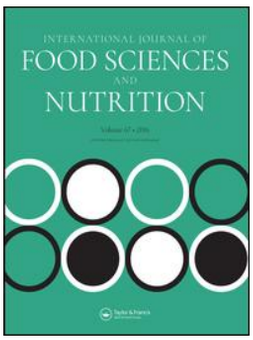

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RESEARCH ARTICLE

Effects of fish protein hydrolysate ingestion on endothelial function compared to whey protein hydrolysate in humans

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ABSTRACT

Fish protein-derived bioactive peptides may improve endothelial dysfunction through an antihypertensive and antioxidant effect. However, few studies have evaluated the bioactive peptides effect on vascular function. Therefore, this study investigates the effect of a single dose of fish protein hydrolysate (FPH) or whey protein hydrolysates (WPH) on endothelium-dependent dilation in nine healthy adults. The subjects ingested a single dose (20 g) of FPH, WHP or placebo (PLA). The endothelium-dependent dilation was evaluated by flow-mediated dilatation before and at 30, 60 and 120 min after supplementation. Total antioxidant capacity (TAC) of the FPH and WPH supplements was evaluated by using the Trolox equivalent antioxidant capacity assay. There was a significant increase of endothelium-dependent dilation at 30 min after WPH but not after FPH as compared to PLA. There was a significant great TAC in FPH than WPH supplement. A single dose of FPH was not able to improve endothelium-dependent dilation compared to WPH.

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Endothelial function; fish protein; nitric oxide; flow-mediated dilatation

Introduction

Oxidative stress (i.e. the excessive and/or dysregulated generation of reactive oxygen species – ROS) is a major contributor to disease pathologies (Taverne et al. 2014). In the vascular endothelium, an excess of superoxide reacts with NO to generate peroxynitrite, decreasing NO bioavailability (which may contribute to endothelial dysfunction). Given the critical roles played by NO and its alterations observed under oxidative stress, regulation of ROS levels by dietary compounds is a key nutritional strategy for enhancing or maintaining normal vascular function.

Recent studies have shown that marine fish are a rich source of bioactive peptides (Li et al. 2017), which may have specific biological activities beyond nutrient supply, including antioxidant activity. García-Moreno et al. (2013) investigated the antioxidant activity of fish protein hydrolysates (FPH) from five discarded species in the Alboran Sea (sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark). The highest DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging activity was found in the hydrolysate of sardines. Bernardi et al. (2016) demonstrated that the hydrolysates of processed tilapia by-products and produced by the enzymatic treatment with alcalase showed high FRAP (ferric reducing antioxidant power) activity and ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) radical cation. Other authors have also shown elevated antioxidant activity in hydrolysates produced with tilapia (Choonpicharn et al. 2015; Yarnpakdee et al. 2015). In addition, whey protein hydrolysate (WPH) has also been shown to have *in vitro* antioxidant activity as well as regulate antioxidant genes and increase glutathione and catalase activity in human umbilical vein endothelial cells (O'Keeffe and FitzGerald 2018).

Although many *in vitro* studies have demonstrated a potential antihypertensive (Kim et al. 2012) and antioxidant properties (García-Moreno et al. 2014; O'Keeffe and FitzGerald 2018) for FPH and WPH, little attention has focussed on the potential impact of these proteins on vascular function. Previous studies have demonstrated that a single oral dose of whey-derived peptide improves vascular endothelial responses in individuals with normal and impaired endothelial function as evaluated by brachial artery flow-mediated dilatation (FMD) (Ballard et al. 2009; Ballard et al.

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2013). Furthermore, Petyaev et al. (2012) reported improvements in FMD after supplementing with whey protein in individuals with normal endothelial function. The results of these studies may suggest that certain peptides can directly induce endothelial NO formation in healthy individuals instead of improving NO bioavailability through antioxidant effects.

In our previous study, we have shown that a single low oral dose (5 g) of FPH was not able to improve the macro- and microvascular function in healthy subjects (Alvares et al. 2018), raising the question of whether a high dose of FPH would be necessary to promote the vascular benefits. Therefore, the present study was developed to investigate the effect of a single oral dose (20 g) of FPH and WPH on endothelium-dependent dilation in healthy adults. The hypothesis of this study was that FPH and WPH ingestion could improve endothelium-dependent dilatation.

Materials and methods

Participants

Nine healthy, young, physically active adults were recruited to participate in this study. An a priori power analysis was conducted for an F test (repeated measures, within–between interaction for four time points) by using G*Power software (version 3.1.9.2). On the basis of a statistical power $(1-\beta)$ of 0.80, a medium effect size ($f = 0.3$), and an overall level of significance of 0.05, at least eight participants were needed to detect a statistical difference in FMD assessment. Exclusion criteria for participants included the presence of chronic diseases as determined by medical history questionnaire, hypertension, smoking, diabetes mellitus, HIV, inflammatory and cardiovascular disease, and use of vasoactive, antioxidant and/or caffeine supplements. Of the nine participants who completed all experimental procedures, three were physically active (involved in resistance training for 3–4 times a week, spending ~50 min per day). Only participants who have ingested animal-derived protein (eggs, milk and/or meats) daily were included in the study. Premenopausal women were studied within the first 5 days of their menstrual cycle, as macrovascular measurements of the brachial artery have been documented to fluctuate with the menstrual phase (Corretti et al. 2002; Thijssen et al. 2011). All participants were fully informed of the nature and purpose of the investigation and provided written consent to participate. All experimental procedures were performed in accordance with the ethical standards of the Declaration of Helsinki and approved by the institutional ethics committee of the Federal University of Rio de Janeiro, Brazil (protocol number: 53392216.0.0000.5699).

Experimental procedures

The study was conducted in a randomised, double-blind, crossover and placebo-controlled design. All subjects reported to the laboratory on four occasions, with at least 1-week interval between visits. The first visit was used to explain the experimental procedures and to collect baseline FMD (Pre), blood sample and anthropometrics measurements. The blood samples were collected (only first visit) for determination of biochemistry characteristics of the subjects. The blood sample was collected in the red top serum tubes (no additive) and lavender-topped plasma tubes (EDTA as additive), followed centrifugation. The serum and plasma were used to determinate biochemistry parameters, which were performed by using a clinical chemistry analyser (Reflotron® Plus, Roche Diagnostic) by using specific test strips for each analysis performed. In the second, third and fourth visits, the participants were randomly divided into either a fish protein hydrolysate (FPH), whey protein hydrolysate (WPH) or placebo (PLA) supplementation and endothelium-dependent dilation was evaluated at different time points over 120 min after nutritional intervention (Figure 1). All visits were held between 07:00 and 11:00 a.m. The participants were instructed to fast for at least 8 h and restrict physical exercise, caffeine consumption, protein and vegetable ingestion for at last 12 h before each visit. One day before each visit, participants were instructed to avoid ingesting foods rich in protein and nitrate and nitrite (a list of foods to be avoided was provided for each participant).

Dietary intervention

In a double-blind and randomised manner, all participants received orally 20 g of FPH, 20 g of WPH or 5 g of sucralose (as placebo - PLA). FPH and WPH were dissolved in 100 mL of water and immediately administered. PLA was administered in six white plastic capsules, together with 100 mL of water. Subjects were informed that all the capsules (PLA, FPH and WHP) contained bioactive peptides, and that the purpose of the study was to compare the effects of bioactive peptides on arterial dilation. The FPH was prepared in our laboratory as previously described (Alvares et al., 2018) and the WPH was purchased from a dietary supplements store. The essential amino acids content of FPH and WPH were

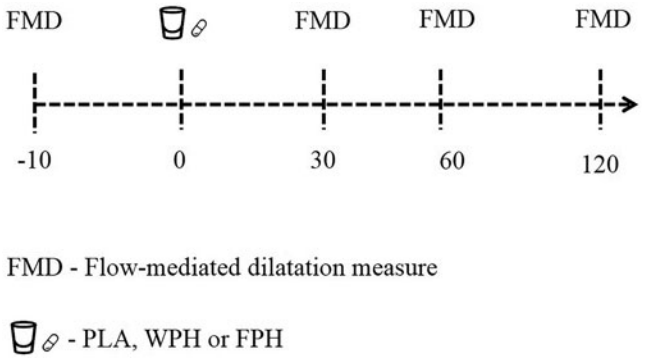

Figure 1. Experimental design. FPH = fish protein hydrolysates; WPH = whey protein hydrolysates; PLA = placebo (sucralose).

analysed according to Gatti et al. (2010) by using a high-performance liquid chromatography system.

Endothelium-dependent dilation measurement

The endothelium-dependent dilation was evaluated by measuring the FMD in the brachial artery as described previously (Thijssen et al. 2011; Alvares et al. 2018). The measurement was performed by using an ultrasound (Prosoundα6; Aloka Co., Tokyo, Japan) with a high-resolution linear array transducer (13 MHz) coupled with computer-assisted analysis software (e-TRACKING system, Aloka Co., Tokyo, Japan) that used an automated edge detection system for measurement of artery diameter and synchronised by 3-lead electrocardiography to the R-wave of the QRS complex. The measurements were performed at Pre (baseline measurement in the first visit to the laboratory), 30, 60 and 120 min after supplementation. FMD was determined by the percentage dilated at the maximum vessel diameter in peak vasodilation (peak brachial artery diameter – PBD) during reactive hyperaemia, relative to maximum vessel diameter in the baseline (baseline brachial artery diameter – BBD). All analyses were conducted by a single investigator who was blind to the participant and nutritional intervention order. In our laboratory the coefficient of variation ($n = 9$) of the FMD measurement was, respectively, 6.6% to intra-subject and 5.2% to inter-subject.

Total antioxidant capacity (TAC)

The TAC from PLA, WPH and FPH supplement were analysed using Trolox equivalent antioxidant capacity (TEAC) assay and expressed as a fresh weight (mM TE/20g fresh weight), as previously described (Arts et al. 2004; Deng et al. 2013; Alvares et al. 2018). The TAC was based on a fresh weight because we were interested to provide antioxidant information (TAC content) present in the supplementation form (FPH, WPH and PLA) that was offered to the participants.

Statistical analysis

The normality, sphericity and homogeneity of variances of the data were examined with the Shapiro-Wilk, Mauchly and Levene tests, respectively. To identify differences in the FMD, BBD and PBD between PLA, WPH and FPH, a two-way ANOVA was used. When significant F value was found, additional post hoc test with Bonferroni were performed. To identify differences in the TAC between PLA, WPH and FPH a one-way ANOVA was used. For all variables, when sphericity was violated, a Greenhouse-Geisser correction was used. In addition, effect size to observe the magnitude of the effect of interventions was calculated by Cohen's f which values 0.1 is considered small effect, 0.25 is a medium effect and $\geq$0.4 is considered large effect size. Statistical significance was set at the 0.05 level of confidence. All analyses were performed using a commercially available statistical package (IBM SPSS Statistics version 23 for Mac, Chicago, IL), and the results were expressed as mean ± standard deviation (SD).

Results

For all variables analysed in this study, the assumptions of normality, sphericity and homogeneity of variances were not violated. The baseline volunteers' characteristics are shown in Table 1 and the amino acids content of FPH and WPH are shown in the Table 2.

Total antioxidant capacity (TAC)

The TAC (mM TE/20 g fresh weight) in the FPH was greater than PLA supplement (FPH: 239.17 ± 3.91 vs. 25.56 ± 0.00, $p \leq 0.001$) and WPH supplement (WPH: 40.09 ± 13.32, $p \leq 0.001$). There was no difference between PLA and WPH supplement ($p = 0.205$).

Endothelium-dependent dilation

The endothelium-dependent dilation (FMD, %) values before and after PLA, WPH and FPH interventions are shown in and Table 3. There was no difference in FMD ($p = 1.000$) between PLA, WPH and FPH at PRE. There was no significant main effect regarding time for FMD ($p = 0.275$). There was a significant treatment × time interaction effect for FMD ($p = 0.044$). Post hoc test revealed that FMD increased at T30 in WPH compared to PLA ($p = 0.029$), but not to FPH compared to PLA ($p = 1.000$). In addition, there was no difference in BBD and PBD before and after PLA, WPH and FPH interventions.

Table 1. Baseline characteristics of the subjects completing the study.

Variables	
Demographics	
N (female)	9 (3)
Age (years)	22.5 ± 3.3
Body mass (kg)	74.3 ± 5.7
BMI (kg/m^2)	25.9 ± 3.0
Biochemistry	
Glucose (mg/dL)	86.6 ± 9.0
Total cholesterol (mg/dL)	164.2 ± 40.5
HDL cholesterol (mg/dL)	36.5 ± 10.9
LDL cholesterol (mg/dL)	97.2 ± 49.7
TG (mg/dL)	107.7 ± 42.6

BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: triglycerides.
The values are mean ± standard deviation.

Table 2. Amino acid profile of the fish protein hydrolysate (FPH) and whey protein hydrolysate (WPH).

Amino acids (mg/g)	FPH	WPH
Alanine	40.51 ± 7.14	31.65 ± 4.13
Arginine	74.08 ± 14.53	24.48 ± 1.89
Aspartic acid	301.00 ± 43.77	385.61 ± 31.53
Glutamic acid	424.97 ± 68.42	497.29 ± 51.61
Glycine	84.61 ± 14.87	39.20 ± 3.33
Histidine	178.81 ± 17.94	151.50 ± 3.93
Isoleucine	46.37 ± 10.17	101.67 ± 16.47
Leucine	75.07 ± 14.24	109.85 ± 12.76
Lysine	77.93 ± 13.68	89.96 ± 11.26
Methionine	66.15 ± 4.29	40.97 ± 4.36
Phenylalanine	91.72 ± 15.81	123.83 ± 19.83
Serine	122.85 ± 20.71	123.72 ± 13.80
Threonine	147.33 ± 27.21	257.39 ± 29.71
Tryptophan	5.72 ± 0.98	3.30 ± 0.85
Valine	82.90 ± 8.16	140.58 ± 5.16
∑EAA	772.01 ± 107.17	1019.05 ± 93.79
BCAA	204.35 ± 32.18	352.09 ± 32.37

The values are mean ± standard deviation.
EAA: essential amino acids; BCAA: branched-chain amino acids.
This table was reprinted with permission from Elsevier (Alvares et al. 2018).

Discussion

This study demonstrated that single dose of the WPH, but not FPH increased the endothelium-dependent dilation in healthy adults. Recently, Alvares et al. (2018) evaluated the endothelium-dependent dilation at 30, 60 and 120 min after single dose (5 g) of FPH or PLA ingestion in healthy adults. No significant difference was observed at any time evaluated. Corroborating Alvares et al. (2018) findings, this study did not observe significant increase in endothelium-dependent dilation after 20 g of FPH ingestion. Therefore, increasing FPH dose from 5 to 20 g does not seem to implicate in significant effect on endothelium-dependent dilation.

In contrast, a significant increase of the endothelium-dependent dilation at 30 min after WPH ingestion was observed. Previous studies have demonstrated the effects of the NOP-47, a supplementation containing whey-derived peptides, on endothelium-dependent dilatation in healthy (Ballard et al. 2009) and overweight, middle-age subjects Ballard et al. (2013). Ballard et al. (2009) demonstrated that unlike the two weeks of NOP-47 supplementation, single dose (5 g) of NOP-47 increased endothelium-dependent dilation at 30-, 60- and 90-min post-ingestion in healthy subjects. Ballard et al. (2009) demonstrated that single dose (5 g) of NOP-47 increased endothelium-dependent dilation at 120 min post-ingestion in overweight, middle-age subjects. Fekete et al. (2016) demonstrated that supplementation with whey protein concentrate (2 × 28 g/day) and calcium caseinate (2 × 27 g/day) ingestion during 8 weeks increase endothelium-dependent dilation in pre-hypertension and mild hypertension adult subjects.

Table 3. Endothelium-dependent dilation (FMD, %) before (PRE) and after PLA, WPH and FPH interventions.

Variable	Intervention	PRE	T30	T60	T120	*f*
FMD (%)	PLA	6.70 ± 2.07	6.02 ± 2.04	5.63 ± 2.21	6.50 ± 2.55	0.32
	WPH		9.02 ± 2.29[a]	6.25 ± 1.76	6.48 ± 2.57	
	FPH		6.41 ± 2.45	6.46 ± 2.07	5.23 ± 1.85	
BBD (mm)	PLA	3.31 ± 0.33	3.56 ± 0.54	3.53 ± 0.38	3.53 ± 0.46	0.00
	WPH		3.55 ± 0.37	3.56 ± 0.38	3.51 ± 0.41	
	FPH		3.40 ± 0.35	3.53 ± 0.39	3.62 ± 0.48	
PBD (mm)	PLA	3.53 ± 0.34	3.76 ± 0.51	3.72 ± 0.43	3.75 ± 0.42	0.05
	WPH		3.86 ± 0.39	3.78 ± 0.39	3.73 ± 0.39	
	FPH		3.61 ± 0.34	3.80 ± 0.42	3.81 ± 0.50	

PLA: placebo; WPH: whey protein hydrolysate; FPH: fish protein hydrolysate; FMD: flow-mediated dilatation; BBD: baseline brachial diameter; PBD: peak brachial diameter. *f*: Cohen's *f*.
[a]Significantly different from PLA.
The values are mean ± standard deviation.

The possible explanation for the improved endothelium-dependent dilation after whey-derived peptides (NOP-47) (Ballard et al. 2009, 2013) and whey protein concentrate ingestion (Fekete et al. 2016) may be related to the bioactive peptides. Bioactive peptides present in dietary proteins either generate naturally in the gut or acquired from the diet as chemical or enzymatic hydrolysed proteins (Chobert et al. 1988; Roslana et al. 2014; Manzanares et al. 2015). Milk proteins are the most important source of bioactive peptides as compared to others animals and plant proteins (Manzanares et al. 2015). These peptides may interact with many systems implicated in arterial dilatation, such as renin-angiotensin, natriuretic peptide and endothelin systems (Manzanares et al. 2015).

Furthermore, this is the first study to demonstrate an increase on endothelium-dependent dilation after single dose of a WPH. Recently, Da Silva et al. (2017) demonstrated that WPH and the branched-chain amino acids (BCAA), leucine, isoleucine and valine, decreased tumour necrosis factor and vascular cell adhesion molecule-1, and increased endothelial nitric oxide synthase (eNOS) enzyme expression in inflamed human umbilical vein endothelial cells. This study observed that BCAA content in WPH (352.1 mg/g of BCAA) was 1.7-fold greater as compared to FPH (204.3 mg/g of BCAA), which might be related to increased NO availability by stimulating eNOS enzyme expression.

Previous study has demonstrated that plasma concentration of BCAA increased at 30 min post-ingestion of WPH, the time at which improvement in endothelium-dependent dilation after WPH ingestion was observed in this study (Tang et al. 2009). In addition, the endothelium-dependent dilation was evaluated through of flow-mediated dilatation (FMD) of the brachial artery by ultrasound technology. The FMD protocol utilised in this study provides an index of endothelium-derived NO function (Green et al. 2014). Thus, these results suggest that WPH ingestion may improve the endothelium-dependent dilation through of the NO bioavailability increase.

The FMD measurement has been used as an independent predictor of cardiovascular disease events in asymptomatic subjects (Green et al. 2014). Likewise, the magnitude of the vasodilatation after the occlusion/reperfusion manoeuvre relates inversely to traditional cardiovascular disease risk factor. Therefore, the FMD measurement has emerged as a clinically relevant tool in predicting future cardiovascular events (Ghiadoni et al. 2008). Although this study has shown an increase in FMD only 30 min after WPH ingestion, this information is clinically relevant as the incorporation of meals enriched with whey protein in the habitual diet may result in the beneficial effects on cardiovascular health. That is, based on the positive acute change observed on vascular function after a single dose of WPH, it may be suggested that the inclusion of this food in daily diet might help to improve long-term cardiovascular health by alleviating or even reversal the endothelial dysfunction that occurs naturally during the aging process and in the presence of cardiovascular diseases risk factors (Ghiadoni et al. 2008).

Additionally, although FPH showed greater total antioxidant capacity, it was not enough to induce significant change in endothelium-dependent dilation regardless of the dose level (5 g and 20 g) in young healthy subjects. In contrast to FPH, a single dose of WPH (20 g) was able to modulate the endothelium-dependent dilation in individuals young and without the presence of cardiovascular disease (healthy people). It is noteworthy that the mechanisms that govern the endothelium-dependent dilatation vary largely in terms of pathophysiology condition, such as age, level of physical activity and disease (Corretti et al. 2002). Thus, individuals who are sedentary, older and/or at high risk for cardiovascular disease would be expected to improve endothelium-dependent dilatation after ingestion of a supplement with antioxidant properties (FPH). As all participants were young ($\leq$28 years) and healthy, improvement in endothelium-dependent dilatation may not have occurred since their vascular function is already near optimal levels (mentioned as a "ceiling effect") (Montero et al. 2014). In addition, three of the nine participants were physically active, which may have contributed even more for this vascular "ceiling effect". Therefore, the results reported in this study should be interpreted with caution, as FPH could still be a relevant nutritional strategy for older individuals at high risk for cardiovascular disease.

Conclusion

This study demonstrated that a single oral dose (20 g) of FPH did not improve endothelium-dependent dilation in healthy young subjects as compared to WPH. Future studies could evaluate the effect of FPH ingestion on vascular function of the others populations, such as in cardiovascular risk factors and elderly subjects, which commonly present oxidative stress.

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Author contributions

GVO, MVS and EMC contributed substantially to volunteers' recruitment and physiological analysis. TSA and CACJ contributed to the development of the fish protein hydrolysate supplement and reviewing the article. GVO contributed substantially to data acquisition, statistical analysis and data interpretation. GVO and MVS wrote the article. All authors read and approved the final article.

Disclosure statement

No potential conflict of interest was reported by the authors.

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