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Research Report

Protective properties of sardine and chickpea protein hydrolysates against lipoprotein oxidative damages and some inflammation markers in hypercholesterolemic rats

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Abstract.
OBJECTIVE: This study evaluated the effect of sardine (SPH) and chickpea protein hydrolysates (CPH) on oxidant stress and inflammatory profile in cholesterol-fed rats.
METHODS: The experiment was undertaken for thirty days on 18 cholesterol-fed Wistar rats (220 ± 10 g) divided into three groups and receiving 1 g/kg of body weight either chickpea protein hydrolysate (CPH), sardine protein hydrolysate (SPH) or casein in water (CG).
RESULTS: Compared to CG, SPH and CPH treatment reduced cholesterol, hydroperoxide and malondialdehyde contents in serum, lipoproteins, erythrocytes and aorta. These same treated groups showed also lower serum isoprostane levels. However, serum paraoxonase activity and HDL-antioxidant property were improved only by CPH compared to CG. SOD activity of aorta and erythrocytes was higher in CPH but in SPH group, SOD activity was lower in these tissues and remained unchanged in serum. Furthermore, CPH and SPH stimulated glutathione peroxidase and catalase activities of aorta and erythrocytes. In CPH group, nitric oxide levels of serum, erythrocytes and aorta were increased by respectively 1.4- to 1.8-fold compared to CG and SPH. In addition, among the three groups, CPH exhibited the best anti-inflammatory effect by lowering serum C reactive protein, uric acid and albumin concentrations.

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CONCLUSIONS: SPH and particularly CPH possess antioxidant and anti-inflammatory properties and could be useful as nutraceuticals for health improving and preventing numerous disorders such as cardiovascular diseases.

Keywords: Marine, leguminous, protein hydrolysates, hypercholesterolemia, antioxidant, anti-inflammation, aorta, erythrocytes, HDL

1. Introduction

Oxidative stress has been strongly implicated in various cardiovascular risk factors such as obesity, hypertension and hypercholesterolemia [1]. Many studies demonstrated that experimental hypercholesterolemia promotes lipid peroxides accumulation in the liver, blood and aorta, which makes it responsible for numerous metabolic and physiologic disorders potentially involved in atherogenesis [2]. Besides, oxidative stress acts by increasing the immune system resulting in the release of pro-inflammatory molecules. Indeed, inflammation is an early event in the development of atherosclerosis [3].

Impaired HDL functionality is also highly involved. Indeed, the HDL possesses a variety of functions that contribute to their atheroprotective effect: macrophage cholesterol efflux, reverse cholesterol transport (RCT), anti-inflammatory, anti-apoptotic, antioxidative and modulating of endothelial function mainly by stimulating endothelial nitric oxide (NO) production [4]. It was reported that HDL-associate proteins such as paraoxonase (PON1), platelet-activating factor-acetylhydrolase, lecithin-cholesterol acyltransferase (LCAT) and glutathione selenoperoxidase, can protect arteries against oxidized-LDL and facilitate the repair of injuries caused by their deleterious effect [2]. Furthermore, apolipoprotein AI prevents inflammation and oxidative stress and promotes cholesterol efflux, which reduces arterial damages [2]. Serum apolipoprotein AI was established to be negatively correlated with the inflammation markers including serum C-reactive protein (CRP) and interleukin levels [5].

The conventional therapeutic modalities that include mainly lipid-lowering drugs are effective. However, compared to bioactive compounds derived from natural substances, these synthetic drugs may cause side effects [6]. Many scientists have reported that bioactive peptides and protein hydrolysates derived from plant, animal and fish proteins possess beneficial properties for human health and food technology due to their particular physiological and bioactive functions: antihypertensive, immunomodulatory, antimicrobial, antithrombotic, hypocholesterolemic, antioxidant and anti-inflammatory [7]. Among these beneficial functions, the antioxidant potential was the most studied. Udenigwe et al. [8] observed that peptides derived from protein hydrolysates stimulate endogenous antioxidant enzymes: such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, as well as the non-enzymatic antioxidant molecules (eg. glutathione). Further researchers attribute the antioxidant potential of these peptides and hydrolysates to their ability for radical scavenging, peroxides-formation inhibition and metal ion chelating properties [9].

It has also been reported an anti-inflammatory effect of protein hydrolysates. For example, it was shown that soybean protein hydrolysate reduces the inflammatory markers in lipopolysaccharide-stimulated RAW 264.7 macrophages [10]. Ktari et al. [11] demonstrated that fish hydrolysates exhibited *in vivo* antioxidant potential.

Even though several studies, carried out separately, have addressed the impact of marine and legume protein hydrolysates utilization on some metabolic disorders, few have compared them. Therefore, the present study aimed to compare the effects of two types of protein hydrolysates prepared from sardine and chickpea on lipoprotein oxidative damages and some inflammation markers in hypercholesterolemic rats.

Table 1a
Composition of cholesterol-enriched diet (g/kg diet)[a]

Ingredients	g
Casein[b]	200
Sunflower oil[c]	100
Cellulose powder[b]	50
Sucrose	40
Vitamin mix[d]	40
Mineral mix[e]	20
Cholesterol[b]	10
Cholic acid[b]	5
Methionine[f]	3
Corn starch[g]	532

[a]The diet contained 16.7 MJ/kg and was given in powdered form. [b]Sigma-Aldrich Chemie, Germany. [c]Commercial product, provides the following fatty acids (saturated: 13%; monounsaturated: 22%; polyunsaturated: 65% of total fatty acids), Cevital, Bejaia, Algeria. [d]UAR 200, (Villemoisson, 91360 Epinay sur Orge, France). [e]UAR 205B, (Villemoisson, 91360 Epinay sur Orge, France). [f]Biochem Chemopharma, Canada. [g]Maghnia Starch, Tlemcen, Algeria.

2. Material and methods

2.1. Preparation of chickpea and sardine proteins

Fish proteins were extracted from fresh sardine (*Sardina pilchardus*) muscle according to Guillaume et al. [12], whereas chickpea (*Cicer arietinum*) proteins were isolated from the defatted grounded-grains according to Boye et al. [13]. The detailed procedures were previously described [14, 15]. Protein concentrations were determined by their nitrogen contents according to Kjeldahl's method. Protein concentrations values represented respectively 87% and 90% in sardine and chickpea proteins.

2.2. Preparation of chickpea and sardine protein hydrolysates

Sardine (SPH) and chickpea (CPH) protein hydrolysates were prepared using a pH-stat method, according to Adler-Nissen [16]. Proteins were solubilized in distilled water (10% w:v) and hydrolyzed with Alcalase 2.4 L (Novozyme, Copenhagen, Denmark) under optimal conditions (pH 8.0 and 50°C). The enzyme was added to the reaction with an enzyme/substrate ratio of 3 U/mg. During the reaction, the pH of the mixture was maintained constant by the continuous addition of NaOH (4 N). When reaching an 8% degree of hydrolysis (DH), the solution was heated to 90°C for 10 min. The protein hydrolysates were then centrifuged at $5000 \times g$ for 20 min and the soluble phase was lyophilized (Christ, ALPHA 1-2 model, Germany). The hydrolysates amino acid composition was analyzed according to Soufleros and Bertrand [17] after hydrolysis with HCl (6 N).

2.3. Animals and diets

Male Wistar rats ($n = 18$) (Animal Research Center, Pasteur Institute, Algiers, Algeria) weighing 220 ± 10 g received a hypercholesterolemic diet (Table 1) containing 20% casein supplemented with 1% cholesterol and 0.5% cholic acid for 30 days. Rats were randomized into three groups of six each: the first (SPH) and second groups

Table 1b
Chemical and amino acid composition of sardine and chickpea protein hydrolysates

	SPH	CPH
Protein[a]	90.0 ± 0.2	90.2 ± 0.8
Fat[b]	2.7 ± 0.5	1.9 ± 0.1
Moisture[c]	5.1 ± 0.2	3.3 ± 0.3
Ash[d]	2.2 ± 0.5	3.2 ± 0.1
Carbohydrates	–	1.4
Amino acid composition[e]		
Isoleucine	4.1 ± 0.0	4.9 ± 0.1
Leucine	8.7 ± 0.1	9.3 ± 0.2
Valine	7.1 ± 0.1	5.6 ± 0.0
Methionine	3.4 ± 0.0	1.2 ± 0.1
Alanine	9.2 ± 0.0	7.2 ± 0.0
Tyrosine	3.1 ± 0.0	1.9 ± 0.0
Histidine	11.0 ± 0.1	3.6 ± 0.4
Tryptophane	5.9 ± 0.1	0.7 ± 0.2
Phenylalanine	12.7 ± 0.0	4.4 ± 0.2
Alanine	9.2 ± 0.0	7.2 ± 0.0
Glycine	8.4 ± 0.1	5.2 ± 0.2
Glutamate	8.9 ± 0.1	10.9 ± 0.6
Serine	3.9 ± 0.0	7.3 ± 0.2
Arginine	5.2 ± 0.1	8.2 ± 0.1
Lysine	8.5 ± 0.9	8.6 ± 0.2
Taurine	3.9 ± 0.1	–

SPH: sardine protein hydrolysate, CPH: chickpea protein hydrolysate. **Notes:** All measurements were performed in triplicate at the Regional Laboratory of Quality Control and Repression of Fraud of Oran, Algeria. a: Total nitrogen contents were determined by using the Kjeldahl method according to the AOAC method number 984.13 (AOAC 2000) and crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. b: Lipids were determined gravimetrically after Soxhlet extraction with *n*-hexane. c: Moisture was determined according to the (AOAC 2000) standard method 930.15. d: Ash was determined according to the (AOAC 2000) standard method 942.05. e: Amino acid composition was determined by HPLC (Soufleros and Bertrand, 1998).

(CPH) received daily 2 ml of an oral dose (1 g/kg body weight) of sardine and chickpea protein hydrolysates, respectively. The control group (CG) was daily administered with 2 ml of distilled water containing casein and treated in the same conditions.

Rats were housed in stainless steel cages at a temperature of 23 ± 1°C, with 55 ± 5% humidity and a 12–hour light/dark cycle and had free access to fresh food and tap water. Animals were kept according to the general guidelines on the use of living animals in scientific investigation [18] and the institutional committee on animal care and use approved the protocol and use of rats (approval number PNR 08/045, 25/11/2012).

2.4. Blood and tissue sampling

At the end of the experiment and after overnight fasting, the animals were anesthetized and blood was collected from the abdominal aorta. Samples were immediately centrifuged at 2000x*g* for 20 min at 4°C. Serum was then

collected and stored at –70°C until analysis. Erythrocytes were washed three times with saline and erythrocyte hemolysates were then prepared and stored.

Aorta was removed, washed with cold saline, quickly blotted in filter paper and immediately frozen at –70°C for subsequent analysis. Aorta was homogenized in 50 mM phosphate buffer saline (PBS, pH 7.2) and centrifuged at 2000x*g*, 15 min at 4°C. The supernatant was recovered and its protein contents were measured according to Lowry et al. [19].

2.5. Determination of serum and lipoprotein fractions cholesterol

Serum total cholesterol (TC) concentrations were estimated spectrophotometrically by an enzymatic colorimetric method using a Biolabo kit (Maizy, France). Serum lipoproteins were separated by fast protein liquid chromatography (FPLC) in Äkta-FPLC (Amersham Pharmacia Biotech, Barcelona, Spain) as described previously [20] using a SuperoseTM 6 10/300 GL column (GE healthcare life sciences, Barcelona, Spain) and a 50 mM PBS as running buffer. TC in the obtained fractions was measured by an enzymatic fluorometric method using cholesterol esterase and cholesterol oxidase enzyme and Amplex Red probe (Molecular Probes, OR, USA).

2.6. Lecithin-cholesterol acyltransferase activity

LCAT is responsible for the conversion of unesterified cholesterol (UC) to esterified cholesterol in circulating plasma HDL. LCAT activity was determined on fresh serum by an endogenous method [21] based on monitoring the change rate in serum UC concentration (Wako Chemicals, VA, USA) after incubation at 37°C. LCAT activity was expressed as μM of serum UC esterified per hour.

2.7. HDL-antioxidant property

The susceptibility of rats LDL to oxidation and the preventive property of HDL were evaluated according to Navab et al. [22] as previously described [20], by using dichlorofluorescein (DCF) (Sigma-Aldrich, MO, USA) as an oxidation detector. The HDL antioxidant property was calculated as:

$$\text{HDL-antioxidant property } (\%) = [1 - \frac{fluorescence_{LDL+HDL}}{fluorescence_{LDL}}] \times 100 \quad (1)$$

2.8. Lipid peroxidation levels

Malondialdehyde (MDA) [23] and lipid hydroperoxides [24] were assayed in serum, lipoprotein fractions and aorta. Serum 15-F_{2t}-isoprostane was determined by a competitive enzyme-linked immunosorbent assay (ELISA) kit from Oxford Biomedical Research (MI, USA).

2.9. Antioxidant enzyme activities

Serum PON1-arylesterase activity was determined according to Kuo and La Du [25] by using phenylacetate as substrate in Tris/HCl (20 mM, pH 8.0) containing 1 mM $CaCl_2$. Phenol formation rate was measured by monitoring the increase of absorbance at 270 nm and 25°C. The activity was calculated based on the molar extinction coefficient of phenol (1310 $M^{-1}.cm^{-1}$). One unit of arylesterase activity is equal to 1 μM of phenol formed per minute.

Superoxide dismutase activity was assayed using nitro-blue tetrazolium (NBT). The NBT is reduced in presence of superoxide anion, which is generated by xanthine oxidase and hypoxanthine couple (Cayman kit, MI,

USA). Results were expressed in units (U) per mg protein, where 1U SOD was designed as the amount of enzyme causing 50% inhibition of NBT reduction. Glutathione peroxidase was assayed with a Cayman kit (MI, USA). Briefly, oxidized glutathione, produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase and NADPH. The NADPH oxidation is accompanied by a decrease in absorbance at 340 nm. The decreased rate of absorbance at 340 nm is directly proportional to the GPx activity in the sample. One unit GPx is defined as the amount of enzyme that causes oxidation of 1 nmol NADPH per minute. Catalase activity assay was performed with a Cayman kit (MI, USA). 1U catalase is defined as the amount of enzyme that causes the formation of 1 nmol of formaldehyde per minute.

2.10. Nitric oxide determination

Nitric oxide determination was performed using Griess reagent [26]. A volume of tissue extract underwent a deproteinization by zinc sulfate solution, then NO_3 was reduced to NO_2 by cadmium overnight at 20°C, under shaking. Samples were added to the Griess reagent and incubated for 20 min at room temperature. Absorbance was measured at 540 nm and sodium nitrite was used as standard.

2.11. Inflammatory markers

Serum albumin and uric acid were measured using commercial kits from Spinreact (Girona, Spain). CRP was assessed with an ELISA kit from RayBio (GA, USA).

2.12. Statistical analysis

Data were expressed as mean ± standard deviation. Statistical evaluation was carried out by STATISTICA (Version 5.1, Stat soft Tulsa, Oklahoma, USA). After one-way analysis of variance, the *post-hoc* comparison was performed using Duncan new multiple range tests. Means with different superscripts were considered significantly different at $p < 0.05$.

3. Results

3.1. Physicochemical and amino acid composition

The protein hydrolysates contained similar calories (383.5 Cal. per 100 g of CPH vs. 384.5 Cal. per 100 g of SPH) and protein contents (90.2 per 100 g of CPH vs. 90.0 per 100 g of SPH).In addition to its taurine content, SPH contains more hydrophobic amino acids (Ala, Val, Ile, Leu, Tyr, Phe, Trp, Pro, Met, Cys; 54%) compared to CPH (35%). Likewise, SPH is richer in aromatic amino acids (Phe, Tyr, Trp) than CPH.

3.2. Serum and lipoprotein cholesterol contents and LCAT activity

Compared to the CG group, serum TC concentrations were respectively 2.9- and 1.4-fold lower in rats administered SPH and CPH. In the SPH group, TC concentrations were 2-fold lower than those of the CPH group. This reduction corresponded to significantly lower VLDL-C contents that were decreased by 78% and 32%, respectively, in SPH and CPH compared to the control (Fig. 1).

CPH supplementation increased HDL-C levels by 59% compared to the CG. The HDL-C levels in CPH rats were 49% higher than in SPH, while all groups exhibited similar LDL-C concentrations. In the CPH group vs.

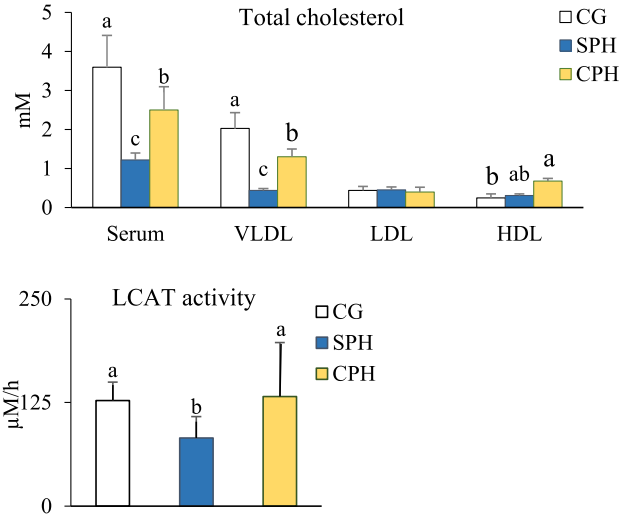

Fig. 1. Serum and lipoprotein cholesterol contents and LCAT activity in hypercholesterolemic rats treated with sardine or chickpea protein hydrolysates. CG: control group, SPH: sardine protein hydrolysate group, CPH: chickpea protein hydrolysate group. Serum lipoproteins were separated by FPLC and collected fractions were analyzed for their cholesterol contents. Data are presented as means ± SD of 6 rats/group. Means with different letters are significantly different at $p < 0.05$.

CG, serum LCAT activity remained unchanged. Whereas, this activity was enhanced by 60% in CPH compared to the SPH group (Fig. 1).

3.3. Lipid peroxidation

In serum, MDA levels were 1.2-fold lower in the SPH group vs. CG. This decrease reflected the lower VLDL- and HDL-MDA contents (–28% and –14%, respectively). The LDL-MDA levels were also significantly reduced by CPH (–50%) compared to CG and SPH. Both SPH and CPH decreased MDA contents in erythrocytes (2.3- and 14-fold, respectively) and aorta (4- and 6-fold, respectively), compared to CG (Table 2).

Serum hydroperoxide concentrations were, respectively, 2.2- and 1.3-fold lower in SPH and CPH rats compared to CG. VLDL-hydroperoxide contents were also lower in both treated groups (1.5- and 2.3-fold, respectively in SPH and CPH) vs. CG, while CPH treatment significantly reduced LDL-hydroperoxide contents in LDL fractions either versus CG (–57%) or vs. SPH (–44%).

CPH rats showed reduced levels of erythrocytes hydroperoxides (–14%) compared to the SPH group, whereas sardine hydrolysate group exhibited the lowest hydroperoxide contents in the aorta (–66%) vs. CG and CPH. Serum isoprostanes were respectively 2.3- and 1.3-fold lower in CPH and SPH groups vs. CG, while in CPH, these levels decreased by 40% compared to SPH (Table 2).

3.4. HDL-antioxidant property

The VLDL-LDL of all the groups had undergone oxidation. However, the degree of oxidation was different. The VLDL-LDL oxidation level was less in the CPH (–31%) and SPH group (–18%), both compared to CG (Fig. 2). When incubated with the respective HDL fraction, the VLDL-LDL oxidation decreased among all the groups. However, the HDL-antioxidant property was higher in the treated groups since it reached 72% in CPH, 64% in SPH and only 26% in CG.

Table 2
Malondialdehyde, hydroperoxide and isoprostane levels in rats fed on high-cholesterol diet treated with sardine or chickpea protein hydrolysates

Tissue	CG	SPH	CPH
		MDA *(nmol/ml)*	
Serum	20.70 ± 0.10^{a}	18.20 ± 0.14^{b}	20.80 ± 1.00^{a}
VLDL	8.45 ± 0.70^{a}	6.07 ± 0.34^{b}	7.75 ± 0.38^{a}
LDL	4.73 ± 0.51^{a}	4.74 ± 0.48^{a}	2.37 ± 0.33^{b}
HDL	2.98 ± 0.21^{a}	2.56 ± 0.14^{b}	3.03 ± 0.45^{a}
Erythrocytes	1.80 ± 0.10^{a}	0.80 ± 0.20^{b}	0.13 ± 0.05^{c}
Aorta *(nmol/g tissue)*	0.80 ± 0.01^{a}	0.20 ± 0.09^{b}	0.12 ± 0.07^{b}
		Hydroperoxides (μ*mol Eq.Cumene-OOH/ml)*	
Serum	168 ± 12^{a}	76 ± 05^{c}	126 ± 28^{b}
VLDL	136 ± 10^{a}	90 ± 08^{b}	59 ± 07^{c}
LDL	69 ± 16^{a}	54 ± 19^{a}	30 ± 07^{b}
HDL	13.6 ± 8.6	9.2 ± 3.8	6.0 ± 2.7
Erythrocytes	89 ± 06^{ab}	93 ± 03^{a}	80 ± 04^{b}
Aorta (μ*mol Eq CuOOH/g tissue)*	1.25 ± 0.06^{a}	0.42 ± 0.04^{b}	1.28 ± 0.11^{a}
		Isoprostanes *(ng/ml)*	
Serum	214.0 ± 0.2^{a}	156.0 ± 0.0^{b}	93.0 ± 0.1^{c}

Data are presented as means ± SD of 6 rats per group. Statistical analysis was performed using Duncan's multiple range test. The means within a row with unlike superscripted letters $^{(a,b,c)}$ are significantly different at $p < 0.05$. CG: control group, SPH: sardine protein hydrolysate group, CPH: chickpea protein hydrolysate group, MDA: Malondialdehyde, Eq.Cumene-OOH: equivalent of cumyl hydroperoxide.

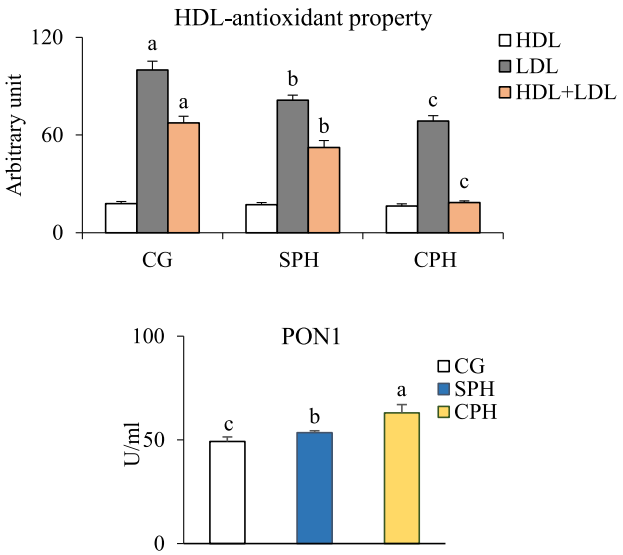

Fig. 2. HDL-antioxidant property and paraoxonase activity in hypercholesterolemic rats treated with sardine or chickpea protein hydrolysates. CG: control group, SPH: sardine protein hydrolysate group, CPH: chickpea protein hydrolysate group. Serum lipoproteins were separated by FPLC and collected fractions were analyzed for their cholesterol contents. Data are presented as means ± SD of 6 rats/group. Means with different letters are significantly different at $p < 0.05$.

Table 3
Antioxidant enzyme activities and NO levels in cholesterol-fed rats treated with sardine or chickpea protein hydrolysates

Tissue/parameters	CG	SPH	CPH
Serum			
SOD *(U/ml)*	55.6 ± 5.4^{b}	90.0 ± 0.2^{a}	61.6 ± 8.7^{b}
GPx *(U/ml)*	110.4 ± 11.2	127.4 ± 15.0	118.9 ± 12.7
NO (μ*mol/ml)*	67.7 ± 5.6^{b}	67.8 ± 6.3^{b}	92.1 ± 2.1^{a}
Erythrocytes			
SOD *(U/mg pt)*	7.3 ± 0.3^{b}	5.8 ± 0.2^{c}	11.0 ± 1.6^{a}
Catalase *(U/mg pt)*	241.3 ± 3.6^{c}	265.8 ± 15.9^{b}	291.9 ± 6.3^{a}
GPx *(U/mg pt)*	101.9 ± 25.5	114.9 ± 1.8	118.9 ± 14.7
NO (μ*mol/ml)*	28.3 ± 5.2^{b}	29.0 ± 6.0^{b}	52.1 ± 0.0^{a}
Aorta			
SOD *(U/mg pt)*	2.61 ± 0.13^{b}	2.41 ± 0.07^{c}	9.85 ± 1.65^{a}
Catalase *(U/mg pt)*	0.14 ± 0.02	0.12 ± 0.05	0.12 ± 0.01
GPx *(U/mg pt)*	250.2 ± 0.1^{c}	340.3 ± 0.1^{b}	390.2 ± 13.5^{a}
NO (μ*mol/g*)	302.5 ± 14.1^{b}	280.0 ± 21.2^{b}	512.5 ± 17.7^{a}

Data are presented as means ± SD of 6 rats per group. Statistical analysis was performed using Duncan's multiple range test. The means within a row with different superscripted letters $^{(a,b,c)}$ are statistically significant at $p < 0.05$. CG: control group, SPH: sardine protein hydrolysate group, CPH: chickpea protein hydrolysate group, SOD: superoxide dismutase, GPx: glutathione peroxidase, NO: nitric oxide, pt: protein.

3.5. Serum, erythrocytes and aorta antioxidant enzyme activities

Serum PON1 was respectively 1.1- and 1.2-fold greater in CPH and SPH groups vs. CG. Likewise, SOD activity was 1.1- and 1.6-fold higher in CPH- and SPH-rats serum, respectively, compared to CG. While GPx activity remained unchanged. In the erythrocytes, CPH rats showed higher antioxidant enzyme activities, where SOD and catalase activities increased in this group compared to CG (1.5- and 1.2-fold, respectively) and SPH (1.9- and 1.1-fold higher, respectively) (Fig. 2).

In the aorta, CPH and SPH treatment increased GPx activity by 55% and 36%, respectively, compared to CG. Meanwhile, the CPH group showed better SOD and GPx activities, which were respectively 4.1- and 1.1-fold higher vs. the SPH group.

3.6. Nitric oxide levels

In serum and erythrocytes, the NO levels were respectively 1.8- and 1.4-fold higher in the CPH group, compared to SPH and CG. In the aorta, CPH increased the NO contents up to 80% compared to CG and SPH rats (Table 3).

3.7. Serum inflammation levels

Compared to CG, UA values decreased by 52% in SPH and by 57% in the CPH group. Serum UA and albumin contents were respectively 1.2-fold greater in rats receiving SPH than those receiving CPH. The level of CRP was 2.2-fold higher in SPH compared to the CPH group (Table 4).

Table 4
Inflammation markers in rats fed on high-cholesterol diet treated with sardine or chickpea protein hydrolysates

Parameters	CG	SPH	CPH
Uric acid (μ*mol/l*)	112.5 ± 13.8^{a}	53.8 ± 5.6^{b}	47.3 ± 7.5^{c}
Albumin *(g/l)*	28.1 ± 1.1^{a}	26.9 ± 1.8^{a}	22.1 ± 1.4^{b}
CRP *(ng/ml)*	0.92 ± 0.04^{c}	2.33 ± 0.13^{a}	1.05 ± 0.01^{b}

Data are presented as means ± SD of 6 rats per group. Statistical analysis was performed using Duncan's multiple range test. The means within a row with unlike superscript letters $^{(a,b,c)}$ are significantly different at $p < 0.05$. CG: control group, SPH: sardine protein hydrolysate group, CPH: chickpea protein hydrolysate group, CRP: C-reactive protein.

4. Discussion

Hypercholesterolemia is involved in numerous metabolic pathologies and it could lead to some irreversible complications, especially when it is associated with oxidative stress and inflammation [27]. Some studies demonstrated that fish and leguminous proteins could protect from hypercholesterolemia and its complications. However, because there is currently limited information about comparative effect of these two sources of protein, this study proposes to investigate this hypothesis by comparing the protective property of chickpea and sardine protein hydrolysates on some oxidative stress and inflammation markers.

The results showed that both hydrolysates decreased hypercholesterolemia in cholesterol-fed rats. The most affected lipoprotein fraction was the VLDL that decreased in the treated rats. SPH decreased efficiently serum cholesterol despite an impaired LCAT activity vs. CPH, whereas, the CPH group showed an increase in HDL-C contents. These results were in agreement with the work of Athmani et al. [28] and Ktari et al. [11] on fish proteins, and Ferreira et al. [29] and Xue et al. [30] on legume proteins. The main mechanism explaining these findings is the reduction of cholesterol and lipids absorption in the intestine and the inhibition of sterol micellar solubility, which could activate the hepatic cholesterol 7α-hydroxylase gene expression [14, 31]. For Liaset et al. [32] and Marques et al. [33], this hypocholesterolemic effect could also be due to an up-regulation of VLDL-receptor expression and down-regulation of hydroxy-methylglutaryl Coenzyme-A reductase and Niemann–Pick C1-like1 expression, respectively. Besides, the high contents in some amino acids like glycine, taurine and hydrophobic amino acids [14, 15] could explain the hypocholesterolemic effectiveness of SPH vs. CPH. Indeed, according to Liaset et al. [32], the ability of liver bile acids to bind to taurine or glycine increases their solubility and affinity for hepatic bile acid transporters and promotes their secretion. Consequently, LDL-receptor activity and LDL uptake increases in the liver [34]. Besides, hydrophobic peptides present in the protein hydrolysates may compete with cholesterol for incorporation into the micelles or displace cholesterol in the bile acid-phospholipid-cholesterol micelles, which affect the micellar solubility and the absorption of cholesterol [6].

According to Chen et al. [1], the high-cholesterol diet increases oxidative stress and inflammation process, which leads to endothelium dysfunction. In this study, CPH and particularly SPH decreased MDA and hydroperoxides in serum. These results could be explained by the low serum- and VLDL-cholesterol levels. Unfortunately, the *in vivo* antioxidative properties of proteins and hydrolysates were rarely tested, particularly from plant sources. Hence, it is difficult to discuss this work results. However, it was suggested that high taurine contents prevent the free radical generation by reacting with hypochlorous acid (HOCl) to create a more stable taurine-chloramine [34]. Taurine could also react with toxic aldehydes such as acetaldehyde and MDA and inhibit oxidative modifications of LDL [35]. Besides, aromatic amino acids (Phe and Trp as donators of protons to electron-deficient radicals) and His (with the hydrogen-donating and lipid peroxyl radical trapping ability of the imidazole group) may also explain the important antioxidant property of SPH vs. CPH [9]. For some researchers, the antioxidant

properties of a protein could be attributed to some antioxidant peptides that are released by hydrolysis and that cross the intestine barrier to reach the systemic circulation where they may exert their action [36]. Besides, the elevated SOD activity in SPH may be responsible of the low serum lipid oxidation by scavenging the superoxide anion and stop the radical chain reaction.

We noticed that CPH, followed by SPH, showed better VLDL and LDL resistance against high-cholesterol oxidative damages. Furthermore, when incubated with HDL, both hydrolysates showed better antioxidant action of HDL than the control. These results were in line with our previous study [20], in which HDL-antioxidant property was enhanced in rats treated with an Esperase-fenugreek protein hydrolysate. HDL are well known for their antioxidant, anti-inflammatory and vasodilator properties and their properties depend mainly on their protein and lipid composition [2]. The elevated PON1 activity, an HDL-associated antioxidant enzyme that catalysis the hydrolysis of oxidized phospholipids in LDL [37], could be responsible for the HDL-antioxidant property. Indeed, PON1 activity was increased in SPH and in particular in CPH group. These finding were consistent with Allaoui et al. [20] and Athmani et al. [28] data. The increased PON1 activity and HDL-antioxidant property in CPH compared to CG and SPH could explain in part the low lipid oxidation in LDL noticed in this group.

In the erythrocytes, the increased SOD and catalase activities in CPH vs. CG were concomitant with a decrease in lipid oxidation. However, SPH administration decreased SOD activity vs. CG, while the hydroperoxide contents remained unchanged. These results were in discrepancy with the work of Athmani et al. [28], who reported an increased SOD activity and low hydroperoxide levels in erythrocytes after a sardine protein hydrolysate supplementation. However, they observed, as in the present data, an increased catalase activity and low MDA contents in the treated groups vs. CG.

In the aorta, both hydrolysates enhanced GPx activity, which is in line with Athmani et al. [28] results. SOD activity also improved in the CPH group vs. CG, while it decreased in SPH rats vs. CG. In contrast, Athmani et al. [28] reported an increased SOD activity with fish hydrolysate.

Lipid oxidation was lower in both treated groups vs. CG. According to Ktari et al. [11], these findings could be attributed to low molecular weight of peptides generated by hydrolysis as well as their amino acid sequence. Oxidative stress and inflammation are tightly related. Effectively, oxidative stress plays a substantial role in both initiating and propagating inflammation. In the present study, we examined whether SPH and CPH decrease inflammation markers such as serum UA, CRP and albumin in hypercholesterolemic rats. Compared to CG, SPH and particularly CPH improved the anti-inflammatory effect by reducing UA and albumin vs. CG. This reduction could be explained by the fact that UA is efficiently metabolized to allantoin through stimulation of uricase by SPH and particularly CPH. Tan et al. [38] have shown that a hypercholesterolemic diet causes a decrease in the levels of allantoin in rats, which is probably due to the inhibition of this enzyme. Ktari et al. [11] reached the same results as well as Athmani et al. [28]. Allaoui et al. [20] conclusions were different as the two parameters were unchanged in treated rats and this could be due to the difference in serum TC levels between these studies.

Another inflammation marker, CRP, is known to facilitate the infiltration of macrophages and promote the development of atherosclerosis [39]. This marker is associated with higher rates of UA that reflect a systemic inflammation and can disrupt reverse cholesterol transport [39, 40]. Besides, plasma isoprostane is also reported to be correlated with plasma CRP levels [1]. Hence, the low isoprostane and UA values found in the CPH group could explain the reduced CRP levels. However, the mechanism explaining the surprising high CRP levels noticed in SPH rats is unclear due to the lack of data treating this topic.

The NO promotes several beneficial physiological effects by enhancing vasodilatation and inhibiting smooth muscle cell proliferation [41]. One of the purposes of the present work was to compare the effect of the two hydrolysates on endothelial dysfunction. This study showed that CPH, and not SPH, increased serum and tissue NO contents, compared to CG. The increased NO by CPH could be explained by the high contents of arginine in this protein hydrolysate or by the HDL capacity to stimulate endothelial NO (the high HDL-antioxidant property is probably an indicator of their ability to stimulate NO production) [41].

5. Conclusion

Sardine and particularly chickpea protein hydrolysates exerted a protective effect against cardiovascular risk factors by improving the anti-atherogenic metabolic pathway of cholesterol. This action was further enhanced by an increased PON1 activity, which could protect from LDL oxidation and resulting in an inhibition of pro-inflammatory factors generation. The study also demonstrated that chickpea protein hydrolysate exerted interesting antioxidant and anti-inflammatory properties and protected better than sardine protein hydrolysate against hypercholesterolemia damages. While, SPH showed a better effect in reducing cholesterol levels, especially in the atherogenic lipoprotein. Hence, SPH and CPH could be useful as nutraceuticals or as ingredients in functional foods to improve human health and prevent some pathologies. Finally, it would be interesting to study the synergistic effect of both protein hydrolysates to check the benefit of their combination.

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Conflict of interest

The authors have no conflict of interest to report.

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