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Effect of Preservation with Formaldehyde Buffered Saline on Viability of Frozen-Thawed Bull Spermatozoa Measured by Propidium Iodide

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Abstract: This study examined the effect of preservation with formaldehyde in buffered saline either at 4 °C or at room temperature on the viability measured by a fluorescent probe propidium iodide (PI) in frozen-thawed bull spermatozoa. Spermatozoa were stained for 5 min with PI (5 µg/ml) and 4 different concentrations (0.00625, 0.05, 0.25 and 1 v/v %) of 37 w/v % formaldehyde commercial solution immobilizing spermatozoa. Samples were examined for percentages of spermatozoa that incorporated PI (PI-positive cells) at 0, 20 and 40 min of staining and neither a significant effect of the formaldehyde concentration or the time for preservation on the percentage of PI-positive cells nor a significant interaction between the two factors was found. When spermatozoa were preserved with formaldehyde at 4 °C before staining with PI, the percentage of PI-positive cells at 4 hrs did not significantly differ from 0 h at any of the formaldehyde concentrations used, while preservation for 24 and 120 hrs increased the percentages except for 0.00625% at 24 hrs. When spermatozoa were stored with formaldehyde at room temperature before staining, at any of the formaldehyde concentrations used, preservation for 4 hrs did not significantly affect the percentage but the proportion was significantly increased after 24 hrs. When the spermatozoa stained with PI AND were stored at room temperature up to 24 hrs, there was a significant interaction between the formaldehyde concentration and the preservation time. The percentage of PI-positive cells did not significantly change after 4 hrs but preservation for 24 hrs significantly increased the percentage at all the formaldehyde concentrations. These results suggest that assessment of viability using PI can be awaited by preserving spermatozoa with formaldehyde (0.00625 ~ 1 v/v % commercial solutions) in buffered saline for up to 4 hrs at 4 ° C or room temperature regardless of the presence of PI.

Key words: Bull · Formaldehyde · Propidium iodide · Spermatozoa · Viability

INTRODUCTION

Semen analysis is an important tool to predict male fertility in mammalian species. One of the most important parameters in the evaluation of fertility in a particular male or in the assessment of methods for semen preservation is the analysis of sperm viability. In addition, studies of sperm function require that quantification of the number of the cells responding to physiological stimuli or other reagents is restricted only to viable cells. The inclusion of non-viable cells will deviate the results and confound the effect of compounds under investigation [1]. Plasma membrane integrity and proper function of spermatozoa are essential for metabolism, capacitation, ova binding and the acrosome reaction. Hence, assessment of plasmalemma characteristics may be useful for predicting the fertilizing ability of spermatozoa [2]. Live–dead stains are necessary for the determination of cell viability [3-5] and integrity of the plasma membrane as shown by the ability of a viable cell to exclude the dye, which will diffuse passively into sperm cells with damaged plasma membranes. Glycerol can interfere with the staining properties of live-dead stains making them less reliable for the evaluation of cryopreserved semen [6]. Recently,

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fluorescent probes have been developed for more accurately assess sperm viability than conventional methods [7] Among these, propidium iodide (PI) or SYBR14/PI stain have been a most powerful method as fluorescent probe because their staining is unaffected by the presence of glycerol in the frozen semen samples [8]. Previous studies suggested that fresh bull spermatozoa could be stored for 48 hrs in formol-citrate at temperatures ranging from 4 to 40°C [9] or up to one month in buffered formol saline at 4°C without major change in sperm morphology before being stained with eosin-nigrosin for live/dead differential stain [10]. One of the critical factors which may influence the proportion of spermatozoa stained by eosin in a given smear is the time elapsing between ejaculation and the preparation of semen-stain mixture. Likewise, preservation of a frozen-thawed semen sample for a period of time prior to staining would be useful when viability is not immediately assessed for an experimental reason. It is extremely convenient to keep sperm samples for a while before they can be stained for viability assessment because spermatozoa could quickly die without any protection. This study sought a possibility to preserve frozen-thawed bull spermatozoa with formaldehyde in buffered saline prior to assessment of viability by the use of PI without significant changes in the measured viability.

MATERIALS AND METHODS

Medium: The standard saline medium used consisted of 142 mM NaCl, 2.5 mM KOH, 10 mM glucose and 20 mM Hepes, adjusted to pH 7.55 at 20 °C with NaOH [11,12]. This medium also contained 0.1 w/v % polyvinyl alcohol (Average molecular weight of 9,000 - 10,000; Aldrich Chemical Co., Milwaukee, WI, USA) and 0.1 w/v % polyethylene glycol (Sigma Chemical Co., St. Louis, MO, USA).

Semen Samples: Frozen semen samples from five Japanese Black bulls were prepared in 0.5-ml straws by the standard method at Hida Beef Cattle Research Department, Gifu Prefecture Livestock Research Institute, Japan and kept in liquid nitrogen until use. Straws were allowed to thaw at 38 °C for 1 min immediately before use.

Staining with PI: The procedure of staining used in this study was described by previous studies [1, 13]. The stock solution of PI (P-4170; Sigma Aldrich, Inc., St. Louis, MO, USA) was prepared by dissolving PI in H_2O (0.5 mg/ml), aliquoted and stored frozen at -30 °C in the dark. A staining medium was prepared by adding the following to each 230 µl of saline medium: 2.5 µl of stock solution of formaldehyde (four formaldehyde concentrations). The final concentrations of the formaldehyde commercial solution (37 w/v % formaldehyde solution; No. 061-00416; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were 0.00625 [13], 0.05 (9), 0.25 and 1 v/v %. Stock solution of PI (2.5 µl; final concentration was 5 µg/ml) and a frozen-thawed semen sample (20 µl) were added to the saline medium. The suspension was incubated for 5 min at room temperature (20 – 25 °C) and 2.5 µl of 100 mg bovine serum albumin (BSA)/ml (Fraction V; A9647; Sigma Aldrich, Inc., St. Louis, MO, USA) was added to avoid spermatozoa from sticking to the glass and producing staining artifacts. Sub-samples (2 µl) of the stained suspension were placed on clean microscopic slides and overlaid carefully with coverslips. Random fields were observed at a 400x magnification with epifluorescence microscope (BX 51-33-PH; Olympus Corporation, Tokyo, Japan) equipped with a green excitation filter block (Mirror unit, U-MW1G2; Dichroic bandpass filter, DM565; excitation filter, BP 520-550; emission filter, BA580 1F; Olympus Corporation, Tokyo, Japan) and examined with phase contrast or epifluorescence illumination, being switched from one to the other. For quantitative assessment of plasma membrane integrity, 100 spermatozoa were counted in each stained sample. Spermatozoa that fluoresced red (PI–positive) were considered as dead and unstained spermatozoa as live and the percentages of PI–positive cells were calculated. Spermatozoa were observed within 5-10 min of preparation. Spermatozoa found to be partially stained were regarded as PI–positive.

Experiment 1: Stability of stained spermatozoa: This experiment was carried out to determine the stability of the stained cells during the first 40 min. Frozen-thawed bull spermatozoa were stained with PI at room temperature for 5 min. immediately after BSA was added, a first sub-sample was taken and examined for viability and the rest was kept at room temperature. Another sub-sample was taken and viability was re-examined at 20 and 40 min after staining.

Experiment 2: Effect of preservation at 4°C on the percentage of PI-positive spermatozoa: Frozen bull spermatozoa from three different ejaculates of two Japanese Black bulls were used to study the effect of preservation with different concentrations of formaldehyde at 4 °C for up to 120 hrs on the percentage

of PI-positive cells. Frozen-thawed spermatozoa were mixed with the 4 different concentrations of formaldehyde in the presence of PI, stained for 5 min and BSA was added. Percentages of PI-positive cells were immediately examined (0 hr). The same sperm sample was mixed with different concentrations of formaldehyde in saline medium and kept at room temperature for 1 hr followed by 4 °C for 3, 23 and 119 hrs Thus, spermatozoa were incubated with formaldehyde for a total of 4, 24 and 120 hrs before being staining with PI and examined for viability as above.

Experiment 3: Effect of Preservation at Room Temperature on the Percentage of PI-Positive Spermatozoa: Frozen bull spermatozoa from 10 different ejaculates of three Japanese Black bulls used to find out the effect of preservation at room temperature with different formaldehyde concentrations in the presence or absence of PI and BSA for 0, 4 and 24 hrs on the percentage of PI-positive cells. Spermatozoa were mixed with PI and the 4 different concentrations of formaldehyde and stained for 5 min and BSA was added. Percentages of PI-positive cells were immediately examined (0 hr) and the rest of the stained sample was kept for 4 and 24 hrs at room temperature. The same semen sample was also mixed with the different concentrations of formaldehyde in saline medium without PI and BSA and kept at room temperature for 4 and 24 hrs. After incubation, PI and BSA were added and percentages of PI-positive cells were examined.

Statistical Analyses: Within each experiment, a two-way analysis of variance (ANOVA) was performed to determine whether there were significant effects of the four formaldehyde concentrations and the periods of the preservation and a significant interaction. When significant effects were found by ANOVA, Bonferroni post-test was used to determine the difference in the percentage of PI-positive cells between the two different conditions. All analyses were carried out using a statistical software program (GraphPad Prism Version 4.0, GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Experiment 1: Stability of Stained Spermatozoa: Table 1 shows the percentages of PI-positive cells in the stained samples preserved with different concentrations of formaldehyde up to 40 min at room temperature. ANOVA revealed that there were no significant effects of either the

Table 1: Percentages of PI-positive frozen-thawed bull spermatozoa after preservation at room temperature with different concentrations of formaldehyde in the presence of PI and BSA for various times up to 40 min.

Concentration (v/v %) of formaldehyde commercial solution	Time (min) for preservation		
	0	20	40
0.00625	38.0±7.6	34.3±3.8	44.7±4.1
0.05	41.0±3.2	40.7±6.9	44.0±4.5
0.25	44.3±4.4	47.7±2.3	47.3±1.8
1.00	39.7±3.8	46.3±1.2	46.7±1.7

Values are mean±SEM (n = 3).

Table 2: Percentages of PI-positive frozen-thawed bull spermatozoa after preservation at 4°C with different concentrations of formaldehyde for various times up to 120 hrs.

Concentration (v/v %) of formaldehyde commercial solution	Time (h.) for preservation			
	0	4	24	120
0.00625	46.0±3.1^{a}	59.6±14.2^{a}	70.6±13.0^{a}	100.0±0.0^{b}
0.05	46.0±1.2^{a}	57.0±12.5^{ab}	74.3±11.9^{b}	100.0±0.0^{b}
0.25	44.3±3.8^{a}	60.3±14.3^{ab}	77.3±11.5^{bc}	96.0±4.0^{c}
1.00	53.0±9.6^{a}	64.0±9.3^{a}	99.0±1.0^{b}	100.0±0.0^{b}

Values are mean±SEM (n = 10).a-c: Different superscripts indicate significant differences within the row (P<0.05; Bonferroni post-test).

Table 3: Percentages of PI-positive frozen-thawed bull spermatozoa after preservation at room temperature with different concentrations of formaldehyde for various times up to 24 hrs.

Concentration (v/v %) of formaldehyde commercial solution	Time (h.) for preservation		
	0	4	24
0.00625	56.0±5.3^{a}	61.8±3.4^{a}	74.1±3.0^{b}
0.05	51.4±3.2^{a}	58.5±2.9^{a}	68.0±4.2^{b}
0.25	53.0±3.2^{a}	58.6±3.5^{a}	75.2±4.2^{b}
1.00	53.0±3.2^{a}	57.9±2.8^{a}	97.3±1.2^{b}

Values are mean ± SEM (n =10). a-b: Different superscripts indicate significant differences within the row (P<0.05; Bonferroni post-test).

Table 4: Percentages of PI-positive frozen-thawed bull spermatozoa after preservation at room temperature with different concentrations of formaldehyde in the presence of PI and BSA for various times up to 24 hrs.

Concentration (v/v %) of formaldehyde commercial solution	Time (h.) for preservation		
	0	4	24
0.00625	56.0±5.3^{a}	59.9±4.3^{a}	71.9±4.2^{b}
0.05	51.4±3.2^{a}	59.6±3.0^{ab}	67.4±5.1^{b}
0.25	53.0±3.2^{a}	57.3±3.2^{a}	75.7±5.0^{b}
1.00	54.2±3.5^{a}	60.8±3.0^{a}	98.3±0.8^{b}

Values are mean ± SEM (n =10).a-b: Different superscripts indicate significant differences within the row (P<0.05; Bonferroni post-test)

formaldehyde concentration or the time for preservation on the percentages of PI-positive cells and that there was no significant interaction between the concentration and the time.

Experiment 2: Effect of Preservation at 4°C on the Percentage of PI-Positive Spermatozoa: When spermatozoa were stored at 4 °C with formaldehyde in the absence of PI (Table 2), neither a significant effect of the formaldehyde concentration nor an interaction between the concentration and the time was observed but the time of preservation significantly increased the percentage of PI-positive cells ($P<0.0001$). Bonferroni post-test revealed that preservation for 4 hrs at room temperature did not alter the percentages of PI-positive cells at any of the formaldehyde concentrations, while preservation for 24 hrs increased the percentages at 0.05 ($P< 0.05$), 0.25 ($P< 0.01$) and 1 % ($P<0.001$). The percentages of PI-positive cells were significantly increased after 120 hrs of preservation at all the formaldehyde concentrations examined ($P< 0.001$, respectively).

Experiment, 3 Effect of Preservation at Room Temperature on the Percentage of PI-Positive Spermatozoa: When spermatozoa were stored at room temperature with formaldehyde before being stained with PI (Table 3), an interaction between the formaldehyde concentration and the time was significant ($P<0.0001$). Bonferroni post-test revealed that preservation for 4 hrs did not significantly affect the percentage of PI-positive cells at any of the formaldehyde concentrations used but the percentage was significantly increased after 24 hrs of preservation at any of the formaldehyde concentrations ($P<0.001$, respectively).

When spermatozoa were stored at room temperature after being stained with PI, there was a significant interaction between the formaldehyde concentration and the preservation time ($P<0.0001$). Bonferroni post-test revealed that the percentage of PI-positive cells did not significantly change after preservation for 4 hrs but preservation for 24 hrs significantly increased the percentage of PI-positive cells at all the formaldehyde concentrations ($P<0.001$, respectively; Table 4).

DISCUSSION

The fluorescent probe PI is employed to detect the proportion of non-viable cells in a population. The use of impermeable fluorescent probes for monitoring the integrity of the membranes is well known in other cell systems and the method of using fluorescent probes for determination of cell viability was also applied previously to the spermatozoa but its efficacy was different among many sperm biologists [13]. Most workers used the flow cytometry which is not available in all laboratories and visual microscopic observation of live sperm populations interacting with the impermeant fluorescent probes enables direct assessment of the individual cells. The use of low concentrations of formaldehyde to immobilize the motile cells for observation does not harm sperm viability [14].

The results of the present study indicate that the percentages of non-viable cells identified by staining with PI at room temperature for at least 5 min showed no significant changes for a period of time up to 40 min. These results are in agreement with the previous results which found that there was no significant increase in the percentage of the PI-positive cells during preservation of fresh ram spermatozoa in the staining solution for a period of time up to 100 min [13]. In that report, after 100 min, there was slight increase in the percentage of PI-positive cells and this increase may be attributed to inherent senescence changes rather than to any detrimental effect of the staining solution. Moreover, fresh human spermatozoa were stained with Hoechst 33258 for 30 min without any significant changes in the percentage of non-viable cells [15].

In our experiments in which frozen-thawed bull semen samples were fixed with formaldehyde in buffered saline and kept at room temperature for up to 24 hrs in the presence or absence of PI, the percentages of PI-positive cells did not significantly differ after 4 hrs of preservation, whereas there were a highly significant increase in the percentages of PI-positive cells after 24 hrs of preservation. At a preservation temperature of 4 °C, the results were similar. The results indicate that there is an increase in the percentage of PI-positive cells with the prolonged time of preservation. Other results [9] reported that the percentage of dead spermatozoa did not differ significantly in the boar, bull, ram and rabbit up to 96 hrs of preservation in formol citrate at 4°C or at room temperature before being stained with eosin-nigrosin stain.

The results of this study revealed that there was no significant difference in the percentage of PI-positive cells after preservation with different formaldehyde concentrations for 4 hrs. All the concentrations used can preserve the sperm viability up to 4 hrs of incubation at 4°C as well as at room temperature. However, the dead spermatozoa in fresh bull semen stained with eosin-nigrosin was increased with the duration of the preservation (2-3 weeks) at room

temperature and this was considered to be attributed to a relatively low formaldehyde concentration used, which could not prevent spermatozoa from becoming eosinophilic [9]. Moreover, bull spermatozoa fixed in 0.00625 % formaldehyde in buffered saline showed motile spermatozoa after the removal of fixative by washing and this is because the fixative inhibits the motility in all spermatozoa but does not affect the staining characteristics of eosin [9].

Our data indicated that fixation of frozen-thawed bull spermatozoa with formaldehyde in buffered saline and staining with PI preserve the viability for a short time in comparison to other stains such as eosin-nigrosin, which can preserve the viability of fresh bull spermatozoa up to 1 week [9] and the eosinophilic prosperities of bull spermatozoa remain unchanged after one month of preservation at 4 °C with formaldehyde in buffered saline as compared to those of fresh semen immediately after collection [10]. It is unclear whey there are differences in the ability of these two dyes to stain sperm cells. It has been recognized that long incubation period leading to senescence of cells can create artifacts and increase the number of cells that incorporate the fluorescent probe [13]. In the present study, the fixation with formaldehyde in buffered saline preserves sperm viability for short time (up to 4 hrs). This difference might be also due to the form of preserved semen (fresh or frozen).

In conclusion, frozen-thawed bull spermatozoa could be preserved with formaldehyde in buffered saline for 4 hrs at 4 °C or at room temperature without significant changes in the percentages of PI-positive cells regardless of whether PI is present or not.

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