CHAPTER IV

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PART – I : EFFECTS OF HORMONAL COMBINATION

This part describes the long-term reversible contraceptive effects of a hormonal combination of depot medroxyprogesterone acetate (DMPA) and testosterone enanthate (TE) in adult male rats. The hormonal regimen consisted of monthly intramuscular injections (im) of depot medroxyprogesterone acetate (DMPA) and testosterone enanthate (TE) at doses of 20 and 40 mg/kg body weight respectively for four months. The combination was used to study its effect on body and organ weights, spermiogram viz. sperm motility, sperm count and sperm viability. The fertility rate was studied. Biochemical parameters like 3β and 17β hydroxysteroid dehydrogenases (HSDs), succinate dehydrogenase (SDH), acid and alkaline phosphatases (ACPase and ALKPase), total ascorbic acid (TAA), cholesterol and proteins were estimated in the testis while adenosine triphosphatase (ATPase), proteins and sialic acids were evaluated in cauda epididymis. In the vas deference SDH, ATPase and proteins while ACPase and proteins in prostate and fructose in seminal vesicles were estimated in all groups of rats. Haematological parameters viz. blood cell counts (RBC and WBC), haemoglobin (Hb) content and serum parameters like protein, cholesterol, transaminases (GPT and GOT), total lipids and testosterone levels were estimated to evaluate toxicity. Histological studies and histocytometry of testis, cauda epididymis and vas deferens too were done in all experimental rats. Recovery studies after 120 days of post-treatment was also carried out.
The average body weights did not alter significantly during the treatment period. No significant changes in body weight were detected in any subjects during testosterone undecanoate (TU) (1000 mg/8 wk) plus 20 mg/d cyproterone acetate (CPA) treatment (Meriggiola et al., 2003). Similarly, there was no significant change in body weight on treatment of Norplant II implants plus TE 100 mg/wk or after treatment (GawGonzalo et al., 2002). A reduction in organ weights viz. testis, cauda epididymis, vas deferens, prostate and seminal vesicles was noted only from 3rd month of hormone injection as a result of fluctuation in circulating androgen level. Different threshold androgen requirement of androgen target tissue in the male was well known and this phenomenon was affected by the hormone injection (Knobil and Neill, 1988). The reduction in testicular weight was further attributed to the loss of spermatogenic elements as a result of local androgen deprivation and reduced level of androgen binding proteins (ABP) in the testis. This is because the biosynthesis and secretion of androgen binding proteins (ABP) appears to be regulated by both FSH and androgen (Tindall and Means, 1976). A decrease in testicular size occurred during experimental period in all men on treatment of CPA (50 mg twice a day, orally) plus intramuscular testosterone enanthate (TE; 100 mg/week) and other combinations (Meriggiola et al., 1996).

Prostate volume decreased by approximately 50% within 8 weeks after castration. Both androgens [Testosterone (T) and 7α-methyl-19-nortestosterone (MENT)] supported prostate growth in a dose-dependent manner, each stimulating the gland to supranormal size at higher doses. Seminal vesicle volumes were affected by both androgens in a manner parallel to that of prostate volumes (Cummings et al., 1998). The growth of seminal vesicles is highly dependent on androgen (Lieber et al., 1980). In rats any
increase in serum testosterone or treatment with androgens was associated with increased seminal weight (Almenara et al., 2000).

A significant depletion in the cauda epididymal sperm count indicated an inhibition of sperm production by this combined (MPA + TE) hormone treatment in the testis. Administration of 200 mg TE, im, weekly induced profound inhibition of spermatogenesis in all men (Anderson et al., 1996). Two major multicentre WHO studies that used as the prototype testosterone regimen, weekly im injections of 200 mg TE, have established that hormonally induced azoospermia or severe oligozoospermia (<3 mol/L/mL) provides highly effective, sustained, and reversible contraception with minimal side effects for 12 months (WHO, 1990; 1996). The combination of 800 mg testosterone with 300 mg DMPA caused a striking fall in sperm output, with 9 of 10 reaching azoospermia and all reaching severe oligozoospermia (<3 mol/L/mL). Thus demonstrated synergism between a depot progestin and a depot androgen in suppressing human spermatogenesis. A greater suppression of sperm output was observed on addition of a depot progestin to a depot androgen (Handelsman et al., 1996). In a study reported, combination of 20 mg CPA and injections of 1000 mg TU every 6 wk induced rapid and profound gonadotropin and sperm suppression in all subjects. At the end of 48 wk of administration, 82% of the subjects were azoospermic and all of the other subjects had achieved severe oligozoospermia (<1 million/ml) (Meriggiola et al., 2003).

McLachlan et al. (2002) reported a study of effect of TE (200 mg im weekly) plus DMPA (300 mg im once) for 2, 6, 12 weeks in human subjects. According to them, failure of spermiation was a feature of both acute and chronic spermatogenic suppression and was characterized by the presence of large numbers of elongated spermatids before
spermiation and of retained spermatids after spermiation in the seminiferous epithelium in the presence of severe oligospermia. The maintenance of normal number of mature elongated spermatids in the testis at 6 wk, marked suppression of sperm counts to less than 1 million per milliliter in the majority of cases in preceding week suggests near-complete spermiation failure within 5 wk of contraceptive treatment. In rodents, spermatids that fail to be released from the seminiferous epithelium during spermiation failure are retained by Sertoli cells and subsequently phagocytosed (Russell, 1991). Sperm motility also declined markedly after steroid combined injections to rats for 120 days. Various hormonal treatments alone or in combination have been reported to suppress sperm motility in rodents, primates and men. Dihydro testosterone (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 by these treatments (Rao et al., 1998). The decline in sperm viability in our study has been correlated with alterations in sperm membrane permeability leading to loss of their morphology and function. In the MPA treated animals, morphological changes were observed mainly in acrosome region of the spermatozoa (Paramo et al., 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 contraceptive hormone injections to rats for 3 months (Kurian, 2000). Accumulation of cytoplasmic droplets on sperm have been reported to indicate disturbances of sperm maturation in the epididymis (Menchini-Fabris, 1986).
These anomalies made these sperm non-mobile 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, 1993; Rao and Shah, 1998). Further MPA + TE treated human spermatozoa were unable to penetrate the hamster-oocyte supporting our data (Wu and Aitken, 1989). Rajalakshmi et al. (1990) and Rao et al. (1994) also reported abnormal sperm morphology with diethylstilbestrol and dihydrotestosterone treatments to monkeys and rats respectively. DHT injections alone also induced ultrastructural deformities and morphological alterations in monkey sperm in addition to a loss of sperm mobility (Ramakrishnan et al., 1989; Rajalakshmi et al., 1990) leading to a contraceptive effect.

Consequently the fertility rate of MPA + TE treated animals was also impaired. The alterations in the spermiogram thus 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 rate in the rats after 120 days of withdrawal of this combined drug treatment in the present study.

The testicular parameters such as SDH, proteins, 3β and 17β HSDs were reduced by MPA + TE injections to rats. Lohiya and Sharma (1984) reported a significant elevation in testicular cholesterol and depleted total proteins on treatment of danazol and testosterone enanthate to rabbits, similar to our study. MPA can suppress hypothalamic-pituitary-gonadal axis and can also directly inhibit gonadal steroidogenesis (Lee et al., 1999). Studies using cultured rodent Leydig cells and testicular homogenates showed that MPA inhibited three enzymatic activities: 17α-hydroxylase, 3β and 17β hydroxysteroid dehydrogenases (Barbieri and Ryan, 1980). MPA is a Δ4-steroid that is structurally...
similar to 17 hydroxyprogesterone, thus the action of MPA to inhibit 3β HSD resembles product inhibition and 3β HSD product inhibition with Δ^4-steroid products has been observed (Lee et al., 1994). The decline in the steroid dehydrogenase levels indicated an affected intatesticular hormone production and hence a decrease in protein levels of the testis of treated animals was also justified. Hypercholesterolaemia has a detrimental effect on Leydig, Sertoli cell secretory function, spermatogenesis, epididymal, sperm maturation process and the overall sperm fertilizing capacity. Alterations in sperm membrane lipid domains induced by hypercholesterolaemia lead to modifications in sperm capacitation and acrosome reaction kinetics (Yamamoto et al., 1999). Testicular total ascorbic acid (TAA) showed an increase in MPA + TE treated animals due to its non-utilization. Ascorbic acid is involved in protein, nucleic acid, steroid, carbohydrate, lipid, mineral and muscle metabolism (Chinoy, 1978). Chinoy and Asok Kumar (1980) have also observed a depletion of testicular ascorbate content concomitant with an increase in serum testosterone levels with the onset of puberty and with passing of the first wave of spermatozoa through the epididymis of rats. A high AA metabolic turnover pattern of sperm has been also correlated with greater sperm motility and metabolism (Chinoy, 1978). The testicular phosphatases did not reveal much variation in our study. The decrease in the testicular SDH enzyme levels were also related to an altered oxidative metabolism and function of testis in our study.

The biochemical effects were supported by histopathological changes observed in the testis of treated rats in this study. The histological alterations include loss of spermatogenic elements, sperm free lumen of seminiferous tubules followed by depopulation of germinal epithelium. The spermatogenic activity seemed to be inhibited
at spermatocyte level. Leydig cell also exhibited mild alterations. These are substantiated by histocytometric data.

The parameters studied in the cauda epididymis were ATPase, proteins and sialic acids. All these parameters declined as a result of MPA + TE treatment. Decrease in ATPase suggests alterations in energy metabolism of cauda epididymis. Local androgen deprivation caused its altered metabolism and secretory activity. The epididymis has been shown to play an important role for sperm maturation including gaining the ability to be mobile (Longo, 1987). The epididymis thus plays a crucial role for acquisition of sperm progressive motility and fertilizing capacity (Cooper, 1996). It receives testicular androgens from the rete testis and the blood circulation. In this organ, testosterone is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase (Castellion and Huidobro, 1999). Under its stimulation, it secretes many products that are involved in sperm maturation. Sialic acid, one of them is derived from neuraminic acid whose main derivative is N-acetyl neuraminic acid which is generally used as the synonym for sialic acid (Ledeen and Yu, 1976). They are widely distributed in nature as non-reducing termini of glycoproteins and glycolipids. About 70% of the total sialic acids of eukaryotic cells are found on the cell surface and the remainder is distributed among the endoplasmic reticulum, mitochondria, lysosomes etc. (Warren, 1976). The microenvironment of epididymis and its relation to sperm maturation are well reviewed (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). All these factors thus generated unsuitable environment for sperm to survive and mature in rats administered MPA + TE injections for 120 days. Similarly proteins

102
secreted by epididymis are also involved in sperm maturation process acting as sperm antigens involved in sperm capacitation during fertilization (Knobil and Niell, 1988). These altered parameters also indicted a loss of epididymal function.

MPA + TE treatment exerted significant changes in proteins, SDH and ATPase levels of vasal tissue. Thus vas deferens metabolic integrity and function were also affected significantly by these combined injections. This tissue is also important for sperm survival and transport in the male. Chinoy (1985) mentioned the contribution of vas in sperm survival, while they are in it. The effect of treatment is also demonstrated by loss of acid phosphatase and fructose levels gradually during later stage of treatment. Secretions of these products by prostate and seminal vesicle (SV) respectively are indicators for androgen sensitivity. Our study clearly reports alteration in these accessory sex organs of treated animals. Serum hormonal levels (androgen) also exhibited alteration by this treatment. All these effects are further supported by histopathology of these reproductive tissue. In epididymis and vas deferens confluence of tubules, pyknotic nuclei in pseudostratified epithelium and degeneration in lamina propria were evident. Tubules had cell debris with no sperm due to treatment. These were well supported by histocytometry in our investigation.

In our study no significant variation in haemoglobin (Hb) or blood cell counts (RBC and WBC) were observed in MPA + TE treated rats. However, much controversy surrounds the clinical significance of an increased concentration of white blood cells (WBC) in the male ejaculate. It is suggested that increased Polymorphonuclear (PMN) concentrations are related to decreased percentages of normal forms of spermatozoa and a significant increase in the percentage mid piece abnormalities was related to an increased
PMN concentration (Fishel et al., 1997). Haemoglobin, hematocrit and red blood cell levels were lower at end of treatment phase in CPA-treated subjects, whereas no significant changes could be detected in the TE alone group (Meriggiola et al., 1996). Other study reported that T routinely increases hematocrit (Sih et al., 1997). Anderson et al. (2002) did not observe any significant change in haemoglobin concentration and haemotocrit through the duration of the study in treated groups (one or two etonogestrel implants with 400 mg depot testosterone pellets on d1 and at 12 wks). Earlier studies from our laboratory also demonstrated no change in blood cell counts and Hb levels in hormone injected rats (Rao and Shah, 1998).

The serum parameters studied were protein, cholesterol, transaminases, total lipids and testosterone. Serum proteins, cholesterol, transaminases and total lipids were unaltered throughout the study period. Therefore, this combination did not produce any toxic effect. Handelsman et al. (1996) reported that DMPA + TE treatment did not cause significant alterations in cholesterol fractions (total, LDL and HDL) and transaminases (SGPT and SGOT). Meriggiola et al. (1996) reported that CPA and TE treatment did not cause any change in lipoprotein levels or liver function tests. No change in HDL-C was seen on treatment of etonogestrel implants with depot testosterone (Anderson et al., 2002). In our study, treatment for 120 days of DMPA + TE showed no change in clinical chemistry profiles was noted as stated in our earlier studies (Rao and Shah, 1998; Rao et al., 1995 and Roy and Rao, 1995).

The reversibility study revealed that all reproductive biochemical parameters, organ weights, spermiogram and fertility of treated rats returned to control levels. The histology of organs also had a recovery after withdrawal of treatment for 120 days.
Androgen levels were almost comparable to control levels, upon withdrawal of treatment. Thus this hormonal combination induces functional sterility in males. At the same time no undesirable side effects were observed after a long-term treatment in rodents and could be useful for human male contraception.

PART – II: EFFECTS OF PLANT PRODUCT

A contraceptive method which is of indigenous plant origin may have particular advantages such as cost effectiveness, less side-effects and orally bioactive. The search for investigating male contraceptive of plant origin is continuing.

In this study, an investigation was done on the anti-fertility effects of alcoholic seed extract of *Abrus precatorius* in adult male mice. Alcoholic (70%) seed extract at doses of 20 and 40 mg/kg body weight for 45 days were studied for its effect and later dose was found to be effective in induction of anti-fertility effect and hence was chosen for further studies. The withdrawal period was of 90 days. The following parameters were studied. Absolute body and organ weights of testis, cauda epididymis, vas deferens and seminal vesicles were noted. Cauda epididymal sperm motility, count and viability were evaluated. Fertility rate was also assessed. In testis, biochemical tests like 3β and 17β hydroxysteroid dehydrogenases (HSDs), succinate dehydrogenase (SDH), acid phosphatase (ACPase), alkaline phosphatase (ALKPase), total ascorbic acid (TAA), proteins and cholesterol were estimated whereas adenosine triphosphatase (ATPase), proteins and sialic acids were studied in cauda epididymis. In vas deferens, SDH, ATPase and protein levels were evaluated. Fructose was estimated in seminal vesicles. For toxicological studies, haemoglobin content and blood cell (RBC and WBC) counts were
performed. In serum, transaminases (GPT and GOT), proteins, cholesterol, total lipids and testosterone were estimated in all groups.

The results of this study indicated that body weights did not show significant alteration upon feeding of the treatment. This indicated that the extract did not promote weight gain causing obesity or water retention. Similar results were obtained by Verma et al. (2002) on feeding *Sarcostemma acidum* stem extract to male albino rats. Weights of testis and cauda epididymis decreased significantly in treated animals whereas weights of vas deferens and seminal vesicle remained similar to that of control animals throughout the study. Rao (1987) also studied the anti-fertility effects of aqueous seed extract of *Arbus precatorius* in rats, wherein reported no effect of the extract in body and organ weights in support of our study. The weights of testis, epididymis, vas deferens and prostate were significantly decreased by feeding of an ethanolic extract of *Bambusa arundinacea* tender shoots for fertility regulation (Vanithakumari et al., 1989). The decrease in weights of testis and cauda epididymis can be attributed to a loss of spermatogenic elements in testis and absence of sperms in cauda epididymis in the present study.

A significant decrease in caudal sperm motility, count and viability was noted as a result of feeding of treatment for 45 days. The decline in sperm count indicated antispermatic nature of this extract. Rao (1987) also obtained a decline in cauda sperm motility and count followed by viability reduction in extract fed rats. Further, according to Rao (1987), scanning electron microscopic study on sperm morphology exhibited decapitation, acrosome damage and formation of bulges on midpiece region of sperms in *Abrus precatorius* Linn. (100 mg/kg body weight) treated rats.
Similar results on spermiogram were found by administration of *Colebrookia oppositifolia* in rats (Gupta et al., 2001) and *Mentha arvensis* in mice (Sharma and Jacob, 2001). Ratnasooriya et al. (1991) reported inhibitory effects of a methanol extract of *Abrus precatorius* seeds on motility of washed human spermatozoa. The extract caused a concentration related impairment of percent sperm motility with 2.29 mg/ml being the EC50. A dose of 20 mg/ml concentration caused onset of antimotility action immediately. This concentration impaired the functional integrity of plasma membrane (hypoosmotic swelling test) and viability (nigrosin-eosin) of spermatozoa. Sinha and Mathur (1990) also reported a decrease in sperm count by treatment of steroidal fraction of seeds of *Abrus precatorius* Linn. in male rats. Sinha (1990) also obtained suppression of sperm motility in cauda epididymis as the most pronounced effect of treatment of 50% ethanol extract of *Abrus precatorius* seeds (250 mg/kg) in albino rats for 30 and 60 days. Changes in spermiogram caused a reduction in fertility rate of the treated animals. In our study also, extract feeding caused a fall in fertility of treated mice in corroboration with the above observation.

In testis the levels of 3β and 17β hydroxy steroid dehydrogenases (HSDs) were significantly decreased by the extract feeding which was similar to results obtained by Sinha and Mathur (1990). It might be that the extract probably exerts its influence directly at the gonadal level resulting in a reduction in production and release of testosterone in testis. However, indirect mechanism through hypothalamo-pituitary-gonadal axis might not be ignored. Protein and SDH levels in testis were also declined in this study which are known to be androgen dependent. Serum androgen level in our study also declined at 40 mg/kg body weight dosage level to support androgen deprivation.

107
effect. Similar result was reported by Rao (1987) and Sinha (1990) with seed extracts of *Abrus* plant in rodents while attributing its mechanism. Jana et al. (2003) reported decreased 3β and 17 β HSDs activity on treatment of supernatant and precipitate parts of aqueous fraction of *Stephania hernandifolia* leaf methanolic extract in rats. They reported that this might be due to similar mechanism as stated in our study. Other biochemical parameters in the testis like TAA, ACPase, ALKPase, cholesterol did not alter significantly from control groups.

Caudal parameters like ATPase and proteins did not alter significantly as a result of treatment but sialic acid levels were found to be depleted. Similar observations were obtained by Rao (1987) in *Abrus* seed extract feed rats. Sinha (1990) also reported a decrease in proteins and sialic acid as a result of *Abrus precatorius* seed extract feeding to rats in support of our data. Gupta et al. (2000) reported a decrease in sialic acid content in epididymides on treatment of *Barleria prionitis* Linn. (100 mg/rat/day) for 60 days to rats. Verma and Chinoy (2001) also reported reduction in total protein and sialic acid content in epididymal fluid on treatment of papaya seed extract (0.5 mg extract/kg body weight; im) for 7 days affecting sperm maturation in albino rats. No alteration in biochemical parameters like SDH, ATPase and proteins were noted in vas deferens after the extract treatment regimen. However fructose content in seminal vesicle had a significant depletion in treated animals. This parameter is an androgen dependent parameter hence a decrease in androgen level in serum is justified as mentioned earlier in our study. Sarkar et al. (2000) reported a decrease in fructose levels in seminal vesicles on treatment of *Piper betle* Linn. to male mice.
Histopathological changes in the testis included confluence of seminiferous tubules, defoliation of spermatogenic elements and loss of sperm in their lumen. Large vacuoles were also conspicuous in certain tubules which support variation in biochemical findings in our study, due to probable androgen deprivation. These histological changes were further supported by histocytometric data, wherein alterations in germinal epithelial height and Leydig cell diameter were noticed in extract fed mice.

In cauda epididymides also, some tubular epithelium exhibited degenerative changes, necrotic stereocilia and lumen had less number of sperm. Some sperm were agglutinated in the lumen. Vas proximal did not show much histological alterations were noticed by 45 days of extract treatment. But in distal vas deferens pseudostratified epithelium exhibited degenerative changes with nuclear pyknosis. Lumen was free of sperm. These were associated with a reduction in histocytometric data indicating androgen deprived effect exerted by the extract feeding to mice. All these effects led to an alteration in fertility rate of these treated mice.

Withdrawal of the treatment for 90 days brought about almost normal levels in all biochemical profiles of testis, epididymis, vas deferens and seminal vesicles and their weights. Histology of these organs were also comparable to control groups after withdrawal of feeding. Reversibility of sperm parameters with regaining of fertility potential of treated mice was also seen after withdrawal of the treatment. Hence, this extract is able to induce functional sterility in mice.

None of the haematological parameters like haemoglobin content, blood cell counts (RBC and WBC) or serum parameters like GPT, GOT, total proteins, cholesterol and total lipids revealed any significant alterations due to the treatment of alcoholic
extract of *Abrus precatorius* seeds in treated groups comparatively. Thus, this study clearly indicated that this plant extract seemed to be a promising male contraceptive of herbal origin which is able to induce functional sterility in animals with no side effects. Further studies need to be carried out to evaluate its anti-fertility potential in other animal models and active principles involved in it.