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NEGATIVE IMPACT OF CRYOPRESERVATION AND CRYODAMAGE ON BOVINE SPERMATOZOA: A REVIEW

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ABSTRACT

Cryopreservation of bovine spermatozoa represents a powerful technology for the distribution of livestock and improvement of the availability and quality of the genetic material in numerous ruminant species. Nevertheless, a significant amount of sperm cells shows typical features of cryodamage, which may affect the quality and fertility potential of thawed samples used for artificial insemination. Low temperatures during the freezing process could cause several issues, which may lead to irreversible cell damage. Most common contraindications related to cryopreservation include the presence of ice crystals, oxidative stress, microbial contamination, cryocapacitation or a possible toxicity of cryoprotectants. However, cryobiology plays an important role in the reproduction of domestic animals and this is the reason why it is necessary to study sperm fertility markers and the effect of cryopreservation on the sperm viability. A more detailed research could improve new protocols and advanced technologies to improve the quality of cryopreserved samples in the future.

Key words: cryopreservation; bovine spermatozoa; cryodamage

INTRODUCTION

At present, cryopreservation along with artificial insemination represent powerful technologies for global livestock breeding. Cryobiology belongs to multidisciplinary sciences studying physical and biological changes in cells and tissues during cryopreservation (Benson *et al.*, 2012). The first attempt to cryopreserve mammalian ejaculates is dating back to 1784, when the Italian scientist Lazzaro Spallanzani used a snow-cooled dog ejaculate for successful insemination of a female dog, which gave birth to three healthy puppies. Later in 1899, the Russian scientist, Ilya Ivanovich Ivanov, developed practical methods for artificial insemination of farm animals, particularly horses. However, one of the largest discoveries in the field of cryopreservation was the application of egg yolk as a cryoprotective additive during the cooling and freezing process of spermatozoa by Philips and Lardy in 1940 (Ombelet and Van Robays, 2015). A few years later in 1949, the cryoprotective effects of glycerol were confirmed (Polge *et al.*, 1949). Nowadays, one of the cryoprotectant molecules, which shows promising results in frozen spermatozoa, is trehalose by the increasing post-thawing motility, acrosomal and plasma membrane integrity (Varela *et al.*, 2020).

Among all species of ruminants, cryopreservation of bull spermatozoa is currently managed the best. Nevertheless, the viability of cells after thawing is still low and different among individual bulls. The elimination of these contraindications in the future could lead to an improvement in the quality of post-cryopreserved samples used for artificial insemination

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(Ugur *et al.*, 2019). The processes responsible for lethal or sub-lethal damage of spermatozoa are still unknown and poorly examined. Low temperatures during the freezing process have a negative effect on the qualitative well as quantitative parameters of bovine spermatozoa. These changes could lead to a decreased motility, viability, membrane integrity, fragmentation of DNA and apoptosis. Following cryopreservation, the sperm cells are exposed to various physiological and structural changes. Based on the previous studies, the most common contraindications associated with cryopreservation of bull spermatozoa include the formation of ice crystals, oxidative stress, bacterial contamination, cryocapacitation and possible cytotoxic effects of cryoaditives (Figure 1; Singh *et al.*, 2014).

Formation of ice crystals

Cryopreservation enables long-term storage of living cells and tissues. The effect of low temperatures during freezing causes a decline of the metabolic and physiological activities of cells. However, freezing can cause irreversible cell damage leading to necrosis or apoptosis (Gao and Critser, 2000; Ozaydin and Celik, 2012). The main problem lies not in the ability of cells to maintain storage at cryogenic temperatures (below -190 °C), because most complications occur in the temperature range from -130 to -15 °C. The cells must undergo this temperature transition twice, once during freezing and later during the thawing process (Öztürk *et al.*, 2019). The control of influx and reflux of water is provided by protein channels called aquaporins (AQP). Many of them, such as AQP7, AQP8 and AQP11 were identified on the sperm membrane. AQP7 is responsible for metabolism of glycerol, while AQP8 affects the water exchange in the cell (Yueng, 2010). During cryopreservation, the formation of extracellular ice crystals increases the concentration of the extracellular fluid outside the cells. Rapid freezing leads to osmotic imbalance and ice crystallization in the extracellular fluid. This phenomenon increases

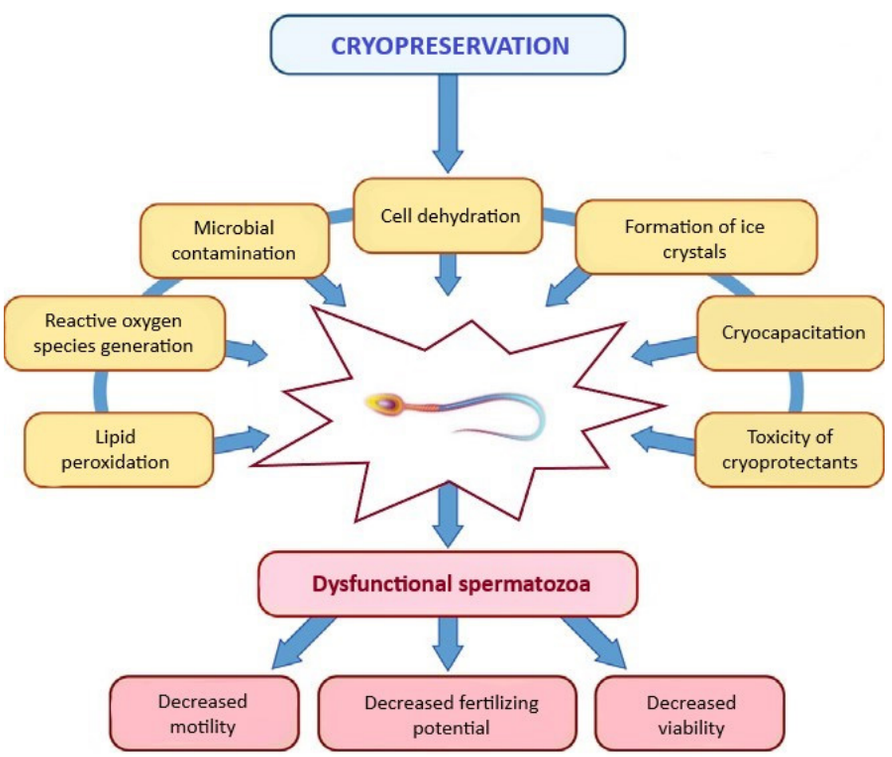

Figure 1. Negative impact of cryopreservation on spermatozoa

the pressure created by ice crystals, which leads to the extension of the subacrosomal region in the sperm membrane area. Changes in the osmolarity and the presence of intracellular ice crystals causes the disruption in the consolidation architecture of proteins and carbohydrates, which may result in damages to the membrane and a reduced sperm viability (Morris *et al.*, 2011; Hezavehei *et al.*, 2018).

The presence of oxidative stress

Oxidative stress can be induced by various mechanisms, such as osmotic stress or increased oxidative metabolism. Oxidative stress is developed due to the imbalance in the reactive oxygen species (ROS) production and the antioxidant capacity of the sperm cells. It is characterized by the induction of sperm membrane alterations and the presence of DNA breaks in the cryopreserved male gametes. The antioxidant properties of the seminal plasma provide a natural defence against ROS and regulate the intracellular redox potential, especially under stress conditions, such as low temperatures during cryopreservation. The increased generation of ROS causes changes in the electron transport chain localized in the mitochondria and synthesis of NADPH oxidase in the plasma membrane (Len *et al.*, 2019; Peris-Frau *et al.*, 2020). That is the reason why oxidative stress is considered to be one of the main causes of structural and molecular cell damages. The reduction in the antioxidant potential is the result of the complete removal or dilution of the seminal plasma before the freezing process. Another complication associated with oxidative stress is the lipid peroxidation. Sperm membranes are characterized by relatively high concentration of polyunsaturated fatty acids, which are sensitive to ROS-induced peroxidation. Lipid peroxidation takes place mainly in the middle (mitochondrial) section of the sperm flagellum, where the main cell respiratory system is localized. This may lead to damage of the lipid layer of cell membranes, depletion of adenosine triphosphate (ATP) formation, loss of motility and DNA damage (Tatone *et al.*, 2010; Ezzati *et al.*, 2020).

Bacterial contamination

The prevalence of microorganisms in the ejaculate is also known as bacteriospermia. This condition affects the sperm function by reducing its motility, viability, causing morphological defects and a premature activation of the sperm acrosome. Bacteriospermia also has a negative effect on the mitochondrial function, increased generation of ROS and an elevated risk of DNA fragmentation. One of the factors responsible for the failure of artificial insemination is the contamination of semen samples with pathogens. The most common microorganisms that contaminate bull ejaculates are *Corynebacterium*, *Staphylococcus*, *Micrococcus*, *Bacillus*, *Escherichia*, *Pseudomonas*, *Klebsiella*, *Streptococcus* and *Enterobacter* species. These pathogens not only reduce the fertilizing ability of sperm but can also infect the female urogenital tract during artificial insemination. Other factors that promote bacterial contamination include an improper manipulation, transport or storage of cryopreserved samples (Sisay *et al.*, 2012; Reda *et al.*, 2020). The pathogens are mainly localized in the genitals of males, where they contaminate the ejaculate and urine of bulls. They are also involved in the occurrence of diseases such as acute pyelonephritis and bovine cystitis. In particular, many studies confirm contamination with aerobic bacteria, which were isolated and identified in thawed semen samples. Current research confirms that cryopreservation does not prevent bacterial contamination of semen, therefore hygiene and sterility during the freezing process play the main role and must be carefully controlled (Morell, 2006).

Cryocapacitation

Under physiological conditions, before the acrosome reaction, sperm cells undergo a series of biochemical and morphological changes, which are also known as capacitation. This process naturally takes place in the urogenital tract of the females, where the environment stimulates the course of the sperm hyperactivation. Major changes include the reflux of cholesterol from the sperm membrane, hyperpolarization of the plasma membrane, increase of pH and the concentration of calcium. Following these processes, spermatozoa are capable of chemotaxis and thermotaxis, allowing them to actively locate the egg, interact with the *zona pellucida* of the egg, and undergo acrosome reaction (Ickowicz *et al.*, 2012).

Cryopreservation of bovine spermatozoa is an important tool for assisted reproduction, but the quality of thawed samples is often reduced by an average of half when compared to the native

sample. Exposure of sperm cells to low temperatures during the freezing process leads to cell membrane destabilization, loss of proteins on the surface of the cell membrane, a decreased motility, viability, mitochondrial activity and an increased ROS production. Cryocapacitation is characterized by the remodelling of the cell membrane, loss of cholesterol and polyunsaturated fatty acids, which play an important role in the maintenance of the natural fluidity and cell membrane integrity. Other contraindications include the inactivation of membrane-bound enzymes and decreased lateral protein diffusion within the membrane. Cryopreserved spermatozoa often start the capacitation process immediately after thawing, which may lead into the loss of the energy required for a successful fertilization. This is the reason why cryocapacitation is a major problem associated with a reduced longevity and poor survivability of cryopreserved bovine spermatozoa (Talukdar *et al.*, 2017; Ledesma *et al.*, 2019).

Cytotoxicity of cryoprotectants

Cryoprotectants are generally divided into membrane-permeable and impermeable. They serve as solvents used for the preservation of sperm cells during freezing by providing them protection against cryogenic damage. Membrane-permeable cryoprotectants include glycerol, dimethylsuloxide, dimethylacetaldehyde, propylene and ethylene glycol, which cross the membrane of cells and replace water. A main disadvantage is their synthetic origin and possible toxic effects especially at higher concentrations, which could dramatically reduce the fertilization potential of spermatozoa. These substances negatively affect the cytoplasm viscosity, alter the diffusion rate and the properties of the cell membrane to shrink at low temperatures (Amirat-Briand *et al.*, 2009; Kumar *et al.*, 2013). The second group of membrane-impermeable cryoadditives consists of raffinose, sucrose, egg yolk, citrate and albumin. Their mechanisms of action lie in the freezing of the extracellular space around the cells and the conservation of cellular structures. However, cryoprotectants like egg yolk are capable to interact with membrane glycoproteins and to decrease the sperm motility by the disruption of the cell membrane (Anand *et al.*, 2015; Sieme *et al.*, 2016).

CONCLUSION

Using the cryopreservation it is possible to store the genetic material for a long period of time. However, this process is also associated with several risks, which may affect the cells before, during and after thawing. Cryogenic damage often leads to physiological, morphological and biochemical changes in male gametes, which reduces the quality and fertilization potential of post-thawed samples used for artificial insemination. Therefore, a detailed research of the changes caused by low temperatures is necessary to understand, reduce or completely eliminate adverse effects of cryopreservation. Nevertheless, cryopreservation plays an important role in the conservation and protection of the genetic heritage of breeding bulls and enables the distribution of quality livestock around the world.

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