Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Jan 01; 10(B):1-12.
https://doi.org/10.3889/oamjms.2022.7543
eISSN: 1857-9655
Category: *B - Clinical Sciences*
Section: *Gynecology and Obstetrics*

Antioxidant and Anti-inflammatory Activity of γ-Oryzanol Compared to Rice Bran Oil to Repair Ovarian Histological Structure from One Push Transfluthrin Exposure Effect

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Edited by: Ksenija Bogoeva-Kostovska
Citation: Lisnawati L, Poeranto S, Endharti AT, Santoso MIE. Antioxidant and Anti-inflammatory Activity of γ-Oryzanol Compared to Rice Bran Oil to Repair Ovarian Histological Structure from One Push Transfluthrin Exposure Effect. Open Access Maced J Med Sci. 2022 Jan 01; 10(B):1-12. https://doi.org/10.3889/oamjms.2022.7543
Keywords: Follicle; Ovaries; γ-Oryzanol; Rice bran oil; Transfluthrin
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Received: 08-Oct-2021
Revised: 11-Nov-2021
Accepted: 22-Dec-2021

Funding: This research did not receive any financial support
Competing Interest: The authors have declared that no competing interest exists

Abstract

BACKGROUND: Fertility is affected by both the reproductive organs and external factors (genetics, hormones, radiation exposure, use of insecticides, and nutrition). The histological structure of the ovaries is an indicator of reproductive function. Insect repellent use with pyrethroid active ingredients and its impact on health has become a discussion in the medical sector for years. Disruption of the reproductive system homeostasis may cause several issues, from disruption of ovarian function to infertility. γ-Oryzanol has higher antioxidants than vitamin E. It is found mostly in rice bran oil (RBO). Researchers have investigated the effectiveness of RBO and γ-Oryzanol, but the number of studies focusing on the reproductive system is very limited. Results of in silico showed anti-inflammatory potential, and nitric oxide γ-Oryzanol is stronger than the antioxidant activity. It also showed γ-Oryzanol bond with Foxo3a and Growth Differentiating Factor 9 (GDF9), indicating the γ-Oryzanol potential for reproductive health (women). Studies also reported that γ-Oryzanol administration caused anti-inflammatory and antioxidant activity compared to RBO in improving ovarian physiological function using tumor necrosis factor α (TNF-α) levels, Foxo3a expression, and GDF9 expression exposed to one push transfluthrin as the parameter.

AIM: We aimed to investigate antioxidant and anti-inflammatory activity of γ-Oryzanol compared to rice bran oil to repair ovarian histological structure from one push transfluthrin exposure effect.

METHODS: Experimental research, post-test only control group design approach, with a completely randomized design, consisted of 6 (six) groups of Wistar strain female rats. They were exposed to one push with the active ingredient of transfluthrin 21.3%, by inhalation for 6 hours, RBO 0.3 mg/g/ body weight (BW)/day, γ-Oryzanol 3.75 ml/g/BW/day. Statistical analysis was done with the Mann Whitney's posthoc Kruskal Wallis test with IBM SPSS version 25 software.

RESULTS: γ-Oryzanol had more potent anti-inflammatory and antioxidant activity than RBO in improving the ovarian histology structure (including maintaining ovarian weight, increasing follicular growth, and suppressing follicular abnormalities) through decreased TNF-α levels and decreased Foxo3a expression, and increased GDF9 expression.

CONCLUSION: The administration of γ-Oryzanol improves the ovarian histological structure from free radicals effects, namely exposure to one push of transfluthrin.

Introduction

Sunaryo's study in 2016 showed that most Indonesians used household insecticides (86.33%) most frequently once a day (85.4%) for more than 5 years (74.51%) [1]. Research of which objective is to identify mosquito repellent distribution showed that spray or aerosols (12.2%) are widely used in Indonesian households [2].

There are three vital processes in the ovaries: oogenesis, folliculogenesis, and steroidogenesis, which play a pivotal role in identifying fertility in the reproduction system. These processes can be measured through the histological structure of the ovaries, while their function can be analyzed through the PI3K-Akt pathway approach.

Exposure to pyrethroid aerosol mosquito repellent during the reproduction cycle may cause cellular damage by increasing Reactive Oxygen Species level and indicating oxidative stress. It will influence oocyte growth and development, including in Growth Differentiating Factor 9 (GDF-9) secretion through Oocyte Secreted Factors. Low GDF-9 expression will cause kit ligand (KL) formation disruption in granulose. KL on the PI3K pathway plays an essential role in the activation of Akt. In normal conditions, activation of Akt on Foxo substrate results in suppression of Foxo3a activation and, as a result, suppresses the negative effects of Foxo3a on oocyte growth, namely

apoptosis and transcription. Through the PI3K-α approach, activation of GDF-9 during folliculogenesis is also associated with extracellular signal-regulated kinase (ERK1/2) activation through the mitogen-activated protein kinase pathway stimulated by tumor necrosis factor α (TNF-α) and epidermal growth factor receptor (EGFR) in granulosa. EGFR activation can only occur if follicle-stimulating hormone (FSH) binds to FSH receptor (FSHR); transfluthrin exposure can be inhibited due to endocrine disrupting chemicals (EDC's) effect, which disrupts gonadotropin function and reduces FSH and luteinizing hormone (LH) levels. It influences the inability of EGFR to activate ERK1/2. The PI3K pathway shows the expression of Foxo3a, GDF-9, and TNF-α; in this condition, the source of antioxidants and anti-inflammatory to repair damaged cells during the folliculogenesis process is necessary [3], [4].

Rice bran has good nutritional content and high bioactive components, and thus, can potentially be used as functional food [5]. One of the less-known products made from rice bran is rice bran oil (RBO). RBO is a source of antioxidants and anti-inflammatory. It contains bioactive compounds, such as ferulic acid, tricin, beta-sitosterol, γ-oryzanol, tocotrienols, tocopherols, and phytic acids that can prevent oxidative stress by reducing damage to plasma lipids and blocking chronic inflammatory responses. Like other products made from rice bran, it also contains a bioactive component, γ-oryzanol. Crude RBO has 62.9% of γ-oryzanol and 35.9% of phenolic acid.

In silico or the preliminary stage of the study showed that γ-oryzanol had anti-inflammatory potential and stronger nitric oxide compared to antioxidant potential [6]. EDCs from one push exposure containing transfluthrin are tested together with γ-oryzanol and RBO application to identify their impact on the histological structure of the ovaries using three parameters. Those parameters are increased TNF-α to measure inflammatory process, Foxo3a expression to identify homeostatic disruption in the ovaries leading to increased Foxo3a expression that influences apoptotic activation and transcription during folliculogenesis, and decreased GDF9 expression playing a vital role in the growth of follicle and oocytes as well as proliferation and differentiation of granulosa cell.

Methods and Material

Design

The study was an experimental study with a post-test only control group design approach.

Setting

Referring to organization for economic co-operation and development (OECD) 412 on sub-acute inhalation procedures, the experimental animals were eight (8) weeks old and weighed 150–200 g. The acclimation process lasted for seven (7) days before the experiment, and samples were selected randomly.

The animal care procedure was as follows. Samples were kept in a plastic container covered with wire with 1.6 cmc^2 holes. The container base was 148.4 $cm^{2,}$ and the height was 17.8 cm. The base was replaced and cleaned every three (3) days. Room temperature was set to 22 ± 3°C, the humidity was 30–70%, 14 h light on, and 10 h light off. The samples were fed with 100 g/body weight (BW)/day/sample of COMFEED starter concentrate/calf starter and 10 ml drink/100 g of BW/day/sample. The samples were monitored before, during (6 h in a glass box), and after treatment.

The study used γ-oryzanol Sigma Aldrich CDS021604_ALDRICH. γ-oryzanol was dissolved using 1% Na CMC with the following requirement: 1.25 g of 1% Na CMC were mixed with γ-oryzanol into 250 ml of homogenous solution. Daily dosage was determined based on BW/week, the highest times conversion. The types of RBO used were RBO OG and purified RBO through "extra cold" filtering. The oral dosage of RBO was determined based on γ-oryzanol content in RBO; 1 kg of RBO dissolved in 1000 cc contained 3137 mg of γ-*Oryzanol,* and thus, 100ml of RBO had 314 mg of γ-*Oryzanol.* RBO dosage based on the samples' BW/kg was 0.3mL/BW/kg.

Study population

Total samples, Wistar strain female rats, were 24 determined based on Federer's formula for experimental testing, namely $(t-1)(n-1)\geq 15$ comprising six (6) groups of samples. (a) Normal control (K1), (b) negative control negative was given one push of transfluthrin (K2), (c) positive control was given γ-Oryzanol (K3) and given RBO (K4), and (d) experiment group was given transfluthrin and γ-Oryzanol (K5), and given transfluthrin and RBO (K6). The rats were from the Bandung Institute of Technology veterinary laboratory and declared healthy based on veterinary certificate Number 524.3/3947-Dispangtan/2019.

Variable, source of data, and data collection

γ-Oryzanol and RBO antioxidant level (1,1-diphenyl-2-picrylhydrazyl [DPPH])

The ability of γ-Oryzanol to inhibit the 50% effect of free radicals was 112 ppm and was classified

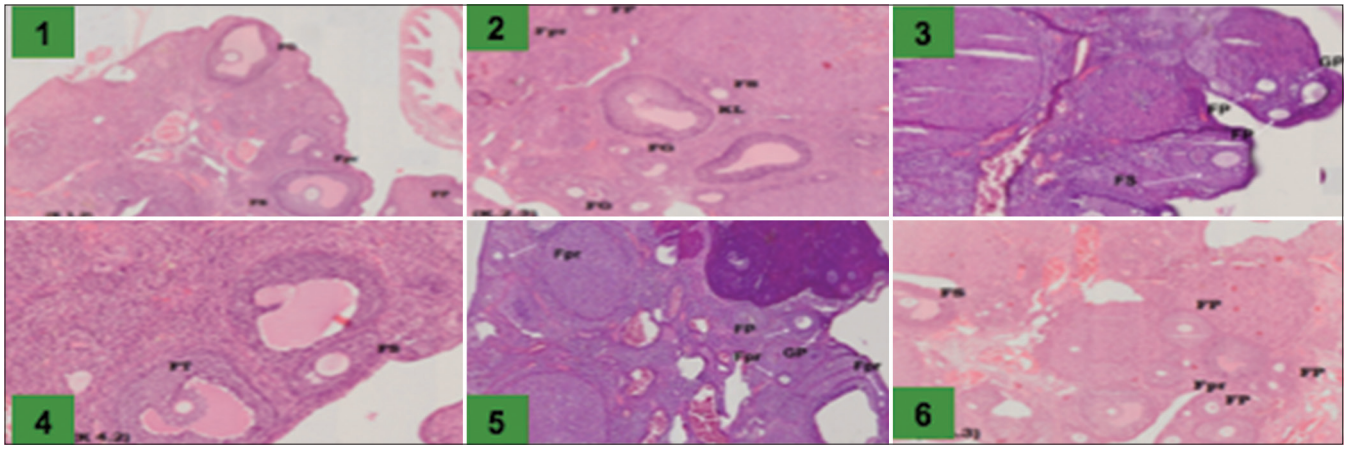

Figure 1: There are 6 pictures showing the growth and development of follicles/folliculogenensis that occurred based on the experiments provided including; picture [1] is folliculogenesis that occurred in the control group, picture [2] is folliculogenesis in the group given transfluthrin exposure, picture [3] is folliculogenesis in the group given gamma oryzanol, pictures [4] is folliculogenesis in the group given RBO, picture [5] is folliculogenesis in the group given exposure and gamma oryzanol, and picture [6] is folliculogenesis in the group given transfluthrin and RBO

as moderate antioxidant potential. The analysis showed that ability of RBO to inhibit 50% free radicals was 1546 ppm or very weak antioxidant potential.

Transfluthrin particle concentration

Measurement toward transfluthrin concentration in one push transfluthrin in three (3) sprays with P-Track UPC 852 showed that ultra particle was 311,500 ± 46,755 particles/cm^3 and fine particle concentration was 12,392 ± 1356 mg/m^3

Ovarian histology

Measurement referred to the qualitative observation of the ovaries to observe cellular damage or damage on the structure of the ovaries due to transfluthrin exposure in particular. The procedure was to observe follicle growth with hematocosilin eosin (HE) stain using an Olympus CX-31 microscope. Quantitative observation of the ovaries, measuring the effect of the treatment, was conducted by weighing the ovaries immediately after surgery.

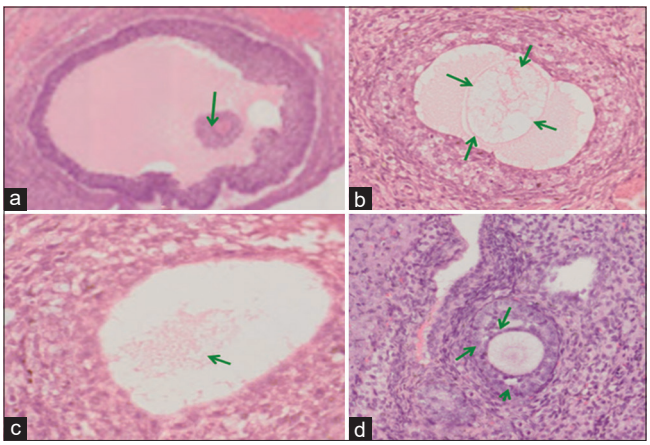

Figure 2: (a-d) Observation of follicle disruption in K2 (Transfluthrin)

Follicle extraction and analysis

The measurement procedures, performed single blindly without knowing which of the exposure and control groups were quantitative observations, were to count primary and secondary follicles, de Graff follicles, and the number of follicular abnormalities.

Reproduction cycle

The procedure was a pre-surgery vaginal swab to identify phases in the reproduction cycle of the rats (estrus, metestrus. diestrus, and proestrus phase).

TNF-α concentration

It referred to the inflammation indicator in the rats and anti-inflammatory activity of γ-Oryzanol in decreasing TNF-α level. TNF-α level or concentration was obtained through ELISA analysis.

Foxo3a expression

It referred to the molecular approach as an indicator of oocyte and follicle growth affecting the reproduction cycle in the ovaries through the intense expression of Foxo3a. Measurement was conducted through immunoflorence (IF) and semi-quantitative Image J.

GDF9 expression

It referred to the parameter used to measure growth factor internal role in the ovaries during the estrous cycle influencing the growth of follicle and oocyte and the differentiation process of granulosa cell proliferation. Measurement was conducted through IF and semi-quantitative Image J.

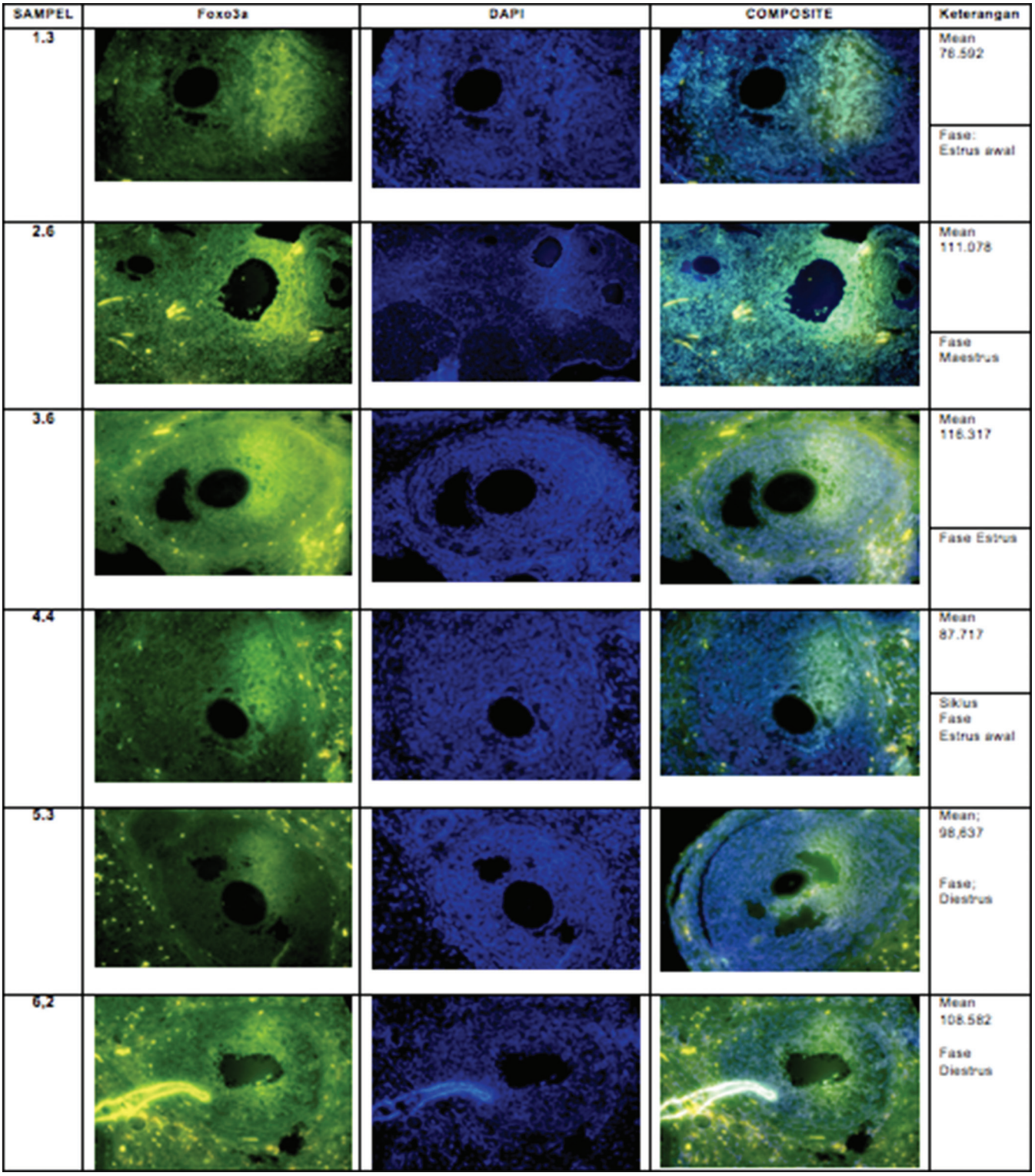

Figure 3: Foxo3a expression measurement with IF method and Image J

Analysis and statistics

It was done using IBM SPSS version 25. The analysis result showed that the variables were not normally distributed or homogeneous. Therefore, based on the type of the numerical dependent variable (ratio) and nominal independent variables, the Kruskal Wallis was used for the hypothesis testing. The objective was to identify a discrepancy between the average scores of all treatments on the histological structure of the ovaries. Furthermore, to determine the significance of the differences between groups according to the objectives of the study, a *post hoc* test, the Mann Whitney test, was conducted. Meanwhile, to determine the effect of the treatment results on TNF levels, Foxo3a expression levels and GDF9 expression levels were used.

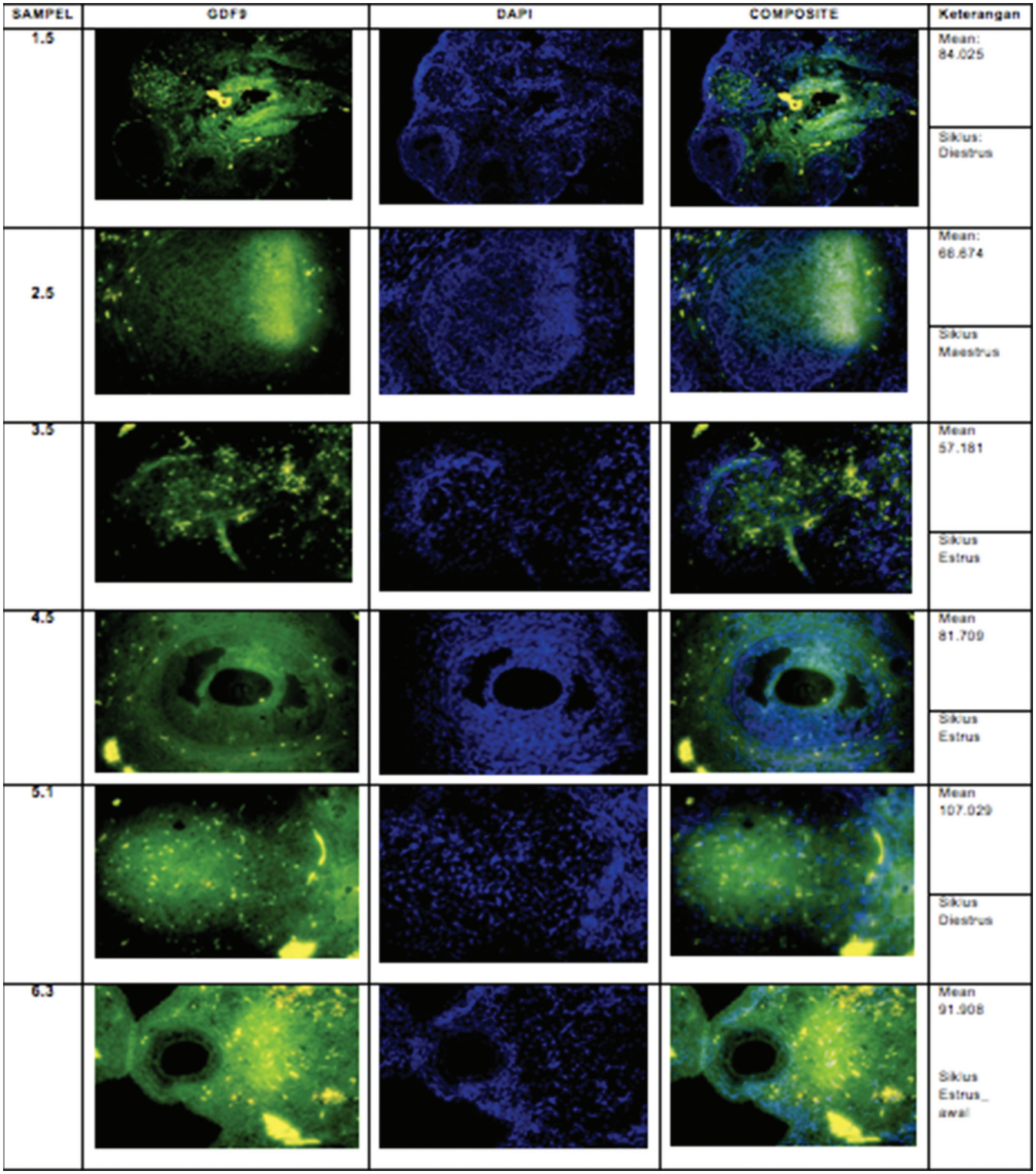

Figure 4: GDF9 expression measurements with IF method and Image J

Ethics appraisal

The administration of one push aerosol inhalation to measure sub-acute toxicity referred to the OECD 412 procedures guidelines for testing the chemical's 28 day (Sub-Acute) inhalation toxicity study. The type of one push exposure was V aerosol, of which the active compound was 21.3% of transfluthrin. It was given through inhalation in one (1) spray and left in the glass box for six (6) h when the samples were first put inside the box until they were returned to the plastic container for 28 days.

The research procedure followed and was approved by the Faculty of Medicine Universitas Brawijaya with ethics appraisal number 38/EC/KEPK-S3/02/2019.

Results

The research was conducted in 2 (two) stages. The results of the study can be explained as follows;

In the first stage of the study, an examination of the condition of the follicles in each experimental group was carried out. At this stage, an examination of Hematocycline Eosin (HE) was performed to calculate quantitatively and qualitatively the number of follicles as an indicator of the ongoing folliculogenesis process in the ovaries. This process of folliculogenesis can be seen in Figure 1, namely that based on the results of the HE examination, the difference in folliculogenesis ability in the control group compared to the experimental group is shown in boxes 2-6, while Figure 2 shows that through the HE examination, the type of follicular abnormality can also be identified. that occurred during folliculogenesis, especially in the experimental group [2], namely the group that was exposed to transfluthrin.

Table 1 shows the results that occurred in the second stage of research (two).

Discussion

Antioxidant and anti-inflammatory activities from γ-oryzanol application compared to RBO affecting the weight of the ovaries through the level of TNF-α, Foxo3a, and GDF9 measurement in transfluthrin-exposed rats (Rattus Novergicus)

The weight of the ovaries was analyzed with Kruskal Wallis. The result was Sig 0.809 ($p > 0.05$), which means there was no difference in the weight gain of the rat ovaries in all sample groups. The Mann-Whitney showed the same result with Sig $p > 0.05$ in all sample groups, showing no significant difference in the average weight of the ovaries between the sample groups.

The statistical analysis showed no significant difference in the average weight of the ovaries among the sample groups. It happened due to several factors, including different estrous phases among the rats. It influenced the weight of the rat ovaries.

Histological structures of the ovaries, for example, changes in weight of the ovaries, depend heavily on hormones. A different phase of the reproduction cycle has a different influence on hormonal activities. When all sample groups were compared at the estrous phase, the weight of the ovaries was as follows K1 = 0.10 K2 = 0.08, K3 = 0.09, K4 = 0.10, K5 = 0.10, and K6 = 0.06 (for the table on the weight of the ovaries or reproduction phase or sample, see the attachment). Based on the data (Table 1), during estrous, the weight of K1 (Control) ovaries was the same as that of K5 (γ-Oryzanol +Tr). The weight of the ovaries increases optimally during the metrestus and estrous phases. However, K6 or the sample group given RBO and transfluthrin had the lightest ovaries, 0.06 g.

In the estrus phase, also called the estrogen phase, there was an increase in estrogen by FSH, which stimulated the growth of selected follicles into de Graff follicles. It was followed by an increase in LH in preparation for pre-ovulation. At this stage, the estradiol produced by de Graff follicles would cause changes in the reproductive tract to its maximum. The anterior pituitary influenced follicular growth and development to increase FSH and LH secretion and then release estrogen [7], [8], [9]. Thus, the change in ovarian weight during the estrous phase was strongly influenced by hormonal factors such as an increase in estrogen and progesterone stimulation that began by increasing LH as preparation for pre-ovulation. It is in line with the opinion [10] that under normal conditions in the estrous phase; there were changes in the reproductive tract and an increase in LH secretion necessary for ovulation. LH would stimulate the production of PGF2alfa, and PGF2alfa would stimulate the collagenase enzyme from follicular cells. This condition played a role in brushing the follicle wall so that the ovum broke and left ovum as a process of ovulation [10].

Based on the measurement, see Table 1, the data shows that through the TNF-α concentration approach in K5 (γ-Oryzanol + Tr) was the same as K1 (Control), which was 1.27pg/mL. It showed that the administration of γ-Oryzanol inhibited the effects of free radicals caused by transfluthrin exposure. In contrast, the high TNF-α concentration in the estrous phase was related to the physiological function of TNF-α for preovulatory preparation. It is in line with [11] that TNF-α played a role in stimulating steroidogenesis by producing progesterone and stimulated apoptotic action in the process of follicular rupture [11], [12].

In K6 (RBO+Tr), the low ovarian weight was followed by a decrease in TNF-α concentration. In the estrous phase, the average TNF-α concentration in K6 was 1.14 pg/mL, the lowest concentration among the sample groups. A low concentration of TNF-α in the estrous phase may prolong the estrous cycle and inhibit the ovulation process. Thus, instead of the low level of TNF-α, the factor causing low ovarian weight in K6 was external factors. Low ovarian weight occurred due to the small number of mature follicles (tertiary follicles and de Graff follicles in the ovaries) [13].

The data in Table 1 and Figure 3 can be analyzed that the Foxo3a expression on K5 (γ-Oryzanol + Tr) and K6 (RBO + Tr) in the estrous and diestrous phases was quite strong.

Foxo3a was vital during the two (2) phases of the cell cycle in the estrous cycle. The first was the

estrous phase. Foxo3a would be activated in regulating apoptosis in follicles not selected to become de Graff. It was related to the preparation of preovulatory follicles. The second was the diestrus phase. Foxo3a played a role in recruiting several primordial follicles to be activated and then stimulated to grow and develop during the estrous cycle. During the transition from diestrus to proestrus, AKT activation occurred. Simultaneously, the phosphorylation inhibition process occurred in Foxo3a, and as a result, this inhibited transcription and apoptosis functions in the ovaries. In this condition, the primordial follicles would develop into primary and follow the development stage of the follicles during the estrous cycle to become pre-antral and antral follicles [4], [14]. Thus, Foxo3a did not have a direct role in stimulating ovarian weight gain but limiting the growth of primordial follicles. The more primordial follicles were activated in each estrous cycle, the shorter the reproductive capacity was.

The presence of data in Table 1 and Figure 4 can be analyzed that ovarian weight through the GDF9 expression level approach is associated with follicular growth and development and the proliferation of granulosa cells. In the estrous phase, GDF9 was influential in stimulating an increase of LH expression by secreting progesterone as negative feedback on the decrease of FSH [15]. Based on the measurement of GDF9 expression in K5 (γ-Oryzanol + Tr) and K6 (RBO + Tr) in the estrous phase, GDF9 was expressed quite strongly. However, the ovarian weight in K5 in the estrous phase was 0.11 g, while in K6 in the same phase was 0.6 g. It indicated that the antioxidant activity formed by γ-Oryzanol was able to maintain GDF9 expression. As a result, it was able to carry out its physiological function in the diestrous phase, namely increasing kit expression, so it bound to KL and stimulated phosphorylation and activation of AKT, an essential part of the cell cycle [14], [16]. At this stage, primary follicles that had not grown were formed and some experienced early growth. GDF9 expression directly affected granulosa cells experiencing proliferation and degeneration and stimulating pre-antral follicle growth by preventing apoptosis in granulosa cells [10].

Based on the explanation, there was a discrepancy in ovarian weight between groups of K5 (γ-oryzanol + Tr), with the average ovarian weight of 0.1 g, and K6 (RBO + Tr), with average ovarian weight of 0.06 g in the estrous phase. When the level of expression of GDF9 was sufficient, this condition means there was a discrepancy in the presence of γ-oryzanol administration compared to RBO on ovarian weight through increased GDF9 expression.

Different ovarian weights in K5 and K6 show discrepancy toward the growth of mature follicles (tertiary and de Graff follicles) [13]. An increase of γ-Oryzanol antioxidant activity was shown from the quite strong intense GDF9 expression capable of increasing kit expression that it could bind to KL and resulted in phosphorylation for activation of AKT, characterized by growth of follicles and proliferation, as well as granulosa cell degeneration [17].

In conclusion, the anti-inflammatory and antioxidant activity of γ-Oryzanol is stronger compared to RBO in increasing weight of the ovaries without being influenced by the decrease in TNF-α level, Foxo3a expression, and quite strong GDF9 expression.

Antioxidant and anti-inflammatory activities from the application of γ-oryzanol compared to RBO in influencing the growth of follicle (number of the follicle) through TNF-α level, Foxo3a expression, and GDF9 in rats (R. novergicus) exposed to transfluthrin

The results of the Kruskal Wallis statistical test on the number of follicles showed a Sig 0.328 ($p > 0.05$), meaning no difference in the mean scores of follicles among the sample groups. In addition, the results of the Mann Whitney test showed a Sig 0.052 in K2 (transfluthrin) and K5 (γ-oryzanol + Tr), indicating a significant effect of sample treatment on the number of follicles, see Table 1.

The growth of pre-antral follicles was higher than antral, and it explained that the primary follicle growth was 43% of the mean score of follicles. K2 (transfluthrin) had the highest number of follicles, followed by K3 (γ-Oryzanol) and K5 (γ-Oryzanol + Tr). On the other hand, in the RBO group, K4 (RBO) and K6 (RBO + Tr), the average follicular growth rate was lower.

The TNF-α concentration in K1 and K5 in the estrous phase (Mean = 1.27 pg/Ml) showed that follicle growth in K5 was higher than that in K1. The condition indicated that transfluthrin exposure did not lead to retardation in follicle growth in the group where γ-Oryzanol was applied. It was different from K6 (RBO + Tr) in the estrous phase, in which it had a lower TNF-α concentration (Mean = 1.14) followed by a low average of follicle growth. It means the antioxidant and anti-inflammatory potential of γ-Oryzanol was stronger than RBO. Transfluthrin exposure disrupted the histological structure of the ovaries. Molavi (2014) reported that the effect of cypermethrin exposure (one of pyrethroid derivatives besides transfluthrin) given to rats at a dose of 0.75 mg/BW for 24 days caused the loss of follicles and oocytes in the ovaries. It indicated that pyrethroid derivatives (transfluthrin or cypermethrin) as a class of EDCs led to disruption in the reproduction system.

Influence of γ-Oryzanol application compared to RBO toward the growth of follicles through Foxo3a expression as a parameter showed that K6 (RBO + Tr) had quite string intense expression (Mean = 114.02), higher than K2 (Mean = 111.08). In the estrous cycle, Foxo3a was integral in the estrous and diestrous phases. In the estrous phase, apoptotic activity occurred

in follicles not selected to be stimulated to become de Graff follicles. In contrast, in the diestrous phase, Foxo3a recruited primordial follicles to be stimulated to grow and develop into primary and secondary follicles entering the proestrus phase. According to the study results, Foxo3a was expressed stronger in the diestrus phase (Mean = 94,667), see Table 1 and Figure 3.

γ-Oryzanol and RBO antioxidant activities could counteract free radicals from EDCs having potentials toward granulosa cell apoptosis. In the pre-antral stage, granulosa cell stimulated kit development and FSHR in the granulosa cell surface, which functioned to stimulate the growth of follicle and oocyte through dimers in KL related to kit receptor resulting in the activation of kit and phosphorylation in PI3K-Akt [3], [4]. Foxo3a expressions depend heavily on the regulation of AKT activation influenced by the ligand and kit binding in granulosa cells. γ-Oryzanol and RBO showed their antioxidant activities in suppressing the effect of free radicals to grow primodial follicles through Foxo3a expression outside the estrous and diestrous phases.

The growth of follicles was affected by the level of GDF9 expression. Kit bonds and ligand in granulosa cells were stimulated by GDF9 paracrine communication with granulosa cells in the pre-antral stage. The intensity of the RBO group (K4 and K5) showed it was still quite strong, namely (Mean = 63–126), see Table 1 and Figure 4, which was the same as the group given γ-Oryzanol (K3 and K5). It means there was a phase difference in the groups. γ-Oryzanol with RBO did not show a significant difference to the level of GDF9 expression. It can be an indicator that the antioxidant potential of RBO was not able to stimulate an increase in GDF9 expression. The results of the GDF9 expression parameter are following the results of HE staining, which showed that follicular growth in the γ-Oryzanol group was higher than RBO.

Expression of GDF9 had an essential role in stimulating the growth of follicles and granulosa cells. The difference in follicle growth rates with the same intense expression of GDF9 (GDF9 was quite strong) on K3 and K4 indicated that the presence of a higher level of antioxidants by γ-Oryzanol affected the level of follicle sensitivity against GDF9 stimulation for follicles to grow and develop. Differences in the sensitivity of receptors RNA messenger in granulosa and theca cells to GnRH and 38-HSD receptors to enzyme steroidogenesis would affect follicle growth. Higher follicle growth conditions were also shown in K5 (γ-Oryzanol + Tr), which increased its endogenous antioxidant activity to neutralize the effects of free radicals from transfluthrin. The difference in the average follicle growth in K3 and K4 measured in the same phase (estrous), and the average follicle growth in K5 and K6 showed that antioxidant activity of γ-Oryzanol through GDF9 expression was able to increase follicle growth.

In conclusion, anti-inflammatory and antioxidant activity from the administration of γ-Oryzanol is stronger than RBO toward the growth rate of follicles through a decrease in TNF-α level, decrease in Foxo3a expression (proestrous phase), and increase of GDF9 expression.

RBO in affecting the number of follicular abnormalities through TNF-α level, Foxo3a, and GDF9 expression in rats (R. novergicus) exposed to transfluthrin

The result of the Kruskal Wallis test, analyzing the influence of the number of follicular abnormalities in the sample groups based on the reproduction cycle, showed that Sig 0.000 ($p < 0.05$). Furthermore, Mann Whitney showed that the sample groups had a significant influence on the number of follicular abnormalities.

K2 (transfluthrin) had the highest number of follicular abnormalitiessee Figure 1 and Figure 2, with a mean score of 19 (stdev = 9.05). Based on HE staining (Figure 2), types of follicular abnormalities identified in the sample group were: Kariolysis (KL), Ruptured Granulosa (RG), and Kariorection (KR). High follicular abnormalities that affected the preparation of the follicles for ovulation were the indicator of disrupted folliculogenesis (growth of follicles).

Implementation of one push inhalation with transfluthrin as the exposure caused toxicity effect on the reproduction organs [19]. The effect occurred through two (2) mechanisms, namely direct influence toward the cells and indirect influence through a biochemical reaction in cell metabolism [20]. In this study, the toxicity that had a direct influence on the cells was shown through disruption of follicular growth (folliculogenesis), which involved: high primary and primordial follicles and other follicles (such as atresia, ruptured/broken granulosa, cariorrexis, karyolysis, and pycnosis), see Figure 2.

Another type of follicle in karyolysis was the loss of the nucleus cells or the waning of the cell nucleus. In contrast, the cariorrexis in the nucleus was divided into several fragments. KL and cariorrexis were conditions in which granulosa cells are undergoing apoptosis [21]. Atrestic follicles or atresia occur due to degeneration of normal follicles characterized by picnosis, reduction of granulosa cells due to proliferation and basement membrane damage, or ruptured granulose [22].

In K3 (γ-oryzanol) and K4 (RBO), in which Sig 0.036 ($p < 0.05$), γ-oryzanol and RBO had a significant influence on the number of follicular abnormalities. Comparing between the influence of K3 and K4 toward follicle disruption, the average score of K4 (mean = 12, stdev = 3.22) was higher than that of K3 (mean = 1,

stdev = 1.22). It means the anti-inflammatory activity of γ-Oryzanol was stronger than RBO. γ-Oryzanol is potential in suppressing the inflammatory effect of transfluthrin exposure as it can repair the reproduction system, particularly folliculogenesis and ovulation.

Another factor was the environment exposed to transfluthrin and the role of γ-oryzanol compared to RBO to maintain folliculogenesis. K3 (γ-oryzanol) and K5 (γ-oryzanol + Transflutrin) were able to suppress the number of follicular abnormalities to the lowest compared to the other sample groups. Compared to K6 (RBO + transflutrin), K5 has stronger anti-inflammatory activities. It is in line with the *in silico* result that γ-oryzanol had a stronger inflammatory activity than antioxidant activity [6].

Another condition showed that γ-Oryzanol had stronger antioxidants. Under normal conditions, the follicle was stimulated to grow and develop during the proestrus phase until it reached its maximum size. The follicle growth in the proestrus phase was supported by an increase in FSH hormone [14]. Therefore, an increase in the number of abnormalities in the proestrus phase, particularly in K5 and KB or the groups die to transfluthrin exposure, indicated that transfluthrin exposure resulted in damage to the ovarian structure, and RBO administration was unable to inhibit this damage, which led to a high number of follicular abnormalities. It may cause the formation of follicular cysts resulting in nymphomania, a condition where the follicles continue to grow but are not released in ovulation so that the estrous cycle is irregular and continuous. In humans, it may cause menstrual cycle disorders and polycystic ovarian syndrome.

Based on the explanation above, the six (6) sample groups had various conditions during folliculogenesis, particularly in each reproduction cycles. This condition was controlled by genetics and modified by external factors such as exogenous hormone, weather, a steroid hormone, and nutrition [23], [24], including exposure to, transfluthrin with the EDCs effect. An attempt to prevent damage in the body is by having additional nutrients such as γ-Oryzanol and RBO that influences folliculogenesis.

An increase in the number of follicular abnormalities was followed by an increase in TNF-α concentration. In K2, an increase of TNF-α concentration and Foxo3a expression occurred during the metestrous phase. TNF-α concentration in K2 (transflutrin), compared to the other group in the same phase (metestrous) such as K4 (RBO) with TNF-α (1.20 pg/mL), was higher. The concentration of TNF-α in K2 was 1.26 pg/mL. The high concentration of TNF-α in the ovaries was the indicator of chronic inflammation [25]. This condition may prolong the luteal phase or in human menstrual cycle disorder.

In Table 1 that the group given γ-Oryzanol, K3 (γ-Oryzanol) and K5 (γ-Oryzanol + Tr) had higher TNF-α concentration compared to the group given RBO, K4 (RBO) and K6 (RBO + Tr). However, the group given RBO had higher follicular abnormalities. Based on Table 1 column 3, row 1, the average follicular abnormalities in K5 was 7 (standard deviation of 4.72), and that in K6 was 16 (standard deviation of 5.75). Furthermore, Table 1 column 3 and row 2, showed an increase of follicular abnormalities in the proestrus and metestrous phases, the two phases K2 and K6 had. Simultaneously, Table 1, column 3 and row 1 indicated that the highest TNF-α concentration in the sample took place in the diestrous, and the proestrus phase had the lowest TNF-α concentration. It can be concluded that there was an increase in TNF-α concentration in K2 and K6 during the proestrus phase.

In the proestrus phase, under normal conditions, TNF-α was not associated with the preparation for ovulation. As the effect, in this phase, the level of TNF-α concentration was low. An increase in TNF-α concentration in K2 and K6 indicated a potential disruption in the ovarian structure, including an increase in follicular abnormalities.

TNF-α facilitated stimulation of steroidogenesis by producing progesterone and stimulated apoptotic action in the process of follicular rupture [11], [12]. Thus, under normal conditions, the estrous phase had higher levels of TNF-α. Still, in the estrous phase, TNF-α was directed to preparation for ovulation through the secretion of LH hormonal function and was not associated with inflammatory follicular abnormalities. Thus, high concentrations of TNF-α outside the diestrous and estrous phases caused follicle growth disorders and the formation of follicles with abnormalities. In conclusion, there is a difference in the effect of anti-inflammatory activity on γ-Oryzanol (K5) compared to RBO (K6) exposed to transfluthrin toward an increase in the number of follicular abnormalities in the proestrus and metestrous phases. This condition is characterized by an increase in TNF-α concentration in the proestrus and metestrous phase.

Therefore, the following conclusions are drawn. (1) The increase in TNF-α concentration outside the estrous and diestrus phases indicated inflammation in the ovarian tissue, marked by an increase in the number of follicular abnormalities. (2) An increase in TNF-α in the estrous phase occurred in K3 and K5 was a physiological condition showing the role of TNF-α in the pre-ovulation stage. (3) The increase of TNF-α in K2 and K6 in the proestrus and metestrous phases showed the effect of increasing TNF-α on the increasing number of follicular abnormalities.

The role of Foxo3a in ovulation in the estrous phase was related to follicular apoptosis as a physiological response to unselected follicles to grow and develop into de Graff follicles. Based on the level of expression of Foxo3a in the group giving γ-Oryzanol, K3 (γ-Oryzanol) (Mean = 86.56) and K5 (γ-Oryzanol + Tr) (Mean = 112.7) and the group

giving RBO, K5 (RBO) (Mean = 112.7) and K6 (RBO + Tr) (Mean = 123.27), the following conclusions are inferred. (1) The intense expression of Foxo3a in K4 (RBO) was higher than K3 (γ-Oryzanol), (2). Transflutrin exposure results in an increase of Foxo3a expression. (3) The administration of γ-Oryzanol in an environment exposed to transfluthrin could inhibit increased expression of Foxo3a and reduce the number of follicular abnormalities.

Increased expression of Foxo3a was an indicator of transfluthrin exposure. Transflutrin as a derivative of pyrethroid compounds has EDCs effect. As a consequence, the use of mosquito repellents containing transfluthrin as the active ingredient continuously for a certain time can cause reproductive cycle disorders (including folliculogenesis, oogenesis, followed by ovulation) [18].

Pyrethroid derivative (EDCs) in the ovaries may cause hormonal and cellular disruption. Examples of the hormonal disruption the compound cause are gonadotropins disruption resulting in apoptosis characterized by abnormal follicular growth, while cellular disruption is damage in the mitochondria. As a result, the x-linked factor is unable to prevent damage in the ovaries [19], [26].

The condition shows RBO antioxidant and anti-inflammatory activity in suppressing the effect of transfluthrin exposure is lower than γ-oryzanol antioxidant and anti-inflammatory in protecting cells and tissue. It is shown in the intense expression of Foxo3a in the same reproduction cycle phase (the estrous phase).

The antioxidant activity of γ-oryzanol used in the study was measured using DPPH, showing that γ-Oryzanol was categorized as a moderate antioxidant and had strong anti-inflammatory activities higher than other types of antioxidants predicted through the *insilico* method previously [6].

The relationship between antioxidant and anti-inflammatory activities of RBO and γ-oryzanol was elaborated by [27] that antioxidant activity caused inhibition in Nuclear Factor-^{K}B, explaining the anti-inflammatory effect mechanism of γ-oryzanol. It is corroborated with [28] conducting an experiment on sample animals to identify the immunomodulatory ability of oryzanol (crude brain oil extract). The experiment showed that oryzanol has the potential to increase immune activity in terms of cellular and humoral mechanisms.

The increasing number of follicular abnormalities through the GDF9 expression approach based on Table 1, column 5,can be analyzed and described as follows. (1) All sample groups had quite strong GDF9, which fell into the intense expression range 126-63. (2) The group given γ-Oryzanol, K3 (γ-Oryzanol) and K5 (γ-Oryzanol + Tr), had lower expression of GDF9 than the groups given RBO, K5 (RBO) and K6 (RBO + Tr). (3) The expression of GDF9 in the group given γ-Oryzanol (K3) decreased when exposed to transfluthrin (K5). (4) The expression of GDF9 in the group given RBO increased once exposed to transfluthrin.

The level of GDF9 expression in the sample groups toward the number of follicles indicated increasing GDF9 expression stimulated the growth and development of follicles. However, an increase of free radical because of transfluthrin exposure resulted in various abnormalities (necrosis) in half of the growing and developed follicles (Figure 2), such as pycnosis (shrinking nucleus and condensation in the chromatin), cariorrexis (a condition in which the nucleus shrink into fragment), and karyolysis (a condition where nuclear chromatin disappeared due to DNA damage by degradation).

The findings of the present study contributed to the body knowledge on RBO as a functional food with antioxidant potential. RBO has a bioactive composition comprising ferulic acid, tricin, beta-sitosterol, gamma oryzanol, tocotrienol, tocopherol, and phytic acid, which can prevent oxidative stress by reducing damage to plasma lipids and blocking chronic inflammatory responses [29]. Based on the study results, the application of RBO is better compared to Poorna Chandar's γ-Oryzanol [30]. The ability of RBO and γ-Oryzanol in inhibiting free radicals shows the anti-inflammatory activity by modulating the expression of NF-κB p65, which ultimately results in decreased secretion of proinflammatory mediators by macrophages.

All in all, based on the application of γ-Oryzanol and RBO to environment exposed to transfluthrin, the following conclusions are presented. (1) There is a difference in the effect of anti-inflammatory activity of γ-Oryzanol (K5) compared to RBO (K6) on the grups exposed to transflutrin toward an increase in the number of follicular abnormalities in the proestrus and metestrous phases. This condition is characterized by the increasing TNF-α concentrations in the proestrus and metestrous phases. (2) There is a difference in the effect of antioxidant activity of γ-Oryzanol compared to RBO in reducing the number of follicular abnormalities by decreasing Foxo3a expression. (3) There is a difference in the effect of antioxidant activity of γ-Oryzanol compared to RBO in reducing the number of follicular abnormalities by maintaining GDF9 expression.

To sum up, anti-inflammatory and antioxidant activities from the γ-Oryzanol are stronger than RBO in decreasing TNF-α concentration and Foxo3a expression (outside the estrous and diestrous phase) and increasing GDF9 expression during the proestrus phase.

Conclusion

Application of the γ-Oryzanol through the decrease of TNF-α concentration and Foxo3a expression and increase of GDF9 expression can repair the histological structure of the ovaries from the effect of free radicals, one push transfluthrin exposure.

Acknowledgments

The researchers would like to thank all parties who have helped the completion of this study, promoters, and co-promoters at Faculty of Medicine-Universitas Brawijaya, Applied Laboratory of Universitas Padjajaran-Jatinangor, Physics Laboratory of Faculty of Mathematics and Natural Sciences-Universitas Brawijaya, Parasites Laboratory of Faculty of Medicine-Universitas Brawijaya, Animal House, Biomedical Laboratory of Faculty of Medicine-Universitas Brawijaya, Anatomical Pathology Laboratory of Faculty of Medicine-Universitas Brawijaya, Saiful Anwar General Hospital-Malang.

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