# CLASTOGENIC AND REPRODUCTIVE EFFECTS OF HEXACONAZOLE IN RATS

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*Thesis submitted in partial fulfilment of the requirements for the degree of*

**DOCTOR OF PHILOSOPHY**
**IN**
**PHARMACOLOGY**

*to the*

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**MADRAS VETERINARY COLLEGE**
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**CHENNAI - 600 007.**

**2001**

*Dedicated*

*to my*

*Beloved Parents*

This is to certify that the thesis entitled **"CLASTOGENIC AND REPRODUCTIVE EFFECTS OF HEXACONAZOLE IN RATS"**, submitted in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** in **Pharmacology** to the Tamil Nadu Veterinary and Animal Sciences University, Chennai, is a record of bonafide research work carried out by **Dr.P.RAVI KUMAR** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journals or magazines.

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(P. RAVIKUMAR)

# Abstract

# ABSTRACT


| | | |
|---|---|---|
| *Title* | : | CLASTOGENIC AND REPRODUCTIVE EFFECTS OF HEXACONAZOLE IN RATS |
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Hexaconazole, an ergosterol biosynthesis inhibiting fungicide, was tested for its clastogenic and reproductive effects in rats. Clastogenicity was studied employing *in vivo* bone marrow chromosomal aberrations and micronucleus assays and *in vitro* lymphocyte cell cultures. Reproductive effects were studied in male and female rats separately.

Hexaconazole at 182, 365 and 730 mg/kg/day in males and 506, 1012 and 2024 mg/kg/day in females in *in vivo* studies and 125, 250 and 500 $\mu$g/ml of total culture in *in vitro* studies did not produce any clastogenic effect.

Male rats that were exposed to the drug at 27.0, 55.0 and 110.0 mg/kg/day *per os*, hexaconazole produced toxic effects at medium and high doses in 30 and 60 days trials. Quality of semen was affected, as was observed by decreased epididymal sperm count and motility and increased percentage of dead and abnormal sperm. There was also testicular atrophy and regression of accessory sex glands. Histologically there were degenerative changes in gonadal cells and hyperplasia of Leydig cells in testis. Hexaconazole also decreased serum testosterone levels at medium and high doses.

Female rats that received the drug at the rate of 27.0, 55.0 and 110.0 mg/kg/day *per os* for 30 days prior to mating and during mating, gestation and lactation (21 days) periods, it was observed that medium and high doses affected the estrous cycle and reproductive indices. The drug also caused atrophy of uterus and vagina and lowered the serum estradiol and progesterone concentrations.

Hexaconazole produced hepatotoxicity as confirmed by the elevated serum transaminases and decreased cholesterol levels along with histopathological lesions.

# *Contents*

# CONTENTS


| Chapter No. | Title | Page No. |
|---|---|---|
| | ACKNOWLEDGEMENT | iii |
| | ABSTRACT | v |
| | LIST OF TABLES | xi |
| | LIST OF FIGURES | xiii |
| | LIST OF PLATES | xv |
| 1. | INTRODUCTION | 1 |
| 2. | REVIEW OF LITERATURE | 4 |
| | 2.1 Triazoles | 4 |
| | 2.1.1 Mechanism of action | 5 |
| | 2.1.1.1 Synthesis of ergosterol by fungi | 5 |
| | 2.1.1.2 Inhibition of ergosterol biosynthesis | 6 |
| | 2.2 Hexaconazole as a fungicide in crop protection | 9 |
| | 2.3 Genotoxicity | 9 |
| | 2.3.1 Types of genetic damage | 10 |
| | 2.3.1.1 Gene mutations or single point mutations | 10 |
| | 2.3.1.2 Structural chromosome aberrations | 11 |
| | 2.3.1.3 Genomic mutations | 12 |
| | 2.3.2 Genotoxicity of triazoles | 12 |
| | 2.4 Reproductive toxicity | 14 |
| | 2.4.1 Synthesis of steroidal reproductive hormones | 15 |
| | 2.4.2 Disruption of reproductive system function | 19 |
| | 2.4.3 Effect of azole antifungals on steroid hormone synthesis in mammals | 21 |
| | 2.4.4 Toxic effects on male reproductive system | 25 |
| | 2.4.5 Toxic effects on female reproductive system | 28 |
| | 2.5 Hepatotoxicity | 31 |
| 3. | MATERIALS AND METHODS | 33 |
| | 3.1 Hexaconazole | 33 |
| | 3.2 Experimental animals | 33 |

| Chapter No. | Title | Page No. |
|---|---|---|
| 3.3 | Clastogenic studies | 34 |
| 3.3.1 | Bone marrow chromosomal aberrations assay | 34 |
| 3.3.1.1 | Treatment of animals | 34 |
| 3.3.1.2 | Grouping of animals | 35 |
| 3.3.1.3 | Harvesting of bone marrow | 35 |
| 3.3.1.4 | Hypotonic treatment | 36 |
| 3.3.1.5 | Fixation | 37 |
| 3.3.1.6 | Slide preparation | 37 |
| 3.3.1.7 | Screening of aberrant cells | 37 |
| 3.3.2 | The micronucleus test | 37 |
| 3.3.2.1 | Extraction of bone marrow and preparation of slides | 38 |
| 3.3.2.2 | Evaluation of slides | 38 |
| 3.3.3 | The lymphocyte cell culture | 38 |
| 3.4 | Male reproductive toxicity | 40 |
| 3.4.1 | Grouping and treatment | 40 |
| 3.4.2 | Collection of blood and organs | 41 |
| 3.4.3 | Epididymal sperm count | 41 |
| 3.4.4 | Sperm motility | 42 |
| 3.4.5 | Live and dead sperm count | 42 |
| 3.4.6 | Abnormal sperm count | 42 |
| 3.4.7 | Histopathological examination | 42 |
| 3.4.7.1 | Quantitative histopathology | 43 |
| 3.4.8 | Assay of serum testosterone, estradiol and progesterone levels | 43 |
| 3.4.8.1 | Hormones and chemicals | 43 |
| 3.4.8.2 | Steroid extraction from serum | 44 |
| 3.4.8.3 | Antisera | 44 |
| 3.4.8.4 | Procedure of steroid RIA | 45 |
| 3.5 | Female reproductive toxicity | 46 |
| 3.5.1 | Grouping and treatment of animals | 46 |

| Chapter No. | Title | | Page No. |
|---|---|---|---|
| | 3.5.2 | Monitoring of estrous cycle | 47 |
| | 3.5.3 | Confirmation of mating | 47 |
| | 3.5.4 | Reproductive indices | 47 |
| | 3.5.5 | Collection of blood and reproductive organs | 48 |
| | 3.5.6 | Estimation of serum estradiol and progesterone levels | 48 |
| 3.6 | Hepatotoxicity | | 49 |
| | 3.6.1 | Serum biochemical profiles | 49 |
| | 3.6.1.1 | Serum aspartate aminotransferase | 49 |
| | 3.6.1.2 | Serum alanine aminotransferase | 49 |
| | 3.6.1.3 | Serum alkaline phosphatase | 49 |
| | 3.6.1.4 | Serum cholesterol | 50 |
| | 3.6.2 | Histopathological examination | 50 |
| 3.7 | Statistical analysis | | 50 |
| 4. | RESULTS | | 51 |
| 4.1 | Clastogenicity of hexaconazole | | 51 |
| | 4.1.1 | *In vivo* clastogenic studies | 51 |
| | 4.1.1.1 | Bone marrow chromosomal aberrations | 51 |
| | 4.1.1.2 | Bone marrow micronuclei | 54 |
| | 4.1.2 | *In vitro* clastogenic study | 61 |
| 4.2 | Effect of hexaconazole on male reproductive system | | 61 |
| | 4.2.1 | Effect on body weight gain | 64 |
| | 4.2.2 | Effect on male reproductive organ weight | 64 |
| | 4.2.3 | Effect on semen characteristics | 73 |
| | 4.2.3.1 | Total epididymal sperm count | 73 |
| | 4.2.3.2 | Motility percentage | 78 |
| | 4.2.3.3 | Dead sperm percentage | 78 |
| | 4.2.3.4 | Abnormal sperm percentage | 79 |
| | 4.2.4 | Serum testosterone levels | 79 |
| | 4.2.5 | Histopathological changes in testis | 80 |
| | 4.2.5.1 | Quantitative analysis | 80 |
| | 4.2.5.2 | Qualitative changes | 80 |

| Chapter No. | Title | | Page No. |
|---|---|---|---|
| | 4.3 | Effect of hexaconazole on female reproductive system | 85 |
| | | 4.3.1 Body weight gain | 85 |
| | | 4.3.2 Maternal body weight gain in pregnant rats | 90 |
| | | 4.3.3 Estrus cycle | 90 |
| | | 4.3.4 Pregnancy and its outcome | 90 |
| | | 4.3.5 Postnatal growth | 93 |
| | | 4.3.6 Reproductive indices | 93 |
| | | 4.3.7 Serum estradiol and progesterone levels | 98 |
| | | 4.3.8 Histopathology of reproductive organs | 98 |
| | 4.4 | Hepatotoxic potential | 103 |
| | | 4.4.1 Serum biochemical profiles | 103 |
| | | 4.4.2 Histopathological changes | 108 |
| 5. | DISCUSSION | | 114 |
| | 5.1 | Clastogenic effect | 114 |
| | | 5.1.2 *In vivo* clastogenic studies | 115 |
| | | 5.1.2.1 Bone marrow chromosomal aberrations | 115 |
| | | 5.1.2.2 The micronucleus test | 117 |
| | | 5.1.3 *In vitro* clastogenic study | 118 |
| | | 5.1.4 Clastogenic effect of hexaconazole - conclusion | 119 |
| | 5.2 | Effect on male reproductive system | 120 |
| | 5.3 | Effect on female reproductive system | 127 |
| | 5.4 | Hepatotoxicity | 132 |
| 6. | SUMMARY | | 134 |
| | REFERENCES | | 136 |

# List of Tables

# LIST OF TABLES


| Table No. | Title | Page No. |
|---|---|---|
| 1. | Effect of hexaconazole (single exposure) on chromosomal aberrations in bone marrow cells of rat | 52 |
| 2. | Effect of hexaconazole (multiple exposures) on chromosomal aberrations in bone marrow cells of rat | 55 |
| 3. | Effect of hexaconazole (single exposure) on the frequency of micronuclei in bone marrow cells of rat | 57 |
| 4. | Effect of hexaconazole (multiple exposures) on the frequency of micronuclei in bone marrow cells of rat | 59 |
| 5. | Effect of hexaconazole on chromosomal aberrations in rat lymphocyte cell cultures | 62 |
| 6. | Effect of hexaconazole (30 days *per os*) on body weight in male rats | 65 |
| 7. | Effect of hexaconazole (60 days *per os*) on body weight in male rats | 67 |
| 8. | Effect of hexaconazole (30 days *per os*) on male reproductive organ weights in rats | 69 |
| 9. | Effect of hexaconazole (60 days *per os*) on male reproductive organ weights in rats | 71 |
| 10. | Effect of hexaconazole (30 days *per os*) on semen characteristics and serum testosterone levels in rats | 74 |
| 11. | Effect of hexaconazole (60 days *per os*) on semen characteristics and serum testosterone levels in rats | 76 |
| 12. | Effect of hexaconazole (30 days *per os*) on seminiferous tubules in rats | 81 |
| 13. | Effect of hexaconazole (60 days *per os*) on seminiferous tubules in rats | 83 |

| Table No. | Title | Page No. |
|---|---|---|
| 14. | Effect of hexaconazole (30 days *per os*) on body weight in female rats | 86 |
| 15. | Effect of hexaconazole on maternal body weight gain in rats | 88 |
| 16. | Effect of hexaconazole on pregnancy and its outcome in rats | 91 |
| 17. | Effect of hexaconazole on litter size and postnatal growth of pups | 94 |
| 18. | Effect of hexaconazole on reproductive indices in rats | 96 |
| 19. | Effect of hexaconazole (30 days *per os*) on estrus cycle and serum estradiol and progesterone levels in female rats | 99 |
| 20. | Effect of hexaconazole (≥ 73 days *per os*) on serum estradiol and progesterone levels in female rats | 101 |
| 21. | Effect of hexaconazole (30 days *per os*) on serum biochemical profiles in male rats | 104 |
| 22. | Effect of hexaconazole (60 days *per os*) on serum biochemical profiles in male rats | 106 |
| 23. | Effect of hexaconazole (30 days *per os*) on serum biochemical profiles in female rats | 109 |
| 24. | Effect of hexaconazole (≥ 73 days *per os*) on serum biochemical profiles in female rats | 111 |

# *List of Figures*

# LIST OF FIGURES


| Figure No. | Title | Page No. |
|---|---|---|
| 1. | Schematic representation of the cascade of abnormalities which ensues when triazoles replace lanosterol as substrate for the cytochrome P450 enzyme lanosterol 14$\alpha$ - demethylase | 8 |
| 2. | Biosynthesis of gonadal steroids | 17 |
| 3. | Effect of hexaconazole (single exposure) on chromosomal aberrations in bone marrow cells of rat | 53 |
| 4. | Effect of hexaconazole (multiple exposures) on chromosomal aberrations in bone marrow cells of rat | 56 |
| 5. | Effect of hexaconazole (single exposures) on the frequency of micronuclei in bone marrow cells of rat | 58 |
| 6. | Effect of hexaconazole (multiple exposures) on the frequency of micronuclei in bone marrow cells of rat | 60 |
| 7. | Effect of hexaconazole on chromosomal aberrations in rat lymphocyte cell cultures | 63 |
| 8. | Effect of hexaconazole (30 days *per os*) on body weight in male rats | 66 |
| 9. | Effect of hexaconazole (60 days *per os*) on body weight in male rats | 68 |
| 10. | Effect of hexaconazole (30 days *per os*) on male reproductive organ weights in rats | 70 |
| 11. | Effect of hexaconazole (60 days *per os*) on male reproductive organ weights in rats | 72 |
| 12. | Effect of hexaconazole (30 days *per os*) on semen characteristics and serum testosterone levels in rats | 75 |
| 13. | Effect of hexaconazole (60 days *per os*) on semen characteristics and serum testosterone levels in rats | 77 |
| 14. | Effect of hexaconazole (30 days *per os*) on seminiferous tubules in rat testis | 82 |

| Figure No. | Title | No. |
|---|---|---|
| 15. | Effect of hexaconazole (60 days *per os*) on seminiferous tubules in rat testis | 84 |
| 16. | Effect of hexaconazole (30 days *per os*) on body weight in female rats | 87 |
| 17. | Effect of hexaconazole on maternal body weight gain in rats | 89 |
| 18. | Effect of hexaconazole on pregnancy and its outcome in rats | 92 |
| 19. | Effect of hexaconazole on litter size and postnatal growth of pups | 95 |
| 20. | Effect of hexaconazole on reproductive indices in rats | 97 |
| 21. | Effect of hexaconazole (30 days *per os*) on serum estradiol and progesterone levels in female rats | 100 |
| 22. | Effect of hexaconazole (≥ 73 days *per os*) on serum estradiol and progesterone levels in female rats | 102 |
| 23. | Effect of hexaconazole (30 days *per os*) on serum biochemical profiles in male rats | 105 |
| 24. | Effect of hexaconazole (60 days *per os*) on serum biochemical profiles in male rats | 107 |
| 25. | Effect of hexaconazole (30 days *per os*) on serum biochemical profiles in female rats | 110 |
| 26. | Effect of hexaconazole (≥ 73 days *per os*) on serum biochemical profiles in female rats | 112 |

# *List of Plates*

# LIST OF PLATES


| Plate No. | Title | Page No. |
|---|---|---|
| 1 | Photomicrograph showing (arrow) a break in metaphase spread of bone marrow cell of a treated rat | 51-52 |
| 2 | Photomicrograph showing (arrow) a gap in metaphase spread of bone marrow cell of a treated rat | 51-52 |
| 3-6 | Photomicrograph showing (arrow) micronuclei in PCEs of bone marrow cells of treated rat | 60-61 |
| 7 | Photomicrograph showing (arrow) a break in metaphase spread of lymphocyte cultured *in vitro* | 61-62 |
| 8 | Photomicrograph showing (arrow) a gap in metaphase spread of lymphocyte cultured *in vitro* | 61-62 |
| 9-12 | Photomicrographs showing (arrow) the abnormal sperm observed in treated rats | 79-80 |
| 13 | Rat-Hexaconazole (High dose-60 days) - Testis-showing intertubular edema H4E 360 | 85-86 |
| 14 | Rat-Hexaconazole (High dose-30 days) - Testis-showing accumulation of round cells with in the human of seminiferous table H & E x 360 | 85-86 |
| 15 | Rat-Hexaconazole (High dose-30 days) - Testis-showing intertubular edema, absence of spermatogenesis and mild Leydig cell hypertrophy H & E x 360 | 85-86 |
| 16 | Rat-Hexaconazole (High dose-60 days) - Testis-showing degenerative changes in the gonadal epithelium with accumulation of giant cells with in the lumen of seminiferous tubule H & E x 360 | 85-86 |
| 17 | Rat-Hexaconazole (High dose - 60 days) -Testis-showing absence of spermatogenesis and Leydig cell hypertrophy H & E x 360 | 85-86 |
| 18 | Rat-Hexaconazole (High dose-60 days)-Testis-showing complete absence of spermatogenesis and accumulation of cellular debris with in the lumen H & E x 300 | 85-86 |

| Plate No. | Title | Page No. |
|---|---|---|
| 19 | Rat-Hexaconazole (High dose - ≥ 73 days)-Ovary-showing elongated glandular epithelial cells in the medulla H & E x 360 | 102-103 |
| 20 | Rat-Hexaconazole (High dose - ≥ 73 days)-Uterus-showing scanty endometrial glands H & E x 100 | 102-103 |
| 21 | Rat-Hexaconazole (High dose - ≥73 days) -Uterus-showing short columnar cells with basally placed nuclei with scanty glands H & E x 360 | 102-103 |
| 22 | Rat-Hexaconazole (High dose - ≥ 73 days) Uterus-lining cells of endometrium showing focal hyperplasia and squamous metaplasia H & E x 360 | 103-104 |
| 23 | Rat-Hexaconazole (High dose - ≥ 73 days) Vagina-showing thinned out epithelium which is 2-3 layered H & E x 360 | 103-104 |
| 24 | Rat (male) Hexaconazole (High dose-30 days)- Liver-showing diffuse degenerative changes around the central vein H & E x 100 | 113-114 |
| 25 | Rat (male)-Hexaconazole (High dose-60 days) -Liver-showing fatty changes H & E 3 320 | 113-114 |
| 26 | Rat (male)-Hexaconazole (High dose - 60 days) - Liver-showing focal necrosis and lymphocytic - plasmocytic infiltration H & E x 100 | 113-114 |
| 27 | Rat (male)-Hexaconazole (High dose 60 days) - Liver-showing focal microgranuloma H & E x 100 | 113-114 |

# *Introduction*

# CHAPTER - 1

# INTRODUCTION

Pesticides are chemical agents that may be classified according to the target pest to be destroyed *viz.*, insecticides, fungicides, molluscicides, bactericides, and herbicides. These have been developed as extremely important aids to world agricultural production over the last few decades. In contemporary situation, chemical control of pests and weeds have become an important and in some circumstances, indispensable measure. Apart from the use in agriculture, horticulture and forestry, pesticides are also used in livestock production and public health programmes.

However, liberal and indiscriminate use of pesticides has immense negative impact on the quality of environment and ultimately on the well being of animal and human population. The translocation of pesticides from soil and water, to plants and aquatic animals, results in their entry into food chain and bioaccumulation. Apart from this, higher living organisms are also directly exposed to the pesticides by way of inhalation and dermal absorption. Chronic exposure of humans and livestock to these xenobiotics results in various deleterious health hazards that are manifested over a time. The resulting toxic effects on various systems of the body have a bearing on the quality of life in humans and the economics in livestock production.

Triazoles are one of the newer and more promising groups of fungicides. They are broad spectrum systemic fungicides that offer both protective and curative effects (Bohmont, 1996). Triazoles are not only used as fungicides in agriculture, but also clinically, to control several important fungal infections. Itraconazole and fluconazole belong to the clinically used triazoles.

Triazoles inhibit the biosynthesis of ergosterol, an essential component of fungal cell membrane, via, inhibition of the cytochrome P450 - dependent enzyme lanosterol 14 α-demethylase. One of the nitrogen atoms in the triazole ring binds to the heme iron of cytochrome P450 thereby inhibiting cytochrome activation and enzyme function. The relative binding efficiency of these azole antifungal agents to cytochrome P450, differs, resulting in variations in antifungal activity, toxicity of the agents and the relative likelihood of drug interactions with other cytochrome P450 metabolized drugs. Cytochromes P450, the ubiquitous heme containing proteins, are found throughout the plant and animal kingdom and play a crucial role in the synthesis of steroidal hormones in mammals. Any interference in the synthesis of sex steroidal hormones will obviously affect the reproductive performance.

Life and propagation of life are determined by the information stored in the genes and the proper functioning of reproductive system. It has long been recognised that several xenobiotics have the potential to alter this genetic information.

Hexaconazole is a triazole fungicide that is widely being used in agricultural and horticultural practices. Besides, it has wood preservative action (PRDD 95-1). Perusal of available literature suggests that not many reports are available on the adverse effects of hexaconazole, with particular reference to its clastogenic and reproductive effects.

In this scenario, the present work was designed on hexaconazole with the following objectives:

1. To study the clastogenic effect.
2. To assess the effect on male reproductive system.
3. To assess the effect on female reproductive system.
4. To assess the hepatotoxic potential.

# CHAPTER - 2

# REVIEW OF LITERATURE

## 2.1 TRIAZOLES

Triazoles, belong to 'azole' group of antifungals, which are stable synthetic compounds with one or more five membered azole rings. Each azole ring may contain either two or three nitrogen atoms. Azoles containing two nitrogen atoms are known as imidazoles and those containing three nitrogen atoms are known as triazoles. Imidazoles are best exemplified by the well known human antifungal compounds clotrimazole, miconazole, ketoconazole etc.

Triazole compounds contain a ring composed of two carbon atoms and three nitrogen atoms. The triazole ring increases tissue penetration, prolongs half life and enhances efficacy of the azoles, while decreasing the toxicity when compared to imidazole antifungal agents (Fromtling, 1988). Some of the antifungal triazole compounds include biloxazole, cyproconazole, diclobutazole, diniconazole, etaconazole, fluotrimazole, flutriafole, flusilazole, furconazole, hexaconazole, myclobutanil, panconazole, propiconazole, terbuconazole, triadimefon, triadimenol, triazbutil, tricyclazole, BAS-45406F, M-14360 and RH-7592 (Vyas, 1993). Apart from the above mentioned triazole fungicides used in agriculture, clinically important compounds consist of fluconazole, itraconazole etc.

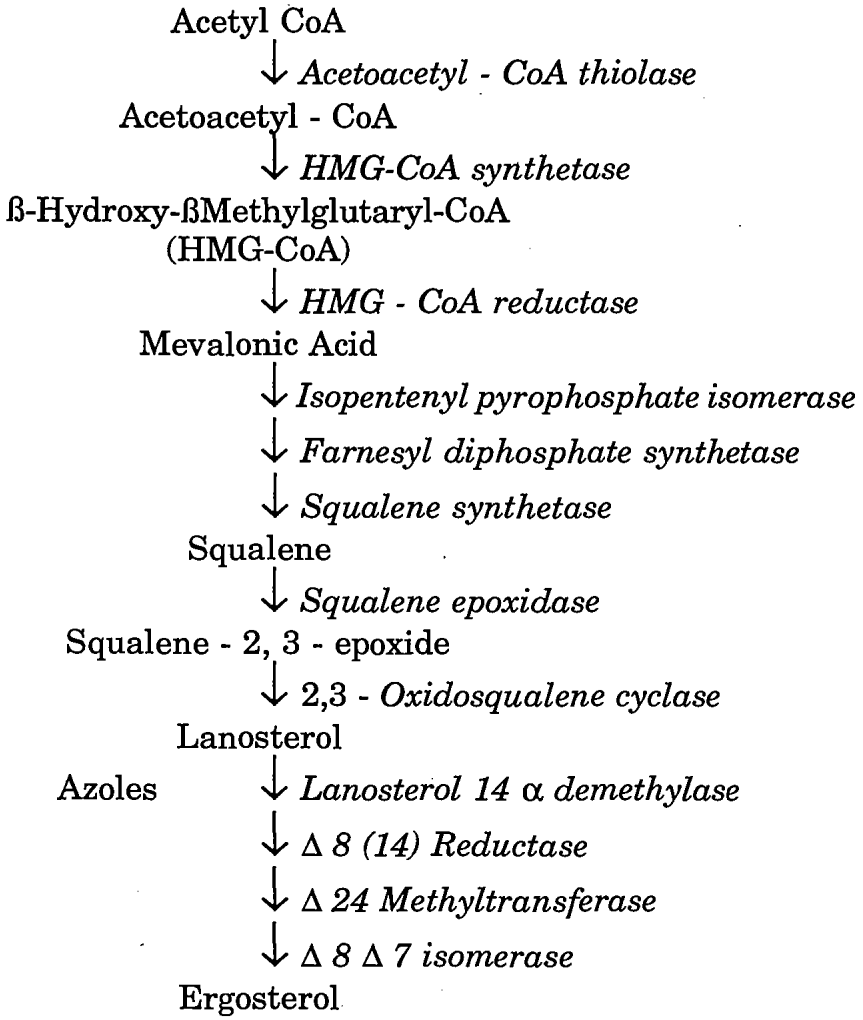
### 2.1.1 Mechanism of action

Fungal organisms consist of cell wall and plasma membrane. The major macromolecular components of cell wall include chitin, ß-glucan and mannoproteins. While chitin and ß-glucan fibrils are responsible for the cell wall's strength and shape, interstitial mannoproteins account for the wall's porosity, antigenicity, and in some like *C. albicans,* adhesion (Debono and Gordee, 1994).

The fungal plasma membrane acts as a permeability barrier, a conduit for the transport of small molecules and signals, and a matrix for proteins.It is mainly composed of sterols, phospholipids and sphingolipids and it is the composition of these lipids that determines the membrane's fluidity, a key factor for its function (Groll *et al.,* 1998).

#### 2.1.1.1 Synthesis of ergosterol by fungi

Ergosterol is synthesized from acetyl - CoA as shown below,m with the help of several enzymes (Groll *et al.,* 1998).




Since fungal sterols are structurally distinct from corresponding mammalian molecules and since their biosynthesis has been elucidated, they have been the focus of rational drug design efforts (Oehlschlaeger and Czyzewska, 1992).

### 2.1.1.2 Inhibition of ergosterol biosynthesis

The mechanism of action of all azole antifungal agents appears to be similar. Azoles inhibit the biosynthesis of ergosterol, an essential component of fungal cell membranes, via inhibition of the cytochromes P450 - dependent

enzyme lanosterol 14α - demethylase. Lanosterol 14α - demthylase is necessary for the conversion of lanosterol to ergosterol. At the molecular level, one of the nitrogen atoms (N-3 in the imidazoles; N-4 in the triazoles) binds to the heme iron of cytochrome P-450, thereby inhibiting cytochrome activation and enzyme function (van den Bossche *et al.*, 1986). Depletion of ergosterol in the fungal cell membrane results in altered membrane fluidity, thereby reducing the activity of membrane - associated enzymes. This leads to increased permeability and subsequent inhibition of cell growth and replication (Como and Dismukes, 1994). In addition, azoles may exhibit other direct effects on cell membrane fatty acids and can inhibit cytochrome P-450 dependent enzymes of the fungal respiration chain (Uno *et al.,* 1982). The sequelae of lanosterol 14α - demthylase inhibition in fungal organisms are shown in Figure.1

The relative binding efficiency of azole antifungal agents to cytochromes P450 differs, resulting in differences in antifungal activity, toxicity of the agents and the relative likelihood of drug interactions with other cytochrome P450-metabolised drugs (van den Bossche, 1987). Cytochromes P450, ubiquitous heme containing proteins found throughout the plant and animal kingdom, act as terminal mono-oxygenases in an electron transport chain mediated via NADPH reductase. The major role of cytochromes P450 is to insert oxygen via electrophilic attack of lipophilic substrates (Groves and Gross, 1995).

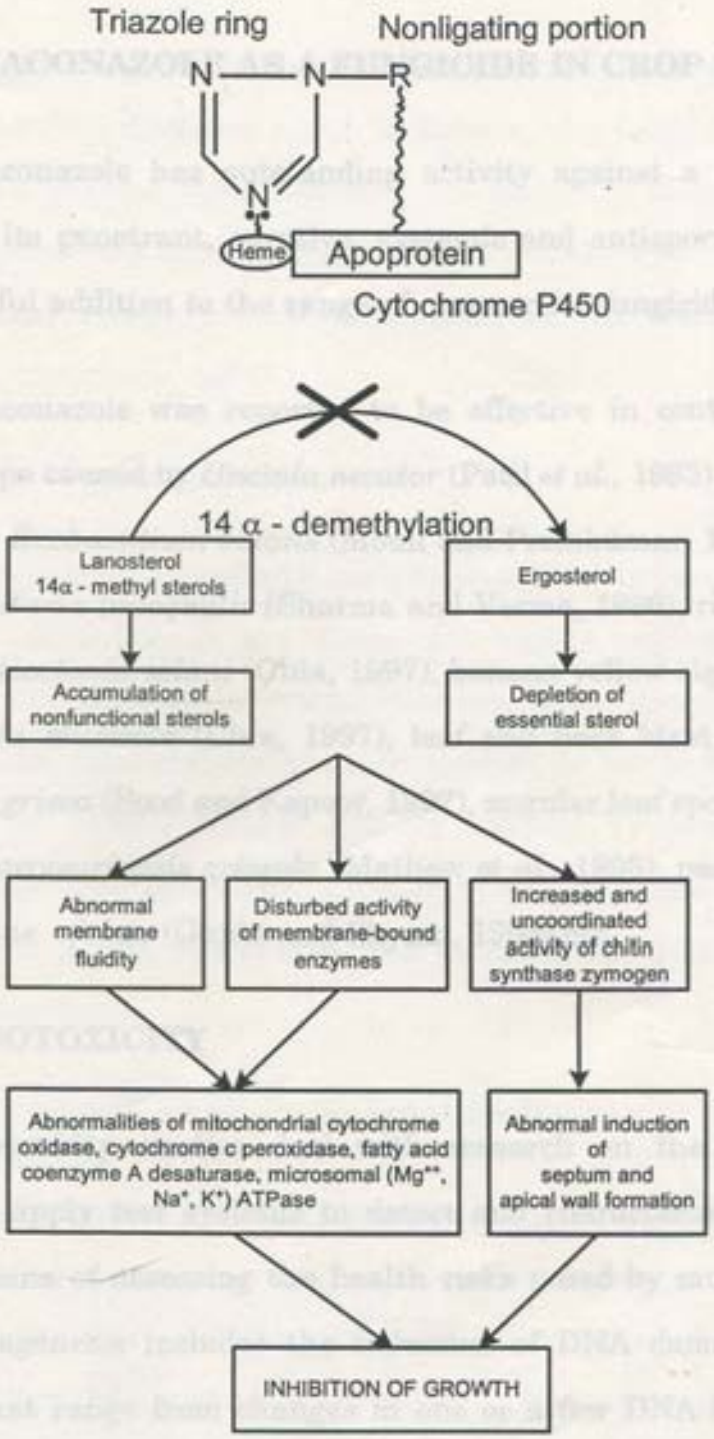



Figure 1 : Schematic representation of the cascade of abnormalities which ensues when triazoles replace lanosterol as substrate for the cytochrome P450 enzyme lanosterol 14α demethylase (after Janssen 1987 ; Janssen and van den Bossche, 1987)

## 2.2 HEXACONAZOLE AS A FUNGICIDE IN CROP PROTECTION

Hexaconazole has outstanding activity against a wide variety of diseases and its penetrant, curative, systemic and antisporulant properties provide a useful addition to the range of commercial fungicides (Vyas, 1993).

Hexaconazole was reported to be effective in controlling powdery mildew of grape caused by *Uncinla necator* (Patil *et al.,* 1993), blister blight of tea caused by *Exobasidium vexans* (Mouli and Premkumar, 1995), apple scab caused by *Venturia inaequalis* (Sharma and Verma, 1996), rice sheath blight caused by *Rhizoctonia solani* (Chia, 1997), banana yellow sigatoka caused by *Mycosphaerella musicola* (Chia, 1997), leaf and neck blast of rice used by *Magnaporthe grisea* (Sood and Kapoor, 1997), angular leaf spot of French bean caused by *Phaeoisariopsis griseola* (Mathew *et al.,* 1998), pea rust caused by *Uromyces viciae - fabae* (Gupta and Shyam, 1998) etc.

## 2.3 GENOTOXICITY

Genotoxicity studies deal with research on the mechanisms of mutagenesis, apply test systems to detect and characterize mutagens and formulate means of assessing the health risks posed by mutagens. Defined broadly, mutagenesis includes the induction of DNA damage and genetic alterations that range from changes in one or a few DNA base pairs (gene mutations) to gross changes in chromosome structure (chromosome aberrations) or number (aneuploidy and polyploidy). Any agent that causes mutations is a mutagen. The more specialised terms "clastogen" and "aneugen" are used for agents that cause chromosome aberrations and aneuploidy, respectively (Hoffmann, 1996).

The first evidence of chemical mutagenesis was obtained in Scotland in 1942 by Charlotte Auerbach and J.M. Robson, who found that mustard gas was mutagenic in *Drosophila.* However, the beginning of the modern era of mutation research was marked by H.T. Muller's discovery in 1927 that X-rays cause sex-linked recessive lethal mutations in the fruit fly *Drosophila molenogaster.* (Auerbach, 1976).

### 2.3.1 Types of genetic damage

Brusick (1987) classified genetic damage broadly into microlesions and macrolesions. Microlesions are those not visible microscopically, while macrolesions are detected in cytogenetics assays as microscopically visible alterations in chromosomal structure or number. As per Hoffman (1996) and Vanparys *et al.* (1996) genetic damage induced by xenobiotics can be categorised into three groups *viz.*, gene mutations or single point mutations, structural chromosome aberrations and genomic mutations or changes in chromosome number.

#### 2.3.1.1 Gene mutations or single point mutations

Gene mutations are small changes in the DNA at the level of bases and arise due to base pair substitution or due to addition or deletion of bases (frame shift mutations) (Grifiths *et al.,* 1993). Base pair substitutions can be of transition type (where purines are replaced by purines or pyrimidines by pyrimidines) or transversion type (where purines are replaced by pyrimidines and *vice versa*). Underlying molecular mechanisms in base pair substitution include, incorporation of base analogs, chemical alteration of normal bases, binding of chemicals to bases (alkylation of bases) and spontaneous base modification (Vanparys *et al.,* 1996).

Frame shift mutations involve the gain or loss of one or two base pairs in a gene and thereby alter the reading frame of the genetic code during the translation of RNA into proteins (Hoffman, 1996).

These various changes occurring in gene mutations may cause the substitution of a new amino acid in the subsequently coded protein molecule or result in a different sequence of amino acids in the protein synthesized. Furthermore, a protein synthesis termination codon may be formed, giving rise to a shortened protein. While the first type may or may not result in a modification of the biologic property of the protein molecule, the latter two types do effect the biological function (Lu, 1996).

#### 2.3.1.2 Structural chromosome aberrations

These involve gross alteration of the genetic material and are generally detected by light microscopic examination of metaphase chromosomes in appropriately prepared cells (Hoffman, 1996). Cytologically detected damage includes chromosome breakage and various chromosomal rearrangements that result from broken chromosomes. Aberrations that involve, only one of the two chromatids in a replicated chromosome are called chromatid type aberrations and those that involve both chromatids are called chromosome type aberrations. Easily scored aberrations include chromatid breaks, chromosome breaks, acentric fragments, chromatid exchanges, dicentric chromosomes, ring chromosomes and some reciprocal translocation (Bender *et al.,* 1988; Kirkland *et al.,* 1990). Ionizing radiation induces chromosome type aberrations when cells are treated before DNA replication and chromatid-type aberrations after

DNA replication (Sankaranarayanan, 1993). However, most chemical clastogens induce only chromatid aberrations, because the aberrations result from DNA synthesis on the damaged DNA template, in the S period of the cell cycle (Bender *et al.,* 1988; Sorsa *et al.,* 1992).

### 2.3.1.3 Genomic mutations

These are also known as numerical chromosome aberrations (Vanparys *et al.,* 1996) and involve a change in the number of chromosomes. Aneuploidy involves the gain or loss of one or a few chromosomes, whereas polyploidy involves complete set of chromosomes. Aneuploids lacking a chromosome are said to be monosomic, whereas those with an extra chromosome are called trisomic (Griffiths *et al.,* 1993).

Aneuploidy results from the loss of a damaged chromosome during cell division or from irregular distribution (nondisjunction) of the chromatids over the two daughter cells during cell division (Vanparys *et al.,* 1996).

## 2.3.2 Genotoxicity of triazoles

Katoh and Takayama (1981) studied the *in vitro* carcinogenic activities of 3-(N-salicylol) amino-1, 2,4-triazole (SAT) and its two compounds, 3-amino - 1,2,4 - triazole and phenyl salicylate in a transformation assay with cryopreserved hamster embryo cells. SAT induced morphological transformation at certain doses but gave a negative result in the *Salmonella* mutation assay.

Treatment of maize kernals with triazole fungicide triticonazole (RP-727) resulted in nuclear alterations (Biradar *et al.,* 1994). Analysis of nuclear DNA content in various maize plant organs by flow cytometry revealed that the chromatin structure was altered with no significant change in nuclear DNA in active meristematic plant organs, such as root tip. But stem and mesocotyl showed both DNA and chromatin condensation. It was further reported that the magnitude of the alterations was minute in different plant organs studied and these alterations did not appear to be deleterious for normal plant development.

Mattioli *et al.* (1994) conducted studies on the carcinogenic effects of amitrole in cultures of human thyroid follicular cells and rat and human hepatocytes *in vitro*. Exposure to amitrole did not increase DNA fragmentation in human thyroid and hepatocyte cultures while a dose dependent increase in the frequency of DNA breaks was seen in cultures of rat hepatocytes. In *in vivo* studies conducted by the same authors in rats, there was no evidence of DNA fragmentation but hepatic cell hyperplasia and decrease in thyroid follicle size were observed. The authors concluded that the mechanism of amitrole carcinogenic activity is not likely due to genotoxicity but due to hormone imbalance.

Triadimefon, a triazole fungicide was tested for its clastogenic and aneugenic activities, *in vivo* and *in vitro* systems by Kevekordes *et al.* (1996). The compound neither increased the frequency of micronucleated polychromatic erythrocytes in bone marrow cells of mice nor had any sister chromatid exchange inducing effect in human lymphocyte cell cultures.

Ohe *et al.* (1999) estimated the levels of the two aromatic amine mutagens, 2-(2-(acetylamino)-4-(bis (2-methoxyethyl) amino)-5-methoxyphenyl)-5-amino-7- bromo-4-chloro-2H-benzotriazole (PBTA-1) and 2-(20 acetylamino - 4- [N-(2-cyanoethyl) ehtylamino]-5-methroxyphenyl] - 5-amino-7-bromo-4-chloro-2H benzotriazole (PBTA-2) in the Yodo river system in Japan and Shiozawa *et al.* (1999) reported that PBTA-1 isolated from water of the Nishitakase river in Kyoto exhibited potent mutagenic activity in *Salmonella typhimurium* TA98 with S9 mix. The compound had the characteristic moieties, including bromo, chloro, acetylamino, bis (2-methoxyethyl) amino and primary amino groups on a 2-phenyl benzotriazole skeleton. When the mutagenicities of PBTA-1, its congeners and five related 2-phenylbenzotriazoles were examined in *S.typhimurium* TA98 with S9 mix, to elucidate the structure activity relationship, the data obtained suggested that a primary amino group, played an essential role in the mutagenic activity. Further the effect of planarity of the 2-phenylbenzotriazole ring was significant.

## 2.4 REPRODUCTIVE TOXICITY

Reproductive toxicity is the occurrence of adverse effects on the reproductive system that may result from exposure of agents from exogenous sources. The toxicity may be expressed as alterations to the reproductive organs, the related endocrine system, or pregnancy outcomes. The manifestation of such toxicity may include adverse effects on sexual maturation, gamete production and transport, cycle normality, sexual

behaviour, fertility, gestation, parturition, lactation, pregnancy outcomes, premature reproductive senescence or modifications in other function that are dependent on the integrity of the reproductive system (Kimmel *et al.,* 1995).

### 2.4.1 Synthesis of steroidal reproductive hormones

Cholesterol is the pivotal building block in steroid biosynthesis and it can be synthesized from acetate or obtained from body cholesterol stores. Under normal circumstances, the majority of cholesterol in steroidogenic tissues is derived from circulating plasma lipoproteins, with only small amounts synthesized *de novo*. In most species LDL acts as the cholesterol donor and one of the actions of the trophic hormones is to stimulate both the LDL receptor and the uptake of LDL cholesterol (Golos *et al.,* 1985).

The uptake of LDL cholesterol esters involves receptor-mediated endocytosis (Brown *et al.,* 1979) but in the rat where the cholesterol donor is HDL, rather than LDL (Andersen and Dietschy, 1978) the process does not involve internalisation of the intact lipoprotein (Gwyne and Hess, 1980, Gwynne and Strauss, 1982 and Glass *et al.,* 1985).

Following the transfer of cholesterol esters they may be stored as such or enzymatically de-esterified to free cholesterol. Cholesterol esters are stored in lipid droplets and the interconversion between free and esterified cholesterol is under the control of two enzymes, cholesterol ester hydrolase and cholesterol ester synthetase. Trophic hormones activate the esterase to ensure adequate supplies of free cholesterol for steroid synthesis within their target tissues (Trzeciak *et al.,* 1979).

As free cholesterol is insoluble in the aqueous medium of cytosol, it is transported to the mitochondria by a steroid carrier protein (Chanderbhan *et al.,* 1983 and Tanaka *et al.,* 1984).

The enzymes involved in the synthesis of steroids are largely cytochromes P450 and the main enzymes involved in gonadal steroid synthesis are shown in Figure 2.

The conversion of cholesterol to pregnenolone is a common step in the synthesis of all steroid hormones. The enzyme is located in the mitochondria and the reaction which is hormonally regulated is a three step rate limiting conversion involving 20α - hydroxylation, 22-hydroxylation and side chain scission between carbons 20 and 22 (Voutilainen *et al.,* 1986). Its activity is inhibited by estrogens (Onoda and Hall, 1987). The enzyme involved is called P 450 scc (side chain cleavage) or 20, 22 desmolase. Though three stages are involved in this reaction, P450 scc has been shown to be a single protein (Simpson, 1979 and Chung *et al.,* 1986) with a single active site (Duque *et al.,* 1978).

From pregnenolone to androstenedione, there are two alternative routes involving either 3 β-dehydrogenation or 17α - hydroxylation as the first step. Pregnenolone can be converted to 17α - hydroxypregnenolone and thence to dehydroepiandrostenone by the enzyme P450*C17* (17α - hydroxylase/17, 20 lyase). This enzyme is bound to smooth endoplasmic reticulum and catalyses the 17α - hydroxylation of pregnenolone, then removes the side chain by clearing the C17, 20 bond from 17α - hydroxypregnenolone to produce

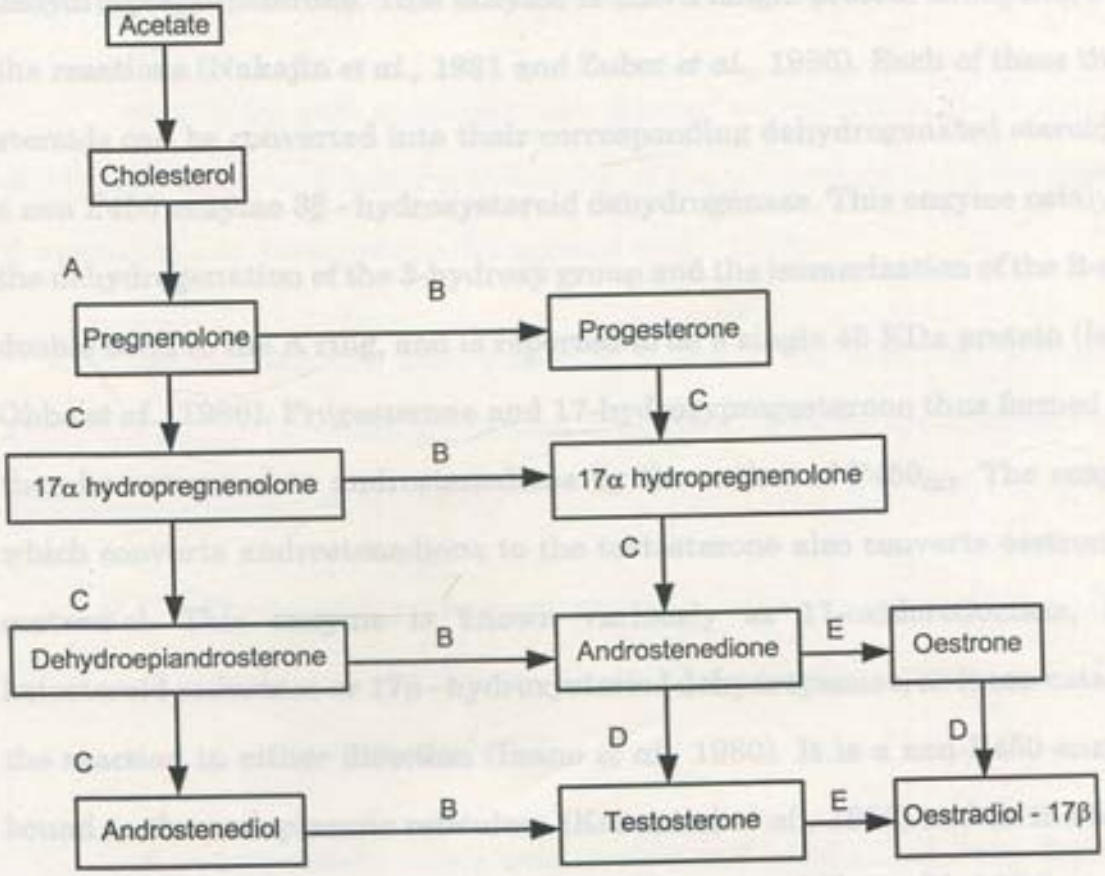



**Figure 2 : Biosynthesis of gonadal steroids**

Enzymes involved are :

A : P450 scc
B : 3β - hydroxysteriod dehydrogenese
C : $P450_{C17}$
D : 17β - hydroxysteroid dehydrogenase
E : P 450 aro
(After Woodman, 1997)

dehydroepiandrosterone. This enzyme is also a single protein catalysing both the reactions (Nakajin *et al.,* 1981 and Zuber *et al.,* 1986). Each of these three steroids can be converted into their corresponding dehydrogenated steroid by a non P450 enzyme 3β - hydroxysteroid dehydrogenase. This enzyme catalyses the dehydrogenation of the 3-hydroxy group and the isomerization of the B-ring double bond to the A ring, and is reported to be a single 45 KDa protein (Ishii-Ohba *et al.,* 1986). Progesterone and 17-hydroxyprogesterone thus formed can then be converted to androstenedione by the action of $P450_{C17}$. The enzyme which converts androstenedione to the testosterone also converts oestrone to oestradiol. This enzyme is known variously as 17-oxidoreductase, 17ß-ketosteroid reductase or 17β - hydroxysteriod dehydrogenase, as it can catalyse the reaction in either direction (Inano *et al.,* 1980). It is a non-P450 enzyme bound to the endoplasmic reticulum (Kurosumi *et al.,* 1986) and in the testis appears to be confined to the Leydig cells (Inano and Tamaoki, 1984).

The conversion of androgens to oestrogen is catalysed by cytochrome P450 aro (aromatase) which is located in the endoplasmic reticulum. This enzyme is a hydroxylase which removes the C-19 methyl group by a two stage hydroxylation, and as a result of a further hydroxylation at C-2, causes the A ring to aromatise (Fishman and Goto, 1981). The conversion of testosterone to its active metabolite dihydrotestosterone is catalysed by 5-α - reductase which occurs in peripheral target tissues rather than the testis (Mooradian *et al.,* 1987). 5α reductase is associated with the nuclear membrane and is NADPH dependent (Moore and Wilson, 1972).

### 2.4.2 Disruption of reproductive system function

Environmental chemicals may disrupt reproductive development by either mimicking or inhibiting the action of the gonadal steroid hormones, 17 β-oestradiol and testosterone (Vinggaard *et al.*, 1999).

Many pesticides have shown a potential for endocrine disruption. Different modes of action have been suggested to be involved in endocrine disruption (LeBlanc *et al.*, 1997).

i. Estrogenicity has been shown *in vitro* for endosulfan,o,p-DDT, toxaphene, dieldrin, methoxychlor, chlordecone (Soto *et al.*, 1995, Jorgensen *et al.*, 1997).

ii. Anti-androgenicity both *in vitro* and *in vivo* have been shown for p,p-DDE, vinclozolin and procymidone (Kelce and Wilson, 1997).

iii. Altered testosterone metabolism either by inhibition of its synthesis or by inducing its degradation e.g.by ketoconazole, endosulfan, mirex, chlordecone and o,p-DDT (LeBlanc *et al.*, 1997).

The mammalian gonad is capable of metabolizing a host of foreign chemicals that have traversed the blood-testis barrier (Thomas, 1996). Cytochromes P-450(arylhydrocarbon hydroxylases, AHH) are present in the testes and are fairly sensitive to the effects of a numbers of chemicals (Lee *et al.*, 1981) consequently, the pathways for steroidogenesis contain a number of enzymes that are affected by chemicals or drugs as shown below.

Inhibitors of steroidogenic enzymes (source:Haney, 1985, modified)

| Enzyme | Inhibitor |
|---|---|
| Cholesterol side chain cleavage | Aminoglutethimide, 3-methoxy benzidine, cyanoketone, azastene, danazol |
| Aromatase | 4-Acetoxy-androstene-3,17-dione, 4-hydroxy-andorstene-3,17-dione, 6-bromoandrostene-3,17-dione fenarimol (Hirsch *et al.*, 1987) |
| 11-Hydroxylase | Danazol, metyrapone |
| 21-Hydroxylase | Danazol, spiranolactone |
| 17-Hydroxylase | Danazol, sprionolactone |
| 17,20-Desmolase | Danazol, sprionolactone |
| 17-Hydroxysteriod dehydrogenase | Danazol |
| 3-Hudroxysteriod dehydrogenase | Danazol |
| C-17-L-20-lyase | Ketoconazole (Effendy and Krause, 1989). |

Apart from the interference in steroidogenesis, several drugs and chemicals also directly target the gonads (Chapman, 1983; Thomas and Keenan, 1986). Even in gonads, different cell populations exhibit some what different thresholds of sensitivity to different toxicants. Germ calls are most sensitive to chemical insult, the Sertoli cells possess an intermediate sensitivity to chemical inhibition and Leydig cells are more resistant to environmental toxicants (Thomas, 1996).

### 2.4.3 Effect of azole antifungals on steroid hormone synthesis in mammals

van den Bossche *et al.* (1980) have reported that a six times higher dose of ketoconazole, necessary to inhibit the growth of candida albicans, were required to effect cholesterol synthesis in female Wistar rats.

3-Amino- 1,2,4-triazole was found to be an inhibitor of fatty acid synthesis by isolated rat hepatocytes (Beynen *et al.*, 1981). Half maximal inhibition of fatty acid synthesis occurred at approximately 20 mM. Acetyl - CoA carboxylase activity in homogenates of hepatocytes was not affected by previous exposure of the intact cells to 3 - amino - 1,2,4 - triazole. The drug opposed the activation of partially purified acetyl - coA carboxylase by citrate, but did not influence enzyme activity in the absence of citrate. As compared to fatty acid synthesis, cholesterol synthesis by the hepatocytes was more drastically depressed by incubation of cells with 3-amino - 1, 2,4 -triazole.

Pont *et al.* (1982) have investigated the effects of ketoconazole on steroid synthesis in healthy male human volunteers and in isolated adrenal cells from rats. In humans the cortisol response to adrenocorticotropic hormone was significantly blunted 4 hours after dosing with ketoconazole, which persisted upto 8 hrs and was absent by 16 hrs. In *in vitro* studies they noted that the therapeutic concentrations of the drug virtually eliminated the production of corticosterone by isolated adrenal cells from rats. They also opined that their findings indicated the reduced adrenal androgen response.

Santen *et al.* (1983) reported decreased serum testosterone levels in ketoconazole treated patients and concluded that the agent blocked the testicular 17, 20 - desmolase activity which converts 17α - hydroxyprogesterone to androstenedione.

Grosso *et al.* (1983) reported that *in vivo* perfusion of canine testes with ketoconazole inhibited the stimulation of testosterone production by human chorionic gonadotropin in a dose dependent manner. Ketoconazole also selectively displaced steroids from serum-binding globulins.

Ketoconazole administered in experimentally high doses in humans, resulting in high serum levels, inhibited adrenal steroidogenesis and this was reversible with discontinuation of ketoconazole therapy (Tucker *et al.,* 1985).

Sikka *et al.,* (1985) studied the *in vitro* effects of ketoconazole on three (17α - hydroxylase, 17, 20 - desmolase and 17β - hydroxysteroid dehydrogenase)of the five enzymatic steps involved in the $^{4}\Delta$ - testosterone biosynthetic pathway using the rat testis as an animal model. With dosages between 10 and 300 µg/ml ketoconazole, they observed a marked inhibition of both the 17α- hydroxylase and 17, 20 - desmolase activities, with no effect on 17β - hydroxysteroid dehydrogenase activity. They further observed that ketoconazole also inhibited the increased activity of these enzymes induced by human chorionic gonadotropin.

In a study in male rats ketoconazole effectively lowered the serum testosterone concentration and accessory sex organs, prostate and seminal vesicle weights (Bhasin *et al.,* 1986). The enzymes that were inhibited were 17-

α- hydroxylase, 17, 20 - desmolase and 17ß - hydroxysteriod dehydrogenase. The authors also stated that these observations differed from the *in vitro* data in that no significant inhibition of 17ß - hydroxysteriod dehydrogenase activity was seen in those *in vitro* studies. They attributed this to difference in the time course of ketoconazole induced inhibition of 17 α hydroxylase and 17,20 - desmolase activities from that of 17ß- hydroxysteroid dehydrogenase.

Studies with high doses of itraconazole in rats have demonstrated a slight decrease in progesterone synthesis and a more significant decrease in corticosterone synthesis (van Cauteren *et al.*, 1987).

Phillips *et al.* (1987) have reported that itraconazole does not share the adverse effect of ketoconazole upon glucocorticoid synthesis in humans treated with maximum doses (400 mg/day) of itraconazole.

Among human volunteers given itraconazole daily for 2 weeks, there was no change in testosterone and cortisol levels compared with pretreatment values. However among 27 male patients treated for histoplasmosis or blastomycosis, 3 reported impotence and/or decreased libido and in all 3, serum testosterone concentrations during therapy were normal (Saag and Dismukes, 1988).

Hanger *et al.* (1988) have studied the effect of fluconazole on testosterone in *in vivo* and *in vitro* systems. In *in vivo* studies consisting of male human volunteers, fluconazole (@ 25 and 50 mg/kg for 28 days) had no significant effect on circulating testosterone levels. In studies with rat Leydig cells *in vitro,* fluconazole at concentrations upto 10 µg/ml was found to be only

a weak inhibitor of testosterone production, whereas ketoconazole caused more than 50 per cent inhibition at 0.1 µg/ml. They concluded that although at relatively high concentrations fluconazole had some ability to inhibit testosterone production *in vitro,* it had less than 1/100th the potency of ketoconazole in that test system.

Studies by Colby *et al.* (1995) revealed that the adrenal enlargement caused by 1-aminobenzotriazole was associated with a decline in plasma corticosterone concentrations, suggesting inhibition of adrenal steroidogenesis. They also reported that, a single injection of 1-aminobenzotriazole (25 or 50 mg/kg body wt) to rats caused concentration dependent declines (60-80%) in adrenal mitochondrial and microsomal cytochrome P450 concentrations accompanied by decreases in steroid hydroxylase activities.

Ketoconazole was administered to rats during days 1-8 of pregnancy by Cummings *et al.* (1997). On day 9, evaluation revealed a reduction in the number of implantation sites and serum progesterone levels. It was further observed by them that, ketoconazole had no effect on long term ovariectomised rats that were hormone supplemented, indicating that the ovary was one site of ketoconazole action on early pregnancy. The authors also noticed a decline in ovarian progesterone production, *in vitro*, from ovaries removed from rats treated *in vivo*, with ketoconazole, which indicated a direct effect of ketoconazole on ovarian steroidogenesis.

Effects of 1-aminobenzotriazole (ABT) on testicular steroid metabolism were evaluated in rats by Soltis and Colby (1998). Administration of ABT to adult male rats caused dose dependent decreases in testicular microsomal and mitochondrial cytochrome P450 concentrations. Declines of testicular P450 content were accompanied by decreases in microsomal 17 alpha-hydroxylase and mitochondrial cholesterol side chain cleavage activities. These results indicted that ABT, *in vivo* caused inactivation of steroidogenic P450 isoenzymes in the testis.

The pesticides propiconazole, triadimenol and triadimefon, all belonging to the group of triazoles, were able to inhibit cytochrome P19 aromatase activity *in vitro,* in human placental microsomes using the classical $(^3H)_2O$ method (Vinggaard *et al.*, 2000).

### 2.4.4 Toxic effects on male reproductive system

Since the literature pertaining to the effects of triazoles on male reproductive system is sparse, the effects of various xenobiotics on it is reviewed hereunder.

A study conducted by Carter *et al.* (1987) in rats showed that treatment with 400mg/kg/day of carbendazim, a methyl -2 benzimidazole carbamate fungicide resulted in severe seminiferous tubular atrophy and infertility.

Dietary administration of cyclohexylamine, a metabolite of non-nutritive sweetener, cyclamate, to rats at 400 mg/kg/day for 3, 7 and 13 weeks resulted in significantly lower tests weight with marked histopathological changes. Though lesions were not apparent at week 3, they were evident by week 7, with partial depletion of the spermatocyte and the round and elongated spermatid populations. More extensive germ cell degeneration and depletion of tubules were noticed at week 13 (Roberts *et al.*, 1989).

Kleeman *et al.* (1990) studied the mechanism by which 2,3,7,8 - tetrachlorodibenzo-p-dioxin (TCDD) decreases testosterone secretion in rats. They reported that TCDD did not inhibit the conversion of pregnenolone to testosterone, but inhibited pregnenolone formation itself. Further they opined that the inhibition of pregnenolone formation by TCDD must be due to a reduction in the activity of the enzyme cytochrome P450 scc, which converts cholesterol to pregnenolone and/or an impairment in the multistep process responsible for mobilizing cholesterol to this enzyme.

Insecticide endosulfan significantly inhibited testicular androgen biosynthesis in adult rats, when fed, *per os*, at 7.5 and 10.00 mg/kg body wt for 15 and 30 days. It also lowered the steroidogenic enzymes, 3ß and 17ß - hydroxy steroid dehydrogenases on longer exposure (Singh and Pandey, 1990).

Decreased gonado-somatic index and decreased weights of epididymis, seminal vesicle and prostate were produced in male rats that received vincristine at dose levels of 10 and 20 µg/day for 15 days. The regression of accessory sex organs was attributed to the effect of vincristine on the androgenic compartment of the testis (Stanley *et al.*, 1993).

Isoproturon, a herbicide was evaluated for its male reproductive toxicity in rats by Sarkar *et al.* (1997). The compound when administered po for 10 weeks @ 200,400 and 800 mg/kg, produced decreased epididymal sperm count percentage of motile sperm and increased the percentage of morphologically abnormal sperm at the highest dose. At the same dose diameter of seminiferous tubules was also reduced, number of tubules per microscopic field was increased and the percentage of tubules with evidence of spermatogenesis decreased.

Benomyl, a benzimidazole fungicide, and its metabolite carbendazim were administered intraperitoneally or directly into the testis in rats by Lim and Miller (1997). By both the routes, while benomyl caused no significant testicular damage in two hours, its metabolite carbendazim produced severe changes with sloughing of the seminiferous epithelium. These results suggested that the benomyl metabolite carbendazim, but not benomyl it self is the mediator of benomyl induced testicular toxicity.

Akbarsha and Sivasamy (1998) reported that phosphamidon in drinking water for 30 days in rats caused vacuolization and pinching off principal cells in caput epididymis. Phosphamidon appeared to affect the principal cells indirectly through its toxic effect on the Leyding cells and the clear cells of the cauda appeared to be directly vulnerable to the toxic action of the pesticide.

Abd-Allah *et al.* (2000) studied the adverse testicular effects of some quinoline members in rats. Ofloxacin, ciprofloxacin and pefloxacin, when given in doses of 72, 135 and 72 mg/kg day for 15 days, caused marked reduction in sperm count, motility and daily sperm production. Oflaxacin also caused marked testicular histopathological changes. It was concluded that these quinolones significantly impaired both testicular function and structure in rats.

Andric *et al.* (2000) studied the acute *in vivo* and *in vitro* effect of a commercially used polychlorinated biphenyl mixture, Arochlor 1248, on testicular androgenesis in rats. Single intraperitoneal, or bilateral intratesticular injections of the compound decreased serum androgen levels. The capacity of the post mitochondrial fractions from testes of intratesticular treated animals to convert pregnenolone to progesterone and progesterone to testosterone was also reduced. The overall results indicated that Arochlor 1248 down regulated the testicular androgenesis by an acute inhibition of 3ß-hydroxysteroid dehydrogenase, 17α- hydroxylase/lyase and 17ß hydroxysteroid dehydrogenase activities.

### 2.4.5 Toxic effects on female reproductive system

The effects of substituted triazole, R151885 (1,1-di(4-fluorophenyl)-2-(1,2,4-triazol-1-yl) - ethanol), on ovulation were investigated in Wistar rats by Middleton *et al.* (1986). A single oral dose of 5 or 25 mg/kg of the drug delayed

the ovulation by 24 or 48 hrs, respectively. The preovulatory peak plasma concentrations of progesterone and estradiol were reduced. The authors concluded that, low levels of plasma estradiol, combined with some antagonism of the estradiol action, might have prevented the adequate priming of the pituitary, which in turn suppressed the preovulatory luteinizing hormone surge required for ovulation.

Milne *et al.* (1987) observed that ovulation in the rat was delayed by a single administration of the substituted triazole R 151885 (1,1-di (4-fluorophenyl) -2- (1,2,4-triazol -l-yl)-ethanol). Plasma levels of estradiol were found markedly reduced (42-45%) 6-12 hr after administration of R 151885. The restoration of ovulation in R151885 - pretreated rats, by administration of exogenous estradiol benzoate, indicated that the delay in ovulation was due to reduced estradiol levels. It was also observed that granulosa cells isolated from rat ovaries, that produce estradiol and progesterone *in vitro,* in the presence of follicle stimulating hormone and testosterone, failed to produce estradiol at normal level, when the triazole R515885 was added to the cultures. Estradiol production was decreased by 69%.

A two generation reproductive study in rats on triadimenol, demonstrated unaffected reproductive endpoints such as fertility, lactation and insemination in doses corresponding to 1,4 and 20 mg /kg/day. However a higher dose (120 mg/kg/day) was found to give rise to an increased rate of resorption of fetuses (WHO, 1989).

Triazole fungicides, flusilazole and bitertanol were given in single oral dosages on days 9,10,11 or 13th of gestation, to pregnant rats (Vergieva,1990). The results demonstrated that both compounds induced congenital anomalies, when given on days 9,10 or 11th.

Lloyd *et al.* (1994) administered ICIA 0282, a triazole fungicide to groups of pregnant rats as a single dose on each of gestation days 7-16 and evaluated the foetuses after c-section on gestation day 22. Examination of pups showed a very high incidence of specific developmental abnormalities like abnormal or absent tympanic annulus, cleft palate and shortened ribs.

Machera (1995) studied the developmental toxicity of cyproconazole in pregnant rats. When pregnant rats were gavaged with cyproconazole daily from day 6 to 16 of gestation, a sharp increase in the number of resorptions and dead fetuses and decrease in fetal body weight and crown/rump length were observed.

*In vitro* teratologic evaluation of triazoles, triadimefon and triadimenol by Menegola *et al.* (1998) using the rat post implantation whole embryo cultures, revealed that, both the triazole fungicides were teratogenic and adversely affected the development of embryo. Highest doses of triazoles reduced the developmental parameters like somite number, crown - rump and head length. Other abnormalities included branchial arch defects, fusions

between first and second aortic arches, agenesis of branches of nerve V and abnormal shape and position of certain cranial nerves.

Moser *et al.* (1999) reported that when tebuconazole (0,6,20, or 60 mg/kg) was given orally to rats from gestational day 14 to postnatal day 7, the pup viability and body weight were found decreased at high dose. However, no differences were observed in the fertility indices.

In an *in vitro* study using the rat post implantation whole embryo culture, the triazole, fluconazole was found teratogenic (Menegola *et al.,* 2000) Embryos cultured in rat serum added with fluconazole showed abnormalities at the level of the branchial apparatus, cellular death on maxillary processes and fusions between aortic arches.

## 2.5 HEPATOTOXICITY

Beynen *et al.* (1981) reported that 3-amino-1,2,4-triazole inhibited fatty acid synthesis by isolated rat hepatocytes. They observed that acetyl coA carboxylase activity in homogenates of hepatocytes was not affected by previous exposure of the intact cells to 3-amino-1,2,4-triazole. The drug opposed the activation of partially purified acetyl coA carboxylase by citrate, but did not influence enzyme activity in the absence of citrate. As compared to fatty acid synthesis, cholesterol synthesis by the hepatocytes is more drastically depressed by incubation of the cells with 3-amino-1,2,4-triazole.

The hepatic cells from liver sections of rats treated with 4-N-butyl-1,2,4-triazole, exhibited hydropic degeneration and swelling, while the nuclei were in various stages of karyolysis (Gajendragad and Seshadri, 1983).

Lewis *et al.* (1984) observed that as may as 10 per cent of patients receiving ketoconazole showed transient and idiopathic elevations of hepatic transaminases.

Transient increases in liver enzymes was reported as one of the adverse effects of long term itraconazole therapy in humans (Grant and Clissold, 1989).

Itraconazole at higher end of the dosage range had elevated liver tansaminases (Tucker *et al.,* 1990) while Lavrijsen *et al.* (1992) reported a few cases of severe hepatic injury or hepatitis.

Increased activity of alanine aminotransferase and alkaline phosphatase in serum of dogs given ketoconazole was reported by Martin *et al.* (1992).

Higher doses of fluconazole caused elevation of hepatic transaminases (Como and Dismukes, 1994) and hepatic toxicity (Anaissie *et al.,* 1995).

Severe hepatic damage was ascribed to the oral use of ketoconazole for the treatment of fungal infections in Netherlands (van Puijenbroek *et al.,* 1998) and in Argentina (Findor *et al.,* 1998).

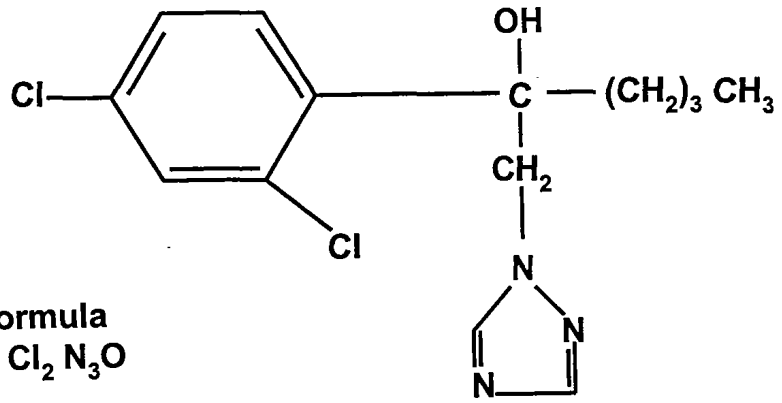
# *Materials and Methods*

# CHAPTER - 3

# MATERIALS AND METHODS

## 3.1 HEXACONAZOLE

Hexaconazole technical grade (92.5%) used in the study was a generous gift from M/s. Rallis India Limited, Agro Chemicals Division, Turbhe, Navi Mumbai 400 703.




**Mol. formula**
$C_{14}H_{17}Cl_2N_3O$

Chemical name: (RS)-2-(2,4-dichlorophenyl)-1-(1H-1,2,4-triazole-1-yl)-hexane-2-ol.

## 3.2 EXPERIMENTAL ANIMALS

Wister strain albino rats were used throughout the study. The rats were obtained from the Department of Laboratory Animal Science, Tamil Nadu Veterinary and Animal Sciences University, Chennai - 7 and were maintained in polyacrylamide cages at ambient temperature. Pelleted feed and clean drinking water were provided *ad libitum*.

## 3.3 CLASTOGENIC STUDIES

*In vivo* and *in vitro* studies were conducted to assess the clastogenicity of hexaconazole. *In vivo* clastogenic studies were carried out employing bone marrow chromosomal aberrations assay and micronucleus test. *In vitro* studies were carried out in lymphocyte cell culture.

### 3.3.1 Bone marrow chromosomal aberration assay

#### 3.3.1.1 Treatment of animals

A total of 60 rats were randomly divided into five equal groups.

Out of twelve rats in each group, six were treated orally once with single dose of hexaconozole where as the remaining six rats were subjected to five oral exposures on five consecutive days. The animals were sacrificed 24 hours after the last dose in either case (Preston *et. al.*, 1987a).

The doses of hexaconazole selected in the present study were 182, 365, and 730 mg/kg for male rats and 506, 1012,and 2024 mg/kg for females. These doses were corresponding to 1/12th, 1/6th and 1/3rd, respectively of earlier reported (Tomlin, 1995) oral LD50 values, which were 2189 mg/kg in males and 6071 mg/kg in females.

The drug was dissolved in dimethyl sulfoxide (DMSO) (S.D.Fine-chem Ltd.) so as to make 12.5, 25 and 50% solutions which enabled to administer an equal volume of the drug solution in relation to their body weight for all the groups of animals.

Cyclophosphamide (Sigma, USA) was used as a positive control at 25 mg/kg body weight, given intraperitoneally, dissolved in distilled water to make a final concentration of 1mg/ml. Solvent control animals received DMSO at the rate of 1ml/kg body weight orally.

### 3.3.1.2 Grouping of animals

Sixty rats of either sex, aged between 10-12 weeks were randomly assigned to five equal groups as detailed below:

| Group No | No.of animals* | Treatment |
|---|---|---|
| I | 12 | Dimethylsulfoxide (solvent control) (Both sexes : 1 ml/kg) |
| II | 12 | Hexaconazole (Low dose) (Male : 182mg/kg Female:506 mg/kg) |
| III | 12 | Hexaconazole (Medium Dose) (Male : 365 mg/kg Female: 1012 mg/kg) |
| IV | 12 | Hexaconazole (High Dose) (Male:730 mg/kg Female:2024 mg/kg) |
| V | 12 | Cyclophosphamide (Positive control) (Both sexes : 25 mg/kg) |

* Six animals in each group were sacrificed 24 hrs after first exposure

### 3.3.1.3 Harvesting of bone marrow

Rats were administered with colchicine (Sigma, USA) @ 2mg/kg intraperitoneally two hours before sacrificing. The rats were sacrificed under ether anaesthesia and the bone marrows were harvested as described by Preston *et al.* (1987a).

Both femurs were quickly removed from the sacrificed animals and were cleaned of muscle mass. The femurs were placed on the edge of a renumbered plastic centrifuge tube which corresponded to the animal number.

Then both ends of the femurs were crushed with bone forceps in the inner lip of the tube and the fragments were washed down into the test tube with little quantity of normal saline prewarmed to 37°C. Now bone marrow cells from the shaft of each femur were flushed thoroughly, with prewarmed (37°C) normal saline using a syringe fitted with a 22-g needle. Flushing was done until no bone marrow remained attached to bone. In all the test tubes final volume of the suspension was adjusted to 10 ml, with the same normal saline.

To ensure free suspension of marrow cells in normal saline, the test tubes were placed on a cyclomixer for about a minute. The test tubes were then left undisturbed for 3 minutes to allow the fragments to settle to the bottom. The suspension was then decanted to the other corresponding tube and centrifuged for about 4 minutes near 800 rpm. The supernatant was slowly removed by gentle aspiration, leaving a small volume over the pellet. Then the pellet was resuspended in the remaining volume.

### 3.3.1.4 Hypotonic treatment

About 5 ml of freshly prepared 0.075M potassium chloride, prewarmed to 37°C was added drop wise to the resuspended pellet by a fine Pasteur pipette with constant agitation. The test tubes were then incubated for 20 minutes at 37°C in a water bath.

### 3.3.1.5 Fixation

Fixative containing methanol and glacial acetic acid mixed in the ratio of 3:1 was prepared freshly. About 0.5 ml of the above prepared fixative was added to each tube, drop wise with agitation. The tubes were allowed to stand at room temperature for 15-20 minutes and were subjected to centrifugation at 800 rpm for 4 minutes to obtain the pellet. The supernatant was gently aspirated out and the pellet was resuspended in the remaining small volume of solution. About 2.0 ml of fixative was added to the tubes drop wise with agitation, centrifuged at 800 rpm for 4 minutes and pellet obtained. The above step was repeated twice and the resuspended pellet was finally made upto a volume of 0.5ml with fixative.

### 3.3.1.6 Slide preparation

Two drops of the suspension were dropped on to a clean chilled wet slide placed at an angle and the slides were dried over slide warmer. The slides were then stained in Giemsa (1:40) for 3 to 4 minutes.

### 3.3.1.7 Screening of aberrant cells

The slides were coded and scored for chromosomal aberrations. About 100 metaphase spreads were analysed for each animal. The aberrations in hexaconazole treated groups were compared with concurrent negative controls.

### 3.3.2 The micronucleus test

Treatment and grouping of animals for the micronucleus test were similar to those described in the bone marrow chromosomal aberrations assay.

#### 3.3.2.1 Extraction of bone marrow and preparation of slides

The bone marrow smears were prepared as per the procedure of Schimid (1975). Both the femurs were removed, cleared of muscle tissue and both the ends of each femur were chipped off with forceps. The marrows were thoroughly flushed into clean test tubes using a syringe and needle, that was loaded with 5 ml of fetal calf serum (Hi-media). The cells were then suspended by vigorous agitation of the tubes on a cyclomixer for 30 sec. After centrifugation at 800 rpm for 5 minutes, the supernatant serum was discarded and the pellet was resuspended in a small volume of fresh serum. A drop of this suspension was smeared onto a clean glass slide and air dried. The slides were then immediately fixed in methanol for 10 minutes and stained in May-Gruenwald stain followed by Giemsa as described by Schimid. (1975).

#### 3.3.2.2 Evaluation of slides

One thousand polychromatic erythrocytes (PCEs) per animal were screened under oil immersion and the micronucleated PCEs (MNPCEs) were counted.

### 3.3.3 The lymphocyte cell culture

The lymphocyte cell cultures were carried out in RPMI 1640 medium as detailed by Preston *et al.,* (1987b).

Blood was collected from the rats, under light ether anaesthesia through heart puncture in heparinised tubes. Cultures were set up in sterile 15 ml culture tubes. RPMI 1640 medium (Sigma USA), 7.5 ml of was taken in culture tubes and 1.5 ml of fetal calf serum (Sigma, USA), 7 drops of PHAP (Sigma USA) and 0.75 ml of heparinized blood were added. To these tubes hexaconaxzole dissolved in DMSO (Sigma, USA) was added to give a final concentration of 125, 250 and 500 µg hexaconazole/ml of total culture. Mitomycin (Sigma, USA) in distilled water was added to another set of tubes to give a final concentration of 0.1µg/ml of total culture, which served as a positive control. Solvent control cultures were added with DMSO. In all the cases the total volume of the drug solution was adjusted to 0.04 ml. All the tubes were then incubated at 37°C in a slanting position in an incubator for 72 hrs. The contents of the tubes were mixed gently, twice daily. Two hours before the harvest of cells (i.e., on 70th hour from the start of culture) all the tubes were added with colchicine (Sigma, USA) to achieve a final concentration of 0.05µg/ml of medium. After the end of 72 hours of incubation the tubes were centrifuged at 1000 rpm for 10 minutes, which gave a small cell pack at the bottom. The supernatant was gently aspirated out and the cells were resuspended in the remaining small volume of medium. The tubes were then added with 7.5 ml of 0.075 M potassium chloride, that was freshly prepared and kept at 37°C. The tubes containing the hypotonic solution were incubated at 37°C for 30 minutes. Following incubation, the tubes were centrifuged at 1000 rpm for 10 minutes to obtain the cell pack. The resuspended cells were added with 5 ml of freshly prepared and chilled fixative (methanol 3 parts and glacial acetic acid 1 part). The tubes containing the cells in fixative were then

left in the refrigerator (4°C) overnight. Next day the tubes were centrifuged at 1000 rpm for 10 minutes and supernatant was gently aspirated out. Fresh fixative washes were given as many times as required to give a whitish cell button at the bottom of the tube. The cell button was resuspended in about 0.5 ml fresh fixative. Three to four drops of the cell suspension from each tube were dropped separately on clean chilled wet slides, from a height of about three feet. The slides were then waved to shake off excess fluid and air dried. The slides were stained with 4% Giemsa for 30 minutes. Two hundred chromosome spreads per each treatment were analysed for aberrations.

## 3.4 MALE REPRODUCTIVE TOXICITY

### 3.4.1 Grouping and treatment

Eighty male rats (10-12 weeks old) were randomly assigned to four groups each consisting of 20 rats. Group I served as control and the rats were given refined ground nut oil, *per os*, @ 1ml/kg body weight. Rats in group II,III and IV were administered hexaconazole, *per os*, as suspension in refined ground nut oil @ 27.0, 55.0 and 110.0 mg/kg body weight respectively. These doses were 1/80th, 1/40th and 1/20th LD 50 (2189 mg/kg) in male rats.

Ten animals in each group received the treatment for 30 days and the remaining ten rats continued to receive the drug for 60 days. During the treatment, weekly body weights were recorded and the animals were observed for any mortality and abnormal signs. At the end of the treatment (i/e., either 30 days or 60 days ) the animals were sacrificed under ether anaesthesia.

### 3.4.2 Collection of blood and organs

Blood was collected through heart puncture under light ether anaesthesia and the serum was decanted off for estimation of testosterone, and other biochemical profiles. Testes, epididymides, prostate and seminal vesicles were collected following a vertical incision on the lower abdomen. Weights of testes, prostate and seminal vesicles were recorded and testes were preserved for histopathological examination. The testes, seminal vesicle and prostate gland were weighed upto the nearest milligram and the organ weights calculated per 100g body weight by using the formula, organ weight/body weight x 100.

### 3.4.3 Epididymal sperm count

Epididymal sperm collection was carried out as per Abd - Allah *et al.* (2000) and counting was done as described by Freund and Carol (1964). The two cauda epididymides from each rat were placed in 4 ml of normal saline prewarmed to 37°C. Small cuts were made in the two cauda epididymides and the spermatozoa were suspended in the saline. A small amount of the diluted suspension was transferred to the chambers of a Neubar haemocytometer using a Pasteur pipette by touching the edge of the cover slip, allowing each chamber to be filled by capillary action.

### 3.4.4 Sperm motility

A drop of the above collected semen was placed on a clean slide and covered with a cover slip. A total of 200 sperm per each animal were counted for motility at x 400.

### 3.4.5 Live and dead sperm count

A drop of the epididymal sperm suspension was placed on one edge of a slide and mixed with a drop of 3 per cent eosin using a glass rod. Immediately this mixture was again mixed with a drop of 10 per cent nigrosin using the same glass rod. A small quantity of this was smeared on another slide and air dried. Two hundred sperm per each animal were counted at x 400 and live and dead sperm numbers were recorded.

### 3.4.6 Abnormal sperm count

A drop of diluted epididymal sperm suspension was smeared on a slide and stained with 3 per cent rose bengal stain for 10 min and two hundred sperm per animal were observed for morphological defects at x 400.

### 3.4.7 Histopathological examination

Testes were fixed in Bouin's fluid for 24 hours and later transferred to 70 per cent alcohol until paraffin embedding and sectioning was done. The sections were stained with hematoxylin and eosin.

### 3.4.7.1 Quantitative histopathology

The slides were analysed for number of seminiferous tubules per microscopic field, mean tubular diameter and percentage of damaged tubules.

Total number of seminiferous tubular cross sections and damaged tubules, per microscopic field were counted in 5 fields/testis at a magnification of 10 x 10. Testicular damage was assessed by determining the percentage of damaged tubules against the total tubules counted. Twenty circular cross sectioned seminiferous tubules per testis were randomly selected and the tubular diameter was measured at a magnification of 10 x 10.

### 3.4.8 Assay of serum testosterone, estradiol and progesterone levels

Serum testosterone, estradiol and progesterone levels were assayed by radio immuno assay (RIA) method as described by Mukku *et al.* (1981) and Jagannadharao *et al.* (1984).

### 3.4.8.1 Hormones and chemicals

The unlabelled testosterone, 17ß - estradiol and progesterone, were obtained from Steroloids Inc, USA. (2,4,6,7) - 3H-estradiol - 17ß testosterone and progesterone were purchased from Radiochemical Center, Amersham, UK. The specific activities of the tritiated testosterone, estradiol and progesterone used in the study were 94 Ci/mmol, 92 Ci/mmol and 99 Ci/mmol respectively. Activated charcoal, 2,5 diphenyl oxazole (PPO), 1,1-bis (1-methyl-5 phenyl-2 oxazolyl) benzene (POPOP), were obtained from Sigma Chemicals, USA.

Gelatin was purchased from Difco Laboratory, USA and Dextran 170 from Pharmacia Fine Chemicals, Uppsala, Sweden. Other chemicals like diethyl ether and methanol were obtained form Sarabhai Chemicals, India.

### 3.4.8.2 Steroid extraction from serum

Serum samples were extracted twice with 100 µl of methanol and 4 ml of diethyl ether, by vortexing vigorously each time for 90 seconds. The lower aqueous phase was frozen by immersing the tubes in a breaker of liquid nitrogen. The upper ether layer was transferred to separate tubes and evaporated to dryness in a water bath at 40°C. The dried residue was reconstituted in 1.0 ml GPBS (0.01 M phosphate buffer, pH 7.4 containing 0.1% gelatin, 0.9% sodium chloride and 0.01% merthiolate). Suitable aliquot from this were used for radio immuno assay.

### 3.4.8.3 Antisera

The testosterone, estradiol and progesterone antisera used in the present study were kind gift from Prof.A.J.Rao (Department of Biochemistry, Indian Institute of Science, Bangalore). The estradiol antiserum cross reacted with estrone and estriol to the extent of 10% and 1% respectively. The progesterone antiserum cross reacted with 17α - hydroxy progesterone and 20α - dihydroxy progesterone to the extent of 3% and 5% respectively.

### 3.4.8.4 Procedure of steroid RIA

The steroid radio immunoassay consisted of incubating the sample aliquot or standards of appropriate steroid in 10µl GPBS (pH 7.4) with 0.1 ml of tritiated testosterone, progesterone or estradiol (10000 cpm/tube) and 0.1 ml of homologous antiserum (diluted to give 25 to 30 % net binding with labeled hormone) in a total volume of 0.4 ml at 4°C for 6 h. Following incubation, 300µl of chilled dextran coated charcoal (10mM phosphate buffer saline, pH 7.4 containing 1% activated charcoal and 0.1% dextran T70) was dispensed to each tube and shaken well. The mixture was incubated at 4°C for 10 minutes. After incubation, the samples were centrifuged at 2500 g for 15 minutes using swing out rotor in a refrigerated centrifuge. The supernatant was then transferred to vials containing 3 ml of scintillation cocktail (5 g PPO, 100 mg POPOP in 1000 ml of toluene containing 2 ml of methanol), shaken well and radioactivity counted in LKB liquid scintillation counter . Each assay consisted of determining nonspecific binding of labeled hormone in the presence of excess cold hormone, specific binding of labelled hormone to antisera in the presence and absence of different concentrations of standard steroid or samples. After deducting the nonspecific binding from all the values, net specific binding was obtained. This was converted into the percentage of net specific binding obtained in the presence of unlabelled hormone. The percentages obtained in presence of standard concentrations of unlabelled steroid were plotted against log concentrations of steroid used to obtain a standard curve. The percentage of specific binding for samples were read on this standard curve to determine the concentration of steroid in the sample.

## 3.5 FEMALE REPRODUCTIVE TOXICITY

Toxicity of hexaconazole to the female reproductive system was assessed through the monitoring of estrus cycles, reproductive indices, serum estradiol and progesterone levels and histopathology of reproductive organs.

### 3.5.1 Grouping and treatment of animals

Adult Wister strain female rats aged 90-110 days, were used in the study. They were randomly divided into four groups, each having 20 rats. All the rats were previously screened for normal estrus cyclicity. One group served as control and received the vehicle, corn oil *per os* @ 1ml/kg body weight and the remaining three groups received hexaconazole suspension in corn oil @ 27.5, 55.0 and 110.0 mg/kg *per os* once daily until the sacrifice. Of the 20 rats in each group, 10 rats were subjected to daily estrus cycle monitoring from day 16 to 30. Following this, these 10 rats were sacrificed on the mid day of proestrus. The remaining 10 rats in all the groups were used for further reproductive studies. These rats were allowed to mate with untreated male rats in the ratio of 1:2. Every morning vaginal smears were examined for the presence of sperm. Those found negative were returned to the male. Male rats were often interchanged among the females. Irrespective of the outcome of mating, the mating was discontinued after two cycles. Treatment was continued through the mating, gestation and postnatally, until the end of lactation and weaning on day 21.

### 3.5.2 Monitoring of estrous cycle

Sterile cotton swabs soaked in sterile normal saline solution were introduced into the vagina and rotated gently along the vaginal walls. Too deep insertion and excessive manipulation inside the vagina were avoided to avert pseudopregnancy and disruption of normal estrus cycle (Manson and Kang, 1989). The swabs were then squeezed on a clean glass slide for microscopic examination of cytology, under low power, as per Manson and Kang (1989). Estrus was characterized by masses of cornified calls with degenerating nuclei, metestrus by many leucocytes and epithelial cells, diestrus by almost exclusively leucocytes and proestrus by nucleated epithelial cells, singly or in sheets.

### 3.5.3 Confirmation of mating

The female was considered mated if sperm were found in the vaginal smear and that day was designed 0 day of pregnancy. (Manson and Kang, 1989).

### 3.5.4 Reproductive indices

All the dams were allowed to deliver naturally. The pups were examined for litter size, viability and weight. Reproductive indices were calculated as per Lu (1996).

a. Fertility index : $\frac{\text{No.of pregnant animals}}{\text{No.of females mated}} \times 100$

b. Parturition index : $\frac{\text{No.of females delivered}}{\text{No.of pregnant animals}} \times 100$

c. Gestation index : $\frac{\text{No.of pups born alive}}{\text{No.of total pups born}} \times 100$

d. Viability index : $\frac{\text{No.of pups alive at day 4}}{\text{No.of pups born alive}} \times 100$

e. Lactation index : $\frac{\text{No.of pups alive at day 21}}{\text{No.of pups alive at day 4}} \times 100$

### 3.5.5 Collection of blood and reproductive organs

Blood was collected through heart puncture under light ether anaesthesia. Reproductive organs, *viz*, ovaries, uteri and vagina were collected through lower abdominal opening.

### 3.5.6 Estimation of serum estradiol and progesterone levels

Serum estradiol and progesterone levels were estimated as described earlier.

## 3.6 HEPATOTOXICITY

Rats used in the male and female reproductive toxicity studies were used for the assessment of hepatotoxic potential. Blood and liver were collected of necropsy for serum biochemical studies and histopathology, respectively.

### 3.6.1 Serum biochemical profiles

#### 3.6.1.1 Serum aspartate amino transferase (AST or GOT)

The activity of serum AST was estimated by the method of Reitman and Frankel (1957) using commercial Span diagnostic kits (Span Diagnostics Ltd.Sachin, Surat)

#### 3.6.1.2 Serum alanine aminotransferase (ALT or GPT)

The activity of serum ALT was estimated by the method of Reitman and Frankel (1957) employing Span diagnostic kits (Span Diagnostics Ltd., Sachin, Surat)

#### 3.6.1.3 Serum alkaline phosphatase (ALP)

Serum ALP was determined by the method of Kind and King (1954) with Span diagnostic kits (Span Diagnostics Ltd, Sachin, Surat)

#### 3.6.1.4 Serum cholesterol

Serum cholesterol was estimated by the method of Wybenga and Pileggi (1970) with Span diagnostic kits ( Span Diagnostics Ltd, Sachin, Surat)

### 3.6.2 Histopathological examination

Liver collected at necropsy was fixed in 10% formalin. Paraffin embedded tissue sections were made and stained with hematoxylin and eosin for histopathological examination.

## 3.7 STATISTICAL ANALYSIS

Data on clastogenic studies and reproductive indices were analysed by chi square test and the data on other parameters were analysed by analysis of variance as described by Snedecor and Cochran (1968).

# Results

# CHAPTER - 4

# RESULTS

## 4.1 CLASTOGENICITY OF HEXACONAZOLE

### 4.1.1 *In vivo* clastogenic studies

#### 4.1.1.1 Bone marrow cell chromosomal aberrations

Bone marrow cell chromosomal aberrations were studied in rats treated with single dose and multiple doses of hexaconazole. One hundred spreads per animal were analysed and the observations were expressed as percentage of aberrant cells in each group. Gaps and breaks are achromatic regions and if the width of the achromatic region is greater than the width of chromatid, it was considered as a break. Though gaps were counted while analysing, they were not considered for calculating the percentage of abnormal cells. The aberrations observed are shown in plates 1 and 2 (X 1000).

In control rats belonging to single exposure study, the percentage aberrant cells were 0.17 while they were 0.33, 0.17 and 0.50 in low, medium and high dose hexaconazole groups. However, in cyclophosphamide treated, positive control animals the percentage of aberrant cells were 11.5 (vide Table 1 and Figure 3). Statistical analysis showed that hexaconazole did not produce any significant increase in the aberrant cells, at the three dose levels studied. The increase in positive control group was highly significant ($P < 0.01$) compared to control animals.

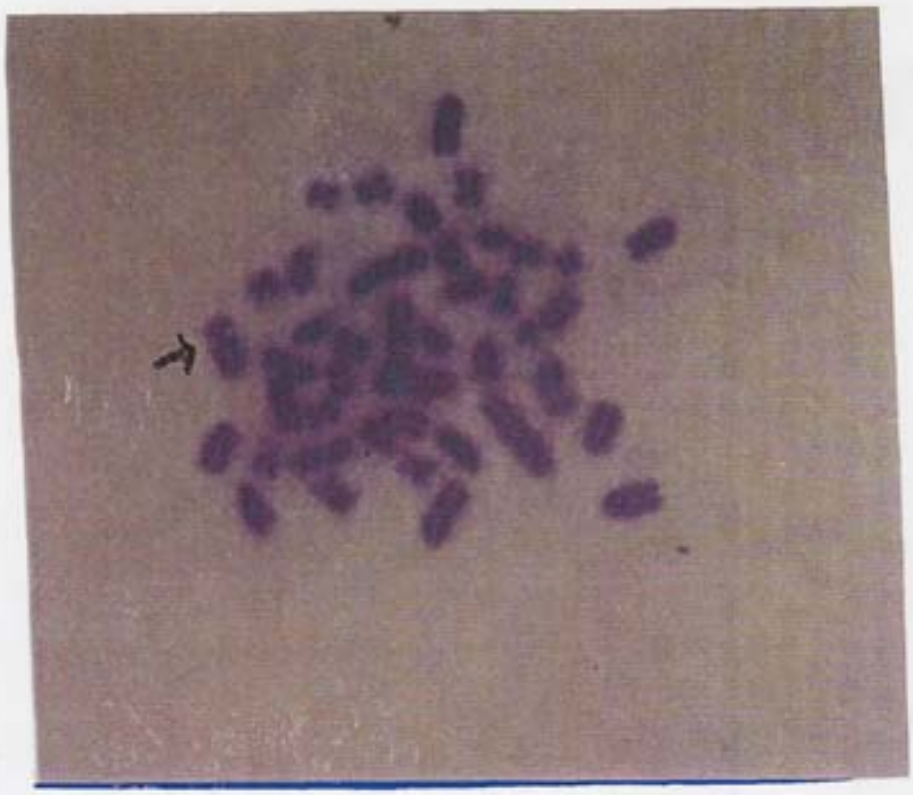


Plate 1 Photomicrograph showing (arrow) a break in metaphase spread of bone marrow cell of a treated rat

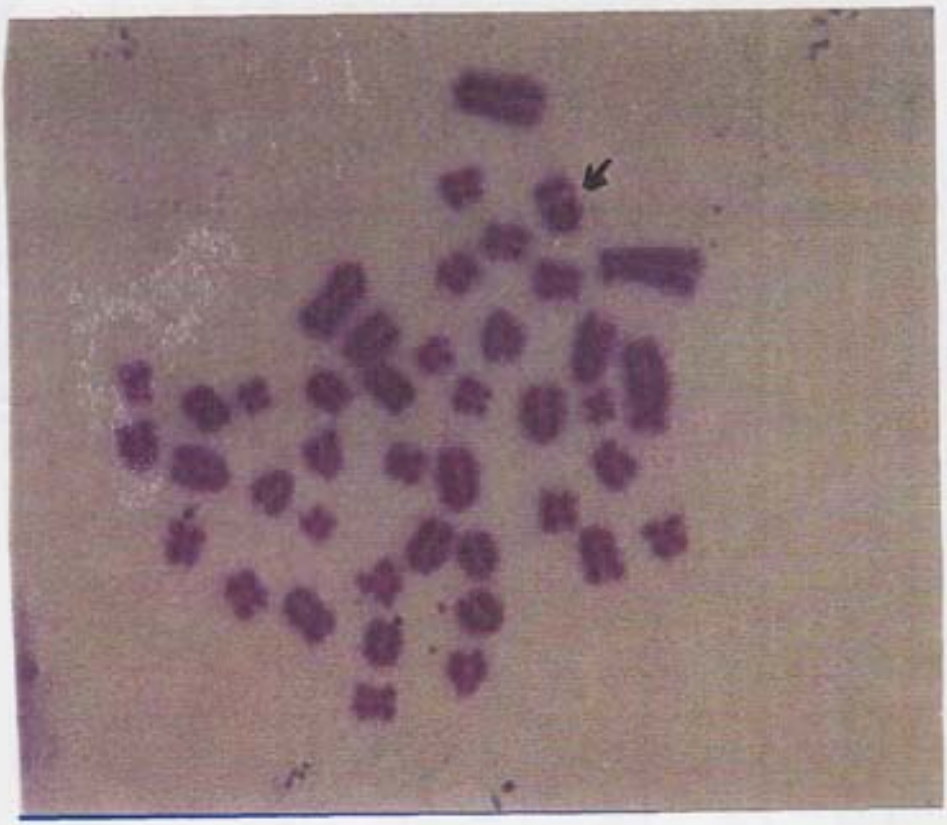


Plate 2 Photomicrograph showing (arrow) a gap in metaphase spread of bone marrow cell of a treated rat

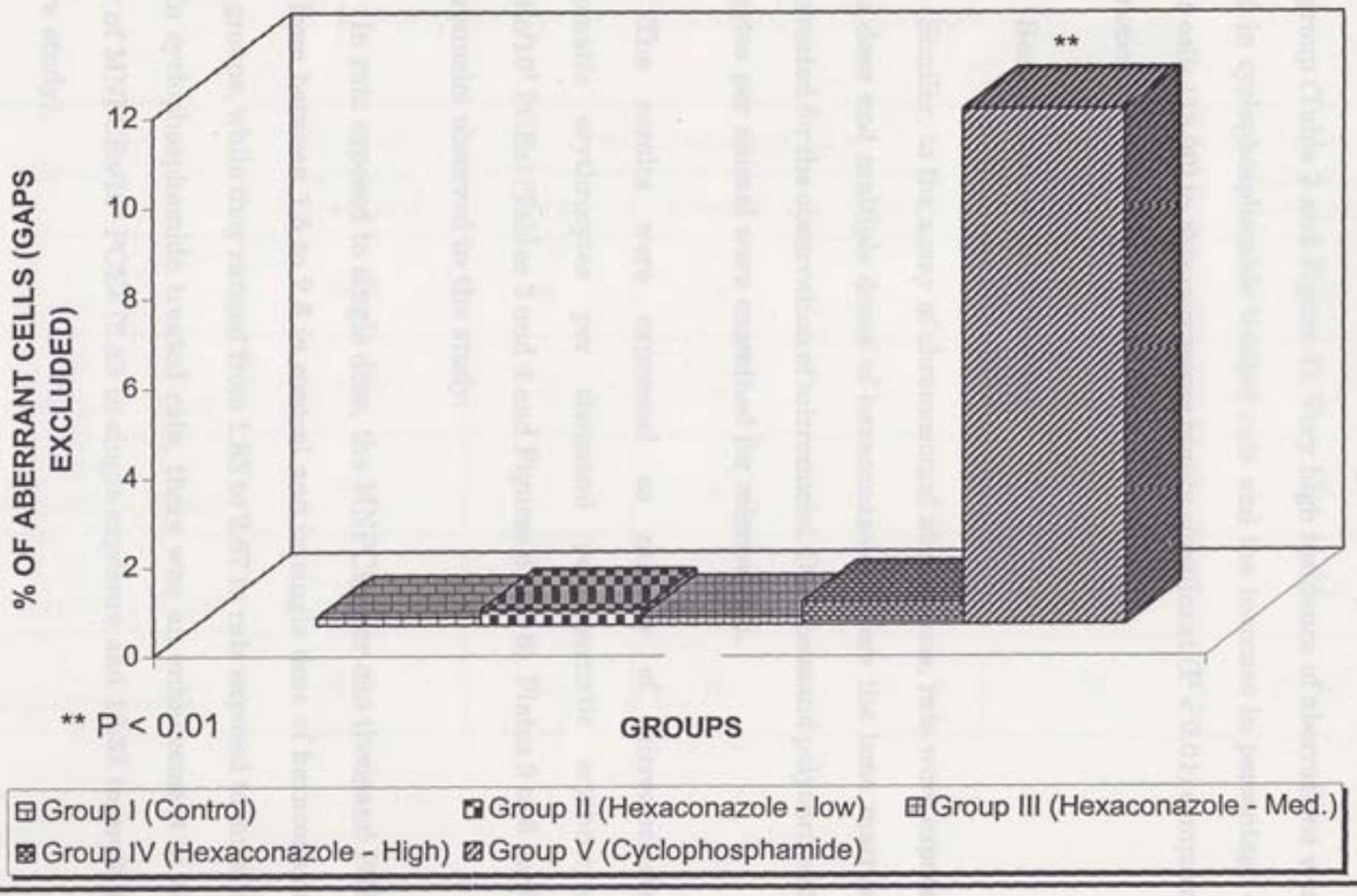
**TABLE 1**

**EFFECT OF HEXACONAZOLE (SINGLE EXPOSURE) ON CHROMOSOMAL ABERRATIONS IN BONE MARROW CELLS OF RAT**

| Group | No. of spreads analysed | No. of aberrations | | | No. of aberrant cells | % of aberrant cells (Gaps excluded) |
|---|---|---|---|---|---|---|
| | | Gaps | Breaks | Others | | |
| I (Control) | 600 | 2 | 1 | - | 1 | 0.17 |
| II (Hexaconazole - low) | 600 | 1 | 2 | - | 2 | 0.33 |
| III (Hexaconazole - Med.) | 600 | 2 | 1 | - | 1 | 0.17 |
| IV (Hexaconazole - High) | 600 | 4 | 3 | - | 3 | 0.50 |
| V (Cyclophosphamide) | 600 | 38 | 69 | - | 69 | 11.50** |

** $P < 0.01$

## FIGURE 3 : EFFECT OF HEXACONAZOLE (SINGLE EXPOSURE) ON CHROMOSOMAL ABERRATIONS IN BONE MARROW CELLS OF RAT



The percentage of aberrant cells in rats exposed to multiple doses of hexaconazole treated rats did not differ significantly with that observed in control group (Table 2 and Figure 4). Very high incidence of aberrations were observed in cyclophosphamide treated rats and the increase in percentage of aberrant cells (13.00) in this group was highly significant ($P < 0.01$) compared to that noticed in control group (0.33).

#### 4.1.1.2 Bone marrow micronuclei

Similar, to the assay of chromosomal aberrations, rats were exposed to single dose and multiple doses of hexaconazole, before the bone marrows were harvested for the observation of micronuclei. One thousand polychromatic erythrocytes per animal were examined for micronuclei.

The results were expressed as number of micronucleated polychromatic erythrocytes per thousand polychromatic erythrocytes (MNPCEs/$10^3$ PCEs) (Tables 3 and 4 and Figures 5 and 6). Plates 3 to 6 depict the micronuclei observed in the study.

In rats exposed to single dose, the MNPCEs per one thousand PCEs ranged from between 1.5 to 2.6 in control and in single dose of hexaconazole treated groups, while they ranged from 1.83 to 2.67 in rats exposed to multiple doses. In cyclophosphamide treated rats, there was an enhancement in the number of MNPCEs/$10^3$ PCEs (8.83 in single exposure and 10.33 in multiple exposure study).

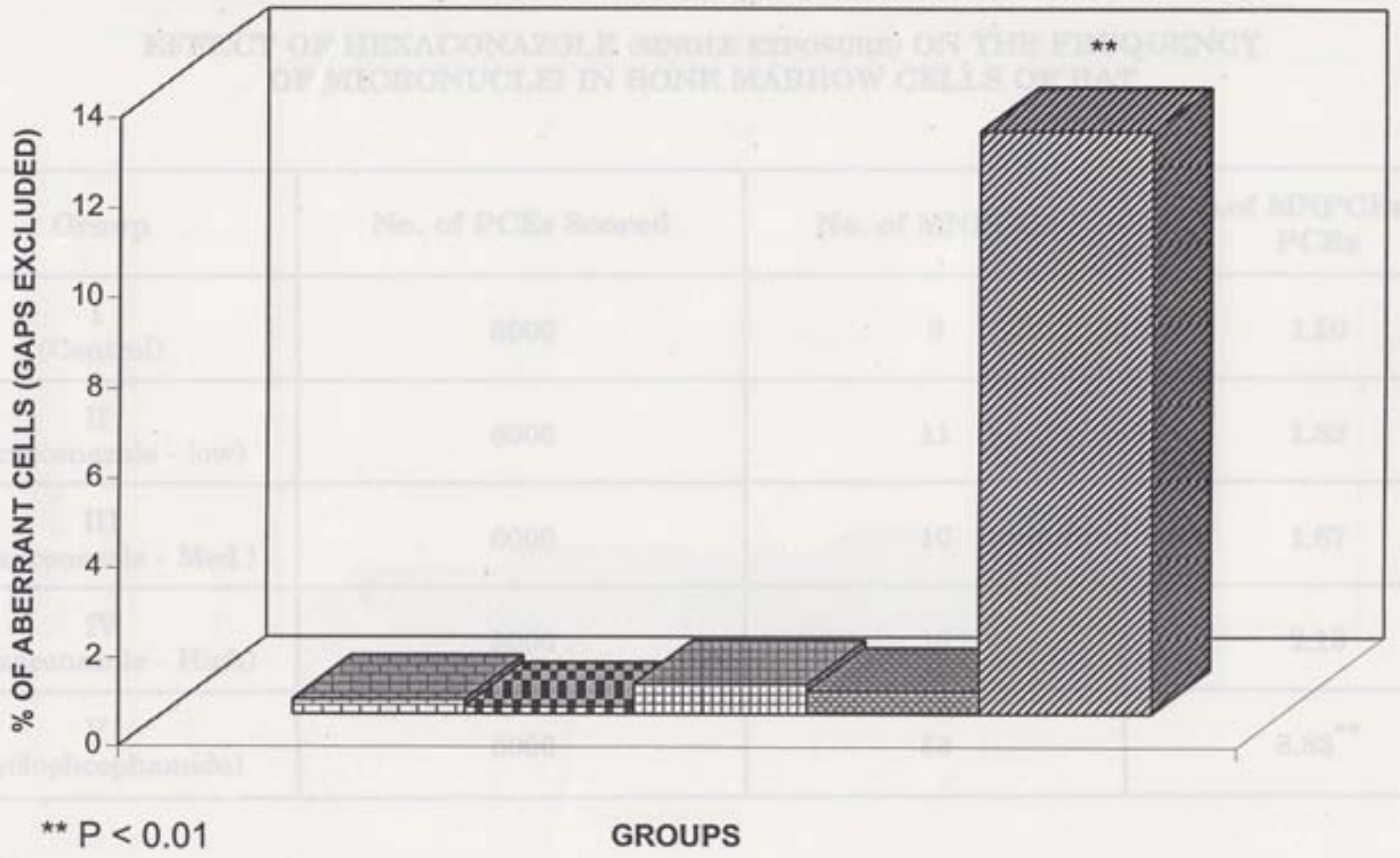
## TABLE 2

## EFFECT OF HEXACONAZOLE (MULTIPLE EXPOSURES) ON CHROMOSOMAL ABERRATIONS IN BONE MARROW CELLS OF RAT

| Group | No. of spreads analysed | No. of aberrations | | | No. of aberrant cells | % of aberrant cells (Gaps excluded) |
|---|---|---|---|---|---|---|
| | | Gaps | Breaks | Others | | |
| I (Control) | 600 | 3 | 2 | - | 2 | 0.33 |
| II (Hexaconazole - low) | 600 | 2 | 1 | - | 1 | 0.17 |
| III (Hexaconazole - Med.) | 600 | 3 | 4 | - | 4 | 0.67 |
| IV (Hexaconazole - High) | 600 | 5 | 3 | - | 3 | 0.50 |
| V (Cyclophosphamide) | 600 | 41 | 76 | 2 | 78 | 13.00** |

** $P < 0.01$

## FIGURE 4 : EFFECT OF HEXACONAZOLE (MULTIPLE EXPOSURES) ON CHROMOSOMAL ABERRATIONS IN BONE MARROW CELLS OF RAT



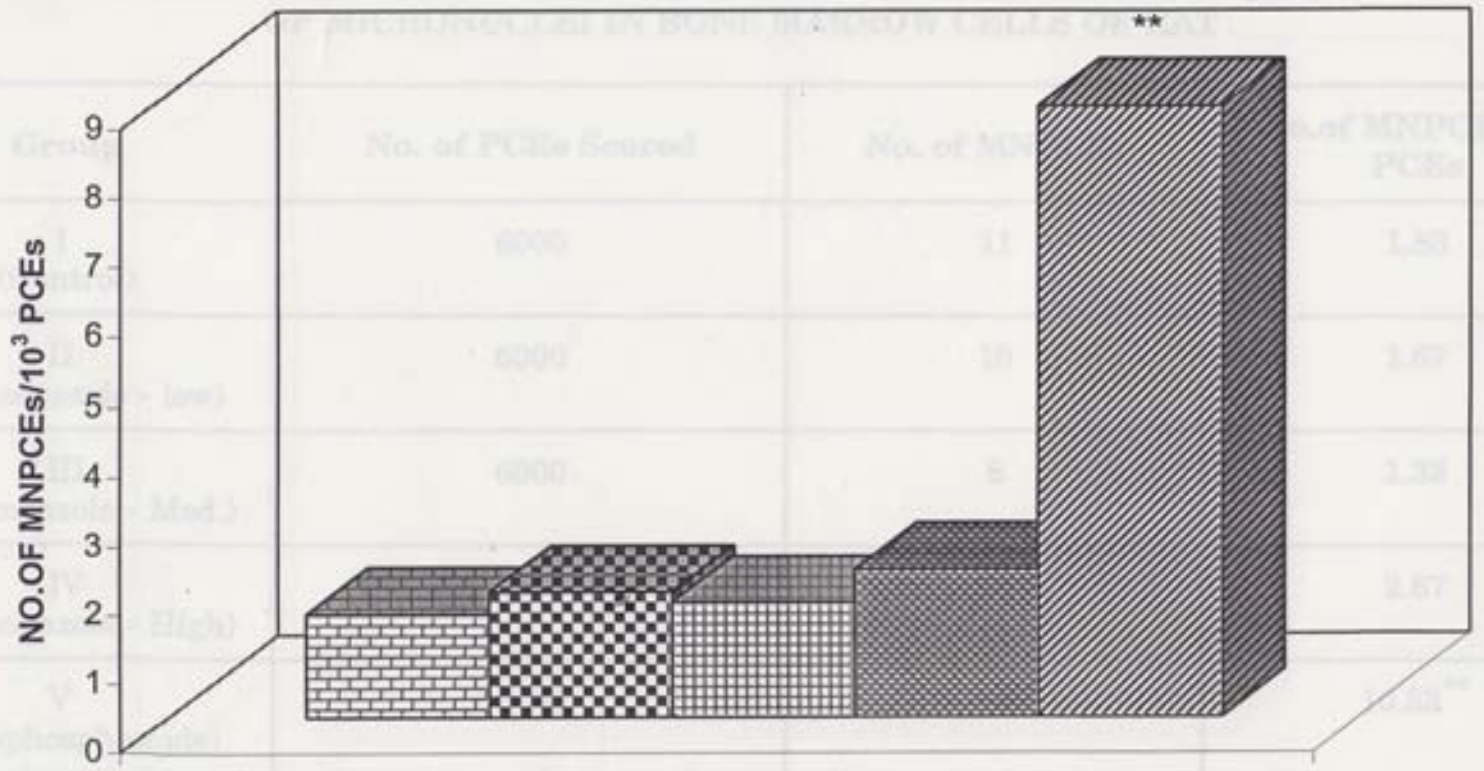
## TABLE 3

## EFFECT OF HEXACONAZOLE (SINGLE EXPOSURE) ON THE FREQUENCY OF MICRONUCLEI IN BONE MARROW CELLS OF RAT

| Group | No. of PCEs Scored | No. of MNPCEs | No.of MNPCEs/$10^3$ PCEs |
|---|---|---|---|
| I (Control) | 6000 | 9 | 1.50 |
| II (Hexaconazole - low) | 6000 | 11 | 1.83 |
| III (Hexaconazole - Med.) | 6000 | 10 | 1.67 |
| IV (Hexaconazole - High) | 6000 | 13 | 2.16 |
| V (Cyclophosphamide) | 6000 | 53 | 8.83** |

** $P < 0.01$

## FIGURE 5 : EFFECT OF HEXACONAZOLE (SINGLE EXPOSURE) ON THE FREQUENCY OF MICRONUCLEI IN BONE MARROW CELLS OF RAT



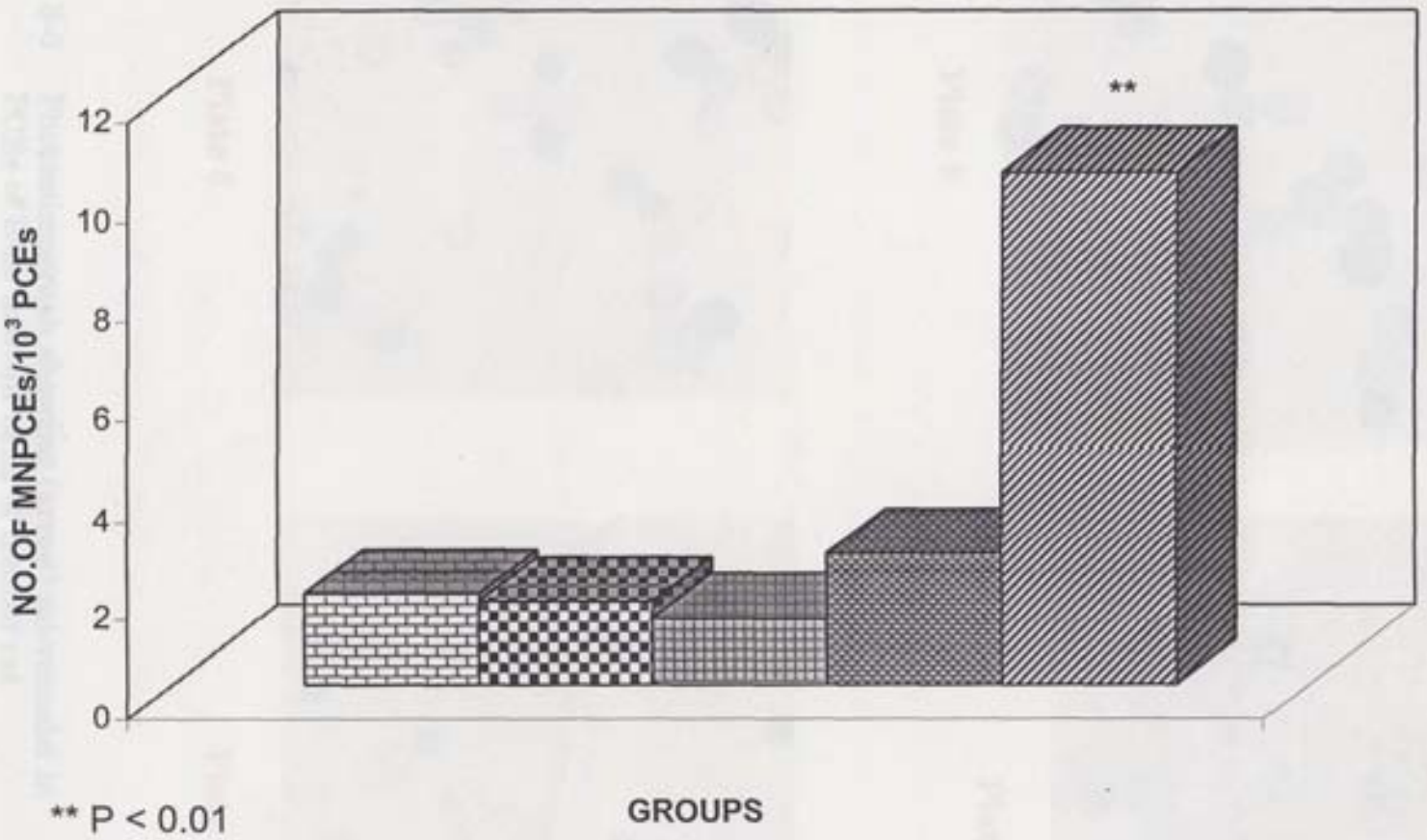
## TABLE 4

## EFFECT OF HEXACONAZOLE (MULTIPLE EXPOSURES) ON THE FREQUENCY OF MICRONUCLEI IN BONE MARROW CELLS OF RAT

| Group | No. of PCEs Scored | No. of MNPCEs | No.of MNPCEs/$10^3$ PCEs |
|---|---|---|---|
| I (Control) | 6000 | 11 | 1.83 |
| II (Hexaconazole - low) | 6000 | 10 | 1.67 |
| III (Hexaconazole - Med.) | 6000 | 8 | 1.33 |
| IV (Hexaconazole - High) | 6000 | 16 | 2.67 |
| V (Cyclophosphamide) | 6000 | 62 | 10.33** |

** $P < 0.01$

## FIGURE 6 : EFFECT OF HEXACONAZOLE (MULTIPLE EXPOSURES) ON THE FREQUENCY OF MICRONUCLEI IN BONE MARROW CELLS OF RAT



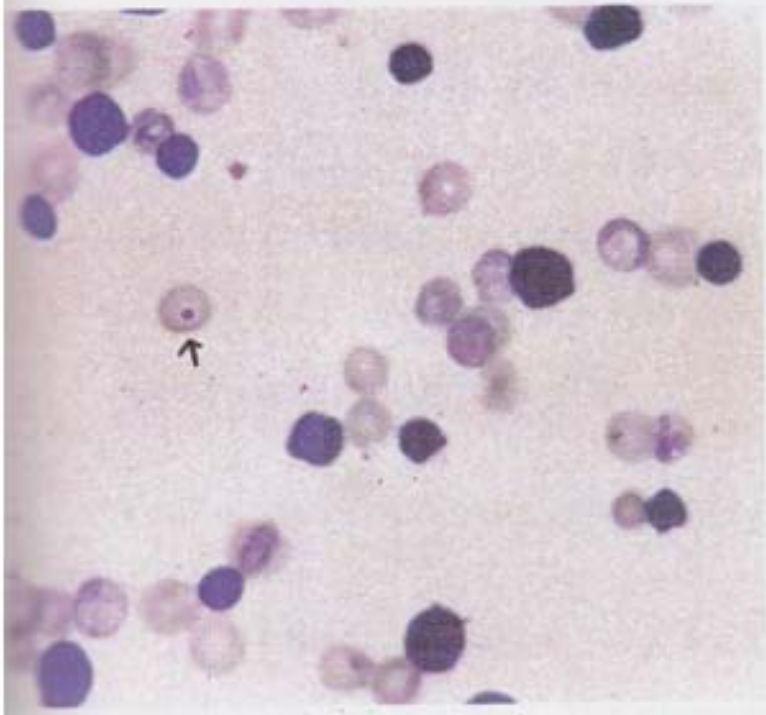


Plate 3

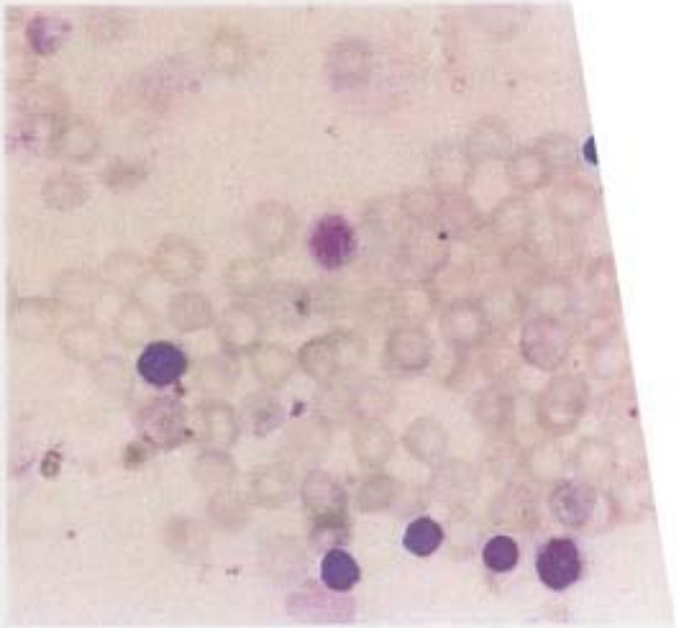


Plate 4

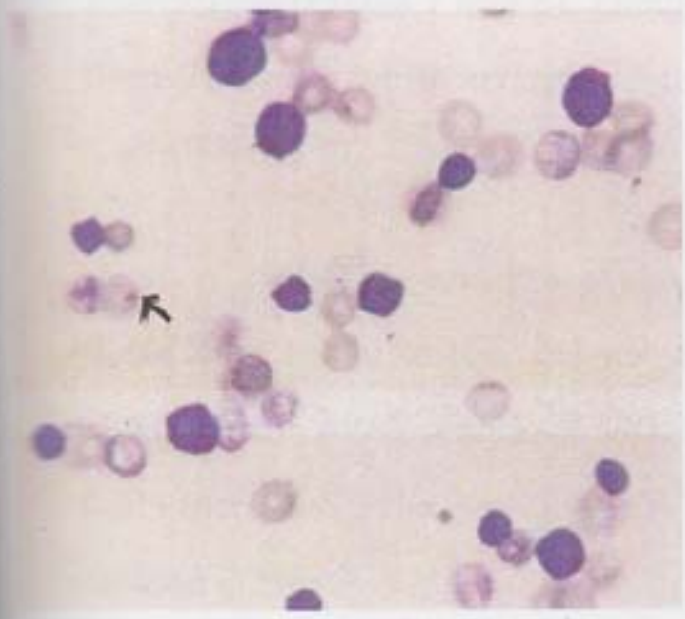


Plate 5

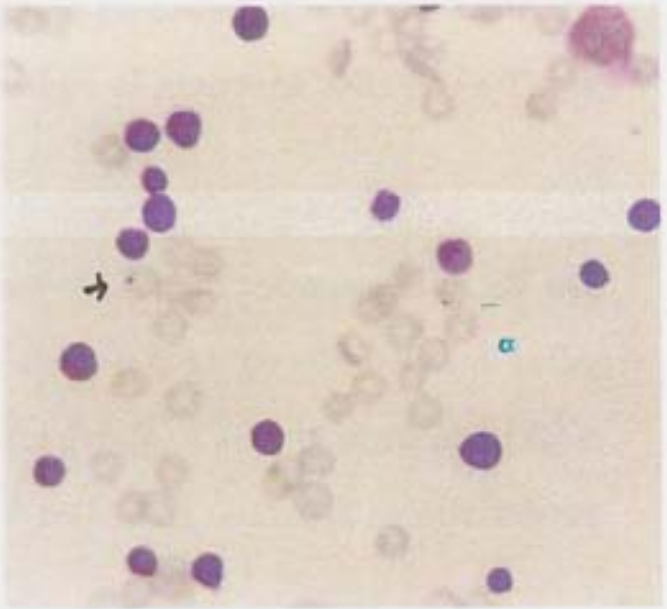


Plate 6

Plate 3-6 Photomicrograph showing (arrow) micronuclei in PCEs of bone marrow cells of treated rat

It is obvious from the results that the exposure of rats to either single dose or to multiple doses of hexaconazole for 5 days did not produce any significant increase in the incidence of micronuclei. As expected, the positive control drug, cyclophosphamide did increase the occurrence of micronuclei highly significantly ($P < 0.01$).

### 4.1.2 *In vitro* clastogenic study

Clastogenicity of hexaconazole, *in vitro*, was studied in rat blood lymphocyte cell cultures. The doses selected were 125, 250 and 500 µg/ml of total culture volume. Mitomycin (0.1 µl/ml) was used as positive control drug. From each group, two hundred chromosomal spreads were analysed for aberrations (Plates 7 and 8). Though gaps were recorded, they were excluded from the study while arriving at the percentage of aberrant cells (vide Table 5 and Figure 7).

Statistical analysis of the data showed that hexaconazole, at all the three dose levels tested, did not increase the percentage of aberrant cells, significantly, when compared with the control group. A highly significant ($P < 0.01$) increase in the percentage of aberrant cells was observed in mitomycin treated group.

## 4.2 EFFECT OF HEXACONAZOLE ON MALE REPRODUCTIVE SYSTEM

Male reproductive toxicity of hexaconazole was assessed in rats that were given the drug *per os* for 30 and 60 days. The three different doses selected were 27.5, 55.0 and 110.0 mg/kg/day.

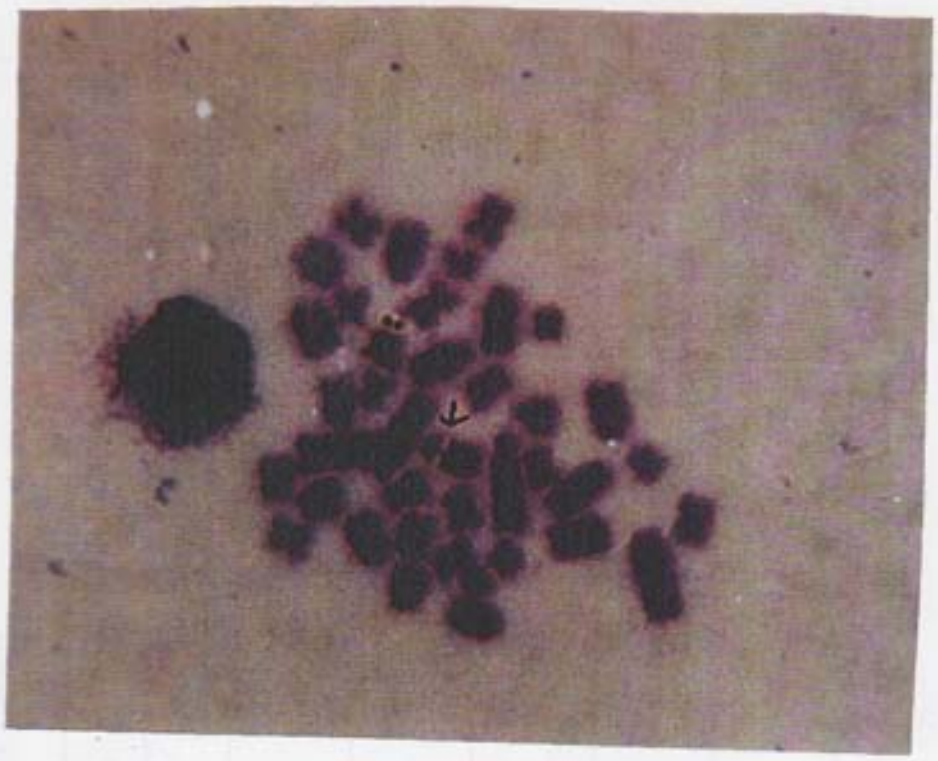


Plate 7 Photomicrograph showing (arrow) a break in metaphase spread of lymphocyte cultured *in vitro*

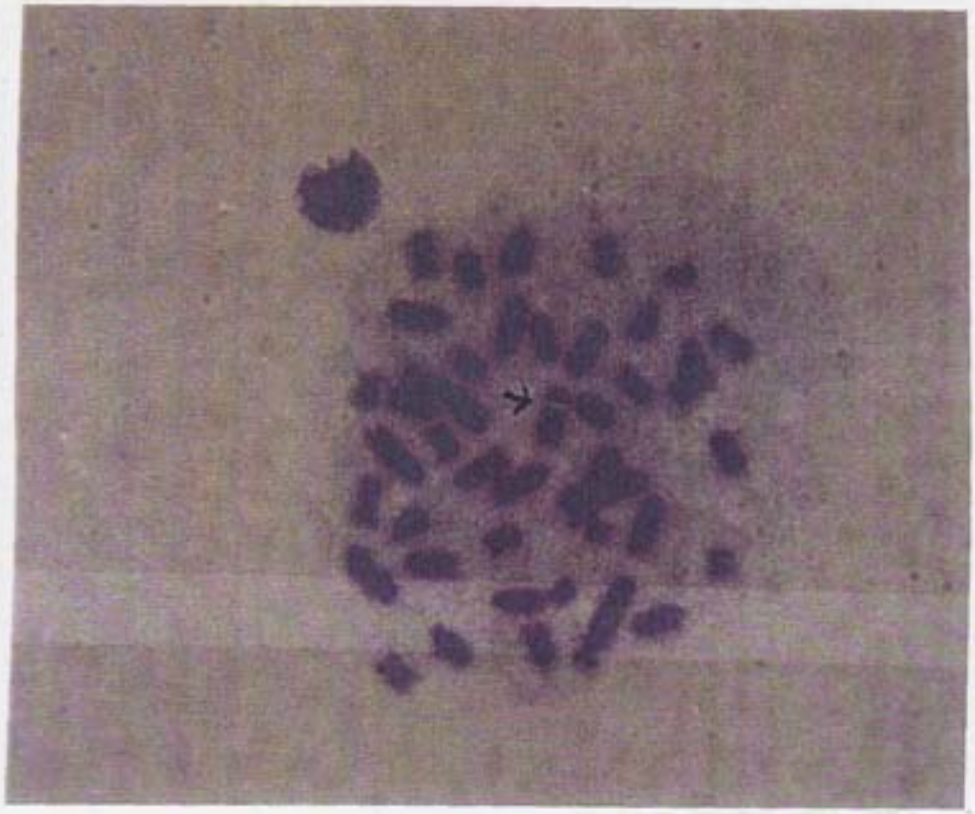


Plate 8 Photomicrograph showing (arrow) a gap in metaphase spread of lymphocyte cultured *in vitro*

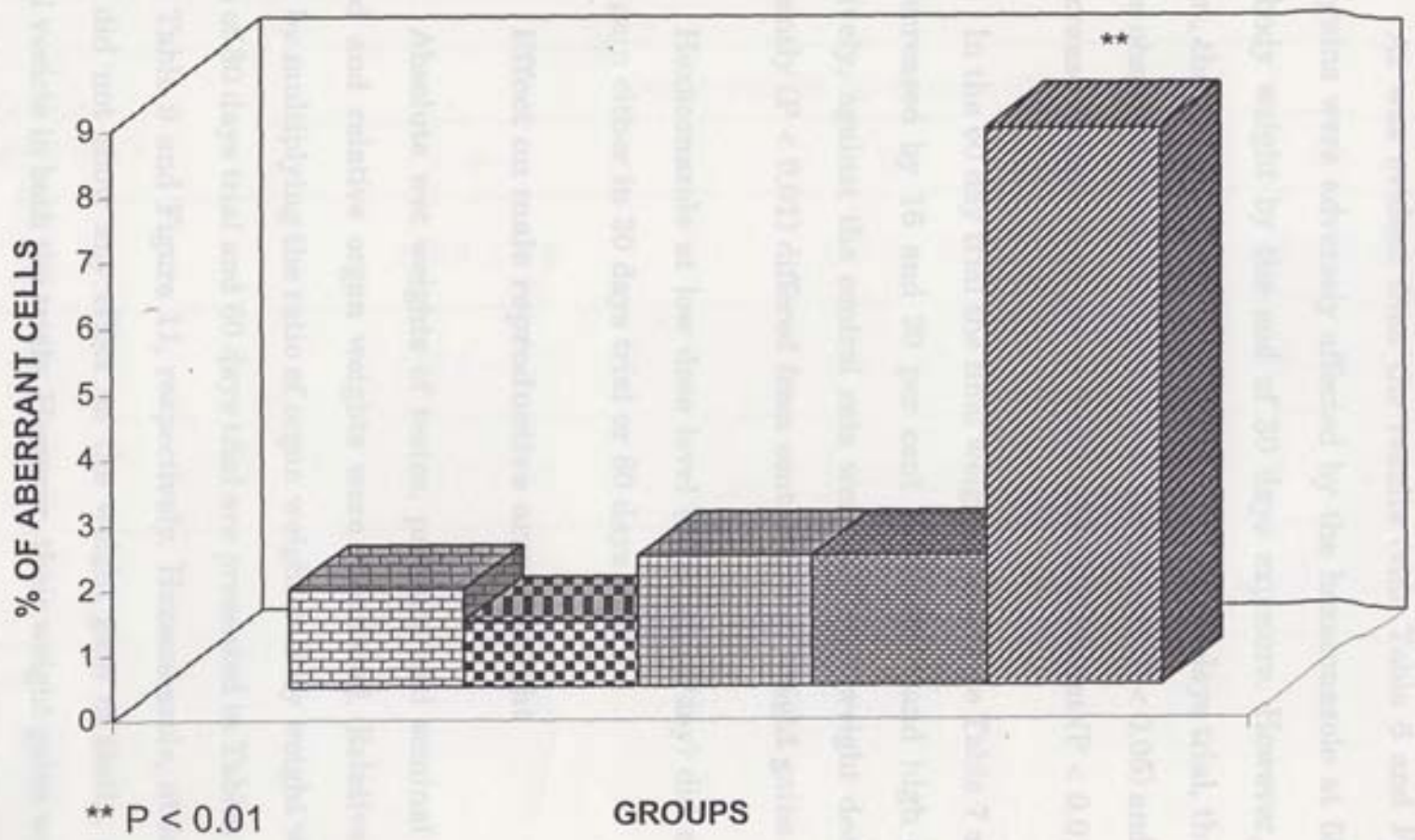
## TABLE 5

## EFFECT OF HEXACONAZOLE ON CHROMOSOMAL ABERRATIONS IN RAT LYMPHOCYTE CELL CULTURES

| Group | No.of spread analysed | No. of aberrations | | | No. of aberrant cells | % of aberrant cells |
|---|---|---|---|---|---|---|
| | | Gaps | Breaks | Others | | |
| I (DMSO) | 200 | 1 | 3 | - | 3 | 1.5 |
| II (125 µg/ml) | 200 | - | 2 | - | 2 | 1.0 |
| III (250 µg/ml) | 200 | 2 | 4 | - | 4 | 2.0 |
| IV (500 µg/ml) | 200 | 1 | 4 | - | 4 | 2.0 |
| V (Mitomycin 0.1ug/ml) | 200 | 5 | 16 | 1 | 17 | 8.5** |

** $P < 0.01$

## FIGURE 7 : EFFECT OF HEXACONAZOLE ON CHROMOSOMAL ABERRATIONS IN RAT LYMPHOCYTE CELL CULTURES



### 4.2.1 Effect on body weight gain

As was evident from the results (vide Table 6 and Figure 8), the weight gains were adversely affected by the hexaconazole at 55.0 and 11.0 mg/kg body weight by the end of 30 days exposure. However, in the first fortnight, the weight gains were not affected. In 30 days trial, the 10 per cent decrease observed in medium group was significant ($P < 0.05$) and the 14.6 per cent decrease in high dose group was highly significant ($P < 0.01$).

In the 60 day trial the final weight gains (vide Table 7 and Figure 9) were decreased by 16 and 20 per cent in medium and high dose groups, respectively, against the control rats weight. These weight declines, highly significantly ($P < 0.01$) differed from control group weight gains.

Hexaconazole at low dose level (27.5 mg/kg/day) did not effect the weight gain either in 30 days trial or 60 days trial.

### 4.2.2 Effect on male reproductive organ weight

Absolute wet weights of testes, prostate and seminal vesicle were recorded and relative organ weights were calculated. Relative weight was arrived by multiplying the ratio of organ weight to body weight with hundred. Results of 30 days trial and 60 days trial are presented in Tables 8 and Figure 10 and Table 9 and Figure 11, respectively. Hexaconazole, at low dose level tested, did not show any effect on the weight gain of testis, prostate and seminal vesicle in both the trials. However, their weight gains were adversely affected at medium and high dose levels. In the 30 days trial, medium dose caused a significant ($P < 0.05$) decrease in the weight of all three organs, while the high dose resulted in a highly significant decrease ($P < 0.01$).

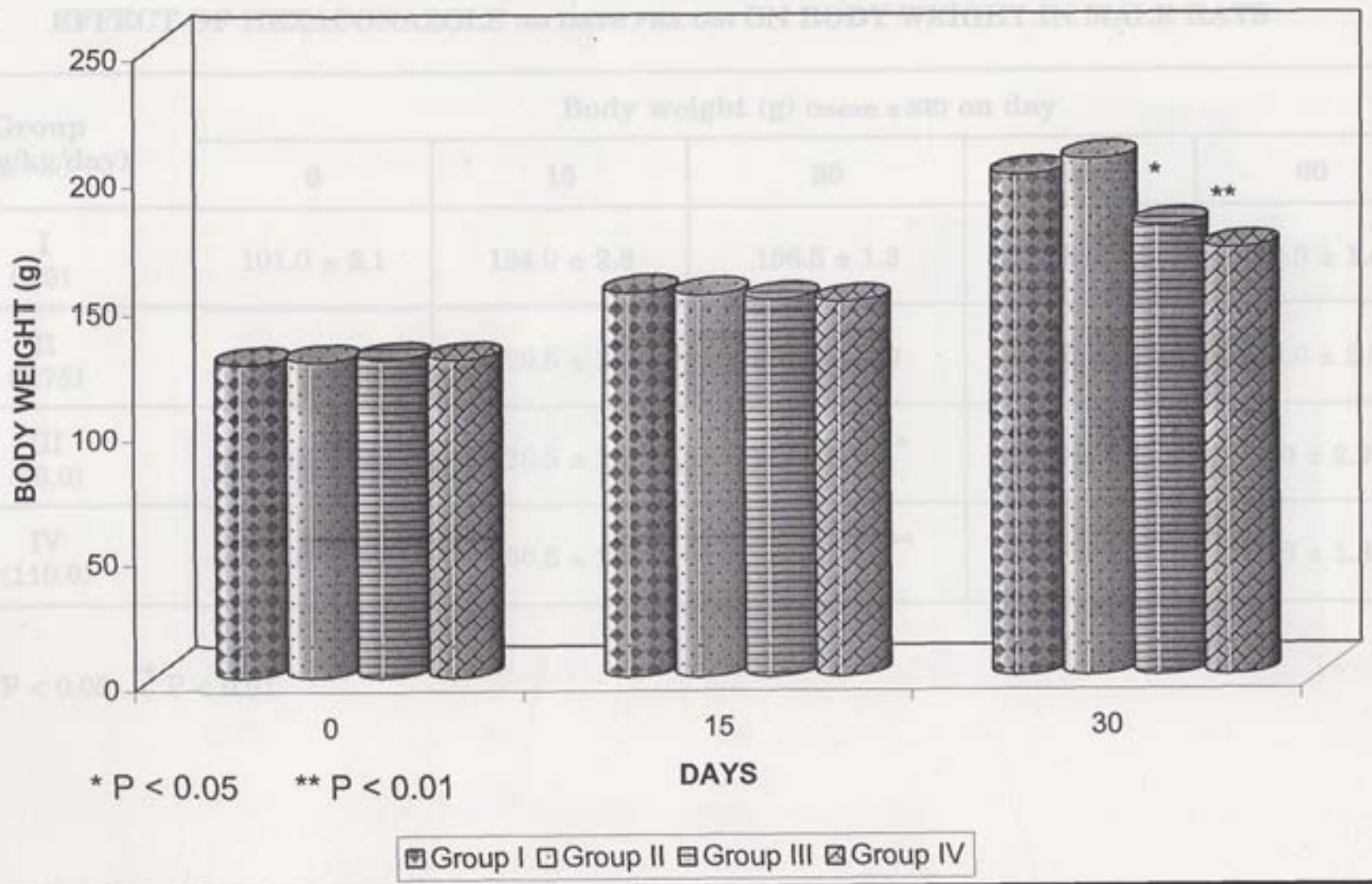
## TABLE 6

## EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON BODY WEIGHT IN MALE RATS

| Group (mg/kg/day) | Body weight (g) (mean ± SE) on day | | |
|---|---|---|---|
| | 0 | 15 | 30 |
| I (0.0) | 125.5 ± 1.4 | 153.5 ± 2.1 | 199.0 ± 3.6 |
| II (2.75) | 126.5 ± 1.1 | 152.5 ± 1.7 | 205.0 ± 2.7 |
| III (55.0) | 127.0 ± 1.5 | 150.5 ± 2.2 | 179.0 ± 3.1* |
| IV (110.0) | 127.5 ± 1.3 | 149.5 ± 2.3 | 170.0 ± 2.4** |

* $P < 0.05$ ** $P < 0.01$

## FIGURE 8 : EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON BODY WEIGHT IN MALE RATS



## TABLE 7

## EFFECT OF HEXACONAZOLE (60 DAYS *PER OS*) ON BODY WEIGHT IN MALE RATS

| Group (mg/kg/day) | Body weight (g) (mean ± SE) on day | | | | |
|---|---|---|---|---|---|
| | 0 | 15 | 30 | 45 | 60 |
| I (0.0) | 101.0 ± 3.1 | 134.0 ± 2.8 | 156.5 ± 1.3 | 178.5 ± 1.3 | 220.5 ± 1.4 |
| II (2.75) | 102.0 ± 2.0 | 129.5 ± 3.0 | 152.5 ± 2.9 | 177.5 ± 2.7 | 218.0 ± 2.0 |
| III (55.0) | 99.0 ± 3.5 | 128.5 ± 2.2 | 143.5 ± 1.5* | 160.5 ± 3.7** | 185.0 ± 2.9** |
| IV (110.0) | 103.0 ± 3.7 | 130.5 ± 2.0 | 140.5 ± 1.3** | 152.0 ± 2.3** | 170.6 ± 1.8** |

* $P < 0.05$ ** $P < 0.01$

## FIGURE 9 : EFFECT OF HEXACONAZOLE (60 DAYS *PER OS*) ON BODY WEIGHT IN MALE RATS

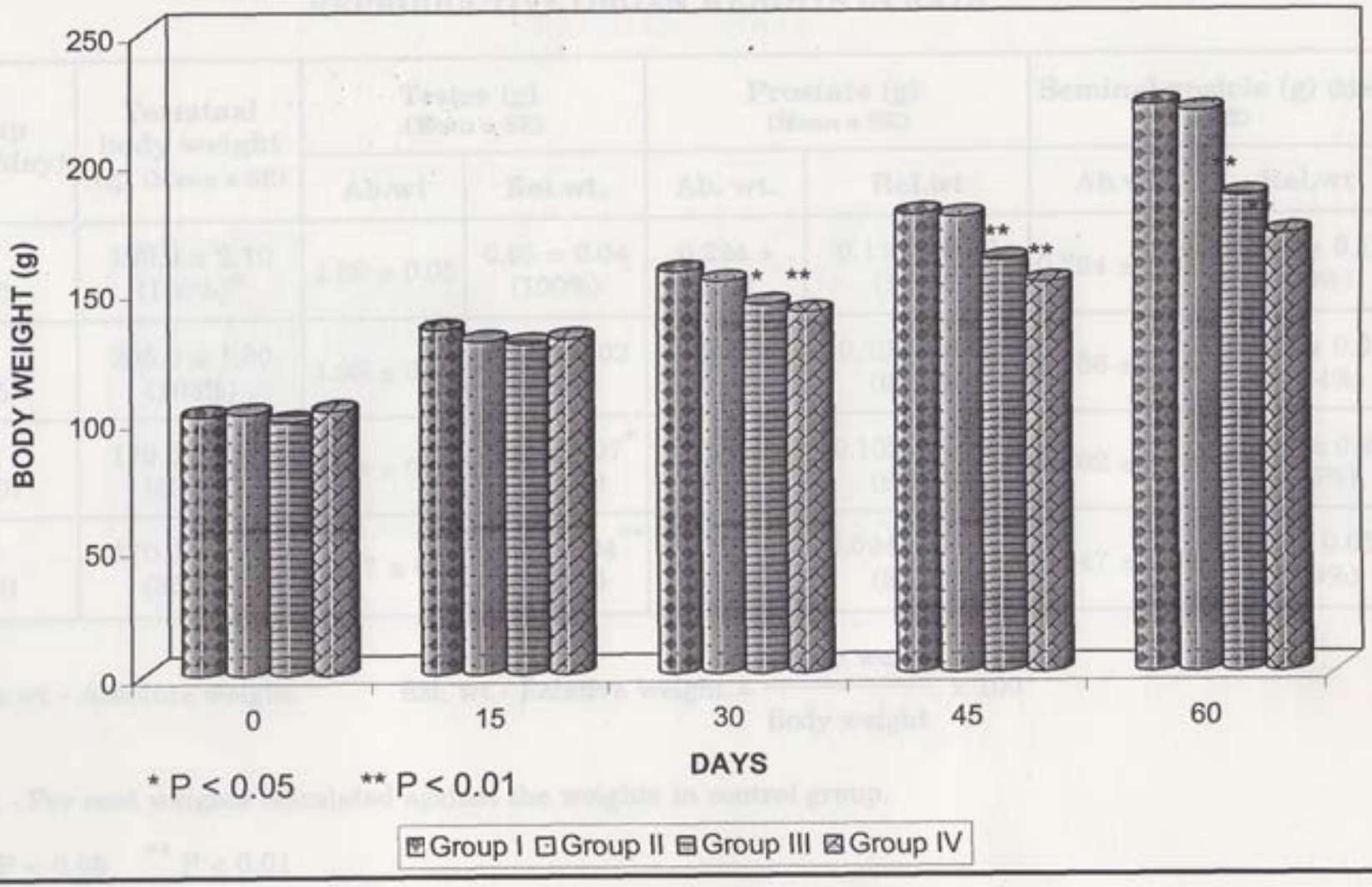


**TABLE 8**

**EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON MALE REPRODUCTIVE ORGAN WEIGHTS IN RATS**

| Group (mg/kg/day) | Terminal body weight (g) (Mean ± SE) | Testes (g) (Mean ± SE) | | Prostate (g) (Mean ± SE) | | Seminal vesicle (g) (Mean ± SE) | |
|---|---|---|---|---|---|---|---|
| | | Ab.wt | Rel.wt. | Ab. wt. | Rel.wt | Ab.wt | Rel.wt |
| I (0.0) | 199.0 ± 2.10 (100%)[a] | 1.89 ± 0.05 | 0.95 ± 0.04 (100%) | 0.224 ± 0.06 | 0.113 ± 0.02 (100%) | 0.724 ± 0.21 | 0.363 ± 0.13 (100%) |
| II (27.5) | 205.0 ± 1.30 (103%) | 1.93 ± 0.10 | 0.94 ± 0.02 (98.9) | 0.218 ± 0.02 | 0.106 ± 0.02 (93.8%) | 0.756 ± 0.15 | 0.368 ± 0.08 (101.4%) |
| III (55.0) | 179.0 ± 1.25 (89.9%) | 1.53 ± 0.08 | 0.86 ± 0.07* (90.5%) | 0.183 ± 0.08 | 0.102 ± 0.03* (90.3%) | 0.602 ± 0.13 | 0.336 ± 0.8* (92.6%) |
| IV (110) | 170.0 ± 2.03 (85.4%) | 1.37 ± 0.5 | 0.80 ± 0.04** (84.2%) | 0.159 ± 0.05 | 0.094 ± 0.02** (83.2%) | 0.547 ± 0.09 | 0.319 ± 0.05** (87.9%) |

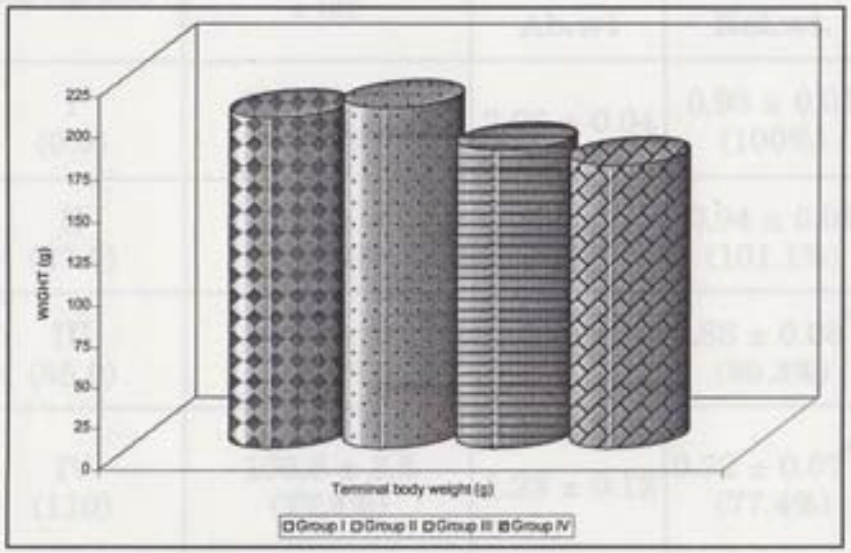
Ab.wt - Absolute weight

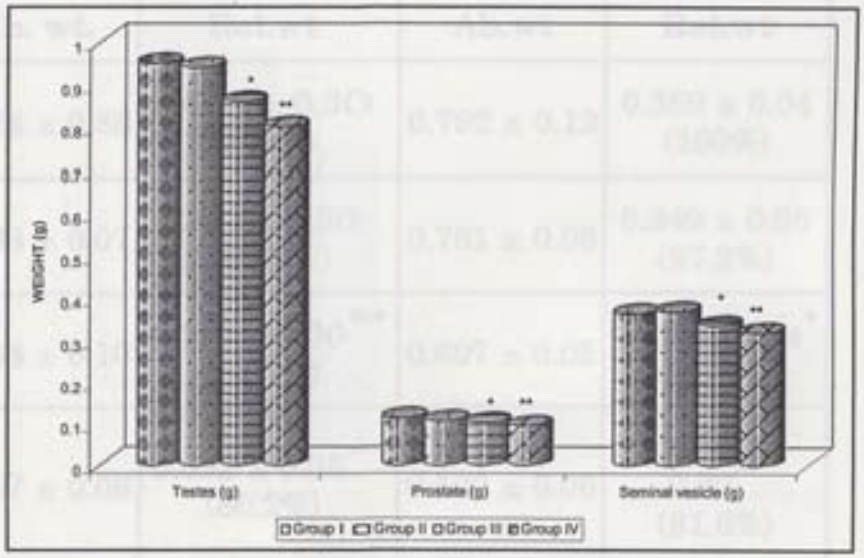
Rel. wt - Relative weight = $\frac{\text{Organ weight}}{\text{Body weight}} \times 100$

**a** - Per cent weights calculated against the weights in control group.

* $P < 0.05$ ** $P < 0.01$

## FIGURE 10 : EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON MALE REPRODUCTIVE ORGAN WEIGHTS IN RATS




* P < 0.05 ** P < 0.01

**TABLE 9**

**EFFECT OF HEXACONAZOLE (60 DAYS *PER OS*) ON MALE REPRODUCTIVE ORGAN WEIGHTS IN RATS**

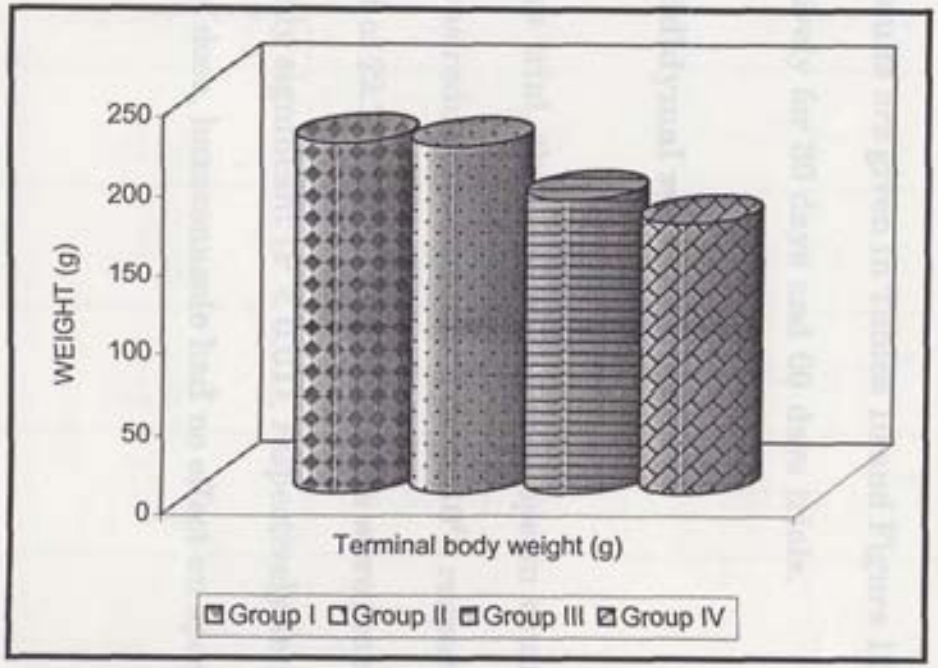
| Group (mg/kg/day) | Terminal body weight (g) (Mean ± SE) | Testes (g) (Mean ± SE) | | Prostate (g) (Mean ± SE) | | Seminal vesicle (g) (Mean ± SE) | |
|---|---|---|---|---|---|---|---|
| | | Ab.wt | Rel.wt. | Ab. wt. | Rel.wt | Ab.wt | Rel.wt |
| I (0.0) | 220.5 ± 2.3 (100% | 2.06 ± 0.04 | 0.93 ± 0.03 (100%) | 0.254 ± 0.88 | 0.115 ± 0.30 (100%) | 0.792 ± 0.12 | 0.359 ± 0.04 (100%) |
| II (27.5) | 218.0 ± 4.5 (93%) | 2.04 ± 0.08 | 0.94 ± 0.06 (101.1%) | 0.268 ± 0.07 | 0.123 ± 0.50 (106.9%) | 0.761 ± 0.08 | 0.349 ± 0.05 (97.2%) |
| III (55.0) | 185.0 ± 2.5 (81.2%) | 1.55 ± 0.10 | 0.83 ± 0.08** (89.3%) | 0.188 ± 0.10 | 0.102 ± 0.06** (88.7%) | 0.607 ± 0.05 | 0.328 ± 0.04* (91.4%) |
| IV (110) | 170.6 ± 3.5 (77.4%) | 1.23 ± 0.12 | 0.72 ± 0.07** (77.4%) | 0.157 ± 0.08 | 0.092 ± 0.05** (80.2%) | 0.500 ± 0.06 | 0.293 ± 0.02** (81.6%) |

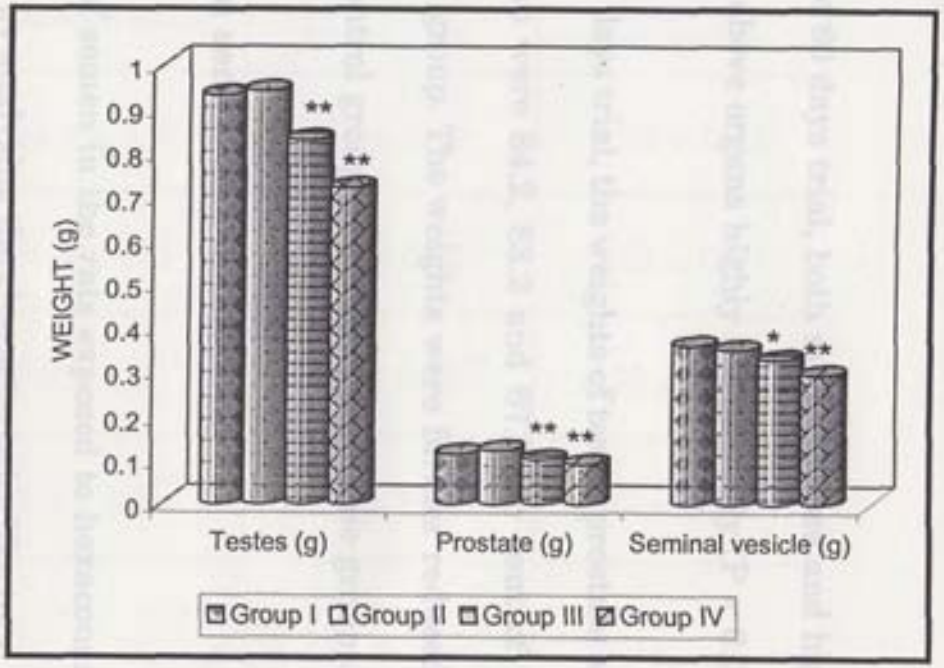
Ab.wt - Absolute weight  Rel. wt - Relative weight = $\frac{\text{Organ weight}}{\text{Body weight}} \times 100$

**a** - Per cent weights calculated against the weights in control group.

* $P < 0.05$  ** $P < 0.01$

## FIGURE 11 : EFFECT OF HEXACONAZOLE (60 DAYS *PER OS*) ON MALE REPRODUCTIVE ORGAN WEIGHTS IN RATS




* P < 0.05 ** P < 0.01

In case of 60 days trial, both medium dose and high dose, decreased the weight of the above organs highly significantly ($P < 0.01$).

In the 30 days trial, the weights of testis, prostate and seminal vesicle in high dose group were 84.2, 83.2 and 87.9 per cent of the corresponding weights in control group. The weights were further reduced to 77.4, 80.2 and 81.6 per cent of control group weights, in high dose group of 60 days trial.

### 4.2.3 Effect on semen characteristics

Quality of semen in the rats exposed to hexaconazole for 30 and 60 days was assessed by analysing the characteristics like total epididymal sperm count, motility percentage, dead sperm percentage and abnormal sperm percentage. The results are given in Tables 10 and Figure 12 and Table 11 and Figure 13, respectively for 30 days and 60 days trials.

#### 4.2.3.1 Total epididymal sperm count

In 30 days trial, the total epididymal sperm count in medium and high dose groups was reduced to 16.8 and 14.8 ($x 10^6$), respectively, against the control group count of 22.2 ($x 10^6$). These decreases were found to be significant ($P < 0.05$) and highly significant ($P < 0.01$), respectively in medium and high dose groups. At low dose, hexaconazole had no effect on sperm count Table 10 and Figure 12.

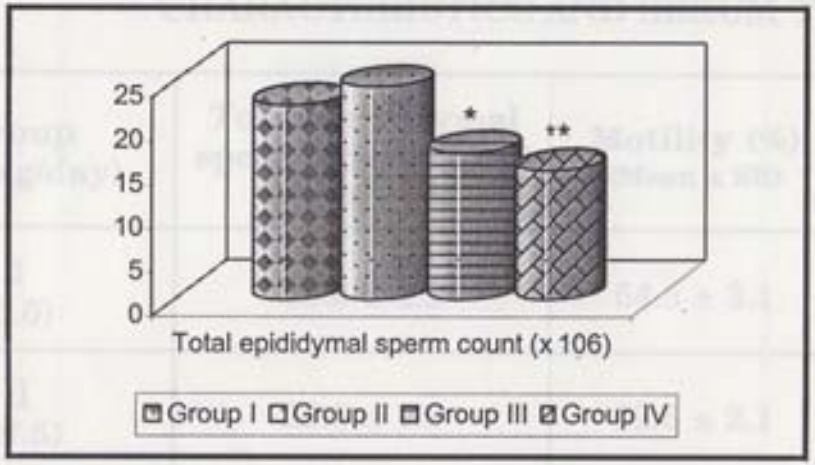
## TABLE 10

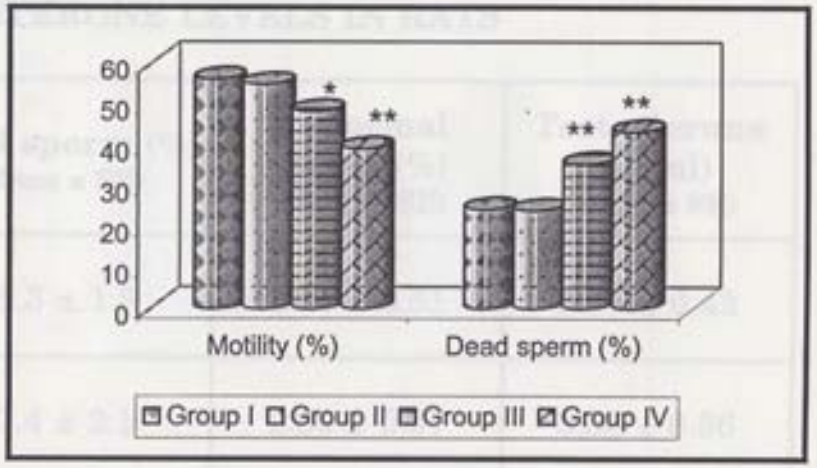
## EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON SEMEN CHARACTERISTICS AND SERUM TESTOSTERONE LEVELS IN RATS

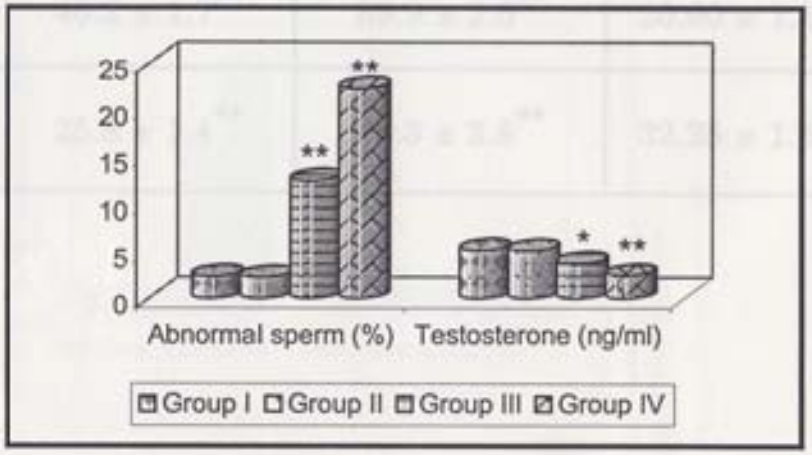
| Group (mg/kg/day) | Total epididymal sperm count (x $10^6$) (Mean ± SE) | Motility (%) (Mean ± SE) | Dead sperm (%) (Mean ± SE) | Abnormal sperm (%) (Mean ± SE) | Testosterone (ng/ml) (Mean ± SE) |
|---|---|---|---|---|---|
| I (0.0) | 22.2 ± 1.2 | 56.2 ± 2.1 | 24.4 ± 1.2 | 2.4 ± 0.30 | 5.18 ± 0.51 |
| II (27.5) | 24.3 ± 1.5 | 54.9 ± 1.5 | 24.1 ± 2.0 | 2.3 ± 0.20 | 5.12 ± 0.31 |
| III (55.0) | 16.8 ± 1.4* | 48.3 ± 1.8* | 35.6 ± 1.3** | 12.5 ± 0.62** | 3.79 ± 0.25* |
| IV (110.0) | 14.8 ± 1.8** | 39.3 ± 1.2** | 43.5 ± 1.7** | 22.3 ± 1.03** | 2.52 ± 0.18** |

*$P < 0.05$ **$P < 0.01$

## FIGURE 12 : EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON SEMEN CHARACTERISTICS AND SERUM TESTOSTERONE LEVELS IN RATS




* P < 0.05 ** P < 0.01

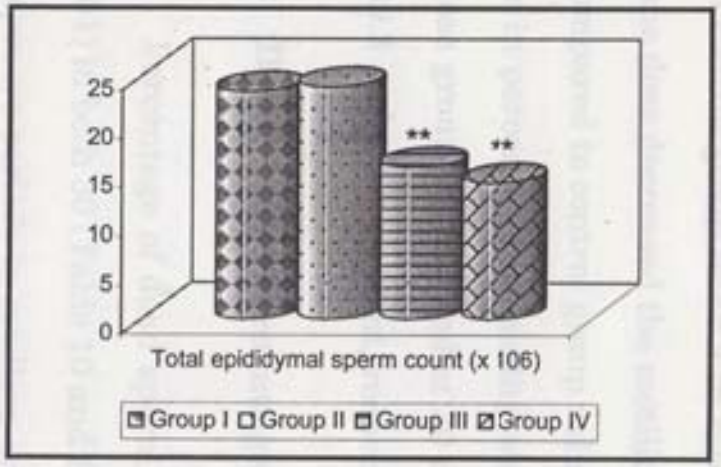
**TABLE 11**

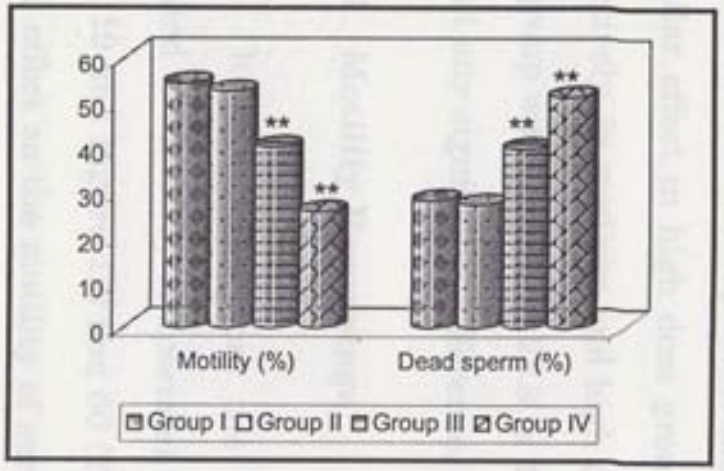
**EFFECT OF HEXACONAZOLE (60 DAYS *PER OS*) ON SEMEN CHARACTERISTICS AND SERUM TESTOSTERONE LEVELS IN RATS**

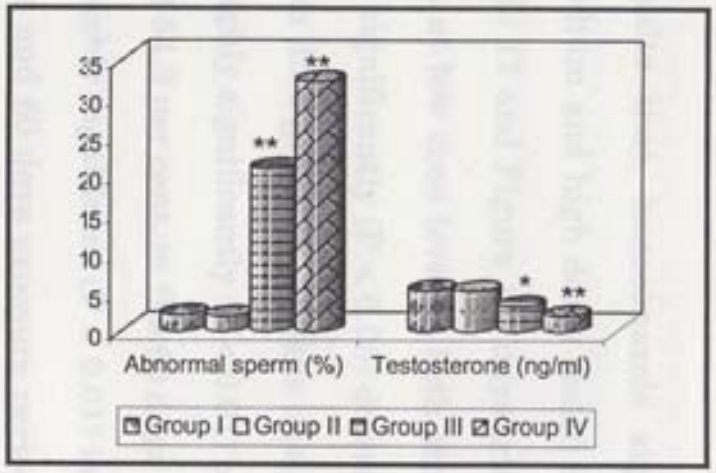
| Group (mg/kg/day) | Total epididymal sperm count ($x10^6$) (Mean ± SE) | Motility (%) (Mean ± SE) | Dead sperm (%) (Mean ± SE) | Abnormal sperm (%) (Mean ± SE) | Testosterone (ng/ml) (Mean ± SE) |
|---|---|---|---|---|---|
| I (0.0) | 23.3 ± 2.1 | 54.3 ± 3.1 | 28.3 ± 1.8 | 2.20 ± 0.51 | 5.37 ± 0.42 |
| II (27.5) | 23.8 ± 1.8 | 52.5 ± 2.1 | 27.4 ± 2.1 | 2.00 ± 0.07 | 5.30 ± 0.56 |
| III (55.0) | 15.5 ± 1.7** | 40.2 ± 1.7** | 39.9 ± 2.5** | 20.90 ± 1.13** | 3.34 ± 0.36* |
| IV (110.0) | 13.9 ± 1.9** | 25.8 ± 1.4** | 51.3 ± 3.8** | 32.25 ± 1.56** | 2.11 ± 0.05** |

*P < 0.05 **P < 0.01

## FIGURE 13 : EFFECT OF HEXACONAZOLE (60 DAYS *PER OS* ) ON SEMEN CHARACTERISTICS AND SERUM TESTOSTERONE LEVELS IN RATS




* P < 0.05 ** P < 0.01

Exposure to hexaconazole for sixty days resulted in a highly significant decrease in sperm count even in medium dose group also along with a similar effect in high dose group. The values were 15.5 and 13.9 (x $10^6$) respectively, in medium and high dose groups. The values in control and low dose group were 23.3 and 23.8 (x $10^6$) respectively (Table 11 and Figure 13), without any significant difference.

#### 4.2.3.2 Motility Percentage

It is evident from the results that, hexaconazole significantly decreased the motility of sperm at medium and high doses tested in both 30 (Table 10 and Figure 12) and 60 (Table 11 and Figure 13) days trials. There was no effect on the motility of sperm at low dose level in both studies. The percentage of motile sperm was found significantly ($P < 0.05$) decreased from 56.2 in control group to 48.3 in medium dose group in 30 days trial, whereas the same dose decreased the motility highly significantly ($P < 0.01$) to 40.2 per cent compared to control group value of 54.3 per cent, in the 60 days trial. The decline in percentage of motility was highly significant ($P < 0.01$) in both the high dose groups at the end of 30 days and 60 days exposure periods, which were 39.3 and 25.8 per cent, respectively.

#### 4.2.3.3 Dead sperm percentage

Percentage of dead sperm was found increased highly significantly ($P < 0.01$) in both 30 (Table 10 and Figure 12) and 60 (Table 11 and Figure 13) days exposure periods in medium and high dose groups. The values were 35.6 and 43.5 per cent in medium and high dose groups, respectively, while in control group the dead sperm were only 24.4 per cent, at the end of 30 day trial.

In 60 days trial, the percentage of dead sperm were 28.3, 39.9 and 51.3 in control, medium and high dose groups respectively. Sperm viability was not affected by the low dose of hexaconazole, given either for 30 days or 60 days.

#### 4.2.3.4 Abnormal sperm percentage

In 30 days trial, hexaconazole produced highly significant ($P < 0.01$) increase in the percentage of abnormal sperm at medium and high dose levels, while the low dose of the drug had no effect (Table 10 and Figure 12). In 60 days trial medium and high doses of hexaconazole produced highly significant ($P < 0.01$) increase in the percentage of abnormal sperm. In this trial too also no significant change in the percentage of abnormal sperm was observed with low dose of hexaconazole (Table 11 and Figure 13). Plates 9 to 12 show the abnormal sperm observed in the treated rats.

### 4.2.4 Serum testosterone levels

Testosterone levels observed in control rats were 5.18 ng/ml in 30 days trial (Table 10 and Figure 12) and 5.37 ng/ml of serum in 60 days trial (Table 11 and Figure 13). In both studies, the serum testosterone levels recorded in low dose groups did not differ significantly with their corresponding control groups. However, medium and high doses decreased the hormone levels significantly to 3.79 and 2.52 ng/ml in 30 days trial and to 3.34 and 2.11 ng/ml in 60 days trial.

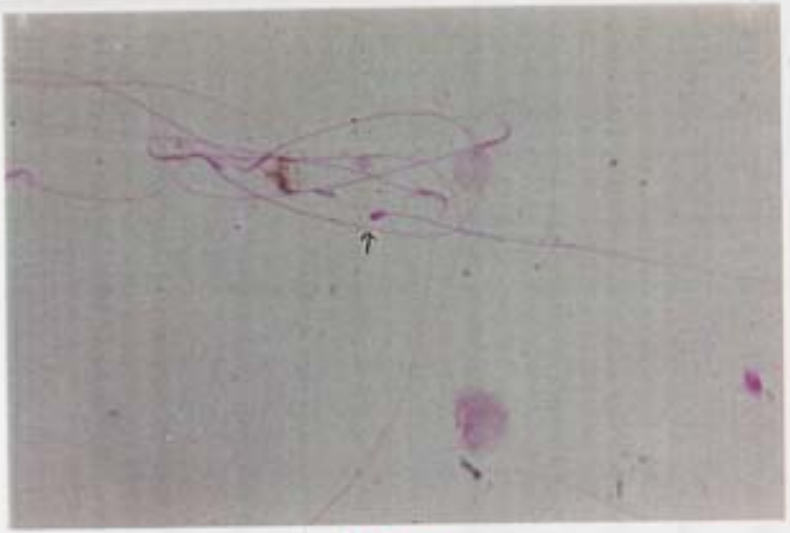


Plate 9

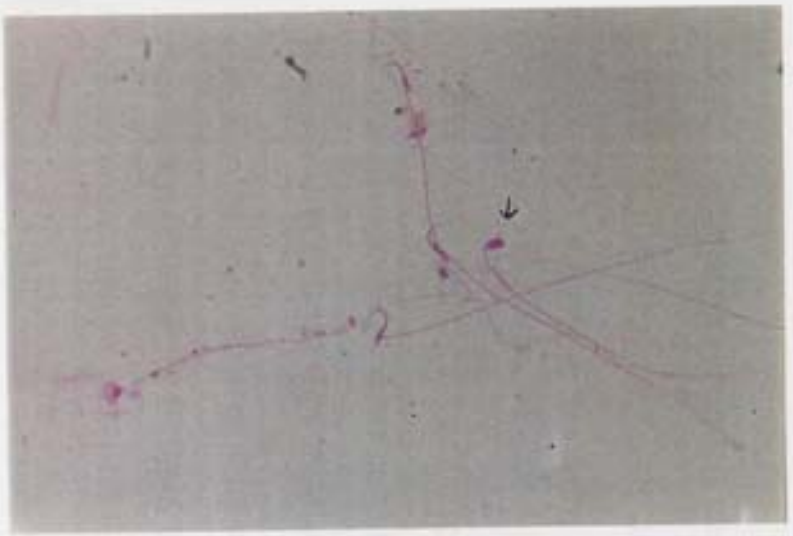


Plate 10

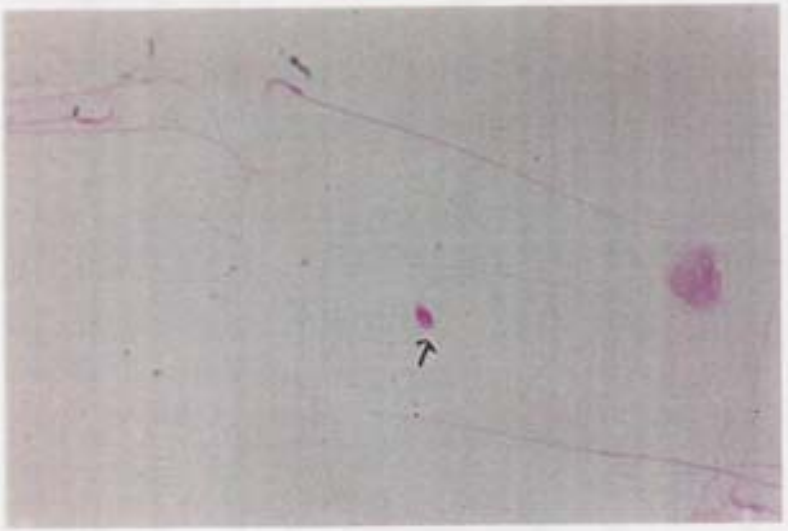


Plate 11

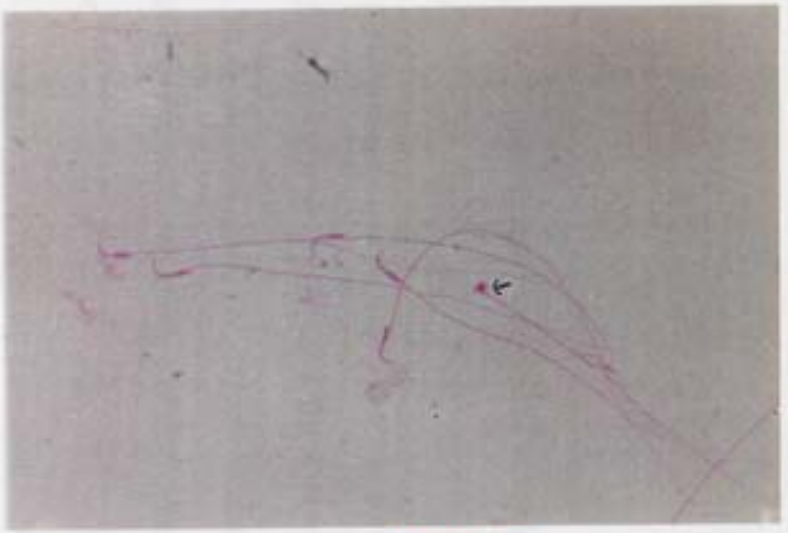


Plate 12

Plate 9-12 Photomicrographs showing (arrow) the abnormal sperm observed in treated rats

### 4.2.5 Histopathological changes in testis

#### 4.2.5.1 Quantitative analysis

In rats exposed to the drug for 30 days, the number of seminiferous tubules observed per microscopic field was found significantly ($P < 0.05$) increased at medium dose and highly significantly ($P < 0.01$) increased in high dose level (Table 12 and Figure 14). This was accompanied by a significant decrease in the tubular diameter in both the groups, which indicated a decrease in the size of tubules. In 60 days trial, both medium and high doses resulted in a highly significant ($P < 0.01$) increase in the number of tubules observed per field with concomitant decline in the tubular diameter (Table 13 and Figure 15).

In 30 days trial low and medium doses did not produce a significant increase in the percentage of damaged tubules, where as in high dose group the percentage of damaged tubules was significantly increased. But, in rats exposed to the drug for 60 days, medium dose also significantly increased the percentage of damaged tubules along with the high dose.

#### 4.2.5.2 Qualitative changes

While the low dose of hexaconazole did not cause any adverse effect on the histological architecture of the testis in rats, medium and high doses of the drug produced moderate to severe histopathological changes, depending on the dose and duration of the trial.

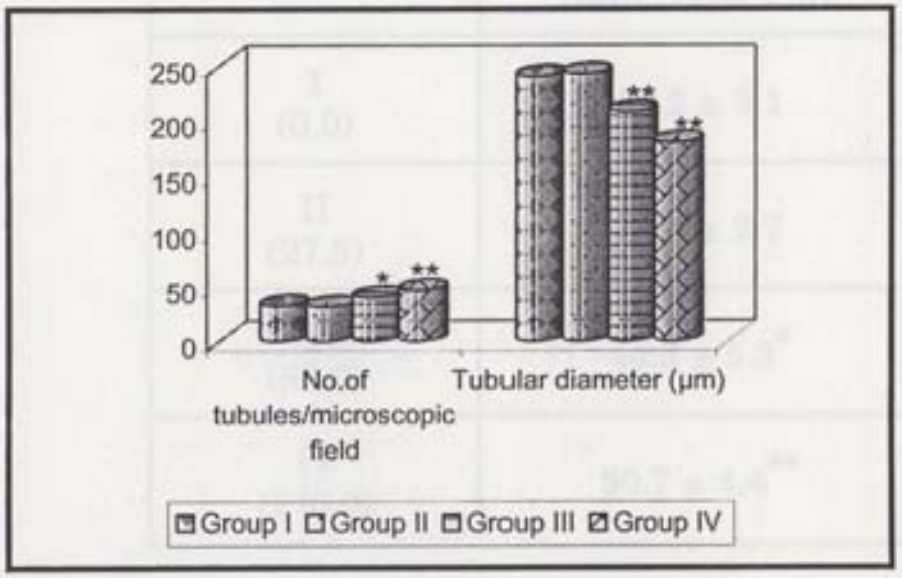
## TABLE 12

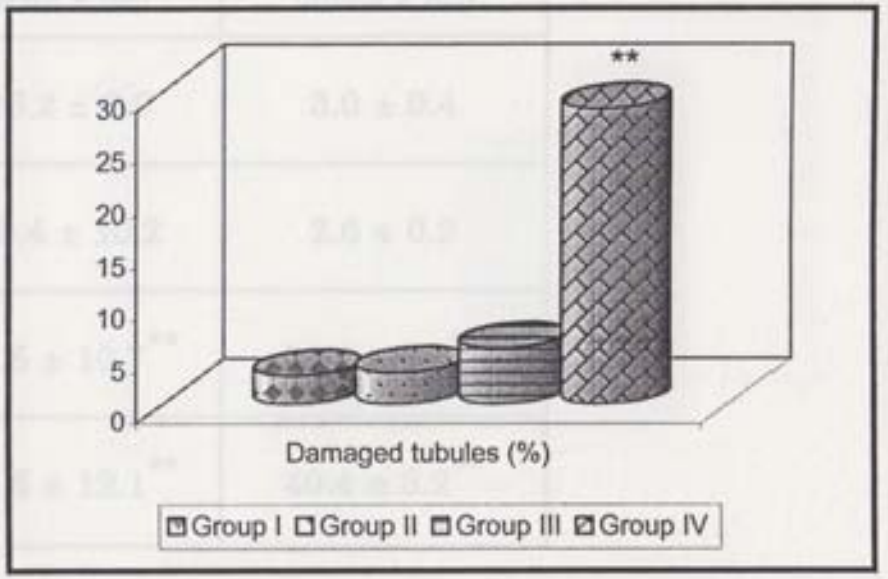
## EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON SEMINIFEROUS TUBULES IN RAT TESTIS

| Group (mg/kg/day) | No. of tubules/microscopic field (Mean ± SE) | Tubular diameter (µm) (Mean ± SE) | Damaged tubules (%) (Mean ± SE) |
|---|---|---|---|
| I (0.0) | 32.4 ± 2.5 | 240.5 ± 11.3 | 2.8 ± 0.2 |
| II (27.5) | 31.6 ± 1.9 | 242.8 ± 9.6 | 3.1 ± 0.7 |
| III (55.0) | 40.2 ± 3.1* | 208.5 ± 7.2** | 5.4 ± 0.4 |
| IV (110.0) | 46.7 ± 3.4** | 180.4 ± 10.1** | 28.3 ± 6.9** |

*P < 0.05 **P < 0.01

## FIGURE 14 : EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON SEMINIFEROUS TUBULES IN RAT TESTIS




* P < 0.05 ** P < 0.01

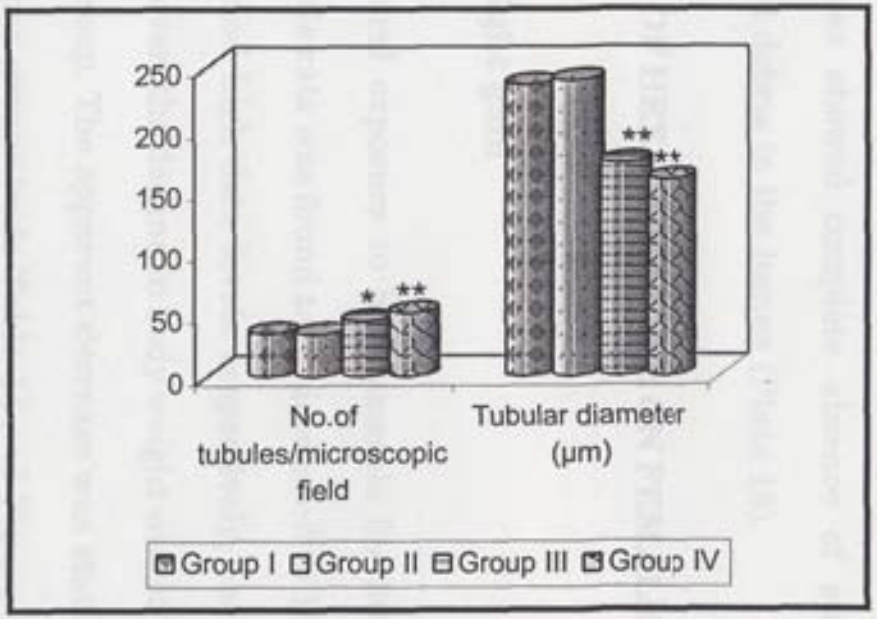
## TABLE 13

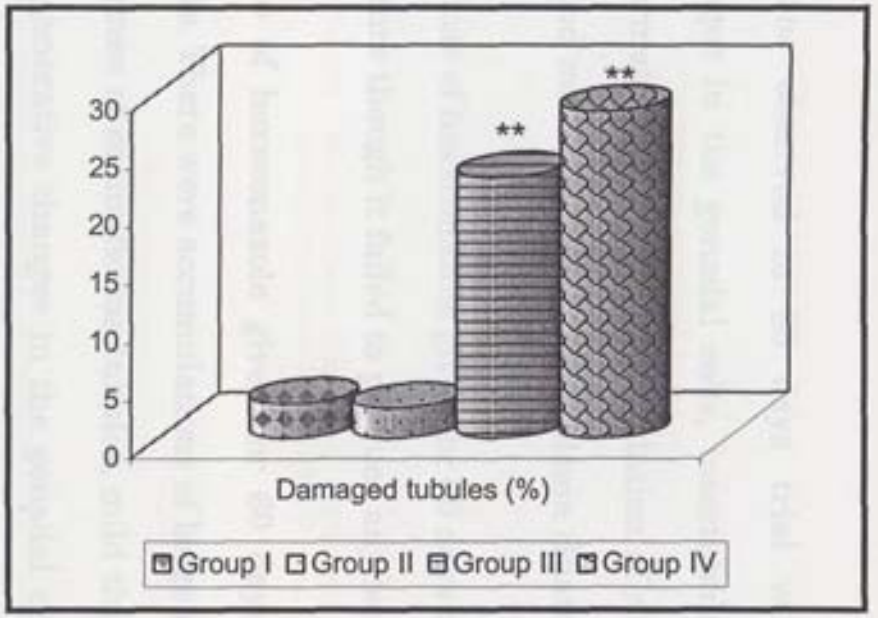
## EFFECT OF HEXACONAZOLE (60 DAYS *PER OS*) ON SEMINIFEROUS TUBULES IN RAT TESTIS

| Group (mg/kg/day) | No. of tubules/microscopic field (Mean ± SE) | Tubular diameter (µm) (Mean ± SE) | Damaged tubules (%) (Mean ± SE) |
|---|---|---|---|
| 1 (0.0) | 34.2 ± 3.1 | 236.2 ± 8.6 | 3.0 ± 0.4 |
| II (27.5) | 33.5 ± 2.7 | 239.4 ± 10.2 | 2.6 ± 0.2 |
| III (55.0) | 44.3 ± 5.3* | 172.5 ± 10.7** | 22.5 ± 1.5** |
| IV (110.0) | 50.7 ± 4.4** | 158.6 ± 12.1** | 40.4 ± 3.2** |

*$P < 0.05$ **$P < 0.01$

# FIGURE 15 : EFFECT OF HEXACONAZOLE (60 DAYS *PER OS*) ON SEMINIFEROUS TUBULES IN RAT TESTIS




* P < 0.05 ** P < 0.01

The lesions observed in 30 days trial with high dose were degenerative changes in the gonadal cells, intertubular edema (Plate 13) impairment of spermatogenesis with accumulation of round cells within the lumen (Plate 14) and mild Leydig cell hyperplasia (Plate 15).

Medium dose of hexaconazole given for 60 days caused damage to the testicular architecture though it failed to produce any lesions in 30 days trial.

High dose of hexaconazole given for 60 days resulted in severe damage to the testis. There were accumulations of large round cells and giant cells with in the lumen of seminiferous tubules, mild thickening of basement membrane and degenerative changes in the gonadal epithelium (Plate 16). Hypertrophy of Leydig cells was more marked in this group (Plate 17) and in some places the Leydig cells showed degenerative and necrotic changes. Some seminiferous tubules showed complete absence of spermatogenesis with accumulation of cell debris in the lumen (Plate 18).

## 4.3 EFFECT OF HEXACONAZOLE ON FEMALE REPRODUCTIVE SYSTEM

### 4.3.1 Body weight gain

Effect of oral exposure to hexaconazole for 30 days on final body weight gain in female rats was found to be decreased to 162.5, 161.5 and 146.0 g at low, medium and high dose levels respectively, compared to 168.5 g in control group. However, the decline in body weight was statistically significant only in high dose group. The apparent decrease was statistically insignificant low and medium dose groups (vide Table 14 and Figure 16).

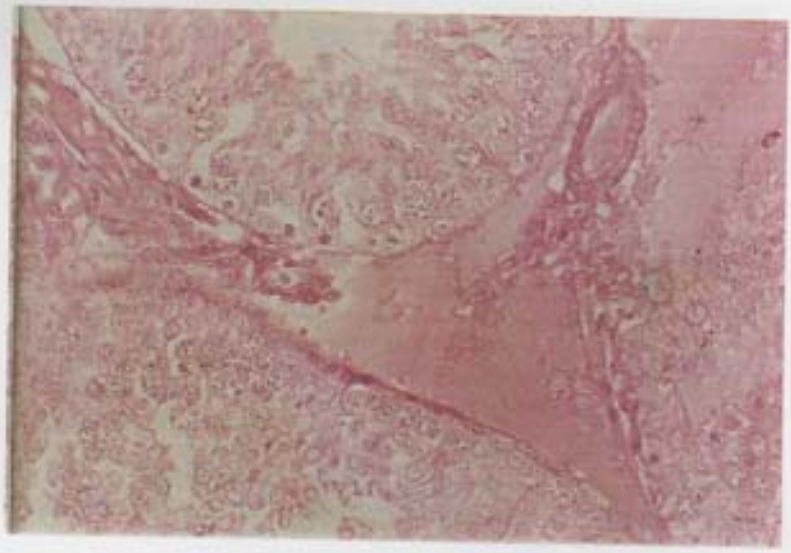


Plate 13 Rat-Hexaconazole (High dose-60 days) - Testis-showing intertubular edema H4E 360

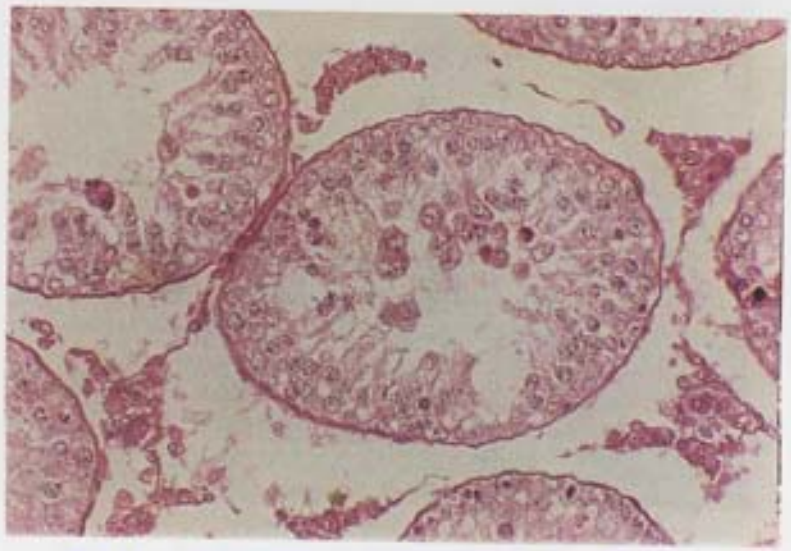


Plate 14 Rat-Hexaconazole (High dose-30 days) - Testis-showing accumulation of round cells with in the l.uman of seminiferous table H & E x 360

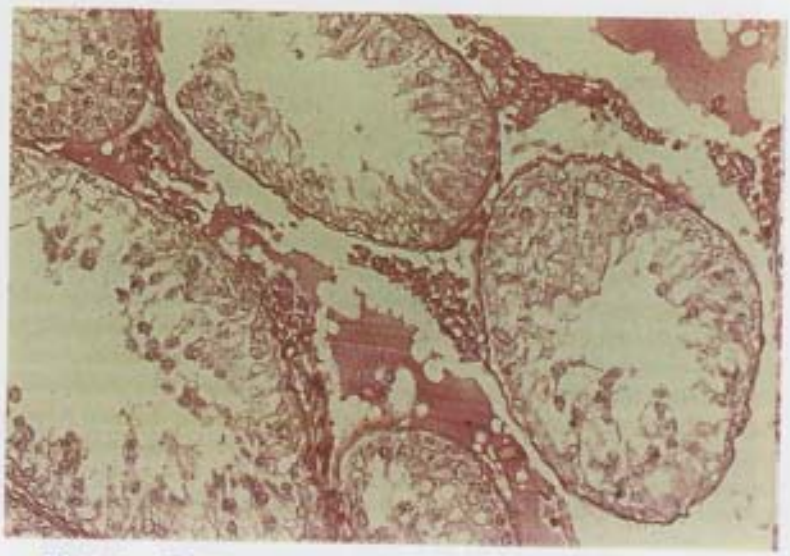


Plate 15 Rat-Hexaconazole (High dose-30 days) - Testis-showing intertubular edema, absence of spermatogenesis and mild Leydig cell hypertrophy H & E x 360

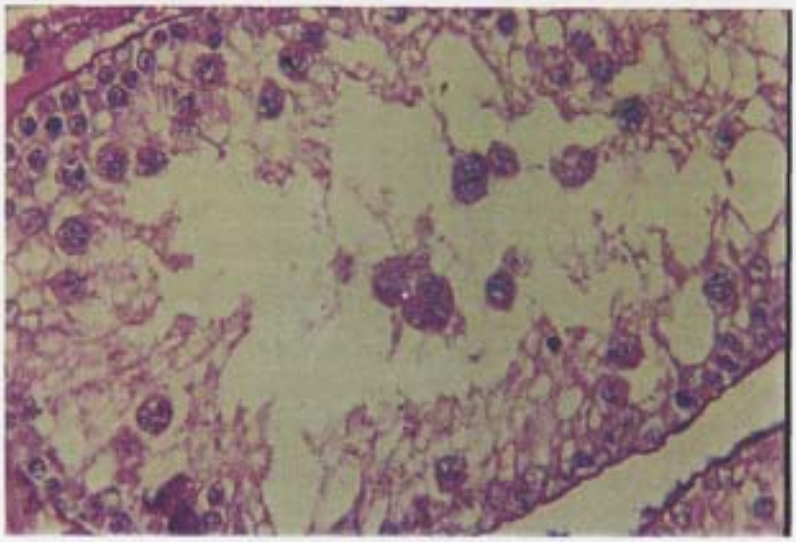


Plate 16 Rat-Hexaconazole (High dose-60 days) - Testis-showing degenerative changes in the gonadal epithelium with accumulation of giant cells with in the lumen of seminiferous tubule H & E x 360

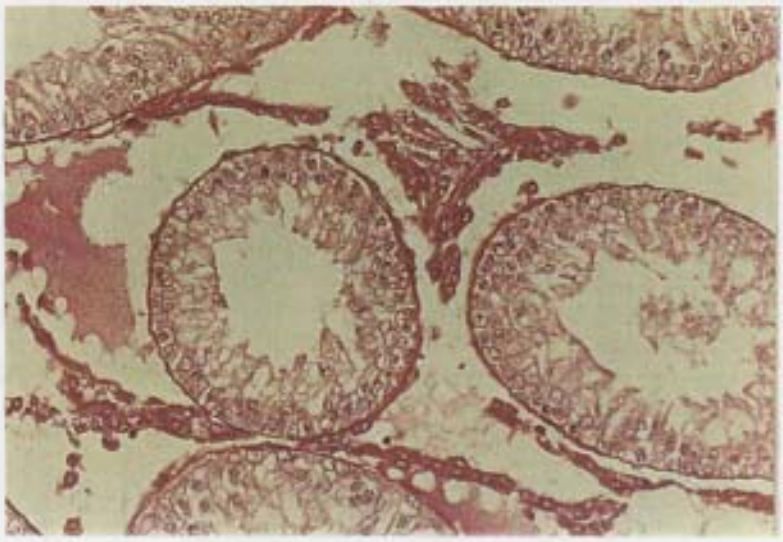


Plate 17 Rat-Hexaconazole (High dose - 60 days) -Testis-showing absence of spermatogenesis and Leydig cell hypertrophy H & E x 360

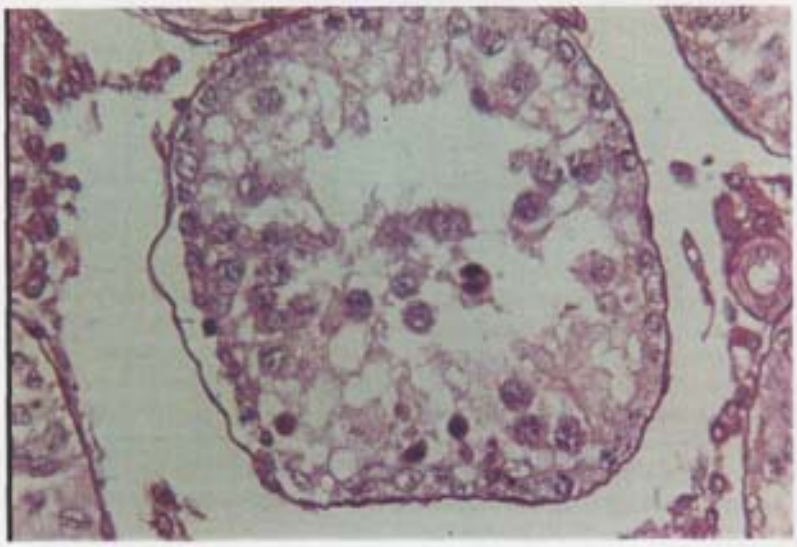


Plate 18 Rat-Hexaconazole (High dose-60 days)-Testis-showing complete absence of spermatogenesis and accumulation of cellular debris with in the lumen H & E x 300

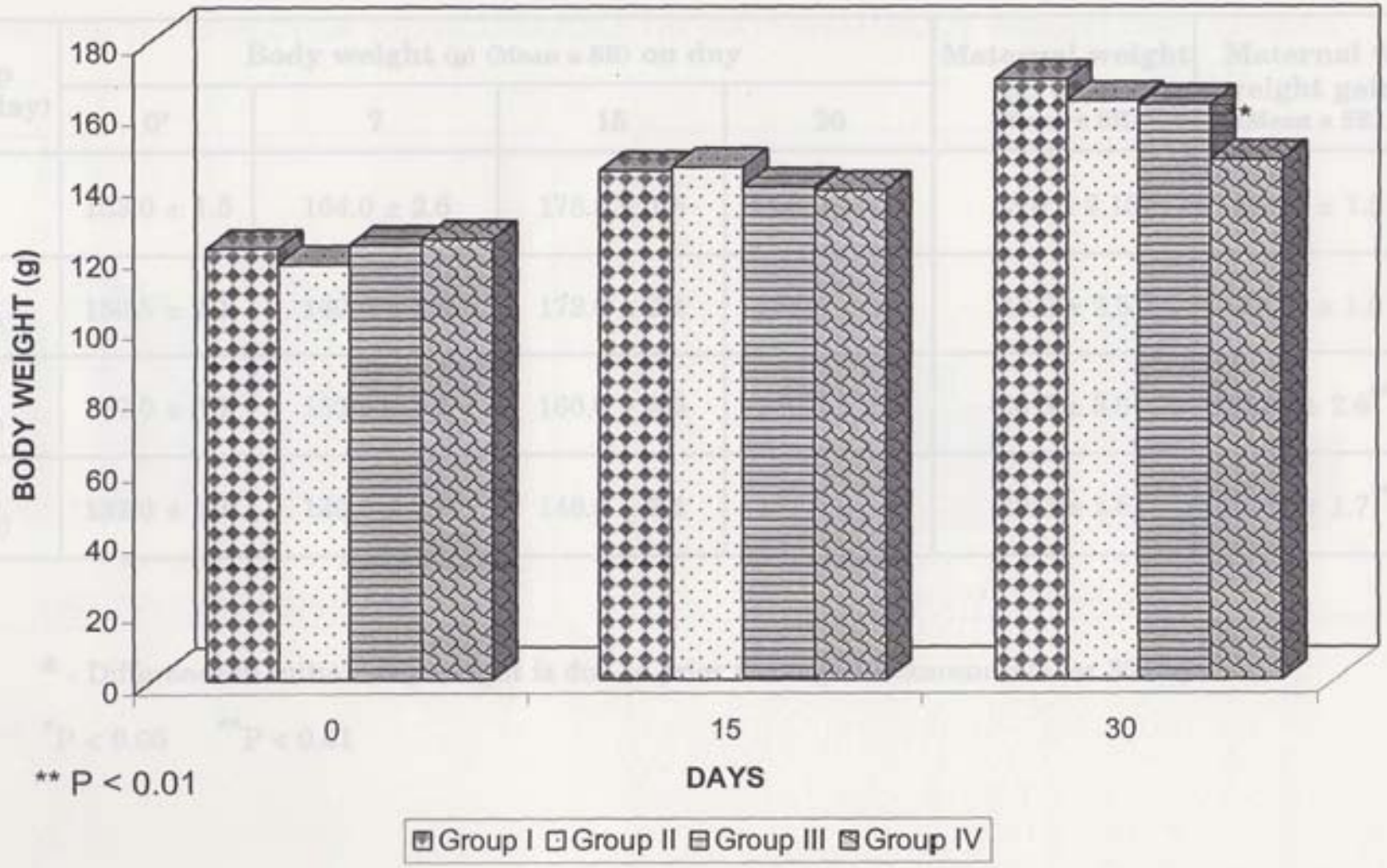
**TABLE 14**

**EFFECT OF HEXACONAZOLE (30 DAY *PER OS*) ON BODY WEIGHT IN FEMALE RATS**

| Group (mg/kg/day) | Body weight (g) (Mean ± SE) on day | | |
|---|---|---|---|
| | 0 | 15 | 30 |
| I (0.0) | 121.5 ± 2.2 | 143.0 ± 3.1 | 168.5 ± 2.5 |
| II (27.5) | 117.0 ± 3.8 | 144.0 ± 2.4 | 162.5 ± 3.6 |
| III (55.0) | 122.5 ± 3.1 | 138.5 ± 2.8 | 161.5 ± 3.5 |
| IV (110.0) | 124.0 ± 2.1 | 137.5 ± 3.6 | 146.0 ± 2.4** |

**$P < 0.01$

## FIGURE 16 : EFFECT OF HEXACONAZOLE (30 DAY *PER OS*) ON BODY WEIGHT IN FEMALE RATS



## TABLE 15

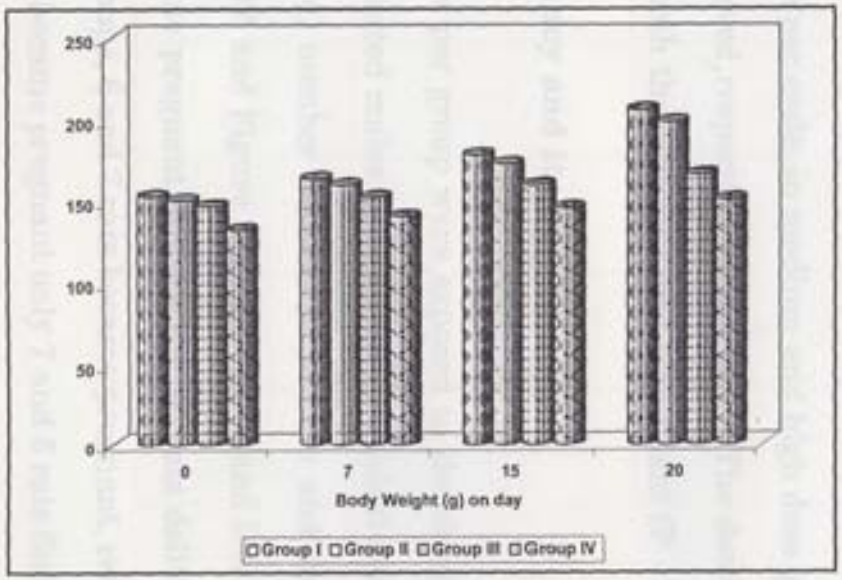
## EFFECT OF HEXACONAZOLE ON MATERNAL BODY WEIGHT GAIN IN RATS

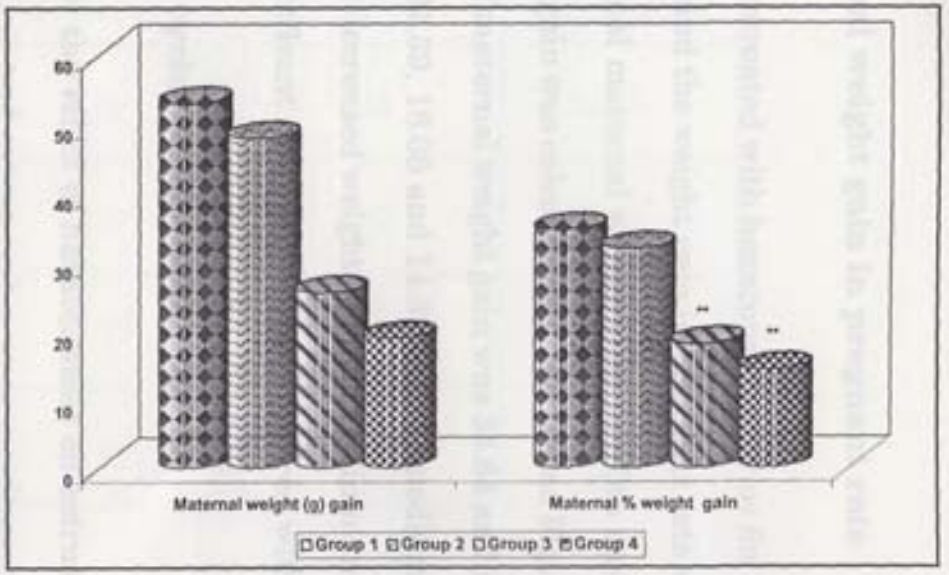
| Group (mg/kg/day) | Body weight (g) (Mean ± SE) on day | | | | Maternal weight (g) gain (Mean ± SE) | Maternal % weight gain (Mean ± SE) |
|---|---|---|---|---|---|---|
| | 0[a] | 7 | 15 | 20 | | |
| I (0.0) | 153.0 ± 1.5 | 164.0 ± 2.6 | 178.5 ± 1.8 | 206.0 ± 3.0 | 53.0 ± 2.10 | 34.64 ± 1.5 |
| II (27.5) | 150.5 ± 2.1 | 160.0 ± 1.8 | 173.0 ± 2.4 | 198.5 ± 2.1 | 48.0 ± 2.5 | 31.89 ± 1.8 |
| III (55.0) | 147.0 ± 3.1 | 152.5 ± 3.2 | 160.0 ± 3.0 | 166.5 ± 1.6 | 25.5 ± 3.5 | 18.08 ± 2.6** |
| IV (110.0) | 132.0 ± 2.1 | 140.5 ± 2.6 | 146.0 ± 2.1 | 151.0 ± 1.5 | 19.0 ± 1.6 | 14.39 ± 1.7** |

[a] - Difference in initial body weight is due to prior feeding of hexaconazole for 30 days.

*P < 0.05 **P < 0.01

## FIGURE 17 : EFFECT OF HEXACONAZOLE ON MATERNAL BODY WEIGHT GAIN IN RATS




** P < 0.01

### 4.3.2 Maternal weight gain in pregnant rats

Rats, pretreated with hexaconazole *per os* for 30 days, were mated to untreated males and the weight gain in pregnant rats was recorded on 0, 7, 15 and 20th day. Final maternal weight gain by 20th day and the percentage of maternal weight gain was calculated (Table 15 and Figure 17). In control group the percentage of maternal weight gain was 34.64 and in hexaconazole treated rats these were 31.89, 18.08 and 14.39 in low, medium and high dose groups, respectively. The decreased weight gain, in medium and high dose groups was found highly significant ($P< 0.01$) when compared with that of control group.

### 4.3.3 Estrus cycle

To know the effect of hexaconazole on estrus cycle, rats were given the drug for 30 days and estrus cycles were monitored from day 16th to 30th. While in control and low dose groups, three cycles were observed with an average of 5 days per cycle, in medium and high dose groups only 2.1 and 1.6 cycles were observed, respectively (Table 19). The decrease in the number of estrus cycles in both these groups was significant ($P < 0.05$).

### 4.3.4 Pregnancy and its outcome

Ten rats per group were exposed to the drug for 30 days and were mated with untreated males. Number of animals found pregnant, number of animals delivered, number of total pups born and average litter size were recorded (Table 16 and Figure 18). In control and low dose groups all the 10 mated rats became pregnant and all the 10 rats delivered pups. In medium and high dose groups, 8 and 7 rats became pregnant, respectively. Out of these 8 and 7 rats that became pregnant only 7 and 5 rats finally delivered the pups.

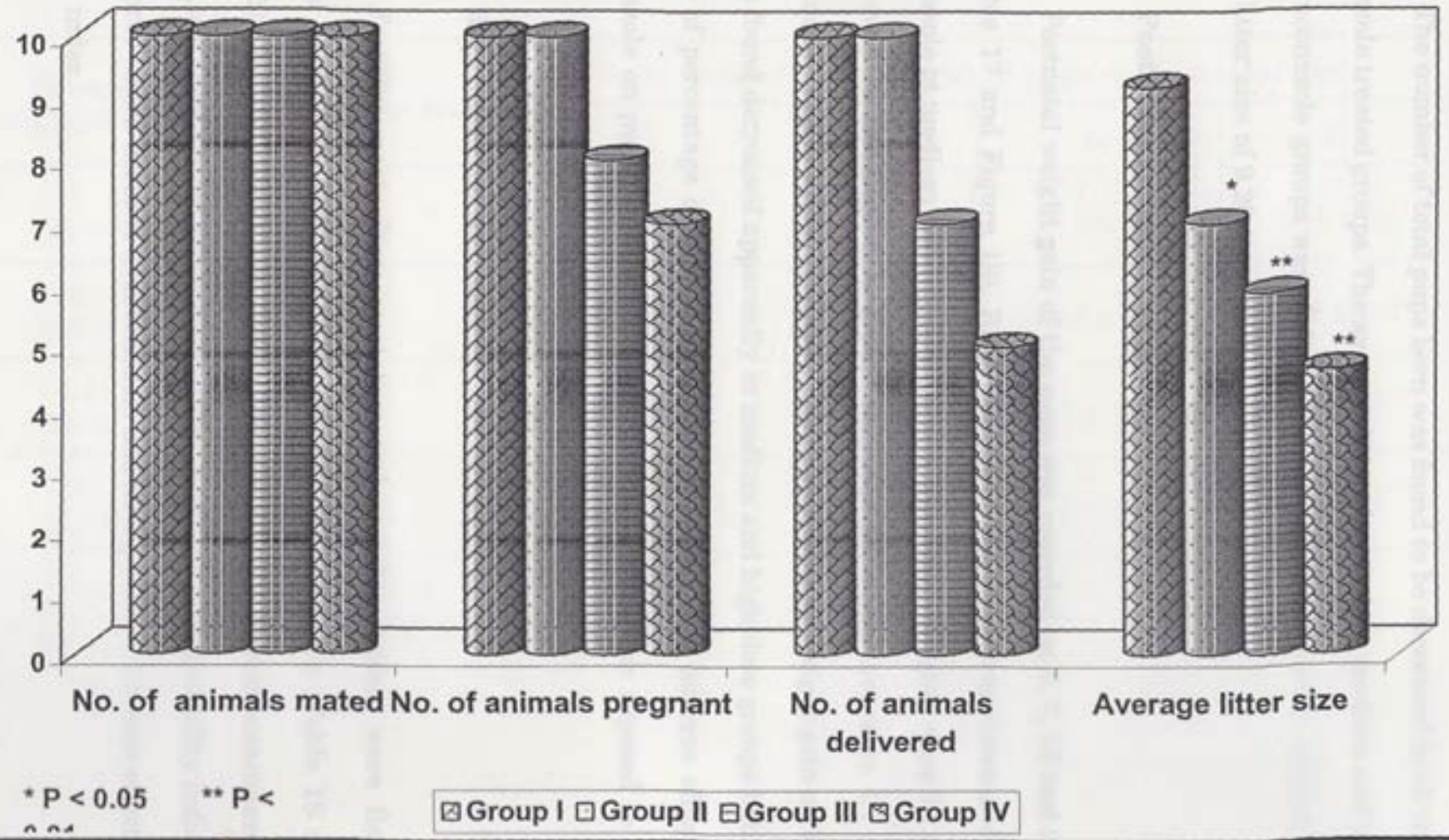
**TABLE 16**

**EFFECT HEXACONAZOLE ON PREGNANCY AND ITS OUTCOME IN RATS**

| Group (mg/kg/day) | No. of animals mated | No.of animals pregnant | No. of animals delivered | No. of total pups born | Average litter size |
|---|---|---|---|---|---|
| I (0.0) | 10 | 10 | 10 | 92 | 9.20 |
| II (27.5) | 10 | 10 | 10 | 70 | 7.00* |
| III (55.0) | 10 | 8 | 7 | 41 | 5.86** |
| IV (110.0) | 10 | 7 | 5 | 23 | 4.60** |

* $P < 0.05$ ** $P < 0.01$

## FIGURE 18 : EFFECT OF HEXACONAZOLE ON PREGNANCY AND ITS OUTCOME IN RATS



The number of total pups born was found to be decreased in all three hexaconazole treated groups. The average litter sizes in low, medium and high dose hexaconazole groups were 7.00, 5.86 and 4.60, respectively against the average litter size of 9.20 in control group.

### 4.3.5 Postnatal growth

Postnatal weight gain of the pups was recorded on 0, 7, 14 and 21st day (Table 17 and Figure 19). Birth weight of the pups was decreased by hexaconazole at medium and high dose levels. The mean weights were 3.1 and 2.9 g in medium and high dose groups respectively, while they were 4.7 and 4.5 g in control and low dose groups respectively. The final weight gain on 21st day, was found decreased apparently in medium and high dose groups but the analysis of percentage of weight gains did not reveal the adverse effect of hexaconazole on pup weight gain, when nursing dams were exposed to the drug.

### 4.3.6 Reproductive indices

Fertility, parturition, gestation and viability indices were found decreased at medium and high dose levels of hexaconazole (Table 18 and Figure 20). Gestation index was lowered even at the low dose of hexaconazole. However, low dose did not affect the fertility, parturition and viability indices. All the three tested doses of hexaconazole had no significant adverse effect on lactation index.

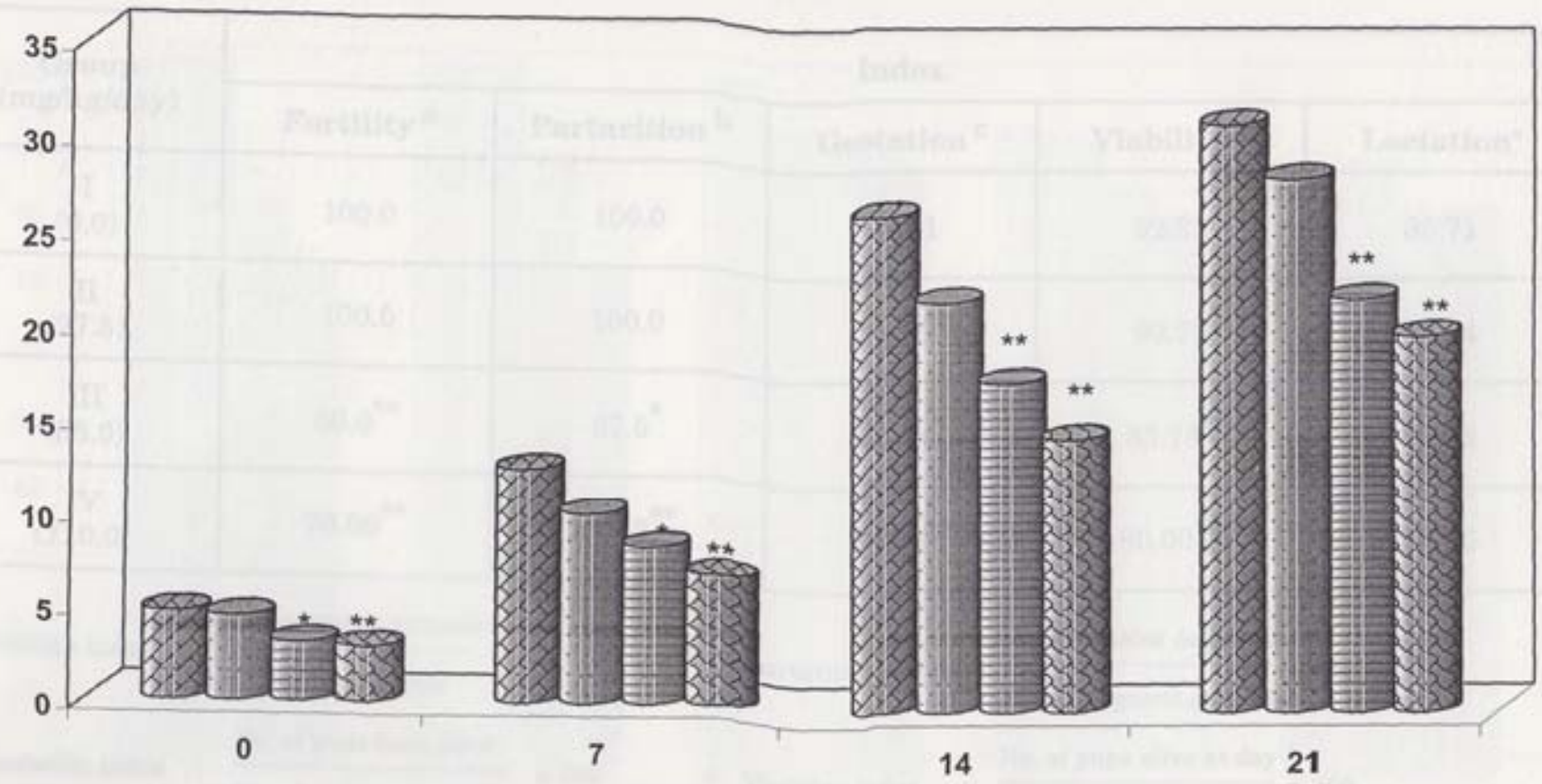
## TABLE 17

## EFFECT OF HEXACONAZOLE ON LITTER SIZE AND POSTNATAL GROWTH OF PUPS

| Group (mg/kg/day) | Average litter size | Body weight (g) (Mean ± SE) of pups on postnatal day | | | |
|---|---|---|---|---|---|
| | | 0 | 7 | 14 | 21 |
| I (0.0) | 9.20 | 4.7 ± 0.5 | 12.6 ± 0.8 | 26.5 ± 1.2 | 31.4 ± 2.1 |
| II (27.5) | 7.00* | 4.5 ± 0.2 | 10.2 ± 1.3 | 22.0 ± 1.3 | 28.5 ± 2.3 |
| III (55.0) | 5.86** | 3.1 ± 0.4* | 8.4 ± 0.6* | 17.7 ± 0.8** | 22.2 ± 1.9** |
| IV (110.0) | 4.60** | 2.9 ± 0.1** | 7.0 ± 0.3** | 14.7 ± 1.3** | 20.2 ± 1.6** |

*$P < 0.05$ **$P < 0.01$

## FIGURE 19 : EFFECT OF HEXACONAZOLE ON LITTER SIZE AND POSTNATAL GROWTH OF PUPS



## TABLE 18

## EFFECT OF HEXACONAZOLE ON REPRODUCTIVE INDICES IN RATS

| Group (mg/kg/day) | Index | | | | |
|---|---|---|---|---|---|
| | Fertility [a] | Parturition [b] | Gestation [c] | Viability [d] | Lactation [e] |
| I (0.0) | 100.0 | 100.0 | 98.91 | 92.31 | 85.71 |
| II (27.5) | 100.0 | 100.0 | 92.86* | 90.77 | 88.14 |
| III (55.0) | 80.0** | 87.5* | 90.24* | 83.78* | 80.65 |
| IV (110.0) | 70.00** | 71.43** | 86.96** | 80.00** | 81.25 |

a. Fertility index : $\frac{\text{No. of pregnant animals}}{\text{No. of females mated}} \times 100$

b. Parturition index : $\frac{\text{No. of females delivered}}{\text{No. of pregnant animals}} \times 100$

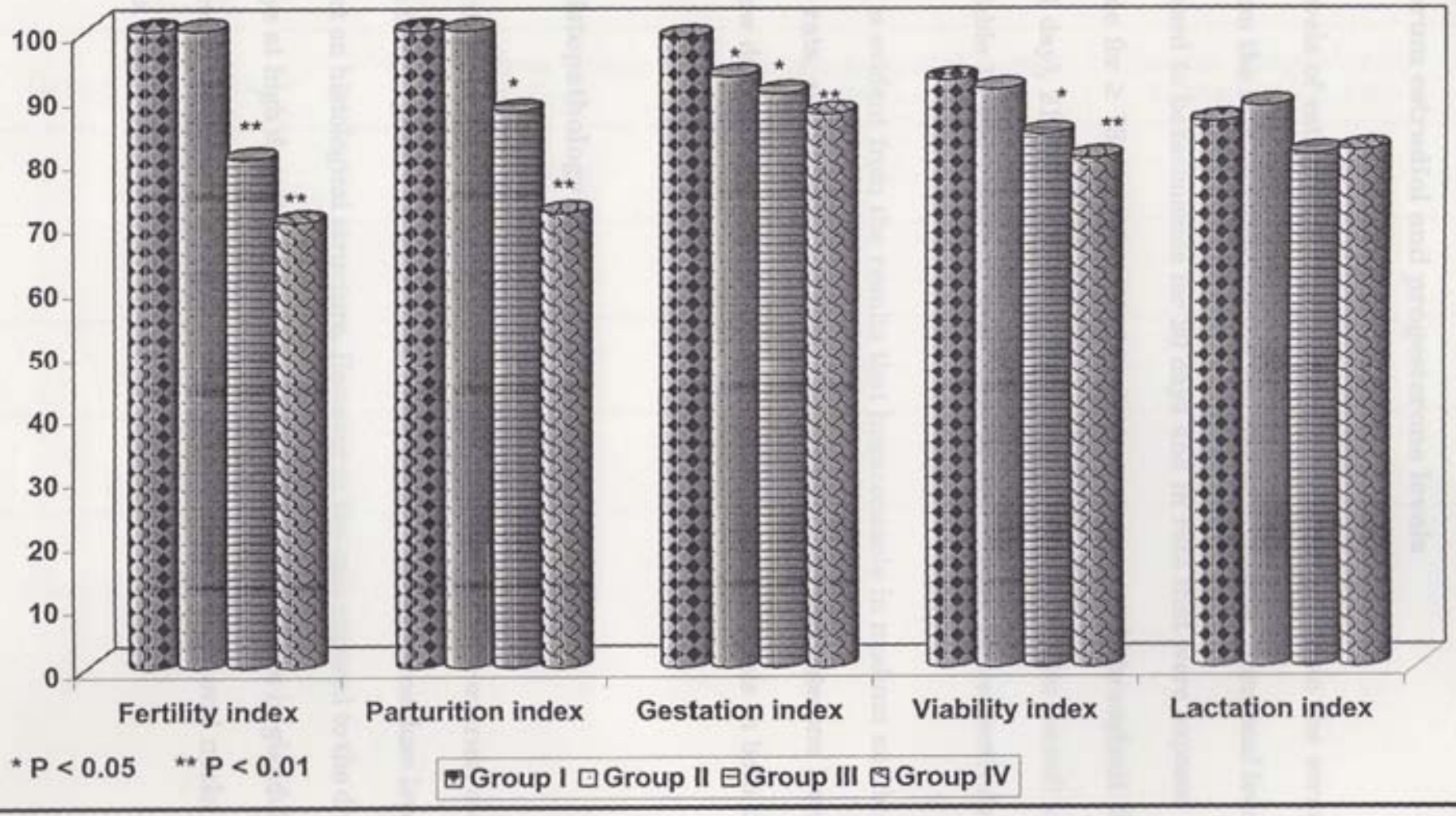
c. Gestation index : $\frac{\text{No. of pups born alive}}{\text{No. of females mated}} \times 100$

d. Viability index : $\frac{\text{No. of pups alive at day 4}}{\text{No. of pups born alive}} \times 100$

a. Lactation index : $\frac{\text{No. of pups alive at day 21}}{\text{No. of pups alive at day 4}} \times 100$

* $P < 0.05$ ** $P < 0.01$

## FIGURE 20 : EFFECT OF HEXACONAZOLE ON REPRODUCTIVE INDICES IN RATS



### 4.3.7 Serum estradiol and progesterone levels

Levels of estradiol and progesterone were estimated in the serum, collected from the rats sacrificed on the mid day of proestrus. Hormonal levels in rats exposed to hexaconazole for 30 days and in rats that were exposed to hexaconazole for ≥ 73 days (i.e. 30 days before mating and throughout the mating (≥ 1 day), 21 days gestation period and 21 days lactation period) are shown in Table 19 and Figure 21 and Table 20 and Figure 22, respectively.

It is evident from the results that hexaconazole in medium and high dose group rats, significantly reduced the estradiol and progesterone levels, while the low dose of drug had no effect on these hormone levels in both the studies.

### 4.3.8 Histopathology of reproductive organs

It is evident based on the microscopic observation of the ovaries, uteri and vagina that exposure to hexaconazole for 30 days at the three dose levels had no effect on histological structure. However in the rats exposed to the drug for ≥ 73 days at high dose level, the ovaries showed mild changes in glandular epithelial cells which were elongated and spindle shaped with oval nuclei in the medulla (Plate 19).

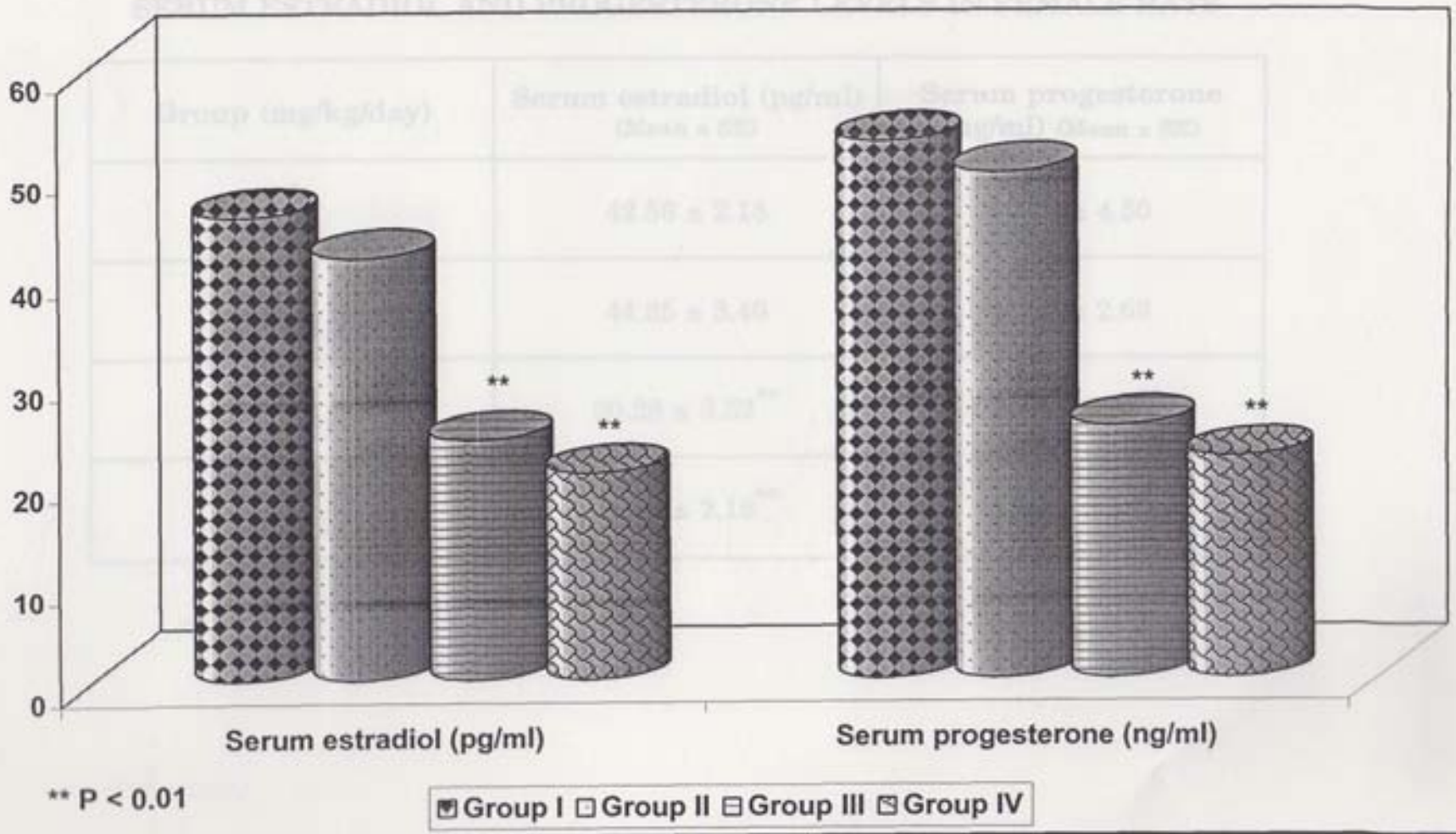
## TABLE 19

## EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON ESTRUS CYCLE AND SERUM ESTRADIOL AND PROGESTERONE LEVELS IN FEMALE RATS

| Group (mg/kg/day) | No. of estrus cycles in 15 days | Serum estradiol (pg/ml) (Mean ± SE) | Serum progesterone (ng/ml) (Mean ± SE) |
|---|---|---|---|
| I (0.0) | 3.3 ± 0.21 | 45.28 ± 4.51 | 52.46 ± 3.57 |
| II (27.5) | 3.2 ± 0.25 | 41.32 ± 3.67 | 49.36 ± 2.19 |
| III (55.0) | 2.1 ± 0.34* | 23.52 ± 4.15** | 24.72 ± 2.23** |
| IV (110.0) | 1.6 ± 0.42** | 20.18 ± 2.78** | 21.64 ± 2.1** |

*$P < 0.05$ **$P < 0.01$

## FIGURE 21 : EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON SERUM ESTRADIOL AND PROGESTERONE LEVELS IN FEMALE RATS



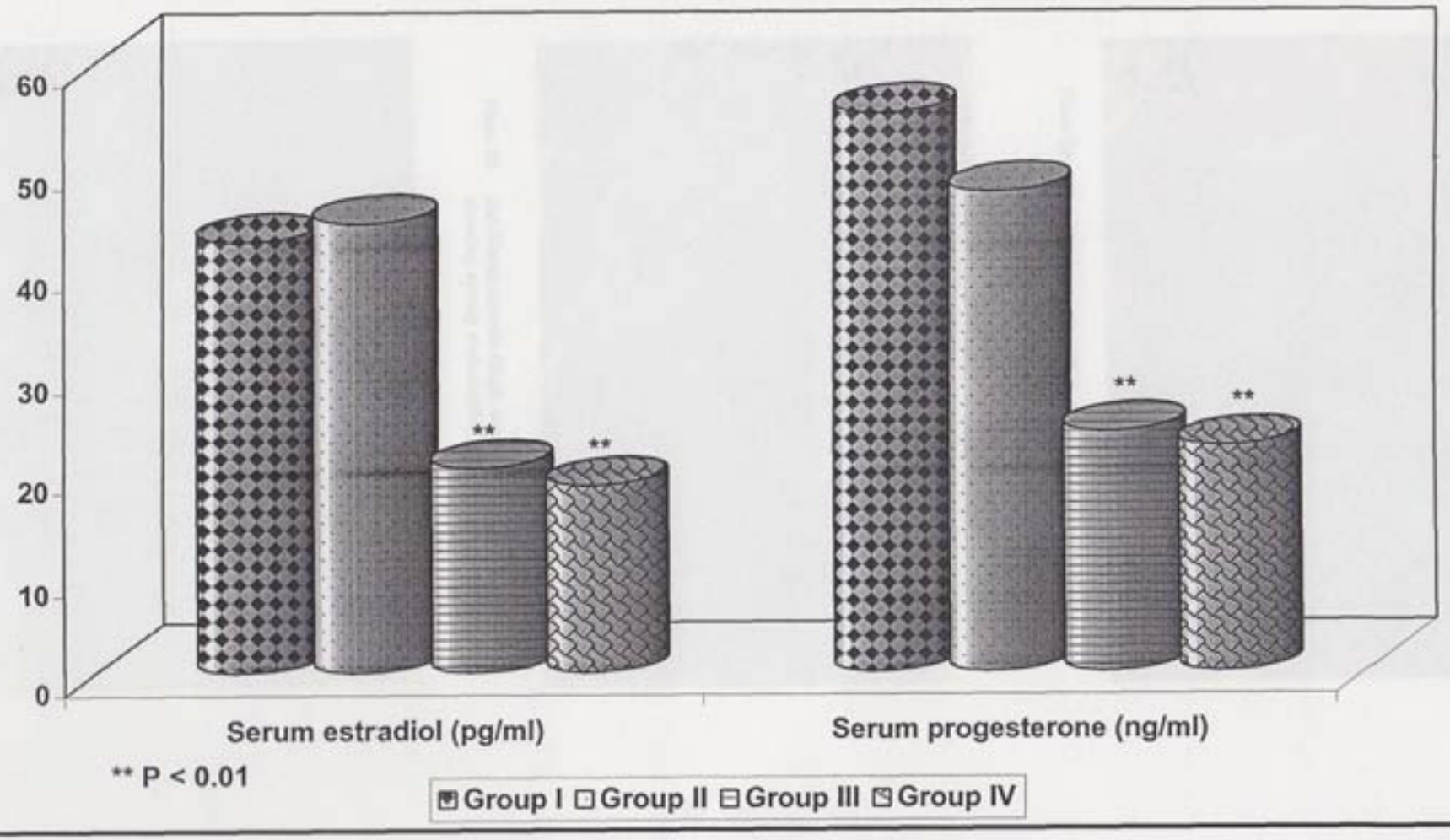
## TABLE 20

## EFFECT OF HEXACONAZOLE (≥ 73 DAYS *PER OS*) ON SERUM ESTRADIOL AND PROGESTERONE LEVELS IN FEMALE RATS

| Group (mg/kg/day) | Serum estradiol (pg/ml) (Mean ± SE) | Serum progesterone (ng/ml) (Mean ± SE) |
|---|---|---|
| I (0.0) | 42.56 ± 2.18 | 55.12 ± 4.50 |
| II (27.5) | 44.35 ± 3.46 | 47.35 ± 2.63 |
| III (55.0) | 20.28 ± 3.52** | 23.78 ± 3.21** |
| IV (110.0) | 18.47 ± 2.16** | 22.35 ± 3.48** |

**$P < 0.01$

## FIGURE 22 : EFFECT OF HEXACONAZOLE (≥ 73 DAYS *PER OS*) ON SERUM ESTRADIOL AND PROGESTERONE LEVELS IN FEMALE RATS



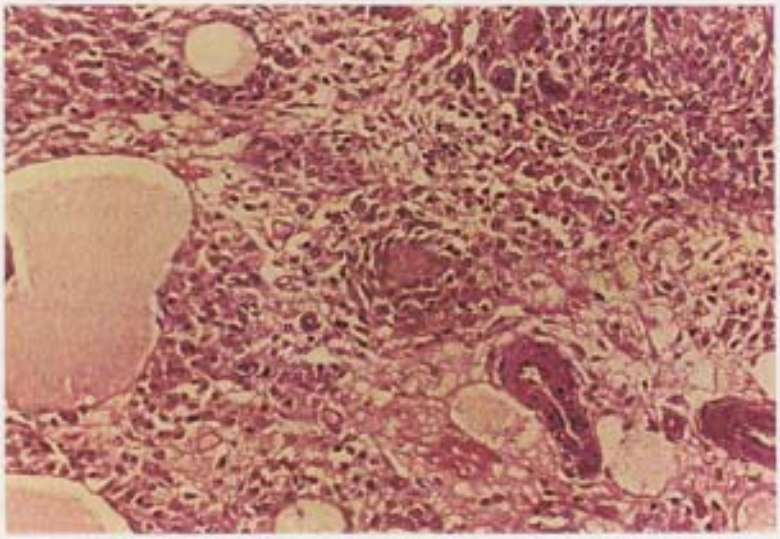


Plate 19 Rat-Hexaconazole (High dose - ≥ 73 days)-Ovary-showing elongated glandular epithelial cells in the medulla H & E x 360

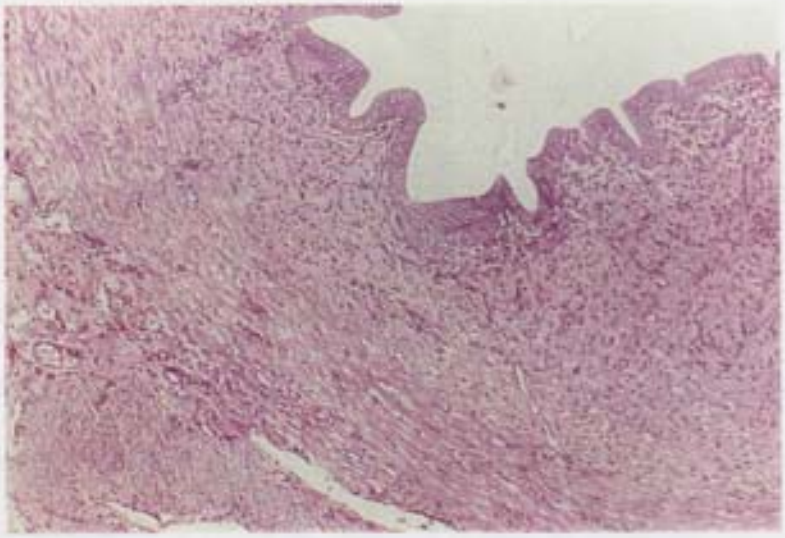


Plate 20 Rat-Hexaconazole (High dose - ≥ 73 days)-Uterus-showing scanty endometrial glands H & E x 100

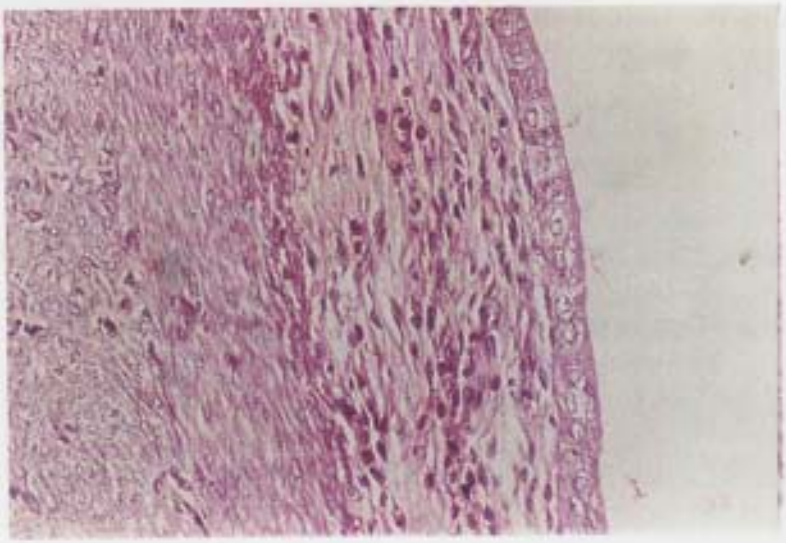


Plate 21 Rat-Hexaconazole (High dose - ≥73 days) -Uterus-showing short columnar cells with basally placed nuclei with scanty glands H & E x 360

Compared to the changes in ovaries, the changes in uteri and vagina were more conspicuous. Uterus in the rats treated with hexaconazole for ≥ 73 days at medium dose showed scanty endometrial glands (Plate 20), while in those treated with high dose the uterus showed small inactive epithelial cells lining the endometrium with large basally placed nuclei, atrophy of myometrium and absence of endometrial glands (Plate 21). In a few cases the endometrium revealed focal hyperplasia and squamous metaplasia (Plate 22). Vagina showed marked atrophy of lining epithelium which appeared only 2-3 layered (Plate 23)

## 4.4 HEPATOTOXIC POTENTIAL

The hepatotoxic potential of hexaconazole was assessed in rats that were exposed to hexaconazole in reproductive toxicity assessment trials. Male rats were exposed to the drug for 30 days and 60 days and females were exposed for 30 days and ≥ 73 days. Serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and cholesterol were estimated. Liver tissues were subjected to histopathological examination.

### 4.4.1 Serum biochemical profiles

In both the trials in male rats, medium and high doses of the drug significantly increased the alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase enzymes and significantly decreased the cholesterol levels in serum (Tables 21 and 22 and Figures 23 and 24).

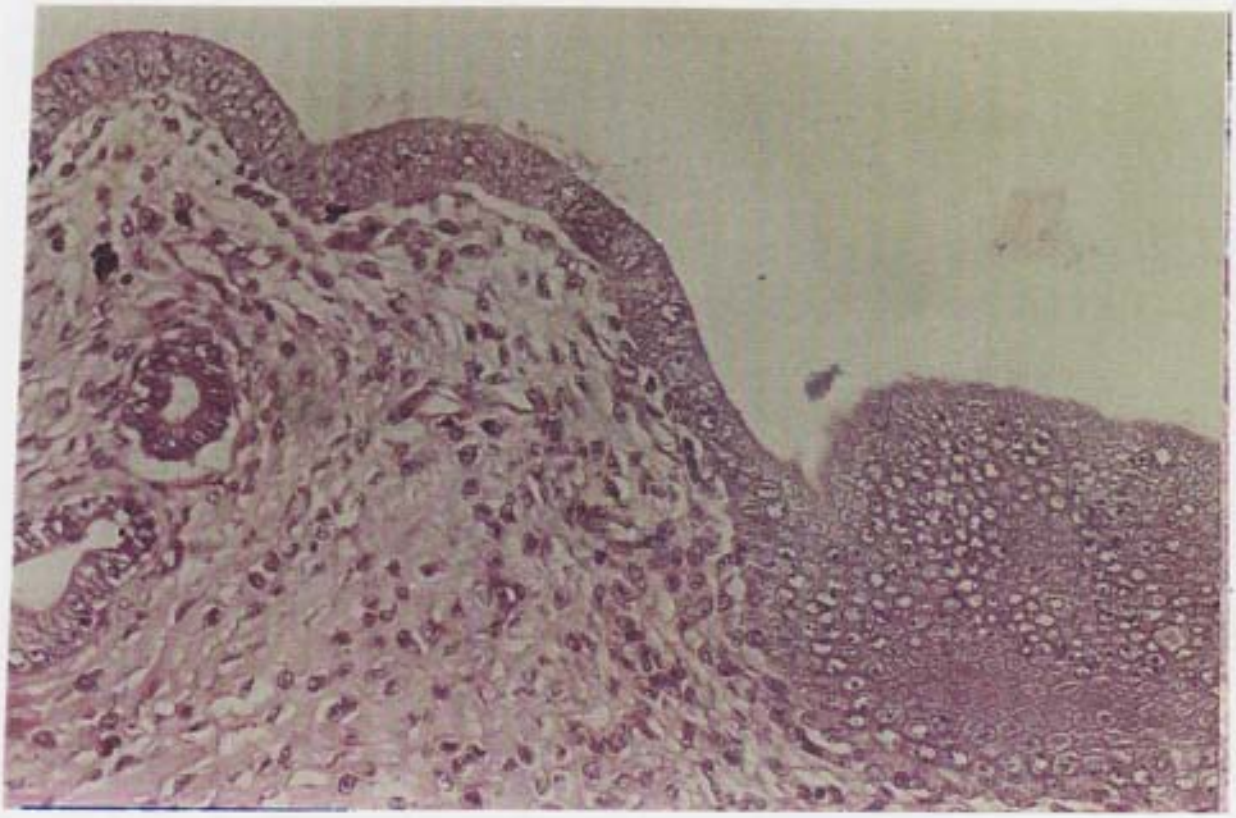


Plate 22 Rat-Hexaconazole (High dose - ≥ 73 days) Uterus-lining cells of endometrium showing focal hyperplasia and squamous metaplasia H & E x 360

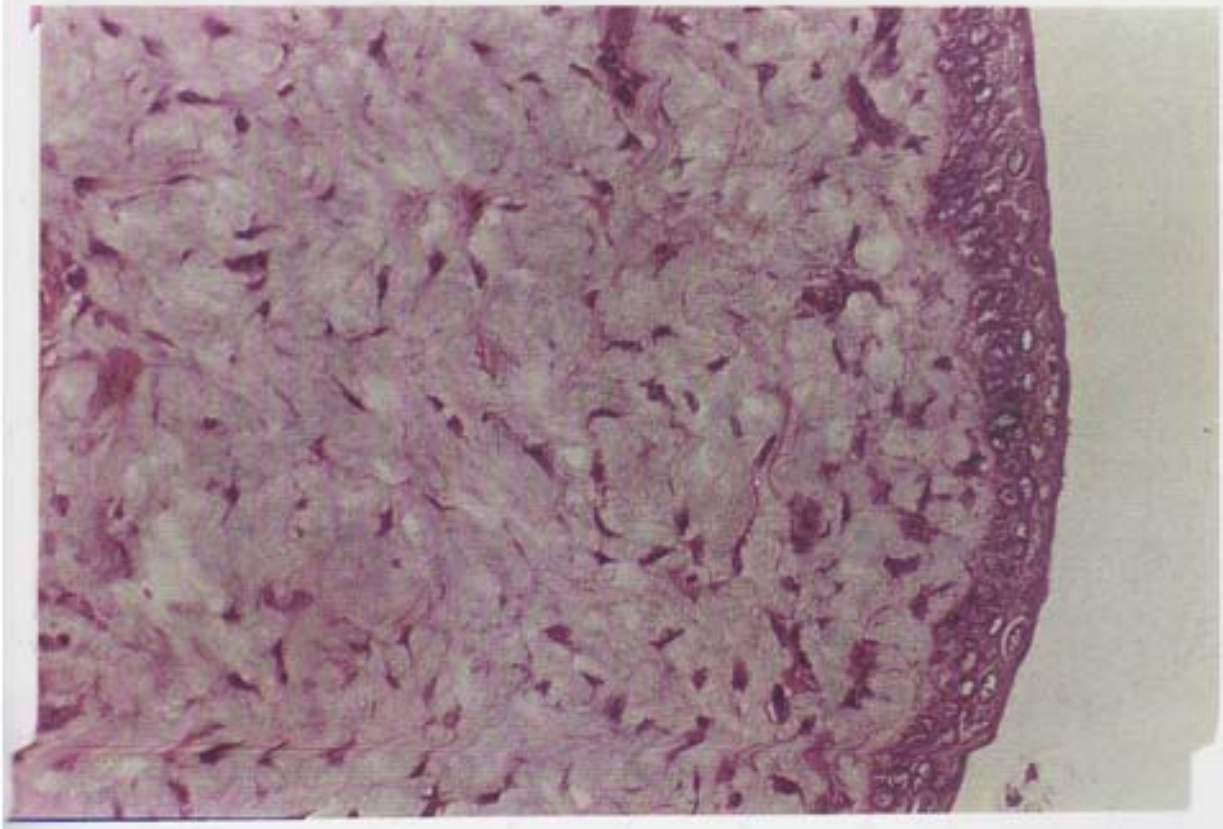


Plate 23 Rat-Hexaconazole (High dose - ≥ 73 days) Vagina-showing thinned out epithelium which is 2-3 layered H & E x 360

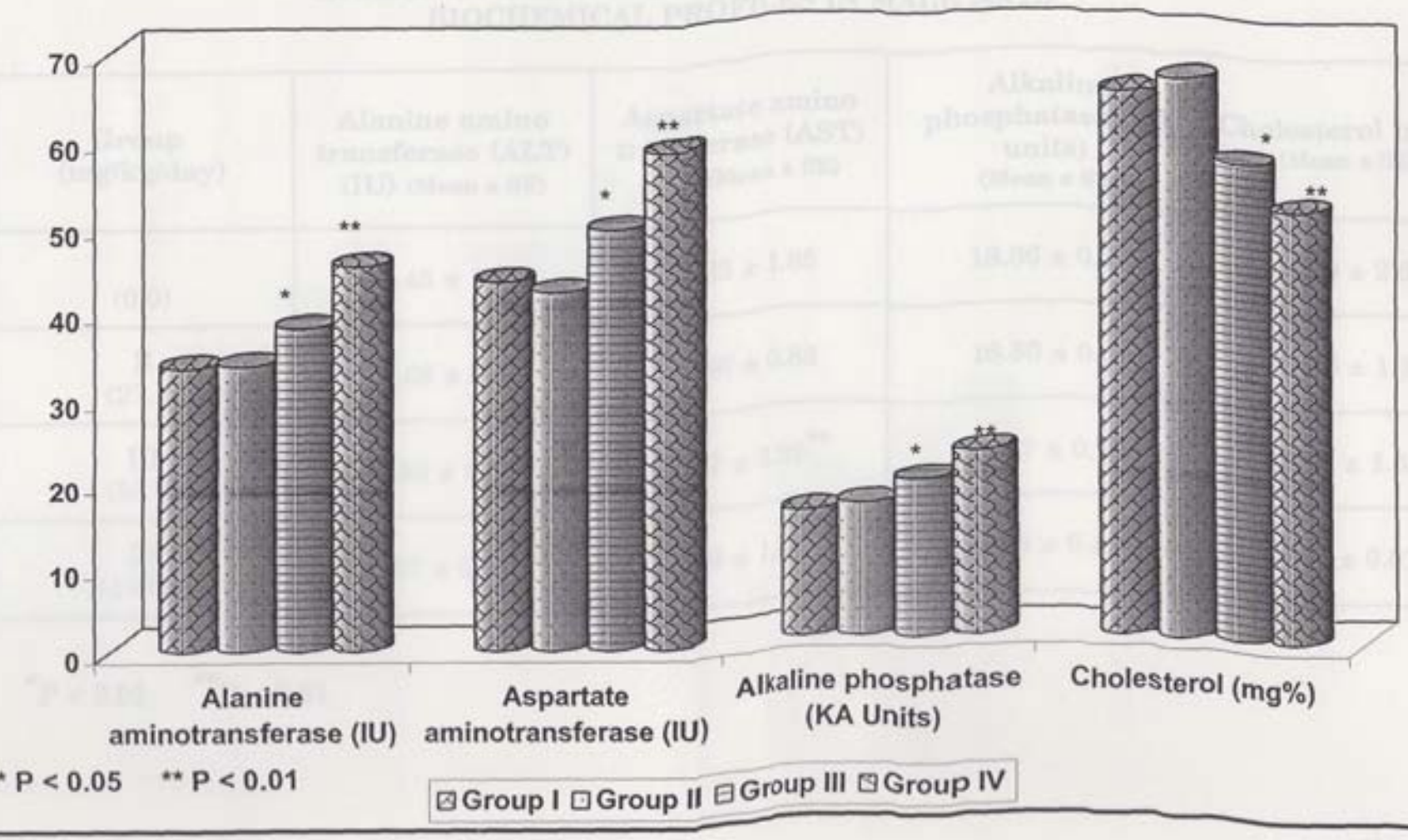
## TABLE 21

## EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON SERUM BIOCHEMICAL PROFILES IN MALE RATS

| Group (mg/kg/day) | Alanine amino transferase (ALT) (IU) (Mean ± SE) | Aspartate amino transferase (AST) (IU) (Mean ± SE) | Alkaline phosphatase (KA units) (Mean ± SE) | Cholesterol (mg%) (Mean ± SE) |
|---|---|---|---|---|
| I (0.0) | 33.39 ± 1.66 | 43.44 ± 0.82 | 14.78 ± 0.34 | 63.81 ± 2.77 |
| II (27.5) | 33.63 ± 1.87 | 42.19 ± 1.73 | 15.57 ± 0.32 | 65.71 ± 3.11 |
| III (55.0) | 38.00 ± 2.59* | 49.50 ± 1.43* | 18.52 ± 1.22* | 56.28 ± 2.77* |
| IV (110.0) | 45.28 ± 2.96** | 58.34 ± 2.79** | 21.71 ± 1.36** | 50.95 ± 2.24** |

*P < 0.05 **P < 0.01

## FIGURE 23 : EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON SERUM BIOCHEMICAL PROFILES IN MALE RATS



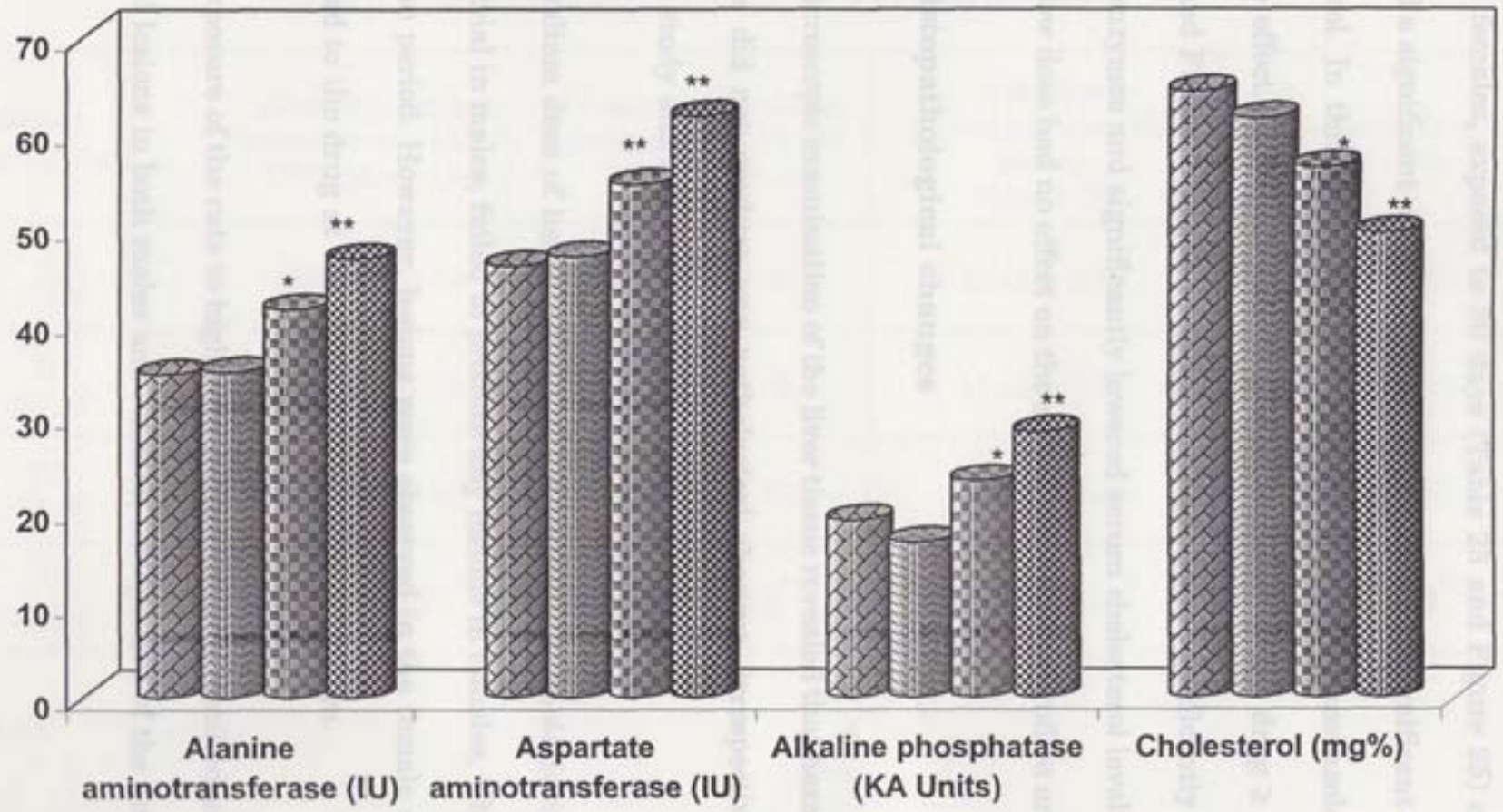
## TABLE 22

## EFFECT OF HEXACONAZOLE (60 DAYS *PER OS*) ON SERUM BIOCHEMICAL PROFILES IN MALE RATS

| Group (mg/kg/day) | Alanine amino transferase (ALT) (IU) (Mean ± SE) | Aspartate amino transferase (AST) (IU) (Mean ± SE) | Alkaline phosphatase (KA units) (Mean ± SE) | Cholesterol (mg%) (Mean ± SE) |
|---|---|---|---|---|
| I (0.0) | 34.45 ± 1.53 | 45.88 ± 1.85 | 18.86 ± 0.23 | 64.29 ± 2.63 |
| II (27.5) | 34.68 ± 0.62 | 46.86 ± 0.82 | 16.50 ± 0.47 | 61.45 ± 1.31 |
| III (55.0) | 41.36 ± 0.61* | 54.47 ± 2.32** | 23.07 ± 0.26* | 56.28 ± 1.62* |
| IV (110.0) | 46.67 ± 0.54** | 61.69 ± 1.57** | 28.25 ± 0.42** | 49.41 ± 0.87** |

*P < 0.05 **P < 0.01

## FIGURE 24 : EFFECT OF HEXACONAZOLE (60 DAYS *PER OS*) ON SERUM BIOCHEMICAL PROFILES IN MALE RATS



In females, exposed to 30 days (Table 23 and Figure 25) only high dose caused a significant elevation of serum enzymes and significant decrease in cholesterol. In this group low and medium dose of hexaconazole had no appreciable effect. In female rats that were exposed to the drug ≥ 73 days, (Table 24 and Figure 26) both medium and high doses significantly elevated the serum enzymes and significantly lowered serum cholesterol levels. In this study also low dose had no effect on the serum biochemical profiles monitored.

### 4.4.2 Histopathological changes

Microscopic examination of the liver tissue revealed that hexaconazole at low dose did not produce any pathological changes, irrespective of the duration of study and sex of the animals.

Medium dose of hexaconazole which produced pathological changes in 30 days trial in males, failed to produce any lesions in females, when given for the same period. However, lesions were observed in the female rats that were exposed to the drug at the same dose level for ≥ 73 days.

Exposure of the rats to high dose of hexaconazole resulted in marked pathological lesions in both males and females, irrespective of the duration of exposure.

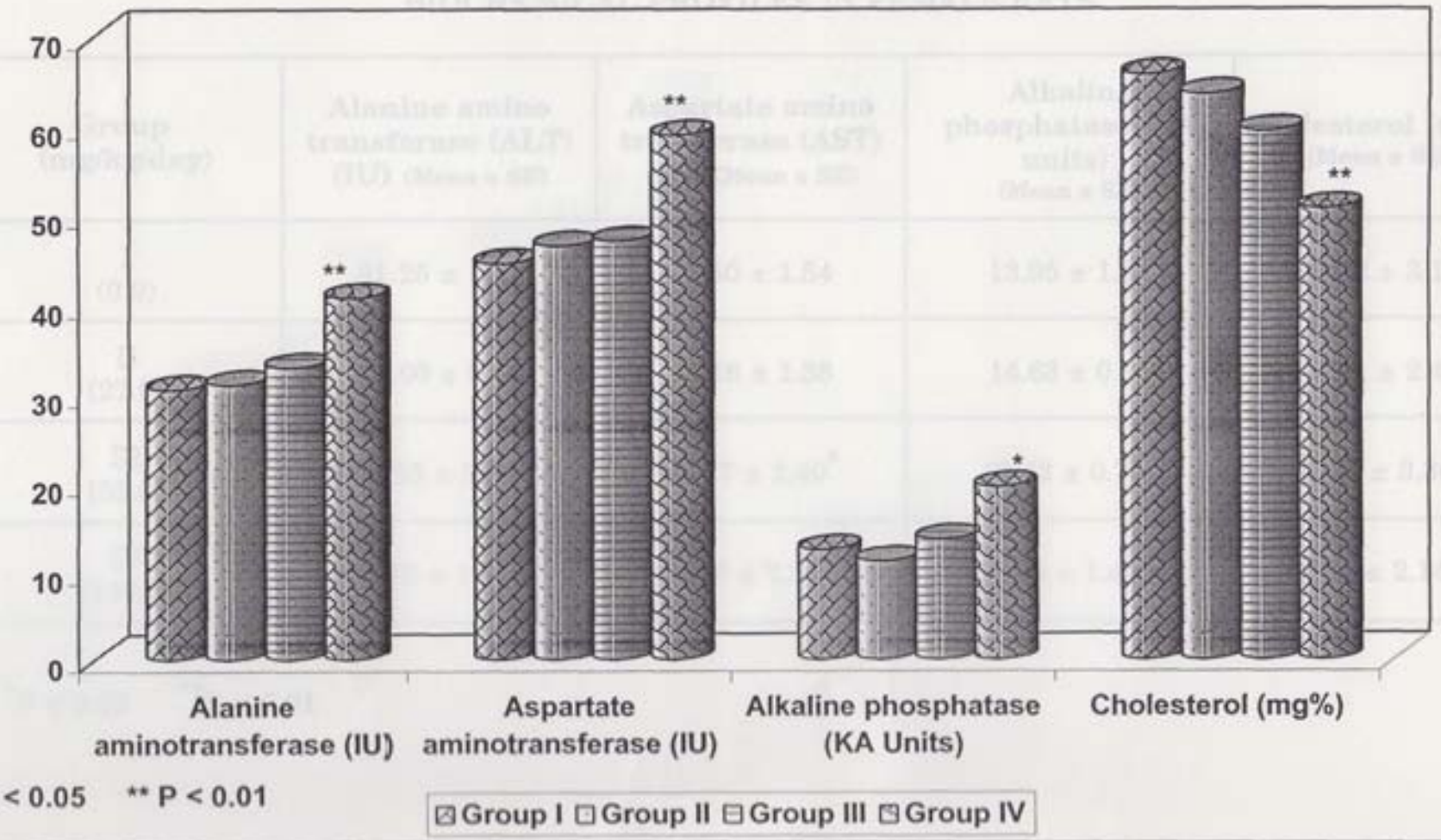
## TABLE 23

## EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON SERUM BIOCHEMICAL PROFILES IN FEMALE RATS

| Group (mg/kg/day) | Alanine amino transferase (ALT) (IU) (Mean ± SE) | Aspartate amino transferase (AST) (IU) (Mean ± SE) | Alkaline phosphatase (KA units) (Mean ± SE) | Cholesterol (mg%) (Mean ± SE) |
|---|---|---|---|---|
| I (0.0) | 30.57 ± 1.51 | 44.62 ± 1.83 | 12.45 ± 0.54 | 65.80 ± 1.52 |
| II (27.5) | 31.10 ± 1.62 | 46.54 ± 0.92 | 11.12 ± 0.71 | 63.62 ± 1.35 |
| III (55.0) | 33.02 ± 1.24 | 47.10 ± 1.43 | 13.60 ± 0.48 | 59.45 ± 1.67 |
| IV (110.0) | 40.81 ± 0.97** | 59.02 ± 1.65** | 19.47 ± 0.59* | 50.78 ± 2.54** |

*P < 0.05 **P < 0.01

## FIGURE 25 : EFFECT OF HEXACONAZOLE (30 DAYS *PER OS*) ON SERUM BIOCHEMICAL PROFILES IN FEMALE RATS



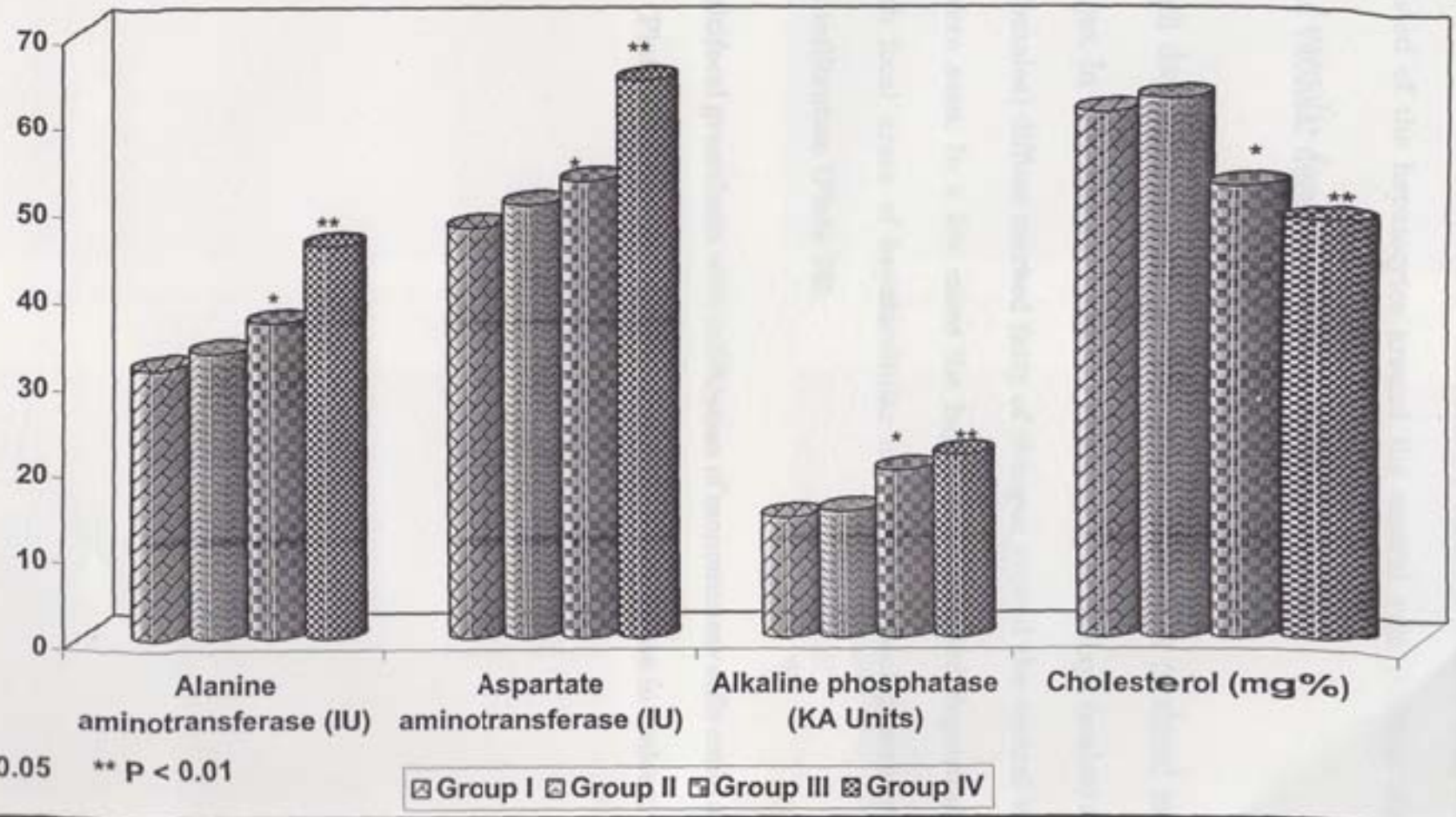
## TABLE 24

## EFFECT OF HEXACONAZOLE (≥ 73 DAYS *PER OS*) ON SERUM BIOCHEMICAL PROFILES IN FEMALE RATS

| Group (mg/kg/day) | Alanine amino transferase (ALT) (IU) (Mean ± SE) | Aspartate amino transferase (AST) (IU) (Mean ± SE) | Alkaline phosphatase (KA units) (Mean ± SE) | Cholesterol (mg%) (Mean ± SE) |
|---|---|---|---|---|
| I (0.0) | 31.25 ± 1.32 | 47.50 ± 1.54 | 13.95 ± 1.21 | 60.72 ± 3.13 |
| II (27.5) | 33.00 ± 0.56 | 50.16 ± 1.38 | 14.63 ± 0.81 | 62.41 ± 2.41 |
| III (55.0) | 36.53 ± 0.81* | 52.87 ± 2.40* | 19.42 ± 0.73* | 52.38 ± 3.36* |
| IV (110.0) | 45.42 ± 1.25** | 64.48 ± 2.58** | 21.25 ± 1.41** | 48.65 ± 2.14** |

*P < 0.05 **P < 0.01

## FIGURE 26 : EFFECT OF HEXACONAZOLE (≥ 73 DAYS *PER OS*) ON SERUM BIOCHEMICAL PROFILES IN FEMALE RATS



The lesions in male rats that received medium dose of drug for 30 days consisted of the hepatocytes around the central vein showing cloudy swelling and vacuolar degenerative changes (Plate 24).

High dose of hexaconazole when given for 30 days produced more severe changes. In rats that were exposed to the drug for 60 days (males) and ≥ 73 days (females) diffuse marked fatty of changes around the central vein (Plate 25) were seen. In a few cases the hepatocytes revealed degenerative changes with focal areas of hepatocellular necrosis and focal plasmocytic-lymphocytic infiltration (Plate 26).

Multifocal granuloma with infiltration of mononuclear cells could also be observed (Plate 27). The lesions were more severe and diffuse in males than in females.

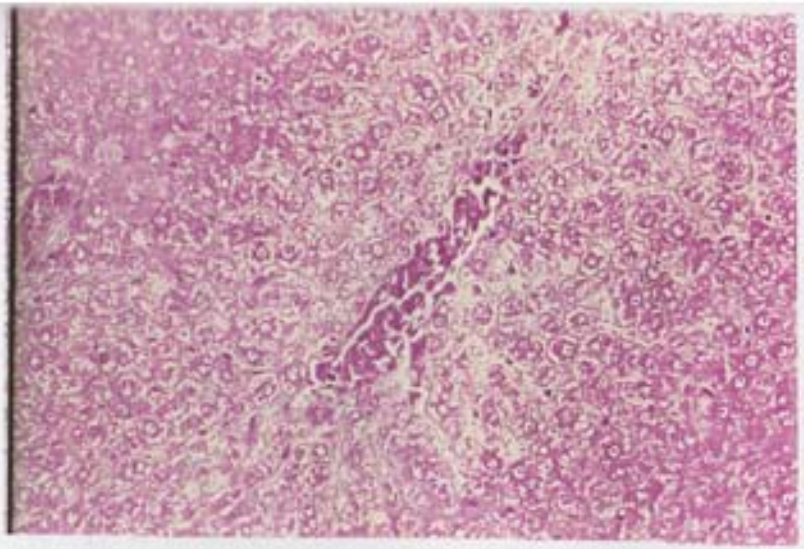


Plate 24 Rat (male) Hexaconazole (High dose-30 days)- Liver-showing diffuse degenerative changes around the central vein H & E x 100

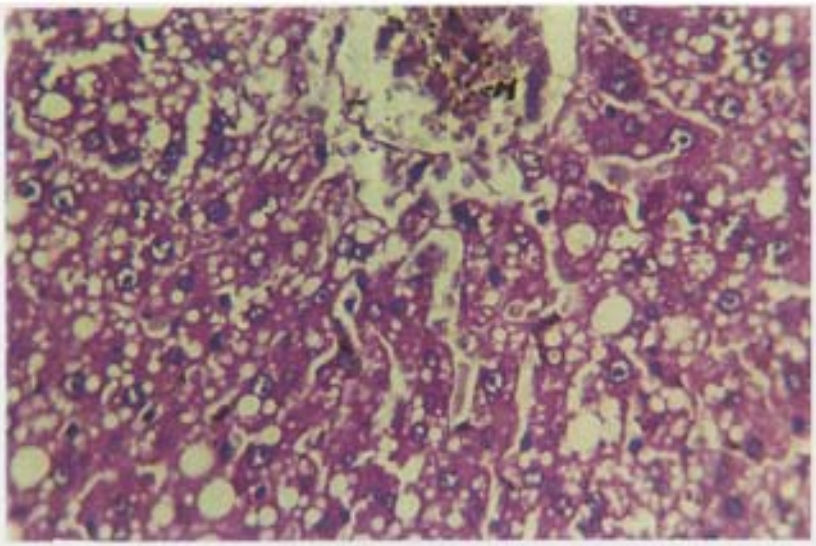


Plate 25 Rat (male)-Hexaconazole (High dose-60 days) - Liver-showing fatty changes H & E 3 320

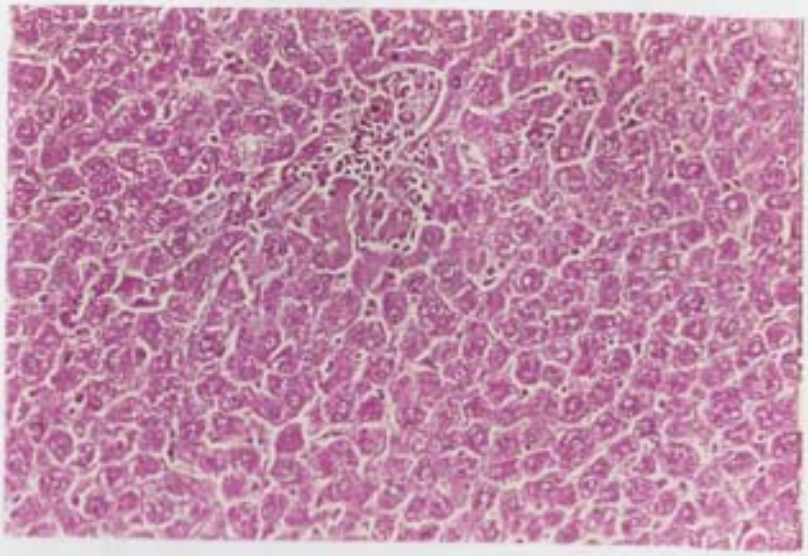


Plate 26 Rat (male)-Hexaconazole (High dose - 60 days) - Liver-showing focal necrosis and lymphocytic - plasmocytic infiltration H & E x 100

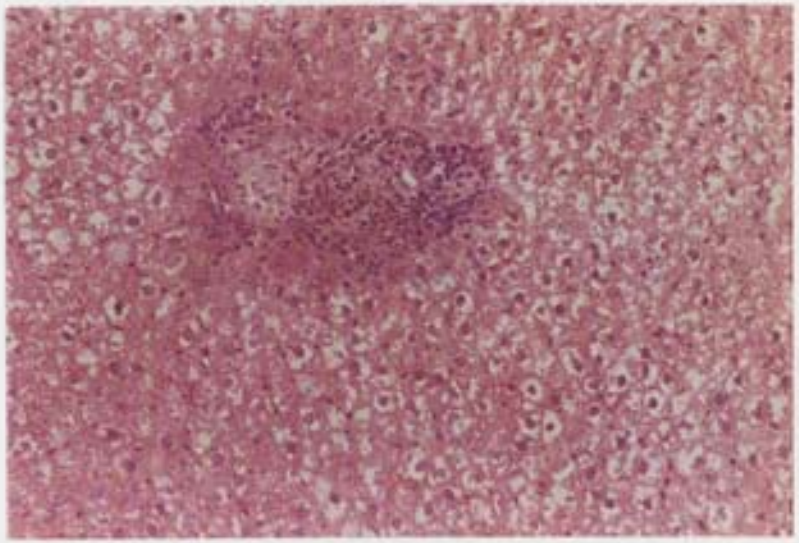


Plate 27 Rat (male)-Hexaconazole (High dose 60 days) - Liver-showing focal microgranuloma H & E x 100

# *Discussion*

# CHAPTER - 5

# DISCUSSION

Several new chemicals continue to enter the environment, as part of man's day to day activities and the presence of many such chemicals in the biosphere is not without any adverse impact on the living things. Intensive agricultural practices are making our delicate environment vulnerable to the accumulation of several pesticides. A number of these pesticides were linked to a host of health hazards in man and animals. Constant screening of these chemicals, particularly the new entrants, is thus highly warranted.

Hexaconazole, a triazole compound, of late is being used extensively as a fungicide in agricultural and horticultural practices. Hence, this compound was selected and its clastogenic potential and reproductive effects were studied.

## 5.1 CLASTOGENIC EFFECT

Among the potential ill effects of pesticides, mutagenicity, clastogenicity and carcinogenicity are especially significant. Genetic alterations of a clastogenic type have been reported to be associated with a variety of human cancer and birth defects (Radman *et al.*, 1982, Brusick, 1984). In order to protect the health and genetic heritage of humans and animals, the evaluation of genotoxic potential of these pesticides used in our environment is necessary.

Hexaconazole was assessed for its clastogenic potential in *in vivo* and *in vitro* systems.

### 5.1.2 *In vivo* clastogenetic studies

#### 5.1.2.1 Bone marrow chromosomal aberrations

In rats exposed to single dose of hexaconazole, the percentage of aberrant cells observed were 0.33, 0.17 and 0.50 with low, medium and high doses respectively, while they were 0.17 in control group. In rats that were exposed to multiple doses, the percent aberrant cells were 0.33, 0.17, 0.67 and 0.5 respectively. In control, low, medium and high dose groups. However in both the studies, cyclophosphamide, which was used as a positive control (Preston *et.al.*, 1987a and Oldham, 1997) produced a very high incidence of chromosomal aberrations.

Results indicated that hexaconazole, in the tested doses did not produce any structural or numerical aberrations in both male and female rats. Exposure to single dose of hexaconazole as well as multiple doses, failed to demonstrate any clastogenic effect. Although a single exposure would, in the majority of cases provide for maximum sensitivity of assay (Preston *et al.*, 1987a), multiple exposures were also followed in the present study, to ensure adequate concentration of the drug in bone marrow. This was felt necessary, in view of the paucity of literature on this particular aspect.

Though gaps (or achromatic lesions) were recorded, they were not included in the calculations, since their cytogenetic significance is questionable (Preston *et al.*, 1987a). Gaps might be actually a single strand break in the DNA double helix as a result of incomplete excision repair and thus might represent a point of possible instability (Bender *et al.*, 1988). Damage to the genetic material, if not repaired rapidly and correctly, changes the DNA sequence. If the changes are not lethal they will lead to heritable changes i.e. mutations. DNA sequence changes can be single nucleotide changes that result in point mutations or multiple nucleotide changes that result in visible chromosomal aberrations (Kramer, 1998). Mutations often result in the elimination or alteration of gene function. The adverse effect of a mutation depends on the gene, the site of the gene and the tissue affected. The most serious effects of mutations in germ cells are birth defects and in somatic cells the neoplasms. Carcinogenesis is often associated with mutations in oncogenes and antioncogenes (tumour-suppressor genes) (Kramer, 1998).

Triazole compounds are often implicated in thyroid tumours. 3-amine -1, 2, 4 - triazole was associated with thyroid tumors in rats (Hiasa *et al.*, 1982, Tsuda, 1975; Steinhoff *et al.*, 1983; Mattioli *et al.*, 1994). Diniconazole was also reported to induce thyroid tumor in rats (Hosokawa *et al.*, 1993). However, the tumorigenic potential of triazoles in thyroid gland was not related to their genotoxic potential, instead, it was linked to hormonal disregulation mechanism (Hosokawa *et al.*, 1993; Mattioli *et al.*, 1994).

### 5.1.2.2 The micronucleus test

The micronucleus test, *in vivo*, is a method devised primarily for screening chemicals for chromosome breaking effects. In the monitoring of chromosome breakage, the test is as sensitive as the metaphase method ; in addition it also detects the effects on the spindle apparatus (Schmid, 1975).

The method is based on the following principle and observations : in anaphase, acentric chromatid and chromosome fragments lag behind when the centric elements move towards the spindle poles. After telophase the undamaged chromosomes, as well as the centric fragments, give rise to regular daughter nuclei. The lagging elements are also included in the daughter cells, but a considerable proportion is transformed into one or several secondary nuclei which are, as a rule, much smaller than the principal nucleus and are therefore called micronuclei. These micronuclei still remain in the immature erythrocytes (polychromatic erythrocytes) even after the extrusion of the regular nucleus (Schmid, 1975). Thus, an increase in the frequency of micronucleated polychromatic erythrocytes indicates that the administered compound had interfered with nuclear division of the bone marrow erythroblasts in such a way that chromatin fragments or whole chromosome had lagged at anaphase and failed to be incorporated into one of the daughter nuclei at the time of cell division.

Analysis of the bone marrow smears of the slides for micronucleated polychromatic erythrocytes revealed no significant increase in their number in hexaconazole treated rats. Multiple exposures also could not enhance their

incidence significantly. These observations confirmed the results of chromosomal aberrations assay in this study, where hexaconazole could not produce any chromosomal damage. It further appears from this study that hexaconazole has no effect on spindle formation, during cell division. A well known clastogen, cyclophosphamide included as a positive control, significantly increased the occurrence of micronucleated polychromatic erythrocytes.

### 5.1.3 *In vitro* clastogenic study

Clastogenic potential, *in vitro*, of hexaconazole was studied in rat blood lymphocyte cell cultures. Hexaconazole was added to the cultures in three different concentrations of 125, 250 and 500 µg / ml of total culture. Mitomycin was used as a positive control drug. This study was conducted without the use of exogenous metabolic activation system, that contains extract of liver microsomal (S9) and $NADPH_2$ as a coenzyme. Some genotoxic chemicals can produce their effects directly, whereas others require metabolism to mutagenically active intermediates. Since, *in vivo* studies, which formed part of the clastogenic assays in the present study, could indicate the effect of mutagenically active intermediates, of hexaconazole metabolism, this *in vitro* study aimed mostly in detecting the inherent ability of intact hexaconazole molecule to cause cytogenetic damage. To meet this requirement, exogenous metabolic activation system was not added to the culture. To establish the sensitivity of the test to detect any clastogenic effect, mitomycin C was used as a positive control drug. Mitomycin is preferred to cyclophosphamide, as the former is a direct-acting clastogen. In contrast, cyclophosphamide requires metabolic activation, before it produces any cytogenetic damage (Oldham, 1997).

It was evident from the careful observation of the metaphase spreads of lymphocyte cell cultures that there was no significant difference in the percentage of aberrant cells between the control and the hexaconazole treated cultures. The percentage of aberrant cells recorded in these groups ranged between 1 and 2, which was well with in the order of normal frequency of aberrant cells in cultures (Preston *et al.*, 1987b).

### 5.1.4 Clastogenic effect of hexaconazole - conclusion

Results obtained in all the three assays, in both *in vivo* and *in vitro* systems, indicated that hexaconazole is a non-clastogen in the tested doses. The negative results obtained in this study in bone marrow chromosomal aberrations, the micronucleus test and *in vitro* lymphocyte cell cultures, are in accordance with the earlier reports on some triazole compounds. Amitrole, a triazole herbicide did not increase mutation frequencies in tests involving two genera of bacteria, *Drosophila* and human lymphocyte cell cultures (Sorsa and Gripenberg, 1976; Laamanen *et al.*, 1976, and Meretoza *et al.*, 1976). WHO (1974) also reported negative results in *Salmonella* strains and yeast cells with amitrole. Further, no cytogenetic effects were observed in bone marrow cells from rats treated with amitrole for 5 days. Mattioli *et al.* (1994) also reported no evidence of DNA fragmentation in *in vivo* studies in rats, with amitrole. 3 - (N-saticylol) amino - 1, 2, 4- triazole (SAT) gave a negative result in *in vitro* *Salmonella* mutation assay (Katoh and Takayama, 1981). Triadimefon neither increased the frequency of micronucleated polychromatic erythrocytes in bone marrow cells of mice nor had any sister chromatid exchange inducing effect in human lymphocyte cell cultures (Kevekordes *et al.*, 1996).

Triazoles are reported as potential carcinogens to thyroid gland and carcinogenesis is often related to mutagenicity. However, none of the studies, established the genotoxic effect of triazoles behind the thyroid tumour induction and rather a hormonal disregulation mechanism was suggested for the said effect (Hosokawa *et al.*, 1993; Mattioli *et al.*, 1994).

## 5.2 EFFECT ON MALE REPRODUCTIVE SYSTEM

Livestock and humans are exposed to potential and known reproductive toxins in the environment. The function of the male reproductive system may often be the most sensitive to toxic effects (Meistrich, 1986). Indeed, azoospermia, in the absence of any other major systemic toxicities, has been documented in workers handling the pesticide dibromochloropropane (Wharton *et al.*, 1977).

In the present study triazole fungicide hexaconazole, was assessed for its potential toxic effects on male reproductive system in rats. Triazoles inhibit the fungal growth through inhibition of ergosterol biosynthesis in fungal cell membrane, via cytochrome P450 containing enzyme inactivation. Several cytochrome P450 containing enzymes take part in mammalian steroid hormone synthesis. Hence, it could be possible that the selected triazole fungicide, hexaconazole may have some effect on steroid reproductive hormone synthesis in mammals.

To test this possible phenomenon, hexaconazole was administered to the rats in three different dose levels for 30 days and 60 days. The 60 days

maximum exposure period was choosen, basing on 48 days as the period of spermatogenic cycle (Clermont and Harvey, 1967) and 9 to 14 days for sperm passage through the epididymis (Robb *et al.*, 1978). Christian (1997) also suggested that males be treated for 60 days, a full cycle of spermatogenesis. Body weights were recorded fortnightly. Blood was collected on 30$^{th}$ and 60$^{th}$ day for assay of serum testosterone levels. At necropsy, testes, seminal vesicle and prostate were collected and weights recorded. Epididymal sperm were analysed for total count, motility percentage, percentage of dead sperm and percentage of abnormal sperm.

There was no appreciable decline in body weights of the treated rats at the end of first fortnight. But, by the end of second fortnight, significant reduction in body weight was observed in medium and high dose groups. Medium dose caused a 10 per cent decline in body weight by 30$^{th}$ day, which further declined to 16 per cent by 60$^{th}$ day, when compared against body weight in control group. The decrease in body weight was highly significant in high dose group, in which it was down by 14.6 per cent of control on 30th day and 22.6 per cent on 60th day.

Decreased feed intake, due to unpalatability of the feed was ruled out in this study, since the drug was given by stomach tube. Hence, the decrease in weight gain observed may be attributed to drug induced anorexia and general toxic effect on various organ systems, particularly in the liver in the treated animals.

Wet weights of testes, seminal vesicle and prostate glands collected at necropsy, were recorded. Since there were differences in the terminal body weights between control and treated rats, comparison of absolute weights of these organs may not give the actual effect of the drug on their development. Hence to derive at a more realistic interpretation, the organ weights were transformed as per cent of the terminal body weight.

It was observed from the results that the relative weights of testes, prostate and seminal vesicle were adversely affected by the end of 30 days, in medium and high dose hexaconazole groups. This effect was more severe by the end of 60 days. However, low dose of hexaconazole tested in this study did not induce any effect on the testes and accessory reproductive organs, the prostate and seminal vesicle. Thus, it was evident that hexaconazole could cause the regression of testes and accessory reproductive organs.

Effect of hexaconazole on the quality of semen was assessed by analyzing the cauda epididymal sperm reserve for total count, motility, dead sperm and abnormal sperm. Results revealed that hexaconazole at medium and high doses, significantly decreased the total count, and motility and significantly increased the dead and abnormal sperm proportions in the epididymal sperm reserve. These changes were dose and time dependent.

Since, testosterone synthesis involves the active role of cytochrome P450 containing enzymes, their likely inhibition by hexaconazole may result in altered circulating testosterone levels. Thus, measurement of serum testosterone levels will indicate any effect of the hexaconazole on this steroid

hormone synthesis. In the treated rats, the testosterone level was not altered significantly in low dose group, either after 30 days or 60 days of exposure. However, testosterone levels were significantly lowered in medium and high dose groups. The decrease was slightly more in the rats that were exposed to the drug for 60 days. These observations demonstrated the ability of hexaconazole to impair the testosterone synthesis in rats.

To know the effect of hexaconazole on testicular tissue, the tissue sections were subjected to quantitative and qualitative screening.

Quantitative analysis consisted of the counting of seminiferous tubules, for their number per microscopic field, diameter and per cent damaged. In medium and high doses of hexaconazole treated groups there was a significant increase in the number of tubules observed per field (x 100) and a significant decrease in the tubular diameter. These changes were more prominent in 60 days trial. The increase in seminiferous tubular number with concurrent decrease in tubular diameter indicated the atrophy of testes, in treated groups. In addition to these atrophic changes, these testes also revealed more number of damaged tubules. However medium dose of hexaconazole given for 30 days, did not cause significant increase in the number of damaged tubules, though atrophic changes were conspicuous. But this same dose of hexaconazole, when given for 60 days resulted in the appearance of significantly higher number of damaged tubules, indicating its cumulative effect.

The qualitative changes consisted of degenerative lesions in gonadolcells, detachment of seminiferous epithelium with impaired spermatogenesis and hyperplasia of Leydig cells. The intensity of these changes increased with dose and time of exposure.

The overall effects of hexaconazole, on male reproductive system observed in the present study include decreased testicular weight, regression of accessory reproductive organs, adverse effects on sperm count, motility, viability and morphology, decreased circulating testosterone and histopathological changes in seminiferous tubules and Leydig cells.

The testicular mass, a valuable index of reproductive toxicity in male animals (Amann, 1982), found decreased in this study is consistent with the atrophic and degenerative changes observed microscopically. The reduction in testis weight can be attributed to a loss of spermatogenic elements histologically and probably reduced levels of androgen binding protein as a result of local androgen deprivation observed in the study. The synthesis and secretion of androgen binding proteins are androgen and follicular stimulating hormone dependent (Tindall and Means, 1976).

The degenerative changes might have resulted from the direct cytotoxic effect of hexaconazole on the seminiferous tubular cells. Another probable reason for the pathological changes may be the appearance of the factor(s) from the peripheral circulation due to hepatic and/or renal toxicity of hexaconazole. Hepatic malfunction and renal failure are known to influence testicular functions (Lim and Fang, 1975; Griffin and Wilson, 1991).

Leydig cell hyperplasia can be attributed to lowered circulating testosterone levels. To maintain functional levels of testosterone, it is probable that, the Leydig cells exhibited hyperplastic changes as a compensatory phenomenon.

The regression of accessory reproductive organs, prostate and seminal vesicle, noted might be due to the decreased levels of circulating testosterone. Male accessory sex organs are essentially under the control of androgen through their specific receptor (Liao *et al.*, 1975). Atrophy of accessory sex glands in the presence of hypoandrogenism was also reported by Ortiz *et al.* (1999) and Menjivar *et al.* (1997).

Lemasters and Selevan (1993) reported that alterations in epididymal sperm count and motility provide a direct measure of fertility in animals. Sperm motility is often used as a marker of chemical induced testicular toxicity (Mori *et al.*, 1991).

Significant depletion in sperm reserves in the cauda epididymis observed in hexaconazole treated rats pointed out the impaired spermatogenic activity in the testis. It is well known that high levels of intra testicular testosterone are necessary, for the proliferation and differentiation of spermatogenic cells and spermatogenesis. Thus the reduced testosterone levels induced by hexaconazole might have contributed in this effect. Moreover spermatogonia lie dormant until puberty and then proliferative activity resumes. These rapidly dividing, developing, and maturing cells are highly susceptible to chemical insult at many stages (Ecobichon, 1995).

In addition to the sperm number, viability, motility and the sperm morphology were also affected in the hexaconazole treated rats. Zenick and Clegg (1989) noted several sources of morphological alterations viz. mutagenic events, nonmutagenic events or cellular degeneration. Non mutagenic events may occur during spermiogenesis or during post testicular development involving disruption of the maturational process.

The biochemical environments in the testes and epididymides are highly regulated to assure the proper development and maturation of the sperm and the acquisition of critical functional characteristics. With the chemical exposures, perturbations of this balance may occur, producing alterations in sperm properties (Zenick and Clegg, 1989). Thus in the present study alterations in sperm morphology and motility appear to be associated with the androgen - deprived maturational anomalies (Zenick *et al.*, 1994; Iwasaki *et al.*, 1995) as well the modified epididymal milieu due to possible presence of hexaconazole and/or its metabolites. High circulating testosterone concentration is required for androgen-dependent sperm maturation process in epididymis (Steinberger, 1971).

Decreased serum testosterone levels observed in treated rats indicated the ability of hexaconazole to impair testosterone synthesis. Decreased androgen levels in medium dose group, without appreciable testicular damage, indicate the noncytotoxic mechanisms behind the androgen synthesis inhibition. Decreased testosterone synthesis was reported with other azoles earlier. Decreased testosterone synthesis in the presence of

ketoconazole, an imidazole, was widely reported (Santen *et al.*, 1983; Hanger *et al.*, 1988; Bhasin *et al.*, 1986). Though triazoles are comparatively less potent than imidazoles in inhibiting steroid hormone synthesis, they are not devoid of this ability at higher doses (Hanger *et al.*, 1988; Soltis and Colby, 1998).

## 5.3 EFFECT ON FEMALE REPRODUCTIVE SYSTEM

Many chemicals in our environment have been shown to interfere with reproductive function in animals. The male is often used to study reproductive toxicity because spermatogenesis is a continuous process. Any interruption of spermatogenesis is manifested as atrophy of the testis or accessory sex glands, which can be easily recognized and assessed both grossly and microscopically. Reproductive toxicity is less often studied in the female because toxicity is more difficult to detect and interpret. Reproductive function in the female is often a noncontinuous cyclic process (Haschek and Rousseaux, 1998). Keeping this in view, the present study on female reproductive system was designed carefully employing necessary parameters.

The three doses of hexaconazole selected in the study were 27.5, 55.0 and 110.0 mg/kg body weight. These were the same doses selected for assessing male reproductive toxicity of hexaconazole. Though the LD50 value of hexaconazole is very high in females, compared to males, the present study was confined to the doses that were used in male reproductive toxicity assessment. This was based on the report that the no observed effect level (NOEL) for reproductive toxicity of hexaconazole in rats is 50 mg/kg body weight per day and 250 mg of hexaconazole per kg body weight per day

increased post implantation loss attributable to an elevation in late intrauterine deaths, when given to mated females from day 7-16 of gestation (PRDD 95). Since the present study involved the administration of the drug for a prolonged period i.e. before mating and through out the gestation period, the highest dose was deliberately chosen as 110 mg / kg / day.

The study was conducted on the mature rats whose normal estrous cyclicity was established, prior to the start of administration of the drug. Each group consisted of 20 rats. Ten rats per each group, were utilised for estrus cycle monitoring from $16^{th}$ to $30^{th}$ day of exposure to the drug and these were sacrificed on the mid day of proestrus for estimation of serum estradiol and progesterone concentrations, apart from the histological examination of ovaries. The remaining 10 rats in each group were allowed to mate with untreated males for further studies like maternal weight gain, reproductive indices and hormonal profiles. The rats that were used for estrus cycle monitoring for 15 days, were avoided for further reproductive studies because continuous manipulation of vagina for such a prolonged period could possibly result in pseudopregnancy and give false interpretation of results (Manson and Kang, 1989).

Body weights recorded on the 30th day, indicated that low and medium doses of hexaconazole tested, did not significantly reduce the body weight. However in high dose group a highly significant decrease was observed. The medium dose of 55.0 mg/kg which resulted in significantly decreased body weight in 30 days, in males, failed to induce the same effect in females suggesting that females are less sensitive than males to hexaconazole toxicity. This is in line with the higher LD50 values of hexaconazole in female rats.

The earliest biomarker that reflects ovotoxicity identified in laboratory animals is disruptions in estrus cyclicity (Hoyer and Sipes, 1996). It was observed that hexaconazole at medium and high doses significantly impaired the estrus cyclicity in rats. Since estrus cycle is a reflection of circulating sex steroids, it appears that hexaconazole caused disruption of normal estrus cycle in rats through impaired steroidogenesis.

Studies on the effect of hexaconazole on the maternal body weight gains in mated female rats revealed that the drug significantly reduced the maternal weight gain, at medium and high doses tested. Medium dose (55.0 mg/kg) of hexaconazole which did not reduce the body weight gain by the end of 30 day exposure period, significantly reduced the maternal weight gain in pregnant rats. This might be due to the prolonged administration of the drug, beyond 30 days. Impaired development of fetuses in them, also might be another reason for this.

When hexaconazole pretreated rats were mated with untreated males, it was recorded that the number of rats that could become pregnant was reduced in medium and high dose groups. It was further observed that all those that became pregnant in these groups, could not deliver at the end of the gestation period. In medium dose group out of 10 animals mated, only 7 rats could deliver the pups, while in high dose group only 5 rats delivered out of the 10 rats mated. Low dose of hexaconazole did not effect the pregnancy and its culmination in delivery. However, all the three doses of hexaconazole reduced the average litter size which was evident from the decreased total number of pups. All the three doses also reduced the birth weight and postnatal day 21 body weight of the pups. However, the effect was significant only in medium and high dose groups.

Reproductive indices in the hexaconazole treated rats revealed that, it affected the fertility and parturition indices at medium and high dose levels. Gestation index was affected by the three doses of hexaconazole. The affected gestation index and decreased average litter size in low dose group, are suggestive of the fetotoxic effect of hexaconazole even at 27.5 mg/kg dose. It is worth noting here that this dose did not cause any adverse effect, other than this, either in male or female rats. Viability and lactation indices were found decreased in high and medium dose groups.

These effects of hexaconazole on the reproductive indices revealed its potential to impair the mating and ovulatory process along with the fetotoxicity. Impairment of ovulation and fetotoxic potentials were previously recorded with other triazole members (Middleton *et al.*, 1986; Milne *et al.*, 1987; Vergieva, 1990; Machera *et al.*, 1995).

Histopathological examination of ovaries revealed no conspicuous changes expect those in glandular cells at high dose level. Uterus and vagina, however revealed atrophic changes.

Recognition of an atrophic ovary may be difficult in short term studies in rats because of continued follicular development. In addition, corporalutea may not be obviously reduced in number or size due to the orientation of the ovary during tissue preparation. However, ovarian interstitial glands, particularly those located in the medulla, offer valuable information about ovarian activity (Haschek and Rousseaux, 1998).

Atrophy of uterus and vagina observed in the study can be considered as secondary to deficiency of ovarian steroid hormones (Haschek and Rousseaux, 1998).

Estimation of serum estradiol and progesterone levels in hexaconazole treated rats showed that these hormone levels were significantly lowered in medium and high dose groups, with no alterations in low dose group.

Decrease in circulating estradiol and progesterone levels by the administration of substituted triazoles was reported by Middleton *et al.* (1986). Milne *et al.* (1987) reported decreased estradiol levels with another substituted triazole. Among the azole antifungals, imidazoles are more prone to inhibit mammalian steroid hormone synthesis and triazoles are less potent in this aspect. However reports are available suggesting that at high doses even triazoles can inhibit the mammalian steroid hormone synthesis (Hanger *et al.*, 1988).

One important enzyme in the steroid synthesis pathway is CYP19 aromatase, which catalyses the conversion of androgens to estrogens. This is an enzyme complex involving an NADPH-Cytochrome C reductase and a cytochrome P450 (Brodie, 1985). *In vitro* inhibition of this aromatase by the triazole fungicides, propiconazole, triadimefon and triadimenol was reported by Vinggaard *et al.* (2000). Hence, the lowered levels of estradiol, observed in hexaconazole treated rats might be due to, the failure of conversion of testosterone and androstenedione to estradiol as well as decreased availability of testosterone itself for conversion. Hexaconazole lowered the testosterone levels in male rats in this study.

Since it is well known that male reproductive tissues express estrogen receptors and that estrogen is present in low concentrations in the blood of males, inhibition of aromatase may have implications on male reproductive system also. Reproductive systems of rats and mice are affected by aromatase inhibition, where as the human system is not (WHO, 1995). In several species, aromatase is present in the tail of newly released sperm and has been found to be active in the epididymal sperm as well as in the developing germ cells of the testis (Hess *et al.*, 1995). Recently, estrogen has been found to regulate fluid reabsorption in the efferent ductules of mice (Hess *et al.*, 1997). These tubules carry the sperm from the testis to the epididymis, and this up-concentrating function of estrogen seems to be essential for fertility and related to sperm concentration. Thus the exact role for estrogen and aromatase in the male has not yet been fully clarified (Vinggaard *et al.*, 2000).

## 5.4 HEPATOTOXICITY

Liver is the major site of metabolism resulting in the activation of exogenous chemicals or xenobiotics to toxic metabolites. Liver is also the first major organ to be exposed to ingested toxins due to its portal blood supply. Therefore, when animals are exposed to xenobiotics, toxic responses occur relatively frequently in the liver compared with other organs.

The results indicated that low dose of hexaconazole had no effect on the liver in both males and females as was evident from the unaltered serum biochemical profiles and histological structure. Medium dose, which showed signs of hepatotoxicity in 30 days trial in males, did not show any such effect

in females. This may be due to the low dose (in relation to the LD50 dose in females) used in females. However the same dose when given for prolonged period i.e. ≥ 73 days caused hepatotoxicity.

Higher dose of hexaconazole produced marked hepatotoxicity in both the trials in males and females.

Determination of the activity of alanine aminotransferase and aspartate aminotransferase indicate the cytotoxic hepatic injury and that of alkaline phosphatase reflects cholestatic injury (Zimmerman, 1978). Elevated level of serum transaminases is in accordance with the histopathological changes observed in liver sections. Increased activity of serum transaminases by the administration of various azoles was reported earlier (Anaissie *et al.*, 1995; Lavrijsen *et al.,* 1992; Findor *et al.,* 1998).

Apart from the elevated transaminase hexaconazole also caused a decline in the serum cholesterol level. Beynen *et al.* (1981) reported that 3-amino-1,2,4-triazole drastically depressed cholesterol synthesis by hepatocytes.

# *Summary*

# CHAPTER - 6

# SUMMARY

Hexaconazole, a triazole fungicide that inhibits ergosterol biosynthesis in fungi, was tested for its clastogenic and reproductive effects in rats. Clastogenicity was studied in *in vivo* and *in vitro* systems and reproductive toxicity was assessed in male and female rats separately.

Hexaconazole at the rate of 1/12, 1/6 and 1/3rd of oral LD50 dose (2189 mg/kg in males and 6071 mg/kg in females) was given *per os*. The doses in male and female rats were 182, 365 and 730 mg/kg and 506, 1012 and 2024 mg/kg, respectively. *In vivo* studies consisted of observation of bone marrow chromosomal aberrations in metaphase spreads and assay of micronucleated polychromatic erythrocytes in the bone marrow cells of rats that were exposed to the drug once or for five consecutive days (multiple exposures). Examination of chromosomal spreads of rat lymphocytes cultured in hexaconazole (125, 250 and 500 µg/ml of total culture) containing medium formed the *in vitro* study. The results revealed that hexaconazole did not cause the chromosomal aberrations in *in vivo* and *in vitro* systems and did not increase the frequency of micronuclei formation in bone marrow cells. Thus it was apparent that hexaconazole is nonclastogenic at the tested doses in rats.

Male reproductive toxicity was assessed in adult rats that were exposed to the drug (27.0% 55.0 and 110.0 mg/kg/day) *per os* for 30 and 60 days. Weight of testes, prostate and seminal vesicles were recorded and semen analysis, histopathology of testis and assay of serum testosterone levels were

carried out at necropsy. Results showed that hexaconazole at medium and high doses studied, caused atrophy of testis and regression of accessory sex glands. Quality of semen was adversely affected, which was indicated by decreased sperm count and motility and increased percentage of dead and abnormal sperm. Histologically the testis revealed degenerative changes in gonadal cells with impaired spermatogenesis and hyperplasia of Leydig cells. Serum testosterone levels were also drastically reduced by the medium and high doses of hexaconazole.

Female reproductive toxicity was assessed in adult female rats that were exposed to the drug (27.0, 55.0 and 110.0 mg/kg/day) *per os* for 30 days before mating and mated to untreated male rats. They continued to receive the treatment during the mating, gestation and lactation (21 days) periods. Estrus cycle was monitored in treated rats and in the mated rats reproductive indices *viz.*, fertility index, parturition index, gestation index, viability index and lactation index and serum estradiol and progesterone levels were recorded. Ovaries, uterus and vagina were examined histologically. Hexaconazole in the medium and high doses tested adversely affected the estrus cycle and reproductive indices, while the serum estradiol and progesterone levels were significantly lowered. The uterus and vagina were atrophied.

Hexaconazole was also studied for its toxic effect on liver. The drug caused on increase in serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase while it decreased the serum cholesterol levels. There were also histopathological lesions in liver.

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