International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG(USA) Volume 4, Issue 4 - 2017

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2017.04.04.025

Effect of addition trehalose and steps of freezing on sperm properties of bull frozen in liquid nitrogen.

[1]Qusay Mohammed Al-Badrany,[1]Kreem Iwaid Al-Badry, [2]Faris Faisel Ibrahim, [2]Wafa'a Yeedam Lateef

[1]Department of Surgery & Obstetrics-College of Veterinary Medicine- University of Baghdad;
[2]Ministry of Agriculture-Department of Artificial Insemination center-Baghdad.
Correspondence | Qusay Mohammed Aboud, Department of Surgery & Obstetrics,
College of Veterinary Medicine, University of Baghdad, Iraq;
E-mail: *qusaymoh988@yahoo.com*

Abstract

Object of study to improve semen preserve ability through the use of trehalose sugar 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 through a period of 16 weeks, were diluted with Tris-based extender containing different Trehalose 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 group 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 to Tris diluents of bull spermatozoa, however, overall, this effect was lower when the 100mM trehalose was added to this diluents. **Conclussion:** The addition of 100 Mm trehalose 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.

Keywords: Trehalose, freezing steps, bull, sperm properties.

Introduction

Artificial insemination is one of the important tools for genetic improvement in modern dairy breeding practices (Rehman *et al.*, 2013). 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(Malo *et al.*, 2010). 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 (Watson, 1995; Salvador *et al.*, 2006).Trehalose, a non-reducing disaccharide which contains two glucose molecules linked together as 1, 1-glycosidic linkage (α-d-glucopyranosyl-l, 1-α-d-glucopyranoside), commonly found in higher concentrations in yeast and fungal spores (Woelders *et al.*, 1997; Aisen *et al.*, 2000).

Trehalose probably plays a vital role in preventing deleterious alteration to the sperm membrane by maintaining the osmotic pressure of the diluent, acting as a non-reducing cryoprotectant and providing energy substrate for the sperm cell during equilibration, cryopreservation and post-thawing (Liu *et al.*, 1998; Uysal and Bucak, 2009). Addition of trehalose with semen extenders is known to improve the motility and viability of sperm cells during cryopreservation (Sztein *et al.*, 2001; Matsuoka *et al.*, 2006). Trehalose, when added in hypertonic condition showed a synergic effect with glycerol and prevented intracellular ice crystal formation (Gutiérrez-Pérez *et al.*, 2009).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 (Woelders *et al.*, 1997; Sitaula *et al.*, 2009; Tuncer *et al.*, 2011), buffalo bull (Badr *et al.*, 2010; Reddy *et al.*,2010), ram (Molinia *et al.*, 1994; Bucak *et al.*, 2007), goat (Aboagla and Terada, 2003; Tuncer *et al.*, 2013), rabbit semen (Dalimate and Graham, 1997).

Although the addition of trehalose to semen diluents has a role in improving the ability of bulls sperms to freeze, but there are no studies in Iraq, especially in the production of semenfrozen bull semen by the artificalinsemination center.So this study aimed toimprove semen preserve ability through the use of trehalose sugar as extender additive expressed in frozen semen and study effect steps of freezing on sperm properties.

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 (3-4year)old and they were kept under identical conditions of management, feeding and watering throughout the study period which starts from October 2016 untill may 2017.Semen was collected from bulls weekly with the aid of an artificial vagina method, Immediately after collection, semen placed in water bath at 37C^{o} untill 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 5 equal aliquots and each one was diluted with Tris-Fructose-Egg Yolk-Glycerol (TFEG) freezing extender containing different Trehalose concentrations viz. T1-50mM, T2-100mM, T3-150mM, T4-200mM and no additive T5-(control) so as to have a final sperm concentration of 80 million sperms per ml, cooled slowly up to 5 C^{o} and equilibrated for 4hrs, semen was packed into 0.25 ml polyvinyl French straw (IMV, France) after equiliberation peroids, the straw were placed horizontally on arack 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 37 C^{o} for 30 seconds in a water bath for evalution, 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

Effect addition trehalose on sperm individual motility, dead and abnormality percentage.

Individual motility: The effect addition trehalose on individual motility percentage of bull sperm frozen in liquid nitrogen are mentioned in Table (1), in which, as comparing between treatments within each step showed :

after dilution the individual motility percentage of sperm in T2-100mM and T3-150mM (55.67 and 55.31) respectively appears significantly ($p<0.05$) higher than T4- 200mM (49.06%), But it was not clear that there were any significant differences between T1-50mM(52.50), T4-200mM and T5-control and among T1-50mM, T2-100mM and T3-150mM. But ***after cooling*** **(Table 1),** sperm motility percentage in treatments T2-100mM(51.33)and T3-150mM (52.50) appears more significant ($p< 0.05$) than T4-200Mm (41.25)and T5- control(45.62), but no any significant differences between T1-50mM, T2-100mM and T3-150mM, In addition to that between T1-50mM and T5- control as well as betweenT4-200mM and control.

In Table (1),the present study revealed that in ***post thawing*** steps individual motility percentage of sperm in T2-200mM(54.67) superior significant ($p< 0.05$) than other treatment T1(47.18),T3(50.31),T4(36.56) and T5 (40.94).

The overall mean of individual motility percentage of sperms Table (1) notice the treatment T2-100mM (54.67) were significant($p< 0.05$)higher than other treatment T1(49.87),T3(50.27),T4(42.32) and T5 (45.85), but not any differences among T2 and T3,also no differences between T1 comparative with T3 and T5.

Table (1) Effect addition trehalose on individual motility percentage of bull sperms frozen in liquid nitrogen. (Mean ±SE)

Treatment	Steps of freezing			Overall Mean
	After dilution	After cooling	Post-Thawing	
TFEG **T1 -50mM/L**	52.50 ± 2.09 **AB a**	49.68 ± **1.40** **AB a**	47.18 ± **1.76** **B a**	49.87 ± **0.89** **BC**
TFEG **T2-100mM/L**	55.67 ± **1.61** **A a**	51.33 ± **1.3** **A a**	54.67 ± **1.03** **A a**	53.67 ± 0.79 **A**
TFEG **T3 -150mM/L**	55.31 ± **1.16** **A a**	52.50 ± **1.12** **A a**	50.31 ± **0.31** **B a**	50.27 ± **0.63** **AB**
TFEG **T4 -200mM/L**	49.06 ± **1.78** **B a**	41.25 ± **1.41** **C b**	36.56 ± **1.09** **D b**	42.32 ± **0.78** **D**
TFEG **T5 –Control**	51.05 ± **2.37** **AB a**	45.62 ± **2.54** **BC b**	40.94 ± **2.29** **C b**	45.85 ± **1.64** **C**
Overall Mean	52.70 ± **1.52** **a**	48.06 ± **1.31** **b**	47.93 ± **1.49** **b**	

TFEG= Tris-Fructose Egg yolk Glycerol T =Trehalose mM/L=milliMole/Liter
Within row different small letters for each parameter means significant at ($p<0.05$).
Within Colum different large letters for each parameter means significant at ($p<0.05$).

Dead sperms: The effect addition trehalose on dead percentage of bull sperm frozen in liquid nitrogen revealed in table (2), in which, as comparing between treatments within each step observed. **After dilution** the treatment T4(33.22) significantly($p< 0.05$) calculated increase in dead percentage of sperms as compared with other treatments T1(24.19),T2(19.36),T3(27.40) and T5(28.03), in addition T2 show decrease significant ($p< 0.05$) in compare with T1, T3 and T5 (Table2), alsoT1 recorded low dead significantly ($p< 0.05$) coparative with T5,but no any significant differences between T1with T3 and between T3 with T5(Table 2). **After cooling**, results in Table (2)T4(37.62) also more significant ($p< 0.05$) in dead percentage of sperm compare with T1 (26.32),T2(21.30),T3(26.15) and T5(31.72), but T2 show less significant ($p< 0.05$) from T1, T3 and T5, otherwise result in Table (2) revealed T1 and T3 recorded decrease in dead sperms comparative with T5, but the differences no significant between T1 with T3.

Table (2) Effect addition trehalose on dead percentage of bull sperms frozen in liquid nitrogen. (Mean ±SE)

Treatment	Steps of freezing			Overall Mean
	After dilution	After cooling	Post-Thawing	
TFEG **T1-50mM/L**	24.19 ± **0.90** **C a**	26.32 ± **0.92** **C a**	26.50 ± **1.24** **C a**	25.67± **0.53** **C**
TFEG **T2-100mM/L**	19.36 ± **1.15** **D b**	21.30 ± **1.72** **D b**	29.43 ± **1.53** **BC a**	23.36± **0.67** **C**
TFEG **T3-150mM/L**	27.40 ± 1.21 **BC a**	26.15 ± **0.67** **C a**	27.59 ± **0.96** **C a**	27.05± **0.47** **B**
TFEG **T4-200mM/L**	33.22 ± **1.33** **A a**	37.62 ± **1.09** **A a**	37.78 ± **1.35** **A a**	36.21± **0.88** **A**
TFEG **T5-Control**	28.03 ± **1.28** **B b**	31.72 ± **1.25** **B ab**	32.13 ± **1.14** **B a**	30.63± **1.03** **B**
Overall Mean	26.44± **1.09** **b**	28.62± **1.33** **ab**	30.69± **1.26** **a**	

TFEG- T=Tris-Fructose Egg yolk Glycerol T =Trehalose mM/L= milli Mole/Liter
Within row different small letters for each parameter means significant at (p<0.05).
Within Colum different large letters for each parameter means significant at (p<0.05).

In step of *Post –Thawing*, the different significant (p< 0.05) between T4 (37.78) compare with T1 (26.50), T2(29.43), T3(27.59) and T5(32.13) which different significantly (p< 0.05) in comparative with T1 and T3 (Table 2),moreover T5 no differ significant from T2 and T1 among T2 and T3.***Overall means*** of dead percentage of bull sperms in Table (2) show that high percentage in T4(36.21) in compare with T1 (25.67), T2(23.36), T3(27.05) and T5(30.63), also T3 and T5 record more significant (p< 0.05) than T1 and T2,but the different no significant between T1 with T2 and among T3 with T5.

***Abnormality sperms*:** The effect addition trehalose 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 semen, the sperm abnormality percentage for T4 (14.78) was increased significant (p< 0.05) compare with T1(10.50), T2(7.97), T3(9.25) and T5(10.90) but T2 recorded less abnormality sperms (p< 0.05) compare with T1 and T4,but the variation no significant between T1,T3 and T5, also among T2,T3 and T5 (Table 3).

Table (3) Effect addition trehalose on abnormality percentage of bull sperms frozen in liquid nitrogen. (Mean ±SE)

Treatment	Steps of freezing			Overall Mean
	After dilution	After cooling	Post-Thawing	
TFEG **T1-50mM/L**	10.50 ± **0.74** **B b**	12.91 ± **0.94** **B ab**	14.91 ± **1.34** **B a**	13.31 ± **0.69** **B**
TFEG **T2-100mM/L**	7.97 ± **0.65** **C b**	9.10 ± **0.78** **C ab**	10.43 ± **0.89** **C a**	8.07 ± **0.72** **C**
TFEG **T3-150mM/L**	9.25 ± **0.65** **BC c**	12.75 ± **0.46** **B b**	15.09 ± **0.69** **B a**	12.00 ± **0.49** **B**
TFEG **T4-200mM/L**	14.78 ± **1.23** **A b**	16.87 ± **0.82** **A b**	21.78 ± **0.97** **A a**	17.97 ± **0.85** **A**
TFEG **T5- Control**	10.90 ± **0.69** **B b**	10.93 ± **0.84** **BC b**	14.25 ± **0.97** **B a**	11.01 ± **0.67** **B**
Overall Mean	10.68 ± **1.07** **c**	12.51 ± **0.73** **b**	15.15 ± **0.94** **a**	

TFEG- T=Tris-Fructose Egg yolk Glycerol T =Trehalose mM/L=milliMole/Liter
Within row different small letters for each parameter means significant at (p<0.05).
Within Colum different large letters for each parameter means significant at (p<0.05).

After cooling diluent semen in Table (3) T2 which causes decrease significant ($p< 0.05$) in abnormal percentage in sperms where it was (9.10) compare with T1(12.91),T3(12.75)and T4(16.87) which give higher significant ($p< 0.05$)in abnormality percentage among all treatments, but the differences no significant between T1, T3 and T5, also the different no significant between T2 and T5(Table 3).

Abnormality percentage in sperms for **Post-Thawing step** (Table 3) revealed that also T2(10.43) give least percentage better significant ($p< 0.05$) than T1(14.91),T3(15.09), T4(21.78) and T5(14.25) besides that T4 give more abnormality percentage significantly ($p< 0.05$) from T1,T2,T3 and T5, but the differences no significant between T1, T3 and T5 (Table 3). ***Overall mean*** which describe in Table (3) explain that the percentage of abnormality sperms inT2(8.07) decrease significantly ($p< 0.05$) in comparative with T1(13.31), T3(12.00), T5 (11.01) and T4(17.97) which causes high value significant ($p< 0.05$) in percentage abnormality compare with T1,T3,T4 and T5, but the differences no significant between T1,T3 and T5 (Table 3).

Effect steps of freezing on sperm individual motility, dead and abnormality percentage .

Individual motility*:** If comparing between individual motility percentage of sperms for steps of freezing in each treatment (Table 1), the differences significant observed only in T4 and T5 whereas the motility decrease significant ($p< 0.05$) after cooling in T4(41.25, 49.06),T5 (45.62, 51.05) respectively, compared with after dilution and the same post-Thawing **T4** (36.56), T5 (40.94) comparative with T4 and T5 after dilution. Results in Table (1) clear that the steps of freezing did not effect on individual motility percentage of sperms after dilution with T1,T2 and T3 whereas donot found any differences significant between these steps, after dilution (52.50,55.67 and 55.31), after cooling T1,T2 and T3(49.68, 51.33 and 52.50) respectively and Post-Thawing T1,T2 and T3 (47.18, 54.67 and 50.31).Present study in Table (1) show that also the different no significant in motility percentage between after cooling and Post Thawing in T4(41.25 and 45.62) and T5(36.56 and 40.94) in alternation. ***The overall mean of individual motility percentage of sperms (Table 1) explain after cooling (48.06) and post thawing (47.93) decrease significant($p< 0.05$) comparative with after dilution (52.70) .

Dead sperms: In comparative between steps of freezing during each treatment which summarized in Table (2) shows that dead percentage during Post—Thawing step for T2 (29.43) increase significant ($p< 0.05$) compare with after dilution (19.36) and cooling (21.30),also Post—Thawing and after cooling for T5(32.13 and 31.72)increase significant ($p< 0.05$) compare with after dilution (28.03) but no significant differences between all steps, after dilution (24.19, 27.40 and 33.22), cooling (26.32, 26.15 and 37.62) and Post-Thawing (26.50, 27.59 and 37.78) alternate for T1 ,T3 and T4 (Table 2).In addition there are no significant differences among after dilution(21.30)and cooling (19.36) for T2 and between after cooling (32.13)and Post-Thawing (31.72) for T5 (Table 2).The present study denote that the **overall mean** (Table 1) of dead percentage of sperms in Post-Thawing(30.69) increased significant ($p< 0.05$) compare with after dilution of semen (26.44),but the different no significant between Post-Thawing with after cooling (28.62) and after dilution semen has no significant differences with after cooling.

Abnormality sperms: Results of effect steps of freezing on abnormality percentage of sperm during each treatment summarized in Table (3). Post-Thawing causes significant ($p< 0.05$)increased in abnormality percentage comparative with after dilution semen for all treatment T1 (14.91, 10.50),T2(10.43, 7.97),T3(15.09, 9.25), T4(21.78, 14.78) and T5(14.25, 10.90) alternate, by the same way, data output in table (3) confirmed that Post-Thawing causes increase significant ($p< 0.05$) in abnormality percentage comparative with after cooling for T3(12.75),T4(16.87) and T5(10.93),also after cooling occur more abnormality sperms ($p< 0.05$) compare with after dilution for T3, but the differences no significant between abnormality in Post–Thawing compare with after cooling inT1(12.91) and T2 (9.10) and between after dilution with cooling in T1,T2,T4 and T5 (Table 3).**Overall mean** in Table (3) appear that the differences significant ($p< 0.05$) between steps of freezing ,after dilution (10.68), cooling (12.51) and Post-thawing (15.15).

Discussion

Effect addition trehalose on individual motility, dead and abnormality percentage of bull sperms frozen in liquid nitrogen

It is clear that the concentration of sugar 100mM of trehalose lead to a significant increase in the

individual motility and there was also a significant in dead and abnormality percentage of the sperms comparison with control and other concentrations 50mM and 200mM (individual motility),150mM, 200mM (dead) and 50mM,150mM, 200mM (abnormality). Our finding supports with (Badr *et al.*, 2010; Hu *et al.*, 2010; Shaikh *et al.*, 2016) reported that the extender supplemented with 100 mM Trehalose resulted in the greater sperm motility, at post-thaw stages of freezing in bovine and buffalo bulls. Aisen *et al.*,(2005) and Jafaroghli *et al.*,(2011) noticed a better cryoprotective effects and a superior recovery of post thawing ram spermatozoa with addition of 3% or 5%glycerol combined with 100 mM trehalose in the extender. Trehalose has a protective action related both to osmotic effect and specific interactions with membrane phospholipids, rendering hypertonic media, causing cellular osmotic dehydration before freezing, and decreasing the amount of cell injury by ice crystallization (Bucak *et al.*, 2007; Badr *et al.*, 2010). Whilst using 100mM Trehalose, lower individual motility per cent have been reported in Karan-Fries bulls (Chhillar *et al.*, 2012) and in bovine bulls (Hu *et al.*, 2010) at post-thaw stage of cryopreservation than that of the present findings in Holstien bulls.

In present study, it was clear that supplementation of trehalose to the extender improved bovine semen quality variables. The current findings were in agreement with many studies and indicated favorable effects of trehalose on post thaw viability of mouse (Storey *et al.*, 1998), goat (Aboagla and Terada, 2003; Bucak and Uysal, 2008), ram (Aisen *et al.*, 2002; Bucak *et al.*, 2007), boar (Hu *et al.*, 2009; Gutierrez-Perez *et al.*, 2009), dog (Yildiz *et al.*, 2000; Yamashiro *et al.*, 2007),bull (Hu *et al.*, 2010). Moreover current study show that 50mMfavored result significant ($p<0.05$) in individual motility compare with 200mM diluents and among Tris diluents in dead percentage.

El-Sheshtawy *et al.*,(2015)find out that addition of 50 mM trehalose or 100 mM trehalose to Tris have a beneficial effect in chilling diluted bull semen, but (Uysal *et al.*, 2007) reported improved individual motility per cent using 50mM Trehalose in bovine bulls in comparison with the control groups at post-thaw stage of cryopreservation. El-Badry *et al.*, (2017) reported that 50 mM trehalose attained the best chilling and post thawing semen quality parameters and the limited number of available mares, so the extender containing only 50 mM trehalose either chilled or frozen was selected to undergo fertility test, so field fertility data revealed that the inclusion of 50 mM trehalose in semen extender improved the pregnancy rate of mares inseminated with chilled and frozen-thawed semen. Similarly, previous studies revealed that supplementation of semen extender with trehalose exerted valuable effects on the in vitro (Badr *et al.*, 2014) and in vivo (Iqbal *et al.*, 2016), fertilizing potentials of the frozen thawed buffalo spermatozoa and lambing rate using frozen-thawed ram spermatozoa (Bucak *et al.*, 2007;Jafaroghli*et al.*, 2011). Cold shock of stallion sperm during storage at 5C^{o} was reduced by adding 50 Mm trehalose (Arifiantini *et al.*, 2013).

A significant improvement in acrosome integrity with the addition of 50-100 mM trehalose, these results are in accordance with the previous studies conducted on bovine and buffalo bull semen (Hu *et al.*, 2010;Cirit *et al.*, 2013;Iqbal*et al.*, 2016). Another authors shows that the optimum concentration of trehalose in freezing extender was 50 mM for rams (Bucak and Tekin, 2007; Dolti*et al.*, 2016), 100 mM for bulls(Hu *et al.*, 2010, Shaikh *et al.*, 2016) and buffalo bulls(Badr *et al.*, 2010; Reddy *et al.*, 2010; Badr *et al.*, 2014; Shahba *et al.*, 2016), 250mM for boars (Gutiérrez-Pérez *et al.*, 2008; Gutiérrez-Pérez *et al.*, 2009) and 370 mM for bucks (Aboagla and Terada, 2003), this lead us to suggest that that the optimum concentration of trehalose differs among species. The results of the current study confirmed that the addition of Trehalose to moderate concentrations have improved the ability of the sperm to freeze through the increase in individual motility and reduce the dead and abnormality of sperm, these are agreement with Trehalose has also been reported to improved post-thaw sperm characteristics in mouse (Story *et al.*, 1998), dog (Yildiz *et al.*, 2000; Yamashiro *et al.*, 2007), and boar (Hu*et al.*, 2009; Gutiérrez- Pérez *et al.*, 2009) semen, but failed to improve fertility in frozen bull (Foote *et al.*, 1993), rabbit (Dalimata and Graham, 1997).

Aboagla and Terada, (2003) reported that the substitution of a Tris–citric acid diluents composition with trehalose only significantly improved sperm motility, acrosome integrity and membrane fluidity of Japanese native miniature goat. The positive role of Trehalose may be due to the positive role of this sugar when adding to diluents semen. The beneficial effects of the addition of trehalose to the extender on the post-thaw motility of mammalian sperm have been reported in many studies (Molinia *et al.*, 1994; Khalili *et al.*, 2009; Reddy *et al.*, 2010; Jafaroghli *et al.*, 2011). Also probably plays a vital role in preventing deleterious

alteration to the sperm membrane by maintaining the osmotic pressure of the diluents, acting as a non-reducing cryoprotectant and providing energy substrate for the sperm cell during equilibration, cryopreservation and post-thawing (Chen *et al.*,1993; Liu *et al*., 1998; Uysal and Bucak, 2009).

Trehalose, when added in hypertonic condition showed a synergic effect with glycerol and prevented intracellular ice crystal formation (Gutierrez *et al.*, 2009; Najafi *et al.,* 2013). Trehalose is not an antioxidant and has been shown to have a protective effect on the deleterious effects of low temperature on the membranes of bulls (Chen *et al.,* 1993; Woelders *et al*., 1997; Uysal *et al.,* 2007; Sariozkan *et al*., 2009; Hu *et al.,* 2010), mice (Storey *et al*., 1998), rams (Lopez-Saaz et al., 2000; Aisen et al., 2002; Bucak amd Tekin, 2007; Bucak *et al*., 2007; 2008; Uysal and Bucak, 2009; Nur *et al*., 2010; Cirit *et al.,* 2013; Najafi *et al.,* 2013), buffalo (Reddy *et al.,* 2010), boars (Funahashi and Sano, 2005; Gutierrez-Perez *et al.,* 2009; Hu *et al.,* 2009), dogs (Yildiz *et al.,* 2000; Yamashiro *et al.,* 2007; Michael *et al.,* 2007; Martins-Bessa *et al.,* 2009), rabbits (Dalimata and Graham, 1997; Kozdrowski, 2009) and goats (Aboagla and Terada, 2003; Atessshin *et al.,* 2008; Khalili *et al.,* 2009; Tuncer *et al.,* 2013).

The current study found that the addition of Trehalose led to a significant($P<0.05$) decrease in the percentage of dead sperms during the various stages of freezing and this may be due to its ability to increase in membrane fluidity resulting from the depression of membrane transition temperature, allowing the sperm membrane to endure low-temperature effects (Aboagla and Terada, 2003) and improved sperm membrane integrity, sperm viability and post-thaw fertility (Aisen *et al.,* 2002; Bucak and Tekin, 2007). Trehalose, acts as non-permeating cryoprotectant which causes dehydration of sperm due to the osmosis of water, due to this mild dehydration, sperm have low water content which results in reduced intracellular ice crystal formation,this might be the reason for improved post-thaw sperm viability of frozen-thawed sperm, diluted with Trehalose in semen extender (Chhillar *et al.,* 2012).Addition Trehalose aid to decrease abnormality in present study may be it was provides effective protection for phospholipid membrane of sperm head (Aisen*et al.,* 2000; Aisen *et al.,* 2002; Aboagla and Terada, 2003; Matsuoka *et al.,* 2006; Farshad and Akhondzadeh, 2008)and it has stabilizing effect on both cellular protein and plasma membrane (Aboagla and Terada, 2003) and reducing the cell injury by ice crystallization (Molinia *et al.,* 1994). Previous researches have shown Trehalose has ability to protect membrane protein deformation caused by temperature changes in spermatozoa (Hami and Molla Hoseyni, 2011).Concentration of 50-100 mM Trehalose in semen extender significantly increases the number of spermatozoa with intact plasma membrane after thawing bull and buck semen (Hu *et al.,* 2010, Atessahin *et al.,* 2008).Furthermore, it has the antioxidative property at lower doses and protects the spermatozoa by reducing lipid peroxidation (Chen and Haddad, 2004; Tuncer *et al.,* 2013).

Antioxidative and cryoprotective effects of additives such as trehalose, which improve sperm function, such as motility, membrane integrity, endogenous antioxidant activities and fertilizing ability, have been demonstrated in various species (Bucak *et al.,* 2013). In the same time present study confirmed that 200mMTrehalose show deleterious in individual motility, dead and abnormality percentage,agreement with Hu *et al.,* 2010 (bull),El-Sheshtawy *et al.,* 2015 (buffalo) and El-Badry *et al.,* 2017 (horse).

The negative results of the 200mM trehalose shown in the current study may be due to the addition of sugar by this concentration lead to a rise in diluted osmolarity, resulting in a decrease in the individual motility and increase dead and abnormality percentage, Spermatozoa motility is susceptible to extracellular osmolarity(Aisen *et al.,* 2002; Bucak *et al.,* 2007; Hu *et al.,* 2009)occurred due to high osmolarity of the extender with latter concentration being deleterious to the sperm cells which were the osmolarity of the solution used in extender preparation influences post-thaw sperm characteristics, This is in confirmation with ram (Aisen *et al.,* 2002) where 200 and 400 mOsm trehalose showed negative influence on post-thaw motility and plasma membrane integrity. Trehalose concentration at more than 100 mM did not improve the post-thaw sperm quality of bull (Hu *et al.,* 2010) and ram (Uysal and Bucak, 2009). The mechanism of this negative influence of high trehalose concentration is not clearly understood; but higher doses may change the media hypertonic and therefore increase the external pressure on sperm and altering cell architecture by membrane protein denaturation, lipid phase transitions and reducing membrane fluidity (Ahmad *et al.,* 2013). Higher concentrations of trehalose increased semen viscosity leading to decreased sperm motility by suppressing sperm flagella movement that was partially recovered by diluting the freezing solution (Woelders *et al.,*1997).

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 and addition Trehalose to Tris diluents of bull spermatozoa, however, overall, this effect was lower when the 100mMtrehalose 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 prefreeze stage (Chaudhari *et al.*, 2015).

In conclusion: The addition of 100 mMtrehalose to TFEG diluents had their benefits on freezing-thawing bull semen for artificial insemination center-Iraqi.A steps 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.

Acknowledgments

This study was carried out in the Artificial Insemination Center, Iraq (AI Center). The authors would like to thank every member in the AI Center, especially to the dear **Dr.Kateg Ehthrir** and **Mr.Omar Adel Mohammad** for their cooperation and afferent we indefinite facilities along experimental period in order to be performed conveniently.

Authors' Contribution

All authors contributed equally in all the efforts for these articles.

References

Aboagla, E.M.E. and Terada, T. (2003). Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. *Biology of reproduction*, *69*(4), pp.1245-1250.

Ahmad, E.; Naseer, Z. and Ahmad, N. (2013). High trehalose concentration in tris-based egg yolk extender has detrimental effect on post-thaw semen quality in Nili-Ravi buffalo bull. *Anim Health Prod Hyg*, *2*, pp.213-215.

Aisen, E.; Quintana, M.; Medina, V.; Morello, H. and Venturino, A. (2005). Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. *Cryobiology*, *50*(3), pp.239-249.

Aisen, E.G.; Alvarez, H.L.; Venturino, A. and Garde, J.J. (2000). Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. *Theriogenology*, *53*(5), pp.1053-1061.

Aisen, E.G.; Medina, V.H. and Venturino, A. (2002). Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. *Theriogenology*, *57*(7), pp.1801-1808.

Arifiantini, R.I.; Purwantara, B.; Yusuf, T.L. and Sajuthi, D. (2013). The quality of stallion semen in skim milk and dimitropoulos extenders preserved at 5 ^{o}c and ambient temperature supplemented with different sugar. *Media Peternakan*, *36*(1), pp.45–51.

Atessahin, A.; Bucak, M.N.; Tuncer, P.B. and Kızıl, M. (2008). Effects of anti-oxidant additives on microscopic and oxidative parameters of Angora goat semen following the freeze–thawing process. *Small Ruminant Research*, *77*(1), pp.38-44.

Badr, M.R.; Azab, A.M.S. and Rawash, Z.M. (2014). Effect of trehalose, cysteine and hypotaurine on buffalo bull sperm freezability, ultrastructure changes and fertilizing potentials. *Assiut Vet Med J*, *60*, pp.38-45.

Badr, M.R.; Mary, G. and Hassan, M.H. (2010). Effect of trehalose on cryopreservation, oxidative stress and DNA integrity of buffalo spermatozoa. *J ReprodInfertil*, *1*, pp.50-57.

Ball, P.J. and Peters, A.R. (2004). *Reproduction in cattle*.(3rd edition). John Wiley & Sons.

Barbas, J.P. and Mascarenhas, R.D. (2009). Cryopreservation of domestic animal sperm cells. *Cell and tissue banking*, *10*(1), pp.49-62.

Batista, M.; Nino, T.; Alamo, D.; Castro, N.; Santana, M.; Gonzalez, F.; Cabrera, F. and Gracia, A. (2009). Successful artificial insemination using semen frozen and stored by an ultrafreezer in the Majorera goat breed. *Theriogenology*, *71*(8), pp.1307-1315.

Bearden, H.J. and Fuquay, J.W. (2000). Semen evaluation. In: Bearden H. J., Fuquay J.W., editors. Applied animal reproduction. New Jersey: Prentice Hall, Upper Saddle River, 168–182.

Bilodeau, J.F.; Chatterjee, S.; Sirard, M.A. and Gagnon, C. (2000). Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Molecular reproduction and development*, *55*(3), pp.282-288.

Bucak, M.N. and Tekin, N. (2007). Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen. *Small Ruminant Research*, *73*(1), pp.103-108.

Bucak, M.N. and Uysal, O. (2008). The role of antioxidants in freezing of Saanen goat semen. *Indian veterinary journal*, *85*(2), pp.148-150.

Bucak, M.N.; Ateşşahin, A. and Yüce, A. (2008). Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze–thawing process. *Small Ruminant Research*, *75*(2), pp.128-134.

Bucak, M.N.; Ateşşahin, A.; Varışlı, Ö.; Yüce, A.; Tekin, N. and Akçay, A. (2007). The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen: microscopic and oxidative stress parameters after freeze–thawing process. *Theriogenology*, *67*(5), pp.1060-1067.

Bucak, M.N.; Keskin, N.; Taşpınar, M.; Çoyan, K.; Başpınar, N.; Cenariu, M.C.; Bilgili, A.; Öztürk, C. and Kurşunlu, A.N. (2013). Raffinose and hypotaurine improve the post-thawed Merino ram sperm parameters. *Cryobiology*, *67*(1), pp.34-39.

Chaudhari, D.V.; Dhami, A.J.; Hadiya, K.K. and Patel, J.A. (2015). Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (− 196 C) of buffalo semen. *Veterinary world*, *8*(2), pp.239-244.

Chen, Q. and Haddad, G.G. (2004). Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals. *Journal of Experimental Biology*, *207*(18), pp.3125-3129.

Chen, Y.; Foote, R.H. and Brockett, C.C. (1993). Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. *Cryobiology*, *30*(4), pp.423-431.

Chhillar, S.; Singh, V.K.; Kumar, R. and Atreja, S.K. (2012). Effects of Taurine or Trehalose supplementation on functional competence of cryopreserved Karan Fries semen. *Animal reproduction science*, *135*(1), pp.1-7.

Cirit, Ü.; Bağış, H.; Demir, K.; Agca, C.; Pabuccuoğlu, S.; Varışlı, Ö.; Clifford-Rathert, C. and Agca, Y. (2013). Comparison of cryoprotective effects of iodixanol, trehalose and cysteamine on ram semen. *Animal reproduction science*, *139*(1), pp.38-44.

Dalimata, A.M. and Graham, J.K. (1997). Cryopreservation of rabbit spermatozoa using acetamide in combination with trehalose and methyl cellulose. *Theriogenology*, *48*(5), pp.831-841.

Dolti, P.; Moghaddam, G.h. and Ahmadian, H. (2016). Evaluation of the effects of cysteine and trehalose on long-term cryopreservation of ram semen. J Agri-Food ApplSci, 4(1), pp.1- 6.

Dorado, J.; Hidalgo, M.; Muñoz, A. and Rodríguez, I. (2009). Assessment of goat semen freezability according to the spermatozoa characteristics from fresh and frozen samples. *Animal reproduction science*, *112*(1), pp.150-157.

Duncan, D.B. (1955). Multiple Rang and Multiple F-test. Biometrics. 11: 4-42.

El-Badry, D.A.; El-Maaty, A.M.A. and Gamal, A. (2017). The Effect of Trehalose Supplementation of INRA-82 Extender on Quality and Fertility of Cooled and Frozen-Thawed Stallion Spermatozoa. *Journal of Equine Veterinary Science*, *48*, pp.86-92.

El-Sheshtawy, R.I.; Sisy, G.A. and El-Nattat, W.S. (2015). Effects of different concentrations of sucrose or trehalose on the post-thawing quality of cattle bull semen. *Asian Pacific Journal of Reproduction*, *4*(1), pp.26-31.

Evans, G. and Maxwell, W.C. (1987). *Salamons' artificial insemination of sheep and goats* (No. Ed. 2). Butterworths, Sydney.

Farshad, A. and Akhondzadeh, S. (2008). Effects of sucrose and glycerol during the freezing step of cryopreservation on the viability of goat spermatozoa. *Asian-Aust. J. Anim. Sci*, *21*, pp.1721-1727.

Foote, R.H.; Chen, Y.; Brockett, C.C. and Kaproth, M.T. (1993). Fertility of bull spermatozoa frozen in whole milk extender with trehalose, taurine, or blood serum. *Journal of dairy science*, *76*(7), pp.1908-1913.

Funahashi, H. and Sano, T. (2005). Select antioxidants improve the function of extended boar semen stored at 10 C. *Theriogenology*, *63*(6), pp.1605-1616.

Gutiérrez-Pérez, O.; de Lourdes Juárez-Mosqueda, M.; Carvajal, S.U. and Ortega, M.E.T. (2009). Boar spermatozoa cryopreservation in low glycerol/trehalose enriched freezing media improves cellular integrity. *Cryobiology*, *58*(3), pp.287-292.

Gutiérrez-Pérez, O.; Uribe, C.S.; Juárez, M.M.L. and Trujillo, O.M.E. (2008). Cryoprotective effects with low glycerol freezing extender for boar spermatozoa added to different trehalose concentrations. *Theriogenology*, *70*(8), p.1398.

Hami, K. and MollaHoseyni, M. (2011). Trehalose production by yeasts and biotechnological effects as feed additive. First National food safety PP 1. (In Persian).

Hu, J.H.; Li, Q.W.; Jiang, Z.L.; Yang, H.; Zhang, S.S. and Zhao, H.W. (2009). The cryoprotective effect of trehalose supplementation on boar spermatozoa quality. *Reproduction in domestic animals*, *44*(4), pp.571-575.

Hu, J.H.; Zan, L.S.; Zhao, X.L.; Li, Q.W.; Jiang, Z.L.; Li, Y.K. and Li, X. (2010). Effects of trehalose supplementation on semen quality and oxidative stress variables in frozen-thawed bovine semen. *Journal of animal Science*, *88*(5), pp.1657-1662.

Hussain, S.O.; Shahad, H.K. and Al-Badry, K.I. (2016). Effect of dilution, cooling and freezing on physical and biochemical properties of semen for holstein bull born in Iraq. *Adv. Anim. Vet. Sci*, *4*(11), pp.575-579.

Iqbal, S.; Andrabi, S.M.H.; Riaz, A.; Durrani, A.Z. and Ahmad, N. (2016). Trehalose improves semen antioxidant enzymes activity, post-thaw quality, and fertility in Nili Ravi buffaloes (*Bubalus bubalis*). *Theriogenology*, *85*(5), pp.954-959.

Jafaroghli, M.; Khalili, B.; Farshad, A. and Zamiri, M.J. (2011). The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. *Small ruminant research*, *96*(1), pp.58-63.

Khalili, B.; Farshad, A.; Zamiri, M.J.; Rashidi, A. and Fazeli, P. (2009). Effects of sucrose and trehalose on the freezability of Markhoz goat spermatozoa. *Asian-australas J Anim Sci*, *22*, pp.1614-1619.

Kozdrowski, R. (2009). The effect of trehalose on post-thaw viability and fertility of European brown hare (Lepuseuropaeus Pallas, 1778) spermatozoa. *Animal reproduction science*, *116*(3), pp.326-334.

Liu, Z.; Foote, R.H. and Brockett, C.C. (1998). Survival of bull sperm frozen at different rates in media varying in osmolarity. *Cryobiology*, 37, pp.219-230.

Lopez Saez, A.; Ortiz, N.; Gallego, L. and Garde, J.J. (2000). Liquid storage (50C) of ram semen in different diluents. *Archives of andrology*, *44*(2), pp.155-164.

Malo, C.; Gil, L.; Gonzalez, N.; Cano, R.; De Blas, I. and Espinosa, E. (2010). Comparing sugar type supplementation for cryopreservation of boar semen in egg yolk based extender. *Cryobiology*, *61*(1), pp.17-21.

Martins-Bessa, A.; Rocha, A. and Mayenco-Aguirre, A. (2009). Effects of taurine and hypotaurine supplementation and ionophore concentrations on post-thaw acrosome reaction of dog spermatozoa. *Theriogenology*, *71*(2), pp.248-253.

Matsuoka, T.; KOHNO, H. and FUKUI, Y. (2006). Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. *Journal of Reproduction and Development*, *52*(5), pp.675-683.

Michael, A.; Alexopoulos, C.; Pontiki, E.; Hadjipavlou-Litina, D.; Saratsis, P. and Boscos, C. (2007). Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology*, *68*(2), pp.204-212.

Molinia, F.C.; Evans, G.; Casares, P.Q. and Maxwell, W.M.C. (1994). Effect of monosaccharides and disaccharides in Tris-based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. *Animal Reproduction Science*, *36*(1-2), pp.113-122.

Najafi, A.; Zhandi, M.; Towhidi, A.; Sharafi, M.; Sharif, A.A.; Motlagh, M.K. and Martinez-Pastor, F. (2013). Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology*, *66*(3), pp.275-282.

Nur, Z.; Zik, B.; Ustuner, B.; Sagirkaya, H. and Ozguden, C.G. (2010). Effects of different cryoprotective agents on ram sperm morphology and DNA integrity. *Theriogenology*, *73*(9), pp.1267-1275.

Reddy, N.S.S.; Mohanarao, G.J. and Atreja, S.K. (2010). Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo (*Bubalus bubalis)* sperm quality following cryopreservation. *Animal reproduction science*, *119*(3), pp.183-190.

Rehman, F.U.; Zhao, C.; Shah, M.A.; Qureshi, M.S. and Wang, X. (2013). Semen extenders and artificial insemination in ruminants. *Veterinaria*, *1*(1), pp.1-8.

Salvador, I.; Yániz, J.; Viudes-de-Castro, M.P.; Gómez, E.A. and Silvestre, M.A. (2006). Effect of solid storage on caprine semen conservation at 5 C. *Theriogenology*, *66*(4), pp.974-981.

Sariözkan, S.; Bucak, M.N.; Tuncer, P.B.; Uiutas, P.A. and Bilgen, A. (2009). The influence of cystein and taurine on microscopic oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. Cryobiol., 58: pp.134-138.

SAS. (2012). Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA.

Shahba, M.I.; El-Sheshtawy, R.I. and El-Azab, A.I. (2016). Improvement of buffalo semen freezability by using TRIS extender enriched with different concentrations of trehalose/sucrose. *J Innovations in Pharmaceuticals and Biological Sci*, *3*, pp.198-203.

Shaikh, K.Q.; Nakhashi, H.C.; Suthar, B.N.; Sutaria, P.T. and Sharma, V.K. (2016). Trehalose as semen diluent additive for cryopreservation of Kankrej bull's semen. Life Sci Leaflets, 71, pp.29 – 40.

Sitaula, R.; Elmoazzen, H.; Toner, M. and Bhowmick, S. (2009). Desiccation tolerance in bovine sperm: a study of the effect of intracellular sugars and the supplemental roles of an antioxidant and a chelator. *Cryobiology*, *58*(3), pp.322-330.

Storey, B.T.; Noiles, E.E. and Thompson, K.A. (1998). Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation. *Cryobiology*, *37*(1), pp.46-58.

Sztein, J.M.; Noble, K.; Farley, J.S. and Mobraaten, L.E. (2001). Comparison of permeating and non permeating cryoprotectants for mouse sperm cryopreservation. *Cryobiology*, *42*(1), pp.28-39.

Tuncer, P.B.; Sarıözkan, S.; Bucak, M.N.; Ulutaş, P.A.; Akalın, P.P.; Büyükleblebici, S. and Canturk, F. (2011). Effect of glutamine and sugars after bull spermatozoa cryopreservation. *Theriogenology*, *75*(8), pp.1459-1465.

Tuncer, P.B.; Taşdemir, U.; Büyükleblebici, S.; Özgürtaş, T.; Coşkun, E.; Erol, H.; Aydın, F.N. and Gürcan, İ.S. (2013). Effects of different doses of trehalose supplementation in egg yolk extender in frozen–thawed Angora buck semen. *Small Ruminant Research*, *113*(2), pp.383-389.

ÜSTÜNER, B.; Nur, Z.; Alcay, S.; TOKER, M.B.; SAĞIRKAYA, H. and Soylu, M.K. (2015). Effect of freezing rate on goat sperm morphology and DNA integrity. *Turkish Journal of Veterinary and Animal Sciences*, *39*(1), pp.110-114.

Uysal, O. and Bucak, N.B. (2009). The role of different trehalose concentrations and cooling rates in freezing of ram semen. Ankara University, *Veterinary Faculty Degree*, 56, pp.99-103.

Uysal, O.; Bucak, M.N.; Yavas, I. and Varisli, O. (2007). Effect of various antioxidants on the quality of frozen-thawed bull semen. *J. Anim. Vet. Adv*, *6*(2), pp.1362-1366.

Watson, P.F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction, Fertility and Development*, *7*(4), pp.871-891.

Woelders, H.; Matthijs, A. and Engel, B. (1997). Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology*, *35*(2), pp.93-105.

Yamashiro, H.; Narita, K.; Sugimura, S.; Han, Y.J.; Sugawara, A.; Morohaku, K.; Nakazato, F.; Konno, T.; Yoshida, M. and Sato, E. (2007). Trehalose enhanced the freezability of Poodle dog sperm collected by an artificial vagina (AV). *Animal reproduction science*, *102*(1), pp.165-171.

Yildiz, C.; Kaya, A.; Aksoy, M. and Tekeli, T. (2000). Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. *Theriogenology*, *54*(4), pp.579-585.

Access this Article in Online	
	Website: www.ijarbs.com
Quick Response Code	Subject: Veterinary Medicine
DOI:10.22192/ijarbs.2017.04.04.025	

How to cite this article:
Qusay Mohammed Al-Badrany,Kreem Iwaid Al-Badry, Faris Faisel Ibrahim, Wafa'a Yeedam Lateef. (2017). Effect of addition trehalose and steps of freezing on sperm properties of bull frozen in liquid nitrogen. Int. J. Adv. Res. Biol. Sci. 4(4): 189-200.
DOI: http://dx.doi.org/10.22192/ijarbs.2017.04.04.025