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The Effects of A Short Period of A Ketogenic Diet on Blood Biochemistry and Immunological Status in Overweight Thai Subjects

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Abstract

Being overweight and obese is a fundamental health problem worldwide and is associated with many diseases. The ketogenic diet (KD) includes high fat and moderate protein, with the daily consumption of carbohydrates amounting to less than 10 % daily intake. KD is a popular choice for weight loss but has benefited many diseases, such as metabolic diseases and neurodegenerative disorders. Nonetheless, the effect of KD on overweight but healthy individuals is limited. Therefore, the objectives of the present study were to evaluate the effects of the KD program on the physical, blood biochemistry, hematological and immunological status of overweight, healthy individuals. The 9 overweight Thai women with body mass index (BMI) values over 25 kg/m^2 participated in a self-conduct KD program for 28 days. The mean age of the group was 28.44 years. With 28-days of KD, a significant ($p < 0.01$) increase in blood ketone confirmed the ketosis status of participants. The mean blood ketone concentration increased from 0.41 ± 0.061 to 1.22 ± 0.267 mmol/L. Most of the participants' physical parameters, such as body mass index (BMI), body weight, chest circumferences, hip circumferences, and arm circumferences, significantly decreased after 28-days of KD. Furthermore, the biochemistry indices (total cholesterol, triglyceride, LDL-C, and HDL-C) did not significantly differ from the baseline. However, fasting blood sugar decreased ($p < 0.01$) from 88.00 ± 1.014 to 83.78 ± 1.289 mg/dL. KD did not influence all hematological and immunological indices such as nitric oxide (NO) production (unstimulated and in 2.5 µg/mL PHA stimulation) and T cell subpopulations. Nonetheless, it increased NO production in 5 µg/mL PHA stimulation and mononuclear cell proliferation capacity (proliferation index). The data revealed that short-term KD in this study decreased participants' physical body compositions and blood glucose levels. Moreover, the immune status after KD proved the immunomodulation effect of KD.

Keywords: Ketogenic diet, Blood biochemistry, Immunological status, Overweight, Obesity

Introduction

During the past 30 years, the incidence and prevalence of overweight and obesity have progressively increased worldwide. The body mass index (BMI) ranging from 25.0 to 29.9 kg/m^2 refers to being overweight, while BMI exceeding 30 kg/m^2 suggests being obese [1]. World health organization (WHO) reported that about 1.9 billion people were overweight and 650 million obese worldwide in 2016 [1]. The actual cause of obesity is a sedentary lifestyle and poor dietary habits, while the second reason is endocrine and genetic disorders [2]. Being overweight and obese may cause hypertension, metabolic syndrome, respiratory diseases, diabetes, cardiovascular diseases (CVDs), non-communicable diseases, autoimmune diseases, cancers, digestive diseases, neurodegenerative diseases, prostate diseases, metabolic syndrome, and mental disorders [3].

The previous retrospective study in the United States (from 2001 - 2016) showed that the annual budget need for obese adults was 100 % more than that for other adults [4]. Many strategies may include losing weight in the short term, such as modifying lifestyle, bariatric surgery, liposuction, medicine, exercise, and restricted diet intake [5,6]. Nevertheless, some weight loss methods may cause adverse effects, such as physical pain, homeostatic imbalance, impaired immune and renal function [6,7].

Currently, people are more interested in consuming healthy food, explicitly resorting to low carbohydrate diets to lose weight [8]. Some of them are the South Beach diet, Atkins diet, and Ketogenic dietary patterns [9]. The Ketogenic Diet (KD) includes consuming a high fat, moderate protein, and low carbohydrate [10-13]. Dr. Russell Morse Wilder coined the term in 1921 to describe foods increasing levels of body blood ketone by higher fat and lower carbohydrate intake [9]. Previous studies have shown varied benefits of KD, including diabetes mellitus control, increased insulin sensitivity, and reduced blood cholesterol levels by decreasing insulin use and fat deposition in the body [14]. Besides its positive effect on weight loss or insulin sensitivity, the ketogenic diet may benefit psychological and neurological disorders such as schizophrenia, Parkinson's disease, Alzheimer's disease, and seizure [15].

Limited research has addressed the effect of KD on the immune system. The current research with KD essentially includes athletes, experimental animals, and pathological conditions [10,13,16-19]. Additionally, no research has investigated the effect of KD on ordinary people, especially Thai people. Therefore, the objective of this study was to assess the impact of the ketogenic diet program on the body mass index (BMI), waist and thorax circumference, blood biochemistry, and blood immunological system of healthy but overweight individuals.

Materials and methods

Schematic overview of the experimental program

Those interested in getting training on keto and project details signed up for it. Nine individuals signed the consent form. Volunteers were provided with knowledge and advice on the food items before participating in the project. The suggested food items in this study were fried eggs, stir-fried pork with kale, and stir-fried basil with pork. The participants had to know the foods allowed and avoided throughout the study. The demographic, baseline physiological data, blood chemistry, and immunological status data were also available. The physiological data contained weight, height, chest circumference, waist circumference, hip circumference, arm circumference, and thigh circumference. After fasting overnight, 30 mL of vein puncture blood was collected for blood chemistry and immunological testing. Three milliliters of sodium fluoride (NaF) blood was for blood glucose, 5 milliliters of clotted blood for cholesterol, triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) tests. Five milliliters of EDTA blood was for blood ketone, complete blood count (CBC), and T cell sub-population tests. Twenty milliliters of heparinized blood were reserved mononuclear cells for *ex vivo* nitric oxide production and proliferative response capacities tests. The subjects ate a ketogenic diet under the advice of experts for 28 days (4 weeks). After 4 weeks of ketogenic diet course intervention, the physiological examination, blood biochemistry testing, and *in vitro* and *ex vivo* immunological testing were conducted. The overview of the experiment is shown in **Figure 1**.

Participants

In this study, 9 Thai women volunteers were healthy individuals with a mean age of 28.44 years (ranged between 21 and 46 years) with a body mass index (BMI) exceeding 25 kg/m^2. They were personnel or students of Walailak University, Nakhon Si Thammarat province, Thailand. All participants gave informed before any measurements. The experimental protocol was per the ethical standards outlined in the declaration of Helsinki in 1975 and approved by the Office of the Human Research Ethics Committee of Walailak University (Approval number WUEC-19-116-01, Approval date July 18, 2019).

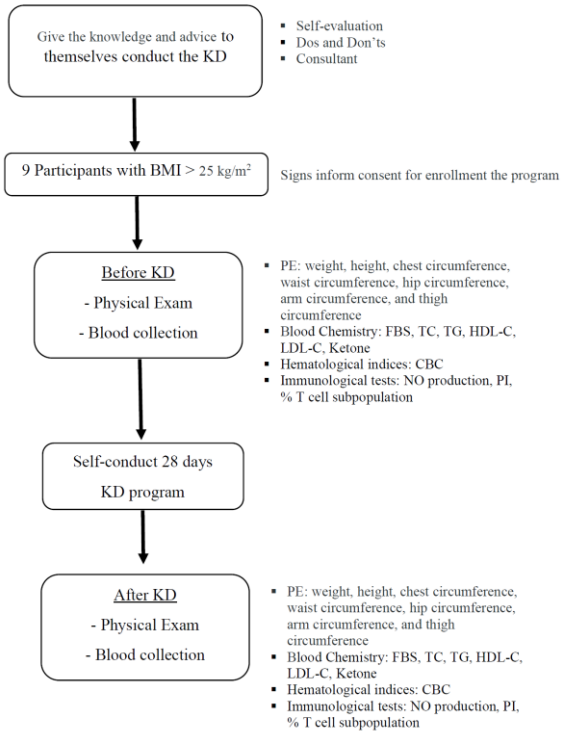

Figure 1 Schematic overview of the experimental program.

Blood biochemistry and hematology determination

The tube of heparinized and clotted blood was centrifuged. Then, the serum was gathered for testing fasting blood sugar (FBS), total cholesterol, triglyceride, HDL-C, LDL-C by BS-800 Mindray™ blood chemistry automation (Mindray Medical International, Shenzhen, China) available in Walailak University Medical Technology Laboratory (WU-MeT), Walailak University, Nakhon Si Thammarat, Thailand. The whole EDTA blood was utilized for the blood ketone test. Testing was accomplished by a portable point-of-care (POC) blood ketone meter MultiSure Ketone Monitoring System (APEXBIO, Hsinchu, Taiwan).

The complete blood count (CBC) was gauged by a BC-5390 Mindray™ hematology analyzer (Mindray Medical International, Shenzhen, China).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by the Ficoll gradient centrifugation method [20] from blood samples in heparinized tubes. Isolated cells were counted, and the cell viability was assessed by trypan blue staining. One hundred microliters of 2×10^6 cells/mL of viable PBMCs were cultured in RPMI 1640 culture medium (Gibco, Thermo Fisher, Waltham, MA, USA) having 10 % fetal bovine serum (Merck, Darmstadt, Germany) and 1 % penicillin-streptomycin Gibco™ (Thermo Fisher Scientific, Massachusetts, USA).

Quantitative analysis of nitric oxide production

One hundred microliters of 2×10^6 cells/mL of PBMC were inoculated into RPMI 1640 culture medium (Gibco, Thermo Fisher, Waltham, MA, USA) having 10% fetal bovine serum (Merck, Darmstadt, Germany) and 1 % penicillin-streptomycin Gibco™ (Thermo Fisher Scientific, Massachusetts, USA). A 100 µg/mL lipopolysaccharide-induced the cells (Merck KGaA, Darmstadt, Germany) incubation at 37 °C, 5 % CO_2 for 24 h pursued. The amount of nitrite in the cultured medium was gauged by the Griess reagent (1 % sulfanilamide and 0.1 % naphthylethylenediamine in 2µ.5 % phosphoric acid). The absorbance measurement was at 570 nm with a microplate reader. A standard nitrite curve gave the amount of nitrite in the sample.

Cell proliferation assay

A total of 2×10^6 cells/mL of PBMC in complete RPMI 1640 medium were grown in 96 well plates and enriched with 5 µg/mL PHA and incubated at 37 °C, 5 % CO_2 for 48 h. After incubation, 3-(4-5-dimethylthaizolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (Merck, Darmstadt, Germany) was added, and the cells were incubated at 37 °C for 4 h. Then, 100 µL of dimethyl sulfoxide (DMSO) was added, and samples were mixed thoroughly by recurring pipetting and incubated at room temperature in the dark for 2 h. The absorbance wavelength was 570 nm measured by a Thermo Scientific™ Multiskan™ GO microplate reader (Thermo Fisher Scientific, Massachusetts, USA). A direct correlation between the absorption value and cell number existed. Each sample was analyzed thrice, and controls and unstimulated blanks were present. The proliferation index was increased in cell number in the culture throughout the experiment.

Determination of T cell subpopulation

Fifty microliters of EDTA blood were utilized for 10 µL of BD Tritest CD4/CD8/CD3 (Becton Dickinson and Company, New Jersey, USA) in a 5 mL Falcon™ polystyrene tube (Becton Dickinson and Company, New Jersey, USA). The method was followed depending on the manufacturer's manual. The BD FastImmune™ γ1 PE/CD45 PerCP control (Becton Dickinson and Company, New Jersey, USA) reacted with the negative control tube. Samples' analyses were performed by flow cytometry. Cytotoxic T (Tc) cells were $CD3^+CD8^+$, while helper T (Th) cells were $CD3^+CD4^+$.

Fifty microliters of a buffy coat of EDTA blood were mixed with 10 µL of isotype control (PE-Cy™ 7 Mouse IgG1κ isotype control, Becton Dickinson and Company, New Jersey, USA) and a cocktail of human regulatory T cells (CD4/CD25/CD127) (Becton Dickinson and Company, New Jersey, USA) antibody in each sample in the 5 mL Falcon™ round-bottom polystyrene tube (Becton Dickinson and Company, New Jersey, USA). The regulatory T (Treg) cells' analyses ($CD4^+CD25^{bright}CD127^{dim}$) were by BD FACSCalibur™ flow cytometry (Becton Dickinson and Company, New Jersey, USA).

Statistical analysis

Data were analyzed using the SPSS software version 18.0, and the values were given as mean ± SD. The normality for the dependent variables was tested using the Kolmogorov-Smirnov (K-S) goodness of fit test. Moreover, the Wilcoxon matched-pairs signed-rank test was used to explore the difference in participants' corresponding values before and after the KD program. Data included body mass index (BMI), blood biochemistry indices, hematological parameters, phagocytic activity, nitric oxide (NO) production, proliferation index, and percentage of T cell subpopulations. The Type 1 error rate (α) was 0.05, meaning that *p*-value less than or equal to 0.05 suggested statistical difference (significance).

Results and discussion

Physical indices

The participants' values before and after the ketogenic diet program were compared. Statistical significance ($p < 0.05$) existed in most physical indices, such as BMI, body weight, chest circumference, hip circumference, and arm circumference. Although waist circumference and upper thigh circumference did not decrease significantly, both values tended to decrease like other physical indices, as depicted in **Table 1**. Our results were in line with those of Hyun-seung Rhyu and Su-Youn Cho (2014), who researched the effect of ketogenic diets on taekwondo athletes. They noted that taekwondo athletes lost their weight, % body fat, BMI, and lean body mass within only 3 weeks [21]. The possible mechanism for this weight loss was the diuretic effect of a ketogenic diet. Some participants' early weight loss was due to water loss, in the beginning, pursued by a fat loss. In the first 2 weeks or less, people on a ketogenic diet initially lost up to 10 lbs [8].

Table 1 Physical index of participants before and after the ketogenic diet program.

Physical index	Mean ± SD		*p*-value
	Before	After	
BMI (kg/m^2)	28.38 ± 5.27	27.37 ± 4.89	< 0.01*
Body weight (kg)	72.68 ± 14.18	69.89 ± 13.46	< 0.01*
Chest circumference (inches)	38.11 ± 4.29	36.94 ± 4.13	0.03*
Waist circumference (inches)	33.83 ± 3.62	32.39 ± 4.12	0.06
Hip circumference (inches)	42.50 ± 4.36	40.94 ± 4.50	0.02*
Arm circumference (inches)	12.44 ± 1.79	11.44 ± 1.49	0.03*
Upper thigh circumference (inches)	21.00 ± 2.46	20.28 ± 1.92	0.06

BMI body mass index, kg/m^2 kilogram/square meter, SD standard deviation, $^*p < 0.05$ statistically significant difference

Biochemical indices

Even though most of the biochemistry indices for the participants before KD were normal, 3 participants had hypercholesterolemia (Participant No.2 and No.5 had 239 and 210 mg/dL, respectively). Two other participants had a slightly higher blood ketone (Participant No.3 and No.7 had 0.8 and 0.6 mmol/L, respectively). However, no participants had a diagnosed metabolic disease by a physician before. After 28-day KD self-conduct, the blood biochemistry indices were determined and compared to those before KD. **Table 1** presents the physical indices of participants before and after KD. The mean blood ketone value for the participants significantly increased, suggesting the ketosis of participants ($p < 0.01$). Moreover, no significant difference ($p > 0.05$) existed in all lipid profiles (total cholesterol, triglyceride, HDL-C, and LDL-C), but FBS significantly decreased with KD ($p < 0.01$). Nonetheless, blood cholesterol, triglyceride, and LDL-C levels tended to decline, while HDL-C levels tended to increase compared to the baseline. The biochemistry indices of participants are in **Table 2** and the average indices are in **Table 3**.

Table 2 The individual biochemistry indices of 9 participants before and after ketogenic diet.

No.	FBS (mg/dL)		Cholesterol (mg/dL)		TG (mg/dL)		HDL-C (mg/dL)		LDL-C (mg/dL)		Blood ketone (mmol/L)	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	87	80	198	176	102	68	52	53	122	119	0.3	2.2
2	92	89	239	218	61	84	49	51	181	160	0.4	0.9
3	92	87	170	163	156	103	67	69	76	88	0.8	2.7
4	84	78	187	181	120	70	65	67	87	80	0.2	1.8
5	90	86	210	198	102	76	56	66	116	133	0.4	0.6
6	86	81	172	141	37	43	49	51	112	85	0.3	0.7
7	86	83	185	218	109	66	42	67	117	135	0.6	0.8
8	90	88	198	190	102	115	56	49	116	128	0.3	0.6
9	85	82	143	140	58	133	52	41	87	85	0.4	0.7

mg/dL milligram/deciliter, mmol/L millimole/liter, FBS fasting blood sugar, TG triglyceride, HDL high-density lipoprotein, LDL low-density lipoprotein

Table 3 Biochemical assay of participants compared before and after ketogenic diet program.

Biochemical index	Mean ± SD		Normal range	*p*-value
	Before	After		
FBS (mg/dL)	88.00 ± 3.04	83.78 ± 3.78	70 - 115	< 0.01*
Cholesterol (mg/dL)	189.11 ± 27.13	180.56 ± 29.02	< 200	0.13
Triglyceride (mg/dL)	94.11 ± 36.35	84.22 ± 27.94	35 - 160	0.43
HDL (mg/dL)	54.22 ± 7.90	57.11 ± 10.20	38 - 40	0.36
LDL (mg/dL)	112.67 ± 30.54	112.56 ± 28.80	< 130	0.97
Blood ketone (mmol/L)	0.41 ± 0.18	1.22 ± 0.80	< 0.6	< 0.01*

mg/dL milligram/deciliter, mmol/L millimole/liter, FBS fasting blood sugar, TG triglyceride, HDL high-density lipoprotein, LDL low-density lipoprotein, SD standard deviation, *$p<0.05$ statistically significant difference

Blood biochemistry indices, cholesterol, triglyceride, HDL-C, LDL-C, and blood ketone were evaluated before and after KD. The values of biochemistry indices indicated that cholesterol, triglyceride, HDL-C, and LDL-C values before and after KD did not differ ($p > 0.05$). However, blood ketone values increased from 0.41 - 1.22 mmol/L after 28 days of KD ($p < 0.01$). Ketone bodies generally occur in people observing intermittent fasting (IF) or a ketogenic diet, a biochemical model of fasting [22]. When you eat a lot of fat and less than 50 g of carbohydrates per day, decreased insulin secretion results in. Afterward, the body enters a catabolic state; then glycogen is converted into less complex sugars but not enough to force the body to break down fat. The liver produces more ketone bodies as an alternative energy source to supplant glucose [23], leading to ketone bodies' accumulation in the blood [22]. However, the production of ketone bodies relies on many factors, including basal metabolic rate (BMR), body mass index (BMI), and body fat percentage [8]. The blood glucose levels (FBS) after KD were significantly lower than before KD ($p < 0.01$). It was in line with a study investigating the effects of exogenous ketone feeding for 28 days in Sprague-Dawley rats which revealed the reduced glucose levels and little change in lipid biomarkers compared to control animals [24].

Complete blood count indices

Data showed insignificant differences in all complete blood count indices ($p > 0.05$) such as white blood cell (WBC) number, red blood cell (RBC) number, hemoglobin (Hb) concentration, hematocrit % (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet number, and the percentage of WBC differentiation (neutrophil, lymphocyte, monocyte, eosinophil, and basophil). Moreover, the results revealed that the 28 days KD program did not influence hematological indices (all $p > 0.05$). These data are discernible in **Table 4**.

Table 4 Hematology index of participants compared before and after ketogenic diet program.

CBC index	Mean ± SD		Normal range	*p*-value
	Before	After		
WBC (cells/μL)	7,085.56 ± 2,003.98	6,475.89 ± 1,968.71	4,000 - 10,000	0.25
RBC (10^6 cells/μL)	4.87 ± 0.44	4.81 ± 0.40	3.50 - 5.50	0.10
Hb (g/dL)	13.49 ± 1.48	13.41 ± 1.23	11.0 - 16.0	0.63
Hct (%)	40.19 ± 4.36	39.31 ± 2.97	37.0 - 54.0	0.76
MCV (fL)	82.89 ± 7.10	82.00 ± 5.96	80 - 100	0.33
MCH (pg)	27.22 ± 2.11	27.56 ± 2.01	27 - 34	0.34
MCHC (pg/dL)	32.78 ± 0.97	33.56 ± 1.01	32.0 - 36.0	0.56
Platelet (cells/μL)	266,777.78 ± 52,528.03	248,555.56 ± 48,260.00	100,000 - 300,000	0.18
Neutrophil (%)	58.22 ± 3.83	59.44 ± 3.36	50 - 70	0.64
Lymphocyte (%)	33.78 ± 3.42	34.11 ± 3.66	20 - 40	0.80

CBC index	Mean ± SD		Normal range	*p*-value
	Before	After		
Monocyte (%)	5.33 ± 1.12	4.44 ± 2.01	3.0 - 12.0	0.14
Eosinophil (%)	2.56 ± 1.01	1.78 ± 1.09	0.5 - 5.0	0.22
Basophil (%)	0.11 ± 0.33	0.22 ± 0.44	0.0 - 1.0	> 0.99

WBC white blood cell, RBC red blood cell, Hb hemoglobin, Hct hematocrit, MCV mean corpuscular volume, MCH mean corpuscular hemoglobin, MCHC mean corpuscular hemoglobin concentration, µL microliter, dL deciliter, pg picogram, SD standard deviation $^{*}p < 0.05$ statistically significant difference

No significant difference with KD existed in WBC number, RBC number, Hb, % Hct, MCV, MCH, MCHC, and percentage of WBC differentiation ($p > 0.05$). Hence, the ketogenic diet did not change in quantity and types of white blood cells and red blood cells in this study. Surprisingly, it was noticeable that the average platelet number decreased from 266,777.78 - 248,555.56 cell/µL, but the decrease was not statistically significant ($p > 0.05$). The previous study of Berry-Kravis and colleagues (2001), who collected the evidence that children treated with the ketogenic diet for epilepsy control, showed 1.4 % of participants (16 of 51 patients) bled [25]. Minor bleeding could occur due to changes in the structure or amount of lipids, the essential component of the platelet membrane. Then, the proteins embedded in the membrane (membrane-embedded proteins) could also malfunction, leading to a small amount of bleeding and may decrease the total platelet number.

Immunological status indices

Before and after the KD, immunological tests indicated significant differences for nitric oxide (NO) production in 5 µg/mL PHA and the mononuclear cell proliferation capacity (shown as proliferation index; PI) having $p = 0.02$ less than 0.05. Nonetheless, other immunological indices, including NO production (both unstimulated and in 2.5 µg/mL PHA), the percentages of T cell subpopulations (T regulatory, T helper, and T cytotoxic cells) were insignificant ($p > 0.05$). The data are in Table 5.

Table 5 Immunological indices of participants compared before and after ketogenic diet program.

Immunological index	Mean ± SD		*p*-value
	Before	After	
NO production unstimulated (µM)	3.89 ± 0.83	2.69 ± 2.03	0.11
NO production in 2.5 µg/mL PHA (µM)	7.50 ± 2.17	6.96 ± 2.06	0.55
NO production in 5 µg/mL PHA (µM)	3.65 ± 0.63	8.89 ± 5.46	0.02*
Cell proliferation capacity (PI)	0.87 ± 0.32	2.25± 0.58	0.02*
T regulatory (%)	8.85 ± 1.60	8.85 ± 2.63	> 0.99
T helper (%)	49.79 ± 7.54	47.9 ± 7.14	0.74
T cytotoxic (%)	35.40 ± 6.39	36.16 ± 5.78	0.92

NO nitric oxide, PHA phytohemagglutinin, µg/mL microgram/milliliter, µM micromolar, PI proliferation index, SD standard deviation, *significantly different (*p*-value < 0.05)

Meanwhile, NO production in 5 µg/mL PHA and the mononuclear cell proliferation capacity (proliferation index; PI) after 28 days of KD increased significantly ($p < 0.05$). Similarly, Noh and his colleagues (2006) reported an increase in NO production with the ketogenic diet. They suggested that neuronal NO synthase (nNOS) activity exerted an antiepileptic effect on induced seizure mice [26]. Increased PI presented a response to a stimulator of mononuclear blood cells. Evidence demonstrated that a diet deficient in carbohydrates could improve human T-cell immunity, probably through immunometabolism reprogramming [27]. The likely mechanisms of the ketogenic diet's effect on physical, chemical, and immunological statuses are shown in **Figure 2**.

Our results suggested that the ketogenic diet assists obese people because it makes weight loss faster and enhances body shape within a short period (28 days in this research). Nonetheless, our data have some limitations as this research does not have many subjects and have a comparatively short KD period. Thus, 28-day-KD may not be adequate to observe crucial changes. Hence, future research should include more volunteers and a more extended KD period, alleviating to see observed changes in weight, body composition, and immunological status better.

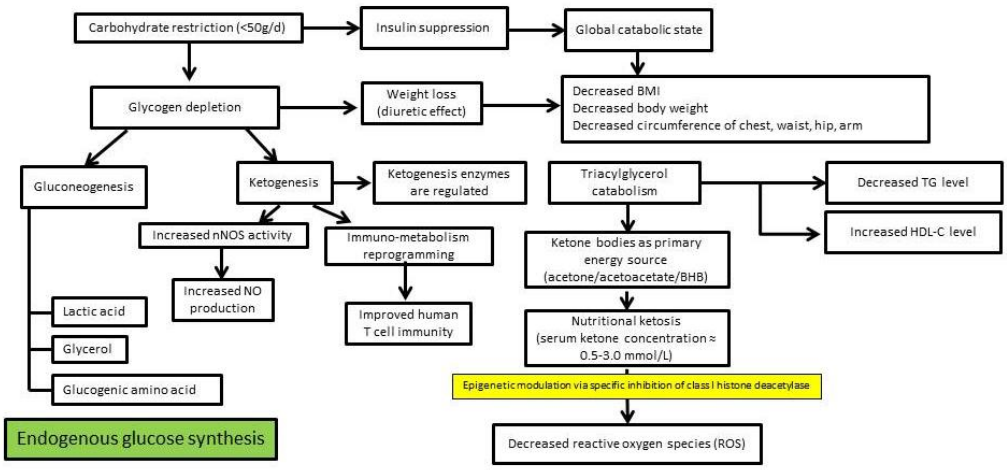

Figure 2 The possible mechanism of ketogenic diet affecting physical, chemical, and immunological statuses.

Conclusions

The 9 overweight Thai participants with a body mass index (BMI) > 25 kg/m^2 have pursued a self-conduct ketogenic diet program for 28 days. All participants are Thai women with a mean age of 28.44. After KD, most physical indices, including BMI, body weight, chest circumference, hip circumference, and arm circumference, have significantly decreased. However, KD has not affected waist and upper thigh circumference considerably. Before KD, most participants were healthy, having average blood chemistry indices. Nonetheless, 2 participants had hypercholesterolemia, one participant had high LDL-C, and 2 had a slightly elevated blood ketone. With 28 days of KD, the mean blood ketone of participants has significantly increased, proving that participants are in ketosis status. Most biochemistry indices of participants after KD are not substantially different from before-KD, including cholesterol, TG, LDL-C, and HDL-C. However, blood glucose levels have reduced considerably. KD has not changed all the hematological indices and limited effect on the immunological indices. However, mononuclear proliferation capacity and nitric oxide production in PHA stimulation have increased substantially.

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