The effect of dietary methionine restriction and the mechanism of glucose and lipid metabolism in obese induced C57BL/6 Mice

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Abstract:

　　Background: In animal study, dietary supplementation of methionine (Met), depending on dosage is beneficial for improving several metabolic health problems, such as obesity, diabetes and cardiovascular disease which potential benefit was investigated on cardiac function in glucose and lipid metabolism. Therefore, in order to effectively complete this work, the following parameters were examined: Growth performance (Weight), fasting blood glucose levels, measurement of malondialdehyde, total antioxidant capacity levels, C-reactive protein and Non-esterified Fatty Acids.

　　Methods: In this study, 42 male C57BL/6 mice were randomly shared into three groups of 18 each and fed a diet comprises of Control (Con) group given 4% fat, 0.86% Met and 67.17 % starch. High Fat + High Met (HF+HM) group 20% fat, 0.86% Met and 51.17%

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starch. While High Fat + Low Met (HF+LM) received 20% fat, 0.17% Met and 51.17% starch respectively.

Result: Met restriction (MetR) showed decreases in body weight gain followed by reduction in fasting blood glucose levels observed throughout the study in both Con and MetR groups. However, noticeable reduction in total antioxidant capacity (T- OAC) was observed in the HM group, but Malondialdehyde (MDA) levels were higher (<0.05) in HM than Con and MetR group suggesting oxidative stress damage. However, changes were observed in Non-Esterified Fatty Acids (NEFA) parameters in the three study groups, though not statistically significant. There was an increase in C- Reactive Protein (CRP) activity of HF+HM fed mice, which was indicative of inflammation, although not specific but found to correlate with risk of cardiovascular disease.

Conclusion: Dietary MetR is effective in regulating weight gain, glucose and lipid homeostasis and restoring oxidative damage during Oxidative stress situations through adaptive changes.

Key words: Dietary Methionine Restriction, Oxidative Stress, Reactive Oxygen Species, Cardiovascular Disease, Essential Amino Acids

1. INTRODUCTION

Amino acids (AAs) play a major role in the mammalian defense mechanism as they are involved in the synthesis of proteins such as antibodies and control of key immune regulatory pathways. Certain essential amino acids can improve health by regulating major metabolic pathways important for improving food utilization, enhancing protein accretion and reducing adiposity [1] . In other words, utilization of nutrient is greatly affected by an imbalance in its consumption, However, in normal physiological process, there is an active equilibrium in the production of Reactive Oxygen Species (ROS) activity and antioxidant defense capacity and when this balance is

compromised in favour of ROS, whether by a decrease in anti-oxidant protection or a rise in ROS production, it consequently results in oxidative stress (OS). However, oxidative stress can cause severe damage to the heart subsequently leading to cell injury and organ damage [2]. The primary source of ROS is mitochondria [3] that involved in cardiac redox signaling predominantly produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) which stimuli is activated by Angiotensin 2 (Ang 2). Thus, ROS occurs as a result of imbalance in fatty acid utilization and oxidative stress when lipid is accumulated within the myocardium [4]. It can also excite cellular dysfunction, myocardial growth and matrix remodeling [5] . In recent years, a considerable body of evidence has been developed to support a key role for free radicals in many fundamental cellular reactions and suggesting that oxidative stress might be important in the pathophysiology of common diseases such as neurodegenerative disorders, diabetes, cancers, cardiovascular disease and autoimmune disorders [6-8].

Met is the first limiting AA considered to be essential and abundant in meat and other animal proteins, commercially available in its synthetic form for use in experimental animals. It has been used to complement cancer treatment in humans [9, 10] and improve metabolic health [1, 11-13]. In addition to its action and as part of the one-carbon metabolic cycle, it can perform a critical role in generating S-adenosylmethionine (SAM) which is the major methyl donor for a variety of biologically important reactions. However, the major intermediary in Met metabolism is Hcy, which is either methylated to Met or enter the transulphuration pathway to form cysteine and later produce glutathione, known to be an important intra-cellular antioxidant. There are still many research evidenced in both cohort and ex vivo study, pointing to high Hcy levels as linked to many vascular abnormalities

increasing susceptibility to disease [14-18]. Glucose and lipids are both important components of energy metabolism. It is therefore not surprising that glucose as well as lipid metabolism are closely linked to each other which has important clinical implications such as dyslipidemia associated with cardiovascular disease causing hypertriglyceridemia. [19] .Interestingly, the reduction of the Met has become the focus of interest as dietary regimen since the incidence of diseases have emerged and [20, 21] are rising at a higher rate and is posing health care challenges worldwide. Early work done by Orentreich together with Richie et al [22] recognized Met reduction technique as a robust dietary manipulator (from 0.86% of the diet to 0.17%) in animal studies [22, 23]. According to these authors, restricting Met in the diet has greatly improved longevity, which further improve insulin sensitivity, reduced weight gain and adiposity relative to animals on a normal diet. In addition, current researchers have demonstrated that dietary essential amino acid (EAA, markers of protein nutrition) restriction or limitations cause profound transcriptional and metabolic responses, which result in notable changes in lipid metabolism in adipose tissue and liver [24-26]. There is still a controversy about whether the reduction of Met in supplemental diet is creating impact in reducing adiposity and improving glucose homeostasis. Main while, the best suitable animal model studied for diet-induced metabolic conditions are the male C57BL/6 mice which characteristics are similar to humans supplemented with a diet high in saturated fat [27] observed to develop lipid metabolic disorder and IR that was initially published by Malloy et al [28]. However, the toxic effect of excessive dietary Met intake is known to exert negative health outcomes. Thus, the exact mechanism (s) through which MetR mediates these varied effects leading to these complications still needs to be investigated and clarified. Therefore, this study, hypothesized

that MetR may possibly improve weight gain, regulate glucose metabolism, reduced lipid peroxidation and enhance antioxidant capacity and immune function in the heart and other vital organs. The aim of the present study was to determine the effects of dietary MetR and examine their metabolic relationship linked to glucose and lipid metabolism in C57BL/6 mice.

2. METHODS AND MATERIALS

2.1. Chemicals
All the chemical reagent products and foodstuffs were procured from the Nanjing Laboratory Animal Center of Chinese Academy Sciences (SLACCAS) for malondialdehyde (MDA), total antioxidant capacity (T-AOC), non - esterified fatty acid (NEFA), and C - reactive protein (CPR). Except for Handheld Ul-trasensitive one touch glucose meter machine and reagent strips provided by the Food Science and Technology Department, Jiangnan University, P.R. China.

2.2. Animal care
Four (4) week old C57BL/6 healthy male mice (19.34±0.44 grams) free from pathogen were purchased from Shanghai Laboratory Animal Center of Chinese Academy (SLACCAS). However, the protocol used in this study was in agreement with the Jiangnan University guidelines for the care and management of animals. Forty two (42) mice were maintained in a ventilated free cage on a 12:12-h light-dark cycle under conditions of controlled room temperature at 23 ± 2^0C with humidity (60%). Moreover, the dietary intervention was maintained for 24 weeks and at the end of the supplemental feeding, the animals were weighed (27.84±2.10 grms) and surgical procedure was followed accordingly by the approval of the ethical committee of the University.

2.3. Experimental diet

Prior to the study, mice were fed normal diets and were acquainted with the Jiangnan University Animal Experimental Facility and the condition for two weeks. Proximate composition of basal diets were water, 10 %; crude protein, ≥ 18 %; crude fat, ≥ 4%; crude fiber, ≤ 5%; crude ash, ≤ 8%; calcium, 1.0-1.2%; total phosphorus, 0.6-1.2%; Lysine, ≥ 1.32%; salt, 0.4%; methionine cysteine, 0.78%; The composition of animal diets is presented in table 1. All the foodstuff ingredients for each diet were powdered and mechanically prepared in a food blender for about 20 minutes. Lard oil and sterile water were added during the course of mixing and dried to approximately 10% moisture, then sealed in plastic-lined bags and stored at 15^{0}C until used. In addition, food and water were provided and all mice were allowed free access to the diet during the experimental period. Body weights were monitored and recorded at the same time of the day throughout the feeding period. Otherwise environmental conditions were favorable.

Table 1.

Ingredient Composition of diets fed to mice			
Ingredients (g per kg diet %)	Normal diet	HF+HM diet	HF+LM diet
Isoleucine	1.22	1.22	1.22
Threonine	1.22	1.22	1.22
Phenylalanine	1.73	1.73	1.73
Leucine	1.65	1.65	1.65
Arginine	1.66	1.66	1.66
Glutamic Acid	4.01	4.01	4.70
Lysine	2.14	2.14	2.14
Methionine	0.86	0.86	0.17
Histidine	0.49	0.49	0.49
Valine	1.22	1.22	1.22
Tryptophan	1.27	1.27	1.27
Glucose	7.44	7.44	7.44
Starch	67.17	51.17	51.17
Lard Oil	4	20	20
Vitamin	0.02	0.02	0.02
Choline	0.3	0.3	0.3
Mineral Substance	0.06	0.06	0.06
NaCl	0.2	0.2	0.2
Calcium Hydrophosphate	0.01	0.01	0.01
CaCO3	1.32	1.32	1.32

Potassium Citrate	1.01	1.01	1.01
Carboxymethyl Cellulose (CMB)	1.00	1.00	1.00
Total	100	100	100

Composition of Control (Con) High Fat /High Methionine (HF+HM) and High Fat/Low Methionine diets. The composition of the diets used in this experiments are identical except for lad oil (4g/kg) and starch (67.17g/kg) in Con. For LM, Met (0.17g/kg) and Glutamic Acid (4.70g/kg).

2.4 sample collection and analysis

2.4.1 Surgical procedure

Mice were placed in metabolic cages at night and starved for 12 hours before sacrifice. Thereafter, nine mice in total per experimental group were sacrificed at 13th, 18th and 24th week. Dissecting stage was prepared and the animals were anaesthetized with isoflurane, sacrificed by cervical dislocation and mice were placed in a supine position and immobilized. However, blood samples were collected from the sinus by using the orbital bleeding technique and transferred into Eppendorf tubes containing heparin, which was used for fasting blood glucose measurement. The thoracic cavity was immediately dissected to remove the heart, excised, washed with phosphate-buffered saline solution at a PH of 7.4, rinsed, dried between folds of filter paper, weighed and then divide into two parts. However, samples were homogenized and centrifuged for 15 min at 12000 rmp and 4^0C to separate the supernatant. The left over homogenate was reserved at 20^{-0}C for the analysis of total antioxidant capacity (T-OAC) and malondialdehyde (MDA). The remaining portion of the heart was quickly frozen in liquid nitrogen and kept in -80°C for later analysis.

2.4.2 Fasting Blood glucose determination

Fasting blood glucose levels were measured using a handheld Ul-trasensitive one touch glucometer machine and reagent strips. The measurements were done by a single measurer to maintain accuracy and eliminate potential operator variability in recording results.

2.4.3 Assessment of antioxidant status

The assessment of antioxidant was done by using glass, Teflon Homogenizer, including already prepared 50 Mm phosphate (PBS) buffer saline at PH of 7.4 to obtain whole homogenate 1:9 (tissue PBS). In addition, the heart tissue supernatant of homogenates was used for measurement of total antioxidant capacity (T-OAC)) with commercially available kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, P.R. China). Measured accordingly by spectrophotometer at 520 nm using their respective kits as used in HIROSHI KOBAYASHI et al 2001 [29].

2.4.4 Lipid Peroxidase determination

The progress of lipid peroxidation was checked by measuring Malondialdehyde (MDA) for free radical damage. The method includes heating and MDA was recognized as product of lipid peroxidation, which was treated with thiobarbituric acid to produce a pink coloured product wherein the optical density (OD) including heart tissue supernatant was measured at 532 nm alongside the blank having the reagents using a spectrophotometer. However, the commercial available, detecting kit for MDA was purchased from the Nanjing Jiancheng Bioengineering Institute, Nanjing Jiancheng Co.Ltd China. The method used was in conjunction with Xue Tang et al 2011 [30] and procedure was followed according to the manufacture's instruction.

2.4.5 Determination of Protein

Determination of protein levels of heart tissue samples was done according to Marion and Bradford et al 1976 [31] method. Protein levels of tissue samples were measured and assayed using commercial kits bought from the previously described company and used according to the instruction giving by the manufacturer. Concentrated Coomassie blue was diluted in

distilled water, and then 2.5mL of this diluted dye was added to 50 µL of diluted tissue supernatant. The mixture was incubated at room temperature for 5 min and the absorbance measurement was taken at 595nm using UV-vis spectrophotometer. However, bovine serum albumin (BSA) was used as protein standard.

2.4.6 C - reactive protein determination

The concentration of CRP was determined by using heart tissue sample and the commercially available mouse CRP ELISA Kits which was assayed with the appropriate test kit obtained from the Nanjing Jiancheng Bioengineering Institute, Nanjing, P.R China. Procedure followed according to the instructions giving by the manufacturer.

2.4.7 Enzyme activity of Non Esterified Fatty Acids (NEFA) concentration

The concentration of NEFA samples were analyzed for all in duplicate by colorimetric method with a microplate spectrophotometer. Thus, all samples were run on three plates measurements of an enzymatic reaction according to the manufacturer's directions.

2.4.8 Statistical Evaluation

Difference between means in the three groups were evaluated by representative mean ± SD and subjected to one-way anova analysis of variance with post-hoc Duncan's test considered statistically significance of differences ($P < 0.05$). All statistical analyses of data were completed using the SPSS soft wear program for windows, version 17.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 5.0.

3. RESULTS

3.1 Effect of MetR on body weight gain, heart weight and ratio of heart weight to body weight.

The result in Fig.1 A,B,C indicates that animals fed on HF+HM diet steadily start to gain weight at 6th week (24.65 ± 1.40g) P<0.05 and continue with this trend throughout the feeding period, compared to Con (22.91 ± 1.29g) and HF+LM (22.91 ± 1.29g) which relatively had a similar weight gain. Similarly to changes observed in Fig1.B as indicated below. By the 13th week of feeding, HF+LM (26.17±1.1.72g) group experienced an elevation (P<0.05) in weight gain over Con (24.62±1.46g) fed group. However, the 18th week of dietary regimen showed that, HF+LM (25.63±1.51g) had decreased weight gain similar to the level in Con (25.40±1.40g), but by the end of 24th week, there was a decrease in Con (26.24±1.41g) over HF+LM (26.49±1.58g) although not statistically important when compared with HF+HM (30.8±4.67g) fed group respectively. In addition, as shown in Fig1.D and E, there was no significant differences observed in Heart weight index among the three groups, but the ratio of heart weight (HW) to total body weight (TBW) was increased P<0.05 in mice fed HF+LM (0.64±0.13%) diet as compared to Con (0.62±0.06%) though not statistically different from HF+HM (0.53±0.0.06%) (p<0.05). Overall, data exhibited that mice in HF+HM fed group gained more weight than HF+LM and Con fed (p<0.05) showing the beneficial effect exerted by MetR in animals.
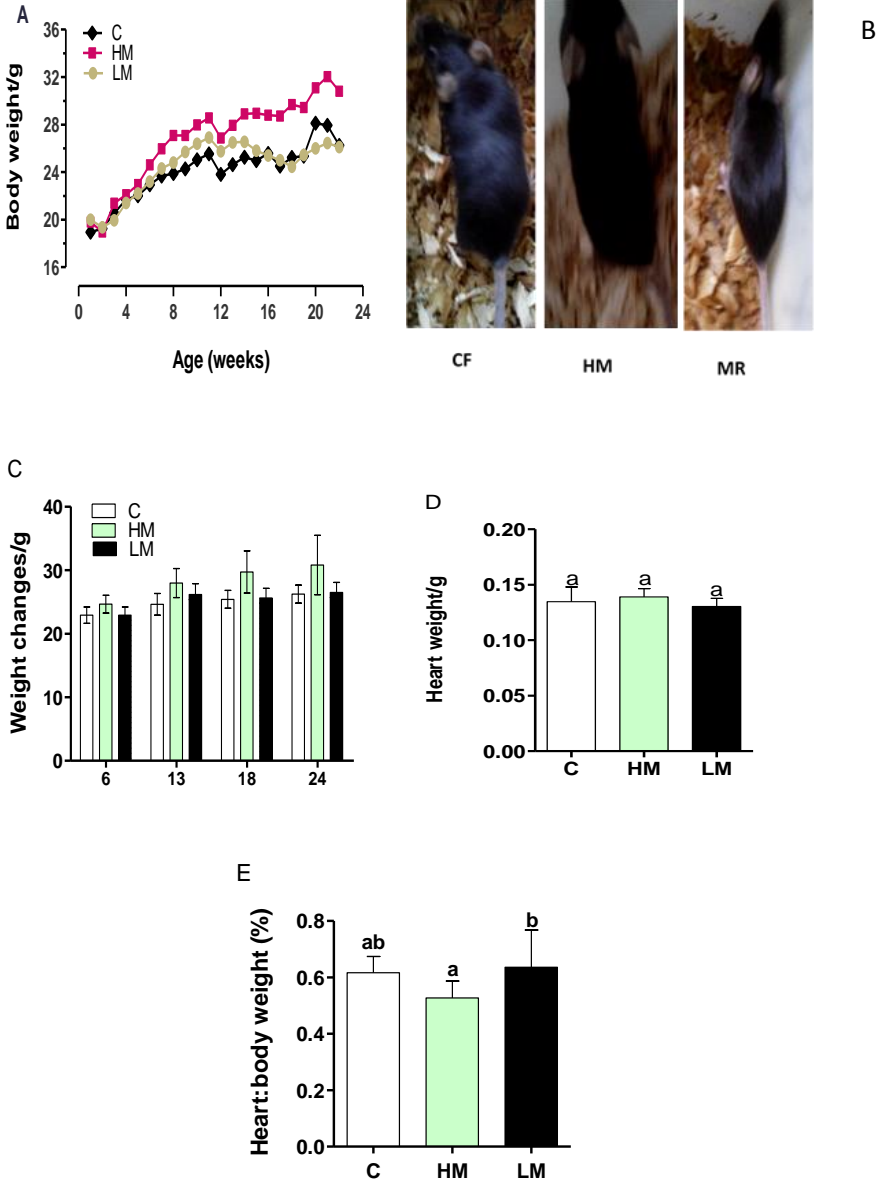
Fig. 1. Body weights in grams (A), physical morphology (B), Changes in weight gain over experimental period (C). Heart weight index (D), Heart weight (HW) and total body weight (TBW) ratio % of mice (E), Data values are presented as mean±SD (n=9), Statistical significance of mean was determined by using one-way ANOVA followed by

Duncan's multiple range post hoc test. Values that does not show the same superscript letters above bars represent significant difference (p<0.05) among groups.

3.2 Effect of MetR on glucose metabolism

The effect of MetR on fasting glucose levels as part of metabolic homeostasis in C57BL/6 mice fed with diet for 24 weeks. Since high fat diet (HFD) feeding induced risk of insulin resistance and MetR has shown reduction in body weight gain [32] as demonstrated in previous studies. To assess the effect of dietary Met supplementation on glucose metabolism, whole blood glucose levels were measured in all groups at different (13th, 18th and 24th week) time points. However, according to the analyses of the results, Mice sampled at 13th week on HF+LM (4.31± 0.42 mmol/l) diet had normal fasting glucose level as well as in Con (4.54±0.52 mmol/l) P<0.05. Whereas, HF+HM (6.08± 0.69 mmol/l) exhibited significant (p<0.05) increase in fasting glucose levels. However, the fasting blood glucose levels continue to rise significantly (p<0.05) in HF+HM (6.44±1.03 mmol/l) fed group than Con (4.64±0.85 mmol/l) with slight elevation over HF+LM (4.16±0.83 mmol/l) following 18th week of dietary supplementation. Moreover, at 24th week, the level of fasting glucose in Con (4.32±0.68 mmol/l) was reduced to that of the13th week in MetR mice fed with HF diet as compared to HF+LM (3.95±0.37 mmol/l) which level was the lowest (p<0.05) over HF+HM (7.01±1.59 mmol/l) which was consistently higher (p<0.05) throughout the testing period. However, a glucose value ranging from 6.08 to 7.01 mmol/l was indicative of prediabetic in HF+HM fed mice (**Fig 2.**).
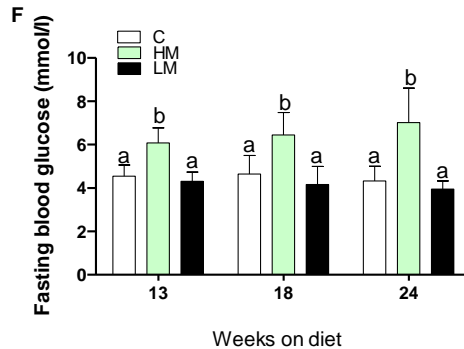
Fig 2. Fasting blood glucose was measured from the orbital sinus of each mice using handheld glucometer. Data are presented as mean ± SD (n=9), Statistical significance of mean was determined by using one-way ANOVA followed by Duncan's multiple range Post hoc test. Values that does not show the same superscript letters above bars represent significant difference (P<0.05) among groups.

3.3 Effect of MetR on oxidative stress markers in heart tissue

The status of antioxidant and MDA indicated in Fig 3. Shows the effect of dietary MetR activities after animals consumed a diet containing Con, HF+HM and HF+LM for 24 weeks. In mice fed with HF+HM diet, the T-OAC was drastically lower (6.44±0.8 U/mg protein) compared to Con (10.16±1.6 U/mg protein) which was slightly higher than the MetR (9.7±0.78 U/mg protein) group (P<0.05) as seen in previous studies . There was an increase in malondialdehyde (MDA) levels which caused significant elevation in the tissue (heart) of mice supplemented with HF+HM (10.93±1.92 mmol/mg protein), an indicator of lipid peroxidation, compared to Con (8.38±0.74 mmol/mg protein) which was lower than MetR (8.63±0.54 mmol/mg protein) group but the respective changes was not statistically different (P<0.05).
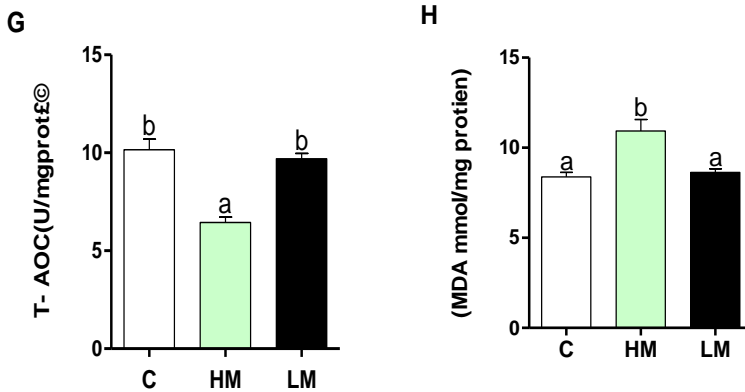
Fig. 3 T-AOC and MDA activity in heart tissues of C5BL/6 mice fed with Con, HM and LM diet. Data are presented as mean ± SD (n=9), Statistical significance of mean was determine by using post hoc test. Values that does not show the same superscript letters above bars represent significant different P<0.05) among groups.

3.4 Effect of Met on non-esterified fatty acids (NEFA) in C57B/6 mice heart tissue

Tissue Non Esterified Fatty Acid (NEFA) concentration after administration of diet**.** Mice who consumed HF+HM (645.80 ± 90.14 µmol/g protein) and HF+LM diet showed no significant difference (p<0.05) in NEFA concentrations. Similarly, neither Con (477.36±65.35µmol/g protein) nor HF+LM (556.92±41.94µmol/g protein) group show significant change (P<0.05). Rather, there was marked significant difference observed between the Con and the HF+HM group. The variation in concentration of NEFA has been linked to rate of changes that occur due to reduced oxidation of fatty acid [33]. However, plasma concentration of NEFA remains comparatively stable in Con over MetR group indicating less CVD risk, but significantly higher in the HF group (**Fig 4 I.**).

3.5 Determination of C-reactive protein (CRP) inflammatory marker

To help determine the risk of heart disease related to inflammation in MetR C57BL/6 mice, CRP was used as marker to establish inflammation in mice heart tissue. According to the analysis result, no significant association was observed between Con (32.39% mol/l)) and HF+LM (31.15% mol/l)) fed group. While in HF+HM (36.46% mol/l), we found significant elevation (<0.05) indicating risk of developing inflammation in the heart of C57BL/6 mice (**Fig 4 J.**).
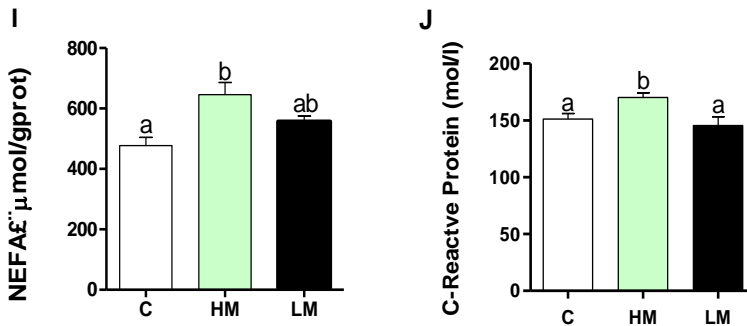

Fig.4 Nonesterified fatty acid (NEFA) and C-reactive protein inflammatory marker of heart tissue measured in C57BL/6 mice fed for 24 wks.

4. DISCUSSION

The present study is the first to validate the effect of dietary MetR on glucose and lipid profile status, antioxidant, inflammation and cardioprotective enzyme activity in C57BL/6 male mice. Met is an essential amino acid extensively used as a dietary supplement in animal diet In our findings, we have shown evidence that MetR was able to decrease body weight gain including animals fed with normal diet observed to be in good physical shape, on the other hand, those that were fed on HF+HM presented higher body weight, which was associated

with over eating compared to Cont. and LM which showed lower body weight, respectively in conjunction with previous study [34]. In earlier studies, the ability of MetR to delay the progression or combat disease is clearly established by providing a series of coordinated biochemical and physiological reactions brought about immediately after the diet is introduced [28, 35, 36]. Thus, the biological importance is shown in animals fed on MetR diets with reduced weight, including overall insulin sensitivity and improved life span [12, 37, 38].

Furthermore, there were no significant changes observed in the heart weight of the three groups. We also detected higher-heart-to-body weight ratio (%) in Low met fed group than Control as demonstrated in previous studies [39, 40]. However, the supplementation with HF+HM leads to physiological changes that prompted excess body fat accumulation shown in Fig 1B. Thus, triggering ROS, which induces OS and contributes to the pathogenesis of inflammation in the heart. Moreover, the vulnerability of oxidative stress in the heart depends on its redox sensitivity and beneficial nutritional therapies are needed to regulate and improve redox imbalance causing injury to the heart. The results have also shown that HM supplemented diet significantly elevated the MDA levels, indicating the degree of lipid peroxidative damage in the heart with noticeable reduction in T-AOC activity in Fig 3 G, H. In this study, mice who were given high-fat diet had increase fasting blood glucose levels at 13th , 18th and 23th weeks respectively which resulted in a glucose concentration that was significantly higher and this trend continues throughout the course of the study. The absence of an increase in glucose levels over the duration of the study indicates the improvements of glucose metabolism in the MetR group over Con. However, these improvements in glucose control were brought about by a change in body weight likely due to

improvement in glucose homeostasis through a reduction in Met supplementation [39]. In the present study, we observed that the NEFA concentrations was associated with inflammation and stress on myocardial performance, which was clearly demonstrated in HF+HM feeding indicative of metabolic disturbance associated with disease risk, compared with the treatment group. However, treatment with Met supplementation (0.17 %) reduced the concentration levels, but the changes did not differ between groups (Con). Moreover, high CRP has been associated with inflammation causing risk of CVD and T2DM [41]. CRP measurement in HF+HM fed mice was elevated in response to inflammation. While, in both Con and LM Met supplementation group showed decrease in the levels of CRP activity. However, the anti-inflammatory effects of MetR diet are intermediated through cytokines (interleukin 6 and tumour necrosis factor alpha) which serves as a molecular messenger between cells and is regulated by the body's response to infection and disease, as well as mediation in normal cellular processes in the body when its consumption is restricted in the diet. In particular, inflammation strongly linked to endothelial dysfunction is accepted as one of the cardiovascular risk factors clustering in the IR syndrome or metabolic syndrome. This result suggests that excess dietary Met consumption like all other nutrients can be toxic and cause metabolic abnormalities and subsequent death.

5. CONCLUSION

In summary, we compare the effect of MetR in Con, HF+HM and HF+LM fed mice. The results of the present study are in support of the metabolic changes induced by the dietary supplementation of MetR responsible for the physiological changes by promoting growth, alleviating inflammatory responses, improving immune function and health status, and

reduce oxidative stress. Thus, MetR has emerged as a favorable method for several kinds of diseases specifically relating to metabolic syndrome. Therefore, the result achieved from this study can serve as a possible benefit in relation to future studies in humans with regards glucose and lipid metabolism which still need further studies.

Acknowledgements

Conflict of interest

The authors report no conflict of interest

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