〔報　文〕

KOR. J. ENVIRON. TOXICOL.
Vol. 6, No. 1~2, 71~82 (1991)

Culture of Rat Embryos During Early Organogenesis

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초기 기관형성기중 랫트배자의 배양에 관한 연구

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ABSTRACT

Rat embryos were cultured out of the mother from the head-fold stages, 9.5 days to 11.5 days during which they start to develop the brain, eyes, ears and cardiovascular system etc. We principally did the basic experiment in order to establish the best condition of the rat whole embryo culture in our laboratory. The temperature in the culture system was maintained 37℃±0.2℃ for 48 hrs. The culture was carried out in humidified atmosphere of the air, intially, the bottles were equilibrated with 5% O_2, 5% CO_2, and 90% N_2 gas mixture. After 22 or 24 and 29 or 30 hrs the cultures were reequilibrated with 20% O_2, 5% CO_2, 75% N_2 and 40% O_2, 5% CO_2, 55% N_2 respectively. Various types of sera were tested and for the purpose of minimizing the quantity of rat serum, artificial medium was also tried and it was determined that rat serum supported normal growth over a period of 48 hrs, based on total growth analysis and structural comparisons with in vivo specimens.

INTRODUCTION

With particular attention to teratogen screening as one of the most hot issues in human reproduction since the episode of thalidomide, painstaking teratogen screening has been carried out to predict and prevent the risk of xenobiotic exposure on human pregnancy out come by many advanced countries. This teratological testing so far has been limited to outcome of in vivo assay which needs a lot of

animals, time and cost. With our remarkable industrial development, the task load to screen environmental teratogens are overweighing rapidly year by year. The main reasons restricted to in vivo teratogen screening by other national agencies may have been attributable to few accomplishment of alternative method and the recognition of its limitation with which the in vitro assay for teratogen has no mediating effect of maternal pharmacokinetics. The organogenic period of mammalian embryological development has been a focal of investigations by embryologists, teratologists and others interested in determining mechanisms of normal and abnormal organ differentiation. However, due to the inaccessibility of the fetus in vitro, problems have arisen in examining and manipulating specific developmental events during critical organogenic stages. Thus, much attention has been directed toward developing life support systems capable of maintaining mammlian embryos in vitro[1~3]. One of the most successful of these techniques has been developed and it is now possible to culture early stage rat embryos, throughout much of the organogenic period[2~4]. Thus rat embryos explanted at the primitive streak or early head-fold stage can be maintained using some kinds of apparatus for approximately 4 days in culture with a rate of organogenesis similar to that observed in vitro [4,5]. Culture of later stage embryos permits some fundamental investigations into embryological events, but rarely affords the opportunity to observe development of an organ system from primordial stages to a definite form. Culture of early stage mouse embryos has also been attempted, but without the success of using rat or later staged mouse.

In these investigations, a large percentage of embryos failed to estalish a normal circulation, rotation, or growth rate compared to similar stage embryos maintained in vivo[6]. Due to the lack of success with rat embryo cultures under some kinds of conditions and because many laboratories employ rats for teratological investigation, it was determined that development of a culture system under basic conditions which was capable of supporting early stage rat embryos it sought to investigate.

MATERIALS AND METHODS

1. Equipments

① Whole embryo culture system

The apparatus consists of a set of motor-driven rollers housed in a standard tissue culture incubator, on which culture bottles are rotated. The bottles are inserted into a hollow rotating drum through which the gas circulated. The rotating rate in the circulation is maintained at 35 rpm and the flow rates of the gas is continued at 150 *ml*/min. Most of the manipulations of embroys and culture serum has done under sterilized condition.

② Stereomicroscope

The microscope used have a good range of magnification (×40) to provide good access for the dissection to the preparation. Illumination of the microscope state is best provided from a fiber optic source.

2. Reagents

Hank's balanced salts, Tris-HCl buffer (pH 7.0), protein-assay reagent were purchased from Sigma Chemical Co. Waymouh 75/B2, newborn calf serum, calf serum were purchased Gibco Chemical Co.

3. Animals

All animals aged 5 weeks were obtained from the SPF animal room of NISR. Animal room was maintained at constant room temperature (22±1℃), relative humidity (55±5%) and photoperiod (12 hr light/dark cycle). They were given continuous access to pellet diet (Shinchon feed Co.) and tap water.

Each male rat had been caged overnight with one proestrus-stage female which was checked for vaginal plugs or sperm at the following morning. Embryonic age 0 is defined as the midpoint of the dark cycle during which copulation take place.

4. Serum preparation

Blood was collected from the abdominal arota of SD-male rats anaesthetized with ether and immediately centrifuged for the precipitation of blood cells at 2,500 rpm, 4℃ for 5 min. After centrifugation, a clear fibrin clot forms separately from the cells in the plasma layer above. The fibrin clot then slowly was squeezed with pinsets and removed to release serum which could eventually be separated by centrifuging (2, 500 rpm, 4℃ for 5 min) and had obtained supernatant using spoid. Resultant supernatant was heat-inactivated at 56℃ for 30 min. This is IC (Immediately Centrifuged) serum and used without dilution for embryo culture. Above serum had to be filtrated using 0.3 μm microfilter for sterilization and stored at −20℃.

5. Statistical analysis

Statistical significance of yolk sac diameter, crown-rump length, total protein content were determined by ANOVA (analysis of variance: One-way). $P<0.05$ was selected as the level of significance.

6. Experimental methods

① Effect of temperature

control group ; 37°±0.2℃

high-temperature group ; 38.5°±0.2℃

The medium was composed of 100% (v/v) rat IC-serum. The culture was carried out in humidified atomosphere of the air described under for 48 hrs. Initially, the bottles were equilibrated with 5% O_2, 5% CO_2, 90% N_2 gas mixture. After 22 or 24 and 29 or 30 hr, the culture was reequilibrated with 20% O_2, 5% CO_2, 75% N_2 and 40% O_2, 5% CO_2, 55% N_2, respectively. After 48 hrs, at the end of the culture period, the embryos was evaluated the growth and development.

② Effect of gassing schedule

Change of gassing schedule and composition were under performed. All gas mixture have pCO_2 of 5%; the balance is N_2.

	time of gas change	0 hrs	22 hrs	29 hrs	44 hrs
pO_2 of gas mixture	A group	5%	20%	40%	40%
	B group	5%	20%	20%	40%
	C group	5%	20%	20%	20%
	D group	5%	5%	20%	40%

③ Difference of rat serum by preparation method

Control group used IC serum. IC-d (Immediately Centrifuged but delayed) serum prepared from blood that had been immediately centrifuged on withdrawal, and after that have left alone for 18 hr in 4℃. DC (Delayed Centrifuged) serum was centrifuged after had left alone for 18 hr in 4℃ (Fig. 1). IC+DC serum mixed each serum 2 *ml*.

④ Serum of different species and artificial medium as culture medium

control group ; rat IC serum

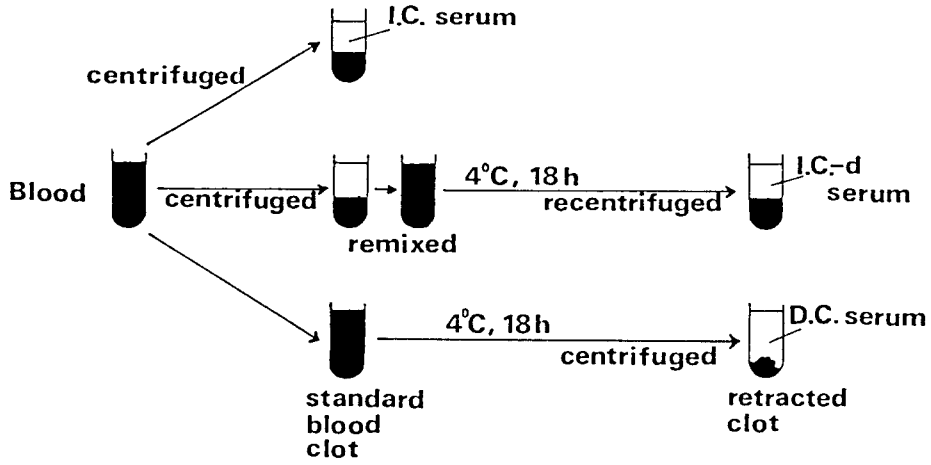

Fig. 1. Diagram of Preparations of 'immediately centrifuged' (I.C.) serum, 'immediately centrifuged but delayed' (I.C.-d) serum, and 'delayed-centrifuged' (D.C.) serum[7].

A group; Waymouth 75/B2 : IC serum (1 : 1)
B group; NCS (newborn calf serum)
C group; CS (calf serum)
D group; Hank's solution : IC serum (3 : 1)

7. Evalution of growth and differentiation of cultured embryo

Cultured embryos were transfered to the petridish containing Hank's balanced salts solution. Heart beat, visceral yolk sac circulation and rotation were then measured on a scale of −, ±, +. The + heart beat, the + circulation, and the + rotation were optimum (rapid, uninterrupted heart beat and circulation, and direction of head to placenta). Yolk sac diameters, crown-rump lengths, somite numbers, and any embryonic abnormalities were noted under the stereomicroscope. Embryos were transfered to Tris-HCl buffer (pH 7.0), washed three times and pipetted by 0.5 *ml* with buffer to determine the protein contents by the method of Lowry *et al*[8].

RESULTS

This study was done in order to set up culture systems an instrumental condition which was suitable for the embryonic growth of rats. Fig. 2. shows the 9.5 days embryo, head fold stage. When cultured at high temperature, yolk-sac diameter, crown rump length, total protein content were significantly decreased compared to the normal temperature groups. Especially at high temperature, blood vessel was not developed in yolk-sac membrane and existed as blood islands of underdeveloped conditions (Table 1), (Fig. 3).

Malformation by gassing schedule and composition appeared when gas composition had not been changed to 40% O_2 (C group) or was supplied 5% O_2 still at 29 hrs (D group). When the bottles were equilibrated with 5% O_2 gas mixture, after 22 and 29 hrs, the culture was reequilibrated with 20% O_2 and 40% O_2 mixture, respectively (A group), the best results were come out (Table 2).

Rat IC serum as the culture medium was suitable for embryonic growth of rat (Fig. 4). Case of another serum (IC-d, DC, IC+DC serum), crown-rump length, somite number, total protein content were shown sigificant reduction.

But, embryos had not appeared any malformations or non-normal shaped, only had some growth retardation (Table 3).

In addition, any heterologous sera except for rat serum were not suitable for the embryonic growth of rats and the addition of artificial medium was not good for rat embryos (Table 4). Fig. 5. shows the comparisons of in vivo and in vitro embryo development.

Table 1. Effects of Temperature on the Development of 9.5 day Rat Embryos.

	No. of embryo	Yolk sac diameter	Heart beat	Rotation (+, ±, −)	Crown-rump length	No. of somites	Protein content (μg/embryo)
Control	7	4.3	+	+	3.25	25	321.34
		4.25	+	+	3.5	24	318.74
		4.25	+	+	3.5	23	285.8
		4.0	+	+	3.5	21	308.4
		4.3	+	+	3.8	23	286.8
		4.0	+	+	3.5	24	272.4
		4.2	+	+	3.8	23	270.0
Mean±S.D.		4.19± 0.13			3.55± 0.19	23.3± 1.3	294.93± 21.24
High temper.	7	3.75	+	+	2.5	23	173.2
38.5℃		4.0	+	+	2.5	21	149.4
		2.5	+	−	2.0	14	119.2
		2.5	+	−	ND	ND	ND
		4.0	+	+	3.0	23	ND
		4.0	+	±	3.25	22	192.5
		2.5	+	+	ND	20	ND
Mean±S.D.		3.32*± 0.77			2.65*± 0.49	20.5± 3.39	158.6*± 31.66

*Significantly different from value with control group, $p<0.05$.

All embryos were cultured for 48 hrs at 37℃ in rat IC serum.

Table 2. Effects of Gassing Schedules on the Development of 9.5 day Rat Embryos.

	No. of embryo	Yolk sac diameter	Heart beat	Rotation (+, ±, −)	Crown-rump length	No. of somites	Protein content (μg/embryo)
Control	9	3.6	+	+	3.2	19	231.3
A		3.8	+	+	3.4	20	270.9
		4.0	+	+	3.5	23	313.3
		4.0	+	+	3.5	24	346.5
		3.6	+	+	3.0	23	309.6
		3.7	+	+	3.0	23	295.9

Table 2. Continued.

		3.6	+	+	3.0	21	298.2
		3.9	+	+	3.5	23	311.1
		3.9	+	+	3.3	20	ND
Mean±S.D.		3.79± 0.17			3.27± 0.22	21.8± 1.79	297.1± 34.0
B	11	4.1	+	+	3.5	24	274.5
		4.1	+	+	3.5	25	306.6
		3.9	−	−	3.5	24	286.1
		4.0	−	±	3.5	24	ND
		4.1	+	+	3.1	20	262.0
		4.0	+	=	3.3	24	280.5
		3.5	+	+	3.3	23	ND
		3.8	+	+	3.2	23	250.6
		3.5	+	+	2.6	20	209.7
		3.6	+	+	3.0	ND	196.9
		3.5	+	+	3.3	22	268.3
Mean±S.D.		3.83± 0.26			3.3 ± 0.28	22.9± 1.73	259.5± 35.63
C	11	3.3	+	+	2.5	21	233.5
		3.3	+	+	2.6	21	212.3
		3.5	±	±	2.0	6	146.4
		3.0	+	+	2.5	18	212.3
		3.3	+	+	2.5	19	220.8
		4.0	+	+	3.6	23	ND
		3.7	+	+	3.5	24	272.5
		3.9	+	+	3.5	22	ND
		4.1	+	+	3.5	ND	ND
		3.7	+	+	3.4	22	ND
		3.5	+	+	3.3	ND	ND
Mean±S.D.		3.57± 0.34			2.94± 0.58	19.3± 5.7	217.1*± 45.8
D	8	3.3	+	+	2.6	21	ND
		3.5	+	+	2.7	16	200.3
		3.3	+	+	2.0	ND	105.0
		3.3	+	±	2.0	5	ND
		ND	+	±	2.0	ND	156.6
		4.2	+	+	3.4	22	260.0
		3.8	+	−	3.0	ND	ND
		4.0	+	+	3.4	25	264.7
Mean±S.D.		3.55± 0.41			2:62*± 0.60	17.8± 7.90	164.77*± 100.45

*Significantly different from value with control group, $p<0.05$.

All embryos were cultured for 48 hrs at 37℃ in rat IC serum.

ND: Nondetermined

Table 3. Effects of Differences of Rat Serum by Preparation Method on the Development of 9.5 day Rat Embryos.

	No. of embryo	Yolk sac diameter	Heart beat	Rotation (+, ±, −)	Crown-rump length	No. of somites	Protein content (μg/embryo)
IC serum	6	3.5	+	+	3.25	22	230.2
		4.3	+	+	3.8	23	237.3
		4.0	+	+	3.5	24	238.5
		4.2	+	+	3.8	23	325.3
		3.7	+	+	3.5	23	235.7
		4.0	+	+	3.5	23	306.4
Mean±S.D.		3.95± 0.30			3.56± 0.21	23± 0.63	233.9± 17.51
IC-d serum	6	3.8	+	+	3.1	20	233.4
		3.8	+	+	3.2	20	220.2
		4.0	+	+	3.5	22	229.3
		4.0	+	±	3.0	22	277.6
		3.7	+	+	3.5	22	201.0
		3.5	+	±	2.9	20	168.1
Mean±S.D.		3.8 ± 0.19			3.2*± 0.25	21*± 0.09	204.93*± 27.41
DC serum	7	3.5	+	+	3.0	ND	232.11
		3.25	+	+	2.5	21	234.4
		3.1	+	+	3.0	21	228.0
		4.0	+	+	3.5	22	329.9
		3.8	+	+	3.5	22	230.5
		3.0	−	+	2.7	ND	ND
		3.0	+	+	ND	ND	225.9
Mean±S.D.		3.38*± 0.40			3.03*± 0.41	21.5± 0.58	210.13*± 16.30
IC-DC serum	5	3.5	+	+	3.0	20	234.3
		4.0	+	+	3.0	20	234.3
		4.0	+	+	3.5	22	234.6
		3.9	+	+	3.4	22	262.6
		3.7	+	+	3.0	24	230.1
Mean±S.D.		3.82*± 0.30			3.18*± 0.24	21.6*± 1.67	231.18*± 39.74
IN VIVO	5	4.5	+	+	3.9	23	333.19
		4.3	+	+	3.7	24	355.25
		4.4	+	+	4.0	25	333.19
		4.0	+	+	4.0	25	398.20
		4.0	+	+	3.5	23	295.91
Mean±S.D.		3.81± 0.39			3.82± 0.21	24± 1.0	323.15± 25.48

*Significantly different from value with control group, $p<0.05$.

All embryos were cultured for 48 hrs at 37℃.

ND : Nondetermined.

Table 4. Effects of Heterologous Serum and Artificial Medium on the Development of 9.5 day Rat Embryos.

	No. of embryo	Yolk sac diameter	Heart beat	Rotation (+, ±, −)	Crown-rump length	No. of somites	Protein content (μg/embryo)
Rat IC	4	4.6	+	+	3.8	24	308.00
serum		4.6	+	+	3.8	25	326.34
		4.6	+	+	3.8	24	ND
		4.4	+	+	3.7	25	292.11
Mean±S.D.		4.55± 1.00			3.77± 0.05	24.5± 0.58	308.85± 17.13
Rat IC+	6	3.6	+	+	3.0	21	229.5
Waymouth's		3.6	+	+	2.5	22	226.2
		3.3	+	+	2.7	21	270.8
		3.7	+	+	2.9	22	201.2
		2.6	−	+	1.5	19	180.9
		3.0	+	+	2.7	16	ND
Mean±S.D.		3.3*± 0.43			2.55*± 0.54	20.17± 2.31	221.72± 33.8
Human serum+ Waymouth's	6	All Dead					
NCS	6	1.5					
		1.6					
		2.1		All Dead			
		2.0					
		1.8					
		1.5					
Mean±S.D.		1.75*± 0.25					
CS	6	1.6					
		2.0					
		1.3		All Dead			
		2.0					
		ND					
		ND					
Mean±S.D.		1.75*± 0.34					
Hank's+	6	3.3	+	+	3.4	23	308.09
Rat IC serum		3.8	+	+	3.1	24	238.43
		3.9	+	+	2.7	16	111.41
		4.0	+	+	3.1	25	303,31
		3.7	+	+	3.9	ND	300.74
		4.7	+	+	3.7	24	315.69
Mean±S.D.		3.9*± 0.46			3.32*± 0.44	22.4± 3.64	257.95± 82.05

*Significantly different from value with control group, $p<0.05$.

All embryos were cultured for 48 hrs at 37℃.

ND; Nondetermined.

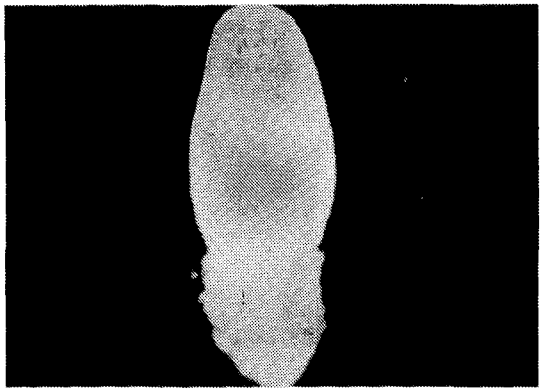

Fig. 2. 9.5 Day Rat Embryo Extracted from Mother (×25).

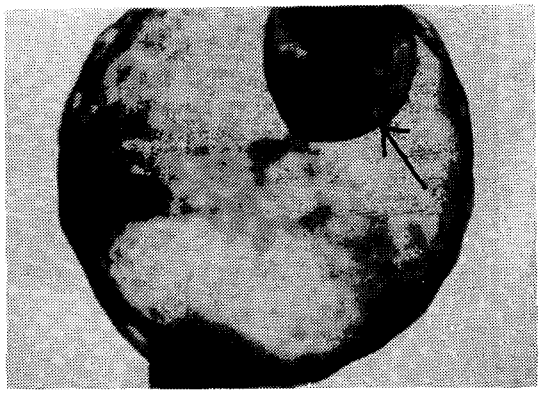

Fig. 3. 11.5 day Embryo Cultured at high temperature.
It showed blood island, not-well developed yolk-sac circulation, small and malformed embryo (the arrow) (×20).

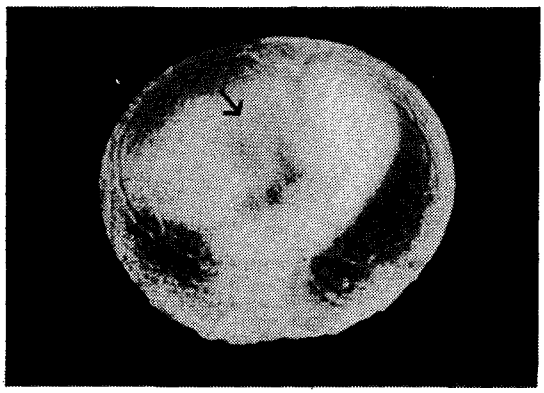

Fig. 4. 11.5 day Cultured from 9.5 day from 48 hrs. It Showed Good Development of Yolk-Sac Circulation.
The arrow shows the heart of embryo (×15).

DISCUSSION

Experiment 1. shows that high temperature can induce the underdevelopment of the blood vessel of yolk-sac and delayed growth. A temporary rise of body temperature occurs in many mammals as a result of infection or a hot environment. Such hyperthermia is often harmless, but in the pregnant female it may be teratogenic with particularly damaging effects on the brain of the developing embryo. The studies that have been made on experimentally induced hyperthermia in pregnant animals have been reviewed[9,10]. The frequency of gross abnormalities also rose sharply above 40℃ ; at 40.5℃ over half the embryos were obviously microcephalic and this proportion increased further at 41℃ together with the frequency of pericardial edema and other abnormalities[11,12]. A notable feature of the results was the very small temperature rise above the normal needed to produce abnormalities of development. In vitro, the yolk sac of the rat embryo acquires a blood circulation and probably becomes an important organ of O_2/CO_2 exchange at about the 10-somite stage (10.5 days). The allantoic placenta acquires a circulation at about the 17-somite stage (11 days), and becomes a second organ of respiratory exchange, taking over an increasing share as gestation continues. In vitro, although the yolk sac may be functional but the allantoic placenta is not, with the result that in embryos explanted after 11 days and cultured in constant oxygen levels, oxygen consumed fall progressively with age and size of embryo. New et al. showed that growth of embryos in culture in 20% oxygen slowed after about the 17-somite stage and 16 hour later the protein content was significantly

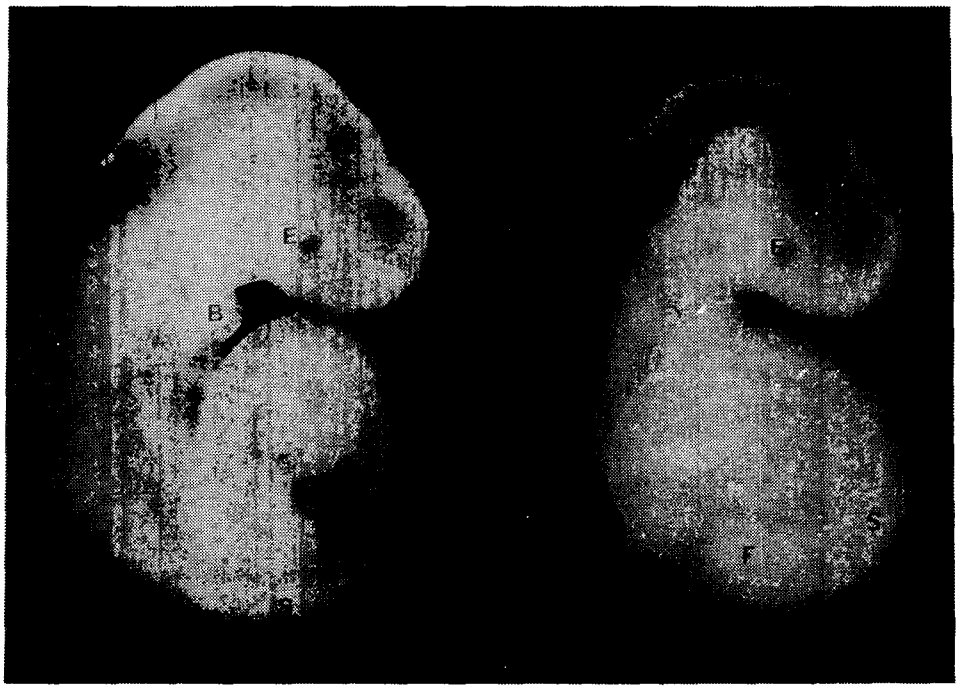

Fig. 5. Comparisons of in Vivo (left) and in Vitro (right).
It showed good development of brain, eye, ear, branchial bar, heart and somites. Average somite number is 23.
F; fore-limb bud E; Eye B; branchial bars S; Somite (×20)

less than that of controls in vivo; when the oxygen level was increased to 40% to compensate for the lack of a functional allantoic placenta, growth over the same period was indistinguishable from that of the controls[3]. According to Freeman *et al.*, gas changes from 20% to 40% O_2 between 10.5 days and 11.5 days had good results[13].

Steel *et al.* made the important observation that embryo growth is affected by the way the blood from which the culture serum is obtained has clotted (Fig. 1)[7]. Overall growth of primitive streak and early somite stage embryos is less significant in DC than in IC serum[7]. Older embryos are less affected[14]. Why does IC serum support better growth than DC serum? A few facts are now known, though the full explanation is still far from clear. Evidently the harmful properties of DC serum are products of normal blood clotting in which the blood cells and platelets are trapped in the fibrin clot and free cells and platelets. The most promising attempt so far to find the chemical basis of the difference between IC and DC serum is that of Klein *et al* [15]. These workers have demonstrated by gel electrophoresis a protein difference between the two sera that could be related to growth-supporting capacity. They have shown that after embryos have been grown in IC serum, it becomes depleted of one protein estimated from its band position on the gel to have a molecular weight of about 125,000. Fresh DC serum differs from fresh IC serum by a reduced concentration of this same protein and by the absence of two others with a molecular weight higher than 200,000[15]. IC-d serum which is finally decanted has

the same growth-supporting capacity as IC serum in this experiment. It was concluded from early studies with somite-stage rat embryos that development was equally good in autologous serum (i.e. obtained from the same rat as the embryos), serum from other pregnant female rats, from non-pregnant females, from males or from rats of a different strain[3,16].

In contrast, heterologous sera are often harmful. Good development of mouse embryos has also been obtained in a 1 : 1 mixture of fetal calf serum and Waymouth's medium[17,18].

No comparison appears to have been made yet of the growth of rat embryos of organogenesis stages in undiluted heterologous and homologous sera. At present it is doubtful growth in heterologous sera is as good as in serum from the same species. Analysis of the nutritive requirements of embryos would be greatly assisted if the culture serum could be replaced by a chemically defined medium. At present no such medium is available. It seems essential that at least part of the culture medium should be serum.

ACKNOWLEDGEMENT

This work was supported by a research grant from the Ministry of Science and Technology.

요 약

포유동물의 최기형성을 연구하는데 가장 많이 사용되는 동물인 설치류중 랫트의 배자를 이용하여, egg-cylinder기를 지나 head-fold가 이루어지는 초기체절기인 9.5일경부터 뇌, 눈, 귀, 심혈관계가 형성되는 시기인 11.5일까지 2일(48시간) 동안, 체외에서 배양하였다.

그 배양의 최적조건은 실험실마다 다르기 때문에 최적조건확립을 목적으로 이 실험을 행하였다. 온도는 37℃±0.2℃로 유지시키고 가스는 배양초기인 9.5일~10.5일 동안을 50% O_2, 5% CO_2, 90% N_2 가스를 공급하여 10.5일부터 6시간 동안은 20% O_2, 5% CO_2, 75% N_2로 하며 나머지 18시간 동안은 40% O_2, 5% CO_2, 55% N_2 가스를 배양계에 공급했을 때 가장 좋은 결과가 나왔다.

배양배지로서는 랫트 IC 혈청을 분리하여 열비동화시켜서 사용하였다. 이종혈청이나 합성배지를 첨가해서 배양할 경우는 랫트 혈청에 비해 성장이 좋지 않은 것으로 관찰되었다.

동물실험법의 대체법으로서 배자배양법의 기본조건을 확립하여 최기형성 평가 방법으로서의 가능성을 제시하였다.

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