Culture of rabbit zygotes into blastocysts in protein-free medium with one to twenty per cent oxygen

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Embryos were collected from superovulated Dutch rabbits 19 h after injection of LH and insemination. The embryos were at the one-cell stage at that time and those judged to be normal by the absence of granular cytoplasm and regular shape were distributed randomly within donors into culture dishes containing 500 μ l of a macromolecule-free medium consisting of RPMI-1640 and low glucose Dulbecco's modified Eagle's medium, 1:1, without a cover of oil. In Expt 1, O₂ concentrations of 5, 10 and 15%, with 5% CO₂ plus 90, 85 and 80% N₂, respectively, were tested. In Expt 2, O₂ concentrations of 1, 5 and 20% were combined with 5% CO₂ and the remaining gas was N₂. After 84 h in culture at 39°C, embryos were examined for stage of development and stained with Hoechst 33342 so that the number of cells could be counted. In Expt 1, the proportion of embryos reaching the hatching blastocyst stage after 84 h in culture in 5, 10 and 15% O₂ was 48, 38 and 21%, ($P < 0.01$) and the cell number per embryo averaged 258, 226 and 188, respectively ($P < 0.01$). In Expt 2, the proportion of hatching embryos after 84 h in culture in 1, 5 and 20% O₂ was 67, 72 and 29% ($P < 0.01$), respectively. Cell numbers in the 1 and 5% O₂ concentrations were higher than in the 20% O₂ concentration ($P < 0.01$). These results indicate that reduction of O₂ concentration to 5%, well below the frequently used concentration of about 20% O₂ in 95% air, is beneficial to rabbit embryo development from the zygote to the blastocyst stage. The O₂ concentration may be more critical with simple defined macromolecule-free media than in media containing serum.

Introduction

Good development of rabbit embryos in culture is possible with the use of a defined protein-free medium (Carney and Foote, 1991). However, development into blastocysts *in vitro* is inferior to preimplantation development *in vivo*, indicating that culture conditions are not optimal. This could be due to many factors, such as (1) deficiencies of some components in the medium, (2) toxic substances or toxic imbalances in the medium, (3) accumulation of toxic end products, (4) a suboptimal gaseous environment or (5) a combination of factors.

A common gaseous environment for embryo culture is 5% CO₂ and 95% humidified air (Wright and Bondioli, 1981; Biggers, 1987). Although O₂ is important for energy producing metabolism, and O₂ uptake increases as the embryo reaches the blastocyst stage in rabbits (Fridhandler, 1961) and mice (Mills and Brinster, 1967; Benos and Balaban, 1980), the O₂ concentration in air exceeds the concentration of 5–8% in the rabbit oviduct (Bishop, 1956; Mastroianni and Jones, 1965; Bavister and Fischer, 1991). The rabbit embryo passes into the uterus before the blastocyst stage, so development during cleavage stages *in vivo* is in the presence of 5–8% O₂.

The use of 5–10% O₂, or lower, with 5% CO₂ has been reported to be advantageous for preimplantation embryos of mice (Auerbach and Brinster, 1968; Whitten, 1971; Quinn and Harlow, 1978; Pabon *et al.*, 1989; Umaoka *et al.*, 1992), hamsters (McKiernan and Bavister, 1990), sheep (Tervit *et al.*, 1972; Thompson *et al.*, 1990), pigs (Wright, 1977) and cattle (Tervit *et al.*, 1972; Wright *et al.*, 1976a; Nakao and Nakatsuji, 1990; Voelkel and Hu, 1992). However, no advantage of the lower O₂ concentration was reported for sheep by Wright *et al.* (1976b) and Betterbed and Wright (1985) or for cats (Johnston *et al.*, 1991). When Voelkel and Hu (1992) cocultured bovine embryos with buffalo rat liver cells, 20% O₂ was superior to 5% O₂. Concentrations of O₂ higher than 20% have been shown to be detrimental to mice (Quinn and Harlow, 1978) and rabbit embryos (Brackett, 1978).

The O₂ concentration in the 95% air:5% CO₂ system approaches 20% and this could result in various types of radical formation which might be toxic to the embryo under some *in vitro* conditions (Ellis, 1991). This factor could cause more detrimental effects in defined protein-free media where there is less chance for effects of free radicals to be ameliorated by high molecular weight proteins. Because of the lack of *in vitro* studies in the rabbits, the known low concentration of O₂ in the rabbit oviduct and the recent ability to culture rabbit one-cell embryos to expanding blastocysts in macromolecule-free media, this study on various O₂ concentrations was initiated.

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Received 12 June 1992.

Table 1. Culture of rabbit zygotes for 84 h in RD medium (RPMI 1640:DMEM, 1:1) with various oxygen concentrations

O ₂ concentration (%)	No. of embryos	Number of embryos at stage of development in culture (%)			Total (%)
		Early blastocyst	Expanding blastocyst	Hatching blastocyst	
5	48	9 (19) ^a	16 (33) ^a	23 (48) ^a	100
10	48	14 (29) ^a	16 (33) ^a	18 (38) ^a	100
15	48	26 (54) ^b	12 (25) ^a	10 (21) ^b	100
		$P < 0.01$	$P > 0.05$	$P < 0.01$	
Number of cells at 84 h*					
5	30	258 ^a			
10	30	226 ^b			
15	30	188 ^c	$P < 0.01$		

^{a,b,c}Numbers within columns with different superscripts are significantly different.

*All embryos from a subset of four rabbits were examined.

Materials and Methods

Medium

Culture medium was similar to the RD medium of Carney and Foote (1991), but it contained the low glucose Dulbecco's modified Eagle's medium (DMEM, 1 g l⁻¹) mixed 1:1 with RPMI 1640, designated as RD medium. The final concentration of glucose was 1.5 g l⁻¹. This medium was stored at 5°C and before use, 2.85 µg sodium bicarbonate was added per millilitre of RD medium. This medium was equilibrated at 39°C overnight in 4-well dishes (Nunc, Kastrup, Denmark) under the same gas conditions used for culturing embryos.

Animals

Dutch-belted rabbits were superovulated by a standard FSH-LH schedule (Kennelly and Foote, 1965) and inseminated at the time of LH injection. This was timed so that 19 h later the oviducts were flushed with phosphate-buffered saline containing 1 mg polyvinyl alcohol (PVA) ml⁻¹ and one-cell zygotes were obtained in an air atmosphere. These zygotes were washed twice with RD medium. Normal zygotes from each donor rabbit, as judged by the absence of granular cytoplasm and presence of sharply defined perivitelline membrane, were randomly transferred as soon as possible into 4-well dishes with each well containing 500 µl of RD medium. Embryos were evaluated briefly after 24 h to determine whether any that were classified initially as zygotes were actually unfertilized oocytes. This occurred infrequently, but any such oocytes were removed.

Culture

The culture dishes were placed in tissue culture chambers with a water-filled dish to supply humidity; the chamber was regassed for 5 min with the appropriate gas, sealed and incubated at 39°C, the approximate body temperature of rabbits.

The medium was replaced with fresh equilibrated medium after 48 h.

Evaluation

After 84 h, embryos were evaluated for stage of development and general appearance. A subset of all embryos from four of six donor rabbits in each experiment was stained with Hoechst 33342 and the number of cells was counted with the aid of an epifluorescent microscope.

Experimental design

In Expt 1 three gaseous environments of 5, 10 or 15% O₂, each with 5% CO₂ and 90, 85 or 80% N₂, respectively, were used. In Expt 2, 1 and 5% O₂, each with 5% CO₂ and the balance consisting of N₂ were compared with the control environment of 5% CO₂:95% air. Each experiment was replicated across six zygote donors, with two donors used per day on three occasions. Zygotes from each donor were randomly distributed across all treatments. Cell number data were analysed by analysis of variance, in a mixed linear model with rabbits a random variable and treatments fixed. Differences among means were compared, using Tukey's HSD for pairwise comparisons. The proportions of embryos developing into blastocysts were analysed by chi-square.

Results

Experiment 1

Embryos developed into blastocysts in all treatments (Table 1). The high development rate occurred when males and females used were very fertile. A higher proportion of embryos cultured with 5 and 10% O₂ reached the hatching blasto-

Table 2. Culture of rabbit zygotes for 84 h in RD medium (RPMI 1640:DMEM, 1:1) with various oxygen concentrations

O ₂ concentration (%)	No. of embryos	Number of embryos at stage of development in culture (%)			Total (%)
		Early blastocyst	Expanding blastocyst	Hatching blastocyst	
1	48	6 (12) ^a	10 (21) ^a	32 (67) ^a	100
5	50	5 (10) ^a	9 (18) ^a	36 (72) ^a	100
20	49	10 (20) ^b	25 (51) ^b	14 (29) ^b	100
		$P < 0.05$	$P < 0.01$	$P < 0.01$	
Number of cells at 84 h*					
1	35	252 ^a			
5	30	262 ^a			
20	32	204 ^b	$P < 0.01$		

^{a,b}Numbers within columns with different superscripts are significantly different.

*All embryos from a subset of four rabbits were examined.

cyst stage during the 84 h culture period, while more embryos cultured with 15% O₂ had developed only to the early blastocyst stage ($P < 0.01$). Thus, there was a difference in rate of development. Cell numbers also decreased as O₂ increased ($P < 0.01$).

Experiment 2

There was no difference in embryo development between treatments containing 1 and 5% O₂ ($P > 0.05$, Table 2). Both treatments resulted in embryos with higher cell counts and more hatching embryos after 84 h of culture than embryos in 95% air:5% CO₂ ($P < 0.01$). Thus, both experiments are in agreement indicating that embryonic growth *in vitro* is more rapid and probably superior in the presence of 5% O₂ than in 20% O₂.

Discussion

The majority of studies on O₂ concentrations for culturing mammalian embryos favour the use of less than 10% O₂ for mice (Auerbach and Brinster, 1968; Whitten, 1971; Quinn and Harlow, 1978; Pabon *et al.*, 1989; Umaoka *et al.*, 1992), or 5 to 10% O₂ for sheep (Tervit *et al.*, 1972; Thompson *et al.*, 1990), pigs (Wright, 1977) and cattle (Tervit *et al.*, 1972; Wright *et al.*, 1976a; Nakao and Nakatsuji, 1990; Voelkel and Hu, 1992). Hamster oocytes were reported to have a distinct optimum at 5% O₂ for maturation (Gwatkin and Haidri, 1974). In a study with rabbits, Brackett (1978) reported no difference in fertilization of oocytes or early development of embryos in the presence of 20 versus 48% O₂. However culture was for 24 h only. Quinn and Harlow (1978) and Pabon *et al.* (1989) observed a delayed inhibitory effect of 20% O₂ on mouse embryos that occurred after culture for more than 24 h. These latter workers found that exposure to 20% O₂ for as little as 1 h in medium

devoid of macromolecules caused inhibition of development of mouse embryos after four to five divisions. In that regard it was similar to the RD medium used for rabbits in the present study.

In other studies no beneficial effect was found when O₂ concentration was reduced (Wright *et al.*, 1976a; Betterbed and Wright, 1985; Johnston *et al.*, 1991). When bovine embryos were cocultured with buffalo rat liver cells, Voelkel and Hu (1992) found 20% O₂ to be superior to 5% O₂. Certain types of coculture system may require different O₂ concentrations for optimal results. In addition, it is likely that the optimal concentrations of O₂ differ according to culture media, species and stage of development. In the present studies the embryos were cultured without oil over the culture medium. However, it is likely that the results presented here apply to embryos cultured under oil, as Baltz and Biggers (1991) found that embryos cultured in microdroplets under silicone oil did not reduce oxygen tension significantly.

The improved development of rabbit embryos when cultured with 5% O₂ is consistent with O₂ concentrations reported in the rabbit oviduct (Bishop, 1956; Mastroianni and Jones, 1965; Bavister and Fischer, 1991). It is also consistent with studies of other cell types (Ellis, 1991) where oxygen free radicals are more prevalent under higher oxygen tension. Umaoka *et al.* (1991, 1992) reported that reduction of O₂ concentration from 20 to 5% and the addition of 500 µg superoxide dismutase ml⁻¹ increased the proportion of pronuclear mouse embryos developing into blastocysts during culture. Exposure of bovine embryos for as little as 1 h to 20% atmospheric oxygen, significantly decreased the rate of blastulation and addition of superoxide dismutase was beneficial (Noda *et al.*, 1991). The handling of embryos in air after removal from the female reproductive system until culture may therefore be crucial.

Blastocyst-stage embryos consume considerable O₂ and by 4 days produce H₂O₂ (Manes, 1992). It is not known whether this is toxic to the embryo, but this oxidizing agent appears to be toxic to totipotent cells in the inner cell mass of mice (Pierce *et al.*, 1991).

High O_2 concentrations may interfere with anaerobic glycolysis. Substrate utilization can be affected by O_2 concentration. Khurana and Wales (1989) reported that 1–5% O_2 in young mouse embryos is required to promote utilization of endogenous glycogen pools.

In the present studies we used a lower glucose concentration of 1.5 g l^{-1} in the RD medium than previously (Carney and Foote, 1991). However, this is still higher than 0.2 g l^{-1} found in oviductal fluid (Iritani *et al.*, 1971). Glucose was inhibitory to development of hamster eight-cell embryos (Seshagiri and Bavister, 1989). Omission of glucose from the media during the period of maternal genomic control of development was beneficial for culturing mouse (Chatot *et al.*, 1989) and cattle embryos (Ellington *et al.*, 1990). The lower glucose concentration was helpful in culturing embryos of these species past the two-cell block. The rabbit embryo does not have this culture block, and appears to be more tolerant to glucose concentration. Glucose utilization by the rabbit embryo is low until embryos reach the blastocyst stage (Fridhandler, 1961), but obviously rabbit embryos develop in the presence of substantial glucose in the medium.

In conclusion, the optimal O_2 concentration for culturing rabbit zygotes to hatching blastocysts in a macromolecule-free medium is about 5%. This concentration is similar to the content in rabbit oviductal fluid. The gaseous environment is a relatively simple component of the culture system to control. Because of the positive results obtained here and in other studies, indicating deleterious latent effects of high O_2 in other species following even relatively short exposure to air, it is suggested that minimal exposure to air during processing is desirable. The frequently used culture system of 5% CO_2 and 95% air should be discontinued for culture of rabbit embryos and should be critically tested in other embryo culture systems where currently used.

The authors acknowledge assistance of M. Simkin and L. Belanger and the NICHD National Cooperative Program on Non-human in Vitro Fertilization and Preimplantation Development, and was funded by the National Institute of Child Health and Human Development NIH, through Cooperative Agreement HD 21939. Vetrepfarm, Inc. (London, Ontario, Canada) kindly provided LH.

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