Evaluation of cockerel spermatozoa viability and motility by a novel enzyme based cell viability assay

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ABSTRACT
1. The results of spermatozoa assessment by the WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) assay, flow cytometry (FC) or computer-assisted sperm analysis (CASA) were compared.
2. Different live/killed ratios of cockerel semen were serially diluted to 120, 60, and 30 x 10^6 cells/ml, and each sample was analysed by (1) WST-8 assay at 0, 10, 20, 30, 40, 50, 60 min, (2) viability with FC, and (3) motility with CASA.
3. The WST-8 reduction rate was closely correlated with spermatozoa viability and motility. The optimal semen concentration for the WST-8 assay was 120 x 10^6 cells/ml, and the standard curves for spermatozoa viability and motility predictions, respectively, were Yviability60 = 162.8x + 104.96 (R^2 = 0.9594) after 60 min of incubation and Ymotility40 = 225.09x + 96.299 (R^2 = 0.8475) after 40 min of incubation.
4. It was concluded that the WST-8 assay is useful for the practical evaluation of cockerel spermatozoa viability and motility. Compared to FC and CASA, the WST-8 assay does not require expensive and complex instrumentation in the lab. Moreover, one well of the WST-8 reaction can be used to predict spermatozoa viability and motility at the same time, which all lead it to be efficient and economical for semen quality assessment.

ARTICLE HISTORY
Received 14 August 2017
Accepted 8 December 2017

KEYWORDS
CASA; flow cytometry (FC); formazan; MTT; reduction rate; standard curve

Introduction
It is necessary to evaluate the quality of semen samples before artificial insemination (Graham and Mocé 2005; Petruninka et al. 2007; Dhurvey et al. 2012; Sikka and Hellstrom 2016). The spermatozoa mass motility test is fast and inexpensive, but is subjective and the evaluation results can differ from one analyst to another. Computer-assisted sperm analysis (CASA) technology was introduced in the late 1980s (Amann and Waberski 1997; Mortimer et al. 2015). With CASA, spermatozoal images are captured by a microscope camera, using the defined software to determine spermatozoa variables, such as motility and morphology (King et al. 2000; Lu et al. 2014; Maroto-Morales et al. 2016; Santiago-Moreno et al. 2016). Flow cytometry (FC) can detect spermatozoa labelled by various specific fluorescent probes and rapidly calculates the relevant numbers of spermatozoa for assessing different functional characteristics (for example, plasma and acrosomal integrity, mitochondrial function and DNA status) (Christensen et al. 2004; Cordelli et al. 2005; Graham and Mocé 2005; Mocé and Graham 2008; Partyka et al. 2010). Both CASA and FC can provide accurate, reliable, objective and efficient analysis for spermatozoa quality; however, they require expensive and complex instrumentation in the laboratory.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay is used to determine mitochondrial dehydrogenase activities in living cells, which is applied extensively in many studies to assess viability in different cells (Holder et al. 2012), including spermatozoa (Hara et al. 1995; Hazary et al. 2001; Nasr-Esfahani et al. 2002; Aziz et al. 2005; Aziz, 2006; Byun et al. 2008). MTT is reduced by NADH to form water-insoluble purple formazan, which has a needle-shaped crystal and accumulates in cells (Berridge et al. 1996; Berridge et al. 2005). It requires an organic solvent to solubilise purple formazan before measuring the absorbance. Needle-shaped crystals and organic solvents both damage cells (Tominaga et al. 1999). The WST-8 assay is another enzyme-based method for determining cell viability (Ishiyama et al. 1997; Holder et al. 2012). However, WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) formazan is water-soluble and results in less damage and less toxicity to viable cells.

The WST-8 assay is a fast, simple and reliable method to evaluate cell viability, however, to our knowledge there is no report describing its application for semen quality evaluation. The purpose of the present study was to establish the value of the WST-8 assay for cockerel spermatozoa quality assessment.

Materials and methods

Animals and semen collection
All experiments were carried out in accordance with the legislation governing the ethical treatments of animals and approved by the LRI IACUC (No. 106–38).

Forty adult Taiwan Native chicken L7 and L12 cockerels of 57–63 weeks of age were used in this study. L7 and L12 chickens are two inbred lines of Taiwan Native chickens, selected for meat production at the Livestock Research...
Institute, Tainan, Taiwan for 20 years. Cockerels were divided into 4 groups (10 cockerels each) and semen was collected routinely by abdominal massage (Burrows and Quinn 1937) once a week. Semen from 10 cockerels of each group was pooled and the spermatozoa concentration was determined immediately by a photometer (ACCUREAD, IMV, L’Aigle, France).

**Experimental design**

In order to obtain the standard curve of the relationship between the WST-8 reduction rate, spermatozoa viability and motility, the frozen-killed procedure was applied as described (Aziz et al. 2005; Byun et al. 2008). Fresh semen samples of 4 cockerel groups were 1:1 diluted with PBS (Lonza, Basel, Switzerland) and divided into viable and killed portions. The viable portion was kept at room temperature, and the killed portion were plunged into liquid nitrogen and thawed at 37°C for two cycles. Different live/ killed semen were prepared by mixing aliquots of viable and killed spermatozoa ratios of 0/10, 2/8, 4/6, 6/4, 8/2, and 10/0 (v/v), respectively. The prepared semen samples were serially diluted to 120, 60, and 30 × 10⁶ cells/ml, and each sample were analysed by (1) WST-8 assay at 0, 10, 20, 30, 40, 50, 60 min (2) viability with FC and (3) motility with CASA.

**WST-8 assay**

Cell Counting Kit-8 (CCK-8®) (Bimake, Houston, USA) was adopted to perform WST-8 assay according to the manufacturer’s instructions. CCK-8® is a redox indicator that takes the highly water soluble tetrazolium salt WST-8 to produce a cell-viable formazan dye upon the reduction by cellular dehydrogenases, which is directly proportional to the number of living cells. For each sample, three repeats of 100 μl semen were prepared in wells of the 96-well microplate (Greiner Bio-One, Kremsmünster, Austria). Then 10 μl CCK-8® solution was added directly to each well and examined by spectrophotometry (BioTek, Vermont, USA) to record the absorbance at a wavelength of 450 nm after incubation at 37°C for 10, 20, 30, 40, 50, and 60 min, respectively.

**FC analysis**

Guava® easyCyte microcapillary FC (Guava Technologies Inc., Hayward, CA, USA; distributed by IMV Technologies) with EasyKit 1 Viability and Concentration (ref. 024708, IMV Technologies) was used to determine cockerel spermatozoa viability according to the manufacturer’s instructions. The kit contains SyBr14 and PI dyes with differential permeability to viable (membrane intact) and nonviable (membrane damaged) cells. Each aliquot of 57000 spermatozoa was placed into the well of EasyKit 1 Viability and Concentration, incubated at 37°C for 10 min, and then passed through the easyCyte. A total of 5000 events were analysed for each sample, and results were expressed as a percentage of viable spermatozoa (Sellem et al. 2015).

**CASA analysis**

Spermatozoa motility was assessed with a CEROS II™ CASA system (Hamilton Thorne Inc., Beverly, MA, USA) with settings of 30 frames to avoid spermatozoa track overlapping, minimum contrast = 50, non-motile head size = 7 pix, and non-motile intensity = 95 (Mocé et al. 2010). Spermatozoa concentration was adjusted to 30 × 10⁶ cells/ml and a 2.6 μl aliquot was placed into a four-chamber Leja counting slide (Leja Products B.V., Nieuw-Vennep, the Netherlands). Motility was evaluated with a minimum 200 spermatozoa per sample, and results were expressed in percentage of motile spermatozoa.

**Statistical analysis**

The CORR procedure of SAS™ package v 9.2 (SAS Institute Inc., Cary, NC, USA) was applied to calculate the Pearson correlation coefficient between WST-8 reduction rate and viability/motility of cockerel semen. P < 0.001 was considered as extremely significant, and P < 0.01 was considered as highly significant. Hereafter, data were analysed by the REG procedure of SAS™ to result in the standard curves for prediction of spermatozoa viability and motility.

**Results**

**Analysis of different ratios of live/killed semen samples**

The viability and motility of semen samples are presented in the Table 1. The viability/motility of 0/10, 2/8, 4/6, 6/4, 8/2, and 10/0 live/killed samples, respectively, were 0.02%/1.18%, 18.8%/20.6%, 30.4%/44.4%, 49.1%/59.5%, 63.9%/67.0%, and 78.2%/70.6%. The results demonstrate that the semen samples prepared for the standard curves exactly reflected the different live/killed ratios.

**Correlation between WST-8 reduction rate and viability/motility**

Pearson correlation coefficients between the WST-8 reduction rate and viability/motility are shown in Table 2. In all groups, the correlation increased gradually with semen concentration and incubation period. When semen concentration increased to 120 × 10⁶ cells/ml, the WST-8 reduction rate and viability are very strongly correlated (r ≥ 0.933, P < 0.001) at 10, 20, 30, 40, 50, and 60 min; the WST-8 reduction rate is also very strongly correlated (r ≥ 0.916, P < 0.001) with motility at 30, 40, 50, and 60 min.

**Effects of WST-8 reduction rate, viability, and motility**

Based on the result of the correlation coefficient analysis, the strongly correlated groups were followed with regression analysis and the three most appropriate standard curves for prediction of spermatozoa viability and motility

<table>
<thead>
<tr>
<th>Live/killed ratio</th>
<th>Viability (%)</th>
<th>Motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/10</td>
<td>0.02 ± 0.01a</td>
<td>1.18 ± 0.54a</td>
</tr>
<tr>
<td>2/8</td>
<td>18.78 ± 5.15b</td>
<td>20.58 ± 1.19b</td>
</tr>
<tr>
<td>4/6</td>
<td>30.08 ± 4.02c</td>
<td>44.35 ± 6.40c</td>
</tr>
<tr>
<td>6/4</td>
<td>49.11 ± 5.84d</td>
<td>59.45 ± 7.75d</td>
</tr>
<tr>
<td>8/2</td>
<td>63.90 ± 3.95e</td>
<td>66.99 ± 7.21e</td>
</tr>
<tr>
<td>10/0</td>
<td>76.12 ± 3.22f</td>
<td>70.63 ± 7.33f</td>
</tr>
</tbody>
</table>

Values are means ± SEM from 24 samples in 4 different experiments. Different superscript letters within a column indicate statistically significant differences between different live/killed sperm ratios (P < 0.01).
Table 2. Pearson correlation coefficients between WST-8 reduction rate and viability/motility in different incubation periods.

<table>
<thead>
<tr>
<th>Incubation period (min)</th>
<th>Viability 120</th>
<th>Viability 60</th>
<th>Viability 30</th>
<th>Motility 10</th>
<th>Motility 20</th>
<th>Motility 30</th>
<th>Motility 40</th>
<th>Motility 50</th>
<th>Motility 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.933***</td>
<td>0.597**</td>
<td>0.214</td>
<td>0.981***</td>
<td>0.633***</td>
<td>0.285</td>
<td>0.958***</td>
<td>0.624***</td>
<td>0.433***</td>
</tr>
<tr>
<td>20</td>
<td>0.933***</td>
<td>0.735***</td>
<td>0.621**</td>
<td>0.896***</td>
<td>0.798***</td>
<td>0.698***</td>
<td>0.916***</td>
<td>0.838***</td>
<td>0.753***</td>
</tr>
<tr>
<td>30</td>
<td>0.958***</td>
<td>0.860***</td>
<td>0.742***</td>
<td>0.921***</td>
<td>0.878***</td>
<td>0.753***</td>
<td>0.916***</td>
<td>0.838***</td>
<td>0.753***</td>
</tr>
<tr>
<td>40</td>
<td>0.975***</td>
<td>0.895***</td>
<td>0.800***</td>
<td>0.919***</td>
<td>0.909***</td>
<td>0.805***</td>
<td>0.919***</td>
<td>0.909***</td>
<td>0.805***</td>
</tr>
<tr>
<td>50</td>
<td>0.979***</td>
<td>0.920***</td>
<td>0.838***</td>
<td>0.918***</td>
<td>0.912***</td>
<td>0.839***</td>
<td>0.918***</td>
<td>0.912***</td>
<td>0.839***</td>
</tr>
<tr>
<td>60</td>
<td>0.997***</td>
<td>0.920***</td>
<td>0.838***</td>
<td>0.918***</td>
<td>0.912***</td>
<td>0.839***</td>
<td>0.918***</td>
<td>0.912***</td>
<td>0.839***</td>
</tr>
</tbody>
</table>

WST-8 reduction rate $= \Delta OD$ at 450 nm.

Data collected from 32 samples in 4 different experiments.

*** Correlation differs significantly from zero at $P < 0.001$.

Discussion

The WST-8 assay has been widely used to determine the viability of various types of cells (Ishiyama et al. 1997; Holder et al. 2012). However, this study is the first report which indicates that this method could be applied to cockerel semen evaluation for both spermatozoa viability and motility. WST-8 is a water-soluble tetrazolium dye, and there are several commercial ready-to-use solution kits available. It offers a simple, rapid, reliable, quantitative, and sensitive measurement of cell viability for use in practical applications. Tominaga et al. (1999) tested WST-8 with microplates on 5 different cell lines of HeLa (human cervical cancer), RC (rabbit cornea), L929 (mouse connective tissue), Balb3T3 (mouse embryo) and HL60 (human acute promyelocytic Leukemia) cells and compared the sensitivity with results obtained with other tetrazolium dyes. Compared with an MTT assay, WST-8 did not form needle-shaped crystals, so the absorbance could be measured directly without solubilisation by an organic solvent. Therefore, the WST-8 assay might be more efficient and reliable than the MTT assay.

Several studies reveal that in vitro analysis of spermatozoa viability and motility are correlated with fertilising potential in vivo (Donoghue 1999; King et al. 2000; Blesbois et al. 2008; Hossain et al. 2011; Farah et al. 2013; Petrunkina and Harrison 2013). It is therefore important to measure spermatozoa viability and motility before artificial insemination in order to maximise fertility. Eosin-nigrosin staining has been extensively used to evaluate spermatozoa viability for decades, but there are reports indicating that double fluorescence SyBr14 and PI staining is more effective than the eosin-nigrosin test for the assessment of cockerel spermatozoa viability (Chalah and Brillard 1998; Chalah et al. 1999). SyBr14/PI double staining is a spermatozoa membrane assay. When PI penetrates membrane-damaged spermatozoa, it displaces Sybr14 fluorescence. Thus, live spermatozoa fluoresce green and dead spermatozoa are red. The spermatozoa membrane is important for many functions such as the ability to maintain cell homeostasis, exercise motility and interact with the female genital tract. In this study, different ratios of live/killed cockerel semen samples were prepared and double staining with SyBr14 and PI was used to determine spermatozoa viability. Each semen sample was, therefore, compared in different live/killed ratios between SyBr14/PI viability and WST-8 reduction rate to obtain the prediction curves for cockerel spermatozoa viability. Thus, the viability prediction curves attained in this study are more accurate than the prediction from eosin-nigrosin staining viability.
Byun et al. (2008) used the MTT assay to evaluate boar spermatozoa viability and the resultant correlation coefficients between MTT at 1 and 4 h of incubation and spermatozoa viability from eosin-nigrosin staining were 0.9493 and 0.9564, respectively. Another study (Aziz et al. 2005) also reported a correlation between MTT reduction assay at 1 and 4 h of incubation and equine spermatozoa viability of $r = 0.954$ and $r = 0.977$, respectively. Compared with their findings, this study indicates that the incubation period could be shortened to 50 min by increasing the semen concentration to $120 \times 10^6$ cells/ml to get a similar result, which means that increasing semen concentration could improve assay efficiency. However, this dissimilarity could also be due to different sensitivities between the MTT and WST-8 assays, or the different dehydrogenase activity between boar, stallion and cockerel spermatozoa.

In conclusion, the WST-8 assay needs only a plate reading spectrophotometer instead of expensive and complex FC or CASA. Moreover, many samples can be tested at the same time. Above all, one well of WST-8 reaction can be used to predict spermatozoa viability and motility simultaneously. All these advantages make the WST-8 assay a simple and practical tool for evaluating cockerel spermatozoa quality in a simply-equipped laboratory or a breeding farm.

**Acknowledgements**

The authors would like to thank Mr Ming-Chuan Chien, Mr Yung-Yao Cheng, and Ms Wen-Tsen Chen of Animal Industry Division at LRI for assistance with animal management and semen collection.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was supported by the Council of Agriculture, Executive Yuan [1062101011003-020501L1].

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