CHAPTER I

Introduction

Part I Study on estrogenic activity in plants

Existence of estrogenic substances in plants has been recognized for a considerable time. However, phytochemical interest in them has remained relatively dormant until the 1950's. Interest in phytoestrogens has been aroused by the following reasons. First, the recognition that infertility in animals and humans occurred following excessive ingestion of plants rich in compound possessing extrogenic activity. Many studies were initiated in Australia as a result of the large economic loses caused by infertility of sheep grazed on pastures of subteranean clover (1). A second reason for interest in phytoestrogens is the realisation that the spring flush (the increased milk yield and improved quality of milk occured in spring) in dairy cows is probably due to the occurrence of estrogens in rapidly growing grass (2). Thirdly, the demonstration that carcass quality may be improved by the adminstration of estrogens, suggested that the feeding of plants rich in estrogen will also improve carcass quality (3). Fourthly, the possibility of obtaining estrogen from plant sources has also been suggested (4). Since then, phytoestrogens have been widely studied both in chemical and biological aspects and a number of plant species which exhibit estrogenic activity or contain estrogenic substances are demonstrated (5, 6, 7, 8).

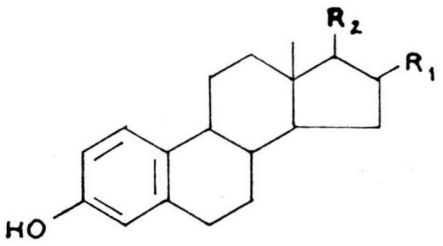
Types of estrogen

Natural estrogens of animal origin so far isolated are all steroids, possessing the cyclopentanoperhydrophenanthrene nucleus. The classical estrogens are estradiol 17-β, estradiol -17 α , estrone and estriol (figure 1p.3). Estradiol-17 β is synthesized mainly by the ovary and is the most potent natural estrogen by nearly all test methods. It is also produced by the adrenals, the placenta, and the testis.

Estrone are formed by the adrenals, and the placenta. In most tests involving parenteral injection it is less active than estradiol-17 B but in intravaginal test of all types it is only a little less active, the ratio of estradiol-17 β : estrone potency being about 1.5.

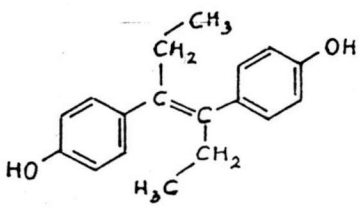
Estriol is metabolite of estradiol and estrone but less active than estrone in all but intravaginal tests . under optimal conditions, the two are of equal potency. Both estrone and estriol are formed in vivo from estradiol and are excreted in the urine mainly as sulfates and glucoronides (9).

There are also a series of synthetic compounds with potent estrogenic activity and with no apparent qualitative differences in action from the natural estrogens. A very large number of such compounds is now known, although many are far less potent than the natural estrogens. The majority of this group are almost certainly



	R_1	R_2
17 α-estradiol	H	----OH
17 β-estradiol	H	——OH
estriol	----OH	——OH
estrone	H	═══O

Figure 1 Structures of Estrogenic sterols.



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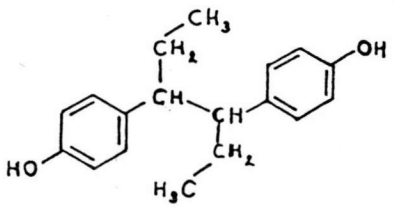

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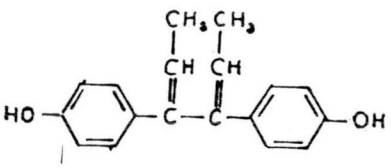


(3)

Figure 2 Three commonly used synthetic estrogens (1) diethylstilbestrol; (2) hexestrol; (3) dienestrol

estrogenic by virtue of their metabolites. Important members of the synthetic series are diethylstilbestrol, hexestrol and dienestrol (figure 2 p. 3).

Diethylstilbestrol, nonsteroidal compound which is active by oral administration as well as by injection in contrast to the natural estrogens which lose much of their potency when given by mount unless they are protected by some means. It has a potency when injected between that of estrone and estradiol, and a potency equal to that of estrone in intravaginal tests. Hexestrol and dienestrol are very similar in structure to diethylstilbestrol and share its properties. They are of about the same potency in most tests, but hexestrol seems to be less potent orally in human than is diethylstilbestrol (10).

Physiological roles, usages and adverse effects of estrogens.

Estrogens are necessary for the growth and function of the female reproductive organs. Many physiological processes are modified by estrogens. These modifications generally results in increased growth or metabolism of tissues receptive to the hormone, and in characteristic sexual behavior pattern. Estrogen sensitive tissues include reproductive tract and associated pelvic structures, the mammary gland, hypophysis and long bones. Cornification of vaginal epithelium in some rodents such as mouse, rat, and guinea pig is a specific action of estrogens. Estrogens act on mammal uterus resulting in hyperemia, followed by an increase in both wet and dry weight,

amino acid incorporation, nitrogen deposition, nucleic acid synthesis, phosphorus uptake and glycogen deposition. An increase in enzyme activity and oxidative metabolism occurs, resulting in a generalized hypertrophy and hyperplasia of both endometrium and myometrium (11).

Today, the medical practitioner prescribes estrogens for a variety of condition which are generally correlated with deficiencies in naturally produced sex hormones. For example, women with menstrual irregularities or delayed menstruation may be given synthetic estrogens. Menopausal women commonly receive estrogens to help allay hot flushes, nausea and other symptoms caused by decrease in the body's natural hormonal level. It is of interest to note that the symptoms of the male counterpart, the male climacteric often respond favorably to estrogen therapy (12, 13).

Estrogens also find increasing application in the meat and poultry industry. Because of its low price, diethylstilbestrol is used almost exclusively. The implantation of diethylstilbestrol pellets under the skin near the head of the chicken produces more tender and juicier meat (11). Diethylstilbestrol is widely used as growth stimulant in raising of cattle in the U.S. although its use was at one time banned by the FDA because of its residues found in liver and other tissues of the treated animals (7).

Prolong treatment with estrogens cause endometrial hypertrophy and endometrial carcinoma (14). A possible association between estrogen treatment and endometrial carcinoma has been suggested

since 1973 (14). It was concluded that estrogens treatment increase the risk of endometrial cancer and degree of risk was in proportion to the length of treatment (14).

Assay of estrogens

Estrogens may be assayed biologically or chemically, and the choice of methods depends on particular requirements of the investigation. Biological assays as such and experiments of the same character, continue still in use when very high sensitivity is needed. They also continue in use in investigations demanding the demonstration of estrogenic activity or its potentiation or inhibition; much new investigation thus demands a biological approach at least in the first instance (10).

Several biological indicators have been developed into standard bioassays for the estrogens. These are cornification of the vaginal epithelium, increase in weight of the uterus of ovarectomized rat or mouse, vagina opening in immature rodents or in immature or ovarectomized guinea pigs, and increase in weight of the chick oviduct (15).

Two responses have been used in the assay of plant estrogens : the occurrence of vaginal conification, the Allen-Doisy Mehtod (16), and the increase in weight of uterus the uterine weight method (17). The former method, a test substance is injected into ovarectomized rats or mice, and vaginal smears are examined at intervals for the appearance of cornified epithelial cells.

This method has become less popular in recent years for somewhat unconvincing reasons. Assays based on cornification of the vagina have been criticised for several years on the grounds that scoring the vagina smears is highly subjective (15), however, can be overcome if the vagina smears are presented to the observer at random so that he does not know which experimental group he is scoring. The most serious economic objection to Allen-Doisy assay is that the response is quantal and about twice the number of animals are required than when a graded response, such as the weight of uterus is employed, in order to attain identical levels of accuracy. Another strong objection to assays based on a quantal response, is that the statistical analysis is laborious, involving interative calculations (17). Uterine weight method, developed originally by Bülbring & Burn (18) depends on the growth response of uterus of immature rats or mice to estrogen treatment. A convenient version of this method takes the advantages of the fact that the uterine weight increased appreciably in 6 hours due to the accumulation of water in endometrial stroma. This method has problem of specificity since substances such as testosterone, progesterone and androsterone could produce an increase in uterine weight (19). Other disadvantages of this method are : a dependable supply of immature female is needed and the animals can be used only once. Of the several advantages, the following may be mentioned. Firstly there is complete objectivity of end point. Secondly, no castrating, priming or other laborious standardization procedures are required. Thirdly, the useful range of

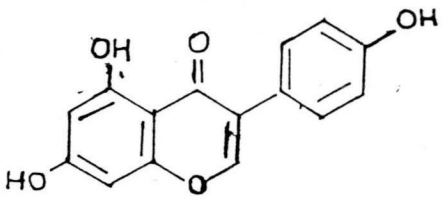
reactivity is large, greatly reducing the number of preliminary assays required. Finally and most important, the uniformity of response make possible relative accuracy consistently when as few as 1-4 animals are used in each assay (20).

The results of bioassays depend not only on the method used, but also on the manner in which the test material is administered and of vehicle used. When the vaginal smear and uterine weight methods are applied, the biological activity of the natural estrogens decreases in the following order : estradiol > estrone > estriol (11).

Plant estrogens

Based on chemical structure, plant estrogens can be classified into two major groups : steroidal estrogens and nonsteroidal estrogens. Original demonstration of the occurrence of estrone in date palm with a structure and activity identical to those of animal hormones (21).has been followed by the identification of several other steroidal estrogens from plant sources. Estradiol, estrone and estriol could be found in Phaseolus vulgaris (22) and estriol was isolated from willow flowers (23). Pomegranate seeds which seem to be the richest plant sources of steroid estrogens, have been reported to contain 17 mg of estrone per kg (24).

Nonsteroidal estrogens which have been so far found are belonged to the isoflavone, coumestan and resorcylic acid lactone groups (figure 3 p. 9).



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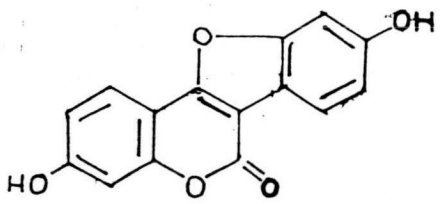

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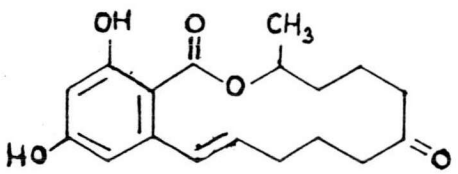


(3)

Figure 3 Structure of nonsteroidal estrogenic agents (1) genistein; (2) coumestrol; (3) zearalenone

Isoflavone

Several isoflavones are known to exist in nature, they may exist either in free state or as glycosides and only limited number of them show estrogenicity. Genistein, prunetin, biochanin A, formononetin and daidzein are five of aglycones which have been reported of estrogenicity and are mostly found in plants of legume family (6). Investigations of the estrogenic isoflavone began in the 1940 as an attempt to explain the causes of clover disease which resulted in infertility in sheep grazing on certain forages. The problem was traced to the presence of estrogenic isoflavones in several common clovers in which genistein and formononetin are the major isoflavones present and responsible for reproductive problems in sheep. Though not a steroid, it has a structure with some resemblance to that of estradiol and diethylstilbestrol. It stimulates uterine growth in ovarectomized mice, sheep and rats. In mice, genistein is roughly 10^5 times less active than diethylstilbestrol in stimulating uterine enlargement (25, 26). Daidzein, biochanin A and formononetin although estrogenic, are very weak estrogens as judged by assay based on either relative affinity for estrogen receptors or assays using mouse uterine weight (7).

Coumestan

Coumestan is another group of nonsteroidal estrogen, having the skeletal structure of 6H-benzofuran (3,2-c) benzopyran-6—one.

The most throughly studied are the estrogenic coumestans from alfalfa (Medicago sativa) and ladino clover (Trifolium repens). Coumestrol, a dominant estrogen of this group, has been detected in several clovers as well as in many other plant products commonly consumed by man such as soybean sprouts and soybean derivatives, green beans, snowpeas, green peas. Based on the dosage required to produce a uterine weight of 25 mg in mice, coumestrol has been estimated to be 30-100 times more active as estrogens than are in isoflavones (7).

Resorcylic acid lactones

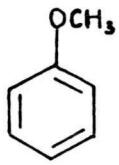
The third class of estrogenic substances which occur in plant foodstuffs are derivatives of resorcylic acid lactones. These involve zearalenone 6 (10-hydroxy-6oxo-trans-1-undecenyl) -B-resorcylic acid lactone and its derivatives. They are mycotoxins synthesized by the mold Fusarium roseum which can infect corn, wheat, barley, sorghum or hay. Surveys in the U.S., corn and wheat crops show a 10-20% incidence of zearalenone contamination as high as 10 ppm. A derivative of zearalenone, zearalanol has been used as growth stimulant in animals. Both zearalenone and zearalanol bind to estradiol-binding sites from several mammalian tissues. Zearalenone also appears to be effective in treatment of symptoms of postmenopausal syndrome in women. Zearalenone and its derivatives are the only naturally-occurring non-steroidal estrogens which have been shown to be estrogenic in man. However, amounts of zearalenone to which human are

likely to be exposed in foods are several orders of magnitude lower than the amounts necessary for contraception (7).

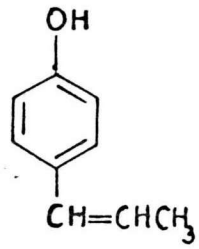
There are additional compounds which exhibit estrogenicity such as anethole, p-anol and anisole (figure 4p.13). These compounds are phenol methyl ether derivative. Anethole is the main constituent of the essential oil of the umbelliferous plants fennel (Foeniculum vulgare), and anise (Pimpinella anism). Both plants have been used as estrogenic agents for millennia. Essential oils of fennel and anise also have been used as flavoring agents in food and beverages. Further studies on estrogenicity of both essential oils suggests that the pharmacologically active agents are polymers of anethole such as dianethole and photoanethole (27) (figure 4p.13). It is note worthy that both of anethole polymers have a structure with some resemblance to that of diethylstilbestrol.

Significance of plant estrogens

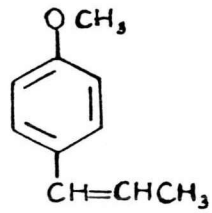
Medicinal uses of plant estrogens have been practised for a long time. In Egypt, the pollen grains of the date palm Phoenix dactylifera var somani (Palmae) which have been reported to contain estrone, were used to induce fertility in women (28). Fennel and anise are plants which have been used as estrogenic agents for millennia. Specifically, they have been reported to increase milk secretion, promote menstruation, facilitate birth, alleviate the symptoms of male climacteric and increase libido in woman (27).



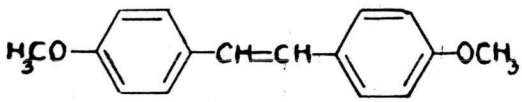
Anisole



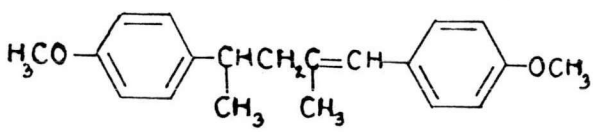
p-Anol



Anethole



Photoanethole



Dianethole

Figure 4 Structures of additional nonsteroidal compounds which possess estrogenic activity.

In the light of a long historical usage of these plant estrogens, it seems likely that estrogens from plants may play a significant role in medical therapy in the near future. As recently noted, traditional medicine is the only mode of health care delivery for 75% of the world's population (20), and plants are the primary therapeutic agents in traditional medicine. Hence, the integration of plants rich in estrogens into medical practices in developing nations, as well as industrial nations, may have merit. Unlike most synthetic estrogens those plants are inexpensive, easily cultivated and yet gently and efficacious in their effects.

A number of commonly consumed vegatables such as carrots, cabbages, peas, soybean sprout and soybean derivatives including onion and garlic have been reported to possess estrogenic activity (5, 7). Unless phytoestrogens are metabolized to more potent estrogens, however, it is clearly not likely that humans are exposed to dietary doses sufficient to cause any major physiological response (7). However, the possibility of metabolic alteration to more or less active forms should not be ignored, since effects of this kind have been demonstrated in experimental animals. The likelihood of human exposure to phytoestrogens is considerably higher when measured in DES equivalents than those of human exposure to DES in liver from cattle treated with DES as a growth stimulant (7). Thus, human should recognize the significance of low-level but long term exposure to those phytoestrogens especially in etiology of carcinoma of

reproductive organs. A man should avoid consuming plants rich in estrogens since they may make him a characteristic of woman and be infertile. Furthermore, researches on plants as a source of fertility regulating agents for male should exclude their estrogenicity. Therefore a knowledge of plant estrogens and their distribution will be useful in selection a promising plant without estrogenicity for antifertility study. On the other hand, estrogen if present in the plant studied, should be get rid of before testing of the antifertility effects.

Part II Study on antispermatogenic effect of various plant extracts in rats and mice.

A world population is now increasing at such a high rate. This made it necessary and urgency for scientists who have taken the responsibility for the problem of population explosion to develop methods or agents to control the birth rate. In the past, emphasis has been placed on the development and use of contraceptive methods for women but, with increasing publicity on problems associated with the use of oral estrogen-gestragen contraceptives in women, the role of the male in contraceptive practice is being re-examined. At present, a man willing to take the responsibility for contraception has these effective methods to choose : sexual abstinence, coitus interruptus, and barrier method which include condoms and vasectomy.

Sexual abstinence should be practised during the unsafe peroid of the wives, but sexual desire of the couple may occur during this period and this may cause psychological stress.

Coitus interruptus is an ancient and probably earliest form of birth control and is widely practised but it has a high failure rate. It may cause nervous disorder in the couple, especially in the female.

Condoms are mechanical barriers used to prevent sperm to enter female genital tract. They are thin bags made of rubber, collagenous tissue or plastic and may be coated with spermicidal agents. They are effective if used properly but lack of care can result in an unacceptable high pregnancy rate. They are widely distributed for their simplicity since no medical personnel are required and also inexpensive.

Vasectomy or male sterilization is a simple operative procedure which can be carried out under a local anesthetic, consisting of cutting the vas deferens and tying off the ends. The principle of this technique is to prevent spermatozoa from reaching the penis, although the man can still ejaculate semen and the testis remain unaffected so that the production of testosterone is not impaired. Failure of this method may be due to spontaneous recanalization and the patient must wait for the seminal reserviors to be emptied since the average man will emptry his seminal reserviors only after 10 ejaculations (30). Though this method is regarded as irreversible, the reversal of fertility can be achieved by means of surgery. However, there is problem related with the rise in sperm antibody titre after occlusion of the vas in 20-40% of vasectomized men (31).

The role of the male in contraceptive practice is still less significant as compared to woman. The previous mentioned methods such as sexual abstinence and coitus interruptus though theoretically effective but have some problems in practice. People in some rural

areas have bad attitude to vasectomy, thinking that it may cause a loss of libido and potency. Moreover, this method is largely unacceptable in cultures such as Islam and sub-Saharan Africa (32). Most of the young men do not practise vasectomy due to its irreversible effect. Thus, it is necessary to develop alternative methods for male contraception. This will provide each man a suitable contraceptive method and make him taking more responsibility for contraception. A number of researches have been done to develop the agents or methods for fertility control in the males. These must meet the requirements as in the females, i.e. they must be effective, relatively safe, preferably self administered, or alternatively, long acting, devoid of adverse effects on libido-perceived or actual, relatively inexpensive and reversible. Therefore, a well understanding in the processes of regulation of the male reproductive system and spermatogenesis makes it possible to interfere these processes thus an effective method or agent for male fertility regulation may be obtained.

Regulation of the male reproductive function

It is obvious that regulation of the male reproductive function is mediated through the hypothalamo-hypophysealgonadal axis which is the same as in the female. The hypothalamus is triggered to release gonadotropin releasing hormone (GnRH) via the portal hypophyseal vessels to the anterior lobe of pituitary gland. These hypothalamic RH has recently been isolated, characterized for

chemical structure and synthesized. It is a decapeptide which is identical in all mammalian and many sub-mammalian species. It causes the release of gonadotropin from pituitary into blood stream to act upon testis. The two gonadotropins i.e. luteinizing hormone (LH) and follicle stimulating hormone (FSH) are essential for maintenance of spermatogenesis in the seminiferous tubules. LH stimulates testosterone production by Leydig cells and by maintaining high levels of intratesticular testosterone, it indirectly stimulates spermatogenesis. FSH act on the seminiferous tubule probably influencing spermatogenesis indirectly by modifying the Sertoli cells which act as supporting cells.

There is negative feedback of these two hormones exerted by these target organ's secretions (fig.5 p.20). For LH, testosterone exerts this effect directly or through conversion to estradiol -17β, but signal for FSH controls is still unclear. It seems likely that the spermatogenic tubules may also produce a selective inhibitor of FSH secretion termed inhibin, which probably synergizes with testosterone in controlling the output of FSH (33).

Inhibin has multiple sites of action and modulates FSH action at the gonadal level. Inhibin like activity has been found in various sources such as human testis, seminal plasma, rete testis, spermatozoa, human prostate, human placenta and bovine testis. Inhibin extracted from testis of Indian water buffalo was shown to

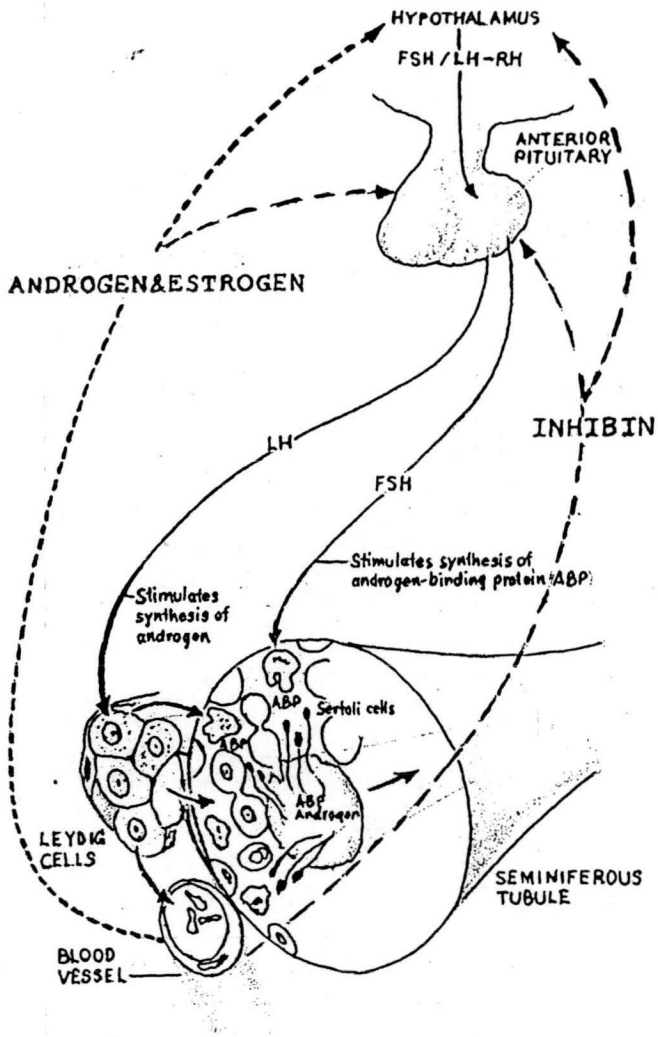

HYPOTHALAMUS
FSH/LH-RH
ANTERIOR PITUITARY
ANDROGEN&ESTROGEN
INHIBIN
LH
FSH
Stimulates synthesis of androgen-binding protein (ABP)
Stimulates synthesis of androgen
ABP
Sertoli cells
ABP
ABP Androgen
LEYDIG CELLS
SEMINIFEROUS TUBULE
BLOOD VESSEL
negative feedback

Figure 5

Figure 5 : A diagram illustrating the probable endocrine regulations of spermatogenesis. The hypothalamo-hypophyseal testicular axis is composed of two axis, one is concerning with Leydig cells and the other with the seminiferous tubules. The first axis is composed of hypothalamus, pituitary gland and Leydig cells along with their respective hormones, LH-RH, LH, androgen and estrogen. The second axis is composed of hypothalamus, pituitary gland and the Sertoli cells of the seminiferous tubules along with their respective hormones, LH-RH, FSH and inhibin.

Based on a diagram from : Bardin, C.W. : Pituitary Testicular Axis., in Yen, S.S. and R.B. Jaffe, ed., Reproductive Endocrinology. W.B. Saunders, Phil., 1978, p. 111 and Setchell, B.P. et al : Inhibin in Johnson, A.D. and W.R. Gomes, ed., The Testis. Vol. 4 Advances in Physiology, Biochemistry and Functions, Academic Press, New York, 1977, p. 191.

drastically diminished the fertility of adult male rats by reducing a litter size (34). The discovery of inhibin has led to the discovery of other small peptides in the testis possibly active in regulating testicular function (35).

Androgen binding protein (ABP) which is found in the testis, rete testis fluid and in the head of epididymis (caput epididymis) is FSH dependently synthesized by Sertoli cells. The decrease in plasma FSH will result in decrease of ABP synthesis. In human, it is still unclear whether ABP synthesis is FSH dependent alone or there may be other products which play important role in the metabolic function of the Sertoli cells in the synthesis of ABP. ABP is speculated to be the factor that causes accumulation of androgens in high concentration in the seminiferous epithelium (36).

Spermatogenesis is the process occurred in the seminiferous tubules under control of male sex hormones, the androgens fig.6p.22). The spermatogenic cycle in the rat is first studied by Leblond & Clermont in 1950s (36). Multiplication of germ cells is syncytial in nature i.e. there still have intercellular bridges among these germ cells which represent incomplete cytokinesis (figure 7 p.23). Germ cells divided by mitotic process from the stage of spermatogonia to primary spermatocytes and by meitotic process in later stage. The spermatids then undergo differentiation to be the spermatozoa. Maturation of spermatozoa takes place as they pass through the epididymis.

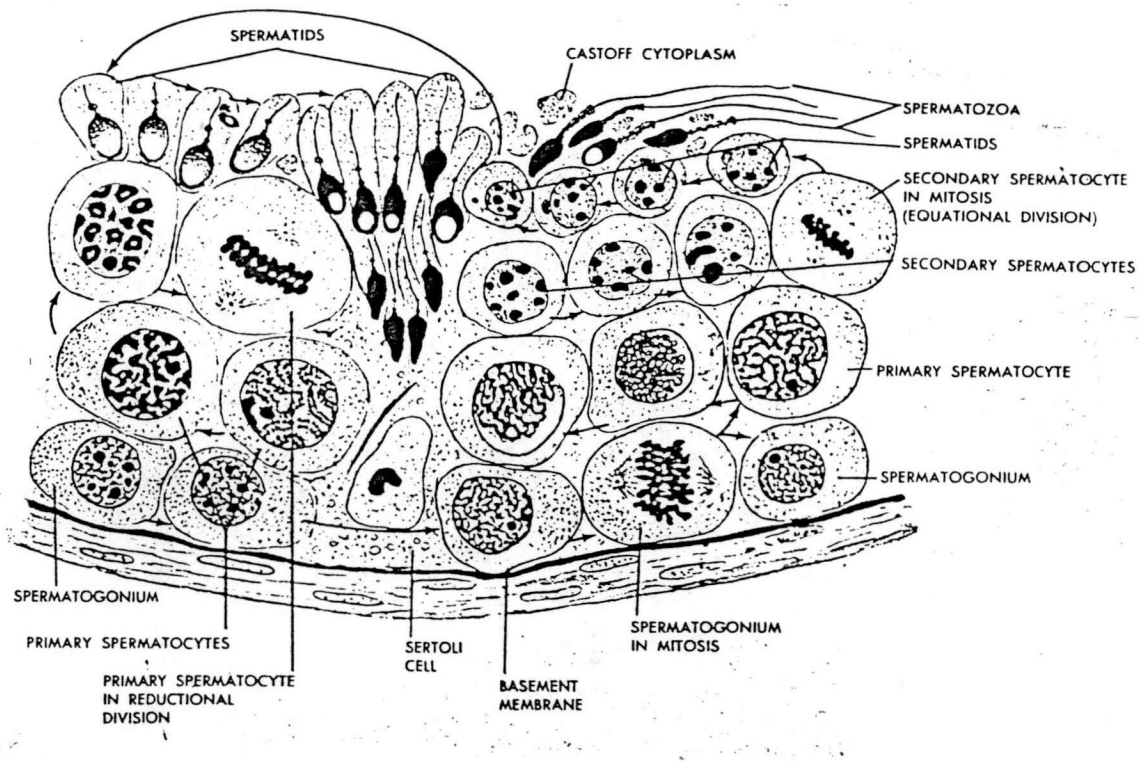


Figure 6 Schematic representation of the spermatogenic elements of spermatogenesis in seminiferous tubule. Spermatogonia, primary spermatocyte, secondary spermatocytes, spermatids and spermatozoa are shown.
(From Neitter, F.H. : The Ciba Collection of Medical Illustrations, vol 2, Reproductive System, CIBA, New York, 1954, p 25)

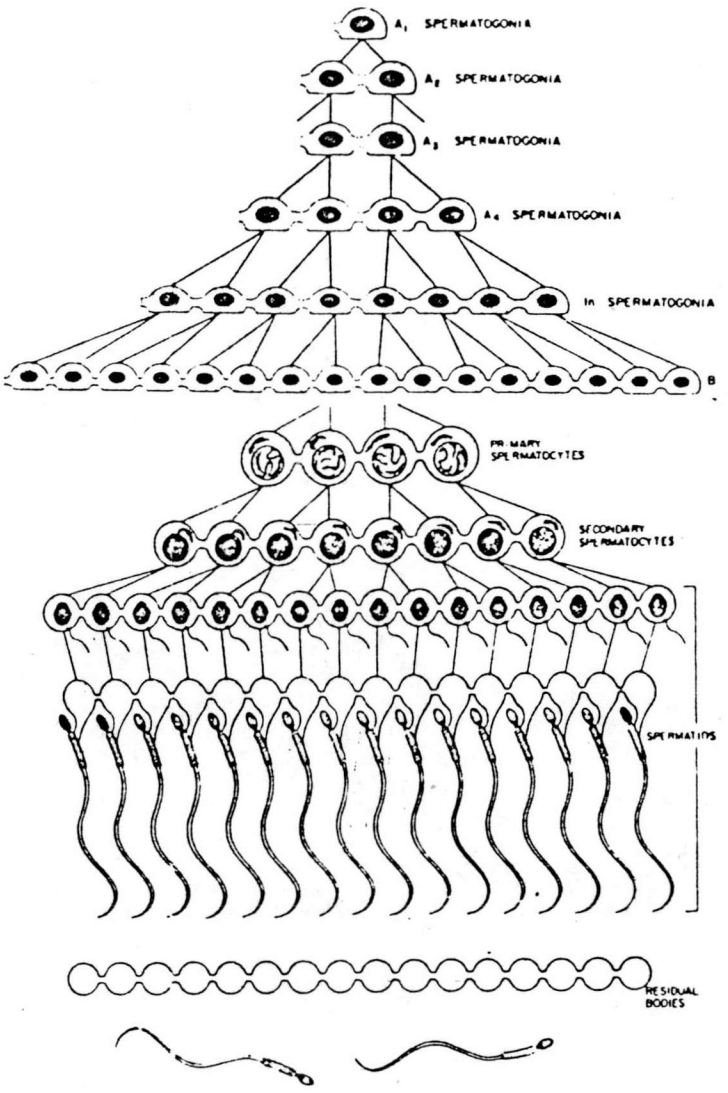


Figure 7 . Schematic representation of the syncytial nature of the mammalian germ cells. Cytokinesis is incomplete in all but the earliest spermatogonial division, resulting in expanding clones of germ cells that remain joined by intercellular bridges.

From Greep, R.O., et al. : Reproduction and Human Welfare : A Challenge to Research. MIT Press, Cambridge, Mass., 1976, p. 187.

Morphological, biochemical and physiological modifications are involved in these maturational changes which are the important factors of fertilizing capacity. In most mammals there is a special region in the epididymal head just distal to the initial segment of the duct, where the epithelial cytology is dominated by large pinocytic vacuoles and numerous lysosomes. Sperm accumulation takes place here, as well as structural maturation of the acrosome and movement of cytoplasmic droplet down to the middle piece. The epithelial alterations are accompanied by abnormal maturation changes in the structure of intraluminal spermatozoa (37). A change at the molecular level within the sperm membrane or possibly the removal of some component from its surface has taken place before the acrosome reaction. The important phenomena of morphological modification are the acrosome reaction. Acrosome reaction is the series of structural changes in the sperm head occurred after the acrosome cap was formed resulting in the loss of the outer acrosomal membrane and the overlying cell membrane. There is evidence that capacitation is a general prerequisite of conception in all mammals including man (36). In the past decade, loss of acrosome was thought to be equivalent to capacitation but now it is believed that capacitation does not involve any visible morphological change in the spermatozoa (36). The prospects on control of capacitation are promising, but this will probably not be achieved in the near future.

The character of the swimming movements of spermatozoa in different regions of the epididymis are important for the fertilizing capacity too. The epididymal epithelium has been found to synthesize glycerophosphorylcholine and sialic acids. It may synthesize steroids but with limited capacity (36). It also is able to accummulate carnitine, a compound which is involved in the transport of certain fatty acids into mitochondria, in high concentration in its lumen. Whether these substances contribute to maturation of spermatozoa during their transit, to their maintenance during storage, or to their activity after ejaculation are important gaps in our knowledge, there is no reason to assume that these compounds are the only products of epididymal synthesis and transport. Others of equal or even greater functional importance may be discovered in the future. The fact that certain exogenous compounds are also accummulated in the corpus and cauda epididymis suggests that it may be possible to take advantage of this property of the epithelium to achieve high intraluminal concentrations of antifertility agents which is directly deleterious to sperm (36).

Another important factor related to fertilizing capacity is the penetration of the spermatozoa into the zona pellucida. The lytic enzymes bound to the inner side of the acrosomal membrane is believed to assist in creating a path for a vigorously motile sperm to penetrate into the zona pellucida of the ovum. Having passed through this barrier, the sperm lies free in the perivittelline

space between the zona pellucida and the surface of the egg, the two gamates fuse and thus fertilization occurs.

Contraceptive methods for male can be achieved by interference with those processes involved in spermatogenesis and sperm maturation. Disruption of sperm formation could be achieved either by interference with hormone melieu necessary for spermatogenesis or by directly suppression of spermatogenic cells. However, these means are not so attractive as the way applying disruption of sperm maturation for several reasons. A time period for assessment of antifertility effect is so long and it may require several weeks. Hormonal interference may cause undesired effect such as loss of libido and potency. A direct suppression of spermatogenic cells may cause mutagenic effect to germ cells which would result in abnormal offsprings. Alkylating agents similar to those used in cancer chemotherapy such as nitrogen mustard, ethyleneimine derivatives, cyclophosphamide, chlorambucil are antispermatogenic compounds because of their inhibition of cell division (38). However, they cannot be used as contraceptive agents because of the likelihood that they would not only affect germ cells but would also interfere with the cell proliferation essential for cell replacement and tissue repair elsewhere in the body. The ideal antispermatogenic agent should have a high degree of selectivity for the later stages of spermatogenesis, that is the stage which spermatids are being transformed into spermatozoa and should certainly not be mutagenic or destructive to the stem cells.

Interference with sperm maturation is very attractive contraception for males since it affords several advantages; rapid effectiveness, rapid reversibility and no interference with spermatogenesis. Thus, the possibility of mutagenic effect is reduced. Moreover, no interference with androgen production by the testis and therefore, effects on libido and potency are avoided (39).

Agents which possess male antifertility effects

LH-RH analogues

By using LH-RH analogues, the release of LH & FSH was blocked. In addition to blocking of LH & FSH release, testicular atrophy was also produced in various species of laboratory animals by its desensitizing action on pituitary and gonadal hormone receptors (35). Since suppression of LH can induce loss of sexual desire due to impairment of testosterone production, the selective inhibition of FSH release would have more advantage than the LH supression (36).

Steroid hormones

Hormonal agents such as gonadal steroid are used as potential male contraceptives to suppress pituitary gonadotropins. This results in inhibition of spermatogenesis and azoospermia. All gonadal steroids, estrogens, progestins, and androgens are capable of this suppression, but the doses required and the

concomitant potential side effect limit their use. This is particular true of estrogens, compounds that suppress gonadotropins and induce azoospermia but result in feminizing side effects, thus, prohibiting their use as male contraceptives. Progestin and androgens offer greater potential as male contraceptives and have been employed in several studies, both alone or in various combinations (40). Of the two progestogen and androgen combinations that have been studied extensively are danazol-testosterone and medroxy-progesterone acetate (DMPA) - testosterone. Danazol is a synthetic steroid which appears to lower gonadotropin release without the undesirable endocrinological and metabolic side effects of estrogen or progestin. Testosterone enanthate is given with it in an amount sufficient to maintain normal libido, that is 200-250 mg/month (40). This approach showed some promise although Danazol is now prohibitively expensive (41). Other synthetic compounds that are less costly and equal effective may be found in the future. DMPA (200 mg) and testosterone enanthate (200 mg) given by monthly injection offered the best potential male contraceptive. However, there is a problem concerning a failure to achieve persistent azoospermia in all men, though oligospermia of less than 5 mill/ml occurred in all subjects (40). On practice with progestogen-androgen combinations, the onset of action was long ranging between 7 and 30 weeks, the degree of spermatogenic suppression varied with the dosage and potency of progestogens used. The time required for recovery is

often prolonged up to 18 months, but in all men full recovery resulted (40). D-homo-steroids which possess both androgenic and progestational effects should be developed for this purpose since it would reduce the costs of toxicity testing to ⅓ of that for a steroid combination (35).

1,2-Dibromo-3-chloropropane

1,2-Dibromo-3-chloropropane (DBCP,fig.8p.34) was developed as a soil fumigant for nematodes. The potential effect of DBCP in humans was realized recently when workers manufactering DBCP discovered that they had reduced sperm counts. Decreased libido, infertility, and impotence were also presenting symptoms. Of the six men who were exposed to DBCP for 2-10 years, all were azoospermic (42). Testicular biopsies indicated atrophy of seminiferous epithelium. Unfortunately DBCP possesses beside antifertility activity, a very toxic and carcinogenic in rats and mice (43, 44) this preclude further testing of this agent in man.

Bis (dichloroacetyl) diamine

Bis (dichloroacetyl) diamine constitute a class of compounds known to have reversible antispermatogenic activity in monkey, man, rat, dog, mouse and guinea pig (45). It has been suggested that one possible mechanism of action for the bis (dichloroacetyl) diamines is that it produces tissue hypoxia, or energy depletion (45).

The most studied analogue of this series in WIN 18, 446 (fig.8p.34). This compound possesses relatively low toxicity and low incidence of side effects so it was introduced to clinical trial in prisoner volunteers and was found to be effective and reversible. Despite its initial promise it was abandoned for human use because it proved to be an incompatibility with alcohol, producing distressing symptoms comparable to those of Antabuse, which is used in the treatment of alcoholism (46).

α-Chlorohydrin

α-Chlorohydrin (3-chloropropan-1, 2-diol, Fig.8p.34) is found to induce temporary sterility in many species of animal including rats, guinea pigs and monkeys by alteration of the epididymal environment (47). Low dose of α-chlorohydrin inhibits the sodium dependent fluid reabsorption in the isolated duct of rat cauda epididymis. This effect could be mediated through an inhibitory action on the active sodium transport across the epididymis (48). It also reduced the percentage of motile sperm in the vas deferens of rat which may be due to decreased glycolysis, oxygen consumption or ATP concentration and arrests spermatogenesis at the primary spermatocyte stage (47). When α-chlorohydrin was added to suspension of rat epididymal spermatozoa in vitro in concentration that did not affect sperm motility, intrauterine insemination of in vitro treated spermatozoa was found to be completely infertile compared to untreated

spermatozoa and there was almost completely absence of oocytes in the flushed ampullas of recipients of α-chlorohydrin treated sperm (49). Thus, α-chlorohydrin has direct effect upon spermatozoal function and also has a possible effect on the female reproductive tract. α-chlorohydrin at low doses appears to be a true functional sterilant, while at high doses it induces a spermatocele in the rat and may have antitesticular effects in several species (50). Although much research has been done with α-chlorohydrin, interest in its further development as a human male contraceptive waned following the discovery at therapeutic doses, of toxic and neurotoxic effect in nonhuman primate (51).

6-chloro-6-deoxy glucose

Modified sugars, especially the chlorinated hexoses were investigated as male antifertility agents through the interference with glycolysis of spermatozoa during sperm maturation. The most studied compound in this series is 6-chloro-6 deoxy glucose (6 DCG, figure 8); 24 mg/kg/day was sufficient to produce infertility in male rats and marmoset monkeys. At 240 mg/kg/day, it was neurotoxic to marmoset monkeys but not to rats. At 480 mg/kg/day, it was neurotoxic to mice but did not affect their fertility. Ratios of the toxic dose to the antifertility dose varied with the structure of 6-chloro-6-deoxyhexose so it is possible that the toxic and antifertility effects are produced by different metabolites (35). 6 CDG has also been shown to penetrate the rat blood brain barrier and inhibit

transport of D-glucose, so its potential as contraceptive for males is deminised (52).

D-thio glucose

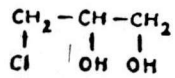
5-thio-D-glucose (5 TDG, figure 8) is analogue of glucose which appears to be safe and effective in male rats (53). However, several studies also showed its diabetogenic effect in rats (52, 54). Oral administration of 5-TDG causes reversible inhibition of spermatogenesis in mice but certain mechanism of action is not known. This may be related to its inhibition of active transport of D-glucose across cell membrane (55).

Gossypol

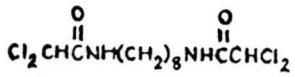
Gossypol, a polyphenolic compound (figure 8) isolated from some cotton plants, has been regarded as a promising male contraceptive (56). Long term treatment with gossypol results in a severe oligozoospermia or azoospermia in most species. In the early studies in China, male rats were given gossypol acetic acid 15-40 mg/kg, by mouth five times a day for 2-4 weeks. This treatment produced decapitated and bent tail spermatozoa. In addition, spermatids and spermatocytes were observed in the cauda epididymis and the vas deferens. The animals became infertile, yet they maintained normal mating behavior. The majority of the treated animals regained fertility following withdrawal of treatment of more than 4 weeks (57).

The site of action of gossypol is believed to be the testis (58). Histological examination reveals a loss of germ cells following chronic treatment. Recent studies have demonstrated that gossypol also has a direct action on spermatozoa. When human spermatozoa were incubated with micromolar concentrations, motility along with glucose and fructose metabolism were inhibited (59). In vitro studies of gossypol acetic acid with human spermatozoa showed a potent inhibitor of acrosin activity (60). The true mechanism is still unknown, however, this approach may lead to development of gossypol as a vagina contraceptive.

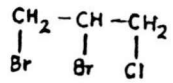
A clinical trial of gossypol as male contraceptive agents was begun in China in 1972. The antifertility efficacy evaluated by semen examinations is 99.98 % (56). In 91.1% of the subject, gossypol was effective in reducing sperm count to less than 4 mill/ml (61). Recovery after withdrawal, however, is incomplete up to 25% of the 2000 subjects followed up (61). Side effects of gossypol are hypokalemia, fatigue, gastrointestinal symptoms and decrease in libido and potency. The effective antifertility dose (about 0.4 mg/kg/day) used for men in China is far below the dose that causes overt toxicity in some species of animals which is 20–40 mg/kg/day (62). In conclusion, gossypol is a promising compound that may be used as a male contraceptive in the future. Since this agent is new, the issues of toxicity, effectiveness, reversibility and mode of action remain serious topics of research.



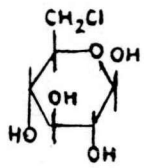
α-CHLOROHYDRIN



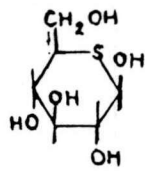
WIN 18 446



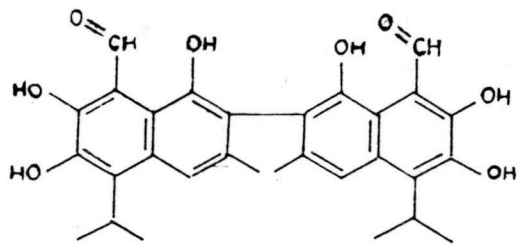
1, 2-DIBROMOCHLOROPROPANE
DBCP



6-CHLORO-6-DEOXYGLUCOSE



5-THIO-D-GLUCOSE



GOSSYPOL

Figure 8 Structure of compounds possessing male antifertility effects.

Antifertility plants in the male

Numerous synthetic compounds have been reported to arrest spermatogenesis or induce functional sterility in rodents (50, 63) but were unsuitable for human use due to toxic manifestations. Attention has therefore been focussed on plant products aiming at a discovery of safe, orally effective antifertility agents. For centuries, virtually every indigenous culture has utilized plants in one form or another to limit its population. Informations acquired by ancient and remote people concerning the uses of herbs and various plant materials for fertility control purposes made it easy to select the proper plants for testing their antifertility effects in animals. Those informations also made available to WHO task force consisted of a list of more than 3000 plant species for which fertility regulating activity in both female and male had been claimed (64).

A number of plants have been investigated for their antifertility effects in male animals during the past decade. Some of them are as follows :

Malvaviscus conzattii : Alcoholic extract of the flowers of this plant when given orally to the dogs in dose of 150 mg/kg/day for 2 months caused testicular lesions resulting in a miss atrophy of the spermatogenic elements, the seminiferous tubules were aspermatogenic and showed disorganized epithelium, the lumen of epididymis and vas deferens were also devoid of spermatozoa (65). Further studies of the same extract in mice in dose of 50 mg/day/mouse for 50 days showed the same results (66).

Hibiscus rosa sinensis

Benzene extract of the flowers of this plant given orally to the male rats in dose of 250 mg/kg/day for 30-60 days resulted in impairment of spermatogenesis and reduction in testicular weight, other accessory sex organs and pituitary. These effects were reversible after discontinuation of treatment. Histologically, the thyroid and adrenal were apparently unaffected. It was proposed that the inhibitory effects of H. rosa sinensis on spermatogenesis are selective; mediated via pituitary, without affecting pituitary-adrenal and pituitary-thyroid axis (67).

Aristolochia indica

The water soluble part of the chloroform fraction of A. indica orally given to mice in dose of 75 mg/kg every 3rd day for 7 times in 19 days cause degenerative changes in the seminiferous germ cell components with prominent nuclear degeneration in all cell types. It also caused a marked decrease in the weight of testis and other accessory genital organs (68).

Ocimum sanctum

Fresh leaves of O. sanctum was investigated for antifertility effects in mice in dose of 465 mg/mouse/day for 30 and 90 days. Histological studies showed some impairment of spermatogenesis i.e. the spermatogonial cells are adhered together in some places of the tubular luminar, the spermatid bundle are not properly developed.

Sperm which are scattered through all the tubular lumen and interstitial cells are very sparse and in degenerate condition. Biochemical studies also showed a decrease in pH of seminal plasma and mucoprotein (69). Further studies of benzene extract of this plant in rats in dose of 100-200 mg/kg/day for 15 days showed a decrease in testicular weight, sperm count and motility (70).

Papaya seed

Oral administration of seed powder of riped papaya fruit to male rats in dose of 20mg/rat/day for 60 days caused 43% of the treated animals infertile. The weight of genital organs, spermatogenesis and sperm motility were not affected. The ripe papaya seed powder, however, might have some toxic effect as demonstrated by the reduction in body weight gain (71).

Solanum xanthocarpum

Solasodine, a nitrogen analogs of Diosgenin, was extracted from berries of this plant. Investigation for its antifertility effect by giving 20 mg./kg. alt day for 30 days to male dogs showed a severe impairment of spermatogenesis. Solasodine given to castrated dogs with testosterone propionate fail to stimulate epididymis growth, indicating its antiandrogenic property (72).

Momordica charantia

Momordica charantia or bitter gourd is a herb belonging

to the Curcubitaceae family. Two varieties of them are commonly found in Thailand. Marachin the large varietyin Thai word and Marakenok also in Thai word the small variety. The fruits of both varieties are consumed as vegetable in many Thai dishes : Fruits, leaves and roots of this plant are used in traditional medicine to cure a number of diseases, including Diabetes millitus. Fruit juice of Marakenokin dose of 6 ml/kg was reported to reduce blood sugar in normal and diabetic rabbits (73). Studies on antifertility effects of M. charantia fruit extract in male gerbils showed a reduction in testicular weights anddisruption of spermatogenesis without significantly affecting seminal vesicle or ventral prostate weight, the extract was found to be active orally and subcutaneously when doses of 200-400 mg/kg were given for a period of two weeks (74). The same extract when given orally to male dogs (1.75 gm/day/dog) for 60 days resulted in a reduction in testicular weights. Histological studies also showed spermatogenic arrest at spermatid stages (74).

Ocimum basilicum

Basil or Horapha (Ocimum basilicum), a plant belongs to the Labiatae family is well known throughout Thailand as flavouring herbs in many native Thai dishes. It possesses a clove-like scent with an aromatic, somewhat a saline taste. Essential oil of basil has many uses such as favouring confectionery, foods, condiments,

dental creams and mount washes. Because of its characteristic odour, it is also used in certain perfume composition. Moreover, basil oil passesses insecticidal and insect repellant properties, it is effective against housefies and mosquitoes. It is also bactericidal (75). Steam distillation of fresh leaves and air-dried leaves gave volatile oil that showed a positive anti-wormal response on earthworm as compared with piperazine citrate (76). Basil was also claimed as a contraceptive plant drug. However, only one report has been found so far and no detail of experiment appeared (77).

Apium graveolens (Koenchai)

Chinese celery or Koenchai as locally called in Thailand is one of the plant species that has been claimed to possess antifertility effect in the male. The plant has been used as a vegetable by the Thai and the Chinese in Thailand. There has been one report on the antifertility effect of Koenchai in Thai human male (78). Koenchai given in a dosage of 85 gm/day in both fresh and cooked forms manifested definite suppression on testicular function in respect to sperm count. Active sperm seemed slightly to be inhibited in most cases. In rats fed either orally or parenterally decreased sperm count, cauda epididymis and prostate and seminal vesicles weights were observed in the group of rats taking aqueous, alcoholic, petroleum ether extracts and aromatic distillate of both leaves and seed with statistical significance. The overall sperm motility was not changed. In rabbits, all preparations used i.e.

ingestion of fresh leaves and stem, petroleum ether extract of both leaves and seed & aromatic distillate of leaves and stem had proved to contain active principles which exerted definite and marked suppressive effect on sperm density (80-99 %). The sperm count went down rapidly to zero level so that the maximum effects were obtained at 1-5 weeks of treatment in most of the animals. The sperm motility was slightly decreased at the peroid of maximum effect in which some degree of morphological change was also observed (79).

Preliminary studies on the antispermatogenic effects of those plants give informations which are advantageous for further studies. Some plants may show toxicity during treatment, thus our attention in developing contraceptive drugs from them are dropped or we might purify the extract to remove the toxic fraction. It is even more interesting that Hibiscus rosa sinensis, Malvaviscus conzattii and Gossypium hirtusum are all belonged to the Malvaceae family which exhibit antispermatogenic effects. Thus plant species which are closely related to each other (same genus and family but different in specie or variety) should be evaluated for antifertility effect.

Aim of studies

The objectives of this study is to extend investigation on male antifertility effects of some plant extracts. The plants selected are commonly found and consumed in Thailand, these are the followings:-

- volatile oil of whole plant (without root) of O. basilicum and O. sanctum.

- alcoholic extract of M. charantia fruit of both large and small varieties.

Studies were emphasized on changes in sperm concentrations, sperm motility in the cauda epididymis, weight of cauda epididymis, testis, seminal vesicle and ventral prostate in male rats and mice.