EVALUATION OF THE VARIATION IN MITOCHONDRIAL FUNCTION OF BOVINE SPERMATOZOA USING THE MEAN OF GREEN FLUORESCENCE OF RHODAMINE 123 (MGF-R123)

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ABSTRACT

Mean of green fluorescence of Rhodamine 123 (MGF-R123) was evaluated flow cytometrically to study the variation in mitochondrial function of bovine spermatozoa and its relation to sperm viability. Fresh semen from two mature bulls with normal seminal characteristics was used in this study. R123 + PI was used to evaluate the mitochondrial activity of sperms and SYBR-14 + PI was used to evaluate the sperm viability. Freeze-killed procedure was used to estimate the relationship between MGF-R123 and the number of viable spermatozoa. Monensin was used as a mitochondrial inhibitor to evaluate the relationship between MGF-R123 and the mitochondrial activity. The results indicated that a highly significant (P< 0.001) correlation (r = 1.00) between percentage of sperms with active mitochondria and percentage of viable sperms. There is no correlation between MGF-R123 of viable sperms and percentage of viable sperms. MGF-R123 was negatively correlated with concentration of monensin (P< 0.001, r = -1.00). These data suggest that the MGF-R123 is well suited for evaluate the variation in mitochondrial function of bovine spermatozoa.

INTRODUCTION

Sperm motility has a strong relationship with fertility (3,6,14,15) and it is a useful indicator of sperm fertilizing capacity in vivo (19). There is a positive correlation between mitochondrial membrane potential and spermatozoal motility (21). Mitochondria of the sperm midpiece generate energy to support motility, therefore changes in mitochondrial membrane potential could be a good indicator of functional impairment without the direct assessment of sperm motility parameters (7).

Flow cytometry has become increasingly useful for assessing morphological and functional parameters of spermatozoa, since it has substantial advantages over epifluorescence or light microscopy methods. Flow cytometry allows parameters related to several sperm functions to be collected from thousands of cells in a short time during the analysis of each sample (13). Another main advantage of the flow cytometry is that only particle-associated fluorescence is detected, and therefore the washing of sperm suspensions to remove free fluorophores is not necessary (13).

Flow cytometry has been used to estimate sperm mitochondrial activities (9,11). The most widely used mitochondrial specific probe, Rhodamine 123 (R123), is a cationic compound that is excited at 488nm, it accumulates in the mitochondria as a function of transmembrane potential (1,5). The R123 accumulated in the mitochondria and emits green fluorescence light, thus identifying the sperm that exhibited a mitochondrial membrane potential.(8). Peak fluorescent channels of R123 (with the EPICS 753 MDADS computer) was used to determination mitochondrial function (9). Other fluorescent dyes such as 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) and MitoTracker Green FM (MITO) have been used to estimate mitochondrial function (11). Methylene blue reduction rates were evaluated spectrophotometrically to study bovine spermatozoal mitochondrial function (4).

The aim of this study was to evaluate the variation in mitochondrial function of bovine spermatozoa using the mean of green fluorescence of R123 and its relation with sperm viability.

MATERIALS AND METHODS

Semen samples

Fresh semen from two mature bulls with normal seminal characteristics was supplied by Rinder Union West EG. Samples were diluted 1:40 in Hepes[1] buffered saline containing 0.1% Bovine Serum Albumin[1] (BSA) (Hepes-0.1%BSA) (10).

Fluorescent staining

Stock solutions of 0.53 mM Rhodamine 123[2] were prepared in DMSO[1] and 2.99 mM Propidium Iodide[1] (PI) in Tyrode's Salt Solution[1]. Final staining solution was prepared which contained 3 µl of R123 stock solution and 12 µl PI stock solution/ml Hepes-0.1%BSA (11). SYBR-14, available together with PI as LIVE/DEAD sperm viability kit[2]. A staining solution was prepared that contained 0.8 µl SYBR-14 and 9 µl PI/ml Hepes-0.1%BSA (11).

Staining procedure

To estimate mitochondrial function, 150 µl of the diluted semen were stained with 300 µl of the final staining solution of R123 (ratio 1:2). The samples were incubated for 30 min before flow cytometry examination. To estimate sperm viability, 100 µl of the diluted semen were stained with 300 µl of the staining solution of SYBR-14 (ratio 1:3). The samples were incubated for 15 min before flow cytometry examination (11).

Flow cytometry

Flow cytometry analysis was conducted using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA 94039, USA). The cell flow rate was placed on the low reading for the machine, which, based on sperm concentration in the solution, resulted in a actual flow rate of 300-400 cell/sec. A total of 10000 events was evaluated for each sample. Measures of green fluorescence (FL1) and red fluorescence (FL3) were collected for each event. The FL1 measure indicated the R123 orSYBR-14 positive and FL3 the PI positive fluorescence for each spermatozoon. Logarithmic amplification was used to collect green and red fluorescence.

To determine the correct side scatter and forward scatter of spermatozoa, the samples that were stained with R-123 or PI only and were backgated from the area in which fluorescently stained populations appeared in the green and red channels. The generated data were analysed using CellQuest software on an Apple Macintosh computer (Becton Dickinson). WinMDI 2.8 software (Joseph Trotter) for PC, MS windows 98 was used to partition and quantify dot-plot sperm populations. The data for each sample in this study include the mean of green fluorescence and the number of events in green and red populations.

Experiments

Freeze-killed procedure (9,17) was used to estimate the relationship between MGF-R123 and the number of viable spermatozoa. 10 ml of diluted semen were divided in two fractions, one fraction was maintain at 37 °C while the sperms 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 sperms in ratios of 10:0, 8:2, 6:4, 4:6, 2:8 and 0:10 (vol:vol), respectively. Monensin[1] was used as a mitochondrial inhibitor (9,17) to evaluate the relationship between MGF and the mitochondrial activity. 10 ml of diluted semen were divided to five groups, first group was used without monensin as control group, samples in the remaining four groups were prepared with monensin to obtain a final concentration of monensin 20, 40, 60, 80, 100 µM, respectively.

Statistical Analysis

Results were expressed as mean ± SD. MGF data were subjected to one way repeated measures analysis of variance and correlation using Jandel Sigmastat statistical software V2.0. P<0.05 was considered as statistically significant.

[1] Sigma Chemicals, St. Louis,MO, USA.

[2] Molecular Probes, Eugene, OR, USA.

RESULTS

Figure 1 shows a representative example of the outcome from flow cytometer used to estimate MGF and percent of active mitochondria.

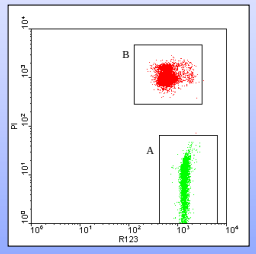

Figure 1: Dot plots resulting from flow cytometric analysis of stained spermatozoa. (A) The spermatozoa with green fluorescent (R123 positive). (B) The spermatozoa with red fluorescent (PI positive).

The data of MGF-R123 and percentage of active mitochondria for different ratios of viable and freeze-killed sperms were summarised in Table 1.

Table 1: The MGF-R123, percentage of sperms with active mitochondria and percentage of viable sperms for different ratios of viable and freeze-killed sperms of bull.

Ratios of viable :freeze-killed sprms	Mean of Green Fluorescent (Mean ± SD)	% of sperms with active Mitochondria (R123 positive) (Mean ± SD)	% of viable sperm (SYBR-14 positive) (Mean ± SD)
10:00	1339 ± 82	77,9 ± 3,2	79,4 ± 3,1
08:02	1364 ± 81	62,9 ± 3,4	64,6 ± 3,3
06:04	1325 ± 74	47,1 ± 3,7	48,1 ± 3,9
04:06	1374 ± 67	34,3 ± 5,3	35,4 ± 5,8
02:08	1315 ± 64	16,5 ± 1,8	16,7 ± 1,9
00:10	1329 ± 75	1,6 ± 0,2	1,7 ± 0,1

There is a highly significant (P< 0.001) correlation (r = 1.00) between percentage of sperms with active mitochondria and percentage of viable sperms (Figure 2). There is no correlation between MGF-R123 of viable sperms and percentage of viable sperms.

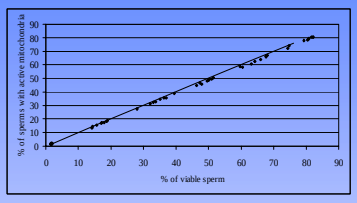

Figure 2: The relationship between percentage of sperms with active mitochondria and percentage of viable sperms for different ratios of viable and freeze-killed sperms of bull.

The relationship between different concentration of mitochondrial inhibitor (monensin) and MGF-R123 were shown in Table 2. MGF-R123 decreased gradually with increasing concentration of monensin, while there is no significant variation in percentage of viable sperms.

Table 2: The MGF-R123 of viable sperms and percentage of viable sperms for different concentrations of monensin.

Concentration of monensin (µM)	Mean of Green Fluorescent (Mean ± SD)	% of viable sperm (SYBR-14 positive) (Mean ± SD)
0 (control)	1342 ± 50	79,6 ± 9,3
20	1214 32	78,4 8,8
40	1167 ± 34	77,6 ± 8,1
60	1123 ± 20	78,9 ± 6,5
80	1120 ± 36	78,9 ± 6,4
100	1100 ± 42	78,2 ± 6,1

The data of MGF-R123 were negatively correlated with concentration of monensin (P< 0.001, r = -1,00) (Figure 3).

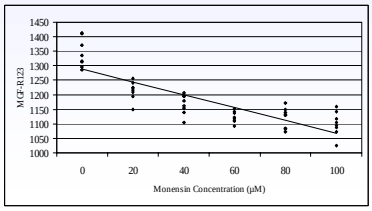

Figure 3: The relationship between MGF-R123 of viable sperms and concentration of mitochondrial inhibitor (Monensin).

DISCUSSION

Rhodamine 123 has been used as a probe of mitochondrial membrane potential in bovine spermatozoa (9,11), Many studies have used R123 with PI to find the percentage of active mitochondria and related that with sperm viability in human, bovine and equine (2,9,11,12,16,17,20) but very few studies have been published using R123 to find the variation in mitochondrial function (9,17).

The aim of the present study was to use MGF-R123 as indicator of mitochondrial function. Therefore, freeze-killed procedure and monensin was used to estimate MGF-R123 and its relation with number of viable sperm and mitochondrial function, respectively.

The result of freeze-killed procedure show a gradually decrease in percent of functional mitochondria of sperm. This results agrees with the findings of Garner et al (11), who found a high correlation between assessment of R123 and sperm viability. Similar observation have been seen in other studies (12,17,18). At the same time the data indicate no significant variation in MGF-R123 of viable sperms with increased numbers of freeze-killed sperms, this result was observed because there is no effects on mitochondrial fuction of viable sperms. Therefore, there is no correlation between MGF-R123 of viable sperms and percentage of viable sperms.

Mitochondrial function and R123 uptake was tested using the mitochondrial inhibitor monensin. The results indicated that there is decrease of MGF-R123 gradually with increase concentration of monensin compared with that of control group, in which the inhibitor was absent. At the same time the percent of viable sperms was not affected. Thus, it was indicated that MGF-R123 is emitted exclusively from the functioning mitochondria. Monensin have been used with bovine (9) and equine (17) spermatozoa and similar conclusions were obtained.

I n conclusion, the MGF-R123 using flow cytometer is well suited to evaluate the variation in mitochondrial function of bovine spermatozoa.

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