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# ASSESSMENT OF MITOCHONDRIAL ACTIVITY OF FRESH AND FROZEN-THAWED BUFFALO SEMEN IN RELATION TO SPERM VIABILITY AND IN VITRO FERTILIZING ABILITY

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## SUMMARY

The present study aimed to validate MTT ( 3 [4,5- dimethylthiazol -2-y1]-2,5 diphenyltetrazoliumbromide test, which determines the spermatozoal mitochondrial activity, for evaluation of the fresh and frozen-thawed buffalo sperm viability with special reference to spermatozoal in vitro fertilizing capacity (IVF). Fresh ejaculates and frozen-thawed semen from six buffalo-bulls were included in this study. The rates of MTT reduction for semen samples were measured in microtiter plates after different periods of incubation using ELISA reader at wave length 550 nm. Simultaneously, sperm individual motility, viability and acrosomal integrity were determined. In vitro fertilizing capacity for fresh and frozen-thawed semen samples were evaluated using vitrified-thawed buffalo mature oocytes. The correlation between obtained results was calculated. The results demonstrated that the correlations among MTT reduction rate, spermatozoal motility and viability and IVF rate of both fresh and frozen-thawed buffalo semen were significantly ($P < 0.01$) positive, with correlation coefficients ranging from 0.590 to 0.825. Conclusively, MTT test was found to be a reliable, relatively rapid and inexpensive tool for predicting fresh and frozen-thawed buffalo semen fertility.

**Key words:** MTT- tetrazolium- mitochondria- IVF- viability- fertility- buffalo bull- semen- cryopreservation.

## INTRODUCTION

Today, there is an increasing trend to use artificial insemination (AI) as a breeding tool. With the development of frozen semen technology in

buffaloes, the demand for outstanding sires has increased considerably. Accurate evaluation of fertility of bulls used for AI is of utmost importance since a single ejaculate provides several insemination doses, and influences the reproductive potential of a herd (Rodriguez-Martinez and Larsson, 1998). This has led to the development of methods for predicting bulls of high semen quality and high fertility.

Numerous methods have been developed over the years for the laboratory evaluation of semen quality and fertility. Some of these measures are general characteristics of sperm (viability, motility patterns, morphology, sperm metabolism, membrane and acrosomal integrities). Estimation of sperm concentration and spermatozoal motility has been routinely used for judging semen quality. There exists a controversy regarding the relationship between fertility and sperm motility. Many researchers have found a strong correlation (Hirao, 1975; Linford et al., 1976), whereas others found poor relationships between these traits (Bishop et al., 1954; Deibel et al., 1976) and fertility.

To assess male fertility by mating or AI is expensive and time consuming, and only allows a limited number of males to be tested. Hence, interest has lately been focused on methods related to in vitro fertilization technique (Larson and Rodriguez-Martinez, 2000).

In recent years, mitochondrial function has been used to assess semen quality (Marchetti et al., 2002). The mitochondrial sheath surrounding the midpiece of the sperm generates the energy that aids transit in the female reproductive tract, penetration, and fertilization of the egg (Windsor, 1997; Kasai et al., 2002). Hence, assessment of the mitochondrial function rather than the standard viability tests has been considered more useful to assess semen quality (Turner, 2006).

Assessment of the ability of spermatozoa to reduce the resazurin redox dye in boar (Foote, 1999; Zrimsek et al., 2004) and methylene blue in bull (Chandler et al., 2000) was used successfully to evaluate semen quality.

MTT (3[4,5 -dimethylthiazol -2-yl ]-2,5-diphenyltetrazoliumbromide) is a yellow water-soluble tetrazolium salt. On reductive cleavage of its tetrazolium ring by the succinate dehydrogenase system of the active mitochondria, the dye is converted to water-insoluble purple formazan. (Slater et al., 1963). The amount of formazan formed can be determined spectrophotometrically which serves as an estimate of the number of mitochondria and hence the number of living cells in a sample (Denizot and Lang, 1986). MTT assay was used in many studies related to viability of different cells (Mosmann, 1983; Levitz and Diamone, 1985; Carmichael et al., 1987; Campling et al., 1988; Freimoser et al., 1999).

The MTT assay is a simple, rapid and reliable method for estimating the percentage of viable spermatozoa in human and boar, depending on the accumulation of formazan grains around the midpiece of sperm tail (Naser-Esfahani et al., 2002; Gaczarzewicz et al., 2003). It was also, considered reliable for objective evaluation of fowl (Hazary and Wishart, 2001; Hazary et al., 2001), bovine (Aziz, 2006) and equine semen (Aziz et al., 2005), depending on the reduction rate and optical density of the MTT.

Perusal of literature revealed that no information is available on the use of MTT sperm viability assay to predict frozen-thawed semen quality and, to our knowledge, it may be the first attempt to assess buffalo semen by this technique. Hence, the aim of the present study was to evaluate a simple and reasonably priced MTT test in determining both fresh and frozen-thawed buffalo sperm viability and to compare the efficiency of this test with spermatozoal in vitro fertilizing capacity.

## MATERIALS AND METHODS

### Semen samples

Semen samples from six buffalo-bulls, maintained on the experimental farm of Animal Reproduction Research Institute (ARRI) under optimum feeding and housing conditions, were used in this study. The age of the bulls ranged between 2.5 and 5.5 years (during the semen collection period). Two ejaculates were collected once a week from each bull with pre warmed artificial vagina (37-40°C for 6 successive weeks. Immediately after collection, semen samples were evaluated for individual motility using a pre-warmed stage of a phase contrast microscope. Two smears of semen, stained with eosin-nigrosin, were prepared (Blom, 1950) and used to determine the percentage of live spermatozoa by counting at least 200 sperm cells. Sperm concentration was measured with a hemocytometer.

### Semen processing:

After the microscopic evaluation, each semen sample was divided into two portions; the first of which was diluted with HEPES 0.1% BSA (Garner et al., 1997) to obtain a concentration of $40x10^6$ sperm/ml.

The second portion was processed for freezing as follows: Semen was diluted with a commercial extender (Triladyl®, Minitub, Germany) at 37°C in an incubator at appropriate dilution rate to obtain a final concentration of 40 x $10^6$ sperm cell/ml. Diluted semen was cooled slowly to 5°C over a period of 1.5 hours. Semen was loaded in 0.25 ml straws (IMV, L'Aigle, France) and placed 4 cm above liquid nitrogen in the vapor phase in a foam box for 15 minutes before being plunged into the liquid phase (Khalifa, 2001). Straws were stored in liquid nitrogen until thawing at 37°C in water bath for 30 seconds. Individual motility and viability were recorded just after thawing, 1, 2 and 3 hours post-thawing. Also, acrosomal integrity was estimated using fast green stain. The post-thawing viability indices were es-

timated according to Milovanov (1962).

**MTT reduction assay**

The MTT assay was performed according to the method of Mosmann (1983). For each fresh or frozen-thawed sample, six wells of a 96-well microplate were used. A 100 µl of semen plus 10 µl of MTT stock solution (5 mg MTT/ml of PBS) was placed in each well. The rates of MTT reduction were determined using an ELISA reader (Versamax Reader ) at wavelength of 550nm. The optical density of fresh samples was measured four times, (immediately after thawing and 1, 2 and 3 hours post-thawing). MTT reduction rates (optical density) for each semen sample was calculated from the difference between the first and each of the second (MTT1), third (MTT2) and fourth (MTT3) readings of the ELISA reader.

In order to determine the relationship between the MTT reduction rate and sperm viability, the freeze-killed procedure (Capkova et al., 2000) was used. Each semen sample was divided into two fractions; the first of which was maintained at 37°C while the spermatozoa in the other fraction were killed by two cycles of plunging into liquid nitrogen and thawing at 37°C. Samples used for analysis were made by combing aliquotes of viable and freeze-killed spermatozoa at ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5; 4:6, 3:7, 2:8, 1:9 and 0:10 v/v, respectively. The prepared samples were analyzed by MTT and sperm viability was evaluated by eosin-nigrosin staining.

In vitro fertilization:

Buffalo cumulus oocyte complexes (COCs) were obtained by aspiration of medium sized follicles (3-8 mm). COCs that have homogeneous ooplasm and surrounded by compact muli-layers of cumulus cells were selected and placed in groups of 10-15/ 50 µL drops of TCM 199, covered with mineral oil and incubated for 24 hours at 39°C in 5% $CO_2$ atmosphere and maximum humidity.

Vitrification and thawing of matured buffalo COCs were performed according to the method applied by Cetin and Bastan (2005) , briefly; matured oocytes were exposed to vitrification solutions of 10% of ethylene glycol (EG) + 10% DMSO containing holding medium (TCM 199 containing HPES + 20% fetal calf serum) for 45 s (step one) and then were moved into a 200 µl droplet of 20% EG + 20% DMSO +1 m sucrose in the same holding medium for 25 s (step two). Oocytes were loaded into straws by aspiration and the straws were plunged directly into liquid nitrogen within 25-30 seconds of beginning with the exposure to step 2 vitrification solution. the straws were stored for 2-3 months in liquid nitrogen. dilution media was prepared by using 10% FCS containing TCM199. the straws were thawed in air for 5 seconds, and immediately plunged into a water bath at 37°C for 10 seconds. in order to dilute the cryoprotectant, oocytes were subsequently washed three times in different solutions containing 0.25, 0.15 and 0 m sucrose for 1.5, 1.5 and 5 min, respectively. cumulus-oocyte complexes were washed three times at 37°C in maturation medium (TCM 199) before being transferred for fertilization drops.

Straws of thawed buffalo semen were thawed in a water bath at 37°C for 30 seconds. The most motile spermatozoa of fresh and frozen-thawed semen of the 6 buffalo bulls used in this study were separated by swim up method in TCM medium containing 6 mg/ml bovine serum albumin (BSA) for 1 hour (Parrish et al., 1988). The uppermost layer of spermatozoa was selected and washed twice by centrifugation at 2000 rpm for 10 minutes. at the end of washing procedure, the resulting sperm pellet was suspended in F-TALP media containing 100 µg/ml heparin and 6 mg/ml BSA. After appropriate dilution, 2 µl of sperm-suspension was added per fertilization drop to obtain a final concentration of 2 x $10^6$ sperm cell/ml.

Spermatozoa were incubated with ova in fertilization drops under mineral oil for 18 to 24 hours at 39°C in 5% $CO_2$ in humified air. After that oocytes were fixed in acetic acid-ethanol (1:3; v/v) and stained with 1% aceto-orcein stain to assess fertilization at 400x magnification. fertilization was defined with the presence of either the sperm tail associated with the male pronucleus or the presence of two pronuclei (Martino et al., 1994).

statistical analysis:

Each experiment was repeated six times (once weekly for 6 successive weeks). Two way analysis of variance and Duncanís multiple range test were done for the data obtained. Correlation coefficients and regression analysis were used to evaluate the efficacy of the MTT test for the assessment of sperm viability of buffalo fresh and frozen-thawed semen. data were analyzed using the 1984-version of costat (Ecosoft, Inc, USA), and $p < 0.05$ was considered as statistically significant.

## RESULTS

The mean MTT reduction rates of the fresh semen samples which contained different proportions of freeze-shocked sperm cells are shown in Fig. (1). With the increase of incubation time, the rate of MTT reduction increased gradually till the 1st hour of incubation, then there was a decline in the MTT reduction rate after 1.5 hours incubation. In all incubation times, increasing the volume of freeze-shocked sperm cells in semen samples resulted in a proportional and significant ($P < 0.001$) decrease in the rate of MTT reduction. In frozen-thawed semen containing different proportions of killed sperm cells in (Fig. 2), by increasing the incubation time, the rate of MTT reduction increased gradually till the 2nd hour of incubation, then declined at the 3rd hour of incubation. Also in all incubation times, increasing the volume of killed sperm cells in semen samples resulted in a proportional and significant ($P< 0.001$) decrease in the rate of MTT reduction.

As shown in Fig. (3), there was a highly positive correlation ($P< 0.001$, $r = 0.906$) between the MTT reduction rates and sperm viability. A regression equation ($y = 69.920x + 5.948$) for the relationship between the MTT reduction rate and sperm viability was calculated. Also in frozen-thawed semen Fig. (4), there was a highly positive correlation ($P< 0.001$, $r = 0.958$) between the MTT reduction rates and sperm viability in-

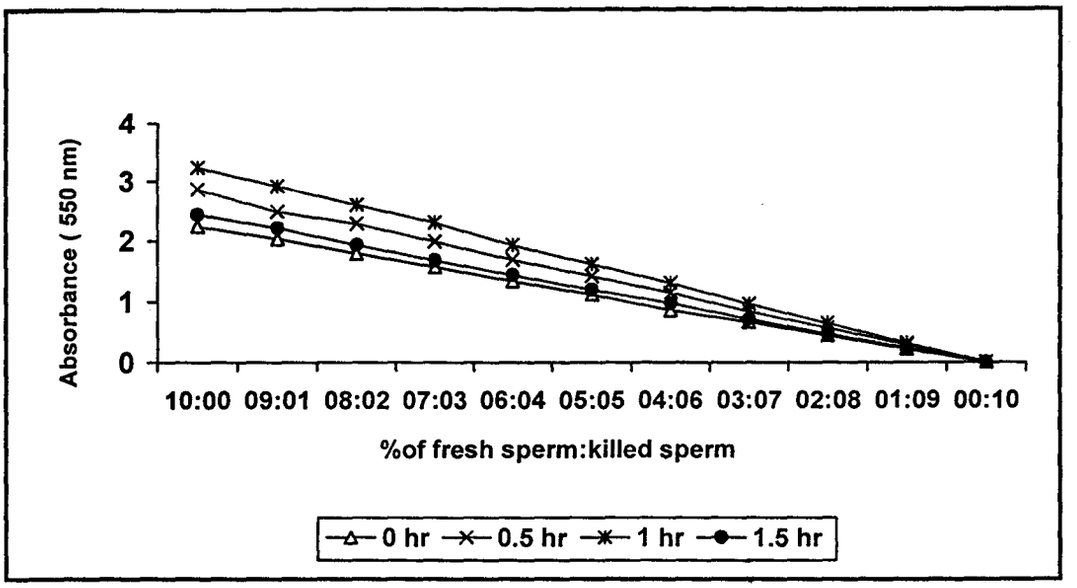


**Fig. (1):** MTT reduction rates of the fresh semen samples containing different proportions of live and freeze-shocked sperms at 0, 0.5, 1 and 1.5 hours incubation times.

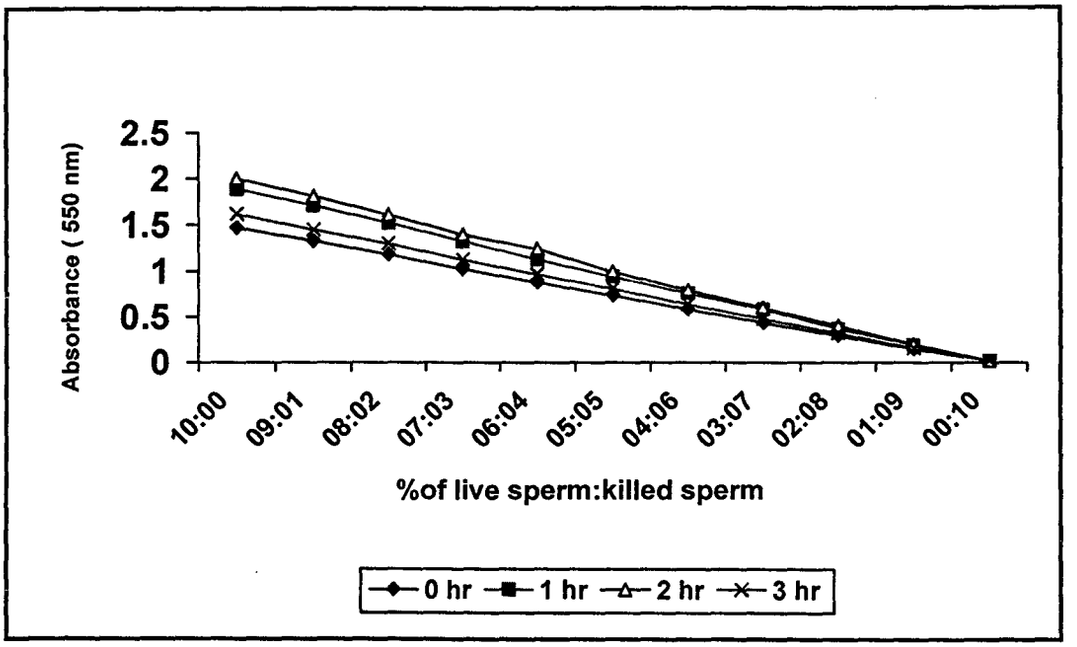


**Fig. (2):** MTT reduction rates of the frozen-thawed semen samples containing different proportions of live and killed sperms at 0, 1, 2 and 3 hours incubation times.

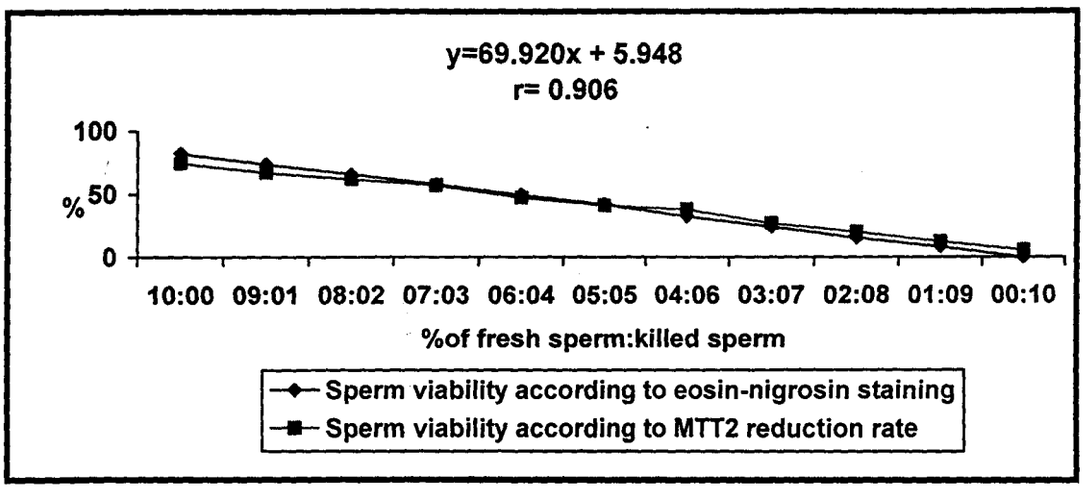


**Fig. (3)**: sperm viability according to both eosin-nigrosin staining and MTT reduction rate of the fresh semen samples containing different proportions of live and freeze-shocked sperm cells at 1 hour incubation time.

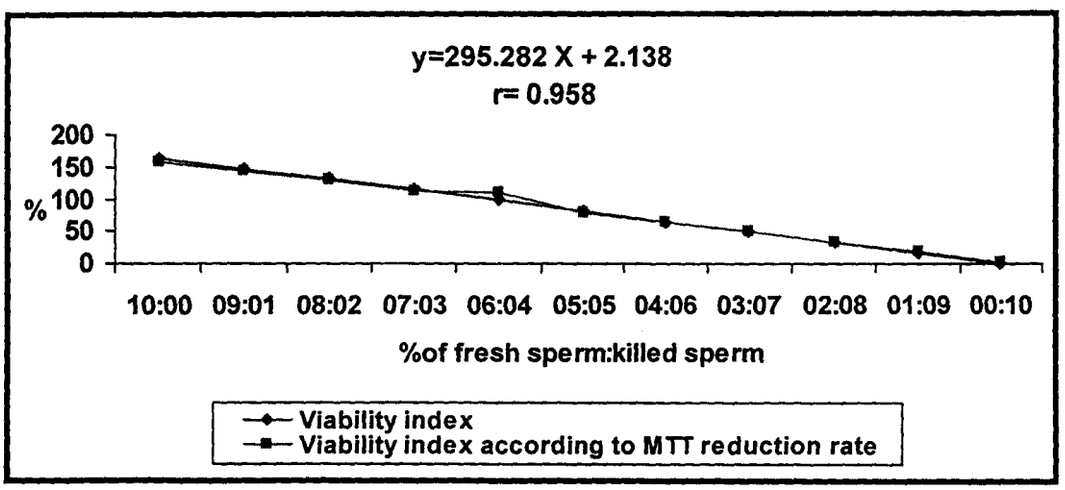


**Fig. (4)**: sperm viability indices of the frozen-thawed semen samples containing different proportions of live and killed sperm cells at 1 hour incubation time.

dex. A regression equation (y = 295.282x + 2.138) for the relationship between the MTT reduction rate and sperm viability index was calculated. These equations were applied later as standards to calculate the sperm viability indices on the basis of MTT reduction rates.

Tables (1 and 2) present the MTT reduction rates for fresh and frozen-thawed buffalo semen obtained after 1 hour of incubation and sperm viability as estimated by eosin-nigrosin staining in the first or by the equation of Milovanov (1962) in the second or calculated according to MTT reduction rate using the above regression equation of samples containing different proportions of live and freeze-killed sperm cells. There was a highly significant decrease ($P < 0.001$) of both estimated (by eosin-nigrosin staining or by the equation of Milovanov (1962)) and calculated sperm viability by increasing the proportion of the freeze-killed spermatozoa.

MTT reduction of fresh (Table 3) and frozen -

**Table (1):** MTT reduction rates and sperm viability (mean ± S.E.) of semen samples containing different proportions of live and freeze-killed sperm cells

| Proportions of live : freeze-killed sperm in semen samples | MTT reduction rate at 1 hour | Percentage of Viable sperm cells according to: | |
|---|---|---|---|
| | | Eosin-nigrosin staining | MTT |
| 10:0 | $0.984\pm0.04^{A}$ | $82.44\pm1.48^{A}$ | $74.74\pm2.66^{A}$ |
| 9:1 | $0.870\pm0.06^{B}$ | $73.83\pm1.41^{B}$ | $66.78\pm3.87^{B}$ |
| 8:2 | $0.797\pm0.03^{BC}$ | $65.72\pm1.11^{C}$ | $61.65\pm2.26^{BC}$ |
| 7:3 | $0.728\pm0.06^{C}$ | $57.67\pm1.09^{D}$ | $56.83\pm4.46^{C}$ |
| 6:4 | $0.587\pm0.02^{D}$ | $49.28\pm0.94^{E}$ | $47.01\pm1.71^{D}$ |
| 5:5 | $0.501\pm0.02^{E}$ | $41.83\pm0.90^{F}$ | $40.95\pm1.39^{E}$ |
| 4:6 | $0.457\pm0.05^{E}$ | $32.50\pm0.82^{G}$ | $37.87\pm3.42^{F}$ |
| 3:7 | $0.300\pm0.01^{F}$ | $24.39\pm0.63^{H}$ | $26.92\pm0.83^{G}$ |
| 2:8 | $0.204\pm0.01^{G}$ | $15.61\pm0.41^{I}$ | $20.24\pm0.53^{H}$ |
| 1:9 | $0.096\pm0.00^{H}$ | $8.33\pm0.97^{J}$ | $12.67\pm0.28^{I}$ |
| 0:10 | $0.00\pm0.00^{I}$ | $0.00\pm0.00^{K}$ | $5.94\pm0.00^{J}$ |

Within column, means with different alphabetical superscripts are significantly different at least at $P<0.01$.

**Table (2):** MTT reduction rates and sperm viability of semen samples (mean ±S.E.) containing different proportions of live and freeze-killed sperm cells

| Proportions of live: killed sperm in semen samples | MTT reduction rate at 1 hour | Viability index | Sperm viability index according to MTT reduction rate |
|---|---|---|---|
| 10:0 | $0.529\pm0.02^{A}$ | $164.44\pm7.00^{A}$ | $158.47\pm4.90^{A}$ |
| 9:1 | $0.482\pm0.01^{B}$ | $146.89\pm6.33^{B}$ | $144.37\pm4.08^{B}$ |
| 8:2 | $0.428\pm0.01^{C}$ | $131.39\pm5.66^{C}$ | $128.62\pm3.91^{C}$ |
| 7:3 | $0.374\pm0.02^{D}$ | $114.94\pm4.88^{D}$ | $112.54\pm5.10^{D}$ |
| 6:4 | $0.364\pm0.02^{E}$ | 98.58±4.29E | $109.59\pm6.96^{E}$ |
| 5:5 | $0.256\pm0.01^{F}$ | $82.56\pm3.47^{F}$ | $77.93\pm2.81^{F}$ |
| 4:6 | $0.213\pm0.01^{G}$ | $66.08\pm2.86^{G}$ | $65.13\pm2.19^{G}$ |
| 3:7 | $0.168\pm0.01^{H}$ | $49.64\pm1.85^{H}$ | $51.84\pm2.69^{H}$ |
| 2:8 | $0.112\pm0.00^{I}$ | $33.03\pm1.65^{I}$ | $35.11\pm0.96^{I}$ |
| 1:9 | $0.055\pm0.00^{J}$ | $16.56\pm0.81^{J}$ | $18.38\pm1.18^{J}$ |
| 0:10 | $0.000\pm0.00^{K}$ | $0.00\pm0.00^{K}$ | $2.14\pm0.00^{K}$ |

Within column, means with different alphabetical superscripts are significantly different at least at P<0.01

thawed buffalo semen (Table 4) and various sperm parameters (sperm motility, viability, acrosomal integrity and in vitro fertilization rate are shown for each of the six buffalo bulls. The fresh semen of bull number 2 had the significantly (P<0.01) highest MTT reduction rate (1.21 ± 0.00), motility (88.33 ± 4.41%), viability (92.00 ± 0.58%) and IVF rate (46.00 ± 0.58%). On the other hand, while fresh bull semen number 5 and 6 had the significantly (P<0.01) lowest MTT reduction rate (0.82 ± 0.00 & 0.89 ± 0.01), motility (76.67 ± 1.67 & 70.00 ± 2.89%), viability (78.33 ± 0.33 & 73.00 ± 0.58%) and IVF rate (31.33 ± 0.88 & 29.00 ± 0.58%, respectively). Concerning the percentage of sperm with intact acrosome, fresh bull semen number 3 had the significantly (P<0.01) highest percentage (82.00 ± 1.15%) and bull number 6 had the lowest one (63.00 ± 1.15%).

In case of frozen-thawed semen bull number 2 had the significantly (P<0.01) highest MTT reduction rate (0.66 ± 0.03), motility (70.00 ± 1.15, 60.00± 1.15, 55.00± 1.15 and 45.00± 1.15 at 0, 1, 2 and 3 hours post-thawing, respectively), viability (72.67 ± 0.33, 62.33 ±0.33, 58.33 ±0.33 and 46.33 ±0.33% at 0, 1, 2 and 3 hours post-thawing, respectively), viability index (202.83 ± 1.74%) and IVF rate (43.33±0.33%). On the other hand, bull number 3 had the significantly (P<0.01) a lowest MTT reduction rate (0.46 ± 0.04) and bulls number 5 and 6 had the significantly (P<0.01) lowest motility, viability, viability indices and IVF rate. Concerning the percentage of sperm with intact acrosome, bull number 3 had the significantly (P<0.01) highest (71.00 ± 0.58%) while bull number 6 had the lowest percentage (58.00 ± 0.58%).

**Table (3)**: MTT reduction rate and fresh sperm parameters of the examined buffalo bull (mean ± se) s:

| Bull no. | MTT2 reduction rate | Sperm motility (%) | Sperm viability (%) according to | | Sperm with intact acrosome (%) | In vitro fertilization rate (%) |
|---|---|---|---|---|---|---|
| | | | Eosin-nigrosin staining | MTT2 reduction rate | | |
| 1 | 1.17±0.00$^{b}$ | 85.00±2.89$^{ab}$ | 86.67±0.33$^{b}$ | 87.75±0.00$^{b}$ | 77.00±1.73$^{b}$ | 39.00±1.73$^{b}$ |
| 2 | 1.21±0.00$^{a}$ | 88.33±4.41$^{a}$ | 92.00±0.58$^{a}$ | 90.55±0.00$^{a}$ | 77.67±0.66$^{b}$ | 46.00±0.58$^{a}$ |
| 3 | 0.82±0.01$^{e}$ | 81.67±1.67$^{ab}$ | 80.67±0.88$^{d}$ | 63.52±0.93$^{e}$ | 82.00±1.15$^{a}$ | 37.00±1.15$^{b}$ |
| 4 | 0.99±0.01$^{c}$ | 81.67±1.67$^{ab}$ | 84.00±0.58$^{c}$ | 75.17±0.40$^{c}$ | 79.33±0.33$^{ab}$ | 38.33±0.33$^{b}$ |
| 5 | 0.82±0.00$^{e}$ | 76.67±1.67$^{bc}$ | 78.33±0.33$^{e}$ | 63.23±0.00$^{e}$ | 77.00±0.58$^{b}$ | 31.33±0.88$^{c}$ |
| 6 | 0.89±0.01$^{d}$ | 70.00±2.89$^{c}$ | 73.00±0.58$^{f}$ | 68.18±0.81$^{d}$ | 63.00±1.15$^{c}$ | 29.00±0.58$^{c}$ |

Within column, means with different alphabetical superscripts are significantly different at least at P<0.01

**Table (4):** MTT reduction rate and sperm parameters (mean ±S.E) of the examined buffalo bull's frozen thawed semen:

| Bull no. | | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| MTT reduction rate at 1 hr post-thawing | | 0.50 ±0.03$^{c}$ | 0.66 ±0.03$^{a}$ | 0.46 ±0.04$^{d}$ | 0.57 ±0.03$^{b}$ | 0.49 ±0.03$^{c}$ | 0.50 ±0.03$^{c}$ |
| Sperm motility (%) at: | Post-thawing | 65.00 ±0.58$^{b}$ | 70.00 ±1.15$^{a}$ | 60.00 ±0.58$^{c}$ | 65.00 ±1.15$^{b}$ | 55.00 ±1.15$^{d}$ | 45.00 ±1.15$^{e}$ |
| | 1 hr | 55.00 ±0.58$^{b}$ | 60.00 ±1.15$^{a}$ | 50.00 ±0.58$^{c}$ | 50.00 ±1.15$^{c}$ | 45.00 ±1.15$^{d}$ | 35.00 ±0.58$^{e}$ |
| | 2 hr | 50.00 ±0.58$^{b}$ | 55.00 ±1.15$^{a}$ | 45.00 ±0.58$^{c}$ | 45.00 ±1.15$^{c}$ | 30.00 ±1.15$^{d}$ | 30.00 ±1.15$^{d}$ |
| | 3 hr | 40.00 ±0.58$^{b}$ | 45.00 ±1.15$^{a}$ | 35.00 ±0.58$^{c}$ | 40.00 ±1.15$^{b}$ | 25.00 ±1.15$^{d}$ | 25.00 ±0.58$^{d}$ |
| Sperm viability (%) at: | Post-thawing | 68.33 ±0.88$^{b}$ | 72.67 ±0.33$^{a}$ | 62.33 ±0.33$^{c}$ | 67.67 ±1.20$^{b}$ | 57.67 ±0.33$^{d}$ | 48.00 ±1.15$^{e}$ |
| | 1 hr | 57.33 ±0.33$^{b}$ | 62.33 ±0.33$^{a}$ | 51.67 ±0.88$^{c}$ | 53.33 ±0.88$^{c}$ | 47.33 ±0.33$^{d}$ | 37.00 ±0.58$^{e}$ |
| | 2 hr | 53.00 ±0.58$^{b}$ | 58.33 ±0.33$^{a}$ | 46.67 ±0.88$^{c}$ | 46.67 ±0.88$^{c}$ | 33.33 ±0.33$^{d}$ | 31.33 ±0.88$^{d}$ |
| | 3 hr | 42.33 ±0.33$^{b}$ | 46.33 ±0.33$^{a}$ | 37.00 ±1.15$^{c}$ | 41.33 ±0.33$^{b}$ | 27.33 ±0.33$^{d}$ | 26.67 ±0.33$^{d}$ |
| Viability index | | 186.33 ±1.74$^{b}$ | 202.83 ±1.74$^{a}$ | 167.00 ±2.02$^{d}$ | 175.50 ±2.02$^{c}$ | 136.00 ±2.02$^{e}$ | 119.00 ±2.02$^{f}$ |
| Viability index according to MTT reduction rate | | 156.17 ±5.88$^{c}$ | 197.39 ±1.89$^{a}$ | 146.92 ±1.89$^{d}$ | 174.68 ±0.00$^{b}$ | 154.49 ±0.00$^{c}$ | 157.01 ±0.00$^{c}$ |
| Sperm with intact acrosome (%) | | 67.00 ±0.58$^{b}$ | 69.00 ±1.15$^{ab}$ | 71.00 ±0.58$^{a}$ | 62.00 ±1.15$^{c}$ | 62.67 ±1.20$^{c}$ | 58.00 ±0.58$^{d}$ |
| In vitro fertilization | | 38.00 ±0.58$^{c}$ | 43.33 ±0.33$^{a}$ | 36.00 ±0.58$^{d}$ | 40.00 ±0.58$^{b}$ | 30.67 ±0.66$^{e}$ | 26.00 ±0.58$^{f}$ |

Within raws, means with different alphabetical superscripts are significantly different at least at P<0.01

As presented in Tables 5 and 6, the MTT reduction rate was significantly ($P < 0.01$) correlated with various sperm parameters. For fresh semen, correlation coefficients of r = 0.825, 0.593 and 0.755 were found between MTT reduction and each of sperm viability, motility and in vitro fertilization rate, respectively. For frozen semen the correlation coefficients between MTT reduction and sperm viability, post-thawing motility and, post-thawing viability and in vitro fertilization rate were 0.598, 0.590, 0.595 and 0.659, respectively. For both fresh and frozen semen there was no significant correlation between the MTT reduction rate and the acrosomal integrity.

**Table (5):** the correlation between MTT reduction rate and sperm parameters of the examined fresh semen:

| *Parameters* | *MTT reduction rate* | *Sperm viability (eosin-nigrosin)* | *Sperm motility* | *Sperm with intact acrosome* | **In vitro fertilization rate** |
|---|---|---|---|---|---|
| Sperm viability (MTT reduction rate) | | 0.825 | 0.593 | 0.105 | 0.755 |
| Sperm viability (eosin-nigrosin) | | | 0.850 | 0.600 | 0.950 |
| Sperm motility | | | | 0.637 | 0.799 |
| Sperm with intact acrosome | | | | | 0.616 |

**Table (6):** the correlation between MTT reduction rate and sperm parameters of the examined fresh semen:

| *Parameters* | *MTT reduction rate* | *Sperm viability index* | *Sperm post-thawing motility* | *Sperm post-thawing viability* | *Sperm with intact acrosome* | In vitro fertilization rate |
|---|---|---|---|---|---|---|
| MTT sperm viability index | | 0.598 | 0.590 | 0.595 | 0.126 | 0.659 |
| Sperm viability index | | | 0.972 | 0.975 | 0.725 | 0.975 |
| Sperm post-thawing motility | | | | 0.995 | 0.702 | 0.983 |
| Sperm post-thawing viability | | | | | 0.678 | 0.980 |
| Sperm with intact acrosome | | | | | | 0.669 |

Within column/raws, means with different alphabetical superscripts are significantly different at least at $p<0.01$

## DISCUSSION

The present study was conducted to determine the reliability of determination of the mitochondrial activity for predicting the quality of fresh and frozen-thawed buffalo semen. This was of interest for accurate evaluation of fertility of bulls used for AI. Mitochondrial activity was previously assessed in spermatozoa of ram (Windsor and White, 1993), turkey (McClean et al., 1993), fowl (Hazary et al., 2001), human (Auger et al., 1989; Naser-Esfahani et al., 2002), boar (Gaczarzewicz et al., 2003), bull (Aziz, 2006) and stallion (Aziz et al., 2005).

Results of the present study demonstrated that the correlations among MTT reduction rate and spermatozoal motility and viability of both fresh and frozen-thawed buffalo semen were significantly ($p < 0.01$) positive; the magnitude of the relationship (r) ranged from 0.590 to 0.825. Similar high correlations between the MTT reduction rate and the result of sperm viability were recorded in cattle (Aziz, 2006) and equines (Aziz et al., 2005), respectively. 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 (1983) 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 (1983), the reduction rate of MTT in the present study was taken successfully after 1 hour of incubation. This was expected because spermatozoa are very active cells and rich in mitochondria; therefore, the reduction of MTT by spermatozoa is expected to be faster than other cells. A similar observation was reported in the studies of Aziz (2006) and Aziz et al. (2005). There was a gradual increase in the reduction rate of MTT till the 1st hour of incubation in case of fresh buffalo semen and till the 2nd hour of incubation in case of frozen-thawed semen. This increase in MTT reduction rate is attributed to the gradual increase in the release of succinate dehydrogenase enzyme and via increase in the amount of formazan formed. Compared to fresh semen, the gradual increase in the rate of MTT reduction of frozen-thawed buffalo semen has longer duration which could be due to extension of the life of spermatozoa by the added extender (Foote, 1978). The later on decline of the MTT reduction rate in the present investigation may be explained by the release of toxic materials by dead and abnormal spermatozoa which as believed by Shannon and Curson (1972) might have adversely affected the vitality of their companion cells.

Sperm viability according to the rate of MTT reduction of fresh and frozen-thawed semen of all examined buffalo-bulls was highly correlated with the estimated sperm parameters. The values of MTT reduction rate for fresh semen tended to

be about two-fold higher than that for the frozen-thawed semen of the same buffalo bulls. This could be explained by reduction of sperm viability by as much as 50% following semen cryopreservation (Watson, 1995) known to exert, chemical, osmotic, thermal and mechanical stress on spermatozoa. Moreover, buffalo spermatozoa are more susceptible to hazards of freezing compared to cattle spermatozoa (Raizada et al., 1990). Thomas et al. (1998) suggested that although the majority of spermatozoa survive cryopreservation, many die in the process and those surviving undergo a certain change in viability, presumably related to changes in the plasma membrane and organelles such as the mitochondria, the acrosome and the tail.

In retrospective studies, Zhang et al. (1998) found significant correlations between in vitro ability to fertilize (IVF) and the field fertility of the same batches of bull frozen semen. Also, Larson and Rodriguez-Martinez (2000) found that in vitro fertilization tests enabled the prediction of the semen fertility. The high values of correlation coefficient between the MTT reduction rate and the in vitro fertilizing capacity of both fresh and frozen-thawed buffalo spermatozoa as obtained in the present investigation indicate that the MTT reduction rate by buffalo spermatozoa may be used as an indicator for the buffalo-bull sperm fertility.

MTT test is simple and inexpensive (Mosmann, 1983) and has the advantages of being fast (1 hour); many samples (up to 10) can be examined at the same time and many replications of each sample can be tested simultaneously (Aziz et al., 2005). The results of our study suggest additional advantage of this test which is the reliability for evaluation of frozen-thawed semen as equal as freshly ejaculated semen.

In conclusion, the MTT test was found to be as a reliable, practical, time and money saver method for an objective evaluation of fresh and frozen-thawed buffalo semen.

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