Research programs in the area of fertility regulation include development of new and improved technologies for regulation of fertility, assessment of safety, efficacy and acceptability of existing methods of fertility regulation enhancing the role and responsibilities of men in reproductive health. Various strategies are being employed to identify molecules relevant to contraception. Studies to elucidate the mechanism of expression of several novel proteins having a role in sperm function, sperm maturation and fertilization are underway. In the area of female fertility regulation, studies on FSH binding inhibitor which blocks FSH binding to granulosa cells are being continued.

1.1 Identification and Characterization of Sperm Antigens using Neonatal Tolerization and Antisperm Antibodies

Principal Investigator: Vrinda Khole
Project Associates: Geeta Vanage, S. Ranpura, S. Joshi, Monali Wakle and Shagufta Shaikh
Duration: 1997-2007

Development of a contraceptive vaccine using sperm as an antigen has been pursued over the last couple of decades, as it has been well documented that sperm antigens play a crucial role in reproduction. Sperm proteins/antigens are mostly acquired either from the testis or the epididymis. Several different approaches and attempts using 1) neonatal tolerization, and 2) using antisperm antibodies (post vasectomy sera) were undertaken for identifying relevant proteins.

a) Using neonatal tolerization:

Conventional immunization using whole sperm followed by hybridoma technology results in antibodies to antigens invariably of testicular origin as these immunization procedures result in preferential production of antibodies to antigenically strong components. In view of this, we exploited the approach of neonatal tolerization where mice were tolerized to testicular sperm protein (tolerogen) at birth and later immunized with epididymal sperm protein (immunogen) for raising an epididymis specific immune response. Serum from one of the tolerized and immunized mouse identified a dominant epididymis specific protein of approximately 27 kDa (Annual Report 1999-2000, p 1) to which a polyclonal antibody was raised and immunochemically characterized (Annual Report 2000-01, p 20-23). The tolerized and immunized mouse was later used for generating monoclonal antibodies. Immunohistochemical analysis indicated that five monoclonal antibodies (mAbs) showed epididymis specific
reactivity (Annual Report 2001-02, p 26). Further immunochemical characterization of these antibodies showed that they exhibited strong reactivity to epididymal sperm in ELISA, were localized on different domains of the spermatozoa, and exhibited radial or comet shaped agglutination patterns. Of the five monoclonal antibodies, V1B8 mAb, was taken up for biochemical characterization of the cognate protein. The molecular weight of the protein, identified was ~ 27kDa. To examine the mode of anchorage of the protein, spermatozoa proteins from cauda epididymis were sequentially extracted and the cognate protein was found present both in the peripheral and integral forms (Annual Report 2002-03, p 25-31). Further characterization of the protein indicated the following:

i) It is epididymis specific

Specificity of the mAb V1B8E10 was checked by immunohistochemistry of somatic and reproductive tissues. The antibody showed no reactivity in any of the somatic tissues tested (Fig.1A) but showed a epididymis specific and cell type specific pattern (Fig. 1B).

Fig. 1A: Tissue specificity of 27 kDa protein by immunohistochemical staining. 1- Thymus, 2- Brain, 3- Spleen, 4- Liver, 5- Heart, 6- Kidney, 7- Vas deference, 8-Prostrate, 9- Seminal vesicle, 10- Testis (Magnification 40X). 1B: Cell type specific staining in the epididymal epithelium. Arrows indicate absence of immunoreactivity. IT- Intertubular space, L- Lumen. Magnification 100X.
It is developmentally expressed

The 27 kDa protein could be localized in the corpus epididymal epithelium by postnatal day 30 (Fig. 2).

![Fig. 2: Developmental expression of 27 kDa protein in rat corpus epididymis](image)

This time frame corresponds to the period during which dramatic alteration in morphology of epididymal epithelial cells takes place. Around this time principal cells become much more columnar, exhibit extensions of the golgi apparatus and have increased number of vesicles indicating upregulation of secretory activity.

iii) It is androgen regulated

Epididymal expression of 27 kDa protein was investigated in adult rat at different times following orchidectomy (Fig. 3).

![Fig. 3: Effect of castration on immunoreactivity of 27 kDa protein in corpus at 1 week castration, 2 week castration, 3 week castration.](image)

Immunohistochemical localization showed decreased protein in the luminal epithelium of corpus after 7 days of castration and no reactivity on days
14 and 21 post castration. Following supplementation with dihydrotestosterone there was gradual reappearance of the protein within 14 days confirming its androgen regulated status. As the 27 kDa epididymis specific protein was found to be androgen regulated it was named AREP-27 i.e. androgen regulated epididymal protein 27.

It is transferred to sperm via epididymosomes.

Western blot analysis indicated presence of AREP-27 in epididymosomes prepared from corpus and cauda epididymal fluid but not from caput. Presence of AREP-27 in the epididymosomes indicated that these membrane vesicles could act as carriers of AREP-27 to the sperm surface. (Fig. 4).

**Fig. 4:** Transfer of AREP-27 from epididymosomes to caput sperm. Lane 1 - caudal sperm protein, lane 2 - caput sperm incubated with epididymosomes, lane 3 - caput sperm, lane 4 - corpus epididymosomes. A: Silver stained protein profile and B: Immunoblot.

Ultrastructural Localization of AREP-27 on sperm and epididymal tissue

Caudal sperm showed gold labeling on the plasma membrane of mid piece and tail region (Fig 5A). Immunogold labeling showed the presence of the reactivity in the apical region of the principal cell as well as on microvilli of the corpus epididymis indicating possible mode of secretion (Fig. 5B).
b) Using antisperm antibodies (post vasectomy sera)

Vasectomy results in the occlusion of testicular outflow leading to autoimmunity characterized by the production of antisperm antibodies (ASA). At puberty, when immune competence is already established, differentiating germ cells commence a new programme that leads to the formation of mature spermatozoa. During this process an array of new surface molecules are expressed on the differentiating germ cells which do not belong to the family of those considered as ‘self’ by the immune system. Following obstruction of male genital tract, spermatozoa/germ cells are no longer sequestered behind the blood-testis and blood epididymis barrier, resulting in an autoimmune response. Using the vasectomised mouse model, monoclonal antibodies were generated and used as a tool to identify and characterize functionally relevant and conserved proteins. Monoclonal antibody D5E5 identified a ~70kDa antigen which is testis specific. The protein is referred to as Testis Specific Autoantigen-70, TSA-70.

TSA 70 is a testis specific autoantigen

Immunochemical localization of antigen in testis and epididymis with the mAb D5E5 is illustrated in (Fig.6). The cognate antigen is expressed postmeiotically in a stage specific manner starting from elongating spermatids step 8 during spermiogenesis upto mature spermatozoa. In the epididymis, the antigen is localized only on the spermatozoa from all the three regions but no reactivity was observed in the epididymal epithelium of caput, corpus or cauda epididymis (Fig. 6A2-A4). No staining was observed with the myeloma cell culture supernatant. (Fig. 6B)
Fig. 6A: The localization of antigen in mouse testis and epididymis (6A). In mouse testis (A1) the antigen is localized postmeiotically starting from elongating spermatid. In the epididymis the reactivity was seen on the spermatozoa in the lumen (L) of caput (A2), corpus (A3) and cauda (A4) but no reactivity was observed in the epididymal epithelium (EE). Absence of reactivity with the myeloma culture supernatant was observed for all the tissues as seen in B1-B4.

Domain specific localization

When domain specificity of the cognate antigen on mouse caudal sperm was studied using IIF with the mAb, it identified the antigen on the principal piece and end piece of the sperm tail and also on the tip of the acrosome. The fluorescence pattern was identical for sperm from the testis as well as those from all three regions of epididymis (Fig. 7). No reactivity was observed with SP2/0 supernatant.

The immunofluorescent labeling on rat caudal sperm showed an identical pattern of domain specificity i.e. on the tip of acrosome and the principal piece and end piece of the sperm tail. It was interesting to note that in bull, marmoset and human sperm it showed species-specific domain localization. In case of bull, it was localized on acrosome and tail. While in marmoset sperm it was seen on sperm tail whereas in human sperm it was restricted to the acrosome (Fig. 8).
Fig. 7: The domain specific localization of the TSA 70 by indirect immunofluorescence on mouse sperm from testis and epididymis. The mAb identified the domains on the tip of the acrosome as well as on the sperm tail (principal piece + end piece). Identical domain specificity was observed for the sperm from testis (A) and caput (B), corpus (C) and cauda (D) epididymis. A1-D1 depicts the corresponding phase contrast images.

Fig. 8: The protein is conserved across the species as seen by the fluorescence localization on rat, bull, marmoset and human sperm. Rat sperm (B) showed identical domain localization as that of mouse spermatozoa (A). But in phylogenetically divergent species the localization was different. In bull sperm (C), it identified domains on the acrosome and principal piece, in marmoset sperm (D) only on the tail while in human sperm (E) the localization was restricted to the acrosomal region. C1-E1 are corresponding phase contrast images.

On SDS-PAGE / WB a species-specific variation in the molecular weights of the conserved antigen was observed (Fig. 9).
Fig. 9: SDS-PAGE Western blot showing the species specific variation in the molecular weight of the protein. The molecular weights of the protein showed downward gradation being highest in rat (~70kDa; Lane 1) followed by human sperm (Lane 4) which was slightly lower. In marmoset (Lane 3) and bull sperm (Lane 2) it was the lowest. This study confirmed that the protein is conserved across the species and has a species specific form.

Role in sperm motility

The effect on motility was studied in terms of loss of progressive motility as well as total sperm motility. The mAb D5E5 showed significant effect on the percentage of progressive motility (Fig.10).

Fig. 10: Effect of the mAb on the sperm motility. The mAb drastically reduced the sperm progressive motility. As seen in the graph, in the presence of the mAb D5E5, the percent progressive motility was reduced to 36% within 15 mins of incubation. The myeloma supernatant had negligible effect on the sperm motility. Each point represents mean of 3 readings ± standard errors.

Significant loss of progressive motility upto 64 per cent was observed within 15 min of incubation. The loss of progressive motility was found to be more
significant and dramatic as compared to total sperm motility. The mAb had no apparent effect on viability when compared to the control at the given time interval (data not shown).

In view of its testis specificity, acrosome and tail localization and likely role in motility, the cognate antigen is likely to play an important role in reproduction and needs to be pursued.

Future Plans: The sequence of the 27kDa epididymis specific protein will be determined. Once this is known, attempts will be made to search for an orthologue for this protein in other species, most important of all, humans. Using specific primers (designed on the basis of the sequence determined), the epididymal cDNA will be amplified, cloned in an appropriate expression vector to obtain high amounts of the gene product which will then be used for further characterization of the protein.

1.2 Role of a Novel Androgen Regulated HoxB2 Containing Gene Expressed in the Epididymis (Funded by Indian Council of Medical Research under Functional Genomics and Molecular Medicine Program)

Principal Investigator: Vijaya Raghavan

Project Associates: A.H. Bandivdekar, E. Prabagaran and Nirmala Nair

Duration: 2002-2005

Spermatozoa acquire motility and fertilization potential in the epididymis, an accessory reproductive organ in male. Epididymal microenvironment is known to play a conducive role in acquisition of the functional motility by spermatozoa. Identification and characterization of proteins secreted by epididymis and regulated by androgens will provide an insight into their role in the cascade of sperm maturation. A cDNA clone showing homology to the conserved region of Hox-B2 was identified in a monkey epididymal cDNA library, screened with monoclonal antibody raised against washed human spermatozoa. The gene was demonstrated to be expressed in adult rat, monkey and human epididymis but not in immature rat epididymis by RT-PCR, suggesting that it is a conserved protein, probably regulated by androgen.

Our earlier studies indicated that the Hox-B2 containing protein is developmentally regulated as its expression has been observed from day 40 onwards in the epididymis concomitant with increase in the levels of androgens in circulation. This was further confirmed in castrated male rats where the protein was undetectable in the epididymis as seen by immunohistochemical localization, and was restored to normal levels in these animals when administered with DHT. It was observed that maximal expression was in the distal corpus and proximal caudal regions of the epididymis. The expression was
specifically localized in the epithelial principal cells and basal cells were not seen in any other reproductive tissues (Annual Report 2002-03, p 32-33).

Expression and Regulation of the Protein following Castration and Chemical Castration in the Rat Model

In addition to the castrated model, studies were carried out in 90-day old rats administered with a single dose of Ethane Dimethane Sulfonate (EDS) (75 mg/kg body weight) intraperitoneally. Since EDS is effective as a Leydig cell specific toxicant, it causes destruction of Leydig cells in the interstitium of testis that results in the depletion of testicular testosterone supply to the epididymis. In the EDS treated rats the level of Hox-B2 containing protein were found to be lower as compared to the untreated group and were found to be restored in the DHT and DHT +β-estradiol supplemented groups (Fig. 11a). This was confirmed using the BIOVIS Image Analysis software (Fig. 11b). The protein was localized in the epithelial principal and basal cells of the epididymis (Fig. 11c).

**Fig. 11a: Immunohistochemical localization of untreated rats (B), the Hox-B2 containing protein in the various segments of the epididymis of rats treated with EDS(C); administered DHT supplementation (D); administered DHT and estradiol supplementation (E), and epididymal sections stained with non-immune serum( are shown in A). Magnification 20X.**
Fig. 11b: Image Analysis of the effect of chemical castration using Ethane Dimethane Sulfonate (EDS) on Hox-B2 expression

Fig. 11c: Immunolocalization of Hox-B2 containing protein in the epithelial principal and basal cells of the rat epididymis (40X)
Northern Blot Analysis of Hox-B2 Containing Gene

Northern blot analysis was carried out using RNA isolated from the caput, corpus and cauda epididymis and using a commercially available multiple tissue northern blot (BD Biosciences). The 591 bp cloned fragment corresponding to the conserved Hox-B2 region non radioactively labeled with DIG was used as a probe (Annual Report 2002- 03, p 31). As seen in Fig. 12 a single transcript of ~2.5 kb was detectable in the epididymis alone. These results indicate that the protein is encoded by a single transcript of ~2.5 kb in size as shown by northern blot analysis only in epididymis but not in any other tissues studied.

Fig. 12: Northern Blot analysis identified a single transcript present in the epididymis but not in any of the other tissues

Multiple Tissue Western Blot Analysis

In an effort to study the tissue specific expression of the Hox-B2 containing protein, protein extracts were prepared from different rat tissues including brain, heart, lung, liver, spleen, kidney, skeletal muscle, small intestine, large intestine, testis, prostate, seminal vesicle, vas deference as well as epididymis 10mM Tris-HCl, 15mM EDTA, 1mM DTT, 1 per cent with 50µl cocktail 25µg protein equivalent of the extracts were loaded and electrophoresed on a 10% SDS gel under denaturing conditions (Fig. 13a), transferred to nitrocellulose membrane, and probed with the antibody to Hox-B2 and detected using the ECL Plus Kit.

Western Blot analysis indicates a single 30 KDa band as visualized only in the lane of epididymis but not in any other tissue extracts as analyzed (Fig. 13b).
a. SDS-PAGE analysis of Rat tissue extracts

Lane 1: Brain
Lane 2: Heart
Lane 3: Lung
Lane 4: Liver
Lane 5: Kidney
Lane 6: Spleen
Lane 7: Skeletal muscle
Lane 8: Small intestine
Lane 9: Large intestine
Lane 10: Testis
Lane 11: Epididymis
Lane 12: Prostate
Lane 13: Seminal vesicles
Lane 14: Vas deferens
Lane 15: Molecular Weight

b. Western blot detection with ECL

i) Western blot analysis probed with specific antibody to Hox-B2

ii) Probed with non-immune serum.

Fig 13 a: Coomassie blue stained proteins extracted from various tissues.
Fig 13 b: i) Western blot analysis probed with specific antibody to Hox-B2
ii) Probed with non-immune serum.
The results obtained so far indicate the presence of a single transcript of approximately 2.5kbp in the epididymis; that the protein is specifically expressed in the principal and basal cells of the epididymis as evidenced by immunohistochemical localization and is androgen regulated. The expressed protein has a molecular weight of ~30kDa as seen by multiple tissue western blot analysis and detected only in the epididymal tissue extract.

Studies are ongoing to localize the protein on sperm, to express the recombinant protein and attempt to elucidate the role of this protein in sperm function.

1.3 Studies on 80 kDa Human Sperm Antigen (80kDa HSA) and its Synthetic Peptides for Immunocontraception (Funded under the Indo-US Programme on Contraceptive and Reproductive Health Research)

Principal Investigator: A.H. Bandivdekar

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

Duration: 1993-2006

An 80kDa human sperm antigen (80kDa HSA) was identified from human sperm extract by Western blot technique using serum of an infertile woman having antisperm antibodies as a cause of infertility. Active immunization with purified antigen induced immunological infertility in male and female rats. Immunohistochemical and immunofluorescent studies showed that 80kDa HSA is sperm specific protein. Antibodies to 80kDa HSA agglutinated human, rat and monkey spermatozoa in vitro. The partial N-terminal amino acid sequence of 80kDa HSA (Peptide NT) and the peptides obtained by digestion with endoproteinase Lys-C (Peptides 1, 2, 3 and 4) and with endoproteinase Glu-C (Peptides 5 and 6) showed no sequence homology with any of the known protein sequences in the gene database.

The synthetic peptides NT, 1, 2 and 4 conjugated to Keyhole limpet haemocyanin (KLH) were demonstrated to be immunogenic in rabbits. Antibodies against these peptides specifically recognized 80kDa HSA in specific ELISA and Western blot and agglutinated human, rat and monkey spermatozoa in vitro. Thus suggesting these peptides immunobiologically mimicked the native protein Peptide 3 failed to elicit a significant antibody titer and hence was not further investigated.

A 540bp partial cDNA sequence obtained using specific primers designed based on the amino acid sequence of peptide NT and peptide 6, showed homology with a hypothetical testicular protein (Annual Report 2002-03, p 33).
Passive administration of antibodies to peptides NT, 1, 2 and 4 to adult male rats resulted in agglutination of epididymal spermatozoa with loss of motility and failure to impregnate normal females and when administered to normal cycling females resulted in infertility, and antibodies to peptides NT and 1 being most effective in inhibiting fertility (Annual Report 2001-02, p 28).

These results were further confirmed by passive administration to male and female rats of the purified IgG fraction of antibodies to peptides 1 and NT (10 and 40µg) as seen in Tables 1 and 2. A dose dependent inhibition of fertility was seen in female and male rats following passive administration of purified IgG fraction of antibodies to peptide NT and 1.

**Table 1: Fertility status of female rats following passive immunization with purified IgG of antibodies to NT peptide and peptide 1 of 80kDa HSA.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pregnant/Total No. Animals (%) Inhibition of Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10µg</td>
</tr>
<tr>
<td>Control (IgG from preimmune serum)</td>
<td>8/ 8 (0)</td>
</tr>
<tr>
<td>Ab. NT Peptide</td>
<td>5/ 8 (37)</td>
</tr>
<tr>
<td>Ab. P1</td>
<td>5/ 9 (45)</td>
</tr>
</tbody>
</table>

**Table 2: Fertility status of male rats following passive immunization with purified IgG antibodies to NT peptide and peptide 1 of 80kDa HSA.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fertile/Total No. Animals (%) Inhibition of Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10µg</td>
</tr>
<tr>
<td>Control (IgG from preimmune serum)</td>
<td>8/ 9 (11.0)</td>
</tr>
<tr>
<td>Ab. NT</td>
<td>5/ 9 (45)</td>
</tr>
<tr>
<td>Ab. P1</td>
<td>4/ 9 (56)</td>
</tr>
</tbody>
</table>

Active immunization with KLH conjugated peptides 1 and NT resulted in agglutination of ejaculated spermatozoa and these rabbits failed to impregnate the normal females. These rabbits regained fertility with decline in antibody titer, following cessation of immunization (Annual Report 2002-03, p 34).
Anti-fertility studies are being extended in nonhuman primates- marmosets. Eleven fertile adult male marmosets have been recruited and nine have been immunized with KLH conjugated peptide-1. A significant antibody titer was observed in 7 marmosets following 2 boosters. In one of these animals with high antibody titer, the epididymal spermatozoa were found completely immotile. Studies are ongoing to assess the fertility of the immunized male marmosets.

In an attempt to assess the mode of action of 80kDa HSA, localization of the protein was studied using specific antibodies and FITC labeled antirabbit gamma globulin. Localization was observed predominantly in the tail region of the marmoset sperm and in a few sperms in the acrosomal region (Fig. 14).

![Fig. 14: Immunofluorescent localization of 80kDa HSA on marmoset sperm using antibodies to 80kDa HSA. Predominant positive stain is seen on the tail region and on the head region of a few sperms. Marmoset sperms stained with preimmune serum did not show any fluorescence (1000X).](image)

In human sperm localization of the antigen was observed predominantly in the post acrosomal region of the acrosome intact and in the acrosomal cap of acrosome reacted sperms (Fig. 15). In rat spermatozoa, the localization was observed in the head and tail regions of the acrosome intact and in the tail region of the acrosome reacted sperm (Fig. 16).
Acrosome Intact         Acrosome Reacted

Fig. 15: Acrosome reaction was induced using calcium ionophore A23187 and incubated with antibodies to 80kDa HSA. Acrosome intact and reacted human sperm showed the localization of 80kDa HSA on post acrosomal and acrosomal region respectively (1000 X).

Acrosome Intact         Acrosome Reacted

Fig. 16: Acrosome reaction was induced using calcium ionophore A23187 and incubated with antibodies to 80kDa HSA. showed the Localization of 80kDa HSA in acrosome intact rat sperm was observed predominantly in the head and tail region, and in acrosome reacted sperm localization was observed in the head region (1000 X).

Developmental expression of 80kDa HSA was observed immunohistochemically in rat testis after day 40 of age (Annual Report 2002-03, p 35). Similar expression has also been observed in the rat epididymis from day 40 of age onwards (Fig. 17). The results with antibodies to peptide-NT in the rat testis (Fig. 18) confirm that the peptide antibodies recognize the native protein.
Fig. 17: Immunohistochemical localization of 80kDa HSA in rat epididymis on day 20, 40, 60 and 90 of age using antibodies to 80kDa HSA and HRP labeled anti rabbit gamma globulin (ARGG). The image analysis data indicate that the expression increases after day 40 of age in caput, corpus as well as in cauda regions of the epididymis (500 X).
Fig. 18: Immunohistochemical localization of 80kDa HSA in rat testis on day 10, 20, 40, 60 and 90 of age using antibodies to peptide-NT and HRP labeled ARGG.

The data obtained so far suggest that 80kDa HSA is developmentally regulated and also confirms that the antibodies to the peptides recognize the native protein, and its possible utility as a candidate for immunocontraception.
1.4 Modulation of c-kit Proto-oncogene Function During Spermatogenesis in Mice (Funded by INDO-US)

Principle Investigator: K.V.R. Reddy
Project Associate: A. P. Sikarwar and Clara Aranha
Duration: 2003-2008

c-kit signaling facilitates germ cell differentiation and maturation during spermatogenesis. Mutation in the c-kit leads to arrest of spermatogenesis and thereby results in infertility in mice. Cryptorchid mouse model was used to study the role of c-kit in spermatogenesis (Annual Report 2003-2004, p 36). These studies were extended further to characterize c-kit gene.

SDS-PAGE / Immunoblot analysis of c-kit expression

The presence of c-kit protein in the testis was determined by SDS-PAGE/immunoblot analysis. Total proteins (10µg) from testis of control, cryptorchid and busulfan treated mice were separated by SDS-PAGE, transferred to nitrocellulose membrane (NC) and probed with anti-c-kit antibody. The antibody recognized a single protein band of ~ 150 KDa in normal and cryptorchid mice. No immunoreactive bands were detected in busulfan treated mice (Fig.19). These studies indicate that busulfan treatment causes complete depletion of germ cells thus enabling the use of these animals as recipients for germ cell transplantation. However, c-kit expression was found to be normal in cryptorchid mice, suggesting no adverse effect on spermatogonial cells and hence they could be used as germ cell donors.

Detection of germ cell apoptosis using TEM

Normal spermatogenesis, the process by which male germ cells mature from spermatogonia to spermatocytes and spermatids to spermatozoa involves a fine balance between the proliferation and death of cells. The involvement of c-kit in testicular germ cell proliferation, maturation and differentiation was demonstrated using cryptorchid mouse model (Annual Report 2003-2004, p-36). This led us to believe that c-kit may influence cell death process during spermatogenesis.

Testicular tissue sections of normal and 3 month old cryptorchid mice were processed by Transmission Electron Microscopy (TEM). Mouse spermatocytes showed compact electron-dense clumps of chromatin in cryptorchid mice compared to the controls, where uniform distribution of chromatin was observed (Fig. 20). Spermatids and spermatozoa were also found to be affected. However, no effect was seen on spermatogonial cells confirming the SDS-PAGE/western blot studies.
Role of c-kit in germ cell apoptosis

The role of c-kit in germ cell apoptosis was studied using serum free in vitro culture model. This model is known to induce germ cell apoptosis. Mouse testicular tissue explants were incubated at 37°C for 24 hrs in an atmosphere of 5 per cent CO₂.
The experimental protocol is as follows:

Group A: Testis explant + culture medium containing 10% FBS + Stem cell factor (SCF- 10ng/ml)

Group B: Testis explant + culture medium containing 10% FBS

Group C: Testis explant + serum free culture medium (DMEM)

Group D: Testis explant + culture medium containing 10% FBS + c-kit antibody (1µg/ ml)

Fig. 21: In vitro detection of apoptosis in the testicular explants of group A, B, C and D by TUNEL assay

Fig. 22: Levels of c-kit in the testicular explants of mouse cultured in vitro
After the culture, a portion of the tissue was cryosectioned to identify apoptotic cells by TUNEL assay and the rest of the tissue was used to determine the levels of c-kit protein by ELISA. Testicular explants of group C showed the highest percentage of apoptotic cells followed by group D and B (Fig. 21). Addition of c-kit ligand, SCF to enriched explant cultures markedly prevented apoptosis by favoring cell survival. Results obtained by ELISA were in agreement with TUNEL studies (Fig. 22).

Expression of c-kit mRNA in mouse testis

The expression of c-kit in spermatogonial cells was studied in cryptorchid mice using IHC (Annual Report 2002-2003, p 36). These studies were further confirmed by RT-PCR using two sets of oligonucleotide primers which were designed on the basis of known mouse c-kit receptor cDNA sequence. The primers 1 and 2 spanning the nucleotide sequence (2485-2703) 5' TAGCCAGAGACATCAGGAATGA-3'(sense), 5'-CTTCCTTGATCATTTGAA AACTT-3' (anti-sense) gave rise to an expected 219 bp PCR product. The primers 3 and 4 spanning the nucleotide sequence (1732-2569) 5'AGGAGATAATGGGAACATTATGT-3'(sense) 5'-ATGCTCTCCGGTG CCATC-3' (anti-sense) gave rise to the expected 838 bp PCR product (Fig. 23), indicating the presence of c-kit gene in testis. Constitutively expressed β-actin gene (457 bp) was used as positive control. The level of gene expression in normal testis was found to be similar to the testis of cryptorchid mouse and reduced in case of busulfan treated mice. Further studies are in progress to determine the stage-specific expression of c-kit during spermatogenesis.

![Fig. 23: RT-PCR amplification of c-kit gene segment from mouse testis.](image)

Lane 1=457bp β-actin control, Lane2=100 bp DNA ladder, Lane 3=219 bp PCR product of normal mice, Lane 4=1 month cryptorchid mice, Lane 5= 3 month cryptorchid, Lane 6=busulfan treated mice, Lane 7= Without primer pair, Lane 8=838 bp PCR product of busulfan treated mice, Lane 9= 3 month cryptorchid mice and Lane 10= normal mice.
In vitro modulation of c-kit expression in spermatogonial cells and transplantation

c-kit gene mutation leads to infertility due to developmental arrest of spermatogonial stem cells (SSCs). SSCs form the foundation of spermatogenesis and their transplantation provides a unique opportunity to study essential role played by c-kit. The present study was initiated to evaluate the efficiency of SSC survival and proliferation in vivo, which were modulated in vitro by SCF and anti-c-kit antibody.

The method to isolate SSCs from donor mice and transfer them into recipient mice has been standardized. In preliminary experiments, SSCs were enriched from three months cryptorchid mice and injected into the busulfan treated (40mg/kg, i.p) recipient mice at a concentration of 10^6 cells via rete testes. At the time of injection trypan blue dye was mixed into the cell suspension to visualize the entry of injected cells in the seminiferous tubules. Further studies are underway to investigate the functionality of the SSCs.

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: Radhika Kelkar, Swati Kulkarni, Sharmila Barve, Gayatri Shinde, SM Rewadekar, ST Ghanekar and Smita Mahale
Project Collaborators: D.S.Joshi, Retd. BARC, Mumbai
Duration: 2003 - 2006

The pituitary gonadotropins FSH and LH play a key role in follicular development. The action of gonadotropins, is in turn, regulated by intraovarian factors. FSHBI (Follicle-Stimulating Hormone Binding Inhibitor, MW<4kDa) an intraovarian factor, identified by our group, inhibits binding of FSH to granulosa cells, and studies involving purification and characterisation have led to the deduction of the N-terminal 8 amino acid sequence of FSHBI hereafter referred to as Octapeptide (OP). The octapeptide inhibits FSH binding to granulosa cells in vitro, and has an antifertility effect in marmosets similar to that observed with the native peptide. Both OP and the partially purified FSHBI (hGF2) inhibit FSH – induced progesterone production by rat granulosa cells in vitro (Annual Report 2002-2003, p 37). Administration of FSHBI to adult female Swiss mice arrested maturation of large follicles propelling them towards atresia while small and medium follicles remained unaffected. Based on its in vivo and in vitro effects, an autocrine role of the peptide can be postulated in follicular maturation and ovulation. Efforts have therefore been directed towards understanding the role of
this factor in normal ovarian folliculogenesis, as well as ovarian disorders such as Polycystic Ovaries (PCO).

Flow cytometry studies with partially purified FSHBI (hGF₂)

To understand the apoptotic events/pathways occurring in cells &/or follicles undergoing atresia two approaches were studied: 1) follicular development and atresia by PMSG in immature mice, 2) treating animals with partially purified fraction of human follicular fluid (hGF₂).

The changes occurring during apoptosis were assessed using flow cytometry. Annexin V antibody tagged to FITC was used to detect cells in early apoptosis, with Rhodamine 123 as an index for the mitochondrial membrane potential and active Caspase 3 an intracellular protein as a marker for late apoptosis.

The number of apoptotic cells (which showed positivity for FITC labeled Annexin V binding to the phosphatidyl serine residues) increased in atretic (22.52%) and hGF₂ (14.23%) treated population in comparison to normal (10.87%) controls (Fig. 24a,b,c). There was an increase in R1 population and a decrease in R2 population (Fig. 25a,b,c) in atretic (R1- 63.23%, R2- 30.23%) and hGF₂ treated cells (R1- 42.73%, R2- 49.05%) compared to normal control group (R1- 12.05%, R2- 80.49%) indicating granulosa cell apoptosis. Increase in active caspase 3 (Fig. 26a,b,c) in cells from atretic (14.55%) and hGF₂ (8.48%) treated population as compared to the normal control population (5.08%) further confirmed the occurrence of late apoptosis in the two groups.

Mechanism of action of OP/FSHBI on granulosa cell apoptosis is being studied at present.
Granulosa cells

a) Normal Follicles  b) Atretic Follicles  c) hG F2 injected

UL = 12.61%
UR = 10.11%
LL = 66.41%
LR = 10.87%

UL = 6.56%
UR = 23.94%
LL = 46.99%
LR = 22.52%

UL = 17.62%
UR = 26.11%
LL = 42.04%
LR = 14.23%

UL = Necrotic population
UR = Necrotic + Late apoptotic
LL = Non apoptotic cell population
LR = Apoptotic cell population

Fig. 24: Detection of early apoptosis using Annexin V - FITC

a) Normal Follicles  b) Atretic Follicles  c) hG F2 treated

R1 = 12.05%
R2 = 80.49%
R1 = 63.23%
R2 = 30.23%
R1 = 42.73%
R2 = 49.05%

R1 = Apoptotic cell population  R2 = Normal cell population

Fig. 25: Estimation of mitochondrial membrane potential using Rhodamine 123
Our earlier studies demonstrated that OP mimics the biological activity of the native protein FSHBI. Attempts were made to generate polyclonal antibodies to OP, with a view to study the expression of FSHBI in ovarian follicles.

The OP was synthesised, conjugated to Diphtheria toxoid and used for immunisation of male rabbits. Antibody titres were determined by ELISA using OP-BSA conjugate as the antigen. A decrease in the optical density was observed when the antiserum was preincubated at 37°C for 2h with serial concentrations of OP.

Cross reactivity of the antiserum with various mouse tissues was studied by immunohistochemistry (IHC). The antibodies to OP/FSHBI were specific to the ovary and did not cross react with any other tissues i.e liver, kidney, spleen, adrenal and uterus (Fig. 27).
The antiserum was used to localise OP/FSHBI in ovarian follicles of human, marmoset and mouse ovarian follicles by IHC, maximum activity was observed in the mouse ovaries. IHC in murine normal and atretic follicles revealed increased expression in atretic follicles as compared to normal (Fig. 28a,b). The peptide was localized in the granulosa cells. Preincubation of the antiserum with 100µg OP at 37°C for 2h abolished staining in the granulosa cells (Fig. 28c). No staining was seen in control sections incubated with preimmune serum instead of the antiserum (Fig. 28d).

Expression of OP/FSHBI in cystic ovaries

Due to the ethical constraints in obtaining human tissue, a murine model for cystic ovaries was developed (Annual Report 2002-03, p 39) by administration of 17β Estradiol (in olive oil) to neonatal mice on day 5 of age. Control animals were
injected with olive oil only. Animals were sacrificed at 12 weeks of age and the presence of cystic ovaries in treated animals was confirmed histologically (Annual Report 2002-03, p 39). Absence of corpora lutea in treated animals indicated anovulation, an important feature of Polycystic ovarian condition.

Localization of Insulin-like Growth Factor-I (IGF-I) and Androgen Receptor (AR) was employed as markers of cystic follicles. No alteration in expression of IGF-I was observed in cystic follicles (Fig. 29a) when compared to cycling controls (Fig. 29b). However, an upregulation of AR expression was observed in granulosa cells of cystic follicles (Fig. 29d) as compared to those from normal controls (Fig. 29e). Immunohistochemical localization of OP/FSHBI using antiserum to OP revealed an increase in the expression of the peptide in cystic follicles (Fig. 30).

Fig. 29: Localisation of Insulin-like Growth factor-I (a-c) and Androgen Receptor (d-f) in cystic follicles (X400) a,d: 12 wks control, b,e: 12 wks E2 treated, c,f: -ve control

Fig. 30: Localisation of OP/FSHBI in mouse ovary (X100) a: normal, b: atretic, c: atretic (X400), d: 12 wks control, e,f: 12 wks E2 treated; a-e: anti-OP, f: NRS
1.6 Studies of Endometrial Proliferation and Apoptosis in Primates

(Partially Funded by DBT)

Principal Investigator: Tarala D. Nandedkar


Project Collaborators: S.V. Parulekar, Seth G.S. Medical College and KEM Hospital, Parel, Mumbai

Duration: 1999-2007

In the human endometrium, proliferation and apoptosis occur in a cyclic manner. It is further postulated that ovarian steroid hormones oestradiol and progesterone are the prime regulators of these processes. Our previous studies in common marmosets indicated that, during the ovarian cycle, proliferation occurs during early follicular phase in the endometrium while apoptosis and expression of apoptosis promoting protein, Bax, was mainly observed during mid-luteal phase (Annual Report 2002-03, p 40-41). Hence it was hypothesized that increased levels of progesterone are responsible for apoptosis during mid-luteal phase and is mediated by Bax. In order to investigate this further, artificial cycles were simulated in ovariectomized female marmosets by exogenous treatment with oestradiol and progesterone according to the previously standardized protocol (Fig. 31).

**Fig 31:** Steroid hormone treatment protocol for ovariectomized female marmosets. The female marmosets were treated in four groups: 1. Oestradiol treatment (10 µg for 3 days, 20 µg for 4 days, 50 µg for 1 day) 2. Oestradiol (10 µg for 3 days, 20 µg for 4 days, 50 µg for 1 day) and progesterone 5 mg for 9 days) treatment 3. Progesterone treatment (5 mg for 9 days) 4. Vehicle (Olive oil) as control. E₂, Oestradiol, P, Progesterone.

In situ localization of apoptosis by TUNEL in the endometrium during artificially simulated cycles

Apoptosis was not observed in the endometrial sections of oestradiol treated animals. (Fig. 32A). In oestradiol and progesterone treated animals, intense
apoptosis was observed mostly in the glandular epithelial cells (Fig. 32B). In the progesterone treated group, weak staining for apoptosis was noted (Fig. 32C). In control animals, glands were few due to lack of hormones. However, some apoptotic cells were observed in the luminal epithelium (Fig. 32D).

Immunohistochemical localization of Bax

Weak expression of Bax was observed in the endometrium of oestradiol treated marmosets Fig. 32E). The oestradiol and progesterone treated group showed intense immunostaining (Fig. 32F) while only progesterone treated group showed moderate expression in the endometrium (Fig. 32G). Bax localization was predominantly present in glandular and luminal epithelial cells and was not observed in the stromal cells in all the three groups. Weak immunolocalization of Bax was observed in endometrial sections of control animals ((Fig. 32H).

Immunohistochemical localization of PCNA

Immunolocalization of the proliferation marker PCNA was studied in ovariectomized marmosets following administration of steroid hormones. PCNA expression was observed in both the stroma as well as glandular and luminal epithelial cells. It was intense in oestradiol treated group in both epithelial and stromal cells (Fig. 32I). Endometrial sections from marmosets treated with progesterone after priming with oestradiol showed absence of PCNA expression (Fig. 32J). However, in animals treated with progesterone alone without oestradiol priming, PCNA expression was moderate (Fig. 32K). No immunostaining of PCNA was observed in endometrial sections of ovariectomized control animals (Fig. 32L).
Fig. 32: Apoptosis, Bax and PCNA expression in endometrium of common marmosets during simulated cycle. Apoptosis (By TUNEL) was scarce in E₂ group (A), high in glands in E₂+P group (B), nearly absent in P₄ group (C) and weakly observed in control (D). Bax expression was moderately seen in E₂ group (E), intense in E₂+P group (F), low in P₄ group (G) and lower in control (H). Proliferation or PCNA expression was highest in E₂ group (I), low in E₂+P group (J) and moderate in P group (K) less in control (L). E₂, Oestradiol treated, E₂ + P, Oestradiol and Progesterone treated, P, Progesterone treated.

Thus, in oestradiol treated group (E₂), which is comparable to proliferative phase, proliferation was high while apoptosis and Bax expression was moderately present. In oestradiol and progesterone treated group (E₂+P) group, proliferation was decreased but apoptosis and Bax expression was increased. These observations were comparable to mid luteal phase of ovarian cycle. However, progesterone treatment alone failed to induce considerable apoptosis in endometrium. Expression of Bax was also reduced although PCNA was moderately present. These observations suggest that in marmoset endometrium, apoptosis is regulated by progesterone following priming with oestradiol when Bax expression is upregulated while proliferation in the endometrium is regulated by oestradiol. Studies are in progress to compare these results with endometrial changes in normally menstruating women.
1.7 Factors Regulating Early Folliculogenesis (Funded by DBT)

Principal Investigator: Tarala D. Nandedkar

Project Associates: Shalmali Dharma, S.T. Ghanekar and Anurupa Maitra

Duration: 2003-2007

Ovarian folliculogenesis is marked by the development of follicles from primordial to preantral and further to preovulatory follicle. The activation of dormant primordial follicles and its development to secondary (preantral) follicles is gonadotropin independent. This process of early folliculogenesis requires bi-directional communication between germ cells and somatic cells. The mammalian ovary contains fixed number of primary oocytes enveloped in primordial follicles. Only few primordial follicles are recruited at a time from the resting pool. The paracrine factors secreted by oocytes and somatic cells possibly regulate many of the events of early follicular development in mammals. The factors responsible for stimulating the follicle for differentiation and development are not well defined. Hence the study was undertaken to delineate the factors that induce transition of primordial follicles to primary and secondary stages using a mouse model.

The primordial follicles were observed on Day 2 of age, while Day 4 and Day 6 showed presence of primary and secondary follicles, respectively. Hence, the neonatal mouse ovaries at Day 2, Day 4 and Day 6 were selected for further studies. Proliferation, differentiation and apoptosis were studied by localization of the markers for proliferation (PCNA), differentiation (GDF-9) and apoptosis (TUNEL). GDF-9 was localized in the oocytes of primary follicles on Day 4 whereas the intense expression of PCNA was observed in the granulosa cells of secondary follicles on Day 6 (Fig. 33). Apoptosis as evaluated by TUNEL staining was negative in neonatal ovaries. These results suggest that GDF-9 secreted by oocytes of primary follicles possibly interact with granulosa cells to induce proliferation. Ovarian follicles in neonatal mice are either quiescent or growing but not apoptotic.

In vitro studies are in progress to elucidate the action of Stem Cell Factor (SCF) and other growth factors on early follicular development.
Fig. 33: The upper panel shows H and E stained sections of neonatal mouse ovaries at Day 2 only primordial follicles (A), Day 4 primary (B) and Day 6 secondary preantral follicles (magnification x 20). The middle panel shows GDF-9 localization in neonatal mouse ovaries. Positive staining appears as a brown stain as indicated by black arrows in the oocytes. (D) Day 4. (E) Day 6 (magnification x 20). The lower panel shows the immunostaining for PCNA in Day 4 (F) and Day 6 (G) mouse ovaries. PCNA is expressed in granulosa cells of secondary follicles on Day 6 as indicated by white arrows (magnification x 40).
1.8 Acceptability and Continuation Rates of Two Monthly Injectable Contraceptive: Norethisterone Enanthate (Funded by Ministry of Health and Family Welfare, Government of India)

Principal Investigator: Shanta Chitlange


Duration: 2001-2006

A multicenter study, including nine centers from different parts of the country representing diverse socio-cultural background, has been initiated with the 2 monthly injectable contraceptive - Norethisterone Enanthate (NET-EN). The data generated by this study will help program managers recommend the introduction of NET-EN into the national program.

The objectives of this study are to 1) assess user acceptability / continuation rates of injectable contraceptive -Norethisterone Enanthate (Net-En) 2) evaluate the incidence of menstrual irregularities and other side effects 3) assess socio-behavioural aspects of volunteers and compare with different regions and cultural settings 4) study the return of fertility following discontinuation of contraceptive use. The major emphasis of this study is on counseling by qualified staff in an attempt to encourage better continuation rates.

Enrollment of 1209 women has been completed and follow up visits for injection are scheduled for 24 months.

A total of 2352 eligible women who met the enrollment criteria were given in-depth information regarding Net-En. Of these, 1209 women accepted this method after having given written informed consent. The enrollment at different centers and the basic characteristics of these women are depicted in Fig. 34 and Table. 3 respectively.
Table 3: Characteristics of Women (n=1209)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± S.D. (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>24.97 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>(19 – 40)</td>
</tr>
<tr>
<td>Parity</td>
<td>1.53 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>(1- 6)</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>20.6 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>(13.6 – 42.3)</td>
</tr>
</tbody>
</table>

Other characteristics

- Interval cases
- Post Partum cases
- Post MTP cases
- Total

The remaining 1143 eligible women did not accept injectable for various reasons as seen in Table 4.

Fig. 34: Number of eligible women Screened and Enrolled for Net-En
Table 4. Eligible women - Reasons for not accepting injection

<table>
<thead>
<tr>
<th>Responses</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not willing for frequent visits</td>
<td>318</td>
<td>27.8</td>
</tr>
<tr>
<td>Menstrual irregularity/ weight gain/ inj. prick not acceptable</td>
<td>275</td>
<td>24.1</td>
</tr>
<tr>
<td>Prefers IUCD</td>
<td>201</td>
<td>17.6</td>
</tr>
<tr>
<td>Family members' objection</td>
<td>132</td>
<td>11.5</td>
</tr>
<tr>
<td>Prefers permanent method</td>
<td>59</td>
<td>5.2</td>
</tr>
<tr>
<td>Prefers OC / Condom</td>
<td>49</td>
<td>4.3</td>
</tr>
<tr>
<td>Fear of side effects</td>
<td>43</td>
<td>3.8</td>
</tr>
<tr>
<td>No response</td>
<td>31</td>
<td>2.7</td>
</tr>
<tr>
<td>No need for regular contraception</td>
<td>20</td>
<td>1.7</td>
</tr>
<tr>
<td>New method</td>
<td>9</td>
<td>0.8</td>
</tr>
<tr>
<td>May develop male characteristics</td>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1143</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

The majority of the women (27.8%) refused to accept this method because of the need for frequent visits to the clinic for the 2 monthly injection schedule, while 24.1 per cent of women perceived the side effects like menstrual cycle disruption and weight gain. As at the initial and discontinuation visits, all the volunteers were again interviewed to obtain information on their perceptions regarding injectable contraceptives.

It is very important for women to remember the schedule for dates of next injection. Many women tend to forget scheduled dates and hence there is a greater risk of pregnancy. In this regard majority of women (89.28%) referred to the written instructions for the dates of next injections on the menstrual diary cards given to them, while 8.39 per cent of women were reminded by clinic staff, if they failed to report on the scheduled date and in a few instances (1.17%) the husbands reminded their wives of the date for injection. Only a few women (1.17%) could memorise the dates of subsequent injections (Fig. 35).
When asked, “Was it convenient for you to take this contraceptive injection”? many women (65.5%) found 2 monthly contraceptive injection as convenient schedule (Fig 36).

The preliminary observation based on 12068 women months of use showed a good cumulative continuation rate. At 12 months, the continuation rate was 66.27 per 100 women (Fig. 37).
The major discontinuations were due to loss to follow up and migration because of floating population (Table 5). The study is ongoing.

**Table 5: Reasons for discontinuation of NET-EN by months of injection use**

<table>
<thead>
<tr>
<th>Reasons</th>
<th>Duration of use in months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Two</td>
</tr>
<tr>
<td>Menstrual disruption</td>
<td>35</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>0</td>
</tr>
<tr>
<td>Weight Gain</td>
<td>1</td>
</tr>
<tr>
<td>Desired Pregnancy</td>
<td>3</td>
</tr>
<tr>
<td>Change of F.P. Method (TL)</td>
<td>5</td>
</tr>
<tr>
<td>No further need for contraception</td>
<td>1</td>
</tr>
<tr>
<td>Personal Reasons</td>
<td>23</td>
</tr>
<tr>
<td>Late for Injection</td>
<td>8</td>
</tr>
<tr>
<td>Lost to follow up / migrated</td>
<td>55</td>
</tr>
<tr>
<td>Other Medical Reasons</td>
<td>7</td>
</tr>
<tr>
<td>Deaths</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>138</td>
</tr>
</tbody>
</table>
1.9 Longitudinal Study of Improving Involvement of Men in the Welfare of Families (Funded by Ministry of Health and Family Welfare, Government of India)

Principal Investigator: D. Balaiah


Collaborators: A. Kanbur, Consultant, Andrologist, Mumbai, H.N. Singh Thakur, Consultant Physician, Mumbai

Duration: 1999-2003

Status: Completed

Male reproductive health involves encouraging a range of positive reproductive health issues to ensure the individuals’ own as well as the family member’s well-being. In India, men play a dominant and many times a decisive role in regulating women’s access to reproductive health. Traditionally, men are not involved in parenting responsibility and often shy away from seeking reproductive health services in public. Under these circumstances, male participation is vital in enabling them to seek reproductive health knowledge and services for themselves and to extend support to their partners or spouses.

The objectives of the study are to: (i) understand family planning knowledge, perception, attitude and practices of men; (ii) plan appropriate intervention strategies for enhancing male involvement; and (iii) evaluate the impact of the interventions on the reproductive health care of the family. The study design included: situational analysis in both control and experimental areas; intervention phase to increase awareness and knowledge regarding reproductive health issues and services in experimental area; and impact evaluation after three years in both the areas.

In continuation of the intervention programme, health talks and street plays were organized during the year on the occasion of World Population Day. Forty people attended for health talks and about one hundred people attended the street play. Services of the Clinic for Men were available between April 2003 to August 2003, forty-three men attended for various problems. Out of these, twenty-five men attended for follow-up whereas six men attended for infertility problem, five for sexual problem, three for contraceptive information, one for renal problem and three for general check-up.

The resurvey was completed following the intervention period i.e., after three years. The results of impact analysis are based on information collected from 1460 men during baseline and 1419 men during resurvey after three years.
in the control area and 1227 men during pre-intervention and 1604 men during post-intervention in the intervention area.

Family size and son preference

Desire for three or more children had increased by 10.7 per cent in control area, whereas it had deceased by 8.1 per cent in the intervention area. An increase of 7.5 per cent was observed among control group who had reported three or more children as an ideal family size whereas it decreased by 6.9 per cent in intervention area. Strong preference for two or more sons had increased by 10.1 per cent in control group; whereas 4.2 per cent decrease was observed in the intervention group. It is surprising to note that the family size and son preference is found higher in resurvey as compared to baseline survey in control area.

Knowledge about contraceptive methods

The results indicate a marginal increase in the knowledge of men about condom among control group, whereas in the interventional group it had increased by 5.1 per cent. Similarly, knowledge about withdrawal method increased by 21.8 per cent in the control area as compared to 49.4 percent in the intervention area, indicating an increase of 27.6 per cent in the intervention area. The knowledge of correct use of rhythm had increased to 24.6 per cent in the control area whereas it was 52.1 per cent in the intervention area indicating an increase of 27.5 per cent due to the interventions.

Knowledge about correct use of contraceptive methods

The knowledge of correct use of condom had increased in control area 37.6 per cent whereas in the intervention area it was 51.7 per cent indicating the 14.1 per cent increase due to the interventions carried out in the area. Similarly the knowledge of correct use of withdrawal method increased by 21.8 per cent in control area as compared to 49.4 per cent in intervention area indicating an increase of 27.6 per cent in the intervention area. The knowledge of correct use of rhythm had increased to 24.6 per cent in control area whereas 52.1 per cent (from 17.6% to 69.7%) in experimental area indicating an increase of 27.5 per cent due to the interventions (Fig. 38).
The knowledge of condom use for dual purpose in prevention of pregnancy and infection had increased in control area 24 per cent whereas in the intervention area it was 30.3 per cent indicating a 6.3 per cent increase due to interventions.

Inter-spouse communication regarding family welfare aspects

Communication between husband and wife about obtaining family planning information increased by 15.2 per cent in control group and it increased by 20.6 per cent in intervention group; regarding permanent methods it increased in control area by 24.7 per cent whereas in intervention group it increased by 28.6 per cent and spacing methods increased by 30.2 per cent in control group and it increased by 30.8 per cent in the intervention area. Significant increase was observed in obtaining family planning information regarding permanent methods; and marginal increase was observed in spacing methods in intervention area as compared to control area (Fig. 39).
Contraceptive use by couples

Overall contraceptive use in control area had increased by one per cent whereas in the intervention area it was 17.1 per cent indicating a 16.1 per cent increase due to interventions (Fig. 40).

* Include permanent methods

Fig. 39: Impact: Inter-spouse communication

Fig. 40: Contraceptive use by couples
The improvement was mainly due to increase in acceptance of condom, withdrawal and rhythm in the intervention area. Female sterilization was widely accepted in control area whereas condom use was higher in experimental area.

1.10 Adolescent Reproductive Health Initiatives

1.10.1 To Assess Knowledge, Attitude and Felt Need of Emergency Contraception among Adolescents and Youth, Health Care Providers and Family Planning Counsellors, Teachers, Parents, NGOs and Women's Health Advocates, Chemists and Drug Store Owners in and around Mumbai (Funded by WHO Country Budget)

Principal Investigator: D. Balaiah
Project Associates: S.L. Chauhan, P. Tapase
Duration: 2001-2003

Status: Completed

Emergency contraceptives (ECs) have an important role in preventing unwanted pregnancies, induced abortions and abortion related mortality and morbidity. In view of this, it is essential that potential users as well as providers are aware of such methods and their timely and correct use. The objective of the study was to assess the knowledge, attitude and felt need of EC among adolescents and youth, health care providers, parents and teachers, woman's health advocates and chemists in and around Mumbai. Qualitative and quantitative information was collected from adolescents and youth (910), service providers and family welfare counselors (200), parents (173), teachers (237), women health advocates (WHA) and non-governmental organizations (NGOs) (27), and drug store (chemist shop) owners/employees (35).

Awareness, attitude and felt need of EC among adolescents and youth

The overall awareness of emergency contraception among adolescents and youth was 4.2 per cent. The main sources of information about EC were medical personnel, relatives, friends and neighbours, newspapers, radio and television. More than two-thirds of young persons reported that they would recommend EC to their friends and relatives. More than four-fifths of the adolescents and youth felt that education about EC should be given in their schools and colleges. Detailed report has been given in (Annual Report 2002-03, p 49-51).

Awareness, knowledge and perceptions of EC among Health Care Providers

The overall awareness of EC among service providers was 48 per cent i.e., 75 per cent among Obstetricians and Gynecologists, 46.7 per cent among other medical practitioners, 40 per cent among family planning counselors and 31 per
cent among nurses. Awareness of EC is higher among younger (66.7%) age group, male (51.5%) and urban service providers as compared to other groups.

Among those who were aware of EC, 90.6 per cent named levonorgestrel, 77.1 per cent mentioned Yuzpe Regimen and 70.8 per cent reported IUD as EC methods (Fig. 41). About 81 per cent of service providers felt that EC services should be made available to rape victims followed by both married and unmarried (73.5%), widowed women (41%), married girls (21%) and unmarried girls (6.5%).

![Fig.41: Knowledge of EC methods among those who were aware of EC (N = 96)](image)

Regarding views about EC, 88.5 per cent of health care providers reported that easy accessibility and availability may increase the use, 81.3 per cent felt that EC should be included in National Family Welfare Programme, 79.2 per cent opined that it should be made available to adolescent girls and 77.1 per cent felt that men should be allowed to obtain pills (Fig. 42).
Health care providers were asked about their course of action in care their client reported an unprotected intercourse. About 37 per cent reported that they prescribed EC pills and other practitioners mentioned that they referred the cases to gynecologists. Other providers reported that they suggested pregnancy test followed by MTP, if delayed, or insert IUD with in three days or start EC pills next day or increase the dose of oral pills. Sixteen per cent could not give any answer. About 20 per cent of health care providers reported that they prescribe EC when the clients miss two or more pills, 28 per cent when condom breaks and 14.5 when late for injection.

Majority (47%) felt that the means of communication for disseminating information about EC was through media, 18.5 per cent said education, 12.5 per cent mentioned community meetings and 6 per cent reported through counselling. About 68 per cent felt EC should be given in the form of oral tablets.

Awareness, knowledge, perceptions and felt need of EC among Teachers

Of the total 237 teachers interviewed, 12.2 per cent were aware of EC. Awareness of EC was found higher among 36-40 years age group (14.6%), males (18.5%) and post-graduates (13.5%). Region-wise distribution indicates that 12.5 per cent of urban, 17.9 per cent of suburban and 4 per cent of rural teachers were aware of EC. Religion-wise distribution of teachers indicates that 12.3 per cent of Hindus and 6.7 per cent of Christians were aware of EC. Thirteen per cent of married teachers heard about EC.
About 21 per cent of teachers viewed induced abortion as a method of contraception and 86 per cent felt it affects woman’s health adversely. Regarding the views of teachers, 79.3 per cent mentioned that EC should be introduced in Family Welfare Programme and similar proportion opined that easy accessibility and availability would increase EC use. More than three-fourth reported that they would recommend the use of EC to their friends and relatives. About 52 per cent feared that easy accessibility and availability may reduce the use of regular contraceptive methods. About 48 per cent felt that EC should be made available to adolescent girls at the same time similar proportion of teachers foresee problem in the use of EC such as sexual activity may increase among adolescents and youth, misused or used as a regular contraceptive method (Fig. 43).

![Graph: Views of teachers about emergency contraception](image)

**Fig.43: Views of teachers about emergency contraception**

Regarding the means of expanding EC services, about 33 per cent of teachers viewed medical personnel could promote EC services, 31 per cent felt that media could disseminate information followed by education (5.5%), and books & magazines (1.7%).

Awareness, knowledge, perceptions and felt need of EC among parents

Of the total 173 parents interviewed, 7.5 per cent of them were aware of EC. Age-wise distribution indicates that the awareness of EC was higher among younger age group (15.4%). Region-wise distribution shows that awareness of EC among rural parents (2.9%) was significantly lower than that of urban (8.7%) and suburban (8.6%) parents (Fig. 44). The awareness of EC is higher among post-graduates (13.9%) and higher income group (12.5%) i.e., more than Rs. 15000.
Fig. 44: Awareness of emergency contraceptive of parents by age, sex and region

About 22 per cent of parents felt that EC should be made available to both married and unmarried whereas 65.3 per cent felt it should be made available only to married persons and 12.7 per cent could not answer.

None of the parents viewed induced abortion as a method of contraception and 84.6 per cent felt it affects woman's health adversely. Majority (38.5%) felt that family planning services should be made available to adolescents and youth. Majority (92.3%) of the parents opined that boys and girls should be allowed to interact and give freedom to select their life partner.

Regarding the views of parents, 76.9 per cent mentioned that EC should be introduced in family welfare programme and similar proportion opined that easy accessibility and availability would increase EC use. About 92 per cent reported that they would recommend the use of EC to their friends and relatives, if needed. About 46 per cent of teachers feared that easy accessibility and availability may reduce the use of regular contraceptive methods. About 38.5 per cent felt that EC should be made available to adolescent girls and 53.8 per cent of parents foresee problem in the use of EC. They felt that sexual activity may increase among adolescents and youth, and it might be misused or used as a regular contraceptive method (Fig. 45).
Fig. 45: Views of parents about emergency contraception

Regarding the means of expanding EC services, 61 per cent felt media would play an important role in disseminating the information, 20 per cent mentioned that through medical personnel that could be expanded, 4 per cent reported that education would be better and rest could not suggest. About 37 per cent felt that EC should be provided on doctor’s prescription, 11.1 per cent reported that EC should be available over the counter and 3.7 per cent said that it should be made available through National Family Welfare Programme.

Awareness, knowledge, perceptions and felt need of EC among NGOs and Women’s Health Advocates

Out of the total, 18.5 per cent of WHAs and NGOs had heard about EC as an emergency effort to protect women at accidental risk of pregnancy. About 14 per cent of female and 33.3 per cent of male respondents heard about emergency contraception. Regarding the question “what should be done to prevent unwanted pregnancy?” Majority (92.6%) reported that promoting safer sex followed by offering family planning services (59.3%), distributing condoms (63%), offering safe abortion services (55.6%) and providing counseling services (18.5%) were means to prevent unwanted pregnancy.

Of the total WHAs and NGOs, 55.6 per cent reported that they foresee problems in the use of EC methods. About 40 per cent felt that there would be misuse of the method and 27 per cent said that it might have adverse effects on women’s health. Others reported that sexual urge will increase among adolescents and youth. When asked about how EC services should be provided,
37 per cent felt that ECs should be provided through doctors’ prescription followed by over the counter (11.1%) and through National Family Welfare Programme (3.7%).

Awareness, knowledge, attitude and felt need of emergency contraception among chemists and drug store owners

Over all, 60 per cent of chemists and drug-store owners were aware of EC i.e. 40 per cent of medical stores personnel, 68 per cent of medical and fancy store persons were aware of EC. Area-wise distribution shows that 58.3 per cent of urban, 58.8 per cent of sub-urban and 66.7 per cent of rural chemists were aware of EC.

Regarding the question “What should be done to prevent unwanted pregnancy?” 25.7 per cent reported of promotion safe sex followed by marriage with the partner (22.8%), avoiding sex (20%) and seeking parent’s advice (8.6%). When enquired about “if contraceptive fails or anybody missed to use contraceptive method, then what do you suggest?” 20 per cent of chemists reported that they suggest the clients to consult a doctor, 28.5 per cent felt that the client should decide whether the pregnancy should be continued or not, 14.3 per cent suggested taking EC pills.

The study shows that awareness of EC among various groups is low, attitude towards EC is positive and felt need of contraception is observed among various groups. All the respondents expressed their desire to know more about contraception and EC. The study underscores the pressing need to increase awareness and knowledge of EC through effective intervention programmes such as audiovisuals (television and radio), print media (posters, pamphlets, brochures and flipcharts), help-line, hotline and internet services, so that unwanted pregnancies could be prevented.

1.10.2 Improving Reproductive Health of Adolescents: An Urban School-based Approach

Principal Investigator: Beena Joshi


Duration: 2002-2005

To operationalise Adolescent Friendly Services in school and college settings and assess the off-take of services provided, an interventional study was taken up in two schools in F-South ward of Mumbai. The specific objectives were to understand sexual and reproductive health (SRH) needs of adolescents and
assess their reproductive health status by carrying out a thorough medical checkup.

After obtaining necessary permissions from various authorities, baseline information was collected using self-administered questionnaires in the local language and through focus group discussions with parents, teachers and adolescent boys and girls in the age group 11-19 years. The survey indicated poor knowledge on reproductive health issues and a number of common reproductive health problems. They also had a lot of exposure to pornographic literature and many had very liberal attitude to sex and sexuality.

Based on the findings of situation analysis, an intervention package was developed. This package consisted of life skill education followed by IEC activities on the SRH issues that emerged from the baseline study. In addition, a letterbox was installed in the school and college premises and the SRH queries answered on a notice board safeguarding the confidentiality of the questioner. An Adolescent Friendly Center manned by a doctor and two social workers was set up in a separate room provided by the school and was kept open twice a week for two hours each. Referral linkages were established with the neighboring Institutions, i.e. KEM hospital and Child Guidance Center of Tata Institute of Social Sciences.

A situational analysis dissemination workshop was held in which the teachers of the school and college participated and their continued association was sought throughout the study. Essay competitions, poster preparation and exhibition and short skits on reproductive health issues were organized as part of IEC activities. Over a period of 8 working months of the school, about 150 adolescents visited the center and availed services on various SRH issues. Through the letterbox, 6 queries per week were answered totaling approximately to 220 over 1 year.

A thorough medical checkup, with a focus on nutrition and reproductive health of 300 students (11-14 years) enrolled in the project, was carried out by a male and female doctor. Laboratory investigation including haemoglobin estimation was carried out and referrals were made for any other investigations as required.

The findings reveal that almost 30 per cent of the boys and 15 per cent of the girls were below the 5th percentile for the Body Mass Index (BMI) as compared to the WHO recommended standards (Fig. 46). Mean BMI in the females was 19.08 (SD±4.89) and in males 17.98 (SD±3.15) and the mean difference was 1.1040 (p < 02)
Medical examination revealed that 36 per cent of girls reported leucorrhoea, 14 per cent among them had pruritis vulvae. On examination majority of them had white watery discharge but in 20 per cent of the cases it was creamish / yellowish and sometimes foul smelling with vulvitis. FGD’S among this group of girls revealed that they called it Saphed Pani or Saphed Pali (white menses) and related it to excessive heat in the body and to general weakness. It was associated with bodyache, backache, abdominal pain, pain in lower extremities and sometimes with itching, foul smell of genital region, small boils on vulva or burning urine. After defecation majority used back to front technique of washing. They used home remedies like drinking sabja seeds soaked in water overnight or application of coconut oil and eating less spicy food, and 20 per cent of them said mother’s had promised to take them to a doctor but only one had seen the doctor who had explained that it was normal. They knew of other female family members like mother, elder sister, aunt who also had similar problems.

The mean age of menarche was 10.8 years, much lower than the reported age in the Indian context. Almost 75 per cent of girls mentioned that they had normal menstrual flow. However 5 per cent had heavy flow and 5 per cent scanty flow and 13.5 per cent complained of irregular menses. Almost 44 per cent of girls who had attained menarche complained of dysmenorrhoea.

Hemoglobin values were below 8 gms (severe anaemia) in 15.5 per cent of girls and 1.8 per cent of boys and 35.5 per cent of girls and 28 per cent of boys had hemoglobin values between 8 and 10gms. (Fig. 47)
Iron treatment was recommended for 3 months to all those who had hemoglobin values below 10 gms. Their parents were counseled regarding change in the dietary habits. Those with genital anomalies were referred to KEM hospital (Table 6). These cases are being followed up.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small testes</td>
<td>6</td>
</tr>
<tr>
<td>Inguinal hernia</td>
<td>1</td>
</tr>
<tr>
<td>Single testes</td>
<td>3</td>
</tr>
<tr>
<td>Retractile testes</td>
<td>1</td>
</tr>
<tr>
<td>Accessory nipple</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 6: Genital Anomalies detected among boys (n=150)

1.10.3 Assessing Reproductive Health Needs of Youth: A Study of Rural College Students in Maharashtra.

Principal investigator: **M.D Ghule**

Project Associates: D. Balaiah, S.L. Chauhan, P. Tapase

Duration: 2003-2004

Adolescents and youth form one of the largest groups (33 percent) with unmet needs for reproductive health services. Existing studies are mainly restricted to the urban, educated upper class and English-speaking students. Detailed studies of sexuality among unmarried youth in rural parts of India are
not available. Knowledge of reproductive health issues is extremely poor but the
desire to learn is greater among the youth. In view of this, a study has been
undertaken with the objectives to ascertain knowledge, understand perception,
and source of information on human reproduction, sexuality, STDs/ HIV/ AIDS,
and contraception, attitude towards sex, and to understand the nature and extent
of premarital sexual activity among rural college youth.

A study including college students in the age group 15-24 years- Junior (11-
12th standard) and senior (13-15th standard) college going youth from Shahapur,
Wada and Murbad of Thane District was undertaken to gain insights into
sexuality of rural college youths. The study population included students from
tribal and non-tribal groups. Qualitative information was obtained through 4
focus group discussions and 3 in-depth interviews among male students, and
provided the range of misconceptions and also indicated the possible reasons for
such misconceptions. The study revealed that male students were exposed to
pornographic material and films and this material was circulated among boys.
Few students, who never had an opportunity to watch these films, had
knowledge about what is shown in these films.

Quantitative data revealed the general level of information from 800 male
and 700 female students on various reproductive health issues including their
knowledge, attitude towards sex, sex practices and their perception about
reproductive health service provision. The knowledge issues included 26
statements on components of reproductive health, adolescent changes, virginity,
menstruation, conception, pregnancy and abortion, safe sex, orgasm, impotence,
homosexuality, heterosexuality, oral sex, contraception, sexually transmitted
diseases (STDs), HIV/ AIDS and risk reduction through advantages of using
condom. Respondents were asked to tick mark correct answers from a choice of
options provided. For every correct answer a score of 2 was assigned and for
every partially correct answer a score of 1, if the student gave wrong answers or
responded as ‘don’t know’ the score assigned was 0. A higher score on the scale
indicates a higher degree of knowledge about reproductive health issues. The
index value ranged from 9 to 101 with a mean 50.77 (SD ± 16.85). The results of
the survey revealed that students lacked scientific information and
misconceptions are widespread on various reproductive health issues. Boys
obtained higher mean scores on reproductive health issues when compared to
girls (Fig. 48). The informal channels that provided information of sex related
issues were peers, pornographic material and media. Over 83 percent boys and
girls felt that schools/ colleges should have counselling facility. These preferences
need to be accommodated.
The attitude towards various aspects of sex and sex related issues were assessed by using the attitude scale. For permissive attitude a score of 3 was assigned and for neutral/ don’t know answer a score of 1 and a score of 0 was provided for every conservative attitude. The sex attitude scale consisted of 23 attitudinal statements, which were related to virginity, premarital sex, extramarital sex relations and double standards. Total score range for attitude towards sex was 25 to 61 and the mean score was 40.20 (SD = 5.89). A higher score on the scale indicates a higher degree of sexual permissiveness. Results revealed that boys had more liberal attitudes towards premarital sex as compared to the girls. Overall results showed that conservatism continues to a large extent (Fig. 49). Many students not only disagreed with casual sex but consider it immoral.
The sexual behaviour was divided into three categories i.e. no sexual experience, non-coital sex, and coital sex. Non-coital sex included kissing, hugging, masturbation, caressing breast, hip/thigh, sex between thighs and fondling partner’s genitals. Coital sex included sexual intercourse, oral sex and anal sex.

Results showed that proportion of students with any sexual experience was much higher in boys (29.8 percent) compared to girls (4.9 percent). Of the total students that had any sexual experience, about 11 percent boys and 1 percent girls reported to have had coital sex experience (Fig. 50). Based on the preliminary results, an intervention program for rural youth will be formulated.

Fig. 50: Percentage distribution of boys and girls by type of sexual contact