CHAPTER IV

DISCUSSION
SECTION I

MPA + DHT COMBINATION

The development of an effective contraceptive has given greater importance to the female system than to the male. This imbalanced emphasis has been to a large extent related to the comparative lack of understanding to the male reproductive processes. Various investigators (WHO, 1994, Puri and van Look, 1994) have claimed several effective and promising experimental approaches for male contraception. The relative vulnerability and susceptibility of spermatogenesis in the testis and sperm maturation in the epididymis (Bennet, 1974) provide us with target sites which may be good indicators for screening the efficacy of male contraceptive drugs, such as hormones and their combinations (Nag and Ghosh, 1979).

This section describes the reversible contraceptive effects of a hormonal combination of medroxyprogesterone acetate (MPA) and non-aromatizable androgen, dihydrotestosterone (DHT) in rats. This hormonal regimen consists of weekly intramuscular injections of medroxyprogesterone acetate (MPA) and dihydrotestosterone (DHT) at doses of 2.5 mg and 500 μg per animal respectively. This combination was given to rats for 60 days to study its effects on body and organ weights, spermiogram viz., sperm count, percent sperm motility and sperm viability. Sperm acrosomal enzymes such as hyaluronidase, acrosin forms and superoxide dismutase (SOD) were also done. Its effects on other organs such as the testis, epididymis and liver functions were also assessed. Haematological parameters viz. blood cell counts (RBC and WBC), haemoglobin (Hb) content and serum chemistry parameters like proteins,
transaminases, cholesterol and testosterone levels were estimated in order to investigate the variations induced by this hormonal combination. Histology of above tissue was done. Finally recovery of these effects 60 and 90 days after discontinuation of this treatment has also been studied in these adult rats.

The average increase in body weight did not alter significantly during this treatment period. But, the reduction in the testis was about 40% and this decline is attributed to loss of spermatogenic elements as a result of androgen deprivation and reduced levels of androgen binding protein (ABP) in the testis. This is because, the biosynthesis and the secretion of ABP appear to be regulated by both FSH and androgen (Tindall and Means, 1976). Other organ weights such as epididymis and vas deferens did not exhibit marked variations in their weights in this study. It is also reported that DHT administration to rats brought about a reduction in testis cellularity leading to atrophy of the rat gonad (Lotz and Krause, 1981). Similarly, MPA injections also resulted in a loss of body and organ weights (Roy, 1994). However, organ weights were not affected by the hormonal combination of MPA + testosterone enanthate (TE) in rats and monkeys (Rao and Roy, 1993; Roy, 1994; Rao et al, 1995b) to support our data. Rao and Roy (1992) also demonstrated no effect of DHT combined MPA injections on body and organ weights. The organ and body weights are indicators of androgen sensitivity (Nieschlag and Behre, 1990). Thus, these data overall did not reveal any significant change in body and organ weights by this antifertility drug regimen.

Significant depletion in the cauda epididymal sperm count indicated an inhibition of sperm production by this hormone treatment. Reduction in sperm numbers or absence of sperm in semen of human volunteers by MPA combined
with TE was noted (WHO, 1993) which support the present data. Rao and Roy (1992) found a reduction of sperm counts by MPA + DHT weekly injections. Thus, these steroidal hormones exert their influence by knocking off pituitary gonadotrophin secretion thereby affecting testicular spermatogenesis due to insufficient intratesticular androgen necessary for this process (Lotz and Krause, 1981) in the present study. Hence, the oligospermic state observed in the present study is justified and is one of the criteria for a contraceptive drug.

Sperm motility was also declined markedly after this steroid injections to rats. Numerous hormonal treatments in alone and combinations, suppressed sperm motility in rodents, primates and man (Wu and Aitken, 1989; Puri and Van Look, 1994; Roy, 1994; Rao et al., 1995a,b; WHO, 1994). The decline in sperm viability has been correlated with alterations in sperm membrane permeability leading to loss of their function. Silver nitrate (AgNO₃) staining of sperm showed defects like decapitation, accumulation of cytoplasmic droplets in mid and tail piece regions and acrosomeless/defective acrosome as a result of this contraceptive injections to rats for two months. These anomalies made these sperm non-motile in these injected rats. Same results were also reported during MPA, TE, DHT and MPA + TE, MPA + DHT treatments in rodents (Rao and Roy, 1992; 1993; Rao, 1991; 1992; WHO, 1994). 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 induced ultrastructural deformities and morphological alterations in monkey sperm in addition to a loss of motility (Ramakrishnan et al., 1989; Rajalakshmi et al., 1990).
The MPA + DHT treatment also induced a significant decline in the acrosomal membrane bound enzymes i.e., hyaluronidase and acrosin in the epididymal sperm. Hyaluronidase is important for the dispersion of cumulus oophorus during its penetration. The acrosin is involved in sperm egg fusion especially during the entrance of zona pellucida. Thus, these enzymes are essential for fertilization process in mammals. Since their levels were altered, the fertilizing capacity of sperm was also lost in these injected animals. Rao and Roy (1992) and Roy (1994) have demonstrated a decrease in these enzymes levels of sperm in MPA + androgen (TE/DHT) injected animals and has been related to low sperm count and loss of their fertilizing capacity. It is further confirmed by in vitro studies of Wu and Aitken (1989) using hamster oocyte penetration (HOP) test in sperm of hormonally induced oligospermic males. Thus, low levels of acrosin system is correlated with infertility in human (Koukoulis et al., 1989). These results also support the present investigation.

The fall of SOD activity in sperm explains also the loss of viability of sperm and induction of abnormal sperm in the cauda epididymis of rats administered with MPA + DHT in this study. The sperm SOD is known to catalyse the dismutation of highly reactive superoxide dismutase radical into less toxic H$_2$O$_2$. The protective role of SOD, in preventing oxidative damage by oxygen free radical species is known (Fridovich, 1981). The increased reactive oxygen species in hormonally suppressed sperm function is demonstrated (Wu and Aitken, 1989) and is probably related to fall of SOD levels in corroboration with our data. Similarly mammalian spermatozoa have become more prone to the oxidative action of superoxide anion radicals as the epididymal maturation progresses (Pradeep et al., 1991). One of such changes include the conversion of sulphydryl group to disulfide in sperm (Huang et al., 1987). Hence, the
reduction in this enzyme activity adversely affected the normal sperm maturation process in the epididymis by this hormonal regimen in this study.

Consequently the fertility rate of these treated animals was reduced to 18%. But, mating rate was comparable to control and other groups. In addition the pregnancy rate of mated females and their litter size also declined. Thus, alterations in spermioagram was correlated with reduced fertility in these animals received MPA + DHT describing its antifertility action. Withdrawal of this combination for 60 and 90 days gradual recovery was noted with respect to sperm parameters and fertility profiles in rats comparable to pretreatment levels.

The testicular biochemical parameters such as SDH and ACPase levels which are androgen dependent were reduced by MPA + DHT injections. Hence, the testicular metabolic activity is affected in these animals. These effects were also supported by a decline in testicular steroidogenic enzymes in MPA + DHT treated rats as noted in our study. Testicular spermatogenesis activity also suppressed by this treatment. Avari (1990) also reported altered testicular biochemical profiles and ultrastructure by MPA and MPA + TE administration with suppressed spermatogenic activity in support of our light microscopic study. Non-aromatizable androgens like DHT alone also affected testis histology in monkeys (Ramakrishnan et al., 1989).

Atrophy of Leydig cells also had been demonstrated in rats in addition to testicular spermatogenic activity (Lotz and Krause, 1981). But, protein and cholesterol levels did not reveal appreciable changes in the testis in this study. Androgen levels were also not significantly altered during this treatment. Effect
of MPA and DHT on pituitary gonadal axis is well established (Lotz and Krause, 1981; Kuhn et al., 1983; Lobl et al., 1983).

The parameters of the epididymis studied were succinate dehydrogenase (SDH), adenosine triphosphastase (ATPase), sialic acid and protein. These enzyme activities were declined in the epididymal tissue as result of local androgen deprivation caused by this hormonal treatment. Hence, the metabolic turnover of the epididymis i.e. caput and cauda epididymides was affected. It has also been demonstrated that an optimal ratio of testosterone : DHT : androstanediol are important to regulate epididymal physiology which is affected by this treatment. Moreover, DHT has caused a down regulation of its own receptors in this study (Rajalakshmi et al., 1990). Declined in the sialic acid levels indicated its altered microenvironment, which is normally under control of androgens (Orgebin-Crist et al., 1975; Robaire and Hermo, 1988). These sialoproteins are important for sperm maturation process in the epididymis. The role of the epididymis in sperm maturation has been well reviewed (Mann and Lutwak-Mann, 1981; Rajalakshmi et al., 1990; Nieschlag and Habenchit, 1992). Fluctuations in circulating androgen level adversely affect microenvironment of the epididymis that, influences the sperm maturation (Soufir et al., 1981; Robaire and Hermo, 1988). Thus, this hormonal contraceptive exerted its influence on the epididymis leading to sperm maturational defects to bring about reduced fertility of these animals in the present study. Withdrawal of the treatment, the epididymal functions seemed to be normal in a phased manner.

The haemopoietic tissue did not exhibit significant variations by this MPA + DHT treatment for 60 days, as the blood cell (WBC and RBC) counts.
haemoglobin and SOD levels were not different in all experimental groups, supporting our earlier data (Rao and Roy, 1992; Bhavita, 1993).

The serum parameters studied were protein, cholesterol and transaminases levels. Most of these parameters were unaffected in all experimental groups indicating normal liver function. However, anabolic steroids are known to be associated with the consistent increase in various enzymes of liver and some plasma proteins showing the liver function impairment. Anabolic steroids also cause alterations in lipid profiles (Haffner et al., 1983; Knuth et al., 1989) which was not observed in cholesterol and protein levels in our observations. This condition indicated non toxicity of this hormone combination. An insignificant elevation in transaminase levels was noticed in animals treated with MPA + DHT combination. Faundes et al. (1981) also observed elevated levels of transaminases in serum of MPA + TE in human volunteers in corroboration with our data.

MPA + DHT treatment also generated an elevation in glycogen levels. Contrarily, the liver phosphorylase activity was found to be decreased in treated animals. This condition resulted in an affected glycogen metabolism in the liver. This could be probably due to altered carbohydrate metabolism and arrest of glycogenolysis in the liver. Hyperandrogenism also causes the promotion of pancreatic insulin secretion for raising glycogen levels (Burghen et al., 1980; Knuth et al., 1989) in support of our data. It is further supported by the fact that the steroids influence the carbohydrate metabolism in human using contraceptives (Hafez, 1980) and animals (Bhavita, 1993) to support our study.
The active site for synthesis of ascorbate in the liver in mammals except in man and guinea pig and its role in overcoming the stress condition is well reported (Kutsky, 1973; Lewin, 1976). In our study the synthesis of ascorbate in liver was not significantly altered by the treatment. However, an insignificant decline in total ascorbic acid, reflects on the role of ascorbate in stress condition (Chinoy, 1978).

These observed effects in liver carbohydrate metabolism, serum parameters and blood were transient and were reversible after withdrawal periods of 60 and 90 days. By 90 days of withdrawal treatment all these parameters appeared to be comparable to the pre-treatment levels.

Thus, in sum up it is mentioned that MPA + DHT combination induced antifertility effects by affecting testicular and epididymal functions thereby leading to reduced fertility rate of these animals. The side effects caused were minimum and were reversible after withdrawal of the treatment. However, this study would be extended to find out the exact duration and dose regimen in order to achieve severe oligospermic or azoospermic state with no adverse effects. In addition supplementation of testosterone or its esters leads to aromatize to estrogens exerting adverse symptoms in males. Therefore, MPA + DHT combination is a better candidate applicable for regulation of fertility in the male in developing countries like India in future.
Although presently available methods for male contraception have risks and benefits, a method of which is of indigenous plant origin may have particular advantages such as cost effectiveness, less or non-toxic and orally bioactive. The combined efforts by investigators during last five decades to develop an ideal male contraceptive of plant origin yielded a very limited success (Puri and Van Look, 1994; WHO, 1994). However, search for investigating male contraceptive of plant origin is continuing.

This section reports the antifertility effects of two plant products viz., alcoholic extracts prepared from *Balanites* fruit pericarp and *Phyllanthus* whole plant in adult male mice. The parameters studied were gravimetry, androgen sensitive parameters of target organs such as succinate dehydrogenase, phosphatases, proteins, cholesterol and ATPase in testis and epididymis. The spermiogram consisted of sperm counts, sperm motility, sperm viability and sperm morphology using respective techniques in normal, extract fed and withdrawal groups. The toxicology parameters studied were haemoglobin and blood cell counts, i.e. RBC and WBC. Serum parameters included were protein, cholesterol, transaminases and serum testosterone levels in all animal groups. The treatment period for mice was 45 days which constitutes the entire spermatogenic - epididymal maturation cycle (Sharma et al., 1995).

The alcoholic extracts of these two plants had good spermicidal activity as these products at 2% concentration brought about instantaneous immobilization of sperm from mice and rat cauda epididymidis *in vitro*. This
spot test was used to confirm spermicidal activity of about 1600 Indian medicinal plants by Setty et al., (1977). Rao (1986; 1987; 1988; 1989) also screened the sperm immobilization effects of seed and fruit extracts of *Arbus Precatorius, Solanum xanthocarpum* and *Terminalia bellirica* on rat and human sperm. *Piper betle* leaf extracts also showed antimotility effects in washed human sperm (Ratnasooriya et al., 1990). Later, these extracts were used for in vivo studies using various doses ranging from 100-500 mg/kg in mice for 45 days. Amongst all, 500 mg/kg dose was comparatively effective to bring down the fertility rate. Hence the same was used for further investigation. The recovery periods were 30 and 45 days after cessation of feeding to mice.

Oral feeding of these unripe fruit pericarp extract of *Balanites* and whole plant extract of *Phyllanthus* for 45 days to mice had no significant effects on whole body and organ weights of mice indicating no effect on general metabolism and growth of exposed mice. Similarly Shah et al. (1994, 1995) also noticed no effect on these parameters in these extracts fed mice. Moreover, it was observed that *carcia papaya* seed extracts also had no effect on general body growth and reproductive organs weights of rodents (Lohiya and Goyal, 1992; Lohiya et al., 1994; Chinoy et al., 1994, 1995). Rao also did not find any effect on body and organ weights in *T. bellirica* fruit and *A. precatorius* seed extracts fed rats.

The spermiogram of the extract fed animals had a significant reduction in epididymal sperm counts, sperm motility and sperm morphology. The decline in sperm count indicated antispermatogenic nature of these extracts. Numerous plants and their products have been shown to possess antispermatogenic activities (Rao, 1988; Adhikari, et al., 1992; Kamal et al., 1993; Arjamand et al., 1994) to suppert these data. The above plant extracts
also affected sperm motility and sperm morphology for exhibiting their contraceptive effects, in addition to their spermatogenic activity inhibition. The abnormal sperm morphology included were decapitation, acrosomal defects, agglutination and other anomalies in extract fed mice. These abnormalities were attributable to a loss of sperm motility in these extracts fed animals. Sperm structural variations have also been demonstrated by Weisan et al., (1994) in glycosides of *Tripterygium wilfordii* fed rats. The loss of sperm motility is also related to inhibition of sperm enzymes essential for flagellar movement and their metabolism.

Rao (1989) and Batla et al. (1990) studied the enzyme profiles of rat and human sperm administered with *bellirica* fruit extract and gossypol acetic acid and correlated the loss of sperm motility with inhibition of sperm enzymes involved for energy production. Alterations in viability of sperm reflected on changes in their plasma membrane, as most of sperm retained Trypan blue in extract fed animals in the present investigation. This led to an alterations in sperm movement thereby also leading to loss of their motility patterns in our study. It has been demonstrated the production of O2 generative free radicals in sperm during hormone treated conditions detrimental to plasma membrane configuration (Fridovich, 1981: Roy, 1994). Thus, changes in sperm count, sperm morphology, sperm viability and percent motility appeared to be responsible for reduced fertility of these animals. Thus, the fertility loss in these mice as a result of extract feeding were comparable to those results reported by combined treatment with gossypol and glycosides of *T. wilfordii* in rats (Xu et al., 1990).
Anthelmenthic, antifeedant and larvicidal effects of extracts derived from *Balanites* species have been demonstrated by Jain and Benerjee (1988) and Sullaiman et al. (1988), Zarragh et al. (1990) and Jain and Tripathi (1991). Antiviral activity of *Phyllanthus* whole plant extract has been reported in chronic carriers of hepatitis virus B in rats and human beings (John and Krishnamurthy, 1993; Thyagarajan et al., 1988) to support our data. Previous studies from our laboratory have also shown the effective induction of infertility in rats by 70% alcoholic extract in comparison to 70% methanolic and 1:1 dichloromethane + methanol extracts of *Balanites* (Rao et al., unpublished data).

The androgen sensitive parameters of the testis such as SDH and alkaline phosphatase declined by feeding these extracts to mice. However, no appreciable changes were noted to protein and cholesterol levels. However, slight increase in cholesterol levels in *P. amarus* extract fed mice testis was evident indicating its probable accumulation in it. The reduction in these enzymes were an indicative of depleted androgenic activity of the testis, which is probably related to dysfunction of Leydig cells. However, the serum testosterone levels remained within normal levels in these animals. This gives evidence that these extracts had probably no effect on hypothalmo-hypophysial-gonadal axis. It requires to estimate the circulating gonadotrophin levels in these mice. Histology of the testis of extracts fed groups exhibited partial depletion of spermatogenic process. Few seminiferous tubules showed absence of sperm in their lumen. In testis of *Phyllanthus* extract fed mice, no active proliferation of germ cells was noticed. Chronic administration of *B. roxburghii* fruit pulp extract to dogs, induced testicular
dysfunction (Dixit et al., 1981) with a significant fall in protein and sialic acids contents in agreement with our data.

The epididymal parameters such as SDH and ATPase levels also declined by extracts feeding to mice. However, the protein levels did not exhibit significant variations in both epididymal regions of mice feeding with these alcoholic extracts. The depletion of these enzymes viz., SDH and ATPase in the epididymis probably affected its oxidative and energy metabolism and became hostile for sperm maturation. The epididymal maturation process is very much essential for sperm function (Knobil et al., 1988; Nieschlag and Habenicht, 1992). The histological features of the caput and cauda epididymi did also not reveal much variation in comparison to that of control groups. Tubular lumen had free or less number of sperm. Epithelium of some tubules had a sign of regressive changes in *P. amarus* extract fed animals.

Since the serum testosterone levels were not altered as mentioned above, it seems that these extracts seem to have their actions at target tissue level, without affecting pituitary gonadal system. Hence, it could be possible that the androgen after reaching to target site does not convert to its active metabolite, dihydrotestosterone (DHT) for its action or there would be likely a configurational changes at receptor level of the target cell. These aspects need to be further evaluated. Thus, the mechanism of action of these crude extracts seem to be similar to those of *Carica papaya* seed extracts in males as reported by others (Lohiya et al., 1994, Chinoy et al., 1994, 1995). But, the observed effects were found to be reversible after 30 and 45 days of withdrawal of feeding.
The haematological parameters such as haemoglobin, RBC and WBC counts were comparable to control and withdrawal groups. Similarly clinical chemistry parameters also did not exhibit appreciable variations comparatively by these alcoholic extracts oral feeding. However, a slight increase in serum SGPT and SGOT levels were observed which might reflect a probable effect on liver function. This need to be further explored.

Recovery studies for 30 and 45 days revealed that the induced contraceptive effects and altered gonadal and vital organ metabolic integrity and functions resumed to normalcy gradually after 30 and 45 days of withdrawal of extract feeding to mice.

Thus, it is concluded that alcoholic extracts of pericarp of *Balanites roxburghii* unripe fruit and *Phyllanthus amarus* whole plant have a definite antifertility effects in mice with no side effects. Moreover, the induced effects seemed to be reversible after cessation of the feeding to animals. Further studies are under way to analyze the active principle(s) involved in these extracts and other crude extracts of these plants, as this is a preliminary report.