THE EFFECT OF N'N-BIS(DICHLOROACETYL)-1,8-OCTAMETHYLENEDIAMINE (WIN 18446) ON THE TESTIS, EPIDIDYMIS, SPERM AND FERTILITY OF MALE CBA MICE

by

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DECLARATION

I, the undersigned, hereby declare that **"The effects of N'-N-bis(dichloroacetyl)-1,8-octamethylenediamine on the testis, epididymis, sperm and fertility of male CBA mice"**, is my own work and has not previously in its entirety, or in part, been submitted at any university for a degree. All the sources I have used or quoted have been indicated and acknowledged by means of complete references.

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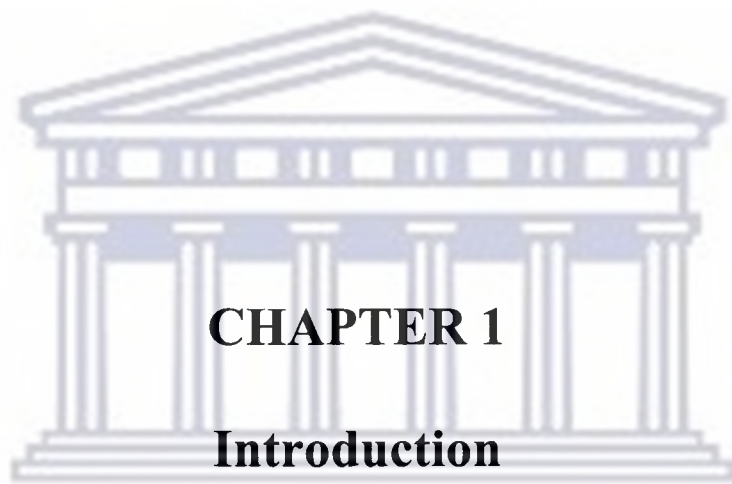
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Abstract

Although the modern era of contraception has focused attention on the female, research into male contraception is continuing on several fronts. Despite the widespread use of premature withdrawal, the condom and vasectomy, there is no acceptable drug for controlling fertility in the male. Research and developments to suppress the sperm production, inhibit the maturation of sperm, and block sperm transport are under investigation. Thus, a strong demand exists for a safe, reversible and effective male contraceptive. A compound N'N-bis(dichloroacetyl)-1,8-octamethylenediamine, commonly known as WIN 18446 used in the treatment of amebiasis was found to possess anti-spermatogenic activity. WIN 18446 (WIN), the most potent of the bis(dichloroacetyl)diamines and one of the least active amoebicidal agents was shown to exert a specific effect on the testes while the Leydig cells were unaffected. Full recovery of spermatogenesis is established upon the withdrawal of WIN. One drawback associated with this compound was that the human consumption of alcohol induced an antabuse effect. However, WIN could prove to be a successful contraceptive for animal and wildlife populations. The aim of this investigation was to quantify the anti-fertility effects of WIN in mice. A group of male CBA mice was exposed to a daily dose of WIN of 125 mg/kg body weight for 42 days. Two other groups of mice received WIN for 42 days followed by a withdrawal period of either 15 or 42 days, while the controls received only 1% gum tragacanth. After treatment, the mice were humanely killed and intact testes and epididymidis were removed. The testis and epididymis fixed in Bouins were processed for light microscopy and stained with a haematoxylin and eosin stain. Cauda epididymal

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sperm were analyzed for sperm count, percentage normal and abnormal sperm using scanning electron microscopy and sperm motion parameters. The fertility indices of the treated male mice and non-treated virgin females were determined by mating experiments. The histologic assessment of the testes for the WIN treated group showed that spermatogenesis was severely affected. The presence of large multi-nucleated forms, vacuolization and the absence of sperm within the testes supports the findings that the testis is the target site for WIN. A significant decrease (P<0.05) in the sperm concentration was observed for the WIN treated group (47.2%) as compared to the controls. The WIN 15 days withdrawal group exhibited a decrease (35.9%) and the WIN 42 days withdrawal group showed a (11.4%) decrease when compared to the control group. Analysis of the normal epididymal sperm showed a significant reduction for the WIN treated group of 29.4% and the WIN 15 days withdrawal group showed a 19.4% reduction in morphologically normal sperm forms. The sperm motion parameters for the control group was compared with the three treatment groups. VSL, LIN, mnALH, VAP, WOB and STR differed significantly (P<0.05) for the WIN only and the WIN 15 days withdrawal groups. VCL, MAD and CURV did not show any significant differences (P>0.05) for each of the treatment groups. DNC and mxALH showed a significant difference (P<0.05) for the WIN treated group. BCF differed significantly (P<0.05) for all the treatment groups when compared to the control group. A significant decrease (P<0.05) existed for the percentage of females which gave birth to pups for the WIN treated group and the WIN 15 days withdrawal group. Also, a significant difference (P<0.05) existed for the male fertility indices for the WIN treated group and the WIN 15 days withdrawal group. Essentially, complete recovery of the above mentioned parameters were evident after 42 days withdrawal of WIN and confirms its potential as a future male contraceptive.

CHAPTER 1

Introduction

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With the human population of the world growing at an explosive rate, the need for additional forms of readily available contraception appears paramount. In contrast to the striking success in the regulation of fertility in females, at present there is no approved method for the pharmacological regulation of male fertility (Bonn, 1998; Frick *et al.*, 1998). Control of fertility by the male is restricted to coitus interruptus (withdrawal), use of the condom and vasectomy which can be attributed to a combination of technical, economic and sociological problems. The notion that contraception is the woman's responsibility is changing and with this change of attitude, along with recent progress in understanding male reproductive physiology, has begun an intensive search for a reversible male contraceptive agent. Interference with fertility has proved to be more difficult in males than in females (Barwin, 1978). Thus, for men to have the same choice as women, there is clearly a need for a safe anti-fertility pill.

The ultimate aim in the regulation of fertility in the male is the development of acceptable methods which should be capable of reversing suppression of sperm production or sperm function without interfering with *libido* or any other short- or long-term features of the health status of men (Schwallie, 1976; Waites, 1986). The prospective sites of action of the contraceptive are directed toward (a) the inhibition of spermatogenesis accompanied with infertility, and (b) interference with sperm transversing the epididymis undergoing

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maturation. Compounds could act directly on the developing germ cells during spermatogenesis or could influence spermatogenesis through the hormonal system (Hinton, 1980).

1.1 Hormonal approach to the inhibition of spermatogenesis

Male fertility is subject to hormonal regulation by the hypothalamic-pituitary testicular system. Secretion of gonadotrophin-releasing hormones (GnRH) regulates the output of follicle-stimulating hormone (FSH) and a luteinizing hormone (LH) from the pituitary. FSH activates the Sertoli cells which line the seminiferous tubules and directly nourish the developing sperm cells, whilst LH stimulates the Leydig cells to secrete testosterone. The presence of androgenic steroid in the testes is essential for spermatogenesis and is also required in the peripheral circulation to support male secondary sexual characteristics, *libido* and potency (Matlin, 1994).

The potential interaction among the cells of the testis is illustrated in Fig. 1.1. The Sertoli cell and their responses to FSH and testosterone are crucial to spermatogenesis. FSH stimulates estradiol production by Sertoli cells from androgen precursors of Leydig cell origin. In response to FSH and testosterone acting synergistically, an androgen-binding glycoprotein (ABP) is synthesized. ABP binds testosterone and estradiol with high affinity thereby regulating their availability to the germ cells. APB also regulates the inhibitory effect of estradiol on Leydig cell testosterone synthesis. Inhibin, activin and other growth factors are also synthesized by the Sertoli cells under the influence of FSH and testosterone.

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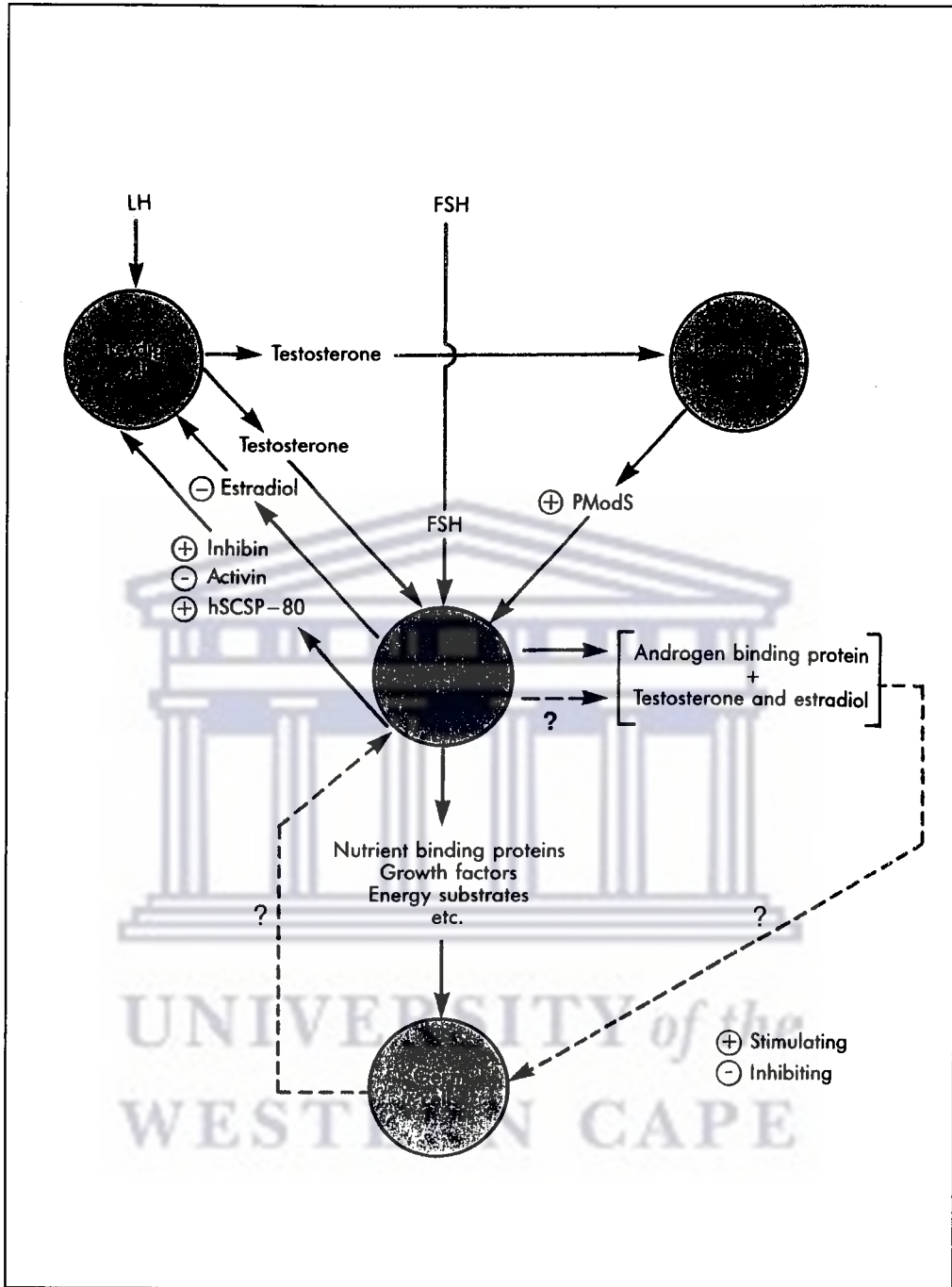
Fig. 1.1: Potential interactions among the cells of the testis (Reproduced from: Berne & Levy, 1993)

Inhibin and activin have reciprocal actions on neighbouring cells. Testosterone secreted by the Leydig cells stimulates the differentiation and proliferation of the peritubular myoid cells. A protein, PModS secreted by these peritubular cells, stimulates Sertoli cell function. Thus, the Sertoli cells secrete products which modulate Leydig cell growth and steroid secretion.

The aim of male hormonal contraception is to block spermatogenesis by suppressing secretion of pituitary gonadotrophins. This can be achieved by giving exogenous sex steroids to stimulate the negative feedback mechanism that regulates the gonadotrophins (Bonn, 1998).

Testosterone

Testosterone given as an ester is an effective contraceptive. Testosterone is inactive when taken by mouth but chemical modification of the molecule, confers oral activity (Jackson, 1972; Barwin, 1978). There are numerous derivatives of testosterone which have been used in man for hormonal treatment, and all are known to have an inhibiting effect on gonadotrophin secretion and consequently on the testes. However, supra-physiological doses suppress spermatogenesis by inhibiting LH and FSH secretion while preserving *libido* and potency. An approach to block spermatogenesis, other than high-dose testosterone which may affect blood-lipid profiles and the prostate is being pursued (Bonn, 1998). This includes the usage of a progesterone or gonadotropin-releasing hormone antagonist or agonist to block spermatogenesis. Gonadotropin Releasing hormone (GnRH) antagonist plus testosterone was shown to suppress LH and FSH levels and inhibit

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spermatogenesis to azoospermia or severe oligozoospermia (Swerdloff *et al.*, 1998). GnRH antagonist combined with testosterone has been proposed as a prototype male contraceptive as azoospermia was induced more rapidly than with testosterone alone. A study was designed to develop a self applicable hormonal male contraceptive regimen by combining transdermal testosterone with an oral gestagen, levonorgestrel (Buchter *et al.*, 1999). Healthy men treated with oral levonorgestrel and transdermal testosterone became azoospermic after 24 weeks of treatment. When these men stopped using the treatment, sperm counts gradually returned to normal.

Testosterone enanthate

Bonn (1998) reported that the use of testosterone enanthate produced azoospermia in 98% of men, with an effect on spermatogenesis. These findings are supported by Matlin (1994) who showed that high doses of testosterone enanthate could not result in azoospermia in all men tested. Testosterone enanthate has a number of undesirable characteristics that limit its suitability for routine use in male contraception. These include injections at seven to ten day frequencies, steep increase in serum testosterone, increase in body weight, aggression and the incidence of severe acne (Matlin, 1994). The raised circulating androgen level for prostate hyperplasia has also been a concern. Thus, due to its short-acting ability of this intramuscular once weekly injection, testosterone enanthate has been rendered unacceptable for long-term use. Healthy men with normal semen analysis treated with testosterone enanthate alone induced azoospermia or severe oligozoospermia (Swerdloff *et al.*, 1998). It was concluded that sperm counts suppressed with GnRH antagonist plus testosterone could be maintained with a relatively low dose testosterone

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enanthate treatment alone.

Testosterone undecanoate

Testosterone undecanoate appears to be more promising as this compound has no adverse effects on sexual function (Bonn, 1998). However, further research is required for this compound to be feasible as a hormonal contraceptive.

Testosterone buciclate

Matlin (1994) reported that the administration of a single muscular injection of testosterone buciclate restored serum testosterone levels in hypogonadal men. Clinical studies for testosterone buciclate needs to be performed to evaluate its contraceptive potential in healthy men. The combination of testosterone buciclate and levonorgestrel butanoate given in a three monthly regimen would be clinically evaluated as a potential contraceptive (Bonn, 1998). Levonorgestrel was shown to reduce high density lipoprotein (HDL)-cholesterol concentrations whereas desogestrel reduces low density lipoprotein (LDL)-cholesterol as well as HDL-cholesterol levels (Bonn, 1998). Thus, desogestrel may prove to be a more desirable component to combine with testosterone buciclate for a male hormonal contraceptive.

7α-Methyl-19-nortestosterone

An androgen, 7α-methyl-19-nortestosterone (MENT) caused an increase in the weights of the ventral prostate and seminal vesicles of castrated rats which was higher than testosterone and also has significant effects on muscle weight (Matlin, 1994). MENT

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possesses a much higher potency in the suppression of serum gonadotropin levels. Thus, the administration of this androgen with a one year duration appears feasible. The result would be a rate of drug release capable of suppressing gonadotropin secretion, while maintaining sexual function and normal muscle mass with no overstimulation of the prostate. Bonn (1998) demonstrated that in monkeys, MENT was far more potent than testosterone in its gonadotropin-suppressing and anabolic effects. Implants containing this androgen appears to have considerable promise for male contraception.

However, the recent interest in the hormonal approach to develop contraceptives for men is encouraging and may result in an acceptable, reversible contraceptive. Clinical trials with these pituitary gonadotropin blocking agents appear to be at an advanced stage of development in this ever expanding area of research, but further testing is of utmost importance for the establishment of a male contraceptive.

1.2   Non-hormonal approaches

Hormones dominate male contraceptive research, but non-hormonal systemic agents are also attracting attention (Bonn, 1998). The development of new lines of approach to the problem of anti-fertility agents involves the use of anti-spermatogenic agents that would eliminate the spermatogenic activity of the testes, without affecting either man's *libido*, ejaculatory process or copulatory behaviour. Several methods are available to cause disruption of spermatogenesis and many compounds have been tested. The mode of action of some of these compounds is understood while many are not. Several studies have shown

chemical control of male fertility to involve the use of many compounds that not only result in irreversible sterility but at the same time often decreases *libido*, cause genetic damage and have toxic side effects when given in high doses (Shandilya *et al.*, 1979).

The available literature on possible male contraceptives is vast and is not within the scope of this dissertation to discuss in detail all the intricate studies that have been performed. Some of the possible contraceptives will be discussed in the next section.

1.2.1 Testicular Anti-spermatogenic Agents

Nitrofurans

The nitrofurans were developed originally as anti-bacterial and anti-cancer agents, and were used in the systematic search for contraceptives for men (Barwin, 1978). These compounds were found to have a direct effect on the seminiferous tubules by halting spermatogenesis and upon withdrawal of this compound these effects were reversible. However, undesirable side effects were produced by the nitrofurans, manifested by gastrointestinal disturbances which prompted the discontinuation of testing in man (Barwin, 1978).

Pyrimethamine

Pyrimethamine (PYR) used clinically in the control of malaria was investigated for reversibility of infertility in mice in a dose dependant manner (Consentino *et al.*, 1990). The findings included a decrease in male fertility, sperm production and seminiferous tubule diameter and with the cessation of PYR, recovery to normal fertility status was

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evident. These findings supports the hypothesis that PYR acts on spermatogenesis while having no effect on testosterone levels or body weight. The action of PYR was suggested to be due to its anti-folate action (Consentino *et al.*, 1990). Further studies are necessary to establish whether lower doses desirable for human consumption would be effective to attain contraception in males. Thus, PYR represents another approach toward the development of a male contraceptive.

Etoprine

Etoprine caused a reduction in testicular weights, seminiferous tubule diameters and epididymal sperm reserves when tested on rodents (Malik *et al.*, 1995). These findings indicate that etoprine is a very potent inhibitor of spermatogenesis. The effectiveness of etoprine was compared to pyrimethamine (PYR) and it was reported that etoprine was a more effective anti-fertility agent than PYR which could be attributed to several factors (Malik *et al.*, 1995). Etoprine has a greater lipophilicity relative to PYR which presumably enhances its ability to penetrate cell membranes and the blood testis barrier and accumulation in the testes. Also, the difference in the structural formulae between etoprine and PYR appear to be responsible for the greater efficacy of etoprine. PYR acts in a dose-dependant manner and could be attributed to the way in which it is transported into the germinal epithelium or a different mode of testicular dihydrofolate reductase (DHFR) inhibition as compared to etoprine. Thus, various compounds show a higher degree of specificity for testicular DHFR and etoprine does appear to have some specificity against this enzyme.

1-Formyl-4-dichloroacetyl piperazine (CDRI-84/35)

Another anti-spermatogenic agent which may hold promise for the development of a male contraceptive is 1-formyl-4-dichloroacetyl piperazine (CDRI-84/35). Gupta *et al.* (1997) found CDRI-84/35 to be anti-spermatogenic in rats with a direct effect on the seminiferous tubules producing complete arrest on spermatogenesis while the Leydig cells remain unaffected. This compound did not show complete reversibility and may be due to its structural formula. Thus, CDRI-84/35 or its analogs with better reversibility could be used for the control of fertility and this will need to be ascertained by further testing of these compounds.

5-Thio-D-glucose

The exciting discovery of the anti-spermatogenic activity of 5-thio-D-glucose (5TDG) was welcomed by many scientists as another approach for non-hormonal male contraception. 5TDG, an anti-metabolite of D-glucose has been reported to have an anti-spermatogenic effect in several mammalian species possibly via the competitive inhibition of glucose utilization (Brady & Majumdar, 1981). Lobl & Porteus (1978) reported that 5TDG inhibited spermatogenesis and fertility in mice. However, the anti-spermatogenic activity and the drug induced infertility were only partially reversible with some apparently intrinsic spermatogenic activity independent of its effect on serum glucose concentrations. An investigation undertaken by Brady & Majumdar (1981) identified the specific germ cells that were affected by 5TDG in mice while the Sertoli and the Leydig cells were unaffected by 5TDG. After 21 days of 5TDG injections, early spermatids and testicular sperm were markedly reduced and after 28 days of 5TDG injections, early and late

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spermatids as well as testicular sperm were affected. When the period was increased to 35 days, virtually no spermatocytes, spermatids and sperm were found in the seminiferous tubules. 5TDG is a competitive antagonist of glucose metabolism in all spermatogenic cells and has an additional effect on spermatid metabolism. Spermatogonia appeared to be relatively protected from the drugs' anti-metabolic action. Administration of 5TDG to mice was followed by transient hyperglycemia (Davies & Meanock, 1981). However the susceptibility of different species to the hyperglycemic effect of 5TDG varies. It is possible that 5TDG damages spermatids by interfering with glycogen metabolism or the anti-fertility action of 5TDG is due to a primary action on protein synthesis. Das & Yanagimachi (1978) revealed that hamsters failed to respond to 5TDG even after continuous release and may be attributed to species variation. Obviously, the possibility of irreversible damage to the germinal tissue and the occurrence of hyperglycemia reduces the likelihood that 5TDG might be used to control fertility in man.

Gossypol

In 1978, a major development took place when Chinese scientists discovered gossypol, a polyphenol pigment isolated from the cotton plant, *Thespesia populnea*. It was established that this compound caused the disruption of spermatogenesis in man and some mammalian species (Sadykov *et al.*, 1985). Clinical trials of gossypol conducted on men for a period of 6 months attained disruption of spermatogenic epithelium which ultimately led to oligo- and azoospermia and restoration of fertility was not fully obtained in all cases. It was concluded that gossypol does not disrupt the integrity of epididymal sperm but is restricted to target cells in the testes. Experiments of gossypol on laboratory animals confirmed that

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species differences were evident (Sadykov *et al.*, 1985). The majority of studies of anti-spermatogenic effects of gossypol have been conducted on rats and sterility was obtained within four months. Disruption in LH secretion and functions of Leydig cells was evident due to the effects of gossypol. A comparative study evaluated the anti-fertility effects of gossypol in rats and hamsters (Srivastava *et al.*, 1989). The effect of gossypol was found however not to be restricted to spermatogenesis since degenerating primary spermatocytes and exfoliated spermatids occurred in the affected seminiferous tubules. Thus, gossypol exerts its action on pre- as well as post-meiotic germ cells. Also, a marked reduction in the secretory function of the cauda epididymidis was observed which may suggest that gossypol has effects on the epididymal epithelium without altering its morphology. These results indicate that gossypol exerts effects on the testes as well as the epididymidis. Albino rats treated with gossypol for eight weeks showed necrotic changes in the seminiferous tubules in rats and effects on the epithelium of the epididymis (Kaur *et al.*, 1988). Reports of the changes induced by gossypol in the testes and epididymis remains quite controversial. Stumpf *et al.* (1988) suggested that gossypol has both direct and indirect effects on testicular function. These findings were elicited when the binding sites of gossypol was studied by means of autoradiography. The administration of gossypol to langurs revealed reversible inhibition of spermatogenesis (Lohiya *et al.*, 1990). The occurrence of hypokalemia was more pronounced in the langurs which were administered gossypol only and extensive renal potassium loss was evident with the renal excretion of sodium markedly decreased. The oligospermia which was achieved was reversible, but further studies are necessary to establish an adequate supplementation dose of potassuim chloride and to evaluate the occurrence of hypokalemia. Although gossypol has been

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considered a very promising anti-fertility agent, the presence of hypokalemia and the delayed recovery of spermatogenesis remains elusive and influences the widespread acceptability of gossypol as a male contraceptive (Faundes & Pinotti, 1990). It seems unlikely that gossypol will ever, be widely used as a male contraceptive, but the work with this agent could provide the starting point either for the production of less toxic analogues of gossypol or for the provision of more basic knowledge on the control of spermatogenesis which could eventually lead to a male pill.

Triptolide

A triptolide extracted from the medicinal herb, *Tripterygium wilfordii* (Vine of the Thunder God) for rheumatoid arthritis, chronic nephritis, chronic hepatitis and various skin disorders has been evaluated as a potential male contraceptive (Zhen *et al.*, 1995). Studies undertaken by Lue *et al.* (1998), revealed that triptolide, at a specific dose induced infertility, but with a much higher dosage this compound has an immunosuppressive effect. Triptolide was shown to have little or no detrimental effect on various structural and endocrine parameters of the testes, but interference by triptolide occurred during spermatogenesis with a significant decrease in spermatids. In support of these findings, Zhen *et al.* (1995), reported that *Tripterygium wilfordii* disturbed the spermatogenic dynamics and recovery of fertility was evident upon withdrawal of this anti-spermatogenic agent. Future studies with *Tripterygium wilfordii* are necessary as there is a striking paucity regarding meticulous safety evaluation of this anti-fertility agent.

The anti-fertility effects of oleanolic acid, a triterpene (closely related to the triptolide isolated from *Tripterygium wilfordii*) isolated from the flowers of *Eugenia jambolana* was evaluated (Rajasekaran *et al.*, 1988). This compound yielded spermatogenic arrest with no abnormalities to the spermatogenic, Leydig and Sertoli cells. Oleanolic acid may prove to be a promising agent without undesirable side effects, but further studies are necessary to establish its success as an anti-fertility agent in man.

1.2.2 Epididymal Agents

Once spermatogenesis is concluded, the sperm are transported from the seminiferous tubules to the proximal epididymal anatomical region (caput) by secretory fluid pressure and efferent duct ciliar activity (Reyes & Chavarria, 1981). Sperm leave the testes neither fully motile nor able to fertilize ova and must transverse a long and tortuous pathway to reach maturity. This transformation occurs in the epididymis and is termed sperm maturation. Concomitant with this functional maturity are changes in sperm morphology, motility, chemistry, permeability, density and metabolism (Consentino & Cockett, 1986). During their development, sperm are continually bathed in fluid provided by epithelial secretions of the seminiferous tubule and the epididymal duct. Hoppe (1975) demonstrated that cauda epididymal sperm of the mouse exhibited optimal fertilizing ability as compared to the other epididymal regions. Thus, the epididymis holds significant promise as the possible site of action for a male contraceptive. This approach is regarded as an ideal target for male contraception, since an epididymal agent would act much more rapidly than one affecting testicular function. Also, there should be less risk of genetic damage, irreversible

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or adverse influence on Sertoli and Leydig cell functions which might feed back to the pituitary or hypothalamic levels. Several different anti-fertility mechanisms can be envisaged for an epididymal agent and therefore numerous compounds have been tested in an attempt to alter epididymal function.

Methylene-dimethanesulphonate (MDS)

The first hint of selective "sterilization" of sperm in the terminal part of the epididymidis came with methylene-dimethanesulphonate (MDS). A single dose for one week, induced sterility in male rats and recovery was attained within a short time (Hinton, 1980; Jackson, 1972). This observation indicated that MDS affected sperm in the distal region (cauda) of the epididymidis while spermatogenesis remains unaffected. MDS was shown to produce anti-fertility effects with predictable periods of sterility in the male animal (Jackson, 1972). Unfortunately, MDS was found to be related to toxic anti-cancer compounds and possessed an insufficient margin of safety.

Cyproterone acetate

Cyproterone acetate implanted capsules was reported to produce sterility in rats which was reversible when these capsules were removed (Jackson, 1972). It was shown by Lewy (1977) that cyproterone acetate administration in rats resulted in non-motile sperm and recovery was achieved within 30 days of withdrawal of cyproterone acetate. Sperm obtained from the epididymis were found to be non-motile blocking androgens at the epididymal level thereby interfering with the androgen-dependant maturation of epididymal sperm and these findings were supported by Barwin (1978). The production of non-motile

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sperm could be an acceptable method to control fertility, but cyproterone acetate has been shown to depress *libido* in man and is therefore not suitable as a male contraceptive.

α-Chlorohydrin

Another anti-fertility compound, α-chlorohydrin (3-chloropropane-1,2-diol) was evaluated as a potential male contraceptive as it has a pronounced effect on epididymal sperm. Jackson (1972) revealed that α-chlorohydrin produced sterility in rats which could be maintained indefinitely and upon cessation of this compound, full recovery was attained. These findings were supported by Barwin (1978) who proposed that α-chlorohydrin induced infertility by damaging the germinal epithelium. A low dose of α-chlorohydrin impaired the motility of the cauda epididymal sperm while higher doses with a longer duration of treatment produced morphologic changes in the epididymal epithelium as luminal blockage was evident (Hinton, 1980). A low dose administration of α-chlorohydrin produced a continuous antifertility response which was reversed upon withdrawal, but high doses led to prolonged or permanent infertility (Jones, 1983). The primary site of inhibition appears to be the enzyme glyceraldehyde-3-phosphate dehydrogenase, also aldolase and triphosphate isomerase were inhibited. The inhibition of these enzymes resulted in a depression of sperm motility (Hinton, 1980). The findings of Brown-Woodman & White (1975) and Reyes & Chavarria (1981) was in agreement with Hinton (1980) as the glycolytic pathway was inhibited at the specific enzyme levels. However, while the primary site of action of α-chlorohydrin is in the glycolytic pathway, higher concentrations were reported to affect reactions involved in the tricarboxylate cycle (Brown-Woodman *et al.*, 1978).

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Due to the dynamic participation of the epididymis in sperm maturation, the anti-fertility effects of α-chlorohydrin may not depend only on its direct action on the sperm cell, but may be partly due to its interference with the normal absorptive processes of sodium and water in the cauda epididymidis of the rat disturbing the critical relationship between sperm and micro environment during the maturation process (Reyes & Chavarria, 1981). The chronic administration of α-chlorohydrin in the rat caused lesions in the testes with degenerative changes in the germ cells, a regressed epithelium and the absence of sperm in the epididymis were observed (Dixit & Agrawal, 1980). The rate of fluid absorption in the cauda epididymidis diminished and these findings support the theory that the normal milieu of the epididymis is disturbed by the administration of α-chlorohydrin. A study undertaken by Tsang *et al.* (1981) examined the effects of α-chlorohydrin on epididymal protein secretion, acquisition of sperm surface proteins and fertility of male rats. These findings concluded that α-chlorohydrin interfered with the acquisition of specific proteins by epididymal sperm hereby decreasing the capacity of epididymal sperm to initiate motility and hence loss of fertilizing capacity. Changes in rat sperm motion parameters after the administration of α-chlorohydrin was studied by Toth *et al.* (1992). The percentage of motile sperm was significantly reduced for the 10 mg α-chlorohydrin/kg level. LIN, VCL, VSL and ALH were significantly different for the 10 mg/kg level when compared to the control animals. These studies confirm that α-chlorohydrin affects sperm motion parameters. The anti-fertility effects of α-chlorohydrin was found to produce serious systemic toxic adverse effects, including neurotoxicity when tested at high doses and no safer analogues have been discovered, thus its use in man has been precluded.

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6-Chloro-6-deoxyglucose

One of the chlorodeoxy sugars studied was 6-chloro-6-deoxyglucose (6CDG) and this compound produced complete infertility when administered at high doses (Heitfeld *et al.*, 1979). When 6CDG was withdrawn, complete recovery of normal fertility was achieved. The action of 6CDG was rapid in onset associated with no effect on testes or accessory organ weight and the site of action was the epididymis. The findings of Hinton (1980) that the anti-fertility effects of 6CDG appeared to be similar in action to those of α-chlorohydrin in that both reduce the ability of mature sperm to oxidize glucose and both lower sperm adenosine triphosphate content. As the action paralleled that of α-chlorohydrin, it has been proposed that the compound must be degraded either by glycolysis or a similar metabolic pathway. Tsang *et al.* (1981) reported that the anti-fertility effects of 6CDG were due to the interference with the acquisition of specific proteins by epididymal sperm as shown with α-chlorohydrin. Unfortunately, 6CDG like α-chlorohydrin was found to produce adverse toxic effects when tested at high doses.

Ornidazole

Ornidazole (ONZ), a 5-nitroimidazole derivative clinically used for the treatment of genital tract infections in men has been shown to cause infertility with no effect on the testes (Oberländer *et al.*, 1994). The effect of ornidazole on rat cauda epididymal sperm motion was investigated by Toth *et al.* (1992). The treatment of rats with 200 mg ornidazole/kg/day for 14 days reduced the percentage of motile sperm. The mean VCL, VSL and ALH were reduced by 400 mg ornidazole/kg/day treatment. No changes were observed in sperm count, sperm morphology, testicular and epididymal weights, and

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testicular histology. Thus, ornidazole treatment brings about a rapid, reversible effect on epididymal sperm function. A study undertaken by Oberländer *et al.* (1994) investigated the effects of 200 and 400 mg ornidazole/kg/day on sperm motility and epididymal secretions of rats. The rats became infertile within 10 days of treatment, precluding an action on the testis and a rapid recovery in fertility after drug withdrawal was demonstrated. A significant difference in VSL, VCL and VAP for epididymal sperm was shown for rats fed 400 mg ornidazole/kg/day. The decline in motility parameters suggests that ornidazole acts on the epididymis and the rapidity of action in inducing infertility is compatible with post-testicular action (Oberländer *et al.*, 1994). Ornidazole caused a dose-dependant reduction in certain kinematic parameters of sperm from the cauda epididymidis (Cooper *et al.*, 1997). The fertility of the rats was significantly reduced with the treatment of ornidazole for 14 days. Ornidazole has some potential as a basis for a human contraceptive, but further knowledge of the action of this drug in inducing infertility needs to be pursued.

Sulfonamide

The discovery of sulfonamide seemed promising as fertility was affected at the epididymal level and it was fully reversible (Consentino & Cockett, 1986). Sulfonamide widely used for the treatment of ulcerative colitis was reported to cause reversible infertility in man and the rat (Waites, 1986). Sperm motility was significantly depressed, abnormal sperm morphology was evident and with a longer-term treatment sperm density was shown to decline. These effects were reversed after the drug was withdrawn. These findings suggest

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that the effect may be on later stages of spermatogenesis as well as sperm in the epididymis.

Sulfasalazine

Sulfasalazine widely used in the treatment of inflammatory bowel disease has been associated with reversible male infertility. The anti-fertility action of sulfasalazine was investigated on sperm density and motility of men with stable inflammatory bowel disease (Wu *et al.*, 1989). Male rats administered with sulfasalazine (SASP) produced alterations in the spermiogenic phase of spermatogenesis (Hoyt *et al.*, 1995). SASP did not directly affect sperm motion as the sperm motion characteristics remained unchanged by SASP treatment. Two weeks after SASP treatment was stopped, adverse effects on sperm motion was evident. Changes in sperm quality two weeks after treatment reflect effects that occurred after the sperm entered the epididymis while testicular effects predominated two weeks after treatment was stopped. Some of the side effects associated with the use of sulfasalazine include vomiting, nausea, headache, fever, reticulocytosis, eosinophilia, bronchospasm and peripheral neuropathy (Zaneveld & Waller, 1989). Screening of these compounds are crucial in the hope of finding the most effective and safe agent for the purpose of reversible contraception.

There is a long way to go for the acceptance of a male contraceptive which exerts a reversible anti-fertility action in the epididymis of the male, without affecting *libido* or testicular and secondary sex organs. However, the most obvious advantage of the epididymal approach is its speed of action, any effect on stored sperm could produce

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infertility within days, whereas an effect on the testes would require several weeks (Reyes & Chavarria, 1981). Success in the rational development of new male anti-fertility agents which would act on the epididymis, will depend on a clear understanding of the factors that regulate epididymal function, as well as the role of epididymal secretions in sperm maturation and survival.

1.3   Agents blocking the vas deferens

The vas deferens is a duct through which sperm is transported into the penis, to be emitted during copulation (Berne & Levy, 1993). An agent which would block the vas deferens would essentially prevent the sperm from reaching the ova thereby inhibiting fertilization. Vasectomy, which is the removal of a portion of the vas and/or by vas ligation is a means of permanent male sterility. Relatively simple chemical and physical techniques are available. The chemical techniques prevent sperm transport by hindering vas contractility or by the occlusion of the vas after injection into the lumen (Zaneveld, 1987).

Phenoxybenzamine

Phenoxybenzamine (PBZ), a α-adrenergic blocking agent administered to men resulted in aspermia and the absence of retrograde ejaculation following male orgasm (Homonnai *et al.*, 1984). PBZ inhibited ejaculation by eliminating contractions of the seminal vesicles, the ampulla and the vas deferens. These effects were fully reversed with the cessation of treatment and the reappearance of normal ejaculation. A study undertaken by Amobi & Smith (1995) investigated the male contraceptive action of PBZ on contractions of the

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muscle of the vas deferens. It was found that PBZ readily inhibited contractions in longitudinal muscle of the human vas deferens, but the contractions of circular muscle were substantially resistant to the antagonist. The physiological consequences of the differential inhibition of longitudinal but not circular muscle contraction will be, the disruption of the propulsive function of the vas deferens. The lumen would be constricted which would impede sperm transport by a sphincter-like effect and inhibit sperm emission. These findings suggest that the muscle types of the human vas deferens differs in physiological and pharmacological properties.

Vasal Occlusion

In the field of vasal occlusion, surgical thread was placed into the vas deferens of dogs (Lewy, 1977). No sperm was present in the ejaculate as long as the thread was in place. Removal of the thread resulted in the presence of sperm in the ejaculate which can be attributed to the thread causing blockage of the vas deferens acting as a mechanical plug. A valve implanted into the vas deferens resulted in the blockage thereby restricting sperm from passing through (Lewy, 1977). Numerous devices have been evaluated for their success in blocking sperm transport in the vas deferens which included plugs, clips, valves, injectable polymers, shunts, intra vasal devices, intra luminal devices and intra vasal extra luminal devices (Zaneveld, 1987). A contraceptive drug named Risug which comprises of styrene maleic anhydride (SMA) in a solvent vechile was injected into the vas deferens of male subjects (Guha *et al.*, 1997). The assessment of the contraceptive effectiveness yielded azoospermia in the male subjects with protection against pregnancy. Investigations need to be pursued to find a suitable blocking agent of the vas deferens where *libido* and

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sexual potency are preserved but ejaculation does not occur.

The future of research into male infertility should not be regarded with gloom as encouraging progress has been made thus far in this ever expanding area of research.

1.4   Current contraceptive methods

A large number of compounds have been studied as possible contraceptive agents in the male. Many of these compounds possess anti-spermatogenic or anti-fertilizing capacities, however, their contraceptive action is invariably overshadowed by observations that the compounds may be toxic, result in unpleasant side effects or affect *libido* or sex accessory glands. Thus, there is a need in the field of male fertility regulation for much more fundamental research. Current possibilities of contraception that can be practiced by the male continue to be based on the barrier concept and coitus interruptus (withdrawal). The two barrier methods: the condom and vasectomy both have a permanent place in contraception. The condom is by far the more popular of the two, additionally providing the greatest capacity to protect against sexually transmitted diseases (STDs) and AIDS. However, consistent and correct use is the most important factor in condom effectiveness. While the search for new and safer chemical and hormonal approaches go on, the recent evidence that vasectomy offers a safe surgical option leaves responsible men with some choice to add to the condom (Waites, 1986).

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1.5 N,-N'-bis(dichloroacetyl)-1,8-octamethylenediamine (WIN 18446)

1.5.1 Why WIN 18446?

In the search for a male contraceptive, numerous compounds have been tested which interferes with spermatogenesis, sperm maturation or sperm transport. The compound N'-N-bis(dichloroacetyl)-1,8-octamethylenediamine, commonly known as WIN 18446 (Fig.1.2) was prepared specifically as an amoebicide in the clinical treatment of amebiasis, but displayed anti-spermatogenic activity (Surrey & Mayer, 1961).

$$Cl \quad Cl$$
$$CH - CO - NH - (CH_2)_8 - NH - CO - CH$$
$$Cl \quad Cl$$
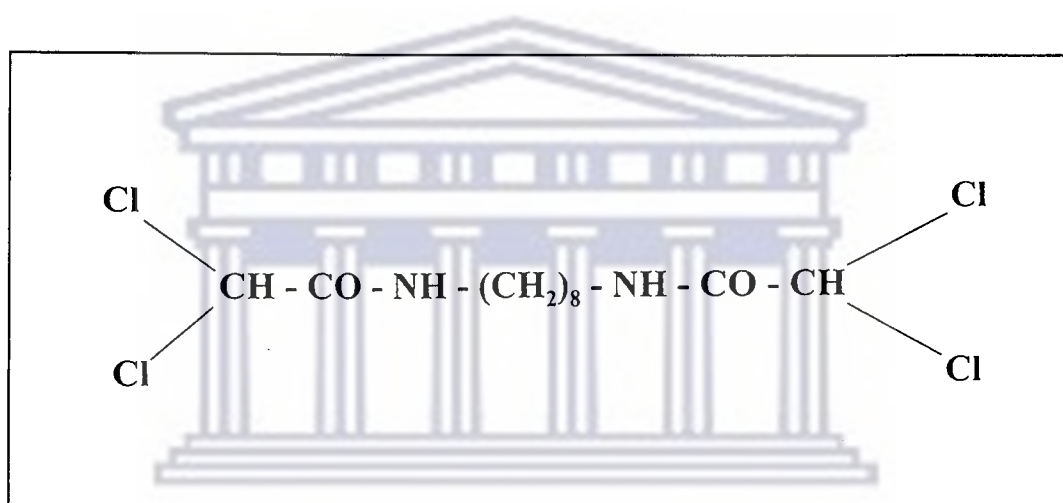
Fig. 1.2:   Chemical formula for N'N,-bis(dichloroacetyl)-1,8-octamethylenediamine (WIN 18446)

The synthesis of the most potent bis(dichloroacetyl)diamines, namely WIN 18446, enabled investigators to examine the biological activity of this potent, reversible anti-spermatogenic agent. This compound was proven to be the most potent of the bis(dichloroacetyl)diamines and one of the less active amoebicidal agents (Surrey & Mayer, 1961).

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1.5.2 Site of action of WIN 18446

The anti-spermatogenic effects of bis(dichloroacetyl)diamines were first reported by Coulston *et al.* (1960). This study undertaken by Coulston *et al.* (1960) evaluated the effects of bis(dichloroacetyl)diamines on the testes of several mammalian species which included: rats, dogs and monkeys. It was found that these relatively non-toxic substances exerted a specific effect on the testes which produced spermatogenic arrest. These effects were completely reversible and the Leydig and Sertoli cells appeared normal under the influence of WIN. These findings were supported by Beyler *et al.* (1961) and Mac Leod (1961) who indicated that the sperm producing cells are the only mammalian tissue elements vulnerable to chemical attack by bis(dichloroacetyl)diamines.

Heller *et al.* (1961) evaluated the effects of WIN 18446 administered to inmates and found that sperm counts approached zero within 11 weeks of treatment, sperm motility also approached zero and abnormal forms were exceptionally high. These effects conclude that the germinal epithelium of the testes is affected by WIN 18446 and its exact mode of action remains to be elucidated. In support of the findings of Heller *et al.* (1961), Mac Leod (1962) also noted a depression in sperm count, inhibition of sperm motility and pronounced aberrations in sperm morphology. A study undertaken by Heller *et al.* (1963) revealed that an inhibition of sperm output in the human ejaculate was evident, with alterations in sperm morphology and the principal point of action in the testes appeared to be the spermatids. Sperm numbers returned to normal after WIN 18446 was withdrawn.

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The effects of WIN 18446 at the ultra-structural level in the guinea pig were investigated in the hope of determining the site of action of WIN 18446 (Flores & Fawcett, 1972). These effects included a distortion in the shape of the acrosome, abnormal cytoplasmic vacuoles of early spermatids, some abnormal sperm retained in the epithelium and spermatocyte numbers were shown to decline. Thus, the effects of WIN 18446 were not confined to the germ cells as multiple large vacuoles appeared early in the Sertoli cells.

The administration of WIN 18446 to rats caused noteworthy metabolic changes in the seminiferous tubules with severe spermatogenic arrest (Kar *et al.*, 1966). The most significant metabolic changes were: a total inhibition of hyaluronidase activity; an increase in glycogen, lactic acid, and lipid concentration; and a decrease in oxygen uptake and bicarbonate level.

Drobeck and Coulston (1962) confirmed the findings that bis(dichloroacetyl)diamines have specificity to affect the testes by a gradual depletion of the germinal epithelium with full recovery attained upon withdrawal of the compound. A prominent characteristic observed was the formation of large multi-nucleated forms termed "fusion bodies" and cap-phase spermatids. The histological findings of Reddy & Svoboda (1967) demonstrated that spermatids and spermatocytes are the cells affected by WIN 18446, thereby preventing further progression to later stages of spermatogenesis. The induction of marked hyperplasia of the interstitial cells was demonstrated due to the cytotoxic effect of WIN 18446 on spermatids and spermatocytes. Several multi-nucleated cells were present in the seminiferous epithelium possibly due to the failure of cytoplasmic division of some of the

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spermatid precursors.

Singh & Dominic (1995) reported that WIN 18446 caused severe atrophic changes in the seminiferous tubules which were in accordance with the findings of Reddy & Svoboda (1967) and Flores & Fawcett (1972). Multi-nucleated spermatidic giant cells were also observed in the seminiferous tubules of mice (Singh & Dominic, 1995). The integrity of the Sertoli cells was affected as cytoplasmic vacuolizations were evident in these cells and these findings supported the findings of Flores & Fawcett (1972) in the guinea pig. The Leydig cells were not altered, however, they were shown to increase in number.

The effects of WIN 18446 on the testes and epididymis of a non-scrotal species, the musk shrew *Suncus murinus* were investigated by Singh & Dominic (1980). Dose-related degenerative changes in the seminiferous tubules were noticed. The failure to induce regressive changes in the Leydig cells, epididymis and accessory sex glands of the musk shrew suggests that the endocrine functions of the testis is unaffected by WIN 18446.

Recently, Zaneveld had success in producing infertility in wolves using the most potent bis(dichloroacetyl)diamine, WIN 18446 (personal communication).

Thus, many studies have been undertaken to elucidate the action of WIN 18446 mainly on the testes as well as on the epididymis. However, a serial mating method was necessary to accurately assess the effects of WIN 18446 on the reproductive structures of male mice (Hershberger *et al.*, 1969). This method which employed male mice and a serial mating

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technique was found to reduce the amount of WIN 18446 required for anti-fertility effects and accurately assess fertility.

These findings of WIN 18446 clearly indicate that the testis is the site of action of this drug and it does not act via the pituitary-testes axis. WIN 18446 was close to being marketed as a male contraceptive, but one drawback associated with this compound was that human consumption of alcohol induced severe vomiting (antabuse effect) which led researchers to terminate interest in this compound as an anti-fertility agent (Jackson, 1972; Bernstein, 1984). These side effects made them undesirable for general clinical application to the human.

1.6   Objectives

The study undertaken examined the effects of N,N'-bis(dichloroacetyl)-1,8-octamethylenediamine (WIN 18446) on the testes, epididymis, selected sperm parameters and fertility of male mice. The evaluation of the contraceptive effect and reversibility of WIN 18446 assessed the following parameters:

- Histology of the testis and epididymis by use of light microscopy
- Epididymal spermatozoa concentration
- Morphological examination of epididymal spermatozoa by use of scanning electron microscopy
- Epididymal spermatozoa kinematics
- Fertility of male mice to impregnate female mice

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WIN 18446 has proved to be an exciting investigative tool in that it affects the function of one portion of the testes, the germinal epithelium while not altering Leydig cell function. The effect of WIN 18446 on the reproductive system of the male mouse has not been studied in great detail. Only one report is available which assesses the effects of 200mg WIN 18446/kg body weight on the mouse for a period of 30 days (Singh & Dominic, 1995). Numerous studies have been undertaken to demonstrate the effects of WIN 18446 on testicular morphology, epididymis morphology, fertility and the chemical composition of the seminiferous tubules. However, no study elucidates the effect of WIN 18446 comprehensively including the effects on sperm characteristics and mating outcome in mice. The purpose of the present study is to demonstrate the effect of 125mg WIN 18446/kg body weight on male CBA mice subjected to prolonged treatment. This study reassesses the reversible anti-spermatogenic effect of WIN 18446 on the male reproductive structures and fertility of male mice, since this compound could prove to be a successful contraceptive for the animal and wildlife population.

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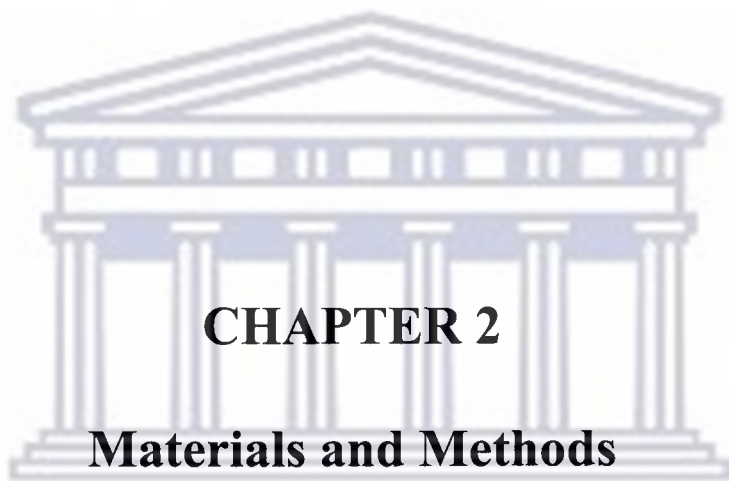
CHAPTER 2

Materials and Methods

CHAPTER 2

Materials and Methods

The activity of WIN 18446 on male reproductive function was examined following the oral administration to male CBA mice. The CBA mice were obtained from the Animal Research Unit of the Medical Research Council [Tygerberg, South Africa]. WIN 18446 was donated by Dr L.J.D. Zaneveld for the experimental procedure at the University of the Western Cape.

2.1 Mice Selection and Husbandry

Adult CBA mice were randomly selected for the determination of the contraceptive effect of WIN 18446 and the reversibility of WIN 18446. The animals were acclimatized to laboratory conditions for one week before experimentation in a housing facility at the University of the Western Cape. These animals were housed five mice per cage prior to the experiment. They were maintained on a diet of commercial mouse feed [Specialist Animal Feeds, Tygerberg] and water was available *ad libitum*. The mouse feed is a diet which is richly supplied with proteins, with fair amounts of fats, fibre, moisture, calcium and phosphorous. The temperature was 22°C to 26°C and the light settings were set for 12 hours light and 12 hours dark in the housing environment.

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2.2   Determination of the Contraceptive Effect of WIN 18446

The intra gastric administration of WIN 18446 to the mice was delivered by means of a rounded metal feeding needle attached to a syringe. A dosage of 125 mg/kg body weight was used as the effective dosage of WIN 18446 for the evaluation of the contraceptive effect and reversibility. Administration of WIN for 42 days was selected since the duration of spermatogenesis in the mouse is 34.5 days [Stages, Version 2.2]. Sperm during all stages in the testes would be targeted as well as sperm transversing the epididymis. Forty randomly selected male CBA mice were used for the determination of the contraceptive effect and reversibility of WIN 18446. Ten male CBA mice served as the control group which received 1% gum tragacanth only and thirty male CBA mice served as the experimental group and received a dosage of 125 mg/kg body weight which was divided into three different treatment periods:

Group 1:   1% gum tragacanth for 42 days

Group 2:   WIN 18446 for 42 days only

Group 3:   WIN 18446 for 42 days followed by a withdrawal of 15 days

Group 4:   WIN 18446 for 42 days followed by a withdrawal of 42 days.

The mice in group two had no withdrawal and the contraceptive effect of WIN 18446 was evaluated while the mice of groups three and four were evaluated for the reversibility of WIN 18446. All these mice were humanely killed 24 hours after the last administration by anaesthetization while placing them in a desiccator with chloroform. It has been shown

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recently that long-term treatment with chloroform for 45 days or more has an effect on sperm concentration and sperm morphology in rabbits (Lohiya *et al.*, 1999). However, the experimental animals in this investigation were only exposed to chloroform for a few minutes. Furthermore, the control and experimental animals were anaesthetized with chloroform in the same way.

2.2.1 Excision of organs

Once the mice were fully anaesthetized, an incision was made through the scrotal skin to expose the reproductive organs. Both the testes and epididymidis were excised, but all excess adipose tissue and blood vessels were removed on a preheated stage of the Zeiss stereo microscope SV8. The testes and epididymidis were rinsed in a solution of Hams-F10 medium [Sigma Chemical Co.] at 32°C and pH 7.4. One epididymis and testis were fixed in Bouins fixative for the histological assessment of the structure of the testis and epididymis. The cauda region of the epididymidis was located, light pressure was applied to this region and sperm were expelled. Cauda epididymal sperm were used for the evaluation of parameters including: sperm concentration, motility assessment and the morphological examination of sperm.

2.2.2 Histological Assessment

The testis and epididymis fixed in Bouins were placed into embedding cassettes and processed in a Histokinette [Type E7326, British American Optical Co. Ltd]. During the processing, the tissues were sequenced through a series of secondary fixation in formalin

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which was followed by a series of dehydration using different alcohol concentrations: 70%, 80%, 90%, 96%, and absolute alcohol. The tissues were then exposed to a clearing process using xylene and finally transferred to molten paraffin wax.

The procedure for processing tissue for light microscopy is outlined below:

Solution	Time
Buffered Formalin	1 hour
70% alcohol	1½ hours
80% alcohol	1½ hours
90% alcohol	2 hours
96% alcohol	2 hours
96% alcohol	2 hours
100% alcohol	2 hours
100% alcohol	1½ hours
Xylene	1½ hours
Xylene	2 hours
Wax	2 hours
Wax	1½ hours

Once the tissues had been sequentially processed, the tissue samples were embedded in wax blocks [Type 4604, Tissue Tek II Embedding Center] and sectioned at 5 μm using a Lab Tek sliding microtome [Reichert Jung, Heidelberg]. The sections were allowed to float in an electro-thermal paraffin section mounting bath and then picked up onto slides. The slides were then incubated [Heraeus incubator] at 80°C for 1 hour to allow the sections to be fixed onto the slides. Staining of these sections was done using a Mayers acid alum haematoxylin [Merck, Darmstadt] and a yellowish eosin [Merck, Darmstadt] stain before the cover slips

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were mounted onto the sections on the slides with E-Z-Mountant-xylene based [Shandon, USA]. The epididymis and testis sections were visually analyzed with a Universal transmitted-light research microscope [Zeiss D-7082, West Germany] with an optovar setting of 1.25. The photographs were captured with an automatic photomicrographic camera [Zeiss MC 63, West Germany]. Black and white films [Ilford FP 4 Plus 125] were used and the DIN / ASA setting was 22 / 125 with a reciprocity setting of 4. The program Stages [Version 2.2] was used to identify the spermatogenic stages in the mouse as outlined by Hess (1990). In essence, this program assists to identify the 12 spermatogenic stages in the mouse by means of testicular histological presentations and tables.

Two dimensions were measured for the six randomly selected seminiferous tubule diameters per testis section for each of the five animals. Thus, an average was obtained for each seminiferous tubule diameter measured. The eyepiece of the Universal transmitted-light research microscope is fitted with a micrometer scale of 0 to 10 units. A stage micrometer (100 x 0.01 = 1mm) [Graticules Ltd, Tonbridge: England] was used to calibrate the ocular micrometer. It was shown that 80 eyepiece units were equal to 350 μm and this equation was used to calculate the seminiferous tubule diameters.

2.2.3 Sperm Concentration

Cauda epididymal sperm from each of the control and experimental mice were evaluated for sperm concentration. A 1:1 dilution of distilled water and single strength gluteraldehyde which was 0.5 ml distilled water and 0.5 ml gluteraldehyde was prepared in eppendorf

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tubes. To each of these distilled water and gluteraldehyde solutions, $10\mu l$ of cauda epididymal sperm was added. The sperm suspension was agitated well using a micropippette. A coverslip was placed onto a haematocytometer and the tip of the micropippette which contained the sperm suspension was held at the junction of the coverslip and the chamber. Fluid filled the chambers due to the capillarity between the coverslip and the chamber. The haematocytometer was placed into a moist, closed petri dish for 5 minutes which ensured that sperm settled before the sperm concentration $(x10^6/ml)$ was determined under a light microscope [Olympus CH, Japan].

2.2.4 Scanning Electron Microscopy

For each of the mice, 20 μl of expelled cauda epididymal sperm was fixed in 1 ml of 2,5% Sörenson phosphate buffered gluteraldehyde. These sperm suspensions were mixed well and fixed for at least 24 hours at 4°C. 15 μl of the buffered gluteraldehyde suspension was added to 0,5 ml of single strength Sörenson phosphate buffer. This sperm suspension was withdrawn into a 1 ml syringe and injected onto a polycarbonate membrane filter with a pore size of 3.0 μm which was placed in the membrane filter holder attached to the syringe. The processing of sperm included washing the sperm with single strength Sörenson phosphate buffer, followed by a post fixation with 1% osmium tetroxide, then washing again and the exposure to a sequential dehydration procedure from 70% to absolute alcohol. The protocol for the processing of tissue for scanning electron microscopy was published by Van der Horst *et al.*, 1989. The filters containing the sperm were placed in small metal baskets and then critical point dried [Hitachi HCP-2 Critical Point Dryer] to remove excess water without distortion. The filters were then sputter coated [Edwards S150B Sputter Coater] for 4 minutes. A Hitachi X-650 Scanning Electron Microanalyzer was used to view

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the specimens and to determine the percentage of normal sperm. The accelerating voltage used was 25 KV. Images were digitally captured on Imageslave for Windows [Version 2.11].

2.2.5 Sperm Motility

The subjective assessment of sperm motility differs greatly between different laboratories and has limitations in that detailed sperm movement characteristics cannot be measured (Van der Horst, 1992). The percentage of motile sperm and the speed of forward progression is assessed on a scale of zero to four with zero indicating non motile sperm and four indicative of an extremely fast forward directed progression of sperm (Windt *et al.*, 1994).

A computer-aided sperm analysis (CASA) system was optimized for objective assessment of the movement characteristics of mouse sperm. The Sperm Motility Quantifier (SMQ) [Version 1.01: Wirsam Scientific, South Africa] is a system which was used for motility analysis of sperm. There are thirteen motility parameters which can be assessed in either the manual or automated mode with the SMQ.

Sperm from the cauda epididymal region was aspirated into a petri-dish containing Hams-F10 medium. The sperm were left to swim out into the medium before the motility patterns were recorded. 5 μl of sperm was pipetted into a motility chamber containing 1 ml of Hams-F10 medium. The chamber was then placed onto a preheated stage of a Zeiss ICM 405 inverted microscope. The cauda epididymal sperm was observed with a 10x objective lens and motility was recorded onto VHS cassettes with a video camera [JVC Avicom

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camera, Model VF-1900E] connected to a video cassette recording system [JVC Model CR-6060ET] attached to the inverted microscope. To ensure that a representative sample of sperm was obtained, sufficient microscopic fields were recorded for at least fifteen seconds to incorporate at least fifty motile sperm per animal. The sperm motility images recorded onto VHS cassettes were analyzed in the manual mode set at 50 Hz for fifty motile sperm for each animal and the motility parameters were assessed. The actual sperm trajectories exhibited of the control and treatment groups were saved as *.TIF files and accordingly printed out for visual inspection.

2.3   Determination of the number of pregnant females

Twenty randomly selected CBA mice were used for the assessment of the ability of the dosed mice to impregnate virgin female mice. The male mice were divided into four groups which consisted of five mice per group and was dosed for the stipulated periods:

Group 1:   42 days of 1% gum tragacanth

Group 2:   42 days of WIN 18446

Group 3:   42 days of WIN 18446 followed by 15 days of withdrawal

Group 4:   42 days of WIN 18446 followed by 42 days of withdrawal

When the treatment periods had elapsed, male fertility was assessed by placing each dosed male with two, virgin female CBA mice and allowed to mate for two weeks. A male mouse was considered fertile if he impregnated any of the females, with which he was housed. Following this period of pairing, the male mice were discarded without further examination. The female mice were monitored to establish whether they were impregnated

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by the males and this continued until the pups were born.

Statistics

All data were statistically analyzed with a computer software package Medcalc [Version 3.00, Belgium] and ANOVA was performed in conjunction with the Student-Newman-Keuls test for all pairwise comparisons where $P<0.05$ was considered significant.

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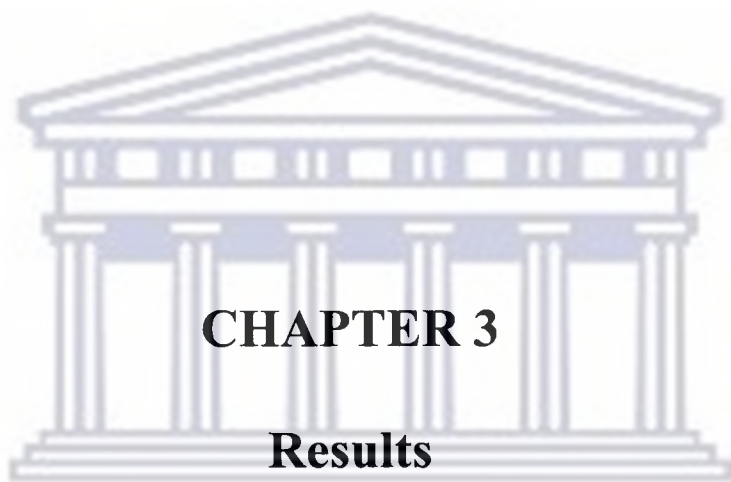
CHAPTER 3

Results

CHAPTER 3

Results

3.1   Histological Assessment

3.1.1 Testis

The histologic examination of the control mouse testis showed the normal pattern of the testis with an orderly arrangement of germ cells in the seminiferous epithelium. Figures 3.1 and 3.2 illustrate full spermatogenic activity in the seminiferous tubules of the control group. The spermatogenic cycle in the mouse seminiferous epithelium consists of 12 stages of which many of these stages were identifiable in the control group. Cross sections of seminiferous tubules were randomly selected in order to recognize the various stages of spermatogenesis in the mouse. The different spermatogenic stages observed were IV, V, VI, VII, VIII, X, XI and XII. Stage XII was identified in many seminiferous tubules and a prominent feature was the presence of meiosis and the abundance of pachytene spermatocytes. Fig 3.2 depicts stage VI, Fig. 3.3 depicts stage VIII and Fig. 3.4 depicts stage XII of spermatogenesis.

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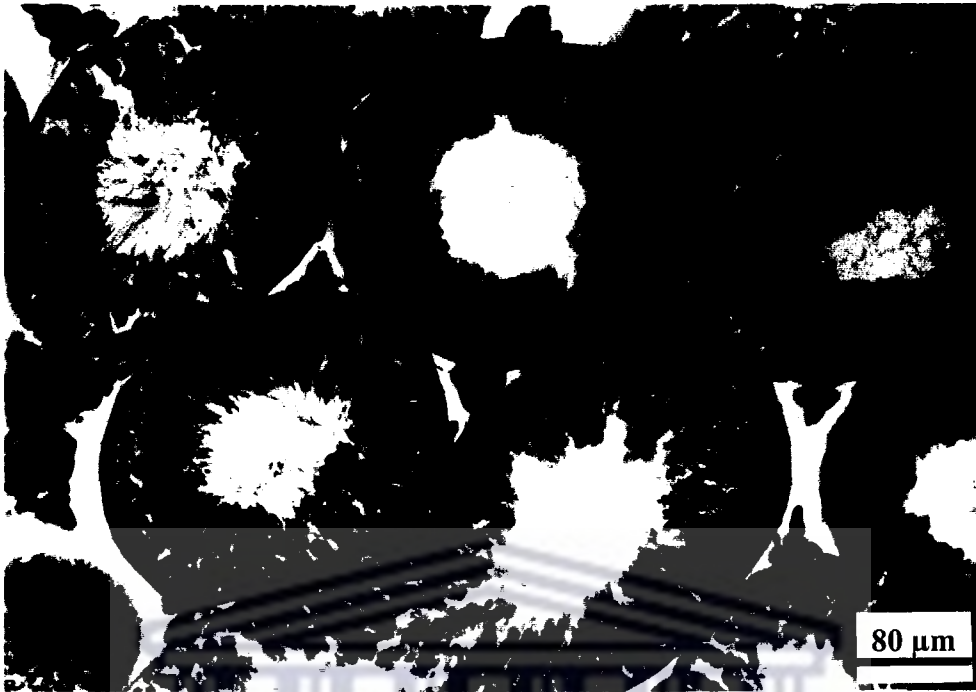
Fig. 3.1: Testicular section of a control mouse illustrating normal spermatogenesis. X190

Fig. 3.2: Testicular section of a control mouse illustrating stage VI of spermatogenesis. X480

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Fig. 3.3: Testicular section of a control mouse illustrating stage VIII of spermatogenesis. X 480

Fig. 3.4: Testicular section of a control mouse illustrating stage XII of spermatogenesis. X480

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The treatment of mice with WIN for 42 days produced a profound impact on spermatogenesis as illustrated in Fig. 3.5 and Fig. 3.6. The spermatogenic cells exhibited severe degenerative changes in most of the seminiferous tubules. Several multi-nucleated cells appeared in the lumen of the seminiferous tubules and the number of sperm within the tubules was reduced. In a few tubules, sperm were found in the lumen of the seminiferous tubules. Various degrees of vacuolization were evident in most of the seminiferous tubules. A high degree of disruption was observed in the seminiferous tubules of the WIN treated group. Thus, it was impossible to identify the various stages in the cycle of the seminiferous epithelium in both the "vacuolated tubules" as well as ones where some spermatogenic cells could be identified.

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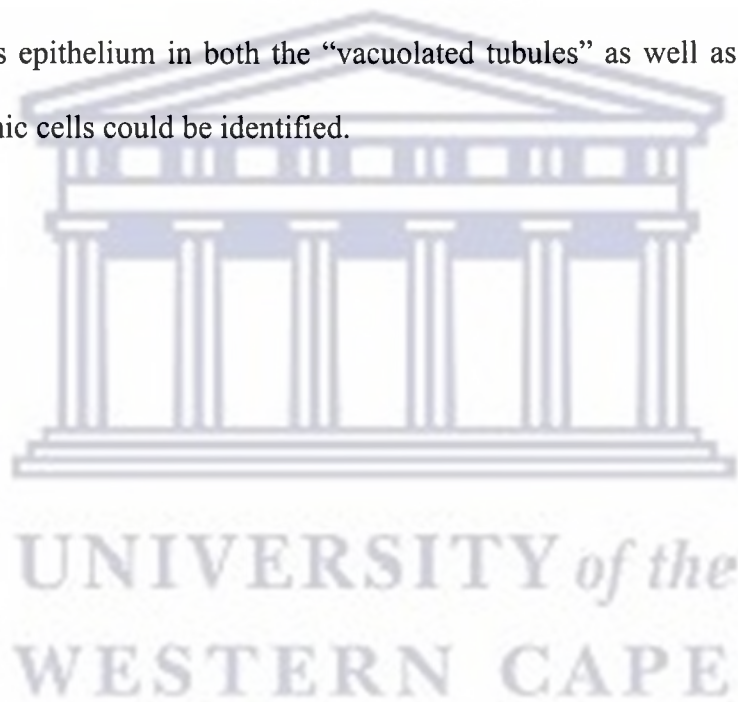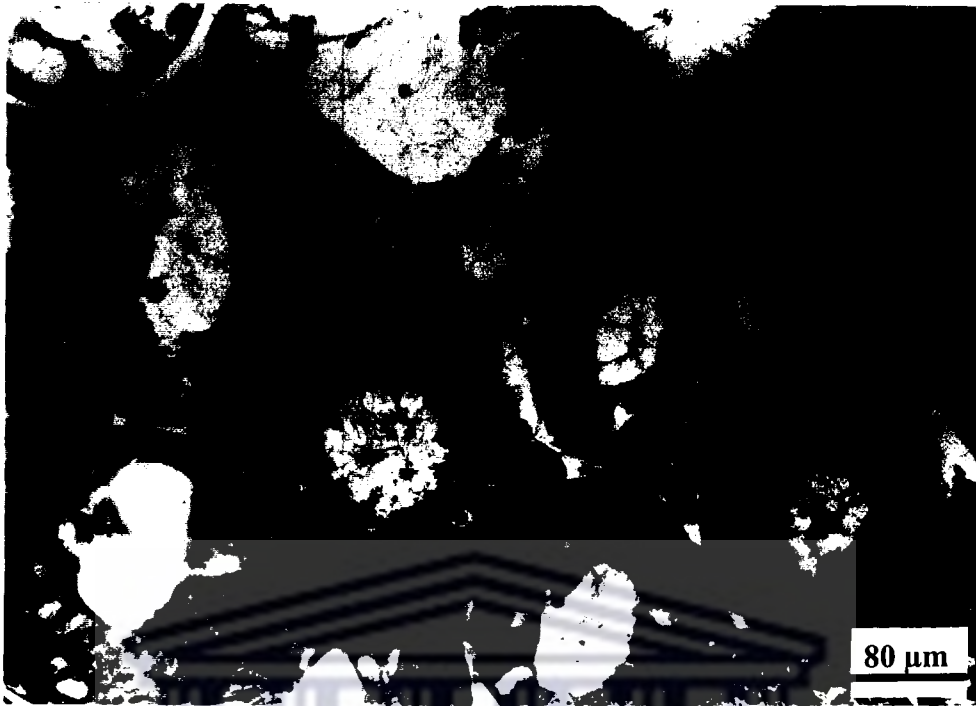
Fig. 3.5: Testicular section of a WIN treated mouse after 42 days. Note vacuolization in most of the seminiferous tubules. X190

Fig. 3.6: Testicular section of a WIN treated mouse after 42 days. Note the presence of multi-nucleated cells and vacuolization. X480

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Mice treated for 42 days with WIN followed by 15 days of withdrawal exhibited recovery of spermatogenesis as shown in Fig. 3.7 and Fig. 3.8. Sperm were present in the lumen in the seminiferous tubules where spermatogenesis progressed. Multi-nucleated cells were still identifiable in some tubules. The degree of vacuolization was reduced as recovery of spermatogenesis progressed and the majority of seminiferous tubules displayed a gradual repopulation of the germinal epithelium. Severely depleted tubules were still present, but considerably less of these tubules were observed. The seminiferous tubules displayed a combination of stages which made it difficult to discern the exact stage in the cycle of the seminiferous epithelium. One stage reasonably assessed from a few cross sections of the testis was stage X. It was clear that spermatogenesis was returning, but 15 days was not sufficient for full recovery of spermatogenesis.

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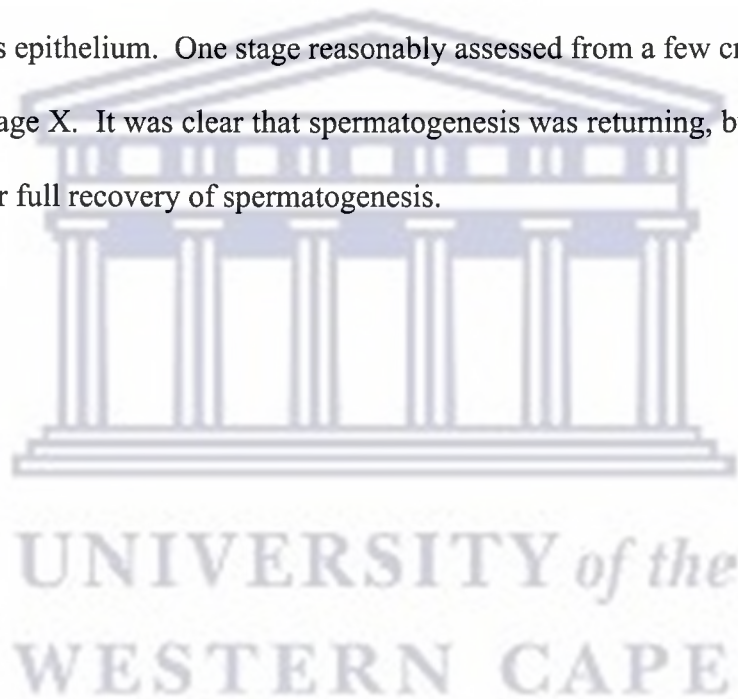
Fig. 3.7: Testicular section of a WIN treated mouse followed by 15 days of withdrawal.

Large dark areas close to the lumen represent multi-nucleated cells. X190

Fig. 3.8: Testicular section of a WIN treated mouse followed by 15 days of withdrawal.

Note the dark areas which represent the multi-nucleated cells. X480

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The mice treated with WIN for 42 days followed by 42 days of withdrawal exhibited a marked recovery of spermatogenesis as shown in Fig. 3.9 and Fig. 3.10. However, spermatogenesis recovery was slow in some tubules as a few partially depleted seminiferous tubules were still identifiable. The degree of vacuolization was drastically diminished and very few multi-nucleated cells were observed in a few seminiferous tubules. Generally, the morphology of the germinal epithelium was normal in most of the tubules when judged by Stages [Version 2.2] (Refer to Materials and Methods). Various spermatogenic stages were identified in the tubules and Fig. 3.10 represents stage III, Fig. 3.11 represents stage VI and Fig. 3.12 represents stage X. Thus, spermatogenesis is normal within most of the seminiferous tubules.

In all the WIN treated and withdrawal groups, the interstitial (Leydig) cells were apparently not affected by WIN and retained their normal histology. It therefore appears that 42 days of WIN treatment produces vast disruption of spermatogenesis. However, 42 days of withdrawal of WIN indicate almost full recovery of spermatogenesis.

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Fig. 3.9: Testicular section of a WIN treated mouse followed by 42 days of withdrawal. Note almost full recovery of spermatogenesis. X190

Fig. 3.10: Testicular section of a WIN treated mouse followed by 42 days of withdrawal illustrating stage III of spermatogenesis. X480

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Fig. 3.11: Testicular section of a WIN treated mouse followed by 42 days of withdrawal illustrating stage VI of spermatogenesis. X480

Fig. 3.12: Testicular section of a WIN treated mouse followed by 42 days of withdrawal illustrating stage X of spermatogenesis. X480

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Table 3.1: The effect of WIN on seminiferous tubule diameters. Values represent

means ± SEM

Parameter	Control	WIN	WIN15dw	WIN42dw
Seminiferous tubule diameter (μm)	228.0 ± 4.87	190.9 ± 4.25*	203.6 ±3.56*	217.0 ±2.41
Range	175 to 284	155 to 252	166 to 243	195 to 245

* Statistically significantly different from the control and 42day withdrawal (42dw)

groups (P<0.05)

Table 3.1 represents the seminiferous tubule diameters for the various treatment groups. Treatment of mice for 42 days of WIN produced a significant decline (P<0.05) in seminiferous tubule diameters from 228.0 μm to 190.9 μm. After 15 days withdrawal of WIN, the seminiferous tubule diameters increased to 203.6 μm which was significantly different (P<0.05) from the other three groups. The seminiferous tubule diameters increased to 217.0 μm after 42 days withdrawal of WIN, but were significantly different (P<0.05) from the WIN and WIN 15 days withdrawal groups. The trend indicates that the seminiferous tubule diameters returned to normal. However, if the withdrawal period was extended, the seminiferous tubule diameters would in all likelihood tend more closely towards the control values.

3.1.2 Epididymis

The histology of the epididymis in all the treatment groups was comparable with that of the control group as shown in Fig. 3.13 to Fig. 3.16. The epididymis in the WIN treated mice presented normal histology. The epithelial cells lining the tubules of the epididymis were not affected and the tubular lumina was filled with sperm. Particularly the principal cells and stereo-cilia of the control group and the treatment groups appeared morphologically similar.

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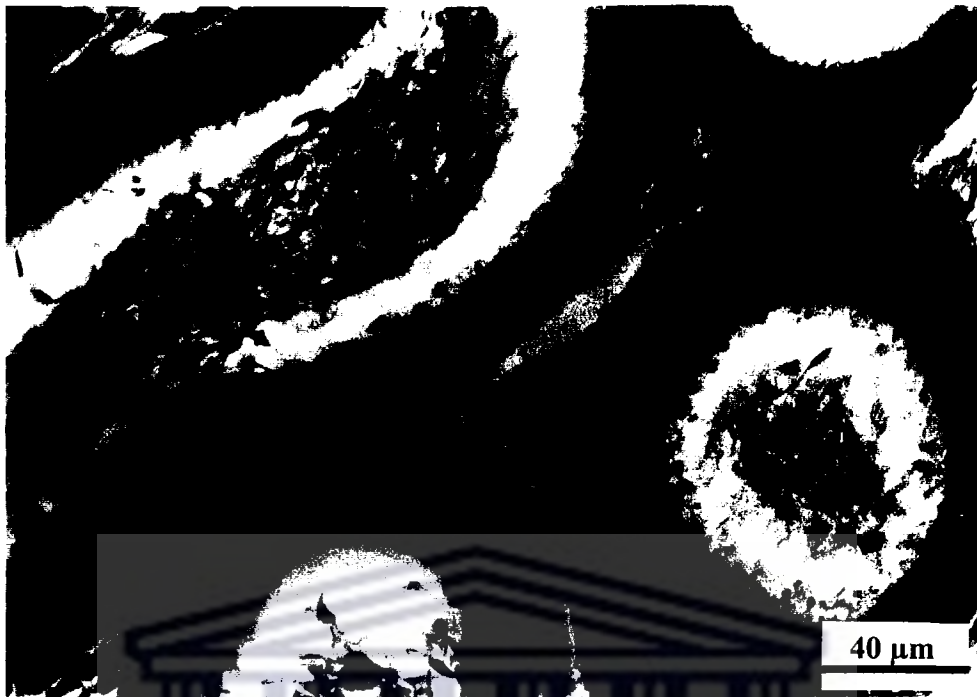
Fig. 3.13: Epididymis of a control mouse illustrating normal histology. X480

Fig. 3.14: Epididymis of a WIN treated mouse. X480

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Fig. 3.15: Epididymis of a WIN treated mouse followed by 15 days of withdrawal. X480

Fig. 3.16: Epididymis of a WIN treated mouse followed by 42 days of withdrawal. X480

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3.2 Sperm Concentration

Fig. 3.17 depicts the sperm concentrations ($\times 10^6$/ml) expressed as means $\pm$ SEM of the cauda epididymidis for the control and treatment groups. The examination of cauda epididymal sperm concentrations showed a significant decrease (P<0.05) in the mice which received WIN for 42 days as compared to the control group which received gum tragacanth only. After 42 days WIN treatment, there was a significant decline in sperm concentration from 60.57×10^6/ml to 13.36×10^6/ml. A withdrawal period of 15 days after WIN treatment yielded a slight recovery in sperm concentration which was still significantly lower than the control group. The sperm concentration of the WIN 42 day withdrawal group approached the normal concentration, but differed significantly from the WIN and the WIN 15 day withdrawal groups. It therefore appears that sperm concentration had essentially returned to normal. The trend indicates that there was a clear recovery of sperm concentration and if the withdrawal period was extended, sperm concentration would in all likelihood fully return to normal.
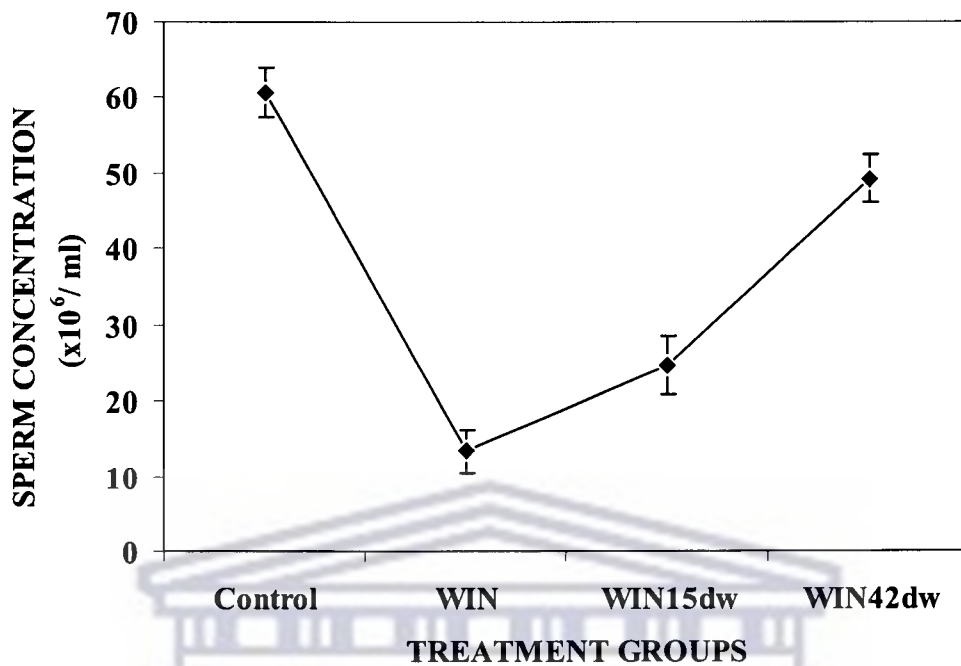
Fig. 3.17: Line graph depicting the sperm concentration ($x10^6$/ml) for the control and treatment groups

3.3 Sperm Morphology

The percentage of morphologically normal cauda epididymal sperm was calculated by manually counting one hundred sperm per animal using the scanning electron microscope. These results are illustrated in Table 3.2 and are represented as the means ± SEM. The mice which received WIN for 42 days only and WIN for 42 days followed by 15 days of withdrawal illustrated a statistically significant difference ($P<0.05$) in percentage normal sperm morphology as compared to the control group. However, the mice which received WIN for 42 days followed by a 42-day period of withdrawal did not show any significant

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difference (P>0.05) in sperm morphology from the control group.

The highest percentage of abnormal forms occurred in the 42-day WIN treatment group (50.6%) followed by the WIN and 15 day withdrawal group (41.0%). The most common abnormal form of sperm observed was deformed heads, detached heads, kinks in the mid-piece and retention of the cytoplasmic droplets. The morphologically normal mouse sperm are illustrated in Fig. 3.18 and Fig. 3.19 as captured using the scanning electron microscope. Some morphological abnormal forms of mouse sperm as captured by the scanning electron microscope are illustrated in Fig. 3.20 and Fig 3.21. The average pore size of the polycarbonate filters is 3.0 μm in diameter and serves as a scale in all the scanning electron micrographs.

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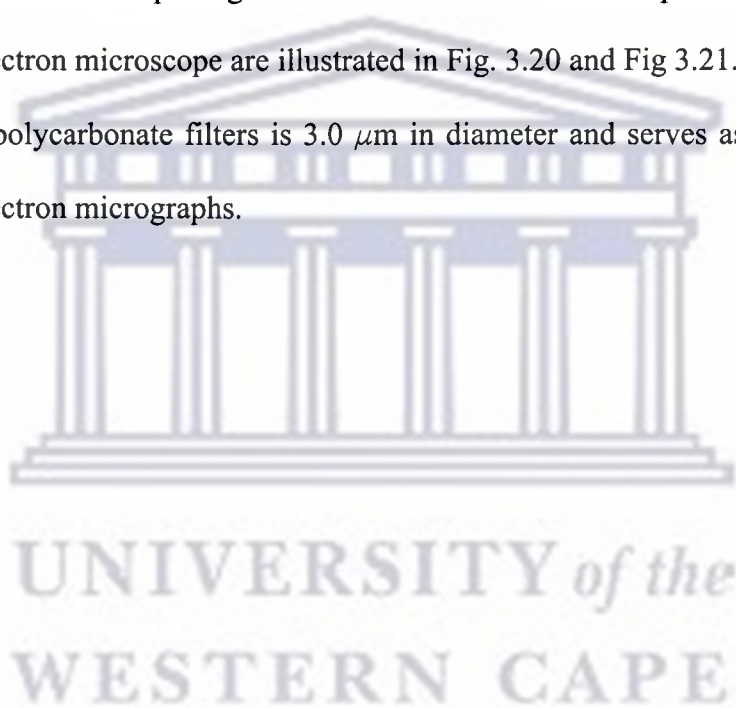
Table 3.2:   Percentages of normal epididymal sperm as calculated by means of scanning electron microscopy. Data is represented as means ± SEM

Parameter	Control	WIN	WIN15dw	WIN42dw
Mean ± SEM	78.4 ± 3.12	49.4 ± 2.38*	59.0 ± 3.61*	70.6 ± 2.84
Range	69 to 87	43 to 57	51 to 70	63 to 80

* Statistically significantly different from the control and 42day withdrawal (42dw) groups (P<0.05)

Fig. 3.19: Normal cauda epididymal mouse sperm of the control group as viewed with the scanning electron microscope. Note the presence of an abnormal sperm with its retained cytoplasmic droplet.

Fig. 3.20: Abnormal cauda epididymal mouse sperm of the WIN treated group as viewed with the scanning electron microscope. Note the presence of the retained cytoplasmic droplet and the deformed head.

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Fig. 3.21:   Abnormal cauda epididymal mouse sperm of the WIN 15 day withdrawal

group as viewed with the scanning electron microscope. Note the deformed

head and the kink in the mid-piece.

3.4   Sperm Motility

Cauda epididymal sperm motion parameter measurements are represented in Table 3.3 and explanations of these parameters are illustrated in Appendix 6.1. The result of each of the three treatment groups was compared to the control group and statistically significant differences (P<0.05) were indicated. No significant differences (P>0.05) were observed between groups for the following parameters: VCL, MAD and CURV when compared to the control group. Comparison of the treatment groups to the control group showed statistically significant differences (P<0.05) for kinematic parameters VSL, LIN, mnALH, BCF, VAP, WOB and STR. DNC and mxALH were significantly different (P<0.05) when the WIN treated group was compared to the control group.

Table 3.4 compares significant differences (P<0.05) which exists between the treatment groups. The following parameters: VSL, LIN, VAP, WOB and STR exhibited significant differences (P<0.05) between the WIN group and the WIN 42 days withdrawal group. Significant differences (P<0.05) existed between the WIN 15 days withdrawal and the WIN 42 days withdrawal groups for VSL, LIN, VAP, WOB and STR. Significant differences (P<0.05) were identified among all the treatment groups for mnALH. DNC and mxALH displayed significant differences (P<0.05) between the WIN group and the WIN 42 days withdrawal group.

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Table 3.3: Comparison of sperm motion parameter measurements of the three treatment groups to the control group. Data represented as means± SEM

Motion Parameter	Control Group	WIN	WIN5dw	WIN42dw
VCL (μm/s)	268.61 ± 22.22	279.66 ± 4.53	269.92 ± 24.75	260.33 ± 3.57
VSL(μm/s)	100.99 ± 11.33	155.60 ± 4.50*	148.10 ± 2.18*	109.33 ± 7.13
LIN (%)	38.99 ± 3.37	56.24 ± 0.81*	57.58 ± 0.98*	42.41 ± 2.79
mnALH (μm)	7.86 ± 0.78	14.15 ± 0.31*	11.84 ± 0.24*	7.41 ± 0.74
mxALH (μm)	19.75 ± 2.63	26.74 ± 0.16*	22.81 ± 0.49	18.25 ± 1.65
BCF (Hz)	20.48 ± 3.90	33.37 ± 1.04*	33.84 ± 1.55*	27.59 ± 1.21*
DNC (μm^2/s)	4533.5±893.61	8067.2±61.84*	6261.2±175.26	4355.5±795.92
VAP (μm/s)	143.53 ± 17.47	269.22 ± 2.93*	244.91 ± 4.36*	147.48 ± 9.38
WOB (%)	53.97 ± 2.58	96.71 ± 0.80*	94.70 ± 1.79*	56.14 ± 1.78
STR (%)	72.18 ± 5.53	57.98 ± 1.06*	60.89 ± 0.64*	74.55 ± 3.15
MAD(radians)	1.84 ± 0.03	1.91 ± 0.02	1.95 ± 0.04	1.91 ± 0.06
CURV	0.49 ± 0.02	0.53 ± 0.01	0.53 ± 0.01	0.52 ± 0.01

* Statistically significant (P<0.05)

Table 3.4:   Statistical comparison of kinematic parameters of the various treatment

groups. Data represented as means ± SEM

Motion Parameter	WIN vs WIN15dw	WIN vs WIN42dw	WIN15dw vs WIN42dw
VSL (μm/s)		P<0.05	P<0.05
LIN (%)		P<0.05	P<0.05
mnALH (μm)	P<0.05	P<0.05	P<0.05
mxALH (μm)		P<0.05	
DNC (μm^2/s)		P<0.05	
VAP (μm/s)		P<0.05	P<0.05
WOB (%)		P<0.05	P<0.05
STR (%)		P<0.05	P<0.05

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Fig. 3.22 to Fig.3.25 are graphic representations of the kinematic parameters of sperm for the various treatment groups. The three measures of sperm vigour (VCL, VSL and VAP) exhibited an increase for the WIN treated group followed by a decline for the WIN 15 days withdrawal group (Fig. 3.22). A further decline for the WIN 42 days withdrawal group was elicited for the measures of sperm vigour. VCL did not display any significant differences (P>0.05) between treatment groups when compared to the controls. VSL and VAP exhibited a significant rise for the WIN group when compared to the control group, with a decline for the WIN 15 days withdrawal group. A further decline was evident for the WIN 42 day withdrawal group. While sperm velocity did not increase, both LIN and WOB suggest that sperm are swimming along a straighter path than in the control group.

The measures of the pattern of sperm motion (LIN, WOB and STR) were illustrated in Fig. 3.23. WOB displayed a sharp rise for the WIN treated group followed by a decline for the WIN 15 days withdrawal group. An even sharper decline for the WIN 42 day withdrawal group existed. LIN followed a similar trend as WOB, an increase followed by a decline for the WIN 42 day withdrawal group. In contrast to this pattern, STR displayed a decline for the WIN group followed by a increase for the WIN 15 day withdrawal group. A further rise existed for the WIN 42 day withdrawal group. These parameters (LIN, WOB and STR) further emphasize that the sperm trajectories become straighter (LIN increases), but more irregular (STR declines).

mnALH and mxALH displayed a similar pattern as that of sperm vigour as shown in Fig. 3.24. An increase for the WIN group, followed by a decline for the WIN 15 day

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withdrawal group was exhibited. This was followed by a further decline for the WIN 42 day withdrawal group. BCF exhibited an increase for the WIN group followed by a further increase for the WIN 15 day withdrawal group (Fig. 3.24). A decline was observed for the WIN 42 day withdrawal group for BCF.

DNC displayed a sharp increase for the WIN group, followed by a significant decline for the WIN 15 day withdrawal group. A further decline was observed for the WIN 42 day withdrawal group (Fig. 3.25).

Fig. 3.26 to Fig. 3.29 are representative of the control and each of the treatment groups depicting actual sperm trajectories. Sperm trajectories illustrated in Fig. 3.26 and Fig. 3.29 display forward progression with a much more regular wave-motion pattern. The sperm trajectories illustrated in Fig. 3.27 and Fig. 3.28 show more variation and irregularities representing a more straight-line sperm motion pattern. These straight-line patterns indicated in Fig. 3.27 and Fig. 3.28 are marked by arrows. Combinations of irregular straight-line sperm trajectories and regular wave-like sperm trajectories are shown in Fig. 3.27. Visual inspection of the actual sperm trajectories may also further assist in establishing differences/ similarities among the control and treatment groups. It is therefore clear that both quantitative parameters of sperm motion kinematics and inspection of sperm trajectories (qualitative) are complementary in establishing differences/similarities amongst the control and treatment groups.
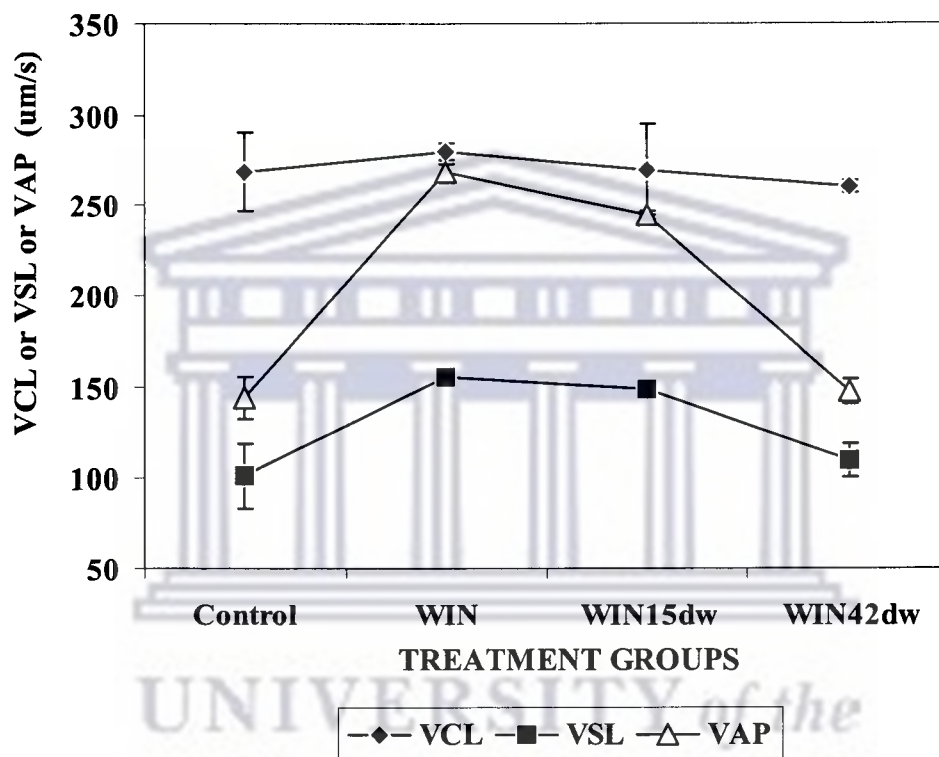
Fig. 3.22:   Kinematic parameters (VCL, VSL and VAP) of cauda epididymal sperm of

the control and treatment groups
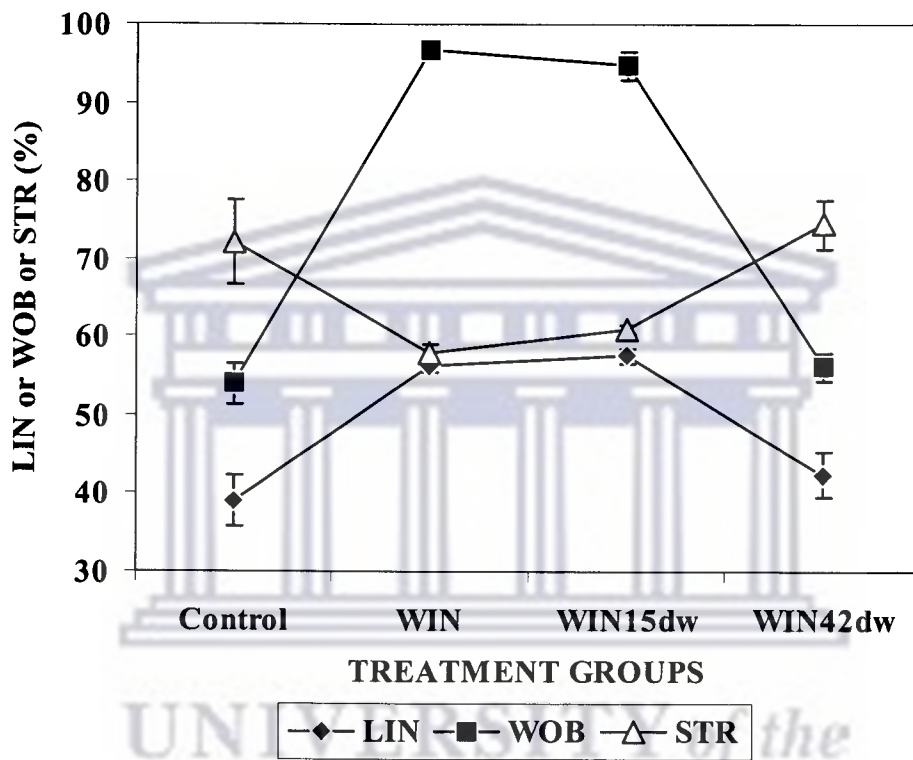
Fig. 3.23: Kinematic parameters (LIN, WOB and STR) of cauda epididymal sperm of the control and treatment groups
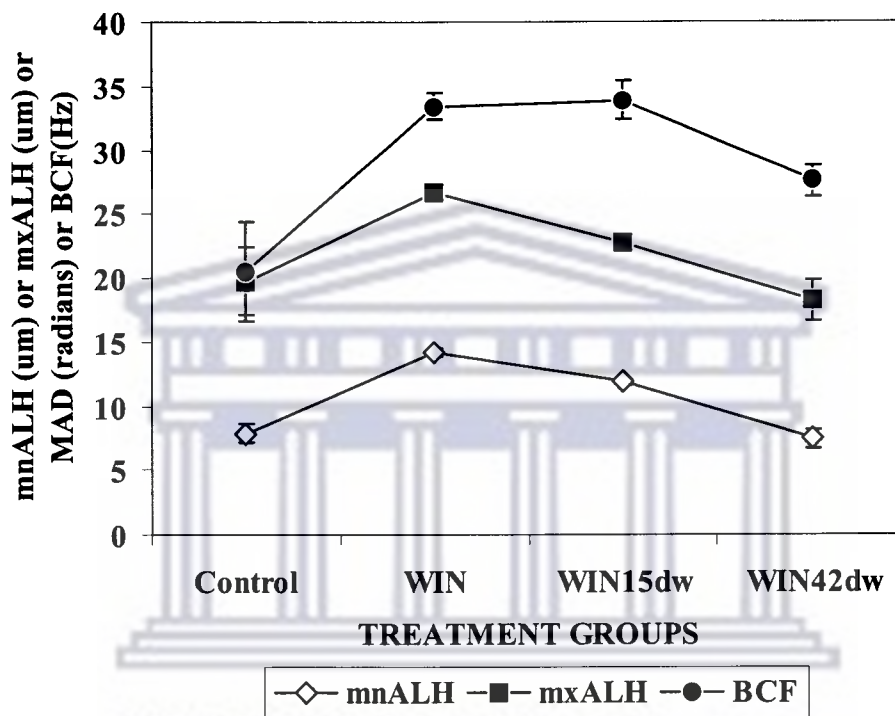
Fig. 3.24:   Kinematic parameters (mnALH, mxALH and BCF) of cauda epididymal

sperm of the control and treatment groups
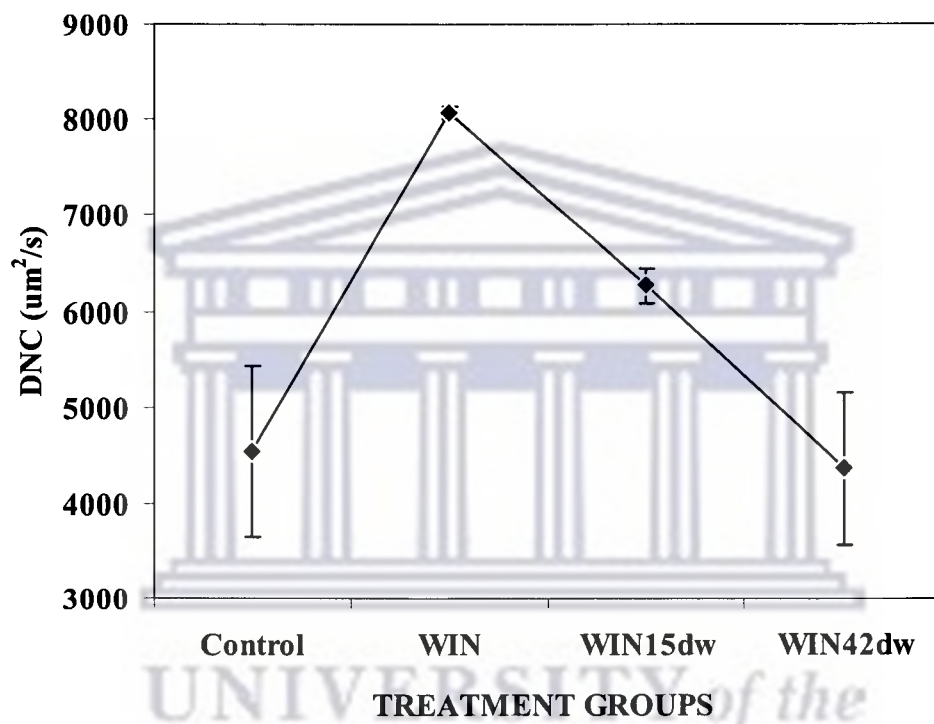
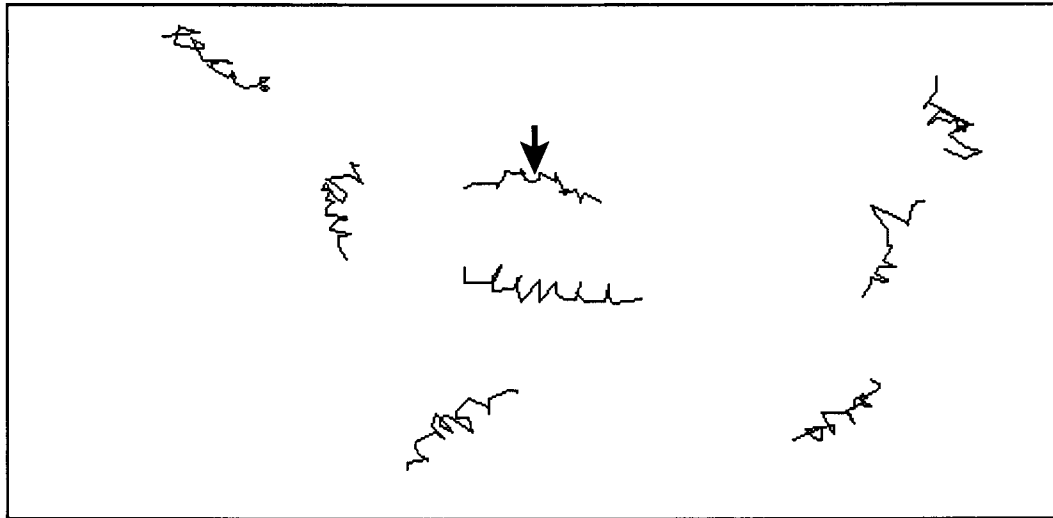Fig. 3.25:   Kinematic parameter (DNC) of cauda epididymal sperm of the control and
treatment groups

Fig. 3.26: Sperm motion tracks collected from the control group. One straight-line sperm trajectory represented by an arrow.

Fig. 3.27: Sperm motion tracks collected from the WIN treated group. Straight-line sperm trajectories are indicated by arrows.
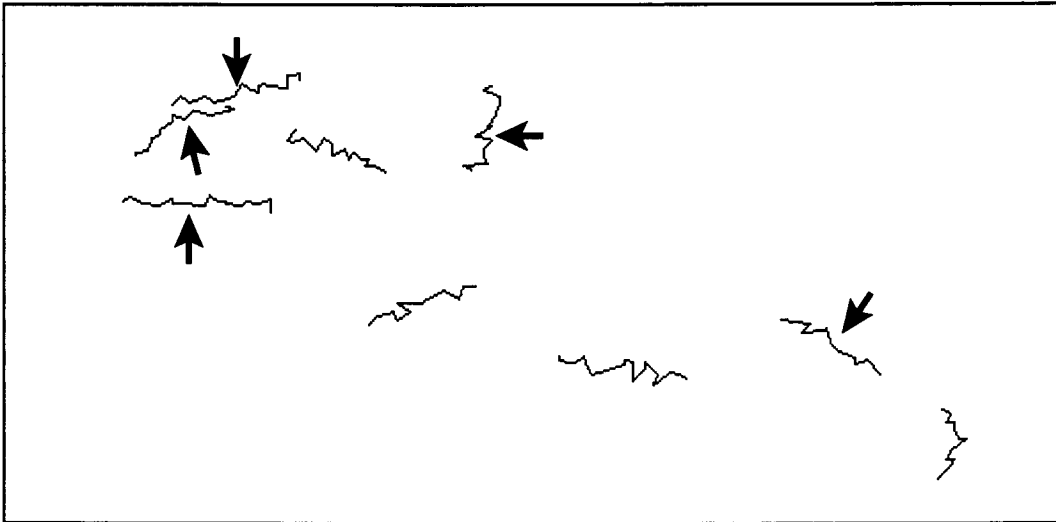
Fig. 3.28:   Sperm motion tracks collected from WIN 15 days withdrawal group. Straight-line sperm trajectories are indicated by arrows.
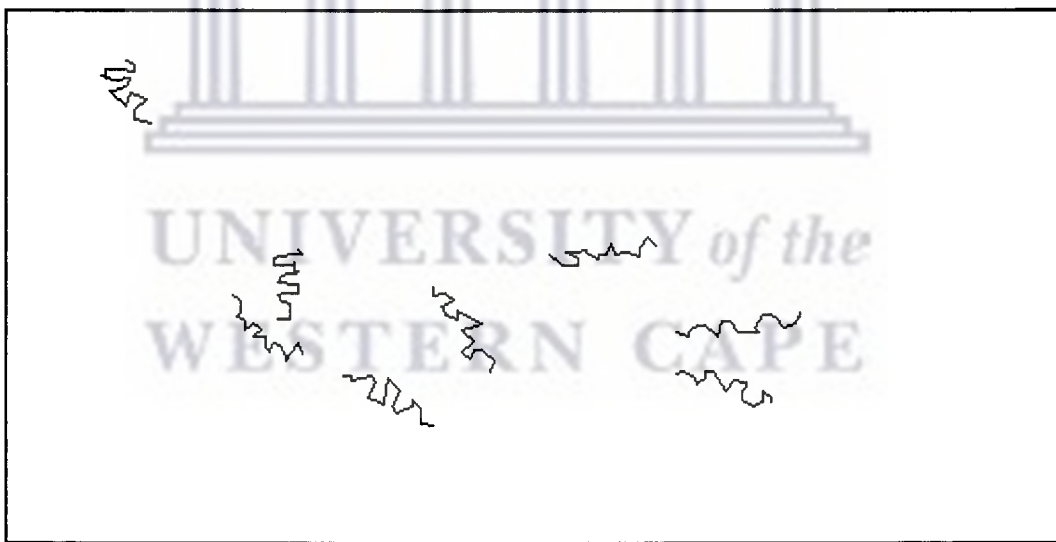
Fig. 3.29:   Sperm motion tracks collected from the WIN 42 days withdrawal group. Note the absence of straight-line sperm trajectories.

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3.5   Effect on males to impregnate females

The percentage of females which gave birth to pups and the male fertility indices are shown in Table 3.5. Females paired with WIN only treated males showed a significant decline (P<0.05) of 65.29% in the pregnant females which gave birth to pups. WIN 15 days withdrawal treated males paired with females resulted in a significant decline (P<0.05) of 42.43% in pregnant females which gave birth to pups as compared to the control group of males. Males from the WIN 42 day withdrawal group paired with females resulted in a lower pregnancy rate of 15.29% when compared to the control group. These results are graphically illustrated in Fig. 3.30. The male fertility index was higher than the pregnant females which gave birth to pups in all the groups. The percentage of fertile males and the females which gave birth to pups in Fig.3.30 was significantly reduced (P<0.05) for the WIN group and the WIN 15 days withdrawal group when compared to the control and WIN 42 day withdrawal groups.
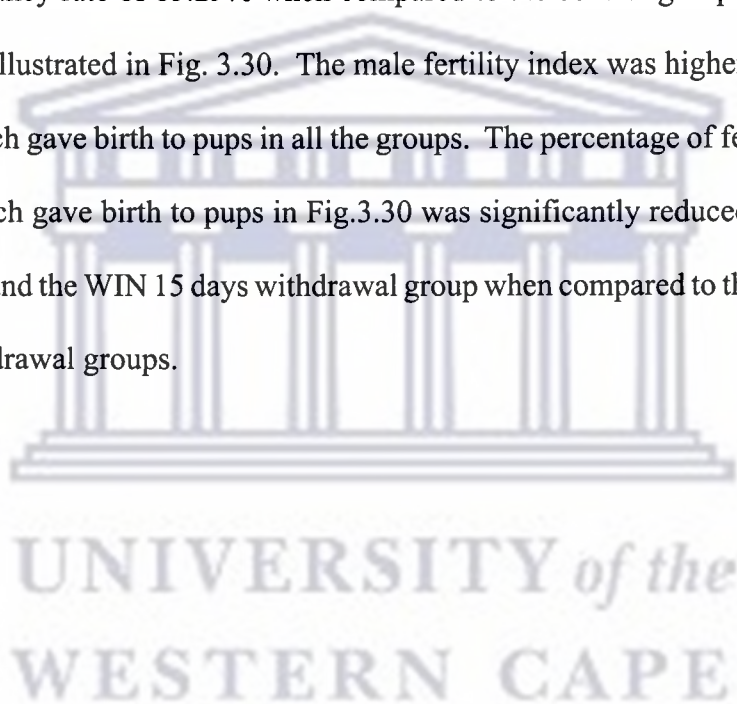
Table 3.5:   Data representing the percentage of females which gave birth to pups (female fertility index) and the male fertility index expressed as a percentage. All treatment groups are compared to the control group and shown as means ± SEM

Parameter	Control	WIN (WIN 0 - 14)	WIN5dw (WIN 15 - 29)	WIN42dw (WIN 42 - 56)
Females which gave birth to pups (%) [a]	85.29 ± 5.70	20.0 ± 12.25*	42.86 ± 17.00*	70.0 ± 20.0
Male fertility index (%)[b]	100.0 ± 0.00	40.0 ± 24.50*	57.14 ± 20.20*	80.0 ± 20.0

* Statistically significantly different from the control and 42day withdrawal (42dw) groups (P<0.05)

[a] Number of pregnant females which gave birth to pups expressed as a percentage equals the female fertility index = (No. of females pregnant / no. of females placed with male) x 100

[b] Male fertility index = (No. of males that became sire / no. of males placed with females) x 100

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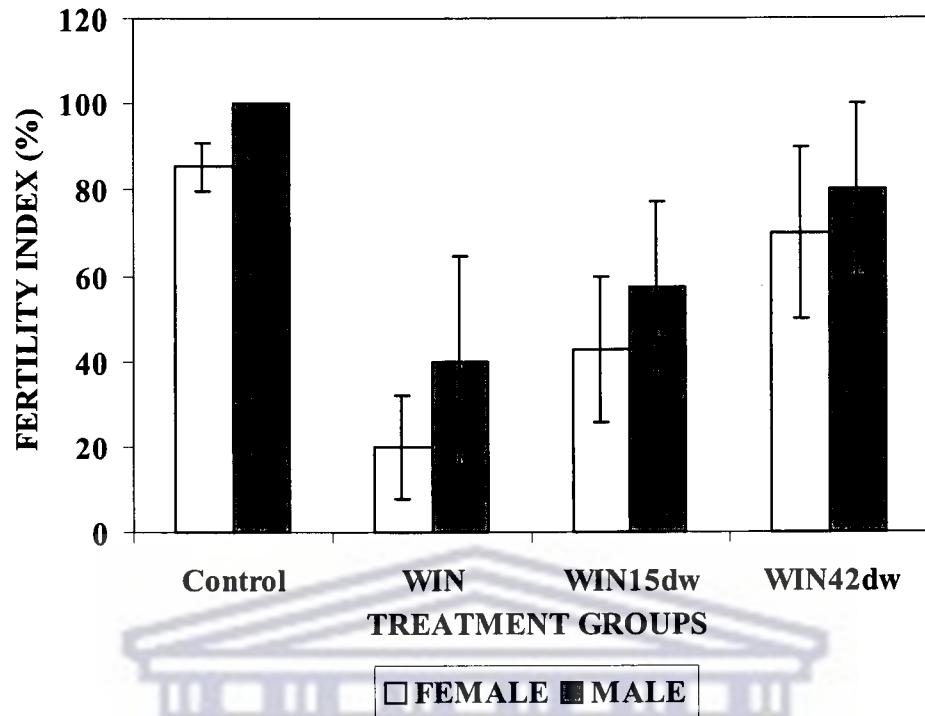
Fig. 3.30:   Bar graph depicting the male fertility index and the percentage pregnant

females of the control and the treatment groups

CHAPTER 4

Discussion

CHAPTER 4

Discussion

4.1 Histological Assessment

The specificity of WIN is very striking as it affects only the testes, essentially the germ cells. The investigation in mice revealed that WIN has a profound impact on the spermatogenic cells while the Leydig cells were unaffected. The observation that severe degenerative changes occurred in most of the seminiferous tubules of the WIN 42 day treated group is consistent with the findings in the rat (Beyler *et* al., 1961; Drobeck & Coulston, 1962; Kar *et al.*, 1966), guinea pigs (Flores & Fawcett, 1972), man (Heller *et al.*, 1963), mouse (Singh & Dominic, 1995) and musk shrews (Singh & Dominic, 1980). However, there was variation in the dosage of WIN used by various researchers which ranged from 62.5 to 500 mg/kg body weight as well as the duration of the dosage of WIN for the various mammalian species. In this investigation, a dosage of 125 mg/kg was used as the effective dose of WIN to exert degenerative changes on spermatogenesis. It is however clear that in all the mammalian species, the testes was the target site for the action of WIN and severe degenerative changes was observed. The various seminiferous tubules produce sperm in different, successive waves, and as seen in the histologic sections some tubules were unaffected by WIN. While some of the seminiferous tubules were not affected by WIN, the majority of tubules showed signs of severe changes. This could be attributed to the fact that a higher dosage of WIN or a longer duration of treatment is

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required to produce damage in all the seminiferous tubules. The occurrence of multi-nucleated cells within the tubules established in this investigation are consistent with the findings in the rat (Beyler *et al.*, 1961; Drobeck & Coulston, 1962; Reddy & Svoboda, 1967) and the mouse (Singh & Dominic, 1995). These multi-nucleated cells termed multi-nucleated giant cells were not seen in the seminiferous tubules of the shrews (Singh & Dominic, 1980). It has been postulated that these multi-nucleated cells possibly arise by the fusion of damaged spermatids or nuclear division without cytoplasmic separation (Singh & Dominic, 1995). It has been previously suggested that they are formed as a result of fusion of spermatocytes, agglutination of spermatids and failure of cytoplasmic division with continued nuclear division (Reddy & Svoboda, 1967; Drobeck & Coulston, 1962). Another prominent feature observed in most of the seminiferous tubules of the WIN treated mice was the appearance of vacuoles. These vacuoles have also been found in the seminiferous tubules of the guinea pig (Flores & Fawcett, 1972), rat (Drobeck & Coulston, 1962) and mouse (Singh & Dominic, 1995). A withdrawal period of 15 days produced slight recovery of spermatogenesis as the number of severely depleted tubules was considerably less. However, multi-nucleated cells and the presence of vacuoles in the seminiferous tubules were still discernable. Drobeck & Coulston (1962) found that after two weeks of withdrawal of WIN, the majority of seminiferous tubules were still severely affected. An extended withdrawal period of 42 days produced a marked recovery of spermatogenesis. There were a few partially depleted seminiferous tubules observed, a diminished degree of vacuolization and a few multi-nucleated cells. Thus, almost complete recovery of spermatogenesis occurred within the seminiferous tubules of the mouse. Extending the withdrawal period by another week, the recovery of spermatogenesis would

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in all likelihood be complete. In the rat, six weeks withdrawal of WIN produced essentially complete recovery of spermatogenesis with only a few partially depleted seminiferous tubules (Drobeck & Coulston, 1962). One week later, the rat testes appeared completely normal both grossly and histologically.

In this study, the interstitial (Leydig) cells were apparently unaffected by WIN and retained their normal morphology. These findings are in conformity with the reported findings of the rat (Coulston *et al.*, 1960; Kar *et al.*, 1966; Beyler *et al.*, 1961) and the shrew(Singh & Dominic, 1980). The interstitial tissue weight recorded after micro dissection was compared between the control and treated rats by Reddy & Svoboda (1967). It was found that the wet weight of the interstitial tissue of the control rats was 85 mg and that of the WIN treated rats was 185 mg. Thus, there is more than 100% increase in the interstitial tissue in rats treated with WIN for eight weeks. It is clear that WIN induced marked interstitial cell hyperplasia in rats. Drobeck & Coulston (1962) found an increase in the number of Leydig cells in the testes of WIN treated rats. An appreciable increase in Leydig cell numbers was noted in mice treated with WIN for thirty days, but the morphology of these cells were unaffected (Singh & Dominic, 1995). Quantitative evaluation of photographs of control versus experimental seem to indicate that Leydig cell numbers remained fairly constant (Refer to Fig. 3.1 to Fig. 3.12). In all instances, Leydig cell numbers appeared in groups of three to five cells. The findings of this investigation appear to be in contrast to the findings of Singh & Dominic (1995). Several studies suggest that Leydig cell morphology remains unaltered by WIN, while other studies propose that Leydig cell numbers are affected.

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Identification of the stages of spermatogenesis in the mouse was observed for the control and the WIN 42 day withdrawal groups only. The WIN only and the WIN 15 day withdrawal groups displayed disruptions to the seminiferous epithelium to an extent that it was difficult to identify the stages of spermatogenesis. The various spermatogenic stages observed in the control mice were IV, V, VI, VII, VIII, X, XI and XII. After 42 days of withdrawal a marked recovery of spermatogenesis was evident and the seminiferous tubules showed all cell type associations. The spermatogenic stages observed was III, VI and X in a few tubules. Singh & Dominic (1995) identified the spermatogenic stages in the laboratory mouse. Their findings conclude that WIN affects specifically the spermatocytes and spermatids with a very slow spermatogenic recovery upon WIN withdrawal.

A decrease in seminiferous tubule diameters was found in the WIN treated mice. However, a withdrawal period of 15 days resulted in a slight increase in seminiferous tubule diameters. After 42 days of withdrawal, the seminiferous tubule diameters increased even more and tended towards the control values. Thus, almost complete recovery of seminiferous tubule diameters was observed. However, the random selection and measurement of a few histologically processed seminiferous tubules suggest similar diameters for control versus WIN 42 day withdrawal mice. A marked reduction in seminiferous tubule diameters was observed in the rat (Drobeck & Coulston, 1962; Beyler et al., 1961; Kar et al, 1966) and in the mouse (Singh & Dominic, 1995) when these animals were treated with WIN.

Mammalian sperm undergo changes in morphology and biochemical composition during

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epididymal transit investing them with the ability for ovum binding and fertilization (Kaur *et al.*, 1991). A key role in the accomplishment of such maturation is therefore played by the epididymis. The histology of the epididymis appeared morphologically similar in all the treatment groups and was comparable with that of the control epididymis. In contrast to the marked degenerative changes induced in the testes by WIN, the epididymis was apparently unaffected and presented normal morphology. The principal cells and the stereo-cilia were comparable in all the treatment groups to that of the controls. The lumen of the epididymis was filled with sperm in all the groups. An investigation of the effects of WIN on the epididymis of the musk shrew was reported by Singh & Dominic (1980). Their findings were that shrews treated for thirty days with 200 mg WIN/kg body weight produced some epididymis tubules which were devoid of sperm, while the seminiferous tubules appeared normal. Thus, WIN does not induce regressive changes in the epididymis of the musk shrew. It is clear that the findings of this investigation proposes that the epididymis of the mouse is unaffected by WIN as no degenerative changes were evident in any of the treatment groups.

It can be concluded that WIN possesses marked anti-spermatogenic activity by impairing spermatogenesis in the mouse. However, the epididymis and the Leydig cells were resistant to WIN treatment. The failure of WIN to induce regressive changes in the Leydig cells may suggest that the endocrine functions of the testes are not affected by WIN. The exact mechanisms by which WIN induces its anti-spermatogenic effects remains to be elucidated.

4.2   Sperm Concentration

The spermatogenic cycle in the mouse is approximately 34.5 days and with 42 days of WIN treatment, the entire spermatogenic cycle is targeted as well as sperm undergoing epididymal maturation. After 42 days of WIN treatment, a marked decline in the sperm concentration of 47.2% was observed. There was a significant decline in sperm concentration since most seminiferous tubules were affected and some sperm eventually reached the epididymis. This result strongly suggests that the epididymis is not involved in altering the function of the sperm. After 15 days of withdrawal, sperm concentration levels recovered slightly, but was still significantly lower than the control. This suggests that the degenerative changes in the testes is slowly returning to normal as more sperm is being produced in the seminiferous tubules which had reached the epididymis. Therefore, the sperm concentration after 15 days of withdrawal of WIN could possibly have been sperm from the unaffected seminiferous tubules which had reached the epididymis. After 42 days of withdrawal of WIN, the sperm concentration approached normal values. Accordingly, sperm that reached the cauda epididymidis after 42 days withdrawal are representative of ones that originated from spermatogonia. It therefore signifies that recovery of spermatogenesis after cessation of dosing with WIN is almost immediate. This indicates that WIN acts at the level of the testes by inhibiting spermatogenesis.

4.3   Sperm Morphology

Accompanying the sperm concentration depression was a profound increase in aberrations in sperm morphology. The percentage of morphologically abnormal epididymal sperm of

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50.6% was significantly higher for the 42 day WIN treated group which indicate that this compound affects the development of sperm. Scanning electron microscopic observations revealed damage to sperm with an abundance of retained cytoplasmic droplets. Since a high percentage of morphologically abnormal epididymal sperm was noted in the WIN 42 day treated group, it can be said that the differential development of sperm in the testes is affected. After 15 days withdrawal of WIN, the percentage of morphologically abnormal sperm decreased to 41.0% and this can be attributed to a slow recovery of spermatogenesis. After 42 days withdrawal of WIN, the percentage of morphologically abnormal sperm diminished to 29.4%. It is evident that spermatogenesis has returned to normal and therefore more morphologically normal sperm was present in the epididymis. The trend in sperm morphology follows a similar pattern as that of the sperm concentration which suggests that a decreased sperm count and sperm abnormality may be associated. Thus, after 42 days of withdrawal of WIN virtually complete recovery was attained and the percentage of morphologically normal sperm returned to normal.

4.4 Sperm Motility

Sperm motility is attained as sperm migrate through the epididymis and therefore motility is chiefly a parameter of post-testicular function (Consentino & Cockett, 1986). Kinematic parameters were evaluated for cauda epididymal sperm of the mouse for the control and treatment groups. Sperm from the control animals displayed forward progression with a regular wave motion pattern. The decreased VSL and LIN of sperm from the control group clearly indicate that they possess less linear trajectories. Sperm from the 42 day WIN

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treated animals exhibited the largest variation in kinematic parameters. The pronounced changes in motion parameters were an increase in VSL, VAP, LIN, ALH, BCF, and DNC, while STR declined. An increase in sperm vigor (VSL, VAP and VCL) indicate that sperm tended to swim more rapidly with greater linearity (increased LIN) which produced more straight-line trajectories. This was confirmed by the trajectories which exhibited an irregular, straight- line motion pattern of the sperm from the 42 day WIN treated mice. There was a significant increase in WOB which indicated little deviation of the sperm head from the path of progression. It is evident that sperm from the 42 day WIN treated group exhibited the largest variations in the sperm kinematics as the development of sperm in the testes had been affected. After 15 days of withdrawal of WIN, VCL, VSL, LIN, ALH, BCF, DNC, VAP and WOB were considerably higher than the control values, but lower than the 42 day WIN treated mice. The increase in LIN clearly indicate that sperm followed a more linear motion pattern and there was less deviation from the average path progression of sperm. The slight increase in STR for the WIN 15 day withdrawal group as compared to the 42 day WIN treatment group seems to indicate that sperm trajectories follow more regular, motion patterns. However, STR was considerably lower than the control indicating that sperm continue to follow an irregular, but straight motion pattern. The decrease in sperm vigor (VCL, VSL and VAP) for the WIN 15 days withdrawal group indicates that sperm tended to swim less rapidly but with greater linearity (increased LIN). After 42 days of withdrawal of WIN, sperm vigor returned to normal. LIN, ALH, DNC, WOB and STR virtually returned to control values. Thus, there was a noticeable improvement in most of the kinematic parameters after 42 days withdrawal of WIN. These parameters clearly resemble closeness to the control values except for BCF which remained

considerably higher. The sperm motion pattern is essentially one which has wavelike, regular trajectories and suggests that almost complete recovery has occurred. Sperm in the epididymis is essentially sperm which had originated from spermatogonia during spermatogenesis. It is important that quantitative kinematic parameters essentially be correlated with the visualization of the trajectories of sperm. The kinematic parameters indicate that regular wavelike trajectories are changed to more irregular, straighter trajectories in mice exposed to WIN treatment. Upon the withdrawal of WIN for 42 days, these regular, wavelike trajectories are then attained. Soler *et al.* (1994) examined several regions of the epididymis of mice in an attempt to pinpoint the site of the most drastic changes in motility and to document the sperm kinematics. Sperm were immotile in the caput epididymidis with slow progression which was transformed to motile, linear progressions in the cauda epididymidis with less deviation from the average path as sperm matured. Despite the lack of studies on the motility parameters of sperm, this study confirms that the determination of sperm kinematic parameters could serve as an important tool in the evaluation of toxicological studies.

4.5 Effect on males to impregnate females

Male mice were cohabited with females for two weeks to minimize stress on the mice as these animals had been excessively handled during the experimental procedure. This period allowed for sperm in the testes to be targeted as well as sperm transversing the epididymis.

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In the present study, the male fertility index was 100% for the controls, which indicated that all these males had impregnated the females with whom it was placed. The females who paired with the males of the control group did not all give birth to pups as 85.29% of them were pregnant. Treatment of male mice for 42 days with WIN resulted in a 60% decrease in the male fertility index when placed with the females. The number of females which became pregnant and gave birth to pups was significantly reduced which indicates that WIN possibly impairs the formation and quality of sperm which reaches the female. After 15 days of withdrawal of WIN, the male fertility index rose which meant that more males were able to impregnate the females and become fathers. The female fertility index increased to 42.86% which confirmed that more females became pregnant as compared to the females paired with WIN treated mice. A withdrawal period of 42 days resulted in 80% of males becoming fathers. The female fertility index increased to 70% which signified that males were able to impregnate most females successfully. This signifies that 42 days recovery is sufficient to yield an almost complete recovery of the normal fertility index. By extending the withdrawal period, the male and female fertility indices is most likely to rise and attain somewhat normal values. It is evident that the inhibition of fertility coincides with the variation in sperm motility, concentration, morphology as observed by scanning electron microscopy and the histology of the testes in response to WIN treatment. These effects were shown to be almost completely recovered following 42 days withdrawal of WIN.

Conclusion

The results of this investigation indicate that WIN 18446 induces marked anti-spermatogenic effects of the testes which is completely reversible upon cessation of treatment. Even though this anti-spermatogenic action of WIN is convincingly demonstrated, the exact mechanism by which WIN induces such effects is not clearly elucidated. The failure of WIN to induce regressive changes in the Leydig cells suggest that the endocrine functions of the testes are not affected by WIN. This was further supported by the absence of regressive changes in the epididymis. Thus, it can be postulated that WIN does not act via the pituitary-testis axis. While WIN may never be an acceptable anti-fertility agent for the human male, further studies are necessary to establish its exact mode of action since it could be used in domestic and wildlife contraception. WIN 18446 could hold promise for the development of a contraceptive for animal and wildlife populations.
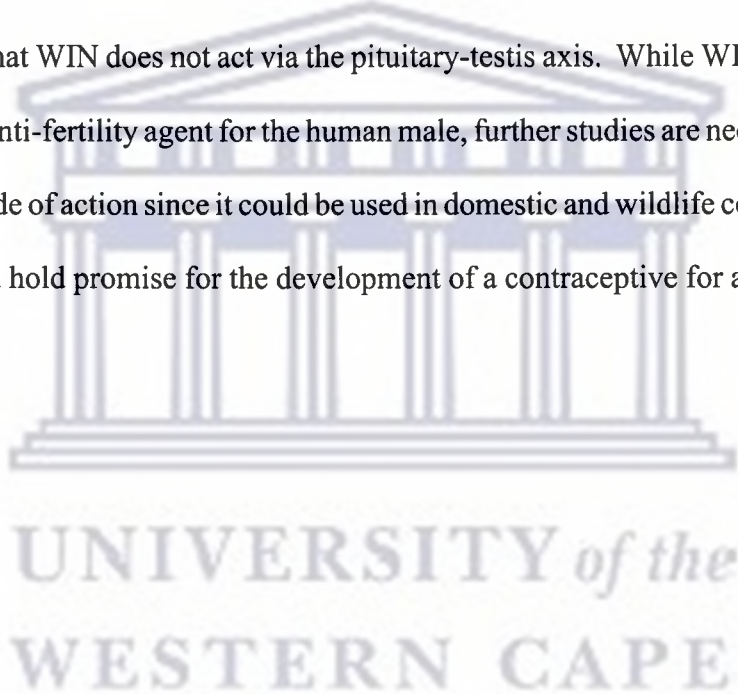
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Appendix

6. *Appendix*

6.1 Sperm Motion Parameters

The motility characteristics of sperm as well as sperm density (concentration) can be expressed in both the automated and manual modes by means of SMQ. The text below describes each of these sperm motility parameters which are internationally accepted terminology according to Katz *et al.* (1991).

I *Velocities*

1. Curvilinear velocity (VCL)

The time-average velocity of the sperm head along its actual precise path or curvilinear trajectory measured in μm/s.

2. Straight-line velocity (VSL)

The time average velocity of the sperm head as projected along the straight line between its first and final detected positions expressed in μm/s.

3. Average Path Velocity (VAP)

The time average velocity of the sperm head as projected along its spatial average trajectory and is expressed as μm/s.

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II   *Ratios, amplitudes and frequencies*

4.   Linearity (LIN)

A ratio of projected length to total length of the curvilinear trajectory and is expressed as a percentage.

5.   Mean amplitude of lateral head displacement (mnALH)

The average amplitude of lateral distances of the actual sperm head trajectory about its spatial average path expressed as μm.

6.   Maximum amplitude of lateral head displacement (mxALH)

The maximum amplitude of lateral distances of the actual sperm head trajectory about its spatial average path expressed as μm.

7.   Beat Cross Frequency (BCF)

The time average rate at which the curvilinear path crosses its average path. This parameter partly describes sperm vigor and is expressed in Hertz (Hz).

8.   Dance (DNC)

This parameter is defined as the product of VCL and mean ALH and describes sperm motion as the space occupied by the sperm head path during 1 second. DNC is expressed as μm^2/s.

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9. **Wobble (WOB)**

This is the ratio of VAP to VCL and is an expression of the degree of oscillation of the curvilinear path about its spatial average path. WOB is expressed as a percentage.

10. **Straightness (STR)**

This is the ratio of VAP to VSL and is an expression of the straightness of the average path. STR is expressed as a percentage.

11. **Mean Angular Deviation (MAD)**

This parameter gives information on the average angle at which a sperm turns when motile and is expressed in radians. [radians = radius/3.14 x 180]

12. **Curvature (CURV)**

This parameter reflects the progressiveness of movement and is reflected in the curve 0 - 1 (1 - straight-line path μm/curvilinear path μm). The smaller the curve 0 - 1, the straighter the sperm path and the higher is progressiveness. This parameter also gives information on the mode of movement and a value > 0.5 will indicate sperm swim in a circular mode.

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