Methionine Supplementation Did Not Augment Oxidative Stress, Atherosclerotic Changes and Hepatotoxicity Induced by High Cholesterol Diet in C57BL/6J Mice

Jale BALKAN1, Semra DOĞRU-ABBASOĞLU1, Uğur ÇEVİKBAŞ2, Gülcin AYKAÇ-TOKER1 and Müjdat UYSAL1,*

1Department of Biochemistry and 2Department of Pathology, Istanbul Faculty of Medicine, Istanbul University, 34093 Çapa-Istanbul, Turkey

Summary The purpose of this study was to investigate the effect of a high-methionine plus cholesterol diet (HM+HC) on plasma, erythrocyte, liver and aorta lipid, lipid peroxide levels, and the liver antioxidant system, as well as hepatic and aortic histopathology in C57BL/6J mice, and to compare these results to those observed following administration of a high-methionine (HM) or high-cholesterol diet (HC) alone. Mice were fed diets containing 1.5% methionine, 1.5% cholesterol and 0.5% cholic acid, or a combination of the two diets, for 4 mo. The HM diet did not alter cholesterol or diene conjugate (DC) levels in the plasma or aorta, but this diet caused increases in cholesterol, triglyceride, malondialdehyde (MDA) and DC levels and a decrease in α-tocopherol levels without any change in the levels of glutathione and ascorbic acid or the activities of superoxide dismutase, glutathione peroxidase and glutathione transferase in the liver of mice. However, the HC diet alone was found to further increase cholesterol, triglyceride, MDA and DC levels in the plasma and liver together with changes in hepatic antioxidant system elements, but aortic cholesterol and DC levels remained unchanged as compared to the control group. There were no changes in blood hemoglobin and erythrocyte MDA levels or erythrocyte hemolysis values in both the HM and HC groups. However, the parameters related to lipid and lipid peroxide and antioxidant systems did not change in the plasma or tissues of the HM+HC and HC groups. Only plasma cholesterol was observed to increase in the HM+HC group as compared to the HC group. In addition, histopathological findings in the liver and aorta were similar in the HC and HM+HC groups. In conclusion, our results indicate that the addition of methionine to the HC diet did not augment oxidative stress, hepatotoxicity or atherosclerotic changes induced by the HC diet in mice.

Key Words cholesterol, methionine, oxidative stress, atherosclerosis, hepatotoxicity, mice

Methionine is an essential amino acid found in high concentrations in animal proteins. It is necessary for the adequate growth and development of mammals. However, excess amounts of methionine can retard growth and damage tissues (1). A high-methionine (HM) diet caused hepatic lesions, such as hepatitis and steatosis (2, 3), and erythrocyte membrane damage (4). In addition, it has been reported that an HM diet led to hypercholesterolemia, hypertriglyceridemia (1, 5, 6) and atherosclerotic changes in rabbits (7, 8) and apolipoprotein-deficient mice (9, 10). However, in rats, an HM diet did not alter cholesterol and triglyceride levels in the plasma and did not produce atherosclerotic changes in the aorta (11, 12). An HM diet has been reported to induce the production of oxidative metabolites resulting from methionine metabolism and to cause defects in the enzymatic antioxidant system as well as iron accumulation in tissues (3, 7, 13–15). Therefore, oxidative stress has been proposed to play a role in the development of hepatic lesions (3, 13), atherosclerotic disturbances (5–8) and erythrocyte hemolysis (4) induced by an HM diet.

On the other hand, a high-cholesterol (HC) diet resulted in a significant increase in serum (16–18), erythrocyte (19), liver (17, 18, 20) and aorta (17, 18, 20) lipid peroxide levels, as well as significant alterations in the antioxidant system and typical atherosclerotic changes in the aorta of rabbits. However, in rats, an HC diet does not induce atherosclerotic changes in the aorta (12, 21) and erythrocyte hemolysis (22), although this diet produces changes in prooxidant and antioxidant balance in some tissues (16, 18, 21).

High-fat and HC diets are well known risk factors for atherosclerosis. Overconsumption of proteins of animal origin may also contribute to the development of atherosclerosis. The atherogenic potential of animal proteins is related to their amino acid composition, especially their methionine amounts (1). Therefore, considering methionine and cholesterol are usually consumed...
together, the combination of these two factors may affect their relative toxic effects. Therefore, it has been postulated that an HM + HC diet may have a synergistic effect in the production of atherosclerotic lesions in the aorta (12, 23, 24). Indeed, some investigators have reported an augmented effect of HM plus HC feeding on plasma cholesterol and triglyceride levels (11, 24) and atherosclerosis (12, 23) in rabbits and rats, compared with effects seen by feeding with each agent alone. However, we recently detected that an HM + HC diet in rats did not cause atherosclerotic changes and oxidative stress in the aorta, although the hepatic prooxidant-antioxidant balance was affected by this diet (25). Therefore, in the current study, we investigated the effect of an HM + HC diet on lipid levels, atherogenicity, hepatotoxicity and oxidative stress in C57BL/6j mice, and compared these results with those observed following an HM or HC diet alone. For this reason, we used C57BL/6j mice, since this strain is relatively more susceptible to the development of hypercholesterolemic atherosclerosis (26).

MATERIALS AND METHODS

Materials. Methionine, cholesterol and cholic acid were supplied from Sigma (USA).

Animals and treatment. Male C57BL/6j mice aged 8 wk and weighing approximately 20 g were used for all the experiments. Animals were obtained from TUBİTAK (Gebze, Istanbul). The animals were divided into four groups:

a) Control group: The animals were fed with commercial mouse chow.

b) HM group: Mice were fed a diet containing 1.5% methionine (w/w).

c) HC group: The animals were fed a control diet enriched with cholesterol (1.5%; w/w) and cholic acid (0.5%; w/w).

d) HM + HC group: The animals were fed a diet containing 1.5% methionine, 1.5% cholesterol and 0.5% cholic acid.

The animals were allowed free access to food and water and were kept in wire-bottomed stainless steel cages. Food intake was controlled periodically to avoid differences between groups in the amount of feed consumed. The experimental procedure used in this study met the guidelines of the Animal Care and Use Committee of the University of Istanbul.

The determinations in plasma and erythrocytes. At the end of the feeding period of 4 mo, the animals were fasted overnight. Blood was collected in tubes containing EDTA by cardiac puncture and plasma and erythrocytes were separated by centrifugation. Plasma cholesterol and triglyceride levels were enzymatically measured with kits from Sigma. The degree of lipid peroxidation was assessed by measuring diene conjugate (DC) formation in the plasma. For this reason, plasma lipids were extracted with a chloroform–methanol mixture (2:1, v/v). After evaporation, lipids were redissolved in cyclohexane and absorbances measured at 233 nm spectrophotometrically and calculated using a molar extinction coefficient of 2.52 × 10^4 M^{-1} cm^{-1} (27).

Erythrocyte susceptibility to lipid peroxidation was measured by the method of Stocks et al. (28). The final composition of the incubation mixture was 5 mM H_2O_2, 2 mM sodium azide, and erythrocyte suspension in phosphate-buffered saline, pH 7.4 (30 mg Hb/mL incubation mixture). Lipid peroxidation was assayed by measurement of malondialdehyde (MDA) production during a 2-h incubation period at 37°C. Values were expressed as nanomoles of MDA per gram of hemoglobin (Hb). Hb concentration in erythrocyte suspensions and blood was measured using Drabkin’s reagent (29). A spontaneous hemolysis test of erythrocytes was done after 4 h of incubation at 20°C in phosphate saline buffer, pH 7.4. The percentage of hemolysis was expressed as the ratio of the absorbance at 410 nm in isotonic buffer to the completely hemolysed samples in water (30).

The determinations in liver and aorta. The livers were rapidly removed, washed in 0.9% NaCl and kept in ice. Liver portions were homogenized in ice-cold 0.15 M KCl (10%, w/w). Lipids were extracted with chloroform: methanol (2:1). After the extraction and evaporation, hepatic lipids were re-dissolved in isopropanol and hepatic cholesterol and triglyceride levels were enzymatically assayed by kits provided from Sigma. The degree of lipid peroxidation was assessed by two different methods in the liver. First, the levels of MDA were measured in liver homogenates by a thiobarbituric acid test according to the method of Ohkawa et al. (31). The breakdown product of 1,1,3,3-tetraethoxypropane was used as a standard. Second, diene conjugate (DC) levels were determined in hepatic lipid extracts at 233 nm spectrophotometrically (27). Liver glutathione (GSH) levels were measured with 5,5-dithiobis-(2-nitrobenzoate) at 412 nm (32). α-Tocopherol and ascorbic acid levels were determined in liver homogenates by a thiobarbituric acid test according to the method of Desai (33) and Omaye et al. (34), respectively. Hepatic superoxide dismutase (SOD) activities were assayed by the ability to increase the effect of riboflavin-sensitized photooxidation of orthodianisidine in postmitochondrial fractions (35). Glutathione peroxidase (GSH-Px) (36) and glutathione transferase (37) activities were measured using cumene hydroperoxide and 1-chloro-2,4-dinitrobenzene as substrates, respectively, in postmitochondrial fractions. Protein levels were determined using bichinchoninic acid (38).

The aorta, from the aortic valve to renal artery, was quickly removed, rinsed and aorta lipids were extracted with chloroform:methanol mixture (2:1). Aortic cholesterol and DC levels were determined in lipid extracts with the respective procedures applied to hepatic tissue.

Histopathological analyses. Liver and aorta were dissected and fixed in 10% buffered formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological studies.

Statistical analyses. The results were expressed as mean±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey test.
RESULTS

Body weight and tissue weight

The weight gain of mice during the 4 mo period was not significantly different among groups. In addition, no differences were detected in liver weights among groups (data not shown).

Lipids and lipid peroxides

The results obtained in this study are shown in Figs. 1, 2 and Table 1. According to these:

a) HM diet did not affect cholesterol, triglyceride or DC levels in the plasma and aorta, but cholesterol, triglyceride, MDA and DC levels increased significantly in the liver as compared to the controls. This diet did not alter blood Hb or erythrocyte MDA levels or erythrocyte...
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Table 1. Glutathione (GSH), α-tocopherol, and ascorbic acid levels and superoxide dismutase (SOD), GSH-peroxidase (GSH-Px) and GSH-transferase (GST) activities in the liver of mice fed high-methionine (HM), high-cholesterol (HC) and high-methionine plus high-cholesterol (HM+HC) diets.1

| Parameter                        | Control       | HM group      | HC group      | HM+HC group   |
|----------------------------------|---------------|---------------|---------------|---------------|
| GSH (µmol/g)                     | 5.18±0.91ab   | 5.09±0.72ac   | 3.80±0.63ab   | 4.38±0.55bc   |
| α-Tocopherol (nmol/g)            | 36.3±4.31ac   | 22.9±4.02bc   | 19.0±5.59bc   | 18.7±4.38bc   |
| Ascorbic acid (nmol/g)           | 371.3±87.3ac  | 385.8±119.9a  | 257.7±61.6a   | 220.2±35.9a   |
| SOD (U/mg protein)               | 12.0±1.52a    | 11.5±1.57a    | 11.1±1.28a    | 10.7±1.73a    |
| GSH-Px (nmol/mg protein/min)     | 698.2±55.9a   | 696.0±53.1a   | 506.2±71.4a   | 531.1±82.2a   |
| GST (nmol/mg protein/min)        | 581.2±62.6a   | 558.3±79.0a   | 455.2±76.8a   | 465.3±80.4b   |

1 Each value is expressed as mean±SD for 10 mice per dietary group. Values not sharing a common superscript letter are significantly different by ANOVA (p<0.05).

hemolysis levels (Figs. 1, 2). The levels of GSH and ascorbic acid and the activities of SOD, GSH-Px and GST remained unchanged, but α-tocopherol levels decreased in the liver (Table 1).

b) Plasma and liver cholesterol and triglyceride levels were significantly increased in the HC group as compared to controls. The HC diet was observed to increase plasma DC and liver MDA and DC levels. Aortic cholesterol and DC levels were not changed. There were no differences in blood Hb or erythrocyte MDA levels or erythrocyte hemolysis values as compared to the controls. This diet lowered GSH α-tocopherol and ascorbic acid levels, as well as GSH-Px and GST activities, but SOD activity remained unchanged in the liver.

c) Although cholesterol and lipid peroxide levels in plasma, liver and aorta were observed to increase in the HM+HC group as compared to the HM group, there were no changes in these values between the HM+HC and HC groups. Only, plasma cholesterol was observed to increase in the HM+HC group as compared to the HC group. No changes were found in GSH, α-tocopherol or ascorbic acid levels, orin the SOD, GSH-Px and GST activities in the liver between the HC and HM+HC groups.

Histopathological assessment

The liver and aorta of mice showed the following hist-
Histopathological changes (Figs. 3, 4). In the control and HM groups, normal hepatic and aortic structures were seen. In the HC and HM+HC groups, mild steatosis, portal and lobular necrosis with inflammatory cellular infiltration and granulomatosis were detected in the liver. In the HC group, no fatty streaks or plaques had developed in the aorta of any mice. In some sections, disarrangement of the endothelial layer was detected in the aortas of mice in the HC group. However, there were no differences in aortic histopathological findings of mice fed the HC and HM+HC diets.

**DISCUSSION**

Excessive dietary amounts of methionine are shown to result in hepatotoxicity (2, 3), atherosclerosis (7–10) and erythrocyte hemolysis (4) in experimental animals. Similar toxic effects are also observed following excess cholesterol intake (17, 19, 39). Free radicals and lipid peroxides have been accepted to be responsible for the toxic effects observed due to excess dietary amounts of both methionine (3, 4, 7, 13, 14) and cholesterol (16–21).

Toborek et al. (3, 7) have reported that a 0.3% methionine-enriched diet feeding for 9 mo resulted in inflammatory infiltration of the portal triads in the liver and atherosclerotic alterations in the aorta of rabbits. These authors also reported a significant elevation in lipid peroxide levels and GSH-Px, catalase and SOD activities in the liver and aorta in rabbits following this HM diet. Although the 2% methionine-containing diet did not produce atherosclerotic changes in the aorta of rats at the end of the 15-wk period (11), Matthias et al. (40) found some atherosclerotic changes in the aorta such as loss of endothelium, degeneration and dissolution of the media cells following the oral administration of methionine in high doses (200 mg/d) for 14 d in rats. Although there is no study concerning prooxidant and antioxidant status in the aorta of rats following an HM diet, Lynch and Strain (13) found that MDA levels and activities of catalase and GSH-Px increased, but SOD activity was reduced in the liver of rats fed a 2% methionine-supplemented diet for 7 wk. Mori and Hirayama (14) also reported that hepatic MDA levels increased, but hepatic antioxidant enzyme activities remained unchanged in rats fed a 1.6% methionine-supplemented diet for 1 mo. In our study, for the first time, we detected plasma and aortic lipids and lipid peroxide levels together with hepatic lipid peroxide levels and antioxidant system in C57BL/6J mice following the feeding of an HM diet. A 1.5% methionine-supplemented diet feeding for 4 mo did not alter the lipid and lipid peroxide levels in the plasma or aorta of C57BL/6J mice, but this diet caused increases in hepatic lipids and lipid peroxidation without any change in antioxidants except a decrease in hepatic α-tocopherol levels.

On the other hand, an HC diet also affects the proox-
identant and antioxidant balance in the tissues (16–21). Indeed, severe hypercholesterolemia and vascular atherosclerotic lesions as well as an increase in oxidative stress in plasma, erythrocytes and several tissues such as liver and aorta have been observed in rabbits following the feeding of an HC diet (16–20). Contrarily, atherosclerotic changes were not observed in the aorta of rats following the feeding of an HC diet (12, 18, 21), although lipid peroxidation increased and the antioxidant system was affected in some tissues (16, 18, 21).

In the current study, an HC diet (1.5% cholesterol-supplemented diet) fed to mice for 4 mo was found to increase lipid, MDA and DC levels in the plasma and liver, and also caused changes in the hepatic antioxidant system. However, the aortic cholesterol and DC levels remained unchanged in the hypercholesterolemic mice as compared to the controls.

It has been reported that the combination of methionine and cholesterol may have a synergistic effect on the development of atherosclerosis. Zulli et al. (11, 12) found that plasma cholesterol and triglyceride levels and aortic wall thickness increased in rats fed a diet containing 2% methionine plus 2% cholesterol for 15 wk, compared with effects seen by feeding with each agent alone, as reported for rabbits (23, 24). However, we recently detected that an HM+HC diet (2% methionine plus 2% cholesterol for 6 mo) diet in rats did not cause any atherosclerotic changes or oxidative stress in the aorta, although the hepatic prooxidant-antioxidant balance was affected by this diet (24). Therefore, in this study, using C57BL/6J mice, a strain of mice susceptible to atherosclerosis, we compared the effect of an HM+HC diet on lipids and, prooxidant-antioxidant status in the plasma, liver and aorta, as well as histopathological findings in the liver and aorta of those observed following the feeding of an HM or HC diet alone. Blood Hb levels, erythrocyte lipid peroxidation and spontaneous hemolysis values were also investigated. Although an HM+HC diet was observed to increase the plasma cholesterol levels, this diet did not exaggerate the alterations observed in cholesterol, triglyceride and lipid peroxide levels and antioxidant system elements in the liver and aorta as compared to the HC group. Similarly, there were no differences blood Hb, erythrocyte MDA or erythrocyte hemolysis values between the HC and HM+HC groups. In addition, histopathological findings in the liver and aorta were similar in the HC and HM+HC groups.

In conclusion, our results indicate that the addition of methionine to the HC diet did not augment oxidative stress, atherosclerotic changes or hepatotoxicity induced by an HC diet in mice.

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