EVALUATION OF MITOCHONDRIAL FUNCTION AND MEMBRANE INTEGRITY BY DUAL FLUORESCENT STAINING FOR ASSESSMENT OF SPERM STATUS IN RATS

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ABSTRACT — Dual fluorescent staining (DFS) with calcein acetoxy methyl ester (CAM), which labels the cellular esterase activity that is a major component of energy metabolism in cellular mitochondria, and with ethidium homodimer-1 (EthD-1) was used to evaluate mitochondrial function and membrane integrity in rat spermatozoa.

The spermatozoa stained by DFS could be classified into three different populations microscopically when excited at 490 nm after 60 min incubation. 1) Spermatozoa, which were stained with CAM alone and had maintained either mitochondrial function or membrane integrity, were identified as live during incubation. 2) Spermatozoa, which were stained with EthD-1 alone and had lost either mitochondrial function or membrane integrity, were identified as already dead at the beginning of incubation. 3) Spermatozoa, which were stained with both CAM and EthD-1 and had maintained mitochondrial function with membrane breached, were identified as having died during incubation.

Two toxicological tests, an in vitro triton X-100 experiment and an in vivo nitrobenzene experiment, were done. All spermatozoa were immobilized and lost either mitochondrial function or membrane integrity by 1.0% triton X-100 treatment. Almost no motile sperm were found at 0.1% in the triton X-100 group and in the groups treated with 60 and 40 mg/kg/day of nitrobenzene, and these spermatozoa maintained their mitochondrial function but had their membrane breached.

In conclusion, the DFS procedure, which uses CAM and EthD-1, can clearly and visually identify the population of viable and dead spermatozoa simultaneously by fluorescence microscopy in rats. This is a useful technique to characterize sperm status, which is determined by the mitochondrial function assessed by CAM and membrane integrity evaluated by EthD-1.

KEY WORDS: Calcein acetoxy methyl ester (CAM), Ethidium homodimer-1 (EthD-1), Mitochondrial function, Membrane integrity, Rat sperm

INTRODUCTION

Reproductive and developmental studies on medical products require functional tests, such as sperm analysis, which is difficult or impossible to do by histological examination of male reproductive organs. Sperm analysis is a useful technique to confirm or characterize the effects of medical products on male fertility (Ministry of Health and Welfare Ordinance No. 21, 1997). In some end-points of sperm analysis, measure-
ment of the sperm motility has been used as an indicator for assessment of male fertility, because some investigators have reported that impaired sperm motion affects fertility index and reproductive status (Mineshima et al., 2000; Ban et al., 2001). Establishment of a technique to discriminate viable and dead spermatozoa is needed for accurate evaluation of spermatozoa which have reduced mobility or have died, when measurement of the sperm motility is decreased by some component.

Some DNA dyes, such as eosin or propidium iodide, have been widely used for viability assay on spermatozoa in humans (Dougherty et al., 1975; Kramer et al., 1993), in bulls, boars, rams, rabbits, and mice (Garner and Johnson, 1995), and in rats (Takizawa et al., 1995; Yamamoto et al., 1998; Vetter et al., 1998). However, in rat spermatozoa, it is difficult to discriminate between viable and dead spermatozoa by eosin, because, as John (1970) reported, both living and dead spermatozoa display a strong affinity for eosin. Propidium iodide has a cytotoxic effect as reported by Yamamoto et al. (1998).

A live-dead viability/cytotoxicity assay employing calcein acetoxymethyl ester (CAM) and ethidium homodimer-1 (EthD-1) was first used for viability analyses of mammalian cells in culture by MacCoubrey et al. (1990) and Moore et al. (1990). In comparison with other dyes currently used, CAM has more intense fluorescence, does not readily lose its fluorescence, and is maintained in cells, and both CAM and EthD-1 are insensitive to pH changes between pH 5.5 and 10 (MacCoubrey et al., 1990; Moore et al., 1990; Kaneshiro et al., 1993). Acquisition of energy by mammalian spermatozoa through endogenous respiration occurs in cellular mitochondria, which are abundantly found in mitochondrial sheaths, especially in the midpiece, and endogenous respiration makes it possible for phosphatidate to be cleaved by cellular esterase activity (Mohri, 1991; Suzuki, 1992). CAM can permeate living cell membranes, is hydrolyzed by esterase which is a phosphatidate metabolism enzyme, and produces green fluorescence (MacCoubrey et al., 1990; Moore et al., 1990; Kaneshiro et al., 1993). Thus, CAM can be an indicator for assessment of mitochondrial function and membrane integrity, which is frequently used to study testicular and reproductive toxicity (e.g. Levin et al., 1988; Linder et al., 1992), were also done. We used these studies of toxicity to test whether our present method of evaluating mitochondrial function and membrane integrity could be used for assessment of male reproductive toxicity.

**MATERIALS AND METHODS**

**Animals**

Sexually mature Sprague-Dawley male rats (Crj:CD) were purchased from Charles River Japan, Inc. Thirteen males aged 20 to 25 weeks were used for the evaluation of membrane integrity and mitochondrial function, five males aged 20 to 25 weeks were used for the in vitro triton X-100 study, and sixteen males 12 weeks of age were used for the 49-day in vivo nitrobenzene study. These animals were kept in an animal room where the temperature (22±2°C), the relative humidity (55±15%), and the light and dark cycle (12 hr each) were controlled. A pellet diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo) and tap water were given ad libitum.

**Sperm samples to evaluate sperm mortality**

To prepare the culture fluid for spermatozoa, 2.4 mM of NaHCO₃ and 0.5% bovine serum albumin (BSA: Fraction V, Katayama Chemical Industries Co., Ltd., Osaka; Lot No. 5637) were added to Hank’s balanced salt solution (HBSS: Gibco Life Technologies, NY, USA; Lot No. 70K5154), and the fluid was adjusted to pH 7.2.

Male rats were euthanized by exsanguination under ether anesthesia, and the vasa deferentia were removed. To prepare the theoretically viable sperm sample (88.8±4.0%, actual value of sperm motility), one vas deferens was suspended for 10 min in 2 mL of culture fluid at 37°C. To prepare the dead sperm sample, the other vas deferens was immersed in a water bath at about 80°C for 10 sec and then diffused in 2 mL of culture fluid at 37°C. The sperm number was...
counted in each of the viable and dead sperm samples using a hemocytometer.

Sperm samples with various levels of mortality were prepared and examined as follows. Viable sperm samples and dead sperm samples were mixed in various volumetric ratios, and the sperm mixtures thus prepared were stained and observed by the dual fluorescent staining (DFS) method described below.

**Fluorescent staining**

Calcein acetoxy methyl ester (CAM: excitation wavelength, 490 nm, and emission wavelength, 530 nm; Molecular Probes Inc., OR, USA; Lot No. 0251-3) staining solution was prepared by dissolving CAM in dimethyl sulfoxide (DMSO: Pure, Wako Pure Chemical Industries Ltd., Osaka; Lot No. TPR7212) at a concentration of 4 mM. Ethidium homodimer-1 (EthD-1: excitation wavelength, 528 nm, and emission wavelength, 617 nm; Molecular Probes Inc., OR, USA; Lot No. 0251-3) staining solution was prepared by dissolving EthD-1 at a concentration of 2 mM in a 1:4 (v/v) mixture of DMSO and distilled water.

Prior to staining, the sperm sample was diluted to a sperm concentration of 4.0 × 10^6–8.0 × 10^6/mL. In order to stain spermatozoa, 2 µL of 4 mM CAM and 2 mM EthD-1 were added to the 1 mL of diluted sperm sample. This sperm suspension was placed in a culture dish and incubated in a CO₂ incubator (5% CO₂ in air at 37°C) for about 60 min. After incubation, a drop of the stained sperm suspension was placed on a slide glass and covered with a cover slip. Three hundred spermatozoa were examined by fluorescence microscopy (OLYMPUS BH2-RFL, OLYMPUS OPTICAL CO. LTD., Tokyo) set on the B excitation unit excited between 460 and 490 nm wavelength and the O530 emission filter passed over 530 nm wavelength. Spermatozoa labeled with CAM and EthD-1 were of two kinds: 1) spermatozoa, which had lost membrane integrity, had red fluorescence in their nucleus; and 2) spermatozoa, which had maintained mitochondrial status, had green fluorescence on their midpiece.

**Comparison with membrane integrity**

The calculated value of sperm mortality was indicated as the number of artificially killed sperm in each of the various mixtures of viable and dead sperm samples [(number of artificially killed sperm in the sperm mixture) / (number of sperm in the sperm mixture) × 100]. In the sperm mixtures, viable sperm, sperm which died during incubation, and artificially killed sperm were considered to be included. The calculated value of sperm mortality, therefore, was compared with the ratio of spermatozoa which lost both mitochondrial function and membrane integrity [(number of sperm with red fluorescence in the nucleus but without green fluorescence on the midpiece) / (number of sperm examined) × 100].

**In vitro triton X-100 study**

Male rats were euthanized by exsanguination under ether anesthesia and the right epididymis was removed. Each cauda epididymis was minced in HBSS with 2.4 mM of NaHCO₃ and 0.5% BSA adjusted to pH 7.2 at 37°C. The sperm sample was diluted to a sperm concentration of 4.0 × 10^6–8.0 × 10^6/mL, 0, 0.01, 0.1, or 1.0% triton X-100 (polyoxyethylene (10) octylphenyl ether X-100, Wako Pure Chemical Industries, Ltd., Osaka; Lot No. 049602) was added to it. This sperm suspension was incubated and stained with CAM and EthD-1 as described above. After incubation, mitochondrial status and membrane integrity were examined by DFS, and sperm motility was analyzed by Computer Assisted Sperm Analyzer (CASA, Hamilton-Thorne Research, MA, USA) for each of the sperm suspensions.

**In vivo nitrobenzene study**

Nitrobenzene (Tokyo Kasei Co. Ltd., Tokyo; Lot No. FIE01) was administered by oral gavage to groups of 4 male rats at doses of 20, 40, or 60 mg/kg/day. A control group of 4 male rats was given corn oil. After dosing for 49 days, all males were euthanized by exsanguination under ether anesthesia and the right epididymis was removed. Each cauda epididymis was minced in HBSS with 2.4 mM of NaHCO₃ and 0.5% BSA adjusted to pH 7.2 at 37°C, and then the sperm sample was diluted to a sperm concentration of 4.0 × 10^6–8.0 × 10^6/mL. This sperm suspension was incubated and stained with CAM and EthD-1 as described above. After incubation, membrane integrity and mitochondrial status were examined by DFS, and sperm motility was calculated by direct microscopic observation [(number of motile sperm) / (number of sperm observed) × 100] for each of the sperm suspensions.

**Statistical analysis**

Data were statistically analyzed between the control group and each of the groups treated with triton X-100 or nitrobenzene as follows. All data were tested by Bartlett’s test (Bartlett, 1973) for homogeneity of variance. When the variance was homogeneous, the data were analyzed by Dunnett test (Yoshida and Hamada,
1992). When the variance was heterogeneous, the data were analyzed by a Dunnett type test (Yoshida and Hamada, 1992). Probabilities less than 5% were considered statistically significant.

Linear correlation and linear regression analysis were used to assess the relationship between the ratio of spermatozoa which lost both mitochondrial function and membrane integrity examined by DFS and the calculated sperm mortality.

**RESULTS**

**Fluorescent staining**

The staining of spermatozoa with the combination of CAM and EthD-1 was observed in three different populations microscopically and could be distinguished when excited at 490 nm (Photo 1). Motile spermatozoa in the samples exhibited no red fluorescence in the nucleus and had green fluorescence on their head and on their midpiece. With the addition of EthD-1, the non-motile spermatozoa showed two different patterns: the artificially killed sperm exhibited red fluorescence in the nucleus and no green fluorescence on their midpiece, and the other spermatozoa exhibited red fluorescence in the nucleus and green fluorescence on their midpiece.

**Comparison with membrane integrity**

The correlation between sperm mortality and membrane integrity is shown in Fig. 1. The ratio of spermatozoa stained with EthD-1 alone had a significant correlation coefficient ($r=0.91$, $n=52$, $p<0.0001$)

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**Photo 1.** Staining of spermatozoa with combination of CAM and EthD-1.

A: Spermatozoa, which were stained with CAM alone and had maintained either mitochondrial function or membrane integrity, were identified as live during incubation.
B: Spermatozoa, which were stained with EthD-1 alone and had lost either mitochondrial function or membrane integrity, were identified as already dead at the beginning of incubation.
C: Spermatozoa, which were stained with both CAM and EthD-1 and had maintained mitochondrial function with membrane breached, were identified as having died during incubation.
Evaluation of mitochondrial function and membrane integrity by dual fluorescent staining of rat spermatozoa.

and a significant regression coefficient ($r^2=0.83$, $p<0.0001$) in relation to the calculated value of sperm mortality.

**In vitro triton X-100 study**

The results of *in vitro* triton X-100 study are shown in Table 1. In the sperm motion analysis by CASA, no motile sperm were observed in the 1.0% group. A significantly decreased sperm motility was observed in the 0.1% group. There were no significant differences in the sperm motility in the 0.01% group.

In the DFS examination, the ratios of CAM-stained sperm and doubly-stained sperm were 0% in the 1.0% group, and the ratio of EthD-1-stained sperm was 100% in this group. A significantly decreased ratio of CAM-stained sperm and doubly-stained sperm was noted in the 0.1% group, and a higher value of the ratio of EthD-1-stained sperm was also noted in this group. There were no significant differences in the ratio of spermatozoa distinguished by DFS in the 0.01% group.

**In vivo nitrobenzene study**

The results of *in vivo* nitrobenzene study are shown in Table 2. In the sperm motion analysis by direct microscopic observation, no motile sperm were observed in the 40 or 60 mg/kg/day group. There were no significant differences in the sperm motility in the 20 mg/kg/day group.

In the DFS examination, the ratio of CAM-stained sperm was significantly decreased (to about 0%) in both the 40 and 60 mg/kg/day groups. In these two groups, the ratios of doubly-stained sperm and EthD-1-stained sperm were higher than the control value. There were no significant differences in the ratio of spermatozoa distinguished by DFS in the 20 mg/kg/day group.

**DISCUSSION**

Kramer *et al.* (1993) reported the relationship among mitochondrial status, membrane integrity, motility, and fertility using CASA and three fluorescent dyes, rhodamine 123 to quantify mitochondrial function, carboxydimethyl fluorescein diacetate, and propidium iodide to assess plasma membrane integrity, in fresh and 24-hr preserved human spermatozoa. They demonstrated that sperm velocity, mitochondrial status, and membrane integrity were important factors in determining fertility. Moreover, Vetter *et al.* (1998) reported that mitochondrial status of sperm may be a useful end-point to include in toxicological studies, especially with compounds that can alter energy metabolism without affecting membrane integrity.

In the present study, we used two fluorescent dyes, CAM to indicate mitochondrial status and EthD-1 to indicate membrane integrity. Spermatozoa could be classified into three different populations: one stained with CAM alone, another stained with EthD-1 alone, and still another stained with both CAM and EthD-1. Both eosin and propidium iodide, widely used for viability assay of mammalian spermatozoa, are indicators for membrane integrity (Dougherty *et al.*, 1975; Garner and Johnson, 1995; Takizawa *et al.*, 1995; Yamamoto *et al.*, 1998), and their mechanism of staining dead sperm is similar to that of EthD-1 (MacCoubrey *et al.*, 1990; Moore *et al.*, 1990; Kaneshiro *et al.*, 1993). When spermatozoa are dead, membrane integrity is lost and affinity for dyes becomes greater (Blom, 1950; Noda, 1992), and energy metabolism, which is determined by mitochondrial function, is also lost. Accordingly, the spermatozoa stained with EthD-1 alone had lost either mito-

![Correlation between sperm mortality and membrane integrity.](image)
Mitochondrial function or membrane integrity, and were identified as already dead at the beginning of incubation. The other spermatozoa stained with CAM alone had maintained either mitochondrial function or membrane integrity, and were identified as alive during incubation. These considerations are supported by our correlative study on sperm mortality; i.e., the ratio of spermatozoa stained by EthD-1 alone was in good agreement with the calculated value of sperm mortality ($r=0.91$, $p<0.0001$). In contrast, the spermatozoa stained with both CAM and EthD-1 were identified as dead at observation, because membrane integrity was lost, as indicated by red fluorescence in their nucleus. However, these spermatozoa maintained mitochondrial function, because green fluorescence produced by CAM appeared on their midpiece. The fluorescence of CAM is not readily lost and is maintained in cells (MacCoubrey et al., 1990; Moore et al., 1990; Kaneshiro et al., 1993). Accordingly, the spermatozoa stained with both CAM and EthD-1 were considered to have been alive at the beginning of incubation. Thus, DFS, the present staining procedure using CAM and EthD-1, is a method which can clearly identify the populations of viable and dead spermatozoa in rats by fluorescence microscopy simultaneously and visually.

In mammalian spermatozoa, cellular esterase activity

### Table 1. Results of in vitro triton X-100 study.

<table>
<thead>
<tr>
<th></th>
<th>Control 0.01 %</th>
<th>Triton X-100 0.1 %</th>
<th>Triton X-100 1.0 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>DFS examination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAM-stained sperm 1)</td>
<td>% 74.6 ± 10.0</td>
<td>72.8 ± 7.7</td>
<td>24.6 ± 23.0*</td>
</tr>
<tr>
<td>Doubly-stained sperm 2)</td>
<td>% 23.6 ± 10.5</td>
<td>23.8 ± 7.7</td>
<td>64.8 ± 21.2*</td>
</tr>
<tr>
<td>EthD-1-stained sperm 3)</td>
<td>% 1.8 ± 1.3</td>
<td>3.4 ± 1.8</td>
<td>10.6 ± 12.0</td>
</tr>
<tr>
<td>Sperm motility</td>
<td>% 61.9 ± 6.0</td>
<td>57.2 ± 8.1</td>
<td>5.9 ± 13.3*</td>
</tr>
</tbody>
</table>

Each value shows mean ± S.D.

Significantly different from control (*: $p<0.05$, **: $p<0.01$).

1) Spermatozoa stained with CAM alone had maintained either mitochondrial function or membrane integrity.

2) Spermatozoa stained with both CAM and EthD-1 had maintained mitochondrial function but had their membrane breached.

3) Spermatozoa stained with EthD-1 alone had lost either mitochondrial function or membrane integrity.

### Table 2. Results of in vivo nitrobenzene study.

<table>
<thead>
<tr>
<th></th>
<th>Control 20 mg/kg/day</th>
<th>Nitrobenzene 40 mg/kg/day</th>
<th>Nitrobenzene 60 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DFS examination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAM-stained sperm 1)</td>
<td>% 73.3 ± 5.5</td>
<td>63.4 ± 11.8</td>
<td>0.1 ± 0.2*</td>
</tr>
<tr>
<td>Doubly-stained sperm 2)</td>
<td>% 10.9 ± 5.7</td>
<td>11.3 ± 7.5</td>
<td>48.5 ± 49.7</td>
</tr>
<tr>
<td>EthD-1-stained sperm 3)</td>
<td>% 15.7 ± 5.8</td>
<td>25.3 ± 8.1</td>
<td>51.4 ± 49.6</td>
</tr>
<tr>
<td>Sperm motility</td>
<td>% 81.3 ± 2.5</td>
<td>78.8 ± 2.5</td>
<td>0.0 ± 0.0**</td>
</tr>
</tbody>
</table>

Each value shows mean ± S.D.

Significantly different from control (*: $p < 0.05$, **: $p < 0.01$).

1) Spermatozoa stained with CAM alone had maintained either mitochondrial function or membrane integrity.

2) Spermatozoa stained with both CAM and EthD-1 had maintained mitochondrial function but had their membrane breached.

3) Spermatozoa stained with EthD-1 alone had lost either mitochondrial function or membrane integrity.
is a major component of energy metabolism, and takes place in the cellular mitochondria which are abundantly in the midpiece, and endogenous respiration makes it possible for phosphatidol to be cleaved by cellular esterase activity (Mohri, 1991; Suzuki, 1992). Therefore, it is considered that the mitochondrial function characterized by CAM and membrane integrity evaluated by EthD-1 provide useful suggestions for assessment of sperm status.

Two toxicological tests, an in vitro triton X-100 study and an in vivo nitrobenzene study, were examined in this report. Some nonionic surfactants, including triton X-100, exert a spermicidal activity (Furuse et al., 1983), and menfegol, a nonionic surfactant widely used, affects both motility and viability of spermatozoa immediately after ejaculation in humans (Oshio et al., 1990). However, depression of sperm motion and viability by triton X-100 is unknown. Nitrobenzene has been studied for testicular toxicity and reproductive toxicity (e.g., Levin et al., 1988). In the testes of male rats subchronically treated with nitrobenzene at 300 mg/kg, degenerating and missing pachytene spermatocytes in stages VII to XIV on Day 2 after administration and maturation depletion of spermatozids in stages V to XIV and multinucleated giant cells on Day 14 after administration were found (Linder et al., 1992). Furthermore, chronic treatment with nitrobenzene at 60 mg/kg/day in male rats reduced sperm motility after 14 days and sperm viability after 21 days (Kawashima et al., 1995). Thus, nitrobenzene affects germ cells in the testes directly, and sperm motility and viability are depressed in the epididymis.

In the triton X-100 study, from the fact that no motile sperm were found in the 1.0% group, and all spermatozoa in this group were stained by EthD-1, it appears that both the mitochondrial function and membrane integrity of treated spermatozoa are lost immediately after incubation. Again the fact that, in the 0.1% group, sperm motility was decreased, spermatozoa stained by CAM alone were decreased, and doubly (CAM and EthD-1) stained spermatozoa were increased, suggests that the membrane integrity by which spermatozoa had maintained their mobility (spermatozoa which kept both mitochondrial function and membrane integrity) is breached during incubation. This is why spermatozoa were immobilized by direct action of triton X-100. In the nitrobenzene study, no motile sperm were found in either the 40 or 60 mg/kg/day groups, and there were almost no spermatozoa stained by CAM alone in these groups. An increase in both doubly-stained spermatozoa and spermatozoa stained by EthD-1 alone was also noted in these groups. Therefore, it appears that some spermatozoa in the rats treated with nitrobenzene at 40 and 60 mg/kg/day have already lost both mitochondrial function and membrane integrity in the cauda epididymis, and the membrane integrity by which some spermatozoa maintained their mitochondrial function is breached during incubation. In other words, spermatozoa are immobilized by the toxicological action of nitrobenzene on the testes. These two experiments demonstrate that the DFS staining procedure is a useful technique for determining the mitochondrial function and membrane integrity of rat spermatozoa in toxicological studies.

In conclusion, the DFS procedure, which uses CAM and EthD-1, can clearly and visually identify the population of viable and dead spermatozoa simultaneously by fluorescence microscopy in rats. This is a useful technique to characterize sperm status, which is determined by the mitochondrial function assessed by CAM and membrane integrity evaluated by EthD-1.

REFERENCES


