Characterization of complement activity in serum of the four sturgeon species, *Acipenser stellatus, A. baeri, A. nudiventris* and *Huso huso* (Chondrostei: Acipenseridae)

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Received: August 2017 **Accept:** November 2017

[Downloaded from ijaah.ir on 2026-09-11]

[DOR: 20.1001.1.2345315.2017.3.2.9.8]

[DOI: 10.29252/ijaah.3.2.98]

Abstract

Serum complement is valuable tool in determining the health status of fish. This study was conducted to examine *in vitro* activity of serum complement as an indicator of innate immunity in four sturgeon species, *Acipenser stellatus, A. baeri, A. nudiventris and Huso huso* (Chondrostei: Acipenseridae). The Effects of different temperatures, concentrations and volumes of serum on alternative complement pathway activity were evaluated using standard haemolytic assays in four sturgeon species (*A. stellatus, A. baeri, A. nudiventris and Huso huso*). Results indicated significant relation between measured hemolysis and concentration, volume and temperature ($p<0.05$). Maximal hemolytic activity was exhibited at 100% serum in four sturgeon species. ($p<0.05$).

Exposure of different volumes of serum from sturgeon species to RBCs exhibited volume dependent hemolysis ($p<0.05$). Maximal hemolytic activity was resulted in 30 µL of serum for all the sturgeon species. The highest hemolytic activity occurred at 4 °C for all the sturgeon species, but the hemolytic activity decreased at 37 °C. The results showed that the alternative complement pathway activity in sturgeon species serum may be affected by temperature, concentration and volume of serum that it caused an increase in non-specific immunity and the resistance to the outbreak of diseases.

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Keywords: Complement, Hemolysis, Immunity, Serum, Sturgeon

Introduction

Complement system activates via the classical, alternative and lectin pathways. (Smith, Azumi & Nonaka 1999).The alternative pathway is

activated non-specifically by various microorganisms. All three pathways lead to phagocytosis and killing of the microorganism (Nakao &Yano 1998). The Red blood cells hemolysis, is a rapid and inexpensive method that is based on the hemolytic disruption of the RBCs by the immunological proteins in the serum (Merchant & Britton 2006a). The fish serum does not distinguish the RBCs from a pathogenic microbe. Alternative complement pathway activity can be measured by the determination of serum hemolytic activity (Merchant & Britton 2006a; Yano, 1992). Sturgeon, family of Acipenseriformes is the most species of fish that are valuable because of caviar and meat production. The culture of these fish under intensive conditions has caused infectious diseases such as vibriosis, yersiniosis and motile *Aeromonas* septicemia that these diseases are economically important. The intensive culture of food fish has led to outbreaks of various bacterial diseases, resulting in annual economic losses to the aquaculture industry. (Hedrick, McDowell, Ahne, Torhy, & Kinkelin 1992; Birstein 1993; Watson, Yun, Groff & Hedrick 1995; Watson, Groff & Hedrick 1998; LaPatra, Groff, Jones, Munn, Patterson, Holt, Hauck & Hedrick 1994; Soltani & Kalbassi 2001; Pridgeon & Klesius 2012). Fish immunity is affected by many parameters in the environment. In several reports, lower values of water temperature cause the suppression of acquired immune system. (Magnadottir, 2006). The innate immunity in the common carp is strongly affected by seasonal changes (Buchtikova, Simkova, Rohlenova, Flajshans, Lojek, Lilius & Hyrsl 2011). In previous studies, the optimum temperature for the assay of the classical complement pathway (CCP) activity of common carp, *Cyprinus carpio,* Porgy, *Pagrus major* and Tilapia, *Tilapia nilotica* by use of sheep red blood cells (SRBC) sensitized investigated with the antibodies of these fishes (Yano, Ando & Nakao 1984; Matsushita, Hirata & Nakao 1985; Yano, Hatayama, Matsuyama & Nakao 1988). Alternative complement pathway is reported to be affected by a range of factors, which include environmental conditions, physiological status and dietary regime (Holland & Lambris 2002). Alternative complement pathway activity was evaluated in Brazilian fish (*Piaractus mesopotamicus*) after being challenged with *A. hydrophila* (Takahashi, Machado, Sabbadin, Sabioni & Criscuolo 2012). This study was undertaken to examine the serum complement system of four sturgeon species, *Acipenser stellatus, A. baeri*, *A. nudiventris* and *Huso huso,* and is the first characterization of an innate immune component in this group of fishes. The aim of this research is an evaluation of the natural immune response in sturgeon species, utilizing the hemolysis of RBC tests, and explores the desirable associations among the outcomes and the natural immunity of the fish.

Materials and Methods

Sample collection

Four sturgeon species, the Stellate sturgeons, *(A. stellatus*) (4 fish), (5.47 ± 0.34 kg), The Siberian sturgeons, *(A. baeri*) (4 fish) (5.27 kg ± 0.87 kg), The Ship sturgeons, *(A. nudiventris*) (4 fish) (5.61 ± 0.96 kg) and the great sturgeons

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Huso huso (4 fish) (10.5 ± 1.56 kg), were captured from a fish farm in Kamfiruz city, Fars province, Iran. Three healthy fish from each species were used. Blood was collected and the sera were separated following centrifugation at 2500 g for 15 min and stored at -20^{o}C until assay.

Serum complement analysis

Whole blood obtained from healthy rabbit was treated with citrate sodium to prevent coagulation. The blood was centrifuged at 3000 g for 10 min and the serum discarded. The RBCs were diluted to 10% (v/v) with PBS pH: 7/2. The fish serum was thawed at room temperature and used for analysis. We evaluated the concentration, volume and temperature dependence of fish serum to unsensitized rabbit RBCs. Agarose hemolytic plates were prepared and wells 3 mm in diameter were cut 14 mm apart into agarose gel in hemolytic plates (Lachmann & Hobart 1978).

Effects of serum concentration, volume and temperature on complement activity

The functionality of the fish serum complement system of proteins was examined using a RBC lysis assay. The sera were mixed for each sturgeon species. Fish serum was diluted to different titers using PBS (25%, 50%, 100%) then 30 µl of serum sample was transferred into each well. Incubation was carried out at room temperature (25 °C) overnight. For the determination of the effect of volume, different volumes of the four sturgeon species serum (10 µl, 20 µl and 30 µl) were used at room temperature (25 °C) overnight. To determine the temperature-dependency of RBC hemolysis, the fish serum was incubated at different temperatures (4 °C, 25 °C, 37 °C) (Buchtikova *et al*., 2011). Each sample was analyzed in quadruplicate so that valid statistical results could be obtained. The zones of hemolysis were clearly visible and were measured manually.

Statistical analysis

All results represent the mean zone of hemolysis beyond well diameter (3mm) in mm ± standard deviation of four independent determinants. Statistical analysis was conducted using SPSS 16.0 for windows package. The statistical comparisons between groups were conducted using analyses of variance with Duncan's post-hoc comparisons, and $p<0.05$ was chosen as the standard for statistical significance.

Results

Incubation of various concentrations of fish serum with RBCs in vitro resulted in hemolytic activity at concentrations 25%, 50% and 100% (v/v). Hemolysis of RBCs by four sturgeon species serum was concentration dependent ($p<0.05$, Figures, 1-4). In this study, maximal hemolytic activity was exhibited at 100% fish serum. This concentration was the best for each species, There was no differences in this regards between species. Exposure of different volumes of serum from sturgeon species (10µl, 20µl and 30µl) to RBCs exhibited volume dependent hemolysis ($p<0.05$, Figures, 5-8). The best volume was 30µl of serum for each species, There was no differences between species. Incubation of serum from sturgeon species with RBCs at different temperatures

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(4 °C, 25 °C, 37 °C) resulted in temperature-dependent hemolysis (p<0.05, Figures, 9-12). The hemolytic activities were similar at (4 °C, 25 °C, 37 °C) for all the sturgeon species. The highest hemolytic activity occurred at 4 °C. The hemolytic activity decreased at 37 °C. Comparison of concentration, volume and temperature dependent hemolysis of RBCs indicated. in four sturgeon species. (p<0.05, Figures, 13-15.

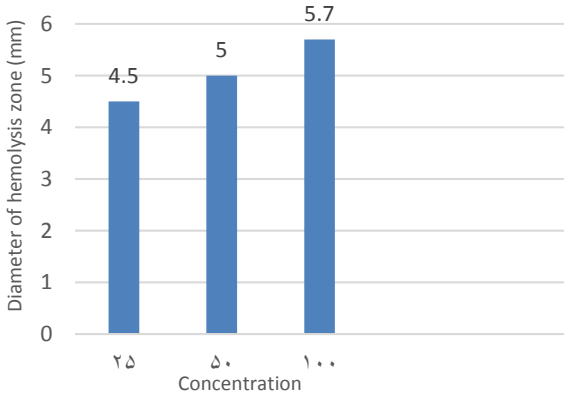

Figure1. Concentration-dependent hemolysis of RBCs by *Acipenser stellatus*. RBCs were incubated with different concentrations of the serum from for *Acipenser stellatus*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, p<0.05).

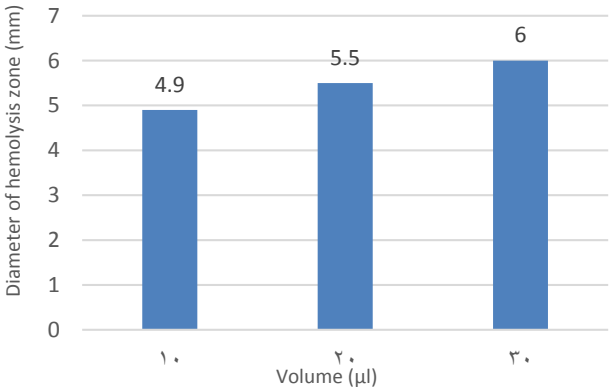

Figure 2. Volume-dependent hemolysis of RBCs by serum of *Acipenser stellatus*. RBCs were incubated with different volumes of the serum from for *Acipenser stellatus*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, p<0.05).

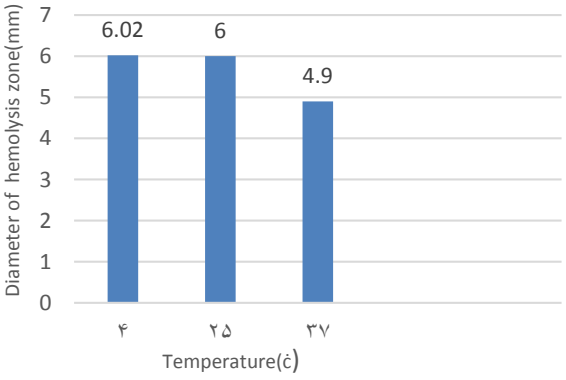

Figure 3. Temperature-dependent hemolysis of RBCs by serum of *Acipenser stellatus*. RBCs were incubated with 100% fish serum at different temperatures. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, p<0.05).

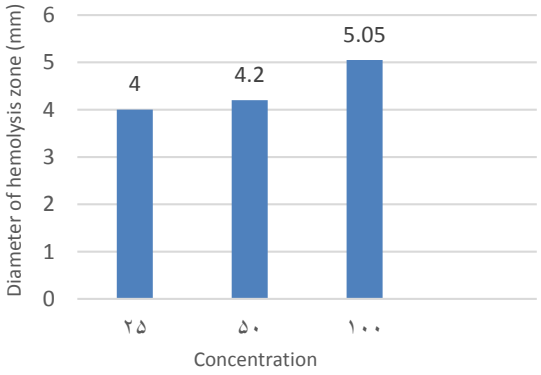

Figure 4. Concentration-dependent hemolysis of RBCs by *Acipenser baeri*. RBCs were incubated with different concentrations of the serum from for *Acipenser baeri*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, p<0.05).

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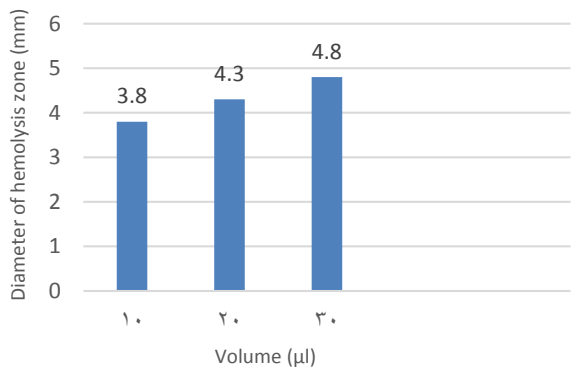

Figure 5. Volume-dependent hemolysis of RBCs by serum of *Acipenser baeri*. RBCs were incubated with different volumes of the serum from for *Acipenser baeri*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, $p<0.05$).

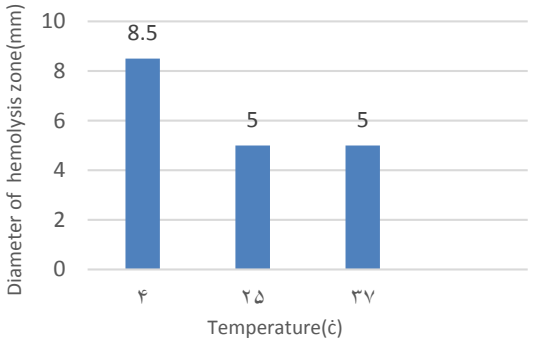

Figure 6. Temperature-dependent hemolysis of RBCs by serum of *Acipenser baeri*. RBCs were incubated with 100% fish serum at different temperatures. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, $p<0.05$).

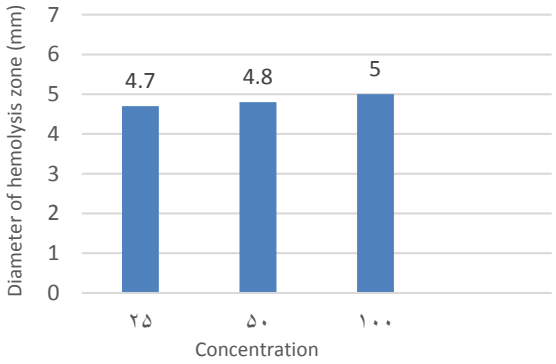

Figure 7. Concentration-dependent hemolysis of RBCs by *Acipenser nudiventris*. RBCs were incubated with different concentrations of the serum from for *Acipenser nudiventris*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, $p<0.05$).

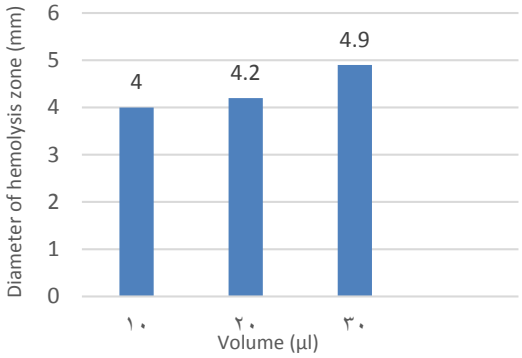

Figure 8. Volume-dependent hemolysis of RBCs by serum of *Acipenser nudiventris*. RBCs were incubated with different volumes of the serum from for *Acipenser nudiventris*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, $p<0.05$).

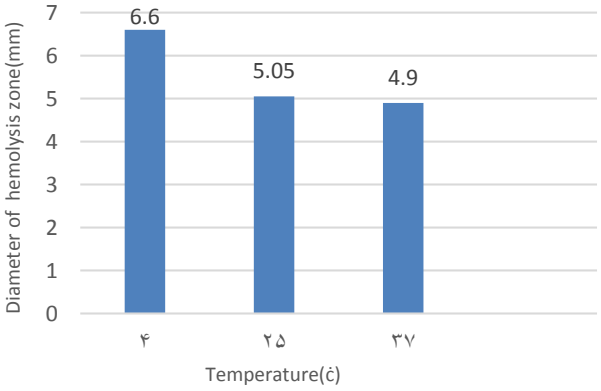

Figure 9. Temperature-dependent hemolysis of RBCs by serum of *Acipenser nudiventris*. RBCs were incubated with 100% fish serum at different temperatures. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, $p<0.05$).

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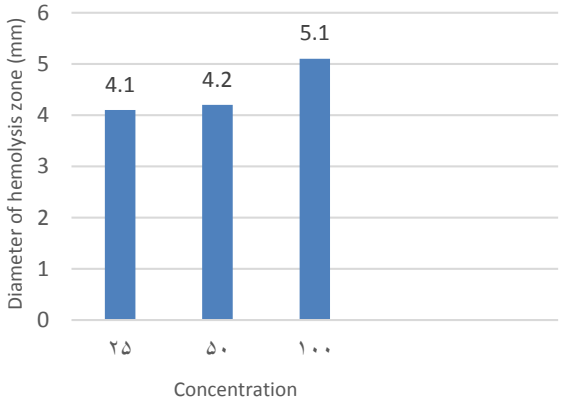

Figure 10. Concentration-dependent hemolysis of RBCs by *Huso huso*. RBCs were incubated with different concentrations of the serum from for *Huso huso*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, $p<0.05$).

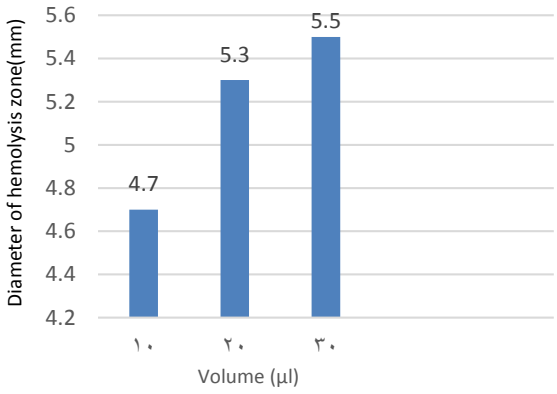

Figure 11. Volume-dependent hemolysis of RBCs by serum of *Huso huso*. RBCs were incubated with different volumes of the serum from for *Huso huso*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences with control group, $p<0.05$).

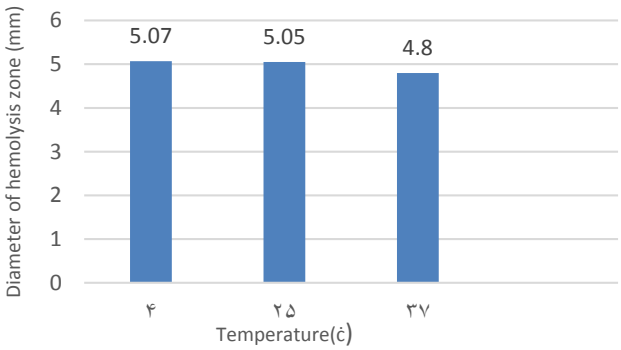

Figure 12. Temperature-dependent hemolysis of RBCs by serum of *Huso huso*. RBCs were incubated with 100% fish serum at different temperatures. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations. (significant differences, $p<0.05$).

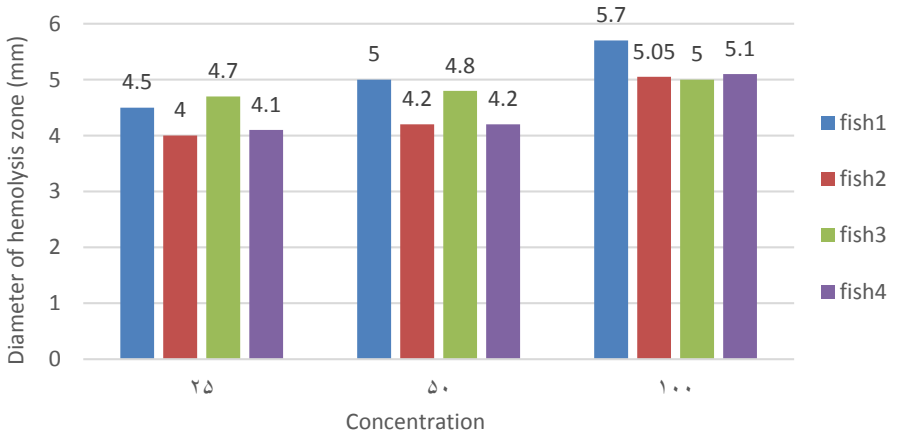

Figure 13. Comparison of concentration-dependent hemolysis of RBCs by four sturgeon species. RBCs were incubated with different concentrations of the serum. Data showed as Mean ± SD. (significant differences, $p< 0.05$). (fish 1*: Acipenser stellatus*, fish 2*: A. baeri*, fish 3*: A. nudiventris,* fish 4 *Huso huso*).

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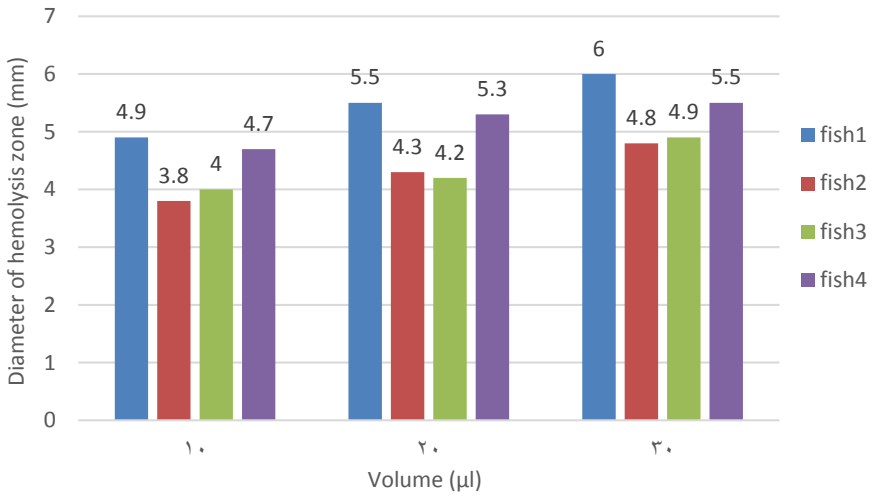

Figure 14. Comparison of volume -dependent hemolysis of RBCs by four sturgeon species, RBCs were incubated with different volumes of the serum. and Data showed as Mean ± SD. (significant differences, p<0.05) (fish 1*: Acipenser stellatus*, fish 2*: A. baeri*, fish 3*: A. nudiventris*, fish 4 *Huso huso*).

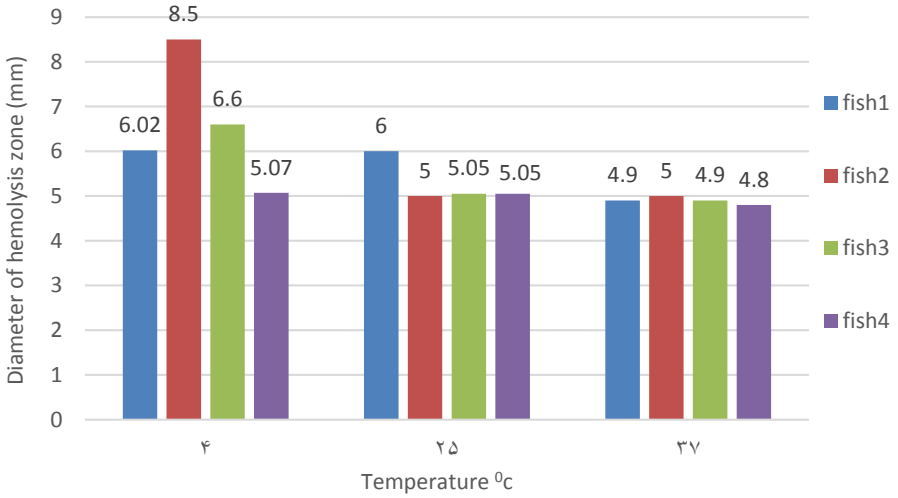

Figure 15. Comparison of temperature -dependent hemolysis of RBCs by four sturgeon species, *Acipenser stellatus, A. baeri, A. nudiventris a*nd *Huso huso.* RBCs were incubated with different temperatures of the serum. Each sample was analyzed in quadruplicate and Data showed as Mean ± SD. (significant differences, p<0.05) (fish 1*: Acipenser stellatus*, fish 2*: A. baeri*, fish 3*: A. nudiventris*, fish 4 *Huso huso*).

Discussion

Few studies have extensively assessed the complement activity of fish, particularly sturgeon, in response to environmental conditions and physiological status. Hemolytic assays are used to assess function of the complement (Kirschfink & Mollnes 2003). The third complement component (C_3) is a central protein of the complement system. The fish and mammals C_3 genes experience different evolutionary patterns for their distinct living environments. C_3 in mammals possess 1 isoform and fish multiple such as 3 in trout (*Oncorhynchus mykiss*) and medaka (*Oryzias latipes*), 5 in seabream (*Sparus aurata*) and carp (*C. carpio*), and 3 loci coding for 3 isoforms in zebrafish *(Danio rerio)* (Gongora, Figueroa &

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Klein 1998; Kuroda, Naruse, Shima, Nonaka, Sasaki & Nonaka 2000; Nakao, Obo, Mutsuro & Yano 1997; Nakao, Mutsuro, Obo, Fujiki, Nonaka &Yano 2000; Sunyer, Zardakis, Sahu & Lambris 1996; Sunyer, Tort & Lambris 1997, Meng, Sun, Liu, Wang, Xu &Wang 2012). Fish immunity is affected by many parameters in the environment, temperature being considered as the leading factor. Difference between fish and mammals is the temperature required for the complement activation (Sakai, 1981). Fish complement has its highest activity at 15-26°C, while the optimum temperature for mammals complement is 37°C (Ingram, 1987). The lowest complement activity in the serum of gilthead sea bream (*Sparus aurata*) were recorded in the coldest months and the highest complement activity were observed in the beginning of autumn when water temperatures reached the maximal values (Hernandez& Tort 2003). Buchtikova. *et al*., (2011) detected the highest activity of alternative complement pathway in the serum of common carp (*Cyprinus carpio*), in spring, which decreased in autumn and the lowest values were found in winter and in summer. Nakao & Yano (1988) reported that the classical complement pathway (CCP) of rainbow trout *(Oncorhynchus mykiss)* had highest hemolytic activity at 20-25 °C. Other results have been reported that classical complement pathway (CCP) activity of common carp *C. carpio*, Porgy *P. major* and Tilapia nilotica, *(Oreochromis niloticus*) showed maximum activity of CCP at 25°C (Yano *et al*., 1984; Matsuyama *et al*., 1985; Yano *et al*., 1988). Yektaseresht, Gholamhosseini, Janparvar& Salighehzadeh 2016; Yektaseresht, Gholamhosseini & Janparvar 2017) reported that the alternative complement pathway (ACP) in common carp, *C. carpio* and koi carp, *C. carpio koi* had highest hemolytic activity at 25 °C. Takahashi *et al*., (2012) showed that alternative complement pathway (ACP) activity in the serum of pacu (*Piaractus mesopotamicus*) was concentration dependent. In the different serum dilutions, the dilution of 1:10 was chosen due to its effectiveness for determining the hemolysis. Yektaseresht *et al*., (2016, 2017) also showed that maximal hemolytic activity was at the highest amount of blood serum in common carp, *C. carpio* and koi carp, *C. carpio koi*. The current study provides valuable information regarding the alternative complement activity of the *A. stellatus, A. baeri, A. nudiventris and Huso huso*. The results presented in this study show that four sturgeon species serum complement activity occurs in a volume, concentration, and temperature-dependent manners. In four sturgeon species, The highest hemolysis occurred at 4°C. Maximal hemolytic activity was exhibited at the highest amount of blood serum (100% fish serum). In conclusion, alternative complement activity of the *A. stellatus, A. baeri, A. nudiventris and Huso huso* may be affected by concentration, volume and temperature that provides useful data for immunological studies and to further our knowledge of the mechanisms of biology of fish.

Acknowledgements

We acknowledge the research vice chancellors of the Shiraz University for financial supporting of this project.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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[DOR: 20.1001.1.2345315.2017.3.2.9.8]

[DOI: 10.29252/ijaah.3.2.98]

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[DOR: 20.1001.1.2345315.2017.3.2.9.8]

[DOI: 10.29252/ijaah.3.2.98]

ارزیابی فعالیت کمپلمان در سرم چهار گونه از ماهیان خاویاری: اوزون برون (*Acipenser stellatus*)، سیبری (*Acipenser baeri*)، شیپ (*Acipenser nudiventris*) و فیل ماهی (*Huso huso*)

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چکیده

ارزیابی فعالیت کمپلمان سرم به عنوان یک ابزار با ارزش برای تشخیص وضعیت سلامت در ماهی محسوب می‌شود. مطالعه حاضر به منظور بررسی فعالیت کمپلمان سرم به عنوان یک شاخص ایمنی ذاتی در چهار گونه از ماهیان خاویاری ازون برون (*Acipenser stellatus*)، سیبری (*Acipenser baeri*)، شیپ (*Acipenser nudiventris*) و فیل‌ماهی(*Huso huso*) در شرایط آزمایشگاهی انجام شد. تأثیر دما، غلظت و حجم سرم بر فعالیت مسیر آلترناتیوکمپلمان با استفاده از روش‌های همولیتیک استاندارد در چهار گونه ماهی خاویاری مورد سنجش قرار گرفت. نتایج نشان داد که ارتباط معنی‌داری بین غلظت، حجم و دما با میزان همولیز گلبول‌های قرمز وجود داشت ($P<۰/۰۵$). بیشترین میزان همولیز در غلظت ۱۰۰ درصد از سرم چهار گونه ماهی خاویاری دیده شد. حجم‌های مختلف (۱۰،۲۰،۳۰ میکرولیتر) سرم ماهیان خاویاری ارتباط معنی‌داری با میزان همولیز نشان دادند. ماکزیمم میزان همولیز در حجم ۳۰ میکرولیتر از سرم چهار گونه ماهی خاویاری نتیجه شد. ($P<۰/۰۵$). حداکثر میزان فعالیت همولیتیک سرم در چهار گونه ماهی خاویاری در دمای ۴ درجه سانتی‌گراد اتفاق افتاد، ولی میزان همولیز در دمای ۳۷ درجه سانتی‌گراد کاهش پیدا کرد. نتایج به دست آمده نشان می‌دهد که فعالیت مسیر آلترناتیو کمپلمان در سرم ماهیان خاویاری ممکن است تحت تأثیر دما، غلظت و حجم سرم قرار گرفته که این سبب افزایش پاسخ ایمنی غیراختصاصی و ایجاد مقاومت به شیوع بیماری‌ها می‌گردد.

کلمات کلیدی: کمپلمان، همولیز, سرم، ایمنی، ماهی خاویاری.

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[DOR: 20.1001.1.2345315.2017.3.2.9.8]

[DOI: 10.29252/ijaah.3.2.98]