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Decreased Insulin and Interleukin-6 Levels in *Rattus Norvegicus* Model of Polycystic Ovary Syndrome-Insulin Resistance Treated with a High Protein Diet

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Abstract

Hypercaloric in women of childbearing age is one of the causes of polycystic ovary syndrome (PCOS) with insulin resistance. Increased inflammation (tumor factor necrosis-α (TNF-α) and interleukin-6 (IL-6)) is the impact of endocrine and metabolic disorders involved due to metabolic syndrome, obesity, and diabetes mellitus. This study aims to evaluate whether a high-protein diet can reduce insulin and interleukin-6 levels, which are key indicators of polycystic ovary syndrome, and whether these changes can help in the treatment of the syndrome. This study used a prepost test only control design, true experimental, with a total sample of 6 female *Rattus norvegicus* rats aged 2–3 years (150–200 g). It consists of 4 groups, namely the negative control group (K-), the positive control group (K+), the treatment group (P), and the group without treatment (P-). The study examined the rat blood serum ELISA measurement of insulin and IL-6 levels. Anova test results of IL-6 levels (p = 0.002). Post-hoc test results of group K- and group P (p = 0.002), group K + and group P (p = 0.037), group K- and group K + (p = 0.437). The Anova test results of insulin levels between groups found a significant difference (p = 0.001). Post-hoc test of insulin levels of group K- and group P (p = 0.002), group K- and group K+ (p = 0.000), and group K+ with group P (p = 0.356). In group K and group P, the results of the ANOVA and post hoc tests on IL-6 levels and insulin levels with the provision of a high-protein diet show significant differences. This study suggests that a high-protein diet may be an effective therapeutic alternative in reducing the symptoms of polycystic ovary syndrome, especially in controlling high insulin and interleukin-6 levels.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a family of endocrine and metabolic disorders often found in women of reproductive age, characterized by follicular changes, anovulation, and menstrual cycle disorders. The prevalence of PCOS in women of childbearing age is about 6–15%, with insulin resistance (IR), and is found in 50–70% of PCOS patients (Cincione et al., 2021). Among these disorders, insulin resistance is an important mechanism that causes glucolipid metabolism and reproductive system dysfunction (Zhao et al., 2023). Obesity increases insulin levels, increases adipogenesis, reduces lipolysis, and increases ovarian androgen production. Ovarian hyperandrogens will worsen reproduction in PCOS patients (Barber et al., 2019). The positive correlation between hyperinsulinemia and androgen levels suggests that insulin contributes to hyperandrogenism in PCOS women (Zeng et al., 2020). The ovaries of PCOS patients usually respond to insulin secretion through the IGF-1 receptor. This binding occurs when insulin reaches high concentrations, which will stimulate tela cell proliferation and hypersecretion of LH levels that can mediate androgen secretion and increase the expression of LH and IGF-1 receptors. In addition, hyperinsulinemia inhibits the production of sex hormone-binding globulin (SHBG) by the liver, leading to increased levels of free testosterone and insulin globulin factor-binding protein-1 (IGFBP-1) (Paoli et al., 2020).

Excessive carbohydrate intake triggers inflammation and interacts with insulin resistance and hyperandrogenism to amplify PCOS (Ma et al., 2022). Acute hyperglycemia is known to increase inflammation such as Tumor necrosis factor -α (TNF-α), C-reactive protein (CRP), interleukin-4 (IL-4), interleukin-1 (IL-1) and interleukin-6 (IL-6) as well as oxidative stress through reactive oxygen species (ROS). Some of these inflammatory markers are cytokines that play a role in the reproductive system, stimulating the secretion of progesterone, estradiol, and follicular development (Butkowski & Jelinek, 2017). The proinflammatory cytokine IL-6 to cause insulin resistance, type 2 diabetes mellitus, and an association with metabolic changes in PCOS women (Amisi, 2022).

Obesity and insulin resistance are the same two factors associated with reduced fertility that can be improved with medication, exercise, and diet (Wondmkun, 2020). Non-pharmacological treatments with healthy lifestyle management, such as diet, exercise, and psychological modification, have been recommended as the first-line treatment for PCOS patients (Cowan et al., 2023). Dietary modification plays an important role in the treatment of PCOS patients (Mizgier et al., 2024). Some researchers state that the diet of PCOS patients tends to consume high calories or high carbohydrates and fat and has an influence on the increase of pro-inflammatory and lipid levels, in addition to promoting the development of several other diseases such as cardiovascular and metabolic syndrome (Goldenberg et al., 2021). Caloric restriction has been recognized as the dietary regimen of choice for PCOS patients according to dietary modification therapies that have been studied, such as low-fat diets, low-carbohydrate diets, pulse-based diets, high-potassium diets, ketogenic diets, and other dietary models (Gray et al., 2020). Then, a low-fat diet can effectively reduce obesity, lower insulin levels, and improve lipid metabolism (Mei et al., 2022). A typical diet with nutritional habits reduces the use of oils, nuts, seafood, fewer complex carbohydrates or higher simple carbohydrates, low fiber, and saturated fatty acids (Liu et al., 2017). This study aims to evaluate whether a high-protein diet can reduce insulin and interleukin-6 levels, which are key indicators of polycystic ovary syndrome, and whether these changes can help in the treatment of the syndrome.

METHODS

2.1 Animals and experimental protocols

The experimental unit in this study was a female white mouse model PCOS-RI (*Rattus norvegicus*), aged 3 months, weighing 100-200 grams. The white mouse was chosen because they have stable genetics, shorter reproduction life, short estrogen cycle, and easy to be handled. Previous research in 2020 also used the white mouse as a PCOS model with insulin resistance. Before the study began, an adaptation period was given for 1 week, with a health condition, normal behavior, and normal vaginal swab result. Exclusion criteria for the white mouse are anatomic abnormalities (ie, ears are injured or not intact, the tail is short or stump, one or all four legs are deformed, cannot stand, have sores on body parts, eyes are not clear) and pregnancy during the adaptation period. All of these procedures have been approved by the ethics committee of the Faculty of Veterinary Medicine, Airlangga University, Indonesia, No. 2. KE.062.04.2019 and the ethics committee of STIKES Husada Jombang, No. 002-KEPKSHJ on

December 6, 2024.

This research was conducted in a laboratory experimental type with a post-test-only control group design. The rat experiment was conducted with the PCOS model. Rats were divided into 3 major groups, namely the normal control group (K-), and PCOS-RI rats model given broiler standard food (K+), and the PCOS-RI rats model given a diet low carb high protein (P). This research was conducted in the laboratory of the Faculty of Veterinary Medicine, Airlangga University, Indonesia.

2.2 Material Protocols

For rat feed using the standard feed, namely standard broiler feed consisting of moisture content of 13%, protein 21.5–23.8%, fat 5, 0%, fiber 5.0%, ash 7, 0%, Ca 0.9%, and P 0.6%, with metabolic energy of 3025–3125 kcal/g,. In this study for the treatment group, feed pellets were made using corn flour, reflecting the low glycemic index content for carbohydrates with a composition of 40%, egg white containing protein with a composition of 30%, and fish oil containing omega-3 with a composition of 30% fat in Itziar (2010 as the composition of a polycystic ovary diet with insulin resistance (carbohydrates 40%, protein 30%, and fat 30% (Aryani et al., 2023). A low-carbohydrate, high-protein diet and standard broiler feed were fed for twenty days.

PCOS-RI modeling was obtained through a 0.1-ml testosterone propionate injection protocol intramuscularly once a day for 28 days (Aryani et al., 2023). Testosterone propionate was used to stimulate PCOS conditiona in *Rattus norvegicus* because another research in 2009 has proved that prolonged exposure to androgens (testosterone propionate) affects insulin resistence index and inflamation in PCOS model rat serum. The determination of the size of the rat sample in each group was calculated based on the formula of Lemeshow (Aryani et al., 2023). A significant increase in insulin resistance index in group that got testosteron propionate for 14, 21 and 28 days was higher than the controls. Before and after treatmnet, a vaginal swab was performed to see changes in the cycle due to tretament. On the 48rd day, the rats were termineted by ester anesthesia and neck dislocation, then the ovaries and blood sample were taken.

2.3 Biochemical assay for insulin and IL-6 levels

Before the experimental animals were terminated, they fasted for 12 hours for blood collection. IL-6 and age levels were measure from the blood taken through rats' blood vessels which are examined using enzyme linked immunosorbent assay (ELISA). ELISA is a labelled immunoassay that is considered the gold standard of immunoassays. The immunological test is very sensitive and is used to detect and quality substances, including antibodies, antigens, proteins. Blood IL-6 and AGEs was taken at the end of the study, the units obtained are ng/ml. The data scale is ratio. Measurement of IL-6 levels was carried out by ELISA examination using Germany Bioassay Kit No. E0177Mo. insulin level was carried out by ELISA examination using Termo scientific US and Canada Kit No 743021419.

2.4 Statistical analysis

This research data will be recorded in a data collection form that is specifically designed for this study, to observe IL-6 and insulin level in PCOS - RI rats model. First, the normality test (Kolmogorov Spirnov test) is carried out. When the distribution is normal, the ANOVA test or analysis of variance was used. However, if the distribution is not normal, the Kruskal Wallis non parametric test or Mann Whitney test will be carried out. Bonferroni Post Hoc test will be carried out when there was a significant difference in variable between groups. Statistical calculations will be performed using SPSS version 22.0 software tools.

RESULTS

Table 1 shows that the data is normally distributed ($p > 0.05$), then proceed with anova analysis to determine whether there are differences between groups, then proceed with post hoc analysis to find out which pair of means is the most different between the existing pairs of groups.

Table 1. Kolmogorov Smirnov normality analysis table

Variable	SD	*Mean*	Minimum	Maximum	*p*
IL-6 level	0,03	0,738	0,35	0,45	0,200*
Insulin level	3.14	14,63	10,59	23,62	0,200*

Source: *Primary Data, 2024*

Insulin and IL-6 levels analysis

Serum insulin levels were taken post-treatment; normality analysis stated a normal distribution with a value of p = 0.200 (table 1). Significant differences in insulin levels occurred between the control group and the treatment group as a result of the ANOVA analysis, with a significance level of p = 0.001. Serum IL-6 levels were taken at the time of post-treatment; normality analysis stated a normal distribution with a value of p = 0.200 (table). Significant differences in insulin levels occurred between the control group and the treatment group as a result of the ANOVA analysis, with a significance level of p = 0.00.Table 1.2 is below.

The post hoc analysis in Table 1.2 below shows that there is a significant difference between the K-group with K+ and the K-group with P, but there is no significant difference between the K+ group and the P group.

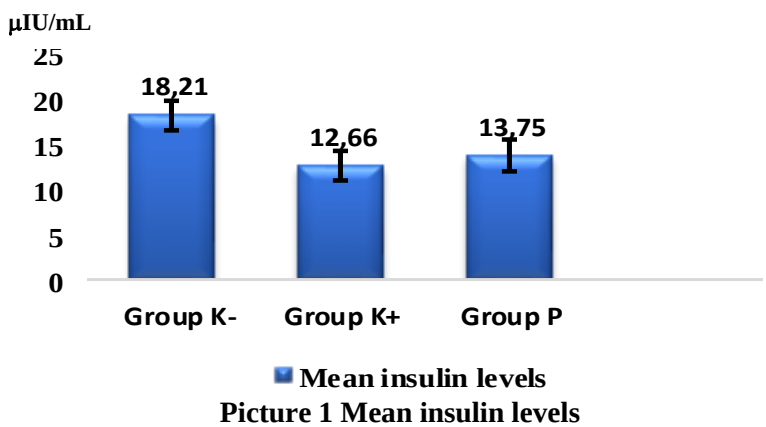
Table 2. ANOVA and Post Hoc analysis of insulin levels anda IL-6 levels

Variabel	Anova						*Post Hoc*	
				Confidence Interval 95%				
	Grup	*Mean* µIU/mL	SD	Lower limit	Upper limit	*P*	Group	*p*
Insulin level	1(K-)	18,21	2,71	15,36	21,06	0,001	K+	0,000 [a]
							P	0,002 [b]
	2(K+)	12,66	1,99	10,57	14,75		K-	0,000 [a]
							P	0,356[c]
	3(P)	13,75	0,76	12,95	14,56		K-	0,002 [b]
							K+	0,356 [c]
IL-6 level	1(K-)	0,358	0,20	0,33	0,37	0,002	K+	0,488 [a]
							P	0,002 [b]
	2(K+)	0,387	0,03	0,35	0,42		K-	0,488 [a]
							P	0,038[c]
	3(P)	0,441	0,42	0,39	0,48		K-	0,002 [b]
							K+	0,038 [c]

Source: *Primary Data, 2024*

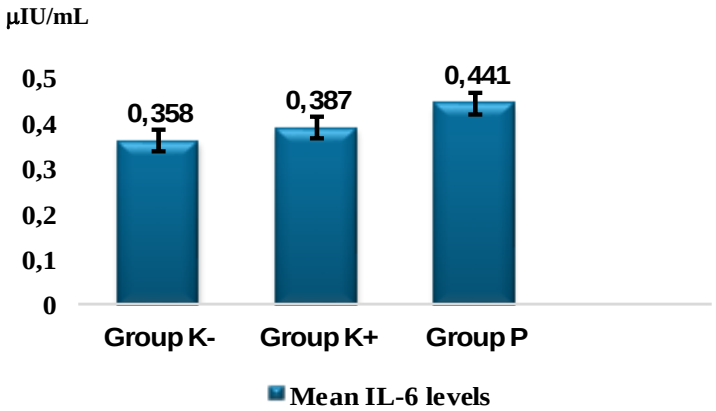
Table 2 shows the shows the results of the post hoc analysis of insulin levels comparison. There is the most significant difference or difference between group K-, with group K + p value = 0.000; group K- , and group P p value = 0.002. The results of the post hoc analysis in this study found insulin levels in groups K+ and P with a value of p = 0.356c, indicating there was no significant difference between groups.

Post hoc analysis of IL-6 levels comparison shows that there is the most significant difference or difference between group K- with a group 2 p-value of 0.000 and group K+ with a group P p-value of 0.038. The results of the post hoc analysis in this study found IL-6 levels in groups K- and K+ with a value of p = 0.488c, indicating there was no significant difference between group.



Picture 1 Mean insulin levels

Picture 1 above shows the lowest mean insulin levels in the K + group = 12.66 IU/mmL; but they are not much different from the mean insulin levels in group P = 13.75 IU/mmL with a difference of 1.09 IU/mL. However, it shows no significant difference in the post hoc analysis of group K + with group P.



Picture 2. Mean IL-6 levels

Picture 2 above shows the lowest mean IL-6 levels in the K + group = 0.387 IU/mL, but they are not much different from the mean IL-6 levels in group P = 0.441 IU/mL, with a difference of 0.05 IU/mL. However, but however it shows no significant difference in the post hoc analysis of group K + with group P.

DISCUSSION

The decrease in insulin levels in the control group and treatment group was found in the Anova analysis, but it was found in this study that insulin levels in the K+ group tended to be lower than group P. The increase in insulin levels in group P found in this study could be possible due to the intervention of giving TP injections for 28 days. Testosterone propionate (TP) injections increase androgens, induce testosterone, and disrupt the development of insulin and glucose metabolism. Directly giving Testosterone propionate injections causes an increase in IRS-1 to phosphorylate serine, causing a decrease in insulin sensitivity through GLUT-4 and triggering hyperinsulin (Hajam et al., 2024). The decrease in insulin levels in this study has not reached the normal limit of insulin, so it has not shown a significant difference between the K+ group and the P group.

The results of the post hoc test were conducted to compare treatments between groups. Group K-, with a group P p-value of 0.002, and group K+, with a group P p-value of 0.038, obtained significant differences in that the provision of a high-protein diet leads to improvements in suppressing proinflammation and does not stimulate ROS, which causes disturbances in the axis of the hypothalamic-pituitary central pathway. Improvement from diet and exercise on the inflammatory process through reducing adipocytes and releasing proinflammation (Scheffer & Latini, 2020). The benefits of a high-protein diet result in low CRP counts by the mechanism of the loss of adipocyte tissue while also providing a satiating effect that has the potential for weight loss (Moon & Koh, 2021). However, it is different in the K- group, with a K + p-value of 0.488, which states that there is no significant difference between these groups. It is possible that the administration of testosterone propionate in the study was not enough to significantly reduce IL-6 levels. By comparing the K-group with the P-group, the difference in increase was also not too much different. A high-protein diet has not been able to improve the state of oxidative stress by inhibiting the activity of the pro-inflammatory cytokine IL-6 involved in the development of hyperandrogen and hyperinsulinemia in PCOS women (Mancini et al., 2021). The novelty of this study is that it shows that a high-protein diet can reduce insulin and interleukin-6 (IL-6) levels in an animal model with PCOS-RI. These results indicate the potential of a high-protein diet as an effective therapeutic alternative for treating PCOS-RI and the limitations of the study. Although the animal model is similar to PCOS in humans, there are significant differences between animals and humans. The generalization of the study results to the human condition should be done with caution and consider different factors.

CONCLUSION

A high-protein diet can be used as a repair modality through inflammatory and glucose pathways. The high-protein diet in this study was favorable in improving IL-6 and insulin levels in insulin-resistant PCOS model mice.

SUGGESTION

Future research is expected to examine more complex nutrients, such as micronutrients.

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CONFLICTS OF INTEREST

The researcher declares that there is no conflict of interest in this research.

AUTHOR CONTRIBUTIONS

In this research, the authors fully contributed by presenting empirical evidence that a high-protein diet may be one of the dietary modality management strategies for PCOS women with insulin resistance. Through the research conducted, the authors provide new insights into alternative diets that can be used in dietary management for insulin-resistant PCOS women.

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