POSSIBILITY OF USING THE BOVINE SEMINAL PLASMA AS A DILUENT FOR CRYOPRESERVATION OF EGYPTIAN BUFFALO SEMEN

H. A. El-Nagar*

Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Dokki, Giza, Egypt, *Corresponding Author's E-mail: elnagar1970@hotmail.com, Cellular phone +201066315955

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SUMMARY

The seminal plasma separated from ejaculates of the same or different animal species has been used as an extender for semen preservation to minimize the negative effects of reduced temperatures and cryo-damage on mammalian spermatozoa. The semen of sexually-matured buffalo bulls (n=5) collected at 3-4 day-interval by artificial vagina was used in this study for a duration of 12 weeks. Spermatozoal mass motility was at least 70%. Semen was collected, pooled, diluted with bovine seminal plasma (BSP) (after adding egg-yolk, glycerin and anti-biotics) or Tris-extender at 3 dilution rates (1:10, 1:15 and 1:20), equilibrated at cool temperature (4-5^{o}C) for 2 h, frozen for one month at -196^{o}C (liquid nitrogen), and thawed at 37^{o}C for 15 s. Semen was evaluated for the percentages of progressive motility (PM), live sperm (LS), sperm abnormalities (SA), acrosome damage (AD), and membrane integrity (MI) in diluted, equilibrated and thawed cases, besides the head to head agglutination (HHA) percentage post-thawing. In the sperm medium of thawed semen, AST, ALT, LDH activities and total antioxidant activity (TAA) levels were also determined. Sperm fertilizability was recorded based on the best results for BSP or Tris-extender. Results showed that freezability parameters (PM, LS, SA, AD, MI and HHA percentages), the activity of AST, ALT and LDH, and TAA and conception rate were improved (P<0.05) for thawed buffalo semen diluted with BSP at a rate of 1:20 in comparison with Tris-extender at 1:10. The bovine seminal plasma of excluded ejaculates for poor quality could be considered as a promising successful extender for cryopreserved buffalo semen.

Keywords: Buffalo semen, bovine seminal plasma, freezability, antioxidant status, fertilizability.

INTRODUCTION

The development of assisted reproductive techniques, including artificial insemination (AI) and *in vitro* fertilization (IVF), are considered the most important means for improving reproductive efficiency and productivity in ruminants (Camargo *et al*., 2006; Srivastava and Kumar,2014). Freezing sperm and artificial insemination (AI) provides many advantages for the livestock industry. However, there are many problems during the conservation process which is the deadly and almost fatal damage to the structure of sperm, which leads to poor fertility of sperm preserved (Watson, 2000).

Seminal plasma (SP) is secreted by the accessory sex glands and mixed with spermatozoa during ejaculation. It is a buffered medium vehicle that is needed for the movement of spermatozoa in the genital tracts of the male and female. This fluid is a complex mixture of proteins, ions and organic substances of low molecular weight, such as free amino acids, monosaccharides, lipids, polyamines, prostaglandins and steroid hormones. Probably more important than components in the fluid are the seminal plasma-derived proteins that attach to sperm cell membranes (Katila and Kareskoski, 2006). The SP interacts with the secretions and the cells of the female genital tract after insemination. It is recognized to have long-lasting effects on sperm mobility (Maxwell *et al*., 2007**)**, capacitation (Manjunath *et al.,* 2007; Leahy and Gadella, 2011), storage of sperm cells in the female reproductive tract (Talevi and Gualtieri, 2010**),** and modulation of the immune response of female to tolerate sperm cells and the conceptus (Robertson, 2007).

The SP and sperm cells in mammals contain several macro- and micro-elements (Marzec-Wroblewska *et al*., 2012). These elements are important components for sperm preservation and fertilization. Elements such as Na, K, Ca, and Mg are essential for proper sperm cell functions; others like Zn, Cu, Mn, Co, Se, and Fe are required in relatively narrow limits (Massanyi *et al*., 2003; 2004). The influence of major biologically active inorganic components on spermatozoa viability parameters has been studied in animals (Massanyi *et al*., 2003). The SP contains ascorbic acid, which represents 65% of the antioxidant capacity. The concentration of this vitamin in SP is ≥10 times more than its blood plasma level (Tariq *et al*., 2015). Early reports (Banerjee and Ganguli, 1973; Reddy and Raja, 1979) reported that the content of ascorbic acid is significantly lower in buffalo semen than in cow bull semen, which might cause poor quality of preserved buffalo semen.

Semen extender is an extracellular sperm fluid which plays an important role as a diluent to transport and protect spermatozoa from cold shock during cryopreservation and artificial insemination. It must possess properties as isotonic (280-310 mOsm/kg), buffering (regulate pH), cryoprotectant, energy (sperm metabolism), antimicrobial, and fertility-preserving properties. Tris-based extenders are commonly used for cooled and frozen semen (Raheja *et al*., 2018). Tris-based extenders are commonly used for semen preservation in most farm animals including buffaloes (Purdy, 2006).

Better understanding of the properties of the extenders used is required for improving

cryopreservation of buffalo semen. During cryopreservation, preventing sperm cells from formation of lethal ice crystal which increases sperm membrane damage is the 1st aim of freezing protocols (El-Nagar *et al*., 2019). The semen extender must has a high dilution rate, nutrients for sperm cells, buffering capacity, balance ion and electrolytes and protectants against cold shock (El-Nagar, 2017).

Lately, there has been a lot of attention toward the role of reactive oxygen species (ROS) in sperm function and their ability to fertilize during conservation. During semen preservation, oxidative damage increases ROS generation, which has been reported to reduce motility and fertility of sperm cells (Roca *et al*., 2005). Since it is rich in polyunsaturated fatty acids, therefore, buffalo spermatozoa are more susceptible to oxidative damage compared to bull sperm (Nair *et al*., 2006). Bovine seminal plasma (BSP) contains proteins, minerals, electrolytes, hormones and enzymes (Poiani 2006), and has been shown to activate and support spermatozoa (Garner *et al*., 2001). Furthermore, BSP is implicated in sperm decapacitation and fertilization (Rodriguez-Martinez *et al*., 2011**)**. The SP of the same or other species, as an extender for cooled or frozen semen, has been reported to minimize the adverse effects of lowered temperature and cryo-damage on sperm cells of mammals (Baran *et al*., 2004**)**. It is well known that in AI centers, many of bull ejaculates were excluded for poor motility (≤70% initial motility). Therefore, the present study was suggested to investigate the potential beneficial usage of the seminal plasma of bovine semen as an extender in cryopreservation of buffalo semen after adding egg-yolk, glycerin and antibiotics recommended for Tris-extender at different dilution rates.

Therefore, this work aimed to investigate the effect of bovine seminal plasma, as an extender at different dilution rates on freezability and fertilizability of cryopreserved semen of Egyptian buffaloes compared with the common use of Tris-extender.

MATERIALS AND METHODS

Animals used in this study were provided from El-Gemmezah experimental Station, Gharbiya Governorate (Animal Production Research Institute, APRI, Agricultural Research Center, Egypt), during the interval from November 2019 till March 2020.

Semen donors:

Semen donors in this study included sexually mature and good healthy Egyptian buffalo-bulls (n=5). They were ranging from 400 to 455 kg of live body weight and 3 to 4 years old. They were individually kept under a semi-open system and received daily concentrate mixture (4 kg), clover berseem (35 kg, *Trifolium alexandrinum*) and rice straw (6 kg). The concentrate mixture contained 25% uncorticated cotton seed cake, 44% coarse wheat bran, 15% corn, 8.5% extracted rice bran, 3% molasses, 3% limestone and 1.5% common salt with the availability of drinking water all times.

Collection of semen:

Semen was ejaculated (twice/week) before feeding at 6-7 a.m. from all bulls (n=5) for 12 weeks by using a teaser and artificial vagina (IMV, France at 40°C). Immediately after the complete collection, semen was transported in a water bath (37°C) to the laboratory for evaluation and freezing processes only for ejaculates with mass motility of ≥70%. On the day of collection, semen of all bulls was pooled and extended with Tris-extender or bovine seminal plasma (BSP) at three dilution rates (1: 10, 1: 15 and 1: 20) for each.

Semen dilution:

Tris-based extender:

For preparation the Tris-egg yolk extender (TEY), Tris (3.63 g), fructose (0.5 g), citric acid (1.99 g), streptomycin (100 mg) and penicillin (100.000 IU) were dissolved in distilled water (100 ml). Egg yolk (10 ml), 7 ml pure glycerol, and vitamin (0.9 mg/ml) were added per 83 ml of the TEY. The value of pH and osmolarity level of the TEY was 6.8 and 280-300 mOsm/k H_2O.,-respectively by (El-Nagar, 2017).

Bovine seminal plasma extender:

Bovine seminal plasma (BSP) used as an extender in this study was prepared from semen collected from bulls and excluded for poor initial motility (≤70%) at the International Livestock Management Training Center (ILMTC), Sakha, belonging to APRI. Semen was centrifuged (4000 rpm for 15-20 min) and stored (-20°C) until used, as an extender. About 100 mg streptomycin and 100.000 IU penicillin were added to 100 ml of BSP, and then egg yolk (10 ml) and pure glycerol (7 ml) were added to 83 ml of BSP with antibiotics.

The prepared BSP was filtered by 0.22 μm millipore filter (milieux GV, millepore, Cooperation Bedford MOA). The pH value of the medium was adjusted to 7.1-7.3 using pH-meter and to the osmolarity of 280-300 mOsmol/kg H_2O using osmometer (Micro-Osmometer, Loser Type 6, Germany).

Semen freezing and thawing processes:

The collected semen was diluted with Tris-extender or BSP at rates of 1: 10, 1: 15 and 1: 20. The extended semen (for each treatment) was packaged in French straws (0.25 ml), sealed with polyvinyl alcohol powder, and then straws were equilibrated (at 5°C for 4 h), were frozen in liquid nitrogen (LN) vapor (at a level of 5 cm above liquid nitrogen surface for 10 min) and then plunged and stored into LN for one month. Frozen straws were thawed at 37 °C for 30 s in a water bath for evaluation and insemination (El-Nagar, 2017) .

Evaluation of semen:

In post-diluted, equilibrated and thawed semen extended with BSP or Tris, sperm progressive motility, live sperm**,** abnormal sperm and acrosomal damage were determined according to Amann and Hammerstedt (1980), Hackett and Macpherson (1965), Blom (1983) and Yanagimachi (1982), respectively. However, the percentage of head to

head agglutination was determined only in post-thawed semen (Senger and Saacke, 1976). Sperm membrane integrity was performed by determining the percentage of sperm with a curled tail at 50 mOsm/kg H_2O for 30 min according to El-Sherbieny (2004).

Enzyme and total antioxidant activity in sperm medium:

After thawing, semen was centrifuged at 3500-4000 rpm for 15 minutes, then sperm medium was separated and stored at -20°C till determination of enzyme activity and total antioxidant activity. Activity of transaminases (aspartate, AST and alanine, ALT), and lactic dehydrogenase (LDH) (Young (1990), as well as total antioxidant activity (TAA, Koracevic, *et al*., 2001), were determined in sperm medium. Enzyme and TAA activities were determined using commercial kits (Salucea, Netherlands) and spectrophotometer (JENWAY-6405UV/Vis).

Fertility trial:

After a synchronization of 35 sexually mature buffaloes, a total of 26 oestrous synchronized buffaloes came into heat were used for fertility trail. These buffaloes (n=26) were randomly allotted to 2 groups (13 animals in each). Buffaloes were synchronized by i.m. injection with 3 ml Estrumate/animal (PGF2α, Essex Animal Health Friesoythe, Germany) and estrus was observed. Animals came into heat within 48-72 h after the 1st and 2nd Estrumate injection were artificially inseminated with semen extended with TEY (n=13) or BSP (n=13) according to the obtained results. On the day of AI, semen straws were thawed using filled plastic AI gun close to the cervix. All inseminations were done by the same inseminator. The inseminated animals were palpated via the rectum for pregnancy diagnosis 45-50 days post-AI.

Statistical analysis:

The statistical analysis comprised factorial ANOVA (Two extenders x 3 dilution rates) within SPSS (2013) program was used to study the effect of type of the extender, dilution rate and their interaction on sperm parameters, enzyme activities, and total antioxidant activity. While, the differences between the extenders were performed using T test. The significant effects at least at P<0.05 were tested (Duncan, 1955**)**. The percentage values were subjected to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from the transformed values to percentages. Fertility data were statistically analyzed by Chi-square student test.

RESULTS

Sperm characteristics after dilution:

Sperm characteristics, including sperm progressive motility (PM), sperm abnormality (SA), acrosomal damage (AD) and membrane integrity (MI) percentages in post-diluted semen, were significantly (P<0.05; P<0.01) improved by BSP in comparison with Tris-extender, while LS percentage was not affected by extender type. However, only percentages of PM and MI were affected significantly (P<0.05) by dilution rate, being the highest with a dilution rate of 1:20. The interaction effect between extender type and dilution rate on all sperm characteristic studied was highly significant (P<0.001), reflecting the best results of dilution of buffalo semen with BSP at a rate of 1:20, followed by those diluted with Tris-extender at a rate of 1:10 (Table 1).

Table 1. Sperm characteristics (M±SE) in post-diluted buffalo semen as affected by type of extender, dilution rates and their interaction

Item	Sperm characteristics (%)				
	PM	LS	SA	AD	MI
Effect of extender type (E)					
Tris	68.04±0.64^{b}	71.08±0.73	16.93±0.60^{a}	18.61±0.66^{a}	64.10±0.73^{b}
BSP	69.83±0.84^{a}	72.56±0.76	15.40±0.63^{b}	16.67±0.65^{b}	66.66±0.81^{a}
P-value$^{(sign,)}$	0.029*	0.068^{NS}	0.038*	0.011**	0.002**
Effect of dilution rate (R)					
R1 (1: 10)	67.54±0.85^{b}	71.42±0.92	16.31±0.70	18.06±0.75	63.92±0.86^{b}
R2 (1: 15)	69.08±0.71^{ab}	71.89±0.78	16.02±0.68	16.89±0.69	66.02±0.83^{a}
R3 (1: 20)	70.19±1.13^{a}	72.15±1.05	16.16±0.90	17.96±0.98	66.21±1.15^{a}
P-value	0.030*	0.751^{NS}	0.948^{NS}	0.382^{NS}	0.048*
Effect of interaction (E x R)					
Tris x R1	71.13±0.93	75.67±1.10	13.58±0.82	15.17±0.96	67.50±1.05
Tris x R2	68.83±0.93	71.42±1.03	16.46±0.92	17.67±0.92	65.21±1.28
Tris x R3	64.17±0.97	66.16±0.86	20.75±0.86	23.00±0.93	59.58±0.88
BSP x R1	63.96±0.98	67.17±0.83	19.04±0.85	20.95±0.80	60.33±0.88
BSP x R2	69.33±1.08	72.37±1.18	15.58±1.03	16.12±1.03	66.83±1.07
BSP x R3	76.21±1.05	78.12±0.81	11.58±0.86	12.91±0.91	72.83±0.89
P-value	***	***	***	***	***

Means (M) in the same column for each factor with different superscripts differ significantly (P<0.05). *** Significant at P<0.001. ** Significant at P<0.01. * Significant at P<0.05. NS: Non-significant. BSP: Bovine seminal plasma. PM: Progressive motility. LS: Live sperm. SA: Sperm abnormality. AD: Acrosomal damage. MI: Membrane integrity.

Sperm characteristics after equilibration:

Semen diluted with BSP significantly increased PM, LS, and MI percentages, while significantly decreased AD percentage in post-equilibrated compared with Tris-extender. The percentage of SA was not affected significantly by the extender. However, only percentages of LS, AD and MI were affected significantly (P<0.05) by dilution rate, being better with a dilution rate of 1:15 or 1:20 than with 1:10. Also, the interaction effect between extender and dilution rate was highly significant (P<0.001) on all sperm characteristic studied, reflecting the best results of equilibration after dilution of buffalo semen with BSP at a rate of 1:20, followed by those diluted with Tris-extender at a rate of 1:10 (Table 2).

Table 2. Sperm characteristics(M±SE) in post-equilibrated buffalo semen as affected by type of extender, dilution rates and their interaction

Item	Sperm characteristics (%)				
	PM	LS	SA	AD	MI
Effect of extender (E)					
Tris	63.51±0.71^{b}	66.82±0.71^{b}	19.13±0.64	22.24±0.64^{a}	60.50±0.72^{b}
BSP	67.58±0.83^{a}	69.32±0.91^{a}	17.92±0.70	18.50±0.72^{b}	64.83±0.96^{a}
P-value	0.0001***	0.002**	0.093^{NS}	0.0001***	0.0001***
Effect of dilution rate (R)					
R1 (1: 10)	64.33±0.80	65.98±0.94^{b}	19.08±0.75	21.56±0.70^{a}	60.48±0.87^{b}
R2 (1: 15)	66.15±0.75	69.31±0.76^{a}	18.04±0.64	19.35±0.73^{b}	63.92±0.81^{a}
R3 (1: 20)	66.17±1.31	68.92±1.23^{a}	18.44±1.04	20.19±1.12^{ab}	63.60±1.41^{a}
P-value	0.081^{NS}	0.0001***	0.722^{NS}	0.045*	0.0001***
Effect of interaction (E x R)					
Tris x R1	67.71±0.91	70.50±0.99	15.91±0.85	18.79±0.77	64.46±0.93
Tris x R2	64.63±1.02	68.37±0.98	17.29±0.81	21.25±0.99	62.08±0.95
Tris x R3	58.21±0.88	61.58±0.90	24.17±0.88	26.67±0.90	54.96±0.94
BSP x R1	60.96±0.88	61.46±0.94	22.25±0.85	24.33±0.86	56.50±0.91
BSP x R2	67.67±1.04	70.25±1.16	18.79±0.98	17.46±0.95	65.75±1.22
BSP x R3	74.12±0.83	76.25±0.81	12.71±0.88	13.71±0.83	72.25±0.86
P-value	0.0001***	0.0001***	0.0001***	0.0001***	0.0001***

Means (M) in the same column for each factor with different superscripts differ significantly (P<0.05). BSP: Bovine seminal plasma. PM: Progressive motility. LS: Live sperm. SA: Sperm abnormality. AD: Acrosomal damage. MI: Membrane integrity. *** Significant at P<0.001. ** Significant at P<0.01. * Significant at P<0.05. NS: Non-significant.

Sperm characteristics after thawing:

Sperm characteristics, including percentages of PM, LS, SA, AD, MI and HHA, were significantly (P<0.001) improved after cryopreservation/thawing in semen diluted with BSP in comparison with Tris-extender. Also, percentages of all sperm characteristics were affected significantly (P<0.001) by dilution rate, being better with a dilution rate of 1:15 than with 1:10 or 1:20. The interaction effect (extender type x dilution rate) was significant (P<0.05; P<0.01; P<0.001) on all sperm characteristics studied, reflecting the best results of semen cryopreservation after dilution of buffalo semen with BSP at a rate of 1:20 (Table 3).

Table 3. Sperm characteristics (M±SE) in post-thawed buffalo semen as affected by type of extender dilution rates and their interaction

Item	Sperm characteristics (%)					
	PM	LS	SA	AD	MI	HHA
Effect of extender (E)						
Tris	54.67±0.79^{b}	57.50±0.87^{b}	28.48±0.79^{a}	29.68±0.82^{a}	51.29±0.85^{b}	50.76±0.64^{b}
BSP	59.98±0.93^{a}	62.88±0.96^{a}	24.58±0.79^{b}	25.84±0.79^{b}	57.01±0.86^{a}	54.47±0.81^{a}
P-value	0.0001***	0.0001***	0.0001***	0.0001***	0.0001***	0.0001***
Effect of dilution rate (R)						
R1 (1: 10)	55.08±0.85^{b}	58.91±0.89^{b}	27.39±0.81^{a}	28.62±0.86^{a}	53.52±0.77^{b}	51.35±0.78^{b}
R2 (1: 15)	59.31±0.80^{a}	61.37±0.81^{a}	24.66±0.68^{b}	25.58±0.69^{b}	55.68±0.76^{a}	53.22±0.72^{a}
R3 (1: 20)	57.58±1.50^{a}	60.29±1.66^{ab}	27.54±1.36^{a}	29.08±1.34^{a}	53.25±1.60^{b}	53.27±1.20^{a}
P-value	0.0001***	0.028*	0.002**	0.0001***	0.020**	0.051*
Effect of interaction (E x R)						
Tris x R1	58.87±0.98	63.54±0.77	23.54±0.77	24.08±0.76	56.75±0.79	54.83±0.75
Tris x R2	56.92±1.07	59.38±1.12	26.08±0.88	27.63±0.92	53.92±0.84	51.29±0.94
Tris x R3	48.21±0.89	49.58±0.81	35.83±0.91	37.33±0.83	43.21±0.93	46.17±0.82
BSP x R1	51.29±0.86	54.29±0.87	31.25±0.88	33.17±0.79	50.29±0.93	47.88±0.92
BSP x R2	61.71±0.98	63.38±1.04	23.25±0.97	23.54±0.87	57.46±1.18	55.17±0.97
BSP x R3	66.96±0.90	71.00±0.79	19.25±0.85	20.83±0.85	63.29±0.94	60.37±0.91
P-value	0.0001***	0.0001***	0.0001***	0.0001***	0.0001***	0.0001***

Means (M) in the same column for each factor with different superscripts differ significantly (P<0.05).***Significant at P<0.001.
** Significant at P<0.01. * Significant at P<0.05. BSP: Bovine seminal plasma. PM: Progressive motility. LS:Live sperm. SA:Sperm abnormality. AD:Acrosomal damage. MI: Membrane integrity. HHA: Head to head agglutination.

Activity of enzymes and total antioxidant in sperm medium of cryopreserved semen:

The activity of AST, ALT and LDH was significantly (P<0.01; P<0.001) reduced, while total antioxidant activity (TAA) was significantly (P<0.001) improved in sperm medium of cryopreserved semen diluted with BSP in comparison with Tris-extender. On the other hand, the activity of AST and LDH as well as TAA level was significantly (P<0.05; P<0.001) improved at a dilution rate of 1: 15, 1: 20, and 1: 10, respectively, as compared to other dilution rates. The activity of ALT was not affected significantly by the dilution rate. The observed significant (P<0.001) interaction (extender type x dilution rate) on the activity of enzymes and TAA reflected the best results of cryopreserved semen after dilution with BSP at a rate of 1:15 or 1:20 (Table 4).

Table 4. Enzyme and total antioxidants activity (M±SE) in post-thawed buffalo semen as affected by type of extender, dilution rates and their interaction

Item	Enzyme activity (IU/l)			Total antioxidants activity (mmol/l)
	AST	ALT	LDH	
Effect of extender (E)				
Tris	27.85±0.79^{a}	19.86±0.75^{a}	234.36±1.79^{a}	2.18±0.07^{b}
BSP	24.61±0.81^{b}	18.09±0.83^{b}	224.29±2.07^{b}	2.41±0.09^{a}
P-value	0.0001***	0.019**	0.0001***	0.0001***
Effect of dilution rate (R)				
R1 (1: 10)	27.69±0.80^{a}	20.02±0.85	230.35±1.65^{a}	2.23±0.07^{b}
R2 (1: 15)	24.33±0.63^{b}	18.25±0.67	230.02±0.78^{a}	2.28±0.04^{ab}
R3 (1: 20)	26.67±1.37^{a}	18.66±1.30	227.60±3.89^{b}	2.38±0.15^{a}
P-value	0.0001***	0.054^{NS}	0.033*	0.018*
Effect of interaction (E x R)				
Tris x R1	24.00±0.83	15.96±0.82	220.50±1.01	2.66±0.07
Tris x R2	24.50±0.95	17.29±0.94	228.91±0.99	2.46±0.03
Tris x R3	35.04±0.86	26.33±0.96	253.66±1.28	1.43±0.04
BSP x R1	31.37±0.86	24.08±0.91	240.21±1.29	1.79±0.04
BSP x R2	24.16±0.86	19.21±0.92	231.12±1.19	2.09±0.05
BSP x R3	18.29±0.91	11.00±0.92	201.54±1.01	3.34±0.08
P-value	0.0001***	0.0001***	0.0001***	0.0001***

Means (M) in the same column for each factor with different superscripts differ significantly (P<0.05). *** Significant at P<0.001. ** Significant at P<0.01. * Significant at P<0.05. NS: Non-significant. BSP: Bovine seminal plasma. AST: Aspartate transaminase. ALT: Alanine transaminase. LDH: Lactic dehydrogenase.

Fertility rate:

The pregnancy rate of estrus synchronized buffalo cows and artificially inseminated with cryopreserved semen extended with Tris-extender at a rate of 1:10 was compared with those extended with BSP at a rate of 1:20 based on the best results of both types of extenders after dilution, equilibration and thawing. Results revealed that pregnancy rate was higher for buffalo cows inseminated with post-thawed semen extended with BSP (11/13, 84.61%) than those inseminated with Tris-extender (10/13, 76.92%), but the difference was not significant (Fig. 1).

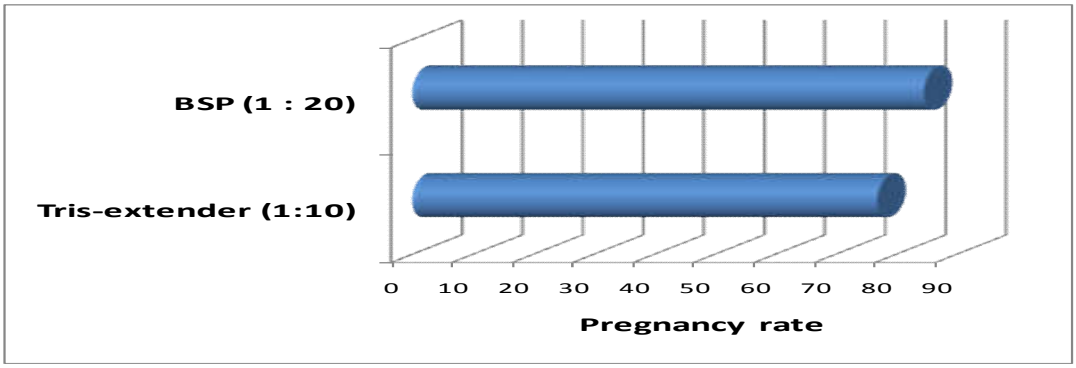

Fig. 1. Pregnancy rate of buffalo cows artificially inseminated with semen diluted with Tris-extender or bovine seminal plasma.

DISCUSSION

Semen extender or diluent is a chemical medium used for preservation, extension and protection of sperm cells against various shocks during processing, storage and transportation used for AI. Diluent properties such as isotonic (280-310 mOsm/kg H_2O), buffering capacity (regulate pH value), cold shock protection, energy source (sperm metabolism), control microbial contamination, protection during freezing and thawing and capability of preserving spermatozoa fertility must be encompassed in any extender used. Generally, Tris-extender is the predominantly one used for semen preservation for different species and for freezing bull semen (Raheja *et al*., 2018) and in buffaloes (Purdy, 2006).

This study aimed to evaluate the freezability and fertilizability of buffalo semen diluted with bovine seminal plasma (BSP), as an extender at different dilution rates, compared with Tris-based extender. The results of the present study revealed many positive aspects of sperm characteristics. Buffalo sperm cells were reported to be preserved with better quality by substitution of buffalo seminal plasma with half the volume of BSP before semen extension (Sahni, 1990; Lodhi *et al*., (1998**)**. The observed increase in the proportion of live sperm in half seminal plasma substituted semen may be due to proper molarity of the mixed seminal plasma than complete one (Lodhi *et al*., (1998**)**. Also, a proportion of 20% seminal plasma (dilution ratio of semen: extender of 1:4) did not have any negative effects on sperm characteristics of equine during storage for 24 or 48 h (Rigby *et al*., 2001**)**. The supplementation of BSP to extender before freezing of washed Angora goat semen had beneficial effects on post-thaw sperm parameters **(**Cagin Umut and Daskin, 2010). The seminal plasma at levels of 20-40% protected sperm motility in cryopreserved ram semen (Mata-Campuzano *et al*., 2015 a**).** In sheep, using 1-10% seminal plasma of rainbow trout improved quality of ram semen incubated for 5 hours at 37^{o}C (Ustuner *et al*., 2016**)**. Several authors indicated that motility, viability, acrosome integrity, capacitation and mitochondrial respiratory activity were improved in post-thawed ram semen by the addition of seminal plasma **(**Leahy *et al*., 2010; Bernardini *et al*., 2011; Rovegno *et al*., 2013**)**. On the contrary, the partial substitution of buffalo seminal plasma with BSP provided no beneficial effects **(**Sianturi *et al*., 2012**)**.

These beneficial results of using BSP as an extender may be attributed to that BSP may act as a buffer, facilitator of sperm transport and sperm movement **(**Maxwell *et al*. 2007**)**, capacitated factor (Manjunath *et al*. 2007; Leahy and Gadella 2011**)**, storage of sperm cells in the female genital tract **(**Talevi and Gualtieri 2010**)** and modulator of the immune response of female tolerance to sperm cells and the conceptus **(**Robertson, 2007**)**. Seminal plasma is a complex mixture of proteins, ions and organic substances of low molecular weight, such as free amino acids, monosaccharides, lipids, polyamines, prostaglandins and steroid hormones. Probably more important than components in the fluid are the seminal plasma-derived proteins that attach to sperm cell membranes **(**Katila and Kareskoski, 2006**).** Also, BSP contains proteins, electrolytes, hormones, and enzymes (Poiani, 2006**)** required for sperm activity and mobility of spermatozoa **(**Garner *et al*. 2001**)**, and fertilization **(**Rodriguez-Martinez *et al*.**, 2011)**. Different macro- and microelements in the seminal plasma and sperm cells in mammalian semen were reported by Marzec-Wroblewska *et al*., (2012). Impact of elements such as Zn, Mg, Se, and Ca on mobility, and morphology was reported by Eghbali *et al*., (2008**)** and Atig *et al*. (2012**)**. It is of interest to note that dilution of buffalo semen with BSP showed significantly ($P<0.05$) positive impact on increasing level of TAA in sperm medium of thawed semen, which may suggest the presence of antioxidant in BSP. Roca *et al*., (2005**)** reported that ROS resulting from oxidative stress plays a vital role in reducing sperm function and their fertilizing ability because the sperm cell membrane is rich in polyunsaturated fatty acids. Therefore, sperm in buffalo are more susceptible to oxidative damage compared to bull sperm **(**Nair *et al*., 2006**).**

The seminal plasma contains some compounds (proteins, enzymes and non-enzyme antioxidants) to protect the sperm cells from ROS and prevent sperm capacitation in ram semen **(**Van Overveld *et al*., 2000; Maxwell *et al*., 2006**)**. Therefore, many authors indicated that the seminal plasma is a good protectant of sperm cells from ROS formation (Agarwal, *et al*., 2004; Tvrda, *et al*., 2011**)**. Ascorbic acid (AA) represents 65% of the antioxidant capacity in the seminal plasma, being higher in the seminal plasma 10 times (364 vs. 40-µmol/L) than in blood plasma (Tariq *et al*., 2015**)**. It was reported that the content of AA in cow bull semen is greater than that in buffalo bull semen (Banerjee and Ganguli, 1973; Reddy and Raja, 1979**)**. This may explain better cryopreservation of buffalo semen extended with BSP in comparison with Tris-extender.

It is worth noting that improvement in enzyme activity (AST, ALT and LDH) in sperm medium of cryopreserved semen was associated with improving all sperm function, particularly sperm membrane integrity and in antioxidant status of sperm medium after thawing. The seminal enzymes play a crucial role in sperm fertility. Enzyme activities are influenced by freezing. For judging the freezability and fertilizing ability of sperm cells, the assessment of transaminases and dehydrogenases activities is essential in the seminal plasma (White, 1958**)**.

Regarding different dilution rates, the obtained results indicated the best results of using BSP when buffalo semen was extended at a rate of 1: 20 versus 1: 10 for Tris-extender. Early researchers employed dilution rates ranging from 11 to 26-fold, which are significantly higher than the 2–5-fold dilution rates

commonly used today (Purdy, 2006**)**. Historically, semen samples in farm animals have been diluted by either diluting semen with specific volumes of diluents or by diluting semen to a specific spermatozoa concentration. Dilution rates of 1:1–1:23 (v/v; semen to diluent) have been used successfully **(**Evans and Maxwell, 1987; Ritar *et al*., 1990a, b**)**. In the present study, dilution of semen at a rate from 1: 10 to 1: 20 indicated a sperm concentration from 400 to 100 $x10^6$/ml, which is within reports of sperm being successfully frozen, and reasonable fertility being achieved, have been obtained with samples ranging from 80 to 500 x 10^6 cells/ml (Ritar *et al*., 1990a, b; Karatzas *et al.,* 1997**)**. In agreement with the high dilution rate of buffalo semen, Akcay *et al*. (2012**)** suggested a marked increase in motility, acrosomal damage and membrane integrity in thawed ram semen diluted at high rates or by decreasing sperm concentration per insemination. D'Alessandro *et al*. (2001) also found a positive effect of high pre-freeze extension of ram spermatozoa, where the highest post-thaw motility and acrosome integrity were observed when semen was diluted to 200 million spermatozoa per ml and lowest after dilution to $800x10^6$/ml. As such, the obtained results proved the successful use of BSP, as an extender, for buffalo semen cryopreservation in terms of sperm freezability. Concerning the fertilizing ability, pregnancy rate of buffaloes inseminated with cryopreserved semen extended with BSP at a rate of 1: 20 was higher than the control semen (extended with Tris at a rate of 1: 10), but the difference was not significant (84.61 vs. 76.92%, $P \geq 0.05$). These results are in parallel with the freezability of both types of semen.

In general, the previous findings revealed that the pregnancy rate was associated with improved sperm parameters, antioxidant status and activity of AST, ALT and LDH in post-thawed buffalo semen. Similarly, El-Nagar (2017**)** recorded a higher pregnancy rate of buffaloes following AI with semen diluted with Tris-extender supplemented with vitamin C as compared to Tris-based extender without supplementation. Also, Lodhi *et al*., (1998**)** found improved fertility of buffalo sperm cells by substituting the half of BSP. The fertility rates of The 30-50% v/v of homologous seminal plasma added to milk-egg yolk extenders for ram semen used as liquid-stored (5 to 15 °C) or frozen-thawed was improved (Belibasaki *et al*., 2000; Lopez-Perez and Perez-Clariget 2012**).** The pregnancy rate of ewes was increased by cervical AI with cryopreserved ram semen supplemented with 40% boar seminal plasma **(**Fang *et al*., 2018**).** As found in the present study BSP added to ram semen improved pregnancy rate of ewes following AI **(**Gunay *et al*., 2006**).**

CONCLUSION

In conclusion, using BSP, as a diluent, in semen cryopreservation of buffalo semen significantly improved freezability and fertilizability in comparison with Tris-Extender. The bovine seminal plasma of excluded ejaculates for poor quality could be considered as a promising successful extender for cryopreserved buffalo semen following the addition of egg-yolk, glycerin and anti-biotics as in Tris-extender. From the economic point of view, it is cheaper than Tris-extender (3.60 vs. 11.85 L.E./100 ml).

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إمكانية استخدام بلازما السائل المنوي البقري كمخفف في حفظ السائل المنوي بالتجميد في الجاموس المصري

حمدي عبد الله النجار

قسم بحوث البيوتكنولوجيا، معهد بحوث الإنتاج الحيوانى،مركز البحوث الزراعية، الدقى، الجيزة، جمهورية مصر العربية.

تم استخدام البلازما المنوية المنفصلة من القذفات المنوية من نفس الأنواع الحيوانية أو المختلفة كمخفف للحفاظ على السائل المنوي لتقليل التأثيرات السلبية لخفض درجات الحرارة والضرر الناجم عن التبريد على الحيوانات المنوية للثدييات. في هذه الدراسة تم إستخدام قذفات السائل المنوي لعدد خمس طلائق جاموسى ناضجة جنسياً التي تم جمعها في فترة ٣-٤ أيام عن طريق المهبل الإصطناعي لمدة ١٢ أسبوع. كانت الحركة الكلية للسائل المنوي ٧٠٪ على الأقل. تم جمع السائل المنوي، وخلطه، وتخفيفه ببلازما السائل المنوي البقري (بعد إضافة صفار البيض، والجليسرين والمضادات الحيوية) أو مخفف الترس عند ثلاث ٣ معدلات تخفيف (١:١٠ ، ١:١٥ و ١:٢٠)، فترة إتزان (٤ - ٥ درجة مئوية) لمدة ساعتين، تجميد لمدة شهر عند -١٩٦ درجة مئوية (نيتروجين سائل)، وإسالة عند ٣٧ درجة مئوية لمدة ١٥ ثانية. تم تقييم السائل المنوي للنسب المئوية للحركة التقدمية (PM)، الحيوانات المنوية الحية (LS)، الحيوانات المنوية الشاذة (SA)، تلف الأكروسوم (AD)، سلامة الغشاء (MI) بعد التخفيف، الاتزان والاسالة، بالإضافة إلى تقابل الرأس بالرأس (HHA) بعد الاسالة. تم تحديد مستويات AST و ALT و LDH وإجمالي مستويات نشاط مضادات الأكسدة (TAA) في بلازما السائل المنوي بعد الاسالة. تم تسجيل القدرة الاخصابية للحيوانات المنوية بناءً على أفضل النتائج لـمخفف بلازما السائل المنوي البقري أو الترس. أظهرت النتائج أن مؤشرات التجمد (PM، LS، SA، AD، MI و HHA)، نشاط AST، ALT، LDH و TAA ومعدل الحمل تم تحسينها ($P < 0.05$) بالنسبة لعينات السائل المنوي الجاموسي المذابة المخففة مع بلازما السائل المنوي البقري عند معدل تخفيف ١:٢٠ بالمقارنة مع الترس عند ١:١٠. تعتبر البلازما المنوية البقريه المنفصلة عن القذف المستبعدة للجودة السيئة مخففا ناجحًا واعدًا في حفظ السائل المنوي بالتجميد في الجاموس المصري.