Development of Sperm MTT Assay for Its Application in Boar Semen

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ABSTRACT

The MTT assay is one of superior evaluation methods widely used to analyze the viability of metabolically active cell. It can be used to determine the percentage of viable sperm through measurement of the reduction of MTT granules at mitochondria in sperm tail. The purpose of this study is to determine the optimal condition of a simple and easy MTT assay to validate boar sperm viability and compare the accuracy of this test with microscopic examination. The MTT reduction rate for sperm viability were analyzed in microtiter plates (96 well) from 1 hr to 5 hr incubation periods at 37℃ using spectrophotometer (microplate reader) at 550 nm wavelength. The remainder of semen sample was simultaneously examined to compare the correlation of accuracy between MTT assay and other sperm parameters. Those sperm parameters were included the motility, survival rates, membrane integrity, mitochondria activity and acrosome integrity. The OD values of MTT assay (MTT reduction rates) did not greatly change at 1 hr to 5 hr incubation periods in different proportion of live and freeze-killed sperms (dead sperm). The MTT reduction rates or survival rates were decreased according to the different concentration of live and dead sperm. The linear regression at 1 hr and 4 hr incubation periods in sperm MTT assay was $y = 291.55x - 72.176$ and $y = 180.64x - 44.569$, respectively. There are high correlation between 1 hr and 4 hr incubation periods ($p<0.001$). The results of MTT assay and other sperm parameters has a positive correlation ($p<0.01$ or 0.05). The correlation coefficients for MTT assay was 0.88115 for motility, 0.89868 for survival rates, 0.91722 for membrane integrity and 0.77372 for acrosome integrity, respectively. In conclusion, the MTT assay can be used as a reliable and efficient evaluation method for boar sperm viability. It can be use practical means to evaluate the quality of boar sperm by a fast, inexpensive and easy method.

(Key words : MTT assay, boar sperm, viability, membrane integrity, mitochondria activity)

INTRODUCTION

The most ideal methods for analyzing of semen quality are to predict accurately, objectively, rapidly and inexpensively the fertilizing ability of semen sample. Most laboratory methods used to evaluate semen quality is basically depend on motilizing property. The semen evaluation methods by the percentage of motile and living sperm using a microscope is fast, easy and inexpensive, but it is subjective and can be influenced by examiner.

To accelerate the accuracy rates for semen quality to be examined, many researchers have been designed the various staining methods either vital stain or fluorescence stain. The various fluorochromes and compounds conjugated to fluorescent probes have made possible a more accuracy means of semen quality analysis than vital stain. However, both staining methods of spermatozoa are also subjective and time-consuming methods which have a limitation to accurately evaluate the sperm viability.

Computer-assisted sperm analysis and combination of organelle-specific fluorescent staining and flow cytometry for the analysis of sperm quality are a more accuracy method to examine a large number of sperm. However it has not been widely accepted because it is very expensive and require special instrument and skilled technician (Gillan *et al.*, 2005).

Therefore, various techniques have been developed to evaluate simply and conveniently the sperm characteristics. Evaluation of metabolic aspect of spermatozoa is one of the useful methods that provide valuable information for predicting fertilizing capacity. There are many methods to estimate the metabolic status, which include the MTT assay, resazurin reduction assay (Zrimsek *et al.*, 2004) and methylene blue reduc-

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tion assay (Chandler *et al.*, 2000). Out of sperm evaluation methods for metabolic measurements, sperm MTT assay measures color change in the tetrazolium reaction to mitochondrial reductase by absorption with spectrophotometer at biochemical and metabolic levels. It is a relatively simple, rapid and reliable method for analyzing the percentage of viable sperms according to the accumulation of formazan grains around the middle piece of sperm tail. The MTT assay have been used to analyze the sperm viability in many species including human, boar, stallion and cattle (Nasr-Esfahani *et al.*, 2002; Gaczarzewicz *et al.*, 2003; Aziz *et al.*, 2005; Aziz, 2006; Iqbal *et al.*, 2010).

The objective of this study was to determine the optimal conditions of MTT assay for evaluating the viability of boar semen and to confirm the accuracy when compared to other evaluation methods.

METERIALS AND METHODS

1. Semen Preparation

Sperm-rich fractions were collected from three pure breed (Duruc, Yorkshire and Landrace) with 85% motile sperm by the grove hand method at the Wonju AI center. After collection, semen was diluted with semen extender (Gene sperm®) and transported to the laboratory within 2 hr at 17℃. All of the experiments were repeated at least three times with semen samples from different boars. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (U.S.A) and were analytical grade.

2. Semen Analysis

The microscopic analysis of semen quality was evaluated based on the motility, survival rates, membrane integrity, mitochondrial activity and acrosome integrity, and examined viability by MTT assay(Jang *et al.*, 2010). A total 200 spermatozoa with two different semen samples in each evaluation method were observed at 400× magnification under an inverted phase contrast microscope (Nikon, Japan). The experiments were repeated at least three times.

1) Sperm Motility, Survival Rates and Membrane Integrity

Sperm motility was subjectively assessed by visual estimations, which was measured by determining the percentage of spermatozoa showing any movement of the flagellum, considering motility of individual spermatozoa from oscillatory to progressive motion. Sperm survival rate was examined by double fluorescence staining that was stained by Hochest 33342 (HO) and propidium iodide (PI). Briefly, a 100 μl aliquot of fresh semen samples was added 10 μl PI (0.5 mg/ml) and incubated for 5 min, and then 10 μl HO (0.5 mg/ml) was mixed and stained for 10 min at 37℃ in the dark condition. After staining, 10～20 μl of stained spermatozoa suspension was placed on clean slide glass and evaluated immediately at 400× magnification by epifluorescence microscope (Zeiss, Germany) equipped with excitation/barrier filter of 460/500 nm. The judges of sperm survived were classified based on the fluorescence emitted. Viable spermatozoa (live) emitted blue, whereas non-viable spermatozoa (dead) emitted red. Membrane integrity of spermatozoal plasma membrane was evaluated by hypo-osmotic swelling test (HOST). The semen sample was incubated for 30 min at 37℃ followed by mixing a 50 μl semen sample with 1 ml of a hypo-osmotic solution (7.35 g Na-citrate and 13.51 g fructose in 1 l of distilled water). Viable spermatozoa (positive) had coiled or swollen tails after incubation when observed under inverted phase contrast microscope at 400X magnification.

2) Mitochondrial Activity

The percentage of live spermatozoa with functional mitochondria was evaluated using a combination of fluorescent stains, Rhodamin 123 (R123) and PI. For this assay, 3 μl of R123 solution (2 mg/ml) were added to 1 ml of semen sample (20 × 10^6 spermatozoa/ml) and incubated for 15 min at 37℃. Subsequently, semen samples were stained with 10 μl of PI for 10 min at 37℃. Following the second incubation, the suspension (10 μl) was placed on clean slide glass and examined at 400× under epifluorescence microscope equipped with excitation/barrier filter of 490/515 nm for R123, excitation/barrier filter of 545/590 nm for PI. Sperm cells displaying only green fluorescence at the middle piece region of tail were considered viable spermatozoa having strong mitochondria activity. Sperm cell displaying red or faint green fluorescence at middle piece were considered non-viable spermatozoa.

3) Acrosome Integrity

Staining with FITC-PNA (fluorescein-labeled peanut agglutinin) was used for evaluation of acrosome integrity. Briefly, an aliquot (10 μl) of the sperm sample was smeared on the glass slide, air-dried and membrane-permeability with 95% ethanol for 30 sec. A 90 μl aliquot of FITC-PNA (100 μl/ml

in PBS) was mixed with 5 μl of PI (340 mM in PBS, final concentration of 18 mM), and 20 μl of this solution was spread over the smeared slide glass. The slide was incubated in a dark humidified chamber at 4℃ for 30 min. After incubation, the slide was rinsed with cold distilled water and air-dried in the dark at 4℃. The semen samples were evaluated under the epiflourescence microscope (excitation 460~500 nm, emission 550 nm) and classified into three categories: intact acrosome (stained bright green from FITC-PNA at the acrosomal cap), damaged acrosome (stained green and red) and missing acrosome (stained red from PI).

4) Sperm Viability by MTT Assay

Sperm MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay that depend on the ability of metabolically active cells to reduce the tetrazolium salt to formazan was used to evaluate sperm viability. Fresh semen samples of three boar semen with good quality (more than 85% motile sperm) were used in this experiments. Sperm MTT assay was performed to validate the relationship between the MTT reduction rates and sperm viability. The semen samples were washed twice with Hepes-BSA sol. and adjusted to 30×10^6 spermatozoa/ml. The 100 μl of semen samples plus 10 μl of MTT stock sol. (5 mg MTT/mg of PBS) was transferred in each well of 96-well microplate and incubated at 37℃ for 1 hr. After incubation, sperm MTT reduction rates was immediately measured at 37℃ in 550 nm wavelength in a microplate reader (Packard, USA).

3. Experimental Design

Experiment I was performed to examine the accuracy between MTT assay and survival rates, and to obtain the standard curve. After dilution of good quality of fresh boar semen (more than 85% progressive motility), diluted semen divided into two groups; One (viable sperm) was maintained at 37℃, while the others (freeze-killed sperm) was killed, which were plunged three times into liquid nitrogen and thawing at 37℃. Semen samples for analysis were made by mixing of viable and freeze-killed sperms at ratios of 10:0, 8:2, 6:4, 4:6, 2:8 and 0:10, respectively. Semen sample containing different concentration of live and freeze-killed sperm were incubated for 1 hr up to 5 hr and analyzed the MTT reduction rates and survival rates.

Experiment II was designed to examine the relationship between sperm MTT assay and sperm characteristics evaluated by microscopic examination. Sperm characteristics that examined at 1 hr after incubation at 37℃ were included motility, survival rates, membrane integrity, mitochondria activity and acrosome integrity.

4. Statistical Analysis

Statistical analysis of replicated experiment results were used for treatment comparisons and were carried out one-way analysis of variance (ANOVA) using SAS program. Pearson correlation coefficients and regression analysis were used to evaluate the efficiency of the MTT assay for assessment of sperm viability of boar semen. All results were expressed as mean ± S.E.M. A p-value below 0.05 was considered significant.

RESULTS

The MTT reduction rates of the boar sperm with different ratio of live and freeze-killed sperms were indicated in Fig. 1. The MTT reduction rates increased gradually according to the incubation periods from 1 hr to 5 hr in all experimental groups (1 hr, 0.23~0.505; 2 hr, 0.217~0.583; 3 hr, 0.229~0.741; 4 hr, 0.220~0.641; 5 hr, 0.207~0.677). After 1 hr up to 5 hr of the incubation periods, the color changes of MTT from yellow to purple was very clear. Increasing the rates of freeze-killed sperm in semen samples and the lapse of incubation periods resulted in slightly decline in the MTT reduction rates.

The results of sperm MTT reduction rates and sperm survival rates of semen samples which contained different proportions of live and freeze-killed sperm were summarized in Table 1. The OD values of MTT reduction rates and percentage of survival rates were shown a similar trends, indicating that increasing of freeze-killed sperm in semen samples was gradually obtained the decreasing viability, especially high pro-

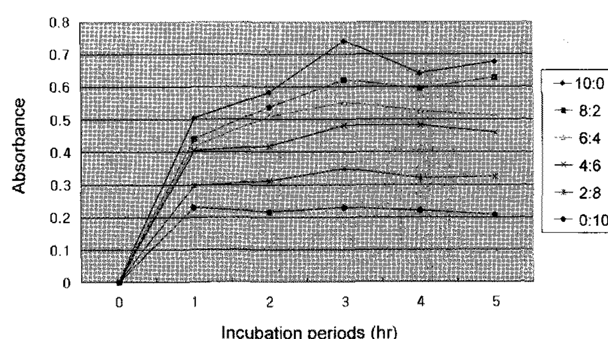

Fig. 1. MTT reduction rates of the semen with different ratio of live and freeze-killed boar sperms. MTT assay was performed after 1 to 5 incubation periods at 37℃ using microplate reader at a wavelength of 550 nm.

Table 1. Comparison of MTT reduction rates and survival rates of boar semen containing different proportions of live and freeze-killed sperm

The ratio of live and freeze-killed sperms	MTT reduction rates[1] (OD[3])	Survival rates[2] (%)
10:0	0.505 ± 0.011	77.3 ± 1.8
8:2	0.440 ± 0.004	60.3 ± 1.8
6:4	0.419 ± 0.004	51.0 ± 1.0
4:6	0.405 ± 0.008	35.7 ± 2.2
2:8	0.298 ± 0.008	10.5 ± 0.5
0:10	0.230 ± 0.005	0.0 ± 0.0

[1] Sperm viability were examined by MTT reduction assay at 550 nm after 1 hr incubation period at 37℃.
[2] Sperm survival rates were evaluated by HO/PI staining methods.
[3] OD : optical values at 550 nm.

portions of freeze-killed sperms ($\geq$ 80%) was shapely decreased both MTT reduction rates (0.298～0.230) and survival rates (10.5～0%).

Two linear regression for relationship between the sperm MTT reduction assay and the sperm survival rates after 1 hr and 4 hr incubation periods were indicated in Figs. 2 and 3, it was applied to calculate the sperm viability against sperm MTT reduction rates. The linear regression at 1 hr and 4 hr incubation periods in sperm MTT reduction assay was $y = 291.55x - 72.176$ and $y = 180.64x - 44.569$, respectively, and both incubation periods in this test had a high corelation (p< 0.001). These two standard curves were used next experiments to obtain the percentage of viable sperm in each sample according to the sperm MTT reduction assay.

The analyzing results for 6 boars obtained by sperm MTT reduction assay at 1 hr and 4 hr after incubation and the results of sperm characteristics such as motility, survival rates, mitochondrial activity, membrane integrity and acrosome integrity were indicated in Table 2. The percentage of viable sperm by MTT reduction assay and the other evaluation methods were shown a similar results, indicating that there were not greatly differ among individuals. Especially, viability by MTT reduction assay measured after 1 hr incubation periods was indicated a similar results of other evaluation methods. The sperm viability by MTT reduction rates calculated against standard curve was slightly decreased at 4 hr incubation period than that of 1 hr incubation period, but OD values at 4 hr incubation period was slightly higher than that of 1 hr incubation period.

To elucidate the relationship between sperm viability by MTT reduction assay and other microscopic examination (motility, survival rate, mitochondrial activity, membrane integrity and acrosome integrity), semen samples collected from 7 boars were incubated 1 h at 37℃ and analyzed the sperm characteristics. Those results were summarized in Table 3 and 4. As shown in Table 3, viability by MTT reduction assay was ranged 53.5 to 96.3%. Sperm viability index such as motility, survival rates and membrane integrity was 73.3～86.7%, 60.0～88.6% and 19.6～35.3%, whereas sperm fertility index such as mitochondrial activity and acrosome integrity was 19.6～33.7% and 75.0～92.3%, respectively. The correlations between the MTT assay and various sperm evaluation methods was analyzed. Among each evaluation methods, the sperm MTT reduction rates posi-

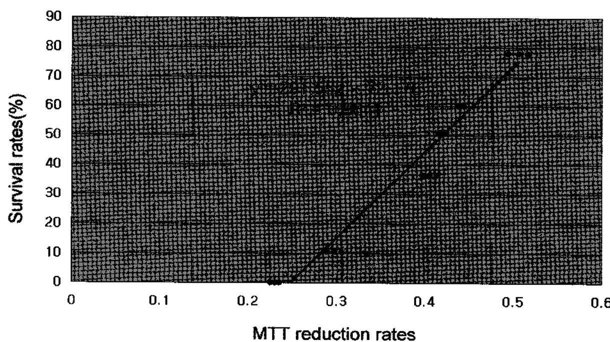

Fig. 2. Linear regression between the MTT reduction rates at 1 hr incubation period and percentage of viable sperm, which was evaluated by HO/PI staining method.

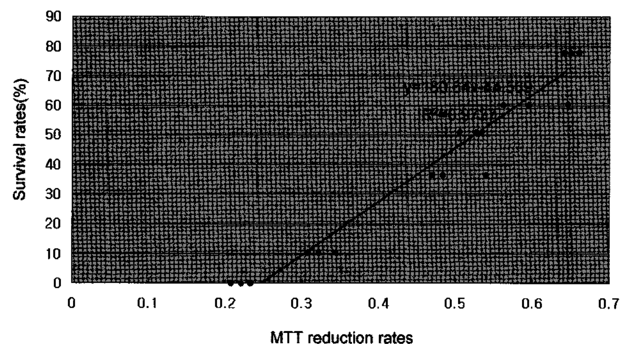

Fig. 3. Linear regression between the MTT reduction rates at 4 hr incubation period and percentage of viable sperm, which was evaluated by HO/PI staining method.

tively correlated in all evaluation methods except for mitochondria activity. The results showed a high correlation (p<0.001) between the sperm viability by MTT reduction assay and motility (r = 0.88115), survival rates (r = 0.89868) and membrane integrity (r = 0.91722), but there were not correlated between sperm viability and mitochondrial activity (r = 0.69481).

Table 2. Comparison of MTT reduction rates and various semen evaluation methods on boar sperm characteristics

Individual number	MTT reduction rates (OD)		Percentage of viable sperms by MTT (%)		Microscopic examination				
	1 h	4 h	1 h	4 h	Motility (%)	Survival rates (%)	Mitochondrial activity (%)	Membrane integrity (%)	Acrosome integrity (%)
1	0.505 ± 0.037	0.611 ± 0.003	75.1	65.8	73.3 ± 4.4	68.0 ± 3.2	77.6 ± 4.9	25.0 ± 2.6	88.8 ± 6.3
2	0.548 ± 0.015	0.674 ± 0.023	87.6	77.2	83.3 ± 2.2	69.8 ± 6.0	71.6 ± 2.1	34.4 ± 4.4	70.0 ± 2.0
3	0.513 ± 0.010	0.613 ± 0.023	77.4	66.2	78.3 ± 2.2	77.5 ± 2.3	79.3 ± 1.1	45.7 ± 1.6	81.5 ± 3.5
4	0.521 ± 0.020	0.653 ± 0.030	79.7	73.4	76.7 ± 2.2	75.3 ± 3.8	79.0 ± 2.5	35.5 ± 0.5	86.8 ± 2.4
5	0.582 ± 0.013	0.799 ± 0.040	97.5	99.8	81.7 ± 2.2	79.0 ± 1.3	86.5 ± 3.5	27.0 ± 2.0	82.0 ± 0.0
6	0.548 ± 0.033	0.707 ± 0.040	87.6	83.1	73.3 ± 2.2	68.0 ± 2.7	85.0 ± 1.3	38.6 ± 0.4	89.0 ± 2.0

Table 3. Analysis of sperm characteristics by various sperm evaluation methods in fresh boar semen

Individual number	MTT assay	Microscopic examination				
	Viability (%)	Motility (%)	Survival rates (%)	Mitochondrial activity (%)	Membrane integrity (%)	Acrosome integrity (%)
1	96.3	86.0 ± 2.7	88.6 ± 1.6	91.3 ± 1.1	35.3 ± 0.4	92.3 ± 0.9
2	53.5	73.3 ± 2.2	60.0 ± 4.0	80.3 ± 3.2	19.6 ± 1.1	75.0 ± 4.0
3	74.5	79.3 ± 2.9	71.6 ± 3.0	79.6 ± 1.7	25.6 ± 1.6	88.0 ± 0.0
4	78.0	85.7 ± 0.9	72.3 ± 1.1	85.3 ± 2.4	30.0 ± 2.0	88.7 ± 1.1
5	74.5	78.3 ± 2.2	69.0 ± 2.0	84.3 ± 1.1	28.0 ± 3.0	85.7 ± 3.8
6	85.8	86.7 ± 2.2	76.0 ± 1.3	89.7 ± 2.1	34.7 ± 3.6	86.0 ± 8.0
7	77.7	80.3 ± 1.8	64.7 ± 2.2	77.3 ± 1.8	33.7 ± 2.5	77.7 ± 3.5

Table 4. Correlation coefficient between MTT assay and various semen evaluation methods on boar sperm characteristics

	Viability	Motility	Survival rates	Mitochondrial activity	Membrane integrity	Acrosome integrity
Viability	–	0.88115**	0.89868**	0.69481	0.91722**	0.77372*
Motility		–	0.78424*	0.73354	0.85293*	0.73131
Survival rates			–	0.83305*	0.67591	0.87125*
Mitochondrial activity				–	0.54602	0.69994
Membrane integrity					–	0.49697
Acrosome integrity						–

Significant level; $^{**}p$<0.01, $^{*}p$<0.05.

DISCUSSION

MTT, an index of cell viability and growth, is based on the ability of viable cells to reduce MTT from a yellow water-soluble dye to a dark-blue insoluble formazan products. The quantity of formazan formed at mitochondria in sperm tail can be estimated using spectrophotometer and the presence of MTT formazan granules in middle piece of sperm identifies that sperm are alive, that is considered as an index of sperm viability and can be predict indirectly the fertilizing ability (Ohtani *et al.*, 2004; Betteridge *et al.*, 2005; Aziz, 2006).

The MTT reduction assay is a spectrophotometric measurement and time dependent method to analysis the cell and sperm viability. In general, sperm has a rich in mitochondria and more active motility than other somatic cells (Twentyman and Luscombe, 1987; Nasr-Esfahani *et al.*, 2002). Accordingly, the sperm MTT assay can be measure faster than somatic cells. In our results, the OD values of MTT assay slightly increased according to the lapse of incubation periods and the score of MTT assay to measure the boar sperm viability decreased greatly according to the increasing ratio of live and freeze-killed (dead) boar sperm. This study also indicated that the results of MTT assay at 1 hr and 4 hr incubaton periods had a high correlation ($p<0.001$). These results are in accordance with the reporting of Nasr-Esfahani *et al.* (2002) and Aziz *et al.*(2005) who MTT reduction rate increased gradually with incubation period and increasing the proportion of freeze-killed sperm resulted significant decrease in MTT reduction rates, and the sperm viability can be evaluate efficiently by MTT reduction assay after 1 or 2 hr of incubation periods. In order to assess the accuracy rates of MTT assay, we compared the accuracy between the viability by MTT assay and other evaluation parameters by microscopic examination with fresh semen of seven boars, which were included motility, survival rates, mitochondrial activity, membrane integrity and acrosome integrity. The present study have obtained a similar results both evaluation methods. The present study indicated that the results of MTT reduction assay was significantly positive correlated to the those results of the motility ($p<0.05$), survival rate ($p<0.05$), membrane integrity ($p<0.05$) and acrosome integrity ($p<0.01$). This results are in agreement with previous findings of Aziz *et al.* (2005) and Aziz (2006) reported that the results of MTT assay showed highly correlated with sperm parameters that were estimated using a flow cytometer. This finding suggest that the MTT assay for boar semen may be used as an index of sperm fertilizing ability in pig. The MTT assay for sperm analysis can not only be a fast, easy and inexpensive methods, but also examine quickly and simultaneously a lot of semen, i.e. more than 10 samples.

In conclusion, present study suggested that MTT assay is superior methods we assess the viability of boar sperm in aspects of practical or easy means. Additionally, the sperm MTT assay revealed to be a reliable and accurate methods in predicting fertilizing ability in pigs.

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