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Equine semen cryopreservation: inter-individual variation, centrifugation processing, protective agents, and freezing protocols

Criopreservação de sêmen equino: variação inter-individual, processamento de centrifugação, agentes protetores e protocolos de congelamento

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Abstract

Stallion sperm cryopreservation allows for long distance transport of sperm insemination doses. In addition, it can be used for long term storage and maintenance of sperm and gene reserves; e.g. in case of stallions participating in competition, endangered breeds, or prior to castration or removal of a stallion from a stud book in case of illness or death. In most countries, stallions used for semen cryopreservation, are held in supervised quarantine during the entire collection and processing period, while taking into account international regulations concerning animal breeding and disease control. Sperm cryopreservation involves collection of semen, dilution in extenders containing nutrients and protectants, packaging in straws, controlled cooling and freezing, and storage in liquid nitrogen. Frozen samples are thawed prior to use. All these procedures expose sperm to stress, which affects survival and fertility rates. In this presentation, the following factors affecting sperm cryosurvival are discussed: (i) inter- and intra-individual variation of semen quality and survival after cryopreservation, (ii) centrifugation processing for sperm concentration and/or selection and removal of seminal plasma, (iii) composition of the extender that is used, for primary dilution as well as cryopreservation, and (iv) cooling and freezing protocols.

Keywords: cryopreservation, cryoprotective agents, spermatozoa, stallion.

Resumo

A criopreservação do esperma de garanhões permite o transporte em longas distâncias de doses de esperma de inseminação. Além disto, pode ser usado para o armazenamento e manutenção de esperma e reservas de genes em longo prazo; e.g. no caso de garanhões participando de competições, raças em extinção ou antes da castração ou remoção de um garanhão dos livros de origem no caso de doença ou morte. Na maioria dos países os garanhões usados para criopreservação de sêmen são deixados em quarentena supervisionada durante todo o período de coleta e processamento, enquanto considerando regulamentos internacionais com relação a reprodução animal e controle de doenças. A criopreservação de esperma envolve a coleta do sêmen, diluição em extensores contendo nutrientes e protetores, o armazenamento em palhetas, resfriamento controlado e congelamento e armazenamento em nitrogênio líquido. Amostras congeladas são descongeladas antes do uso. Todos estes procedimentos expõem o esperma ao estresse, que afeta taxas de sobrevivência e fertilidade. Nesta apresentação são discutidos os seguintes fatores que afetam a criosobrevivência de esperma: (i) variação inter e intra individual da qualidade de sêmen e sobrevivência após criopreservação, (ii) processamento de centrifugação para concentração de esperma e/ou seleção e remoção de plasma seminal, (iii) composição do extensor usado para diluição primária além da criopreservação, e (iv) protocolos de congelamento e resfriamento.

***Palavras-chave**: agentes crioprotetores, criopreservação, espermatozoides, garanhão.*

Introduction

Cryopreserved stallion sperm is of great importance for the breeding industry, but reputed for its high degree of inter-individual variability. Differences in stability of sperm towards cryopreservation processing have been suggested to be related to cellular damage compromising sperm function. Membranes are thought to be the primary site of cryopreservation injury. In this review, we discuss factors affecting the success of stallion sperm cryopreservation including: inter-individual variation, centrifugation processing, protective agents and freezing protocols.

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Inter- and intra-individual variation of semen quality and cryosurvival

Not every stallion fulfills the prerequisites that are needed for successful sperm cryopreservation. Cryopreserved stallion sperm is of great importance for the breeding industry, but reputed for its high degree of inter-individual variability with respect to sperm quality after freezing and thawing, and pregnancy rates when used for inseminating mares (Vidament et al., 1997; Loomis and Graham, 2008).

Semen collection for use for cryopreservation typically takes place outside the breeding season. First, after a period of sexual rest, extragonadal sperm reserves should be depleted by repeated semen collections. Furthermore, regular semen collection intervals (e.g. every 48 h) should be employed in order to ensure sperm quality and minimize differences among ejaculates. However, different individuals/sires may require optimization of semen collection intervals/protocols (Sieme et al., 2004). For animals like stallions, which exhibit a breeding season, the optimal period for manufacturing cryopreserved sperm is a matter of debate. Whereas some authors reported no noteworthy seasonal effects on the quality of frozen-thawed sperm, others reported higher sperm cryosurvival rates for cryopreservation performed during the non-breeding season (Janett et al., 2003).

It is generally believed that sperm cryosurvival rates are dependent on individual characteristics. Sires that are fertile under normal field conditions can produce semen that, when used for cryopreservation and insemination results in low pregnancy rates. The mechanisms underlying differences in stability towards cryopreservation damage are poorly understood but can be genetic in origin. With the increasing number of studies on individual genomic sequences, specific mRNA and protein expression levels, and their correlations with specific traits including sperm cryosurvival, selection of sires for semen cryopreservation programs based on their genetic properties may become possible in the future (Pérez-Rico et al., 2014; Jobim et al., 2011).

In addition to genetic factors, factors of non-genetic origin may be involved in determining sperm function before and after cryopreservation (Katila, 2001). Warmblood stallions are typically suitable for participation in semen cryopreservation programs when they produce ejaculates containing more than 200 x 10^6 sperm ml^{-1}, with more than 50% progressively motile sperm and 70% morphologically normal sperm (Sieme, 2011). Post-thaw motility is typically higher when using ejaculates with high sperm concentrations, as compared to ejaculates with low sperm concentrations. Therefore, the number of mounts taken by the stallion per ejaculate should be kept as low as possible, since more mounts result in an increase of the gel-free volume of an ejaculate coinciding with decreased sperm concentration and motility (Kalmar et al., 2014).

It has been proposed that the ability of sperm to survive cryopreservation correlates with their ability to withstand stress (Vidament et al., 1989), and temperature-induced membrane reorganizations (Oldenhof et al., 2012). Pre- and post-freeze sperm progressive motility and plasma membrane integrity typically correlate: the higher the number of viable sperm prior freezing, the higher the number of sperm surviving cryopreservation (Oldenhof et al., 2015).

Centrifugation processing for sperm selection and removal of seminal plasma

Presence of seminal plasma has been described to be harmful for stallion spermatozoa when stored at 5°C or cryopreserved. Semen can be subjected to centrifugation processing for minimizing deleterious effects of seminal plasma on semen quality and to increase the sperm concentration. Retention of 5-20% (v/v) seminal plasma after centrifugation, however, has been described to result in increased cryosurvival rates (Moore et al., 2005).

Directly after collection, semen should be diluted with at least one volume of primary extender of 37°C, or to 50-100 x 10^6 sperm ml^{-1}. Ordinary centrifugation is typically done at 400-600 x *g* for 10 min. Higher speeds for longer duration result in lower sperm losses, but packing of sperm in a dense pellet together with cellular debris can be detrimental. Alternatively, high-speed cushioned centrifugation can be performed for improving sperm recovery and/or quality. With such an approach, a dense so-called cushion medium (e.g. 60% iodixanol) is layered on the bottom of centrifuge tube, under the sperm sample, on which the spermatozoa will 'float' during centrifugation (20 min, 1000 x *g*). Also, glass nipple-bottom tubes with a small volume of cushion fluid can be used, without the need of removal of cushion fluid after centrifugation before cryopreservation (Waite et al., 2008). Recently, a seminal plasma removal method referred to as 'SpermFilter' has been developed (Ramires-Neto et al., 2013).

Density gradient centrifugation can be performed for enrichment of samples with increased numbers of morphologically normal and motile sperm with increased chromatin intactness (Sieme and Oldenhof, 2015b). Such 'good quality' sperm are separated from seminal plasma as well as immature and dead sperm and microorganisms based on differences in density/weight. Removal of cellular debris might eliminate sources for damaging reactions affecting sperm quality during preservation. Selecting sperm via density centrifugation prior to cryopreservation has been described to be particularly beneficial for so-called poor freezer stallions (Stuhtmann et al., 2012).

In case of delayed cryopreservation after one day refrigerated storage, performing centrifugation processing directly after collection resulted in higher percentages of post-thaw membrane integrity compared to samples that were subjected to centrifugation processing after storage (Heutelbeck et al., 2015). Thus, it is best to perform sperm selection procedures as soon as possible, thereby limiting the damages as accumulate during refrigerated storage.

Composition of the extender used, for primary dilution as well as cryopreservation

As a primary extender, for diluting raw semen, either buffered saline/sugar (e.g. glucose-EDTA), or skim milk/sugar solutions (e.g. INRA-82) can be used. This primary extender protects sperm and maintains sperm viability during centrifugation processing.

Freezing extenders for stallion sperm typically contain skim milk, egg yolk, and glycerol as cryoprotective agents (Sieme and Oldenhof, 2015a). Skim milk can be replaced with defined milk proteins like phosphocaseinate and ß-lactoglobulin (Batellier et al., 1998). Egg yolk is generally used in extenders at various concentrations of 2-25% (v/v). Clarified egg yolk and/or detergents can be added to solubilize egg yolk lipids and lipoproteins. Egg yolk can be substituted by defined phospholipids of non-animal origin. Furthermore, low-density lipoproteins, isolated from egg yolk, have been described to effectively replace egg yolk. In addition to glycerol, other permeating agents like ethylene glycol or dimethyl formamide can be used (at concentrations ~2.5%). Membrane impermeable disaccharides such as sucrose or trehalose, as well as polysaccharides and proteins have also been employed for cryopreserving sperm. Cryoprotective agents protect sperm during cryopreservation by minimizing exposure to osmotic stress, affecting ice formation, stabilizing biomolecules and cellular structures, and limiting the damaging effects of reactive oxygen species (Oldenhof et al., 2013).

For commercial purposes, regulations on minimum values for numbers and/or percentages of motile and morphological sperm which need to be present in a dose for use for artificial insemination have to be taken into account, as well as antibiotics use. Generally the total number of sperm per straw or insemination dose is calculated. For stallions, 4-8 straws of 0.5 ml containing ~50-100 x 10^6 sperm typically result in ~250 x 10^6 progressively motile sperm post-thaw.

Cooling and freezing protocols

Cooling-induced membrane domain reorganizations, irreversible conformational changes of membrane proteins and leakage of intracellular solutes, have been implicated in causing chilling and freezing injury, impairing sperm function. After adding freezing extender, sperm samples are typically slowly cooled from room temperature to 5°C (i.e., at 0.1°C min^{-1}; Salazar et al., 2011), after which semen is packaged into (0.5 ml) straws while maintaining sperm at 5°C.

The optimal cooling rate during freezing is defined as the rate where damage due to intracellular ice formation associated with rapid cooling and osmotic dehydration associated with slow cooling is minimal. For sperm, an optimal cooling rate of 40-60 °C min^{-1} has been determined. Such a cooling rate can be obtained when straws are placed at a predetermined defined distance (2.5-5 cm) above liquid nitrogen for 20 min, after which they can be plunged and stored in liquid nitrogen. Alternatively, a controlled rate freezing machine can be used. Thawing is typically done rapidly, by incubating straws for 30 s at 37ºC.

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