Assessment of bovine sperm viability by MTT reduction assay

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Abstract

The MTT reduction assay depends on the ability of metabolically active cells to reduce the tetrazolium salt (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to formazan. This study was conducted to examine and validate a simple and less costly MTT test to determine bovine sperm viability and compare the efficiency of this test with a flow cytometer. Fresh ejaculates from eight bulls were included in this study. Semen sample was diluted to $30 \times 10^6$ sperms/ml in a Hepes 0.1% BSA. The rates of MTT reduction were measured in microtiter plates after incubation for 1 h at 37 °C using spectrophotometer (MS2 Reader) at wave length 550 nm. Simultaneously split samples of the same semen were tested, using a flow cytometer for sperm viability, mitochondrial activity, and acrosomal integrity using SYBR-14, Rhodamine 123 and LysoTracker Green DNA-26, respectively. The correlation between the results of these tests was calculated using the Pearson correlation coefficients. The results revealed a strong correlation ($P < 0.001$) between the results of MTT reduction rate and the results that simultaneously determined by flow cytometer, yielding correlation coefficients of $r = 0.950$ for sperm viability, of $r = 0.926$ for mitochondrial activity and of $r = 0.959$ for acrosomal integrity. The same correlation coefficient was observed between the values of sperm viability calculated on the basis of MTT reduction rates and the results of flow cytometer. In conclusion, the MTT reduction test was found to be a reliable method in evaluating bovine semen viability and can be used successfully, especially in routine analysis, where practical aspects such as time, costs and practicability are important.

Keywords: Bovine; Sperm viability; Mitochondrial activity; Acrosome integrity; MTT; Reduction test

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1. Introduction

Several methods are currently available to evaluate the quality of semen samples. Visual estimation of the percentage of motile sperm is the most common mode of analysis. This method is fast and inexpensive, but it is subjective and can be influenced by the experience of the analyst (McNiven et al., 1992). Computer-assisted sperm analysis is also based on motility characteristics. The system uses a camera, computer, and software to record and analyse sperm motility (Jasko et al., 1988). Flow cytometry in different technical applications offers many advantages for the analysis of sperm quality. It provides an objective and rapid evaluation of individual cells, and estimates thousands of cells per sample quantitatively and qualitatively (Magistrini et al., 1997). The latter methods are expensive and require special instrumentation.

Therefore, various analytical techniques have been developed to evaluate sperm quality. Assessment of metabolic status of spermatozoa is one of these techniques, which provide valuable information for predicting sperm fertilizing capacity. Reduction activity of spermatozoa is one of these methods that depend on the ability of metabolically active spermatozoa to reduce specific stains. The ability of spermatozoa to reduce the resazurin redox dye (Foote, 1999; Zrimsek et al., 2004) and methylene blue (Chandler et al., 2000) was used successfully to evaluate semen quality of boar and bull.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a yellow water-soluble tetrazolium salt. The dye is converted to water-insoluble purple formazan on the reductive cleavage of its tetrazolium ring by the succinate dehydrogenase system of the active mitochondria (Slater et al., 1963). Thus, the amount of formazan formed can be determined spectrophotometrically and serves as an estimate of the number of mitochondria and hence the number of living cells in the sample (Denizot and Lang, 1986). MTT assay was used in many studies, which were related to viability of different cells (Campling et al., 1988; Carmichael et al., 1987; Freimoser et al., 1999; Levitz and Diamon, 1985; Mosmann, 1983).

The MTT assay is a simple, rapid and reliable method for estimating the percentage of viable sperms in human and boar, depending on the accumulation of formazan grains around the midpiece of sperm tail (Gaczarzewicz et al., 2003; Naser-Esfahani et al., 2002). And in our previous study (Aziz et al., in press), the MTT test was reported to be a reliable method for an objective evaluation of equine semen, depending on the reduction rate and optical density of the MTT.

The aim of the present study was to examine and validate a simple and less costly MTT test in determining bovine sperm viability and to compare the efficiency of this test with a flow cytometer.

2. Materials and methods

2.1. Semen samples

Fresh semen samples from eight bulls, maintained at Rinder Union West e.G. (Borken, Germany) under uniform feeding and housing conditions, were used in this study. The age of the bulls ranged between 1.2 and 6 years (during the semen collection period). Two
ejaculates were collected from each bull with pre warmed artificial vagina (37 °C). The volume of collected semen samples ranged between 2 and 8 ml with an initial sperm motility of 45–80% and a total concentration of 0.81–1.10 × 10⁹ sperms/ml. After the microscopic evaluation, each semen sample was diluted with Hepes 0.1% BSA (Garner et al., 1997) to obtain a concentration of 30 × 10⁶ sperms/ml.

2.2. Semen analysis

All the laboratory examinations were conducted in the physiology laboratory of the Institute of the Anatomy, Physiology and Hygiene of Domestic Animals, University of Bonn (Bonn, Germany).

Flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) was performed to determine sperm viability, mitochondrial activity and acrosomal integrity. A total of 10,000 events were analysed for each sample.

To evaluate the sperm viability, LIVE/DEAD sperm viability kit (L-7011, Molecular Probes, Eugene, OR, USA) was used. A staining solution was prepared that contained 0.8 μl SYBR-14 and 9 μl PI/ml Hepes-0.1% BSA. One hundred microliters of the diluted semen was stained with 300 μl of the staining solution of SYBR-14. The samples were incubated at 37 °C for 15 min prior to flow cytometric examination (Garner et al., 1997).

To estimate the sperm mitochondrial activity, stock solutions of 0.53 mM Rhodamine 123 (R-302, Molecular Probes, Eugene, OR, USA) were prepared in DMSO and 2.99 mM propidium iodide (PI) (P-4170, Sigma, Deisenhofen, Germany) in Tyrode’s Salt Solution (T-2397, Sigma, Deisenhofen, Germany). The final staining solution contained 3 μl of R123 stock solution and 12 μl PI stock solution/ml Hepes-BSA (Garner et al., 1997). One hundred and fifty microliters of the diluted semen was stained with 300 μl of the final staining solution of R123. The samples were incubated at 37 °C for 30 min before flow cytometric examination.

The acrosomal integrity of spermatozoa was analysed using a stock solution of 1 mM LYSO-G (L-7526, Molecular Probes, Eugene, OR, USA), which was prepared in DMSO (D-8779, Sigma, Deisenhofen, Germany), and 2.99 mM propidium iodide in Tyrode’s Salt Solution. Final staining solution contained 5 μl of LYSO-G stock solution and 12 μl PI stock solution/ml Hepes-BSA (Garner et al., 1997). One hundred and fifty microliters of the diluted semen was stained with 300 μl of the final staining solution of LYSO-G. The samples were incubated at 37 °C for 30 min before flow cytometric examination.

2.3. MTT reduction assay

The MTT assay was performed according to the method of Mosmann (1983). For each sample, 6 wells of the 96-well microplate were used. One hundred microliters of semen sample plus 10 μl of MTT stock solution (5 mg of MTT/ml of PBS) were placed in each well. According to our previous study (Aziz et al., in press), the optical density of samples were measured immediately and after 1 h of incubation at 37 °C using a spectrophotometer (MS2 Reader) at a wave length of 550 nm. MTT reduction rate (optical density) for each sample was calculated by concurring the difference between the first and second reading of the spectrophotometer.
2.4. Experiments

Firstly, to obtain the standard curve and the relationship between the MTT reduction rate and sperm viability, the freeze-killed procedure (Capkova et al., 2000) was used. Fresh semen of three bulls with good semen quality (about 80% viable sperms) was used. After the dilution of semen with Hepes-BSA, 6 ml of the diluted semen were divided in two fractions; one fraction was maintained at 37 °C while the sperm in the other fraction were killed by two cycles of plunging into liquid nitrogen and thawing at 37 °C. Samples for analysis were made by combining aliquots of viable and freeze-killed sperm in ratios of 10:0, 8:2, 6:4, 4:6, 2:8 and 0:10 (v/v), respectively. The prepared samples were analysed by: (1) MTT and spectrophotometer and (2) SYBR-14 and flow cytometer.

Secondly, the MTT test was applied to evaluate the sperm viability in fresh semen of the eight bulls. After the dilution of semen samples in Hepes-BSA, samples and MTT stock solution were distributed in the wells of the 96-well microplate. The rates of MTT reduction were taken immediately and after 1 h incubation at 37 °C. Simultaneously split samples of the same semen were tested using the flow cytometer to evaluate the mitochondrial activity, sperm viability and acrosomal integrity using Rhodamine 123, SYBR-14 and Lyso Tracker Green DNA-26, respectively (Garner et al., 1997).

2.5. Statistic analysis

Pearson correlation coefficients and Regression analysis were used to evaluate the efficacy of the MTT test for the assessment of sperm viability of bovine semen. Data were analysed using SigmaStat (Jandel scientific software V2.0), and $P < 0.05$ was considered as statistically significant.

3. Results

Table 1 shows the MTT reduction rates, which were obtained after 1 h of incubation time, and sperm viability, which were estimated by flow cytometer, of samples containing different proportions of live and freeze-killed sperm cells.

<table>
<thead>
<tr>
<th>Semen samples containing different proportions of live and freeze-killed sperms</th>
<th>MTT reduction rate (optical density) at 550 nm after 1 h incubation time at 37 °C</th>
<th>Sperm viability obtained by flow cytometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:0</td>
<td>0.850 ± 0.05</td>
<td>79.43 ± 3.12</td>
</tr>
<tr>
<td>8:2</td>
<td>0.690 ± 0.03</td>
<td>64.60 ± 3.32</td>
</tr>
<tr>
<td>6:4</td>
<td>0.529 ± 0.07</td>
<td>48.14 ± 3.89</td>
</tr>
<tr>
<td>4:6</td>
<td>0.417 ± 0.02</td>
<td>35.36 ± 5.77</td>
</tr>
<tr>
<td>2:8</td>
<td>0.264 ± 0.02</td>
<td>16.71 ± 1.88</td>
</tr>
<tr>
<td>0:10</td>
<td>0.094 ± 0.01</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>
MTT reduction rates were decreased significantly \( (P < 0.001) \) by increasing the portion of dead sperm cells. There was a highly negative correlation \( (P < 0.001, r = 0.979) \) between the MTT reduction rates and sperm viability.

A regression equation \( (y = 104.583x - 8.877) \) for the relationship between the MTT reduction rate and sperm viability were calculated; the corresponding correlation curve is presented by Fig. 1. This curve was applied later as standards to calculate the percentage of viable sperm cells on the basis of MTT reduction rates.

Table 2 contains the diagnostic results for all semen samples of the eight bulls that obtained by MTT reduction test and the results of sperm parameters (mitochondrial activity, viability and acrosomal integrity), which were evaluated simultaneously using a flow cytometer.

The MTT reduction rate was significantly \( (P < 0.001) \) correlated with the results that simultaneously determined by flow cytometer, yielding correlation coefficients of \( r = 0.950 \) for sperm viability, of \( r = 0.926 \) for mitochondrial activity and of \( r = 0.959 \) for acrosomal integrity. The same correlation coefficient was observed between the values of sperm viability that calculated on the basis of MTT reduction rates and the results of flow cytometer.

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**Table 2**

Analysis of the bovine semen samples using MTT test and flow cytometer

<table>
<thead>
<tr>
<th>Bull number</th>
<th>MTT test</th>
<th>Flow cytometer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT reduction rate (optical density) at 550 nm</td>
<td>Percentage of viable sperms according to MTT</td>
</tr>
<tr>
<td>1</td>
<td>0.602 ± 0.05</td>
<td>54.06 ± 4.90</td>
</tr>
<tr>
<td>2</td>
<td>0.675 ± 0.03</td>
<td>61.72 ± 2.71</td>
</tr>
<tr>
<td>3</td>
<td>0.499 ± 0.01</td>
<td>43.31 ± 1.07</td>
</tr>
<tr>
<td>4</td>
<td>0.772 ± 0.04</td>
<td>71.83 ± 4.15</td>
</tr>
<tr>
<td>5</td>
<td>0.839 ± 0.07</td>
<td>78.89 ± 6.95</td>
</tr>
<tr>
<td>6</td>
<td>0.889 ± 0.05</td>
<td>84.11 ± 4.87</td>
</tr>
<tr>
<td>7</td>
<td>0.667 ± 0.02</td>
<td>60.91 ± 1.96</td>
</tr>
<tr>
<td>8</td>
<td>0.624 ± 0.02</td>
<td>56.38 ± 2.51</td>
</tr>
</tbody>
</table>
Table 3
Correlation coefficient between the results of MTT test and flow cytometer

<table>
<thead>
<tr>
<th></th>
<th>Flow cytometer</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sperm viability</td>
<td>Mitochondrial activity</td>
<td>Arcosomal integrity</td>
</tr>
<tr>
<td>MTT test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTT reduction rate</td>
<td>1.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.950, 0.000302</td>
<td>0.959, 0.0000000685</td>
</tr>
<tr>
<td></td>
<td>1.443 × 10&lt;sup&gt;−45&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.926, 0.000971</td>
<td>0.000166</td>
</tr>
<tr>
<td>Sperm viability based on MTT results</td>
<td>0.950, 0.000302</td>
<td>0.926, 0.000971</td>
<td>0.000166</td>
</tr>
<tr>
<td>Flow cytometer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm viability</td>
<td>0.949, 0.000320</td>
<td>0.997, 0.0000000685</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial activity</td>
<td>0.958, 0.000181</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arcosomal integrity</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Correlation coefficient.

<sup>b</sup> P-value.

Additionally, the results showed a high correlation (\( P < 0.001, r \geq 0.949 \)) between sperm parameters that evaluated by the flow cytometer (Table 3).

4. Discussion

The MTT assay was used in many studies to evaluate the viability of different cells (Campling et al., 1988; Carmichael et al., 1987; Freimoser et al., 1999; Levitz and Diamone, 1985; Mosmann, 1983). This test depends on the ability of viable cells to reduce the MTT (Mosmann, 1983). In this study, the diagnostic value of the MTT reduction assay to evaluate the bovine sperm viability was investigated by comparison of those results with sperm parameters that were simultaneously achieved using a flow cytometer.

Results of the experiments indicate a high correlation between the MTT reduction rate and the result of sperm viability. Furthermore, the reduction rate of MTT decreased significantly with an increasing proportion of killed sperm. These results are in agreement with the findings of Mosmann (1983) who concluded that the MTT reduction rate depends strongly on the number of viable cells in the sample.

In contrast to the procedure that was published by Mosmann (1983), the reduction rate of MTT was taken successfully after 1 h of incubation time. This was expected because spermatozoa are very active cells and rich in mitochondria; therefore, the reduction of MTT by spermatozoa is faster than other cells. A similar observation was reported in our previous study (Aziz et al., in press).

Sperm viability of all semen samples of the eight bulls that obtained according to the rate of MTT reduction were highly correlated with sperm parameters, which were estimated using a flow cytometer, a similar high correlation was also reported in a previous study for equine semen (Aziz et al., in press). Also the results showed a high correlation between
sperm parameters that evaluated by the flow cytometer, these results agreed with previous studies in bulls (Graham et al., 1990; Garner et al., 1997; Thomas et al., 1997).

The viability, mitochondrial activity and acrosomal integrity of sperm cells correlate positively with fertility (Garner et al., 1997); therefore, our results suggest that the MTT reduction rate by the bovine spermatozoa may be used as an indicator for the bovine sperm fertility.

The advantages of the MTT test are that it is simple and inexpensive (Mosmann, 1983). Additionally, results from this study suggest other advantages of this test in evaluating the bovine semen. Firstly, this test is fast (1 h); secondly, many samples (up to 10) can be examined at the same time; and finally, many replications of each sample can be tested simultaneously.

5. Conclusion

The MTT reduction test was found to be a reliable method in evaluating bovine semen viability and can be used successfully, especially in routine analysis, where practical aspects such as time, costs and practicability are important.

References