III MISCELLANEOUS PROJECTS

(a) *Monitoring of Organic Chemical Pollutants in Placental Tissue: A New Approach to HEBM*

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Technical Staff	:	Mr, S. D. Joshi, Mrs. Kamlesh Sharma
Duration	:	2002-2005

Aims, Objectives and Background:

Of late there has been a lot of awareness about pollution, its adverse effects and sources and efforts are being made to control and bring down the levels of pollutants in the environment. Green as well as industrial revolution so as the increasing numbers of automobiles on road have been worldwide recognized to be the major contributor to environmental pollution. For the implementing agencies to take any decision, it is mandatory that background data on the levels of polluting chemicals should be available through various monitoring programmes. In order to get dynamic information about the changes in the environment, regular monitoring of pollutants at various levels of ecosystem and biosphere assumes great significance. While some attempts are being made to assess levels of pollutants in air, water or vegetables, there have been negligible attempts at Human Environmental Bio-monitoring in India or elsewhere in the world for want of a suitable monitoring model. The limitations faced by HEBM Program and advantages of using Human Placenta for HEBM have already been enumerated in the last report.

This is an extramural research project sanctioned by Department of Biotechnology with an aim to establish the utility of human placenta as a tool for comprehensive bio

monitoring for organic pollutants and to demonstrate the feasibility of monitoring region specific organic pollutants in placenta. It also aims at establishing standard operating procedures (SOP) for the above two objectives.

Work done during the year:

Sample Collection:

During the year under report approximately 10000 pregnant women attending Antenatal Out Patient Department Clinic of Safdarjang Hospital were screened for possible exposure to pollutants during the year. In addition to seventeen women who were enrolled for collection of samples last year, 73 who reported exposure to agricultural chemicals during pregnancy due to their involvement in agricultural activity by themselves or their family members were selected for the purpose of collection of samples during this year. Their OPD forms have been stamp marked and the cases are being followed up for subsequent collection of placental sample at the time of delivery. So far a total of 45 samples have been collected which include 30 random samples and 15 samples from high-risk population.

Apart from collection of samples, recovery experiments were conducted using matrix blank, fortified samples and known standards for commonly used pesticides (Endosulphan, Monocrotophos and Deltamethrin).

Efforts were made for standardising the procedures for extraction of pesticides; cleanup of co-eluents and interfering compounds from the extracts and analysis of pesticides in the extracts. A total of 30 extracts were prepared and 12 extracts were analysed using Gas Chromatography.

Preparation of Standard Solutions:

Stock standards (1 mg/ml) were prepared individually and working standards (10µg pesticide/ml) were prepared by appropriately diluting 1ml of stock standards in n-hexane. For calibration of GLC instrument a mixed standard solution containing each pesticide at 1ppm concentration was prepared by mixing 0.5 ml each of Endosulphan, Deltamethrin and Monocrotophos (10 ppm working standard)

Recovery Experiments:

The pesticide recovery experiments were conducted in placental extract for checking the efficiency of the operating procedure using pesticide standards.

Extraction Procedure

For the purpose of extraction, approximately 5.0 grams of placental tissue was macerated with 30-40 grams of anhydrous sodium sulphate in a Teflon vessel using Teflon pestle till the tissue became free flowing. If required more anhydrous sodium sulphate was added. The extraction was carried out by column extraction method using packed columns. Solvents used for extraction were n-hexane, acetonitrile, acetone, and dichloromethane. For recovery experiments, the organic chemicals extracted from placental tissue were divided in two groups (A) Fortified sample and (B) Matrix blank. A Third group consisting of only Pure Standard was also concurrently run. Each experiment was run in duplicate.

Clean up Procedure:

Clean up was done in extract by partitioning technique. The dried extract residue were dissolved in 25 ml of Acetonitrile and partitioned three times with 20 ml of n-hexane using Separating funnel. The separating funnel was shaken and swirled thoroughly and left for separating the layer. The acetonitrile and n- hexane layer was collected in separate vessels The Acetonitrile layer was diluted 5 times with 10 % Sodium chloride solution. This aqueous phase was partitioned thrice with 50 ml of chloroform using separating funnel. The chloroform layer was collected after separation in a flask. The collected chloroform layer was passed through anhydrous sodium sulphate taken in a funnel to remove the traces of moisture. The chloroform layer kept for evaporation and concentrated up to dryness. The dried extract was reconstituted with 4ml of n-hexane. In another experiment Florisil was used for clean up.

Gas Chromatography:

Samples were analysed for pollutants by Gas-liquid chromatography with electron capture detector. The operating condition of the instrument was as follows:

GC-	Hewlett Packard 5890 series II
Detector	ECD
Carrier gas	Nitrogen
Column	HP-1 (10m x 0.53mm ID)
Film thickness	0.25µm

Flow rate	20ml/minute
Sample volume injected	3 µl
Analysis condition	220^0C for 7 minutes
	Followed by ramp @ 30^0 C / minute up to 260^0C
	Maintained at 260^0C for 15 minutes

Table 1: Percentage Recovery of Pesticide by different extraction procedures

Method	Clean up	Solvent	Percentage Recovery Range		
			Monocrotophos	Deltamethrin	Endosulphan
Method 1		Hexane	94-100	127	106
Method 2		Acetonitrile	57.16-57.46	41.88-44.87	43.66-43.78
Method 3	Without Clean up	Acetone	7.71-7.73	4	36.46-36.65
		DCM	5.45-5.57	83-87	82.4-84.7
	With Clean up	Acetone	19.3-19.6	27	26.69-21.71
		DCM	3.6	183-188	59-61
Method 4	Florisil Clean up	DCM	84 -86	122 -126	88 -93
		Acetonitrile	52 -59	68 -70	101

The recovery experiment conducted by method 1,2 and 3 did not show satisfactory recovery. The recovery in method 4 was found to be satisfactory in the range of 52-120 % with both the solvent. In this method the tissue was homogenized and clean up was done by florisil column chromatography. The over all results of the method used for conducting recovery experiment method 4 was found suitable and showed satisfactory recovery percentage.

Sample analysis:

The five placental tissues extract were analysed using Hewlett Packard Model 5890 series II with Ov-5 column (30m x 0.25mm ID; 0.25um film thickness) with Electron capture Detector.

The operating conditions of the instrument for sample analysis were as follows:

GC	Hewlett Packard 5890 series II
Detector	ECD
Carrier gas	Nitrogen
Column	OV-5
Flow rate	1ml/minute

Injector temp. 250^0C

Detector 280^0C

Sample volume injected 3µl

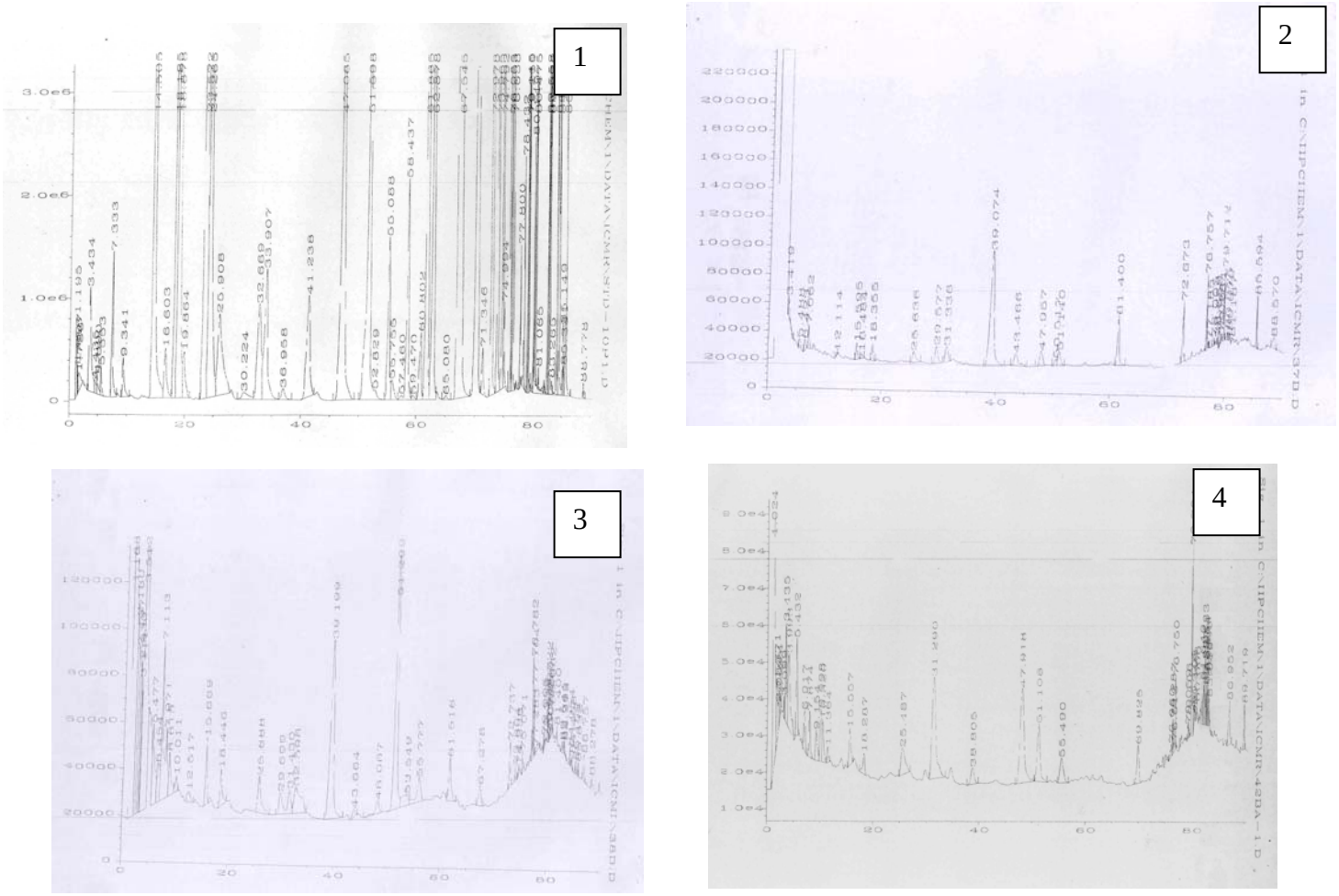
Oven Temperature 140^0Cfor 45 minutes increased @ 20^0C/minute till 170C increased @10^0C/ minute till 270^0C increased @20^0C till 280^0C and maintained for 10 minutes

Pesticides were identified in the samples by comparing the retention time of the peaks in the sample chromatogram with those of the peaks in standard chromatogram. The pesticides detected in placental extracts (Table 2).

Pesticide			Sample No.				
			37	38	40	41	42
Class	List	Ret Time	Concentration in ppb				
Fungicide	Tebuconazole	78.432	21		52.8	58.6	
	Thiafluzamide	73.965		9.53	81.3		43
Herbicide	Dithiopyr	47.065	22.7	14.6	0.95	17.4	85.6
	Flufenacet	55.088		141	240	45.9	97.8
OC	DDE-pp	71.346					20.6
	DDT-op + Iprodion	76.055		36	74.4	8.22	14.4
	DDT-pp	77.8	69	22.2	9.58	68	
	Dieldrin+ Hexaconazole	67.545		24.4	127		39.1
	Endosulphan Sulphate	76.638	70.3	60.4	77.8	42.6	24.2
	Endosulphan- ?	61.865	64.4	42.9	11.9		
	Endosulphan-?	72.978	56	27.8	41.8		11.5
	HCH-?	18.185	234	459	59.8	166	181
OP	Chlorpyriphos	51.598	22	276	11.6	40.6	28
	Dimethoate	16.603	59.5			48	
	Edifenphos	82.875		13.8	32.9	31.5	70.7
	Metribuzin	32.669		249	160	170	
Pyrethroide	Cyhalo-L	84.122		14.1	69.4		20.9
	Cypermethrin	80.475	20.3	27.3	513	6.43	25.2
	Deltametherin	88.778	1768	279	1194	580	3800
	Fenoprop-1	79.115	0.2	8.17	65.1	4.31	8.01
	Fenoprop-2	79.259	55	19	2570	19.5	199
	Fenvel-1	85.149		23.7	25.8		
	Fenvel-3	85.659	20.5	12.8	12.1	3.85	

Table 2: Gas Chromatography analysis of Pesticides in Placental Extracts

Chromatogram of 33-pesticide standards is shown in Fig.1. Representative Chromatograms of placental samples are shown in Figs. 2 – 4.



Figs. 1 – 4: GLC Chromatograms from standard and Placental Samples.

Preliminary analysis of the results has revealed presence of pyrethroids, active ingredient of commonly used mosquito repellent in significantly large number of samples. Some other organo-chlorine and organo-phosphorus compounds have also been observed rather infrequently. Further extraction, analysis and reconfirmation of observed compounds by GC-MS are being carried out.

(b) *In vitro cultivation of differentiated epidermis from human Keratinocytes suitable for autologous grafting in burns patients.*

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Technical Staff	:	Mr. Dinesh Kumar
In collaboration with	:	Dr. R. P. Narayan, Safdarjang Hospital, New Delhi
Duration	:	2000-2004

Aims, Objective & Background

The project (ICMR funded scheme) was aimed at the development of methodology for cultivation of differentiated epidermis from human keratinocyte culture and application of the epidermal sheets in burns patients as autologous graft material. So far the basic method of culturing and large-scale expansion of keratinocyte cultures were standardized.

Work done during the year

The clinical application of cultured epidermal sheets in patients was planned in phased manner as per the ethics committee's recommendations. In order to identify the optimal factors for better graft take the first phase would include only preliminary grafting with cultured epidermis on 50-to 500-cm^2 area in 10 patients. It is only after analysing the outcome in phase-I trial, the phase-II & subsequently phase-III trials involving 500 to 6400 cm^2 or more, would be initiated for better acceptance of this technique.

The cultured epidermal sheets were used to graft the raw wounds of total five patients. The grafted patients were kept on oral antibiotic therapy with Amicacin and Cefotaxime. The following are the details of graft and graft take in these patients.

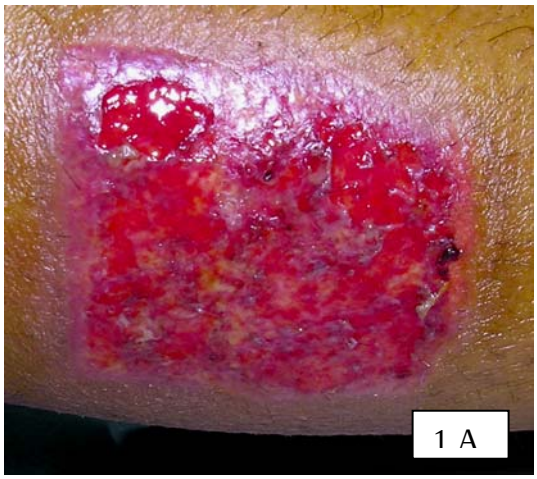
Patient No	Age/Sex	Type & Area of burn	Degree of burn	Area grafted with cultured epidermis cm^2	Day of opening the dressing	Average Percent of graft take*
01	22/Male	Scald 30%	2^{nd} & 3^{rd}	130	Day 6	64%
02	24/Female	Thermal 50%	2^{nd} & 3^{rd}	390	Day 4	72%
03	28/Female	Thermal 50%	3^{rd}	130	Day 6	82% #
04	5/Male	Thermal 40%	2^{nd} & 3^{rd}	195	Day 6	Graft ** rejected
05	2/Male	Thermal 15%	2^{nd} & 3^{rd}	260	Day 6	Graft** rejected

Table.1: The details and the outcome of grafting with cultured epidermis in five burns patients.

*** On the day of 1^{st} change of dressing after grafting.**

 # The graft was found to be destroyed on the subsequent dressing and the microbiological investigation of the dressing material revealed nosocomial infection (Pseudomonas sp. streptococcus aureus, Klebsiella sp.)

**** The microbiological investigation of the graft dressing material revealed nosocomial infection (Pseudomonas sp, Staphylococcus sp.)**



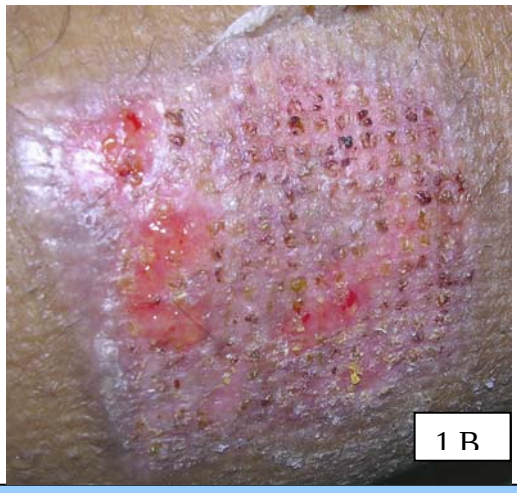
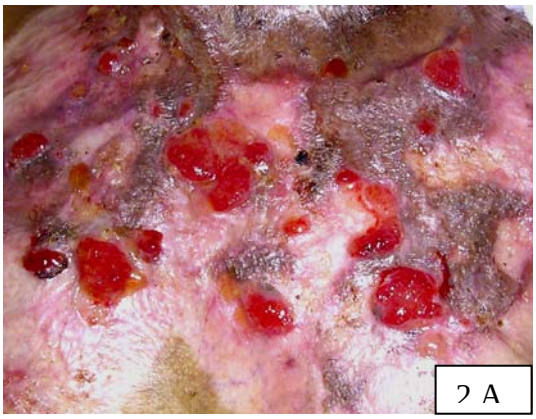
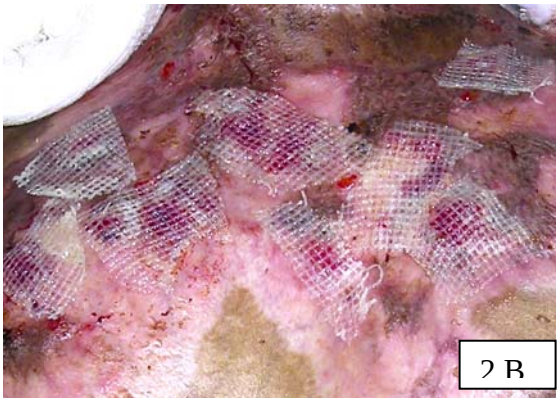


Fig. 1 :The left arm wound of patient 02 with 50% thermal 3rd degree burns grafted with cultured epidermis. 1A: The raw wound before grafting. 1B: Same wound on day 4 showing 86.7% wound healing.





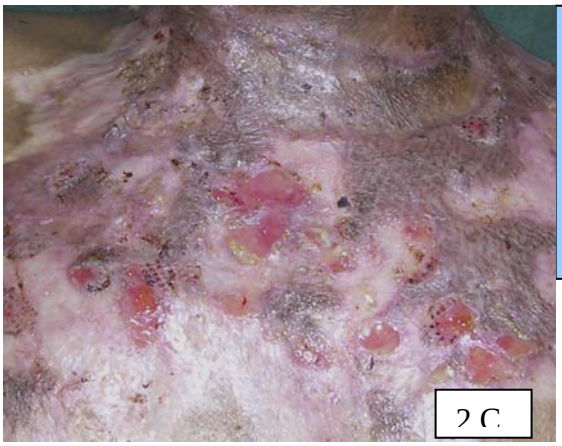


Fig. 2. The raw wounds on chest of patient 02 with 50% thermal 3rd degree burns grafted with cultured epidermis.
2A: The raw wounds before grafting.
2B: The wounds with cultured epidermal grafts.
2C: Same wounds on day 4 showing 74.5% wound healing.

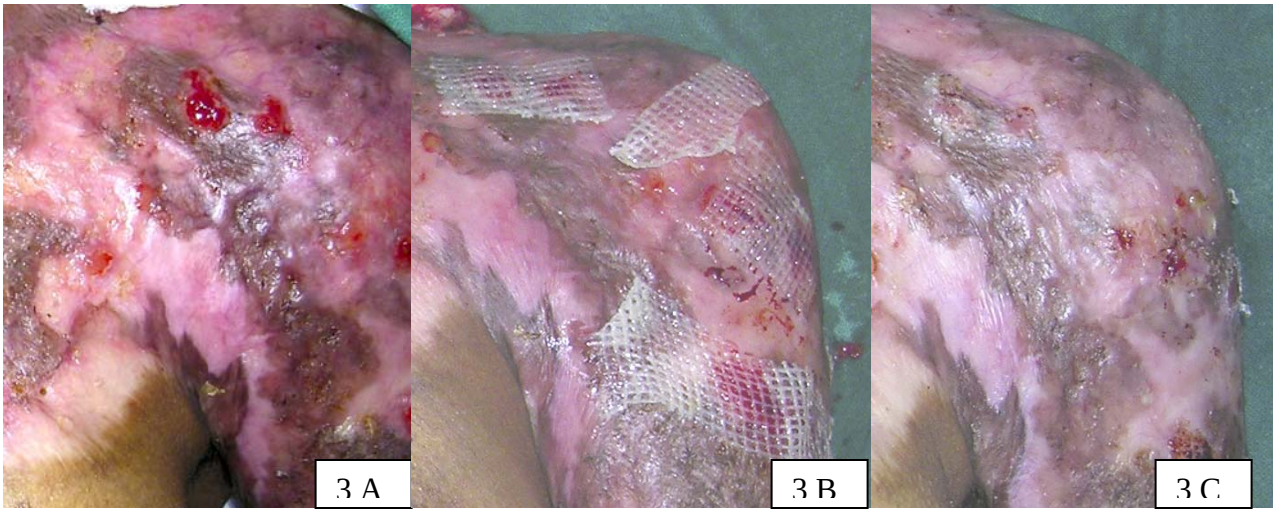


Fig. 3. The raw wounds on left shoulder and upper arm of patient-02 with 50% thermal 3rd degree burns grafted with cultured epidermis.
3A: The raw wounds before grafting.
3B: The wounds with cultured epidermal grafts.
3C: Same wounds on day 4 showing 84.8% wound healing.

In three out of five patients' cultures it has been experimented to observe the maximum potential of keratinocyte amplification through serial passages by the use of appropriate seeding densities. An amplification of keratinocytes to prepare epidermal sheets to the order of 6400 cm^2 surface area (suitable for 40% burn cover) was achieved in 29-31 days by these experiments. Additionally, the formation of epidermal sheets originating from a frozen stock of keratinocytes was demonstrated. The epidermal sheets by this method could be grown in half the usual time required. This method could be applied in keratinocyte banking.

Future Plan of action

A project proposal on clinical application of autologous cultured human epidermis in burns patients has been submitted to ICMR for support of funds.

Another project proposal for Identification of optimal cell proliferation stimulants for human keratinocyte-epidermal culture system: a comprehensive approach has been submitted to DRDO for support of funds.

(c) Pathophysiological Role of Estrogen in males

(i) Flow cytometric Evaluation of Estrogen Receptor α in the ejaculated human spermatozoa.

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Technical Staff	:	Mr. Dinesh Kumar
Duration	:	2003-2004

The earlier studies in our laboratory have indicated the immunohistochemical localization of Estrogen Receptor (ER) and a number of estrogen modulated proteins in the ejaculated human spermatozoa.

In the fertile subjects, most of the spermatozoa in the ejaculate appear to show the presence of ER; however there seems to be heterogeneity in the type of localizations.

In the infertile subjects, a number of individuals show a ER positivity in majority of spermatozoa, however some percentage of individuals either show a less concentration or total absence of ER in their spermatozoa. But because of the smaller size of the spermatozoa and their high numerical density in the ejaculate, the heterogeneity could not be evaluated objectively. Unfortunately the image analysis software available (to us) is not in a position to improve our knowledge in the area. So investigations were undertaken to investigate the localization of ER in the spermatozoa using flow cytometry. The preliminary studies have indicated that ER α can be evaluated flow cytometrically.(Fig.1). The studies are in progress to understand the possible implication of IH localization in fertile and infertile subjects.

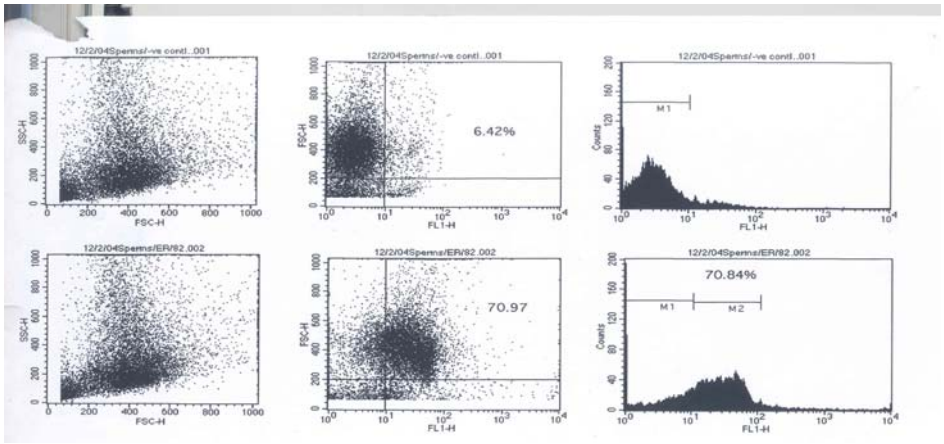

Fig. 1 : The flow cytometric separation of ERα labelled human ejaculated spermatozoa. .The top panel shows the control and the lower ERα labelled with FITC. The % of unlabelled / labelled / sperms are represented as M1 and M2.

(ii) Flow cytometric Evaluation of Estrogen Receptor α in the rat testicular germ cells.

The earlier investigations in the laboratory indicated the presence of at least one of the isoforms of Estrogen Receptor (ER) or one of the estrogen induced protein, namely Progesterone Receptor (PR) in almost all the testicular cellular components of mouse, rat, monkey and human testicular germ cells. It was difficult to hypothesize the possible role of Estrogen in the testicular germ cells, as ours were the only report in the literature and each stage in the germ cell development is unique and serves it's own purpose. Investigations were undertaken to investigate the localization of ER α in the isolated rat germ cells. The

a b

c d

e f

g h

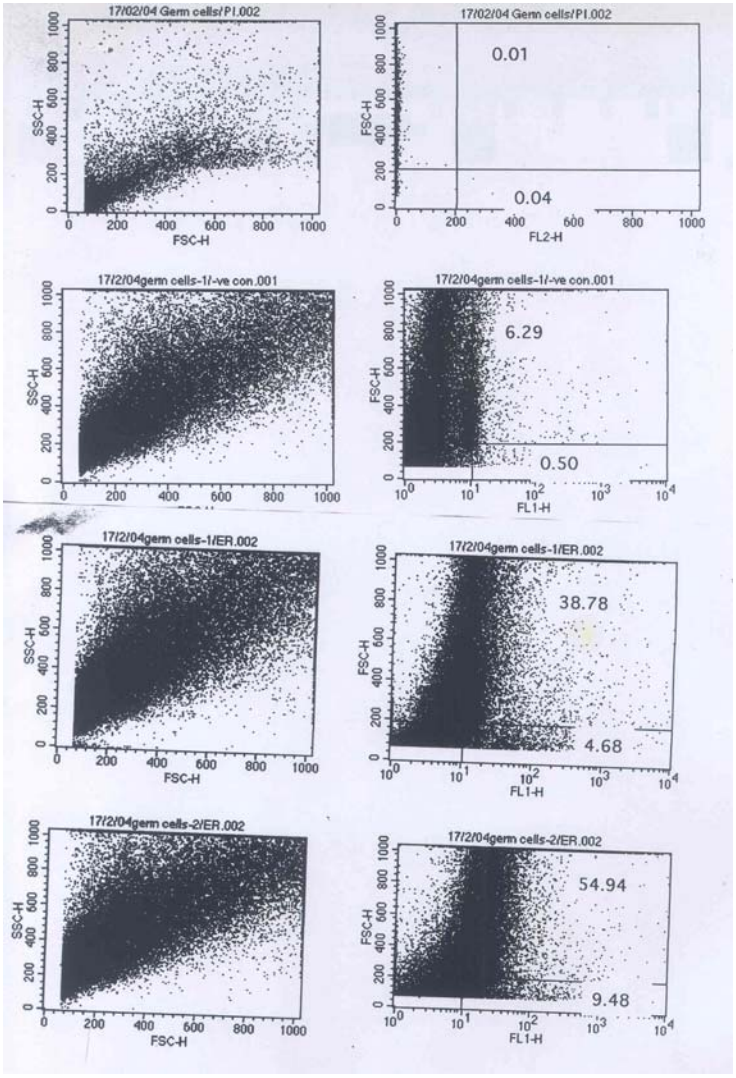

Fig. 2 : The flow cytometric separation of ERα labeled rat testicular germ cells.
a-d : The panels indicate the controls and the
e-h : from the FITC labeled ERα samples

preliminary studies have indicated that it is possible to evaluate the role of ER in the germ cells by using flow cytometry. However, since the germ cells lose a good amount of their morphological identity following isolation, it may be difficult to identify the individual germ cell type. Attempts will be made to use differential centrifugation cellular for separation before evaluating the different fractions through flow cytometry.

The preliminary results of ER α localization are presented in Fig.2. As indicated in the Fig.2, perhaps different germ cell population of testes seem to demonstrate the localization. The studies need to be confirmed in additional batches using the differential centrifugation of protocols followed by flow cytometry.

(iii) Detection of mRNA transcripts of Estrogen Receptor (ER α & β), Progesterone Receptor (PR), and Cytochrome P450 from human spermatozoa.

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In collaboration with	:	Dr. MM Misro, SJ Hospital, New Delhi
Technical Staff	:	Mr. Dinesh Kumar
Duration	:	2004

Our earlier studies have indicated heterogeneity in the immunohistochemical localization of Estrogen Receptors. In addition some infertile subjects showed different profiles in the immunohistochemical expression of the same. Other literature reports have indicated the presence of splice variants in the Estrogen Receptor in some subjects with breast cancer. So attempts were made to evaluate the presence of splice variant of ER and PR, if any, in the human ejaculated spermatozoa from fertile subjects and to investigate correlation if any, of the same with male infertility.

The pilot studies were initiated in 20 semen samples, collected from the infertility clinic. Ten samples with higher sperm count (>70 million / ml) and motility (> 70 %) were considered to be in the fertile range and ten samples in the less sperm counts (<20 million / ml.) and with less motility (< 30%) were arbitrarily classified as infertile, for the purpose of comparison of results.

RNA was isolated using the One Step Invitrogen TRIzol reagent. The amount and integrity of RNA was checked. Formation of cDNA was checked by RT- PCR, using Primers specific for transcripts of house keeping genes and β actin and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase Fig. 3 and 4.

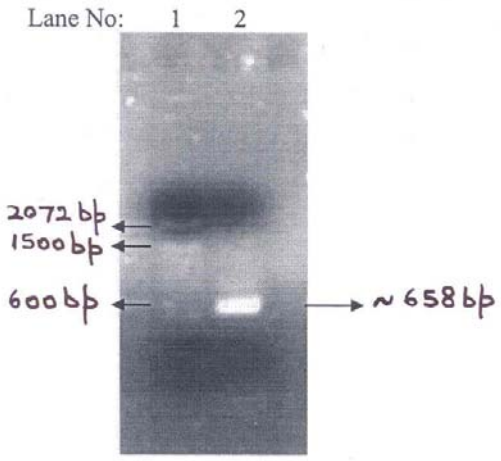

Fig. 3 : RT-PCR detection of β actin transcript from RNA isolated from human ejaculated spermatozoa. Lane1: 100bp DNA ladder. Lane 2: shows presence of the PCR band of β actin of around 658bp in the sample.

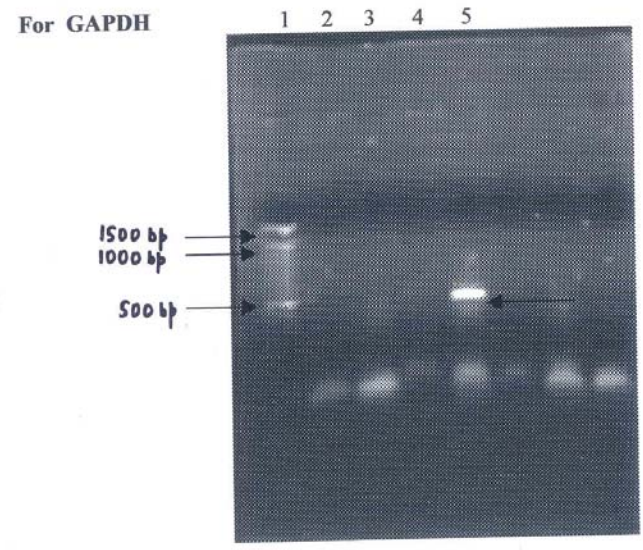

Fig. 4 :Showing band of GAPDH as a positive control. Lane–1 shows the 100 bp marker. Lane-2 shows the negative control (without cDNA), Lane-3 shows PCR done with RNA to detect any DNA contamination, Lane-5 shows the GAPDH band of Mw~600 bp.

The PCR for ER α (primer specific for Exon-5 ,HBD) were carried out in 5 samples (2 from the 'fertile' and 3 from the 'infertile' Group) showing amplification with positive controls. No transcripts were detected in the semen samples from the two 'fertile' semen samples. One sample from the 'infertile' sample showed a single band at 468 bp. position (Fig.5).

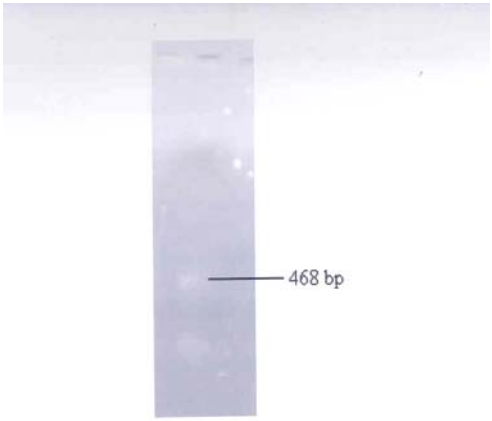

Photo 4

Fig. 5 Lane 2. PCR product of ER-α (sample 112)

The PCR studies carried out with Primers specific for Exon 5 of ER-β demonstrated the presence of ER-β transcripts in all the above mentioned 5 samples, the 2 'fertile' and one' infertile' sample showed the product size of approximately 429 bps (Fig.2) and the other two 'infertile' samples showed the PCR – products in the range of approx. 150 bp and 250 bp.(Fig. 6)

Fig. 6

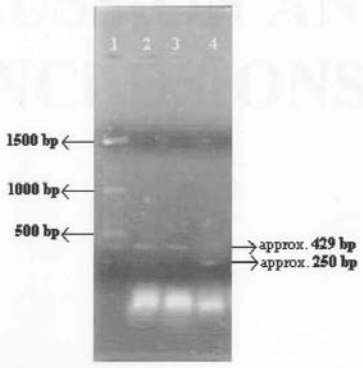

Fig.16 Gel for PCR of ERβ (sample 2, 3, 5)

Lane 1. 100bp DNA ladder
Lane 2. PCR of ER-β (sample 2) at annealing temp. of 58ºC (band of ~429bp size)
Lane 3. PCR of ERβ (sample 3) at annealing temp 58ºC (band of ~429 bp size)
Lane 4. PCR of ERβ (sample 5) at annealing temp.58ºC (band size of ~250bp)

mRNA transcripts for the Progesterone Receptor (PR) could not be detected by standard PCR protocols from the ejaculated spermatozoa of fertile individuals. It is possible that the methodology adopted was not sensitive enough to detect the low number of transcripts present in the ejaculated spermatozoa from fertile individuals. (It is to be mentioned that the usual alterations in the standard PCR protocols were tried before arriving at this conclusion). However in four infertile subjects, transcripts of (500 bp (wild type) and 250bp (variant) Progesterone receptor were detected (Fig.7). Similar splice variants were reported by Sachdeva et al. (2000) from the spermatozoa from one subject, though the fertility status of the subject was not mentioned.

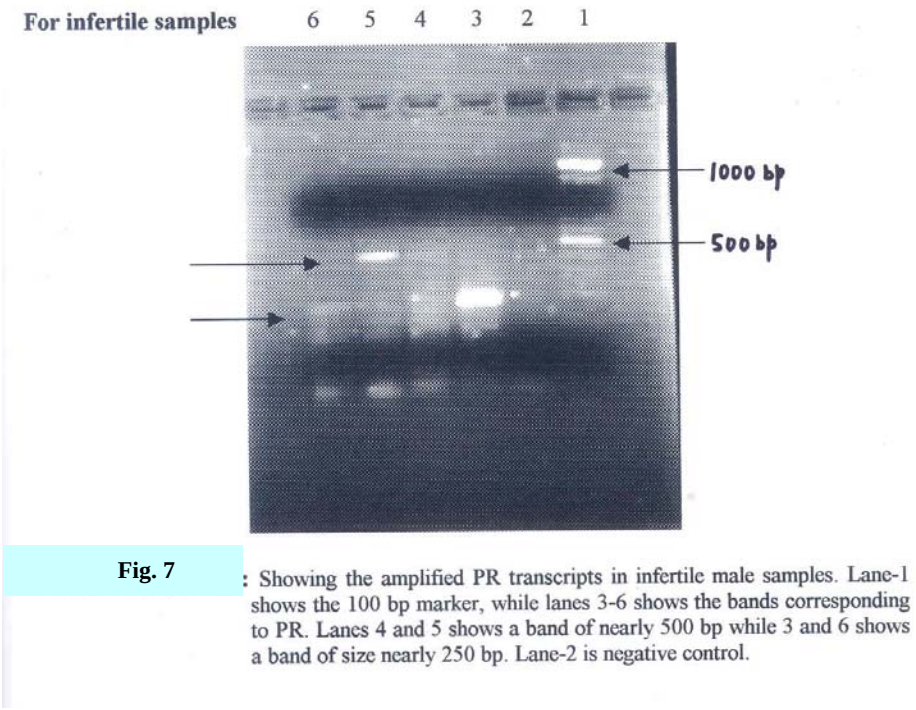

Fig. 7 : Showing the amplified PR transcripts in infertile male samples. Lane-1 shows the 100 bp marker, while lanes 3-6 shows the bands corresponding to PR. Lanes 4 and 5 shows a band of nearly 500 bp while 3 and 6 shows a band of size nearly 250 bp. Lane-2 is negative control.

None of the 24 semen samples (14 ‘fertile and 10’infertile’) demonstrated the presence of mRNA transcripts of Cytochrome P450, with RT-PCR using primers specific to the conserved regions of the P450 aromatase gene. The cDNA synthesis was proper, but the amplification of the product could not be achieved in spite of the alterations in the annealing temperature, magnesium ion concentrations, amount of template, number of cycles, primer concentrations etc. in the standardization of the PCR protocol. It is possible that further standardization of the technique may be essential.

Sperms were generally thought to be transcriptionally inactive because of the condensed nucleus and the immunohistochemical localization of ER and the different ER induced proteins were possibly contemplated to have steroidal action through non-genomic pathway. It is possible that the good yield of mRNA obtained in both ‘ fertile’ and ‘infertile’ subjects may be from mitochondria as this organelle of the sperm is highly active and may involve active transcription. However the possibility that this RNA in the sperm has been synthesized during maturation and retained till ejaculation may have some credibility, only if it could be demonstrated that spermatozoa have some mechanism to enhance the stability of stored RNA.

The results demonstrating the presence of isoforms / splice variants of ER and PR in infertile subjects perhaps need further clarifications in additional subjects. The presences of the ER and PR splice variants, if confirmed, may through new insights into the role of these steroid receptors in fertility regulation and may have diagnostic and therapeutic implications.

The demonstrations of ER/ PR transcripts in some individuals also indicate that the ejaculated spermatozoa are not totally inactive transcriptionally and that these steroids may possibly act through the ‘genomic pathway ’ in addition to the ‘non-genomic’ route.