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Effect of ethinylestradiol on sperm quality of the tropical fish *Barbodes binotatus*

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This study was aimed to evaluate the in vitro toxicity of ethinyl estradiol (EE_2) in different concentrations (0; 10; 15; 25; 50 IU/mL) using sperm cells of model organism tropical fish, *Barbodes binotatus*. Sperm quality parameters, including mass and individual time of sperm motility (second), viability (%), and sperm velocity (µm/s) were measured by digital inverted microscope and DNA fragmentation of sperm after exposure to EE_2 was examined with fluorescence microscopy.*Barbodes binotatus* sperm was collected by stripping then exposed to EE_2. The results showed that EE_2 exposure could negatively affect some sperm quality parameters, which might significantly reduce the mass and individual time of sperm motility, sperm viability and sperm velocity rate of this animal. DNA fragmentation in sperm of *B. binotatus* was increased after EE_2 exposure compared to the control group. We conclude that administration of EE_2 concentration 10 IU/mL decreased mass and individual motility time, velocity, viability of sperm and increased DNA fragmentation in sperm of *B. binotatus*

Keywords: *Barbodes binotatus* ethinyl estradiol, fish, sperm quality.

INTRODUCTION

Over the last 50 years, the use of chemical pollutants and their releases in the environment has increased and affected the wildlife, such as fishes. Industrial development, agrochemicals and human chemical consumption produce an increasing amount of chemical pollutants into the environment, especially in surface water (Lecomte et al., 2017). Among these contaminants, estrogenic compound represent a significant proportion.

Estrogenic chemicals synthesized in pharmaceuticals raise exposure levels of estrogenic chemical in living things to the naturally occurring estrogen. The most pronounced effect occurred in aquatic species that make their homes in waters with elevated levels of estrogens. Many researchers have found estrogenic compound in streams, rivers, and lakes throughout the world, as well as in the effluent of wastewater treatment plants in the United States, Europe, Asia, South America, and Australia. Synthetic estrogens from contraceptive pills, hormone-replacement therapy all end up in wastewater and can be discharged into rivers and lakes (Lundgren and Novak, 2010). Estrogens have been detected in numerous studies of wastewater influents and effluents, specifically estrone (E_1), 17β-estradiol (E_2), estriol (E_3), and ethinylestradiol (EE_2) (Caldwell et al., 2010).

Ethinyl estradiol is an active component of drugs including oral contraceptives. This compound is also used as a cattle growth regulator and can be used to evaluate the estrogenic effects on the reproductive system. Ethinyl estradiol affects the reproductive system through the mediation pathway of the estrogen receptor (Metcalfe et al., 2001).

In fish, E_2 promotes spermatogonium to reform. In some fishes species, high concentrations of synthetic estrogens produce inhibitory effects. However, E_2 itself is unable to induce all stages of spermatogenesis. Interestingly, in some species, E_2 levels correlate with spermatogenesis and spawning, when spermatogonia are the main cell type in the testes. Receptors of reproductive hormones in fish leukocytes have provided evidence supporting the role of immunoregulatori for these steroids in fish. In fish, E_2 was injected to inhibit the immune response (Wang and Belosevic, 1994; Watanuki, 2002), while E_2 affect the function of fish in vitro leucocytes (Chaves et al., 2001).

Ethinyl estradiol is distributed on the water surface as a waste with concentrations ranging from 1-831 ng/L (Wise et al., 2011). The limit of international bargaining quality standards for EE_2 was 0.035 ng/L (Gilbert, 2012; Owen and Jobling, 2012). Humans and farm animals excrete EE_2 in waters through urine and feces in conjugated form with sulfate and glucoronide (Heberer, 2002). Then directly reach the aquatic environment or waste treated at waste treatment stations (Wu et al., 2014; Esteban et al., 2014; Jin et al., 2013). It is important to note that conventional waste treatment technologies have limited ability to remove estrogens (Mills et al., 2015; Chong et al., 2014; Fent et al., 2006). The situation is exacerbated by the fact that EE_2 has a half-life in water about 17 days and low levels of photo degradation that make it persistent estrogenic (Jurgens et al., 2002).

Sperm fish can be used as a bio monitor and an indicator of toxicity parameters (Kime et al., 2001), so that in this study, we used sperm of *Barbodes binotatus* fish to analyze the effect of EE_2 in vitro. *Barbodes binotatus* lives in fresh water. The population of *B. binotatus* in nature or in aquaculture is affected by the quality of sperm (Ochokwu et al., 2015). The quality of fish sperm was determined by the motility of spermatozoa. According to Wolf and Smital (2009), the factors that affect egg quality and fish sperm are environmental conditions.

This fish do external fertilization (Islam and Akhter, 2011). The sperm is inactive and immotile in the seminal fluid, but moves when the water contacts. When it is activated, it will move only a few minutes (Chapman, 2016). The sperm that is ejaculated into the waters will be in contact with EE_2 which reduces of the sperm motility and then causes the failure of fertilization. The early stages of embryonic development are very sensitive to pollutants (Brion et al., 2004). Due to dangerous effect of EE_2, this study was conducted to examine the sperm quality of *Barbodes binotatus* exposed with to EE_2.

MATERIALS AND METHODS

Animals

Male *Barbodes binotatus* (8-12 cm in body length and 12-18 g in body weight) were chosen and purchased from Fish Cultivation (Pandaan-Indonesia). In this study, *B. binotatus* were not anesthetized, but they remained quiet out of the water by using a cloth to cover their eyes while stripping. After rinsing with distilled water, the genital area was careful dryed. Sperm was collected in 1-ml syringes by a gentle pressure along the anterior and posterior abdomen until the pore of urogenital. After that, the sperm was placed in 1.5 ml micro tubes. The sperm suspension was made by mixing fish sperm with 0.9% physiological saline solution with a ratio of 1:6 (v:v). Sperm was activated using water dissolved in Ethinylestradiol (EE_2) with five different concentrations (0; 10; 15; 25; 50 IU/mL).

Motility

The sperm motility was examined by determination of the mass and individual sperm motility time (seconds) and the velocity of motility (µm/s) with different EE_2 concentrations. Sperm suspension were pipetted into a single concave microscope glass slides (depth 0.5 mm to 0.8 mm) and immediately assessed. Each slide was measured by digital inverted microscope (Olympus). Motility parameters were analyzed and 5x100 sperms were evaluated per sample. Motion parameters included mass and individual time of sperm motility (second) and straight line velocity of sperm (µm/s).

Viability

The percentage of viability of sperm was assessed with 40 magnification of objective lens under light microscope. Sperm viability was examined by supravital staining with 1% aqueous eosin Y and 10% aqueous nigrosin solution (Sigma, USA). A drop of fish sperm suspension was placed on a spot plate and mixed with one drop of eosin solution. After 15 seconds, two drops of 10% nigrosin solution was added and thoroughly mixed. A drop of this mixture was transferred to a clean glass slide. A thin smear was made and then air dried. The smears were examined under light microscope. Viable sperm

cells were appeared white and dead sperms were appeared pink. At least 5x100 spermatozoa were counted and the result was expressed as the percentage of viable sperm.

DNA fragmentation

For assessment of sperm DNA fragmentation, Acridine Orange (AO) staining was used. In brief, for AO staining, a dried smear fixed in Carnoy's solution (methanol and glacial acetic acid in 3: 1 proportions) for core fixation (Sigma Chemicals, St Louis, MO, USA) for at least 2 hours and air dried again. Then the sperm smears were stained with AO solution (10 mL AO 1%, 40 ml of citric acid, 2.5 mL $Na_2HPO_4.7H_2O$ 0.3 M pH 2.5). After 5 minutes, the smear was washed with distilled water, covered with a coverslip and sealed with a nail polish to protect the smear from drying. Smears were examined using a fluorescence microscope (Olympus-FSX100, Japan) with the following filter combination: 450-490 nm excitation, 510 nm reflector and 520 nm barrier filter. The nuclei of 200-300 spermatozoa from each smear were examined and scored as green or red. Normal sperm heads showed green tingle whereas fragmentation or single stranded DNA was stained red and the result was expressed as percentage of unchanged nucleus sperms (green).

Data analysis

The data was analyzed using ANOVA followed by LSD test by Statistical Package for Social Studies (SPSS software version 17). A comparison was considered significantly different when $p<0.05$.

RESULTS

Effect of EE_2 on sperm time motility

Sperm was exposed to EE_2 in different concentrations (0; 10; 15; 25; 50 IU/mL) in vitro. Control group showed significantly difference of mass sperm motility time compare to other group ($P<0.05$). Mass sperm motility time in control group was the highest (310±26 seconds). The higher of the concentration exposed, the lower time of mass motility. They were 205; 179; 143; and 131 seconds, respectively.

Similarly, individual sperm motility time in the control (153.08±19.07 seconds) was higher than the treatment groups. There was a significant decrease in the time of individual motility after exposed of EE_2 in different concentrations ($P<0.05$). Significant decreases occured from concentrations of 10 to 50 IU/mL (119; 99; 85; 66; and 63 seconds, respectively) (Figure 1).

The time of mass sperm motility after exposure of 10 IU/mL EE_2 showed significant difference with control ($P< 0.05$), but when exposed with increased concentration (more than 10 IU/mL), there was a significant difference too. Increased EE_2 concentration exposure to sperm caused a decrease in time of mass sperm motility. Likewise with the time of individual sperm motility after EE_2 exposure, the higher the concentration of EE_2 decreased the time of individual sperm motility.

Effect of EE_2 on sperm velocity

The velocity of sperm motility was calculated by measuring the distance of movement per second using an inverted microscope. The observed sperm motility was a straight forward movement. The result showed that sperm velocity of control group was 173.43±10.54 µm/s. When exposed to 10 IU/mL EE_2, the result showed a decrease in motility velocity significantly ($P<0.05$). When the EE_2 concentration was increased (15, 25 and 50 IU/mL), the motility velocity of sperm were decreased (73.92 ± 17.89; 68.64 ± 11.44; 58.82 ± 13.95; and 48.81 ± 5.76 µm/s, respectively) (Figure 2).

Effect of EE_2 on sperm viability and DNA fragmentation

The viability of sperm effectively measures the number of live sperm. Measurement of viability is very important because sperm viability is an important parameter of fertility. The results of the collection of sperm fish showed that control group contained many live, but not all of these sperm will live. The percentage of live sperm is determined by identifying sperm with intact cell membranes. This determination is made using the dye method (eosin and nigrosin), in which the dye (red or pink) enters the non-vital cell (die) due to the damaged plasma membrane. Therefore, the viable cell will be clear, but the dead cells will absorb the color (Figure 3).

Exposure of different EE_2 concentrations caused many dead sperm, the higher EE_2 concentration, lower percentage of live sperm. The sperm viability of the controls was 97±0.5% and decreased to be 94±1.2% after 10 IU/mL EE_2 exposure. The result showed a significant different ($P <0.05$) and the sperm viability continued to decrease at the higher concentrations of 15, 25, and 50 IU/mL EE_2. They were 83±1; 82±2.2; and 72±1%, respectively

(Figure 4).

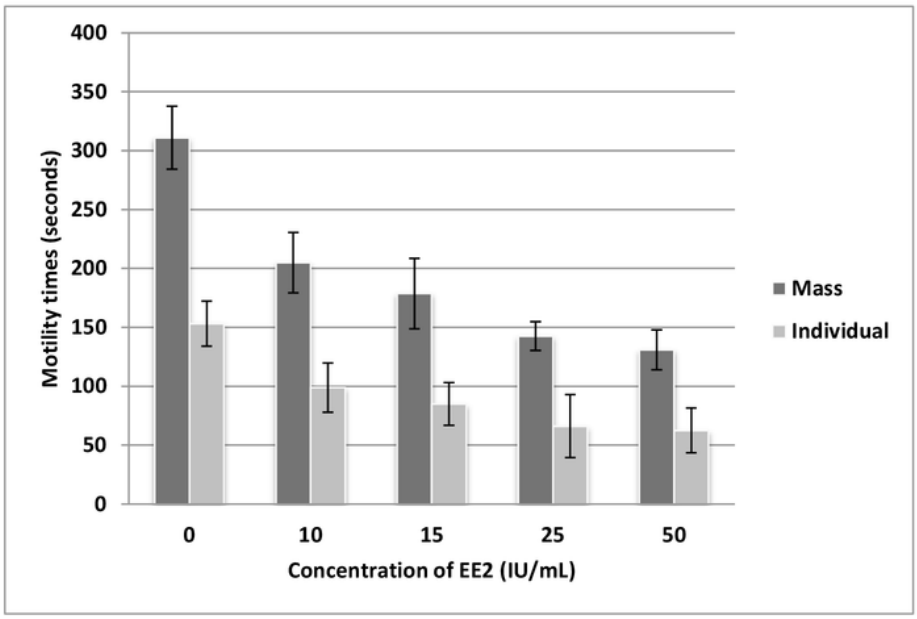

Figure. 1 The time of mass and individual motility of *B. binotatus* sperm after exposure to EE_2

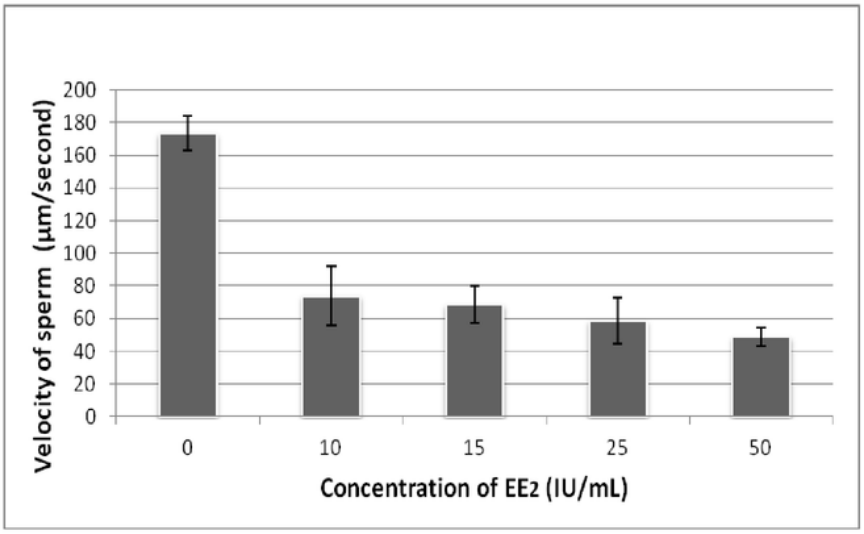

Figure. 2 Velocity of *B. binotatus* sperm motility after exposed of EE_2

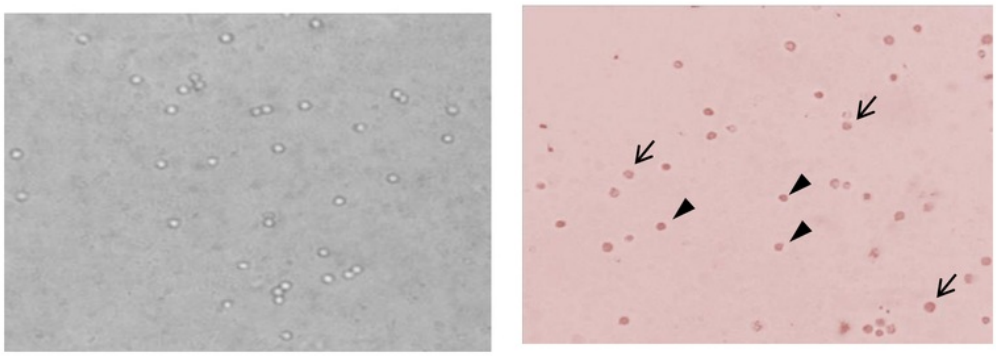

Figure. 3. Sperm viability of *B. binotatus* after exposed of EE_2. Live sperm (◄, clear) and dead sperm (↓, red or pink), 400x

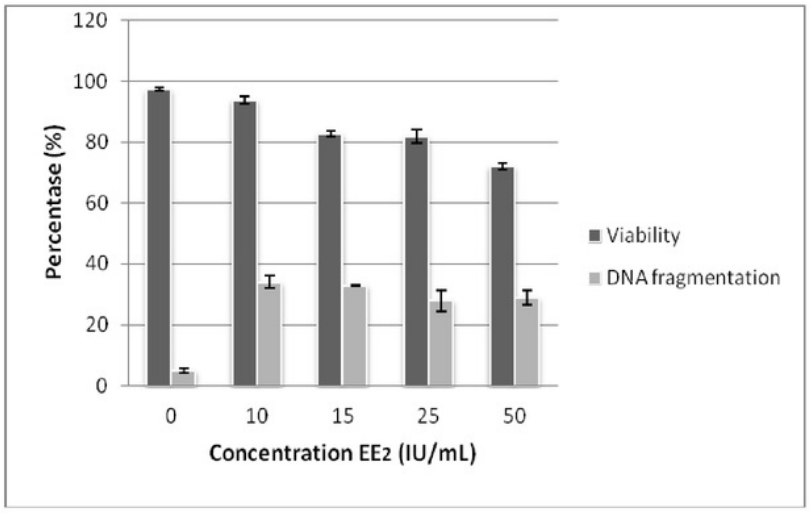

Figure. 4 Viability and DNA fragmentation of *B. binotatus* sperm after exposed of EE_2

The results of evaluation of DNA fragmentation showed that EE_2 exposure increased DNA fragmentation of sperm. Exposure of 10 ppm EE_2 (34±1.9%) increased fragmentation significantly ($P<0.05$) compared to control group (5±0.7%). Increase of EE_2 concentrations (15, 25, and 50 ppm) caused rising percentage of sperm DNA fragmentation, 33±0.2; 28±3.5; and 29±2.5%, respectively. There was no significant difference ($P> 0.05$) against DNA fragmentation for all EE_2 treatment groups (Figure 4).

Many studies have shown high levels of estrogen in aquatic environments could affect adverse reproductive effects in fish populations. The exposure of these compounds would affect the reproductive behavior of male fish and the expression of gonad aromatase, as well as the quality of sperm in fish species. Study using fluorescent microscope, observed the damage or fragmentation of sperm DNA of fish after EE_2 exposure (Figure 5).

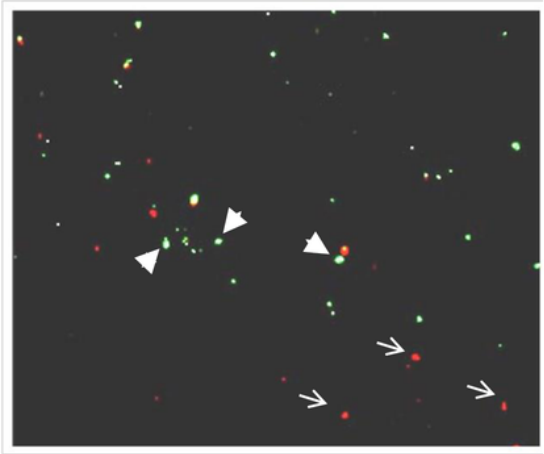

Figure. 5 DNA fragmentation of *B. binotatus* sperm after exposed to EE_2. Normal sperm DNA (◄, green) and DNA fragmentation (↓, red), 400x

DISCUSSION

Ethinylestradiol (EE_2) was an estrogen that was used extensively in regulating the number of child births. EE_2 waste contaminated freshwater and affected the reproductive health of freshwater biota including *B. binotatus*. Based on the research, the presence of EE_2 affects the quality of fish sperm. EE_2 exposure has resulted in decreased sperm motility. The effect of EE_2 as an estrogenic compound inhibits the motility of spermatozoa by binding to estrogen receptors. Estrogen receptor was ESR_1 and ESR. Estrogen receptor was strongly expressed in the middle piece of the sperm. Assuming that ESRs were present in sperm mitochondria, their mitochondria function can be affected by estrogen. (Gavrilova et al., 2007; Tavares et al. ,2009; Rajender et al., 2010).

It has been observed previously that once estrogen binds to receptors, it stimulates increased concentrations of free calcium ions (Ca^{2+}) in mitochondrial spermatozoa. As a consequence of increased concentrations of free calcium ions in the mitochondria, activity of mitochondrial nitric oxidase synthase (mtNOS) is stimulated which leads to increase the synthesis of reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydrogen peroxide hydrogen peroxide (H_2O_2) and hydroxyl OH^-) and cause cytochrome c oxidase activity inhibition. Cytochrome-c-oxidase was an essential enzyme for cellular respiration processes in the electron transport chain in the mitochondria. Inhibition of this enzyme will lead to decreased ATP production and decrease sperm motility.

The binding of estrogens to estrogen receptors that increase the concentration of free calcium ions and increase ROS synthesis is responsible for mitochondrial and cell membrane damage. Mitochondrial damage results in the formation of canals in mitochondrial membranes called mitochondrial permeability transition pore. The opening of this channel causes loss of mitochondrial potential membrane loss. Research conducted by Kotwicka, et al. (2016) stated that 17β-estradiol causes significant changes in mitochondrial membrane potential. 17β-estradiol concentrations of 10-6 M induce a significant decrease in the percentage of mitochondria sperm function. It has been previously described that there is a positive correlation between decreased effectiveness of mitochondria with sperm motility and poor egg fertilization capacity. The sperm cells exposed to 17β-high concentrations of estradiol decreased mitochondria function while increasing the superoxide anion level. Decrease of mitochondrial function will decrease oxidative phosphorylation processes thereby decreasing ATP synthesis (Gharagozloo and Aitken, 2011; Kim et al., 2010).

The live sperm is a healthy sperm and without any kind of defect that might prevent conception. This defect may include damage to DNA and its substrate, or other chemical problems that will prevent sperm fertilization. To get an accurate fertility picture, a decent fish sperm is usually seen

from the time and speed of motility and sperm viability.

In this study, spermatozoa viability parameters presented EE_2 decreased significantly at 10 IU/mL concentration. Damage to spermatozoa membranes can be caused by phospholipase and protease enzymes that are activated by increased concentrations of free calcium ions. Phospholipase degrades the membrane phospholipids and proteases degrade the membrane proteins. Increased ROS can also cause damage to lipids, proteins, and DNA so that the viability of sperm cells decreases.

Ethinyl estradiol was a group of estrogen steroid hormones used as birth control drugs also found in fresh water. The presence of these compounds in waters affects the reproductive behavior of fish and other biota. The presence of an estrogenic signal will be passed on by the estrogen receptor (ER) to the mitochondria. Mitochondria are organelles that play a role in energy synthesis, in sperm help for motility. Existence of estrogen receptors in this mitochondrion could relate with aging. In ideal concentrations, these steroid hormones trigger complex molecular mechanisms involving mitochondria, nuclei, and plasma membranes, and the cytoskeleton that plays a role in cell life. The result of this signaling was protection against mitochondria. Therefore, the molecular component of a pathway activated by a sexual steroid can protect cells from the aging process (Vasconsuelo et al., 2013).

This steroid affects the growth and function of different cells in some organs, because ER could be found everywhere. Estrogen receptor also has intracellular localization in the plasma membrane, mitochondria and endoplasmic reticulum. In addition to the modulation of gene transcription by direct interaction with its receptors, steroids can rapidly activate the signal pathway by a non-genomic mechanism mediated by ER that were identical or different from steroid receptors. Among the various functions, EE_2 could regulate apoptosis through the pathway. In mitochondria, the presence of ER could protect mitochondria against cell death due to apoptosis (Vasconsuelo et al., 2011). However, when estrogenic levels increase, it was thought to affect mitochondrial function, thus increasing the production of ROS. Increased ROS caused the oxidation of proteins, lipids, and sperm DNA. The oxidized DNA causes damaged or DNA fragmentation.

CONCLUSION

Based on the results of this study was concluded that in vitro administration of 10 IU/mL concentration of EE_2 exposure to *Barbodes binotatus* sperm decreased the mass and individual motility duration, velocity of motility, and viability of sperm, but DNA fragmentation increased after EE_2 exposure at concentrations of 10 IU/mL.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

AH and LS designed the experiments. AS, DSA, EW, and NF performed animal treatment, motility assessment, viability assessment and DNA fragmentation assessment. All authors collected and performed data analysis. AH and LS wrote the manuscript. All authors read and approved the final version.

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