



Observational Effect of Chitin and Chitosan from Horseshoe Crab's Exoskeleton on the Test Animal, Zebrafish *Danio Rerio*, Under the Laboratory Condition

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Abstract

Being a component of crustacean's exoskeleton, chitin is one of main carbohydrate found on earth. Its derivative, chitosan can be isolated through deacetylation process of chitin. Due to their potential in pharmaceutical field, this study is using chitin and chitosan isolated from horseshoe crab to be tested on zebrafish (*Danio rerio*) developing embryo at various volumes. By using Danioscope, morphological defects on zebrafish embryos and larvae has been observed. We found out that both chitin and chitosan affect the zebrafish in several morphological abnormalities including dented tail, accumulation of blood, stunted development, enema and no pigmentation observed. Percentage of mortality was also determined for three different concentrations of chitin and chitosan which are at 10 μ l, 50 μ l and 100 μ l that been exposed to the embryo. Chitosan shows higher mortality rate compared to chitin. Both observations and data on morphological defects and mortality rate help in determining the toxicity level of chitin and chitosan on zebrafish and their effects. Our results suggested that both compounds are toxic at certain amount, hence having a high capability to be used in combating pathogens and tumour.

Keywords: Chitin; Chitosan; Zebrafish; Horseshoe Crab

Introduction

Chitin or poly- β -(1 \rightarrow 4)-*N*-acetyl-D-glucosamine is a polysaccharide that functions as the component of crustacean's exoskeleton. For laboratory and industrial use, chitin usually prepared using crustacean's exoskeleton. The crustacean's are members of arthropods, which constitute of 10⁶ species from 1.2 x 10⁶ total species in animal Kingdom Tolaimate, et al. [1]. Chitosan is the *N*-deacetylated derivative of chitin, produced after deacetylation process.

The monomer of chitosan is D-glucosamine and *N*-acetyl-D-glucosamine that linked by β - (1,4)-glycosidic bond Hamed, et al. [2]. The chitin and chitosan have specific chemical properties which lead to varieties of applications such as in cosmetics, water engineering, food processing, wound dressing, and drug delivery system Rinaudo, et al. [3]. Chitin and chitosan from the horseshoe crab could be exploited more as it was found to have antibacterial and antioxidant activity Ismail N, et al.; Pati, et al. [4,5] as well as potential biomaterials Wardiatno, et al. [6].

Zebrafish is a freshwater fish, belongs to the family Cyprinidae together with goldfish Meyer, et al. [7]. Natively, it can be found in South Asia from India to Thailand, Malay Peninsula and up to Yunnan. The use of zebrafish as an animal model serves many advantages because of their high human genetic homology, transparent embryos and larvae that allow easier observation Benchoula, et al. [8]. Hu, et al. [9] works on chitosan nanoparticle that used as drug or gene delivery vehicles in human gene therapy. When zebrafish embryos were exposed to chitosan nanoparticle, these embryos perform delay hatching and as the concentration of the nanoparticles increases, more embryos died. More information and observation should be gathered to be a reference for those who are working with the chitin and chitosan from the horseshoe crabs as to avoid unsustainable exploitation of this living fossil.

This study is aimed to observe the effect of the different concentration of raw chitin and chitosan from horseshoe crab carapace on the developing embryo of zebrafish under laboratory condition.

Methodology

A group of healthy adult wild type Zebrafish from Central Research and Animal Facility (CREAM) of the International Islamic University Malaysia (IIUM) were used

for egg production. Fishes were maintained under standard laboratory conditions, which are at $27 \pm 1^\circ\text{C}$ with 12h light: 12h dark cycles [10]. They were feed three times a day with freshly hatched *Artemia*. Both male and female zebrafish that aged nine months old were put in spawning tank overnight with ratio of 1:2. The next morning, eggs were harvested. They will then be put in petri dishes containing embryo water tank that consist of methylene blue. They were carefully washed to remove debris. Any dead embryo or unfertilized eggs will be discarded by aspiration using plastic pipette.

Medium has been prepared for zebrafish embryo to hatch. The recipe for media per 1 litre is 5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl and 0.33 mM MgSO₄. The organic solvents 0.1% dimethyl sulfoxide (DMSO) from Fisher Scientific was used. Since chitin is insoluble in water, it is needed to be diluted using this organic solvent. Healthy fertilized embryos were transferred into 24-well plate where each well contained two embryos. Treatments were carried out using two set of plates in each cycle. In total, 96 embryos were used. The total volume of each well was 1000µl, consisting of media and chitin or chitosan with respectable volume of 10µl, 50µl and 100µl. Positive control was 30µl of AlCl while and negative control was culture media itself. Figure 1 shows the volume and distribution of content in each well. All plates were incubated at $27 \pm 1^\circ\text{C}$ in a temperature-maintained room.

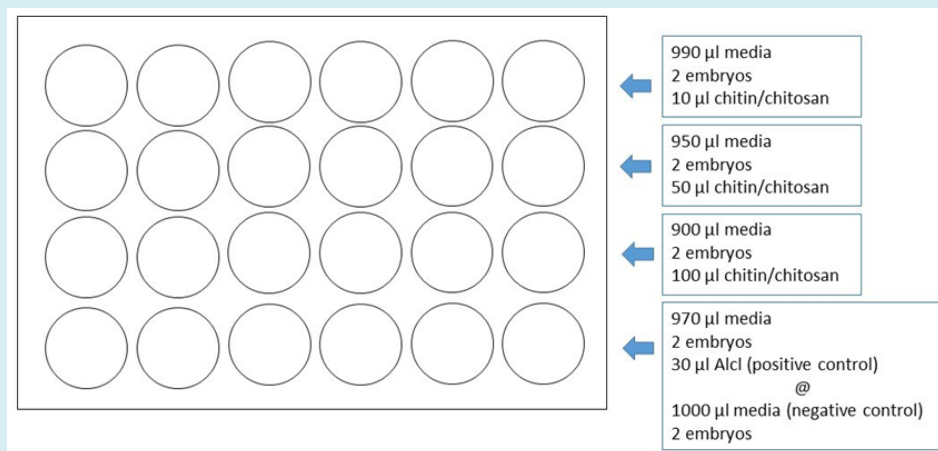


Figure 1: The zebrafish embryos and solutions on 24-well plate. A total of 12 larvae involved in the replicate experiment of five replication exposures.

The effects of treatments on the embryo were observed 24, 48 and 72hours post fertilization (hpf). Their mortality rates were evaluated, together with their morphological development defects. Software called 'Danioscope' is applied to gather all important data needed. The software is developed by a research company called Noldus Information Technology. Danioscope can help to examine the morphology of zebrafish embryo larvae by using a few parameters

including their activity, measurement on cardiovascular, blood flow and basic morphology. By using Danioscope, any developmental defects can be identified. The mortality rates were determined using the following equation:

No. *ooo* dead embryos

$$\text{Percentage } \textit{ooo} \text{ mortality}(\%) = \frac{\textit{ooo} \times 100}{\text{Total embryos}}$$

Results

Morphological Defects of Zebrafish Embryos

Three dilutions of chitin, three dilutions of chitosan, AlCl₃ as positive control and the media itself as negative control were applied onto the zebrafish embryos to investigate the

effect of these eight different solutions on their development. Observations on morphological effects were determined using Danioscope, shown in Table 1 (chitin treatment), (Table 2) (chitosan treatment) and (Table 3) (positive/negative control).

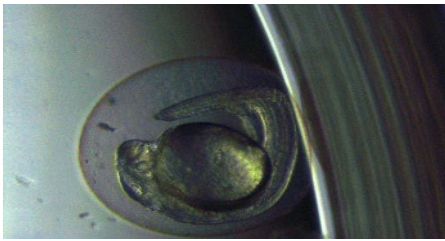
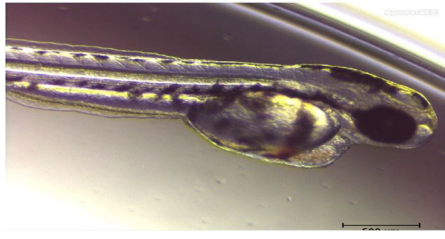

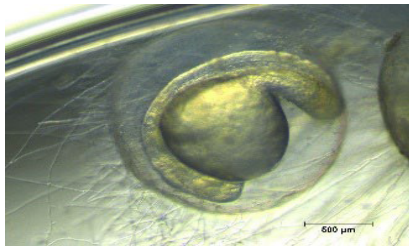

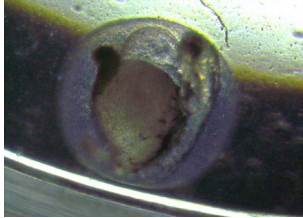

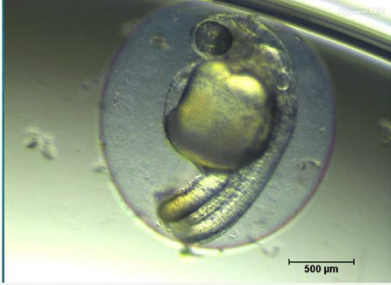

Treatment	Morphological defect
10 μ l chitin	
	Dented tail at 24 hpf
	
	Accumulation of blood in the yolk sac at 72 hpf
50 μ l chitin	
	Stunted development at 48 hpf
100 μ l chitin	
	Stunted development at 48 hpf

Table 1: Morphological observation of *Danio rerio* embryos and larvae that has been treated with different dilution of chitin from horseshoe crab.

Treatment	Morphological defect
10 μ l chitosan	
	Stunted development at 48 hpf
	
Yolk sac enema at 72 hpf	50 μ l chitosan
	
Accumulation of red blood cell in the head at 72 hpf	
	
Early pericardial enema at 48 hpf	
	
Stunted development at 48 hpf	


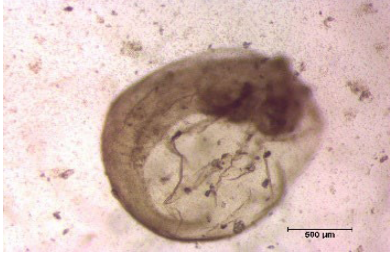
100 μ l chitosan	
	No pigmentation at 72 hpf
	
	Stunted development at 72 hpf

Table 2: Morphological observation of *Danio rerio* embryos and larvae that has been treated with different dilution of chitosan from horseshoe crab.



Treatment	Morphological defect
30 μ l AlCl	 Accumulation of red blood cell in abdomen at 24 hpf
	 Normal embryo at 24 hpf



Table 3: Morphological observation of *Danio rerio* embryos and larvae that has been treated with 30 μ l AICI and 1000 μ l media.

Mortality Rate of Zebrafish Embryos

Six different dilutions of chitin and chitosan were applied onto zebrafish embryo at 0 hpf. The number of embryos that

died was recorded three times, at 24 hpf, 48 hpf and 72 hpf. The percentage of mortality amongst zebrafish embryos was presented in Figure 2.

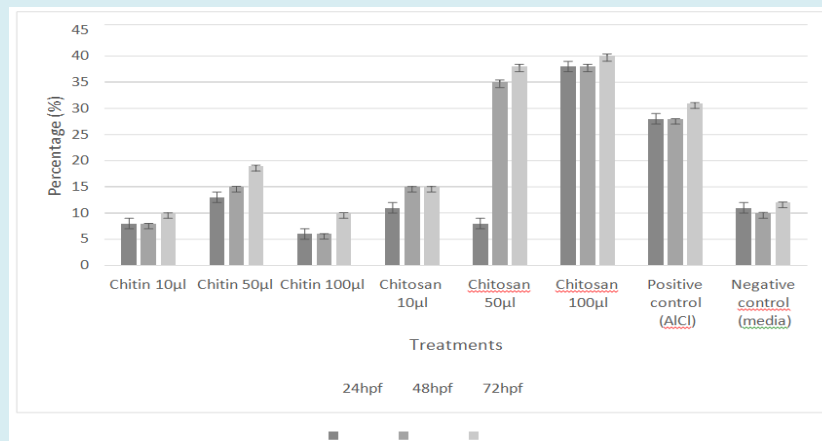


Figure 2: Percentage of mortality of the developing embryo in three period (in hours post fertilization) when treated with different dilution of chitin and chitosan. (Different capital letters in the same column indicate the significant difference 127 ($p < 0.05$))

Treatments	Mean
Chitin 10 μ l	1.08 \pm 0.289 ^A
Chitin 50 μ l	1.83 \pm 1.267 ^B
Chitin 100 μ l	0.92 \pm 0.669 ^C
Chitosan 10 μ l	1.58 \pm 0.793 ^D
Chitosan 50 μ l	3.08 \pm 3.8485 ^E
Chitosan 100 μ l	4.33 \pm 4.097 ^F
Positive control (AICI)	3.44 \pm 0.726 ^G
Negative control (media)	1.27 \pm 1.223 ^H

Table 4: The mean of mortality developing embryos in three period (in hours post fertilization) when treated with different dilution of chitin and chitosan.

Table 4 showed the mean of mortality on developing embryos when treated with different dilution of chitin and chitosan. Obviously, the mortality rate is the highest on chitosan at highest concentration. There was a significant different between treatments as determined by one-way 132 ANOVA [$F(7,88) = 4.096$, $p = 0.001$, $p < 0.05$].

Discussion

Morphological Defects of Zebrafish Embryos

These are overall morphological defects were observed on various dilutions of chitin and chitosan applied. Normal embryo showed when using 1000 μ l media as how should it develop in optimum condition. The same treatment also showed the normal larvae that show perfect organogenesis

as the organs developed well to help the larvae body system functions effectively.

Dented tail was shown by embryo that treated with 10 μl chitin, as there was kink at the tail part. This is known as kyphosis Pamanji, et al. [11]. The condition will affect its swimming ability when it hatched. Dented tail can also be observed as one of the defects. These malformations may due to decrease in collagen located in spinal column Murugesu, et al. [10] or some alteration in the composition of amino acid Celik, et al. [12] and maybe because of the inhibition of specific gene regulator named protein tyrosine kinase 7 (ptk7) [13].

Accumulation of red blood cell in the yolk sac was shown in 10 μl chitin treatment, in the head when treated with 50 μl chitosan and in the abdomen when treated with 30 AlCl. This condition will of cause let to death soon due to inefficient blood circulation to the whole body. Accumulation of red blood cell also can be seen as a morphological defect that happens on zebrafish. When the accumulation occurs in the head, it may be due to serotonergic stimulation that will results in intravascular coagulation, together with cerebral haemorrhage. This was based on a study done by Andrade, et al. [14]. However, further study should be done to confirm the situation.

Stunted development was shown in embryos in many treatments including 50 μl chitin, 100 μl chitin, 10 μl chitosan and 100 μl chitosan where its development stops at certain age. These embryos were found in 72 hpf, with no eye pigmentation, less developed backbone, and delay hatching. They probably stop its development at 21-somite stage to prom-6 stage, that refers to development between 19.5 to 25 hpf. The prediction is based on comparison been done on the embryos observed with embryo sketches by Kimmel, et al. [15]. The situation occurred is known as stunted development. Yolk sac enema was shown by embryo treated with 10 μl chitosan enema occurs due to the accumulation of body fluid. In this case, the accumulation occurs in the yolk sac. Pericardial oedema can occurs due to accumulation of body fluid that originated from the failure of its osmoregulatory system [11]. If accumulation of water occurs in the head region, it will result in hydrocephalus. Murugesu, et al. [10] stated that enema can increase significantly with increase in concentration of toxicants. On the other hand, the condition of pericardial enema in the embryo was found when treated with 100 μl chitosan.

No pigmentation has also been observed as one of the morphological defects on zebrafish. Generally, pigmentation should start at retinal epithelium and the skin dorsolateral as early as 24 hpf Kimmel, et al. [15]. Pigment cell that controls colour changes in fish skin is known as melanophores.

Sugimoto, et al. [16] in their research reported that density of melanophores decreased by sporadic cell death, which also known as apoptosis. They suggested that the apoptosis process is controlled by sympathetic nervous system through sympathetic neurotransmitter, norepinephrine. The absence of pigment on zebrafish in this study probably due to the apoptosis to melanophores, due to the treatments that performed on them.

Mortality Rate of Zebrafish Embryos

Mortality can be indicated by the absence of heartbeat. Hu, et al. [9] suggested that chitosan is able to induce cellular apoptosis. The study proposed the usage of acridine orange staining, that will present a bright green fluorescent spot which indicates the occurrence of apoptosis. Chou, et al. [17] did a research on effect of low molecular weight chitosan on zebrafish. This substance showed high toxicity towards both zebrafish larvae and adults by attacking and damaging the cell membranes. Efflux of ions from the cell was induced those results in ion imbalance. Zebrafish larvae showed breakage of yolk, while the adult display damaged gill lamella that leads to hypoxia, then their death. Cheng, et al. [18] suggested that there are pores at the size of 0.17 μm^2 on the chorion. These pores obviously responsible for the exposure of chemicals to the embryo at their early stage.

The outcome of this research showed that both chitin and chitosan effect the zebrafish negatively although it is known that both compounds are used widely in pharmaceutical industry. Stine, et al. [19] stated that zebrafish is very sensitive at low concentration of chitin and chitosan that at last may leads to mortality. By determining the toxicity of chitin and chitosan, toxic dose that effect the development zebrafish can be recorded. This can be done using median lethal concentration (LC50). However, LC50 that can be construct using probit analysis needs a huge number of different concentrations of treatments up to 7 concentrations Wang, et al. [20] and 9 concentrations Ismail HF, et al. [21]. Since this study only use three different concentrations at 10 μl , 50 μl and 100 μl for each compound, probit analysis cannot be constructed. Based on previous study on chitin and chitosan, LC50 has been recorded at 105 mg/L in and 548 mg/L Stine, et al. [19], 270 mg/L Wang, et al. [20], 200 mg/L [22].

This research is using chitin and chitosan with concentration of 2.5 mg/mL. This concentration is very high compared to Wang, et al, (2016) [20] and Chou, et al. [17] that using 250 mg/L and 0.2 mg/L. This is the main factor that contributes to very high mortality rate on zebrafish in this study. Size of particles also should be taken into consideration. The lower the molecular weight of compound, the higher the rate of toxicity. Most study are using nanoparticle weight that ranging from 107 Da Wang, et al. [20] 3.6⁴Da and 1.2⁴

Da Chou, et al. [17] and size at 200 nm by Hu, et al. (2011) [9]. This study is using chitin and chitosan at 250µm in size that considered as big which gives less impact on zebrafish toxicity. Because of its toxicity at certain concentration, chitosan is widely used to eradicate tumour. A study by Jiang, et al. (2020) [23] used chitosan derivative, carboxymethyl chitosan oligosaccharide (CM-COS) as the inhibitor in growth of hepatocellular carcinoma. This study resulted in CM-COS as cytotoxic and able to inhibit tumour. At the same time, they suggested that positive charge CM-COS is attracted to negatively charged tumour cell as the main reason why this chitosan derivative only attack cancer cell but not normal cell, and it has been shown by MTT essay test conducted. Chiu, et al. (2017) [24] in their study used the combination of glycated chitosan and radiofrequency ablation on breast tumour. The combination showed a significant antitumor immune response by the rat and abled to eradicate the growth of tumour, together with suppressing its metastasis. This is supported by Zaharoff, et al. [25] whom demonstrated that chitosan enhanced humoral mediated immune response by increasing IgG production and induced cell-mediated immune response by stimulating CD4+ proliferation.

Conclusion

The effect of the different concentration of raw chitin and chitosan from horseshoe crab carapace on the developing embryo of zebrafish could be observed and recorded under a laboratory condition. Defects were shown by several characters such as reduced number of pigments, oedema and stunted development when larvae are exposed to as little as 10 µl chitin or chitosan extract. Mortality was significantly high (35-40%) after 24 hours exposure to 50 and 100 µl chitosan. This data could contribute to the knowledge gap on the concentration of chitin and chitosan used for any laboratory experiment on a model animal such as zebrafish. Further study particularly on the lethal concentration must be carried out to complete the understanding on the effect of the concentration of extract thus manipulation can be made for the target cells in study.

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