Science Arena Publications
Specialty Journal of Biological Sciences
Available online at www.sciarena.com
2017, Vol, 3 (1): 1-6

Evaluation of the effect of temperature, concentration and volume of serum complement on alternative complement pathway activity in koi carp (*Cyprinus carpio koi*)

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Abstract: The aim of this study was to estimate the effect of concentration, volume and temperature of koi carp (*Cyprinus carpio koi)* serum complement on unsensitized rabbit red blood cell hemolysis. The complement system is known to be a critical component of innate immunity. Serum complement is valuable tool in determining the health status of fish. The alternative pathway activity of complement system can be measured by serum hemolytic activity activated in foreign red blood cells. The zones of hemolysis were clearly visible and were measured manually. The results indicated that Serum concentrations of 12.5% v/v produced 3.9±0.05mm, 25%, 4.1±0.115mm, 50%, 4.9±0.05 mm and 100%, 6.8±0.4 mm hemolysis. 10µL volume of serum resulted in 5.25±0.28mm, 20µl, 6.1 ±0.23 mm and 30µL 6.7±0.25mm hemolysis. Incubation of sera at 4°C produced 6±0.08mm, 25°C, 6.25±0.19mm but at 37°C 5.8±0.25mm hemolysis. From the result of the present study, we concluded that the alternative complement pathway activity in *Cyprinus carpio koi* serum could be affected by concentration, volume and temperature. This is the first report on the innate immune activity of *Cyprinus carpio koi*, and provides useful data for future assessments of fish health.

Key words*: Cyprinus carpio koi*, complement, hemolytic assay, serum.

INTRODUCTION

The complement system is one of the central immune responses in fish. The complement system, is composed of about 35 proteins in blood that can be activated by three pathways: classical pathway, activated by antigen-antibody complex; alternative pathway, activated by molecules of surface microorganisms; and lectin pathway, activated by bacterial surface carbohydrate (Holland & Lambris, 2002). The alternative pathway is activated non-specifically by various microorganisms. All pathways generate factor C3, which has been described from teleost species (Nakao and Yano, 1998). The RBCs hemolysis assay, used for humans in the clinical setting has recently been adapted to measure the innate immune activity of fish (Lachmann et al., 1978). The alternative pathway activity of complement system can be measured, among other techniques, by the determination of serum hemolytic activity, when this pathway is activated by foreign red blood cells (Yano, 1992). This analysis can be used to evaluate the effects of several factors such as infections, environmental impact and nutrition on the lytic activity of complement system (Holland & Lambris, 2002). Common carp, *Cyprinus carpio*, an economically fish species cultivated mainly in Asia and Europe. It is a

warm water freshwater fish species that is native to Asia. It is cultivated commercially in other parts of the world, including Australia and South America, because of its fast growth rate, facile cultivation and high feed efficiency ratio (Cao et al., 2013; Tokur et al., 2006). More recently, in the early 19th century, the Japanese ornamental carp (koi), a colored variant of the common carp, was developed in Japan, as documented by breeders in the Niigata. koi carp (*Cyprinus carpio koi*) is an ornamental strain of common carp *(Cyprinus carpio)* and differ widely in color and hues, color pattern types and orientation, scale types and fin length (Balon, 1995; Gomelsky, 2011). The aim of this study was to investigate the effect of concentration, volume and temperature of serum complement of *Cyprinus carpio koi*, on alternative pathway activity to examine the possible relationships between the results and the mechanisms of immunity available to fish.

Materials and Methods

Sample collection

Three healthy koi carp *Cyprinus carpio koi* were obtained from Department of Aquatic Animal Health and Diseases, School of Veterinary Medicine, Shiraz University, Iran. Blood was taken and allowed to clot at room temperature and then centrifuged at 2500×*g* for 15 min. The serum was removed and pooled for subsequent 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 plasma discarded. The rabbit red blood cells were resuspended in phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 3000×g. After one more PBS resuspension and centrifugation, the RBCs were diluted to 10 % (v/v) with PBS (Lachmann et al., 1978).

Serum complement analysis

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 RBCs. For preparation of hemolytic plates barbital buffer (5.1 ml) was mixed with 2% agarose (4 ml) at 56°C. This mixture was then cooled to 45°C and mixed with 0.5 ml of a 10% suspension of rabbit erythrocytes which had previously been washed with PBS buffer. The final mixture (10 ml) was poured onto plates. Wells (diameter, 3 mm) separated by 14 mm were cut into the agarose (Lachmann et al., 1978). Fish serum was diluted to different titers using PBS (12.5%, 25%, 50%, 100%) then 30 µL of each serum sample was transferred into each well. Incubation was carried out at room temperature (25°C) overnight. Another assay was performed to evaluate the effect of volume on the complement system of *Cyprinus carpio koi* serum, different volumes of serum (10, 20, 30µL) were used at room temperature (25°C) overnight. To determine the temperature-dependency of RBC hemolysis, the fish serum was incubated at different temperatures (5-35°C). The zones of hemolysis were clearly visible and were measured manually.

Statistical analysis

Each sample was analyzed in quadruplicate so that valid statistical results could be obtained. Any test value with P value less than 0.05 was considered significant. 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.

Results

Incubation of various concentrations of fish serum with RBCs in vitro resulted in hemolytic activity at concentrations 12.5 % (3.9± 0.05mm), 25% (4.1±0.115mm), 50% (4.9±0.05mm) and 100% (6.8±0.4mm). In this study, maximal hemolytic activity was exhibited at 100% fish serum. Hemolysis of RBCs by *Cyprinus carpio koi* serum was concentration dependent (Figure.1, $p<0.05$). Exposure of different volumes of serum from *Cyprinus carpio koi* to RBCs resulted in volume-dependent hemolysis (Figure. 2, $p<0.05$). 10µL of serum resulted in 5.25±0.28mm. Increased volumes, 20 and 30µL of serum produced 6.1±0.23mm and 6.7±0.26mm

hemolysis, respectively. Incubation of fish serum from *Cyprinus carpio koi* with RBCs at different temperatures showed temperature-dependent hemolysis. Hemolysis zone at 4°C 6±0.08mm was exhibited. The hemolytic activity increased at 25°C, 6.25±0.19mm. However, the activity at 37°C decreased to 5.8±0.25, (Figure. 3, $p<0.05$).

Discussion

Hemolytic assays are used to assess function of the complement (Kirschfink and Mollnes, 2003). Hemolytic activity of complement in fish is much higher than mammals (Yano, 1996). C3 is the central component in the complement system where in mammals possess 1 isoform and fish multiple such as 3 in trout (*Oncorhynchus mykiss*) and medaka (*Oryziaslatipes*), 5 in seabream (*Sparus aurata*) and carp (*Cyprinus carpio*), and 3 loci coding for 3 isoforms in zebrafish (Daniorerio) (Gongora et al., 1998; Kuroda et al., 2000; Nakao et al., 1997, 2000; Nonaka et al., 1985; Sunyer et al., 1996, 1997). The classical complement pathway (CCP) of rainbow trout *Salmo gairdneti* had highest hemolytic activity at 20-25°C (Nakao et al., 1988). Classical complement pathway (CCP) activity of common carp *Cyprinus carpio*, porgy *Pagrus major* and tilapia *Tilapia nilotica* showed maximum activity of CCP at 25°C (Yano et al., 1984; Matsuyama et al., 1985; Yano et al., 1988). We found alternative complement activity of *Cyprinus carpio koi* serum at 12.5% showed some hemolysis increasing in 25% and 50% and maximum at 100%. Exposure of different volumes of serum from *Cyprinus carpio koi* to RBCs resulted in volume-dependent hemolysis. The hemolysis of RBCs by *Cyprinus carpio koi* serum in vitro depended on the temperature at which it was incubated. The highest hemolytic activity occurred at 25°C.

Conclusion

From the results of the present study, we observed that the alternative complement hemolytic pathway activity of the *Cyprinus carpio koi* serum complement may be affected by concentration, volume and temperature. In the present study, concentration- and volume-dependent assays were conducted at room temperature. Future studies should compare concentration- and volume-dependent analysis at various temperatures. This study provides useful data for comparative immunological studies and to further our knowledge of the mechanisms of immunity of fish.

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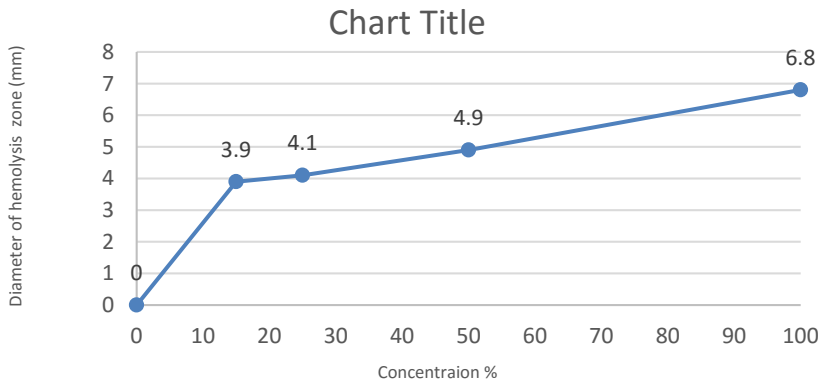

Figure 1: Concentration-dependent hemolysis of RBCs by koi carp *Cyprinus carpio koi*. RBCs were incubated with different concentrations of the serum from for *Cyprinus carpio koi*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations

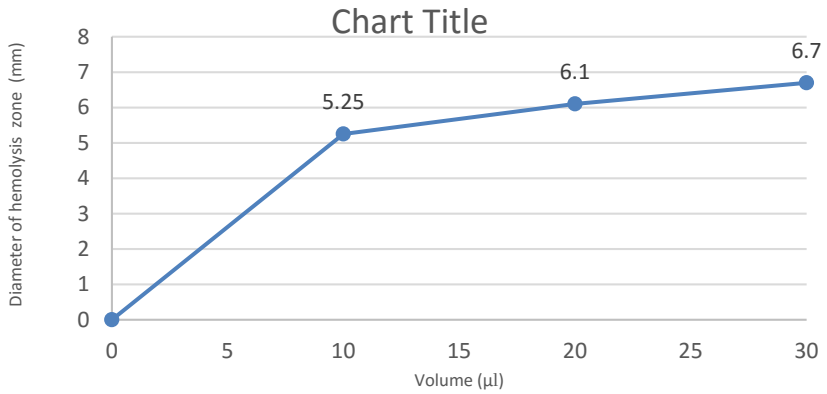

Figure 2: Volume-dependent hemolysis of RBCs by serum of *Cyprinus carpio koi*. RBCs were incubated with different volumes of the serum from for *Cyprinus carpio koi*. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations

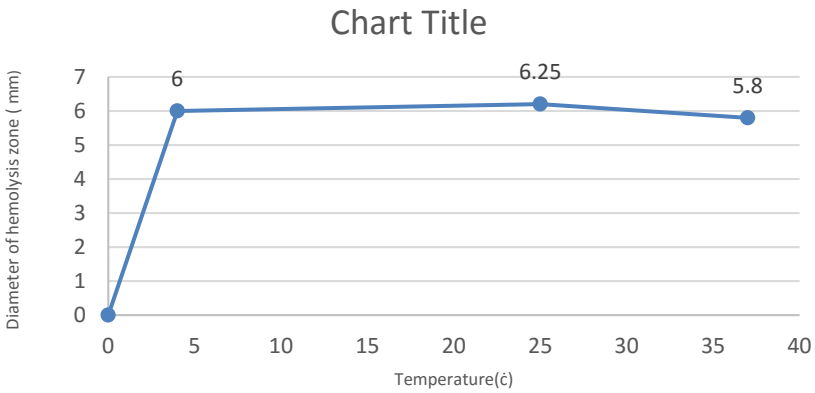

Figure 3: Temperature-dependent hemolysis of RBCs by serum of *Cyprinus carpio koi*. RBCs were incubated with 100% fish serum at different temperatures. Each sample was analyzed in quadruplicate and the results represent the mean ± standard deviations