CHAPTER IV

DISCUSSION

PART A: (MPA TREATMENT AND ITS RECOVERY).

This part of the chapter describes the reversible effects of medroxyprogesterone acetate (MPA) on sperm function in adult male rats. This contraceptive drug was administered weekly at a dosage of 5 mg per rat intramuscularly for 60 days. The parameters studied were spermiogram consisting of sperm counts, sperm motility, sperm viability, sperm morphology, sperm acrosome integrity and their nuclear integrity. The viability, nuclear and acrosome integrity were done using trypan blue, acridine orange (AO) and silver nitrate (AgNO₃) staining methods respectively. Sperm morphology was studied on scanning electron microscope. Sperm biochemical indices such as SDH, ATPase, sialic acid and total proteins were estimated along with their acrosomal enzymes viz., acrosin, hyaluronidase and superoxide dismutase (SOD), to assess their function, using standard techniques. Fertility rate of these animals was also evaluated using WHO protocols to assess its contraceptive nature. In order to find out its adverse effects, haematological parameters such as blood cell counts (RBC and WBC) and haemoglobin (Hb) levels and clinical parameters viz., serum proteins, serum cholesterol, serum transaminases and SOD from blood cell were estimated in all experimental groups. The metabolic function and histology of the vas deferens, liver and adrenal were also evaluated in addition to the adrenal
catecholamine concentrations. Contractility pattern of the vas deferens with graded doses of nor-adrenalin was also studied. In order to study the reversibility of the effects of this antifertility agent, 90 and 120 days after cessation of the treatment were also chosen.

The treatment brought about a significant decline in body growth of these animals. The weights of the testes, epididymis, and vas deferens also showed the same trend. This decrease in these organ weights is attributed to androgen deficit condition in this study. These organ and body weights are androgen sensitive parameters which are used to identify androgen antagonistic effect of a compound (Nieschlag and Behre, 1990). The loss of body and organ weights in estrogenized animals has also been demonstrated in animals due to androgen deprivation (Chinoy and Rao, 1982; Rao and Chinoy, 1983; Rao and Mathur, 1987). Previous reports with MPA also described reduction in the testis and epididymal weights in rats and other animals (Lobl et al., 1983; Frick et al., 1982; Brenner et al., 1977). This loss of weights in these organs reflects on the reduced cellularity of the seminiferous tubules and spermatozoa in the epididymis and vas deferens, as these organs are androgen dependent (Frick et al., 1982; Lobl et al., 1983; Rao and Roy, 1993).

The study also revealed a decline in the epididymal sperm counts to indicate suppression of spermatogenic activity in the testes. Hence, the MPA treated animals became oligospermic. The antispermatogenic activity of MPA is well documented in rats earlier, to support our data (Satyaswaroop and Gurpide, 1978; Flickinger, 1977; Worgul et al., 1979; Barbieri and Ryan, 1980; Lobl et al., 1983; Albin et al., 1973; Brenner et al., 1977; Rao et al., 1994b), acting both indirectly
by inhibiting gonadotropin secretion and directly by blocking Leydig cell function. Similar effects were also reported with other antiandrogens in rats, primates and men (Kaur et al., 1992; Rao et al., 1993a; Neumann et al., 1978).

Sperm motility was also suppressed by MPA injections in the present study. Loss of sperm motility by MPA treatment has also been demonstrated like those of other progestins, both in vivo and in vitro conditions (Cheng and Boettcher, 1979; Knuth et al., 1989b). MPA with androgen co-treatment also affects sperm motility characteristics in men (Wu and Aitken, 1989). Along with, sperm viability also affected in MPA injected rats indicating loss of their membrane permeability. The changes in the sperm viability was correlated with alterations in their morphology.

The sperm anomalies like decapitation, accumulation of cytoplasmic droplets on mid and tail piece regions, acrosomal loss and other head defects were also noted in MPA treated rat cauda epididymal sperm using the SEM. These defects probably suggest that the sperm maturation and fertilizability are affected in the epididymis as a consequences of androgen deprivation. Antiandrogens like cyproterone acetate, also induced abnormal sperm morphology in a variety of animals including man (Kaur et al., 1992). Rao et al. (1993a, 1994a) and Rajalakshmi et al. (1990) also reported abnormal sperm morphology with diethylstilbestrol and dihydrotestosterone treatments in rats and monkeys respectively. Combination of MPA and TE also affected sperm morphology in rats and human beings, in addition to its effects on sperm motility, viability and spermatogenesis, corroborating with the present data (Wu and Aitken, 1989; Rao and Roy, 1992; 1993; Rao et al 1994b). But the nuclear integrity was not affected
in this study as all sperm in various experimental groups showed green fluorescence with acridine orange stain. However, Huang and Nieschlag (1984) reported an impaired chromatin condensation in androgen deprived state induced by testosterone propionate injections in rats.

The caput epididymal sperm parameters, viz., ATPase, sialic acid and protein reduced in rats, as their epididymal function is affected due to androgen deficiency, generated by MPA injections. However, in the cauda epididymis, slight increments with respect to these parameters were observed due to their improper maturation in epididymis. It is likely, that abnormal sperm had altered metabolic activity leading to accumulation of metabolic enzymes due to MPA treatment. The augmented proteins in sperm explains the increased lysosomal secretions during their degeneration by this treatment (Rajalakshmi and Prasad, 1975; Kaur et al., 1992). Sialic acids are reduced during sperm maturation in normal animals. The slight increment in its level in our study indicated accumulation of sialoprotein due to abnormal maturation process in the epididymis, affected by antiandrogenic effect. Moreover, high levels of sialic acid was correlated with increased frequency of decapitated sperm in the present study and those of others in human beings (Toit et al., 1992; Toowicharanol et al., 1979). These effects reflects on the direct effect of MPA on the residual sperm. The direct effect of MPA on human sperm and/or through the epididymis has been suggested in support of these results (Knuth et al; 1985; Wu and Aitken, 1989; Cheng et al., 1981).

The MPA regimen to rats for 60 days brought about a significant decline in the acrosomal membrane bound enzymes i.e., hyaluronidase and acrosin activities.
in the epididymal sperm. These enzymes are essential for fertilization process in mammals. Hyaluronidase is important for the dispersion of cumulus oophorus and its penetration by the sperm. The acrosin system is involved in sperm egg fusion especially during the penetration of zona pellucida. Since, their levels are altered, the fertilizing capacity of sperm was also lost in these injected animals. Hence, the fertility rate of these animals severely affected. It is further confirmed by in vitro studies of Wu and Aitken (1989) using hamster oocyte penetration (HOP) assay in sperm of hormonally induced oligozoospermic human males.

The sperm SOD is known to catalyse the dismutation of highly reactive superoxide radical into less toxic \( \text{H}_2\text{O}_2 \). The deleterious effects of superoxide anion radicals on cellular system are well known (Wu and Aitken, 1989). This enzyme, together with other catalases and peroxidases is believed to protect from oxidative damages that could be caused by this oxygen free radical species (Fridovich, 1981). In the present study, the observed reduction in the SOD enzyme probably, could lead to an altered membrane permeability mediated via peroxidation of phospholipid due to an increased production of superoxide anion. This is further confirmed by the trypan blue staining, in which an increased percentage of non viable sperm which took up the stain due to its altered membrane permeability, as mentioned earlier. Similar findings were also reported by Wu and Aitken (1989) who, found an enhanced production of reactive oxygen species (superoxide anions) by the sperm in the semen of MPA + TE treated individuals, which could probably due to a reduced activity of superoxide dismutase. Similarly, mammalian spermatozoa become more prone to the oxidative action of superoxide anion radicals as the epididymal maturation progresses.
One of such changes includes the conversion of sulphydryl group to disulfide in sperm (Calvin et al., 1975; Huang et al., 1984). Moreover, the increase in the level of unsaturation of the phospholipid of the sperm membrane (Scott et al., 1967) and the decline in phospholipid content (Dacheux, 1977; Evans and Setchell, 1979a,b) are associated with epididymal maturation, mediated by superoxides and regulated by the enzyme superoxide dismutase (Pradeep et al., 1991). Hence the reduction in this enzyme activity adversely affected the normal sperm maturation process in the epididymis by this hormonal regimen.

The mating rate was also reduced markedly along with the fertility rate of these animals received the MPA treatment. This can be explained by diminution of circulating levels of testosterone as observed by our study in MPA injected rats. The direct and indirect effects of MPA on the testis and pituitary gonadal axis have been well documented in rat and other animals with in vivo and in vitro conditions (Rao et al., 1994b; Lobl et al., 1983; Satyaswaroop and Gurpide, 1978; Worgul et al., 1979) as cited previously.

The degenerative and involutory changes observed in the testes, vas deferens, caput and cauda epididymides were indicative of antiandrogenic effect of MPA in our study, and in the observations of other investigators (Lobl et al., 1983) in MPA treated rats, and are comparable with the androgen antagonistic effects induced by other antifertility agents (Rao et al., 1994a; Rao et al., 1993; Kaur et al., 1992). These effects induced by MPA seem to be recovered gradually, 90 and 120 days after withdrawing the treatment. The animals regained fertility with sperm function comparable to control animals. Testosterone level in blood...
was also in normal range.

The metabolic function of the vas deferens was affected since, its androgen sensitive parameters viz., SDH, ATPase and sialic acid contents reduced by these MPA injections to rats. The androgenic control of vas deferens in rat is also well reviewed (Chinoy, 1985). As its physiology is affected, its contractility pattern to various doses of nor-adrenalin was also altered. This probably explains the physiological and pharmacological alterations of the vas deferens during this treatment period. These changes were not prominent in the vas deferens of rats treated with MPA and TE combination (Chapter IV, Part B).

The histology of adrenal medulla revealed hypertrophy of chromaffin cells. The spaces were filled with secretions in rats treated for 60 days. This is supported by alterations in catecholamine levels in our study. Moreover, it appears to be an increased conversion of epinephrine to nor-epinephrine as former exhibited a decrease by MPA injections. Same condition exists in stress condition as reported by Selye (1971). Moreover, progestagens could exert an estrogenic effect on the rat adrenal gland (Baker et al., 1973).

Other side effects on the liver are its morphological changes and increased levels of glycogen in this observation. Contrarily, its phosphorylase activity lowered. These conditions imply the effects of MPA in glycogen metabolism of the liver. It is well known that the steroids influence carbohydrate metabolism of the contraceptive users in human beings (Kalkhoff, 1975; Briggs, 1976; Hafez, 1980). Estrogens are also able to upset glucose metabolism in rats (Rao et al., 1993b).

The data on clinical chemistry reflects on rising levels of transaminases by
MPA treatment which further reveals abnormal function of the liver in these treated animals. Accumulation of cholesterol in serum indicated alteration in lipid metabolism. Since, MPA induces androgen deprivation effect, it is likely that its transport could have been affected in our study. Contrarily, a reduction in plasma HDL cholesterol was observed by Hedman et al. (1988) in human beings with MPA injections. This variance might be related to dose, duration and other experimental conditions. No changes were observed in SOD levels of blood. Haematological data showed no significant changes in their levels. However, an increase in WBC counts indicated probably alterations in immune function of the body of these MPA treated animals, as steroids are known to affect normal immune function (Luster et al., 1985).

Recovery data on these induced effects indicated the return of vas deferens and liver functions to normal. Haematology and serum parameters also seem to be comparable to those of normal levels. However, the recovery of these parameters in these animals is slow as compared to those of MPA + TE treated animals. This implies that higher doses of MPA takes more time to be eliminated and hence the recovery period was extended to 120 days in this study. The slow elimination rate of MPA was also reported in human beings of both sexes (Hedman et al., 1988; Brenner et al., 1977; Lan et al., 1984) in agreement with the present observations.

In conclusion, it may be mentioned that MPA induced contraceptive effect by generating oligospermic state with altered sperm function in rats. The side effects of this antifertility drug are related to androgen deprivation leading to altered physio-metabolic state and regressive changes in target organs. The side...
effects induced in other vital organs appear to be more or less similar as reported in combined MPA and androgen treatment in animals. The recovery of these effects induced by MPA injections seem to be slow in comparison to a combination of MPA + TE treatment. Hence, the present study suggests that MPA with androgen supplementation is better for induction of oligospermic state with suppressed sperm function in steroid hormone contraception.
PART B: MPA + TE COMBINATION

This part of the chapter describes the reversible effects of a hormonal combination of medroxyprogesterone acetate (MPA) and testosterone enanthate (TE) on residual sperm function in adult male rats.

Intramuscular injections of MPA (3 mg/rat) and TE (2 mg/rat) combination were given to rats weekly for two months to study spermiogram viz., sperm counts, percent motility, viability, sperm acrosomal integrity and sperm morphology using standard techniques. In addition, sperm acrosomal enzymes, such as hyaluronidase, acrosin and other biochemical parameters like superoxide dismutase (SOD), succinate dehydrogenase (SDH), ATPase, proteins and sialic acids were also estimated. The contraceptive effect of this antifertility agent (combination) and its side effects on other organs, such as the liver, adrenal and vas deferens morphology and their metabolic functions were also assessed. Haematological parameters viz., blood cell counts (RBC and WBC), haemoglobin levels (Hb), and clinical chemistry parameters like proteins, transaminases, cholesterol and testosterone levels were estimated in serum, in order to investigate the adverse effects of this steroid hormonal combination. Recovery of these effects, 60 and 90 days after discontinuation of hormonal treatment has also been studied.

The data on sperm counts in the cauda epididymis revealed a significant reduction indicating inhibition of sperm production by MPA combined with TE treatment to rats for 60 days. Similarly, this combination at varied dose levels also brought about a reduction in sperm number or absence of sperm in semen of human volunteers (WHO report, 1991, 1992, 1993) to support the present data. Thus,
these hormones exert their influence by knocking off pituitary gonadotropin secretion thereby blocking Leydig cell function to suppress spermatogenic activity in the testis. Androgenic control of spermatogenesis is an established fact and is well known (Mann and Lutwak-Mann, 1981; Nieschlag and Behre, 1990; Nieschlag and Habenicht, 1992). Moreover, the reduction in sperm number in the present study fell to oligospermic state, one of the necessities for a contraceptive drug.

In addition, sperm motility also markedly declined in this investigation. Studies have been published to support suppression of sperm motility by numerous hormonal treatments in animals, primates and man (Knuth et al., 1985; 1989; Wu and Aitken, 1989; Rao, 1991; 1992). A direct effect of MPA on human sperm motility in vitro has been demonstrated by binding of gestagens to sperm (Cheng et al., 1981; Kessrü et al., 1975; Cheng and Boettcher, 1979). Computerized sperm motion analysis also revealed that sperm motility characteristics in the residual sperm were reduced in combined androgen - progestin injected human beings (Knuth et al., 1989; Wu and Aitken, 1989). The viability of sperm in our study also declined. This has been correlated with alterations in sperm membrane permeability leading to loss of their function, by these contraceptive injections to rats. The sperm morphology studied by scanning electron microscope (SEM) and silver nitrate (AgNO₃) staining technique showed defects like decapitation, accumulation of cytoplasmic droplets in mid and tail piece regions and acrosome less/defective acrosome, as a result of this hormonal regimen in rats. These anomalies made these sperm non-motile in these injected rats. Same results have been reported by others during MPA, TE, dihydrotestosterone (DHT) and MPA+TE contraceptive treatments in animals (Rao and Roy, 1992, 1993; Rao, 1991, 1992;
WHO 1993). Rajalakshmi et al. (1990) also reported DHT induced abnormal sperm morphology in monkeys. The increase in morphologically abnormal forms was positively correlated with loss of their function (Hafez, 1980). The sperm nuclear integrity was unchanged, as all the sperm of treated rats emitted green fluorescence as those of other groups. This condition indicated that the double stranded DNA is intact and no denaturing effects are presumed in this study.

Sperm acrosomal enzymes viz., acrosin and hyaluronidase are involved in fertilization process. These enzyme levels in residual sperm also exhibited alterations during this hormonal injections to animals for 60 days. Rao et al., (1994b) have demonstrated a decrease in these enzyme levels of sperm in MPA and TE injected animals and has been related to loss of their fertilizing capacity. It is also likely that the sperm in our study groups were also incapable of fertilizing the ovum. The fall of SOD activity in sperm explains also the loss of viability of sperm and induction of abnormal sperm in the cauda epididymidis of rat administrated with MPA + TE in this study. 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. These effects of sperm were also correlative of their metabolic changes in these animals received hormonal combination. Hence, the alterations in metabolic enzymes such as SDH and ATPase were explained in the epididymal sperm. The slight changes in protein and sialic acid in sperm indicated probably the effect of MPA and TE combination on the micro-environment of the epididymis (Rao and Roy, 1993; Rajalakshmi et
Soufir et al., (1983) found a reduction in seminal carnitine levels reflecting on the effect of MPA in the epididymal function, in human to support the present observation. The role of the epididymis in sperm maturation has been well reviewed (Mann and Lutwak-Mann, 1981; Lamming, 1990; Rajalakshmi et al., 1990; Nieschlag and Habenicht, 1992). Fluctuations in circulating androgen level adversely affect micro-environment of the epididymis that influences the sperm maturation in it (Soufir et al., 1981b; Robaire and Hermo, 1988). In the present study, also it is suggestive that this hormonal contraceptive exerted its influence on the epididymis leading to sperm maturational defects (Rao, 1991; 1992).

The fertility of these animals also severely affected, with normal mating rate in our study. But the pregnancy rate of mated females and their litter size were very much diminished in the present study. The suppression of fertility rate in these animals was directly proportional to loss of sperm function by this hormonal contraceptive administration. These results are well matched with the suppressed sperm fertility potential subjected to hamster oocyte penetration (HOP) assay in human administered with MPA combined with TE (WHO, 1993). Wu and Aitken (1989) and Knuth et al., (1989b) also suggested the impaired sperm function assessment by zona free hamster ovum penetration test in steroid contraception. The WHO is also currently testing this hypothesis in a multi-center fertility trial (Waites, 1993; WHO, 1993). Thus our study emphasize that the generation of antifertility action is not only due to inhibition of sperm production (spermatogenesis) but also due to suppression of sperm function during oligospermic state in steroidal contraception.
The libido of the treated animals was unaltered as the mating rate was not affected in our study and the testosterone level did not alter appreciably during treatment. An insignificant increment in its level may be supra-physiological event which also found in human males injected with MPA+TE (WHO 1990). However, we were unable to measure MPA, estradiol and other gonadotropin profiles during treatment. The slight increment in testosterone levels in this report, might probably have a protective role on MPA induced effects during treatment as suggested by Wu and Aitken (1989). Recently, a new testosterone ester (20 Aet -1) has been identified which releases constant levels of serum testosterone and proves to be a good choice over TE for supplementation with progestin (Rajalakshmi and Ramakrishnan, 1989).

The testicular histology indicated spermatogenic arrest in MPA + TE treated groups for two months. Seminiferous tubular size also partially regressed with altered Leydig cell morphology. Bhiwgade et al. (1991) also reported changes in the ultrastructure of testis and other organs in MPA + TE treated rats in accordance with our data. However, the vas deferens during treatment period did not reveal any significant changes. Similarly the contractility pattern of the vas deferens was found to be unaffected with different doses of nor-adrenalin as compared to other groups. The histology of the caput and cauda epididymis also did not seem to be affected adversely. But the lumen of each tubule had less number of sperm or free of sperm due to this hormonal regimen.

Recovery studies were extended for 60 and 90 days after discontinuation of hormonal combination. The results revealed a gradual restoration of sperm parameters with respect to the concentration, motility, viability and morphology.
in the cauda and caput epididymides. The acrosomal enzymes and metabolic parameters also exhibited a recovery, 90 days after cessation of the injections. The testicular, vas deferens, epididymal morphology and the physio-metabolic functions also turned back to those of normal rats. As a result, the fertility of these animals also regained and was comparable to control animals.

In the present study, the observed side effects are minimum. No significant changes were detectable in haemoglobin levels and blood cell counts. Clinical parameters, viz., serum proteins were within the normal range. An insignificant elevation in transaminase levels were evident. This could be probably due to a slight increase in the serum androgen level in animals treated with MPA + TE combination. Faundes et al. (1981) also observed elevated levels of transaminase in serum of MPA + TE in human volunteers as in this study. Serum cholesterol levels was also significantly increased in this study revealing the effect of progestagens. Progestagens have been implicated in changing serum lipids in woman using oral contraceptives and have cardio-vascular risk over a long period (Friedl et al., 1985). Moreover, positive association between androgen and lipid profile has been suggested in azoospermic and oligospermic men with hyper-triglyceremia in support of our data (Mendozo et al., 1981).

The liver glycogen content elevated in MPA and TE combined treatment followed by a decline in phosphorylase activity. It means that the carbohydrate metabolic pathway is affected. Similar reports have also been reported in respect to carbohydrate metabolism in steroid contraceptive users (Hafez, 1980, Soufir et al., 1983). However, much changes were not observed in histoarchitecture of liver during this treatment. Similarly, adrenal morphology with respect to cortex was
unaffected. But medullary region had increased secretions in the spaces present between chromaffin cells during treatment. The levels of epinephrine and nor-epinephrine in this tissue (treated animals) were also at higher levels. This would imply that the adrenal medulla might synthesize and accumulate more of these hormones as a result of hormonal injections. MPA alone also affect the adrenal medullary hormones as mentioned earlier. The reason for these biogenic amines accumulation is difficult to explain at present, and probably might be related to the stress condition (Selye, 1971).

The body weight did not alter significantly during this treatment. But the reduction in the testis, cauda and caput epididymis was 46% and 25% respectively and was explained due to loss of spermatogenic elements in the testis and subsequent loss of sperm density in the epididymis as a result of this MPA + TE injection. However, the vas deferens weight was unchanged. The organ and the body weights are the indicators for androgen sensitivity (Neischlag and Behre, 1990). Thus, this data overall did not exhibit any significant change both in organ and body weights, by this antifertility agent.

Recovery data after discontinuation of the hormonal regimen, showed normal function of vital and reproductive organs. Serum chemistry and haematological parameters were also within the normal levels in the these animals. The serum testosterone level were also comparable to normal levels. These results suggest that the observed side effects seem to be transient and are completely reversible upon the withdrawal of this hormonal contraceptive.

In summary, it is evident that this hormonal combination induces oligospermic state with suppressed sperm function in rats. This suppressed sperm function is
another action of this contraceptive regimen in addition to inhibition of sperm production (spermatogenesis). Moreover, the required hormonal doses are minimum and side effects are also limited, where the complete azoospermic state is not achievable (WHO, 1993).