Amino Acids (2010) 38:1323–1331
DOI 10.1007/s00726-009-0340-x

ORIGINAL ARTICLE

Oral supplementation with L-aspartate and L-glutamate inhibits atherogenesis and fatty liver disease in cholesterol-fed rabbit

Amalia E. Yanni · George Agrogiannis · Tzortzis Nomikos · Elisabeth Fragopoulou · Alkisti Pantopoulou · Smaragdi Antonopoulou · Despoina Perrea

Received: 25 May 2009 / Accepted: 8 August 2009 / Published online: 23 August 2009

Abstract Previous studies have shown that dietary supplementation with L-aspartate and L-glutamate inhibits fatty streak initiation in cholesterol-fed rabbit. The present study investigates the role of dicarboxylic amino acids on the progression of fatty streaks and the development of fatty liver disease, which were caused in New Zealand White rabbits after a 0.5% w/w cholesterol diet for 7 weeks. A group of animals additionally received a combination of 12.5 mM L-aspartate and 12.5 mM L-glutamate per day through drinking water. Total cholesterol (TC), high-density lipoproteins cholesterol (HDLC), non-HDLC and triacylglycerol (TAG) concentrations were measured in plasma. Serum gamma-glutamyl transferase (γ-GT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were also determined. At the end of dietary intervention, animals were sacrificed. Aortic, hepatic and brain lesions were evaluated after staining with hematoxylin and eosin. Supplementation with dicarboxylic amino acids inhibited the progression of aortic intima thickness ($P < 0.05$) and the development of liver lesions ($P < 0.05$). TC, non-HDLC and TAG were similarly increased in both cholesterol-fed groups. Serum γ-GT and AST activities elevated during the study in all cholesterol-fed animals but the elevation of γ-GT was milder and significantly lower in rabbits treated with L-aspartate and L-glutamate ($P < 0.05$). ALT activity was not affected by cholesterol feeding. In conclusion, oral supplementation with L-aspartate and L-glutamate inhibits the progression of atherogenesis and the development of fatty liver disease in the animal model of cholesterol-fed rabbit. The beneficial effects of dicarboxylic amino acids reflect the limited elevation of serum γ-GT activity.

Keywords Aspartate · Glutamate · Atherogenesis · Fatty liver disease · Gamma-glutamyl transferase

A. E. Yanni (✉) · T. Nomikos · E. Fragopoulou · S. Antonopoulou
Department of Dietetics and Nutrition, Harokopio University of Athens, 70 El. Venizelou Avenue, 17671 Athens, Greece
e-mail: ayanni@hua.gr

A. E. Yanni · G. Agrogiannis · A. Pantopoulou · D. Perrea
Medical School, National and Kapodistrian University of Athens, Athens, Greece

Introduction

Hyperlipidemia is one of the main causes leading to atherogenesis and fatty liver disease (Cohn et al. 1999; Glass and Wiztum 2001; Yesilova et al. 2005; Yen and Brunt 2007). Hypolipidemic diet and physical exercise are usually recommended to encounter these conditions; however, the design of new therapeutic strategies remains a priority together with the necessity of appropriate animal models.

It is widely known that feeding rabbit with high cholesterol diet leads to the formation of lesions in the aorta that their extent is proportional to the amount of cholesterol consumed (Finking and Hanke 1997; Yanni 2004). Rabbit has been extensively used for the study of the pathogenesis of atherosclerosis and also for testing new drugs and nutrients with antiatherogenic properties. In recent years, it is also being used as animal model for the study of fatty liver disease related to hyperlipidemia. It has been observed that cholesterol feeding leads to characteristic fibrosis, ballooning, activation of hepatic stellate cells and enhanced oxidative stress (Kainuma et al. 2006; Birkner

et al. 2007; Kawada and Otogawa 2007; Fujimoto et al. 2008; Yoko et al. 2008; Fu et al. 2009).

Previous studies have shown that oral supplementation with dicarboxylic amino acids, L-aspartate and L-glutamate inhibits fatty streak initiation in cholesterol-fed rabbit. The combination of the two amino acids attenuates mononuclear cell adhesion to the endothelium and the formation of foam cells in the intima of thoracic aorta (Yanni et al. 2003). The mechanism of the protective effect exhibited in these initiating stages of the disease was not explained. It was partially attributed to increase of high-density lipoproteins (HDL) (Yanni et al. 2005) and was hypothesized that it could be related to the enhancement of endogenous antioxidants (Yanni et al. 2003, 2005). A number of studies support the antioxidant properties of aspartate and glutamate. In particular, supplementation of these amino acids in the cardiopulmonary bypass circuit prime prevents the deleterious consequences of reoxygenation in hypoxemic piglets. Myocardial dysfunction and reduced tolerance to oxidative stress were significantly restricted in animals, which were treated with the combination of the two dicarboxylic amino acids (Morita et al. 1995), while supplementing cardioplegic formulation with aspartate and glutamate additives reduces conjugated diene levels and enhances antioxidant reserve capacity (Ihnken et al. 1995). Glutamate loading has been shown to act against intracellular reactive oxygen species generation in adult cardiomyocytes through the stimulation of glutathione (GSH) peroxidase activity (King et al. 2003). In addition, intraperitoneal treatment with aspartate or glutamate maintains normal mitochondrial function during myocardial infarction in experimental rats through reduction of lipid peroxides and enhancement of GSH in the mitochondria (Sivakumar et al. 2008).

On the other hand, serum gamma-glutamyl transferase (γ-GT), an enzyme that elevates in all forms of liver disease, has been recently implicated in cardiovascular disease and high mortality risk (Grundy 2007; Hozawa et al. 2007; Lee et al. 2007). The mechanism that connects γ-GT with atherogenesis is not elucidated but it has been postulated that contributes to oxidative stress and is inversely correlated with levels of antioxidants (Lee et al. 2004; Paolicchi et al. 2006; Grundy 2007; Hozawa et al. 2007; Lee et al. 2007; Wannamethee et al. 2008).

The present study was carried out to examine the effect of oral supplementation with L-aspartate and L-glutamate on rabbit aortic lesions, which are one step closer to human fatty streaks compared to the early lesions studied (Yanni et al. 2003). Since oxidative stress hold key role also in fatty liver disease (Pessayre 2007), the effect of dicarboxylic amino acids on the extent of liver lesions, which are caused in the rabbit by high cholesterol diet, was investigated.

Materials and methods

Chemicals

L-Aspartic acid sodium salt monohydrate ($\geq$ 98% purity), L-glutamic acid monosodium salt monohydrate ($\geq$ 98% purity) and cholesterol (approximately 95% purity) were obtained from Sigma-Aldrich (Germany). The diagnostic kits for the determination of plasma total cholesterol (TC), HDL cholesterol (HDLC) and triacylglycerol (TAG) concentrations and for serum γ-GT, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were obtained from Wassermann Diagnostics.

Animals and experimental design

Eighteen male New Zealand White rabbits (body weight 2.54 $\pm$ 0.13 kg, mean $\pm$ SD) were individually housed in stainless steel wire-bottom cages. At the beginning of the experimental period the animals were 2 months old and before the intervention they remained for 2 weeks in the laboratory for acclimatization. Animals were kept in a temperature-controlled environment (19 $\pm$ 1°C with 55 $\pm$ 5% humidity) with a 12-h light/dark cycle (5:30 a.m. to 5:30 p.m.) in an air-conditioned room and had free access to standard rabbit chow and tap water. A veterinarian was observing their health and general condition during the entire experimental period. The animal care facility and all the procedures were reviewed and approved by the Veterinary Directorate of the Athens Prefecture and conducted in compliance with the European Convention in Animal Care.

After the 2 weeks of acclimatization, animals were randomly separated in three groups. Animals in Chol group ($n = 8$) received standard commercial rabbit chow supplemented with 0.5% w/w cholesterol while animals in Chol + AspGlu group ($n = 8$) received the same diet and 12.5 mM L-aspartic acid monosodium salt (L-aspartate) and 12.5 mM L-glutamic acid monosodium salt (L-glutamate) in drinking water using conditions previously described by our study (Yanni et al. 2003) as well as other research groups in similar studies (Boger et al. 1995; Behr-Russel et al. 2000; Hayashi et al. 2005). A group of animals ($n = 4$) served as control. The duration of the intervention was 7 weeks. Rabbit chow consisted of (w/w): 37% carbohydrates, 16% proteins, 4% fat, 15% fiber, 11% moisture, 8% ash and an appropriate mixture of minerals and vitamins for the healthy subsistence of the animals in the laboratory (added to the premix by the manufacturer). The atherogenic food was prepared by dissolving cholesterol in diethyl ether (without butylated hydroxytoluene as inhibitor) and thoroughly coating the pellets of chow with the mixture. After ether evaporation, it was stored at −20°C

until use. Fresh food from the freezer was provided to the animals every morning between 8:30 and 10:30 a.m. while food remaining from the previous day was removed from the cages. Amino acids salts' solution was prepared using tap water as solvent.

Food and amino acid consumption was recorded daily and body weight was measured every 2 weeks. Due to the fact that dietary stress severely affects immune and cell functions in rabbits (Franci et al. 1996), the amount of provided food was not restricted. Blood samples were collected from the central ear artery without anesthesia after 14-h fast and restriction from amino acid consumption at 0, 4 and 7 weeks of dietary intervention. At the end of the study, animals were anesthetized by intramuscular injection of ketamine and xylazine (35 and 10 mg/kg of body weight, respectively). After anesthesia, animals were given an overdose of sodium pentobarbital, intravenously. Right brain hemisphere, right liver lobe and aorta from the arch to the iliac bifurcation were quickly removed.

Blood biochemical analyses

Blood samples were collected in pre-cooled tubes containing EDTA as anticoagulant to determine TC, HDLC and TAG concentrations. A separate blood sample portion was transferred into tubes with no-additives for serum preparation needed for γ-GT, AST and ALT determinations. Plasma and serum samples were stored at $-80°C$ until analysis and the hemolyzed ones were discarded. All samples were measured in the same batch in an automated analyzer (Schiapparelli Biosystems Inc) using commercially available kits.

Histochemistry

The isolated aorta was quickly transferred in ice-cold 0.9% w/v NaCl solution, cleaned of surrounding tissue and cut open along its length. The vessel was then fixed in neutral 10% v/v formalin. Liver and brain were also fixed in the same solution. An expert blinded to the treatment groups performed the histological preparation. Two days after fixation in formalin, representative sections of aorta (thoracic and abdominal), liver and brain were obtained and processed for paraffin incubation. Sections of 5 μm thickness were cut and stained with eosin and hematoxylin for microscopic examination.

Image analysis was performed in order to evaluate intimal thickening in aorta specimens. Slides were digitized using a microscope Eclipse 80i (Nikon Corp., Tokyo, Japan) attached to a digital camera (DS-2 MW, Nikon Corp.). The images were transferred to computer equipped with the appropriate software (Image ProPlus v 5.1, Media Cybernetics, MD, USA) and the layers of the vessel wall (intima, media, serosa) were traced. The intimal thickening was then calculated automatically.

Table 1 Food consumption of Control, Chol and Chol + AspGlu groups of rabbits during the dietary intervention of 7 weeks (g/kg body weight, mean ± SEM)

Group	2 Weeks	4 Weeks	7 Weeks
Control	54.77 ± 2.26	50.89 ± 4.07	43.03 ± 4.27
Chol	55.66 ± 1.72	53.17 ± 2.43	36.97 ± 1.87
Chol + AspGlu	49.73 ± 2.16	48.50 ± 0.70	36.63 ± 1.52

P = NS

The liver was evaluated as it has been previously described by the Pathology Committee of non-alcoholic steatohepatitis Clinical Research Network (Kleiner et al. 2005). Both eosin–hematoxylin and Masson's trichrome stains were used. The histological features were grouped into five broad categories: steatosis, ballooning, portal inflammation, lobular activity and fibrosis. A score from 0 (absence) to 3 (severe lesion) was assigned to each parameter. The brain was studied for interstitial edema, inflammation, apoptotic figures and neuron chromatin condensation.

Statistical analysis

Values were expressed as mean ± SEM. Comparisons were made by ANOVA. Liver lesions were demonstrated as mean score ± SEM for steatosis, ballooning, portal inflammation and lobular activity individually, for each experimental group. Fibrosis was also expressed as mean score ± SEM. The effect of dietary amino acids on intima thickness was tested by ANCOVA using 7th week plasma cholesterol concentration as covariate. $P < 0.05$ was considered statistically significant.

Results

Blood biochemical data

There were no statistical differences in food consumption between the three groups of animals during the entire experimental period ($P > 0.05$, Table 1). The amount of each amino acid received by the animals of Chol + AspGlu group ranged between 1.19 ± 0.09 mmol/kg of body weight at 2 weeks of the study and 0.90 ± 0.10 mmol/kg of body weight at 7 weeks (mean ± SEM). Total plasma cholesterol was increased significantly in Chol and Chol + AspGlu groups ($P < 0.05$ vs. Control, Table 2) but without statistical difference between the two groups ($P > 0.05$). Similarly, non-HDLC was increased in both cholesterol-fed groups during the study ($P < 0.05$ vs.

Table 2 Plasma total cholesterol (TC), HDL-cholesterol (HDLC), non-HDL cholesterol (non-HDLC) and triacylglycerol (TAG) concentrations (mean ± SEM) of Control, Chol and Chol + AspGlu groups of rabbits

	Group	0 Weeks	4 Weeks	7 Weeks
TC (mg/dl)	Control	57.13 ± 9.59	77.38 ± 8.75	66.43 ± 7.90
	Chol	59.69 ± 10.57	640.94 ± 111.60[a,b]	982.50 ± 171.55[a,b]
	Chol + AspGlu	58.44 ± 6.20	894.88 ± 91.02[a,b]	1285.63 ± 140.09[a,b]
HDLC (mg/dl)	Control	23.88 ± 6.00	30.38 ± 2.51	24.60 ± 4.19
	Chol	28.86 ± 8.08	26.43 ± 3.89	33.81 ± 5.06
	Chol + AspGlu	27.84 ± 5.02	28.34 ± 2.11	40.84 ± 3.52
Non-HDLC (mg/dl)	Control	33.25 ± 3.60	44.00 ± 6.80	41.81 ± 6.62
	Chol	34.36 ± 4.90	665.36 ± 11.29[a,b]	1010.47 ± 180.64[a,b]
	Chol + AspGlu	33.34 ± 3.38	752.92 ± 61.79[a,b]	1274.17 ± 170.10[a,b]
TAG (mg/dl)	Control	74.81 ± 7.14	59.31 ± 5.13	58.68 ± 4.20
	Chol	71.25 ± 11.13	85.31 ± 10.79[a,b]	96.21 ± 10.21[a,b]
	Chol + AspGlu	60.71 ± 5.26	91.79 ± 12.07[a,b]	103.21 ± 9.97[a,b]

[a] $P < 0.05$ vs. baseline

[b] $P < 0.05$ vs. Control group

Table 3 Serum γ-GT, AST and ALT activities of Control, Chol and Chol + AspGlu groups of rabbits

	Group	0 Weeks	4 Weeks	7 Weeks
γ-GT (U/L)	Control	14.75 ± 2.29	12.93 ± 0.71	12.60 ± 1.54
	Chol	16.63 ± 1.56	21.00 ± 5.46	51.50 ± 9.84[a,c]
	Chol + AspGlu	21.75 ± 3.39	31.00 ± 8.65	31.88 ± 6.28[b,c]
AST (U/L)	Control	23.00 ± 2.27	22.00 ± 2.83	20.10 ± 0.93
	Chol	27.25 ± 5.07	26.38 ± 4.78	38.25 ± 5.14[c]
	Chol + AspGlu	21.00 ± 1.99	21.63 ± 4.40	54.25 ± 11.47[c]
ALT (U/L)	Control	48.00 ± 9.20	54.75 ± 8.04	56.10 ± 1.84
	Chol	50.63 ± 7.29	52.50 ± 10.33	59.50 ± 16.06
	Chol + AspGlu	33.38 ± 4.08	60.13 ± 17.14	54.25 ± 5.44

[a] $P < 0.05$ vs. Control group

[b] $P < 0.05$ vs. Chol group

[c] $P < 0.05$ vs. baseline

Control, Table 2) without difference between them ($P > 0.05$). On the other hand, HDLC did not significantly changed in the duration of the study since there was no difference in HDLC levels of animals in Chol and Chol + AspGlu groups in comparison to animals of Control group ($P > 0.05$ vs. Control, Table 2). TAG levels elevated in both groups during the intervention ($P < 0.05$ vs. Control, Table 2) but without statistical difference between the two groups ($P > 0.05$).

Serum γ-GT activity was increased in both Chol and Chol + AspGlu groups but the increase was significantly lower in animals treated with L-aspartate and L-glutamate ($P < 0.05$ between the two groups in the 7th week vs. baseline). Table 3 represents alterations in γ-GT activity, which were expressed during the time of intervention in the three groups of rabbits and also changes in AST and ALT activity. AST was increased in Chol and Chol + AspGlu groups ($P < 0.05$ in comparison to baseline values) but without difference between the two groups ($P > 0.05$). ALT was not affected by cholesterol feeding since there was no difference in ALT activity of Chol and Chol + AspGlu groups when compared to Control group in the entire experimental period (Table 3).

Histochemistry

Intima thickness was measured in thoracic and abdominal aorta and the results are presented in Fig. 1. Animals of Chol + AspGlu group had lower intima thickness in comparison to animals of Chol group in both parts of the aorta, which reached significant difference between the two groups in the thoracic part ($P < 0.05$).

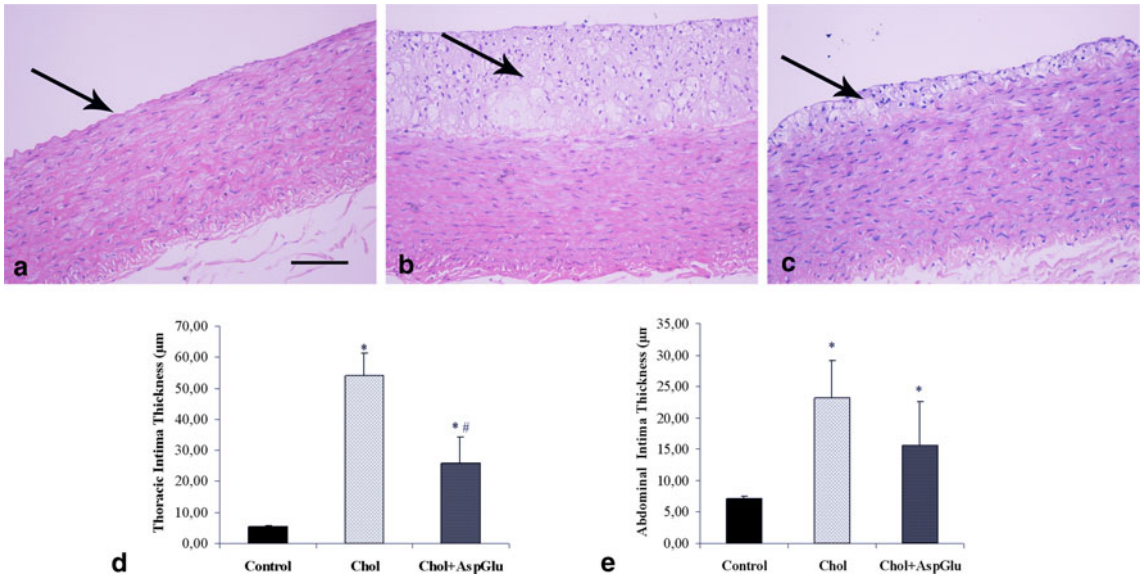

Fig. 1 Effect of cholesterol and Asp/Glu intake in the intimal thickness (*arrows*). *Photomicrographs* of representative aortic sections of Control (**a**), Chol (**b**) and Chol + AspGlu (**c**) groups are presented. Hematoxylin–eosin stain, scale bar = 100 μm. The *graphic forms* of thoracic (**d**) and abdominal (**e**) aortic thickness are also presented. $^{*}P < 0.05$ vs. Control group, $^{\#}P < 0.05$ vs. Chol group

Table 4 Scores for steatosis, ballooning, portal inflammation and lobular activity of Control, Chol and Chol + AspGlu groups of rabbits

Group	Steatosis	Ballooning	Portal inflammation	Lobular activity
Control	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00
Chol	1.75 ± 0.16[a]	1.75 ± 0.31[a]	1.38 ± 0.18	0.88 ± 0.30
Chol + AspGlu	0.88 ± 0.23[a,b]	1.75 ± 0.16[a]	0.38 ± 0.18	0.25 ± 0.16

Values are expressed as mean score ± SEM

[a] $P < 0.05$ vs. Control group

[b] $P < 0.05$ vs. Chol group

Liver lesions were expressed as mean score ± SEM. Scores for steatosis, ballooning, portal inflammation and lobular activity (Brunt and Tiniakos 2002; Kleiner et al. 2005) are presented in Table 4. Fibrosis score is demonstrated in Fig. 2. Steatosis was significantly attenuated in animals of Chol + AspGlu group in comparison to animals of Chol group ($P < 0.05$). Fibrosis was also attenuated in the same group of animals ($P < 0.05$) and the score was similar to that of Control animals. Portal inflammation and lobular activity were also more pronounced in Chol group of rabbits.

The grade of fatty liver disease was considered as "mild" in the seven of the eight animals of Chol + AspGlu group and "moderate" in one animal. In Chol group, the grade was "severe" in four animals, "moderate" in three animals and "mild" in one animal (with lower cholesterol levels compared to the others). Photomicrographs of representative aortic and liver sections are shown in Figs. 1 and 2.

Cross-sections of brain tissue did not reveal any lesions in both treatment groups (Fig. 3).

Discussion

The present study shows that oral supplementation with L-aspartate and L-glutamate inhibits the progression of fatty streaks and the development of fatty liver disease in the animal model of cholesterol-fed rabbit.

Previous studies have shown that dietary supplementation with L-aspartate and L-glutamate inhibits fatty streak initiation in rabbits fed with a diet enriched with 0.25% w/w cholesterol for 4 weeks (Yanni et al. 2003). The protective effect of the two amino acids in the initiating stages of the disease was partially attributed to a mechanism that includes HDL (Yanni et al. 2005) and was hypothesized that it could be associated with the enhancement of endogenous antioxidants (Yanni et al. 2003, 2005) since

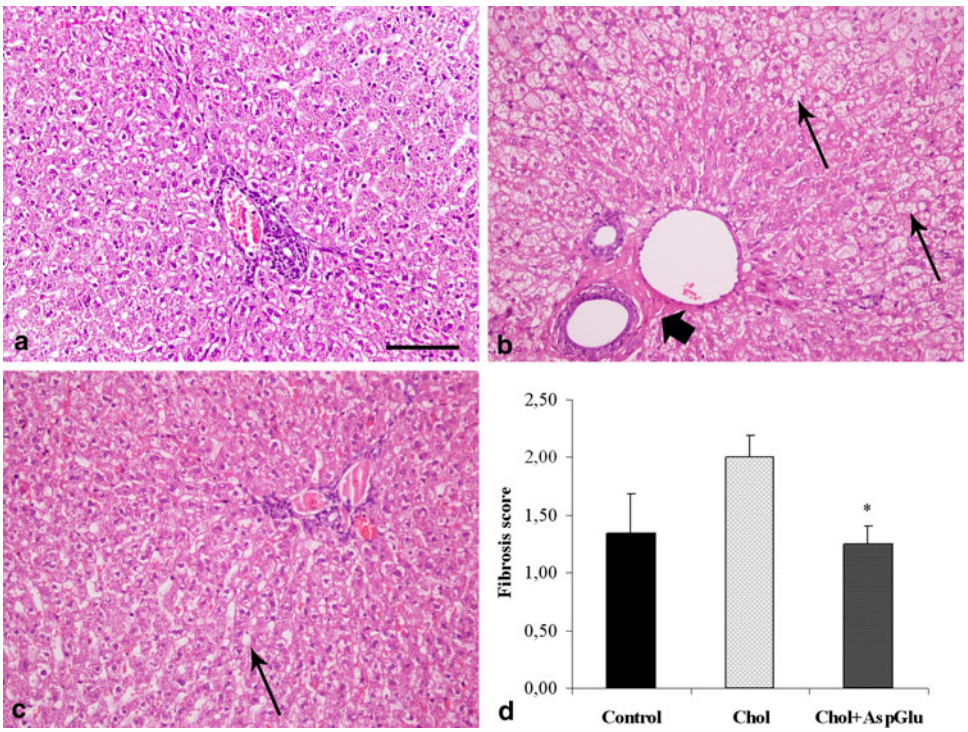

Fig. 2 *Photomicrographs* of representative liver sections from animals of Control (**a**), Chol (**b**) and Chol + AspGlu (**c**) groups. *Arrows* in **b** and **c** indicate hepatocyte ballooning and lipid inclusions (steatosis) which is more obvious in **b**. *Arrowhead* in **b** indicates also mild periportal fibrosis. Hematoxylin–eosin stain, scale bar = 100 μm. The *graphic form* (**d**) of fibrosis is also presented. $*P < 0.05$ vs. Chol group

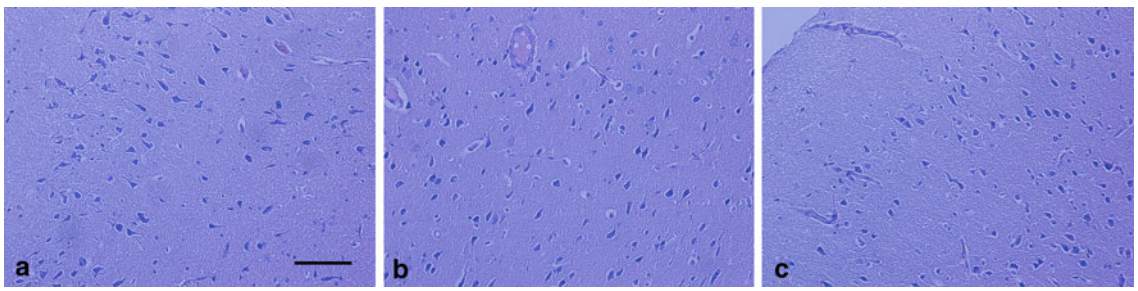

Fig. 3 *Photomicrographs* of representative brain sections from animals of Control (**a**), Chol (**b**) and Chol + AspGlu (**c**) groups. Hematoxylin–eosin stain, scale bar = 100 μm

bibliographic data support antioxidant effects of aspartate and glutamate (Morita et al. 1995; Ihnken et al. 1995; King et al. 2003; Sivakumar et al. 2008).

The present study was designed in order to investigate the effect of dicarboxylic amino acids on the progression of fatty streaks and the development of fatty liver disease. For this reason, the cholesterol-fed rabbit was used as animal model. It is known that feeding rabbit with high cholesterol diet leads to the formation of aortic lesions, which are proportional to the amount of consumed cholesterol. The role of the liver in the formation of these lesions is crucial: hypercholesterolemia induces the production of large amounts of atherogenic lipoproteins, low-density lipoproteins (LDL) and β-very LDL, which remain in the blood circulation for extended time inducing endothelial dysfunction (Kolodgie et al. 1996). In recent years, the cholesterol-fed rabbit is also being used as animal model for the study of fatty liver disease related to hyperlipidemia. It has been observed that cholesterol feeding leads to characteristic fibrosis, ballooning, activation of hepatic stellate cells and enhanced oxidative stress (Kainuma et al. 2006; Birkner et al. 2007; Kawada and Otogawa 2007; Fujimoto et al. 2008; Yoko et al. 2008; Fu et al. 2009). In the present study, hepatocellular fat deposition, ballooning, lobular

inflammation, fibrosis and steatosis were observed in the liver (Fig. 2) and also the formation of early lesions rich in foam cells in the aorta (Fig. 1).

Our results revealed that the oral supplementation with L-aspartate and L-glutamate inhibits the progression of fatty streaks and the development of fatty liver disease. Thoracic intima thickness was significantly reduced in animals, which received the combination of the two dicarboxylic amino acids in comparison to the animals that received only the cholesterol-enriched diet. Interestingly, supplementation with L-aspartate and L-glutamate also inhibited the development of liver lesions. Seven rabbits of Chol group exhibited fatty liver disease from "moderate" to "severe" grade while lesions of Chol + AspGlu group were classified as "mild" except from one animal, which were classified as "moderate". Score for steatosis was significantly lower in animals treated with the dicarboxylic amino acids' combination and the score for fibrosis was similar to that of normally fed animals.

In our previous study (Yanni et al. 2003), we showed that oral supplementation with L-aspartate and L-glutamate inhibits mononuclear cell adhesion to the endothelium and the formation of foam cells in the intima of thoracic aorta, the initiating stages of atherogenic process. In the present study, we made on more step in order to come closer to human fatty streaks by causing in rabbit aorta, intimal thickening. For this reason, we used a different dietary intervention, with higher cholesterol concentration (0.5% w/w instead of 0.25% w/w in our previous experiment) and longer duration (7 weeks instead of 4). The results showed that the protective effect of the combination of dicarboxylic amino acids was also insisted in more advanced lesions. As far as the effect on the liver concerns, this has not been assessed in our first study because at the time it was designed, cholesterol-fed rabbit was not used as animal model for fatty liver disease. In any case, higher cholesterol amounts and longer durations of dietary interventions than the one was used (Yanni et al. 2003) are needed in order to cause liver lesions characteristic to non-alcoholic fatty liver disease (Kainuma et al. 2006; Birkner et al. 2007; Kawada and Otogawa 2007; Fujimoto et al. 2008; Yoko et al. 2008; Fu et al. 2009).

Another observation of the present work was that serum γ-GT activity increased in all cholesterol-fed animals during the intervention but the increase was milder and significantly lower in the animals treated with L-aspartate and L-glutamate. Serum γ-GT originates from hepatobiliary system and its activity is elevated in all forms of liver disease. Recently, it has been implicated in cardiovascular disease and high mortality risk (Grundy 2007; Hozawa et al. 2007; Lee et al. 2007; Wannamethee et al. 2008). The mechanism that connects γ-GT with atherogenesis is not elucidated. It has been reported that the enzyme is absorbed in LDL particles and catalyzes their oxidation leading to atherosclerotic lesion formation (Paolicchi et al. 2006). It has also been postulated that contributes to oxidative stress and is inversely correlated with levels of antioxidants (Lee et al. 2004). The limited elevation of enzyme activity that caused by dietary dicarboxylic amino acids probably reflects the milder liver lesions, which were observed in the treated animals but also represents means of vessel protection. Accordingly, the implication of γ-GT in oxidative stress supported by bibliography reinforces the hypothesis that dicarboxylic amino acids may protect vessel and liver through the enhancement of endogenous antioxidants. However, further studies are needed in order to elucidate the mechanism of their action and its association with γ-GT.

As far as the concentrations of blood parameters concern, we observed that cholesterol feeding caused increase in the concentration of TC, non-HDLC and TAG in both treatment groups. These were presumable results, which were caused by the high availability of substrates. In our previous study on dicarboxylic amino acids (Yanni et al. 2005), we found that their administration causes significant elevation in HDLC levels. Such an elevation was not observed in the present study. Probably, this is due to higher amount of cholesterol and longer duration of dietary intervention, which was used that leads to very high cholesterol concentration in plasma and possibly counteracts the beneficial effect on HDLC. As far as AST concerns, it was increased in both cholesterol-fed groups without difference, meaning that amino acids are not implicated in its activity. It is known that AST is a marker of fatty liver disease (Mardini and Record 2005) and it could be expected that the protective effect of aspartate and glutamate on fatty liver must also reflect maintenance of AST activity. Such a property was not observed and it cannot be explained by the available data. However, it has to be mentioned that in the cholesterol-fed rabbit the association of AST activity with the severity of liver lesions is not clear. There are studies showing no effect on enzyme activity by cholesterol feeding although characteristic liver lesions are present (Kainuma et al. 2006; Kawada and Otogawa 2007) and other studies showing a significant elevation of the enzyme activity when the animal is under high cholesterol diet and characteristic liver lesions are also present (Fang et al. 2008; Fu et al. 2009). Probably this is due to the different experimental conditions used. On the other hand, ALT did not change during the intervention in any group and cholesterol feeding did not impair its activity in serum, something that has also been observed in other studies (Kainuma et al. 2006; Kawada and Otogawa 2007). Further research work will set light on the implication of aspartate and glutamate in fatty liver disease and the association with liver enzymes.

Compiling the experimental results, we conclude that the combination of dicarboxylic amino acids inhibits the progression of fatty streaks and the development of fatty liver disease causing a limited elevation in serum γ-GT activity. The mechanism of their action is not attributed to reduction of serum lipids and remains to be elucidated. It is probably related to the maintenance of endogenous antioxidants (i.e., glutathione) since aspartate and glutamate hold key roles in energy generation through the production of Krebs' cycle intermediates.

The applied dose of amino acids was based on previous research work performed by Yatzidis (2002) as well as on personal observations (unpublished data), which guided in a daily amount of 1 mmol/kg of body weight of each amino acid that could be effective.

In our previous study (Yanni et al. 2003), we used the certain amount of the combination of the two amino acids and the results were encouraging. Although the percentage of cholesterol in the present study leads to more advanced lesions, it was undesirable to exceed the amount of 1 mmol/kg/day of each amino acid due to the long discussion about the neuroexcitory effects of aspartate and glutamate. However, to our knowledge, there is no acceptable daily intake for these amino acids (Walker 1999; Yanni et al. 2003). Especially for glutamate consumption, the Consensus meeting of Hohenheim in 2007 made clear that it does not cause adverse effects except if it is consumed in extremely high amounts (Beyreuther et al. 2007). However, due to the fact that the combination of these amino acids was administered, we proceeded to brain histological examination. Cross-sections of brain tissue did not reveal lesions in any group of rabbits.

There is a line of argument about the effects of amino acid supplements. However, it is generally accepted that in cases of increased needs non-essential amino acids become essential. Amino acids perform extremely important roles except from their participation in protein synthesis, such as the provision of intermediates for metabolic routes and the production of energy.

In conclusion, dietary supplementation with dicarboxylic amino acids, L-aspartate and L-glutamate inhibits the progression of fatty streaks and the development of fatty liver disease in the animal model of cholesterol-fed rabbit. The data of this study indicate that their protective action resulted in limited γ-GT elevation in serum. However, studies are needed in order to elucidate the underlying mechanism.

The beneficial effects of L-aspartate and L-glutamate on the complicated condition of atherogenesis and fatty liver disease related to hyperlipidemia, which are exerted in an animal model, open a field of investigation in humans, especially due to restricted known therapeutic interventions and virtually the inexistence of side effects.

Acknowledgments We thank Perikles Kakias and Vissaris Kakias for their excellent technical assistance. The Medical School of National and Kapodistrian University of Athens and the Department of Dietetics and Nutrition of Harokopio University of Athens supported this work.

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