



EFFECT OF TURMERIC EXTRACT AND DIMETHYL SULFOXIDE ON BUFFALO SEMEN FREEZABILITY AND FERTILITY

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Summary

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The freeze-thaw process results in structural and functional damages caused by overaccumulation of reactive oxygen species (ROS). Addition of antioxidants to semen extenders is of a great importance to overcome this oxidative damage. The study objective was to evaluate the consequence of using Tris-citric acid fructose egg yolk (TCFY) extender supplemented with a combination of turmeric extract and dimethyl sulfoxide (DMSO) [TTD] on sperm freezability and fertility. From five tubes (each containing 5 mL TCFY), the first tube contained neither turmeric extract nor DMSO and was kept as control. The other four tubes contained 1.5% DMSO plus 100 μ L/5 mL, 200 μ L/5 mL, 300 μ L/5 mL and 400 μ L/5 mL turmeric extract. Semen samples were pooled and extended to reach an application of 60×10^6 sperm/mL (TT₁D to TT₄D, respectively). Diluted semen was exposed to the freezing protocol. The post cooling results revealed significant improvement in percent of alive spermatozoa in TT₁D, significant decrease in sperm abnormalities in all concentrations used, significant improvement in intact acrosome percentage in TT₁D, TT₂D and TT₄D. The post thawing results exhibited significant improvement in sperm motility in TT₁D, TT₂D and TT₄D; significant amelioration of sperm membrane integrity (HOST) in TT₁D, TT₂D, TT₃D and TT₄D. Acrosome integrity was maintained in all concentrations as in the control. It was concluded that TT₁D revealed the best semen quality in cooled semen; and TT₁D, TT₂D exhibited the superior post thawing semen quality. In addition, conception rate (CR) of the post-thawed semen was ameliorated in TT₁D, TT₂D, TT₃D and TT₄D with TT₁D being the best one with this regard.

Key words: buffalo, cryopreservation, dimethyl sulfoxide, semen, turmeric

INTRODUCTION

The composition of the freezing extender, dilution method, cooling and thawing rates are different factors that influence the post semen freezability (Eiman & Terada, 2004). The penetrating cryoprotectant

reduces the physical and chemical alterations resulting from the freezing process (Purdy, 2006).

DMSO is a permeable cryoprotectant that penetrates easily through the sper-

matozoal membrane to replace the water content of the sperm cell and decrease the cryoinjury caused by ice crystallisation (Rasul *et al.*, 2007).

Supplementation of the extended semen with antioxidants improves buffalo bulls' semen cryopreservation. Plant extracts are considered a major category to fulfill this purpose. Turmeric extract contains curcumin which is a main ingredient acting as antioxidant in semen extenders (Petruska *et al.*, 2014). Turmeric is a useful plant. Curcumin is a phytochemical having antioxidant and anti-inflammatory effect, extracted from the rhizome of *Curcuma longa*. It is demonstrated to have an *in vitro* protective effect for spermatozoa depending on its concentration where low concentrations improved sperm motility while high concentrations decreased it (Glombik *et al.*, 2014). Curcumin is a polyphenolic compound insoluble in water that scavenges free radicals (Sharma, 1976) through decreasing generation of reactive oxygen species (ROS), as H₂O₂ and nitrite. Curcumin is the major fraction of curcuminoids of turmeric (*Curcuma longa*), a member of ginger family (Kim *et al.*, 2019). Curcuminoids are natural phenols in charge of the turmeric yellow colour (Nelson *et al.*, 2017). Turmeric extract contains curcumin with other curcuminoids and essential oils which were found to be bioactive (Kulkarni *et al.*, 2012).

Addition of curcumin to raw bull semen markedly ameliorated sperm output post thawing (Bucak *et al.*, 2012). Supplementation of curcumin to male rodents improved testicular function and fertility (Sahoo *et al.*, 2008; Mathuria & Verma, 2008).

The current study aimed to evaluate the effect of Tris citric acid fructose egg yolk (TCFY) extender supplemented with

a combination of turmeric extract and dimethyl sulfoxide on buffalo semen freezability and fertility.

MATERIALS AND METHODS

Semen extenders

TRIS base extender. Tris-citric acid-fructose diluent (TCF) was prepared according to Foote *et al.* (2002) and 20% whole egg yolk (TCFY) was added. TCF contained 3.029 g Tris, 1.679 g citric acid monohydrate, 1.259 g fructose, 6.4% glycerol in 100 mL distilled H₂O.

Preparation of turmeric extract. Four grams turmeric powder + 60 mL ethanol were mixed in a test tube, while 4 g turmeric powder + 60 mL distilled water – in another tube using a stirrer for mixing. After filtration, the filtrate was left at 40 °C for 24 h till evaporation. The residues in both tubes were mixed together and dissolved in 2 mL Tris and kept as a stock solution from which the different dilutions were done (Kim *et al.*, 2019).

Turmeric and DMSO enriched extender (TDEE). From five tubes (each containing 5 mL TCFY), the first tube contained neither turmeric extract nor DMSO and was kept as control. The other four tubes contained 1.5% DMSO+100 µL/5 mL turmeric extract (TD₁), 1.5% DMSO+200 µL/5 mL turmeric extract (TD₂), 1.5% DMSO+300 µL/5 mL turmeric extract (TD₃) and 1.5% DMSO+400 µL/5 mL turmeric extract (TD₄).

Semen collection and preliminary evaluation

Semen from five mature buffalo bulls kept at Semen Freezing Center, Veterinary Services Organisation, Egypt, were used. Ejaculates were collected using artificial vagina at weekly intervals for 8 weeks.

Semen samples were initially evaluated for subjective sperm motility and sperm concentration. Ejaculates satisfying at least sperm motility of 70% and normal sperm morphology were pooled in order to have sufficient semen and to exclude the individual variation. Semen was held for 10 minute at 37 °C in the water bath pre processing. The experimental design was approved by the Medical Research Ethics Committee of the National Research Centre, Dokki, Egypt (registration number 19/104 from 10/10/2019).

Semen processing

Semen samples were diluted with TCFY extender and used as control. Other aliquots of pooled semen samples were diluted with TCFY extenders containing 1.5% DMSO plus the different concentrations of turmeric extract to reach concentration of 60×10^6 sperm/mL (TT₁D to TT₄D, respectively). Extended semen was cooled slowly (approximately for 2 h) to 5 °C and equilibrated for 2 h. Semen was filled into 0.25 mL polyvinyl French straws. After this period, the straws were placed horizontally on a rack and frozen in vapour 4 cm on the top of liquid nitrogen for 10 minutes and then were plunged in liquid nitrogen (Khan & Ijaz, 2007).

Evaluation of semen quality parameters

The assessment was implemented post cooling and on freeze thawed bull spermatozoa. Frozen straws were thawed at 37 °C/1 minute. The studied parameters were subjective semen characteristics: motility, alive, abnormality, hypoosmotic swelling test (HOST) and acrosome status.

Sperm motility. Sperm motility was examined and recorded using a pre-warmed stage of phase contrast microscope (200×) just after thawing (Salisbury *et al.*, 1978).

Sperm livability. Smears were stained with Eosin-Nigrosin (Campbell *et al.*, 1956).

Sperm morphology. Smears were stained with Eosin-Nigrosin (Campbell *et al.*, 1956).

Sperm membrane integrity. It was assessed using the hypoosmotic swelling test (HOST) as described by Jeyendran *et al.* (1984). The hypoosmotic solution consisted of sodium citrate (7.35 g/L; Sigma chemical Co.) and fructose (13.51 g/L; Sigma chemical Co.). The final osmolarity was adjusted to 150 mOsm/L with pH 7.2. An amount of 50 µL semen was mixed with 500 µL of the pre-warmed hypoosmotic solution in 1.5 mL tubes and incubated at 37 °C for 45 min. After incubation, a total of 10 µL was pipetted on a slide, a cover slip was placed on top of the droplet, and the preparation was observed by phase-contrast microscopy at 400×. A total of 200 spermatozoa in at least five different fields were examined in each preparation. The swollen spermatozoa characterised by coiling of the tail were considered to have an intact plasma membrane.

Sperm acrosome integrity. Acrosomal integrity was tested by Giemsa's staining. The stock Giemsa's stain was prepared and kept at 37 °C in an incubator for 7 days in amber coloured bottle for maturation with intermittent shaking. The working Giemsa solution was prepared mixing 3 mL Giemsa's stock, 2 mL phosphate buffer and 45 mL Milli-Q water in a cup licker and warmed at 37 °C for 30 min. The smeared slides of spermatozoa were put into the working solution and kept at 37 °C for 2 h. The slides were removed from the stain and washed in running tap water and finally air dried. The counting of intact, partially damaged and fully damaged acrosomes was carried out in oil

immersion Olympus microscope (BX51) at 1000× (10×100) magnification (Chowdhury *et al.*, 2014).

In vivo fertility rate

Buffalo females (n=320) were inseminated with the treated post-thawed semen and with the post-thawed semen extended in TCFY (control group). Pregnancy was recorded by rectal palpation after 2 months from insemination. The inseminated cows were used via the cooperation in Beni-Suef Governorate. Conception rate (CR) was computed as (No. of pregnant buffaloes/Total No. of inseminated buffaloes) ×100.

Statistical analysis

Data were analysed using the analysis of variance (ANOVA) (SPSS v. 14.0) to compare the different parameters between control and additives replications. Significant difference between means was calculated using Duncan test at P<0.05.

RESULTS

The post cooling results (Table 1) revealed that sperm motility was not significantly changed in all concentrations as compared to the control. Significant (P<0.002) improvement in percent of alive spermatozoa was detected in TT₁D, significant (P<0.000) decrease in sperm abnormalities occurred in all concentrations used, sperm membrane integrity was not significantly altered in all concentrations and was nearly equal to the control. Significant (P<0.004) improvement in intact acrosome percent in TT₁D, TT₂D and TT₄D was found out compared to the control.

The post thawing results (Table 2) exhibited significant (P<0.001) improvement in sperm motility in TT₁D, TT₂D and TT₄D; and significant (P<0.001) amelioration of sperm membrane integrity (HOST) in TT₁D, TT₂D, TT₃D and TT₄D relative to the control. Acrosome integrity was significantly decreased in the TT₄D group compared to the control and all other concentrations.

Table 1. Effect of Tris extender enriched with turmeric extract and dimethyl sulfoxide on the cooled extended buffalo bull semen (mean ±SEM, n=40)

Extender	Motility %	Alive %	Abnormality %	HOST %	Acrosome integrity %
TT ₁ D	91.66±1.66 ^a	91.66±1.66 ^c	11.00±0.57 ^{ab}	83.78±0.61 ^a	86.00±1.00 ^b
TT ₂ D	91.66±1.66 ^a	86.33±1.33 ^b	8.33±0.33 ^a	80.56±5.41 ^a	86.00±1.00 ^b
TT ₃ D	90.00±5.00 ^a	86.33±1.33 ^b	11.33±0.33 ^b	84.43±1.53 ^a	81.00±1.00 ^a
TT ₄ D	88.33±3.33 ^a	80.66±0.66 ^a	11.66±0.88 ^b	84.69±1.97 ^a	86.33±1.33 ^b
Control	88.33±1.66 ^a	85.66±1.20 ^b	18.33±1.66 ^c	80.69±0.73 ^a	81.00±1.00 ^a
P-value	NS	0.002	0.000	NS	0.004

TT₁D=Tris+100 µL turmeric extract+ 1.5% DMSO; TT₂D=Tris+200 µL turmeric extract+1.5% DMSO; TT₃D= Tris+300 µL turmeric extract+1.5% DMSO; TT₄D= Tris+400 µL turmeric extract+1.5% DMSO; control=Tris-citrate-fructose-egg yolk-glycerol (TCFYG); HOST=hypoosmotic swelling test. Means bearing different superscripts (a, b, c) within columns differ at P<0.05; NS=non significant.

Table 2. Effect of Tris extender enriched with turmeric extract and dimethyl sulfoxide on the post-thawed extended buffalo bull semen (mean \pm SEM, n=40)

Extender	Motility %	Alive %	Abnormality %	HOST %	Acrosome integrity %
TT ₁ D	58.33 \pm 1.66 ^b	80.66 \pm 0.66 ^a	11.33 \pm .33 ^b	79.38 \pm 1.15 ^b	86.66 \pm 1.66 ^b
TT ₂ D	51.66 \pm 1.66 ^b	86.33 \pm 1.33 ^a	11.66 \pm .66 ^b	79.01 \pm 1.04 ^b	85.66 \pm 0.66 ^b
TT ₃ D	41.66 \pm 1.66 ^a	83.00 \pm 3.00 ^a	7.33 \pm 1.20 ^a	82.68 \pm 5.01 ^b	84.66 \pm 2.60 ^b
TT ₄ D	51.66 \pm 1.66 ^b	84.66 \pm 2.60 ^a	10.33 \pm 0.33 ^b	80.59 \pm 0.07 ^b	79.33 \pm 0.66 ^a
Control	43.33 \pm 3.33 ^a	86.66 \pm 3.33 ^a	6.66 \pm 0.33 ^a	48.66 \pm 7.51 ^a	87.50 \pm 2.50 ^b
P-value	0.001	NS	0.001	0.001	0.048

TT₁D=Tris+100 μ L turmeric extract+ 1.5% DMSO; TT₂D=Tris+200 μ L turmeric extract+1.5% DMSO; TT₃D= Tris+300 μ L turmeric extract+1.5% DMSO; TT₄D= Tris+400 μ L turmeric extract+1.5% DMSO; control=Tris-citrate-fructose-egg yolk-glycerol (TCFYG); HOST=hypoosmotic swelling test. Means bearing different superscripts (a, b, c) within columns differ at P<0.05; NS=non significant.

Table 3. Effect of Tris extender enriched with combination of turmeric extract and dimethyl sulfoxide in a field conception rate test in buffaloes

Treatment	<i>In vivo</i> fertility rate (%)
TT ₁ D	65%
TT ₂ D	62%
TT ₃ D	60%
TT ₄ D	64%
Control	55.2%

TT₁D=Tris+100 μ L turmeric extract+1.5% DMSO; TT₂D=Tris+200 μ L turmeric extract+1.5% DMSO; TT₃D=Tris+300 μ L turmeric extract+1.5% DMSO; TT₄D=Tris+400 μ L turmeric extract+1.5% DMSO; control=Tris-citrate-fructose-egg yolk-glycerol (TCFYG); HOST=hypoosmotic swelling test. Means bearing different superscripts (a, b, c) within columns differ at P<0.05; NS=non significant.

Conception rate (Table 3) of the post-thawed semen was the best in TT₁D (65%), TT₂D (62%) and TT₄D (64%) followed by TT₃D (60%) when compared to the control (55.2%).

DISCUSSION

There is a great worldwide interest to the beneficial synergistic effects of natural supplements and their multiple ingredients as compared to the single active fractions (Seeram *et al.*, 2004). Semen freezing causes damage to spermatozoa leading to reduction in semen quality, but it is essential to conserve the supergenetic characters of our local buffalo breeds (Watson, 2000). Semen freezing is associated with cryodamage caused by overproduction of oxygen free radicals (Agarwal *et al.*, 2005). So, the natural additive to the extender ameliorates the antioxidant effect with consequent improvement of the fertilising potential of post-thawed frozen spermatozoa (Gadea *et al.*, 2007).

The post cooling and post-thawing semen characteristics in our study were improved. The post cooling features revealed significant improvement in percentage of alive spermatozoa in TT₁D as well as significant decrease in sperm ab-

normalities in all concentrations used, significant improvement in intact acrosome percentages in TT₁D, TT₂D and TT₄D compared to the control. The post-thawing characteristics comprised marked amelioration in sperm motility and sperm membrane integrity in TT₁D, TT₂D and TT₄D. The conception rate was the best in TT₁D, TT₂D and TT₄D. These results coincide with the best sperm motility at these concentrations and are in accordance with those of Mahmoud *et al.* (2013) who showed that motility may be an applicant indicator for semen characteristics, where significant correlations were found between motility and each of sperm abnormalities and membrane integrity. Ramos & Wetzel (2001) reported that motility may be related to DNA status of the sperm cells. Vale (1997) recorded a pregnancy rate over 50% as a good consequence after artificial insemination with post-thawed frozen semen in buffaloes. Al Naib *et al.* (2011) categorised bulls with pregnancy rate of about 50% to be highly fertile, and the sperm of high fertility bulls was highly efficient in penetrating artificial mucus and had a high potential to fertilise oocyte *in vitro*. The improved sperm quality and fertility are attributed to the presence of curcumin in the turmeric extract. Curcumin is the major ingredient of turmeric, a lipophilic polyphenol insoluble in water that scavenges free radicals, significantly inhibits the generation of ROS (Petruska *et al.*, 2014). Curcumin increases significantly the sperm GSH content, thus improving the antioxidant capacity of the semen extender (Bucak *et al.*, 2012). Curcumin shows antioxidant activity through binding with egg and soy phosphatidyl choline which in turn binds divalent metal ions and has antibacterial and antiviral effects (Bhowmik *et al.*, 2009). The antioxidant effect of curcumin

is referred to its unique conjugated structure which includes two methoxylated phenols and an enol form of b-diketone; this structure reveals an ideal free radical trapping ability as a chain breaking antioxidant (Bagchi, 2012). Turmeric contains essential oils like polyunsaturated fatty acids which interact with sperm membrane and increase the polyunsaturation of spermatozoa rendering it more stable and resistant to cold shock and damage during cryopreservation (Maldjian *et al.*, 2005). DMSO is an agent permeating the sperm cells which can move across cellular membranes and modulate the rate and extent of cellular dehydration during freezing-induced membrane phase transitions. Permeating protectants provide intracellular protection because they are preferentially excluded from the surface of biomolecules thereby stabilising the native state (Sieme *et al.*, 2016). DMSO is a permeable cryoprotectant penetrating easily through the spermatozoal membrane to replace the water content of the sperm cell and decrease the cryoinjury caused by ice crystallisation (Rasul *et al.*, 2007).

El-Harairy *et al.* (2011) found that the frozen-thawed semen diluted with 3.5% glycerol plus 3.5% DMSO when added with GSH at levels of 0.2, 0.4 and 0.8 mM increased markedly ($P < 0.05$) the percentage of frozen-thawed sperm motility and spermatozoal freezability and reduced ($P < 0.05$) the proportion of acrosomal damage of spermatozoa and level of extracellular AST, ALT, ACP, ALP and LDH enzymes released into the extracellular medium. They added that the highest pregnancy rate ($P < 0.05$) was observed in the cows artificially inseminated with the frozen-thawed bull semen processed with a combination of 3.5% glycerol and 3.5% DMSO. Farshad *et al.* (2009) postulated

that post-thaw sperm motility, viability and intact acrosome was improved using 1.75% DMSO in goat semen extender. It could be concluded that TT₁D revealed the best semen quality in cooled semen and that TT₁D and TT₂D exhibited the superior post-thawing semen quality. Conception rate (CR) of the post-thawed semen was the best in TT₁D, TT₂D and TT₄D.

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