Development of the rat conceptus
in vitro and associated changes in components of culture medium

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SUMMARY

Rat conceptuses of pregnancy day 11 (embryonic age approximately 10.5 days) with embryos within the yolk and amniotic sacs and polar allantoic placental tissues were cultured by the ‘New system’ utilizing homologous serum in 60 ml roller bottles with 20% O₂, 5% CO₂ and 75% N₂ gas environment (five conceptuses/10 ml serum). Embryonic growth and development was assessed by external morphology and somite counts, and by measurement of DNA, RNA and protein contents. The embryonic growth was considerably retarded beyond 24 h when culture was extended to 48 h.

During continuous culture of conceptuses for 48 h, the partial pressure of O₂ in the medium decreased gradually with simultaneous increase of PO₉₅ and decline of pH values. Glucose level was depleted considerably and small quantities of urea, uric acid and creatinine accumulated in the medium. The concentrations of electrolytes (Na⁺, K⁺, Cl⁻) and total Ca and P, and lactic dehydrogenase activity in the medium increased during the latter part of the 48 h culture period.

The growth and differentiation of embryos were significantly improved by transferring conceptuses to fresh serum medium with 40% O₂, 5% CO₂ and 55% N₂ gas phase after 22–24 h culture and continuing the culture for an additional 24 h. Embryos grown in vitro for 48 h developed 39 somites and showed a tenfold increase in DNA content and a five- to seven-fold increase in RNA and protein contents, over initial values.

INTRODUCTION

New and his associates have developed a system for in vitro culture of rat conceptuses with embryos within the yolk and amniotic sacs and polar ecto-placental cones during the process of major organogenesis of the embryo (New, 1973, 1978; Steele, 1975). Embryo-placental complexes of pregnancy days 10, 11 or 12 (embryonic age 9.5, 10.5, 11.5 days respectively) could be conveniently cultured for 1–3 days in roller bottles with medium of heat-inactivated homologous serum under adequate oxygen tension (New, Coppola

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& Terry, 1973; New & Coppola, 1977; New, Coppola & Cockroft, 1976a, b). The embryonic growth and differentiation in vitro closely resembled those in vivo in the mother during the same period. The placental differentiation, however, was marginal in the conceptuses grown in vitro.

Development of conceptuses in this in vitro system for a prolonged period, with continued growth and differentiation of foetal and placental tissues, has not been possible. We found that rat conceptuses of pregnancy day 11 grew optimally for 24 h in adult male rat serum with a gas phase of 20/40 % O\textsubscript{2}, 5 % CO\textsubscript{2} and 75/55 % N\textsubscript{2}, paralleling in vivo development (Sanyal & Wiebke, 1979). Embryonic growth in vitro was significantly retarded when the culture period was extended to 48 h. The possibility that depletion of nutrients or accumulation of unfavourable substances in the culture medium may partially prevent continued in vitro embryo development has been explored in the present study. Progressive changes in biochemical constituents, partial pressures of gases and pH of the culture medium were recorded in order to determine the basic nutritional and gaseous requirements for in vitro growth and differentiation of pregnancy-day-11 rat embryos.

MATERIALS AND METHODS

Dissection of conceptuses

Conceptuses were obtained from adult pregnant rats (Charles River, CD-strain) maintained with balanced rat chow and tap water and bred under normal laboratory conditions of 14 h of daily illumination (05.00-19.00 h). Pro-oestrous rats were placed with proven breeder males in the afternoon for mating. The sperm-positive females next morning were considered pregnant, and this day was designated day 1 of pregnancy (embryonic age approximately 0-5 day at noon).

During the afternoon (15.00-18.00 h) of pregnancy day 11 the donor rats were sacrificed by a blow to the head. The conceptuses were dissected out of the uterus in Trowell T8 tissue culture medium (GIBCO, Grand Island, N.Y.) in a sterile environment by the methods described previously (New, 1973). The external decidual tissues of the implantation sites were removed by fine watch maker's forceps under a binocular microscope at magnification ×20. Reichert's membrane was torn, exposing the yolk sac. Trophoblastic tissue over the Reichert's membrane was then pushed gently towards the polar ectoplacental cone (allantoic placenta). The yolk-sac diameter was measured by an eye-piece micrometer. The embryos were at Witschi stage 16 of development, fully curved ventrally with 14-15 somites and beating hearts (Witschi, 1962).
Preparation of culture medium

The culture medium consisted of fresh rat serum prepared by immediate centrifugation of blood at 3500 rpm (1000 g) according to the methods described earlier (Steele & New, 1974). The blood was collected from the dorsal aorta of ether-anaesthetized 75- to 80-day-old male rats (Charles River, CD-strain). Pooled serum from several rats was heated in a water bath at 56 °C for 30–40 min. Antibiotics were added to give final concentrations of 50 μg streptomycin and 50 IU penicillin per ml before utilization for culture.

Culture of conceptuses

Five conceptuses were suspended in sterilized 60 ml roller bottles containing 10·5 ml of culture medium as soon as they were dissected out. The bottles were sealed with rubber stoppers after equilibration with a gas mixture of 20 % O₂, 5 % CO₂ and 75 % N₂. The bottles were rotated (40 rev/min) in a Roller culture apparatus (Bellco Glass Co., Inc.), similar to that described by Kochhar (1975), placed within an incubator maintained at 38 °C. The apparatus consisted of a roller drum measuring 35 cm in diameter to which the bottles were fixed horizontally at the periphery. Rotation of the bottles produced gentle agitation and uniform mixing of gases with the culture medium.

The conceptuses were cultured continuously for 6, 12, 18, 24 and 48 h. In one series of experiments after 22–24 h of culture in 20 % O₂, 5 % CO₂ and 75 % N₂ the conceptuses were transferred to bottles with 10 ml fresh serum medium equilibrated with 40 % O₂, 5 % CO₂ and 55 % N₂ gas atmosphere, and the culture was continued until 48 h. At the end of the culture period conceptuses were removed from the bottle and washed in 5 ml Ringer's solution. The yolk-sac diameters were measured by an eye-piece micrometer. The number of somites and gross anomalies of the embryos were also noted. The spontaneous beating of the heart or beating in response to gentle prick of a forceps was considered to be the primary vital sign of the cultured embryo. Embryos or ectoplacental and trophoblast tissues (placentas) of five conceptuses from a bottle were frozen together in liquid nitrogen. The frozen tissues and culture medium were stored at −10 °C until utilized for nucleic acid and protein estimations of these tissues and biochemical measurements of the medium within 2–3 weeks.

Measurement of pO₂, pCO₂ and pH of the medium

The partial pressures of O₂ and CO₂ and pH of the culture medium were estimated by a Micro Blood Gas Monitor (Radiometer, Copenhagen). The instrument was calibrated with precision buffer solutions, pH 6·841 and 7·383, and certified standard gases, 12 % CO₂, 21 % O₂, 67 % N₂ and 5 % CO₂, 0 % O₂, 95 % N₂. In each experiment, following equilibration for 1–2 h in the roller apparatus at 38 °C, 0·5 ml aliquots were withdrawn from bottles for
estimation of initial pH and partial pressures of gases in the medium. Another aliquot of 0.5 ml was withdrawn for measurement of changes in these parameters at the end of the culture period.

Nucleic acid and protein estimation of conceptus

Nucleic acid and protein contents of embryos and placentas were quantified by a procedure adapted from Shibko et al. (1967) for measurements in small quantities of tissues. Five cultured embryos or five placentas from individual bottles, or controls (20 each for day 11, 5 each for day 12 or 3 each for day 13) were homogenized in 1 ml of 0.25 M sucrose by a sonifier (Branson Sonic Power Co.). Nucleoproteins were precipitated by addition of 100 µl concentrated (60–62%) perchloric acid (PCA). The precipitates were dissolved with 0.3 N sodium hydroxide at 37 °C for 30–60 min and reprecipitated by adjusting PCA concentration to 5–6% on ice. The supernatant was used for spectrophotometric (260 nm) estimation of RNA. The precipitate was homogenized again by sonification in 1.5% PCA and heated for 15 min in a water bath at 90 °C for extraction of DNA. Finally, the solution was chilled on ice and proteins were precipitated by increasing PCA concentration to 5%. DNA was determined in the supernatant by diphenylamine colour reaction (Burton, 1956) and protein in the precipitates by the method described by Lowry, Rosebrough, Farr & Randall (1951).

Biochemical analysis of the medium

A high-speed-computer-controlled biochemical analyser (SMAC, Technicon Instruments Corporation, Tarryton, N.Y.) was programmed to estimate the following groups of biochemical correlates in the culture media: (1) nutrients and substrates – glucose, albumin, triglycerides, and cholesterol; (2) excretory products of intermediary metabolism – urea nitrogen, uric acid and creatinine; (3) enzyme activities – alkaline phosphatase (AP), lactic dehydrogenase (LDH), glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT) and creatinine phosphokinase (CPK); and (4) electrolytes and elements – Na⁺, K⁺, Cl⁻, Ca and P. Methods of analysis of these biochemical constituents have been outlined in detail in the Technicon SMAC brochure released March 1976. There was reduction in the volume of culture medium which was estimated to be approximately 5% of the initial volume in 18 h, 7.5% in 24 h and 10% in 48 h of culture. Accumulation of excretory products and increase of enzyme activity were calculated by subtraction of initial concentration from values obtained after culture in each experiment.

Statistical analysis

The nucleic acid and protein contents of embryos and placentas and the concentration of biochemical constituents or enzyme activities in the culture
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medium have been computed as mean ±S.E.M. The data were statistically evaluated by Fisher's least significant difference test comparing the initial nucleic acid and protein values of embryos and placentas or concentrations of biochemical components of culture medium with those after culture in each experiment and by Student's t test to establish statistical significance of the data (Miller, 1966).

RESULTS

Growth and viability of embryos in culture

Conceptuses grew progressively in serum culture medium under the present conditions of culture. The diameters of yolk sacs increased from 2·6 mm to 6·4 mm during 48 h of culture. The number of somites increased gradually from 14 to 31–32 somites during 24 h of continuous culture. Embryos cultured for 24 h were similar in appearance to those grown in vivo during the same period and all of them had vigorously beating hearts when retrieved from the culture bottles. At the end of the 48 h continuous culture period, the number of somites had not increased further and only 40 % of the embryos had beating hearts. However, when the conceptuses were transferred to fresh medium with 40 % O₂, 5 % CO₂ and 55 % N₂ gas phase after 22–24 h culture in 20 % O₂, 5 % CO₂ and 75 % N₂, growth and differentiation were markedly stimulated and the number of somites increased from 31–32 to an average of 39.

During the afternoon of pregnancy day 11 when the cultures were initiated the embryos had 3·4 µg DNA which rose to 19·7 and 24·0 µg at 24 and 48 h of culture respectively. Gradual increase of RNA content from 11·6 to 61·7 µg per embryo followed an identical pattern of DNA increase during the 48 h culture period. The amount of protein synthesized by embryos was approximately sixfold within 48 h of culture; the values increased from 42·5 to 244·2 µg/embryo during this period. Transfer of conceptuses to fresh medium with 40 % O₂ markedly improved nucleic acid and protein synthesis in the embryo. DNA content increased from 24·0 µg per embryo in continuous culture for 48 h to 35·5 µg with change of medium and higher O₂ atmosphere, RNA from 61·7 to 72·3 and protein from 244·2 to 278·8 µg per embryo. In vitro growth and development of the embryo during latter 24 h period of culture were markedly retarded compared to those in vivo even after transfer to fresh medium and higher O₂ environment; DNA content was 75 %, RNA 29 %, and protein 28 % of the embryos in vivo during the same period.

The placental differentiation and growth were marginal or absent in the present system of culture. Transfer of conceptuses to fresh medium did not stimulate placental development. DNA, RNA and protein contents were approximately 25, 18, and 19 % of in vivo placentas in 24 h and 7, 6 and 3 % in 48 h of culture.
Fig. 1. Changes of pH (■) and partial pressures of O₂ (▲) and CO₂ (●) in the medium during continuous culture for 46-48 h with initial gas environment of 20% O₂, 5% CO₂ and 75% N₂. Mean ± s.e.m.; n, within parentheses.

Changes in partial pressures of O₂ and CO₂ and pH of the medium

Progressive alteration of partial pressures of O₂ and CO₂ in the culture medium is depicted in Fig. 1. The average initial partial pressure of O₂ after equilibration with 20% O₂, 5% CO₂, and 75% N₂ was 141 mmHg and it decreased considerably to 106 mmHg after 48 h of continuous culture. The partial pressure of CO₂, however, gradually increased from 42 to 85 mmHg during the same periods. The decline of pH values from 7·4 to 6·9 and associated increase of the partial pressure of CO₂ in the medium samples after 48 h of culture was significant (P < 0·01).

Depletion of nutrients in the medium

The initial glucose concentration was 172 ± 4 mg/100 ml in serum culture medium obtained from 75 to 80-day-old male rats. The glucose concentration in the medium gradually declined through the culture period (Fig. 2). During the first 24 h of culture it was reduced to one half (86 mg/100 ml) and it was decreased further (34 mg/100 ml) during the second 24 h of continuous culture. The rate of glucose utilization was greater during the first 24 h period compared with the second 24 h period in continuous culture for 48 h. Triglyceride and cholesterol concentrations were also measured, but no significant alteration was observed in these medium components. Albumin concentration increased marginally, presumably due to reduction of the medium volume.
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Accumulation of excretory products in the medium

Accumulation of urea (estimated as urea nitrogen), at various time periods of culture is shown in Fig. 3. The initial concentration of urea was 16 mg/100 ml of serum. This value increased to 19–20 mg/100 ml medium during culture for 24 h. The increase in concentration of urea was significant even after 6 h of culture \((P < 0.01)\). The uric acid concentration in the serum was 1.5 mg/100 ml, which increased slightly to approximately 2 mg/100 ml of the medium in 24–48 h of culture. The creatinine values were low, 0.5 mg/100 ml of serum, and the accumulation was marginal during the culture (Fig. 3).

Enzyme activities of the medium

Increases of enzyme activities of lactic dehydrogenase, alkaline phosphatase, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and creatine phosphokinase are shown in Fig. 4. The LDH activity increased 39–50 U/l after 24 h and 75 U/l after 48 h of continuous culture when assessed from individual initial values for each experiment \((259 \pm 18 \text{ U/l, } n = 9)\). Alkaline phosphatase activity was also elevated within 12 h by 12–17 U/l from the initial values \((65 \pm 8 \text{ U/l, } n = 9)\), and remained unaltered throughout the culture period until 48 h. Increases in AP, GOT, GPT and CPK enzyme
activities were small and without significant change during different time periods of culture.

Electrolytes (Na\(^+\), K\(^+\), Cl\(^-\)) and Ca and P levels of the medium

The electrolyte concentrations progressively increased during culture, Na\(^+\) from 142 to 160, K\(^+\) from 4-4 to 5-2 and Cl\(^-\) from 106 to 124 m-equiv/l in 48 h culture period. Calcium and phosphorus concentrations in the medium were 10-3 and 7-3 mg/100 ml, which rose to 12-8 and 7-6 mg/100 ml during the 48 h culture period respectively. These increases during different time periods resulted from a decrease of medium volume presumably due to a small degree of evaporation of the medium and transfer of water into distended yolk and amniotic sacs of the conceptuses.

Metabolic activity of conceptuses when transferred to fresh medium

Comparison of metabolic activity of conceptuses during the first 24 h of culture with that in the second 24 h, after conceptuses were transferred to fresh medium with 40 % \(O_2\) gas phase, is given in Table 1. Changes from the initial values represent actual utilization or accumulation of biochemical components in the media. Glucose utilization and accumulation of urea, uric acid and
creatinine due to metabolic processes of the embryo and other conceptus tissues were less in the second half compared to the first half of the 48 h culture period. The enzyme activities were, however, not markedly different during the two culture periods.

**DISCUSSION**

In this culture method with sealed serum bottles, the oxygen tension gradually declined from 141 to 120 mmHg in 24 h of continuous culture (Fig. 1). The embryos at this stage of development (pregnancy day 11) grew optimally for 24 h in initial $P_{O_2}$ of 140 mmHg or more in the medium produced by equilibration with 20–40% $O_2$ in the gas phase (Sanyal & Wiebke, 1979). Buffering capabilities of the medium were also partially depleted; the number of $HCO_3^-$ ions available for maintenance of constant pH was low. The lowering of pH from 7.4 to 6.9 in the medium during a 48 h culture may be an adverse factor for overall embryo-placental growth and differentiation. Thus, continuous infusion of gases with opportunity for $CO_2$ escape from the bottles will presumably improve embryo-placental development *in vitro* (New, 1978).

During gestation *in vivo*, glucose freely diffuses from the mother into the embryo, and a constant infusion of glucose from the maternal system is required for embryo survival (Rosso, 1975; Gabbe & Quilligan, 1977). The data in the present study show that conceptuses *in vitro* also utilize glucose...
Table 1. Concentration of constituents of the culture medium after 24 h culture and after culture of the same conceptuses in fresh medium for additional 24 h in higher O₂ atmosphere (mean ± S.E.M., n = 6)

<table>
<thead>
<tr>
<th>Medium constituents</th>
<th>Culture period (0–24 h)</th>
<th>Culture period (24–48 h)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>Difference</td>
</tr>
<tr>
<td>Glucose (mg/100 ml)</td>
<td>78.43 ± 1.60</td>
<td>-89.57</td>
</tr>
<tr>
<td>Albumin (g/100 ml)</td>
<td>6.74 ± 0.08</td>
<td>+1.14</td>
</tr>
<tr>
<td>Triglycerides (mg/100 ml)</td>
<td>165.71 ± 1.28</td>
<td>+30.71</td>
</tr>
<tr>
<td>Cholesterol (mg/100 ml)</td>
<td>79.43 ± 0.48</td>
<td>+11.43</td>
</tr>
<tr>
<td>Urea nitrogen (mg/100 ml)</td>
<td>18.71 ± 0.18</td>
<td>+2.71</td>
</tr>
<tr>
<td>Uric acid (mg/100 ml)</td>
<td>2.22 ± 0.06</td>
<td>+0.62</td>
</tr>
<tr>
<td>Creatinine (mg/100 ml)</td>
<td>0.80 ± 0.03</td>
<td>+0.20</td>
</tr>
<tr>
<td>Alk. phosphatase (U/l)</td>
<td>48.43 ± 1.06</td>
<td>+14.43</td>
</tr>
<tr>
<td>Lactic dehydrogenase (U/l)</td>
<td>276.71 ± 3.21</td>
<td>+51.71</td>
</tr>
<tr>
<td>GOT (U/l)</td>
<td>100.71 ± 0.84</td>
<td>+9.71</td>
</tr>
<tr>
<td>GPT (U/l)</td>
<td>36.14 ± 1.79</td>
<td>+7.14</td>
</tr>
<tr>
<td>CPK (U/l)</td>
<td>7.43 ± 1.00</td>
<td>+2.43</td>
</tr>
<tr>
<td>Na⁺ (m-equiv/l)</td>
<td>175.43 ± 1.84</td>
<td>+28.43</td>
</tr>
<tr>
<td>K⁺ (m-equiv/l)</td>
<td>5.43 ± 0.09</td>
<td>+0.53</td>
</tr>
<tr>
<td>Cl⁻ (m-equiv/l)</td>
<td>126.71 ± 1.70</td>
<td>+16.71</td>
</tr>
<tr>
<td>Ca (mg/100 ml)</td>
<td>12.41 ± 0.14</td>
<td>+2.31</td>
</tr>
<tr>
<td>P (mg/100 ml)</td>
<td>8.70 ± 0.07</td>
<td>+1.20</td>
</tr>
</tbody>
</table>

* Differences from the initial values represent depletion (−)/accumulation (+) of constituents. Depletion and accumulation values of the two culture periods are significantly different (P < 0.05) except those marked NS.

from the culture medium (Fig. 2). Glucose levels were depleted to approximately one half the initial level in 24 h culture. Reduction in glucose level continued during 48 h culture without significant embryonic growth. The requirement of high oxygen concentration in the medium for in vitro development of embryos at this stage (New, Coppola & Cockroft, 1976a, b; Sanyal & Wiebke, 1979), and the rapid depletion of glucose from the culture medium, as observed in the present study, indicate that aerobic glycolysis may be one of the major processes of energy generation in these embryos. Previous studies have also suggested that glucose is an essential requirement for energy metabolism in the embryo during organogenesis (Shepard, Tanimura & Robkin, 1970; Tanimura & Shepard, 1970; Gunberg, 1976). Hyperglycaemia, however, is injurious. In a recent study, Cockroft & Coppola (1977), employing embryo culture techniques similar to the present study, have shown that excess glucose in the medium is deleterious to embryo development, inducing retardation of growth and anomalous differentiation.

Beneficial effects due to change of medium support the hypothesis that embryo-placental growth and differentiation could be improved when adequate
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amounts of nutritional and other requirements are provided in the culture medium (Table 1). In the present study the absence of simultaneous differentiation of the allantoic placenta compartment of the conceptuses presumably also limited the growth and viability of the embryos. The conditions for placental differentiation and development in vitro in the present system of culture is unknown. However, in a recent study, New & Coppola (1977) discovered that by removal of most of the ectoplacental trophoblast in conceptuses explanted at head-fold and early somite stages (comparable to days 10 and 11 of pregnancy), the growth and differentiation of preplacental tissues into chorioallantoic placenta could be improved. Development of chorioallantoic placenta in vitro would, perhaps, substantially enhance embryonic growth and differentiation.

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