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Short Communication

Influence of temperature, concentration and volume of serum on alternative complement pathway activity in European pond turtle (*Emys orbicularis*)

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ABSTRACT:

Little is known about complement system as a component of innate immunity of the ectothermic vertebrates such as turtles. Serum complement is a valuable tool in determining the health status of turtles. In this article, the activation of alternative complement pathway of European pond turtle *(Emys orbicularis)*, using standard haemolytic assays was done. Effect of concentration, volume and temperature of serum complement of *E. orbicularis,* on unsensitized rabbit red blood cell hemolysis was measured. Serum concentrations of 25% v/v produced 4.17±0.23mm, 50%, 5.05±0.057mm, and 100%, 6.25±0.5 mm hemolysis. 10µL volume of serum resulted in 4.9±0.05mm, 20µl, 5.4±0.52mm and 30µL 6.2±0.19mm hemolysis. Incubation of sera at 5-15°C produced 5.02±0.05mm, 25°C, 5.17±0.095mm and at 35°C produced 6±0.05mm hemolysis. In this research, clear data about the significant effect of concentration, volume and temperature of serum complement on alternative complement pathway activity in turtles were presented. These data suggested that the increased innate immunity induced by high developmental concentration, volume and temperature strength rise the resistance of turtles to the outbreak of diseases.

Keywords:
Emys orbicularis, serum, complement, reptilian, innate immunity.

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INTRODUCTION

The immune system defends organism against pathogenic diseases. Innate immunity is nonspecific, so, a strong innate answer can improve an antigen-specific response of received immunity. Serum complement operation, an essential part of the innate immune way, is an old mechanism of innate immunity that could be detected in the whole vertebrates and several early invertebrates (Dalmasso *et al.,*1989; Merchant *et al.,* 2006). The complement method, made of approximately 35 proteins in blood in inactive mode that could be activated by three pathways: classic pathway, activated with antigen-antibody system; alternative pathway, activated with molecules of surface microorganisms; and lectin pathway, activated by bacterial surface carbohydrate (Holland and Lambris, 2002). The RBCs hemolysis assay, applied for people in the clinical setting has lately been changed to measure the natural immune action of reptiles (Lachmann and Hobart, 1978). The choice pathway action of complement method could be contained, between different methods, with the purpose of serum hemolytic action, at this pathway is activated with different red blood cells (Yano, 1992). The report could be utilized to assess the influences of many factors such as diseases, environmental impact and food on the lytic action of the complement method (Holland and Lambris, 2002). Turtles apply to the reptilian level and three sorts of freshwater turtles relating to the families Emydidae and Trionychidae are found in Iran (Kami *et al.,* 2006). European pond turtle *Emys orbicularis* belongs to the family Emydidae and are defined as one of the Palearctic vertebrates. It has been described from western North Africa, over utmost of southern, central and Eastern Europe to Asia and the Caspian and Aral seas in the east (Fritz and Havas, 2007). This research is an evaluation of the natural immune answer of *Emys orbicularis*, utilizing the hemolysis of RBCs test, and explores the desirable associations among the outcomes and the natural immunity of the tortoise.

MATERIALS AND METHODS

Collection of blood sample

In this study seven adult turtles belonging to the variety European pond turtle *Emys orbicularis* were captured using earthy fishing basins at Zarghan region (29º46'25"N, 52º43'14"E), Fars of Iran. Blood was taken and made to clot at room temperature and centrifuged at 2500×g for 15min. The serum was then removed and merged for the following investigation. Whole blood obtained from the healthy white New Zealand rabbit were supplemented with citrate sodium to inhibit condensation. The blood was centrifuged at

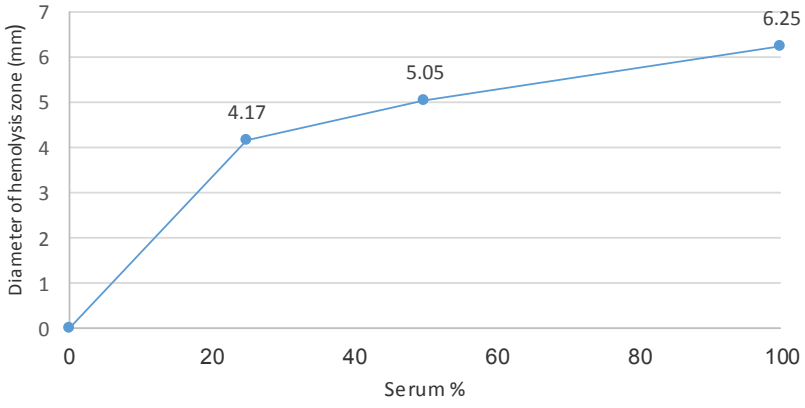

Figure 1. Concentration-dependent hemolysis of RBCs by European pond turtle *Emys orbicularis*

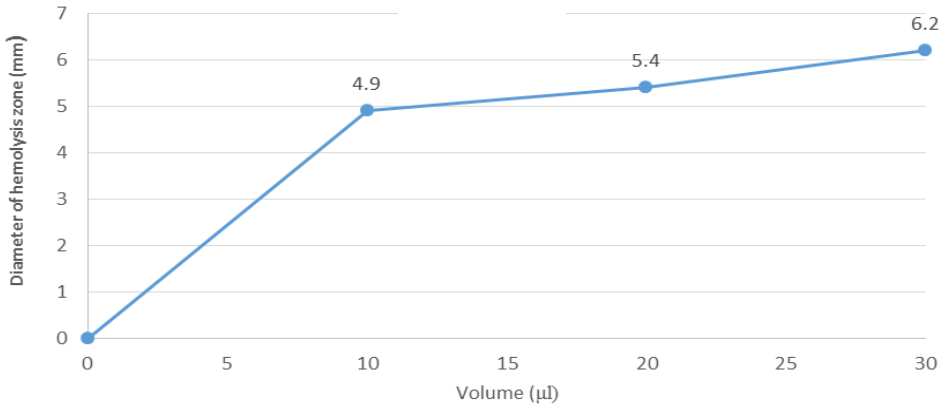

Figure 2. Volume-dependent hemolysis of RBCs by the serum of European pond turtle *Emys orbicularis*.

3000x g for 10 min and the plasma is discarded. The animal red blood cells were suspended in the phosphate-buffered salt (PBS, pH7.4) and centrifuged at 3000×g. One more PBS suspension and centrifugation were carried out and, the RBCs were diluted to 10% (v/v) with PBS (Lachmann and Hobart, 1978).

Serum complement assay

The turtle serum is melted at room temperature and utilized for investigation. We assessed the concentration - volume and temperature dependency of turtle serum to unsensitized RBCs. For the preparation of hemolytic plates, barbital buffer (5.1ml) is combined with 2% agarose (4ml) at 56°C. The mix was next cooled to 45°C and combined with 0.5ml of a 10% suspension of white New Zealand rabbit erythrocytes that had earlier been removed with PBS buffer. The last mix (10ml) is poured on the plates. Wells (diameter, 3mm) separated by 14mm were cut into the agarose gel (Lachmann and Hobart, 1978). The turtle serum is reduced to various titers utilizing PBS (25%, 50%, 100%), then 30 µL of any serum specimen was placed inside the well. Incubation was done at room temperature (25°C) overnight. Different test is performed to assess the effect of volume on the complement method of *Emys orbicularis* serum, various amounts of serum (10, 20, 30µL) were used. To ascertain the temperature dependency of RBC hemolysis, the turtle serum was incubated at different temperatures (5-40°C). These zones of hemolysis are certainly distinct and are aligned manually (Lachmann and Hobart, 1978) (Figure 4).

Statistics and controls.

Specimens were examined in quadruplicate therefore that actual analytical outcomes can be verified and standardized. As a positive control, 30 µL from a solution containing 1% (v/v) Triton X-100 was added until complete hemolysis had been achieved. For negative control only PBS was used. Those control samples are utilized as a judgment for whole another samples. All results represent the mean zone of hemolysis beyond well diameter (3mm) in mm ± standard deviation of four independent determinants. For statistical analysis of data SPSS software version 16 was used.

RESULTS AND DISCUSSION

Incubation of various companies of turtle serum by RBCs in vitro resulted in hemolytic action at concentrations 25% turtle serum (4.17±0.23 mm), 50% (5.05±0.057 mm) and 100% (v/v) (6.25±0.5 mm). In this study, maximal hemolytic activity was exhibited at 100% turtle serum. Hemolysis of RBCs by *Emys orbicularis* serum was concentration dependent ($p<0.05$) (Figure 1).

Exposure of different volumes of serum from *Emys orbicularis* to RBCs exhibited volume dependent

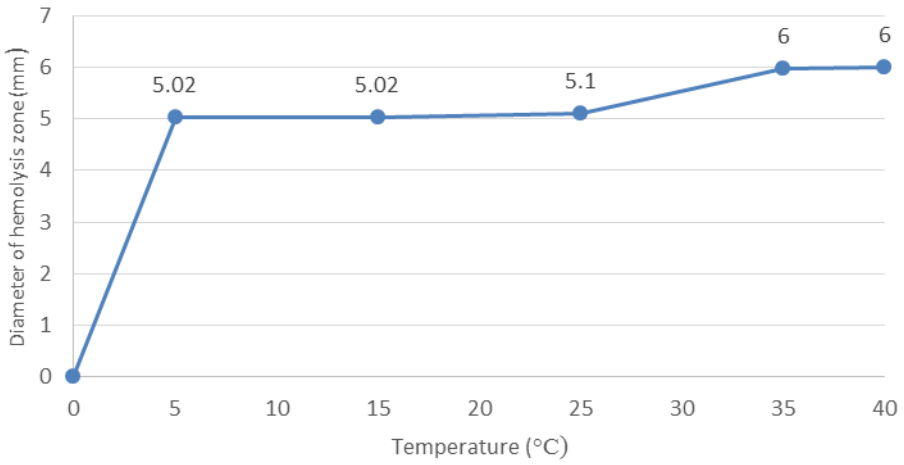

Figure 3. Temperature-dependent hemolysis of RBCs by serum of European pond turtle *Emys orbicularis*.

hemolysis ($p<0.05$). 10µL of serum resulted in 4.9±0.05 mm hemolysis. Increased volumes of 20 and 30 µL of serum produced 5.4±0.52 mm and 6.2±0.19 mm hemolysis, respectively (Figure 2).

Incubation of serum from *Emys orbicularis* with RBCs at various temperatures occurred in the temperature dependent hemolysis ($p<0.05$) (Figure 3). Hemolysis zone at 5°C-15°C was exhibited at 5.02±0.05 mm. The hemolytic action increased at 25°C, 5.17±0.09 mm, and maximum activity was observed at 35°C (6±0 05 mm).

Serum complement is recognized to be a critical part of natural immunity and could be observed in any vertebral (Nair *et al.,* 2000; Smith *et al.,* 1999). The appearance of complement system has been reported in reptiles (Koppenheffer, 1986 and 1987; Sunyer *et al.,* 1998). The results of this study showed that the hemolysis of RBCs could be favorably utilized for the evaluation of the natural immune method of the tutle, *Emys orbicularis*. In this investigation the hemolytic activity of European pond turtle *Emys orbicularis* serum on unsensitized rabbit red blood cells were characterized. We found alternative complement activity of *Emys orbicularis* serum at 25% showed some hemolysis increasing in 50% and maximum at 100%. Expression of various volumes of serum from *Emys orbicularis* to RBCs appeared in volume dependent hemolysis. The hemolysis of RBCs by turtle serum in vitro depended on the temperature at because it was produced. The greatest hemolytic action happened at 35°C. The activity of *Emys orbicularis* serum is similar to that of *Phrynops geoffroanus* (Geoffroys side-necked turtle) (Ferronato *et al.,* 2009). The action is far smaller than that recognized in crocodilian kinds (Merchant *et al.,* 2006; Merchant *et al.,* 2005; Siroski *et al.,* 2010). Previous studies of innate immune system from other reptiles such as the American alligator, *Alligator mississippiensis* is (Daudin, 1801); the Australian freshwater, (Krefft, 1873), *Crocodylus porosus* (Schneider, 1801), crocodiles; and the broad-snouted caiman, *Caiman latirostris* (Daudin, 1801), reported concentration dependent action. 20% American alligator serum showed 90.4% hemolysis action (Merchant *et al.,* 2006). The freshwater turtle, *Phrynops geoffroanus* plasma presented fewer than 10% of the action by 80% of serum concentration. But, a little amount of hemolysis is recognized at 90% tortoise plasma that raised to 25% in 100% plasma (Ferronato *et al.,* 2009). The concentration of serum complement proteins, raised crocodilians than in turtle (Merchant *et al.,* 2005b). The volume-dependent hemolysis was observed in the serum of Komodo dragon (*Varanus komodoensis*), *Amphiuma tridactylum* plasma

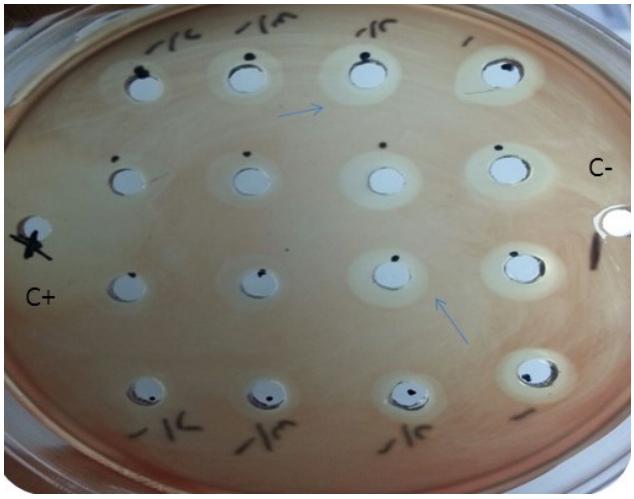

Figure 4. Formation of zones of hemolysis in a hemolytic agarose plate. (Positive control (C+), negative control C-)

and the serum of *Mecistops cataphractus* and *Osteolaemus tetraspis*. The freshwater turtle *P. geoffroanus* exhibited maximal activity at 35ºC (Ferronato *et al.,* 2009). Komodo dragon serum showed maximum activity at 35°C (Merchant *et al.,* 2012). In another kind of crocodilians, the top serum complement action is recognized at 30°C in the freshwater crocodile, 25°C in the saltwater crocodile and 35°C in the American alligator (Merchant *et al.,* 2006). The ability of *Amphiuma tridactylum* plasma to hemolyse RBCs is also temperature dependent, by maximal action at 30°C (Major *et al.,* 2011). It is important to know when the immune system answers to environmental differences to learn the effectiveness of vertebral protection.

CONCLUSION

In this investigation, we discovered the influence of concentration, volume and temperature of serum complement of turtles, *Emys orbicularis* on the immune function. Results showed that the alternative complement hemolytic pathway activity of *Emys orbicularis* serum complement may be affected by concentration, volume and temperature. These data provide new insights into the innate immunity of *Emys orbicularis* against pathogens and reveal the value of serum complement as a component of innate immunity to control microbial infections.

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