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The Repairing Effect of *Γ-Oryzanol* on the Ovarian Histological Structure of Transfluthrin-Exposed Female Rats (*Rattus Norvegicus*) from Endocrine-Disrupting Chemicals (EDCS)

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Abstract: Rice bran contains many bioactive compounds of γ-Oryzanol. We must reduce the use of mosquito repellent due to its impact on reproduction. One of the active compounds in mosquito repellent is pyrethroid derivatives known as transfluthrin, which belongs to the Endocrine-Disrupting Chemicals (EDCs). Therefore, the purpose of this study is to analyze the effect of γ-Oryzanol administration on repairing the ovarian histological structure from EDCs in transfluthrin-exposed female rats. This study used Foxo3a expression and TNF-α concentration as parameters. Moreover, the study went through the following stages. (1) In silico method consisted of (a) the effect of γ-Oryzanol on EDCs and (b) the effect of γ-Oryzanol on reproductive function through TNF-α concentration and Foxo3a expression. (2) In vivo method included a post-test only control group design applied using Wistar females divided into 4 (four) groups. The groups were exposed to one push of 21.3% transfluthrin for 6 hours and were administered with γ-Oryzanol for 28 days. The analysis was done with Kruskal Wallis's statistical analysis and Mann Whitney's posthoc with IBM SPSS Statistics 25 software. Findings confirm the following. (1) There is an effect of γ-Oryzanol on EDCs through adenylate cyclase activation (HTR1A and HTR1B, serotonin receptor genes). Y-oryzanol also has an inflammatory activity and nitric oxide scavengers, which influence TNF-α concentration and Foxo3a expression through APP and AKT1. (2) γ-Oryzanol stimulates follicle growth and effectively reduces the number of follicular abnormalities. (3) γ-Oryzanol is found to have the potential of maintaining a normal estrous cycle by controlling Foxo3a expression and TNF-α concentration. From these results, it can be concluded that γ-Oryzanol is effective to counter EDCs effects, reduce follicular abnormalities, and reduce Foxo3a expression and TNF-α concentration.

Keywords: *EDCs,* Foxo3a, γ-Oryzanol, *Ovarium,* TNF-α, Transfluthrin.

1. Introduction

Fertility illustrates the function of the reproduction organs to work optimally in carrying out reproductive functions. In females, fertility is closely related to folliculogenesis, the reproductive cycle, and ovulation (Leung and Adashi, 2004). Several factors can influence reproductive functions, such as endocrinology, gonadal development, and nutritional conditions [1,2].

Research has been conducted to determine the types of mosquito repellent use where it was found that spray or aerosol is the most widely used mosquito repellent in households (12.2%). [3] The active ingredients in aerosols that are commonly found in Indonesia are permethrin and pyrethrin. Both of which are medium-grade pesticides that are permitted to be used in Indonesia. Also, permethrin and pyrethrin are classified as a synthetic active pyrethroid. [4] The high use of mosquito repellent to prevent the spread of mosquito-borne diseases has become a community lifestyle. Without realizing it, every one-push aerosol spray contains the active ingredients of Endocrine-Disrupting Chemicals (EDCs) [2].

The toxicity of pyrethroid can affect reproduction organs both hormonally and cellularly. Hormonally, it reduces LH-FSH's secretion (Luteinizing and Follicle Stimulating Hormones) and gonadotropin deficiency, which results in apoptosis and is marked with follicle growth inhibition. Cellularly, the toxicity of pyrethroid will increase the damage in the mitochondria. This condition makes the x-linked factor unable to block the damage in ovaries [5,6]. Transfluthrin, as an active compound derived from pyrethroid, has fast-acting properties so that it is widely used in every type of mosquito repellent, including the one-push aerosol [7]. The toxicity of pyrethroid on ovum cells can be seen during folliculogenesis, including impaired integration of extra ovarian and intrafollicular signals [8], endocrinology, and gonad development [9].

The findings above demonstrated that the effective use of mosquito repellent to prevent the spread of mosquito-borne diseases such as dengue and malaria is contrary to the nature of EDCs. As explained in the previous section, EDCs are the active ingredient of pyrethroid that implies human health, especially the reproductive system. Several hormonal regulations for ovarian follicular development (folliculogenesis) studies have been widely conducted. Most of the studies focused on the development of antral follicles from the early-stage to the re-ovulation stage. For example, a study examined an intraovarian mechanism through exogenous FSH stimulation was performed on patients diagnosed with primary ovarian insufficiency, polycystic ovary syndrome (PCOS), and infertile women (secondary) in the advanced reproductive age group [10]. This condition is a long-term effect due to the damaged/impaired follicular growth. Therefore, a preventive effort needs to be taken into account to control folliculogenesis through a healthy lifestyle.

The lifestyle and the development of the food processing industry nowadays offer various natural potentials such as a source of antioxidants that can prevent oxidative stress due to exogenous factors. Besides tocopherol (Vitamin E) and β-carotene, γ-Oryzanol is one bioactive component contained in rice bran [11]. Rice bran contains γ-Oryzanol (62.9%) and phenolic acids (35.9%) as antioxidants [12]. In other words, γ-Oryzanol is a major antioxidant in rice bran.

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The active ingredient of γ-Oryzanol in rice bran oil acts as an antioxidant that can reduce cholesterol levels and menopausal disorders. On in-vitro cholesterol oxidation, the antioxidant activity of γ-Oryzanol is stronger than vitamin E [13]. The role of γ-Oryzanol as an anti-inflammatory content is also effective in preventing the activation of nuclear factor kappa-B (NF_KB). The presence of γ-Oryzanol in the cytoplasm separates the NF_KB through a family inhibitor known as Kappa-B (I_KB) inhibitor. The induction of I_KB is not influenced by cells but by signals from outside the cells, namely the inflammatory effect of γ-Oryzanol [14, 15].

The antioxidant potential of γ-Oryzanol has been broadly tested in health and medical research. However, the anti-inflammatory potential of γ-Oryzanol on maintaining the physiological functions of the reproductive system is still not studied much.

2. Methods and Material

2.1 Research Design

This study was conducted in two stages as follows:

1. Stage I (In Silico)

The researchers of this study established a target selection with Small Incision Lenticule Extraction (SMILE) techniques. The target proteins for γ-Oryzanol were obtained from the SMILES PubChem server (http: //pubchem.ncbi.nlm.nih.gov/), which provides a list of target proteins for γ-Oryzanol. The data were then compared to the target proteins to determine which section has the most potential structure of γ-Oryzanol ligands (PubChem ID 5282164). Further predictive analyzes were performed using the Pass Server and STRING & STITCH approach.

2. Stage II (In Vivo),

This study is an experimental research that was conducted with a post-test only control group design.

2.2. Setting

Following OECD 412 Test Guidelines regarding sub-acute inhalation procedures, which include an experiment on animals aged 8 weeks weighing 150-200 grams, the acclimation process was carried out for 7 days before the research began. After that, the samples were selected randomly.

The cage for the rats in this study was made of a 148.4 cm^2 (width) x 17.8 cm^2 (length) plastic box covered with a hollow wire of 1.6 cm^2. The cage mats were replaced and cleaned once every 3 days. The room temperature was set at 22° ± 3°C with a relative humidity of 30–70% and of 14 hours bright and 10 hours dark. As for the feed, the rats were given a Comfeed calf starter and pap milk as much as 5-10g per 100g/body weight/day/rat and water as much as 10 ml per 100g/body weight/day/rat. The rats' activities were observed before, during (6 hours in a glass box), and after the exposure was given.

This study used γ-Oryzanol Sigma Aldrich (CDS021604_ALDRICH) and diphenyl-picryl-hydrazyl-hydrate (DPPH) method to determine the antioxidant activity in γ-Oryzanol. The DPPH test on γ-Oryzanol showed a level of 122 ppm, which illustrated a strong antioxidant level. A solution of 1% NaCMC was administered to dissolve γ-Oryzanol by taking 1.25g of 1% NaCMC and mixed with a dose of γ-Oryzanol to make a 250 ml homogeneous solution. Daily doses were based on weight gain/week results by multiplying the heaviest weight with the conversion number. Every week, the weight was observed to determine the need for RBO. The weighing was carried out every morning at 09.00 before the administration of OG-RBO and one-push aerosol.

2.3. Population

A total of 24 female Wistar rats were used as the samples in this study. The samples were divided into 4 control groups (positive, negative, transfluthrin treatment (Tr), and (Tr) + γ-Oryzanol treatment) and determined by using the Federe formula ($(t-1)(n-1) \geq 15$). The researchers obtained the rats in a healthy state, complete with a veterinary certificate number 524.3/3947-Dispangtan/2019 from the Animal Laboratory of Bandung Institute of Technology (*Institut Teknologi Bandung* or ITB).

2.4. Data Variable, Source, and Data Collection

2.4.1. Transfluthrin Particle Concentration

The results of the test highlighted that at one impulse of transfluthrin and 3 sprays of P-Track UPC 852 were an ultra particle concentration of 311,500 ± 46,755 particles/cm^3 and a fine particle concentration of 12,392 ± 1,356 mg/m^3.

2.4.2. Ovarian Histology

In this study, the assessment was done by observing the ovaries qualitatively to see the damage of ovarian structures caused by transfluthrin exposure. A Hematocosillin Eosin (HE) staining was also performed to examine the follicular development through Olympus CX-31 microscope. After that, a quantitative assessment of the ovaries took place to measure the treatment's effect and then weigh the ovaries immediately after surgery.

2.4.3. Follicle Extraction and Analysis

The procedure was completed in a single-blind manner without knowing which of the exposure and control groups that were analyzed. A quantitative observation was taken by counting the primary, secondary, de graff, and abnormal follicles in this stage.

2.4.4. Reproduction Cycle

A vaginal swab was implemented at the time of the surgery by determining the phase they were experiencing (estrous phase, metestrous phase, diestrous phase, and proestrus phase) to determine the reproductive cycle of the rats.

2.4.5. TNF-α Concentration

The inflammation indicators in rats and γ-Oryzanol inflammatory activity in reducing TNF-α levels were obtained through enzyme-linked immunosorbent assay (ELISA) analysis.

2.4.6. Foxo3a Expression

A molecular approach was chosen to verify the growth of oocytes and follicles, which then will affect the reproductive cycle of the ovaries through an intense expression of Foxo3a. The examination was executed through Imunoflorence (IF) and Image J semi-quantitative method.

2.5. Analysis and Statistics

A statistical significance was analyzed using IBM SPSS version 25. The results were not normally distributed and were not homogeneous following the type of numeric dependent variables (ratio) and nominal independent variables. Henceforth, a hypothesis test was done using the Kruskal Wallis test to know the difference between the overall treatments. Not only that, but a posthoc test was also carried out with Mann Whitney test to find out the significance of differences between control groups.

2.6. Ethics Appraisal

In this study, the administration of one-push aerosol to measure sub-acute toxicity refers to the OECD 412 guidelines for testing chemicals: a 28day (sub-acute) inhalation toxicity study. To be noted, the researchers used V aerosol brand, which contained 21.3% transfluthrin. The aerosol was given to the rats through inhalation in 1 spray and left in a glass box for 6 hours. After that, the rats were placed back in the cage for 28 days.

The research procedure was based on and approved by the ethic code number 38/EC/KEPK-S3/02/2019 from the Faculty of Medicine, Universitas Brawijaya.

3. Results

3.1. In Silico Results

3.1.1. The Effect of γ-Oryzanol on EDCs from Transflutrin Exposure

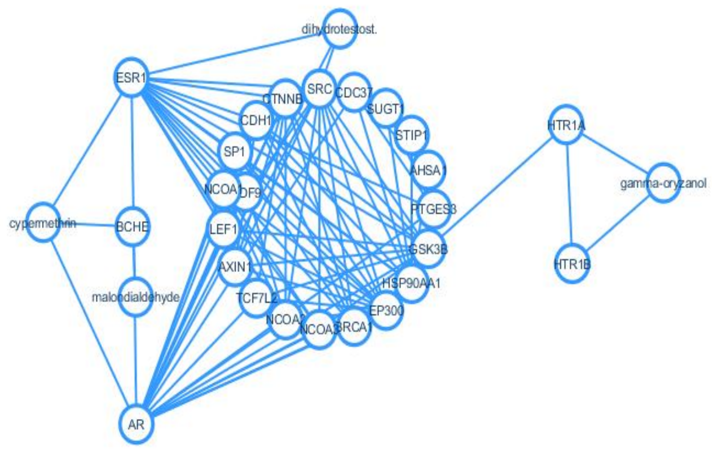

Figure 1. The Pathway of γ-Oryzanol to the Effect of EDCs

The toxicity effect of cypermethrin (CPM) or transfluthrin as Endocrine-Disrupting Chemicals (EDCs) interacts with estrogen receptors 1 (ESR1) to regulate the activation of ESR1. Exposure to CPM/transfluthrin also inhibits the androgen

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receptors (AR) hormone. Thus, exposure to CPM/transfluthrin as a pyrethroid derivative will interfere with these two hormones.

3.1.2. The Effect of γ-Oryzanol on Reproductive Function through STRING and STITCH Approach

The effect of γ-Oryzanol on reproductive function in this study is investigated with Foxo3a expression and TNF-α concentration. The presence of protein interactions between γ-Oryzanol with Foxo3a and TNF-α shows the potential of γ-Oryzanol to repair rat (*Rattus Novergicus*) folliculogenesis from the effects of EDCs due to transfluthrin exposure. The analysis of protein interactions using the STRING approach shows that the active compound of γ-Oryzanol can interact with Foxo3a and TNF-α target proteins through the intermediary of several proteins. In detail, the results are presented in Table 1.

Table 1. Protein analysis in biological processes

Compound	Protein	Interaction	Biological Processes	Description
γ-Oryzanol	Amyloid Precursor Protein (APP)	Direct Activation –	Foxo3 signaling pathway	Interacts with AKT1, Mtor, Foxo1 to go to Foxo3 Bind with AKT1, AKT3, Pik3cg, Smad2 to go to Gdf9 Bind with AKT1, AKt3, Akt2, IkbkB, Casp3, Fadd, Tradd, Tnfrsf1a , Tnfrsf1b, Traf3, Birc2, Ikbkg to bind with TNF
			Foxo signaling pathway	Interacts with Scn8a, Sgk1, Foxo1, AKT2, Ikbkb, AKT1, AKT3 to go to Foxo3
Transfluthrin	SCN1A	Direct	Foxo signaling pathway	Interacts with Scn8a, Sgk1, Foxo1, AKT2, IkbkB, Casp3, Fadd, Tradd, Tnfrsf1a, Tnfrsf1b, Traf3, Birc2, Ikbkg to bind with TNF Interacts with Scn8a, Sgk1, Foxo1, AKT2, Creb1, Smad2 to bond with Gdf9.

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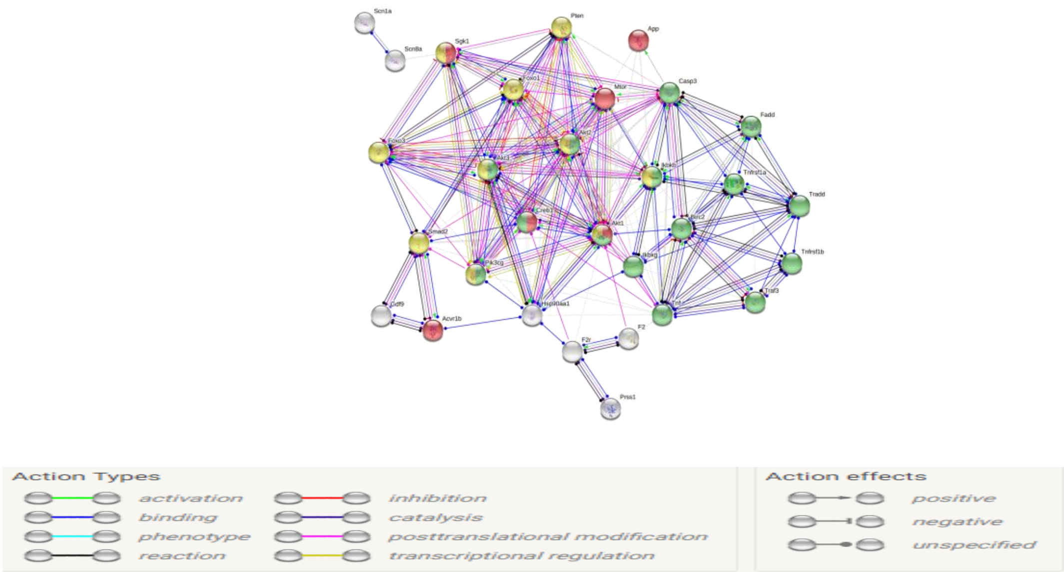

Figure 2. STITCH Analysis: The Effect of γ-Oryzanol on TNF-α and Foxo3a Expression to Follicle Growth.

Description: Yellow (Foxo3a Signaling), Green (TNF-α Signaling), Red (Growth Regulation)

Figure 2 shows that more proteins activate growth regulation than bonds that inhibit it. It shows γ-Oryzanol compounds' potential to influence follicle growth through the binding of Foxo3a and TNF-α proteins on the regulation of follicle growth during the reproductive cycle.

3.2. **In Vivo Results**

3.2.1. The Effect of EDCs on the Histological Structure of Ovaries

3.2.1.1. Ovarian Weight

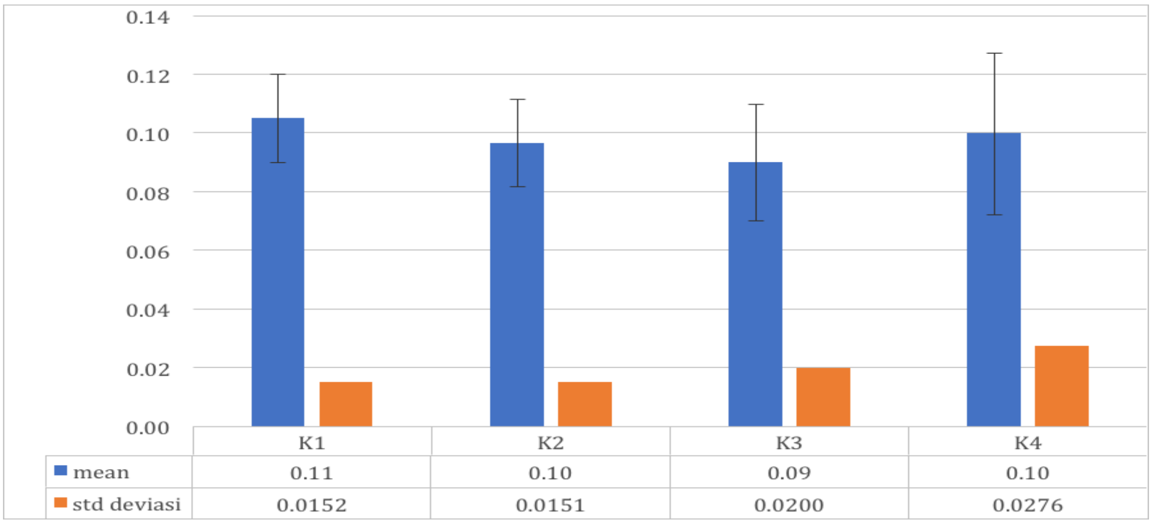

Figure 3. Ovarian weight histogram from the sample group

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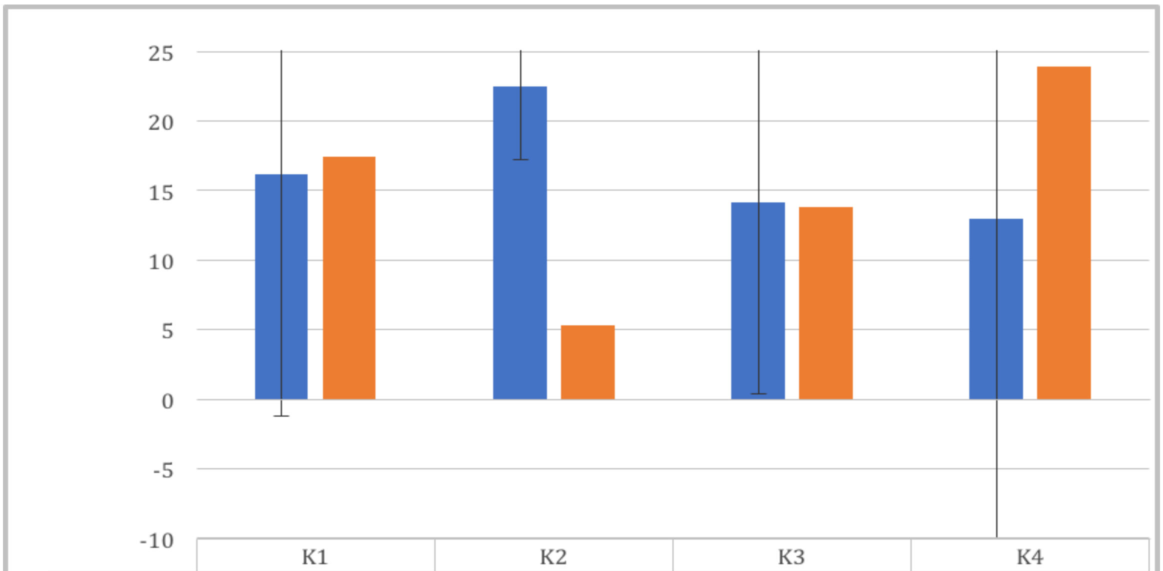
Figure 3 illustrates that the average ovarian weight at K3 (γ-Oryzanol) is 0.09 grams, and K4 (γ-Oryzanol + Tr) is 0.10 grams. K4 has the largest ovarian weight, which is 0.14 grams.

3.2.1.2. Rat Weight Gain



	K1	K2	K3	K4
mean	16	23	14	13
std deviasi	17.45183849	5.282045058	13.80458861	23.93324048

Figure 4. The Effect of Treatment Group on Rat Weight Gain

Based on Figure 4, it can be seen that there is a decrease in body weight in K3 (γ-Oryzanol) and K4 (γ-Oryzanol + Tr). The most significant decrease occurs in K4 when compared to other sample groups. This condition contrasts with K2 (transfluthrin), which has a relatively high body weight gain.

3.2.1.3. Number of Follicles and Follicular Abnormalities

The following figure compares the number of follicles and abnormalities in each sample group and reproductive cycle group.

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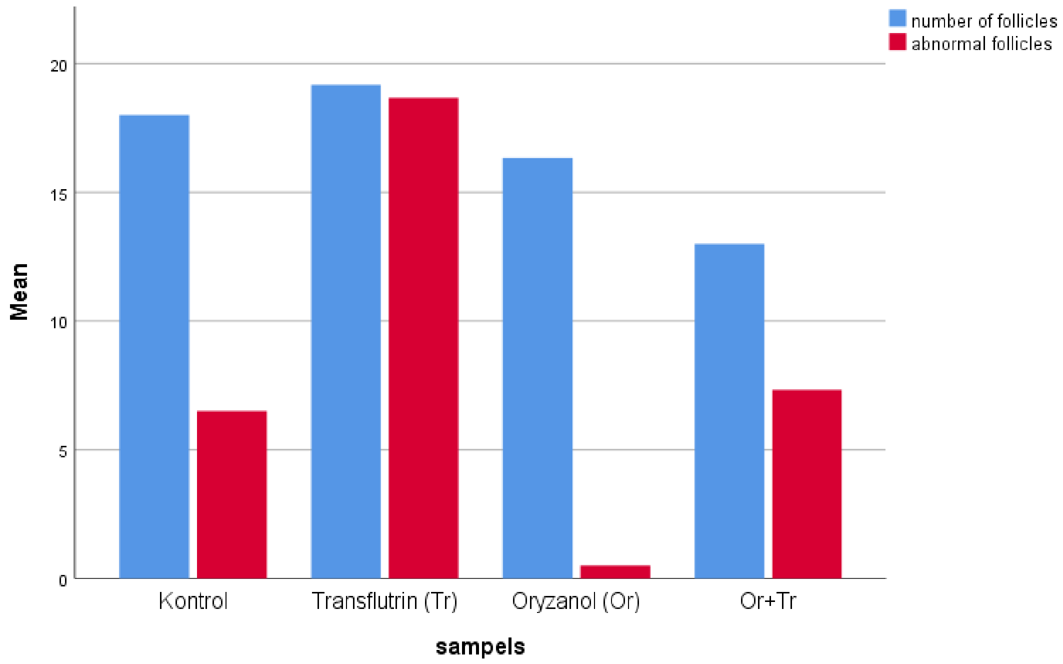

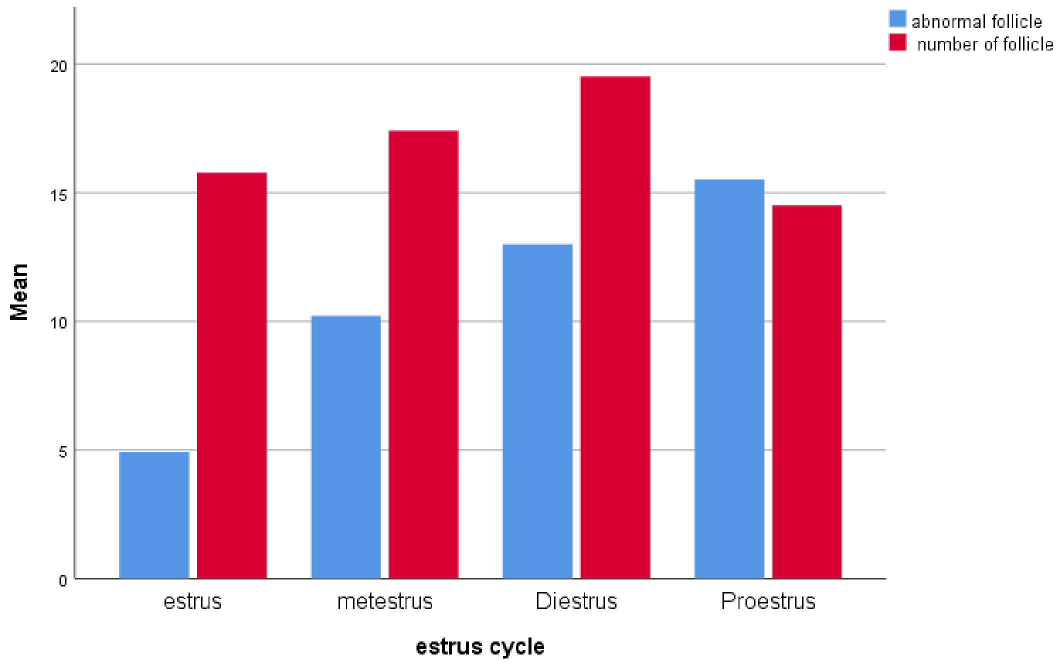

(a)
(b)

Figure 5. Diagram of the average number of follicles and follicular abnormalities in the sample group (1) and reproductive cycle group (2)

Figure 5 (a) explains that K3 (γ-Oryzanol) has a higher number of follicles with the least number of follicular abnormalities than other groups. In K2, the high number of follicles is followed by a high number of follicular abnormalities. Whereas, Figure 5

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(b) points out that in the diestrus phase, the number of follicles is higher than the other phases of the reproductive cycle but followed by an increase in follicular abnormalities.

Here is a picture of the ovarian histological structure that was examined using hematoxylin-eosin (HE) examination to see the follicular growth (Figure 6) and the type of follicular abnormalities that appear on the estrous phase for each sample group (Figure 7).

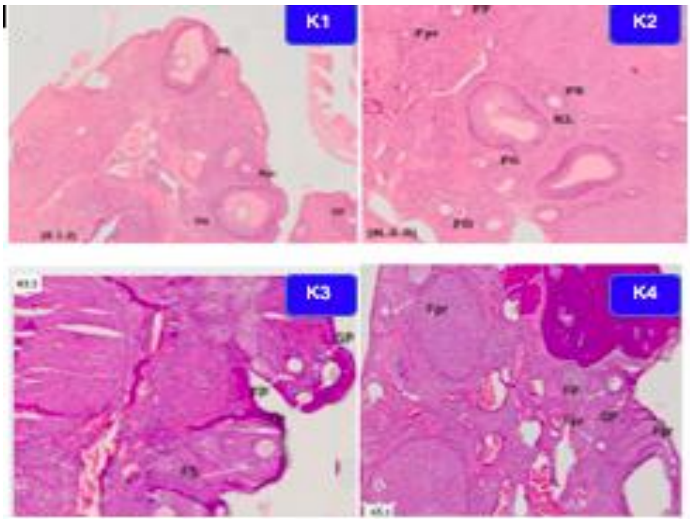

Figure 6. Follicular growth in the sample group; control (K1), Transflutrin (K2), γ-Oryzanol (K3), and γ-Oryzanol +Tr (K4)

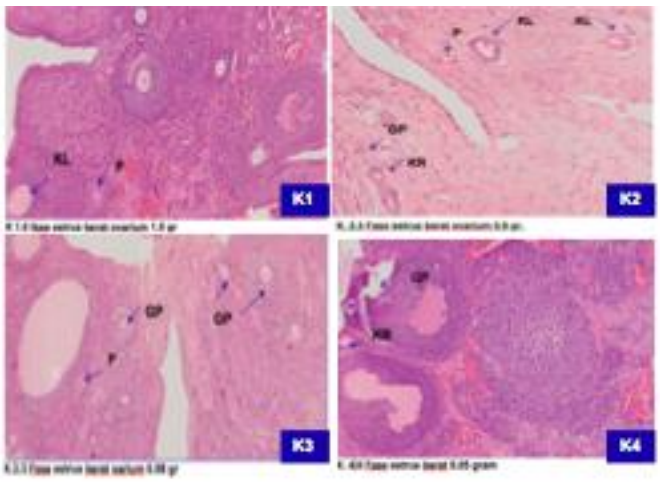

Figure 7. Follicular abnormalities in the sample group; control (K1), Transflutrin (K2), γ-Oryzanol (K3), and γ-Oryzanol +Tr (K4)

The results of HE examination demonstrate that K2 had the highest follicular abnormalities, such as karyolysis (KL), broken granulosa (BG), and karyorrhexis (KR), than other groups. It indicates folliculogenesis (follicular growth), which is disturbed by a high number of follicular abnormalities and affected the follicle preparation for ovulation.

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3.2.2. γ-Oryzanol Activity to EDCs in the Reproductive System

3.2.4.1. TNF-α Concentration

The ELISA analysis results on TNF-α concentration levels in the sample group (treatment) and reproductive cycle group are presented in Figure 8 below.

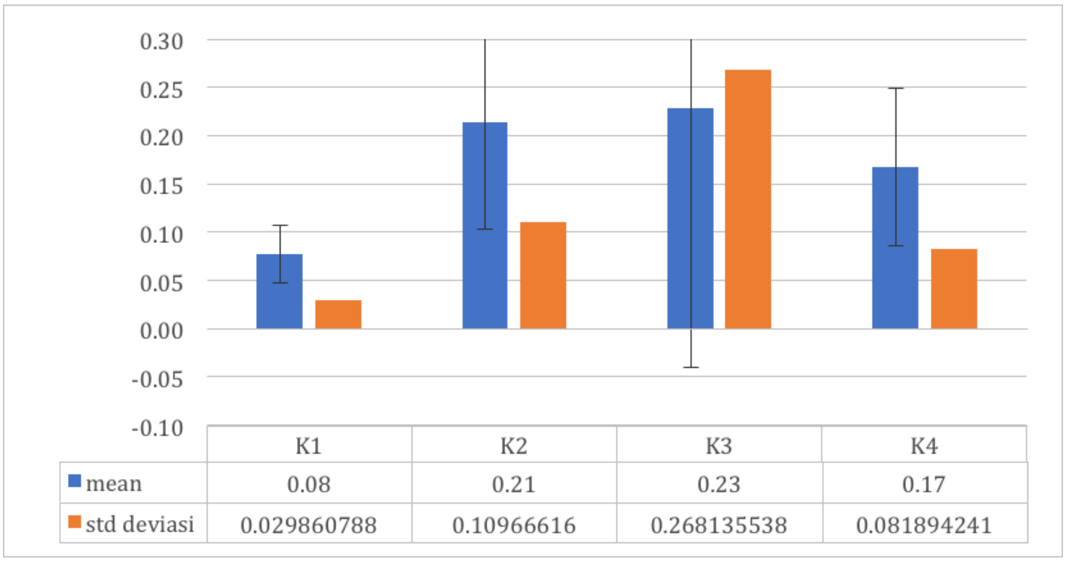

Figure 8. TNF-α Concentration Histogram in Sample Group

With a standard deviation of 0.08, K3 (γ-Oryzanol) has the highest TNF-α concentration compared to other groups. Meanwhile, the lowest level of TNF-α occurs in K1 (control) with a standard deviation of 0.02.

3.2.4.2. Foxo3a Expression

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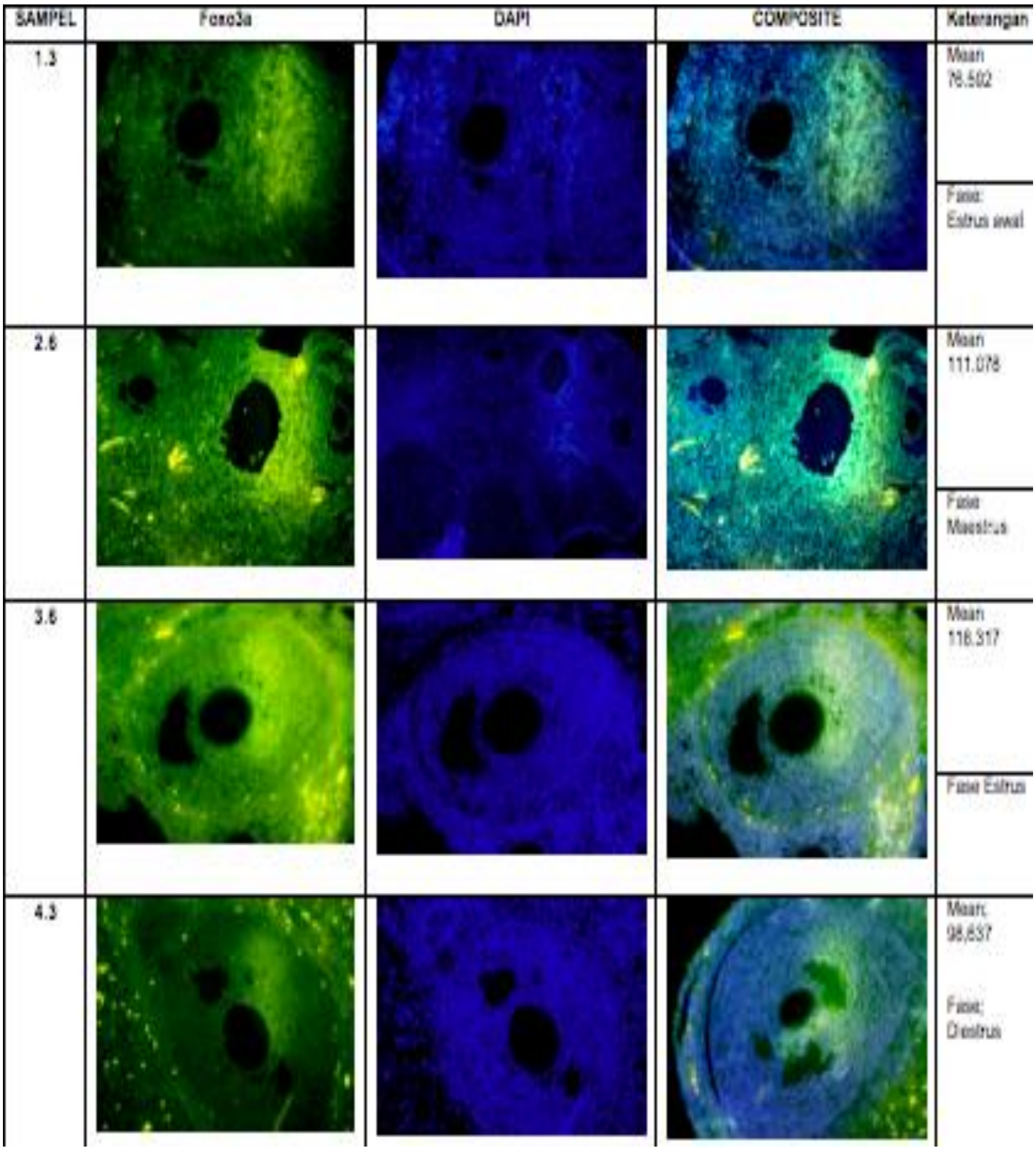
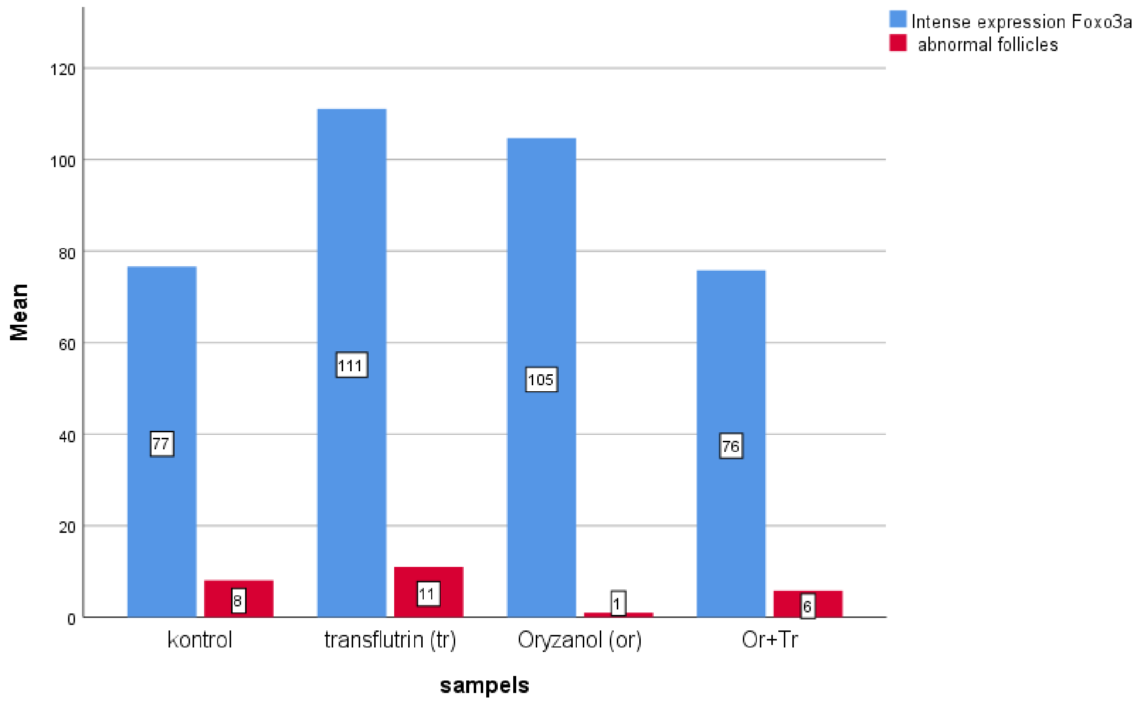
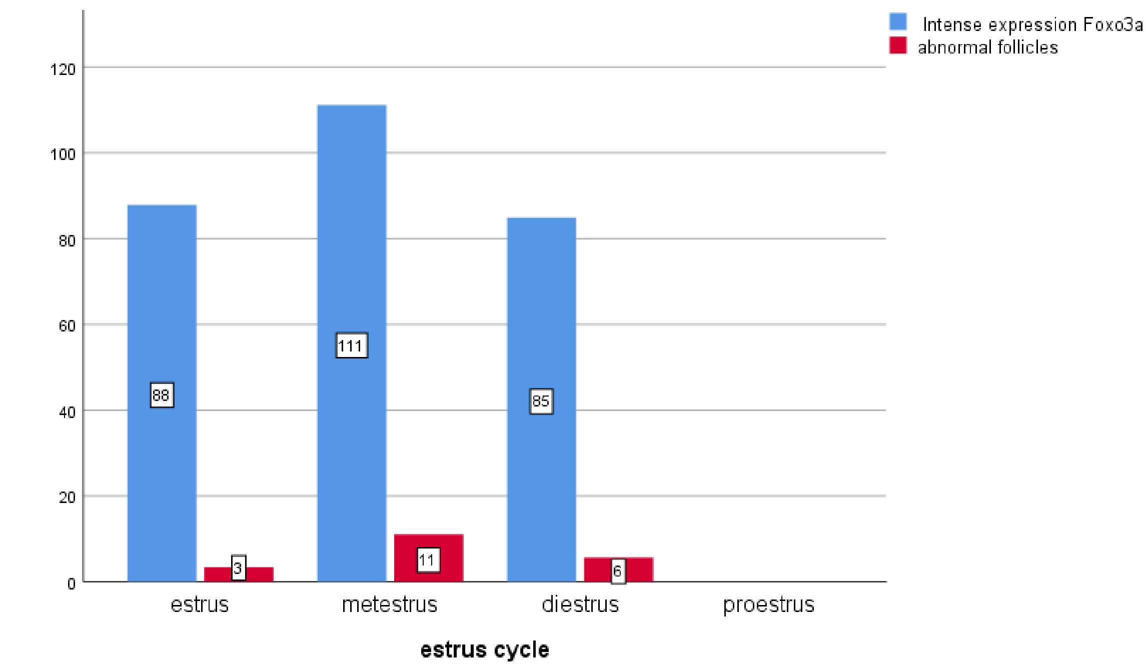


Figure 9. Foxo3a expression based on sample group: K1 (control), K2 (Transfluthrin), K3 (γ-Oryzanol), and K4 (γ-Oryzanol + Transfluthrin) and phase in the reproductive cycle

The degree of grayness from ImageJ portrays the mean indicator of Foxo3a expression, which is quantitatively equal to 0-255. As a result, Figure 9 signifies that the expression level in each sample is in the <255 categories and is in a different reproductive phase.





(a) **(b)**

Figure 10. Diagram of Foxo3a expression on the number of follicular abnormalities in the sample group (a) and reproductive cycle group (b)

K3 (γ-Oryzanol) has a fairly strong Foxo3a expression (105 on average) that is equal to K4 (γ-Oryzanol + Tr), which has sufficient Foxo3a expression (76 on average). This condition underlines the potential of γ-Oryzanol to protect oocytes and follicles from tissue damage caused by exposure to transfluthrin in reducing Foxo3a

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expression. It can be concluded that the number of follicular abnormalities in K3 (γ-Oryzanol) has the least number than other groups.

4. Discussion

4.1. In Silico Results

Figure 1 depicts the effects of γ-Oryzanol on EDCs from transfluthrin exposure. It proves the effect of γ-Oryzanol through HTR1A and HTR1B activation. The antioxidant potential of γ-Oryzanol is assessed by increasing the activation of endogenous antioxidants (SOD, CAT, and Gx) as a radical scavenger. Together, with the anti-inflammatory function of γ-Oryzanol through the blocking of NF_KB, γ-Oryzanol can reduce the level of follicle and oocyte damage by inhibiting ESR1 activation and increasing the AR hormone. The role of γ-Oryzanol through adenylate cyclase activation will increase cyclic adenosine monophosphate (cAMP) and activate protein kinase A (PKA), so the steroidogenesis process can run normally. It makes the follicles grow and develop due to the conversion of androgens to estrogen. In line with previous research on the potential of γ-Oryzanol conducted using Pass Server, the results of this study predict that γ-Oryzanol can be used to treat the exposure of insecticides because it can act as an anti-inflammatory agent and lipid peroxidase inhibitor [16].

Insecticides are believed to trigger oxidative stress. This oxidative stress can induce the presence of lipid peroxidase. Lipid peroxidase changes vasodilation due to an increase in selectins, intercellular adhesion molecules (ICAM), and vascular cell adhesion molecules (VCAM), resulting in the transfer of pro-inflammatory molecules, such as cytokines, macrophages, low-density lipoproteins (LDL), and immune cells. This molecule then undergoes oxidation and continues to inflammation. As a result, endothelial cells become damaged, and inflammation occurs. It leads to the appearance of plaques and other disorders because the barrier is dysfunctional. The end lipid peroxidase products are MDA (reactive aldehyde/malondialdehyde) and 4-HNE (4-Hydroxynonenal). These two molecules can cause the DNA adduct to happen. DNA adduct is the attachment of a DNA segment with a chemical substance that can induce mutations. A DNA mutation itself can cause the emergence of certain diseases like cancer. In this case, γ-Oryzanol has a role that is predicted to neutralize oxidative stress because it can act as an antioxidant and lipid peroxidase inhibitor [15, 17].

Based on the examination of protein interactions using STRING and STITCH analysis, several active compounds of γ-Oryzanol are found to interact with Foxo3 target proteins through the mediation of other proteins. The pathway prediction results from STRING analysis and HitPick Server reveal that γ-Oryzanol can bind APP, interact with AKT1, influence Mtor expression, and modify post-translation on Foxo1 to affect Foxo3. Firstly, APP binds with AKT1, AKT3, and AKT2 and then activates IkbkB. It then binds with Casp3, Fadd, Tradd, Tnfrsf1a, Tnfrsf1b, Traf3, Birc2, and Ikbkg to bind with TNF. It is in line with the in silico results from previous studies that the potency of γ-Oryzanol using the Pass Server approach demonstrates an anti-inflammatory potential. Besides that, the nitric oxide scavenger is stronger than the antioxidant compound, shown by its ability to activate IkbkB [18, 19].

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The anti-inflammatory function is supported by the interaction between APP and AKT1, which helps regulate metabolism, proliferation, cell survival, growth, and angiogenesis. AKT1 is mediated by phosphorylation of serine and/or threonine and is responsible for regulating glucose uptake by insulin-induced translocation [20, 16].

4.2. In Vivo Results

4.2.1. The effect of EDCs on the ovarian histological structure

4.2.1.1. Ovarian weight

In this study, the ovarian weight was analyzed using the Kruskal Wallis statistical test. The result was Sig 0.598 ($P > 0.05$), which means that the rats' ovarian weight experienced no significant increase in the treatment group. A post hoc analysis with the Mann-Whitney test $p > 0.05$ also showed that the treatment did not significantly affect the sample group. Meanwhile, the ovarian weight in the reproductive cycle group, assessed using Kruskal Wallis analysis, showed a Sig of 0.05 ($p=0.05$). It means a change in the ovarian weight of the rats. The examination then continued with Mann Whitney post hoc test and proved that the estrous and diestrous phases received a significant effect of Sig 0.02 ($p < 0.05$) from the treatment. It is noted that the highest ovarian weight occurs in the diestrus phase while the lowest is in the estrous phase.

There is a decrease in ovarian weight in the early estrous phase due to follicle growth cessation. It is found that one selected follicle grows and develops into de graff follicle. The changes in ovarian weight during the estrous phase are influenced by FSH, which stimulates the growth of selected follicle into de graff follicle and is followed by an increase in LH in preparation for pre-ovulation. At this stage, the estradiol produced by the de graff follicle will cause changes in the reproductive tract to its maximum. The growth and development of follicles are influenced by anterior pituitary stimulation to increase FSH and LH secretion, followed by the release of the estrogen hormone [21, 22, 23]. Consequently, it can be said that the changes in ovarian weight during the estrous phase are strongly influenced by hormonal factors such as estrogen.

There is an increase in ovarian weight in the diestrus phase due to an increase in the progesterone hormone. If pregnancy occurs, progesterone will enlarge and thicken the reproductive organs, including the uterus and maintain the continued function of the corpus luteum in the ovaries.

A decrease in the estrous phase and an increase in the diestrous phase in K4 (γ-Oryzanol + Tr) can be controlled by γ-Oryzanol with its antioxidant and anti-inflammatory functions by preventing ovarian damage due to transfluthrin exposure. Chronic inflammation can be indicated if there is a decrease in ovarian weight and failure of oocyte growth and development [24].

4.2.1.2. Bodyweight gain

Several previous studies regarding the use of γ-Oryzanol in weight loss have proven that γ-Oryzanol can inhibit cholesterol absorption in the intestine and increase the amount of fat excreted through feces [25]. Moreover, the γ-Oryzanol content in rice

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bran oil can reduce the lipase enzyme activity in the intestines by inhibiting the absorption of fat in the intestines [26]. It indirectly causes a reduction of the fat stored.

The increase in body weight on K2 (transfluthrin) is related to the phase experienced by each member of the K2 sample, which is generally in the metestrous phase as shown in Figure 1 (b). In normal conditions, the ovary is characterized by the formation of the corpus luteum, which contains lutein cells and small follicles. In this phase, the progesterone hormone is secreted from LH, which plays a role in repairing the damage caused by ovulation. When fertilization occurs, progesterone will thicken the walls of the uterus and ovaries. This condition provides the potential for an increased body weight of the rats [27].

In K2, the weight gain of the rats is followed by an increase in the number of follicular abnormalities. The high number of atretic follicles and an enlarged ovary can have follicular cysts in the ovaries. Follicular cysts will form when a follicle does not release an egg during ovulation. Instead, follicles grow and turn into cysts. Follicular cysts usually have no symptoms and do not require intervention as the cysts will disappear on their own [28]. This condition is riskier to experience a prolongation of the leukocyte phase or post-estrus phase (metestrus-diestrus) than the epithelial phase. It explains that the estrous cycle in K2 has already interfered; there is a prolongation of the leukocyte phase caused by the disruption of LH and FSH stimulation or hormonal stimulation of follicular maturation for the next estrous cycle [29, 9].

4.2.1.3. Number of follicles and follicular abnormalities

The effect of EDCs on the histological structure of the ovaries is related to follicular development (folliculogenesis) and oocyte growth (oogenesis), which occur simultaneously and play an important role in the ovulation process.

The results of the Kruskal Wallis test analysis in the sample group on the number of follicles showed a Sig of 0.15. A Mann Whitney test was performed and proved that K2 (transfluthrin) and (γ-Oryzanol + transfluthrin) had a Sig of 0.05. It means that the treatment is given to the sample group significantly influenced the number of follicles. On the other hand, the Kruskal Wallis test on the reproductive cycle group obtained a Sig of 0.526 ($p > 0.05$). Thus, there was no significant change in the number of follicles. A Mann Whitney test also took place and showed a Sig of $p > 0.05$. This indicates that the reproductive cycle group did not experience a significant change in the number of follicles.

The high number of follicles on K2 (transfluthrin) is followed by many follicular abnormalities, as shown in Figures 5 and 7. Based on the Kruskal Wallis test results, the Sig for follicular abnormalities in the sample group was 0.000, which means a significant increase in the number of follicular abnormalities. It is also shown by the Mann Whitney test on K1-K3 (Sig 0.03), K2 -K4 (Sig 0.016), and K3-K4 (Sig 0.04).

In summary, the treatment is proven to affect the increase and/or decrease of follicular abnormalities. K2 signified that the exposure of one-push aerosol with transfluthrin as its active ingredient could increase follicular abnormalities due to toxicity effects on the reproduction organs [7]. The toxicity effect emerges through 2

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mechanisms: the direct effect on cells and the effect of biochemical reactions on cell metabolism [14]. In this study, the toxicity condition, which directly affects cells, is indicated by follicle growth disorders (folliculogenesis). The folliculogenesis form includes primordial and primary follicles and follicular abnormalities, such as atresia, granulosa rupture or damage, karyorrhexis, karyolysis, and pyknosis.

Karyolysis is the loss or the waning of the nucleus, while karyorrhexis is the destruction of several fragments in the nucleus. Kariolysis and karyorrhexis are granulose cells that undergo apoptosis [30]. Finally, atresia occurs due to degeneration of normal follicles characterized by pyknosis, reduced granulosa cells due to proliferation, and basement membrane damage or glucose rupture [31].

Figures 5, 6, and 7 show that γ-Oryzanol can maintain folliculogenesis continuity in an environment exposed to one-push transfluthrin. γ-Oryzanol suppresses the number of follicular abnormalities by maintaining the continuity of the follicular growth process following the reproductive cycle. K4 (γ-Oryzanol + Transfluthrin) has illustrated the strong anti-inflammatory activity of γ-Oryzanol, indicated by the number of abnormal follicles less than K2 (Transfluthrin). It is consistent with the in silico test results that γ-Oryzanol has a stronger anti-inflammatory activity than its antioxidant activity [19].

As we can see in the discussion above, 4 study groups have presented varying folliculogenesis conditions, especially in each reproductive cycle. This condition is controlled by genetics and is modified by external factors such as exogenous hormones, weather, steroid hormones, and nutrition [29], including transfluthrin exposure with its EDC effects. The efforts to prevent the damaging effects of EDCs on the body require additional or supporting nutritional intake [32, 33]. In this study, the administration of γ-Oryzanol is confirmed to control folliculogenesis.

4.2.2. The activity of γ-Oryzanol on EDCs in the reproductive cycle through TNF-α concentration and Foxo3a expression

4.2.2.1. TNF-α Concentration

Based on the Kruskal Wallis test, the TNF-α concentration of the sample group showed a Sig of 0.223. A further test was conducted with Mann Whitney parameters on K1 (control) and K2 (Tr) and generated a Sig of 0.025. It proposes a significant effect on the sample group regarding its TNF-α concentration. Nevertheless, in the reproductive cycle group, the Kruskal Wallis test showed a Sig of 0.480, and the Mann Whitney test produced a Sig of $p > 0.05$. It explains that the reproductive cycle did not affect TNF-α concentration.

The results above are not similar to previous studies' results, emphasizing that the reproductive cycle affected the increase and decrease of TNF-α concentration. In this study, the absence of reproductive cycle influence on TNF-α concentration is caused by several possibilities: the high number and variation of samples in each group. It creates a small average value and rate of each sample, and thus, the effect becomes not significant.

In Figure 8, K3 (γ-Oryzanol) is shown to have the highest value and levels of TNF-α concentration compared to other groups. The high average value of TNF-α

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concentration at K3 is affected by the phases of the reproduction cycle, such as estrous and diestrus (highest concentration) phases. It supports the theory that during the folliculogenesis process, TNF-α plays a role in the ovaries when the primordial follicle is developing (in the final diestrus phase). During the transition period of diestrus to proestrus, Foxo3a and TNF-α together suppress the ovarian response to gonadotropins. The goal of this process is to limit the primordial follicles that are activated as negative feedback in maintaining the continuity of reproductive function.

In the estrous phase (pre-ovulation), TNF-α concentrations are also high. TNF-α plays a role in stimulating steroidogenesis by producing progesterone as a progesterone mediator and stimulating apoptotic action in the process of follicular rupture [34,35].

In Q4 (γ-Oryzanol + transfluthrin), most of the samples are found to be in the diestrus phase. γ-Oryzanol exhibits its antioxidant activity by neutralizing reactive oxygen species (ROS) caused by EDCs effects. It can be seen in the increase of TNF-α concentration in K4, even though the increase is not higher than K2. From here, the researchers underlined that the presence of γ-Oryzanol could maintain the physiological function of TNF-α. In other words, an increase of TNF-α concentration in the diestrus phase suppresses the ovarian response to gonadotropins that the goal is to limit the primordial follicles as negative feedback in maintaining the process of reproductive function. The anti-inflammatory activity of γ-Oryzanol can also be measured from the low number of follicular abnormalities in the γ-Oryzanol treatment group.

4.2.2.2. Foxo3a Expression

The result of the Kruskal Wallis test was Sig 0.376, while the Mann Whitney test was $p > 0.05$. This proves that the sample group did not affect Foxo3a expression. Whereas, in the group that is based on the reproductive cycle, it is known that the result of the Kruskal Wallis test was Sig 0.845 with the Mann Whitney test of $p > 0.05$. It confirms that the reproductive cycle did not significantly influence Foxo3a expression.

In Figure 9, it is displayed that K2 has the highest Foxo3a expression compared to other groups. But, based on its reproductive phase, K2 is stated to be in the metestrous phase. In normal conditions, there is no follicle growth process in the metestrous phase. Foxo3a, whose function is to maintain follicular reserves in the ovaries, is not activated in this phase. This condition continues until the middle of the diestrus phase (if conception does not occur). Henceforth, if there is an increase in Foxo3a expression at this stage, it can be predicted that there is an abnormality of function/overexpression of Foxo3a. This process will occur outside the cycle of primordial follicle activation. As a result, the potential of follicular cysts formation and oocyte abnormalities is higher [28].

The role of Foxo3a in mammals can have positive and negative effects on the continuity of cell function. The positive role of Foxo3a occurs in the cell cycle, particularly at the G2-M stage. Foxo3a is also able to stimulate the repair of damaged DNA. DNA damage itself is caused by oxidative stress due to increased ROS. The relationship between protein kinase B (PKB) activation and the transcription factor of Foxo3a serves to protect cells from oxidative stress directly through increased manganese superoxide dismutase (MnSOD), messenger RNA (mRNA), and protein. The increase can protect cells from ROS against apoptosis that is caused by glucose loss. This mechanism requires Foxo3a to activate the transcription of MnSOD so that there is a reduction in ROS. On the other hand, the negative role of Foxo3a can be found in cell mutations, which lead to abnormalities in oocytes [36,37].

The continuous use of mosquito repellent containing the active ingredient of transfluthrin for a certain period can result in reproductive function (ovary) disorders, including folliculogenesis, oogenesis, and ovulation issues. Transfluthrin as a pyrethroid derivative of EDCs, has a hormonal and cellular impact on ovaries. Hormonally, it reduces the secretion of LH-FSH hormones and disrupts gonadotropins. It will cause apoptosis that is marked by follicular growth inhibition. As for the cellular effect, transfluthrin is believed to increase the damage in mitochondria. This condition makes the x-linked factor unable to prevent damage in the ovaries [38, 9]

In this study, the antioxidant and anti-inflammatory potentials of γ-Oryzanol in controlling Foxo3a expression are measured by 2 groups, namely K3 and K4 (Figure 11). Both K3 (γ-Oryzanol) and K4 (γ-Oryzanol + Tr) are in the estrous phase where Foxo3a is poorly expressed. This condition indicates that the antioxidant and anti-inflammatory activity of γ-Oryzanol maintains the physiological role of Foxo3a during the reproductive cycle (estrous phase). This process is done by maintaining a hormonal balance as a resistant effect and suppressing the uncontrolled follicle and oocyte necrosis activity from Foxo3a due to EDCs (transfluthrin) exposure.

Based on the results of DPPH measurements, the antioxidant activity of γ-Oryzanol is classified as moderate. Whereas, compared to its antioxidant potential, the anti-inflammatory potential of γ-Oryzanol in controlling the reproductive cycle is found to be strong and high [27]. The relationship between the antioxidant and anti-inflammatory activity of γ-Oryzanol is explained by Tantamango et al. [39] that antioxidant activity of γ-Oryzanol can lead to the inhibition of $NF_{K}B$. This can explain the mechanism of anti-inflammatory effects from γ-Oryzanol. Ghatak & Punchal [40] also conducted experiments on animals to determine the immunomodulatory ability of γ-Oryzanol (crude rice bran oil extract). Their experiments proved that γ-Oryzanol has sufficient potential to increase immune activity, both cellularly and hormonally.

5. Conclusion

In conclusion, the inflammatory activity of γ-Oryzanol is capable of controlling the reproductive cycle of the rats from the effects of EDCs that are characterized by a decrease in the number of follicular abnormalities. γ-Oryzanol can also control TNF-α concentration at each phase of the reproductive cycle and is effective in reducing Foxo3a expression by suppressing the number of follicular abnormalities.

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