Research Article

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Comparative *in vivo* antioxidant capacity of DL-2-hydroxy-4-methylthiobutanoic acid (HMTBA) and DL-methionine in male mice fed a high-fat diet

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Abstract

BACKGROUND: In animal diets, methionine (Met) is considered to be the first limiting amino acid, and the activity of synthetic Met is typically added either as DL-methionine (DLM) or as DL-2-hydroxy-4-methylthiobutanoic acid (HMTBA). It has been demonstrated that HMTBA exhibits a higher antioxidant capability *in vitro* as compared to DLM. However, the difference in antioxidant capability between DLM and HMTBA *in vivo* is unknown.

METHODS: In the present study, 60 male C57BL/6 mice were randomly divided into six groups and fed either a normal diet (NFD, 5.37% fat) or a high-fat diet (HFD, 19.7% fat) in conjunction with 0.2% DLM, 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA for 4 weeks.

RESULTS: HFD supplemented with 2% DLM and NFD with 2% HMTBA both induced adverse affects in relation to serum lipid parameters and depressed antioxidant defense systems in the digestive system. However, these changes were restored in the 0.2% HMTBA-treated HFD group. Furthermore, no significant differences were found in the lipid parameters and antioxidant status in the NFD and HFD group supplemented with 0.1% DLM and 0.1% HMTBA.

CONCLUSION: HMTBA restored oxidative redox status under OS conditions and its antioxidant properties were positively correlated with the dosage included in diet.

Keywords: 2-hydroxy-4-methylthiobutanoic acid; DL-methionine; high-fat diet; antioxidant capacity; mice

INTRODUCTION

Synthetic sources of dietary methionine (Met), such as DL-methionine (DLM) or its corresponding hydroxy analogue, 2-hydroxy-4-methylthiobutanoic acid (HMTBA), are commonly added to commercial animal diets to satisfy the current total sulfur amino acid requirements for growth and maintenance. The bioefficacy of HMTBA compared with DLM has been the subject of numerous studies[1–4] and remains controversial today.

Although DLM and HMTBA provide methionine activity, they are structurally different molecules with different physiological characteristics until they are converted to L-methionine. They exert different influence on animal performance depending on the dietary level used or depending on environmental conditions (e.g. under heat stress). A study by Vázquez-Añón *et al*.[5] revealed that HMTBA and DLM have different dose–response patterns. HMTBA out-performed DLM at levels used in commercially available feed whilst DLM out-performed HMTBA at reduced levels. Feeding HMTBA at commercial levels resulted in a greater weight gain response than that predicted by the dose–response curve with the best goodness of fit being linear for HMTBA and quadratic for DLM. Under the average conditions described in the database literature, the predicted responses for the weight gain and feed conversion models suggest that HMTBA provides greater benefits than DLM at commercially relevant levels of supplementation.[6] Under chronic heat stress conditions broilers fed HMTBA with a low Arg : Lys ratio utilised dietary protein better than those fed L-Met only.[7]

Differences in the absorption of HMTBA and DLM may be the reason for the variation in growth performance. It has been demonstrated that HMTBA is absorbed mainly by monocarboxylate transporter 1, while absorption of DLM takes place via active transport and carrier-mediated uptake.[8] HMTBA is completely absorbed along the entire gastrointestinal tract, especially the upper gastrointestinal tract.[9] The rates of HMTBA absorption were significantly greater than those of DLM during periods of heat stress.[10] Many studies have described the main metabolic fate of these two

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Met sources to be transmethylation to homocysteine which was found to induce the formation of oxygen free radicals (ROS) in the mitochondria.[11,12] The production of homocysteine within the intestinal mucosa may contribute to oxidative stress (OS) which would mediate an inflammatory response and weaken the mucosal barrier.[13] Restricting the supply of methionine may alter claudin composition and thus improve the function of the tight junction barrier.[14] Xie *et al*.[15] reported that supplementation with HMTBA produced less homocysteine in the plasma than DLM supplementation. Homocysteine is a toxic methionine metabolite and the mechanism of homocysteine toxicity has been reviewed by Perna *et al*.[16] In contrast, the cysteine and taurine content of chicken enterocytes was higher when HMTBA was used as the source of Met.[17] Cysteine and taurine derived from HMTBA are functional constituents of intestinal antioxidant systems and would impact several elements of redox status that regulate epithelial intracellular signaling, proliferation and survival. Our previous study (unpublished results) demonstrated that *in vitro* HMTBA had greater free radical scavenging ability than DLM, especially in relation to hydroxy radicals. There is now considerable evidence that an increased production of ROS plays a major role in heat stress and OS and is one of the earliest identifiable inducers of the heat shock response.[18] Therefore, HMTBA might not only provide methionine but also serve as a source of antioxidants under OS conditions.

Furthermore, recent research has focused on the dose–response of the two Met sources to improve the growth performance in animal. There is a need for studies to identify dietary supplementation of HMTBA that may be serve as a potential antioxidant to regulate redox status. We hypothesised that HMTBA could restore oxidative redox status under OS conditions and its antioxidant properties may be positively correlated with the dosage included in diet. Therefore, the objective of the present study was to elucidate the impact of different levels of HMTBA and DLM on the antioxidant capacity of the blood and digestive system in male mice fed either a normal diet or a high-fat diet.

EXPERIMENTAL

Animals

Sixty male C57BL/6 mice (6 weeks old, 18 ± 1 g) were obtained from the Shanghai Laboratory Animal Center, Chinese Academy Sciences. The animals were housed under conditions of controlled temperature (23 ± 2 °C) and humidity (60%) and were exposed to natural light. The experimental protocol was developed according to the institution's guideline for the care and use of laboratory animals by Jiangnan University.

Experimental design

Before the study commenced, the test animals were initially fed standard diets for 1 week. After this period of adaptation the animals were then randomly divided into six groups each fed with a different diet (10 mice per group). These feeding regimes included a normal-fat diet (NFD) containing 5.37% fat supplemented with 0.2% DLM (control), 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA, or a high-fat diet (HFD) containing 19.7% fat supplemented with 0.2% DLM, 0.2% HMTBA, or 0.1% DLM and 0.1% HMTBA (Table 1). All mice were allowed free access to the test diets and deionised water throughout the 4-week test period.

At the end of the experimental periods, the mice were deprived of food for 12 h, lightly anaesthetised and sacrificed by decapitation. Blood was collected in microcentrifuge tubes containing heparin and used immediately for the ROS assay. Plasma samples were prepared from the blood and stored at −70 °C until further analysis. The liver, kidney, duodenum, jejunum and pancreas were immediately dissected, weighed, and processed for ROS and other biochemical assays. Abdominal adipose tissue and leg muscle were also removed and weighed.

Table 1. Compositions of the diets

	Diet	
Ingredient (%, w/w)	Normal-fat diet (NFD)	Higt-fat diet (HFD)
Cornmeal	52.94	29.75
Soybean meal	25.00	28.00
Wheat flour	9.00	11.32
Wheat bran	6.50	9.20
Lard[1]	2.80	18.00
NaCl	0.20	0.20
$CaHPO_4$	1.15	1.25
$CaCO_3$	1.65	1.60
AIN-76 minerals	0.06	0.06
AIN-76 vitamins	0.02	0.02
Lysine	0.30	0.21
DLM or HMTBA	0.20/0.10	0.20/0.10
Sucrose	0.10	0.10
Sinkaline	0.10	0.10
Fat (%, w/w)	5.37	19.70
Methionine[2] (%, w/w)	0.20	0.21
Energy (kcal g^{-1} diet)	3.93	4.78

[1] Lard provides the following (g 100 g^{-1} lard): 14 : 0, 2.0; 14 : 1, 0.3; 15 : 1, 0.1; 16 : 0, 26.5; 16 : 1, 3.7; 17 : 0, 0.5; 17 : 1, 0.4; 18 : 0, 12.1; 18 : 1, 42.5; 18 : 2(ω-6), 9.8; 18 : 3(ω-3), 0.7; 20 : 0, 0.2; 20 : 1, 0.6; 20 : 4(ω-6), 0.3.
[2] Analysed values from the common basal diet.

Measurement of serum lipids

For determination of serum total triglyceride (TG), cholesterol (TC), high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C) concentrations, the corresponding diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, P.R. China) were used according to the manufacturer's instructions. The lipoproteins LDL-C and HDL-C were fractionated using a dual precipitation technique.[19] After fractional precipitation, lipoprotein cholesterol was estimated. The atherosclerosis index (AI) was calculated using the following formula (TC – HDL-C)/HDL-C.

Assay for reactive oxygen species

ROS levels were measured by chemiluminescence in the presence of luminal (0.5 mmol L^{-1}) and horseradish peroxidase (12 U mL^{-1}) using a thermostatically (37 °C) controlled luminometer (MPI-B Multiparameter Chemiluminescence Analysis System, Xian Ruimai Analytical Instruments Co., Ltd, Xian, China) as described previously.[20] The area under curve was integrated to give the total intensity of the chemiluminescence response. Results were expressed as the area under curve per millilitre of plasma or milligram of protein.

Determination of lipid peroxidation

Free radical damage was determined by specifically measuring malondialdehyde (MDA). The method was as described

previously.[21] MDA which is formed as an end product of lipid peroxidation was treated with thiobarbituric acid to generate a coloured product that was measured at 532 nm (MDA detecting kit purchased from Jiancheng Bioengineering Institute, Nanjing, China).

Measurements of antioxidant status

Total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activity in serum and tissue were assayed with the appropriate test kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, P.R. China).

Statistical analysis

Data are representative of mean ± SD for 10 animals with two repeats, in total. Comparisons across groups were carried out using one-way analysis of variance with the post-hoc Duncan's test. $P < 0.05$ was considered to be statistically significant. Analysis of the data was achieved using SPSS 13 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Performance parameters

As shown in Table 2, HFD-fed groups supplemented with 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA exhibited significantly lower food intake and daily weight gain as compared to the control group. A similar tendency was also observed in NFD-fed group supplemented with 0.2% HMTBA. When the HFD groups (0.2% DLM, 0.2% HMTBA, or 0.1% DLM and 0.1% HMTBA) were compared, 0.2% HMTBA supplementation caused a significant decrease in abdominal fat pads. There was no significant difference in weight gain or the percentage of leg muscle across the six groups. However, under OS conditions the HMTBA-containing diet led to improved feeding and weight gain compared to diets containing DLM mainly due to the overall lower feed intake under these conditions.

Plasma lipids level and atherosclerosis index

Table 3 shows the effect of DLM and HMTBA on the serum lipid status in male mice. Feeding the experimental animals 0.2% DLM in HFD and 0.2% HMTBA in NFD for 4 weeks resulted in the development of hyperlipidaemia. There were significant increases in TC, TG, LDL-C and AI. All these abnormalities were considerably reduced when the HFD diet was supplemented with 0.2% HMTBA and a marked increase in the level of HDL-C was also observed. Treatment with 0.1% DLM and 0.1% HMTBA partially restored the serum lipid status to that observed in animals fed a diet supplemented with 0.2% DLM but remained higher than that in the control animals.

Table 2. Effect of DL-methionine (DLM) and 2-hydroxy-4-methylthiobutanoic acid (HMTBA) on growth performance and carcass composition of male mice

Group	DWG (g mice d^{-1})	DFI (g mice d^{-1})	Feed/gain (g g^{-1})	PDL (%)	PDAT (%)
NFD + 0.2% DLM	0.53 ± 0.04	4.78 ± 0.30^{b}	8.95 ± 0.85^{bc}	9.91 ± 1.00	1.61 ± 0.33^{a}
NFD + 0.2% HMTBA	0.49 ± 0.08	3.53 ± 0.44^{a}	6.84 ± 0.74^{a}	9.83 ± 1.31	1.81 ± 0.53^{a}
NFD + 0.1% DLM + 0.1% HMTBA	0.51 ± 0.04	4.53 ± 0.31^{b}	9.68 ± 0.69^{c}	11.27 ± 0.38	2.03 ± 0.57^{a}
HFD + 0.2% DLM	0.50 ± 0.06	4.32 ± 0.24^{b}	8.61 ± 0.47^{b}	10.67 ± 1.41	2.17 ± 0.41^{a}
HFD + 0.2% HMTBA	0.53 ± 0.08	3.44 ± 0.25^{a}	6.54 ± 0.46^{a}	10.79 ± 1.22	1.66 ± 0.44^{a}
HFD + 0.1% DLM + 0.1% HMTBA	0.54 ± 0.04	3.35 ± 0.21^{a}	6.25 ± 0.24^{a}	10.62 ± 2.46	2.53 ± 0.52^{b}

Sixty male C57BL/6 mice were randomly divided into six groups and fed either a normal diet (NFD, 5.37% fat) or a high-fat diet (HFD, 19.7% fat) in conjunction with 0.2% DLM, 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA for 4 weeks.
DWG, daily weight gain; DFI, daily feed intake; PDL, leg muscle weight (without bone and skin)/final body weight; PDAT, abdominal fat weight/final body weight.
Data are representative of mean ± SD for ten animals.
[a,b,c,d] Means with different superscript letters within a row are significantly different ($P < 0.05$).

Table 3. Effects of DL-methionine (DLM) and 2-hydroxy-4-methylthiobutanoic acid (HMTBA) on serum lipid status in male mice

Group	HDL-C (mmol L^{-1})	LDL-C (mmol L^{-1})	TG (mmol L^{-1})	TC (mmol L^{-1})	AI
NFD + 0.2% DLM	2.66 ± 0.49^{b}	1.87 ± 0.54^{a}	1.21 ± 0.21^{ab}	3.25 ± 0.57^{a}	0.30 ± 0.10^{a}
NFD + 0.2% HMTBA	1.90 ± 0.45^{a}	3.44 ± 0.98^{c}	1.62 ± 0.43^{c}	3.43 ± 0.14^{ab}	0.5 ± 0.19^{bc}
NFD + 0.1% DLM + 0.1% HMTBA	2.69 ± 0.40^{b}	1.88 ± 0.53^{a}	1.23 ± 0.19^{b}	3.22 ± 0.43^{a}	0.34 ± 0.07^{a}
HFD + 0.2% DLM	2.83 ± 0.36^{b}	2.71 ± 0.45^{b}	1.33 ± 0.38^{b}	3.93 ± 0.76^{b}	0.62 ± 0.22^{c}
HFD + 0.2% HMTBA	3.13 ± 0.22^{c}	1.80 ± 0.42^{a}	0.93 ± 0.10^{a}	3.29 ± 0.41^{a}	0.28 ± 0.03^{a}
HFD + 0.1% DLM + 0.1% HMTBA	2.89 ± 0.31^{b}	2.52 ± 0.54^{b}	1.27 ± 0.24^{b}	3.89 ± 0.63^{b}	0.44 ± 0.12^{b}

Sixty male C57BL/6 mice were randomly divided into six groups and fed either a normal diet (NFD, 5.37% fat) or a high-fat diet (HFD, 19.7% fat) in conjunction with 0.2% DLM, 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA for 4 weeks.
HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; AI, atherosclerosis index.
Data are representative of mean ± SD for 10 animals.
Means with different superscript letters within a row are significantly different ($P < 0.05$).

Table 4. Effects of DL-methionine (DLM) and 2-hydroxy-4-methylthiobutanoic acid (HMTBA) on ROS levels in male mice

Group	Plasma (AUC μL^{-1})	Liver (AUC mg^{-1})	Kidney (AUC mg^{-1})	Duodenum (AUC mg^{-1})	Jejunum (AUC mg^{-1})	Pancreas (AUC mg^{-1})
NFD + 0.2% DLM	1202.2 ± 144.4^{a}	2604.2 ± 218.9^{a}	5042.5 ± 561.5^{ab}	2553.6 ± 146.3^{c}	1954.7 ± 144.8^{b}	6365.3 ± 762.5^{b}
NFD + 0.2% HMTBA	2319.6 ± 229.4^{c}	$4821.2 \pm 563.9.2^{c}$	6201.6 ± 414.3^{c}	2639.6 ± 207.2^{c}	2479.4 ± 394.1^{c}	6589.6 ± 407.2^{b}
NFD + 0.1% DLM + 0.1% HMTBA	1239.6 ± 207.2^{a}	2501.9 ± 286.7^{a}	5165.9 ± 260.7^{ab}	1880.6 ± 88.4^{a}	1655.5 ± 222.1^{a}	6449.5 ± 692.5^{b}
HFD + 0.2% DLM	1542.7 ± 261.3^{b}	3458.5 ± 534.0^{b}	5552.8 ± 334.8^{b}	2558.1 ± 359.6^{c}	2366.8 ± 259.4^{c}	6733.0 ± 695.5^{b}
HFD + 0.2% HMTBA	1178.4 ± 184.3^{a}	2452.7 ± 460.1^{a}	4580.1 ± 407.9^{a}	2059.2 ± 217.8^{ab}	1945.4 ± 199.3^{b}	5221.1 ± 366.6^{a}
HFD + 0.1% DLM + 0.1% HMTBA	1500.2 ± 267.6^{b}	3270.5 ± 296.8^{b}	5110.4 ± 543.2^{ab}	2139.6 ± 161.0^{b}	1969.7 ± 180.8^{b}	8230.3 ± 387.9^{c}

Sixty male C57BL/6 mice were randomly divided into six groups and fed either a normal diet (NFD, 5.37% fat) or a high-fat diet (HFD, 19.7% fat) in conjunction with 0.2% DLM, 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA for 4 weeks.
Data are representative of mean ± SD for 10 animals.
Means with different superscript letters within a row are significantly different ($P < 0.05$).

Table 5. Effects of DL-methionine (DLM) and 2-hydroxy-4-methylthiobutanoic acid (HMTBA) on level of malondialdehyde (MDA) in male mice

Group	Serum (nmol mL^{-1})	Liver (nmol mg $prot^{-1}$)	Kidney (nmol mg $prot^{-1}$)	Duodenum (nmol mg $prot^{-1}$)	Jejunum (nmol mg $prot^{-1}$)	Pancreas (nmol mg $prot^{-1}$)
NFD + 0.2% DLM	24.77 ± 2.04^{ab}	0.78 ± 0.14^{ab}	1.73 ± 0.27^{a}	1.50 ± 0.26^{a}	1.70 ± 0.23^{b}	2.47 ± 0.26^{a}
NFD + 0.2% HMTBA	46.10 ± 5.48^{c}	0.99 ± 0.15^{b}	2.19 ± 0.34^{b}	3.12 ± 0.47^{c}	2.32 ± 0.63^{c}	3.38 ± 0.39^{c}
NFD + 0.1% DLM + 0.1% HMTBA	21.98 ± 2.13^{a}	0.69 ± 0.11^{a}	1.60 ± 0.19^{a}	1.23 ± 0.28^{a}	1.36 ± 0.28^{ab}	2.19 ± 0.28^{a}
HFD + 0.2% DLM	27.52 ± 2.60^{b}	2.44 ± 0.41^{d}	2.51 ± 0.30^{c}	3.27 ± 0.41^{c}	2.10 ± 0.11^{c}	3.52 ± 0.41^{c}
HFD + 0.2% HMTBA	21.18 ± 5.45^{a}	2.09 ± 0.42^{c}	2.10 ± 0.26^{b}	1.40 ± 0.41^{a}	1.22 ± 0.30^{a}	2.91 ± 0.41^{b}
HFD + 0.1% DLM + 0.1% HMTBA	26.61 ± 3.77^{b}	2.22 ± 0.21^{cd}	2.16 ± 0.13^{b}	2.43 ± 0.42^{b}	1.64 ± 0.38^{b}	3.30 ± 0.42^{bc}

Sixty male C57BL/6 mice were randomly divided into six groups and fed either a normal diet (NFD, 5.37% fat) or a high-fat diet (HFD, 19.7% fat) in conjunction with 0.2% DLM, 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA for 4 weeks.
Data are representative of mean ± SD for 10 animals.
Means with different superscript letters within a row are significantly different ($P < 0.05$).

Levels of reactive oxygen species and malondialdehyde levels in plasma and the digestive system

As shown in Tables 4 and 5, there was an increase in reactive oxygen species (ROS) and malondialdehyde (MDA) levels in the plasma and digestive system (liver, kidney, duodenum, jejunum and pancreas) of the HFD-fed group treated with 0.2% DLM. A similar tendency was also observed in the NFD-fed group supplemented with 0.2% HMTBA. Supplementation with 0.2% HMTBA in the HFD group reduced ROS and MDA production, which suggested that the decrease in ROS and MDA release may be related to the HMTBA-induced antioxidant activity under OS.

Antioxidant status in plasma and the digestive system

The antioxidant status is shown in Tables 6, 7, 8 and 9. There was a decrease in TAC, SOD, CAT and GSH-Px activity in the HFD group fed 0.2% DLM and the NFD group fed 0.2% HMTBA. However, supplementation of 0.2% HMTBA brought about a significant improvement in antioxidant defences in the HFD-fed mice. Supplementation with 0.1% DLM and 0.1% HMTBA partially restored the antioxidant capability to that produced by supplementation with 0.2% DLM but remained lower than that in the control group.

DISCUSSION

Methionine is a limiting essential amino acid in animal diets. To meet the nutritional requirements for this amino acid, dietary supplementation with synthetic sources of Met, such as DLM or HMTBA, is common practice in the production of animal feed. It has been demonstrated that HMTBA which is considered to be an organic acid, differs from Met in having a hydroxyl group on the alpha carbon rather than an amino group.[22] Because HMTBA contains a hydroxyl group instead of an amino group, it may exhibit antioxidant properties and serve as an antioxidant source under conditions of OS. Our previous study (unpublished results) indicated that *in vitro* HMTBA had a superior ability to scavenge free radicals compared with DLM, especially hydroxy radicals. Thus, the present study explored the effect of DLM and HMTBA on the antioxidant capacity of blood and the digestive system of mice fed an HFD.

Previous studies have demonstrated that HFD-mediated oxidative stress diminished the antioxidant capacity of the blood and digestive system, thus resulting in abnormal lipid metabolism and consequently dyslipidemia.[23,24] In the present study, results of the serum lipid status of HFD-fed mice treated with 0.2% DLM for 4 weeks showed increased concentrations of serum TC, TG and LDL-C, whereas the concentration of HDL-C was decreased as compared to the control group. The elevation in serum total TC and TG levels observed in our study following a HFD are in agreement with the results of several other studies,[25,26] which showed that abnormal lipid metabolism was observed in growing rats fed with diets containing high cholesterol and fat. However, treatment of HFD-fed rats with 0.2% HMTBA showed a considerable restoration of these parameters to the levels observed in animals fed a NFD. The AI, defined as the ratio of (TC – HDL-C)/HDL-C, is believed to be an important risk factor for atherosclerosis. Our results demonstrated that 0.2% HMTBA significantly decreased this ratio. It has been shown that abnormally high serum levels of LDL-C and low serum levels of HDL-C are associated with an increased

Table 6. Effects of DL-methionine (DLM) and 2-hydroxy-4-methylthiobutanoic acid (HMTBA) on total antioxidant capacity (TAC) in male mice

Group	Serum (U mL^{-1})	Liver (U mg prot^{-1})	Kidney (U mg prot^{-1})	Duodenum (U mg prot^{-1})
NFD + 0.2% DLM	1.23 ± 0.13^{cd}	0.15 ± 0.01^{cd}	0.16 ± 0.03^{c}	1.32 ± 0.10^{ab}
NFD + 0.2% HMTBA	0.38 ± 0.70^{a}	0.05 ± 0.02^{a}	0.029 ± 0.01^{a}	1.15 ± 0.37^{a}
NFD + 0.1% DLM + 0.1% HMTBA	1.31 ± 0.22^{d}	0.16 ± 0.02^{d}	0.17 ± 0.05^{c}	1.45 ± 0.18^{ab}
HFD + 0.2% DLM	0.74 ± 0.16^{b}	0.14 ± 0.022^{c}	0.11 ± 0.01^{b}	1.26 ± 0.13^{a}
HFD + 0.2% HMTBA	1.35 ± 0.33^{d}	0.23 ± 0.022^{e}	0.22 ± 0.05^{d}	1.53 ± 0.30^{b}
HFD + 0.1% DLM + 0.1% HMTBA	1.09 ± 0.18^{c}	0.10 ± 0.01^{b}	0.17 ± 0.02^{c}	1.31 ± 0.18^{ab}

Sixty male C57BL/6 mice were randomly divided into six groups and fed either a normal diet (NFD, 5.37% fat) or a high-fat diet (HFD, 19.7% fat) in conjunction with 0.2% DLM, 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA for 4 weeks.
Data are representative of mean ± SD for 10 animals.
Means with different superscript letters within a row are significantly different ($P < 0.05$).

Table 7. Effects of DL-methionine (DLM) and 2-hydroxy-4-methylthiobutanoic acid (HMTBA) on activity of superoxide dismutase (SOD) in male mice

Group	Liver (U mg prot^{-1})	Kidney (U mg prot^{-1})	Duodenum (U mg prot^{-1})
NFD + 0.2% DLM	17.04 ± 4.08^{b}	12.98 ± 1.76^{b}	11.17 ± 2.53^{ab}
NFD + 0.2% HMTBA	12.16 ± 0.72^{a}	13.35 ± 2.58^{b}	10.28 ± 1.14^{ab}
NFD + 0.1% DLM + 0.1% HMTBA	17.46 ± 2.24^{b}	11.35 ± 0.86^{a}	12.34 ± 3.70^{b}
HFD + 0.2% DLM	16.77 ± 2.32^{b}	10.29 ± 0.87^{a}	10.63 ± 1.60^{ab}
HFD + 0.2% HMTBA	21.57 ± 3.62^{c}	13.77 ± 1.07^{b}	9.96 ± 2.25^{ab}
HFD + 0.1% DLM + 0.1% HMTBA	19.64 ± 3.44^{ab}	9.87 ± 0.77^{a}	8.41 ± 1.41^{a}

Sixty male C57BL/6 mice were randomly divided into six groups and fed either a normal diet (NFD, 5.37% fat) or a high-fat diet (HFD, 19.7% fat) in conjunction with 0.2% DLM, 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA for 4 weeks.
Data are representative of mean ± SD for 10 animals.
Means with different superscript letters within a row are significantly different ($P < 0.05$).

risk for atherosclerosis.[27,28] The increased HDL-C concentrations and decreased LDL-C concentrations in HFD-fed mice provides an indication of the anti-atherogenic properties of HMTBA.

In the present study, consumption of a HFD induced an increase in the weight of the abdominal adipose tissue in C57BL/6 mice. Excessive fat intake can induce the hypersecretion of insulin which then increases nutrient uptake and, subsequently, an increased electron flow along the mitochondrial respiratory chain resulting in an increase in ROS generation and further inducing oxidative stress.[29] Our data indicate that ROS production in the blood and digestive system (liver, kidney, and jejunum) significantly increased in HFD-fed mice supplemented with 0.2% DLM. On the other hand, the presence of MDA which is a major reactive aldehyde that is formed during peroxidation of polyunsaturated fatty acids in the biological membrane is an indicator of lipid peroxidation and oxidative damage.[30] In our previous studies, HFD-mediated oxidative stress diminishes the antioxidant defense system thus leading to damage of the lipids, namely peroxidation.[23,24] As expected, a significant increase in MDA concentration in the digestive system (liver, kidney, duodenum, jejunum and pancreas) was also observed in the HFD-fed mice supplemented with 0.2% DLM. Conversely, the group fed a HFD in conjunction with 0.2% HMTBA exhibited a pronounced decreased in ROS and MDA levels as compared to the control group. Currently, many antioxidants have been reported to prevent or at least attenuate damage caused by a HFD-induced oxidative stress.[31,32] These effects of HMTBA could be attributed to its antioxidant properties which regulate redox status, thus maintaining normal tissue and cellular function.

ROS are produced by aerobic organisms in which low concentrations are beneficial and are related to several cellular processes. However, an increase in ROS levels leads to oxidative stress which may cause oxidative modifications to lipids, nucleic acids and proteins. Tissue or cellular antioxidant capacity can act as a defence system against ROS-mediated damage.[33] These systems include scavenger enzymes, such as SOD, CAT and GSH-Px.[34] The present results show that HFD treatment caused a significant down-regulation of SOD, CAT and GSH-Px activities in serum and the digestive system, as well as in the antioxidant capacity marker TAC, which is in keeping with the results from our previous studies in rats and mice.[23,35] Conversely, supplementation with 0.2% HMTBA instead of DLM to HFD-fed mice could prevent the build-up of oxidative stress which is apparent from the enhanced TAC, SOD, CAT and GSH-Px activities. TAC is a component of the antioxidant system and is considered to be an important indicator of antioxidant capacity. SOD destroys the radical superoxide by catalysing its breakdown to produce hydrogen peroxide, which can be eliminated by CAT or GSH-Px. Many processes can lead to the modification of the antioxidant enzymes including ageing, nutrition and drugs.[36] The molecular mechanisms by which HMTBA circumvents the synergistic effects of the antioxidant enzymes activity are not fully understood. Homocysteine, a toxic methionine metabolite, acts as a toxicity biomarker indicating the presence of excess methionine *in vivo*.[16] Evidence has indicated that supplementation with HMTBA does not produce as much plasma homocysteine as supplementation with DLM.[15] In contrast, concentrations of cysteine and taurine which are derived from HMTBA are higher in chicken enterocytes when HMTBA is used

Table 8. Effects of DL-methionine (DLM) and 2-hydroxy-4-methylthiobutanoic acid (HMTBA) on activity of catalase (CAT) in male mice

Group	Serum (U mL^{-1})	Liver (U mg prot^{-1})	Kidney (U mg prot^{-1})	Duodenum (U mg prot^{-1})
NFD + 0.2% LM	5.82 ± 0.39^{b}	42.83 ± 4.55^{b}	85.80 ± 5.73^{c}	14.57 ± 0.88^{c}
NFD + 0.2% HMTBA	6.16 ± 0.90^{bc}	31.39 ± 2.48^{a}	68.05 ± 7.82^{a}	8.26 ± 3.09^{a}
NFD + 0.1%DLM + 0.1% HMTBA	7.51 ± 0.43^{d}	45.61 ± 1.86^{b}	85.99 ± 7.79^{c}	17.80 ± 1.52^{d}
HFD + 0.2% LM	4.76 ± 0.53^{a}	41.96 ± 3.09^{b}	76.25 ± 6.41^{ab}	10.73 ± 0.83^{b}
HFD + 0.2% HMTBA	6.80 ± 0.71^{c}	70.10 ± 2.75^{c}	84.88 ± 8.48^{c}	12.42 ± 0.44^{c}
HFD + 0.1%DLM + 0.1% HMTBA	5.65 ± 0.50^{b}	42.88 ± 3.84^{a}	80.76 ± 4.01^{bc}	13.53 ± 1.87^{c}

Sixty male C57BL/6 mice were randomly divided into six groups and fed either a normal diet (NFD, 5.37% fat) or a high-fat diet (HFD, 19.7% fat) in conjunction with 0.2% DLM, 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA for 4 weeks.
Data are representative of mean ± SD for 10animals.
Means with different superscript letters within a row are significantly different ($P < 0.05$).

Table 9. Effects of DL-methionine (DLM) and 2-hydroxy-4-methylthiobutanoic acid (HMTBA) on activity of glutathione peroxidase (GSH-Px) in male mice

Group	Serum (U mL^{-1})	Liver (U mg prot^{-1})	Kidney (U mg prot^{-1})	Duodenum (U mg prot^{-1})
NFD + 0.2% DLM	0.10 ± 0.15^{b}	0.23 ± 0.03^{c}	0.158 ± 0.01^{bc}	0.65 ± 0.08^{b}
NFD + 0.2% HMTBA	0.93 ± 0.13^{b}	0.14 ± 0.01^{a}	0.06 ± 0.01^{a}	0.54 ± 0.03^{a}
NFD + 0.1% DLM + 0.1% HMTBA	1.01 ± 0.07^{b}	0.33 ± 0.02^{d}	0.158 ± 0.01^{bc}	0.87 ± 0.05^{c}
HFD + 0.2% DLM	0.62 ± 0.08^{a}	0.185 ± 0.02^{b}	0.142 ± 0.01^{b}	0.57 ± 0.06^{a}
HFD + 0.2% HMTBA	1.17 ± 0.06^{c}	0.350 ± 0.02^{d}	0.168 ± 0.02^{c}	0.61 ± 0.05^{ab}
HFD + 0.1% DLM + 0.1% HMTBA	0.63 ± 0.04^{a}	0.226 ± 0.02^{c}	0.123 ± 0.03^{b}	0.61 ± 0.04^{ab}

Sixty male C57BL/6 mice were randomly divided into six groups and fed either a normal diet (NFD, 5.37% fat) or a high-fat diet (HFD, 19.7% fat) in conjunction with 0.2% DLM, 0.2% HMTBA or 0.1% DLM and 0.1% HMTBA for 4 weeks.
Data are representative of mean ± SD for 10 animals.
Means with different superscript letters within a row are significantly different ($P < 0.05$).

as a Met source.[17] As a glutathione precursor, cysteine plays a key role in antioxidant functions in the intestinal epithelial, and it may also regulate epithelial cell proliferation via modulation of redox status.[37] Taurine is involved in many physiological functions including osmoregulation, detoxification, and antioxidation.[38] These findings indicate that HMTBA has an effect on these enzyme activities, probably via its metabolic pathways and that further investigation is clearly required. Moreover, supplementation with 0.1% DLM and 0.1% HMTBA in the NFD and HFD groups did not produce any significant differences in the lipid parameters or antioxidant status as compared to the control group. From the above findings, it is important to note that HMTBA was able to restore oxidative redox status under OS conditions and that its antioxidation properties were positively correlated with its concentration in the diet.

Another interesting observation from this study was that the NFD-fed group supplemented with 0.2% HMTBA showed increased ROS and MDA levels and decreased antioxidant status in plasma and the digestive system, as compared to the NFD group supplemented with 0.2% DLM. The results indicate that mice fed a normal-fat diet containing 0.2% DLM showed better growth performance and antioxidant capacity than those fed HMTBA only and that HMTBA may act as pro-oxidant under normal conditions. The relative importance of the antioxidant and pro-oxidant activities are an area of current research. Antioxidants that are reducing agents can also act as pro-oxidants. For example, vitamin C has antioxidant activity as shown by its ability to reduce oxidising substances such as hydrogen peroxide; it will, however, also reduce via the Fenton reaction metal ions which generate free radicals.[39] Vázquez-Añón *et al*.[5] reported that HMTBA and DLM exhibit different dose–response patterns. HMTBA outperforms DLM at levels used in commercial feed production whilst DLM outperforms HMTBA at reduced levels. However, neither the mechanism nor the function of the HMTBA-dependent antioxidant activity is known. Further study is needed to more precisely validate this hypothesis.

CONCLUSION

The present results indicate for the first time that HMTBA not only serves as a source of Met but can also serve as a dietary supplement to considerably improve certain metabolic disorders and address the redox imbalance induced by HFD.

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