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Low Carbohydrate High Protein Diet Reduces Levels of Tumour Necrosis Factor-α (TNF-α) in Rats (*Rattus norvegicus*)

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Abstract

The purpose of this study is to explain the effect of a low-carbohydrate-high-protein (LCHP) diet on reducing TNF-α levels. The study was carried out with true experimental post-test only control design, using 3-month-old females rats (*Rattus norvegicus*) with a bodyweight of 200-300 g, which were divided into three groups: Negative control (K-) (rats without treatment were fed with broiler standard feed), Positive control (K+) (PCOS-IR model rats were fed broiler standard feed), and Treatment (P) (PCOS-IR model mice were fed with LCHP diet). Serum taken from the rats' heart on day 49 was examined for TNF-α levels using Bioassay Germany ELISA kit. Statistical analysis was carried out using ANOVA and Post Hoc tests. ANOVA analysis of TNF-α resulted in a p value of 0.0017, indicating significant difference of TNF-α levels when LCHP diet was applied, followed by Post Hoc test found that the TNF-α level of the treatment group ($p = 0.006$) is lower than the positive control group ($p = 0.038$). One of the roles of a low-carbohydrate diet in the pathogenesis of PCOS-IR is to provide an inflammatory repair effect by increasing insulin sensitivity, improving insulin levels, and stimulating an increase in steroidogenesis synthesis. Increased breakdown of fatty acids is inhibited, so that it does not cause a build-up of fatty acids and does not induce an increase in proinflammatory cytokines against gonadotropins, increasing FSH secretion and effecting follicular development.

Keywords: *low-carbohydrate-high-protein diet (LCHP), polycystic ovary syndrome, and TNF-α levels*

Introduction

Infertility is a problem, especially in couples who are in their reproductive years and have not yet borne and given birth to a child which could become perilous if proper treatment is not administered. Many things can cause infertility, one of which being PCOS. 5%-10% incidence of infertility in the world are caused by PCOS. PCOS often occurs in women of reproductive age, around 4-18% in the world. Lifestyle changes that increases the risk of PCOS, high calorie consumption, and low physical activity which amplifies the risk of obesity[1,2,3].

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Low carbohydrate diets (low glycaemic index) are proven to reduce body weight with no increase in lipolysis and increase in lipogenesis, thus increasing insulin sensitivity through GLUT-4 which will reduce insulin levels, increase SHBG in the liver, suppress the production of androgens in the ovaries and increase the aromatisation of testosterone in theca cells to estrogen in ovarian granulosa cells, inciting folliculogenesis. The addition of protein intake as a counterweight for other nutrients in a diet, where increasing the amount of protein intake can stimulate insulin secretion, benefits anabolism which causes satiety, stimulates glucagon secretion from α cells in the pancreas, and stimulates gluconeogenesis. Protein uptake through protein breakdown called proteolysis, will suppress lipolysis activity and increase insulin sensitivity through improvements in inhibiting

the cytokine TNF-α[4,5,6,7,8].

We suspected that a LCHP diet can improve TNF-α levels by reducing proinflammatory cytokines TNF-α which will inhibit the inflammatory pathway that will increase insulin sensitivity, suppress insulin secretion, and suppress ovarian androgen production which will affect the growth and development of follicles in the ovary[9]. This study was conducted to determine whether LCHP diet can reduce TNF-α levels in PCOS-IR rats. The purpose of this study is to observe and compare the effects of two types diet, namely LCHP and without a diet, on TNF-α levels which certainly changes inflammatory factors that have an effect on reproductive hormones in PCOS which until now remains unexplainable.

Materials and Methods

Research Design

This study is a true experimental laboratory study with a post-test only control group design, consisting of two control groups and one treatment group. The ethical eligibility test for this study was carried out by the ethics committee at Faculty of Veterinary Medicine, Universitas Airlangga with certificate number 2.KE.062.04.2019

Experiment Unit

This study used healthy 2-3 months old female rats (*Rattus norvegicus*) with a bodyweight of 100-200 grams prepared by the Embryology laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga, which was chosen as a model of PCOS-IR. Female rats were acclimated for 2 weeks to adapt and fed broiler standard feed. Rats were divided into 3 (three) groups, namely the negative control group (K-), the positive control group (K+) and the treatment group (P), each group consisted of 6 rats. The K-group did not receive treatment (normal) injected with placebo intramuscularly for 28 days and given broiler standard feed. The K+ rat group were induced by testosterone propionate (TP) as much as 0.1 ml intramuscularly for 28 days to act as a PCOS-IR model, and were given broiler standard feed. The P group of PCOS-IR models and given the LCHP diet. The LCHP diet is given for 20 days on an *ad libitum* basis.

Materials and Instruments

The materials used in this this research includes mice, broiler standard feed, testosterone propionate ampoules, aquadest, low-carbohydrate-high-protein pellet (LCHP) (consisting of 40% low glycaemic index carbohydrate from corn starch as a substitute for carbohydrate, 30% protein from egg whites, and cow oil as a substitute for fat containing omega-3). The instruments used includes experimental animal cages (plastic tubs), sipper water bottles, digital scales, cloths, blenders, flour sieves, microscopes, insulin spuits, 3 ml spuit, 10 ml measuring cups, object glass, label, rotary evaporators, spectrophotometer, 500 ml beaker glass, and bioassay Germany brand TNF-α ELISA Kit no. E0177Mo.

Table 1. LCHP feed composition (RKTP).

Nutrient	Ingredient	%
Carbohydrate	Corn starch	40%
Protein	Egg whites	30%
Fat	Fish oil	30%

PCOS-IR Inducement

Determination of the PCOS-IR rats model was done by means of a vaginal swab by wetting the cotton soaked and then inserted into the vagina and gently rotated, then smeared on the glass object. It is then fixated with 70% alcohol for 5 minutes. Furthermore, the object glass is dripped with Giemsa colouring, left for 2-3 minutes, and then washed with water and dried. Afterwards, it is examined under a microscope to determine the stages of the estrus cycle, namely proestrus, estrus, metestrus and diestrus. Vaginal swab was done to determine what cycle is taking place before testosterone administration. If the experimental animal is not in the metestrus or diestrus phase (showing anovulation) then the experimental animal is excluded. Testosterone (Wonder brand, PT. Wonderindo Pharmatama, Jakarta, Indonesia) is a hormone that is used to induce PCOS-IR models in this study. This hormone can be given intramuscularly or subcutaneously on the left thigh at a dose of 1 mg/100 gr BW. The volume that was injected into each rat was 0.1 cc/100 gr BW, given once daily for 28 days until the PCOS-IR model was obtained. In Belooseky *et al.* (2004), research on rats injected with testosterone 0.1 mg per day for 14 days showed an ovarian appearance of large follicular cysts with thickening of the stroma

and accumulation of preantral follicles coated on day 14. On day 16, the increasing number of preantral and antral follicles diminished as do preovulation follicles and corpus luteum number. Continued injection of testosterone until day 21 causes a 50% lower insulin ratio and subsequently decreases drastically describing the state of insulin resistance[10].

Data Analysis

Data were analysed statistically using SPSS. Analysis of normality was done with Kolmogorov-Smirnov. If the value of $p > 0.05$, the analysis was continued by ANOVA to determine the statistical difference between groups, and post hoc comparison testing to see more clearly the differences between each group.

Results and Discussion

Data normality analysis is performed to determine whether the research data obtained follows or approaches the normal distribution, i.e. distribution of data in the shape of a bell (bell shape). Data was tested for normality using Kolmogorov Smirnov in the control group and the treatment group (Table 1). The table above shows that the data are normally distributed ($p > 0.05$) which were then followed by the ANOVA test.

Tabel 1. Kolmogorov smirnov normality analysis.

Variable	Sample	Mean	Minimum	Maximum	*p*
TNF-α levels	18	45.65	36.85	54.13	0.064*

p= significance, * normally distributed $p > 0.05$

TNF-α levels analysis

Data on TNF-α levels taken at the end of the treatment is presented in table 5.4. The TNF-α level was tested for normality in all groups and it resulted in a significance value of 0.064 which indicated the data to be normally distributed, followed by the ANOVA analysis there were significant differences ($p = 0.017$).

Table 2. ANOVA analysis of TNF-α levels.

Variable	Group	Mean	SD	95% Confidence level		*p*
				Lower limit	Upper limit	
TNF-α levels	K-	50.43	3.09	47.18	53.67	0.017
	K+	44.48	5.53	38.67	50.28	
	P	42.06	4.63	37.19	46.92	

p= significance, SD= Standard deviation

Based on the table above, the average TNF-α levels at the end of the 8th week of treatment in the control group had the highest value compared to the treatment group and showed significant differences between groups ($p = 0.017$). The process of ovulation, fertilization and implantation of the embryo is influenced by TNF-α, as an important factor in the pathogenesis of PCOS. Higher androgen level increases TNF-α release from mononuclear

cells (MNC)[11]. This study endeavoured to determine the effect of the LCHP diet on TNF-α levels in biochemical parameters with the aim of reducing the production of proinflammatory cytokines and the accumulation of free fatty acids (FFA) in adipose and muscle tissue so as to suppress TNF-α hypersecretion in laboratory animals.

Post hoc analysis

Significant differences in TNF-α and insulin levels were observed between the three groups (p <0.05). The analysis was continued by using the post hoc analysis of least significant difference (LSD) as a test between groups in the TNF-α, insulin and free testosterone levels which can be seen in the table below.

Table 3. Least significant difference (LSD) analysis of TNF-α levels.

Variable	Group	Sample	Mean	p
TNF-α levels	1	K+	5.94*	0.038
		P	8.36*	0.006
	2	K-	-5.94*	0.038
		P	2.42	0.370
	3	K-	-8.36*	0.006
		K+	-2.42	0.370

p=significance, *mean difference with p<0,05

The table above shows the results of post hoc analysis of TNF-α levels in the control and treatment groups and significant differences were observed. The most difference can be seen between the positive control group (K +) and the treatment group (P) with a significance value of 0.038 and 0.006. Giving LCHP diet had an effect on reducing TNF-α levels in the treatment group.

Effect of treatment on tumour necrosis factor-α (TNF-α) levels

This study used laboratory animals to facilitate the management and supervision of the LCHP diet, given the limitations of supervision when done on humans. According to Szczuko *et al.* (2018), increased synthesis of TNF-α by visceral adipose tissue in PCOS women contributes to the development of glucose intolerance and type II diabetes mellitus. TNF-α will inhibit FSH synthesis, deactivate follicular growth, and correlate with the synthesis of dehydroepiandrosterone (DHEA) [11].

Retardation of TNF-α proliferation on a low-carbohydrate diet. This is due to the fact that glucose metabolism provides the right amount of ATP, which is needed for TNF-α proliferation. Reduction of fat mass in foods with low glycaemic index suppresses excessive adipocyte accumulation by increasing glycogen, lipid, and protein degradation, which are important in ovarian hormone-dependent malignancies, which often accompanies overweight women due to increased estrogen levels (androgen conversion by aromatase) in adipose tissue. Estrogen is responsible for the production of higher free radicals, which are mutagenic agents. In addition, increased TNF-α correlates with the occurrence of hyperinsulinaemia, insulin resistance and hypertriglyceridemia, which is produced by excessive amounts of body fat in patients with diabetes mellitus[13,14].

High protein intake can induce glucose tolerance, decrease insulin resistance, reduce TNF-α, leptin and FFA levels accompanied by a reduction in adiposity, decreasing fat mass so that muscles shrink because of the formation of fat accumulation in muscles and adipose tissue. In addition, there was an absence of oxidative stress increase and ROS formation. Therefore, protein can provide beneficial effects for the treatment of insulin resistance and type 2 diabetes caused by high fructose diets[13].

Conclusion

In summary, low-carbohydrate-high protein (LCHP) diet significantly reduce TNF-α levels with the p value of 0.0017 (p <0.05) which shows there is a significant effect. It can be inferred that the LCHP diet suppresses the inflammatory pathway which in turn is likely to improve the insulin signaling pathway.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Ethical Approval: This study was approved by the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6