1985

Baboon Embryo Culture As A Model For Primate Embryology: Functional And Histological Differentiation In Vitro.

Valerie Z. Pope
University of Alabama at Birmingham

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The University of Alabama at Birmingham PH.D. 1985

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BABOON EMBRYO CULTURE AS A MODEL FOR PRIMATE EMBRYOLOGY: FUNCTIONAL AND HISTOLOGICAL DIFFERENTIATION IN VITRO

by

VALERIE Z. POPE

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Biology
in the Graduate School, The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

1985

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree PhD Major Subject Biology

Name of Candidate Valerie Z. Pope

Title Baboon Embryo Culture as a Model for Primate Embryology: Functional and Histological Differentiation in Vitro

A culture method for culture cleavage stage baboon embryos was developed to allow investigations of early primate development. The differentiation achieved, and the ability of embryos to secrete baboon chorionic gonadotropin (bCG), placental protein SP-I, progesterone (P4) and estradiol (E2) following removal from the maternal environment were evaluated. Cultured embryos were recovered nonsurgically from 59 female baboons at the 2-cell stage to hatched blastocyst stage. Most embryos were cultured individually in CMRL-1066 medium supplemented with human cord serum (HCS), although several were switched to different serum supplementation after hatching. More embryos achieved posthatching stages when the media containing the embryo was incubated under mineral oil (43.5%) compared to culture plate wells (21.4%); however, insignificantly fewer embryos reaching posthatching stages underwent normal differentiation of the inner cell mass when cultured under oil (32%) than without an oil overlay (49%). The trophoblast acquired functional capacity for secreting both bCG and SP-1 during in vitro development. BCG Secretion profiles were similar to those in vivo, with a peak followed by a precipitous decline to undetectable levels. SP-1 was frequently present in ug quantities in media from a single day's culture, and may provide a marker for

trophoblast health. Both proteins were undetectable before hatching. Significant increases in spent media P4 content occurred after attachment in many embryo cultures regardless of normality of embryonic development. Increases of up to 500 ng over P4 available were measured in the daily media harvested from a single embryo. Levels of E2 were subject to less variation, although some embryos had an increase in media E2 around the time of hatching. Histological methods were used to verify morphological observations of differentiation of embryonic structures as well as trophoblast. Three of five embryos had differentiation of the bilaminar embryonic disc and definitive amnion. One additionally had a presumptive secondary yolk sac forming beneath the hypoblast of the embyronic disc. A fourth embryo had degenerated embryonic structures, and the fifth embryo was a solid cell mass. The results achieved with baboon embryo culture suggest that this model has significant potential to provide new information on early primate embryology.

Abstract Approved by: Committee Chairman Gary R Grower

Program Director Elaine R. Jones

Date 8/23/85 Dean of Graduate School

DEDICATION

To my parents, Carroll and Mildred Zimmerman.

ACKNOWLEDGEMENTS

I would like to acknowledge the assistance of my faculty advisor, Dr. Gary R. Poirier, and the rest of my graduate committee: Drs. C. Pat Dagg, Larry Boots, Lee Beck, Ron Gettinger and Mel Kunkle. Each member was empathetic and supportive of my commitment to not only accumulate sufficient material for a dissertation, but to help establish an important model for early primate embryology that could provide a career's worth of opportunity and challenge. They were also reassuringly sympathetic with my inability to convince NIH of the latter.

Dr. Beck was particularly helpful by providing baboons for embryo recovery and allowing me to use animals in their control cycles between experiments. Additionally, he generously provided laboratory space and materials to allow me to do my research while working full-time on his funded projects.

My colleague and husband, Earle, was a constant source of moral support and a 24-hour sounding board for problem-solving sessions. Our son, Mike, was incredibly patient throughout, and a valuable assistant in the preparation of this dissertation, during which time he has acquired some interesting skills on a word processor.

Finally, I give my thanks to Dr. Bill Thurmond who, in his embryology class at Cal Poly, San Luis Obispo, 1966, inspired my interest in embryology.

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CHAPTER I

INTRODUCTION

Early implantation is one of the least understood stages in mammalian ontogeny. Improved histologic technology and availability of culture systems have allowed much progress in the last twenty years, mainly using nonprimate models. Studies involving human embryos went through a period of ethical restrictions, which have been relaxed in many countries with the advent of the clinical application of in vitro fertilization and embryo transfer to treat several forms of infertility in humans. Experimentation with human embryos is controversial, which limits the number of embryos available for experimental use.

Nonhuman primates have been used as models of human reproduction for decades. Studies of early primate embryonic development have been limited by restricted availability and the cost of keeping primates. Recovery of embryos using surgical methods is expensive, and repeated surgery limits the embryo donor's performance. Recovery of embryos at necropsy is even more expensive, and conservationally negligent unless the procedure can be performed in association with another project that is necessarily terminal. Concomitant use of primates in terminal experiments and for embryo recovery is essentially limited logistically to large primate centers, and by design must ensure that one procedure does not interfere with the protocol or results of another.

The baboon colony at the University of Alabama at Birmingham has been used for reproductive investigations for 12 years. The program to develop the baboon as a model for the scientific investigation of early embryonic development was carried out under the administrative direction of Dr. Lee Beck. Technical and supervisory reponsibilities for the program were shared by the author and Dr. Earle Pope. The body of work presented in this dissertation was performed during the period from 1981 through 1985. It was agreed that the achievements of this program would be published in peer review journals, and that the information resulting either directly or indirectly from this author's efforts would constitute the subject matter of this dissertation.

Dr. Beck, as administrative director of the program and a member of the author's graduate committee, was responsible for supervising the research efforts of the author and for determining those aspects of the overall research program that constitute independent research performed by the author, collaborative research performed by the author, and research performed by the author but directed by Dr. Beck. This dissertation includes both independent and collaborative research performed by the author, but not research performed by the author under the direction of Dr. Beck.

Four scientific publications have resulted from the author's independent and collaborative research. These are included as Chapters III through VI. An additional manuscript in preparation is included as Chapter VII. A background for the subject matter in Chapters III through VII comprises Chapter II; while Chapters III through VII, in traditional manuscript style, include background, methods, results and discussion sections specific to the subject matter of each chapter. The general review of the literature in Chapter II is in some instances repetitious to information included in the individual chapters. The author believes that this repetition is necessary to give continuity to this dissertation.

Shortly after the author joined the primate reproduction laboratory in 1979, a method was developed in this laboratory to recover baboon embryos nonsurgically (Pope et al., 1980). Over 600 eggs and embryos have been collected from 71 baboons using this procedure. The findings based on 498 eggs and embryos relative to species of baboon, day post ovulation ("deturgescence") that the recovery procedure was performed, and cell stage at recovery have been published (Chapter III; Pope et al., 1983).

The early objective of this research was to establish an in vitro model for baboon pre- and periimplantation development. This required that we develop a culture system that allows the embryos to proceed through implantation or attachment in vitro. A detailed description of the system has been published (Chapter IV, Pope et al., 1982a). This culture system has given us the potential to: 1) compare in vitro and in vivo development using biochemical, histological, and temporal criteria; 2) provide new data on periimplantation events made possible by media sampling and embryo evaluation at discrete intervals; 3) determine, through media modifications, the optimum environment for maximum normal development in vitro; 4) further expand the criteria used to evaluate embryonic development; and, 5) evaluate the effects of altering the environment on embryo viability in vitro and possibly in vivo.

Specific goals, in addition to the development of the culture system, that have been achieved to date include: 1) measurement of baboon chorionic gonadotropin in media samples obtained from cultured embryos (Chapter V; Pope et al., 1982b); 2) development of an assay for quantitation of baboon placental protein SP-1, and measurement of SP-1 levels in media samples obtained daily from cultured embryos (Chapter VI; Pope et al., 1984); 3) the development of methods for processing baboon embryos for histological evaluation (Chapter VII); and, 4) the quantitative determination of estradiol and

progesterone levels in fresh and spent culture media, and the relationship of this endocrine data to the morphological and histological observations made on embryos in different culture environment (Chapter VII).

CHAPTER II

BACKGROUND

Culture Systems for Mammalian Embryos

Rodents and lagomorphs. The importance of culture systems for mammalian embryos is demonstrated by the number of laboratories using cultured embryos as a model for studying reproductive mechanisms. In vitro development of mouse embryos beyond the blastocyst stage was first reported by several groups in the mid-1960's (Mintz, 1964; Cole and Paul, 1965; and Gwatkin, 1967). Since that time, it has been shown that postimplantation development in vitro resembling that which occurs in vivo can be achieved using various media and culture conditions depending upon the objective of the particular laboratory. Although mice are the only species whose embryos can reliably be cultured to postimplantation stages (Spindle and Pederson, 1973; Rizzino and Sherman, 1979; and Hsu et al., 1974), some success has been reported with the guinea pig (Ilgren, 1981), and rabbit (Daniel, 1965).

Hsu and colleagues spent 12 years (Hsu, 1971; Hsu, 1972; Hsu, 1973; Hsu, 1979; Libbus and Hsu, 1980; Gonda and Hsu, 1980; Hsu, 1980; and Chen and Hsu, 1982) developing a mouse embryo culture system in which 10% develop from the blastocyst to the limb bud stage in vitro (Chen and Hsu, 1982). In one study (Hsu, 1973), mouse blastocysts cultured in minimal Eagle's medium plus

heat inactivated fetal calf serum attached to a collagen substrate and formed an early egg cylinder. When fetal calf serum was replaced by human cord serum, development to the early somite stage was achieved. Hsu concluded that frequent medium changes were critical to the success of the culture. He later determined that in vitro development from the 2-cell to the early somite stage was possible without collagen (Hsu, 1979), and cultured mouse blastocysts to somite stages in CMRL-1066 medium containing appropriate serum supplements necessary for provision of embryonic differentiation and growth factors required at each stage (Hsu, 1980; Herken and Hsu, 1983).

Spindle and Pederson (1973) studied the fixed nitrogen requirements for embryonic growth in the mouse in an effort to eliminate exogenous sources of macromolecules from the culture medium while maintaining a high rate of hatching, attachment and outgrowth. Although maximal hatching occurred in a chemically defined medium (Eagle's Basal Medium) containing the appropriate amino acids, they found that for attachment and outgrowth to occur a macromolecular component--fetal calf serum--was required.

Other investigators used the cultured mouse embryo and its microsurgically isolated parts to characterize differentiation and origin of the various structures during the early postimplantation period. This included many developmental events including induction (Atienza-Samols and Sherman, 1979), cell migration (Surani and Handyside, 1983), and comparative mitotic indices (Handyside and Hunter, 1984).

The in vitro development of cultured mouse embryos was described as a direct parallel of that which occurs in vivo, except for a 180 degree rotation of the egg cylinder (Libbus and Hsu, 1980), and a reduced rate of protein synthesis and cell division (Sellens and Sherman, 1980). The elimination of serum from the culture medium either prevented or retarded postattachment

development compared to that in vivo. Failure of embryoblast development occurred when there was a deficiency of the growth factors in human cord serum during the period of culture from stage 11 to 15. This resulted in an empty yolk sac or "blighted ovum" which continued to grow for a week or two (Hsu, 1981).

More thorough comparisons of development of postimplantation mouse embryos in vitro to those in vivo were accomplished using light and electron microscopy (Gonda and Hsu, 1980; Wiley and Pedersen, 1977). Mural trophoblast and associated extraembryonic structures did not develop normally, as the attaching mural trophoblast formed a monolayer on the plastic culture dish, and this outward growth caused collapse of the inner cell mass, essentially obliterating the blastocyst cavity. The parietal endoderm had not yet completely lined the blastocyst cavity at the time of the collapse, and was lost as development proceeded. The embryo proper erupted through a break in the trophoblast, forming a normal egg cylinder lacking the outer membranes comprising mural trophoblast, Reichert's membrane, and parietal endoderm. The displacement of the trophoblast, therefore, demonstrates the adaptability of the placental tissues, allowing normal development of the critical embryonic structures in an 'abnormal' environment.

Ilgren (1981) cultured guinea pig embryos to the egg cylinder stage in alpha modified Eagle's medium supplemented with 10% fetal calf serum (FCS), nucleosides and antibiotics. This system was selected after several others, including some successfully used for mouse embryos in other laboratories, failed to support postimplantation development of guinea pig embryos in his laboratory.

Culture of primate embryos. In vitro culture of nonhuman primate embryos has not been as widely pursued nor as extensive in accomplishments as

work with rodents. This is due primarily to the difficulty in obtaining embryos from primates. Initially, embryos from all monkeys, even those with relatively straight cervices, were obtainable only by surgical methods (Hendrickx and Kraemer, 1968; Hurst et al., 1978). Obtaining preovulatory oocytes via laparoscopy with subsequent in vitro fertilization prior to culture attempts results in lesser trauma to the animal, but also requires extensive technical training and expertise. More recently, a nonsurgical method has been used to recover fertilized baboon embryos (Pope et al., 1980).

In one 5 year study, Kuehl and Dukelow (1979) reported fertilization of 78 of 608 squirrel monkey ovarian oocytes recovered via laparoscopy, with some proceeding through three cleavage divisions during in vitro culture. The fertilization procedure was improved significantly in the succeeding 3 years (Dukelow, 1982) to the extent that up to 90% fertilization was achieved, although development past the 16-cell stage has yet to be reported (Dukelow et al., 1983).

Gould et al. (1973) report fertilization rates of up to 50% of 22 squirrel monkey oocytes, with one reaching an apparent 8-cell stage of development following in vitro culture. In vitro development past early cleavage stages, has been reported in a single instance in in vitro fertilized nonhuman primate embryos (Morgan et al., 1984).

Hearn and colleagues have achieved postattachment development of some surgically recovered marmoset embryos (Hearn, 1983; Summers et al.,1984), and have demonstrated chorionic gonadotropin secretion by cultured marmoset embryos. Additionally, trophoblast pieces were subcultured, with some lines retaining gonadotropin production capability for over one year in vitro.

In humans, where in vitro fertilization has proven to be a much simpler technique than that for nonhuman primates, there are two reports of posthatching development (Edwards and Surani, 1977; Fishel et al., 1984). The latter measured chorionic gonadotropin levels in spent culture media and demonstrated that two human embryos were capable of secreting this protein in vitro. The zonae pellucidae were removed by chemical means, precluding definition of an association between escape from the zona and initiation of gonadotropin secretion.

Pre- and Periimplantation Development in Primates

Embryos from several primate species have been evaluated histologically, and as the order is ascended, increasing similarity to human development is observed. Availability of embryos from the great apes is limited. Accordingly, early embryos of the rhesus monkey and baboon have been compared to human embryos in an attempt to define the suitability of these species as models for studying human embryonic development following implantation.

Several reports on primate preimplantation embryos, usually comprising small numbers of specimens, have been published in the last few years. Panigel et al. (1975) evaluated six baboon embryos at estimated fertilization ages from 2 to 8 days. The day 7 and 8 embryos were appreciably retarded when compared to the cleavage stages of baboon embryos recovered nonsurgically (Pope et al., 1983). According to their description, 7 to 8 day embryos were equivalent to nonsurgically recovered embryos 5 to 6 days postdeturgescence. This discrepancy was most likely due to inaccurate assessment of the time of ovulation.

Enders and coworkers, using surgical methods of retrieval, have studied 11 normal (Enders and Schlafke, 1981) and five abnormal (Enders et al., 1982)

oviductal and uterine rhesus monkey embryos. The 26% incidence of abnormality in these embryos was similar to the 24% (6/25) reported by Hurst et al. (1980) in the same species, and suggests a possible parallel between early pregnancy loss in monkeys and humans, where preimplantation loss is estimated to be as high as 40 to 60 percent. Histological preparations evaluated by light and electron microscopy allowed a more thorough interpretation of the cellular events associated with the various gross morphologies observed, and a variety of abnormalities associated with presumed degeneration were described (Enders et al., 1982). Isolated and abnormal cells in normal embryos were not uncommon, however, and occasional grossly abnormal embryos were histologically normal. The possibility that the latter, including collapsed blastocysts, could have been normal in the reproductive tract and developed their abnormality as a result of recovery and handling was not mentioned. In general, abnormal embryos were characterized by greater numbers of cells with large vacuoles, cells of variable sizes, and primitive or degenerating organelles that frequently clustered near the cell nucleus.

The difficulty of accurately identifying normally developing embryos was stressed by Enders' laboratory. They reported finding two "mock blastocysts"--spherical structures complete with cavity--in the monkey uterine washings. Following fixation and sectioning, however, the epithelium of these 'eggs' was not typical of trophoblast, and the inner cells were determined to be fibroblasts.

There are several developmental similarities among primate species; but interspecific differences, particularly when compared to nonprimates, support the importance of in-depth studies on primate development. For example, cleavage stage rhesus monkey embryos (Enders and Schlafke, 1981; Hurst et al., 1978, 1982), unlike early embryos of other laboratory animals that have been

studied by electron microscopy, possess only primitive junctional complexes, rather than the gap junctions and focal-tight intercellular junctions characteristic of the 8-cell mouse embryo (Ducibella et al., 1975; Magnusun et al., 1977). Fully developed intercellular associations including apical junctional complexes containing desmosomes and possibly gap junctions were not apparent in the rhesus monkey embryo until the blastocyst stage was reached.

Evaluating normally developing rhesus monkey blastocysts, Enders and Schlafke (1981) found no evidence of endodermal differentiation in an early blastocyst but did observe cellular changes suggestive of early endodermal formation in a larger zona-enclosed blastocyst. A basal lamina was being formed under mural, but not polar, trophoblast at this time, and patches of basal lamina were evident between epiblast and hypoblast. All zona-free blastocysts exhibited a similar stage of endodermal differentiation. The endodermal layer had spread peripherally beyond the inner cell mass, and a discontinuous basal lamina was present under the polar trophoblast.

Although there are a few reports of early implantation in primates comprising evaluation of one to three embryos (Knoth and Larson, 1972; Reinus et al., 1973; Enders et al., 1983), the major source of information on early postimplantation embryonic development in primates has been from the Carnegie Institute of Washington. This classic collection includes 34 serially sectioned human embryos and over 50 rhesus monkey embryos. Several embryologists and anatomists have evaluated the slides and reported their interpretations of the progression of differentiative events and cellular origin of the early fetal and placental structures (Hertig et al., 1956; Hertig, 1968; Heuser and Streeter, 1941; Wislocki and Streeter, 1938; Luckett, 1975; and, Luckett, 1978). Hendrickx and Houston (1971) studied 23 baboon embryos

comprising five similar stages of early implantation development. Inavailability of intermediate stages in all three species has hindered these studies. As a result, several hypotheses were proposed for the mechanisms of early embryonic development. Some of these have been disproven with further evaluation, although many conflicting theories still persist.

The primary yolk sac in both rhesus monkeys and humans is thought to develop by a peripheral spread of extraembryonic endoderm by day 10 and 9, respectively (Heuser and Streeter, 1941; Luckett, 1978; Heuser et al., 1945; and Enders et al., 1983). A difference between higher primates and monkeys is the presence of extraembryonic endoderm filling the primary yolk sac of human and chimpanzee embryos in a mesh-like arrangement (Luckett, 1978). This temporary structure may be associated with interstitial implantation. A restriction on expansion imposed on the newly implanted embryo could force the proliferating endoderm to fold over upon itself until expansion of the blastocyst resumes and results in a breakdown of the meshwork. Endodermal strands have not been observed in the primary yolk sac of rhesus monkey (Luckett, 1978) or baboon (Hendrickx and Houston, 1971) embryos.

One theory for secondary yolk sac formation in the rhesus monkey is that it occurs rapidly late on day 12 by an orderly folding and pinching off of the primary yolk sac endoderm near its 'roof' on the bilaminar embryonic disc (Luckett, 1978). The primary yolk sac remains as a single large vesicle that may contain additional smaller endodermal vesicles, all of which begin degeneration on day 17 to 18, and are completely degenerated by day 23. In the human, secondary yolk sac formation is thought to occur as a result of a disorganized collapse and fragmentation of the primary yolk sac by day 13 (Luckett, 1978). A mechanism for the formation of the primary and secondary yolk sac in the baboon has not been postulated.

Formation of the primitive amnion in humans and rhesus monkeys has been described as occurring by cavitation of the epiblast of the embryonic disc, with subsequent lateral spread of the epiblast to form a transitional trophoepiblastic cavity. The epiblast rejoins to form the definitive amnion by day 9 in humans and 11 in rhesus monkeys. This mechanism for amniogenesis has been suggested to be critical for species with persistence of the polar trophoblast, by allowing normal formation of the primitive streak (Luckett, 1975). Heuser and Streeter's (1941) hypothesis, however, that the amnion is not of embryonic origin, but is created by delamination of the cytotrophoblast, has also received some acceptance. In the baboon, for example, Hendrickx and Houston (1971) observed that a single day 9 embryo had not undergone amniogenesis, and in 10 to 15 day embryos amniogenesis was complete, with the amnion roof comprised of a single squamous cell layer resembling cytotrophoblast.

In a recent study of single in situ implanting rhesus monkey embryos obtained at necropsy on days 9.5, 10.0 and 10.5 of pregnancy (Enders et al., 1983), the inner cell mass on day 9.5 was composed of radially arranged epiblast cells covered by a layer of cuboidal endoderm, the latter acquiring a stellate appearance during migration over the trophoblast. By day 10 the inner cell mass had not undergone much additional development, although it was further compacted and isolated from the cytotrophoblast, with a small central cavity presumed to be a potential amnion, or the primitive amnion that forms by cavitation. The "parietal endoderm" lined only one-third of the blastocyst cavity in both the day 9.5 and day 10 embryos. They claimed the day 10.5 embryo contained a distinct amniotic cavity, although this structure was not shown in the figures.

Secretory Products of the Conceptus

Placental proteins. Chorionic gonadotropin (CG) was the first placental protein identified. It is a sialoglycoprotein with a molecular weight of 47,000, that is first detectable in maternal serum soon after implantation (Catt et al., 1975). Its early secretion by the conceptus provided the basis for pregnancy diagnosis, with chemical assays replacing the original bioassays. In humans, levels peak at 8-12 weeks of gestation (150-200 U/ml), then decline until the 18th week, with a slight rise toward the end of pregnancy.

Although its presence was initially described as being limited to primates, several investigators have claimed that chorionic gonadotropin-like activity is associated with preimplantation stages of nonprimate embryos (sheep, Wintenberger-Torres, 1978; mice, Wiley, 1974; and rabbits, Haour and Saxena, 1974; and Varma et al., 1979).

In primates, rhesus monkey (Macaca mulatta) morulae and blastocysts maintained (ie, development did not progress beyond the stage at recovery) in vitro also secreted a substance capable of inducing luteinization of monkey granulosa cells in vitro (Batta and Channing, 1979). The secretion of chorionic gonadotropin from cleavage stage embryos would fulfill a criterion for the immune-suppressor of pregnancy, ie, that it should be available prior to implantation in order to inhibit rejection of the conceptus by the maternal host.

The essential role of CG in maintaining corpus luteum function in early pregnancy is universally accepted; additional roles are still being evaluated as the maternal-fetal relations of critical early implantation stages are further investigated.

Several additional proteins that were initially referred to as being pregnancy specific have been identified in placental extracts and serum from pregnant women. With the development of highly sensitive assay techniques for

some of these proteins, their occurrence in low levels in fluids and cells of nonpregnant primates has been demonstrated (Rosen et al., 1979; Inaba et al., 1981a; Heikinheimo et al., 1982). Although extensive immunological and biochemical characterization has been completed for these proteins, their involvement with normal fetal and placental development remains unknown (Engvall, 1980; Bohn, 1971; Lin et al., 1974a,b; Lin and Halbert, 1975).

The study of placental proteins has relevance to clinical as well as biological science. For example, quantification of proteins for which significant functions have not been determined can nonetheless become a valuable index for monitoring fetal well-being. Additionally, studying the similarities between placental and cancer cells, including the secretion of specific proteins, could provide insight to mechanisms for controlling the growth of these invasive tissues. Finally, assuming that the presence of these proteins is not vestigial, determining their role in the progression of a successful pregnancy can expand our understanding, and subsequently control, of mammalian reproduction.

One of these proteins, SP-1, has been the subject of several investigations within the last decade. The independent identification of this protein by several laboratories has resulted in at least four names and abbreviations being used to indicate this compound. Pregnancy specific $beta_1$-glycoprotein (Towler et al., 1977), specific protein 1 (Bohn, 1971), trophoblast specific $beta_1$ globulin (Tatarinov, 1978) and pregnancy associated plasma protein C (Lin et al., 1974c) were all applied to what was subsequently proven to be the same protein. SP-1 has an isoelectric point between 3.6 and 6.5, and a molecular weight of approximately 90,000 to 120,000 daltons. Molecular heterogeneity is common with various preparative and analytical procedures due to complex formation and possibly altered carbohydrate content (Tatarinov, 1982; Bischof, 1984).

With at least one placental protein (PP5) studying the biochemical variants has provided insight into the overall biological significance of the protein. Studies of the different forms of SP-I have not, however, contributed significant new information (Chard, 1982; Bischof, 1984).

SP-1 can be detected in the serum of pregnant women usually within 18 to 23 days after the LH peak (Grudzinskas et al., 1977b). The minimum value reported for positive pregnancy detection has been approximately 10 ng/ml (Elvers et al., 1980; Anthony et al., 1980), as SP-I levels of 3 to 6 ng/ml are detected in serum of 29% of the normal population (Tatarinov, 1982). Concentrations in pregnancy plasma then undergo an exponential rise to 10 ng/ml at 40 to 70 days after the LH surge (Elvers et al., 1980; Grudzinskas et al., 1979a). Levels continue to increase until a plateau of 100 to 150 ug/ml is reached during the 36th or 37th week of gestation (Tatra et al., 1974; Gordon et al., 1977b; Lee et al., 1979; Elvers et al., 1980). At this time, it appears in greater concentration in the serum than any other placental protein (Bohn, 1979; Klopper et al., 1979).

SP-I of human placenta is localized on the syncytiotrophoblast (Horne et al., 1976; Lin and Halbert, 1976; Tatarinov et al., 1976) and cytotrophoblast (Tatarinov et al., 1976) of human placenta. Localization of proteins with immunohistochemical techniques, however, cannot be used to definitively demonstrate site of production as the presence of a protein in a tissue does not preclude a possible storage function for that tissue.

As with other placental proteins, SP-I has been detected in neoplastic tissue (Tatarinov et al., 1976; Skinner et al., 1981) and serum from patients with trophoblastic (Tatarinov et al., 1977; Elvers et al., 1980) as well as nontrophoblastic (Tatarinov et al., 1977) malignancies. The ability of specific cell types to produce placental proteins is controversial. Cancer cells that

produce and secrete proteins similar to those of placental cells may have undergone dedifferentiation as a result of a discrete gene effect, such as derepression. Alternatively, cell location could be the determining factor for production and secretion of some proteins. For example, many normal cell types may be capable of SP-I secretion. Its presence on cells and in biological fluids and culture media of cells not related to pregnancy (cerebrospinal fluid, Heikinheimo et al., 1982; peripheral blood cells, Inaba et al., 1981a; lymphocytes, Inaba et al., 1981b; brain cells, Heikinheimo et al., 1981; fibroblasts, Rosen et al., 1979) supports this suggestion. The production and secretion of significant quantities of SP-I and other proteins by placental, and in some instances cancer, cells may be related to the almost "infinite sink" achieved when cells are directly exposed to the blood stream (Chard, 1982). The continual removal of the protein may effect its production and secretion, which could be inhibited when a cellular or membranous barrier separates the potential synthesizing cell from a fluid environment.

Although early investigators reported that such fetal compartments as umbilical cord vessels and amniotic fluid did not contain significant SP-I, initial quantitation was accomplished using radial immunodiffusion and Laurel rocket techniques (Tatra et al., 1974; Bohn, 1974; Smith et al., 1979). The development of sensitive radioimmunoassays for SP-I allowed detection of much smaller quantitites of the protein permitting its evaluation in fluids containing what were previously considered to be undetectable concentrations (Towler et al., 1977; Grudzinskas et al., 1977a; Sorensen et al., 1977). Cord serum and amniotic fluid also show an increase in SP-I throughout pregnancy, with concentrations significantly less than those of maternal circulation. Grudzinskas et al. (1978) reported values of 100 ng/ml or greater in cord serum at the time of delivery, or approximately 0.1% of the concentration in maternal

peripheral blood. Similar observations were made by Towler et al. (1977), and Elvers et al.(1980) reported levels of 100 to 200 ng/ml in cord serum compared to 100 ug/ml in maternal serum.

An early controversy arose regarding the source of SP-I, when a reverse gradient in SP-I concentrations was demonstrated between maternal peripheral blood and retroplacental blood (Smith et al., 1979; Grudzinskas et al., 1979b; Klopper, 1980). Klopper et al. (1979), however, concluded that if SP-I was produced by invasive trophoblast, entry into the maternal circulation might bypass villous lacunae. Lin et al. (1976), found a higher concentration of SP-I in extracts of term placentae than could be accounted for by contributions from the maternal plasma, and Horne et al. (1976) have demonstrated, using immunoperoxidase staining of placental tissue at recovery and after 7 days of culture, that SP-I appears on the cell surface in the syncytiotrophoblast. Klopper (1980) has sugested that these placental specific proteins may act at the trophoblast-decidual interface.

The application of SP-I quantitation in diagnosing pregnancy disorders is being evaluated, and relationships between SP-I levels and fetal and placental well-being have been described (Tatra et al., 1974; Gordon et al., 1977a; Tatarinov, 1978). Although only 11% of subjects giving birth to normal weight infants had depressed SP-I levels during pregnancy, 71 and 80% of normotensive and hypertensive women, respectively, with low birth weight babies had concomitant low SP-I. Lin et al. (1974a) and Gordon et al. (1977b) also showed a direct correlation between SP-I concentration in late pregnancy and birth weight. Hughes et al. (1980), however, failed to observe such a correlation. Although Rh incompatability and diabetes had no effect on serum SP-I levels in late gestation (Tatra et al., 1974),SP-I levels in amniotic fluid were elevated under these conditions (Tatra, 1979).

Determination of luteal phase SP-I in cycles with subsequent menses has been suggested as being indicative of early spontaneous abortion (Anthony et al., 1980; Seppala et al., 1978). SP-I determinations could be used to supplement hCG assays or replace them in pregnancy determinations of subfertile women treated with gonadotropins (Seppala et al., 1979). First trimester spontaneous abortion has been shown to be preceded (Tatarinov, 1978) and accompanied (Jandial et al., 1978) by a decrease in maternal SP-I.

Analogues to SP-I have been identified in sera from pregnant rodents and lagomorphs with hemochorial placentation including the guinea pig, rat, and rabbit (Tatarinov, 1982). SP-I has been isolated from placental extracts of chimpanzees, baboons, cynomolgous and rhesus monkeys (Bohn and Sedlacek, 1975) and squirrel monkeys (Lin and Halbert, 1978). Amino acid composition and sugar content of rhesus SP-I has been evaluated, and a close similarity to the human protein was observed (Bohn and Sedlacek, 1975).

Passive immunization of pregnant cynomolgous monkeys on 3 consecutive days between days 19 to 55 of a 165 day gestation with anti-human SP-I IgG resulted in abortion of eight of 10 monkeys. Pregnancy was unaffected in two monkeys treated with the same amount (750 mg) of IgG from a non-immunized rabbit (Bohn, 1976). Fertility was slightly decreased following active immunization with human SP-I, and there was a significant abortion rate in immunized animals that became pregnant (Bohn and Weinmann, 1974, 1976).

Stevens et al. (1976) quantified serum SP-I during pregnancy in the baboon, and reported a rapid increase from detection at day 17 until day 50 of gestation (late in the first trimester), followed by a slight increase until parturition. The detection limit of the heterologous radioimmunoassay used was 1.8 ng/ml, and the maximum value achieved, when compared to a human standard, was 25 ng/ml. The apparent concentration difference between

baboons and humans was due to low cross-reactivity of the anti-human SP-I with the baboon protein. Pregnancy sera assayed with anti-rhesus SP-I resulted in considerably higher values (Stevens, personal communication).

Steroid hormones. One of the most significant controversies in peri-implantation development is the involvement of steroid hormones in the maternal recognition of pregnancy and implantation. Early work demonstrated the requirement for a maternal estrogen surge in rats and mice to allow implantation to occur (Psychoyos, 1973). Steroid replacement experiments following ovariectomy demonstrated in many species that progesterone therapy alone was sufficient to maintain pregnancy (rabbit, Denker, 1972; hamster, Harper et al., 1966; ferret, Wu and Chang, 1973; sheep, Moore, 1975; and monkey, Bosu and Johansson, 1975; and Meyer, 1972).

Lack of maternal or exogenous estrogen does not preclude a role of estrogen at the time of implantation. The blastocyst may be a source of estrogen capable of effecting the necessary local changes in the endometrium that allow implantation to occur. Mouse embryos cultured in media supplemented with 2 ug estradiol--17 beta/ml, and then transferred to nonpregnant uteri, were capable of effecting an increase in local capillary permeability not accomplished by control embryos (Dickmann et al., 1977). Estradiol did not show any evidence of toxicity to mouse embryos when it was present at the same concentration (2 ug/ml) in culture medium from the 4-cell to the blastocyst stage. Steroid metabolizing enzyme activity demonstrated by steroid interconversion in vitro (Wu and Lin, 1982) and histochemical labeling (Dickmann et al., 1976) has been reported in several species. George and Wilson (1978) suggested that rabbit blastocysts may be capable of producing estrogen around the time of implantation, as estradiol was formed from labeled steroid precursors during a 1 hour in vitro incubation interval.

Sherman and Atienza (1977) concluded that steroid biosynthesis by mouse embryos is not involved in implantation because delta-5-3,beta-hydroxysteroid dehydrogenase activity was only detectable after attachment of cultured blastocysts. Angle and Mead (1979) and Bleau (1981) could not detect delta-5-3,beta-hydroxysteroid dehydrogenase activity in preimplantation rabbit blastocysts, although progesterone uptake by rabbit embryos from culture media occurred during 2- and 4-hour incubations (Angle and Mead, 1979). Borland et al. (1977) demonstrated progesterone and estradiol in rabbit blastocyst fluid if the steroid was present in the maternal uterine fluid, suggesting a maternal source for blastocyst steroids. Accumulation of maternal steroids in the blastocyst cavity with subsequent release around the time of implantation, therefore, is a possible mechanism for concentrating steroids at the implantation site.

Carson et al. (1982) demonstrated little or no steroid synthesis in mouse blastocysts in vitro until after implantation, when first gradual and then rapid increases occurred to the early somite stage. Addition of inhibitors of progesterone and pregnanedione synthesis to the culture media on day 8 (early somite stage) severely repressed the production of these steroids; however, neither embryo nor trophoblast development was affected. They concluded that embryonic steroid secretion at this stage functions as a chemical signal to maternal tissues.

CHAPTER III

A 4-YEAR SUMMARY OF THE NONSURGICAL RECOVERY OF BABOON EMBRYOS: A REPORT ON 498 EGGS

V.Z. Pope, C.E. Pope, and L.R. Beck

American Journal of Primatology 5:357-364 (1983)

Abstract

A nonsurgical embryo recovery procedure, developed to allow the economical acquisition of cleavage stage baboon embryos, has been successfully used for 4 years. With this technique, 498 eggs have been recovered from 979 uterine flushes (50.9%) on 71 baboons. Of 467 eggs recovered from mated baboons, 290 (62.1%) were fertilized. Papio anubis females provided a higher percentage of fertilized eggs (75.3%) than did Papio hamadryas (47.8%) or Papio cynocephalus (44.3%) females following exposure to males during estrus, although sexual preference may be responsible for the reduced fertilization rate in the P. cynocephalus females. Recovery rates from individual baboons ranged from 0% (n = 11) to between 66% and 93% for ten baboons from each of which 12-33 eggs have been recovered. Fertilized eggs were at the two-cell (n = 23) to blastocyst (n = 53) stage at recovery 1-6 days postdeturgescence(PD) of the sex skin, with morulae (n = 84) being the most frequent cell stage recovered (305). The optimum time for performing the procedure was the third day PD, when 113 (40%) embryos were recovered. The abilities of baboons to become pregnant and to provide fertilized embryos were significantly related ($P < 0.005$), allowing the embyro recovery technique to be used as a screening procedure for evaluating baboon fertility.

Introduction

We have previously described instrumentation and procedures for the nonsurgical recovery of baboon embryos (Pope et al., 1980). In the initial series, 37 eggs were recovered using this technique. Since then, over 400 additional eggs have been recovered. Although this technology was originally

developed to provide embryos for in vitro culture (Pope et al., 1982), its use has been expanded to service a variety of investigations of reproduction in our laboratory. The purpose of this communication is to provide a more detailed description of the baboon breeding program for embryo recovery, to provide an update of the embryo recovery results, and to allow comparison of our observations on baboon reproduction and fertility with those of other investigators in the field.

Methods

Subjects. The baboons used in this study were taxonomically identified using the criteria of Hill (1967). Although interbreeding occurs in the wild and unequivocal determination of species purity is not possible, we nonetheless defined the 71 female baboons used in this study as consisting of 28 Papio anubis, 16 Papio cynocephalus, 19 Papio hamadryas, and eight baboons whose phenotypes were not distinct. The female baboons were purchased from Primate Imports (n = 35), University of Florida (n = 20), Buckshire Corporation (Pennsylvania) (n = 8), McGill University (n = 5), St. Louis University (n = 2), and Harvard University (n = 1).

The baboons used as embryo donors had been in the colony for 1.50-10.16 years ($\bar{x}$ = 4.16). During this time, they had been used for a variety of reproductive investigations, which included such procedures as multiple endometrial biopsies, chronic uterine catheterization, chronic cervical transmittor implantation, mechanical and chemical pregnancy termination, and midventral laparotomies and laparoscopies.

The adult males used included one feral born P. anubis acquired in 1974, and two captive-reared P. cynocephalus X anubis hybrids 11 and 13 years of

age. Additionally, a 5-year-old captive-reared P. hamadryas, which had been in the colony for 1.5 years, was used in some trials.

Mating Procedure. Females to be mated were housed in individual cages in one of five rooms containing 10-24 additional baboons. Males were added to the females' cages for mating, and the females were routinely exposed to a male at least once every 48 hours during the period of maximum sex skin turgescence. Depending on the number of females to be mated, pairs were occasionally allowed to cohabit for the entire turgescent phase, unless either partner found the situation disagreeable.

Although definite male/female preferences were displayed, these were susceptible to change. Cyclical variations within animals cause different groups of baboons to be estrus at the same time. Accordingly, it was important, when a single male had several females to breed in 1 day, to pair him with females in order of descending interest. Some males were found to mate certain females only when they were left together overnight.

It was not possible to discern a "dominant male" and an accurate social ranking of the females in the caged environment of the laboratory; therefore at no time were two adult males in a room at the same time to avoid hierarchial interferences with the imposed mating schedule.

Although evidence of mating was usually obvious (observation of completed mating or semen plugs extending from the vagina), on some occasions a more subtle indication--caked fluids on the penis--was relied upon. Infrequently, successful mating occurred without detection. An ejaculate resulting from masturbation was characterized by opaque semen plugs solidified in the shape of pooled drops following deposition on the cage pan, whereas semen plugs following copulation occurred as the polymorphous clumps that formed in the vagina of the female.

Ratings of sex skin turgescence on a scale from 0 to 4 were recorded daily between 7 and 9 AM. Following deturgescence of the sex skin, the females were scheduled for embryo recovery. Initial recovery attempts were performed 2 to 4 days following deturgescence. If an embryo was not recovered on the first attempt, the procedure was repeated 2 days later.

Several matings were performed without subsequent embryo recoveries for the following purposes: 1) to provide control pregnancies for experimental investigations; 2) to evaluate fertility following various drug treatments; 3) to provide parallel control groups for treatment groups in 2; and 4) to determine fertility of specific baboons.

Nonsurgical Embryo Recovery. The procedure for the nonsurgical recovery of baboon embryos has been described (Pope et al., 1980). The female is anesthetized with an IM injection of ketamine hydrochloride (Parke-Davis, 10 mg/kg) and xylazine hydrochloride (Haver-Lockhart, 0.5 mg/kg), placed in lateral recumbancy on a tiltable table, and caudally elevated. After the perineal area was washed, an adolescent-sized vaginal speculum was inserted to allow visualization of the cervix, and the nonsurgical embryo recovery device, an Isaacs endometrial cell sampler (Curity 4860) modified to permit bidirectional medium flow, was inserted across the undilated cervix into the uterine lumen. Ten milliliters of 37° C sterile culture medium was flushed through the device at a rate of 2 ml/min and collected in a sterile centrifuge tube. One milliliter of a protein-free phosphate-buffered saline solution was then flushed into a separate tube. After 5 minutes, an additional 4 ml of saline rinse was injected through the device. These saline rinses were saved for the eventual evaluation of uterine secretory proteins present at the time of the flush. The recovered medium was decanted into a 10 x 35 mm culture dish and

was evaluated with a stereomicroscope. All statistical comparisons were made using 2 x 2 chi-square analysis.

Results

Four hundred ninety-eight eggs have been recovered from 979 flushes (50.9%) on 71 baboons, most of which were exposed to males during estrus. This rate has increased from 42% in 1980 to 52% in 1981 and 60% in 1982. No eggs were recovered following 35 flushes on 11 (15%) of the baboons, although six of the 11 were flushed only one or two times.

The overall fertilization rate was 62.1%, or 290 fertilized eggs out of 467 eggs recovered from females exposed to males. Species variation in the recovery of eggs and their respective rates of fertilization are shown in Table I. Thirty-four of the 49 (69.4%) unfertilized eggs recovered from P. cynocephalus females were from cycles where none of the previously mentioned criteria for mating were met. If these unfertilized eggs are not included in the total, the fertilization rate increases to 72.2% (39/54), which is similar to that experienced with the P. anubis females (75.3%). The percent of fertilized eggs recovered from P. hamadryas females (47.8%) was also significantly less than from P. anubis (P. 0.005), although 17 of the 48 unfertilized P. hamadryas eggs (35%) were recovered from two (10.5%) of the P. hamadryas females.

Ten or more eggs were recovered from 22 baboons, for a total of 322 eggs out of 507 recoveries on these animals (63.5% recovery rate). Therefore, 64.7% of the 498 eggs have been recovered from 31.0% of the baboons which have been flushed. Ten of these 22 most reliable embryo donors have provided 182 eggs following 240 flushes (Table II), for an average of 18.2 eggs per baboon in this group.

TABLE I. Egg Recovery Results by Species

Eggs recovered from total flushes	P. anubis	P. cynocephalus	P. hamadryas	Other	Total
No. of baboons	28	16	19	8	71
Eggs	260	96	94	48	498
Flushes	454	171	223	131	979
% Rec	57.3	56.1	42.2	36.6	50.9
Eggs recovered from flushes on mated control animals					
Unfert	59	49	48	21	177
Fert	180	39	44	27	290
Total	239	88	92	48	467
% Fert	75.3	44.3	47.8	56.3	62.1

Fert, Unfert = No. of fertilized and unfertilized eggs, respectively.
% Rec = Eggs recovered as a percent of the total number of flushes.

TABLE II. Ten Most Successful Baboon Embryo Donors

Name	Species	No. Embryos	No. Flushes	Percent Recovery
O	A	13	18	72.2
KA	A	27	41	65.9
MA	A	18	26	69.2
NA	A	18	24	75.0
VA	C	33	40	82.5
TB	H	20	26	76.9
DC	A	16	23	69.6
RC	A	13	14	92.9
SC	A	12	14	85.7
PD	A	12	14	85.7
Total		182	240	75.8

A = Papio anubis; C = Papio cynocephalus; H = Papio hamadryas.

Almost half (40.1%) of the fertilized eggs were recovered 3 days postdeturgescence (Table III), at which time 55.8% were at the 16-cell to morula stage of development. Blastocysts were not recovered until at least 3 days following deturgescence, and they were the dominant developmental stage recovered 4 and 5 days postdeturgescence. Twin embryos, both blastocysts, were recovered on only one occasion.

Fifty-nine eggs were recovered from 366 (16.1%) second recoveries performed when an egg was not recovered on the first attempt (Table IV). Fourteen of 31 fertilized embryos (45.2%) were at the blastocyst stage of development at recovery 2 days following the initial attempt, compared with 18.5% (39/211) blastocysts recovered from first recoveries (P 0.005) or from second recoveries performed 3 or more days following the first recovery procedure. Eight of the 14 blastocysts were expanded, of which two were azonal when recovered in the second recovery. Twelve of the fertilized eggs recovered on second recoveries were either present but not flushed from or were not yet in the uterus 3 days postdeturgescence when the initial recovery attempt was performed. When embryos were not recovered on either recovery attempt, 13 pregnancies resulted, accounting for 3.3% of all recovery attempts. Seven of these 13 pregnancies occurred in three of the nine baboons that became pregnant.

In our limited breeding program, 70 pregnancies resulted when 55 baboons were mated 206 times (34.0% conception rate). Thirty-six of the 55 baboons (64.5%) have had pregnancies (fertility rate). This conception rate includes all cycles in which females were exposed to males where there were no subsequent experimental interventions until a new cycle had begun. It does not exclude matings of animals with suspected reduced fertility due to one or more of the following reasons: 1) first cycle following abortion; 2) first cycle following

TABLE III. Number of Embryos Recovered Days Postdeturgescence vs Developmental Stage

Days PD	Developmental Stage: Number (%)[a] 2-5	6-12	16-32	Morula	BC	Total
1	6 (43)	4 (29)	3 (21)	1 (7)	0 (0)	14 (5)
2	6 (10)	21 (33)	21 (33)	15 (24)	0 (0)	63 (22)
3	7 (6)	30 (27)	23 (20)	40 (35)	13 (12)	113 (40)
4	4 (6)	9 (14)	5 (8)	23 (35)	25 (38)	66 (23)
5	0 (0)	3 (15)	2 (10)	5 (25)	10 (50)	20 (7)
6	0 (0)	1 (17)	0 (0)	0 (0)	5 (83)	6 (2)
Total	23 (8)[b]	68 (24)[b]	54 (19)[b]	84 (30)[b]	53 (19)[b]	282(100)[b]

[a]Percent of total for that day PD.

[b]Percent of total eggs recovered.

TABLE IV. Number of Embryos Recovered on Second Recoveries Days Postdeturgescence vs Developmental Stage

	Developmental stage: No.					
Days	2-5	6-12	16-32	M	BC	Total
1/3	0	2	1	4	2	9
2/4	0	2	1	1	4	8
3/5	0	1	1	3	6	11
4/6	0	1	0	0	2[a]	3
Total	0	6	3	8	14	31

Days = Days postdeturgescence that the flushes were performed.

[a]Both were azonal expanded blastocysts.

long-term reproductive steroid treatment; 3) genital-tract adhesions diagnosed via laparoscopy; 4) inconsistent or poorly timed acceptance of the male; or 5) treatment with compounds suspected of reducing fertility. All matings were included as some pregnancies have occurred in all of the above categories, and not including those cycles would significantly reduce the number of matings to report. Fourteen baboons were mated during only one cycle, with a 57% pregnancy rate (8/14).

When the nonsurgical embryo recovery results from baboons that had and had not been pregnant in our laboratory were compared (Table V), the percentage of fertilized eggs recovered was 1.67 times greater in the group that had been pregnant (72.6% vs 43.4%, respectively, $P < 0.005$). If data from one of the six baboons that were mated only one time without a resulting pregnancy were eliminated from the embryo recovery totals for the nonpregnant group (SC, Table II), the fertilization rate of embryos recovered from baboons without pregnancies was reduced to 36.6%, or half that of the group with pregnancies.

Discussion

These results indicate that the nonsurgical recovery technique is an effective and reliable method for obtaining preimplantation baboon embryos. The overall recovery rate (50.9%) was similar to that achieved when surgical procedures were used (Hendrickx and Kraemer, 1968), but it is unlikely that the results that have been obtained with individual baboons using our procedure would be possible using surgical recovery techniques, which result in adhesion development and require a recuperative period after surgery. Repeated use of

TABLE V. A Comparison of Pregnancy and Embryo Recovery Results

	No pregnancies			Pregnancies		
	Mated 1X	Mated > 1X	Total	Mated 1X	Mated > 1X	Total
Mating:						
No. baboons	6	13	19	8	28	36
No. pregnancies	0	0	0	8	62	70
No. matings	6	51	57	8	141	149
Pregnancy rate[a]	0	0	0	100	43.9	47.0
Embryo recovery:						
No. flushes	76	102	178	101	521	622
No. eggs	45	38	83	65	245	310
Percent recovery	59.2	37.2	46.6	64.4	47.0	49.8
No. fertilized	23	13	36	47	178	225
Percent fertilized	51	34.2	43.4	72.3	72.7	72.6

[a]No. pregnancies/No. matings.

individual baboons allows more efficient use of valuable animals and permits identification of baboons which excel as donors of fertilized embryos.

The overall fertilization rate of 62.1% is similar to that reported by Hendrickx and Kraemer (1968), where 71% of surgically recovered eggs were fertilized. The reduction in fertilization rate in the P. cycnocephalus females appears to be the result of sexual incompatibility between many of the P. cynocephalus females and the P. anubis hybrid males. We have recently been able to improve the mating performance of several of the P. cynocephalus females with one of our hybrid males by pairing the female with one of two 3-year-old males for 8-24 hours before putting the adult male with the female. We are optimistic that this procedure will prove to be successful at increasing the sexual activity of the majority of our P. cynocephalus females.

The reliability of timing the embryo recoveries based entirely on the initiation of sex skin deturgescence eliminated the necessity of time-consuming and expensive blood sampling and hormone assays to determine the time of ovulation. The baboon embryo is reported to enter the uterus 3 days following ovulation (Eddy et al., 1976), at the 16-cell stage of development (Hendrickx and Houston, 1971). It was initially reported that ovulation in the baboon occurs 3 days prior to deturgescence (Hendrickx and Kraemer, 1971). Later observations involving laparoscopy and hormone determinations (Shaikh et al., 1980) have demonstrated that at least one-third of ovulations occur 1 day before deturgescence. The results of the present embryo recoveries support these recent observations that ovulation in the baboon occurs nearer the time of sex skin deturgescence than previously believed.

The recovery of 23 uterine embryos at the five cell stage or earlier suggests that baboon embryos may arrive in the uterus at earlier cell stages than first reported. Although it was previously unknown if these early embryos were capable of normal development, we have recently achieved a viable male

offspring following nonsurgical embryo transfer of a nonsurgically recovered four-cell uterine embryo (Pope et al., 1983). Additionally, embryos recovered at the six-cell stage have proceeded through postimplantation stages of development during in vitro culture, albeit at a reduced rate when compared to that of embryos recovered at later cleavage stages (Pope et al., 1982, and unpublished observations).

Twinning is a rare phenomenon in baboons, with the incidence of twin births reported at 0.23-0.50% (Hendrickx and Nelson, 1971). Our recovery of a single set of twins suggests a similar incidence (0.35%).

When blastocysts are recovered 2 days following an unsuccessful recovery attempt, it is not known if at the time of the first flush, the embryo was still in the oviduct. Based on the cell stages of embryos recovered on reflushes (Table IV), we believe that, in some instances, the embryo has not yet arrived in the uterus when the first flush is performed. The baboon embryo is at the blastocyst stage for at least 2 days prior to hatching (Pope et al., 1982), which raises speculation as to whether the eight expanded blastocysts, some without zonas, recovered on reflushes were perhaps achieving the late morula to early blastocyst stage while still in the oviduct.

The fact that pregnancies following unsuccessful flushes occurred at one-tenth the overall conception rate (3.3% vs 34.0%) suggests that the flushing procedure itself is mildly abortifacient, and further enhances the economic feasibility of the technique.

When selecting baboons for use in fertility trials, initial screening for reliability as donors of fertilized embryos has allowed selection of baboons with optimum fertility to be included in experimental groups. This biased selection allows acquisition of embryos with fewer animals than would be necessary when the objective of a study is to demonstrate a significant antifertility effect of an

experimental treatment. The 34% conception rate for all baboons mated is within the range reported by other investigators. Dmowski et al. (1980), using a control group of 20 baboons of proven reproductive performance, achieved 14 pregnancies (70%) out of 110 matings (13% "fertile matings"). Castracane and Goldzieher (1981) reported 25% conception in 20 mated control baboons, and Shaikh et al. (1976) reported that 50% of 60 baboons with sperm in a vaginal smear became pregnant. When 17 baboons of proven fertility were mated for 57 cycles, 18 pregnancies resulted (32% conception - Kraemer and Vera Cruz, 1972). Goldberg et al. (1981) achieved a much greater pregnancy rate, however, with 21 pregnancies resulting from 29 matings of 21 baboons 72%. This wide range of fertility and conception rates (pregnancies per mating from 13% to 72%) could conceivably be increased and abbreviated if baboons to be used in fertility studies would routinely be selected using fertilized embryo recovery as a criterion.

Conclusions

1. The nonsurgical recovery technique has proved to be an efficient and reliable method for obtaining large numbers of live embryos from a small number of baboons.

2. The procedure can be used to screen baboons for their suitability for inclusion in reproductive investigations requiring fertile animals, without compromising their fertility or resulting in costly time delays required for completion of control pregnancies.

3. Efficiency of animal usage is maximized, and the usable life span of individual baboons is greatly extended.

4. The ability to acquire viable embryos from individual baboons over several years further broadens the scope of studies that are possible using this procedure.

Acknowledgements

The authors gratefully acknowledge the support of the NIH (HD 15358), the Alcoa Foundation, The Lalor Foundation, and the Southern Medical Association, which made these studies possible.

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CHAPTER IV

DEVELOPMENT OF BABOON PREIMPLANTATION EMBRYOS TO POST-IMPLANTATION STAGES IN VITRO[1]

C.E. Pope, V.Z. Pope and L.R. Beck

Biology of Reproduction 27:915-923(1982)

[1]This research has been supported by a grant from NIH (HD15358) and by a grant from the Southern Medical Association

Abstract

Preimplantation baboon embryos were recovered nonsurgically and cultured in vitro to postimplantation stages. Most embryos (61/69) were cultured in CMRL-1066 medium supplemented with glutamine (1 mM), pyruvate (0.37 mM) and antibiotics; plus 20% heat-inactivated human cord serum (HCS) or fetal calf serum (FCS). Embryos were cultured in microtiter plate wells containing approximately 250 ul of medium in a gas atmosphere of 5% O_2, 5% CO_2 and 90% N_2, at 37° C and 100% relative humidity. Initially, embryos were cultured in CMRL medium plus 20% FCS until after hatching occurred (4/14), at which time FCS was replaced with HCS. When medium contained HCS which had been heat inactivated and filtered prior to freeze storage, 12 of 35 embryos developed to the expanded blastocyst stage and hatched from the zona pellucida. Seven of 12 (56%) embryos hatched when medium contained unfiltered HCS which was heat inactivated after thawing. Hatching occurred after 4 to 7 days in culture following recovery at the 8-cell to morula stage and blastocysts hatched on the second day after recovery. Hatching occurred rapidly, i.e., the zona was completely evacuated within 2 to 4 h after hatching had started. Most blastocysts underwent further expansion and attached to the culture dish within 5 days after hatching. Coincident with attachment, a second cell layer was formed around the inner periphery of the blastocyst cavity, and trophoblast outgrowth was initiated 1 to 3 days after attachment. In 12/20 embryos, the primary yolk sac greatly increased in diameter, and a structure identified as the embryonic disc was observed in 7 of 12 embryos. The interval from recovery to maximal post-attachment development ranged from 10 to 16 days, depending upon the stage of development at recovery; and up to a 7- to 8-fold increase in size was achieved. These post-attachment

embryos correspond in size and morphological appearance to 11- to 12-day (Stage Vc-VI) human and rhesus monkey embryos after fixation.

Introduction

The *in vitro* development of early cleavage stage embryos to the blastocyst stage has been achieved in several mammalian species, and culture requirements for preimplantation embryos have been most completely defined for the mouse and rabbit. One-cell mouse embryos will develop to the blastocyst stage in vitro in a bicarbonate-buffered salt solution supplemented with bovine serum albumin, lactate and pyruvate (Whitten and Biggers, 1968).

The addition of fetal calf serum to the culture medium will allow mouse blastocysts to hatch from the zona pellucida, attach to the culture dish and undergo embryonic development up to the egg cylinder stage *in vitro* (Hsu, 1972; Spindle and Pederson, 1973, and Sherman, 1975). Furthermore, growth and differentiation achieved by the mouse blastocyst *in vitro* can be extended up to the early somite stage by using human placental cord serum in place of fetal calf serum in the culture medium after hatching has occurred (Hsu, 1973).

Most attempts to culture ova from nonhuman primates have been made with oocytes following *in vitro* fertilization. The cleavage rate and preimplantation development stages achieved in culture have not approached that which has been reported in humans. Squirrel monkey oocytes fertilized *in vitro* have been cultured only up to the 8-cell stage using tissue culture medium with serum added (Gould *et al.*, 1973; Kuehl and Dukelow, 1979). In contrast, human embryos develop to the blastocyst stage in 4 to 5 days after fertilization *in vitro* in medium containing either fetal calf serum or the donor's own serum (Steptoe *et al.*, 1971; Edwards *et al.*, 1981).

Although the development of a successful culture system for primate embryos has been inhibited by the lack of availability of early cleavage stage embryos, a reliable method is now available for the nonsurgical recovery of preimplantation embryos from the baboon (Pope et al., 1980). The current study was undertaken in an effort to develop an in vitro culture system for baboon embryos and to determine the extent of development which can be achieved in vitro.

Materials and Methods

Nonsurgical recovery of baboon embryos. The procedure for recovering baboon embryos was as previously described (Pope et al., 1980). Briefly, an endometrial cell sampler (Curity) modified to permit bidirectional medium flow was inserted across the cervix of the anesthetized animal until the tip was situated in the uterine lumen. Ten ml of recovery fluid were injected through the catheter and collected in a sterile 15 ml centrifuge tube. In addition, after the uterus was flushed with recovery medium, 0.5 ml of warm sterile saline was injected and collected into a separate tube. After 5 min an additional 4.5 ml of saline was flushed through the uterus and collected into a 12 x 75 mm culture tube in an effort to obtain uterine secretory products by diffusion. The embryo recovery medium was a bicarbonate buffered salt solution containing crystalline bovine serum albumin, energy sources (glucose, lactate and pyruvate) and penicillin/streptomycin (BWW, Biggers et al., 1971). Medium was prepared fresh each week using triple distilled water, sterilized by positive pressure filtration through a 0.22 um filter (Millipore Corp.) into sterile serum bottles, stoppered, gassed with 5% CO_2, 5% O_2 and 90% N_2, and stored at 5° C until use.

Embryo recoveries were performed on mated baboons at 0 to 8 days following deturgescence of the sex skin. Initially, embryo recoveries were performed within the first 2 days following deturgescence, as published reports (Hendrickx and Kraemer, 1971) indicate the baboon ovulates 3 days prior to deturgescence. In our laboratory, however, it became apparent that ovulation occurs more closely to the time of deturgescence, based on the recovery of early cleavage stage embryos (8-cell to morulae) 3 to 4 days following deturgescence (Pope et al., 1980). If an embryo was not recovered on the first attempt, the procedure was repeated after approximately two days. Most of the embryos (61%) were at the 12 to 16-cell or morula stage at recovery but the stages of development of embryos placed in culture varied from the 2-cell to expanded blastocyst.

Embryo culture. Seven embryos were cultured in Ham's F-10 (Gibco) plus 10% fetal calf serum (FCS) and one was cultured in Whitten's medium. Sixty-one embryos were cultured in CMRL-1066 medium (Gibco) supplemented with glutamine, (1 mM), pyruvate (0.37 mM), penicillin (100 U/ml) and streptomycin (50 ug/ml) or gentamycin sulfate (50 ug/ml) and 20% human placental cord serum (HCS) or FCS. CMRL medium was purchased every 6 to 8 weeks and dry supplements were added to a previously unopened 100 ml bottle of medium approximately every 10 days. The supplemented medium was resterilized by positive pressure filtration (0.45 um, Millipore) into a 100 ml serum bottle after discarding the first 20 ml to pass through the filter. The prepared medium was stored at 5^{o} C until use within 10 days. A portion of the supplemented medium was transferred to a 10 ml serum bottle every 2 to 4 days and FCS or HCS was added to achieve a final concentration of 20% serum.

Human placental cord serum was obtained by drawing blood from the umbilical cord at the time of delivery. Serum was separated from the clot by

centrifugation within 24 hours of collection. Initially, serum was placed in a sterile 12 x 75 mm culture tube, heat treated at 56° C for 30 min and stored at -20° C. Later, suitable blood samples collected less than 24 h earlier were pooled after centrifugation, filtered (0.45 um, Millipore) and aliquoted prior to storage (1 to 2 ml/tube). Only clear, yellow serum was used for embryo culture and any samples which appeared even slightly hemolyzed were discarded. During the latter part of the study, 12 embryos were cultured in CMRL medium with 20% unfiltered serum that was heat treated after thawing immediately prior to use.

All embryo observation and manipulation was carried out under a lexan hood sterilized with ultraviolet (G15T8 tube) light before use. When an embryo was located in the recovery medium it was transferred to a culture dish (10 x 35 mm, Falcon) containing warm BWW medium and held at 37° C. The embryo culture vessel was prepared by placing 200 ul of culture medium in a well of a flat-bottomed microtiter plate (Falcon 3042) and this was allowed to equilibrate at 37° C in a gas atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The embryo was then aspirated into a finely drawn pasteur pipet using mouth-controlled suction and transferred to the well containing equilibrated culture medium.

Embryos were cultured at 37° C in an incubator (Thelco, Model #4) adapted for continuous gas flow and 100% relative humidity. The gas mixture of 5% CO_2, 5% O_2 and 90% N_2 flows at a rate of approximately 2.8 l/h through a gas dispersion tube in a flask containing sterile distilled water, and exits the flask via tubing on a side arm port and enters a glass and stainless steel dessicator (Fisher Scientific Co.) which serves as the culture chamber. An atmosphere of 100% humidity was ensured by filling the top and bottom of a 100 mm sterile petri dish with sterile water and placing them on the lower shelf

of the culture chamber. The water and petri dish were changed weeky to prevent mold contamination of the culture chamber.

Each embryo was evaluated and photographed (Ektachrome 200) daily using an inverted phase contrast microscope. Medium was changed each day during the culture period by adding fresh medium to an empty well in the same microtiter plate and allowing it to equilibrate for at least 1 h in the culture chamber. The spent medium was removed from around the embryo using a finely drawn pasteur pipet with a 60^{o} angle approximately 1 cm from the tip. The equilibrated fresh medium was gently transferred to the well containing the embryo and the culture vessel was returned to the incubator. Spent medium was labelled and stored at -20^{o} C.

Results

Culture medium effect. Eight embryos cultured in Ham's F-10 or Whitten's medium supplemented with 10% FCS did not progress beyond a few cleavage divisions. Although one embryo recovered as an early blastocyst underwent expansion, it did not hatch from the zona pellucida. The other six embryos were at the four-cell to morula stage at recovery, and although one reached the early blastocyst stage *in vitro*, the remainder underwent only limited cleavage.

Four of 14 embryos (29%) cultured in CMRL-1066 containing 20% FCS (Table 1, Group I) hatched from the zona pellucida *in vitro*. One of these was lost shortly after hatching, but the other three, after being changed to medium with 20% HCS, attached to the culture vessel and development continued for 9 to 10 days. When CMRL medium with heat treated, filtered HCS was used during the entire culture period (Table 1, Group II), hatching occurred in 12 of 35

TABLE I

DEVELOPMENT OF BABOON EMBRYOS THROUGH HATCHING FROM THE ZONA PELLUCIDA IN DIFFERENT CULTURE CONDITIONS

	Cell Stage at Recovery (#)		Stage Achieved[a]	Number (%) Hatching	
Group I[b]	2-cell	(0)	--	--	--
	4-6	(2)	8, BC	0	(0)
	7-10	(2)	M, HBC	1	(50)
	12-16	(5)	32, BC, BC, BC, BC	0	(0)
	M	(3)	M, BC, HBC	1	(33)
	BC	(2)	HBC, HBC	2	(100)
Total		14		4	29
Group II[c]	2-cell	(1)	M	0	(0)
	4-6	(2)	M, HBC	1	(50)
	7-10	(8)	8, 16, M, BC, BC BC, BC, OG[e]	0	(0)
	12-16	(12)	16, M, BC, BC, BC HBC, HBC, HBC, HBC, HBC, HBC, HBC	7	(58)
	M	(10)	M, M, M, BC, BC, BC HBC, HBC, HBC	3	(30)
	BC	(1)	HBC	1	(100)
Total		34		12	35

Table 1, Continued

Group III[d]	2-cell	(0)	--	--	--
	4-6	(1)	M	0	(0)
	7-10	(2)	10, BC	0	(0)
	12-16	(3)	HBC, HBC, HBC	3	(100)
	M	(4)	M, M, HBC, HBC	2	(50)
	BC	2	HBC, HBC	2	(100)
Total		12		7	58
TOTAL		60		23	38

[a]M = Morula; BC = Blastocyst; HBC = Hatched Blastocyst

[b]Group I = 20% FCS

[c]Group II = 20% HCS filtered, heat-treated prior to storage

[d]Group III - 20% HCS unfiltered, heat-treated immediately prior to use

[e]OG = This embryo achieved outgrowth through a slit in the zona pellucida without actually escaping from the zona.

(34%) embryos. Attachment and further embryonic development occurred in 11 of those embryos after hatching. Seven of 12 (56%) embryos cultured in CMRL supplemented with 20% unfiltered HCS which was heat treated immediately prior to use (Table 1, Group III) hatched from the zona pellucida. The percent hatching of the limited number that have been cultured in serum so treated was not significantly different from the other groups, which do not differ from each other ($P > 0.05$).

Hatching. Overall, 50% of the embryos recovered at the 12-cell stage or greater (21/42) continued development to the expanded blastocyst stage in vitro and hatched from the zona pellucida. One of these embryos, which was recovered at the 16-cell stage, is shown in Fig. 1A. Only 11% (2/19) of those embryos recovered at or before the 10-cell stage were able to develop to the hatched blastocyst stage.

Hatching from the zona pellucida occurred after 4 to 7 days ($\bar{x} = 5.3 \pm .81$) in culture when the embryos were recovered at the 8-cell to morula stage of development. Three embryos recovered as blastocysts hatched 2 days after recovery. Part of the actual hatching process was observed in three embryos. At the initial observation of these embryos, when fresh medium was being added to a separate well to equilibrate, they had started to hatch with up to approximately one half of the blastocyst already protruding from the zona pellucida (Fig. 2A). When changed to fresh medium 2 to 3 h later, the hatching process was complete and expansion was continuing (Fig. 1B and 2B).

Attachment. Twenty of 22 (91%) blastocysts attached to the culture dish within 5 days ($\bar{x} = 3.3 \pm 2.2$) after hatching from the zona pellucida. At about the time of attachment, most embryos appeared to flatten and a second distinct layer of cells developed around the inner periphery of the blastocyst cavity (Fig. 1C). The inner cavity formed in these bilaminar blastocysts represents the

FIGURE 1

Pre- and post-attachment development of a baboon embryo during a 12-day period of in vitro culture

A. Day of recovery. The embryo is at the 16-cell stage. Numerous spermatozoa are visible in the zona pellucida. X 250.

B. Day 5. The blastocyst has completely hatched from the zona pellucida and has undergone further expansion. Clumps of cellular debris remain inside the ruptured zona pellucida. X 175.

C. Day 10. Bilaminar blastocyst on the day of attachment to the culture dish. The distinct inner cell mass is seen in the upper portion of the blastocyst. X 175.

D. Day 12. Yolk sac expansion and early trophoblast outgrowth is evident. The dark area of cells in the upper portion is identified as the embryonic disc.
X 50.

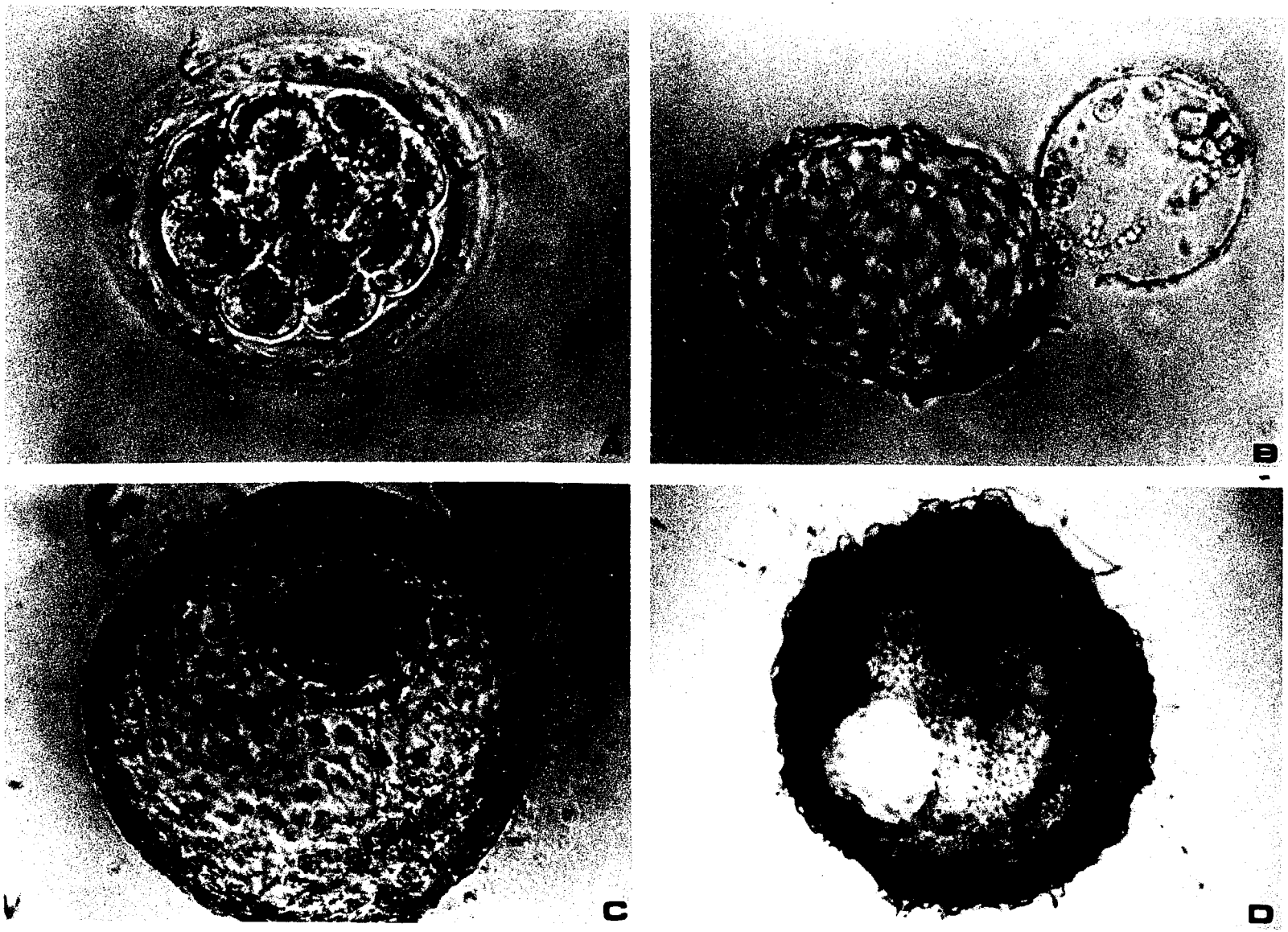
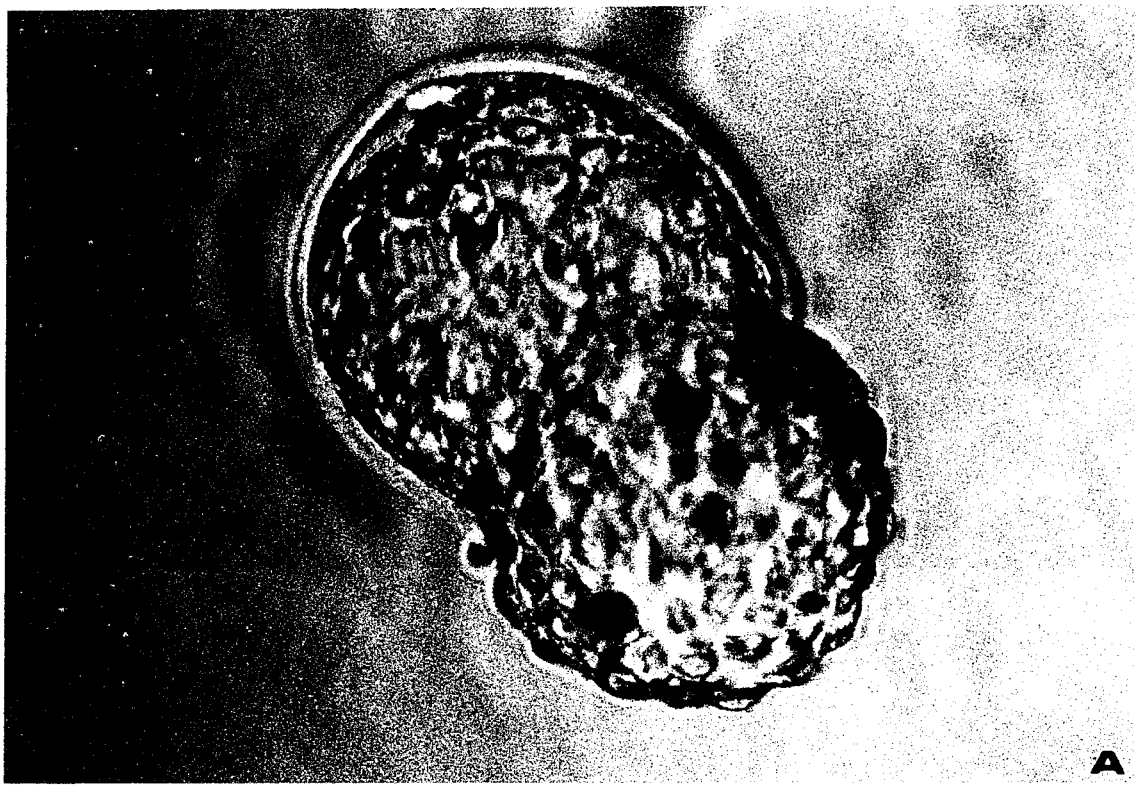

A
B
C
D

FIGURE 2

A blastocyst on Day 5 of culture after recovery at the 16-cell stage of development.

A. The blastocyst in the process of hatching from the zona pellucida.
B. The completely hatched blastocyst shown 3.75 h later. A few clumps of cellular debris remain in the zona pellucida. X150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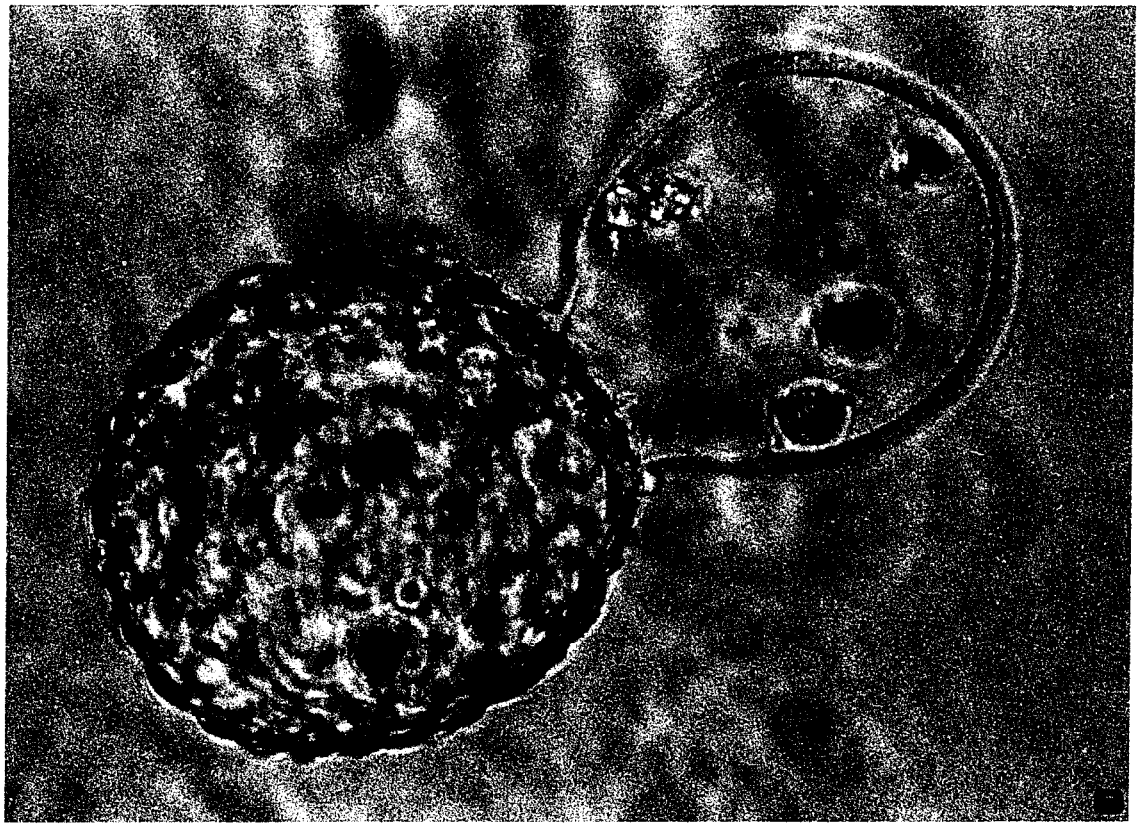


early formation of what will later become the primary yolk sac. The presumed inner cell mass (Fig. 3) at this stage of in vitro development was usually evident as a compact dark clump of cells.

Trophoblast outgrowth was initiated from 1 to 3 days ($\bar{x}$ = 2.1) after attachment and coincided closely with expansion and growth of the primary yolk sac (Figs. 1 D and 4).

Post-attachment development. For the purpose of classifying the post-attachment stages of in vitro development, embryos were categorized into one of two groups--either 'organized' or 'unorganized.' Twelve (60%) embryos were classified as having undergone organized post-attachment development. In these embryos the primary yolk sac cavity expanded and greatly increased in diameter during the following 6 to 8 days. Also during this period trophoblast outgrowth was rapidly progressing to eventually form a continuous sheet of cells surrounding the embryo.

A structure which appeared morphologically to be the embryonic disc (Figs. 1 D) was observed in 7 of 12 (58%) embryos undergoing organized post-attachment growth. Whether such a structure was present but not detectable in the other embryos is not known. For the embryos which were successful in hatching and developing to post-attachment stages the interval from recovery to the maximal development attained in vitro ranged from 10 to 16 days ($\bar{x} = 13.9 \pm 1.8$). As expected, this interval was closely related to the stage of development at recovery, i.e., expanded blastocysts reached their peak after approximately 10 days in culture while 8 to 16-cell embryos required 15 or 16 days to reach a similar stage of development.

After reaching their maximal growth as judged by yolk sac expansion and trophoblast outgrowth, the embryo gradually shrank in size and turned darker while the trophoblast outgrowth contracted back toward its origin. These

FIGURE 3

A post-attachment embryo shown with the empty zona pellucida on Day 7 of culture after recovery at the early morula stage.

The blastocyst on the third day after hatching at the initial stage of attachment. The inner cell mass is the dark area of cells in the middle. X 40.

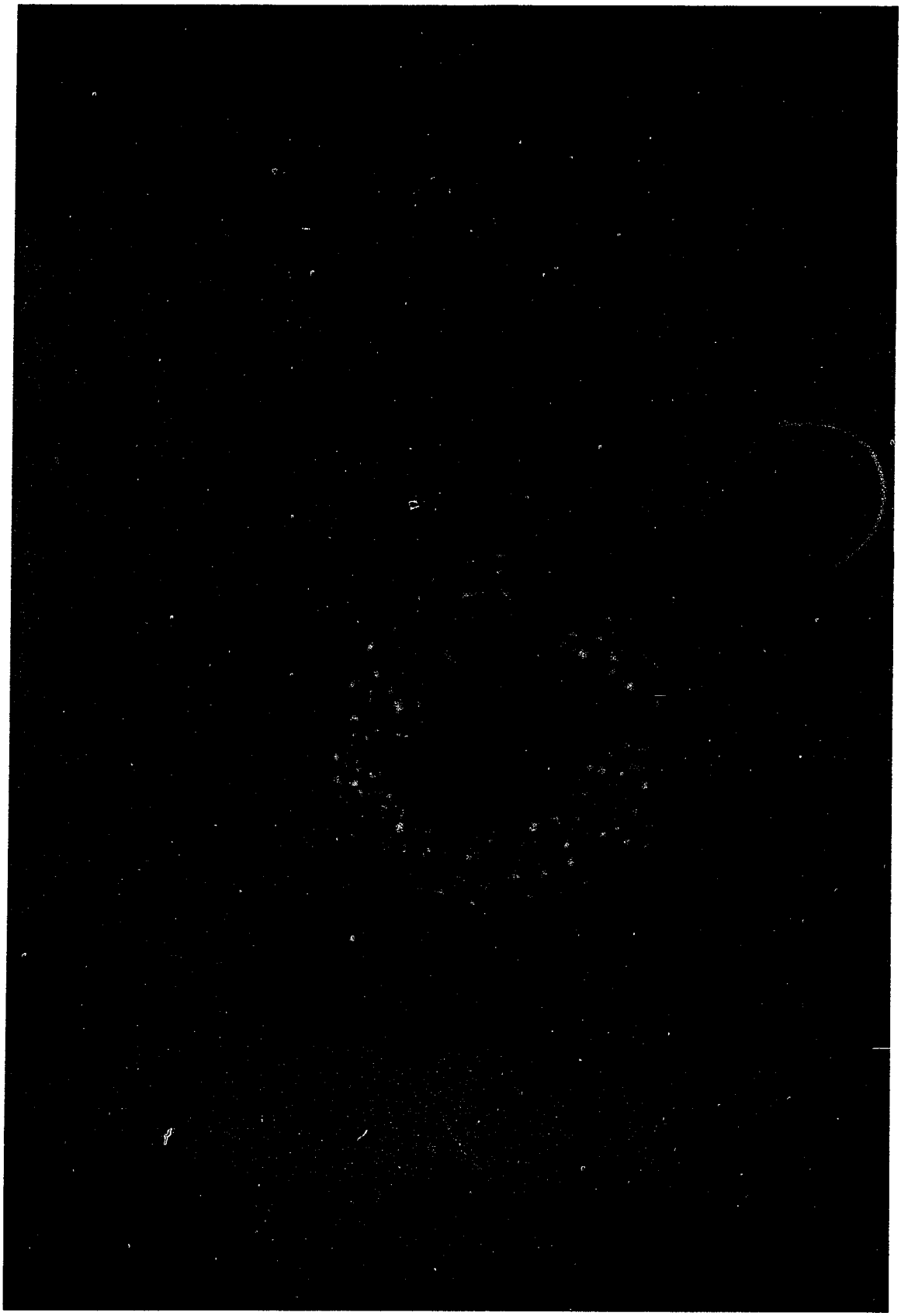
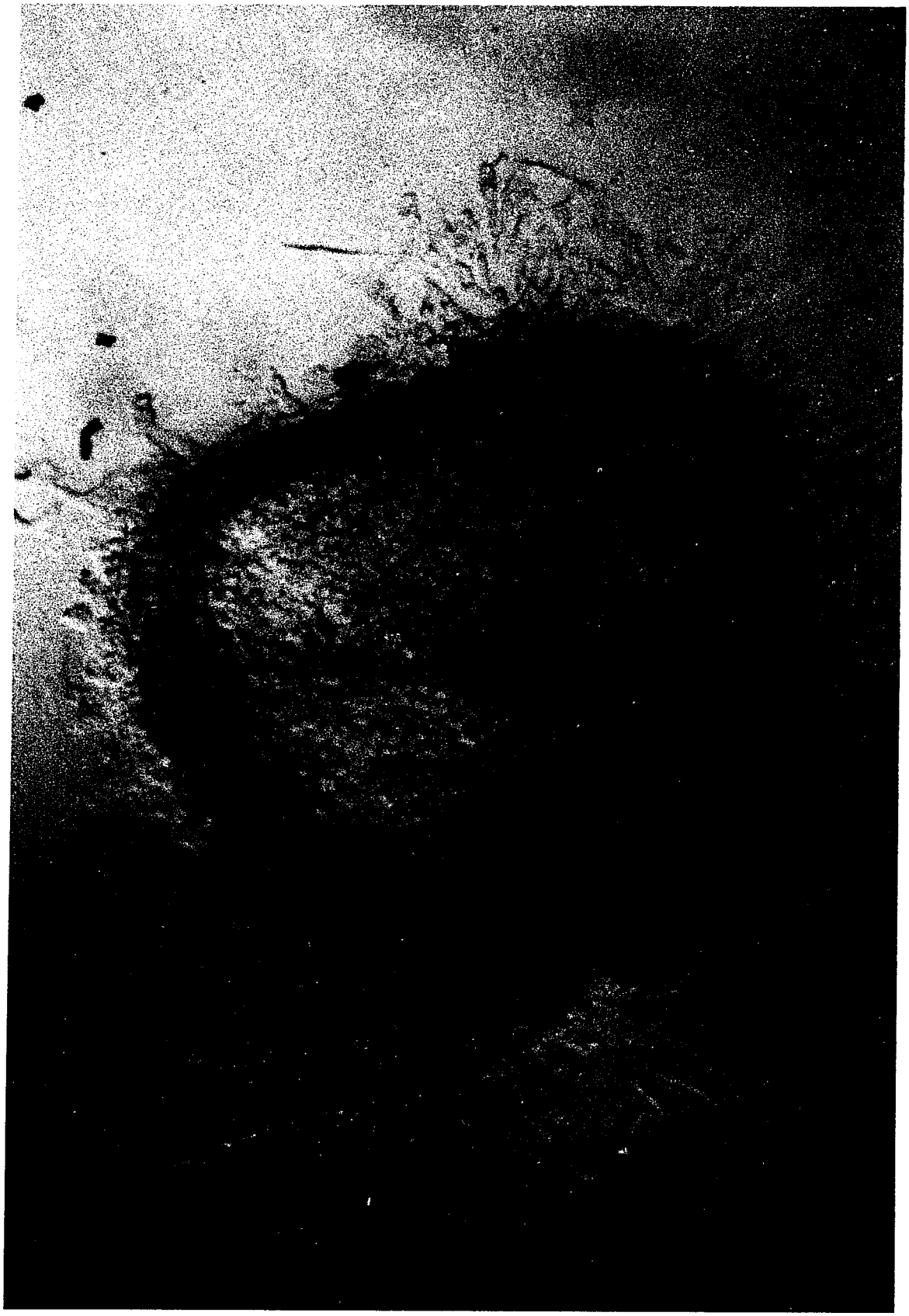


FIGURE 4

A 'flattened' embryo at Day 4 of post-attachment development after recovery at the expanded blastocyst stage: the second day of trophoblast outgrowth. The embryonic disc is seen in the center. X 80.



regressive changes were followed by dislodgment of the embryo from the dish. A few embryos which were left in culture for an extended period were able to reattach to the dish with some resumption of trophoblast outgrowth and a slight reexpansion of the yolk sac.

Measurements made from photographs taken each day of culture were used to provide a profile of the growth of several embryos during the culture period (Fig. 5). Prior to hatching, the measurements include the zona pellucida, with post hatching values indicating the mean external diameter of the blastocyst cavity or the primary yolk sac. During the early cleavage stages, prior to expansion and thinning of the zona pellucida, embryos were approximately 170 um in diameter. Embryos ranged from 190 to 270 um (216 $\pm$ 31) the day before hatching and 265 to 410 um (314 $\pm$ 51) the day that hatching occurred. The day following hatching the embryos measured 240 to 520 um (340 $\pm$ 71) in diameter, and continued growing to a 7 to 8-fold increase in size (1313 $\pm$ 188 um diameter) before maximum development was reached on Day 10 after hatching.

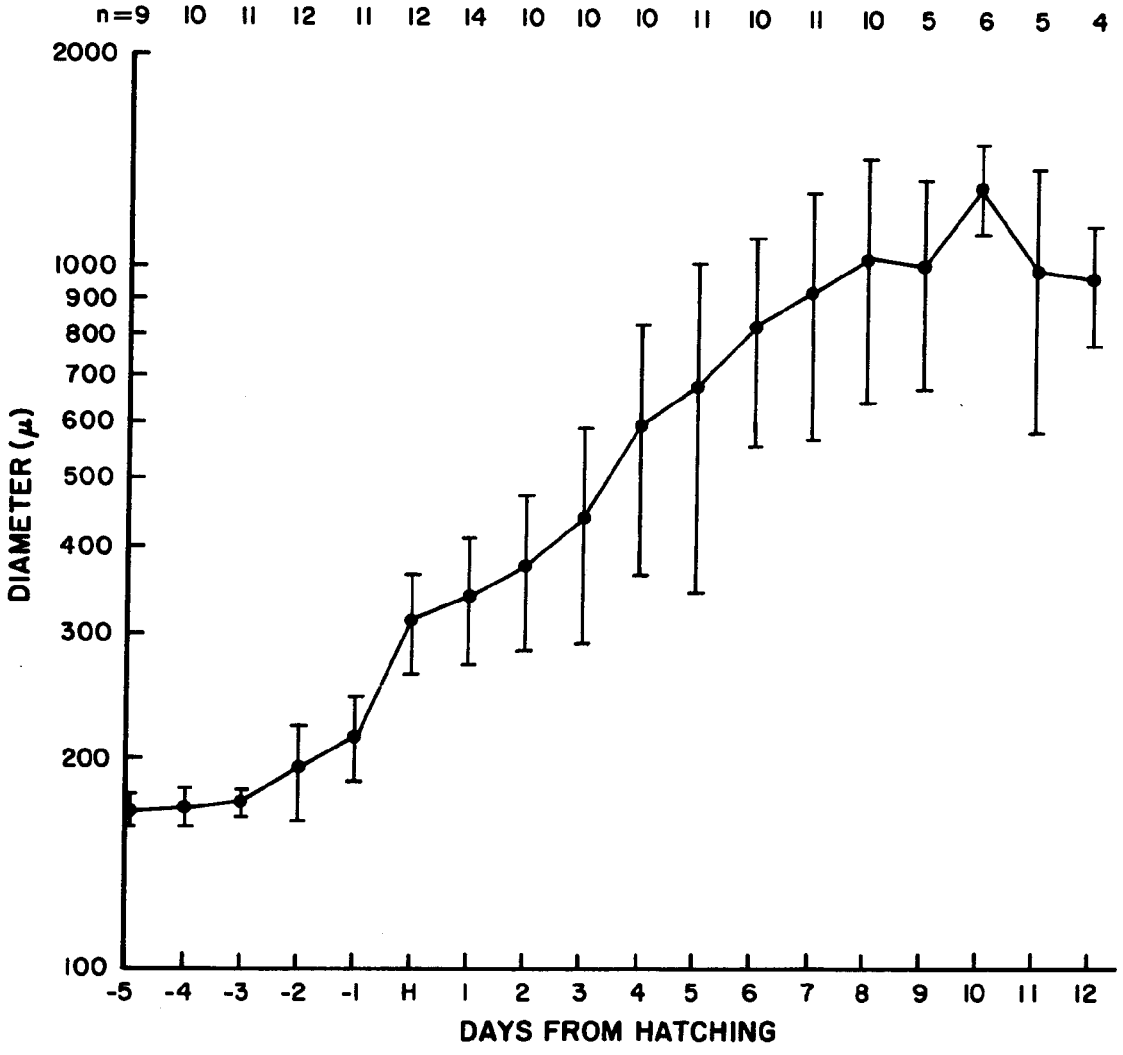
Discussion

This initial demonstration of the ability of primate embryos to undergo development to post-attachment stages in vitro indicates the potential utility of the baboon as a model for studying early embryonic growth and differentiation. Previously, the mouse has been the principal species from which cleavage stage embryos have been cultured to post-implantation stages with any degree of success. Several groups have shown that mouse embryos can be cultured to post-implantation stages in vitro with media and culture conditions adapted to the objectives of the particular laboratory. For example,

FIGURE 5

Growth rate, as measured by external diameter, of baboon embryos during the in vitro culture period.

Day of hatching from the zona pellucida is designated as Day 0


n=9 10 11 12 11 12 14 10 10 10 11 10 11 10 5 6 5 4
2000
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300
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DIAMETER (μ)
-5 -4 -3 -2 -1 H 1 2 3 4 5 6 7 8 9 10 11 12
DAYS FROM HATCHING

Spindle and Pederson (1973) studied the fixed nitrogen requirements for embryonic growth in the mouse in an effort to eliminate undefined sources of macromolecules from the culture medium while maintaining a high rate of hatching, attachment and outgrowth. Although maximal hatching occurred in a chemically defined medium (Eagle's Basal Medium) containing the appropriate amino acids, they found that for attachment and outgrowth to occur a macromolecular component--fetal calf serum--was required.

Sherman (1975) developed a system for the post-blastocyst development of mouse embryos with the primary objective being to examine the potential for development of individual cell types in vitro. The optimal medium for their requirements was NCTC-109 to which carefully selected, heat inactivated FCS was added (10%). Under these conditions hatching and attachment were observed in more than 90% of the embryos. The trophoblast cells grew out as a monolayer while the inner cell mass initially remained as a solid clump of cells that occasionally developed into egg cylinder like structures.

The greatest extent of embryonic development in vitro and that which more nearly reproduces normal events in vivo has been achieved in the experiments of Y.C. Hsu. It was shown that mouse blastocysts cultured in minimal Eagle's Medium plus heat-inactivated FCS would attach to a collagen substrate and form the early egg cylinder (Hsu, 1971, 1972). When FCS was replaced by HCS just before attachment, some blastocysts developed to the early somite stage (Hsu, 1973). Frequent medium changes were thought to be critical to the success of the culture. More recently, these investigators have found that in vitro development from the 2-cell to the early somite stage can be accomplished (Hsu et al., 1974), that collagen is not required, and that mouse blastocysts can be cultured singly in CMRL-1066 medium containing 20% HCS (Hsu, 1979).

It was on the basis of the latter results that a similar culture system for baboon embryos was tested in our laboratory, and the greatest success achieved in this preliminary study was obtained by using CMRL-1066 with 20% HCS.

Since, at present, it is necessary to use an undefined (serum added) culture medium, identification of the embryotrophic factors in sera and their role at various stages of development is essential. Hsu (1980) found that three embryo growth and differentiation factors (EGDF) were required for mouse blastocysts to develop to the early somite stage in vitro. FCS was found to have two of the factors, but the third factor which was necessary for development beyond the egg cylinder stage was present only in HCS. The first two factors were also present in HCS since blastocysts developed to the early somite stage with HCS as the only macromolecular source.

Tam and Snow (1980) have shown that embryotrophic properties are destroyed if serum to be used in the culture medium is heat inactivated prior to storage at -20^{o} C. They found that good development of primitive streak stage mouse embryos for 24 to 48 h could be obtained only if the serum was heat inactivated immediately before its addition to the culture medium. The observation was also made that filtration of fresh serum lowers its embryotrophic potency. Although baboon embryos are being cultured in medium with HCS treated using their recommended procedure to study the effect on post-attachment development, initial results do not indicate a significant improvement. Our recent evidence suggests that certain other aspects of serum preparation, such as interval from collection to centrifugation, may be of more vital importance in determining the extent of growth and differentiation of baboon embryos in vitro.

Comparing the extent of differentiation and growth achieved by cultured baboon embryos with the developmental stages of primate embryos in vivo

provides an indication of the temporal and morphological relationship between growth in utero and in vitro. The flattened appearance taken on by the cultured baboon blastocysts shortly after attachment has been previously observed in fixed human (Hertig et al., 1956), rhesus monkey (Wislocki and Streeter, 1938) and baboon embryos (Hendrickx and Houston, 1971) during implantation. This collapsing phenomenon has been described in some early reports (Wislocki and Streeter, 1938 and Hertig et al., 1956) to be a natural part of the implantation process, while it has later been described as an artifact of fixation (Luckett, 1978).

In the current study the dimensions of the baboon embryos at the time of maximal development in vitro after 10 to 16 days in culture are similar to those (0.9 to 1.1 mm) of fixed rhesus monkey and human embryos at approximately 11 to 12 days of gestation (Luckett, 1978). Embryos 8 days after hatching in addition to being of a similar size, appear to be comparable to 11- to 12-day human and monkey embryos after fixation and staining (Luckett, 1978). Although based solely on morphological observations, the degree of differentiation and growth achieved by baboon embryos in vitro in the current study corresponds to Streeter's Horizon Vc that has been established for human embryonic development (Hertig et al., 1956). The extended interval required for the cultured baboon embryos to reach a similar stage indicates they are developing at a slower rate in vitro than in vivo, as Hendrickx and Houston (1971) observed stage VI embryos at 11 to 15 days after the estimated day of fertilization.

As environmental factors required for optimal development of baboon embryos in vitro are further defined, the stage will then be set for critical studies of peri-implantation events in a primate model. While it has been suggested (Edwards et al., 1981) that human embryos should be the basis of such

studies to be of clinical value, baboon embryos offer the advantage of greater availability without the disadvantage of ethical considerations.

Indirect evidence suggests that a rather high rate of embryonic mortality occurs in primates, particularly around the time of implantation. Furthermore, chromosomal analysis of spontaneous human abortuses have revealed an imbalance in almost two-thirds of the cases (Carr, 1971 and Boue et al., 1975). Although of obvious importance, a systematic cytogenetic examination of early primate embryos has not been possible in a practical sense. The feasibility such studies should be greatly increased by having the dual capability of a nonsurgical embryo recovery technique and a culture system that allows development to post-attachment stages in vitro.

Our laboratory is currently measuring baboon chorionic gonadotropin production by cultured baboon embryos (Pope et al., 1982), and further investigations are planned which will include identification and quantitation of other products of the conceptus. By studying the products of the developing baboon embryo in vitro, and the requirements for their production and secretion, it may be possible to obtain pure compounds unaffected by maternal metabolism and to determine the temporal initiation of their production and secretion.

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CHAPTER V

GONADOTROPIN PRODUCTION BY BABOON EMBRYOS IN VITRO

V.Z. Pope, C.E. Pope and L.R. Beck

IN:

In Vitro Fertilization and Embryo Transfer
E.S.E. Hafez and K. Semm, eds.
MTP Press, Ltd, England
1982
pp 129-134

Introduction

Despite the greatly increasing clinical interest in techniques of embryo manipulation, there is still a paucity of information on the environmental requirements of early primate embryos. Parameters indicative of normal development have not been established, with the result that the suitability of conditions in which embryos scheduled for transplantation are kept during their transient period *in vitro* cannot be retrospectively optimized.

Cleavage of undifferentiated embryos before transplantation can occur in situations that will not permit further development. With the many ethical restrictions on wastage of human embryos, there is a need for a primate in vitro embryo culture model which can be used to define environmental conditions that will allow a majority of embryos to develop to post-implantation stages.

A system for the culture of baboon embryos has been established, and criteria that could potentially be used to evaluate the normality of development are being investigated. The current study was undertaken to determine if baboon embryos are capable of producing and secreting baboon chorionic gonadotropin (bCG) *in vitro*, in an attempt to compare events occurring in culture with those of in situ pregnancies. Additionally, if embryos recovered at early cleavage stages secrete bCG after being removed from the maternal environment, defining the temporal initiation of that secretion could additionally provide information regarding the relationship of gonadotropin secretion to the implantation process.

Chorionic gonadotropin (CG) is attributed with effecting rescue of the corpus luteum during pregnancy in primates. Controversy exists as to whether it is used in the early implantation process, or if its secretion is initiated after implantation. Saxena *et al.* (1974) have described hCG-like activity in human

pregnancy plasma 4 days after ovulation using a radioreceptor assay; while Catt et al. (1975) detected no CG activity in plasma until after implantation.

In the past, luteotropins originating from the conceptus were considered to be limited to primates; there is now evidence, however, of a gonadotropin-like substances being on mouse and rabbit morulae and in rabbit blastocysts (Wiley, 1974 and Asch et al., 1978, 1979). Using a radioreceptor assay, Haour and Saxena (1974) and Varma et al. (1979) have demonstrated approximately 10 to 16 mIU of a CG-like substance per embryo in rabbit blastocyst fluid. Rabbit blastocyst fluid is also capable of stimulating progesterone production by monkey granulosa cell cultures (Channing et al., 1978). More recently, rhesus monkey morulae and blastocysts maintained in vitro have been shown to secrete a substance capable of inducing luteinization of monkey granulosa cells in vitro (Batta and Channing, 1979).

Baboon Chorionic Gonadotropin Radioimmunoassay Procedure

Twelve embryos recovered nonsurgically at the 6-cell to blastocyst stage of development from seven baboons were cultured in vitro as described later (Chapter 31). To summarize the culture technique, individual embryos were maintained in wells of microtiter plates, with 200 ul of CMRL-1066 supplemented with glutamine, pyruvate, antibiotics and 20% fetal calf serum (FCS) or human cord serum (HCS) serving as the culture medium. Embryos were cultured at 37° C, in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. The medium was changed daily, and stored at -20° C until bCG content was determined by radioimmunoassay (RIA).

The radioimmunoassay for bCG is a double antibody precipitation technique using anti-ovine beta-luteinizing hormone (serum H-26, courtesy Gary

Hodgen, NIH) as the first antibody. Urinary bCG previously standardized against human chorionic gonadotropin (HCG) is used as standard, and the sensitivity of the assay approximately 0.4 mIU. For these experiments, 200 ul of culture medium from each embryo was initially diluted 1:5 with assay buffer and 200 and 100 ul duplicates of each sample were assayed. Further dilutions were made when necessary. Additionally, a sample of complete, control culture medium was similarly assayed for each batch of FCS or HCS used, to determine the extent of interference by gonadotropins present in the serum. Of 52 batches of HCS or FCS used in the culture medium, only three displayed significant cross-reactivity in the RIA for bCG. Values obtained from embryos cultured in cross-reacting media have been excluded.

BCG Production In Vitro

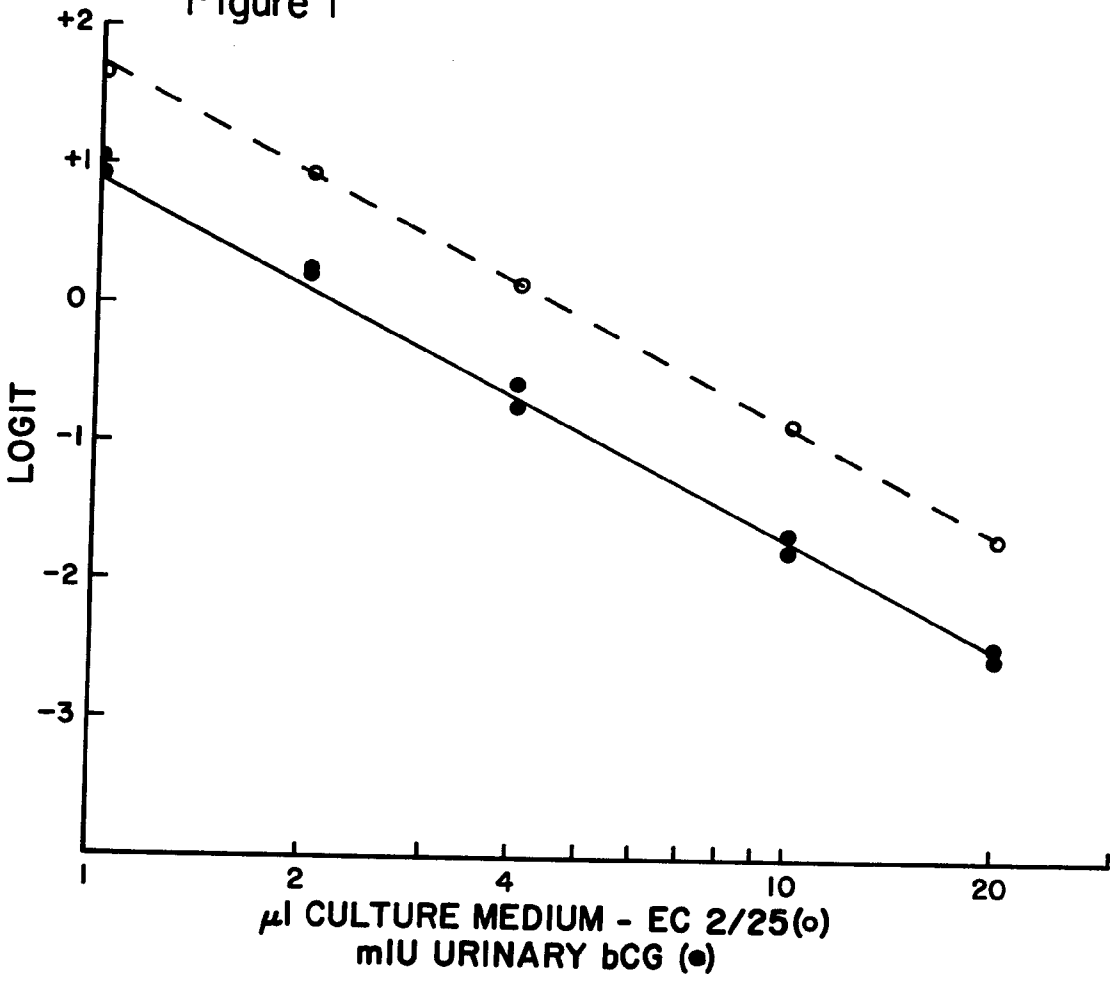
All 12 embryos in this investigation secreted gonadotropin in vitro; with measurable quantities of bCG detectable by RIA at or following attachment of the hatched blastocyst to the culture vessel. Similarity of this hormone to the urinary standard is shown in Figure 1. Secretion increased to a peak of up to 7 IU/day at 7-12 days after hatching from the zona pellucida, and continued for up to 27 days or longer. Of four embryos judged as having extensive trophoblastic outgrowth, two in which the embryo proper appeared morphologically "normal" had a maximum production of 5 and 7 IU bCG/day, while two in which the yolk sac and inner cell mass were disorganized with poor differentiation of embryonic structures peaked at approximately 2 IU/day. It appears, therefore, that the integrity of the embryo proper is related to the total amount of bCG produced.

Ten of the 12 embryos were visually determined as having good trophoblastic outgrowth regardless of the appearance of the developing embryo

FIGURE 1

Comparison of culture medium bCG and urinary bCG.

Figure 1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µl CULTURE MEDIUM - EC 2/25(o)
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proper, and had significantly greater bCG secretion than two that underwent less extensive trophoblastic development. The well containing one of the latter embryos also contained a piece of fibrous debris to which the embryo attached, and attempts to rescue it apparently resulted in its retarded and disorganized development, with no more than 50 mIU of bCG being produced per day throughout the culture period. An additional embryo that had appeared to be retarded in growth from early in the culture period secreted only 240 mIU of bCG from days 9 to 11 post hatching, which was the maximum produced by the embryo.

In the heterologous bCG assay used in our laboratory, the baboon conceptus does not secrete measurable quantities of bCG throughout gestation. In our colony, peak serum bCG concentration is reached in pregnant baboons by day 22-24 of pregnancy. There is usually no measurable serum bCG by 40-50 days. Baboon embryos are thought to hatch from the zona pellucida in vivo on day 8, and a comparison of maximum bCG production in vitro (Figure 2) with serum peaks in vivo (Figure 3) reveals the cultured embryos produce less hormone a few days before the decline in serum gonadotropin concentration in pregnant baboons. Considering the difference in the volume of the receiving fluid, more frequent medium changes or a continuous-flow culture system could potentially alter bCG quantities produced in vitro.

To determine the ability to maintain the cells in culture, the trophoblast cells from two embryos were trypsinized when outgrowth was still occurring. The cells from one embryo were lost owing to contamination of the culture medium, and those of the second embryo were pooled with cells from a third embryo trypsinized when further outgrowth was not apparent. Three additional embryos were trypsinized following termination of additional visible outgrowth.

FIGURE 2

bCG produced by baboon embryos in vitro

Figure 2 **CULTURE MEDIA bCG**

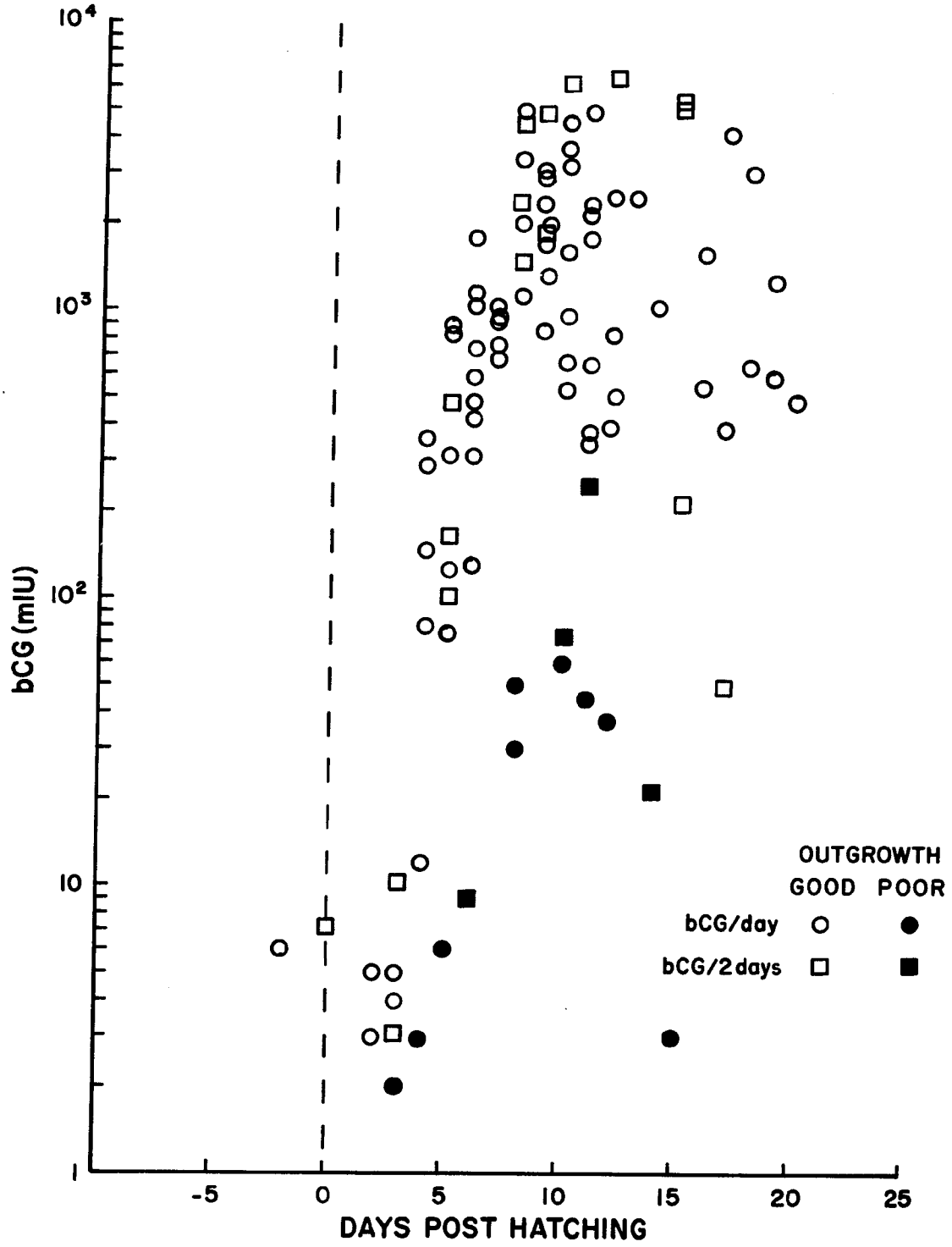
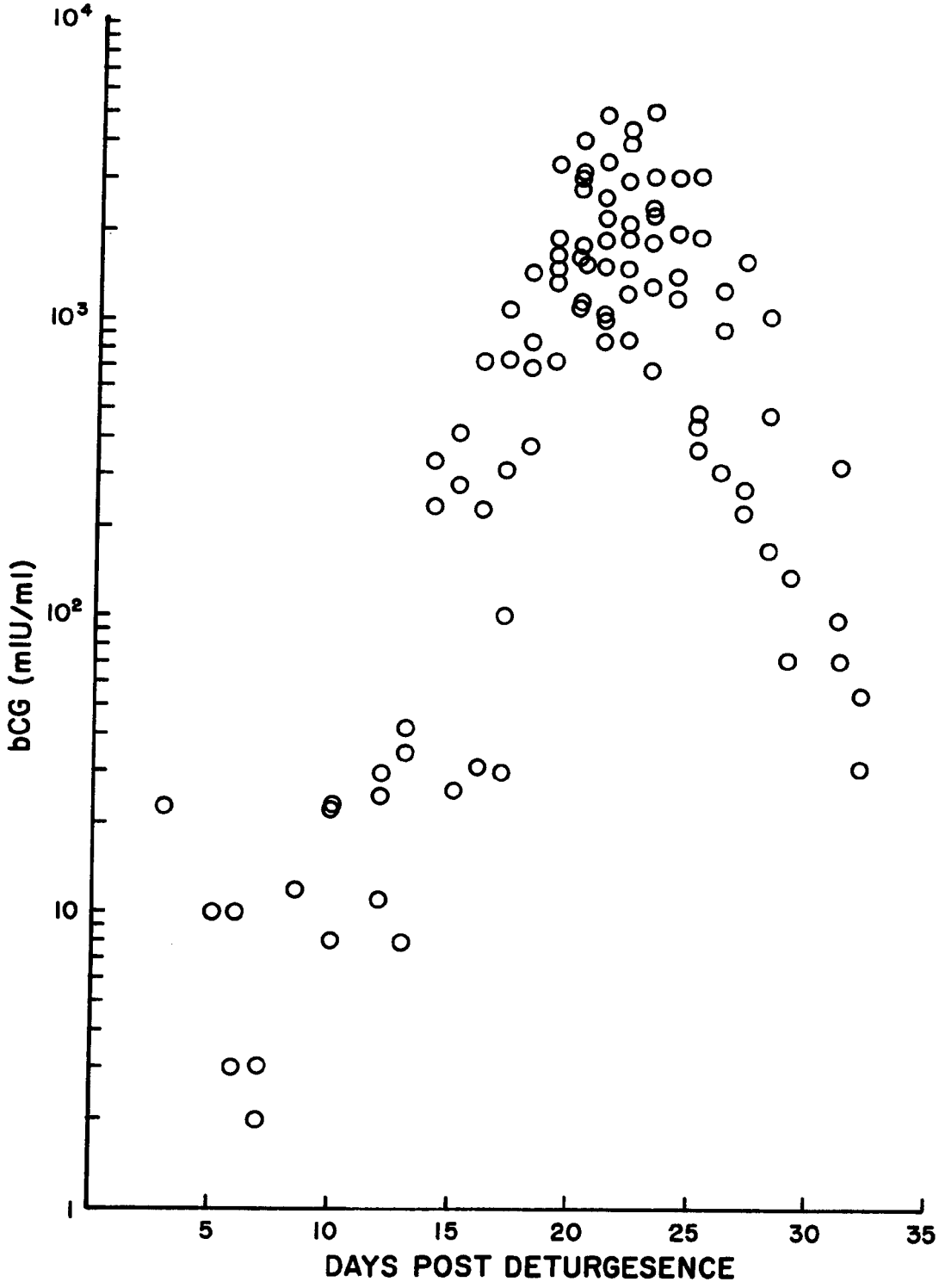


FIGURE 3

Serum bCG levels in pregnant baboons

Figure 3 **SERUM bCG**



No significant bCG production occurred in the latter three cultures, but the culture consisting of the pooled cells from two embryos continued to secrete up to 200 mIU bCG/day for at least 10 days following trypsinization.

These results indicate that in vitro, the baboon embryo produces and secretes chorionic gonadotropin in a pattern similar to that obtained from serum bCG levels in pregnant baboons. The fact that embryos recovered as early as the six-cell stage of development are capable of producing bCG in vitro indicates that the uterine environment is not necessary for the initiation of hormone secretion.

In view of the sensitivity limitation of the currently used assay procedure, to determine whether the actual initiation of secretion occurs before the embryo has hatched from the zona pellucida will require pooling of media from several embryos with subsequent dialysis to remove interference in the assay experienced with undiluted culture medium. Additionally, although Haour and Saxena (1974) reported approximately 16 mIU of HCG-like material in rabbit blastocysts, the hormone might be concentrated in the blastocoele at this stage of development, with only small quantities being secreted.

Significant stimulation of granulosa cell progesterone production by media from monkey morulae and blastocysts cultured in vitro occurred only after a culture period of several days (Batta and Channing, 1979), which may indicate the presence of extremely low quantities of gonadotropin beyond the detection limit of the heterologous radioimmunoassay system used in this study.

Conclusion

We believe that a culture system that allows primate embryos to undergo normal development to postimplantation stages in vitro, if used for the culture

of human embryos before their transfer to recipient women, could have a beneficial effect on the establishment of normal pregnancies.

Cultured baboon embryos produce and secrete chorionic gonadotropin in vitro, in a pattern which closely resemble that which occurs in vivo. Additionally, the amount of bCG secreted is related to the morphological development and integrity of the embryo and trophoblast during the culture period. The quantitation of this hormone, therefore, should be included as a criterion for normal development, and will enhance the utility of the baboon embryo model for studying early primate embryogenesis.

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CHAPTER VI

SP-I SECRETION BY BABOON EMBRYOS IN VITRO

Valerie Z. Pope, C. Earle Pope and Lee R. Beck

Placenta (1984), 5, 403-412

Introduction

Pregnancy-specific B_1-glycoprotein (SP-I), one of the several 'new' placental proteins isolated in the last fifteen years, is a product of the syncytiotrophoblast of primate placentae (Horne et al., 1976, 1977; Lin and Halbert, 1976; Heikinheimo et al., 1981). Although no specific function has been identified for this protein, its concentration in pregnancy serum is greater than that of any other placental protein (Bohn, 1979; Klopper, Smith and Davidson, 1979). Sp-I can be detected 18 to 23 days (Grudzinskas et al., 1977b) or as early as seven days (Grudzinskas et al., 1977a) following the LH peak in the serum of pregnant women.

Placental extracts from chimpanzees, baboons, cynomolgous and rhesus monkeys have been shown to contain SP-I (Bohn, 1976; Bohn and Sedlacek, 1975), and Stevens, Bohn and Powell (1976) have demonstrated SP-I in the serum of baboons from day 17 of pregnancy through to parturition, using an heterologous radioimmunoassay based on cross-reaction of baboon SP-I with human SP-I antibodies.

An in vitro culture system has been developed in our laboratory which allows achievement of postimplantation stages by nonsurgically recovered cleavage-stage baboon embryos (Pope, Pope and Beck, 1982a). We have demonstrated that the capability for baboon chorionic gonadotrophin (bCG) production and secretion develops in these embryos in the absence of the maternal environment (Pope, Pope and Beck, 1982b), and we aspire to establish whether secretion of other significant protein products of the conceptus occurs during the culture period. To initiate these studies, we utilized an heterologous enzyme-immunoassay (EIA) to determine whether SP-I is being produced and

secreted into the culture medium by baboon embryos that develop to postimplantation stages in vitro.

Materials and Methods

Baboon embryo recovery. The procedure for the nonsurgical recovery of baboon embryos has been described in detail (Pope, Pope and Beck, 1980). Briefly, a Curity endometrial cell sampler (Curity 4860), modified to permit bidirectional medium flow, is inserted across the undilated cervix of the anaesthetized female baboon an estimated three to four days following ovulation, as determined by loss of sex skin turgescence. Ten millilitres of a balanced salt solution supplemented with bovine serum albumin or 10 per cent serum is flushed through the device and collected in a centrifuge tube. The contents are decanted into 10 X 35 mm petri dishes and examined, using a stereomicroscope, for the presence of an embryo. Using this procedure, eggs are recovered from over 50 per cent of baboons flushed, with an approximate 62 per cent fertilization rate in baboons exposed to fertile males during estrus. Fertilized embryos are at the two-cell to blastocyst stage at the time of recovery, with half being at the 16-cell to morula stage of development.

Embryo culture. The embryo culture procedure and extent of development obtained have also been described in detail (Pope, Pope and Beck, 1982a), and will be summarized here. The medium used is CMRL-1066 (Gibco) with glutamine, pyruvate, antibiotics and 20 per cent fetal calf serum (FCS), human cord serum (HCS) or baboon serum (BS). If a serum source is changed during culture, the embryo is first rinsed in the new medium before being put back in culture.

Embryos are cultured in flat-bottomed microtiter plate (Falcon 3042) wells in 200 ul of medium, under an atmosphere of 5 per cent CO_2, 5 per cent O_2 and 90 per cent N_2 at 37° C. The embryos are photographed and the culture medium is changed daily in most instances by withdrawing the 'spent' culture medium from around the embyro, using a finely drawn pasteur pipette controlled with mouth suction. Individual spent media samples are stored at -20° C. Under these conditions, approximately 30 per cent of all embryos cultured have hatched from the zone pellucida in vitro, with attachment to the culture dish usually occurring one to three days later. Based on evaluation using phase contrast microscopy (Pope et al., 1982a), and, more recently, histological evaluation of fixed embryos (Pope, C.E. and Kunkle, H.M., unpublished observation, 1982), approximately 67 per cent of embryos that attach appear to undergo further differentiation of the embryonic disc, primitive amniotic cavity and primary yolk sac.

SP-I assay. The SP-I assay used in our laboratory has been an heterologous double antibody precipitation EIA utilizing rabbit anti-rhesus monkey SP-I as first antibody, and alkaline phosphatase-labelled rhesus SP-I as tracer. Purified rhesus monkey SP-I, or an equivalent 1:1000 diluted pregnant baboon serum sample, was used as standard with a detection range of 2.5-100 ng/tube. All samples were assayed in duplicate sets of at least two dilutions for a minimum of four tubes per sample to verify parallelism with the standard curve. Aliquots of complete unused culture media, supplemented with each serum or batch of serum that was used for embryo culture, were assayed as negative controls, and a serum sample obtained from the uterus of a pregnant baboon during a dilatation and evacuation procedure was included as a positive control in each assay.

Sample values were accepted if the standard deviation of replicate values did not exceed 15 per cent, although for 70 per cent of the values it was less than 10 per cent. Four sample values, however, have been included, in spite of a 16 to 19 per cent within-sample variation, in order to fill out profiles where sufficient media were not available for reassay (indicated by a superscript "b" in Table I).

bCG assay. Baboon chorionic gonadotrophin was measured using an heterologous radioimmunoassay (Pope, Pope and Beck, 1982b) in which anti-ovine beta-LH (Gary, Hodgen, NIH), was the first antibody, and ^{125}I-hCG (New England Nuclear) served as tracer. Values are expressed as mIU equivalents to a standard hCG preparation (NIH).

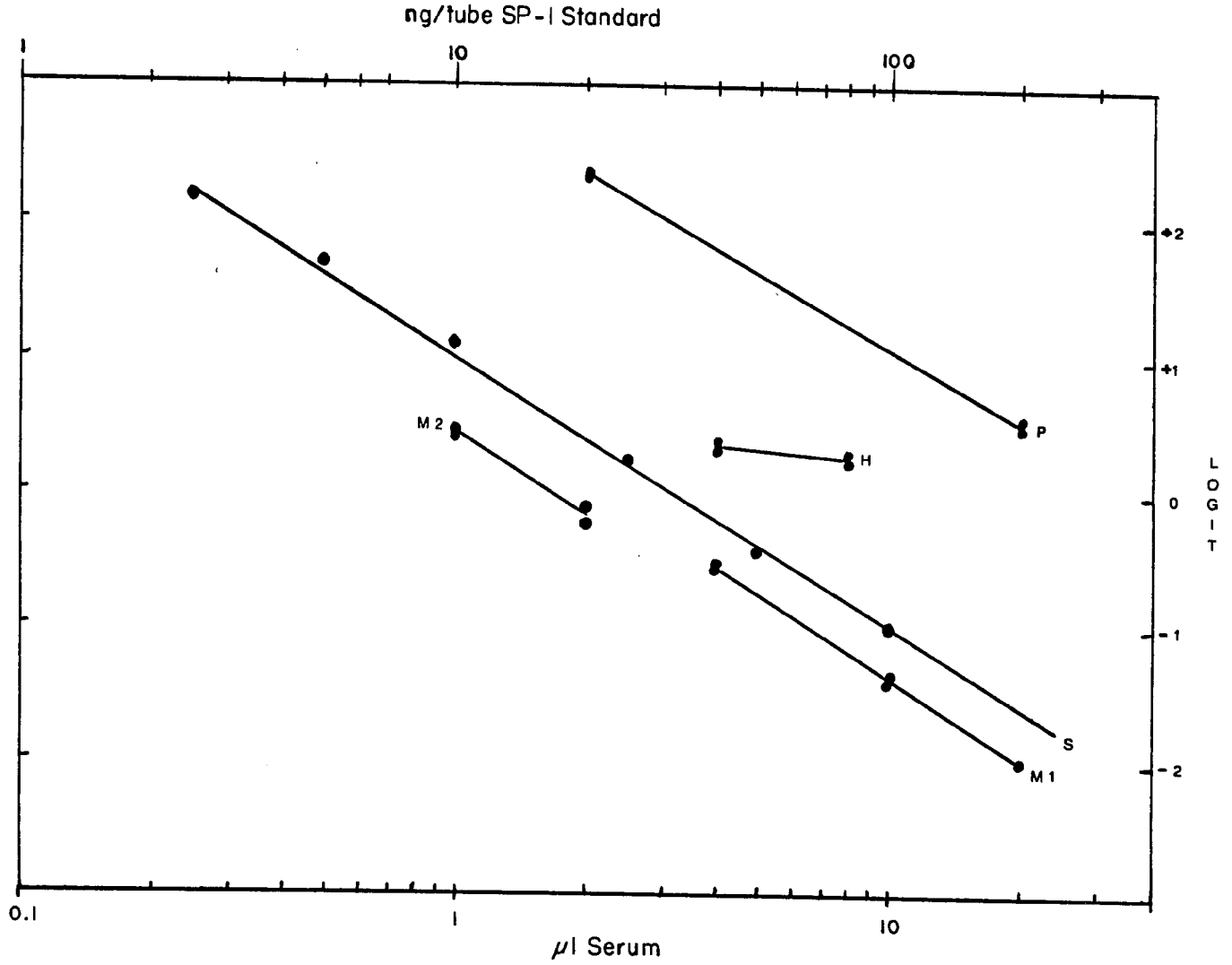
Results

Trace binding in the EIA for SP-I was unaffected by sera from nonpregnant primates. The inter-and intra-assay variations of the positive control sample were both 9.4 per cent (n = 12).

Our culture medium was initially supplemented almost exclusively with human cord serum (HCS). Therefore, before attempting to determine SP-I secretion by embryos cultured in HCS-supplemented medium, it was necessary to determine the extent of cross-reactivity of the anti-rhesus SP-I antibody with human SP-I present in the cord serum-containing medium. Of 14 samples assayed, six (43 per cent) showed sufficient cross-reactivity to inhibit maximum label binding in the EIA (Figure I, line H). Media samples from embryos cultured in those sera were not assayed. Spent media samples from three embryos cultured in medium containing noncross-reacting quantities of human SP-I were found to contain measurable SP-I starting from four to seven days

FIGURE 1

Enzyme-immunoassay for SP-1 using anti-rhesus antibody. S = average standard curve (2.5 to 100 ng/tube) from the two assays in which displayed samples were included; H = cross-reacting unused medium sample containing 4-8 ul human cord serum batch YJ; P = 2-20 ul 1:1000 diluted pregnant baboon serum sample; M1 = 4-20 ul spent culture medium with 20 per cent (0.8 to 4.0 ul) non-cross-reacting human cord serum; M2 = 1-2 ul spent culture medium with 20 per cent (0.2 to 0.4 ul) non-pregnant baboon serum.


ng/tube SP-I Standard
1
10
100
+2
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H
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0.1
1
10
μl Serum

FIGURE 1

Table I. SP-I Secretion by baboon embryos in vitro

			Medium with 20% BS				Medium with 20% HCS			
Embryo	1	2	3	4	5	6	A	B	C	
Stage at recovery	14-cell	morula	BC	BC	BC	BC	12-cell	16-cell	BC	
Culture day hatched	7	5	3	6	4	3	7	4	4	
Culture day attached	10	6	5	7	5	6	9	6	6	
Days PA	ng/day SP-I									mIU/day bCG[a]
0									<25	
1		<12.5				<12.5			<25	< 5
2		<12.5			<12.5	<12.5	< 25		@@@	@@@
3		<12.5	<12.5	< 12.5	<12.5	<12.5	< 25	< 50	<25	76
4	<12.5	5[6]	41	4[6]	120[b]	47		125	51	855
5	<12.5	247		224	277	52[6]	@@@	12.5	102	653
6	22	364	756[b]	719	774	1109	@@@	101	310	1298
7	177	376	1201	786	399	903	841	852	831	2832
8	dead	OC	472		488		@@@	1399	1058	3059
9			321		1082	2497	1109	2805	1003	1894
10					1279	3598	@@@	> 4000	OC	
11			417	787[b]	4773	636	OC			
12						@@@				
13					@@@	6168	OC			
14					1253	3377				
15						3738				
16						OC				
17					@@@					
18					856[b]					
19					OC					

PA = post attachment of the embryo to the culture dish; OC = out of culture; BC = blastocyst; HCS = human cord serum; BS = baboon serum; @@@ = culture medium not changed that day, therefore subsequent values represent secretion over greater than one day; 'No.' = lower limit of sensitivity for the assay, and measurable SP-I was not present.

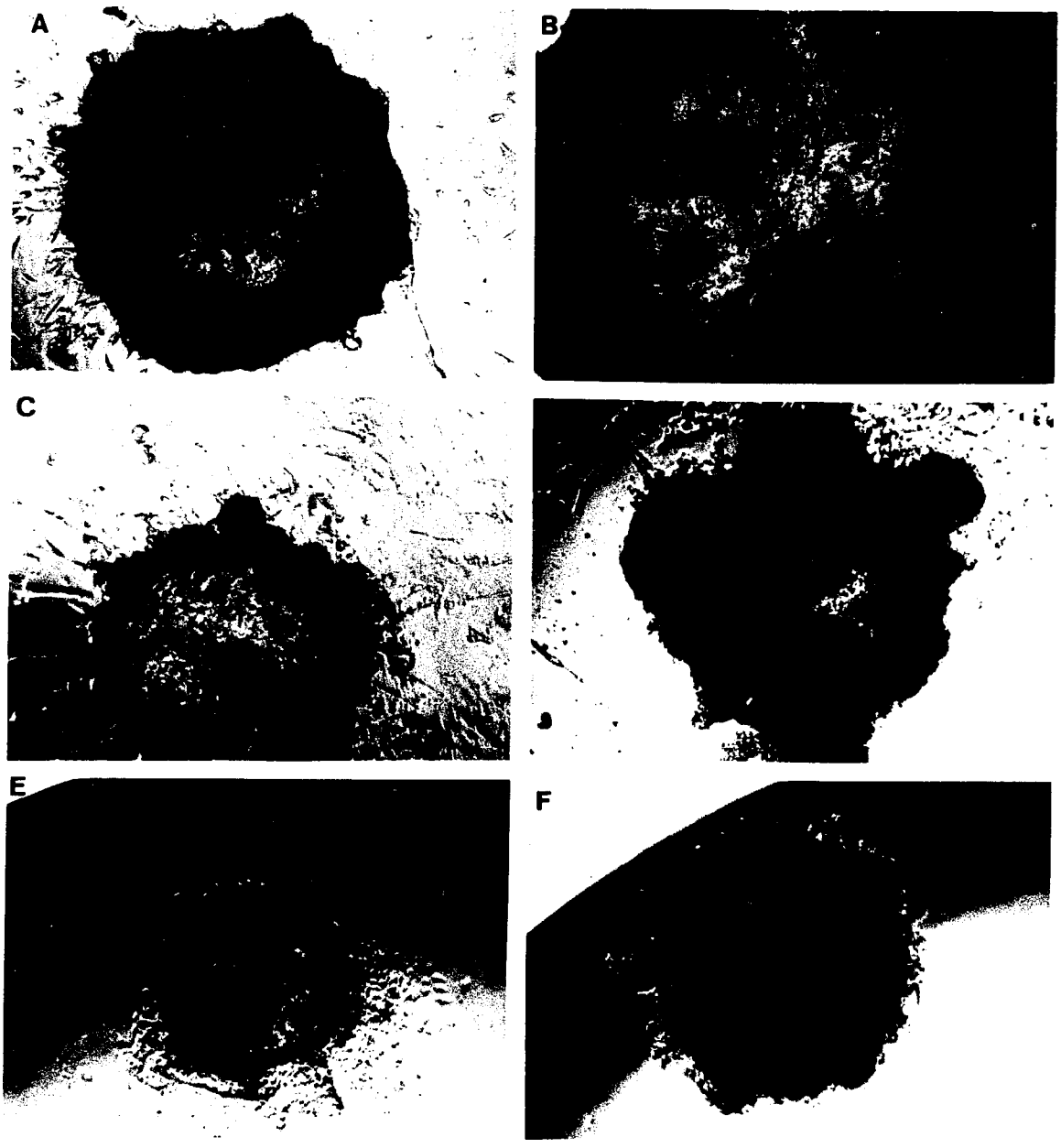
[a] bCG = mIU baboon chorionic gonadotrophin/day for embryo C.

[b] Sample variation 16-19 per cent, therefore approximate value.

after attachment to the culture dish (Table I), with a peak of over 4000 ng/day occurring in one embryo (embryo B) 10 days after attachment. Throughout the entire culture period negligible trophoblast outgrowth was visible on the bottom of the culture well containing this embryo, and blebs of trophoblast appeared to be protruding from several locations around the exterior of the embryo. These results prompted us to change to media supplemented with nonpregnant baboon serum instead of human cord serum following attachment of the embryo to the culture dish. This would allow us to verify that molecular conversion of human SP-I by baboon embryos to a form more recognizable by the anti-rhesus SP-I antibody was not occurring in culture. Media samples were assayed for nine embryos which were switched from human cord serum to baboon serum-supplemented CMRL medium, and all were shown to secrete SP-I into the medium. Secretion was initiated by the fourth day post attachment in five of six embryos for which profiles have been obtained (Table I). One of the embryos that first had measurable SP1 in the culture medium four days post attachment (PA) was similar to other embryos studied; outgrowth was apparent around the embryo during the first week of the attachment stage (Figure 2e), but then receded off the plastic over the next two to three days. During this period the trophoblast continued to grow around the embryos, and SP-I secretion had increased to 719-852 ng/day by day six to seven post attachment in five embryos (Figure 2b, d, f). In most embryos this trophoblast growth surrounded the entire embryo to such a thickness that it became difficult to discern previously-apparent embryonic structures (i.e., embryonic disc and amniotic cavity) under phase-contrast microscopy (Figure 2f). After day seven post attachemnt, SP-I present in spent media from embryos cultured in CMRL supplemented with baboon serum continued to increase in two of the three embryos left in culture, with peaks of up to almost 5 ug SP-I/day being

FIGURE 2

Baboon embryos in culture, x 123. (a) Embryo 2; four days post attachment, 56 ng SP-I. (b) Embryo 5; six days post attachment, 774 ng SP-I. (c) Embryo 3; four days post attachment, 41 ng SP-I. (d) Embryo 6; seven days post attachment, 903 ng SP-I. (e) Embryo 4; four days post attachment, 46 ng SP-I. (f) Embryo 4, seven days post attachment, 786 ng SP-I.


A
B
C
E
F

secreted. Spent media samples from a 12-day culture period for one embryo (embryo 6, Table I) contained a total of over 26 ug SP-I. An embryo was taken out of culture on day seven post attachment (Figure 2f), fixed, and processed for light microscopy (Figure 3). The structures visible in this histological section support our evaluation, made under phase-contrast microscopy, that a bilaminar embryonic disc, amniotic cavity and primitive yolk sac were forming in this embryo. Additionally, the thick extraembryonic layer surrounding the embryo had the staining characteristics and appearance of human syncytiotrophoblast.

Our most successful rate of postimplantation development has been achieved in baboon embryos initially cultured in human cord serum-supplemented medium. One embryo, however, recovered as a blastocyst, hatched from the zona pellcida in a balanced salt solution supplemented with 15 percent fetal calf serum. Following attachment, this embryo was switched to the same medium supplemented with 40 to 50 per cent autologous baboon serum, so that throughout the entire culture period the embryo was not exposed to sera from a pregnant primate. This embryo secreted measurable SP-I by three days post attachment, with 695 ng SP-I being secreted on day five post attachment, at which time the embryo was taken out of culture. Both SP-I and baboon chorionic gonadotrophin profiles were obtained on one embryo (embryo C, Table I), allowing a comparison of the time during culture at which maximum secretion of the two placental proteins was achieved.

Discussion

The demonstration that baboon embryos developing to postimplantation stages in vitro are capable of SP-I secretion suggests that the cultured baboon

FIGURE 3

Section through baboon embryo number 4 on day seven post attachment. A = amniotic cavity; D = bilaminar embryonic disc; Y = primary yolk sac cavity; T = trophoblast; P = site of attachment of embryo to plastic substrate. X 110.


P
A
D
Y
f

embryo could become a useful model for studying the production and secretion of this protein. The quantity secreted in vitro, based on a rhesus monkey standard, compares favorably with SP-I secretion rates reported for cultured human placenta. Chou, Rosen and Mano (1981) have established cloned virus-transformed human placental cell lines, capable of producing SP-I in vitro, which secreted up to 10 ng SP-I per million cells per day when cells obtained from term placentae were cultured under a restrictive temperature (40° C). Virus-transformed cells obtained from first-trimester placentae were less productive, although sodium butyrate induced an increase in SP-I secretion by both first-trimester and term placentae to up to 100 to 300 ng per million cells per day, respectively, over a seven day culture period. Rosen et al. (1979), however, have shown that up to 120 ng SP-I per million cells per day was secreted by 19 out of 20 fibroblast cultures, although they were unable to demonstrate detectable SP-I production in lung fibroblast cultures from baboons and other monkeys. Their study is one of several indicating that production of this protein is not limited to cells of the placenta, and stresses the importance of establishing an appropriate model if a true relationship between SP-I production and specific events in developmental biology are to be determined. Significant in vitro production of SP-I by placental cells was achieved by Heikinheimo et al. (1981), who were able to successfully plate 50 to 70 percent of syncytiotrophoblasts from 8-to 11-week human placentae; these remained viable for one week before being overgrown by other cell types. Up to 9150 ng SP-I per milligram of protein was present in the medium after 96 h in culture, with intracellular SP-I approximately two to three times greater.

The cross-reactivity of the rhesus monkey antibody with baboon SP-I, compared to that of anti-human SP-I antibody, resulted in an approximate 2000-fold increase over the reported SP-I levels (Stevens, Bohn and Powell, 1976) in

sera of pregnant baboons (Vernon Stevens, personal communication, 1980). The quantitites of SP-I secreted by baboon embryos in vitro, as measured in an heterologous assay, are still probably less than the actual amounts that will be accurately determined when an homologous assay is developed. The presence of at least microgram quantities of SP-I in the spent culture medium from single culture days from individual embryos as early as six days following 'implantation' in vitro (Table I), and the continuation of secretion for up to two weeks, should result in the availability of sufficient material to allow extensive biochemical characterization of the SP-I being produced in vitro.

We have been unable to discern any visible criteria that can be used to predict the rate of SP-I secretion in vitro. For six embryos, similar patterns were followed with regard to initiation of SP-I secretion and its increase over the next few days, based simply on the temporal relationship to attachment to the culture dish. The apparent quantity of trophoblast outgrowth onto the plastic substrate (Figure 2) does not appear to be related to the quantity of SP-I secreted, raising the question whether these cells are even involved in its production. Although it appears that this system has potential as a model for studying SP-I production and secretion by primate placentae in vitro, we also hope to utilize quantitation of SP-I routinely in our effort to monitor the progression of development in the cultured embryos. Baboon chorionic gonadotrophin secretion in vitro reaches a peak followed by a rapid decline, much like that which occurs in serum of pregnant baboons (Pope, Pope and Beck, 1982b), making quantitation of that placental protein a poor indication of the health of the embryo. Serum SP-I in pregnant baboons continues to rise throughout the first trimester of pregnancy (Stevens, Bohn and Powell, 1976; Pope, V.Z., unpublished observation, 1983), which suggests that SP-I secretion profiles may prove to be reliable indicators of the health of the trophoblast. As

mentioned earlier, the thickness of the trophoblast usually renders the embryo fairly opaque by the seventh day following attachment, making it particularly difficult to evaluate the viability of the culture by visual observation. We hope to acquire the ability to quantitate additional placental and embryonic protein to further enable us to evaluate the progression of early developmental events in vitro. Recently we have utilized human alpha-fetoprotein (AFP) antibody (DAKO) and label (Cambridge Medical Products) in a radioimmunoassay for AFP which cross-reacts with the analogous baboon protein. Although we are just starting to evaluate spent baboon embryo culture media for the presence of AFP, embryo number 6 (Table I) had no AFP in the medium on day seven post attachment, while I.736, 0.443 and 0.022 ug of AFP were present on days nine, ten, and eleven post attachment, respectively (Pope, V. Z., unpublished observation, 1983). These results indicate that an homologous assay for baboon SP-I, which would allow accurate quantitation of SP-I production by the cultured baboon embryos, as well as permit isolation of the protein for further studies, should be developed. Additionally, the actual site of synthesis of SP-I should be determined by immunohistochemical techniques. The values obtained in the current study suggest that SP-I is not secreted until after implantation, but determination of the actual time of initiation of secretion will require a more sensitive assay.

Summary

Baboon embryos cultured to postimplantation stages have been shown to secrete the placental protein SP-I into the culture medium in quantities of up to almost 5 ug/day, based on a rhesus monkey standard. Twelve embryos, for

which spent media samples have been assayed, have been shown to secrete this protein, with measurable quantities usually being present on day four following attachment of the zonaless embryo to the culture dish. Secretion has continued for up to 14 days, with over 26 ug total SP-I secretion from one embryo. These observations further enhance the utility of the baboon embryo culture system as a model for studying early placental development in the primate.

Acknowledgements

The authors wish to thank Dr Vernon C. Stevens, Ohio State University, for his generous donation of both purified rhesus monkey SP-I and specific anti-rhesus SP-I antibody, and Dr Mel Kunkle, Anatomy Department, University of Alabama in Birmingham, for performing the histology on the baboon embryo. This research has been supported by grants from the NIH (HD 15358) and the Alcoa Foundation.

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CHAPTER VII

OBSERVATIONS ON THE MORPHOLOGY, HISTOLOGY, AND ENDOCRINE FUNCTION OF PERI-IMPLANTATION BABOON EMBRYOS IN VITRO

V. Z. Pope, C. E. Pope, L. R. Beck and G. R. Poirier

Abstract

Nonsurgically recovered baboon embryos were cultured in vitro in different environments to determine the extent of postimplantation differentiation that could be achieved. Embryos (n = 378) were recovered at the 2-cell to hatched blastocyst stage, and 25% developed posthatching. The extent of development in vitro was similar for embryos recovered at the 16-cell through blastocyst stage. Embryos were cultured in petri dishes under oil or in culture plates, and CMRL-1066 medium supplemented with human cord serum (HCS) was the main media used through hatching from the zona pellucida. Following hatching, some embryos were cultured in media supplemented with baboon serum (BS). Approximately 50% of embryos in culture plates in media supplemented with HCS or BS after hatching underwent normal differentiation of the bilaminar embryonic disc. When the embryos were cultured under oil, 32% underwent differentiation of embryonic structures posthatching, regardless of serum supplement used. Histological evaluation of five embryos verified the accuracy of morphological evaluations made during culture. One embryo was a solid mass of cells; two developed a definitive amnion, bilaminar embryonic disc and primary yolk sac; one perhaps developed a secondary yolk sac, and one had normal appearing trophoblast with a degenerate inner cell mass. Estradiol and progesterone measurements made on spent media demonstrated progesterone synthesis and secretion by postimplantation embryos, and possibly a release of estradiol around the time of hatching from the zona pellucida.

Introduction

The early development of rodent embryos has been studied in great detail using an *in vitro* culture system. The culture of primate embryos has not been as widely pursued due to limited availability of embryos. In vitro development through escape or "hatching" from the zona pellucida has been reported for rhesus monkey (*Macaca mulatta*; Morgan *et al.*, 1984), marmoset (*Callithrix jacchus*; Summers *et al.*, 1984), human (Fishel *et al.*, 1984), and baboon (*Papio sp*; Pope *et al.*, 1982a) embryos. The small number of embryos achieving posthatching stages in these studies did not permit determination of the optimal environmental conditions for maximum growth and differentiation to be determined. Improving the efficiency of embryo recovery, defining the essential components of the culture environment, and comparing *in vitro* with *in vivo* development are, therefore, primary objectives of primate embryo culture laboratories.

As a step toward fulfilling these objectives, 378 embryos recovered nonsurgically from 59 adult female baboons were cultured in various environments. In the present study we compare the morphology, histology, and endocrine function of these embryos *in vitro*.

Materials and Methods

Baboon embryo recovery. The nonsurgical procedure developed in this laboratory was used as previously described (Pope *et al.*, 1980). Some baboons were specifically allocated as embryo donors, and some were in control cycles between studies evaluating long-acting contraceptives. Up to 32 embryos were from a single donor, with 15 baboons providing 10 or more embryos for culture.

Embryo culture. Embryos were cultured in several environments in an attempt to increase the percentage achieving posthatching development. The procedure for media preparation, embryo handling, and the culture apparatus was as described (Pope et al., 1982a, Chapter IV). The basic medium for baboon embryo culture was CMRL-1066 (Gibco) supplemented with glutamine, pyruvate, penicillin and streptomycin or gentamycin sulfate. Human cord serum (HCS) was the basic serum supplementation used for cleavage stage embryos, although this was subject to variation. For some embryos, baboon serum (BS) replaced HCS as supplement after hatching. Other serum sources evaluated included fetal calf serum (FCS), a combination of HCS and BS, baboon pregnancy serum, human male serum, and rat serum. Pooled or individual samples of serum were stored at -20° C in 1 ml aliquots, and heat treated (56° C, 30 minutes) prior to addition to the medium at 20 to 40% by volume. Some serum samples were filtered (0.22 or 0.45 um).

Embryos were cultured in 35 mm plastic petri dishes (Falcon 1008 or 3801) under mineral oil, or in flat bottom culture plate (Falcon 3008) wells. Additionally, one embryo was transferred to a culture chamber on a glass slide (Lab-Tek; Miles Laboratories 4838) after hatching. Embryos were cultured in 200 to 1000 ul media in a humidified atmosphere usually comprising 90% N_2, 5% O_2, and 5% CO_2. Used, or "spent," media was usually replaced with fresh media daily, occasionally every other day, and rarely at greater intervals. Harvested spent media was stored at -20° C.

In an additional experiment, the effect of incubating culture medium under oil on the estradiol and progesterone content of harvested medium not exposed to an embryo was determined. Normal CMRL medium supplemented with 20% HCS was kept in culture, and for each of the first 5 days, the medium was withdrawn from under the oil and replaced with fresh medium. The next

five medium samples were left in culture for 1 to 3 days before being removed and stored at -20° C until E2 and P4 levels were determined. In a separate dish, aliquots from a different medium sample were incubated and removed at 2-day intervals.

Morphological observations. The gross morphology of the embryos was evaluated, and in most cases documented by photography, when the media was changed. Notes were made on the morphological changes as an index of development and the apparent health of the culture. Criteria for assessing the normality of morulae and blastocysts included uniform density of cytoplasm, rate of cleavage, incidence of fragmenting and abnormally large and/or small cells. Evaluation of development during early postimplantation stages in vitro required presumptuous definition of structural differentiation based on the presence or absence of an oval opaque mass within the embryo (ICM or embryonic disc), a membranous- appearing lining of the perimeter of the blastocyst cavity (primary yolk sac), and a membranous or sac-like structure (definitive amnion) surrounding the embryonic disc (Fig. 1). In some embryos, the trophoblast thickening around the embryo obscured any embryonic differentiation or degeneration that may have occurred during the final few days in culture.

Following the termination of culture, information on each embryo was summarized into at least 13 fields of a data base file to allow grouping of similar field entries. The first two fields identified the embryo using the unique one- or two-letter initials of the donor baboon's name and the recovery date. Embryo stages at recovery were divided into eight classifications (Table 1) used by the International Embryo Transfer Society (Seidel, 1983). The stage achieved in culture included the ninth classification for posthatching development. For embryos reaching this ninth stage, the morphology was

FIGURE 1

Postimplantation Morphology of Baboon Embryos In Vitro

A. Embryonic disc (a)
Yolk sac endoderm (b)
X86.

B. The embryonic disc is the opaque central mass, surrounded by the amnion X182.

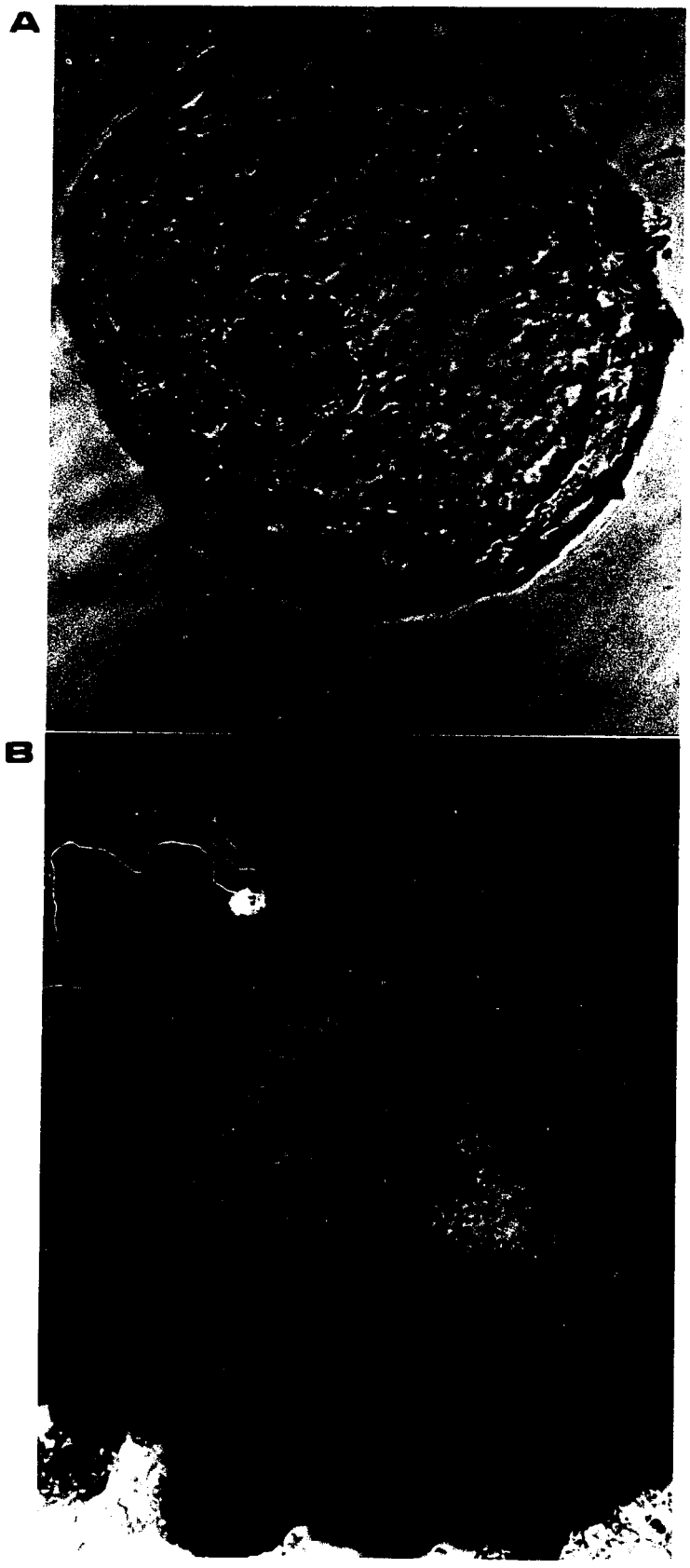

A
B

TABLE 1

Embryo Classification

Code	Stage	Abbreviation and/or Explanation
1	Unfertilized	Unf
2	2-12 cell	2-12
3	Early Morula	Erl M
4	Morula	M
5	Early Blastocyst	Erl BC
6	Blastocyst	BC
7	Expanded Blastocyst	Exp BC
8	Hatched Blastocyst	HBC
9	HAOG	H = Hatch A = Attach OG = Outgrowth

Modified from Seidel (1983).

further described by one of 13 additional categories (Table 2). Of these categories, embryos in groups 2, 3, 9 and 10 were considered to have undergone normal differentiation of early embryonic structures; ie, amniotic cavity and/or bilaminar embryonic disc formation. Embryos in groups 4, 5 and 11 were considered to be abnormal, characterized by failure of the inner cell mass (ICM) to differentiate into an embryonic disc. A few embryos developed without hatching from the zona pellucida, and were classified as indicated.

The number of days in culture was included in the records, as well as the day perceived to be that on which development peaked, or the point following which no additional differerentiation occurred. On most occasions, cell division and growth continued past the subjectively determined peak, but the integrity of the differentiated structures began to deteriorate.

The medium and gas mixture used were two additional fields, although CMRL-1066 and 5% O_2, 5% CO_2, and 90% N_2 comprised the entries in 92.6% and 88.3% of the records, respectively. The serum supplement, volume percent, and day the supplement was changed, if applicable, were recorded for each embryo.

Histology. Embryos (n = 5) selected for histological evaluation were rinsed in buffer at the termination of culture, and fixed in 2.0% glutaraldehyde. Embryos were post-fixed in OsO_4, dehydrated, and embedded in Poly Bed 812 or Durcopan resins. Sections (1.5 to 2.0 um) were stained with toluidine blue and permanently mounted.

Assays. Estradiol (E2) and progesterone (P4) were determined in unextracted (Fortune and Armstrong, 1978) media samples using standard RIA procedures (Beck et al., 1985). Unused media samples prepared from each batch of serum were assayed to determine exogenous steroids available. Logit transformation for standard curve regression and quantitation of sample steroid

TABLE 2

Differentiation Scores for Baboon Embryos in Vitro

Score	Explanation
0	Development stopped pre-culture
1	Identified by cell stage (ie, prehatch)
2	Hatch (H), Attach (A), Yolk Sac Expansion (YSE), Bilaminar Embryonic Disc (ED) and Amniotic Cavity Formation (AC)
3	H, A, YSE, ED
4	H, A, YSE - "Blighted"
5	H, A, No Organized YSE - "Abnormal"
6	H, then died
7	No H, "lysed zona," A, YSE
8	No H, A, YSE
9	H, YSE, ED, AC, no A
10	H, YSE, ED, no A
11	H, YSE, no A
12	Unintentionally stopped - lost, etc.
13	Intentionally stopped culture

concentrations was performed using a spread sheet program developed for use on a microcomputer.

Results

Embryo morphology in relation to culture conditions. Baboon embryos placed in culture ranged from the 2-cell to the blastocyst stage at the time of recovery, with the morula stage comprising 50% of embryos cultured (Table 3). Regardless of the stage at recovery, over 30% (122/389) of embryos developed at least to the zonaless blastocyst stage, and 25% (97/389) underwent posthatching development. The extent of posthatching development achieved was similar when embryos were recovered at the early morula, (16- to 32-cell stage), morula, or early blastocyst stages; i.e., approximately 23% (51/218) of embryos recovered at these stages progressed beyond hatching from the zona pellucida.

Significantly ($p < .005$) more embryos achieved posthatching development under oil, with 43.5% (27/62) of embryos cultured under oil developing beyond hatching in vitro, compared to 21.4% (70/237) of embryos cultured without an oil overlay. After hatching, embryos under oil did not attach as firmly to the culture dish as those in culture plate wells; in fact, the turbulence caused by daily medium changes was frequently sufficient to dislodge the embryos.

In all culture conditions evaluated (Table 4), 22.9% of the embryos did not develop at all. Development stopped before escape from the zona pellucida in 44% of the embryos, while 33% proceeded to hatching and beyond. The total number of embryos comprising these groups (350) is less than in the groups evaluated by recovery stage (Table 3), due to exclusion of embryos cultured in

TABLE 3

Embryo Culture Success by Cell Stage at Recovery

Rec Stage[a]	OIL 8[b]	OIL 9[c]	OIL Total	NO OIL 8	NO OIL 9	NO OIL Total	TOTAL 8	TOTAL 9	TOTAL
2-12	0	0	8	2	10	95	2	10	103
16-32	0	9	17	8	17	91	8	26	108
M	0	5	16	5	15	71	5	20	87
Erl BC	1	3	7	2	2	16	3	5	23
BC	0	3	6	4	8	21	4	11	27
Exp BC	0	6	7	3	14	26	3	20	33
HBC	X	1	1	X	4	7	X	5	8
TOTAL	1	27	62	24	70	327	25	97	389

[a] M = Morula; Erl BC = Early Blastocyst; Exp = Expanded; HBC = Hatched Blastocyst

[b] 8 = Reached Hatched Blastocyst Stage (see Table 2)

[c] 9 = Underwent Further Development Posthatching

Table 4

Baboon Embryo Culture - Development Achieved in Different Media

Group	Descript	Culture Stage[a] 0 1	H 2	Differentiation[b] H only 3	Norm 4	Abn 5	Misc 6	TOTAL 4-6 7	TOTAL 3+7 8	TOTAL EMBRYOS 9	
A	HCS	20	30	4	11	6	2	19	23	73	
					57.9	31.6	10.5				% of 7
		27.4	41.1	5.5	15.1	8.2	2.7	26.0	31.5		% of 9
B'	HCS to HCS-P	1	5	2	4	4	0	8	10	16	
					50	50	0				% of 7
		6.3	31.3	12.5	25.0	25.0	0	50.0	62.5		% of 9
B	HCS-P	37	84	12	8	9	4	21	33	154	
					38.1	42.9	19.0				% of 7
		24.0	54.5	7.8	5.2	5.8	2.6	13.6	21.4		% of 9
A-B	All HCS	58	119	18	23	9	6	48	66	243	
					47.9	39.5	12.5				% of 7
		23.9	49.0	7.4	9.5	7.8	2.5	19.8	27.2		% of 9
C	HCS-P Under Oil	14	19	0	2	3	0	5	5	38	
					40.0	60.0					% of 7
		36.8	50.0	0	5.3	7.9		13.2	13.2		% of 9
D	HCS to BS	NA	NA	2	10	8	2	20	22	22	
					50	40	10				% of 7
				9.1	28.6	57.1	9.5	95.2	100		% of 9
E	HCS to BS Under Oil	NA	NA	1	6	12	2	20	21	21	
					30	60	10				% of 7
				4.8	28.6	57.1	9.5	95.2	100		% of 9
F	FCS	8	15	1	0	1	1	2	3	26	
						50	50				% of 7
		30.8	57.7	3.8	0	3.8	3.8	7.7	11.5		% of 9

Table 4, Continued

Total Oil	14	19	1	8	15	2	25	26	59	
				32.0	60.0	8				% of 7
	23.7	32.2	1.7	13.6	25.4	3.3	42.4	44.1		% of 7
Total No Oil	58	119	20	33	27	8	68	88	265	
				48.5	39.7	11.8				% of 7
	21.9	44.9	7.5	12.5	10.2	3.0	25.7	33.2		% of 9
TOTAL	80	153	22	41	43	11	95	117	350	
				43.2	45.3	11.6				% of 7
	22.9	43.7	6.3	11.7	12.3	27.1	33.4			% of 9

[a] 0 = No Development Occurred
H = Some Development Occurred; Stopped Prehatching
H Only = Embryo Hatched from Zona Pellucida, Then Died

[b] = Differentiation (See Table 2 for codes):
Norm = Normal - Differentiation scores of 2,3,9 or 10
Abn = Abnormal - Differentiation scores 4,5, or 11
Misc = Differentiation scores of 7,8,12 or 13

HCS = Human Cord Serum supplementation
HCS-P = HCS Pool
BS = Baboon Serum
FCS = Fetal Calf Serum

media supplemented with serum obtained from sources other than those listed in Table 4.

Approximately 10% (23/243) of embryos cultured without oil in media supplemented with HCS throughout the culture period progressed through hatching from the zona pellucida and achieved differentiation of at least the bilaminar embryonic disc (Groups A-B, Table 4). Twenty of 22 (95.2%) embryos switched to media supplemented with baboon serum following hatching and not cultured under oil (Group D, Table 4) developed further, and 50% (10/20) of those undergoing further development appeared to be normal. When the medium containing the embryo was incubated under mineral oil, only 30% (6/20) of embryos continuing to develop in BS-supplemented medium underwent ICM differentiation. Regardless of the serum supplement used, only 32% (8/25) of embryos achieving posthatching development cultured under oil underwent differentiation of embryonic structures, while 49% (33/68) of those cultured without oil appeared to develop at least a bilaminar embryonic disc from the inner cell mass. This difference, however, was not significant ($p = 0.10$).

When serum used as media supplement was filtered (0.45 um) before use, 19 of 50 (38.0%) embryos developing beyond hatching from the zona pellucida underwent normal embryonic differentiation. Medium prepared with unfiltered serum supported normal ICM differentiation in 56.5% (26/46) of posthatching embryos ($p = 0.074$).

Histology. Table 5 summarizes the culture conditions, time of hatching, length of culture and extent of differentiation for embryos that were subjected to histological evaluation. Embryo 1 (SC 8/82 - Group D) was recovered at the early blastocyst stage and cultured in 200 ul of CMRL-1066 medium supplemented with 20% HCS for the first 10 days. Autologous BS replaced the HCS supplement during the last 3 days in culture. On culture day 5, the embryo

TABLE 5

Baboon Embryos Evaluated by Histology

Embryo	Recovery Stage	Culture Day: Hatch	Attach	Peak	Days Cult.[a]	Diameter (um)	ID
1	Blastocyst	5	6	13	13	1100	SC820813
2	Morula	5	9	12	12	950	ZB850311
3	Exp. BC[b]	5	9	12	12	1440	XC841024
4	Exp. BC	1	6	6	10	965	VA850123
5	Exp. BC	4	6	5	14	1340	BC850301

[a] No. of days in culture

[b] Expanded Blastocyst

hatched from the zona pellucida, and attached to the culture dish one day later. By day 7 the embryo had assumed a flattened appearance, and trophoblast outgrowth was evident. The ICM was continuously visible from before hatching until its transition to become the bilaminar embryonic disc during the latter part of the culture period (Fig. 2). The amnion was also first apparent when initial yolk sac expansion began on day 10 of culture. At the end of the 13-day culture period, 7 days following attachment to the culture dish and 17 days following presumed ovulation (Pope et al., 1983, Shaikh et al., 1980) the embryo was fixed in glutaraldehyde and embedded in Durcopan.

Histological evaluation revealed a fully expanded primary yolk sac lined by a layer of extra-embryonic endoderm (Fig. 2). The bilaminar layer of trophoblast surrounding the yolk sac had an inner layer consisting of irregular thicknesses of pale staining cuboidal cells. In some areas, masses of this cytotrophoblast streamed through the outer layer of syncytiotrophoblast to form mushroom-shaped clumps (Fig. 3). The thin outer layer of syncytiotrophoblast was composed of darkly staining homogeneous cells that followed the projecting contours of cytotrophoblast. Numerous spaces were present within the syncytiotrophoblast, but coalescence of the spaces had not occurred. Attachment to the plastic substrate occurred at the embryonic pole, the orientation assumed by implanting primate embryos in vivo (Enders et al., 1983).

The bilaminar embryonic disc consisted of a hypoblast, or primitive endoderm, loosely arranged on top of the pseudostratified layer of columnar cells comprising the epiblast (Fig. 3). These epiblast cells had apparent continuity with those forming the lining of the amniotic cavity.

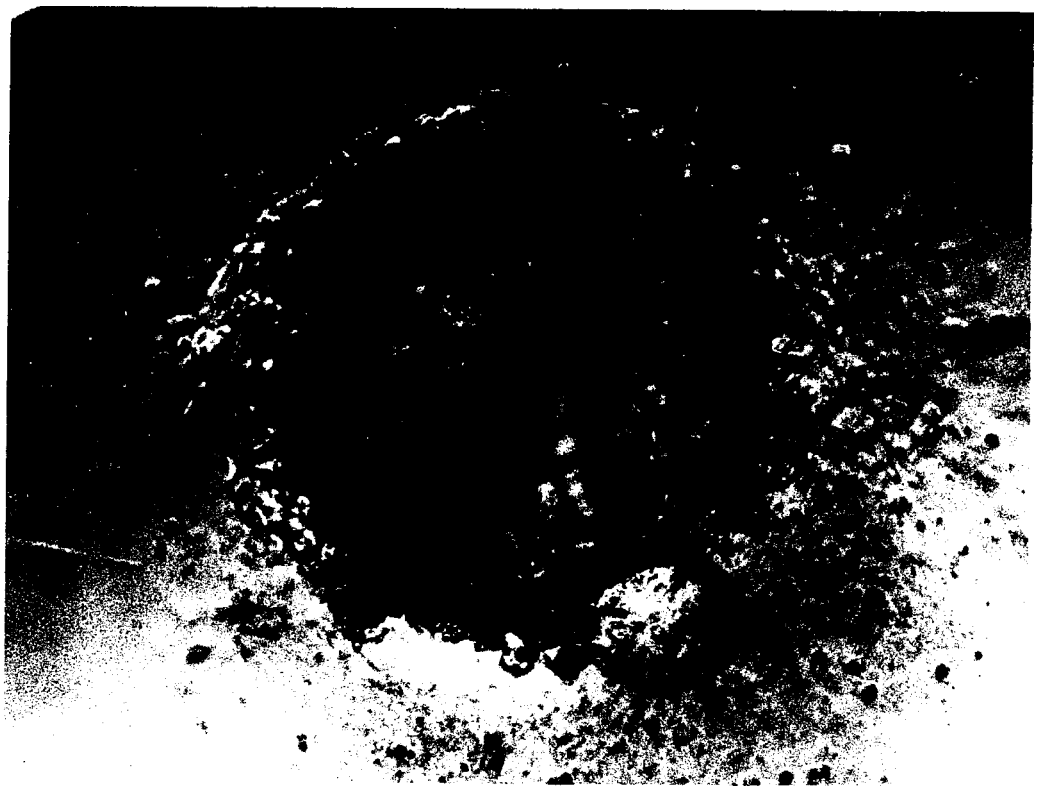
Embryo 2 (ZB-3/85) was recovered as a morula and cultured in Biggers-Whittin-Whittingham (BWW) balanced salt solution supplemented with

FIGURE 2

Embryo 1 (SC-8/82)

A. Morphology 2 days pre-sacrifice. X138.

B. Bilaminar embryonic disc (a)
Amnion (b)
Primary yolk sac (c)
Extraembryonic endoderm (d)
Primitive villus (e)
"Lacuna" (h)
Attachment orientation (p)
X192




d
c
b
h
p

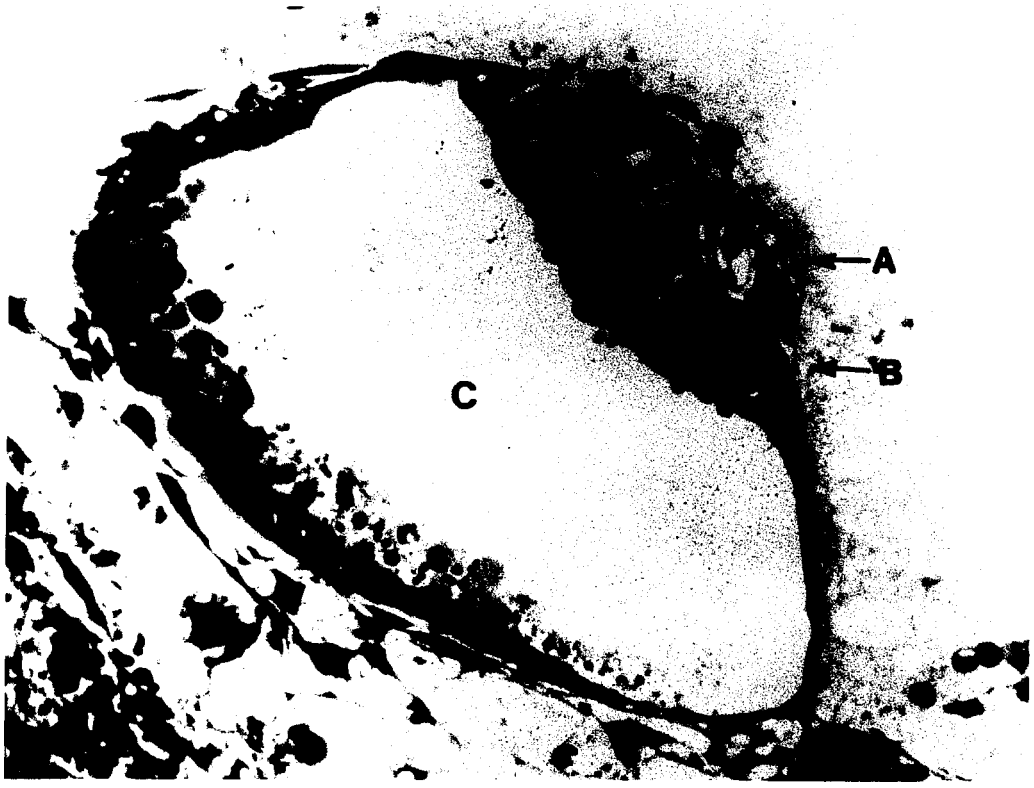
FIGURE 3

Embryo 1 (SC-8/82)

A. Early formation of chorionic villus, illustrating the streaming of cytotrophoblast (a) through a channel in the syncytiotrophoblast (b). A trophoblastic "lacuna" (c) is visible in the upper right. X785.

B. A cross-section of the bilaminar embryonic disc consisting of an endodermal cap (a) and a larger area of columnar epiblast cells (b) from which the wall of the amniotic cavity (c) develops. X524.


C
A
B


A
B
C

0.3% bovine serum albumin (BSA) and 20% HCS filtered through a 0.22 um low protein adsorbing Durapore filter (Millipore). Escape from the zona pellucida occurred on day 5, and the HCS supplement was increased to 40% the following day. The embryo remained floating until day 9, and the next day was switched to basic CMRL medium supplemented with 20% unfiltered normal human male serum. After 12 days in culture, while it still appeared healthy and with good integrity of the differentiated structures, the embryo was fixed in 2% glutaraldehdye.

Histological evaluation confirmed that this embryo had undergone extensive differentiation of the ICM (Fig. 4). Serial sections revealed an organized epiblast layer of cuboidal cells, and an obliquely sectioned amnion. A smaller cavity was present on the distal surface of the embryonic disc, and endodermal strands were present in the blastocyst cavity.

The embryonic structures were eccentrically located with regard to the site of attachment to the plastic substrate. The attachment area was characterized by an absence of central trophoblast, being sealed by a ring of outgrowth surrounding the extraembryonic endodermal layer that formed a continuous covering on the attachment surface.

Embryo 3 (XC 10-84) was recovered as an expanded blastocyst, but did not escape from the zona pellucida until 5 days later. HCS provided the serum supplement throughout culture in a 35 mm Primaria petri dish under mineral oil. Attachment occurred on day 9, with growth and expansion continuing. The embryonic disc was still visible at the time of fixation on culture day 12. The embryo was still extremely flattened and firmly attached to the culture dish at the time of sacrifice.

This embryo had no trophoblast above the attached embryo, so that the blastocyst cavity was lined by a single layer of cells. The trophoblast had

FIGURE 4

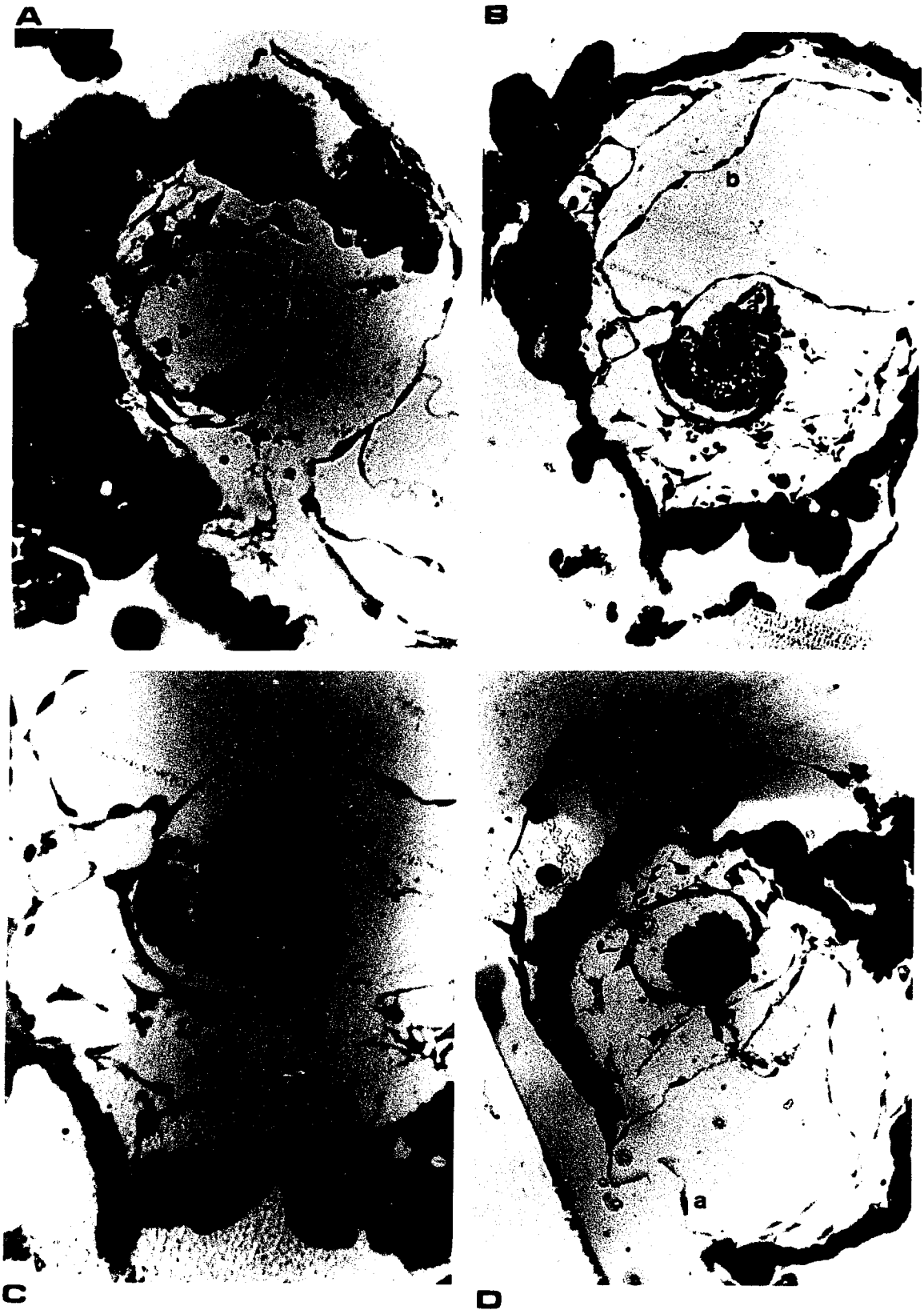
Embryo 2 (ZB-3/85)

A. Embryonic disc (a)
Amnion (b)
X400

B. Possible secondary yolk sac (a)
Endodermal strand (b)
X325

C. Higher magnification of B
X585

D. Endoderm in attachment area (a)
X325


A
B
b
C
D
a

greater thickness in the area of the differentiated ICM (Fig. 5). A definite amnion, pseudostratified epiblast, and loosely arranged hypoblast comprised the embryonic structures. The extraembryonic endoderm was continuous with the hypoblast, and formed the single cell layer covering the embryo. A second cell layer was present between the endoderm and trophoblast.

Embryo 4 (VA 1-85) was recovered as an expanded blastocyst that had temporarily contracted, with a crack in the zona pellucida suggesting it was in the process of hatching at the time of recovery. The embryo hatched the first day in culture, and on day 2 was transferred to a well of an eight-compartment Tissue-Tek glass slide in 400 ul medium with 20% unfiltered HCS. By the time attachment occurred on culture day 6, the ICM was of questionable integrity. Four days later the embryo was fixed in glutaraldehyde.

This embryo was similar to embryo 2 in that it had only peripheral trophoblast at the site of attachment, and a single endoderm layer covered the slide to which the embryo was attached (Fig. 6). The blastocyst cavity was lined with endoderm, and an area of degenerating cells was apparent lateral to the attachment site, that was presumed to be remnants of the embryonic disc. Irregular clumps of indistinct cytoplasm with a small cavity comprised what resembled a small amnion beneath the degenerating embryonic disc.

Embryo 5 (BC 3-85) was recovered at the expanded blastocyst stage and was cultured in 200 ul of BWW supplemented with 10% HCS filtered through a Durapore filter. The initial medium became contaminated with microorganisms, and the embryo contracted on the first day in culture. Fresh medium was prepared and the embryo re-expanded but did not hatch until culture day 4. On the second day the ICM appeared to be dispersed within the blastocyst cavity, as there were focal points with greater cell density scattered around one pole of the blastocyst. The next day (day 3) the ICM cells appeared

FIGURE 5

Embryo 3 (XC-10/84)

A. Embryonic disc in vitro X112

B. Embryonic disc, amnion and trophoblast X72

C. Embryonic disc
Hypoblast (a)
Epiblast (b)
Amnion (c)
X284

D. Continuity of hypoblast with extraembryonic endoderm X284

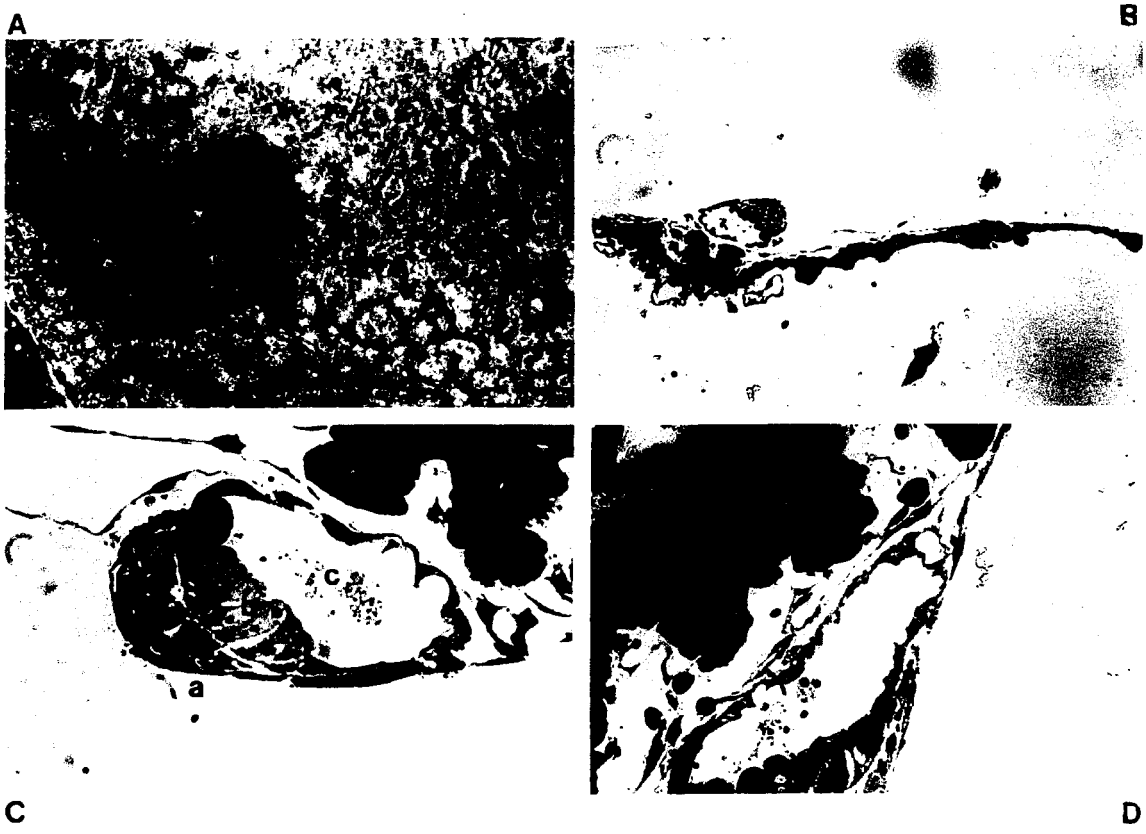

A
B
c
a
C
D

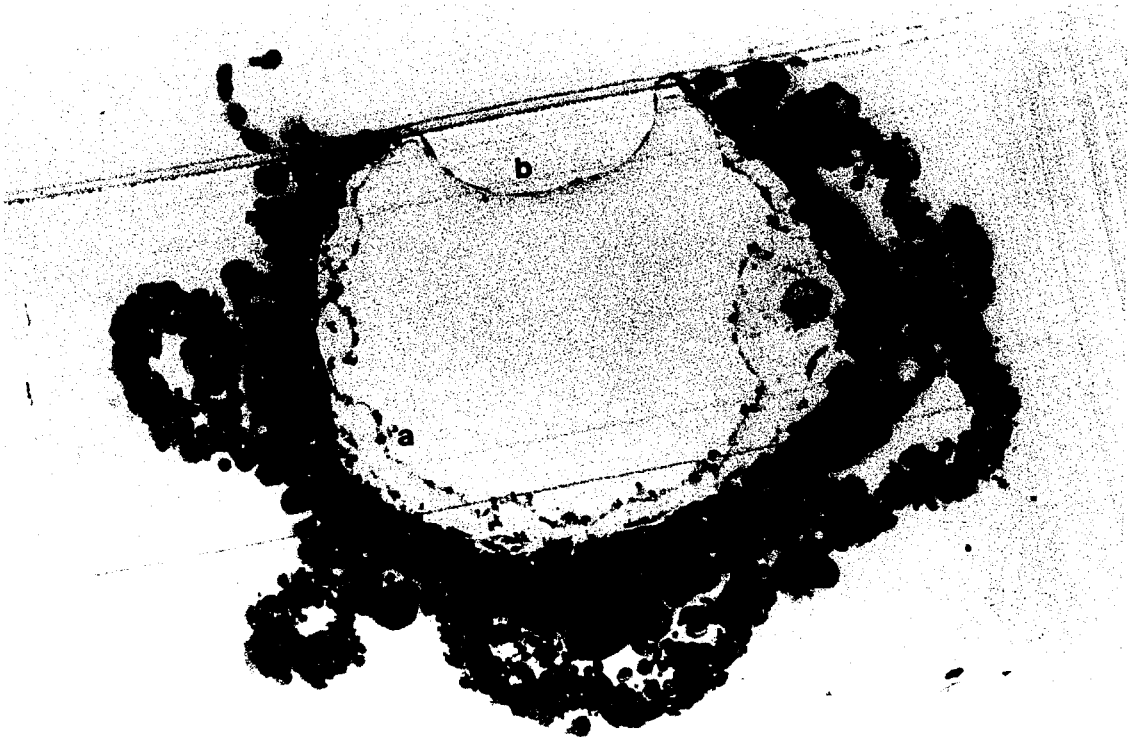
FIGURE 6

Embryo 4 (VA-1/85)

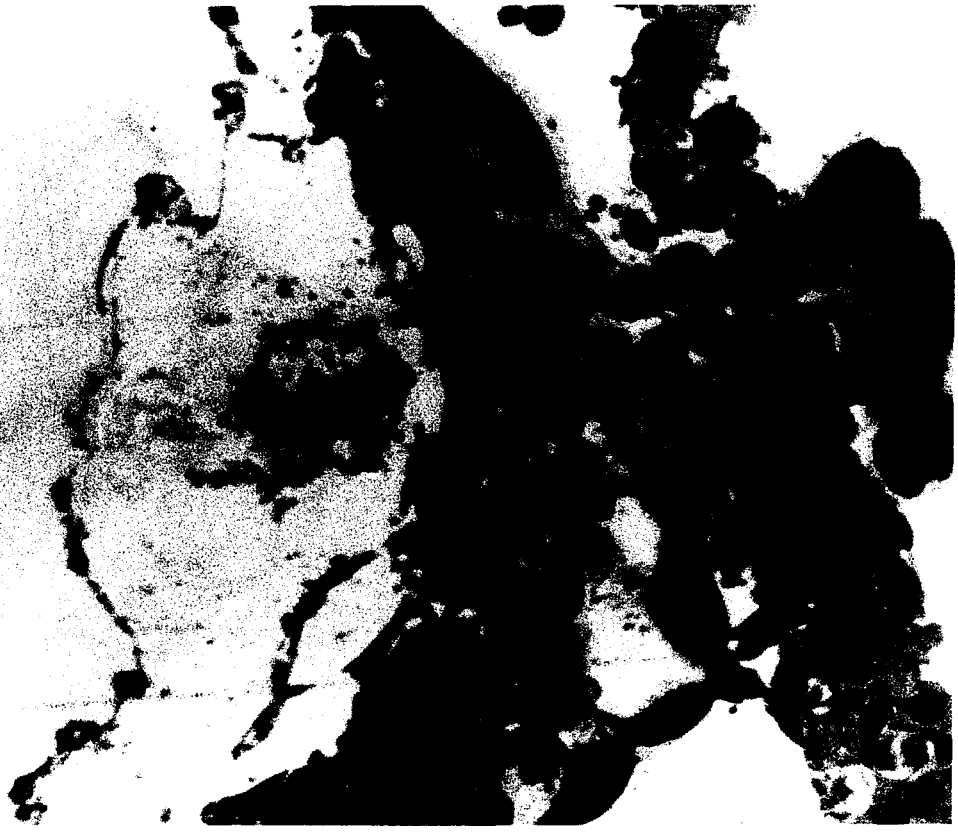
A. Yolk sac endoderm (a)
Attachment site (b)
X178

B. Degenerating embryonic disc (a)
X606

A



B



to be coming together, yet by day 10 the ICM or its derivatives were no longer visible. Following attachment on day 5, the HCS supplement was increased to 20%, and to 40% on day 8. A broad layer of outgrowth was apparent by day 7, but the embryo itself underwent little increase in diameter. On day 10 basic CMRL with 20% baboon serum was used to allow harvesting of medium uncontaminated by human placental proteins. The small embryo became fairly opaque, and was fixed after 15 days in culture. The morphological description of this embryo as a solid ball of cells was upheld by histological evaluation, as no cavity was present throughout the entire embryo (Fig. 7).

Endocrine function in relation to culture environment and morphology. Estradiol levels in spent media varied slightly from the amount available, while P4 levels consistently increased following hatching. When HCS was the serum supplement, at least 20 ng and occasionally 100 ng or more P4 was available (Tables 6-7), while 200 ul of 20% BS-supplemented media usually contained less than 2 ng P4. A single P4 value in spent media from an embryo cultured in HCS-supplemented medium was 460.1 ng, representing a more than 10-fold increase over available P4 (31 ng: Table 6). When BS provided the serum supplement, up to 153 ng P4 was measured in the medium from a single day's culture when less than 0.5 ng P4 was available (Table 8). Progesterone content at the end of culture in medium harvested from eight embryos cultured in HCS medium without oil was between 66 and 582 ng; while when BS was the supplement, P4 content of medium from seven embryos on the last day of culture ranged from 25 to 153 ng.

Seven embryos in HCS-supplemented media without an oil overlay (Groups A-B) had 1456 to 10,583 pg E2 in the culture media harvested from the embryos on the last day of culture (Table 6). Some media samples contained less E2, and some more, than that available in the culture media, although the maximum

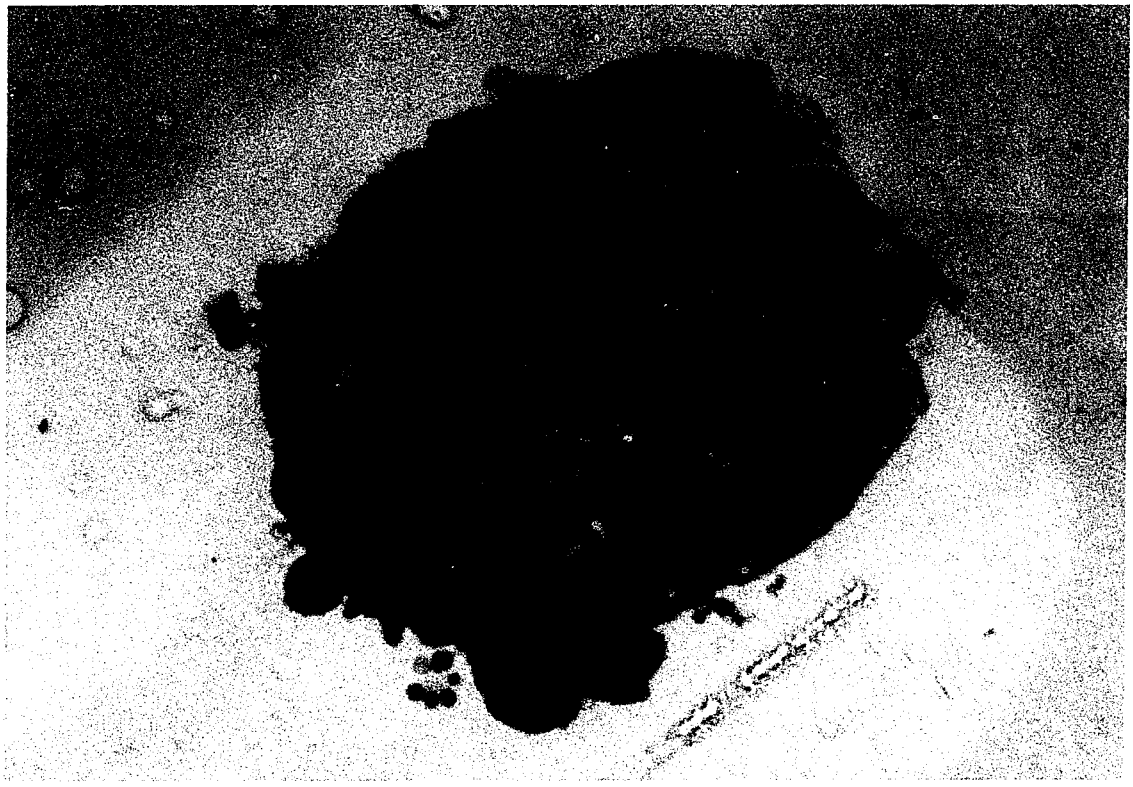
FIGURE 7

Embryo 5 (BC-3/85)

A. Disorganized mass of cells resulting from embryo initially cultured in media contaminated with microorganisms. X303

B. X610

A



B

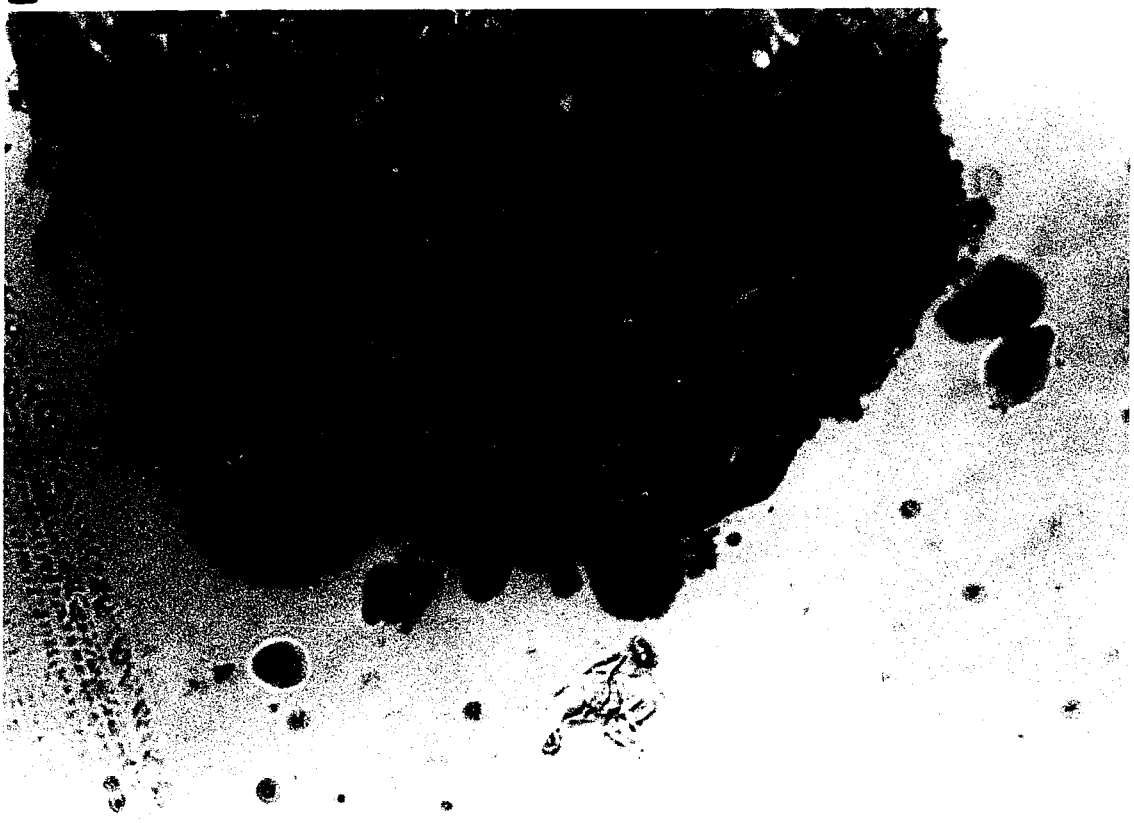


TABLE 6
Group A-B - HCS Only Without Oil

Embryo ID	Rec Stg	Day H[a]	Days C[c]	Diff ID[d]	Media Content on Last CD[e] (E2, P4 available) E2 pg	P4 ng	Days Prechange
VA810727	4	na	16	2	3829= (3550)	243= (72.8)	1
SC810820	2	6	34	10	9135+ (8503)	260- (158.5)	2
KA810828	2	9	18	3	1456+ (500)	103= (8.38)	1
ZB811021	2	4	16	4	2440+ (1623)	460.1= (31.0)	1
W801024	3	6	16	5	3299= (2122)	119.5 (46.23)	2
VA810116	3	5	17	4	2500= (4631)	214- (78.3)	2
QA810121	7	1	12	2	3716= (4631)	189- (78.3)	2
DC810228	7	2	12	2	10683- (4631)	581.7= (54.1)	2

[a] = Day in Culture Hatched from Zona Pellucida

[c] = Total Number of Days Cultured

[d] = Differentiation Score (Table 3)

[e] - = daily compound concentration appears to be decreasing
+ = daily compound concentration appears to be increasing
= = daily compound concentration not changing

Rec Stg = Coded stage at Recovery (Table 2)

TABLE 7
Group C - HCS Serum Supplementation Under Oil

Embryo ID	Rec Stg	Day H[a]	Days C[c]	Diff ID[d]	Media Content on Last CD[e] (E2, P4 available) E2 pg	P4 ng	Days Prechange
RB830806	3	15	18	4	793- (1058)	5.07+ (32.6)	1
CB831014	7	0	11	3	1151 (1058)	11.66+ (32.6)	1
SC831024	3	10	28	4	1355 (839)	4.7= (83.5)	2
XC841024	7	5	12	2	988 (1379)	2.58 (32.4)	1
ZB841130	3	5	20	5	810 (1379)	2.32 (32.4)	4

[a] = Day in Culture Hatched from Zona Pellucida

[c] = Total Number of Days Cultured

[d] = Differentiation Score (Table 3)

[e] - = daily compound concentration appears to be decreasing
+ = daily compound concentration appears to be increasing
= = daily compound concentration not changing

Rec Stg = Coded stage at Recovery (Table 2)

TABLE 8
Group D - HCS to BS Serum Supplementation Without Oil

Embryo ID	Rec Stg	Day H[a]	Day S[b]	Days C[c]	Diff ID[d]	Media Steroid Content on Last CD[e] (E2, P4 available) E2 pg	P4 ng	Days In Sample
VA821226	3	6	14	19	2	49= (<50)	93.6= (0.4)	2
CC830106	4	4	11	14	3	50= (<50)	118 (<0.5)	1
SC830115	7	4	7	23	2	100= (<50)	134+ (<.8)	2
CB830121	4	4	9	17	11	26= (<50)	25+ (<.8)	2
VB830118	8	0	6	12	1	106= (<15)	84.7+ (<.4)	1
NA831208	7	7	9	27	3	118 (<25)	153 (<.4)	1

[a] = Day in Culture Hatched from Zona Pellucida

[b] = Day Serum Source Switched from HCS to BS

[c] = Total Number of Days Cultured

[d] = Differentiation Score (Table 2)

[e] - = daily compound concentration appears to be decreasing
+ = daily compound concentration appears to be increasing
= = daily compound concentration not changing

Rec Stg = Coded stage at Recovery (Table 1)

TABLE 9
Group E - HCS to BS Serum Supplementation Under Oil

Embryo ID	Rec Stg	Day H[a]	Day S[b]	Days C[c]	Diff ID[d]	Media Content on Last CD[e] (E2, P4 available) E2 pg	P4 ng	Days Prechange
XA830428	4	3	11	18	4	77= (<50)	7.2+ (0.84)	1
DB830604	6	3	10	13	2	100= (<25)	24.75= (<.1)	1
CC830605	4	4	9	12	5	88 (<25)	2.0 (<.1)	1
ZB830811	4	5	12	14	2	129= (<10)	7.35+ (<.1)	1
GC830818	3	10	13	19	7	44= (<10)	11.32+ (<.10)	1
CB830909	3	5	11	18	11	106= (<15)	6.35= (<.1)	1
EC830926	4	4	11	18	11	90= (<15)	3.85= (<.1)	1
VA831008	3	5	11	16	11	72 (<15)	9.1 (<.1)	1

[a] = Day in Culture Hatched from Zona Pellucida

[b] = Day Serum Source Switched from HCS to BS

[c] = Total Number of Days Cultured

[d] = Differentiation Score (Table 2)

[e] - = daily compound concentration appears to be decreasing
+ = daily compound concentration appears to be increasing
= = daily compound concentration not changing

Rec Stg = Coded stage at Recovery (Table 1)

variation between available and spent media E2 quantities was two-fold. When the serum supplement for baboon embryos in culture plates was changed to BS after hatching (Group D), E2 was rarely present in measurable quantities in the daily-acquired spent medium samples, and E2 levels were less than 120 pg on the last culture day in seven embryos.

An embryo in Group A (HCS only, no oil) cultured in 1 ml of medium from days 8 through 34 of culture had P4 levels in harvested medium up to five-fold greater than the amount available, while E2 levels were consistently lower in spent than in control medium (Table 10). Development peaked on day 20 of culture, with obvious presence of a bilaminar embryonic disc, although the amnion was never distinctly visible under phase contrast microscopy.

An embryo (ZB-811021) in Group B that failed to undergo differentiation of the ICM during 16 days in vitro in medium supplemented with an HCS pool had over 10-fold greater P4 than that available on the last day of culture (Table 10). The E2 level in spent medium, which had remained similar to that in control medium for the 5 days prior to the end of culture, was increased to 1.5 times that available on the last culture day.

Medium samples obtained daily from a normal embryo in Group D contained less than 60 pg E2 (Table 10). For the 5 remaining days of the 27 day culture period, the embryo was left in a single medium sample that contained 118 pg E2 at the time of harvest. The P4 concentration in spent medium from this embryo remained fairly stable at approximately 100 ng/day during the second week following hatching. This embryo was recovered at the expanded blastocyst stage, but did not hatch from the zona pellucida until after 1 week in culture.

Embryos cultured in HCS-supplemented media under oil (Group C), had a greatly reduced P4 content. The media P4 content at the termination of

Table 10

E2 and P4 in Baboon Embryo Culture Medium

Embryo ID	Gp[a]	Diff[b]	Steroid Avail[c]	Day Post Hatching 3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
SC	A	3	E2	11027						4913	5300	NC	4206	5705	5591	7193	7284	6112
810820			P4	176.1						382.9	469.9		1361	884.7	618.9	471.7		685.4
ZB	B	4	E2	1623					1789	1985	1635	1358	1711	2440				
811021			P4	23.2					34.2	54.4	146.0		427.4	460.1				
RB	C	5	E2	1058		1674	1508	1380	1365	2248	793							
830806			P4	32.6		2.7	1.9	2.1	1.8	2.0	5.1							
NA	D	3	E2	∠ 25	26	∠50	∠50	∠50	∠50	∠50	∠50	55	∠50	∠50	∠50	∠50	∠50	
831208			P4	∠0.4	67.4	65.8	125.9	60.5	47.2	82.9	114.8	144.7	116.0	98.5	91.2	108.2	109.5	
V	E	2	E2	∠15							77	68		75	51	NC	76	56
830923			P4	0.1							2.8		4.2	4.2	2.7		1.8	1.5

[a]Group - See Table 3

[b]Differentiation Score - See Table 2

[c]E2(pg)/P4(ng) in medium

culture on day 11 to 28 for five embryos ranged from 2.32 to 11.65 ng (Table 7). One of two embryos with normally developing embryonic structures had 11.66 ng P4, while the other (embryo 3, section 2) had 2.58 ng P4 in the medium on the final culture day. Occasionally E2 was increased in spent media over than available to embryos cultured under oil (Table 10). Similar levels of E2 and P4 were present when embryonic development was abnormal or normal.

Eight embryos cultured under oil in BS-supplemented media after hatching (Group E) had media P4 content at termination of culture ranging from less than 2.0 to 25.0 ng/day (Table 9). One embryo had P4 concentrations between 1.5 to 4.2 ng and E2 levels of approximately 50 to 77 pg on days 13 through 21 of culture, 9 to 17 days posthatching. Both compounds were present in greater quantity than that available in the culture medium. This embryo had undergone normal differentiation of the embryonic disc and amnion by culture day 12, but when culture was terminated on day 21 these structures had degenerated and were no longer visible under phase contrast microscopy.

When HCS-supplemented medium samples alone were cultured under oil (Table 11), E2 levels in the first five samples acquired from daily changes were 47.3 to 84.9% of the original amount available in the culture medium. Concentrations of E2 in the remaining samples, 7 to 15 days after initiation of the medium incubation under oil, ranged from 68.3% of initial E2 on day 7 to 97.4% on day 15. P4 levels in the first 5 days were less than 4.0 to 4.6% of the concentration in the complete medium; thereafter they ranged from 4.0 to 8.3%. In the second experiment three samples cultured for 2 days had 46.9% to 57.9% E2 compared to the original value, and P4 was reduced to 0.6 to 0.8% of its original concentration.

Spent media harvested from several embryos in all environments on the first few days after hatching contained increased quantities of E2. One embryo

TABLE 11

Effect of Incubating CMRL-1066 Media with 20% HCS
Under Mineral Oil

Day in Culture	E2 pg/0.2 ml	%	P4 ng/0.2 ml	%
STUDY 1:				
0	1878	100	25.4	100
0-1	933	49.7	<1.0	<4.0
1-2	888	47.3	ca. 1.0	4.0
2-3	1167	62.1	ca. 1.0	<4.0
3-4	1513	80.6	1.2	4.6
4-5	1594	84.9		
5-7	1283	68.3	1.37	5.4
7-8	1225	65.2	1.02	4.0
8-11	1609	85.7	1.74	6.9
11-13	1692	90.1	1.50	5.9
13-15	1830	97.4	2.10	8.3
STUDY 2:				
0	1462	100	25.11	100
0-2	811	55.5	0.21	0.8
2-4	686	46.9	0.15	0.6
4-6	847	57.9	0.20	0.8

was one-half in the zona on the second day of culture, and did not completely escape until 2 days later (Fig. 8). The P4 levels at the time of hatching were usually less than or equal to that in the unused medium (Fig. 9).

Discussion

The extent and frequency of postimplantation development reached by baboon embryos *in vitro* has not been achieved with any other primate species. Fishel *et al.* (1984) described two human embryos developing for up to 13 days post-insemination of the laparoscopically recovered oocytes. Following acid removal of the zonae pellucidae 5 to 6 days post-insemination, secretory trophoblast cells were described for one embryo at 7 days, with visible endoderm at approximately 8 days post-insemination.

Attachment of the human embryo to the petri dish occurred at 9 days, with "fibroblastic cells migrating from the inner cell mass" at 10 days. At 13 days, after undergoing extensive outgrowth, the tissue collapsed and was fixed for analysis. The results of this analysis are as yet unreported, but the embryonic organization was lost as the outgrowth formed in the early post-attachment stages (Fishel *et al.*, 1984). This human embryo secreted up to 4500 mIU hCG into the culture medium (Earle's medium supplemented with 15% maternal serum, under oil) the day before it was taken out of culture.

Other investigators have also achieved postimplantation development of primate embryos without evidence of integrity of embryonic structures. Morgan *et al.* (1984) fertilized rhesus monkey oocytes *in vitro* and cultured them in CMRL medium supplemented with 20% HCS. Development occurred for up to 9 days posthatching, or up to 20 days post-insemination. Although the

FIGURE 8

Estradiol (E2) and Progesterone (P4) in Medium from Cultured Baboon Embryos:

Legend:

● = E2 (% of Available)

o = P4 (% of Available)

↘ = Ng E2 Available

H = Day hatched in vitro

↓ = Day attached in vitro

Figure 8

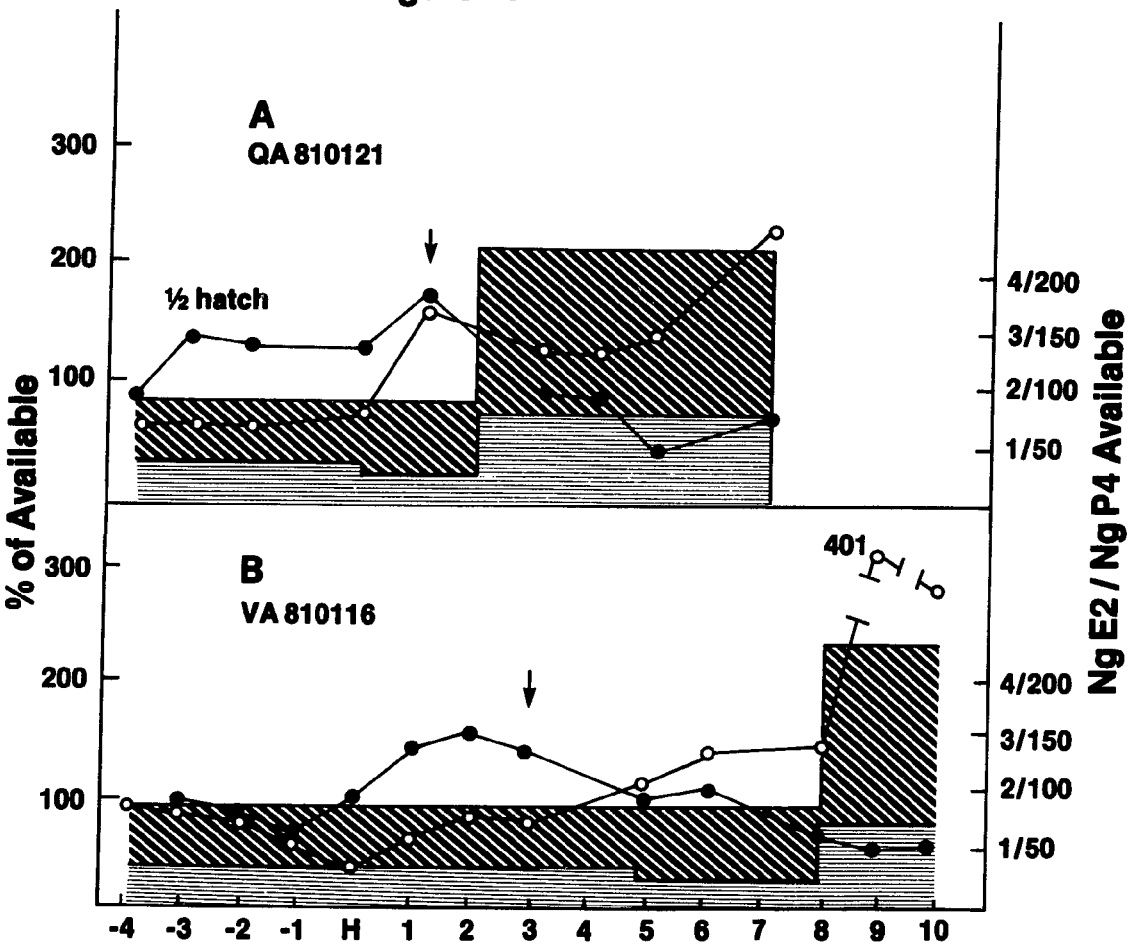

A
QA 810121
½ hatch
300
200
100
B
VA 810116
401
300
200
100
% of Available
4/200
3/150
2/100
1/50
Ng E2 / Ng P4 Available
-4 -3 -2 -1 H 1 2 3 4 5 6 7 8 9 10

FIGURE 9

Estradiol (E2) and Progesterone (P4) in Medium from Cultured Baboon Embryos:

Legend:

● = E2 (% of Available)

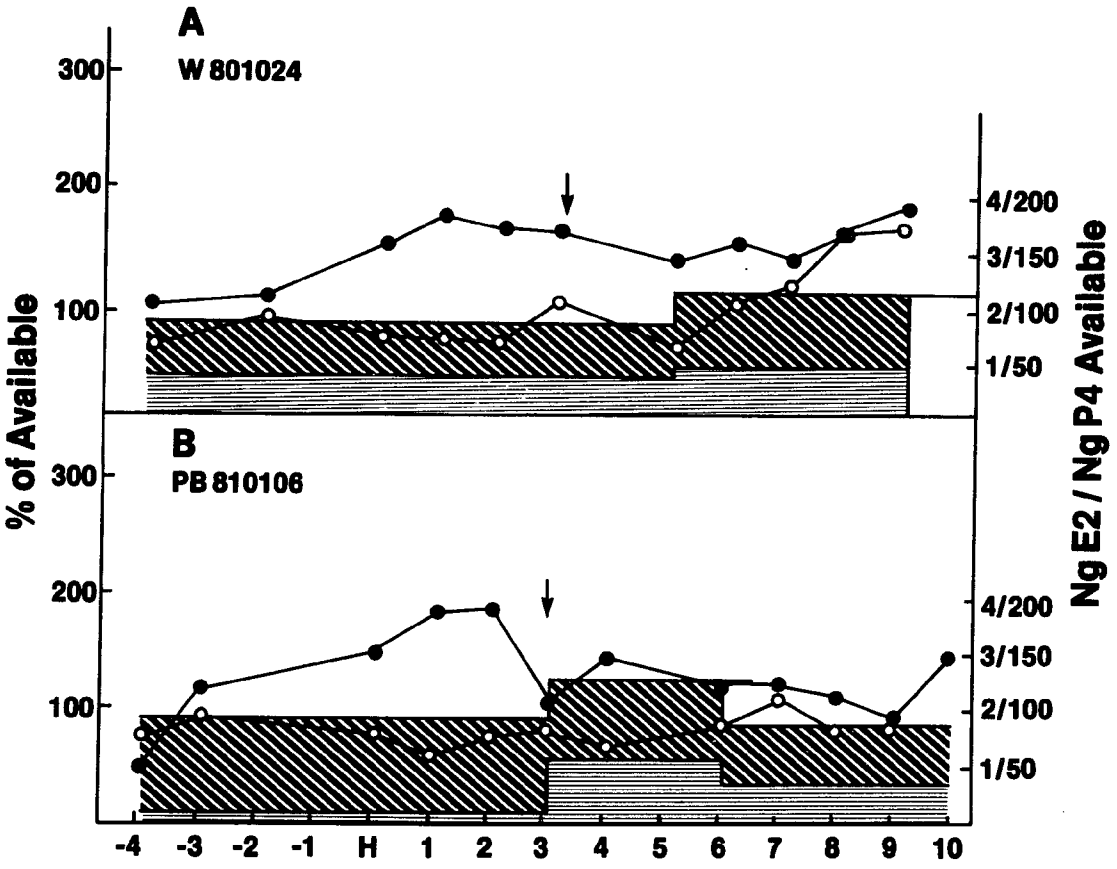
o = P4 (% of Available)

↖ = Ng E2 Available

H = Day hatched in vitro

↓ = Day attached in vitro

Figure 9


A
W 801024
300
200
100
B
PB 810106
300
200
100
% of Available
4/200
3/150
2/100
1/50
Ng E2 / Ng P4 Available
-4 -3 -2 -1 H 1 2 3 4 5 6 7 8 9 10

number of embryos achieving these posthatching stages was not reported, it was mentioned that a total of four embryos hatched from the zona pellucida.

Summers et al. (1984) report postattachment development of marmoset preimplantation embryos, with the blastocysts collapsing and inner cell mass derivatives degenerating 4 to 7 days after attachment to the petri dish. Trophoblast subcultures were made by cutting the 1.5 to 3.0 mm diameter tissue into two to eight pieces and dividing the pieces among new dishes. Pieces composed only of syncytiotrophoblast failed to develop. Cell lines were successfully established from three of 13 embryos, with viability in one line continuing for an excess of 16 months. The cell lines continued to secrete "abundant quantities" of chorionic gonadotropin when subcultured using this procedure. Trypsinization was ineffective at establishing functional cell lines, which has been our limited experience with baboon embryo trophoblast in vitro (Pope et al., 1982b). The mechanical propogation method of Summers et al. (1984), therefore, appears to have tremendous potential as a means of maintaining trophoblast function in vitro.

A model for evaluating trophoblast function in the absence of embryonic tissue is indeed useful; however, this development should not detract from the importance of continued efforts to determine the environment necessary for the normal differentiation of embryonic structures. In the present study, baboon embryos 1 and 3 appear to have progressed to the equivalent of stage 5B to 5C of human development at 11 to 12 days of gestation (O'Rahilly, 1973). Estimation of equivalence to in vivo development in the baboon is made difficult by the paucity of available material. An amniotic cavity was not yet formed in a single day 9 baboon embryo fixed in situ in one study, but was present by day 10 to 15 (Hendrickx and Houston, 1971). The remaining description of this time period of development in the baboon is not sufficiently

complete, however, to allow an accurate comparison with the in vitro development achieved in this study.

The arrangement of the extraembryonic endoderm in embryo 2, and the formation of a discrete small cavity beneath the embryonic disc, suggests that this embryo may have reached stage 6, with development of the secondary, or definitive, yolk sac. This occurs late on day 12 in the rhesus monkey. In ape and human embryos a meshwork of extraembryonic endoderm fills the primary yolk sac (Heuser, 1940; Luckett, 1978), but this phenomenon has not been described for monkey embryos. Although there is a similarity between the endodermal strands in embryo 2 with this meshwork, its absence in monkey embryos in vivo supports the conclusion that this embryo possesses a secondary yolk sac. In monkeys, the primary
yolk sac persists for several days after formation of the secondary yolk sac, and vesiculation of the primary yolk sac endoderm has been described (Luckett, 1978) similar to the strands in embryo 2.

It was recently reported that there is an increase in uterovarian vein androgens beginning on day 10 of pregnancy in the baboon (Hodges et al., 1984), which prompted our switching this embryo to medium supplemented with human male serum on the last 2 days of culture. The embryonic development achieved suggests that further evaluation of media supplemented with human male serum alone or in combination with serum from other sources may be warranted.

The bilaminar embryonic disc evident in embryos 1, 2 and 3 consists of endoderm or hypoblast fitting the description of human embryonic endoderm by O'Rahilly (1973) and Hamilton et al. (1943) as comprising a cap-like mass of darkly-staining, cuboidal, vesiculated and vacuolated cells without any specific arrangement. In nonhuman primates in vivo, these loosely arranged cells

proliferate on days 10 and 11, but do not become organized until day 16 (Hendrickx, 1971).

The epiblast in these embryos is a pseudostratified layer of columnar cells similar to that described for other primates (Heuser, 1940; Hendrickx, 1971; O'Rahilly, 1973). In monkeys a transition is made from cuboidal to columnar on day 11 (Hendrickx, 1971). In human embryos in vivo, the epiblast is convex into the amniotic cavity (Hamilton et al., 1943) until the early primitive streak stages when it assumes a concave appearance (Luckett, 1975).

The apparent differentiation of syncytial trophoblast from cytotrophoblast was accompanied by protrusions of cytotrophoblast through the syncytial layer similar to the early chorionic villi described for human embryos in vivo (O'Rahilly, 1973; Hamilton et al., 1943). Structures reminiscent of lacunae were present in the trophoblastic labyrinthe; contradicting theories of a maternal requirement for lacunae formation. The squamous endothelial lining of the trophoblast remained intact even in situations without further differentiation of the embryonic disc, or where degeneration of the embryonic disc had occurred. Although Hertig and Rock (1944) noted that trophoblast developed only where it had adequate contact with maternal tissue, baboon embryos in vitro usually develop a trophoblast shell of apparent randomly varying thickness around the entire embryo. The stimulus for trophoblast differentiation and proliferation, therefore, remains to be determined.

Spherical embryos 2 and 4 had no trophoblast at the central attachment area, which was covered by a single layer of extraembryonic endoderm. The periphery of the attachment site, as well as the rest of the embryo, was covered by a thick layer of trophoblast. Attachment was not at the embryonic

pole, as the embryonic structures were lateral to the attachment site in both embryos.

Embryo 3 also lacked circumferential trophoblast development; in fact, this embryo was essentially two-dimensional. A single cell layer provided the uppermost boundary of the entire embryo, and the blastocyst surface lacked the thickness usually seen at this stage in vitro regardless of the integrity of the embryonic structures. Mouse embryos in vitro undergo protrusion of the embryo through gaps in the trophoblast created as the mural trophoblast spreads upon the culture dish (Gonda and Hsu, 1980). The trophoblast of baboon embryo 3 may have had a similar affinity for the plastic substrate, also resulting in gaps in the trophoblast through which the yolk sac protruded. Alternatively, attachment may have been inverted, with the amnion directed away from the attachment site, and endoderm separating the embryo from the culture dish as seen in embryos 2 and 4. Embryo 3 became dislodged during processing, and it was not obvious from the serial sections whether attachment was oriented from the trophoblast or endoderm side of the flattened embryo. This embryo was included in an initial evaluation of the Primaria dishes, which are a modified plastic with the polymer surface simulating collagen I, IV and polylysine to enhance cellular attachment and growth.

These presumed deviations from normal development perhaps reflect inadequacies in the culture environment, or the development of barriers to nutrient transport that inhibit cell growth in an area of trophoblast isolated from direct contact with the culture media. Embryo 4 had undergone degeneration of the embryonic disc while trophoblast development continued. Death of embryonic tissues was apparent from morphological evaluations made during culture, and could be readily distinguished from situations where the ICM failed to differentiate.

Formation of a solid ball of cells (Embryo 5) was not a common occurrence during culture, although determination of actual cavity obliteration instead of extremely thick trophoblast growth requires histological verification. Coincidentally, the previous embryo from this donor assumed a similar morphology in vitro.

Abnormalities described for primate embryos in vivo are not dissimilar to those seen in baboon embryos in vitro. One of two early implantation chimpanzee specimens available was an abnormal day 12 embryo measuring 270 um in diameter (vs 720 um diameter of the normal 10.5 day embryo). The cytotrophoblast was vesiculated with distended nuclei, but the syncytium appeared normal. Embryonic differentiation of the inner cell mass had not occurred. Hertig and Rock (1944) evaluated a series of five abnormal human embryos included in their 12 specimens ranging in age from 7 to 14 days. Three of the five abnormal embryos had normal embryonic structures with placental defects, one had a 'mesoblast deficiency,' and one lacked differentiation of the ICM. A later series (Rock and Hertig, 1948) of 26 embryos revealed 12 (47%) with abnormalities, including three with undifferentiated cleavage. Of implanted embryos, two consisted of only trophoblast, and one had a defective embryo. Two had embryonic and trophoblast abnormalities, and four had normal embryos with defective 'accessory structures.'

The rates of abnormal embryonic development in the present study when baboon embryos were cultured with and without an oil overlay were not significantly different. The ratios of normal to abnormal embryonic development in embryos cultured with and without oil, however, were 0.52 and 1.22, respectively. This reduction in normal embryonic differentiation of embryos cultured under oil could be due to removal of essential factors with greater solubility in organic than aqueous solvents. Further efforts to define

the requirements for embryonic differentiation might involve specific evaluation of oil soluble compounds, such as the fat soluble vitamins.

The reduced medium steroid content in embryos cultured under oil is assumed to be due to diffusion of media steroids into the oil, as steroid content was also reduced in control media incubated under oil (Table 11). This reduction was less dramatic with time, suggesting an equilibrium may be established between the culture medium and the oil. The source of steroids in spent embryo culture media under oil, therefore, cannot be defined by the results of this study. Diffusion of previously sequestered E2 and P4 from the oil into the medium could occur concomitantly with diffusion of steroids secreted by the embryos into the oil from the culture medium.

A comparison of the range of available E2 and P4 in the serum supplement and the normality of embryonic differentiation fails to reveal a direct relationship between availability of these steroids and inner cell mass development. Additionally, embryonic contribution to media E2 and P4 appears to be independent of embryo normality. The P4 in media from embryos in culture plates can be assumed to be of embryonic origin, and the quantities attained suggest a need for investigation of the relationship of P4 production to other criteria during early implantation development. Carson et al. (1982) concluded that postimplantation embryonic progestins in mice are in some functional capacity related to maternal biochemical communication, since inhibition of progestin sythesis in vitro did not decrease the integrity of either trophoblast or embryonic differentiation.

The increase in E2 in relation to the amount available around the time of hatching is consistent with the hypothesis that blastocyst estrogen may have a role in implantation (Dickmann et al., 1976). Sherman and Atienza (1977), however, concluded that steroid biosynthesis by mouse embryos is not involved

in implantation, when delta-5-3-beta-hydroxysteroid dehydrogenase activity could not be detected until after attachment in cultured blastocysts.

Borland et al. (1977) demonstrated P4 and E2 in rabbit blastocyst fluid if the steroid was present in the uterine luminal secretions. A maternal source for the steroids was therefore assumed. Baboon embryos in vitro may also be accumulating exogenous E2, to be released around the time of hatching, as seen in the present study. In vitro, total media removal is not effected with each change, and actual volume recovered is not determined with precision. Steroid levels less than 100% of available could reflect, therefore, this lack of complete media harvesting and/or steroid uptake by the embryo. The latter could be assumed when E2 and P4 are not present in harvested media at the same ratio, although production and secretion of steroids by the embryo could also affect their concentration and ratio. Experiments using radiolabeled steroids and precursors would assist in defining the steroid production and secretion capabilities of early baboon embryos.

In summary, postimplantation differentiation of ICM occurs in vitro when baboon embryos are cultured with medium supplemented with HCS or BS. Although hatching occurs more frequently when embryos are cultured under oil, a nonsignificant reduction in normality of embryonic structures occurs in posthatching embryos cultured under oil. Increased E2 levels in spent media around the time of hatching suggest a release of estrogen by the embryo; radiolabeling experiments could possibly resolve the validity of this assumption as well as the source of the steroid. The levels of P4 in spent media indicate secretion and imply synthesis by the embryo.

The histological descriptions of embryos 1 through 5 corroborate the accuracy of the morphological descriptions made under phase contrast microscopy, and indicate that the culture systems being evaluated are capable

of supporting development approaching at least the equivalent of day 13 in vivo. It is presumed that the termination of development in vitro is due to limitations of the culture environment. Attempts to further increase the extent and rate of postimplantation development should focus on indentifying specific media constituents necessary for maximum normal differentiation. Considering the inefficiency of natural development, primate embryos in vitro should provide experimental potential similar to those established for other species.

Acknowledgements

The authors would like to thank Dr. Mel Kunkle for performing the histology on Embryo 1.

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CHAPTER VIII

SUMMARY

The results of these investigations indicate that baboon embryos *in vitro* are a useful model for studying early implantation development in primates. Nonsurgical embryo recovery is the most economically feasible method of acquiring baboon embryos, and results obtained from all species studied indicate that embryos fertilized *in vivo* have a better potential for development *in vitro* than do oocytes fertilized *in vitro*.

The demonstration that bCG and SP1 are secreted by baboon embryos provides the rationale for studying other placental proteins *in vitro*. Dr. Hans Bohn (Behring-Werke, West Germany) has provided antisera and standard for two additional proteins, PP11 and PP12, making them a logical next choice for study. The fact that functional differentiation occurs to the extent of secretion of these proteins is significant, but the opportunity exists to further evaluate temporal and biochemical relationships of these proteins to embryonic development. It would be interesting to employ the method used by Summers *et al.* (1984) to propagate marmoset trophoblast to determine if baboon embryos will likewise continue to secrete placental proteins for extended periods *in vitro*. This would be a desirable method for acquiring material for purification and characterization studies.

The initial demonstration of steroid synthesis by primate embryos in vitro will allow more detailed, planned evaluations of relationships between E2 and P4 levels and all aspects of trophoblast and embryonic differentiation, including histology and secretory function. Studies with labelled precursors might define at what stage synthesis begins, and what precursors must actually be exogenously provided at each stage. Additionally, it would be useful to identify a component in the serum supplement or a media additive that is metabolically inactive when exposed to baboon embryos and that can be efficiently and accurately quantitated to provide a base to which harvested media volumes and component quantities can be adjusted.

Pursuant to our interest in establishing a relationship among the secretory activities for E2, P4, SP-1, and bCG and the in vitro development achieved by baboon embryos, preliminary comparisons have been made of the levels of these compounds in spent media samples (Appendix 1). The information currently available suggests that chorionic gonadotropin secretion was not affected by culture under oil. Removal of compounds more readily soluble in oil than aqueous media did not, therefore, appear to affect the ability of trophoblast to produce and secrete this hormone. Additionally, a firm attachment to the culture dish was not required for unit levels of bCG secretion, since embryos cultured under oil were easily dislodged from the petri dish.

There was a tendency toward decreased SP1 levels in media obtained from embryos cultured under oil. Embryo 3 (XC841024: Chapter VII), unlike most embryo under oil, attained a firm attachment to the petri dish that resisted release even through the addition of fixative and dehydration reagents in preparation for histological evaluation. This embryo, like others under oil, secreted little SP1 into the culture medium. Measurable SP1 was present only on the last 3 days in culture, with a peak of only 112 ng.

No association between SP1 and steroid levels has been determined from this data, although steroid, especially P4, levels were also lower in embryos cultured under oil. One proposed function of SP1, based on preliminary evaluation of immune precipitates, is steroid binding (Bohn and Kranz, 1973; and Bohn, 1974; both as cited by Bishof, 1984), but the current data do not provide support for this theory. Additionally, an association between SP1 secretion and normality of embryonic development cannot be inferred from this preliminary comparison, as the embryo secreting the greatest quantity of SP1 (4800 ng/day) failed to undergo differentiation of the embryonic disc, and low SP1 levels (a peak of 100 ng/day) were measured in an embryo undergoing normal bilaminar embryonic disc development.

It is unfortunate that more embryos were not subjected to histological evaluation. The material that has been processed to date suffers mainly from a lack of technical experience, yet is sufficient to prove that embryonic, and not just trophoblastic, differentiation does occur in baboon embryos in vitro. Now that the initial experience has been acquired, all embryos will be fixed at the termination of culture, to prevent a recurrence of the loss of almost 100 post-implantation specimens that occurred in the initial years of the culture system development.

The rapidity with which accomplishments have been made with baboon embryo culture is particularly interesting in view of the relatively small number of embryos involved. There are still several laboratory species, in fact, for which posthatching development in vitro is currently unobtainable (eg rat, hamster, rabbit).

In summary, the noninvasive method for acquiring cleavage stage baboon embryos and the culture method capable of supporting development through attachment stages in vitro should continue to provide useful information on

periimplantation development in primates. If additional necropsy specimens are evaluated in other laboratories, an excellent basis will exist for further histological comparisons of in vitro with in vivo development. More post-implantation embryos per baboon can be studied using our culture methods instead of sacrificing valuable animals, enhancing the practicality of primate embryo culture from a conservational point of view. Attempts to develop regimens for superovulation induction in the baboon have been unsuccessful, which is not too disappointing in light of the refractoriness that quickly develops in rhesus monkeys stimulated to produce multiple embryos or mature oocytes (B. Bavister, personal communication).

The observations made thus far on the morphology, histology, and secretory capabilities of cultured baboon embryos provide the rationale for a more thorough characterization of the secretion pattern of these and additional compounds, particularly in relation to normal embryonic development. The recent demonstration that baboon embryos can be successfully frozen should further enhance the utility of the model (Pope et al., 1984). Length of storage of embryos at -196^{o} C does not affect viability, so sibling groups could be formed for use in controlled experiments on the effects of various environmental treatments on the normality of growth and differentiation.

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APPENDIX

The following data are included to allow comparison of the four compounds that have been measured in spent media from several embryos. Appendix Tables 1 through 9 include daily levels of bCG, SP-1, E2 and P4 in spent media from embryos in the culture conditions outlined in Chapter 7. Tables 10 through 13 repeat the steroid levels in the media at the end of culture for several embryos in the various culture groups, with SP-1 and bCG levels as well.

Appendix Table 1

E2,P4,bCG and SP1 for Embryo SC810820
Group A (HCS)
Recovered at 12-Cell Stage
Differentiation Score = 3 (H,A,YSE,ED)

Culture Day	PH	Avail E2 (pg)	E2 (pg)	Avail P4 (ng)	P4 (ng)	bCG (mIU)	SP1 (ng)
11	5	5378	3902	94.8	74.1	171	< 125
12	6		4409		162.6	813	< 125
13	7		4660		203.0	1908	< 125
14	8		3947		214.3	3761	195
15	9	11027	4913	176.1	382.9	9300	614
16	10		5300		469.9	12604	
17	11		---		---	---	--
18	12		4206		1361	24283	2060
19	13		5705		884.7	15161	1300
20	14		5591		618.9	10503	1881
21	15		7193		471.7	7503	2273
22	16		7284			10540	1614
23	17		6112		685.4	9588	1470

Appendix Table 2

E2, P4, bCG and SP1 for Embryo KA810828
Group A (HCS)
8-Cell at Recovery
Differentiation Score = 3 (H,A,YSE,ED)

Culture Day	PH	Avail E2 (pg)	E2 (pg)	Avail P4 (ng)	P4 (ng)	bCG (mIU)	SP1 (ng)
2	-8	1076	893	19.0	20.3	< 40	< 25
3	-7		946		19.5	< 40	< 25
4	-6		904		22.9	< 40	< 25
5	-5		642		23.1	< 40	< 25
6	-4		1504		17.4	< 40	< 25
7	-3		624		14.6	< 40	< 25
8	-2	2205	1548	35.2	26.1	< 40	< 25
9	-1		---		---	---	--
10	0		1807		26.7	< 40	< 25
11	1		1905		24.5	< 40	< 25
12	2		3280		35.6		< 25
13	3		2302			212	< 25
14	4		1291		50.0	905	< 25
15	5		994			2284	< 25
16	6		---		---	---	-
17	7	2500	878	41.9	199.8	4509.5	101
18	8		1456		103.1		35

Appendix Table 3

E2,P4,bCG and SP1 for Embryo ZB811021
Group B (HCS Pool)
12-Cell at Recovery
Differentiation Score = 4 (H,A,YSE - Blighted)

Culture Day	PH	Avail E2 (pg)	E2 (pg)	Avail P4 (ng)	P4 (ng)	bCG (mIU)	SP1 (ng)
11	7	1623	1789	23.2	34.2	<500	<12.5
12	8		1985		54.4	716	101
13	9		1635		146.0	2122	852
14	10		1358			2946	1399
15	11		1711		427.4		2805
16	12		2440		460.1	539	4800

Appendix Table 4

E2, P4, bCG and SP1 for Embryo XC841024
Group C (HCS Under Oil)
Differentiation Score = 2 (H,A,YSE,ED,AC)

Culture Day	PH	Avail E2 (pg)	E2 (pg)	Avail P4 (ng)	P4 (ng)	bCG (mIU)	SP1 (ng)
2	-3	1379	544	32.4	< 1.0	< 50	
3	-2		840		< 1.0	< 50	
4	-1		---		---	---	--
5	0		1120		< 2.0	< 50	< 25
6	1		---		---	---	--
7	2		1308		< 2.0	< 20	< 25
8	3		730		< 0.4	< 20	< 25
9	4		657		< 0.4	< 50	< 50
10	5		877		.162	128	112
11	6		981		0.772	644	27
12	7		988		2.583	1234	30

Appendix Table 5

E2, P4, bCG and SP1 for Embryo RB830806
Group C (HCS Under Oil)
Recovered at 16-20 Cell Stage
Differentiation Score = 5 (H,A,No Organized YSE)

Culture Day	PH	Avail E2 (pg)	E2 (pg)	Avail P4 (ng)	P4 (ng)	bCG (mIU)	SP1 (ng)
6	-3	1150	1313	16.27	1.65	< 50	< 25
7	-2		1732		2.14	< 50	< 25
8	-1		1146		1.05	< 50	< 25
9	0		1311		1.45	< 50	< 25
10	1		1588		1.31	< 20	< 25
11	2		1034		< 1.0		< 25
12	3		1729		1.75	< 50	< 25
13	4*	1058	1674	32.6	2.70	< 50	< 25
14	5		1508		1.90	< 50	< 25
15	6		1380		2.12	20	< 25
16	7		1365		1.81	80	< 25
17	8		2248		2.03	320	< 25
18	9		793		5.07		< 25

Appendix Table 6

E2, P4, bCG and SP-1 in NA831208
Group D (HCS to BS)
Expanded Blastocyst at Recovery
Differentiation = 3 (H,A,YSE,ED)

Culture Day	PH	Avail E2 (pg)	E2 (pg)	Avail P4 (ng)	P4 (ng)	bCG (mIU)	SP1 (ng)
6	-1	950	833	28.4	26.3	< 25	< 25
7	0		805		26.9	79	< 25
8	1	1014	973	31.4	54.1	570	68
9	2	< 25	< 50	< 0.4	34.3	570	68
10	3		26		67.4	1527	
11	4		< 50		65.8	1203	120
12	5		< 50		125.9	1791	141
13	6	< 25	< 50	< 0.4	60.5	414	69
14	7		< 50		47.2	452	78
15	8		< 50		82.9	887	224
16	9		< 50		114.8	1035	195
17	10		55		144.7	827	213
18	11		< 50		116.0	839	226
19	12		< 50		98.5	587	175
20	13		< 50		91.2	508	191
21	14		< 50		108.2	340	217
22	15		< 50		109.5	222	262

Appendix Table 7

E2,P4,bCG and SP1 for Embryo VB830118
Group D (HCS to BS)
Recovered at Hatched Blastocyst Stage
Differentiation Score = 2 (H,A,YSE,ED,AC)

Culture Day	PH	Avail E2 (pg)	E2 (pg)	Avail P4 (ng)	P4 (ng)	bCG (mIU)	SP1 (ng)
6	NA	<25	<100	<0.4	7.32	702	<50
7			<100		10.4	1032	<50
8			<100		32.1	4986	70
9					54.6	3075	96
10					66.3	2109	104
11					89.7	5209	161

Appendix Table 8

E2, P4, bCG and SP1 for Embryo CB830121
Group D (HCS to BS)
Recovered at Morula Stage
Differentiation Score = 11 (H,No A, YSE)

Culture Day	PH	Avail E2 (pg)	E2 (pg)	Avail P4 (ng)	P4 (ng)	bCG (mIU)	SP1 (ng)
9	5		1298		22.9	223	< 75
10	6		285		11.6	778	< 75
11	7		<100		14.8	1597	< 75
12	8		---		---	---	--
13	9		<100		57.0	3575	131
14	10		<100			1446	60
15	11		<100		10.6	672	70
16	12		---		---	---	--
17	13		26		24.9	1700	170

Appendix Table 9

E2,P4,bCG and SP1 for Embryo V830923
Group E (HCS to BS Under Oil)
Recovered at Early Blastocyst Stage
Differentiation Score = 2 (H,A,YSE,ED,AC)

Culture Day	PH	Avail E2 (pg)	E2 (pg)	Avail P4 (ng)	P4 (ng)	bCG (mIU)	SP1 (ng)
13	9	<15	77	<.1	2.8	2527	78
14	10		68			723	34
15	11				4.2	1580	118
16	12		75		4.2	1118	162
17	13		51		2.7	685	116
18	14		--		--	--	--
19	15		76		1.8	558	119
20	16		--		--	--	--
21	17		56		1.5	482	24

Appendix Table 10

Group A-B - HCS Only Without Oil

Embryo ID	Rec Stg	Day H[a]	Days C[c]	Diff ID[d]	Media Content on Last CD[e] (E2, P4 available) bCG mIU/d	SP1 ng/d	E2 pg/d	P4 ng/d	Days Prechange
VA810727	4	na	16	2	947+	305+	3829= (3550)	243= (72.8)	1
SC810820	2	6	34	10	500-	912-	9135+ (8503)	260- (158.5)	2
KA810828	2	9	18	3	1000-	35-	1456+ (500)	103= (8.38)	1
ZB811021	2	4	16	4	539-	4800+	2440+ (1623)	460.1= (31.0)	1
W801024	3	6	16	5	1000-	422=	3299= (2122)	119.5 (46.23)	2
VA810116	3	5	17	4	3121-	439-	2500= (4631)	214- (78.3)	2
QA810121	7	1	12	2	7208-	386=	3716= (4631)	189- (78.3)	2
DC810228	7	2	12	2	2000+	658=	10683- (4631)	581.7= (54.1)	2

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[a] = Day in Culture Hatched from Zona Pellucida

[c] = Total Number of Days Cultured

[d] = Differentiation Score (Table 2 - Chapter 7)

[e] - = daily compound concentration appears to be decreasing
+ = daily compound concentration appears to be increasing
= = daily compound concentration not changing

Rec Stg = Coded stage at Recovery (Table 1 - Chapter 7)

Appendix Table 11

Group C - HCS Serum Supplementation Under Oil

Embryo ID	Rec Stg	Day H[a]	Days C[c]	Diff ID[d]	Media Content on Last CD[e] (E2, P4 available) bCG mIU/d	SP1 ng/d	E2 pg/d	P4 ng/d	Days Prechange
RB830806	3	15	18	4	320+[f]	25	793- (1058)	5.07+ (32.6)	1
CB831014	7	0	11	3	5515+	174+	1151 (1058)	11.66+ (32.6)	1
SC831024	3	10	28	4	85-	116=	1355 (839)	4.7= (83.5)	2
XC841024	7	5	12	2	1234	30	988 (1379)	2.58 (32.4)	1
ZB841130	3	5	20	5	483	50	810 (1379)	2.32 (32.4)	4

---

[a] = Day in Culture Hatched from Zona Pellucida

[c] = Total Number of Days Cultured

[d] = Differentiation Score (Table 2 - Chapter 7)

[e] - = daily compound concentration appears to be decreasing
+ = daily compound concentration appears to be increasing
= = daily compound concentration not changing

Rec Stg = Coded stage at Recovery (Table 1 Chapter 7)

[f] = bCG value for next to last CD for this embryo

Appendix Table 12

Group D - HCS to BS Serum Supplementation Without Oil

Embryo ID	Rec Stg	Day H[a]	Day S[b]	Days C[c]	Diff ID[d]	Media Content on Last CD[e] (E2, P4 available) bCG mIU/d	SP1 ng/d	E2 pg/d	P4 ng/d	Days Prechange
KA821018	4	6	10	23	5	500-	158-	50=	50.4-	1
VA821226	3	6	14	19	2	474-	46=	49= (<50)	93.6= (0.4)	2
CC830106	4	4	11	14	3	6439-	153=	50= (<50)	118 (0.5)	1
SC830115	7	4	7	23	2	2281+	856=	100= (<50)	134+ (<.8)	2
CB830121	4	4	9	17	11	1700-	170=	26= (<50)	25+ (<.8)	2
VB830118	8	0	6	12	1	5209+	161+	106= (<15)	84.7+ (<4)	1
NA831208	7	7	9	27	3	745	478	118 (<25)	153 (<.4)	1

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[a] = Day in Culture Hatched from Zona Pellucida

[b] = Day Serum Source Switched from HCS to BS

[c] = Total Number of Days Cultured

[d] = Differentiation Score (Table 2 - Chapter 7)

[e] - = daily compound concentration appears to be decreasing
+ = daily compound concentration appears to be increasing
= = daily compound concentration not changing

Rec Stg = Coded stage at Recovery (Table 1 - Chapter 7)

Appendix Table 13

Group E - HCS to BS Serum Supplementation Under Oil

Embryo ID	Rec Stg	Day H[a]	Day S[b]	Days C[c]	Diff ID[d]	Media Content on Last CD[e] (E2, P4 available) bCG mIU/d	SP1 ng/d	E2 pg/d	P4 ng/d	Days Prechange
XA830428	4	3	11	18	4	1180-	222=	77= (<50)	7.2+ (0.84)	1
DB830604	6	3	10	13	2	2299-	164=	100= (<25)	24.75= (<1)	1
CC830605	4	4	9	12	5	390=	25=	88 (<25)	<2.0 (<1)	1
ZB830811	4	5	12	14	2	3064=	462+	129= (<10)	7.35+ (<1)	1
GC830818	3	10	13	19	7	3062-	569=	44= (<10)	11.32+ (<.10)	1
CB830909	3	5	11	18	11	1007	278-	106= (<15)	6.35= (<1)	1
EC830926	4	4	11	18	11	2578	288	90= (<15)	3.85= (<1)	1
VA831008	3	5	11	16	11	3103-	250-	72 (<15)	9.1 (<1)	1

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[a] = Day in Culture Hatched from Zona Pellucida

[b] = Day Serum Source Switched from HCS to BS

[c] = Total Number of Days Cultured

[d] = Differentiation Score (Table 2 - Chapter 7)

[e] - = daily compound concentration appears to be decreasing
+ = daily compound concentration appears to be increasing
= = daily compound concentration not changing

Rec Stg = Coded stage at Recovery (Table 1 - Chapter 7)

Table 1, Continued

Group III[d]	2-cell	(0)	--	--	--
	4-6	(1)	M	0	(0)
	7-10	(2)	10, BC	0	(0)
	12-16	(3)	HBC, HBC, HBC	3	(100)
	M	(4)	M, M, HBC, HBC	2	(50)
	BC	2	HBC, HBC	2	(100)
Total		12		7	58
TOTAL		60		23	38

[a]M = Morula; BC = Blastocyst; HBC = Hatched Blastocyst

[b]Group I = 20% FCS

[c]Group II = 20% HCS filtered, heat-treated prior to storage

[d]Group III - 20% HCS unfiltered, heat-treated immediately prior to use

[e]OG = This embryo achieved outgrowth through a slit in the zona pellucida without actually escaping from the zona.

Table 1, Continued

Group III[d]	2-cell	(0)	--	--	--
	4-6	(1)	M	0	(0)
	7-10	(2)	10, BC	0	(0)
	12-16	(3)	HBC, HBC, HBC	3	(100)
	M	(4)	M, M, HBC, HBC	2	(50)
	BC	2	HBC, HBC	2	(100)
Total		12		7	58
TOTAL		60		23	38

[a]M = Morula; BC = Blastocyst; HBC = Hatched Blastocyst

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Valerie Z. Pope

Major Subject Biology

Title of Dissertation Baboon Embryo Culture as a Model for Primate Embryology: Functional and Histological Differentiation in Vitro

Dissertation Committee:

Gary R Bowers, Chairman

Charles F Wagg

Ronald L Cotsinger

Larry Boots

Lee A Beck

Director of Graduate Program Daniel D. Jones

Dean, UAB Graduate School Kenneth J Ruzen

Date June 25, 1985

PS-1420