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Current aspects of boar semen cryopreservation

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Abstract. Advances in cryobiology and semen evaluation techniques stand for offering new perspectives in boar semen cryopreservation, showing a great potential to reveal aspects of sperm cells still unknown. The new prospects on boar germplasm cryobiology seems to be related to some issues which may encode key factors for semen cryopreservation, referring to the sperm cell population cryosensitivity, the effects of oxidative stress, the sperm vacuoles, the toxic effect of cryoprotectants, the new cryoprotectant alternatives and the use of various compounds for supplementing the cryopreservation media (e.g. CLC, IGF-I, GSH, alginate, next to many other antioxidants and other compounds, which may be not yet addressed or known). Recent reproduction research indicates the great potential of genetics in boar sperm cryopreservation progress. DNA damage after cryopreservation, for instance the breakup on crucial genes involved in fertilization and embryo development (e.g. ADD1, ARNT, BIK, FSHB, PEG1/MEST, PRM1, SNORD116/PWSAS, UBE3A), the relationship between miRNAs and fertilizing ability, and definitely other molecular events of the omics, concur to find out solutions to boar semen cryopreservation and to the reproductive area development, especially in swine. Isn't it that all these could provide future challenges of using the -omics to address the reproduction field in more specific terms, marking up the development of the omics era of reproduction?
Key Words: boar sperm, freezing-thawing, DNA damage, media supplements, cryosensitivity.

Rezumat. Progresele în domeniul criobiologiei și al tehnicilor de evaluare a spermei oferă noi perspective asupra crioconservării meterialului seminal de vier, având un mare potențial de a releva aspecte încă necunoscute ale celulelor spermatice. Noile perspective ale criobiologiei germoplasmei la vier se referă la realizări cu potențial de codificatoare a unor factori cheie pentru crioconservarea spermei, respectiv la criosensibilitatea populațiilor de celule spermatice, efectele stresului oxidativ, vacuolele spermatozoizilor, efectul toxic al agenților crioprotectori, noi alternative pentru substanțele crioprotectoare și utilizarea diferiților compuși pentru suplimentarea mediilor de crioconservare (CLC, IGF-I, GSH, alginat, alături de o serie de antioxidanți și alte substanțe, care nu au fost încă studiate sau cunoscute). Cercetările recente din domeniul reproducției indică potențialul mare al geneticii în ceea ce privește progresul realizat în crioconservarea gameților de vier. Altearea ADN în urma crioconservării, cum ar fi de exemplu alterarea ale unor gene cruciale, implicate în fertilizare și dezvoltarea embrionară (ADD1, ARNT,BIK, FSHB, PEG1/MEST, PRM1, SNORD116/PWSAS, UBE3A), relația dintre ARNmi și capacitatea fertilizantă, precum și alte procese moleculare din domeniul omicii, concură la identificarea soluțiilor pentru crioconservarea materialului seminal de vier și dezvoltarea sferei reproducției, în special la porcine. Nu-i așa că aceasta ar putea oferi viitoare provocări pentru utilizarea –omicii în domeniul reproducției în termeni mult mai specifici, marcând dezvoltarea erei omicii în reproducție?
Key Words: spermă de vier, congelare-decongelare, alterare ADN, substațe de suplimentare a mediului, criosensibilitate.

Introduction. Semen cryopreservation greatly facilitates the distribution of agriculturally desirable genes, rapidly increasing herd productivity. A pool of cryopreserved semen would minimize the effects of a sudden outbreak of a contagious illness or a natural disaster (Honaramooz et al 2002; Bailey et al 2008). Gene banking is enabled through the available and the potential of the reproductive technologies, cryopreservation and molecular genetics techniques, assuring animal genetic resources conservation (FAO 2012; Agca 2012; Socol et al 2012). Except biodiversity preservation, frozen semen is essential in breeding and selection schedules contributing to increase production of domestic species (Barbas & Mascarenhas 2009; Criste 2011; Kasso & Balakrishnan 2013; El-Sheshtawy et al 2014) and the number of animals through artificial reproductive technologies; also frozen germplasm availability can be considered for facilitating ART implementation. There are still some answers required for germplasm preservation in

various species, finding alternatives and optimization of currently available imperfect semen cryopreservation and evaluation methods (Criste 2011).

Since the first recorded offspring produced by artificial insemination using frozen–thawed semen in pig (Hess et al 1957*)* relevant studies were conducted in cryopreservation techniques, progress still being required for semen cryopreservation purposes in boars. Cryopreservation technology is not sufficient to yield reliable fertility following AI in commercial swine production systems (Bailey et al 2008ș Rodriguez-Martinez 2012). Semen cryopreservation is not showing satisfying results in all species, as those registered in bull. Boar cryopreservation is still pointing out substantial differences between individuals on semen quality and cryopreservation ability. Both, storage and freezing-thawing processes causes a physical and chemical stress on sperm plasma membrane level. This stress is often associated with loss of sperm motility and its fertilizing ability (Aitken 200; Rusu 2011). The fertilizing capacity of stored or frozen semen presents differences between species. One of the major issues involved in improving the process of storage and cryopreservation of semen is the identification and use of new substances and additives (Koskinen et al 1989; Sanchez-Partida et al 1992). The choice of cryoprotectant seems to have been a matter of trial and error in nearly all investigations; this is partly because a complete and satisfactory explanation for the action of cryoprotectants does not exist (Fernandez-Santos et al 2006). Cryopreservation success depends on several factors, including the initial semen quality, the cryopreservation protocol, the specific susceptibility of sperm cells in cold shock, mechanic and osmotic stress conditions, and also the sperm cells capacity to interact with different extender's compounds during freezing and thawing processes (Fraser et al 2007; Rusu 2011). Since considerable advances in boar semen cryopreservation were made, the present study aim to summarize some valuable data achieved in boar semen cryopreservation.

Material and Method. The paper is an assesment of the boar semen cryopreservation status, carried out to point the advances achieved and some relevant aspects to consider in swine male germplasm preservation.

Results and Discussion. One of the major causes of semen quality decrement during cryopreservation is considered to be the temperature decrease from 15°C up to 5°C. During cryopreservation, even in the incipient stages, spermatozoa membrane is exposed to low temperatures, which are causing a fluidity state much decreased, restricting in the same time the free movement of membrane proteins. Thus, the membrane structure and functionality is altered. Beside this, there are other several factors influencing cryopreservation such as cryopreservation protocol, cryopreservation media, cryoprotectants, semen dilution, cooling and thawing rate specific, etc. Detecting sperm cell alterations is essential in germplasm preservation or cryopreservation. The success of semen cryopreservation is mainly based on choosing "good freezers", due to the existing variation between individuals. As ejaculate consists of a heterogeneous population of cells, the freezing–thawing process results in a sperm cell population, which may be more or less functionally compromised. Post-thaw sperm survival may be consistently poor for certain individual animals even though pre-freeze parameters appear normal. The mechanisms that may underlie such differences in cryosensitivity remain unclear (Curry 2000; Rusu 2011). The heterogeneity of sperm cells population of an ejaculate through its role in fertilization is an important factor in cryopreservation. The nature and importance of sperm populations are still unclear, it is apparent that there are changes in these subpopulations following the freeze-thaw process (Curry 2000). The freezing process might differentially affect a particular population of cryosensitive cells other freeze–thaw process might affect all cells to such an extent that pre-existing more subtle differences are masked giving the appearance of a more homogeneous population (Curry 2000). Referring to boar sperm cryopreservation damages at the perinuclear theca level leads the sperm nucleus to decondense (Gutiérrez-Pérez et al 2011). Mitochondrial activity is reduced after cooling and freeze-thawing in boar sperm (Flores et al 2010). Effects of cryopreservation on ROS production in boar sperm being less clear than in

other species (Guthrie & Welch 2006; Guthrie & Welch 2012). The different vacuoles found in sperm that are observed in the acrosome and cytoplasm droplets during spermatogenesis, are less frequent in pigs, where they can only be detected under transmission electron microscope (Briz & Fàbrega 2013). There are no studies about the effects of cryopreservation on sperm vacuoles in pigs, relevant studies proving that freeze-thawing procedures increased sperm nuclear vacuolization (Boitrelle et al 2012).

The combinations of storage temperature, cooling rate, chemical composition of the extender, cryoprotectant concentration, seminal plasma composition and hygienic control are the key factors that affect the life-span of spermatozoa (Barbas & Mascarenhas 2009). Fertility of extended boar semen declines within the first 72 h of storage in vitro and conventional sperm assessment often lacks standardization and does not allow identification of sub-lethal changes of sperm quality during the initial 72 h of storage (Waberki et al 2011). Securing isoosmotic/isotonic conditions, adequate buffering capacity, the necessary energy supply, the provision of a plasma-membrane protecting agent is of vital importance in cryopreservation (Pesch & Hoffmann 2007). Also sperm centrifugation before freezing seems to influence boar sperm cryosurvival (Carvajal et al 2004). Moreover, the assessment of the specific responsiveness to capacitating stimuli by monitoring intracellular calcium with flow cytometry proved to be a sensitive tool for detection of extender-dependent alterations in the functionality of liquid-preserved boar spermatozoa (Schmid et al 2013a).

Even if there are many cryoprotectants and potential cryoprotective agents, glycerol has remained, almost without exception, the cryoprotectant of choice for spermatozoa from all species; the basis of the cryoprotective properties of glycerol and precisely why it should be more effective, remains unclear (Curry 2000). The sensitivity of spermatozoa to the toxic effects of different cryoprotectants varies with species (Curry 2000) and individuals. Extenders for freezing sperm cells contain buffers, carbohydrates (glucose, lactose, raffinose, saccharose and trehalose), salts (sodium citrate, citric acid), egg yolk and antibiotics (Barbas & Mascarenhas 2009). Generally an antibiotic is added to the extender (procain penicilline, ampicillin, gentamicin sulphate, lincomycin hydrochloride) to prevent contamination (Pesch & Hoffmann 2007). In general, semen cryopreservation protocols, and also in boar, involves the use of an egg yolk and lactose based extender, which are having a well known protective effect. Lactose could have a relatively important role in boar semen cryopreservation and maintaining its quality in the freezing-thawing stages. It seems that it could be involved by raising the water percentage that is not forming ice crystals (at any temperature) or by reducing salts concentration in unfrosted water solutions (Holt 2000). This effect of lactose is beneficial for boar sperm cells even in abnormal pH and osmolarity conditions. The high osmolarity of LEY extender could also explain the positive influence of lactose in cryopreservation.

When sperm cells are exposed to low temperature, the processes that normally are keeping cellular homeostasis are altered. Some of the cell properties such as striking out the oxidant compounds are slowed down (Yoshida 2000). Also, loss of calcium homeostasis proved to be a more sensitive parameter of chilling associated injury in hypothermically stored boar spermatozoa compared with conventional sperm quality traits (Schmid et al 2013b). The intracellular environment is exposed for a long time to high concentrations of toxic compounds. Some studies support the hypothesis that the use of a high value cooling rate could decrease this effect. In general, by supplementing cryopreservation media with antioxidants has been reported to be positive effects, those listed in pigs include L-cysteine, alpha tocopherol, lutein, butylated hydroxytoluene, Trolox and ascorbic acid, the results of the latter being better when combined with Trolox or with reduced glutathione (GSH) (Giaretta et al 2015; Tomás et al 2013; Kaeoket et al 2010; Jeong et al 2009; Roca et al 2004). Cholesterol-loaded cyclodextrins (CLC) supplementation improves post-thaw sperm quality and in vitro–fertilizing ability without affecting capacitation status and DNA integrity (Tomás et al 2013; Blanch et al 2012), but high glycerol concentrations being required (Blanch et al 2014). Even if the role of insulin growth factor I (IGF-I) seems to be a key factor in cancer and other human diseases, reliable studies proved it benefic effect in semen cryopreservation (Padilha et al 2012), studies in pigs have not been yet reported (Yeste 2015). Also addition of various

compounds in semen extenders such as GSH (Estrada et al 2014; Yeste et al 2013) and alginate (Hu et al 2014) improved semen quality in cryopreservation process.

The mechanisms underlying the increase in DNA fragmentation after cryopreservation are still unknown and appear to be related to the increase of oxidative DNA damage (Paoli et al 2014), being and it seems that induces lesions on crucial genes involved in fertilization and early embryo development (ADD1, ARNT, BIK, FSHB, PEG1/MEST, PRM1, SNORD116/PWSAS, and UBE3A) (Thomson et al 2009). Such studies were not yet conducted in pigs (Yeste 2015), further results may have the potential to give answers for some boar semen cryopreservation issues. Moreover, the relationship between miRNAs and fertilizing ability have not yet addressed in pigs (Curry et al 2011) Future studies should address whether sperm miRNAs degraded by cryopreservation are relevant for fertilization success (Yeste 2015).

Adequate data analysis is always crucial for providing conclusive results, facilitating an appropriate interpretation of the data (Chervitz et al 2011; Altmae et al 2014) and could reveal to new research directions; accurate data and results can be provided by applying advanced technologies enabled throughout the omic sciences. Considering the molecular advances and the related data provided by implementing such techniques into the reproduction field, definitely a larger area seems to be not yet approached. Next to the molecular events applied so far, new ones from the omics could stand for elucidating unknown role of various phenomena, relevant for boar semen cryopreservation and the reproductive area development, especially in pigs. However, new “omic” sciences such as the transcriptomics, proteomics, metabolomics, glycomics, lipidomics, fluxomics and interactomics (D’Alexandri et al 2010) and its challenges will stand for successful achievements in the reproductive area.

Some of the researchers consider that the cryogenic damages are due to irreversible destruction in single components of the structural organization of sperm cells. Both osmotic and temperature gradients cause changes in the molecular organization and function of the plasma membranes and the membranes of the intracellular organelles at low temperature influences on spermatozoa (Sabev et al 2006). This level of complexity and the number of unknown factors make it extremely difficult to assess the potential fertility of spermatozoa effectively both within the ejaculate and after freezing and thawing (Curry 2000). Spermatozoon in many species is still a cryobiological enigma related its cryosensitivity and functionality, further studies being required.

Conclusions. The accurate approaches for defining the optimal methods and conditions for semen freezing, thawing and preservation are showing an impressive dynamic and flexibility, being directed and adapted to the new challenges and advances of the research area. The variation in sperm cell cryosensitivity, the effects of cryopreservation on ROS production, the increased sperm nuclear vacuolization, the optimal cryoprotectant and media supplementation choice, the role of new compounds such as IGF-I, the increment in DNA fragmentation, the relationship between miRNAs and the fertilizing ability are some of the new approaches that should be addressed for finding the key of boar semen cryoconservation. The new perspectives in semen cryopreservation and techniques compiled in this paper point out both the actual high efficiency and precision requirements for boar semen cryopreservation, even if conclusive results have been achieved so far and further more still needed.

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