SCREENING AND BIOLOGICAL CHARACTERIZATION OF SOME NATURAL/SYNTHETIC ENDOCRINE DISRUPTORS

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree

of

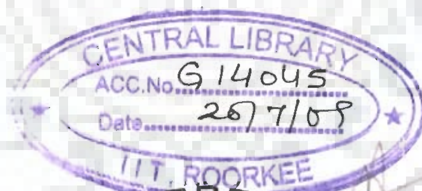
DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

VIKAS KUMAR



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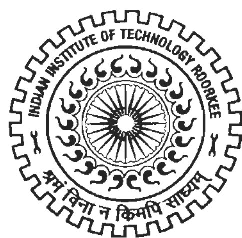

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **SCREENING AND BIOLOGICAL CHARACTERIZATION OF SOME NATURAL/ SYNTHETIC ENDOCRINE DISRUPTORS** in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during the period from July 2004 to June 2008 under the supervision of Dr. Partha Roy, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(VIKAS KUMAR)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

Date: 27/6/08

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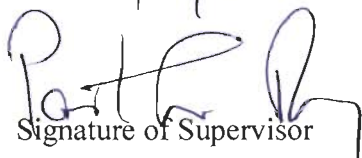

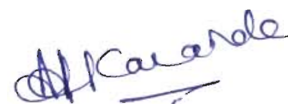

Signature of Supervisor

Signature of External Examiner

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CONTENTS

	Page No.
CANDIDATE DECLARATION	
ACKNOWLEDGEMENT	**I-III**
CONTENTS	**IV-IX**
LIST OF FIGURES	**X-XIII**
LIST OF TABLES	**XVI-XV**
LIST OF ABBREVIATION	**XVI-XVIII**
CHAPTER 1: INTRODUCTION	
1.1 General Introduction	1
1.2 Aims and Objectives	5
CHAPTER 2: REVIEW OF LITERATURE	
2.1 The Endocrine System	9
2.1.1 Reproductive endocrine system	
2.1.1.1 Introduction	10
2.1.1.2 Hormones involved in reproduction	11
2.1.1.2.1 Gonadotrophic hormones or gonadotropins	11
2.1.1.2.1.1 Follicle stimulating hormone (FSH)	12
2.1.1.2.1.2 Luteinizing Hormone	12
2.1.1.2.1.3 Prolactin or Luteotropin Hormone (LTH)	12
2.1.1.2,2 Steroid hormones	13
2.1.1.2, 2.1 Female sex steroids (estrogen and progesterone)	13
2.1.1.2,2.2 Male Sex Steroids	14
2.1.1.2.3 Hypothalmic-Pitutarty-Gonadal Axis	14
2.1.2 Dynamics of Steroidogenesis	16
2.1.2.1 The conversion of cholesterol to pregnenolone	17
2.1.2.2 Stepwise conversion of pregnenolone to testosterone: Δ^4-3-ketone pathway	18
2.1.2.3 Stepwise conversion of pregnenolone to testosterone: Δ5-3β-hydroxy Pathway	19
2.1.2.4 Aromatization	19
2.1.2.5 17β-hydroxysteroid dehydrogenation	20
2.1.3 Steroid hormone receptors	20
2.1.3.1 Androgen Receptor (AR)	21
2.1.3.2 Estrogen receptors (ER)	23
2.1.4 Dynamics of Steroid Action	24
2.2 Endocrine Disruption	27
2.2.1 Decent of the Concept and Definition	27
2.2.2 Historical Prospective 29	
2.2.3 The complexity of endocrine disruptors	31
2.2.3.1 Adverse in vivo effects of EDCs to human health	35

2.2.3.1.1 Sperm quality deterioration 36
2.2.3.1.2 Testicular cancer 37
2.2.3.1.3 Congenital abnormalities **Page**
(cryptorchidism and hypospadias) 38
2.2.3.1.4 Changes in sex ratio 39
2.2.3.1.5 Effects on central nervous system 39
2.2.3.2 Tiered Bioassays for the assessment of endocrine disruptors 40
2.2.3.2.1 In vitro Assays: 42
2.2.3.2.1.1 Receptor binding assay 42
2.2.3.2.1.2 Cell proliferation assay 43
2.2.3.2.1.3 Reporter gene assays 43
2.2.3.2.2 In vivo Assays: 45
2.2.3.2.2.1 (Anti) Estrogenic assays 45
2.2.3.2.2.2 (Anti) Androgenic assay 45
2.2.3.2.2.3 (Anti) Progesterogenic Assay 46
2.2.3.2.2.4 Other in vivo assays using non-mammalian vertebrates 47
2.2.3.3 Analytical methods for the determination of potential EDC in test samples: GC-MS and HPLC 48
2.2.3.3.1 Gas chromatography-mass spectroscopy (GC-MS) 49
2.2.3.3.2 High Performance Liquid Chromatography (HPLC) 50
2.3 Endocrine Disruptors: Different Sources and Categories (types) and biological relevance 51
2.3.1 Steroids
2.3.1.1 Phytoestrogens 53
2.3.1.2 Synthetic Steroids 54
2.3.2 Pesticides and antimicrobials 55
2.3.3 Industrial Chemicals
2.3.3.1 Phthalates 58
2.3.3.2 Bisphenol 58
2.3.3.3 Polychlorinated dibenzodioxins and furans 59
2.3.3.4 Alkylphenol ethoxylates 59
2.3.3.5 Biphenyls 60
2.3.3.6 Polycyclic aromatic hydrocarbons 61
2.3.4 Anti-androgenic Mixtures (Combinational Effect) 61
2.3.5 Pharmaceutical Products 62
2.3.6 Endocrine Disruptors vs. aquatic system: sewage water and industrial Effluents 63

CHAPTER 3: MATERIALS AND METHODS
3.1 Material 66
3.2 Methdology 66
3.2.1 Samples Preparation
3.2.1.1 Sewage water samples 66
3.2.1.2 Leather industry effluents 67
3.2.1.3 Triclosan, Ibuprofen, Tetracycline and Hexachlorobenzene 68
3.2.2 Experimental Models (Animals) 68

3.2.3 Treatment
3.2.3.1 Triclosan (TCS) 69
3.2.3.1.1 Screening of TCS for anti-androgen and anti-estrogen activity
3.2.3.1.2 Intact assay
3.2.3.2 NSAID and antibiotic 71
3.2.3.2.1 Screening of test samples for anti-androgen and anti-estrogen activity
3.2.3.3.2 Intact assay
3.2.3.3 Leather Industry Effluent 72
3.2.3.3.1 Screening of test samples for androgenicity by Hershberger assay and uterotorphic assay
3.2.3.3.2 Intact assay
3.2.3.4 Sewage water 73
3.2.3.4.1 Screening of test samples for androgenicity by Hershberger assay and uterotorphic assay.
3.2.3.4.2 Effects of WWTP effluents in intact rats.
3.2.3.5 Hexachlorobenzene (HCB) 75
3.2.3.5.1 In vivo study: Hershberger and Intact assay
3.2.3.5.2 In vitro study: HCB Treatment of Leydig Cells
3.2.4 Courtship behavior 76
3.2.5 Daily sperm production (DSP) 77
3.2.6 Sperm structural analysis 77
3.2.7 ELISA to measure concentration of various hormones in animal sera and culture medium 78
3.2.8 Histopathology of various tissues 79
3.2.9 Immunohistochemical analysis (IHC) 80
3.2.10 Western blot analysis 81
3.2.11 RNA Extraction 83
3.2.12 Estimation of purity of RNA samples by formaldehyde gel 84
3.2.13 Semiquantitative RT – PCR analysis 85
3.13.1 First strand cDNA synthesis (reverse transcription) 85
3.13.2 PCR Amplification 86
3.2.14 Serum Alkaline Phosphatase, Acid Phosphatase, SGPT and SGOT Level 90
3.2.15 Assay of anti-oxidant enzymes 92
3.2.16 Reversed phase HPLC fractionation of the WWTP inlet and outlet samples 93
3.2.17 Physicochemical analysis of WWTP inlet and outlet water and leather industry effluents samples 94
3.2.18 Leydig cell isolation, purification, Immunocytochemical Identification and assessment of viability (MTT assay) 95
3.2.19 Steroidogenic enzyme activity in vitro. 98
3.2.20 Adenylyl Cyclase activity in isolated Leydig cells 99
3.2.21 MTT Assay in isolated leydig cells 99
3.2.22 Statistical analysis 100

CHAPTER 4: IN VIVO CHARACTERIZATION OF SOME ENDOCRINE DISRUPTING CHEMICALS

Chapter 4.1 An antimicrobial Chemical as EDC

4.1.1 Introduction 101
4.1.2 Results
4.1.2.1 Endocrine disrupting effects of TCS in male reproductive system
4.1.2.1.1 Weights of SATs in castrated rats (Hershberger Assay) 103
4.1.2.1.2 Effects on courtship behavior 103
4.1.2.1.3 Weight of testis and SATs in intact rats 104
4.1.2.1.4 Gene expression analysis 104
4.1.2.1.5 Testicular 3β-HSD and 17β-HSD) levels in vitro 106
4.1.2.1.6 Testicular StAR and AR protein immunoblot analysis 106
4.1.2.1.7 Immunohistocemical detection of testicular StAR protein 106
4.1.2.1.8 Serum hormone levels 107
4.1.2.2 Endocrine disrupting effects of TCS in female reproductive system
4.1.2.2.1 Effect of TCS on the weight of uterus of ovarectomised rats (uterotrophic assay) and intact female rats 107
4.1.2.2.2 Gene expression analysis 111
4.1.2.2.3 Serum Estradiol and other hormone levels 111
4.1.2.2.4 Ovarian 3β-HSD(I) and 17β-HSD(III) levels in vitro 111
4.1.2.3 Toxicological evaluation of TCS
4.1.2.3.1 Body weight 112
4.1.2.3.2 Toxicity marker enzymes: hypothalamic SOD, CAT, GPx and GR 112
4.1.2.3.3 Histopathology of male reproductive system (Testis and SATs) 115
4.1.2.3.4 Histopathology of female reproductive system (uterus) 115
4.1.2.3.5 Sperm Toxicity 116
4.1.3 Discussion 123

Chapter 4.2 Two common pharmaceuticals: NSAID and antibiotic as EDC

4.2.1 Introduction 129
4.2.2 Results
4.2.2.1 Endocrine disrupting effect of ibuprofen and oxytetracycline in male reproductive system
4.2.2.1.1 Determination of anti-androgenicity of test drugs by Hershbergar assay 131
4.2.2.1.2 Effects on courtship behavior in intact animals 134
4.2.2.1.3 Gene expression analysis 135
4.2.2.1.4 Testicular 3β-HSD and 17β-HSD levels in vitro 135
4.2.2.1.5 Testicular immunoblot analysis 136
4.2.2.1.6 Serum hormone levels 136
4.2.2.2 Endocrine disrupting effect of ibuprofen and oxytetracycline in female physiological system 136
4.2.2.2.1 Determination of (anti)estrogenicity of test drugs by uterotrophic assay 136
4.2.2.2.2 Gene expression analysis 140
4.2.2.2.3 Ovarian 3β-HSD and 17β-HSD levels in vitro 141
4.2.2.2.4 Serum Hormone Level 141
4.2.2.3 Toxicological evaluation of the ibuprofen and tetracycline

4.2.2.3.1 Effects on body weight 141
4.2.2.3.2 Histopathologiacl Analysis 141
4.2.2.3.3 Assay of toxicity marker enzymes- : SOD, CAT, GPx and GR assay 144
4.2.3 Discussion 145

Chapter 4.3 Industrial effluents as EDC: Leather industrial effluents
4.3.1 Introduction 151
4.3.2 Results
4.3.2.1 Endocrine disrupting effects of leather industry effluents samples in the male reproductive system
4.3.2.1.1 Sex accessory tissue weight (Hershberger Assay) 153
4.3.2.1.2 Daily Sperm Production 154
4.3.2.1.3 Gene expression profile 154
4.3.2.1.4 3β-HSD and 17β-HSD levels 158
4.3.2.1.5 Testicular 3β-HSD and AR protein immunoblot analysis
4.3.2.1.6 Serum Hormone levels
4.3.2.2 Endocrine disrupting effects of leather industry effluents samples in the female reproductive system
4.3.2.2.1 Determination of estrogenicity of leather effluents by uterotrophic Assay 159
4.3.2.2.2 Gene expression analysis 163
4.3.2.2.3 Ovarian 3β-HSD and 17β-HSDlevels in vitro 163
4.3.2.2.4 Serum Estradiol and gonadotropin levels 163
4.3.2.3 Toxicological evaluation of the test sample 164
4.3.2.3.1 Effects on Body Weight 164
4.3.2.3.2 Histopathological analysis 164
4.3.2.3.3 Toxicity marker enzymes 167
4.3.3 Discussion 168

Chapter 4.4 In vivo characterization of endocrine disrupting behavior of a WWTP inlet and outlet water samples and their comparative analysis
4.4.1 Introduction 171
4.4.2 Results
4.4.2.1 Endocrine disrupting effects of wastewater samples in male reproductive system 173
4.4.2.1.1 Determination of androgenicity of effluents by Hershberger assay 173
4.4.2.1.2 Gene expression profile of steroidogenic enzymes. 174
4.4.2.1.3 Testicular 3β-HSD and 17β-HSD levels in vitro. 177
4.4.2.1.4 Testicular 3β-HSDand AR protein immunoblot analysis 177
4.4.2.1.5 Serum Hormone levels 177
4.4.2.2 Endocrine disrupting effects of wastewater samples in female reproductive system 178
4.4.2.3 Toxicological evaluation of the test samples 178
4.4.2.3.1 Body and organ weights of effluent treated intact rats 178
4.4.2.3.2 Histopathology of sex accessory tissues 178

4.4.2.3.3 Histopathology of kidney and liver effluent treated rats
4.4.2.3.4 Analysis of systemic toxicity in WWTP treated animals 180
4.4.3 Discussion 184

Chapter 5 : Physicochemical characterization of WWTP water and leather industry effluent samples
5.1Introduction 189
5.2 Results 190
5.2.1 WWTP water sample
5.2.1.1 HPLC Analysis 190
5.2.1.2 GC-MS Analysis 192
5.2.2 Leather industry effluents analysis 192
5.3 Discussion 193

Chapter 6: In-vivo and In-vitro characterization of HCB as EDC
6.1 Introduction 198
6.2 Results
6.2.1 (Anti)androgenic characterization of HCB: in vivo
6.2.1.1 Sex accessory tissue weight (Hershberger Assay) 199
6.2.1.2 Gene expression profile 201
6.2.1.3 3β-HSD and 17β-HSD levels 201
6.2.1.4 Testicular StAR and AR protein immunoblot analysis 201
6.2.1.5 Serum Hormone levels 201
6.2.2 (Anti) androgenic characterization of HCB: in vitro
6.2.2.1 Leydig cell testosterone metabolism 205
6.2.22 Gene Expression Analysis 205
6.2.2.3 Steroidogenic enzymes activities 206
6.2.2.4 Immunoblot analysis 206
6.2.2.5 Adenylyl cyclase activity 206
6.2.2.6 Leydig cell cAMP production 206
6.2.2.7 Effect of adenylyl cyclase activator/ inhibitor on LH induced testosterone production and their cross-talk with HCB 212
6.2.3 Toxicological evaluation of the HCB 214
6.3 Discussion 214

CHAPTER 7: CONCLUSION **219**
CHAPTER 8: REFERECE **229**

RESEARCH PUBLICATIONS

LIST OF FIGURES

Figure No.	Figure Title	Page No.
1	Endocrine organs of human body	10
2	Hypothalmic-pituatry-gonadal axis showing the Feedback pathway that controls the level of steroids in blood	15
3	An overview of steroidogenic pathway showing the production of different intermediates	17
4	General structure of a steroid receptor showing different domains	21
5	Schematic illustration of the location, exon structure and protein domain structure of the AR gene	22
6	A schematic structural comparison of human ERα and ERβ	24
7	Schematic representation of the genomic action of a steroid in the target cell	25
8	The comparative representation of different agents involved in genomic and non-genomic action of steroids	26
9	Representative Structure of few common Phytoestrogens	53
10	The chamber used to analyze the effect of test sample induced changes in the courtship behavior in male rats	76
11	The representative formaldehyde gel showing the distinct ribosomal bands in the RNA samples extracted from the testis of control and treated rats	85
12	Assessment of purity of Leydig cells by 3β-HSD staining	96
13	Chemical structure of the representative test chemical, triclosan	102
14	Effect of TCS on the reproductive behavior of adult intact male rats. The reproductive behavior was started to be taken into account from day 31 of dosing as described in materials and methods	105
15	RT-PCR analysis of testicular mRNA expression of P450scc, 3β-HSD(I), 17β-HSD(III), P450C-17, AR, StAR and SRB-1 genes in rats treated daily with test chemical at a dose of 20 mg/kg/day	105
16	Effects of TCS on testicular level of 3β-HSD(I) and 17β-HSD(III) enzyme activity in vitro	108
17	Western blot analysis of testicular 3β-HSD enzyme (approximately 45 kD), StAR protein (approximately 30 kD) and AR protein (approximately 100 kD) in response to test chemical treatment	109
18	Immunolocalisation of StAR in Leydig cells from testis of control and rats treated daily with TCS at a dosage level of 20 mg/kg/day	110
19	RT-PCR analysis of ovarian mRNA expression of P450scc, 3β-HSD(I), 17β-HSD(III), P450C-17, StAR, aromatase, ER, and SRB-1 genes in rats treated daily with test chemical at a dose of 20 mg/kg/day	113
20	Effects of TCS on ovarian level of 3β-HSD and 17β-HSD enzyme activity in vitro	114
21	Decrease in the enzyme activity of the hypothalamic detoxifying	114

	enzymes in rats treated with 5, 10 and 20 mg/kg/day doses of TCS for 60 days	
22	Histopathological analysis of testis and daily sperm production/gm of testis weight (DSP/gm) in vehicle treated (control) and TCS treated (20 mg/kg/day) rats	117
23	Photomicrographs of rat cauda epididymes showing different degenerative histopathological malformations developed as a result of 60 days consecutive TCS treatment at a dosage level of 20 mg/kg/day	118
24	Effects of TCS treatment on the structure of vas deferens and ventral prostrate glands	119
25	Effects of TCS on the structure of seminal vesicle. Sections of vas deferens from control	120
26	TCS induced uterine histopathological malformations.	121
27	Toxic effect of TCS on the sperms, comparative analysis by the routine eosine staining	122
28	Chemical structure of the representative test chemicals used to gavage the animals in the study	130
29	The effects of sample drugs on the structure of some of the SATs of castrated rats treated with ibuprofen and tetracycline at doses of 40 and 500 mg/ kg of body weight respectively (simultaneously treated with TP).	133
30	Effect of test drugs on the reproductive behavior of adult intact male rats	134
31	RT-PCR analysis of testicular mRNA expression of P450scc, 3β-HSD(I), P450C-17, 17β-HSD, androgen receptor (AR) and StAR genes in rats treated with the two highest does of sample drugs	137
32	Effects of treatment of three different doses of ibuprofen	138
33	Western blot analysis of testicular 3β-HSD enzyme (approximately 45 kD), StAR protein (approximately 30 kD) and AR protein (approximately 100 kD) in response to test drugs treatment	139
34	The effect of sample drugs on the size of uterus of the ovarectomised rats treated with ibuprofen and tetracycline at doses of 40 and 500 mg/ kg of body weight respectively (simultaneously treated with estradiol), 10X. C, control; P.C., positive control (treated with E_2); I, ibuprofen; T, tetracycline	142
35	RT-PCR analysis of ovarian mRNA expression of cytochrome P450scc, 3β- HSD(I), 17β-HSD, cytochrome P450C-17, aromatase, StAR and ER genes in rats treated with the two highest does of sample drugs	143
36	Effect of treatment of three different doses of ibuprofen	144
37	The effect of leather industry effluents on the structure of some of the SATs of castrated rats	155
38	Daily sperm production/gm of testes weight (DSP/gm) at 13 weeks	156

	of age in vehicle and water sample (300 ml equivalent) treated rats	
39	RT-PCR analysis of testicular mRNA expression of cytochrome P450scc, 3β-HSD, 17β-HSD, cytochrome P450C-17 and AR genes in rats treated with 300 ml equivalent of leather industry effluents	157
40	Effects of leather industry effluents on testicular levels of 3β-HSD and 17β-HSD enzyme activities in vitro	160
41	Western blot analysis of testicular 3β-HSD enzyme and AR protein in response to treatment with 300 ml equivalent leather industry effluents. C, control; T, effluent treated	161
42	The effect of leather industry effluents on the size of uterus of ovarectomised rats	162
43	RT-PCR analysis of ovarian mRNA expression of cytochrome P450scc, 3β-HSD, 17β-HSD, P450C-17, aromatase and ER genes in rats treated with 300 ml equivalent of leather industry effluents	165
44	Effects of leather industry effluents on ovarian levels of 3β-HSD and 17β-HSD enzyme activity in vitro	165
45	Photomicrograph of testicular seminiferous tubule showing fusion of tubules indicating the symptoms of hyperplasia in the testes of the rats treated with 300 ml equivalent of leather industry effluent for 20 days	166
46	The effects of WWTP inlet and outlet water samples on the structure of some of the SATs. Adult male rats were treated with 200 ml equivalent of inlet and outlet water samples for 20 days	175
47	RT-PCR analysis of testicular mRNA expression of P450scc, 3β-HSD, P450C-17, 17β-HSD and AR genes in control and 200 ml equivalent of WWTP inlet and outlet water sample treated rats	176
48	Effects of WWTP water samples from inlet and outlet on testicular level of 3 β-HSD(I) and 17 β-HSD(III) enzyme activity in vitro	179
49	Western blot analysis of testicular 3β-HSDenzyme, and AR protein in response to treatment with 200 ml equivalent outlet water	180
50	Photomicrograph of liver sections. H&E. 40X. Blue arrows represent the dissolution of cytoplasm and appearance of purple colored granules. BV, Blood Vessel.	181
51	Photomicrograph of kidney sections of the rats treated with the 200 ml equivalent of WWTP inlet and outlet water. Asterisk represents appearance of vacuolar degeneration. BC, Bowman's capsule	182
52	Effects of WWTP inlet and outlet water samples (200 ml equivalent) on SGOT, SGPT, ALP and ACP levels in rats	183
53	Comparative analysis of high performance liquid chromatograms of inlet and outlet water samples of WWTP treatment plants	191
54	Representative GC-MS Chromatograms of final WWTP effluent sample	194
55	Representative GC-MS chromatograms of the extracted leather	195

industry effluent sample

56 RT-PCR analysis of testicular mRNA expression of P450scc, 3β-HSD, 17β-HSD, cytochrome P450C-17, StAR and AR genes in control and HCB (4.0 mg/kg/day) treated rats 202

57 Effects HCB treatment on testicular levels of 3β-HSD and 17β-HSD enzyme activity in vitro 203

58 Western blot analysis of testicular 3β-HSD enzyme, StAR, and AR protein in response to HCB treatment (4.0 mg/kg/day) 204

59 Dose dependent effect of HCB on Leydig cell testosterone production. Leydig cells were treated with different concentrations of HCB with/ without LH for 2 hrs and testosterone level was measured. * indicates significant level of differences as compared to only LH treated group ($p<0.05$). 207

60 RT-PCR analysis to demonstrate the changes in the mRNA expression of P450scc, 3β-HSD, 17β-HSD and StAR gene in Leydig cells treated with different concentration of HCB in the presence of 100 ng/ml of LH for 2 hrs. 208

61 Effects of HCB on activity of P450C-17 (A), 3β-HSD (B) and 17β-HSD (C) enzyme activity in vitro 209

62 Effects HCB treatment on testicular levels of 3β-HSD and 17β-HSD enzyme activity in vitro 210

63 Western blot analysis of testicular 3β-HSD enzyme, StAR, and AR protein in response to HCB treatment (4.0 mg/kg/day) 211

64 Dose dependent effects of HCB on Leydig cell cAMP production 212

65 Alteration in LH stimulated testosterone production in Leydig cell treated with adenylyl cyclase activator (forskolin) and adenylyl cyclase inhibitor (SQ22536), for 2 hrs with/without HCB at a concentration of 1.0 μM 213

66 Representative diagram showing the action of HCB at the adenylyl cyclase enzyme leading to a depressed synthesis of testosterone 218

67 Summary of the ultimate effects of test samples used in this thesis on the estrogen and androgen production 220

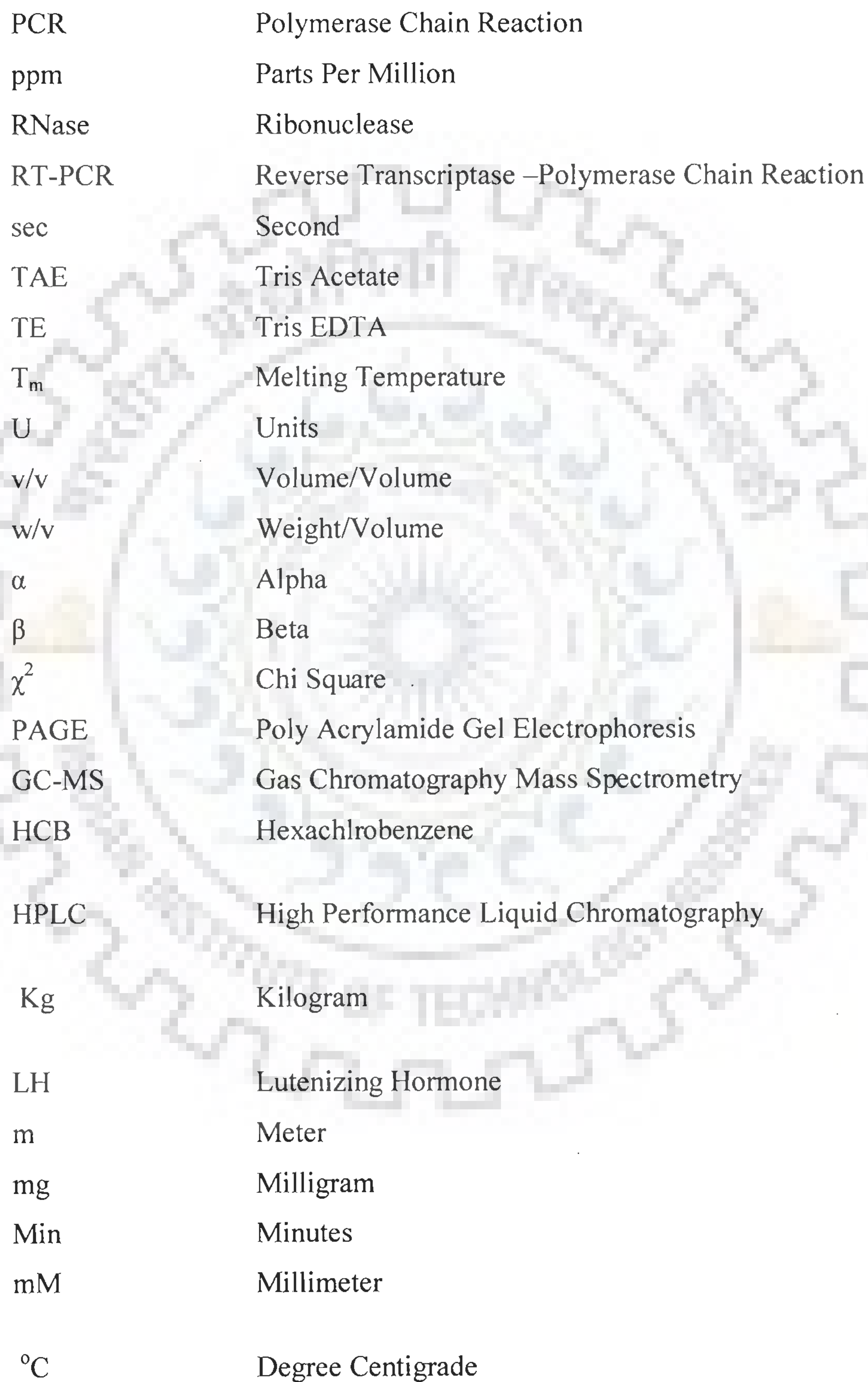
68 A hypothetical diagram (based on the results of this thesis) representing the various sites of the action of different test samples 225

LIST OF TABLES

Table No.	Table Title	Pg no.
1	Mode of actions for various EDCs	52
2	Primers used for semi-quantitative RT-PCR	88
3	Effects of three different dosage levels of TCS on the weights of testis and SATs from rats given 60 days treatment	104
4	Serum levels of LH, FSH, cholesterol, pregnenolone and testosterone from untreated (control) and TCS treated (20 mg/kg/day) male rats	107
5	Comparative uterine weight of the intact rats treated with the three different indicated doses of TCS for a period of 60 days	111
6	Serum levels of LH, FSH, cholesterol and estradiol from intact (female) control rats and rats treated with the indicated doses of TCS for a period of 60 days	112
7	Effects of three increasing doses of ibuprofen and tetracycline on the weights of SATs from castrated male rats given 14 days of consecutive treatments	132
8	Serum levels of LH, FSH and testosterone from intact rats given 14 days treatments with sample drugs	140
9	Comparative uterine weight of the rats treated with the test drugs at two higher dosage levels. Rats treated with only estradiol served as positive control	140
10	Serum levels of LH, FSH and estradiol from intact (female) control and rats treated with the indicated doses of ibuprofen and tetracycline for a period of 14 days	145
11	Effects of leather industry effluents on weights of SATs from castrated rats given 20 consecutive daily treatments	154
12	Effects of extracted leather industry effluents on the weights of testes and SATs from intact rats given 20 consecutive daily treatments	156

13	Dose dependent changes in serum levels of LH, FSH and testosterone from intact rats given 20 consecutive daily treatments of water sample of indicated dose	159
14	Comparative uterine weight of the ovarectomised rats treated with the three indicated concentrations of leather industry effluents	161
15	Comparative uterine weight of the intact rats treated with the three indicated concentrations of leather industry effluents	165
16	Serum levels of LH, FSH and estradiol from intact control and treated (300ml equivalent of leather industry effluents) rats	164
17	Effects of WWTP inlet and outlet water samples on weights of SATs from castrated rats given 20 consecutive daily treatments with WWTP water samples	174
18	Serum levels of LH, FSH and testosterone from intact rats given 20 consecutive daily treatments with water sample of indicated dose	178
19	A comparative analysis of peak area from WWTP inlet and outlet samples showing the reduced level of contamination of the five common peaks.	190
20	Concentration (µg/l) of 4-nonylphenol, Hexacholrobenzene, Dehydroepiandrosterone and isoandrostenedione in 200 ml equivalent outlet water sample determined by the selected ion monitoring mode (SIM) GC-MS analysis	192
21	Concentration (µg/l) of 4-nonylphenol, hexacholrobenzene, benzidine and 4-aminobiphenyl in 300 ml equivalent leather industry effluent sample determined by the selected ion monitoring mode (SIM) GC-MS analysis	193
22	Effects of HCB on weights of SATs from castrated rats given 20 consecutive daily treatments	200
23	Effects of HCB on the weights of testis and SATs from intact rats given 20 consecutive daily treatments	200
24	Dose dependent changes in serum levels of LH, FSH and testosterone from intact rats given 20 consecutive daily treatments of HCB of indicated dose	205

ABBREVIATIONS USED



PCR	Polymerase Chain Reaction
ppm	Parts Per Million
RNase	Ribonuclease
RT-PCR	Reverse Transcriptase –Polymerase Chain Reaction
sec	Second
TAE	Tris Acetate
TE	Tris EDTA
T_m	Melting Temperature
U	Units
v/v	Volume/Volume
w/v	Weight/Volume
α	Alpha
β	Beta
χ^2	Chi Square
PAGE	Poly Acrylamide Gel Electrophoresis
GC-MS	Gas Chromatography Mass Spectrometry
HCB	Hexachlrobenzene
HPLC	High Performance Liquid Chromatography
Kg	Kilogram
LH	Lutenizing Hormone
m	Meter
mg	Milligram
Min	Minutes
mM	Millimeter
^oC	Degree Centigrade

μ	Micron
μg	Microgram
μm	Micrometer
bp	Base Pair
cDNA	Complimentary DNA
kD	Kilo Dalton
DEPC	Diethyl Pyrocarbonate
DNase	Deoxyribonuclease
EDTA	Ethylene Diamine Tetraacetic Acid
Fig.	Figure
g	Gram
hr	Hour
l	Litre
M	Molarity
mg	Milligram
min	Minute
ml	Millilitre
mm	millimetre
mM	millimole
mRNA	Messenger Ribonucleic acid
NCBI	National Center for Biotechnology Information
ng	Nanogram
°C	Degree Centigrade
PAGE	Poly-acrylamide Gel Electrophoresis
3 β-hsd	3 beta Hydroxysteroid Dehydrogenase
RT-PCR	Reverese Trnscription PCR
SDS	Sodium Dodecyl Sulphate
STAR	Steroidogenic acute regulatory Protein
17 β-HSD	17 beta Hydroxysteroid Dehydrogenase
P450scc	P450 side Chain Clevage Enzymes
P450C-17	P450 C-17 Enzymes

PCR	Polymerase Chain Reaction
AR	Androgen Receptor
ER	Estrogen Receptor
cDNA	Complementary DNA
FSH	Follicle Stimulating Hormone
EDC	Endocrine Disrupting Chemical
TCS	Triclosan
cAMP	Cyclic AMP

CHAPTER - 1

INTRODUCTION

Chapter 1: Introduction

1.1 General Introduction

Endocrine system and nervous system are two major modes of communication that coordinate and control different body functions. While the dynamics of nervous system is maintained by conduction of electrical impulses through the complex circuits, the messengers of the endocrine system are hormones that are synthesized and excreted at very low quantities from specialized glands and transported to the target organ(s) via the bloodstream. A tuned functioning of endocrine orchestra is necessary for sustained maintenance of different pivotal functions in human or animal body like reproduction and development, growth and maturation, energy production, electrolyte balance etc. However, increasing scientific evidences depict the existence of a newly defined category of environmental contaminants which may have diverse chemical structures and can alter the normal functioning of the endocrine and reproductive systems (Cargouët et al., 2004). These chemicals mimic/ inhibit the actions of endogenous hormones or modulate the synthesis of latter and have been named as 'endocrine disrupting chemicals' (EDC) (Sonnennschein and Soto, 1998). In this way EDC may interfere with usual hormonally regulated biological processes and thus, may adversely affect the development and reproductive function in wildlife, experimental animals, and humans (Satoh et al., 2001). In general EDC have been defined as-

"An exogenous agent that interferes with the synthesis, storage/release, transport, metabolism, binding, action or elimination of natural blood-borne hormones responsible for the regulation of homeostasis and of developmental processes".

Depending on the mode of action, EDC can be broadly categorized as androgenic or anti-androgenic chemicals, causing disturbances in production of androgens or estrogenic/ anti-estrogenic chemicals, when they affect the production of estrogens. EDC may enter into the body of animals/humans through the diet, contaminated water or occupational exposure and may lead to the generation of an agonistic or antagonistic effect (Kumar et al., 2008a, Kumar et al., 2008b). Once inside the physiological system the EDC may exert their effect by acting at one or more of the following steps: (i) through arylhydrocarbon receptor (AhR) (Indarto and Izawa, 2001) (ii) direct binding of these chemicals to steroid receptors, steroidogenic enzymes and proteins associated with steroidogenesis (like StAR protein) (Walsh *et al.*, 2000, Sanderson and Vanden Berg, 2003; Rice *et al.*, 2006), and (iii) increasing the stability of transcripts and transcriptional rate of the promoter of steroidogenic enzymes (Lin *et al.*, 2006; Lyssimachou *et al.*, 2006).

An increasing body of evidences reveals association between various therapeutic/environmental compounds that act as EDC and many sex hormone-sensitive disease/disorders (Colborn and Clement, 1992; Satoh et al., 2001; Sone et al., 2005; Guillette, 2006; Massart et al., 2006; Chen et al., 2007). A probable link have been proposed between exposure to EDC and production of a number of diseases like reduced fecundity, abnormal fetal development, delayed onset of puberty, cryptorchadism, abnormal lactation, testicular dysfunction and even various types of cancers (Sharpe and Irvine, 2004; Roy *et al.*, 2005; Buck et al., 2006; Darbre, 2006; Guillette, 2006; Maffini *et al.*, 2006). In the past decade, the utilization of many chemicals (including pesticides and persistent organic pollutants) has declined worldwide diminishing the chances of

human/ animal exposure to them. However, the chemicals having endocrine disrupting (ED) potential are being used directly or indirectly for diverse purposes in different sectors of life ranging from the simple household activities like detergents, cosmetics and toilet articles to specialized applications viz. pharmaceuticals, insecticides and pesticides. Many of these chemicals are being released into the environment from various sources (household activities, industrial manufacturing, agricultural applications etc) without prior evaluation of their endocrine activity at the molecular levels. Once these chemicals reach the environment they may enter into the food chain and finally reaching the animal/human systems (Roy et al., 2005). Although the carcinogenic potential of these compounds are evaluated by routine mutagenicity testing or simple biophysical tests but the concentrations necessary to disrupt endocrine regulation may be lower than required to act as a carcinogen. Life long intake of even very low levels of these compounds may disturb the delicate hormone balance and compromise the reproductive fitness and health of many species (Ralph *et al.*, 2003). All this has led to the widespread occurrence of EDC in food chain and different strata of environment in many forms like persistent organic pollutants (POPs) such as the insecticide dichlorodiphenyl-trichloroethane (DDT) and its metabolites, the industrial by-product dioxins, the industrial compounds polychlorinated biphenyls (PCB), several agrochemicals, pesticides and biocides (e.g. chlorinated insecticides, organotins, imidazoles, triazoles, etc.) and other industrial compounds (several phenol compounds such as bisphenol A) (Mantovani et al., 1999). According to one of the enviornmetal scientist (Trivedi, 2007)-

"Today and every day, you can expect to be exposed to some 75,000 artificial chemicals. All day long you will be breathing them in, absorbing them through your

skin and swallowing them in your food. Throughout the night they will seep out of carpets, pillows and curtains, and drift into your lungs. Living in this chemical soup is an inescapable side effect of 21st-century living. The question is: is it doing us any harm?"

Once used in various household activities, different types of chemicals or their byproducts are discharged finally to sewage water making it a complicated broth of chemicals having diverse category of chemicals which may have different effects on biological organization including endocrine system (Darbre, 2006; Heidler et al., 2006; Sarmah et al., 2006). Thus sewage water receives of a number of chemicals which may act as potent EDC, however, worst part is that when sewage water is canalized into the WWTP treatment plants (WWTP) for removal of harmful contaminants, the WWTP outlet still contains a number of potential EDC in spite of the rigorous treatment process (Kumar et al., 2008a, 2008b). From the treated or untreated sewage water, EDC are finally discharged into open water channels (canals, rivers etc) which may be very harmful for reproductive and general health (Kumar et al., 2008a). Majority of the effects observed in the aquatic environment concerning the reproductive system, for instance, the feminization of male fish with sewage treatment plant effluents, are attributed to the presence of EDC (Sumpter and Jobling, 1995; Sumpter, 1998; Ternes et al., 1999). It has been hypothesized that the statistically derived decrease in sperm counts over the last decades, increasing incidents of testicular cancer and other disorders regarding male infertility may be caused by the intake of these chemicals via food or drinking water (Sharpe and Skakkebaek, 1993). Industries are another source of EDC in the environment especially polluting the water channels (canals, rivers) through their harmful effluents.

Waste water effluent of leather industry is of great concern to agencies responsible for environmental management. Some of the authorities consider it to be one of the ten most harmful industrial effluents to the environment, responsible for extreme pollution of water resources and generating substances that may lead to several health complications and even death of wide range of organisms (Aragon, 1990; Junior et al., 2007).

Besides, substances used as preservatives in cosmetics or beverages, drugs, anabolic agents and phytoestrogens (e.g. isoflavones, lignans; present in some food items such as soy and in cosmetics with active ingredients of vegetal origin) may also act as potent EDC. There are several reports which have included to some of the commonly used NSAID and antibiotics in the list of probable EDC (Ingerslev et al., 2003; Hontela, 2006). Several personal care products and cosmetics have been demonstrated to be preoccupied with chemicals that may exert endocrine disrupting effect (Daughton et al, 1999). Triclosan, a very common antimicrobial used widely in cosmetics, toothpaste etc., has been demonstrated to be an EDC (Foran et al., 2000).

Further, EDC can also work in synergistic or combinational manner i.e. some chemicals may be present in too low concentration to elicit any ED response alone but when present with other chemicals (e.g. estrogens vs. anti-androgens or androgens vs. anti-estrogens) they may behave as potent EDC (Birkhoj et al., 2004; Kortenkamp, 2007).

1.2 Aims and Objectives

The present work endeavored to adopt a holistic approach for accomplishing both biological and chemical characterization of EDC from diverse potent sources accessing their impact on both male and female reproductive systems. In addition through this study attempt was also made to analyze the toxicological effects exerted by the test chemicals

in the exposed physiological system. Investigations were carried out to scrutinize the EDC from few of the sources that suspected to act as potential EDC based on the information available in the literature - antimicrobial chemicals (Triclosan), non-steroidal anti-inflammatory drugs (ibuprofen), antibiotics (tetracycline), a common site receiving effluents form many leather industries and outlet and inlet water streams of a waste water treatment plant (WWTP). First, different dilutions of antimicrobial chemical (Triclosan), NSAID (ibuprofen) and antibiotic (tetracycline) samples and non-polar extracts from WWTP inlet/ outlet streams and leather industry effluents samples were screened for their endocrine disrupting potential by using a combination of Hershberger assay and Utreotrophic assys. Once the foresaid assay systems confirmed that samples under consideration are behaving as EDC, exact mode of their action was assessed by investigating *modus operandi* of these chemicals in intact male and female rats. Following the *in vivo* characterization, crude non-polar extracts of WWTP water and leather water were subjected to physicochemical characterization using a combination of HPLC and GC-MS analysis to identify the offending molecules responsible for their ED nature. In the concluding part of the thesis, approaches were devoted to elucidate the detailed downstream molecular mechanism of endocrine disruption of one of the offending molecules, hexachlorobenzene (HCB), demonstrated by physicochemical characterization to be present in both WWTP inlet and outlet and leather industry effluents samples, using a combination of *in vivo* and *in vitro* studies. Overall, this study attempted to achieve the following objectives -

1. To extract the non-polar contaminants from the test samples by solvent extraction and solid phase extraction.

2. To screen the samples for their (anti) androgenic or (anti) estrogenic nature by carrying out Hershberger assay and Uterotrophic assay respectively.
3. To investigate the molecular mechanism of the endocrine disrupting effect of test samples by performing *in-vivo* study on male and female rats. For achieving this task following parameters were taken into account-

3.1 Assays of the enzymes involved in steroidogenesis for measuring the test sample induced changes in the enzyme activity.

3.2 Western blot and immunohistochemical (IHC) analysis to measure test sample induced alterations in the level of proteins involved in steroidogenesis.

3.3 Enzyme linked immunosorbent assay (ELISA) to measure the changes in the serum levels of gonadotrophins (LH and FSH) and steroids (testsosterone, pregnenolone etc).

3.4 Histopathological analysis to examine the malformations in the liver, kidney, testis, uterus and SATs, caused by the test samples induced hormonal balance or toxicity.

4. HPLC and GC-MS analysis of the WWTP water and leather effluent samples for the identification and quantification of the offending molecules accountable for the endocrine disrupting nature of these samples.
5. To evaluate the overall effect of the foresaid parameters on reproductive status of animals through courtship behavior and sperm count analysis.
6. To elucidate the detailed molecular mechanism of endocrine disrupting action of one of the molecules identified in both WWTP and leather industry effluents by using a combination of *in vivo* and *in vitro* studies.

7. Last but not the least to assay the enzymes involved in the detoxification system for assessing the toxic effects of test samples.

CHAPTER - 2

REVIEW LITREARTURE

Chapter 2: REVIEW LITERATURE

2.1 The Endocrine System

In vertebrates various key physiological functions are regulated by a tuned performance of the endocrine system in a stringent systematic manner much like an orchestra. In general, endocrine system can be defined as a highly organized system composed of different types of ductless glands, called endocrine glands, which synthesize and release the specific chemical messengers (hormones) into the body fluids from where these messengers are transported to the distal part of body to exert their specific physiological effects (Hadley, 2000). The primary function of endocrine system is to respond to various exogenous or endogenous stimuli by secreting one or more specific hormones which initiate specific physiological events at target sites causing the expression of the appropriate gene/s and finally the synthesis of proteins or the activation of already existing tissue-specific enzyme systems. Figure 1 demonstrates the general localization of various types of endocrine glands present in the body. The endocrine system can be designated to be composed of the following components-

- Specific type of ductless glands, designated as endocrine glands, located throughout the body.
- Hormones (chemical messengers) that are synthesized in the glands and released into the bloodstream or the fluid of surrounding cells.
- Receptors in cells of target organs and tissues that recognize their respective hormones and recruits different downstream agents to perform various biochemical reactions so that hormonal signal may be manifested into specific biological functions.

Based on the objectives of the present thesis major emphasis has been given to reproductive endocrinology in this review literature along with endocrine disruptors.

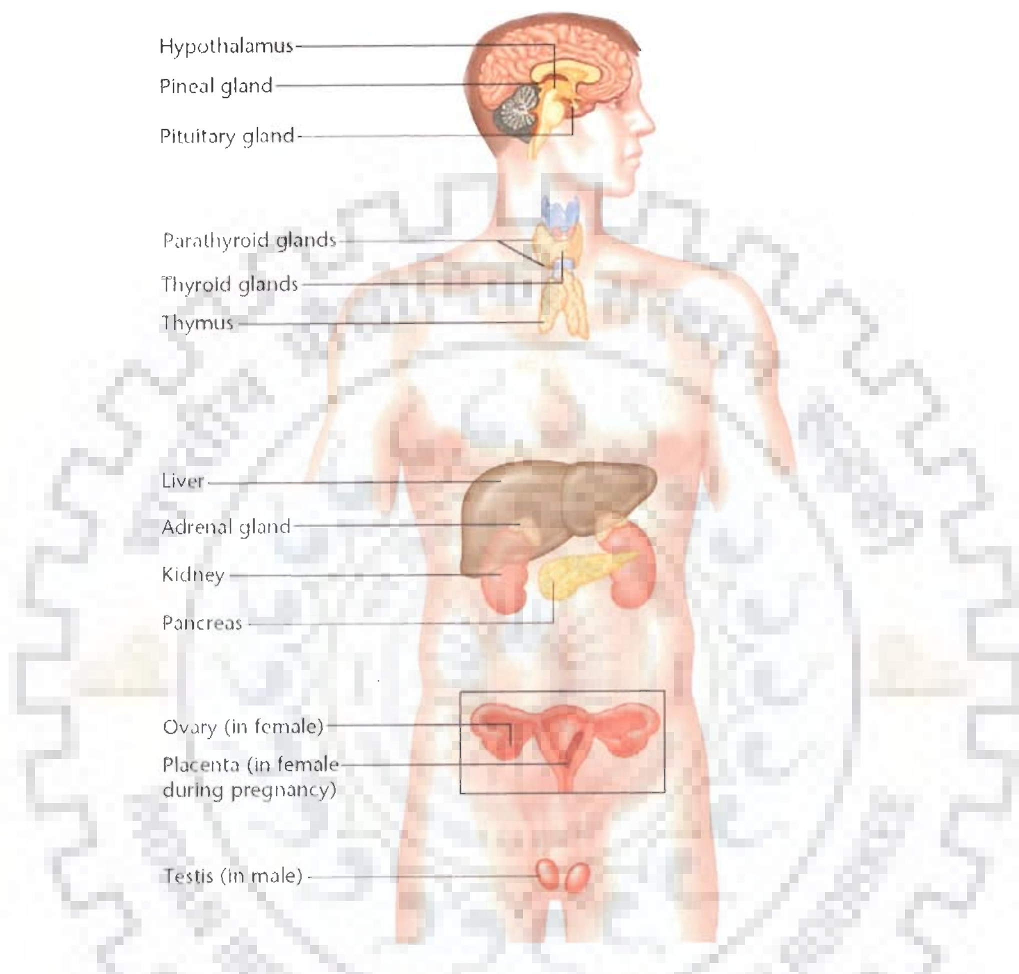


Figure 1. Endocrine organs of human body

2.1.1 Reproductive endocrine system

2.1.1.1 Introduction

Reproduction is an essential process of life since it involves the formation of new organisms of the same type by the parents either by sexual or asexual means. The asexual mode of reproduction is accomplished by mitosis or budding (for example Hydra, jelly fishes and corals etc.) or by development of new organisms from the broken part of the

parent body like Planaria, star fishes etc. (Parker and Haswell, 1995). However, when we discuss about the sexual reproduction, we find it highly complex process consisting of various steps starting from the production of specialized cells, designated generally as male and female gametes and culminating with the union of latter. In case of higher vertebrates, process of sexual reproduction involves the origin of gametes, i.e. sperm and ovum, from gonads by reduction division or meiosis followed by the union of opposite partners for subsequent fusion of sperm and ovum which results in the formation of the zygote (Parker and Haswell, 1995). This zygote ultimately develops into the offspring similar in phenotype and genotype to the parents. These various events are coordinated by a tuned performance of reproductive endocrine system of body. In case of higher mammals the reproductive endocrine system consists of an organized interplay of various hormones secreted form various sites viz. hypothalamus, pituitary gland, gonads (testis and ovary) and placenta. The interrelation among the hormones secreted form the foresaid sites is so much well-knit that it is designated as to constitute an axis, designated as hypothalmic-pitutarty-gonadal axis, described in the latter section.

2.1.1.2 Hormones involved in reproduction

In mammals, reproductive endocrine system chiefly consists of testis in male and ovary and placenta in female, which secretes their respective hormones under the active stimulation of gonadotropins released by the pituitary gland (Klinefelter et al., 1987).

2.1.1.2.1 Gonadotrophic hormones or gonadotropins

The follicle-stimulating hormone (FSH), luteinizing hormone (LH) or interstitial cell stimulating hormone (ICSH) and prolactin or luteotropin are collectively called gonadotropins because they stimulates various activities of gonads; the testis in males and

the ovaries in females and also control the reproductive cycles. These are secreted by the pituitary gland or hypophysis which is situated in the hypophyseal fossa (sella turcica) of the spheroid bone at the base of the brain and has two distinct parts, the anterior pituitary gland or adenohypophysis and posterior pituitary parts or neurohypophysis. Gonadortropins are derived from the anterior lobe of pituitary gland.

2.1.1.2.1.1 Follicle stimulating hormone (FSH)

It is a water soluble glycoprotein whose molecular weight ranges from 30 to 67 kD. This acts through G-protein coupled receptors present in the testis and ovaries. In females, FSH initiates the growth of follicles in the ovary. This further regulates the development and maturation of ovarian follicles till its ovulation. The FSH receptors are solely expressed in granulosa cells of ovaries. It also induces the ovaries to secrete estrogen, a major female sex hormone from the ovaries. In the male, FSH stimulates the germinal epithelium in the testis to promote the development of sperm and also regulates the maturation of sperms.

2.1.1.2.1.2 Luteinizing Hormone

The molecular weight of LH (glycoprotein) ranges from 26 to 30 kD and like that of FSH, it also acts through G-protein coupled receptors present in the testis and ovaries. It stimulates secretion of sex steroids from the gonads in both the sexes. In the testis, LH binds to receptors on Leydig cells, stimulating synthesis and secretion of testosterone and is designated as interstitial cell stimulating hormone (ICSH) while in female it helps in the maturation of ovarian follicles and is required for continues development and function of corpora lutea.

2.1.1.2.1.3 Prolactin or Luteotropin Hormone (LTH)

It has diverse functions out of which most important is its role in the development of mammary glands in female in conjunction with the estrogen and progesterone and has a role in the secretion of progesterone by the corpus luteum of ovary.

2.1.1.2.2 Steroid hormones

All the steroids hormones are derived from a single precursor cholesterol, using a complicated enzymatic cascade and all of them bearing a cyclopentanohydrophenanthrene ring (Nelson and Cox, 2005; Chatterjee, 1994). The major sites of synthesis of the sex steroids are corpus luteum for progestagens, testis for androgens, and ovaries for estrogens. Five major types of steroids are derived from the cholesterol which can be classified as follows, depending on the number of carbon atoms–

(1) C21: derivatives of pregnane, so-called progestagens, glucocorticoids, and mineralo-corticoids

(2) C19: derivatives of androstane, so-called androgens

(3) C18: derivatives of estrone, so-called estrogens

2.1.1.2.2.1 Female sex steroids (estrogen and progesterone)

Estrogens (estradiol, estrone, estriol, equilin, equilenine) have been defined as compounds which can produce estrous in ovarectomised females. They play many important tasks in female system in conjunction with other hormone progestagens. e.g. development of female secondary sex characteristics and regulation of reproduction; helps in maintaining pregnancy and prepare the breasts for lactation, regulate the changes that occur during menstruation and influences the development of fetal membranes and mammary glands during pregnancy.

2.1.1.2.2.2 Male sex steroids (androgen)

Androgens are steroids having the masculinising properties. The principal androgens or male hormones are testosterone and dihydrotestosterone and the latter is more potent then former. They promote the development and differentiation of the reproductive organs before and after birth, determine secondary male sex characteristics and are required for sperm production.

2.1.1.2.3 Hypothalmic-Pitutarty-Gonadal Axis

The vertebrate forebrain has two major divisions – cerebellum and diencephalons. The latter consists of dorsal thalamus and ventral hypothalamus. Besides, controlling several important physiological functions like maintaining body temperature, water balance, feeding etc. hypothalamus regulates reproductive behavior by releasing a series of major neurosecretory substances (neurohormones) that have regulatory influence on the endocrine function of the pituitary gland. One of the major hypothalamic hormone related to the reproductive process is gonadotropin releasing hormone (GnRH) (Sridaran et al., 1999; Sengupta et al., 2008). It is a decapeptide and acts through the membrane receptor that is present in the gonadotropic cells of the anterior lobe of the pituitary gland and gonads and stimulates the secretion of a number of reproductive hormones (gonadotropins) from the anterior pituitary gland (Chakrabarti et al., 2008). Gonadotropins in turn simulates the secretion of sex steroids form the gonads. When the concentration of a sex hormone (e.g. testosterone or estradiol) exceeds to a certain limit in the blood, the secretion of GnRH form the hypothalamus is retarded and thus that of gonadotropins from the pituitary. This finally slows down the production of steroids in the gonads. On other hand, when level of steroids declines to that of a certain threshold

level in the blood, the above depicted pathway flows in the opposite direction leading to the production of more steroids from the gonads. This complicated arrangement maintains an appropriate level of sex steroids in the blood and known as Hypothalmic-Pitutarty-Gonadal Axis (Fig 2). This leads to the pulsatile secretion of LH and to a much lesser extent FSH. Numerous hormones influence GnRH secretion, and positive and

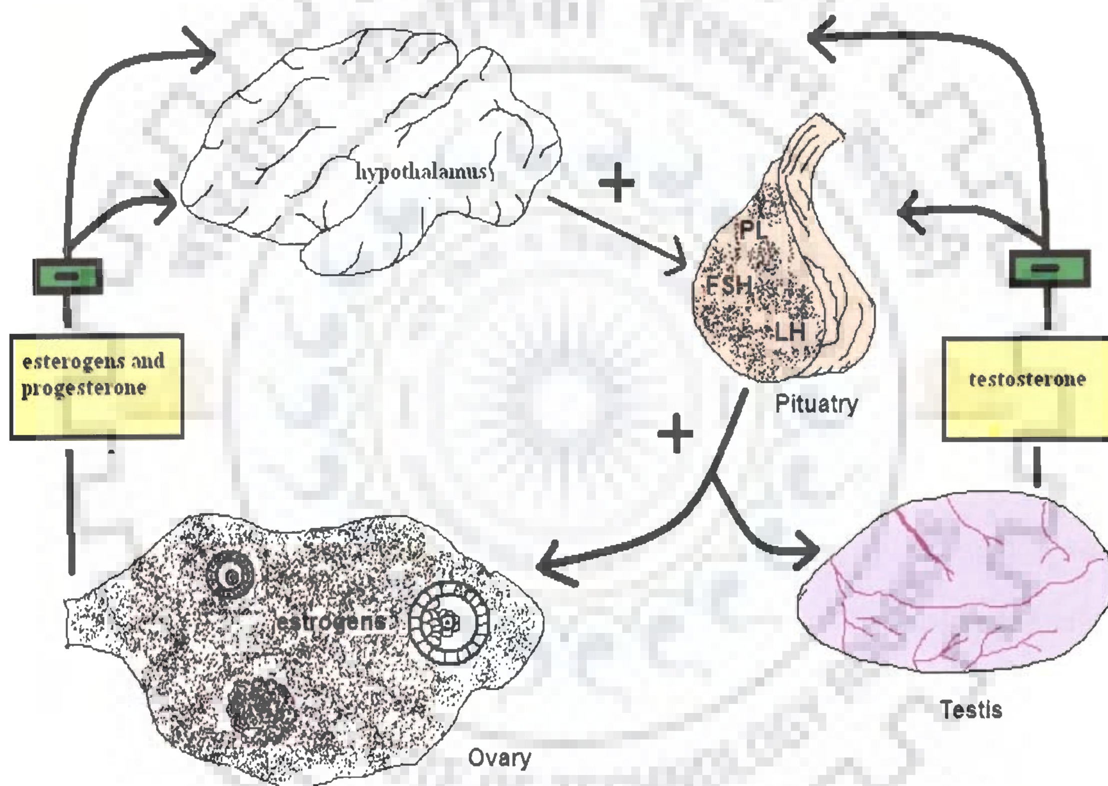


Figure 2. Hypothalmic-pituatry-gonadal axis showing the Feedback pathway that controls the level of steroids in blood.

negative control over GnRH and gonadotropin secretion is actually considerably more complex than depicted in the Fig. 2. For example, the gonads secrete at least two additional hormones; inhibin and activin which selectively inhibit and activate FSH secretion from the pituitary.

2.1.2 Dynamics of Steroidogenesis

Steroids are produced in the active steroidogenic cells, under the active stimulation of gonadotropins, through a complicated cascade which starts with the side chain cleavage of cholesterol, the parent molecule for all the steroid hormones (Eacker et al., 2008). Steroidogenic cells acquire cholesterol either from *de novo* synthesis or from the lipoprotein, both high density lipoprotein (HDL) and low density lipoprotein (LDL) circulating in the blood. But it has been shown that active steroidogenic glands preferentially use cholesterol for steroidogenesis from lipoproteins (Andersen and Dietschy, 1978; Liscum and Dhal, 1992; Reavan et al, 1995; Brown et al., 1997; Cao et al., 1999). HDL and LDL are recognized on the surface of steroidogenic cells by a number of receptors (LDL and SRB-1 etc.) and then transport cholesterol esters to these cells where subsequent steps of steroidogenesis takes place (Barlow et al., 2003). Fig. 3 displays a brief schematic presentation of the entire steroid biosynthesis pathway showing the role of different steroidogenic enzymes and production of different intermediates. This pathway is based primarily on the pioneer work of Kenneth. J. Ryan and his co-workers (Ryan 1959; Ryan and Smith, 1965) and represents to a fundamental pattern displayed by all steroid hormone producing organs. Generally, steroidogenic enzymes are either dehydrogenases or members of the cytochrome P450 group of oxidases. Mostly during steroidogenesis, the number of carbon atoms in cholesterol or any other steroid molecule can be reduced but never increased. Steroidogenesis requires the contribution of the following reactions-

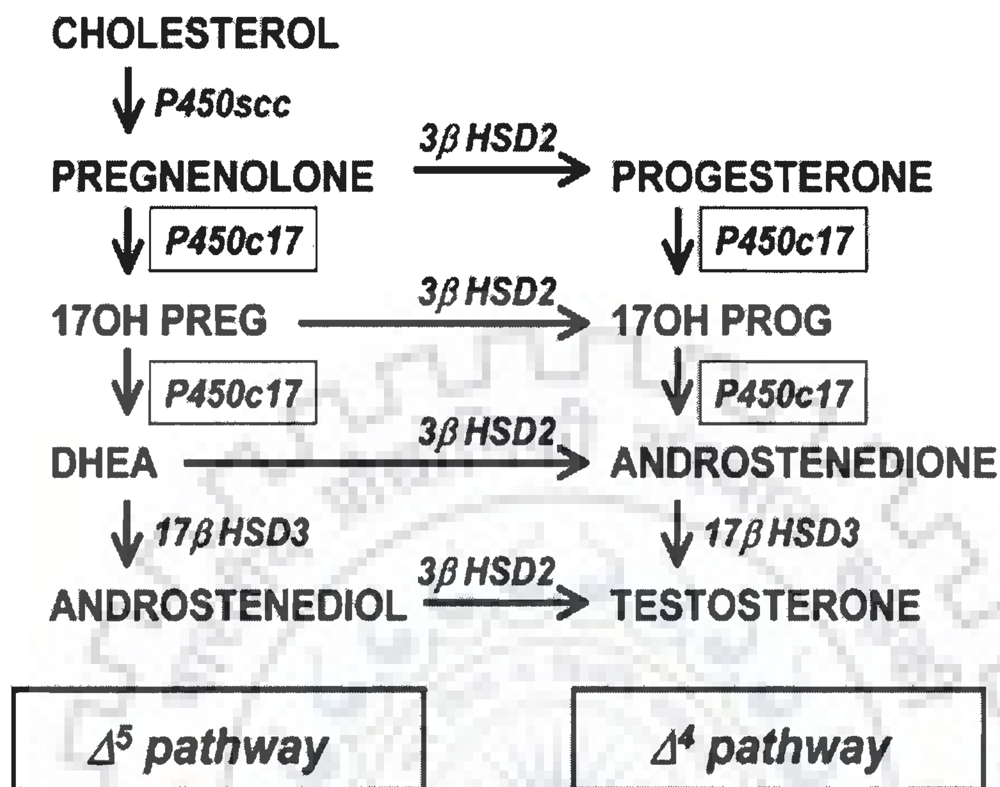


Figure 3. An overview of steroidogenic pathway showing the production of different intermediates.

1. Cleavage of side chain (desmolase reaction).
2. Conversion of hydroxyl groups into ketones or ketones into hydroxyl groups (dehydrogenase reaction).
3. Addition of OH group (hydroxylation reaction).
4. Creation of double bonds (removal of hydrogen).
5. Addition of hydrogen to reduce double bonds (saturation).

Briefly, the various events of steroidogenesis can be depicted as follows-

2.1.2.1 The conversion of cholesterol to pregnenolone

The conversion of cholesterol to pregnenolone takes place within the inner mitochondrial membrane. The transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane is the rate-limiting step of steroidogenesis and several

proteins have been characterized and proposed as regulators of acute intracellular cholesterol transfer. Sterol carrier protein 2 (SCP2), steroidogenesis activator polypeptide (SAP), and peripheral benzodiazepine receptor (PBR), are the factors which play a role in the transfer of cholesterol to inner mitochondrial membrane (Papadopoulos et al., 1999; Zhou et al., 2004; Hauet, 2005; Martin et al., 2008). Out of all these, the most important factor responsible for acute cholesterol transfer is steroidogenic acute regulator (StAR) protein (Clark et al, 1994, 1995; Hesgawa et al., 2000; Manna et. al., 2001). StAR protein is regarded as pacemaker of the steroidogenesis and the level and activity of StAR protein mainly depends on LH hormone (Arakane et al. 1997; Stocco 2000, 2001). In a number of studies decreased expression of StAR has been shown to severely depress the steroidogenesis (Arukwe, 2005; Clark and Cochrum, 2007; Murugesan et al., 2007). Following the transfer of cholesterol to the inner mitochondrial membrane, it is acted upon by the enzyme P450scc to produce pregnenolone. The level and activity of P450scc is also maintained by gonadotropins (Miller, 1988; Omura and Morohashi, 1995; Fauser, 1996).

2.1.2.2 Stepwise conversion of pregnenolone to testosterone: Δ^4-3-ketone pathway

Once pregnenolone is formed, further steroid synthesis in the ovary or testis can proceed by one of two pathways, either via Δ^5 -3β-hydroxysteroids or via the Δ^4-3-ketone pathway. The former proceeds by the production of pregnenolone and dehydroepiandrosterone (DHEA) and the latter via progesterone and androstenedione.

In Δ^4-3-ketone pathway, the conversion of pregnenolone to progesterone involves two steps; the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzymes and 4-5 isomerase

reactions that leads to the formation of a ketone product, progesterone (Berube et al., 1989; Lorence et al., 1990; Rheaume 1991 and 1992; Chang et al., 1993). Once the progesterone is formed, it is hydroxylated at the 17 position to form 17α-hydroxyprogesterone which further is converted to androstenedione, both the reactions takes place in the presence of a single enzyme, P450C-17. Androstenedione may be further reduced to testosterone by the action of 17β-hydroxysteroid dehydrogenase enzyme (17 β-HSD) (Langer, 1957; Talalay, 1957). The activity of 17- β-HSD can be modulated by a number of agents other than animal steroids (Krazeisen, 2001).

2.1.2.3 Stepwise conversion of pregnenolone to testosterone: Δ5-3β-hydroxy Pathway

As an alternative to Δ^4-3-ketone pathway, pregnenolone may be subjected to Δ^5-3β hydroxysteroid pathway. It is first converted to 17α-Pregnenolone and then to dehydroepiandrosterone (DHEA), again both the reactions performed by a single enzyme, P450C-17. DHEA is further metabolized into androstenediol by 17β-hydroxysteroid dehydrogenase enzyme (17β-HSD) (Langer, 1957; Talalay, 1957). It is thought that conversion of each of the Δ^5 compounds to their corresponding Δ^4 compounds can occur at any step, however, the principal pathways are via progesterone and DHEA.

2.1.2.4 Aromatization

Both C-19 steroids (androstenedione and testosterone) are rapidly converted to corresponding C-18 phenolic steroids, estrogens (estrone and estradiol), by a microsomal reaction known as aromatization (Kellis and Vickery, 1987; Simpson et al., 1989 and 1994; Naftolin, 1994). The reaction is accomplished by the elimination of the C-19 angular methyl group of the substrate as formic acid. Aromatization is mediated by cytochrome P450aromatase (P450arom) found in the endoplasmic reticulum (Simpson et

al., 1994). A variety of hormones stimulate P450arom mRNA accumulation in granulosa cells including gondadotropins (FSH), insulkin like growth factor-1, cAMP analogs, phorbol esters and glucocorticoids (Fauser et al., 1999).

2.1.2.5 17β-hydroxysteroid dehydrogenation

The 17β-hydroxysteroid dehydrogenase is bound to the endoplasmic reticulum and converts estrone to estradiol, androstenedione to testosterone, and DHEA to androstenediol and vice-versa. The 17β-HSD reaction is due to non P450 enzymes (Talalay, 1957, 1958; Langer, 1957, 1958; Talaly et al., 1958; Levy and Talaly, 1959). The metabolic processes by this enzyme are mediated by four known 17β-HSDs designated types I-IV, named according to chronological order in which their respective cDNAs were cloned (Fauser et al., 1999).

2.1.3 Steroid hormone receptors

The receptors for steroid hormones are found in the cytoplasm or nucleus of eukaryotic cells and regulate the transcription of DNA under the ordinance of steroid hormones. The steroid receptors can be classified into three functionally distinct subfamilies: Type I receptors- classical steroid hormone receptors which are represented by the glucocorticoid receptors (GR), androgen receptors (AR), mineralcorticoid receptors (MR), and progesterone receptor (PR); Type II receptors- represented by thyroid hormone related receptors that includes T3R, RAR, RXR, and VDR; and Type III receptors - represented by the estrogen receptor (ER) and a few orphan receptors.

Generally, Steroid receptors share regions of close structural and/or functional homology that are termed as domains. A general steroid receptor will have 4 distinct

domains: N-terminal region (A/B domain), DNA binding zinc finger region (DBD, C domain), hinge region (D domain) and C-terminal ligand binding domain (LBD, E/F domain) (Fig. 4). These domains are conserved in all superfamily members. The steroid-receptor complex binds to the specific sites on DNA which are designated as hormone response elements (HREs). These response elements bind two receptors at a time forming a dimer which can be either homodimer (same receptor proteins), or heterodimer (different receptor proteins).

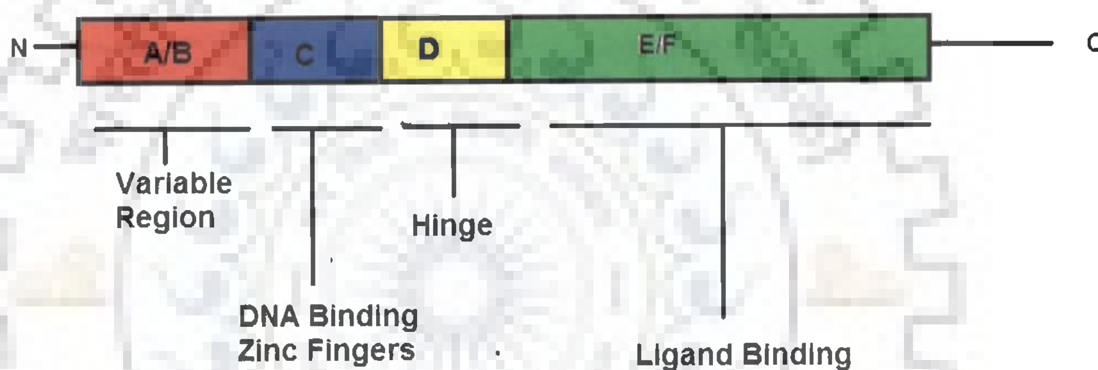


Figure 4. General structure of a steroid receptor showing different domains.

Type I (e.g. AR) and estrogen (type III) receptors remain coupled to heat shock proteins (HSPs) in the absence of hormone but on approaching the appropriate ligand (hormone) they get disillusioned from HSPs, homodimerize and bind to their DNA response elements. In contrast, type II receptors (thyroid/retinoic acid receptors) do not associate with HSPs and can bind to the DNA even in the absence of hormone (ligand) either as homo or heterodimers.

2.1.3.1 Androgen Receptor (AR)

The androgen receptor is a polypeptide product of around 910-919 amino acids, encoded by the AR gene, which is located in Xq11-12 (Fig. 5) (Yuan-Shan Zhu, 2005). The AR

gene is a single-copy gene that braces about 90 kilobases of genomic DNA within the long arm of the X chromosome and its encoding region consists of around 8 exons and 7 introns. As foresaid, the AR can be divided into distinct domains: A/B domain, C domain (DBD), D domain, and E/F domain (LBD). The NH_2 domain comprises nearly half of the AR molecule and is encoded by exon 1. It is involved in the transcriptional activation

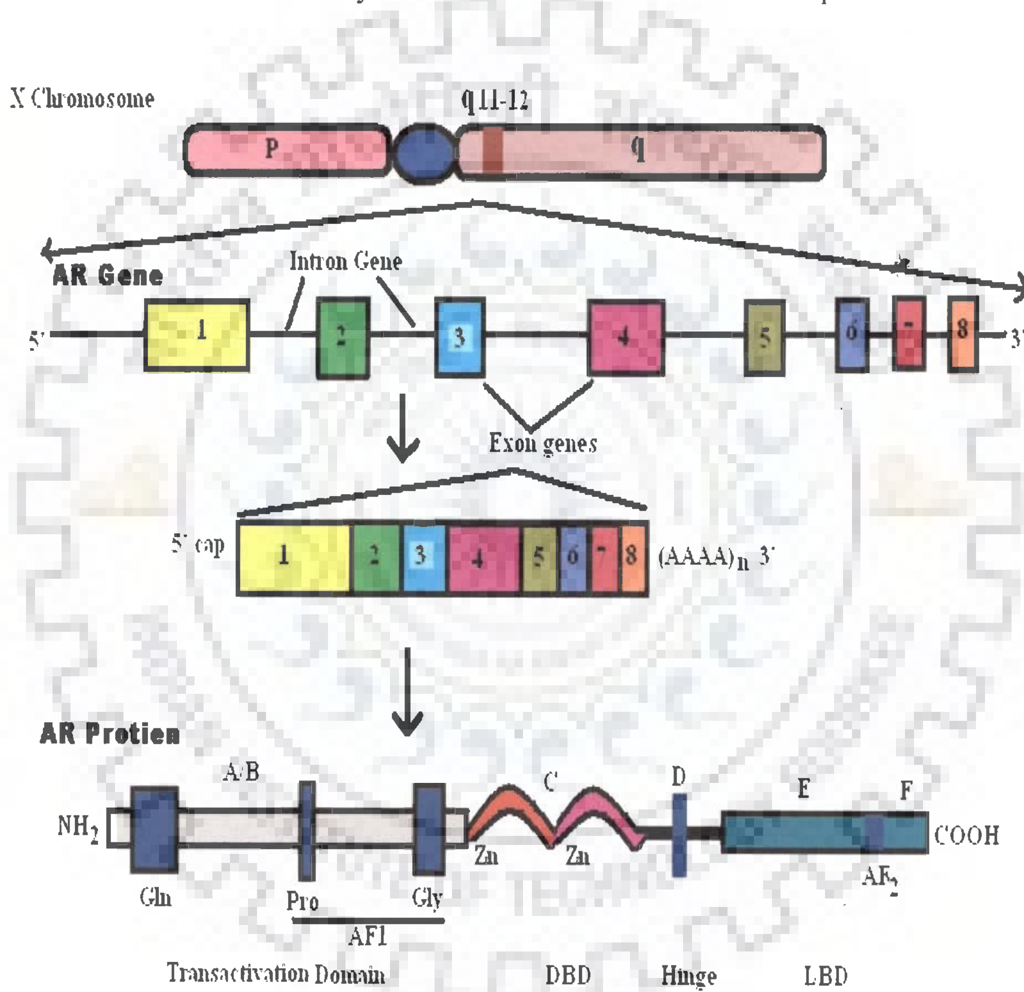


Figure 5. Schematic illustration of the location, exon structure and protein domain structure of the AR gene. (Top) The location of AR gene at the q11-12 of X chromosome. (Middle) The AR gene and its mRNA. The AR gene consists of 8 exons (boxes) and 7 introns (line). (Bottom) The AR protein. The domains of AR are indicated. Relative positions of glutamine (Gln), proline (Pro) and glycine (Gly) repeats within the N-terminal domain are shown by the indicated boxes. The transactivation function domains, AF-1 and AF-2 are located within the N-terminal domain and ligand-binding domain, respectively. Two zinc fingers in the DNA binding domain and a PEST sequence in hinge region are indicated.

of target genes, plays an integral role in AR functions via intramolecular and/or intermolecular interaction with other co-regulators and contains a transactivation domain, known as activation function 1 (AF-1). The DNA binding domain is encoded by exons 2 and 3 and consists of two "zinc finger" motifs that are hallmarks of all nuclear steroid receptor; the most highly conserved region within this family and are responsible for specific interactions with the cognate DNA of target genes (Freedman, 1992). The C-terminus of the AR is represented by the LBD, encoded by the 3'-portion of exon 4 and exons 5-8, and intervene the specific high-affinity ligand binding through the hydrogen bonding and hydrophobic interaction (Matias et al, 2000; Sack et al, 2001). Besides LBD also envisages the second transactivation function (AF-2) domain of AR resides and interact with co-regulators such as coactivators to affect AR function (Gronemeyer 1991; Glass, 1997; Heinlein, 2002). Further AR-AF2 domain may also contribute to the stability of the overall structure of the receptor (Chang, 2002). Between DNA-binding domain and the steroid-binding domain hinge region lies which contains the nuclear translocation signal.

2.1.3.2 Estrogen receptors (ER)

Like that of AR, ER interacts with the estrogen response element (ERE) of the target genes in the presence of estrogens. Besides, similar to that of the AR, ERs also consists of LBD, DBD, dimerization and transactivation domains (Fig. 6). Both agonists and antagonists bind to the ligand-binding domain but produce differential conformational changes (Katzenellenbogen et al, 1996; Brzozowski et al, 1997). Similar to the AR, ER can also interact with co-regulators which modulate estrogen actions (Glass et al, 1997; Norris et al, 1998). There are at least two ER isoforms encoded by two distinct genes

ERα (Greene etr al, 1986) and ERβ (Kuiper et al, 1996; Mosselman et al, 1996, Ogawa et al, 1998) in mammals, including humans (Fig. 6).

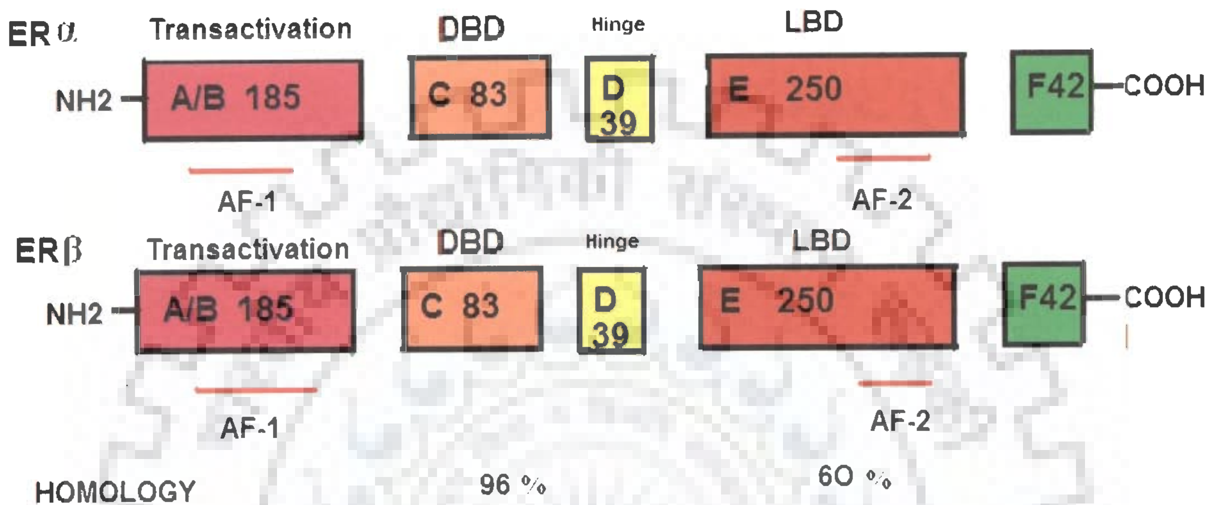


Figure 6. A schematic structural comparison of human ERα and ERβ. Receptor domains are illustrated with different colored boxes, and he approximate size of each domain is indicated.

2.1.4 Dynamics of Steroid Action

The above description dealt with the types of steroid hormones, their relation with the gonadotropins and the structure of the two important receptors responsible for the action of steroid hormones. This section will deal about the general mechanism of action of a steroid hormone i.e. how a steroid acts in the target cells. Once released from the endocrine glands, steroid hormones travel throughout the body in search of matching receptors localized either in the cytoplasm or nucleus and/or in some rare cases in the plasma membrane of the target cells. Once it reaches to the specific receptors, the hormone binds with the receptors much like a key would fit into a lock to unlock a door (Fig. 7). The unliganded steroid receptors remain inactive due to their interactions with

proteins like HSP 90. The binding of steroid on the receptor results in a conformational change that promotes the dissociation of chaperone proteins and facilitates receptor dimerization.

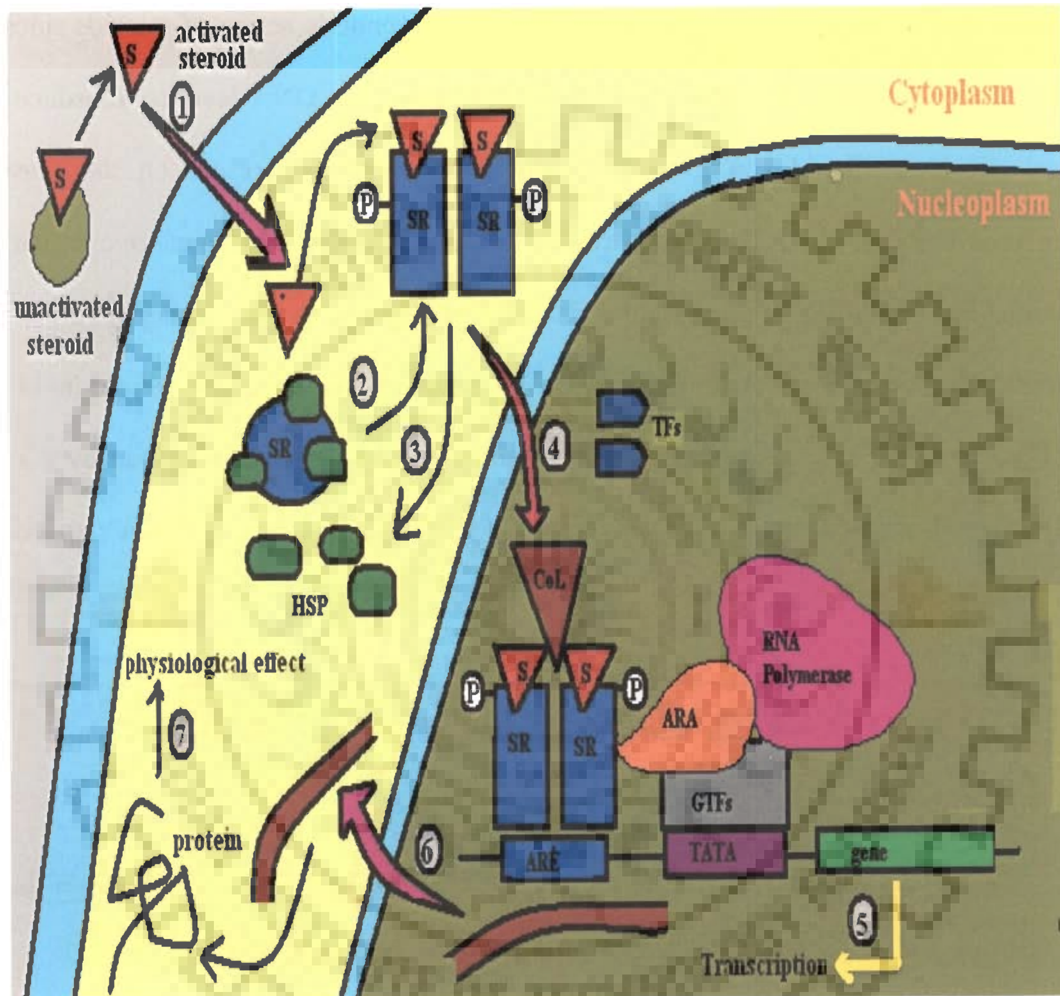


Figure 7. Schematic representation of the genomic action of a steroid in the target cell (1) dissociation of steroid from binding protein, transport of steroid into cell, formation of binding Steroid (2) Binding of steroid to cytoplasmic receptor with bound heat shock protein (3) Dissociatiuon of Heat Shock Protein forms "activated" Receptor (4) Activated cytoplasmic receptor enters nucleus, binds DNA response elements as homodimers, (5) DNA transcribed into messenger RNA (6) mRNA leaves nucleus, translated into protein on cytoplasmic ribosomes (7) Newly synthetic proteins elicit biological response.

The receptor hormone complex is then transported to nucleus where it gets phosphorylated and binds to the specific hormone response element (HRE). Now

different co-regulators and other transcription factors are recruited and this leads to the changes in cellular function either by altering the cell's existing proteins or by turning on the genes that will build a new protein, which in turn will activate the existing genes. The foresaid mechanism of action of steroids is known as genomic action of steroids since it involves the mediation of a pathway that directly acts at the DNA level to transduce its signal. Besides, steroid can also act through the receptors present on the plasma membrane and this type of steroid hormone action is known as non-genomic action of steroids. Fig. 8 shows a comparative analysis in regards to the agents involved in genomic and non-genomic action of the steroids. Non genomic action of steroid involves the binding of the steroids on plasma membrane receptors which is followed by the up regulation of the second messenger system like MAP Kinase or calcium dependent pathway (Falkenstein et al., 2000).

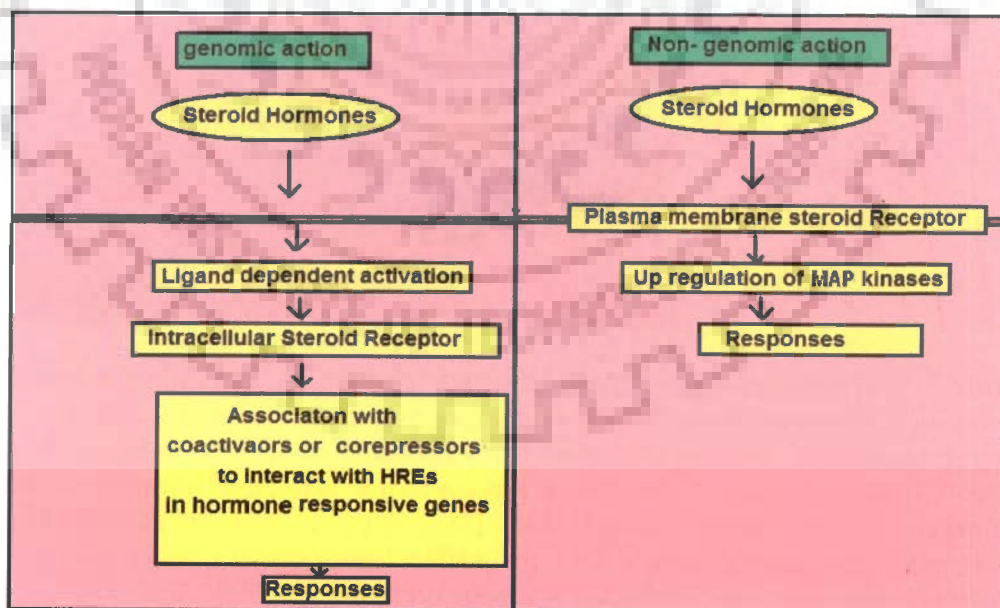


Figure 8. The comparative representation of different agents involved in genomic and non-genomic action of steroids.

While genomic action of steroids is manifested after a considerable periods of time, the non genomic actions are almost instantaneous (Falkenstein et al., 2000; Schmidt et al., 2000).

2.2 Endocrine Disruption

2.2.1 Decent of the Concept and Definition

A balanced functioning of the endocrine system, as described above is essential for controlling different pivotal functions in the human or animal body. However, endocrine system is highly sensitive toward factors which leads to partial or complete paralysis of physiological system or may severely impair even the whole development of the organism. In recent years a number of studies have been conducted which indicate that various environmental chemicals may interfere with the normal functioning of the endocrine system of humans and wildlife (Witorsch, 2002; Roy and Pereira, 2005; Roy et al., 2004, 2006; Rogan and Ragan, 2007; Gore, 2008; Kumar et al., 2008a, 2008b; Suresh and Sikha, 2008). These chemicals have been designated as EDC. In general EDC are the substances that may lead to the generation of the adverse effect by obstructing the body's hormonal or chemical messengers. Although a chemical can be either a toxic chemical or an EDC, but may just as easily be both (Vogel, 2004, Kumar et al, 2008a), however, generally an EDC is different from classical toxicants such as carcinogen, neurotoxin and heavy metals in the sense that it interferes with the normal hormonal functions, but doesn't have a classical toxic effect (Roy and Pereira, 2005). A number of descriptions have been proposed to define EDC and during the Weybridge Conference (1996) the European scientific and regulatory community has agreed on the following definition of a potential endocrine disruptor (Weybridge, UK, 1996)-

"An endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function." or "A potential endocrine disruptor is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism."

In May 1997, the U.S. Environmental Protection Agency (EPA) task force on endocrine disruption (EDSTAC) agreed on the following operational definition:

"An endocrine disruptor is an exogenous chemical substance or mixture that alters the function(s) of the endocrine system and thereby causes adverse effects to an organism, its progeny, or (sub) population."

However, the usage of the word "adverse" effect in the foresaid definition created ambiguity and thus, in order to achieve consensus, the EDSTAC finally agreed to the following general description (EPA Final Report, 1998)-

"The EDSTAC describes an endocrine disruptor as an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, the populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle."

In a special report, EPA has stated that, "when we consider the existing scenario of the science, we can't consider the endocrine disruption to be an adverse end point per se, instead it can be seen as a mode or mechanism of action which may lead to other outcomes, for example, carcinogenic, reproductive, or developmental effects" (Crisp et al, 1997).

2.2.2 Historical Prospective

Although the concept of endocrine disruption received prominence in the 1990s only but its history can be dated back up to 1930s when Sir Edward Charles Dodds, a British scientist, collected the scientific evidence on endocrine-disrupting effects, ultimately developing diethylstilbestrol (DES), a 'synthetic estrogen' eventually banned in U.S. (Krimsky, 2000). Soon after that in 1950, it was demonstrated that exposure of chickens to DDT, a chemical very similar to DES and a well known pesticide, resulted in sexual underdevelopment and suppressed expression of secondary sexual characteristics (Burlington and Lindeman, 1950). Burlington and Lindeman contended that there is a correlation between DDT and development of estrogenic effects in chicken and they finally hypothesized that pesticides may have adverse effects on the hormonal systems, however, this hypothesis remained scientifically dormant for decades. Again, the issue related with the adverse effects of chemicals on endocrine system received attention in late 1970s and early 1980s in conjunction of two initially isolated pathways: human health effects and wildlife biology. While John McLachlan showed the adverse effects of DES and DDT on human health (McLachlan, 1980, 1985; McLachlan and Newbold, 1987); wildlife biologists commenced to accredit the harmful effect of chemicals on wildlife populations following Rachel Carson study in 1962 which was based on the harmful effects of pesticides on wildlife. The foresaid study showed that the use of pesticides can be related with the following adverse effects in wildlife viz. widespread population declines of song birds, eggshell thinning in predatory birds (most notably the bald eagle), reproductive failure and declining populations of otters, dolphins, other sea mammals, alligators, sea gulls, and other ecosystem consumers (Carson, 1962). This

author wrote a book *Silent Spring*, published in 1962, where she attempted to raise an alarm on the impacts of certain chemicals on wildlife biology (Carson, 1962). Although this book was severely criticized for the industrial interests but later researches proved that she was largely correct. Finally, in 1990s it was Colborn who can be accredited with preaching the agenda of endocrine disruption in detail. He endeavored to amalgamate the science of wildlife ecology with the human health studies after being a part of the study of environmental degradation in the Great Lakes basin (Colborn et al., 1990). According to the references of Science Citation Index (ISI Web of Science), the word 'endocrine disruption' (ED) was first of all used by Colborn in a work which depicted the idea of ecosystem-wide endocrine disruption due to environmental contamination (Colborn and Clement 1992, Vogel et al 2004). Colborn tried to bring together the researchers working in the fields of wildlife and human toxicology, endocrinology, anthropology, ecology, immunology, histopathology, anthropology, and wildlife management by convening the wingspread work sessions. In this convention, experts from the foresaid fields conversed together on a common agenda, the endocrine disruption, shared evidence across disciplines and finally drafted a detailed statement, wingspread consensus statement, on the issue of endocrine disruption (Bern et al., 1991; Krimsky, 2000). The wingspread consensus statement focused attention on the previously overlooked effects of chemical contamination besides cancer. In March 1996, Colborn published a book called, *Our Stolen Future,* which can be regarded as a continuation of Rachel Carson's *Silent Spring.* By the end of 1996, scientists were able to distinguish at least 51 chemicals which were suspected to be EDC including DES, DDT, PCBs, dioxins, and furans and this number increased up to 87 by the end of 2003 (Colborn et al., 1996; Myers, 2003). In May 2002,

the usage of the search term “endocrine disruption” with the Science Citation Index (ISI Web of Science) brought up 1346 research article references which used the term endocrine disruption. With the approach of 21st centaury, the agenda of the endocrine disruption has become the topic of serious discussion among the academicians and researchers equally. Different efforts are being carried out worldwide to screen as well as to investigate the mechanism of action of various EDC and the list is increasing day by day. In United States, the endocrine disruption screening and testing advisory committee (EDSTAC) formed under the auspices of U.S. Environmental Protection Agency (EPA) is working exhaustively in this area. EDSTAC consists of the representatives from the industries, academic and research arenas who help in devising the strategies for screening and studying various EDC having diverse chemical structures and belonging to different classes. Thus, it can be said that earlier researches prepared the ground for awakening the peoples towards the problem of endocrine disruption which became the topic of serious discussion in 1990s.

2.2.3 The complexity of endocrine disruptors

The endocrine disrupting chemicals are believed to exert their effect by (1) mimicking normal endogenous hormones such as estrogens and androgens; (2) antagonizing endogenous hormones; (3) altering the pattern of synthesis and metabolism of endogenous hormones; and (4) modifying the hormone receptor levels. Natural endogenous estrogens are involved in the development and maintenance of the female reproductive tract, secondary sexual characteristics and regulation of the menstrual cycle, pregnancy and lactation. At the cellular level, these endogenous hormones mediate cell proliferation, and also the synthesis and secretion of cell-specific proteins in reproductive

tissues such as the ovary, oviduct, uterus, vagina, hypothalamus, pituitary and mammary gland. These effects are mediated for the most part by estrogen receptors (ER) α and β in the female. The expression of these receptors has also been observed in male reproductive organs like the prostate and non-reproductive organs such as the cardiovascular system, bone, thyroid gland etc. indicating the vast reach of these synthetic estrogenic chemicals (Couse & Korach, 1999). It is apparent that the chemical structures of xenoestrogens are diverse differing not only from endogenous estradiol but also from each other. Similarly, the endogenous steroid androgen and its receptors are present in testis, prostate and other parts of the male reproductive tract. In addition, some other non-gonadal tissues like larynx and facial hair follicles also possess the androgen receptors. The synthetic androgens or xenoandrogen or androgen like molecules can mimic the action of endogenous androgen. These chemicals act as the ligand not only for estrogen but also for androgen, progesterone or arylhydrocarbon receptors thereby exerting a combined action (Norgil et al., 2002; Eertmans et al., 2003).

Following the hormone-receptor interaction, a variety of biochemical reactions occur within the target cells, collectively referred to as intracellular signaling leading to a characteristic biological response (Witorsch, 2002). The molecular mechanisms triggered by hormonal substances from the environment can be subdivided into two categories: nuclear receptor mediated responses and direct effects. In the case of environmental estrogens, the effects are triggered by the binding to ER and finally culminating in genomic responses through alteration of gene expression. Over the last few years the picture of receptor mediated estrogen action has become rather complex due to the identification of two receptor subtypes ER-α, ER-β and several splice variants thereof. In

addition, several ligands have different binding affinities to the two receptor subspecies α and β. Most prominent in this regard is the phytoestrogen genistein with its clear-cut preference for ER-β. Upon ligand binding they can form homo and heterodimers with different transcriptional activity (Cowley et al., 1997; Pettersson et al., 1997). To make things even more complicated, the steric confirmation of the ligand-binding domain is dependent on the bound ligand (Hubbard et al., 1997; Pike et al., 1999 and 2000). This is important because ligand-binding domain (LBD) harbors the activation function-2 (AF-2), which in turn is necessary for the interaction with the so-called co-activators or co-repressors. In other words, the bound ligand determines whether the hormone receptor complex is capable of enhancing or suppressing the transcriptional activity (Bramlett et al., 2001; Nilsson et al., 2001; Wong et al., 2001).

Molecular modes of action of environmental chemicals comprise interactions with key enzymes of steroid metabolism like sulfotransferase, sulfatase, 3β-HSD, 17β-HSD and aromatase (Le Bail et al., 2000; Kirk et al., 2001 Krazeisen et al., 2001 Mueller et al., 2003). Another point of concern is that environmental chemicals can get metabolized in animal cells and the potency of metabolites to cause endocrine disruption may change. Further they are suspected to bind to steroid binding proteins such as sex hormone binding globulin and α-fetoprotein. This alters the ratio of free (bioactive) and protein bound hormone (Milligan et al., 1998). Furthermore, natural and synthetic hormones in some cases directly trigger rapid responses thereby circumventing receptor-mediated mechanism of gene expression. Signal transduction pathways involved are those leading to an increase of intracellular calcium levels and to activation of mitogen activated protein kinases (MAP-kinases) (Abraham et al., 2003; Fin et al., 2003). These

mechanisms lead to functional consequences such as alteration in energy metabolism, oxidative, inflammatory and angiogenic pathways and inhibition of tyrosine kinases. In addition, a cross talk between steroid receptors and other factors (IGF-I, EGF) and has also been reported (Turner et al., 2003). Further the actions of the EDC may be executed at another places then the steroid tissues like in the hypothalamus or piturary gland through the alteration in the gonadotropins production (Sridaran et al., 2003, Muthuvel et al., 2006; Murgesan et al., 2007).

Although the environmental chemicals are usually less potent than the endogenous hormones, such as estradiol, it is now known that they act additively with them. This explains how a low, seemingly insignificant level of xenoestrogens, such as the weak "bisphenol A" (BPA), may have a dramatic impact by magnifying the response in target tissues (Silva et al., 2002). In addition, several endocrine disruptors have unequivocally been shown to induce non-monotonic dose-response curves of biological effects that is lower doses induce a more profound effect than a higher dose "low dose effect". This phenomenon, which was known for sex steroids since long was found for the first time in mouse prostate in relation to EDC (Markey et al., 2003). Fetal exposure to increasing doses of estradiol and diethylstilbesterol (DES) and some weak estrogens (example BPA), was shown to induce the prostate weight that persisted into adulthood. Exposure of the pre-pubertal mouse to a range of BPA doses induces a U-shaped curve for the end points of vaginal opening and uterine weight change, two parameters that are the hallmarks of estrogen action. This means that a more apparent effect is observed in the lower and higher doses relative to the medium range of doses (Vom et al., 1997; Nagel et al., 1997; EDSTAC, 1998). Endogenous hormones are also

known to act on hormone-sensitive endpoints in an inverse manner, as evidenced by the observation that prostate cells undergo increased cell proliferation in response to low doses of androgen but decreased cell proliferation in response to high doses of androgens (Sonnenschein et al., 1989). Depending on these data, it has been postulated that this phenomenon is due to the operation of different and discrete pathways at low and high doses (Geck et al., 1989). In some of the other cases, the low dose effects have been observed to occur due to down-regulation of the receptor by the ligand. Considering all these facts it seems that the endocrine activity of EDC is quite complicated and not so straightforward as it seems to be.

2.2.3.1 Adverse in vivo effects of EDC to human health

Up to now laboratory experiments with animals and field observations on wild life have strengthened the endocrine disruption hypothesis (Andersson et al., 2008a, 2008b, Kumar et al., 2008a, 2008b). In the recent decade, several instances of environmental chemicals hampering endocrine function in human males have come to light Epidemiological findings of genital malformations in children of workers exposed to pesticides (Weidner et al., 1998) and the clustering of cryptorchidism cases in areas of intensive agriculture (Garcia-Rodriguez et al., 1996), gives credence to the theory of endocrine disruption. In the past few years, an increase in the incidence of poor semen quality, testis cancer, undescended testis and hypospadias has been noticed in several parts of the world, a condition collectively referred to as testicular dysgenesis syndrome (TDS). Experimental and epidemiological studies suggest that TDS is a result of disruption of embryonic programming and gonadal development during fetal life. The increasing incidences of such cases point to the environmental influences as the potential cause rather than the

accumulation of genomic structural defects. Statistical data on TDS in many industrialized nations is also disturbing (Skakkabaek et al., 2001). But relatively few chemicals have so far been closely examined for their bioactivity in disrupting the hormonal balance of the populations. Flutamide and diethylstilbestrol has been shown to have harmful impact on fetal testicular steroidogenesis in the rat (Adamsson et al., 2008).

2.2.3.1.1 Sperm quality deterioration

The concern over decreased sperm counts and male reproductive capacity was triggered by a paper on the meta-analysis of 61 sperm count studies which concluded that there is a genuine decline in semen quality over the last 50 years (Carlsen et al., 1992). However this study was questioned (Olsen et al., 1995) but reanalysis (Swan et al., 1997) confirmed the originally described time trend and pointed out that, regional factors could affect sperm quality and/or concentrations. This has been substantiated by mono- and multilaboratory studies conducted in Belgium (Van Waeleghem et al., 1996), Finland (Pajarinen et al., 1997), France (Auger & Jouannet, 1997) and Denmark(Andersen et al., 2001). As per a recent summary (Safe, 2002), substantial variations in semen quality do exist in samples from different geographical locations. Vinclozolin is known to be a potential anti-androgenic substance and exposure to this chemical during testis differentiation alters programming of germ cells and/or Sertoli cells, leading to germ cell apoptosis and reduced sperm motility later in adult life (Uzumcu et al, 2004). Similarly, in sons born to women treated with DES during pregnancy a decrease in ejaculate volume, semen concentration and percentage of normal motile sperm have been recorded (Bibbo et al, 1998). An inverse correlation between the concentration of PCB metabolites in blood, seminal plasma and sperm motility concentration has been found (Dallinga et

al., 2002). A statistical correlation between poor semen quality and high levels of alachlor, diazinon, atrazine, metalachlor and 2,4-D (2,4-dichlorophenoxyacetic acid) suggested that pesticide residues may be a factor for the difference in semen quality between American population residing in rural and urban areas (Swan et al., 2003).

2.2.3.1.2 Testicular cancer

Testicular cancer is often quoted as the most common type of cancer in young men. The secular trends across Europe and USA show that it is increasing in incidence in Caucasian men (Bergstrom et al, 1996; SEER, 2003; Fisher, 2004). The obvious regional differences in incidence and the association with birth cohort suggest a possible role of environmental factors in the development of testicular cancers (McKiernan et al, 2003). Testicular cancer arises from the carcinoma in situ (CIS) cells, which should have their origin in fetal life, whereby subnormal androgen and/or an increased estrogen exposure are potentially important factors (Eertmans et al, 2003). The main risk factor for testicular cancer is cryptorchidism, followed by hypospadiasis (Sharpe, 2003). A study conducted on workers of the plastic industry exposed to polyvinyl chloride (PVC) demonstrated a significantly increased risk of seminoma (cancer of semniferous tubules) (Ohlson & Hardell, 2000). The mycotoxin ochratoxin A, a naturally occurring contaminant of cereals, pig meat and other foods is known to be a genotoxic carcinogen in animals. It has been hypothesized that ochratoxin A could be a cause for development of testicular cancer based on the data obtained on the per-capita consumption of coffee and pig meat, the principal dietary sources of ochratoxin A (Schwartz, 2002). These evidences strongly suggest that occupational and dietary exposure could lead to cancer of reproductive tissues.

2.2.3.1.3 Congenital abnormalities (cryptorchidism and hypospadias)

Cryptorchidism and hypospadias are abnormalities normally detected at birth (congenital abnormalities). Cryptorchidism occurs when testis do not descent into scrotal sac, which is usually unilateral. Hypospadias is a developmental abnormality of the penis in which the urethral opening is not located at the tip of the glans penis but elsewhere along the shaft. The prevalence studies of cryptorchidism and hypospadias are difficult to compare due to differences in screening techniques (Toppari, et al., 2001). Despite this, cryptorchidism has been recognized as the most common congenital abnormality of the newborn (2-4% incidence). Based on registry data, hypospadias is the second most common (0.3-0.7% at birth) congenital malformation (Sharpe et al., 2003). Exposure assessment of selected organochlorine compounds has revealed that in adipose tissue of boys undergoing correction for cryptorchidism there is a significant increase in heptachloroepoxide and hexachlorobenzene residues when compared with adipose tissue from children undergoing surgery for other reasons (Hoise et al., 2000). Thus, despite multiple causes there are clear indications of a rise in incidence of these abnormalities in a number of European countries, the United States and Japan (Jensen et al., 1995; Paulozzi et al., 1997; Dearnaley et al., 2001). It has been known that there is an increased transgenerational risk of hypospadiasis in sons of women exposed in utero to DES (Klip et al., 2002). However, caution needs to be exercised since the registry data for both cryptorchidism and hypospadias could be highly unreliable due to different diagnostic approaches and there is need for prospective studies to make trustworthy conclusions (Sharpe, 2003). Nevertheless, proliferation of xenobiotic chemicals can produce

potentially disastrous unintended consequences for the male gender development (Steinhardt, 2004).

2.2.3.1.4 Changes in sex ratio

Under "normal" conditions, the ratio of newborn boys to girls is higher than one. Several studies reported a small but significant decrease in the sex ratio in Canada and the United States (Davis, 1998), the Netherlands, Denmark and several other European countries (Martuzzi, 2001). A report from Turkey suggests that, mothers who were exposed to hexachlorobenzene during their fertile period showed a lower percentage of male births as compared to female ones (Jarrell, 2003). A remarkable decrease in the birth of male children was noticed especially during the Yu-Cheng disaster when fathers were exposed to PCBs at an age below nineteen (Del Rio-Gomez et al., 2002). Sex ratios were determined in families who had been accidentally exposed to high levels of dioxin (in Zone A) as a result of an industrial accident that occurred in Seveso, Italy, in 1976. In a small group of highly exposed families, a decrease in male/female sex ratio at birth was observed (Mocarelli et al., 2000). Paradoxically, in another report, changes in sex ratio were not observed in the offspring of parents who were occupationally exposed to relatively high dose of dioxin (Schnorr et al, 2001).

2.2.3.1.5 Effects on central nervous system

Central nervous system (CNS) development is acutely sensitive to sex hormone levels, particularly in the perinatal period just prior to and after birth. The expression of both ERα and ERβ has been shown distinctly in mammalian CNS, with β showing a wider distribution and expression of several splice variants. Although not substantiated, there is suspicion that EDC may pose a risk for the developmental neurotoxicity during this

vulnerable period by interacting with these ERs. Few studies have addressed this issue, but epidemiological associations have linked perinatal exposure to PCBs, pesticides, and polychlorinated dibenzofurans with cognitive and behavioral deficits (Singleton & Khan, 2003). PCBs are known to activate estrogen receptors, alter thyroid hormone status, and affect dopamine signaling together with related behavior in rodents (Laessig et al., 1999). In another study, male monkeys were exposed for 20 weeks following birth to a mixture of PCBs at concentrations similar to that found in human breast milk (50 parts per billion). These animals showed marked behavioral abnormalities 2.4-5 years after exposure (Rice, 1999). Another study has also indicated that male rats exhibit permanent changes in motor function and emotional behavior following exposure *in utero* to certain classes of PCBs (Hany et al, 1999). In addition, there are reports that EDC such as bisphenol A transfer from the maternal rat to the fetus and that the chemicals affect the developing brain, leading to behavioral alternations such as impulsive or aggressive behaviour (Adriani et al, 2003). Similarly, exposure to p-Nitrotoluene, a potent EDC, has been shown to cause hyperactivity in the rat (Ishido, 2004). It should be emphasized that PCBs can affect several signaling mechanisms and therefore the phenotypic effects described above may not entirely be linked just to ERs (Singleton & Khan, 2003).

2.2.3.2 Tiered Bioassays for the assessment of endocrine disruptors

As a result of the numerous field observations, epidemiologic reports, laboratory studies, magazine articles, books, ongoing controversy, and congressional hearing, the issue of endocrine disruption was incorporated into the Food Quality Protection Act (FQPA) and amendments made to the Safe Water Drinking Act (SWDA) in 1996. This mandated the U.S. Environmental Protection Agency (EPA) to develop and implement a screening

program to determine whether hormonal activity existed in environmental chemicals. Consequently, they established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), which strongly recommended setting up a program with tiered approach to evaluate potential endocrine activity of chemicals. At the same time Organization of Economic Cooperation and Development (OECD) developed the conceptual framework for the testing and assessment of endocrine disrupting chemicals consisting of several levels of screening and testing (OECD report, 2003). Although the mandate dealt mainly with environmental estrogens and androgens, thyroid hormone activity was also considered. The "tiered approach" proposed by EDSTAC starts with identification of substances that exhibit hormonal activity using a variety of *in vitro* and *in vivo* bioassay systems (Tier 1 Screening) and then determines which of these could evoke detrimental effects in test animals (Witorsch et al., 2002) (Tier 2 Testing). Marked species differences have been observed in the endocrine disruptive activities of environmental chemicals and therefore multi-tiered approaches are also being undertaken. Tier I screening battery, proposed by the US-EPA detects the hormonal activity of the compounds and Tier II characterizes and quantifies those effects (Eertmans et al., 2003).

Today several *in vitro* and *in vivo* bioassays are available to screen EDC's. These assays are based on different principles right from binding assays to *in vivo* models. Of course the *in vitro* assays are sensitive, high throughput and easy to perform, but the importance of *in vivo* assays cannot be ignored since they can truly assess the actual risk of the compounds. The problem with the *in vitro* assays is that the tests are carried out on an isolated system and do not take into consideration all the factors that add to the

complexity of the organism as a whole (Eertmans et al., 2003). Added to this, many a time the parent compound may not have endocrine disruptive activity but could be metabolized by animals into compounds that disturb endocrine function. Thus, *in vitro* assays could give 'false negative' responses (Gray et al., 2002). It is desirable therefore that whole animal testing should follow up the results obtained with *in vitro* assays. Again, because of species differences, the results of *in vivo* bioassays used for risk assessment studies should be extrapolated to the human population with discretion. In order to give an idea of the practices, some *in vitro* and *in vivo* assays that are currently used to screen these EDC are described.

2.2.3.2.1 In vitro Assays:

2.2.3.2.1.1 Receptor binding assay

This assay is used to detect if the concerned chemical interacts directly with the steroid receptors, like ER, AR or progesterone receptor (PR) and determines the affinity of a particular chemical for the receptors. Briefly, the chemical is made to compete with the radiolabelled ligands (like estradiol, androgen) for binding a particular receptor isolated from the nuclear or cellular extract of the target tissue or cells (Wong et al., 1995). Recently, there are few reports on the development of *in vitro* binding assays for estrogen (Wong et al., 1995; Gee et al., 2008), androgen (Scippo et al., 2004; Freyberger & Ahr, 2004) and progesterone (Scippo et al., 2004), where recombinant receptor proteins synthesized in bacterial systems have been used. However, the major drawback of these assays is that they do not provide information regarding hormonal activity and thus they cannot discriminate between agonistic and antagonistic actions of particular substances.

2.2.3.2.1.2 Cell proliferation assay

The E-Screen assay is best known for identifying estrogenic chemicals resulting in the proliferation of the human breast cancer cell line (MCF-7) in response to estrogen or estrogen like chemicals (Lippman & Huff K, 1976; Soto et al., 1995; Hong et al., 2005; Menendez et al., 2005; Zhao et al., 2008). A MCF-7 based proliferation assay has been developed and optimized after thorough considerations of different factors involved in this assay (Desaulniers et al., 1998). This assay is one of the most simple and sensitive assays available for estrogenic compounds. A detailed protocol including the critical parameters that should be taken into account for performing the E-Screen assay is now available (Rasmussen & Nielsen, 2002). Recently, there is a report of screening androgenic compounds using the proliferation of human mammary carcinoma cell line stably transfected with human AR (Körner et al., 2004). However, this type of assay is also not free of disadvantages; first, batch-to-batch variation of cells result in change of cell proliferation rates (Zachaarewski, 1992) Secondly, this test is difficult to perform for environmental samples containing constituents that are toxic to cells.

2.2.3.2.1.3 Reporter gene assays

This assay principally measures the level of expression of the reporter genes in response to the induction by some ligands or ligand like molecules. For these assays either the mammalian cell lines (MCF7, COS1, CHO etc.) or yeast strains such as *Saccharomyces cerecisiae* are transfected with a reporter plasmid and the receptor plasmid (in case the cell line does not have the endogenous receptor). The reporter plasmid consists of hormone response element (HRE) coupled to the reporter gene, such as luciferase (mostly in the case of mammalian cells) and β-galactosidase (Sohini & Sumpter, 1998; Metzger et

al., 1998; Hoogenboom et al., 2001; Roy et al., 2004, 2006; Chaterjee et al., 2007). The rationale behind this assay is that exposure of the cells to a hormonally active compound, will result in the binding of this compound to its receptor. The ligand receptor complex then dimerizes and binds to the HRE resulting in the transcription and expression of the reporter genes. β-galactosidase metabolizes the substrate generating a red color that can be estimated with a spectrophotometer (Sohini & Sumpter, 1998). For luciferase as the reporter, the substrate is luciferin and this generates a flash/glow of light, which can be determined using a luminometer (Roy et al., 2004, 2006). Cell based reporter gene assays are getting extremely popular day by day with its application not only in endocrine disruptor research but also in drug screening programs of pharmaceutical industries. Both yeast and mammalian cell lines can be stabilized with the transformed genes and therefore easy to use. This avoids the batch-to-batch variations as has been seen in transient expression of reporter and receptor genes in cells (Vinggaard et al., 1999). Towards the development of high throughput screening system a recent report shows the use of green fluorescent protein as the reporter gene expressed in yeasts (Bovee et al., 2004; Chaterjee et al., 2007). This assay bypasses the cell lysis step as done in other reporter-based assays thus reducing the time and labor to a great extend. However, in spite of all these advantages there is still a question about the efficiency of this assay for screening toxic chemicals without damaging the normal physiology of the cells. Several laboratories are working on this aspect so that it can evolve into one of the best models for identification and screening of EDC. Recently a number of assays have been developed to screen the (anti)andorgenic and (anti)estrogenic compounds (Sonneveld, et

al., 2005; Simon and Mueller, 2006; Xu et al., 2005, 2006; Chaterjee et al., 2007; Paris et al., 2007; Sun et al., 2007; Xu et al., 2008).

2.2.3.2.2 In vivo Assays:

2.2.3.2.2.1 (Anti-) Estrogenic assays

The rodent uterotrophic assay has been used as a tool since 1930 to screen (anti-) estrogenic chemicals. This involves the *in vivo* binding of the chemicals to the estrogen receptor. The basic idea behind this assay is, the exogenous estrogen or estrogen like chemical can cause the hypertrophy of the uterus in female immature rats or ovariectomized rats. The rats are treated for 3 days with the chemicals and then killed to collect the uterus, which is physiologically characterized. This test has been extensively evaluated and found to be reliable for detecting estrogenic chemicals (Laws et al., 2000; Nakagawa et al., 2002; Owens & Ashby, 2002; Andersen et al., 2006; Schmidt et al., 2006; Koda et al., 2007). In another set of assays, the abnormalities associated with the development of female sex organs and sex characters in immature animals (mainly rats) have been studied. For this, the immature female animals are exposed to the chemicals for 20 days and after that the response of their reproductive organs are evaluated. This test is yet to get the proper standardization and validation from EPA (Gray et al., 2002). This test if validated and approved finally can be used to screen compound with estrogen and thyroid hormone like activities.

2.2.3.2.2.2 (Anti-) Androgenic assay

Assays to detect androgenic properties of different chemicals have been developed since 1930s, but Hershberger and his collegues demonstrated the best among them (Hershberger et al., 1953). They analyzed in castrated rats, the response of the ventral

prostrate, seminal vesicles and coagulating glands to exposure of several chemicals, mostly androgenic and some estrogenic and even progesterogenic. In this assay, among all parameters tested the capacity of the exogenously administered chemicals to restore the weight of the accessory sex organs of the castrated male rats was determined and validated. It has been used widely to screen different (anti)androgenic compounds (Yamada et al., 2001, 2003; Freyberger, 2007; Tinwell et al., 2007). Yamasaki et al., 2004 performed a detailed study to identify twelve different chemicals for their androgenic activities (Yamasaki, 2004). Recently OECD validated this assay using three reference compounds (Shine et al., 2007). Yamada et al. (2004) validated successfully for screening the thyroid hormone disrupting compounds also. An additional test has been developed that utilizes immature male rats for identifying and screening androgenic properties of chemicals. This assay is based on the same principle as the rodent pubertal female assay, used for estrogenic chemicals. The assay examines abnormalities with development of the male sex organs and secondary sex characteristics. This test is also undergoing standardization and validation by the EPA (Gray et al., 2003).

2.2.3.2.2.3 (Anti-) Progesterogenic Assay

Not many chemicals have yet been identified with progesterone like activities. One of the recent reports utilizes the rabbit endometrial transformation test for identifying the *in vivo* activity of progesterogenic chemicals (Tabata et al., 2002). In this assay, immature female rabbits are administered with these chemicals for 5 consecutive days. The rabbits are sacrificed and uterine sections are then studied histologically for their extend of transformation in response to the chemicals.

2.2.3.2.2.4 Other in vivo assays using non-mammalian vertebrates

Fish is one of the most popular and easily used models listed in this category. A fish reproduction assay, is used to test agents for estrogenic and androgenic effects. Briefly, the adult reproductively active fish are exposed to the chemicals for 21 days and abnormalities associated with survival, reproductive behavior, secondary sex characteristics and fecundity (number of spawns, number of eggs per spawn, fertility and development of offspring) are examined. This assay is being proposed to be included in the high tier screening program (Hutchinson et al., 2000). Another end point that is monitored in the fish model is vitellogenin (vtg) protein regulation and its plasma clearance rates. Vtg is a protein expressed mostly in female fish. Male fish also possess this gene, but under normal condition it is not expressed in a level equal or greater than female fish. Thus, this has become an excellent marker for estrogenic chemicals in aquatic environment. A recent investigation of vtg regulation and plasma clearance in male sheepshead minnows reports that liver vtg levels returns to the base line in estradiol-exposed fish, but remains elevated in nonylphenol-treated fish (Hemmer et al., 2002). Furthermore, plasma vtg clearance is both concentration and time dependent and may be detected even many months after exposure (Hemmer et al., 2002).

Many man-made chemicals disrupt thyroid function either by influencing the synthesis of thyroid hormone or by adversely affecting their peripheral metabolism. Measurement of thyroperoxidase (TPO) activity is considered to be a valuable parameter to detect disruption of thyroid function (Capen, 1998). Frog metamorphic assay is another assay, which is performed in relation to the regression of the tadpoles tail during metamorphosis in *Xenopus laevis.* This test is based on the fact that thyroid hormone

causes the metamorphosis in frogs and the assumption that exposure to the environmental chemicals would interfere with this process. Along with other tests and assays this test is also undergoing validation by EPA (Yaoita & Nakajima, 1997).

2.2.3.3 Analytical methods for the determination of potential EDC in test samples: GC-MS and HPLC

When a single chemical is concerned, then biological assays can yield a reliable information about the endocrine disrupting nature of the test chemical. However, situation becomes complicated when endocrine disruption is induced by complex mixture of chemicals. These sorts of complexities are encountered when sewage water or any industrial effluents are tested for its endocrine disrupting potential. In this case, the biological assays can indicate only the total effect of test sample on the endocrine orchestra of exposed animal but does not explain much about the chemical configuration of test samples and thus the offending chemicals responsible for the EDC nature remain unresolved. This problem can be sorted out by the chemical analysis of the test samples which involves the usage of high performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS). These are the indispensable tool for the analysis of test samples, having the mixtures of many chemicals, which have demonstrated positive responses in biological assays performed for measuring endocrine disruption. Besides, they can also indicate the exact concentration of contaminating chemicals in test samples like natural or synthetic steroids and pesticides (Kumar et al., 2008b).

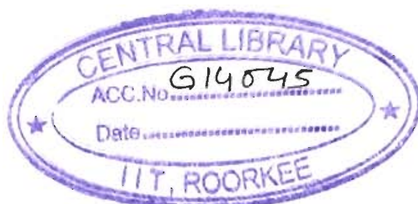
Prior to GC-MS or HPLC analysis, the test samples are subjected to extraction methods so that the concentration of the particular chemical (to be analyzed) increases

and other contaminating chemicals are removed upto the maximum possible extent. The extraction may be either by the use of solid phase extraction method or by the solvent to solvent inter-exchange type (Lee et al., 2005). For example, to analyze the non-polar contaminates in a test sample (viz. sewage water), contaminates are first extracted by a polar solvent like dicholoromethane or non- polar column (e.g. C-18 column) and are finally analyzed by HPLC and GC-MS (Lee et al., 2005; Kumar et al., 2008, 2008b).

2.2.3.3.1 Gas chromatography-mass spectroscopy (GC-MS)

As the name implies, gas chromatography-mass spectroscopy (GC-MS) are practically two techniques that are combined to form a single method of analyzing mixtures of chemicals. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. By combining these two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing a number of chemicals. The GC-MS is used extensively in the medical, pharmacological, environmental, and law enforcement fields.

In general, chromatography is used to separate mixtures of chemicals into individual components. Once isolated, the components can be evaluated individually. In all chromatography, separation occurs when the sample mixture is introduced (injected) into a mobile phase. In liquid chromatography (LC), the mobile phase is a solvent. In gas chromatography (GC), the mobile phase is an inert gas such as helium. The mobile phase carries the sample mixture through what is referred to as a stationary phase. The stationary phase is a usually chemical that can selectively attract components in a sample mixture. The stationary phase is usually contained in a tube of some sort. This tube is referred to as a column. Columns can be glass or stainless steel of various dimensions.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As the individual compounds elute from the GC column, they enter the electron ionization (mass spectra) detector. In latter, they are bombarded with a stream of electrons causing individual compounds to break apart into fragments. These fragments can be large or small pieces of the original molecules and are actually charged ions with a certain mass. The mass of the fragment divided by the charge is called the mass to charge ratio (M/Z). GC-MS has been extensively used for the detection and quantification of numerous natural and synthetic EDC like steroids, industrial chemicals and pharmaceuticals form different sources like urine, blood, sludge, sewage water, industrial effluents (Whorwood et al., 1990; Azevedoa et al., 2001; Hilton and Thomas, 2003; Kumar et al., 2008).

2.2.3.3.2 High Performance Liquid Chromatography (HPLC)

HPLC is a refined technique which works exactly on the basics of the column chromatography, with the difference that its column is formed of higher-quality chromatographic material which can withstand high pressure which makes the chromatographic separation very fast. From the detection point of view, HPLC consists of two parts- the column and detector. The different fractions of the applied samples are retained for a particular time period (retention time), after which they are eluted and finally detected by the detector at a particular wave length. Thus, presence and concentration of a contaminant in any test sample can be known by the use of an appropriate standard. It has been used successfully in various applications like identification of EDC form diverged samples for example blood, sewage water, industrial effluents and milk (Suzuki et al., 2004; Main et al., 2007; Kumar et al, 2008a, 2008b).

2.3 Endocrine Disruptors: Different Sources and Categories (types) and biological relevance

The modern life style compels us to use a number of synthetic chemicals, intentionally or unintentionally, for diverse purposes in different sectors of life ranging from the simple household activities like detergents, cosmetics and toilet articles to specialized applications viz. pharmaceuticals, insecticides and pesticides. Besides, during the manuf acturing of various commercial products, a number of byproducts are released from the industries into air and water. All of these chemicals or their by products may have various, direct or indirect, adverse consequences on the wildlife and human health. One of the prominent side effects of these chemical is their contrary effect on endocrine system, popularly known as endocrine disruption (Keller et al., 1996; Ternes et al., 1999; Roy et al., 2004 and 2006; Roy and Preira, 2005; Rogan and Ragan, 2007; Gore, 2008; Suresh and Sikha, 2008; Kumar et al., 2008). Once any such EDC gets entry into the environment, it may undergo various fates such as: additional distribution between other environmental strata like water, air, and soil/sediment or may be changed into other by products by the action of different environmental agents. The prominent sources and categories of EDC can be listed as follows-

2.3.1 Steroids

The natural steroids are the derivatives of a common precursor, cholesterol, in addition some synthetic as well as some compounds of plant origin (phytoestrogens) also may mimic the structure and effects of natural steroids. Both these classes of steroids whether synthetic steroids or phytoestrogens may add to the menace of endocrine disruption.

Table 1- Mode of actions for various EDC.

Compound	Use	Receptor	References
DES (diethylstilbesterol)	Medicine	ERα, ERβ	Kuiper GGJM et al. (1998), Newbold R. (1995).
Bisphenol A		ERα, ERβ	Kuiper GGJM et al. (1998). Sonnenschein C, Soto AM. (1998)
Nonylphenol		ERα, ERβ	Kuiper GGJM et al. (1998). Sonnenschein C, Soto AM. (1998).
PAHs :HCB, HCH	Pesticide	AhR	Safe S. et al.(1998), Loaiza-Perez AI (1999)
o,p'-DDT		ERα, ERβ	Kuiper GGJM et al. (1998).
p,p'-DDT		ERα, ERβ	Kuiper GGJM et al. (1998).
Methoxychlor		ERα, ERβ	Kuiper GGJM et al. (1998).
Endosulfan		ERα, ERβ	Kuiper GGJM et al. (1998).
Chlordecone		ERα, ERβ	Kuiper GGJM et al. (1998).
p,p'-DDE		AR	Gray LE Jr. et al.(1999a,b) Ostby J et al.(1999)
Linuron		AR	Gray LE Jr. et al.(1999a,b) Ostby J et al.(1999)
Vinclozolin		AR	Gray LE Jr. et al.(1999a,b) Ostby J et al.(1999)
Procymidone		AR	Gray LE Jr. et al.(1999a,b) Ostby J et al.(1999)
PCBs	Industrial waste	ERα, ERβ	Gray LE Jr. et al.(1999b), Kuiper GGJM et al. (1998).
Bisphenol A		ERα, ERβ	Kuiper GGJM et al. (1998). Sonnenschein C, Soto AM. (1998)
4 Tert-octyphenol		ERα, ERβ	Kuiper GGJM et al. (1998). Sonnenschein C, Soto AM. (1998)
4-Octyphenol		ERα, ERβ	Kuiper GGJM et al. (1998). Sonnenschein C, Soto AM. (1998)
TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin).		AhR	Safe S. et al.(1998), Lee et al. (2003).
Nonylphenol			

2.3.1.1 Phytoestrogens

Phytoestrogens are naturally occurring substances having estrogenic activity and belong to flavinoids family of plant secondary metabolites. The main known phytoestrogens are the isoflavones daidzein, β-sitosterol, campasterol, stigmasterol, genistein, formononetin, biochanin A, equol, coumestan, coumestrol, and lignans *seco*-isolariciresinol (SECO) and matairesinol (MAT). Figure 9 shows the structure of some of the common phytoestrogens. Exposure to phytohormones depends either direct on the uptake of plants containing these natural hormones (Adams et al., 1998) or on account of their presence in the effluents of plant based industries like paper and pulp industries (Maria et al., 2004; Nair et al., 2006). They have been detected even in tap water and surface water (Stahlschmidt-Allner et al., 1997; Robakowski et al., 2000). Mazur and Adlercreutz

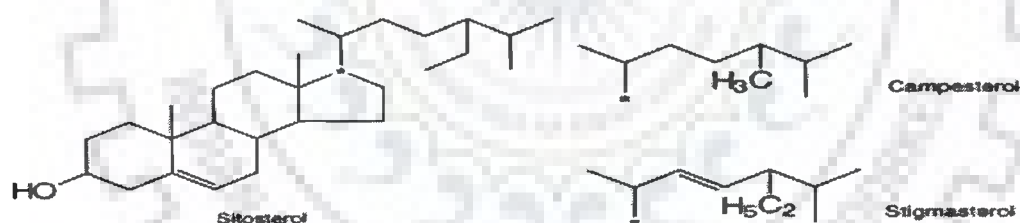


Figure 9. Representative Structure of few common Phytoestrogens

analyzed the phytoestrogen content in many food plants such as legumes, oilseeds, nuts, grains, cereals, vegetables, berries, fruits, and others (Mazur and Adlercreutz, 1998). β-concentrations (Stahlschmidt-Allner et al., 1997) and have been shown to be converted into androgens under environmental conditions (Stahlschmidt-Allner et al., 1997). Since β-sitosterol is used in the biotechnological production of androgens by mycobacteria, it an be assumed that in animals, β-sitosterol can be directly converted to steroid hormones

such as pregnenolone (Liehr et al., 1998). Clover is historically known for containing phytoestrogens like genistein, biochanin A, and formononetin which have been confirmed to have estrogenic potential by *in vivo* studies (Adams et al., 1998; Milligan et al., 1998; Wenzel et al., 2000). Geneistin have been shown to cause malformations in female reproductive system of rats (Schmidt et al, 2006; Jefferson et al., 2007). *In vitro* binding assays showed that many phytoestrogens bind to estrogen receptors as agonists like that of estrogens, but with a lower affinity (Miksicek et al., 1995). β-Sitosterol is capable of inducing vitellogenin in male fish and evokes an estrogenic response in MCF-7 and T47D cells (Mellanen et al., 1996; Tremblay and Van Der Kraak et al., 1999). β-Sitosterol containing paper mill wastewater showed androgenic effects in fish. Female live-bearing Poecilid fish of the genus *Gambusia* developed male reproductive organs. These androgenic effects only appeared after biotransformation of the sterols in wastewater. Diethylstilbestrol (DES) or genistein have been shown to induce uterine adenocarcinoma in neonatal mice and to disrupt testicular steroidogenesis (Newbold, 2001; Adamsson et al., 2008).

2.3.1.2 Synthetic Steroids

The steroids used for curing the complicacies during menopause as well as oral contraceptives (ovulation-inhibiting hormones) can be included in the list of EDC. Widely used synthetic steroids include the norgestrel or norethisterone which are progestagenic component in oral contraceptives and ethinylestradiol and mestranol, the estrogen-like synthetic steroids. Another important group of synthetic hormones are steroids used for growth and milk enhancement in cattle farming. Different regularly used natural and synthetic steroids and phytohormones (viz. estradiol, testosterone,

progesterone, trenbolone and zeranol, derivative of zearalenone) have been shown to induce adverse effects on reproductive system (Römbke et al., 1996; Menendez et al., 2005). Besides synthetic steroids are also excreted with urine along with the natural steroids thus increasing the hormonal load of surface water demonstrating slow rate of degradation as compared to natural steroids (Wenzel et al., 1998; Desbrow et al., 1998; Rombke et al., 1996; Ternes et al., 1999c). Gestational and lactational exposure to ethinyl estradiol (synthetic estrogen) have been shown to produce potent anti-androgenic effects and showed a reduced androgen-dependent reproductive organ weights and epididymal sperm abundance (Howdeshell et al, 2008). Diethylstilbestrol (DES), once considered as an excellent drug for pregnant women to prevent miscarriages, has been banned since it demonstrated a number of health complications in the children whose mother got DES exposure during pregnancy (Gill et al., 1976; Appendix, 1999). It has been shown to function as potent anti-androgen causing gross abnormalities in male reproductive tracts (McKinnell et al., 2001).

2.3.2 Pesticides and antimicrobials-

Pesticides and antimicrobials consist of thousands of chemicals with active ingredients in use. The continuous use of these chemicals has led to their occurrence at detectable concentrations in different places like in ground water and soil, in human blood, milk, various organs and tissues (Hovander et al., 2002; Kolpin et al., 2002; Darbre, 2006; Dayan, 2006; Heidler et al., 2006; Nakada et al., 2006). There are several indications that many pesticides and antimicrobial chemicals may function as potential EDC (Mendola et al., 2008). Among all the pesticides, most notorious are DDT and its dehydrochlorination product DDE. Although DDT was banned in 1970 but still its presence can be detected in

river waters, sea water and even up to distant islands. DDE showed potent anti-androgenic activity in androgen receptor reporter gene assay and has been demonstrated to retard the growth of male sex accessory tissues in weanling rats (Tinwell et al., Xu et al., 2006). Further *in utero* exposure to the DDE indicates its anti-androgenic nature as it decreases anogenital distance in male newborns (Longnecker et al., 2007). *In vitro* binding assays revealed estrogenic potential of DDT and DES or estradiol (McBlain, 1987; Kelace, 1995). Pyrethroids pesticides, another widely used category of pesticides, displayed anti-androgenic activity and significantly suppressed the luciferase expression in reporter gene assay (Kim et al, 2004; Sun et al., 2007). Linuron, a widely used herbicides, has been shown to function as a potent anti-androgen as it causes the regression of sexual organs in male rats and alters the expression of androgen-regulated genes (Cook et al., 1993; Lambright et al., 2000; Lambright et al., 2000). Mtehoxycholr is another insecticide which has been demonstrated to posses endocrine disrupting (estrogenic) activity as it increased uterine weight in uterotrophic assay and increased MCF7-cell proliferation in an E-screen test (Gulden and Turan, 1998).

There are a number of reports which indicate the potent endocrine disrupting nature of several fungicides. In male rat offspring perinatal exposure to vinclozolin (a fungicide) has been shown to produce anti-androgenic effects which are displayed in the form of hypospadias, ectopic testis, vaginal pouch formation, agenesis of the ventral prostate, and nipple retention and affect the hypothalamic-pituitary-gonadal axis (Wong et al., 1995; Shono et al., 2004; Loutchanwoot et al., 2008). It also disturbs the expression of male steroidogenic genes (Nellemann et al., 2003; Mitchell et al., 2005) as well as alters sex-differentiated social play and sexual behaviors in rat (Colborn et al., 2005).

Another fungicide, fenarimol has also been shown to work as potent anti-androgen and reduces weights of sex accessory glands in castrated rats and decreases the expression of androgen-regulated genes (Vinggaard, 2005). Prochloraz, a potent fungicide, also demonstrated antiandrogenic effects in male rats *in utero* (Laier et al., 2006). Similarly, myxothiazol, a fungicide commonly used as preservative has been shown to depress the LH stimulated testosterone synthesis in male rats (Midzak et al., 2007).

Triclosan (TCS; 2,4,4'-trichloro-2'-hydroxydiphenyl ether), a chlorophenol by chemical nature, is an antimicrobial agent widely used as a preservative in toothpaste, soap, shampoo, and cosmetics (Black et al., 1975). It has been included in the probable list of endocrine disruptors on account of its resemblance with known non-steroidal estrogens (e.g. Diethylestradiol, Bisphenol A). The nature of the endocrine disrupting effect of TCS is controversial, while some studies demonstrated it to be an estrogenic (Ishabishi et al., 2004), others report it to act as weak androgen (Foran et al., 2000) and still others report it as anti-androgen (Chen et al., 2007). Fourteen days exposure of TCS in Japanese medaka fry (*Oryzias latipes*) showed a weak androgenic effect instead of any estrogenic effect (Foran et al., 2000). On the other hand, Ishibashi et al. (2004) reported that the metabolite of TCS may be behave as weak estrogen with the potential to induce vitellogenin in male medaka while in female medaka it decreased the hatchability as well as delayed the hatching (Ishibashi et al., 2004).

2.3.3 Industrial Chemicals

A number of industrial chemicals are used for various applications and many of these products may behave as EDC. Some of the commonly used industrial chemicals, having the potential to behave as EDC, are phthalates used in cosmetics, polychlorinated

biphenyls used in electrical appliances, dioxins byproducts formed during formation of herbicides, polyaromatic halogens formed during incomplete combustion of fossil fuels, different pharmaceuticals and so on (Lintelmann et al., 2003). The prominent industrial chemicals, demonstrated to behave as EDC, are listed as follows-

2.3.3.1 Phthalates

Phthalates are the chemicals used as plasticizing agents in cosmetics and other commercial preparations. These chemicals or their derivatives have been detected in the urine of men and women (Becker et al., 2004; Koch et al., 2004, 2005b; Koch and Angerer, 2007). Several reports indicate the anti-androgenic nature of these compounds e.g. their inhibitory effects on testicular steroidogenesis and development of male reproductive system (Akingbemi et al., 2001; Fisher, 2004; Foster, 2006; Hallmark et al., 2007; Matsumoto et al., 2008). However, in MCF-7 cell proliferation assay phthalates showed estrogenic effect (Hong et al., 2005).

2.3.3.2 Bisphenol

Several reports demonstrate that Bisphenol A is a potent EDC. It belongs to the bis(hydroxyphenyl)methane group of chemicals used worldwide. It is used for several application: as an intermediate in the manufacture of polymers; epoxy resins; polycarbonates; fungicides; antioxidants; flame retardant and dyes; (Health Council of The Netherlands, 1999). Besides, it is also used as a resin in plastic dental fillings and in the inner coating of food cans indicating the possible mode of transmission of bisphenol A to food chain (Franse and Voogt, 1997). Recent reports have indicated that bisphenol can affect secondary sexual organs and reduce sperm count in rats (Nagel et al, 1997; Saal et al., 1998). Many recent studies has confirmed that bisphenol demonstrates both

anti-androgenic and estrogenic actions (Perez et al., 1998; Kloas and Einspanier, 1999; Lindholst et al., 2000; Pawlowski et al., 2001; Sanger et al., 2001; Andersen et al., 2006; Xu et al., 2006).

2.3.3.3 Polychlorinated dibenzodioxins and furans

The polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are two chemically similar groups of chlorinated aromatic compounds. Both the PCDDs and PCDFs are not main commercial products, but are the byproducts formed during diverse industrial operations (e.g., chlorine synthesis, production of hydrocarbons etc.), during pyrolysis and uncompleted combustion of organic materials in the presence of chlorine. All these operations form mixtures of different PCDD and PCDF congeners, which are characterized by a special pattern typical for the respective generation process. The experiments have demonstrated that these chemicals functions as anti-estrogens and reduce uterine weights and down regulates the number of estrogen and progesterone receptors in organisms (Krishnan and Safe, 1993; Ahlborg et al., 1995; Romkes et al., 1998). In MCF-7 cell lines, TCDD inhibits the estradiol-induced cell proliferation and progesterone biosynthesis (Biegel and Safe, 1994).

2.3.3.4 Alkylphenol ethoxylates

Alkylphenol ethoxylates (APEs) are used during production of phenol resins, as plastic additives, emulsifiers, wetting agents, dispersing agents in household products, in agricultural and industrial applications, and as spermicides in contraceptive applications. Their alkyl group is branched having nonyl, octyl, or dodecyl chain, and accordingly they are chemically known. Nonylphenol ethoxylates (NPEs) are the most common of the APEs, constituting approximately about 82 % of the world production of APEs. APES, as

such are not harmful but their derivatives such as *p*-octylphenol and *p*-nonylphenol are potential EDC. These metabolites are formed from APEs during the treatment in wastewater treatment plants and by microbial degradation in the environment. APEs have been frequently detected in human blood, urine, mussels and fetal tissues, (Ahel et al., 1993; Thomsen et al. 2001). 4-octylphenol (4-*tert*-OP) and 4-nonylphenol have been shown to be potent anti-androgen as they inhibit the effect of testosterone (Vom Saal and Hughes 2005; Xu et al., 2005). In caged male rainbow trout, exposure to 4-octylphenol (4-*tert*-OP), 4-nonylphenol, 4-nonylphenol acetic acid, and 4-nonylphenoldiethoxylate induced the production of vtg. NP has also been shown to induce estrogenic effects in ***in vivo*** and ***in vitro*** studies (Soto et al., 1995; Shelby et al., 1996; Lee et al., 1996; Alonso-Magdalena et al., 2006).

2.3.3.5 Biphenyls

Biphenyls serves as the precursors for the synthesis of hydrocarbons (with hydroxy or chlorine groups) used for different industrial purposes like as educts for plasticizers, pesticides, and disinfectants. Besides, mono- and dihydroxy biphenyls are also generated during the degradation of biphenyls. On account of being highly chlorinated, PCBs are persistent compounds and accumulate heavily within the food chain (Gülden et al., 1998). They are released into the environment due to inappropriate disposal, accidents, and leaks from industrial facilities, leakage from old equipment, building materials, stockpiles, and landfill. Several reports confirmed the endocrine disrupting nature of these groups of chemicals for example adverse effect on male reproductive system, increased production female offspring in turtles (Bergeron et al., 1994; Soto et al., 1995; Korach et al., 1998; Pflieger-Bruss et al., 2006).

2.3.3.6 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are produced during incomplete combustion operations of organic matter (e.g., coal, oil, petrol, wood), occurring either from anthropogenic sources or natural sources like natural combustions such as forest fires and volcanoes. It's noteworthy that the most PAHs investigated have shown mainly estrogenic properties (Schneider et al., 1976) and only some compounds like methylcholanthrene, benzo[*a*]pyrene, 1,2-benz[*a*]anthracene, and 7,12-dimethyl-1,2-benzo[*a*]anthracene showed anti-estrogenic effects in *in vitro* tests (Chaloupka et al., 1992).

2.3.4 Anti-androgenic Mixtures (Combinational Effect)

Berenbaum (1981), Loewe and Muischnek (1986), Nellemann et al. (2003) found that procymidone and vinclozolin, both AR antagonists, additively inhibited testosterone binding to the AR. Administration of a 1:1 mixture of both fungicides to castrated and testosterone treated male rats led to dose-additive alterations in reproductive organ weights, androgen levels and AR-dependent gene expression. An equimolar mixture of the three pesticides deltamethrin, methiocarb and prochloraz additively suppressed AR activation *in vitro* (Birkhoj et al., 2004). In a separate experiment, a combination of these three chemicals along with simazin and tribenuronmethyl to castrated testosterone-treated rats, showed changes in the weight of adrenal gland, levator ani muscle, and an alteration in expression of AR-associated genes. The effects shown by the combination of all these five chemicals were not found when they were tested individually; however, their additive effects could not be assessed (Kortenkamp, 2007). A mixture of the AR antagonists, procymidone and vinclozolin, was evaluated in the Hershberger assay, where

they acted additively in reducing ventral prostate and levator ani weights (Gray et al. 2001). A combination of procymidone and dibutyl phthalate, an inhibitor of androgen synthesis, significantly enhanced the occurrence of hypospadias in male offspring when given to pregnant rats during gestational days 14–18 (Gray et al. 2001). Wolf et al. (2004) observed that vinclozolin and testosterone proprionate, two chemicals with opposing effects on male sexual differentiation, antagonized each other during sexual development of the male rats. A mixture of butyl benzyl phthalate, an inhibitor of testosterone synthesis, and linuron, an AR antagonist, decreased testosterone production and caused alterations of androgen-organized tissues in a dose-additive fashion (Hotchkiss et al. 2004). Jarfelt et al. (2005) studied changes in anogenital distance and retained nipples of male rat offsprings when treated with di-(2-ethylhexyl) phthalate and di-(2-ethylhexyl)adipate, but the effects of the mixture were not different from those of each chemical alone.

2.3.5 Pharmaceutical Products

Pharmaceuticals are the therapeutics that includes drugs, antibiotics, hormones, and veterinary feed additives and many of them have been shown to behave as EDC (Ingerslev et al., 2003; Lintelmann et al., 2003). Some of the very commonly used non-steroidal anti-inflammatory drugs (NSAIDs) and antibiotics have been included in the list of probable EDC (Ingerslev et al., 2003; Hontela, 2006). Ibuprofen, a potent NSAID, has been shown to block the effects of the estrogen agonist, tamoxifen and 17ß-estradiol in a study related to bone metabolism in rats, thus confirming its anti-estrogenic action (Sibonga et al., 1998). In rainbow trout administration of ibuprofen disrupts steroidogenesis in the inetrrenal tissues, an organ homologous to the adrenal gland of

mammals (Gravel and Vijayan, 2006). Similarly oxytetracycline has been shown to be anti-estrogenic causing developmental toxicity and decreased uterine weights in treated CD rats and CD-1 mice (Morrissey et al., 1986).

2.3.6 Endocrine Disruptors vs. aquatic system: sewage water and industrial effluents

All the chemicals or their degradation products, depicted to behave as EDC in the foresaid section, are released with the industrial effluents or sewage drainage, depending on the site of their usage, and finally reach to the open water bodies. One of the potential water bodies to receive EDC is waste water treatment plant (WWTP).

WWTP receives a broad spectrum of molecules from domestic and/ or industrial wastes, which, are not eliminated completely during the treatment process (Ternes et al., 1999; Hovander et al., 2002; Cargouët et al., 2004; Darbre, 2006; Heidler et al., 2006; Kumar et al, 2008a, 2008b). WWTP discharges are considered a major source of EDC rich pollution that plays a significant role in environmental contamination. Several studies have reported the correlation between reproductive abnormalities in fish and exposure to WWTP effluents even several kilometers downstream from outfalls (Jobling et al., 1998; Sumpter, 1998) and these are required in a minor quantities to cause endocrine disruption (Sarmah et al., 2006). For instance, concentrations as low as 1 ng/l of estrogen led to induction of vitg in male trout (Hansen et al., 1998). Similarly Metcalfe et al. (2001) observed the formation of ova in the testis of Japanese medaka even at a concentration of 4 ng/l of estradiol and 0.1 ng/l of 17α-ethnylestradiol. All these indicate that even a minor quantity of the contaminants is sufficient to bring about changes in the endocrine physiology of wild life and human beings. Domestic, workplace and industrial discharges contribute a vast quantity of human excreta,

detergents and other substances (pharmaceuticals) into the nation sewers. These discharges are also a possible source of contamination. In order to evaluate the potential risk of the endocrine disruption, the occurrence of individual compounds needs to be documented. Numerous studies have been reported to detect and quantify the estrogenic potency of water samples either by targeting their research on specific molecules, such as natural (E1, E2 and E3) and synthetic (EE2) hormones and/ or alkylphenols (Barontoni et al., 2000) or by evaluating estrogenic activities (Gray et al., 1997). However, except for a few reports, little is known about the androgenic contaminants in the WWTP effluents and there is a need to consider this effect in environmental risk assessment (Svenson and Allard 2005; Leusch et al., 2006a; Subramanian and Amutha, 2006). Further, besides hormonally active substances, sewage water also consists of many toxic substances like nitrates, phenols which are toxic to the human/animal health There are a number of reports which suggests histopathological malformagtions in the different organs like kidney and liver of animals exposed to sewage water (Bucher and Hofer, 1993; Bernet et al., 2004).

In addition, various industrial effluents are of great concern to agencies responsible for environmental management. For example, leather industry effluents remains heavily polluted with chemicals like formaldehyde, chromates and bichromate salts, aniline, benzene based dyes, other different organic chemicals (butyl acetate, ethanol, benzene, toluene, nonylpenols, polychlorinated phenols), dimethyl formamide, sulphuric acid, ammonia, hydrogen sulphide and others (Levin et al., 1987; Sole et al, 2000). Some of the authorities consider the leather industrial effluent to be one of the ten most harmful industrial effluents to the environment, responsible for extreme pollution of

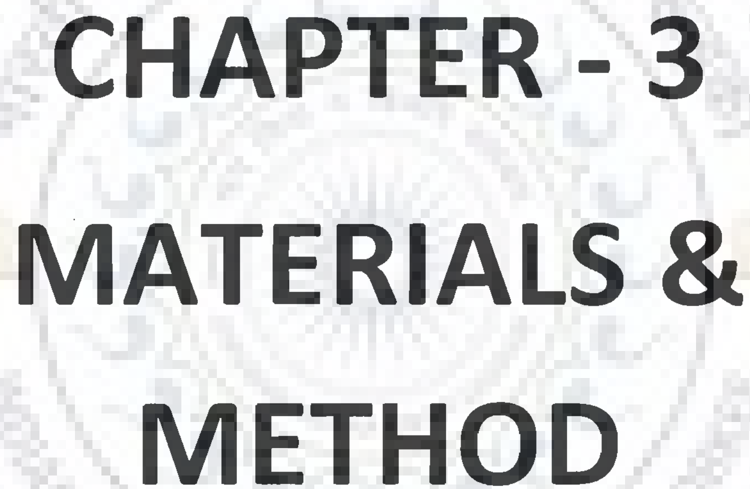
water resources and generating substances leading to disease and death of wide range of organisms (Aragon, 1990; Junior et al., 2007). Similarly paper and pulp effluents have showed to be heavily loaded with a number of phytoestroegens that have been reported in the earlier section as potent EDC once entered into the food chain (Wei and Jianying, 2006).

Concluding remark

Environmental toxicology studies have revealed that EDC could be real threats to human health and wild life in the near future. Although concerns have been raised among scientists and public officials, many countries in the world have yet to take serious action to handle this environmental problem. One impediment to achieve proper management of this major problem is the general belief that environmental endocrine disruptors, particularly those that are steroid hormone agonists and antagonists, are less potent than their natural counterparts, i.e. endogenous sex steroids and therefore do not have the capacity to cause health-threatening effects. However, it has been shown that when these EDC act through the same receptor pathway as endogenous sex steroids, they produce a magnified response. For example, xenoestrogens, which have been deemed of low potency relative to estradiol, are able to shift the dose response curve for estradiol to the left. Moreover, endogenous hormones and their environmental mimics show non-monotonic dose-response curves resulting in different effects at low and high doses. Considering these facts, it seems that the endocrine disruptors are not the class of chemicals that can be ignored and therefore proper risk assessment strategies for potential adverse effects on human health need to be established.

CHAPTER - 3

MATERIALS & METHOD



CHAPTER 3: MATERIALS AND METHODS

3.1Material

The routine chemicals used in this thesis were purchased from the SRL, Mumbai; SD fine Chemicals, Mumbai and Rankem Mumbai. The chemicals and enzymes used in the molecular biology were purchased from Bangalore Genei, Bangalore and Genescript, Bangalore. The medium used in the cell culture was obtained from Himedia India. The kits used for ELISA were obtained from DRG Diagnostics, Germany and Transasia Biomedical, Mumbai. Enzyme assay kits were obtained from Calbiochem, USA. Radioisotopes were obtained with the kind assistance of Dr. Subeer Majumder, Scientist VI National Institute of Immunology, Delhi.

3.2 Methodology

3.2.1 Samples Preparation

In our study, investigations were carried out on six samples viz. TCS, ibuprofen, tetracycline, leather industry effluent, sewage inlet and outlet water and HCB. Out of these samples, first four were used in the form of pure chemicals (at the concentrations described in the animal treatment sections) while the sewage inlet and outlet water and leather industry effluents were used in the form of crude non-polar extracts.

3.2..1.1 Sewage water samples

Samples were collected monthly from May to November 2006, in amber colored glass bottles, rinsed initially with acetone (SRL Chemicals, Mumbai, India) and MilliQ water (Millipore, India) from the inlet and final effluent site of a WWTP and stored at 4 ^{o}C. A total of 250 l of water sample was collected and extraction was performed within 48 h of sampling. All the glassware to be used in the experiment was rinsed with

dichloromethane (DCM) (SRL Chemicals, Mumbai, India) with 0.6% concentrated HCl to prevent the clinging of the steroids to the glass walls. Samples were collected in amber colored glass bottles, immediately placed on ice and brought to the laboratory. Collected water samples were aliquoted into four parts viz. 24, 48, 72 and 96 l. All sample aliquots were filtered and were subjected to organic phase extraction by adding DCM according to the method described earlier (Soto et al., 2004) with slight variations as per our laboratory conditions. For the organic phase extraction, DCM was added to each aliquot at the ratio of 60 ml/l of crude sample and mixed thoroughly for 2 min, left for 10 min for settling down of organic phase and finally the aqueous phase was separated by separating funnel. The procedure was repeated thrice to extract organic phase and all aqueous phase was poured off. Extracted organic phases were mixed and concentrated under reduced pressure on a Bucchi rotatory evaporator to 2 ml/l of crude sample and concentrated organic phase was solvent exchanged with 10% ethanol. At the final preparation, single dose per animal consisted of 150 μl of prepared sample and this single dose per animal was equivalent to 200, 150, 100 and 50 ml for 96, 72, 48 and 24 l of crude water samples respectively. Prepared doses were stored at -20 °C until gavaged to animals.

3.2.1.2 Leather industrial effluents

Samples were collected monthly from December to June, 2007, in amber colored glass bottles, rinsed initially with acetone and MilliQ water from the site receiving effluent from a number of leather industrial units and stored at 4 °C. The total samples collected during this period were pooled together to reduce the chances of variations arising during sampling. Collected water samples were aliquoted into three parts viz. 18, 36 and 72 l. All sample aliquots were filtered and subjected to organic phase extraction by adding

DCM according to the method described in the case of sewage water. At the final preparation, single dose per animal consisted of 150 μl of prepared sample and this single dose per animal was equivalent to 300, 150, and 75 ml for 72, 36 and 18 L of crude water samples respectively. Prepared dose was stored at -20 °C until gavaged to the animals.

3.2.1.3 Triclosan, Ibuprofen, Tetracycline and Hexachlorobenzene

A uniform suspension of all of these chemicals was made in phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), prepared fresh everyday immediately before the treatment according to the concentration levels as described in the treatment section.

3.2.2 Experimental Models (Animals)

The *in vivo* study was performed using adult male and female albino rats, *Rattus norvegicus,* of age group almost 6-7 weeks. Animals were purchased from the animal house facility of All India Institute of Medical Sciences (New Delhi, India) and Zamia Hamdard University (New Delhi, India) and were in healthy condition at the time of purchasing. They were housed in a well-ventilated animal house at a temperature of 22–23 °C, humidity 50–55% and lighting cycle of 14 h light: 10 h dark. The animals were fed with a balanced animal feed (Ashirwad Animal Feed Industries, Punjab, India) and had access to hygienic drinking water *ad libitum.* The animals were acclimatized to the animal house condition for 10 days prior to beginning of each of the experiment. All the procedures were approved by the Institutional Animal Ethics Committee and confirmed to the UFAW Handbook on the Care and Management of Laboratory Animals.

For Hershberger and uterotrophic assay male and female rats were castrated (by removing testis and epididymis) or ovarectomised (by removing ovary) respectively at

the age of almost 6 weeks and recovered for 10 another days before initiating the experiment.

3.2.3 Treatment

The study was carried out in two parts – first part dealt with Hershberger and uterotrophic assay to screen the test samples for their (anti)androgenic and (anti)estrogenic potential and second part dealt with analyzing the mode of endocrine disruption of test samples in intact male and female rats. Different dose levels, used for various samples, were below LD_{50} according to the information available in the literature and as worked out by us.

3.2.3.1 Triclosan (TCS)

Both castrated and ovarectomised as well as intact male and female rats were gavaged orally with the three dosage levels of TCS (5, 10 and 20 mg/Kg of body weight per day, mg/Kg/day) for a period of 60 days. All the three doses selected were found to be below LD_{50} as demonstrated by earlier studies (Bhargava et al., 1996). A uniform suspension of TCS in PBS was made fresh everyday just before intubations and 200 μl of each was used.

3.2.3.1.1 Screening of TCS for anti-androgen and anti-estrogen activity

Both castrated and ovarectomised rats were grouped (n = 8) as follows

Group Ia and Ib: Treated with 10% alcohol (control).

Group IIa and IIb: Positive controls for castrated and ovarectomised rats i.e. receiving the subcutaneous injections of testosterone propionate (0.4 mg/Kg/day) and estradiol (0.2 mg/Kg/day) respectively.

Group IIIa and IIIb: castrated and ovarectomised rats treated with 5 mg/Kg/day of TCS.

Group IVa and IVb: castrated and ovarectomised rats treated with 10 mg/Kg/day of TCS.

Group Va and Vb: castrated and ovarectomised rats treated with 20 mg/Kg/day of TCS.

Animals in group “a” indicate male rats while in “b” indicates female rats.

Rats in group III-V received 0.4 mg/Kg body weight of TP and estradiol (0.2 mg/Kg/day) in castrated and ovarectomised rats respectively along with TCS.

The test chemical was gavaged daily for 60 days to each group. Approximately after 24 h of final treatment, rats were sacrificed; testis were collected, weighed and immediately placed in liquid nitrogen till further analysis. Testis and androgen-dependent SATs (seminal vesicles, cauda epididymis, vas deferens and prostate) from male rats and uteri from female rats were carefully removed, weighed and documented by photography.

3.2.3.1.2 Intact assay

Rats were grouped (n = 8) as follows and treatment was initiated when rats were around 10 weeks old:

Group I: Treated with 10% alcohol (control).

Group II: Treated with 5 mg/Kg/day of TCS.

Group III: Treated with 10 mg/Kg/day of TCS

Group IV: Treated with 20 mg/Kg/day of TCS

The test chemical was gavaged daily (single time a day) by the intraperitoneal injections and treatment was continued for 60 days. Approximately after 24 h of final treatment, rats were sacrificed and testis, ovaries, liver and hypothalamus were collected, weighed and immediately placed in liquid nitrogen till further analysis. In addition Liver, kidney, testis, and androgen-dependent SATs (seminal vesicles, cauda epididymis, vas deferens, prostate and uteri) were carefully removed, weighed and were fixed in Bouins solution for immunohistochemical and histopathological studies.

3.2.3.2 NSAID and antibiotic

For this section we selected two well known synthetic analogs depending on their frequency of applications in pharmaceutical industry and their probable potency to behave as EDC. The analogs under study were– ibuprofen (NSAIDs) and tetracycline hydrochloride (antibiotic) and the three dosage levels were decided for the administration of each of the test chemicals to castrated and ovarectomised rats. The highest dosage level for ibuprofen, 40 mg/Kg of body weight (mg/Kg/day) was adopted from earlier literatures (Richardson *et al.*, 2002). Two more dosage levels used for this chemical were 10 and 20 mg/Kg/day. On the other hand, tetracycline was gavaged initially at a dose of 1350 mg/Kg/day according to an earlier report (Morrissey *et al.,* 1986) and then scaled down to 500 mg/Kg/day through 700 mg/Kg/day. Two more dosage levels for tetracycline were 125 and 250 mg/Kg/day. Daily a uniform suspension of each of these chemicals was formed in PBS just before administration.

3.2.3.2 1 Screening of test samples for anti-androgen and anti-estrogen activity

Both the castrated and ovarectomised rats were grouped (n = 8) as follows:

Group Ia and Ib: castrated rats (a) and ovarectomised (b) rats respectively as control without any treatment.

Group IIa and IIb: Positive controls for castrated and ovarectomised rats i.e. receiving the subcutaneous injections of testosterone propionate (0.4 mg/Kg/day) and estradiol (0.2 mg/Kg/day) respectively.

Group IIIa and IIIb: Castrated and ovarectomised rats respectively treated with varying doses of ibuprofen (10, 20, 40 mg/Kg/day).

Group IVa and IVb: Castrated and ovarectomised rats respectively treated with varying doses of tetracycline hydrochloride (125, 250, 500 mg/Kg/day).

The castrated and ovarectomised rats in group III and IV received 0.4 mg/Kg/day of TP and estradiol (0.2 mg/Kg/day) respectively along with the test drugs. Doses were administered via gavaging for 14 consecutive days. Approximately after 24 h of final treatment, androgen-dependent SAT (seminal vesicles, cauda epididymis, vas deferens and glans penis) and uteri were carefully removed, weighed and were documented by photography.

3.2.3.2. 2 Intact assay

Intact rats were grouped (n = 8) as follows and treatment was initiated when rats were 10 weeks old:

Group I: Intact male and female rats used as control.

Group II: Intact male and female rats treated with ibuprofen (40 mg/Kg/day).

Group III: Intact male and female rats treated with tetracycline hydrochloride (500 mg/Kg/day).

Doses were administered for a period of 14 days. After final dose, rats were sacrificed and testis, ovaries, liver and hypothalamus were collected and immediately placed in liquid nitrogen till further analysis. In addition, liver, kidney, testis, and androgen-dependent SATs (seminal vesicles, cauda epididymis, vas deferens, prostate and uteri) were carefully removed, weighed and were fixed in Bouins solution for immunohistochemical (IHC) and histopathological studies.

3.2.3.3 Leather Industry Effluent

All of the animals were provided with three doses of leather industry effluent according to the following plan:

3.2.3.3.1 Screening of test samples for androgenicity by Hershberger assay and uterotorphic assay

Castrated and ovarectomised rats were divided into four groups each (n = 8) out of which one was kept as control while others were gavaged orally with 75, 150 and 300 ml equivalent of leather industry effluents sample respectively, for a period of 20 days. The doses used in the experiment were optimized earlier and were below the LD_{50} concentration. Approximately after 24 h of final treatment, SATs viz. ventral prostrate, seminal vesicles, glans penis, vas deferens, cowper's gland and uteri were carefully removed and weighed and documented by photography.

3.2.3.3.2 Intact assay

Intact male and female rats were divided into four groups each (n = 8) out of which one group represented control while others were gavaged orally with 75, 150 and 300 ml equivalent of leather industry effluents samples respectively for a period of 20 days. After final dose, the rats were sacrificed and testis, ovaries, liver and hypothalamus from each of control and treated group were collected and immediately placed in liquid nitrogen for further analysis. In addition liver, kidney, testis, SATs and uteri from the same groups were weighed and fixed in the Bouins solution for histopathological studies.

3.2.3.4 Sewage water

All of the animals were provided with four doses of each of sewage inlet and outlet water as described below-

3.2.3.4.1 Screening of test samples for androgenicity by Hershberger assay and uterotorphic assay.

The animals were grouped (n = 8) as follows:

Group Ia and Ib: castrated and ovarectomised rats respectively; treated with only alcohol (10%) as vehicle (control).

Group IIa and IIb: castrated and ovarectomised rats respectively; treated with 50 ml equivalent of inlet (IIai, IIbi) and outlet (IIaii and IIbii) WWTP sample respectively.

Group IIIa and IIIb: castrated and ovarectomised rats respectively; treated with 100 ml equivalent of inlet (IIIai, IIIbi) and outlet (IIIaii and IIIbii) WWTP sample respectively.

Group IVa and IVb: castrated and ovarectomised rats respectively; treated with 150 ml equivalent of inlet (IVai, IVbi) and outlet (IVaii and IVbii) WWTP sample respectively.

Group Va and Vb: castrated and ovarectomised rats respectively; treated with 200 ml equivalent of inlet (Vai, Vbi) and outlet (Vaii and Vbii) WWTP sample respectively.

Animals in group "a" indicate male rats while in "b" indicate female rats and (i) and (ii) indicates inlet and outlet samples repectively.

Vehicle and extracted leather industry effluents sample were administered via gavaging to 52 days old castrated and ovarectomised rats for 20 consecutive days. The doses used in the experiment were optimized earlier and were below the LD_{50} dose. After approximately 24 h of final treatment, SATs from the male rats and uteri from the female rats were carefully removed, weighed and some of these were documented by photography.

3.2.3.4.2 Effects of WWTP effluents in intact rats.

A similar scheme of treatment was followed in intact male and female rats (n = 8) as that for castrated and ovarectomised animals (as described above).

After final dose, the rats were sacrificed and testis, liver, hypothalamus and ovaries from control and treated groups of both inlet and outlet water samples were collected and immediately placed in liquid nitrogen for further analysis. In addition, hypothalamus, liver, kidney, SATs and Uteri from the same groups were weighed and fixed in the Bouins solution for histopathological studies.

3.2.3.5 Hexachlorobenzene (HCB)

The characterization of HCB induced endocrine disrupting effects was carried out using both *in vivo* and *in vitro* studies.

3.2.3.5.1 In vivo study: Hershberger and Intact assay

Castrated and intact male albino rats were gavaged with three doses of HCB viz. 1, 2 and 4 mg/Kg/day (a uniform suspension was prepared fresh in PBS daily, just before treatment) for a period of 20 days. After final dose, all control and treated rats were sacrificed; testis, liver and hypothalamus were collected and immediately placed in liquid nitrogen till further analysis. In addition liver, kidney, testis, and androgen-dependent SATs were carefully removed, weighed and were fixed in Bouins solution for histopathological studies.

3.2.3.5.2 In vitro study: HCB Treatment of Leydig Cells

The isolated Leydig cells (as described in the latter section of this chapter) were plated in 24 well plates (Axygen, India) ($1X10^5$ cells/well) at a volume of 1 ml per well. Throughout the experiment, culture conditions (if otherwise not stated) consisted of M-199 medium supplemented with 2.2 g/l $NaHCO_3$, 2.4 g/l HEPES, 0.1% BSA, 0.25 g/l

bovine lipoprotein and 25 mg/l gentamicin, pH 7.4 for 2 hours at 34 ^{0}C in 5% CO_2. After 24 h of plating, the cells were treated with varying concentrations of TCS (0.001, 0.01, 0.1, 1.0 and 10 μM) for 2 h in the presence and absence of LH (100 ng/ml) or various other test chemicals.

32..4 Courtship behavior

The time dependent changes in the sexual behavior of the male rats, following their exposure to test samples, were studied by placing the single male rat at a time with female rat (in proestrous phase as determined with microscopic examination of vaginal smears) in a specially designed sex behavior analysis chamber (Fig 10). The attempt of male rat to invade into the female rat chamber was considered as an approach of

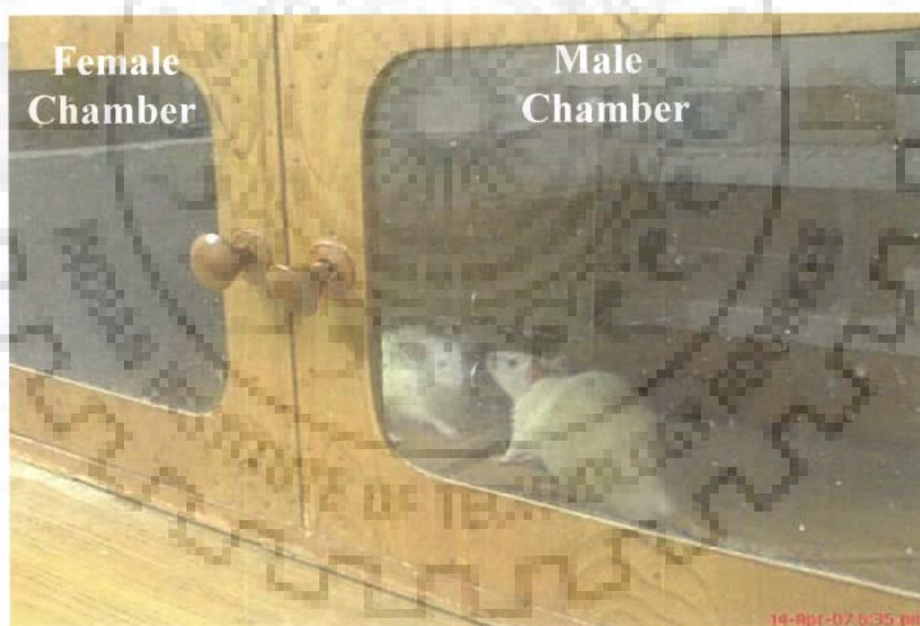


Figure 10: The chamber used to analyze the effect of test sample induced changes in the courtship behavior in male rats. At a time, individual male and female rats were placed separately in the two chambers of the box arbitrarily designated as 'male' and 'female' chamber of the box. The number of times the male rats approaching the female rats were scored as "number of attempts".

courtship behavior and the experiment was repeated for all the rats of control and treated groups. The average of the number of attempts shown by all the male rats of a group, on any particular day, was counted as an attempt of that group on that day. A group of separate female rats were devoted for courtship behavior analysis and they were selected at random and used only single time a day for the analysis to avoid the experimental error caused by the courtship behavior induced changes in the hormonal and pheromonal level in female rats.

3.2.5 Daily sperm production (DSP)

Testicular sperm content and DSP/gm testis were determined as per the methods described earlier with slight modifications (Robb et al., 1978; Cooke, et al., 1993). Briefly, the testis of control and all the treated groups were weighed and homogenized for 3 min in 25 ml of PBS containing 0.05% (v/v) Triton X- 100 (Sigma, St. Louis, MO, USA) using a semimicro waring blender. Step 14-16 spermatids (stages II-VIII) survived this homogenization and their nuclei could be counted using a hemocytometer to determine average number of spermatids per sample. These values were used to obtain total number of spermatids per testis; this was then divided by the testis weight to determine the number of spermatids per gram of testis. Developing spermatids spend 4.84 days in steps 14-16 during spermatogenesis in rats (Oakberg et al., 1956). Thus, the values for the number of spermatids per testis and spermatids per gram testis were divided by 4.84 to obtain DSP and efficiency of sperm production (DSP/g testis), respectively (Oakberg et al., 1956).

3.2.6 Sperm structural analysis

This analysis was done to observe the toxic effect of test samples at the level of sperm's superficial structure; and the analysis involved fluorescent staining of sperms based on the method as described by Lee et al. (1987). Sperms were collected from fresh epididymis with the gentle flow of PBS and 2.5 μl sperm suspension was placed on a slide at 37 ^{0}C followed by the addition of chlorotetraccyline (CTC) (5 mM CTC, 20 mM Tris, 5 mM cystine and 130 mM NaCl; pH 8.0). Multiple slides were formed according to the method described as follows; within 10 seconds, 0.25 μl glutaraldehyde fixative (Himedia, India) was added and mixed with tip of micro-syringe dispenser. The slide was transferred to a humified chamber until ready to use and was observed under fluorescent microscope. The sperm suspension as described earlier was also stained by eosine staining by simply placing 20 μl of sperm suspension with diluted solution of eosine stain for 5 minutes and then observing the sperms under microscope using 100x objective.

3.2.7 ELISA to measure concentration of various hormones in animal sera and culture medium

For the determination of serum hormones, blood was collected by cardiac puncture from all intact male and female rats on completion of the treatment and allowed to clot at 4 ^{0}C overnight. The serum was then aspirated, centrifuged at 2000Xg for 10 min and the clear supernatant was used for hormone assays. The assays were performed using the commercial enzyme immunoassay kits as per manufacturer's instructions (Omega Diagnostics, UK and Transasia Biomedical, Mumbai, India). The hormones that were assayed included; cholesterol, pregnenolone, testosterone (male rats), estradiol (female rats), luteinizing hormone (LH) and follicle stimulating hormone (FSH). The intrassay

and interassay coefficient of variations for all the assays were below 6 and 12% respectively and the assays were highly specific for each protein/steroid hormones tested.

To observe the effect of HCB on testosterone production, testosterone level in the medium (after 2 hrs culture of Leydig cells with or without maximally stimulating LH) was assayed using the commercial enzyme immunoassay kits as per manufacturer's instructions (Omega Diagnostics, UK). Each experiment was performed in quadruplicates to avoid statistical errors and cells having 1% ethanol in the medium were used as vehicle treated control, throughout the study.

3.2.8 Histopathology of various tissues

For histopathological staining, the basic protocol by Mukherjee et al. (2003) was followed with modifications according to our laboratory conditions. Briefly, the liver, kidney, testis, ovary, SATs and uterus were separated and fixed in Bouins solution (saturated aqueous solution of picric acid, 75%; Formalin, 25%; glacial acetic acid, 25%) for 4 h. Following the fixation, sections were washed thoroughly in 30% alcohol until all the colour of picric acid disappeared. This was followed by gradual dehydration of the samples by placing them in ascending series of alcohols (each change for 1 hr) viz. 30% - 50% - 70% - 80% (2 changes) - 95% (2 changes) - 100% (3 changes) – xylene (3 changes). The tissues were then placed in wax, made into blocks and finally cut into sections of 5 micron thickness. Sections were adhered on the glass slide treated with Mayer's solution (per 100 ml having 50 ml egg albumin, 50 ml glycerin, 1.0 gm sodium salicylate,), stretched at approximately 40 ^{0}C temperatures and preserved. The sections were then stained in haematoxylin and eosin by placing the slide in different solutions in a sequential manner as described below:

xylene (2 changes, each 5 min) followed by 1 min change each in 1:1 (v/v) xylene and 100% alcohol - 100 % alcohol (2 changes) – 90% alcohol – 80% alcohol – 70% alcohol – washing in tap water followed by gentle tapping of slide – haematoxylin solution (5min) – washing in running water – 3 to 4 dippings of slide in 0.5% HCl – wash in distilled water (1 min) – several dippings in ammonia water (section changes to blue color) - wash in distilled water (1 min) – 80% alcohol (1 min) – 95% alcohol (1 min) – eosine staining (2 to 3 min) – 70% alcohol – 95% alcohol– 100% alcohol (1 min each) – xylene (two changes, 1 min each). This was followed by the mounting of tissue with canada balsam and preservation with cover slip.

3.2.9 Immunohistochemical analysis (IHC)

Testicular sections or isolated Leydig cells were subjected to StAR IHC analysis by the procedure described by Kroft et al., (2000) with some modifications as standardized in the lab. For testicular sections, the paraffin sections of the testis of control and treated rats were prepared according to the procedure described in the earlier section and then deparaffinized by incubating twice in xylene (5 min each time) and then rehydrated by incubating twice, for each time, 3 min in 100% (v/v) ethanol, 3 min in 95% ethanol, 20 min in 70% ethanol containing 1% (v/v) H_2O_2 (to inactivate endogenous peroxidase activity), 20 min in 70% ethanol saturated with Li_2CO_3 (to neutralize picric acid from the fixative), 3 min in 50% ethanol, 10 min in double distilled water, 10 min in phosphate-buffered saline (PBS) (pH 7.2), and 5 min in 300 mM of glycine in PBS. Similarly the isolated Leydig cells were fixed and prepared for IHC. Following to this, sections were incubated overnight in blocking buffer (3% BSA and 0.1% Tween-20 in 1X PBS) in a humified chamber with continuous shaking, then incubated with primary antibody

solution diluted in blocking buffer (1:1000 dilution), then washed 3 x10 min with blocking buffer and finally incubated in secondary antibody tagged with alkaline phosphatase () diluted in blocking buffer (1:1000). Sections were then washed 3 x 10 min in PBS and incubated in the substrate solution until the development of color. Then the sections were passed through 95 % (2 x 5min) and 100% (2 x 5 min) alcohol followed by incubation in xylene (15 min) and finally mounted in canada balsam and enclosed with cover slip. No counter stain was used for background staining.

3.2.10 Western blot analysis

The testis from treated and control male rats of various treatment groups were homogenized in phosphate buffer saline containing 20% v/v glycerol and 1mM EDTA and centrifuged at 10,000xg for 30 min at 4 ^{0}C. In case of Leydig cells, control and treated cells were collected by trypsinization followed by brief spinning. The pellet was lysed in 100 µl of lysis buffer (20 µl for 5 x 10^5 cells) on ice for 10 min and then both testicular homogenates and Leydig cell suspension were centrifuged at 14,000 rpm (16,000x g) for 10 min at 4 ^{0}C. The supernatants were then transferred to a new tube and pellet was discarded. Supernatants separated from testis and Leydig cells were quantified for their protein content by Bradford assay using commercially available kit (Bangalore Genei, Bangalore). An equal quantity of protein was loaded in 12% polyacrylamide gel to visualize any changes in total protein profiling in the treated rats/ cells as compared to control according to the method described earlier (Laemmli et al., 1970, Sambrook and Rusell, 2001).

For preparation of 5 ml of 12 % resolving agarose gel, 2 ml of 30% acrylamide mix (acrylamide, 29 % w/v in warm water and N-N'–methylene-bis-acrylamide, 1% w/v

in warm water) was mixed with 1.3 ml of 1.5 M Tris (pH 8.8), 50 µl of each 10% SDS (w/v) and 10% ammonium per sulphate (w/v) and volume was made with deionised water. 20 µl of TEMED (Sigma, USA) was added to the solution which was then rapidly poured (with continuous swirling) into the gel casting tray. Once the resolving gel was cast, the top of the resolving gel was washed several times with deionised water to wash off any unpolymerised acrylamide. A 5% stacking gel (containing 170 µl of 30% acrylamide mix, 130 µl of 1.0 M Tris buffer, 10 µl each of 10% SDS and 10% APS, 1 µl of TEMED per 1 ml of solution) was overlayed above the resolving gel. For sample preparation, the samples were mixed with 1x SDS gel loading buffer (50 mM Tris-Cl, pH 6.8; 100 mM dithiothreitol; 2% SDS, w/v; 0.1% bromophenol blue, 10% glycerol, v/v) in 1:1 proportion and heated at 100 ^{0}C for 3 min to denature the proteins. About 15 µl of the sample was loaded in the gel and separated using Tris-glycine buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS).

After the proteins of different sizes were separated by polyacrylamide gel electrophoresis, they were transferred to PVDF membrane (Himedia Chemicals, Mumbai) (pre wetted for about 30 min in methanol and then transferred to 1x blotting buffer until ready to use). The blot was electro-transferred using transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol, pH 8.5) for 2 hr at 200 volts. The membranes were then immersed in blocking buffer having 1x TBS (2.42 g tris base and 8.0g NaCl/ l of water give), 0.1% Tween-20 and 5% w/v nonfat dry milk and blocked overnight to obstruct non specific membrane binding sites. Blotted membranes were then probed with various antibodies depending on the experiments (for example, StAR, AR and 3β-HSD) antibodies (dilutions 1:1000) in blocking buffer followed by washing with 3 x 10 min

with 0.05% Tween 20 in PBS. Goat anti-rabbit IgG antibodies (Bangalore Genei, Bangalore) conjugated to alkaline phosphatase was then used to probe the primary antibodies (1:1000 in blocking buffer) and again washed 3 x 10 min with 0.05% Tween 20 in PBS. Colour development was performed in 30 ml AP-buffer (100 mM Tris Cl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), with 200 µl nitroblue tetrazolium (NBT), 50 mg/ml and 100 µl 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), 50 mg/ml. Finally, the developed blots were subjected to densitometry using the beta-actin as internal control.

3.2.11 RNA Extraction

This was performed in highly aseptic conditions and all the glassware used in the experiment was baked at 250 ^{0}C for 6 h and then treated with DEPC treated water before autoclaving. The RNA was extracted by the methods described earlier (Chomczynski and Sacchi, 1987). In case of Leydig cells, total RNA was extracted from the cells treated with or without different concentrations of TCS in presence of LH (100 ng/ml). Briefly the medium was removed and the cells were rinsed with ice cold PBS and directly lysed in the well with solution D (4 M guanidium thiocynate; 25 mM sodium citrate; 0.5%, w/v, sodium lauryl sarcocinate, 1M β-mercaptaethanol) (1 ml per well). The cell lysate was transferred to a polypropylene snap cap tube and homogenized with micropestle for 15-20 seconds. In case of testis and ovaries, they were transferred from the liquid nitrogen to mortar and pulvarized in presence of liquid nitrogen. The powdered tissue was transferred to polypropylene tube having 3 ml of solution D and was homgenised for 1 min. The homogenate was transferred to a fresh tube and 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml of water saturated phenol and 0.2 ml of chloroform-isoamyl alcohol (per ml of solution D used) were sequentially added to the tube with through mixing (by

inversion) with each addition. The homogenate was vortexed vigorously for 10 seconds and incubated on ice for 15 min (to permit the complete dissociation of nucleoprotein complexes) followed by centrifugation at 10,000xg for 20 min (4 ^{0}C) and the upper aqueous phase containing extracted RNA was transferred to a fresh tube. An equal volume of isopropanol was added to the extracted RNA, the solution was mixed well and allowed to precipitate for 1 hr at -80 ^{0}C. The precipitated RNA sample was collected by centrifugation, carefully decanted and was dissolved in 0.3 ml of solution D for every 1 ml of this solution used in step 1. The precipitated RNA sample was again collected by centrifugation at maximum speed for 10 min and pellet was washed twice with 75% ethanol and any remaining ethanol was removed with a disposable pipette tip. The tube was stored at room temperature for few minutes to allow the ethanol to evaporate. The RNA pellet was then dissolved in 50-100µl of DEPC treated water and was stored at -70 ^{0}C till use. Before use the RNA was quantified in an UV/Vis spectrophotometer (Perkin Elmer, USA) reading at 260/280 nm.

3.2.12 Estimation of purity of RNA samples by formaldehyde gel

Following denaturation reaction was set up in a sterile centrifuge tube –

20 µl RNA (up to 20 µg)

2.0 µl 10x MOPS electrophoresis buffer (0.2 M MOPS, pH 7.0; 20mM sodium acetate; 10mM EDTA, pH 8.0)

4.0 µl Formaldehyde

10.0 µl Formamide

1.0 µl Ethidium Bromide (200 µg/ml)

The tubes were incubated for 60 min at 55 ^{0}C, then chilled in ice water for 10 min finally 2 µl of 10x formaldehyde gel loading buffer (50% glycerol, diluted in DEPC treated water; 10 mM EDTA; 0.25% w/v bromophenol blue; 0.25% xylene cyanol FF) was added to each sample and tubes were returned to an ice bucket. A 1.5% of agarose/ formaldehyde (2.2 M) gel was prepared by melting 1.5 g of agarose in 72 ml of sterile water which was further cooled to 55 ^{0}C before the addition of 10 ml of 10x MOPS buffer (0.2 M MOPS, 20 mM sodium acetate, 10 mM EDTA) and 18 ml of deionised formaldehyde. The solution was finally allowed to set in a horizointal electrophoresis box.

The prepared gel was covered with sufficient amount of 1x MOPS buffer and the prepared RNA samples were loaded on the gel. The gel was ran at 4-5 V/cm until the bromophenol blue has migrated to approximately 8 cm followed by their visualization in UV light. All the samples were found to contain two distinct ribosomal bands (28s and 18s) as showed in following figure (Fig 11)

3.2.13 Semiquantitative RT – PCR analysis

Total extracted RNA from the testis and ovaries of all the control and treated animals as well as Leydig cells were quantified and tested as described above and an equal amount

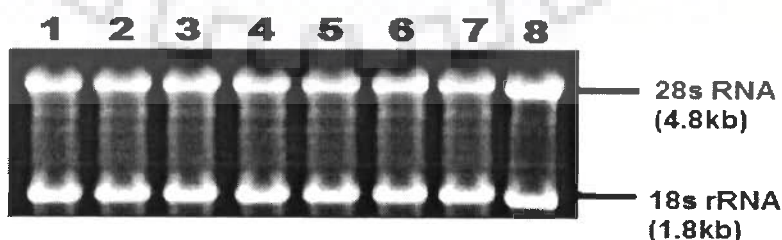


Figure 11. The representative formaldehyde gel showing the distinct ribosomal bands in the RNA samples extracted from the testis of control and treated rats. 1, control; 2, triclosan; 3, ibuprofen; 4, tetracycline; 5, sewage inlet; 6, sewage outlet; 7, leather industry effluent; 8, hexachlorobenzene treated groups.

of it was reverse transcribed. The reaction was carried out in two steps: cDNA synthesis and PCR amplification.

3.2.13.1 First strand cDNA synthesis (reverse transcription)

As the first step, total RNA was reverse transcribed to form cDNA by adding approximately 100 ng of RNA sample form all the groups in individual 0.2 ml tubes and sterile water was added to bring volume to 9 µl. To this, 1 µl of Oligo $(dT)_{18}$ primer was added and the vial was placed at 65 ^{0}C for 10 min and then at room temperature for another 2 min (to remove any secondary structure). Vial was spun briefly and following agents were added sequentially in the following order-

1 µl RNAse inhibitor (10 U/µl)

1 µl DTT (0.1 M)

4 µl RT Buffer (5x)

2.0 µl dNTP mix (30 mM)

0.5 µl M-MuLV Reverse Transcriptase (50 U/µl)

1 µl sterile water

The solutions were mixed well and incubated at 37 ^{0}C for 1 h and followed by incubation at 95 ^{0}C to dentaure RNA-cDNA hybrids. The samples were then spun briefly and quickly placed on ice.

3.2.13.2 PCR Amplification

PCR amplification was done in a 25 µl reaction volume with the desired number of cycles for each product. The reaction mixture consisted of following:

14.2 µl Sterile water

2.5 µl 10x PCR buffer (Bangalore Genei, Bangalore)

1 μl 30mM dNTP mix

1 μl Forward Primer (100ng/μl)

1 μl Backward Primer (100ng/μl)

0.3 μl Taq Polymerase (0.3U)

4 μl cDNA strand (as obtained above)

PCR was performed by denaturing at 94 °C for 60 seconds, annealing for 30 seconds and by extension at 72 °C for 60 seconds. Primer sequence, gene bank accession number, annealing temperature and number of cycles used for amplification for each gene is mentioned in the table 2. The primer sequences, annealing temperature and number of cycles for PCR were all designed according the earlier report by Ohsako et al. (2003)

Table 2. Primers used for semi-quantitative RT-PCR

except for steroidogenic acute regulatory protein (StAR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and scavenger receptor class B-1 (SRB-1). Primer sequence for StAR was adopted from Murugesan *et. al.* (2007). Primers for SRB-1 and GAPDH were designed with the help of Primer3 software (*Steve Rozen, Helen J. Skaletsky, 1998, Primer3*) and standardized in the lab.

Agarose gel (2%) was prepared by melting 2 gm agarose in 100 ml of 1 x TAE buffer (50X stock having 242 gm of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA). The gel was allowed to cool up to a temperature of approximate 55 °C and ethidium bromide was added to a final concentration of 0.5 μg/ml. The solution was poured into a tray and gel was allowed to set and finally dipped with sufficient amount of 1x TAE buffer. The DNA samples were mixed with 0.20 volume of 6X gel loading

Table 2 - Primers used for semi-quantitative RT-PCR

Gene	Primer Sequence	Product Size	Cycle Used	Annel Temp	Gene Bank Accession No.
P450scc (F)	CgCTCAgTgCTggTCAAAA	688	26	55	J05156
P450scc (R)	TCTggTAgACggCgTCgAT				
P450C17 (F)	GACCAAGGGAAAGGCGT	302	28	55	M22204
P450C17 (R)	GCATCCACGATACCCTC				
3 β –HSD (F)	CCgCAAgTATCATgACAgA	547	28	55	M38178
3 β –HSD (R)	CCgCAAgTATCATgACAgA				
17 β-HSD (F)	TTCTgCAAggCTTTACCAgg	653	28	55	AF035156
17 β –HSD(R)	ACAAACTCATCggCggTCTT				
AR (F)	TTACgAAgTgggCATgATgA	570	28	55	M20133
AR (R)	ATCTTgTCCAggACTCggTg				
SRB-1 (F)	CCATTCATGACACCCGAATCCT	100	26	60	AY682847
SRB-1 (R)	TCGAACACCCTTGATTCCTGGT				
StAR (F)	TTgggCATACTCAACAACCA	389	30	58	NM031558
StAR (R)	ATgACACCgCTTTgCTCAg				
Aromatase (F)	gTgCCTgCAACTACATACAATAAg	521	28	55	M33986
Aromatase (R)	CTCATACTTTCTgTAgAgCCAAg				
ERα (F)	TTACgAAgTgggCATgATgA	711	28	56	Y00102
ERα (R)	ATCTTgTCCAggACTCggTg				
GAPDH (F)	AgACAgCCgCATCTTCTTgT	207	21	58	NM017008
GAPDH (R)	CTTgCCgTgggTAgAgTCAT				

buffer (0.25% bromophenol blue, 40% sucrose w/v in water), loaded on the gel and electrophoresis was carried out. The intensity of the bands on gels was converted into digital image with a gel analyzer and the amounts of RT-PCR products were quantified

with Scion Images software (Scion Corporation, Fredrick, MD, USA) against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene used as internal standards.

3.2.14 Serum Alkaline Phosphatase, Acid Phosphatase, SGPT and SGOT Level.

Serum from all the animals was subjected to assay of the four enzymes present in serum viz. Alkaline phosphatase (ALP), acid phosphatase (ACP), serum aspartate aminotransferase (AST) or glutamate oxaloacetate transaminase (SGOT) and alanine aminotransferase (ALT) or serum glutamate pyruvate transaminase (SGPT) by the methods as described in Mukherjee, 2003.

3.2.14.1 Serum acid phosphatase

First of all following reagents were prepared-

- Citrate buffer (0.09 gm/L)- 18.9g of citric acid monohydrate, 500 ml H_2O + 180 ml 1N NaOH + 100 ml 0.01 N HCl, adjust the pH to 4.85 and dilute to 1000 ml.
- Tartrate – Citrate buffer (pH 4.85), 0.09 mole citrate and 0.04 mole tartrate/L- 1.5 g of L-tartrate in 250 ml of citrate buffer (1).
- Stock substrate of PNPP (4 mg/ml)
- Working buffered substrates- mix equal volumes of acid buffers and substrate:

  (a) Citrate buffer and stock substrate (solution 1 and 3)

  (b) Tartrate – Citrate buffer and stock substrate (solution2 and 3)
- Store both solutions in 1 ml aliquots in test tubes (15 ml)Sodium hydroxide (0.1N).

After the reagents are prepared frozen substrate solutions were taken -2 citrate substrate tubes and one tartrate substrate tube. They were marked as "CT" (citrate total), "CSB" (citrate serum blank) and "T-NP" (tartrate non-prostatic). Two more tube of one each

citrate substrate and one tartrate were taken and labeled as CB and TB. They were used as blank. And all tubes were incubated at 37^0C for 5 min. After exactly 5 min 0.2 ml serum was added to CT and T-NP test tubes and continues incubation at 37^0C for 30 min after which 4 ml of 0,05 M NaOH was added to both test tubes and mixed well to stop the reaction. Tubes were removed from water bath and 0.2 ml serum was added to the tube marked as 'CSB' and 0.2 ml water was added to the substrate blank tubes marked as 'CB' and 'TB'. The solution in "CB" tube was used to zero "CT" and "CSB" tube and "TB" tube was used to zero "T-NP" tube. Calibration curve was referred for reporting the enzyme activity in international units (U/L).

3.2.14.2 Serum Alakline Phosphatase

Two test tubes were taken and were labeled as T and B for test and blank respectively. 1 ml working substrate (equal volume of p-nitrophenyl disodium phosphate, 4 mg/ml and glycine buffer) was taken and incubated at 37^0C for 5 min. After exactly 5 min 0.5 ml serum was added to test tube marked as 'T' and incubation was continued at 37^0C. After 30 min 10 ml of 0,05 M NaOH was added to both test tubes and was mixed well to stop the reaction. Tubes were removed from water bath and 0.5 ml serum was added to the tube marked as 'B' (for blank). Absorbance was recorded against water at 405 nm and determine the change in absorbance by subtracting the absorbance of tube 'B' from 'T'. Calibration curve was referred for reporting the enzyme activity in international units (U/L).

3.2.14.3 Serum SGOT

Two test tubes were taken and were labeled as T and B for test and blank respectively. 1 ml substrate (200mM/L DL-aspartate + 2mM/L alpha-ketoglutarate in 0.1M phosphate

buffer, pH 7.4) was taken and incubated at 37^0C for 5 min. After exactly 5 min 0.2 ml serum was added to test tube marked as 'T' and incubation was continued at 37^0C After 60 min 1.0 ml of hydrazine was added to both test tubes (1mM/L prepared in 1N HCl) and mixed to stop the reaction and develop the colour. Tubes were removed from water bath and 0.2 ml serum was added to the tube marked as 'B' (for blank). After 20 min 10 ml of 0.4N NaOH was added and mixed by inversion and reaction was continued for 5-20 min. Absorbance was recorded against water at 505nm and the change in absorbance was determined by subtracting the absorbance of tube 'B' from 'T'. Calibration curve was referred for reporting the enzyme activity in international units (1U).

3.2.14.4 Serum SGPT

Two test tubes were taken and labeled them as T and B for test and blank respectively. 1 ml substrate was taken (200mM/L DL-alanine + 2mM/L alpha-ketoglutarate in 0.1M phosphate buffer, pH 7.4) and incubate at 37^0C for 5 min. After exactly 5 min 0.2 ml serum was added to test tube, marked as 'T' and incubation was continued at 37^0C. After 30 min 1.0 ml of hydrazine was added to both test tubes (1mM/L prepared in 1N HCl) and mixed to stop the reaction and develop the colour. Tubes were removed from water bath and 0.2 ml serum was added to the tube marked as 'B' (for blank). After 20 min 10 ml of 0.4N NaOH was added and mixed by inversion and reaction was continued for 5-20 min. Absorbance was recorded against water at 505 nm and the change were determined in absorbance by subtracting the absorbance of tube 'B' from 'T'. Calibration curve was referred for reporting the enzyme activity in international units (1U).

3.2.15 Assay of anti-oxidant enzymes

This section was aimed to measure four major enzymes as markers of oxidative stresses, viz. superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) in the hypothalamus of the rats treated with test chemicals. The major aim for this section was based on the fact that some animals demonstrated lower level of LH and FSH in response to treatment. Hypothalamus from the control and treated animals were homogenized in the five-fold volume of ice cold Tris buffer (20 mM, pH 7.4) using a potter homogenizer with 10 strokes at 1200 rpm. Homogenates were centrifuged at 8500xg for 10 min at 4 ^{0}C. Supernatants were collected and stored at -20 ^{0}C until assaying the enzymes.

The assay of SOD (EC 1.15.1.1) was based on the method of Nebot et al. (1993) utilizing the commercially available assay kit from Calbiochem (Calbiochem San Diego, USA). For elimination of interfering substances and Mn-SOD-activity, Cu/Zn-SOD enzyme activity was assayed after extraction with chloroform and ethanol according to the supplier's manual. SOD activity was determined from the Vs/Vc ratio (Vs, in the presence and Vc, absence of sample) of the autoxidation rates of the chromophore BXT-01050 at 37 ^{0}C measured in the presence and absence of sample. One SOD activity unit was defined as the activity that doubles the autoxidation background (Vs/Vc = 2).

The assay of GPx (cytosolic GPx, EC 1.11.1.9) was based on the reaction described by Paglia and Valentine (1967) using the commercially available GPx assay kit from Calbiochem. Tert-butylhydroperoxide was used as substrate (as provided in the kit). One unit of GPx was defined as the activity that converts 1 mM of reduced glutathione per litre per minute at 25^{0}C.

The GR activity (EC 1.8.1.7.) assay was based on the method of Mizuno and Ohta (1986). Enzymatic activity was assayed photometrically by measuring NADPH consumption during the enzymatic reaction: in the presence of GSSG (oxidized glutathione) and NADPH, GR reduces GSSG and oxidizes NADPH to yield NADP resulting in a decreased absorbance at 340 nm. We used the commercially available GR assay kit from Calbiochem. One unit of GR was defined as the activity that converts 1 mmol of NADPH per litre per minute at 25^0C.

Similarly CAT (EC 1.11.1.6) activity was assayed from the tissue supernatant by the method of Sinha and was expressed as U/mg protein with the help of commercially available kit form calbiochem [12]. One unit of enzyme was considered as the amount of enzyme that utilizes 1 μM of hydrogen peroxide/min.

3.2.16 Reversed phase HPLC fractionation of the WWTP inlet and outlet samples.

HPLC analysis of the WWTP water samples was carried out according to the earlier described methods with some modifications (Brett et al., 2003). HPLC fractionation of WWTP influent and effluent extracts were performed on a Synergi Max-RP C12 column with 4 μm particle size (Phenomenex, Torrance, CA, USA). The HPLC system consisted of Agilent (Palo Alto, CA, USA) G1312A binary gradient pump. A gradient consisting of 0.1% formic acid (v/v) in water (A) and 100% methanol (B) at a flow rate of 700 μl/min was used. The gradient was as follows: 5% B held for 3.5 min, increased linearly to 80% by 10 min and held for 3 min, and stepped to 100% and held for 8 min. A 9 min equilibration step at 5% B was used at the beginning of each run to bring the total run time per sample to 30 min. 10 μl of influent and effluent extracts were injected and the

compounds were monitored by fluorescence with 229 nm excitation/ 310 nm emission wavelengths or 210 nm wavelength.

3.2.17 Physicochemical analysis of WWTP inlet and outlet water and leather industry effluents samples

One litre of collected WWTP inlet and outlet and leather industry effluents samples were extracted with DCM in same ratio as prepared for gavaging to the rats, concentrated to 1 ml and then evaporated to dryness in a 1.5 ml vial. Further, extract was derivatized in same vial by addition of N,O bis(trimethylsilyl) trifluoroacetamide (BSTFA) (Sigma Aldrich, USA) as a silylation agent. The derivatization was performed by reconstituting the dried elute samples with 110 μl of acetone:hexane (1:1, v/v) and 20 μl of BSTFA. Vials were then capped and placed in a water bath at 65 ^{0}C for 30 min to ensure complete derivatization of the compounds of interest. The derivatization led to the silylation of all the target compounds except hexachlorobenzene. On completion of derivatization, 2 μl of the reaction mixture of each of inlet and outlet and leather industry effluents sample were injected (in splitless mode) into the GC-MS system. GC-MS analysis was performed using the protocols described earlier (Liu et al., 2004; Leusch et al., 2006b) with some modifications. EI-MS analysis was performed on a Perkin-Elmer Clarus 500 gas chromatograph coupled with a mass spectrometer and an autosampler. An Elute -1 Crossbond® (5% Diphenyl – 95% Polysiloxane) column of 30 m x 0.25 mm i.d. x 0.25 μm film thickness was used. The GC column temperature ranged from 75 ^{0}C (initial equilibrium time for 2 min) to 155 ^{0}C at a temperature increase of 10 ^{0}C/min, 155 ^{0}C-260 ^{0}C at a temperature increase of 15 ^{0}C/min and 260 ^{0}C -300 ^{0}C at a temperature increase of 12 ^{0}C/min.. The mass spectrometer was operated in the full acquisition electronic impact

mode (70 eV) for the qualitative analysis. The presence of the compounds was confirmed by matching the retention time of the standards with that of corresponding peaks in the chromatogram of sample and further by analyzing mass spectra of the matching peaks.

Quantification of contaminants was performed using selected ion monitoring mode (SIM); the *m/z* values of the ions monitored are listed in physicochemical analysis section. Quantification was done using the external standards of nonylphenol, hexachlorobenzene, DHEA, isoandrosterone, 4-aminobiphenyl and benzidine which were spiked in acetone: hexane (1:1, v/v) in concentrations ranging from 0.2 to 2.0 μg/ml. Calibration curves were prepared by linear regression of peak areas of standard solutions against their respective concentrations.

3.2.18 Leydig cell isolation, purification, imunocytochemical identification and assessment of viability (MTT assay)

Rat testicular Leydig cells were isolated by the method described earlier (Murugesn et al, 2007) with some modifications as standardized in the lab. Highly aseptic conditions were maintained throughout the experiment. The capsule coverings of testis were removed and decapsulated testes were digested in M-199 medium containing 0.25 mg/ml collagenase at 34 ^{0}C with constant shaking. On completion of incubation, same volume of DMEM-F12 (without collagenase) was added to the tubes to stop collagenase activity and allowed to stand for 10 min followed by careful aspiration of supernatant. The last procedure was repeated once more for removing additional Leydig cells, both the supernatants were combined and centrifuged at 2500 x g for 10 min at 4 ^{0}C. The pellet was then resuspended in 1 ml DMEM-F12 which represented a crude testicular suspension. A discontinuous percoll gradient was maintained carefully in a 15 ml

centrifuge tube having 75% at the bottom followed by 60%, 45%, 30%, 15% and finally 5% at the top (each 2 ml). The crude testicular suspension was layered over the gradient followed by centrifugation at 3000 x g for 30 min (4 ^{0}C). After centrifugation, most of the purified cells were observed in between 30% and 45% gradient. This portion was transferred carefully to a centrifuge tube containing M-199, mixed and centrifuged at 2500 x g for 10 min to remove excess percoll. The procedure was repeated three times more and finally Leydig cells were suspended in 1 ml M-199 medium. The purity of Leydig cells was assessed by 3β-HSD staining (Fig. 13) and viability was checked by routine trypan blue exclusion method, both of which were found to be 88% and 92% respectively.

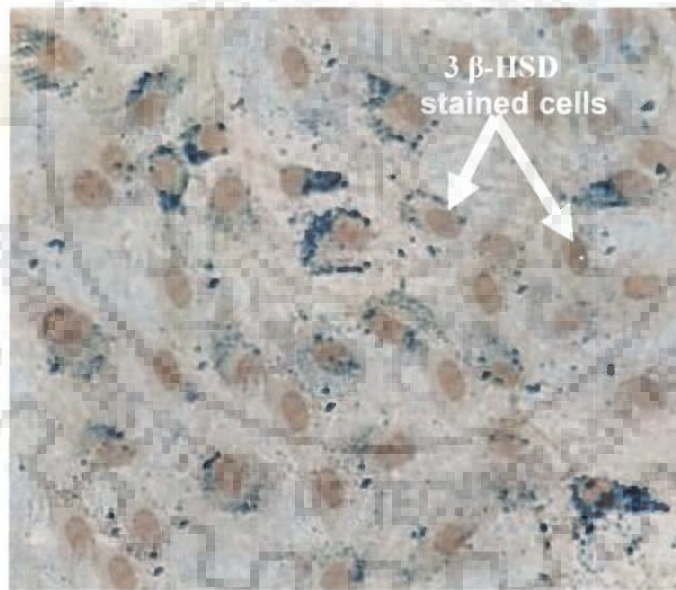


Figure 12. Assessment of purity of Leydig cells by 3β-HSD staining

For immunocytochemical stainig, protocol of Shiraishi and Ascoli (2007) was used with some modification standadised in the lab. Leydig cells were fixed using 4% paraformaldehyde dissolved in 10 mm sodium phosphate, 150 mm NaCl (pH 7.4) (PBS) for 10 min at 4 ^{0}C. After washing twice with 10 mm Tris, 150 mm NaCl (pH 7.4)

Trisbuffered saline (TBS), the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. The cells were processed with the avidin-biotin complex method using the Vectastain ABC kit (Vector Laboratories, Burlington, CA) according to the instructions of the manufacturer. The fixed cells were incubated for 60 min at room temperature in a solution of 0.5% goat serum, 0.1% Triton X-100 in TBS. The cells were then incubated overnight at 4 ^{0}C with a 1:1000 dilution of normal rabbit serum in 0.1% Triton X-100 in TBS. After washing twice with 0.1% Triton X-100 in TBS, the cells were treated with biotinylated antirabbit IgG (1:500 dilution) in TBS containing 3% BSA for 1 h at room temperature. This was followed by 30 min incubation with 0.6% hydrogen peroxide in TBS. Then, the avidin-biotin complex reagent was applied for 1 h, and the immune complexes were revealed with 3,3'-diaminobenzidine chromogen (prepared according to the instructions of the manufacturer) for 10 min at room temperature. The reaction was stopped by adding 1 ml of water, and the cells were examined and photographed with a phase contrast microscope.

The effect of TCS on viability of cells was estimated by MTT assay. In this assay reduction of MTT (a yellow tetrazolium salt) to a blue formazan product by the viable cells was measured (West et al, 2001). For the assay, Leydig cells which were previously incubated for 2 h with different concentrations of TCS were treated with 100 μl fresh medium containing 0.5 mg/ml MTT for 1 hr. Following 1 hr, the medium was removed and the reduced formazan was dissolved in 100 μl acidified (0.04 N HCl) isopropanol at room temperature for 25 min. The dissolved formazan concentration was then measured in a Beckman plate reader at 562 nm wavelength. Control (blank) wells contained only isopropanol.

3.2.19 Steroidogenic enzyme activity in vitro.

3β-HSD and 17β-HSD are the two crucial enzymes in the steroid biosynthesis pathway and their activities have been found to be affected by some of the EDC. These two enzymes were assayed according to the methods described earlier (Talalay, 1962; Sarkar et al., 1991; Shukla et al., 2001; Krazeisen et al., 2001). Briefly, the testis and ovaries removed from the different groups of intact animals were homogenized in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1mM EDTA followed by centrifugation at 10,000Xg for 10 min at 4 °C. For 3β-HSD activity, 1 ml aliquot of the supernatant was mixed with 100 μM sodium pyrophosphate buffer (pH 8.9), 0.9 ml double distilled water and 30 μg DHEA making up the incubation mixture to a volume of 3 ml. Enzyme activity was measured at 25 °C after the addition of 0.5 μM of NAD^+ to the mixture against a blank (without NAD^+). For the determination of 17β-HSD (type III) activity, 1 ml aliquot of the above centrifuged supernatant was mixed with 400 μM sodium pyrophosphate buffer (pH 10.2), 25 mg bovine serum albumin, and 0.3 μM testosterone bringing the total reaction volume to 3 ml. Enzyme activity was measured after the addition of 1.1 μM NADP to the mixture against a blank without NADP. By this experiment, the activity of reverse reaction catalyzed by 17β-HSD (conversion of testosterone to androstenedione) was determined. The forward reaction (conversion of androstenedione to testosterone) was performed under almost similar condition using 50 mM phosphate buffer, 30 nM androstenedione and 7 mM NADPH. One unit of enzyme activity was equivalent to a change in the absorbance of 0.001units/min at 340 nm.

An HPLC based method was used to measure activities of three steroidogenic enzyme in case of Leydig cells (Darney et al, 1993) with some modifications in the lab.

The cells were incubated with pregnenolone (25 μM), progesterone (12.5 μM), or androstenedione (12.5 μM), substrates for 3β-HSD, cytochrome P450C-17 and 17β-HSD, respectively, in the presence or absence of TCS for 2 h with maximum stimulation of LH (100 ng/ml). The steroid products of each enzyme were then extracted from culture medium with hexane and solvent exchanged with methanol which was finally reduced to 50 μl. Steroid products were quantified by a reverse-phase HPLC C18 column (Waters-Millipore Associates Inc., Milford, MA, USA) using 11 β-Hydroxy-androstenedione (200 ng/tube) as internal standards. Peaks of ketosteroids were detected by an online UV absorbance detecting system at 240 nm. The integrated peak areas were used to determine individual steroids. The samples were eluted with the following solvent system at a flow rate of 1 ml/min: 0–4.0 min, 10% acetonitrile in methanol/water (80/20, V/V); 4.0–7.0 min, a linear gradient of 10% to 100% acetonitrile in methanol/water (80/20, V/V); 7.0–50.0 min, 100% acetonitrile.

3.2.20 Adenylyl Cyclase activity in isolated Leydig cells

Cells from both TCS treated and vehicle treated Leydig cell culture were scrapped off the plates and activity of adenylyl cyclase enzyme was assayed by the methods as described earlier (Salomon et al., 1974) with variations according to our laboratory conditions. Unless otherwise stated, the assay of homogenate was performed in a final volume of 200 μl in a medium containing 40 mM Tris/HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.5 mM cyclic AMP, 0.1% bovine serum albumin, 10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, 1 mM [α-^{32}P]ATP (approximately 2 μCi) and 0.25 mM methylisobutylxanthine. Incubation was carried out at 32 °C for 15 min and the reaction was stopped by immersing the incubation tubes in a boiling water bath for 5 min.

Approximately 30,000 cpm cyclic $[^{3}H]$ AMP was added for recovery and water was added to a final volume of 1 ml. The mixture was then clarified by centrifuged for 10 min at 7000xg (4 °C). The supernatants were subjected to chromatography on Dowex-AG50-WX4 columns followed by chromatography on neutral alumina. The radioactivity in the elutes was then determined in a Beckman LS-100C Liquid Scintillation counter (Beckman, USA).

3.2.21 MTT Assay in isolated leydig cells

The effects of TCS on viability of cells were estimated by MTT assay. In this assay cell reduction of MTT (a yellow tetrazolium salt) to a blue formazan product by the viable cells was measured (West et al, 2001). For the assay, Leydig cells which were previously incubated for 2 h with different concentrations of TCS were treated with 100 µl fresh medium containing 0.5 mg/ml MTT for one hour. Following one hour, the medium was removed and the reduced formazan was dissolved in 100 µl acidified (0.04 N HCl) isopropanol at room temperature for 25 min. The dissolved formazan concentration was then measured in a Beckman plate reader at 562 nm wavelength. Control (blank) wells contained only isopropanol.

3.2.22 Statistical analysis.

Origin 6.1 software, Origin Corporation Lab, USA and MS excel software (Microsoft USA) were used for statistical analysis and plotting the graph. Data were expressed as mean ± SEM. For statistical analysis of data, ANOVA followed by multiple two-tail comparison *t*-test was used and $P<0.05$ was considered significant.

CHAPTER - 4

CHAPTER 4: IN VIVO CHARACTERIZATION OF SOME ENDOCRINE DISRUPTING CHEMICALS

4.1 An antimicrobial Chemical as EDC

4.1.1 Introduction

In modern life different types of synthetic chemicals are used directly or indirectly for diverse purposes and their extensive use may have adverse physiological consequences. Out of these chemicals antimicrobial agents and preservatives plays an important role due to their common use in toiletries like soap, shampoo, detergents, disinfectants, cosmetics and pharmaceutical products and their continuous use has led to their accumulation at detectable concentrations in different body parts like human blood, milk, various organs and tissues (Darbre et al., 2006; Dayan et al., 2006; Heidler et al., 2006; Lakeram et al., 2006; Nakada et al., 2006; Cabana et al, 2007). Triclosan (TCS; 2,4,4'-trichloro-2'-hydroxydiphenyl ether; a chlorophenol) is an antimicrobial agent widely used as preservative in toothpaste, soap, shampoo, and cosmetics (Fig. 13). In general TCS has been known to be a highly toxic chemical for aquatic flora and fauna (Tatarazako et al., 2004). TCS and its chlorinated derivatives are readily converted into various chlorinated dibenzo-p-dioxins by heat and ultraviolet irradiation which, further, may be harmful for body system (Kanetoshi et al., 1987, 1998a, 1998b). Besides, TCS has been also included in the probable list of endocrine disruptors on account of its structural resemblance with known non-steroidal estrogens (e.g. Diethylestradiol, Bisphenol A). The mode of endocrine disrupting actions of TCS is controversial and various studies indicate it to be of different nature viz. estrogenic or weak androgenic or anti-androgenic. Fourteen days exposure of TCS to Japanese medaka fry (Oryzias latipes) induced a weak androgenic

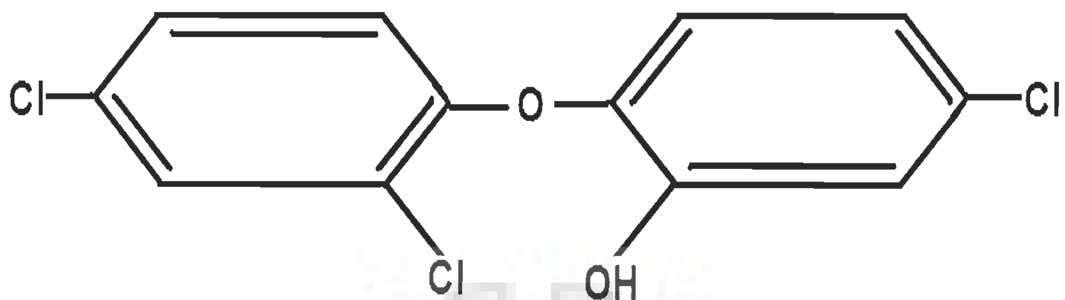
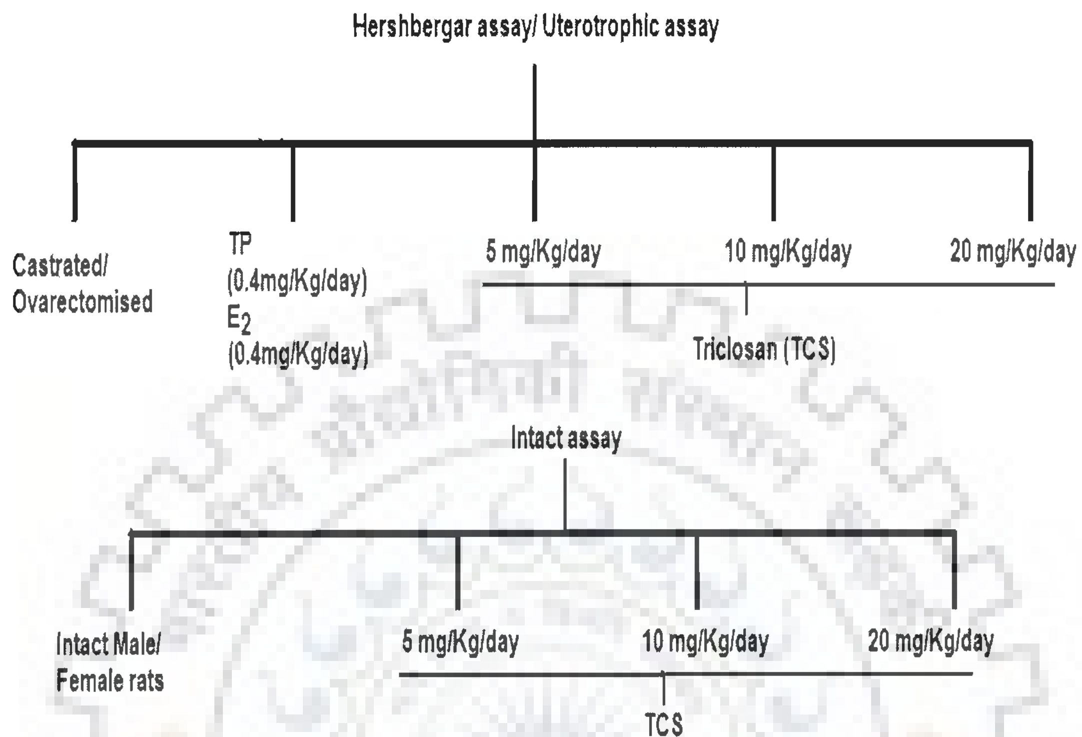


Figure 13. Chemical structure of the representative test chemical, triclosan.

effect (Foran et al., 2000). On the other hand, Ishibashi *et al.*, (2004) showed that metabolite of TCS may be a weak estrogenic compound with the potential to induce vtg in male medaka while in female it decreased the hatchability and delayed the hatching (Ishibashi et al., 2004). TCS has also been shown to function as an anti-androgen since it inhibits transcriptional activity induced by testosterone (Chen et al., 2007). Exposure of TCS to the human and wildlife may be a consequence of its presence in the cosmetics and other human use products and it has been frequently detected in wastewater effluents (Heidler et al., 2007).

The main objective of this section of the thesis was to confirm as well as to elucidate the mechanism of action of endocrine disrupting effects of one of the widely used antimicrobials, TCS, using male and female albino rats as models. The castrated, ovarectomised and intact male and female rats were treated with three dosage levels of TCS for a fixed period of time and on completion of treatment serum and tissue samples were analyzed for assessing (anti)androgenic and (anti)estrogenic effects in response to test chemical. Following schematic representation depicts the summary of treatment plan used for gavaging the TCS to male and female rats as discussed in the methodology section..



4.1.2 Results

4.1.2.1 Endocrine disrupting effects of TCS in male reproductive system

4.1.2.1.1 Weight of SATs in castrated rats (Hershberger Assay)

TCS did not show any sign of the endocrine disruption in castrated rats as weight of the SATs of treated rats was not significantly different from that of the control rats.

4.1.2.1.2 Effects on courtship behavior

The treatment of rats with TCS caused a decrease in the number of attempts by male rats to invade into the female rat's chamber. The control rats showed an average 12 numbers of attempts per day while it decreased to an average of 8 in TCS treated group of rats on completion of dosing (Fig. 14). Further, the decrease was found to be dose dependent i.e. there was a consistent decrease in the number of attempts with the increase in the number of days of dosing.

4.1.2.1.3 Weight of testis and SATs in intact rats

The administration of TCS did not cause any significant change in the weight of testis and SATs at a dose of 5 mg/kg/day, however, the two higher doses (10 and 20 mg/kg/day) induced a significant decrease in the weight of testis and SATs. At a dose of 10 mg/kg/day decrease in the weight of testis, epididymis, ventral prostate, vas deference and seminal vesicles was 28%, 25%, 36%, 50% and 20% respectively (Table 3). At dose of 20 mg/kg/day decrease was 35%, 37%, 45%, 49% and 35% in the case of testis, epididymis, ventral prostate, vas deference and seminal vesicles respectively (Table 3).

Table 3. Effects of three different dosage levels of TCS on the weights of testis and SATs from rats given 60 days treatment.

Groups	Testis (gm±S.E.)	Seminal Vesicle (gm±	Ventral Prostate (gm±S.E.)	Epididymis (gm±S.E.)	Vas defrentia (gm±S.E.)
Control	2.334 ± 0.089	462.2 ± 15	133.4 ± 4	868.3 ± 10	125.5 ± 6
Triclosan (5 mg/Kg)	2.111 ± 0.067	446.3 ± 21	128.4 ± 9	852.2 ± 12	118.5 ± 5
Triclosan (10 mg/Kg)	1.709 ± 0.060*	374.3 ± 19*	99.2 ± 5*	651.2 ± 11*	85.2 ± 6*
Triclosan (20 mg/Kg)	1.524 ± 0.051*	302.6 ± 22*	74.3 ± 8*	549.3 ± 10*	60.4 ± 7*

(Each value denotes mean ± S.E.M. of eight animals)
* Significantly different from control group receiving TP at $p<0.05$ level

4.1.2.1.4 Gene expression analysis

Rats treated with a dose of 20 mg/kg/day showed a statistically significant down regulation in the testicular levels of mRNA for cytochrome P450scc (P450scc), P450C-17, StAR and SRB-1 mRNA decreased up to 33, 54, 46, 58, 54 and 41% Rats treated with a dose of 20 mg/kg/day showed a statistically significant down regulation in the testicular levels of mRNA for cytochrome P450scc (P450scc), cytochrome P450C-17

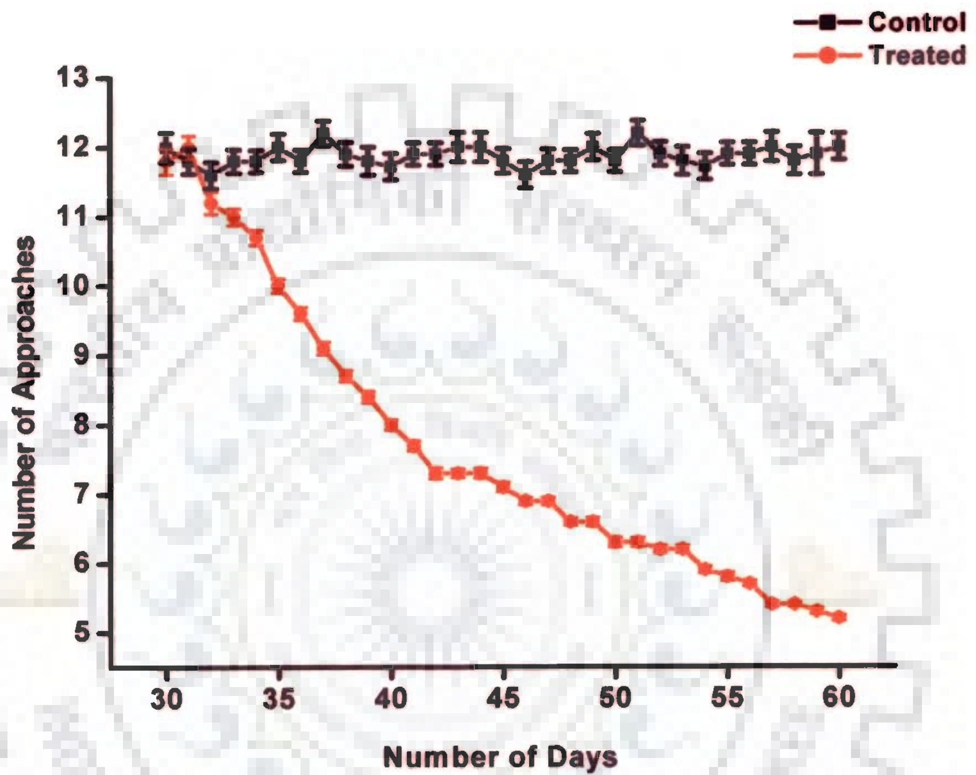


Figure 14. Effect of TCS on the reproductive behavior of adult intact male rats. The reproductive behavior was started to be taken into account from day 31 of dosing as described in materials and methods.

(P450C-17), 3β-HSD, 17β-HSD, StAR, Scavager receptor class B-1 (SRB-1) and AR as compared to control. Level of P450scc, 3β-HSD, 17β-HSD, respectively while expression of AR transcript increased up to 133% as compared to control (Fig. 15).

4.1.2.1.5 Testicular 3β-HSD and 17β-HSD) levels in vitro

In vitro spectrophotometric enzyme assays for 3β-HSD and 17β-HSD demonstrated that the treatment of animals with test chemical caused a statistically significant decrease in the activity of both the testicular steroidogenic enzymes at two higher dose levels (10 and 20 mg/kg/day) ($p<0.05$). The decrease was not significant at a dose of 5 mg/kg/day for both the enzymes. Doses of TCS at a level of 10 and 20 mg/kg/day decreased 3β-HSD(I) enzyme activity up to 27% and 39% respectively while that of 17β-HSD(III) enzyme activity up to 31% and 46% respectively when compared with control (Fig. 16).

4.1.2.1.6 Testicular StAR andAR protein immunoblot analysis

Rats treated with TCS at a dose of 20 mg/kg/day showed reduced translation of StAR (30 kD) and 3 β-HSD (45 kD) while an increased translation of AR (100 kD) protein as compared to control (vehicle treated animals) and this decrease was statistically significant ($p<0.05$) (Fig 17). The uniform band intensities of β-actin in all the wells indicated equal gel loading.

4.1.2.1.7 Immunohistocemical detection of testicular StAR protein

As shown in Fig. 18, there was a decreased localization of StAR protein in testicular Leydig cells as determined by immunolocalization, indicating a reduced expression of this protein in animals treated with TCS as compared to control. The reduced expressionof StAR could also be correlated to the reduction in Leydig cell number. In

order to confirm this fact, an *in vitro* MTT assay was performed using isolated Leydig cells treated with TCS which did not show any significant cell death even at a concentration of 10 μM TCS treatment (data not shown).

4.1.2.1.8 Serum hormone levels

There was a statistically significant decrease in the serum LH (38.5%), FSH (17%), cholesterol (35%), pregnenolone (31%) and testosterone (41%) levels in male rats treated with TCS at a dose of 20 mg/kg/day as compared to control. ($p<0.05$) (Table 4).

Table 4. Serum levels of LH, FSH, cholesterol, pregnenolone and testosterone from untreated (control) and TCS treated (20 mg/kg/day) male rats

	Control	Treated
LH (ng/ml)	1.04 ± 0.054	0.64 ± 0.056*
FSH (ng/ml)	8.12 ±0.168	6.82 ± 0.045 *
Cholesterol (mg/dl)	93 ± 7.00	61 ± 5.00*
Pregnenolone (ng/ml)	0.26 ± 0.008	0.18 ± 0.007*
Testosterone (ng/ml)	6.60 ± 0.130	3.94 ± 0.077*

(Each value denotes mean ± S.E.M. of eight animals)
*Significantly different from vehicle control group at $p<0.05$ level.

4.1.2.2 Endocrine disrupting effects of TCS in female reproductive system

4.1.2.2.1 Effect of TCS on the weight of uterus of ovarectomised rats (uterotrophic assay) and intact female rats

Like that of Hershberger assay, TCS did not elicit any response in the uterotrophic assay at any of the three dosage levels tested. However, TCS caused a decreased weight of uterus in intact female rats at the two higher dosage levels (10 and 20 mg/kg/day) zhereas the uterine weight of the rats treated with the lowest dose (5 mg/kg/day) was almost similar to that of control rats (Table 5).

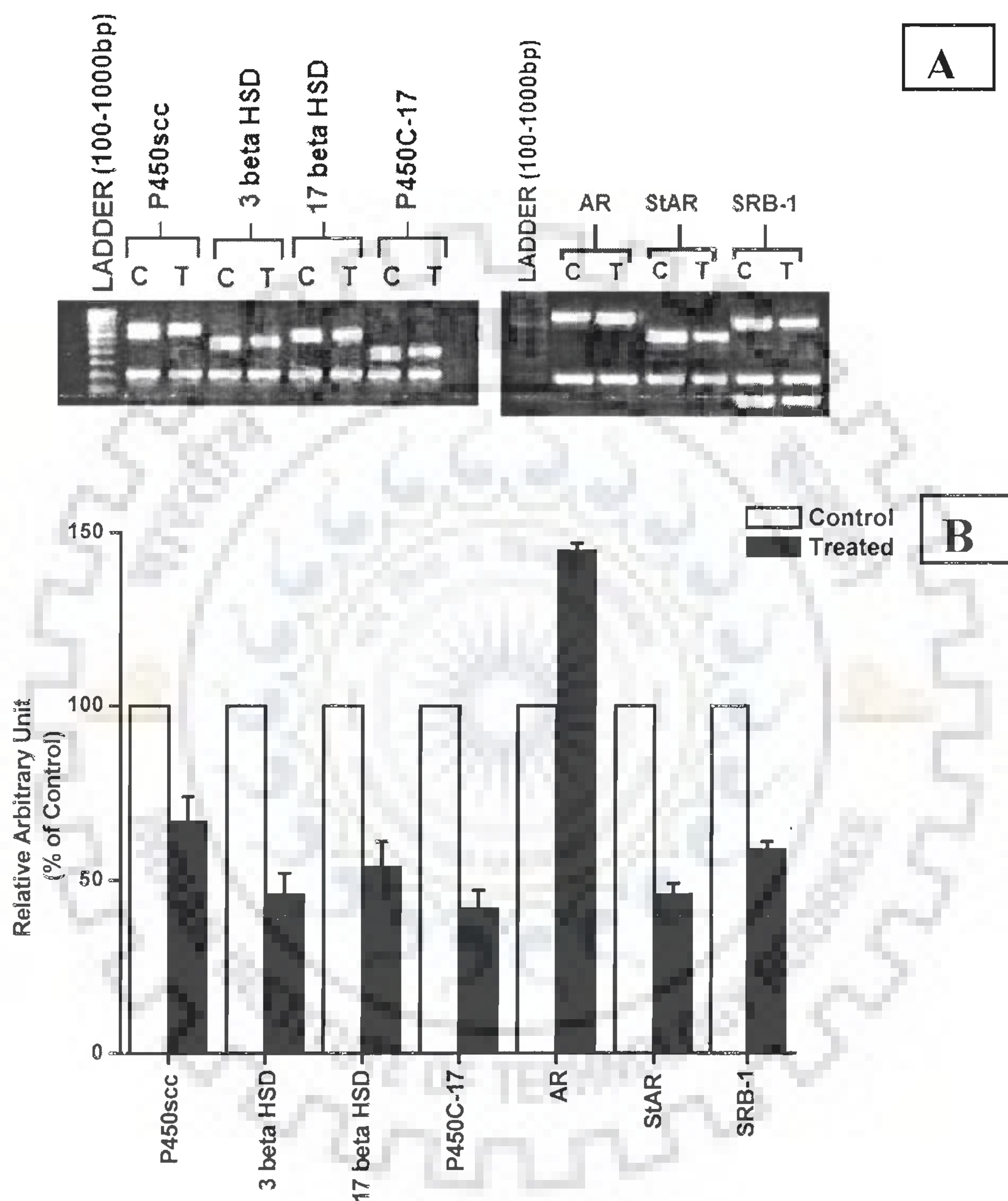


Figure 15. RT-PCR analysis of testicular mRNA expression of P450scc, 3β-HSD(I), 17β-HSD(III), P450C-17, AR, StAR and SRB-1 genes in rats treated daily with test chemical at a dose of 20 mg/kg/day . (A) The total RNA isolated from testis was reverse transcribed and cDNA obtained was subjected to PCR (B) The relative intensity of the signals were quantified by densitometer and normalized against the internal control (GAPDH). The values are mean ± S.E.M. of RT-PCR reactions run for eight RNA samples for each group.

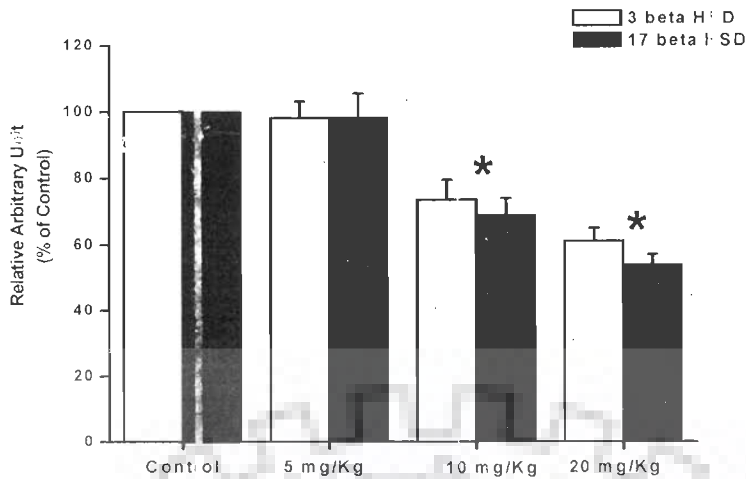


Figure 16. Effects of TCS on testicular level of 3β-HSD(I) and 17β-HSD(III) enzyme activity *in vitro*. The crude enzyme extract was isolated from the testis of vehicle and TCS treated rats and incubated in the presence of respective substrates as described in materials and methods. The results are expressed as percent increase of enzyme activity over vehicle treated groups (control). Data are mean ± S.E.M ; n = 6. * indicates the significant level of difference in enzyme levels as compared to vehicle treated groups for both the enzymes ($p<0.05$).

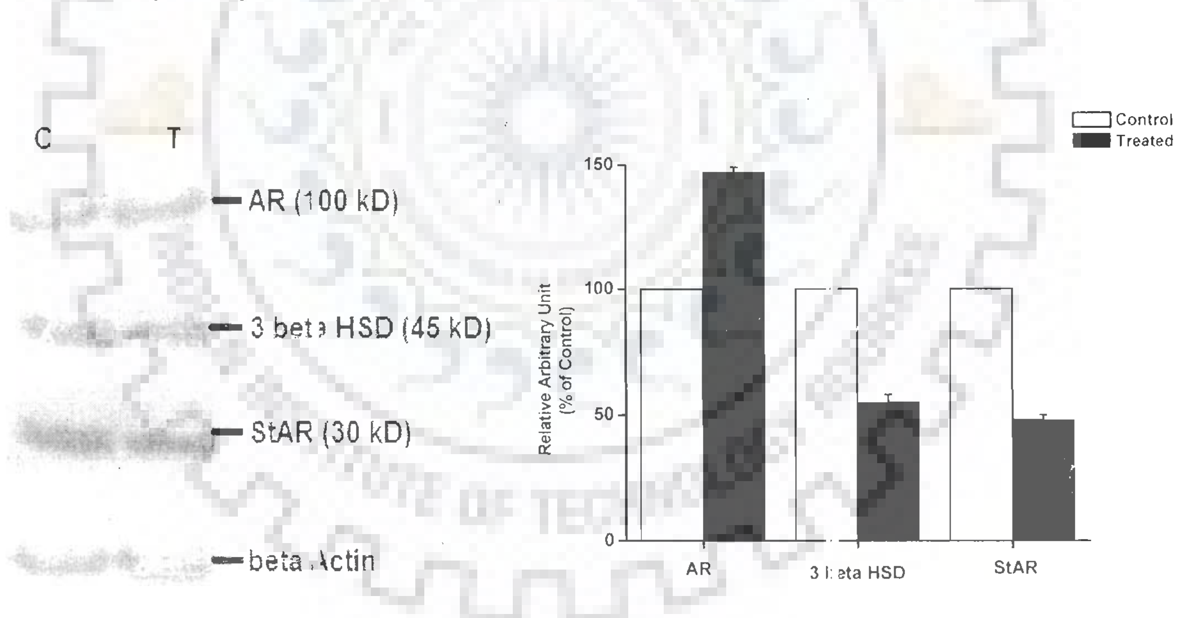
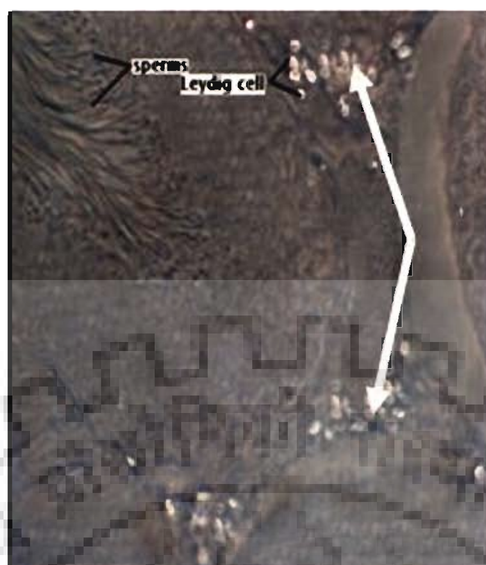
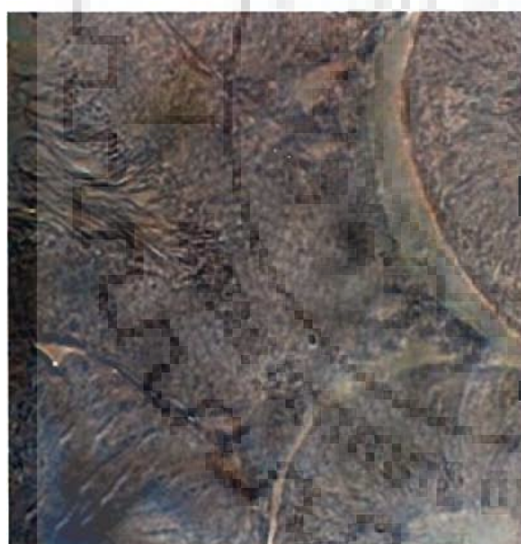


Figure 17. Western blot analysis of testicular 3β-HSD enzyme (approximately 45 kD), StAR protein (approximately 30 kD) and AR protein (approximately 100 kD) in response to test chemical treatment. Protein extracts from vehicle treated rat testes were used as control (C) and β-actin was used as loading control. C, control; T, treated rats (A). The relative intensity of the bands were quantified by densitometer and normalized against the internal control (β-actin) (B). The values are mean ± S.E.M. of three separate experiments.



(A)



(B)

(C)

Figure 18. Immunolocalisation of StAR in Leydig cells from testis of control and rats treated daily with TCS at a dosage level of 20 mg/kg/day. (A) Leydig cells from vehicle treated rats showing expression of StAR (B) Testis section from a vehicle treated rat incubated with non fat milk without primary antibody (negative control). (C) Testis section from the rats treated with TCS showing a decreased staining of Leydig cells (shown by arrow) indicating a decreased level of StAR. No background staining was provided to the sections and photographs were taken in phase contrast mode leading to the appearance of violet colored spot (developed as result of StAR antigen-antibody complex) in the form of whitish violet colored spots. 40x.

Table 5. Comparative uterine weight of the intact rats treated with the three different indicated doses of TCS for a period of 60 days.

Group	Uterine weight (g)
Control	389±16
5mg/Kg	390±12
10mg/Kg	312±15*
20mg/Kg	198±9.0*

(Each value denotes mean ± S.E.M. of eight animals)
*Significantly different from vehicle treated (control) group at $p<0.05$ level.

4.1.2.2.2 Gene expression analysis

Like that of male rats, TCS treatment induced a significant alteration in all of the ovarian steroidogenic genes (except ER) tested in intact rats. Rats treated with a dose of 20 mg/kg/day showed a statistically significant down regulation in the ovarian mRNA levels of cytochrome P450scc, 3β-HSD(I), 17β-HSD(III), cytochrome P450C-17, StAR, aromatase, ER and SRB-1, as compared to control. Level of P450scc, 3β-HSD(I), 17β-HSD(III) and P450C-17 mRNA decreased up to 50, 48, 40 and 48% respectively as compared to control (Fig. 19). In the case of StAR, aromatase and SRB-1 the expression decreased up to 45-50% respectively while that of ER increased up to 130% as compared to control (Fig.19).

4.1.2.2.3 Serum Estradiol and other hormone levels

There was a statistically significant decrease in the serum estradiol levels in female rats treated with TCS at a dose of 20 mg/kg/day as compared to control. ($p<0.05$) (Table 6).

4.1.2.2.4 Ovarian 3β-HSD(I) and 17β-HSD(III) levels in vitro

Like that of male rats, *in vitro* spectrophotometric enzyme assays for 3β-HSD(I) and 17β-HSD(III) demonstrated that the treatment of animals with test chemical caused a statistically significant decrease in the activity of both the ovarian steroidogenic

enzymes at two higher dosage levels (10 and 20 mg/kg/day) ($p<0.05$). The decrease was not significant at a dose of 5 mg/kg/day for both the enzymes. Doses of TCS at a level of 10 and 20 mg/kg/day decreased 3β-HSD(I) enzyme activity up to 29% and 42% respectively (Fig. 20) while that of 17β-HSD(III) enzyme activity up to 39% and 47% respectively (Fig. 20) when compared with control.

Table 6. Serum levels of LH, FSH, cholesterol and estradiol from intact (female) control rats and rats treated with the indicated doses of TCS for a period of 60 days.

	Control	Treated
LH (ng/ml)	0.89 ± 0.054	0.54 ± 0.056 *
FSH (ng/ml)	7.32 ±0.168	5.82 ± 0.045 *
Esradiol (pg/ml)	42.5 ± 0.130	25.4 ± 0.077*
Cholesterol (mg/dl)	75 ± 7.00	43 ± 5.00*

(Each value denotes mean ± S.E.M. of eight animals)
*Significantly different from vehicle control group at $p<0.05$ level.

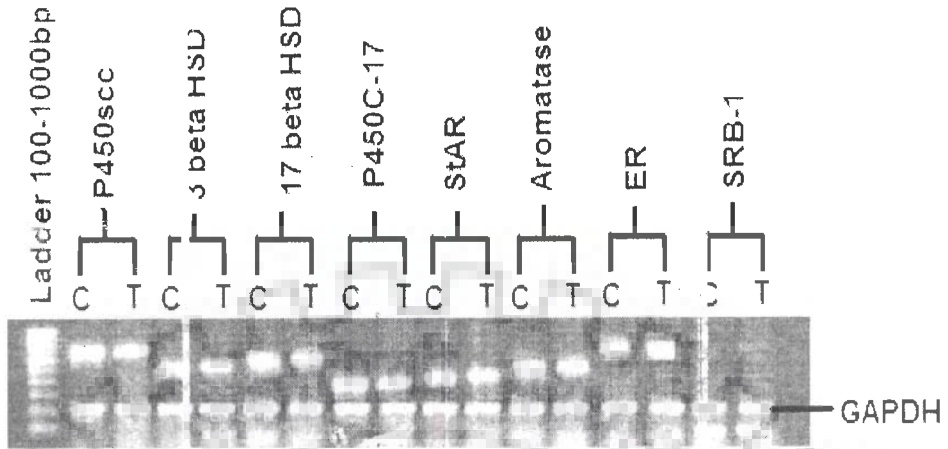
4.1.2.3 Toxicological evaluation of TCS

4.1.2.3.1 Body weight

Treatment of rats with test samples did not induce any significant changes in the body weight at all three doses in the case of both male and female rats (data not shown).

4.1.2.3.2 Toxicity marker enzymes: hypothalamic SOD, CAT, GPx and GR

TCS did not induce noticeable oxidative stress in hypothalamus of treated rats at a dose of 5 mg/kg/day indicated by an unchanged activity of SOD, CAT, GPx and GR enzymes in treated rats as compared to control. However doses of 10 and 20 mg/kg/day induced significant alteration in the activities of the foresaid enzymes in male rats (Fig. 21). At a dose of 10 mg/kg/day, the decrease in the activity of SOD, CAT, GPx and GR was 35%, 29%, 39% and 42% respectively (Fig. 21). The dose of 20 mg/kg/day caused a further



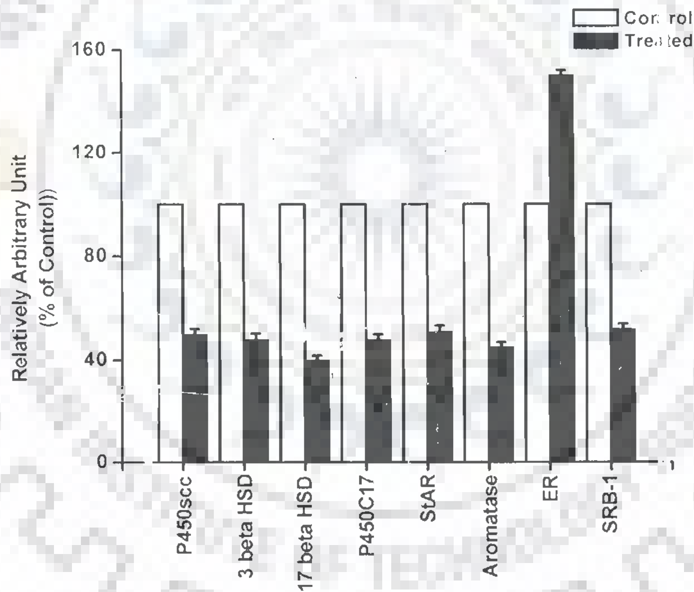


Figure 19. RT-PCR analysis of ovarian mRNA expression of P450scc, 3β-HSD(I), 17β-HSD(III), P450C-17, StAR, aromatase, ER, and SRB-1 genes in rats treated daily with test chemical at a dose of 20 mg/kg/day . (A) The total RNA isolated from ovaries were reverse transcribed and cDNA obtained was subjected to PCR (B). The relative intensity of the signals were quantified by densitometer and normalized against the internal control (GAPDH). The values are mean ± S.E.M. of RT-PCR reactions run for eight RNA samples for each group.

significant decrease of 42%, 49%, 58% and 60% in the activity of SOD, CAT, GPx and GR respectively (Fig. 21).

Female rats showed almost same pattern of the reduction in the activities of all the four enzymes (SOD, CAT, GPx and GR) at the two higher dosage levels (10 and 20 mg/kg/day) while no change in the activity of these enzymes was noticed at a dose of 5 mg/kg/day . However data for effect of TCS on female SOD, CAT, GPx and GR isn't presented here, since it displayed almost the similar pattern of hypothalamic toxicity as obsereved in the male system.

Further a similar pattern of oxidative stress (as tested by the estimation of foresaid enzymes) was observed in the liver of treated rats at the highest dosage level only (data not shwn).

4.1.2.3.3 Histopathology of male reproductive system (Testis and SATs)

A number of histopathological malformations were observed in the testis and SATs of 20 mg/kg group as compared to control. Analysis of the testis of 20 mg/kg group displayed noticeable histopathological malformations, reveled in the form of a decrement of spermatogonia, spermatocytes and spermatids, as compared to control (Fig. 22A and 22B). This was further supported by a 34% decrease in the DSP/gm of testis weight ($p<0.05$) in treated group as compared to control (Fig. 22C). The cauda epididymis (CE) from control rats showed a normal structure and sperm density (Fig. 23A) while a reduced sperm density was observed in the lumina of epididymal tubule from the treated rats (Fig. 23B). Epididymal tubule from control rats showed a normal epithelium and a normal distribution of epithelial nuclei (Fig. 23C) while necrosis was evident in tubular

epithelium of the treated rats (Fig. 23D). Further the TCS treatment resulted in the appearance of scattered nuclei due to the degeneration of tubular epithelium (Fig. 23D).

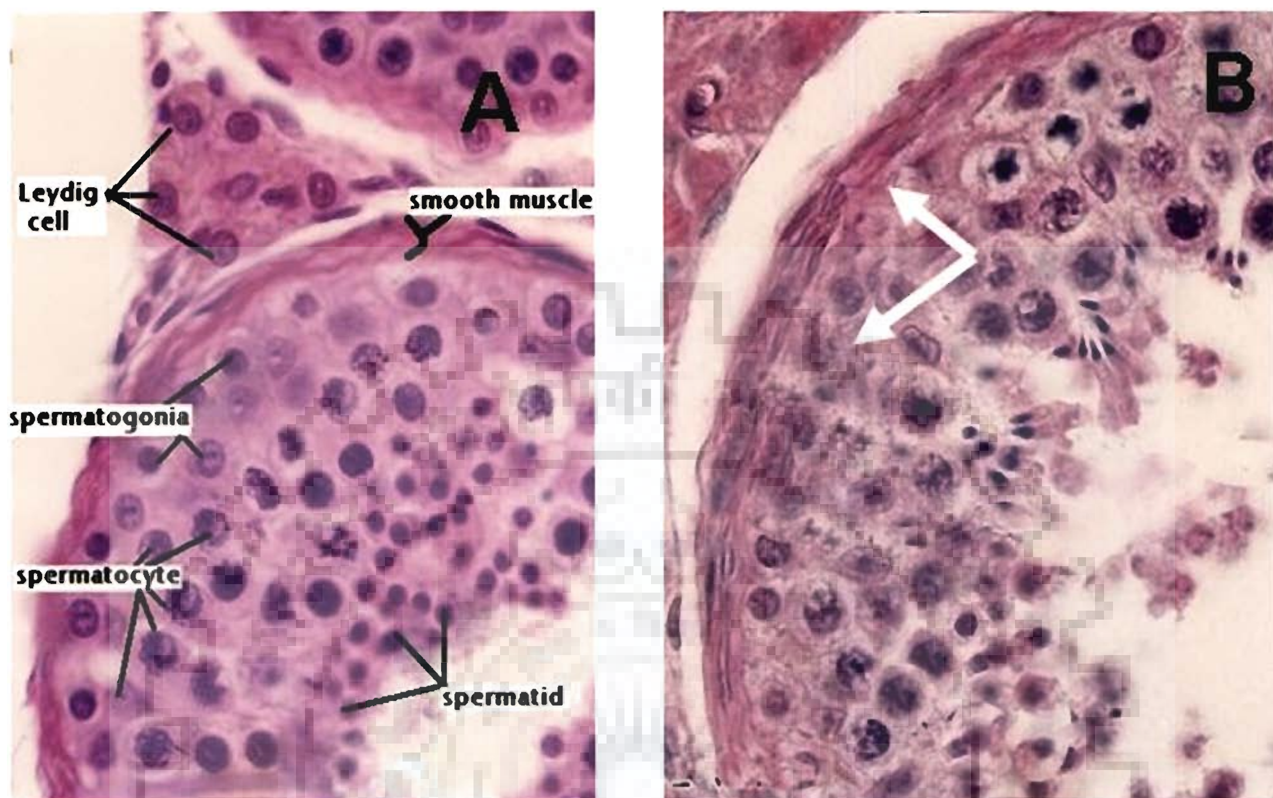
A normal thickness as well as arrangement of ciliated brush border was observed in vas deference from control rats (Fig. 24A) while several malformations were observed in vas deference from treated rats (Fig. 24B). Lumen of vas deference from the treated rats showed presence of sterocilia detached from the epithelium and presence of eosinophillic bodies (Fig. 24B). The stereocilia were found to be thin, few or absent in the epithelium of treated rats (Fig. 24B). In the case of prostate tissue, folliculli appeared to be normal and large sized in control rats (Fig. 24C) while in treated rats the folliculli were found to be comparatively degenerated, empty and with thin follicular wall (Fig. 24D). Seminal vesicle did not display any noticeable histopathological changes in control and treated rats (Fig. 25A and 25B).

4.1.2.3.4 Histopathology of female reproductive system (uterus)

In the control group, the endometrium was composed of distinct layers of columnar epithelium and lamina propria. The myometrium and uterine serosa were found to be normal structurally (Figure 26A). However intact female rats treated with TCS at a dosage level of 20 mg/kg/day displayed severe histopathological malformations in the form of appearance of apoptotic cells within lamina propria and myometrium (Fig. 26B).

4.1.2.3.5 Sperm Toxicity

Eosine and CTC staining displayed that TCS was also adversely affecting the sperm's integrity and its exposure was leading to the fragmentation of the sperms as evident by the detachment of sperm's head from its body (indicated by the arrow) (Fig 27).



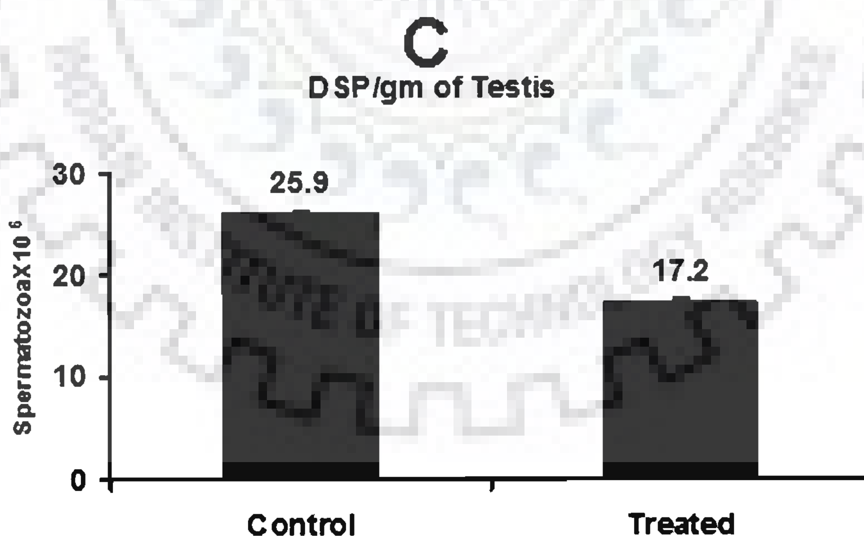


Figure 22. Histopathological analysis of testis and daily sperm production/gm of testis weight (DSP/gm) in vehicle treated (control) and TCS treated (20 mg/kg/day) rats. Testicular histopathology of (A) control and (B) TCS treated rats. (C) Daily sperm production (expressed as DSP/gm of testis weight) in control and TCS treated rats. Data are presented as mean ± SEM of duplicate determinations from individual testis; n = 8 for control and treated testis. DSP was significantly decreased ($p<0.05$) in the treated group as compared to control.

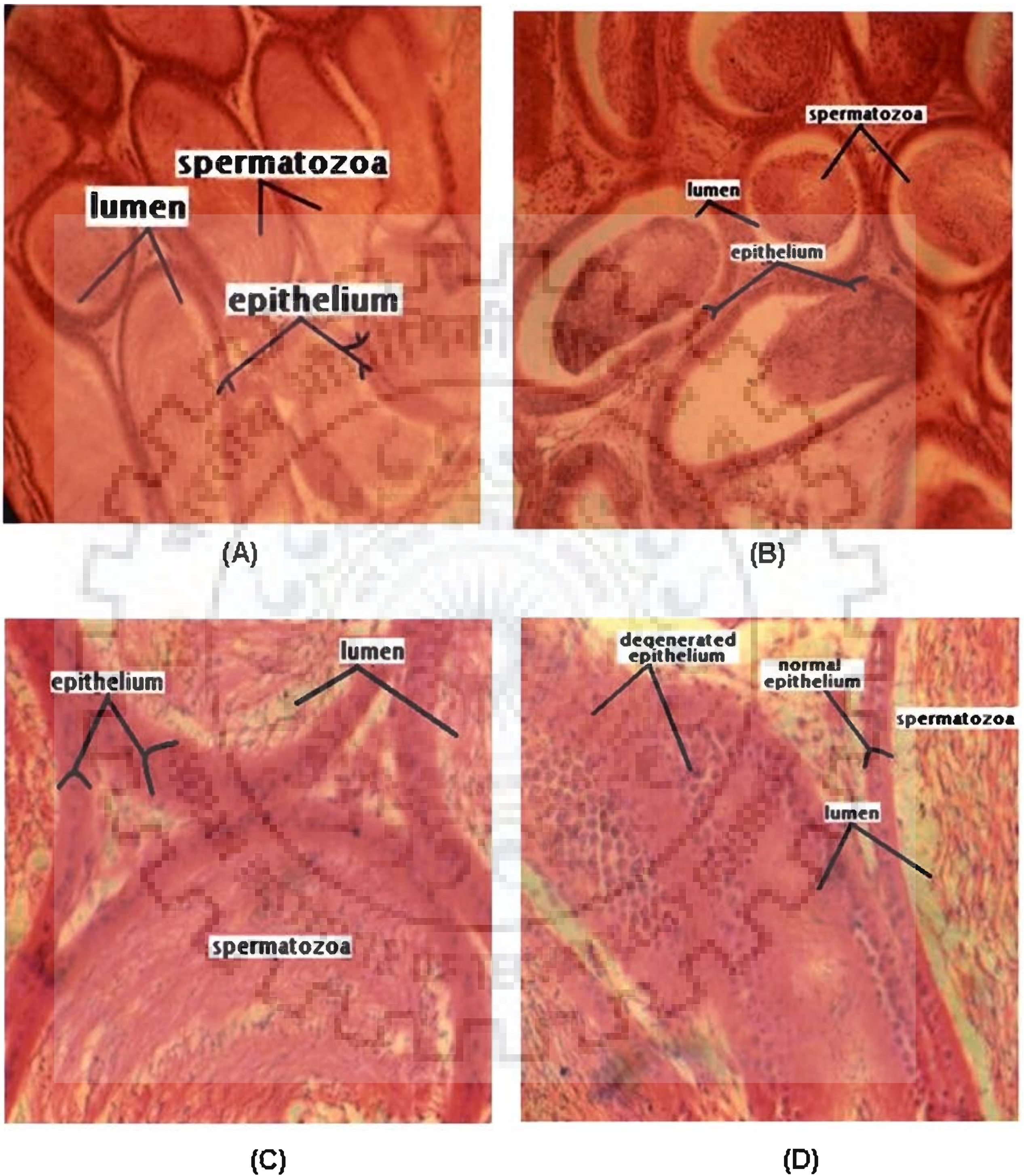
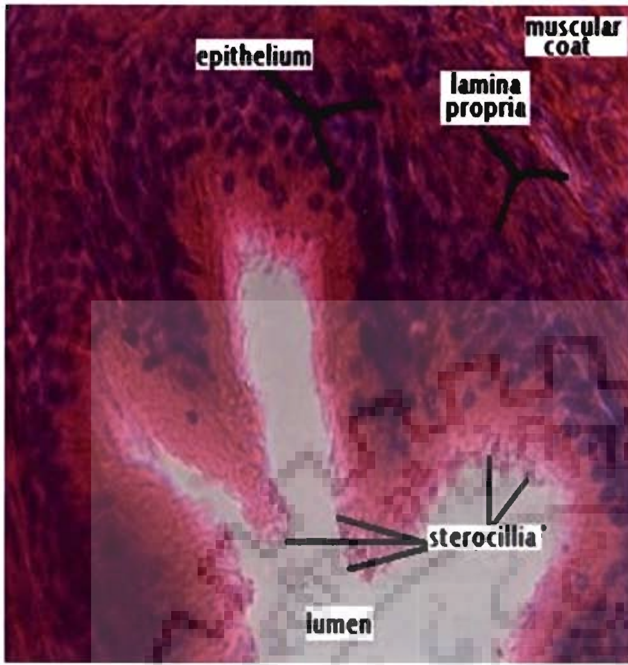
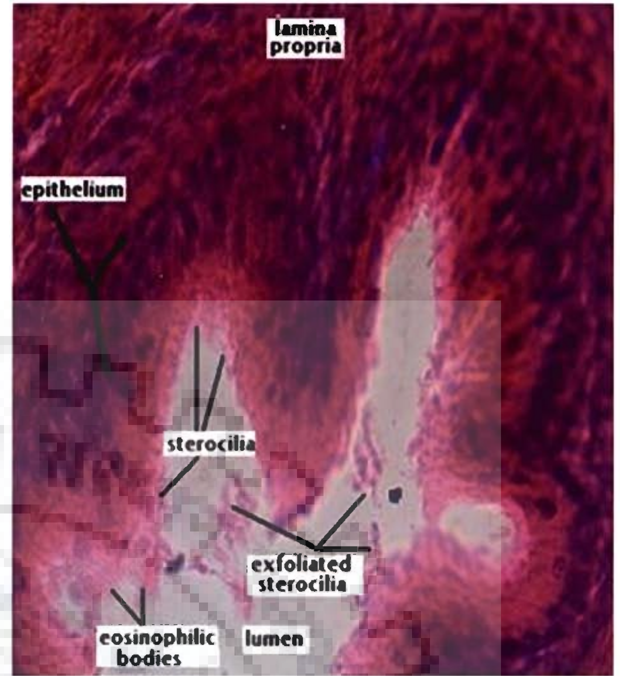


Figure 23. Photomicrographs of rat cauda epididymes showing different degenerative histopathological malformations developed as a result of 60 days consecutive TCS treatment at a dosage level of 20 mg/kg/day. Section of cauda epididymes of control (A) and treated rats (B). H&E, 20×. Epididymal tubule from control (C) and treated rats (D). H&E. 63x.



(A)



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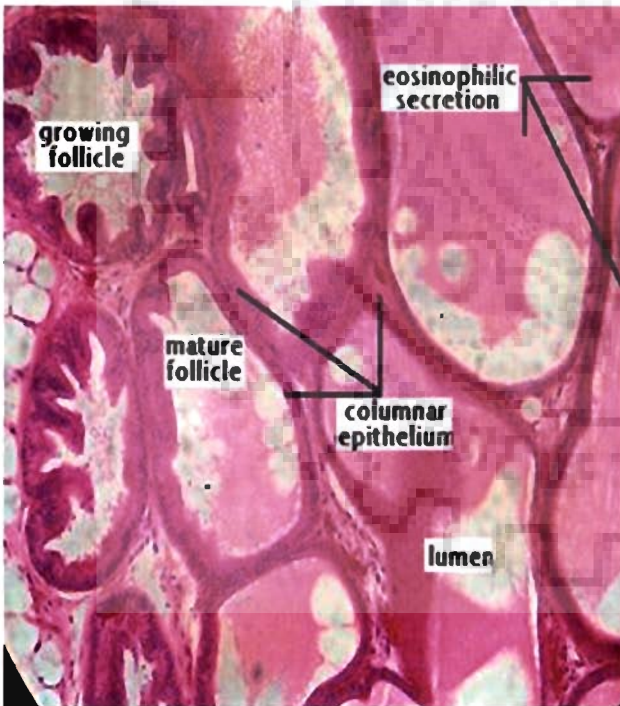

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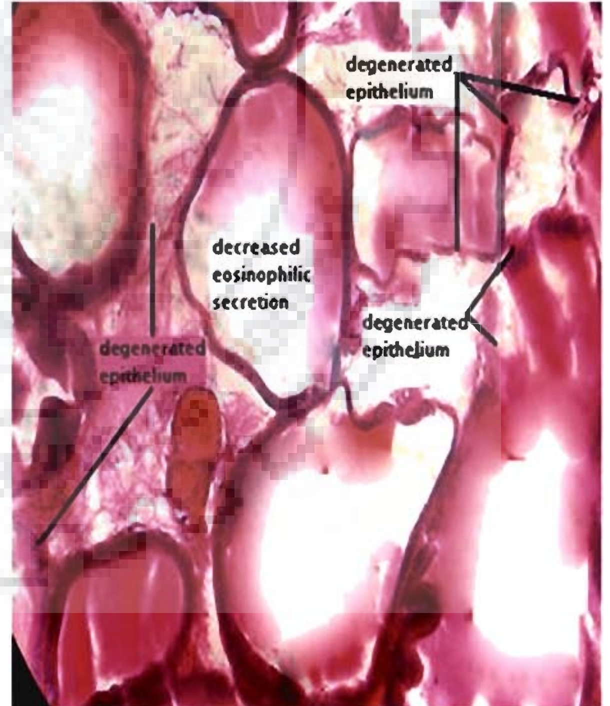
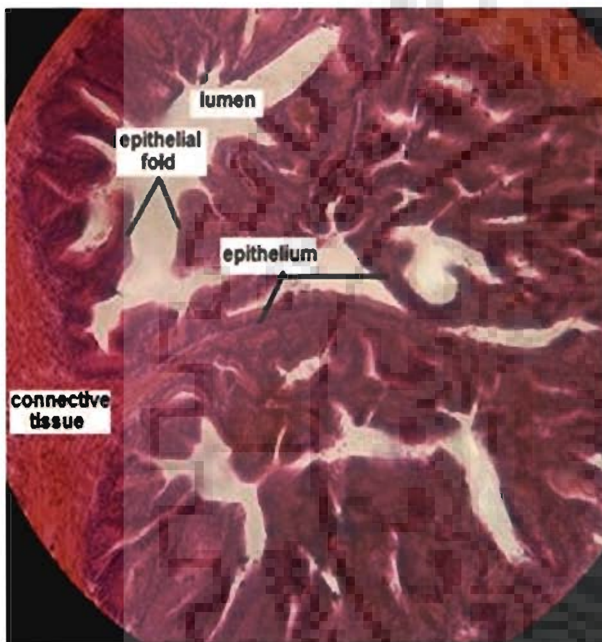
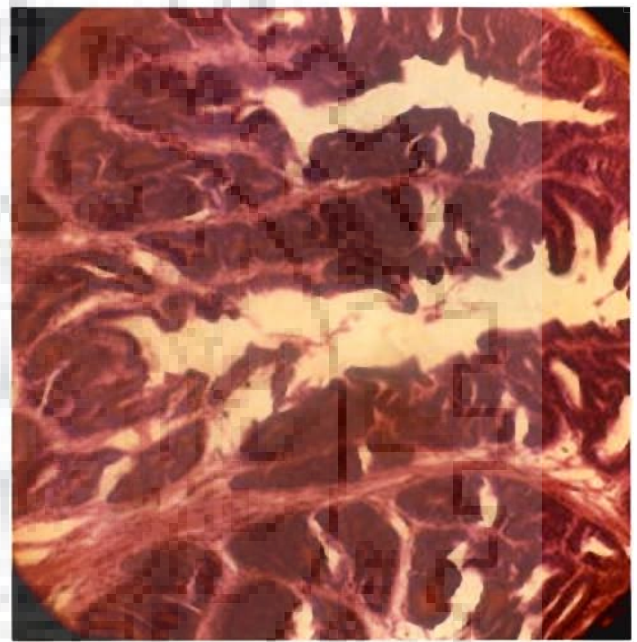


(D)

Figure 24. Effects of TCS treatment on the structure of vas deferens and ventral prostrate glands. Section of vas deferens from control (A) and treated (B) rats. H&E. 63 x. Section from a prostate of control and (C) treated(D) rats. H&E. 40x.



(A)



(B)

Figure 25. Effects of TCS on the structure of seminal vesicle. Sections of vas deferens from control (A) and treated rats (B). H&E. 63x.

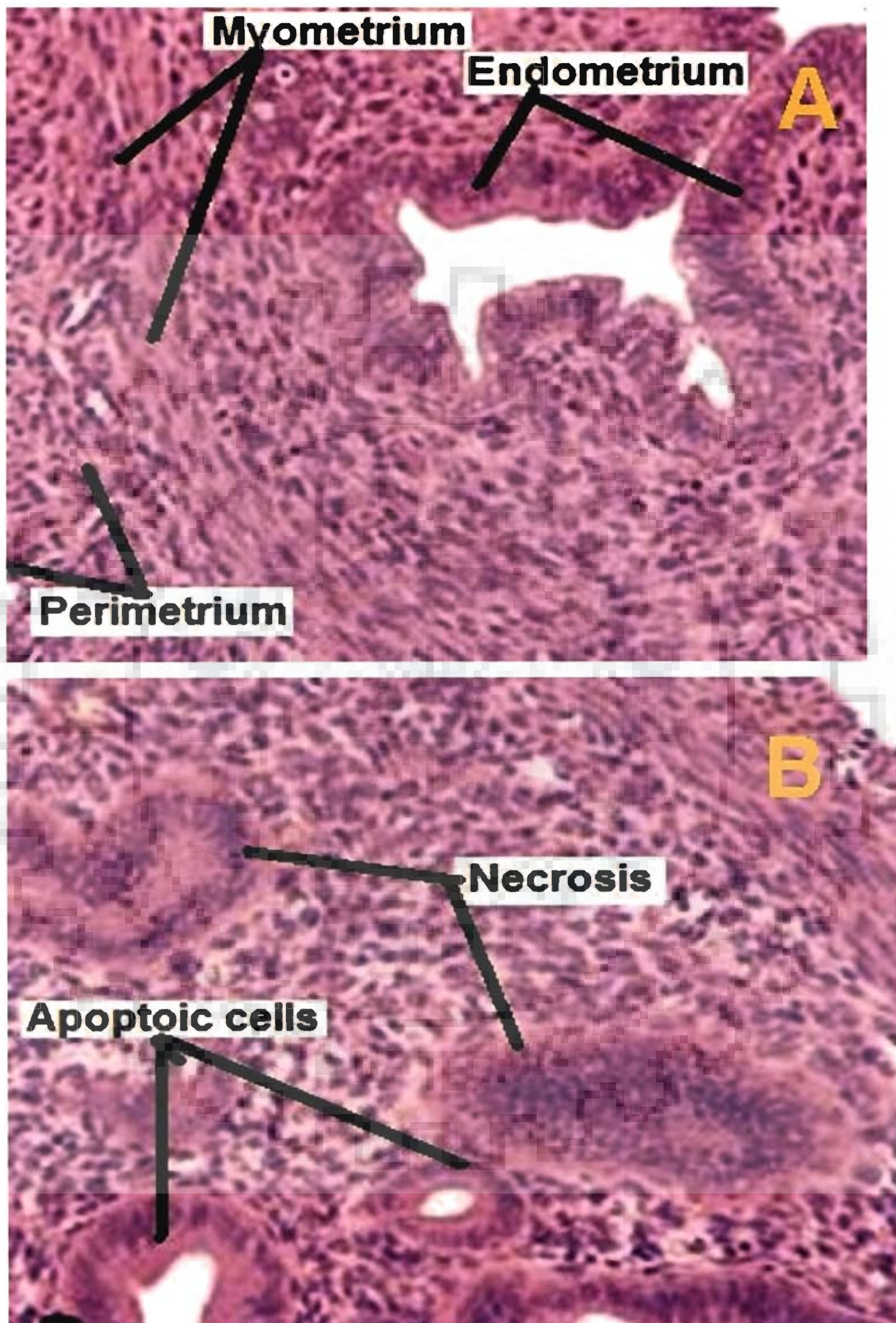


Figure 26. TCS induced uterine histopathological malformations. Uterus of control (A) and TCS treated (20 mg/kg/day) (B) female rats. (H&E, 20x).

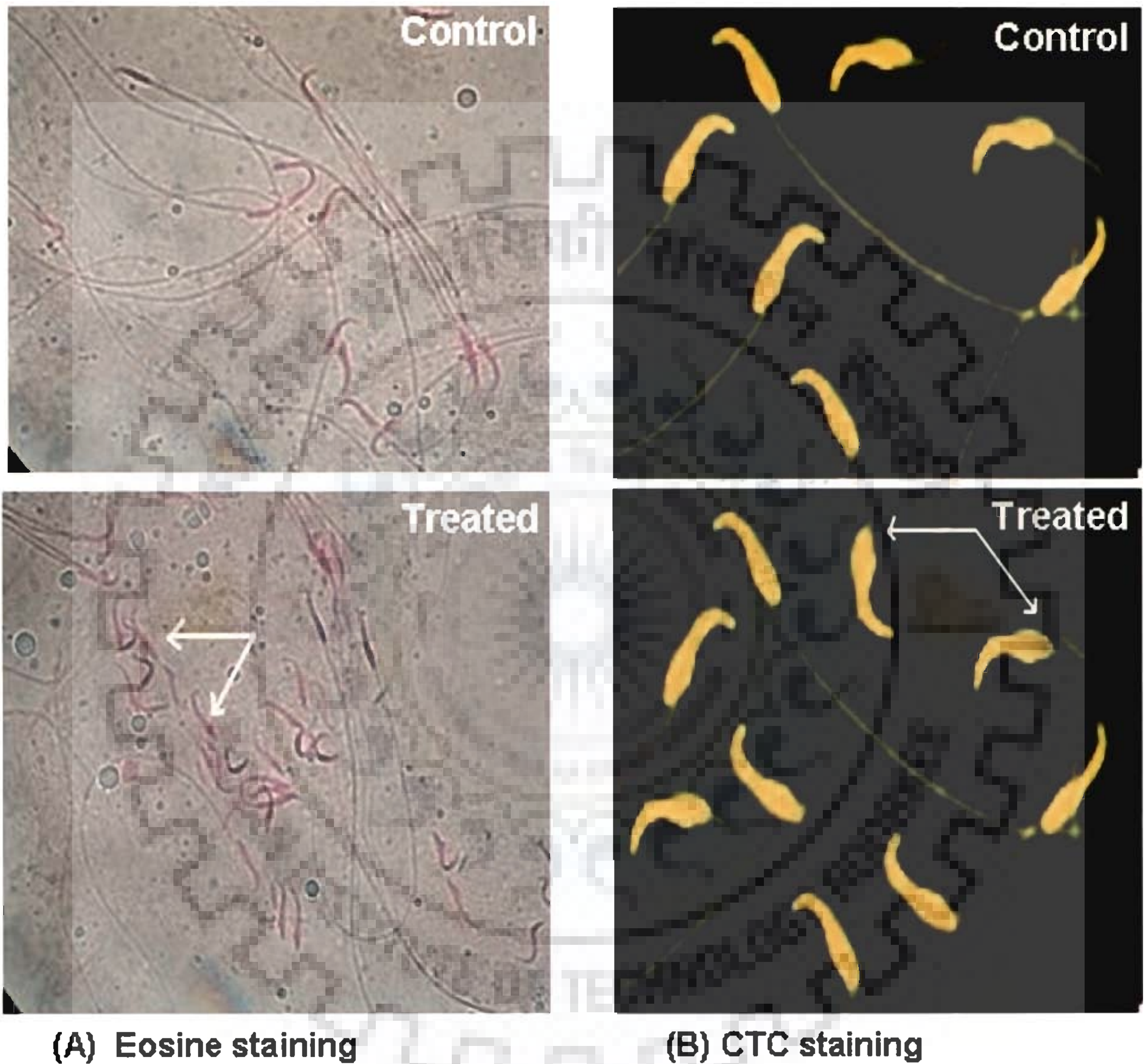


Figure 27. Toxic effect of TCS on the sperms, comparative analysis by the routine eosine staining (A) and CTC staining (A). Heads of the sperm getting detached from the main body of the sperm.

4.1.3 Discussion

TCS is a synthetic chemical widely used as an antimicrobial agent in different commercial preparations (Black et al., 1975). Since TCS possess a phenolic moiety like many of the common EDC it could be presumed to display similar activities as demonstrated by other EDC of the same chemical family (Kitamura et al., 2005). The dosage for the test chemicals used in this study were decided based on LD_{50} values available in the literature and also an earlier report where a similar compound, triclocarbon, was used in rat models (Nollen et al., 1979; Chen et al., 2008). Further, available data have demonstrated that the exposures to these sorts of chemicals either by dermal or oral routes in human and rats lead to similar metabolic profiles (Hiles et al., 1977). Based on these reports, in the present study 5, 10 and 20 mg/kg/day of TCS were administered by oral routes.

One part of this study focused on the TCS induced oxidative stress in hypothalamic region of brain in both male and female rats. Brain is highly sensitive to oxidative stress due to its high concentration of unsaturated lipids and high rate of oxidative metabolism (Pajovic et al., 2003). Like any other tissues brain also has its own detoxifying system composed of the enzymes SOD, CAT, GPx and GR which prevents the generation of reactive oxygen species (ROS) (Muthuvel et al., 2006). ROS are continuously generated by normal metabolism and neuronal activity in the nervous system and attacks phospholipids membrane of brain causing LPO (Allen and Treseni, 2000). GPx is main scavenging enzyme of brain which detoxifies H_2O_2 preveting the generation of ROS (Arivazhagan et al., 2002). Other main detoxifying enzymes responsible for keeping ROS at a lower level are GR, SOD and CAT (Kono and

Fridorich, 1982; Shila et al., 2005). The decreased activities of these enzymes point to a state of oxidative stress as it indicates increased LPO caused by an increased level of ROS (Muthuvel, 2006). In this study, results indicated a decreased level of CAT, SOD, GPx and GR in hypothalamus of both male and female rats treated with two higher doses of TCS (10 and 20 mg/kg/day). This shows that the administration of TCS is leading to development of oxidative stress in treated rats of both the sexes. The generation of oxidative stress is further supported by the malfunctioning of hypothalamus leading to a decreased production of gonadotropins (LH and FSH) as demonstrated by serum hormone analysis in both the sexes. It has been shown elsewhere that production of LH is reduced in hypothalamus due to oxidative stress induced by arochlor 1254 (a PCB) which returns to normal level leading to a normal testosterone synthesis when is supplemented with vitamin C, a well known antioxidant (Murugesan et al., 2007; Muthuvel et al., 2006).

Besides the results indicated that TCS treatment, at two higher dosage levels (10 and 20 mg/kg/day) results in drastic down regulation of both androgen and estrogen synthesis in male and female animals respectively. In active steroidogenic glands, steroidogenesis (androgen and estrogen synthesis) involves several crucial steps starting with the synthesis of cholesterol, the parent molecule for all the steroid hormones, to its transport within the steroidogenic tissues and then its further metabolism to form steroids. Cholesterol is acquired by steroidogenic cells either from de novo synthesis or from the high density/ low density lipoprotein (HDL and LDL) circulating in the blood although former method is preferred (Cao et al., 1999). In this study 20 mg/kg/day group demonstrated a decreased level of serum cholesterol in both the treated male and female

rats. Results also indicated a significantly reduced level of SRB-1 (a type of lipoprotein receptor present on surface of steroidogenic cells to recognize HDL and LDL) in treated animals of both the sexes which indicated a decreased uptake of cholesterol by the steroidogenic cells. Both of these events might have been achieved by a reduced LH production (as demonstrated by serum hormone analysis) since this hormone promotes the synthesis of cholesterol by activating the enzymes of cholesterol synthesizing machinery (like cholesterol ester hydrolase) and also regulates the uptake of cholesterol esters by steroidogenic cells by stimulating the expression of receptors to recognize LDL and HDL e.g. SRB-1 (Fauser et al., 1999). Serum hormone analysis further demonstrated decreased serum testosterone level in male rats while a reduced serum estradiol in female rats which could be attributed to TCS induced decreased synthesis and availability of cholesterol for steroidogenesis. It has been shown earlier that a decreased cholesterol synthesis results in the down-regulation of steroidogenesis (Barlow et al., 2003).

Once the cholesterol is synthesized, StAR is a factor that plays a crucial role in regulating steroidogenesis by transporting cholesterol to inner mitochondrial membrane (IMM) (Hasegawa et al., 2000; Manna et al., 2001, Barbara and Conchrum, 2007). The present findings indicated a decreased transcription and translation of StAR in testis as well as ovary of the treated (20 mg/kg/day) male and female rats (as evident by RT-PCR, immunoblot and immunohistochemical analysis). This might have been caused by a decreased level of serum LH since it regulates steroidogenesis mostly by regulating the level and activity of StAR protein in the steroidogenic cells (Arakane et al., 1997; Murugesan et al., 2007). Several studies have demonstrated that steroidogenesis is

severely decreased by a reduced StAR activity (Kitamura et al., 2005; Murugesan et al., 2007).

In IMM cholesterol is acted upon by P450scc enzyme to produce pregnenolone, one of the major enzymes regulating steroidogenesis (Miller, 1988; Omura and Morohashi, 1996). This study showed a significantly decreased level of P450scc enzyme in both male and female treated rats which might have been caused by a decreased availability of its substrate (cholesterol) in IMM due to reduced level of FSH and LH, since both regulates the level of P450scc (Fauser 1995). Thus, a significantly decreased expression of P450scc enzyme might have been another contributor for attenuating testosterone and estradiol synthesis in male and female rats respectively. This decreased level of P450scc enzyme further supports another finding of this study viz. a decreased level of serum pregnenolone in male rats, which is produced by this enzyme from cholesterol. In similar studies chemicals like nonylphenol and DBP have been shown to decrease steroidogenesis due to a reduced expression of StAR and P450scc enzyme (Arukwe, 2005; Kitamura et al., 2005; Lyssimachou et al., 2006).

Expression profiles and activity of two major steroidogenic enzymes, 3β-HSD(I) and 17β-HSD(III), were also found to be significantly decreased in the treated rats of both sexes. This finding is supported by some recent studies that showed direct effect of endocrine disruptors at enzyme levels (Andric et al., 2006; Lyssimachou et al., 2006; Kumar et al., 2008a, 2008b). According to literature xenobiotics dependent direct up/down regulation of steroidogenic enzymes and steroidogenesis can be affected at several levels viz: direct binding of these chemicals to steroid receptors, steroidogenic enzymes and proteins associated with steroidogenesis e.g. StAR protein (Rice et al.,

2006) and increasing the stability of transcripts and transcriptional rate of the promoter of steroidogenic enzymes (Lin et al., 2006).

Further, the expression patterns of AR and ER was found to be increased in treated male and female rats as demonstrated by RT-PCR and immnunoblot analysis respectively. This might have been achieved by autologous regulation of AR and ER genes by a reduced level of testosterone and estradiol (in the testis and ovary respectively).

Another interesting finding of this study was the development of extensive histopathological malformations in testis and SATs (CE, ductus deference and prostate) and uterus of 20 mg/kg/day TCS treated male and female rats respectively. The decreased testosterone level in treated rats (as indicated above) might have led to the degenerative changes and atrophy in the SATs as supported by their decreased weight and size (data not shown) in this study. Results also demonstrated a decreased sperm count in the testis of treated rats as compared to control caused by a lowered testicular spermatogenesis due to toxicity induced by TCS (Ku et al., 1993; Poon et al., 2004). This might have been responsible for a lowered sperm density in cauda epididymis of treated rats as demonstrated by histopathology. Serum ELISA demonstrated a reduced level of serum FSH which also might have been a reason for reduced spermatogenesis since FSH in conjunction with testosterone have been shown to maintain spermatogenesis (Plant et al., 2001). Other degenerative changes observed in the cauda were occurrence of epithelial degeneration in the form of nuclear karyolysis and pyknosis. These types of histopathological changes in cauda have been reported by others also (Chitra et al., 2003; Narayana et al., 2006). Similar degeneration and atrophy were also found in the other

SATs like ductus deferens and prostate glands. All these could be attributed to the decrease in the level of testosterone as it is known to support the functioning and continuous persistence of these organs. Similarly a pertinent amount of estradiol is required for the maintenance of uterus and a TCS induced dwindled synthesis of estradiol might have led to the development of histopathological malformations in the uterus of female rats (as demonstrated by their decreased weight in TCS treated rats) (Guney et al., 2006).

In conclusion, TCS, a commonly used chemical in various cosmetics and other applications, acts as an endocrine disruptor in both male and female rats and has the potential to impair the entire pituitary-gonadal pathway by acting at various levels. We demonstrated that once TCS inhibited the production of LH and FSH due to the induction of oxidative stress in hypothalamus of both male and female rats, its may act as an anti-androgen and anti-estrogen by various modes of action - firstly, by depressed cholesterol synthesis, secondly, by reduced uptake of HDL by a depressed expression of SRB-1, a key receptor for HDL; thirdly, by depressed StAR expression, one of the crucial protein responsible for cholesterol transport to inner mitochondrial membrane for its utilization by steroidogenic enzymes, and last but not the least, by the down regulation of several key steroidogenic enzymes like P450scc, P450C-17, 3β-HSD(I), and 17β-HSD(III). A decreased level of testosterone and estradiol led to the increased production of gonadotropins and AR and ER respectively in treated rats. Altogether, gonadotropins, steroidogenic enzymes, StAR, SRB-1, ER and AR, key factors involved in androgen and estrogen production and maintenance of SATs and uteri, are targets for TCS mediated oxidative stress and impairment of steroidogenesis.

Chapter 4.2 *Two common pharmaceuticals: NSAID and antibiotic as EDC*

4.2.1 Introduction

Scientific advancements in pharmaceutical sectors have led to the synthesis of a number of compounds to be used by humans as therapeutics for fighting against various diseases. However, the concern regarding the usage of these therapeutics becomes important when administration of them may have some harmful side effects like abnormal fetal development, pregnancy loss and ailing reproductive health. Ibuprofen is such a chemical that is used in preparation of non-steroidal anti-inflammatory drugs (NSAIDs) which are used for management of fever, pain and inflammation (Vane, 1971; Sibonga et al., 1998). Ibuprofen, naproxen, sulindac and aspirin are some of the commonly used NSAID. Another class of widely used pharmaceutics is the antibiotics and one of the main constituent of a number of well known antibiotics is tetracycline which acts by inhibiting the action of the prokaryotic 30S ribosome by binding aminoacyl-tRNA.

A number of synthetic chemicals used in the preparation of commonly used therapeutics like drugs, antibiotics, hormones, and veterinary feed additives may behave as endocrine disrupting chemicals (EDC) leading to production of a number of sex related disorders (Satog et al., 2001; Ingerslev et al., 2003; Lintelmann et al., 2003; Guillette, 2006; Massart et al., 2006). Ibuprofen has been shown to affect the actions of tamoxifen and 17ß-estradiol in a study related to bone metabolism in rats (Sibonga et al., 1998). In rainbow trout administration of ibuprofen disrupts steroidogenesis in the inetrrenal tissues, an organ homologous to the adrenal gland of mammals (Gravel and Vijayan, 2006). Similarly oxytetracycline has been shown to be anti-estrogenic causing

developmental toxicity in CD rats and CD-1 mice and treated groups demonstrated decreased weight of uterus (Morrissey et al., 1986).

This section of the thesis was aimed to find out the endocrine disrupting effect of some of the synthetic chemicals employed for the preparation of commonly used pharmaceutical products. We selected two synthetic analogs depending on their frequency of applications in pharmaceutical industry, and their probable potency to behave as EDC. The analogs under study were – Ibuprofen and Oxytetracycline (Fig. 28).

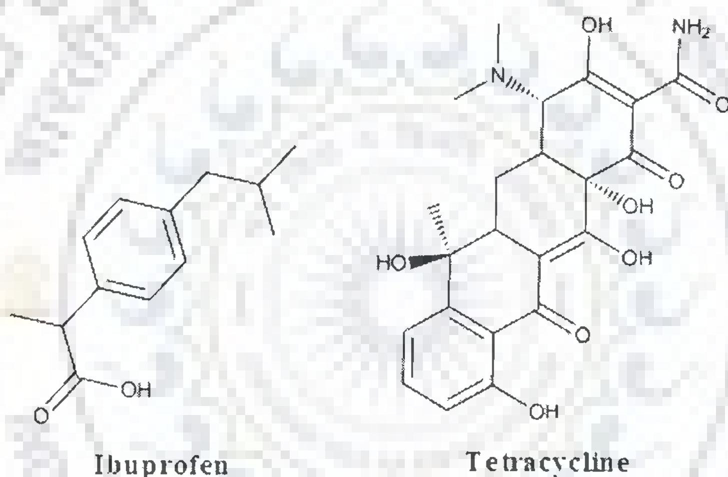
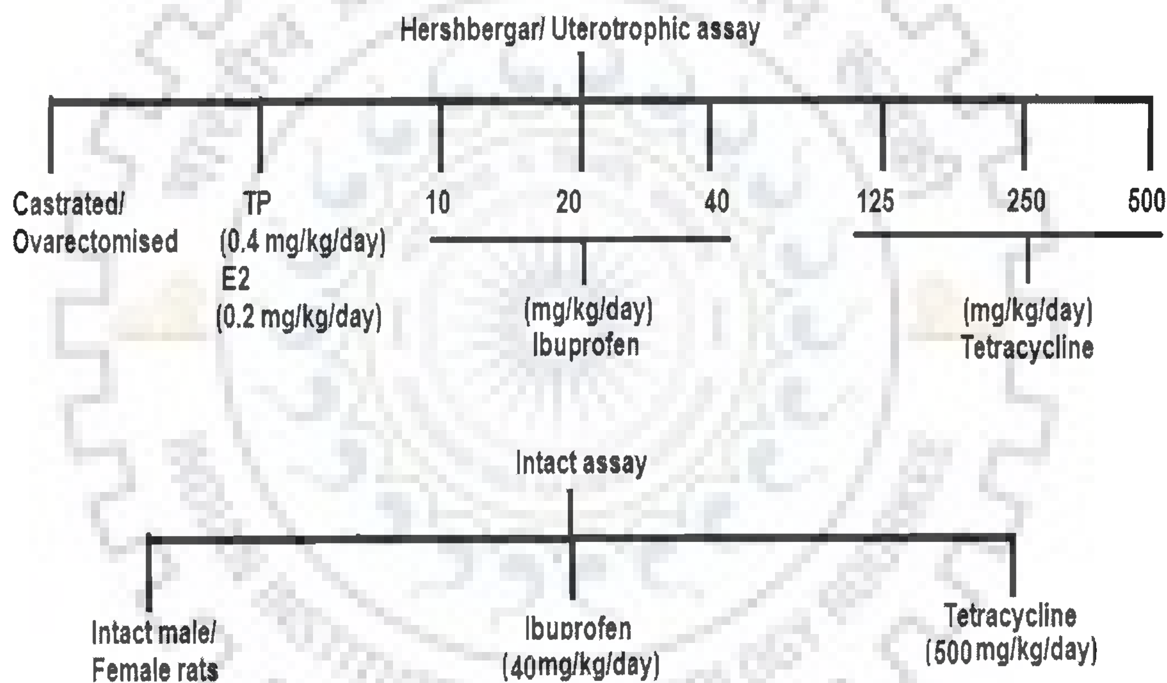


Figure 28. Chemical structure of the representative test chemicals used to gavage the animals in the study.

Although these two chemicals are suspected to behave as EDC still not many information are available on the extent of endocrine disrupting effects caused by them. In the present study, an integrated approach was adopted to study the endocrine disrupting effect of ibuprofen and oxytetracycline. First, the test chemicals were screened for their (anti)androgenicity and (anti)estrogenisity using Hershberger assay and uterotrophic assay by gavaging them with three dosage levels (below LD_{50}) for a fixed period of time. The doses used here were based on the amount consumed by human being and scaled

down to rat system. Once the chemicals under consideration were found to be anti-androgenic and anti-estrogenic, their mode of endocrine disruption was determined by treating the intact male and female rats with the dosage level found most effective in Hershberger assay and uterotrophic assay for the same duration of time. Schematic representation, given immediately after this paragraph, depicts the summary of dosing plan used for gavaging the different doses of both the drugs to male and female rats as described in the methodology section..



4.2.2 Results

4.2.2.1 Endocrine disrupting effect of ibuprofen and oxytetracycline in male reproductive system

4.2.2.1.1 Determination of anti-androgenicity of test drugs by Hershbergar assay

The administration of both the test chemicals at the highest and moderate dosage levels used in the study (20 and 40 mg/kg/day for ibuprofen, and 250 and 500 mg/kg/day for tetracycline) caused a significant decrease in the weight of, seminal vesicle, vas deference, glans penis and cauda epididymis in the case of castrated male rats as compared to respective positive controls ($p<0.05$) (Table 7). This can be attributed to the specific anti-androgenic effect induced by these chemicals in the castrated rats. The decrease in the weight of sex accessory tissues in castrated male rats is also supported by their decreased size (Fig. 29). However, no significant decrease in the weight of SATs was observed at the lowest dosage level (10 and 125 mg/kg/day for ibuprofen and tetracycline respectively) for each of the two test chemicals (Table 7).

Table 7. Effects of three increasing doses of ibuprofen and tetracycline on weights of SATs from castrated rats given 14 days consecutive treatment.

	Androgen (0.4 mg/kg)	Seminal Vesicle (mg±S.E.)	Vas defrence (mg±S.E.)	Glans Penis (mg±S.E.)	Cauda Epididymis (mg±S.E.)
Control	No	56 ± 1.10	72 ± 2.64	52 ± 1.30	3.6 ± 0.12
Control	Yes	413 ± 2.05	278 ± 2.73	87.5 ± 1.23	32.16 ± 1.57
Ibuprofen (10 mg/kg/day)	Yes	412 ± 2.76	280 ± 2.48	87.0 ± 1.63	32.02 ± 0.70
Ibuprofen (20 mg/kg/day)	Yes	309 ± 2.76	208 ± 2.48*	71 ± 1.63*	21 ± 0.70*
Ibuprofen (40 mg/kg/day)	Yes	109 ± 2.76*	158 ± 2.48*	53 ± 1.63*	9.75 ± 0.70*
Tetracycline (125 mg/kg/day)	Yes	414 ± 3.39	276 ± 2.27	88 ± 1.45	31.8 ± 0.76
Tetracycline (250 mg/kg/day)	Yes	287 ± 3.39*	211 ± 2.27*	69 ± 1.45*	25 ± 0.76*
Tetracycline (500 mg/kg/day)	Yes	114 ± 3.39*	170 ± 2.27*	57 ± 1.45*	14.5 ± 0.76*

(Each value denotes mean ± S.E.M. of eight animals)
* Significantly different from control group receiving TP at $p<0.05$ level

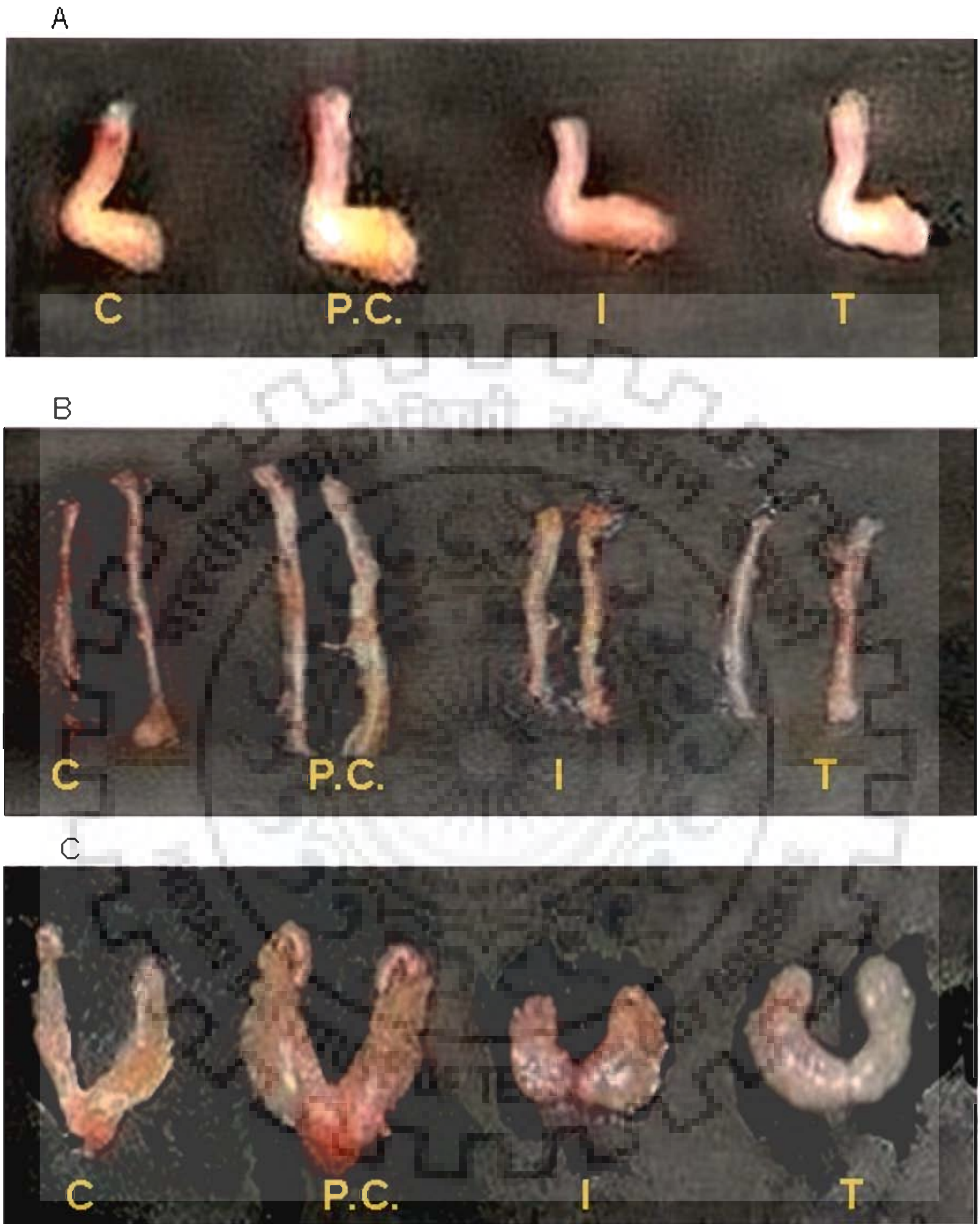


Figure 29. The effects of sample drugs on the structure of some of the SATs of castrated rats treated with ibuprofen and tetracycline at doses of 40 and 500 mg/ kg of body weight respectively (simultaneously treated with TP). A, glans penis; B, vas deference; and C, seminal vesicle. The organs were collected, weighed and were documented by photography under a stereo zoom microscope with 10X magnification. C, control; P.C., positive control (treated with TP); I, ibuprofen; T, tetracycline.

4.2.2.1.2 Effects on cour ship behavior in intact animals

The treatment of rats wi h sample drugs caused a decrease in th number of attempts by male rats to invade into he female chamber. In the present stud courtship behavior was taken into consideratio from 8th day of dosing and continued till the end of dosing period. The control rats showed an average 11 to 12 number of attempts per day (Fig. 30). The number of atter pts were found to be approximately 8 i case of rats treated

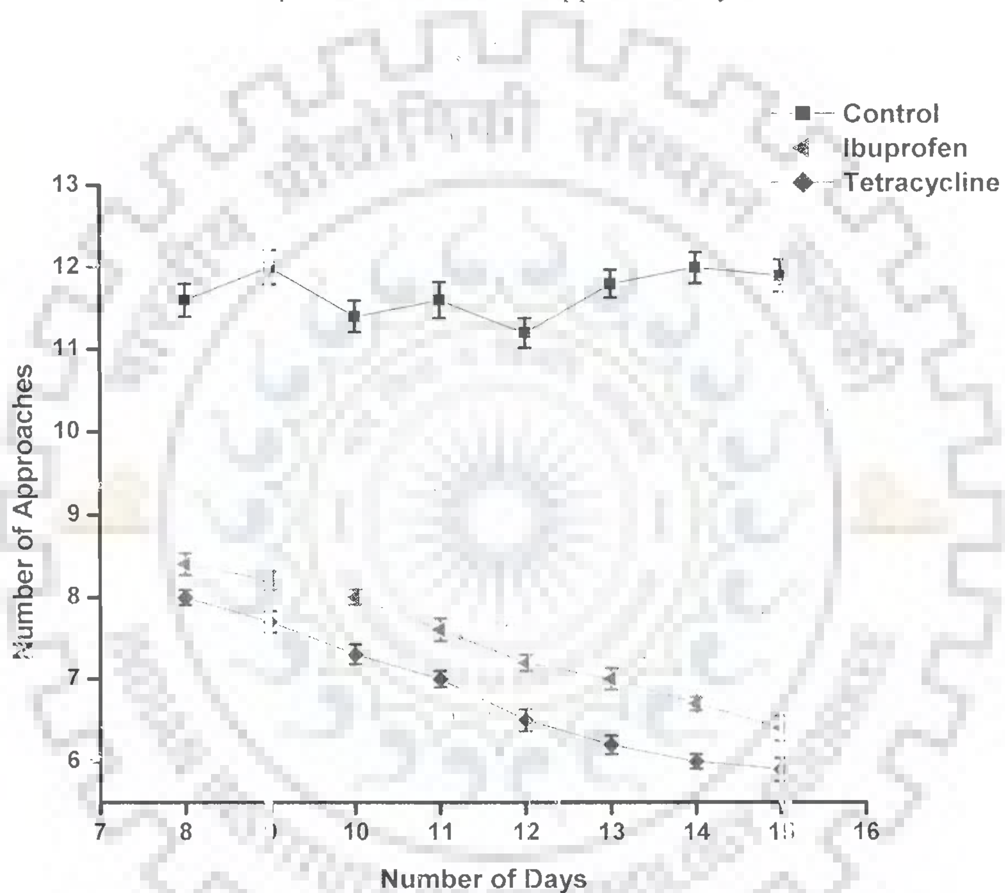


Figure 30. Effect of test drugs on the reproductive behavior of a lult intact male rats. The reproductive behavior w is observed from 8th day of dosing as described in materials and methods.

with both the chemical on 8th day of dosing (the first day o studying the courtship behavior). There was a consistent decrease in the number of atte npts with the increase in the number of days of dc sing. On the 14th day of dosing the num er of attempts decreased

up to 6 and 5 (average for whole group) in case of tetracycline and Ibuprofen treated rats respectively.

4.2.2.1.3 Gene expression analysis

A marked alteration in the testicular levels of mRNA for P450scc, 3β-HSD, 17β-HSD, P450C-17, StAR, and AR was detected in both the treated groups of male rats as compared to control. In testis of ibuprofen and tetracycline treated male rats level of P450scc decreased up to a level 54 and 60 % of control, 3β-HSD up to 75 and 54% of control while that of P450C-17 decreased up to 56 and 51% of control respectively (Fig. 31). Further testicular 17β-HSD and StAR mRNA levels were also decreased significantly by approximately 50% as compared to control in response to treatment with both the drugs (Fig. 31). Expression of AR was increased by a value of 150% in treated rats. The treatment did not alter the expression of SRB-1 gene in the treated rats in case of both the drugs (data not shown).

4.2.2.1.4 Testicular 3β-HSD and 17β-HSD levels in vitro

In vitro spectrophotometric enzyme assays for 3β-HSD and 17β-HSD demonstrated that the treatment of animals with both the test drugs caused a significant decrease in the activity of both the testicular steroidogenic enzymes at the two higher dosage levels (20 and 40 mg/kg/day for ibuprofen and 250 and 500 mg/Kg body weight for tetracycline) ($p<0.05$) (Fig. 32). There was no significant decrease in the activity of both the enzymes by the lowest doses of both tests drugs. The treatment of animals with ibuprofen decreased 3β-HSD activity by 36 and 68% while 17β-HSD activity by 25 and 61% as compared to control, at dosage levels of 20 and 40 mg/kg/day respectively. In case of tetracycline the decrease was 31 and 70% for 3β-HSD while 32 and 69% for 17β-HSD

as compared to control, at dosage levels of 250 and 500 mg/Kg body weight respectively (Fig. 32).

4.2.2.1.5 Testicular immunoblot analysis

A decreased translation of testicular 3β-HSD and StAR while an increased level of AR proteins was found in both the treated groups of animals as seen by the changed band intensities as compared to control (vehicle treated animals) in western blot analysis (Fig. 33A). The uniform band intensities of β-actin in all the wells indicated equal gel loading Western blot strips were examined by reflectance densitometry (Fig. 33B) with a densitometer (BioRad, USA).

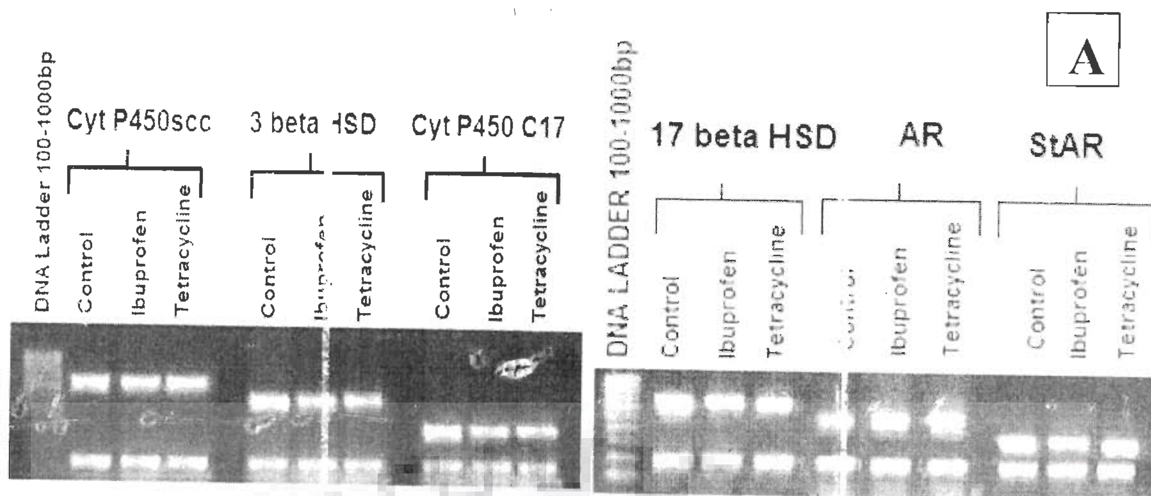
4.2.2.1.6 Serum hormone levels

There was a significant decrease in the testosterone levels and a marginal non significant change in the level of LH and FSH in treated male rats ($p<0.05$) (Table 8).

4.2.2.2 Endocrine disrupting effect of ibuprofen and oxytetracycline in female physiological system

4.2.2.2.1 Determination of (anti)estrogenicity of test drugs by uterotrophic assay

Like that of male rats, administration of both the test chemicals caused a significant decrease in the weight of uterus of the ovarectomised rats at the two higher dosage levels (20 and 40 mg/kg/day for ibuprofen, and 250 and 500 mg/kg/day for tetracycline) as compared to positive controls (treated simultaneously with estradiol, 0.2 mg/kg/day) ($p<0.05$) (Table 9). This can be attributed to the specific anti-estrogenic effect of the test chemicals. The decrease in the weight of uterus is also supported by their decreased size (Fig.34). However, no significant decrease in the weight of uterus was observed at the lowest dosage level (10 and 125 mg/kg/day s for ibuprofen, and tetracycline respectively) for each of the two test chemicals (data not shown).



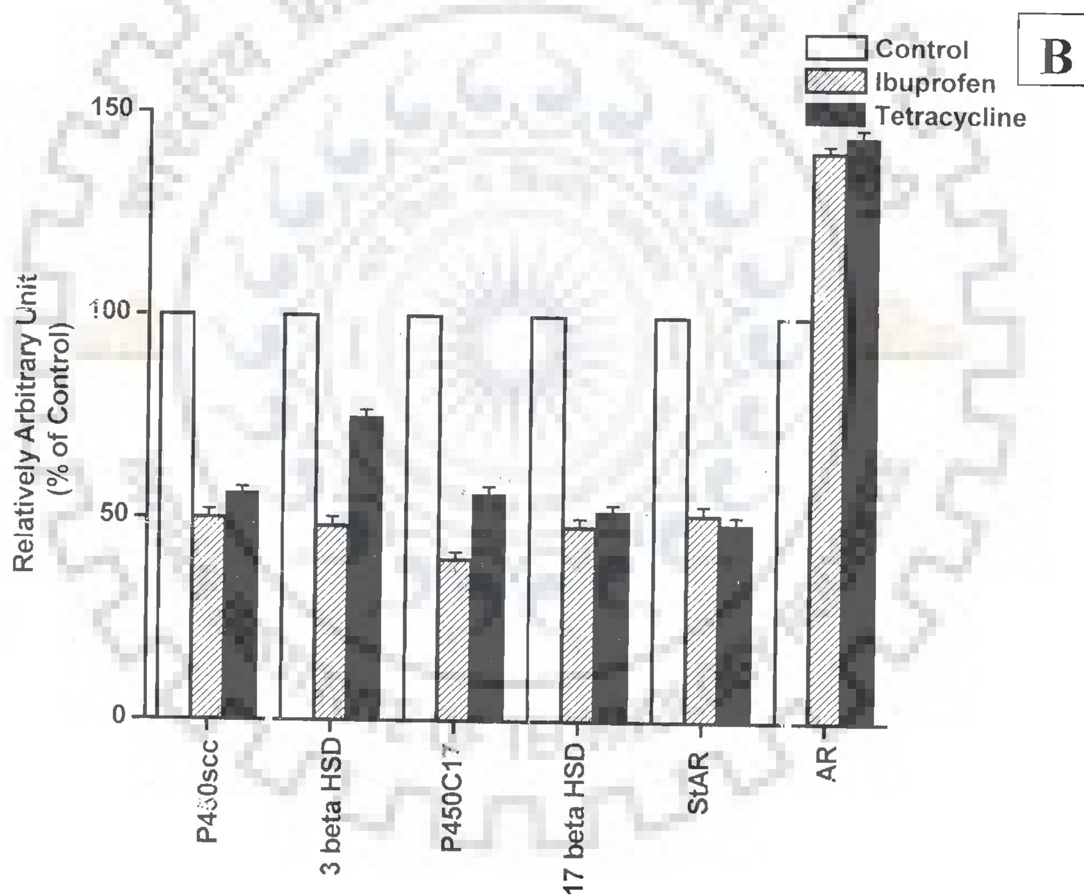
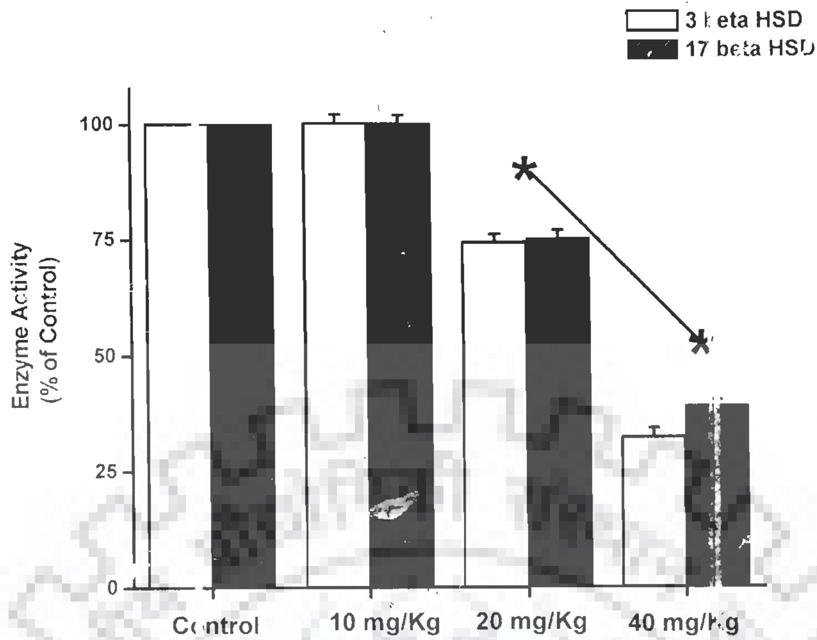


Figure 31. RT-PCR analysis of testicular mRNA expression of P450scc, 3β- HSD, P450C-17, 17β-HSD, androgen receptor (AR) and StAR genes in rats treated with the two highest does of sample drugs (A). Densitometric analysis against the internal control (GAPDH) (B). The values are mean ± S.E.M. of eight RT-PCR reactions for each group tested.



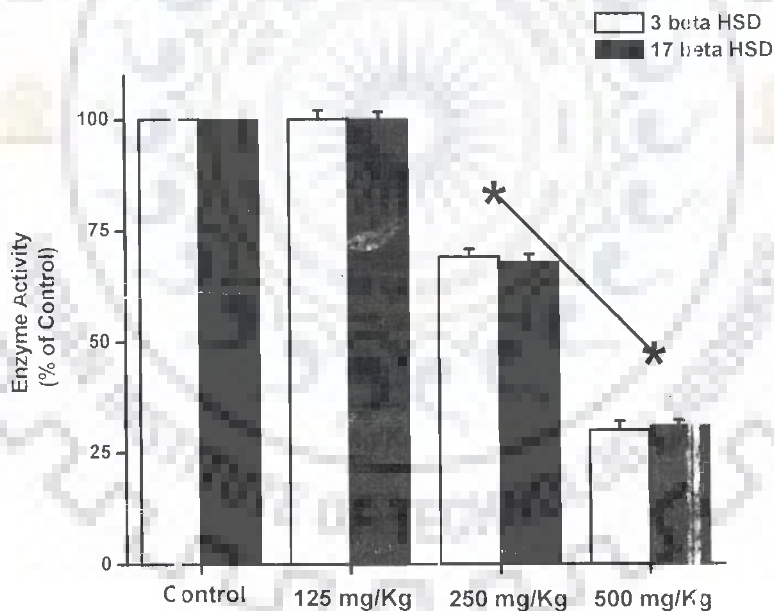


Figure 32. Effects of treatment of three different doses of ibuprofen (A) and tetracycline (B) on testicular level of 3β-HSD (open bars) and 17β-HSD (solid bars) enzyme activity *in vitro*. The results are expressed as fold increase of enzyme activity over vehicle treated groups. Data are mean ± S.E.M.; n = 5. * indicates the significant level of difference in enzyme levels as compared to vehicle treated groups for both the enzymes ($p<0.05$).

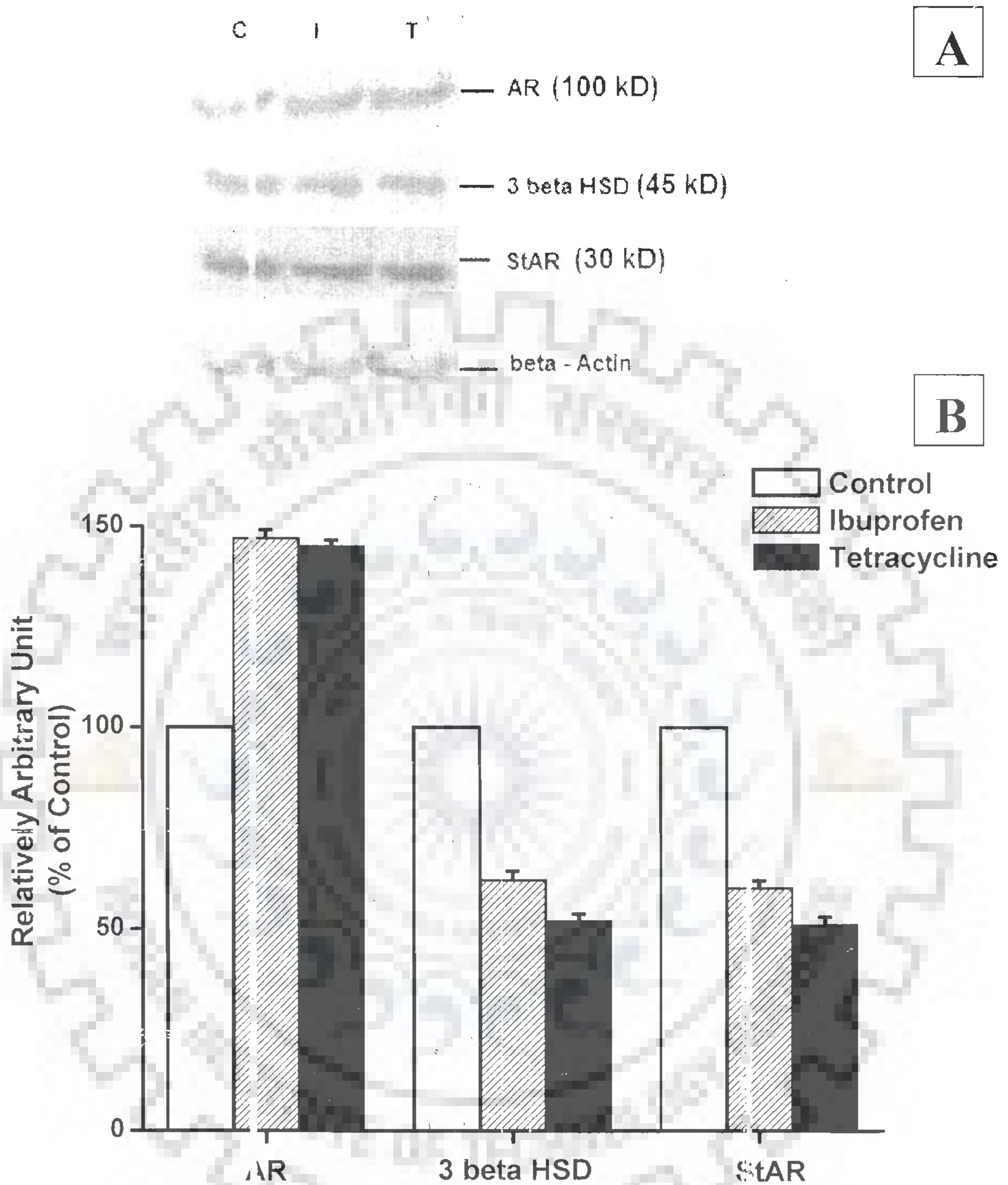


Figure 33. Western blo analysis of testicular 3β-HSD enzyme (approximately 45 kD), StAR protein (approximately 30 kD) and AR protein (approximately 100 kD) in response to test drugs treatment. Protein extracts from vehicle treated rat testis was used as control and β-actin was used as loading control. C, control; I, ibuprofen; T, tetracycline (A). Denestometric analysis against the internal control (β-actin) (B). The values are mean±S.E.M. of three separate experiments.

Table 8. Serum levels of LH, FSH and testosterone from intact rats given 14 days treatments with sample drugs.

	LH (ng/ml)	FSH (ng/ml)	Testosterone (ng/ml)
Control	1.56±0.56	8.00±0.18	4.07±0.16
Ibuprofen	1.47±0.48	8.16±0.21	2.28± 0.28*
Tetracycline	1.41±0.32	8.27±0.14	2.33±0.11*

(Each value denotes mean ± S.E.M. of eight animals)
*significantly different from vehicle control group at $p<0.05$ level

Table 9. Comparative uterine weight of the rats treated with the test drugs at two higher dosage levels. Rats treated with only estradiol served as positive control.

Group	Uterine weight (mg)
Control	78.00±2
Estradiol (0.2 mg/kg/day)	389±16
Ibuprofen (+ E_2) (20 mg/kg/ day)	282±14*
Ibuprofen (+ E_2) (40 mg/ kg/ day)	176±9*
Tetracycline (+ E_2) (250 mg/ kg/ day)	277±15*
Tetracycline (+ E_2) (500 mg/ kg/ day)	163±8*

(Each value denotes mean ± S.E.M. of eight animals); bw-body weight
*Significantly different from estradiol treated group at $p<0.05$ level.

4.2.2.2.2 Gene expression analysis

A marked down regulation in the ovarian mRNA of P450scc, 3β-HSD, 17β-HSD, cytochrome P450C-17, aromatase and StAR while an increased transcription of ER was detected in both the treated groups of female rats as compared to control. In ovaries of ibuprofen and tetracycline treated female rats level of P450scc decreased up to a level 60 and 45%, 3β-HSD up to 49 and 51%, 17β-HSD up to 45 and 49%, while P450C-17 decreased up to 55 and 52% as compared to control respectively (Fig.35). Further

ovarian aromatase and StAR mRNA levels were also decreased significantly by approximately 40-50% as compared to control in response to treatment with both the drugs (Fig. 35). However, the expression of ER was increased by the treatment with both the drugs.

4.2.2.2.3 Ovarian 3β-HSD and 17β-HSD levels in vitro

Like that of testicular steroidogenic enzymes, *in vitro* spectrophotometric enzyme assays for 3β-HSD and 17β-HSD demonstrated that the treatment of animals with both the test drugs caused a significant decrease in the activity of ovarian steroidogenic enzymes at the two higher dosage levels (20 and 40 mg/kg/day for ibuprofen and 250 and 500 mg/Kg body weight for tetracycline) ($p<0.05$). No decrease was observed for both enzymes by the treatment with 10 and 125 mg/kg/day for ibuprofen and tetracycline respectively (Fig. 36).

4.2.2.2.4 Serum Hormone Level

There was a statistically significant decrease in the serum levels of estradiol in female rats treated with highest doses of ibuprofen and tetracycline (40 and 500 mg/kg/day respectively) as compared to control. ($p<0.05$) (Table 10).

4.2.2.3 Toxicological evaluation of the ibuprofen and tetracycline

4.2.2.3.1 Effects on body weight

Test samples did not induce any significant change in body weight of the treated rats as compared to control (data not shown).

4.2.2.3.2 Histopathological Analysis

Test sample did not induce any histopathological malformations in the liver, kidney, ovary, uterus, testis and none of the sex accessory tissues in the treated rats.

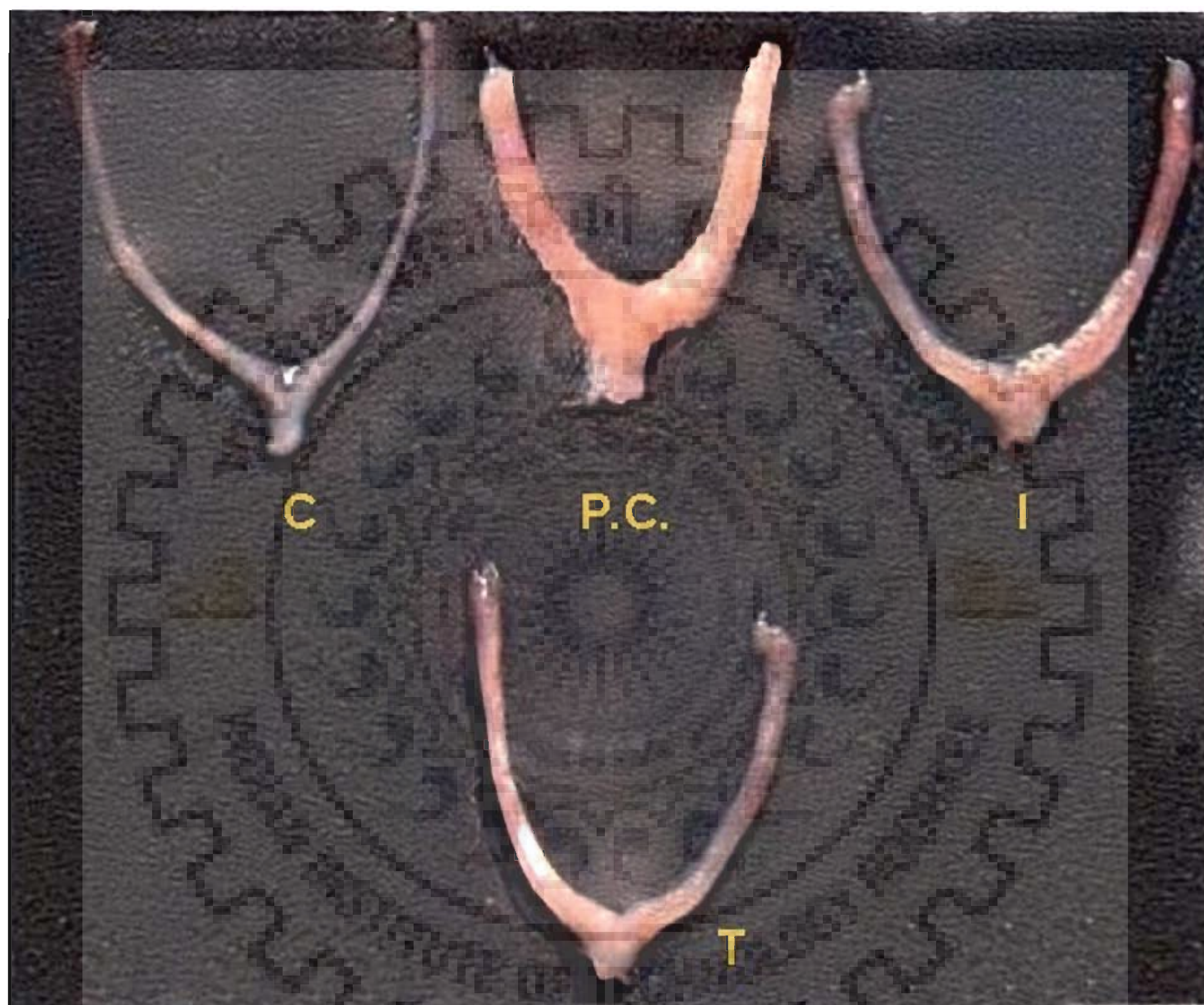
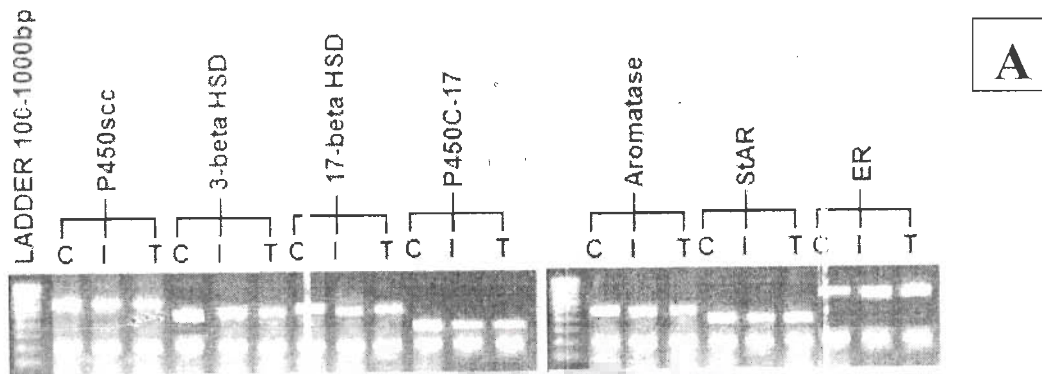


Figure 34. The effect of sample drugs on the size of uterus of the ovarectomised rats treated with ibuprofen and tetracycline at doses of 40 and 500 mg/ kg of body weight respectively (simultaneously treated with estradiol), 10X. C, control; P.C., positive control (treated with E2); I, ibuprofen; T, tetracycline.



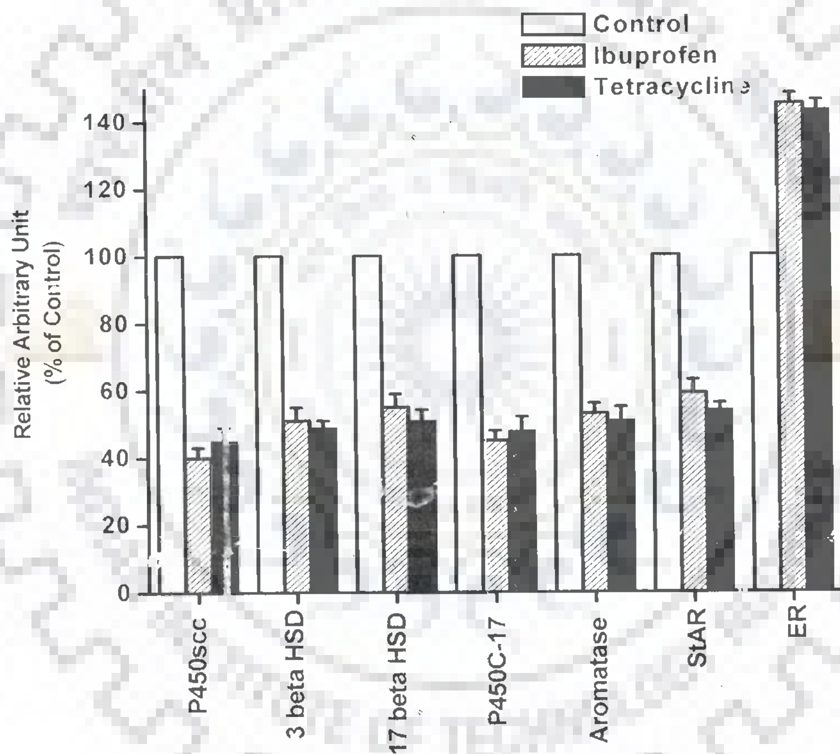


Figure 35. RT-PCR analysis of ovarian mRNA expression of cytochrome P450scc, 3β-HSD, 17β-HSD, cytochrome P450C-17, aromatase, StAR and ER genes in rats treated with the two highest does of sample drugs. The total RNA isolated from ovary was reverse transcribed and cDNA obtained was subjected to PCR (A). Densitometric analysis against the internal control (GAPDH) (B). The values are mean ± S.E.M. of eight RT-PCR reactions for each group.

4.2.2.3.3 Assay of toxicity marker enzymes- : SOD, CAT, GPx and GR assay

The activities of the foresaid enzymes did not alter at hypothalamic or hepatocyte level.

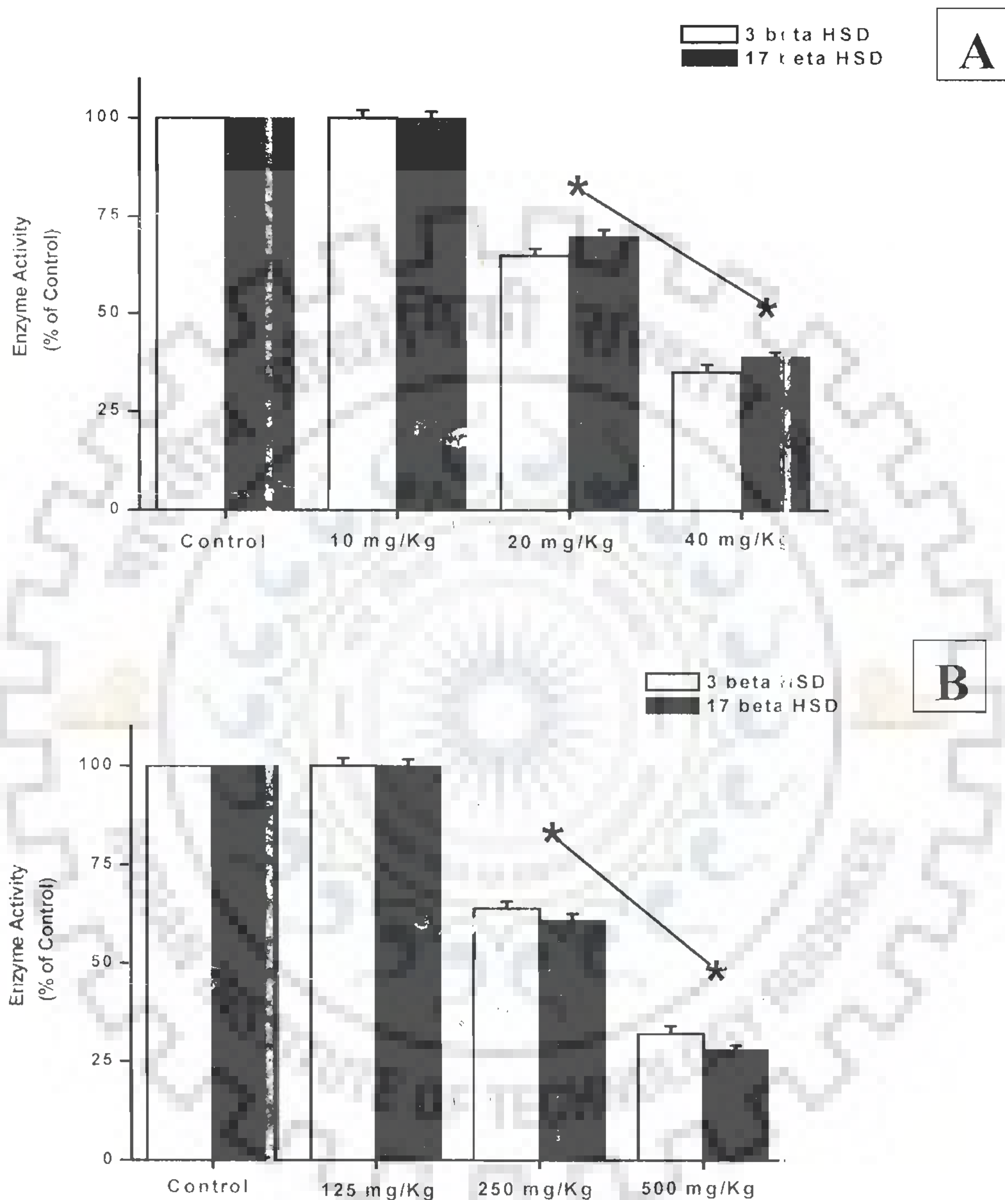


Figure 36. Effect of treatment of three different doses of ibuprofen (A) and tetracycline (B) on ovarian level of 3β-HSD (open bars) and 17β-HSD (solid bars) enzyme activity *in vitro*. The results are expressed as percent increase of enzyme activity over vehicle treated groups. Data are mean ± S.E.M.; n = 5. * indicates the significant level of difference in enzyme levels as compared to vehicle treated groups for both the enzymes ($p<0.05$).

Table 10. Serum levels of LH, FSH and estradiol from intact (female) control and rats treated with the indicated doses of ibuprofen and tetracycline for a period of 14 days.

	Control	Ibuprofen (40 mg/ kg/ day)	Tetracycline (500 mg/ kg/ day)
LH (ng/ml)	0.89 ± 0.064	0.85 ± 0.046	0.91 ± 0.028
FSH (ng/ml)	7.32 ±0.218	7.09 ± 0.035	7.56 ± 0.020
Estradiol (pg/ml)	42.5 ± 0.190	25.4 ± 0.067*	28.2 ± 0.39*

(Each value denotes mean ± S.E.M. of eight animals)
*Significantly different from vehicle control group at $p<0.05$ level.

4.2.3 Discussion

Understanding the factors which governs the pathogenesis of adverse side effects associated with drug therapy is essential if we are able to design drugs composed of safer and more effective constituents. This section of the thesis attempted to investigate the mode of endocrine disruption by two synthetic chemicals used widely in the preparation of pain killers and antibiotics thus resulting in hormonal dysfunction. There are a number of reports which indicates various adverse effects of these chemicals on hormonal system of body. But these conclusions may need to be revalidated with the recent reports that ibuprofen disrupts the function of tamoxifen and 17β-estradiol (Sibonga et al., 1998), oxytetracycline can interact with hormone metabolism (Hamalainen et al., 1987), and acetyl salicylic acid binds to estrogen receptor of MCF-7 cells (Aswegen et al., 1992). As a whole, considering the recent impact of various chemicals used in pharmaceutics on endocrine system resulting in endocrine dysfunction, it is important that these daily usable drugs need to be revalidated for their mode of actions. This further becomes more important since several reports have demonstrated the presence of a number of

pharmaceutically active compounds in various strata of the environment from where these compounds may enter into food chain thus posing an indirect threat to the animal/human health. (Gross et al., 2004; Glassmeyer et al., 2005; Lindqvist et al., 2005; Nakada et al., 2006).

When subjected to Hershberger assay in this study, both the test chemicals caused a dose dependent decrease in the weight of sex accessory tissues of TP treated male rats at the two higher dosage levels (10 and 20 mg/kg/day for ibuprofen and 250 and 500 mg/Kg/day for tetracycline). This may be explained by the fact that both of the test chemicals under study are impeding the action of TP on the sex accessory tissues and thus are acting as anti-androgenic compounds (Kennel et al., 2004). However, both the test drugs at further lower doses were below their effective limits necessary for causing a decrease in the weight of sex accessory tissues. Thus, in the present study, Hershberger assay demonstrated that both the test chemicals are acting as potential anti-androgens at the moderate as well as highest dosage level.

When all the three doses of each ibuprofen and tetracycline were administered to the ovarectomised rats (simultaneously treated with estradiol), results indicated that the two higher doses of both the drugs were antagonizing the action of estradiol leading to a decreased weight of uterus as compared to positive control (treated with estradiol only). Thus uterotrophic assay indicated that besides being anti-andorgenic in male system, both the drugs are acting as anti-estrogens in the female system as well. Besides, it was also confirmed that the dosage level i.e. 40 mg/kg/day for ibuprofen as adopted from literature and 500 mg/kg/day for tetracycline as worked out by us were well below the LD50 as

histopathological analysis and measurement of toxicity marker enzymes did not display any sign of toxicity in treated rats.

Following the Hershberger assay and uterotrophic assays, the next obvious question to be answered was to understand the simultaneous mechanism of action of these drugs as anti-androgens and anti-estrogens. When we discuss about the role of various steroidogenic enzymes and other proteins involved in steroidogenesis, StAR protein is one of the major factors that comes into play. Regardless of organ or tissue, the delivery of cholesterol from the outer mitochondrial membrane by the StAR protein to the inner mitochondrial membrane (where it is acted upon by enzyme P450scc to produce pregnenolone to be further metabolized by the steroidogenic cascade) is the pivotal rate-limiting step in steroid hormone biosynthetic process and this process may potentially affect testicular, ovarian and adrenocortical functions (Stocco, 2000; Stocco, 2001; Clark and Cochrum, 2007). In our study, treatment of drugs led to a decreased level of StAR protein in both testis and ovary which is resulting in a reduced transport of cholesterol into inner mitochondrial membrane in all treated male and female rats as compared to control which may have further down regulated the downstream steroidogenic cascade (Sanderson and Vanden Berg, 2003). Recently, lindane and organophosphate insecticide dimethoate has also been reported to block steroid hormone biosynthesis by reducing StAR protein expression (Arukwe, 2005).

Once cholesterol is transported to the IMM, alterations of rate-limiting enzymes could further influence the production of testosterone and estradiol, especially reduction in the conversion of cholesterol to pregnenolone, as discussed in the preceding section of the thesis also. In order to gain further insight into this fact, the transcript levels for

various testicular and ovarian steroidogenic enzymes were determined in control and treated male and female rats. Results indicated that the transcripts of all major steroidogenic enzymes namely, P450scc, P450C-17, 17β-HSD and 3β-HSD were down regulated in testis and ovary (including aromatase). This notion finds support from some recent study that showed the direct effect of endocrine disruptors at enzyme levels (Ohsake et al., 2003; Andric et al., 2006; Lyssimachou et al., 2006; Kumar et al., 2008a and 2008b). Besides, it has been shown recently that salicylates and nonylphenol modulates steroidogenesis by decreasing the expression of StAR and P450scc (Arukwe, 2005; Gravel and Vijyan, 2006). At this point, it is difficult to make a precise conclusion on how the test chemicals decreased the steroidogenic enzyme levels, since the transciption of steroidogenic enzymes is a very complex process and xenobiotics dependent direct up/down regulation of steroidogenic enzymes and steroidogenesis could be attributed to several factors (Walsh et al., 2000; Indarto and Izawa, 2001; Eertmans et al., 2003; Sanderson and vanden Berg, 2003; Kang et al., 2005; Andric et al., 2006; Rice et al., 2006; Lin et al., 2006; Lyssimachou et al., 2006). Further our results indicated that the levels of gonadotropins did not change significantly in both the sexes of treated animals and the decrease in the steroidogenic cascade was independent of this. This may be explained by the fact that our test drugs are acting directly on the steroidogenic enzymes. LH independent regulation of steroidogenesis has also been reported earlier by some authors where treatment of rats with testosterone or its analogues resulted in up regulation of steroidogenesis even without any significant change in LH levels (Kennel et al., 2004) and significant amount of testosterone production in LH receptor knockout mice and mice lacking genes for pituitary developments (Pakarinen et al., 2002). All

these further supports our finding that even without any significant changes in levels of LH, the rate of steroidogenesis was down-regulated. However, the exact cross talk between the gonadotrophic hormone, the xenobiotcs and testosterone remains an intriguing question which needs further research in these directions (Kumar et al., 2008a).

These data were further supported by the in vitro estimation of testicular 3β-HSD and 17β-HSD enzyme activities in testis and ovaries, which showed a significant decrease in enzyme activities in all treated groups of intact rats as compared to control. This part of analysis further proved that the enzymes are regulated both at transcription and translation levels. Thus, a decreased level of StAR, P450SCC, 3β-HSD and 17β-HSD, both at transcriptional and translational points, might have led to a decreased synthesis of testosterone and estradiol in testis and ovaries of treated rats (indicated by serum hormone analysis). Further, the increased level of AR and ER in treated rats is achieved through autologus regulation caused by a decreased level of testosterone and estradiol.

Although the mechanism involved in steroidogenic disruption by the ibuprofen and tetracycline tested by us is not clear, few likely scenarios emerge. For example, the NSAID inhibits cyclooxygenases (Sibonga et al., 1998) and therefore, will reduce the formation of arachidonic acid and metabolites, including prostaglandins (PG). It is well known that PG modulates the functioning of hypothalamic-pitutary-gonadal axis in animals (Davis et al., 1999; Reese et al., 2001; Balaji, 2007). Together, these results point to a role for PG as a link for impact of these chemicals on testosterone and estradiol production and functions. However, recent studies have also demonstrated that several NSAIDs exhibit anti-inflammatory effects independent of cyclooxygense activity

(Amann and Peskar, 2002; Gravel and Vijayan, 2006). This raises the possibility that other pathways, in addition to PG metabolism, may be involved in the test chemical mediated disruption of steroidogenesis in both the sexes of animals. Specifically, according to a recent report, NSAIDs can also affect the activation of transcription factors, including the peroxisome proliferator-activated receptor (PPARs), a key regulator of steroidogenesis (Komar, 2005; Gravel and Vijayan, 2006). One report suggests that PPARα stimulation depressed peripheral-type benzodiazepine receptor (PBR) (one of the protein involved in steroidogenesis along with StAR) transcript levels in mammalian cell model (Gazouli et al., 2002).

In conclusion, ibuprofen and tetracycline are most likely behaving as endocrine disruptors in both male and female rats and have the potential to impair the androgen and estrogen dependent functions in the body. We further demonstrated that the inhibition of testosterone and estradiol production by these chemicals involved the depression of StAR transcripts, one of the most crucial proteins for cholesterol transport to the inner mitochondrial membrane for its (cholesterol) utilization by steroidogenic enzymes. This action was also followed by subsequent down regulation of testicular and ovarian major steroidogenic enzymes like P450scc, P450C-17, 3β-HSD and 17β-HSD. Taken together, StAR, some of the major steroidogenic enzymes, and AR and ER, the key proteins involved in the activation of testicular and ovarian steroid functions, are the targets for some of the synthetic analogs used in production of NSAIDs and antibiotics as tested by us. Thus it leads to a decreased synthesis of androgen and estrogen leading to the reproductive dysfunction.

Chapter 4.3 Industrial effluents as EDC: Leather industrial effluents

4.3.1 Introduction

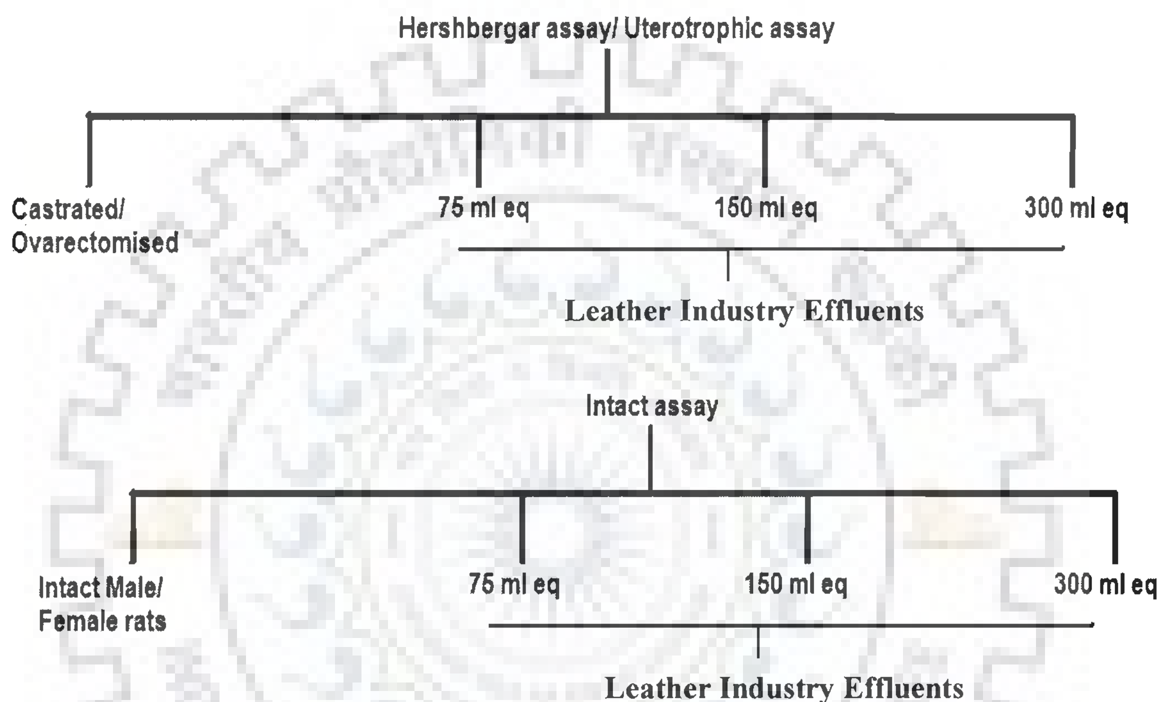
In the previous sections of the thesis it has been described that various synthetic products like antimicrobials or pharmaceutics may be harmful for the physiological system of the body on account of their direct applications. However, there may be still another category of chemicals which are released into the environment as byproducts/ waste products with the effluents released from various industries (Nair et al., 2006; Chatterjee et al., 2008; Kumar et al., 2008b). This is followed by the entry of these chemicals into the food chain causing adverse physiological consequences in the organisms including humans (Alil and Soltan, 1996; Sole et al., 2000; Selim et al., 2003; Vanderford et al., 2003; Batta et al., 2006; Sparovek et al., 2006; Kumar et al., 2008b). Besides industrial workers are exposed to different types of chemicals (occupational exposure) during the manufacturing of various products which results in disastrous health consequences (Golec et al., 2003; Hooiveld et al., 2006; Jensen et al., 2006; Perry, 2008).

This part of the thesis dealt with the endocrine disrupting action of leather industry effluents, one of the ten most harmful industrial effluents to the environment, responsible for extreme pollution of water resources and generating substances leading to detoriation and death of wide range of organisms (Aragon et al., 1990; Junior et al., 2007). Several chemicals are used in various steps of leather processing at different time period giving leather effluent a very composite nature. Effluents of leather industry have been reported to contain formaldehydes, chromates and bichromate salts, aniline, benzene based dyes, other organic chemicals (butyl acetate, ethanol, benzene, toluene, nonylphenols, polychlorinated phenols), dimethyl formamide, sulphuric acid, ammonia,

hydrogen sulphide and others (Levin et al., 1987; Solé et al., 2000). A number of studies have shown that these chemicals have harmful effects on development and reproduction of animals. However, in majority of cases only physical and chemical tests have been used to evaluate the impact of these industrial effluents. These tests satisfy the criteria set up by state agencies for environmental control, but several authors have recommended that the physical and chemical analysis must also be supplemented with biological assessment to evaluate toxicity of the compounds resulting from anthropogenic activity (Bertoletti et al., 1992; Smaka-Kincl et al., 1995). What ever data obtained till date on the endocrine disrupting nature of leather industry effluents, they give a superficial idea about their toxic and disrupting effects. However a detailed study is needed which may pinpoint the effect of different contaminants on the physiological system of body including reproductive system. This is critical from the Indian environmental scenario since leather industry is one of the major industries in India.

In this section efforts were made to understand the molecular mechanism of (anti)androgenic and (anti)estrogenic action of the leather industry effluents. Water samples were collected from a common site receiving leather effluents from more than 100 small and big leather industrial units in Northern part of India. Since during leather processing various chemicals are used at different times so samples were collected from a site which was supposed to represent the chemicals involved in almost all steps of leather processing. Here an integrated approach was adopted, first, screening the samples for their (anti)androgenic and (anti)estrogenic nature by Hershberger and uterotrophic assay respectively and when samples were found positive for androgenicity and estrogenesity, molecular mode of action of probable EDC was studied by various parameters after

gavaging the samples to intact male and female rats. Schematic representation, given immediately after this paragraph, depicts the summary of dosing plan used for gavaging the different doses of Leather industrial effluents to male and female rats as described in the methodology section.



4.3.2 Results

4.3.2.1 Endocrine disrupting effects of leather industry effluents samples in the male reproductive system

4.3.2.1.1 Sex accessory tissue weight (Hershberger Assay)

In castrated rats at a dose of 75 ml equivalent of water sample there was no significant change in the weight of SATs. However, at a dose of 150 ml equivalent and higher (300 ml equivalent) of water samples, a statistically significant increase in the weight of all accessory sex organs viz. prostate gland, glans penis, cowper's gland, seminal vesicle and

deference, was observed in all treated groups as compared to the reference vehicle control ($p<0.05$) (Table 11 and Fig 37). The significant increases in absolute SAT weights could be attributed to the androgenic effects by some or all of the contaminants (combinational effect) present in the leather industry effluents. A similar pattern of dose dependent increase in the weights of SAT and testes was observed in intact rats in response to the effluent treatments (Table 12).

Table 11. Effects of leather industry effluents on weights of SATs from castrated rats given 20 consecutive daily treatments.

	Ventral prostate (mg)	Glans penis (mg)	Cowper's gland (mg)	Seminal vesicle (mg)	Vas deference (mg)
Control	11.66±0.55	162.91±2.81	4.58±0.16	47.81±0.17	65.33±0.57
75ml/150µl	12.00±0.72	162.86±3.85	4.62±0.18	47.92±0.31	64.83±1.29
150ml/150µl	20.38±0.60*	208.50±0.42*	7.56±0.13*	86.43±1.22*	108.40±0.36*
300ml/150µl	27.83±0.94*	256.83±3.77*	10.12±0.11*	108.13±0.72*	138.92±0.51*

(Each value denotes mean ± S.E.M. of six animals)
* Significantly different from vehicle control group at $p<0.05$ level

4.3.2.1.2 Daily sperm Production

There was 92 % increase in the DSP/gm of testes weight ($p<0.05$) in treated group as compared to control at 13 weeks of age (Fig. 38).

4.3.2.1.3 Gene expression profile

There was a marked up regulation of mRNA of all the steroidogenic enzymes (except AR) under study in testes in treated group of intact male rats. There were almost 2.9, 2.4, 2.7 and 2.1 folds up regulation of cytochrome P450scc, 3β-HSD, 17β-HSD and cytochrome P450C-17 respectively while a 2.4 fold down regulation of AR mRNA was noticed in the treated rats (Fig. 39). The expression of StAR and SRB-1 protein remain unchanged in the treated rats as compared to control.

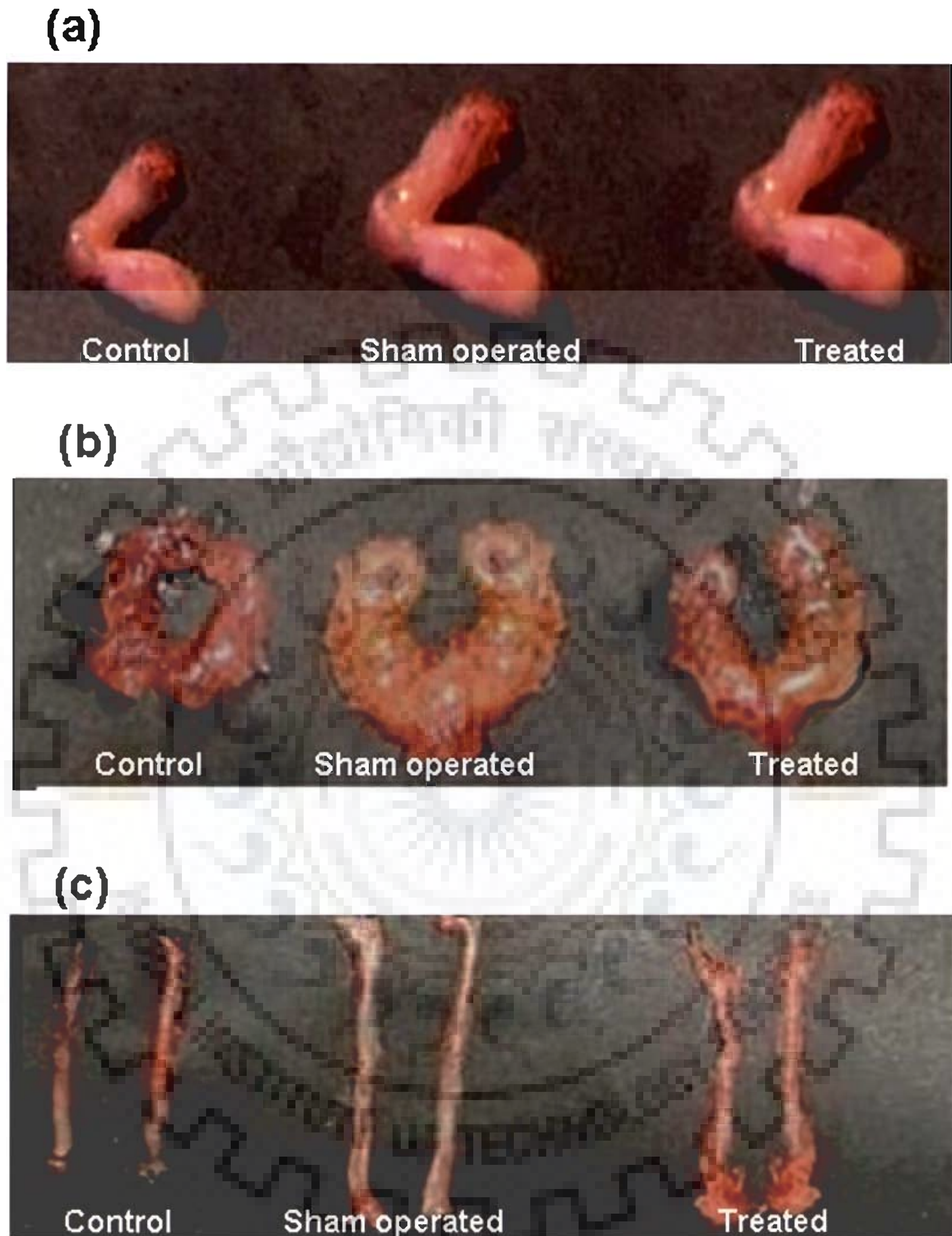


Figure 37. The effect of leather industry effluents on the structure of some of the SATs of castrated rats. Adult castrated rats were treated with 300 ml equivalent of leather industry effluents for 20 days. The organs were then collected and photographed under a stereo zoom microscope with 10X magnification. a, glans penis; b, vas deference; c, seminal vesicle.

Table 12. Effects of extracted leather industry effluents on the weights of testes and SATs from intact rats given 20 consecutive daily treatments.

	Testes (mg)	Ventral Prostate (mg)	Glans Penis (mg)	Cowper's Gland (mg)	Seminal Vesicle (mg)	Vas Deference (mg)
Control	3321±30.11	36±0.45	319±2.58	11.7±0.65	147±2.49	173±1.66
75 ml equivalent	3231±25.41	41±0.77	330±1.42	14.7±0.5[illegible]	153±1.98	185±1.89
150 ml equivalent	5403±19.76*	49±1.3*	456±2.56*	19±0.36*	209±2.89*	256±1.93*
300 ml equivalent	7259±35.12*	67±0.90*	607±3.99*	21±0.55*	289±4.58*	382±3.33*

(Each value denotes mean ± S.E.M. of six animals)
* Significantly different from vehicle control group at $p<0.05$ level

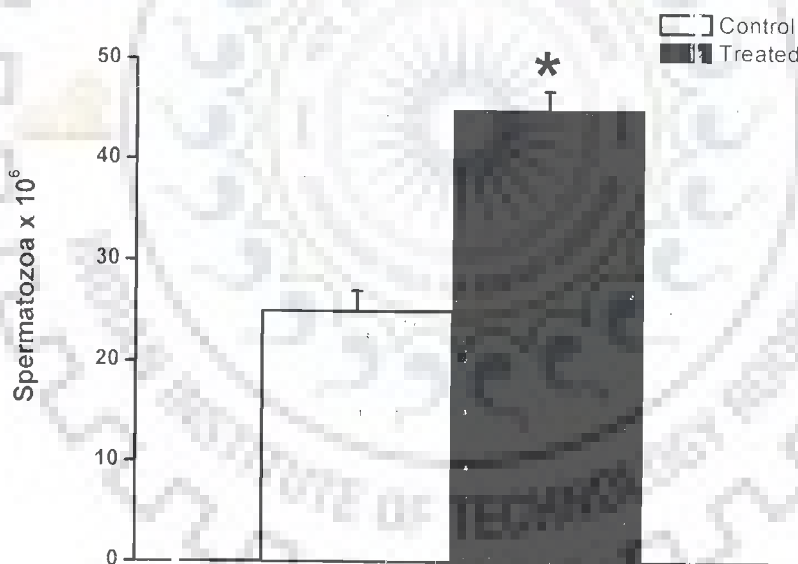


Figure 38. Daily sperm production/gm of testes weight (DSP/gm) at 13 weeks of age in vehicle and water sample (300 ml equivalent) treated rats. Data are presented as mean ± SEM of quadruplet determinations from individual testes; n = 6 for control and treated testes. DSP was significantly increased ($p<0.05$) in the treated group compared to controls. * indicates the significant level of differences.

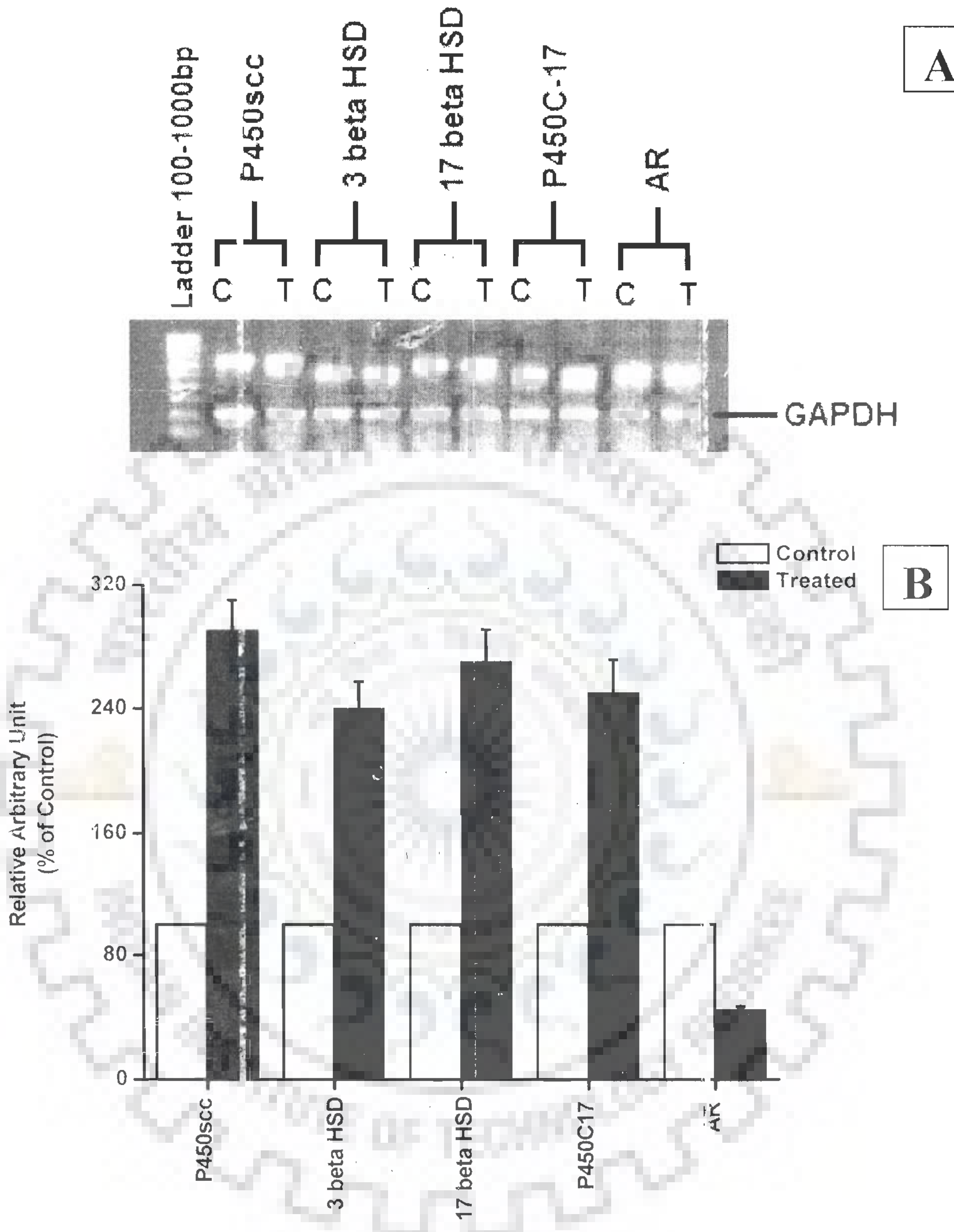


Figure 39. RT-PCR analysis of testicular mRNA expression of cytochrome P450scc, 3β-HSD, 17β-HSD, cytochrome P450C-17 and AR genes in rats treated with 300 ml equivalent of leather industry effluents (A). Densitometric analysis against the internal control (GAPDH) (B). The values are mean ± S.E.M. of eight RT-PCR reactions for each group tested. C, vehicle treated; T, effluent treated animal groups.

4.3.2.1.4 3β-HSD and 17β-HSDlevels

Spectrophotometric enzyme analysis for 3β-HSD and 17β-HSD in testes of intact male rats demonstrated that although 75 ml equivalent of water samples could not induce any additional enzyme activities, yet 150 ml equivalent resulted in significant up regulation of enzyme activities (Fig 40) ($p<0.05$). This was further elevated dramatically with 300 ml equivalent of water sample treatments. The enzyme activity of intact animals treated with150 and 300 ml equivalent effluents resulted in almost 2 and 3 folds increase over vehicle treated groups respectively for both the enzymes (Fig. 40). *4.3.2.1.5 Testicular 3β-HSD and AR protein immunoblot analysis*

Rats treated with 300 ml equivalent of leather industry effluents showed an increased translation of 3β-HSD while a decreased translation of AR protein as compared to control (vehicle treated animals) and this increase was statistically significant (Fig 41).

4.3.2.1.6 Serum Hormone levels

Estimation of serum testosterone levels in intact male rats treated with 150 and 300 ml equivalent concentrations of effluents resulted in significant increase in serum testosterone levels. However, the lowest concentration of water extract (75 ml equivalent) did not show any significant response which could be attributed to extremely low levels of contaminants at that dilution. The apparent increase in the level of testosterone was 200% in case of rats treated with 300 ml equivalent of water samples over the vehicle treated groups (Table 13). As expected the levels of serum LH and FSH also decreased by a value of 33% and 29% respectively in animals treated with 300 ml equivalent of water samples while remain unchanged at a dose of 75 ml equivalent of leather industry effluent ($p<0.05$) (Table 13). Decrease in the levels of LH and FSH could be attributed to

the initiation of feed back mechanism by the androgenic contaminants in the water samples and (or) increased serum androgen levels.

Table 13. Dose dependent changes in serum levels of LH, FSH and testosterone from intact rats given 20 consecutive daily treatments of water sample of indicated dose

	LH (ng/ml)	FSH (ng/ml)	Testosterone (ng/ml)
Control	0.86 ± 0.11	6.11 ± 1.53	4.50 ± 0.39
75 ml equivalent	0.81 ± 0.06	6.15 ± 1.48	4.96 ± 0.42
150 ml equivalent	0.67 ± 0.05*	5.58 ± 1.23*	6.23 ± 0.89*
300 ml equivalent	0.61 ± 0.09*	4.50 ± 0.62*	9.53 ± 0.92*

(Each value denotes mean ± S.E.M. of six animals)
* Significantly different from vehicle control group at $p<0.05$ level

4.3.2.2 Endocrine disrupting effects of leather industry effluents samples in the female reproductive system

4.3.2.2.1 Determination of estrogenicity of leather industry effluents by uterotrophic assay

In ovarectomised rats at a dose of 75 ml equivalent of water sample, there was no significant change in the weight of uterus. However, at a dose of 150 ml and 300 ml equivalent effluents samples, a 47 and 93% increase in the weight of uterus was observed in all treated groups as compared to the reference vehicle control ($p<0.05$) (Table 14 and Fig. 42). This increased weight of uterus could be attributed to the estrogenic effects by some of the contaminants (combinational effect) present in the leather industry effluents. A similar pattern of dose dependent increase in the weight of uteri of intact rats was observed by an increase of 37 and 68% at two highest dose levels (150 and 300 ml equivalent respectively) as compared to control (Table 15).

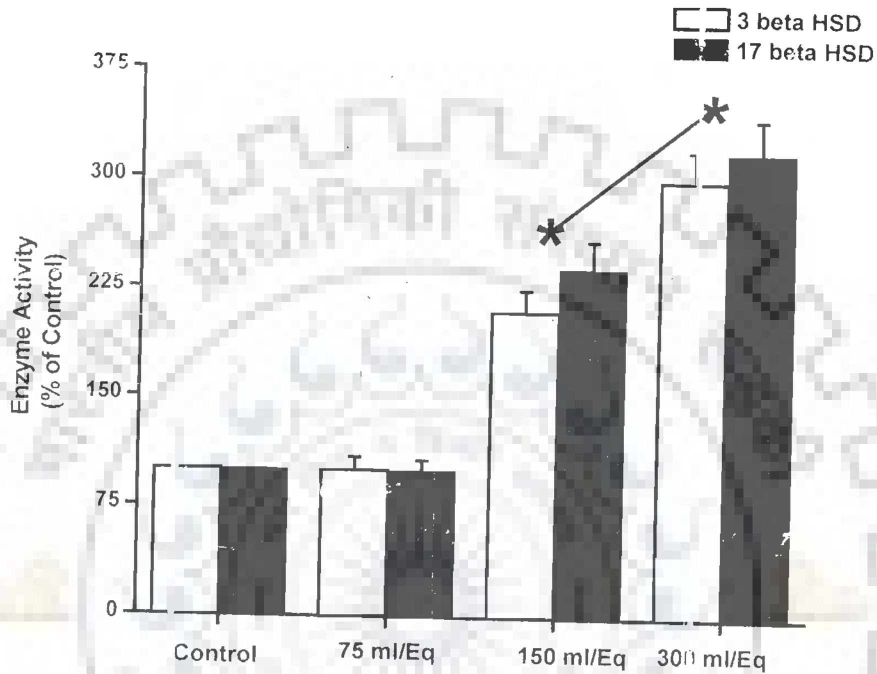
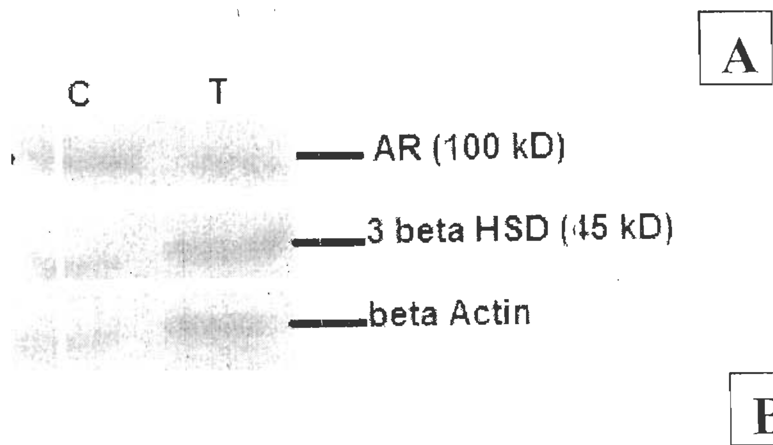


Figure 40. Effects of leather industry effluents on testicular levels of 3β-HSD and 17β-HSD enzyme activities *in vitro*. The crude enzyme extracts were isolated from testes of vehicle and effluent treated rats and incubated in the presence of respective substrates as described in materials and methods. The results are expressed as percent increase of enzyme activity over vehicle treated groups. * indicates significant level of differences as compared to vehicle treated control ($p<0.05$). Eq, Equivalent.



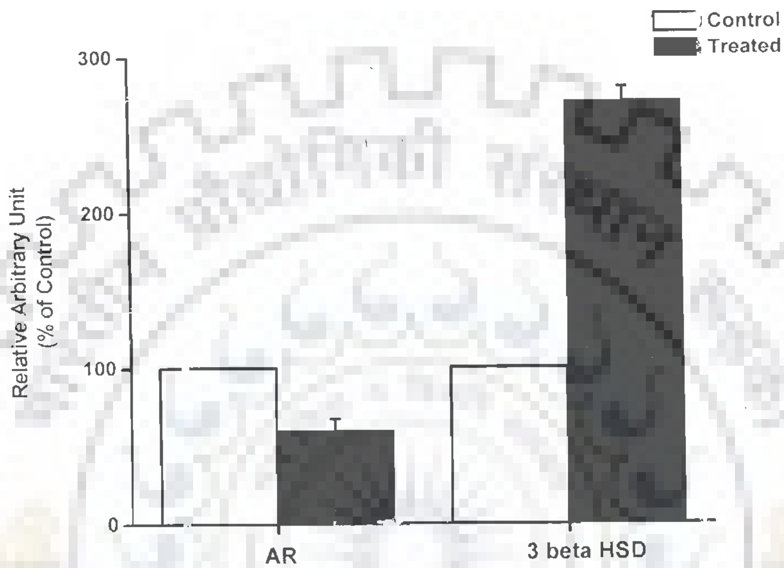


Figure 41. Western blot analysis of testicular 3β-HSD enzyme and AR protein in response to treatment with 300 ml equivalent leather industry effluents. C, control; T, effluent treated (A). Derestometric analysis against the internal control (β-actin) (B). The values are mean ± S.E.M. of three separate experiments.

Table 14. Comparative uterine weight of the ovarectomised rats treated with the three indicated concentrations of leather industry effluents.

Group	Uterine weight (mg)
Control	81.0±2.4
75 ml equivalent	84.1±2.3
150 ml equivalent	121±15*
300 ml equivalent	159±15*

(Each value denotes mean ± S.E.M. of eight animals);
*Significantly different from vehicle treated group at $p<0.05$ level.

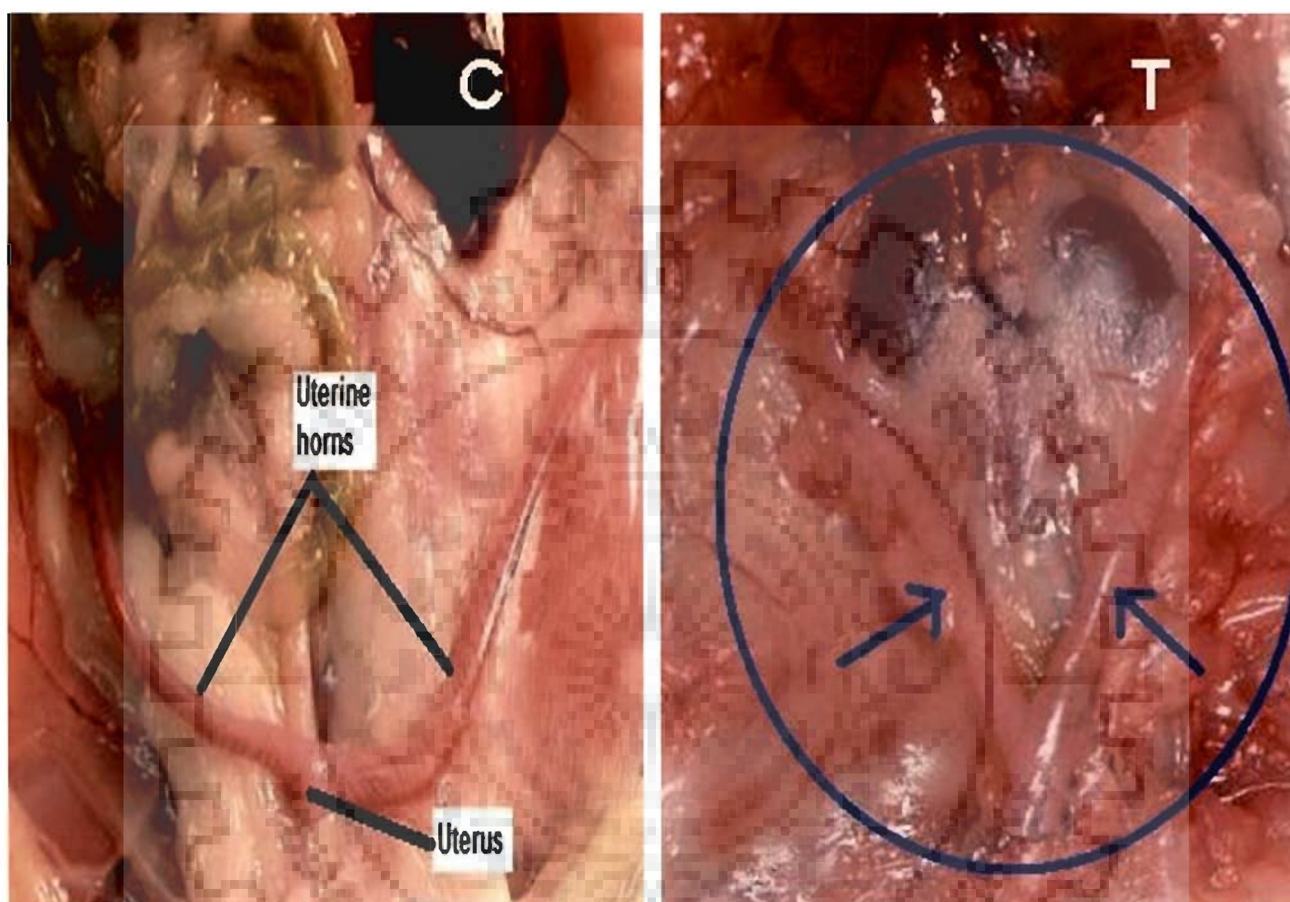


Figure 42. The effect of leather industry effluents on the size of uterus of ovarectomised rats as observed *in situ*. Adult female rats were treated with 300 ml equivalent of leather industry effluents for 20 days. The uteri were then collected and photographed. 10X. C, control; T, treated.

Table 15. Comparative uterine weight of the intact rats treated with the three indicated concentrations of leather industry effluents.

Group	Uterine weight (mg)
Control	301±14
75 ml equivalent	306±14
150 ml equivalent	412±15*
300 ml equivalent	506±15*

(Each value denotes mean ± S.E.M. of eight animals)
*Significantly different from vehicle treated group at $p<0.05$ level.

4.3.2.2.2 Gene expression analysis

There was a marked up regulation of mRNA transcripts (except ER) of all the steroidogenic enzymes under study in ovaries in treated group of intact rats except ER. There was almost 2.6 folds up regulation of cytochrome P450scc, 3β-HSD, 17β-HSD, cytochrome P450C-17 and aromatase while a significant downregulation of ER mRNA in300 ml equivalent group as compared to control (Fig. 43). The expression of the StAR and SRB-1 protein remain unchanged in the treated rats as compared to control.

4.3.2.2.3 Ovarian 3β-HSD and 17β-HSD levels in vitro

Spectrophotometric enzyme analysis for 3β-HSD and 17β-HSD in ovaries of intact rats demonstrated that although 75 ml equivalent of water samples could not induce any change in enzyme activities, yet 150 ml equivalent resulted in significant up regulation of enzyme activities (Fig 44) ($p<0.05$). This was further elevated dramatically with 300 ml equivalent of water sample treatments. The enzyme activity of intact animals resulted in almost 2.5 fold increase at 150 ml equivalent of test sample and almost 3.4 folds increase at 300 ml equivalent of test sample for both 3β-HSD and 17β-HSD respectively (Fig. 44).

4.3.2.2.4 Serum Estradiol and gonadotropin levels

There was a statistically significant increase in the serum levels of estradiol while a significant decrease in gonadotropin levels in female rats treated with two highest concentration of leather industry effluent (300 ml equivalent concentration) as compared to control ($p<0.05$) (Table 16).

Table 16. Serum levels of LH, FSH and estradiol from intact control and treated (300ml equivalent of leather industry effluents) rats.

	Control	Treated
LH (ng/ml)	0.64 ± 0.064	0.48 ± 0.057 *
FSH (ng/ml)	7.54 ±0.207	6.12 ± 0.043 *
Estradiol (pg/ml)	41.2 ± 0.310	75.4 ± 0.085*

(Each value denotes mean ± S.E.M. of eight animals)
*Significantly different from vehicle control group at $p<0.05$ level.

4.3.2.3 Toxicological evaluation of the test samples

4.3.2.3.1 Effects on Body Weight

Test sample did not induce any significant change in the over all body weight as compared to the reference vehicle control group. Even at the highest concentration of water samples (300 ml equivalent), the terminal body weight was not significantly different from those of reference control groups in both castrated, ovarectomised and intact animals (data not shown).

4.3.2.3.2 Histopathological analysis

Histopathological studies of testes were carried out in leather industry effluents treated animals which were sacrificed after 20 days. As shown in Fig. 45B, the animals receiving water extract from leather industry effluents demonstrated deformed seminiferous tubules where individual tubules seems to fuse with each other representing testicular hyperplasia, while the vehicle treated groups showed a normal testicular morphology

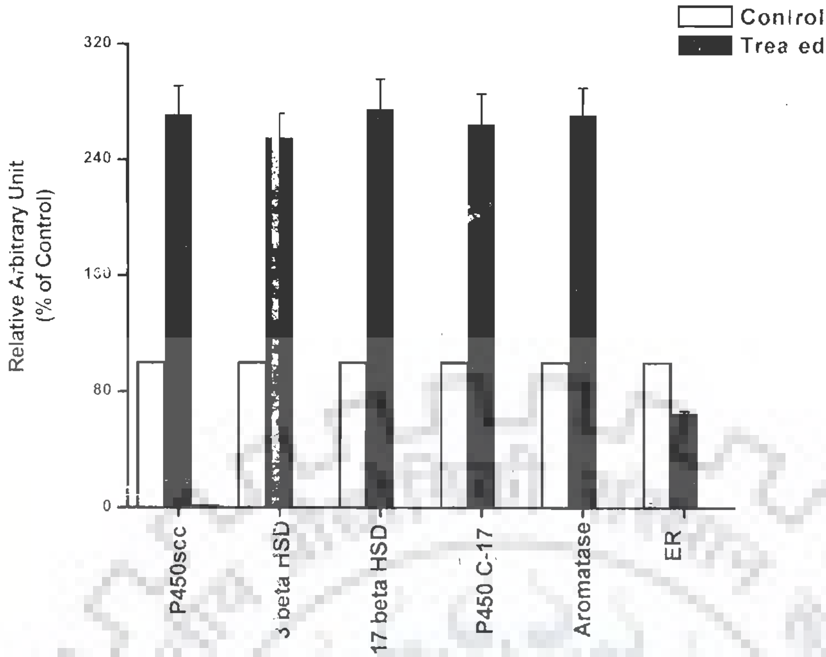


Figure 43. RT-PCR analysis of ovarian mRNA expression of cytochrome P450scc, 3β-HSD, 17β-HSD, P450C-17, aromatase and ER genes in rats treated with 300 ml equivalent of leather industry effluents. The values are mean ± S.E.M. of eight RT-PCR reactions for each group tested.

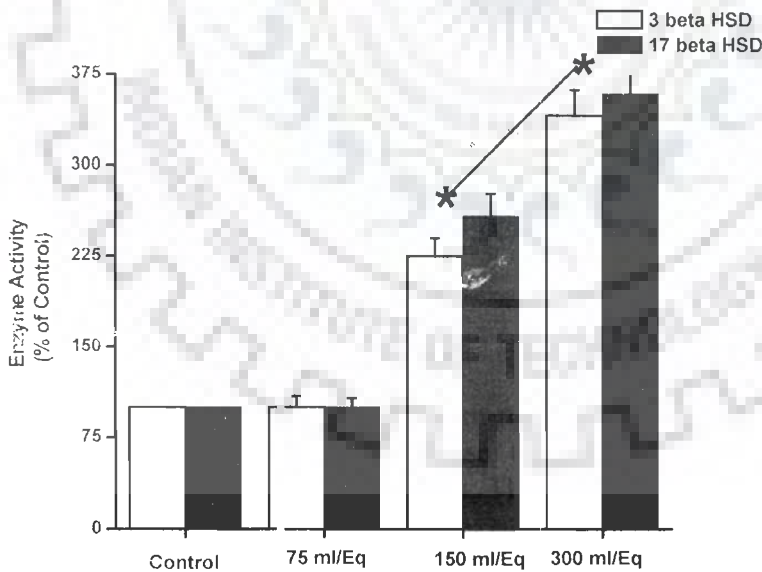
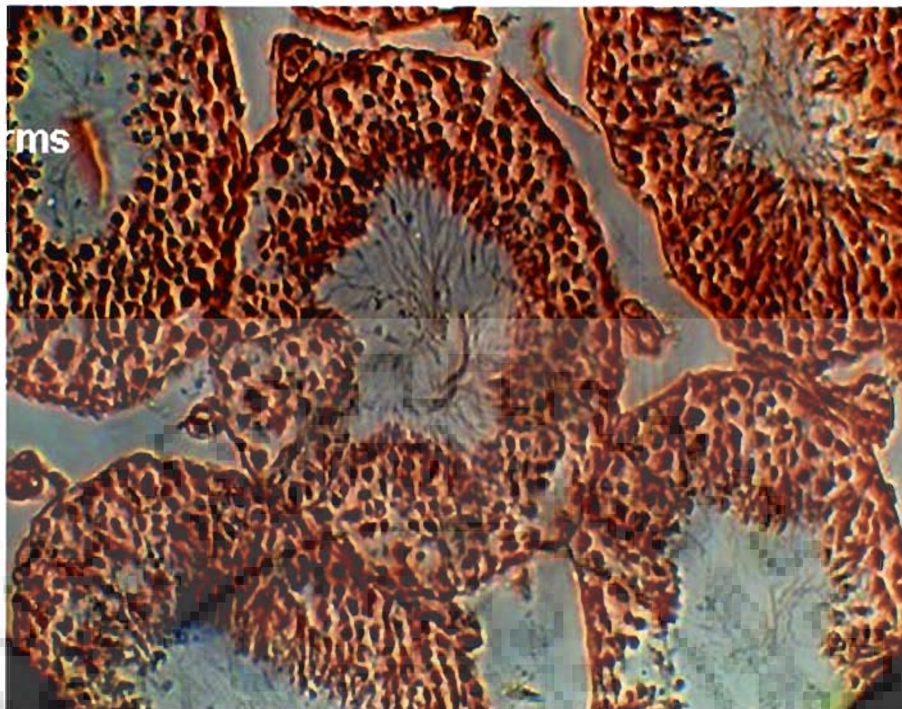


Figure 44. Effects of leather industry effluents on ovarian levels of 3β-HSD and 17β-HSD enzyme activity *in vitro*. Data are mean ± S.E.M.; n = 5. * indicates significant level of differences as compared to vehicle treated control ($p<0.05$). Eq, Equivalent.

(A)



(B)

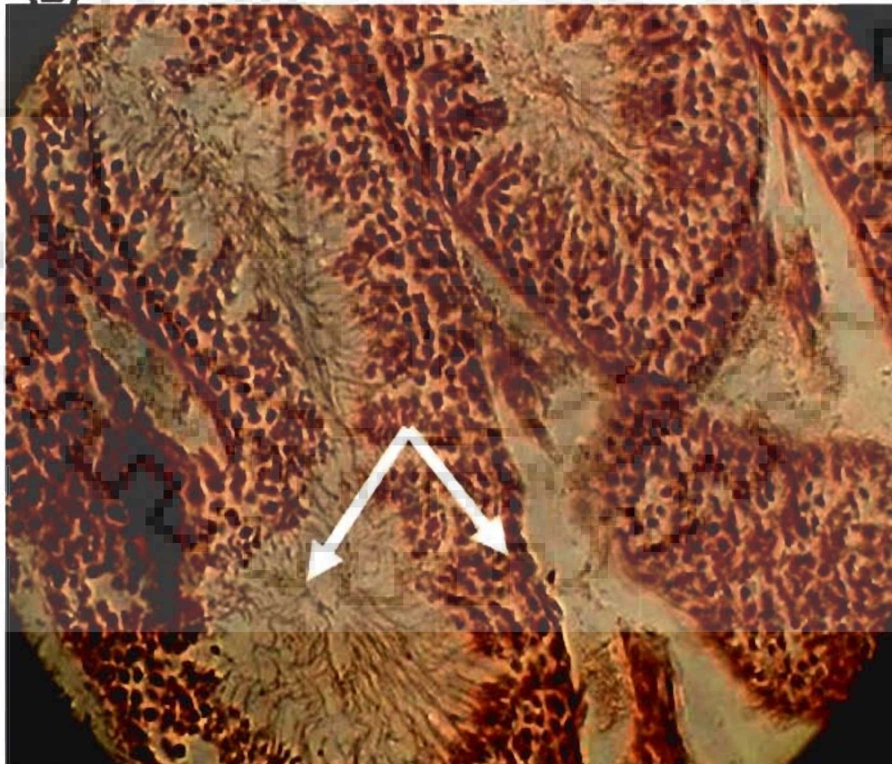


Figure 45. Photomicrograph of testicular seminiferous tubule showing fusion of tubules indicating the symptoms of hyperplasia in the testes of the rats treated with 300 ml equivalent of leather industry effluent for 20 days. H &E. 40X. a, vehicle treated; b, effluent treated.

(Fig. 45A). No histopathological malformations were observed in the SATs of males, uterus of female and liver and kidney of both the sexes.

4.3.2.3.3 Toxicity marker enzymes

There was no change in the activity of the enzymes considered as markers of the toxicity in hypothalamus, liver and kidney.

4.3.3 Discussion

An extensive body of evidence has associated a number of health hazards with the leather and tanning industry, including both occupational exposures, and water and land contamination (Bianchi et al., 1997; Chang et al., 2004; Meriç et al., 2005; Gerein, 2006). This resulted in a number of health effects, affecting crops, aquatic and terrestrial biota (Barnhart., 1997; Jochimsen and Jekel., 1997; Tisler et al., 2004; Riva et al., 2005; Veyalkin and Gerein, 2006; Júnior et al., 2007). All these reports emphasizes on the fact that the toxic effects be also analyzed in terrestrial animals and thus, the aim of this section was to assess the degree of endocrine disrupting effects caused by the leather industry effluents on the male and female reproductive system.

When tested by the rodent Hershberger assay, weights of all or some of the accessory sex organs increased significantly in rats gavaged with extracts of leather industry effluent samples as compared to vehicle treated control groups and they were almost of the same size as that of sham operated animals used as positive control. This clearly indicated an androgen agonistic activity of the leather industry effluents. Thus, the present Hershberger assay was capable of detecting a clear dose-related androgen agonist effect of leather industry effluents on accessory reproductive organs. The Hershberger assay data was further supported by the serum levels of LH, FSH and testosterone. In our

study the leather industry effluents demonstrated a tendency towards increasing the plasma testosterone level and a decrease in LH and FSH level (Kennel *et al.*, 2004), however, there was a marginal increase in serum testosterone level in castrated animals at the lowest dilution (75 ml equivalent) of sample which could be considered as adrenal androgen (data not shown). Further, an increased weight of the SATs in intact animals also indicated an androgen agonistic activity present in the leather effluent and this was supported by increased level of serum testosterone. Besides results indicated a more than 200% increase in the weight of testes which may be due to induction of testicular hyperplasia as demonstrated by histopathology. Though there are several reports on the presence of various classes of compounds in the leather industry effluents and majority of them have been shown to have toxic effects as discussed earlier but none of them reported the effects of these chemicals on endocrine system except two studies by Chang et al. (2004) and Fail et al. (1998). The former group reported a severe infertility in leather industry employees due to change in motility and morphology of sperm. In a similar type of study the latter group demonstrated a significant reduction in sperm count in N,N-dimethylformamide treated rats, which is one of the major component part of leather industry effluents. Both these reports supported our data on the change of seminiferous tubule structure in effluent treated rats resulting in testicular hyperplasia (Kumar et al., 2008b). Though at this point it is difficult to predict the exact reason for this hyper plastic nature of these contaminants, but development of cancer and tumor has already been reported by several authors in leather industry employees which provides some support to our this finding (Menza et al., 1992; Saber et al., 1998).

Similarly uetrotrophic assay demonstrated a significant increase in the uterine weight of both ovarectomised and intact rats treated with the 150 and 300 ml equivalent of effluent samples displaying estrogen agonistic activity (Andersan et al., 2006; Koda et al, 2007). This demonstrated that the leather industry effluents have dual regulatory system in males as well as females.

Once the androgenic and estrogenic nature of the leather industry effluents were established, efforts were made to investigate the mechanism of endocrine disrupting effects of leather industry effluents in intact male and female rats. For this we determined the expression profiles of some of the major steroidogenic enzymes in testes and ovaries of male and female rats using reverse transcriptase polymerase chain reaction (RT-PCR). As shown in the result section, three major steroidogenic enzymes, cytochrome P450scc, 3β-HSD and 17-βHSD mRNA levels in testes and ovaries (including aromatase) were up-regulated in both testes and ovaries of treated group of animals. This was further supported by *in vitro* estimation of testicular and ovarian 3β-HSD and 17-βHSD enzyme activities which also showed a significant increase in the activity over the control group of animals. The up regulation of steroidogenic enzymes like P450scc, 3β-HSD by xenobiotics and low dose of testosterone has already been reported by some groups earlier (Satoh et al., 2001; Kim et al., 2007; Kortner et al, 2007). The rise in serum level of androgen and estrogen could be attributed to the increased level of expression and activity of these steroidogenic enzymes. However, possibility of binding of these contaminating chemicals to these enzymes directly to regulate their expressions may also be considered as one of the reason as has been reported for some non steroidal compounds binding to 5α reductase and regulating their expressions (Liu et al., 2007).

Another interesting feature of this study is the down regulation of androgen and estrogen receptor expressions in testes and ovaries of treated group of animals respectively indicating autologus regulation (Ohsake et al., 2003; Andersan et al., 2006; Koda et al, 2007).

In order to gain further insight into the exact interfering components in the samples, we performed the GC-MS analysis of the water extracts to characterize some of the compounds that may be responsible for the endocrine disrupting nature of the test samples, as described in the next chapter of the thesis.

Thus, it can be concluded that the synthesis of steroid hormones is one of the crucial processes in the endocrine regulation. It consists of sensitivity regulated steps and it may be affected by different endocrine disrupting chemicals. Various classes of contaminants of leather industry effluents can interfere with the transcriptional activity of major steroidogenic factors and the downstream effects, thus amplifying its potential endocrine-disrupting impact. The fact, that some of its components may affect the androgen and estrogen signaling pathways in a different manner depending on the dose, should reinforce the concept that environmental xenobiotics (though present at low doses) may pose a threat to human health.

Chapter 4.4 *In vivo* characterization of endocrine disrupting behavior of a WWTP inlet and outlet water samples and their comparative analysis

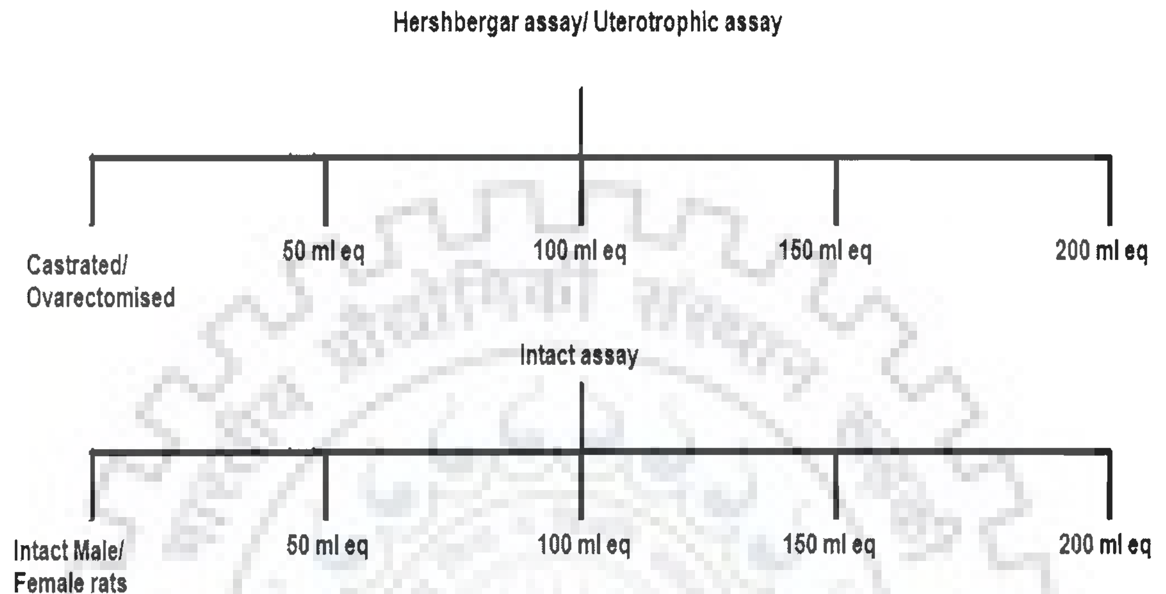
4.4.1 Introduction

When we discuss about different potential sources of endocrine disrupting chemicals the significance of sewage water can not be neglected out as it is expected to receive a broad spectrum of molecules from domestic and/or industrial waste (some of which as discussed in the earlier sections of this chapter) which are not completely eliminated during the treatment process in wastewater treatment plants (WWTP) (Ternes et al., 1999; Cargouët et al., 2004, Kumar et al., 2008a). In this context, WWTP discharges are considered as a major source of EDC rich pollution that plays a significant role in environmental contamination. Majority of the effects observed in the aquatic environment concerning the reproductive system, for instance, the feminization of male fish with WWTP treatment plant effluents, are attributed to the presence of EDC (Sumpter, 1998; Ternes et al., 1999; Mendola et al., 2008). Many substances like nonylphenols, phthalic esters, PCBs, dioxins, polycyclic aromatic hydrocarbons (PAHs), phytoestrogens, estrogens, androgens, and progesterone have been reported to be present in different water bodies (Vanderford et al., 2003; Leusch et al., 2006a; Oh et al., 2006; Xue and Xu et al. 2006; Mendola et al., 2008; Kumar et al., 2008a, 2008b; Chatterjee et al., 2008). It has been hypothesized that the statistically derived decrease in sperm counts over the last decades, increasing incidents of various cancer and other disorders regarding male infertility may be caused by the intake of these chemicals via food or drinking water (Sharpe and Skakkebaek, 1993; Phillips and Tanphaichitr, 2008). Several studies have reported the correlation between reproductive abnormalities in fish and exposure to WWTP effluents even several kilometers downstream from outfalls (Jobling et al., 1998;

Sumpter, 1998; Metcalfe et al., 2001; Sarmah et al., 2006). Domestic, workplace and industrial discharges contribute a vast quantity of human excreta, detergents and other substances (pharmaceuticals) into the nation's sewers (Vanderford et al., 2003). These discharges are also a possible source of contamination. One well-known example is the contraceptives utilized as ingredients of birth control pills. In general, municipal sewage and therefore excreted human pharmaceuticals and natural hormones have to pass through WWTP before entering rivers or streams. Another factor involved here is the potential contamination of soil and ground water, caused by the application of digested sludge from municipal WWTP on agricultural fields (Ternes et al., 1999). In order to evaluate the potential risk of the endocrine disruption, the occurrence of individual compounds needs to be documented. Further, besides hormonally active substances, sewage water also consists of many toxic chemicals like nitrates and phenols which are toxic to the human/animal health (Bucher and Hofer, 1993; Bernet et al., 2004).

This section of thesis aimed to examine and compare the (anti)androgenic and (anti)estrogenic endocrine disruption caused by the contaminants present in the inlet and outlet water streams of a WWTP. Water samples were collected from inlets and outlet streams of one of the major WWTP localized in the northern region of India. This area was chosen as representative since a major portion of drinking water is produced from surface waters in this area. Here, an integrated approach was adopted, first screening the sample for their androgenicity and estrogenesity by the Hershberger assay and uterotrophic assays. Once samples were found positive in Hershberger assay and uterotrophic assays, probable mode of action of these were studied by evaluating various parameters on animals. Schematic representation, given immediately after this paragraph,

depicts the summary of dosing plan used for gavaging the different doses of inlet and outlet water samples to male and female rats as described in the methodology section.



4.4.2 Results

4.4.2.1 Endocrine disrupting effects of wastewater samples in male reproductive system

4.4.2.1.1 Determination of androgenicity of effluents by Hershberger assay

At a dose of 150 and 200 ml equivalent of water samples both WWTP inlet and outlet samples induced a statistically significant increase in the weight of all accessory sex organs viz. prostate gland, glans penis, cowper's gland. seminal vesicle and vas deference in all treated groups as compared to the reference vehicle control ($p<0.05$) (Table 17). The significant increase in the weight of these SATs have been further supported by their increased size (Fig. 46). Thus, the increase in absolute sex accessory tissue weights was attributable to the specific androgenic effects of the WWTP inlet and outlet water samples. Water samples at a concentration of 100 ml equivalent per day, induced a significant increase only in ventral prostrate, glans penis and Cowper's gland, while no

Table 17. Effects of WWTP inlet and outlet water samples on weights of SATs from castrated rats given 20 consecutive daily treatments with WWTP water samples

	Ventral prostate (mg)	Glans penis (mg)	Cowper's gland (mg)	Seminal vesicle (mg)	Vas deference (mg)
1. Control	10.55±0.35	44.55±0.12	4.40±0.18	47.85±0.18	64.45±1.23
2. 50ml/150μl.					
a-inlet treated	13.25±0.26	44.32±0.39	4.72±0.22	48.37±0.16	63.30±1.27
b-outlet treated	11.35±0.23	44.77±0.47	4.30±0.19	47.95±0.29	63.0±1.46
3. 100ml/150μl					
a-inlet treated	15.0±0.35*	48.02±0.27*	5.45±0.17*	48.62±0.24	63.52±0.95
b-outlet treated	14.0±0.35*	46.47±0.31*	4.37±0.18*	47.75±0.28	63.22±0.97
4 150ml/150μl					
a-inlet treated	30.35±0.70*	54.32±0.23*	8.38±0.20*	67.72±0.24*	98.52±0.28*
b-outlet treated	27.80±0.25*	52.55±0.21*	8.17±0.11*	63.47±0.19*	92.82±0.27*
5- 200ml/150μl					
a-inlet treated	90.50±0.28*	62.0±0.13*	12.72±0.24*	89.1±1.23*	111.0±0.41*
b-outlet treated	86.30±0.55*	59.25±0.22*	11.10±0.17*	80.85±0.23*	102.0±0.48*

(Each value denotes mean ± S.E.M. of six animals)
*Significantly different from vehicle control group at $p<0.05$ level

change was observed in other SATs. However, on treatment of the animals with 50 ml equivalent of water samples did not result in any significant changes in the weight of the SATs.

4.4.2.1.2 Gene expression profile of steroidogenic enzymes.

As shown in Fig 47, although there was a marked up regulation of transcription of P450scc, P450C-17, 3β-HSD and 17β-HSD but a significant down regulation of AR transcript was observed in the case of both inlet and outlet treated rats. It is to be noticed here that although effect on expression is less evident in outlet treated group as compared to inlet treated group, the former was around two fold more as compared to control. Testis of inlet and outlet treated group showed a 2.5 to 3.5 folds up/ down regulation of transcripts of the foresaid genes.

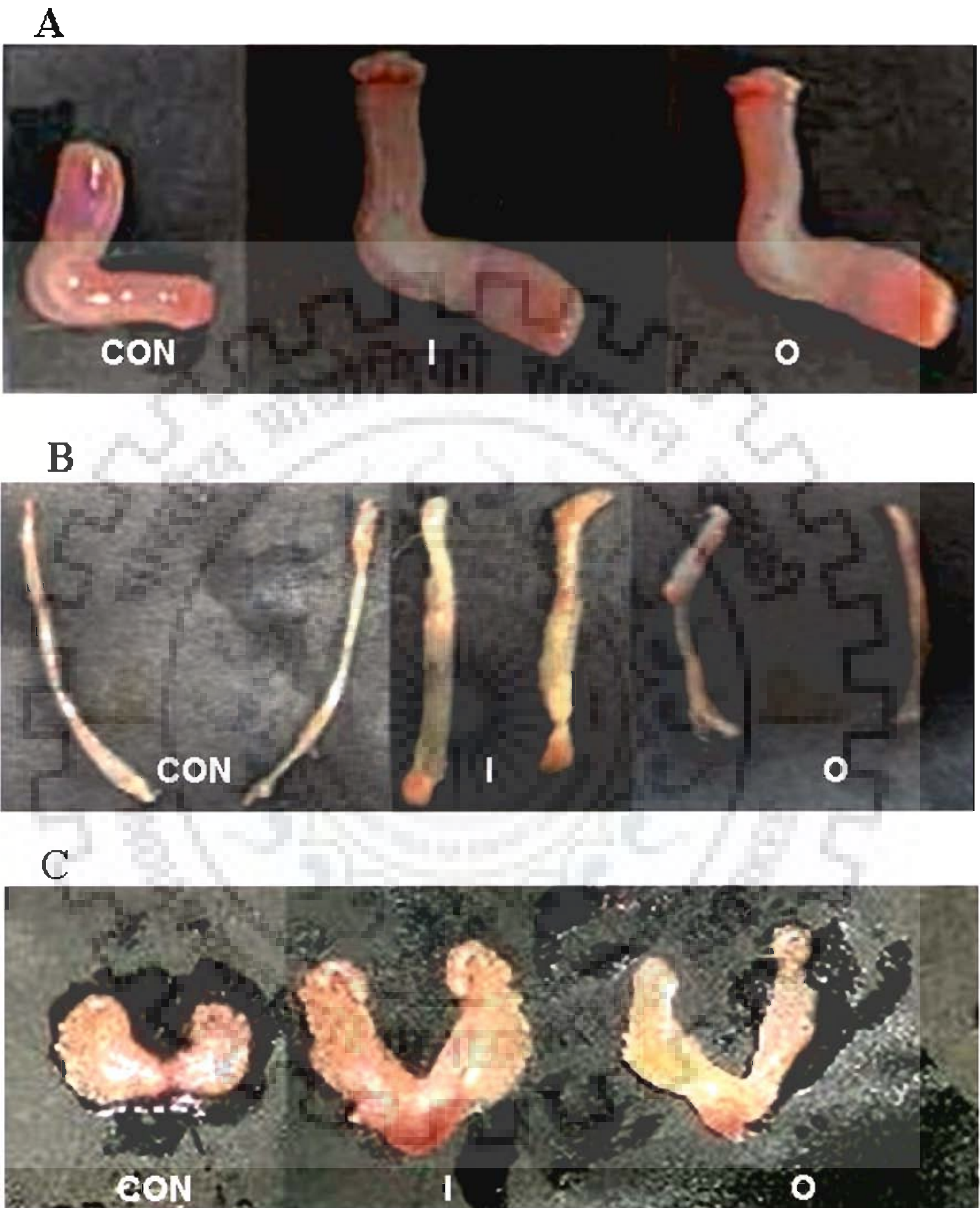


Figure 46. The effects of WWTP inlet and outlet water samples on the structure of some of the SATs. Adult male rats were treated with 200 ml equivalent of inlet and outlet water samples for 20 days.. A, glans penis; B, vas deference; C, seminal vesicle. CON, vehicle treated animals; I, inlet effluent treated animals and O, outlet effluent treated animals. 10X.

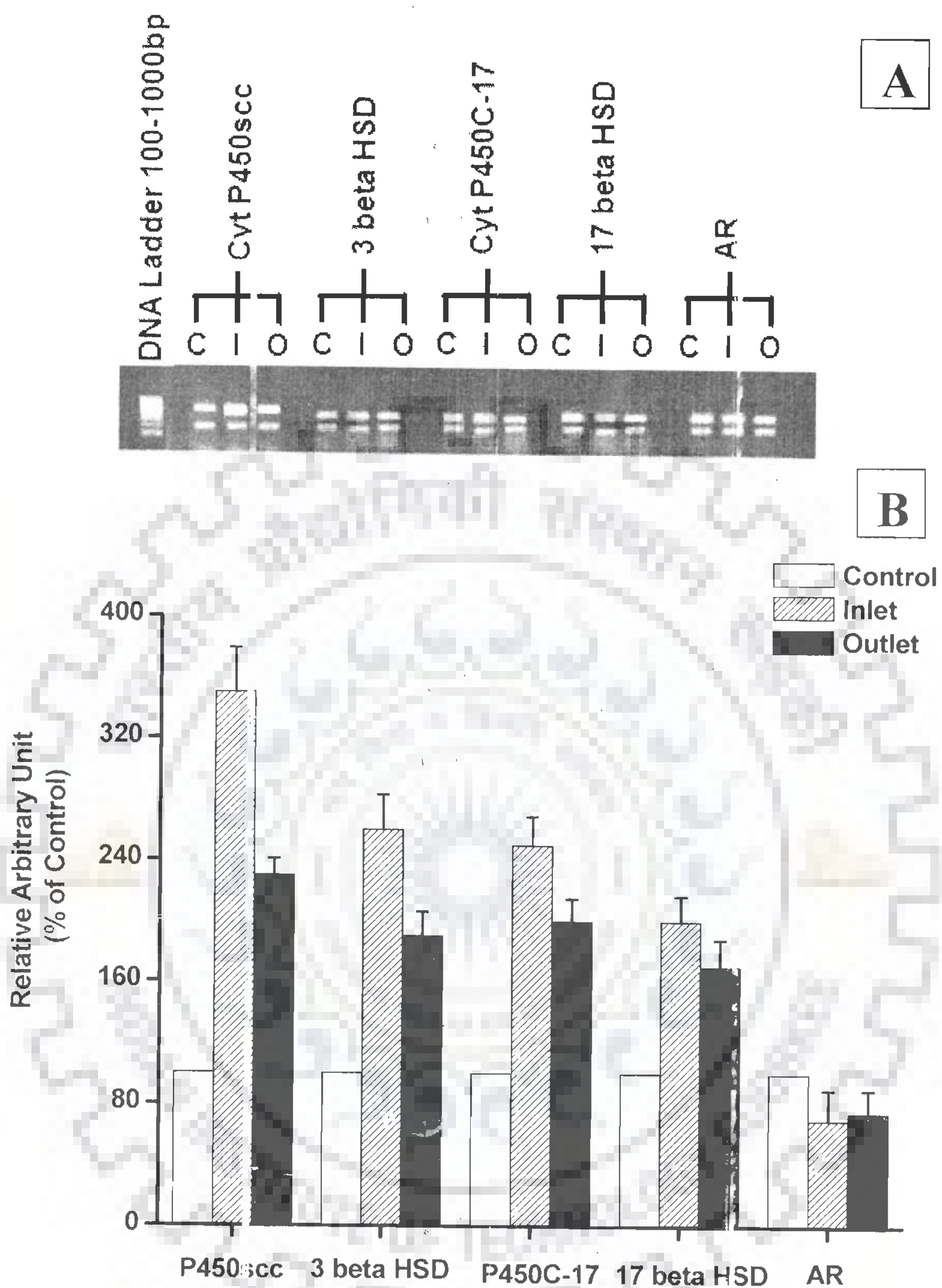


Figure 47. RT-PCR analysis of testicular mRNA expression of P450scc, 3β-HSD, P450C-17, 17β-HSD and AR genes in control and 200 ml equivalent of WWTP inlet and outlet water sample treated rats (A). Densitometric analysis against the internal control (GAPDH) (B). The values are mean ± S.E.M. of eight RT-PCR reactions for each group tested. Open, thatched and solid bars represent the vehicle, inlet and outlet sample treated groups respectively. The values are mean ± S.E.M. of eight RT-PCR reactions for each group.

4.4.2.1.3 Testicular 3β-HSD and 17β-HSD levels in vitro.

Spectrophotometric enzyme assays for 3β-HSD and 17β-HSD demonstrated that 50 and 100 ml equivalent of water samples could not induce any significant change in the level of these two enzymes (Fig. 48). However, 150 ml equivalent of the samples demonstrated a significant increase which was further up regulated (about3 fold) by 200 ml equivalent of the samples in case of both 3β-HSD and 17β-HSD activities. Finally, treatment with 200 ml equivalent of inlet water sample induced a dramatic effect causing a 2.5 and 3 folds of increase in testicular 3β-HSD and 17β-HSD respectively. In outlet treated groups, the effect was 2.3 fold and 2.5 fold respectively for 3β-HSD and 17β-HSD respectively (Fig. 48).

4.4.2.1.4 Testicular *3β-HSDand AR protein immunoblot analysis*

Rats treated with 200 ml equivalent of WWTP outlet (O) and inlet (I) samples showed increased translation of 3β HSD (45 kD) while a decreased translation of AR (100 kD) protein as compared to control (vehicle treated animals) and this increase was statistically significant (Fig 49) ($p<0.05$). The uniform band intensities of β-actin in all the wells indicated equal gel loading.

4.4.2.1.5 Serum Hormone levels

As shown in Table 18, there was an apparent increase in the level of testosterone in the intact rats treated with WWTP effluents (almost 152% in inlet and 127% in outlet treated groups) as compared to control, Also, there was a decrease in the levels of LH (24% in case of inlet and 20% in case of outlet) and FSH (34% in case of inlet and 25% in case of outlet) in the serum samples, confirming further the androgenicity of the contaminants in

the WWTP (Table 18). Decrease in the levels of LH and FSH could be attributed to the initiation of feed back mechanism by the androgenic contaminants in the water samples.

4.4.2.2 Endocrine disrupting effects of wastewater samples in female reproductive system

To our surprise no significant changes were observed in the female reproductive system at the highest concentration of the test sample. Hence data not produced.

Table 18. Serum levels of LH, FSH and testosterone from intact rats given 20 consecutive daily treatments with water sample of indicated dose

	LH (ng/ml)	FSH (ng/ml)	Testosterone (ng/ml)
1. Control	0.83±0.09	6.52±1.79	4.86±1.10
2. 200ml/150µl			
a-inlet treated group	0.61±0.08*	4.32±0.94*	7.40±1.70*
b-outlet treated group	0.67 ±0.02*	4.93±1.27*	6.20±1.60*

(Each value denotes mean ± S.E.M. of six animals)
* Significantly different from vehicle control group at $P<0.05$ level

4.4.2.3 Toxicological evaluation of the test samples

4.4.2.3.1 Body and organ weights of effluent treated intact rats.

There was no significant change in the body weight of the animals treated with both of the WWTP inlet and outlet samples as compared with the control.

4.4.2.2.2 Histopathology of sex accessory tissues

There were no remarkable histopathological deformations in the testis or any of the SATs of both inlet and outlet water treated groups as compared to control.

4.4.2.3.3 Histopathology of kidney and liver effluent treated rats

Histopathological studies demonstrated a considerable damage to the liver (Fig. 50) and kidney (Fig. 51) of treated rats. In liver of rats treated with 200 ml equivalent of inlet water sample there was a fragmentation of nucleus (pyknosis and karyorrhexis) and

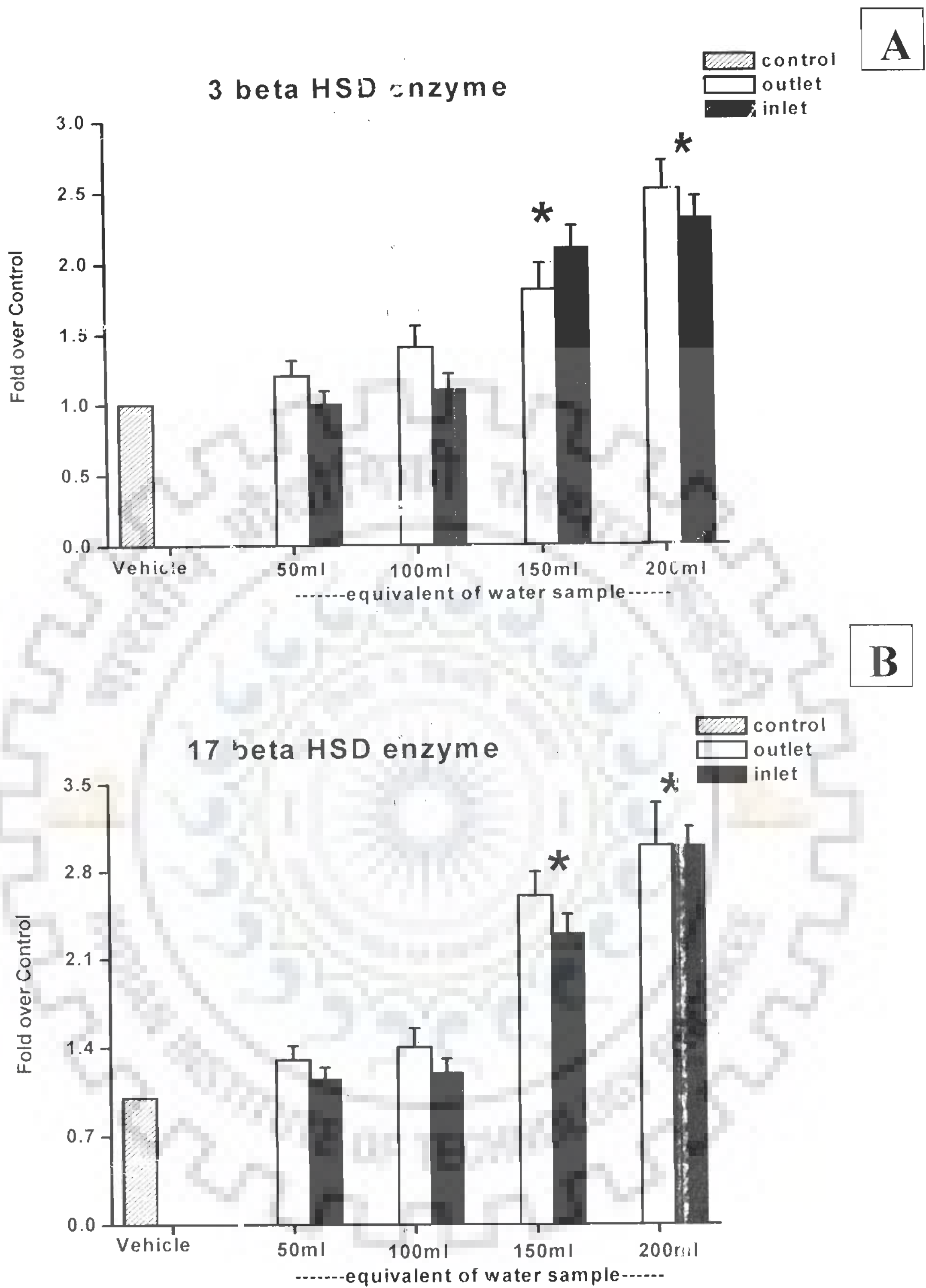


Figure 48. Effects of WWTP water samples from inlet and outlet on testicular level of 3 β-HSD and 17 β-HSD enzyme activity *in vitro*. The results are expressed as folds increase of enzyme activity over vehicle treated groups which was given a value of 1. Data are mean ± S.E.M.; n = 5. * indicates the significant level of difference in enzyme levels as compared to vehicle treated groups for both the enzymes ($p<0.05$).

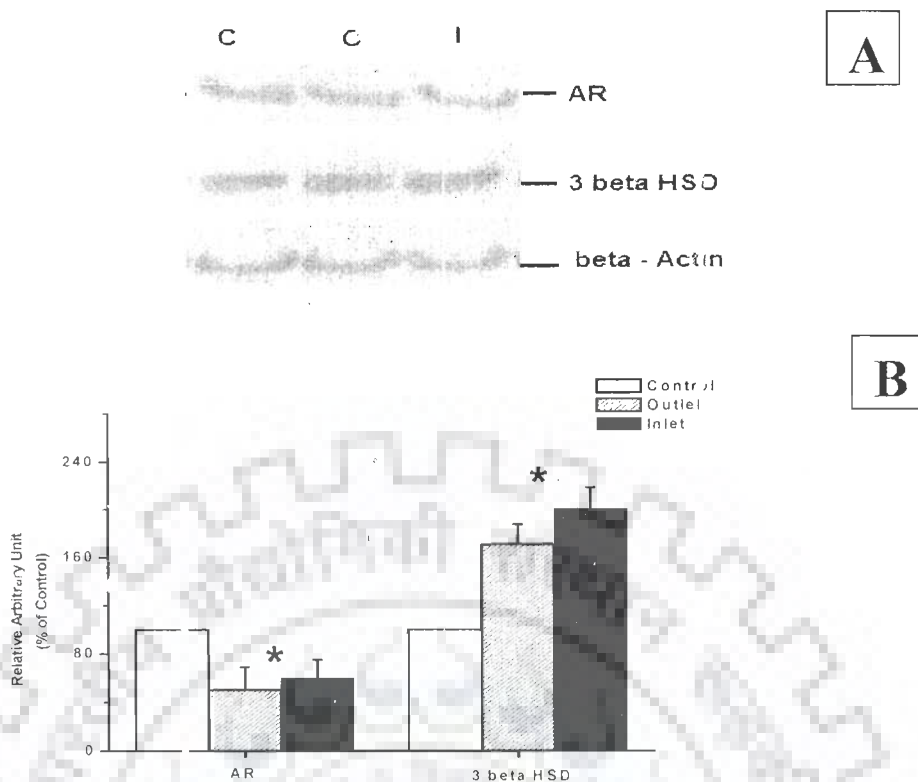


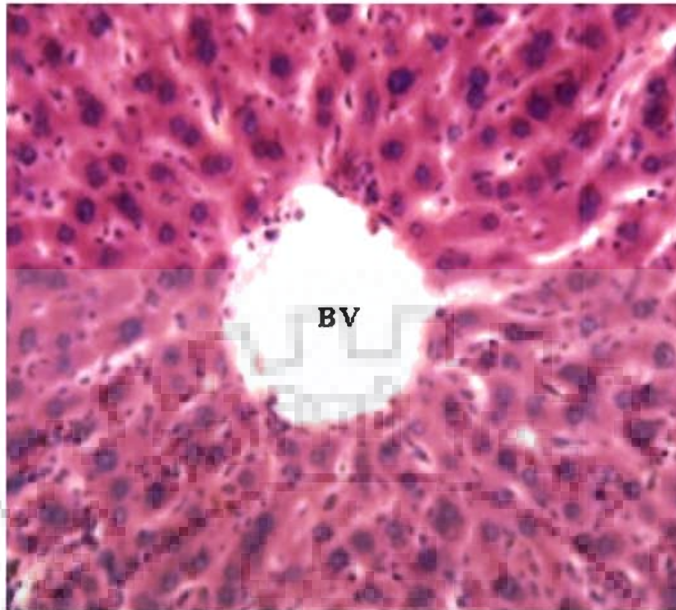
Figure 49. Western blo analysis of testicular 3β-HSD enzyme and AR protein in response to treatment with 200 ml equivalent of outlet water samples (A). Denestometric analysis against the internal control (β-actin) (B). The values are mean ± S.E.M. of three separate experiments C,control; I, Inlet; O, outlet treated rats. * indicates significant level of difference over vehicle treated group.

disappearance of the cellular cytoplasm. This led to the appearance of purple colored granules in the cytoplasm (Fig. 50). This indicates the preliminary stage of necrosis which was more pronounced around the blood vessels. In case of kidney, extensive perforation was observed in the form of vacuolar degeneration in the animals treated with both inlet and outlet samples (Fig. 51).

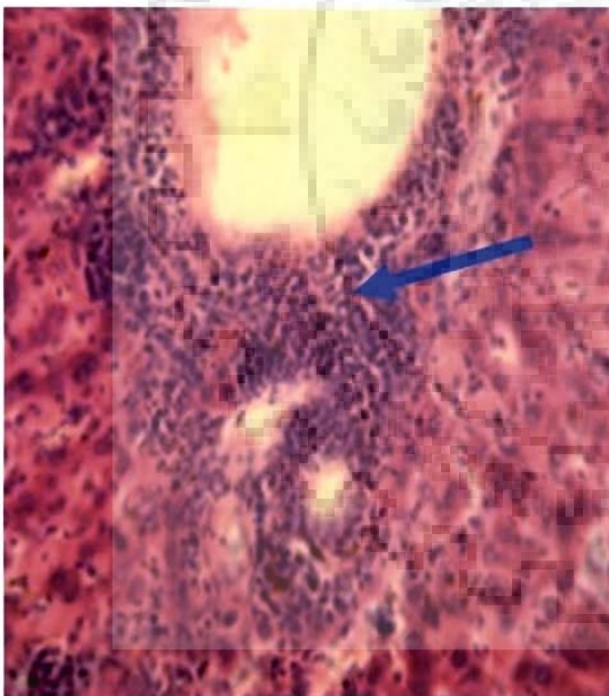
4.4.2.3.4 Analysis of systemic toxicity in WWTP treated animals

Systemic toxicity was confirmed by about 2-3 fold increase in the serum level of alkaline phosphatase, SGPT and SGOT in both inlet and outlet water treated groups of rats and the effect was more pronounced in inlet stream (Fig. 52). However, no significant change in the levels of the ACP was observed.

Control



Inlet Treated



Outlet Treated

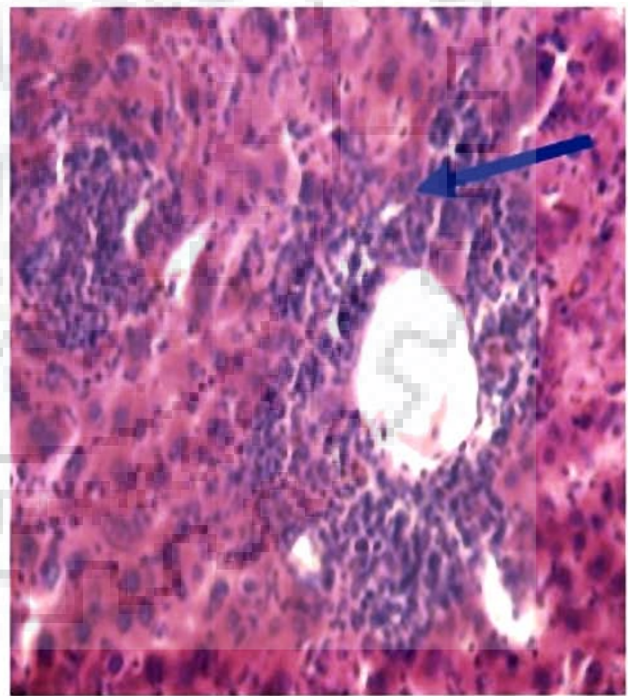
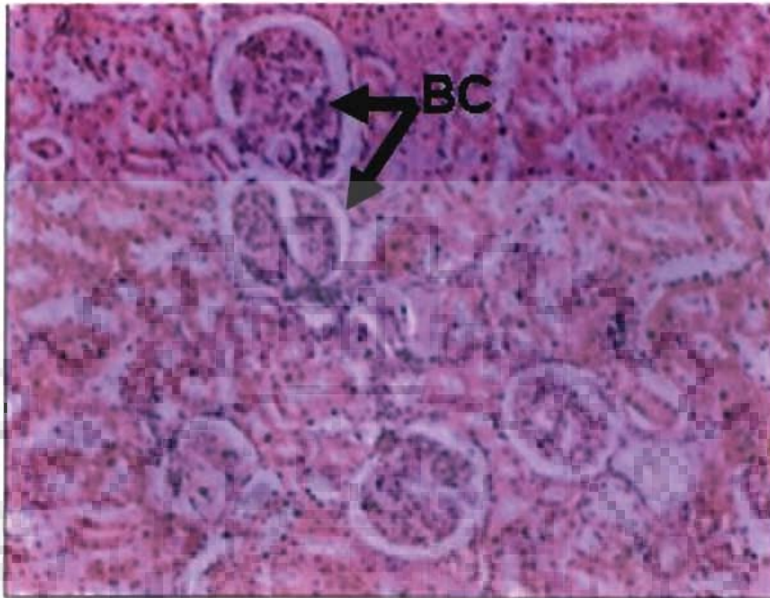
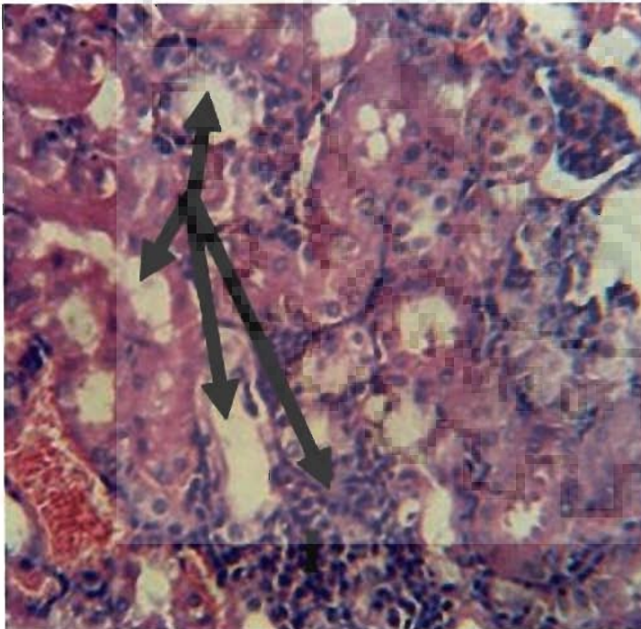


Figure 50. Photomicrograph of liver sections. H&E. 40X. Blue arrows represent the dissolution of cytoplasm and appearance of purple colored granules. BV, Blood Vessel.

Control



Inlet Treated



Outlet Treated

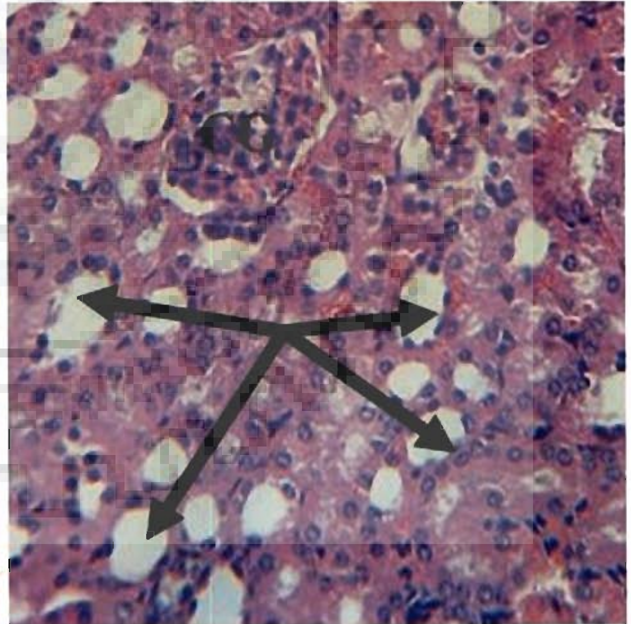


Figure 51. Photomicrograph of kidney sections of the rats treated with the 200 ml equivalent of WWTP inlet and outlet water. Arrow represents appearance of vacuolar degeneration. BC, Bowman's capsule. HE 32X.

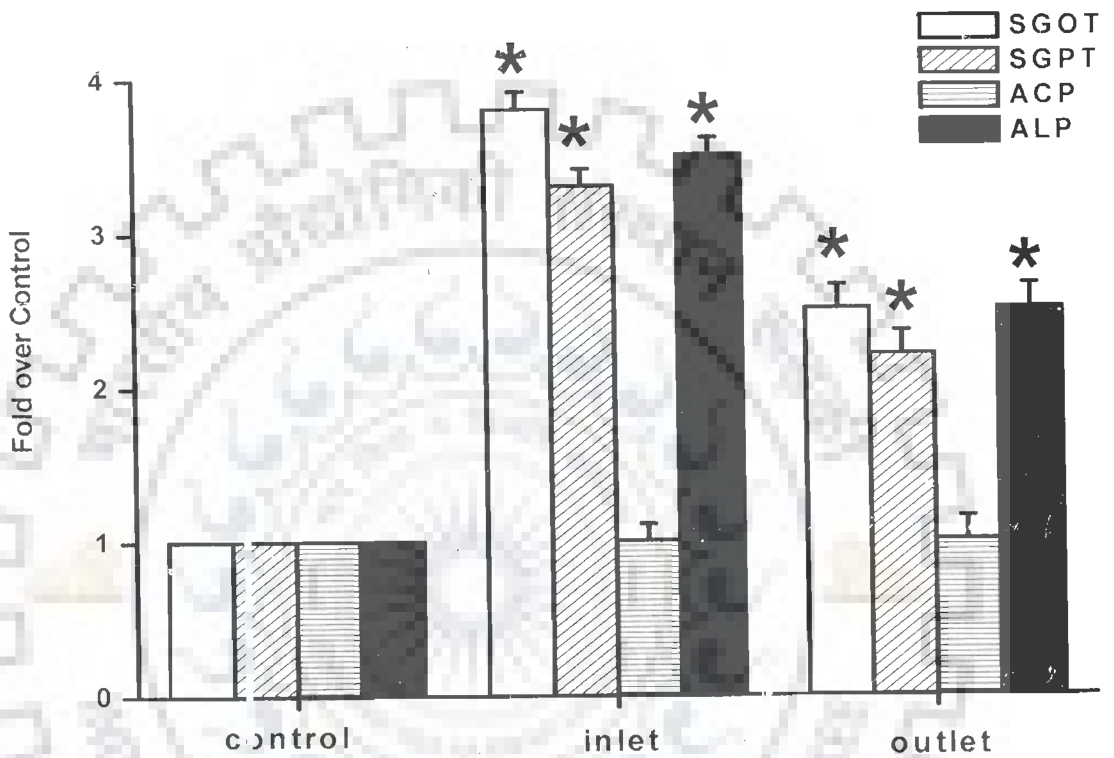


Figure 52. Effects of WWTP inlet and outlet water samples (200 ml equivalent) on SGOT, SGPT, ALP and ACP levels in rats. The values are mean ± S.E.M. of three separate experiments. * indicates significant increase over control group at $P<0.05$ level.

4.4.3 Discussion

It is not surprising that the inlet water in WWTP is loaded with several contaminants from the domestic applications but incomplete/partial treated final effluent from these plants also contaminates the receiving water bodies (river, estuaries, canals) with the chemicals harmful for general health, aquatic animals and plants in a gradual process of accumulation. Several reports suggests the presence of a number of potential EDC as well as toxicants in finally treated sewage effluents and in receiving water bodies like synthetic and natural androgens/estrogens, insecticides, pharmaceuticals,, phenols, detergents etc. (Thomas et al., 2002 ; Oh et al., 2006). The dramatic effects at the endocrine disruption as well as toxicity level have been shown in the aquatic animals exposed to the sewage water (Svenson and Allard, 2005; Leusch et al., 2006a; Sarmah et al., 2006; Subramanian and Amutha, 2006). Some activities, like the usage of water contaminated with sewage effluent for irrigation purpose as well as that of heavily contaminated sludge produced during treatment of inlet water for agricultural applications and production of drinking water from sewage water also poses a threat for general health of terrestrial animals/humans (Aparicio and Santos, 2007). Thus, there is a need to analyze the endocrine disrupting and toxic effects of offending chemicals present in finally treated effluent of WWTP on terrestrial animals. In this study, we executed a comparative evaluation of the endocrine disrupting activity and general toxicity caused by the complex mixture of chemicals from inlet and outlet water samples of WWTP in male rat reproductive system.

First, both inlet and outlet water samples were screened by Hershberger and uterotrophic assays to determine whether they have any (anti-)androgenic and (anti-)

estrogenic effect on the male and female reproductive system respectively. Although Hershberger assay showed a dose dependent increase in the weight and size of the SATs depicting a clear androgen agonistic activity in both inlet and outlet treated castrated rats, uterotrophic assay did not show any significant changes. Since the water samples tested by us contained some androgen precursors or its analogs (DHEA and isoandrosterone as demonstrated in the next chapter), it could be argued that they might interfere in the androgen bioassay in the male system, thus, demonstrating an increased serum androgen levels in castrated treated animal groups (data not shown).

Once both the inlet and outlet water samples tested positive as androgen agonist in Hershberger assay, their probable mechanism of action was determined by administrating the samples to intact male and female rats. RT-PCR analysis of the major steroidogenic enzymes namely: P450scc, P450C-17, 17β-HSD and 3β-HSD demonstrated a significant up-regulation of testicular mRNA transcripts while no remarkable changes were observed in ovarian mRNA transcripts in treated male and female rats respectively as compared to control. This was further supported by the *in vitro* estimation of testicular and ovarian 3β-HSD and 17-HSD enzyme activities, which also showed a significant increase in enzyme activity in testis while no significant change in ovaries as compared to their respective controls. The alteration in the activities of steroidogenic enzymes like P450scc, P450C-17, 3β-HSD by androgenic and estrogenic xenobiotics has already been reported by some groups earlier (Satoh et al., 2001; Lin et al., 2006; Kim et al., 2007; Kortner and Arukwe, 2007). Another interesting finding of this study was the up regulation of the serum testosterone levels even in absence of elevated gonadotrophins (LH and FSH) in the case of both inlet and outlet WWTP treated

groups. This showed that although the negative feed back mechanism was operative yet there was a sustained testosterone production. All these further support our finding that the low levels of LH did not interfere with alteration in steroidogenesis. However, the exact cross talk between the gonadotropic hormone, the xenobiotcs and steroidogenic cascade remains an intriguing question which needs further research in these directions. There are also few reports which indicate that sewage sample may affect both androgenic and estrogenic system depending on the nature of contaminants in WWTP (Spengler et al., 2001; Bandelj et al., 2006; Leusch et al., 2006). Absence of significant (anti)estrogenic effects by the WWTP samples in our study could be attributed to two factors: first there may be a extremely low level of (anti)estrogenic chemical and second, there may be estrogenic chemicals in the samples but their effects may be hindered by the other compounds (combinational effect).

The next obvious question was to check if in addition to endocrine disruption, whether the effluents also demonstrated the toxic effects in treated animals. On performing the toxicity analysis it was found that there were evident histopathological malformations with up regulations of serum alkaline, SGOT and SGPT(Sato et al., 2004; Bhattacharjee and Sil, 2006). These findings were not surprising since production of pyknosis, karyorrhexis and vacuolar degeneration in liver and kidney has been reported earlier also by the exposure of animals to the sewage water and other toxicants (Bucher et al., 1993; Bernet et al., 2004; Roy et al., 2005; Bhattacharjee and Sil, 2006). It confirmed that the contaminating chemicals present in both the inlet and outlet stream of WWTP were causing the hepatotoxicity as well in addition to endocrine disruption in male rats. It could be argued that the increased testosterone levels in animals treated with water

extract is simply secondary to xenobiotic induced liver/kidney injury there by changing testosterone metabolism. However, at this point, this fact could not be over ruled completely, but earlier reports confirmed that the systemic toxicity had also been noted in rats treated with methyl testosterone (MT) and paper mill effluents (rich in several anti-androgenic chemicals) along with significant change in absolute SAT weights and other male reproductive structures. This could be attributed to the specific (anti)androgenic effect of MT and contaminants of leather industry effluents rather than systemic toxicity (Kennel et al., 2004, Rana et al., 2004). In the present investigation, out of the four chemicals identified by GC-MS (as described in the next chapter), dehydroepiandrosterone (DHEA) and isoandrosterone are androgen precursors or testosterone equivalents which are very well known to exert androgen agonistic behavior in male system. Based on these reports and our data, it is probable that the androgenic effects as demonstrated by effluents here are due to the presence of these chemicals in significant amount and their androgenic actions rather than the inhibition of testosterone metabolism due to liver and kidney injury.

In conclusion, the synthesis of steroid hormones is one of the crucial processes in the endocrine regulation. It consists of sensitivity-regulated steps and different endocrine disrupting chemicals may affect it. The data presented here conclusively provides evidence of WWTP effluents (both inlet and outlet) acting as endocrine disruptors in male rats and demonstrates its potential impact on human androgen axis. The content of this effluent can interfere with transcriptional activity of major steroidogenic enzymes and the downstream effects, thus amplifying its potential endocrine-disrupting impact. At this point it could be speculated that the offending chemicals act through AR as agonistic

ligands, as has been reported earlier by several authors (Ralph et al., 2003; Kortner and Arukwe, 2007). This may in turn alter the expression patterns of steroidogenic enzymes including its own receptor up-regulation. Further at a high concentration of these chemicals, especially the NP, HCB and androgen agonists (DHEA and isoandrosterone) may result in systemic toxicity as has been demonstrated by our study and some earlier reports. However, given the complexities in the steroid synthesis pathways and biological activities of hormones, together with unknown biokinetic properties of the complex mixture of EDC for systemic toxicity, further investigations with *in vivo* and *in vitro* experimental models are required to define a clear-cut picture on this aspect of endocrine disruptor research. We have tried to explore the detailed *in vivo* and *in vitro* mechanism of one of the androgenic chemicals identified in both WWTP water and leather industry effluents samples which is described in the latter part of this thesis.

CHAPTER - 5

CHAPTER 5: PHYSICOCHEMICAL CHARACTERIZATION OF WWTP WATERAND LEATHER INDUSTRY EFFLUENTS SAMPLE

5.1Introduction

The preceding chapter (including the subchapters) of the thesis described the endocrine disrupting action of some commercially available synthetic chemicals due to their direct application or environmental presence. Out of all the samples tested for *in vivo* study, some of them were in the form of pure chemicals (triclosan, ibuprofen and tetracycline) while the others were a complex mixture of environmental chemicals (WWTP water and leather industry effluents). It can be deduced from the results that all the tested samples were acting at various sites to induce the endocrine disruption in the exposed physiological system. However, when we discuss about the latter category of samples (WWTP water and leather industry effluents), it can be noticed that they were tested in the form of crude non-polar extracts which was a complex mixture of various compounds representing a combinational effects (that may be additive, synergistic or independent) in regards to their endocrine disrupting activities (Nellemann et al. 2003; Birkhoj et al., 2004). In order to resolve this issue, this chapter describes the identification of few of the offending molecules that may be responsible for the endocrine disrupting potential of the WWTP and leather industry effluents using the combination of two modern techniques viz. HPLC and GC-MS. These two techniques have been widely used for the identification and quantification of insecticides, pharmaceuticals, natural and synthetic steroids, phytoestrogens in various sources like industrial effluents, sewage water, river water, wetlands, marine water, milk, blood etc. (Hilton and Kevin 2003; Vanderford, et al., 2003; Cargouet et al., 2004; Suzukia et al., 2004; Hill et al., 2005; Hoogmartens,

2006; Lee et al., 2006; Nair et al., 2006 ; Tagawa et al., 2006; Kumar et al., 2008a and 2008b).

In this section while both WWTP water and leather industry effluents were subjected to the GC-MS analysis for the identification of the potential contaminates, the WWTP inlet and outlet water samples, additionally, were subjected to the HPLC for a comparative scrutiny of the contaminants present in the inlet and outlet streams of the WWTP water.

5.2 Results

5.2.1 WWTP water sample

5.2.1.1 HPLC Analysis

It can be deuced from the chromatograms that 20 contaminants were within the detection limit in inlet water samples while only 13 in case of outlet water samples and out of them 5 peaks (at retention times 4.32, 6.98, 15.88, 18.55, 19.88 min) overlapped in both chromatograms, as shown by arrows, indicative of their persistence in the outlet water even after the waste water treatment process (Fig. 53). Besides an analysis of the peak areas of 5 common contaminants in the chromatograms of inlet and outlet water samples indicates a reduced level of contamination in outlet as compared to inlet sample which points to their partial removal in treatment porcess (Table 19).

Table 19. A comparative analysis of peak area from WWTP inlet and outlet samples showing the reduced level of contamination of the five common peaks.

Retention time	Peak Area (Inlet)	% Area (Inlet)	Peak Area (Outlet)	% Area (Outlet)
4.32	23198	0.15764	12143	0.09142
6.98	141534	0.96547	88632	0.41760
15.88	55887	0.41234	25021	0.19823
18.55	33779	0.23451	14158	0.11597
19.88	44539	0.31093	21428	0.14876

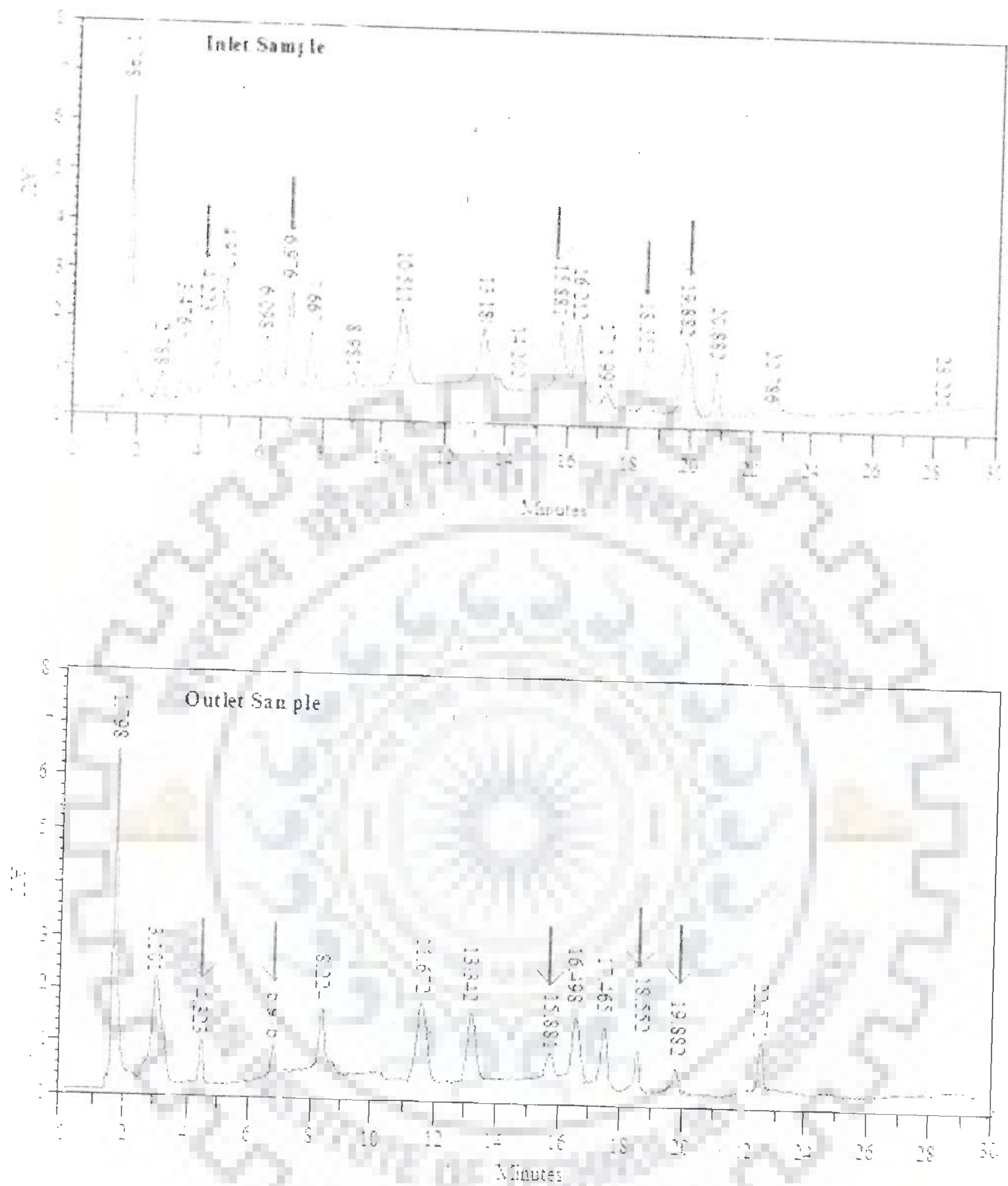


Figure 53. Comparative analysis of high performance liquid chromatograms of inlet and outlet water samples of WWTP treatment plants. The arrows indicate the common peaks in both inlet and outlet water samples having same retention time.

5.2.1.2 GC-MS Analysis

Four compounds viz 4-nonylphenol, hexachlorobenzene, dehydroepiandrsterone and isoandrostenedione having retention times 10.72, 11.80, 16.60 and 17.65 min respectively were identified by GC-MS of both inlet (data not shown) and outlet samples as shown in Fig. 54A. Further, like that of HPLC analysis, the peak area of the common peaks (identified by GC-MS analysis) was higher in inlet sample as compared to outlet sample. The inset shows the structures of the derivatized compounds. The elution profile of the outlet sample matched closely with the known standards (Fig. 54B). All the detected contaminants were well in the detection limit. Table 20 shows the concentration of the four identified offending molecules determined by the selected ion monitoring mode (*SIM*) GC-MS analysis.

Table20. Concentration (μg/l) of 4-nonylphenol, Hexacholrobenzene, Dehydroepiandrosterone and isoandrostenedione in 200 ml equivalent outlet water sample determined by the selected ion monitoring mode (*SIM*) GC-MS analysis.

	Concentration (μg/l)	Molecular Mass	Retention Time (min)	Selected ions (m/z)
Nonylphenol	3.1	220.35	10.72	162
Hexachlorobenzene	3.8	284.8	11.80	284
Isoandrosterone	2.9	290.4	16.6	73
Dehydroepiandrosterone	3.1	28.4	17.65	129

5.2.2 Leather industry effluents analysis

Four compounds viz 4-nonylphenol, hexachlorobenzene, 4-aminobiphenyl and benzidine having retention times 7.25, 11.03, 15.71 and 16.78 min respectively were identified by GC-MS as shown in Fig. 55A. The inset shows the structures of the derivatized compounds. The elution profile of the effluent samples matched closely with the known standards (Fig. 55B). All the detected contaminants were well in the detection limit. The

concentrations of contaminants in leather industry effluents extract, analyzed by the GC-MS quantification, are presented in Table 21.

Table 21. Concentration (µg/l) of 4-nonylphenol, hexacholrobenzene, benzidine and 4-aminobiphenyl in 300 ml equivalent leather industry effluent sample determined by the selected ion monitoring mode (*SIM*) GC-MS analysis.

	Concentration (µg/l)	Molecular Mass	Retention Time (min)	Selected ions (m/z)
Nonylphenol	4.6	220.35	11.25	162
Hexachlorobenzene	3.3	284.8	11.03	284
4-aminobiphenyl	2.1	169.20	15.71	184
Benzidine	2.2	184.25	16.78	169

5.3 Discussion

The *in vivo* characterization of the inlet and outlet WWTP water samples demonstrated clearly that both the samples were inducing androgenic response although the androgenic potential of the outlet samples was comparatively lesser as compared to the inlet water samples. This gave us an idea that in spite of rigorous treatment process of the inlet water samples, WWTP inlet and outlet is still having some chemicals that are common in both of them. For confirming this notion, both the inlet and outlet samples were subjected to the HPLC analysis under similar sets of condition and this analysis demonstrated the presence of about 20 and 13 different contaminating peaks in inlet and outlet water samples respectively. Further, five peaks were found common in both inlet and outlet water samples, although peak area of these common peaks was less in outlet water as compared to inlet water samples. This was indicative of the fact that although the WWTP was effective enough in removing the contaminants from the inlet water streams (as

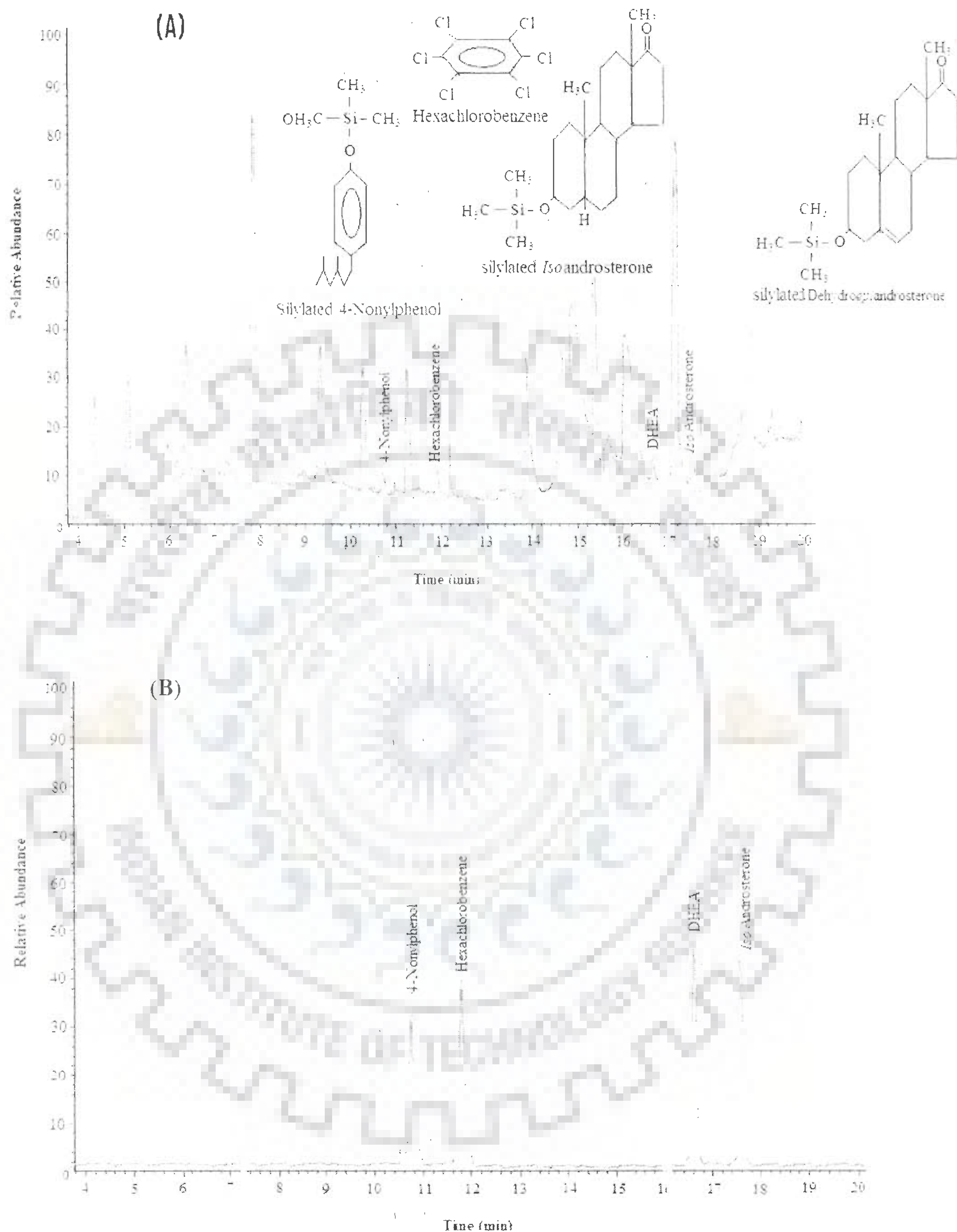


Figure 54. Representative GC-MS Chromatograms of final WWTP effluent sample (a) and its comparison with solutions of known standards (B) in full acquisition mode. The WWTP outlet effluents were derivatized as described in materials and methods and analyzed by GC-MS. The insets in (A) shows the structure of derivatized (silylated) forms of target chemicals except hexchlorobenzene that is not derivatized by BSTFA.

(A)

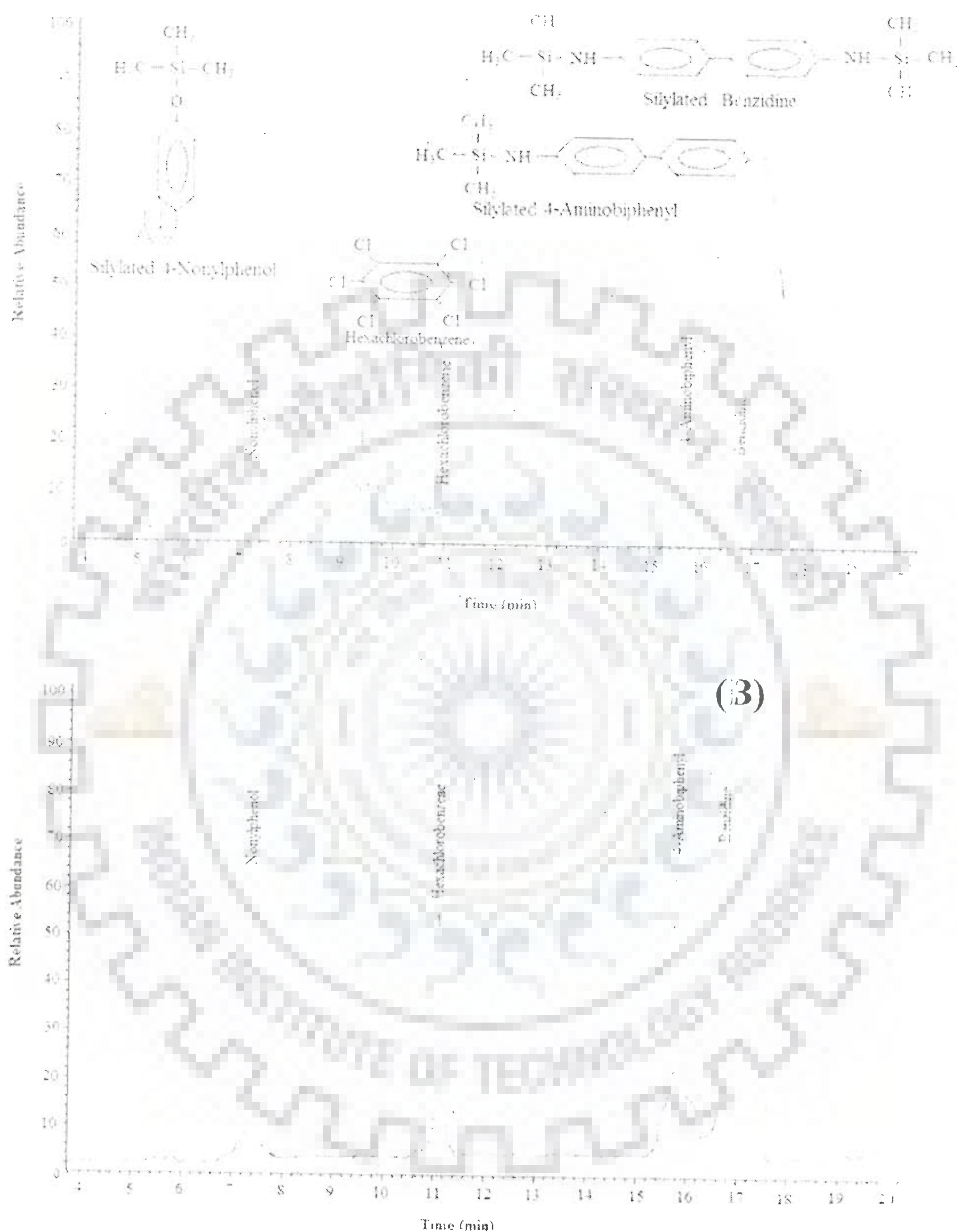


Figure 55. Representative GC-MS chromatograms of the extracted leather industry effluent sample (A) and its comparison with solutions of known standards (B) in full acquisition mode. The insets in (A) shows the structure of derivatized (silylated) forms of target chemicals except hexchlorobenzene that is not derivatized by BSTFA.

denoted by the significantly reduced peak area of the common contaminates), however, the clean up was not effective enough to make it mostly free from the contaminants. Further, the GC-MS analysis of both inlet and outlet water sample confirmed the presence of 4 interfering molecules in the samples albeit at a lesser level in outlet sample. Both inlet and outlet samples were found to contain several aromatic compounds like nonylphenol (NP), hexachlorobenzene (HCB), isoandrosterone and dehydrepiandrosterone (DHEA) and they were well in the detection limit. All the chemicals identified by the GC-MS analysis of the WWTP water have been known to be used in various household and industrial applications i.e. HCB is a very commonly used insecticide, NP is a breakdown compound of different synthetic product and last but not the least isoandrosterone and DHEA are natural androgens found in human body and percolates to the WWTP through human/animal use or excretion in municipality area (Soto *et al.* 2004, Kumar et al., 2008a). All these four compounds identified by us have already been well documented as androgen receptor agonists in various reports (Sohoni and Sumpter, 1998; Sonnennschein and Soto, 1998; Satoh et al., 2001; Ralph et al., 2003; Leusch et al., 2006a; Kortner and Arukwe, 2007, Kumar et al., 2008a, 2008b). Similarly in the case of leather industry effluents, GC-MS analysis revealed the presence of several aromatic compounds like nonylphenol, hexachlorobenzene, aminobiphenyl and benzidine. All these chemicals have been known to be used at various phases of leather processing and some of them are even known to be AR agonists which further supported our findings (Sonnenschein et al., 1998; Satoh et al, 2001; Ralph et al., 2003; Kumar et al., 2008a and 2008b).

Quantification of the identified chemicals demonstrated that in an average their concentration ranges between 2-4.5 μg/l in the samples tested by us. There could be variations in the concentration of these chemicals from effluent to effluent which could be attributed to several factors like, type of process used in leather manufactures, kind of treatment processes used in the in various WWTP, living life style of the population close to WWTP, extent of industrialization of an area and so on (Chatterjee et al., 2008; Kumar et al., 2008a, 2008b).

Thus, the present physicochemical characterization led to the identification of the four molecules each in the WWTP outlet and leather industry effluents. Further there may be still other chemicals in the both the WWTP and leather industry effluentsse samples which are not identified by the present physicochemical characterization and may add into the androgenic potential of the test samples. Hence, it can be argued from the above discussion that the androgenic potential of the WWTP inlet and outlet water samples and leather industry effluent is the outcome of a number of contaminating chemicals present in them. This combined effect of many chemicals together is known as combinational effect of EDC (Nellemann et al., 2003; Birkhoj et al., 2004; Hass et al., 2007) and depicts to the fact that these chemicals may exert an altogether different effect when they are present in the form of a mixture as compared to their exclusive effects.

CHAPTER - 6

CHAPTER 6: *IN-VIVO* AND *IN-VITRO* CHARACTERIZATION OF HCB AS EDC

6.1 Introduction

The preceding sections of the thesis dealt with exploring the detailed *in vivo* molecular mechanism of action of few potent endocrine disrupting samples ranging from pure chemicals to crude non-polar extracts viz. sewage water and leather industry effluents. This was followed by identification of some of the contaminating molecules responsible for endocrine disrupting nature of crude non-polar extracts (sewage water and leather industry effluents). We identified four molecules each in sewage outlet and leather industry effluents out of which two were found to be common in both of them viz. hexachlrobenzene (HCB), a commonly used insecticide and nonylphenol, a breakdown compound of different synthetic products. However, the *in vivo* characterization of crude extracts of sewage and leather industry effluents although demonstrated potential androgenic activities yet it could not provide the explicit information regarding mechanism of endocrine disrupting action of the individual molecules involved. Hence, once these chemicals were identified as discussed in earlier chapter, attempts were made to explore the detailed mechanism of action of one of the common contaminating molecules, HCB, identified in both the sewage outlet and leather industry effluents, using a combination of *in vivo* and *in vitro* methods. NP was not included in this thesis since a large number of reports exist on endocrine disruption activity of nonylphenol (NP). On Other hand there are very few reports which may suggest clearly the endocrine disrupting nature of HCB.

There are several recent reports from different parts of world suggesting the presence of HCB in various sites like sewage water, river water, marine water, wetlands and so on (Sapozhnikova et al., 2005; Yuan et al., 2006; Almeida et al., 2007; Xue et al., 2005, 2006, 2008). HCB originally was introduced in the 1940s as a seed grain antifungal. Although its use was discontinued by 1975 in USA but it continues to be produced in large quantities as a byproduct in the manufacture of chlorinated solvents and also exists as an impurity of several pesticides (Tobin, 1986). Thus, HCB continues to pose a risk to human health due to its continued entry into the environment, resistance to degradation, and propensity to bioaccumulate. Several reports demonstrated that HCB functions as a potent endocrine disruptor in regard to estrogen cross talk (Foster et al., 1992; Foster et al., 1995; Alvarez et al., 2000; Munoz-de-Toro et al., 2006; Lelli et al., 2007; Li et al., 2008) but there are very limited reports explaining its (anti)androgenic action (Ralph et al., 2003).

This section of the thesis starts with exploring the (anti)androgenic effects of HCB in rats at three dosage levels (1, 2and 4 mg/kg/day) and then culminates with exploring the *in vitro* molecular mechanism of androgenic action of HCB in primary culture of Leydig cells at six different concentrations (0.001 to 10 μM).

6.2 Results

6.2.1 (Anti)androgenic characterization of HCB: in vivo

6.2.1.1 Sex accessory tissue weight (Hershberger Assay)

In castrated rats at the lowest dose of HCB (1 mg/kg/day), there was no significant change in the weight of SATs. However, at a dose of 2 and 4 mg/kg/day of HCB, a statistically significant increase in the weight of SATs were observed in all treated groups

as compared to the reference vehicle control ($p<0.05$) (Table 22). The significant increase in absolute SATs weight could be attributed to the androgenic effects induced by HCB in the treated rats. A similar pattern of dose dependent increase in the weights of SATs and testis was observed in intact rats in response to the HCB treatments (Table 23).

Table 22. Effects of HCB on weights of SATs from castrated rats given 20 consecutive daily treatments

	Ventral prostate (mg)	Glans penis (mg)	Cowper's gland (mg)	Seminal vesicle (mg)	Vas deference (mg)
Control	12.0 ±0.46	165 ±3.2	5.5 ±0.23	49±0.32	70±0.64
1 mg/Kg/day	12.09±0.72	165.8 ±3.85	5.7±0.28	49.8±0.43	69.6±1.21
2 mg/Kg/day	22.00±0.56*	212±0.32*	8.9±0.42*	89.25±1.56*	110.40±0.42*
4 mg/Kg/day	31.45±0.88*	259.45±3.9*	12.2±0.34*	111.43±0.45*	141.67±0.46*

(Each value denotes mean ± S.E.M. of six animals)
*significantly different from vehicle treated control group at $p<0.05$ and $p<0.01$ level

Table 23. Effects of HCB on the weights of testis and SATs from intact rats given 20 consecutive daily treatments.

	Testis (mg)	Ventral prostate (mg)	Glans penis (mg)	Cowper's gland (mg)	Seminal vesicle (mg)	Vas deference (mg)
Control	3561±32.23	41±0.63	378±3.67	14.9±0.78	159±2.57	198±1.23
1 mg/kg/day	3562±26.36	41±0.98	376±1.56	15.0±0.69	161±2.11	192±1.65
2 mg/kg/day	5891±21.34*	53±2.4*	501±3.06*	21±0.42*	229±3.78*	279±2.74*
4 mg/kg/day	7467±38.34*	72±0.80*	632±4.76*	25±0.65*	301±5.11*	412±4.56*

(Each value denotes mean ± S.E.M. of six animals), BW- body weight
* Significantly different from vehicle control group at $p<0.05$ level

6.2.1.2 Gene expression profile

There was a marked up regulation of mRNA of all the steroidogenic enzymes under study in testis of treated intact male rats as compared to control. There were almost 2-2.5 folds up regulation of P450scc, 3β-HSD, 17β-HSD, P450C-17 and StAR mRNA respectively while almost 2 folds down regulation of AR transcripts (Fig. 56). The expression of the SRB-1 protein remained unchanged in the treated rats as compared to control.

6.2.1.3 3β-HSD and 17β-HSD levels

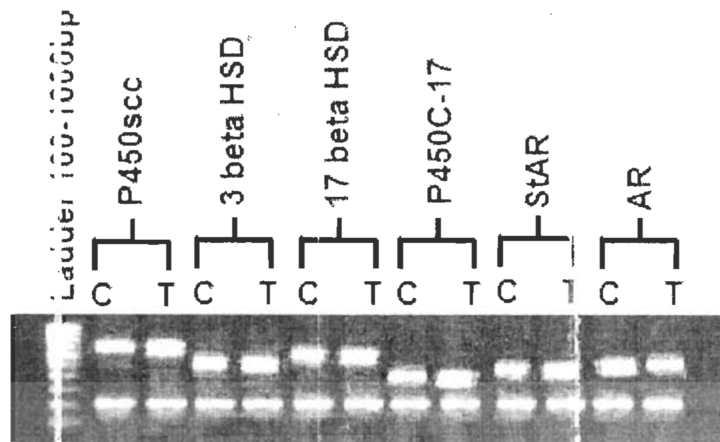
Spectrophotometric enzyme analysis for 3β-HSD and 17β-HSD in testis of intact male rats demonstrated that although HCB at a dosage level of 1.0 mg/kg/day could not induce any additional enzyme activities, yet a dose of 2.0 mg/kg/day resulted in significant up regulation of enzyme activities (Fig 57) ($p<0.05$). This was further elevated dramatically by the treatment with a dose of 4 mg/kg/day.

6.2.1.4 Testicular StAR and AR protein immunoblot analysis

4 mg/kg/day HCB treated groups showed increased translation of StAR and 3 β-HSD and while a markedly decreased translation of AR protein as compared to control (vehicle treated animals) and this increase was statistically significant ($p<0.05$) (Fig 58). The uniform band intensities of β-actin in all the wells indicated equal gel loading.

6.2.1.5 Serum Hormone levels

Estimation of serum testosterone levels in intact male rats treated with HCB at doses of 2 and 4 mg/kg/day showed a significant increase in serum testosterone levels. However, the lowest concentration of HCB (1 mg/kg/day) did not show any significant response which could be attributed to extremely low levels of contaminants at that dilution (Table 24). As expected the levels of serum LH and FSH was also decreased by a value of



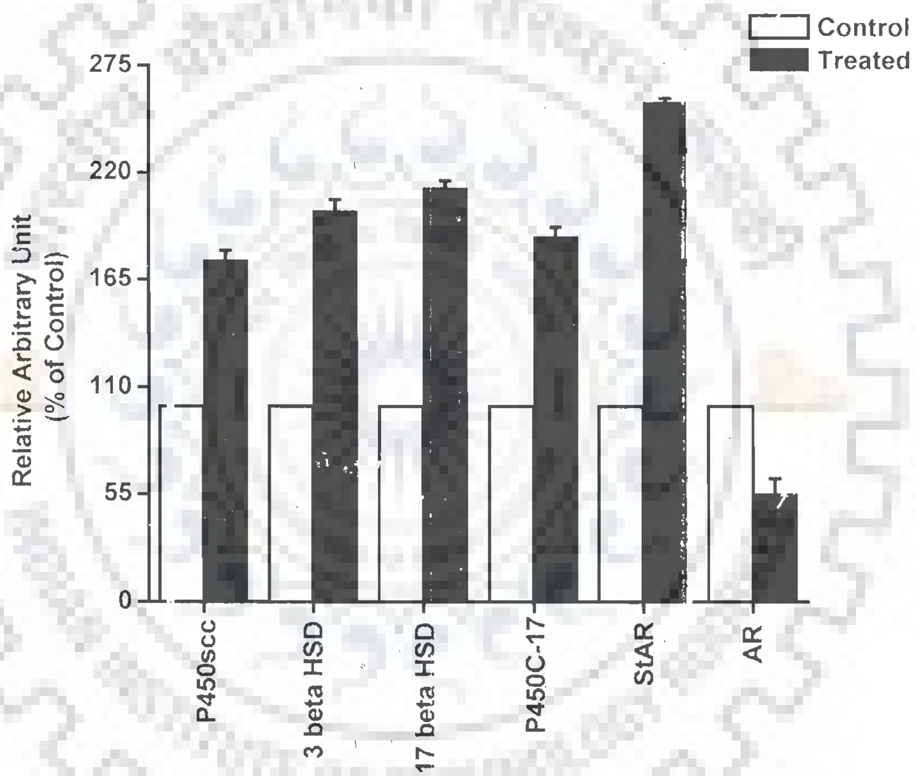


Figure 56. RT-PCR analysis of testicular mRNA expression of P450scc, 3β-HSD, 17β-HSD, cytochrome P450C-17, StAR and AR genes in control and HCB (4.0 mg/kg/day) treated rats (A). Densitometric analysis against the internal control (GAPDH) (B). The values are mean ± S.E.M. of eight RT-PCR reactions for each group tested. C, vehicle treated: T. HCB treated animal groups.

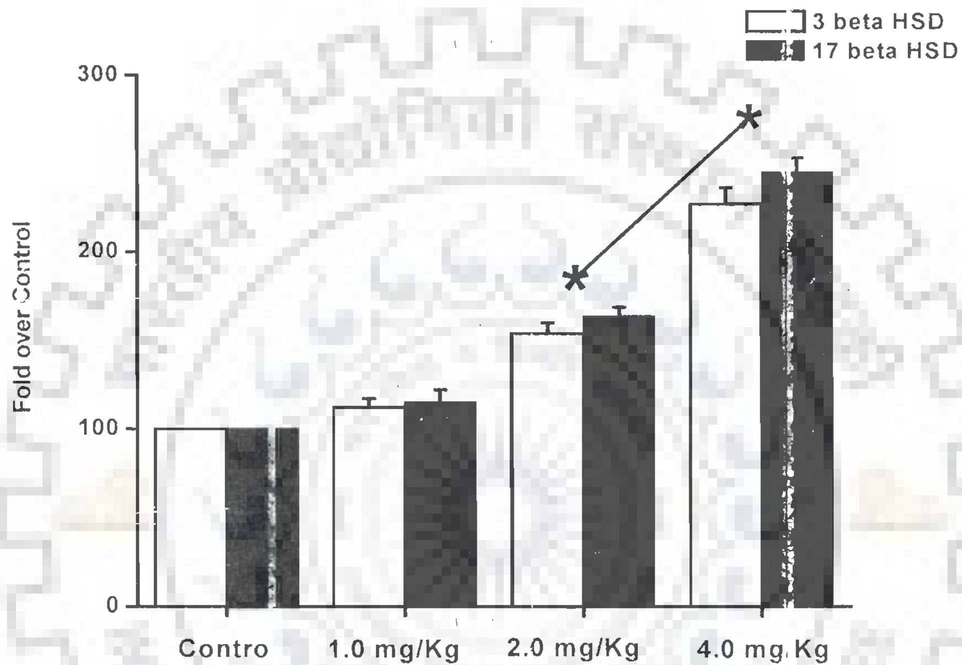


Figure 57. Effects HCB treatment on testicular levels of 3β-HSD and 17β-HSD enzyme activity *in vitro*. The results are expressed as fold increase of enzyme activity over vehicle treated groups. Data are mean±S.E.M.; n = 5. * indicates significant level of differences as compared to vehicle treated control ($p<0.05$).

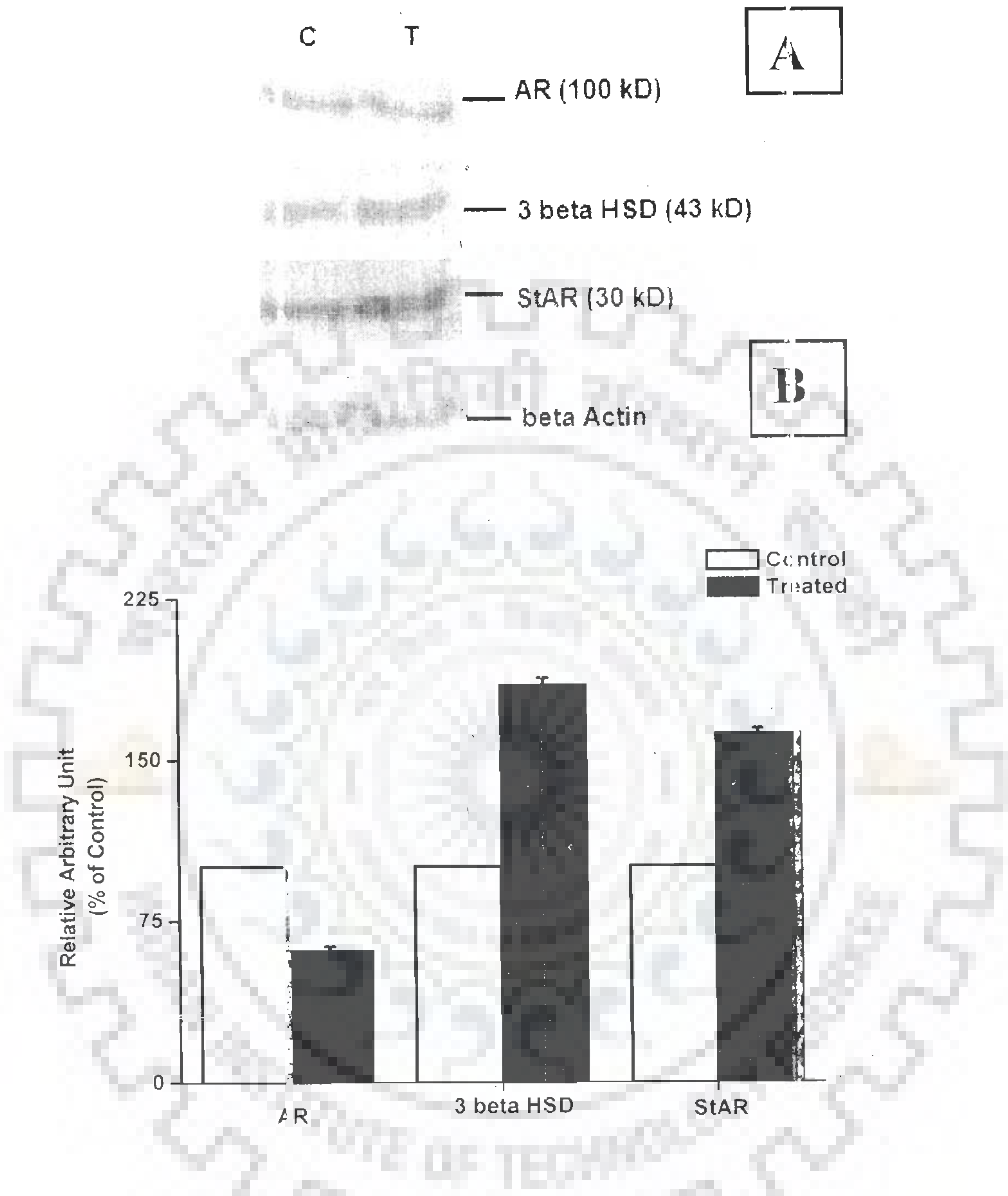


Figure 58. Western blot analysis of testicular 3β-HSD enzyme, StAR, and AR protein in response to HCB treatment (4.0 mg/kg/day) (A). Denestometric analysis against the internal control (β-actin) (B). The values are mean ± S.E.M. of three separate experiments C, control; T, HCB treated.

around 25-40% in animals treated with HCB at a dose 2 and 4 mg/kg/day ($p<0.05$) (Table 24). Decrease in the levels of LH and FSH could be attributed to the initiation of feed back mechanism by the increased serum androgen levels.

Table 24. Dose dependent changes in serum levels of LH, FSH and testosterone from intact rats given 20 consecutive daily treatments of HCB of indicated dose

	LH (ng/ml)	FSH (ng/ml)	Testosterone (ng/ml)
Control	0.91 ± 0.15	6.77 ± 2.1	4.96 ± 0.55
1 mg/kg/day	0.90 ± 0.07	6.98 ± 1.98	5.10 ± 0.51
2 mg/kg/day	0.72 ± 0.08*	5.79 ± 1.68*	6.34± 0.77*
4 mg/kg/day	0.51 ± 0.04*	4.68 ± 1.23*	7.34± 0.89*

(Each value denotes mean ± S.E.M. of six animals)
Significantly different from vehicle control group at $p<0.05$ level

6.2.2 (Anti) androgenic characterization of HCB: in vitro

6.2.2.1 Leydig cell testosterone metabolism

HCB greatly enhanced both basal as well as LH stimulated testosterone production in Leydig cells in a dose dependent manner (Fig 59). At a concentration of 1 μM it resulted in about 50% stimulation of LH induced testosterone production which did not show any further significant up regulation with increase in the concentration of HCB ($p<0.05$) (Fig 59) .

6.2.22 Gene Expression Analysis

The semi quantitative RT-PCR analysis of mRNA, extracted from Leydig cells treated with increasing concentrations of HCB (0.001, 0.01, 0.1, 1.0 and 10 μM) in the presence of 100 ng/ml of LH, demonstrated that the transcription of P450scc, 3β-HSD, 17β- HSD and StAR increased dramatically in a dose dependent manner as compared to only LH treatment (Fig. 60). However, at the lowest concentration of HCB treatment (0.001 μM) no significant change in transcription was observed for all the four genes tested. Further,

the HCB induced response stabilized at a concentration of 1μM without further change in the expression of the genes at higher concentrations (Fig 60).

6.2.2.3 Steroidogenic enzymes activities

Incubation of Leydig cells with increasing concentrations of HCB induced a statistically significant increase in the activities of P450C-17, 3β-HSD, and 17β-HSD enzymes ($p<0.05$) (Fig 61). The increasing doses of HCB resulted in almost 50% increase in enzyme activities for the all the three enzymes up to a concentration of 1 μM after which it leveled off.

6.2.2.4 Immunoblot analysis

The immunoblot analysis of proteins extracted from Leydig cells treated with three different concentrations of HCB (0.01, 0.1, 1.0 μM) demonstrated that there was a dose dependent increase in the expression of 3β-HSD enzymes and StAR proteins induced by only LH (Fig. 62A). Fig 62B demonstrates the denesitometric data of the blot.

6.2.2.5 Adenylyl cyclase activity

As shown in fig. 63, HCB increased the activity of adenyl cyclase enzyme in a dose dependent manner. Although it could not up regulate the enzyme activity at the lowest concentration tested (0.001 μM), it significantly up regulated it right from the 0.01 μM concentration and this trend continued further till 1.0 μM ($p<0.05$).

6.2.2. 6 Leydig cell cAMP production

The incubation of Leydig cells with increasing concentrations of HCB resulted in a gradual increase in cAMP production. The increase was 27%, 47%, 51%, 70% and 71% at the concentrations of 0.001, 0.01, 0.1, 1.0 and 10 μM respectively (Fig. 64).

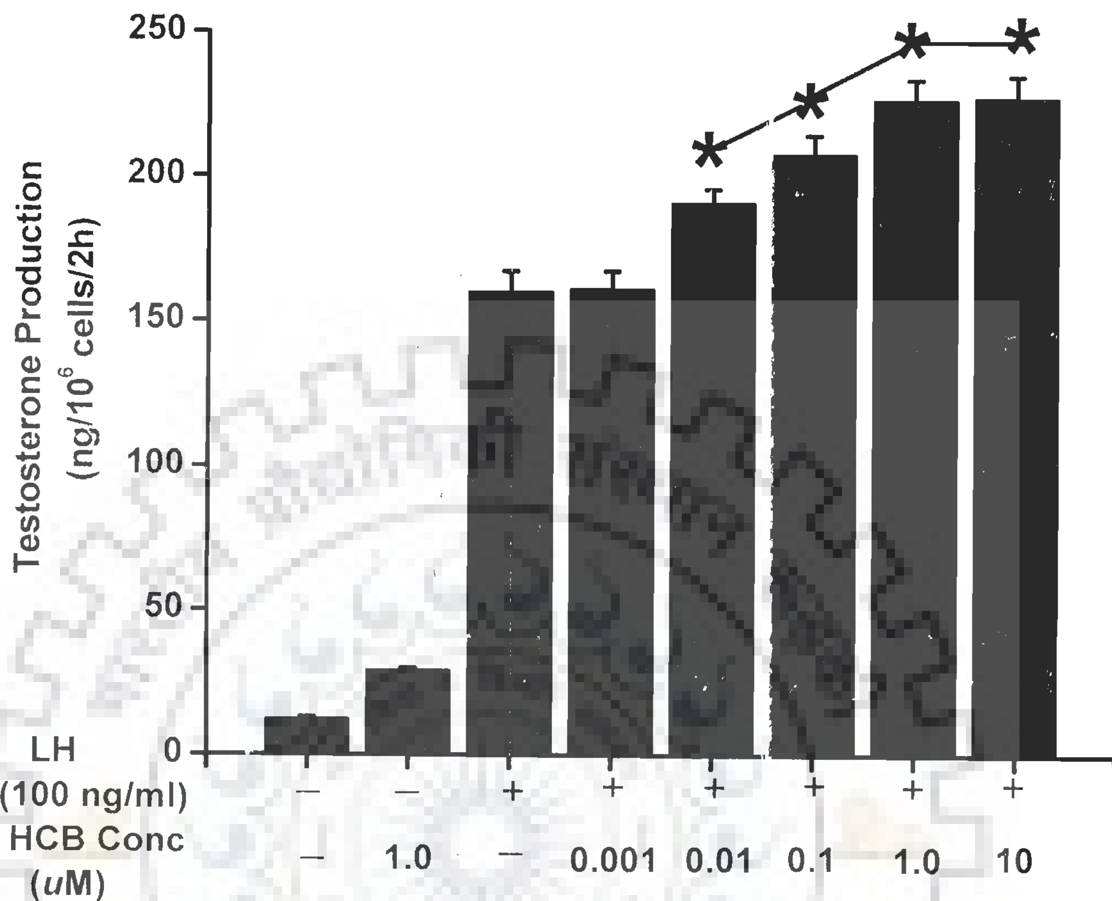


Figure 59. Dose dependent effect of HCB on Leydig cell testosterone production. Leydig cells were treated with different concentrations of HCB with/ without LH for 2 hrs and testosterone level was measured. * indicates significant level of differences as compared to only LH treated group ($p<0.05$).

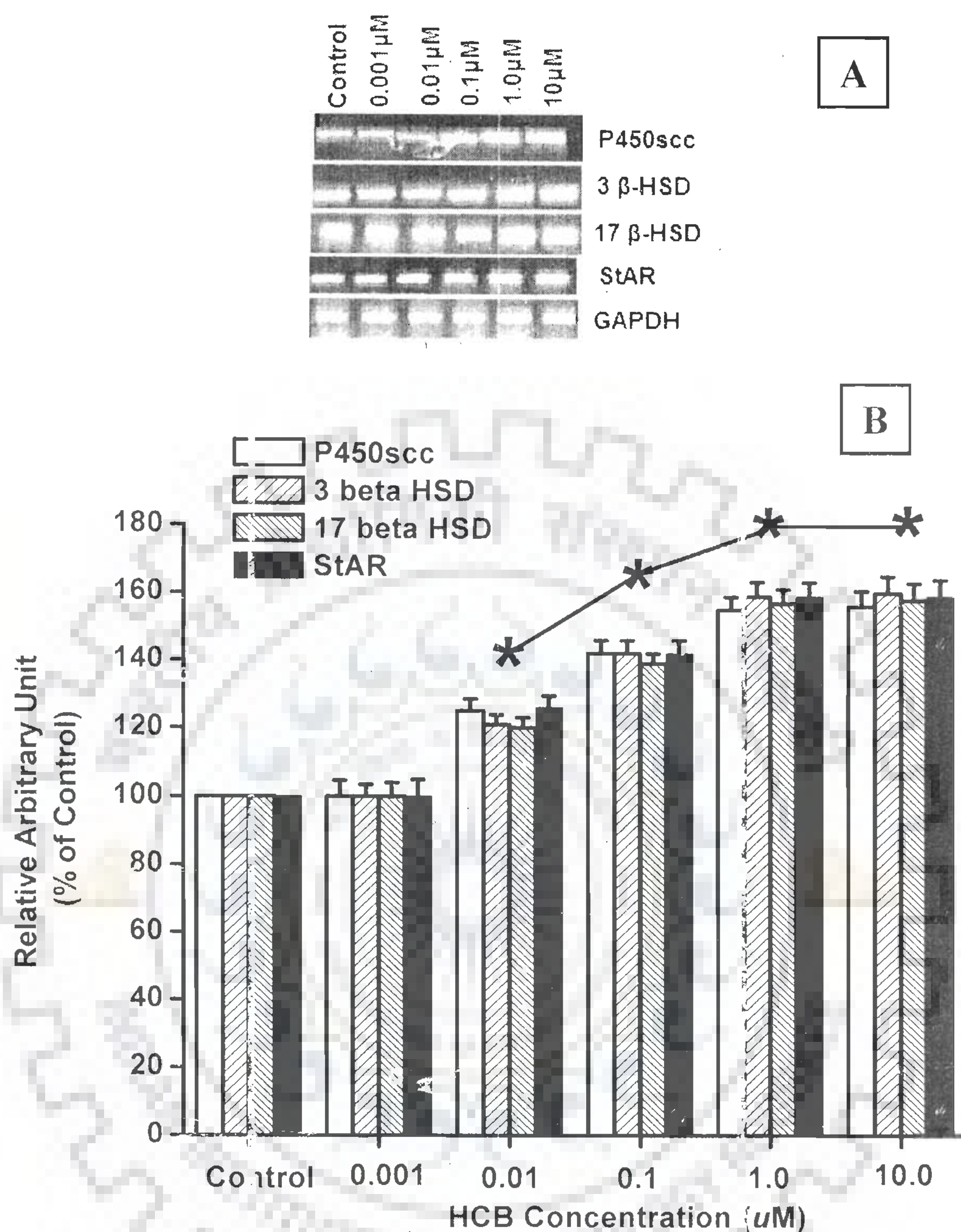


Figure 60. RT-PCR analysis to demonstrate the changes in the mRNA expression of P450scc, 3β-HSD, 17β-HSD and StAR gene in Leydig cells treated with different concentration of HCB in the presence of 100 ng/ml of LH for 2 hrs. The total RNA isolated from Leydig cells were reverse transcribed and cDNA obtained was subjected to PCR (A). The relative intensity of the signals were quantified by densitometer and normalized against the internal control (GAPDH) (B). Control indicates Leydig cells treated with only LH. *Significant level of difference as compared to control group ($p<0.05$).

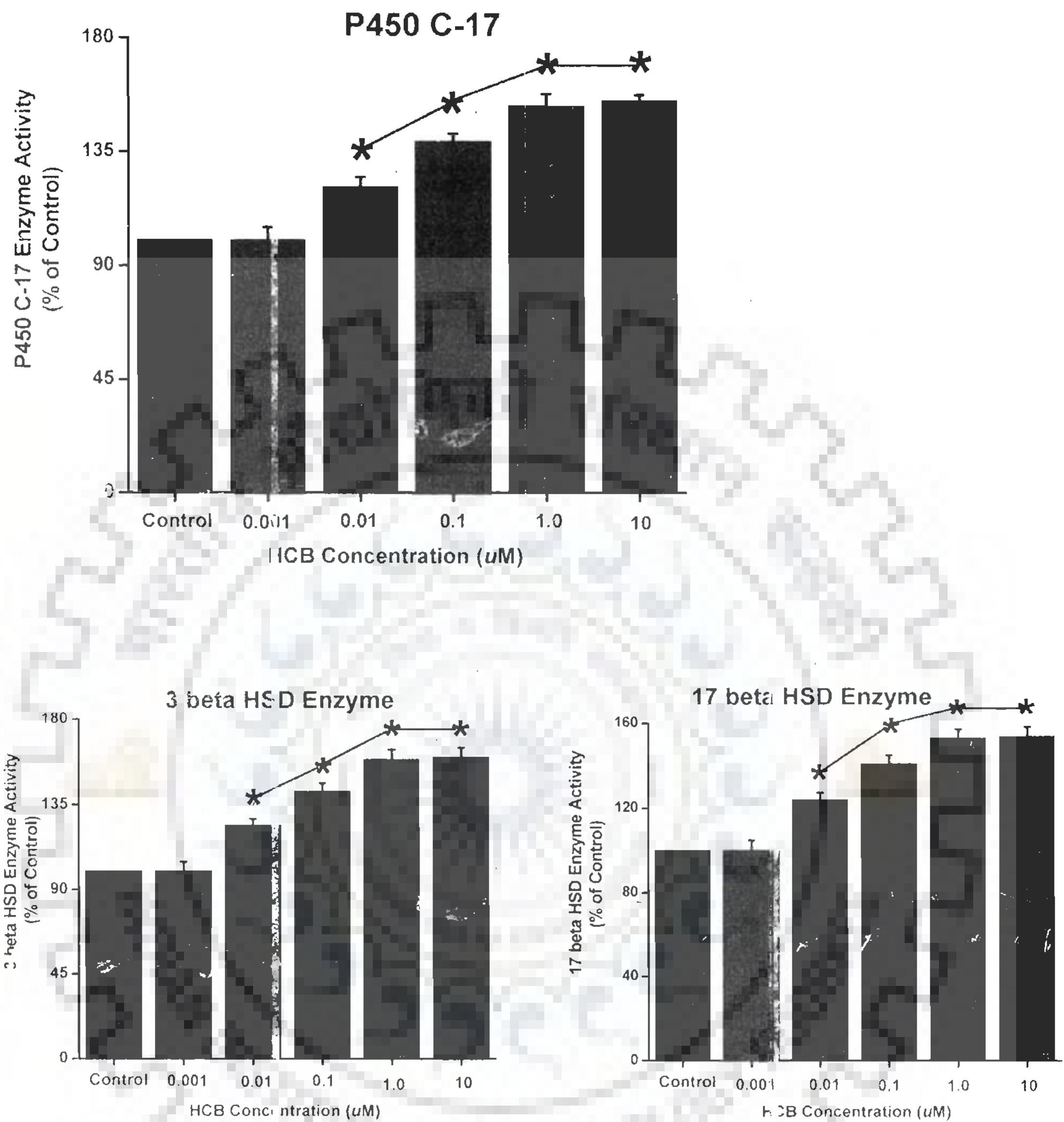


Figure 61. Effects of HCB on activity of P450C-17 (A), 3β-HSD (B) and 17β-HSD (C) enzyme activity *in vitro*. The results are expressed as percent decrease of enzyme activity over control group (treated with only LH) which was given a value of 100. Data are mean ± SEM of four enzymatic reactions of each concentration tested for all these enzymes. Control indicates treatment of cells with only LH. *Significant level of difference in enzyme levels as compared to control group ($p<0.05$).

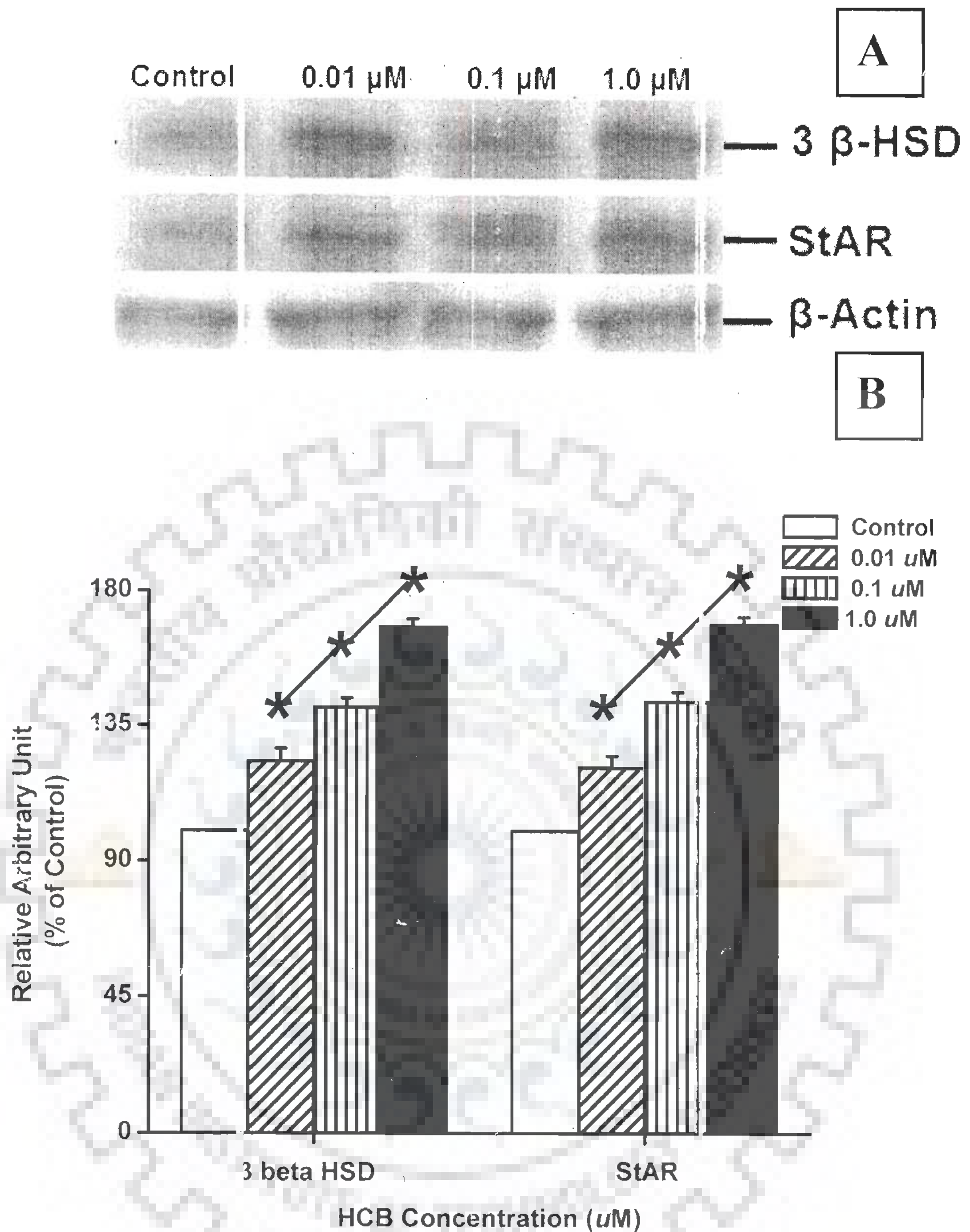


Figure 62. Immunoblot analysis of 3β-HSD and StAR protein from Leydig cells treated with different concentrations of HCB for 2 hours in the presence of 100 ng/ml of LH (A). Densitometeric analysis against the internal control (β-actin) expressed as percent over control (B). Control indicates treatment of cells with only LH. *Significant level of difference in expression patterns as compared to control group ($p<0.05$).

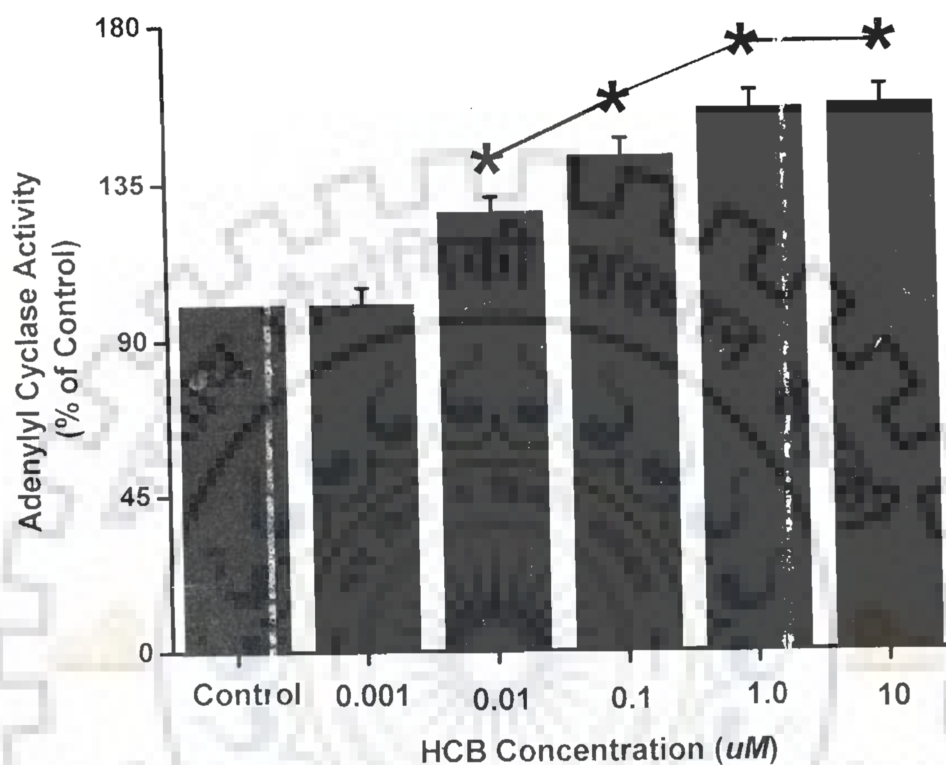


Figure 63. Dose dependent effects of HCB on Leydig cell adenylyl cyclase enzyme activity *in vitro*. Each column represents mean ± SEM (n = 4). Control indicates treatment of cells with only LH. *Significant level of difference in adenylyl cyclase levels as compared to control ($p<0.05$).

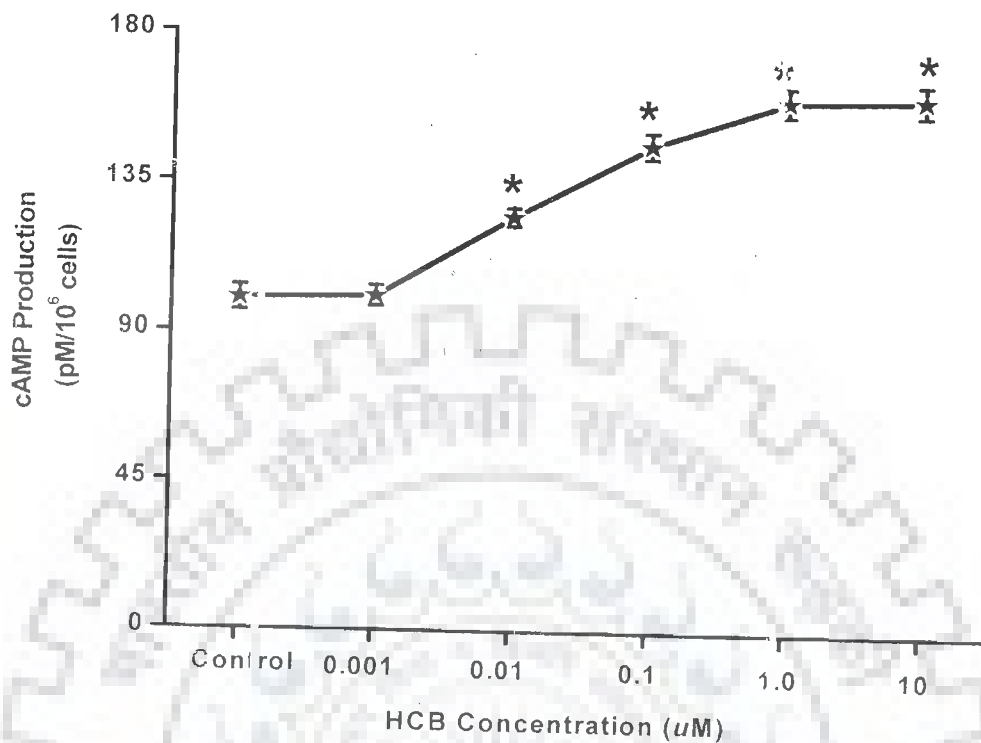


Figure 64. Dose dependent effects of HCB on Leydig cell cAMP production. Each column represents mean ± SEM (n = 4). Control indicates Leydig cells treated with only LH. *Significant level of difference in cAMP levels as compared to control group ($p<0.05$).

6.2.2.7 Effect of adenylyl cyclase activator/ inhibitor on LH induced testosterone production and their cross-talk with HCB

On incubation of LH stimulated Leydig cells with forskolin (adenylyl cyclase activator) in absence or presence of HCB, testosterone production did not show any elevation. This confirms that HCB failed to induce forskolin induced testosterone production.

However, HCB greatly improves SQ22536 (a well known adenylyl cyclase inhibitor) induced decrease in the testosterone production, indicating that it was somewhat masking the effect of SQ22536 (Fig. 65).

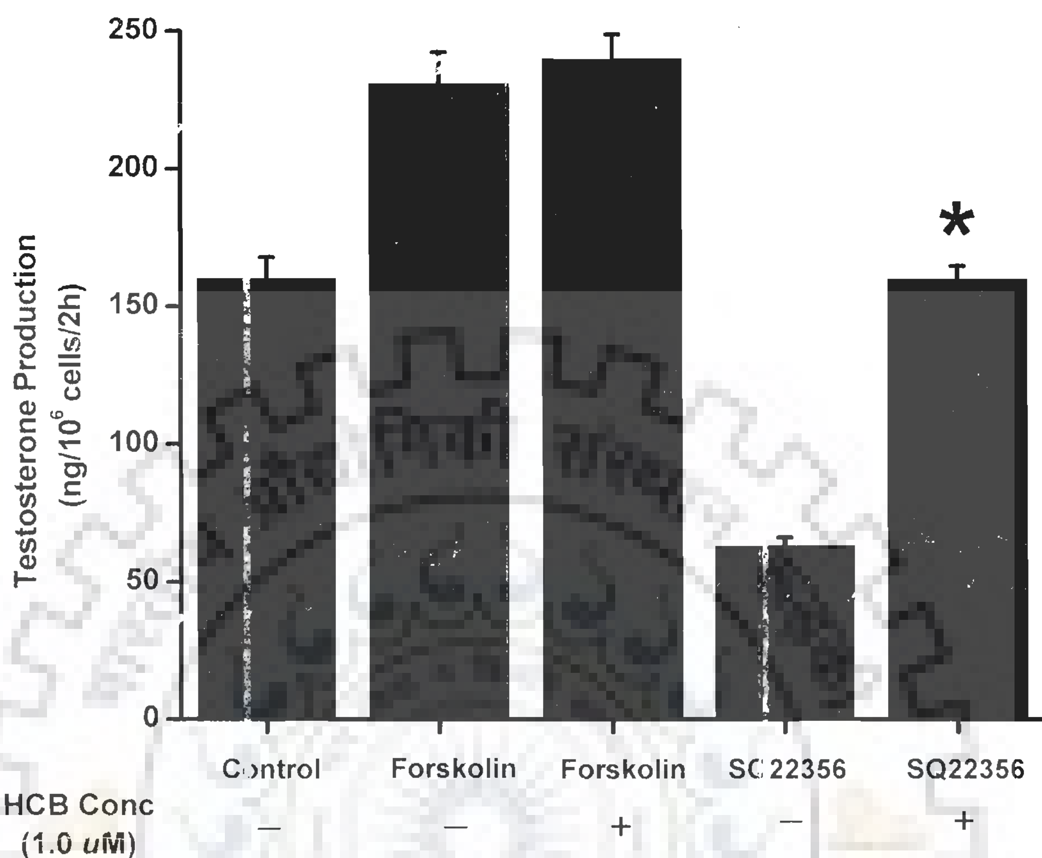


Figure 65. Alteration in LH stimulated testosterone production in Leydig cell treated with adenylyl cyclase activator (forskolin) and adenylyl cyclase inhibitor (SQ22536), for 2 hrs with/without HCB at a concentration of 1.0 μM. Results are mean ± SEM (n = 4). * indicates significant increase in the production of testosterone when Leydig cells were coincubated with HCB and SQ22536 as compared to incubation with SQ22536 alone.

6.2.3 Toxicological evaluation of the HCB

In the case of *in vivo* study, no histopathological malformations in liver, kidney or SATs were observed and also there was no sign of toxicity as indicated by unchanged activities of the enzymes regarded as markers of toxicity (SGPT, SGOT, ALP and ACP) in the treated animals as compared to control. Further, in MTT assay on treatment of the Leydig cells with HCB, there was no significant change in the cellular proliferation even at the highest concentration used in the experiment (data not shown).

6.3 Discussion

In the present study, Hershberger assay demonstrated that HCB treatment resulted in a dose dependent increase in the weights of all the SATs as compared to vehicle treated control groups and SATs of the treated rats were almost of the same size as that of sham operated animals (positive control). This clearly indicated that HCB treatment was clearly inducing androgen agonistic activity in castrated rats. HCB treatment followed same trend of androgen agonistic behavior in intact rats and results displayed an increased weights of SATs in treated rats. This might have been achieved by an increased production of testosterone as indicated by an increased serum testosterone level (Kennel et al., 2005). Further results indicated a decreased serum LH and FSH level in intact rats which might have been achieved by the feedback mechanism induced by an abnormally increased serum testosterone. Results also indicated that treated intact rats displayed completely stimulated steroidogenic machinery as evidenced by an increased up regulation of the prominent steroidogenic genes (P450scc, 3β-HSD, 17-βHSD, P450C-17, StAR) and an increased activity of the two prominent steroidogenic enzymes viz. 3β-HSD and 17-βHSD. These two events (decreased level of gonadotropins and an increased

steroidogenic cascade) are somewhat contradictory as studies indicate that an increased steroidogenesis is supported by an enhanced level of gonadotropins (Bousfield et al., 1994; Fauser, 1999; Muthuvel et al., 2006 ; Murugesan et al., 2007). As described in the earlier chapters of the thesis also, many of the EDC and steroid agonists acts directly on the receptor and steroidogenic enzymes and it is possible that the increase in the expression and activity of these steroidogenic enzymes in this study are independent of the levels of LH and FSH which are known to play a significant role in steroidogenesis in normal instances (Kumar et al., 2008a). However, the exact cross talk between the gonadotropic hormone, the xenobiotcs and testosterone remains an intriguing question which we tried to answer through the in *vitro* studies, preformed by incubating the Leydig cells with varying concentrations of HCB.

In Leydig cells, steroidogenesis is accomplished in several steps most of which are dependent on appropriate levels of cAMP (Beavo and Brunton, 2002, Lin et al., 2001). cAMP is produced from ATP by the action of enzyme adenylyl cyclase, hence, a proper functioning of the enzyme adenylyl cyclase is crucial for maintaining a balanced steroidogenesis. In active steroidogenic cells, the expression and activation of StAR is maintained by cAMP modulated PKA under maximal stimulation of LH (Andrew et al., 2007). The present study demonstrated that HCB treatment affected both the foresaid events resulting in an increased cAMP level followed by an increased expression as well as an enhanced level of StAR protein in treated Leydig cells. Further, the increase was found to be dose dependent. This increased availability of cAMP could be attributed to two factors, either HCB treatment was enhancing the production of ATP which was then stimulating the activity of adenylyl cyclase enzyme leading to the production of excess

cAMP, or HCB was directly interacting with the activity of adenylyl cyclase itself. However our results ruled out the first possibility as HCB treatment was not affecting the ATP generating system since ATP level was same in both treated and untreated group of Leydig cells (data not shown). Thus the increased activity of the adenylyl cyclase enzyme might have been achieved by direct interaction of the test chemical with this enzyme.

Once StAR protein transports cholesterol to the inner mitochondrial membrane, expression and availability of P450scc enzyme is one more regulating step of steroidogenesis (Miller, 1988; Omura and Morohashi, 1995). In our study, results indicated a significant increase in level of P450scc enzyme in both *in vitro* and *in vivo* studies and this increase was demonstrated to be dose dependent through *in vitro* study. In addition, another factor which might have contributed to this event is the stimulated cAMP level, since it is also responsible for the phosphorylation of the components of side chain cleavage system (Fauser, 1996) in spite of a decreased level of LH in case of *in vivo* study . This study also demonstrated a dose dependent increase in the activity of the other steroidogenic enzymes viz. P450C-17, 3β-HSD and 17β-HSD in both *in vivo* and in *vitro* studies. Besides transcriptional profiles of 3β-HSD and 17β-HSD were also found to be significantly increased (in a dose dependent manner) both *in vitro* and *in vivo*. The probable explanation for these findings could be attributed to the increased delivery of the intermediates resulting in an up regulated expression of all enzymes of steroidogenic cascades since it has been known that the precursors substrates also plays an important role in these enzyme biosynthesis (Sanderson and Vanden Berg, 2003).

Another interesting finding of the study was a dose dependent increase in the synthesis of testosterone by HCB. This might have been achieved by an increased

expression of StAR protein as well as an increased expression and activity of P450scc, P450C-17, 3β-HSD and 17β-HSD enzymes since a balanced status of all of them is required for an optimum testosterone synthesis in active steroidogenic cells.

Further, results showed that if the cells are treated with HCB in presence of forskolin (adenylyl cyclase activator), HCB treatment failed to further enhance forskolin induced testosterone synthesis even at highest effective HCB concentration (1 μM). The probable reason for this could be attributed to the fact that forskolin and HCB do not have any additive effects. However HCB was able to revert SQ22536 depressed testosterone synthesis since in this case it was able to mask the depressant effect of SQ22536. All these data indicated that the main factor responsible for HCB induced increased synthesis of testosterone is through cAMP stimulation which is in turn is caused by an increased activity of adenylyl cyclase enzyme.

Thus, our results demonstrated that HCB is acting as a potent androgenic endocrine disruptor in both intact animals and in isolated Leydig cells and it acts by interacting with one of the key enzyme (adenylyl cyclase) indirectly related with steroidogenesis (Fig 66). This interaction results in a perturbed performance of the enzyme adenylyl cyclase leading to an abnormally enhanced level of cAMP. This abnormal level of cAMP boosts the entire steroidogenic pathway leading to an unusual testosterone level in the treated cells and treated intact animals concomitant with downfall of gonadotropins. This particular study paves the way for understanding the crosstalk of various EDCs with varied factors of steroidogensis including adenylyl cyclase.

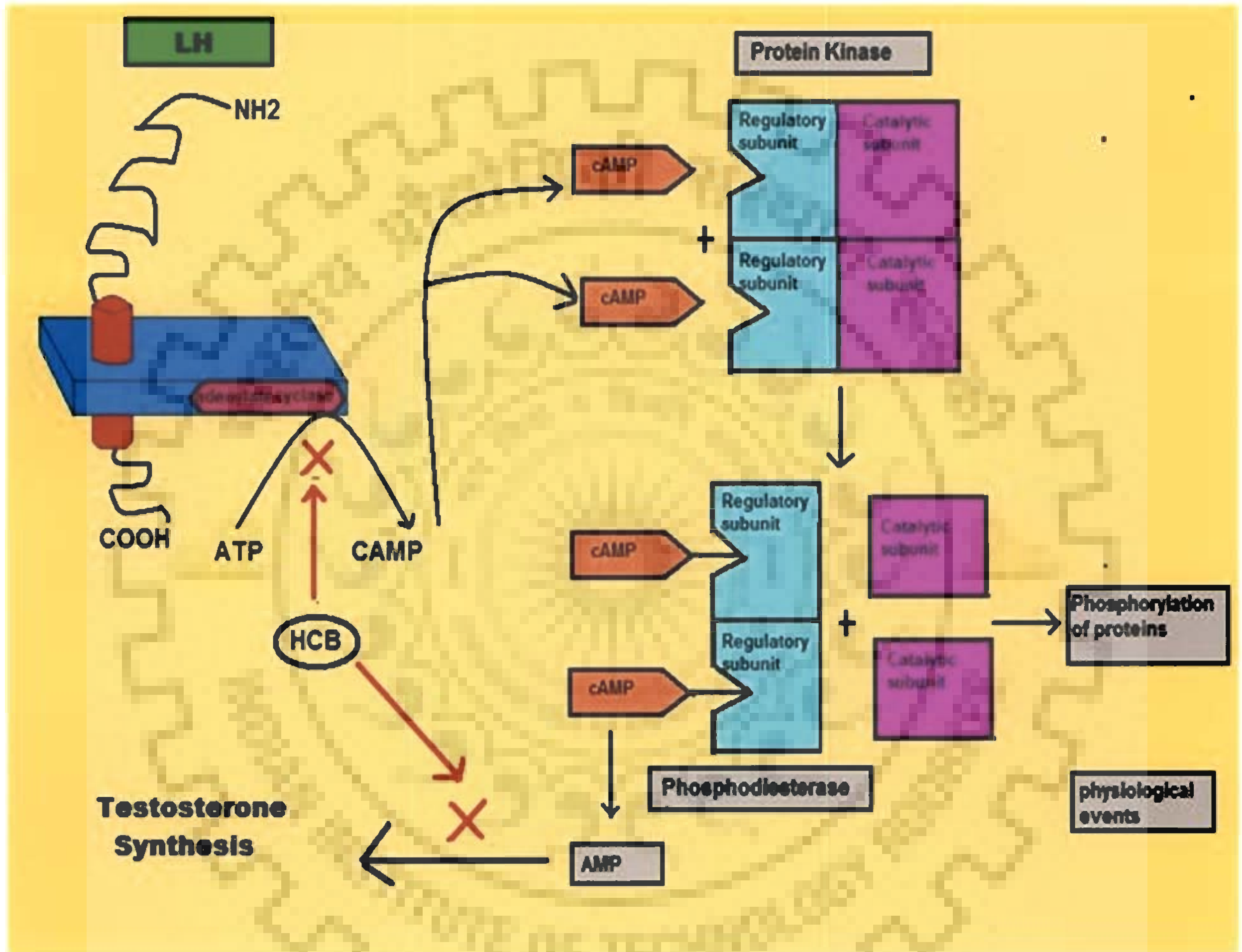
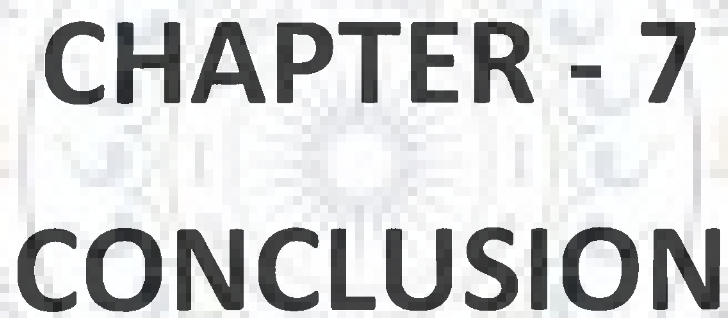


Figure 66- Representative diagram showing the action of HCB on adenylyl cyclase enzyme leading to an increased synthesis of testosterone.



CHAPTER - 7

CONCLUSION

Chapter 7: Conclusion

Endocrine disruptors are named so because of their ability to modulate or deregulate the endogenous endocrine system. In humans, potential adverse health effects include (1) infertility (fecundity, reduced semen quality, endometriosis), (2) abnormal prenatal and childhood development (spontaneous abortions, male reproductive tract abnormalities, and other birth defects, altered sex ratios, precocious puberty, and (3) reproductive cancers of prostate, breast, ovaries, endometrium, or testes (National Research Council, 1999; U.S. EPA, 1997; EM-COM, 2002; IPCS, 2002; Phillips et al., 2003; Phillips et al., 2008; Kumar et al., 2008a, 2008b). Although the risk posed by endocrine disruptor represents an important area of environmental health, yet it represents a considerable controversy regarding the nomenclature of the term "endocrine disruptor". An increasing number of chemical compounds from the environment have been identified as endocrine disruptor utilizing *in vitro* and *in vivo* bioassays. These includes pesticides, industrial chemicals, waste water effluents and pharmaceuticals that behave like ligands for the estrogen, androgen, progesterone or aryl hydrocarbon receptors producing effects that mimic the natural hormone (Roy et al., 2005).

The present thesis describes the endocrine disrupting action of some synthetic and environmental chemicals using *in vivo* and *in vitro* models. The chemicals or environmental samples, used for studying in this thesis, were selected keeping in consideration to cover a wide range of sources that the human populations are exposed to. For example, some of the common drugs used in day to day life like ibuprofen and antibiotics (tetracycline) are sold many a times over the counter even without the prescription of clinicians (at least in India). This has been further supported by heavy

accumulation of these and other drugs and their metabolites in surface waters of various countries (Kasprzyk-Hordern et al., 2008). Similarly, TCS one of the chemical tested by us, is used in various cosmetics as preservatives regularly. The common use of these chemicals necessitates that they should be checked for their potential threats as endocrine disruptors. Figure 67 depicts the summary of the actions of different test chemicals (based on the results of this thesis) as potential inductor of endocrine disruption by regulating androgen and estrogen production.

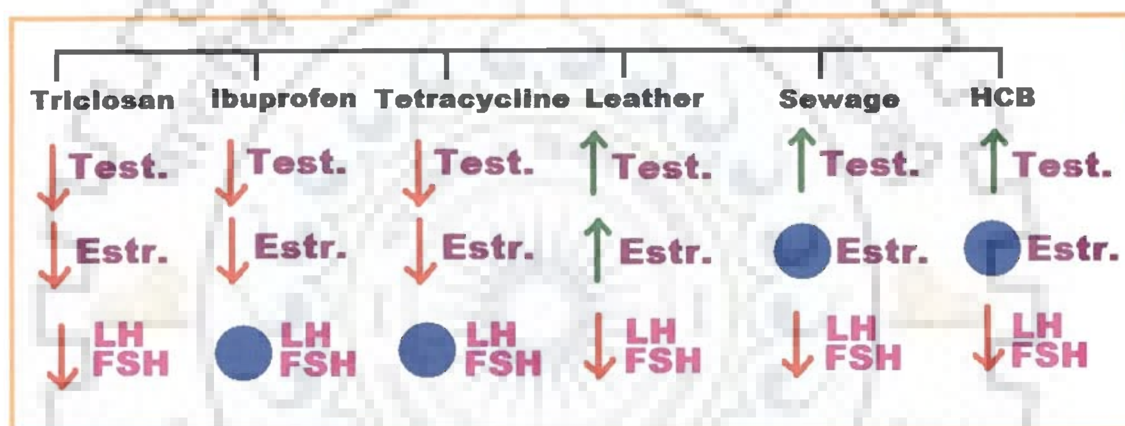


Figure 67. Summary of the ultimate effects of test samples used in this thesis on the estrogen and androgen production. Figure also shows the changes in the serum level of gonadotropins as a result of the treatment with the test samples. Red arrow, decreased level; green arrow, increased level and blue dot, no change, as compared to control animals. Test., Testosterone; Estr., Estrogen.

Further, one of the biggest challenges in endocrine disruptor research is to distinctly designate a compound as EDC because the effects shown by these chemicals are largely dependent on several factors and one of the most prominent of them is the dose used in the assay system. Same chemical may or may not show any response depending on the dose being used. For example, since the discovery that pharmaceutical usage leads to the occurrence of these substances in natural waters, numerous research

activities have focused on revealing the extent and consequences of these increasingly emergent findings (Ingerslev et al., 2003). The occurrence of these substances in the environment is linked to usage in either a veterinary or human context. A number of papers regarding general eco toxicology show that these substances generally occur at nontoxic concentration levels (Stuer-Lauridsen et al., 2000; Ingerslev et al., 2003). However, due to their continuous introduction into the environment and synergic effects through combined parallel action, even compounds of a low persistence might cause unwanted effects in the environment (Fent et al., 2006; Kaspryzk-Hordern et al., 2008). In this context a more interesting issue is thedetailed biological analysis which may address the questions related to endocrine disrupting activities of various drug additives, personal care components and pharmaceutics like antibiotics, analgesics, anti-inflamatorics. Although there are several reports on the presence of varying amount of pharmaceuticals in aquatic environment but only few studies have actually addressed the impact of these drugs on its users. Pharmaceuticals are administered in many different formulations and often in combination with other chemicals that are included for purposes such as preservation, or as vehicles to aid administration/uptake in tablets, or for adjusting the ion strength and pH in solutions (Ingerslev et al., 2003). Many of these agents are also used in personal care products. Apparently, there are almost no reports on nonhormonal drugs as endocrine disruptor except few recent studies (Gravel and Vijayan, 2006; Sibonga et al., 1998; Ingerslev et al., 2003; Chen et al., 2007). This is not surprising, considering the thorough approval procedures that exists before the drug is being marketed. Naturally it is important to decide the correct dosage and treatment schemes to prove the endocrine disrupting nature of these common chemicals. In our

study the doses used for the pure chemicals (phramaceutics, personal care products or environmental chemicals like HCB), were decided mainly based on three factors: firstly already existing reports, secondly, LD_{50} values and thirdly, probable daily rate of accumulation of these chemicals within an individual. In our study, we did not measure the serum levels of those administered drugs (ibuprofen, tetracycline, TCS or HCB). However, studies in which nearly identical dose of these chemicals were administered to rats (Morrissey et al., 1986; Kimmel et al., 1992; Richardson et al., 2002) resulted in peak blood levels of some of these chemicals that were almost similar to those reported in subjects receiving a single dose of those drugs (Sibonga et al., 1998). This further confirmed that the endocrine disrupting activities as shown by the test chemicals in the present thesis is closely related to the physiological system and not mere effects of toxicity. Naturally the question remains that in spite of a rigorous approval procedure involving through testing for unwanted side effects including endocrine disruption on numerous levels, how these chemicals show these effects. There can be several arguments in suggesting that pharmaceutical compounds used in normal therapy or personal care may be endocrine disruptor. Firstly, the medicinal substances may be transformed to endocrine active metabolites during long term usages within the individual exposed to them intentionally (as medical therapy) or unintentionally. Secondly, the endocrine system in the target organs may react differently than that of patients where drug was used. This matter still remains an intriguing question to be answered.

Another major source of EDC that was discussed in this thesis was the WWTP and leather industry effluents. The main reason for selecting those two sources of

effluents are that, almost no information was available on the endocrine disrupting contaminants from those two sources in Indian context except one by Senthilkumar et al. (1999) which demonstrated the presence of several potential EDC like PCBs, DDT, hexachlorocyclohexane isomers (HCHs), chlordane compounds (CHLs), and HCB in dolphins, fishes, benthic invertebrates and sediments collected from the river Ganges, one of the major rivers in India. This prompted us to characterize some of the EDC from WWTP effluents of Northern India which is one of the most populated regions of India. On the other hand the leather industry effluents were selected to be tested based on the importance of this industry in Indian economy and also rich source of pollutants released out of this industry. Our data showed that both these effluents contained a complex mixture of various aromatic compounds exerting androgenic and estrogenic activities. Our study also demonstrated that the average levels of androgenic and estrogenic compounds in WWTP effluents were in the range of 100 ng/l and 15 ng/l respectively as determined by *in vitro* assays (data not shown). Earlier several authors showed although not same yet almost similar levels of these contaminants from various WWTP effluents across the world (Kaspryzk-Hordern et al., 2008; Van Der Linden et al., 2008). Whatever marginal variations obtained they could be attributed to the life style of the population of the area under consideration. The androgenic activities found in these studies could be almost completely explained by the presence of some of the known androgens like DHEA, isoandrosterone. Further, hexachlorobenzene, a common pesticide, is also known to exert androgenic activities by its interaction with androgen receptor (Ralph et al., 2003). Taken together, all these chemicals combinedly exerted the androgenic activities both in leather and WWTP effluents. At this point it is difficult to explain the estrogenic

potentiality of these effluents in spite of the fact that none of the potential estrogenic chemicals was identified by GC-MS in our study. One probable reason for this disparity could be their existence in extremely low concentration and the effect as shown by the test chemicals could be by the combined effect of various identified chemicals (including androgenic chemicals) and some unidentified chemicals (Nellemann et al., 2003; Birkhoj et al., 2004). Therefore effort should be made to separate these compounds using approaches like effect directed analysis, to get a more clear picture about the identity of the compounds involved.

When we discuss the mode of action of various chemicals tested by us, they were demonstrated to act at various levels to disrupt the normal endocrine orchestra of the body viz. at enzyme level (3 β-HSD, 17 β-HSD, adenylyl cyclase), at receptor level (ER, AR), at protein level (StAR) (Fig. 68). Although we estimated the levels of androgen and estrogen as end markers but their ultimate effects are further upstream of them. While some chemical (TCS) was found to act at hypothalamic level causing oxidative stress ultimately jeopardizing the whole steroidogenic cascade, others blocked the functions of StAR protein which was found to be independent of gonadotropic hormone levels. There are several reports on various other endocrine disruptors (in addition to tested by us) affecting those two above mentioned target sites (Stocco, 2001; Arukwe, 2005; Muthuvel et al., 2006; Murugesan et al., 2007; Barbara and Cochrum, 2007). Further, WWTP and leather industry effluents acted at various steroidogenic enzyme levels resulting in the up regulation of the androgen biosynthesis irrespective of the levels of LH and FSH (Fig. 68). The LH and FSH independency by some of the endocrine disruptors has already

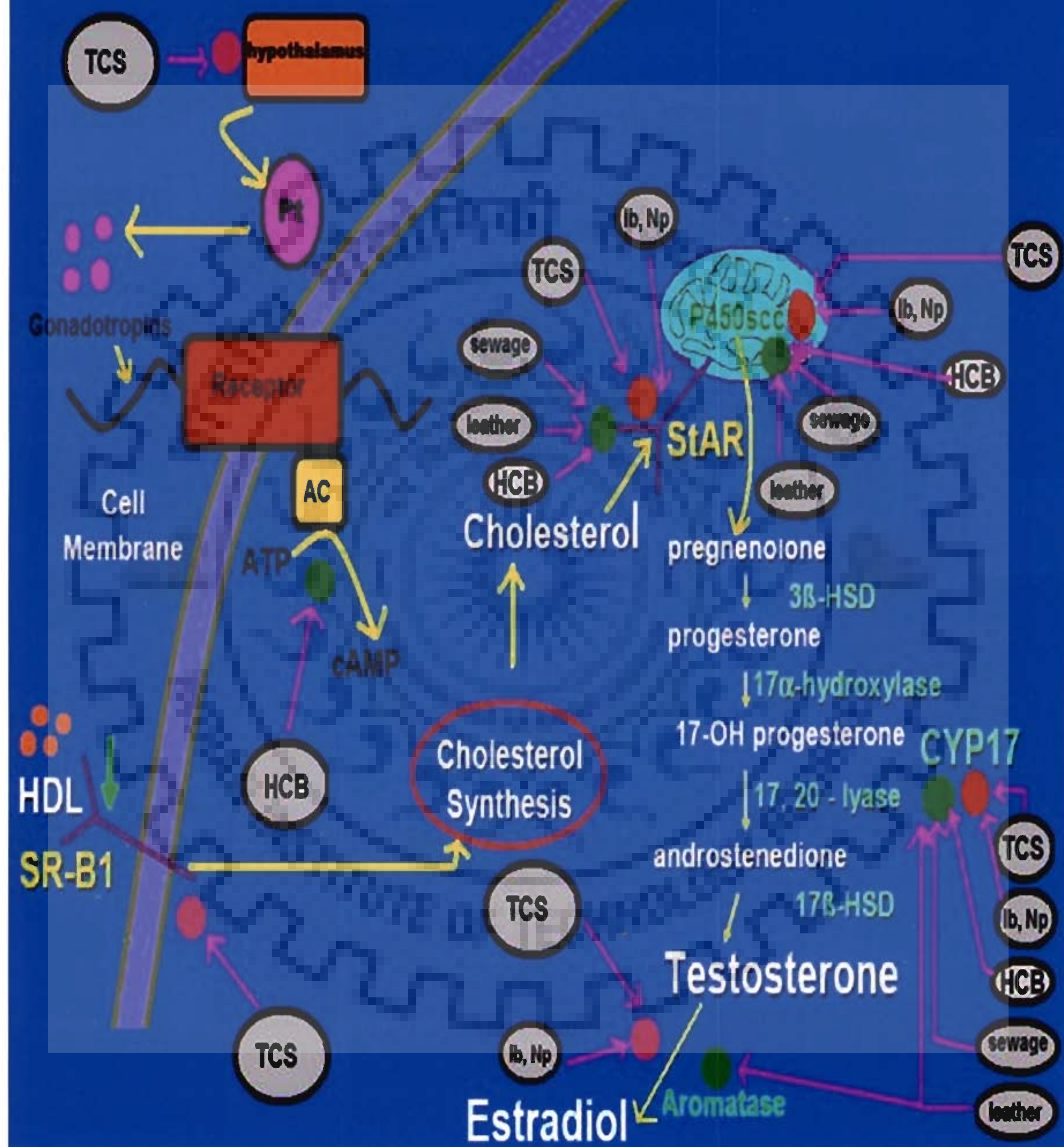


Figure 68. A hypothetical diagram (based on the results of this thesis) representing the various sites of the action of different test samples.

been discussed in earlier sections of this thesis. Besides, although many endocrine disruptors have been reported to demonstrate an inverted-U dose response for FSH and LH secretion (Li et al., 2006), but it was not detected in our study even at the highest concentrations of the effluent treatments, the mechanisms for which is still unclear to us. Another intriguing fact that came out of our study is that majority of the test chemicals demonstrated both (anti)androgenic as well as (anti)estrogenic activities although the general notions of EDC are that most of the estrogenic chemicals are anti-androgenic and vice versa. But in our study, at least in case of leather industry effluents, it was not clear why the same extract behaved as both androgenic and estrogenic. One probable explanation could be the use of extracted water samples which is a complex mixture of various chemicals where, probably, the presence of both agonists and antagonists in the same extract may mask each individual contribution (Urbatzka, et al., 2006; Van der Linden et al., 2008). This makes it important to determine all biological responses of these complex mixtures at various locations, since compounds responsible for the different biological responses observed might have different degradation speeds (Labadie and Budzinski, 2005) or different distribution between water and sediment (Kuster et al., 2004). This was further supported by the fact that some other endocrine disruptors like 4-nonylphenol also demonstrates both estrogenic and androgenic activities (Satoh et al., 2001). Therefore effort should be made to separate these compounds using approaches like effect directed analysis, to get more clear picture about the identity of the compounds involved.

In conclusion, the synthesis of steroid hormones is one of the crucial processes in the endocrine regulation. It consists of sensitivity regulated steps and different endocrine-

disputing chemicals may affect it. The data presented here conclusively provides evidence that the broad range of chemicals as tested by us in the present thesis acts at various target sites for steroid metabolism and thus confirms the potential impact on human androgen axis. These various chemicals can affect hypothalamus, cholesterol biosynthesis and interfere with transcriptional activity of major steroidogenic enzymes and the down stream effects, thus amplifying their potential endocrine-disrupting impact. At this point, it could be speculated that majority of the offending chemicals act through their cognate receptors as has been reported earlier by several authors (Ralph et al., 2003; Chen et al., 2007; Kortner ans Arukwe, 2007). These may either up or down regulate the expression patterns of various steroidogenic factors. In addition they may also activate the LH dependent pathways involving adenylyl cyclase and cAMP as shown by HCB in our study. All these various pathways thus depict a complex mode of action of these EDC which further warrants a serious in depth study of these chemicals at various levels like measuring their effects during critical windows of exposure, dosage of exposure and so on (Phillips et al, 2008). Further, at a high concentration, majority of these chemicals, especially, HCB, androgen agonists (DHEA and isoandrosterone), TCS may result in systemic toxicity as has been demonstrated in our study and some earlier reports also. However, given the complexities in the steroid synthesis pathways and biological activities of hormones, together with unknown biokinetic properties of these EDC for systemic toxicity, further investigations with *in vivo* and *in vitro* experimental models are required to define a clear-cut picture on this aspect of endocrine disruptor research. The holistic approach as presented in this thesis in understanding the mode of action of a diverse variety of EDC using *in vitro* and *in vivo* models and varying doses should

reinforce the concept that environmental xenobiotics or various daily usable chemicals (pharmaceutics, cosmetics and toiletries), though present in low doses, yet may pose a serious threat to human health. Based on these informations and considering the serious impact of these chemicals on human health, further investigations with adequate screening systems and *in vivo* confirmation are urgently needed to fully appreciate the spectrum of these endocrine disrupting properties.

CHAPTER - 8

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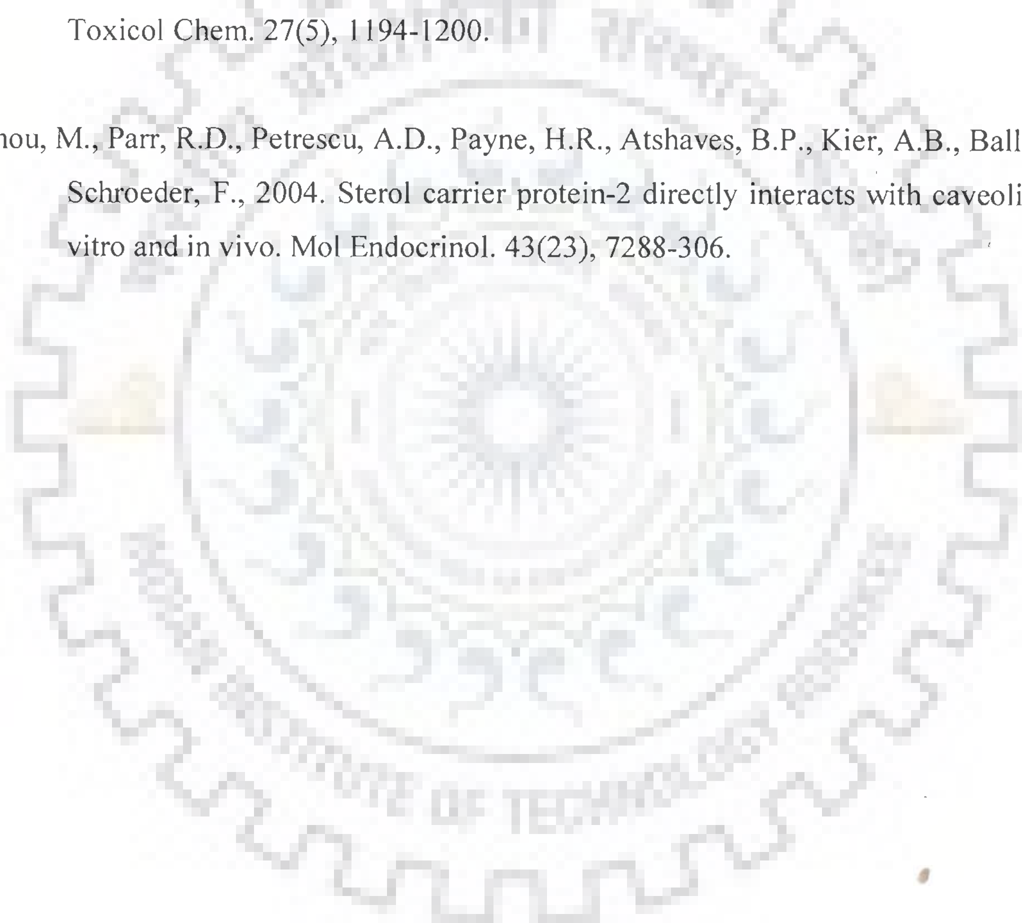

PAPERS PUBLISHED/ COMMUNICATED OUT OF THIS THESIS WORK

1. **Kumar Vikas**, Chakraborty Ajanta, Viswanath Gunda and Roy Partha (2008). Androgenic endocrine disruptors in wastewater treatment plant effluents in India: their influence on reproductive processes and systemic toxicity in male rats. Toxicology and Applied Pharmacology 226(1):60-63.

2. Chatterjee Shamba, **Kumar Vikas**, Majumder Chandrajeet Bala and Roy Partha. (2008) Screening of some anti-progestin endocrine disruptors using a recombinant yeast-based in vitro bioassay. Toxicology i*n vitro* 22:788-798.

3. **Kumar Vikas** and Roy Partha. Assessment of endocrine disrupting activity of leather industry effluents from Northern India in male rats. Journal of Steroid Biochemistry and Molecular Biology (in press, **10.1016**/j.jsbmb.2008.06.005).

4. **Kumar Vikas** and Roy Partha. Triclosan induced cAMP deprivation disrupts in vitro leydig cell androgenesis. Toxicology *In Vitro* (in press).

5. **Kumar Vikas,** Kural Moolraj and Roy Partha. Speramint induced hypothalamic toxicity and testicular anti-androgenesity- altered genes, enzymes and hormones. Food and Chemical toxicology **(under revision).**

6. **Kumar Vikas**, Rana Manish, Parekh Mrinal and Roy Partha 'Naproxen, Ibuprofen and Tetracycline disrupt testicular steroidogenesis in male albino rats: probable mechanism of action'. Life Science **(submitted).**

7. **Kumar Vikas**, Chakraborty Ajanta, Kural Moolraj and Roy Partha. Alteration of testicular steroidogenesis and histopathology of reproductive system in male rats treated with Triclosan. Reproductive Toxicology **(submitted).**

8. **Kumar Vikas** and Roy Partha. Spearmint: Dose dependent depressed steroidogenic cascade in isolated Leydig cells. Toxicology *In Vitro* **(submitted)**.