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Evaluation of Rabbit Semen Quality Using Resazurin Reduction Test

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Abstract: The resazurin reduction test (RRT) depends on the ability of metabolically active cells to reduce the resazurin dye to resorufin. In the current study, spectrophotometric evaluation of resazurin reduction test (RRT) to assess the color changes of resazurin reduction in butanol extracted color was used to evaluate rabbit semen quality.RRT was performed on one hundred rabbit semen samples in this investigation. The results of RRT were determined visually using resazurin colour chart and again the extent of resazurin reduction in each sample was additionally read by spectrophotometer to assess the quality of samples. Absorption was read at 580 nm and 615 nm. Results indicated that RRT ratios decreased as the preservation time increased. and the highest correlation was observed with sperm motility ($r=0.975$, $P<0.0001$) and acrosomal integrity ($r=0.864$, $P<0.0001$). In conclusion, RRT could be used as a tool for evaluating the quality of rabbit semen.

Key words: Resazurin · Semen · Diluent · Rabbit

INTRODUCTION

Various analytical techniques have been developed to evaluate sperm quality, including sperm concentration, motility, viability and morphology [1, 2]. Metabolic tests such as fructolysis and oxygen consumption are important measures of sperm function [3, 4], but these tests are not done routinely because of their complexity [5].

The resazurin reduction test (RRT) depends on the ability of metabolically active cells to reduce the non-fluorescent dye resazurin (Alamar blue) to fluorescent resorufin and thus it can be used to monitor cell viability as well as rabbit spermatozoa [6]. The RRT requires little equipment and is simple to apply.

Many workers reported a significant correlation between RRT and fertility as RRT evaluates the metabolic status of active spermatozoa and it is associated with the concentration of motile sperms [6, 7]. RRT has been used successfully in assessing fertility potential in human [7, 8], bulls [7, 10], rams [11, 12], stallions [13] and most recently with boars [14].

The aim of the present study was to evaluate diagnostically the spectrophotometric application of RRT to assess rabbit semen quality

MATERIALS AND METHODS

This study was carried out in the experimental rabbitry of the Animal House, National Research Center.

Experimental Animals: Five mature Californian rabbit bucks were used in this investigation.. Rabbits were kept in commercial cages (40 x 86 x 32 cm). A food hopper was used to feed the animals. The cages had an automatic watering system with nipple drinkers. Cages were provided with a feet rest. A commercial formula, which had 16% of protein, was offered *ad libitum*. Clean and cool water was always available.

Experimental Materials: Reagents required for the RRT (resosurin dye and n-butanol) were purchased from Bio-Diagnostic corporation, Cairo, Egypt.

Semen Collection and Evaluation: Semen was collected from bucks twice weekly via artificial vagina (IMV, l'Aigle Cedex, France). Immediately after collection, semen was keep at 35°C in water bath in order to be evaluated. Visual motility of each ejaculate was assessed at 37°C by

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usingmicroscope. Sperm concentration was determined in a hemocytometer in a 1:200 dilution. Ejaculates possessing more than 60% visual motility were used.

Sperm Functional Assays: These assays were conducted in fresh and chilled rabbit spermatozoa.

Visual Motility: A drop of the diluted semen sample was placed on a pre-warmed glass slide and cover slipped. Sperm motility was evaluated at 400× magnification based on the visual estimation of the percentage of sperm possessing progressive motility and the percentage was rounded to nearest 5%.

Sperm Morphology: Total sperm morphological Rabbit buck spermatozoa was determined using Eosin-Nigrosin.

Percent Live Sperm Cells: Percents were evaluated using Eosin–Nigrosin staining method [15].

Semen Diluent: galap® extender (IMV, France) was purchased from France.

RRT Quality Test: The RRT test was carried out to assess rabbit semen quality. Semen samples (100) were divided in two aliquots after being diluted (1 : 3) at 30 °C with galap® extender. Twenty µl of resazurin dye were added to 400 µl of each extended semen sample. After mixing, the samples were incubated at 37 °C for one hour and then 2 ml n-butanol were added, vortexed and centrifuged for 10 min at 2000 rpm. The cleared colored upper layer of n-butanol was transferred into glass cuvette. Optical densities of the samples were measured at 580 nm and 615 nm against blank using spectrophotometer. The RRT ratio was calculated by dividing the absorption at 580 nm by the absorption at 615 nm according to Reddy and Bordekar [16].

Acrosomal Integrity: Acrosomal staining procedure followed the method of Kovacs and Foote [17]: equal drops of trypane blue and diluted semen were mixed at room temperature on slides at the edge of another slide and smeared; semen smears were air dried, slides were fixed for two minutes and then rinsed with tap and distilled water. The spermatozoa were stained in Geimsa for 3.5 h. Slides were rinsed with tap and distilled water and then immersed for two min in a jar of distilled water for the best differentiation. Finally the slides were dried in air and then examined after covering with a cover slide. A total of 200 spermatozoa/smear were evaluated with light microscopy at x 1000 magnifications.

Data Analysis: Data are presented as mean ± standard error (SEM). Statistical analysis was carried out using one-way ANOVA, followed by multiple comparison LSD range test. Probability values < 0.05 were considered significant. The statistical analysis was computed using SPSS software. Pearson correlation coefficient among the semen quality ratio, motility and acrosomal integrity were also computed at least for $P<0.0001$.

RESULTS

Table 1 showed that there was a significant ($P<0.05$) difference in semen quality by RRT in relation to time. According to these results, after 72 hours, a decrease in sperm activity was found.

Table 2 declared that RRT was significantly correlated to the acrosomal integrity ($r=0.864$, $P<0.0001$) and sperm motility ($r=0.975$, $P<0.0001$).

DISCUSSION

Results of the present work revealed that there was a high significant ($P<0.0001$) correlation between RRT and sperm motility% as this ratio decreased with the regression of time and decline of sperm motility %. Among the many tests of semen quality studied the most commonly used ones for many years have been sperm concentration, total sperm% and sperm morphology [5].

The RRT showed that there was a significant ($P<0.05$) difference in relation to time. According to these results, after 72 hours, a decrease in sperm activity was found. The RRT was significantly correlated to the acrosomal integrity ($r=0.864$, $P<0.0001$) and sperm motility ($r=0.975$, $P<0.0001$). The RRT using visual detection of color change is quite subjective and varies between evaluators [12]. Spectrophotometric measurement of resazurin reduction provides quantitative and objective method. Following Zalata *et al.* [18], who developed a spectrophotometric method of resazurin reduction to evaluate human semen, Zrimset *et al.* [14] extracted the developed color after the assay of boar semen with butanol and measured the absorbance in the clear upper layer of butanol. There was minimal overlapping between absorption peaks of resazurin and resarufin at 610 nm.

Table 1: Semen quality (resazurin reduction test, RRT) differentiation through chilling of extended rabbit semen in Galap within 3 days period

	Hours		
	After chilling at 5°C		
Semen quality	24	48	72
RRT (Resazurin reduction test)	$2.332^{a} \pm 0.037$	$1.875^{b} \pm 0.017$	$0.811^{c} \pm 0.018$

The same superscript does not differ significantly (P<0.05)

Table 2: Correlation among the RRT, acrosomal integrity and sperm motility of chilled rabbit semen at 5°C

Correlation parameters	RRT	Absorption at 580 nm	Absorption at 615 nm	Acrosomal integrity	Sperm motility
RRT	1.000				
Absorption at 580 nm	0.29912				
	P<0.0017	1.000			
Absorption at 615 nm	-0.54715	0.58855			
	P<0.0001	P<0.0001	1.000		
Acrosomal integrity	0.86371	0.33113	-0.39200		
	P<0.0001	P<0.0005	P<0.0001	1.000	
Sperm motility	0.97453	0.33853	-0.53262	0.82994	
	P<0.0001	P<0.0003	P<0.0001	P<0.0001	1.000

Results of the present work revealed that there was a significant (P<0.0001) correlation between RRT ratios and sperm motility as these ratios decreased with the time and the decline of sperm motility.

Among the many tests of semen quality studied, the most commonly used ones for many years have been sperm concentration and sperm morphology [5]. The RRT using visual detection of color change is quite subjective and varies between evaluators [12]. However, spectrophotometric measurement of resazurin reduction provides a quantitative and objective method. Following Zalata *et al.* [18]. who developed a spectrophotometric method of resazurin reduction to evaluate human semen, Zrimset *et al.* [14] extracted the reduced resaruzin after the assay of boar semen with butanol and measured its absorbance in the clear upper layer of butanol. There was minimal overlapping between absorption peaks of resazurin and resarufin at 610 nm.

The current results are in accordance with those of Glass *et al.* [8], Mahmoud *et al.* [9] and Dart *et al*. [7] who reported that the RRT was highly correlated with sperm concentration and the percentage of motile sperm of humans and bulls, respectively. Moreover, our results coincide with those of Zrimsek *et al.* [14] who observed the highest correlations of the RRT with sperm concentration followed by percentage of motile sperm.

As an indicator of dehydrogenase activity with high sensitivity, the RRT is a better metabolic assay than measuring ATP [9]. Zalata *et al.* [18] found that the RRT could distinguish between semen samples in which sperm produced varying amounts of reactive oxygen species that cause lipid peroxidation of sperm membrane leading to poor sperm function.

In conclusion, RRT could be used as an additional diagnostic tool for evaluating the quality of rabbit semen.

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