Availability of Serum from Clotted Rat Blood on Embryogenesis

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SUMMARY

There are two ways to isolate serum from blood. One is to isolate so-called IC-serum by centrifuging immediately after drawing blood. Another is to isolate serum (called DC-serum) from blood 18 h after clotting. IC-rat serum has been used as the most beneficial medium for *in vitro* culture of rat whole embryos. Rat embryos are known to develop well and normally in the IC-serum, but not with the DC-serum. In our experiments, serum was isolated from rat blood I h after clotting. This serum (3-h-DC-serum) seemed to be one of the DC-sera. Embryogenesis in whole embryo culture with 3-h-DC-serum occurred as normally as it did in the IC-serum. Development of rat embryos cultured in 50% 3-h-DC-serum was compared with that in 50% IC-serum, knowing that all embryos cultured in 30-100% IC-sera diluted with Dulbecco' s Modified Eagle medium revealed normal development. As a result, it was found that the promoting effect of 3-h-DC-serum on embryogenesis was indistinguishable from that of the IC-serum. Moreover, hemolysis of rat serum within 5% was also found not to affect embryogenesis.

INTRODUCTION

In a study on the development of rat embryos at the egg-cylinder stage in the whole embryo culture system, Steel and New (1974) found that most of the explanted embryos developed with a failure in the fusion of the heart primordia. DC-rat serum (delated-centrifuged rat serum) used in those cultures was prepared from freshly extracted blood 18 h after clotting. Up to this time, therefore, IC-rat serum (immediately centrifuged rat serum) has been used as the medium for rat whole embryo culture. Since the obtainable quantity of serum is so small, diluting it with artificial culture medium is highly desirable provided that such dilution dose not negatively effect on normal embryonic development. The effects of dilution of the serum on embryogenesis were then examined. However, preparation of the IC-serum requires rapidity and is labor intensive. We obtained 3-h-DC-serum from clotted

blood 3 h after drawing it from male rats. There were no detectable differences in protein components between 3-h-DC-serum and the IC-serum. We then tested the efficiency of 3-h-DC-serum as a medium for rat whole embryo culture. Upon preparation of rat serum, hemolysis occasionally takes place. We also examined the effects of hemolysis on rat embryogenesis.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle (DME) medium and L-glutamine were obtained from Nissui Pharmaceutical Co., Ltd. , Tokyo. Sodium bicarbonate, penicillin and streptomycin were from Nakarai Chemicals, Osaka. Filter units (0.22 mm) were from Millipore Products Division, Bedford, HA. Other chemicals used were of the purest grade available from regular commercial sources.

Animals

SD-rats were used throughout our experiments.

Isolation of rat serum

Three kinds of sera were isolated from the freshly drawn blood of adult male rats. First, IC-serum was prepared by the method of Steele (1972). Secondly, we separated the 3-h-DC-serum from clotted blood by centrifuging and decanting 3 h after drawing blood. Finally, we obtained serum hemolized from blood which was vigorously pipetted in and out as was done in preparing 3-h-DC-serum. These pooled sera were filtrated through a filter unit (0.22 mm) after heating at 56°C for 30 min and then stored at -80° C until use.

Whole embryo rolling culture

SD-rats were mated overnight, and pregnancy was confirmed by the presence of a vaginal plug. Whole rat embryos were dissected at day 9. 5 of gestation. Two embryos were immediately suspended in each roller bottle containing 4 ml of culture medium. They were then cultured for 48 h according to the method developed by New (1978). Culture media were 100% rat serum and diluted with DME medium. Supplemental penicillin and streptomycin were added at 31.2 mg/ml and 100 mg/ml to the rat serum and DME medium, respectively. Embryonic development was examined by the morphological scoring system of Brown and Fabro (1981).

RESULTS

Effects of dilution of rat serum on rat embryonic development

The IC-serum was diluted with DEM medium to levels of 10, 15, 20, 25, 30, 40, 50 and 75%. These diluted sera were then tested for the promoting effects on embryogenesis. Table I shows the measured values of the parts of whole embryos developed in each diluted IC-serum. All of the measured values of embryos separately cultured in 30, 40, 50 arid 75% IC-sera were identical to those of embryos cultured in 100% IC-serum, whereas embryonic development gradually worsened in 25% and under IC-sera. It was thus concluded that a threefold dilution of IC-serum was effective as a culture medium.

Table I

Development of rat embryos in IC-serum diluted with DME medium

Serum (%)	n	Yolk sac diameter (mm)	Crown-rump length (mm)	Head length (mm)	No. of somites	Score by HSS[1]
100	37	3.8±0.4	3.5±0.3	1.7±0.2	24.1±1.3	40.1±1.1
75	16	3.6±0.3	3.4±0.2	1.7±0.1	24.3±1.0	40.1± 0.7
50	19	3.6±0.2	3.4±0.2	1.7±0.1	24.2±0.9	40.1± 0.5
40	20	3.7±0.3	3.3±0.3[2]	1.7±0.2	24.4±1.4	40.1± 0.4
30	23	3.7±0.3	3.3±0.2[2]	1.7±0.1	24.1± 1.4	40.1± 0.5
25	24	3.5±0.4[2]	3.2±0.3[3]	1.6±0.2[2]	23.5± 1.7	39.0± 1.7[2]
20	24	3.6±0.5	3.3±0.4[3]	1.6±0.2[2]	23.0±2.1[2]	38.6± 3.2[3]
15	13	3.4±0.5[3]	3.0±0.3[4]	1.5±0.3[3]	20.3±5.5[4]	35.4± 4.9[4]
10	13	2.5±0.6[4]	2.1±0.8[4]	1.1±0.4[4]	13.2±6.7[4]	20.7± 1.8[4]

1)Morphological scoring system by Brown and Fabro.

2) P<0. 05 3) P<0.01 4) P<0.001 . Significant difference against control value by the Student's t-test. Values are mean ± SD.

Proteins in IC- and 3-h-DC-sera on SDS-PAGE

SDS-PAGE was examined using 8 and 10% gels by the method of Laemmlie (1970) . The 25% solutions of fresh IC- and 3-h-DC-sera were treated with the same volume of the denatured solution. Two, four and six ml of these mixtures were

separately electrophoresed. No visible differences were detected among the proteins separated from both sera on the gels (data not shown). Consequently, we inferred that the protein components of both sera were almost identical.

Development of 9. 5-day rat embryos cultured in rat serum obtained by our preparation

Development of rat embryos cultured in 3-h-DC-serum was the same as that cultured in the IC-serum. Embryogenesis in 50% 3-h-DC-serum (DC-50) was then compared with that in 50% IC-serum (IC-50). The average values of yolk sac diameter, crown-rump length, head length, number of somites and morphological scores of rat embryos cultured in DC-50 and IC-50 are shown in Table 2. It was found that there were no differences in development between rat embryos cultured in DC-50 and IC-50. Furthermore, of 90 embryos cultured in DC-50, none showed any malformation.

Table 2
Development of 9.5-day rat embryos in IC- and DC-sera

Medium	n	Yolk sac diameter (mm)	Crown-rump length (mm)	Head length (mm)	No. of somites	Score by MSS[1)]
IC-50	23	3. 72	3. 44	1. 75	25	40. 6
DC-50	90	3. 76	3. 46	1. 78	25	40. 5

IC-50: 50% IC-serum diluted with DME medium. DC-50: 50% DC-serum diluted with DME medium. [1)]Morphological scoring system (MSS) by Brown and Fabro.

Effects of hemolysis of serum on embryogenesis

We examined the harmful effects of 5%-hemolyzed 3-h-DC-serum diluted 1:1 with DME medium compared with the absorbance of 100%-hemolyzed serum at 550 nm. The results are shown in Table 3. As compared with embryonic development in DC-50 (Table 2), rat embryos developed in 5%-hemolyzed DC-50 had higher average values for almost all items (Table 3). Moreover, all 20 embryos cultured in 5%-hemolyzed DC-50 had no malformation.

Effects of the storage of serum on embryogenesis

We surmised that one of the causes of embryonic malformations due to the DC-serum would be the storage rather than the preparation of serum. Upon a whole embryo culture with 3-h-DC-serum stored at 4°C for one month, all embryos had a morphological score of 0 (data not shown). This suggested that cold storage of rat

Table 3
Development of 9. 5-day rat embryosin 50% DC-serum hemolysis

n	20
Yolk sac diameter (mm)	3.80
Crown-rump length (mm)	3.62
Head length (mm)	1.86
Number of somites	26. 2
Score by MSS[1)]	41. 1

[1)]Morphological scoring system by Brown and Fabro.

Table 4
Morphological score for development of 9. 5-day rat embryo with 5% in stored serum

Month	DC-20	DC-50	
	at -20°C	at -20°C	at -80°C
0	39 37	40 40	40 41
1	33 17	37 33	40 40
2	14 13	28 23	40 40

DC-20: 20% DC-serum diluted with DME medium. DC-50: 50% DC-serum diluted with DME medium. Morphological scoring system by Brown and Fabro.

serum should be avoided. Subsequently we examined the harmful effects of frozen storage of 3-h-DC-serum on embryogenesis. Morphological scores of rat embryos cultured in diluted sera stored at -20°C or -80°C were shown in Table 4. The diluted sera used in this test were DC-20 (20% serum) and DC-50, both of which were stored at -20°C for one or two months; resulting embryonic development was poor. Results with other groups in this experiment were similar to those in Table 4. Rat embryos cultured in DC-50 stored at -80°C for 4 months developed normally (data not shown). It was found that rat serum remained stable for a long time at-80°C.

DISCUSSION

Rat whole embryo culture has attracted special interest recently as one system for assaying developmental toxicity. *In vitro* embryonic development of rats in rat

serum has been described by New (1966). He found that rabbit serum was inferior to rat serum as a culture medium. Furthermore, New et al. (1973) have established a rotating culture method of rat whole embryos. In a rat whole embryo culture, egg cylinders regularly develop into well-formed embryos with 30-40 somites; and the average rates of protein synthesis and differentiation over 48 h in embryos at the head-fold stage are indistinguishable from those *in vivo*. On the other hand, most of the embryos cultured in rat serum developed abnormal double hearts (Steel and New, 1974; New and Daniel, 1969). The serum used in those cultures was the DC serum. Steele (1972) obtained improved embryonic development with the formation of normal single hearts in cultivation with the IC serum. For this reason, IC-rat serum is now widely used in rat whole embryo culture. However, since an adult male rat (250g) yields only about 5ml of serum, many rats are needed to collect the required amount. The usefulness of rat serum will increase, if diluted sera can promote embryogenesis. Our results (Table 1) show that 25% and under rat sera resulted in poor embryonic growth and that, as a result, sera diluted to those levels were of no practical use in rat embryo culture. But there were no differences among embryos grown in 30% and over rat sera, i.e., fresh rat serum diluted threefold is still effective. New (1966) also reported that 10. 5-day rat embryos grew normally in 25% rat serum with 199 medium.

Large quantities of IC-serum must be prepared Quickly using labor-intensive ways. However we were easily able to prepare large quantities of rat serum by isolating the serum after clotting. As clotting was complete practically 1 h after drawing blood, we could isolate serum within 3 h. Protein components of IC- and 3-h-DC-sera heated at 56°C on a gel after SDS-PAGE showed no detectable differences. If the protein contents of 3-h-DC-serum differed from IC-serum, increases in dilution would have magnified the effects of both sera on embryogenesis. However, the development of rat embryos cultured in 50% 3-h-DC-serum inactivated at 56°C was indistinguishable from that of 50% IC-serum inactivated at 56°C (Table 2). Furthermore, no malformations were detected in any of 90 embryos cultured in 50% 3-h-DC-serum inactivated at 56°C. These findings suggested that the effects of 3-h-DC-serum on embryogenesis are identical to those of IC-serum.

Regarding the storage of rat serum, we found that IC- and 3-h-DC-sera stored at 4°C for one month barely grew 9.5 day rat embryos. A decline in the effects of 50% DC-serum on embryogenesis stored at -20°C was barely detectable at one month but was pronounced at two months. However, the storage of 50% DC-serum at -80°C for four months and over was not found to change its initial promoting effects on embryogenesis. These findings suggested the existence of serum components which would be unstable under conditions at -20°C and over. It, therefore, seemed likely that a decline in the promoting effects of rat serum on embryogenesis resulted from

conditions of its storage rather than its preparation.

We feared the negative effects of hemolysis on embryogenesis, since blood has a tendency to hemolyze under rough handling. However, it turned out that embryonic development in 3-h-DC-serum with 5% hemolysis was superior to that in unhemolyzed serum. Thus, it seems that about hemolysis are unfounded.

In conclusion, it is desirable that the serum isolated should be stored at -80°C as fast as possible, although serum as a culture medium does not always need to be prepared with IC-treatment.

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