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# Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos

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**Summary.** Two-cell sheep embryos and 2–4-cell and 8-cell cow embryos were cultured for 5 days in stoppered test-tubes in Synthetic Oviduct Fluid supplemented with 32 mg BSA/ml. The medium had been previously equilibrated with one of the following $O_2$ concentrations (sheep: 0, 2, 4, 6, 8, 10, 12, 17, 20%; cow: 0, 4, 8, 12, 17, 20%). At the end of culture embryos were examined for morphology and stained to assess numbers of nuclei. Mean (±s.e.m.) nuclei/embryo was highest at 8% $O_2$ for sheep embryos (23·6 ± 3·1), 4% for 2–4-cell cow embryos (23·2 ± 6·1) and 8% for 8-cell cow embryos (29·6 ± 5·2). The minimum number of nuclei/embryo occurred at 20% $O_2$ in each case (10·3 ± 0·9, 10·3 ± 2·7, 14·5 ± 2·4, respectively) with similar values also recorded at 0% $O_2$ (10·8 ± 1·9, 16·5 ± 6·0, 14·6 ± 2·4, respectively). Analysis of the proportion of embryos reaching at least the morula stage demonstrated a significant quadratic component for the different oxygen concentrations for sheep ($P < 0·01$) and cow ($P < 0·05$) embryos. A number of sheep and cow embryos showed abnormalities, suggesting that the culture conditions require further refinement.

The results confirm that, under lowered oxygen levels, development of sheep and cattle embryos can occur through the 8- to 16-cell block in a simple defined medium without somatic cell support.

*Keywords:* sheep; cattle; embryos; oxygen; culture

## Introduction

Little is known of the requirements for development of preimplantation sheep and cattle embryos *in vitro*. It has been established that embryos from these species often fail to develop past the 8- to 16-cell stage in defined media under standard incubation conditions such as 37–39°C under 5% $CO_2$ in air (Wright & Bondioli, 1981). Thus the fourth cell cycle appears to be critical for development and does not proceed normally under in-vitro conditions (Gandolfi & Moor, 1987). Successful development through this stage can be achieved when embryos are incubated in co-culture with somatic cells, in particular oviduct epithelial cells (Gandolfi & Moor, 1987; Rexroad & Powell, 1988; Eyestone & First, 1989). However, it has also been reported that development of sheep, goat and cattle embryos can be achieved without somatic cell support if incubated in defined media under a gas atmosphere of 5% $CO_2$, 5% $O_2$, 90% $N_2$ (Tervit *et al.*, 1972; Tervit & Rowson, 1974; Quinn *et al.*, 1984; Walker *et al.*, 1988). Indeed, Tervit *et al.* (1972) reported that oxygen concentrations of 0, 5 or 10% were superior to air in terms of embryo development. However, other investigators have shown no beneficial effects of incubating sheep embryos in a reduced oxygen atmosphere (Wright *et al.*, 1976; Betterbed & Wright, 1985).

In this paper we examine the development of sheep and cattle embryos cultured in stoppered tubes under various gas atmospheres with a reduced oxygen concentration relative to air.

## Materials and Methods

***Media and chemicals.*** The basic medium used for all embryo culture work was Synthetic Oviduct Fluid supplemented with 32 mg crystallized and lyophilized bovine serum albumin/ml (Sigma, St Louis, MO, USA; SOF + BSA) as described by Tervit *et al.* (1972). The medium used for the recovery and handling of ova was an *N*-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes; Sigma)-buffered SOF supplemented with 3 mg BSA/ml (Hepes-SOF), in which the $NaHCO_3$ concentration was reduced from 25 to 5 mM and replaced with 20 mM-Hepes. Sodium pyruvate, sodium lactate and $CaCl_2$ were from the Sigma Chemical Company. All other components were from BDH Chemicals (Poole, Dorset, UK).

***Preparation of culture vessels.*** Gases containing oxygen concentrations of 0, 2, 4, 6, 8, 10, 12, 17 and 20% plus 5% $CO_2$ with the balance made up with $N_2$ were supplied as individual cylinders of gas (NZIG, Wellington, New Zealand). To equilibrate the medium with the appropriate gas atmosphere, 1-ml samples of SOF + BSA in 5 ml Pyrex test-tubes (BDL, Lincoln Park, NJ, USA) were vigorously gassed for 2 min. Gases were passed through a 0·2 μm filter (Millipore, Bedford, MA, USA) and a 20-gauge hypodermic needle positioned inside the test-tube but above the surface of the medium to prevent excessive bubbling. At the end of each gassing, the test-tube was tightly stoppered with a rubber bung. Tubes were then incubated overnight at 39°C before embryo culture. Once embryos were allocated to test-tubes for culture, each tube was regassed for 30 sec with the appropriate gas mixture.

***Collection and culture of sheep embryos.*** Embryos for culture were obtained from mature, mixed breed ewes superovulated and mated to entire rams. On Day 2–2·5 after detection of oestrus, ewes were slaughtered at an abattoir and the reproductive tracts were immediately flushed with warm Hepes-SOF. Ova were isolated from flushings and 2-cell embryos pooled and transported to the laboratory. On arrival, embryos were washed four times in Hepes-SOF, then once in SOF + BSA before random allocation to a gas atmosphere.

The 2-cell embryos ($n$ = 126, 4 replicates per treatment of 3–4 embryos per tube) were incubated for 5 days at 39°C under the 9 different oxygen concentrations. At the end of culture, embryos were recovered and general morphology was assessed under a dissecting microscope. Embryos were then stained with Hoechst 33342 (Pursel *et al.*, 1985) or aceto-lacmoid to evaluate numbers of nuclei. The proportion of embryos that had reached at least the morula stage (24 nuclei or more) was examined by regression analysis after logit transformation.

***Collection and culture of cow embryos.*** Embryos for culture were obtained from superovulated Friesian and Jersey cows artificially inseminated with frozen–thawed semen (Livestock Improvements Association, Newstead, New Zealand). On Day 2–3 after detection of oestrus the cows were slaughtered and embryos were collected and handled in the same manner as for sheep embryos, with the exception that 4- and early 8-cell embryos were obtained and utilized as well as 2-cell embryos.

The 2–4-cell embryos ($n$ = 61, 3 replicates per treatment of 3–4 embryos per tube) and early 8-cell embryos ($n$ = 99, 3 replicates per treatment of 5–7 embryos per tube) were incubated for 5 days at 39°C under 6 different oxygen concentrations (0, 4, 8, 12, 17 and 20%). At the end of culture, embryos were evaluated as for sheep embryos. The proportions of 2–4-cell embryos that had reached at least the morula stage (24 nuclei or more) and of 8-cell embryos that were even more advanced (32 nuclei or more) were examined separately by regression analysis after logit transformation.

***Determination of $O_2$* concentration in media.** To determine the effectiveness of the gassing regimen, an oxygen electrode (Diamond Electro-Tech, Ann Arbor, MI, USA) was used to evaluate oxygen concentration in culture media after gassing for 30 sec or 2 min with 0 or 12% $O_2$ gas mixtures. A further experiment was conducted to determine whether oxygen levels in the medium changed after incubation for 5 days. Tubes were equilibrated with 0% ($n$ = 8) or 12% ($n$ = 7) $O_2$ for 2 min, then tightly stoppered. Determination of $O_2$ levels with an oxygen electrode was performed following incubation for 5 days at 39°C.

## Results

### Culture of sheep embryos

Development of sheep embryos from the 2-cell stage to morula/blastocyst stages was observed after incubation under reduced oxygen concentrations in SOF (Fig. 1). Using logit-transformed proportions, regression analysis demonstrated a significant quadratic component for the different oxygen concentrations ($\chi^2_1$ = 10·4, $P < 0·01$, Fig. 1). Concentrations of 6–10% appeared to be superior for supporting embryo development to the morula stage. Poor development was observed at the 0% as well as the 20% $O_2$ concentration, indicating that oxygen is required for development, but at levels lower than that found in air. Mean (±s.e.m.) nuclei/embryo were 10·8 ± 1·9,

20·7 ± 5·2, 23·0 ± 2·7, 19·1 ± 2·2, 23·6 ± 3·1, 19·2 ± 2·2, 17·7 ± 2·0, 19·7 ± 2·1 and 10·3 ± 0·9 for the 9 oxygen concentrations from 0 to 20% respectively.

The morphological appearance of embryos after in-vitro culture was usually poorer than for equivalent stages *in vivo*. Abnormalities observed included anucleate fragments, degenerate blastomeres, and many compact morulae with blastomeres outside the cell mass and lower than expected numbers of cells making up the mass.

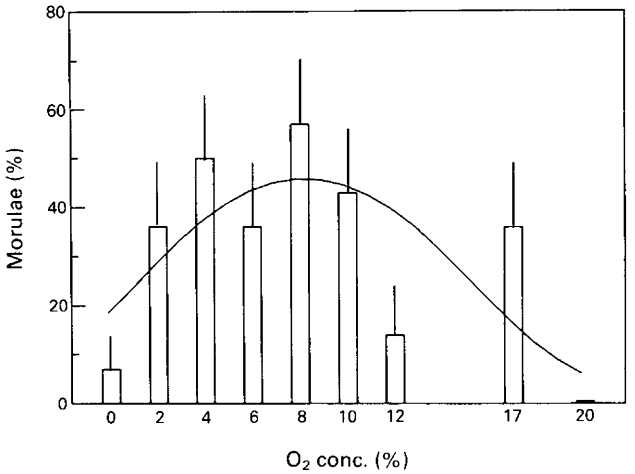


**Fig. 1.** Proportion of sheep 2-cell embryos developing to morulae (≥24 nuclei) during culture for 5 days in SOF + 32 mg BSA/ml under different oxygen concentrations. Bars represent standard errors. The line represents the back-transformed estimates from the quadratic regression.

### Culture of cattle embryos

As with sheep embryos, development of 2–4-cell and early 8-cell cattle embryos to morula/blastocyst stages occurred under reduced oxygen atmospheres (Fig. 2). Using logit-transformed proportions, regression analysis demonstrated no difference between the proportion of 2–4-cell embryos reaching the morula stage and 8-cell embryos reaching the compacting morula stage. As for sheep embryos, there was a significant quadratic component for the different oxygen concentrations ($\chi^2_1 = 4{\cdot}3$, $P < 0{\cdot}05$, Fig. 2). Concentrations of 4–12% oxygen in the atmosphere appeared best to support development to the morulae and compacting morulae stages. Oxygen levels of 0 and 20% did not readily support development. Mean (±s.e.m.) nuclei/embryo were 16·5 ± 6·0, 23·2 ± 6·1, 16·3 ± 2·9, 15·4 ± 2·2, 13·5 ± 2·4 and 10·3 ± 2·7 for the 6 oxygen concentrations from 0 to 20% respectively for 2–4-cell embryos and 14·6 ± 2·4, 20·8 ± 3·3, 29·6 ± 5·2, 21·9 ± 3·9, 20·2 ± 2·5 and 14·5 ± 2·4 for the 8-cell embryos. As with the sheep embryos, some embryos showed abnormalities.

### Oxygen equilibration after gassing

Measured oxygen concentrations (% mean ± s.e.m., $n = 10$) after gassing for 30 sec and 2 min were 5·1 ± 0·8 and 0·3 ± 0·2 with 0% $O_2$, and 15·1 ± 0·2 and 12·9 ± 0·3 with 12% $O_2$, respectively. The pH of the medium (7·3–7·5) had equilibrated after gassing for 2 min. Furthermore, incubation for 5 days at 39°C did not affect oxygen tension. Measured oxygen levels for media gassed with 0% $O_2$ were approximately 0% in all cases and ranged from 11 to 12·5% for media gassed with 12% $O_2$.

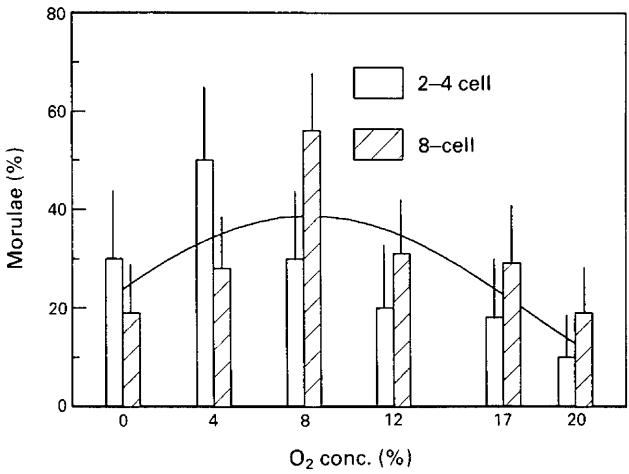


**Fig. 2.** Proportion of cow 2–4-cell embryos developing to morulae (≥24 nuclei) and 8-cell embryos developing to compacting morulae (≥32 nuclei) during culture for 5 days in SOF + 32 mg BSA/ml under different oxygen concentrations. Bars represent standard errors. The line represents the back-transformed estimates from the overall quadratic regression.

## Discussion

The results of our experiments demonstrate that, under the incubation conditions described here, oxygen concentration in the atmosphere is a factor involved in the development of sheep and cattle preimplantation embryos. Furthermore, our work supports the original observations of Tervit *et al.* (1972) that an oxygen concentration around 5–10% is optimal for development of sheep and cattle embryos *in vitro*. Similar studies utilizing early mouse embryos demonstrated that 5% $O_2$ was optimal for development (Whitten, 1971; Quinn & Harlow, 1978). However, other studies have not been able to demonstrate an effect of reduced oxygen concentration (5%) on the development of sheep embryos (Wright *et al.*, 1976; Betterbed & Wright, 1985). Differences between media and incubating conditions (i.e. culturing in microdrops under oil) may explain the different findings between the present study and those of others.

To our knowledge, the $pO_2$ tension in the sheep oviduct is not known. However, Mastroianni & Jones (1965) reported that the $pO_2$ tension in the rabbit oviduct is approximately 60 mmHg (7·8%), which falls within our suggested range of 6–10%.

We have found that gassing for 2 min was adequate to equilibrate oxygen levels (and pH) in the media. However, it is unlikely that true anaerobic conditions were created by gassing with 0% $O_2$ for this period of time. This may explain why development did occur in some replicates of this treatment.

The mechanism by which oxygen concentration in the atmosphere affects development *in vitro* remains to be elucidated. There is some evidence that developmental blocks during in-vitro culture are associated with metabolic processes. In hamsters, Schini & Bavister (1988) have demonstrated that the 2-cell block to development can be overcome if glucose and inorganic phosphate ions are deleted from the medium. Increased development of 8-cell embryos to blastocyst stages is also achieved in the absence of glucose and inorganic phosphate (Seshagiri & Bavister, 1989a, b). These authors suggest that inadequate energy generation is the cause of retarded development and is due to the Crabtree effect, whereby phosphate ions stimulate glycolysis and thus compete with mitochondrial respiration. There is also evidence that oxygen concentration may affect energy substrate

utilization in embryos. Khurana & Wales (1989) have demonstrated that a reduced oxygen atmosphere (1–5%) is necessary to promote the utilization of endogenous glycogen pools in cultured mouse morulae.

Auto-oxidation by oxygen-free radicals and lipid radicals is a major cause of cell damage and retarded cell growth during culture (Joenje, 1989). Lipid radicals can alter metabolic pathways, inhibiting proliferation of cells in culture (Hornsby & Gill, 1981). Lowering the oxygen concentration, or providing protection by the addition of antioxidants, reduces this effect (Hornsby & Gill, 1981; Hornsby, 1982). We suspect that a similar process occurs in cultured embryos. Incubating under reduced oxygen concentrations may reduce the auto-oxidative damage and provide adequate energy production to support development. Sheep and cattle embryos may be particularly susceptible to oxidative damage at the 8-cell stage, when transcription of the embryonic genome is activated (Crosby *et al.*, 1988).

Attention is drawn to the observation that, although development was improved under physiological oxygen concentrations, embryos cultured in SOF + 32 mg BSA/ml appeared to show more abnormalities than did embryos of equivalent stage recovered after development *in vivo*. Improved culture conditions, such as incubating in multiwell dishes (Thompson *et al.*, 1989), alterations to the chemical composition of the medium, or the addition of human serum as described by Walker *et al.* (1988), growth factors or possibly other mitogenic factors associated with oviduct epithelial co-cultures (Gandolfi & Moor, 1988), may improve the morphological appearance, and probably the viability, of cultured sheep and cattle ova.

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