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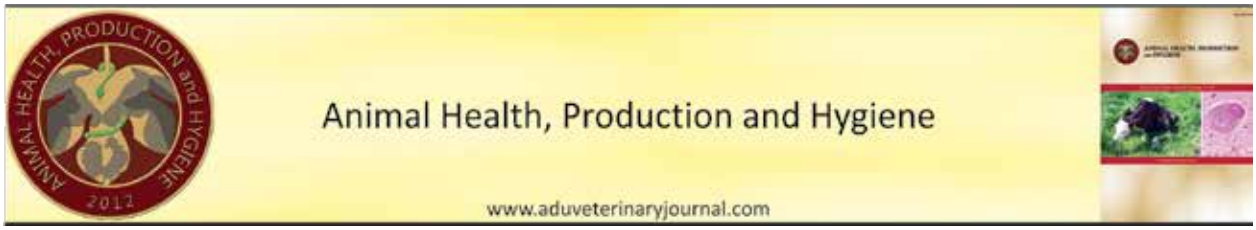

Review

Trehalose as a Cryoprotective Agent for the Sperm Cells: A Mini Review

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A B S T R A C T

Although many protocols have been developed for semen cryopreservation, sperm cryosurvival rate is still not optimum in most of the species. Factors responsible for low cryosurvival rate are; changes in temperature, ice formation, lipid peroxidation, alterations in sperm membrane, toxicity of cryoprotectants and osmotic stress. Moreover, cell dehydration during freezing process is one of the factors altering the structural and functional integrity of the plasma membrane. Sugars have several functions in sperm extender, including providing energy substrate for the sperm cell during incubation, maintaining the osmotic pressure of the diluents, and acting as a cryoprotectant. Trehalose, (α-D-glucopyranosyl-α-D-glucopyranoside) a non-permeable sugar, has a significant role to prevent deleterious effect of cell dehydration on plasma membrane. Recent reports have shown encouraging data regarding the use of trehalose for the cryopreservation of sperm cells. Therefore, in this review, information is presented related to the use of trehalose for cryopreserving sperm from different species.

Keywords: Cryoprotective agent, Sperm cell, Trehalose

Spermatozoon Dondurulmasında Koruyucu Bir Madde Trehalose: Kısa Derleme

Ö Z E T

Spermanın dondurularak saklanmasına yönelik bir çok yöntemin geliştirilmiş olmasına rağmen, bir çok hayvan türünde çözüm sonu yaşama oranları henüz istenen düzeye ulaşmamıştır. Sıcaklık değişimleri, buz oluşumu, lipidoksidasyonları, membran yapısındaki değişiklikler, kriyoprotektanların toksik etkileri ve ozmotik stres bu düşük yaşama oranlarına yol açan temel faktörlerdir. Bunların yanı sıra dondurma sırasında hücrenin su kaybetmesi de plazma membranının yapısal ve fonksiyonel bütünlüğünü bozan bir faktördür. Sperma sulandırıcılarına eklenen şekerler spermatozoonlar için enerji kaynağı oluşturmanın ötesinde ozmotik basıncı ayarlar ve kriyoprotektan olarak etki gösterirler. Non-permeable bir şeker olan trehaloz (α-D-glucopyranosyl-α-D-glucopyranoside) su kaybı sırasında hücre membranında oluşan olumsuz etkilerin önlenmesinde önemli bir etkiye sahiptir. Son yıllarda yapılan çalışmalar spermanın dondurularak saklanması sırasında trehaloz kullanmayı teşvik eden bulgular sunmaktadır. Bu nedenle, bu derlemede çeşitli hayvan türlerinde sperma dondurma işlemleri sırasında trehaloz kullanımına ilişkin bilgiler sunulmuştur.

Anahtar Kelimeler: Kriyoprotektan maddeler, Spermatozoon, Trehaloz

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Introduction

Artificial insemination has an important role in domestic animal breeding to improve the production in term of milk, beef, mutton, leather, wool and hair. The success of an AI program depends on the proper management of semen collection, storage and use (Leboeuf et al., 2000). Although, many protocols have been developed for semen cryopreservation, sperm cryosurvival rate is still not optimum in most of the species. Cryopreservation induces some irreversible damages in sperm cells, which have been reviewed previously (Bailey et al., 2000; Medeiros et al., 2002). Factors responsible for these damages includes; changes in temperature, ice formation, lipid peroxidation, alterations in sperm membrane, toxicity of cryoprotectants and osmotic stress (Watson, 1995, 2000). It is generally believed that cryopreservation induces cell dehydration by removal of water, which results in a higher solute concentration, reduced cell volume, and ultimately alters the structural and functional integrity of the plasma membrane. Furthermore, access of reactive oxygen species and lipid peroxidation leads to premature acrosomal reaction, loss of intracellular components, reduced motility and low fertility of sperm during the freezing-thaw process (Beconi et al., 1991, 1993). To keep the cell alive during freezing-thawing process, plasma membrane is a key component that must be maintained (Aboagla and Terada, 2003).

Sugars have several functions in sperm extenders, including providing energy substrate for the sperm cell during incubation (Fukuhara and Nishikawa, 1973), maintaining the osmotic pressure of the diluents (Salamon and Ritar, 1982; Aboagla and Terada, 2003), acting as a cryoprotectant and decreasing the extent of cell injury by reducing the intracellular ice formation (Liu et al., 1998). Among sugars, trehalose, a non-permeable sugar, has a significant role to prevent deleterious effect of cell dehydration on plasma membrane (Aboagla and Terada, 2003). During the last two decades, several studies have been conducted on trehalose supplementation to the freezing extenders of semen in bovine (Chen et al., 1993), buffalo (Reddy et al., 2010), ram (Aisen et al., 2000; Bucak et al., 2007), goat (Aboagla and Terada, 2003; Ateşşahin et al., 2008), boar (Gutiérrez-Pérez et al., 2009, Hu et al., 2009), dog (Yamashiro et al., 2007) and rabbit (Dalimata and Graham, 1997) aiming to improve post-thaw semen characteristics. This review will summarize the outcome of studies conducted on the supplementation of trehalose to semen extenders as a strategy to improve the post-thaw semen quality.

Why Trehalose

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide consisting of two glucose moieties joined together by an alpha-1, 1 glucosidic bond (Patist and Zoerb, 2005). It exists naturally in insects, plants and fungi as well as some bacteria (Sussman and Lingappa, 1959; Aisen et al., 2000). It has stabilizing effect on both cellular protein and membrane; however, nature of this mechanism is still unclear. Probably, there is formation of a hydrogen bond between the sugar hydroxyl group and the phospholipid polar head group during dehydration which substitutes for water molecules in the membrane, which helps to maintain membrane integrity during dehydration conditions (Crowe et al., 1984; Woelders et al., 1997). Some authors reported that trehalose renders the hypertonic media causing cellular osmotic dehydration before freezing. This osmotic effect decrease intracellular freezable water and then decrease the amount of cell injury by ice crystallization (Storey et al., 1998; Schmehl et al., 1986; Molinia et al., 1994).

Comparison between different concentrations of trehalose in semen extenders

Most of the previous studies were designed to compare the efficacy of different concentrations of trehalose on post-thaw semen characteristic (Aisen et al., 2002; Aboagla and Terada, 2003; Matsuoka et al., 2006). To the best of author's knowledge, trehalose concentrations in extenders for semen cryopreservation of different species range between 25-400 mM. The influence of different concentrations of trehalose on frozen-thawed sperm parameters in different species is presented in Table 1. Generally in protocols where 100 mM and 70 mM trehalose concentrations were used, best post-thawing semen parameters were recorded in ram (Aisen et al., 2002; Bucak et al., 2007; Jafaroghli et al., 2011), goat (Khalili et al., 2009), boar (Hu et al., 2009) and in dog semen (Yildiz et al., 2000). Furthermore, Aisen et al. (2002) reported that high concentration of trehalose (200 and 400 mOsm) has deleterious effect on sperms during cooling process and concluded that this might be due to the incompatibility between water efflux and cellular integrity. In contrast, some authors found significant protection against freezing damage by using high concentrations of trehalose (0.35 M and 0.375 M) in mouse (An et al., 2000) and goat (Aboagla and Terada, 2003) spermatozoa. The variable results of these studies might be due to different extender compositions used or different species exhibiting different tolerance level for trehalose. Therefore, the effect of different concentrations of trehalose should be reevaluated across the species.

Comparative cryoprotective efficacy of trehalose with other sugars and additives

The cryoprotective ability of sugars may differ according to storage temperature, molecular weight of sugar and type of buffer used (Yildiz et al., 2000). Attempts has been made to compare the cryoprotective efficacy of trehalose in semen extenders with different sugars (fructose, galactose, glucose, xylose, lactose, maltose, sucrose, mannose, raffinose) and some other additives (taurine, cysteamine, hyaluronan) in ram (Bucak et al., 2007), dog (Yildiz et al., 2000), goat (Khalili et al., 2009), bulls (Woelder et al., 1997), stallion (Squires et al., 2004) and red deer (Fernández-Santos et al., 2007) . Raffinose compared to trehalose showed a significantly better

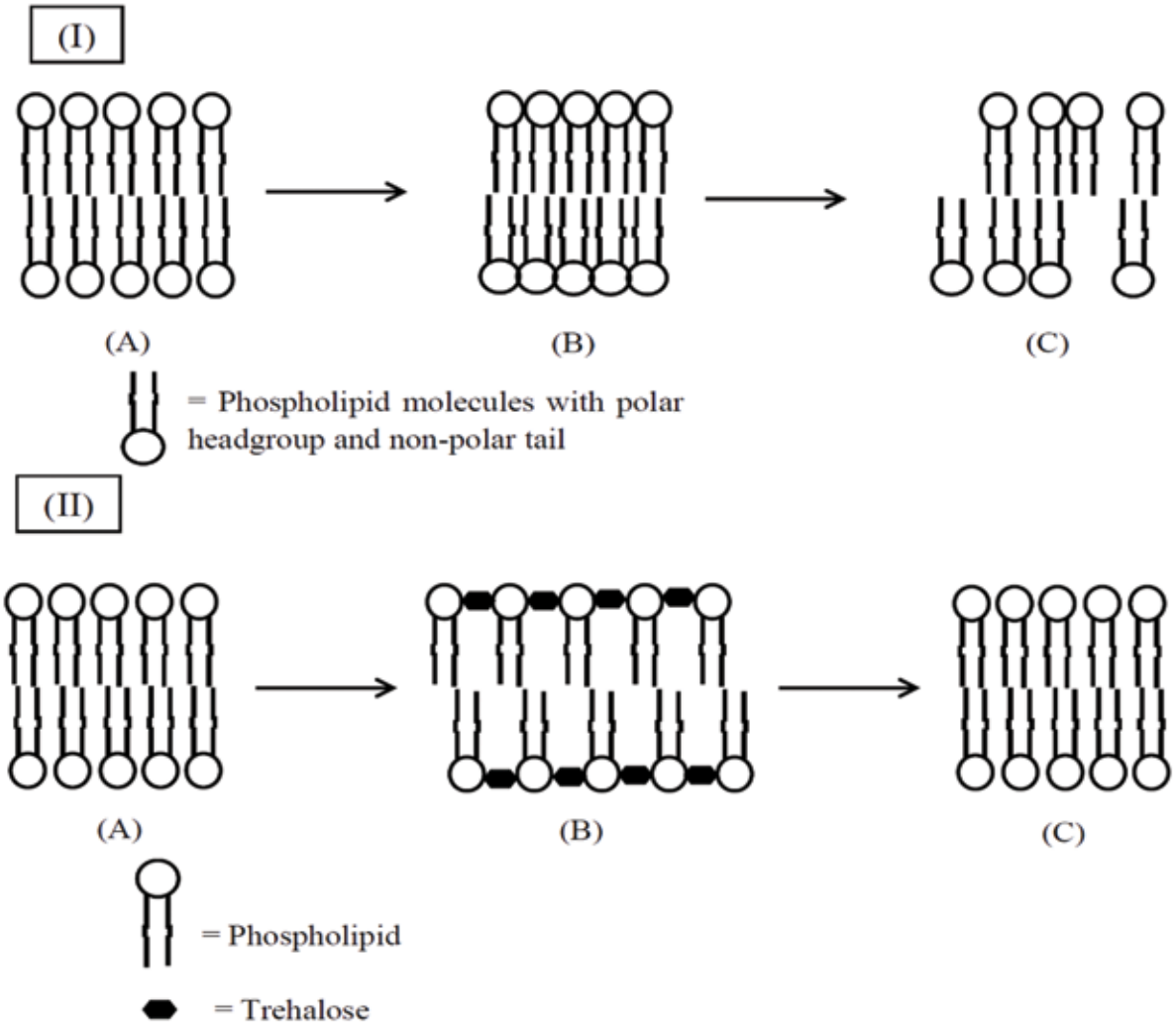

Figure 1. (I)"Biological membrane (phospholipid bilayer) in lamellar phase (A) showing transition to gel phase upon drying (B) and subsequent dehydration (C) causing packing defects making the membranes leaky. **(II)** Proposed mechanism by which trehalose preserves biomaterials. Trehalose shows a direct interaction with the head groups during drying, reducing the van der Waals interactions among the hydrocarbon chains (A → B). Upon rehydration the membrane integrity remains intact (C) (Patist and Zoerb 2005)"
Şekil 1 (I) "Lamellar fazdaki biyolojik membran (çift katlı fosfolipit) (A) kurumaya bağlı olarak jel fazına dönüşmesi (B) ve daha sonraki dehidrasyon (C) membranın yeniden düzenlenmesi sırasında oluşan sorunlar membranı delikli bir hale getirir. **(II)** Trehalozun biyolojik materyalleri koruma mekanizmasını açıklayan hipotez. Trehaloz hidrokarbon zincirleri arasındaki van der Waals çekimini azaltarak (A → B) kuruma sırasında hidrokarbon başları ile direk bağlantı kurar. Membranın tekrar su almasından sonra membrane bütünlüğü bozulmaz (C) (Pattist ve Zoerb 2005)"

recovery rate in intact mouse spermatozoa (Storey et al., 1998). Molinia et al. (1994) concluded that sucrose or trehalose are more suitable to preserve the motility of frozen-thawed ram spermatozoa than glucose in the absence of glycerol., However, glycerol incorporation in the diluents with various sugar types furnishes similar results.

While monosaccharides (glucose, galactose) showed almost no protective effect on the cryosurvival rate of mouse spermatozoa, disaccharides (sucrose, trehalose) and trisaccharides (raffinose, melezitose) resulted in higher survival rates (An et al., 2000). In a similar study in red deer, Fernández-Santos et al. (2007) suggested that monosaccharides especially fructose may enhance red deer epididymal sperm cryopreservation, whereas, disaccharides and trisaccharides may not be adequate. More recently Jafaroghli et al. (2011) reported that extenders containing of sucrose, raffinose and trehalose improve post-thawing semen quality of goat semen; however, trehalose or raffinose is more effective than sucrose and significantly decreases the sperm abnormalities. Reddy et al. (2010) observed similar post-thaw motility, viability and membrane integrity by addition of 50 mM taurine or 100 mM trehalose to the freezing extender. Bucak et al. (2007) studied the influence of trehalose, taurine, cysteamine and hyaluronan on ram semen and found that all these additives except hyaluronan improve the motility of ram spermatozoa, but none of them gives significant positive

Table 1 Different concentrations of trehalose used for cryopreservation of sperm cells and observed frozen-thawed sperm quality of different species.

Tablo 1. Sperma dondurulmasında kullanılan farklı trehaloz yoğunlukları ve çeşitli hayvan türlerinde çözüm sonu sperma kalitesi

Species	Studies	Conc.	Motility		Membrane integrity		Acrosome Integrity	
			Treatment	Control	Treatment	Control	Treatment	Control
Bovine	Woelder et al. 1997	0.236 M	3	57	-	-	59	57
	Hu et al., 2010	25 mM	38	37	37	36	55	53
		50 mM	44	-	39	-	59	-
		100 mM	47	-	44	-	65	-
		200 mM	35	-	34	-	51	-
Buffalo	Reddy et al. 2010	100 mM	38	30	50	35	-	-
Ram	Aisen et al. 2002	50 mOsm	50	50	55	40	70	70
		100 mOsm	70	-	60	-	75	-
		200 mOsm	30	-	40	-	60	-
		400 mOsm	20	-	35	-	55	-
	Aisen et al. 2005	76g/L	-	-	64	38	-	-
	Matsuoka et al.2006	437 mM	50	38	Similar		Similar	
	Bucak et al. 2007	50mM	59	48	49	44	5 DA	10 DA
		100 mM	56	-	45	-	6 DA	-
	Soylu et al. 2007	62.5 mM	17	4	27	17	26 DA	28 DA
	Tonieto et al. 2010	100 mM	44 - 49	23	35 - 37	16	56 - 65	49
	Jafaroghli et al. 2011	50 mM	50	40	50	38	12 DA	14 DA
		70 mM	55	-	55	-	8 DA	-
		100 mM	60	-	58	-	5 DA	-
Goat	Aboagla and Terada 2003	0.375 M	78	62	-	-	65	40
	Khalili et al. 2009	50mM	44	35	46	36	10 DA	16 DA
		75mM	50	-	49	-	9 DA	-
		100mM	52	-	55	-	8 DA	-
Dog	Yildiz et al. 2000	70 mM	59	50	-	-	58	45
	Yamashiro et al. 2007	0.375 M	68-72	55-63	-	-	76-83	77-81
Stallion	Squires et al. 2004	9 mM	66	63	-	-	-	-
Boar	Gutiérrez-Pérez et al. 2009	250 mM	19	20	-	-	70	36
	Hu et al. 2009	25 mM	37	30	36	30	54	37
		50 mM	47	-	38	-	63	-
		100 mM	50	-	45	-	67	-
		200 mM	38	-	39	-	65	-
Rabbit	Dalimata and Graham 1997	-	46	43	-	-	53	41
Hare	Kozdrowski 2009	50 mM	12	28	Similar		Similar	
		100 mM	8	-				
Deer	Fernández-Santos et al. 2007	400 mM	49	61	55	56	50	52
Mouse	Sztein et al., 2001	0.3 M	60	40	40	20	-	-

DA= Defected Acrosome

effect on viability, morphological abnormality, and membrane integrity by HOST after thawing. In another study, Yildiz et al. (2000) concluded that trehalose, xylose and fructose significantly increased total active sperm rates in frozen thawed dog semen samples compared to other sugars and the sugar-free control. These evidences showed that the addition of trehalose in semen extenders may improve the post thaw semen quality but its influence is variable compared to the other sugars.

Effect of trehalose on sperm functionality

Effect of trehalose on different sperm functionality parameters during and after cryopreservation is discussed in the following sections.

Membrane integrity and fluidity

Sperm plasma membrane is one of the primary sites of damage induced by cryopreservation (Hammerstedt et al., 1990; Parks and Graham, 1992; Watson, 1995). It is assumed that sugars enable the plasma membrane less vulnerable to cryo-damage during freezing and thawing process. Most of the previous studies in ram (Tonieto et al., 2010; Jafaroghli et al., 2011), goat (Khalili et al., 2009), bulls (Hu et al., 2010) and boar (Hu et al., 2009) revealed that trehalose supplementation in semen extender enhances the membrane integrity. The presence of trehalose in extenders is likely to modulate membrane fluidity by inserting itself into membrane phospholipids

bilayer, thus it renders membrane more stable during freezing (Aboagla and Terada, 2003). Sperm's osmotic regulation at low temperatures may impair motility and membrane integrity due to the structural damages in the lipid chain of the membrane (Meyers, 2005; Patist and Zoerb, 2005). It is speculated that trehalose shows a direct interaction with the phospholipid polar head groups during dehydration reducing the van der Waals interactions among the hydrocarbon chain and upon rehydration the membrane integrity remains intact. This process has been schematically illustrated by Patist and Zoerb, 2005 (Figure 1). During freezing, the extracellular fluid becomes hypertonic due to the dramatic increase in osmotic pressure exerted by extracellular solutes (Holt and North, 1994; Watson, 1995). In response to this insult, the sperm cell loses water and shrinks in volume and conversely during thawing process, when cell exposed to hypotonic media its volume increases by passive diffusion of water (Meyers, 2005). The exposure of sperm cell to these anisosmotic conditions during freezing and thawing process is the potential factor that renders the plasma membrane more labile and might have harmful consequences on the viability of the cells. It is expected that reducing the osmotic stress or extending the osmotic tolerance of cells would improve their cryotolerance against different solute concentrations. To the author's knowledge there is no study available, which describes the influence of trehalose on osmotic tolerance of the sperm cells. Therefore, future studies should be designed to elucidate this effect of trehalose.

Acrosome Integrity

For successful penetration into oocyte, the spermatozoa require the intact acrosome. It has been reported that freezing and thawing process induce capacitation-like changes such as plasma membrane modification and fluidization, calcium influx, protein tyrosine phosphorylation and reduce the fertilizing ability of the sperm cells (Bailey et al., 2000; Thomas et al., 2006; Watson et al., 1995). In the recent study of buffalo bull semen, Reddy et al. (2010) reported that trehalose has the capacity to reduce this cryocapacitation and maintains the acrosomal integrity. They used lysophosphatidylcholine (LPC) to induce acrosome reaction in trehalose treated frozen-thawed buffalo semen to assess cryocapacitation. The results of this study showed that 100 mM trehalose supplementation to the cryopreservation medium could significantly reduce ($P<0.05$) cryocapacitation after freezing and thawing. Similarly, the studies in dog (Yildiz et al., 2000) and boar (Gutiérrez-Pérez et al., 2009; Hu et al., 2009) concluded that trehalose help to reduce the acrosomal abnormalities occurring during cryopreservation. In spite of these evidences, in favor of the beneficial effect of trehalose on acrosomal integrity, there are some studies in ram (Soylu et al., 2007; Tonieto et al., 2010), bull (Woelder et al., 1997) and rabbit (Dalimata and Graham, 1997) demonstrating its minor or no impact on acrosomal integrity. However, by induction of artificial acrosomal reaction in fresh semen either by calcium ionophore or lysophosphatidylcholine the acrosome integrity maintaining capacity of trehalose should be illuminated.

Biochemical parameters of sperm cells

Production of reactive oxygen species during freezing and thawing process leads to lipid peroxidation and consequently results in impaired cell functions, low sperm fertility (Lenzi et al., 2002; Bucak et al., 2007). It has been well elucidated that reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) in the seminal plasma form an antioxidant system and inhibit the lipid peroxidation (Aitken and Baker, 2004; Gadea et al., 2004). Trehalose supplementation in bovine semen increased significantly CAT, GSH-Px, and GSH activity compared with the control group (Hu et al., 2010). Similarly, total antioxidant level was also improved in trehalose treated buffalo semen when it was measured by ferric reducing/antioxidant power (FRAP) assay after freezing and thawing (Reddy et al., 2010). Aisen et al. (2005) quantified GSH as acid-soluble thiols (AST) in frozen-thawed ram semen and found higher AST level both immediately after thawing and 3 hr incubation post-thawing. However, no pronounced antioxidative effect of trehalose was observed except minor elevation of vitamin E production in frozen-thawed ram semen (Bucak et al., 2007). All of above studies concluded that antioxidant properties of trehalose may be related to its effectiveness in membrane cryopreservation.

Effect of trehalose on sperm fertility

Because of the fact that trehalose help to diminish the acrosomal damage and enhance the membrane fluidity during cryopreservation, it would ultimately affect the fertility. Limited number of studies has been conducted to evaluate the fertility of sperms frozen with trehalose. The frozen/thawed sperm cryoprotected with trehalose retained significantly better in-vitro fertility (79%) ($P<0.001$) than control (11%) in mouse; however, percentage of offspring born was the same (Sztein et al., 2001). Similarly, 45 to 47% lambing was obtained in ewes inseminated with sperms cryopreserved in 100 mM trehalose extender, which was 2.5 times higher than control ($P<0.05$) (Aisen et al., 2002). In another study, a significant difference was recorded between fertility rates (46.8% vs. 16.7%) in ewes following AI with frozen-thawed 100 mM trehalose treated or control semen (Jafaroghli et al., 2011). In contrast, the addition of trehalose to an extender did not improve the fertility of frozen-thawed bull semen (Foote et al., 1993) and European brown hare semen (Kozdrowski, 2009). Therefore, future studies should be designed to evaluate more pronounced effect of trehalose.

Conclusion

In summary, the outcomes of these studies (Table 1) evidenced that 100 mM and 70 mM trehalose concentrations can provide best cryoprotection but different species exhibit different tolerance ranges to the freezing-thawing process. These variations might be due

to different compositions of extenders used in different species; therefore, the effect of various trehalose concentrations should be reevaluated. Moreover, it can be speculated that trehalose supplementation to the extender improves the fertility of spermatozoa. Further studies such as effect of trehalose on osmotic tolerance, acrosomal reaction and biochemical parameters of spermatozoa are warranted to elucidate the mechanisms by which trehalose function as cryoprotectant for sperm cells.

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