Improved development of head-fold rat embryos in culture resulting from low oxygen and modifications of the culture serum

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Previous work has shown that rat embryos can be grown in culture for periods of 1–3 days at any time during the 2nd week of gestation, i.e. throughout the period of major organogenesis. The cultured embryos undergo extensive growth and differentiation, and many of them compare well with embryos of equivalent age *in vivo*. But the rate of growth, as determined by assays of total protein, has usually been found to be lower than *in vivo* (Berry, 1968; Shepard, Tanimura & Robkin, 1970; Payne & Deuchar, 1972; Robkin, Shepard & Tanimura, 1972; New, 1973; Cockroft, 1973, 1976; Steele, 1975) and a higher proportion of the embryos show malformations (Morriss & Steele, 1974).

This paper examines three modifications of the previous culture methods which appeared in preliminary trials to give improved development of embryos explanted at the early head-fold stage (9½ days *post coitum*). Such embryos have previously been cultured in a gas phase of 5% CO₂ in air, and in a nutrient medium of rat serum prepared by standard methods of decanting from clotted blood. The modifications were (i) to obtain the culture serum from blood centrifuged immediately after extraction from the rat and before clotting had occurred, (ii) 'heat-inactivation' of the serum, and (iii) reduction of the oxygen concentration in the gas phase to 5% during the first 24 hr of the culture period.

Embryos of CFHB-strain rats were explanted with the visceral yolk sac and ectoplacental cone intact. The explants were incubated at 38°C in serum in small rotating culture bottles (method of New, Coppola & Terry, 1973). In all the 48-hr cultures, the culture bottles contained 1 ml serum/embryo. The serum was prepared from the blood of non-pregnant female or male CFHB rats and the serum samples from different rats were pooled before use. In comparing different culture treatments, embryos from each litter were divided between the different treatments to eliminate any effects of inter-litter variation.

Comparison of 'immediately centrifuged' and 'delayed centrifuged' serum

'Immediately centrifuged' (I.C.) serum was prepared as described by Steele & New (1974). Blood was centrifuged immediately after withdrawal from the rat to precipitate the cells. A white fibrin clot then rapidly formed in the supernatant. Then either (i) the clot was removed with forceps, the fluid being allowed to drain from it back into the centrifuge tube, which was recentrifuged and the I.C. serum decanted, or (ii) the entire contents of the tube were remixed and allowed to stand for 18 hr before recentrifuging and decanting to give the serum designated 'I.C.-(d)'. 'Delayed centrifuged' (D.C.) serum was prepared from blood allowed to clot and stand for 18 hr before centrifuging.

All the sera in this experiment were heat-inactivated (at 56°C for 30 min) before use. The culture period was 48 hr and the culture bottles were gassed with 5% O₂/5% CO₂/90% N₂ initially, followed by 20% O₂/5% CO₂/75% N₂ after 24 hr.

Table 1 shows that the embryos grown in I.C. serum developed better and synthesized more protein than those in D.C. serum. The *P* value for the difference in mean protein of both groups in I.C. serum compared with the group in D.C. serum is between 0.01 and 0.02, but the reasons for this difference are unknown. That length of time in contact with cells and clot is insufficient explanation for the harmful effects of D.C. serum is shown by the good growth obtained in I.C.-(d) serum.

Table 1. Effects of 'immediately centrifuged' (I.C.) serum, 'immediately centrifuged but delayed-decanted' (I.C.-(d)) serum, and 'delayed centrifuged' (D.C.) serum on the growth and development of 9½-day rat embryos cultured for 48 hr

Serum	Total no. of embryos	No. of embryos showing:				Mean yolk-sac diam. (mm)	Mean somite no.	Embryo protein* (µg) (mean ± S.E.M.)
		Blood circ.	Turning complete	Fused allan.	Closed N.T.			
I.C.	12	10	12	11	12	3.7	23.5	137 ± 9
I.C.-(d)	12	12	11	12	12	3.6	23.2	142 ± 10
D.C.	12	7	10	10	12	3.1	20.4	108 ± 11

Explanation of terms: blood circ. = a normal vigorous blood circulation in both embryo and yolk-sac at the end of the culture period; turning complete = rotation to the fetal position with the ventral surface concave; fused allan. = the extension of the allantois from the hind end of the embryo and its fusion with the chorion; closed N.T. = normal closure of the neural tube to form the brain and spinal cord.

* Embryo protein (without the membranes) was determined by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951).

Comparison of heat-inactivated and unheated serum

In this experiment only 'immediately centrifuged' sera were used. The culture period was 48 hr. The effects of heat-inactivating the serum (56°C for 30 min) were examined both in cultures gassed with 5% O₂/5% CO₂/90% N₂ initially followed by 20% O₂/5% CO₂/75% N₂ at 24 hr, and in cultures gassed with 20% O₂/5% CO₂/75% N₂ initially and at 24 hr.

Table 2 shows that the embryos grown in heat-inactivated serum developed better and synthesized more protein than those in the unheated control serum, under both conditions of oxygenation. Typical embryos after growth in the two types of serum are shown in Pl. 1, Fig. 1.

We conclude that the beneficial effects of heat-inactivation are independent of those of immediate centrifugation.

Table 2. Effects of heat-inactivation of the culture serum, and reduced oxygen, on the growth and development of 9½-day rat embryos cultured for 48 hr

Serum	O ₂	Total no. of embryos	No. of embryos showing:				Mean yolk-sac diam. (mm)	Mean somite no.	Embryo protein (µg) (mean ± S.E.M.)
			Blood circ.	Turning complete	Fused allan.	Closed N.T.			
Heat inactivated	5→20%	24	23	23	20	24	3.8	23.9	152 ± 8*
	20%	24	23	23	24	22	3.9	24.4	159 ± 8†
Control	5→20%	24	24	17	19	20	3.2	21.9	113 ± 7*
	20%	24	22	16	16	9	3.3	21.7	124 ± 8†

Abbreviations as in Table 1.

*† Significantly different, $P < 0.01$

Comparison of 5% O₂ and 20% O₂

Table 2 also compares the effects of exposure to 5% O₂ and 20% O₂ during the first 24 hr of culture. No significant difference was found in the development of the blood circulation, yolk sac and allantois, in the turning of the embryo, in the number of new somites formed or in the amount of protein synthesized, but a striking difference was noted in the development of the neural tube, particularly in the embryos grown in control serum. Of these, 15/24 showed a failure of closure of the neural tube in 20% O₂ but only 4/24 in 5% O₂. In heat-inactivated serum, the neural tube failed to close in 2/24 embryos in 20% O₂ but in none in 5% O₂. In the abnormal embryos, usually only the brain was affected and the malformation varied from a small hole in the roof of the hind brain to wide-open neural folds extending for most of the length of the head (Pl. 1, Fig. 2). In a few very deformed embryos, in which turning had also failed and the posterior part of the embryo was bent back on the anterior

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Fig. 1. Rat embryos explanted at 9½ days of gestation and grown for 48 hr in heat-inactivated serum (top row) and untreated serum (bottom row). The gas phase contained 20% O_2 throughout the culture period. The embryos at bottom left and right have failed to adopt the normal fetal position; in the one on the right the anterior and posterior parts of the trunk have fused together dorsally.

Fig. 2. Failure of closure of the heart folds in embryos grown in untreated serum in a gas phase containing 20% O_2 throughout the culture period. The middle embryo is normal. Photographed after fixation.

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Fig. 3. Three embryos from the same rat, explanted at 9½ days of gestation. (a) At explantation; (b) after 48 hr in culture; (c) after 72 hr in culture. All extraembryonic parts have been removed and the embryos have been photographed after fixation and clearing.

Fig. 4. Two rat embryos explanted at 9½ days of gestation and grown in culture in heat-inactivated, 'immediately centrifuged' serum for 95 hr.

(Pl. 1, Fig. 1), the neural folds of each side remained widely separated in the mid-line over much of the length of the spinal cord while fusing together anterior to posterior.

These results confirm the earlier, unpublished, observations of M. H. Jacobs who found the same abnormalities in 15/31 rat embryos cultured in 20% O₂. Moreover, Ferm (1964) has reported exencephaly, spina bifida and other fetal abnormalities in pregnant golden hamsters exposed to hyperbaric oxygen at stages of gestation equivalent to those of our rat embryos.

The beneficial effect obtained by reducing the oxygen of the gas phase to 5% raises the question whether this resembles more closely the oxygen concentration surrounding the conceptus *in vivo* than the gas mixture containing 20% O₂ that has been used previously. Yochim & Mitchell (1968) found oxygen tensions of around 80 mmHg in the rat aorta, 30 mmHg in the abdominal vena cava, and in the uterine lumen a maximum of 58 mmHg (on Day 7) during pseudopregnancy or 40–45 mmHg after conception and decidualization. These values may be slightly low because they were obtained from anaesthetized animals. Comline & Silver (1975) give values of 95–99 mmHg for the uterine artery and 49–59 mmHg for the uterine vein of the conscious sheep, cow and mare. An estimate based on all these data suggests that the blood in the decidua surrounding the rat conceptus has an oxygen tension in the range 40–90 mmHg. The oxygen tension in the serum in our culture bottles was calculated to be around 40 mmHg when the gas phase contained 5% O₂ and around 160 mmHg when the gas phase contained 20% O₂.

Development in culture for 72–95 hr

Twenty embryos were explanted at the head-fold stage and grown in culture for 72–95 hr. The culture medium throughout was heat-inactivated 'immediately centrifuged' serum. The culture bottles were gassed initially with 5% O₂/5% CO₂/90% N₂, then at 24, 32 and 48 hr with 20% O₂/5% CO₂/75% N₂ and at 54–56, 72 and 82 hr with 95% O₂/5% CO₂. The embryos were incubated for the first 48 hr in 30-ml culture bottles, each bottle containing 4 embryos and 4 ml serum. At 48 hr, each group of 4 embryos was transferred to a 60-ml bottle containing 8 ml fresh serum.

Table 3. Growth and development of 9½-day rat embryos cultured for 72–95 hr in heat-inactivated 'immediately centrifuged' serum

Culture duration (hr)	Total no. of embryos	No. of embryos showing:				Mean yolk-sac diam. (mm)	Mean somite no.	Embryo protein (µg) (mean ± S.E.M.)
		Blood circ.	Turning complete	Fused allan.	Closed N.T.			
72	19	13	19	12	19	5.5	35.2	474 ± 38
82	4	2	4	4	4	5.5	39.0	794
95	2	0	2	2	2	6.4	45.0	1570

Abbreviations as in Table 1. The data for yolk-sac diameter, somite number and protein at 72 hr exclude the 4 embryos that were returned for further incubation; similarly these data at 82 hr exclude the 2 embryos that were returned for incubation to 95 hr.

The 19 surviving embryos (one was damaged during transfer) were examined at 72 hr. The results are summarized in Table 3. All the embryos were well formed with an average of 35 somites; 13 still had a good blood circulation in the yolk sac, and a few showed circulation in the allantois. Four of the embryos were returned for further incubation in fresh serum. By 82 hr the circulation in two had failed but the other two continued to develop until examined at 95 hr (Pl. 2, Fig. 4). The blood circulation in the yolk sac of both embryos had ceased but one still had a circulation in the allantois. Both embryos appeared to be well developed, with 45 somites and prominent limb rudiments, the anterior limb being at the early 'paddle' stage.

Relative to the protein content at explantation, very large amounts of new protein were synthesized during the culture period. The embryonic part of the 9½-day explant contains less than 5 µg protein; this was increased by about 100-fold at 72 hr and over 300-fold at 95 hr (Table 3). The relative sizes

and overall development of three embryos explanted from one rat at 9½ days gestation and fixed at 0, 48 and 72 hr of culture are shown in Pl. 2, Fig. 3.

Some of the explanted embryos developed a blood circulation in the allantois in addition to that in the yolk sac. But although this represents the beginnings of an allantoic placenta, the structure lacks any maternal component and can provide little if any support for embryonic growth. This is probably the main reason why the embryos cultured for 72 hr or longer have a lower protein content than those of equivalent age or stage of development *in vivo* (New, 1973). However, it is possible that during the first 30–40 hr, before the embryo has attained the stage at which the allantoic placenta acquires a supportive function, the rates of growth *in vitro* and *in vivo* may be the same (New, Coppola & Cockroft, 1976).

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