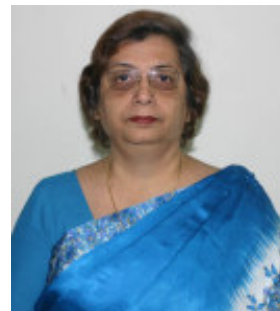


Executive Summary

The Institute, a premier research organization of the Indian Council of Medical Research addresses problems in Reproductive health and continues to strive for excellence in its scientific endeavors.



The Institute has, during the last few years, made progress in terms of infrastructure, modernization and capacity building efforts to meet the challenges of scientific output. The enormous task, with the unstinting support of the council is being undertaken with great zeal and perseverance.

During the reporting year, the Institute has made remarkable contributions in the area of microbicide development, HIV pathogenesis, RTI/STIs. Scientists at the Institute earned many firsts to their credit. It has been demonstrated that the HIV binding 160 kDa protein is a mannose receptor on sperm/vaginal cells through which HIV binding occurs in these cells. The Institute also played a key role in development of the National Guidelines for RTI/STIs including HIV/AIDS to be incorporated in RCH2 programme. Stem cell research gained momentum and in the next few years we will be ready to harness the benefits of embryonic stem cell lines for fundamental research. Studies to elucidate the genetic determinants of various reproductive disorders such as polycystic ovarian syndrome, premature ovarian failure and congenital adrenal hyperplasia have provided some interesting leads. These programmes are being expanded to explore the clinical significance of these observations.

A National Centre for Preclinical Reproductive and Genetic Toxicology, funded jointly by the Department of Science and Technology and the ICMR, has been established. Research facilities to carry out general reproductive and genetic testing, teratogenicity, prenatal and postnatal developmental toxicity and mutagenicity have been established. The center would evaluate new products and devices for their safety. The center would also work towards training manpower and disseminate information on related issues. These services are being offered to scientists at the Institute, industry and other organizations.

The laboratory, equipped with various biosafety measures for experimentation with HIV and other viruses, has also been made functional. This will expedite the pace of our research programmes on elucidation of the mode of sexual transmission of HIV.

It is indeed commendable that scientists at the Institute were bestowed with several prestigious awards – Swaran Kanta Dingley Oration Award and the Nations Academy of Medical Sciences Award during the reporting year. The Institute scientists, as in previous years, were instrumental in organizing several training courses for WHO In-Country fellows and other professionals and briefed them with the latest technical and conceptual advancements in the area of reproductive biology.

1 Expanding Contraceptive Choices

1.1 Identification and Characterization of Sperm Antigens Using Multifaceted Approach

Several different approaches have been exploited to identify sperm specific proteins, which could be used as targets for contraception or as markers for infertility diagnosis.

Neonatal Tolerization

Indirect immunofluorescence was done with sera of animals immunized with different immunogens, to elucidate regionalized distribution of antigen on rat sperm as well as to check if the animals showed reactivity in the domains with which the immunogens were prepared. Group HI and HS show staining on the acrosomal cap, indicating domain specific immune response, to the immunogen (sperm head) used for immunization. Sera of animals from group FI immunized with intact fraction of flagella showed fluorescence staining on the midpiece and principal piece. However, sera of animals immunized with soluble protein of flagellar fractions (FS), showed staining on the acrosome, midpiece and principal piece. Post tolerization sera used, as negative control did not show any staining. Indirect immunofluorescence using non-permeabilized human sperm was done to determine whether the cognate proteins are conserved and show similar domain specificity as in the rat sperm. Group HI, FI and FS showed localization on the domain with which the animals were immunized. Group HS showed fluorescence on either acrosome or equatorial region. PT sera did not show any fluorescence staining.

Antisperm Antibodies

Using vasectomy induced autoimmunity approach a sperm auto antigen termed TSA70 which on sequencing indicated homology with cenexin molecule has been identified. During the reporting period experiments were carried out for molecular characterization of this molecule. *In silico* analysis of peptides

following tryptic digestion exhibited number of predicted phosphorylation sites at 5 serine residues, 2 threonine residues and 1 tyrosine residue. The analysis on leucine zippers showed the presence of two leucine zippers further reiterating the fact that TSA70 belongs to Odf2 family. Based on *in silico* predictions of potential phosphorylation sites, we determined whether TSA70 is a phosphoprotein by probing with antibodies to phosphorylated residues. Immunoblotting of TSA70 with anti-phospho serine, threonine or tyrosine antibodies indicated that the cognate protein is phosphorylated at serine, threonine as well as tyrosine residues. Northern blotting results using multiple tissues showed testis specific presence of a transcript of expected size (1.518kb) and two more transcripts of ~ 2.4 kb and 2.2 kb. The additional transcripts could be splice variants.

1.2 Role of a Novel Androgen Regulated HoxB2 Containing Gene Expressed in the Epididymis

An epididymis-specific sperm protein designated HOXBES2 was identified by screening an epididymal cDNA library using an agglutinating antibody raised against washed human sperm. The positive clone (AF255949) showed homology to the conserved region of transcription factor HOXB2. Using a DIG-labeled DNA fragment including the conserved HOXB2 region and a polyclonal antibody raised to the conserved region as probes; presence of its single 2.5kb transcript, epididymis-specific expression of the 30kDa protein, regionalized and cell-type specific expression, androgen dependency, postnatal-developmental expression, species conservation and its association to sperm were established. Acrosome reaction indicates a probable role for HOXBES2 in sperm-egg interaction and sperm maturation. The data further indicated that HOXBES2 shares epitope similarity with embryonic HOXB2 transcription factor. The 1.657 kb full-length cDNA sequence of Hoxbes2 gene was obtained from rat epididymis using 5'RACE-PCR approach. The full length mRNA (DQ399532) as well as its putative protein (ABD73307) sequences were submitted to the NCBI GenBank and rat genome database. Sequence analysis of Hoxbes2 reiterated the previous observation on its sequence similarity to HOXB2 transactivator with an upstream extension of 560 bp. These observations confirm further that HOXBES2 has a probable role to play in sperm function during fertilization. A 95% pure, 25 amino acid peptide (N-FQNRRMKHKRQTQHREPPDGEPACP-C) corresponding to the highly hydrophilic region, most antigenic and immunodominant region of HOXBES2 protein was commercially synthesized. It was conjugated to Keyhole Lymphocyte Haemocyanin (KLH), as a carrier molecule for immunization in

rabbits and the molecular mass of the peptide confirmed by Mass-spec analysis. Preimmune serum has been collected prior to initiation of immunization with the conjugated peptide.

The data obtained from immunofluorescence indicated that HOXBES2 protein is localized on the sperm surface undergoes modification in presence of various energy substrates such as ATP, cAMP, Ca²⁺ and creatine phosphate, suggesting that HOXBES2 has an indirect role to play in the process of sperm capacitation, hyperactivation and acrosome reaction. To obtain the recombinant HOXBES2 protein, the ORF of the Hoxbes2 gene constructed in Pmt/v5-His-TOPO vector was induced with a combination of 0.2 mM CuSO₄ and 0.5 mM IPTG. Expression of the recombinant HOXBES2 was initiated 3 hrs post induction and reached maximum levels by 6 hr. The protein expressed was analyzed and confirmed by dot-blot and Western blot analysis. The anti-HOXBES2 peptide antibody raised will be used to characterize and to define the role of HOXBES2 protein in epididymis with respect to sperm maturation.

1.3 Studies with 80kDa Human Sperm Antigen (80kDa HSA) and its Synthetic Peptides for Immunocontraception

80kDa human sperm antigen (80kDa HSA) is proved to the potential candidate for development of antifertility vaccine. Active immunization with purified antigen induced reversible infertility in male and female rats. Immunohistochemical, immunofluorescent studies using specific antibodies to 80kDa HSA demonstrated that it is sperm specific protein. Immunofluorescent studies demonstrated that 80kDa HSA is localized predominantly in the tail region and also acrosomal region in few marmoset sperm. In the case of human spermatozoa the immunofluorescent localization was found predominantly in the post acrosomal region of the acrosome intact and in the acrosomal cap of acrosome reacted human sperm. While in rat the localization was in the head region of the acrosome intact and in the tail region of the acrosome reacted sperm. Incubation of anti 80kDa HSA antibodies with human, rat and monkey spermatozoa, in-vitro resulted in agglutination

The peptides corresponding to partial N terminal amino acid sequence of 80kDa HSA (Peptide NT) and its peptides obtained by enzymatic digestion with endoproteinase Lys C (Peptides 1, 2 and 4) were synthesized, conjugated to keyhole limpet haemocyanin (KLH) and raised the antibodies in rabbits. Peptide 3 failed elicit significant antibody titer and hence was not further investigated. Peptides NT, 1, 2 and 4 immunobiologically mimic native protein. Passive

administration of antibodies to these peptides resulted agglutination of rat epididymal spermatozoa with loss of motility and impaired fertility in male and female rats. Antibodies to peptides NT and 1 were found to be most effective in inhibiting fertility. Passive administration of purified IgG of antibodies to peptides 1 and NT in male and female rats also resulted in dose dependent inhibition of fertility in male and female rats.

Active immunization of male rabbits with KLH conjugated peptides 1 and NT elicited gradual increase in antibody titer. The ejaculated spermatozoa collected using artificial vagina showed agglutination with complete loss of motility and these rabbits failed to impregnate the normal females. Active immunization with peptide 1 induced 100 percent infertility while with peptide NT induced 60 percent infertility in male rabbits. The fertility was regained with decline in antibody titer, following cessation of immunization.

The antifertility effect of active immunization with peptide 1 was evaluated using no-human primate model. The normal fertile adult male marmosets were actively immunized with KLH conjugated peptide-1, which showed gradual increase in antibody titer. The epididymal spermatozoa of these animals showed complete loss of sperm motility. Six out of seven animals (85%) showed the significant antibody titer and these animals failed to impregnate normal female. All the infertile animals regained their fertility with decline in antibody titer following cessation of immunization. The antifertility effect of active immunization with peptide 1 in male marmoset is being confirmed independently by another investigator. Antifertility studies are also being further investigated in male bonnet monkeys. Five male monkeys and ten females are being investigated for their fertility status. Sperm count and morphology of these males were found normal. Females were found normal cycling. The proven fertile males will be immunized with KLH conjugated peptide 1 and antifertility effect will be evaluated.

Immunohistochemically developmental expression of 80kDa HSA has been detected in rat testis and epididymis after day 40 of age. Similar results were also obtained in the rat testis using antibodies to peptide-NT. Treatment with Ethane Dimethane Sulphonate (EDS), an antiandrogen suppressed testosterone levels which resulted in decreased expression of 80kDa HSA in rat testis and epididymis and the supplementation of EDS treated rats with testosterone restored the immunolocalization of 80kDa HSA. The data suggest that the expression of 80kDa HSA is androgen regulated.

Using the primers designed based on the partial amino acid sequence of peptide NT and peptide 6, 540bp partial cDNA sequence was determined which showed the homology with hypothetical testicular protein. Attempts are being made to determine full-length cDNA sequence of 80kDa HSA and cloning into appropriate expression vector to obtain recombinant protein.

The data suggest that 80kDa HSA is developmentally regulated and its peptides immunobiologically mimic the native protein and are immunogenic. Thus suggesting the utility of 80kDa HSA and its synthetic peptides as candidate for development of antifertility vaccine.

1.4 Modulation of c-kit Proto-Oncogene Function During Spermatogenesis in Mice

The rising incidence of male infertility is a cause of great concern. Approaches to this problem will need a basic understanding of the process of spermatogenesis at the molecular level. In our earlier studies, that c-kit/SCF plays a critical role in the differentiation of germ cells and their survival *in vitro* has been shown. Studies were undertaken to determine the role of c-kit in PGCs formation, proliferation and migration using c-kit. The number of PGCs positive for AIP and Brdu were drastically reduced after c-kit antibody treatment, suggesting c-kit plays a critical role in the formation, proliferation and migration of PGC.

During the year, the cell specific expression of different genes in the normal testis and in the testis of mice born to the mothers who had received c-kit antibody was also studied. The marker genes of spermatogonia (c-kit, OCT-4, Nucleostemin, c-ret, Ep-CAM), sertoli cells (SCF, AR, PEM-I, Inhibin- α , Gata-I, SF-I, SGP-2, FSHr) and leydig cells (AR, LHR, β -HSD, SF-I) were determined. The results indicated that the expression of several of the genes was altered between the two groups.

C-kit gene silencing using RNAi interference: Chemically synthesized, target specific 21 nt SiRNA (sense and antisense) with 2-nt 3' overhangs of identical sequence were used for targeting c-kit mRNA and β -actin. The SiRNA was tested in c-kit expressing P815 (mouse mastocytoma origin) cell lines for determining its ability to silence the endogenous c-kit gene expression. With this approach, we were able to generate a highly efficiency SiRNA specific for c-kit which shows potent knockdown (~80%) of c-kit at concentration 37.5 ng.

1.5 Studies with FSH Binding Inhibitor: Functional Significance of FSH Modulators from Follicular Fluid in Ovarian Pathophysiology

The follicle-stimulating hormone binding inhibitor (FSHBI) has been purified from human ovarian follicular fluid by our group. The partially purified fraction (hGF2) of FSHBI was further purified, its partial N – terminal 8 amino acid sequence was obtained which was termed as Octapeptide (OP). hGF2 and OP have exhibited antiovarulatory / antifertility activity in rodents and non – human primates. However, the treatment in monkeys was administered for 8 – 10 days. To avoid multiple injections, an approach of slow release of the peptide by nano drug delivery system has been attempted. Aim of the present study was therefore to develop hGF2 / OP - nanoparticles exhibiting a slow release profile. Nanoparticles (NP) were prepared by double emulsion methodology using Alkyl Poly-Glucoside (APG) / Poly vinyl alcohol (PVA) as surfactants. On the basis of physical properties, PVA was found to be better than APG. Further characterization was done based on drug loading and encapsulation efficiency. The release profile studies in vitro are being conducted.

1.6 Factors regulating early Folliculogenesis in Mouse Ovary

The follicle of the mammalian ovary develops in several distinct stages during folliculogenesis. The ovary contains a pool of primordial follicles containing oocytes arrested in meiosis, which are the source of developing follicles for the female. Transition of arrested primordial follicles to primary and secondary follicles is a crucial process in female fertility. Relatively few genes are known which control these events, thus identification of additional genes expressed during follicular development may reveal key players in this process. To investigate this issue, we extracted RNA from the ovaries of neonatal mice on day-2 and day-4 and the gene expression profiles by cDNA arrays was analysed. The results revealed that 30 percent genes were differentially expressed during the transition of primordial to primary follicles. Amongst these genes, the up regulation of cell cycle regulators, neurotransmitters, signal transducers, growth factors was predominant. Apart from these genes, differential expression of several molecules that have not been reported previously in the ovary but associated with follicle development were identified. These include the members of olfactory receptor gene family which are G-protein coupled receptors. This is the novel finding. The olfactory receptor gene family was found to be up regulated in day-4 ovaries compared to day-2 ovaries. We validated the presence of olfactory receptors by in-situ hybridization. We observed that the transcripts were found in the granulosa cells of day-4 ovaries but absent in day-2 ovaries.

Our results suggest that specific members of olfactory receptor gene family may have a function in proliferation.

1.7 Acceptability and Continuation Rates of 2 Monthly Injectable Contraceptive: Norethisterone Enanthate

By and large, it is felt that there is a need to increase the range of contraceptive choices to facilitate eligible couples to fulfill their unmet need for contraception. A multicentre study among 1209 women from diverse socio-cultural backgrounds has been initiated to obtain recent data on the injectable contraceptive, Norethisterone Enanthate (Net-En). The findings will be important for recommendation to programme managers before its introduction into the National programme. The duration of the injection is for two years and thereafter women are being followed up for return to conception for one to two years.

The objectives of the study are to i) assess user acceptability/ continuation rates of injectable contraceptive, Norethisterone Enanthate ii) evaluate the incidence of menstrual irregularities and other side effects iii) assess socio-behavioural aspects of users and compare with different regions and cultural settings iv) study the return of fertility following discontinuation of injection.

Salient observations are based on 17268 women months of injection Net-En use enrolling 1209 women. Continuation rates using a life-table method of calculation at the end of 12, 18 and 24 months are 65 percent, 53.6 percent and 48.3 percent respectively Majority of discontinuations were due to personal reasons and lost to follow up / migration because of floating population.

Participants were interviewed after discontinuation of injection about their views and attitudes towards this method. Over 79 percent of women were satisfied with the method. 88.5 percent said that the 2 monthly schedule of injection is “convenient to comply with”. Over 89 percent of women expressed that the method should be available in our National Family Welfare Programme (NFWP) as the “bimonthly schedule is convenient as against daily intake like oral pills”, “majority of women can afford and access through NFWP” “It does not have effect on breast milk”. About 39 percent of women expressed that they would like to use this method beyond 2 years.

1.7.1 Acceptability and Continuation Rates of 2 Monthly Injectable Contraceptive - Norethisterone Enanthate: Protocol Amendment for DEXA Study

In May 2005, a women's health group had raised issues about bone mass density (BMD) among long-term users of 3 monthly injectable contraceptive Depo-Provera. In view of this, Institute's Ethics Committee (IEC) recommended and approved BMD evaluation by DEXA among users of 2 monthly injectable contraceptive *Norethisterone Enanthate* (Net-En). The study has been initiated at 6 centres where DEXA facility is locally available. During the reporting period, a total of 142 active users of injection Net-En underwent initial DEXA scans. The use of injection varied between 12 to 16 months at the initial scan. Of these 73 eligible women still using injection Net-En underwent repeat DEXA at 6 months interval (i.e. after receiving 3 more injections). The final DEXA scans have been initiated one year after drug discontinuation i.e. during recovery phase. So far 69 women have been enrolled for final DEXA. Preliminary observations showed that there was marginal increase in mean BMD at both femoral neck and lumbar spine between initial and second DEXA scans. These observations are reassuring.

Return to fertility: Women who wished to conceive following drug discontinuation is being followed for 12 to 24 months for return to conception. During the reporting period a total of 135 women were eligible for return to conception and of these 92 percent conceived during the first 12 months following drug discontinuation.

1.8 Re-acceptance of contraceptives an intervention study

Two social workers in the clinical division attended INSA (International Services Association) training at Bangalore, and have carried out their projects. One is on 'Re-acceptance of contraceptives an intervention study' among 200 past users who discontinued for various reasons

It is a one-year study, (from March 2006) in which, following intervention, 84 percent women (N=166) accepted a spacing method and 16 percent underwent tubal sterilization. Program Officers from INSA came from a 2 days followup visit. Interventions included interpersonal, group and couple IEC and counseling; community activities were organized on Diwali, Children's day, World AIDs day to emphasize the relevant themes, share experiences and build rapport. Continuation rates of contraceptives accepted is high so far (16% have completed 9 months of use, 24% up to 6 months, 34% up to 3 months and 16% have initiated 1 month ago).

1.8 Interventions in Urban Slums for Enhancing Participation of Men in Reproductive Health

Male reproductive health involvement implies encouraging a range of positive reproductive health behaviours of men to help ensure women's and children's well-being as well as their own. There has been a general agreement among countries of the world, particularly after the International Conference on Population and Development (ICPD) held in Cairo in 1994, that there is a need for ensuring participation of men in family planning and reproductive health with a view to promote gender equality, sharing of reproductive responsibilities and also to meet men's own reproductive health needs. In India, the National Population Policy, the Reproductive and Child health Programmes (RCH) I and II, envisage the necessity of male involvement in improving reproductive health of men as well as women. The major emphasis is on improving vasectomy and involving men in safe motherhood. If men are brought into a wide range of reproductive health services in such a way that they are supported as equal partners and responsible parents, as well as clients in their own right, better outcomes are expected in reproductive health indicators such as contraceptive acceptance and continuation, safer sexual behaviour, use of reproductive health services, and reduction in reproductive morbidity and mortality. Hence, there is a need to address reproductive health concerns of men, as also of the couples for improving the reproductive health seeking behaviour of couples.

The overall objective of the study is to identify programme strategies contributing to effective participation for men in programmes aimed at improving reproductive health. Specific objectives of the project are to i) study the knowledge, perception and practices among married men and married couples regarding safe motherhood and family planning; to assess knowledge regarding RTIs/STIs and HIV/AIDS; to determine decision making process on issues related to safe motherhood, family planning, RTIs/STIs and HIV/AIDS; to investigate the reproductive health seeking behavior and the support they had received from their spouses; ii) plan appropriate intervention for enabling couples to gain correct knowledge about reproductive health issues concerning men and take appropriate actions to seek and avail reproductive health services and iii) evaluate the impact of interventions addressed to married men and married couples on their reproductive health seeking behavior.

Three comparable slum areas were selected (on the basis of similar population, infant mortality rate and infrastructure) with the help of Municipal Corporation of Greater Mumbai (MCGM) after surveying 12 health post areas

for the purpose of this research study. One is area-1 (Mohili Village) where intervention would be addressed to husbands only, second is area-2 (Bail Bazar) where intervention would be addressed to couples and third is control area (Asalfa Village) where no intervention is proposed. The ongoing government reproductive health and family welfare programmes will continue in all three-study areas. The information presented below is from the ongoing intervention in area-1 (Mohili Village) and intervention area-2 (Bail Bazar).

Intervention programmes

The findings of the baseline study suggested that there needs to be intervention in the areas of a) attaining gender equality, b) preventing unwanted/unplanned pregnancy by means of promoting knowledge and correct use of contraceptives including non-scalpel vasectomy, c) promoting safe abortion practice, d) promoting early registration of couples for ANCs and safe delivery, e) promoting spacing between two children, f) enhancing knowledge regarding RTIs/STIs and HIV/AIDS and providing counseling to people affected, g) promoting health seeking behaviour with regard to all the reproductive health problems, h) strengthening spousal communication, and i) enhancing male responsibility in reproductive and sexual health matters.

Accordingly, various interventions programmes have been carried out for husbands only in intervention area-1 (Mohili Village) and for couples in intervention area-2 (Bail Bazar) during the period April 2006 to February 2007. The intervention programmes have been through Information, Education and Counseling (IEC). The services provided at the MCGM health posts supported by the staff from the institute. Several gender cross cutting issues were addressed through the various programmes. Decision making process in each of the issues concerning reproductive health was enquired. This has helped in integrating this component in the various intervention programmes and thereby improving couple communication and involving men in decision-making process. The various programmes addressing each of the issues raised at the baseline survey and the respective services provided is given below:

Street Plays

Five street plays were organized in both areas on issues concerning safe motherhood (Attendance - 1975 persons). Nine street plays in Mohili Village and 10 street plays in Bail Bazar were organized on family planning and contraception (Attendance: 4495 persons). Five street plays in Mohili Village and 6 street plays in Bail Bazar were organized on RTIs/STIs (Attendance - 1875 persons). Ten street plays were organized in both areas HIV/AIDS, (Attendance - 4725 persons).

Smaller Group Meetings

Information on safe motherhood, family planning, RTIs/STIs and HIV/AIDS were provided in the smaller group meetings. In the area-1, 285 group meetings were organized for husbands for which, 2967 husbands attended. Whereas, in the area-2, 95, 84 and 15 meetings were organized for husbands, wives and couples respectively for which, 695 husbands, 686 wives and 55 couples attended respectively.

Inter-personal communication (IPC)

Husbands from Mohili Village, as well as husbands, wives and couples from Bail Bazar were contacted personally and information on four issues (i.e. safe motherhood, family planning and contraception, RTIs/STIs and HIV/AIDS) of reproductive health was provided to them. Information on family planning was provided to 2359 husbands from Mohili Village and 939 husbands, 1161 wives and 1172 couples from Bail Bazar. Information on correct use of condom was provided to 2475 husbands from Mohili Village and 1023 husbands, 1260 wives and 1379 couples from Bail Bazar. Information on non-scalpel vasectomy (NSV) was provided to 1358 husbands from Mohili Village, and 902 husbands, 1139 wives and 1146 couples from Bail Bazar. Information on RTIs/STIs was provided to 2501 husbands from Mohili Village and 980 husbands, 1396 wives and 1379 couples from Bail Bazar. Information on HIV/AIDS was provided to 2310 husbands from Mohili Village and 910 husbands, 1051 wives and 1135 couples from Bail Bazar. Information on Antenatal Care (ANC) was provided to 2235 husbands from Mohili Village and 962 husbands, 1285 wives and 1355 couples from Bail Bazar.

IEC material (Self learning material)

Pamphlets containing information with messages for men on safe motherhood, family planning, RTIs/STIs and HIV/AIDS were prepared and distributed to 400 husbands in Mohili Village and 400 couples in Bail Bazar. 2700 pamphlets giving information regarding Pap smear screening test and camps were distributed in each area. 1000 pamphlets giving information regarding clinic for men were distributed in Mohili Village and another 1000 pamphlets giving information regarding clinic for couples were distributed in Bail Bazar along with the messages on safe motherhood, family planning, RTIs/STIs and HIV/AIDS.

Educational Programmes on Reproductive Health

Audio-visual programmes were organized for providing information on safe motherhood, family planning, RTIs/STIs and HIV/AIDS. Sixty five programmes were organized for husbands in Mohili village for which 1806 husbands attended. In Bail Bazar, 23 programmes were organized, 143 wives and 147 couples attended these programmes.

Orientation Programmes

Orientation programmes on four issues (i.e. safe motherhood, family planning and contraception, RTIs/STIs and HIV/AIDS) of reproductive health were organized for Integrated Child Development Scheme (ICDS) project staff (i.e. anganwadi teachers and workers) in Mohili Village and for peer volunteers from Bail Bazar. Fifteen ICDS project staff from Mohili Village and 10 peer volunteers from Bail Bazar respectively, participated in these programmes.

Contact Visits

Visits to General physicians, Local mandal/NGOs and Volunteers were made to solicit their co-operation in fulfilling the aim of the project.

Cases Identified during intervention programmes

28 husbands, 13 wives and 6 couples from Mohili Village and 6 husbands, 80 wives and 37 couples from Bail Bazar reported RTIs/STIs, 4 husbands and one couple from Mohili Village as well as 8 couples from Bail Bazar reported infertility problem during intervention programmes.

Pap smear screening for Cervical Cancer

Nine camps each in Mohili Village and Bail Bazar, were conducted. A total of 157 and 189 pap smears were collected from women in Mohili Village and Bail Bazar respectively during these camps.

Counselling clinic

As part of the intervention, counselling services were provided in the Mohili Village municipal health post and Bail Bazar municipal health post. Counselling on problems such as white discharge, infertility, sexual problems, sexually transmitted infections, gynecological problems, burning micturition as well as information on reproductive health issues such as ANC/PNC, spacing contraceptive methods and Non Scalpel Vasectomy (NSV), were provided in these clinics once in a week on Monday at Mohili Village and Tuesday at Bail

Bazar health post. Suspected or HIV positive cases were also provided counseling and referral. 163 husbands, one wife and 31 couples attended counseling clinic at Mohili Village and 63 husbands, 227 wives and 40 couples attended the counseling clinic at Bail Bazar.

Services for treating Reproductive Health Problems

As a part of intervention programme, a Clinic for Men was initiated at the Mohili Village Municipal health post in the month of May 2006 and a Clinic for Couples was initiated at Bail Bazar Municipal health post for couples in the month of June 2006. Both clinics function on Sundays between 10.00 am to 1.00 pm.

Services for reproductive health problems such as white discharge, primary and secondary infertility, sexual problems, sexually transmitted infections, gynecological problems, burning micturition, information on ANC/PNC, motivation for NSV and post NSV follow up, counselling for HIV positive and suspected cases, motivation for condom use and information on reproductive health issues are provided along with the services in these clinics once a week on Sunday. Husbands, wives, couples and girls attended the clinics at Mohili Village and at Bail Bazar. Follow up services were also provided to them.

Informal discussions with beneficiaries reveal satisfactory results regarding the intervention programmes conducted and the services provided. Nevertheless, to understand the change that has taken place in the overall reproductive health knowledge, attitude and practice and the support provided by men to their spouse, a post intervention survey needs to be conducted and the planning and preparation for the post-intervention survey is underway.

2 Infertility and Reproductive Disorders

2.1 Studies on Genetic Aspects of Polycystic Ovary Syndrome

Polycystic Ovary Syndrome (PCOS) is one of the most common endocrinopathies affecting about 5-10 percent of women in their reproductive age. It is marked by anovulation manifested as amenorrhea, clinical and/ or biochemical evidence of hyperandrogenemia and presence of cystic ovaries on ultrasound. Altered LH: FSH ratio and obesity are other important features of PCOS. A genetic basis underlying PCOS is now well recognised.

Observations of familial segregation as well as increased prevalence of symptoms in first-degree relatives suggest a genetic trait to be involved in

pathophysiology of PCOS. A study has therefore been undertaken to elucidate this genetic basis of PCOS in a cohort of Indian women diagnosed with the syndrome. As obesity is one of the hallmark features of PCOS, it is logical to speculate that leptin, the product of obesity gene, could play an important role in pathophysiology of the syndrome. No data is yet available in the Indian scenario on genetic analysis of leptin in PCOS. Putative genetic variations in the leptin gene are therefore being investigated in the cohort of Indian women to determine their association with PCOS. Analysis of 60 women with PCOS and 30 age-matched controls showed a significant increase in testosterone levels in the former as compared to controls ($p < 0.001$), confirming hyperandrogenic profile of the PCOS cases. Also, leptin levels were increased significantly in the PCOS group as compared to controls ($p < 0.05$). In an attempt to assess the genotype phenotype association of leptin with PCOS, entire coding region of the gene was analyzed in PCOS cases vis-à-vis controls. No variation in the coding region has been observed so far. In one of the grossly obese subjects however a substitution variant A>G was seen which has not been reported earlier. A Genebank Accession number has been obtained for this variant (DQ054472). Analysis of the non coding sequences is in progress.

2.2 A Genetic Analysis of Polycystic Ovary Syndrome with Special Emphasis on Genes involved in Insulin Resistance

Polycystic ovary syndrome (PCOS) is a common heterogeneous disorder characterized by hyperandrogenism and chronic anovulation. The syndrome is frequently associated with an increased risk of insulin resistance and type 2 diabetes mellitus (T2DM); obesity exacerbates insulin resistance and favors the progression from impaired glucose tolerance to diabetes in these patients. The consequences of polycystic syndrome extend beyond reproductive axis: women with the disorder are at substantial risk of developing metabolic and cardiovascular abnormalities. The familial clustering of women with PCOS suggests that heredity is implicated in the origin of the syndrome. PCOS is a polygenic disorder. Studies indicate that PCOS and T2DM could share genetic susceptibility factors. The present study has been undertaken to determine the role of variants in the genes associated with insulin resistance like insulin receptor, insulin receptor substrates, calpain 10, calpain 5, peroxisome proliferator-activated receptor gamma and paraoxonase in the pathogenesis of PCOS.

Forty controls and sixty PCOS women have been evaluated so far after excluding diabetic subjects. Blood samples were collected from enrolled women,

at fasting state and 2hrs after 75g of glucose load. Glucose, insulin and other hormonal estimations were carried out in recruited subjects. Conventionally fasting glucose to insulin ratio < 4.5 is a marker of insulin resistance has been detected in 55 percent PCOS subjects.

Exon 17 of INSR gene have been amplified and subjected to direct sequencing. Analysis of sequence revealed the presence of one polymorphism C/T at His 1058 in both PCOS and controls. However, frequency of polymorphic T allele was found to be higher in PCOS women (49%) compared to control (25%). Screening also revealed the absence of the T/C polymorphism at Cys 1008 site as reported in Chinese population. Study subjects were screened for a major variant Pro12Ala SNP in PPRg gene. Eighteen control and twenty PCOS samples were sequenced. The preliminary data showed absence of Ala variant, which protects insulin sensitivity. All samples were found to be of wild type.

Variations in the gene encoding a cysteine protease CAPN10 are also associated with insulin resistance and influence predisposition to T2DM. To investigate role of CAPN10 in PCOS, two SNP sites i.e. SNP 19 and SNP 63 were been amplified. SNP 19 contained an insertion deletion polymorphism, allele1 (two repeats of 32 bp sequence) was 155bp and allele 2 (three repeats) was 187 bp. The preliminary data showed the frequency of allele 1 was 0.522 and 0.461 in control and PCOS subjects respectively.

2.3 Genetic Studies in Women with Premature Ovarian Failure

Genetic studies on premature ovarian failure were carried out with the objective to determine mutations/premutations in the genes associated with the disorder. Mutational analysis has revealed a mutation in the FOXL2 gene in 2.5 percent of the POF cases. This encouraged us to look for other genes involved in Premature Ovarian Failure and a new gene, NOBOX was incorporated in the study. Eighty women under the age of forty years with secondary amenorrhoea, elevated serum FSH levels (>40 mIU/ml) and normal karyotype, and sixty-six regularly cycling fertile women have been studied, along with twenty-five family members of nineteen index cases. Thirteen women with poor ovarian reserve (POR) were also enrolled and studied.

Mutational analysis of the NOBOX gene was carried out in the study and control groups. NOBOX is a homeobox gene expressed in the ovaries at high levels. The entire NOBOX gene in humans has not yet been identified. So far 5 exons of this gene have been predicted in humans. Hence primers for the NOBOX gene from the predicted exons, i.e., exons 2 - 6, were designed and this region

was PCR amplified. Using heteroduplex analysis this region was screened for mutations. No mutations have been detected in the control and study groups.

In continuation of the work being carried out on the Inhibin, FMR1 and FOXL2 genes, in addition to the C>T transition that was detected earlier, we also detected a 773C>G transversion in the coding region of the FOXL2 gene. The study is being continued with recruitment of more cases and controls.

2.4 Assessment of Gene Mutations Associated with Congenital Adrenal Hyperplasia

Congenital adrenal hyperplasia (CAH) due to deficiency of 21-hydroxylase enzyme is the most common autosomal recessive disorder, with a wide range of clinical manifestations ranging from severe, which can be lethal, to a mild form. The disease is commonly attributed to mutations in the 21-hydroxylase gene (CYP21), encoding 21-hydroxylase enzyme. CYP21 is located in the major histocompatibility complex (MHC) locus on chromosome 6p21.3 in the HLA class III region. In close proximity to active CYP21, the pseudogene CYP21P is present. The sequence similarity and the proximity of CYP21 and CYP21P predispose to exchange of material between these two genes as a consequence of misalignment during meiosis followed by reciprocal recombination. This can generate variation in copy number among individuals. In addition, about 95 percent of mutations causing CAH are transferred from pseudogene to active gene by a process called gene conversion. Rest 5 percent of mutations are rare and population specific. A total of 19 common mutations and 29 polymorphisms have been identified in CAH patients specific to different populations, apart from about 100 rare mutations. Data in Indian population is however, sparse.

The present study has been undertaken with the specific objective to identify mutations and polymorphisms in the 21-hydroxylase gene in Indian cases with classical CAH. The approach involves selective amplification of active CYP21 gene followed by multi-step sequencing and identification of the variant by automated DNA analysis, against the reference sequence. Analysis of 30 CAH subjects so far has shown different kinds of mutations in our population viz. homozygous gene deletion, intron-2 splice, non-sense, missense and frameshift mutations. Further analysis is ongoing to determine frequencies of these mutations.

The approach is useful for confirmation of CAH diagnosis and their clinical management. It is also useful in genetic counseling, prenatal diagnosis and

treatment of the affected cases. It can be used as a tool for neonatal screening, thus helping in reducing mortality associated with this disorder.

2.5 Antigens involved in Ovarian Autoimmunity

Repeated IVF attempts have been speculated to induce antiovarian antibodies (AOA), both by repeated hormonal stimulation and by repeated microtrauma during oocyte retrieval that can cause the release of altered immunogenic proteins from internal ovarian layers. Most couples seeking children attempt to do so by visiting IVF clinics. The presence of AOA could impair the development of the egg and embryo, downstream to oocyte collection, and thus after its removal from the ovary. Identifying women with AOA before recruiting them in the IVF-ET program would not only benefit the treating clinician in disease management but also aid the registered couple investing a large amount of money for the procedure.

A non-invasive, simple, specific and sensitive novel test to identify women with AOA has been reported earlier. Using this test (Western blotting and Immunohistochemistry) a large number of these women with AOA have been identified. Of the 581 serum samples screened, 121 were AOA positive (22%). Majority of these antibodies reacted to the oocyte although some of them did show reactivity to various other gonadal somatic cells. There is involvement of not just a single antigen but multiple antigenic targets are involved. Of these 121 AOA positive patients 60 of them had antibodies to a 90 kDa immunodominant protein. This protein shows predominant ooplasm of the oocyte localization.

Future plans are to re-screen the serum of these AOA positive women, who will now be treated with corticosteroids to check whether there is a fall in the AOA titre and thereby successful IVF-ET. Further characterization of the 90 kDa antigen is being pursued.

2.6 Expression of Integrins on Human Endometrium: A Possible Marker for Uterine Receptivity and Implantation

Integrin $\alpha_v\beta_3$ has long been proposed to be the prime candidate for endometrial maturation and receptivity. Our earlier studies have shown that $\alpha_v\beta_3$ and its ligand vitronectin are co-expressed in mouse decidual tissue on day 8.5 of pregnancy, indicating their role in decidual function. In the previous year we used an in-vitro model where $\alpha_v\beta_3$ integrin-vitronectin interaction was blocked by a function-blocking antibody. The differentially expressed genes were analyzed by cDNA array analysis. It was observed that blockade of $\alpha_v\beta_3$

integrin by function-blocking antibody resulted in differential expression of the various genes.

Downregulated genes

Genes encoding various transcription factors. Eg. E2F,
Cell cycle proteins. eg. Cyclin D3, Cyclin F, Cyclin G.

Upregulated genes

Proto-oncogenes. Eg. Shc, b-raf, p53.

The results obtained from cDNA array analysis by real time PCR, was validated this year. They exhibited a similar pattern of expression after blockade of $\alpha_v\beta_3$ integrin; ie, cell cycle regulatory proteins and transcription factors were downregulated and proto-oncogenes were upregulated.

To study whether similar mechanisms of $\alpha_v\beta_3$ signaling exist *in vivo* also, integrin $\alpha_v\beta_3$ and its ligand vitronectin was localized in pregnant mouse endometrium during different stages of pregnancy (6.5, 8.5 and 13.5 dpc). The receptor and its ligand exhibited a similar pattern of expression in the decidual zone of pregnant endometrium. The genes that were differentially expressed after blockade of $\alpha_v\beta_3$ were also analyzed by real time PCR. It was observed that cell cycle proteins and transcription factors were upregulated on day 8.5 when the decidual function is maximum and $\alpha_v\beta_3$ expression is highest. The expression of protooncogenes was downregulated on 13.5 dpc, the day when expression of $\alpha_v\beta_3$ goes down. This hints towards an important role for $\alpha_v\beta_3$ integrin in decidual function and regression of the decidua. An aberrant expression of $\alpha_v\beta_3$ integrin at any stage of pregnancy may lead to consequent changes in expression of the downstream genes, leading to improper functioning of the decidua. This indicates that $\alpha_v\beta_3$ may be a major regulator of decidual function, thus underlining its importance in a successful pregnancy.

2.7 Studies to Elucidate the Mechanisms of Endometrial Receptivity and Implantation in Primates

Last year studies were conducted to decipher the structural and molecular profile of the bonnet monkey endometrium in response to embryonic stimuli on day 6 of pregnancy, a time point when there is no physical contact between the embryo and the endometrium. This year we examined these profiles in bonnet monkeys on day 7 of pregnancy, a time point when embryo is anticipated to initiate physical contact with the endometrium. The endometrial expression of

molecular factors like Cyclooxygenase 2 (COX-2), Insulin-like growth factor binding protein-1 (IGFBP-1), Interleukin -1 beta (IL-1beta), Alpha smooth muscle actin (alpha SMA) was studied by immunohistochemistry. The endometria of pregnant animals exhibited a characteristic differential expression profile in terms of the cellular distribution pattern and intensity of localization of these molecular factors as compared to non-pregnant animals. In pregnant animals, a differential pattern of expression was observed throughout the endometrium. Intense localization of COX-2, IGFBP-1, IL-1 beta and alpha SMA was observed specifically in the stromal cells surrounding the implantation site. In the adjoining areas, COX-2, IGFBP-1 and IL-1 beta expression was localized to endometrial glandular epithelial cells while alpha SMA was localized to the blood vessels. In non-pregnant animals, only the glandular epithelial cells expressed IGFBP-1, COX-2 and IL-1 beta with the intensity of localization being less than that observed in pregnant monkeys. These studies indicate that it is the stromal compartment of endometrial tissue, which first responds to embryonic stimuli.

2.8 Proteomic Approach for the Assessment of Pathophysiology and Progression of Endometriosis

Endometriosis is one of the most common gynecological disorders associated with infertility and chronic pelvic pain. It is defined as the presence of functional endometrial glands and stroma in ectopic locations outside the uterine cavity. The most common sites are ovaries, peritoneum, uterosacral ligaments, pouch of Douglas and rectovaginal septum. It may also be found at extra pelvic sites like intestinal tract, urinary tract, surgical scars, lungs, thorax, peripheral nerves and central nervous system. The precise etiology and pathophysiology of endometriosis remains unknown despite many years of research.

The study was undertaken with the objective to i) generate proteome for eutopic and ectopic endometrium and identify proteins associated with endometriosis ii) identify and characterize the eutopic endometrial proteins showing differential expression pattern in women with and without endometriosis iii) identify endometrial antigens present in the sera of endometriosis patients and determine their relevance in the pathology of endometriosis.

A total of 10 women with endometriosis and 4 women without endometriosis as controls were recruited during the reporting year. Using Western blot the prevalence of anti endometrial antibodies (both IgM and IgG isotype) in sera of endometriosis patients and involvement of multiple endometrial targets

was determined. It was seen that 13/40 were positive for IgG isotype (Prevalence 33%) and 10/27 IgG negative patients were positive for IgM isotype (Prevalence 37%). The reactivity was specific and targeted to the endometrial antigens of different molecular weights in the range of 30-160 kDa. Of the various antigens, two endometrial antigens, 30 and 45 kDa, were found to be predominant. Immunohistochemical analysis showed reactivity in luminal, glandular epithelium and stroma. Dot blot analysis using sera from positive patients showed reactivity also with ovary, adrenal and thyroid. We carried out 2D western blot with the sera showing reactivity on 1D western blot and identified 5 spots RG-1, 2, 3, 4 & 5. Out of these 3 spots RG1, 3 & 5 have been sequenced. RG-1 (Mol wt 29 kDa, PI 4.7), RG-3 (Mol wt 38.5, PI 6.8) RG-5 (Mol wt 39.5, PI 5.07) were determined as Tropomyosin-3, Stomatin like protein-2 and Tropomodulin-3 respectively. The role of these proteins in endometriosis is unknown and is currently being investigated.

2.9 Deciphering the Role of Homeobox Protein HOXA10 in Endometrial Receptivity and Decidualization

Homeobox genes are developmentally regulated transcription factors and play a very crucial role in body patterning and cell differentiation. Amongst these homeobox genes, HOXA10 is reported to play a crucial role in development of the mammalian uterus. In addition murine studies have indicated HOXA10 may also play an important role in the adult uterus particularly during embryo implantation and decidualization. However, molecular cascades regulated by HOXA10 in the adult endometrium are unknown. The aim of our study is to examine the expression profile of HOXA10 in primate endometrium and to decipher the stromal cell transcriptome regulated by HOXA10 in the human endometrium.

The expression profiles of HOXA 10 in endometria of mated and antiprogestin treated bonnet monkey to determine if HOXA10 in the primate endometrium is regulated by progesterone and embryonic stimulus was compared. Results revealed that HOXA10 expression was downregulated in the functionalis zone of the endometria of monkeys treated with antiprogestin, but as unregulated in the mated animals. These results indicate the important roles of progesterone and embryonic stimulus in regulation of HOXA10 *in vivo*.

Studies were also conducted to determine the definite role of HOXA10 in the human endometrium. For this purpose, primary cultures of human endometrial stromal cells were setup and these could be maintained in the

laboratory upto eight weeks. These cells were characterized based on the expression of various markers and it was evident that almost 99 percent of cells expressed vimentin (a stromal cell marker) and were negative for cytokeratin (epithelial cell marker) indicating their purity. Immunofluorescence studies demonstrated that these cells also express HOXA10. In an attempt to knockdown HOXA10 in the stromal cells siRNA probes against HOXA10 was used and it was evident that the transfection efficiency varied from 85-90 percent in repeated experiments. Two independent siRNA duplexes targeted to different regions of HOXA0 RNA were transiently transfected and the knockdown efficiency was studied by real time RTPCR using SYBR green chemistry. It was observed that duplex 1 gave almost 50 percent knock down efficiency whereas duplex 2 gave a knockdown by almost 70 percent after 48 hours for transfection. Currently we are using duplex 2 probe and studies are ongoing to determine the time course of the HOXA10 knockdown and expression of HOXA11 and IGFBP1. We subsequently plan to use this experimental strategy for microarrays to dissect out the HOXA10 regulated transcriptome.

2.10 Genomic Study of Y Chromosome Microdeletion in Severe Oligoasthenozoospermic and Non-obstructive Azoo-spermic males

Genetic factors are seen to play an important role in the regulation of spermatogenesis. The role of genes in aberrant spermatogenesis is complex and needs to be further elucidated. 700 infertile males were screened to enroll 210 infertile men according to the inclusion criteria; this year 30 new patients were recruited. Objective 1 and 2 viz. frequency of micro deletion and phenotype/genotype variation of the above study has been completed. Frequency of micro deletions were seen to be 3.2 percent and abnormal karyotype was seen in 9 out of 204 subjects (4.4%) of infertile men. Mosaicism for Klinefelter's Syndrome was seen in 7 and inversion of Y was seen in 1 and Robertsonian in one. Objective three of the project envisages studying the impact of microdeletions on spermatogenesis at the molecular level viz. the gene and the protein expression leading to aberrant spermatogenesis.

A comprehensive evaluation encompassing hormone estimation, sperm FISH analysis and at the molecular level, protein and gene expression in those cases with aberrant spermatogenesis were carried out. Informed consent was taken from all the participants. Gonadotropin assays were carried out in 180 subjects. FSH and LH were significantly high in non-obstructive azoospermic as compared to severe oligo-asthenozoospermic males ($p < 0.001$). Testosterone levels did not show any significant difference. Aneuploidy rates are known to

be high in severe infertility. These genetic aberrations affect fertilization rate and embryo quality and subsequently pregnancy rate in male factor cases undergoing ICSI. We are looking at aneuploidies in sperm of infertile males using X, Y 13 and 18 chromosome probes shows normal signal intensities in men with severe oligozoospermia. So far, 10 subjects in whom aneuploidies were not seen have been screened and the study is ongoing to include a larger number of subjects. Immunohistochemistry was carried out to understand the role of RBMY protein in cases with aberrant spermatogenesis. Twenty-six testicular biopsies with spermatogenic arrest or hypospermatogenesis were screened. The role of RBMY protein in spermatogenesis needs to be further documented.

2.11 Human Semenology: Sperm Function Tests, Biochemical Analysis and Ultrastructural Details

2.11.1 Studies on Sperm Chromatin Integrity in Male Infertility

Infertility affects approximately 10-15 percent of all couples. Male factor infertility is a primary problem in about half of these subjects. Paternal contribution to a successful gestation depends on several factors and one of the crucial factors is the packaging of sperm nuclear chromatin. During spermiogenesis, histones (somatic nuclear proteins) are replaced with small highly basic proteins, protamines, leading to chromatin packaging. Chromatin packaging anomalies in spermatozoa can arise because of defective protamination, and/ or the presence of breaks in DNA molecule. The overall aim of the study is to assess the sperm chromatin packaging with respect to status of basic nuclear proteins and DNA integrity in well-defined groups keeping in mind the different etiologies of male infertility viz. male partner of a couple with unexplained infertility, males whose wives had recurrent pregnancy loss during first trimester, BOH and males with Varicocele (study groups). Recently proven fertile males served as controls. The specific objectives of the study are to i) assess the chromatin integrity in sperm obtained from fertile and infertile group, ii) study chromatin packaging with respect to basic nuclear proteins (protamine 1 and protamine 2) at protein and gene level and iii) correlate the status of protamination with DNA integrity.

Our earlier study indicated an overall decrease in protamination in study groups as compared to fertile controls. These results suggested that proper protamination is essential for normal sperm chromatin integrity and disorders of chromatin structure can cause infertility or lower the fertilization potential. Thus, the way in which sperm DNA has been packed during spermiogenesis

will ultimately influence the potential of sperm to achieve fertilization. During the reporting year, 219 semen samples including study groups i.e. BOH (male partner whose spouse underwent repeated early pregnancy loss, i.e. Bad Obstetric History (BOH), n=59), unexplained (male partner of unexplained infertile couple, n= 91) and Varicocele (men with varicocele, n= 47) and control group (n=22, recently proven fertile males) were analyzed for routine semen analysis (Volume, motility, normal morphology and count) *in vitro* nuclear chromatin decondensation test, protamine status at the protein and gene level, sperm DNA integrity and ultra structural morphology. The current report describes the data on chromatin status as determined by Acridine Orange staining, ultra structural morphology of sperm using transmission electron microscopy and its correlation to number of sperm undergoing apoptosis by TUNEL assay.

TUNEL assay also demonstrated an increased number of sperm undergoing apoptosis in study groups as compared to fertile group (dark blue sperm heads indicative of apoptosis, and among them, varicocele group exhibited highest number of apoptotic sperm.

At the ultra structural level a significant increase in sperm head abnormalities especially with respect to nuclear chromatin were observed in study groups as compared to fertile group. An improper sperm nuclear chromatin condensation was observed in a majority of the sperm nucleus affecting nuclear chromatin integrity. Varied abnormalities were observed in study groups as compared to the fertile group. These results support our hypothesis that protamine protects the DNA.

2.12 Studies on the Molecular Characterization of Human Sperm Progesterone Receptor

Ejaculated spermatozoa are not capable of fertilization. It has to undergo the process of capacitation and acrosome reaction. Progesterone in the female reproductive tract is one of the physiological initiators of acrosome reaction. A membrane bound sperm protein that interacts with progesterone, but not to antiprogesterin has been previously identified. That a member of protein disulphide isomerase (PDI) family is a ~55kDa protein with an expected pI of 4.6-5.2 and is localized on the acrosomal region of digitonin treated spermatozoa has been reported. In ligand blot assays, immunopurified PDI demonstrated progesterone binding and antibodies against PDI block progesterone mediated sperm functions. These experiments are convincing evidences to equate PDI as a candidate membrane progesterone receptor in spermatozoa.

PDI has been successfully cloned in an expression vector with 6X histidine tags for purification. After validating the clones for their orientation, these were transformed in BL21 host cells and the protein was expressed using IPTG induction. Time kinetics based experiments indicated that the protein is maximally expressed at four hours post induction. The recombinant protein has been successfully purified to 95 percent homogeneity using nickel NTA columns. The identity of the purified recombinant protein was confirmed by Western blotting. Approximately 30-60ug of the purified protein per liter of culture is now available with us. Studies are in progress to determine ligand specificity and binding kinetics of progesterone to the bacterially recombinant protein. In addition, the cognate cDNA sequences encoding for the different domains of the human PDI protein also have been cloned and studies are underway to determine the progesterone-binding domain in the human PDI.

2.13 Generation of Transgenic Mouse Model of Male Infertility to Study the Molecular Mechanism of Block of Spermatogenesis

During spermatogenesis a number of genes are expressed in a cell and stage specific manner. Among them the c-kit proto-oncogene plays a decisive role right from the differentiation of male gonads to the formation of spermatogonial stem cells. However, factors that regulate differentiation of gonads and spermatogonial stem cells have been poorly understood.

During the year attempts were made to determine the efficient promoter region of c-kit using CpG Island approach. To identify promoter region in murine c-kit gene, the presence of DH sites in the 5'-flanking region, exon 1, and part of intron I of the c-kit gene, and in the 5'-flanking region, coding region and 3'-flanking region of the intronless c-kit gene, in spermatogonial cells were investigated. Our results indicated that c-kit gene DH site 1 may be critical for tissue specific regulation as examined by Northern blot and RT-PCR analysis.

2.14 Regulation of Spermatogenesis by Estrogen

2.14.1 Effect of Estrogen/Antiestrogen on Germ Cell Maturation in the Testis

Spermiation is the final step of spermatogenesis whereby spermatozoa are released into the seminiferous tubular lumen. Any interference in the process of spermiation leads to a failure of spermatozoa to be released into the lumen, which is ultimately phagocytosed by the Sertoli cells. Hence, spermiation failure could serve as a potential target for contraception however not much is known about the molecular events during spermiation.

Spermiation occurs in specific stage of the seminiferous epithelium cycle. In rat it occurs in stage VIII, while in human it occurs in stage III of the seminiferous epithelium cycle. It is a complex process involving displacement and removal of spermatid cytoplasm, formation and degradation of tubulobulbar complexes and progressive loss of adhesive junctions including ectoplasmic specializations and subsequent phagocytosis of residual bodies by the Sertoli cell.

Studies done in our laboratory to understand the effect of increased intratesticular estrogen on spermatogenesis revealed failure of spermatozoa to be released into the lumen following exogenous estradiol treatment (20 µg/kg/day for ten days) to adult male rats. It was observed that the spermiation failure was dependent on testosterone and FSH as reported in literature. Hence, this treatment model has been used in the present study to deduce the molecular events during spermiation and their regulation by hormones.

Studies done by others have indicated involvement of cell adhesion molecules, viz., integrin and cadherins, their associated molecules and cytoskeletal proteins (actin, vimentin and tubulin) to be involved in adhesion of spermatids to Sertoli cell and thereby in spermiation. Beta 1 integrin and N-Cadherin have been found to be associated with failed spermatids. The integrins and cadherin have been shown to be associated with various cytoskeletal proteins in the Sertoli cells through various cross-linking and capping protein. Studies have shown that as much as 50 percent of the E- and N- cadherins were associated with vimentin in the testis. Cytoskeletal proteins present in the Sertoli cell may be important in the control of adhesion as well as for subsequent disengagement of the spermatid during spermiation. One such cytoskeletal protein vimentin plays an important role in the adaptation of Sertoli cell to varying configurations of neighboring cells during spermatogenesis. In the rat during stages XII-V (before spermiation), Sertoli cells showed perinuclear area of vimentin positive extension, projecting towards the developing spermatid bundles. During stages VI - XI (during spermiation and after) this projections were narrow and almost localized to perinuclear area. Vimentin staining has been found radiating from the perinuclear area towards desmosomes like junction with the early spermatogenic cells, Sertoli germ cell junction and ectoplasmic specializations.

The study is based on the hypothesis that dynamic processes such as protein phosphorylation and dephosphorylation regulate Sertoli cell cytoskeletal elements thereby maintaining it in a polymerized form and inhibit premature release of elongated spermatids. The reverse would hold true during

spermiation where deactivation results in release of mature step-19 spermatids. Hence in case of spermiation failure the phosphorylation/ dephosphorylation of the cytoskeletal proteins may be affected thereby retaining them in polymerized form thus causing spermiation failure.

In the current year the distribution pattern of vimentin in Sertoli cell was studied in control and estradiol treated animals. The stage specific distribution pattern as described above was studied using confocal microscopy. Z sections were taken which revealed a stage specific pattern of distribution. Stages I – V showed an extensive branched appearance extending from the Sertoli cell nucleus towards the lumen, where as stages VIII – IX showed a perinuclear distribution around the Sertoli cell nucleus, stages X- XIV revealed branched appearance however the degree of branching was not as extensive like that seen in stage I-V. In the treated animals stages I-V and X-XIV were not affected, however stages VIII-IX showed a branched appearance as compared to the control and similar to that seen in stages before spermiation. The results obtained suggested stage specific effect on vimentin polymerization status, which may have caused spermiation failure. Studies on phosphorylation status of vimentin are on to confirm confocal findings.

2.14.2 Genomic Imprinting: A Paternal Contribution to Embryogenesis

Epigenetic changes are stable and heritable (or potentially heritable) alterations in gene expression that are independent of nucleotide sequence. The epigenome is dynamic and comprises of two different components, the chromatin structure associated with DNA and a pattern of DNA methylation. DNA methylation is extensively studied epigenetic modification characterized by covalent addition of a methyl group to the carbon 5 of the cytosine residue in CpG dinucleotides. Recently, several studies have shown alterations in DNA methylation upon exposure to chemicals, food and supplements demonstrating dynamic character of DNA methylation patterns and their susceptibility to environmental agents.

Genomic imprinting is an epigenetic mechanism whereby gene or genomic domain exists in a state of epigenetic differentiation that depends upon its parent of origin resulting into monoallelic gene expression. Parental methyl imprints are established during gametogenesis so that in offsprings, they will be exclusively expressed from either the paternal or maternal allele. Genomic imprinting could be one of the paternal factors involved in embryogenesis regulating fetal growth, and development. A number of genes regulated by

imprinting have been shown to be essential for fetal growth and placental function. We are working on two reciprocally imprinted neighboring genes, Insulin-like growth factor 2 (Igf 2) and H19. Igf2 is a paternally expressed imprinted gene, involved in early embryo growth and development where as H19 is expressed from maternal allele and is thought to be growth inhibitory. These genes are coordinately regulated by DNA methylation at Imprinting Control Region (ICR) positioned between Igf2 & H19 and interaction of ICR with common enhancer elements. In the spermatozoa, ICR is methylated while oocyte shows unmethylated ICR.

Our previous studies demonstrated an increase in pre- and post-implantation loss following tamoxifen (a selective estrogen receptor modulator, SERM) treatment to adult male rats at a dose 0.4 mg / kilogram body weight/ day, for 60 days. The post-implantation loss was observed in the form of resorption characterized by marked decrease in the embryo size. Histology of the resorbed fetus revealed that post implantation loss occurred during midgestation i.e. around day 9-11. Further, studies on the protein and mRNA expression of insulin-like growth factor 2 (Igf2), a major growth controlling system in the early embryo revealed significant decrease in both the transcripts in the resorbed embryos. Since paternal specific expression of Igf2 is dependent on methylation of ICR, we investigated methylation status of CpG island at Igf2-H19 ICR in spermatozoa of F0 rats in control and tamoxifen treated group using methylation specific PCR and bisulphite sequencing. Also, analysis of methylation status at Igf2-H19 ICR of normal and resorbed F1 fetuses was performed by MS-PCR.

Our results show a correlation between methylation at Igf2-H19 ICR in the spermatozoa and embryo loss. This signifies Igf2-H19 ICR methylation in spermatozoa as a predictive factor to assess embryonic development. Secondly, the study also shows a correlation of methylation at Igf2-H19 ICR in spermatozoa and tamoxifen treatment indicating an effect of tamoxifen in the establishment and/ or maintenance of methyl imprint during spermatogenesis.

Since tamoxifen is a selective estrogen receptor modulator, the effects could be mediated through estrogen associated signaling pathways. Flow cytometric analysis for global methylation of spermatozoa did not show any correlation either with resorption or tamoxifen treatment suggesting locus specific effect of tamoxifen. In conclusion, the study shows that errors in establishment and/or maintenance of paternal imprints during spermatogenesis at Igf2-H19 ICR locus affect embryo development.

3 Reproductive Tract Infections

3.1 Human Papillomavirus Infection in Women with Different Clinical Manifestations

Human Papillomavirus is sexually transmitted and causally related to cervical cancer. This infection is highly prevalent as clinically latent infection in general population. Few reports are available on clinic-based studies on this infection. Our study objective was to standardize a sensitive technique to screen and type HPV infection and to correlate with clinical manifestations. Polymerase chain reaction was standardized to diagnose this infection followed by southern hybridization with general and type specific probes (16,18,6,11). Restriction fragment length polymorphism analysis was carried out in the amplified specimens followed by sequencing for confirmation of the diagnosis. Four hundred seventy specimens, collected from women attending the Gynecology OPD of Seth G.S. Medical College were screened for the infection. The average infection rate was found to be 8.1 percent. HPV types 16,18,6 and 11 were found in 10 (26.3%), 4 (10.5%), 14 (36.84%) and 2(5.2%) women respectively. Mixed infection was found in 6 (15.78%) women. But all these women were with normal cytology as detected by Pap smear, highlighting the need to follow up these women for further preventive measures.

3.2 Purification and Characterization of CD4 Independent 160kDa Sperm Receptor for HIV

Seminal leukocytes and cell free virus was initially considered to be the sole source for sexual transmission of human immunodeficiency virus (HIV). However, later it was realized that HIV binds and enters into spermatozoa and the sperm bound virus facilitates the infection into urogenital cells such as Langerhan cells and Macrophages. The recent studies using monkey model demonstrated that due to acidic vaginal pH the survival of seminal leukocyte as well as cell free virus is difficult. Moreover, the cell free virus need for efficient infection through vaginal route is very high as compared to that of systemic route. This suggests that the sperm is possibly a major source in sexual transmission of HIV. However the modality of HIV entry into spermatozoa was not known due to absence of conventional CD4 receptors and CCR5 and CXCR4 co-receptors on spermatozoa as well as testicular germ cells, thus, suggesting the presence of alternate receptors on the spermatozoa.

CD4 independent 160kDa sperm protein has been identified for the first time by Western blot technique. It was observed that both gp120 HIV envelope

glycoprotein as well as cell free HIV binds specifically to 160kDa human sperm protein. Further characterization demonstrated that 160kDa HIV binding protein is distinct from conventional CD4 receptors and binding of gp120 to 160kDa protein can be displaced specifically by unlabelled gp120. It was also demonstrated that 160kDa protein is not associated with glycolipid, which has also been reported to be involved in binding of HIV in neuronal cells. The protein was partially purified from human sperm extract by ion exchange chromatography on Mono Q column using fast protein liquid chromatography (FPLC). From about 600 sperm samples about 10mg of partially purified protein was isolated. Partial N-terminal amino acid sequence of 160kDa HIV receptor protein and its peptides obtained by enzymatic digestion showed partial sequence homology (85% and 63% respectively) with human mannose receptors (hMR). Presence of mannose receptor in the testis was confirmed by PCR amplification and sequencing of human testicular cDNA which showed 99 percent homology with macrophage specific hMR. Identity of 160kDa protein with mannose receptor was further confirmed by PCR amplification of human testicular cDNA using degenerate deoxyinosine primers corresponding to the peptide sequences of the 160kDa protein, which resulted in 850bp product. The cDNA sequence of this 850bp PCR product exhibited homology with the corresponding region of the human mannose receptor transcript.

The preliminary studies demonstrated the differential expression of this protein in sperm samples from individual donors. By SDS-PAGE eight out of ten sperm samples showed the presence of 160kDa protein band and the reactivity of gp120 to 160kDa sperm protein by Western blot analysis and by flow cytometry, while the remaining two samples did not show presence of 160kDa protein and did not show reactivity with gp120. Interestingly two sperm samples of two serodiscordant husbands of HIV positive wives did not show presence of hMR. This suggests the possible involvement of hMR in risk of sexual transmission of HIV.

These observations suggest the need for renewed efforts towards the development of modalities in prevention of sexual transmission of HIV and also need to understand mechanism of CD4 independent interaction of HIV.

3.3 Nisin: The Antimicrobial Peptide for the Control of Fertility and Sexually Transmitted Infections

Microbicides are now recognized as the most promising prevention technologies to combat the pandemic of STIs / AIDS. Several anionic polymers

and detergent-based microbicides are undergoing preclinical development, but their long-term mucosal safety is a cause for concern. Therefore, immediate priority lies in the development of safe, efficacious, non-detergent type mechanism-based microbicides.

A group of compounds exhibiting the above properties are naturally occurring antimicrobial peptides (AMPs). One of the well-characterized AMPs is Nisin, a 34 amino acid cationic, amphiphelic peptide belonging to the lantibiotic family. Our earlier studies have shown that Nisin possesses antibacterial and spermicidal properties and is found to be safe. Since Nisin did not inhibit growth of HIV, a combined gel formulation of Nisin has been prepared with a known anti HIV compound, carrageenan (NisCar gel). The gel showed activity against HIV-1 and 2. Contraceptive, antimicrobial and safety studies with NisCar gel are in progress.

3.4 Identification, Purification and Characterization of Antifertility Compounds with Microbicidal Activities

In the search for the identification of newer vaginal compounds having microbicidal/spermicidal properties, a 12 kDa protein (SSP12) exhibiting antimicrobial and spermicidal activities from the hemocytes /hemolymph of Indian mud crab, *Scylla serrata* has been identified. During the year the full-length ORF of SSP12 from *S. serrata* has been cloned, sequenced and its expression examined in different tissues.

Results revealed that SSP12 gene expression was detected in several tissues of both male and female *S. serrata*. A predominant homogeneously expressed SSP12 transcript was observed in the gut, heart, testis, muscle, pituitary, gills and ovary, but not in the eyestalk and hepatopancreas both in male and female animals. The production of SSP12 by recombinant method is currently in progress.

3.5 Development of National Guidelines for Management of Reproductive Tract Infections including Sexually Transmitted Infections

In the Phase-1 of the National Reproductive and Child Health (RCH) program in India, STI/RTI services could not be operationalised below district levels. Therefore management of RTIs is the most needed inclusion in the Phase-2 of the RCH programme and the upcoming phase 3 of National AIDS Control Programme. The Institute has developed the National Guidelines for Management of RTIs/STIs in collaboration with the ministry (Division of maternal health and the NACO), WHO, and UNFPA.

The input required for framing management guidelines for RTIs/STIs was drawn from desk research and primary data collection, which was collected through a countrywide Rapid Assessment Survey (RAS) on the representative samples of facilities selected from six zones in the country. The RAS assessed the situation on programme management at state and district levels, infrastructure, availability of equipments, supplies and drugs, availability of IEC material, record keeping and accessibility of RTI/STI services to the community. Needs and preferences of the community for RTI services in terms of place, service provider and cost were also assessed.

Major gaps observed were inadequate coordination between HIV/AIDS and RCH programme, inadequate space for examination in privacy and confidentiality, facilities to conduct laboratory tests for RTIs, inadequate knowledge on RTI management among service providers, poor follow-up and partner management and unavailability of IEC material. Community level data indicated poor knowledge on causation and prevention of RTIs as compared to HIV/AIDS.

Simultaneously, working groups had prepared clinical management guidelines, highlights of which included an overview of RTI/STI epidemiology; approach to comprehensive RTI/STI case management; user friendly management flowcharts including syndrome-specific partner management and management of pregnant women; effective drug regimens with single oral dosages wherever possible; issues of privacy and confidentiality, and partner management given special focus; opportunities and approach for detection of asymptomatic RTIs/STIs by screening tools; dual protection options and integration of RTIs/STIs assessment into FP services; special emphasis on RTIs in the context of pregnancy; RTIs/STIs among special populations like neonates and adolescents; infection control and universal safety precautions; procedures for simple laboratory tests which can be done at PHC level.

Government of India has approved the guidelines in November 2006 for mainstreaming RTI/STI management, prevention, and control in the health care delivery system through RCH 2 and NACP 3 under the umbrella of National Rural Health Mission.

4 Menopause and Osteoporosis

4.1 Biochemical Markers for Early Diagnosis of Osteoporosis

Biochemical markers of bone turn over reflect changes in bone metabolism earlier and aid in the management of osteoporosis. Since referent database for Indian women is lacking, the study was initiated to establish the same. The levels of the bone turnover markers and ovarian hormones FSH and estrone glucuronide (E1G) were measured in 365 Indian women (20-70 years) and correlated with BMD measurements by dual energy absorptiometry (DXA). The mean levels of bone resorption markers; CTX-I and DPD increased significantly across the age showing a negative correlation with BMD. The increase in levels of CTX-I and DPD was significantly higher as compared to the femoral and spinal BMD, which dropped only 30-36 percent. The levels of bone turnover markers and FSH decreased across the four quartiles of spinal and femoral BMD showing a negative correlation whereas E1G levels increased across the four quartiles. The levels of hormones and bone markers can aid in identifying women at risk for osteoporosis. The bone turnover markers were comparatively low in cohort of Indian women studied.

4.2 Genetic Factors Contributing to Osteoporosis: Study of Gene Polymorphism in Vitamin D Receptor Gene and Estrogen Receptor Genes in Indian Population

We had studied association between BMD and polymorphism in vitamin D receptor and estrogen receptor genes in Maharashtrian- Indian postmenopausal women. Since BMD and fracture incidences vary across racial and ethnic groups, the study was extended to Parsee-Indian community, another ethnic group. The frequency of VDR genotype aa, bb, TT and ER genotype XX and PP was significantly higher in Parsee women as compared to women from other Indian-ethnic group.

The study suggests that certain alleles of ER and VDR gene polymorphisms seem to play a dominant effect on BMD. However, their frequency varies amongst people from different ethnicities.

4.3 Osteoporosis in Men: To Study Impact of Genetic and Environmental Factors on Bone Health in Indian Men

Osteoporosis not less common in men is now considered a major public health issue. Effects of lifestyle factors, predominantly alcohol intake, smoking and physical exercise was assessed in 330 young Indian army men. BMD at the

spine and hip were related to lifestyle factors, particularly the level of alcohol intake and the degree of physical activity. Higher alcohol intake had a protective effect on femoral neck BMD. Exercise was also protective. In stepwise linear regression, age and alcohol intake were the most important predictors for femoral neck BMD, while age and BMI were more important at the spine. It is concluded that in physically active men with a defined nutritional intake, modest increase in alcohol intake was associated with beneficial effects on BMD particularly at the hip.

5 Prevention of Unsafe Abortions

5.1 A Study of Psychosocial and Service Dynamics of Illegal Abortion in Rural Areas of India: ICMR Multi-Centric Study

Abortion is a sensitive issue impacting crucial aspect of women's health and reproductive rights. Maternal mortality attributable to abortions in India is 12-18 percent and is mostly contributed by illegal abortions nationwide. More so, abortion, willing or unwilling, is invariably a traumatic experience for any woman. Even after three decades of legal intervention, women continue to resort to illegal abortion for several reasons, which ultimately drag them mostly to the hands of untrained personnel.

In order to ensure that women who desire termination of unwanted pregnancies have easy access to safe and hygienic facilities, there is a need to explore the type of facilities available, whether they are adequately equipped and how well they are utilized. Such information would help strengthen existing MTP services in the country. The proposed study is an attempt to carry out research in various parts of India and analyze the reasons for illegal abortion from the seekers' and providers' perspectives and related issues. The objectives of the study are to i) find out the perception and awareness of the community about abortion related issues ii) understand the factors responsible for choosing illegal source of abortion iii) explore the decision making process regarding induced abortion and source iv) study the profile of providers v) find out the methods used for such abortions and complications faced and remedies sought and vi) carry out a situation analysis of service provisions for induced abortion

The study is being conducted in seven states i.e. Haryana, U.P, Rajasthan, Maharashtra, Tamil Nadu, Orissa and Assam using both qualitative and quantitative methods. Institute has been given the responsibility of carrying out research in Maharashtra. According to the methodology, within each state, two

districts i.e. one better performing in RCH and one poor performing will be selected. Within each district, two PHC villages will be selected randomly and within each PHC, two sub-centre villages and two remote villages will be selected by simple random sampling method. In each of these five villages in a PHC area, all women of reproductive age (15-49 years) will be screened for socio-demographic and pregnancy history. In-depth study will be carried out among all those women who had undergone induced abortion. In addition, 50 percent of the screened women in the village will be studied in detail regarding perception and knowledge issues etc. Further, in each village 50 adult married men will be studied in detail for their awareness and views in relation to induced abortion related issues. In addition to the community, all the providers of induced abortion in these PHC areas including nearby town/district will be studied.

In Maharashtra, two districts were selected for the study viz. Satara and Ahmednagar. Fieldwork in Satara has been carried out in five villages. The two PHC villages selected are Limb and Umbraj. In Limb, 550 females and 50 males were interviewed. The two selected sub-centres are Wadooth and Malgaon and remote villages selected are Rautwadi and Borkhal. In Wadooth, 365 females and 50 males were interviewed. In Malgaon, 378 females and 50 males were interviewed. In Rautwadi, 176 females and 50 males were interviewed. In Borkhal, 210 females and 50 males were interviewed. In Umbraj, 585 females and 56 males were interviewed. The two selected sub-centres are Andharwadi and Talbid and remote villages selected are Korti and Taswade. In Andharwadi, 393 females were interviewed. In Talbid, 424 females were interviewed. In Korti, 364 females were interviewed. In Taswade, 181 females were interviewed. A total of 10 Focus Group Discussions (FGDs) were held in Limb, Wadooth, Malgaon, Rautwadi and Borkhal, one for each male and female group.

In Umbraj, a total of 5 FGDs were held in villages Andharwadi (1 male and 1 female), Talbid (1 male and 1 female) and Korti (1 female). From the two PHC areas, a total of 15 MTP providers were interviewed. Interviews for the male and 7 FGDs will be completed during the next visit to Satara; this would be followed by the interviews in Ahmednagar district.

The study is expected to provide estimates regarding illegal abortion in rural areas, the type of provider, the perspective of the community, and factors responsible for abortion. In addition, it is expected to suggest measures to prevent illegal abortion by understanding various factors (medical, sociological and psychological) linked to the occurrence of abortion.

6 Maternal and Child Health

6.1 Transfer of Therapeutic Drugs from Maternal Circulation to Breast Milk: A Study with Specific Reference to Anti-tuberculosis Drugs

Breastfeeding provides the mother and her infant with complete emotional satisfaction creating a life-long bonding between the mother and her infant. It provides the infant with nutrients tailor made for its growth and survival. However, in developing countries like India, poverty, illiteracy, un-hygienic conditions and multiple pregnancies result in maternal ill-health requiring medication even if she is breastfeeding. The infant may thus become an unintended recipient of these maternal drugs, which could be potentially harmful. In an effort to minimize this exposure of the infants, studies have been undertaken to assess transfer potentials from circulation to breast milk of some of the chronically administered drugs and factors that may influence metabolism and transfer of these drugs from circulation to breast milk. Studies are presently being focused on the first line anti-tuberculosis drug Isoniazid, which needs chronic long-term administration even if the mother is breast-feeding. Transfer pattern of the drug as well as genetic factors viz. polymorphisms in NAT2 gene that may influence its metabolism are being studied.

Ten exclusively breast feeding women were assessed for plasma and milk levels of Isoniazid over a four hour period by reverse phase HPLC. A peak level of 3-8 $\mu\text{gm/ml}$ of the drug was seen in plasma with a milk-plasma ratio of 0.55 to 2.5. Pattern of appearance of the drug in breast milk was found to be similar to that in circulation. Analysis of NAT2 gene in a group of 50 subjects indicated a high prevalence of its polymorphisms in the population. The study so far also indicates its association with a slow metabolizing pattern of Isoniazid.

6.2 Cause of Death by Verbal Autopsy Assessing Magnitude and Determinants of Chronic Obstetric Morbidities in Nasik District in Maharashtra

The overall objective of the project is to assess the magnitude and determinants of defined chronic obstetric morbidities (Vesico-vaginal fistula/ Recto-vaginal fistula, Uterine/Vaginal Prolapse, Pelvic Inflammatory Disease, Secondary infertility) in the study area. It is a community based cross-sectional study wherein a sample of 1560 women meeting the eligibility criteria of the study will be included. Data collection tools include focus group discussions, household interviews, clinic schedules, in-depth interviews of eligible women and also in-depth interviews of service providers at district hospitals and

personnel conducting deliveries such as dais and ANMs and local dais. Six Primary Health Centre (PHC) areas – 3 tribal and 3 non tribal, are included in the study.

Data collection has been completed in four PHCs and is in progress in the fifth PHC. The methodology for data collection in the PHC areas was as follows i) FGDs were conducted in the PHC areas ii) house-listing was done following which household interviews were taken iii) camps were conducted at PHC/ subcentre/ Anganwadi and v) women detected with any obstetric morbidities were referred to the Civil hospital at Nasik and some were also given symptomatic treatment. Field investigators revisited the households and motivated the remaining women to undergo examination. 1300 household interviews and clinical examination of 775 women have been conducted in five PHCs. In depth interviews of the Medical Officer/ Gynaecologist/ Surgeon (10), ANMs (5), Dais (14) and women suffering from chronic obstetric morbidities (12) have been conducted. Data entry is ongoing.

Emergency Contraception (EC)

IEC material in the form of poster and pamphlets were developed in English and the local language (Marathi). Training on Emergency Contraception was organized for the service providers - Medical Officers (MOs) and Public Health Nurses (PHNs) of the urban Health Posts of the MCGM.

7 Adolescent Reproductive Health

7.1 Improving Service Utilization by Adolescents Through Urban Health Posts in Mumbai

The overall objective of the project is to create an adolescent friendly environment at the Urban Health Posts as well as in the community and improve their service utilization through networking within the existing health care infrastructure. Within the study, a comprehensive adolescent health care program has been conceptualised in collaboration with the Municipal Corporation of Mumbai service units to meet multiple needs of adolescents, particularly those for prevention of STIs and contraception. After completing situational analysis of adolescents during the preparatory phase, the second phase of intervention was initiated by starting Adolescent Friendly Centers named as JAGRUTI and providing specialized quality sexual and reproductive health (SRH) care services including IEC activities to improve awareness on various SRH issues and dispelling myths and misconceptions.

Following interventions were undertaken during the period under report: (i) provision of SRH services through Adolescent Friendly Centers (ii) dissemination of information on services being provided at the center through street plays, poster exhibitions, health talks, group discussions, pamphlet distributions, wall posters and health checkup camps (iii) training programs for health post staff including community health volunteers and peer volunteers (iv) undertaking a house to house survey to create a detailed database of adolescent population in the defined area and their profile (iv) involvement in the activities of yuvak mandals and local NGOs such as in blood donation camps, HIV/ AIDS related activities etc. (v) meetings with stake holders and committee members.

Almost 12,000 adolescents in the area were reached through various outreach activities. About 50 percent of the 700 adolescents who approached the center in one year out were married. Girls and women outnumbered boys and men. Nearly 60 percent of them were below 24 years of age and 20 percent between 25-30 years. Married adolescent women mainly sought services for family planning methods, pregnancy detection, and complaints of vaginal discharge, menstrual complaints, infertility and antenatal care. Adolescent unmarried girls approached the center for menstrual complaints, RTI/STI related symptoms, pregnancy detection, premarital counseling, breast related problems, acne and queries on sexual and reproductive health. Those girls found pregnant were counseled and referred to appropriate centers for medical abortion with involvement of their male partners in the entire process. Adolescent boys (n=164) approached the center mainly to procure condoms and also for information on a number of their sexual and reproductive health problems. Some accompanied their girl friends for pregnancy detection or counseling.

7.2 Evolving a Model for Improving Reproductive Health among Rural College Youth in Maharashtra

The study was based on developing and applying information, education and communication (IEC) and counseling intervention backed up by referral linkages with the district health services. The study was based on linking the education and the health sector within the district for providing reproductive health (RH) services to college youth. The study also tested the feasibility of provision of RH services within the existing primary health care system. The main objective of the study was to develop a replicable and sustainable model for provision of sexual and reproductive health services to college-based youth in Thane district. The study was of 30 months duration and was conducted in

three phases. In order to assess impact of interventions, pre and post intervention data (quantitative) was collected. A self-administered, semi-structured questionnaire was used for survey, which included a sample of 1500 (800 male and 700 female) in baseline and 1953 (1022 male and 931 female) college going students in post intervention, in the age groups of 15-24 years.

Results of the post intervention data showed large gain in knowledge (mean score 56.2 and 62.2 among male students and 46.4 and 53.9 among female students in control and experimental area respectively). It was observed that misconceptions on various RH issues such as virginity, conception, oral sex and orgasm were reduced greatly. Results showed that there was not much difference in male and female student's attitudes towards sexuality in baseline and post intervention between experimental and control area. In the control area physical closeness and sexual relationship has increased between sexes. Data indicated that coital sex activity has increased by 4.2 percent in control area compared to 1.7 percent decrease in experimental area among male students. Similarly among the female students, in control area the increase in coital sex activity was 3.7 percent, compared to 1.4 percent in experimental area. A significant improvement was noted in the usage of condom during their sexual intercourse in experimental group.

The interventions have greatly facilitated in developing understanding on all major aspects of sexual and reproductive health. The IEC programs, provision of IEC material in college settings, peer leader training and orientation to teachers helped students and teachers to initiate a dialogue on RH issues. The letterbox approach helped in determining the concerns of the study population where it was feasible to address common concerns of college going youth. This approach maintained confidentiality. The interventions, which were carried out in college setting, helped them to share their concerns and problems. The outcomes of the interventional activities on sexual and reproductive health promotion were quite significant. College authorities and teachers response to build awareness for reproductive and sexual health among male and female students was overwhelming. The interactions with teachers and students have helped in creating an enabling environment for interactions with college youth. Over 200 male and female students and 100 faculties directly participated in orientation programs. The trained peer leaders and teachers independently started providing IEC and counseling services to the students. A large proportion of male and female students were consulting 'Peer Leaders' for the information on sexual and reproductive health issues. A total of 776 male and female students

visited youth friendly centers in the colleges. Over four-fifth liked this program to be continued in their colleges.

Overall results showed that there was tremendous need for reproductive health information. IEC programs in classroom settings separately for boys and girls were most preferred. Question answer sessions provided them detailed information. IEC material can be made available free of cost if properly linked with government/non-government agencies. Use of different approaches in selection of interested teachers and students to be trained as 'educators' is very important. Youth Friendly Centers in colleges can function as the primary point of entry especially for information, counseling and referral. Teachers (both male and female) can be given responsibility to run the Youth Friendly Center.

The utilization of medical services at rural hospitals was very good and majority of male and female students (195), who were referred for medical services, attended the facility. Two-third of them were satisfied with the services provided.

8 Genetic Disorders

8.1 Genetic Heterogeneity of MTHFR: Its Implication as a Risk Factor for Neural Tube Defects

Neural Tube Defects (NTD) are common and severely disabling central nervous system (CNS) malformations with worldwide distribution and is most common in Sikhs and uncommon in South Indians. It is multifactorial in inheritance, arises due to incomplete closure of neural tube in early embryogenesis (21-26 days). Many of these SNPs have proved useful as markers to identify certain phenotypes. Certain SNPs in MTHFR (C677T, A1298C) have been associated with a variety of multifactorial disorders like Neural Tube Defects, vascular disorders, Down syndrome, osteoporosis, bad obstetric history and male infertility. Results of the ICMR study and MRC trial highlighted the role of pre-conceptional folic acid (4 mg) in cases at risk for NTDs however it gave protection only in 70 percent of the cases. Thirty percent of NTD cases did not respond to administration of folic acid. MTHFR gene polymorphism is being studied in those families who are non-responders to folic acid.

Additionally, MTHFR gene polymorphism is being studied in normal population, in a cohort of NTD patients. Genetic heterogeneity is being investigated with respect to the C667T & A1298C polymorphisms as a risk factor. Preliminary data on 154 subjects screened revealed 122 (80%) were

homozygous(CC), 32 (20%) were heterozygous(CT). No homozygous TT was found.

The study will help in genetic counseling and will also highlight the role of MTHFR gene in the etiology of NTD & other genetic disorders.

8.2 Genomic Analysis of Sex Reversed Individuals

If we are to attain health for all by 2015 the best strategy is prevention of genetic disorders is by counseling, screening and prenatal diagnosis. Therefore, Genetics has a pivotal role to play in reproductive health whether it is birth defects or genetics of HIV. Genetics cuts through all specialities and plays centre stage.

Clinical genetics is the most rapidly advancing field in medicine; the most important outcome of the human genome project has been the discovery of single nucleotide polymorphism (SNPs). Over the last year our efforts have been concentrated on gene polymorphism studies (MTHFR / CCR5), genetic studies in repeated spontaneous abortions using T-FISH, Sperm FISH and M-FISH, preimplantation aneuploidy screening, molecular mechanisms in Fragile X syndrome.

8.3 Preimplantation Genetic Diagnosis (PGD)

Molecular Characterization of Fragile X Syndrome

Fragile X Syndrome is common cause of mental handicap. It is a dynamic mutation of the CGG repeat. The Fragile X gene exhibits characteristic hypermethylation. Methylation of cytosines located 5' to guanosine is known to have a profound effect on the expression of several eukaryotic genes. In normal cells, methylation occurs predominantly in CG-poor regions, CG-rich areas called CpG islands, remain unmethylated. Methylation specific PCR is a new technology for sensitive detection of abnormal gene methylation using small amounts of DNA. This process employs an initial bisulfite reaction to modify the DNA, followed by PCR amplification with specific primers designed to distinguish methylated from unmethylated DNA. We are standardizing the technique by using commercially available kit supplied by Chemicon International. (CpG WIZ Fragile X amplification Kit 37807). It will help us identify the methylation status of FMR1 gene in normal and Fragile X patients.

Preimplantation Aneuploidy PGD screening

PGD represents a “state of the art” procedure, which avoids the need to terminate affected pregnancies through identification and transfer of only unaffected embryos. PGD involves testing one or two cells taken from a recent embryo of eight cells. PGD excludes therapeutic abortion. Preimplantation Genetic Diagnosis (PGD) represents an additional prenatal service for couples at high risk. FISH studies in 20 blastomeres have been performed using visas aneuvysion probe sets for 13,18,21 X & Y no aneuploidies have been detected so far. These stored blastomeres were given for studies after obtaining written consent of the couple by our collaborator Dr Sadhana Desai. Results could be obtained in 75 percent of the cases.

8.4 The Role of CCR5 alleles in HIV Transmission, Progression and Prevention

The highest HIV prevalence rates are found in Maharashtra Andhra Pradesh and Karnataka. The CCR5 D32 deletions prevent entry of HIV into the cell and provide complete resistance to HIV in homozygous individuals and partial resistance in the heterozygous state. Global survey indicates 13 percent Caucasians have mutant allele while in Africans Eurasians and Indians the mutant allele is found in 5-10 percent. No data is available from Maharashtra.

Aims and objectives are to study the distribution of CCR5 polymorphism in different ethnic groups in Maharashtra, to study the CCR5 protective haplotype in the high-risk group and to study the CCR5 haplotype in those patients who are exposed to HIV but are not infected. Preliminary data revealed all 150 subjects were homozygous normal as indicated by a band of 193bp.

8.5 Cryptic Chromosomal Rearrangements in Couples with Three or more Recurrent Abortions

The technique of M-FISH has been applied in couples with RSA. Images are collected with fluorescent microscopes that have filter sets and combinatorial labelling algorithm. This allows separation & identification of all chromosomes visualized in pseudocolours. This procedure is being standardized and it will help in detection of balanced or unbalanced cryptic chromosomal rearrangements.

9 Structural Biology

9.1 Immunology of the Peptides Corresponding to the Ligand Binding Regions from the Extracellular Domain of Rat Follicle Stimulating Hormone Receptor

Several proteins originating from different reproductive organs have been implicated to play a crucial role in various reproductive processes. Peptides 9-30, 216-235 and 285-309 from the extracellular domain of human follicle stimulating hormone receptor (FSHR-ECD) were shown to modulate hormone binding as well as signal transduction by *in vitro* methods earlier. Corroboration of the results with these peptides using an *in vivo* rat model has been undertaken. Our studies have led to the identification of region 285-309 as a bionutralizing epitope and an active hormone-binding site. Studies have been undertaken to delineate the role of the three extracellular loops of FSHR in initial hormone binding and the subsequent signal transduction.

9.2 Mutational Analysis of Gonadotropin Receptor Genes and its Implications in Physiology and Pathophysiology of Pituitary Gonadal Function

Studies on FSHR gene polymorphisms *viz.* Thr³⁰⁷Ala and Asn⁶⁸⁰Ser in subjects undergoing ovarian hyperstimulation in IVF programme revealed that Ala³⁰⁷Ala variant subjects required significantly high amount of exogenous FSH for ovulation induction and the peak estradiol levels before and on the day of hCG administration were significantly high as compared to TT and TA variants.

9.3 Studies on β -Microseminoprotein: Biochemical, Molecular and Bioinformatics Approaches

Another protein being studied is the human β -microseminoprotein (β -MSP) also known as prostate secretory protein 94 (PSP94). Structure-function studies with respect to the delineation of its disulphide bond pairings and to understand the importance of these bonds were undertaken. Reduction of the disulphide bonds of this protein causes a conformational change but does not affect its IgG binding property. Studies are on to determine which of the domains of human IgG are involved in binding to PSP94.

10 Stem Cell Biology

10.1 To Establish Human Embryonic Stem Cell Lines in Xeno-free Environment

At present about 200 hES cell lines have been derived worldwide of which 78 hES cell lines have been registered on NIH registry. Only 10 lines of the 78 registered with NIH are available at present for research purpose. All the cell lines registered with NIH were grown on mouse embryonic feeder layers, using fetal calf serum and also reagents for immuno-surgery were of animal origin, which are a potential source for animal pathogens and thus these cell lines are not optimal for clinical use. In general, human ES cell lines are extremely sensitive, have poor survival and revival rates, cryopreservation and thawing is a problem, undergo mutational changes and genetic drift in culture. Thus one can not depend on commercially available cell lines for use as a source to develop a national regenerative medicine program. Aim of this ongoing project is to develop the technology for hES cell lines derivation in a xeno- free environment using human feeders.

A total of 50 spare human embryos were used for the derivation of hES cell lines, of which first 32 were cryopreserved embryos, frozen at 4- 6 cell stage. Only 8/32 formed blastocysts post- thaw and after removal of inner cell mass by laser micromanipulation, none of the inner cell mass attached to the feeder layer. Remaining 18 embryos were fresh, spare embryos. Ten of them developed into blastocysts, from which we successfully obtained 4 cell lines, additional three blastocysts have attached on feeder layer and are in early stages of culture.

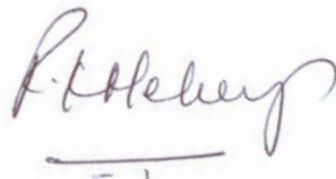
The long-term goal of this program is to cryopreserve gonadal tissue under liquid nitrogen for individuals with gonadal insufficiency including cancer survivors. The tissue could later be thawed and germ cells matured in vivo by transplantation or in vitro maturation to achieve genetic parenthood.

Fertility conservation, for individuals with gonadal insufficiency including cancer survivors, is a challenging and upcoming area of research. Heterotypic transplantation of cryopreserved- thawed ovarian cortical strips, as a source of eggs for assisted conception, will help such individuals to achieve genetic parenthood, get over the stigma of infertility induced by aggressive chemotherapy and radiotherapy and lead a quality life. Massive loss of primordial follicles occurs prior to neovascularization in the transplanted ovaries. Present study was aimed to conserve the primordial follicles (PF) reserve in the transplanted ovaries by treating with VEGF; since VEGF is known to induce early angiogenesis

thereby may reduce the loss of follicles due to hypoxia and ischemic insult. Various strategies have been used to expedite angiogenesis in ovarian grafts with the objective to lengthen the life of the transplanted tissue. Antioxidants like vitamin C, vitamin E and recently transplanting the ovarian xenografts into a granulation tissue bed appear to have a beneficial effect.

We carried out bilateral ovariectomy in immature, 20 - 21 days old Holtzmann rats and the removed intact ovaries were immediately autotransplanted subcutaneously on the ventral surface of the animals. VEGF (200ng) was injected subcutaneously at the site of transplant. The rats were sacrificed 3 and 7 days after transplantation (n=6 per group), with and without VEGF treatment.

Our results indicate that VEGF not only helped to prevent PF loss, but also induced de novo assembly of PF in the ovarian surface epithelium. The study has further provided direct cellular evidence in support of the earlier reports in literature, that postnatal mammalian ovary is a dynamic organ and is not endowed with fixed number of eggs since birth.



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