RESAZURIN REDUCTION TEST AS A TOOL FOR ASSESSMENT OF RABBIT SEMEN QUALITY

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ABSTRACT

In the present investigation, 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. One hundred samples of rabbit semen were included in this study and the 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

Metabolic tests such as fructolysis and oxygen consumption are important measures of sperm function (Van Demark *et al*., 1945; Braton *et al*., 1956), but these tests are not done routinely because of their complexity (Seed *et al*., 1996).

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 (Erb *et al*., 1950; 1952). 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 (Erb *et al*., 1952; Dart *et al*., 1994). RRT has been used successfully in assessing fertility potential in human (Glass *et al*., 1991; Mahmoud *et al*., 1999), bulls (Dart *et al*., 1994; Foote, 1999), rams (Cooper *et al*., 1996; Wahg *et al*., 1998), stallions (Carter and Ericsson, 1998) and most recently with boars (Zrimsek *et al*., 2004).

The objective of the current investigation was to evaluate the spectrophotometric application of RRT to assess rabbit semen quality.

MATERIALS AND METHODS

This study was carried out in the Experimental rabbitry of the department of Animal Reproduction and A.I, 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 waterbath in order to be evaluated. Macroscopic and microscopic characteristics were evaluated: mass motility (scale from 0 to 3), sperm motility (%), and acrosomal integrity (%).

Semen diluent

TGGY (Tris-glucose-glycerol–egg yolk) diluent was used for preserving rabbit semen. TGGY was prepared according to Roca *et al*. (2000) adding 10% egg-yolk and 6.7% glycerol according to Chauhan and Anand (1990).

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 (1:2) at 30°C with TGGY. 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 (1994).

Acrosomal integrity

Acrosomal staining procedure followed the method of Kovacs and Foote (1992): 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 of the mean (SEM). Statistical significance was assessed 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 AND DISCUSSION

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 (Table 1). The RRT was

significantly correlated to the acrosomal integrity (r=0.864, P<0.0001) and sperm motility (r=0.975, P<0.0001) as shown in Table 2.

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

Hours / Semen quality	After chilling at 5°C		
	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)

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 (Seed *et al*., 1996). The RRT using visual detection of color change is quite subjective and varies between evaluators (Wang *et al*., 1998). However, spectrophotometric measurement of resazurin reduction provides a quantitative and objective method. Following Zalata *et al*. (1998), who developed a spectrophotometric method of resazurin reduction to evaluate human semen, Zrimset *et al*. (2004) 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.

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 P<0.0001	0.58855 P<0.0001	1.000		
Acrosomal integrity	0.86371 P<0.0001	0.33113 P<0.0005	-0.39200 P<0.0001	1.000	
Sperm motility	0.97453 P<0.0001	0.33853 P<0.0003	-0.53262 P<0.0001	0.82994 P<0.0001	1.000

The current results are in accordance with those of Glass *et al*. (1991), Mahmoud *et al*. (1994) and Dart *et al*. (1994) 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*. (2004) 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 (Mahmoud *et al*., 1994). Zalata *et al*. (1998) 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.

CONCLUSIONS

In conclusion, RRT could be used as a tool to evaluate the quality of rabbit semen.

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REFERENCES

Bratton R.W., Foote R.H., Henderson C.R., Musgrave S.D., Dunbar R.S., Dunn H.O. 1956. The relative usefulness of combinations of laboratory tests for predicting the fertility of bovine semen. *J. Dairy Sci., 28, 1542.*

Carter R.A., Ericsson S.A. 1998. Assessing the fertility potential of equine semen samples using reducible dyes methylene green and resazurin. *Arch. Androl., 40, 59.*

Cooper T.A., Wang S., Liu Y., Bunch T., Holyoak G.R. 1996. A new method to evaluate the viability of cryopreserved ram semen using a resazurin reduction assay. *Theriogenology, 45, 313.*

Dart M.G., Mesta J., Cremshaw C., Ericsson S.A. 1994. Modified resazurin test for determining the fertility potential of bovine spermatozoa. *Arch. Androl., 33, 71.*

Foote R.H. 1999. Resazurin reduction and other tests of semen quality and fertility of bulls. *Asian J. Androl., 1, 109.*

Glass R.H., Ericsson S.A., Ericsson R.J., Drovin M.T., Marcoux L.J., Sullivan J. 1991. The resazurin reduction test provide an assessment of sperm activity. *Fert. Steril., 56, 743.*

Kovacs A., Foote R.H. 1992. Viability and acrosome staining of bull, boar and rabbit spermatozoa. *Biotech. Histochem., 67, 119.*

Mahmoud A.M., Comhaire F.H., Vermeulen L., Andreou E. 1994. Comparison of the resazurin test, adenosine triphosphate in semen and various sperm parameters. *Hum. Reprod., 9, 1688.*

Reddy K.V., Bordekar A.D. 1999. Resazurin reduction test. *Ind. J. Exp. Biol., 37, 782.*

Roca J., Martinez S., Vazquez J.M., Lucas X., Parilla I., Martinez E.A. 2000. Viability and fertility of rabbit spermatozoa diluted in tris-buffer extenders and stored at 15°C. *Anim. Reprod. Sci., 64, 103.*

Seed J., Chappin R.E., Clegg E.D., Postal L.A., Foote R.H., Hurtt M.E. 1996. Methods for assessing sperm motility, morphology and counts in the rat, rabbit and dog: A consensus report. *Reprod. Toxicol., 10, 237.*

Shaffer H.E., Almquist J.O. 1948. Vital staining of bovine spermatozoa with an eosin-aniline blue staining mixture. *J. Dairy Sci., 31, 677.*

Van Demark N.L., Mercier E., Sailsbury G.W. 1945. The methylene blue reduction test and its relation to other measures of quality in bull semen. *J. Dairy Sci., 28, 121.*

Wang S., Holyoak G.R. Panter K.E., Liu Y., Evans R.C., Bunch T.D. 1998. Resazurin reduction assay for ram sperm metabolic activity measured by spectrophotometry. *Proc. Soc. Exp. Med., 217, 197.*

Zalata A.A., Lammertijn N., Christoper A., Comhaire F.H. 1998. The correlates and alleged biochemical background of the resazurin reduction test in semen. *Int. J. Androl., 21, 289.*

Zrimsek P., Kurc J., Kosec M., Mrkun J. 2004. Spectrophotometric application of resazurin reduction assay to evaluate boar semen quality. *Int. J. Androl., 27, 57*.