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EXTENDERS IN BOAR SEMEN PRESERVATION

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Introduction. Cryopreservation induces wide-range changes in the structural membrane integrity of spermatozoa, resulting in a reduction in their fertilizing ability (Yeste, 2016; Yeste et al., 2017; Fraser et al., 2018). Moreover, the specific susceptibility of spermatozoa to cold shock-induced damage has been shown to be a major factor responsible for compromised post-thaw (PT) semen quality (Pettitt & Buhr, 1998; Fraser et al., 2010;

Alkmin et al., 2014). Cryobiochemical analysis of frozen-thawed spermatozoa has demonstrated that ultrastructural modifications in the sperm plasma membrane play a key role in the reduced quality of PT semen (Ekwall, 2009; *Yeste, 2016)*. Furthermore, evidence has indicated that cryo-induced damage significantly compromises the functions of spermatozoa, which are manifested in alterations in their membrane constituents (Pettitt & Buhr, 1998; Fraser et al., 2011; Leahy & Gadella, 2011), and might be associated with pro-apoptotic changes (Trzcińska et al., 2015; Wasilewska et al., 2016), probably due to the excessive production of reactive oxygen species (ROS). The results of the above-mentioned studies have confirmed that the maintenance of the sperm membrane properties during semen preservation is crucial for spermatozoa to successfully participate in the fertilization-related events.

Recently, more detailed study on the sperm physiology and the impact of cryo-induced stress on sperm function have contributed to renewed interest in developing an optimal cryopreservation protocol for boar semen. The cryopreservation of boar semen consists of a multi-step procedure, including dilution, cooling, and freezing and thawing (Johnson et al., 2000), and many efforts have been made to modify the initial stage of the cryopreservation procedure (Guthrie & Welch, 2005; Casas & Althouse, 2013; Alkmin et al., 2014; Yeste et al., 2014; Frydryová et al., 2015). The use of different long-term extenders, in combination with an extended holding time (HT) period of cooled semen at the initial stage of the freezing procedure, has been incorporated in the cryopreservation protocol to improve sperm cryo-survival (Wasilewska et al., 2016; Wasilewska & Fraser, 2017*)*. In recent years many semen extenders have been developed to enhance the protective action of spermatozoa against cold shock-induced damage (van den Berg et al. 2014; Weitze, 2014). It is noteworthy that the ability of stored semen to withstand the preservation conditions depends on the components of the extenders (short-term, middle-term and long-term) to provide a favorable environment for the sperm cells during the different cooling regimens (Guthrie & Welch, 2005; Weitze, 2014; Frydryová et al., 2015; Karunakaran et al., 2016). Even though a large number of boar semen extenders are available, there are marked differences among the extenders, in terms of viability and fertilizing ability of spermatozoa following liquid storage (Dziekońska et al., 2013; Schulze et al., 2013; Karageorgiou et al., 2016; Karunakaran et al., 2016) or cryopreservation (Kaeoket et al., 2011; Dziekońska et al., 2015; Frydryová et al., 2015; Schäfer et al., 2018*)*. The standard cryopreservation protocol of boar semen incorporates a HT period of 2h at 17°C (HT 1), but in our laboratory we have sought to mimic this step through the application of a modified protocol based on the incorporation of a 24-h period at 10°C (HT 2), in

combination with long-term extenders (Fraser et al., 2015; Wasilewska et al., 2016; Wasilewska & Fraser, 2017). This review gives a brief outline of the effects of some selected semen extenders on PT quality of boar semen. In this review we have focused mainly on the effects of three semen extenders, Androhep® Plus (AHP, Minitübe), Androstar® Plus (ASP, Minitübe, Tiefenbach, Germany) and TRIXcell® Plus (TCP, IMV Technologies, France) on the quality of pre-freeze and PT boar semen.

Extender effects on sperm function during semen preservation. Most semen extender comprise sugars, proteins and buffering agents, which are found to be indispensable for providing extracellular protection to spermatozoa during preservation (Johnson et al., 2000; Gadea, 2003; van den Berg et al., 2014). Moreover, much effort has been sought to prolong the sperm viability in liquid-stored boar semen (Dziekońska et al., 2013; Schulze et al., 2013; Karageorgiou et al., 2016; Karunakaran et al., 2016), and to optimize the cryopreservation protocol using different long-term extenders prior to freezing (Kaeoket et al., 2011; Casas & Althouse, 2013; Frydryová et al., 2015; Schäfer et al., 2018). The results of these studies have confirmed that the components of the semen extenders are indispensable for providing protection to spermatozoa against cold shock-induced damage, consequently preserving their function. Besides our studies (Fraser et. al., 2015; Wasilewska et al., 2016; Wasilewska & Fraser, 2017), previous findings have confirmed that overnight cooling of extended semen at 15°C or 17°C had beneficial effects on PT semen quality (Guthrie & Welch 2005; Alkmin et al., 2014; Yeste et al., 2014). We have provided strong evidence that the incorporation of a 24-h cooling period at 10°C, in combination with different long-term extenders, significantly improved the sperm cryotolerance.

Due to the extent of cryo-induced damage to spermatozoa, specific molecular markers have be used to provide reliable assessment of the different sperm attributes prior to and after freezing-thawing, and a plethora of sperm tests have been used to assess the quality of pre-freeze and PT semen (Kordan et al., 2013; Daigneault et al., 2015; Yeste, 2016; Fraser, 2017). Sperm motility and motion characteristics, analyzed by the CASA (computer-assisted semen analysis) system, include a wide range of parameters, such as total motility (TMOT), progressive motility (PMOT), velocity straight line (VSL), velocity curvilinear (VCL), velocity average pathway (VAP), path straightness (STR, VSL/VAP×100), linearity (LIN, VSL/VCL×100), mean amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Our studies have shown that there were wide variations in the CASA-analyzed motility and motion characteristics among the extenders and between the holding time periods (Wasilewska et al., 2016; Wasilewska

& Fraser, 2017). Furthermore, there were no consistent differences in the pre-freeze and PT sperm path characters, STR and LIN among the extenders, or between the HT periods (Tables 1-2). At this point we are unable to explain these inconsistencies in the sperm path characters in the pre-freeze and PT semen. However, it was reported that boars with good freezability ejaculates exhibited greater STR and LIN than those with poor freezability ejaculates following storage at 5°C, prior to freezing (Casas et al., 2009). It should be emphasized that higher values of STR might indicate that the trajectory of the spermatozoa is uniform, whereas higher LIN values indicate a lesser amplitude of the VCL path (Casas et al., 2009; Perumal et al., 2014). As shown in Table 2, even though there was a tendency for greater STR and LIN values in frozen-thawed spermatozoa, the changes were not significant.

In our studies, variations in ALH and BCF of the pre-freeze and frozen-thawed boar spermatozoa were more marked among the extenders in HT 1 period (Table 1 and Table 2, respectively). Moreover, we have shown that there were marked variations among the extenders, with respect to the velocity parameters (VCL, VSL and VAP), particularly in the pre-freeze semen (Wasilewska et al., 2016; Wasilewska & Fraser, 2017). It is interesting to note that these changes were concurrent with higher ALH values in the pre-freeze semen, irrespective of the extender. However, it should be emphasized that even though the CASA-analyzed motility (TMOT and PMOT) and motion characteristics in PT semen are essential for spermatozoa to achieve fertilization, their threshold levels on fertility have not yet been established (Casas et al., 2009; Perumal et al., 2014). However, Casas et al. (2009) suggested that the susceptibility of boar spermatozoa to cold shock-induced damage could be predicted by the analysis of the CASA motion parameters. It is noteworthy that BCF, a motion parameter associated with the fertilization-related events, indicates the number of times the sperm track crosses the smoothed path, and is associated with linear progression (Perumal et al., 2014). Our findings have shown that there were lower BCF values in the HT 1 period of the pre-freeze and PT semen treated with AHP extender compared with the TCP extender, indicating the differential effects of the components of the extenders on the sperm motion pattern. Differences in the sperm motion characteristics between the pre-freeze and PT semen, and a wide range of motile sperm subpopulations with different values for velocity parameters, ALH and BCF, have been detected in frozen-thawed boar spermatozoa (Rodríguez-Martínez et al. 2008; Wasilewska et al., 2016; Wasilewska & Fraser, 2017).

The integrity of the sperm plasma membrane is an important parameter due to its major role in cellular protection and in cell-cell interaction, and its integrity is a prerequisite for successful sperm-egg fertilization (Leahy &

Gadella, 2011). Moreover, the plasma membrane of boar spermatozoa, with regard to its specific lipid-protein composition, is exceptionally susceptible to disturbances in its molecular mechanisms, mainly to the action of the different cooling periods on their specialized domains (Ekwall, 2009). Furthermore, cryo-induced changes in the lipid-protein composition of the sperm membranes have been shown to impair motility, decreased ATP production and reduced the sperm membrane integrity (Fraser et al., 2011; Alkmin et al., 2014; Daigneault et al., 2016; Yeste, 2016; Fraser et al., 2018). Several florescent probes have been used for the evaluation of the sperm quality characteristics to provide more detailed information about the different attributes of sperm cells following semen preservation.

Besides the CASA-analyzed sperm motility and motion assessments, microscope and fluorescent analysis commonly used to assess the semen quality include SYBR-14/PI (propidium iodide) assay (plasma membrane integrity), JC-1/PI and R123/PI assays (mitochondrial membrane function), FITC-PNA (fluorescein isothiocyanate conjugated peanut agglutinin) and the Giemsa staining technique (acrosome membrane), *Vybrant Apoptosis assay (*viability), Comet assay, SCSA (sperm chromatin structure assay) and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end) assay (DNA and chromatin integrity), and TBARS assay (thiobarbituric acid reactive substances) assay (Kordan et al., 2013; Trzcińska et al., 2015; Daigneault et al., 2016; Yeste, 2016; Fraser, 2017; Fraser et al., 2018). We have shown that the proportions of PT spermatozoa with functional mitochondrial membrane and intact plasma membrane are highly correlated in each extender (Figures 1-2). Such correlations were more marked in semen samples of the HT 1 period, irrespective of the extender (Figures 1-2), indicating the varying effects of the extender components on the mitochondrial membrane potential, plasma membrane integrity and viability of frozen-thawed spermatozoa after a 24-h storage at 10°C. It is well known that the composition of most long-term extenders includes a complex buffered system and protein additive, such as bovine serum albumin (BSA) or whey protein Porex (Gadea, 2003; Waberski et al., 2011; van den Berg et al., 2014; Weitze, 2014). Both the ASP and AHP extenders comprise a non-animal origin substance, which confers protection to membranes of boar spermatozoa during their preservation (Waberski et al., 2011; Weitze, 2014). Furthermore, the results of our study confirmed that there were high percentages of *viable frozen-thawed spermatozoa (*YO-PRO-1^{-}/PI^{-}*) in the extended semen samples stored for 24h at* 10°C, suggesting increased sperm cryo-tolerance (Fraser et al., 2015; Wasilewska et al., 2016; Wasilewska & Fraser, 2017). Such enhanced sperm cryo-tolerance was more marked in the semen samples cooled in the AHP and AHS extenders, reaffirming that the

specific composition of these extenders have a significant protective effect on the sperm membrane structures against cold shock-induced damage.

Boar variability in PT semen assessment. Even though studies have been shown that storage of boar semen in long-term extenders before freezing have beneficial effects on PT semen quality (Kaeoket et al., 2011; Frydrychová et al., 2015; Schäfer et al., 2017), the success in sperm cryosurvival is partially dependent on the response of individuals to the cryopreservation conditions (Holt et al., 2005; *Casas et al., 2009;* Alkmin et al., 2014; Yeste, 2016). These findings corroborate those of our studies (Wasilewska et al., 2016; Wasilewska & Fraser, 2017), which confirmed that type of extender had varying effects on the quality of PT semen among individual boars. The main reason for the boar-to-boar variations in the response to the freezing-thawing procedure is still not fully understood, but seem to be related to molecular markers linked to genes controlling semen freezability (Thurston et al., 2002; Holt et al., 2005; Fraser et al., 2008). Furthermore, there are variations in the composition of boar seminal plasma (SP) among individuals (Strzeżek et al., 2005; Fraser et al., 2016), and their interactions with the extender components appear to have a positive effect on sperm viability during preservation (Plante et al., 2016). Overall, the role of boar SP and its interaction with the extender components and spermatozoa is still unclear.

In our recent study (Wasilewska & Fraser, 2017), we have confirmed that differences in the sperm treatments (HT 1 and HT 2), prior to freezing, had a significant effect on the membrane proteome of frozen-thawed spermatozoa, irrespective of the extender. *The sperm electrophoretic profiles in samples treated with various extenders differed significantly among the boars within the HT period, particularly in boars with poor semen freezability (*Wasilewska & Fraser, 2017). Substantial changes in the sperm membrane proteome following cryopreservation have been shown to compromise the sperm fertilizing ability (*Casas et al., 2009;* Leahy & Gadella, 2011; Chen et al., 2014; Yeste, 2016). Boar spermatozoa comprise numerous proteins, in which their susceptibility to the freezing-thawing process and role in cryo-tolerance have not been elucidated as yet (Wasilewska & Fraser, 2017; Yeste, 2016). Since we did not characterize and analyze the level of expression of the identified sperm membrane-associated proteins, we are unable to specify their significant relevance either in the pre-freeze semen or PT semen. In a previous study, it was confirmed that boar spermatozoa could modulate their functions during the cooling period, prior to freezing, as indicated in the changes in the levels of heat shock protein 70 (Yeste et al., 2014). There is growing evidence indicating that protein phosphorylation or carbonization of membrane proteins, caused by oxidative stress dur-

ing the cryopreservation process, is associated with cryo-capacitation, resulting in the reduced lifespan of spermatozoa within the female reproductive (Leahy & Gadella, 2011; Mostek et al., 2017). It should be noted that the mechanism of cryo-damage to sperm membrane proteins is still unclear.

Conclusions. The use of long-term extenders to store boar semen overnight at 10°C has modulated the functions of spermatozoa, rendering them less susceptible to cryo-induced damage. Furthermore, the performance of the PT semen varied significantly among the boars, suggesting that the type of extender used for semen cryopreservation is also an important factor affecting sperm cryo-survival at different cooling regimens.

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Table 1 – Pre-freeze CASA-motion parameters of boar spermatozoa held in different extenders in holding time periods. Values represent the means ± SEM (Duncan multiple comparison test).

Extender	Straightness (STR, %)		Linearity (LIN, %)		ALH (μm))		BCF (Hz)	
	HT 1	HT 2	HT 1	HT 2	HT 1	HT 2	HT 1	HT 2
AHP	75.7 ± 1.7	70.9 ± 1.4	44.9 ± 1.4	41.2 ± 1.1	6.9 ± 0.2^{a}	6.9 ± 0.3	31.9 ± 0.6^{a}	31.5 ± 0.6
ASP	76.2 ± 1.8	72.0 ± 1.1	44.7 ± 1.4	43.0 ± 0.9	6.3 ± 0.2^{b,x}	7.1 ± 0.2^{y}	33.5 ± 0.7^{b,x}	31.3 ± 0.5^{y}
TCP	76.6 ± 1.5	74.2 ± 1.3	43.2 ± 1.1	42.4 ± 1.0	6.6 ± 0.3^{ab}	6.9 ± 0.1	32.0 ± 0.5^{ab}	31.7 ± 0.4

Within extender, values with different superscripts (a-b) are significant at P < 0.05. Values with different letters (x-y) are significantly differed between the HT1 and HT 2 periods at P < 0.05.

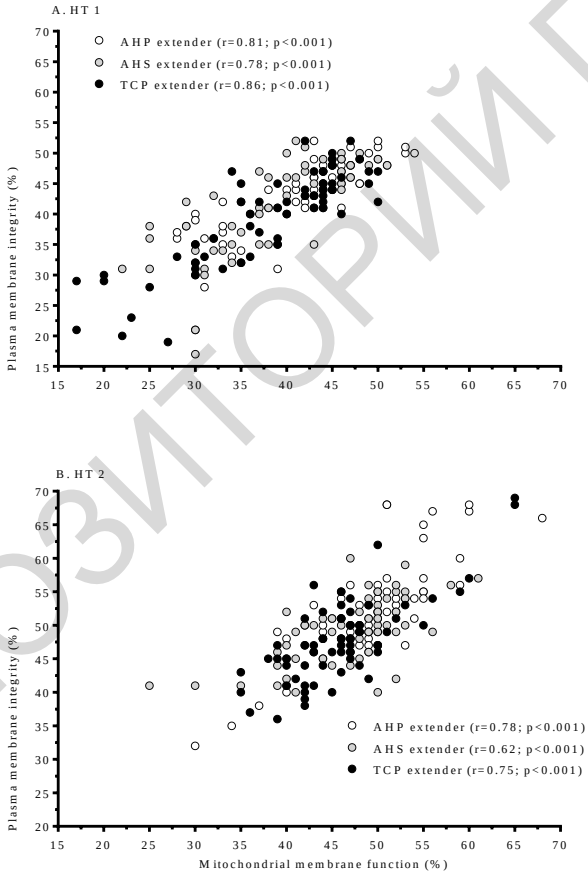
HT 1–2h/17°C; HT 2–24h/10°C; AHP–Androhep® Plus; ASP–Androstar® Plus;TCP–TRIXcell®Plus; ALH–amplitude of lateral head displacement; BCF–beat cross frequency.

Table 2 – Post-thaw CASA-motion parameters of boar spermatozoa held in different extenders in holding time periods. Values represent the means ± SEM (Duncan multiple comparison test).

Extender	Straightness (STR, %)		Linearity (LIN, %)		ALH (μm)		BCF (Hz)	
	HT 1	HT 2	HT 1	HT 2	HT 1	HT 2	HT 1	HT 2
AHP	84.4 ± 1.1	86.3 ± 0.9	55.9 ± 1.3	58.7 ± 1.7	5.6 ± 0.2	5.5 ± 0.1	24.3 ± 0.7^{ax}	27.5 ± 0.7^{y}
ASP	85.8 ± 0.9	86.2 ± 1.1	54.7 ± 1.2	57.1 ± 1.3	5.5 ± 0.3	5.6 ± 0.2	26.6 ± 0.8^{b}	27.6 ± 0.6
TCP	85.6 ± 0.7	87.0 ± 0.8	53.1 ± 1.0^{x}	58.5 ± 1.6^{y}	5.3 ± 0.1	5.2 ± 0.1	28.1 ± 0.7^{b}	26.8 ± 0.8

Within extender, values with different letters (a-b) are significant at P < 0.05. Values with different letters (x-y) are significantly differed between the HT 1 and HT 2 periods at P < 0.05. HT 12h/17°C; HT 2–24h/10°C; AHP–Androhep® Plus; ASP–Androstar® Plus; TCP–TRIXcell® Plus. ALH–amplitude of lateral head displacement; BCF–beat cross frequency

Figure – Scatter plots showing the relationships between post-thaw plasma membrane integrity (SYBR-14/PI assay) and mitochondrial membrane function (JC-1/PI assay) of boar spermatozoa held in different extenders for (A) 2h at17°C (HT 1) and 24h at 10°C (HT



2).

Conclusions. The use of long-term extenders to store boar semen overnight at 10°C has modulated the functions of spermatozoa, rendering them less susceptible to cryo-induced damage. Furthermore, the performance of the PT semen varied significantly among the boars, suggesting that the type of extender used for semen cryopreservation is also an important factor affecting sperm cryo-survival at different cooling regimens.

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