Hypotonic resistance of boar spermatozoa: sperm subpopulations and relationship with epididymal maturation and fertility

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Abstract

Hypotonic resistance of boar spermatozoa was investigated by measuring the ratio of live/dead spermatozoa (SYBR-14/propidium iodide) by flow cytometry after hypotonic stress. The survival rate of ejaculated spermatozoa incubated in hypotonic solutions ranging from 3 to 330 mmol/kg followed a sigmoid curve that fitted a simple logistic model. The critical osmolality value (Osm_{crit}) at which 50% of spermatozoa died was determined with this model. Hypotonic resistance of spermatozoa increased with temperature between 15 and 39 °C and decreased after hydrogen superoxide treatment, but was not modified during 8 days of preservation in Beltsville thawing solution. Hypotonic resistance markedly decreased during epididymal maturation and after ejaculation as Osm_{crit} at 15 °C was 54.7 ± 3.2 , 68.5 ± 10.6 , 116.7 ± 2.1 and 194.3 ± 3.7 mmol/kg for the caput, corpus, cauda and ejaculated spermatozoa respectively. Hypo-osmotic stress of 100 mmol/kg revealed a sperm subpopulation exhibiting increased hypotonic resistance compared with the whole ejaculate ($Osm_{crit} = 67.8 \pm 2.1$ mmol/kg). Consistent differences were observed between lean and standard breeds (Pietrain versus Large White) and between boars within the same breed. According to data collected by artificial insemination centers during a large-scale field trial, hypotonic resistance of ejaculates was found to be positively correlated with *in vivo* fertility.

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Introduction

Owing to volume-regulatory processes, animal cells are able to withstand substantial osmotic stress without permanent damage (Okada *et al.* 2001, Stutzin & Hoffmann 2006). When subjected to moderate hypotonic conditions, spermatozoa undergo initial swelling, caused by the entry of external water through the plasma membrane, followed by a decrease in volume to the initial value through a process known as regulatory volume decrease (RVD; Yeung *et al.* 2006, Petrunkina *et al.* 2007a). When hypotonic stress overwhelms the RVD process, swelling of the spermatozoa induces coiling of the flagella and ultimately disrupts the plasma membrane, leading to cell death (Curry *et al.* 2000). Although understanding the metabolic and signaling pathways involved in sperm RVD is increasing (Yeung *et al.* 2003, 2005, Klein *et al.* 2006, Petrunkina *et al.* 2007b), the distinct role of membrane mechanical properties allowing resistance of spermatozoa to hypotonic stress remains to be fully elucidated. The relationship between hypotonic resistance and cryopreservation has been

studied intensively (Curry & Watson 1994, Gilmore *et al.* 1998, Watson 2000, Petrunkina *et al.* 2005), but the impact of hypotonic resistance of the membranes of fresh spermatozoa on fertility remains unclear. In a limited study performed in porcine species, the resistance of the acrosomal sperm membrane to breakage under hypotonic conditions was reported to be associated with *in vivo* fertility (Schilling & Vengust 1987).

Sperm fertility is acquired during epididymal maturation, together with concomitant modifications of membrane properties such as membrane fluidity and lipid composition (Nikolopoulou *et al.* 1985, Rana & Majumder 1990, Rana *et al.* 1991). Information regarding the changes in hypotonic resistance of spermatozoa during epididymal maturation might help to understand how osmotic resistance may affect fertility. Moreover, spermatozoa are subjected to changes in lipid composition after ejaculation, as well as several stresses such as lipid peroxidation that affects membrane fluidity (Christova *et al.* 2004, Brouwers *et al.* 2005), and these may result in decreased fertility after *in vitro* preservation. Oxidation and long-term preservation might thus

modify the properties of the sperm membrane and its hypotonic resistance.

The aims of this study were therefore to identify factors affecting the hypotonic resistance of boar spermatozoa and to evaluate the relationship between hypotonic resistance and fertility.

Results

Modeling of hypotonic resistance of boar spermatozoa

In order to measure solely the impact of hypotonic resistance on spermatozoa diluted in BTS and stored at 15 °C, the hypotonic media with osmolality ranging from 3 to 330 mmol/kg were based on BTS and the incubation was performed at 15 °C. The proportion of live boar spermatozoa after incubation at 15 °C in these media showed a sigmoid curve (Fig. 1). Several nonlinear regression models such as sigmoid, logistic, and Weibull models could also fit this variation. The sigmoid model was chosen because only three parameters had to be calculated with regard to the experimental data available for each curve (see Materials and Methods).

Effects of temperature, oxidation, and storage duration on hypotonic resistance

Hypotonic resistance was positively correlated with temperature (Fig. 2): Osm_{crit} was 194.3 ± 3.7 , 170.2 ± 3.1 , and 132.9 ± 4.6 mmol/kg at 15, 25, and 39 °C respectively ($P < 0.01$). Lipid peroxidation of spermatozoa was induced in order to evaluate the potential

Figure 1 Effects of hypo-osmotic stress on membrane integrity of boar-ejaculated spermatozoa. Data are presented as percentage of the initial percentage of live cells under iso-osmotic conditions. Error bars not seen are within the symbol. Solid curve shows the logistic model of the percentage of live sperm after hypo-osmotic stress ($n = 12$ boars). Critical osmolality (Osm_{crit}) was defined as the osmolality at which 50% of the cells died due to osmotic stress and was calculated from the model.

relevance of lipids in membrane resistance to hypotonic stress. When hydrogen peroxide was used to produce rapid oxidation of the spermatozoa, the proportion of live spermatozoa in the iso-osmotic medium (BTS) was not different between treatments ($P < 0.01$) but the hypotonic resistance was greatly reduced (Fig. 3). The Osm_{crit} value increased from 185 ± 5.3 to 211 ± 1.9 , 235 ± 3.9 , and 248 ± 3.9 mmol/kg for 0, 0.1, 1, and 10 mM hydrogen peroxide respectively ($P < 0.01$). By contrast, the hypotonic resistance of ejaculated spermatozoa was not significantly modified after 0, 1, 3, and 8 days of storage in BTS at 15 °C (199 ± 4.6 , 195.1 ± 4.7 , 196.5 ± 10.0 , and 196.5 ± 9.6 mmol/kg respectively).

Hypotonic resistance and epididymal maturation

Epididymal maturation was associated with a significant decrease in hypotonic resistance (Fig. 4): Osm_{crit} was 54.7 ± 3.2 for caput spermatozoa, and increased to 68.5 ± 10.6 for the corpus and 116.7 ± 2.1 mmol/kg for the cauda ($P < 0.01$). Hypotonic resistance was further decreased after ejaculation, reaching an Osm_{crit} of 194.3 ± 3.7 mmol/kg for ejaculated spermatozoa.

Isolation of a subpopulation of spermatozoa which increased hypotonic resistance

Boar spermatozoa were diluted in control iso-osmotic medium (330 mmol/kg) and subjected to hypotonic stress at 100 mmol/kg at 15 °C. The proportion of live spermatozoa after 25 min was $87.7 \pm 2.6\%$ in control medium and $19.6 \pm 1.5\%$ in 100 mmol/kg medium.

Figure 2 Effects of temperature on hypotonic resistance of ejaculated spermatozoa. Spermatozoa were subjected to hypo-osmotic stress at 15, 25, and 39 °C. Data are presented as the percentage of the initial percentage of live cells under iso-osmotic conditions ($n = 13$ boars). Critical osmolality (Osm_{crit}) was linearly dependent on temperature between 15 °C and 37 °C.

Figure 3 Effects of oxidation on hypotonic resistance of ejaculated spermatozoa. Spermatozoa were incubated with hydrogen peroxide (0.1, 1, and 10 mM) and subjected to hypo-osmotic stress at 15 °C. Data are presented as the percentage of the initial percentage of live cells under iso-osmotic conditions ($n=3$ boars).

After incubation, live spermatozoa were sorted by flow cytometry based on live/dead staining with SYBR-14/PI and returned to iso-osmotic conditions. The proportions of live spermatozoa for the live sorted population were $99.7 \pm 0.1\%$ and $97.6 \pm 0.4\%$ in control and hypotonic conditions respectively. After the re-equilibration step in iso-osmotic conditions, sorted spermatozoa were again subjected to hypotonic stress over the 3–330 mmol/kg

Figure 4 Effects of epididymal maturation on hypotonic resistance. Epididymal spermatozoa from caput, corpus, and cauda regions, and ejaculated spermatozoa were subjected to hypo-osmotic stress at 15 °C. Data are presented as the percentage of the initial percentage of live cells under iso-osmotic conditions ($n=3$ boars). Critical osmolality (Osm_{crit}) increased during epididymal maturation and after ejaculation. Values with different letters (a, b, c, d) are significantly different ($P<0.05$).

range of osmolality (Fig. 5). The control spermatozoa (330 mmol/kg-sorted) exhibited the typical sigmoid evolution as a function of osmolality ($Osm_{crit} = 186.7 \pm 6.0$ mmol/kg), while the spermatozoa sorted for their hypotonic resistance (100 mmol/kg-sorted) were not affected between 330 and 100 mmol/kg ($P<0.01$), showing that this subpopulation was much more resistant to hypotonic stress ($Osm_{crit} = 67.8 \pm 2.1$ mmol/kg).

Figure 5 Identification of a subpopulation of ejaculated spermatozoa with increased hypotonic resistance. (A) Effects of hypo-osmotic stress on sperm membrane integrity. Example of representative individual boar showing the subpopulation of spermatozoa with increased hypotonic resistance ($n=3$ ejaculates). Data are presented as the percentage of the initial percentage of live cells under iso-osmotic conditions. (B) Live spermatozoa were sorted by FACS after iso-osmotic incubation at 330 mmol/kg (330 mmol/kg-sorted) or after initial hypo-osmotic stress at 100 mmol/kg (100 mmol/kg sorted). These two populations of live spermatozoa were then re-equilibrated in the iso-osmotic buffer (330 mmol/kg) and subjected to further hypo-osmotic stress over the complete 0–330 mmol/kg range. Data presented are raw percentages of live spermatozoa after sorting and the second hypo-osmotic stress ($n=4$ boars).

Effects of breed

Genetic origin affected the hypotonic resistance of spermatozoa (Fig. 6): spermatozoa from Pietrain boars showed greater hypotonic resistance than Large White boars ($\text{Osm}_{\text{crit}} = 148.6 \pm 2.3$ vs 188.4 ± 1.2 mmol/kg respectively, $n=48$ and $n=77$, $P<0.01$).

Hypotonic resistance and fertility

The relationship between hypotonic resistance and fertility of the spermatozoa was evaluated during a field trial involving 11 Pietrain boars and 922 Pietrain sows housed in 12 different commercial farms. After correction for the farm effect, farrowing rate and litter size were not significantly different between boars. No significant correlation was observed between the mean hypotonic resistance value for each boar and fertility (farrowing rate and litter size). The relationship between hypotonic resistance and fertility was further investigated after grouping the total of 133 ejaculates into classes according to their Osm_{crit} . The ejaculates were grouped in three classes, with $\text{Osm}_{\text{crit}} > 170$ mmol/kg for class 1, between 150 and 170 mmol/kg for class 2, and < 150 mmol/kg for class 3. Mean Osm_{crit} was 191.3 ± 0.8 , 160.5 ± 0.4 , and 128.8 ± 1.4 mmol/kg for classes 1, 2, and 3 respectively. Although litter size was not significantly different between classes, the farrowing rate was significantly different ($P<0.05$), being 88.7 ± 3.6 , 92.1 ± 3.0 , and $95.4 \pm 2.4\%$ for classes 1, 2, and 3 respectively (Fig. 7). Sperm viability and mobility were not significantly different between classes.

Discussion

In our experimental conditions, the hypotonic resistance of boar spermatozoa was evaluated by the resistance of the plasma membrane to rupture in hypotonic media. Between 3 and 330 mmol/kg, the proportion of spermatozoa showing intact membranes was not linearly

related to osmotic pressure. Instead, the hypotonic resistance followed a curve that was appropriately fitted by several non-linear models. Models such as sigmoid, logistic or Weibull models, often used for failure analysis (Weibull 1951), could be used for fitting the rupture resistance of the boar sperm membrane. The logistic model was chosen because only a limited number of parameters were needed to fit the experimental curves. This modelization therefore allowed the use of a single value, Osm_{crit} , defined as the osmolality at which 50% of the spermatozoa remain alive (Noiles *et al.* 1997) and which, in the case of this logistic curve, is the x-coordinate of the curve point of inflexion. This parameter was used throughout the study to summarize the logistic curve and to study the impact of several factors on hypotonic resistance.

Hypotonic resistance was linearly dependent on incubation temperatures between 15 and 39 °C. It has been shown that the fluidity of the plasma membrane of boar spermatozoa increases with temperature (Canvin & Buhr 1989). The increase in hypotonic resistance could be explained by increased fluidity and greater ability of spermatozoa to tolerate swelling. This is also supported by the fact that oxidative treatment of spermatozoa with cumene hydroperoxide has been shown to reduce the diffusion of membrane lipids (Christova *et al.* 2004), and in our study hydroperoxide also decreased hypotonic resistance. The genetic impact on hypotonic resistance observed between the Pietrain breed and the Large White breed might also be related to the composition of sperm lipids since Pietrain boars are leaner than Large White boars (Quinious & Noblet 1995). The relationship between the body lipid composition and the sperm lipid composition in these two breeds has not yet been established, but it can be hypothesized that these breeds produce spermatozoa with different lipid compositions and hence different physical properties. Our findings showed clearly a progressive decrease in the hypotonic resistance of boar spermatozoa during their transit from

Figure 6 Effects of breed on hypotonic resistance of ejaculated spermatozoa. Semen from Large White and Pietrain boars were subjected to hypo-osmotic stress at 15 °C. Data are presented as the percentage of the initial percentage of live cells under iso-osmotic conditions. (A) Large White breed ($n=77$ boars). Each spot is the mean value for one boar (two ejaculates per boar). (B) Comparison of Large White and Pietrain breeds. (C) Pietrain breed ($n=48$ boars).

Figure 7 Effects of hypotonic resistance on fertility of ejaculated spermatozoa. Values represent farrowing rate for three classes of ejaculates with different critical osmolality. Hypotonic resistance of sperm was positively correlated with *in vivo* fertility as low critical osmolality was associated with high farrowing rate. Osm_{crit} was over 170 mmol/kg for class 1, between 150 and 170 mmol/kg for class 2, and less than 150 mmol/kg for class 3. Values with different letters (a, b, c) are significantly different ($P < 0.05$).

the caput to the cauda of the epididymis. The resistance of osmotically swollen spermatozoa to rupture by detergent was reported to be higher for caput than cauda spermatozoa in the ram (Schweisguth & Hammerstedt 1992), and higher hypotonic resistance was also reported for caput than cauda spermatozoa in the kangaroo (McClean *et al.* 2006). During epididymal maturation, boar spermatozoa undergo modifications of lipid membrane composition (Evans & Setchell 1979, Nikolopoulou *et al.* 1985) and this lipid membrane remodeling leads to the appearance of domains with different diffusional properties (James *et al.* 1999, Bruckbauer *et al.* 2007, Jones *et al.* 2007). During this epididymal transit, spermatozoa also undergo changes in protein composition related to sequential changes in epididymal fluid proteins due to epididymal epithelium secretion (Dacheux *et al.* 1989, 1998, 2005, Syntin *et al.* 1996). Decreases in hypotonic resistance during epididymal maturation could thus be associated with these changes in lipid and protein compositions, resulting in different physical properties of the membrane.

We observed that hypotonic resistance of boar spermatozoa was markedly reduced after ejaculation. During ejaculation, large amounts of boar seminal plasma proteins, such as spermadhesins, bind to the sperm membrane (Dostalova *et al.* 1994, Jonakova *et al.* 2000). Bull seminal plasma protein PDC 109, related to spermadhesins, binds with high affinity to sperm membrane lipids (Ramakrishnan *et al.* 2001, Tannert *et al.* 2007) and decreases the mobility of spin-labeled phospholipids in epididymal sperm membranes (Greube *et al.* 2001). SP $1/2$, a stallion seminal plasma protein related to PDC 109, also induces immobilization of epididymal sperm phospholipids (Greube *et al.* 2004). Bovine seminal plasma proteins, such as NPC2, induce cholesterol efflux from cauda epididymal spermatozoa and affect the combination of lipids and membrane

proteins (Girouard *et al.* 2008). The role of seminal plasma proteins on hypotonic resistance remains to be fully determined, but the coating of seminal proteins on epididymal spermatozoa might decrease hypotonic resistance through a decrease in fluidity of the sperm membrane lipid phase. Taken together, our results suggest that the hypotonic resistance of boar spermatozoa at 15 °C could be explained by mechanical properties of the membrane related to membrane fluidity.

Evidence of a subpopulation of spermatozoa with increased hypotonic resistance has already been suggested by repeated hypotonic stress on ram spermatozoa, showing that the proportion of live spermatozoa stabilized after the first osmotic stress (Curry & Watson 1994). The distribution of boar sperm volume after moderate hypotonic stress also suggested sperm subpopulations (Petrounkina *et al.* 2000). Our study revealed the existence in the boar ejaculate of a subpopulation of spermatozoa resistant to hypotonic stress at 15 °C. We hypothesized the existence of this subpopulation when the proportion of live spermatozoa observed after incubation in 60–100 mmol/kg media was always higher than expected with non-linear models. The resistant spermatozoa could then be effectively separated by cell sorting from the other populations and could withstand subsequent hypotonic stress without affecting their viability. The mechanisms underlying this hypotonic resistance are unknown, but it could be hypothesized that these spermatozoa might have a different lipid composition resulting in increased membrane fluidity, as a subpopulation of boar spermatozoa with a lower cholesterol/phospholipids ratio has been described (Flesch *et al.* 2001). It is of note that the resistant subpopulation was only observed for ejaculated spermatozoa and not for epididymal spermatozoa. The effects of the interaction between seminal plasma and epididymal spermatozoa might vary according to different epididymal sperm subpopulations. The increased hypotonic resistance of this subpopulation could therefore be explained as being a consequence of a different interaction of seminal plasma with an epididymal subpopulation with distinct properties.

Throughout this study, several boars with spermatozoa showing extremes of hypotonic resistance (high or low) were used weekly over long periods (1 or 2 years) and they always exhibited the expected hypotonic resistance, despite the fact that variations in viability and motility were observed. Moreover, during the fertility study (which extended over 13 weeks), the hypotonic resistance of extreme Pietrain boars remained without significant variation between 13 consecutive ejaculates, and this parameter appears to be a consistent semen characteristic which is male dependent.

The relationship between boar semen quality and fertility has been widely documented, (Rodriguez-Martinez 2006) without consensus regarding effective fertility predictors. In commercial sow farms, the

prediction of fertility by semen parameters is limited by the use of high numbers of spermatozoa per dose and high quality of semen (Gadea *et al.* 2004). As we observed that hypotonic resistance showed greater repeatability and variability among artificial insemination (AI) boars than classical criteria, the aim of the fertility trial was first to characterize boars on their hypotonic resistance and then to relate this parameter to fertility. Despite the scale of this trial, and probably because of the high standard of AI conditions (3 AI per cycle with 3.10^9 of spermatozoa showing 80% normal morphology and motility) and the design of the trial correctly evaluating farm and parity effects, no significant difference in fertility was found between males. However, when ejaculates were grouped by class according to their hypotonic resistance, we were able to demonstrate a significant positive correlation between hypotonic resistance and fertility, even in the narrow range of 85–95% farrowing rate.

The mechanisms underlying the relationship between hypotonic resistance and fertility remain unclear. It has been proposed that the ability to regulate sperm volume through RVD processes in the female genital tract is important for fertility because spermatozoa undergo hypotonic stress due to the lower osmolality of uterine fluid compared with those of cauda epididymal fluid and seminal plasma (Yeung & Cooper 2001). Indeed, RVD has been reported to be associated with *in vivo* fertility in boar species (Petrunkina *et al.* 2004). Although it has been reported that the RVD process was acquired together with fertilizing ability during mouse epididymal maturation (Yeung *et al.* 2002), this study clearly showed that hypotonic resistance decreased during the epididymal maturation in the boar. Different mechanisms could possibly be involved, as accumulation of osmolytes could be involved in the acquisition of RVD during maturation (Yeung *et al.* 2004). Our findings also suggest that the decrease in hypotonic resistance might be associated with structural changes in the sperm membrane. The relationship between RVD and the hypotonic resistance measured in our conditions, based on mechanical resistance of the membrane to rupture, require further investigation and suggest the use of this parameter as a fertility predictor in the boar.

Materials and Methods

Reagents and media

The live/dead sperm viability kit was obtained from Molecular Probes (Eugene, OR, USA). All chemicals were of reagent grade and purchased from Sigma. The medium used was BTS (Beltsville Thawing Solution extender) and hypo-osmotic solutions were prepared by diluting iso-osmotic BTS (330 mmol/kg) with Millipore purified water (3 mmol/kg) to produce solutions of 3–330 mmol/kg. Actual media osmolalities were determined with a vapor osmometer (VAPRO,

Westcor Inc., Logan, UT, USA) and osmolalities were accurate to ± 3 mmol/kg.

Animals and semen collection

Large White and Pietrain boars were housed under standard conditions. Semen was collected by masturbation using the gloved-hand technique. The whole ejaculate was collected and filtered through gauze. Semen was diluted in 35 °C BTS at 3.10^7 spermatozoa per ml, packaged in 100 ml insemination doses in plastic bottles, and allowed to cool at 15 °C. All ejaculates showed high motility (>80% progressive motile spermatozoa) and normal morphology (>80% normal spermatozoa). Epididymides were obtained from three adult Large White boars. Spermatozoa from the different epididymal regions (caput, corpus and cauda) were collected by microperfusion (Dacheux 1980).

Assessment of motility

Sperm motility was determined after 10 min incubation at 37 °C using a computer-assisted analysis system (Hamilton-Thorn, Beverly, MA, USA). A 2 μ l aliquot was transferred to a preheated slide and a minimum of 300 cells were analyzed over 10 fields. Spermatozoa were classified as progressive spermatozoa when VAP > 80 μ m/s and LIN > 60.

Flow cytometric assessment of hypotonic resistance

The osmotic resistance of spermatozoa was estimated by the proportion of cells with intact plasma membranes after 20 min incubation in a hypo-osmotic medium. Aliquots of BTS-diluted semen (33 μ l, 3.10^7 cells/ml) were transferred into 1 ml iso- or hypo-osmotic BTS solution, ranging from 3 to 330 mmol/kg, containing 10 μ l SYBR-14 at 10 μ g/ml (final concentration of 100 ng/ml), and 2 μ l propidium iodide at 2 mg/ml (final concentration of 4 μ g/ml). SYBR-14 stained the nuclei of living spermatozoa bright green whereas PI stained red only dead spermatozoa that had lost their membrane integrity (Garner & Johnson 1995). The proportions of live/dead spermatozoa exhibiting SYBR-14 (green) and PI (red) fluorescence respectively were measured with an EPICS-XL flow cytometer (Beckman-Coulter, Villepinte, France) using an air-cooled argon laser operating at 488 nm and 15 mW. Forward scattering and side scattering were recorded to define the size characteristics and provide gating parameters for selection of the sperm population. A minimum of 10^4 cells was counted for each analysis.

Determination of Osm_{crit}

Critical osmolality (Osm_{crit}) was defined as the osmolality at which 50% of the cells died due to osmotic stress (Noiles *et al.* 1997) and was used as a parameter to characterize the osmotic resistance of the whole sperm population. This parameter was calculated from a sigmoid function model describing the relationship between sperm viability and osmolality by three parameters:

$$Y = b_0 / (1 + (e^{-(OS-b_2)})/b_1)$$

where Y is the percentage of live spermatozoa normalized to the percentage of live spermatozoa in control iso-osmotic medium and OS is the osmolality. The three parameters (b_0 , b_1 , and b_2) were calculated using the NLIN procedure of the SAS package (SAS Institute, Cary, NC, USA). Osm_{crit} was then determined using the above described model and calculated as:

$$Osm_{crit} = b_2 - b_1 \log((b_0 - 50)/50)$$

This non-linear model and the calculation of Osm_{crit} were validated using the first set of data generated from the analysis of hypotonic resistance at 15 °C of semen collected from 12 Large White boars.

Effects of several physical environmental and animal parameters on sperm hypotonic resistance

To evaluate the effects of temperature, incubation of spermatozoa in iso- and hypo-osmotic media (3–330 mmol/kg) was performed at 15, 25, and 39 °C in incubators. The hypotonic resistance of boar spermatozoa was analyzed during long-term preservation. BTS-diluted spermatozoa were stored at 15 °C for 8 days (Day 0 = collection day) and hypotonic resistance was assessed at 15 °C on days 0, 1, 3, and 8. The influence of oxidation status of spermatozoa was estimated by the addition of hydrogen peroxide to BTS-diluted spermatozoa (3.10^7 /ml) at final concentrations of 0.1, 1, and 10 mM, and spermatozoa were incubated overnight at 15 °C. Hypotonic resistance was then assessed at 15 °C.

The influence of the genetic origin of boars on osmotic resistance was studied in Large White boars and Pietrain boars housed under identical conditions in one commercial semen production center (Cobiporc, St Gilles, France). Hypotonic resistance was analyzed for 158 ejaculates from 77 Large White boars and 98 ejaculates from 48 Pietrain boars. Individual differences between males and ejaculates were further investigated in 11 Pietrain boars from which semen was collected once a week for 15 weeks, and hypotonic resistance was assessed at 15 °C.

Flow cytometric sorting of spermatozoa with increased hypotonic resistance

Flow cytometric sorting was used to separate spermatozoa with increased hypotonic resistance from the whole spermatozoa population. BTS-diluted semen (300 µl) was transferred and incubated for 20 min at 15 °C either in 10 ml BTS (330 mmol/kg-treated sample) or in 10 ml hypo-osmotic solution (100 mmol/kg-treated sample). Both solutions contained SYBR-14 (100 ng/ml) and PI (4 µg/ml). At the end of the incubation period, spermatozoa from 330 mmol/kg-treated samples and 100 mmol/kg-treated samples were subjected to flow cytometric analysis. Live spermatozoa, i.e. SYBR-positive and PI-negative spermatozoa, were identified and sorted on the basis of their green fluorescence. Sorted sperm from both samples were returned to iso-osmotic conditions by collection after sorting in a 15 ml conical tube containing 5 ml BTS (330 mmol/kg). The Cell sorter, an ALTRA cell sorter (Beckman-Coulter) with EXPO32 software,

operated at low pressure (12 psi) and moderate sorting rate (1000 cell/s) to assure high purity and maximum preservation of integrity after sorting. 10^7 live spermatozoa were sorted from 330 mmol/kg and 100 mmol/kg-treated samples. Sorted spermatozoa were then centrifuged (800 g, 5 min) and the pellet was gently re-suspended in 1 ml BTS. To check the efficiency of sorting, a 10 µl aliquot was immediately transferred to a 1 ml BTS tube and live/dead proportions were determined using the same flow cytometric procedure. The hypotonic resistance of live spermatozoa sorted from 330 mmol/kg and 100 mmol/kg treated samples was then measured. Live sorted spermatozoa were subjected to further hypotonic stress by transferring 10 µl aliquots to a series of tubes containing 1 ml BTS hypo-osmotic solution (3–330 mmol/kg) with SYBR/PI. After 20 min incubation at 15 °C, sperm membrane integrity was again assessed with the previously described flow cytometric procedure.

Hypotonic resistance and fertility

A fertility trial was designed to study the relationship between hypotonic resistance of spermatozoa and fertility. Semen was collected weekly over a 15-week period from 11 Pietrain boars housed in a commercial artificial insemination center. Doses of 3.10^9 total spermatozoa were produced and one dose per ejaculate was sent to the laboratory in isothermal boxes at 15 °C where motility and hypotonic resistance were assessed. A total of 133 ejaculates were used to inseminate 922 sows housed in 12 different farms under commercial management. Sows showing estrus 4 days after weaning were inseminated thrice: after the detection of standing heat and 12 and 24 h later. Sows ranged in parity from 2 to 11.

Statistical analyses

Statistical analyses were carried out using the SAS software (SAS Institute, USA). The percentage of intact spermatozoa in a given sample after exposure to hypo-osmotic media was normalized to the percentage of intact spermatozoa of the same sample in iso-osmotic control medium. This normalized percentage of intact spermatozoa was subjected to arc sine transformation and analyzed by standard ANOVA procedures using the GLM procedure in SAS software. Differences between Osm_{crit} determinations were obtained by the same procedures.

In order to assess only the effects of semen quality on fertility, the fertility trial was designed to evaluate side effects, such as farm and parity. Data collected included any observations at insemination, parity, insemination times and dates, farrowing, total litter size, number of live piglets born, dates of return to estrus or abortion, death or culling.

Fertility data were analyzed with a general linear model (GENMOD) for an estimation of the effects of farm, parity, boar, and ejaculate parameters on fertility. Ejaculates were grouped in 3 classes according to their Osm_{crit} . Individual farrowing data for each sow were linked with a logit link to the other terms of the model, including the farm, boar, and Osm_{crit} class. Litter size was introduced with a linear link in the same model including parity, boar, and Osm_{crit} class.

Declaration of interest

The authors declare there is no conflict of interest that would prejudice the impartiality of this scientific study.

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