated oocyte activation and cell cleavage in bovine embryos. Much work has been published on the negative effects of ROS production during in vitro embryo production (e.g. reviews by Johnson and Nasr-Esfahani, 1994; Guérin et al., 2001; Bedaiwy et al., 2004). Nevertheless, this work demonstrates that changes in ROS production during development are positively associated with early development, and therefore the use of significant levels of antioxidants during fertilization and culture should be considered judiciously.

Authors’ roles

A.S.L., M.L. and J.G.T. were equally involved in the literature review, design of the experiment, data acquisition, interpretation and analysis, manuscript preparation and approval.

Acknowledgements

The authors would like to thank Annie Whitty for their technical assistance. The current study was performed in collaboration with Unisense Fertilitetch, Aarhus, Denmark who provided the Nanorespirometer and the Embryoscope for the oxygen measurements. Dr Henrik Callesen is thanked for consent to print unpublished data from his laboratory, Dr Gabriel Dalvit for critical discussions of the manuscript and Dr Niels Rasmig (Unisense) is acknowledged for expert assistance with both technologies and data analyses.

Funding

This work was funded by the National Health and Medical Research Council (NHMRC) of Australia. A.S.L. was supported by a grant from FCT, Ministry of Science and Technology, Portugal (SFRH/ BPD/26397/2006).
References


Lopes AS, Dalvit GC, Lane M, Thompson JG. Peaks of respiration and ROS production coincide with the time of fertilization and cell cleavage in bovine oocytes. Hum Reprod 2008;23(Suppl. 1):152.[abstract].


Trimarchi JR, Liu L, Porterfield DM, Smith PJ, Keefe DL. Oxidative phosphorylation-dependent and—independent oxygen consumption...
by individual preimplantation mouse embryos. Biol Reprod 2000; 


