



The use of açai as a potential antioxidant for bovine semen cryopreservation

[Uso do açai como potencial antioxidante para a criopreservação de sêmen bovino]

"Revisão/Review"

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Abstract

Semen cryopreservation is an important tool for animal reproduction due to its potential for fast dissemination of genetics from males with high genetic merit. However, cryopreservation protocols continue to provoke negative effects on sperm cell structure and function, thus leading to lower viability. This reduction in viability is correlated with excessive production of reactive oxygen species (ROS) that generate oxidative stress, which is responsible for lowering sperm motility, alteration in plasma membrane fluidity, and lipidic peroxidation. Due to these facts, it becomes necessary to add substances with the antioxidant potential for semen cryopreservation to confer additional protection to sperm cells. A variety of compounds from plants and fruits have been incorporated to such media due to their antioxidant potential. In such context, açai has gained attention by researchers due to its substantial antioxidant capacity, particularly attributed to its polyphenolic fraction, which is rich in anthocyanins. Moreover, it also contains resveratrol and quercetin in its composition, which has activity against free radicals. In general terms, the antioxidant of natural sources (i.e., açai) deserves special attention since it contributes to the preservation of the national flora, also displays a significant potential to improve the quality of frozen-thawed bovine semen.

Keywords: bull; *Euterpe oleracea* Martius; freezing; spermatozoa; membrane.

Resumo

A criopreservação do sêmen é uma ferramenta importante para a reprodução animal devido propiciar rápida disseminação de reprodutores com elevado mérito genético. Entretanto, os protocolos de criopreservação ainda provocam efeitos negativos a estrutura e a funcionalidade das células espermáticas, com conseqüente redução de sua viabilidade. Essa redução, está relacionada com a produção excessiva de espécies reativas ao oxigênio (EROs) que geram o estresse oxidativo, responsável pela redução da motilidade espermática, alteração na fluidez da membrana plasmática e peroxidação lipídica. Diante disso, é necessário adicionar substâncias com ação antioxidante aos meios diluidores de congelamento do sêmen para garantir proteção adicional aos espermatozoides. Uma variedade de compostos oriundos de plantas e frutos têm sido incorporados a esses meios por possuírem potencial antioxidante. Nesse contexto, o açai tem despertado interesse de pesquisadores por possuir expressiva capacidade antioxidante, especialmente atribuída à sua fração polifenólica, rica em antocianinas, além de possuir, em sua composição, quercetina e resveratrol que também apresentam ação contra radicais livres. De um modo geral, os antioxidantes de origem natural, como o açai, merecem atenção especial tendo em vista que, além de contribuir para preservação da flora nacional, apresentam significativo potencial para a melhoria da qualidade do sêmen bovino criopreservado.

Palavras-chave: congelamento; espermatozoides; *Euterpe oleracea* Martius; membrana; touros.

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Introdução

Semen cryopreservation was initially described during the fifties since it was found that glycerol displays cryoprotectant potential for sperm cells (Holt, 2000; Kaka et al., 2015). This biotechnology is important for animal production because it contributes to the rapid dissemination of genetic material of high genetic merit to increase milk and meat productions. Additionally, it favors the global commercialization of animals of high genetic merit and further allows the establishment of crossbreeding zebu cows with taurine bulls by artificial insemination programs using frozen-thawed semen. Furthermore, it allowed the usage of animals that could not be used under natural mating conditions (Artmann et al., 2015; Moore e Hasler, 2017) due to acquired lesions without genetic compromise (Sousa et al., 2012).

Despite the fact that cryopreservation is a routine for bovine reproduction, the advances in the cryopreservation process of bovine semen during the last few decades have been slow (Layek et al., 2016). The currently used protocols continue to provoke structural and functional damage to sperm cells, such as alterations on semen homeostasis, reduction in sperm motility and plasma membrane integrity after cryopreservation (Celeghini et al., 2008).

The reduction of sperm viability during cryopreservation is related to the excessive production of reactive oxygen species (ROS) that generate oxidative stress (Büyükleblebici et al., 2014). The oxidative stress diminishes sperm fertilizing-ability because motility is reduced and further provokes alterations in both plasma membrane fluidity and integrity. Moreover, it also leads to DNA damage and lipidic peroxidation (Cocchia et al., 2011).

With the aim to minimize the impact of such effects on sperm cells, antioxidants have been added to the semen cryopreservation media. More recently, a variety of compounds derived from plants and fruits (Bucak et al., 2015; Sapanidou et al., 2015; Sobeh et al., 2017) have been added to such media due to their antioxidant potential. For these reasons, the aim was to describe the antioxidant potential of açai (*Euterpe oleracea* Martius) for bovine semen cryopreservation.

Bovine semen cryopreservation

General facts

The principle of cryopreservation is to block the metabolic processes in sperm cells and

maintain its properties for long periods of time under low temperatures (Vishwanath and Shannon, 2000). However, the currently used protocols still allow the occurrence of both structural and functional damage to sperm cells, with diminished motility and plasma membrane integrity after cryopreservation (Celeghini et al., 2008).

The cryopreservation protocols for bovine semen include the cooling step, in which the temperature is lowered to 4 - 5 °C, followed by a stabilization period that is necessary for sperm membrane adaptation to low temperatures. Depending upon diluent choice, this period (also known as equilibrium period) may vary from 30 minutes to 72 hours (Leite et al., 2010; Fleisch et al., 2017). The cooling step induces the onset of the thermal stress, also described as a thermal shock (Watson, 2000).

The heat shock is the result of thermal stress that occurs during cryopreservation and generally provokes alterations in plasma membrane permeability due to phospholipids' rearrangements. These molecules transit from the clear liquid state to a gel state, thus reducing cellular metabolism, causing loss of intracellular components, loss of sperm motility, and diminished fertilizing-ability (Watson, 2000; Patel et al., 2016).

The ideal conditions are those that allow sufficient cellular dehydration but not excessively, thus avoiding the formation of intracellular ice crystals. Moreover, the freezing curve must be fast enough to reduce the exposure of sperm cells to hyperosmotic conditions, minimizing osmotic stress and thus proportionate good conditions for survival of sperm cells after thawing (Mazur, 1970; Medeiros et al., 2002; Peña et al., 2011).

The success of cryopreservation depends on the bull-dependent sensibility of sperm cells to cryopreservation (Gürler et al., 2015). Studies in horses (Hartwig et al., 2014; Ramires Neto et al., 2014; Ferreira et al. 2018; Ferreira-Silva et al., 2018a,b) and swine (Yeste et al., 2013; Yeste, 2016) have demonstrated the actual response to cryopreservation varies among animals of the same species. For this reason, males with the semen of high and low tolerance to cryopreservation, vary in the lipidic composition of the sperm plasma membrane, their diet or breed (García et al., 2011; Gürler et al., 2015; Yeste, 2016).

Under such context, many additives that do not show improvements for semen freezing from

bulls of high tolerance to cryopreservation, within the requirements preconized by CBRA (2013), may be beneficial for semen of bulls of low tolerance to cryopreservation. Such additives that may play important roles to allow the usage of such bulls of low freezing potential but with high genetic merit.

Cryoprotectants in diluents

There is a variety of diluents that exert influence on the survival of sperm cells during cryopreservation since those reagents play important roles by minimizing the deleterious effects on sperm cells provoked by freezing. However, it is necessary to address the interaction of media composition and freezing/cooling rates to ensure a greater viability of sperm cells after cryopreservation (Chaveiro et al., 2006).

The components used in freezing diluents are used for the maintenance of its own osmolarity and pH and avoidance of the growth of microorganisms by addition of antibiotics. Sources of lipoproteins or high molecule weight compounds are added to diluents to prevent thermal shock, and the addition of glucose and fructose as energy sources. Moreover, there are also cryoprotectants and antioxidants in its composition (Oliveira et al., 2013; Souza et al., 2017).

The cryoprotectants are added to diluents can be classified as non-penetrating (extracellular) or penetrating (intracellular), based on their mechanisms of action. The extracellular cryoprotectants are represented by high molecular weight macromolecules, such as sugars, the egg-yolk lipoprotein, milk proteins, and aminoacids (Lima-Verde et al., 2017). Such substances act via the osmotic effects by increasing the exit of water from cells and thus preventing intracellular ice crystal formation and further promote greater plasmatic membrane stability (Avila-Portillo et al., 2006).

On the other hand, penetrating cryoprotectants are of low molecular weight compounds, high solubility in aqueous solution, and cellular toxicity (Nash, 1966). In general terms, these cryoprotectants mechanistically act by their properties to connect with water molecules that modify the orientation of intracellular ice crystals and generate a less toxic environment for sperm cells (Nash, 1966; Holt, 2000).

Oxidative stress

During semen cryopreservation, several deleterious effects are observed in sperm cells, such as reduction of motility and mitochondrial activity. Moreover, damage to the plasmic membrane can be detected, thus resulting in diminished fertilizing-ability (Hu et al., 2010). One of the main factors associated with lowered sperm viability during cryopreservation is the ROS production (Büyükleblebici et al., 2014).

In bovine semen, ROS can be generated by physiological sperm metabolism and by the presence of dead and immature cells (Sariözkan et al., 2009). According to Bansal and Bilaspuri (2011), the ROS with a greater effect on sperm cells are superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2).

ROS produced in small quantities are physiologically important for their importance in sperm capacitation, acrosome reaction, and maintenance of fertilizing-ability (Burnaugh et al., 2010). Therefore, minimum ROS concentrations are indispensable for maintaining normal cellular function (Cocchia et al., 2011). However, when ROS production exceeds the defense mechanism by antioxidant systems that are naturally found in seminal plasma and on diluents used for cryopreservation then occurs oxidative stress (Andrade et al., 2010).

The oxidative stress diminishes the sperm fertilizing-ability because it reduces sperm motility, provokes deleterious alterations in both plasma membrane integrity and fluidity, thus resulting sperm DNA damage and lipidic peroxidation (Tuncer et al., 2010; Cocchia et al., 2011).

The lipidic peroxidation occurs due to the high quantities of polyunsaturated fatty acids found in sperm plasma membrane (Cocchia et al., 2011). The ROS react with sperm membrane-bound polyunsaturated fatty acids and reduces membrane permeability that ultimately leads to the loss of sperm function (Aitken and Krausz, 2001).

Sperm cells do not display an efficient antioxidant system that could be capable to protect them from excessive ROS production. This limitation is due to the small cytoplasm volume, which holds limited endogenous antioxidant capacity (Du Plessis et al., 2008). The main enzymes found in seminal plasma with the capacity to naturally protect sperm cells against ROS are

catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (Bansal and Bilaspuri, 2011).

The dilution step during the semen cryopreservation process leads to lower protective capacity to sperm cells. Due to the high concentration of polyunsaturated fatty acids, sperm cells can be inefficient in preventing lipidic peroxidation, due to its limited antioxidant system (Sapanidou et al., 2015). Moreover, ROS production and oxidative stress are inevitable during both freezing and thawing steps. For this reason, it becomes necessary to promote an additional protection additional during semen cryopreservation. This additional protection can be accomplished by adding substances with antioxidant activity to diluents (Souza et al., 2017).

Antioxidants

The process of cryopreservation reduces the viability of sperm cells due to the changes in temperature that provoke the thermal shock, determining alterations in plasma membrane permeability, loss of intracellular components, and motility reduction. Semen cryopreservation also contributes to increased ROS production that favors the occurrence of both lipidic peroxidation and diminished sperm cell quality (Patel et al., 2015).

The research on semen cryopreservation continues to be carried out to validate new cryoprotectants or other substances to be added to diluents to further reduce the cryoinjuries to sperm cells (Moraes et al., 2010; Takahashi et al., 2012; Kandelousi et al., 2013; Mocé et al., 2014; Kaka et al., 2015; Patel et al., 2016). However, recent studies have focused on the search for substances with antioxidant potential for minimizing the oxidative stress (Patel et al., 2016).

As previously described, sperm cells are vulnerable to oxidative stress during cryopreservation due to their limited antioxidant capacity (Du Plessis et al., 2008). In light of these facts, various strategies have been developed for reducing this damage and to improve semen quality after thawing. Therefore, it is important to supplement diluents with substances that carry antioxidant potential, such as enzymes, vitamins, and minerals (Shah et al., 2017). In general terms, these antioxidants can be added directly to cryopreservation diluents or be included in the diet of the animals before semen collection (Maia and Bicudo, 2009; Sobeh et al., 2017).

Antioxidants are classified by their enzymatic activity (enzymatic or non-enzymatic) and source (produced by the organism or not), according to Barreiros et al. (2006). Within antioxidants produced by the body, three are more prominent, the enzymatic superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Among the non-enzymatic antioxidants, the reduced glutathione (GSH), histidine peptides, iron-conjugated proteins (transferrin and ferritin), dihydrolipoic acid, and coenzyme q10 (CoQH) can be highlighted (Barreiros et al., 2006). The non enzymatic antioxidants that can be obtained from the diet are efficient against ROS production, such as α -tocopherol (vitamin E), β -carotene (vitamin A precursor), ascorbic acid (vitamin C) and phenolic compounds (Barreiros et al., 2006; Vasconcelos et al., 2007), among others.

Plants synthesize hundreds of phenolic compounds, which exert various functions and are distinguished as the most abundant and potent antioxidants found in the diet (Cerqueira et al., 2007; Maia and Bicudo, 2009; Sobeh et al., 2017). The antioxidant activity of such compounds is primarily due to their oxide-reductive properties, a fact that allows absorbing and neutralizing ROS, to chelate triplet and singleton oxygen, and further decomposing peroxides (Degáspari and Waszczynskyj, 2004).

The phenolic compounds obtained from plants can be divided into two groups: flavonoids and non-flavonoids, where both classes are secondary metabolites (Degáspari and Waszczynskyj, 2004). The capacity of plant polyphenols to act as antioxidants in biological systems was discovered in the thirties (Benthath et al., 1936). However, the consumption of polyphenol-rich foods, their bioavailability, and other interfering factors are being largely investigated in more recent times (Fiorani et al., 2002; Behling et al., 2004).

The flavonoids are a class of natural compounds of considerable scientific and therapeutic interests (Behling et al., 2004). Among almost 4,000 members already described, the major classes are of flavonoids, catechins or flavones, and isoflavones, which display great structural variation, depending on the molecule hydrogenation, hydroxylation, methylation, and sulfonation levels. Despite these facts, flavonoids hold ideal structures (C6-C3-C6), composed of hydroxyl groups (-OH) bound to two aromatic

rings by an oxygenated heterocyclic (Cerqueira et al., 2007).

The chemical structure of flavonoids allows an efficient activity against ROS, in a more effective manner than both C and E vitamins. The number and position of -OH groups and by glycosylation sites (Barreiros et al., 2006; Cerqueira et al., 2007) influence its antioxidant capacity. This fact is due to its intrinsic capacity for electron transfer, the stability of flavonoid radical formation, and its reactivity compared to other antioxidants. Moreover, these compounds also carry chelating capacity, solubility and cellular membrane interacting-capacity (Barreiros et al., 2006). Under this context, flavonoids notoriously act upon -OH and superoxide anions (O₂⁻), which are considered two reactive species involved in the initiation of lipidic peroxidation (Behling et al., 2004).

Katequines are flavonoids that are highlighted by their biological activities, including antioxidant activity, anti-inflammatory roles, anti-carcinogenic, lipolytic activities, and DNA damage inhibition, when these processes are caused or mediated by ROS production (Guaratini et al., 2007). These compounds are abundant in green tea, which account for 90 % of the polyphenolic fraction (Belitz and Grosch, 2004). Due to these beneficial effects and no record of potentially damaging effects, Katequines have been used in treatments several pathologies (Vaccari et al., 2009) and for testicular hyper-thermal damage recovery (Abshenas et al., 2011).

Plant-derived substances with antioxidant potential, such as carotenoid crocin, have been associated with reductions in intracellular ROS levels with a significant increase in sperm fertilizing-ability. These findings were attributed to crocin activity that may lead to free radical removal, which may ultimately lead to greater protection against lipidic peroxidation (Sapanidou et al., 2015).

Lycopene is a carotenoid found in tomatoes and red fruits, while resveratrol is a polyphenol mainly found in grapes. Both compounds have been added to bull sperm cryopreservation media, due to their antioxidant potential (Bucak et al., 2015). These substances have been linked to free radical removal thus possibly conferring greater protection to sperm cells against oxidative stress during cryopreservation (Bucak et al., 2015).

Açai

The açai (*Euterpe oleracea* Martius) has gained interest in animal reproduction due to its chemical composition, mainly associated with its polyphenolic fraction that confers antioxidant protection (De Lima Yamaguchi et al., 2015). The açai is a palm tree found throughout the North of Brazil, where it holds a relevant economic importance. The fruits have dark violet coloring and are found in bunches (Sun et al., 2010; Dias-Souza et al., 2018).

From a commercial standpoint, the açai-based industry has been significantly expanded (Heinrich et al., 2011). This expansion is attributed mainly to the food industry that relies on the açai fruit for energy drinks. This growth in interest in açai-derived products is due to its known benefits for human health. Açai has been characterized as a functional food since it contains sugars, fatty acids, and especially polyphenols that act as excellent antioxidants (Bonomo et al., 2014).

From a bromatological standpoint, açai contains proteins (8.1 g/ 100 g), total lipids (32.5 g/ 100 g), cholesterol (13.5 mg / 100 g), sugars (7.93 g / 100 g), carbohydrates (52.2 g / 100 g), vitamins (A, C, and E), beta-carotene, ferulic acid, gallic acid, catechins, and fibers. The most abundant sugars in açai are glucose and fructose, fatty acids [saturated (26.0 %), monounsaturated (60.2 %), polyunsaturated (13.3 %)], mainly linoleic and linolenic. The açai also contains flavonoids, phenolic, polyphenolic, and lignoid compounds (Schauss et al., 2006; Rufino et al., 2011; Yuyama et al., 2011).

The most abundant polyphenols in açai are anthocyanins, and most notably, cyanidin-3-glucoside and cyanidin-3-rutinoside. Moreover, there is also quercetin and resveratrol in the açai composition (Del Pozo-Insfran et al., 2004; Schauss et al., 2006).

The anthocyanins are a sub-group of flavonoids that are responsible for determining fruit and vegetable coloring (e.g., açai), and further carry antioxidant activity (Del Pozo-Insfran et al., 2004). The antioxidant protection conferred by anthocyanins is directly linked to the number of hydroxyl groups found in its chemical structure. This fact allows the transfer of electrons or hydrogen atoms to neutralize ROS (Volp et al., 2008). Therefore, the increasing number of OH in flavonoids is proportional to its capacity for both

electron and H⁺ transfer (Barreiros et al., 2006).

The antioxidant activity of açai can also be conferred due to the presence of other flavonoids, such as quercetin, which is a natural antioxidant capable of reacting to free radicals (superoxide and hydroxyl), and thus acts as a metallic ion chelator and blocks lipidic peroxidation. This protection is due to the lipophilic potential of such flavonoids, which are capable of interacting with the plasmatic membrane lipid bilayers. This fact further allows both H⁺ and electron transfers to free radicals, further promoting the stabilization of such radicals, and thus impeding the continuity of the chain reaction (Barreiros et al., 2006; Rufino et al., 2011).

The resveratrol, found in açai, has been used in animal semen cryopreservation media. Some reports have shown that this compound play role as a free radical remover, has effects on lipidic peroxidation, and diminishes DNA damage (Bucak et al., 2015; Longobardi et al., 2017). The açai contains other components that can be linked to their antioxidant potentials, such as C and E vitamins, ferulic acid, gallic acid, and beta-carotene. There are encouraging results from adding some of these compounds for semen cooling and freezing (Hu et al., 2011; Kutluyer et al., 2014; Affonso et al., 2017).

The antioxidant activity of açai was confirmed by *in vitro* studies. These reports found antioxidant activity against peroxy radicals, peroxide-nitrite, superoxide, and hydroxyl (Lichtenthaler et al., 2005; Schuss et al., 2006a). Moreover, these studies also found activities against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, and further demonstrated ROS removal activity in a similar fashion to the mechanisms of both superoxide dismutase and catalase (Spada et al., 2008). The antioxidant potential of açai was also investigated in isolated tissue from rat brain cortex, hippocampus, and cerebellum, and collectively showed the oxidative damage induced by hydrogen peroxide in lipids and proteins (Spada et al., 2009).

The supplementation with extra-aqueous açai extracts increased the resistance of *Caenorhabditis elegans* (*C. elegans*) to both oxidative and osmotic stresses by a decrease in ROS production and pathway activation that is connected to antioxidant enzyme expression (Bonomo et al., 2014). The anthocyanins-enriched açai extract promoted greater resistance to oxidative stress in *C. elegans* and also modulated

stress-response gene expression, demonstrating antioxidant and anti-aging activities (Peixoto et al., 2016).

In humans, the consumption of açai increased the total antioxidant capacity, suggesting that it's a functional food that can promote protection against degenerative diseases that are associated to oxidative stress (Pala et al., 2018). The consumption of açai pulp also increased catalase activity, overall antioxidant capacity, and reduced ROS production in polymorphonuclear cells (Barbosa et al., 2016). These authors further concluded that açai can be considered a functional food that contributes to disease prevention, such as cancer. The use of anthocyanins-rich açai extracts displayed increased anti-proliferative capacity in a brain glioma cell line (i.e., C6), possibly due to induction of apoptosis in such cells. However, no effect of the açai extract was found on breast cancer cells (Hogan et al., 2010).

The açai also displays polyunsaturated fatty acids in its composition. These compounds can be used in semen cryopreservation media due to the potential to incorporate these acids into sperm plasma membrane and confer greater protection during the process of cryopreservation (Nasiri et al., 2012).

Final Considerations

Semen cryopreservation is an important tool both for the dissemination of genetic material of bulls of high genetic merit and for the maintenance of germplasm banks. However, the oxidative stress continues to be a limiting factor in increasing cryopreservation efficiency. In such context, antioxidants of natural sources such as the açai deserve special attention due to the potential to preserve of the national flora and due to its potential for improving the quality of bovine frozen-thawed semen.

Conflict of Interest

The authors declare no conflict of interest.

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