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AN OVERVIEW OF ADDING RHO-ASSOCIATED COILED-COIL KINASE AND KNOCKOUT SERUM REPLACEMENT WITH TREHALOSE TO A LOW GLYCEROL TRIS-BASED SEMEN EXTENDER

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Abstract: It is known that livestock animal semen is very sensitive to cold shock during freezing processes, and this sensitivity directly affects post-thaw sperm qualities which are progressive motility, mitochondrial membrane potential, sperm nuclear DNA integrity and in vitro spermatological parameters such as plasma membrane and acrosome integrity, and sperm fertility. In addition, with the sudden decrease in the total antioxidant level of the semen after thawing, the sperm cells are insufficient to tolerate their damage. Consequently, significant losses occur in sperm fertility. For this reason, researches on freezing the semen of livestock animals include semen processing; cryopreservation/cryogenic damage – thawing methods - sperm extenders, added antioxidants, the mechanisms of action and metabolic pathways of these antioxidants and physiological and metabolic parameters such as sperm fertility. It has been explained that low dose glycerol (trehalose added to increase the cryoprotectant effect) added to the extender in the freezing of livestock animal semen, knockout serum replacement (KSR) and Rho-associated coiled-coil kinase (ROCK), which are antioxidant additives, can increase the in vitro quality parameters of frozen thawed semen.

Keywords: Sperm, Extender, Glycerol, Trehalose, Antioxidant, KSR, ROCK

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1. Introduction

The membranes of spermatozoa are physiologically fluid mosaics. This membrane structure consists of two sequential phospholipid layers surrounded by glycolipids, glycoproteins and proteins (Wolf et al., 1984). Since these thermodynamic structures consist of 65-70% unsaturated fatty acids, sperm membranes are vulnerable to cryo-shock damage. In addition, irreversible phase changes occur during the freezing of semen (transition from a liquid phase to gel phase) (Khan et al., 2021; Keles et al., 2021). The crystallization that occurs in the membrane due to the temperature change, especially between 5 °C and -5 °C, damages the sperm cells during the freezing process, (Said et al., 2010). Moreover, these phase changes in the membrane cause changes in the amount and kinetics of the enzymes in the membrane, resulting in a decrease in sperm viability and plasma membrane integrity (Gürler et al., 2016). Antioxidant and oxidant balance during and after sperm freezing is vital for sperm survival and function. Antioxidant structures protect cells against sperm dysfunction and developing peroxidative damage (Alvarez et al., 1983; Bilodeau et al., 2000,

Bilodeau et al., 2001, Arslan et al., 2019). As a result of the deterioration of the stabilization of the sperm membrane, the antioxidant - oxidant balance of the cells is disrupted and oxidative stress damages occur (Watson, 1995; Watson, 2000; Gürler et al., 2016).

2. The Usage of Antioxidants and Cryoprotectants

Antioxidant agents and cryoprotectants have recently started to be used in sperm extenders against the decrease in sperm quality parameters (motility, plasma membrane and acrosome integrity, acrosome reaction, vitality, DNA break, lipid peroxidation, total antioxidant capacity) (Maxwell and Watson 1996; Gadea et al., 2004; Bucak et al., 2007; Bucak and Uysal 2008; Amini et al., 2019; Arslan et al., 2019). Sperm cells are equilibrated with the cryoprotectants contained in the extender before the freezing process. Because they must be stabilized intracellularly. It has been observed that they are based on two bases when the mechanisms of action of cryoprotectants are examined; These additives reduce the number of ions in the environment and increase the amount of the unfrozen fraction. Furthermore, structural and molecular cryoprotectants have two basic properties. These have low molecular weight and create toxic effects only when they are added at high rates, respectively (Palasz and Mapletopt 1996). To reduce the toxicity of cryoprotectants; applications such as using low amounts in the extender, shortening the exposure time of sperm cells (incubation/equilibrium) and using non-permeable cryoprotectants should be done (Massip 2001). The cryoprotectants used in the extender as a preservative are biochemically divided into two main groups. They are divided into permeable and non-permeable. For those with permeable cryoprotectant properties; examples include glycerol, ethylene glycol, formamide, and dimethylsulfoxide (DMSO). Permeable ones show their effects by penetrating the cell membrane as they are permeable and 'colligative'. The working mechanism of cryoprotectants, which are in permeable structure, is as follows; they minimize the osmotic shrinkage caused by low temperatures, reduce the electrolyte density formed in the environment during the cryopreservation process, and regulate the dehydration that will occur during the freezing process and create protection in the protein structures of the sperm (Mcgann 1978; Leeuw et al., 1993; Holt, 2000a).

3. Use of Glycerol, Trehalose and Antioxidant Substances in Semen Extenders

Glycerol, which is widely used in sperm extenders and provides high success; It is a hydrophilic polyol compound. One of the main reasons that glycerol is successful in the freezing process, as the C/OH ratio is equal in biochemical structure. However, glycerol creates a toxic effect above a certain concentration in diluents. These effects are as follows; they cause osmotic stress and change the membrane bioenergy balance (Katkov et al., 1998; Woods et al., 2000; Alveranga et al., 2005). The toxic effect of glycerol is species-specific, it can irritate in the female genital tract when used in extenders of rabbit, fish and poultry semen. This irritation can have a contraceptive effect (preventing fertilization and pregnancy) in sperm fertilization. The glycerol used in the extender has threshold values for animal species. This threshold value is 4-5% in stallion sperm, 4-8% in ruminants, 5% in bucks, 3% in pigs and 1.75% in mice. Toxic effects can be seen when these threshold values are exceeded. Especially these toxic effects can change the membrane structure, protein and glycoprotein structures. In addition to these, decreases may occur in mitochondrial membrane potential due to the toxic effect, and serious declines may be observed in total and progressive motility. Also, these undesirable effects have been reported to cause irreversible damage to the acrosomal region of the sperm (Hammerstedt et al., 1990; Sinha et al., 1996; Katkov et al., 1998; Morrell and Hodge 1998; Holt, 2000b; Alvarenga et al., 2000). External cryoprotectants used in the extender try to reduce the peroxidative damage (lipid peroxidation) that occurs during freezing/thawing in the cell. Because these structures provide the cell membrane to gain a flexible structure. These external cryoprotectants cause increased membrane permeability to cations. If these structures are used in the diluent, a low rate of permeable cryoprotectant (glycerol, ethylene glycol, etc.) is used, and as a result of this process, the toxic effects of internal cryoprotectants will be minimized (Arav et al., 1993; Cabria et al., 2001). Another substance used as an external cryoprotectant in sperm extenders is trehalose. As a biochemical structure, it is a disaccharide compound and is formed by the bonding of two D-glucose molecules (Richards et al., 2002). The working mechanism of trehalose is not known exactly. However, they penetrate the sperm plasma membrane and expand the surface area by forming hydrogen bonds with the polar head groups of the phospholipids in this region during freezing/thawing. It is also thought that they act as a buffer and exert an osmotic protective effect and reduce the release of free oxygen radicals formed during freezing (Mcwilliams et al., 1995; Gao and Crister 2000; Aisen et al., 2005; Purdy 2006). Studies conducted in recent years have shown that the use of trehalose in mammalian sperm extenders and the end-of-solution quality parameters of sperm have increased. These are, respectively, increasing sperm motility, plasma membrane and acrosome integrity, protecting the total antioxidant capacity and reducing the lipid peroxidation that occurs (Aisen et al. 2000, 2002; Aboagla and Terada 2003; Bucak et al. 2007). Cryoprotective agents are known to have an indirect and direct effect on the fertility of frozen thawed semen. Due to the contraceptive effects of structures such as glycerol, it should be added to diluents in lower amounts and supplemented with trehalose to freeze sperm cells. Antioxidant additives must be added to the extender to achieve high sperm fertility (Hammerstedt

1993; Purdy 2006; Bucak et al., 2021). Since oxidative stress damage (superoxide, hydrogen peroxide, hydroxyl radicals, etc.) is thought to minimize and increase the total antioxidant capacity of semen due to lipid peroxidation that develops during freezing and thawing. However, the release of ROS products at the basal level is a positive effect on sperm fertility (for spermatozoa fusogenetics and necessary membrane lubrication). For this reason, it is not desired to eliminate ROS products in the environment and to increase the total antioxidant capacity excessively (Sies 1993). Classical extenders such as Tris-egg yolk, which are not commercial preparations and prepared by enterprises and laboratories, are also very sensitive to freezing damage due to the presence of traces and/or low antioxidants (Parrish et al., 1986). Antioxidant agents minimize cold shock damage, prevent intracellular crystal formation and have a protective effect against decrystallization and destabilization of the developing plasma membrane during solution. For this purpose, one of the additives with high antioxidant properties, which is extremely new today; it is thought to have a positive effect on the freezing of livestock animal sperm by adding Knockout Serum Replacement (KSR) and Rho-associated coiled coil kinases (ROCK). Also, these additives could increase motility values and mitochondrial membrane potential, reduce plasma membrane acrosome integrity damage, protect sperm viability and DNA integrity, suppress apoptosis and activate antioxidant response signalling pathways. For this, it is of great importance to use additional doses with an optimum effect so that antioxidants do not have a toxic effect on semen and adversely affect sperm fertility (Alvarez and Storey 1984; Aitken and Baker, 2004; Uysal et al., 2005; Bucak et al., 2007).

4. Knockout Serum Replacement

Knouckout Serum Replacement (KSR) is a rich source of antioxidants, vitamins, proteins, amino acids and trace elements. Recent studies have shown that it is a necessary serum substitute for mammalian cell survival, growth and development in vitro (Sato et al., 2011; Aoshima et al., 2013; Zhang et al., 2016, Taher-Mofrad et al., 2020). In addition, the use of KSR, which has an antioxidant effect, in the cryopreservation of different cell types has been reported and positive results have been obtained (Lee et al., 2014; Ha et al., 2016; Park et al., 2018). KSR provides activation of a protein structure, AKT (Protein Kinase B), as a working mechanism. AKT enables the glucose transport protein (GLUT-4) in the cell cytosol to move towards the plasma membrane and allows glucose, which is its main task here, to enter the cell (Ishii et al., 2015). In addition, KSR BIM (BCL 2-like protein 11) inhibits cytochrome C (electron-carrying structures for oxidative phosphorylation) originating from and secreted by mitochondria, positively affecting cell viability and preventing apoptosis (Ishii et al., 2015). Studies have shown that KSR stimulates the formation of BIM-resistant mitochondria and KSR has a direct and indirect effect on the energy pathways of the cell (Ishii et al., 2015). Adding 10% KSR to the extender had a positive effect on sperm acrosome and DNA integrity (Taher-Mofrad et al., 2020).

5. Rho-Associated Coiled-Coil Kinase (ROCK)

The Rho-associated coiled coil is a small G protein that mediates and/or transports intracellular signaling mechanisms. One of the effectors of this material is the Rho-associated helix-helix kinase (ROCK) (Ark et al., 2010; Karaşör et al., 2022). ROCKs have a multifunctional effect on the cell. It has numerous effects on cell cycle regulation, increased motility, regulation of actin dynamics that cause cell invasion, cell adhesion, migration, and inflammatory response (Ark et al., 2010; Schofield et al., 2012). ROCKs exert their primary inhibitory effects in the phagocytosis of apoptotic (programmed death of cells) cells because ROCKs act as complementary receptors in cell phagocytosis. Cells producing apoptosis need mature phagosomes to be cleared by fibroblasts and/or macrophages. This maturation pathway is the Rho-ROCK-ERM pathway (Tosello- Trampont et al., 2003; Erwig et al., 2006). The addition of 10, 20, and 40 μM ROCK to frozen-thawed cat sperm had a positive effect on sperm motility and membrane function.

In a study conducted on cats, the best sperm quality parameters were obtained with the addition of 10 μM ROCK (Tharasanit et al., 2020). Studies have shown that ROCKs contribute to increasing the in vitro fertilization ability of sperm cells (Tharasanit et al., 2020).

6. Conclusion

In the light of literature studies, successful freezing of sperms that are difficult to freeze will be achieved with future studies. In addition, it will be possible to benefit from reducing the number of spermatozoa used in insemination doses and benefiting from the breeders at the highest level.

Author Contributions

The percentage of the author(s) contributions is present below. All authors reviewed and approved final version of the manuscript.

	H.O.A.	E.K.	B.R.	D.A.A.	A.S.	O.B.	A.A.	M.R.	I.A.
C	20	10	10	10	10	10	10	10	10
D	20	10	10	10	10	10	10	10	10
S	20	10	10	10	10	10	10	10	10
L	20	10	10	10	10	10	10	10	10
W	20	10	10	10	10	10	10	10	10
CR	20	10	10	10	10	10	10	10	10
SR	20	10	10	10	10	10	10	10	10

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

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