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Common bean protein hydrolysate modulates lipid metabolism and prevents endothelial dysfunction in BALB/c mice fed an atherogenic diet

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e-NOS

Abstract *Background and aims:* Common beans (*Phaseolus vulgaris* L.) protein hydrolysate is a source of bioactive peptides with known health benefits. The aim of this study was to evaluate the effect of common bean protein hydrolysate on lipid metabolism and endothelial function in male adult BALB/c mice fed an atherogenic diet for nine weeks.
Methods and results: Male adult mice were divided into three experimental groups (n = 12) and fed with normal control diet; atherogenic diet and atherogenic diet added with bean protein hydrolysate (700 mg/kg/day) for nine weeks. Food intake, weight gain, lipid profile, Atherogenic Index of Plasma, inflammation biomarkers and endothelial function were evaluated. APH group presented reduced feed intake, weight gain, lipid profile, tumor necrosis factor-α, angiotensin II (94% and 79%, respectively) and increased endothelial nitric oxide synthase (62%).
Conclusions: Protein hydrolysate showed hypocholesterolemic activity preventing inflammation and dysfunction of vascular endothelium, in addition to decreasing oxidative stress, indicating an adjuvant effect on reducing atherogenic risk.

Introduction

The excess of saturated fat, cholesterol and sugars in the diet affects lipid metabolism, stimulates oxidation of low-density lipoproteins (LDL) and leads to the development of cardiometabolic diseases [1], being responsible for 17.9 million deaths worldwide in 2016 [2].

Atherogenic diets promote a pro-inflammatory environment, increasing the concentration of oxidized LDL in the inner layer of blood vessels and contributing to the development of lesions in vascular endothelium, characterizing the first physiological manifestation of atherosclerosis. Increased endothelial permeability allows the migration of LDL molecules to the tunica intima, where oxidation and phagocytosis of those molecules activate the immune system and induce the release of tumor necrosis factor α (TNFα), an early marker of endothelial activation [3].

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Common bean (*Phaseolus vulgaris* L.) is a widely consumed legume in Brazil and other countries [4]. Its protein hydrolysate has demonstrated *in vitro* anti-hyperlipidemic, anti-inflammatory and antihypertensive properties [5,6], being related to the sequences of antioxidant bioactive peptides and blood pressure regulators. Some of these peptides are able to block the action of angiotensin-converting enzyme (ACE), preventing the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor. Other studies suggest that ACE inhibition may stimulate the expression of endothelial nitric oxide synthase (e-NOS) enzyme, increasing synthesis and release of nitric oxide in vascular endothelium and promoting vasodilation and reduction of blood pressure [7,8]. In addition, nitric oxide can regulate coronary blood flow and protect endothelial layer from cell adhesion and platelet aggregation.

In this regard, some studies evaluated the effects of several protein hydrolysates *in vivo* in spontaneously hypertensive rats (SHR). Evaluation of chicken skin protein hydrolysate (100 mg/kg), mung bean protein hydrolysate (600 mg/kg) and rice protein hydrolysate (600 mg/kg) in SHR rats showed a reduction in blood pressure [9–11]. This effect has been attributed to the ability of peptides to inhibit ACE. In addition, administration of a black bean protein hydrolysate to hyperglycemic rats (hyperglycemic rat model) at a concentration of 200 mg/kg showed a hypoglycemic effect [12].

Despite the available knowledge about the effect of bean protein hydrolysate on ACE inhibition and blood pressure reduction, the mechanism regulating the pathway of atherosclerosis is not well understood. Thus, the role of bioactive peptides on protecting the endothelial barrier and preventing the deleterious effects of an atherogenic diet needs to be investigated.

Therefore, the aim of this study was to evaluate the role of bean protein hydrolysate on endothelial dysfunction and its impact on prevention of atherosclerosis in BALB/c mice fed an atherogenic diet.

Methods

Sample material

Common bean (*P. vulgaris* L.), cultivar BRSMG Madreperola, was cultivated and harvested by EMBRAPA Rice and Bean (Santo Antônio de Goiás, GO, Brazil). The fresh beans were cooked under pressure (1:2 beans/water) for 50 min at 120 °C. After soaking, boiled beans were oven-dried for 8 h at 60 °C and then crushed (sieve of 600 μm aperture size, 30 mesh; Grinder Vertical Rotor MA 090 CFT, Marconi Equipment, Brazil). The protein hydrolysate (PH) was obtained by a simulated gastrointestinal digestion process according to Alves et al. [5] using pepsin and pancreatin [13]. Peptides were previously identified and characterized by Alves et al. [6] using size exclusion chromatography and high-performance liquid chromatography-electrospray-ionization-mass spectrometry (HPLC–ESI–MS). In the aforementioned study, the bioactive peptide sequences were confirmed using UniProt database from BLAST® tool (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi), and potential biological activity of peptides was predicted by using BIOPEP database (http://www.uwm.edu.pl/biochemia). The protein hydrolysate were packed under vacuum and kept at −20 °C until analysis.

Phenolic compounds

Extract from bean protein hydrolysate was obtained according to Bloor [14]. Total phenolic compounds were determined using Folin-Ciocalteau reagent [15]. Results were quantified using a standard curve ranging from 0 to 250 ppm of gallic acid and expressed in milligrams of gallic acid equivalents per gram of dry sample (mg GAE g^{-1}) (standard curve: $y = 0.0009x + 0.0046$; $R^2 = 0.9975$).

Animals and diets

Male adult BALB/c mice (*Mus musculus*, class Rodentia) were obtained from the Central Animal Facility of the Center for Life Sciences and Health at Federal University of Viçosa (Viçosa, MG, Brazil). This model can be used to evaluate the earliest stages of atherogenesis [16,17]. At 60 days of age, 36 male mice were randomly allocated into three groups (n = 12 each). The animals were allocated in individual stainless-steel cages under controlled temperature environment (22 ± 2 °C) and a 12 h photoperiod. Experimental diets were based on AIN-93M standard diet for rodents (Table 1) [18]. The groups received deionized

Table 1 Composition of experimental diets (g/kg of diet).

Ingredients (g/Kg)	NC	AD	APH
Casein[a]	170.73	218.19	218.19
Dextrinized starch	155.00	105.50	105.50
Sucrose	100.00	300.00	300.00
Lard	0.00	200.00	200.00
Celulose	62.01	62.01	62.01
Soy oil	40.00	40.00	40.00
Mineral mix	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00
Cholesterol	0.00	20.00	20.00
Choline bitartrate	2.50	2.50	2.50
L-cystine	1.80	1.80	1.80
Colic Acid	0.00	5.00	5.00
Corn starch	422.96	0.00	0.00
Carbohydrate (%)	76.29	45.89	45.89
Protein (%)	19.21	24.69	24.69
Lipids (%)	4.50	29.42	29.42
Fiber (%)	6.2	6.2	6.2
Energy (kcal/kg)	3754.76	4834.76	4834.76
CD (kcal/g^{-1})	3.75	4.83	4.83
Bean protein hydrolysate (mg/Kg body weight)	–	–	700.00

[a] Purity of 82%. NC: normal control diet; AD: atherogenic diet; APH: atherogenic diet added with bean protein hydrolysate; CD: caloric density.

water and the respective experimental diets weekly and *ad libitum* for nine weeks.

Experimental groups received the following diets: normal control diet (NC); atherogenic diet (AD) and atherogenic diet added with bean protein hydrolysate (APH). The atherogenic diet were based on AIN-93M [18] and high fat high cholesterol diet [19,20]. The bean protein hydrolysate (700 mg/kg/day) was formulated according to Mojica et al. [12] We know that the bean protein hydrolysate has a yield of 51.2% from the whole bean flour [5], thus, considering the dose of 700 mg per kg of body weight, we can assuming that a 70 kg individual would have to consume about 95.7 g of beans. The 6-propyl-2-thiouracil (PTU) (10 mg/kg/day) was used according to Panda and Kar [21] and Panda et al. [22] PTU is a thyreostatic agent that inhibits thyroperoxidase enzyme, acts in thyroid hormone synthesis, and increases weight gain, total cholesterol, LDL-c and triglycerides [23]. The diet and PTU were intragastrically administered by oral gavage. All experimental procedures using animals were performed in accordance with the ethical principles for animal experimentation and the study protocol was approved by the Ethics Committee of the Federal University of Viçosa (Protocol No. 97/2015).

Body weight and feed intake were monitored weekly. Adiposity was measured by Lee index, calculated by the ratio between the cube root of body weight (g) and naso-anal length (cm) x 1000 [24]. The food efficiency ratio (FER) was calculated by the ratio between weight gain (g) and food intake (g). On the 63rd day, after 12 h fasting, animals were anesthetized with isoflurane (Isoforine®, Cristália, Brazil) and euthanized by cardiac puncture. Blood was collected in BD Vacutainer® tubes, centrifuged at 1006 *g* for 10 min for serum separation and then stored in microtubes at −80 °C. Cardiac tissue was collected, immediately frozen in liquid nitrogen and stored at −80 °C for analysis.

Biochemical analysis

An aliquot of 0.5 mL of serum from each animal was used for biochemical analysis. Total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and triacylglycerides (TGL) levels were measured by colorimetric method, using commercially available kits according to the manufacturer's instructions (Bioclin, Brazil). Analyses were performed on a BS-200 Chemistry Analyzer (Bioclin, Brazil). The Atherogenic Index of Plasma (AIP) was determined by the following equation: log (triacylglyceride/HDL cholesterol) [25].

Lipid peroxidation and oxidative stress levels

Antioxidant capacity

Aliquots (10 μL) of serum were added to plate wells with 20 μL of metmyoglobin reagent and 150 μL of 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) solution. Then, 10 μL of increasing concentrations of trolox standard (1.5 mM) were pipetted into the wells, in triplicate, to obtain a standard curve. The plate was incubated at room temperature for 5 min, and then absorbance (405 nm) was read with a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, USA). Results are expressed as mmol of trolox equivalents per liter of serum.

Malondialdehyde

Malondialdehyde (MDA) in serum was determined by thiobarbituric acid reactive substances (TBARS) method [26,27]. MDA was calculated using molar absorptivity coefficient ($E0 = 1.56 \times 10^5\ M^{-1}cm^{-1}$) [28]. Results are expressed as μmol/L of serum.

Nitric oxide

Nitric oxide analysis was performed by mixing 30 μL of serum with solution A (1% sulfanilamide in 2.5% H_3PO_4) and B (0.1% naphthyl l ethylene diamide dihydrochloride in 2.5% H_3PO_4) in a 1:1 ratio in a microtiter plate; then incubated under dark condition for 10 min. Absorbance was read using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, USA) at 570 nm. Results are expressed as μmol/L of serum [29].

Angiotensin II quantification

Serum angiotensin II was quantified by *Angiotensin II EIA Kit* (Sigma–Aldrich, USA). Absorbance was read using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, USA) at 450 nm and quantification carried out using a standard curve with SigmaPlot®, a Systat Software. Results are expressed as pg/mL of serum.

Extraction of mRNA from cardiac tissue and cDNA synthesis

One hundred mg of cardiac tissue were ground under low temperature condition and homogenized under RNAse-free conditions. Total RNA was extracted with a TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. A 2 μg portion of mRNA extracted was used to synthesize cDNA using a M-MLV reverse transcription kit (Invitrogen, USA) according to the manufacturer's protocol [30].

Determination of gene expression of proteins involved with endothelial function by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

The mRNA expression levels from cardiac tissue proteins involved in endothelial function were analyzed by using RT-qPCR. The SYBR Green PCR Master Mix (Applied Biosystems, USA) was used and analyses were performed on StepOne™ Real-Time PCR System (Thermo Fisher Scientific, USA) using the measurement system by SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, USA). The RT-qPCR involved a single initial denaturation cycle at 95 °C (20 s), 40 denaturation cycles at 95 °C (3 s each), then an annealing cycle at 60 °C (30 s),

followed by a standard dissociation curve. Sense and antisense primer sequences (Integrated DNA Technologies, USA) were used to amplify tumor necrosis factor α (TNFα); angiotensin II; endothelial nitric oxide synthase (e-NOS); vascular cell adhesion molecule 1 (VCAM-1) and matrix metalloproteinase 9 (MMP-9). The relative expression levels of mRNA were normalized by endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Supplementary Table 1). All steps were performed under open conditions with RNase.

Statistical analysis

Data were initially submitted to a Kolmogorov–Smirnov normality test and then ANOVA test was applied, followed by the Newman–Keuls test for parametric variables. Experimental treatments were arranged in a completely randomized design with twelve repetitions. The significance level was established for all tests at 5%. All statistical analyzes of biological data were performed using GraphPad Prism® (GraphPad Software, USA), version 5.0.

Results

Identification of bioactive peptides and determination of phenolic compounds content in bean protein hydrolysate

Bioactive sequences from bean protein hydrolysate were mainly related to the inhibition of angiotensin converting enzyme (ACE), dipeptidyl peptidase IV (DPP-IV), stimulating glucose uptake (GUSP) and antioxidative activity (Table 2). The concentration of phenolic compounds found in bean protein hydrolysate was 1.06 ± 0.17 mg GAE per gram of sample.

Table 2 Bioactive peptides identified by HPLC–ESI–MS/MS in BRSMG Madreperola hydrolysate fractions.

Peptide sequence[a]	Biological activity	Parental protein
LVTTTVDL	GUSP, DPP-IV inhibitor	Phytohemagglutinin
QTSTPLFS	ACE inhibitor, DPP-IV inhibitor	Alpha-amylase inhibitor 1
VELVGPK	ACE inhibitor, antioxidative, DPP-IV, GUSP, PRSM, PEI	Alpha and beta phaseolin
TRGVLV	ACE inhibitor, DPP-IV Inhibitor, GUSP	Alpha-amylase inhibitor 2

GUSP: glucose uptake stimulating peptide; DPP-IV inhibitor: dipeptidyl peptidase IV inhibitor; ACE inhibitor: angiotensin-converting-enzyme inhibitor; PRSM: peptide regulating the stomach mucosal membrane activity; PEI: prolyl endopeptidase inhibitor.

[a] Peptides sequenced by HPLC–ESI–MS/MS with intensity at least 50% and 70% of probability. Biological activities were obtained from the BIOPEP database; Highlighted and underlined portion of the sequence refer to part of the peptide with reported antioxidant and anti-inflammatory activity, respectively (BIOPEP database). Only sequences of main proteins of *Phaseolus vulgaris* L. are presented in the table and were confirmed with BLAST® tool (QC > 60%). The amino acids are presented in one letter nomenclature.

Indicators of food consumption, body weight and adiposity in adult BALB/c mice

The consumption of atherogenic diet added with 700 mg/kg of body weight per day of bean protein hydrolysate (APH group) presented some variation during the nine weeks, with a decrease in the week one, two, three and six ($p < 0.05$) and no change in the week four, five, seven, eight and nine ($p > 0.05$) as compared to the atherogenic diet group (AD) (Fig. 1A). The daily consumption and the total consumption in APH group was lower ($p < 0.05$) than in control groups (Table 3). The average consumption of total phenolic compounds was 0.028 ± 0.002 mg GAE per day and bean protein hydrolysate was 26.69 ± 1.73 mg per day (700 mg/kg body weight). Then, the APH group reduced the weight gain ($p < 0.05$), becoming similar to the normal control group (NC) (Fig. 1B). In addition, the Lee index was lower in APH group compared to AD group ($p < 0.05$) (Table 3). The ratio between weight gain and food intake (food efficiency ratio) did not differ ($p > 0.05$) among groups.

Lipid profile and Atherogenic Index of Plasma in adult BALB/c mice

The APH group showed a reduction in total cholesterol levels, triglycerides and HDL-c levels ($p < 0.05$) and no changes in LDL-c levels and Atherogenic Index of Plasma (AIP) ($p > 0.05$) when compared to AD group (Table 3). The consumption of bean protein hydrolysate associated with atherogenic diet did not prevent the decrease of HDL cholesterol compared to control groups.

Lipid peroxidation and oxidative stress levels in adult BALB/c mice

The total antioxidant capacity (TAC) did not differ ($p > 0.05$) between APH group and control groups (NC and AD) (Fig. 1C). In comparison to lipid peroxidation, it was observed that APH group showed reduced MDA levels ($p < 0.05$) than control groups (Fig. 1D).

Effects of bean protein hydrolysate consumption on inflammation and endothelial dysfunction in adult BALB/c mice

The APH group showed reduced expression of TNFα gene (94%) compared to AD group ($p < 0.05$) (Fig. 2A). The angiotensin II (ang II) gene expression and protein concentration in APH group was lower (79% and 50%, respectively) than AD group ($p < 0.05$) (Fig. 2B and C). The endothelial nitric oxide synthase (e-NOS) expression and the nitric oxide serum concentration increased in APH group (62% and 57%, respectively) compared to AD group ($p < 0.05$) (Fig. 2D and E).

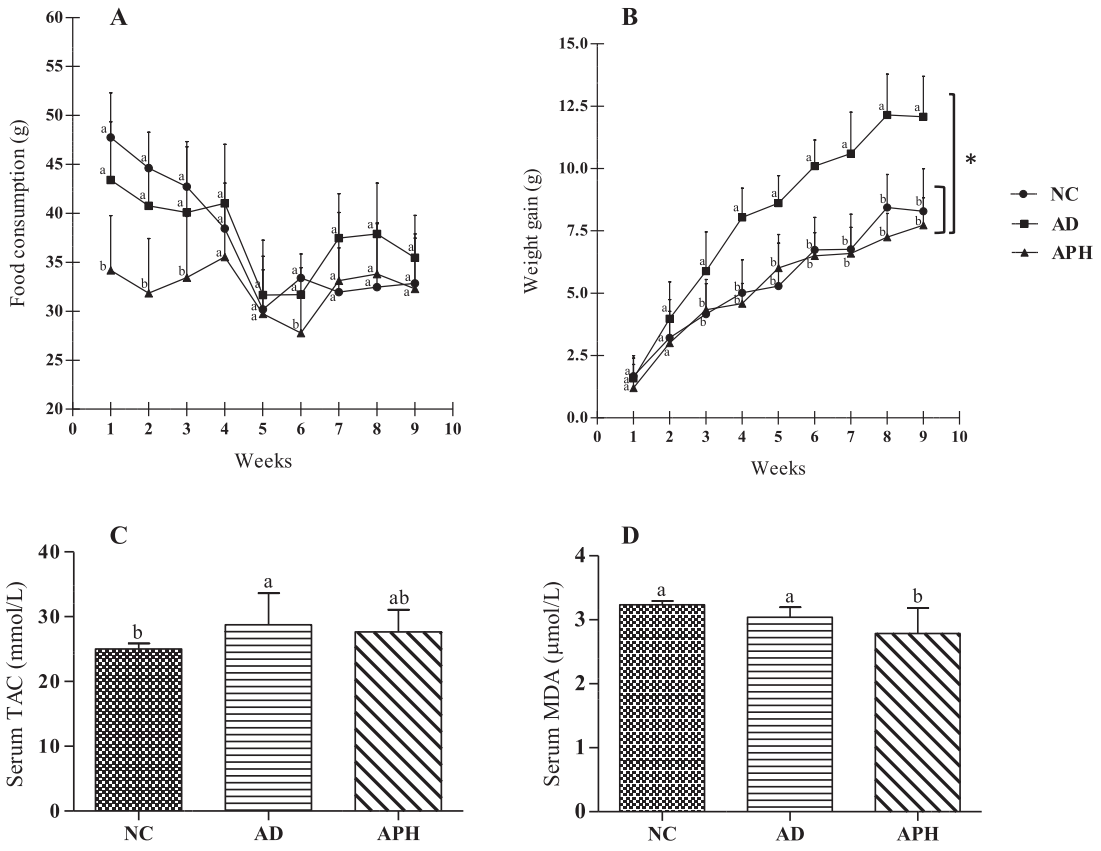

Figure 1 Effect of bean protein hydrolysate intake on food consumption, weight gain and oxidative stress in adult BALB/c mice (n = 12) for nine weeks. A: weekly food consumption; B: weekly weight gain. C: total antioxidant capacity (TAC); D: malondialdehyde (MDA). NC: normal control diet; AD: atherogenic diet; APH: atherogenic diet added with bean protein hydrolysate. Mean followed by different letters in column and the symbol [*] differed by Newman–Keuls test ($p < 0.05$).

Discussion

The present study evaluated the protective effect of common bean protein hydrolysate on the vascular endothelium of BALB/c mice fed an atherogenic diet. Common beans and their protein hydrolysate were regarded as source of phenolic compounds and bioactive peptides with antihyperlipidemic, anti-inflammatory and antihypertensive effects [5,6,31,32].

In this study, the APH group received 700 mg/kg of body weight per day of bean protein hydrolysate by intragastric gavage associated with an atherogenic diet,

Table 3 Effect of bean protein hydrolysate intake on biometric and biochemical variables in adult BALB/c mice (n = 12) for nine weeks.

Groups	NC	AD	APH
Total consumption (g)	334.39 ± 32.20^{a}	329.54 ± 40.48^{a}	291.77 ± 30.96^{b}
Food consumption (g/day)	5.31 ± 0.51^{a}	5.23 ± 0.64^{a}	4.63 ± 0.49^{b}
Body weight gain (g)	8.29 ± 1.7^{b}	12.08 ± 1.62^{a}	7.30 ± 1.6^{b}
Phenolic consumption (mg GAE/day)	–	–	0.028 ± 0.002
FER (g)	0.03 ± 0.00^{a}	0.03 ± 0.00^{a}	0.02 ± 0.00^{a}
Lee index	329.63 ± 8.45^{b}	345.00 ± 14.84^{a}	331.42 ± 9.35^{b}
TC (mg dL^{-1})	124.17 ± 11.61^{b}	140.71 ± 21.14^{a}	115.33 ± 17.15^{b}
HDL (mg dL^{-1})	68.00 ± 4.00^{a}	55.00 ± 7.00^{b}	47.00 ± 9.00^{c}
LDL (mg dL^{-1})	12.00 ± 1.00^{b}	32.00 ± 9.00^{a}	34.00 ± 4.00^{a}
TGL (mg dL^{-1})	45.89 ± 7.85^{a}	25.20 ± 4.82^{b}	18.66 ± 5.66^{c}
AIP	-0.18 ± 0.06^{a}	-0.32 ± 0.07^{b}	$-0,40 \pm 0.14^{b}$

NC: normal control diet; AD: atherogenic diet; APH: atherogenic diet added with bean protein hydrolysate; FER: food efficiency ratio (weight gain/food intake); GAE: gallic acid equivalent. TC: total cholesterol; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; TGL: triacylglyceride; AIP: Atherogenic Index of Plasma: log (TGL/HDL cholesterol). Mean followed by different letters in line differed by Newman–Keuls test ($p < 0.05$). * Total phenolic compounds concentration in protein hydrolysate: 1.06 ± 0.17 mg GAE/g of sample.

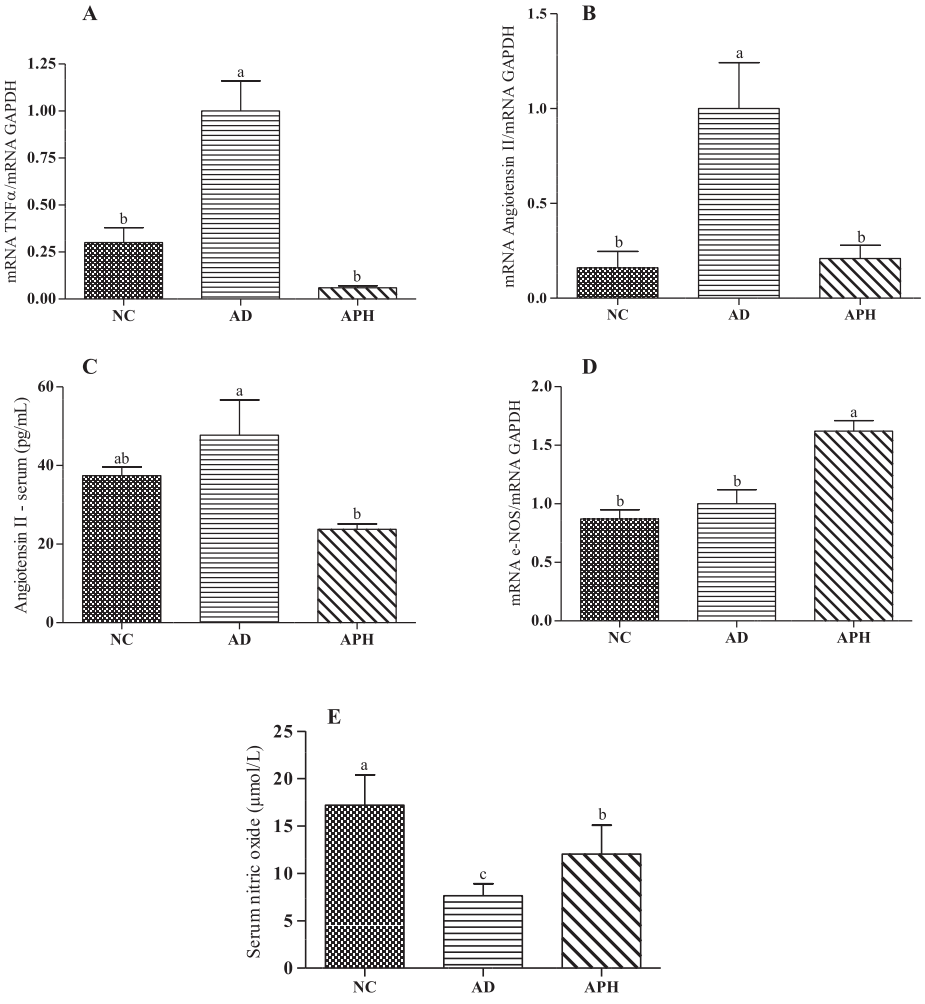

Figure 2 Effect of bean protein hydrolysate intake on endothelial dysfunction in BALB/c adult mice (n = 8) for nine weeks. A: tumor necrosis factor α (TNFα) in cardiac tissue; B: angiotensin II in cardiac tissue; C: angiotensin II quantification in serum; D: endothelial nitric oxide synthase (e-NOS) in cardiac tissue; E: nitric oxide in serum. NC: normal control diet; AD: atherogenic diet; APH: atherogenic diet added with bean protein hydrolysate. Mean followed by different letters differed by Newman–Keuls test ($p < 0.05$).

since proteins have good stability in diet. The bean protein hydrolysate used in this study was a source of phytochemicals and its characterization identified antioxidative peptides (VELVGPK), related to inhibition of dipeptidyl peptidase IV (DPP-IV) (LVTTTVDL; QTSTPLFS; TRGVLV), and inhibition of angiotensin converting enzyme (ACE) (QTSTPLFS; VELVGPK; TRGVLV).

The biological activity of bean protein hydrolysate from parental proteins (phytohemagglutinin, alpha and beta phaseolin, alpha-amylase inhibitor 1 and alpha-amylase inhibitor 2), associated with the presence of phenolic compounds possibly stimulated the release of cholecystokinin, a gastrointestinal hormone that regulates serotonin secretion and increases satiety. In addition, the mechanism of food intake control may be related to inhibition of enzyme DPP-IV by peptides, which may maintain the physiological release of Glucagon-Like Peptide 1 (GLP-1), the gastrointestinal hormone responsible for slowing gastric emptying and increasing insulin secretion [12,31,33]. Thus, the mechanism of action of protein hydrolysate possibly consists in modulation of hunger and the satiety center in hypothalamus.

The APH group did not prevent the decrease of HDL cholesterol compared to control groups and it is commonly observed in animals with this dietary pattern [25,34,35]. However the APH group presented lower HDL cholesterol than AD and NC groups. Although it was not expected, some studies have been observed similar results [36,37].

The improvement in total cholesterol and triacylglyceride (TGL) profile may be linked to the hypolipidemic and antioxidant properties of phytochemicals and peptides present in bean protein hydrolysate, which reduce the micellar solubilization of cholesterol probably by hydrophobic interaction [38–41]. The Atherogenic Index of Plasma (AIP) was higher in the NC group compared to the other groups, since the TGL concentration was also higher in this group. High TGL has been related

with a increased LDL cholesterol and cardiovascular risk [42]. NC group received the standard diet for adult rodents (AIN-93M) [18] containing 76% of carbohydrates and the groups that received atherogenic diet consumed about 45% of carbohydrates and had a lower TGL and AIP concentration.

MDA concentration in APH group was lower than in AD group, indicating that treatment could attenuate oxidative stress. It occurs possibly due to the action of bean protein hydrolysate, phytochemicals and bioactive peptides with antioxidant and anti-inflammatory action, which may neutralize free radicals and prevent lipid peroxidation caused by the atherogenic diet [5,6,43,44]. The total antioxidant capacity (TAC) in APH group serum was similar to the AD group. This may be attributed to the short experimental time, as the TAC is dependent on the type of treatment provided, intervention time and concentration of antioxidant compounds present in the diet [45]. We evaluated the effects of the bean protein hydrolysate on prevention of endothelial dysfunction and the lowest MDA levels can indicate a reduction in oxidative stress.

Bean protein hydrolysate in APH group prevented the increase of TNF-α expression and possibly modulated vascular permeability and migration of LDL to the subendothelial space. This mechanism of endothelial protection may be related to the action of phytochemicals and antioxidant peptides, which possibly minimized the exposure of LDL to transition metal ions, enzymes and other catalysts, preventing their oxidation and the activation of inflammatory cascade [46]. In addition, the APH group reduced angiotensin II (ang II) expression and serum concentration and increased e-NOS expression in comparison to AD group. This mechanism of endothelial control observed even with the administration of atherogenic diet was possibly triggered by the anti-inflammatory and antioxidant action of bean protein hydrolysate, attributed to the presence of VELVGPK bioactive sequence, and to other bioactive sequences with a high potential of ACE inhibition (LVTTTVDL; QTSTPLFS; VELVGPK; TRGVLV).

These sequences were identified and well characterized by Alves et al. [5] according to respective biochemical properties and biological potential. The biological potential of these sequences to inhibit ACE has been previously identified and can be found at BIOPEP®, a database that contains information about the bioactivity of peptides and supports analyses of proteins as potential precursors of bioactive peptides [47]. Most of the effects of ACE-inhibitor peptides from common bean proteins are demonstrated *in vitro*, mainly by enzymatic/biochemical assays, demonstrating that hydrolysis conditions, thermal treatments and hydrolysis time, can be useful to enhance the ACE-inhibition properties by allowing enzymes to perform the cleavage of denaturalized proteins efficiently [39]. In general, ACE inhibitors are able to reduce the activity of ACE, preventing the conversion of ang I into ang II (active form), thereby promoting a vasorelaxant effect on blood vessels [39,48]. The lower expression of ang II in APH group highlights the great potential on cardiovascular control and protection by bean protein hydrolysate, as ang II is involved in production of reactive oxygen species by

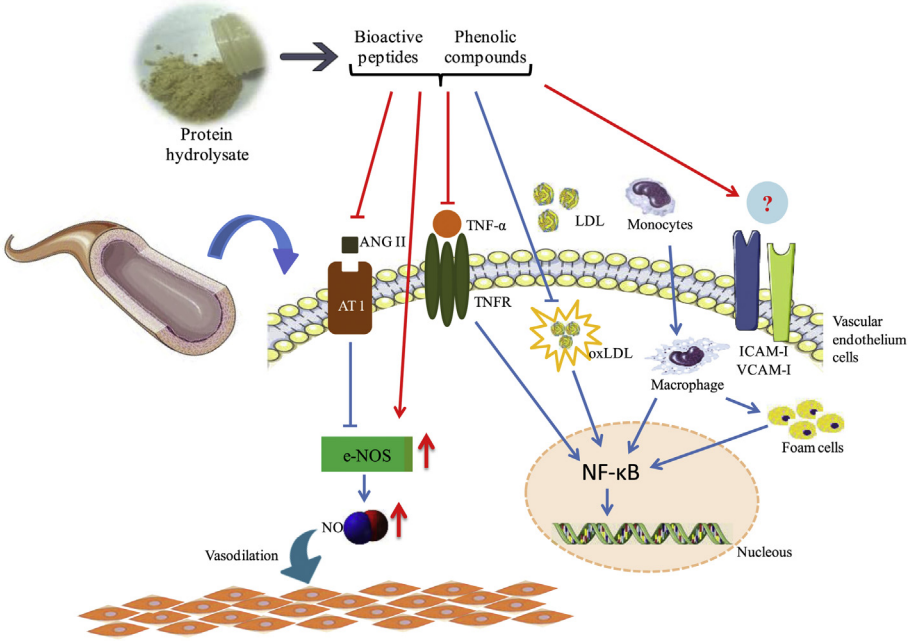

Figure 3 Potential mechanism of action of bioactive peptides and phenolic compounds from common bean protein hydrolysate in the dysfunction of vascular endothelium. Red arrows and lines indicate in which steps common bean protein hydrolysate modulated the pathway in this study. Ang II: angiotensin II; AT1: angiotensin II receptor type 1; TNF-α: tumor necrosis factor-α; TNFR: tumor necrosis factor receptor; LDL: low-density lipoprotein; oxLDL: oxidized LDL; NF-κB: factor nuclear kappa B; VCAM-1: vascular cell adhesion molecule-1, ICAM-1: intracellular adhesion molecule-1; e-NOS: endothelial nitric oxide synthase; NO: nitric oxide. The images were from smart.servier.com. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

activation of NADPH oxidase and in reduction of nitric oxide bioavailability. Nitric oxide has a role on development of chronic diseases such as hypertension and atherosclerosis [49,50].

The increase of e-NOS in APH group demonstrates the protective mechanism of bean protein hydrolysate on endothelium of animals fed with atherogenic diet. The proposed regulatory mechanism suggests that ACE inhibition may favor the expression of e-NOS, which in turn stimulates the synthesis and release of nitric oxide in vascular endothelium and contributes to vasodilatory action [7,8]. Other authors also found bioactive peptides inhibiting ACE in legumes and proposed their action in regulatory pathway of atherosclerosis [5,6,51,52], but without *in vivo* evidence.

Serum nitric oxide concentration was higher in APH group in comparison to AD group, but lower than in NC group. Despite being a free radical, nitric oxide acts as a key regulator in cardiovascular control at physiological concentration [53]. The antihypertensive and antioxidant bioactive peptides of bean protein hydrolysate possibly prevented damage to the vascular endothelium by stimulating the release of guanylate cyclase enzyme, which synthesizes cyclic guanosine monophosphate (cGMP), a calcium channel activator nucleotide present in vascular endothelium that controls the relaxation of smooth muscle and promotes vasodilation [53].

The vascular cell adhesion molecule-1 (VCAM-1) and the matrix metalloproteinase 9 (MMP-9) expression did not differ ($p > 0.05$) among the groups, possibly because they are atherogenesis late markers. Thus, our study confirmed that nine weeks is not sufficient to observe difference in these markers. We suggest that bean protein hydrolysate modified the endothelium permeability and prevented the development and progression of atherogenic lesions, so it is probable that there was no migration of cell adhesion molecules, smooth muscle cells, nor atherosclerotic plaque formation [46].

Considering the experimental model used in this study, we proposed a mechanism of action of common bean protein hydrolysate in the dysfunction of vascular endothelium (Fig. 3). The bioactive peptides and phenolics compounds may act on decreasing markers of inflammation, endothelial dysfunction and may alter the lipid metabolism that appear in the early stages of atherogenic process. The final markers of atherogenic pathway showed no change. Therefore, we suggest evaluating other types of atherogenic diet, different doses of bean protein hydrolysate and a longer experimental period.

Conclusion

This work opens a new perspective of *in vivo* research on the effects of bean protein hydrolysate on endothelial dysfunction and associates the specificity of bean peptides with weight control, lipid metabolism and vascular homeostasis. The results found in this investigation suggests that nutritional supplementation with bean protein hydrolysate, as source of bioactive peptides, prevented inflammation and dysfunction of vascular endothelium, reducing the risk of developing cardiovascular diseases.

Author contributions

M.J.C.G and H.S.D.M conceived of the presented idea. M.J.C.G, S.L.S.L and N.E.G.A wrote the manuscript with support from H.S.D.M, E.G.M, P.Z.B and C.O.B.R.; S.L.S.L, A.A., M.E.C.M., R.C.L.T, O.R.T. processed the experimental data and performed the analysis, H.S.D.M. and S.L.P.M supervised the experiment. All authors discussed the results and commented on the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2019.07.020.

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