

Cytokine profile of rats fed a diet containing shrimp

Perfil das citocinas de ratos alimentados com dieta de camarão

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ABSTRACT

Objective

Studies have shown that shrimps reduced the tensile strength of scars in rat skin. The aim of the present study was to assess the cytokine profile of rats fed shrimp.

Methods

Group 1 (control) received a regular diet and Group 2 (experimental) received a diet containing 33% shrimp for nine days. The two diets contained the same amounts of proteins, fats and carbohydrates. Serum cytokine levels were determined by ELISA and a segment of the jejunum was taken to investigate its histological morphology and eosinophil infiltrate.

Results

The experimental group had lower serum levels of interleukin-4 (IL-4) (14.4 ± 1.9 versus 18.11 ± 2.6 pg/mL; $p < 0.05$) and IL-10 (5.0 ± 0.98 versus 7.5 ± 1.2 pg/mL; $p < 0.05$) and higher levels of IL-6 (17.8 ± 2.3 versus 3.2 ± 0.4 pg/mL, $p < 0.001$) than controls. Morphologically, the shrimp-based diet caused an architectural disorganization of the intestinal mucosa and a greater amount of eosinophils in the jejunal villus.

Conclusion

Our data suggests that shrimp consumption leads to a significant increase in the cytokine IL-6, a decrease in the immunomodulatory cytokine IL-10 in the serum of rats, and high eosinophil infiltration in the jejunum. The cytokine profile typical of inflammation and the histological aspect of the jejunum are compatible with food allergy.

Indexing terms: Cytokines. Diet. Eosinophils. Interleukins.

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RESUMO

Objetivo

Estudos mostraram que a dieta suplementada com camarão reduziu a resistência cicatricial na pele de ratos. Nesse contexto, o objetivo do presente estudo foi avaliar o perfil das citocinas de ratos que receberam dieta adicionada com camarão.

Métodos

Foram comparados um grupo controle e um grupo experimental, que receberam uma dieta enriquecida com camarão (33%) durante nove dias. As duas dietas continham quantidades semelhantes de proteínas, lipídeos, e carboidratos. Os níveis séricos de citocinas foram avaliados por ELISA, assim como um segmento de jejuno foi obtido para exame histológico da morfologia e infiltrado de eosinófilos.

Resultados

A dieta adicionada com camarão diminuiu os níveis séricos de IL-4 ($14,4 \pm 1,9$ versus $18,11 \pm 2,6$ pg/mL, $p < 0,05$) e IL-10 ($5,0 \pm 0,98$ versus $7,5 \pm 1,2$ pg/mL, $p < 0,05$ e aumentou os níveis séricos de IL-6 ($3,2 \pm 0,4$ versus $17,8 \pm 2,3$ pg/mL, $p < 0,001$) quando comparada com os animais controle. Morfologicamente, a dieta adicionada com camarão causou uma desorganização da arquitetura da mucosa intestinal, juntamente com uma abundância de eosinófilos nas vilosidades jejunais.

Conclusão

Os dados sugerem que a ingestão de dieta adicionada com camarão leva a um aumento significativo da citocina IL-6, juntamente com uma diminuição da citocina imunomoduladora IL-10 no soro de ratos e um infiltrado de eosinófilos no jejuno. O padrão inflamatório das citocinas e o aspecto histológico do jejuno são compatíveis com alergia alimentar.

Termos de indexação: Citocinas. Dieta. Eosinófilos. Interleucinas.

INTRODUCTION

Allergen-specific T cells can be isolated from the blood, skin and mucosal sites of patients with food allergy and they characteristically express the allergen-reactive type-2 T helper (Th2) cell phenotype, releasing interleukins (IL) IL-4, IL-5, and IL-13¹. These cytokines play a central role in the induction and maintenance of allergic responses by regulating IgE synthesis, and in the chemoattraction of inflammatory cells, such as mast cells and eosinophils²⁻⁵. IL-10 can be cross-regulated by Th1-related cytokines, such as IL-1 β , IL-6, inflammatory cytokines. Concurrently, the inflammatory cytokine IL-6 is capable of promoting Th2 differentiation dependent upon endogenous IL-4⁶. On the other hand, IL-10, initially considered a Th2-related cytokine, is a regulatory cytokine known to inhibit allergic and inflammatory events⁷⁻⁹.

Clinical manifestations of allergic reactions to food may also be seen in the gut because of morphological changes in the intestinal mucosa. Migration leads to significant T lymphocyte

accumulation in villus microvessels and in the Peyer's patches via mucosa, in a cell adhesion molecule 1-dependent process. The increased migration of lymphocytes to the intestines might play a key role in the development of intestinal mucosal injury in food allergies^{10,11}.

The influence of a shrimp-based diet on the skin wound healing resistance in rats has already been studied¹². The tensile strength of the scar on the fifth postoperative day was lower in the animals fed shrimp than in the controls. However, the mechanism by which shrimp ingestion affects tensile strength is not clear. The present study considered the hypothesis that this effect may be associated with an allergic process. Therefore, the aim of this study was to determine if the cytokine pattern of rats fed shrimp suggests an allergic process.

METHODS

Housed Wistar rats weighing 177-302g had access to food and water *ad libitum*. The

animals were maintained under standard laboratory conditions of a 12/12-hour light-dark cycle and temperature of $23\pm 2^{\circ}\text{C}$. The present investigation was in agreement with the Ethical Principles for Animal Experimentation, used by the local Ethics Committee for Animal Experimentation. The composition of the experimental and control diets was described by Borges *et al.*¹² and is shown in Table 1. The protein concentration in the dry shrimp flour (made using the shell and flesh of the shrimp) was 33.5mg/100mg according to the Lowry *et al.*¹³ method. Both diets contained the same amounts of protein, fats and carbohydrates. The shrimp flour was made by drying commercial shrimp and grinding it until a fine flour was obtained, which was then added to the other components of the diet. The diets were prepared sanitarilly, stored at -20°C and removed from the freezer right before use.

Table 1. Diet composition (%), nutrient contents (g/100g) and energy density (kcal/g).

Ingredients	Control diet	Shrimp-based diet
<i>Diet composition</i>		
Corn starch	57	46.9
Casein	20	9.1
Cellulose	10	1.0
Soybean oil	5	3.0
Vitamin mix ^a	1	1.0
Mineral mix ^b	5	5.0
Choline	1	1.0
Dry shrimp flour	0	33.0
<i>Macronutrient contents</i>		
Proteins	16.00	18.33
Fats	5.00	5.33
Carbohydrates	52.00	42.80
Energy density	3.25	3.00

^aComposition: 30mg niacin, 15mg pantothenic acid, 6mg vitamin B6, thiamin, 6mg riboflavin, 2mg folic acid, 750µg vitamin K, 200µg D-biotin, 25µg vitamin B12, 4000µg; vitamin A, 1000µg vitamin D3 and 75µg vitamin E.

^bComposition of the essential minerals: 35mg iron, 5000mg calcium, 1561mg phosphate, 3600mg potassium, 300mg sulfur, 1019mg sodium, 1571mg chlorine, 507mg magnesium, 30mg zinc, 10mg manganese, 5mg copper, 0.2mg iodine, 0.15mg molybdenum, 0.15mg selenium and potentially beneficial minerals: 5mg silicone, 1mg chromium, 1mg fluorine, 0.5mg nickel, 0.1mg lithium and 0.1mg vanadium.

Fourteen male Wistar rats were randomly divided into two groups: Group 1 (control) received a regular diet and Group 2 (experimental) received a diet containing 33% shrimp flour for nine days. Intake of food and water was assessed during the entire experimental period. On the ninth day after initiation of the shrimp-based diet, all rats were anesthetized with thionembutal (40mg/kg intraperitoneally) and submitted to intracardiac puncture. Serum samples were collected and kept frozen at -80°C until analysis. Then, the animals were submitted to laparotomy and segments of the jejunum, below the duodenojejunal ligament were removed. Tissue fragments were rinsed with 0.9% saline and collected for histological studies after fixation in 10.0% PBS-buffered formalin.

Cytokines

Serum samples were quantitatively assayed for IL-1 β , IL-4, IL-6, and IL-10 by capture Enzyme-Linked Immunosorbent Assay (ELISA) using eBioscience (Iceland, Ireland, United Kingdom) kits. Wells coated with capture antibodies (100µL per well at appropriate dilution) were incubated with premixed standards or sample supernatants (50µL) in 96-well filter plates. Plates were shaken for 30 sec at 1000rpm and then incubated at room temperature for one hour at 300rpm. After incubation, detection antibodies (1µg/mL) were added and the plates were shaken and incubated as before. After rinsing with a vacuum device (Millipore Corp., Billerica, MA), avidin-HRP (2µg/mL) was added to the wells, and the plates were shaken for 30 minutes at room temperature. A substrate solution was added (100µL per well at appropriate dilution) and incubated at room temperature for another 15 minutes. After addition of a stop solution, the optical density was determined at 450nm.

Histological analysis of the jejunum

A segment of the jejunum was stored in 10% buffered formalin and embedded in paraffin.

Sections of 4 μ m were prepared and stained with hematoxylin-eosin.

Statistical analysis

Results are presented as mean \pm Standard Error of Measurement (SEM). The groups were compared by the Student's *t* test for unpaired data and the level of significance was set at $p \leq 0.05$.

RESULTS

The shrimp-based diet did not change food intake (37.04 \pm 4.09 *versus* 47.22 \pm 24.86g) for the control and experimental groups, respectively, ($p > 0.05$) or animal weight (254.0 \pm 38.2 *versus* 264.0 \pm 40.6g) for the control and experimental groups, respectively ($p > 0.05$). The nutritional status of the animals fed shrimp did not change. There was no weight loss, stunting, thinner and more fragile skin, lethargy or hair loss.

Figure 1 shows the serum concentrations of IL-4 and IL-10. The levels of IL-4 (14.4 \pm 1.9pg/mL) and IL-10 (5.0 \pm 0.98pg/mL) in the serum of the experimental rats were significantly lower than those of the controls (18.11 \pm 2.6pg/mL and 7.5 \pm 1.2pg/mL for IL-4 and IL-10, respectively; $p < 0.05$).

Figure 2 shows the serum levels of the inflammatory cytokine IL-6. The levels of IL-6 in samples from the experimental group (17.8 \pm 2.3pg/mL) are significantly higher than those of the control group (3.2 \pm 0.4pg/mL, $p < 0.001$).

The shrimp-based diet did not change the serum levels of IL-1 β (53.1 \pm 4.8 and 50.4 \pm 5.2pg/mL for the control and shrimp-based diet, respectively).

Figure 3 illustrates the histological assessment of the control group's jejunum (Figures 3A and 3B) and experimental group's jejunum (Figures 3C and 3D). The shrimp-based diet caused an architectural disorganization of the intestinal

mucosa, with inflammatory infiltrate, stressed hyperplasia of calyciform cells (short arrows in C and D) and greater abundance of eosinophil cells in the jejunal villi (long arrows in D) in comparison with the control rats.

DISCUSSION

The main findings of the present study are the increase in the inflammatory cytokine IL-6 and

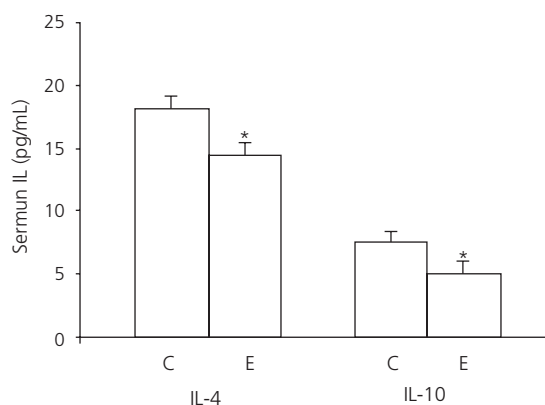


Figure 1. Serum concentrations of IL-4 and IL-10 in rats fed a regular diet (C) or a shrimp-based diet (E).

Note: The data are expressed as means \pm Standard Error of Measurement (SEM) of 7 animals per group. * $p < 0.05$ vs. control group.

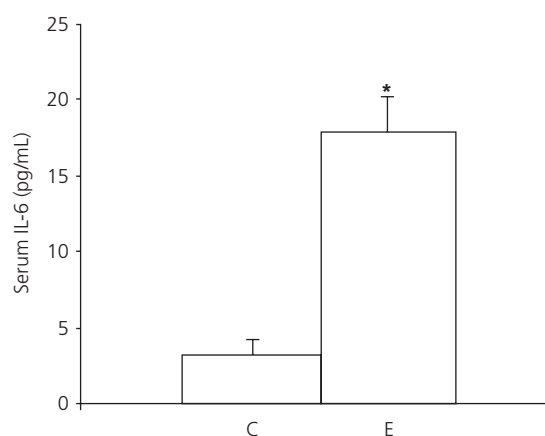


Figure 2. Serum concentrations of IL-6 in rats fed a regular diet (C) or a shrimp-based diet (E).

Note: The data are expressed as means \pm Standard Error of Measurement (SEM) of 7 animals per group. * $p < 0.001$ vs. control group.

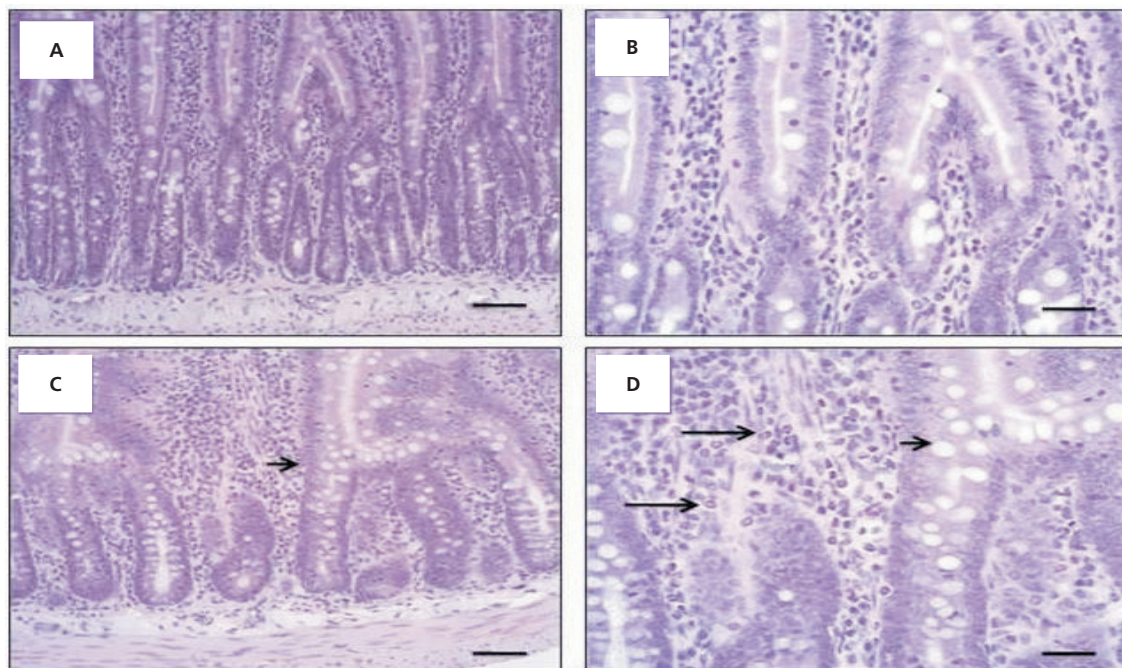


Figure 3. Photomicrographic assessment of rat jejunal mucosa. Histological sections stained with hematoxylin–eosin after control (A and B) and shrimp-based diet (C and D). Inflammatory infiltrate showing hyperplasia of calyciform cells (short arrows in C and D) and greater abundance of eosinophil cells (long arrows in D) in comparison with controls. Original magnification x10 (A and C); x20 (B and D). Bar=25 μ m.

the decrease in IL-10, an important regulatory cytokine, in the serum of rats after daily consumption of a shrimp-based diet. No difference in food intake or body weight was observed between the groups and the diet caused a jejunal histological aspect compatible with food allergy.

The high levels of the proinflammatory cytokine IL-6 in the serum of animals in the experimental group could be explained by a number of possibilities, such as an immune-specific effect of some shrimp component or infection by intestinal microbiota. Among these possibilities, the most plausible would be an allergic sensitization caused by the diet. Regulatory T-cells (Treg) might represent a normal braking mechanism leading to tolerance. An emerging hypothesis is that allergic sensitization may result partially from a lack of appropriate IL-10-producing Treg activity, which is either defective or is overcome in those who develop an allergy¹⁴. Accordingly, an interesting role for IL-6 in

determining Th2 effector and Treg cell function was described by Doganci *et al.*¹⁵, who identified IL-6 as an important factor that may expand Th2 responses to allergen and prevent the proliferation of Treg cells in the allergic airway. On the other hand, the allergy in rats fed shrimp-based diet could be facilitated by intestinal bacterial penetration due to intestinal rupture that may accompany a food allergy process as evidenced by the jejunal histology of those animals.

Contrary to IL-6, IL-10 is a regulatory cytokine known to inhibit allergic and inflammatory events⁷⁻⁹. IL-10 suppresses macrophage activity and reduces their cytokine production¹⁶. Temporally, IL-10 expression follows the early proinflammatory cytokine response, probably to limit both the magnitude and the duration of the inflammatory response¹⁷. In addition, IL-10 may protect children from allergic polysensitization¹⁸. Therefore, the development of new sensitizations as that caused by the shrimp-based diet may be

encouraged by low production of some cytokines, especially IL-10, and possibly be related to functional defects of the Treg cells¹⁸.

While a greater abundance of eosinophils in the jejunal villi of rats in the experimental group was seen, suggesting the occurrence of an allergic food process, there were also low levels of the Th2-related cytokine IL-4. The lower levels of IL-4 could be accounting for an impairment of Treg activity. Indeed, it has been shown that IL-4 can induce the proliferation, as well as prevent Treg apoptosis^{19,20}.

Proinflammatory cytokines, including IL-1 α and IL-1 β , IL-6, and TNF- α , play an important role in wound repair, influencing processes at the wound site, including stimulation of keratinocyte and fibroblast proliferation, synthesis and breakdown of extracellular-matrix proteins, fibroblast chemotaxis, and regulation of the immune response²¹. Polymorphonuclear leukocytes and macrophages have been shown to be the major source of these cytokines^{22,23}. Normal repair seems to occur by the coordinated expression of these cytokines, since expression of these genes was strongly reduced in healing-impaired glucocorticoid-treated mice²².

It has been shown that wounds of IL-6-knock-out animals took up to three times longer to reepithelialize than those of wild-type controls²⁴. The delay in that process and the impairment of granulation tissue formation were probably caused by IL-6 deficiency²⁵. Conversely, the opposite effect was observed by administration of recombinant murine IL-6 protein one hour before wounding, eliminating wound healing impairment, and producing a mitogenic effect on keratinocytes and a chemoattractive effect on neutrophils. Thus, it appears that IL-6 is crucial for kick-starting the healing response. Interestingly, excessive levels of IL-6 have been associated with skin scarring, and this cytokine increased the most with the shrimp-based diet.

Anti-inflammatory cytokines have also been shown to be important regulators of wound repair. In particular, IL-10 is thought to play a major

role in the healing response. This cytokine acts in inflammatory response termination. Moreover, it regulates growth and/or differentiation of various immune cells, but also of keratinocytes and endothelial cells²⁶. Based on these activities, a role of IL-10 in wound healing appeared later. In the present study, its serum levels in the experimental rats were lower, which would correspond to the earliest phase of wound repair.

In conclusion, rats receiving a shrimp-based diet display a cytokine profile compatible with allergic processes. This could be contributing to the lower tensile strength of scars observed in those animals after the ingestion of a shrimp-based diet.

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CONTRIBUTORS

EL Borges coordinated and designed the study, performed the statistical analysis and data interpretation, and prepared the manuscript. DR Oliveira prepared the diets and took care of the animals. LS Barcelos aided in the discussion and preparation of the manuscript. JL Pesquero performed the biochemical analysis and preparation of the manuscript.

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