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ACKNOWLEDGEMENTS The research studies reported in this review were supported by grants from the Indian Council of Medical Research, Government of India, Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization, Geneva, and the National Institute of Health, USA under the United States held India Rupee Fund (USIF)

Secretion of endocrine signals by the primate embryo during the peri-implantation period

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Development of preimplantation embryos and blastocyst implantation are critical early events in the establishment of pregnancy. In primates, embryonic signals, secreted during the peri-implantation period, are believed to play a major role in the regulation of embryonic differentiation and implantation. However, only limited progress has been made in the molecular and functional characterization of embryonic signals, partly due to severe paucity of primate embryos and the lack of optimal culture conditions to obtain viable embryo development. Two embryonic (endocrine) secretions, i.e. chorionic gonadotrophin (CG) and gonadotrophin releasing hormone (GnRH) are being studied. This article reviews the current status of knowledge on the recovery and culture of embryos, their secretion of CG, GnRH and other potential endocrine signals and their regulation and physiological role(s) during the peri-implantation period in primates, including humans.

BLASTOCYST implantation and early establishment of pregnancy in primates relies on endocrine mechanisms that are distinct from those in non-primate species. Embryo-specific endocrine signals are believed to control embryonic differentiation and implantation in primates. But, very little is known of their activation and sequential expression. In contrast, sufficient knowledge is available on placental endocrine secretions and their

Table 1. Secretion of embryonic signals during the peri-implantation period

Type signal	Species	Earliest stage detected	Reference number
CG	marmoset	prehatching blastocyst	3, 4
	rhesus	prehatching blastocyst	7
	baboon	attached blastocyst	5, 6
	human	prehatching blastocyst	8, 9, 23, 24
GnRH	rhesus	prehatching blastocyst	10, 11, 25
Estrogen	mouse	prehatching blastocyst	27–29
	rat	prehatching blastocyst	27–29
	human	preattached blastocyst	30
Progesterone	mouse	prehatching blastocyst	27–29
	rat	prehatching blastocyst	27–29
	human	preattached blastocyst	30
Inhibin	mouse	cleavage stage embryo	31
	human	cleavage stage embryo	32
Activin	mouse	cleavage stage embryo	31
PAF	mouse	prehatching blastocyst	12, 37
	marmoset	preattaching blastocyst	38
	human	prehatching blastocyst	12
Histamine	mouse	prehatching blastocyst	34
Prostaglandin	mouse	prehatching blastocyst	35
	human	prehatching blastocyst	36
Trophoblastic protein	sheep	prehatching blastocyst	39
	cattle	prehatching blastocyst	39
Growth factors	mouse	cleavage stage embryo	40
	cattle	cleavage stage embryo	40

Abbreviations used are CG chorionic gonadotrophin, GnRH gonadotrophin releasing hormone, PAF platelet activating factor

function[1, 2]. This, however, will not be discussed here since it is beyond the scope of this article.

The first clear embryo-derived endocrine signal during peri-implantation period in primates is chorionic gonadotrophin, CG[3–9], which is structurally and functionally similar to pituitary luteinizing hormone (LH). Its major role is to 'rescue' the corpus luteum and sustain its functional life-span during early pregnancy. Recent studies[10, 11] show that primate embryos can also secrete gonadotrophin releasing hormone (GnRH), the known hypothalamic regulator of pituitary gonadotrophin secretion. Although other embryonic endocrine signals have been reported for non-primate species[12], they remain to be clearly established in primates (Table 1). Major limitations to analysing embryo-derived secretory products in primates are: a) inadequacy of embryos, b) lack of optimal culture systems that support complete peri-implantation embryo development, c) lack of sensitive and specific analyses to measure minute quantities of embryonic secretions with a 'high noise to background signal' and d) the cost to maintain primate facility and to carry out embryology research. This article examines the status on the recovery and culture of primate embryos, embryonic endocrine secretory signals and their regulation and function.

Development of primate embryos *in vitro*

Major limitations to studying the embryonic endocrine secretions in primates, which are mostly monotocous, are the lack of adequate number of embryos and optimal culture conditions that maximally support normal and viable development of embryos through the peri-implantation (attachment) period. Availability of *in vivo* fertilized tubal (cleavage) stage embryos are very limiting since its recovery depends mostly on invasive and traumatic procedures (laparotomy) which cannot be repeated on the same animal. Although IVF methods can produce the desirable number of early embryos, their quality and viability are inferior to those produced *in vivo*. A repeatable, non-invasive, atraumatic non-surgical uterine flushing procedure is successfully developed for a few macaques[13, 14], baboons[15] and squirrel monkeys[16] and also for humans[17]. It provides *in vivo* produced uterine-stage embryos, i.e. non-compact morulae to various stages of blastocysts, from either non-stimulated or PMSG/FSH stimulated embryo donors. Moreover, their quality is far superior to that produced by IVF methods, an important factor to be considered while analysing embryo-secretory products during culture.

Freshly recovered *in vivo* produced uterine stage baboon[18], rhesus[7] and marmoset[19] embryos have been successfully cultured through the post-attachment blastocyst stages with extensive trophectodermal outgrowths. Human IVF embryos have been successfully cultured through post-attachment stages[9]. Figure 1 depicts the quality of a rhesus morula (*a*), freshly recovered by non-surgical uterine flushing[7], a hatching (*b*) and a hatched blastocyst (*c*), cultured from the

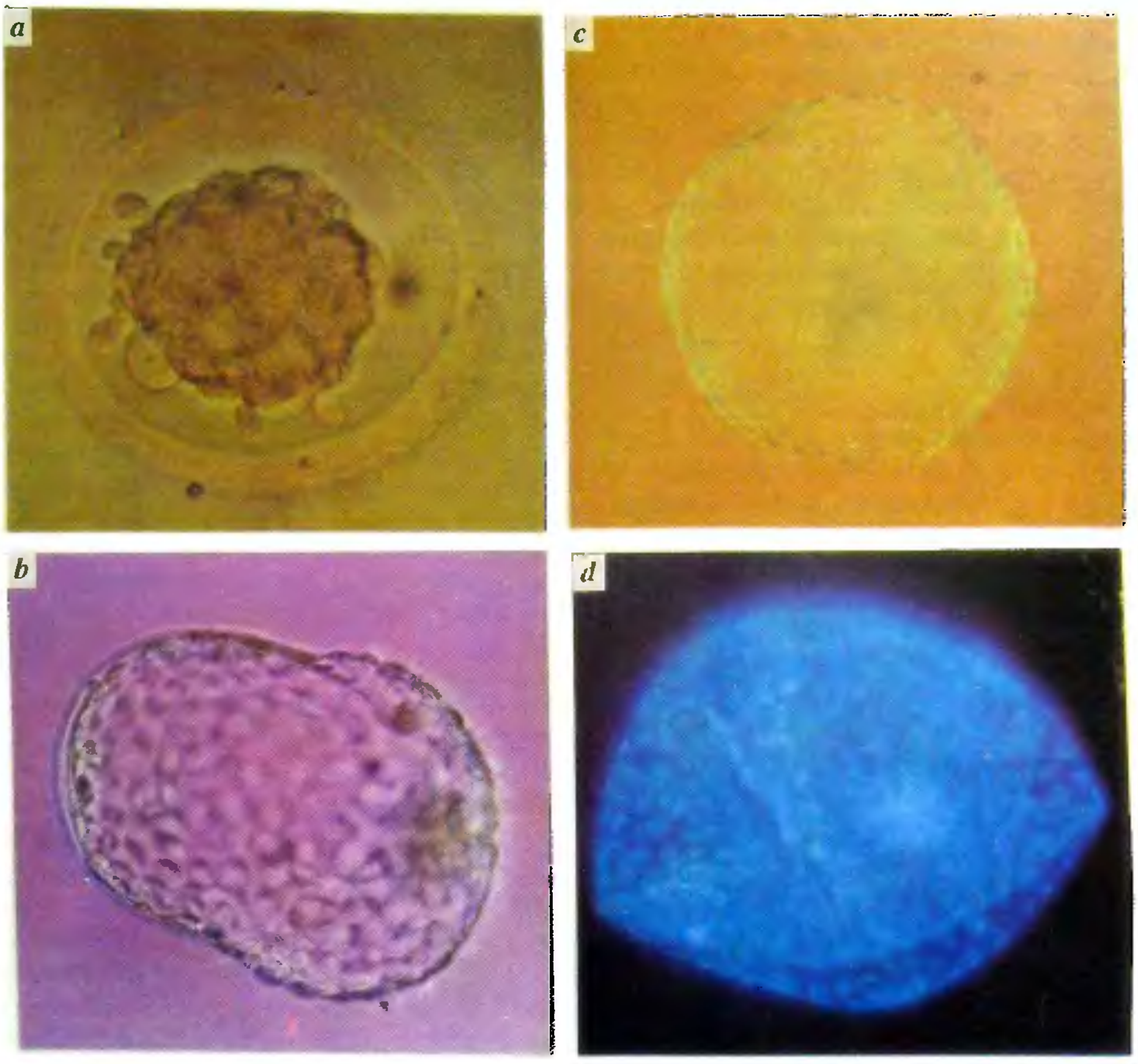

Figure 1. Photomicrograph of a freshly recovered rhesus monkey morula (*a*) and a hatching (*b*) and hatched blastocyst (*c*) cultured from the morula stage. Panel *d* shows the Hoechst stained fluorescent nuclei of the hatched blastocyst. Optical magnification: × 200. Reproduced with permission from *Hum. Reprod*., 1993, **8**, 279–287.

Table 2. *In vivo* and *in vitro* secretion of chorionic gonadotrophin in various primate species*

	In vivo embryo		*In vitro* embryo	
Species	Day of attachment	Day of CG secretion	Day of attachment	Day of CG secretion
Human	7–9	9–12 (IA)	8–10	6 (IA)
Chimpanzee	7–9?	10–11 (IA)	–	–
Rhesus	8–10	11–12 (IA)	6–8	4 (BA)
Baboon	8–10	11–12 (IA)	7–12	8 (IA)
Marmoset	11–12	14–17 (IA)	7–10	6 (BA)

*The information provided here is obtained from reference numbers 3–9, 23–25, 39. Abbreviations used are CG: chorionic gonadotrophin, IA: radioimmunoassay or immunoradiometric assay; BA: mouse Leydig cell bioassay.

morula stage using CMRL-1066 medium supplemented with 20% bovine fetal serum[7]. Hoechst-stained nuclei counts indicate that cultured hatched blastocysts contain approximately 531 cells (panel *d*, Figure 1). Blastocyst-derived trophoblasts could be maintained in culture through several passages. They continue to differentiate into cyto- and syncytiotrophoblasts[7]. These culture systems[7, 9, 18, 19] have provided an excellent opportunity

to analyse embryonic endocrine secretions (Table 1) through the entire period of peri-attachment development which otherwise would not be feasible during the same period *in vivo*.

Embryonic secretion of chorionic gonadotrophin

CG is one of the hormones required for the establishment of early pregnancy in primates. It is secreted by the syncytiotrophoblasts of embryonic or placental origin[1, 9, 20]. The genes for CG are thought to be expressed only in primates[21] and its transcripts are detected as early as the 8-cell stage[22]. Although the embryonic secretion of CG is being studied[3–9], its regulation and function during the peri-implantation development remain to be established.

The profiles of CG in blood circulation in most primates studied indicate that it is detectable 2–3 days after implantation (Table 2). *In vitro*, the earliest time of embryonic secretion of CG detectable during pre- and peri-implantation development is variable, depending on the species (Table 2). In the rhesus monkey, the cumulative concentration of bioactive CG increases soon after hatching but prior to attaching and raises exponentially after the attachment of blastocyst. The rise of CG secretion is positively correlated with the development of blastocysts[7]. A similar profile of bioactive CG secretion by marmoset embryos is observed with very low to undetectable levels prior to embryo attachment and exponential rise immediately after attachment[3, 4]. Reports from Pope *et al.*[5] and Bambra and Tarara[6] indicate that baboon embryos secrete immunoreactive CG at or following hatched blastocyst attachment and the secretion continues for more than 20 days. Reports from Lopata[8, 23, 24] and Dokras[9], in which the secretion of CG by cultured human (IVF) blastocysts were studied, indicate that the immunoreactive CG dimer or its subunits can be secreted by the expanded prehatching blastocysts prior to attachment and the levels increase exponentially after attachment.

The available information on the timing of embryonic secretion of CG (Table 2) indicates that blastocysts can actively secrete CG either prior to its attachment as in the rhesus monkey[7, 25] and humans[8, 9, 23, 24] or immediately after its attachment as in the baboon[5, 6]. There appear to be some species variations in the dynamics (timing) of CG secretion (Table 2). A strict comparison of CG profiles among different primates is difficult since measurements of the hormone are carried out by either a bioassay (rhesus and marmoset monkeys) or an immunoassay (baboons and humans). While the former detects the biologically active form of the hormone, the latter can only detect immunoreactive CG of either the α or β subunits or both which, in all cases, may not represent the biopotency of the hormone. In view of the limitations of the studies inherent to primates, both immuno- and bio-assays may be difficult to perform, although it is desirable.

Secretion of gonadotrophin releasing hormone (GnRH)

In recent studies using rhesus monkey embryos, Seshagiri *et al* [10, 11, 25] have demonstrated that the secretion of GnRH by blastocysts commenced from low levels (0.32 ± 0.05 pg/ml) during the pre-hatching blastocyst stage to 2–4 fold higher (0.7 ± 0.08 to 1.3 ± 0.23) at > 6 days of hatched blastocyst attachment. GnRH secretion was low or undetectable when embryos failed to hatch. There was a positive correlation between profiles of CG and GnRH. Immunocytochemical data, in addition to confirming the RIA data, showed that GnRH positive staining was seen in intact and hatched blastocysts including the inner cell mass (ICM) and trophoblast outgrowths. Moreover, immunopositive staining was localized exclusively in cytotrophoblasts but not in

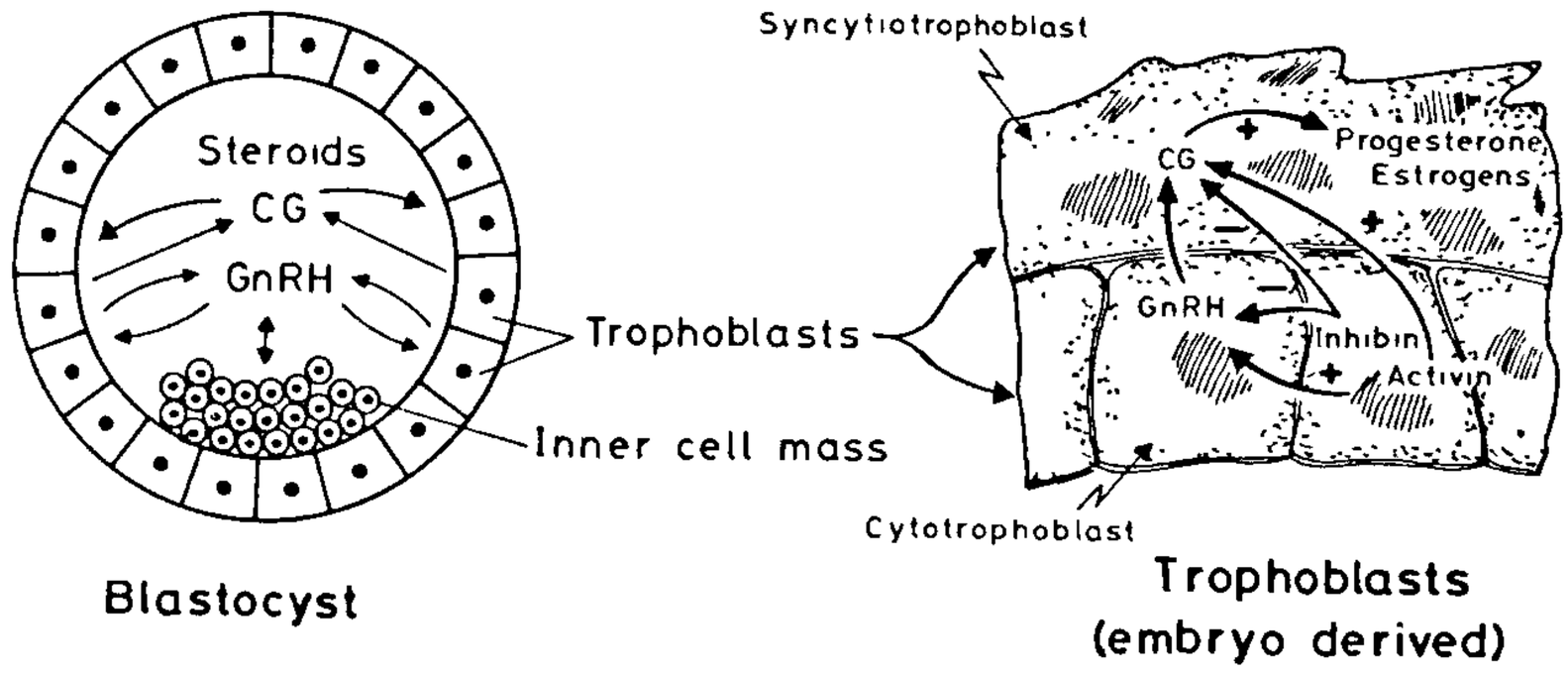

Figure 2. Schematic representation of cell-specific production of a few embryonic endocrine signals during pre- and post-attachment blastocyst stages Abbreviations used are CG chorionic gonadotrophin, GnRH gonadotrophin releasing hormone.

syncytiotrophoblasts[10, 11, 25]. These observations provide evidence, for the first time, that immunoreactive GnRH is produced and secreted by primate (rhesus) blastocysts and their cytotrophoblasts (Figure 2), as seen in placental trophoblasts[1, 2, 26]. Similar discovery is awaited in other primate embryos.

Secretion of other endocrine signals

Although several endocrine secretions of placental trophoblasts are well described[1, 2, 20, 26], they remain to be investigated in detail in peri-implantation embryos (Table 1). Activities of key steroidogenic enzymes and synthesis and secretion of sex-steroid hormones in the rodent preimplantation embryos have been documented[27, 28]. Progesterone metabolizing enzymes have also been demonstrated in mouse embryos[29]. A recent report[30] shows that cultured human (IVF) embryos are capable of synthesizing and secreting both progesterone and estrogen. Such data are currently very limiting for primate embryos.

Very recent discoveries indicate that both rodent[31] and primate[32, 33] preimplantation embryos have the ability to secrete gonadotrophin regulating polypeptides, inhibin and activin (Table 1; Figure 2) which are generally thought to be of gonadal origin. Several other autocrine and/or paracrine signals (Table 1) such as histamine[34], prostaglandins[35, 36], platelet activating factor[12, 37, 38], trophoblastic proteins[39] and growth factors[40] are also secreted by preimplantation embryos[12]. Since it is beyond the scope of this article, these non-endocrine factors will not be discussed here.

Regulation of secretion of endocrine signals

The regulation of placental trophoblastic CG secretion by GnRH, steroids, calcium, etc. is well documented[1, 2, 20, 41, 42]. However, the regulation of embryonic secretion of CG during peri-implantation period is yet to be understood. Secretion of CG by cultured human blastocysts could be modulated by the addition of 1% human cord serum or growth factors such as insulin, transferrin and PDGF and PAF either alone or in combination[24]. Seshagiri *et al.*[10, 11, 25] have suggested the possible involvement of GnRH in the regulation of CG secretion (Figure 2). A recent report shows that the efficient production of CG by trophoblasts of marmoset blastocysts is dependent on the presence of the ICM; bisected blastocysts without the ICM secrete very low levels of CG[43].

Based on the limited data, it can be speculated (Figure 2) that the secretion of GnRH by the blastocyst (ICM?) may regulate the trophoblastic production of CG prior to blastocyst attachment. After the attachment of the blastocyst, the differentiation of trophoblasts could confer cell-specific expression of GnRH in cytotrophoblasts and CG in syncytiotrophoblasts. They may be regulated by a paracrine mechanism leading to steroid production (Figure 2). A circumstantial indication to this is the finding that primate embryos can secrete estrogen and progesterone[30]. Moreover, CG/GnRH in turn may be modulated by inhibin, activin and steroids (Figure 2). The possible regulation of CG secretion by GnRH in peri-implantation embryos, which appears to be analogous to that occurring in placental trophoblasts in culture[20, 37, 38], requires detailed experimentation using GnRH agonists and antagonists.

The evolutionary advantage of primate embryos having adapted the hypothalamo-pituitary mechanism to the regulation of embryo implantation involving CG, GnRH and steroids may be significant, particularly due to the fact that only primates are believed to express the genes for CGβ[21]. Further research on the modulation of embryonic endocrine secretions is necessary to establish the casual relationships of GnRH, CG, steroids and gonadal factors which could regulate peri-implantation embryo development in primates (Figure 2).

Physiology of endocrine signals

The important role of CG in embryo development and implantation is being studied. Hearn and coworkers showed that when marmoset monkeys were immunized actively or passively against CGβ, there was disruption of implantation and termination of pregnancy. Presence of anti-CGβ antibodies in culture inhibited development and attachment of marmoset embryos[4, 38]. These observations indicate that CG, in addition to its well accepted role in rescuing and supporting the corpus luteum, could have a role in blastocyst development and intraembryonic differentiation, in initiating and regulating implantation, the attachment process itself and in the establishment of trophoblast-endometrial vascular links. The observed early secretion of CG prior to embryo attachment *in vitro*[7–9, 23–25] may be occurring *in vivo* wherein CG may diffuse locally from the uterine lumen to the maternal compartment, as observed in the rabbit and human[44] and rescue the corpus luteum prior to embryo implantation. This may be followed by an exponential rise of CG secretion by the rapidly invading/proliferating trophoblasts of the implanting blastocysts. In view of the critical involvement of CG for the early establishment of pregnancy, CGβ has been developed as a contraceptive vaccine[45].

The potential roles of GnRH and sex-steroids during peri-implantation embryo development are to be established. The requirement of GnRH for rat blastocyst implantation has been demonstrated[46]. Recent observation of GnRH secretion by rhesus embryos[10, 11, 25] indicates that this peptide may be required for embryo

development and implantation in primates. A GnRH-based contraceptive vaccine is also being considered[45]. Culture of mouse blastocysts in the presence of an anti-estrogen reduces their implantation rates[47]. Treatment of anti-estrogen or an aromatase inhibitor to proven fertile bonnet monkeys consistently offers pregnancy protection[48]. Antiprogestins prevent implantation and early pregnancy in macaques[49, 50]. During preimplantation period, increases in total steroid binding capacity and occupancy of estrogen and progesterone to nuclear receptors in rhesus endometrium are observed[51]. These observations testify the potential roles(s) played by GnRH and sex-steroids during the peri-implantation period, which remain to be experimented in primates.

Extensive research needs to be done on the secretion and regulation of some of the key endocrine principles such as CG, GnRH, sex-steroids and gonadal factors in the primate embryo (Figure 2). The available information strongly indicates that the above hormones, secreted in a coordinated, time-dependent manner during the pre- and peri-implantation period, are crucial for embryonic development, blastocyst implantation and for the maternal recognition of pregnancy prior to implantation. Potential intervention of these endocrine mechanisms by suitable means may increase or decrease embryonic survival and viability during the peri-implantation period in primates.

Summary

The culture systems now achieved for development of morulae and blastocysts through the entire peri-attachment period should greatly facilitate studies on the endocrine regulation of pre- and peri-implantation embryo development that govern successful establishment of early pregnancy in primates. Embryonic endocrine factors can mutually influence their own synthesis, release and function. They could likely be playing important role(s) in embryonic differentiation and implantation. They could potentially be exploited for the regulation of fertility. A few of these (CG/GnRH) may be involved in the maternal recognition of early pregnancy prior to implantation. New approaches to fertility regulation require knowledge of the precise molecular and functional characteristics of the endocrine principles of the primate embryo during the peri-implantation period.

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ACKNOWLEDGEMENTS. We thank colleagues at the University of Wisconsin Primate Research Center for their expert animal care and assistance with the complex infrastructural requirements for studies in primate embryology. We acknowledge program grant support from NIH grant RR-00167.

Medically assisted reproductive technologies – A clinical perspective

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The birth of Louise Brown by *in vitro* fertilization and embryo transfer in 1978 marked the beginning of Reproductive Medicine. Since then several new medically assisted reproductive technologies have been introduced. Every aspect of *in vitro* fertilization and embryo transfer has undergone major changes. The probability of achieving parenthood for many barren couples has improved significantly during the last few decades. India is a late entrant to this field but has the potential of being in the forefront of research and development because of the vast opportunities available for such endeavours here.

THE world's first test tube baby, Louise Brown is today a 16-year-old bubbling teenager, just as any other teenager of her age. Her mother, Lesley Brown, was told by her gynaecologist that she had a one in a million chance of ever having a baby, she was not only lucky at the first attempt at *in vitro* fertilization and embryo transfer but also for the second time and have two babies in succession. The credit for this pioneering work goes to Edwards, Steptoe and their colleagues at the Oldham General Hospital, UK. The field of reproductive medicine has come a long way since Louise Brown was born. This article gives a clinical perspective of medically assisted reproductive technologies.

Medically assisted reproductive technologies

Medically assisted reproductive technologies (MARTs) are defined as techniques which assist a couple in achieving parenthood by the manipulation of the oocytes and spermatozoa. The currently available MARTs include: *In vitro* fertilization and embryo transfer (IVF-ET), Gamete intra-fallopian transfer (GIFT), Zygote intrafallopian transfer (ZIFT), Tubal embryo stage transfer (TET), Pronuclear stage tubal transfer (PROST), Transcervical oocyte and sperm transfer (TOAST), Peritoneal oocyte sperm transfer (POST), Direct intra-follicular transfer (DIFT), Subzonal insemination (SUZI), Micro insemination sperm transfer (MIST), Intra-cytoplasmic sperm injection (ICSI), Micro insemination fallopian transfer (MIFT), Zona drilling, Zona cutting, Partial zona dissection (PZD), Micro epididymal sperm aspiration (MESA), Sperm retrieval from the testes (SPERT), Oocyte donation (OD) and Embryo donation (ED).