



Evaluation of an MTT reduction assay for the analysis of equine semen by comparison with flow cytometric results

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INTRODUCTION

Semen evaluation is primarily performed to give an indication of the fertilizing capacity of spermatozoa produced by a certain stallion. This may be done as part of an assessment process prior to purchase, at the beginning and at set stages throughout the breeding season, or to test the suitability of a certain stallion for an inclusion in an artificial insemination programme.

Several methods are currently available to evaluate the quality of semen samples. Visual estimation of the percentage of motile sperm is the most common mode of analysis. This method is fast and inexpensive, but it is subjective and can be influenced by the experience of the testing person (1). Computer-assisted sperm analysis is also based on motility characteristics (2). Flow cytometry in different technical applications offers many advantages for the analysis of sperm quality. It provides an objective and rapid evaluation of individual cells, and allows to estimate thousands of cells per sample quantitatively and qualitatively (3). But the last two

methods are expensive and need special instruments.

Recently the MTT test revealed as a simple, rapid and reliable method for evaluation of human sperm viability (5) and other cells (6,7). MTT is a yellow water-soluble tetrazolium salt (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). The dye is converted to water-insoluble purple formazan on the reductive cleavage of its tetrazolium ring by the succinate dehydrogenase system of active mitochondria (4). Thus, the amount of formazan formed is directly proportional to the number of metabolically active cells in the sample, which can be simply quantified spectrophotometrically after dissolving the formazan in an organic solvent.

In this study we investigate the suitability of MTT test for the evaluation of equine semen by comparison the results of this test with those simultaneously achieved flow cytometrically.

MATERIALS AND METHODS

Experiment 1: Definition the relation between the MTT reduction rate and the viability of sperms. For this purpose the freeze-killed procedure (8) was used. Fresh semen of one stallion was diluted with Hepes-BSA. 6 ml of diluted semen was divided in two fractions; one fraction was maintained 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. The MTT assay was performed according to the method of Mosmann (4). 6 wells of the 96-well microplate were used for each group. 100 µl of semen sample plus 10 µl of MTT stock solution (5 mg of MTT/ml of PBS) were placed in each well. The rates of MTT reduction were taken immediately and after incubation at 37°C every hour till 4 hours using a spectrophotometer (MS2 Reader) at wave length 550 nm.

Experiment 2: Correlation between the results of MTT test and those of flow cytometry. Fresh semen was collected from 11 stallions (warm blood). Semen was diluted with Hepes 0.1% BSA to obtain a concentration of 100 million cells/ml. 6 wells of the 96-well microplate were used for each stallion. 100 µl of semen sample plus 10 µl of MTT stock solution was placed in each well. The rates of MTT reduction were taken after 1h and 4h incubation at 37°C. Simultaneously split samples of the same semen were tested flow cytometrically to evaluate the mitochondrial activity, sperm viability and acrosomal integrity using Rhodamine 123, SYBR-14 and Lyso Tracker Green DNA-26, respectively (9).

The correlation coefficient between the results of each test was calculated using the SPSS statistical program.

RESULTS AND DISCUSSION

Experiment 1:

Addition of different volumes of dead freeze-shocked sperm cells to fresh semen and the effects on MTT reduction at the different incubation times are shown in Figure 1.

In all groups, the rate of MTT reduction increased gradually with incubation time. The addition of varying proportions of freeze-shocked sperm cells to fresh semen resulted in a proportional and significant ($P < 0.01$) decrease in the rate of MTT reduction. At each time of measurement, a high negative correlation ($r = 0.99$, $P < 0.001$) was observed between the MTT reduction rate and the amount of freeze-shocked sperms. These results agree with the findings of Mosmann (4), who concluded that MTT reduction rate depends strongly on the number of viable cells in the sample.

Experiment 2:

Table 1 and 2 presents the results of the evaluation of semen from 11 stallions, which were tested simultaneously by the MTT test and by a flow cytometric procedure.

The presented correlation coefficients (Table 2) indicate:

1. A remarkable high correlation between the MTT reduction at 1 and 4 hours of incubation time.
2. An even remarkable high correlation between the MTT reduction rates at the two times of incubation and the corresponding results, determined by flow cytometry.

Because the mitochondrial activity, viability and acrosomal integrity of sperms have a positive relationship with fertility (9), we suggest that, the MTT reduction rate by the equine spermatozoa has also a positive correlation with the fertility of equine sperms.

In conclusion, the MTT-test has been proven as a reliable method for an objective evaluation of equine semen. It can be used successfully in routine analysis, where practical aspects as time, costs and practicability are important.

Table 2: Correlation coefficients between the results of the two test methods.

		MTT at 4 hours	Mitochondrial activity	Sperm viability	Acrosomal integrity
MTT at 1 hours	r	0,814	0,817	0,727	0,666
	P	0,00230	0,00215	0,0113	0,0258
MTT at 4 hours	r		0,718	0,723	0,659
	P		0,0129	0,0120	0,0274
Mitochondrial activity	r			0,977	0,926
	P			0,00000223	0,0000429
Sperm viability	r				0,913
	P				0,0000854

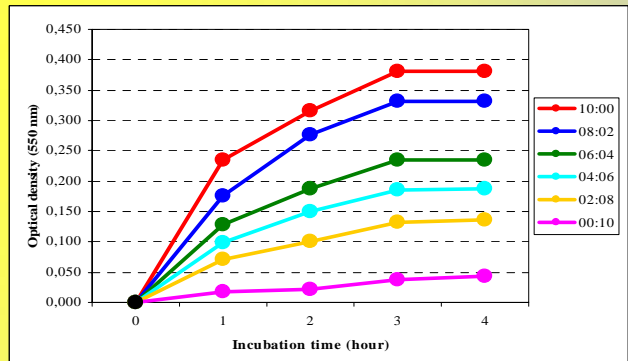


Figure 1: Results of MTT reduction of the semen samples after the addition of freeze-shocked cells in different proportions.

Table 1: Mean values of the optical density (MTT test) and of different sperm parameters (flow cytometer).

No. of stallions	MTT		Flow Cytometer		
	Optical density at 550 nm		% of sperm with active mitochondria (R-123)	% of viable sperm (SYBR-14)	% of sperm with intact arcosomes (Lyso-TG)
	1 hour	4 hours			
1	0,715	0,697	81,08	75,44	60,70
2	0,453	0,501	51,53	53,55	42,20
3	0,363	0,442	45,80	48,37	39,87
4	0,456	0,450	59,23	58,31	51,71
5	0,489	0,474	77,13	76,52	65,27
6	0,594	0,683	77,94	80,09	59,79
7	0,623	0,775	77,20	76,66	70,86
8	0,611	0,553	69,93	64,21	60,21
9	0,570	0,603	80,01	78,63	69,85
10	0,573	0,610	79,07	78,15	71,79
11	0,547	0,483	63,02	62,38	49,78

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