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The Effect of Protein on Preimplantation Mouse Embryo Development in Vitro

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The effects of supplementing culture medium with protein for the culture of mouse embryos from two cells to blastocysts were examined in vitro. The proportion of embryos developing was affected by the type of protein and concentration. The highest rates were obtained in protein-free medium, fetal calf serum, and A Grade bovine serum albumin at all concentrations tested (2–16 mg/ml). Reduced rates of embryo development were observed in proteins of human origin, particularly at the highest concentrations tested. Purification of human and bovine serum albumin resulted in a marked reduction of embryo development. Significantly more normal fetuses were found in pregnant mice receiving transferred embryos grown in protein free-medium than in medium containing fetal calf serum. It is concluded that protein supplementation of culture medium may adversely affect embryo development and viability. These observations could have important implications for human in vitro fertilization.

KEY WORDS: mouse; embryos; protein; culture; in vitro.

INTRODUCTION

The development of mouse embryos from single-cell fertilized eggs to blastocysts will occur in a number of chemically defined media (1–4). Bovine serum albumin (BSA) is the most common protein added to culture media as a fixed nitrogen source for embryos, however, combinations of essential amino acids have been successfully used in vitro to enable development of mouse embryos to the blastocyst stage (5).

Cholewa and Whitten (6) have shown that mouse embryos will develop from two cells to blastocysts in the complete absence of any exogenous fixed nitrogen source, although a high molecular weight colloid (polyvinylpyrrolidone) was added to the culture media as a replacement for BSA. Although albumin is not essential for the development of mouse embryos to the blastocyst stage, it has been found to be crucial for enabling the blastocyst to hatch from the zona pellucida (7). Albumin may also have beneficial effects in the culture medium for chelation of toxic metal ions and for cell membrane stabilization (6). Kane and Headon (8) credit commercial BSA with at least two functions; the provision of energy for embryos from fatty acids bound to albumin and the promotion of blastocyst hatching.

In the present study, we have examined mouse embryo development in the presence of different albumin preparations, of both bovine and human sources, as well as heat-inactivated sera. Culture medium containing no exogenous fixed nitrogen source was used as a control for the experiments.

MATERIALS AND METHODS

Two-cell embryos were collected from superovulated F_1 hybrid (CBA × C57) female mice which were sacrificed by cervical dislocation approximately 36 hr after mating. The embryos were recovered by flushing the oviducts with a syringe attached to a 30-G needle. Any embryos appearing degenerate or abnormal were discarded.

The proteins to be examined were prepared in modified Whitten's medium (9) and the pH was ad-

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justed to 7.4. Normal two-cell embryos were washed in culture medium, then placed into 5-ml tissue culture tubes (12 × 75-mm 2003 tube, Falcon Plastics, Oxnard, CA) containing 1 ml of the appropriate culture medium and gassed under a mixture of 5% oxygen, 5% carbon dioxide, and 90% nitrogen in a humidified atmosphere at 37°C with the tubes in an upright position for approximately 96 hr. At this time, development to the fully expanded blastocyst stage was recorded.

The eight different proteins examined were human serum albumin (HSA) A Grade (Sigma, St. Louis, MO), bovine serum albumin (BSA) A Grade (Calbiochem–Behring, San Diego, CA), HSA Fraction V (Sigma, St. Louis, MO), BSA Fraction V (Flow Laboratories, Sydney, Australia), human serum from female donors obtained during the follicular phase of the menstrual cycle, 1 to 5 days before the preovulatory LH surge (pooled and heat inactivated at 56°C for 30 min), and heat-inactivated fetal calf serum (FCS) (Flow Laboratories, Sydney, Australia). Purified BSA was prepared from a Fraction V extract by Quinn and Stanger (10) and purified HSA was prepared from HSA A Grade (Sigma) using the same method. Each of the protein preparations was examined at concentrations of 2, 4, 8, and 16 mg protein/ml culture medium (9). The protein content of human and fetal calf serum was determined by the method of Bradford (18) and the appropriate volume of serum was added to achieve the equivalent protein concentrations as used for the dried preparations. In each protein solution five or six replicates of 20 two-cell mouse embryos (total of 100 to 120 embryos) were cultured in vitro to test the capacity of the solution to enable embryo development to the blastocyst stage.

Apparently normal blastocysts which had developed from two cells in protein-free medium and medium containing 4 mg/ml FCS were transferred to recipients to assess their viability. Six to eight blastocysts were transferred to each uterine horn of recipient mice 3 days after mating with vasectomized males. The reproductive tracts of all recipient mice were examined 11 days after transfer and the number of normal and resorbing fetuses was recorded.

Data were analyzed using chi-square tests.

RESULTS

The proportion of two-cell mouse embryos developing to blastocysts (Table I) was significantly affected by the type of protein ($\chi^2_7 = 266.1$; $P < 0.001$) and protein concentration ($\chi^2_3 = 60.3$; $P < 0.001$). The only proteins, irrespective of concentration, which did not reduce the percentage of embryos developing to blastocysts below that obtained in protein-free medium were fetal calf serum (FCS) and A Grade BSA. However, at the lowest protein concentration tested (2 mg/ml), reduced embryo survival was noted only with human serum ($\chi^2_1 = 9.8$; $P < 0.01$) and purified HSA ($\chi^2_1 = 18.1$; $P < 0.001$). A toxic effect on embryo development of both purified BSA and HSA was evident at 4 and 8 mg/ml, respectively (Table I). Reduced embryo development also occurred in all the other three human protein preparations at high concentrations (16 mg/ml) and in Fraction V BSA (Table I).

Table I. Percentage of Two-Cell Mouse Embryos (N = 100–120) Developing to Expanded Blastocysts

Protein concentration (mg/ml)	Protein type									
	No protein	Human serum	HSA V	HSA A Grade	HSA purified	FCS	BSA V	BSA A Grade	BSA purified	Mean
2	—	71	92	90	64	92	87	87	93	85
4	—	76	92	74	50	88	88	90	14[a]	72
8	—	68	90	81	17[a]	91	82	90	10	68
16	—	52[a]	80[a]	52[a]	0	89	68[a]	92	0	55
Mean	88	67	89	74	33	90	81	90	31	
				Type of protein: $\chi^2_7 = 266.1$; $P < 0.001$						
				Protein concentration: $\chi^2_3 = 60.3$; $P < 0.001$						

[a] Significant reduction in embryo development ($P < 0.01$) compared with lower protein concentration.

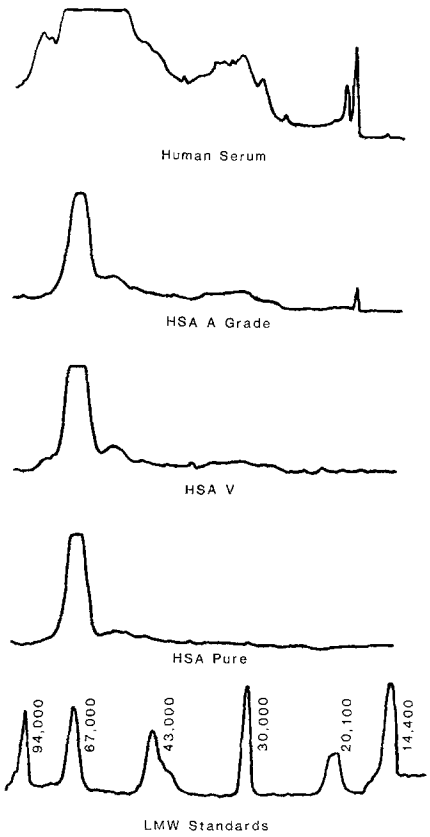

Fig. 1. Laser scanning densitometer profiles of SDS–polyacrylamide gels of the proteins used for culture of mouse embryos. LMW, low molecular weight standards.

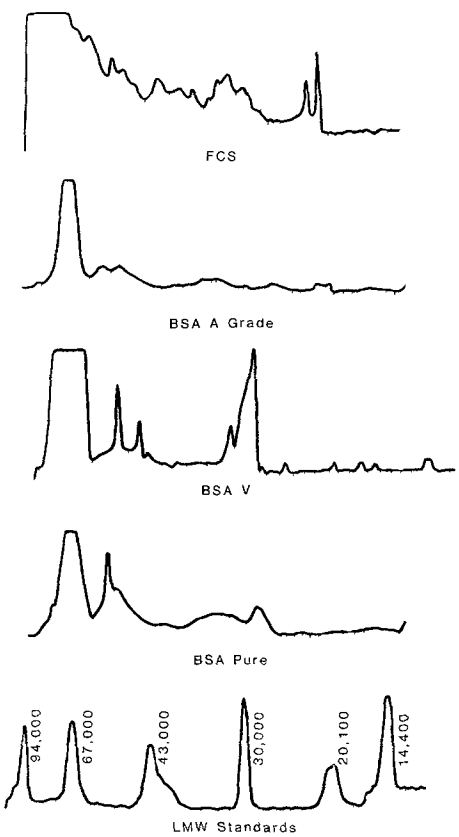

Fig. 1. Continued.

Examination of the molecular weight profile of each protein solution used for culture by SDS gel electrophoresis (Fig. 1) showed that all three human albumin preparations consisted of the one major albumin constituent. Small quantities of lower molecular weight constituents were removed by purification. Fraction V BSA contained at least three major protein constituents besides albumin. Two protein bands close together were identified in purified BSA and only one major albumin band was identified in A Grade BSA.

The number of normal (Fig. 2) and resorbing fetuses in mice receiving transferred embryos cultured in medium containing FCS (4 mg protein/ml) and protein-free medium are shown in Table II. There were significantly more normal fetuses ($\chi_1^2 = 5.59$; $P < 0.025$) in pregnant mice receiving transferred embryos grown in protein-free medium. The only resorbing embryos were found in mice receiving transferred embryos grown in medium containing FCS.

DISCUSSION

The three culture media which resulted in the maximum rate of embryo development were the extremes according to protein constitution. Medium containing no protein resulted in the same percentage of embryos developing to blastocysts as medium containing fetal calf serum or A Grade BSA. The latter had only a single major band of protein identified in SDS gel electrophoresis. Furthermore, we found no indication of reduced development of embryos grown in protein-free medium, to advanced fetuses when transferred to recipients, compared to embryos grown in medium containing fetal calf serum. These results show that neither a fixed nitrogen source nor a high molecular weight colloid is essential for mouse embryo growth and development from the two-cell to the blastocyst stage in vitro. These results support the observations of Cholewa and Whitten (6) that protein is not essential for mouse embryo development prior to

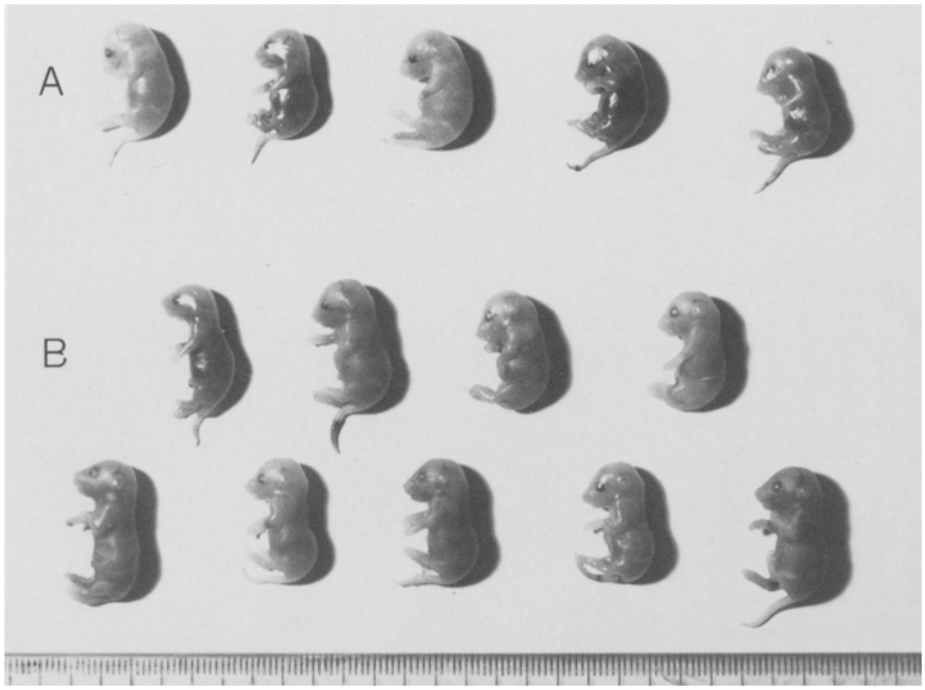

Fig. 2. Normal fetuses (scale in mm) obtained 11 days after transfer of embryos grown in medium containing 4 mg/ml FCS (A) and in protein-free medium (B).

the blastocyst stage. It is apparent from the present study that unless the protein type and concentration are very carefully selected, the addition of protein to culture media may have a detrimental effect on the in vitro development of two-cell embryos. Even when embryo development in vitro is satisfactory, the use of protein may reduce the capacity of embryos for normal development to advanced fetal stages. These observations could be important for human in vitro fertilization. Human embryos are always fertilized and cultured in medium containing either human or bovine albumin or serum (adult human serum, human cord serum, or fetal calf serum). The use of these proteins could be detrimental to embryo viability and the use of protein-free medium for embryo culture in vitro may significantly improve the development of normal embryos and pregnancy success rates. Studies on this are underway in our own laboratory.

The effect of the different protein types on embryo development did not show any consistent trends according to their origin (human or bovine) or to their purity. Of the two sera, human serum reduced the rate of embryo development, but fetal calf serum had no detrimental effect on embryo development, even at 16 mg/ml. The relatively crude Fraction V serum albumins supported high rates of embryo development except at the highest concentration tested. The more highly purified commercial A Grade bovine preparation gave results similar to those with FCS. Except for the lowest concentration of purified BSA, both purified albumin preparations were toxic to embryo development. This may be due to traces of chemicals remaining from the purification procedure, even though both preparations were thoroughly dialyzed against several changes of distilled water at 4°C for 48 hr. Alternatively, the purification procedure may expose chemical components of BSA which are directly toxic to embryos or may enable chemical reactions which produce embryo toxins. The purification procedures used would reduce fatty acids, proteins other than BSA, and nonprotein components from the cruder preparations. It is of interest to note that Quinn and Stanger (10) used the same preparation of purified BSA at a concentration of 3 mg/ml in culture medium for their studies, which is below the level of toxicity observed in the present study. Given these results we would not be prepared to risk the use of this preparation in the culture of human embryos in vitro.

It is known that exogenous proteins (7) or, more specifically, the essential amino acids histidine, methionine, threonine, tryptophan, tyrosine, and va-

Table II. Viability of Blastocysts Cultured from Two Cells to Blastocysts in Protein-Free Medium and Medium Containing 4 mg/ml FCS

Protein (mg/ml)	Number of recipients		Total Number of embryos transferred	Number of embryos transferred to pregnant mice	Number of fetuses		
	Transferred	Pregnant (%)			Normal (%)	Resorbing (%)	Total (%)
FCS (4)	9	8 (89)	144	128	31 (24)	11 (9)	42 (33)
Nil	9	7 (78)	124	98	39 (40)	0	39 (40)

line (11) are required for the hatching of mouse blastocysts in vitro. The protein content of mouse embryos decreases by 26% during the first 3 days of embryonic development (12) and the endogenous taurine and glycine pools in mouse eggs decrease to about 50 and 10%, respectively, during development to the blastocyst stage (13). These results indicate that the embryo preferentially utilizes endogenous fixed nitrogen sources during the early cleavage stages of preimplantation development, although it is well established that exogenous radioactive amino acid precursors can be incorporated into a large number of embryonic proteins during this time (14–17). It is not essential for embryonic protein synthesis or for embryo viability prior to the hatching blastocyst stage that exogenous amino acids are available, but they may be utilized if present. The increase reported (13) in the total amino acid pool size in the blastocyst compared with the oocyte may be due primarily to the metabolism of endogenous protein or to the conversion of exogenous lactate and pyruvate to the amino acids glutamate, aspartate, and alanine. These energy sources are included in the basic culture medium used in the present study (9).

It is concluded that protein is not necessary for mouse embryo development from the two-cell to the expanded blastocyst stage and that protein may have a detrimental effect on embryonic development depending on the type and the concentration used for culture in vitro. Even if the protein type does enable normal rates of embryo development in vitro, culture in the presence of protein supplements may reduce the capacity for embryos to develop to advanced fetal stages in utero.

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