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Effect of L-Methionine Feeding on Serum Homocysteine and Glutathione Levels in Male and Female Wistar Rats

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Abstract: Homocysteine (Hcy) is a critical indicator of cardiovascular disease. High levels of Hcy have now been recognised as a risk factor for the development of a wide range of diseases. Hyperhomocysteinemia (Hhcy) can be induced by methionine or Hcy supplementation. On the other hand, Glutathione (GSH) is a major antioxidant in the body and also an important compound for oxidative defence. It is composed of 3 amino acids: cysteine, glutamate, and glycine. Interestingly, methionine is also a crucial compound in GSH synthesis. This study aims to assess the impact of 1% L-methionine feeding (10 or 30 weeks) on the body weight and serum Hcy and GSH levels of young adult (16 weeks) and middle-aged (36 weeks) Wistar rats of both sexes. Serum was analysed for Hcy and reduced GSH levels by liquid chromatography mass spectrometry (LCMS) in response to 1% L-methionine feeding. One percent L-methionine feeding decreased body weight in all conditions investigated, although this only reached significance in males after 10 weeks supplementation and females after 30 weeks supplementation. It also induced a significant increase in the serum Hcy levels of male Wistar rats, whilst having no significant effect on Hcy serum levels in female rats. Finally, we also observed a small increase in serum GSH levels in female Wistar rats but no change in serum GSH levels in the males. These results suggest that methionine feeding affects body weight homeostasis and alters by products of methionine catabolism.

Keywords: Methionine, Homocysteine, Reduced Glutathione, Body Weight

1. Introduction

Elevated concentrations of serum homocysteine (Hcy) have recently been shown to be a high risk factor for cardiovascular diseases [1]. Normally Hcy is biosynthesised during methionine metabolism [2-3]. The harmful effect of a high methionine diet is due to the conversion of methionine into Hcy, which in turn induces endothelial and oxidative stress [4-5].

The correlation between Hcy and cardiovascular diseases has been known since the 1960s [6]. A disturbance in the Hcy metabolic pathway causes Hcy accumulation leading to Hyperhomocysteinemia (Hhcy) [7-9]. In addition, common causes for Hhcy are: renal disease [10], insufficiency of vitamins contributing to Hcy metabolism [11], excess amount of dietary methionine [12-13] and also deficit of enzymes involved in Hcy metabolism [3]. It has been reported that in atherosclerotic patients with high levels of cholesterol, there was a considerable elevation in plasma Hcy concentrations [14].

There is abundant evidence indicating that high levels of Hcy induce damage to the heart and blood vessels. Boushey *et al.* [15] found that an elevation of 5 μmol/L in total serum Hcy (tHcy) concentration increases the odds of developing coronary artery disease (CAD) by 1.6 in males and 1.8 in

females. They further state that 10% of the population's risk of CAD is attributable to Hhcy [15]. Also a lack of one or more of the vitamins (B6, B12, folic acid) may disturb methionine metabolism, resulting in Hhcy [16]. Normally, Hcy is converted into two critically important compounds: S-adenosyl methionine (SAM) and glutathione (GSH). This pathway is vital to maintain low levels of Hcy [17]. Also three specific vitamins B6, B12 and folate as well as zinc and tri methyl glycine (TMG) are involved in the conversion of Hcy to SAM [18]. Vitamins B6 and B12 and zinc also contribute in the conversion of Hcy to GSH [19]. The concentration of Hcy in the blood increases when the body does not efficiently convert Hcy into SAM and GSH [3, 20-21]. It has been shown that, in the elderly, non-fasting tHcy levels are increased and contribute to the increasing rates of cardiovascular disease mortality [22].

GSH, a sulfhydryl (SH)-containing tripeptide, is composed of three amino acids (glutamate, glycine and cysteine) [23-24] and is found in all mammalian cells [25]. In particular, it acts as an antioxidant, detoxifying agent and a free radical scavenger. Our working hypothesis is that methionine feeding affects body weight and causes changes in serum Hcy and serum GSH levels. To test this hypothesis, body weight and serum Hcy and serum GSH concentrations of young adult (16 weeks) and middle-aged (36 weeks) (female and male) Wistar rats were measured in control and those fed with 1% L-methionine.

2. Materials and Methods

2.1. Chemicals

Standards, Hcy, GSH, and dithiothreitol (DTT) were from Sigma-Aldrich (Sydney, NSW, Australia), formic acid was from Fluka, LC-MS grade acetonitrile and H_2O were from Burdick and Jackson.

2.2. Experimental Animals

Young post-weaned Wistar rats (6 weeks old) (body weight, 130 - 190 gm) (n = 48) of both sexes were divided into 2 sets of four groups (n=6 in each group) according to sex and diet. The control groups routinely received standard rat chow and water ad libitum while the test groups received standard rat chow and water supplemented with 1% L-methionine [26] for 10 weeks for the first set and 30 weeks [27-28] for the second set of animals (rats fed for 10 weeks are called young adult and rats fed for 30 weeks are called middle-aged). Regular checks were made of the rats' weight, general health and well-being. At the end of this time the rats were sacrificed by stunning and cervical dislocation. The rats were then decapitated and trunk blood samples collected into Eppendorf tubes according to guidelines from the National Centre for the Replacement, Refinement and Reduction of animals in Research ($NC3R^S$). Blood specimens were left to coagulate at room temperature and then centrifuged for 10 minutes at 4000 rpm using a tabletop centrifuge (Sigma, Mode No. 1-15, Germany). The serum was then removed and transferred into clean Eppendorf tubes and stored frozen at –80˚C until used. This study was approved by the Animal Ethics committee of the University of New England and followed international guidelines.

2.3. Measurement of Serum Hcy with Liquid Chromatography Gas Spectrometry

The samples were prepared according to Shimadzu Application News No. C92. The serum samples were thawed. 100 µl of serum was put into a labelled Eppendorf tube and 20 µl (1mg/ml) DTT added. The solution was vortexed on high and allowed to stand at room temperature for 10 minutes (2x). After that 300 µl of 0.2% (1:500 v/v) $HCOOH\text{-}CH_3CN$ (formic acid/acetonitrile) was added and the solution was vortexed on high. Then the solution was centrifuged (Sigma, Mode No. 1-15, Germany) at 12000 rpm (9659.52 g) for 2 minutes and 150µl of supernatant transferred to a labelled vial.

Serum Hcy concentrations were quantified in young adult rats (n=24) and middle-aged rats (n=24) of both sexes using high-performance liquid chromatography-triple quadrupole mass spectrometer (Shimadzu, LCMS-8050, Japan). Both control of instrumentation and data analysis were performed using standard Shimadzu software (Lab Solutions v.5.8). The MS parameters were set at – m/z 136.00>90.10 and 136.00>56.10 for Hcy. Standard Hcy was made up into serial dilutions ranging from 62.5-2000 ng/ml in LC-MS grade water, analysed and plotted using Shimadzu Lab Solutions.

2.4. Measurement of Serum GSH with Liquid Chromatography Mass Spectroscopy

The serum GSH concentrations were measured on the same samples as the Hcy. The MS parameters were modified to m/z 308.00>179.10 in order to detect GSH. For quantification, a standard curve of GSH solutions ranging from 62.5-2000 ng/ml was determined.

2.5. Data Analysis

Statistical analyses were expressed as Mean (SD). Student T tests were used for significance of difference between control and treated groups. Statistical significance was considered at $p<0.05$.

3. Results

3.1. Determination of Body Weight

Table 1. *Effect of L-methionine feeding on growth in (young adult) male and female Wistar rats. Data are means ± SD body weight (n=6). *p<0.05 vs. control.*

Sex	Body weight (g/10 weeks)	Body weight (g/10 weeks)
	Control	1% Methionine
Male	350.46±26.75	314.45±28.37*
Female	154.90±20.30	141.98±14.15

The weight gain from young adult male Wistar rats fed 1% L-methionine was found to be significantly less at 314.45

(28.37) grams mean (SD) ($p<0.05$) after 10 weeks than the control group at 350.45 (26.75) grams mean (SD). Although the same trend was observed for the young adult female Wistar rats, the difference did not reach significance (see Table 1).

For the middle-aged rats, we observed a lower weight gain of 680.03 (64.34) grams mean (SD) in males fed 1% L-methionine during the 30 weeks when compared to controls which increased to 752.89 (62.08) grams mean (SD), however this change was not significant. On the other hand, there was a highly significant lower weight gain for the female rats with 257.88 (50.80) grams, mean (SD) ($p<0.001$) when compared to controls with 376.14 (15.50) grams (see Table 2).

3.2. Measurement of Serum Hcy and Serum GSH

Young adult and middle-aged data were merged together because there were no differences between the two population values.

Serum Hcy and GSH levels for the female rats increased in 1% L-methionine rats to 7.819 (2.91) µM [mean (SD)] and 32.303 (10.83) µM when compared to control at 4.87 (1.497) µM and 21.38 (8.88) µM respectively, although they were not statistically significant (Figure 1).

Serum Hcy levels of male Wistar rats were significantly higher in 1% L-methionine rats at 11.32 (7.05) µM [mean (SD)] $p<0.05$ when compared to control at 5.33 (1.26) µM.

Table 2. *Effect of L-methionine feeding on growth in (middle-aged adult) male and female Wistar rats. Data are means ± SD body weight (n=6). *p<0.001 vs. control.*

Sex	Body weight (g/30 weeks)	Body weight (g/30 weeks)
	Control	1% Methionine
Male	752.89±62.08	680.03±64.34
Female	376.14±15.50	257.88±50.80*

Serum GSH levels were only slightly lower in 1% L- methionine rats at 24.89 (7.56) µM when compared to control at 27.53 (7.84) µM (Figure 2).

4. Discussion

Our results have shown that 1% L-methionine supplementation in the drinking water caused a significant decrease in the body weight of young adult male Wistar rats compared to control and a similar trend in the body weight of young adult female Wistar rats in comparison to control (Table 1). Also, there was a trend towards a reduction in the body weight in the middle-aged male rats compared to control and a significant reduction in the middle-aged female rats compared to control (Table 2). This could be correlated with an increase in the rate of methionine metabolism due to excessive dietary methionine. It has been found that the amount of methionine required from the diet is less than 1% and excess amounts of methionine can be toxic [29]. Another study reported that excessive levels of methionine may convert into S-methyl-L-cysteine which is a toxic compound and leads to growth reduction [30]. These findings were similar to that obtained by Herrmann *et al*. [31] who found that supplementation with 2.4% methionine, 1% Hcy and 2% Hcy in female Wistar rats reduced body weight significantly. In addition, findings from other studies indicated that 1.6 gm/kg L-methionine feeding led to significantly slower growth rate of experimental rats compared to control [27]. However, El Aty *et al*. [32] found a significant increase in rats' body weights at tested concentrations (1%, 2% and 4%) of DL-methionine and also another study by Hegedus *et al*. [33] indicated that body weight increases at high dietary protein diet. The disagreement between the findings may possibly be due to various factors. For example, animal strain, feeding period, isomeric forms of methionine, examined concentration of methionine and mode of treatment, injection (subcutaneous or intraperitoneal (IP)) or supplementation.

This is the first study to investigate the effects of methionine supplementation in female rats. Our results indicated that the serum Hcy values of control groups are in agreement with those reported by Durand *et al*. [12] and Meng *et al*. for the male rat [34]. Serum Hcy values in the methionine groups are in agreement with that found by Meng *et al*. for the male rat [34]. We also report increased serum concentrations of Hcy in 1% L-methionine fed rats compared to control, which while not statistically significant for young adult and middle-aged (female) rats (Figure 1) became significant in young adult and middle-aged (male) rats (Figure 2). Dietary methionine plays an important role in the generation of Hcy through the demethylation pathway. This is a recognised method for inducing Hhcy [12].

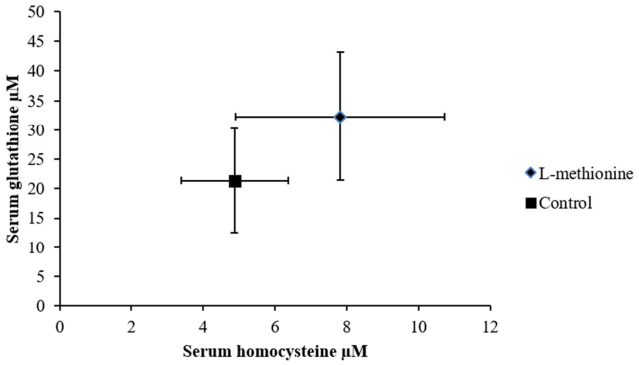

Figure 1. *Serum Hcy concentration (µM) versus serum GSH concentration (µM) in female rats fed with 1% L-methionine in comparison to control. Data shown are the means (SD).*

Our results are in agreement with de Rezende & D'Almeida [35] who found that 0.5% methionine supplementation in water increased plasma homocysteine concentration after 2 and 6 months and also that 1% methionine supplementation in water, increased plasma homocysteine concentration after 2, 4 and 6 months in male C57BL/6 mice. Results from other studies have also indicated that methionine or Hcy supplementation significantly raised plasma total Hcy levels, which accelerates plaque growth and boosts plaque fibrosis in apoE-/- mice [36] and also influences myocardial brain natriuretic peptide (BNP) concentrations in rats [31]. A study by Nygård *et al.* [37] revealed that human plasma Hcy levels are higher in men than in women and increases with age. Our results also indicated that males have higher serum Hcy levels

than females, although the difference was not significant in the rat. We did not observe any differences in Hcy levels with age. The mechanism of sex differences in Hcy concentrations may be due to alterations in rates of Hcy remethylation [38]. A small increase in serum GSH concentrations in 1% L-methionine fed rats of young adult and middle-aged female rats, although they were not statistically significant (Figure 1). This finding suggested that methionine loading improves GSH synthesis, which is in agreement with Bianchi *et al*. [39] who found that in human cirrhosis methionine flux is reduced through the transmethylation/transsulfuration pathway, which in turn reduces GSH synthesis. Mosharov *et al*. [40] who reported that Hcy dependent transsulfuration pathway is vital in sustaining the intracellular GSH pool. In males, there were no changes in serum GSH concentrations in 1% L-methionine fed rats of young adult and middle-aged male rats compared to controls (Figure 2). Given the significant increase in serum Hcy concentrations, changes in redox balance and impaired antioxidant defence mechanisms may be involved in this group. Indeed, Vyas *et al*. [41] found that GSH concentrations can significantly decrease during oxidative stress (increased free radical generation) in osteoarthritis patients. Moreover, another finding by Pastore *et al*. [42] demonstrated that in patients with non-alcoholic fatty liver diseases an increase in oxidative stress can be associated with rising plasma Hcy and cysteine levels and depletion of GSH levels. Changes in GSH levels can be critical in many cases such as inherited or acquired defects in the transporters, enzymes, transcription factors that are essential in its homeostasis, signalling molecules, or exposure to reactive chemicals or metabolic intermediates [43].

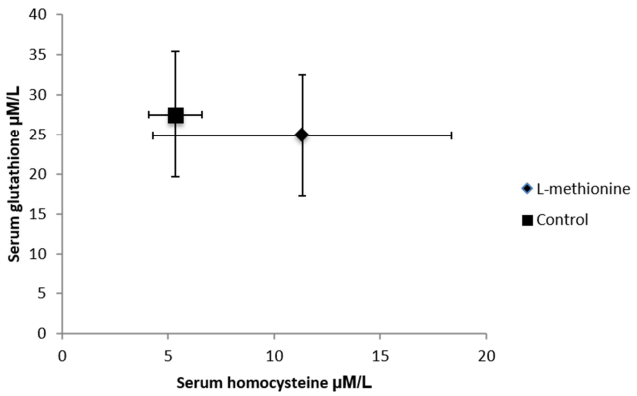

Figure 2. *Serum Hcy concentration (µM) versus serum GSH concentration (µM) in male Wistar rats fed with 1% L-methionine in comparison to control. Data shown are the means (SD).*

5. Conclusion

The results presented here show that L-methionine supplementation affects methionine-homocysteine metabolism cycle resulting in increased serum Hcy and serum GSH levels. Male rats have higher serum Hcy levels than females. The positive interactions between serum Hcy and serum GSH concentrations propose a possible common or analogous controlling mechanism in females.

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