Immunological findings in shrimp *Litopenaeus vannamei* exposed to attenuated WSSV vaccine produced by Gamma irradiation

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Abstract

In this research, White spot syndrome virus (WSSV) with $10^{5.4}LD_{50}.mL^{-1}$ inactivated with gamma irradiation at a dose rate of 14-15 $kGy.sec^{-1}$ was used as a vaccine. Three hundred and sixty shrimp of *Litopenaeus vannamei*, medium body weight (MBW) 1.0±0.17g), were prepared and experimentally exposed. They were distributed among four groups, inclusing vaccinated-virus exposed (T1), vaccinated-nonvirus exposed (T2), virus exposed (Positive control as T3) and non-vaccinated-non-virus exposed (Negative control as T4). The mortality percent of T1 started from day 5 and reached the highest (50%) on day 11 and remained constant until end of the experiment. In T2, no mortality was observed until the 3rd day and the maximum mortality (20%) was observed on day 9. On the other hand, no remarkable mortality was recorded for T4 unlike T3 in which the mortality started on day 2 and stopped on day 7. The minimum density of hyalinocyte was calculated in T4 but the maximum one was found in T3 (17.77±0.33) with no significant differences (p<0.05) compared to T2 (16.59±0.96). A significant difference (p<0.05) was observed between the values of Total Haemocyte Count (THC) before and after exposing to virus in T1. Total Protein Plasma (TPP) decreased to 29.03±3.41, while it was higher than 25.96±2.75 in T3 after 7 days. Such results might be responsible for developing resistance against WSSV in gamma irradiant vaccine in *L. vannamei* in comparison to T3 while the cause of mortality in T2 could be due to the lower effectiveness.

Keywords: WSSV, Gamma irradiant, Haemolymph parameters, Mortality rate

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Introduction

White Spot Syndrome Virus (WSSV) is one of the most dangerous pathogens in shrimp farms and has been responsible for a major proportion of wrathful outbreaks and huge economical losses in shrimp culture (Lightner, 1996 ; Flegel, 1997). In 1992-93, it was the first time that WSSV was reported from Southeast Asia and then it spread to several countries throughout the world in the last decade (Chou *et al*., 1995). In Iran, WSSV disease first occurred in cultured shrimp in Khuzestan Province in 2001, followed by other provinces namely Bushehr Province in 2003 and 2005, and it occurred in Sistan and Baluchestan Province in 2005, 2007, 2008, 2011 and 2013. It is interesting that it has not been reported yet from Hormozgan Province located between the two last mentioned provinces in the east of the Persian Gulf (Afsharnasab *et al*., 2014) which might be due to greater level of pond water temperature with no remarkable fluctuation in comparison to the other provinces (Kakoolaki *et al*., 2014). This pathogen is a large DNA virus (Van Hulten *et al*., 2001) and virus particles contain at least five major virion proteins, of which three (VP26, VP24 and VP15) are present in rod-shaped nucleocapsid and two others (VP28 and VP19) reside in envelope. The causative agent is a highly contagious virus and cumulative mortality reaches 100% in rearing shrimp ponds within 3 to 10 days (Escobedo-Bonilla *et al*., 2008 ; Afsharnasab *et al*., 2009). Different strategies have been applied to prevent WSSV outbreak in shrimp farms such as enhancing immunity, stocking specific pathogen free (SPF) postlarvae and ultimately vaccination (Kumar *et al*., 2008). Several types of vaccine such as DNA vaccine, recombinant vaccine (Rout *et al*., 2007 ; Haq *et al*., 2012) and inactivated virus or protein "sub- unit" vaccine (Johnson *et al*., 2008) were investigated to prevent the WSSV. The recombinant proteins vaccine consists of r-VP26 or r-VP28 protein induced resistance against WSSV (Namikoshi, *et al*., 2004; Jha et al. 2006). WSSV envelope proteins (VP19 and VP28) were applied as bacterial expression procedure through injection, oral and immersion vaccines in *P. monodon*. (Witteveldt *et al*., 2004). Westenberg *et al*. (2005) used large dsRNA molecules as well as siRNAs to induce a sequence-independent anti-viral immunity when injected to shrimp. Namikoshi *et al*. (2004) indicated the possibility of vaccination for kuruma shrimp with recombinant proteins against WSSV. Many researchers have reported the development of vaccine against WSSV using glucan (Namikoshi, *et al*., 2004) peptidoglycan and lipopolysaccharide (Itami *et al*., 1998). The preparation of inactivated vaccines which are suspensions of microorganisms exposed to ionized radiation has been reported for bacteria, viruses, and parasites *(*Heidarieh *et al*., 2014). The applied short wavelength electromagnetic rays like χ and γ have high penetrating

ability and the desired characteristic of not imparting radioactivity to the exposed material (Motamedi Sedeh *et al*., 2012). Gamma ray have been widely used by many researchers to inactivate virus, instead of thermal or chemical conventional methods of inactivation. It is known in advance that shrimp and other crustaceans lack a truly adaptive immune response system but have innate immune response systems which contain humoral factors to efficiently recognize and wipe out ‘‘non-self’’ agents such as viruses (Tonganunt *et al*., 2008).

These mechanisms led us to assess the application of a WSSV vaccine using gamma irradiation and evaluate its protective characteristics such as the immunological changes of Total Haemocyte Count (THC), Total Protein Plasma (TPP), Differentiated Haemocyte Count (DHC) and cumulative mortality percent in *L. vannamei* through an experimental infection.

Material and methods

Virus purification

Following the procedure explained by Heidareh *et al.* (2014) and Motamedi Sedeh *et al.* (2012), WSSV (IRWSSVBU1) was isolated from infected *L.vannamei* juvenile tissues, homogenized in TNE buffer (0.05 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.4) and centrifuged at 4000 g for 15 min at 4 °C. The supernatant was filtered through a 0.45-µm Millipore. Finally, one hundred healthy crayfish, *Astacus leptodactylus* were intramuscularly inoculated with 100 µl of the filtrated supernatant. Gill tissues of newly dead crayfish were gathered, homogenized and filtered as mentioned above and the collected supernatant was stored at -70°C prior to experiment.

In order to determine the dilution resulting in 90-100% mortality in *L. vannamei*, the stock solution was diluted using sterile PBS at required concentrations of 10^0-10^5 and then injected intramuscularly to 14 shrimp kept in each group at the aforementioned concentrations. In addition, the virus stock was diluted in steps from 1/2 till 1/32 times in the sterile PBS and injected intramuscularly to *A. leptodactylus.* Ultimately, the LD_{50} of live virus stock sampled from *A. leptodactylus* and *L. vannamei* were calculated to $10^{3.29}$ and $10^{5.35}$ ml^{-1}, respectively using the Karber method. Thirty milliliters of the infected crayfish haemolymph was diluted in the sterile TN buffer (20 mmol L^{-1}Tris-HCl, 400 mmol L^{-1} NaCl; pH 7.4; 1:5 w/v) and centrifuged at 1700 *g* for 10 min at 4°C. The collected supernatant was consequently centrifuged at 112400 *g* for 1 min at 4°C (Beckman, L2-6sB). The pellet of each tube was dissolved in sterile TN buffer.

Vaccine preparation

Discontinuous sucrose gradient from 15 to 45% was prepared and stored at 4°C, overnight. One ml of the dissolved viral pellet was layered in the sucrose gradient tubes and finally, it was

centrifuged at 153200 *g*, 4°C for 2h. Consequently, The visible virus bands were gradually observed and isolated (Poulos *et al*., 2001). A gamma cell instrument Nordian, model 220 with a dose rate of 4.8 $Gy.sec^{-1}$ and activity of 20469 Ci was applied to inactivate the virus (Nuclear Science and Technology Research Institute (NSTRI), Karaj, Iran; 2011). Different doses of gamma ray consisting of 1, 3, 5, 10, 15, 20, 25, 30, and 35 kGy were used to irradiate the viral samples. Three viral samples were subjected to each irradiation gamma dose and the process was performed on frozen samples held on dry ice.

Eventually, virus with titre value of $LD_{50}= 1\times10^{5.4}$ ml^{-1} was calculated with the dose of 15 kGy Gamma irradiation used in this experiment. The LD_{50} was calculated using Van Hultenet *et al*. (2001) and Karber methods (Heidarieh *et al*., 2014).

Experimental design and mortality assessment

Three hundred and sixty shrimp, *L. vannamei*, (MBW=1.0±0.17 g) were collected from quarantine farms and transferred to the Iran Shrimp Research Center located in the south of Iran in Bousher Province, to examine the experimental objective for 14 days. The shrimp were previously screened based on the OIE viral pathogens list, consisting of WSSV, TSV, IHHNV, IMNV, HPV and YHD using the IQ2000 TM nested-PCR kit (GeneReach Biotechnology Corp., Taiwan, China) in order to use healthy shrimp in the experiment. They were maintained in fiberglass tanks (500 L) prefilled with the flow-through aerated and de-chlorinated seawater with the flow rate of 0.5 $L.s^{-1}$, water temperature 27.0±1.0°C, dissolved oxygen 5.0- 5.5 ppm, pH 7.5-7.8 and salinity 28.0±1.0 ppt for 7 days and fed with commercial pellet and *Artemia franciscana* three times a day. Healthy WSSV-free shrimp were divided into four groups including 30 shrimps in each triplicate. All the groups were intramuscularly injected in the sinus situated between the fourth and fifth abdominal segments. The T1 was vaccinated with 4 μg on the 1st day, kept for 7 days before experiment and were then exposed to WSSV. The T2 group of shrimp was similarly vaccinated on the first day of the experiment without exposing to WSSV. The T3 group was only exposed to WSSV at the same time as the Positive control (C+) and the shrimp in T4 were injected only with PBS and named Negative control (C-). Each group was maintained individually in separate areas to prevent probable cross-infection. Viral challenge was performed intramusculary after vaccination identically and the experiment was carried out in triplicate.

Mortality was recorded twice a day (morning and night) and dead shrimps were tested for the presence of WSSV using nested-PCR kit (Gene Reach Biotechnology Corp., Taiwan, China).

Haemolymph sampling

Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment of *L. vannamei*, and prepared as mentioned by Rodriguez *et al.*, (1995). Haemolymph was obtained during days 0, 1, 3, 5, and 7 and transferred to individual gamma plastic tubes for measuring THC (Total Haemocyte Count), TPP (Total Protein Plasma) and DHC (Differentiated Haemocyte Count).

Total Haemocyte Count (THC)

One hundred µl of haemolymph was drawn directly from each animal into a 1.0 ml syringe prefilled with 0.4 ml cold modified Alsever's solution and THC *(Quantity× 10^6)* was measured as mentioned in Jiang *et al.* (2004). The remaining mixture was used for measuring TPP.

Total Plasma Protein (TPP)

TPP (mg mL^{-1}) was measured according to the modified Lowry-method, using bovine serum albumin (BSA) as a standard (Braak, 2002).

Differentiated Haemocyte Count (DHC)

DHC *(%)*was carried out using a slide, a drop of mixture solution and stained with May-Grundwald Giemsa (MGG) following the method of Kakoolaki *et al.* (2011).

Statistical analysis

To compare the variations in the selected variables, TPP, THC and DHC; One Way Analysis of Variance (ANOVA) statistical tests were performed. Subsequently, the mean values of the selected variables were compared pairwise using the Bonferroni test. All tests were undertaken using SPSS 18 software. The statistical analysis was performed to see significant differences in cumulative mortality between the groups. The mean ± SE of the treatments were reported for all parameters measured at the level of $\alpha = 0.05$.

Results

WSSV infection

As Nested PCR results shown in Fig. 1, the samples were used for WSSV stock and viral propagation in crayfish *A. leptodactylus* were infected by WSSV. The viral titration was done using Kerber methods and in 1 ml of solution, a virus count of $10^{5.4}$ was calculated.

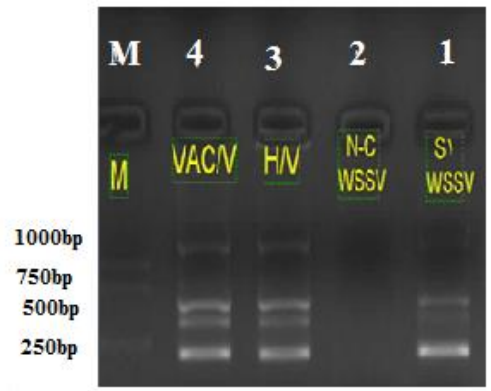

Figure 1: PCR assay of WSSV DNA from 5-pool crayfish (A.leptodactiylus) samples. Lane M: 1kb plus DNA ladder; lane 1: Haemolymph; lane 2: Negative control; lane3: Gill tissue; lane 4: Positive control.

Cumulative mortality

The cumulative mortality showed a significant difference ($p<0.05$) between the shrimps of T1 and that of T3. The cumulative mortality in T3 started on day 3 and total mortality occurred after day 7 of the experiment. On the other hand, the mortality of T1 started from day 5, reached a peak (50%) on day 11 and remained constant until the end of experiment (Fig. 2). In T2, no mortality was seen until the 5th day and the maximum mortality (10%) was observed on day 9. The mortality in this group was lower than that of the other groups (T1 and T3). The mortality in T4 was 3% at the end of experiment. The cumulative mortality in T2 started at approximately 2% and remained constant at 8-9% till the end of experiment.

Analysis of THC

According to the Fig. 3 and Table 1, on day 0, the THC did not show any significant difference between the groups ($p= 0.975$) while on days 1, 3, 5, and 7 significant differences ($p= 0.000$) were observed. Accordingly, the values of THC in T4 and T1 showed the maximum (42.23±0.42) and minimum (20.26±0.25), on day 1, which showed a lower value compared to that on day 0 (39.26±0.40) in T1 but with no significant change in T4 (40.35±0.46). In case of THC calculated for T1, a remarkable difference was observed before and after exposing to virus. The maximum level of THC was obtained from T4 (on days 3, 5 and 7) but the level of THC reached the minimum on the first day in group T1and this descending trend continued up to days 5 and 7. However, the maximum value of THC was obtained on days 3 (16.93±1.90), 5 (15.03±0.27) and 7 (14.96±0.12) in T3.

Analysis of DHC

The DHC (hyaline, semi-granular, granular) of the shrimps among four treatments and controls is given in Table 2 and Fig. 4, in which shows a significant differences ($p<0.05$) among them. The semi-granular cell had a maximum mean value (85.33±0.31) in T4 but this value did not show a significant differences ($p>0.05$) among T3 (46.14±0.56), T1 (46.09±2.09) and T2 (46.01±1.34) groups. In case of granulocyte a notable statistical lower value was considered in T4 (8.36±0.49) compared to the other groups, which reached the maximum level in T2 treatment (40.83±0.70) followed by T1 (37.31±1.13) and T3 (36.08±0.24), with no significant difference ($p>0.05$) between the two former groups. The minimum level of hyalinocyte was calculated for T4 and the maximum one was found for T3 (17.77±0.33) with no significant difference ($p<0.05$) to the T1 (16.59±0.96) slightly greater ($p<0.05$) than that of T2 group (13.15±0.65).

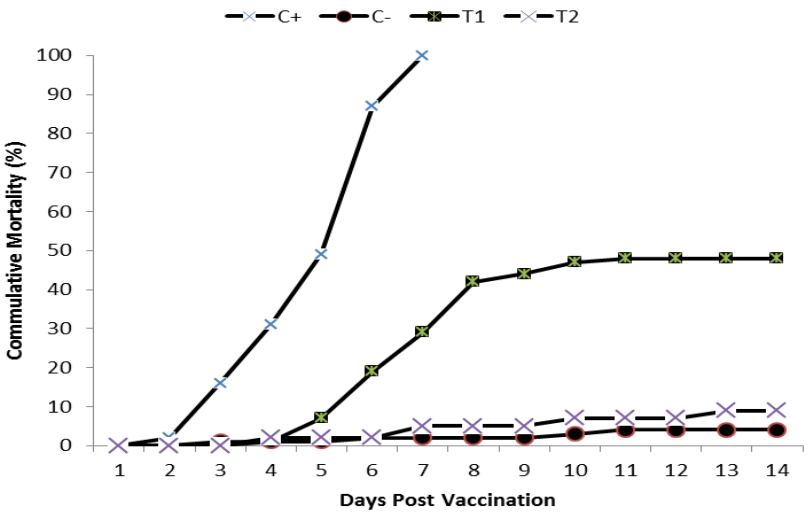

Figure: 2 Cumulative mortality of shrimp vaccinated with gamma irradiant vaccine (T1) compared with the other treatments and controls.

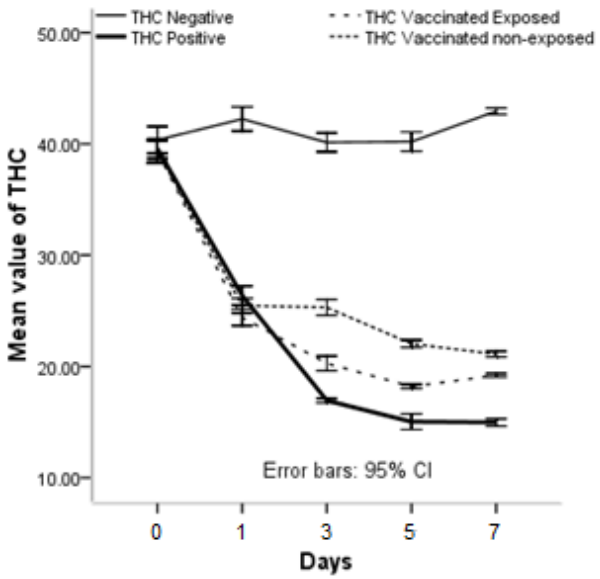

Figure 3: The trends of THC mean values among different treatments.

Table 1: Total Haemocyte Count ($\times 10^6$.mL^{-1}) in different treatments and sampling days (n=6).

	Negative Control	Positive Control	Vaccinated Exposed	Vccinated non-exposed	Sig.
Day 0	40.35±0.46^{a}	39.56±0.33 ^{a}	39.26±0.40 ^{a}	39.33±0.36 ^{a}	.975
Day 1	42.23±0.42 ^{a}	26.36±0.07^{b}	20.26±0.25^{c}	25.46±0.25^{bc}	.000
Day 3	40.13±0.32 ^{a}	16.93±1.90^{b}	39.94±2.82^{a}	25.30±0.27^{c}	.000
Day 5	40.20±0.33 ^{a}	15.03±0.27^{b}	18.20±0.07^{c}	22.03±0.12^{d}	.000
Day 7	42.93±0.11 ^{a}	14.96±0.12^{b}	19.20±0.06^{c}	21.10±0.09^{d}	.000

In each row, similar superscripts show no significant difference (α= .05) among the groups.

Table 2: Differentiated Haemocyte Count (DHC in percent) in different treatments after 7th day (*n*=6).

	Negative Control	Positive Control	Vaccinated Exposed	Vccinated non-exposed	Sig.
Semi Granulocyte	85.33±0.31[a]	46.14±0.56 [be]	46.09±2.09[ce]	46.01±1.34 [de]	.000
Granulocyte	8.36±0.49 [a]	36.08±0.24[be]	37.31±1.13[ce]	40.83±0.70[d]	.000
Hyalynocyte	6.30±0.20 [a]	17.77±0.33[be]	16.59±0.96[ce]	13.15±0.65[d]	.000

In each row, similar superscripts show no significant difference (α= .05) between the groups.

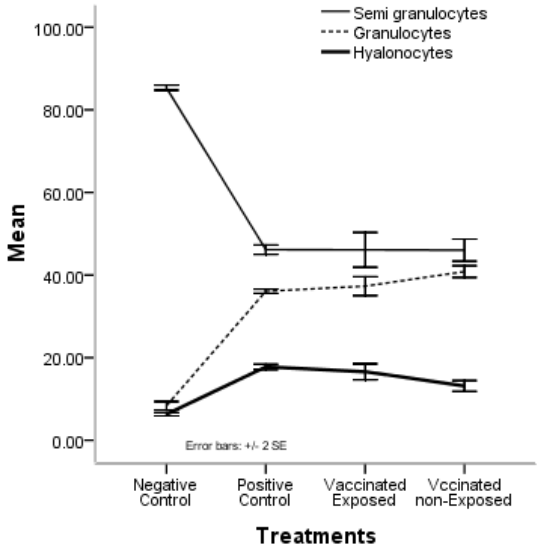

Figure 4: Lines show the status of haemocyte means for different groups.

Analysis of TPP

Table 3 and Fig. 5 showed the TPP or haemolymph protein activity in different treatments and controls in which no significant differences (p=0.975) were observed between the minimum of TPP value (70.37±4.91) in T4 and in other groups, on day 0. A significant variation (p=0.000) was found between values in T4 and that in other treatments but no statistical differences were observed among values in T3, T1 and T2 on day 1 as well as on other sampling days of 3, 5 and 7. The TPP value of the white leg shrimp in T1 was 71.73±0.95 on day 0 with no statistically significant differences (p>0.05) with that in other groups. However there was a sudden decrease in TPP value in T1 on day 1 (50.12±3.99).

Table 3: Total Protein Plasma (TPP) in different treatments and sampling days (*n*=6).

	Negative Control	Positive Control	Vaccinated Exposed	Vccinated non-exposed	Sig.
Day 0	70.37±4.91[a]	71.06±0.69[a]	71.73±0.95[a]	72.33±1.38[a]	.975
Day 1	74.36±3.63	51.47±4.08[a]	50.12±3.99[a]	53.64±0.84[a]	.000
Day 3	76.46±3.48	38.13±1.90[a]	39.94±2.82[a]	44.08±3.98[a]	.000
Day 5	70.43±2.39	27.03±3.34[a]	30.33±3.65[a]	37.17±3.77[a]	.000
Day 7	70.76±2.89	25.96±2.75[a]	29.03±3.41[a]	35.60±3.21[a]	.000

In each row, similar superscripts show no significant difference (α= .05) among the groups.

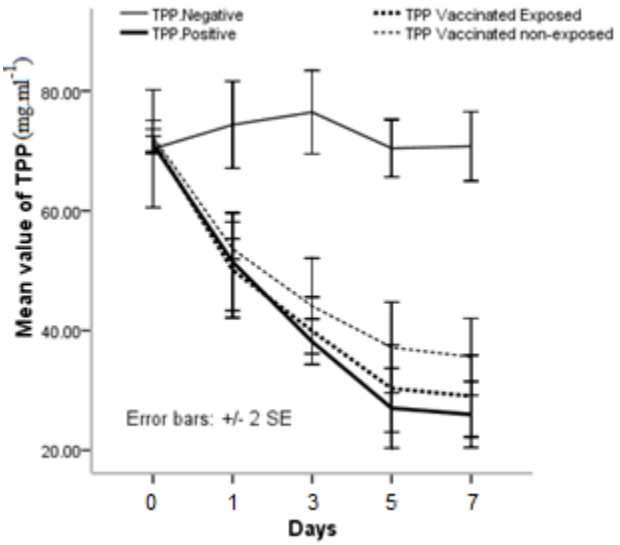

Figure 5: Total amount of TPP (mgml^{-1}) in different experiments and controls.

Discussion

The potential application of Gamma irradiated WSSV intramuscular injection to white leg shrimp *L. vannamei* to increase disease resistance was explored in this study. US Food and Drug Administration (FDA) on 2 December 1997 approved the use of Gamma irradiation (Raffi 1998). Moore (2012) explained that enveloped and non-enveloped viruses, RNA, and DNA viruses, including HIV, Porcine Parvovirus (PPV) and Bovine Viral Diarrhea Virus (BVDV) can be inactivated with a low dose of gamma irradiation.

In our study, the positive control white leg shrimp, *L. vannamei* was assayed and the mean cumulative mortality rate after 7 days exposure contamination with WSSV virus was calculated as 100%. Cumulative mortality (%) of *P. monodon* treated with inactivated vaccine with immune-stimulants applied after 30 days post vaccination (dpv) was less effective than that of 60 dpv, which was 38-45% against 70-100% on the 14th day (Yogeeswaran *et al.*, 2012). The vaccination indicated a significant lower mortality percent in shrimp vaccinated with pVP28 (44.4%) in comparison to control group which showed 100% mortality (Kumar *et al.*, 2008). Similarly, our result showed that the cumulative mortality percent for vaccinated-virus exposed group (T1) reached 50% after 14 days, which was

significantly different in survival rates (p<0.05) from the Positive control group.

This result could be due to the effect of WSSV-Gamma irradiated vaccine that saved half of the population, where the cumulative mortality for T2 reached 8-9% at the end of experiment. Vaccination with formalin-inactivated *Vibrio penaeicida* (strain KH-1) and h-1, 3-glucan from yeast after 10 days resulted in 36 and 45% cumulative mortality, respectively, that were less than the results of the present study in the same time period (Namikoshi *et al*., 2004). Chakraborty and Ghosh (2014) reported that THC value decreased in the first-24 h of the experiment, when exposed to a mixed extraction from four selected marine plants to prevent the mortality of *L. vannamei*. Likewise; our results indicated that THC values decreased suddenly after 24 h in the vaccinated groups either in T1 or T2 groups. Similarly, the decrease of THC, which prevailed up to 48 h after the beginning of the experiment gradually increased and reached the optimal level of the experiment on day 7 in the vaccinated group (Yogeeswaran*et al*., 2012). This was also supported by (Chang *et al*., 2003) who confirmed the drastic reduction of THC to 60% of its pre-infection level within 24 h after WSSV infection in *P. monodon*.

THC values showed a slight descending trend in T1 treatment after 24 up to 72 h. The reduction in the Positive control (exposed to virus) and vaccinated-nonvirus exposed group was similar up to 24 h but this trend was steeper for T4 even at the end of experiment. However the descending curve of THC for T2 was gradually modified after 48 h, which was in the line with the results of Mohajeri *et al*. (2011). Their findings revealed that the THC could increase after a sudden reduction at 48th h when exposed to *V. harveyi*. Those of T3 were lysed remarkably compared to THC of both T1 and T2. Of course, the maximum reduction of THC after 48 h was related to group of T1. These changes could be involved in the lack or existence of the WSSV or vaccine. Sudden decrease in T3 and slight fall in the T2 group could be due to the lack of vaccine and virus, respectively. This phenomenon was similar to that observed by Pipe and Cole (1995) who showed the decrease of the THC could be due to haemocyte lysis. On the other hand, lack of the virus in the "Negative control" (T4) group of this study resulted in consistent THC values after 24 h and up to the end of the study. It seems that THC value increased in T1 in 72 h after a sudden decrease at the end of 1st day showing that haemocytes were confronted with the virus through the immunological procedure on day 5 and later but more than the previous days of the experiments. Moreover, haemolymph of the survivors were able to deactivate the virus up to 60 days after infection. Therefore, a lower number of circulating haemocytes in crustaceans reduces resistance to pathogens (Yogeeswaran, *et al*., 2012). They showed that glucan also enables

the shrimp to increase the haemocyte count. The Semi- granulocyte in T4 was varied from all others, where the lower value in other groups could be due to experimentally exposing the shrimp to WSSV or the vaccine implying the use of semi-granulocyte at the beginning of an ongoing immunological defense reaction. This suggestion is in agreement with findings of Aladaileh *et al*. (2007) who indicated that the increased percent of particular haemocyte types were due to induced cellular proliferation, recruitment of cells from non-circulating compartments of the haemolymph, or rapid cellular differentiation in response to antigenic challenge. In T4, the density of granulocyte and hyalinocyte were at a minimum value among the groups, which was similar to the phenomenon in some other studies. It can thus be derived that the role of the mentioned cells is very crucial in the continuity of the immune system (Martin *et al*., 1985 ; Kakoolaki *et al*., 2010). The definite decrease in the percent of semi-granular cells was compensated by a proportional increase in the percents of granulocytes and hyaline population.

In T1 (Fig. 5) the descending trend of the TPP value continued until the last day and the pattern for the T3 and T2 treatments were similar to that of the T1. Lo *et al*. (1997) reported that, the high concentrations of protein and aminoacids in the haemolymph of crustaceans are due to heavy viral load. Their results revealed that, the protein and glucose levels increased in the haemolymph on the earlier days of infection due to heavy viral load. TPP composition changes in the blood were affected by shrimp size, sex, nutritional behavior, non-pathogenic factors (such as temperature, salinity) and the molting stage but this was contrarily involved with haemolymph volume (Prasetio *et al*., 2013). This phenomenon was in accordance with our clinical symptoms and was indicative of the fact that as the experiment proceeded, the volume of haemolymph increased. This result was inversely correlated to the TPP value.

It is noteworthy to be mentioned that Gamma irradiated WSSV solution may be useful. Thus, our results especially on decreasing the cumulative mortality might be responsible for developing resistance against WSSV for Gamma irradiant vaccine in shrimp, *L. vannamei* in comparison to the Positive control (T3) while the cause of mortality in vaccinated- nonvirus exposed (T2) treatment was low and a natural one.

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