Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading doi:10.12980/JCLM.2.201414J30

Hemolymph cells apoptosis in imported shrimp *Litopenaeus vannamei* from Hawaii to Iran, exposed to white spot virus

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated that crossing among the specific pathogen free generations could induce the increasing immunity level through apoptosis to protect them against WSD. In this study, the authors provided evidence that oxidative stress plays a crucial role in the induction of apoptosis, especially in viral infection.
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ABSTRACT

Objective: To show hemolymph apoptosis in imported shrimp *Litopenaeus vannamei* from Hawaii to Iran.

Methods: One hundred and eighty shrimps [(7.98±0.54) g] which were collected from a research shrimp farm located in Heleh site in north of Bushehr Province were distributed equally to 6 glass aquariums (50 cm×50 cm×60 cm) as group A in triplicate (imported batch in 2011, without crossing with other generations) with well clean aerated sea water (100 L per aquarium), salinity of 40 ‰ and temperature of 29 °C. Shrimps of group B (produced by crossing the adults of imported batches in 2009 up to 2011) were distributed also among 6 aquariums with the same conditions. Both shrimp groups were injected with concentration of $LD_{50}=1\times10^{5.4}$ white spot virus.

Results: The results showed that in group A, the mortality began approximately 24 h after exposure and reached 100% after 36 h but no mortality was occurred up to 15 d in shrimps of group B. The slide evaluation of hemolymph of group B showed an increasing trend of apoptosis occurrence in all three types of hemolymph cells, hyalinocytes, semi-granulocytes and granulocytes from 24 h to 72 h in contrary to group A that not any apoptosis was observed during the course of the study (15 d).

Conclusions: It is concluded that crossing among the specific pathogen free generations could induce the increasing immunity level through apoptosis to protect them against white spot disease.

KEYWORDS

Apoptosis, Hemocytes, Necrosis, Oxidative stress, Shrimp, White spot virus

1. Introduction

White shrimp [*Litopenaeus vannamei* (*L. vannamei*)] is a kind of significant shrimp in aquatic areas in the world. In recent years, the morbidity increased in cultured shrimp due to virus and bacterial diseases. Aquaculture is the fastest developing food generating sector in the world, with an average annual buildup rate of 8.9% since 1970. Shrimp culture is the most significant trade area in South-East Asia. Shrimp culture begins in Iran in 1994 and has quickly enlarged throughout last years. But intense culture systems causing stress to the animals predispose them to infection. In crustaceans, the constituents of immune system are detected chiefly in hemolymph which includes three forms of hemocytes: hyaline (agranular), semigranular (small granular) and granular (large granular) hemocytes. Shrimp

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Article history:
Received 4 Apr 2014
Received in revised form 18 Apr, 2nd revised form 25 Apr, 3rd revised form 4 May 2014
Accepted 23 May 2014
Available online 17 Jun 2014

has not obtained immune response but active. Shrimp blood is known as hemolymph. It includes hemocyanin as oxygen-carrying particles and lectins as immunoreactive glycoproteins (sugar+protein). Lectins are glycoproteins (sugar+protein) that bind with the sugar portion of other molecules with a broad range *e.g.* lipopolysaccharides or beta-glucans in Gram negative bacteria or yeast[1,2]. White spot virus (WSV) is a member of genus Whispovirus of family Nimaviridae. It indicates round white spot, which has prevalent, very large, enveloped, double-stranded DNA. The life phases of shrimp with white spot virus are nauplii and larvae. Postlarvae and juvenile are susceptible to the virus. The high incidence of salinity and occurrence of white spot diseases (WSD) may cause oxidative damages including apoptosis and necrosis in hemolymph of shrimp. Oxidative stress has been reported to implicate in pathogenesis of many diseases including viral infection. During viral infection, the antigenic stimulation activates the immune system of the body thereby causing release of reactive oxygen species (ROS). Oxidative stress benefits the host cells to fight against the viruses in viral infection. The host cells and tissues are damaged when ROS is excessive producted by host's immune system[3]. ROS, *i.e.*, superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\cdot-}$) are toxic and harmful substance that oxidize all biological matter. Oxidants that are produced by admonished neutrophils and macrophages throughout the so-called respiratory burst participate in the destroy of microorganisms and induce cellular injury by stander cells. It has been considered that ROS generation was responsible for the induction of apoptosis. ROS-dependent apoptosis is shown to be independent of cytokines such as tumor necrosis factor-α (TNF-α). TNF-α enhances ROS production of neutrophils and monocytes has been shown to be associated with the beginning of apoptosis. Therefore, it can be possible that TNF-α interacts with ROS mediated apoptosis because both inducers are produced by the same cells, *i.e.*, macrophages by the same stimulation, *e.g.*, upon microorganism infection. ROS may behave independently of TNF-α as an inducer of apoptosis[4]. Apoptosis can begin by several mechanisms, such as defects in activation signalling and cytokine imbalance. Furthermore, some studies express that oxidative stress mediated by the generation of ROS induces apoptosis[4]. Apoptosis is a physiological mechanism that conserves homeostasis in the turnover of normal tissue. Apoptosis, which programmed cell death of hemocytes was observed in virus infected shrimp. It could be connected to oxidative stress. Therefore, it has been investigated that whether ROS induce apoptosis and which might contribute to the cell loss during progression of viral infection. The fact that many agents, which induced apoptosis are either oxidants or activators of cellular oxidative metabolism. It suggests that generation of ROS could induce apoptosis[4,5].

In the study, the evidence has been provided that oxidative stress played a crucial role in the induction of apoptosis, especially in viral infection.

2. Materials and methods

2.1. Shrimps and experimental design

One hundred and eighty shrimps [(7.98±0.54) g] which were collected from a research shrimp farm located in Heleh site in north of Bushehr Province were distributed equally to 6 glass aquariums (50 cm×50 cm×60 cm) as group A in triplicate (imported batch in 2011, without crossing with other generations) with well clean aerated sea water (100 L per aquarium), salinity of 40 ‰ and temperature of 29 °C. Shrimps of group B (produced by crossing the adults of imported batches in 2009 up to 2011) were distributed also among 6 aquariums with the same conditions. Both shrimp groups were injected with concentration of $LD_{50}=1\times10^{5.4}$ WSV. The shrimps were reared in the current condition for 40 d. They were being fed with a commercial dry diet twice a day and residual feed were removed daily by siphon.

2.2. Preparation of WSV stock solution

The infected hemolymph of *L. vannamei* with the code No. of wssv/irn/1/2011 has already been prepared by investigation lab in Iran, in which it was studied to produce a vaccine of WSD. Virus with the titre of $LD_{50}= 1\times10^{5.4}$ were offered to this research and used as virus in challenging with the treatments but control groups were left unchallenged with WSV. The mixture was stored at −80 °C until use.

2.3. Histopathological examination

After the mortality observation, 3 moribund shrimp from each treatment in which the mortality occurred, were prepared to be subjected to histopathology. The remaining tissues were placed in tubes containing Davidson's fixative. They were then transferred to the lab. The tubes containing Davidson's fixative were discarded and replaced with ehanol 70% after 48-72 h in the lab and stained by hematoxylin and eosin stain method[6]. In hemolymph analysis, the hemolymph was collected after the mortality observation. A total of 0.2 mL of hemolymph of 3 moribund shrimp from each triplicate in treatments (hemocytes after exposure to virus) and the controls (normal hemocytes before exposure to virus) were withdrawn from the second leg's basement of ventral segments using 1 mL syringe along with 26 gauge needle. Each syringe was pre-filled with 0.8 mL Alsever solution as anticoagulant[7].

3. Results

In this study, in group A, the mortality began

approximately 24 h after exposure and reached 100% after 36 h but no mortality was occurred up to 15 d in shrimps of group B. The slide evaluation of hemolymph of group B showed an increasing trend of apoptosis occurrence in all three types of hemolymph cells, hyalinocytes, semi-granulocytes and granulocytes from 24 h to 72 h in contrary to group A that no any apoptosis was observed during the course of the study (15 d). Therefore, the figures belong to the group B. Normal hemocytes before exposure to virus with normal size are showed in Figures 1-3.

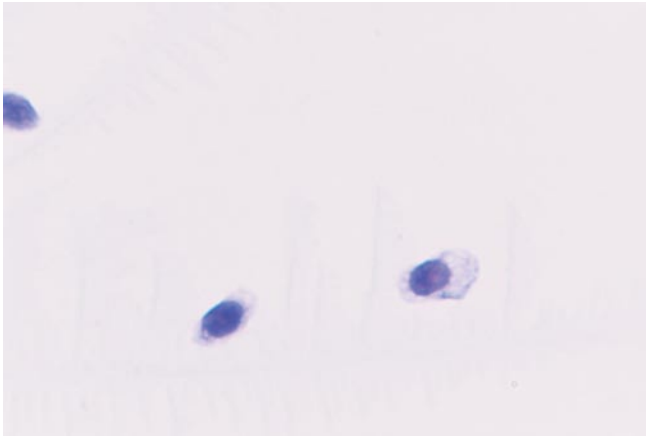

Figure 1. Normal hemocytes before exposure to virus with normal size (×1 000).

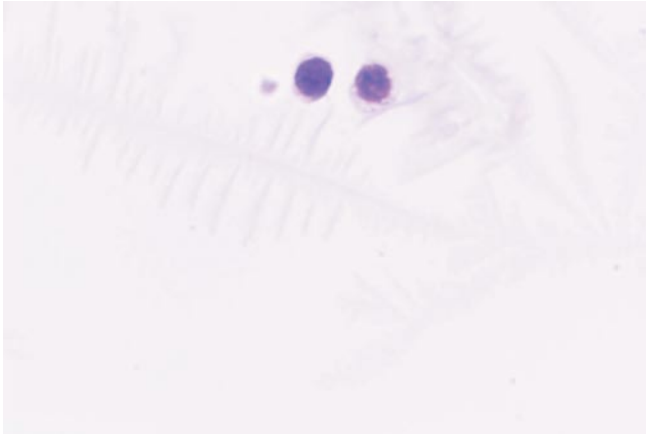

Figure 2. Normal hemocytes before exposure to virus with normal size (×1 000).

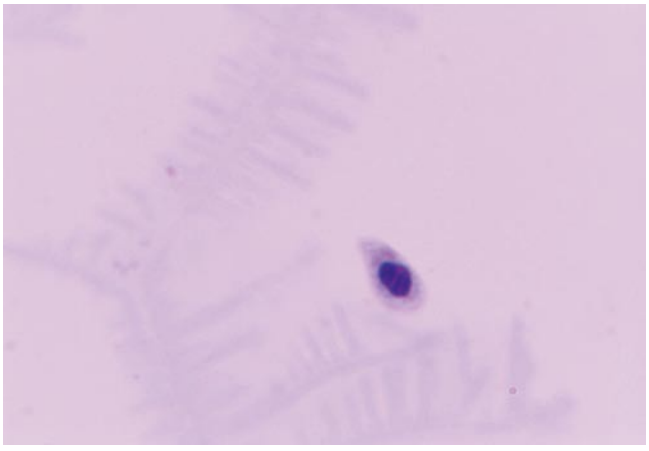

Figure 3. Normal hemocytes before exposure to virus with normal size (×1 000).

The cells normal size obtained some changes after sufferring virus infection. Occured changes after 24 h exposured to virus were showed in Figures 4-6. Some cells with normal size were observed and form morphologically. Pale discoloration, cellular dense and rarely cell debris in some hemocyte cells were also observed (Figures 4-6).

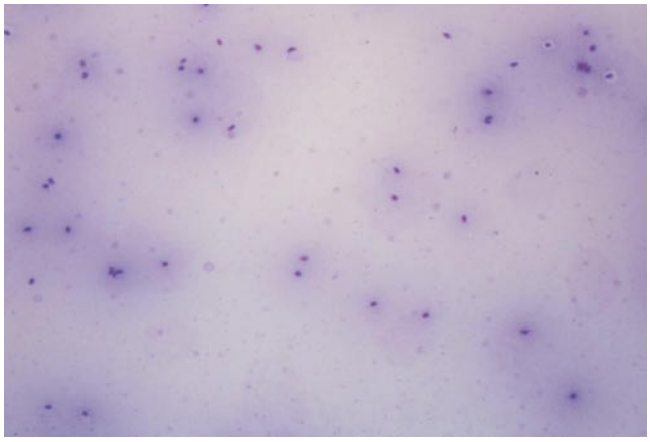

Figure 4. Hemocytes with normal and pale discoloration after 24 h (×100).

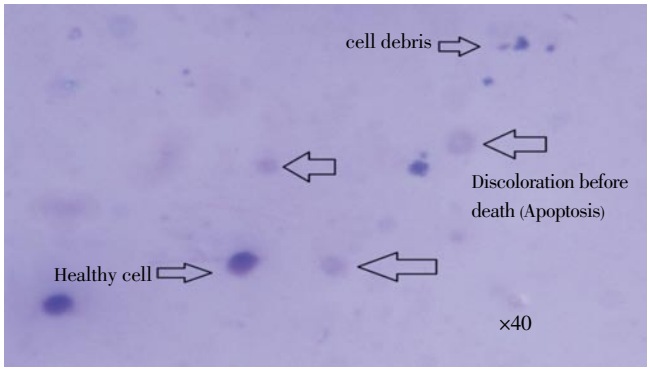

Figure 5. Hemocytes with pale discoloration and a little smaller, dense hemocytes than normal hemocytes after 24 h (×40).

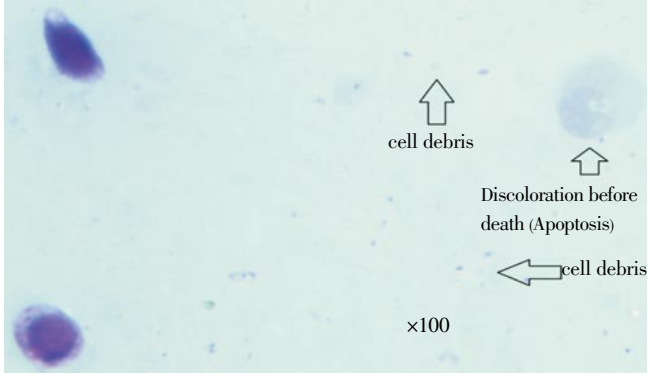

Figure 6. Hemocytes with pale discoloration and a little smaller, dense hemocytes than normal hemocytes after 24 h (×100).

After 48 h from viral infection, the changes, which occured in hemocyte cells were demonstrated in Figures 7 and 8. After 48 h from infection, the hemocyte cells were appeared bigger than normal hemocytes and released the hemocyte content. In addition, large amount of cell debris were observed (Figures 7 and 8). After 72 h from treatment, the changes, which occured in hemocyte cells were showed in Figures 9 and 10. After 72 h, the cell debris were

decreased. The hemocyte content was significantly gathered and smaller and dense hemocytes than normal hemocytes were observed (Figures 9 and 10).

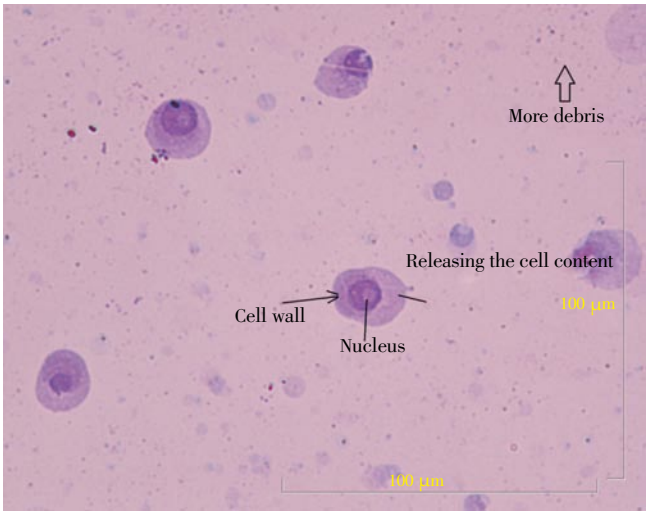

Figure 7. After 48 h, hemocytes releasing the hemocyte content and a little larger hemocytes than normal hemocytes (×100).

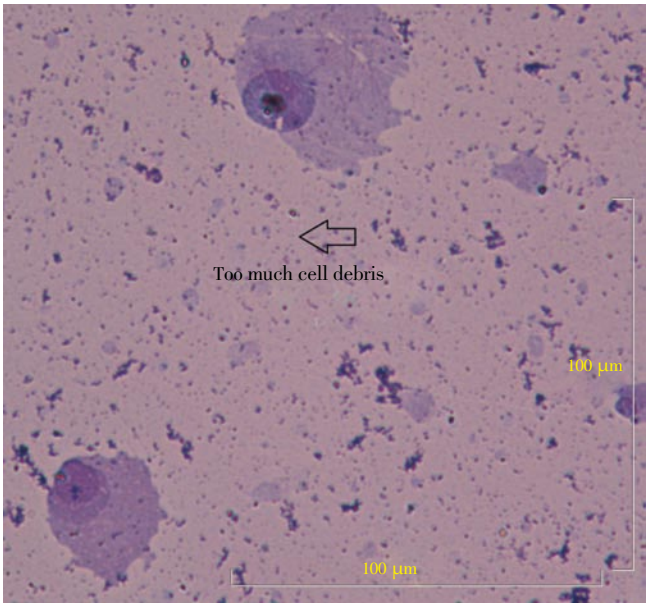

Figure 8. After 48 h, hemocytes releasing the hemocyte content and too much cell debris (×100).

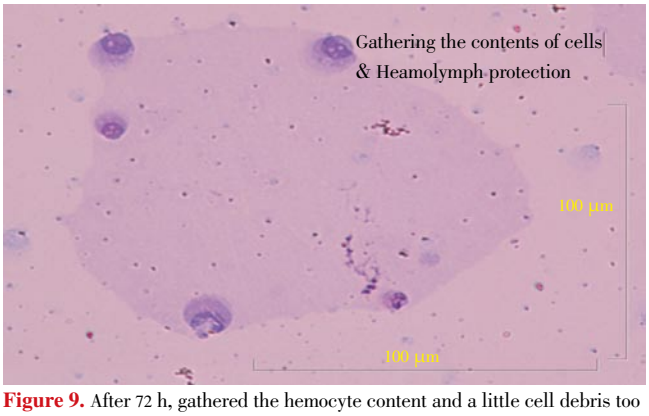

Figure 9. After 72 h, gathered the hemocyte content and a little cell debris too (×100).

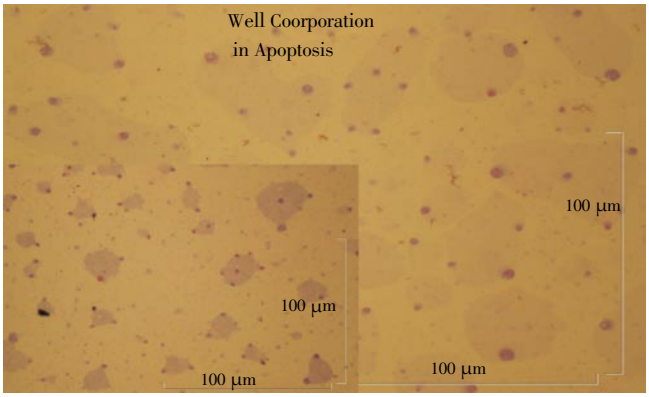

Figure 10. After 72 h, smaller and dense hemocytes than normal hemocytes too (×100).

4. Discussion

In caution to the actions of climate changes on the fluctuations in physico-chemical features of the water areas during the shrimp culture especially in stocking phase and on the time of harvesting, the yield resulted in a few epizootic of WSD in Iran in the last decade[2,8]. Shrimp farmers have been suffering from loss of generation because of the WSD[9]. Suitable shrimp welfare administration applied in Choybdeh shrimp site in southwest Iran in 2010 resulted in a relative control of the WSD[1]. WSD results in heavy mortality and kills the cultured shrimps throughout 3-10 d[6]. Marine penaeid shrimps are sensitive to be infected by the WSV[10], and other marine and freshwater crustaceans are thought to be sensitive as well[11]. The effect of salinity on hemolymph cells was studied in shrimp[2,12]. Rate of salinity is one of the most significant environmental agents that affects the consistent of WSD[13]. Some investaigations determined the effect of salinity factor on the occurrence of viral diseases of the shrimp[13-15]. The cells take action for their defense mechanisms against these foreign infectious agent when organisms and cells are infected by an foreign agent such as microorganisms and toxic substances. They produce ROS to kill and destroy these foreign miroorganisms[3-5]. As a result, oxidative stress develops with the mechanism that stimulated by infectious agents. So, the early days of viral infection lead to the death of hemeocytes when ROS is produced. Especially, deaths of hemocyte cells were types of apoptotic cell death after 24 h suffered-viral infection. But result of growing ROS production against the increasing expose to viral infection has been occured as cellular damage and then the cell death is observed as necrotic type. Particularly, alterations that observed in hemocyte cells were necrotic type after 48 h suffered-viral infection. In the study, deaths are not observed in hemocyte cells after 72 h of viral infection. Morphologically normal aspect

cells were observed. As a result, infection was reduced and deaths in hemocytes cells was also decreased. The case may be due to the excessive production of ROS against viral infection and acquired resistance of immune system. Apoptotic cell death has been observed in hemocyte cells by applying an infective agent for 48 h. The cells and the nucleus of cells have been observed to be compact and smaller. In this situation, the cell nucleus was pointed dark and that is visible in light microscope. In apoptotic death, vesicul in cytoplasm and relation with membranit were observed. But at the end of 48 h, ROS produced was increased for the continue of the infection. As a result, necrotic death appeared. Necrotic cell death occurs due to toxic and infective agents (such as viruses), cell explodes and cell containing are released to extracellular area. The cells produced increased ROS when they were infected with an infective agent and as a result of increased ROS produced, cell deaths occurred. The result of ROS produced cellular damages and necrotic cell deaths increase parallelly when time of exposed to infective agent (virus) increased. But on the other hand, immune system of the cells reinforces against infective agents a time later. Immune resistance developes at the beginning of viral infection after 72 h apoptotic and necrotic cell deaths were not observed. In addition, more studies that use different assay methods for better determination of apoptotic and necrotic cell deaths are needed. Espically, fluorescent and immunohistochemical method is able to be used to determine cell pathways in getting immunity-cells. It was observed that oxygen radicals were produced under the circumstances that opportunistic infections mediate apoptosis. Taking the diminished oxidative resistance of virus-infected cells into account, the results might contribute to the understanding of the pathophysiological mechanims of hemocytes loss. In the view of the diminished oxidative resistance of virus-infected cells, the results suggested that ROS-mediated apoptosis might contribute to the deletion of hemocytes and the pathogenesis of the disease. Apoptosis is a physiological mechanism that preserves homeostasis in the turnover of normal tissue. Some studies suggested that oxidative stress mediated by the generation of ROS induced apoptosis[3,4,16,17]. In the present study, increased ROS production, oxidative stress and mitochondrial pathway of apoptosis in viral infected cells are in accordance with the earlier studies, where it has been documented that the generation of ROS and associated oxidative stress are involved in mitochondrial pathway of apoptosis[3,18,19]. T he study showed that reactive oxygen intermediates produced under circumstances that reflect physiological conditions are able to induce high rates of apoptosis. Induction of apoptosis could be blocked by the reactive oxygen radical scavengers glutation, superoxide dismutase and catalase. The apparent paradox could be explained by the fact that superoxide dismutase extracellularly degrades membrane-impermeable $O_2^{\cdot-}$ to membrane-permeable H_2O_2, which can then easily penetrate into the cell where causes apoptosis. The study done by Dobmeyer *et al*.[4] interestingly showed that lymphocytes of HIV-infected individuals even at very early stages of infection were more susceptible to oxidative stress-mediated apoptosis than lymphocytes isolated from healthy subjects. The data that obtained by Dobmeyer *et al*.[4] indicated that oxidative stress might play an important role in the initiation of lymphocyte loss during HIV infection. Kung *et al*.[20] defined that the death receptor apoptosis and necrosis pathways were intimately connected through multiple shared constituents such as ligands and receptors. They differ with respect to some of the complexes that were formed. In the death receptor pathway, necrosis is a default outcome when apoptosis is inhibited. The obtained date also observed both apoptotic and neucrotic deaths. Particularly, the apoptotic deaths was detected after 24 h from viral infection. The necrotic deaths was observed after 48 h. But after 72 h the normal cells was detected. As Kung *et al*. explained[20], apoptosis and necrosis can be identified in cell culture systems using established morphological and biochemical markers. For apoptosis, these included chromatin condensation, cell shrinkage and fragmentation. Necrosis indicators include cell and organelle swelling, loss of membrane integrity and release of endogenous proteins. Even in cultured cells, however, the separation between apoptosis and necrosis is not as clean as it might be expected. For example, cytochrome c release can result from both apoptosis and necrosis. Although the furthermost results of apoptosis and necrosis are the same with the death of the affected cells, the two processes differ significantly. In apoptosis, the influenced cells actively join by activating a cascade of biochemical reactions that result in cell death. Consequently, apoptosis has been called as cell suicide. In necrosis, however, cell death appears because of the adverse conditions or changes in the cell's environment. The released cellular content subsequently induces an inflammatory response in the effected tissue. That response is mediated by three components: (1) certain cells of the immune system, (2) small molecules called cytokines that are involved in cell communication and (3) ROS. The subsequent inflammatory response is often considered as an integral part of necrosis[20,21]. As Nanji and Sturmhöfel defined[21] that

numerous factors and mechanisms can stimulate apoptotic and necrotic cell death. Some of these factors and mechanisms contribute to both apoptosis and necrosis, whereas others play a role only in one of these processes. It is important to recognize that apoptosis is not always a destructive process. Besides, it generally helps to eliminate damaged or malfunctioning cells from the body. So apoptosis acts a vital role in ensuring the proper functioning of many organs such as the immune system[20,21]. Most studies expressed that more detailed studies have revealed that when a cell dies by a typical apoptotic process, usually there occurs a late-phase necrosis characterized by spillage of cell content[22-24]. In the studies of Chautan *et al*.[25] on the removal of interdigital cells in the developing mouse embryo, when apoptosis was prevented, the cells died by necrosis, and even under normal conditions a certain percentage of the cells are removed by necrosis; but both conditions may be evidence for an inflammatory response. These cases also shown availability of this study. In reality, apoptotic and necrotic cell death corresponding to noninflammatory and inflammatory outcomes, respectively, are extreme examples of a wide spectrum of cellular responses. When a cell is attacked by a pathogen or sustains chemical or physical insults, it is advantage to the organism that signals are sent out, so that proper specific and non-specific defences are set up, in short, an inflammatory response. As an auto-immune response, apoptosis and necrosis is achieved. This case that Fiers *et al*. explained supports the data[24]. Fiers *et al*.[24] implies that the key oxidative step that accomplish the cell to necrotic death is specific and localized and it is not because of the accumulative and generalized oxidative damage. The recognition that not only apoptosis but also necrosis may be actively mediated has renewed interest in the role of regulated cell death in infective diseases[20]. In conclusion, the data and the methods used in this study can not be enough to determine whether the type of death is necrotic or apoptotic. Because of these issues, electron microscopy is used sometimes. This allows apoptosis and necrosis to be assessed using a single technique although sensitivities of detection of apoptotic and necrotic cells are different[20]. However, there is still a need for further studies with the different assay methods for certainly confirm necrotic or apoptotic cell deaths. Despite that, the study will shed light on new researches, allowing different studies in this field.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors thank for Dr. Shapour Kakoolaki at Department of Aquatic Animal Health, Iranian Fisheries Research Organization, Tehran, Iran for helping in animal research.

Comments

Background

L. vannamei is significant shrimp in aquatic areas in the world. In recent years, the morbidity increased in cultured shrimp due to virus and bacterial diseases. Intense culture systems causing stress to the animals predispose them to infection.

Research frontiers

This study aimed to show hemolymph apoptosis in imported shrimp *L. vannamei* from Hawaii to Iran. In crustaceans, the constituents of immune system are detected chiefly in hemolymph which includes three forms of hemocytes: hyaline (agranular), semigranular (small granular) and granular (large granular) hemocytes.

Related reports

In the present study, Fiers *et al*. (1999) implies that the key oxidative step, which accomplish the cell to necrotic death is specific and localized and not because of the accumulative, generalized oxidative damage.

Innovations and breakthroughs

The author's results may contribute to the understanding of the pathophysiological mechanisms of hemocytes loss. In view of the diminished oxidative resistance of virus-infected cells, results of the present study suggest that ROS-mediated apoptosis might contribute to the deletion of hemocytes and the pathogenesis of the disease.

Applications

In the present study, it has been found that increased ROS production, oxidative stress and mitochondrial pathway of apoptosis in viral infected cells were in accordance with earlier studies, where it has been documented that the generation of ROS and associated oxidative stress are involved in mitochondrial pathway of apoptosis.

Peer review

This is a valuable research work in which the authors have demonstrated that crossing among the specific pathogen free generations could induce the increasing

immunity level through apoptosis to protect them against WSD. In this study, the authors provided evidence that oxidative stress played a crucial role in the induction of apoptosis, especially in viral infection.

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