Gelatin intake increases the atheroma formation in apoE knock out mice

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Abstract

The effect of gelatin ingestion on cholesterol metabolism and on atheroma formation was evaluated in both wild type (n = 14) and apoprotein E (apoE) knock out (apoE−/−) (n = 20) C57BL/6 7-week-old mice. Animals were fed a cholesterol-free isoproteic semi-purified diet containing 20% of casein (control diet) or 10% of casein plus 10% of gelatin (gel diet) for 8 weeks. In wild type mice, dietary gelatin caused a reduction in the serum triacylglycerols levels associated with an increase in the fecal excretion. No difference in blood cholesterol was seen at the sixth week of experiment. At the eighth week of experiment, there was a modest but significant reduction of serum total and high density lipoprotein (HDL) cholesterol in apoE−/− mice fed on gel diet compared to the control. Total cholesterol/HDL cholesterol ratio was 2-fold higher in the gel group than that seen in the control group (14.39 and 7.84, respectively). Histological analyzes showed a 2.2-fold increase in the dimension of the atherosclerotic plaques in the proximal aorta in apoE−/− mice fed on a gel diet compared to those fed on a control diet. The gel diet also promoted a reduction in the fecal excretion of bile acids. Hepatic cholesterol was similar in both groups. In conclusion, although gelatin reduced total serum cholesterol, this reduction was associated to a decrease of HDL cholesterol and consequent increase of total cholesterol/HDL cholesterol ratio, resulting in an acceleration of atherogenesis. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Gelatin; Dietary protein; Cholesterol metabolism; Atherosclerosis; ApoE−/− mice

1. Introduction

Animal and vegetal proteins influence cholesterol metabolism in humans and in experimental models [1]. It has been reported that animal proteins such as casein induce hypercholesterolemia, whereas vegetal proteins such as soybean protein have a hypocholesterolemic effect [2]. The mechanisms of these effects are still unclear and include: (i) alterations in digestion and intestinal absorption of steroids due to the amino acid composition of proteins or to physico-chemical activities of either proteins or their constituent amino acids [3]; and (ii) metabolic factors related to differences in serum concentration of amino acids [4] or in lipoprotein metabolism [5].

Gelatin is a mixture of small and large peptides with a typical amino acid composition: 30% glycine, 30% proline and hydroxyproline and absence of tryptophan [6]. Notwithstanding the consumption of gelatin all over the world, very few studies deal with the effect of its ingestion on lipid metabolism [3,7–10] and there is no consensus about its role in hypercholesterolemia and atherosclerosis. Aust et al. [7] described serum triacylglycerols lowering the effect of a mixture of casein and gelatin (1:1) in rats. With a similar protein mixture, Gibney [8] found a hypocholesterolemic effect in rabbits. In contrast, Popescu et al. [9] reported a decrease of hepatic triglycerides, total- and free-cholesterol in
rats fed on a diet containing gelatin (12%) and casein (8%) as the protein source. Ratnayake et al. [10] evaluating the effect of different protein sources on the rat serum lipids and liver polyunsaturated fatty acids, found that gelatin had a hypocholesterolemic effect and also suppressed the incorporation of arachidonic and docosahexaenoic acids in the liver phospholipids. In that study, the levels of serum triacylglycerols and high density lipoproteins (HDL) were 5 and 30% lower in rats fed on the gelatin diets compared with those fed on the casein diets.

Apoprotein E (apoE) is a glycoprotein that mediates a high affinity binding of remnant chylomicrons and very low density lipoprotein (VLDL) and a HDL subclass to two different receptors: LDL receptor (B, E receptor) and apoE receptor [11]. ApoE deficient mice (apoE \(^{-/-}\)) fed on a low fat, low cholesterol diet show serum cholesterol levels six to eight times higher than the wild type mice due to a massive accumulation of remnant lipoproteins [12]. Consequently, these animals spontaneously develop atherosclerotic plaques, which were similar to human lesions [13]. To obtain further information on the effects of gelatin on the levels of total cholesterol and lipoproteins, wild type and apoE \(^{-/-}\) mice were fed a gelatin rich diet for 8 weeks. The influence of gelatin ingestion on atherogenesis was studied by analyzing lesions development in the proximal aorta of apoE \(^{-/-}\) mice. The results showed that gelatin reduced the serum levels of total and HDL cholesterol and accelerated atheroma formation in apoE \(^{-/-}\) mice when compared to mice in the control diet (20% of casein).

2. Materials and methods

2.1. Diets

The composition of the diets is shown in Table 1. Diets were prepared every 2 weeks and stored at \(-4^\circ C\).

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize starch</td>
<td>533</td>
<td>533</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>Gelatin(^a)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Vitamin mixture(^b)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mineral mixture(^b)</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Choline</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) Sigma, St. Louis, MO.  
\(^b\) AOAC [14].

2.2. Animals and experimental design

The homozygous apoE deficient (apoE \(^{-/-}\)) mice (in C57BL/6 background) [12,15] were purchased from Jackson Laboratory (USA). Wild type C57BL/6 mice were obtained from the ‘Instituto de Ciências Biológicas’, UFMG, Brazil. Fourteen female wild type and 20 apoE \(^{-/-}\) 7-week-old female mice were used in the experiment. Wild type mice were fed the control diet (\(n = 7\)) or gelatin (gel) diet (\(n = 7\)) for 8 weeks. ApoE \(^{-/-}\) mice were fed a control diet (\(n = 10\)) or gel diet (\(n = 10\)) and were distributed in two identical experiments using five animals in each group. The animals were distributed based on their initial weight (16.5 \pm 1 g for the wild type groups and 18 \pm 1 g for apoE \(^{-/-}\) groups) and initial serum cholesterol (96 \pm 15 mg/dl for the wild type groups and 470 \pm 60 mg/dl for apoE \(^{-/-}\) groups). The mice were housed in plastic cages and kept in a room with a controlled light/dark cycle (12:12 h). Free access to food and water was provided. Individual weight and food intake were recorded weekly. Blood samples from the ocular plexus were collected for cholesterol analysis at the beginning and sixth week of the diet feeding experiment. At the end of the experiment, the animals were fasted for eight hours and killed under ether anesthesia. Blood was collected from the axillary plexus for lipoprotein fractions and amino acid determinations. The liver was removed, weighted and frozen at \(-20^\circ C\). The aorta was removed from the root at the heart to the iliac bifurcation and fixed for histopathological analysis. The relative liver weight (RLW) ratio (liver weight/body weight \times 100) was determined and used as an indirect parameter of nutritional status. The animal feeding and treatment protocol was reviewed and approved by the Animal Care Committee of the Instituto de Ciências Biológicas, UFMG, Brazil.

2.3. Analytical methods

Blood samples were centrifuged at 12,000 \(\times g\) (3500 rpm) for 10 min and the sera was used for determination of lipids, protein and free amino acids.

Total cholesterol [16] and triacylglycerols [17] in serum, liver and feces were determined enzymatically using commercial kits (kits Analisa-Belo Horizonte, Brazil). Sera of apoE \(^{-/-}\) mice were diluted 1:2 in 0.85% NaCl before cholesterol determination to keep the absorbance in the proper range of the test. Total protein and albumin were measured using commercial kits from Analisa (Belo Horizonte, Brazil).

Three samples from serum from each group (pooled serum of animals 1 and 2, pooled serum of animals 3 and 4 and serum of animal 5) were used for free amino acids and lipoprotein determination.
For free amino acid analysis, 10 μl of serum and 50 mg of liver (perfused with phosphate buffered saline solution and dried on filter paper) were precipitated in methanol (1:4) and, after ultracentrifugation, the supernatant was diluted 1:1 in milliQ water, filtered, vacuum dried and frozen for HPLC analysis [18]. To obtain better resolution, liver samples were diluted 10 times in run buffer (sodium acetate 0.14 M, pH 5.8, containing 0.05% of triethylamine) just before HPLC.

Serum lipoproteins were separated by FPLC analysis as described by Fazio et al. [19]. Cholesterol concentration was determined in each fraction using commercial kits (Analisa) adapted to a microplate assay [19].

Feces collected during the last 7 days of the experimental period, were pooled, dried, weighed and powdered. Fecal bile acids were extracted by the method of van der Meer et al. [20] and the supernatants were evaluated morphometrically using an image analyzer (KS 300 program) attached to a microcamera and Zeiss microscope.

### 2.4. Statistical analysis

Evaluation of differences between diets was analyzed within the genotype (wild type or apoE\(^{-/-}\) mice). Statistical analysis were performed using the unpaired 2-tailed Student’s \(t\)-test. A value of \(P < 0.05\) was used to define statistical significance. The results were considered significant at \(P < 0.05\) using unpaired Student’s \(t\)-test.

### 3. Results

There were no differences in food intake (data not shown) or RLW ( = liver weight/body weight \(\times 100\)) between groups (Table 2). Animals had a good nutritional development, as documented by weight gain and serum protein levels (Table 2). No differences were seen in serum total protein and albumin levels (Table 2). No differences were seen in total serum cholesterol at the beginning and sixth week of experiment when apoE\(^{-/-}\) – gel and wild type gel groups were compared to their respective controls (data not shown). At the end of the experiment, apoE\(^{-/-}\) mice fed on the control and gel diets had serum cholesterol levels 5.6 and 4.6 times higher than the wild type group that received the same diet. By 8 weeks, gelatin ingestion reduced triglyceridemia in wild type mice and total cholesterol in apoE\(^{-/-}\) mice (Table 2). Lipoprotein profiles were similar in both wild type groups (Fig. 1A). However, when the lipoprotein profile was determined in apoE\(^{-/-}\) – mice, the reduction in total cholesterol was found to be mainly due to a reduction in the HDL fraction (Fig. 1B, fractions 20–26). The total cholesterol concentration in atherogenic fractions (VLDL + intermediate density lipoproteins (IDL) and low density lipoproteins (LDL)) were similar in both apoE\(^{-/-}\) groups (Fig. 1B, fractions 6–19). Consequently, total cholesterol/HDL cholesterol ratio was approximately the twofold higher than that seen in control group (14.39 and 7.84, respectively). The reduction in HDL-cholesterol and increase of total cholesterol/HDL ratio paralleled to a 5-fold increase in atherosclerotic lesions observed in apoE\(^{-/-}\) mice fed the gel diet compared to their respective control (26 200 ± 3889 vs. 11 800 ± 2967) (Fig. 2). Animals from the gel group showed fatty streaks that contained more fat deposition and macrophage infiltration (foam cells) when compared to the control group.

### Table 2

Weight gain (g), relative liver weight (RLW) (liver weight/body weight \(\times 100\)) and blood parameters of wild type and apoE\(^{-/-}\) mice fed diets containing 20% casein (control) or 10% casein + 10% gelatin (gel) as the protein source for 8 weeks.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type(^a)</th>
<th>ApoE(^{-/-})(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Gel</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>4.8 ± 0.7</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>RLW (g/dl)</td>
<td>3.5 ± 0.6</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>Total proteins (g/dl)</td>
<td>5.4 ± 0.2</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.7 ± 0.1</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>125 ± 8</td>
<td>121 ± 7</td>
</tr>
<tr>
<td>Triacylglycerols (mg/dl)</td>
<td>99 ± 20</td>
<td>62 ± 12*</td>
</tr>
</tbody>
</table>

\(\text{a} n = 7 \text{ in each group.}\)

\(\text{b} n = 5 \text{ in each group. A second experiment using the same number of animals yielded similar results.}\)

\(\text{c Average ± S.D.}\)

\(\text{* Significant difference (}P<0.05\text{) from the corresponding control.}\)

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Weight gain and relative liver weight (RLW) were measured in order to evaluate growth and liver weight in two different experimental groups. The sample was analyzed for its content of total and free cholesterol, and triacylglycerols. The results obtained were compared with those from wild type mice and apoE\(^{-/-}\) mice on a control diet, and those from apoE\(^{-/-}\) mice on a gelatin diet. The data were analyzed using a two-tailed Student’s \(t\)-test, and a value of \(P < 0.05\) was considered significant.
Fig. 1. Cholesterol distribution in serum lipoprotein of wild type (A) and apoE\(^{-/-}\) mice (B) fed diets containing 20% casein (control) or 10% casein + 10% gelatin (gel). A second experiment using the same number of apoE\(^{-/-}\) mice yielded similar results. Total high density lipoproteins (HDL) cholesterol (sum of fractions 20–25) and very low density lipoproteins (VLDL)/intermediate density lipoproteins (IDL)/low density lipoproteins (LDL) cholesterol (sum of fractions 6–19) are: 38 and 8 \(\mu\)g in wild type control group; 38 and 13 \(\mu\)g in wild type gel group; 25 and 200 \(\mu\)g in apoE\(^{-/-}\) control group and 11 and 185 \(\mu\)g in apoE\(^{-/-}\) gel group, respectively. For apoE\(^{-/-}\) and wild type groups \(n = 3\).

(Fig. 3A and B), suggesting an enhanced lesional development in the mice that consumed gelatin. The histological analysis of livers of both groups showed fat accumulation, but no significant differences were found (data not shown).

Lesions were found in the seven first sections from the aortic root in both apoE\(^{-/-}\) groups. Morphological and morphometrical analysis was performed using the largest lesion area from each animal. Control and gel groups showed that largest lesions were located 400 and 200 \(\mu\)m from the aortic root, respectively. The gel group had a 5-fold increase in atherosclerotic lesion size, which were closer to the aortic root when compared with the lesions of the control group (\(P = 0.003\)).

Amino acid levels were determined in blood and liver from mice in an attempt to clarify whether the effect of gelatin was related to differences in amino acid profiles. Compared to the control group, apoE\(^{-/-}\) mice fed the gel diet had a 38% increase on serum and a 40% reduction of liver concentrations of total free amino acids (23,872 vs. 32,933 pmol/ml serum and 329.61 vs. 235.50 nmol/g of liver, respectively). In wild-type mice, those differences were not seen. The concentrations of amino acids described as having an effect on serum cholesterol (i.e. methionine, arginine, and lysine) were similar in both apoE\(^{-/-}\) groups.

Differences in the excretion of fecal steroids could contribute for the effects of dietary intervention. There was a lower excretion of bile salts in both wild type and...
knock out mice fed on gelatin, suggestive of a higher absorption or a lower bile acid production. There were no differences in liver and fecal concentrations of total cholesterol. However, in the liver, the gel diet promoted a non-significant reduction in free cholesterol in apoE \(^{-/-}\) mice and of cholesteryl ester in wild type mice (Table 3).

4. Discussion

Although gelatin can reduce the weight gain in rats and rabbits [7–9,24], in these experiments, the addition of 10% of gelatin to the diet did not affect the nutritional status of both apoE \(^{-/-}\) and wild-type mice as measured by weight gain, liver weight/body weight ratio and serum total protein and albumin levels.

Regarding triglycerides, gelatin ingestion did not affect the hepatic concentrations in wild-type mice but caused a reduction in serum triglycerides levels that can be explained by the rise in the fecal excretion. In apoE \(^{-/-}\) mice, gelatin ingestion showed a different pattern; i.e. the serum levels of triacylglycerols were not affected whereas hepatic concentrations were significantly reduced. Since there was a lower fecal excretion in apoE \(^{-/-}\) mice of gel group, one may suggest that the uptake of triacylglycerols by extra hepatic tissues was increased. Since hepatic synthesis of triacylglycerols is not affected by the addition of gelatin to the diet [7], the lower levels of total free amino acids in liver of apoE \(^{-/-}\) mice could have led to a lower protein synthesis, including apolipoproteins or membrane receptors involved on the lipoprotein uptake.

Wild type mice are highly resistant to the development of atherosclerosis, due to the low levels of total serum cholesterol, a high HDL fraction (70%) and very low levels of LDL fraction [12,26–30]. ApoE \(^{-/-}\) mice develop atherosclerotic lesions similar to those found in humans, mainly in the proximal aorta [12,28,30]. Therefore, the use of apoE \(^{-/-}\) mice, as a model for studies of gelatin on atherosclerosis development, is justified.

To the authors’ knowledge, this is the first study that shows the effect of gelatin on the atherogenesis, using apoE \(^{-/-}\) animals. Some authors reported the hypocolesterolemic effect of gelatin in rats and rabbits [8,10] and assumed that gelatin can protect against atherosclerosis. However, those studies did not include analysis of

Table 3

| Biochemical parameters determined in feces and liver from wild type and apoE \(^{-/-}\) mice fed diets containing 20% casein (control) or 10% casein + 10% gelatin (gel) as the protein source for 8 weeks  |
|---|---|---|---|---|---|
| **Feces**  |  |  |  |  |
| Bile acids (\(\mu \text{mol/g}\)) | Control | Gel | Control | Gel |
| 8.5 ± 0.2 | 6.8 ± 0.2* | 7.0 ± 0.5 | 3.7 ± 0.6* |
| Total cholesterol (mg/g) | 3.0 ± 0.1 | 2.9 ± 0.1 | 2.05 ± 0.3 | 2.15 ± 0.2 |
| Triacylglycerols (mg/g) | 1.2 ± 0.4 | 2.4 ± 0.3* | 2.5 ± 0.4 | 1.9 ± 0.3 |
| **Liver** (mg/g) |  |  |  |  |
| Total cholesterol | 5.7 ± 1.2 | 4.9 ± 1.0 | 8.6 ± 1.8 | 7.7 ± 0.9 |
| Cholesterol ester | 1.6 ± 0.5 | 1.3 ± 0.2 | 2.2 ± 0.8 | 3.2 ± 0.9 |
| Free cholesterol | 4.1 ± 1.0 | 3.9 ± 0.8 | 6.7 ± 1.8 | 4.5 ± 0.8* |
| Triacylglycerols | 33.7 ± 11.3 | 33.1 ± 7.8 | 44.4 ± 6.8 | 31.4 ± 5.7* |

\(\text{a}\) The results are the average ± S.D.

\(\text{b}\) For the hepatic parameters: \(n = 7\) in each group.

\(\text{c}\) For the hepatic parameters: \(n = 5\) in each group.

\(\text{d}\) Samples were pooled and three different extractions were performed.

* Significant difference \((P<0.05)\) from the corresponding control.
atherosclerotic lesions or amino acid profile in animal tissues. In rats, like in these experiments, this effect of gelatin was associated with a decrease in serum triglycerides and in serum total cholesterol (due to HDL cholesterol) [10]. The authors assumed that differences of amino acid composition in gelatin compared to casein are responsible for these actions in lipid metabolism. Appreciable differences were not found in free amino acids composition in the liver and serum of mice fed the gel diet, especially regarding amino acids that influence cholesterolemia (methionine, lysine and arginine). It must be pointed out that the amino acid determinations were done in fasting mice. The levels reached in post absorptive state were not analyzed in this work. These results are in agreement with those reported by Kurowska and Carrol [25] who found no correlation between serum levels of cholesterol and specific amino acids either in starved or fed rabbits.

The lipoprotein pattern in the wild-type mice in this study is in agreement with those reported in the literature [12,26,30]: HDL is the main cholesterol carrier in the blood and VLDL, IDL and LDL fractions were found only in trace amounts. In these animals, dietary gelatin did not affect the distribution of cholesterol in lipoprotein fractions. However, gelatin ingestion promoted a lower fecal excretion of bile acids with no alterations in serum cholesterol or plaque formation in wild type mice. It is possible that these animals have an efficient compensation mechanism that involved enzymes of hepatic metabolism of cholesterol, such as cholesterol 7-α-hydroxylase and β-hydroxy-β-methyl-glutaryl CoA reductase. Although the activities of these enzymes were not measured in the present study, it is well known that bile acid synthesis is regulated by bile acids returning to the liver, in order to maintain the bile acid homeostasis [31].

ApoE−/− mice fed the control diet showed a plasma lipoprotein pattern characterized by high levels of atherogenic fractions and lower levels of HDL compared to wild-type mice (Fig. 1). In apoE−/− mice ingesting gelatin, the decrease of HDL cholesterol accelerated plaque formation, which can be explained by the reduced efflux of cholesterol from subendothelium, which contribute to a faster foam cells formation.

The reduction of HDL cholesterol (and the consequent increase of total cholesterol/HDL ratio) was probably the main factor involved on the increase of lesional area in gelatin fed mice. The mechanism of action of gelatin in reducing cholesterol in HDL fraction and on increasing the size of atherosclerotic lesions has not been well explained. Saeki and Kiriama [32] report that dietary protein-induced alterations in the levels of HDL were directly related to the LCAT activity involved in free cholesterol esterification by HDL. These authors concluded that the effect of dietary proteins on serum cholesterol is not explained by the differences in intestinal absorption and fecal excretion of steroids or by the modification in lipoprotein (mainly HDL) metabolism. Some amino acids can also affect cholesterol metabolism in rabbits, partly through alteration of liver phospholipids [33], an important component of HDL. Differences may not have been detected under the present conditions, because the animals were fasting when they were sacrificed. The results also revealed that gelatin ingestion causes a reduction in the fecal excretion of bile acids, which may reflect a higher intestinal absorption, or a lower conversion of cholesterol into bile acids and its secretion in the bile, both contributing for the faster development of aorta lesions. The hepatic levels of total cholesterol were not increased in these animals, probably due to some compensating mechanism such a higher hepatic excretion of VLDL or a lower HDL uptake. The LDL/HDL ratio is also in agreement with the higher risk of atherosclerosis development. It was concluded that gelatin ingestion has a negative effect on cholesterol metabolism, reducing bile acid excretion and HDL cholesterol, resulting in a faster lesional development.

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References


