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Research Paper

Immunological effects of fish protein supplementation on healthy adults

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ABSTRACT

The purpose of this study was to test the influence of a fish protein hydrolysate (FPH) on immune parameters of healthy volunteers. In this placebo-controlled double-blind parallel study, participants were randomized to receive either FPH (n=23; 300mg daily) or placebo (n=24) for 4 weeks. Blood and saliva samples were collected before and during the supplementation period (week 2 and 4), and analyzed for total serum IgA concentrations and secretory IgA in saliva. There was no significant difference in serum or saliva IgA concentrations between groups at any time point during the study. However, there was a significant gender by treatment interaction for the change in serum IgA concentrations (P=0.05). After 4 weeks, a tendency toward a significant treatment effect on serum IgA concentrations was observed in men receiving FPH compared with control (P=0.07) but not in women (P=0.72). No adverse events were detected and no safety concerns were presented. In conclusion, these data suggest that 4 week supplementation with a daily dose of FPH may influence the serum IgA concentration in men. Further studies are needed to examine further this apparent gender specific response to FPH.

Keywords: Clinical trial; Fish protein; IgA; Nutraceuticals.

INTRODUCTION

Enzymatic hydrolysis of fish proteins is a valuable and little-exploited source of potentially bioactive peptides. It has been shown that FPH displays hypocholesterolemic (Bergeron and Jacques, 1989; Zhang and Beynen, 1993; Wergedahl, et al., 2004) and antioxidative (Kim, et al., 2001; Je, et al., 2008; Theodore, et al.; 2008 Klompong, et al., 2009) peptides have been identified in fish proteins. Peptides derived from fish proteins are also claimed to be beneficial for a variety of gut conditions including the damage effects induced by non-steroidal anti-inflammatory drugs (Fitzgerald, et al., 2005; Marchbank, et al., 2008; Marchbank, et al., 2009). Like peptides derived from other food sources such as milk (Gill, et al., 2000; Mercier, et al. 2003; Politis and Chronopoulou, 2008; Saint-Sauveur, et al., 2008; Jacquot, et al., 2010;), bioactive peptides from fish proteins can influence host defense mechanisms. In aquaculture, where FPH are widely used as an ingredient for diets, some studies have demonstrated the effects of FPH on nonspecific immunity in

several fish species (Bogwald, et al., 1996; Gildberg, et al., 1996; Liang, et al., 2006; Tang, et al., 2008). In rodents, Duarte *et al*. showed that fermented fish protein concentrate modulates gut-associated non specific immunity by enhancing phagocytic activity and the number of IgA-secreting cells in the mouse small intestine lamina propria (Duarte, et al., 2006). Other fish peptides have also shown effects on lymphocyte functions in mice (Yang, et al., 2009).

Peptides derived from fish proteins are commercially available, and they are claimed to be beneficial for a variety of health conditions including effects on the immune system. Unfortunately, current evidence of the scientific validity of many of these commercial compounds is severely limited. The present study aimed to determine whether a newly available FPH, PeptiBal™, can modulate some immune parameters in a healthy population.

MATERIAL AND METHODS

***Subjects*:** Participants have been recruited from the general population. Inclusion criteria were: women and men aged between 18 and 60 years, non-smokers, with a body mass index (BMI) between 20 and 30 kg/m^2. Female volunteers of childbearing age were eligible if they were using contraceptive methods (oral contraceptives, condoms, etc.) for the duration of the study. Potential volunteers were excluded from the study if they were allergic to fish or to the enzymes trypsin or chemotrypsin, suffering from gastro-intestinal problem, irritable bowel syndrome, gut inflammatory diseases (Crohn's disease or ulcerative colitis), thyroid dysfunction, liver dysfunction or other disease that could compromise their safety. Volunteers with untreated hypertension (systolic blood pressure (SBP)>140mm Hg and/or diastolic blood pressure (DBP)>90mm Hg) as well as those with a SBP>130mm Hg and/or DBP>85mm Hg along with ≥ three other cardiovascular risk factors according to the Canadian Hypertension Education Program (Program C.H.E, 2009) were excluded from the study. Volunteers who had undergone major surgery (including surgery of the digestive tract) were also excluded. Breastfeeding or pregnant women as well as those using unrecognized contraceptive methods were not eligible. Volunteers consuming more than two alcoholic drinks per day or more than 9 alcoholic drinks per week for the duration of the project were excluded. Subjects using probiotic products or fermented beverages with known immunomodulating properties (yogurt, kefir, etc.) two weeks prior to study were also excluded.

***Fish Protein Hydrolysate (FPH)*:** The FPH preparation studied, PeptiBal™, was provided by innoVactiv Inc. The product is a patent-pending blend of small peptides generated by a trypsin-chemotrypsin hydrolysis process of purified shark proteins. The enzymatic hydrolysate was clarified by filtration and centrifugation to remove insoluble material (i.e. non-hydrolysated protein). The filtrate was then ultrafiltered using two subsequent cut-off membranes to remove high molecular weight polypeptides including residual enzymes. After ultrafiltration, the retentate was discarded, whereas permeate, so-called total hydrolysate, was pasteurized in order to inactivate residual enzymes. Spray-drying was performed on the pasteurized total hydrolysate.

The test product was delivered as a 629mg opaque capsule comprising 300mg of the FPH, 124.65mg of microcrystalline cellulose, 3mg of croscarmellose sodium, 100mg of calcium phosphate dibasic, 5mg of magnesium stearate, 0.35mg of silicon dioxide, 1.8mg of titanium dioxide, and 94.2mg of hypromellose. The placebo was provided in an opaque capsule containing 211mg of microcrystalline cellulose, 317mg

of calcium phosphate dibasic, 5mg of magnesium stearate, 1.8mg of titanium dioxide, and 94.2mg of hypromellose. The FPH dose was chosen by extrapolating the amount (4.5mg/kg) showing immunological changes in preliminary studies in mouse.

The dose (300mg) selected for this study have been recommended by the sponsor (innoVactiv). Unpublished studies in animals (mice) conducted in their laboratory have indicated a dose of 4.5mg/kg *per os* was sufficient to induced immunological changes in the animal model. A simple extrapolation was then made for the dose to give to human.

Study design: The study protocol had been approved by the Research Ethical Board of Université Laval, 2009. The study was conducted between May 2009 and August 2009 and was registered at clinicaltrials.gov as NCT00906438. The study was performed using a randomized double-blind, placebo-controlled parallel design. Assignment of treatment was conducted via the use of random sequence of numbers. Subjects visited the clinical investigation unit at week 0, 2 and 4, after a >12h overnight fast for anthropometric characteristics (weight, height), blood pressure and heart rate measurements, blood and saliva collections. Compliance and side effects were assessed at week 2 and 4. The first capsule has been consumed under supervision at the clinical investigation unit and volunteer kept under observation for a 60min period. Participants had then to consume daily one capsule (test or placebo) *per os* for 4 weeks.

Volunteers were asked to refrain from using probiotic products or fermented beverages with known immunomodulating properties (yogurt, kefir, etc.) during the entire period of the study, including a 2week run-in period. A maximum of 9 alcoholic beverages per week were permitted. Subjects were invited to keep their normal physical activities and instructed to avoid intense physical exercise 48 hours before test visits.

Sample collection**:** Biological samples were collected just before the beginning of FPH supplementation (week 0), and at week 2 and week 4. Collection of whole saliva samples was performed under resting conditions in a quiet room during the morning, between 7:00 and 10:00 following an overnight fast. The subjects sat in an upright position with the head inclined forward. The volunteer first rinsed their mouth with sterile water, and unstimulated saliva was collected for a single 4min period by the subject leaning forward and letting the saliva drain into a sampling tube. The subjects were told not to force salivation. Immediately after, the saliva sample was kept on ice and then centrifuged for 5 min at 2000 *x g* and 4°C. Supernatants were aliquoted and frozen at -80°C for subsequent analyses. Fasting blood samples were drawn by venipuncture immediately after saliva collection and the serum samples from all subjects were stored at -80°C until use.

IgA determination**:** Concentrations of secretory IgA (sIgA) antibodies found in saliva samples were determined using a commercial kit (ALPCO immunoassays, Salem, NH). This assay is specially designed to quantify human sIgA through its secretory piece. After thawing on ice, saliva samples were centrifuged (5min, 2000 *x g*, 4°C) and the supernatants were used for the assay. The immunoassay was done following manufacturer's instructions.

Total IgA in the serum was measured by standard ELISA method using the Human IgA Quantitation Set (Bethyl Laboratories, Montgomery, TX). The assay was performed according to manufacturer's instructions. The capture antibody was coated into microtitration plates (Greiner Bio-one, Germany). Bovine serum albumin (1%) in Tris buffered saline solution was used as blocking reagent. All the sera were assayed

at a dilution of 1:4000. Tetramethylbenzidine (SureBlue Reserve, KPL, Gaithersberg, MD) was used as the enzyme substrate and the final optical density was read at 450nm using a Genious plate reader (Tecan, Austria).

***Intolerance symptoms*:** Subjective tolerance scaled ratings of the frequency and intensity of side effects were obtained at week 2 and 4. Side effects of interests were headache, anxiety, fatigue/exhaustion, lack of energy, tendency to become exhausted quickly, decreased appetite, increased appetite, hiccup, nausea, vomiting, indigestion, stomach or abdominal pain, constipation, diarrhoea, flatulence, abdominal bloating, palpitations, balance disorders, decreased ability to concentrate, internal pressure, flushing, feeling cold, joints or members pain, numbness, dark or depressing thoughts. Participants had to indicate whether each side effect was absent (0), of mild intensity (1), of moderate intensity (2) or of severe intensity (3).

***Data analysis and statistics*:** The impact of the FPH on immune parameters in men and women compared with placebo was investigated using the PROC MIXED procedure for repeated measures with the Statistical Analysis Software (version 9.2, 2008, SAS Institute Inc, Cary, NC). Treatment interactions were tested using appropriate terms in the mixed models. Variables not distributed normally were log-transformed prior to analysis. The frequency and intensity of side effects between the two treatments were examined using Wilcoxon non-parametric test. Values of $P \leq 0.05$ were considered statistically significant. Power calculation indicated that a sample of 20 subjects per group would provide 80% power ($P=0.05$) to detect a 10% difference in serum IgA between groups based on an estimated variability of 12.5%.

RESULTS

A total of 47 healthy subjects were randomized, 24 to the placebo group and 23 to the FPH group. Clinical characteristics of each group are summarized in Table 1. Briefly, the proportion of men and women was similar in both groups. Mean body mass index and blood pressure were also comparable between the two groups. The proportion of subjects who have experienced side effects was the same in both groups, at 2 weeks and at 4 weeks, and the severity of side effects was also similar in both groups. Compliance to treatments was assessed by counting the number of capsules not taken by participants. On a total of 1316 capsules (28 capsules to be taken over the course of the study x 47 subjects), 8 capsules were not taken, yielding a compliance of 99.4%.

***IgA*:** Serum and salivary IgA concentrations were measured before the beginning of the trial, as well as 2 weeks and 4 weeks after initiating treatment with FPH or placebo. Serum IgA concentrations at baseline were not significantly different between groups ($P=0.44$) (Table 2). Serum IgA concentrations were also comparable between the FPH and control groups during and after treatment ($P=0.85$ for the treatment*time interaction). sIgA concentration was measured as an indicator of mucosal immune competence and there was no difference between treatment groups during and after treatment ($P=0.42$ for the treatment*time interaction, Table 3).

Sex specific response: Analyses revealed that there was a significant sex by treatment interaction for the change in serum IgA concentrations ($P=0.05$) (Table 4). Over the 4-week period, a tendency toward a significant treatment effect on serum IgA concentrations was observed in men assigned to FPH in comparison to men in the placebo group (+11.5% vs. -5.5% respectively, $P=0.07$). No such treatment effect was seen among women ($P=0.72$). For sIgA concentrations, a gender by treatment by time interaction was seen ($P=0.05$). However, when men and women were analyzed

separately, no treatment effect was observed (*P*-value of the interaction between time and treatment was 0.19 and 0.14 respectively).

DISCUSSION

Most of the studies on the effects of proteins and peptides on the normal immune system have been conducted *in vitro* or in animal and few studies exist on the immune function in humans. To our knowledge, this is the first clinical study to assess the efficacy of FPH in some immune parameters of healthy adults. FPH- and placebo-treated subjects exhibited a comparable side-effect profile indicating that the hydrolysate is well-tolerated for human consumption.

Studies in mice have shown that administration of protein hydrolysates prepared from different fish species increases the number of IgA+ cells in the small intestine (Duarte, et al., 2006) and production of different cytokines such as IL-6 and IFN-γ (Duarte, et al., 2006; Yang, et al., 2009). In the present study, no significant change in serum IgA or in sIgA levels was observed after 4 weeks of FPH consumption. It is difficult to compare the results of the present study with animal studies since different types of FPH preparations (enzymatic digests vs fermented) have been used for those studies.

However, we identify that gender may have an impact of serum IgA concentrations upon FPH intake. When men and women were analyzed separately, a tendency toward a significant treatment effect on serum IgA concentrations was observed in men but not in women. This means that at all time points, men in the FPH group tended to have higher concentrations of serum IgA compared to men in the control group but that it was not the case in women. Looking only at the gender level, it reduces considerably the number of subjects included in statistical analyses (10 men for FPH group *vs* 12 subjects in the placebo group). With a larger number of subjects, one can speculate that a significant difference between both groups could be seen. As regards sIgA in saliva, a time by treatment by sex interaction was observed but because of lack of power, we did not observe a treatment effect in men and women analyzed separately.

Since serum IgA and sIgA are molecules with different properties and produced by cells with different organ distributions, it is possible that the FPH intake would affect serum IgA production only. It is also possible that sIgA production was increased in other compartments, such as the intestine. However it would have been more appropriated to measure secretion of IgA in the intestinal secretions to evaluate any effect of FPH on sIgA production. For practical reasons, we decided to measure sIgA in saliva. Since IgA plasma cells generated at one mucosal site are known to populate remote mucosal sites as well (McDermott and Bienenstock, 1979), it seems conceivable that sIgA responses at other mucosal sites may correlate to those in the intestine. Although this study was not specifically designed to dissect out gender differences in response to FPH, these results must be interpreted with caution. Nevertheless, previous studies have emphasized the effect of sex on immune functions (Fish, 2008). As a general rule, females exhibit more robust cell-mediated and humoral immune responses to antigenic challenges. In women, there is increased expression of some cytokines in peripheral blood and vaginal fluids during follicular phase of the menstrual cycle and with the use of hormonal contraceptives (Brabin, 2002). The effects of estrogens on cytokine production promote an anti-inflammatory environment and are associated with T_H2-cell polarization and the production of cytokines that promote humoral immunity. On another hand, it has been shown that

androgens, such as testosterone, on the immune function are largely suppressive, thereby leading to a decreased of cytokine production (Olsen and Kovacs, 1996). Therefore, sex hormones have an influence on immune functions in both women and men. One may speculate that FPH act on cytokine production such as TGF-β, which is involved in IgA production, and this effect has greater impact on men. Further studies are definitely needed to investigate further the potential sex-specific response to FPH

CONCLUSION

This double-blind study showed that consumption of FPH was well tolerated in humans. The FPH doses used in this study did not show effects on the production of secretory and serum IgA in healthy subjects after 4 weeks of daily consumption. Although this study was not designed to evaluate gender differences in IgA production, the results may suggest that the FPH influence the serum IgA concentrations in men

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Table-1: Characteristics of subjects[a] in each treatment group at baseline and analysis of side effects during the study.

	FPH (N=23)	Placebo (N=24)	P-value
Gender	N=13 women; N=10 men	N=12 women; N=12 men	0.65
Age (yr)[b]	42.9 ± 12.4	40.2 ± 14.7	0.49
Body mass index (kg/m^2)[b]	25.1 ± 2.6	25.4 ± 3.3	0.71
Diastolic blood pressure (mm Hg) [b]	69.9 ± 5.6	70.5 ± 6.7	0.74
Systolic blood pressure (mm Hg) [b]	106.6 ± 9.1	108.1 ± 11.1	0.65
Any side effects[c]			
At 2 weeks	69.6%	62.5%	0.30
At 4 weeks	65.2%	66.7%	0.16

- [a]Subjects who completed the study; [b]Values are presented as means ± SD; [c]% of subjects who reported any of the 25 side effects

Table-2: Baseline and 4-week serum IgA concentrations in the Fish Protein Hydrolysate (FPH) and placebo groups.

	FPH	Placebo
Week 0	3.46 ± 0.29 [a]	3.11 ± 0.35
Week 2	3.66 ± 0.36	3.19 ± 0.39
Week 4	3.64 ± 0.33	3.17 ± 0.36

- [a]Mean concentration (mg/mL) ± S.E.

Table-3: Baseline and 4-week salivary IgA concentrations in the Fish Protein Hydrolysate (FPH) and placebo groups.

	FPH	Placebo
Week 0	0.95 ± 0.12 [a]	1.19 ± 0.20
Week 2	1.02 ± 0.15	1.09 ± 0.13
Week 4	0.96 ± 0.12	1.22 ± 0.14

- [a]Mean concentration (mg/mL) ± S.E.

Table-4: Baseline and on-treatment serum IgA concentrations in the Fish Protein Hydrolysate (FPH) and placebo groups according to the gender.

	Women		Men	
	FPH	Placebo	FPH	Placebo
Week 0	3.25 ± 0.43 [a]	3.15 ± 0.35	3.74 ± 0.39	3.07 ± 0.61
Week 2	3.30 ± 0.47	3.44 ± 0.50	4.12 ± 0.55	2.94 ± 0.60
Week 4	3.23 ± 0.44	3.44 ± 0.52	4.17 ± 0.48	2.90 ± 0.51

- [a]Mean concentration (mg/mL) ± S.E.