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EFFECT OF DIFFERENT CONCENTRATION OF TREHALOSE OR SUCROSE TO TRIS DILUENTS ON SOME PROPERTIES OF SPERMS FOR HOLSTEIN BULLS DURING DILUTION, COOLING AND FREEZING

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Abstract

Objective of study to improve semen preserve ability through the use of trehalose/ sucrose sugars as extender additive expressed in frozen semen and education effect steps of freezing on sperm properties. The research trail was carried on the semen samples collected from 4 bulls, were diluted with Tris-based extender containing different trehalose/ sucrose concentrations (*viz*. 50, 100, 150, 200)mm and control, evaluated for semen characteristics at steps of freezing (after dilution, after cooling and Post-Thawing).

Results clearly indicated that, 100mm trehalose/ sucrose groups had significantly ($P<0.05$) higher individual motility in comparison to the 50mm, 150mm, 200mm and control group. Moreover, the sperm dead and abnormality was significantly ($P<0.05$) lower, when compared to the control group. The freezing process negatively affects ($P < 0.05$) the sperm parameters (individual motility, dead and abnormality), but the current study revealed that this effects were changes from treatment to another's, it means there is interaction between effect steps of freezing and addition trehalose/ sucrose to Tris diluents of bull spermatozoa. In conclusion**,** the addition of 100 mm trehalose and sucrose to TFEG diluents had their benefits on freezing-thawing bull semen. A step of freezing process was detrimental to bull sperm properties, but this effect was less when adding 100mm trehalose to the diluted.

Key words : Trehalose, sucrose, freezing steps, bull, sperm properties.

Introduction

Artificial insemination (AI) used in a proper way to increases the breeding capacity of the males, permitting a higher degree of selection and an extended use of animals with a high breeding value as well as reducing the risk of spreading infectious (Saacke *et al*., 1994; El-Sheshtawy *et al*., 2015). It is well known that the composition of the extender, suitable cryoprotectants and optimal freezing and thawing rates are important factors for successful semen cryopreservation (Woelders *et al*., 1997; Malo *et al*., 2010). The quality of frozen semen is the most influencing factor for conception rate (Saacke *et al*., 1994). It has been reported that cryopreservation process leads to the generation of reactive oxygen species (ROS) that impair sperm motility, membrane integrity and fertilizing ability (Bilodeau *et al*., 2000; Hu *et al*., 2010). These changes are due to oxidative and osmotic stresses (Hammerstedt *et al*., 1990; Watson, 1995). Trehalose and sucrose are non-penetrating disaccharides that seems to protect cells both by increasing the tonicity of the extender and by stabilizing the plasma membrane, possibly due to direct interaction with phospholipid polar head groups of membrane phospholipids (Crowe *et al*., 1987). Trehalose seems to be more efficient than other sugars for protection of spermatozoa in cryopreservation media, and many authors have reported its beneficial effect for semen cryopreservation in different species, such as bull (Sitaula *et al*., 2009; Tuncer *et al*., 2011), buffalo bull

(Reddy *et al*., 2010, Badr *et al*., 2010), ram (Molinia *et al*., 1994; Aisen *et al*., 2002), goat (Aboagla and Terada, 2003; Tuncer *et al*., 2013), rabbit semen (Dalimata and Graham, 1997). Disaccharides are effective in stabilizing biomembrane bilayers and the sperm metabolism can be better sustained in diluents containing degradable sugar (Aisen *et al*., 2005). Lactose, sucrose, raffinose, trehalose and dextrans are not able to diffuse across the plasma membrane, creating an osmotic pressure that induces cell dehydration and a lower incidence of intracellular ice formation. These sugars interact with phospholipids in the plasma membrane, increasing sperm survival to cryopreservation (Aisen *et al*., 2002). The addition of high concentrations of trehalose to sperm extender provides the best protection with regard to post-thaw motility parameters, recovery rates, thermal resistance, and acrosome integrity. This disaccharide increases membrane fluidity before freezing, leading to greater resistance of spermatozoa against freeze thawing damage (Aboagla and Terada, 2003). On the other hand, addition of sucrose and trehalose for freezing of bull semen resulted in an improvement of the sperm survival (Woelders *et al*., 1997). This study aimed to examine the effect of different concentrations of trehalose or sucrose (50 or 100 or 150 or 200 mm) on diluted, cooled and post-thawed quality of bull semen, preserved in Tris-fructose-egg yolk-glycerol (TFEG).

Materials and Methods

Semen collection and initial evaluation

This study was carried out at the Artificial Insemination Center, Abou-Ghareeb Western of Baghdad, on (4) four Holstein bull born in Iraq, all bulls age (3-4 year) old and they were kept under identical conditions of management, feeding and watering. Semen was collected from bulls weekly with the aid of an artificial vagina method, immediately after collection, semen placed in water bath at 37ºC until their assessment in the laboratory, ejaculates of semen with more than (55) percent initial motility was used for the trail.

Semen processing

Each semen samples were split further into 10 equal aliquots and each one was diluted with Tris-Fructose-Egg Yolk-Glycerol (TFEG) freezing extender containing different trehalose (T_1- 50mm, T_2- 100mm, T_3- 150mm and T_4- 200mm/L) and sucrose (T_1- 50mm, T_2- 100mm, T_3- 150mm and T_4- 200 mm/L) concentrations and no additive C1-(control) so as to have a final sperm concentration of 80 million sperms per ml, cooled slowly 1.5-2 hr. up to 5ºC and equilibrated for 4hrs, semen was packed into 0.25 ml polyvinyl French straw (IMV, France) after equilibration periods, the straw were placed horizontally on a rack and frozen in a vapor 4cm above liquid nitrogen (LN2) for (9 min) and were then dipped in liquid LN2.

Semen quality assessment

These assessments were undertaken on fresh semen, after dilution, after cooling and post- thawing of bull spermatozoa, frozen straws were thawed at 37ºC for 30 seconds in a water bath for evaluation, the parameter studies were the sperm individual motility, dead and sperm abnormality percentage. Individual motility was subjectively evaluated using the standard method described by (Bearden and Fuquay, 2000). The dead and sperm abnormality were calculated using eosin-nigrosin stain as per the method described by (Evans and Maxwell, 1987).

Statistical analysis

Statistical analysis was performed according to (SAS, 2012), followed by (Duncan test) to determine significant differences in all the parameter among all groups ($P< 0.05$) was considered statistically significant.

Results

The effect of trehalose or sucrose and steps of freezing on individual motility percentage of bull sperms

The effect addition trehalose and sucrose on individual motility percentage of bull sperm frozen in liquid nitrogen mentioned in table 1, in which, as comparing between treatments within each step showed:

After dilution, the individual motility percentage of sperm in T2 (70.83%) appears significantly ($p<0.05$) higher than T1, T3, T4, S1, S3, S4 and C (61.94, 56.94, 53.33, 51.83, 52.72, 45.00 and 63.33%) respectively, while the treatment S4 (45.00%) appears significantly ($p<0.05$) lower than other treatment, but it recorded no significant differences among T2 and S2 (70.83 and 66.38%) respectively and between C, S2 and C1, T1 and T3, T4, S3 and T4, S1, S3, respectively. After cooling, sperm motility percentage in treatments T2 (66.38%) showed significantly ($p> 0.05$) more than C, T1, T3, T4, S1, S2, S3 and S4 (53.77, 57.33, 45.00, 45.50, 43.61, 59.44, 46.05 and 36.66%), respectively, but the treatment S4 (36.66%) appear significant ($p<0.05$) less than other treatments, whereas no significant differences between T1 with S2 and between T3, T4, S1 to S3. At post thawing steps individual motility percentage of sperm in T2 (65.55%) observed superior significantly ($p>0.05$) than other treatment T1 (53.33%), T3 (46.66%), T4 (46.61%), S1

(41.11%), S2 (59.77%), S3 (41.21%), S4 (36.94%) and C (52.55%), whereas no significant differences between C1 and T1, T3 and T4, S1 and S3. Present study shows that cooling and post–thawing caused decrease significantly($p>0.05$) in individual motility compare with after dilution in C, T2, T3, T4, S1, S2, S4 (Table 1) and in T1, S3 individual motility during post thawing less significantly ($p>0.05$) compare with after cooling and dilution were the differences between them significantly ($p>0.05$), but the different no significant between cooling and post thawing for C, T2, T3, T4, S1, S2, S4.

The overall mean of individual motility percentage of sperms notice that the treatment T2 (67.59%) was significantly ($p> 0.05$) higher than T1 (57.53%), T3 (49.53%), T4 (48.48%), S1 (45.52%), S2 (61.87%), S3 (46.63%), S4 (39.54%) and C (56.56%), also there is no significant differences between T1, S2, C and between T3, T4, S3 and between T4 to S1. If comparing between the individual motility percentages of sperm for steps of freezing in each treatment (table 1) observed the significant differences ($p> 0.05$) in which the motility decreased significantly ($p> 0.05$) after cooling and post thawing in all treatments.

The effect addition trehalose or sucrose and steps of freezing on dead percentage of bulls sperms

The effect addition trehalose and sucrose on dead percentage of bull sperm frozen in liquid nitrogen revealed in the table 2 in which, as comparing between treatments within each step observed.

After dilution the treatment T4 and S4 (36.28 and 36.95%) respectively were increased significantly ($p>0.05$) in dead percentage of sperms as compared with other treatments T1 (19.18%), T2 (14.15%), T3 (27.24%), S1 (27.30%), S2 (24.03%), S3 (33.63%) and C (22.68%), in addition T2 was decreased significantly ($p> 0.05$) in comparison with T1, T3, T4, S1, S2, S3, S4 and C. At after cooling, the treatment T4, S3 and S4 (37.43, 37.18 and 38.52%) respectively appears significantly ($p> 0.05$) higher than T1 (25.38%), T2 (21.80%), T3 (32.15%), S1 (30.21%), S2 (27.38%) and C (27.73%), but no recorded significant differences between T1, S2 and C1 and T3 among S1. In post thawing step, the dead percent in treatment S4 (42.27%) appear significantly ($p> 0.05$) higher compare with T1 (30.06%), T2 (25.96%), T3 (38.92%), T4 (41.60%), S1 (34.03%), S2 (29.35%), S3 (39.08%) and C (35.99%), moreover C did not differ significantly with T3, T4 and S3 among T1 and S2 and between C compare to S1. Overall means of dead percentage of bull sperms show that high percentage in T3, T4, S3 and S4 (32.77, 38.43, 36.63 and 39.25%) compared with T1 (24.87%), T2 (20.64%), S1 (30.51%) and S2 (26.92%), but no significant differences between C, T1, S1 to S2 and among T1 to T2. In comparative between freezing steps during each treatment which summarized in table 2 explain during Post thawing step the T1, T2, T3, S1, S2, S3, S4 and C increase significantly ($p> 0.05$) compare with after dilution and cooling, but no significant differences between all steps in T4. In addition, there are no significant differences between after dilution and after cooling for S4, also no significant differences between after cooling and post thawing of the treatment S2 and S3.

The effect addition trehalose or sucrose and steps of freezing on abnormality percentage of bulls sperms

The effect addition trehalose, sucrose on abnormality percentage of bull sperm frozen in liquid nitrogen observed in table 3, in which, as comparing between treatments within each step revealed that:

After dilution, the sperm abnormality percentage in T4 (12.65%), S3 (11.84%) and S4 (13.00%) were increased significantly($p> 0.05$) compared with T1 (6.25%), T2 (4.22%), T3 (8.04%), S1 (9.21%), S2 (6.29%) and C (6.99%), but the T2 recorded less abnormality percentage ($p> 0.05$) compared with T3, T4, S1, S3, S4 and C1, but there are no significant variation between T1, T2 and S2, also among T1, T3, S2 and C1. After cooling, the abnormality percent in T2 which decreased significantly ($p> 0.05$) where it was (5.77%) compare with T1 (10.56%), T3 (10.75%), T4 (15.66%), S1 (15.12%), S2 (11.30%), S3 (15.26%), S4 (22.55%) and C(9.55%). But no significant differences between C1, T1, T3 and S2 and between T4, S1 and S3. Abnormality percentage in sperms for post thawing step revealed that also T2 (10.04%) shows significant differences ($p> 0.05$) less than T1 (13.50%), T3 (15.63%), T4 (21.12%), S1 (18.20%), S2 (14.41%), S3 (20.68%), S4 (26.17%) and C(14.67%). Besides that S4 give significantly ($p> 0.05$) more than T1, T2, T3, T4, S1, S2, S3 and C1, but no significant difference between T1, T3, S2 and C1 and between T4 to S3.

Overall mean which describe in table 3 apparent that the percentage of abnormal sperms in T2 (6.67%) decrease significantly ($p> 0.05$) in comparative with T1 (10.10%), T3 (11.47%), T4 (16.47%), S1 (14.17%), S2 (10.66%), S3 (15.92%), S4 (20.57%) and C(10.40%). but show no significant differences between T1, T3, S1, S3 and C1. The results of effective steps of freezing on abnormality percentage of sperm during each treatment summarized in table 3. In post thawing steps the all

Table 1 : The effect of trehalose or sucrose on individual motility % of bulls sperms after dilution ,cooling and post- Thawing (Mean ± SE).

Sugar	Concentration	After dilution	After cooling	Post- Thawing	Overall mean
Control (C)		63.33±0.57 **BCa**	53.77±0.75 **Cb**	52.55±0.62 **Cb**	56.56±0.76 **B**
Trehalose	**T1**	61.94±0.59 **Ca**	57.33±0.81 **Bb**	53.33±0.90 **Cc**	57.54±0.65 **B**
	T2	70.83±0.83 **Aa**	66.38±0.88 **Ab**	65.55±0.89 **Ab**	67.59±0.59 **A**
	T3	56.94±0.91 **Da**	45.00±0.80 **Db**	46.66±0.70 **Db**	49.53±0.86 **C**
	T4	53.33±0.57 **DEa**	45.50±1.31 **Db**	46.61±1.32 **Db**	48.48±0.80 **CD**
Sucrose	**S1**	51.83±0.56 **Ea**	43.61±1.26 **Db**	41.11±0.76 **Eb**	45.52±0.81 **D**
	S2	66.38±1.13 **Aba**	59.44±1.135 **Bb**	59.77±0.77 **Bb**	61.87±0.73 **B**
	S3	52.72±0.67 **DEa**	46.05±0.92 **Db**	41.21±0.64 **Ec**	46.63±0.78 **C**
	S4	45.00±0.70 **Fa**	36.66±0.53 **Eb**	36.94±0.59 **Fb**	39.54±0.63 **E**

Within row different small letters for each parameter means significant at ($p<0.05$). Within Column different large letters for each parameter means significant at ($p<0.05$).

Table 2 : The Effect of trehalose or sucrose on dead % of bulls sperms after dilution ,cooling and post- Thawing (Mean ± SE).

Sugar	Concentration	After dilution	After cooling	Post- Thawing	Overall mean
Control (C)		22.68±3.42 **Ea**	27.73±0.27 **Cb**	35.99±1.24 **BCc**	28.80±0.90 **AB**
Trehalose	**T1**	19.18±5.49 **Fa**	25.38±0.37 **Cb**	30.06±0.17 **Dc**	24.87±0.76 **BC**
	T2	14.15±5.36 **Ca**	21.80±0.33 **Db**	25.96±0.54 **Ec**	20.64±0.81 **C**
	T3	27.24±1.46 **Da**	32.15±0.55 **Bb**	38.92±0.47 **Bc**	32.77±0.47 **A**
	T4	36.28±2.99 **Aa**	37.43±0.41 **Aa**	41.60±0.66 **Ba**	38.43±0.47 **A**
Sucrose	**S1**	27.30±2.43 **Da**	30.21±0.35 **Bb**	34.03±0.47 **Cc**	30.51±0.46 **B**
	S2	24.03±2.50 **Ca**	27.38±0.43 **Cb**	29.35±0.39 **Db**	26.92±0.41 **B**
	S3	33.63±2.55 **Ba**	37.18±0.28 **Ab**	39.08±0.65 **Bb**	36.63±0.43 **A**
	S4	36.95±2.16 **A**	38.52±0.31 **Aa**	42.27±0.38 **Ab**	39.25±0.38 **A**

Within row different small letters for each parameter means significant at ($p<0.05$). Within Column different large letters for each parameter means significant at ($p<0.05$).

Table 3 : The Effect of trehalose or sucrose on abnormality % of bulls sperms after dilution ,cooling and post- Thawing (Mean ± SE).

Sugar	Concentration	After dilution	After cooling	Post- Thawing	Overall mean
Control (C)		6.99±0.55 **Cc**	9.55±0.47 **Cb**	14.67±0.45 **Da**	10.40±2.25 **B**
Trehalose	**T1**	6.25 ±0.24 **CDc**	10.56±0.37 **Cb**	13.50±0.47 **Da**	10.10±2.10 **B**
	T2	4.22±0.32 **Db**	5.77±0.27 **Db**	10.04±0.31 **Ea**	6.67±1.74 **C**
	T3	8.04±0.32 **BCc**	10.75±0.58 **Cb**	15.63±0.56 **Da**	11.47±2.22 **B**
	T4	12.65±0.43 **Ac**	15.66±0.57 **Bb**	21.12±0.31 **Ba**	16.47±2.47 **A**
Sucrose	**S1**	9.21±0.51 **Bc**	15.12±0.53 **Bb**	18.20±0.48 **Ca**	14.17±2.63 **BC**
	S2	6.29±0.39 **CDc**	11.30±0.45 **Cb**	14.41±0.42 **D**	10.66±2.63 **C**
	S3	11.84±0.56 **Ac**	15.26±0.64 **Bb**	20.68±1.11 **Ba**	15.92±2.57 **B**
	S4	13.00±0.32 **Ac**	22.55±0.74 **Ab**	26.17±0.62 **Aa**	20.57±3.02 **A**

Within row different small letters for each parameter means significant at ($p<0.05$). Within Column different large letters for each parameter means significant at ($p<0.05$).

Table 4 : Overall means of trehalose and sucrose on individual motility, Dead and abnormality percentage of bulls sperms during dilution, cooling and post-thawing.

Characteristics	Sugar	After dilution	After cooling	Post- Thawing	Overall mean
Individual motility %	**Trehalose**	61.27±2.98 **Aa**	53.59±3.98 **Ab**	52.94±3.45 **Ab**	55.78±4.42 **A**
	Sucrose	53.98±4.47 **Ba**	46.44±4.76 **Bb**	44.73±5.11 **Bb**	48.38±4.75 **B**
Overall mean		57.62±2.14 **a**	50.01±2.44 **b**	48.83±1.97 **b**	
Dead %	**Trehalose**	24.21±4.84 **Ab**	29.19±3.48 **Aab**	33.09±3.06 **Aa**	28.82±3.70 **A**
	Sucrose	30.47±2.93 **Ab**	33.32±2.69 **Aab**	35.70±2.71 **Aa**	33.16±2.75 **A**
Overall mean		**27.34±2.65 b**	**31.25±2.32 ab**	**34.39±2.54 a**	
Abnormality %	**Trehalose**	7.79±1.79 **Ab**	10.68±2.01 **Aab**	13.07±2.32 **Aa**	10.07±2.03 **B**
	Sucrose	10.81±2.49 **Ab**	16.05±2.35 **Aa**	19.86±2.46 **Aa**	15.33±2.06 **A**
Overall mean		9.30±2.56 **b**	13.36±2.50 **ab**	16.46±2.50 **a**	

Within row different small letters for each parameter means significant at ($p<0.05$). Within Column different large letters for each parameter means significant at ($p<0.05$).

treatments T1 ,T2 , T3 , T4 , S1 , S2 , S3 , S4 and C were increased significantly ($p> 0.05$) comparative with that after dilution and after cooling for all treatment. but no significant differences in sperm abnormality between T2 in after dilution and after cooling.

Overall means of effect Trehalose or sucrose on individual motility, dead and abnormality percentage of bulls sperms during dilution, cooling and post thawing

Individual motility percentage : In comparative between freezing steps during each treatment which summarized in table 4 explain during after cooling (53.59, 46.44) and Post thawing step (52.94, 44.73) causes decrease significant ($p<0.05$) in individual motility compare with after dilution (61.27, 53.98), whether in the case of adding trehalose or sucrose, but the differences in individual motility no significant between after cooling and post thawing for both trehalose and sucrose.

Dead sperms percentage

Result indicated that dead percentage during post thawing in addition trehalose (33.09) and sucrose (35.70) causes increase significant ($p<0.05$) compare with after dilution, but the differences no significant between the step of after cooling with post thawing and between after cooling and after dilution for both addition trehalose or sucrose (table 4).

Abnormality sperms percentage

Present study shows that abnormality percentage sperm for step after cooling (16.05) and post thawing (19.86) increase significant ($p<0.05$) abnormality percentage compare with after dilution(10.81) expect during addition sucrose, but containing the diluted trehalose only led to a rise significant ($p<0.05$) the abnormality sperm percentage in post thawing (13.07) compare with after dilution (7.79), but the difference no significant between after cooling and post thawing and between after cooling and dilution for both sugars (table 4). Results about comparison between Trehalose and sucrose were different significant ($p<0.05$) and trehalose (55.78) show up high significant ($p<0.05$) in individual motility and less abnormality percentage (10.07, 15.33%) respectively compare with sucrose, but the differences in dead percentage for both sugars (28.82, 33.16) no significant.

Discussion

The effect trehalose or sucrose on individual motility, dead and abnormality percentage of bull sperms frozen in liquid nitrogen

The results of the present study revealed an improving effect of trehalose and sucrose supplemented to a basic tris extender on bull semen quality (sperm motility, viability, total sperm abnormalities) after cooling and freezing. Our results exhibited improved sperm motility, viability and decreased abnormalities. These results are in accordance with the results obtained by Reddy *et al*. (2010) in buffalo, Aisen *et al*. (2002) in ram, Aboagla and Terada (2003) in goat, Hu *et al*. (2010) in boar. The improved quality of cooled and post-thaw sperm on adding trehalose or sucrose to the extender is due to reducing all injury caused by ice crystallization as trehalose and sucrose are non-permeable sugars render hypertonic media decreasing intracellular freezable water (Bakás and Disalvo, 1991). Aboagla and Terada (2003) and Reddy *et al*. (2010) referred this reduction in cryodamage of spermatozoa to the interaction of these sugars with phospholipids in plasma

membrane and increases membrane fluidity leading to greater resistance of spermatozoa against freeze-thawing damage. While, another argument proposed that probably trehalose protects biomolecular structures through both, the replacement of water in hydrogen bonds (Chen *et al*., 2000) and trapping essential hydration water molecules (Patist and Zoerb, 2005). Also, a role of viscosity in the maintenance of the biomolecular structure has been proposed. Trehalose has indirect antioxidant effect by increasing the level of glutathione and reduced level of lipid peroxide (Aisen *et al*., 2005). Trehalose might have displayed cryoprotective effect on the functional integrity of acrosome and mitochondria that is responsible for the generation of energy from intracellular stores of ATP leading to improved post-thaw sperm motility (Reddy *et al*., 2010). Trehalose is able to protect the integrity of cells against a variety of environmental stresses such as dehydration, heat, cold and oxidation (Chen and Haddad, 2004). It had the remarkable stabilizing properties due to the formation of a non-hygroscopic glass state and protected protein and lipids membranes from degradation during the freeze–drying process (Hu *et al*., 2010). Furthermore, trehalose had been extensively used to improve sperm quality parameters in semen cryopreservation and its protective effects significantly improved the freezability of goat spermatozoa due to increase in membrane fluidity resulting from the depression of membrane transition temperature, allowing the sperm membrane to tolerate low temperature effects (Aboagla and Terada, 2003). The extender containing trehalose improved antioxidant action and reduced the oxidative stress provoked by cryopreservation in bull (Hu *et al*., 2010; Tuncer *et al*., 2011), buffalo bull (Badr *et al*., 2010), ram (Bucak and Tekin, 2007; Bucak *et al*., 2007; Aisen *et al*., 2005) noticed that the extender supplemented with 100 mm trehalose did not affect superoxide dismutase (SOD) levels but catalase (CAT) and glutathione peroxidase (GSH-Px) activities were greater with the supplementation of trehalose at 100 and 200 mm. Sitaula *et al*. (2009) studied the effect of sorbitol and trehalose on sperm motility following partial dehydration and showed a much improved bovine sperm motility in the presence of sorbitol and trehalose. Tuncer *et al*. (2011) evaluated the effects of the addition of different sugars (25 mm raffinose, 25 mm sucrose, and 25 mm trehalose) on bull spermatozoa cryopreserved in a commercial extender (Optidyl) supplemented with 3 mm glutamine on semen parameters, fertilizing ability and superoxide dismutase (SOD) activity. However, Khalili *et al*. (2009) obtained the highest post-thawing quality when combining nearly 200 mm of trehalose (198.24mm) and 8% glycerol. This suggests both that there may be important differences between species regarding the optimal trehalose/sucrose concentrations. This could explain, apart from species differences, why several studies have reported non-positive effects of trehalose and sucrose and even negative effects at some concentrations (Atessahin *et al*., 2008). The antioxidant characteristics of some disaccharides as trehalose may be related to its effectiveness in membrane cryopreservation (Hu *et al*., 2010; Tuncer *et al*., 2011). Trehalose has indirect antioxidant effect by increasing the level of glutathione and reduced level of lipid peroxide (Aisen *et al*., 2005). Chhillar *et al*. (2012) reported that both trehalose and taurine decreased H_2O_2 and MDA in frozen- thawed bull semen to the levels of fresh semen and Badr *et al*. (2010) reported similar results in buffalo semen. Therefore, the effect of trehalose on the oxidative stress concomitant to sperm cryopreservation seems to vary with species, and possibly with the application of different protocols. Also, trehalose might have displayed cryoprotective effect on the functional integrity of acrosome and mitochondria that is responsible for the generation of energy from intracellular stores of ATP leading to improved post-thaw sperm motility. Ours results revealed that trehalose and sucrose at high concentration (200 mm/L) reduced sperm membrane integrity. These results are in close relation to that of Fernandez –Santosa *et al*. (2007), who proved that membrane integrity and mitochondrial status after thawing depend on osmolarity as low osmolality (hyposmotic extenders) produce a higher percentage of spermatozoa with intact spermatozoa membrane. Jafaroghli *et al*. (2011) showed that ram sperm can tolerate hyperosmotic diluents at a range of sugar concentration (50–100 mm/L) with improved post-thaw semen quality.

The effect steps of freezing on individual motility, dead and abnormality of bull sperms frozen in liquid nitrogen

The freezing process negatively affects ($P < 0.05$) the sperm parameters (individual motility, dead and abnormality), agreement with Üstüner *et al* (2015). But the current study revealed that this effects were changes from treatment to another's, it means there is interaction between effect steps of freezing, addition Trehalose and sucrose to Tris diluents of bull spermatozoa, however, overall, this effect was lower when the 100mm trehalose was added to this diluents. These effects were also observed in the studies of Barbas and Mascarenhas (2009) and Dorado *et al* (2009). The kidding rate after artificial insemination with frozen and thawed semen is poorer than with fresh or chilled semen (Batista *et al*., 2009), but most properly freezing and thawing procedures had

negative effects on motility and acrosome integrity (Üstüner *et al*., 2015). Hussain *et al.* (2016) reported that significant decrease in individual motility and increase in dead and abnormalities percentage for both poor and good ejaculate during different steps, dilution, cooling and freezing of bull semen, this might be attributed to the fact that lactic acid which produced as an end product of sperm metabolism, resulting in harmful lowering of PH which exerts toxic effect on sperm cell (Ball and Peter, 2004). The considerably reduced values for sperm motility, viability, morphology and plasma membrane/acrosome integrity observed after cryopreservation of semen over fresh or pre-freeze stage (Chaudhari *et al*., 2015).

Conclusion

The addition of 100 mm trehalose/ sucrose to TFEG diluents had their benefits on freezing-thawing bull semen for artificial insemination center, Iraqi. A step of freezing process (dilution, cooling and Post-Freezing) was detrimental to bull sperm properties, but this effect was less when adding 100mm trehalose to the diluents.

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