Sperm Quality Assessment of Banana Shrimp *Fenneropenaeus merguiensis* (De Man, 1888) from Ultraviolet Irradiation for Initial Development of Gynogenesis Application

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ABSTRACT

Gynogenesis was considered as powerful tools for producing all female larvae. One of the most critical point in gynogenesis was studied in banana shrimp, *Fenneropenaeus merguiensis*. The aim of this study was to determine the effect of different UV induction on *F. merguiensis* sperms quality for initial development of gynogenesis protocol. Fresh sperms taken from male’s broodstock were diluted 1:10 with Ringer’s solution and UV irradiated with doses at 254 and 365 nm from 20-80 sec. Sperms viability was determined using modified eosin-nigrosin staining method. Irradiation for 20 and 40 sec of 254 and 365 nm exposures were optimum doses to achieve haploid gynogenesis. Microscopy image showed destruction of the sperm in the UV irradiated sperms. The sperms abnormalities could be identified by damage on malformed bodies or by a bent, short or missing spike. The results indicated that viability of sperms generally decreased with increased UV dose and time of exposure. Although, the sperm abnormalities present in all treatments with morphologically missing spike, bent spike and malformed body but still the number of abnormal sperm present was less critical. The application of comet assay for detecting the DNA damage was applied on ultraviolet irradiated sperm of *F. merguiensis*. Sperms DNA damage was highly scored at 60 and 80 sec of ultraviolet irradiation exposures.

Key words: *Fenneropenaeus merguiensis*, UV irradiation, sperm viability, sperm morphology, comet assay

INTRODUCTION

*Fenneropenaeus merguiensis* is an important catch in Malaysia, commercially important cultured marine shrimp especially in west coastal water of Peninsular Malaysia. *Fenneropenaeus merguiensis* which also have been known as banana shrimp is one of the important penaeid species in Malaysia due to its good price and faster growth. *Fenneropenaeus merguiensis* also known as udang kaki morah can grow up to a maximum standard length of 24.0 cm (Gibson et al., 2005) and normally harvested from the wild. However, the number of wild broodstocks were decreasing due to rapid harvest activity from natural resources. By culture the *F. merguiensis*, wild shrimp source dependable could be minimize.
Serious occurrences of disease outbreaks occurred to *Penaeus monodon* and *Litopenaeus vannamei* in captivity has also trigger the shrimp industry to search for alternative local shrimp species. Then, farming the *F. merguiensis* is expected to become an alternative resource for the shrimp industry. According to Hoang et al. (2002), *F. merguiensis* is a good candidate for prawn farming and domestication or selective breeding program. However, the market demand caters for uniform sizes. Thus monosex shrimp farming would be a great effort to produce shrimp with larger tails (Gomelsky, 2003). Producing all female penaeid shrimp populations is required especially in countries in which economically valuable crustaceans constitute an important source of income (Sagi and Aflalo, 2005) reducing mortality due to cannibalism, minimizing labour requiring for size grading and eventually providing higher return on investment (Felip et al., 2001; Hulata, 2001).

Biotechnology was widely applied in aquaculture including gynogenesis. Gynogenesis is a technique used to produce all female postlarvae. The first step is producing a gynogenetic haploid, which requires the inactivation of sperm genomic DNA by UV irradiation meanwhile keeping the spermatozoa able to activate the eggs. The second step is to re-establish diploidy in the eggs by blocking the extrusion of second polar body by external shock (thermal, pressure or chemical) (Devlin and Nagahama, 2002). Gynogenesis has been established in many fish species (Yamamoto, 1999; Felip et al., 2001; Piferrer et al., 2004; Luckenbach et al., 2004) but not yet in *F. merguiensis*. However, to produce gynogenesis larvae of *F. merguiensis*, inactivation of sperm DNA should be focus to ensure production of 100% female postlarvae.

In this study, ultraviolet irradiation had been applied due to lower cost and easy maintenance. Good sperms quality must be obtain to produce good quality and quantity larvae. Consequently, the objectives of this study is to determine the effect of different UV induction on *F. merguiensis* sperms quality for initial development of gynogenesis protocol through; (1) Viability test, (2) External morphological changes and (3) DNA damage of comet assay.

**MATERIALS AND METHOD**

**Animals:** Mature male of banana shrimp (*F. merguiensis*) were captured from Kedah coastal water, Malaysia (5°39"N, 100°19"E) then transported to hatchery Institute of Tropical Aquaculture, Universiti Malaysia Terengganu where they were maintained in circular 500-l tanks. Shrimp were fed with fresh squid (*Loligo sp.*) with 10% biomass daily twice a day, during morning (0600 h) and evening (2100 h). Water salinity and temperature in the maturation tank was maintained at 28-30 ppt and 28-32°C and water depth 60 cm. Approximately 70% of the tank water was changed daily.

**Spermatophore collection:** Males with milky whitish spermatophore were selected from the maturation tank. Spermatophore located at the base between the 4th and the 5th walking legs. Spermatophores were removed with fine forceps (Memon et al., 2012). Then, the males were released back into the maturation tank.

**UV irradiation on sperms:** Each spermatophore was weighed. To release sperm from the post-thaw spermatophore, the tissue was gently grinded with 1 mL calcium free (Ca-F) saline by using pastel and mortar. The sperms were transferred to 3 cm diameter glass petri dish that
contained Ringer’s solution (1:10) and kept on crush ice to a depth of approximately 1 cm. Spermatophore was exposed to ultraviolet duration 20, 40, 60 and 80 sec by using UV viewing cabinet (Fisher scientific) with wavelength 254 and 365 nm.

**Sperm viability test:** Sperm viability was determined by a modified Eosin-nigrosin staining method (Nimrat et al., 2005; Memon et al., 2012). A sample of this sperm suspension (6 μL) was then micropipetted onto a double neubauer counting chamber haemacytometer that had been covered with a coverslip. The number of sperm in each of the five larger squares on the haemacytometer was counted. There are 25 of these larger squares has 16 smaller squares within it. Sperm were counted in the four large corner squares and the large center square. The mean number of sperm per large square count (i.e., mean of the five counts) was multiplied by 25 (to obtain the mean per 5×5 large-square grid) by 10 (the depth of the chamber in mm) and then the initial volume of the sample to estimate the sperm viability (Memon et al., 2012). Viable sperms were observed microscopically and estimated visually under (Fig. 1) Advance Research Microscope (EPI-Fluorescence; Nikon 80i, Japan) and then determined by following equation:

\[
\text{Live sperm} (\%) = \frac{\text{Total number of live sperms observed}}{\text{Total number of overall sperms observed (live+dead)}} \times 100
\]

**Sperm morphology assessment:** The viable sperms samples were fixed following modified Ro et al. (1990) method. Coated cover slips were short dipped into poly L-lysine solution (0.01%) and dried completely at room temperature. Cell fixation was carried out using 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 for 30 min and then rinsed with buffer for 10 min with two changes. Sperms cell suspension were drop onto the coated cover slips. After 10 min, cell suspension on coated cover slips were fixed with 1% osmium tetraoxide, pH 7.4 for 30 min at room temperature,
washed in deionized water for 10 min with two changes at room temperature, after that dehydrated in 50, 70, 95 and 100% of ethanol series and HMDS dry agent. Samples were mounted on the stub then coated with gold for the Scanning Electron Microscope observation (scanning electron microscope; JEOL 6360 LA Tokyo, Japan). About 50 cells were used to distinguish the morphology assessment of irradiated sperm.

**Comet assay:** DNA damage on irradiated viable sperms samples from the viability test were evaluated following modified Singh et al. (2003) method. Briefly, sperm cells were mixed with melted; low melting agarose and an aliquot of the sperm mixture were transferred to frosted end microscope slide. The prepared slides were stored in cold lysing solution (4°C) for 1-48 h before transferring to warm lysing solution (37°C) with proteinase-K for 2-24 h. Following cell lysis, all slides were washed three times with deionized water at 10 min interval to remove salt and detergent from the microgels. All slides were transferred into electrophoresis unit for equilibration for 20 min and then electrophoresed (20 min, 12 V, ~250 mA) at 100 mL min⁻¹ and DNA was allowed to unwind in alkaline solution. After electrophoresis, slides were neutralized in neutralizing buffer for 30 min and DNA fluorochrome SYBR Safe was applied.Slides were covered with cover slips and were viewed and analyzed using Advance Research Microscope (EPI-Fluorescence; Nikon 80i, Japan). The resulting images were captured. Comets were scored visually using a scale from 0-4 (Green et al., 1996). Approximately 25 comets were measured per sample. For comet image scoring, the original comet image scoring by Collins (2002) had been referred.

**Statistical analysis:** All measurements were subjected to one way analysis of variance (ANOVA) to determine differences among treatments and Tukey's test to determine, which treatments were significantly different from one another. The level of significance of the results was set at p<0.05 and data is reported as Mean±SD.

**RESULTS AND DISCUSSION**

**Mean viability assessment of irradiated sperm:** Figure 2 show the effect of different dose and duration of UV light exposure on sperm viability. Thinly spread semen diluted to 1:10 of Ringer's solution allowed adequate penetration of UV light. Thinly spread dilute milt is likely critical to providing sperms full exposure to UV light needed to inactivate the DNA. In this study, percentage of viable sperm was evaluated from a maximum of 100 sperm cells from each slide. Higher UV exposure treatments were significantly decreased sperm viability. However those treatments were effective in inactivated the sperms DNA. Treatments with 20 and 40 sec of UV irradiation with 254 nm produced 81.67 and 81.07% of viability respectively (Table 1). The other treatments also showed high viability percentages except for the 80 sec treatment for dose 365 nm that was obtained significantly at lower viability as compared to control (p<0.05). More sperms died when exposed to higher doses and 365 nm wavelength UV light through the spermatophore in penaeus orientalis kishinoue for the duration of 5-8 sec (Chen et al., 1997).

**Sperm morphology assessment of irradiated sperm:** Observation on sperm morphology using three different microscopes shows that the sperms consists almost spherical shape main body (head) and short spike (tail) extends from the main body (Fig. 3). About 50 viable sperm cells were used
Fig. 2: Mean viability percentages of *Fenneropenaeus merguiensis* sperms with different UV duration and wavelength. Different letters indicate significant difference among treatments (p<0.05).

Fig. 3(a-d): Sperms morphology of *Fenneropenaeus merguiensis* observed with scanning electron microscope (JEOL 6380 LA Tokyo, Japan), (a) Normal sperm with spike in spermatophore structure, (b) Abnormal sperm with malformed head and spike in spermatophore structure and (c, d) Normal sperms
Table 1: Mean viability percentages of irradiated sperm using sets of male spermatophore of *Penneropenaeus merguiensis*

<table>
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<tr>
<th>Treatment</th>
<th>Mean viability from right spermatophore</th>
<th>Mean viability from left spermatophore</th>
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<tbody>
<tr>
<td>Control</td>
<td>98.00±1.000</td>
<td>98.00±1.000</td>
</tr>
<tr>
<td>20 sec 254 nm</td>
<td>81.07±0.033</td>
<td>81.07±0.023</td>
</tr>
<tr>
<td>20 sec 365 nm</td>
<td>81.00±0.050</td>
<td>78.09±0.013</td>
</tr>
<tr>
<td>40 sec 254 nm</td>
<td>81.07±0.050</td>
<td>81.07±0.013</td>
</tr>
<tr>
<td>40 sec 365 nm</td>
<td>78.93±0.082</td>
<td>78.38±0.030</td>
</tr>
<tr>
<td>60 sec 254 nm</td>
<td>77.58±0.018</td>
<td>77.58±0.019</td>
</tr>
<tr>
<td>60 sec 365 nm</td>
<td>76.00±0.010</td>
<td>76.47±0.022</td>
</tr>
<tr>
<td>80 sec 254 nm</td>
<td>76.87±0.029</td>
<td>76.87±0.008</td>
</tr>
<tr>
<td>80 sec 365 nm</td>
<td>70.00±0.022</td>
<td>72.40±0.007</td>
</tr>
</tbody>
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Table 2: Sperm morphology assessment from irradiated sperms of *Penneropenaeus merguiensis*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Right spermatophore</th>
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<th>Left spermatophore</th>
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<tbody>
<tr>
<td></td>
<td>Normal sperm</td>
<td>Abnormal sperm</td>
<td>Normal (%)</td>
<td>Normal sperm</td>
</tr>
<tr>
<td>Control</td>
<td>49</td>
<td>1</td>
<td>98</td>
<td>49</td>
</tr>
<tr>
<td>20 sec 254 nm</td>
<td>41</td>
<td>9</td>
<td>82</td>
<td>41</td>
</tr>
<tr>
<td>20 sec 365 nm</td>
<td>40</td>
<td>10</td>
<td>80</td>
<td>39</td>
</tr>
<tr>
<td>40 sec 254 nm</td>
<td>39</td>
<td>11</td>
<td>78</td>
<td>39</td>
</tr>
<tr>
<td>40 sec 365 nm</td>
<td>39</td>
<td>11</td>
<td>78</td>
<td>38</td>
</tr>
<tr>
<td>60 sec 254 nm</td>
<td>39</td>
<td>11</td>
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<td>60 sec 365 nm</td>
<td>39</td>
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<td>78</td>
<td>37</td>
</tr>
<tr>
<td>80 sec 254 nm</td>
<td>35</td>
<td>15</td>
<td>70</td>
<td>35</td>
</tr>
<tr>
<td>80 sec 365 nm</td>
<td>32</td>
<td>18</td>
<td>64</td>
<td>35</td>
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</table>

To distinguish the morphology assessment of irradiated sperm. The overall sperm were in length about 10 μm. The sperm structure of *F. merguiensis*, particularly in the spike possessing tubular-like structure (Memon et al., 2012). The external morphology of abnormal sperms was found with malformed head and spike lost or bent which is same as was reported by Wang et al. (1995) and Leung-Trujillo and Lawrence (1987). Table 2 shows the number of abnormal sperm increased significantly to higher UV light exposure. The number of abnormal sperm present were critical except for control and treatment, or 20 sec at 254 nm. Total numbers of normal and abnormal sperms in sets of spermatophore (right spermatophore and left spermatophore) were not significantly difference. The lowest percentage of normal sperms were found in treatment for 80 sec at 365 nm with 67% (Fig. 4).

**Comet assay:** In this study, comet assay analysis was used to detect DNA damage occurred on ultraviolet irradiated sperm cells. The comet assay is a sensitive rapid method for DNA strand break detection in individual cells (Fairbairn et al., 1995). Advantage of comet assay is its application to any eukaryotic organism and cell types and give results within a few hours (De Silva et al., 2000). In this study, the irradiated sperm cells showed comet tails but the images on the microscope were blurry and hazy due to thick microgel coating and spermatophore tissue. There was significantly greater DNA damage score in the irradiated sperms than in the control group (Table 3). The SCG method was sufficiently sensitive to detect naturally occurring level of UV irradiation in this study. Nuclei from higher UV dose and exposure gave distinctive longer comet tailing. The treatments
Fig. 4: Mean normal sperm percentages of *Fenneropenaeus merguiensis* exposed to UV radiations at different durations and wavelengths. Different letters indicate significant difference among treatments (p<0.05)

Fig. 5: Comet tailing pattern was performed after exposure of *Fenneropenaeus merguiensis* sperms under UV irradiation. Each nucleus was scored according to Collins (2002)

60 sec 365 nm, 80 sec 254 nm and 80 sec 365 nm had highest scores (Class 4). The percentages of nuclei with scores more than 2 were 20, 28, 28, 36 and 48% for treatment 40 sec 365 nm, 60 sec 254 nm, 60 sec 365 nm, 80 sec 254 nm and 80 sec 365 nm, respectively (Fig. 5). By determining DNA damage level on irradiated sperms cell, it is proven that no genetic from male will be transfer to the gynogens larvae.
### Table 3: Scoring for comet assay

<table>
<thead>
<tr>
<th>Damage score</th>
<th>Comet assay image</th>
<th>Description</th>
</tr>
</thead>
</table>
| Score 0      | ![Image](image)   | Head: Normal nuclei size  
Tail: no tail  
Relative head intensity: 100%  
Relative tail intensity: None |
| Score 1      | ![Image](image)   | Head: same as above  
Tail: very short tail, shorter than head size  
Relative head intensity: >75%  
Relative tail intensity: <25% |
| Score 2      | ![Image](image)   | Head: same as above  
Tail: tail length same with head  
Relative head intensity: 50% - 75%  
Relative tail intensity: 25% - 50% |
| Score 3      | ![Image](image)   | Head: More than half size of normal nuclei  
Tail: tail length a bit longer than head size  
Relative head intensity: 20% - 50%  
Relative tail intensity: 50% - 75% |
| Score 4      | ![Image](image)   | Head: < half size of normal nuclei  
Tail: long and wide extend from head size  
Relative head intensity: <25%  
Relative tail intensity: >75% |

Comet assay was performed after exposure of *Penaeopenaeus merguiensis* sperms under UV irradiation. Scoring for comet assay was based on description given by Collins (2002).

**CONCLUSION**

High percentages of sperms viability, more physical damage and DNA damage occurred on the structure of sperms when exposed to higher dose levels and longer durations of UV. Treatment with exposure 60 sec 365 nm gave the best result with 76.2% of mean viability percentage, produced 76% of normal sperm morphology and obtained high scores (score 3 and 4) of sperm DNA damage. By determine viability, morphology and DNA damage of treated sperms with UV irradiation, the best sperms will be used in the first step of gynogenetic application.

**ACKNOWLEDGMENTS**

This study was supported by Exploratory Research Grant Scheme (ERGS) ERGS/1/2013/STG07/UMT/02/01 from the Ministry of Higher Education (MOHE). The authors wish to thank all the members and staff contributed to this project.
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