# Culture of Two- and Four-Cell Rabbit Embryos to the Expanding Blastocyst Stage in Synthetic Media[1] (34595)

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The successful continuous culture of mammalian ova from zygote to blastocyst in what has been referred to as a "chemically defined medium" has been limited to the mouse (1). Purshottam and Pincus (2) reported no growth of rabbit embryos placed in protein-free Waymouth medium in the two-cell stage up to the blastocyst stage, and found it necessary to add serum to Eagle's basal medium for morulae to become early blastocysts. Daniel (3) modified the amino acid content of Ham's F10 so that it could support growth of rabbit zygotes to the morula stage. Krishnan and Daniel (4) reported that protein-free F10 would allow rabbit embryos collected as 3-day morulae to form the blastocoele cavity, but a much smaller proportion did so than when maternal serum proteins or uterine protein was added. A uterine protein was found necessary for blastocyst expansion to take place.

It had been observed in our laboratory that the addition of 1.5% of 4X crystallized bovine serum albumin (BSA) obtained from Nutritional Biochemicals Corp. to Ham's F10 medium (5) could permit culture of two- to four-cell rabbit ova to the expanding blastocyst. This investigation was planned to ascertain the constituents of the F10 medium that were necessary for blastocyst formation under these conditions.

*Materials and Methods.* Three types of media used in the experiments were (a) a simple synthetic medium containing 1.5% BSA (Table I) modified from Brinster (6), (b) a complete synthetic medium containing supplementary nutrients added to the simple synthetic (Table I), and (c) F10 medium (DIFCO) used as a control. Three experiments were carried out. In Expt. 1 there were seven treatments as follows: Ham's F10 with 1.5% BSA as a control, simple synthetic, complete synthetic, and complete synthetic with groups of nutrients omitted, as shown in Table II. Expts. 2 and 3 were designed to gain insight into the amino acid requirements for blastocyst formation in the absence of the vitamins, trace elements, and nucleic acid precursors. In Expt. 2 the 20 amino acids were omitted by groups of four and in Expt. 3 eight amino acids from the two groups whose omission in Expt. 2 resulted in essentially no blastocyst formation were omitted individually (Table III).

Two- and four-cell ova were recovered from Dutch-Belted rabbits superovulated (7) and inseminated 28 to 30 hr previously. The medium used for flushing was similar to the simple synthetic one except that it had only 0.1% BSA. The ova from each rabbit were washed as a group three times in approximately 2 ml of the flushing medium and then distributed as equally as possible among the treatments. In Expt. 1 ova were cultured in 0.6-ml droplets of medium under 10 ml of light-weight paraffin oil in Falcon 60-X 15-mm plastic tissue culture dishes. Subsequently, ova were cultured in 2-ml capacity individual cups of Linbro Disposo trays (Model 96CV) because the oil available at the time caused the droplets of media to spread and run together in the conventional tissue culture dishes. In these cups 0.8 ml of medium was used under 0.2 ml of

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TABLE I. Composition of Simple Synthetic Medium and Concentrations of Supplementing Nutrients.

| Simple synthetic medium[a] | | | |
|---|---|---|---|
| Component | Concentration (g/liter) | Supplementary nutrients (μg/liter) | |
| NaCl | 6.683 | Amino acids[b] | |
| KCl | 0.356 | Vitamins | |
| $CaCl_2 \cdot 2H_2O$ | 0.251 | Biotin | 24 |
| $KH_2PO_4$ | 0.162 | Calcium pantothenate | 715 |
| $MgSO_4 \cdot 7H_2O$ | 0.294 | Choline chloride | 698 |
| $NaHCO_3$ | 2.106 | myo-Inositol | 541 |
| Glucose | 1.801 | Niacinamide | 615 |
| BSA | 15.000 | Lipoic acid | 200 |
| | | Pyridoxine HCl | 206 |
| | | Riboflavin | 376 |
| | | Thiamine HCl | 1012 |
| | | Folic acid | 1320 |
| | | Vitamin $B_{12}$ | 1360 |
| | | Nucleic acid precursors | |
| | | Hypoxanthine | 4080 |
| | | Thymidine | 727 |
| | | Trace elements | |
| | | $FeSO_4 \cdot 7H_2O$ | 834 |
| | | $CuSO_4 \cdot 5H_2O$ | 2.5 |
| | | $ZnSO_4 \cdot 7H_2O$ | 28.8 |

[a] Also potassium penicillin G, 100,000 IU/liter and streptomycin sulfate 500,000 μg/liter.
[b] See Ham (5).

peanut oil. Wherever possible 10 ova were placed in each drop or cup. Cultures were incubated for 4 days at 37° in a humidified gas phase of 5% $CO_2$ and 95% air.

Ova were examined at 35 and 100X daily for 4 days after being placed in culture. Embryos were classified and scored for purposes of statistical analysis according to the stage

TABLE II. Response of Two- to Four-Cell Ova to the Omission of Different Nutrient Groups from the Synthetic Culture Medium.

| | % Ova in different stages after culture | | | | |
|---|---|---|---|---|---|
| Treatments[a] | Pre-blastocysts | Early blastocysts | Blastocysts | Expanding blastocysts | Mean score per treatment |
| Ham's F10 + 1.5% BSA | 16 | 19 | 23 | 42 | 19[b] |
| Simple synthetic | 100 | 0 | 0 | 0 | 0 |
| Complete synthetic | 18 | 23 | 18 | 42 | 18[b] |
| No amino acids | 100 | 0 | 0 | 0 | 0 |
| No vitamins | 46 | 42 | 7 | 5 | 7[c] |
| No trace elements | 21 | 26 | 14 | 39 | 17[b] |
| No nucleic acid precursors | 19 | 5 | 12 | 63 | 22[b] |

[a] No. of ova cultured per treatment was 57.
[b] Mean scores with different superscript letters from the complete synthetic differ significantly from it ($p < .01$). Mean scores with the same superscript do not ($p > .05$). The two treatments with zero mean scores were excluded from the analysis.

TABLE III. The Response of Two- to Four-Cell Ova when Amino Acids Were Omitted from the Synthetic Culture Medium.

| Exp. no. | Treatment[a] | % Ova at different stages after culture: Pre-blastocysts | Early blastocysts | Blastocysts | Expanding blastocysts | Mean score per treatment |
|---|---|---|---|---|---|---|
| 2[b,c] | Simple synthetic plus all amino acids | 27 | 17 | 37 | 20 | 15 |
| | No group 1 amino acids | 63 | 30 | 3 | 3 | 5 |
| | No group 2 amino acids | 100 | 0 | 0 | 0 | 0 |
| | No group 3 amino acids | 97 | 3 | 0 | 0 | 0 |
| | No group 4 amino acids | 77 | 20 | 3 | 0 | 3 |
| | No group 5 amino acids | 60 | 33 | 7 | 0 | 5 |
| | No amino acids | 100 | 0 | 0 | 0 | 0 |
| 3[b] | Simple synthetic plus all amino acids | 60 | 15 | 5 | 20 | 8 |
| | No methionine | 100 | 0 | 0 | 0 | 0 |
| | No phenylalanine | 55 | 25 | 10 | 10 | 8 |
| | No tyrosine | 65 | 25 | 10 | 0 | 4 |
| | No cysteine | 65 | 30 | 5 | 0 | 4 |
| | No alanine | 40 | 25 | 10 | 25 | 12 |
| | No glycine | 55 | 20 | 5 | 20 | 9 |
| | No serine | 90 | 10 | 0 | 0 | 1 |
| | No threonine | 85 | 15 | 0 | 0 | 2 |
| | No amino acids | 100 | 0 | 0 | 0 | 0 |

[a] Sodium pyruvate at a level of 0.11 mg/ml was present in all treatments.

[b] No. of ova cultured per treatment was 30 in Exp. 2 and 20 in Exp. 3.

[c] The amino acid groups are: 1, arg, his, lys, try; 2, met, phe, tyr, cys; 3, ala, gly, ser, thr; 4, asp, asp-$NH_2$, glu, glu-$NH_2$; 5, ileu, leu, pro, val.

reached after 4 days in culture as follows: a score of 0 was assigned to any embryo not reaching the blastocyst stage, a score of 1 to early blastocysts with the blastocoele just visible, a score of 2 to blastocysts with the blastocoele cavity at least half the size of the embryo, and a score of 3 to clearly expanding blastocysts. Scores per drop were obtained by totaling up the scores for all the embryos in each drop. Occasionally there were less than 10 embryos per drop and the drop scores were corrected to a base of 10. The scoring system provided weighted means to take into account all information regarding stages of development attained in culture.

*Results.* The results of Expt. 1 are shown in Table II. The simple synthetic medium and the complete medium minus the amino acids gave a zero response. There was no blastocoele formation whatsoever. Analysis of variance of the drop scores of the remaining treatments revealed a significant difference due to treatments ($p < .005$). A Dunnet's test in which the control complete synthetic medium was compared with other treatments showed that without vitamins development was depressed ($p < .01$). No other treatments differed significantly from the control ($p > .05$). Surprisingly, the percentage of expanding blastocysts in the medium without nucleic acid precursors (63%) was considerably higher than for the complete synthetic or Ham's F10 + BSA medium (42%). Also, the blastocysts in this treatment looked better upon microscopic examination than in the other treatments. One blastocyst in this treatment was "hatched" completely out of the zona pellucida by the fourth day of culture and had expanded to over 400 $\mu$ in diameter.

The results of Expts. 2 and 3 are given in Table III. From Expt. 2 it is clear that omission of each group of amino acids decreased growth, but omission of groups 2 and 3 resulted in practically no response. In

Expt. 3, individual omission of the eight amino acids comprising these two groups showed that methionine, serine, and threonine were most important, as the single omission of any of these three drastically curtailed development. Other amino acids in these groups, especially alanine and glycine were of little importance.

*Discussion.* It is clear from Expt. 1 that Ham's F10 + 1.5% BSA, or a medium such as the complete synthetic, will give good growth of rabbit embryos *in vitro* from the two- and four-cell stage to expanding blastocysts. This is contrary to the work of Krishnan and Daniel (4) who found that a uterine protein component was necessary for expansion of blastocysts cultured from 3-day morulae. The only macromolecule included in the present studies was highly purified BSA. Its mechanism of action cannot be deduced from these experiments, but since it is markedly beneficial when added to synthetic media containing a complex of growth promoting substances, its action was not likely the result of some trace contaminant with growth promoting properties.

The most essential group of nutrients was the amino acids. In their complete absence no blastocysts were formed either in the presence (Table II) or absence of cofactors (Table III). Expts. 2 and 3 showed that many amino acids were essential for blastocyst growth in the absence of cofactors. However, methionine, serine and threonine, followed by cysteine and tyrosine, were most important as judged by the restriction of blastocyst development in their absence. Alanine, glycine or phenylalanine were not required for the development of expanding blastocysts. However, further experimentation is required to rank them all in order of importance.

Miller and Reimann (8) reported that the addition of methionine to serum enabled 10- to 12-cell rabbit embryos to grow rapidly to blastocysts. Also, methionine, serine, and threonine are 3 of the 10 amino acids found by Daniel and Krishnan (9) to be essential for growth of 5-day rabbit blastocysts. Methionine and threonine were reported to be necessary for zygotes to cleave beyond the four-cell stage in culture by Daniel and Olson (10). In the present experiments two- to four-cell ova routinely cleaved to the morula stage in the simple synthetic medium containing 1.5% BSA without any amino acids.

An important point to be noted from Expts. 2 and 3 is that blastocyst formation and growth can be achieved in a very simple medium with only amino acids added. Also, it is possible that increasing the amino acid level might improve growth since the levels of amino acids used here were generally lower than in many other tissue culture media.

The presence of vitamins in the medium was not essential for blastocyst formation, but they materially aided blastocyst development and were essential for good blastocyst growth and expansion. This is in agreement with a report by Daniel (11) that pyridoxine, thiamine, niacinamide, and folic acid were necessary for growth of 5-day blastocysts.

Omission of the trace elements did not cause a significant decrease in response, but this does not prove their nonessentiality. The salts used to prepare the simple synthetic medium were analytical grade and may have contained sufficient trace elements for the needs of the embryos.

The omission of the nucleic acid precursors, hypoxanthine and thymidine, appeared to give a very noticeable improvement in growth even though the difference in mean scores was not significant. In no other treatment of Experiment 1 did the blastocysts look so well formed or show such a tendency to escape from the zona pellucida. Further investigation of this effect is warranted.

*Summary.* Development of two- to four-cell rabbit ova to the expanding blastocyst stage occurred in a simple glucose-salt solution supplemented with amino acids, and with 1.5% BSA as the only macromolecule. However, optimum growth required the presence of vitamins. These experiments would indicate that although a uterine protein component may be necessary for blastocyst growth and expansion *in utero*, it is not necessary in the *in vitro* culture system used. Hypoxanthine and/or thymidine may be inhibitory to good blastocyst development, as

the highest percentage of expanding blastocysts (63%) was obtained when the nucleic acid precursors were omitted.

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