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REVIEW ARTICLE

RESEARCH IN REPRODUCTION : THE INDIAN SCENARIO IN THE LAST DECADE [I]

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Abstract : The physiology of reproduction attempts to decipher the biological basis of procreation. Fundamental advances in the biology of reproduction are essential to decipher this process. In this article, an attempt has been made to chronicle some recent advances in our understanding of the biology of reproduction during the last decade based on the contributions of scientists working in India.

Key words : embryo, epididymis, fertilization, implantation, ovary, spermatogenesis, testis, testosterone, uterus

INTRODUCTION

Is there a need to focus our attention, energy and investment towards understanding the biology of reproduction? The new millennium brings with it the technology to develop a cloned Dolly, debates upon the need to produce cloned human baby, or even the right to use fetuses in organ transplantation. Yet at the same time, society continues to bear the stigma of deaths due to abortion following unplanned pregnancies and the plight of infertile couples. As biologists we must strive to make science a part of the life of the people and it is through science we can bring about transformation of society using knowledge as a means of progressive change.

The physiology of reproduction attempts to decipher the biological basis of procreation. Indeed there are two sides to this coin, to foster fertility and to inhibit fertility, both being closely interrelated. Fundamental advances in the biology of reproduction are essential in order to possess better regulation of either event. In this article, I have attempted to chronicle some recent advances in our understanding of the biology of reproduction during the last decade (1990-2000) based on the contributions of scientists working in India.

Cellular and molecular basis of hormone action

The biological action of hormones in their target cells is generally mediated by receptors that transduce extracellular

signals into intracellular biochemical processes. Estrogen receptors belong to a protein superfamily, the function of which is dependent upon their interaction with corresponding response elements as transcription factor at the genetic level to trigger a cascade of biological actions. Thampan and Clark first reported a cytosolic protein-estrogen receptor activation factor (E-RAF) in the rat (1). Subsequently, E-RAF was purified from goat uteri in three molecular forms E-RAF I, IIa and IIb at the School of Life Sciences, University of Hyderabad (2, 3). E-RAF from rat uterus was shown to be regulated by estrogen and progesterone (4). A novel steroid receptor form was then identified and referred as the non-activated estrogen receptor (naER) which binds estradiol with high affinity, but does not possess any capacity to bind to DNA (5). The naER heterodimerises with E-RAF and it is a protein tyrosine kinase with molecular mass of 66 kDa and sediments at 4.2S. Further characterization revealed that uterine nuclear estrogen receptor RII is the deglycosylated naER (6). Karthikeyan and Thampan (7) identified the plasma membrane as the cellular site of localization of naER; progesterone, dexamethasone and tamoxifen did not facilitate the dissociation of naER from plasma membrane; estradiol mediated dissociation was inhibited by tamoxifen. Estrogen was found to inhibit tyrosine kinase activity of naER, and this was reversed by tamoxifen. The naER to R-II conversion is brought about by a nuclear glycopeptidase (8). Through the isolation and identification of 55 kDa protein of cytosolic origin and a group of 14-11 kDa proteins presumably associated with nuclear pore complex/nuclear membrane, Thampan and colleagues outline the mechanism of ER transport from cytosol, its site of synthesis to nucleus for mediating gene transcription (8, 9). Thus, the model proposed by Thampan et al. (8) to explain the cellular basis of estradiol action is that of an initial binding of estradiol to plasma membrane bound naER causing its entry into cytosol where it dimerises with a 66kDa protein, estrogen receptor activation factor (E-RAF). E-RAF is a DNA binding protein with no capacity to bind estradiol. This dimerisation has been shown to be essential for migration to nuclear compartment. The nuclear localization sequence binding proteins (NSLBPs) which are putative transport proteins having in their primary structure a signal that enables them to accumulate selectively into the nucleus (10, 11). In the case of goat uterine estrogen receptor proteins, the NLS regions are shared by both naER and E-RAF supporting their total interdependence for nuclear entry as a heterodimer. After entry into the nucleus, naER is deglycosylated into R-II by a glycopeptidase and the dimer dissociates; E-RAF binds to DNA and influences transcription while R II binds to RNA polymerase II (8).

A concept proposing plasma membrane receptor for steroid hormones is not novel. In a study of progesterone induced resumption of meiosis in frog oocytes, Baulieu et al. (12) reported that the steroid acts at the level of the cell surface and suggested that an induced change in Ca^{++} distribution triggers a cascade of cytoplasmic events including protein synthesis and germinal vesicle breakdown. The authors further suggested that the molecular aspects of progesterone-membrane interaction may provide an opportunity to study a new type of target site for steroid hormones in somatic cells. For example, Astwood in 1938 had reported for the first

time the very early effects of estrogen on membrane permeability in uterine cells. Using endometrial cell suspensions isolated from uteri of ovariectomized rats, Pietras and Szego (13) reported that physiological levels of estradiol-17β influences rates of cellular Ca^{++} exchange as early as 2.5 min after *in vitro* addition of the hormone. Increments in cellular calcium accumulation are known to precede and/or trigger cell division and growth. Indeed, several reports now support the existence of a membrane surface receptor for estrogen (14, 15). The studies reported by Thampan and colleagues help in describing the sojourn of estrogen from membrane to nuclear compartment in inducing its cellular actions.

The intracellular role of calcium in inducing specific actions of estrogen have been further explored by Gupta and his group at the Centre for Cellular and Molecular Biology in Hyderabad. Using rat vaginal epithelium as an experimental model, Gupta et al. (16) examined the cellular basis of action of estradiol-17β. Following a single injection of estradiol-17β (E2) there was a rapid (within 15 min) rise in intracellular levels of calcium in immature rat vaginal epithelial cells (VEC) which was also observed electron microscopically as aggregates of calcium oxalate in the inner nuclear membrane, nucleolus and mitochondria, while progesterone priming reversed this event. Induction of phosphoinositide signal transduction pathway along with increased intracellular calcium levels in rat VEC by E2 has been reported by Singh and Gupta (17). The authors have evidence to suggest that E2 action led to induction of second messenger pathway in the VEC through specific membrane bound receptors. It is known that keratins help in providing cytoarchitecture and a definite cellular organization to tissue as a whole by controlling morphogenetic migration of cells (18). In rat VEC, the maintenance of cell shape, cellular integrity and differentiation have been shown to depend upon a novel association of calcium dependent cross linking enzyme, transglutaminase essential for target-organ specific keratin filament aggregation (19). *In vivo* and *in vitro* experiments led to defining the role of transglutaminase (EC 2.3.2.13) in rat vaginal epithelial cell differentiation. Increased transglutaminase activity following estradiol action led to transamidation of keratins by the formation of an isopeptide sigma (tau-glutamyl) lysine which led to aggregation of keratin filaments, an action reversed by the addition *in vitro* of a specific inhibitor, 2,4-pentanedione (20, 21).

The nongenomic action of progesterone (P) on capacitated spermatozoon in mediating acrosomal exocytosis through transmembrane signal transduction involving increased intracellular calcium ions and modulation of protein kinases and phospholipid metabolism through a second messenger pathway has been investigated by Laloraya, Kumar and colleagues in Devi Ahilya University, Indore. It is suggested that cell surface receptor having the properties of a calcium channel, chloride channel, bicarbonate/chloride exchanger and gamma aminobutyric acid type A may help to explain the nature of nongenomic receptor of P in mammalian cells. Purohit et al. (22) observed bicarbonate dependent lipid ordering and protein aggregation as a part of the nongenomic action of progesterone on capacitated sperm. Spin labeling studies of lipophilic domains of human spermatozoa during capacitation

and acrosome reaction revealed high level of superoxide anion radical (O2.-) in capacitated spermatozoa while there was a sudden drop in the levels of O2.- in spermatozoa following induction of acrosome reaction (23). Over-expression of superoxide dismutase, poor generation of superoxide anion radicals and lack of surface thiols were found to be inherent defects in oligospermia (24). Abnormal physical architecture of lipophilic domains of human sperm membrane in oligospermia were correlated with low fertility profiles (25). Vasectomy has been shown to induce an immediate biophysical incompetence in epididymal spermatozoa in terms of erroneous execution of membrane configuration processes, premature membrane melting and untimely protein gyration (26). Using dicholorodihydrofluorescein diacetate (DDF) as a reporter, intracellular O2*-levels have been examined within spermatozoa of mice during normal and altered situations of epididymal maturation. Testicular spermatozoa exhibited regional heterogeneity in DDF fluorescence patterns and vasectomy resulted in significant reduction in the O2*-levels of spermatozoa at all levels of maturation leading the authors to suggest a programmed production of reactive oxygen species in specific domains of spermatozoa during various stages of development (27). Supraphysiological doses of testosterone and its derivatives are known to suppress spermatogenesis in mammals by inhibiting the hypothalamic-pituitary-testicular axis leading to oligospermia. While motility and viability of such sperms remains unaltered, they exhibit reduced zona binding resulting in infertility. Laloraya and coworkers hypothesized that such decreased zona binding resulted from perturbations in the mechanical properties of sperm membrane (28). The molecular dynamics of sperm membrane were examined by employing a lipophilic spin label (16-doxyl stearate) and a protein-binding label (Mal-Net). The rotational freedom of lipophilic molecules was reduced significantly within one week of testosterone treatment, and during weeks 1-4, protein rotation was retarded significantly along with sharp increase in ascorbyl radical, suppression in superoxide radical generation in cauda epididymal spermatozoa and in epidydimal fluid with increased levels of glutathione. The perturbed redox status of spermatozoa and epididymal fluid may help to contribute to the physiologically altered functional status of sperms following testosterone exposure.

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are glycoproteins and in many species prolactin (PRL) is found in glycosylated form. In addition to pituitary and placenta, many malignant trophoblastic cells and ectopic tumour cells secrete these gonadotrophins *in vitro*. Muralidhar and coworkers at the Department of Biochemistry, University of Delhi, Delhi, developed procedures to isolate these gonadotrophins from various species and demonstrated microheterogeneity as a common structural feature of the three gonadotrophins (29, 30). It has also been established from immunoassays and bioassays that immunologically active and biologically inactive isoforms of these hormones exist in circulation (31). At the National institute of Immunology, New Delhi, Salunke and coworkers (32) have developed a computer modelling approach suitable for the structure analysis of small bioactive peptide hormones. This involves identification of conformational patterns in protein structure data bank based on the

sequence homology with the bioactive peptide. Application of this procedure to gonadotrophin releasing hormone (GnRH) resulted in a library of possible structures for GnRH, 9 among which share a common beta-turn. The topology of the putative receptor binding site of GnRH was defined by a contiguous surface formed through an appropriate juxtaposition of the N-terminal pGlu-1, the guanidyl group of Arg-8, aromatic side chain of Trp-3 and the Gly-10 NH_2 at the C-terminal end. A tertiary structural model for the mode of recognition between human chorionic gonadotrophin and its receptor has been proposed (33).

The hormone specific bioneutralization epitopes of human FSH on its beta subunit were studied by Iyer and colleagues at the Institute for Research in Reproduction, Mumbai. Predictive methods were used to identify the potential surface-oriented regions of FSH-beta. Peptides corresponding to three regions, 31-52, 66-75 and 88-95 of hFSH beta were synthesized and anti-peptide antibodies were generated and their bioneutralizing actitivities were assessed. The 31-52 hFSH beta fragment was found to be specific antigenic determinant of FSH as it greatly enhanced receptor recognition, while 66-75 hFSH beta may be involved in hormone-receptor interaction (34).

Testicular physiology

While the need for follicle stimulating hormone in initiating spermatogenesis is well accepted, its requirement for the maintenance of spermatogenesis in adulthood is questioned. Moudgal and colleagues working at the Indian Institute of Science in Bangalore provide critical leads to this fundamental question based on studies using rodent and primate models.

The specific effect of deprivation of LH or FSH on testicular function in the adult rat was examined by Vaishnav and Moudgal (35). Deprivation of either gonadotrophin significantly affected RNA and protein synthesis by testicular cells; FSH deprivation specifically affected RNA and protein synthesis of germ cell and not Leydig cell fraction. LH, but not FSH on the other hand, inhibited thymidine incorporation into DNA which was overridden by testosterone (T) supplementation. Lactate dehydrogenase X (LDH-X) and hyaluronidase activities of testicular homogenates of adult rats showed significant reduction following deprivation of FSH or LH, T supplementation reversed the LH effect. Aravindan et al. (36) investigated the role of FSH and diurnal rhythms of serum T levels on specific germ cell transformation during spermatogenesis using DNA flow cytometry and morphometry of seminiferous epithelium of adult male bonnet monkey. Endogenous hormone profiles were altered using two protocols. Firstly, active immunization for 290 days using ovine FSH adsorbed on alhydrogel resulted in neutralization of endogenous FSH sparing LH and diurnal T levels were normal. Secondly, desenstization of pituitary gonadotrophs by chronic infusion of GnRH analogue, burselin, via Alzet pump implant resulting in significant reduction in LH, FSH and abolition of T rhythm. Both treatments led to nearly identical reduction in testicular biopsy scores, mitotic indices and daily sperm production rates compared with respective control groups. However, fundamentally significant different profiles of germ cell populations were observed in the two treatment groups. Abolition of T rhythm led to an arrest of meiosis with decrease in round spermatid population, increases in (2C) spermatogonial cells while

preleptotene spermatocytes (S phase) and (4C) primary spermatocyte populations were normal. At day 80 when FSH deprivation was total the primary block appeared to be in the conversion of spermatogonia (2C) to cells in S-phase and primary spermatocytes (4C). The authors concluded that FSH deprivation led to blockade of spermiogenesis and spermiation. This question was further examined to test whether FSH and T act synergistically on Sertoli cells to control spermatogenesis or they influence independently the transformation of specific germ cell types during spermatogenesis in the adult. Bonnet monkeys were used to study this problem. LH/T deprivation led to specific blockade of meiosis and production of spermatids, while FSH deprivation led to significant reduction in the proliferation of spermatogonial cells and a marked inhibition in the transformation of spermatogonia to primary spermatocytes (37). Das and co-workers at the National Institute of Health and Family Welfare in New Delhi, however, observed that in the rat, testosterone (T) alone had a positive effect in terms of germ cell development, while FSH without T was detrimental to maturing germ cells (38, 39).

It is interesting to note that after nearly a decade or more of investigations to study the question of a true requirement for FSH in promoting spermatogenesis and fertility in primates, Moudgal and Sairam (40) comment that the requirement for FSH in promoting fertility in the male bonnet monkey is reasonably well established, however, in humans the evidence currently available in favour of this concept is only circumstantial, and that further studies are needed to examine this hypothesis in proactive way.

The seminiferous tubular fluid provides the microenvironment necessary for spermatogenesis in the adluminal compartment of the seminiferous tubule primarily through secretions from Sertoli cells. At the National Institute of Immunology in New Delhi, Shaha and colleagues have advanced our understanding of Sertoli cell - germ cell interactions that are essential for spermatogenesis. A 24 kDa protein isolated from seminiferous tubular fluid of the rat showed time-dependent accumulation in spent culture medium and shared similarity to the NH_2 terminus of glutathione S-transferase (GST)-mu subunits in a 20-amino acid overlap. Anti-24-kDa antibodies inhibited murine sperm-oocyte binding *in vitro* (41). While there exists close structural similarity of glutathione S-transferase (GST) isolated from liver and seminiferous tubular fluid (STF) the evidence that immunostainable GST was present in Sertoli cells and that radiolabelled GST was found in Sertoli cell culture media indicate Sertoli cells as secretors of seminiferous tubular fluid GST (42). Functionally STF-GST appeared to serve as a steroid binding protein by its ability to bind testosterone and estradiol, two important hormones essential for spermatogenesis. Inhibition of GST activity led to interference with normal motility, acrosome reaction and fertilizing ability of goat sperms. It has been argued that these functional impairments were due to membrane changes from alterations in the lipid peroxidation status of these cells after GST inhibitor treatment. Increased reactive oxygen species production by the cell which occurs when GST activity was suppressed may be a mediator for membrane damages (43).

The concept that a close interaction exits between epididymal microenvironment and spermatozoa allowing for sperms to acquire forward motility, recognize homologous zona pellucida, bind to it and initiate fertilization-associated events has come under increasing challenge with the development of new andrology techniques like subzonal insemination of spermatozoa (SUZI) and intracytoplasmic sperm injection (ICSI). Indeed such techniques raise the question whether in the human the epididymis is a redundant organ. The relevance of the epididymis has been explored through research conducted by Rajalakshmi and colleagues at the All India Institute of Medical Sciences, New Delhi, using primate as well as data from normal human male to determine how information is skewed when examining data from pathological tissues like obstructed epididymis or epididymis from subjects with vasal agenesis are extrapolated to normal men.

Ultrastructural studies have revealed that spermatozoa undergo structural changes during sperm maturation with reorganization of plasma lipids and stabilization of disulphide likages during epididymal transit (44). In order to evaluate the changes in glycoconjugates on sperm surface during maturation, ejaculation and capacitation, quantitative analysis of lectin binding by live spermatozoon was performed which revealed discernible variations in lectin labeling. Redistribution of sperm surface sugars or membrane damage during maturation and capacitation may be associated with their role in ovum recognition and fusion (45). Aravindan and coworkers (46) in a study of sperm chromatin condensation and compaction noted a role of epididymis in sperm maturation. They observed progressive increase in the formation of inter- and intra-protamine disulphide bridges as sperms transit through caput, corpus and cauda of epididymis. Subsequently it was observed that FSH deprivation in monkeys resulted in the production of sperms with limited potential for disulfide formation and chromatin stability (47).

At the Indian Institute of Chemical Biology in Calcutta, the major biochemical alterations occurring in spermatozoa during epididymal maturation in intact animals and *in vitro* in the initiation of flagellar motility are being investigated by Majumder and colleagues (48). Experimental evidence from studies using goat sperms revealed that external sperm surface possesses a novel coupled ecto-cAMP independent protein kinase (CIK) and ecto-phosphoprotein phosphatase (ecto-PPase) enzyme system that regulates the phosphorylated states of intact sperm ecto-protein phosphorylation and dephosphorylation (49). The activities of this enzyme system increase markedly during forward progression suggesting that they may play a role in flagellar motility (50). A heat-stable glycoprotein, flagellar motility protein, derived from epididymal plasma was isolated which together with elevated intrasperm pH and cAMP initiates flagellar motility through activation of cAMP dependent protein kinases (51, 52). Using purified preparations of immature and mature sperm membranes, isoenzymes of cAMP dependent protein kinases (RC) were identified and a stage specific distribution was observed with type II RC being found only in mature epididymal sperm surface (53, 54). Further purification and characterization of sperm membrane protein kinase revealed that it induces phosphorylation of serine and threonine

residues of several endogenous plasma membrane proteins in maturing epididymal spermatozoa in a stage-specific manner (55, 56). Maturing epididymal goat sperms showed increasing levels of cAMP during their transit from caput to proximal cauda with minimal degree of forward progression. The last phase of its transit in epididymis, that is from proximal to distal cauda, was associated with sharp rise in cAMP level, decreased activity of sperm cAMP phosphodiesterase (PDE) and flagellar motility. Indeed, theophylline, a specific inhibitor of PDE was shown to initiate forward motility in goat and ram spermatozoa obtained from rete testis *in vitro* suggesting that acquisition of motility by sperms is not dependent on motility-promoting protein(s) in epididymal plasma alone (57). Pentoxifylline (PF) is used to enhance motility of spermatozoa from infertile human subjects. Seshagiri and co-workers at the Indian Institute of Science, Bangalore, explored the PF induced changes in motility kinematics of hamster spermatozoa by a computer aided sperm analyser to determine the timing of onset of hyperactivation and acrososme reaction in spermatozoa (58). Pentoxifylline stimulated early onset of sperm capacitation may be mediated by an early rise in cAMP and intracellular calcium and involves protein kinase A activity. Furthermore, pentoxifylline stimulated acrosome reaction may require phospholipase A2 and protein kinase C activity (57, 58).

An anti-androgen, cyproterone acetate, has been employed to assess its effects on testicular and epididymal structure and function in a non-human primate species (59, 60). Low dose administration of cyproterone actetate (CPA) for 70 days caused extensive degenerative changes in the morphology of seminiferous, efferent duct and epididymal epithelia including decrease in the diameter of seminiferous and epididymal tubules and their lumen, height of epididymal epithelium and an increase in inter-tubular connective tissue. Significant changes were noted in sperm protein profile during their transit through epididymal lumen, CPA resulted in the appearance of 14 new polypeptides, probably of lysosomal origin, in epididymal cytosol and luminal fluid. The authors conclude that low dose exposure to CPA causes spermatogenic arrest, degenerative changes in epididymal structure along with alterations in epididymal and sperm protein profile. Suppression of serum T levels indicates the need for androgen supplementation if CPA is to be used for male contraception. The role/need for estrogen in regulating testicular function of adult male bonnet monkeys has also been examined (61). Oral administration of CGP 47645, a long acting non-steroidal aromatase inhibitor once every 5 days for 150 days resulted in a 10 fold increment in nocturnal serum T levels and a 2 fold increase in basal T levels throughout the treatment period. Analysis of sperm scores revealed 90% decrease in sperm counts in 4 out of 5 monkeys between days 55-85 of treatment and loss of sperm motility. Flow cytometric analysis of testicular germ cells from biopsy tissue on days 63 and 120 suggested inhibition in spermiogenesis process and epididymal sperm maturation was also inhibited leading the authors to suggest that estrogen may play a role in providing normal testicular and sperm function in the primate.

At the Institute for Research in Reproduction in Mumbai, Hedge, Iyer and coworkers intensified the search for sperm

antigens and developed monoclonal antibodies against them for pursuance of studies to elucidate their structure-function relationship. Three types of cell membrane antigens have been detected on mammalian spermatozoa. Intrinsic antigens present at the time of spermatogenesis in germ cells, maturation antigens acquired by spermatozoa during their transit through epididymis or coating antigens present only in ejalculated spermatozoa (62). Antigens detected by a monoclonal antibody (D2G4) were identified as two androgen dependent glycoproteins of 26 and 45 kDa expressed during epididymal maturation only in cauda spermatozoa. Antigens recognized by D2G4 were shown to play a critical role in reproduction as antifertility was observed in mice injected with the antibody on proestrous (62) and this could be due to with agglutination of sperms in the female genital tract, inhibition of fertilization and failure of embryo development (63). An antigen was detected by monoclonal antibody, D7G3 in testicular germ cells from spermatogonial stage onwards, and during transit of sperms through epididymis antigen reorganization was clearly evident (63-66). The changes which appeared during epididymal transit caused antigen expression to be restricted to specifics domains of sperm membrane. It is indeed tempting to propose that sperm-epididymal interactions offers a scope for the selection of most suitable spermatozoon for fertilization (67).

Ovarian physiology

The role of estrogen in inducing follicular maturation as an autocrine and paracrine regulator has been examined by Moudgal and colleagues using CGS 16949A, fadrozole hydrochloride, a non-steroidal aromatase inhibitor. A species-specific requirement of estrogen to support reproductive cycle in the female was observed (68, 69). At the Institute for Research in Reproduction, Mumbai, Nandedkar and colleagues observed suppression of antral follicle formation in ovarian follicles of marmosets by human ovarian follicular fluid peptide (hGF2) (70). Disrupted steroidogenesis with decreased levels of progesterone and estrogen were observed in bonnet monkeys injected with partially purified human ovarian follicular fluid peptide (71). Using a polyclonal antibody raised against hGF2, the peptide was detected in medium, but not in large follicles of human ovary (72). Further purification of ovarian folliclular fluid peptide (OFFP) using ultrafiltration, gel chromatography and fast performance liquid chromatography and reversed phase-high pressure liquid chromatography revealed that OFFP is a small (<5 kDa) peptide that competes with FSH for binding to granulosa cells *in vitro* and inhibits progesterone secretion by granolas cells in culture. The peptide is reported to induce granulosa cell apoptosis and follicular atresia (73).

Fertilization, embryogenesis, implantation and placentation

The zona pellucida surrounding mammalian oocyte mediates the initial recognition and binding of spermatozoon in a relatively species-specific manner. The zona pellucida contains three biochemically and immunologically distinct glycoproteins, ZP1, ZP2 and ZP3. The critical role of zona proteins in reproduction, together with their tissue-specificity allow them to be considered as potential candidate antigens for immunocontraception. At the National

Institute of Immunology, New Delhi, Gupta and colleagues have characterized human zona pellucida glycoproteins in order to understand their functions at fertilization (74). A 978 bp corresponding to bonnet monkey ZP3 excluding the N-terminus signal sequence and the C-terminus transmembrane domain was used to generate cDNA clone and antisera raised against the protein r-ZP3 tested by indirect immunofluorescence revealed positive fluorescence with zona pellucida in ovarian sections of bonnet monkey. It is anticipated that the availability of r-ZP3 will be helpful towards understanding auto-immune oophoritis associated with ZP3 immunization in non-human primates (75). It has been suggested that the sequence of bonnet monkey ZP1 and the availability of recombinant protein will help towards a better understanding and evaluation of the contraceptive potential of homologous immunization in a non-human primate model (76). To avoid the problems associated with autoimmune oophoritis and ovarian dysfunction synthetic peptides corresponding to B cell epitope(s) and devoid of oophorogenetic T cell epitopes as immunogens have been proposed. The immunoreactivity and *in vitro* effect on human sperm-egg binding was successfully tested using antibodies against peptides corresponding to bonnet monkey ZP3 glycoproteins (77). DNA-recombinant technology has thus made it possible to generate large quantities of zona proteins and of antigens devoid of other ovarian-associated proteins for their use in the development of contraceptive vaccine (78, 79).

A Ca^{++}-dependent sialic acid-binding protein (SABP) of human endometrium has been identified by Chowdhury and colleagues (80) at the Indian Institiute of Chemical Biology, Calcutta, which specifically bound to human sperm head plasma membrane *in vitro* and was found to increase percentage motility and acrososme-reacted pattern of uncapacitated spermatozoa. This protein was synthesized in the endometrium and secreted into uterine fluid. This intra-uterine factor may play a significant role in promoting post-release maturation of ejaculated spermatozoa by enhancing ^{45}Ca uptake into spermatozoa by a pathway which is insensitive to calcium-channel blockers. Human endometrial SABP was shown to be a Ca^{++}-binding protein inducing sperm capacitation and subsequent acrososme reaction *in vitro* by increasing intracellular Ca^{++} concentration.

The influence of various biomolecules secreted by oviductal and endometrial cells in creating the appropriate milieu facilitating embryo growth and viability and the definition of the metabolic consituents for optimal embryo growth *in vitro* are being debated by scientists across the world. Seshagiri and colleagues at the Indian Institute of Science, Bangalore identified that non-glucose oxidizable energy substrates such as succinate and malate supplemented to chemically defined, protein-free modified hamster embryo culture medium-2 (HECM-2) led to 100% development of hamster 8 cell embryos to high quality viable blastocysts (81). Supplementation of succinate, amino acids, vitamins such as inositol, pantothenate, choline chloride and bovine serum albumin (BSA) to HCEM-2 supported 100% development of zona-escaped blastocysts bearing higher mean cell number than those developing in BSA-free HECM-2. Trophoblast outgrowth and their attachment *in vitro*, however, required the presence of

factors present in bovine fetal serum (82).

Growth factors contributed by oviductal and endometrial cells are considered necessary for optimal growth and differentiation of embryonic cells during the pre- and peri-implantation stages of development (83). Studies conducted by Ghosh, Sengupta and colleagues at the All India Institute of Medical Sciences in New Delhi investigated preimplantation embryo growth and viability in the rhesus monkey with or without early luteal phase exposure to a single, low dose of mifepristone, an anti-progestin which effectively blocks progesterone action at the endometrial level (84). Exposure of the mother to anti-progestin on day 2 post-ovulation resulted in failure of morula to blastocyst transition stage and loss of viability following transfer of embryos to synchronous, non-mated, cyclic recipients (85). Ultrastructural examination of morulae and blastocysts revealed that the observed failure in embryo development, differentiation and viability following mifepristone exposure was associated with loss of cell polarity, lack of mitochondrial maturity, and lack of differentiation in trophoblast cells (86). The precise nature of causative factors responsible for the observed developmental defects in preimplantation stage embryos leading to their loss of viability following mifepristone exposure *in situ* remains to be investigated. Kamboj and colleagues at the Central Drug Research Institute, Lucknow observed that a pure non-steroidal anti-estrogen, CDRI-85/287 administered post-coitally to rats led to slightly faster rate of tubal transport but rates of embryo cleavage, blastocyst formation and zona shedding remained unaffected; anti-estrogen exposed embryos when transferred to host uteri yielded normal term fetuses (87).

At the Indian Institute of Chemical Biology, Calcutta, Chowdhury and colleagues investigated the ovarian steroid hormonal regulation of a sialic-acid binding protein (SAS) synthesized in rat uterus (88). The molecular characteristics of SAS agglutinins isolated from rat uterus at different stages of estrous cycle indicated that they are glycoproteins of molecular weights ranging between 28-31 kDa. Functionally and immunologically these proteins were grouped as estrogenic and progestogenic SAS agglutinins (89). A mannose-containing glycoprotein/lectin named as uterine agglutinin (UA) was purified from rat peri-implantation uterine tissue having mannose-6-phosphate binding sites (90). Experiments conducted to study the functional significance of this protein found in rat uterus on day 4 post-coitum revealed that UA may not be directly involved in sugar-sugar interactions with embryo since it is not present in any significant amount in pregnant uterus from day 5 onwards. The authors suggest that UA may act through some other factor which from preliminary studies is suggested to be TGFβ3 (91).

Ghosh and Sengupta working at the All India Institute of Medical Sciences in New Delhi used timed, proven-fecund and non-fecund endometria from rhesus monkey to demonstrate that the presence of a viable, developing preimplantation stage embryo is associated with endometrial glandular hyperplasia, stromal edema, capillary endothelial differentiation favouring transcytosis, vascular permeability and a 'switch off of lysosomal enzymic machinery to inhibit the onset of menstruation were observed as features supporting receptivity for implantation (92). The hypothesis that differential responses occur during the

luteal phase in endometrium functionalis in the presence of preimplantation stage blastocyst established a new paradigm in implantation biology. The presence of embryonic inputs resulted in increased retention of nuclear receptors for estrogen and progesterone, and an altered ratio of estrogen to progesterone in timed, proven-fecund, endometrial tissue samples (93-95). Though the nature of embryonic paracrine factors influencing endometrial responses is not known for certain, data suggest that prostaglandin E2 of embryonic origin could be one such factor (96).

Scientists have for long been intrigued to investigate the endocrine requirements for implantation especially in primate species since this basic information can be applied to the development of agents aimed to regulate fertility and embryo implantation. At the All India Institute of Medical Sciences, using the rhesus monkey as an experimental animal, Ghosh and colleagues provided conclusive evidence that luteal phase estrogen of ovarian origin is not essential for progesterone-dependent endometrial receptivity and blastocyst implantation and pregnancy maintenance (97). Long-term ovariectomized and acutely ovariectomized recipients were used for the transfer of viable preimplantation stage embryos and progesterone supplementation alone supported implantation, placentation and normal delivery of infants. At the same time, there is however insufficient evidence to rule out the possibility that estrogen from local sources such as embryo (98, 99) and endometrial cells (100) does indeed play a role in the implantation reaction. It has been suggested that the presence of a naive capacity or an inducible capacity in preimplantation blastocyst towards biotransforming sex steroids can give rise to a field property that interacts with the field induced by progesterone and locally creates an activity towards implantation (101, 102).

It is well established that for most mammalian species progesterone is required to support embryo implantation and decidualization of the uterus. Using a potent antiprogestin, mifepristone (RU486) Ghosh and Sengupta established an experimental model to investigate endocrine and paracrine regulation of endometrium leading to receptivity for implantation in proven-fecund cycles of the rhesus monkey. A single dose administration of RU 486 on day 2 post-ovulation resulted in failure of implantation with no alteration in the peripheral levels of estrogen and progesterone, and in the profiles of menstrual cyclicity in three consecutive cycles suggesting endometrium as the site of action of the drug (103). Failure of implantation associated with developmental defects in pre-implantation stage embryo and loss of embryo viability was also marked with significant morphological defects in endometrial growth and differentiation (84-86). The paracrine network of vascular endothelial growth factor (VEGF) - leukemia inhibitory factor (LIF) - transforming growth factor β1 (TGFβ1) in glandular, stromal and vascular cells of receptive and non-receptive (RU486-exposed) stage endometria from proven-fecund cycles of the rhesus monkey was significantly altered suggesting a modulatory role of progesterone (104). Puri and colleagues at the Institute for Research in Reproduction in Mumbai have reported that in the marmoset monkey, endometrial LIF is under maternal control and is secreted in response to the increased concentration of progesterone in circulation. High concentrations of glandular LIF at the

mid-luteal phase may prepare the endometrium for blastocyst implantation (105). Placental protein 14 (PP14), a glycoprotein secreted during the secretory stage of cycle has been implicated in the process of endometrial preparation for blastocyst implantation under progesterone dominance (106). The molecular mechanisms for the function of PP14 are now being studied at the Indian Institute of Science, Bangalore by Karande and colleagues. Using recombinant DNA technology PP14 cDNA was isolated from first trimester human endometrium and after confirming the identity of the gene, recombinant proteins were immunocharacterized and was found to be present in human amniotic PP14 at molecular weight 24 kDa in Western blot analysis (107). In a collaborative study between AIIMS and IISc, the contragestional effect of early luteal phase mifepristone treatment was associated with significant decline in the concentration of immunodetectable PP14 in implantation stage endometrial glands and its secretion in the rhesus monkey (108).

The rescue of corpus luteum by chorionic gonadotropin secreted by trophoblast cells at blastocyst implantation leads to increased secretion of ovarian steroid hormones, estrogen and progesterone into peripheral circulation. The specific sites in the pathway of placental progesterone biosynthesis that underlie the action of human chorionic gonadotropin (hCG) has been examined by Das and colleagues at the All India Institute of Medical Sciences. A stimulatory effect of hCG on normal trophoblast cells at the level of low density lipoprotein (LDL) utilization and cytochrome P450 SCC enzyme has been reported, dibutryl cAMP could mimic these actions of hCG suggesting a possible autocrine-paracrine role of hCG on trophoblast cells (109). Estrogen was found to induce GnRH receptors in trophoblast cells of term placentae (110). In a study designed to examine the maternal and embryonic signal-response mechanisms during embryo implantation, Ghosh et al. (111) examined the circulatory profiles of estrogen, progesterone, chorionic gonadotropin (CG) and relaxin during natural pregnancy cycles and in embryo transfer cycle in the rhesus monkey. A delay of about three days was observed in the appearance of CG and relaxin in circulation between natural mated and embryo transfer conception cycles. The experimental model used to collect and transfer embryos in the laboratory macaque with high efficiency and without the use of exogenous hormone therapy provides a new avenue to study natural and induced early pregnancy loss in the primate.

The endocrine equivalents of trophoblast invasion and placentation have recently been reported for the macaque. Ghosh et al. (112) analyzed the immunocytochemical distribution of receptors for estrogen (ER) and progesterone (PR) at lacunar and villous stages of implantation and placentation in the rhesus monkey, and observed that in pre-villous and villous stages, cytotrophoblast and syncytiotrophoblast cells were PR positive and ER negative. Maternal endometrial cells were ER negative while a heterogenous pattern of PR immunopositivity was obtained which correlated well with glandular hyperplasia and differentiation, stromal-decidual transformation and vascular response seen at implantation sites. However, the authors address a number of issues as areas for further detailed investigations which include the physiological significance of differential immunostaining of PR in glands

of basalis region as compared to the superficial zone; the stage-specific expression of PR in trophoblast cells at pre-villous and villous stages and in different trophoblast cell populations *vis-a-vis* their cell polarization and invasive functions. Among other factors, it has been demonstrated recently that nitric oxide may play significant role in the process of trophoblast proliferation and differentiation (113).

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