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Insecticides Exposure during Early Life Alters Prostate Cells Differentiation in Adulthood Spraque Dawley Rat

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Abstract: Exposure of endocrine disruptor chemicals during early life may alter prostate morphogenesis and cellular differentiation, associated with an increased risk of prostatic intraepithelial neoplasia (PIN). This study was designed to elucidate the effect of early insecticide exposure in the prostate gland. Newborn male Spraque Dawley rats were allocated into untreated (control) and treated groups, including estrogen potent (25 μg β estradiol 3-benzoate), mosquito coil repellent, and liquid mosquito repellent. Prostate luminal epithelial and periductal stromal cells were significantly altered among mosquito insecticide groups [mosquito coil repellent ($p < 0.001$), 3-ml ($p < 0.002$), and 4-ml liquid mosquito repellent ($p < 0.009$)] and [mosquito coil repellent ($p < 0.001$), 3-ml ($p < 0.05$) and 4-ml liquid mosquito repellent ($p = 0.05$)] compared to those of control group, respectively. Mosquito insecticide exposure during early life leads to an alteration of prostate cellular differentiation in adulthood that may predispose to prostate cancer.

Keywords: early life exposure, endocrine disruptor chemicals, insecticides exposure, mosquito repellent, prostate cancer.

生命早期的杀虫剂暴露会改变成年斯普拉格·道利大鼠的前列腺细胞分化

摘要：早期暴露于内分泌干扰物的化学物质可能会改变前列腺形态发生和细胞分化，从而增加前列腺上皮内瘤变（别针）的风险。该研究旨在阐明早期杀虫剂在前列腺中的暴露作用。将新生的斯普拉格·道利雄性大鼠分为未治疗组（对照组）和治疗组，包括雌激素有效（25微克雌二醇3-
苯甲酸酯），驱蚊剂和驱蚊剂。蚊虫杀虫剂组之间的前列腺腔上皮和导管周间质细胞发生了显着变化[蚊香驱蚊剂（p <0.001），3-毫升（p <0.002）和4-ml液体蚊香剂（p <0.009）]和[蚊香驱蚊剂（p <0.001），3毫升（p <0.05）和4毫升液体蚊香（p = 0.05）]。生命早期暴露于蚊虫杀虫剂会导致成年期前列腺细胞分化的改变，这可能导致前列腺癌。

关键字：早期生活暴露，内分泌干扰物化学物质，杀虫剂暴露，驱蚊剂，前列腺癌。

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1. Introduction

Over the past decades, there has been increasing concern on the impact of environmental contaminants exposure known as endocrine disruptor chemicals (EDCs), chemicals that may interfere with the biosynthesis, metabolism, and/or action of endogenous hormones. Alterations of male reproductive development and health that have been reported to be associated with EDCs range from testicular dysgenesis syndrome (TDS), which includes demasculinization or feminization, cryptorchidism, hypospadias, in situ germ cell testicular carcinoma, reduced semen quality, and progression of prostate cancer [1], [2], [3].

Prostate cancer is the most common malignancy in men and the second cause of cancer deaths in developed countries. In the United States, the incidence (1.6-fold) and mortality rate (2.4-fold) among the African-American population were reported higher compared to the white population [4]. Gene polymorphisms, environmental factors (EDCs), lifestyle, and endogenous factors (age, race, and family history) have been studied to comprehend the discrepancy. Prins S reported increasing evidence both from epidemiology studies and animal models determining the role of EDCs that may interfere with the development and progression of prostate cancer [5]. In humans, common agricultural pesticide exposure is associated with an increased rate of prostate cancer in men with a positive family history, suggesting genetic-environmental interactions [6]. An increased risk of prostate cancer was reported in numerous studies related to populations exposed to long-term and low-dose insecticides (organophosphate and organochlorine) [7]. Testosterone plays the primary role in prostate cancer; however, early-life exposure to estrogenic compounds, including EDCs, may also enhance prostate susceptibility to hormonal carcinogens in adulthood [8], [9].

Every tissue has a specific window of development, which refers to the critical period when the developing tissue is susceptible to the effects of EDC exposure [10]. The critical window of reproductive tract development is between 7 and 40 weeks of gestation, during which cells undergo rapid mitotic division and differentiation, eventually resulting in puberty and maturation. The exposure of EDCs during the critical window period will affect cell reprogramming [11]. Therefore, besides the dose and potency of EDCs, the timing of exposure is also very critical, as exposure during the window of development likely leads to irreversible effects. In contrast, the effects of exposure outside of the window of development can be reversed or mitigated with the removal of exposure [12]. Additionally, EDCs were designed to have a longer half-life as an industrial use benefit. Still, their longevity is detrimental to wildlife and humans, and some chemicals that were banned decades ago remain at high levels in the environment.

Tropical countries such as Indonesia have been affected more rigorously by infectious diseases than temperate countries due to biological and environmental factors. Mosquitoes are the most common transmitters of vector-borne human diseases in tropical countries due to environmental conditions (temperature and humidity) conducive for reproduction. In addition, human cultural behavior also plays a role in disease-transmission control programming. Children under the age of 5 have a higher risk of mosquito-borne diseases. Accordingly, to protect children from mosquito bites, mosquito repellent usage is prevalent, often without considering potential side effects [13], [14]. This study was aimed to elucidate the effect of early life insecticide exposure on prostate gland alteration in rats.

2. Methodology

2.1. Animal and Housing

A posttest-only control group was designed to assure the objectivity of the study. Male one-day-old Sparque Dawley (SD) rats were acclimatized for 2 days in standard cages under standard conditions (room temperature at 26 ± 3°, 12-hour light/ dark cycle) and kept in standard cages at the Animal Care Unit in Gadjah Mada University, Yogyakarta. The rats were randomly selected and allocated into 5 groups: control (untreated), treated groups including 25 μg β estradiol 3-benzoate, and insecticide groups [spiral mosquito coil repellent contains transfluthrin 0.03%/coil; 3-ml and 4-ml mosquito liquid repellent contains transfluthrin 0.162 mg and propoxur 4.05 mg/ml]. After weaning, the rats were housed up to 5 per cage. Drinking water was available in plastic bottles, and the rats were fed a pellet diet *ad libitum* from weaning to postnatal day (PND) 100. Pesticide exposure was designed to resemble the natural setting of mosquito repellent usage.

2.2. Administration of β Estradiol 3-Benzoate and Mosquito Repellent

Twenty-five μg of β estradiol 3-benzoate was diluted in 0.02 ml of sesame oil and administered subcutis in single doses with a non-traumatic needle during alternate days for 20 days. The exposure of mosquito insecticide was performed in a different room out of proximity to prevent effect interference. Multilevel cages were used to expose the rats to mosquito coil repellant fumes, which last up to 8 hours. Coils were burnt once a day for 20 days on the ground level of the cage. The rats were placed on the upper level of the cage to allow maximum exposure to the rising repellent fumes. Liquid mosquito repellent groups were divided into two groups, 3-ml/day and 4-ml/day. For 20 days, liquid repellant was sprayed using a nebulizer (Bremmed®), a drug delivery device used to administer medication in the form of a mist inhaled

into the lungs. Twenty days of exposure in a rat is equal to 18 months in humans [15]. The walls of the cages were covered, and wire mesh was placed at the top to allow for ventilation. After exposure, all groups were maintained under standard conditions until the age of 100 days.

2.3. Histopathology

Prostate glands were removed and fixed in 10% buffered neutral formaldehyde solution for 24 hours at room temperature before being embedded into paraffin blocks. After paraffin embedding, serial of 5 µm thickness sections was then stained. Haematoxylin and eosin (HE) staining method was used to explore luminal epithelial cells, while the Masson's Trichrome staining method was used to explore periductal stromal cells [16]. Six sections and 5 fields per section were randomly chosen in every single rat and examined at magnification ×10 of light microscopy. Histopathological features were examined using a single-blind method by covering the labels at the light microscope level, which an experienced pathologist checked.

HE sections were used to assess the degrees of hyperplasia, cellular polarity (good-bad), epithelial cell polymorphism (negative-positive), chromatin pattern (smooth-rough), and nucleolus presentation (negative-positive) in luminal epithelial cell compartments. The Masson's Trichrome technique differentially stains stromal components and is useful in distinguishing prostate smooth muscle cells (red) from collagen fibers (blue) [17], and was used to assess fibroblast proliferation, smooth muscle arrangement (continuous-rare), and prostate gland secretory function.

2.4. Statistical Analysis

The histopathological characteristics of the prostate gland are defined in frequency, and percentage terms, so histopathological characteristics were analyzed as a composite score. The Shapiro-Wilk procedure showed non-normally distributed data, so group comparisons were made using the Mann-Whitney test. In contrast, chi-squared tests were used to assess the differences in distribution frequency between groups. P-value less than or equal to 0.05 were considered significant. The power of this study was 80%, with a 95% confidence interval (CI).

3. Results and Discussion

The histopathological section was focused on the alteration of luminal epithelial and periductal stromal cells of the prostate gland. HE sections used to assess luminal epithelial cells revealed some interesting results regarding insecticide exposure during early life. Among the estrogen group, degrees of epithelial hyperplasia were the highest compared to those of the control and insecticide groups. In contrast, the degrees of hyperplasia in the insecticide groups were significantly higher than those of the control group. The cellular polarity of epithelial cells was bad in estrogen groups. The highest percentage of bad cellular polarity among insecticide groups was found in the mosquito repellent coil group (27.8%). Epithelial cell polymorphism was found in most subjects of the estrogen group (93%). Surprisingly, polymorphism was not significantly found among the insecticide groups. Chromatin patterns were significantly altered in all treated groups compared to those of the control group: severely rough in all (100%) epithelial cells of the estrogen group; alteration of chromatin pattern was found in 77.7%, 55.5%, and 50% of mosquito coil, 4-ml, and 3-ml mosquito liquid repellent, respectively. Nucleolus was found in all subjects (100%) of the estrogen group. Among the insecticide groups, nucleolus was found in 44.4% of the mosquito repellent coil group, in 44% of 4-ml mosquito repellent liquid, and 25% in the 3-ml mosquito repellent liquid group. Luminal epithelial cells were significantly altered in all treated groups compared to those of the control group (see Table 1). The histopathological features of epithelial cell alteration can be seen in Fig. 1.

Table 1 Alteration of a luminal epithelial cell of untreated/control group compared to treated groups (P-value ≤ 0.05 was considered significantly different)

Experimental Group	Control	Estrogen	Coil repellent	Liquid 3-ml	Liquid 4-ml
Control	-	< 0.001*	< 0.001*	0.002*	0.009*
Estrogens	< 0.001*	-	< 0.001	< 0.001	< 0.001
Coil repellent	< 0.001*	< 0.001	-	0.04	0.622
Liquid 3-ml	0.002*	< 0.001	0.04	-	0.473
Liquid 4-ml	0.009*	< 0.001	0.622	0.473	-

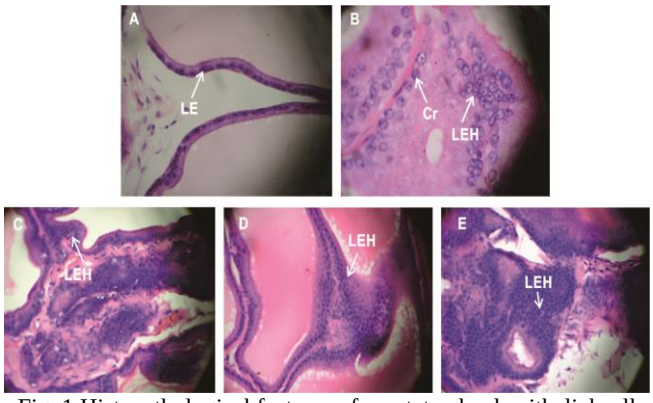

Fig. 1 Histopathological features of prostate gland epithelial cell using HE staining method (x100). Hyperplasia of luminal epithelial cells was obviously shown in estrogen [B] (x400), mosquito coil repellent [C], 3-ml [D], and 4-ml mosquito liquid repellent group [E]. Severe chromatin pattern was also observed in the estrogen group [B] (x400).LE=Luminal Epithelial; LEH = luminal epithelial hyperplasia; Cr = chromatin

Many studies have been carried out to seek the effects of estrogenic compound exposure on the prostate gland during pre- and postnatal period [8], [18], [19], [20]. This study used both potent (β estradiol 3-

benzoate) and weak daily used estrogenic compounds, i.e., mosquito repellent coils (transfluthrin 0.03%/coil) and mosquito repellent liquid (transfluthrin 0.162 mg/ml and propoxur 4.05 mg/ml) which caused hyperplasia in the epithelial cells of prostate gland [13], [14].

Prostate formation and differentiation require communication and interaction between epithelial and stromal cells in the prostate. Initially, androgen acts on the mesenchyme to produce a signal for prostate cell stimulation and growth. Subsequently, androgen focuses on epithelial cells for the secretory function of differentiated cells. Reciprocal homeostatic interactions between prostate epithelium and stromal cells are essential for normal prostate growth, development, and function, so disturbing these interactions plays a significant role in carcinoma [21], [22]. In addition to androgens, prostate development is very responsive to estrogenic compounds, especially from environmental EDCs. Estrogenic compound exposure during early life will increase the expression of androgen receptors in the prostate gland and may result in prostate hyperplasia [23]. Studying the effect of EDCs (Bisphenol A) exposure on prostate gland alteration shows some degree of alteration, which includes hyperplasia, inflammatory cell infiltration, and prostatic intraepithelial neoplasia (PIN) [24]. Jarred RA et al. shows that estrogen will alter androgen-regulated prostate development. Thus the evidence reinforces this finding [25].

Cellular alteration was divided histopathologically into three grades depending on the severity of the following changes, i.e., cell crowding and stratification (cellular polarity), nuclear enlargement, cellular polymorphism, chromatin pattern, and nucleolus appearance [26]. The HE sections show that high exposure to potent estrogenic compounds results in severe hyperplasia of luminal epithelial cells with bad cellular polarity, cellular polymorphism, a rough chromatin appearance, and a nucleolar appearance. Therefore, the histopathological characteristic was considered pre-malignant lesions as the main precursor of invasive carcinoma of the prostate, also called PIN [27], [28]. Luminal epithelial cells were significantly altered in all treated groups than untreated groups with *P* value <0.05. Prostate carcinogenesis involves multiple processes from hyperplasia through dysplasia (pre-cancerous) to carcinoma ranging from low to high histopathological grade, and finally to a cancerous stage (cellular atypia). The HE sections in this study prove that the exposure of estrogen (insecticides) in early life alters the histopathological grading in correlation to the potency of estrogenic compounds.

Insecticide exposure during early life was assessed using the Masson's Trichrome sections, which were particularly focused on periductal stromal cells (fibroblast proliferation, smooth muscle arrangement, and prostate gland secretory function). Fibroblast proliferation was dominantly found in all (100%) subjects in the estrogen group, 80% of the mosquito repellent coil group (see figure 2), and rarely found in both the 3-ml and 4-ml liquid mosquito repellent groups. Smooth muscle differentiation was found normal (thin and continuous) in the control group. In contrast, the discontinuity of periductal smooth muscle was found in all treated groups, i.e., 100% in both the estrogen and mosquito coil repellent groups, 83.3% in the 4-ml liquid mosquito repellent group, and 60% in the 3-ml liquid mosquito repellent group. Masson's Trichrome stained light blue for normal secretory function and red to none when the secretory function was abnormal or diminished. Alteration of periductal stromal cells was significantly found in all treated groups compared to those of the control group. (see Table 2) The histological features of periductal stromal cell alteration can be seen in figure 2.

Table 2 Alteration of periductal stromal cell untreated/control group compared to those of treated groups (*P* value ≤ 0.05 was considered significantly different)

Experimental Group	Control	Estrogen	Coil repellent	Liquid 3-ml	Liquid 4-ml
Control	-	< 0.001*	< 0.001*	< 0.05*	0.05*
Estrogen	< 0.001*	-	0.185	< 0.05	0.001
Coil repellent	< 0.001*	0.185	-	0.095	<0.05
Liquid 3-ml	< 0.05*	< 0.05	0.095	-	0.639
Liquid 4-ml	0.05*	< 0.001	<0.05	0.639	-

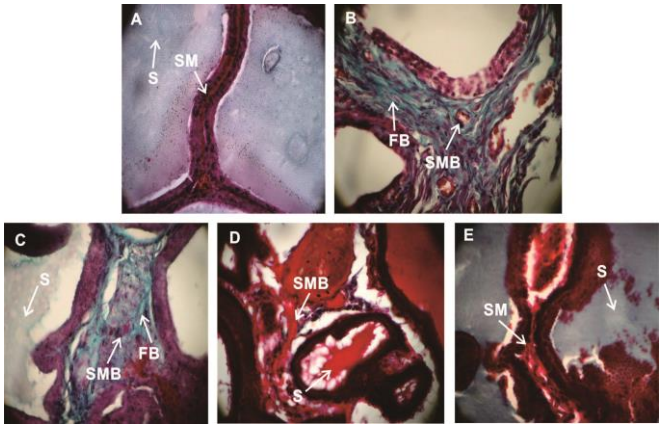

Fig. 2 Histopathological features of prostate gland periductal stromal cell using Masson'sTrichrome staining method (x100). In the control group [A], the smooth muscle was thin and continuous. Smooth muscle bundle and blue reactive stroma were shown in the estrogen group [B] and mosquito coil repellent group [C]. Smooth muscle was decreased and discontinuous in both 3-ml [D] and 4-ml mosquito liquid repellent group [E]. Fibroblast proliferation (blue reactive stromal) was found dominantly in estrogen [A] and coil mosquito repellent group [C]. The secretory function was normal (stained in blue) in the control group [A] and 4-ml mosquito liquid repellent[C], abnormal (stained red) in both mosquito coil repellent [C] and 3-ml mosquito liquid repellent group [D]. Depletion of secretory function was stained none in the estrogen group [A].FB = fibroblast proliferation; SM = smooth muscle were stained red; SMB = smooth muscle bundle; S = secrete

Masson's Trichrome staining method has been used to observe the alteration of periductal stromal cells.

This method can also be used to identify alterations in prostate cancer-reactive stroma (blue reactive/highly collagenous stroma), a modified stromal microenvironment created in response to prostate cancer that accompanies biochemical recurrence and tumor progression [29], [30]. This study shows that neonatal estrogen exposure stimulates the proliferation of periductal fibroblasts, which are clearly visible in the estrogen and mosquito coil repellent groups (See figure 2); additionally, highly collagenous stroma was also identified. Smooth muscle was observed discontinuous in all periductal stroma among treated groups; smooth muscle bundles were found in both the estrogen and mosquito repellent coil groups. Fibroblast proliferation corresponds to a physical barrier that may inhibit branching morphogenesis and interfere with paracrine communication between smooth muscle-epithelial interactions in the postnatal prostate. Paracrine communication plays a key role in regulating epithelial differentiation, proliferation and determining the rate of prostate cancer initiation, growth, and progression. Thus, the regulation of cell differentiation will be disrupted [31]. Under the influence of androgen signals towards the prostatic epithelium, prostatic smooth muscles maintain epithelial differentiation and repress epithelial proliferation [32]. In human prostatic adenocarcinoma, loss of smooth muscle cells combined with elevated collagen levels was observed [32], [33]. In the case of cancer, molecular mechanisms of EDCs are in cell cycle regulation and ER-dependent pathways during carcinogenesis [34]. Estrogen exposure during early life will reprogram the prostate gland, causing a permanent alteration in structure and gene expression that leads to an increased susceptibility to prostate lesions or hormonal carcinogenesis by aging [8], [35], [36].

4. Conclusion

In conclusion, early exposure to widely-used insecticides that were considered is safe. However, this study proves the disruption of reciprocal communication between periductal stromal and luminal epithelial cells due to fibroblast proliferation and alteration of smooth muscle alignment. Accordingly, it may lead to a predisposition towards neoplastic development in adulthood. Molecular biomarkers of prostate cancer-associated reactive stroma such as thioredoxin reductase 2 protein, the presence of which distinguishes the stroma associated between benign prostatic hyperplasia and prostate cancer, is needed in future studies [37]. Stromal changes might also play a critical role in regulating local cancer growth, invasion, progression, and distant metastasis [38], [39]. Early mosquito insecticide exposure leads to prostate cells differentiation that may predispose to prostate cancer development in later life.

4.1. Limitation of Study

This study did not measure the exact concentration of active ingredients of mosquito insecticides, air transfluthrin concentration inside coil repellent group cage, and air transfluthrin and propoxur concentration inside 3-ml and 4-ml liquid repellent group cages. This is an animal study; thus, generalization regarding humans should be considered with caution.

5. Highlights

- Early exposure of pesticides in male rats alters prostate gland cause fibroblast proliferation and smooth muscle alignment
- Early exposure of pesticides in male rats alters reciprocal communication between periductal stromal and luminal epithelial cells, thus, increase the risk of prostate cancer in later life
- Further study needs to be done in animal and human (case-control retrospective cohort design) with the molecular specific marker of prostate cancer

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