

**EVALUATION OF THE PERCENTAGE OF LIVE SPERM IN RAM SEMEN BY USING THE MTT REDUCTION ASSAY**

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**ABSTRACT**

This study was conducted to evaluate the percentage of viable sperm in ram semen by using the MTT reduction assay. Twenty ejaculates from 5 rams were included in this study. Semen samples were diluted with skim milk-glucose diluent to obtain a concentration of  $30 \times 10^6$  sperms/ml. The rates of MTT reduction were taken in microtiter plates after one hour of incubation at  $37^\circ\text{C}$  using a Microplate Reader (DNM-9602) at a wave length of 550 nm. Simultaneously, split samples of the same semen were tested using the microscope and eosin-nigrosin stain. The correlation between the results of these tests was calculated using the Pearson correlation coefficients and Regression analysis. Results of the present study indicate that the values of sperm viability which calculated on the basis of MTT reduction rates were significantly ( $P < 0.001$ ) correlated with the results that simultaneously determined by the microscope and eosin-nigrosin stain, yielding regression coefficients of  $r^2 = 0.979$ . In conclusion, the MTT reduction test proved applicable as diagnostic tool for the quality evaluation of ovine semen. It can be used successfully in routine analysis, where practical aspects as time, costs and practicability are important.

تقدير نسبة الحيامن الحية في السائل المنوي للكباش  
باستعمال قياس اختزال الـ MTT

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**الخلاصة**

اجريت هذه الدراسة لتقدير نسبة الحيامن الحية في السائل المنوي للكباش باستعمال طريقة قياس اختزال الـ MTT. عشرون قذفة من خمسة كباش استخدمت في هذه الدراسة. خففت عينات السائل المنوي بمخفف حليب الفرز-الكلوكوز للوصول الى تركيز  $10 \times 30 \times 10^6$  حيمن/مل. تم قياس معدل اختزال الـ MTT بعد حضن العينات لمدة ساعة تحت درجة 37 م باستخدام جهاز (DNM-9602) Microplate Reader وعلى طول موجي مقداره 550 نانوميتر. في نفس الوقت جزء اخر من العينات تم تحليلها

باستخدام المجهر وصبغة الايوزين-نكروسين. تم حساب الارتباط ما بين النتائج المأخوذة بكلا الاختبارين باستخدام اختبار معامل الارتباط ومعامل الانحدار. بينت النتائج ان قياس حيوية الحيامن بطريقة اختزال الـ MTT ارتبطت معنويا ( $P < 0.001$ ) مع النتائج المحسوبة بنفس الوقت بطريقة المجهر وصبغة الايوزين-نكروسين، حيث كان مقدار معامل الارتباط  $R^2 = 0.979$ . نستنتج من هذه الدراسة ان اختبار اختزال الـ MTT بالامكان استخدامه كطريقة تشخيصية للسائل المنوي في الاغنام حيث يمكن استخدامه في التقييم الروتيني للسائل المنوي لكونه اختبار غير مكلف ولا يحتاج لوقت طويل.

## INTRODUCTION

Several methods are currently available to evaluate the quality of semen samples. Visual estimation of the percentage of motile sperm is the most common method of semen analysis. This method is fast and inexpensive, but it is subjective and can be influenced by the experience of the analyst (1). Computer-assisted sperm analysis is also based on motility characteristics. The system uses a camera, computer, and software to record and analyse sperm motility (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 for estimation of thousands of cells per sample quantitatively and qualitatively (3). Both latter methods are expensive and require special equipments.

Therefore, various analytical techniques have been developed to evaluate sperm quality. Assessment of metabolic status of spermatozoa is one of these techniques which provide valuable information for predicting sperm fertilizing capacity. Reduction activity of spermatozoa is one of these methods that depend on the ability of metabolically active spermatozoa to reduce specific stains. The ability of spermatozoa to reduce the resazurin redox dye (4,5) and methylene blue (6) was used successfully to evaluate semen quality. Recently the MTT assay was revealed as a simple, rapid and reliable method to estimate the percentage of viable sperm in human and boar, depending on the accumulation of formazan grains around the midpiece of sperm tail (7,8). And in our previous studies (9,10), the MTT test was reported to be a reliable method for an objective evaluation of equine and bovine semen, depending on the reduction rate and optical density of the MTT.

MTT (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) is a yellow water-soluble tetrazolium salt. The dye is converted to water-insoluble purple formazan on the reductive cleavage of its tetrazolium ring by the succinate dehydrogenase system of the active mitochondria (11). Thus, the amount of formazan formed can be determined spectrophotometrically and serves as an indicator of the number of mitochondria and hence the number of living cells in the sample (12). MTT assay was used in many studies, which were related to viability of different cells (13-17).

The aim of the present study is to investigate the diagnostic value of the MTT reduction assay to evaluate the percentage of live sperm, and to compare the results of this test with those that were simultaneously achieved by the microscope and eosin-nigrosin stain.

## **MATERIALS AND METHODS**

### **Animals:**

Five adult rams, aged between 1.5-2 years (during the semen collection period), were used in this study. The rams were maintained at the animal house of the College of Veterinary Medicine, University of Mosul under uniform feeding and housing conditions.

### **Semen samples:**

Twenty ejaculates (4 ejaculates from each ram) were collected from rams with pre warmed artificial vagina (37 °C). The volume of collected semen samples ranged between 0.7 and 1.5 ml with an initial sperm motility of 70-90% and a total concentration of 2-2.2 x 10<sup>9</sup> sperms/ml. After the microscopic evaluation, each semen sample was diluted with skim milk-glucose diluent to obtain a concentration of 30 x 10<sup>6</sup> sperms/ml.

Some of semen samples were left at the room temperature for about one hour or more to reduce the percentage of live sperms in the sample, and increase the degree of variation between the samples.

### **Semen analysis:**

Eosin-nigrosin stain was used to evaluate the percentage of live sperm. The stain was prepared according to Dott and Foster (18). To stain a semen sample, one drop of diluted semen was placed at one end of clean, dry and pre-warmed microscope slide alongside of which was placed two drops of stain. The stain and semen were mixed and left for 5 minutes. A thin film of the stained sample was drawn along the slide. The slide was left to dry. A sample of 200 spermatozoa was evaluated under a light microscope at a magnification of 1000X, and the number of spermatozoa stained and unstained was noted, then the percentage of live sperm was calculated (19).

### **MTT reduction assay:**

The MTT assay was performed according to the method of Aziz et al. (9). For each sample, 6 wells of the 96-well microplate were used. 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 one hour of incubation at 37°C using a Microplate Reader (DNM-9602, Beijing Prolong New Technology Co., LTD., Nanjing, China) at a wave length of 550 nm.

### **Experiments:**

Firstly, fresh semen of three rams with good semen quality (about 85% viable sperms) were used to obtain the standard curve of the relationship between the MTT reduction rate and percentage of live sperm that estimated by microscope and eosin-nigrosin stains. The semen samples were diluted with skim milk-glucose diluent, then 10 ml of the diluted semen were 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 water path at 60°C. Samples for analysis were made by combining aliquots of viable and killed sperms in ratios of 10:0, 8:2, 6:4, 4:6, 2:8 and 0:10 (vol:vol), respectively. The percentage of live sperm in the prepared samples was evaluated by using the microscope and eosin-nigrosin stain.

Simultaneously each sample was analyzed by using the Microplate Reader and MTT.

Secondly, the MTT test was applied to evaluate sperm viability in 20 ejaculates that were collected from the 5 rams. After dilution of semen samples with skim milk-glucose diluent, samples and MTT stock solution were distributed in the wells of the 96-well microplate. The rates of MTT reduction were taken immediately and after one hour incubation at 37°C. Simultaneously split samples of the same semen were tested using the microscope and eosin-nigrosin stain.

Statistical analysis:

Pearson correlation coefficients and Regression analysis were used to evaluate the efficacy of the MTT test for the assessment of sperm viability of ram semen. Data were performed using SigmaStat (Jandel scientific software V3.1), and  $P < 0.05$  was considered as statistically significant.

### RESULTS

Table 1 showed the MTT reduction rates, which were obtained after one hour of incubation time, and percentage of live sperm, which were estimated by microscope and eosin-nigrosin stain, of samples containing different proportions of live and killed sperm cells.

MTT reduction rates were decreased significantly ( $P < 0.001$ ) by increasing the proportion of dead sperm cells. There was a high negative ( $R^2 = -0.983$ ,  $P < 0.001$ ) correlation between the MTT reduction rates and the amount of dead sperms.

Regression equation ( $y = 65.335x - 33.227$ ) of the relationship between the MTT reduction rate and percentage of live sperm was calculated; the corresponding correlation curve was presented by Figure 1. This curve was applied later as standards to calculate the percentage of live sperm cells on the basis of MTT reduction rates.

Table 1: MTT reduction rates and sperm viability of semen samples containing different proportions of live and killed sperm cells.

semen samples containing different proportions of live and killed sperms	MTT Absorbance rate at 550 nm after one hour incubation time at 37 C	Percentage of live sperm obtained by microscope and eosin-nigrosin stain
10:00	1.833 ± 0.033	85.75 ± 1.71
08:02	1.506 ± 0.035	67.60 ± 2.38
06:04	1.239 ± 0.043	50.80 ± 2.65
04:06	1.074 ± 0.059	34.40 ± 2.38
02:08	0.797 ± 0.048	16.80 ± 2.22
00:10	0.498 ± 0.027	0.00 ± 0.00

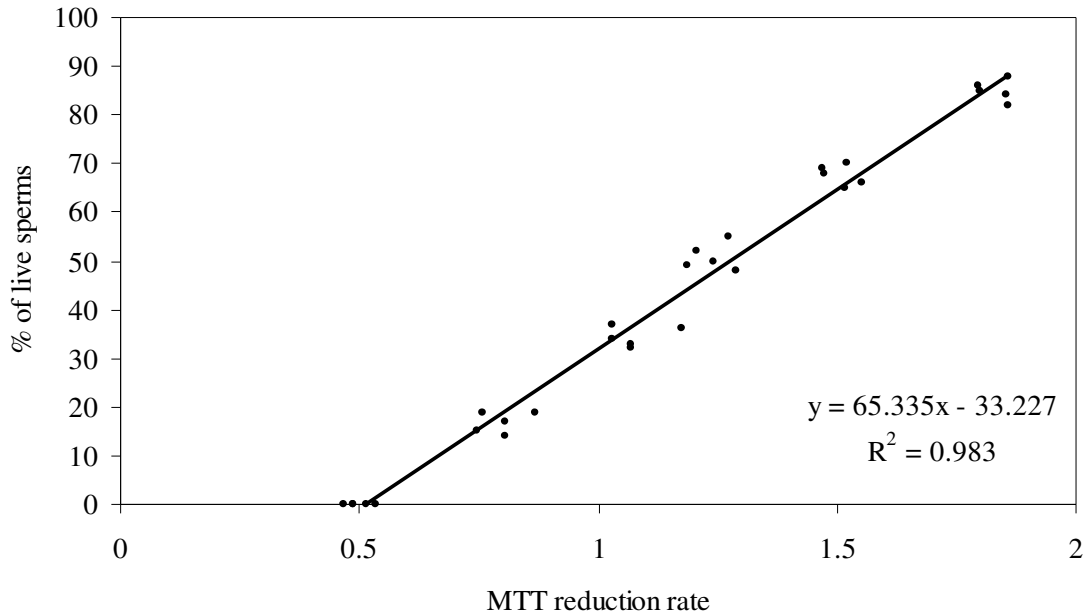


Figure 1: The corresponding correlation curve and Regression equation of the relationship between the MTT reduction rate and percentage of life sperms.

Table 2 contains the diagnostic results for all semen samples of the 5 rams that obtained by MTT reduction test and the percentage of live sperm, which was evaluated simultaneously using a microscope and eosin-nigrosin stain.

The percentage of live sperms that calculated on the basis of MTT reduction rate was significantly ( $P < 0.001$ ) correlated with the result that simultaneously determined by microscope and eosin-nigrosin stain, yielding a regression coefficients of  $R^2 = 0.979$  (Table 2).

Table 2: Analysis of the bovine semen samples using MTT test and eosin-nigrosin stain.

Ejaculates No.	MTT test		Eosin-nigrosin stain
	MTT Absorbance rate at 550 nm	% of live sperms according to MTT	% of live sperms
1	1.094 ± 0.016	38.25 ± 1.05	40.83 ± 6.71
2	1.147 ± 0.029	41.73 ± 1.88	39.39 ± 5.48
3	1.783 ± 0.071	83.28 ± 4.63	85.72 ± 2.03
4	1.636 ± 0.058	73.63 ± 3.82	75.89 ± 2.96
5	1.711 ± 0.044	78.53 ± 2.85	81.06 ± 3.45
6	1.730 ± 0.053	79.80 ± 3.49	78.25 ± 3.97
7	1.448 ± 0.025	61.40 ± 1.66	59.21 ± 4.31
8	1.204 ± 0.023	45.41 ± 1.51	48.09 ± 7.42
9	1.600 ± 0.041	71.33 ± 2.66	73.38 ± 5.33
10	1.096 ± 0.019	38.38 ± 1.22	39.70 ± 10.96
11	1.806 ± 0.096	84.78 ± 6.27	79.81 ± 4.00
12	1.733 ± 0.048	80.00 ± 3.15	76.10 ± 5.67
13	1.556 ± 0.038	68.45 ± 2.48	65.20 ± 11.70
14	0.722 ± 0.047	13.92 ± 3.10	18.84 ± 6.81
15	1.535 ± 0.021	67.03 ± 1.35	63.36 ± 3.17
16	1.222 ± 0.079	46.60 ± 5.13	44.78 ± 5.88
17	1.145 ± 0.033	41.56 ± 2.14	38.41 ± 4.17
18	1.803 ± 0.056	84.56 ± 3.68	78.45 ± 4.34
19	0.836 ± 0.036	21.38 ± 2.32	24.60 ± 6.06
20	1.854 ± 0.044	87.87 ± 2.87	80.13 ± 1.91

### DISCUSSION

The MTT assay was used in many studies to evaluate the viability of different cells (13-17). This test depends on the ability of viable cells to reduce the MTT (17). In this study, the diagnostic value of the MTT reduction assay to evaluate the percentage of live sperms in semen of rams was investigated by comparison of results with those obtained by using the microscope and eosin-nigrosin stain.

Results of this study indicate a high correlation between the MTT reduction rate and the result of microscope. Furthermore the reduction rate of MTT

decreased significantly with an increasing proportion of killed sperm. These results are in agreement with the findings of Mosmann (17) who concluded that the MTT reduction rate depends strongly on the number of viable cells in the sample.

In contrast to the procedure that was published by Mosmann (17), the reduction rate of MTT was taken successfully after one hour of incubation time. This was expected because spermatozoa are very active cells and rich in mitochondria; therefore the reduction of MTT by spermatozoa is faster than other cells. A similar observation was reported by our previous studies (9,10).

The percentage of live sperm in all tested semen samples that obtained according to the rate of MTT reduction were highly correlated with those results which were estimated using the microscope and eosin-nigrosin stain. Therefore our results suggest that the MTT reduction rate by the spermatozoa may be used as an indicator for the sperm viability in ram semen.

The advantages of the MTT test are simple and inexpensive (17). Additionally results from this study suggest other advantages of this test in evaluating the ovine semen. Firstly, this test is fast (one hour); secondly, many samples (up to 10) can be examined at the same time; and finally, many replications of each sample can be tested simultaneously.

### **CONCLUSION**

The MTT reduction test proved to be applicable as diagnostic tool for the quality evaluation of ovine semen. It can be used successfully in routine analysis, where practical aspects as time, costs and practicability are important.

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