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The Effect of *Moringa oleifera* Leaf Extract on Theca Cell in Polycystic Ovary Syndrome Model with Insulin Resistance

L P Wulandari[1], A Hapsari[2)]
[1)] Midwifery Department, Politeknik Kesehatan Kemenkes Malang, Malang, Indonesia.
[2)] Public Health Department, Faculty of Sport Science, Universitas Negeri Malang, Malang, Indonesia.
Email: anindya.hapsari.fik@um.ac.id

Abstract: Polycystic ovary syndrome (PCOS) is known as the commonest endocrine disorder in women of reproductive age and often associated with insulin resistance, hyperandrogenemia, chronic inflammation, and oxidative stress. The use of *Moringa oleifera* as an antioxidant should be investigated as an alternative treatment of follicular refinement in PCOS with insulin resistance. We aimed to prove the effect of *Moringa oleifera* leaf extract in various dosages to decrease the theca cell thickness of PCOS female rat with insulin resistance. This study was a laboratory experimental research. Three month old *Rattus norvegicus stran Wistar* rat weighing 100-130 grams were divided into 5 groups (n = 8). PCOS model obtained by giving injection of testosterone propionate for 28 days, followed by metformin therapy and *Moringa oleifera* leaf extract at 250 and 500 mg/KgBW for 14 days. Then, we analyzed the thickness of theca cell. Statistical analysis used One Way Anova that was performed with IBM SPSS Statistics versions 24.00. The examination of ovarium histology showed that leaf extract *Moringa oleifera* 500 mg/KgBW (0.931±0.457) significantly decreased the thickness of theca cells ($p < 0.05$) compared to the PCOS control group. The conclusions was *Moringa oleifera* leaf extract as an antioxidant proven to decrease the thickness of theca cell of the female rat model of PCOS

Keywords: Moringa oleifera, Polycystic Ovary Syndrome, Theca Cell Thickness.

1. Introduction

Polycystic ovary syndrome (PCOS) is known as the commonest endocrine disorder in women of reproductive age[1]. It affects about 5 to 10% of women of reproductive age[2]. The definition of PCOS includes both clinical and biochemical criteria as well as ovarium morphology. Women with regular cycles and recognized hyperandrogenism could be part of this syndrome. Polycystic ovary syndrome during adolescence can be diagnosed when there were 2 of the following 3 classic features are confirmed[3]. These features are oligo/amenorrhea, androgen excess (can be seen by hirsutism), and polycystic ovaries [(1)].

People who are confirmed to have PCOS commonly associated with insulin resistance (50-70%), endometrial hyperplasia (35%) that 5-3 times could occur endometrial cancer, recurrent pregnancy loss (36-56%), obesity, and future metabolic disease [(1,2)]. Polycystic ovary syndrome have been regarded as a chronic systemic disease rather than a simple local disease, and it was often associated with chronic inflammation and oxidative stress (OS), although the pathogenesis has not been well defined.

Reactive Oxygen Species (ROS) that are found higher in PCOS' women because of oxidative stress, could induce the release of inflammatory factors and inflammatory responses through the activation of nuclear factor-kB (NF-κB), activated protein-1 and hypoxia-inducible factor-1. Further oxidative stress with inflammation could induce insulin resistance through post-insulin receptor signalling pathway that is insulin receptor substrate 1-phosphatidylinositol 3 kinase-protein kinase B pathway [(4)].

Insulin resistance will cause GnRH frequency and LH pulsation secretion to increase. This condition leads to hyperinsulinemia compensation and increased androgen production in the ovaries [(5)]. Then, ovarian theca androgen production will increase and production of SHBG by the liver decrease due to hyperinsulinemia [(6)]

In both mouse and human, normal androgen production of theca cells maintains follicular growth via promotion of early-stage folliculogenesis and prevention of follicular atresia. So, androgen excess will lead to abnormal follicular growth and infertility [(7)]. Patients with syndromes of severe insulin resistance often demonstrate ovarian hyperthecosis, a pathologic finding characterized by islands of hyperplastic luteinized theca cells located throughout the stroma and the presence of relatively few and small atretic follicles [(6)].

Metformin is the first line of PCOS obese treatment by inhibiting hepatic glucose absorption, increasing peripheral glucose uptake, reducing peripheral insulin levels, and improving GLUT-4. However, clinically it was finding long-term metformin treatment results with indigestion, diarrhoea, and other effects [(8)]. Metformin treatment might not be suitable for a long-term PCOS treatment.

The search for herbs that have potential capabilities as preventative and scientifically proven could be used for treatment alternative a much-needed. *Moringa oleifera* (drumstick tree, horse-radish tree, miracle tree) was known in Indonesia as one of the plants that contain antioxidants. Phytochemical studies of the *Moringa oleifera* plant that revealed large polyphenols such as quercetin glucoside, routine, kaempferol glycoside, and chlorogenic acid in *Moringa oleifera* flour via HPLC analysis [(9)]. Quercetin exhibits activity as an antioxidant by decreasing lipid peroxidation (MDA) and increasing antioxidant enzyme activity in STZ-induced diabetic-induced mouse mellitus [(10)].

In this study, we aimed to determine that giving *Moringa oleifera* leaf extract as an antioxidant could decrease the follicle repair in PCOS with insulin resistance. This plant was an original plant in various Asian countries, abundant and cheap as a food source. Thus, every health benefit of this plant will reach most of the population

2. Research Methodology

2.1. Plant material

Moringa oleifera commonly referred as the miracle tree that was a family of Moringaceas originating from Southern Asia. The leaves of this tree are rich in minerals, vitamins and other important phytochemicals [(11)]. Moringa oleifera extract (Kelorina, Moringa Indonesia, Blora, Indonesia) in powder form, all the process was done according to standard to obtain Moringa oleifera extract. The Moringa oleifera leaf was also used in several studies to determine its effectiveness in chronic hyperglycemia and dyslipidemia [(12)].

2.2. Animals and Experimental protocol

This study has been received approval ethical clearance letter of animal subjects from Faculty of Veterinary Medicine Universitas Airlangga with number 705-KE. This study was a laboratory experimental research. The female rat of Rattus norvegicus strain Wistar (Biochemistry Laboratory, Faculty of Medicine, Airlangga University, Surabaya, Indonesia) was 3 months old and weighed 100-130 grams. These rat were also used in research as animal models of diabetes [(13)]. Before the study began, there was a week period of adaptation to maintain the rats were in healthy condition, in normal behaviour and resulting in normal vaginal swab. We excluded rat with anatomical abnormalities and in pregnant during the adaptation. All procedures described were approved by the ethics committee of the Faculty of Veterinary Medicine of Airlangga University.

2.3. Treatment protocol

The white Wistar strain female rats (Rattus norvegicus) of 40 samples were divided into 5 groups randomly (n = 8), which were: negative control group (K1), positive control group (K2), and treatment groups (K3, K4, K5). Negative control group (K1) was only given aquades, while the other four groups were PCOS model. Preparation of PCOS with insulin resistance model was done by using testosterone propionate injection (Testohormon, Wonderindo Pharmatama, Jakarta, Indonesia). This hormone was given intramuscularly in the thigh with a dose of 1 mg/100grBW once a day for 28 days until PCOS-resistance insulin model obtained. Furthermore, the positive control group was (K2) given aquades only, the third group (K3) was followed by giving metformin therapy (2mg/100gBW, orally), the fourth and fifth group followed by giving Moringa oleifera extract orally (250 mg/KgBW for K4) and (500 mg/KgBW for K5) for 14 days. Before and after the study period, vaginal swab were done to

know what cycle was ongoing before and after the study. Before the animal was sacrificed, it has fasted for 12 hours and then ovarium removal were done to measure the thickness of the theca cell.

2.4. Estimation of histological parameters

The thickness of theca cells follicle was examined by HE staining (Hematoxylin-Eosin). Later on, HE was a colouring method widely used in tissue staining, aiming to make it easier to see changes in the tissue. The preparation of ovarium organs was coloured with a hematoxylin-eosin dye, so it could be clearly seen the shape of each cell. The coloured tissue was then placed on object glass which was covered with cover glass that has been previously spilled with entellan, then the tissue was observed under a microscope.

2.5. Statistical analysis

Normality test using Shapiro-wilk test and Levene test was used to know the homogenity of data. If distribution and homogenity of data were normal ($p>0.05$), One Way Anova test was conducted, followed with posthoc Bonferroni, but, if the data distribution or homogenity wasn't normal, Kruskall-Wallis test followed with Mann Whitney test, were conducted. All results were statistically analyzed using SPSS statistical software package version 24.0 (SPSS, Inc., Chicago, IL). The data were considered statistically significant at value $p <0.05$.

3. Result and Discussion

We conducted vaginal swab examination before and after the treatment.Vaginal swab before treatment showed that most female rats in all groups were in the estrous phase, none of which were in the diestrus phase in Figure 1. The results of the vaginal swab after treatment showed that there was a diestrus phase in the group receiving 1 mg/100grBW injection treatment intramuscular for 28 days in Figure 2

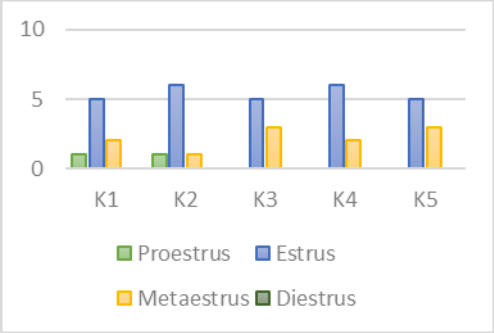

Figure 1 Graph of vaginal swab data of the experimental unit before treatment. The results of the vaginal swab in the experimental unit before treatment showed that most of the female rat in all groups were in the estrous phase, none of which was in the diestrus phase.

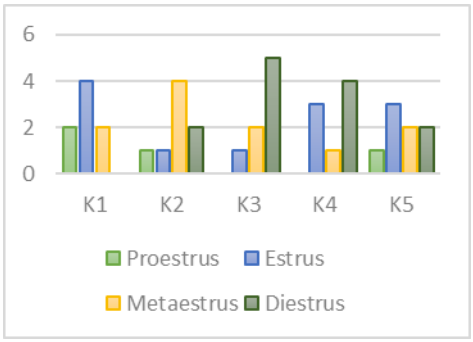

Figure 2 Graph of vaginal swab data of the experimental unit after treatment. The results of vaginal swab after treatment showed that there was a diestrus phase in the group treated with injection of Testosterone Propionate for 28 days (K2, K3, K4 and K5).

The results of measurements of theca cell thickness on ovary samples of control female rats and treated groups using the HE method in Figure 3 showed that the PCOS control group had higher cell-density cells than the other groups. The group treated with the leaf extract of Moringa oleifera at the dose of 500 mg/KgBW had a lower theca cell thickness than the other group in Figure 3. Metformin and Moringa oleifera leaf extracts showed the significant decrease in the thickness of theca cell ($p <0.05$) compared to the PCOS-insulin resistance control group in Table 1

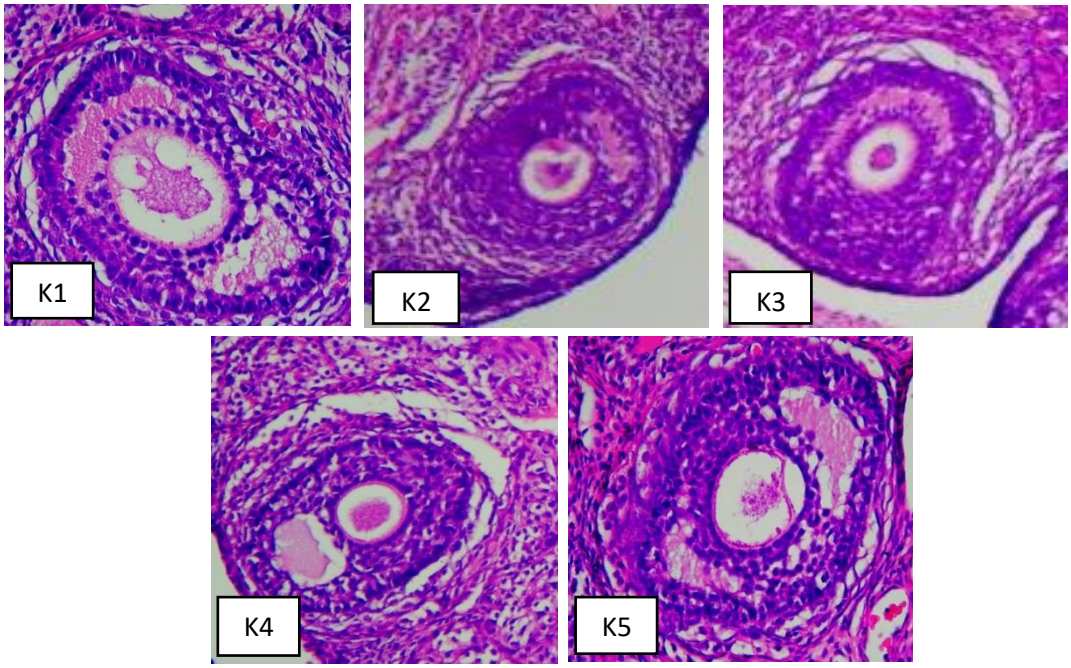

Figure 3 Graphs average follicle theca cell thickness of PCOS model (in µm). The highest covariate cell thickness was obtained in group 2 (PCOS insulin resistance control group), whereas the lowest cell thickness was obtained in group 5 (PCOS insulin resistance with *Moring a oleifera* leaf extract 500mg/KgBW group).

Tabel 1. Effect of Treatment on Histological Parameters

Sample	**Group**				
	K1	K2	K3	K4	K5
Theca Cell Thickness	1.573±0.551	0.000±.000*	1.950±0.577**	2.187±0.860**	0.931±0.457**

*significantly different from normal control ($p < 0.05$)

** significantly different from PCOS control-insulin resistance ($p < 0.05$)

K1: negative control group; K2: positive control group; K3: PCOS insulin resistance metformin group; K4: PCOS insulin resistance Moringa oleifera leaf extract 250mg/KgBW group; K5: PCOS insulin resistance Moringa oleifera leaf extract 500mg/KgBW group.

The PCOS model in this study increased the thickness of the theca cell. The PCOS control group significantly increased the thickness of the theca cells compared with the normal control group. Provision of Testosterone Propionate of 1 mg/grBW in female rats in this study for 28 days was able to increase tertiary theca cell thickness (7.77 µm) in PCOS control group compared to tertiary theca cell thickness (5.77 µm) of the normal control group).

Women with PCOS syndrome usually have an enlarged ovary with an increased number of follicles and volume of the stroma. The treatment of high-dose androgens causes suppression of gonadotropin, but their ovaries were not depressed but enlarged by the increasing number of "cystic" follicles and theca-interstitial hyperplasia, meeting the PCOS morphology criteria. These observations show that androgens could cause growth of ovarian and theca-interstitial follicles[14]. The previous studies whose using rat that was given testosterone injections for 28 days also showed a change in ovarian morphology including the presence of thickening of theca cells [(15)].

The Moringa oleifera extract could decrease the thickness of theca cells follicles in the PCOS model. The thickness of the tertiary theca cell follicles given Moringa oleifera leaf extract at doses of

250 mg/kgBW and 500 mg/kgBW, as well as the metformin group, significantly decreased the thickness of the theca cells compared to the PCOS group.

Additionally, giving Moringa oleifera leaf extract on a female mouse model of PCOS as antioxidant could decrease oxidative stress level. The reduction of oxidative stress might inhibit the release of inflammatory factors and the inflammatory response by inhibiting the activation of nuclear factor-kB, activated protein-1 and hypoxia-inducible factor-1 [(4)]. The decrease in inflammation might decrease induction of insulin resistance through post-insulin receptor signalling the pathway and the insulin receptor of substrate 1-phosphatidylinositol 3 kinase-protein kinase B pathway. Decreased insulin resistance in the peripheral tissue will lead to a decrease in androgen production in the ovaries itself. Decreased insulin levels and IGF-1 indirectly could also decrease androgen levels by increasing SHBG production. This androgen decline will affect the ovarium environment and morphological changes of the ovaries [(5)].

Furthermore, the drumstick tree was a rich plant in nutrients as well as macro, micronutrients, minerals, and vitamins. The nutrient content of the powder of the drumstick tree leaf was Vitamin A 16,3 mg / 100gr, vitamin C 17,3 mg / 100gr, vitamin E 113,6 mg / 100gr, flavonoid 473,3 mg / gr also selenium 0,9 µg / 100gr. Vitamin E was the most important fat-soluble antioxidant and protects against lipid membranes from oxidative damage. Vitamin E has a major function as a fat-soluble antioxidant and it was easy to provide hydrogen from the hydroxyl (OH) groups in the ring structure to free radicals. Vitamin E improves the potential for free radical defene systems and has a beneficial effect in the improvement of glucose transport and insulin sensitivity. Previous research conducted by Rzepczynska et al (2011) proves that administration of anti-oxidants (vitamin E) could improve the theca cell in rats induced by 17β estradiol [(16)].

Phytochemical studies of Moringa oleifera plants reveal large polyphenols such as quercetin, glucoside, routine, kaempferol glycoside, and chlorogenic acid in Moringa oleifera flour via HPLC analysis[9]. Study of antioxidant effects (routine flavonoids) in PCOS showed that there was an improvement in theca cell and oxidative stress cells in letrozole-induced rat [(17)].

We conducted vaginal swab examination before and after the treatment.Vaginal swab before treatment showed that most female rats in all groups were in the estrous phase, none of which were in the diestrus phase in Figure 1. The results of the vaginal swab after treatment showed that there was a diestrus phase in the group receiving 1 mg/100grBW injection treatment intramuscular for 28 days in Figure 2.

4. Conclusion

Moringa oleifera leaf extract as an antioxidant proven to decrease the thickness of theca cell of the female rat model of PCOS.

5. Thank You Note

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