The Effect of Early Mosquito Insecticides Exposure on Spraque Dawley Rat Testis: A Histopathological Feature Towards Malignancy?

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Abstract. The incidence of health problems associated with endocrine-disruption have increased. Many studies suggesting that endocrine disrupter chemicals (EDC) do contribute to cancer through estrogen-related receptors. Many chemicals have EDCs properties including insecticides. Early life exposure to EDCs can increased the risk of testicular cancer have been reported in the last decade. This study was aimed to determine the effect of insecticides exposure on histopathological tumor cell development of germ and Leydig cell. True experiment research design with posttest only control group design was applied. Sprague Dawley (SD) rat (n = 25) were randomly divided into 5 groups (control group, 25 mg β estradiol 3-benzoate, spiral mosquito coil repellent, 3 ml of liquid mosquito repellent, and 4 ml of liquid mosquito repellent). The exposure were administered for 20 days started at aged 3 days. At the age of 100 days (older adult), testis was stained using Hematoxyllin Eosin (HE) and histological features predicting malignancy were observed. The number of tumor cell development in both testicular germ cells and Leydig cells significantly increased in all treated group compared to those of control and the changes towards malignancy were also observed in all treated group. Exposure to mosquito insecticides causes significant changes in testicular germ and Leydig cell histological features that leads to malignancy.

Keywords: endocrine disrupting chemicals, insecticides, testicular germ cell, Leydig cell, malignancy.

1. Introduction

Since the late 80s, there has been increasing concern about the impact of environmental pollutants, called endocrine disrupter chemicals (EDCs), substance that may interfere with the biosynthesis, metabolism, and/or an action of endogenous hormones. Alterations of male reproductive development and health that have been reported associated with EDCs, which includes demasculinization or feminization, cryptorchidism, hypospadia, in situ germ cell testicular carcinoma, reduced semen quality, and progression of prostate cancer[1-5]. Skakkebaek proposed the existence evidence called testicular dysgenesis syndrome (TDS) which includes poor semen quality, hypospadia, undescensus testis, and testicular cancer[6], which may increased due to adverse environmental exposure[3, 6]. The most common form of TDS is a mild form which may present with slight impairment of spermatogenesis counted as much as 20% [7], in contrast, the most severe form of TDS is testicular cancer appear to be uncommon (~5%)[6]. Although it appear to be uncommon form, testicular cancer (TC) is the most common malignancy in 20 to 30-year-old male. With prevalence rate is diverse among countries (Finland 2.5/100,000 cases versus Denmark 9.2/100,000)[8]. Numerous publications have shown an...
increasing incidence of testis cancer in the last 50 years. The increase in the TC incidence was associated with environmental factor with a key role of endocrine disrupters chemicals (EDCs) has been point it out by several group of study. Recent increase in the TC rate in most industrialized countries should lead more attention to the association of EDC and testicular cancer[9, 10].

In tropical country like Indonesia, 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 diseases-transmission control programming. Children under the age of 5 have a higher risk to mosquito-borne diseases. Accordingly, to protect children from mosquito bites, mosquito repellent usage is prevalent, often without consideration of potential side effects[11, 12]. Based of epidemiological studies, suggest that TDS is a result of adverse environmental exposure during fetal and early life. Every organ has a specific window of development, which refers to the critical period when the developing tissue is susceptible to the effects of EDC exposure[13]. The critical window of reproductive tract development is between 7 and 40 weeks of gestation, in which cells undergo rapid mitotic division and differentiation, eventually resulting maturation. The exposure of EDCs during the critical window period will affect cell reprogramming[14]. This study was done to elucidate the adverse effect of mosquito insecticides exposure in early life on testis spraque dawley rat.

2. Materials and methods

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 (C), treated groups including 25 μg β estradiol 3-benzoat (P1), and insecticide groups [spiral mosquito coil repellent (P2) contains transfluthrin 0.03%/coil; 3-ml (P3), and 4-ml mosquito liquid repellent (P4) 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 pellet diet ad libitum from weaning to postnatal day (PND) 100. Pesticide exposure was designed to resemble the natural setting of mosquito repellent usage. Testis were removed and fixed in 10% buffered neutral formaldehyde solution for 24 hours at room temperature before being embedded into paraffin blocks. The haematoxylin and eosin (HE) staining method was used to explore tumor cell development in both testicular germ cells and Leydig cells (large nucleus, having an irregular size and shape, the nucleoli are prominent and irregular, the cytoplasm is scarce and intensely colored or, on the contrary, is pale)[15]. HE slides were assessed by two experienced pathologist using microscope Olympus CX21 in a blinded manner. Kruskal-Wallis test was used to determine the difference between groups, while Mann Whitney test was used to compare the difference between groups.

3. Results

Tumor cell development features were observed significantly in both germ and Leydig cells with p=0.024 and p=0.001, respectively. The difference number of germ cell development between groups can be seen in Table 1. Germ tumor cell development in estradiol group was significantly increased compare to those of control group (p=0.008), the same result was seen also in mosquito repellent (p=0.019) and 4-ml mosquito liquid repellent (p=0.025)

Table 1. The difference number of germ cell tumor development between groups.

| Group                              | P1              | P2              | P3              | P4              |
|------------------------------------|-----------------|-----------------|-----------------|-----------------|
| C (Control)                        | p = 0.008*      | p = 0.019*      | p = 0.589       | p = 0.025*      |
| P1 (Estradiol)                     | -               | p = 0.207       | p = 0.059       | p = 0.142       |
| P2 (Spiral mosquito coil repellent)| -               | -               | p = 0.246       | p = 0.674       |
| P3 (3-ml mosquito liquid repellent)| -               | -               | -               | p = 0.295       |

p < 0.05 = significance (*)
The difference number of Leydig cell between groups can be seen in Table 2. Leydig tumor cell development in estradiol group was significantly increased compare to those of control group (p=0.007), the same result was seen also in mosquito repellent (p=0.008) and 4-ml mosquito liquid repellent (p=0.008).

**Table 2.** The difference number of Leydig cell tumor development between groups.

| Group                        | P1            | P2            | P3            | P4            |
|------------------------------|---------------|---------------|---------------|---------------|
| C (Control)                  | p = 0.007*    | p = 0.008*    | p = 0.827     | p = 0.008*    |
| P1 (Estradiol)               | -             | p = 0.119     | p = 0.008*    | p = 0.013*    |
| P2 (Spiral mosquito coil repellent) | -             | -             | p = 0.011*    | p = 0.197     |
| P3 (3-ml mosquito liquid repellent) | -             | -             | -             | p = 0.026*    |

p < 0.05 = significance (*)

**Figure 1.** Histological feature of tumor germ cell development (A) and (B) normal germ cell. A: Germ cell with large, irregular size and shape nucleus, the nucleoli are prominent and irregular with rough chromatin, the cytoplasm is scarce and pale (arrow). B: Germ cell with regular size and shape nucleus with smooth chromatin (arrow)

**Figure 2.** Histological feature of tumor cell development of Leydig cells. Leydig cell with large, irregular size and shape nucleus, the nucleoli are prominent and irregular with rough chromatin, the cytoplasm is scarce and pale (indicate by arrows).
4. Discussion
These study indicated that there was significant number of both germ and Leydig cells lead towards malignancy in experimental groups i.e. estradiol, spiral mosquito coil repellent, and 4-ml mosquito liquid repellent. The change of Sprague Dawley rat germ cells toward malignancy may due to neonatal exposure of the mosquito insecticide repellent that act as a EDC, where EDCs bind to estrogen receptor (ER)-β in spermatogonia cells. This binding can also cause oxidative damage to deoxyribonucleic acid (DNA), it may results germ cells changing towards malignancy. This result is consistent with previous research conducted by Anja Wellejus et al in 2002 [16], and Vernon Pais et al in 2003[17]. ER-β has an important role in the process of change into a germ cell malignancy, it’s due to the exposure to EDCs[17]. The exposure of estrogen to the ER-β can mediate the carcinogenic effects through the oxidative damage process to DNA with a marked by increasing significant levels of 8-OxodG. This damage also more commonly found in spermatogonia cells, because these cells express many sort of estrogen receptor and are located outside of the blood testis barrier[16, 17]. The time of exposure during early critical window period of development, when the cells undergone differentiation and maturation, has also been found will affect cell reprogramming[14]. Beside of those, the exposure during early postnatal period also give a greater impact other area such as environmental induced disease etiology, epigenetic transgenerational inheritance, and the general systems biology of organisms and evolution[18].

The number of germ cell that lead to malignancy was found higher in the group of spiral mosquito coil repellent compared to those of liquid repellent. This may also cause due to the additional exposure of smoke of mosquito coil that can cause increase level of free radicals, it can create damage lipid membranes of cells and integrity of the nucleus. This damage can modulate germ cells exposure to the chemical compounds. Thus, all together stimulate the changes in histopathological features of germ cells towards malignancy[19].

The exposure of EDCs can cause disturbing negative feedback mechanism of hypothalamic-pituitary-adrenal (HPA) axis, resulting an adverse effect of hormonal balance including the excessive production of luteinizing hormone (LH) that results Leydig cell hyperplasia. If the exposure is persistence and within the critical period, the change of Leydig cells can cause of severe form of hyperplasia ranging from dysplasia to malignancy[20]. Mostly, EDCs was designed have longer half-life for industrial benefit, it will unfortunately give long-term effect on disturbing negative feedback mechanism[21]. In previous studies, chronic progressive of excessive LH production can cause the Leydig cell more easily enters into the mitosis phase. The role of LH in mitogenic process also observed, wherein an administration in rats for chronic period will cause Leydig cell adenoma[22].

Limitation of study. Active ingredients of mosquito insecticides both air transfluthrin concentration inside coil repellent group cage; and air transfluthrin and propoxure concentration inside 3-ml and 4-ml liquid repellent group cages are not measured.

5. Conclusions
Mosquito insecticides exposure in early life will leads histopathological features of tumor cell development in both germ and Leydig cells in adult life. With regards to this study, mosquito insecticides usage in a daily basis should accompany with precautions especially when it exposed to the infant.

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References
[1] Carlsen E, Giwercman A, Skakkebaek NE. Declining sperm counts and increasing incidence of testicular cancer and other gonadal disorders: is there a connection? 1993 Ir Med J 86 85.
[2] Jacobsen R, Bostofte E, Skakkebaek NE, Hansen J, Moller H. Offspring sex ratio of subfertile men and men with abnormal sperm characteristics. 2000 Hum Reprod 15 2369.
[3] Schiffer C, Muller A, Egeberg DL, Alvarez L, Brenker C, Rehfeld A, et al. Direct action of endocrine disrupting chemicals on human sperm. 2014 EMBO Rep.
[4] Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? 1993 Lancet 341 1392.
[5] Prins GS, Tang W-Y, Belmonte J, Ho S-M. Perinatal Exposure to Oestradiol and Bisphenol A Alters the Prostate Epigenome and Increases Susceptibility to Carcinogenesis. 2008 Basic & Clinical Pharmacology & Toxicology 102 134.

[6] Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. 2001 Hum Reprod 16 972.

[7] Andersen AG, Jensen TK, Carlsen E, Jorgensen N, Andersson AM, Krarup T, et al. High frequency of sub-optimal semen quality in an unselected population of young men. 2000 Hum Reprod 15 366.

[8] Huyghe E, Matsuda T, Thonneau P. Increasing Incidence of Testicular Cancer Worldwide: A Review. 2003 The Journal of Urology 170 5.

[9] Soto AM, Sonnenschein C. Environmental causes of cancer: endocrine disruptors as carcinogens. 2010 Nat Rev Endocrinol 6 363.

[10] McGlynn KA, Quraishi SM, Graubard BI, Weber JP, Rubertone MV, Erickson RL. Persistent organochlorine pesticides and risk of testicular germ cell tumors. 2008 J Natl Cancer Inst 100 663.

[11] Ohta K, Ogawa T, Suzuki T, Ohta S, Endo Y. Novel estrogen receptor (ER) modulators: carbamate and thiocarbamate derivatives with m-carborane bisphenol structure. 2009 Bioorg Med Chem 17 7958.

[12] Go V, Garey J, Wolff MS, Pogo BG. Estrogenic potential of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. 1999 Environ Health Perspect 107 173.

[13] Calafat A, Needham L. Human Exposures and Body Burdens of Endocrine-Disrupting Chemicals. In: Gore A, editor. Endocrine-Disrupting Chemicals. Contemporary Endocrinology: Humana Press; 2007. p. 253.

[14] UNEP, WHO. State of the science of endocrine disrupting chemicals 2012 Geneva, Switzerland: WHO Press; 2013.

[15] Baba AI, Catoi C. Tumor Cell Morphology. Comparative Oncology. Bucharest: The Publishing House of the Romanian Academy; 2007.

[16] Wellejus A, Loft S. Receptor-mediated ethinylestradiol-induced oxidative DNA damage in rat testicular cells. 2002 FASEB J 16 195.

[17] Pais V, Leav I, Lau KM, Jiang Z, Ho SM. Estrogen receptor-beta expression in human testicular germ cell tumors. 2003 Clin Cancer Res 9 4475.

[18] Skinner MK. Role of epigenetics in developmental biology and transgenerational inheritance. 2011 Birth Defects Res C Embryo Today 93 51.

[19] Madhubabu G, Yenugu S. Effect of continuous inhalation of allethrin-based mosquito coil smoke in the male reproductive tract of rats. 2012 Inhal Toxicol 24 143.

[20] Naughton CK, Nadler RB, Basler JW, Humphrey PA. Leydig cell hyperplasia. 1998 Br J Urol 81 282.

[21] Patiasaul HB, Adewale HB. Long-term effects of environmental endocrine disruptors on reproductive physiology and behavior. 2009 Front Behav Neurosci 3 10.

[22] Clegg ED, Cook JC, Chapin RE, Foster PM, Daston GP. Leydig cell hyperplasia and adenoma formation: mechanisms and relevance to humans. 1997 Reprod Toxicol 11 107.