EVALUATION OF RAT SPERM BY FLOW CYTOMETRY: SIMULTANEOUS ANALYSIS OF SPERM COUNT AND SPERM VIABILITY

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ABSTRACT — In this study, we conducted a simultaneous analysis of sperm count and viability in rats by flow cytometry (FCM). Epididymal fluids were taken from the caudal epididymis of 12 to 13 week-old Sprague-Dawley rats. The fluids were weighed and mixed with Dulbecco's phosphate buffered saline (D-PBS). Propidium iodide, which can stain only dead sperm, was used to distinguish viable and dead sperm. The sperm count and viability analyzed by FCM were 1.28 × 10⁷/mg and 78.0%, respectively. These values were consistent with the corresponding values (1.39 × 10⁷/mg and 81.0%) that were directly determined microscopically in the fluids of the same sample. In addition, when the original mixture containing sperm was diluted two times and four times with D-PBS, or was diluted two times with D-PBS containing only killed sperm, the sperm count and viability determined by FCM also correlated well with the sperm count (r=0.96, P<0.01) and sperm motility (r=0.99, P<0.01) by direct microscopic observation, respectively. In conclusion, the present flow cytometric analysis would be practical for the simultaneous determination of sperm count and viability in rat epididymal fluids.

KEY WORDS: Flow cytometry (FCM), rats, Simultaneous analysis of sperm count and viability

INTRODUCTION

Sperm analysis is considered to be a useful technique to confirm or characterize an effect of medical products on male fertility (Ministry of Health and Welfare Ordinance No.21, 1997). Some procedures, including direct microscopic observation (Fukazawa and Kotosai, 1987) and computer-assisted sperm analysis (Chapin et al., 1992; Hara et al., 1995), have been used for detecting the effect of medical products on sperm. A flow cytometric analysis is an objective method to evaluate sperm quality. In mice, cattle and humans, various flow cytometric techniques are already available to evaluate sperm count and viability (Tao et al., 1993), acrosome integrity in sperm (Garner et al., 1986), spermatogenesis in the testis (Johnson, 1986), and the portions of X- and Y-chromosome-bearing sperm (Spano and Evenson, 1993). However, flow cytometers have rarely been used for testing rat sperm. In rats, only one procedure has been used to evaluate sperm count and viability; this procedure analyzed the viability, followed by determination of the sperm count after adding the standard fluorescent article (Takizawa et al., 1995). Consequently, there is no research in which the number and viability of rat sperm in the same sample are simultaneously analyzed by FCM. In the present study, we investigated a simultaneous analysis of sperm count and viability in rats using a flow cytometer, and confirmed its precision by comparison with direct microscopic observation.

MATERIALS AND METHODS

Animals and animal husbandry

Male Sprague-Dawley rats (Crl:CD) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). After quarantine and acclimatization, the healthy males (12 to 13 weeks-old) were chosen for experiments. The males were kept in a barrier system room, maintained at 24 ± 1℃, 55 ± 10% (relative...
humidity), 10-15 times/hr ventilation and 12-hr lighting (6:00-18:00), and housed individually in suspended metal cages. Solid food (CRF-1, Oriental Yeast Inc., Tokyo) and tap water were given ad libitum.

Sperm preparation

The males were anesthetized with ether and sacrificed. Both epididymides were removed and the caudal epididymis was cut with a razor blade. Three to 9 mg of epididymal fluids, which leaked out, was taken from the cross section in the caudal epididymis with a stainless steel spatula. The fluids were weighed and immersed into 8 ml of Dulbecco’s phosphate buffered saline (D-PBS) containing Ca\(^{2+}\) and Mg\(^{2+}\) supplemented with 5 mg/ml of bovine serum albumin, 1.65 \(\mu\)l of 1.1% sodium pyruvate solution and 2 \(\mu\)l of 60% DL-sodium lactate (Wako CHEMICALS, Japan), which was kept at 37°C in a tube. The fluids were carefully mixed and diffused by inversion of the tube.

Sperm analysis by FCM

13.3 \(\mu\)l of 0.58 mM propidium iodide (PI) (Sigma, USA) solution was added to the mixture containing sperm; PI can permeate the impaired plasma membrane and stain only dead sperm by intercalation into the double helical structure of deoxyribonucleic acid. After 10 min of incubation at 37°C in a water bath, the sperm count and viability in 20 \(\mu\)l of each sample were simultaneously analyzed using a stop on volume function with a COULTER EPICS XL flow cytometry system (COULTER Corporation, USA). Approximately 10,000 to 20,000 sperm were analyzed by FCM in each sample. Sperm count/epididymal fluid (mg) was calculated using the following formula; (sperm count/20 \(\mu\)l) \(\times\) 8 ml/epididymal fluid (mg).

Sperm analysis by direct microscopic observation

Each sperm count and motility in approximately 100 - 200 sperm was assessed by the modified method

![Dot-plot](image1)

![Histogram](image2)

![Non-treatment](image3)

![Treatment with vortex mixer](image4)

**Fig. 1.** Flow cytometric patterns of rat sperm. Top: Dot-plot of sperm populations. Box A represents sperm populations. Bottom: Histogram of non-treated (left side) sperm and sperm killed with vortex mixer (right side). Each peak 1 and peak 2 represents viable and dead sperm.
of Suzuki (1994) as follows. The same sample used in the flow cytometric analysis was placed in a Toma hemocytometer. Non-motile sperm was counted using a microscope with a stage warmer at 37°C. Afterward, the total sperm count was determined by direct microscopic observation after immobilization of the sperm using a vortex mixer for 30 seconds. The motile sperm (the total sperm count minus the immobile sperm count) divided by the total sperm count was expressed as a sperm motility (percentage). Sperm count/epididymal fluid (mg) was calculated using the following formula: total sperm count × 10³ × dilution/epididymal fluid (mg).

Optimal staining time with the PI staining solution

Because PI is cytotoxic, the effect of PI on sperm viability was investigated. After addition of PI staining solution, sperm viability was determined at 10, 20, 30, 60, 120 and 240 min. Sperm viability at each point was compared with the sperm viability at 10 min after addition of PI staining solution.

Samples with low sperm count or low viability

At the next stage, on the assumption of an encounter with a decreased count or lowered viability, the precision of this flow cytometric analysis was investigated using samples with decreased sperm count or lowered viability. The original mixture containing sperm in D-PBS was diluted two times and four times with D-PBS to give other analytical samples. To produce an additional sample, the original mixture containing sperm was diluted two times with D-PBS containing only sperm killed by the vortex mixer. These samples were subjected to the sperm test by FCM and microscopic observation. The correlation between the viability by FCM and the motility by microscopic observation, and between the number by FCM and the number by microscopic observation, were investigated, respectively.

Statistical analysis

Two-way ANOVA followed by Dunnett's test (Dunnett, 1955 and 1964) was used to compare the values in the decision of optimal PI staining time. Linear correlation and linear regression analysis were used to assess the relationship between the values determined by FCM and those determined by microscopic observation.

RESULTS

Sperm analysis by FCM

As shown in Fig. 1, within 30 min after addition of PI staining solution, the histograms of the non-treated sperm clearly showed two distinct peaks, a negative fluorescent peak (peak 1) and strong fluorescent peak (peak 2), and the killed sperm showed only one strong fluorescent peak (peak 2). These indicate that peak 1 and peak 2 represent viable and dead sperm, respectively. At around 60 min, a third peak (peak 3), which was weakly fluorescent, appeared between peak 1 and peak 2 (Fig.2).

To investigate the effect of PI on sperm, an optimal staining time with the PI staining solution was investigated. The high viability was kept for 30 min (Fig. 3). Thereafter, sperm viability declined in a gentle curve. At 60 min after addition of the PI solution, the viability was lower than the values determined at 10 min. Moreover, at 60, 120 and 240 min, the viability was significantly (P<0.01) decreased as compared with the values at 10 min. Therefore, from the next experiences, sperm viability was determined at 10 min after adding the PI staining solution.

Validation of FCM

To validate this flow cytometric analysis, the sperm count by FCM was compared with the sperm count by microscopic observation, and also the viability by FCM was compared with the motility by microscopic observation. In the original mixture containing sperm, the number and viability determined by FCM were 1.28 × 10⁶/mg and 78.0%, respectively (Table 1).
These values were in agreement with the corresponding values (1.39 × 10^6/mg and 81.0%) determined microscopically.

In the samples with various concentrations of sperm, the sperm count by FCM linearly increased with the sperm count by microscopic observation in the range from 0.13 × 10^6/mg to 1.49 × 10^6/mg (Fig. 4). Moreover, there was a statistically significant correlation between the number obtained by FCM and that by microscopic observation (r=0.96, P<0.01). Likewise in samples containing viable and dead sperm in different ratios, the sperm viability by FCM correlated well (r=0.99, P<0.01) and increased linearly with sperm motility by microscopic observation in the range from 1.9% to 89.6% (Fig. 5).

**DISCUSSION**

Sperm viability remained high for 30 min, but at 60 min after the addition of the PI solution, sperm viability decreased, and the weak fluorescent peak appeared. Such a peak has already been reported in other studies, in which the peak was recognized as partially stained moribund sperm (Tao et al., 1993; Takizawa, et al., 1995). We therefore recommend that sperm viability should be analyzed within 30 min after addition of the PI solution because the quality of rat sperm would deteriorate beyond 30 min.

The sperm count and viability by the present flow cytometric analysis were in good agreement with the corresponding values of microscopic observation in the original mixture containing sperm. The viability was also approximately in accordance with literature values (77.1% to 95.0%: Fukazawa and Kotosai, 1987; Takizawa et al., 1995; Kawashima et al., 1995; Katoh, et al., 1995; Hoshino et al., 1995). Moreover, in the case of decreased sperm count or lowered sperm viability, the sperm count and viability by the present flow cytometric analysis were in good agreement with the corresponding values of microscopic observation in the original mixture containing sperm.

![Fig. 3. Effect of PI staining time on sperm viability by FCM. 0.58 mM PI was added to the medium at 0 min, which is indicated as an arrow. Sperm viability was analyzed by FCM at 10, 20, 30, 60, 120 and 240 min after addition of PI solution (n=5). Data were analyzed by two-way ANOVA followed by Dunnett's test. Significant difference from the viability at 10 min after addition of PI solution is marked: ** P<0.01.](image)

**Table 1. Comparison of values by flow cytometry and values by microscopic observation.**

<table>
<thead>
<tr>
<th></th>
<th>No. of rats</th>
<th>Flow cytometry</th>
<th>Microscopic observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count (× 10^6/mg)</td>
<td>6</td>
<td>1.28 ± 0.22</td>
<td>1.39 ± 0.24</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>6</td>
<td>81.6 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>Sperm viability (%)</td>
<td>6</td>
<td>78.0 ± 5.9</td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean ± S. D. Sperm count is represented per mg of epididymal fluids taken from caudal epididymis. Each sperm motility and viability was determined at 10 min after addition of PI to the medium.
Evaluation of rat sperm by flow cytometry.

cytometric analysis correlated closely with the corresponding values determined microscopically. These results have confirmed that the present flow cytometric analysis is sufficiently precise for measuring sperm count and viability in rats.

By FCM, the sperm count has been determined by adding standard fluorescent particles, which are known to be in a constant concentration, because flow cytometry is incapable of measuring the whole volume of the sample being analyzed (Takizawa et al., 1995; Toppari, et al., 1986). A problem with the previous studies was that the presence of fluorescent particle decreased the viability of sperm. In another study, the sperm count was determined by adding the standard fluorescent particle after the viability was analyzed using a flow cytometer (Takizawa et al., 1995). We solved this problem by avoiding the use of the standard fluorescent particle. Therefore, sperm count and sperm viability could be analyzed simultaneously by this flow cytometric analysis.

Sperm viability is usually determined by microscopic observation after viable or dead sperm are stained by specific fluorophores, eosin-Y (Fukazawa and Katosai, 1987) or ethidium homodimer and calcein AM (Katoh et al., 1995). With the present flow cytometric analysis, the number and viability in approximately 10,000 to 20,000 sperm can be analyzed in a min. Consequently, this procedure has the advantages of objectivity, reliability and efficiency compared with direct microscopic observation of sperm count, motility and viability.

In conclusion, the present flow cytometric analysis would be practical for the simultaneous determination of the sperm count and viability in rat epididymal fluids, and also suitable for male reproductive toxicity studies.

REFERENCES


Ordinance for the conduct of non-clinical studies for the safety of medical products (Ministry of Health and Welfare Ordinance No. 21, effective March 26, 1997)


