DISCUSSION
CHAPTER IV

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PART - I EFFECT OF HORMONAL COMBINATION

This part describes the reversible contraceptive effects of a hormonal combination of medroxyprogesterone acetate (MPA) and non-aromatizable androgen, dihydrotestosterone (DHT) in adult male rats. The hormonal regimen consists of bimonthly intramuscular injections of depot medroxyprogesterone acetate (DMPA) and dihydrotestosterone (DHT) at doses of 20 mg.kg\(^{-1}\).d\(^{-1}\) and 1000 μg.kg\(^{-1}\).d\(^{-1}\) respectively. This combination was given to rats for 90 days to study its effects on body and organ weights, spermogram viz., sperm count, sperm motility, and sperm viability. Sperm acrosomal integrity using silver nitrate stain was also carried out. The biochemical parameters like 3β, 17β hydroxysteroid dehydrogenases (HSDs), succinate dehydrogenase (SDH), alkaline and acid phosphatases (ALKpase and ACPase) and protein levels in the testis, SDH, ALKpase, ACPase, Protein and sialic acid levels in the epididymis and glycogen, phosphorylase and protein levels in the vas deferens were assessed in all groups of rats. Haematological parameters viz., blood cell counts (RBC and WBC), haemoglobin (Hb) content and serum parameters like protein, cholesterol transaminases and testosterone levels were estimated to evaluate toxicity. Electrophysiological studies of the vas deferens was carried out to study the contractility pattern of this tissue in all experimental groups. Histological studies as well as histocytometry of the above tissue were done in all
experimental rats. Finally the recovery studies after 90 days of post-treatment was also carried out.

The average increase in the body weights did not alter significantly during the treatment period. But the reduction in the testicular weight was found to be significant (56%) and this reduction was attributed to the loss of spermatogenic elements as a result of local androgen deprivation and reduced levels of androgen binding protein (ABP) in the testis. This is because the biosynthesis and the secretion of androgen binding protein (ABP) appear to be regulated by both FSH and androgen (Tindall and Means, 1976). Loss of large proportion of germ cells reduces the apical secretion of ABP selectively (Gunsalus et al., 1987). Similarly testosterone enanthate (TE), MPA and their combined regimen in animals also exerted the same effect (Rao and Roy, 1993; Rao et al., 1996). It has been reported that MPA + DHT injections for 60 days brought about a significant reduction in testicular weight (Rao and Roy, 1992; Rao and Shah, 1998). The testis weight also decreased significantly in rats implanted with capsule of danazol and dihydrotestosterone (Benghuzzi et al., 1994) in light of our present data. However, in this study the other organ weights such as the caput and cauda epididymides and vas deferens did not exhibit marked variations in their weights. MPA injections further resulted in a loss of body and organ weights (Roy and Rao, 1995). However, these organ weights were not affected by hormonal combination of MPA + testosterone enanthate (TE)/DHT in rats and monkeys (Rao and Roy, 1992; 1993; Manjramkar, 1995; Avari and Bhawgade, 1999). Body and organ weights are indicators of androgen sensitivity (Nieschlag and Behre, 1990; Rao et al., 1998). The overall data showed that the gravimetric data were not
A significant depletion in the cauda epididymal sperm count indicated an inhibition of sperm production by this combined (MPA + DHT) hormone treatment. Reduction in sperm number or absence of sperm in semen of human volunteers by TE, MPA combined with TE regimen was noted (WHO, 1993) which support the present data. A combination of DMPA + TE weekly were reported to suppress spermatogenesis to near azoospermia levels (WHO, 1993; 1996). A significant reduction in sperm counts by weekly injections of MPA + DHT for 60 days was also observed (Rao and Roy, 1992; Rao and Shah, 1998) in our laboratory in rats. Thus, these steroidal hormones exert their influence by knocking off pituitary gonadotrophin secretion and maintaining androgen levels at the physiological range thereby affecting testicular spermatogenesis (Waites, 1993; Cummings and Bremner, 1994). Hence, the oligospermia state observed in the present study is justified. WHO (1996) also studied the degree of suppression of spermatogenesis needed to ensure protection against pregnancy with TE in humans. It was chosen that the degree of sperm suppression needed for contraceptive efficacy by hormonal drugs has to be 0 to 3 x 10⁶/ml semen (Waites, 1999). Consequently the encouraging results were also obtained with new long-acting drugs of testosterone esters such as testosterone undecanoate or testosterone buciclaté and non-aromatizable, dihydrotestosterone and its esters combined with progestagens such as DMPA and levonorgestrel or desogestrel or with GnRH analogues (Waites, 1999; Behre et al., 1995). Sperm motility also declined markedly after steroid combined injections to rats for 60 days. Various hormonal treatments alone or in combination have been reported to
suppress sperm motility in rodents, primates and men. DHT administration to monkeys also affected sperm motility by altering their morphology (Ramakrishnan et al., 1989; Rajalakshmi, 1994). MPA and DHT combinations to rats for 60 days also affected sperm motility and morphology followed by their viability as these are essential for sperm-egg union (WHO, 1998). Loss of sperm motility in vas deferens was also reported (Rao et al., 1998) by these treatments to support our findings. The decline in sperm viability has been correlated with alterations in sperm membrane permeability leading to loss of their function. In the MPA treated animals, morphological changes were observed mainly in the acrosome region of the spermatozoa (Paramo, 1993). Silver nitrate (AgNO₃) staining of sperm showed defects like decapacitation, accumulation of cytoplasmic droplets in mid and tail piece regions and acrosomeless defective sperm as a result of this contraceptive drug injections to rats for 3 months. Cytoplasmic droplets have been reported to indicate disturbances of sperm maturation in the epididymis (Menchini Fabris, 1986). These anomalies made these sperm non-motile in combined drug injected rats. Similar results were also reported during MPA, TE, MPA + TE, MPA + DHT treatments in rodents (Rao and Roy, 1992; WHO, 1994; Rao and Roy, 1995; Rao and Shah, 1998). Further MPA + TE treated human spermatozoa were unable to penetrate the hamster oocytes supporting our data (Wu and Aitken, 1989). Rao et al. (1994) and Rajalakshmi et al. (1990) also reported abnormal sperm morphology with diethylstilbestrol and dihydrotestosterone treatments to rats and monkeys respectively. DHT injections alone also induced ultrastructural deformities and morphological alterations in monkey sperm in addition to a loss of sperm motility (Ramakrishnan et al., 1989; Rajalakshmi et al., 1990) leading to
a contraceptive effect.

Consequently the fertility rate of these treated animals was also impaired by MPA + DHT treatments and the litter size was also diminished. The alterations in the spermiogram could be correlated with the loss of fertility in these animals explaining its antifertility action. A gradual recovery was noted with respect to sperm parameters and fertility profiles in the rats after 90 days of withdrawal of this combined drug treatment.

The testicular parameters such as SDH, protein levels, 3β and 17β HSDs activities were reduced by MPA + DHT injections to rats. The decrease in the testicular SDH enzyme levels were also related to an altered testicular oxidative metabolism. The decline in the steroid dehydrogenase levels indicated an affected intratesticular hormone production and hence a decrease in the protein levels of the testis of treated animals was also justified. Studies using cultured rodent Leydig cells and testicular homogenates showed that MPA inhibited three enzymatic activities i.e. 17α hydroxylase, 3β hydroxysteroid dehydrogenase and 17β hydroxysteroid dehydrogenase (Barbieri and Ryan, 1980; Lee et al., 1999). This intratesticular androgen deficit was also contributory to a loss of spermatogenic activity in our study. DHT treatment also revealed that the spermatogenic elements were affected at spermatocyte level (Lotz and Krause, 1981). MPA is also known to affect testicular spermatogenesis at primary spermatocyte/spermatid level (Frick et al., 1982). Further MPA + DHT injections in our study too affected testicular physiology and histomorphology due to modified intragonadal androgen levels to support our earlier findings (Rao and Roy, 1992; Rao and Shah, 1998; Rao et al., 1998). But serum testosterone levels were not significantly varied by these injections to
indicate maintenance of normal libido. Further, the testicular phosphatases did not reveal much variations in this report.

The parameters studied in the epididymis were succinate dehydrogenase (SDH), ALKpase, ACPase, sialic acids and total protein levels. The succinate dehydrogenase enzyme activity in the epididymal tissue was declined as a result of local androgen deprivation caused by this hormone treatment leading to an altered metabolism of these tissue. Exogenous administration of DHT and MPA induced a change in ratio of testosterone : DHT : androstanediol available to testis and epididymis to regulate their functions (Rajalakshmi et al., 1990; Rao and Shah, 1998). Variation in the sialic acid and protein levels too indicated changes in its secretory functions. Decline in the sialic acid levels exhibited its altered microenvironment, which is normally under control of androgen (Orgebin-Crist et al., 1975; Orgebin-Crist and Olson, 1984; Robaire and Hermo, 1988). The sialoproteins are important for sperm maturation process in the epididymis. The microenvironment of epididymis and its relation to sperm maturation are well reviewed (Tuck et al., 1970; Rajalakshmi et al., 1990; Nieschlag and Habenicht, 1992; Kirchhoff et al., 1995; WHO, 1998). Further the interaction between sperm surface changes and epididymal fluid is well demonstrated and is strictly controlled by androgens (Cooper and Yeung, 1999). Fluctuations in the circulating androgen level adversely affects micro environment of the epididymis that influences the sperm maturation (Soufir et al., 1981; Robaire and Hermo, 1988). All these factors thus generated unsuitable environment for sperm to survive and mature in the rats administered with MPA + DHT injections for 90 days. The histological features of both regions of epididymis also exhibited few
changes correlative to biochemical variations, due to local androgen deprived effect in our study. Local androgen deprivation effect was also demonstrated in the epididymis by MPA + TE/DHT administration for 60 days in rats (Rao et al., 1998; Rao and Shah, 1998).

MPA + DHT treatment did not exert significant changes in the protein glycogen and phosphorylase levels of vasal tissue as a result this combined drug treatment. But a reduction in the protein levels of the vas deferens was observed after injections of MPA to rats for 60 days (Rao et al., 1998). Thus, vas deferens metabolic integrity and function were significantly affected only in MPA treated rats, but not by combined treatment. Similarly vas deferens did not manifest any regressive changes histologically. But the lumen of the vas deferens had free or less number of sperm, with a decline in the muscular layer thickness. However, the contractility pattern of the isolated vas deferens from rats was altered by MPA + DHT injections for 90 days as evidenced by its percent response to different adrenalin concentrations. But Rao et al. (1998) have not observed significant variations in contractility pattern of vas deferens by MPA + TE injections. These variations may be due to difference in duration, type of catecholamines used, type of combination and other factors. But withdrawal of MPA + DHT regimen to rats for 90 days, the above changes in the epididymis and vas deferens biochemical data and histological changes alongwith histocytometry showed a recovery. Most of the parameters were also comparable to those of control animals after cessation of the treatment indicating its reversible contraceptive efficacy.

The toxicological studies also revealed that the haemopoietic tissue did not exhibit
significant variations by MPA + DHT treatments to rats for 90 days as blood cell counts (RBC and WBC) and haemoglobin (Hb) levels were not different in all experimental groups supporting our earlier data (Rao and Roy, 1992; Rao and Shah, 1998).

The serum parameters studied were protein, cholesterol, transaminases and testosterone levels in this study. All these parameters were unaffected in all experimental groups indicating normal liver functions. Therefore, this combination did not produce any toxic effect since the peripheral androgen levels appeared to be normal (Rao and Roy, 1992). Anabolic steroids are known to be associated with the consistent increase in various enzymes of liver and some plasma proteins showing liver dysfunction. These are also known to cause alterations in lipid profiles (Haffner et al., 1983; Knuth et al., 1989). The serum cholesterol levels were augmented after 60 days of TE treatment to rats (Rao et al., 1996). However, this increase was not observed in serum cholesterol levels in our present observations. Although TE is envisaged as a good contraceptive drug, its long-term effects with regard to cardiovascular risk factors, the prostate and maintenance of bone mineral density (BMD) are to be kept in mind (Gooren and Nguyen, 1999). Hence TE in combination with progestogen (MPA) is advocated. In this regard, MPA + DHT combination may be helpful as no significant elevation in transaminases was noticed in animals treated with this (MPA + DHT) combination for 90 days in our study. Faundes et al. (1981) observed elevated levels of transaminase in serum of MPA + TE injected human volunteers. Hence, these studies are to be further extended in rats to assess toxicity on long-term basis. Since the serum testosterone levels were not significantly affected, the libido was normal and no significant effects were found in other blood parameters of
treated groups as compared to control in our investigation as cited above.

Thus, bimonthly MPA + DHT combined injections to rats for 90 days induced antifertility effect by affecting probably testicular and epididymal functions, leading to a loss of fertility in these animals. It has been reported that progestogen when combined with testosterone esters can cause azoospermia or severe oligozoospermia state and does so more rapidly and completely than does testosterone alone or progestogen alone (Christensen et al., 1994; Goncharov et al., 1995). However the study could be extended to find out the duration and dose regimen in order to achieve complete consistent azoospermia state with no adverse effects or severe oligospermia state with altered sperm function. As supplementation of testosterone esters lead to aromatize to estrogens exerting adverse symptoms and moreover androgens like testosterone buciclate failed to maintain peripheral levels of testosterone in normal range (Goncharov et al., 1995), it was therefore concluded that MPA + DHT drug combination, might satisfy the criterion for an effective hormonal method of contraception for men. Behre (1995) also suggested DHT esters may be better drugs for combination to male contraception.
PART - II EFFECTS OF PLANT PRODUCTS

Although presently available methods for female contraception have risk and benefits, a method which is of indigenous plant origin may have particular advantages such as cost ineffectiveness, less or non-toxic nature and orally bioactive. The need for a cost ineffective, safe and effective oral contraceptive is an urgently felt need to control over-growth of world population. It should also be easily available and should not have any side effects. Hence the search for such a suitable product from indigenous medicinal plants was undertaken.

This section refers to the reversible antifertility effects of two plant products viz. alcoholic extract (each at dose of 100 mg.kg$^{-1}$.d$^{-1}$) prepared from whole plant, *Phyllanthus amarus* and from the pericarp of *Balanites roxburghii* unripe fruits, in adult, cyclic female mice for 30 days. This dose was selected as it exhibited a better contraceptive effect amongst other doses used. The LD$_{50}$ values were more than 1 gm.kg$^{-1}$ for both these extracts. The parameters studied were gravimetry, biochemical parameters of the hormone target organs such as 3β and 17β hydroxysteroid dehydrogenase (HSDs), ascorbic acid, glutathione, cholesterol and protein levels in the ovary and glycogen, phosphorylase, protein and total lipid levels in the uterus of mice. These changes were compared with histology of these tissues. The toxicological parameters studied were haemoglobin (Hb) and blood cell (RBC and WBC) counts. The serum parameters carried out were protein, cholesterol and transaminase levels in all experimental groups. The changes in the estrous cycle of the mice (cyclicity) as well as the spontaneous contractility pattern of the mouse uterus were also studied after extract feeding. The recovery studies were carried out for
30 and 45 days after withdrawal of feeding.

Oral feeding of unripe fruit pericarp extract of Balanites and whole plant extract of Phyllanthus for 30 days to mice had no significant effects on the whole body and organ weights indicating no effect on general metabolism and growth. Similar effects were observed by Rao et al. (1997a,b) in the male mice after 45 days of these extracts feeding. Some of the other plant products which showed no effect on general body growth and reproductive organ weights in rodent are *Carica papaya* (Lohiya and Goyal, 1992; Lohiya et al., 1994; Chinoy et al., 1994, 1995). *T. bellirica* fruit and *A. precatorius* seed extracts (Rao, 1989) in various other rodents. However, the Phyllanthus and Balanites extract fed mice brought about a significant decrease in the uterine weights. A typical progesterone is also an active anti-estrogen which is reported to decrease the uterine weight (Mitra et al., 1999). This decrease in the uterine absolute weight in this report could be attributed to the distorted uterine growth as a result of hormonal imbalance exerted by these plant products. It is known that body and organ weights are good indices of hormonal activity (Chinoy et al., 1995; Lobo et al., 1997).

The study on the estrous cycle in the Phyllanthus and Balanite extracts fed mice revealed an irregular cyclicity with the predominance of diestrous phase. Rao and Alice (1999) showed irregularity in the cyclicity of Phyllanthus and Balanites extracts fed mice. Similarly irregularity in cyclicity with predominance of diestrous stage was observed in *Carica papaya* seed extract fed mice (Chinoy et al., 1995; 1997). The extract of *Momordia charantia* seeds exerted irregular cyclicity with metaestrous stage continuously (Sharanabasappa and Saraswati, 1999) in rodents in light of our results. This irregular
cyclicity in estrous cycle of rodents was attributed to alterations in the hormones prevailed in the extract fed mice. The interplay between hypothalamus-pituitary-gonadal axis and reproductive organ function is well established and studied in diverse physiological conditions (WHO, 1998) to avert unwanted pregnancies and to achieve contraceptive effects in laboratory animals and human beings (Hafez, 1981; Lobo et al., 1997; Puri and Van-look, 1994). The fertility rate in these extract fed females was found to be zero (100% negative). As a result, the litter size was also nil in this investigation. Rao et al. (1997a,b) observed a significant decline in the fertility rate and litter size of females cohabited with Phyllanthus and Balanites extract fed male mice. In our study also, loss of fertility and irregular cyclicity in extract fed female mice clearly demonstrated the contraceptive effect of these plant products. Chinoy et al. (1995) also demonstrated various extract types of *Carica papaya* seed for induction of contraceptive effects in female mice and rats in light of our data by affecting the ovarian functions. After withdrawal of extract feeding for 30 days the animals showed partial recovery in the fertility and litter size while a significant recovery was noted after 45 days of post-treatment.

The ovarian parameters such as 3β and 17β hydroxysteroid dehydrogenases (HSDs) revealed a significant reduction by Phyllanthus and Balanites extracts feeding to mice for 30 days. 17β-estradiol is the principle estrogen during the second phase of growth of the follicles in mammals (Martin and Barry, 1980). The decrease in 17β HSD may result in a decreased production of estradiol that affects the follicular development (Bard and Thong, 1994). Follicular phase of menstrual cycle in human beings is also
affected due to a reduction in E$_2$ levels. The decrease in 3B and 17B HSDs may further account for the altered ovarian steroidogenesis leading to an altered hormonal balance. This can be correlated with a significant increase in the cholesterol levels of the extracts fed mice ovary as it is a precursor for hormonogenesis in this endocrine gland. Changes in the ovarian hormonal production completely depends on the gonadotrophic hormones viz. FSH and LH secreted by anterior pituitary gland. Both FSH and LH stimulate ovarian target cells by combining with highly specific FSH and LH receptors present in their membranes (Vijaya, 1999). LH alone showed inductional effect on oocyte maturation while FSH showed a marginal effect on oocyte maturation (Moses, 1999). However the glutathione and ascorbic acid levels in the ovary did not exhibit any significant changes and this seems that the effect of these extracts was probably at the target system viz. hypothalamo-pituitary-ovarian axis. However the protein levels in the Balanites extract fed mice was significantly declined indicating that protein synthesis was probably inhibited. Rao (1987) also noticed inhibition of proteins in testis of rat fed with alcoholic extract of Solanum seeds thereby affecting steroidogenic enzymes and subsequent hormone production. These biochemical alterations in ovary were in correlation with histological and histocytometry studies where ovary indicated regressive changes in respect to follicular growth in Phyllanthus fed mice. These histomorphological changes were more evident in the ovary of mice fed with Balanites extract where inhibition of follicular development and necrosis occurred in cortex and medulla regions of it.

In uterine tissue the biochemical parameters studies were glycogen, phosphorylase, total lipids and proteins. The glycogen and phosphorylase levels in the uterus of
Phyllanthus and Balanites extracts fed mice remained unaltered indicating that the carbohydrate metabolism was not affected. The protein levels in the Balanites extract fed mice uterus had a significant reduction. This decrease in the protein levels could be attributed to the decreased levels of hormones action on it. Generally estrogens have an anabolic effect. Estrogens could also cause an increase in the total body proteins (Guyton, 1998). Under the influence of estrogen and progesterone, the uterus secretes a number of regulatory proteins and growth factors (Seppala et al., 1992; Biro and Eneroth, 1990). Probably the alterations in the hormonal production might be a possible factor for reduction of protein synthesis in the uterus. Chinoy et al. (1997) observed a significant decline in the uterine protein levels 30 days after alcoholic papaya seed extract feeding to mice. Moreover, the total lipid levels also rendered a significant decrease in both extracts fed mice. Progesterone has a special effect on the endometrium to convert the endometrial stromal cells into large swollen cells (Decidual Cell Reaction) that contains extra quantities of glycogen, protein, lipids and some necessary minerals (Guyton, 1998). Since hormonal milieu was altered, these alterations in the mouse uterus are justified. Further, fluidity of the lipid bilayer is known to show a significant decrease affecting lipid profiles by neem oil treatment in mice uterus (Monzy, 1999) to support the present data. These effects were further related to histological and histocytometric alterations in the uterus of the extracts fed mice. The regressive changes with respect to histology of uterus are seemed to be more in the Balanites extract fed animals.

All these reproductive effects observed, returned to normal state after 45 days of withdrawal of extract feeding. The fertility rate of the extracts fed animals was also
comparable to control animals revealing normal functioning of the reproductive tissues. Thus these extracts induced transient effects and seem to be reversible leading to a functional sterility in the females.

The spontaneous uterine contractility pattern of the uterus in the estrous stage was compared to that of the extract fed mouse in the same phase. There was a significant increase in the contractility pattern of the uterus in the extracts fed mouse comparatively. This increase may be attributed to the altered hormonal milieu of uterine tissue by the extracts feeding. Uterine contractions are known to be under the control of hormones and their mechanisms (Csapo, 1977; 1981). These mechanisms probably might have been affected by the extracts in myometrium leading to an increase in spontaneous contractions. Estrogens are known to sensitize myometrium to the action of oxytocin which promotes uterine contractility while progesterone decreases the frequency and amplitude of myometrial contractions (Vijaya, 1999). It is known that estradiol under physiological conditions stimulates the synthesis of receptors for both estrogen and progesterone in primate endometrium (Clark and Markaverich, 1988). Their regulatory mechanisms might have been affected by these extracts leading to altered uterine myometrium. Moreover, these induced uterine contractility exhibited a recovery in Phyllanthus extract fed female mice, whereas no significant recovery was found with respect to Balanites extract fed animal uterus explaining differential effects of the extracts on myometrium. This indicates that both these extracts might possess different active principles in them, which need to be explored.

The toxicological study revealed that the haematological parameters such as
haemoglobin, RBC and WBC counts in both extract fed mice were comparable to that of control and withdrawal groups. Similarly serum parameters like SGPT, SGOT, protein and cholesterol levels did not exhibit significant changes as compared to the other groups by these extracts feeding. Moreover liver histology was not affected by these extract feeding separately for 30 days. Thus the above observations demonstrate non-toxicity of the extracts of *P. amarus* and *B. roxburghii*. Similar results of these extracts were also observed by Rao et al. (1997a,b) in male mice by these crude extracts.

In conclusion, alcoholic extract feeding of Phyllanthus and Balanites to adult cyclic mice brought about a contraceptive effect by altering cyclicity and fertility. These changes are attributed to malfunctioning of ovary and uterus via affecting ovary directly or indirectly through hormonal regulatory mechanisms. However, these effects seem to be transient and reversible after withdrawal of feeding. Comparatively, Phyllanthus extract induced effects were fastly reversible. Further studies are underway to isolate and characterize the active principles in them and their mechanisms in rodent model. Such studies have a significant bearing to develop a herbal product for regulation of fertility in the female in developing countries like India.