

1 EXPANDING CONTRACEPTIVE CHOICES

Various events like gametogenesis, fertilization, implantation and embryogenesis are regulated by hormones and factors secreted by reproductive and accessory organs. Identification and characterization of these regulatory factors could provide a better understanding of various reproductive processes and help in identifying newer targets for contraception. Research programmes to expand contraceptive choices i) development of improved and new technologies for fertility regulation ii) assessment of the safety, efficacy and acceptability of existing methods of fertility regulation and iii) expanding contraceptive choices amongst men and young couples are being pursued with vigor.

1.1 Development of New and Improved Technologies

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.

1.1.1 Identification and Characterization of Sperm Antigens Using Multifaceted Approach

Principal Investigator: **Vrinda Khole**

Project Associates: Priyanka Parte, Shagufta Shaikh, Monali Wakle, A. Suryavanshi, M. Ghosalkar, S. Jadhav, M. Merchande

Duration: 1997-2007

Neonatal Tolerization-Immunoproteomics

Spermatozoa are morphologically distinct haploid gametes which differentiate from precursor germ cells in the testis during the process of spermatogenesis. However, it is intriguing that they are functionally inert. The acquisitions of functional competence occur in two phases, namely, during the journey of sperm through epididymis and later following capacitation. The sperm proteins acquired in the epididymis are domain specific and have been shown to be essential for motility and capacity to bind and fuse the egg. In the present study we have adopted a high throughput approach to identify epididymal maturation proteins specific to sperm head or flagellum using neonatal tolerization protocol. The tolerization immunization protocol (TI) and initial characterization of proteins has been described earlier (Annual Report 2004-05, p 2 and Annual Report 2005-06, p 17-21). The sera not only showed reactivity with human sperm but the domains were also identical.

During the reporting period experiments were carried out using immunoproteomics approach to identify immunoreactive human sperm proteins. Washed human spermatozoa from normozoospermic men were subjected to protein extraction using 2D lysis buffer containing 8M urea, along with CHAPS, a non-ionic detergent and DTT as reductant. After overnight incubation at 4°C the spermatozoa were sonicated for 5 mins and centrifuged at 10,000 g for 15 mins, supernatant was collected and protein concentration was measured using commercial kit. Immobiline pH gradient strips (3-11NL, 13cm) were rehydrated overnight with protein sample (250µg). Rehydrated strips were then subjected to isoelectric focusing to separate proteins based on their pI (isoelectric pH). Then strips were subjected to second dimension SDS polyacrylamide gel electrophoresis (2D SDS-PAGE). The gels were silver stained or blotted on nitrocellulose membrane, followed by immunodetection.

Immunoblots were compared with silver stained gel. Several proteins were immunoreactive to sera of animals immunized with intact sperm head or intact sperm flagellum as immunogen. With intact sperm flagellum as immunogen, the serum identified proteins in the range of 45-80 k Da (Fig.1), with intact sperm head, the reactivity was seen in the region of 30-80 kDa (Fig.2). Some of these proteins were cored and subjected to MALDI TOF-MS and are being analysed for their tissue specificity and involvement if any in different metabolic pathways. A comprehensive analysis of domain specific sperm proteins is essential for understanding the relevance of these proteins in fertilization.

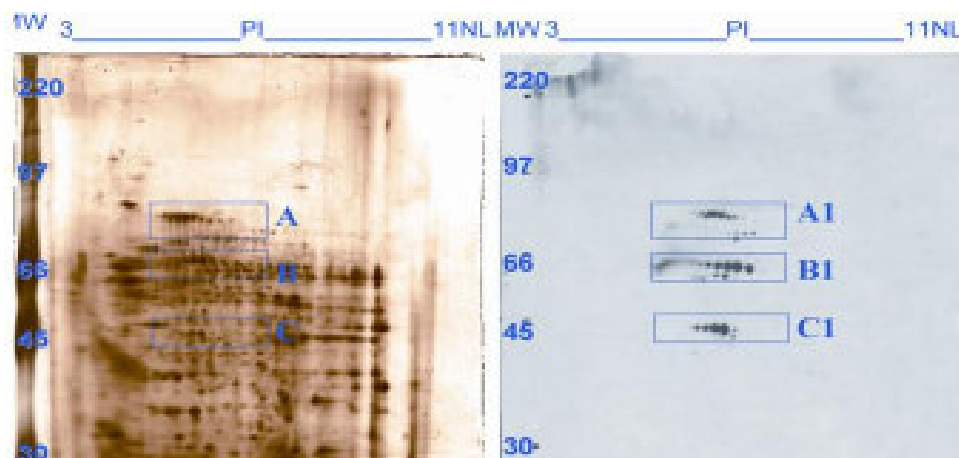


Fig. 1: Reactivity of T1 sera (Immunogen-Intact Flagella) with human sperm proteome Left panel - Gel profile of human sperm proteome; Right panel - Immunoblot of human sperm proteome probed with T1 sera showing reactivity at A1, B1, C1 which corresponds to A, B, C proteins on gel.

Antisperm Antibodies

Using monoclonal antibody generated from post-vasectomized mouse model we identified a testis specific sperm auto-antigen called TSA70. The immunochemical characterization of this protein carried out revealed that it is post-meiotically expressed and plays a role in sperm motility and capacitation-acrosome reaction. Based on its resistance to various high ionic salt solutions it was shown to be a cytoskeletal protein. TSA70 was shown to be developmentally regulated and appears postpubertally (Annual Report 2004-05, p 26-31). 2D SDS PAGE and Western blot followed by sequencing employing LC MS/MS identified 2 spots which showed sequence homology to Cenexin/odf2 indicating that the two are isoforms of the same protein. The immunoreactivity of TSA70 with anti-Cenexin antibody substantiated its homology with Cenexin/odf2 (Annual Report 2005-06, p 22-25).

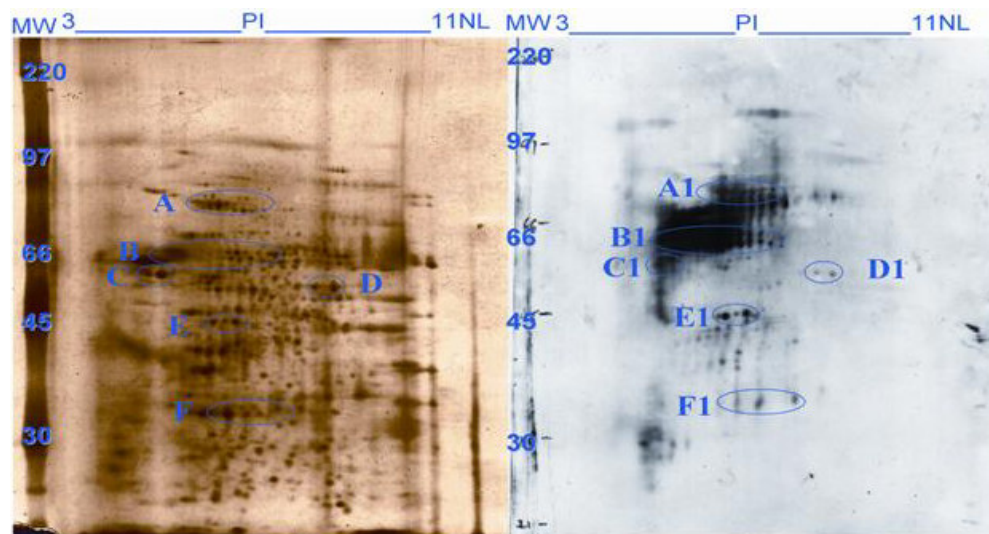


Fig. 2: Reactivity of T1 sera (Immunogen – Intact Head) with human sperm proteome. Left panel – Gel profile of human sperm proteome; Right panel – Immunoblot of human sperm proteome probed with T1 sera showing reactivity at A1, B1, C1, D1, E1, F1 which corresponds to A, B, C, D, E, F proteins on gel.

During the reporting period experiments were carried out for molecular characterization of this molecule. *In silico* analysis of the peptides following tryptic digestion exhibited number of predicted phosphorylation sites at 5 serine residues, 2 threonine residues and 1 tyrosine residue (Fig. 3). 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 (Fig. 4). This is a first report indicating that a rat sperm auto-antigen that belongs to odf2 family is a phosphoprotein. The analysis on leucine zippers showed the presence of two leucine zippers, which further increases the confidence that TSA70 belongs to Odf2 family. Our earlier report indicated likely involvement of TSA70 in capacitation/AR and sperm motility and it could now be attributed to its phosphorylated nature. Cenexin/odf2 proteins have not been shown to be phosphorylated nor involved in capacitation/AR or sperm motility. The phosphorylation of TSA70 may have led to its functional divergence. We further investigated the transcript of TSA70 by Northern blotting (Fig 5). Northern blot analysis revealed that the transcript is testis specific and showed presence of three transcripts of varying sizes of 2.4 kb, 2.2 kb and the expected 1.5 kb transcript. This indicates alternate splicing and may suggest that TSA70 could be result of these splice variants.

In conclusion, for the first time, we report a new member of Cenexin/odf2 family called TSA70 that has functional relevance in sperm motility as well as in capacitation/AR.



Fig. 3: In silico analysis of TSA70 sequence. The peptides of TSA70 displayed 5 serine (S), 2 threonine (T) and 1 tyrosine (Y) phosphorylation sites denoted by different colors. Note the two leucine zippers highlighted in grey box.

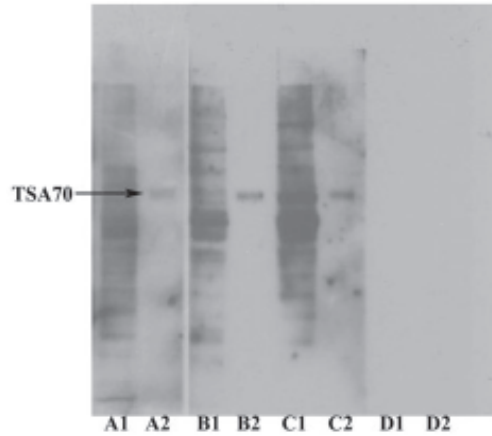


Fig. 4: The phosphorylation status of TSA70. Immunoblotting of electro eluted TSA70 and rat caudal sperm protein was carried out with anti phosphoserine (5A), threonine (5B) mAbs and anti phospho-tyrosine pAb (5C). The three antibodies namely anti phospho-tyrosine (A1), anti phosphoserine (B1) and anti phospho-threonine (C1) identified number of phosphorylated proteins in caudal sperm protein. The three antibodies reacted with electro eluted TSA70 indicating that the cognate protein is phosphorylated at its tyrosine (A2), serine (B2) and threonine (C2) residues.

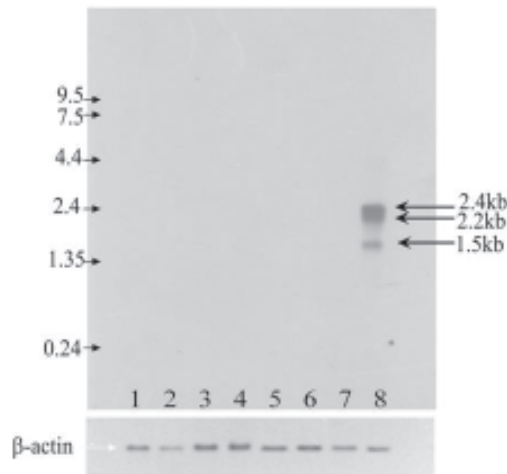


Fig. 5: The Multiple tissue Northern blot analysis of TSA70. The blot was probed with a 598bp DIG labeled probe. The transcript was observed to be testis specific (lane 8). Three splice variants of different sizes such as 2.4 kb, 2.2 kb and 1.5 kb were observed in the testicular RNA. The transcript was absent from all the other tissues such as heart (Lane 1), brain (Lane 2), spleen (Lane 3), lung (Lane 4), liver (Lane 5), skeletal muscle (Lane 6) and kidney (Lane 7). The lower panel shows β -actin control that indicates the integrity and the equal loading of RNA.

1.1.2 Role of a Novel Androgen Regulated HoxB2 Containing Gene Expressed in the Epididymis (*Funded by Indian Council of Medical Research under the Research Program on Functional Genomics*)

1.1.2.1 Development of specific antibodies as a tool to study the functional role of HOXBES2 in sperm maturation

Principal Investigator: **Vijaya Raghavan**

Project Associates: E. Prabakaran, A. H. Bandivdekar

Duration: 2006-2008

An epididymis-specific sperm protein designated HOXBES2 was identified by screening an epididymal cDNA library using an agglutinating monoclonal antibody generated 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 expression, species conservation and its association to sperm were established. An effect on capacitation and acrosome reaction in spermatozoa suggests a probable role for HOXBES2 in motility, sperm-egg interaction and fertilization. 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.

HOXBES2 was localized on rat sperm using anti HOXB2 antibody, in an attempt to study its probable role in the motility of spermatozoa in the presence of various energy substrates *in vitro* conditions. Immunofluorescence indicated that the localization of HOXBES2 protein on spermatozoa surface undergoes redistribution following supplementation with energy substrates such as ATP, cAMP, Ca²⁺ and creatinine phosphate, suggesting that HOXBES2 has an indirect role to play in the process of sperm capacitation, hyperactivation, sperm motility and acrosome reaction (Fig. 6).

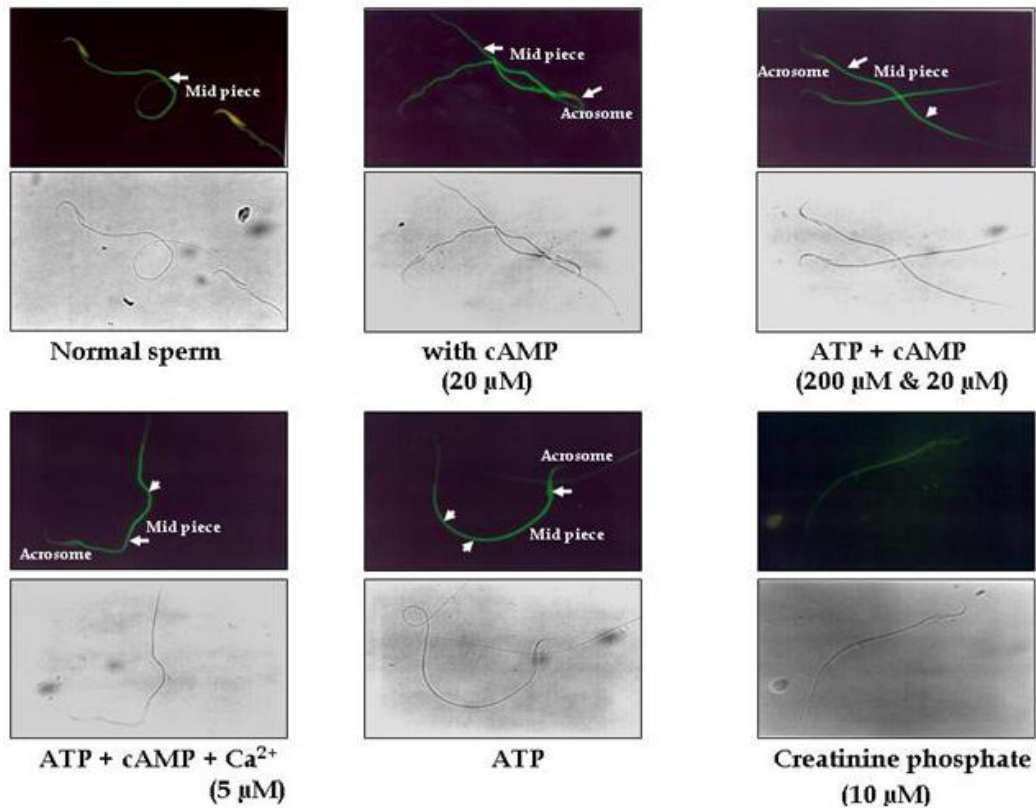


Fig. 6: HOXBES2 localization in presence of energy substrates

Based on our earlier findings (Annual report 2005-2006, p 26-28), a 95 percent pure, 25 amino acid peptide (N-FQNRRMKHKRQTQHREPPDGEPACP-C) predicted by the laser gene software, and corresponding to the highly hydrophilic region, most antigenic and immunodominant region specific to HOXBES2 was commercially synthesized. It was conjugated to keyhole limpet hemocyanin (KLH), as a carrier molecule for immunization in female rabbits and the molecular mass of the peptide was confirmed by Mass-spectrometry (Fig. 7). Studies are ongoing with immunization of rabbit with the conjugated peptide.

Arrows indicate the differential localization of HOXBES2 protein on rat spermatozoa in presence of energy substrates such as ATP, cAMP, Ca^{2+} and creatinine phosphate.

Molecular mass of the peptide was confirmed by Mass-spectrometric analysis. The chromatogram demonstrates the purity (>95%) (Arrow represents the single peak) of the HOXBES2 peptide conjugated to KLH.

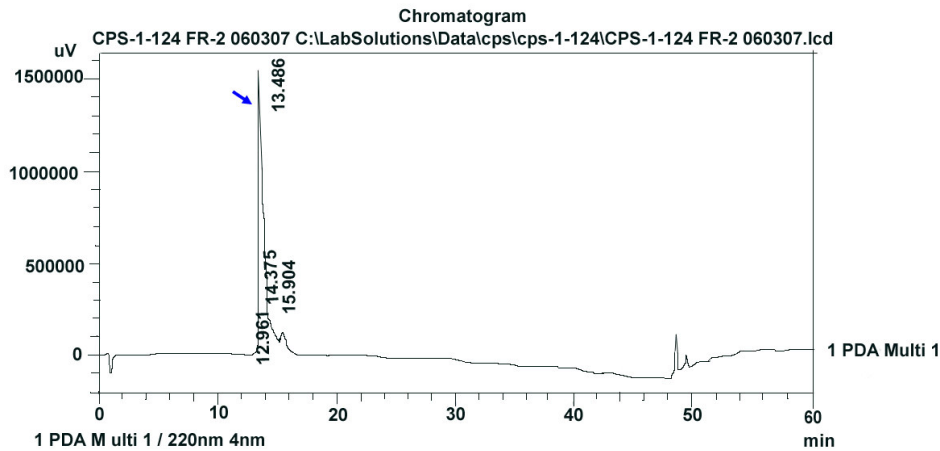


Fig. 7: Chromatogram - Peptide synthesis

In an attempt to obtain the recombinant HOXBES2 protein, the ORF of the *Hoxbes2* gene was constructed in Pmt/v5-His-TOPO vector and then transformed into the overexpressing host BL21 *E. coli* cells. Following characterization of the positive clone, it was induced with a combination of 0.2 mM CuSo4 and 0.5 mM IPTG. Expression of the induced protein (recombinant HOXBES2) was initiated 3 hrs post induction and the expression level of the protein reached its maximum by 6 hr post induction. The protein expressed was extracted, analyzed and confirmed by dot-blot and Western blot analysis. Western blot revealed a single band of the His-tagged HOXBES2 protein with a molecular weight of ~35 kDa in the induced cell extract (Fig. 8).

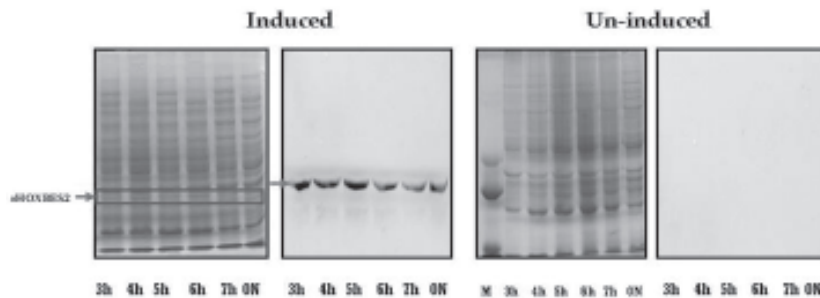


Fig. 8: Western blot analysis of recombinant HOXBES2 protein expression

Western blot analysis using anti-HOXB2 antibody demonstrates the presence of a protein band representing the His-tagged ~ 35 kDa recombinant HOXBES2 protein from the induced cell extract. No band was detected in the un-induced cell extract. Arrows indicate the protein band in the SDS-PAGE and Western blot.

Based on the His-tag on the extracted protein as receptor, the recombinant protein was eluted using Ni^{2+} affinity column from the cell extract at low pH phosphate elution buffer (pH 4.0). The purity of the protein was confirmed by Western blot using anti-HOXB2 antibody (Fig. 9).

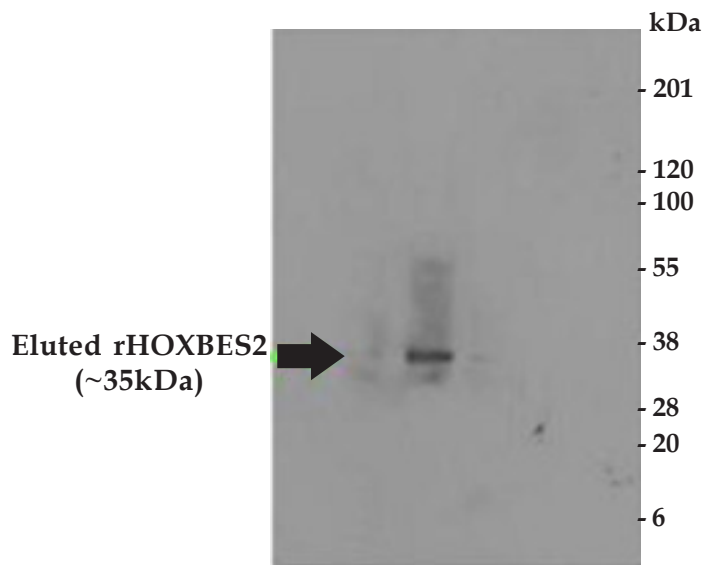


Fig. 9: Western blot analysis of eluted recombinant HOXBES2 protein.

Western blot probed using anti-HOXB2 antibody demonstrates the presence of a protein band represents the ~ 35kDa eluted recombinant HOXBES2 (rHOXBES2) protein. Molecular weight marker is represented on the right side of the blot.

The amount of purified recombinant HOXBES2 protein obtained was 50 mg /gram cell extract. Studies are ongoing to further elucidate the role of HOXBES2 in sperm maturation in epididymis and sperm function during fertilization.

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

Principal Investigator: **A.H. Bandivdekar**

Project Associates: Vijaya Raghavan, Vandana Vernekar,
Jacintha Pereira, Bharati Khobarekar and
R. B. Kadam

Duration: 1993 - 2009

80kDa human sperm antigen (80kDa HSA) is a sperm specific protein responsible for inducing immunological infertility. Active immunization with purified 80kDa HSA rendered reversible infertility in male and female rats. Incubation of human, rat and monkey spermatozoa with anti 80kDa HSA antibodies, *in-vitro* resulted in agglutination. Immunofluorescent studies demonstrated the localization of 80kDa HSA on head region of human, rat and monkey spermatozoa while in the case of marmoset the localization was predominantly on tail region and also on head region in few sperm (Annual report 2004-05, p 37-39). Electron microscopic studies using immunogold technique demonstrated localization of 80kDa HSA on acrosomal and post acrosomal-neck region of human, acrosomal and tail region of rat and acrosomal region of monkey spermatozoa (Annual Report 2005-06, p 29-35).

80kDa HSA has been found to be developmentally expressed in the rat testis and epididymis (Annual report 2003-04, p 46-47). Expression of 80kDa HSA has been demonstrated to be androgen regulated in rat testis and epididymis (Annual report 2005-06, p 30-35). Partial N-terminal amino acid sequence of 80kDa HSA (Peptide NT) and its peptides obtained by digestion with endoproteinase Lys-C (Peptides 1, 2, 3 and 4) and with endoproteinase Glu-C (Peptides 5 and 6) did not show sequence homology with any of the known protein sequences in the gene database. 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 (Annual Report 2003-04, p 43-47).

The peptides NT, 1, 2 and 4 were synthesized, conjugated to Keyhole limpet haemocyanin (KLH) and used as an immunogen to raise the antibodies in rabbits. Peptide 3 did not elicit significant antibody titer and hence was not further investigated. The anti-peptide antibodies recognize the native protein in ELISA and by Western blot analysis of human sperm extract, these anti-peptide antibodies react specifically with 80kDa HSA. Moreover, incubation of the antibodies to these peptides with human, rat and monkey spermatozoa resulted in agglutination. Thus suggesting that the antibodies to peptides NT, 1, 2 and 4 immunobiologically mimic the native protein. Passive administration of antibodies to peptides NT, 1, 2 and 4 caused agglutination of rat epididymal spermatozoa with loss of motility and these rats failed to impregnate normal females. Passive administration of these anti-peptide antibodies in female rats also resulted in infertility. Antibodies to peptides NT and 1 were found to be most effective in inhibiting fertility (Annual Report 2002-03, p 33-35). Passive administration of 10 and 40 μ g of purified IgG fraction of antibodies to peptides

NT and 1 also resulted in dose dependent inhibition of fertility in female and male rats (Annual Report 2004-05, p 37-39).

Active immunization of male rabbits with KLH conjugated peptides 1 and NT elicited gradual increase in antibody titer with agglutination of ejaculated spermatozoa with complete loss of motility and these rabbits failed to impregnate the normal females. These rabbits regained fertility with decline in antibody titer, following cessation of immunization (Annual Report 2003-04, p 43-47).

The antifertility studies were further extended in non-human primates (marmoset-*Callithrix jaccus*). Nine normal fertile adult male marmosets were immunized with KLH conjugated peptide-1. Seven animals showed gradual increase in antibody titer. Six of the seven animals having antibody titer greater than 1:400 failed to impregnate the normal females (Annual Report 2005-06, p 29-35). Epididymal spermatozoa of the immunized animals showed complete loss of motility. One animal with antibody titer 1:400 was found to be fertile following mating with normal fertile female and this animal did not show further increase in antibody titer with additional booster injections of KLH conjugated peptide 1 (Annual Report 2004-05, p 37-39). The histological sections of the testis, epididymis, prostate, spleen, kidney and liver of the immunized animals did not show any changes as compared to that of control group. All these animals regained fertility with decline in antibody titer, following cessation of immunization (Annual Report 2005-06, p 29-35).

Effect of active immunization with synthetic peptide 1 on fertility of male marmoset was independently confirmed by Dr Vrinda Khole, NIRRH. Three male marmosets were actively immunized with KLH conjugated 100µg of peptide 1 with subsequent three boosters at four weeks interval as reported earlier (Annual Report 2004-05, p 37-39). Active immunization with peptide 1 elicited gradual increase in antibody titer in all three animals. Two weeks after the third booster, animals were mated with fertile females and it was observed that two out of three animals did not impregnate the females while the remaining one animal impregnated the female ten weeks after last booster.

Effect of active immunization with KLH conjugated peptide 1 with detailed toxicological studies are being further investigated in male bonnet monkeys. Five male and ten female bonnet monkeys have been recruited for the study. The males were evaluated for detailed semen analysis and found normal. The females were investigated for cyclicity by estimation of serum progesterone levels on alternate days and found to be cycling normally. Males and females were found to be normally fertile following mating and males are being

immunized. Three out of five males were actively immunized with KLH conjugated 100µg of peptide 1 and two with KLH alone, emulsified with 100µg muramyl di peptide (MDP) in 100µl of saline and 100µl Arlacel and Squalene (1:4). Further studies are in progress.

The data suggests that peptides of 80kDa HSA are immunogenic and immunobiologically mimic the native protein. Active/passive immunization with these synthetic peptides resulted in reversible infertility in rats, rabbits and marmosets. This indicates the potential of 80kDa HSA and its synthetic peptides as candidates for development of antifertility vaccine.

1.1.4 Modulation of c-kit Proto-Oncogene Function During Spermatogenesis in Mice (*Funded by Indo-US Program*)

Principal Investigator: **K.V.R. Reddy**

Project Associates: A. P. Sikarwar and M. Rambabu

Duration: 2003 - 2008

Preparation of GFP positive green SGCs

In mice, the proliferation of spermatogonial cells (SGCs) occurs soon after birth, at around 5 day post partum (dpp) and this is followed by first wave of spermatogenic differentiation, which gives rise to primary spermatocytes at around 10 dpp, haploid round spermatids at 20 dpp and mature spermatozoa at 35 dpp. Through the continuous proliferation of SGCs and subsequent differentiation, functional spermatozoa are produced throughout male life. Several genes are known to regulate this process in a stage and cell specific manner. C-kit is one of the autosomal genes that play a key role in spermatogenesis.

In the previous year we have reported the construction of a 4.6kb *pkit-GFP* gene (Annual Report 2005-06, p 103). During the reporting period, attempts were made to produce GFP positive green SGCs using these construct. Cryptorchidism was induced in one-month-old C57BL/6 mice to enrich the SGCs and served as donor. F1 progeny of 30 days old (C57 BL/6 X DBA2) male mice were given busulfan (40mg/kg) (i.p) to destroy the endogenous SGCs and used as recipient. Plasmid DNA was introduced into the SGCs (5µg /10⁵ cells) by electroporation. The cells were cultured for 24 hr in DMEM consisting of 10percent FBS, SCF (10ng/ml) TGF-β (10ng/ml) and LIF (10ng/ml). These GFP positive green SGCs were further used for transplantation studies.

Characterization of GFP positive green germ cells

For this study, thirty days following busulfan treatment, testes were pulled out from the abdominal cavity of anaesthetized busulfan mice and *pkit*-GFP positive cells (10^5) in $10\mu\text{l}$ of DMEM were injected into the seminiferous tubules via rete testes. After three months, mice were sacrificed and the GFP positive green germ cells were isolated (Fig. 10) and characterized by determining various germ cell specific marker genes such as *c-kit* and Nucleostemin and RNA binding motif (Rbm) (SGCs), GATA-1 (Sertoli cells), SF-1 (Leydig cells) and transition protein-2(Tp₂) (Spermatids).

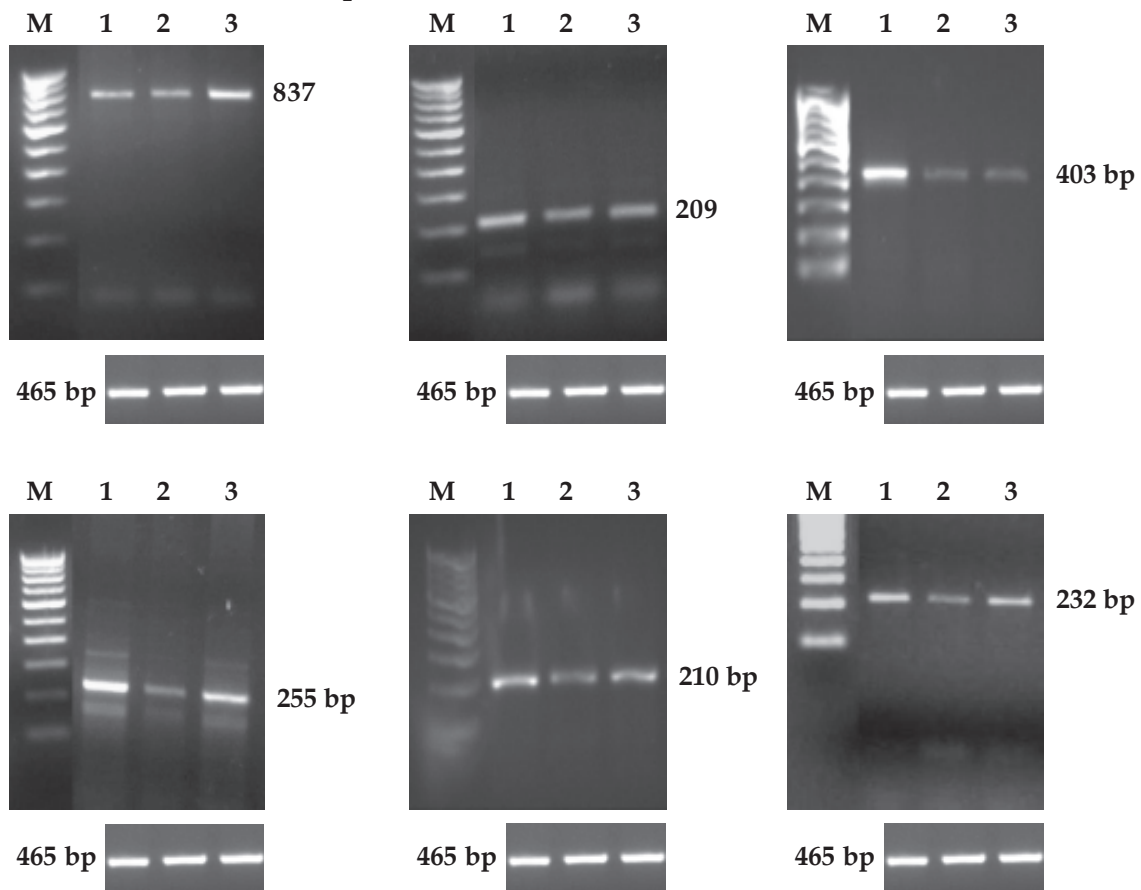


Fig. 10: Expression of cell specific marker genes such as nucleostemin, RNA binding motif protein (Rbm), *c-kit* (SGCs); Transition protein-2 (Tp₂) (spermatid); steroidogenic factor-1 (SF-1) (Leydig cell) and GATA-1 (sertoli cells) in wild type (1), busulfan treated (2) and after germ cell transplantation (3). Cyclophilin-A (465 bp) used as a loading control.

The results revealed that expression of these genes in transplanted mice were similar and comparable with the age matched wild type control mice. However, the expression was negligible in busulfan treated mice (Fig. 11).

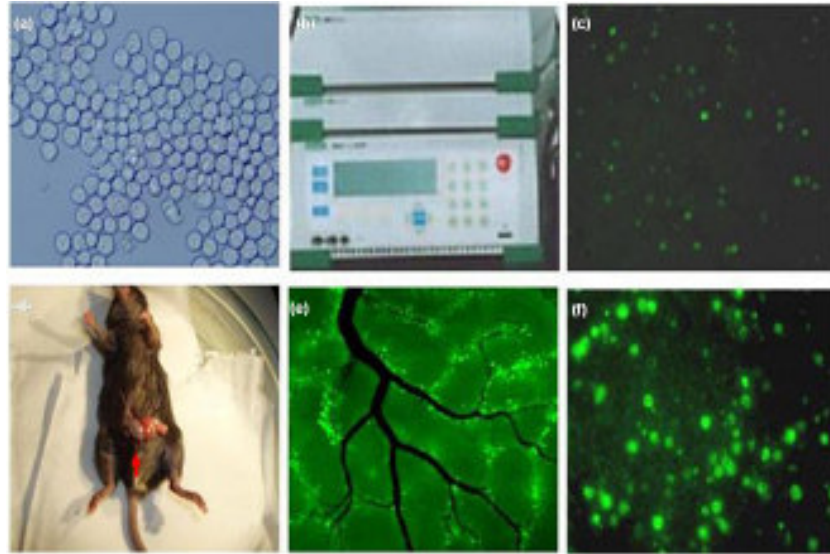


Fig. 11: Electroporation of SGCs with plasmid DNA (c-kit /GFP) and transplantation (a= SGCs; b= electroporator; c= SGCs in culture 24 hr after electroporation; d= one month post busulfan treated recipient mice after GCT (); e= testis expressing GFP positive SGCs on the basement area of seminiferous tubules three months post GCT and f= GFP positive SGCs.

Role of c-kit/SCF in SGCs proliferation and differentiation *in vivo*

The role of c-kit/SCF (stem cell factor) signaling in postnatal gonadal development and spermatogenesis is not clearly known, although unsuccessful attempts were made by several investigators in the past using c-kit (w/w -/-) and SCF (*Sl/Sl* -/-) mutant mice which were devoid of germ cells in their gonads. In the previous year, we showed that c-kit/SCF signaling plays an indispensable role in the migration and proliferation of PGCs during gestation in mice (Annual Report 2005-06, p 36).

During the year, using germ cell transplantation approach, we were able to demonstrate that SCF indeed is required for differentiation of SGCs but not for their proliferation. GFP positive green SGCs (10^5) were incubated at 37 °C and 5 percent CO₂ for 1 hr in the presence of 1 µg/ml of anti- SCF antibody for 10 min. After washing, these cells were transplanted into the seminiferous tubules of busulfan treated infertile mice (one month post treatment) via rete testis. As a

positive control, GFP positive green SGCs (10^5) were incubated at 37 °C and 5percent CO₂ for 1 hr in the presence of SCF (1µg/ml) and used for transplantation. Immunofluorescence studies revealed that, three months post transplantation, all the green SGCs were differentiated as these cells were found to be c-kit negative. In contrast, SGCs in control samples were undifferentiated and hence expressed c-kit, suggesting SCF is a prerequisite for maintenance of differentiated SGCs but not for proliferation of undifferentiated SGCs. These results were further confirmed by RT-PCR for SGC specific markers viz; c-kit and Ep-CAM. The expression of both genes was significantly down regulated in anti-SCF antibody treated group than in the transplanted control group (Fig. 12).

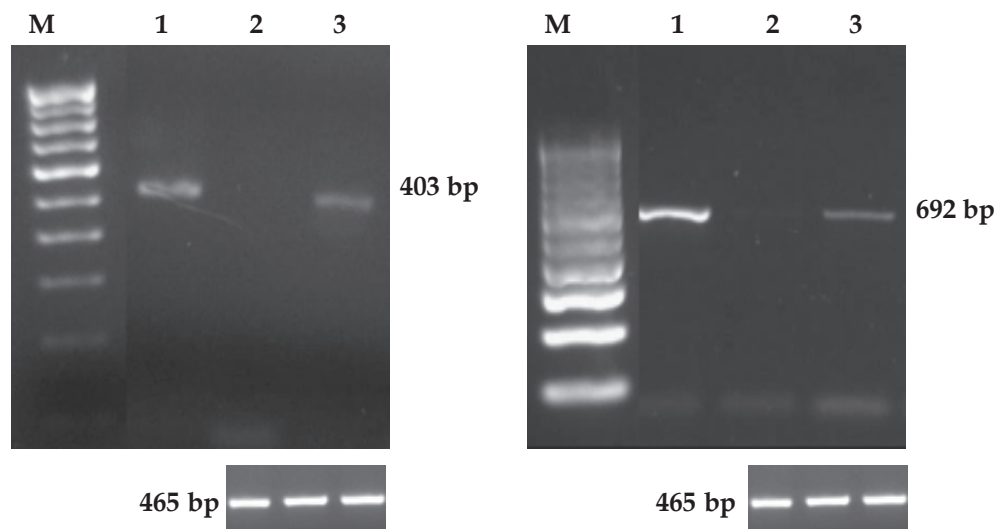


Fig. 12: RT-PCR analysis of SGCs specific marker genes (c-kit and Ep-CAM) in wild type (1), anti-SCF antibody treated (2) and after germ cell transplantation (3). Cyclophilin-A (465 bp) used as a loading control.

Effect of endogenous SGCs of busulfan treated mice on the niche/colonizing efficiency of transplanted SGCs

To evaluate the effect of endogenous germ cells on the transplantation efficiency, green SGCs (10^5) were transplanted into the right seminiferous tubule of GFP positive mice (n=9) via rete testis after 10, 20 and 30 days of busulfan treatment (each group consists of three mice). In each animal the left testicle received 10 µl of DMEM and served as control. Mice were autopsied three months post transplantation. The testicular serial cryosections from 3 testes were viewed under fluorescence microscope for colonization of SGCs. More than 200 tubular cross sections of each mouse were counted. The results indicated that, 30 days post busulfan treatment, the mice showed more than 85percent of GFP positive

cells predominantly in the basement area of seminiferous tubule where the tubules were almost devoid of endogenous SGCs. However, in the other two groups only 10 and 40 percent of SGCs were observed in the basement area, suggesting that the endogenous SGCs may hinder the anchorage of the exogenously transplanted SGCs.

1.1.5 Studies with FSH Binding Inhibitor: Functional Significance of FSH Modulators from Follicular Fluid in Ovarian Pathophysiology (*Partly funded by CONRAD*)

Principal Investigator: **Tarala D. Nandedkar**

Project Associates: Swati Kulkarni, Rajshri Navlakhe and Smita Mahale

Collaborators: Ashok Patel, Pradeep Vavia,
Pharma Division, University Institute of
Chemical Technology, Mumbai

Duration: 2006-2009

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 antiovaratory / antifertility activity in rodents and non - human primates. However, in monkeys the treatment is for 8 - 10 days. To avoid the 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 poly vinyl alcohol (PVA) / alkyl poly glucoside (APG) as surfactants. On the basis of physical properties APG (Fig. 13a) was found to be better than PVA (Fig. 13b). Particle size determination was carried out on freeze dried nanoparticle (after re-dispersion) using Malvern Meta-sizer equipped with 2000 Hydro MU. The size of nanoparticles and encapsulation efficiency are depicted in Table 1. The immunological activity was determined by ELISA using polyclonal antibodies to OP and it was observed that ~90percent activity was retained by the peptide nanoparticles. Biological activity was elucidated by in-vivo administration of the peptide nanoparticles in Swiss mice. Flow cytometric analysis showed that the biological activity was also restored when compared to the control animals (Fig. 14). The release profile studies in vitro are being conducted.

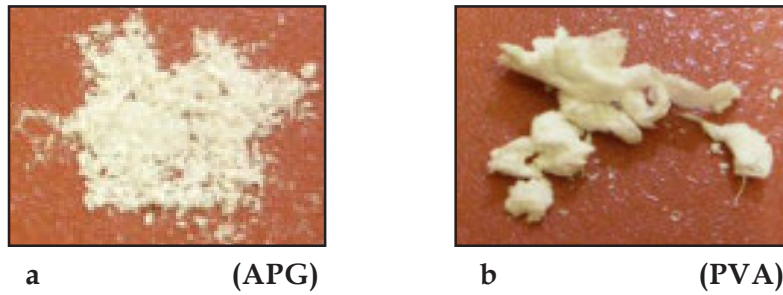


Fig. 13: Physical appearance of the hGF₂ nanoparticles (a) amorphous powder (b) fluffy aggregates.

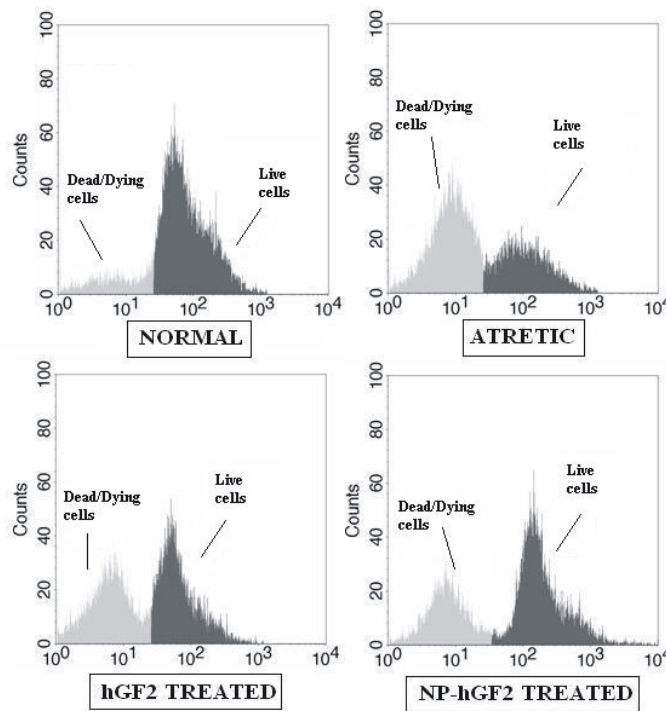


Fig. 14: Flow cytometric analysis showing the biological activity in terms of fractions of live and dead/dying cell population in various experimental groups.

Study of apoptotic pathways

The apoptotic pathways in normal, atretic and hGF₂/ OP treated mice were studied earlier by flow cytometry (Annual Report 2004-2005, p 43-45). It has shown that expression of Fas was predominant in granulosa cells of hGF₂/ OP treated group whereas the mitochondrial membrane potential alteration is more in atretic group, suggesting two different pathways in atretic and treated

groups. In the present study, the results are validated by immunohistochemical localization of various apoptotic markers.

Table 1: Comparative data of particle size and encapsulation efficiency obtained at various concentrations of PVA and APG

Batches	Surfactant used	Concentration of surfactant (% w/v)	Particle size (nm)	Encapsulation efficiency (%)
NPVA1	Poly Vinyl Alcohol	0.5	345.0 ± 2.60	65.60 ± 1.20
NPVA2		1.0	232.0 ± 1.10	68.70 ± 1.12
NPVA3		1.5	210.0 ± 3.26	73.60 ± 1.22
NPVA4		2.0	196.7 ± 5.06	75.16 ± 1.56
NPVA5		3.0	180.7 ± 2.87	77.05 ± 1.21
NAPG1	Alkyl Polyglucoside	0.05	419.0 ± 1.82	70.85 ± 3.12
NAPG2		0.1	394.0 ± 3.47	74.21 ± 1.08
NAPG3		0.25	351.0 ± 1.20	73.90 ± 1.32
NAPG5		0.5	200.0 ± 3.44	74.99 ± 1.53
NAPG6		0.75	184.6 ± 5.07	76.94 ± 2.34
NAPG7		1.0	102.3 ± 1.97	76.99 ± 0.97

For Immunohistochemical (IHC) localization, ovaries were fixed in Bouin's fixative for 24 hrs. Then processed for histology and embedded in paraffin. The paraffin sections (5µ) were used for apoptotic markers Fas and caspase 8 for membrane receptor while Cytochrome C and caspase 9 for mitochondrial pathway.

Expression of the apoptotic markers in all the treated groups was in oocyte as well as in granulosa cells. Localization of the death receptor Fas was observed to be more in hGF₂ / OP treated ovaries whereas the mitochondrial cytochrome c was more in atretic control ovaries. However, Caspase 9 which is supposed to be activated by the cytochrome c released from mitochondria, was found to be more in treated ovaries (Fig. 15). Thus, a shift from Fas - Fas L receptor pathway to mitochondrial pathway can be hypothesized may be through the linker molecule BID which is being elucidated.

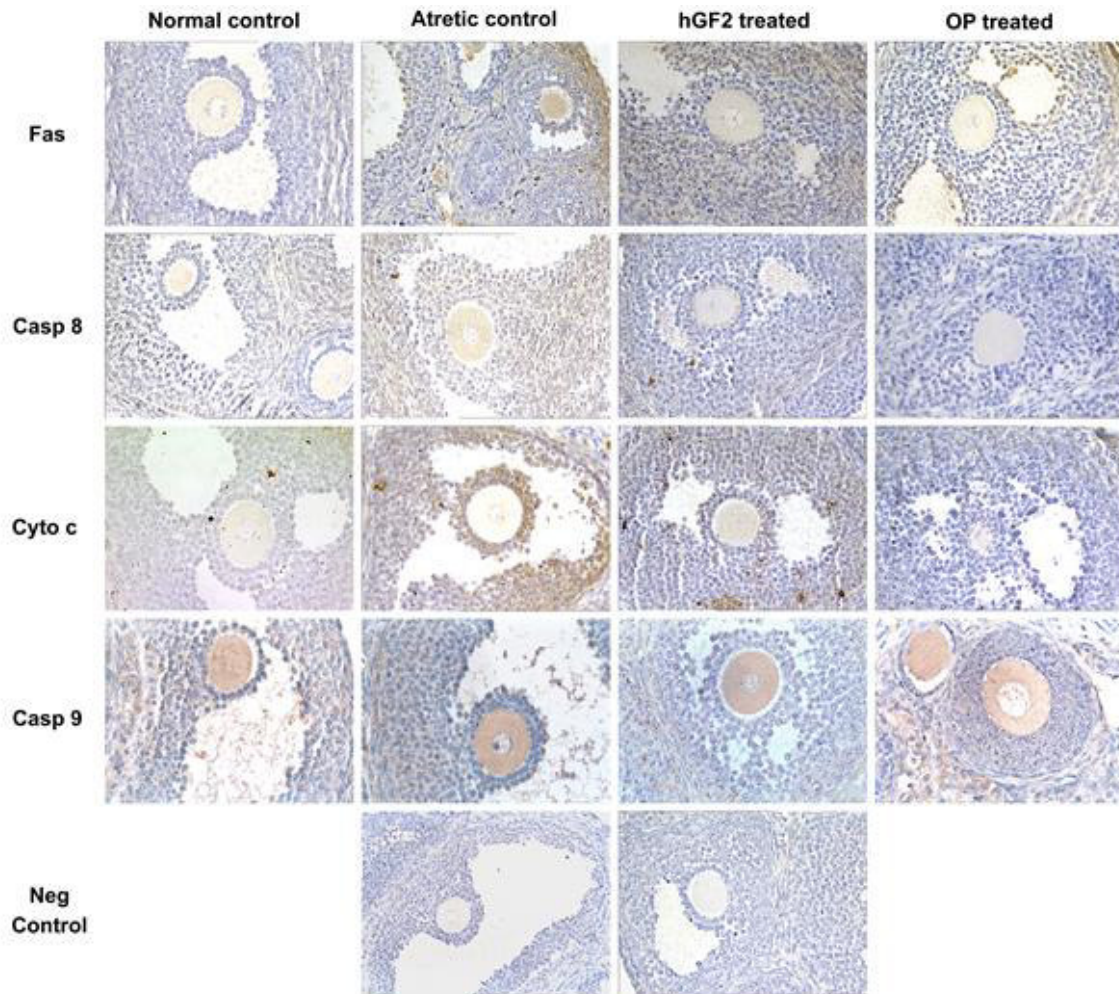


Fig. 15: Immunolocalization of Fas, Caspase 8, Cytochrome c and Caspase 9 in normal atretic and hGF2 / OP treated mouse ovaries. In absence of primary antibody negative controls were incubated with norm.

1.1.6 Factors Regulating early Folliculogenesis in Mouse Ovary (Funded by Department of Biotechnology)

Principal Investigator: **Tarala D. Nandedkar**

Project Associates: Shalmali Dharma, Deepak Modi

Duration: 2003-2007

The follicle of the mammalian ovary develops in several distinct stages during folliculogenesis. The recruitment of primordial follicles into primary and

secondary follicles is a crucial process in folliculogenesis. Relatively few genes are known which control these events; thus identification of specific genes expressed during early follicular development may reveal key players in this process.

To investigate this, RNA from the ovaries of neonatal mice was extracted on day-2 (only primordial follicles) and day-4 (primary follicles) and the gene expression profiles by cDNA arrays were analyzed. Results revealed that thirty percent genes were differentially expressed during transition from primordial to primary follicles. Amongst the up regulated genes, members of distinctly diverse pathways such as growth factors, hormone receptors, transcriptional regulators, signal transducers etc. were found to be expressed. Apoptotic genes showed down regulation suggesting the possibility of modulation of programmed cell death.

Immunohistochemical studies were performed for growth factors like Anti Mullerian Hormone, c-kit and Stem Cell Factor (Annual Report 2005-06, p 45-46). Apart from these genes, differential expression of few molecules that have not been reported previously in the ovary were identified. These include members of olfactory receptor gene family, which are G-protein coupled receptors. The quantitative measurements of transcript levels revealed that olfactory receptor mRNA is up regulated in day-4 ovaries as compared to day-2 ovaries. Hence we validated the presence of olfactory receptors by PCR and in situ hybridization.

Detection of Olfactory receptor (OL) transcripts in neonatal mouse ovaries

The presence of olfactory receptor transcripts was examined by RT-PCR of total RNA from day-2 and day-4 mouse ovaries. Olfactory receptor mRNA was expressed at the stage of day-4. RT-PCR was performed using specific primers of olfactory receptors H12, B12, OL10, OL30 and OL33. After PCR amplification, the preliminary results detected products of expected sizes in RNA isolated from day-4 mouse ovaries (Fig.16). OL10 showed a band of 130bp in addition to 236bp. RNA extracted from day-2 ovaries was devoid of olfactory receptor transcripts (Data not shown). The data needs to be confirmed by sequencing the bands.

Cellular localization of olfactory receptor in neonatal mouse ovaries

Cellular distribution of olfactory receptor mRNA was studied by non-radioactive in-situ hybridization using digoxigenin labeled olfactory receptor specific nucleotides as probes. Olfactory receptor mRNA signals were evident as a brown purple precipitate in the nucleus and cytoplasm of the granulosa cells.

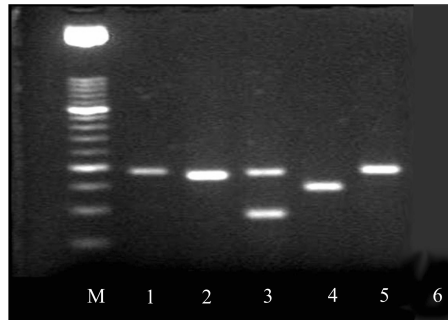


Fig.16: Ethidium bromide stained agarose gel from RT-PCR of RNA extracted from day-4 mouse ovary using primers of olfactory receptors.
 Lane 1. 50-kb DNA standard marker (Gibco BRL),
 Lane 2. Olfactory receptor H12 (214 bp),
 Lane 3. Olfactory receptor B12 (229 bp),
 Lane 4. Olfactory receptor 10 (236 bp, splice variant : band of 130 bp),
 Lane 5. Olfactory receptor 30 (199 bp),
 Lane 6. Olfactory receptor 33 (232 bp),
 Lane 7. Negative control (RT-ve).

The specific signals for olfactory receptor mRNA were detected in the granulosa cells of day-4 (Fig. 17) ovaries whereas day-2 ovaries were devoid of any staining. These signals were specific as no signals were detected in negative control when a sense probe was used for hybridization.

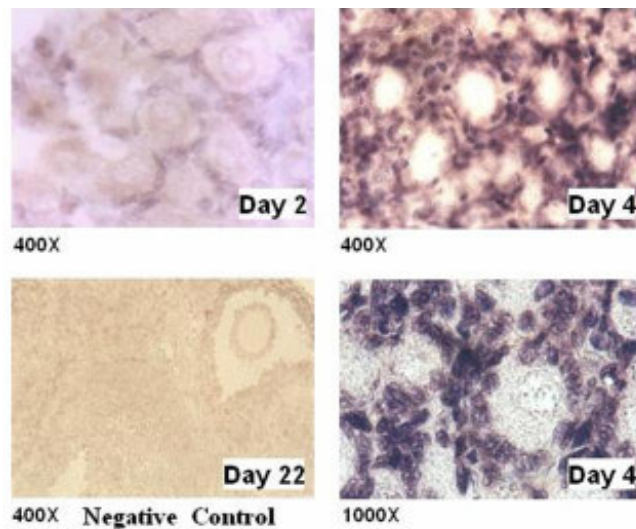


Fig. 17: mRNA localization of Olfactory receptor in day 2 and day 4 mouse ovaries. Note the presence of MRNA in granulose cells of day 4 ovaries. Day 22 is negative control showing no staining.

Our results suggest that the specific members of olfactory receptor gene family may have a function in proliferation, thus follicle undergo transition from primordial to primary stage.

1.2 Assessment of Safety, Efficacy and Acceptability of Existing Methods of Fertility Regulation

India offers five contraceptive options through its National Family Planning Program (NFFP) viz. vasectomy and condom for men, intrauterine contraceptive devices, low dose combined oral contraceptive choices and tubal ligation for women. With the objective of expanding contraceptive choice, a multicentric study evaluating the safety and acceptability of Norethisterone Enanthate (NET-EN), a two monthly injectable contraceptive was initiated with the objective to i) assess user acceptability/ continuation rates of NET-EN ii) evaluate the incidence of menstrual irregularities and other side effects iii) study the return of fertility following discontinuation of contraceptive used and iv) assess socio-behavioural aspects of volunteers and compare with different regions and cultural settings. The main emphasis of this study is on good counseling by qualified and trained staff to enhance better continuation rates. The multicentric study was conducted at nine centers from various parts of our country involving 1200 volunteers.

1.2.1 Acceptability and Continuation Rates of 2 Monthly Injectable Contraceptive: Norethisterone Enanthate: Protocol amendment –DEXA study. (Funded by Ministry of Health and Family Welfare)

Principal Investigator: **Shanta Chitlange**

Project Associates: Kamal Hazari, D. Balaiah,
Lalita Savardekar, Chitra Thosar,
Neha Minde, Shalini Baji,
Pratibha Kokate, Deepika Belekar,
P. Tapase and Kranti Paradkar

Participating Centers: Prof. H. Saini, Baroda
Prof. C. Alexander, Chennai
Prof. A. Bhargava, Jaipur
Prof. V. Salvi, KEM Hospital, Mumbai
Prof. S. Salhan, New Delhi

Duration: 2005-2007

A two-year follow-up of women on injection NET-EN was completed and observations based on 17268 months of use was reported last year (Annual Report 2005-06, p 46-50) Dr Jaya Lalmohan, Consultant, Department of Family Welfare, Government of India, New Delhi reviewed the entire study on March 16, 2007, visited the three NIRRH clinics and interviewed users of injections. During the reporting period, BMD evaluation of women who discontinued Net-En injection at least one year before was carried out (i.e. after the effect of injection was over). Thus a total of 98 women underwent DEXA scans at lumbar spine and femoral neck. They were in the age group of 23-36 years.

The findings show that there was significant increase in mean BMD by 2.7 percent at femoral neck among injection discontinuers compared to injection users. Similarly significant increase in mean BMD was observed at lumbar spine by 5.2 percent (Figure 18a & 18b). The other variables like Body Mass Index (BMI) had positive correlation with BMD. It was observed that women having BMI of less than 20 had low BMD compared to women having BMI of 20 and above and this was significant at femoral neck ($P=0.001$) and lumbar spine ($P<0.001$, Fig. 19). Also, the mean BMD among women having one or two children was significantly higher ($P<0.05$) compared to women having three or more children. There was no correlation between BMD and duration of injection Net-En use. The study is on-going.

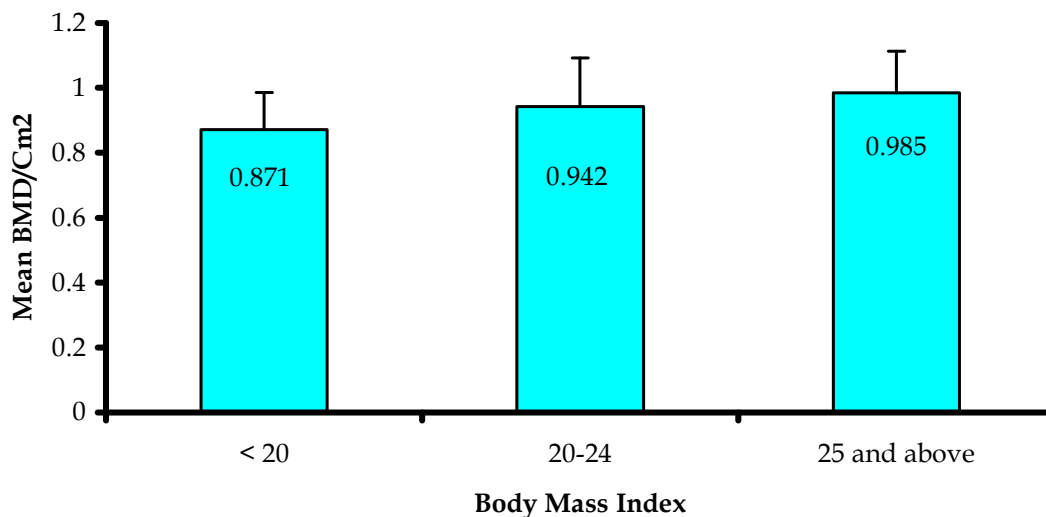


Fig. 18a: Bone Mass Density at Lumbar Spine compared with Body Mass Index (n=142).

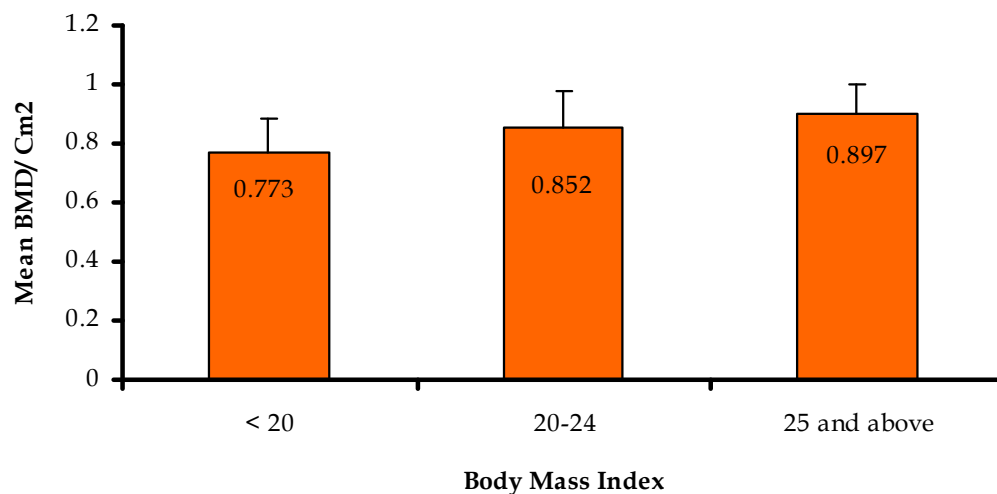


Fig. 18b: Bone Mass Density at Femoral Neck compared with Body Mass Index (n=142).

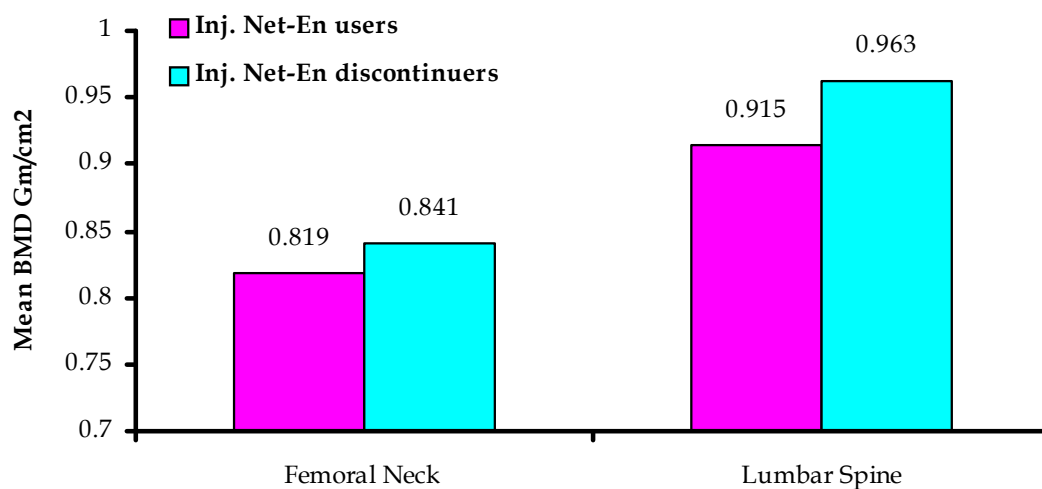


Fig. 19: BMD values by DEXA among injection Net-En users compared with injection discontinuers (n=98).

1.2.2 Reacceptance of Contraceptive: An Intervention Study

Coordinator: Kamal Hazari
 Principal Investigator: **Pratibha Kokate**
 Project Associate: Virginia Kiro
 Duration: 2006 - 2007

The PI underwent 6-week training in Community Health and Development Training programme for Trainers at International Service Association (INSA), Bangalore, which was followed by this study over a period of 1 year. The aim of this study was to identify 200 women from the NIRRH Abhyudaya Nagar Family Welfare clinic and surrounding residential areas, who had discontinued contraceptives and motivate at least 50 percent of these women to re-accept regular and reliable contraceptives

Methodology

From the records of women who had used and discontinued contraceptives 264 women were identified. Among these, 64 were excluded from the study intervention either because they had started another method (n=25) or they were not eligible for contraception (n=39). Only the 200 women eligible for the study were contacted through house visits or by telephone (1st contact) and called to the clinic for interview (2nd contact) to update their socio demographic data, determine details of reasons for discontinuation of the last contraceptive and non-acceptance of another method so far. Based on common reasons for discontinuation, IEC material for motivation and counseling for contraceptives were prepared. Appropriate strategies for individual women/couples were utilized. Women who accepted a method at this visit were given the necessary instructions.

Those who did not accept any method were again contacted and requested to attend the clinic (3rd contact) to discuss with satisfied users and share personal experiences, clarify myths and misconceptions. At this visit additional interventional strategies included couple counseling, group discussions and interpersonal communication (IPC).

All the new accepters were followed up, to note their continuation, satisfaction and provide suitable management for any side effects or problems experienced.

Community activities were arranged on various topics of general and reproductive health on important festivals and occasions, including:

1. 'International Labour Day' programme on hygiene and sexual health
2. Drama on Family Management by women of tailoring class (100 audience)
3. Talk on Nutrition and Parent- Teacher Relationship on Children's day (100 children and 5 teachers participated)
4. Diwali function, invited experts, Topic : Children's mental health & social problems (how to deal with emotionally weak children)

5. Rangoli competition 12 women participated, 4 were awarded cash prizes
6. On HIV/AIDS day posters were displayed, CHV, clinic staff and community women participated

These activities helped to sensitize, involve and promote interaction between community members and clinic staff. A mid-study site visit by INSA faculty in October 2006 evaluated the progress as good.

Results:

Projected Target	200
No. of discontinuers	264
No. not eligible for study	64 (25 already accepted another method and 39 for personal reasons/shifted residence)

Most of the women were from lower middle class, of different ethnic, traditional socio cultural background and practicing different religions (Hindu & Muslim), staying in slum (kuchha), chawl (pucca) or rooms in the housing board buildings. The literacy rate was 80 percent, majority up to 10th Standard. The involvement of men is low, majority were in low paid temporary jobs or unemployed.

Reasons for Discontinuation of last contraceptive (N=200)

Reasons	Number	Reasons	Number
Planning pregnancy	39	Pricking by IUD thread	4
Wants change of method	37	Irregular delayed periods	4
Wants more reliable method	25	Wants tubal ligation	3
Heavy bleeding	24	Amenorrhoea with injection	2
Completed IUD life span	15	Temporary separation	2
Personal reasons	13	IUD thread not seen	1
Study completed Injection	12	Vaginal discharge	1
Other medical reasons	12		
Partial displacement of IUD	4		

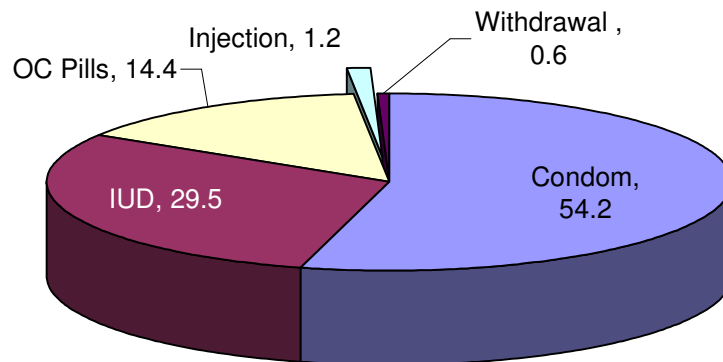


Fig 20: Contraceptive method re-accepted N=200

Among the 200 women eligible for this study, all accepted some contraceptive method following the interventions. 83 percent accepted spacing methods and 17 percent underwent tubal ligation as permanent contraception. Vasectomy was not accepted as a choice. Among the temporary methods initiated 54.2 percent took condoms, 29.5 percent had IUD insertions, 14.4 percent started OC pills, 1.2 percent took injections and 1 couple (0.6%) practiced withdrawal. All women who accepted temporary spacing methods are still continuing the same (satisfied users). The study data was presented at follow-up workshop in March 2007 with all the INSA participants.

Conclusion:

1. The study shows that with proper counseling, reassurance and repeated contacts 100 percent of previous contraceptive users accepted temporary or permanent contraceptive method.
2. Good rapport enhances counseling and helps past users in decision making for re-acceptance of contraceptive.
3. Among the non-eligible couples the major reasons are shifted residence and lost to follow-up, which is a unique feature of frequently mobile urban slum population.
4. The 54.2 percent condom use indicates male involvement and sustained motivation though couples do not easily accept vasectomy.
5. 29.5 percent women accepted IUDs.
6. The 6 monthly contraceptive continuation is high due to regular counseling, follow up and necessary treatment.

1.3 Enhancing the Role and Responsibilities of Men in Reproductive Health

Male reproductive health involvement implies encouraging a range of positive reproductive health behaviours by men to help ensure women's and children's well-being as well as their own. There has been a general agreement among the countries in the world, particularly after the International Conference on Population and Development (ICPD) held at 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, envisages the necessity of male involvement in improving the 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 behaviours, use of reproductive health services, and reduction in reproductive morbidity and mortality. Hence, there is a need to address the reproductive health concerns of men, as also of the couples for improving the reproductive health seeking behavior of couples.

1.3.1 Interventions in Urban Slums for Enhancing Participation of Men in Reproductive Health *(Funded by Department of Family Welfare, Ministry of Health and Family Welfare, Government of India)*

Principal Investigator: **Donta Balaiah**

Project Associates: D.D. Naik, U. Iddya, Saritha Nair and P. Tapase

Collaboration: Municipal Corporation of Greater Mumbai

Duration: 2004-2007

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 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; determine decision making process on issues related to safe motherhood, family planning, RTIs/STIs and HIV/AIDS; 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.

Methodology

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 regarding the ongoing intervention in the intervention 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 only married men in intervention area-1 (Mohili Village) and for couples in

intervention area-2 (Bail Bazar) during the period April 2006 to March 2007. The intervention programmes have been through Information, Education and Counseling (IEC). The services provided at the MCGM health posts is 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:

1. Street Plays:

- a. Five street plays were organized in both areas on issues concerning safe motherhood, for these 1100 persons from Mohili Village and 875 persons from Bail Bazar attended.
- b. Nine street plays in Mohili Village and 10 street plays in Bail Bazar were organized on family planning and contraception, for these 2475 persons from Mohili Village and 2025 persons from Bail Bazar attended.
- c. Five street plays in Mohili Village and 6 street plays in Bail Bazar were organized on RTIs/STIs, for these, 1375 persons from Mohili Village and 1500 persons from Bail Bazar attended respectively.
- d. Ten street plays were organized in both areas (i.e., Mohili Village and Bail Bazar) on HIV/AIDS, for these, 2300 persons from Mohili Village and 1425 persons from Bail Bazar attended respectively.

2. 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, 329 group meetings were organized for husbands for which, 3402 husbands attended. Whereas, in the area-2, 99, 100 and 18 meetings were organized for husbands, wives and couples respectively and for these meeting, 719 husbands, 810 wives and 65 couples attended respectively.

3. 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 2557 husbands from Mohili Village and 959 husbands, 1201 wives and 1185 couples from Bail Bazar. Information on correct use of condom was provided to 2673 husbands from Mohili Village and 1043 husbands, 1300 wives and 1392 couples from Bail Bazar. Information on non-scalpel vasectomy (NSV) was provided to 1556 husbands from Mohili Village, and 922 husbands, 1179 wives and 1159 couples from Bail Bazar. Information on RTIs/STIs was provided to 2966 husbands from Mohili Village and 1000 husbands, 1436 wives and 1392 couples from Bail Bazar. Information on HIV/AIDS was provided to 2508 husbands from Mohili Village and 930 husbands, 1091 wives and 1148 couples from Bail Bazar. Information on Antenatal Care (ANC) was provided to 2252 husbands from Mohili Village and 962 husbands, 1325 wives and 1368 couples from Bail Bazar.

4a. 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. 3000 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.

4b. Educational Programmes on Reproductive Health Issues:

Audio-visual programmes were organized for providing information on safe motherhood, family planning, RTIs/STIs and HIV/AIDS. Seventy-three programmes were organized for husbands in Mohili Village for which 1196 husbands attended. In Bail Bazar, 43 programmes were organized, 215 married women and 275 couples attended for these programmes.

5. 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 and 10 peer volunteers from Mohili Village and 16 ICDS project staff and 10 peer volunteers from Bail Bazar were oriented towards the importance of involving men in reproductive health issues.

6. Contact Visits:

In Mohili Village, project team along with Community Health Volunteers (CHVs) made 198, 151 and 433, visits respectively to General physicians, Local mandal/NGOs and Volunteers. The corresponding figures for Bail Bazar are 138, 91 and 271 visits respectively. The purpose of the visit was to solicit General physicians, Local Mandal/NGOs and Volunteers co-operation in fulfilling the aim of the project.

7. Cases Identified during Intervention Programmes:

Twenty eight married men, 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.

8. Counselling Clinic:

As part of the intervention, counselling services were provided since September 2005 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. 169 husbands, 3 wives, 33 couples and two boys attended counseling clinic at Mohili Village and 68 husbands, 255 wives, 45 couples and one girl attended to counseling clinic at Bail Bazar. About 16 per cent husbands from Mohili Village and 12.2 percent husbands from Bail Bazar accompanied wife for counselling.

9. Pap Smear Screening:

Ten camps each in Mohili Village and Bail Bazar were conducted. A total of 183 and 222 women from Mohili Village and Bail Bazar attended these camps respectively. Of these 175 women from Mohili Village and 208 women from Bail Bazar underwent Pap smear test. Pap smear test was not done in nine women from Mohili Village and four women from Bail Bazar as they were in menstrual period and one woman from Mohili Village was pregnant during the time of Pap smear camps. Four women from each Mohili Village and Bail Bazar were not willing to undergo Pap smear test due to fear of instruments and shyness. Pap smear findings indicate high prevalence of abnormal smears i.e.,

pre-cancerous and cancer is observed in 12 out of 383 women. Only 1.8 per cent smears were inadequate. Trichomonas vaginitis was noted in 0.8 per cent women. Pap smear is useful in detecting six STIs. HPV infection was observed in 7.6 per cent women and Bacterial vaginosis was observed in 13.3 per cent. Multiple infections were observed in 6.8 per cent women (Table 2, 3). About 3 percent of husbands accompanied wife for the Pap smear.

10. Services for Treating Reproductive Health Problems (Sunday Clinic):

As a part of intervention, clinic for Reproductive Health Services, i.e., **Clinic for Men** was initiated at Mohili Village Municipal health post in the month of May 2006 and **Clinic for Couples** was initiated at Bail Bazar Municipal health post for couples in the month of June 2006. Both the clinics function on every Sunday between 10.00 a.m. to 1.00 p.m.

Table 2: Pap smear findings

Result	Exp - 1 (Mohili Village)		Exp - 2 (Bail Bazar)		Total	
	Number	%	Number	%	Number	%
Negative	64	36.6	91	43.8	155	40.5
Inflammatory	103	58.8	106	51.0	209	54.6
Atypia	4	2.3	0	0.0	4	1.0
CIN* I	0	0.0	1	0.5	1	0.3
CIN II	3	1.7	2	0.9	5	1.3
Carcinoma	0	0.0	2	0.9	2	0.5
Inadequate	1	0.6	6	2.9	7	1.8
Total	175	100	208	100	383	100

CIN - Cervical Intraepithelial Neoplasia

Table 3: Infections detected by Pap smear

Result	Mohili Village (N=175)		Bail Bazar		Total (N=383) (N=208)	
	Number	%	Number	%	Number	%
No infection	106	60.6	131	63.0	237	61.9
Bacterial vaginosis	20	11.4	31	14.9	51	13.3
Trichomonas vaginitis	2	1.1	1	0.5	3	0.8
Monilia	8	4.6	9	4.3	17	4.4
Chamydia tracomatis	12	6.9	6	2.9	18	4.7
Human papuloma virus	13	7.4	16	7.7	29	7.6
Ovum- Entrobilus vermicularis	1	0.6	1	0.5	2	0.5
Multiple infections	13	7.4	13	6.2	26	6.8

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 counselling for suspected HIV infected cases, motivation for condom use and information on reproductive health issues are provided in these clinics once in a week on Sunday.

During June 2006 to March 2007, 174 beneficiaries attended the Clinic for Men at Mohili Village and 585 beneficiaries attended the Clinic for Couples at Bail Bazar first time (New Cases); i.e., 101 husbands, 30 wives, 40 couples, two girls and one boy attended clinic at Mohili Village and 39 husbands, 217 wives, 184 couples, 17 boys and 28 girls attended clinic at Bail Bazar. Follow up services were provided to 21 husbands, 25 wives, 28 couples and one girl at clinic in Mohili Village and 23 husbands, 234 wives, 103 couples, 2 boys and 37 girls at clinic in Bail Bazar.

Fig. 21, Fig. 22 and Fig. 23 illustrate complain wise clinic attendance of Clinic for Men (Mohili Village) and Clinic for Couples (Bail Bazar) respectively. About 23 per cent husbands from Mohili Village and 38 per cent husbands from Bail Bazar accompanied wife for reproductive health services.

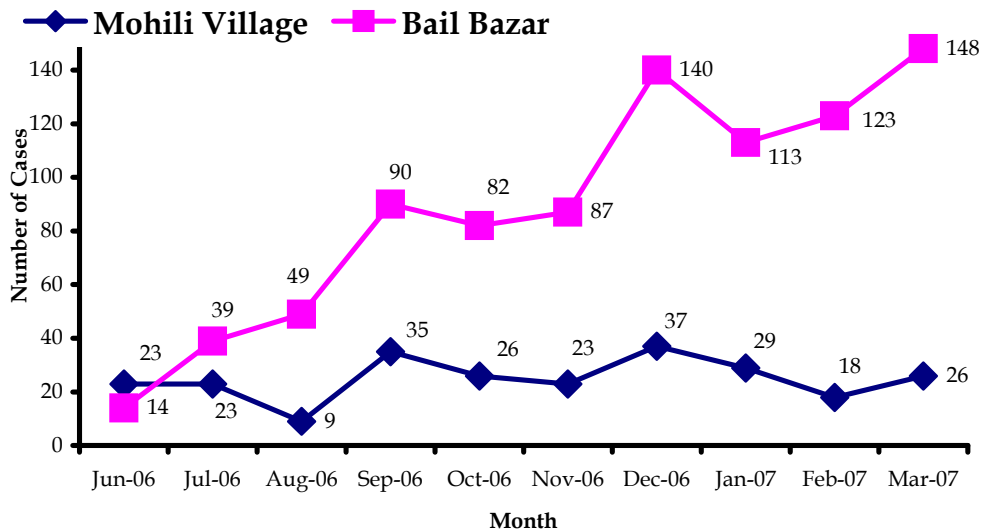
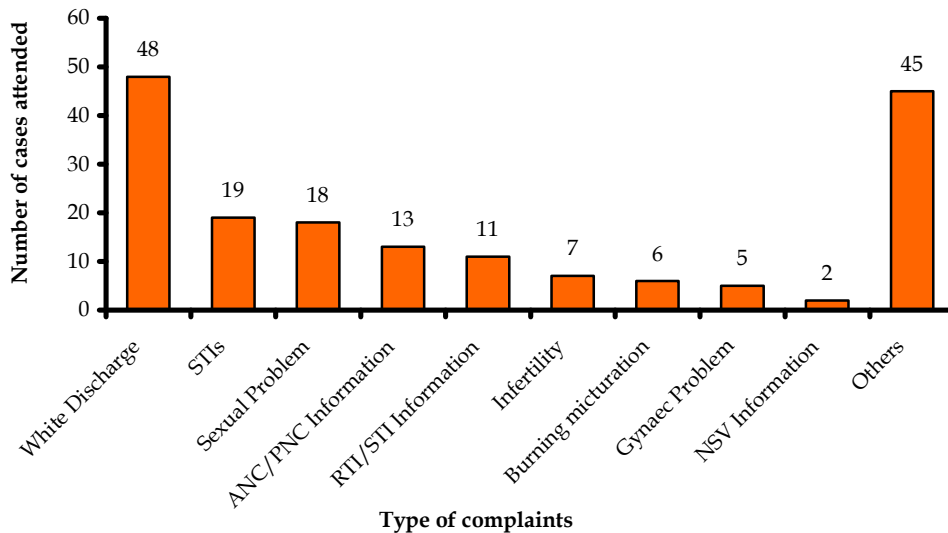


Fig. 21: Depicts clinic wise number of cases attended to Clinic for Men (Mohili Village) and Clinic for Couples (Bail Bazar).



Others include fever, scabies, piles, hernia, boils, chest pain and back pain

Fig. 22: Complain wise clinic attendance of Clinic for Men (Mohili Village).

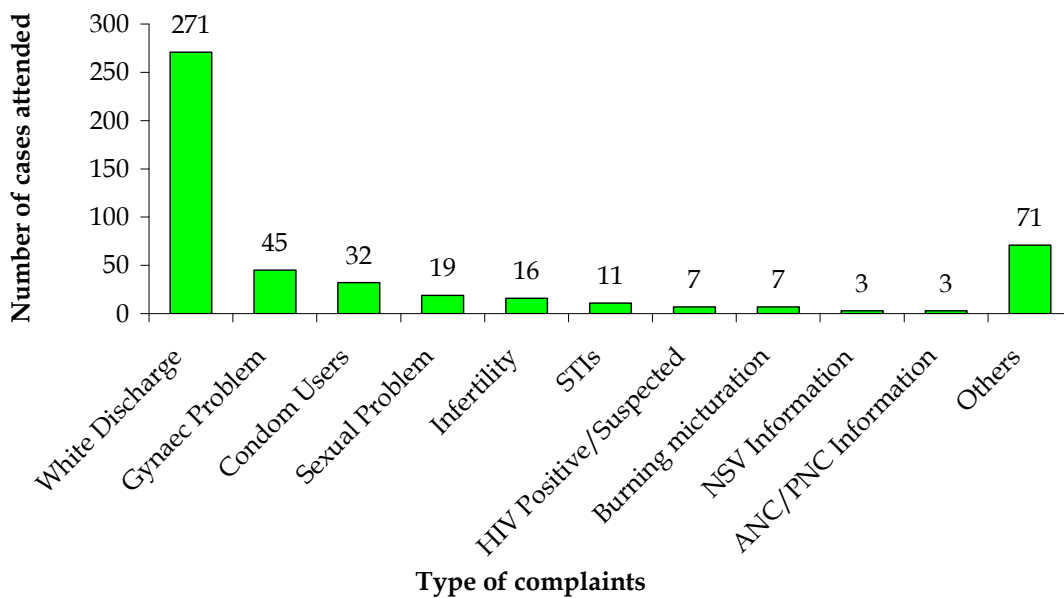


Fig. 23: Complain wise clinic attendance of Clinic for Couples (Bail Bazar).

Informal discussion with the beneficiaries reveals satisfactory results regarding the intervention programmes conducted and the services provided. Nevertheless, to understand the change that has taken place the overall reproductive health knowledge, attitude and practice and the support provided by men to their spouse, the post intervention survey has to be conducted. The planning and preparation for the resurvey is going on and will be initiated in July 2007.