Effects of High Methionine Diet on Oxidative Stress in Serum, Apo-B Containing Lipoproteins, Heart, and Aorta in Rabbits

Seda Yalçınkaya-Demirsöz, Bilge Depboylu, Semra Doğru-Abbasoğlu, Yeşim Ünlüçerçi, and Müjdat Uysal
Department of Biochemistry, Faculty of Medicine, Istanbul University, Istanbul, Turkey

Abstract. This study investigated in rabbits whether a high methionine (HM) diet influences oxidative stress parameters in serum, apo-B containing lipoproteins (LDL+VLDL), heart, and aorta. Rabbits received a normal commercial chow supplemented with 2% L-methionine (w/w) for 6 mo (approximately 1 g/kg body wt/day). Serum homocysteine (HCys), malondialdehyde (MDA), diene conjugate (DC), and cholesterol levels were found to be increased, but protein carbonyl (PC) and triglyceride levels remained unchanged in the HM group as compared to controls. Cholesterol, endogenous DC, and copper-induced MDA levels were significantly higher in the LDL+VLDL fraction of plasma lipoproteins in the HM group. MDA and DC levels were found to be increased in homogenates of heart and aorta in the HM group. The HM diet caused significant increases in cardiac glutathione peroxidase activity, but glutathione, vitamin E, and vitamin C levels and superoxide dismutase and glutathione transferase activities remained unchanged. There were no significant differences in the cholesterol levels and histopathological findings in the aortas of the control vs the HM group. This study demonstrates that a HM diet induces oxidative stress in serum, apo-B containing lipoproteins, heart, and aorta in rabbits.

Keywords: methionine, homocysteine, oxidative stress, apo-B containing lipoproteins, heart, aorta

Introduction

The composition and amount of diet influence the pro-oxidant/antioxidant balance in organisms [1,2]. The effects of dietary proteins on pro-oxidant/antioxidant balance is related to their amino acid composition, especially their methionine content [1-3]. A high methionine (HM) diet could be detrimental due to the conversion of methionine to homocysteine (Hcys), since methionine metabolism is the only known source for Hcys in mammals [1,4,5]. On the other hand, methionine residues of proteins are among the amino acids most susceptible to oxidation by reactive oxygen species (ROS) and the sensitivity of proteins to oxidative stress increases according to their number of methionine residues [1-3]. Oxidation of methionine residues generates methionine sulfoxide in proteins and may lead to loss of their biological activity [3]. Although it is debatable whether the harmful effects of HM diet on tissues are related to hyperhomocysteinemia (HHcys) or to HM [1-3,5], HM diet is usually used to produce HHcys in experimental animals [1].

Excessive methionine uptake might lead to the various pathophysiological consequences associated with HHcys, such as cardiovascular disorders [4,5], hepatic lesions [6,7], and neurologic disturbances [8]. Although the underlying mechanisms of toxic effects of HHcys are poorly understood, oxidative stress has been proposed as playing a role in the development of these disturbances [6-8].

HHcys is considered to be a risk factor for atherosclerotic cardiovascular disease [4,5]. However, the role of HHcys in the development of
atherosclerosis has been disputed. In addition, HHcys has been reported to have direct toxic effects on the heart [9,10]. Some investigators have reported that a HM diet causes oxidative stress in the heart [9,10] and aorta [11,12], leading to structural and functional disorders of the heart and atherosclerotic changes in the aorta of experimental animals [9-12]. However, other investigators have found that a HM diet neither alters oxidative stress parameters nor produces atherosclerotic changes in the aorta or heart of experimental animals [13-15].

There is increasing evidence that lipoprotein oxidation is a primary event in atherogenesis [16]. However, there is inadequate knowledge about lipoprotein oxidation in HHcys. Although in vitro experiments showed that Hcys increases LDL oxidation [17,18], LDL oxidation was found not to change in patients with severe HHcys [19,20]. One group of investigators has reported an increase in LDL oxidizability in gerbils following a HM diet [21]. For these reasons, in this study we investigated in rabbits the effects of HM diet on oxidative stress parameters in serum, in apo-B containing lipoproteins (low- and very low-density lipoproteins, LDL+VLDL), and in cardiac and aortic tissues.

Materials and Methods

Animals and diets. Male New Zealand white rabbits (6 mo old; body wt 2.0–2.5 kg) were obtained from the Experimental Medical Research Institute of Istanbul University. The animals were allowed free access to diet and water and were kept in wire-bottomed stainless steel cages. The rabbits were divided into the two groups: (a) The control group (n = 6) was fed a normal commercial chow containing 11% moisture, 10% crude ash, 15% protein, 3.5% crude fat, 47% carbohydrate, 7.5% cellulose, 3.5% salt mixture (AIN 76), and 1% vitamin mixture (AIN 76). (b) The HM group (n = 6) received a normal commercial chow supplemented with 2% L-methionine (w/w) for 6 mo (approximately 1 g/kg body wt/day). Methionine and other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA). The chows were stored at 4°C. Food intake was controlled periodically to avoid differences between the 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.

Blood and tissue samples. At the end of the feeding period of 6 mo, the rabbits were fasted overnight and blood was taken by cardiac puncture into dry and ethylenediaminetetraacetic acid (EDTA)-containing tubes. EDTA-plasma and serum were obtained by centrifugation at 1500 x g for 10 min. Heart and aorta (from the aortic valve to the renal artery) were quickly removed, rinsed, and cut into small segments. All materials were stored at -80°C until they were analyzed.

Determinations in serum. Serum Hcys levels were determined by HPLC [22]. Serum total cholesterol and triglyceride levels were determined by enzymatic methods using an automatic analyzer. The degree of endogenous lipid peroxidation in serum was assessed by two methods: (a) Serum diene conjugate (DC) formation was determined spectrophotometrically at 234 nm [23]. For this assay, serum lipids were extracted with chloroform/methanol (2:1, v/v) mixture. The extracted lipids were redissolved in cyclohexane and the approximate amounts of hydroperoxides were calculated using a molar extinction coefficient of 2.52 x 10^{-4} M^{-1} cm^{-1}. (b) Malondialdehyde (MDA) levels were assayed using thiobarbituric acid according to the method of Buege and Aust [23]. The oxidation of serum proteins was measured by spectrophotometric detection of the reaction of 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyls (PC) to form protein hydrazones [24].

Determinations in apo B 100-containing lipoproteins. Apo B 100-containing lipoproteins (LDL+VLDL) were precipitated from EDTA-plasma by dextran sulfate and MgCl2 solution, pH 7.0. The pellet was suspended in 0.9% NaCl and reprecipitated by adding precipitation reagent, vortexing, and centrifuging in order to remove EDTA from the non-HDL fraction. The pellet was redissolved with phosphate buffered saline (0.68 M NaCl, 10 mM NaH2PO4, pH 7.0) to obtain the LDL+VLDL fraction [25], and protein [26] and cholesterol levels were determined. Endogenous DC and Cu-induced MDA levels were measured in this fraction as previously reported by us [27]. In brief, lipids were extracted from LDL+VLDL samples with chloroform-methanol (2:1), dried under nitrogen, then redissolved in cyclohexane, and analyzed spectrophotometrically at 234 nm for the determination of endogenous DC levels. Absorbance units were converted to molar units using the molar extinction coefficient 2.95 x 10^{-4} M^{-1} cm^{-1}. To determine Cu-induced MDA levels, the LDL+VLDL fraction (200 μg protein) was incubated with copper sulfate (final copper concentration 50 μM) at 37°C for 3 hr and MDA produced during this period was estimated by the difference in MDA levels from 0 hr.

Cholesterol, lipid peroxides, and protein carbonyls in heart and aorta. Heart and aorta tissues were homogenized in ice-cold 0.15 M KCl (10% w/v). MDA levels were determined in these tissue homogenates according to Ohkawa et al [28]. To determine DC levels in the heart and aorta, lipid extracts of these tissues were prepared and DC levels were assayed according to Buege and Aust [23]. In addition, cardiac protein carbonyl groups [24] and aortic cholesterol levels were determined as mentioned above.

Non-enzymatic and enzymatic antioxidants in heart. Glutathione (GSH) levels were measured with 5,5-dithiobis-(2-nitrobenzoate) at 412 nm in heart homogenates [29]. Vitamin E and vitamin C levels were measured in heart homogenates by the methods of Desai [30] and Omaye et al
Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione transferase (GST) activities were determined in postmitochondrial fraction of heart tissue, which was separated by sequential centrifugation. Tissue homogenates were centrifuged at 600 x g for 10 min at 4°C to remove crude fractions. Then, supernatants were centrifuged at 10,000 x g for 20 min to obtain the postmitochondrial fraction. SOD activity was assayed by its ability to increase the effect of riboflavin-sensitized photooxidation of o-dianisidine [32]. GSH-Px [33] and GST [34] activities were measured using cumene hydroperoxide and 1-chloro-2,4-dinitrobenzene as substrates, respectively. Protein levels were determined using bicinchoninic acid [26].

Histopathological studies. Pieces of aorta from the control and experimental groups were fixed in 10% buffered formaldehyde and processed for paraffin sectioning. Sections 5 μm thick were stained with H&E for histological studies.

Statistical analysis. Results were expressed as mean ± SD. Comparisons between groups were performed by the Mann-Whitney U-test.

Results

The weight gain of rabbits during the 6 mo period was not significantly different between the control and HM groups (data not shown).

Serum Hcys (50.0%), cholesterol (21.0%), MDA (20.2%), and DC (23.4%) levels were increased in the HM group as compared to the controls, but serum triglyceride levels remained unchanged. Although serum PC levels were higher (14.5%) in the HM group, this increase was not statistically significant (Table 1).

The cholesterol (19.0%), endogenous DC (25.8%), and copper-induced MDA (27.3%) levels were significantly higher in the LDL+VLDL fraction of rabbits fed the HM diet as compared to the controls (Table 1).

MDA (15.6%), DC (25.3%), and PC (22.8%) levels were increased significantly in heart homogenates in the HM group. GSH, vitamin E, and vitamin C levels and SOD and GST activities remained unchanged in the hearts of rabbits in the HM group compared to controls. However, the HM diet caused a significant increase (18.3%) in heart GSH-Px activity (Table 2).

The HM diet did not alter cholesterol levels in the aorta. However, MDA (26.7%) and DC (28.2%) levels were increased in aortas of the HM group as compared to controls (Table 2).

### Table 1. Homocysteine (Hcys), cholesterol, triglyceride, malondialdehyde (MDA), diene conjugate (DC), and protein carbonyl (PC) levels in serum, and cholesterol, endogenous DC, and Cu-induced MDA levels in the LDL+VLDL fraction of rabbits in the control and high methionine (HM) groups (mean ± SD; n = 6 in each group).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>HM-diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SERUM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hcys (μmol/L)</td>
<td>10.6 ± 1.27</td>
<td>15.9 ± 4.46*</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>2.71 ± 0.31</td>
<td>3.28 ± 0.18**</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>0.67 ± 0.10</td>
<td>0.65 ± 0.13</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>2.92 ± 0.23</td>
<td>3.51 ± 0.35*</td>
</tr>
<tr>
<td>DC (μmol/L)</td>
<td>103.7 ± 13.5</td>
<td>128.0 ± 14.4**</td>
</tr>
<tr>
<td>PC (nmol/mg protein)</td>
<td>0.96 ± 0.09</td>
<td>1.10 ± 0.13</td>
</tr>
<tr>
<td><strong>LDL+VLDL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>0.79 ± 0.09</td>
<td>0.94 ± 0.07**</td>
</tr>
<tr>
<td>Endogenous DC (nmol/mg protein)</td>
<td>21.7 ± 2.37</td>
<td>27.3 ± 5.51**</td>
</tr>
<tr>
<td>Cu-induced MDA (nmol/mg protein)</td>
<td>9.66 ± 1.64</td>
<td>12.3 ± 2.19**</td>
</tr>
</tbody>
</table>

*p< 0.001; ** p<0.05 vs controls (Mann Whitney U-test).

### Table 2. Malondialdehyde (MDA), diene conjugate (DC), protein carbonyl (PC), and cholesterol levels in homogenates of heart and aorta, as well as glutathione (GSH), vitamin E, and vitamin C levels and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione transferase (GST) activities in heart of rabbits in control and high methionine (HM) groups (mean ± SD; n = 6 in each group).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>HM-diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HEART</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (nmol/g)</td>
<td>111.8 ± 7.78</td>
<td>129.3 ± 8.52*</td>
</tr>
<tr>
<td>DC (μmol/g)</td>
<td>0.87 ± 0.11</td>
<td>1.09 ± 0.08*</td>
</tr>
<tr>
<td>PC (nmol/mg protein)</td>
<td>1.27 ± 0.26</td>
<td>1.56 ± 0.40*</td>
</tr>
<tr>
<td>GSH (μmol/g)</td>
<td>2.21 ± 0.10</td>
<td>2.06 ± 0.08</td>
</tr>
<tr>
<td>Vitamin E (nmol/g)</td>
<td>94.0 ± 5.63</td>
<td>86.4 ± 16.8</td>
</tr>
<tr>
<td>Vitamin C (nmol/g)</td>
<td>329.0 ± 30.7</td>
<td>337.8 ± 56.0</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>17.8 ± 2.29</td>
<td>19.8 ± 3.19</td>
</tr>
<tr>
<td>GSH-Px (nmol/min/mg protein)</td>
<td>115.0 ± 12.0</td>
<td>136.0 ± 16.0**</td>
</tr>
<tr>
<td>GST (nmol/min/mg protein)</td>
<td>199.1 ± 58.8</td>
<td>6.0 ± 33.5</td>
</tr>
<tr>
<td><strong>AORTA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (μmol/g)</td>
<td>4.11 ± 0.37</td>
<td>4.25 ± 0.41</td>
</tr>
<tr>
<td>MDA (nmol/g)</td>
<td>62.5 ± 9.02</td>
<td>79.2 ± 13.1**</td>
</tr>
<tr>
<td>DC (μmol/g)</td>
<td>0.71 ± 0.07</td>
<td>0.91 ± 0.16**</td>
</tr>
</tbody>
</table>

*p< 0.001; ** p<0.05 vs controls (Mann Whitney U-test).
There were no differences in histopathological findings in the aortas of rabbits fed the HM diet as compared to controls (not shown).

Discussion

Administration of a HM diet to experimental animals is generally used to produce HHcys and to investigate the effects of HHcys on tissues. Several researchers have investigated the effects of HM diet on oxidative stress in body fluids and tissues of animals [11,14,21,35,36]. The conflicting results obtained in these studies may be related to the methionine content of the diet, the duration of the treatment, and the experimental animals. In the current study, methionine (1 g/kg body wt/day) was given for 6 mo to rabbits. Serum cholesterol and LDL+VLDL-cholesterol levels were found to increase following the HM diet, in agreement with previous experimental studies [13,21,37]. Indeed, HM diet was reported to induce hepatic cholesterol biosynthesis by transcriptionally regulating 3-hydroxy-3-methylglutaryl coenzyme A reductase activity [37]. In our study, the serum lipid peroxide level, but not the protein carbonyl level, increased in the HM group. These results indicate that pro-oxidant/antioxidant balance is disturbed in favor of pro-oxidation in the serum of rabbits in the HM group. In the literature, oxidative stress parameters were found to be increased [10,11,36], decreased [35], or unchanged [15,21] in serum of experimental animals following a HM diet.

The apo-B containing lipoproteins, LDL and VLDL, are atherogenic, and oxidized LDL and VLDL both play roles in the development of atherosclerotic lesions [25,38]. Assays of oxidized lipoproteins in plasma may provide information related to the oxidation that takes place in the vessel. In this study, baseline DC and copper-induced MDA levels were determined in the LDL+VLDL fraction and were increased in the HM group as compared to the control group. In the literature, there is one study of LDL oxidizability following a HM diet. Hidiroglu et al [21] reported that ingestion of 1% methionine-supplemented diet for 3 mo increased total diene production in the LDL fraction and also increased plasma Hcys and total cholesterol levels in male gerbils.

Although there have been studies of the effects of HM diet on cardiac oxidative stress in rats [9,10], no study is available in rabbits. Hagar [9] reported that daily intake of methionine (1 g/kg) in drinking water for 4 weeks gave rise to increased MDA and decreased GSH levels in heart of rats. Chang et al [10] reported that a HM diet (1%) for 6 weeks stimulated the production of reactive oxygen species (ROS), increased MDA levels, and inhibited SOD activity in myocardial mitochondria [10]. In addition, a 2% methionine-containing diet for 49 days was reported to decrease in cardiac SOD activity [39]. In the current study, lipid peroxide and PC levels were increased in heart homogenates of rabbits following the HM diet, as reported in rats [9,10]. However, we could not find any changes in non-enzymatic antioxidant levels or in SOD and GST activities. Cardiac GSH-Px activity was increased in the HM group, which may be a response to enhanced levels of ROS and/or lipid peroxides.

Researchers have studied the effects of HM diet on aortic oxidative stress and atherogenesis in rabbits [11,13]. Toborek et al [11] reported that feeding a 0.3% methionine-enriched diet for 9 mo resulted in intimal thickening, deposition of cholesterol, and calcification in the aorta together with significant elevations of aortic lipid peroxide levels and GSH-Px, catalase, and SOD activities in rabbits. Fujimoto et al [13] reported that light microscopic examination did not reveal any apparent difference between controls and HM rabbits fed a 3% methionine-supplemented diet for 22 weeks. However, these authors reported that the histopathological examination by immunostaining for eNOS and tissue factor showed significant increases in their endothelial expression in the HM group before atherosclerotic changes appeared [13]. In the current study, MDA and DC levels were increased in aortic homogenates of rabbits in the HM group. However, there were no significant changes in aortic cholesterol levels, and atherosclerotic lesions could not be detected in the aortas by histological examination.

In conclusion, our results indicate that ingestion of a HM diet stimulates oxidative stress in serum, in apo-B containing lipoproteins, as well as in the heart and aorta tissues of rabbits.
Acknowledgement

This study was supported by grant YOP-10/27052004 from the Research Fund of the University of Istanbul.

References

35. Mori N, Hirayama K. Long-term consumption of a methionine-supplemented diet increases iron and lipid peroxide levels in rat liver. J Nutr 2000;130:2349-2355.