ALTERATION OF RAT REPRODUCTIVE ORGAN IN ADULTHOOD CAUSED BY THE EXPOSURE OF FOREIGN ESTROGENIC COMPOUNDS (MOSQUITO INSECTICIDES) DURING EARLY LIFE

Thesis of Master Program in Biomedical Science

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APPROVAL SHEET

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TABLE OF CONTENTS

LIST OF TABLE

Table 16. The p-value of comparison prostate gland characteristics using Masson's Trichrome staining methods between experimental groups.

LIST OF FIGURE

LIST OF ABBREVIATION

1. AMH = Anti Mullerian Hormone
2. ARs = Androgen Receptor
3. BPA = Bispherol A
4. DDT = DichloroDiphenylTrichloroetane
5. DES = Diethylstilbestrol
6. DHT = Dihydrotestosterone
7. ECM = Extra-Cellular Matrix
8. ERE = Estrogen Response Element
9. ERs = Estrogen Receptor
10. FGF = Fibroblast Growth Factor
11. FSH = Follicular Stimulating Hormone
12. G1 = Growth phase (cell cycle phase)
13. HAA = Hormonally Active Agent
14. HE = Haematoxyllin Eosin
15. hCG = Human Chorionic Gonadothropin
16. Id-1 = Inhibitor of Differentiatio
17. LH = Luteinizing Hormone
18. MMP-7 = Matrix Metalloproteinase-7)
19. PAP = Prostate Acid Phospatase
20. PCBs = Polychlorinated Bisphenyls
21. PIN = Prostate Intraepithelial Neoplasia

22. PRL = Prolactine

23. PSA = Prostate Specific Antigen

24. S = Syntesa phase (cell cycle phase)

25. SRY = Sex-determining Region of the Y Chromosome

26. TGFβ = Transforming Growth Factor β

27. TRPM-2 = Testosterone Repressed Prostatic Message-2

LIST OF SUPPLEMENT

ALTERATION OF RAT REPRODUCTIVE ORGAN IN ADULTHOOD CAUSED BY THE EXPOSURE OF FOREIGN ESTROGENIC COMPOUNDS (MOSQUITO INSECTICIDES) DURING EARLY LIFE

Tri Indah Winarni

ABSTRACT

Background : Over the past decades, there has been increasing concern about the impact of environmental estrogen that act like hormone in human reproductive development and health. Its compounds may occur not only through industrial and agricultural activities, but also come from household product such as insecticides.
Objectives : to elucidate the clinical effect of proposed estrogenic compound during young period of male rat on inducing alteration of reproductive organ development in adult
Methods : Design of this study is true experimental design with the post test only control group design. Newborn male SD rats (n=88) were randomised allocated into 6 groups (negative control (n=15), positive control (n=11), estrogen 25 μg (n=15), burn insecticides (n=18), liquid insecticides 3 ml (n=20), and liquid insecticides 4 ml (n=9)). Treatment was given for 20 days. At 100^{th} days old, all rats were killed, testis, penis and prostate gland were removed for further measurement. Recorded data were testis volume and weight, penis length and diameter. Histopathological section of prostate gland were examined under HE and Masson's Trichrome staining.
Result : β-estradiol-3-benzoat (a high potent estrogen) treatment in alternate days for 20 days caused significant lower testicle volume (0.3±0.08 ml) and weight (0.66±0.17 mg), shorter (0.84±0.08 cm) and smaller (0.29±0.05 cm) penis compare to positive control group ($p<0.05$). Exposure of burn mosquito insecticides caused significantly exhibit shorter (1.1±0.07 cm) and smaller (0.4±0.03 cm) of penis compare to negative control group ($p<0.05$). Exposure of 4 ml liquid insecticides caused significant smaller (0.41±0.03) penis diameter compare to negative control group ($p<0.05$). Both β estradiol 3-benzoat and foreign estrogenic compunds caused alteration of histopathological characteristics of the prostatic gland in adulthood.
Conclusion : In general, exposure of burning mosquito insecticide coil, liquid mosquito insecticide and administering of 25 μg β estradiol 3-benzoate to neonatal male SD rat for 20 days can cause alteration of reproductive organ in adult.

Keywords: β-estradiol-3-benzoat, foreign estrogenic compound, insecticide, penis, testis, prostate, HE, Masson's Trichrome

Chapter 1

INTRODUCTION

1.1. Background

Over the past decades, there has been increasing concern about the impact of environmental compounds that act like hormone in human development and reproductive health. There have been several reports concerning adverse effects in male reproductive health and the possible role of such environmental chemicals (hormone-like) also postulated. Exposure of the compounds may occur not only through industrial and agriculture activities, but also come from compounds used in food production and food packaging such as pesticides residues consisted in food and vegetables and food contaminated by compounds found in can lining and plastic wrapping.[1] Total amount of environmental contaminated by synthetic chemicals that gives an estrogen signal is not known; however 45.000 metric tons of the weak estrogen such as p-nonyl-phenol were produced in 1976, and by 1982 the total annual production of all alkyl phenol polyethoxilates was estimated at 140.000 metric tons. In 1993 bispherol A (BPA) production in the United States was 640.000.000 kg/44.000 kg (0,10%) and were reported recycled, land filled, incinerated, or released to the environment.[2]

There are numerous reports of reproductive and developmental abnormalities in species that have been associated with exposure to environmental hormone/estrogen like hormone. Environmentally estrogenized phenotypes may differ depending upon the time of exposure, i.e., whether the exposure occurred at a developmental (organizational and irreversible) or post-developmental

(activation and reversible) stage.[2] Reports of reproductive and developmental abnormalities are ranging from snail species to humans that have been associated with exposure to environmental hormones. Perinatal and neonatal exposures to various concentrations of natural and synthetic estrogens cause irreversible organizational changes in the developing male rodent reproductive tract. [3] Reduction of phallus size and alteration of estrogen-testosterone level seen in alligators in lake Apopka Florida suggest an abnormal developmental duo to exposure to the environmental estrogen/anti-androgen.[4] In human, there have been reported on declining sperm count during the past 50 years. Man-made chemicals that mimic the sex hormone estrogen may incriminate for the falling sperm counts and other reproductive problem. [5]

Research studies concerning exposure to estrogen compound that altered reproductive organ behavior such as bispherol (a monomer polycarbonate plastic), pimazide (dopamine antagonist), methoxychlor and DDT (an organochlorine pesticide), estradiol-17-β, estrogen benzoate, actylphenol (emulsifiers are found in detergents, paints, plastic wraps, textiles and cosmetics) have been done in many countries and regions. [6] Most of people in developing country, such as Indonesia, lives in rural area and less education. There are so many advertisements, especially on television that usually mislead the people to consume the products without any concern against its side effect. Such advertisements are an effective tools and very easy to attempt people adopting and implementing the advertisement proposed as daily living behavior, for example: using insecticides, plastic wraps, cosmetics and detergent.

Many pesticides as well as insecticides have been reported and classified as endocrine disruptors such as DDT, dieldrin, toxaphene, and endosulfan. [7,8] To overcome the mosquito resistancies, recently, the use of combination of pyrethroids and carbamate insecticides in agriculture and in household has been encouraged by promotion of laboratory evidence's, suggesting that they are relatively safe to humans and wildlife.[7] Some of the pyrethroids and carbamate insecticides are believed to be toxic to the reproductive system and disruptive to endocrine function. [9] however little has been done to assess their hormonal potential activities *in vivo* as well as *in vitro* especially to focus on estrogenic activities.

1. 2. Research Statement

Since the turn of the century, manufacture and the use of synthetic chemicals has been rapidly increased. However the capacity to measure the extremely low level exposure of the chemicals, especially toxicological effect on human hormones's pattern has outpaced the ability. Based on the above statement, the safety daily used of insecticide products as seen on television could be questionable, especially when it is used for young children that may alter reproductive organ development in the near feature.

1. 3. Aim of the Study

1. To elucidate the clinical effect of proposed estrogenic compound during young period of male rat on inducing alteration of reproductive organ development in adult.

2. To investigate whether burning mosquito insecticide coil and using liquid mosquito insecticide have the same effect as β-estradiol-3-benzoate that cause alteration reproductive organ development and behavior.

1. 4. Benefit of the Study

This experiment is expected to increase awareness and alertness concerning insecticide daily usage in the family in order to assure normal reproductive development and health of the children and discourage from abnormal sexual differentiation, alteration behavior, infertility and the development of disease/malignancy in their adult life.

Chapter 2

LITERATURE REVIEW

2. 1. Endocrine Disruptor

Endocrine disruptor has been defined as an exogenous agent that interfere with the synthesis, secretion, transport, and action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or organ's behavior. There are a number of term that have been applied to the chemicals in the environment that are believed to affect the endocrine system. These include endocrine disruptor, hormonally active agent (HAA), environmental estrogen, endocrine modulating substance, eco-estrogen, xeno-estrogen, and endocrine active compound.[10]

Environmental chemicals can produce endocrine disruptors in a variety of ways. They may affect hormone secretion from endocrine gland, alter the rate of elimination from the body, and may disrupt regulatory feedback. [11] Finally they may mimic a hormone by binding to its receptor, block and alter endogenous hormone binding to hormone receptor or influencing cell signaling pathways, stimulate the production and the function of hormone receptor that can amplify or alter the effect of the endogenous hormones.[12] Some of them occur naturally as phytoestrogens and some of which are synthetic chemicals or called man-made. The former are presence in grains, legumes, grasses, herbs, nuts, fruits and vegetables, while the later are presence in soil, water, air, food, pharmaceuticals (birth control, Diethylstilbestrol/DES), product associated with plastics (bispherol

A, phthalate), pesticides, fungicides, insecticides, ordinary household product (nonylphenol, octylphenol), and industrial chemicals (dioxine, polychlorinated bisphenyls/PCBs).[12] Although most of man-made estrogens is weaker than natural or endogenous estrogen, they have several characteristic such as lipophilicity and long half-lives that allow them to accumulate and persist in fatty tissues of the body's fat of animal and human. They do not break down readily in the environment, they can accumulate in soil and sediment, which serve as continued sources of exposure to all species. Consequently, species feeding at highest of the food chain are the most vulnerable to the adverse effect of environmental pollutant [12,13]

Because of the endocrine system is the most important integrating and regulatory systems, disruptions could potentially affect development, growth, behaviors, sexual differentiation, fertility, and also might play a role in the development of disease such as prostate and testicular cancer.[14] The body carefully controls synthesis, secretion, release, and elimination of hormones, and uses regulatory mechanism such as feed-back mechanism to control modest fluctuations of hormone concentrations.[14] Normally, absorption and detoxification of endocrine disruptors would be expected to further decrease circulating levels of substances.[14] The developing embryo, fetus and young children may not have the same regulatory capacities as adult, because they have immature immune system and rapid growth cycles. Therefore they may become more sensitive to the effect of endocrine disruptor.[9,14]

2. 2. Male Genital Development

2. 2. 1. Testis

2. 2. 1. 1. Testis Development

The sex of an embryo is determined by X and Y chromosomes. The presence of Y chromosome leads to maleness regardless of the number of X chromosome. In the opposite, the absence of Y chromosome will result in female pattern development.

Testis develops from undifferentiated gonad. The gene called SRY gene (Sex–determining Region of the Y chromosome) is responsible for gonad differentiation.[15] Expression of SRY gene leads to the medulla of undifferentiated gonad develop into testis. Subsequently, differentiation into the male is induced by the production of two factors i.e. (1) Leydig cells begin to produce testosterone, leading to stimulation of the Wolfian ducts to form the male internal genitalia; (2) Sertoli cells produce AMH (Anti Mullerian Hormone) which causes regression of the Mullerian ducts.[16] Testis is divided into two compartments that are morphologically and functionally distinct from one another. One compartment is responsible to the processes of gamete production called spermatogenesis, and the other is responsible to the production of male steroid hormone called steroidogenesis. These are the tubular compartment, consisting of the seminiferous tubules and the interstitial compartment between the seminiferous tubules. Spermatogenesis takes place in the tubular compartment and steroidogenesis in the interstitial compartment. The integrity of both compartment is absolutely needed for the quantitatively and qualitatively normal sperm

production. The two essential function of testis is principally govern by structure and function of hypothalamus and pituitary gland. [17] Tubular compartment consists of germ cells and supporting cells. The supporting cells include the sustentacular cells of the basement membrane and the Sertoli cells. The germ cells consist of epithelial cells population, including slowly dividing primitive stem cells population, the rapidly proliferating spermatogonia, spermatocytes that undergoing meiosis, and the metamorphosing spermatids. The seminiferous tubule is surrounded by several layers of peritubular tissue. These consist of peritubular myoid cells which contain actin type of typical smooth muscle, which confers contractile capacity on the seminiferous tubules.[17,18]

Sertoli cells proliferation occurs on day 16 of embryonic life in the rat and reach a maximum in adulthood. The testis is approximately consists of 1 million Sertoli cells on 2 days before birth. The number will increase approximately to around 40 million at days 15 of postnatal life. After day 15th, proliferation ceases and the number of the Sertoli cells in the testis remains stable throughout adulthood. Proliferation of Sertoli cells are principally regulated and induced by Follicle Stimulating Hormone (FSH), that are secreted from pituitary gland. FSH secretion is depended on balance of the hypothalamo-pituitary-testis axis, which is maintained by negative feedback action of testosterone. It is becoming clear that negative feedback action of testosterone on gonadotropin or FSH secretion is mediated via aromatization testosterone to estrogen. [19] Estrogen administration can cause decreases circulating gonadotropin levels, while administration of aromatase inhibitor cause increases circulating gonadotropin. FSH secretion in

neonatal life appears to be extremely sensitive to estrogen exposure [19,20] The Sertoli cell is characterized by its irregularly shaped nucleus, prominent nucleolus, low mitotic index or mitotically inactive in adulthood, Sertoli–germ cells connections, has an unique tight junctional complexes between adjacent Sertoli cell membrane. The Sertoli cells rest on the basement membrane of the seminiferous tubules and extend filamentous cytoplasmic ramification toward the lumen of the tubule. Germ cells are arranged between these Sertoli cell cytoplasmic projections. The undifferentiated spermatogonia are located near the basement membrane of seminiferous tubule, whereas the more mature spermatocytes and spermatids are arranged at higher level in this epithelial near the lumen of the tubule. The two important function of Sertoli cells are: 1) build tight junction between each other called blood-testis barrier, the physical isolation of haploid male gamete which is not recognized by the immune system and for preparation of the special milieu for the meiotic process and sperm development, and 2) synthesis and secretion a number of substance such as Androgen-binding protein, the growth factor inhibin and activin, extracellular matric component, and protein. [17,18]

Interstitial compartment of the testis contains blood vessel, lymph vessel, fibroblastic supporting cells, macrophages, mast cells and Leydig cells. Leydig cells develop from mesenchymal cells and from fibroblast-like cells in the interstitium, and the differentiation of these cells is induced by LH (Luteinizing Hormone). The proliferation rate of the Leydig cells in the adult testis is low and is influenced by LH. The adult Leydig cells are rich in smooth endoplasmic

reticulum and mitochondria with tubular crists. These physiological characteristics are typical for steroid-producing cells. The regulation of testosterone production is dependent on LH.[17,18]

Human testicular volume is determined by several factors i.e. the interstitial compartment represent 12%-15% of total testicular volume, 10% - 20% of which is occupied by Leydig cells. The tubular compartment represents about 60%-80% of testicular volume, 35% - 40% of which is occupied by Sertoli cells.[17]

2. 2. 1. 2. The role of estrogen in male

Estrogen exerts a wide range of biological effect on large variety of cell type, for example it regulates cell growth and apoptosis, major regulator of many physiological functions in the adult, especially those associated with reproduction. Furthermore estrogen is involved in the organization and differentiation of the developing organism, notably of the endocrine system, nervous system and peripheral reproductive structure.[21] Interesting about the role of estrogen is increasing in recent years, especially in male reproduction tract. The largely interesting of estrogen role in male reproduction tract is mainly due to the demonstration that male fertility is impaired in mice by lack of estrogen receptor and enzyme aromatase.[19] Concentrations of estrogen receptor (ERs) are higher throughout the male reproductive tract than in the other organ. [22] The number of male tissues have the capacity to express aromatase and synthesize estrogens, these include: testis, adipose tissue, chondrocytes and osteoblasts of bone, and numerous site in the brain including several areas of hypothalamus, limbic

system, and cerebral cortex. Estrogen has been recognized being synthesized in male. That biosynthesis it self was catalyzed by a microsomal member of the cytochrom P 450 superfamily, namely aromatase cytochrome P 450. [19]

ERs and aromatase are found at all stages of testicular development in the rodent. Around the time of birth, the testis continuous expressing both ERs and aromatase. Neonatal Sertoli cells are more active in producing estrogen than neonatal Leydig cells, suggesting that the Sertoli cells are an important source of estrogen in the postnatal testis. At this stage, germ cells have been reported not to contain detectable aromatase. During day of 10-26th, Leydig cells and Sertoli cells are divided and undergo functional maturation. During adulthood, aromatase activities in the Leydig cells are higher than in Sertoli cells. Decreasing in aromatase activity during Sertoli cells maturation into the adult form may be related to the control of Sertoli cells division and adult function. In adult germ cells, aromatase activity is found from pachytene spermatocytes stage into around spermatids. When round spermatids begin the morphological transformation into elongated spermatids, aromatase continuous to be found in the cells. Its suggesting that the adult germ cells are an important source of estrogen in the testis and so do adult Leydig cells. Summaries of the reviewed above, it seem likely that the testis has capability to regulate "supply and demand" by synthesizing and responding to estrogens throughout all stages of development. [19] The role of estrogen in hypothalamo-pituitary-testis axis is a major component of negative feedback regulator of gonadotropin secretion. It is becoming clear that the negative feedback action of testosterone on gonadotropin secretion is mediated

by estrogen via aromatization. Estrogen administration and deficiency can affect hypothalamo-pituitary-testis axis balance [19]

The fetal Leydig cells presented at birth are not progenitor of the adult Leydig cells population. During pre-pubertal period, there is rapid growth of Leydig cells, which arises from mesenchymal precursor cells. More mature Leydig cells have high level of aromatase activity, postulated that the mature Leydig cells may produce estrogen that inhibit precursor Leydig cells development. Inappropriate exposure to estrogen during Leydig cells development (pre-pubertal) can cause permanent changes to Leydig cells function, to produce androgen that is necessary for spermatogenesis. [19] Proliferation rate of sertoli cells in the rat reach a maximum 2 days before birth. Sertoli cells produce considerable amount of estrogen during the period of division/proliferation, decline as Sertoli cell mature. Inappropriate exposure to estrogen during differentiation can cause delay of Sertoli cells maturation. Inhibition of Sertoli cells maturation can cause impairing of spermatogenesis in adult.[19]

The development of germ cells is well known to be dependent on the action of Follicular Stimulating Hormone (FSH) and testosterone on the Sertoli cells. Both hormones have been shown to prevent germ cells apoptosis as well as to promote division and differentiation. Aromatase activity and ERs are found in various stages of germ cells development. There is accumulation evidence that estrogen has predominantly stimulatory effect on germ cells development.[19] Recently, estrogen has been proven to act as germ cells survival factor that preventing germ cells from apoptosis. *In vitro* studies using human adult

seminiferous tubules cultured, pointing that estrogen is a potent inhibitor of germ cells apoptosis instead of testosterone.[23]

Although the action of estrogen on Leydig cells and Sertoli cells appear to be mainly inhibitory, there is accumulating evidence that estrogen has a stimulatory effect on germ cells.[19]

2. 2. 1. 3. Estrogenic exposure on testis development

There is growing interest that estrogen plays a role in normal male reproductive development and function. This is based on information such as the widespread distribution of estrogen receptor in the testis and reproductive tract fetal life through adulthood, in the contrary, it is clear that exposure of the developing male to exogenous estrogen either *in utero* or neonatally can result in a range of abnormalities of reproductive development and function. There are difficult to relate concerning about whether weakly estrogenic environmental estrogens can induce impairment of spermatogenesis and whether human exposure to such compound will cause falling in sperm count or disorders of male reproductive health [20]

Perinatal and neonatal exposures to various concentrations of natural and synthetic estrogens cause irreversible organizational changes in the developing male rodent reproductive tract. In particular, high doses of estrogens have been demonstrated to cause such profound permanent effect as feminization, reduction of organ weight, reduced sperm production, and fertility. In the contrary, low doses of natural estrogen cause the inverted manifestation of some of these effect,

which in some cases are present only transiently in the younger animals but in others remain into adulthood.[3]

There has been proved that the estrogens derive from local aromatization of androgens or produce by testis, can exert feedback effect on the neuroendocrine component of the male reproductive axis.[24] Exposure of high dose of potent estrogens can cause significant suppression of FSH level consistently,[20] although exposure of low dose of potent estrogen or high dose of weak estrogen can cause significant increase in FSH production.[25] Elevated FSH level in normal range of adulthood may improve the efficiency of spermatogenesis.[20]

Regardless of various treatment on pubertal spermatogenesis and testicular development suggesting that only exposure to high doses of potent estrogen is able to induce long-term adverse changes in testis size, matting, or fertility. However, numerous reports in the literature show that neonatal administration of estrogen to rodents results in delayed development and /or permanent impairment of spermatogenesis, because administration of potent estrogens in neonatal, could cause suppression FSH secretion at the time when this hormone is playing an important role in both Sertoli cells development and the expansion of spermatogenesis. This suppression explains the consequences of neonatal estrogen treatment.[26]

Exposure of 10 μg DES/Diethylstilbestrol (high dose of potent estrogen) to neonatal rat can causes reduction of adult testis weight about 60%, with many Sertoli cell-only tubules in histopathological section, and very low daily sperm production.[26] Testis weight in adult rat provides a reliable guide to total germ cell

number per testis, this end point was used as quantitative measure of any changes to spermatogenesis.[25] Furthermore, environmental estrogen exposure during developmental stage cause decreasing sensitivity to androgens, leading to the risk of testicular malignancy.[27] Adverse effect of estrogenic compounds to male reproductive differentiation and function has been reinforced by the fact that several group of compounds are used daily in industry, agriculture, and also produced at home. So, the incidence of testicular cancer has been significant increased, being 2-3 times higher than 3-4 decades ago.[27]

2. 2. 2. Penis

2. 2. 2. 1. Penis Development

The external genital organ develops from an indifferent state before distinguishing sexual appearance. In the third week of development, external genitalia primordial composed of proliferating mesenchyme covered with ectoderm, arise around the cloacal membrane. Located between the primitive umbilical cord and the tail, to form the pair of slightly elevated cloacal fold. Cranial to the cloacal membrane the fold unit to form the genital tubercle, and caudally, the fold are subdivided into urethral folds anteriorly and anal folds posteriorly. Cloacal membrane is divided by urorectal septum into a cranial urogenital membrane and caudal anal membrane. Elongation of the genital tubercle, urogenital membrane and the urethral folds produces a primitive phallus. The urogenital sinus (contiguous with the internal aspect of the urogenital membrane) becomes attenuated within the elongating phallus forming the

primitive urethra. In about seventh week, the urogenital membrane breaks down allowing communication of ectoderm and endoderm at the edge of the disrupted membrane and continuity of the urogenital sinus with the amniotic cavity. The endodermal layer of attenuated distal portion of the urogenital sinus displayed on the caudal aspect of the phallus is termed to be the urethral plate. With the proliferation of mesenchyme within the urethral folds, the urethral plate sinks into the body of the phallus forming primary urethral groove. The urethral folds meet proximally in the transverse ridge immediately ventral to the anal membrane.[16,28]

Development of genitalia external requires fetal secretion of dihydro testosterone (DHT) that converted to testosterone by the enzyme 5α-reductase in certain tissues.[16] Fetal production of androgens, especially testosterone, is necessary for normal male development. Early in gestation, placental chorionic gonadothropin (hCG) stimulates the developing testis to produce testosterone. Later in gestation after organogenesis has occurred, the fetal pituitary takes control through production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Failure of adequate gonadothropin stimulation or testosterone production, or both, toward the end of gestation can cause in inadequate penis growth.[29] The hipothalamo-pituitary-gonadal axis is active in late fetal life, shortly after birth, gonadothropin and testosterone production begin fall. By age 6 months, testosterone level increases in pre-pubertal age, the penis growth occur during this time.[29] **The penis remains infantile if hypogonadism becomes manifest before onset of normal puberty**. If hypogonadism appears before

puberty, changes in penis size will be manifest. However, if hypogonadism appears after puberty, changes in penis size my slightly occur due to apoptosis.[17]

Growth and differentiation of the penis is stimulated by the presence of androgen (androgen dependent) especially dihydrotestosterone (DHT) and inhibited by estrogens by means of negative feed back.[4] Alligators in Florida's Lake Apopka were exposed to DDT have significant smaller penis size, lower plasma testosterone concentration, and lack of responsiveness of the penis to the plasma androgen present. Therefore penis size seems likely an obvious marker of abnormal androgen concentration or function before puberty.[4]

2. 2. 2. 2. Estrogenic exposure on penis development

The development of the male reproductive ducts and genital external in vertebrates is dependent on androgen level during embryonic development and the period of post-natal growth.[4] Androgen hormones especially testosterone are responsible for differentiation of Wolfian duct into male reproductive duct, while dihydrotestoterone (DHT) is importance for formation of external genitalia.[4] The increasing frequency of developmental abnormalities of male phallic structures is reported in humans, fish and alligators. Environmental estrogen exposure during embryonic development, directly influences the development of copulatory organ and lacking of androgen during developmental period can result abnormalities of penis differentiation, such as cryptorchismus, hypospadia, small penis size, and testis cancer.[4]

Several hypothesis can be developed to explain the decreasing penis size in the juvenile male alligators from Lake Apopka: (1) males may have decreased plasma androgen concentrations that will result in reduction stimulation of the penis development and therefore reduces penis growing; (2) the relative ratio of testosterone to dihydrotestosterone can be altered by the suppression of the enzyme 5α-reductase, resulting in reducing penis size; (3) reduced numbers of androgen receptors on phallic tissue will also result in a reduction of the penis size; (4) the presence of an androgen antagonist that will effectively compete for the androgen receptor and block normal androgen stimulation that produce inhibition of phallus growth; and (5) the presence of a xeno-biotic estrogen could shift the ratio of estrogen to androgen toward a feminizing environment in the developing embryo, thus blocking phallic development or inducing phenotype poorly responsive to androgens later in life.[4]

2. 2. 3. Prostate

2. 2. 3. 1. Prostate development

The prostate arises from interactions between urogenital sinus mesenchyme and the endoderm of the proximal part of urethra.[16] Urogenital sinus is the origin of prostate that form during embryogenesis through epithelial budding. The most rostral part of urogenital sinus forms the urinary bladder, whereas the most caudal part forms the penile urethra. The prostate gland originates from the intermediate region of urogenital sinus. In the mouse, prostatic buds first emerge at the rostral end of urogenital sinus at approximately 17,5 days

of gestation, toward the end of gestation. Subsequently, the prostatic epithelial buds undergo extensive ductal outgrowth and branching into the surrounding mesenchyme during the first three weeks postnatal development.[30] Prostate formation and differentiation requires interaction between epithelial and mesenchymal/stromal tissue. Firstly, androgen has been acting on the mesenchyme in order to produce signal for prostatic induction and growth. Subsequently, androgen acts on the epithelium for secretory function of differentiated cell types. Because interaction between the epithelial and stromal components are essential for all stage of normal prostate growth and development, disturbing of its interaction may plays a significant role in developing carcinoma. The signals that mediate such mesenchymal-epithelial interaction includes Fibroblast Growth Factor (FGF) and transforming Growth Factor-β (TGF β) families. [30,31] In the normal developing prostate, periductal stromal cells differentiate to form a multicellular layer of smooth muscle cells and a thin layer of fibroblasts that maintain intimate contact with the basal membrane of luminal epithelium. Signals from the stromal are believed to be critical in determining the decision to epithelial cells to undergo proliferation, apoptosis, or differentiation.[32]

The rat prostate gland is rudimentary at birth and undergoes extensive branching morphogenesis followed by functional differentiation during the first 15 days of life.[33] Androgen induced development, includes branching morphogenesis, cellular differentiation, smooth muscle differentiation, and segregation of the epithelial into luminal as well as basal subtypes and ductal canalizations.[31] Developing rodent prostate is also sensitive to other hormones

including estrogen. As male reproductive tract development is depended on estrogen hormones, reducing these hormones may affect genetically males develop into male phenotypic, but infertile.[31]

Within the prostatic epithelium, there are at least three distinct epithelial cell types that can be distinguished by their morphological characteristic, functional significance, and relevance for carcinogenesis. First, the predominant cell type is the secretory luminal cell (a differentiated androgen dependent cell that produces prostatic secretory proteins). The luminal cells are characterized by their expression of androgen receptors. Second, the major epithelial cell type is the basal cell (which are found between the luminal cells and the underlying basement membrane) and which form a continue layer in the human prostate, but not in the mouse prostate. The basal cells express low level of androgen receptor and do not produce prostatic secretory proteins. Finally, the third prostatic epithelial cell types is the neuroendocrine cell, a minor population of uncertain embryological region, which is believed to provide paracrine signals that support the growth of luminal cells. Neuroendocrine cells are androgen-independent cells, dispersed throughout the basal cell layer which express chromogranin A, serotonin, and various neuropeptides.[30]

The prostate gland is one of the male accessory glands. It encircles the part of urethra just inferior to the bladder, and enclosed by a thick connective tissue capsule. Inside the tissue's capsule are tubular-alveolar glands that are embedded in a stromal of smooth muscle and dense connective tissue. The rat prostate gland is divided into four lobes: the anterior lobes, dorsal lobes, lateral lobes and ventral

lobes. There is no clear analogy between the lobular structures of the rodent prostate and the zonal architecture of the human prostate, indeed, although several studies assert that the dorsolateral lobe is most similar to the human peripheral zone. [30]

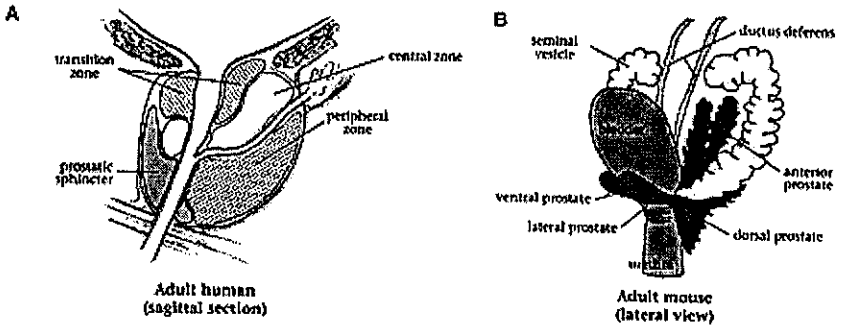


Figure 1. Schematic illustration of the anatomy of the human (*A*) and mouse (*B*) prostate gland.[30]

Prostatic secretion contributes 30% of ejaculate. The main function of the prostate gland is to secrete a milky, alkaline fluid into urethra at the point of ejaculation. The prostatic fluid has a pH of 7,5-7,7 and contains citrate (a nutrient source), bicarbonate buffer, fibrinogen, fibrinogenase, fibrinolysin, hyaluronidase, prostate acid phosphatase (PAP), and prostate specific antigen (PSA). Soon after the semen is ejaculated, fibrinogen in the prostatic fluid is converted into fibrin to form coagulum, after that liquefies after 30 minutes period. The liquefaction of the semen is responsible by the action of fibrinolysin. Coagulation of the semen appeared soon after ejaculation and liquefaction 30 minutes after ejaculation is an important role of successfully fertilization process.[17, 18, 34]

2. 2. 3. 2. Estrogenic exposure on prostate development

Prostate development is androgen dependent, that primary mediates the prostate differentiation. The possibility that estrogen may also involved in both the normal process of prostate development and subsequent adult prostate disease was raised up since 60 years ago. Modulating role for estrogen in prostate development was based on the expression of estrogen receptors in the mesenchyme of embryonic tissues. The increasing number of reproductive organ disorders have been linked to *in utero* exposure to endocrine-disrupting estrogenic chemicals in the environment. The elevation level of estrogen (natural or man-made) during fetal life may alter development of reproductive organs, (including prostate) that may be predisposed them for abnormal function and diseases in later life. During critical period in cell differentiation, hormones are involved in "imprinting" specific genes in the cells that encode receptors for the hormones.[35]

The stromal microenvironment is also an important determinant for the progression from normal prostatic epithelium to an invasive carcinoma. Alteration in the stromal compartment of prostatic tumors may enhance the invasiveness and /or malignancy of the nascent epithelial tumors. Elevation of estrogen level during critical differentiation period may affect the expression of gene involved in the morphogenesis of the gland. That in turn, resulting in persistent changes of the histological architecture of the gland and epithelial secretory pattern of prostate spesific acid phosphatase (PAP), finally altered the glandular cells function at puberty. Prenatal expose to weak environment estrogen affect the differentiation pattern of the periductal stroma cell of the ventral prostate. The presence of the

thicker layer of fibroblast, the reducing number of smooth muscle cells, and the significance of lowering percentage of stromal cells were demonstrated androgen receptor (ARs) in Bispherol A (BPA) treated animals. Present of thicker layer of fibroblast and reduction of smooth muscle cells on periductal stromal cells can act like "physical barrier" that influences branching of morphogenesis. The physical barrier may eventually block paracrine communication between smooth muscle and epithelial cells (which normally regulate differentiation) and enhance the invasiveness and/or malignancy potential of the nascent tumor. On the other hand, decreasing of ARs expression in periductal stromal cells, may also alter the androgen-signaling pathway.[32]

Several studies in neonatal rats have been exposed to higher dose of estrogen resulted in reducing responsiveness to androgen, permanent suppression of prostate growth, retard branching morphogenesis and epithelial differentiation during development, permanent alteration secretory function, and induction of epithelial hyperplasia in adulthood.[31] [36] This process referred as neonatal imprinting that associated with an increasing incidence of prostatic lesions upon aging which includes hyperplasia, inflammation, and dysplasia similar to intraepithelial neoplasia. [37] In male mice, exogenous administration of estrogens could cause alteration hypothalamic-pytuitary-gonadal axis and reduces androgen level, leading to regression of the prostatic epithelium or induces of epithelial squamous metaplasia.[38] The effects of estrogens are not only mediated by changes in androgen level via suppression of the hypothalamic-pituitary-gonadal axis and

subsequent reduction of androgen level, but also by additional direct effect on prostatic growth.[31]

Unlike human, rodent prostate development commonly occurs during 15 days of postnatal life, thus providing a window of susceptibility to hormonal disruption in early life. Brief exposure cause additional direct effect on prostate gland that induces:

1) Up-regulates estrogen receptor expression and down regulates androgen receptor expression [31, 36, 37]
2) Reduced responsiveness to androgen in adulthood.[31, 36, 37]
3) Alter epithelial cell adhesion and gap junction proteins. Junction proteins are macromolecular structures that are, essential for intercellular adhesion and communication. Signal transduction through cell junction-associated adhesion molecules has been implicated in the control of proliferation and differentiation of epithelial cells during normal and neoplastic development. [36, 39]
4) Alter the Transforming Growth Factor-β signaling system in the proatate development and block the transient $p21^{cip1/waf1}$ expression associated with epithelial differentiation. Normal temporal expression of TGFβ by prostate is only transient and peaking between day 10 and 15 and declining thereafter. This temporal expression with the transient expression of $p21^{cip1/waf1}$ (a cyclin-dependent kinase inhibitor) is happened within differentiation of prostatic epithelial cells. Elevation

of $p21^{cip1/waf1}$ in adult prostate cells, inhibits passage of cells from G1 to S phase in the cells cycle that preventing cell proliferation.[36,40]

5) Proliferation of periductal fibroblast and alter the ECM composition in the rat prostate. As mentioned before, proliferation of a multi-cellular layer of periductal fibroblasts in "estrogenized" prostate results in a physical barrier that influences branching morphogenesis and blocks paracrine communication between smooth muscle and epithelial cells which normally regulate differentiation.[36, 41] Thus referred to developmental "estrogenization", exogenous estrogen exposure, are associated with an increasing incidence of prostatic lesion upon aging and reduction of secretory function.[36]

Brief exposure of rat to high doses of natural or man-made estrogen early in life resulting in permanent alterations of the prostate gland, which includes differentiation defects, altered gene expression and dysplasia in conjunction with aging phenomenon. Whether low-dose treatments can cause similar effect in the development of prostatic gland, remains controversial. [3] Exposure of rat to high doses of exogenous estrogen during critical period exhibited a permanent alteration of the prostatic gland, including: Marked development and differentiation abnormalities; Dose-dependent reduction in adult prostate size; Altered secretory function during adulthood; Permanent alteration, particularly down regulation of androgen receptors (ARs) expression and up regulation of estrogen receptors (ERs) expression within the prostatic cells, and pertubation of the TGF-β signaling system of multiple cellular level in the prostate.[3] Fetal

exposure to a very small supplement of estradiol is associated with significant enlargement of the prostate (and changes in behavior) in adulthood. Certain studies have been proved that there is extremely strong relationship between the level of natural or man-made estrogen during developmental period (fetal and postnatal life) and prostate size as well as number of prostatic androgen receptor (ARs) during fetal life and in adulthood.[35] There is an inverted-U relationship between dose and response. The effect on prostate differentiation of high doses of natural or man-made estrogens are thus opposite to effect of low doses. Numerous studies have shown that exposure to high dose of Dietylstibestrol (DES) during prostate development result in an abnormality small prostate size and decrease the number of prostatic androgen receptors (ARs) in adulthood. While exposure of rat to very low doses of estrogenic compounds (estradiol and diethylstilbestrol (DES)) during critical period had been studied can cause enlargement of prostatic size permanently.[35]

2. 2. 3. 3. Prolactinemia during estrogenic exposure and prostate gland

Normal development, growth, and function of the prostate gland throughout life are dependent on androgens that act in synergy with the other modulating hormones such as estrogen and prolactine. In the rat model, prostate development is initiated late in the fetal life and undergoes extensive branching morphogenesis and cellular differentiation during 15 days life. Hormonal modulation during this critical development period can result a permanent and

irreversible effect on the gland's morphology, cellular organization, secretory function, and decreasing responsiveness to androgens in adulthood.[42]

Estrogen exposure in the neonatal rat has been shown to disrupt the normal morphology, development and the function of the prostate gland. The result to this exposure will manifest in the adulthood as epithellial hyperplasia, dysplasia, and chronic inflammation. The inflammatory responses consist of infiltrating T-lymphocytes and macrophages, that is typically observed in chronic prostatitis in both rodents and humans.[42, 43] These effects may deducted as a result from a transient period of hyperprolactinemia just prior to puberty. [6] Over expression of prolactine (PRL) can result in prostatic enlargement and dysplasia. This phenomenon indicates that PRL provides an additional growth regulatory mechanism for the prostate. [44]

Several environmental estrogens have been reported to show estrogenic activity. Pituitary lactotroph is well known established as estrogen's responsive cell. Estrogen exposure can affect releasing PRL by acting directly on the lactotroph cell the in pituitary anterior or indirectly via hypothalamus-pituitary's factor that regulate the lactotroph. The direct acting on the lactotroph is regulation of the transcription through on ERE (estrogen response element) that binds to estrogen receptor in the anterior pituitary, on the other hand, the indirect action is executed through inhibition of hypothalamic dopaminergic suppression pathways. [41,45] Thus, exogenous estrogenic exposure will result in relative hyperprolactinemia for variable lengths of time. Interestingly, estrogen-induced hyperprolactinemia in the adult rat has been shown to induce prostatic

inflammation. These mentioned findings are particularly significant that in neonatal transient estrogen exposure will increases pre-pubertal circulating prolactine level by altering development of dopaminegic neurons in the arcuate nucleus.[42] Thus, it could be deducted that the prostatic inflammation induced by neonatal estrogens is indirectly mediated through transient pre-pubertal hyperprolactinemia.[6, 42]

The effect of hyperprolactinemia in the prostatic gland development was lobe specific. The ventral lobe being most prominently affected with regards to growth, secretory function inhibition, and dysplasia.[42] In addition, another study showed that lateral lobe was being more affected with transient hyperprolactinemia phenomenon.[6] The basis for chronic inflammatory response in the neonatal estrogenized prostatic gland is not well understood. However, it was known both estrogen as well as prolactine have distinct effects on the immune system, particularly on the development of prostatic gland inflammation. It was known for sure that estrogen-induced immune response is mediated through hyperprolactinemia. Lymphocytes own prolactine receptors while prolactine it self act as comitogen for T cell proliferation as well as an induction of cytokine and antibody production.[42] The indirect action seems through inhibition of hypothalamic dopaminergic suppression pathways. [41,45]

Androgen and PRL had been shown to be involved in the developmental of prostatic hyperplasia in adulthood. It seems likely, it was due to the increasing prolactine levels that concordance with the advancement of age, whereas at the time testosterone levels decrease approximately.[43] Prolactine (PRL) up-regulates

5α-reductase activity, resulting an increased local level of dihydrotestosterone (DHT) and up regulation of ARs (androgen receptor) level in prostate, leading an increased response to androgen. However during the period of hiperprolactinemia, the absence of estrogen does not result in induction of malignancy in the prostate gland. [44]

Chronic or recurrent inflammation probably has a role in the development of many types of cancer in human, including prostate cancer. Inflammatory cells elaborate numerous microbicidal oxidants that might cause cellular or genomic damage in the prostatic cells. Prostatic lesion called proliferative inflammatory atrophic is apparently become a precursor of prostatic intraepithelial neoplasia and prostatic cancer. Focal areas of epithelial atrophy in the prostatic gland have long been noticed and as an important factor in developing prostatic carcinogenesis. Such atrophic areas, containing proliferative epithelial cells that fail to differentiate into columnar secretory cell, tend to occur in the periphery areas of the prostate, where the prostate cancers most commonly occurs in human. Proliferative inflammatory atrophic applies to focal atrophic lesion always associated with chronic inflammation and often directly related to prostatic intraepithelial neoplasia and/or prostate cancer.[46]

2. 2. 3. 4. Carcinoma of the prostate

Cells always constantly adapt to their environmental changing. These physiologic adaptations usually represent a response of cells to normal stimulation by hormones or endogenous chemical mediators. Pathologic adaptation uses the

same underlying mechanism, but they allow the cells to modulate their environment and escape from the injury that may happen. Some adaptive responses involve up or down- regulation of specific cellular receptors, induction of new protein synthesis by the target cell, switch from producing one type of protein to another type, and marked over production of a specific protein. Thus cellular adaptive response can occur at any steps of cells development and proliferation, including receptor binding, signal transduction, protein transcription, and translation. The adaptive changes of cell growth and differentiation are particularly important in developing pathologic conditions. These include atrophy (decrease in the size of cell by loss of cell substance), hyperthropy (increase in the size of cell), and anaplasia (change in the cells type). [47]

In general pathology, there are two classical and fundamental concepts of a disease, namely aetiology and pathogenesis. Aetiology refers to the agent that evokes disease, while pathogenesis refers to the mechanism through which the causative agent evokes the histopathological lesion, the clinical symptoms and the laboratory sign of disease. In cancer pathology, the patogenesis of neoplastic disease is often better more understandable its aetilogy. In recent years, it has become increasingly evident that various kinds of hormones can become both real aetiological agents of benign and/or malignant tumor that be actively involved in the pathogenesis of the neoplastic lesion. [48]

The role of hormones in regulation of growth and development has been known for a long time. Hormones are important regulators of growth by

stimulating proliferation. However hormones may increase the risk of mutation and at the same time stimulate the replication of the mutated cell. Thus hormones may become such carcinogens. In the other hand, the importance of sex hormones in prostatic glands development is clearly understood. [48]

Carcinogenesis is multi step process involving mutations, gene regulating growth control, and resulting in increased stimulation (oncogenes) or removal of inhibitor (tumor suppressor genes). Mutagen agents increase the risk of mutation via direct effect on the genes. While hormone may stimulate the growth of cells including mutated cells. Therefore, hormones have been though to be an important factor as co-carcinogens. By stimulating mitosis, hormone increases the risk of mutation by increasing number of division. Thus, hormone not only become co-carcinogens, but also the most important carcinogens by means of increasing the risk of mutation in the normal organ and so stimulating the growth of the mutated cells between the normal organ or tissues.[48] The lipid soluble steroid hormones affect the growth of their target cells by interaction with hormone responsive elements (receptors) in the nucleus, affecting the regulation of gene expression, While peptide hormones (water soluble) influence cellular growth by interacting with their cell membrane receptor through cascade reaction in the cytoplasm and affects gene expression. [48]

Estrogen (as a lipid soluble steroid hormone), through interaction with their receptors, plays an important role in the control of cellular growth and differentiation. They play a role in the development of the male reproductive

system. Recently, the effect of estrogen on prostate growth and differentiation has been proved. [49]

Prostate cancer is primer cause of illness and death among men in United States and Western Europe. In 2002, it was estimated that around 189.000 men received diagnosis of prostate cancer, and there were also estimated that 30.200 of them will die due to prostate cancer metastasis.[46] It was found that over 95% of prostate cancers are adenocarcinomas. On the other hand, prostatic intraepithelial neoplasia (PIN) is concidered to be pre-malignant lesion and the main precursor of invasive the prostatic carcinoma.[50,51] Prostatic intraepithelial neoplasia is currently preferred term for a process involving prostatic intraductal or ductal acinar dysplasia. Its had been divided into three grades, depending on the severity of the following alteration i.e. cell crowding and stratification (cellular polarity), nuclear enlargement, cellular polimorphism, as well as chromatin pattern and nucleolar appearance.[52]

One of the most risk factors is alteration of testosterone and estrogen ratio with advancing age. This phenomenon was proved by long-term and short-term treatment with combination of testosterone and 17-βestradiol in animal models. During short-term treatment (4 months) animal models consistently induced a proliferative through dysplasia lesion, whereas long-term treatment (6-12 months) produced carcinoma in the prostate. [53]

Human prostate carcinogenesis is believed involving multiple process from hyperplasia through dysplasia (pre-cancerous) to carcinoma, from low histological grade to high grade and finally metaplastic carcinoma. Prostatic

hyperplasia was observed by increasing luminal epithelial thickness, infolding (papillomatous), dysplasia, and was characterized by the appearance of multiple layers of luminal epithelial cells and variable degree of cytological atypia (polimorphism).[53]

Several studies in early 1990s demonstrated that the level of prostate specific antigen (PSA), a serine protease, is elevated in most men with clinically prostate cancer. PSA is a substance that produced by normal epithelial prostate. PSA function is to cleave and liquefy the seminal coagulum form after ejaculation. Most of PSA is secreted out of the body through ejaculated semen and only very small amount will escapes into the blood stream. Serum PSA test has sensitivity up to 80% in detecting prostate cancer. However, it may lack of its specificity and false positive results due to the presence of benign prostate hyperthropy or prostatitis. Measuring PSA density (the PSA concentration divided by the volume of the gland) has to be done to improve the accuracy. Recently, the best way to reduce the frequency of false positive, PSA test should be combining with the digital rectal examination and transrectal ultrasonography. As a screening and diagnostic test founded an abnormal result, its usually require additional testing such as prostatic biopsy. Level above 4 ng/ml but less than 10 ng/ml are suspicious, if the level increase above 10 ng/ml, the probability of prostate cancer increases dramatically.[51, 52, 54]

infertility.[4] Organophosphate pesticides have also been associated with male infertility and reducing numbers of normal morphology and live spermatozoa.[56]

Fetuses, infants, and children are especially vulnerable to the effect of pesticides. Small amounts of pesticides, may not affecting adult, however it could have prominent effects on fetus, infant, or children development, several reasons of it could be postulated such as: their organs are develop rapidly; babies and children always take more air, food, and water per unit of body weight than adult; babies and children surely have less ability than adult to excrete toxic substance; babies and children may play on the floor, chew or suck on toys, and put their hands into their mouths. Hence they will likely take more pesticides found on household surface than adult.[55]

2. 3. 1. Propoxur

Propoxur is a non-systemic insecticide that introduced in 1959. Propoxur is developed from the carbamate group of agents which has a broad-spectrum action and used on variety of insect pests such as chewing and sucking insects, ants, cockroaches, crickets, flies, mosquitoes, and may also be used for control agricultural's insect as Baygon® for agricultural applications.[58] Propoxur is one of the *N*-methylcarbamate esters that inhibits cholinesterase in insect. Due to its low toxicity to mammals and other vertebrates, propoxur is an economically important insecticide and manufactured in very large quantities. It is widely used as aerosol to control agricultural and household insect pests. However, several studies found that propoxur is carcinogenic agent which act through inhibition of gap junction

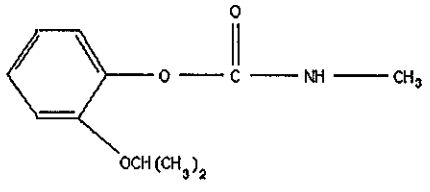
intercellular communication (*GJIC*) and was proved as mutagenic substance.[59] Study for biochemical aspect of propoxur in rats indicated that 24 hours after exposure, the radio-labelled propoxur concentrations had declined markedly in most tissues. The autoradiographs study showed a rapid excretion, mostly in urine and a small amount was also excreted in feces. Since carbamate is rapidly secreted, generally they do not accumulated in mammalian tissue.[60] Half-live propoxur is reported around 14 to 50 days. Propoxur is moderate to low persistence in soil environment, and has low affinity for soil binding. It is highly soluble in water, moderately persistent, and has potential for ground water penetration.[58] **The physical properties of propoxur are as follows:**

Chemical name : 2-isopropoxy-phenyl-N-methylcarbamate

2-(1-methylethoxy)-phenylmethylcarbamate

Common name : Propoxur

Structural formula :



Empirical formula : $C_{11}H_{15}NO_3$

Appearance : Colourless crystals

Molar mass : 209.2 g/mol

Melting point : 90.7° C (pure agent), 86-87 ° C (technical product)

Vapour pressure : < 105 mbar at 20 °C

Solubility : Water 1.9

n-hexane 1 - 10

Methylene chloride >1000

Isopropanol 100 - 1000

Toluene 100 – 1000 (g/1000 ml solvent at 20 °C)

Stability : In alkaline conditions subject to hydrolysis. Half-lives in aqueous solution at 20 °C: pH 10.8 = 40'; pH 11.8 = 11.5'; pH 12.8 = 1'

The agent dissolved in water at pH 7 breaks down at a rate of about 1.5 % per day.

Toxicity (single dose)

Oral : LD_{50} Rat (Male) 116,0 mg/kg

LD_{50} Rat (Female) 95,0 mg/kg

Dermal : LD_{50} Rat (Male and Female) > 2400mg/kg

2. 3. 2. Transfluthrin

Transfluthrin is the latest pyrethroids agent developed for Baygon®. It is one of the best-tested insecticides agents, and has been incorporated in household product against flying insects such flies, mosquitoes, and cockroaches since 1996, and regarded as one of the fast-acting pyrethroids with low persistency.[61]

Pyrethroids is synthetic esther compound, contains of acid and alcohols derived from the natural pyrethrum extract. Their advantage is higher effectiveness against a wide spectrum of pest in agriculture, households, and store products compared to other insecticides.[62] Although they are based on the chemical structure and biological activity of pyrenthrum, an extract from

chrysanthemum, the development of synthetic pyrethroids has involved extensive chemical modifications to make compounds more toxic and less rapidly degraded by light. Insecticides that classified in pyrethroids groups are permethrin, cypermethrin, fenvalerate, flucythrinate, deltamethrin, cyfluthin, *d*-phenothrin, sumithrin, *d-trans*allethrin, and transfluthrin.[63]

In the environment, pyrethroids are usually degraded by biotic and a-biotic process i.e. metabolic degradation by plants, microorganisms, and light (photolysis). The rate of degradation depends on the type of pyrethroids, soil type, climate, the species of microbes present, and the size of their population. Fenvalerate and deltamethrin are the most persistent pyrethroids use for commercial purposed. Both can accumulate to the levels of ten times over the initial concentration if they are repeatedly applied at rates higher than their degradation rate. Pyrethroids are also highly lipophilic, they are adhere strongly to any organic matter in water, easily absorbed into the waxy layer of the plants, and are strongly absorbed by the soil particles. The persistence of residues in soil, water, and plat tissues are varies considerably. The half-life of pyrethroids in soil ranges from day 1 to 16 weeks.[63] It also noted that pyrethroids become more toxic for mammal and insect at low temperature than at high temperatures.[64]

Many pesticides have been estimated to have hormonal activity and classified as endocrine disruptors. Pyrethroids are commonly used insecticides worldwide, but little has been done to characterize their hormone disruptive potential. Fenvalerate and sumithrin demonstrated significant estrogenicity at concentration of 10μM.[7] It was well known that estrogen, whether natural or

synthetic, clearly influence reproductive development, senescence, and carcinogenesis. Using the MCF-7 human breast carcinoma cell line, estrogenic potential activity of synthetic pyrethroids such sumithrin, fenvalerate, *d-trans*allethrin, and permethrin was studied *in vitro* using pS2 mRNA levels as the end point. These findings suggest that pyrethroids should be considered to be hormonal disruptor, and have potential to effect to disrupt endocrine function in humans and wildlife.[65]

Exposure to the pesticide cypermethrin proved has adverse effect on fertility and reproduction of adult male rats. Fertility is significantly reduced in male rats ingesting cypermethrin at a concentration of 13.15 and 18.93 mg and the number of implantation embryo are significantly reduced in female mated with males that has ingested cypermetrin at a concentration of 39.66 mg. Epididymal and testicular sperm concentration as well as daily sperm production were significantly decreased in exposed males. The serum level of testosterone, follicular stimulating hormone (FSH) and luteinizing hormone (LH) are significantly reduced in males exposed to 39.66 mg per day. Histopatological section of the testis of adult male rat after ingestion of cypermethrin in drinking water were caused increasing amount of connection tissue between seminiferous tubules and having a significant number of immature spermatids that observed in lumen of some seminiferous tubules. This research also noted that the testicle weight was also significantly increased.[66]

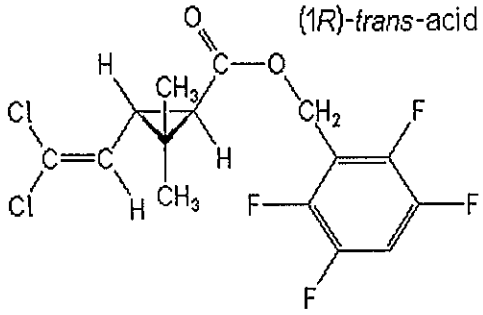
Long term pyrethroids effect had been proved could cause disruption of endocrine system by mimicking the female hormone (estrogen) that causes

excessive estrogen level in female. In male, its estrogenizing (feminizing) effects could cause decreasing sperm production. In both female and male, pyrethroids known carcinogen.[62] All the way through this hormonal pathways, exposure to certain pyrethroids ended on reproductive dysfunction, development impairment, and cancer. **The physical properties of transfluthrin are as follows :**

Chemical name : 1R-trans)-(2,3,5,6-tetrafluorophenyl)-methylester 3 - (2,2-dichloroethenyl) -2,2 - dimethyl - cyclopropane carboxylic acid

Common name : Transfluthrin

Structural formula :



Empirical formula : $C_{15}H_{12}Cl_2F_4O_2$

Molar mass : 371.2 g/mol

Appearance : Colourless crystals

Density : 1.5072 g/cm³ at 23 °C

Vapour pressure : $4.0 \cdot 10^{-6}$ hPA at 20 °C

Melting point : 32 °C

Solubility : Water 5.7 · 10-5
Hexane >200
Isopropanol >200

Toluene >200

Dichloromethane >200

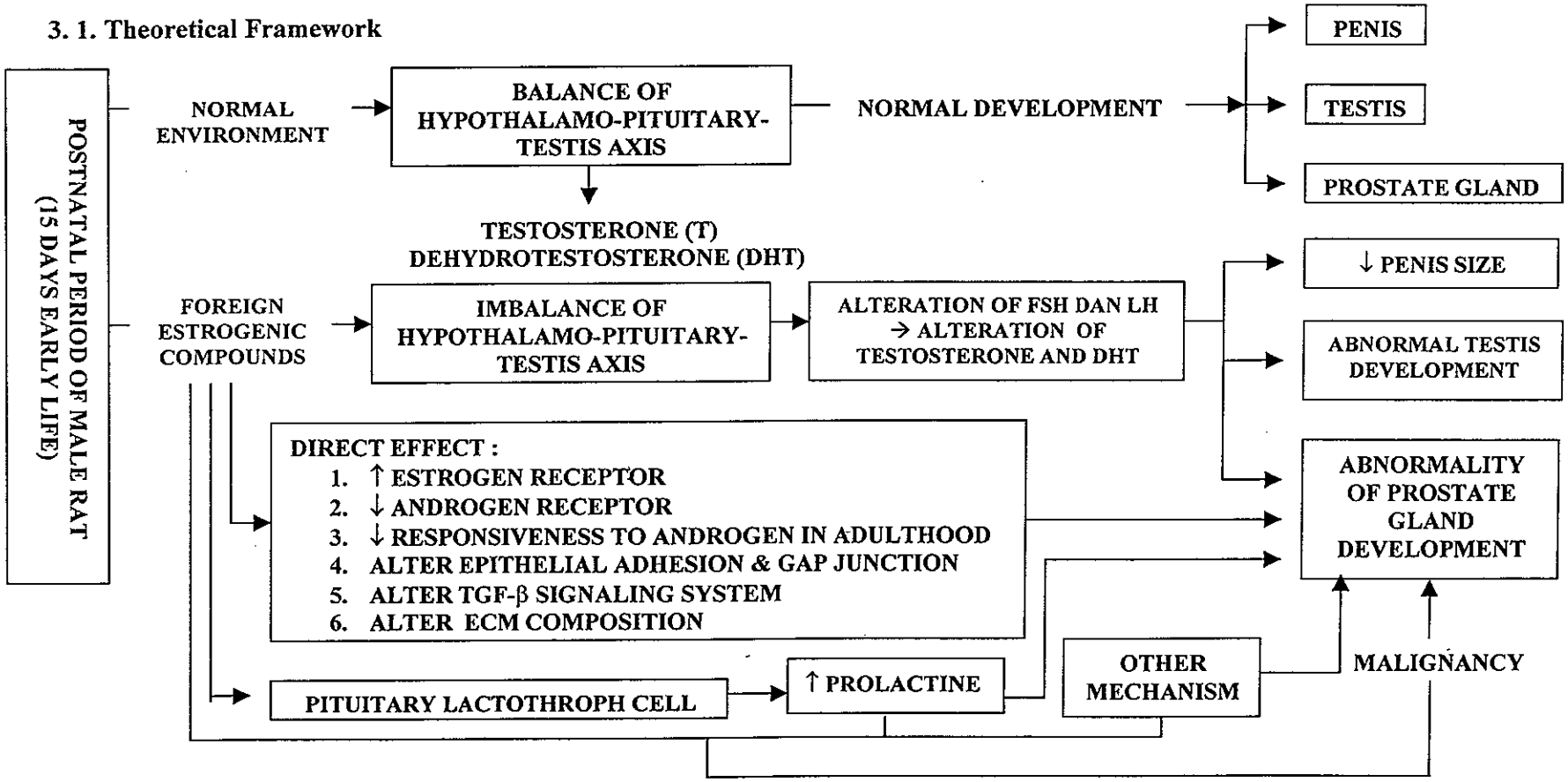
Toxicity :

Oral : LD 50 (Rat) > 5000 mg/kg

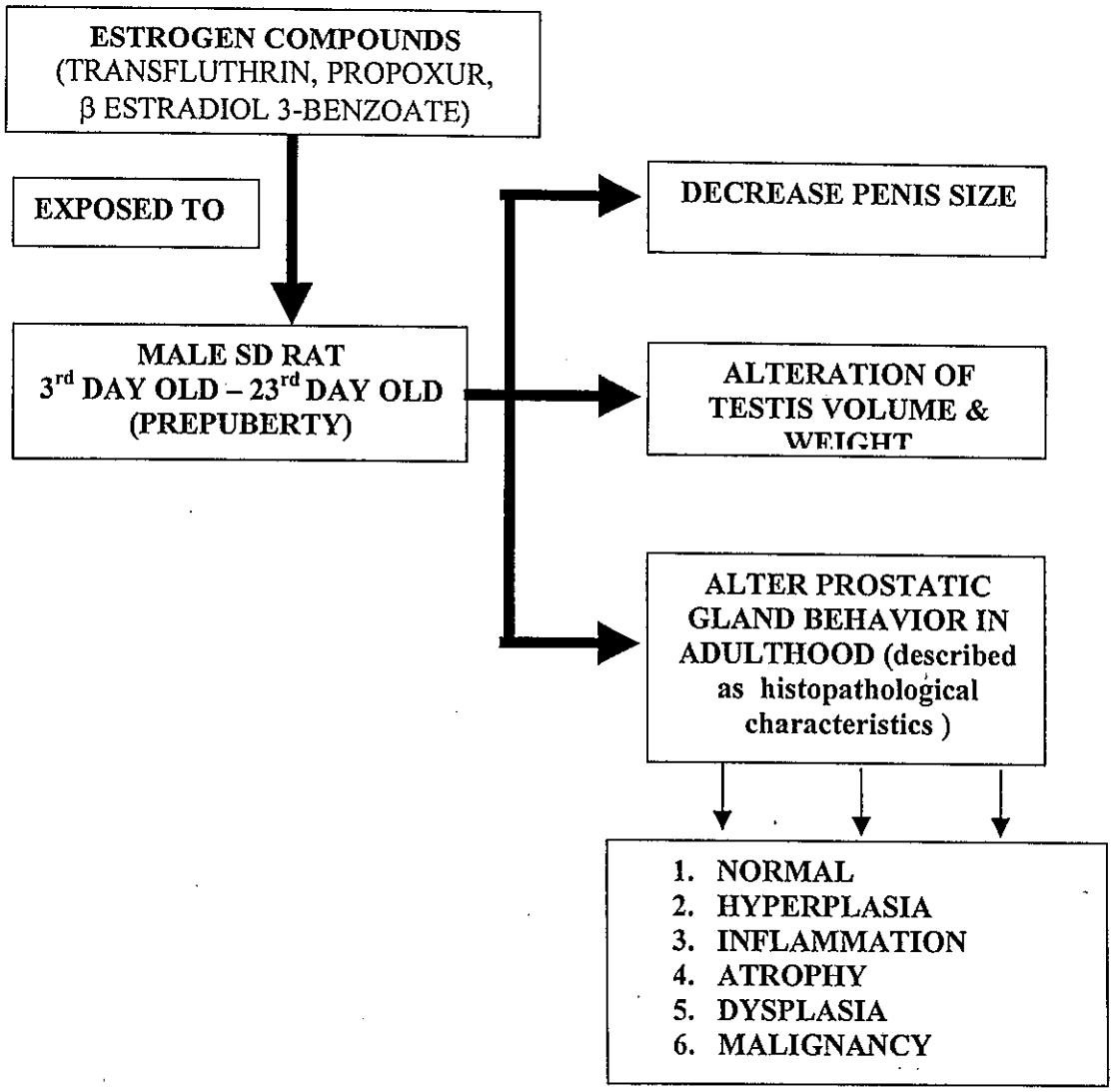
Chapter 3

THEORETICAL FRAMEWORK, CONSEPTUAL FRAMEWORK AND HYPOTHESIS

3. 1. Theoretical Framework



3. 2. Conceptual Framework



3. 3. Hypothesis :

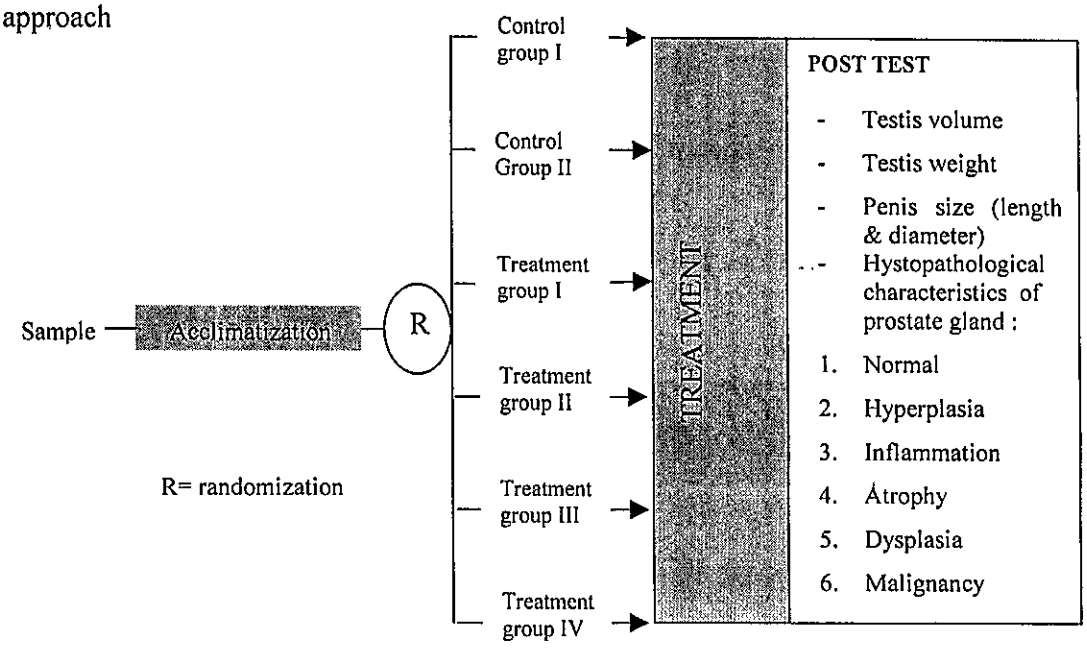
Exposure of burning mosquito insecticide coil (transflutrin 0.03%), liquid mosquito insecticide (transflutrin 0.162 g/l and propoxur 4.05 g/l) and administering 25 μg β estradiol 3-benzoate to neonatal male Spargue Dawley rat for 20 days can cause alteration of reproductive organ in adult.

Chapter 4

RESEARCH METHOD

4.1. Research Design

True Experimental design with The Post Test Only Control Group Design approach



Control groups I = negative control group
Control group II = positive control group (injection 0.02 ml 0.9 % sodium chloride s.c single dose given in alternate days for 20 days)
Treatment group I = injection 25 μg β estradiol 3-benzoat diluted in 0.02 ml sesame oil s.c single dose given in alternate days for 20 days
Treatment group II = exposed to smoke from burned mosquito insecticide coil for 8 hours a day for 20 days
Treatment group III = exposed to 3 ml liquid mosquito insecticide that sprayed to the cage air by nebulizer once time a day for 20 days
Treatment group IV = exposed to 4 ml liquid mosquito insecticide that sprayed to the cage air by nebulizer once time a day for 20 days

4.2. Population And Samples

4.2. 1. Population

Male Sprague Dawley (SD) rats were obtained from Balai POM, Jakarta.

4. 2. 2. Samples

Male Sprague Dawley (SD) rat, age Postnatal day-3, weight 6-8 grams. All rats were group and caged on UPHP (Unit Pemeliharaan Hewan Percobaan) Gadjah Mada University in Yogyakarta.

4. 2. 3. Sampling Method

All rats were allocated in to 6 groups by simple random sampling method by consecutive random sampling.

4. 2. 4. Sample Size

Minimal samples was calculated by using Freeder experimental sample size formula: $t\,(n-1) > 15$

t = number of treatment

n = number of sample in an each group.

There are 4 group of treatment and 2 group of control, therefore $t = 6$

$\rightarrow 6\,(n-1) > 15 \rightarrow n > 4$ for each group

4.3. Spatial and Temporal of the Research

Research was done at UPHP (Unit Pemeliharaan Hewan Percobaan) Gadjah Mada University in Yogyakarta, started on June 2003 until December 2003.

4.4. Research Variables

4.4.1. Independent variables :

a. β estradiol 3-benzoat 25 μg diluted in 0.02 ml sesame oil s.c single dose

b. 0.9 % Sodium Chloride Injection (PT. Otsuka Indonesia)

c. Mosquito insecticide coil (Bayer Co. Indonesia)

d. Liquid mosquito insecticide (Bayer Co. Indonesia)

4.4.2. Dependent variables :

a. Testis volume and weight, measured in mm. Continuous scale

b. Penis size (length and diameter), measured in ml. Continuous scale

c. Histopatholgical section of prostate gland. Ordinal scale classified as:

c.1. Normal

c.2. Hyperplasia

c.3. Inflammation

c.4. Dysplasia

c.5. Malignancy

4.4.3. Controlled variables :

a. Rat age: postnatal day-3

b. Sex: male

c. Weight: 6-8 grams

4.5. Materials and Instruments

4.5.1. Materials

- Rat's penis, testis and prostate gland had been collected after rats were killed by cervical dislocation
- β estradiol 3-benzoat (Sigma Co., USA)
- sesame oil
- 0.9 % Sodium Chloride Injection (PT. Otsuka Indonesia)
- Burn mosquito insecticide coil (contain transflutrin 0.03%) (Bayer Co. Indonesia)
- Liquid mosquito insecticide (contains transflutrin 0.162 g/L and propoxur 4.05 g/L) (Bayer Co Indonesia)
- Rodent feed pellet: 521 (PAU Yogyakarta)
- Buffered neutral formaldehyde solution 10%
- Ethanol 96%, 80%, 70%, 60%, 50%, 40%, 30%
- Xylol solutions
- Paraffin wax
- Aquadest
- Albumin solution
- Haematoxyllin dan Eosin

- Canada Balsam
- Van Gieson A and B solution
- Acetic acid 1%
- Masson Goldner II and III solution

4.5.2. Instruments

- Stable/cage rat specially designed for mosquito repellent treatment
- BD® syringe with non-traumatic needle
- Nebulizer (Bremmed®)
- Pincers
- Scissor
- Scalpel
- Stopper glass jar
- Calibrated beaker with carefulness ± 0.1 ml
- GMP® Calipper
- Sartorius® balance
- Beaker glass
- Autoclave /oven
- Paraffin embedding equipment
- Microtome
- Object glass
- Microscope

4.6. Data Collecting Procedures

4.6.1. Rat preparation and allocation

Fifty male SD rat postnatal day 1 were acclimatized under standard condition at UPHP for 2 days and allocated into 6 groups by simple random sampling. During treatment, rats were received breast-feeding by rat mother until weaning time (postnatal day 22). Weaning time was followed by standard condition. During standard condition all rats received rat feed and water *ad libitum*.

4.6.2. Group Intervention

4.6.2.1. Group I: Control group I (n=15). Rats in control group received breast feeding until 22 days than followed by rat feed and water *ad libitum* until rat aged 100 days

4.6.2.2. Group II: Control group II (n=11). At day-3 postnatal all rats in this group were received 0.02ml 0,9% Sodium Chloride (Otsuka Indonesia) s.c single dose with BD non-traumatic needle on alternate days for 20 days and maintained under standard conditions in UPHP Yogyakarta until aged 100 days.

4.6.2.3. Group III: Treatment Group I (n=15). At day-3 postnatal all rats in this group were received 25 μg β estradiol 3-benzoat diluted in 0.02 ml sesame oil s.c single dose with BD non-traumatic needle on alternate days for 20 days and maintained

under standard conditions in UPHP Yogyakarta until aged 100 days.

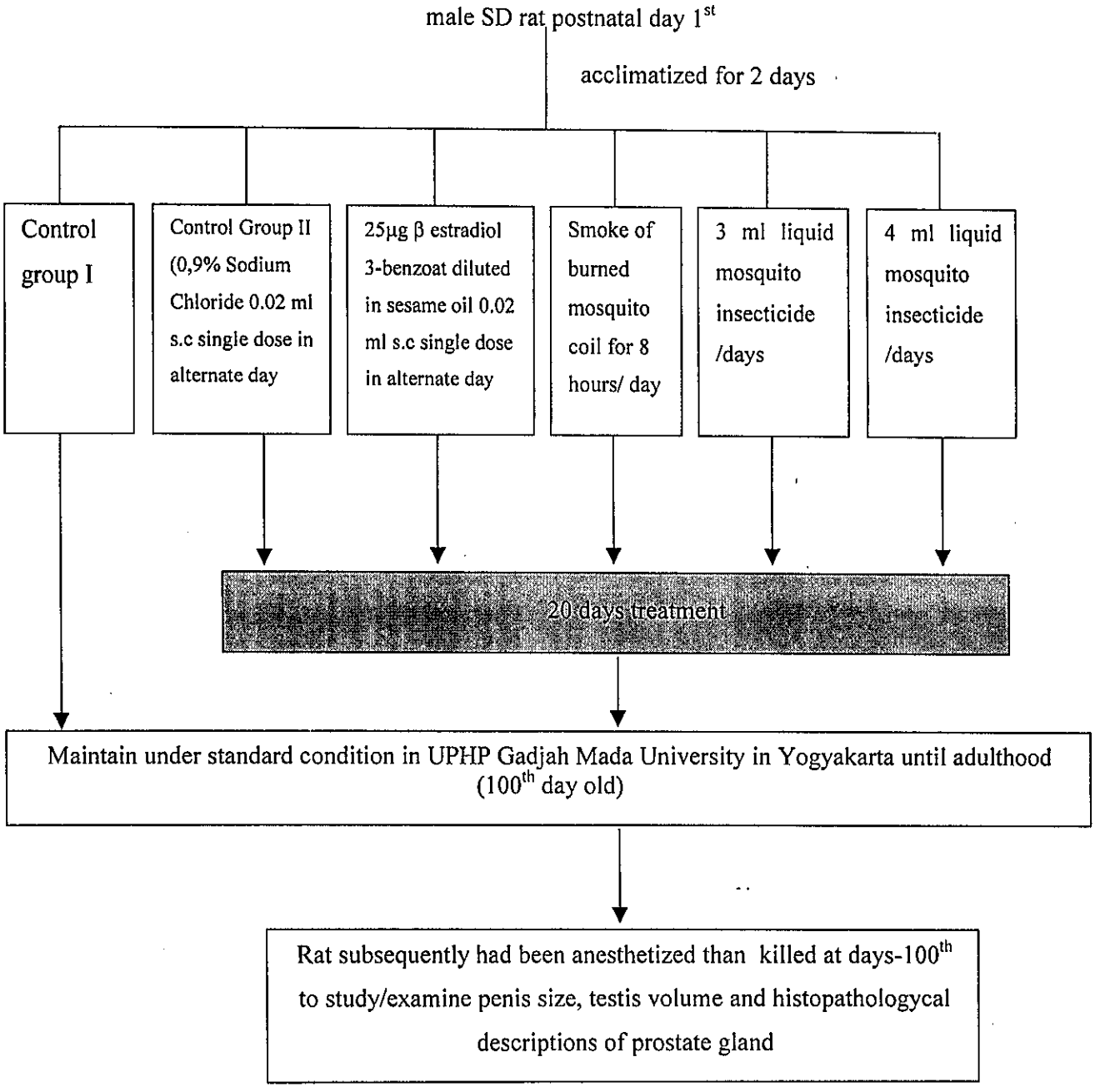
4.6.2.4. Group IV: Treatment Group II (n=18). All rats in this group were exposed to smoke of burned mosquito insecticide coil (contain transflutrin 0.03%) for 8 hours a day for 20 days and maintained under standard conditions in UPHP Yogyakarta until aged 100 days.

4.6.2.5. Group V: Treatment Group III (n=20). All rats in this group were exposed to 3 ml liquid mosquito insecticide (contains transflutrin 0.486 mg and propoxur 12.15 mg) that sprayed to cage air by nebulizer once time a day for 20 days and maintained under standard conditions in UPHP Yogyakarta until aged 100 days.

4.6.2.6. Group VI: Treatment Group IV (n=9). All rats in this group were exposed to 4 ml liquid mosquito insecticide (contains transflutrin 0.648 mg and propoxur 16.20 mg) that sprayed to cage air by nebulizer once time a day for 20 days and maintained under standard conditions in UPHP Yogyakarta until aged 100 days..

After treatment, on aged 100^{th} days old all rats were anesthetized by ether than killed by cervical dislocation. The penis, testis and prostate gland were measured/examined approximately.

4.7. Research Plot



4.8. Measurement methods

4.8.1. Testis sized measurement

Testis was removed from the body of killed rat by minor surgery instruments. The testis volume includes epididymis and lamina visceralis tunica vaginalis were measured by calibrated beaker and were weighed by Sartorius® balance, soon after dissected.

4.8.2. Penis sized measurement

A single researcher to minimize and standardize measurement error measured penis rat, without the preputium. GPM® calliper was used to measure the length of the penis (from tip of the penis glans to bifurcatio of crus penis) and the width (diameter) of penis cuff.

4.8.3. Histopathological characteristic of prostate gland

Examination of prostate gland was focused on alteration of luminal epithelial cell and periductal stromal cell. Haematoxyllin Eosin staining method was used for exploring luminal epithelial cell characteristic, while Masson's Trichrome staining method was used for periductal stromal cell exploration.

Prostate glands were removed from the rat body by minor surgery instruments. Prostate gland were fixed in buffered neutral formaldehyde solution 10% for 24 hours at room temperature before being transferred into ethanol 70% for 1.5 hours, ethanol 80% for 1.5 hours, and transfers into ethanol 90% for 1.5 hours at room temperature in order to dehydration. After dehydration process, the prostate gland was transferred into xylene solution in

stages for clearing the tissues. After cleared, the tissues were fixed into paraffin wax to make a rigid block in order to be cut uniformly. The tissues in the paraffin wax block were cut by microtome. Paraffin sections are located over the bath warm water, separated each other, and located on slides centrally. The slides were dried in 60°C oven for 30 minutes. Firstly, the section stain was Haematoxyllin for 15 minutes, rinse in tap water, differentiate in acid alcohol (three to ten quick dips), washed in tap water, dip in ammonia water until section were bright blue (three to five dip), washed in running tap water for 10 to 20 minute. After stain with Haematoxyllin, the sections were stained with Eosin for 15 seconds to 2 minutes. Finally, the sections were dehydrated, cleared, and covered by covered glass.[67]

Alteration within periductal stromal cell could be seen by Masson's Trichrome staining methods. Firstly, prostate gland sections were deparafinized. After deparafinized, the sections were dripped by Van Giesson solution (Van Giesson A and B 1:1) for 5 minute, solution was discarded by rinsing in warm water for 5 minute, then rinse in aquadest for a while. After rinsing in aquadest, the sections were dripped by Masson Goldner I (MG I) solution for 5 minute, solution was discarded, rinse in acetic acid 1% for 2 minute, the sections were dripped by Masson Goldner II (MG II) solution for 5 minute, rinse in acetic acid 1% for 2 minute, dripped the sections by Masson Goldner III (MG III) solution for 5 minute, rinse in acetic acid 1% for 2 minute. Finally, the sections were dehydrated in 95% absolute alcohol, and cleared in xylene, two changes each, and then covered by covered glass. [67]

Prostate glands histophatological characteristic was examined using single blinding method i.e. covering the label of prostate gland stain section and was checked by pathologist.

4.9. Data Analysis.

Collected data were checked for data cleaning, coding and scoring, then entered to computer. Data analysed for descriptive analysis and hypothesis testing. Penis size and testicle weigh/volume were presented as mean, standard deviation and median. The histopathological characteristics of prostate gland were described as distribution frequency and percentage. Histopatological characteristics are luminal epithelial, epithelial secretion, and periductal stromal cell of prostate gland. Since the changing of histopathological prostate glands were simultaneously occurred, the histopathological characteristic were analysed as a composite score (numeric scale). There were 6 experimental groups, should be examined the differences of penis size and testis volume between groups. One Way ANOVA procedure was subjected to normally distribution and homogeneity of variance that were checked prior to analysis. Normality test by Shapiro-Wilk procedure showed that the data were not normally distributed. Therefore, Kruskall-Wallis test were used to examined the difference of testis volume/weight and penis size between group. Group comparisons were done by Mann-Whitney test. While χ^2 test were used to assess the difference of distribution frequency of histopathological characteristics between groups. P-value less than or equal to 0.05 were considered as significant. The power of this study was 80% with

95% confidence interval. All analysis was conducted by using SPSS for Windows v.11.5. [68]

4.10. Limitation of Research

Estradiol benzoate administration through sub-cutan injection causes physical stress to the SD rat, this situation can result in increasing prolactin level. Based on that fact, positive control group was used for comparison for estradiol benzoate group using normal saline with the same volume and frequency

Since our animal's research were inbreed which assume have homogeneous characteristic, concentration of plasma rat hormonal level was not measure during research.

WHO has a regulation for special examination i.e. each group should consist of at least five animal. [69] The animal was inbreeding strain, since this research was regulated for experimental animal purposes including age, and sex. Histopathological examination of periductal stromal prostate gland was done after Masson's Trichrome staining method. Simple random sampling was used for choosing five prostates on each group.

Chapter 5

RESULT AND DISCUSSION

5.1. RESULT

The average of rat testis, penis, and prostate in all group experiment are shown in table1.

Table 1. The average ± SD of testis volume and weight; penis length and diameter; prostate HE and Masson's Trichrome score in experiment groups. The value in the bracket is the median. The number of animal in each experimental group as mention in N column, except only 5 SD rats in control (-), control (+), Estrogen, burn insecticides and 3 ml liquid insecticides group and 6 rats in 4 ml liquid insecticides for prostate Masson's Trichrome examination.

Groups/ Variable	N	Testis		Penis		Prostate	
		Volume	Weight	Length	Diameter	HE Score	Trichrome Score
Control (-)	15	1.487±0.31 (1.40)	1.665±0.28 (1.61)	1.168±0.04 (1.17)	0.439±0.02 (0.44)	12.066 ±2.31 (12)	4.60±1.52 (5)
Control (+)	11	1.664±0.29 (1.50)	1.912±0.23 (1.82)	1.139±0.06 (1.13)	0.433±0.02 (0.44)	14.091 ±3.65 (14)	7.40±2.88 (9)
Estrogens	15	0.300±0.08 (0.30)	0.664±0.17 (0.65)	0.840±0.08 (0.87)	0.296±0.05 (0.30)	24.533±0.83 (25)	13.40±3.05 (15)
Burn Insecticide	18	1.500±0.13 (1.53)	1.806±0.18 (1.85)	1.111±0.07 (1.11)	0.395±0.03 (0.40)	17.111 ±3.68 (16)	11.20±2.49 (11)
Liquid insecticide 3 ml	20	1.345±0.15 (1.30)	1.527±0.22 (1.52)	1.140±0.05 (1.16)	0.423±0.04 (0.43)	14.950±3.12 (14)	8.40±2.79 (9)
Liquid insecticide 4 ml	9	1.522±0.11 (1.50)	1.818±0.13 (1:83)	1.207±0.17 (1.16)	0.413±0.03 (0.43)	16.555±4.36 (16)	7.67±2.34 (8)

Boxplot diagram of the average of testis volume on experimental groups were shown on figure 2.

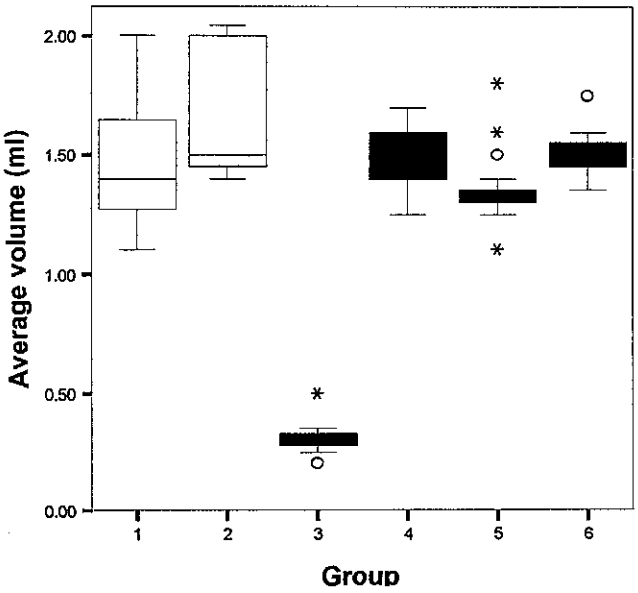


Figure 2. Boxplot diagram of average of rat testis volume on control negative (□), control positive ( ), estrogen (■), burn insecticide (■), liquid 3 ml (■), liquid 4 ml (■) groups

Data on figure 2 show the median of the average of testis volume on negative control group was 1.400 ml, first quartile was 1.275 ml and third quartile was 1.650 ml. The median of testis volume on positive control group was 1.500 ml, first quartile was 1.450 ml and third quartile was 2.000 ml. In estrogen group the median was 0.300 ml, first quartile was 0.275 ml and third quartile was 0.325 ml. In burn insecticide group , the median of testis volume was 1.525 ml, first quartile was 1.400 ml and third quartile was 1.588 ml. In 3 ml liquid insecticide, the median was 1.300 ml, first quartile was 1.300 ml and third quartile was 1.350 ml. In 4 ml liquid insecticide, the median was 1.500 ml, first quartile was 1.450 ml and third quartile was 1.550 ml.

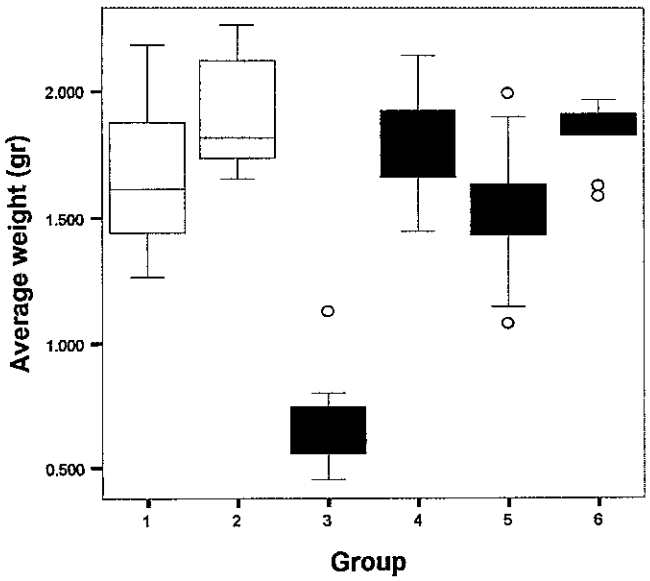


Figure 3. Boxplot diagram of average of rat testis weight on control negative (□), control positive ( ), estrogen (■), burn insecticide (■), liquid 3 ml (■), liquid 4 ml (■) groups

Data on figure 3 show the median of the average of testis weight on negative control group was 1.612 ml, first quartile was 1.436 ml and third quartile was 1.878 ml. The median of testis weight on positive control group was 1.817 ml, first quartile was 1.736 ml and third quartile was 2.117 ml. In estrogen group the median was 0.650 ml, first quartile was 0.556 ml and third quartile was 0.744 ml. In burn insecticide group, the median of testis weight was 1.848 ml, first quartile was 1.667 ml and third quartile was 1.925 ml. In 3 ml liquid insecticide, the median was 1.516 ml, first quartile was 1.435 ml and third quartile was 1.619 ml. In 4 ml liquid insecticide, the median was 1.832 ml, first quartile was 1.821 ml and third quartile was 1.907 ml.

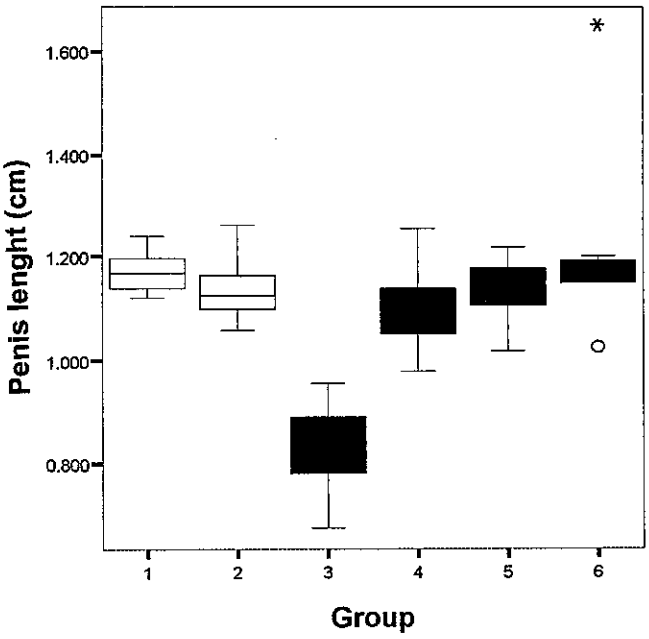


Figure 4. Boxplot diagram of average of rat penis length on control negative (□), control positive ( ), estrogen (■), burn insecticide (■), liquid 3 ml (■), liquid 4 ml (■) groups

Data on figure 3 show the median of the average of penis length on negative control group was 1.170 ml, first quartile was 1.140 ml and third quartile was 1.198 ml. The median of penis length on positive control group was 1.125 ml, first quartile was 1.098 ml and third quartile was 1.165 ml. In estrogen group the median was 0.870 ml, first quartile was 0.780 ml and third quartile was 0.890 ml. In burn insecticide group, the median of penis length was 1.110 ml, first quartile was 1.060 ml and third quartile was 1.139 ml. In 3 ml liquid insecticide, the median was 1.155 ml, first quartile was 1.108 ml and third quartile was 1.180 ml. In 4 ml liquid insecticide, the median was 1.160 ml, first quartile was 1.150 ml and third quartile was 1.195 ml.

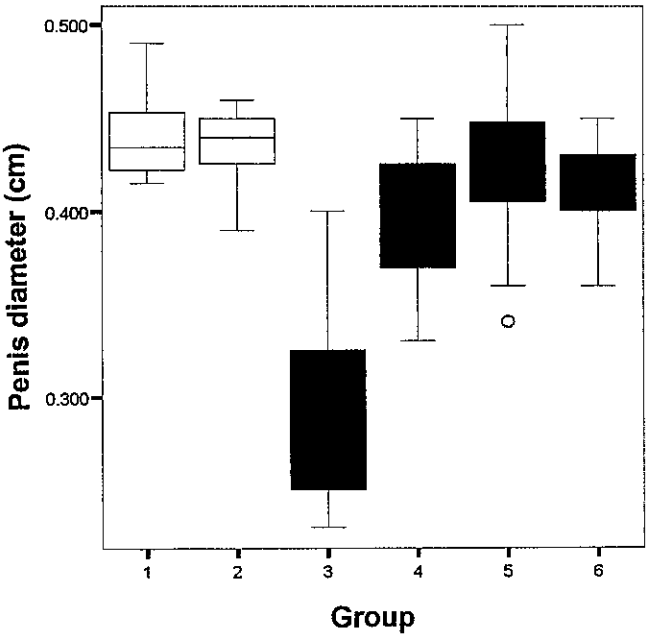


Figure 5. Boxplot diagram of average of rat penis diameter on control negative (□), control positive ( ), estrogen (■), burn insecticide (■), liquid 3 ml (■), liquid 4 ml (■) groups

Data on figure 3 show the median of the average of penis diameter on negative control group was 0.435 ml, first quartile was 0.4225 ml and third quartile was 0.453 ml. The median of penis diameter on positive control group was 0.440 ml, first quartile was 0.425 ml and third quartile was 0.450 ml. In estrogen group the median was 0.300 ml, first quartile was 0.250 ml and third quartile was 0.325 ml. In burn insecticide group, the median of penis diameter was 0.400 ml, first quartile was 0.371 ml and third quartile was 0.423 ml. In 3 ml liquid insecticide, the median was 0.430 ml, first quartile was 0.408 ml and third quartile was 0.446 ml. In 4 ml liquid insecticide, the median was 0.425 ml, first quartile was 0.400 ml and third quartile was 0.430 ml.

5.1.1. Effect of treatment on testis volume

Data on table 1 show the average of testis volume on positive control group (1.664 ml) was significantly higher than negative control group1.487 ml (p= 0.025).

The average of testis volume on estrogens group was significantly lower (0.3 ml) than positive control group (p=0.0005). In other hand, on burn insecticides group the average of testis volume (1.500 ml) was slightly higher than negative control groups (p=0.108), similar finding was also found on 4 ml liquid insecticides group (1.522 ml). Opposite finding was found on 3 ml liquid insecticides group, the average of testis volume (1.345 ml) was slightly lower than negative control group (p=0.085). The difference (p value) of testis volume between groups was showed on table 2.

Table 2. The p-value of comparison testis volume between experimental groups (2 tail). The p-value < 0.05 and < 0.001 were considered as significantly different. While one tail are in the bracket.

Experimental Group	Control (-)	Control (+)	Estrogen	Burn Insect	Liquid 3 ml	Liquid 4 ml
Control (-)	-	0.05 (0.025)	0.001 (0.0005)	0.216 (0.108)	0.17 (0.085)	0.159 (0.0795)
Control (+)	0.05 (0.025)	-	0.001 (0.0005)	0.353 (0.1765)	0.001 (0.0005)	0.729 (0.3645)
Estrogen	0.001 (0.0005)	0.001 (0.0005)	-	0.001 (0.0005)	0.001 (0.0005)	0.001 (0.0005)
Burn Insect	0.216 (0.108)	0.353 (0.1765)	0.001 (0.0005)	-	0.001 (0.0005)	0.815 (0.4075)
Liquid 3 ml	0.17 (0.085)	0.001 (0.0005)	0.001 (0.0005)	0.001 (0.0005)	-	0.001 (0.0005)
Liquid 4 ml	0.159 (0.0795)	0.729 (0.3645)	0.001 (0.0005)	0.815 (0.4075)	0.001 (0.0005)	-

5.1.2. Effect of treatment on testis weight

The average of testis weight on positive control group (1.912 gram) was significantly higher than negative control group (1.665 gram) with p=0.025. On estrogens group, the average of testis weight (0.664 gram) was significantly lower than positive control group (p=0.0005). The average of testis weight on burn insecticides group (1.806 gram) was slightly higher than negative control groups (p=0.052). Similar finding was also found on 4 ml liquid insecticides group (1.818 gram) with p=0.115. Consistent with testis volume, testis weight on 3 ml liquid insecticides group (1.527 gram) was also slightly lower than negative control group (p=0.072). The p value of all groups' comparison of testis weight was showed on table 2.

Table 3. The p-value of comparison testis weight between experimental groups (2 tail). The p-value < 0.05 and < 0.001 were considered as significantly different. While one tail are in the bracket.

Experimental Group	Control (-)	Control (+)	Estrogen	Burn Insect	Liquid 3 ml	Liquid 4 ml
Control (-)	-	0.05 (0.025)	0.001 (0.0005)	0.104 (0.052)	0.23 (0.115)	0.144 (0.072)
Control (+)	0.05 (0.025)	-	0.001 (0.0005)	0.345 (0.1725)	0.001 (0.0005)	0.732 (0.366)
Estrogen	0.001 (0.0005)	0.001 (0.0005)	-	0.001 (0.0005)	0.001 (0.0005)	0.001 (0.0005)
Burn Insect	0.104 (0.052)	0.345 (0.1725)	0.001 (0.0005)	-	0.001 (0.0005)	0.959 (0.4795)
Liquid 3 ml	0.23 (0.115)	0.001 (0.0005)	0.001 (0.0005)	0.001 (0.0005)	-	0.05 (0.025)
Liquid 4 ml	0.144 (0.072)	0.732 (0.366)	0.001 (0.0005)	0.959 (0.4795)	0.05 (0.025)	-

5.1.3. Effect of treatment on penis length

The average of penis length on positive control group (1.139 cm) was tended to be shorter than negative control group (mean=1.168 cm) with p=0.086. On estrogen group, the average of penis length (0.840 cm) was significantly shorter than positive control group (p=0.0005).

The average of penis length on burn insecticide group (1.111 cm) was significantly shorter than negative control group. The average of penis length on 3 ml liquid insecticide group (1.140 cm) was slightly shorter than negative control group (p=0.0995). While, on 4 ml liquid insecticide group the average of penis length (1.207 cm) was slightly longer than negative control group (p=0.476). The p value of all groups' comparison of penis length was showed on table 4.

Table 4. The p-value of comparison penis length between experimental groups (2 tail). The p-value < 0.05 and < 0.001 were considered as significantly different. While one tail are in the bracket.

Experimental Group	Control (-)	Control (+)	Estrogen	Burn Insect	Liquid 3 ml	Liquid 4 ml
Control (-)	-	0.086 (0.043)	0.001 (0.0005)	0.05 (0.025)	0.199 (0.0995)	0.952 (0.476)
Control (+)	0.086 (0.043)	-	0.001 (0.0005)	0.368 (0.184)	0.591 (0.2955)	0.158 (0.079)
Estrogen	0.001 (0.0005)	0.001 (0.0005)	-	0.001 (0.0005)	0.001 (0.0005)	0.001 (0.0005)
Burn Insect	0.05 (0.025)	0.368 (0.184)	0.001 (0.0005)	-	0.082 (0.041)	0.05 (0.025)
Liquid 3 ml	0.199 (0.0995)	0.591 (0.2955)	0.001 (0.0005)	0.082 (0.041)	-	0.333 (0.1665)
Liquid 4 ml	0.952 (0.476)	0.158 (0.079)	0.001 (0.0005)	0.05 (0.025)	0.333 (0.1665)	-

5.1.4. Effect of treatment on penis diameter

The average of penis diameter on positive control group (0.433 cm) was tended to be smaller than negative control group (0.439 cm) with p=0.715. On estrogen group the average of penis diameter (0.296 cm) was significantly smaller than positive control group (p=0.0005). The average of penis diameter on burn insecticides group (0.395 cm) was significantly smaller than negative control group (p=0.0005). On 3 ml liquid insecticide group, the average of penis diameter (0.423 cm) was tended to be smaller than negative control group (p=0.1345). The average of penis diameter on 4 ml liquid insecticides group (0.413 cm) was significantly smaller than negative control group (p=0.025). The p value of all groups' comparison of penis diameter was showed on table 5.

Table 5. The p-value of comparison penis diameter between experimental groups (2 tail). The p-value < 0.05 and < 0.001 were considered as significantly different. While one tail are in the bracket.

Experimental Group	Control (-)	Control (+)	Estrogen	Burn Insect	Liquid 3 ml	Liquid 4 ml
Control (-)	-	0.715 (0.3575)	0.001 (0.0005)	0.001 (0.0005)	0.269 (0.1345)	0.05 (0.025)
Control (+)	0.715 (0.3575)	-	0.001 (0.0005)	0.05 (0.025)	0.547 (0.2735)	0.115 (0.0575)
Estrogen	0.001 (0.0005)	0.001 (0.0005)	-	0.001 (0.0005)	0.001 (0.0005)	0.001 (0.0005)
Burn Insect	0.001 (0.0005)	0.05 (0.025)	0.001 (0.0005)	-	0.05 (0.025)	0.162 (0.081)
Liquid 3 ml	0.269 (0.1345)	0.547 (0.2735)	0.001 (0.0005)	0.05 (0.025)	-	0.285 (0.1425)
Liquid 4 ml	0.05 (0.025)	0.115 (0.0575)	0.001 (0.0005)	0.162 (0.081)	0.285 (0.1425)	-

5.1.5. Effect of treatment on prostate gland histopathological characteristic

Examination of prostate gland was focused on alteration of luminal epithelial and periductal stromal cells. Haematoxyllin Eosin staining method was used for exploring luminal epithelial cell characteristic, while Masson's Trichrome staining method was used for periductal stromal cell exploration.

5.1.5.1. Using Hematoxyllin Eosin staining

Luminal epithelial cell mostly (87%) found as normal condition on negative control group (see Table 6).

Table 6. Frequency distribution of prostate luminal epithelial cells category according to the percentage of hyperplasia. The severity of cell abnormality was graded from normal (0-25%), light hyperplasia (26-50%), Mild hyperplasia (51-75%), severe hyperplasia (76-100%) and atrophy (the most severe abnormality). The worth in the bracket shows the percentage of animal number within group and within epithelial cells category respectively.

	Prostate Luminal Epithelial Cell Category				
Group	Normal	Light Hyperplasia	Mild Hyperplasia	Severe Hyperplasia	Atrophic
Negative control	7 (46.7; 87.5)	5 (33.3; 14.7)	3 (20.0;11.5)	0 (0.0; 0.0)	0 (0.0; 0.0)
Positive control	1 (9.1; 12.5)	6 (54.5; 17.6)	3 (27.3; 11.5)	1 (9.1; 9.1)	0 (0.0; 0.0)
Estrogens	0 (0.0; 0.0)	0 (0.0; 0.0)	0 (0.0; 0.0)	7 (46.7, 63.6)	8 (53.3, 88.9)
Burn Insecticides	0 (0.0; 0.0)	8 (44.4; 23.5)	10 (55.6; 38.5)	0 (0.0; 0.0)	0 (0.0; 0.0)
Liquid Insect 3 ml	0 (0.0; 0.0)	10 (50; 29.4)	7 (35.0; 26.9)	2 (10; 18.2)	1 (5.0; 11.1)
Liquid insect 4ml	0	5	3	1	0

(0.0; 0.0) (55.6; 14.7) (33.3; 11.5) (11.1; 9.1) (0.0; 0.0)

On positive control group, 54.5% of luminal epithelial cell was categorised as light hyperplasia. On estrogens group, 46.7% of luminal epithelial cell was severe hyperplasia and 53.3% was atrophic. On burn insecticides group, 44.4% was categorised as light epithelial hyperplasia and 55.6% was mild hyperplasia. On 3-ml liquid insecticides group, 50% of luminal epithelial cell was categorised as light hyperplasia and 35% mild epithelial hyperplasia. On 4-ml liquid insecticides group, 55.6% of luminal epithelial cell was categorised as light hyperplasia and 33.3% was mild epithelial hyperplasia. There was significantly different ($p < 0.001$) on frequency distribution of hyperplasia of luminal epithelial prostate gland across of experimental groups (see in supplement)

Prostate gland epithelial layer composition was showed on table 7.

Table 7. Frequency distribution of the thickness of prostate gland epithelial layer (HE) according to number of layers category. The thickness was graded as normal (≤2 layers) to abnormal (5-6 layers). The worth in the bracket shows the percentage of animal number within group and within epithelial cells category respectively.

	Number of epithelial layers		
Group	≤2 layers	3-4 layers	5-6 layers
Negative control	6	8	1
	(40; 50)	(53.3; 13.1)	(6.7; 6.7)
Positive control	1	8	2
	(9.1; 8.3)	(72.7; 13.1)	(18.2; 13.3)
Estrogens	4	5	6
	26.7; 33.3)	(33.3; 8.2)	(40; 40)
Burn Insecticides	0	14	4
	(0; 0)	(77.8; 23)	(22.2; 26.7)
Liquid insecticides 3 ml	1	19	0
	(5; 8.3)	(95; 31.1)	(0; 0)
Liquid insecticides 4 ml	0	7	2
	(0; 0)	(77.8; 11.5)	(22.2; 13.3)

Prostate gland luminal epithelial layers on negative control group, 53.3% were composed of 3-4 epithelial layers. Most of (72.7%) prostate gland luminal

epithelial layers on positive control group composed 3-4 layers (see Table 7). On estrogens group, 40% of prostate gland luminal epithelial layers composed 5-6 layers, and 26.7% was less than 2 layers (atrophic). The number of epithelial layer on burn insecticide group, 77.7% composed 3-4 layers. On 3-ml liquid insecticides group, 95% of prostate gland luminal epithelial layer composed 3-4 layers. On 4 ml liquid insecticides group, 77.8 % of prostate gland epithelial layer composed 3-4 layers and 22.2 % was 5-6 layers. There was significantly different ($p < 0.001$) on frequency distribution of prostate gland epithelial layer category across of experimental groups (see in supplement).

Prostate gland luminal epithelial cellular polarity on experimental groups was showed on table 8.

Table 8. Frequency distribution of prostate gland luminal epithelial cellular polarity (HE) category. The worth in the bracket shows the percentage of animal number within group and within epithelial cells polarity category respectively.

	Cellular Polarity	
Group	Good/Normal	Bad/Abnormal
Negative control	15 (100; 24.2)	0 (0; 0)
Positive control	10 (90.9; 16.1)	1 (9.1; 3.8)
Estrogens	0 (0; 0)	15 (100; 57.7)
Burn Insecticides	13 (72.2; 21)	5 (27.8; 19.2)
Liquid insecticides 3 ml	17 (85; 27.4)	3 (15; 11.5)
Liquid insecticides 4 ml	7 (77.8; 11.3)	2 (22.2; 7.7)

Data on table 8 shows that all (100%) of the luminal epithelial cells on negative control group have good polarity. While, on positive control group mostly (90.9%) having a good polarity. In other hand, all (100%) of the cellular

polarity of luminal epithelial cells on estrogens group were categorised as bad polarity. Table 8 also shows that 72.2 % cellular polarity of prostate gland luminal epithelial was categorised as a good polarity after exposed to burn insecticides. Eighty five percent (85%) of cellular polarity of luminal epithelial cells were categorised as good polarity after exposed to 3-ml liquid insecticides. On 4-ml liquid insecticide group, 77.8 % of cellular polarity were categorised as good. The frequency distribution of prostate gland cellular polarity category of luminal epithelial cells across of experimental groups was significantly different ($p<0.001$) (see in supplement).

Table 9. Frequency distribution of prostate gland luminal epithelial cellular polymorphism (HE) category. The worth in the bracket shows the percentage of animal number within group and within epithelial cells polymorphism category respectively.

Group	Epithelial cell polymorphism	
	Negative/Normal	Positive/Abnormal
Negative control	15 (100; 23.1)	0 (0; 0)
Positive control	10 (90.9; 15.4)	1 (9.1; 4.3)
Estrogens	1 (6.7; 1.5)	14 (93.3; 60.9)
Burn Insecticides	14 (77.8; 21.5)	4 (22.2; 17.4)
Liquid insecticides 3 ml	18 (90; 27.7)	2 (10; 8.7)
Liquid insecticides 4 ml	7 (77.8; 10.8)	2 (22.2; 8.7)

Mostly (93.3%), the luminal epithelial cells of prostate gland on estrogens group were polymorphs. While, the luminal epithelial cells of prostate gland on other experimental group mostly were recognised as monomorphs (see Table 5). There was significant different ($p < 0.001$) on frequency distribution of luminal epithelial cell types across of experimental groups (see in supplement).

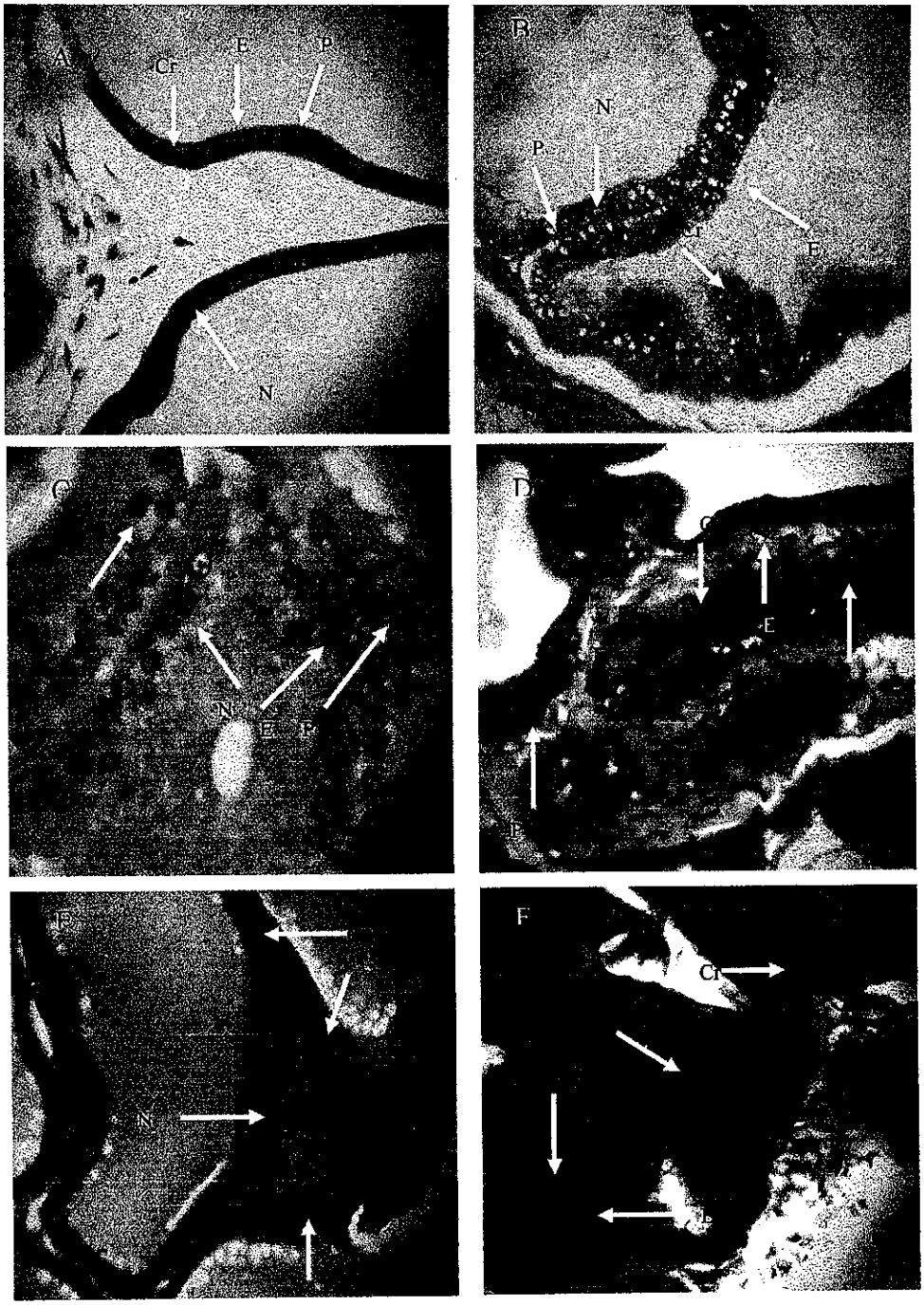


Figure 6. Prostate luminal epithelial cells stained by Haematoxyllin Eosin
In control negative group (**A**): epithel layer was thin and good in polarity, chromatin appearance was smooth. In control positive group (**B**): epithel layer was thicker and polarity still good, chromatin apperance was smooth. In estrogen group (**C**): luminal epithelial was hyperplasia , with bad polarity, polimorphism was noted, epithel layer was thick, chromatin appearance was rough, nucleolus was found in luminal epithelial. In burn insecticides group (**D**): luminal epithelial was hyperplasia (thickness) with good polarity; chromatin appearance was smooth. In 3 ml liquid insecticides group (**E**): some of luminal epithelial hyperplasia and some of its was not (thin layer) with good polarity, chromatin appearance was smooth. In 4 ml liquid insecticides group (**F**): luminal epithelial was thick and hyperplasia with good polarity,and chromatin appearance was smooth**Abbreviations** : E=epithel layer, Cr=Cromatin appearance, N=nucleolus, P=polarity and

Table 10. Frequency distribution of prostate gland luminal epithelial cells (HE) chromatin appearance category. The severity of cell chromatin appearance abnormality was graded a normal (smooth) to abnormal (rough). The Worth in the bracket shows the percentage of animal number within group and within epithelial cells chromatin appearance category respectively.

	Chromatin appearance		
Group	**Smooth**	**Smooth-rough**	**Rough**
Negative control	12	1	2
	(80; 33.3)	(6.7; 5.9)	(13.3; 5.7)
Positive control	6	2	3
	(54.5; 16.7)	(18.2; 11.8)	(27.3; 8.6)
Estrogens	0	0	15
	(0; 0)	(0; 0)	(100; 42.9)
Burn Insecticides	4	(6	8
	(22.2; 11.1)	33.3; 35.3)	(44.4; 22.9)
Liquid insecticides 3 ml	10	7	3
	(50; 27.8)	(35; 41.2)	(15; 8.6)
Liquid insecticides 4 ml	4	1	4
	(44.4; 11.1)	(11.1; 5.9)	(44.4; 11.4)

Estrogens treatment causes chromatin pattern of luminal epithelial cells become 100% rough. In negative control group, chromatin pattern of epithelial cells mostly (80%) smooth. In positive control group, 54.5% chromatin pattern of epithelial cells smooth, even though 27.3% rough. On burn insecticides group, 44.4% chromatin pattern of epithelial cells rough. Exposure of 3-ml liquid insecticides, causes alteration of chromatin pattern i.e. 50% smooth and 35% between smooth to rough. While the exposure of 4-ml liquid insecticides causes alteration chromatin pattern of epithelial cells in between smooth to rough (44.4%), but 44.4% chromatin appearance remain smooth. There was significantly different ($p < 0.001$) on frequency distribution of chromatin appearance category prostate gland luminal epithelial cell across of experimental groups (see in supplement).

Table 11. Frequency distribution of nucleolus of prostate gland luminal epithelial cells (HE) category. The worth in the bracket showing the percentage of animal number within group and within epithelial cells nucleolus category respectively.

Group	Nucleolus	
	Negative/Normal	Positive/Abnormal
Negative control	15 (100; 27.8)	0 (0; 0)
Positive control	9 (81.8; 16.7)	2 (18.2; 5.9)
Estrogens	0 (0; 0)	15 (100; 17)
Burn Insecticides	10 (55.6; 18.5)	8 (44.4; 23.5)
Liquid insecticides 3 ml	15 (75; 27.8)	5 (25; 14.7)
Liquid insecticides 4 ml	5 (55.6; 9.3)	4 (44.4; 11.8)

Nucleolus could not found in epithelial cells of prostate gland on negative control group. On estrogens group, nucleolus presented in all (100%) prostate gland epithelial cells. On burn insecticides group, nucleolus was found in 44.4% of prostate gland epithelial cells. On 3 ml liquid insecticides group, nucleolus presented in 75% of prostate gland epithelial cells. On 4 ml liquid insecticides group, nucleolus presented in 44.4% of prostate gland epithelial cells (see Table 7). There was significantly different ($p < 0.001$) on frequency distribution of nucleolus category in prostate gland luminal epithelial cell across of experimental groups (see in supplement).

Special characteristic was found in estrogens group, there are a lot of lymphocyte and polymorphonuclear leukocyte infiltration in prostate gland. This characteristic was not found in other groups.

Figure 7. Histopathological characteristics of prostate gland in estrogens Group. Infiltration of polymorphonuclear leukocyte and Lymphocyte were found in the tissue. Abbreviation: PMN= Polymorphonuclear leukocyte, L= lymphocyte

5.1.5.2. Using Masson's Trichrome staining

Table 12. Frequency distribution of prostate gland luminal periductal fibroblast (Masson's Trichrome) cell amount and continuity category. The severity of abnormality was graded from normal (little) to the most abnormal (thick-continued). The value in the bracket shows the percentage of animal number within group and within fibroblast cell category respectively.

	Fibroblast				
Group	Little/ Normal	Not much	Discontinued	Thin continued	Thick continued
Negative control	4 (80; 50)	1 (20; 12.5)	0 (0; 0)	0 (0; 0)	0 (0; 0)
Positive control	1 (20; 12.5)	2 (40; 25)	2 (40; 40)	0 (0; 0)	0 (0; 0)
Estrogens	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	5 (100; 16.1)
Burn Insecticides	0 (0; 0)	1 (20; 12.5)	0 (0; 0)	3 (60; 75)	1 (20; 16.7)
Liquid insecticides 3 ml	2 (40; 25)	1 (20; 12.5)	2 (40; 40)	0 (0; 0)	0 (0; 0)
Liquid insecticides 4 ml	1 (16.7; 12.5)	3 (50; 37.5)	1 (15.7; 20)	1 (16.7; 25)	0 (0; 0)

A thicker layer of periductal fibroblast was found in 100% stromal periductal of prostate gland after estrogens treatment. Exposed to burn insecticides, a thin layer of periductal fibroblast was found in 60% prostate gland, while a thicker layer of periductal fibroblast was found in 20% prostate gland.

Exposure of 3 ml liquid insecticides, of periductal fibroblast was found as a discontinued layer in 40% prostate gland. While on 4 ml liquid insecticides group, 50% of periductal fibroblast was rarely found. (see Table 8). There was a significant different ($p < 0.001$) on frequency distribution of periductal fibroblast across of experimental groups (see in supplement).

Table 13. Frequency distribution of prostate gland luminal periductal smooth muscle (Masson's Trichrome) cell continuity category. The severity of abnormality was graded from normal (continue) to the most abnormal (rare). The value in the bracket shows the percentage of animal number within group and within smooth muscle cell category respectively.

	Smooth muscle			
Group	**Continue**	**Continue-discontinue**	**Discontinue**	**Rare**
Negative control	5 (100; 41.7)	0 (0; 0)	0 (0; 0)	0 (0; 0)
Positive control :	3 (60; 25)	0 (0; 0)	1 (20; 14.3)	1 (20; 16.7)
Estrogens	0 (0; 0)	1 (205; 16.7)	1 (20; 14.3)	3 (60; 50)
Burn Insecticides	0 (0; 0)	1 (20; 16.7)	2 (40; 28.6)	2 (40; 33.3)
Liquid insecticides 3 ml	2 (40; 16.7)	1 (20; 16.7)	2 (40; 28.6)	0 (0; 0)
Liquid insecticides 4 ml	2 (33.3; 16.7)	3 (50; 50)	1 (16.7; 14.3)	0 (0; 0)

Reducing of smooth muscle cells on periductal stromal cells were found in estrogens group. After burn insecticides exposure, reduction of smooth muscle cell on periductal stromal cells as discontinuity presented in 40% samples and reduction of smooth muscle in 40% samples was worse (found rarely). On 3 ml liquid insecticides group, smooth muscle cells on periductal stromal reduced (discontinue) in 40%. While on 4-ml liquid insecticides group, smooth muscle cells on periductal stromal were found discontinue on 16.7% (see Table 9). There was a significant different ($p=0.045$) on frequency distribution of periductal

stromal smooth muscle cells category across of experimental groups (see in supplement).

Table 14. Frequency distribution of prostate gland secretes colour cell continuity category. The severity of abnormality was graded from normal (light blue) to the most abnormal (none). The value in the bracket shows the percentage of animal number within group and within secretes colour category respectively.

	Secrete colour					
Group	Light blue	Blue dominant	Blue-red Equal	Red dominant	Red	None
Negative control	2 (40; 50)	0 (0; 0)	2 (40; 22.2)	1 (20; 14.3)	0 (0; 0)	0 (0; 0)
Positive control	1 (20; 25)	0 (0; 0)	3 (60; 33.3)	0 (0; 0)	1 (20; 20)	0 (0; 0)
Estrogens	1 (20; 25)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	4 (80; 80)
Burn Insecticides	0 (0; 0)	0 (0; 0)	1 (20; 11.1)	(60; 42.9	0 (0; 0)	1 (20; 20)
Liquid insecticides 3 ml	0 (0; 0)	0 (0; 0)	1 (20; 11.1)	1 (20; 14.3)	3 (60; 60)	0 (0; 0)
Liquid insecticides 4 ml	0 (0; 0)	1 (16.7; 100)	2 (33.3; 22.2)	2 (33.3; 28.6)	1 (16.7; 20)	0 (0; 0)

Forty percent (40%) of prostate gland secretion colour on negative control group was light blue. A half (50%) of prostate gland secrete colour was blue in negative control group. On positive control group, 60% of prostate gland secrete colour was equal between blue and red.

On estrogens group, 80% prostate gland luminal epithelial cells did not produce secrete. Alteration of prostate gland secrete colour was red dominant in 60% samples after exposed to burn insecticides. On 3 ml liquid insecticides group, 60% samples of prostate gland secrete colour was red. Table 10 shows, on 4 ml liquid insecticides group, 33.3% of secrete colour was equal between blue and red, 33.3% was red dominant, and 16.7% was red. There was a significant

different (p=0.045) on frequency distribution of secrete colour category across of experimental groups (see in supplement).

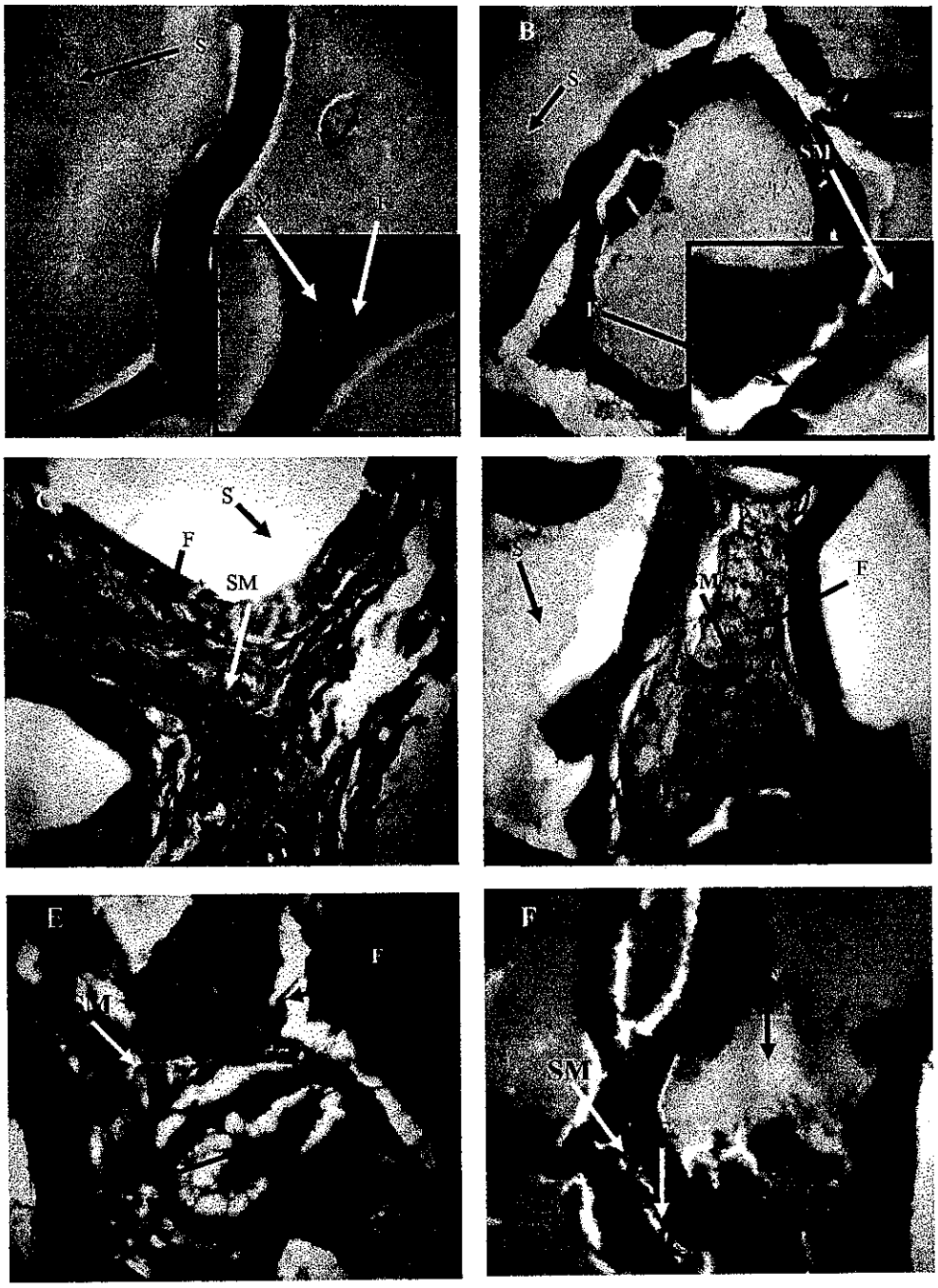


Figure 8. Prostate periductal stromal cells stained by Trichrome. In control negative (A) and control positive (B) groups, smooth muscle was continous (inset: SM, arrow), fibroblast was rare and discountinuous (inset: F arrow). In estrogen group (C) fibroblast was continuous and thick, smooth muscle was rare and discontinuous. In burn insecticide group (D), the histopathologic feature was similar to estrogen group. In liquid insecticide 3 ml group (E), smooth muscle was still continuous, fibroblast was frequently found. Similar feature also found in liquid insecticide 4 ml group (F). Secrete was colored blue in negative control group, in positive control group was colored graded equal red-blue. The secrete was not found in estrogen and burn insecticide groups. Secrete color in liquid insecticide 3 ml group was colored red, however, in liquid insecticide 4 ml group was colored graded equal red-blue. **Abbreviation** : F=periductal fibroblast, S= secret, SM=periductal smooth muscle

5.1.2. Effect of treatment on prostate gland histopathological characteristic (Composite score)

Examination of prostate gland was focused on alteration of epithelial ductile cell and periductal stromal cell. Haematoxyllin Eosin staining method was used to exploring epithelial ductal cell characteristic, while Masson's Trichrome staining method was used for periductal stromal cell exploration.

5.1.2.1. Haematoxyllin Eosin staining methods

Table 15. The p-value of comparison prostrate gland characteristics using HE staining methods between experimental groups. The p-value < 0.05 and < 0.001 were considered as significantly different.

Experimental Group	Control (-)	Control (+)	Estrogens	Burn Insect	Liquid 3 ml	Liquid 4 ml
Control (-)	-	0.067	< 0.001	< 0.001	0.002	0.009
Control (+)	0.067	-	< 0.001	0.019	0.361	0.260
Estrogens	< 0.001	< 0.001	-	< 0.001	< 0.001	< 0.001
Burn Insect	< 0.001	0.019	< 0.001	-	0.04	0.622
Liquid 3 ml	0.002	0.361	< 0.001	0.04	-	0.473
Liquid 4 ml	0.009	0.260	< 0.001	0.622	0.473	-

Estrogen treatment causes significant alteration of histopathological characteristic ($p < 0.001$) compared to positive control group. Histopathological section of prostate gland presented significant alteration on burn insecticides group ($p < 0.001$), liquid insecticides 3 ml group ($p = 0.002$), and liquid insecticides 4 ml group ($p = 0.009$) compared to negative control group (see Table 15).

5.1.2.2. Masson's Trichrome staining methods

Table 16. The p-value of comparison prostate gland characteristics using Masson's Trichrome staining methods between experimental groups. The p-value < 0.05 and < 0.001 were considered as significantly different.

Experimental Group	Control (-)	Control (+)	Estrogen	Burn Insect	Liquid 3 ml	Liquid 4 ml
Control (-)	-	0.095	< 0.001	< 0.001	< 0.05	0.05
Control (+)	0.095	-	< 0.001	< 0.05	0.541	0.864
Estrogens	< 0.001	< 0.001	-	0.185	< 0.05	0.001
Burn Insect	< 0.001	< 0.05	0.185	-	0.095	<0.05
Liquid 3 ml	< 0.05	0.541	< 0.05	0.095	-	0.639
Liquid 4 ml	0.05	0.864	< 0.001	<0.05	0.639	-

Estrogen treatment causes significant alteration of histopathological characteristic ($p<0.001$) compared to positive control group. Histopathological section of prostate gland presented significant alteration on burn insecticides group ($p<0.001$), liquid insecticides 3 ml group ($p<0.05$), and liquid insecticides 4 ml group ($p=0.05$) compared to negative control group. See Table 16.

5.2. DISCUSSION

5.2.1. Testis

In the recent years, it has been proved that the concentration of ERs is much higher throughout fetal life to adulthood in the male reproductive tract. The number of male tissues has the capability to express aromatize and synthesize estrogen. Estrogen plays in hypothalamic-pituitary-testis axis and act as a major regulator of negative feedback control. Its administration or deficiency could cause hypothalamic-pituitary-testis axis imbalance.

Development and differentiation of the Leydig, sertoli and germs cells are estrogen dependent, that it was synthesized through aromatization activity by them self. Thus, it could be deducted that the testis has capability to regulate/manage "supply and demand" (homeostasis mechanism) by synthesizing and responding to estrogen concentration. Administration of estrogen during developmental and differentiation of the testis could causes interruption in such mechanism (regulation of supply and demand).

Exposure of high dose of potent estrogens can cause significant suppression of FSH level consistently,[20] although exposure of low dose of potent estrogen or high dose of weak estrogen can cause significant increase in FSH production.[25] Elevated FSH level in normal range of adulthood may improve the efficiency of spermatogenesis.[20] Environmental estrogen exposure during developmental stage cause decreasing sensitivity to androgens, leading to the risk of testicular malignancy.[27] Adverse effect of estrogenic compounds to male reproductive differentiation and function has been reinforced by the fact that several group of

compounds are used daily in industry, agriculture, and also produced at home. So, the incidence of testicular cancer has been significant increased, being 2-3 times higher than 3-4 decades ago.[27]

Compared to positive control group, male rat treated with S.C. single dose of 25 μg β estradiol 3-benzoat diluted in 0,02 ml sesame oil in alternate days for 20 days, causes significant lowering of testis volume with p=0.0005 (see Table 11), as well as testis weight with p=0.0005 (see Table 12). It also proved by other researcher that perinatal and neonatal exposure of high dose of potent estrogens (25 μg β estradiol 3-benzoat) causes permanent effect of reduction of testis weight, reduced sperm production, and infertility.[3]

Exposure of new born rat to burn mosquito insecticides coil (transflutrin 0,03%) and 4 ml liquid mosquito insecticides (transflutrin 0.162 g/l as well as propoxur 4.05 g/l) for 20 days causes slightly but not significantly higher testis volume and testis weight compared to negative control groups (see Table 1). On the opposite, lowering on testis volume was noted after exposed to 3 ml liquid mosquito insecticides i.e. lowering of testis volume and testis weight (see Table 1). Propoxur (carbamate insecticides group) has never been proved as estrogen disruptor, and so does transfluthrin (pyrethroid insecticides group). But fenvalerate and sumithrin that are classified in pyrethroid group had been proved as estrogenic disrupter. [7] Exposure to weak estrogen during perinatal and neonatal could causes inverted manifestation i.e. increase testis weight compared to those of thus of exposed to potent estrogen.[3] In this study, exposure to 3 ml liquid mosquito insecticides causes slightly decrease of testis volume and testis weight,

but not significantly.[7] The effect of 3 ml liquid insecticides compare to those of 4 ml liquid insecticides and burn mosquito insecticides may be explained with several mechanism as bellow:

1. The degree of testis inflammation on 3 ml liquid insecticides group were quickly disappear and follow by atrophic reaction while on 4 ml insecticides group and burn mosquito insecticides, the inflammation process may be more severe and longer duration and therefore the atrophic process may or may not happened due to other process such hyperplasia, dysplasia, and change to malignancy.
2. The homeostasis of the organ by means of testis, may behave in other mechanism that could not be explained yet. Change or modification molecules that act as receptor, messenger or other downstream mechanism of cell activation and or apoptosis may exist. This possibility, however, still need further investigation.

It seems likely that other factor may contribute to the final affect, such as time exposure, concentration of the chemicals, and the hair contribution of the skin surface. Therefore it might better to be postulated that burn mosquito insecticides do have a detrimental effect of insecticides daily use if other study will do concern with other factors that mention before.

5.2.2. Penis

Developmental of genitalia external requires fetal secretion of dihydrotestosterone that converted to testosterone by the enzyme 5α-reductase. Failure of adequate gonadothropin stimulation or failure of testosterone production, or both, toward the end of gestation causes in adequate penis growth.[29] Shortly after birth, neonatal gonadothropin and testosterone production begin to fall. By age of 6 months, testosterone level increases in prepubertal age and, the penis growth occurs during this periods.[29] The penis remains infantile if hypogonadism becomes manifest before the onset of normal puberty and ended in smaller penis size (micropenis). Although, if hypogonadism appears after puberty, the penis size remain normal.[17] It also might be notice that penis size may decrease depend on the apoptosis process that may happen in secondary hypogonadism (in adult). Growth and differentiation of the penis as well as enlargement was stimulated by the presence of androgen especially dihydrotestosterone and inhibited by estrogen by means of negative feed back.[4,19]

Several hypothesis can be developed to explain the decreasing penis size such as: (1) males may have decreased plasma androgen concentrations that will result in reduction stimulation of the penis development and therefore reduces penis growing; (2) the relative ratio of testosterone to dihydrotestosterone can be altered by the suppression of the enzyme 5α-reductase, resulting in reducing penis size; (3) reduced numbers of androgen receptors on phallic tissue will also result in a reduction of the penis size; (4) the presence of an androgen antagonist that will effectively compete for the androgen receptor and block normal androgen

stimulation that produce inhibition of phallus growth; and (5) the presence of a xeno-biotic estrogen could shift the ratio of estrogen to androgen toward a feminizing environment in the developing embryo, thus blocking phallic development or inducing phenotype poorly responsive to androgens later in life.[4]

The result of this study clearly showed that the penis length and penis diameter were smaller significantly after 20 days estrogens treatment ($p=0.0005$) compared to positive control group. Exposed to burn insecticides for 20 days on newborn rats causes smaller penis length significantly ($p=0.025$) and shorter penis diameter ($p=0.0005$) significantly compared to negative control group. Exposed to 3 ml liquid insecticides did not really affect on penis length and penis diameter compared to negative control group. Twenty days exposure of 4 ml liquid insecticides also did not really affect on the alteration of penis length, but cause smaller penis diameter compare to negative control group with $p=0.0025$ (See Table 13 and 14). It is well known that administration of 25 μg β estradiol 3-benzoat (high dose of potential estrogens) will causes imbalancing of hypothalamic-pituitary-testis axis through negative feed back, that produced decreasing in testosterone production.

As mention before, propoxur (carbamate insecticides group) and transfluthrin (pyrethroid insecticides group) have never been proved founded as estrogens disruptor. On the other hand, fenvalerate and sumithrin, that classified as pyrethroid group that had been proved as estrogenic diruptor.[7] Ingredient of burn mosquito insecticides coil (Bayer Co.) contains transflutrin 0.03% that is suspected as an estrogens disruptor. Most of residual smoke on the air and other

pollutants had been proved and acted as an active foreign estrogens agent. Smoke that produced by burning of burn mosquito insecticides coil by means pollutant. In combination with burn insecticides chemicals characteristic can worsen the effect of residual smoke on the development of penis.

Ingredient of liquid mosquito insecticides contains transflutrin 0.162 g/L and propoxur 4.05 g/L. Exposed to 3 ml liquid insecticides was not really enough to alter the penis length and penis diameter. However, exposed to 4 ml liquid insecticides seem enough in altering the penis size. For simplification the examination of penis length was measure from the tip of the penis to bifurcation of crus penis, even it was not really the penis length. While measuring the penis diameter, calliper was clipped at penis cuff, and seems as it really penis diameter. Therefore, if the alteration of the penis size was not markedly different, the variability of penis length could not be presented, while the variability of penis diameter seems more prominent and easily being presented.

5.2.3. Prostate

Baygon mosquito insecticides were chosen in this study because it is widely used as household insecticides in Indonesia. In this study, examination of prostate gland was focused on alteration of luminal epithelial cells and periductal stromal cell. The prostate gland originally arises from interaction between urogenital sinus mesenchyme and endoderm of the proximal part of urethra. In the mouse, prostatic buds first emerge at the rostral end of urogenital sinus at approximately 17.5 days of gestation and continuous by develop toward the end

of gestation. Subsequently, the prostate epithelial buds undergo extensive ductal outgrowth and branching into the surrounding mesenchyme during the first three weeks postnatal development.[30] and undergo functional differentiation during the first 15 days of life. [33] Prostate formation and differentiation require interaction between epithelial and mesenchymal tissue. Firstly, androgen has been acting on the mesenchyme in order to produce signal for prostate cell stimulation and growth. Afterwards, androgens will acts on the epithelium for secretary function of differentiated cell types. Interaction between epithelial and stromal components are essential for all stage of normal prostate growth and development, disturbing of its interaction plays a significant role in carcinoma.[32] In the normal developing prostate, periductal mesenchymal cells differentiate to form a multicellular layer of smooth muscle cells and a thin layer of fibroblast that maintain intimate contact with the basal membrane of ductal epithelium. Signal from stromal are believed to be critical in determining the decision to epithelial cells to undergo proliferation, apoptosis, or differentiation. [32]

Androgen induced development includes branching morphogenesis, cellular differentiation, smooth muscle differentiation, and segregation of the epithelial into luminal and basal subtypes and ductal canalizations. [31] The prostate development is dependent to androgen stimulation, which is primary mediator of prostate differentiation. However 60 years ago, the possibility that estrogen is also involved in both the normal process of prostate development and subsequent adult prostate disease was introduced. The increasing of reproductive organ disorders has been linked to *in utero* exposure to endocrine-disrupting estrogenic chemicals

in the environment. The elevation level of estrogen (natural or man-made) during fetal life may alter development of reproductive organs (including prostate) may be predisposed for abnormal function and disease in later life. During critical period of cell differentiation, hormones are involved in "imprinting" specific genes in cells to provide receptors for the hormones. [35] Elevation of estrogen level during critical differentiation period may affect the expression of gene involved in the morphogenesis of the gland. Those in turn, result in persistent changes in the histological architecture of the gland and epithelial secretary pattern of prostate specific acid phosphatase (PAP), and altered in glandular cell function at puberty. It blocking of the process may include paracrine communication between smooth muscle and epithelial cells (which normally regulate differentiation) and may enhance the invasiveness and/or malignant potential of the nascent tumor.[32] Several studies in neonatal rats that had been exposed to higher dose of estrogen resulted in reducing responsiveness to androgen, permanent suppression of prostate growth, retard branching morphogenesis and epithelial differentiation during development, permanent alter secretary function, and an induction of epithelial hyperplasia in adulthood.[31,36] This process referred to as neonatal imprinting is associated with an increasing incidence of prostate lesions upon aging which includes hyperplasia, inflammation, and dysplasia similar to intraepithelial neoplasia. [37]

Effects of estrogen seems likely not only mediated by changes in androgen level via suppression of the hypothalamic-pituitary-gonadal axis and reduction of androgen level, but also by additional direct effect on prostate growth.[31] The

direct effect on prostate gland induces: (1) Up-regulates estrogen receptor expression and down regulates androgen receptor expression, (2) Reduced responsiveness to androgen in adulthood, (3) Alter epithelial cell adhesion and gap junction proteins, (4) Alter the Transforming Growth Factor-β signaling system in the developing prostate and block the transient $p21^{cip1/waf1}$ expression associated with epithelial differentiation, and (5) Proliferation of periductal fibroblast and alter the ECM composition in the rat prostate.[31,36,37,39-41] Prostate gland histopathological characteristic was significantly altered after exposure to estrogen compared to those of those of positive control group, as well as after exposed to burn mosquito insecticides coil, 3 ml liquid mosquito insecticides, and 4 ml liquid mosquito insecticides. This result has been proved using both Haematoxyllin Eosin staining for exploration of luminal epithelial characteristics and Masson's Trichrome staining for periductal stromal cells exploration. (See Table 15 and 16)

Exposed to high dose potent estrogen (25 μg β estradiol 3-benzoat) causes alteration of luminal epithelial cells and periductal stromal cells. Estrogen (as a lipid soluble steroid hormone), via interaction with their receptors, plays an important role in the control of cellular growth and differentiation. [49] Their result seems in line with other former research which proved that exposed to higher dose of estrogen resulted in altering prostate histopathology and function as well as behavior.[31,36] Haematoxyllin Eosin staining methods showed that exposure of estrogen for 20 days causes severe hyperplasia of luminal epithelial cells and atrophic (see Table 2), the cellular polarity was 100% bad (see Table 4), most of

prostate gland luminal epithelial cells were polimorphic (see Table 5), chromatin appearance was 100% rough (see Table 6), and nucleolus was found in 100% of prostate gland luminal epithelial cells (see Table 7). Thus, histopathological characteristic is considered to be **pre-malignant lesion and the main precursor to invasive carcinoma of the prostate and also called as prostate intraepithelial neoplasia (PIN).** [50,51] The histopathological alteration has been divided into three (3) grades, depending on the severity of the following change i.e. cell crowding and stratification (cellular polarity), nuclear enlargement, cellular polymorphism, chromatin pattern, and nucleolus appearance.[52] Human prostate carcinogenesis was believed to involve multiple process from hyperplasia through dysplasia (pre-cancerous) to carcinoma, from low histological grade to high grade, and finally anaplastic. [53] Beside of that, the periductal stromal cells which were examined by Masson's Trichrome staining methods and exhibited that 100% of periductal fibroblast became thick and continues (see Table 8), 60% of smooth muscle become rarely present (see Table 9), and 80% of prostate gland did not produce luminal secretion (see Table 10). Presentation of a thicker layer of fibroblast and reduction of smooth muscle cells on periductal stromal cells will influences branching morphogenesis and may end up to undergo proliferation, apoptosis, or differentiation. [32]

Histopathological characteristics of luminal epithelial cells and periductal stromal cells of prostate gland expressed to burn mosquito insecticides, 3 ml, and 4 ml liquid mosquito insecticides groups was significantly altered (see Table 15 and 16). Burn mosquito insecticides and liquid mosquito insecticides are

suspected act as a foreign estrogen (as a weak estrogen). Exposure of these insecticides causes prostate gland epithelial hyperplasia (see Table 2), thickening of luminal epithelial layer (see Table 3), alteration of chromatin appearance (see Table 6), presenting less than a half of nucleolus (see Table 7), thickening of periductal fibroblast (see Table 8), periductal smooth muscle cell become more thin and discontinues (see Table 9), and alteration of prostate gland's secretion pH (see Table 10). Normal pH of prostate gland secretion was high (alkali), while abnormal pH of prostate gland secretion was low (acid). Using Masson's Trichrome staining methods, alkali would be presented as blue color, and acid would be showed as red color. [70]

Expose to estrogen in the neonatal rat cause disrupting the normal morphology, development, and the function of the prostate gland. Pituitary lactothroph is well known established as estrogen's responsive cells. Estrogen exposure can affect releasing prolactine by acting directly on the lactothroph cells in pituitary anterior, and indirectly through inhibition of hypothalamic dopaminergic suppression pathways.[41,45] Alteration become manifest in adulthood such as epithelial hyperplasia, dysplasia, and inflammation. The inflammatory responses consist of infiltrating T-lymphocytes and macrophages are typically observed in chronic prostatitis in both rodent and human prostate. [42,43] Thus effect may deducted as a result from transient period of hyperprolactinemia just prior to puberty. [6] These mention finding are particularly significant that in neonatal transient estrogen exposure will increase pre-pubertal circulating prolactine level indirectly by altering development of dopaminergic neuron in the arcuate nucleus.

[42] Directly, lymphocytes be composed of prolactine receptors and prolactine it self is comitogen to T cells proliferation as well as an induction of cytokine and antibody production. [42] In this study, infiltration of lymphocyte and polymorphonuclear leukocyte in prostate gland was found in certain histopathological section of prostate gland in estrogen group. Chronic or recurrent inflammation probably has role in the development of many types of cancer in human, including prostate cancer.[46]

Chapter 6

CONCLUSION AND SUGGESTION

6. 1. CONCLUSIONS

1. β estradiol 3-benzoat (a high potent estrogen) treatment in alternate days for 20 days caused significant lower testicle volume and weight, shorter and smaller penis compare to positive control group. Those change were consistent with histopathological characteristics in adult hood.
2. Opposite to estrogen treatment, exposure of estrogenic compounds (burn mosquito insecticides and liquid mosquito insecticides those were considered as weak estrogen) caused slightly higher testicle volume and weight compare to negative control group.
3. Exposure of burn mosquito insecticides, 3 ml and 4 ml liquid mosquito insecticides did not significantly alter testicle volume and testicle weight.
4. Exposure of burn mosquito insecticides caused significantly exhibit shorter and smaller of penis compare to negative control group.
5. Exposure of 3 ml liquid insecticides did not significantly alter penis length and penis diameter
6. Exposure of 4 ml liquid insecticides did not significantly alter penis length, but caused significantl smaller penis diameter compare to negative control group.

7. Burn mosquito insecticides, 3 ml and 4 ml liquid mosquito insecticides caused alteration of histopathological characteristics of the prostatic gland of adulthood.
8. There is a permanent enlargement of prostate gland as well as alteration in the functioning of estrogens responsive organ (testis and penis) in rat, could be happened due to exposure to low weak estrogenic compounds during developmental period
9. Generalisation

   Since the effect of foreign estrogenic compounds had been recorded such as the human sperm production falls during fifty years by Skakkebaek and the incidence of testicular cancer has been significant increased being 2-3 times higher than 3-4 decades ago by Giwercman, that most of scientist incriminated the foreign estrogen as the main cause that play in important role of those alteration

   Hence we make deduction that the effect of the chemical used in this studies also affect to human as well. This deduction seems to be supported by unofficially founding of the increasing number of children having micropenis and young adult human male that suffer inflammation and or infection of the prostatic gland

6.2. SUGGESTION

1. Testicle volume and testicle weight are the variables that less sensitive for a little affection and disruption. Further study is absolutely needed to

investigate the possibility of alteration of testicle organ through examination of testicle histopathological section.

2. Examination of penis length that measured from the tip of penis through bifurcatio penis may not represent the real penis length, therefore, for further research that examine the penis length should be measure from the tip of penis through base of the radix penis
3. For more understanding the this theoretical research, it suggested that this study should be continued with:
    a. Examining of estrogen receptor and androgen receptor
    b. Examining of epithelial cell adhesion and gap junction protein
    c. Examining of Transforming Growth Factor-β
    d. Examining expression of $p21^{cip1/waf1}$ (a cyclin-dependent kinase inhibitor) within differentiating prostatic epithelial cell
4. Concern with the permanent enlargement of prostate gland as well as alteration in the functioning estrogens responsive organ in rat, that could occur due to exposure to low weak estrogenic compounds during developmental period, we proposed the possibility of hazardous effect of using insecticides at home especially for our babies and children
5. Using mosquito net is suggested in order to prevent mosquito bite related diseases.

Chapter 7

SUMMARY

Over the past decades, there has been increasing concern about the impact of environmental compounds that act like hormone in human development and reproductive health. There are numerous reports of reproductive and developmental abnormalities in species ranging from snail to humans that have been associated with the exposure of environmental hormone/estrogen like hormone. Environmentally estrogenized phenotypes may differ depending upon the time of exposure, i.e. whether the exposure occurred at developmental causes irreversible alteration, while its occurred at post-developmental causes reversible alteration.

Exposure to estrogen compound may occur not only through industrial and agricultural activities, but also come from compounds that used in food production and as well as food packaging such as pesticides. Studies concerning the exposure of estrogen compounds that caused the alteration on reproductive organ behavior have been done in many countries and region. Many pesticides aa well as insecticides have been reported and classified as endocrine disruptor such as DDT, dieldrin, toxaphene, and endosulfan. Recently, to overcome the mosquito resistant, the use of combination of pyrethroid and carbamate insecticides in agricultural and in household has been encouraged by promotion of laboratory evidence, suggesting that they are relatively safe to human and wildlife. Some of the pyrethroid and carbamate insecticides are believed to be toxic to the reproductive system and disruptive to endocrine function. However little has been

done to assess their hormonal potential activities *in vivo* as well as *in vitro* especially to focus on estrogenic activities.

The aim of this study is to elucidate the clinical effect of proposed estrogenic compound during young period of male rat on inducing alteration of reproductive organ development in adult and investigate whether burning mosquito insecticide coil and using liquid mosquito insecticide have the same effect as β estradiol 3-benzoate that cause alteration reproductive organ development and behavior. This study is expected to increase awareness and alertness concerning insecticide daily usage in the family in order to assure normal reproductive development and health of the children and discourage from abnormal sexual differentiation, alteration behavior, infertility and the development of disease/malignancy in their adult life.

Endocrine disruptor has been defined as an exogenous agent that interfere with the synthesis, secretion, transport, and action or elimination of natural hormones in the body that are responsible for the maintenance of homeostatis, reproduction, development and or organ behavior. They can act as endocrine disruptor in a variety of way. Some of them occur naturally as phytoestrogens and some of which are synthetic chemicals or called man-made. Most of man-made estrogen is weaker than natural estrogen, but they have several characteristic such as lipophilicity, has a long half-lives, and do not break down readily in the environment. Thus, allow them to accumulate and persist in fatty tissues. Consequently, species feeding at highest of the food chain, such as humans, are the most vulnerable to the adverse effect of environmental pollutant.

Interesting about the role of estrogen is increasing in recent years, especially in male reproduction tract. The largely interesting of estrogen role in male reproduction tract is mainly due to the demonstration that male fertility is impaired in mice by lack of estrogen receptor and enzyme aromatase. Concentrations of estrogen receptor (ERs) are higher throughout the male reproductive tract than in the other organ. The role of estrogen in hypothalamo-pituitary-testis axis is a major component of negative feedback regulator of gonadotropin secretion. Perinatal and neonatal exposures to various concentrations of natural and synthetic estrogens cause irreversible organizational changes in the developing male rodent reproductive tract. Exposure of high dose of potent estrogens to neonatal rat can cause reduction of adult testis weight, matting, or fertility, in the contrary, low doses of weak estrogens cause the inverted manifestation of these effect. Exposure of high dose of potent estrogens can cause significant suppression of FSH level consistently, although the exposure of low dose of potent estrogens or high dose of weak estrogens can cause significant increase in FSH production. Elevated FSH level in normal range of adulthood may improve the efficiency of spermatogenesis.

The development of the male reproductive ducts and genital external in vertebrates is dependent on androgen level during embryonic development and the period of post-natal growth. Androgen hormones especially testosterone are responsible for differentiation of Wolfian duct into male reproductive duct, while dihydrotestoterone (DHT) is importance for formation of external genitalia. Development of genitalia external requires fetal secretion of dihydro testosterone

(DHT) that converted to testosterone by the enzyme 5α-reductase in certain tissues. Fetal production of androgens, especially testosterone, is necessary for normal male development. Early in gestation, placental chorionic gonadothropin (hCG) stimulates the developing testis to produce testosterone. Later in gestation after organogenesis has occurred, the fetal pituitary takes control through production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Failure of adequate gonadothropin stimulation or testosterone production, or both, toward the end of gestation can cause in inadequate penis growth. The hipothalamo-pituitary-gonadal axis is active in late fetal life, shortly after birth, gonadothropin and testosterone production begin fall. By age 6 months, testosterone level increases in pre-pubertal age, the penis growth occur during this time. The penis remains infantile if hypogonadism becomes manifest before onset of normal puberty. If hypogonadism appears before puberty, changes in penis size will be manifest. However, if hypogonadism appears after puberty, changes in penis size my slightly occur due to apoptosis.

Growth and differentiation of the penis is stimulated by the presence of androgen (androgen dependent) especially dihydrotestosterone (DHT) and inhibited by estrogens by means of negative feed back. Alligators in Florida's Lake Apopka were exposed to DDT have significant smaller penis size, lower plasma testosterone concentration, and lack of responsiveness of the penis to the plasma androgen present. Therefore penis size seems likely an obvious marker of abnormal androgen concentration or function before puberty.

The increasing frequency of developmental abnormalities of male phallic structures is reported in humans, fish and alligators. The presence of a xeno-estrogen could shift the ratio of estrogen to androgen toward a feminizing environment in the developing embryo, thus blocking phallic development or inducing phenotype poorly responsive to androgens later in life.

The rat prostate gland is rudimentary at birth and undergoes extensive branching morphogenesis followed by functional differentiation during the first 15 days of life. Prostate formation and differentiation requires interaction between epithelial and mesenchymal tissue. Firstly, androgen has been acting on the mesenchyme in order to produce signal for prostatic induction and growth. Subsequently, androgen acts on the epithelium for secretory function of differentiated cell types. Interaction between the epithelial and stromal components are essential for all stage of normal prostate growth and development, disturbing of its interaction may plays a significant role in developing carcinoma. In the normal developing prostate, periductal mesenchymal cells differentiate to form a multicellular layer of smooth muscle cells and a thin layer of fibroblasts that maintain intimate contact with the basal membrane of ductal epithelium. Signals from the stromal are believed to be critical in determining the decision to epithelial cells to undergo proliferation, apoptosis, or differentiation. During critical period in cell differentiation, hormones are involved in "imprinting" specific genes in cells with receptors for the hormones. Elevation of estrogen level causing by the exposure of environmental estrogen during critical differentiation period may affect the expression of gene involved in the morphogenesis of the

gland. That in turn, result in persistent changes in the histological architecture of the gland and epithelial secretory pattern of prostate specific acid phosphatase (PAP), and altered in glandular cells function at puberty. Prenatal exposure to weak environment estrogen alters the differentiation pattern of the periductal stroma cells by presenting of a thicker layer of fibroblast, the reducing number of smooth muscle cells were demonstrated androgen receptor (ARs) in Bispherol A treatment. Present of thicker layer of fibroblast and reduction of smooth muscle cells on periductal stromal cells can act like "physical barrier" that influences branching morphogenesis. The physical barrier may eventually blocks paracrine communication between smooth muscle and epithelial cells (which normally regulate differentiation) and enhance the invasiveness and/or malignant potential of the nascent tumor. On the other hand, decreasing of ARs expression in periductal stromal cells, may also alter the androgen-signaling pathway.

Exposed to higher dose of estrogen resulted in reduced responsiveness to androgen, a permanent suppression of prostate growth, retard branching morphogenesis, epithelial differentiation during development, permanent alteration secretory function, and induction of epithelial hyperplasia in adulthood. his process referred as neonatal imprinting that associated with an increasing incidence of prostatic lesions upon aging which includes hyperplasia, inflammation, and dysplasia similar to intraepithelial neoplasia. In male mice, exogenous administration of estrogens could cause alteration hypothalamic-pytuitary-gonadal axis and reduces androgen level, leading to regression of the prostatic epithelium or induces of epithelial squamous metaplasia. The effects of

estrogens are not only mediated by changes in androgen level via suppression of the hypothalamic-pituitary-gonadal axis and subsequent reduction of androgen level, but also by additional direct effect on prostatic growth.

Estrogen exposure can affect releasing PRL by acting directly on the lactotroph cell the in pituitary anterior or indirectly via hypothalamus-pituitary's factor that regulate the lactotroph. The direct acting on the lactotroph is regulation of the transcription through on ERE (estrogen response element) that binds to estrogen receptor in the anterior pituitary, on the other hand, the indirect action is executed through inhibition of hypothalamic dopaminergic suppression pathways. Estrogen exposure in the neonatal rat has been shown to disrupt the normal morphology, development and the function of the prostate gland. The response to this exposure is manifest in the adulthood as epithellial hyperplasia, dysplasia, and chronic inflammation. The inflammatory responses consist of infiltrating T-lymphocytes and macrophages, which is typically observed in chronic prostatitis in both rodents and humans. These effects may have resulted from a transient period of hyperprolactinemia just prior to puberty. Over expression of prolactine (PRL) can result in prostatic enlargement and dysplasia, indicating that PRL provides an additional growth regulatory mechanism for the prostate. The basis for chronic inflammatory response in the neonatal estrogenized prostate is not understood. However, it is known that both estrogen and prolactine can have marked effects on the immune system in general and prostatic inflammation in particular. In certain studies, demonstrated that estrogen-induced immune response is mediated through hyperprolactinemia. Lymphocytes own prolactine

receptors while prolactine it self act as comitogen for T cell proliferation as well as an induction of cytokine and antibody production. The indirect action seems through inhibition of hypothalamic dopaminergic suppression pathways. Thus possibility suggesting that the prostatic inflammation induced by neonatal estrogens is indirectly mediated through transient prepubertal hyperprolactinemia. Chronic or recurrent inflammation probably has a role in the development of many types of cancer in human, including prostate cancer. Inflammatory cells elaborate numerous microbicidal oxidant that might cause cellular or genomic damage in the prostatic cells. Prostatic lesion called proliferative inflammatory atrophic is apparently become a precursor of prostatic intraepithelial neoplasia and prostatic cancer. Focal areas of epithelial atrophy in the prostatic gland have long been noticed and as an important factor in developing prostatic carcinogenesis.

Pesticides are man-made in order to kill living things such as plants, fungi, insects or rodents. Unfortunately, chemicals designed to kill plants or animals are often dangerous for other living thing including human. Fetuses, babies, and children are especially vulnerable to the toxic effect of pesticides and insecticides. Around 2,5 million tons of pesticides used worldwide each year, less than 0,1 percent reaches the target pest. Thus 99 percent of currently applied pesticides or being released indiscriminately into the environment, many of them will persist for years and may travel far from the point of application. Some pesticides can act as carcinogens by causing mutation in cell's genetic material, by changing the activity of hormones, or by interfering with the action of system that normally prevent tumors. Some of them act as endocrine disruptors, by means of interfering

of hormonal action in our bodies. Exposure of endocrine disruptors to fetuses, infants, and children could promote development of hormone-responsive tumors, and may interfere the sexual development in adulthood. It also may cause a variety of detrimental health effect including genital development, behavior, and reproductive hazardous.

Propoxur is one of the *N*-methylcarbamate esters that inhibits cholinesterase in insect. Due to its low toxicity to mammals and other vertebrates, propoxur is an economically important insecticide and manufactured in very large quantities. Its widely used as aerosol to control agricultural and household insect pests. However, several studies found that propoxur is carcinogenic agent which act through inhibition of gap junction intercellular communication (*GJIC*) and was proved as mutagenic substance.

Transfluthrin is the latest pyrethroid agent developed for Baygon®. It is one of the best-tested insecticides agents, and has been incorporated in household product against flying insects such flies, mosquitoes, and cockroaches since 1996. In the environment, pyrethroids are usually degraded by biotic and a-biotic process i.e. metabolic degradation by plants, microorganisms, and light (photolysis). The rate of degradation depends on the type of pyrethroids, soil type, climate, the species of microbes present, and the size of their population. Pyrethroids are commonly used insecticides worldwide, but little has been done to characterize their hormone potential. Fenvalerate and sumithrin demonstrated significant estrogenicity at concentration of 10μM. Long term pyrethroid effect has been proved can cause disruption of endocrine system by mimicking the

female hormone (estrogen) thus causing excessive estrogen level in female. In male, its estrogenizing (feminizing) effects include lowered sperm production. In both, female and male, it is known carcinogen. Through this hormonal pathways, exposure to certain pyrethroids ended on reproductive dysfunction, development impairment, and cancer.

The hypothesis of this study is exposure of burning mosquito insecticide coil (transflutrin 0.03%), liquid mosquito insecticide (transflutrin 0.162 g/l and propoxure 4.05 g/l) and administering 25μg β estradiol 3-benzoate to neonatal male Spargue Dawley rat during 25 days can cause alteration of reproductive organ in latter life.

The design of this study is true experimental design with the post test only control group design. Study was done at UPHP (Unit Pemeliharaan Hewan Percobaan) Gadjah Mada University in Yogyakarta, started on June 2003 until December 2003.

Male Sprague Dawley (SD) rat, age postnatal day-3, weight 6-8 grams were allocated in to 6 groups by simple random sampling method by using random event. There are 4 groups of treatment and 2 groups of control. Group I was negative control group I ; groups II was positive control, rats in this group were received 0.02ml 0,9% Sodium Chloride (Otsuka Indonesia) s.c single dose with BD non-traumatic needle in alternate day during 20 days; group III was treatment group I, rats in this group were received 25 μg β estradiol 3-benzoat diluted in 0.02 ml sesame oil s.c single dose with BD non-traumatic needle in alternate day during 20 days; group IV was treatment group II, rats in this group

were exposed to smoke from burned mosquito insecticide coil (contain transflutrin 0.03%) for 8 hours a day during 20 days; group V was treatment group III, rats in this group were exposed to 3 ml liquid mosquito insecticide (contains transflutrin 0.486 mg and propoxur 12.15 mg) that sprayed to cage air by nebulizer once time a day during 20 days; group VI was treatment group IV, rats in this group were exposed to 4 ml liquid mosquito insecticide (contains transflutrin 0.648 mg and propoxur 16.20 mg) that sprayed to cage air by nebulizer once time a day during 20 days.

After treatment, all rat was maintained under standard conditions in UPHP Yogyakarta until aged 100^{th} days old. On aged 100^{th} day old, all rats were anesthetized by ether then killed by cervical dislocation. The penis, testis and prostate gland were examined. Testis volume includes epididymis and lamina visceralis tunica vaginalis were measured by calibrated beaker and were weighed by Sartorius® balance. Penis size (without preputium) was measured by GPM® calliper. Examination of prostate gland was focused on alteration of luminal epithelial cell and periductal stromal cell. Haematoxyllin Eosin staining method was used for exploring luminal epithelial cell characteristic, while Masson's Trichrome staining method was used for periductal stromal cell exploration.

Collected data were checked for data cleaning, coding and scoring, then were entered to computer. Data analyses were descriptive analysis and hypothesis testing. Since there were 6 experimental groups, to examine the difference penis size and testis volume between groups. One Way ANOVA procedure was subjected to normally distribution and homogeneity of variance that were checked

prior to analysis. Normality test by Shapiro-Wilk procedure showed the data were not normally distributed. Therefore, Kruskall-Wallis test were used to examine the difference of testis volume and penis size between group. Pair comparison was done by Mann-Whitney test. While χ^2 test were used to assess the difference of distribution frequency of histopathological characteristics between groups.

β estradiol 3-benzoat (a high potent estrogen) treatment in alternate days for 20 days caused significant lower testicle volume and weight, shorter and smaller penis compare to positive control group. Those change were consistent with prostatic gland histopathological characteristics in adult hood. Opposite to estrogen treatment, exposure of estrogenic compounds (burn mosquito insecticides and liquid mosquito insecticides those were considered as weak estrogen) caused slightly higher testicle volume and weight compare to negative control group. Exposure of burn mosquito insecticides, 3 ml and 4 ml liquid mosquito insecticides did not significantly alter testicle volume and testicle weight. Exposure of burn mosquito insecticides caused significantly exhibit shorter and smaller of penis compare to negative control group. Exposure of 3 ml liquid insecticides did not significantly alter penis length and penis diameter. Exposure of 4 ml liquid insecticides did not significantly alter penis length, but caused significantl smaller penis diameter compare to negative control group. Burn mosquito insecticides, 3 ml and 4 ml liquid mosquito insecticides caused alteration of histopathological characteristics of the prostatic gland of adulthood.