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Circulating levels of apelin, glucagon-like peptide and visfatin in hypercholesterolemic–hyperhomocysteinemic guinea-pigs: their relation with NO metabolism

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Abstract The aim of this study was to determine the levels of regulatory peptides apelin, glucagon-like peptide (GLP-1) and visfatin in hypercholesterolemic and hyperhomocysteinemic state and to examine their relation with nitric oxide (NO) metabolism. 32 Male guinea pigs were divided into four groups and each group was fed as follows: (a) commercial chow, (b) cholesterol (*chol*)-rich diet, (c) methionine (*meth*)-rich diet, and (d) *chol* + *meth*-rich diet. Blood samples were drawn at the end of 10 weeks, and abdominal aorta was dissected for histopathological examination. Serum insulin, GLP-1, apelin, visfatin, and nitrotyrosine concentrations were measured by the manufacturer's kits based on ELISA; asymmetric dimethylarginine (ADMA) and arginine levels were measured by the high performance liquid chromatography. Homocysteine level was measured by the chemiluminescence immunoassay; glucose, total *chol* and triglyceride levels were measured by the autoanalyzer. The microscopic examination of aorta indicated varying degrees of vascular disturbance in *chol*- and *chol* + *meth*-fed groups. High levels of *chol* and homocysteine, accompanied with significantly low levels of apelin and GLP-1 were detected in the plasma. Visfatin, ADMA, and nitrotyrosine levels both in *chol*- and *chol* + *meth*-fed groups were significantly higher than those in control animals, whereas arginine and arginine/ADMA ratio were lower. This study indicated that circulating levels of apelin, GLP-1, and visfatin are markedly altered during the development of atherosclerotic changes in close association with *chol*, homocysteine, NO, and ADMA levels. The measurements of these peptides in serum may help for the diagnosis and follow-up of vascular dysfunction.

Keywords Apelin · Visfatin · Glucagon-like peptide · Hypercholesterolemia · Nitric oxide · Hyperhomocysteinemia · Asymmetric dimethylarginine

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Introduction

High plasma cholesterol (*chol*) and homocysteine, the major risk factors for the development of atherosclerosis, are known to cause impairments in endothelial-dependent relaxation which is linked a decrease in the bioavailability of nitric oxide (NO) [1]. NO is a major endothelium-derived relaxing substance synthesized from L-arginine by the activity of NO synthase (NOS). In order to elucidate the mechanisms through which *chol* and homocysteine exacerbate the development of atherosclerosis, either genetic modifications or dietary regimens enriched with *chol* and methionine (*meth*), the precursor of homocysteine, have commonly been used in animal experiments.

Atherosclerotic and inflammatory conditions are always accompanied by irregular synthesis of adipocyte-derived substances. The synthesis of pro-inflammatory mediators by the increased bulk of adipose tissue may further induce inflammatory changes in obesity, and cause a vicious circle for the production of several hormones and adipokines. Apelin is one of these regulatory peptides synthesized in adipocytes as well as other organs, and mainly in endothelial cells [2, 3]. It has been identified as an endogenous

ligand of the G-protein-coupled receptor [4]. The effects of apelin have been demonstrated in cardiovascular and immune functions and energy homeostasis [5, 6]. Previous observations indicated that apelin lowers the blood pressure via a NO-dependent mechanism [7].

Visfatin, another novel peptide synthesized in visceral adipocytes, is expressed mostly in macrophage-infiltrating adipose tissue during inflammatory response [8]. Plasma visfatin levels are known to increase progressively with the degree of obesity, and associate with insulin resistance [9]. Significantly elevated visfatin levels were observed in coronary artery disease, suggesting the involvement of visfatin in the pathogenesis of atherosclerosis [10].

Glucagon-like peptide (GLP-1), a member of the proglucagon incretin family, has been shown to play a role in atherosclerotic process through inducing eNOS [11]. GLP-1 receptors are abundantly expressed in endothelial cells, monocyte/macrophages, and smooth muscle cells. Recent studies suggested that the anti-inflammatory, antiproliferative, and vasodilatory properties of GLP-1 signaling may protect the vascular wall against atherogenesis [12]. In contrast, some researchers have reported positive associations of circulating GLP-1 levels and the development atherosclerosis [13, 14].

Although several animal models have been used to investigate mechanisms of homocysteine and/or *chol*-induced vascular dysfunction, experimental studies related to the role of regulatory peptides in this process are limited. In this study, we used the combination of high dietary *chol* and *meth* in order to develop atherosclerotic changes and endothelial dysfunction in guinea pigs, and measured the circulating levels of apelin, GLP-1, and visfatin together with the biochemical parameters of NO metabolism in order to evaluate their involvement with vascular dysfunction.

Materials and methods

Study groups

Male Dunkin–Hartley guinea pigs, 4–6 months old, and weighing 695 ± 38.6 g, were used. The animals were obtained from the Experimental and Medical Research Institute, Istanbul University. They were kept in steel wire cages at room temperature (25 °C) and maintained on a 12-h light/dark cycle. The study protocols were approved by the Animal Care and Use Committee, Istanbul University.

Thirty-two animals were divided into four groups, eight animals in each. Group 1 (control) was fed a commercial laboratory chow. For the other three groups, a diet chow was prepared by the addition of *chol* (Alfa Aesar A11470) and/or L-methionine (Sigma). Group 2 (*chol*) received a diet supplemented with 1.5 % (w/w) *chol*, while group 3 (*meth*) had a diet containing 2 % (w/w) *meth* only. Group 4 (*chol* + *meth*) was fed with *chol* (1.5 %) + *meth* (2 %)-supplemented diet [15, 16]. Food and water were supplied ad libitum.

Methods

At the end of 10 weeks, animals were anesthetized by sodium thiopental following an overnight fasting and blood samples were drawn by the cardiac venipuncture. Aliquots of serum and plasma were stored at −80 °C until studied, and used for the biochemical analyses. Serum insulin and apelin levels were measured by the competitive binding enzyme immunoassay kits (Wuhan EIAab Science, and Novateinbio, Cambridge, USA, respectively), and serum GLP-1 levels were determined by sandwich enzyme-linked immunosorbent method (Wuhan EIAab Science, Wuhan, China).

Glucose, total *chol* and triglyceride levels were carried out on the same day by using Roche autoanalyzer. Homeostasis model assessment (HOMA-IR) was calculated by the formula of insulin (mU/L) × glucose (mmol/L)/22.5 [17].

Homocysteine concentrations were measured by the chemiluminescence immunoassay using Immulite 2000 XPI (Siemens Medical Solutions Diagnostics, IL, USA).

Serum asymmetric dimethylarginine (ADMA) and L-arginine concentrations were determined using high performance liquid chromatography following pre-column derivation with *o*-phthalaldehyde [18].

Nitrotyrosine levels were measured by the enzyme-linked immunosorbent assay (Cell Biolabs, Inc.). NO levels were estimated as total nitrite + nitrate using spectrophotometric commercial kit (Oxford Biomedical Research, Oxford, USA).

Histopathological studies

Pieces of abdominal aorta from the control and experimental groups were removed immediately and fixed in 10 % buffered formaldehyde and processed for paraffin sectioning. Sections 5 μm in thickness were stained with haematoxylin and eosin (H&E) using a standard protocol and analyzed by the pathologist on the light microscopy.

Statistical analysis

The data were analyzed using SPSS 15 (SPSS, Chicago, IL, USA). The results were expressed as mean ± SD. One-way analysis of variance followed by Tukey's post-hoc test was used for equal variances. Kruskal–Wallis variance analysis and a post-hoc analysis using Mann–Whitney *U*-test were performed for unequal variances. In all cases, a

Table 1 Effects of high cholesterol and/or high methionine diet on the biochemical parameters in plasma of guinea pigs (mean ± SD; $n = 8$ in each group)

Groups	Control	*Chol*	*Meth*	*Chol + meth*
Insulin (mIU/L)	1.78 ± 0.39	1.40 ± 0.32	1.38 ± 0.42	1.41 ± 0.66
HOMA-IR	0.61 ± 0.19	0.45 ± 0.23	0.45 ± 0.17	0.45 ± 0.26
Cholesterol (mg/dL)	46.7 ± 11.4	246 ± 29.6[a]	80.0 ± 9.16[a,b]	288 ± 42.9[a–c]
Homocysteine (μmol/L)	4.48 ± 1.02	6.30 ± 1.56[a]	17.1 ± 5.31[a,b]	22.2 ± 5.74[a,b]
NO (μmol/L)	19.3 ± 2.99	15.8 ± 6.53	13.2 ± 6.37	14.3 ± 3.16
Nitrotyrosine (nmol/L)	23.0 ± 2.17	30.3 ± 3.18[a]	26.7 ± 4.92	46.9 ± 3.23[a–c]

$p < 0.05$ in comparison with: [a] control, [b] cholesterol, [c] methionine groups

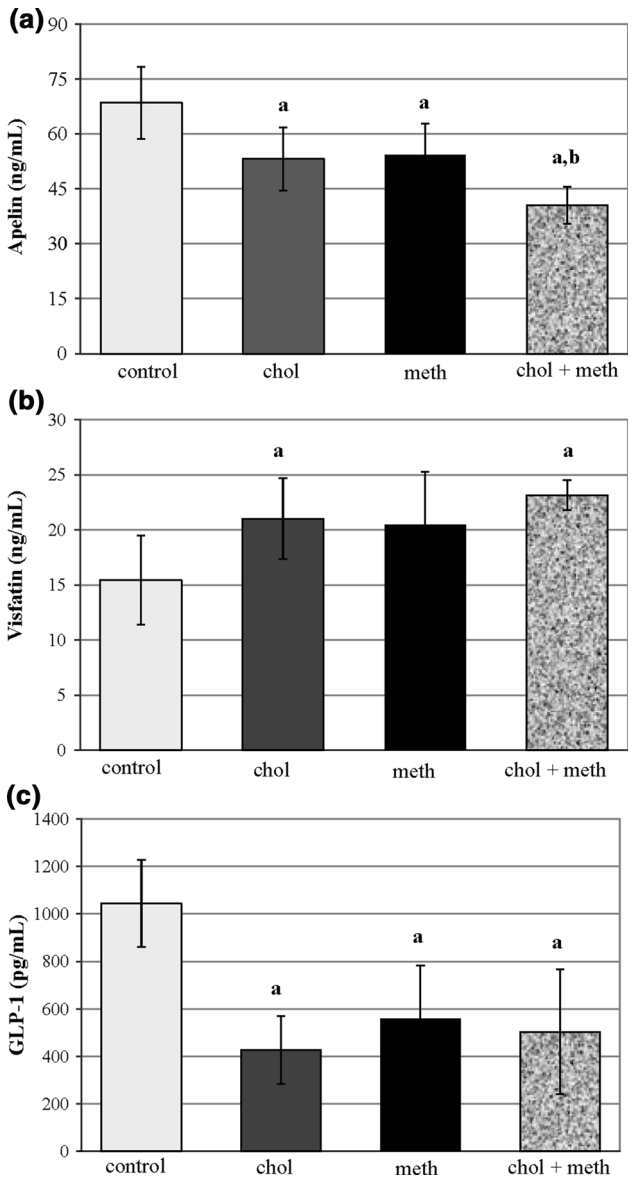

Fig. 1 Effects of high cholesterol and/or high methionine diet on serum apelin (**a**), visfatin (**b**), and GLP-1 (**c**) levels in the study groups (mean ± SD; $n = 8$ in each group). $p < 0.05$ as compared with: (*a*) *control*, (*b*) *cholesterol* groups

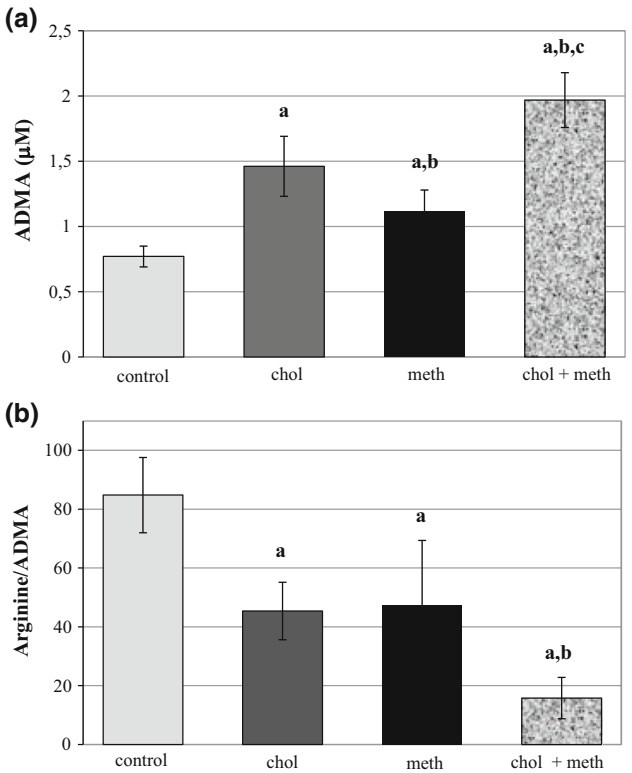

Fig. 2 Effects of high cholesterol and/or high methionine diet on plasma ADMA (**a**) and arginine/ADMA ratio (**b**) in the study groups (mean ± SD; $n = 8$ in each group). $p < 0.05$ as compared with: (*a*) *control*, (*b*) *cholesterol*, and (*c*) *methionine* groups

difference was considered significant when $p < 0.05$. Correlation analyses were carried out by the Pearson test.

Results

The biochemical data are presented in Table 1. Levels of glucose and insulin in *chol*, *meth*, and *chol + meth* groups were not different than those from the control group, and therefore similar HOMA-IR values were obtained in all

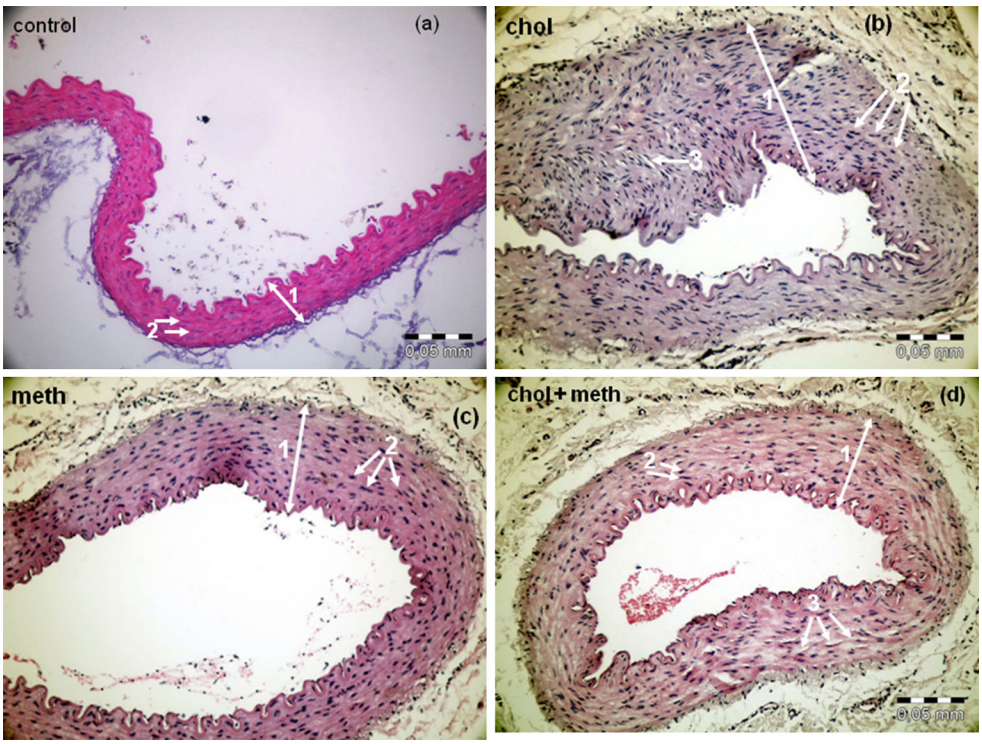

Fig. 3 Histopathological examination of the aortic sections from the animals in the study groups (H&E, magnification 200). **a** *Control*, **b** *chol* increased intima-media thickness (*1*), smooth muscle cell proliferation (*2*) and lipid vacuoles with fatty streaks resembling cholesterol crystals (*3*), **c** *meth* only slight increase in intima-media thickness and smooth muscle cell proliferation, and **d** *chol* + *meth* similar to *chol* group

groups. Serum *chol* levels were higher in all diet-fed groups than those in control animals. Homocysteine levels were high in *chol*, *meth*, and *chol* + *meth* groups.

Serum NO levels were slightly lower in *chol*-fed (18 %) and *chol* + *meth*-fed animals (26 %) than in controls. However, the difference with regard to control group was not significant. Nitrotyrosine levels were significantly high in *chol*- and *chol* + *meth*-fed animals (Table 1).

Significantly lower levels of apelin were found in *chol*, *meth*, ($p < 0.02$) and *chol* + *meth* groups ($p < 0.01$) in comparison to controls; apelin levels being markedly lower in the *chol* + *meth* group than the other groups ($p < 0.05$; Fig. 1a).

Serum visfatin levels in *chol* and *chol* + *meth* groups were significantly high compared to the control group ($p < 0.05$ and <0.01, respectively; Fig. 1b). Serum GLP-1 concentrations were significantly lower in these groups as well as in the *meth* group than in controls ($p < 0.01$; Fig. 1c). No difference was noticed between the diet-fed groups.

ADMA levels in all diet-fed groups were significantly high as compared to the control group ($p < 0.01$). Addition of *meth* to the high-*chol* diet caused more drastic increment in ADMA concentrations (Fig. 2a), together with significant decrements in the arginine/ADMA ratio (Fig. 2b).

Correlation analyses

Significant correlations were obtained as follows: apelin positively with GLP-1 ($r = 0.44$), NO ($r = 0.41$, $p < 0.05$); and negatively with visfatin ($r = -0.44$, $p = 0.05$), ADMA ($r = -0.76$), nitrotyrosine ($r = -0.55$), *chol* ($r = -0.64$, $p < 0.001$); and homocysteine ($r = -0.50$, $p < 0.01$).

Visfatin levels were associated negatively with GLP-1 ($r = -0.44$) and NO ($r = -0.41$, $p < 0.05$); and positively with *chol* ($r = 0.65$), homocysteine ($r = 0.59$), and ADMA ($r = 0.52$, $p < 0.01$). GLP-1 levels were associated negatively with *chol* ($r = -0.55$) and ADMA ($r = -0.51$, $p < 0.01$).

ADMA and nitrotyrosine levels were correlated positively ($r = 0.73$, $p < 0.01$).

Histopathological findings

Examination of aorta revealed some pathological changes in the diet-fed groups (Fig. 3). *Meth* feeding caused slight increases in intima-media thickness and muscle cell proliferation. In the *chol* and *chol* + *meth* groups, increased intima-media thickness, smooth muscle cell proliferation, lipid vacuoles, and in some areas fatty streaks resembling *chol* crystals were seen.

Discussion

In the present study, we fed the guinea pigs with high *meth* and *chol* diet for a 10-week period in order to stimulate hyperhomocysteinemia and hypercholesterolemia. The microscopic examination of aortic sections indicated an early phase of vascular disturbance which was accompanied by significantly high *chol* and homocysteine levels in the plasma. These alterations were more prominent in the *chol* + *meth*-fed group. *Meth* load is known to cause hypercholesterolemia by stimulating several mechanisms. Firstly, increased *meth* concentration in liver enhances the bioavailability of methyl groups for the methylation of phosphatidylethanolamine, thereby leading to increases in phosphatidylcholine:phosphatidylethanolamine ratio which has a regulatory function in *chol* metabolism [19]. More importantly, homocysteine induces 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in *chol* biosynthesis, by activating transcription factors [20, 21]. Therefore, *chol* + *meth* load would be expected to have a more profound effect on plasma *chol*. Our findings in the animals fed *chol* + *meth* diet is in good agreement with the previous reports.

The decreased activity of NOS and impaired NO bioavailability are prominent events leading to vascular dysfunction [22, 23]. ADMA, an endogenous inhibitor of NOS, is a major determinant of NO production [24]. It has been reported that plasma ADMA levels are increased in the presence of hypercholesterolemia [25, 26]. In our study, markedly elevated ADMA levels in hypercholesterolemic and hyperhomocysteinemic animals confirm the relation of ADMA to the development of endothelial dysfunction. Additionally, plasma arginine/ADMA ratios were found significantly decreased in all diet-fed groups, the degree of decrease being more prominent in the *chol* + *meth* group.

A positive correlation between plasma ADMA and homocysteine levels has been well-documented [27]. Reduced dimethylarginine dimethylaminohydrolase (DDAH) activity is considered as the major factor for the elevation of ADMA [28, 29]. In patients with peripheral arterial disease, *meth* load caused elevations in plasma homocysteine and ADMA levels [30]. Homocysteinylation of lysine residues in DDAH protein due to hyperhomocysteinemia may result in inactivation of the enzyme, thereby leading to increases in circulating ADMA [31, 32]. As summarized in Fig. 4, homocysteine itself not only induces *chol* synthesis, but also alters ADMA metabolism in the liver. A profound increment in ADMA levels in the *chol* + *meth* group is likely to be resulted from dual effect of hyperhomocysteinemia.

Elevated ADMA levels are indicative of decreased NO formation. We measured both NO and nitrotyrosine levels in order to detect the bioavailability of NO. Decreased NO levels accompanying to markedly elevated nitrotyrosine

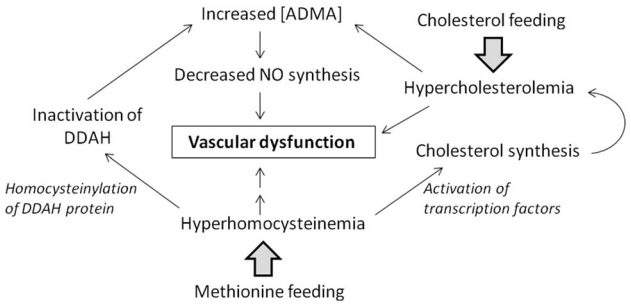

Fig. 4 The relationship between homocysteine, cholesterol, NO, and ADMA with vascular dysfunction

seemed to be due to superoxide radical generation in *chol* and *chol* + *meth* groups. It is known that conditions of oxidative stress promote S-glutathionylation of cysteine residues in endothelial NOS, which causes decreased NO synthesis and increased superoxide generation from the reductase domain of the enzyme [33]. An excess generation of superoxide radical can scavenge NO, thus decreasing its bioavailability and increasing nitrotyrosine formation [34, 35].

One of the main purposes in our study was to see the possible relation of apelin with early vascular lesions. In a previous study, exogenous apelin administration to rats caused elevations in plasma NO concentrations. Also, apelin exerted a hypotensive effect which was abolished by the presence of NOS inhibitor [7]. In our study, plasma apelin levels were significantly decreased in *chol*- and *chol* + *meth*-fed animals. Apelin levels were negatively correlated with those of ADMA, suggesting a possible involvement of this peptide in vascular changes. Several clinical studies have focused on the relation of apelin with hypercholesterolemia and cardiovascular disease [6, 36, 37]. The decrease in apelin levels was thought to be associated with insulin resistance in these patients. Therefore, in our study, we evaluated the HOMA-IR to see whether any changes occurred in glucose homeostasis. Neither glucose nor insulin levels seemed to be affected during atherogenic regiments. Decreased apelin levels were negatively correlated with both *chol* and homocysteine. Furthermore, serum apelin levels were decreased more drastically when *meth* was added to the atherogenic diet. To our knowledge, there is no study with regard to the effect of hyperhomocysteinemia on apelin synthesis or secretion. The decrement in apelin levels seems to be related to the ongoing atherogenic process with an additive impact of hyperhomocysteinemia.

Many experimental studies revealed that GLP-1 and related drugs exert protective effects on atherosclerosis, hypertension and cardiac dysfunction [38, 39]. In a mouse model of obesity, GLP-1-based therapy activated several cardioprotective pathways, as well as it prevented obesity-

induced insulin resistance and inflammation [40]. In clinical trials, treatment with GLP-1 analogs not only had the ability to reduce blood glucose, but also exerted several cardioprotective effects, by influencing positively some risk factors, and improving endothelial function. GLP-1 analogs increased the eNOS expression [41] and decreased the number of inflammatory cells and ROS production [42]. In our study, GLP-1 levels were decreased in *chol*- and *chol* + *meth*-fed animals. A negative association between GLP-1 and ADMA levels was observed, suggesting a possible involvement of ADMA on GLP-1 secretion.

As a potential inflammatory mediator, visfatin plays a role in chronic inflammation, thus contributes to the pathogenesis of atherosclerosis and cardiovascular disease. A positive association between visfatin levels and coronary atherosclerosis has been observed [43]. Moreover, visfatin impairs microvascular endothelium-dependent relaxation through a mechanism involving NADPH oxidase stimulation [44]. Serum visfatin levels were found markedly elevated in both in hypertensive and prehypertensive patients [45]. Uslu et al. have observed high visfatin levels in type 2 diabetic patients which was associated with hyperhomocysteinemia, suggesting a role of visfatin in endothelial dysfunction [46]. In our study, visfatin levels were significantly elevated both in *chol* and *chol* + *meth* groups, and correlated negatively with GLP-1 levels. Moreover, significant correlations between visfatin levels and the markers of endothelial dysfunction were observed.

The roles of apelin, visfatin, and GLP-1 in cardiovascular dysfunction have been investigated previously in clinical studies and their physiological effects have been noted. In this study, their relation with ADMA metabolism was searched in an experimental model of atherogenesis. Our results indicated that levels of apelin, GLP-1, and visfatin are markedly altered during the development of atherosclerotic changes in close association with *chol*, homocysteine, NO, and ADMA levels. According to the results of the present study, measurement of these peptides in circulation may help to assess the development of vascular dysfunction in patients with metabolic abnormalities.

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