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Sperm Quality Assessment of White Shrimp (*Litopenaeus Vannamei*) Broodstock using Comet Assay

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Abstract

The quality of sperms is considered one of the factors determining fertilization success of white shrimp, *Litopenaeus vannamei*. Generally, conventional methods such as sperm count and viability are used for determining sperm quality. Occasionally, the fertilization rate does not correlate with what conventional parameters indicate. Presently, it has become increasingly evident that sperm DNA damage is a potential cause of an unexplained fertilization incompetence of the sperm. Therefore, the present study aimed to determine the feasible application of the comet assay for measuring sperm quality from different male broodstock and to analyze the correlation between sperm DNA integrity and fertilization efficacy of the shrimp. Male and female shrimp sampled from 4 different broodstock ponds were mated in experimental tanks (n = 5). Sperm were collected and subjected to alkaline comet assay. Sperm count, sperm viability, and Gonadosomatic index(GSI) were analyzed for comparison. Viability of the obtained nauplii were recorded and used as larvae production efficiency. The results revealed that nauplius numbers were significantly different among treatments ($6.78\pm1.61\times10^4$, $12.18\pm1.17\times10^4$, $16.76\pm0.97\times10^4$ and $21.38\pm1.14\times10^4$ cells/spawn in treatment 1, 2, 3 and 4, respectively), indicating the different fertilization rate between treatments. The average body weights, lengths, and GSIs of broodstock were not significantly different, indicating that the morphological factors were not major causes of the different nauplii production among treatments. The results of comet measurements clearly showed that the levels of comet parameters correlated with the numbers of nauplii obtained from each treatment while the conventional sperm analyses were unable to detect the different quality of the sperm among treatments. This indicates that the degree of sperm DNA damage correlates with the fertilization rate of the shrimp and can be used as a sensitive indicator for determining sperm quality of broodstock shrimp.

Keywords: *Litopenaeus vanamei*, Captive breeding, Comet assay, Sperm quality assessment, DNA damage

Introduction

Ever since the spawning of penaeid shrimp was achieved using wild-caught broodstock and following the discovery of unilateral ablation for promoting maturation, commercial culture of *L. vannamei* and other penaeid shrimp has increased rapidly in most coastal countries from Central and South America and throughout Asia [1-3]. Subsequent development of domestication programs of *L. vannamei* have been established successfully in most areas [4,5]. However, the drawback of using domesticated broodstock is the low reproductive performance [6-8]. To overcome this problem, most shrimp reproduction research has focused mainly on female maturation [9-14] while the impact of male reproduction is still uncertain. Currently, increasing evidence suggests that the reproductive potential of males also plays an important role in the productivity of captive penaeid shrimp [15-19].

The quality of sperms is one of the factors determining the success of fertilization, and thus the selection of appropriate male broodstock with optimal sperm quality for reproduction is highly desirable. Sperm quality in penaeid shrimp was firstly evaluated by determining sperm count and percentage of live and abnormal sperm [20]. The same parameters have still been used regularly for studying sperm quality under a wide variety of conditions [18,21-27]. Although these conventional analyses give considerable information, it does not assess the damage of genetic materials in spermatozoa, which may be partially responsible for the low fertilization. Numbers of studies have reported the correlation of sperm quality with their physical appearances such as number, size, and age [28-31], very few studies describing sperm quality in relation to DNA integrity of penaeid shrimp were reported. Although, the healthiest sperm (with intact DNA) will ideally fertilize the egg, it is more likely that sperm with normal appearance but contain partially damaged DNA may also accomplish fertilization and eventually result in impaired larval development [32, 33] which could be the potential cause of a decline in the hatching rate. Increasing evidence has confirmed that sperm DNA damage can be a significant cause of an unexplained fertilization incompetency [34-37]. Therefore, alternative parameters are required to determine the actual fertilization performance.

Among quantitative methods used for examining DNA damage, the comet assay has become the method of choice for analyzing DNA strand breaks in most cell systems of various organisms [38-41]. The comet assay, also known as single-cell gel electrophoresis, is based on the ability of damaged DNA from a single cell to migrate out of the cell under electrophoresis while the intact DNA remains within the cell. This creates a comet-like shape after staining with fluorescent dye. The damage of DNA is determined by the relative intensity of the fluorescence pattern of the comet. Various patterns for evaluating and quantifying comet formation have been adopted. These include % DNA in head, % DNA in tail, tail length, head diameter, %tail moment, mean heat intensity, mean tail intensity, mean comet intensity, and many more [42,43].

Many studies have demonstrated that DNA fragmentation of germ cells from a number of invertebrates can be reliably detected using the comet assay [44-48]. Shrimp sperm integrity can be easily disturbed by various environmental factors and husbandry practices which may potentially impact fertilization success [49-51], therefore, the quality of sperm cannot be verified entirely by external morphological characteristics. The present study aimed to determine the feasible application of the comet assay for measuring sperm quality of *L. vannamei* from different culture conditions, and to analyze the influence of sperm DNA integrity on fertilization efficiency in *L. vannamei* male broodstock using the comet assay in comparison with conventional techniques.

Materials and methods

Animal preparation

Male *L. vannamei* broodstock (42 - 45 g bodyweight) obtained from 4 different commercial farms, which were located within eastern and southern areas of Thailand. Hundred shrimp from each farm were reared separately in 4 indoor maturation tanks (5×5 m^2) while female broodstock were collected from 1 farm where their qualities were presumably similar and reared altogether in another tank (n = 100). Both males and females were not previously used as broodstock. Each tank contained seawater at the depth of 60 cm and salinity of 30 psu at ambient temperature (27 - 29 °C). Water was 100 % flow-through exchanged daily. All broodstock were fed 6 times a day (06.00, 09.00, 12.00, 15.00, 18.00, 21.00 h) with commercial feed pellet (80 %) mixed with polychaete (*Perinereis sp*.) (10 %) and squid (*Loligo vulgaris*) (10 %).

Shrimp breeding condition

All male broodstock from 4 separate maturation tanks were tagged using embossing tape glued to the carapace and subjected to spermatophore extrusion (n = 5). Both spermatophores present in the terminal ampulae were removed manually and the regeneration of spermatophore was monitored. Mature females from the maturation tank were subjected to unilateral eyestalk ablation. The eyestalk-ablated females from each stock (n = 5) were tagged and transferred to each of male maturation tanks. Mating between males and females was monitored. Immediately after a pair of spermatophores was ejaculated from the male and attached to the abdomen of the female, both male and female were removed from the tank and their weights and lengths were recorded. One of the spermatophores that attached to the telycum of the female was removed and subjected to sperm evaluation processes. Female shrimp with an attached spermatophore were individually transferred to a 200 L spawning tank. After spawning, viable spawns were transferred to 500 L fiberglass tank for hatching. Water quality of these tanks were maintained as similar condition as maturation tank. On the next day, nauplii were randomly collected 3 times using 50 mL beaker. The number of nauplii counted from each sampling was used for estimating the total number of nauplii.

Sperm preparation

The spermatophore collected from the mated female was mixed with dilution buffer (5 mL of Phosphate-buffered saline (PBS) containing 10 % sodium citrate) and homogenized with a glass homogenizer. The spermatophore debris was allowed to precipitate for 5 min at room temperature while spermatozoa suspended in the solution were transferred to a 15 mL tube and used for further examination.

Morphological examination of sperm

Quality and quantity of shrimp sperm were measured according to the methods described by Leung-Trujillo and Lawrence [21]. Total sperm and viable sperm counts were determined using a Neubauer hemocytometer (BOECO, Germany). Sperm viability was determined using a trypan blue exclusion test. The method was carried out by adding the mixture containing spermatozoa with one tenth volume of 0.4 % trypan blue dye in calcium free saline solution (270 - 300 mOsm/L). After 10 min at room temperature, the mixture (150 µL) was loaded onto the hemocytometer. The numbers of dead sperms, identified by the blue color inside the cells, were recorded. Gonadosomatic index (GSI) examined from each experimental group was calculated as the percentage of spermatophore weight per gram body weight of broodstock.

Single cell gel electrophoresis analysis (comet assay)

DNA fragmentation of shrimp sperm was determined using single cell gel electrophoresis. This method was performed according to the alkaline comet assay described by Singh *et al.* [52] with modification. Sperm (1×10^6 cell/mL) in 10 µL of dilution buffer (5 mL of PBS containing 10 % sodium citrate) was mixed with melted 1 % low melting point agarose (80 µL), subsequently layered on the 1 % agarose pre-coated microscope slide, covered with cover slip, and allowed to solidify at 4 °C for 30 min. Then, the cover slip was removed and a 2nd layer of melted 1 % low melting point agarose was placed on top of the solidified layer. Again, the gel was covered by cover slip and left to solidify at 4 °C. After cover slip removal, the slide was subjected to a lysis step by placing the slide into chilled lysis solution (2.5 M sodium chloride (NaCl), 10 mM Tris hydrochloride (Tris-HCl), 100 mM ethylene diamine tetraacetic acid (EDTA), 1 % Triton X-100, 10 % dimethyl sulfoxide (DMSO), 1 % sodium lauryl sulfate (SLS), pH 10) for 1 h at 4 °C. After that the slide was placed in an electrophoresis chamber containing alkaline solution (0.3 M sodium hydroxide (NaOH), 1 mM Na_2EDTA, 0.2 % DMSO, pH >13) for 10 min. Electrophoresis was conducted using volt constant at 25 V (300 mA) for 30 min. Then, the slide was neutralized by immersing (3 times, 2 min each) in neutralization buffer (400 mM Tris-HCl pH 7.5) and dried by immersing in absolute ethanol followed by distilled water (2 min each) before staining with 50 µL of 20 mg/mL of ethidium bromide (EtBr). The slide was dried at room temperature for 30 min. For DNA damage visualization, microscopic analysis was conducted using an Olympus BX 50 epifluorescence microscope under excitation filter at 510 - 560 nm UV light, barrier filter 590 nm, at ×100 magnification, using an ocular micrometer. Randomly chosen nuclei image ($n \geq 50$) from each slide were categorized into ghost cells (cytotoxic damage cells), comet cells (genotoxic damage cells), and healthy cells (no cytotoxic and genotoxic damage). The comet cells were analyzed using comet score software version 1.5 (TriTek Corp).

Statistical analysis

All quantitative data were expressed as mean ± standard deviation (SD). Statistical differences of male and female body weight, GSI, spermatophore formation, spermatophore weight, average total sperm count, viable sperm, and number of nauplii were estimated by one-way analysis of variance (ANOVA) with normality and homogeneity of variance (SAS version 6.12.0.1). The statistical procedures used for comet analysis was Tritek Comet [Score] Freeware version 1.5 (USA).

Results and discussion

Results

Morphological properties

Morphological data of male and female *L. vannamei* broodstock used in the experiment are shown in **Table 1**. There were no significant differences ($p > 0.05$) in the mean of body weight (g) of males and females between groups 1, 2, 3, and 4 of *L. vannamei* broodstock. There were no significant differences ($p > 0.05$) in average total sperm counts, viable sperms, GSI, and spermatophore formation rate between male broodstock from groups 1, 2, 3, and 4. Similar result of GSI from female broodstock was observed. There was no significant difference between female broodstock from groups 1, 2, 3, and 4 ($p > 0.05$). However, there were significant differences between average numbers of nauplii obtained from broodstock in groups 1, 2, 3, and 4 ($p < 0.05$). The highest number of nauplii was obtained from broodstock in group 4

(21.38±1.14×10^4), followed by group 3 (16.76±0.97×10^4), 2 (12.18±1.17×10^4), and 1 (6.78±1.61×10^4), respectively.

Table 1 External morphological examination data in *L. vannamei* broodstock obtained from groups 1, 2, 3, and 4.

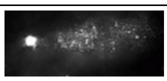
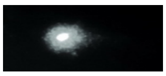
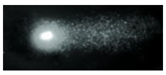
Various parameters	Group 1	Group 2	Group 3	Group 4	F / Pr > F
Male body weight (g)	41.52±2.34^{a}	42.52±1.54^{a}	43.62±1.11^{a}	43.0±0.61^{a}	0.21/0.881
Female body weight (g)	68.24±2.06^{a}	68.06±2.91^{a}	69.90±1.43^{a}	68.82±0.89^{a}	0.40/0.754
Male GSI	0.69±0.15^{a}	0.85±0.03^{a}	0.88±0.05^{a}	0.88±0.04^{a}	0.32/0.807
Female GSI	5.38±0.19^{a}	4.60±0.53^{a}	5.40±0.82^{a}	5.65±0.70^{a}	1.10/0.376
Spermatophore formation rate (day)	5.60±0.55^{a}	4.40±0.55^{a}	4.60±0.55^{a}	3.60±0.55^{a}	1.19/0.346
Spermatophore weight (g)	0.202±0.0^{a}	0.202±0.0^{a}	0.201±0.0^{a}	0.203±0.0^{a}	5.78/0.007
Average total sperm counts (10^6)	4.12±0.65^{a}	4.72±0.94^{a}	4.87±0.25^{a}	4.26±0.31^{a}	0.84/0.489
Viable sperm (%)	65.60±7.04^{a}	63.10±11.95^{a}	62.40±10.58^{a}	61.00±10.30^{a}	0.75/0.538
Number of nauplii (10^4)	6.78±1.61^{d}	12.18±1.17^{c}	16.76±0.97^{b}	21.38±1.14^{a}	0.40/0.717

Data are presented as means (±SD). Different superscripts within rows indicate significant differences ($p < 0.05$).

Comet cell identification

Percentages of dead sperm, healthy sperm, and comet cells are shown in **Table 2**. There were significant differences of the percentages of the comet cells found among the sperms of the experimented shrimp. The highest percentages of comet cells were found in shrimp from groups 1 and 2, while those of groups 3 and 4 were significantly lower than the first 2 groups ($p < 0.05$). Similar result was obtained from the percentages of dead sperm among the experiment groups. In contrast, the highest percentages of healthy cells were found from shrimp in group 4, followed by those of groups 3, 2, and 1, respectively. It appeared that increasing number of healthy cells was corresponding to the number of nauplii while ghost cells and comet cells were in contrast with the number of nauplii. This indicated that the number of comet cells can infer to the fertilization success of the shrimp.

Table 2 Identification of comet cells (percentages of ghost dead, comet cell, and healthy cell) found in the spermatophore of male *L. vannamei* from groups 1, 2, 3, and 4 (n = 100).

Types of comet cells	Images of comet cells	Number of sample cells identified (%)				
		Group 1	Group 2	Group 3	Group 4	F / Pr > F
Ghost cell		32.00±10.20^{a}	27.80±10.73^{a}	14.20±6.06^{b}	9.20±5.22^{b}	0.28/0.838
Healthy cell		38.40±15.34^{c}	37.00±14.27^{c}	70.00±4.06^{b}	82.40±8.29^{a}	0.23/0.873
Comet cell		30.80±10.10^{a}	29.60±10.13^{a}	13.40±6.11^{b}	8.40±4.16^{b}	0.14/0.988

Data are presented as means ±SD. Within rows, different superscripts indicate significant differences ($p < 0.05$).

Comet cell analysis

Comet parameters including % DNA in head, % DNA in tail, tail length, % tail moment, mean comet intensity, and mean head intensity were measured in sperm of male *L. vannamei* broodstock for groups 1, 2, 3 and 4. The results are shown in **Table 3**. There were significant differences between the levels of DNA damage in sperm of males from the 4 groups ($p < 0.05$). Similar results were obtained from all comet parameters which indicated that the highest DNA damage of sperm was observed from males in group 1, followed by groups 2, 3, and 4, respectively. The increasing levels of DNA damage of sperm were in inverse correlation to the decreasing numbers of nauplii obtained from the females mated by the corresponding males in those 4 experiment groups. This indicated that the levels of sperm DNA damage were associated with the fertilization rate of the experimented shrimp.

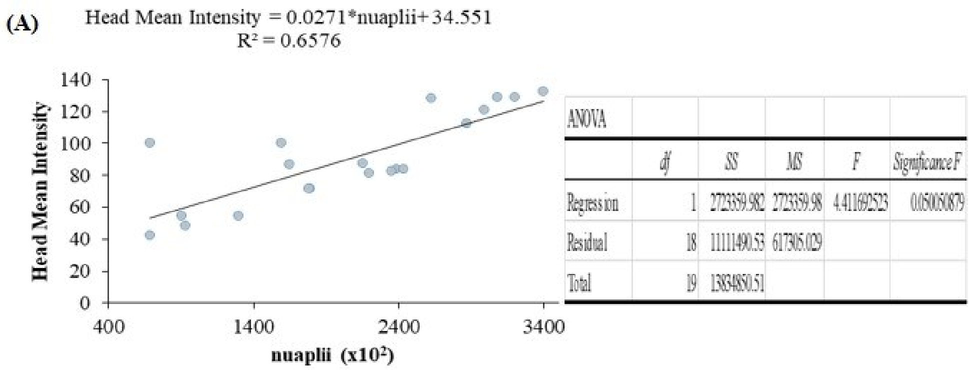
Table 3 Comet parameters including % DNA in head, % DNA in tail, tail length, % tail moment, mean comet intensity, and mean head intensity analyzed in spermatozoa of *L. vannamei* male broodstock from groups 1, 2, 3, and 4.

Comet parameters	Group1	Group 2	Group 3	Group 4	F / Pr > F
% DNA in head	83.1±10.1^{c}	93.5±4.9^{b}	93.7±3.6^{b}	96.7±3.5^{a}	0.04/0.988
%DNA in tail	16.8±10.1^{a}	16.8±10.1^{a}	6.2±3.6^{b}	3.2±3.5^{c}	0.26/0.851
4Tail length	110.708±97.93^{a}	77.848±41.27^{b}	50.988±18.17^{c}	44.18±21.83^{c}	0.33/0.806
% tail moment	23.93±16.87^{a}	6.10±1.18^{b}	3.17±0.28^{c}	1.52±1.016^{c}	0.11/0.952
Mean comet intensity	115.274±28.89^{a}	94.17±22.82^{b}	87.757±11.57^{c}	70.93±26.08^{d}	0.46/0.717
Mean head intensity	59.957±26.21^{d}	82.441±12.04^{c}	88.477±23.39^{b}	112.06±29.81^{a}	0.75/0.539

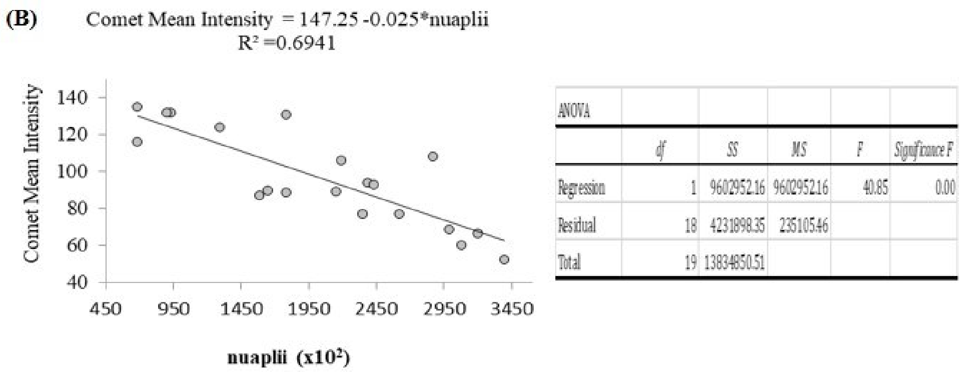
Data are presented as means (± SD). Different superscripts within row indicate significant difference ($p < 0.05$).

Correlation between comet parameters and number of nauplii

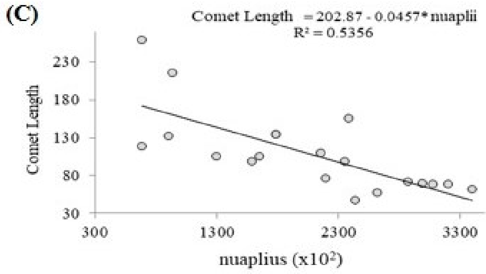
Correlations between comet parameters including head mean intensity (**Figure1(A)**), comet mean intensity (**Figure1(B)**), comet length (**Figure1(C)**), and comet area (**Figure1(D)**) of sperm of male *L. vannamei* broodstock and numbers of nauplius were analyzed and showed in **Figure 1**. All 4 comet parameters were correlated with numbers of nauplius produced by males from 4 experiment groups ($p < 0.05$). Head mean intensity, was positively correlated while comet mean intensity, comet length, and comet area were negatively correlated to number of nauplius ($p < 0.05$).



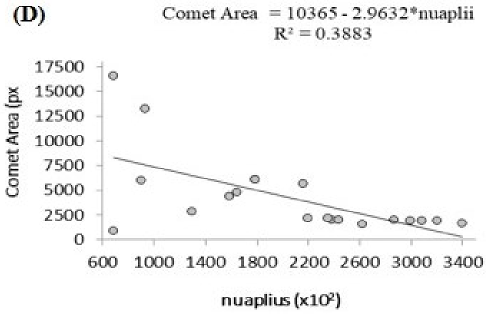
ANOVA					
	df	SS	MS	F	Significance F
Regression	1	2723359.982	2723359.98	4.411692523	0.050050879
Residual	18	11111490.53	617305.029		
Total	19	13834850.51			



ANOVA					
	df	SS	MS	F	Significance F
Regression	1	9602952.16	9602952.16	40.85	0.00
Residual	18	4231898.35	235105.46		
Total	19	13834850.51			



ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	741011558	741011558	20.76	0.00
Residual	18	642473493	35692972		
Total	19	1383485051			



ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	537211369	537211369	11.43	0.00
Residual	18	846273682	47015205		
Total	19	1383485051			

Figure 1 Analysis of correlation between comet parameters including comet mean intensity (A), head mean intensity (B), tail mean intensity (C), and percentage of tail moment (D) of sperm from male *L. vannamei* broodstock and number of nauplii. Data are presented as means ± SD.

Discussion

Reproduction plays crucial roles in aquaculture. The quality of male and female gametes holds the key to reproductive success [53]. Gamete quality is highly vulnerable because they can be influenced by many factors which are not always well characterized. In this study, sperm quality was assessed in male broodstock obtained from 4 different environmental conditions using the comet assay and in comparison, with conventional sperm analyses. Female broodstock with proximities of weight, length and ovary stage were carefully selected to prevent discrimination caused by physical variations.

Sperm quality has been typically assessed by morphological analyses such as sperm counts, viability, and mobility for decades. However, sperm quality of decapods is difficult to measure due to numbers of significant differences between the structure of decapod spermatozoa and other marine invertebrates. Most decapod spermatozoa have no flagella, are non-motile, have unique acrosome vesicle and nuclear composition, and mitochondria are degenerate in mature sperm cells [54-57]. In the case of *L. vannamei*, they are classified as unistellate spermatozoa with non-motile spike [58,59]. These make sperm quality of the shrimp assessed by the aforementioned techniques difficult when compared to other groups of animals [60]. It is confirmed by the results of this study where the morphological parameters including total sperm count, sperm viability, GSI, spermatophore weight, and spermatophore formation rate fail to indicate the differences of sperm quality among treatments while the different nauplii production from 4 experimental groups clearly shows that there are significant differences in the quality of broodstock among the experimental groups.

In this study, the results of comet measurements clearly show the significant differences among treatments and the levels of comet parameters were correspondent to the numbers of nauplii obtained from each treatment. This indicates that the degree of sperm DNA damage inversely correlates with the fertilization rate of the shrimp. This result is in agreement with previous reports showing that sperm DNA fragmentation is associated with fertility rate of the shrimp [47,48,61,62]. The studies provide evidence that DNA damage within sperm could potentially affect the ability of the sperm to properly fertilize an egg, leading to lower fertilization success.

In the comet assay process, DNA damage is determined by the amount of DNA that moves away from the nucleus under electric charge. The alkaline comet assay [52] and modifications [63] can detect a broad spectrum of DNA damage including DNA single- and double-strand breaks, alkali-labile sites, and crosslinks (DNA–DNA or DNA–protein). It is a sensitive method and is recommended for genotoxicity testing [18,64-66]. The comet assay is a simple, rapid, and sensitive techniques for detecting DNA damage which can be analyzed at the level of the individual cell with small amounts of sample, and the cost of performance is considerably low. Additionally, advanced image analyses greatly facilitate the possibility of comet measurements in a variety of applications [39,67,68]. It is to be noted that the comet assay also has limitations. There are some concerns over its accuracy, reliability, and comparability between laboratories [67,69-71]. Thus, standard methodologies and proper interpretation of the comet assay are important when the results between a wide variety of applications from different laboratories are to be compared [71,72].

In visual scoring methods of comet assay, comet cells are generally categorized into 4 and 5 classes based on gradual increase in the length and intensity of the comet tail in parallel with a decrease in the nuclear DNA content. This scoring technique is very useful in situations like acute toxicity in the environment or the exposure of severe pollutants where DNA damage is relatively high [73-75].

In this study, all comet parameters mentioned above provide similar results. Mean comet intensity and mean head intensity shows higher sensitivity than the other parameters as they identify completely different results of DNA damage between 4 samples. The visual identification of comet cells obtained in this study (30.8, 29.6, 13.4, and 8.4 % in groups 1, 2, 3, and 4, respectively) also demonstrates considerable efficacy on detecting the different degrees of DNA damage from the samples. These results indicate that visual identification and computer-based image analysis provide comparable results for determining shrimp sperm integrity.

It is important to note that ghost cells (also called cloud or hedgehog cells), which are comet cells with no or small head and a large bright tail, may arise when performing the comet assay. These atypical comets are considered to be necrotic and/or early apoptotic cells that occur under alkaline condition during sample collection and preparation [75-78]. This kind of cell death could lead to DNA degradation and the comet assay has the potential to detect both cytotoxic and genotoxic effects. To avoid misleading conclusion of the experiment that emphasize on detecting genotoxicity, it is recommended that the number of ghost cells should be recorded, especially if they are significant difference between samples [66,67].

The number of ghost cells in this study were significantly different between samples (32.0, 27.8, 14.2, and 9.2 % in groups 1, 2, 3, and 4, respectively). The numbers of ghost cells were in contrast with the numbers of nauplii indicating that the effects on fertilization rates of the tested shrimp may be influenced by both cytotoxic and genotoxic factors. When the percentages of comet and ghost cells between samples are compared, genotoxic factors appear to be the main effect on sperm integrity of the shrimp. Sperm viability measured in this study showed no significant difference between samples (65.6, 63.10, 62.4, and 61.0 % in groups 1, 2, 3, and 4, respectively), indicating that sperm samples from all groups were in satisfactory condition for the assay.

Conclusions

In the present study sperm quality assessments were carried out in male broodstock from 4 different culture conditions. The comet assay was analyzed in comparison with conventional sperm analyses including sperm count, sperm viability, spermatophore weight and formation rate, and GSI. The results of conventional parameters revealed no significant differences of sperm morphological properties obtained from experimented male broodstock while the numbers of hatched nauplii were significantly different among experimented broodstock. This indicates that the different hatching rate and fertilization success obtained from different broodstock are the influences of cellular and/or molecular integrities of the sperms. While conventional sperm analyses are unable to detect the differences of sperm quality among the male broodstock, comet parameters have shown significant variations of sperm characters among the experimented broodstock and these variations are in correlation with spawning rates. Therefore, these results indicate that comet assay can be an alternative and effective method for detecting the sperm quality and assessing the fertilization success of male broodstock shrimp. The application of comet assay in assessing sperm quality of the male broodstock shrimp will benefit shrimp broodstock production.

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