Original Article

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COMPARATIVE STUDY OF TRIS TURMERIC, TRIS TURMERIC DIMETHYL SULFOXIDE AND TRIS TURMERIC ETHYLENE GLYCOL EXTENDERS ON THE CRYOSURVIVABILITY, SPERM RESISTANCE, IN- VIVO FERTILITY AND ANTIOXIDANT STATUS IN BUFFALO BULL SEMEN

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ABSTRACT

The freeze-thaw process leads to structural and functional damage due to excessive accumulation of reactive oxygen species (ROS). The addition of exogenous antioxidants to sperm diluents is of great importance to overcome oxidative damage during freezing. The purpose of this study was to explore the effects of three diluents Tris Turmeric, Tris Turmeric Dimethyl Sulfoxide and Tris Turmeric Ethylene glycol on the cold survival ability of buffalo sperm. Semen was collected from five local adult male buffalo breeds. A base diluent of Tris-citric acid-fructose (TCF) was prepared, adding 20% whole egg yolk (TCFY). The Tris extender without turmeric, without DMSO, and without EG was kept as a control. Other extenders are Tris containing turmeric TT (100 ml/5 ml Tris), Tris containing turmeric dimethyl sulfoxide TTD (100 ml/5 ml Tris + 1.5% DMSO) and Tris containing turmeric and ethylene glycol TTE EG (100 ml/5 ml Tris + 1.5% EG). Semen samples were added and a pure sperm concentration of 60 × 10^6/ml was achieved. Frozen buffalo sperm after thawing showed significant improvements in all research parameters of the three breeding samples compared to the control. Tris Turmeric Ethylene was the type that best improved sperm survival under frozen conditions, followed by Tris Turmeric and Tris Turmeric Dimethyl Sulfoxide compared to the control. A significant decrease in sperm motility after thawing was evident as usage time increased in all expanders. There was a significant increase in total antioxidant content (TAC) and insignificant change in malondialdehyde (MDA) of the diluent used compared to the control. Conception rate (CR) was higher in Tris Turmeric Ethylene glycol (65.2%), followed by Tris Turmeric (60.3%) and Tris Turmeric Dimethyl Sulfoxide (55.9%) compared to the control (36, 7%). It can be concluded that Tris Turmeric Ethylene Glycol is considered the best agent for improving cold survival and sperm fertility, followed by Tris Turmeric and Tris Turmeric Dimethyl Sulfoxide.

Keywords: *Bubalus bubalis*, buffaloes, Tris turmeric, Tris turmeric dimethyl sulfoxide, Tris turmeric ethylene glycol, Giza, Egypt

INTRODUCTION

Artificial insemination (AI) is considered the main tool for dissemination of the supergenetic

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characters to improve the genetic constitution of the livestock (Vishwanath and Shannon, 2000; Durrant, 2009).

The subfertility of bulls used in AI program is a causative factor for great economic losses especially when the sub fertile bulls are genetically superior (Kuroda *et al*., 2007). The normal capacitation and capability to fertilize the oocytes occurs during the journey of the spermatozoa in the female genital tract after various alterations including rearrangement of the spermatozoal membrane and changes of sperm motility and metabolic activities (Yanagimachi, 1994). Capacitation is promoted in the female reproductive tract by the effect of bicarbonate and calcium ions (Visconti *et al*., 1998). The premature capacitation and spontaneous acrosome reaction occurring during cryopreservation is related to protein and lipid changes of the sperm membrane resulting from uncontrolled influx of calcium ions with consequent lower in-vitro fertilizing capacity (Bailey *et al*., 2000). The laboratory evaluation for sperm capacitation is of a great importance for detection of normality of spermatozoa after cryopreservation (Kuroda *et al*., 2007).

About half of the preserved spermatozoa are damaged during cryopreservation (Watson, 2000), mostly as a result of intracellular ice crystallization after freezing (Watson, 2000; Akhter *et al*., 2008). Reactive oxygen species (ROS) build up excessively during the freeze-thaw process, causing structural and functional impairments (Guthrie and Welch, 2006). Because the spermatozoa membrane contains polyunsaturated fatty acids, it is prone to lipid peroxidation. This can cause oxidative damage, which in turn reduces the spermatozoa membrane's motility, viability, and DNA integrity (Nair *et al*., 2006; Andrabi, 2009). Thus, in order to minimize such damage, the dilator's composition is crucial (Curry *et al*., 1994; Andrabi, 2009). Sperm from farmed species must be preserved using extenders that have the proper osmolarity, pH, and buffering capacity. They also need to shield sperm from freezing injury (Barbaras and Mascarenhas, 2009; Salamon and Maxwell, 2000). Enhanced domestic species sperm cryopreservation is a significant goal that can be attained by supplementing semen with antioxidants for an extended period of time. Plant extracts are thought to be a crucial category in achieving this objective. Curcumin, a vital component for sperm enhancers that functions as an antioxidant, is found in turmeric extract (Petruska *et al*., 2014). The primary curcuminoid found in turmeric (*Curcuma longa*), a plant related to ginger, is called curcumin. Turmeric gets its yellow hue from naturally occurring phenols called curcuminoids (Nelson *et al*., 2017). Curcumin, along with other curcuminoids and essential oils, are present in turmeric extract and have demonstrated biological activity (Kulkarnietal, 2012).

An effective herb is turmeric. One phytochemical substance that has been taken from the rhizome of *Curcuma longa* and has anti-inflammatory and antioxidant properties. Depending on the concentration, curcumin has been demonstrated to have a sperm-protective effect *in vitro*; sperm motility is improved at low concentrations while it is decreased at high doses (Głombik *et al*., 2014). Water-insoluble polyphenol curcumin scavenges free radicals by lowering the production of reactive oxygen species (ROS) like nitrite and H2O2 (Sharma, 1976). After being thawed, adding curcumin to fresh bull semen greatly boosted sperm production (Bucak *et al*., 2012). Male rat testicular function and fertility were enhanced by curcumin administration (Sahoo

et al., 2008; Mathuria and Verma, 2008).

Ethylene glycol is a low molecular weight (EG) cryoprotectant that is less stressful to sperm than glycerol, as its low molecular weight gives it the ability to more easily pass through the sperm membrane and reduce damage to the sperm membrane by lowering ice crystals. Training (Moore *et al*., 2006). Ethylene glycol has less deleterious effects on acrosome viability, motility, and condition than glycerol (Ball and Vo, 2001). EG also showed superior motility of bull sperm after thawing compared to glycerol and dimethyl sulfoxide, due to reduced osmotic damage (Guthrie *et al*., 2002).

Penetrating cryoprotectant helps reduce physical and chemical stresses caused by the freezing process (Purdy, 2006). DMSO is a sperm penetrant. Penetrating agents can move across cell membranes and regulate the rate and extent of cell dehydration during gel-induced membrane phase transitions. Penetrating protectants provide intracellular protection because they are preferentially excluded from the surface of biomolecules, thereby stabilizing the native state (Siemea *et al*., 2016). DMSO is a cryoprotectant that can permeate and easily penetrate the sperm membrane to replace the water content in sperm and reduce cold injury due to ice crystallization (Rasul *et al*., 2007).

MATERIALS AND METHODS

Male buffalo

Five male buffaloes (from 3 to 5 years old) were raised at the Abassia Buffalo Sperm Freezing Center, Central Veterinary Services Organization, Ministry of Agriculture, Egypt, was chosen as the supply source of sperm. Buffaloes are maintained according to uniform standards of nutrition and management. They have good overall health (body weight from 600 to 800 kg), do not suffer from physical and genital diseases. Feeding: During the summer, bulls are kept cool and comfortable by splashing water at least 3 to 4 times/day, avoiding direct wind, and being raised in a place with a comfortable micro-environment, with minimal humidity, for Eat at cool times and have free time. Access to cool drinking water. Feeding: 6 kg dry matter + 2 kg roughage and 3.5 kg dry food/bird/day in summer. In winter, 6 kg of dry matter + 2 kg of roughage and 28 kg of barsim/head/day. Temperature and humidity index: 72 to 78.

Semen collection and preliminary assessment

Semen of 5 local adult male buffalo breeds (from 3 to 5 years old, weighing 600 to 800 kg) is preserved at the Semen Freezing Center of the General Department of Animal Health. Semen samples were collected using a prepared artificial vagina weekly for eighteen weeks. Ejaculation is primarily evaluated for sperm motility and sperm concentration. Semen samples with at least (70%) normal sperm motility and normal morphological sperm percentage were pooled to provide enough sperm to eliminate individual bull variations. Sperm are kept in a water bath for ten minutes at 37°C before stretching.

Sperm extenders and treatments

The basic extender is Tris-Citric Acid-Fructose Glycerol (TCFG) prepared according to Foote (1970), adding 20% pure egg yolk (TCFY). Tris extender with Zero turmeric, Zero DMSO and Zero EG were kept as controls. Turmeric extract: 4 g ground turmeric powder + 60 ml ethanol in a test tube. Add 4 g ground turmeric powder + 60 ml distilled water to the test tube. Mix well in

each tube with a stirrer then filter. The filtrate was maintained at 40°C for 24 h for evaporation. The remaining two tubes were mixed simultaneously and dissolved in 2 ml Tris and set aside as stock solution.

The remaining tubes were Tris containing crude turmeric extract (stock solution) TT (100 ml /5 ml Tris), Triscontaining turmeric and dimethyl sulfoxide TTD (100 ml/5 ml Tris + 1.5% DMSO) and Tris containing turmeric and ethylene glycol TTE EG (100 mL/ 5 ml Tris+1.5 %EG) as test diluents. English Semen samples were added and a pure sperm concentration of 60 × 10^6/ml was achieved. Semen samples diluted with zero turmeric, zero DMSO and zero EG represented the control and the other diluents were kept as assays. The diluted sperm was slowly cooled (about two hours) to 5°C and left to equilibrate for two hours. Sperm were packed in 0.25 ml polyvinyl French straws, and then the straws were placed horizontally on a special shelf and exposed to steam 4 cm above the surface of liquid nitrogen for ten minutes and immediately immersed in liquid nitrogen (Vishwanath and Shannon, 2000).

Evaluation of sperm quality parameters

Evaluation is performed after cooling and freezing of bull sperm. Frozen straws were thawed at 37°C for one minute. Characteristics tested are (motility, liveliness, morphological abnormalities, integrity of sperm membrane and acrosome)

Movement. Incremental motility was subjectively estimated via a drop of semen diluted with prewarmed dehydrated 2.9% sodium citrate solution. The drop was placed on a clean coverslip and then examined under a microscope (X400). At least 200 sperm from at least four microscopic fields were examined. Flexibility was estimated on a continuous scale from zero to 100% (Memon *et al*., 2011).

Percentage and abnormalities of viable sperm. The percentage and abnormalities of viable sperm were assessed by eosin-nigrosin staining in homogenized smears using bright-field optics (X400). Abnormal sperm are counted in the same smear; at least 200 sperm are counted on 5 microscopic fields (Bearden and Fuquay, 1980).

Sperm membrane integrity (Hypoosmolar swelling test (HOST)). Hypoosmotic solution (125 mOsm/1) was prepared by dissolving 6.25 g sodium citrate dihydrate and 11.25 g fructose in 1000 ml distilled water. A volume of 10 µl of spermatozoa was gently mixed with 1 ml of solution and incubated for 60 minutes at 37°C. After incubation, a drop of the spermatozoa solution was placed on a glass slide, covered with a coverslip, and examined under a microscope (X400). A total of 200 spermatozoa were counted; the proportion of HOST-positive spermatozoa (with swollen or curved tails) was determined (Revell and Mrode, 1994).

"Acrosome morphology and motility". Trypan blue/Giemsa staining. Semen samples were analyzed using the Trypan blue/Giemsa staining method with minor modifications (Serafini *et al*., 2014). For staining, Trypan blue was used at a concentration of 0.27%, one drop (5 µl) of diluted spermidine and one drop (5 µl) of Trypan blue were mixed on a glass slide and two smears were prepared using a drop of semen. Slides were air-dried in an upright position and then placed in 10% formalin-buffered saline (9 g Nacl, 6.5 g Na2H PO4, 4 g NAH2PO4 for fixation at 370°C for 30 minutes. Slides were placed in a vial containing Giemsa solution and left overnight. Giemsa staining solution was freshly prepared by adding 14.3% (v/v) Giemsa stock solution (Sigma GS -500) with water. Rinse the specimens again

with distilled water, let them air dry in an upright position and cover with distilled water the intact acrosomes are purple, the anterior part of the sperm head without acrosome, the color is light purple.

Viability index: Motility of frozen sperm after thawing was studied and recorded by hot contrast microscopy (200x) after defrosting for 1, 2 and 3 h. The viability index after thawing was calculated as noted by Milovanov (1962) as half the sperm motility after thawing plus the total sperm motility after the first hour, the second hour second and third after defrosting.

Determination of oxidation/antioxidant parameters

Sperm were collected and then centrifuged at 2,773 × g for 5 minutes at 40°C using a cooled centrifuge (Sigma 3 to 18 KS, Germany). Seminal plasma was collected and stored at -80°C. The level of total antioxidant capacity (TAC) in seminal plasma was determined according to the method of Koracevic *et al*. (2001) and lipid peroxidation content in the form of malondialdehyde (MDA) according to the method of Satoh (1978) using test kits provided by Biodiagnostic Co., Egypt. All measurements were performed using a UV/Visible dual beam spectrophotometer, model T80, UK.

***In vivo* conception rate (CR)**

No. Female buffaloes (n = 260) were artificially inseminated with frozen TCFY as control and other test extenders. Conception rates were recorded by rectal palpation two months after artificial insemination. The inseminated females were used thanks to the cooperation with the Beni-Suef administration. Insemination of females was performed with an insemination gun and spermatozoa were introduced into the uterus. Inseminated females were examined by rectal palpation two months after insemination. CR is calculated according to the equation:

CR = (number of pregnant buffaloes)/(total number of buffaloes inseminated) ×100

Statistical analysis

Statistical analysis data were analyzed using the computer program SPSS (2005) v. 14.0 to calculate analysis of variance (ANOVA) for different parameters between control and additive replicates. Significant differences between mean values were calculated using the Duncan test at $P<0.05$.

Ethics statement

The study was approved by the Medical Research Ethics Committee of Dokki National Research Center, Egypt. The study registration number is 19/104 and the registration date is October 10, 2019.

RESULTS

The frozen post-thawed extended buffalo semen (Table 1) revealed significant amelioration in all the studied parameters of the three extenders Tris Turmeric (100 ml/5 ml Tris, Tris Turmeric Dimethyl Sulfoxyde (100 ml/5 ml Tris + 1.5% DMSO) and Tris Turmeric Ethylene Glycol (100 ml/5 ml Tris+1.5%EG) if compared to the control. Tris Turmeric Ethylene was the best ameliorating of sperm cryosurvivability (sperm motility, alive, abnormalities, sperm membrane integrity (HOST), acrosome integrity ,viability index and capacitation) followed by Tris Turmeric and Tris Turmeric Dimethyl Sulfoxide if compared to the control.

Table 2. Exhibited significant decrease in

post-thawing sperm motility with the advance of time in all the extenders used.

Table 3. Showed significant elevation of the total antioxidants (TAC) and non- significant alteration of malondialdehyde (MDA) of the used extenders relative to the control.

Table 4. Showed the superior conception rate(CR) in Tris Turmeric Ethylene (65.2%) followed by Tris Turmeric (60.3%) and Tris Turmeric Dimethyl Sulfoxyde (55.9%) if compared to the control (36.7%).

DISCUSSION

Several factors have been reported to influence sperm cold survival, including osmotic stress, ice crystal formation, cryoprotectant toxicity, and individual variation (Neild *et al*., 2003; Ferrusola *et al*., 2009). Among various causes, oxidative stress has been reported to affect the fertility and physiology of frozen/thawed sperm (Agarwal *et al*., 2008; O'Flaherty, 2014; Smith *et al*., 2006). Oxidative stress results from an imbalance between the level of reactive oxygen species (ROS) production and the cell's antioxidant capacity (Halliwell, 2006). Excessive amounts of ROS are harmful to sperm (Halliwell and Gutteridge, 2007), low levels of this molecule are needed to induce sperm motility in men, a process necessary for sperm to acquire sperm motility fertilization (O'Flaherty *et al*., 2003). Under oxidative stress, spermatozoa suffer significant damage such as membrane lipid peroxidation, DNA fragmentation (Barroso *et al*., 2000), low mitochondrial membrane activity (Gallon *et al*., 2006; Koppers *et al*., 2006; Koppers *et al*. , 2008) and enzyme inactivation related to motility (de Lamirande and Gagnon, 1992).

Maintaining sperm motility during thawing is directly related to the viability index and is considered a good indicator of sperm resistance. In this regard, Li *et al*. (2016) noted a significant correlation between sperm motility and IVF.

Curcumin is the main extract of turmeric, it is a lipophilic polyphenol that is insoluble in water and has anti-inflammatory properties, eliminating free radicals and significantly inhibiting (ROS) formation (Petruska *et al*., 2014). Curcumin significantly increases the GSH content in sperm, thereby improving the antioxidant capacity of sperm stimulants (Bucak *et al*., 2012). Curcumin exhibits antioxidant activity by binding to egg and soybean phosphatidylcholine, which in turn binds to divalent metal ions and has antibacterial and antiviral effects. (Bhowmik *et al*., 2009). The antioxidant effect of curcumin refers to its unique conjugated structure consisting of two methoxylated phenols and the enol form of b-diketone, which exhibits ideal free radical scavenging ability as an antioxidant. chain-breaking chemistry (Bagchi, 2012). Turmeric contains essential oils. The polyunsaturated fatty acids present in the essential oil interact with the sperm membrane, making it more stable and resistant to thermal shock during cryopreservation (Singh *et al*., 2012).

Ethylene glycol has a small molecular weight and is less toxic, has a higher potency and permeability to sperm than glycerol (Soares *et al*., 2002; Massip, 2001) with reduced osmotic damage to sperm during storage (Gilmore *et al*., 1995). These results coincide with the improvement in sperm motility when ethylene glycol is used. These results are consistent with those of Mahmoud *et al*. (2013), who showed that motility could be a potential marker of sperm quality, considering that a significant correlation was found between motility and both sperm abnormalities and membrane

integrity. Ramos and Wetzel (2001) reported that motility may be an indicator of DNA integrity in sperm. However, Buyukleblebici *et al*. (2014) did not report any improvement in sperm motility after thawing in bulls when ethylene glycol was used for cryoprotection.

DMSO is a sperm penetrant that can move across cell membranes and modulate the rate and extent of cellular dehydration during gelation and phase transitions. Penetrant cryoprotectants provide intracellular protection because they are preferentially excluded from the surface of biomolecules, thus stabilizing their native state (Siemea *et al*., 2016). DMSO is a permeable cryoprotectant that easily penetrates the sperm membrane to replace the water content of the sperm and reduce cryoinjury due to ice crystallization (Rasul *et al*., 2007).

El-Harairy *et al*. (2011) found that frozen-thawed sperm diluted with 3.5% GL plus 3.5% DMSO when spiked with GSH at 0.2, 0.4, and 0.8 Mm ($P<0.05$) significantly increased the motility rate of frozen and thawed sperm and reduced ($P<0.05$) ,the incidence of sperm polar damage and the levels of extracellular enzymes AST, ALT, ACP, ALP and LDH released into the extracellular medium. They added that the highest pregnancy rate ($P<0.05$) was observed in cows artificially inseminated with frozen bull sperm treated with a combination of 3.5% glycerol and 3.5% DMSO. Farshad *et al*. (2009) recognized that sperm motility, viability and fornix integrity after thawing were improved when using 1.75% DMSO in goat sperm enhancer.

CONCLUSION

It can be concluded that Tris Turmeric Ethylene glycol is considered the best agent to improve sperm survival and fertility, followed by Tris Turmeric and Tris Turmeric Dimethyl Sulfoxide.

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Table 1. Comparative study of Tris Turmeric, Tris Turmeric Dimethyl Sulfoxyde and Tris Turmeric Ethylene glycol extenders on frozen post-thawed extended buffalo bull semen (Mean±SE).

Diluent	Motility	Alive	Abnormalities	HOST	Acrosome	Viability index	Capacitation
Control (Ttris extender)	43.60±.97^{a}	85.4000±1.11^{b}	10.0000±.63^{b}	57.3000±.20^{a}	84.5000±1.59^{b}	85.93±0.93^{a}	22.67±1.20^{c}
TT Tris Turmeric (100 ML/5 ml Tris)	62.00±1.22^{c}	84.400±1.12^{ab}	7.3000±.37^{a}	80.8820±3.19^{c}	91.400±.40^{c}	145.00±0.57^{d}	18.33±.88^{a}
TTD Tris Turmeric Dimethyl Sulfoxyde (100 ML/5 ml Tris+1.5% DMSO)	57.60±1.12^{b}	81.4000±.60^{a}	11.5000±.22^{c}	79.2700±.64^{bc}	86.5400±.92^{b}	139.00±0.57^{c}	7.33±.67a
Tris Turmeric Ethylene glycol (100 ML/5 mlTris+1.5% EG)	62.00±1.22^{c}	82.4000±1.28^{ab}	10.3000±.20^{b}	75.1040±.98^{b}	70.4000±1.50^{a}	114.00±1.00^{b}	19.50±.50^{b}
P-value	0.0001	0.064 (NS)	0.0001	0.0001	0.0001	0.000	0.000

Means bearing different superscripts (a, b, c) within column differ at $P<0.05$; non significant (NS).

Control: Tris-citrate-fructose-egg yolk-glycerol (TCFYG); TT: Tris Turmeric (100 ML/5 mlTris); TTD: Tris+100 µl turmeric extract+1.5%DMSO; Tris Turmeric Ethylene glycol (100 ML/5 mlTris+1.5% EG).

Table 2. Effect of different extenders on post-thaw total motility % of frozen-thawed bull spermatozoa.

Hours	Control (tris extender)	Tris turmeric	Tris turmeric dimethyl sulfoxyde	Tris turmeric ethylene glycol	P-value
0	43.60±0.97^{a}	62.00±1.22^{c}	57.60±1.12^{b}	62.00±1.22^{c}	0.000
1	27.00±1.20^{a}	55.00±3.16^{b}	50.00±5.48^{b}	27.50±2.50^{a}	0.000
2	23.00±1.20^{a}	29.00±1.80^{ab}	27.00±3.74^{a}	35.00±2.04^{b}	0.032
3	16.00±2.50^{a}	30.00±6.30^{ab}	34.00±4.00^{b}	20.00±4.08^{ab}	0.039

Table 3. Effect of different extenders on antioxidant concentration-TAC (mM) and MDA concentration (μM).

Diluent	TAC	MDA
Control (tris extender)	0.22±0.02[b]	8.60±0.05[a]
Tris turmeric	0.29±0.00[c]	8.34±0.61[a]
Tris turmeric dimethyl sulfoxyde	0.14±0.01[a]	8.44±0.24[a]
Tris turmeric ethylene glycol	0.28±0.02[c]	7.55±0.11[a]
P-value	0.001	0.201

Table 4. Effect of different extenders on a field conception rate test in buffalo.

Treatment	No of inseminated	No of conceived	*In vivo* fertility rate (CR, %)
Control (TCFYG)	60	22	36.7 %
Tris turmeric	63	38	60.3%
Tris turmeric dimethyl sulfoxyde	68	38	55.9%
Tris turmeric ethylene glycol	69	45	65.2%

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