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# Assessment of Nili-Ravi buffalo (*Bubalus bubalis*) semen by MTT reduction assay

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## Abstract

MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is commonly used to validate the viability of metabolically active cells. The study was conducted to examine and validate the MTT test to assess the sperm viability of Nili-Ravi buffalo bulls and compare the efficiency of the test with the supra-vital staining technique (eosin-nigrosine) and hypo-osmotic swelling test. Fresh semen samples from breeding Nili-Ravi buffalo bulls (n = 20) were collected using an artificial vagina. After assessing the quality of semen for normal parameters, the MTT assay was carried out in phosphate buffer saline. Results revealed a high significant correlation (r = 0.995) between the viability of sperm and the rate of reduction of MTT. The other proportions of some semen samples showed a weak relationship between the eosin-nigrosine method (r = -0.32), hypo-osmotic swelling test (r = -0.12) and motility (r = -0.08). However, the MTT assay was found to be superior to other tests as it was able to determine those sperm which were more than 90% viable. In conclusion, the MTT assay is a simple, robust test that can be used to select Nili-Ravi buffalo bulls on the basis of sperm quality.

**Keywords:** MTT, Nili-Ravi, buffalo, sperm, viability
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## Introduction

It is very important to have high quality semen as sperm quality correlates positively with the fertility in bovines (Garner *et al.*, 1997). Most of the methods that are currently used to evaluate the quality of semen such as supa-vital staining and the hypo-osmotic swelling test (HOST) are subjective in nature and can easily be influenced by the experience of the analyst (McNiven *et al.*, 1992). On the other hand, computer-assisted analysis of sperm requires special instruments and software that provide rapid and objective evaluation of the semen quality. However, these techniques are expensive and sometimes not readily available on average breeding farms. Therefore, it has becomes imperative to look for some other techniques that may be cheap, objective and can easily be performed without the assistance of any sophisticated instrument.

In this regard various analytical techniques have been developed to evaluate the sperm quality in bovines. Among others, assessment of metabolic status of sperm is one of the techniques that can provide valuable information regarding the sperm characteristics. Reduction activity of sperm depends on the ability of metabolically active sperm to reduce the specific stains. The ability of sperm to reduce the resazurin redox dye (Foote, 1999; Zrimsek *et al.*, 2004) and methylene blue dye (Chandler *et al.*, 2000) had been employed to evaluate the semen quality in boars and bulls, respectively. MTT (3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide), a yellow water-soluble tetrazolium salt dye, is converted to water-insoluble purple formazan by the succinate dehydrogenase system of an active mitochondria by the reductive cleavage of its tetrazolium ring (Slater *et al.*, 1963). The amount of formazan formed can, thus, be determined spectrophotometrically and it serves as an estimate of the number of active mitochondria and hence the living cells in a sample (Denizot & Lang, 1986; Song *et al.*, 2007).

Although the MTT assay has been evaluated successfully in different animal species (Aziz *et al.*, 2005; Aziz, 2006; Byun *et al.*, 2008) the literature pertaining to the use of this technique for buffalo semen is still lacking. Therefore, we evaluated the viability of buffalo sperm using the MTT reduction assay and compared it with eosin-nigrosin staining (E&N), HOST and motility.

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## Materials and Methods

Nili-Ravi buffalo bulls from the Semen Production Unit, Qadirabad, Punjab-Pakistan were used in the study. The bulls have been used routinely for collection of semen, which was supplied to various Artificial Insemination Centres throughout the Punjab, Pakistan. The breeding bulls were divided into two groups. The age of group A bulls (n = 13) was about five years, while the bulls in group B (n = 7) ranged between 6 - 8 years. The circumferences of both testes of breeding buffalo bulls were measured. The experimental bulls were maintained under naturally prevailing climatic conditions with free access to drinking water. Each bull was fed seasonal green fodder (Berseem) and wheat straw.

Semen from the experimental bulls was collected once a week with two ejaculates per collection using an artificial vagina. Each bull was given sufficient time for sexual preparation before semen collection, while one to two false mounts were allowed for sexual stimulation. Each collection that comprised of two ejaculates was pooled and divided into various aliquots. Following the determination of ejaculate volume and the concentration of sperm in each semen sample, sperm motility, plasma integrity of sperm in terms of hypo-osmotic swelling (HOS) test, live and dead ratio of sperm and MTT reduction assay of each ejaculate were determined.

Plasma membrane integrity of fresh sperm was assessed using a hypo-osmotic swelling (HOS) assay as described earlier (Adeel *et al*., 2009: Ijaz *et al*., 2009;). Briefly, the hypo-osmotic solution was prepared by dissolving 0.735 g of sodium citrate and 1.351 g fructose in 100 mL distilled water (osmotic pressure = 190 mOsm/kg). For assay, 50 µL of semen was mixed with 500 µL of the pre-warmed (37 °C) HOS solution and incubated at 37 °C for 45 minutes. Two slides for each sample were prepared and examined under a phase contrast microscope (X 40). Two hundred sperm were counted per sample and the number of sperm showing characteristic swelling of tail, an indicative of intact plasma membrane, was recorded.

To determine the spermatozoal viability, a drop of semen sample was mixed with a larger drop of the eosin (1%; Merck, Germany) and nigrosin (5%; Merck, Germany) stains on a pre-warmed slide using applicator stick and a thin smear by smooth drawing of the edge of another slide (Khan & Ijaz, 2008). After air-drying, the smear was studied under a phase contrast microscope (X 400) for unstained heads of sperm (live) and stained/partial stained heads of sperm (dead). A total of 200 sperm was counted to determine live and dead percentages of sperm.

The MTT assay was performed according to the method of Mosmann (1983). The semen samples were diluted with phosphate buffer saline (PBS) to obtain a concentration of $30 \times 10^6$ sperm/mL. Six wells of the 96-well microplate were used. A 100 µL of semen sample plus 10 µL of MTT stock solution (5 m MTT/mL of PBS) was placed in each well. Six replicates of each collection were placed in six different wells of the same plate. The rates of MTT reduction were taken immediately and after incubation at 37 °C for one hour (Naser-Esfahani *et al*., 2002; Aziz, 2006) using a spectrophotometer (MS2 Reader) at a wavelength of 550 nm. A small drop of the semen sample after the measurement of second optical density (OD) was observed under the microscope (X 1000) for the viable cells that gained coloured formazan upon the reduction of MTT solution. MTT reduction rate (optical density) for each sample was calculated by concurring the difference between the first and second reading of the spectrophotometer.

The statistical programme, SPSS for window (version 10.0.1, SPSS Inc., Chicago, Illinois, USA) was used for data analysis. The Kolmogorov Smirnov test was used to test the normal distribution of the data. Results are expressed as means ± s.e. Pearson correlation coefficients and regression analysis were used to evaluate the efficacy of the MTT test for the assessment of sperm viability of buffalo semen. The independent Student's t test was used to compare the means between two groups for each parameter. Probability value at $P < 0.05$ was considered significant.

## Results

The volume of semen collected was more in group A (7.12 ± 0.5) compared to that from bulls of group B (5.29 ± 0.6). However, concentration of sperm (562.5 ± 31.6 *vs*. 642.2 ± 66.4 millions/mL) and circumference of testes of bulls (34.7 ± 0.14 *vs*. 34.47 ± 0.2 cm) were comparable ($P > 0.05$) in both groups. There was no significant difference between the two groups for the remaining spermatozoal variables studied. Therefore, data were pooled for both groups (n = 20) for subsequent analysis. The means of percentages of live sperm for MTT, E&N, HOS and motility were 74.7 ± 1.9, 68.8 ± 0.7, 59.5 ± 1.0 and 72.3 ± 0.5, respectively. After incubation of semen samples for one hour, a regression equation for the

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relationship between MTT reduction rate and the percentage of viable sperm was calculated (y = 90.403x -0.109); the corresponding curve is presented in Figure 1 that was later used to determine the viability of the sperm based on other diagnostic tests.

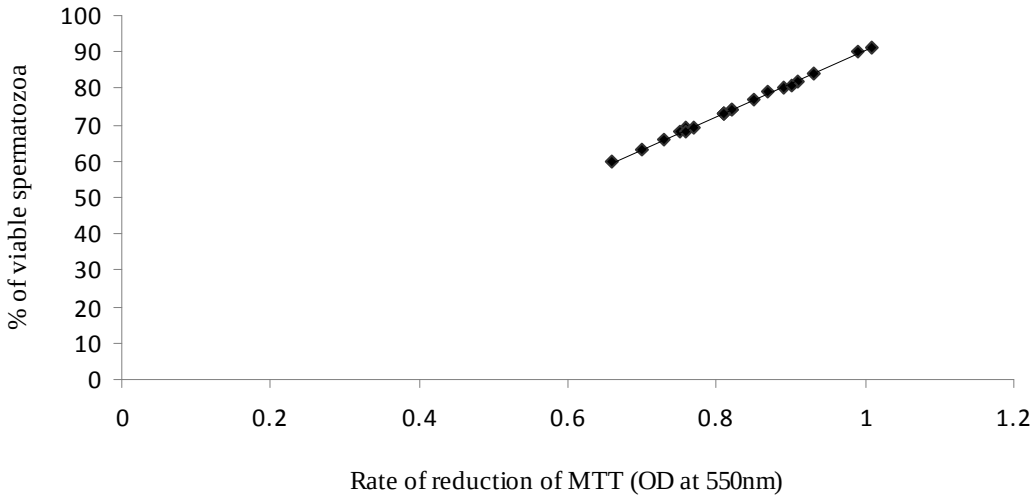


**Figure 1** Relationship between MTT reduction rate and percentage of viable spermatozoa. The regression curve shown is y = -0.109+90.402x; r =0.995; n = 20; OD = optical density.

The optical density (OD) was significantly (P <0.001) correlated (r = 0.995) with the percentage of viable sperm. Results showed a very low negative correlations (P >0.05) between the results of MTT reduction rate and percentage of viable sperm as determined by either eosin and nigrosin staining (r = -0.32, Figure 2), HOST (r = -0.12; Figure 3) or percentage motility (r = -0.08; Figure 4).

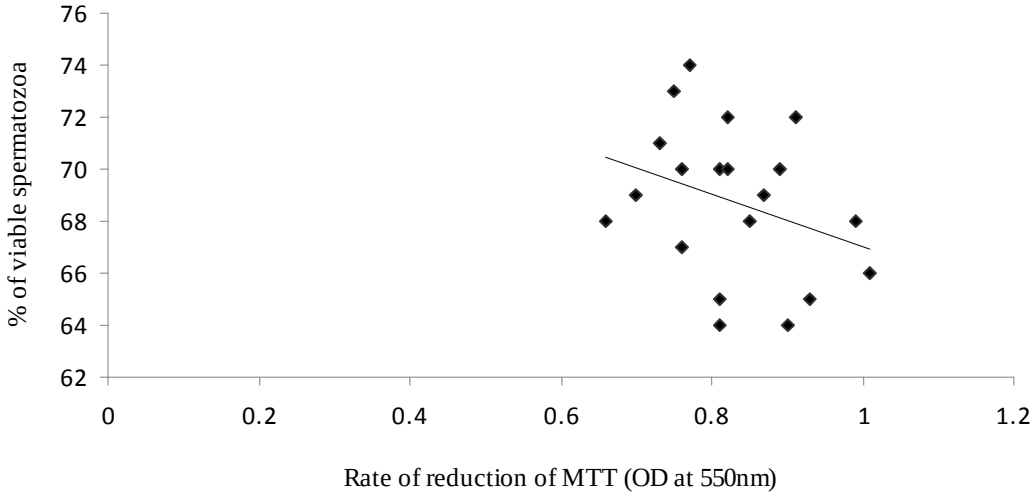


**Figure 2** Relationship between MTT reduction rate and percentage of viable sperm as determined by eosin and nigrosin staining. The regression curve shown is y = 77.15-10.15x; r =- 0.32; n = 20; OD = optical density.

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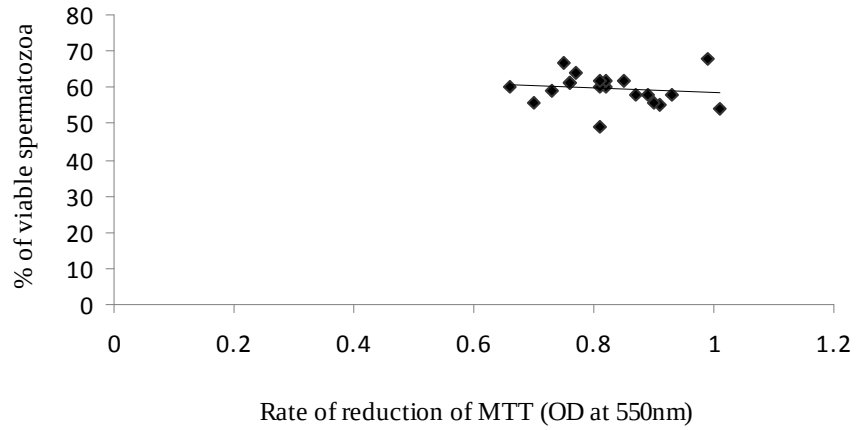


**Figure 3** Relationship between MTT reduction rate and percentage of viable sperm as determined by HOST. The regression curve shown is y = 64.74-6.36x; r =-0.12; n = 20; OD = optical density.

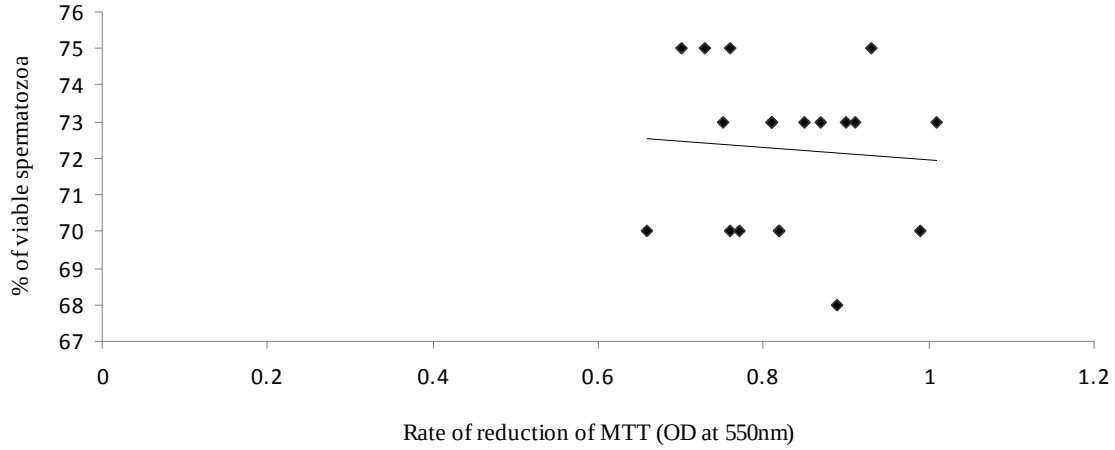


**Figure 4** Relationship between MTT reduction rate and percentage of viable sperm as determined by motility. The regression curve shown is y = 73.56-1.592xl; r =-0.08; n = 20; OD = optical density.

The distribution of experimental breeding bulls based on various percentages of viable sperm determined by different diagnostic tests is presented in Table 1. Most of the animals exhibited the presence of viable sperm at the range of 60 - 80% in all the conducted tests. However, MTT was able to determine the >90% viable spermatozoa in two bulls (Table 1).

## Discussion

The data presented here provide evidence of a relationship between MTT and semen quality. Different tests or methods have been developed for differentiation and selection of viable sperm (WHO, 1992). Some of the tests have only diagnostic value like dye exclusion tests while others carry clinical application such as HOST. The dye exclusion tests such as eosin–nigrosin are based on the cell permeability and, therefore, the viable sperm remain colourless. The non-viable sperm, on the other hand, either stain accordingly or red. However, in HOST, sperm are exposed to a hypo-osmotic condition, thus an influx of water results in swelling of the cytoplasmic spaces causing curling of viable sperm tail fibres (Hossain *et al.*, 1998). Therefore, we investigated the diagnostic value of MTT to select the viable sperm in Nili-Ravi buffalo bulls and compared it with E&N, HOST and motility. Mosmann (1983) used MTT tetrazolium salt

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**Table 1** Distribution of breeding bulls based upon the percentage of viable spermatozoa as determined by different diagnostic tests

| Percentage of viable spermatozoa | Diagnostic tests | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MTT | | E & N | | HOST | | Motility | |
| | No. | %age | No. | %age | No. | %age | No. | %age |
| 50-60 | 0 | 0 | 0 | 0 | 9 | 45 | 0 | 0 |
| 60-70 | 7 | 35 | 11 | 55 | 11 | 55 | 1 | 5 |
| 70-80 | 7 | 35 | 9 | 45 | 0 | 0 | 19 | 95 |
| 80-90 | 4 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| 90-100 | 2 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 2** Distribution of breeding bulls based upon the percentage of viable spermatozoa as determined by different diagnostic tests

| Percentage viable spermatozoa | Diagnostic tests | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MTT | | E & N | | HOST | | Motility | |
| | No. | %age | No. | %age | No. | %age | No. | %age |
| 50-60 | 0 | 0 | 0 | 0 | 9 | 45 | 0 | 0 |
| 60-70 | 7 | 35 | 11 | 55 | 11 | 55 | 1 | 5 |
| 70-80 | 7 | 35 | 9 | 45 | 0 | 0 | 19 | 95 |
| 80-90 | 4 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| 90-100 | 2 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |

for assessment of cellular viability as well as for proliferation and cytotoxicity assay of lymphocytes. Additionally, the MTT assay has also been used in many studies to evaluate the viability of different cells (Carmichael *et al.*, 1987; Campling *et al.*, 1988; Freimoser *et al.*, 1999). This study hopes to present a new diagnostic test using MTT for sperm viability in Nili-Ravi buffalo bulls. Formation of MTT formazan granules or spikes around the sperm mid-piece showed that sperm mitochondria contained succinate dehydrogenase system which is capable of converting MTT to formazan. The presence of formazan granules in the mid-piece region identifies the viability of sperm. Results indicate a high correlation ($r = 0.995$) between the MTT reduction rate and sperm viability (Figure 1). High correlation between MTT and sperm viability has also been found in bovine (Aziz, 2006), stallions (Aziz *et al.*, 2005), boars (Byun *et al.*, 2008), fowls (Hazary *et al.*, 2001) and humans (Naser-Esfahani *et al.*, 2002). The MTT reduction rate was determined after one hour of incubation that was shorter than the method of Mosmann (1983). It seems logical as sperm are highly active cells that contain more mitochondria. Therefore, less time may be needed for the reduction of the dye. A similar observation was also noted in bovines (Aziz, 2006) and equines (Aziz *et al.*, 2005) where optimal time for formazan reading was one hour. Naser-Esfahani *et al.* (2002) found that the optimal time for formazan reading was between 1.5 and 2.5 h after addition of sperms to MTT.

As E&N and HOST are based on the functional aspect/integrity of sperm, while MTT is based on the mitochondrial activity of the sperm, high significant correlation coefficients were expected between these tests and sperm motility. However, very weak relationships were noted between MTT and other tests (Figures 2; 3 & 4). There is a dearth of reports regarding the relationship between MTT and other tests used in the present study. A high correlation ($P < 0.001$) between MTT test and E&N was noted in boar semen

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extended with the Beltsville thawing solution. On the other hand, a weak correlation (r = 0.4) was noted between these two variables in the semen samples of humans in HAM'S F10 solution instead of PBS that was used in the current study. It seems that the nature of the medium in which the MTT is carried out also affects the outcomes of the test. Similarly, the lack of any correlation between MTT and HOST might also be due to the nature of the HOST test. It has been suggested that during the HOST procedure, hypo-osmotic shock on its own induces membrane damage and, therefore, increases the percentage of false positive sperm, which means that the percentage of dead sperm is higher than the expected value (Ramirez *et al.*, 1992).

The results of the present study also suggest that MTT can be used to evaluate the sperm quality in Nili-Ravi buffalo bulls. An additional advantage of this test is that it can determine the sperm having more than 90% viability that could not be observed with other traditional tests used for the evaluation of buffalo semen (Table 1). This characteristic may be exploited to increase the doses of the semen to be used for insemination.

## Conclusion

In conclusion, the MTT test was found to be a reliable method for the evaluation of semen in buffalo bulls and can be used successfully in routine analysis, where practical aspects like time, costs and practicability are important.

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