The effect of created hemolymph apoptosis on WSSV Gama-vaccinated shrimp, *Litopenaeus vannamei* in WSSV disease control

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Abstract

White spot syndrome virus (WSSV) is the causative agent responsible for huge-shrimp viral epidemics in shrimp farms throughout the world. Our study was aimed to determine the effect of WSSV Gamma-vaccinated *Litopenaeus vannamei* on the occurrence of apoptosis. One thousand and twenty PL_{15} were randomly distributed among 2 treatments and two control groups. Gama-Vaccinated shrimp and non-Gamma-vaccinated ones were our treated and untreated groups. Based on our results significant differences (p<0.05) were observed in survival percent between vaccinated-exposed group (82.33±2.51) and non-vaccinated exposed group (26.00±10.00). It is concluded that apoptosis can be a helpful process in enhancing the immune response in shrimp especially against WSSV.

Keywords: Apoptosis, Hemolymph, WSSV, *Litopenaeus vannamei*

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Introduction

White spot syndrome virus (WSSV) is the causative agent of huge-shrimp viral epidemies, which can reach a cumulative mortality of 100% within several days (Lightner, 1996) in crustacean, particularly in cultured penaeid shrimp (Afsharnasab, 2007). Since its first identification in 1992 (Lightner, 1992), so many farmers have suffered from yield losses due to serious economic damage to the shrimp aquaculture industry worldwide (Flegel and Alday-Sanz, 1998; Lightner, 2011;Afsharnasab, 2012). There is no evidence regarding treatment strategies available against WSSV (Witteveldt *et al.*, 2007) and studies that address the application of chemotherapy as control are few (Park *et al*., 2004). Temperature strategy was studied to decrease the mortality of shrimp when water temperature rose to over 29˚C (Kakoolaki *et al.*, 2011b). An intramuscular injection of inactivated WSSV vaccines in kuruma shrimp produced mixed protection results, while heat-inactivated WSSV did not induce resistance in shrimp (Namikoshi *et al.*, 2004). Apoptosis, a process expressing programmed cell death, is considered as an important hemocyte defense mechanism that prohibits viral replication and eliminates infected cells in multicellular organisms (Everett and Mcfadden, 1999). Apoptosis occurs through energy-dependent biochemical reactions accompanied by distinctive morphological traits, including chromatin denseness, cell plica and fragmentation of the cell body (Kerr *et al.*, 1972). The natural pathway of apoptosis is initiated by signals created within the cells (Wang *et al.*, 2008) Examples of the signals include oxidative stress, viral infection and decline of cell survival factors. All these signals are led through the mitochondria, causing changes in the inner mitochondrial membrane, and ultimately, followed by the release of separated pro-apoptotic proteins from the inter-membrane space of the mitochondria into the cytoplasm (Saelens *et al.*, 2004). According to Jiann-Horng Leu (2013) there are at least two anti-apoptotic proteins in WSSV to prohibit the mechanism in the infected cells. AAP-1, which acts as carapace inhibitor, and WSV222, which is an E3 ubiquitin ligase that prevents apoptosis through the declining of TSL protein (an apoptosis inducer). WSSV also induces the expression of a shrimp anti-apoptosis protein, Pm-fortilin, which can act on Bax to inhibit mitochondria-triggered apoptosis. As other animal pathogens, WSSV induces apoptosis that occurs in cells without WSSV virions (Best, 2008). The first observation of apoptosis was recorded in moribund and infected shrimp, many years ago (Henderson and Stuck, 1999). The objective of our study was to determine the effect of WSSV Gamma-vaccinated *L. vannamei* on the occurrence of apoptosis.

Materials and methods

Animals and experiment protocol

Some post larvae (PL_{12}) shrimp, *L. vannamei* with negative WSSV PCR result were obtained from a research hatchery and transferred to Iran Shrimp Research Center located in Bushehr Province, southern Iran. Shrimp were then acclimated to the optimum conditions (Kakoolaki *et al.*, 2013) for 3 days. One thousand and twenty PL_{15}were randomly collected and distributed among 2 treatments as group 1(Gama-Vaccinated, Exposed to WSSV), group 2(Gama-Vaccinated, Non-Exposed to WSSV), group3 (Non-Vaccinated, Exposed to WSSV) as the positive control and group 4(Non-Vaccinated, Non-Exposed to WSSV) as the negative control.Twelve100 l-fiberglass tanks were used for rearing the shrimp during the experiment (20 days).

Collected infected hemolymph was gamma irradiated to 15 kGy to inactivate virus as vaccine and stored at -70ºC until the experiment.The vaccine with $LD50=1\times10^{5.4}.mL^{-1}$ was added to water (1:20 ratio as a volume of vaccine per 20 g weight of shrimp) of the vaccinated treatments.

The survival rate of shrimp was calculated daily by recording the shrimp mortality.

Preparation of WSSV stock solution

Virus with the titre of $LD_{50}= 1\times10^{5.4}$and code no. WSV/irn/1/2011 prepared in Motamedi Laboratory in Iran was used in challenging our treatments but control groups were left untreated. The mixture was stored at -80°C until use (Motamedi Sedeh *et al.*, 2012). After acclimation, Shrimps viruses were added to water reaching the volume to 10^2.LD50.

Hemolymph examinations

After mortality observation, 0.2mL of Hemolymph was withdrawn from the basement sinus of the second leg of 3 moribund shrimp from each of the replicates in treatments and controls, using 1 mL syringe along with 26 gauge needle. Each syringe was pre-filled with 0.8 mL Alsever solution as anticoagulant (Kakoolaki *et al.*, 2010; Kondo, 2003).

Differentiated Hemocyte Count (DHC)

0.2 mL of withdrawn hemolymph was pre-filled with 0.1 mL fixative. DHC was carried out using a slide; a drop of mixture solution was then placed on it and stained with May-Grundwald Giemsa (MGG) method. The method for fixation and staining of the hemolymph was carried out based on the new methods given in previous studies(Kakoolaki *et al.*, 2011a)

Statistical analysis

One Way-ANOVA and Bonferroni multi-comparison tests were carried out to determine the differentiated hemocyte percents among the whole groups when parametric data were applied but the survival percents of different groups were comparedusing the nonparametric Kruskal- Wallis test.

When the Kruskal-Wallis test showed the significant differences between the groupsthe Mann-Whitney test was used to compare the differences between two independent groups.

Results

The different types of hemocytes with each dedicated percent are listed in Table 1. The percent of semi-granular cellswas the highest in the non-vaccinated- non-exposed group (85.00±1.87) and minimum percent belonged to the vaccinated –exposed group (31.40±3.04). The maximum percent for granular cell belonged to vaccinated–exposed group (51.40±2.07) and the minimum percent was seen in the non vaccinated- non exposed group (9.20±1.92) but in case of hyalinocyte the maximum percent reached was 17.60±2.07 in the vaccinated–exposed group and the minimum percent was observed in the non vaccinated- non exposed group (5.80±0.83). Based on Fig. 3 the hemocytes of vaccinated shrimp were more induced to defend against pathogens. No apoptosis were observed in Fig. 4.

According to Table 1 no severe apoptosis was observed in shrimp hemolymph of group 1.Based on Fig.2, it seems an extensive apoptosis occurredin shrimp hemolymphin the vaccinated group.This type of apoptosis (extensive) can cause a hemolymph disorder and may lead the immune system to a deficientstatus that make shrimp susceptible to WSSV or other probable disease. There were significant differences (K=7.20, $df=2$, p=0.027) among the survival percent-rank orders of groups. According to our results (Table 2) the survival percent of the non vaccinated-exposed group showedthe lowest value and the difference between the survival percent of this group and that of the vaccinated-exposed group was 56%, approximately.This percent indicates that vaccinated shrimp are more resistant to WSSV.

Discussion

Apoptosis can be an effective process against WSSV (Wang *et al.*, 2002). Kakoolaki *et al.* (2012) found out 10µL of active WSSV with the titre of $LD_{50}= 1\times10^{5.4}$ induces apoptosis with very low mortality rate occurring after 20 days while 50 µL of that causes severe mortality beginning 36 hours after injection in specific pathogen free (SPF) shrimp, *L.vannamei*. With regards to WSSV- infected cells, apoptosis can serve as a two-edged blade so that if occurrence of apoptosis is limited, it can eliminate the virions and cell debris but if it is extensive, apoptosis will be harmful for cells and tissues and cause death among the infected hosts (Wang *et al.*, 2008; Leu *et al.*, 2013). Our results lead us to suppose that the extensive type of apoptosis is harmful to imune system against WSSV. In this case, our result is in agreement with the findings of other researchers who showed that if occurrence of apoptosis occurred in the late phase of viral infection,it causes mortality and distribution of infection in the host body and population without triggering inflammatory responses (Everett and Mcfadden, 1999;Best, 2008).

Table 1: The percent of differentiated hemocytes of in different treatments and controls (Mean±SEM, *n*=10).

	Vaccinated. Exposed*	Vaccinated. Non Exposed*	Non Vaccinated. Exposed**	Non Vaccinated. Non Exposed
Semi-granular	31.40±3.04[a]	33.00±4.41[a]	66.00±3.80	85.00±1.87
Granular	51.40±2.07[a]	50.60±2.50[a]	24.80±1.92	9.20±1.92
Hyaline	17.60±2.07[a]	16.40±2.19[a]	9.20±2.58	5.80±0.83

Similar superscripts in each row show no changes observed between values (α=.05)
*This shrimp were accompanying with mild apoptosis
**This shrimp were accompanying with severe or extensive apoptosis

Table 2: The survival percent of different treatments and controls 20 days after inoculation given as Mean±SD, *n*=3.

	Vaccinated.Exposed	Vaccinated.Non Exposed	Non Vaccinated.Exposed	Non Vaccinated.None Exposed
Percent	82.33±2.51	97.33±2.51[a]	26.00±10.00	93.00±1.00[a]

Similar superscripts in each row show no changes between values (α=.05).

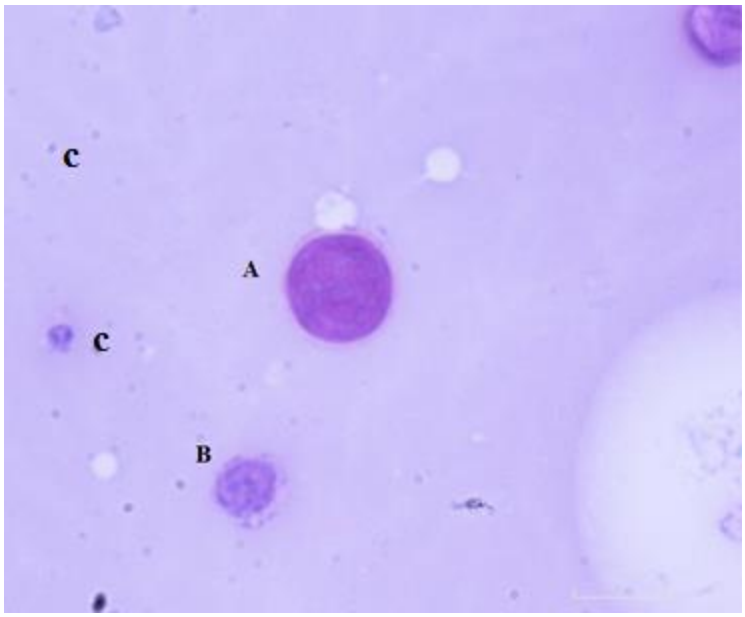

Figure 1: A photograph shows both infected(A) and not infected(B) hemocytes. Mild apoptosis (c) were observed in hemolymph of shrimp group 1 MGG, ×100.

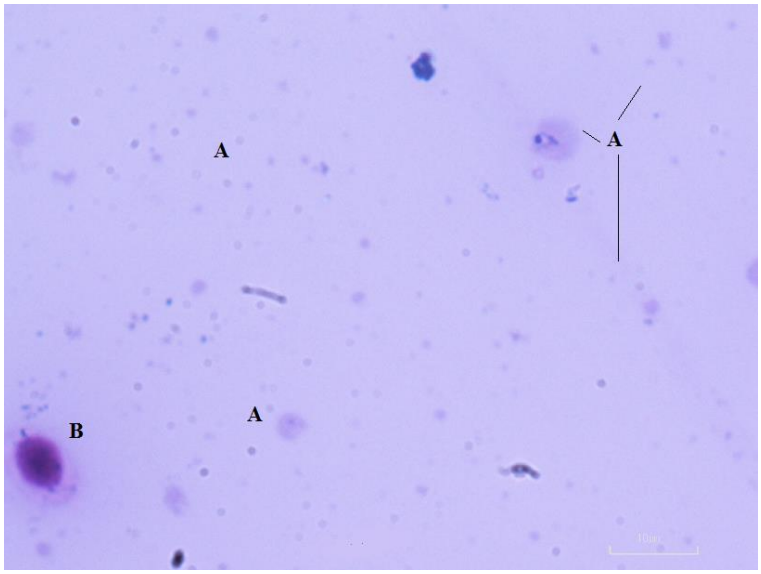

Figure 2: A photograph shows eather extensive apoptosis (A) and not infected hemocyte (B). in hemolymph of shrimp group 3. MGG, ×100.

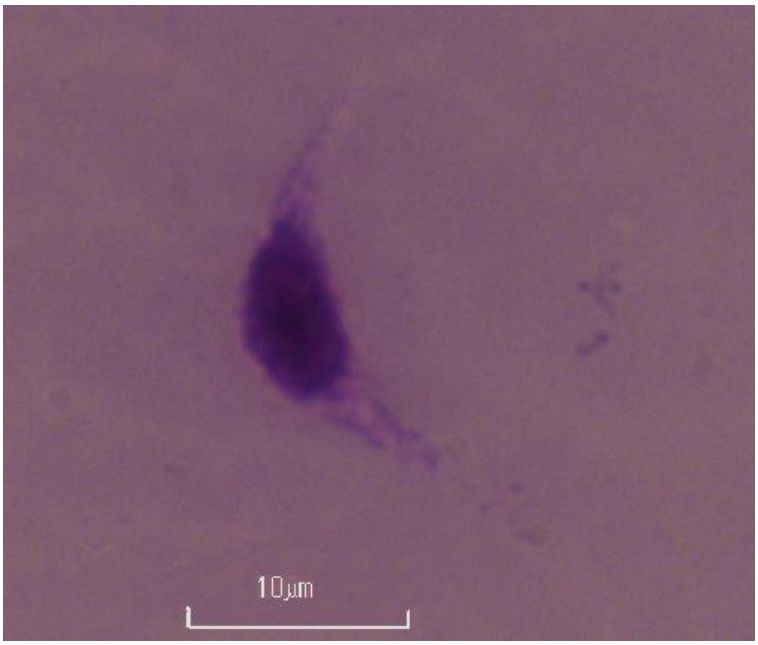

Figure 3: A photograph shows an active hemocyte in group vaccinated-non exposed(group 2) . MGG, ×100.

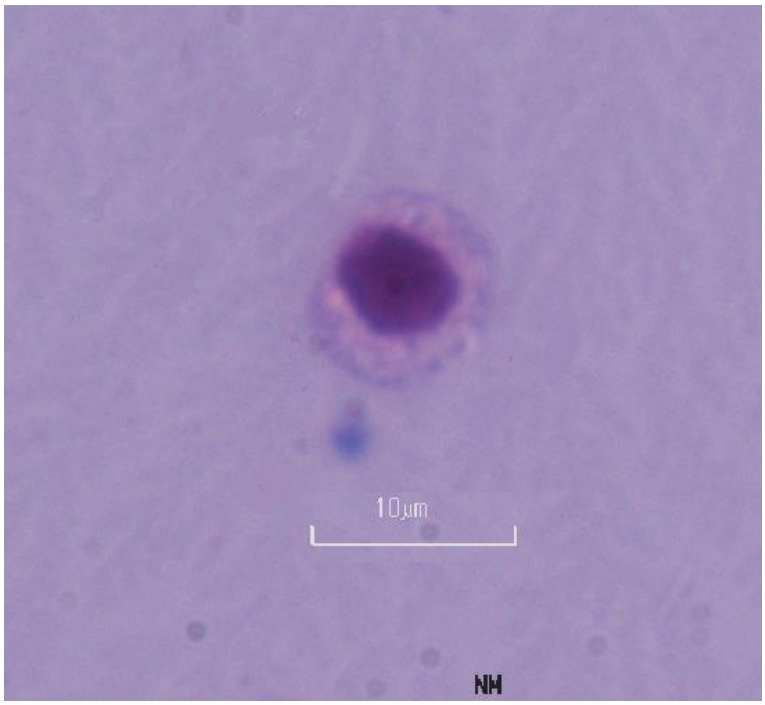

Figure 4: A photograph shows a hemocyte in group non-vaccinated-non exposed (group 4) . MGG, ×100.

Many studies carried outon immunity system showed that shrimp have no acquired but innate immune system, which includes either cellular or humoral factors. Of course, a quasi-immune system has been detected in shrimp that shows which survivors from the last WSSV epidemic can resist the next outbreak so that survival percent reached 94%(Venegas *et al.*, 2000; Witteveldt *et al.*, 2004). This result impliesthe existence of an immune like system in shrimp. Some of these results suggesting the existence of an adaptive immune response in invertebrates, is similar to that observed in vertebrates but it seems the evidence was not sufficient to confirm the existence of an adaptive immune system in shrimp (Wu *et al.*, 2002).

Based on the results of Table 1, semi-granular cells were lesser than that inthe non vaccinated- non exposed group. It seems granular cells and hyalinocytesincreased in percent when exposed to WSSV especially in the vaccinated groups. The most interesting finding was that granular and hyaline cells are more activated against WSSV. Other researchers(Wang *et al.*, 2002) confirmed that WSSV was observed in nucleus and vacuoles of semi-granular cells as well as in vacuoles of granular cells. No viruses were found in hyalinocytes. It seems semi-granular cells as host cellsare more susceptible to WSSV and hyalinocyte and granular cells are more active against WSSV.A mild type of apoptosis mightgive an opportunity to hemocytes to be willing

to go against WSSV. Table 2 simply on higher survival percent in comparison to either none vaccinated groups. This result is accordant with other scientists (Everett and Mcfadden, 1999; Hay and Kannourakis, 2002) that showed apoptosis occurred in the early phase of infection.

It is concluded that apoptosis can be a helpful process as immune function in shrimp specially against WSSV but if it occurred in extensive situation can be harmful for shrimp and lead to huge mortality in shrimp population.

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