Comparison of plasma malondialdehyde and glutathione levels between low calorie high protein diet to standard protein in obese individuals with weight cycling – a randomised trial [version 1; peer review: peer review discontinued]

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Abstract

Background: Increasing BMI increases the risk of developing cardiovascular and metabolic diseases. Oxidative stress is believed to be the underlying mechanism. A significant proportion of people who have previously succeeded in reducing body weight acquire subsequent weight gain (weight cycling). The current study aimed to evaluate the effects of low calorie diets, either with high or standard protein on plasma malondialdehyde (MDA) and glutathione (GSH) levels in obese people with a history of weight cycling.

Methods: A clinical trial was conducted in a worker’s health centre in Jakarta. Participants were assigned to a caloric restriction diet, with two different intervention groups; a high protein/HP group (22-30% of total caloric intake from protein) and a standard protein/SP group (12-20% protein). The diet programme was applied for 8 weeks with daily reminders and weekly counselling. 61 participants were recruited. 54 participants completed the programme but only 15 subjects had their MDA and GSH level measured pre- and post-treatment. Plasma MDA and GSH levels were measured according to Will's method and Ellman's method, respectively.

Results: The mean changes of MDA levels after completing the diet programme in the HP and SP group were 0.031 ± 0.124 and -0.034 ± 0.363 nmol/ml plasma, respectively. Meanwhile, the mean changes of GSH levels in the HP and SP group were -0.059 ± 0.1673 and -0.034 ± 0.363 µg/ml plasma, respectively. No statistical significance were found between the mean difference of plasma MDA and GSH level changes among both groups.

Conclusions: Changes in MDA and GSH levels after high protein or
standard protein low-calorie diet intervention for 8 weeks were not significantly different. Protein proportion in the low calorie diet does not affect the change in oxidative stress state for obese individuals with weight cycling.

**Trial registration number:** NCT03374150

**Keywords**
high protein diet, standard protein diet, oxidative stress, weight cycling, malondialdehyde, glutathione

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**Associated Research Article**

Paranoan AN, Jutamulia J, Prasetya SI et al. » Comparison of hs-CRP level between low calorie high protein to standard protein diet in obese individuals with weight cycling - a randomised trial, F1000Research 2018, 7:447 (https://doi.org/10.12688/f1000research.13342.1)

Jutamulia J, Paranoan AN, Prasetya SI et al. » Comparison of body composition changes between low calorie high protein diet to standard protein in obese individuals with weight cycling – a randomised trial, F1000Research 2018, 7:445 (https://doi.org/10.12688/f1000research.13300.1)

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**Author roles:** Prasetya SI: Formal Analysis, Investigation, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; Jutamulia J: Data Curation, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Validation; Paranoan AN: Data Curation, Investigation, Project Administration, Resources; Witjaksono F: Conceptualization, Funding Acquisition, Methodology, Supervision; Mudjihartini N: Conceptualization, Investigation, Methodology, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed.

**Grant information:** Publikasi Internasional Terindeks Untuk Tugas Akhir Mahasiswa (PITTA) grant 2017, Directorate of Research and Community Services (DRPM) Universitas Indonesia.

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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**How to cite this article:** Prasetya SI, Jutamulia J, Paranoan AN et al. Comparison of plasma malondialdehyde and glutathione levels between low calorie high protein diet to standard protein in obese individuals with weight cycling – a randomised trial [version 1; peer review: peer review discontinued] F1000Research 2018, 7:446 https://doi.org/10.12688/f1000research.13227.1

**First published:** 11 Apr 2018, 7:446 https://doi.org/10.12688/f1000research.13227.1
Introduction

In 2014, more than 1.9 (39%) billion adults aged 18 years and older were overweight with 600 (13%) million among them obese\(^1\). Meanwhile in Indonesia in 2013, the prevalence of overweight adults was 13.5% and adults with obesity 15.4%\(^2\). Approximately, 2.8 million people die each year due to obesity-associated diseases as well as causing an estimated 35.8 million (2.3%) global disability-adjusted life years\(^3\).

Oxidative stress is regarded as the principal underlying mechanism regarding the development of chronic diseases resulting from being overweight or obese\(^4\). Fat accumulation, in addition to a rise in BMI, as occurs in obesity, was proven to be correlated with systemic oxidative stress\(^5\). Accumulated fat showed a correlation with an increase in oxidative stress, which induces metabolic syndrome through several ways, most probably by causing impaired regulation in adipocytokine production and a selective increase in reactive oxygen species (ROS) generation. Within accumulated fat, the increase in NADPH oxidase and concurrent decrease in antioxidant enzymes result in oxidative stress. Oxidative stress exerts its effect on adipocytokine production by increasing the production of plasminogen activator inhibitor-1 and TNF-\(\alpha\), which in turn participates in the formation of thrombosis and insulin resistance, respectively. Oxidative stress also dampens the plasma adiponectin, which exerts insulin-sensitising and anti-atherogenic effects. Systemic oxidative stress elicited by fat accumulation causes an increase in ROS in the vascular wall, which later initiates atherosclerosis formation and subsequently cardiovascular diseases\(^6\).

Dietary programmes as one of mainstays in body weight reduction has proven to bring about improvements in pro-oxidant – antioxidant balance by reducing markers of oxidative stress, such as \(\text{F}_{2\text{-iso}}\) prostanes\(^7\) and lipid peroxidation\(^8\), in addition to increasing antioxidant activity, for instance catalase, glutathione reductase\(^9\) and glutathione peroxidase\(^9\). However, many people who had succeeded in reducing their body weight failed to maintain it at the desired level and later undergo subsequent weight gain. A state of weight gain after a marked reduction from weight loss attempts, which occurs alternately, is known as weight cycling. Regaining body weight after previous weight loss is dominated by an increase in fat mass rather than an increase in lean mass; thus, increasing fat mass percentage\(^10\). This condition is undesirable, since free fat mass that is predominantly muscle mass is an essential part of the body for various activities, while greater fat mass percentage results in the increase of oxidative stress.

The objective of this study was to evaluate the effect of a low calorie high protein diet compared to a low calorie standard protein diet on oxidative stress and antioxidant status, which were measured by plasma malondialdehyde and glutathione levels, respectively.

Methods

Study design

This is an open-randomised clinical trial as a part of larger study comparing the effects of low calorie with either high protein or standard protein diet conducted through dietary consultation on body composition, oxidative stress markers, inflammation markers and metabolic syndrome parameters in obese with weight cycling. There are two parallel intervention group with the allocation ratio of 1:1, namely high protein (HP) and standard protein (SP) groups. There were no changes made to the methods after the study had been started (original trial protocol can be found in our sister article\(^11\)).

The research has been approved by the Health Research Ethical Committee of the Faculty of Medicine Universitas Indonesia – Cipto Mangunkusumo Hospital with letter approval number of 237/UN2.F1/ETIK/2017. All participants in this study were treated based on Declaration of Helsinki and gave spoken and written informed consent before participating this study. A completed CONSORT checklist can be found in Supplementary File 1.

Trial registration number: NCT03374150

Registry name: Clinicaltrials.gov

Date of trial registration: 12/03/2017

Trial link: https://clinicaltrials.gov/show/NCT03374150

Participants

This study was conducted at the province’s Civil Workers’ Health Service Centre of the Special Capital Region of Jakarta (Pusat Pelayanan Kesehatan Pegawai Provinsi DKI Jakarta). Initially, the candidates of participants were obtained from the list of workers attending the health service centre in the previous year and were asked to participate by phone or directly while he/she attended the clinic to do the screening process. Since the number of participant from the worker health center did not meet the sample size, the information of study recruit were also disseminated through social media for people in the greater Jakarta region. Those who wish to participate were asked to attend the worker health center to be screened for eligibility. The inclusion criteria were men or women more than 20 years old with BMI ranging from 25 – 35 kg/m\(^2\) and with a history of weight cycling. In this study, weight cycling was defined as a history of weight loss \(\geq 2\) kg and regaining weight or exceeding its initial body weight at least twice in the last five years. The weight cycling history of each potential participant was firstly self-reported for screening purposes based on a history of body weight changes; nevertheless, in the latter recruitment it was confirmed via interview with the nutritionist (investigator JJ and ANP). Exclusion criteria were diabetes mellitus, a history of gastrointestinal tract
resection, hormonal disorders, such as abnormal thyroid function, hormonal contraception user, menopause, and abnormal kidney function (if the serum creatinine is beyond 0.9–1.3 mg/dl for male or 0.6 to 1.1 mg/dl for female) and blood urea nitrogen beyond 8 to 24 mg/dl for males or 6 to 21 mg/dl for female). The recruitment and intervention were conducted in two periods, the first one was between May and July, while the second period was between July and September of 2017.

Interventions
The date at which the intervention was started to be given for each subject at any period was unique according to the subject’s date of attendance for the initial screening. Two weeks before being given the diet intervention, subjects were interviewed for 24-hour food recall to determine baseline daily caloric intake and their anthropometric data, in addition 1.5 ml of vein blood samples were taken after 8 hours-fasting. Subjects were instructed to discontinue all previous diet programmes and to maintain their daily physical activity at their usual level.

Each participant from both groups were given a nutritional consultation addressing the diet plan related to reduce the daily caloric intake by 1000 kcal from the usual daily caloric intake before joining the study. The amount of previous daily caloric intake was obtained from 24-hour food recall based on food photo books issued by Tim Survey Konsumsi Makanan Individu, the Ministry of Health, Indonesia (see supplementary file on our sister article19). Subjects in the HP group were instructed to decrease their daily caloric intake by 1000 kcal and the remaining amount of daily caloric intake was divided based on the macronutrient source with a composition of 22–30% protein, 50–55% carbohydrate and 20–25% fat. On the other hand, the SP group received a 1000 kcal caloric restriction diet plan with an energy source comprised of 12–20% protein, 55–60% carbohydrates and 20–30% fats. Subjects were taught about appropriate sorts and amounts of foods for daily consumption to comply with the assigned amount and source of caloric intake along with eligible cooking methods. Subjects were also provided with a menu book, which explained things in more detail. The diet programmes were conducted over 8 weeks. Subjects were asked to self-report daily food consumptionin a logbook (see supplementary material on our sister article19). Subjects were also followed up daily by cellphone texting and and weekly counselling by direct meeting with each participant to ensure adherence to the diet plan.

Outcomes
The primary outcomes of this study are the plasma level of malondialdehyde (nmol/ml plasma) and glutathione (µg/ml plasma), while the secondary outcomes are the dietary profile of the treatment groups comprising the mean daily caloric intake and the mean proportion of carbohydrate, protein, and fat in contributing to the total daily caloric intake during the course of the intervention. No changes were made to the type as well as the method of analyzing and providing the primary outcome data. The anthropometric data of the participants were collected during the first encounter and subjects’ blood samples taken on the first day as well as the last day of the 8 weeks diet programme for the measurement of plasma MDA and GSH. As much as 1.5 ml of venous blood samples were taken and then collected in heparinized vacutainer tubes. Next, the samples were centrifuged and stored frozen until further use. Measurement of the MDA level was performed using Will’s method20, while the plasma GSH measurement was conducted according to Ellman’s method13.

Sample size and randomization
In order that this study could achieve a two-tailed α of 0.05 as well as a power of 80%, 13 samples for each group were needed to detect mean differences between these groups. The allocation of participants to the intervention arms was determined by block randomization with a block size of two. The random allocation sequence was obtained from a random number generation method in which an even random number represents an arrangement of HP-SP for a block and vice versa. A participant took an envelope to define the group they will belong to. Hence, during the allocation process, the sequence was concealed from the participant. The investigator JJ assigned subjects to intervention arms based on the group name appeared from the envelope that had been picked by the participant.

Statistical analysis
The mean differences significance in baseline characteristics related to age, BMI and number of weight cycling cycles between the HP and SP groups were assessed by means of independent samples t-test. Meanwhile, Fisher’s exact test was used to determine the proportion difference significance of gender between both groups. Furthermore, the significance of the intra-group mean difference of MDA and GSH before and after the 8-weeks diet were evaluated by paired samples t-test, if the data were normally distributed, as indicated by P>0.05 in the Shapiro-Wilk test. If the distribution of the data was abnormal, the Wilcoxon test as the non-parametric test was used instead. In contrast, independent sample t-test was used to determine the intergroup statistical significance of the mean difference of the change rate in MDA and GSH between the HP and SP groups, if the data distribution was normal. If the data was not normally distributed, the Mann-Whitney test was used for this purpose. Data were analyzed using SPSS for Windows version 20.

Results
The flow diagram of the participants is provided in Figure 1. Initially, there were 61 participants who were randomly assigned to these groups and received the intended intervention. Due to several reasons, only 54 subjects completed the intervention. However, 7 subjects from HP group and 8 from the SP group did not attend the lab for the blood sample taking after the intervention so their oxidative stress markers were not measured after completing the programme. Hence only 15 subjects from each group who were analyzed for the primary outcome of oxidative stress markers.

Table 1 provides the baseline characteristics of the subjects who completed the intervention and were analyzed. Table 2
provides the dietary profile of the subjects. All the subjects were analyzed in the group to which they were originally assigned. During the course of the treatment, no clinically important adverse event nor harms were reported by the subjects. Only a few subjects complained nausea and classic symptoms of lack of energy, such as lethargy and mild dizziness.

Table 3 summarizes the primary results of this study. Before and after the diet intervention, mean concentration of plasma malondialdehyde (MDA) of the HP group was lower than that of the SP group \((p=0.023\) and \(p=0.116\)). Interestingly, after completing the 8-weeks diet programme, the plasma MDA level increased slightly in the HP group, but it decreased very slightly in the SP group. Plasma MDA level in the high protein group indicated that there was probably still an ongoing oxidative stress process. Conversely, the measurement of glutathione (GSH) level after subjects had completed the diet programme indicated that the GSH level decreased in both the HP and SP groups. However, it should be noted that the degree of reduction in the GSH level in the HP group was slightly lower than that of in the HP group. Possibly, since a state of oxidative stress was still occurring in the high protein group, GSH as an endogenous non-enzymatic antioxidant was consumed to balance increasing formations of pro-oxidants. Hence, the rate of GSH depletion was higher...

**Figure 1. Participant allocation and participation.**
Table 1. The characteristics of subjects prior to the low calorie-diet, divided according to the intervention with high protein and standard protein. Only subjects who completed the study were included.

| Variable                        | High protein (n=15) | Standard protein (n=15) | P-value* |
|---------------------------------|--------------------|------------------------|----------|
| Age (years)                     | 35.00 ± 7.892      | 33.47 ± 8.634          | 0.61     |
| Gender (n, %)                   |                    |                        |          |
| Male                            | 3 (20)             | 1 (7)                  | 0.59     |
| Female                          | 12 (80)            | 14 (93)                |          |
| Education level (n, %)          |                    |                        |          |
| High school                     | 0 (0)              | 3 (20)                 | 0.43     |
| Undergraduate                   | 13 (87)            | 11 (73)                |          |
| Postgraduate                    | 2 (13)             | 1 (7)                  |          |
| Body mass index (kg/m²)         | 29.14 ± 3.771      | 29.94 ± 2.793          | 0.51     |
| Fat mass percentage (%)         | 37.27 ± 7.04       | 40.48 ± 5.29           | 0.233    |
| Muscle mass percentage (%)      | 58.89 ± 7.18       | 55.95 ± 5.13           | 0.208    |
| Number of weight cycling history (n, %) | | | |
| 2–3 times                       | 12 (80)            | 11 (73)                | 0.90     |
| 4–5 times                       | 3 (20)             | 4 (27)                 |          |
| Normal daily caloric intake (kcal) | 1864 ± 459        | 1759 ± 345             | 0.484    |
| Level of physical activity (n, %) |                   |                        |          |
| Very low                        | 0 (0)              | 4 (27)                 | 0.167    |
| Low                             | 9 (60)             | 7 (46)                 |          |
| Moderate                        | 6 (40)             | 4 (27)                 |          |

*significant value was set at P<0.05

Table 2. The dietary profile of the subjects during the course of the intervention.

| Variable                                    | Standard protein (n=15) | High protein (n=15) | P-value* |
|---------------------------------------------|------------------------|---------------------|----------|
| Mean daily caloric intake (kcal; mean±SD)   | 964.72 ± 83.34         | 1041.96 ± 175.66    | 0.135    |
| Mean protein proportion of total daily caloric intake (%; mean (range)) | 21.25 ± 2.57           | 27.56 ± 3.49        | <0.01    |
| Mean carbohydrate proportion of total daily caloric intake (%; mean±SD) | 50.73 ± 4.23           | 40.63 ± 5.31        | <0.001   |
| Mean fat proportion of total daily caloric intake (%; mean±SD) | 21.05 ± 3.49           | 24.36 ± 3.63        | 0.017    |
| Number of days with diet programme compliance (mean±SD) | 21 ± 9                | 27 ± 12             | 0.09     |
in the HP group, since it was utilised to counter the increase of pro-oxidants, while the SP group underwent a lower GSH reduction given that the oxidative stress condition seemed to be less profound as indicated by the depletion in the MDA level.

Oxidative stress implies that the rate of pro-oxidant generation exceeds the capability of endogenous and exogenous antioxidants to remove it. We assumed that the excess of protein intake in the HP group was responsible for generating more pro-oxidants; thus, causing a state of oxidative stress. An intake of protein exceeding the required amount with a concurrent deficit in carbohydrates caused a shift in catabolism where proteins were utilised to harvest energy. There will be a state of imbalance in macromolecule composition in which the amino acids supply was excessive, although carbohydrate derivatives were lacking. In a body that is attempting to fulfil its energy needs due to caloric restriction, consumption of amino acids will increase as it is available in high amounts. However, the high protein supply gives rise to an increase in thermogenic response\textsuperscript{14}, which is accompanied by a lower efficiency of food energy utilisation, an increase in oxygen consumption and impaired oxidative phosphorylation capacities\textsuperscript{15}. The combination of excessive amino acids availability and utilisation as a consequence of increased body thermogenic response, results in an increase in electron flow along the mitochondrial respiratory chain that eventually produces oxidative stress\textsuperscript{16}. Oxidised amino acids from the breakdown of protein will enter the Krebs cycle, then advance to the mitochondrial respiratory chain to generate energy in the form of ATP. Furthermore, oxidation of the amino acids as substrates is accompanied by the generation of reducing equivalents that will be re-oxidised in the mitochondrial electron transport chain. This will enhance the electron flow in the respiratory chain that eventually produces reactive oxygen species in mitochondria, such as superoxide in coenzyme Q. Additionally, the rate of ROS formation is so high that endogenous besides exogenous antioxidants could no longer counteract; thus, ROS begins to have a deleterious effect through oxidative stress\textsuperscript{17,18}. One possible mechanism of cell injury by means of oxidative stress is by the cell membrane’s lipid peroxidation with MDA as the end product of the reaction.

The result of this study was coherent with several experimental studies in rats suggesting that a high protein diet enhances oxidative stress especially when measured in a specific organ. A study by Camiletti-Moirón (2015) confirmed that treating rats with 45% high protein intake for 12 weeks significantly increased brain thiobarbituric acid-reactive substances (TBARs, p=0.042), brain protein carbonyl content (PCC, p=0.006), and brain antioxidant enzymes activity (p<0.01), which were measured by total SOD, manganese superoxide dismutase, copper/zinc superoxide dismutase and catalase activity when compared to the normal 10% protein group\textsuperscript{19}. Another study by Sophia et al. (2012), suggested that a high protein diet (100% raw soy flour) for 30 days resulted in significantly higher levels of lipid peroxidation and a decrease in antioxidant enzymes including SOD, catalase and glutathione peroxidase in pancreatic tissue\textsuperscript{20}. Another experiment in relation to high protein intake in rats for 2 weeks showed that the diet significantly increased MDA and decreased T-AOC and activities of

### Table 3. Plasma malondialdehyde and glutathione levels of subjects before and after completing the 8-week low calorie diet with either high protein or standard protein.

| Oxidative stress marker | High protein (n=15) | Standard protein (n=15) | P-value* |
|-------------------------|---------------------|------------------------|----------|
| **Plasma malondialdehyde/MDA (nmol/ml plasma; mean±SD)** | | | |
| Before intervention | 0.446 ± 0.156 | 0.631 ± 0.254 | 0.023\textsuperscript{f} |
| After intervention | 0.482 ± 0.278 | 0.603 ± 0.199 | 0.116\textsuperscript{f} |
| Pre- and post- treatment mean difference significance | 0.353\textsuperscript{f} | 0.427\textsuperscript{g} | |
| Change in plasma MDA level | 0.036 ± 0.146 | -0.028 ± 0.133 | 0.218\textsuperscript{f} |
| Mean difference of change (95% CI) | 0.065 (-0.04 – 0.169) | | |
| **Plasma glutathione/GSH (µg/ml plasma; mean±SD)** | | | |
| Before intervention | 2.485 ± 1.327 | 3.197 ± 0.954 | 0.161\textsuperscript{m} |
| After intervention | 2.439 ± 1.312 | 3.164 ± 1.105 | 0.126\textsuperscript{m} |
| Pre- and post- treatment mean difference significance | 0.400\textsuperscript{m} | 0.667\textsuperscript{m} | |
| Change in plasma GSH level | -0.045 ± 0.139 | -0.034 ± 0.363 | 0.912\textsuperscript{m} |
| Mean difference of change (95% CI) | -0.011 (-0.217 – 0.194) | | |

*significant value was set at P<0.05
\textsuperscript{f}independent samples t-test
\textsuperscript{g}Mann-Whitney test
\textsuperscript{h}Fisher exact test
SOD and GSH-Px in the pancreas of the rats compared to standard protein control (all p<0.05)\textsuperscript{21}. However, a study by Petzke \textit{et al.} (2000), yielded a slightly different result suggesting that a high protein intake on rats on a long-term basis (15 weeks) did not result in a consistently significant difference between different amounts of protein groups in different organs. In that study, adequate protein (13.8\%) and high protein without RRR-a-tocopherol acetate (HP-toc) resulted in a higher plasma protein carbonyl concentration and liver lipid peroxides (TBARs) levels compared with medium protein (25.7\%) and high protein (51.3\%) diets (p<0.05). Besides, no significance was established in the difference in plasma GSH concentration among various protein intakes; however, total blood GSH concentrations were found to be significantly lower in the HP-toc diet compared with the other diets (p<0.05). Furthermore, the GSH concentration in the liver was significantly lower in the adequate protein diet compared with the other diet (p<0.05)\textsuperscript{22}.

A randomised controlled trial conducted by Kitabchi \textit{et al.} (2013), which provided a 6-month diet intervention of high protein composition similar to this study (30\% protein, 40\% carbohydrates and 30\% fats) with 500 kcal of caloric restriction, resulted in a somewhat contradictory conclusion regarding this study. The previous study suggested that the subjects on the high protein diet underwent improvements in markers related to oxidative stress and lipid peroxidation. The dichlorofluorescein level, as a marker of oxidative stress underwent a reduction from 3.2 ± 0.1 µmol/l in the baseline to 2.4 ± 0.1 µmol/l after 6 months of intervention. Additionally, the MDA level also decreased from 1.1 ± 0.06 to 0.7 ± 0.05 µmol/l\textsuperscript{23}. Presumably this discrepancy was primarily caused by a different duration with regards to the diet intervention, where that specific study was conducted over 6 months and restricted participants to a smaller amount of daily caloric intake of only 500 kcal/day based on resting energy expenditure.

The main limitation of this study is the inability to maintain the compliance of the participants. A significant amount of subjects were missing for the measurement of primary outcome leading to potential selection bias. Potential recall bias upon daily intake exists because several participants did not fill the food logbook everyday. Besides, we are unable to explain the oxidative stress state of the participants during the course of the intervention. Furthermore, there are other oxidative stress markers that were not studied in this research. The sample of this research is predominantly female, well-educated, and moderate to high socioeconomic status, which may have a better diet performance than the general population\textsuperscript{24}.

**Conclusions**

The low calorie diet with either high protein or standard protein proportion did not result in significant changes in plasma MDA and GSH levels after 8 weeks of application. The low-calorie diet with high protein proportion resulted in a slight increase in plasma MDA and GSH levels, while the standard protein diet brought about a subtle decrease in both plasma MDA and GSH, although the mean difference of change in the MDA and GSH levels between both groups was not statistically significant. An increase in MDA as a marker of lipid peroxidation with a concurrent decrease in GSH as endogenous antioxidant after the high protein diet indicated that the high protein diet possibly enhanced oxidative stress.

**Data availability**

Dataset 1: Harvard Dataverse. Replication Data for Comparison of low calorie-standard protein or high protein diet on body composition, malondialdehyde and glutathione, and hs-CRP level, http://dx.doi.org/10.7910/DVN/7H5FP\textsuperscript{25}

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

**Competing interests**

No competing interests were disclosed.

**Grant information**

\textit{Publikasi Internasional Terindeks Untuk Tugas Akhir Mahasiswa} (PITTA) grant 2017, Directorate of Research and Community Services (DRPM) Universitas Indonesia.

\textit{The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.}
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