The potential use of fish collagen as a new functional materials due to its good immune-compatibility

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Abstract. As a marine-derived biological macromolecule, fish collagen is attracting more and more attention for its potential application in the biomedical field. Currently, there is no systematic evaluation of immuno-compatibility of fish collagen in vivo. In the present study, 200 mg/kg hydrolyzed tilapia fish collagen (HTC) was injected subcutaneously into mice for 21 consecutive days. To evaluate the effects of HTC on spleen lymphocytes proliferation, cell counting assay (CCK-8) was performed. Flow cytometry was applied to study the effect of HTC on the apoptosis of spleen lymphocytes. Mouse serum immunoglobulin G (IgG) and immunoglobulin M (IgM) were determined by Enzyme-linked immunosorbent assay (ELISA). The results showed no significant difference for the proliferation and apoptosis of spleen lymphocytes between the HTC group and the control group (subcutaneously injected with 0.9% NaCl for 21 consecutive days). And there was no significant difference for serum IgG and IgM levels between the control group and HTC group. The above results showed that the hydrolyzed tilapia fish collagen possesses good immune-compatibility, which can potentially be used as biocompatible implanted biomaterials in vivo.

1. Introduction

Marine organisms have been recognized as encouraging sources of collagen, fish collagen comes from aquatic organisms. Compared with terrestrial organisms, there is less pollution and higher safety for fish collagen. Currently, fish collagen is mainly used in cosmetics, food, and health products. Furthermore, there is a low risk of disease transmission for fish collagen, and it is expected to be used as a new collagen in the field of biomedicine. Besides, fish collagen can effectively avoid religious barriers in Islam and other regions, with the lower religious ethics risk. Collagen extracted from the skin of codfish showed a dose-dependent effect in metabolism and on cell adhesion of lung fibroblast MRC-5 cells[1]. Fish collagen from tilapia can inhibit the excessive inflammatory response of macrophages and umbilical vein endothelial cells (HUVEC) in vitro[2,3]. Yamada et al. found that fish collagen peptides have biological activities such as promoting collagen synthesis and mineralization in MC3T3-E1 cells[4]. More interestingly, it has been suggested that there is an effect on the immunoregulation of mesenchymal stem cells by fish collagen [5,6]. All the above-mentioned studies showed that fish collagen can be potentially used as biomedical materials. However, there is no systematic evaluation of its immune-compatibility in vivo.

Immune rejection occurs when the immune system resists foreign substances. In clinical practice, hyper-acute rejection usually damages the implant. For example, in cardiac transplantation, hyper-acute rejection will cause the death of the patients[7,8]. Acute rejection belongs to the most common
immune rejection, of which the main symptom is characterized by the sudden deterioration of implant function, while chronic rejection is characterized by the fibrosis and dysfunction of the implant[9,10]. It is an effective method to evaluate the immune-compatibility of implanted biomaterials for reducing clinical adverse reactions. However, there is no systematic study on the immune-toxicity of fish collagen in vivo until now.

In the present study, the hydrolyzed tilapia fish collagen (HTC) was selected as the experimental object, and the HTC was implanted subcutaneously into the mice. The effects of HTC on the proliferation and apoptosis of spleen lymphocytes in mice were investigated, and the levels of IgG and IgM in serum samples were evaluated by ELISA. The effects of HTC on the immune system of mice were elucidated, which will provide further basis for the application of hydrolyzed tilapia fish collagen in the biomedical field.

2. Materials and Methods

2.1. Fish collagen (HTC)
Hydrolyzed tilapia fish collagen (HTC) was generously gifted by the Shanghai Fisheries Research Institute.

2.2. Animal experiment
The present study was approved by the Ethics Committee of Shanghai Ninth People's Hospital, affiliated with the School of Medicine, Shanghai Jiao Tong University (Shanghai, China). 20 female 5-6-week-old BALB/c mice were used in the animal experiment (n=10 per group). Before the experiment, 20 mice were randomly divided into the following two groups, control group and experimental group. In the control group, 50 ml/kg 0.9% NaCl was injected subcutaneously into mice; in the experimental group, 200 mg/kg HTC was injected subcutaneously for 21 consecutive days. During the experiments, all animals receive water and food ad libitum. Euthanasia was performed by using carbon dioxide asphyxiation followed by cervical dislocation. Serum and spleen were collected.

2.3. Cell proliferation assay.
Cell proliferation assays were carried out using a CCK-8 assay (Dojindo Molecular Technologies, Tokyo, Japan) following the manufacturer's instruction. the spleen was cut into 2 mm × 2 mm and gently pressed on a stainless-steel sieve (200-mesh), after centrifugation at 1000rpm for 5 minutes at room temperature, the supernatant was discarded, and the red blood cell lysis buffer was added. The mixture was incubated for 3 min at room temperature and centrifuged at 1000 rpm for 3 min. RPMI1640 containing 10% FBS was used to resuspend spleen lymphocytes. The cells were placed into 96-well plate at 100 μL/well for overnight culture. 5 mg/L Concanavalin A (Con A) was added into each well. CCK-8 was added at 10 μL/well after incubation for 24h at 37 ℃. 450 nm absorbance value was detected with a microplate reader after incubation for 4 hours.

2.4. Measurement of apoptosis
Cells were collected and washed with phosphate-buffered saline for 3 times, after centrifugation at 2000r/min for 5min, the cells were resuspended in 400 μL Annexin V-FITC binding buffer, followed by adding of 5 μL Annexin V-FITC, after incubation at 4 ℃ in dark for 10 min, 5 μL PI solution was added, followed by incubation at 4 ℃ in dark for 10 min. Apoptosis was detected by flow cytometry. The experiment was repeated three times.

2.5. ELISA
Serum was collected from both the control group and HTC group, then the ELISA method was applied to quantify IgG and IgM levels using a commercial kit (ab157719 and ab133047, Abcam, Cambridge, MA, USA) following manufacturer's protocol.
2.6. Statistical Analysis
Statistical analysis was performed using SPSS 22.0 software. The experimental data were expressed as mean ± standard deviation, and the intergroup difference was analyzed by one-way ANOVA. P < 0.05 means that the difference was statistically significant.

3. Results

3.1. Splenic lymphocyte proliferation
As shown in Figure 1, during the experimental period, no obvious abnormality was found in all animals. There was no significant difference in lymphocyte proliferation between the HTC group and the control group.

![Figure 1](image1.png)
Figure 1. Effect of HTC on the lymphocyte proliferation. Data are expressed as the mean ± SEM, n ≥ 3.

3.2. Apoptosis of splenic lymphocytes
We next evaluated the effect of HTC on the apoptosis of spleen lymphocyte, after continuous injection for 21 days, there was no significant difference between the HTC group and the control group for the apoptosis of splenic lymphocytes (Figure 2).

![Figure 2](image2.png)
Figure 2. Effect of HTC on apoptosis of cultured splenic lymphocytes. Data are presented with the mean ± standard deviation.

3.3. Serum IgG and IgM level
As shown in Figure 3, after continuous injection for 21 days, there was no significant difference in IgG and IgM level between the HTC group and the control group.
4. Discussion
Currently, medical devices prepared with collagen have been widely used in clinical practice. Although the effectiveness of collagen has been widely recognized, its safety, especially immunogenicity and immuno-compatibility, remains in doubt. Clinically, the immune response caused by implants may result in serious consequences. Although it is believed that collagen has low immunogenicity, there are some adverse reactions in clinical practice after using collagen products, such as local anaphylaxis [11]. Therefore, for any new collagen products, it is necessary to study its immune response. At present, fish collagen has drawn increasing attention because of its excellent biocompatibility and low price. In recent years, it has been found that fish collagen can induce the osteogenic differentiation of stem cells [12,13], which suggests that fish collagen could potentially serve as bone implantation biomaterials. In this study, we comprehensively evaluated the in vivo immune response of HTC, which will lay a foundation for the future clinical application of hydrolyzed tilapia fish collagen.

When the antigen enters the human body, T-lymphocytes and B-lymphocytes will be stimulated and proliferate rapidly, which initiates the acquired immune response. Lymphocyte proliferation and differentiation is an important stage of the immune response. Therefore, the detection of lymphocyte proliferation levels is a common method for cellular immune and clinical immune function evaluation. Specifically, there are receptors for recognizing antigens on the surface of T cells and B cells, which can lead to lymphocyte proliferation under the stimulation of specific antigens such as Con A. It has been suggested that collagen-derived bio drug does not stimulate lymphoproliferation or DNA damage in vitro[14]. The spleen is the largest secondary lymphoid organ, which contains a major number of macrophages and lymphocytes, therefore spleen has been considered as the center of humoral and cellular immunity[15]. In this study, there was no significant difference in the proliferation of spleen lymphocytes between the HTC group and the control group, therefore the continuous administration of HTC could not cause the over-proliferation of spleen lymphocytes. The changes in apoptosis can reflect the state of the internal environment. The lymphocyte is one of the important components of the immune system. The determination of lymphocyte apoptosis in mouse spleen can evaluate the overall immune status of mice. In this study, HTC did not affect the apoptosis of mouse spleen lymphocytes, and there was no significant difference between the HTC group and the control group, indicating that HTC did not cause excessive apoptosis.

Immunoglobulin is produced by plasma cells, which is an important effector of humoral immunity[16]. As an important immune effector molecule in the humoral immune system as well as a substance with biological activity in the body, immunoglobulin plays an important role in preventing bacterial invasion, enhancing the immunity of the body, activating complement, and killing tumor cells. The content of IgG and IgM in the organism can directly reflect the occurrence of the immune response[17]. IgG is the most important biological reaction index of humoral immunity, which is the main antibody component in serum, representing around 75% of the immunoglobulin in serum. Its main function is to play a protective role in the immunity of the body, such as anti-bacteria, anti-virus,
against measles, hepatitis A, which can effectively prevent the corresponding infectious diseases[18]. IgM can be used as a diagnostic indicator for early infection[17]. In the present study, the level of IgG and IgM of HTC group were similar to those of control, suggesting the immuno-compatibility of HTC.

5. Conclusion
A certain concentrations of hydrolyzed fish collagen did not cause abnormal proliferation, apoptosis, IgG, and IgM production of spleen lymphocytes, indicating good biocompatibility and immune-compatibility. In conclusion, HTC has the potential to be used as biomaterials in vivo.

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