Changes in responsiveness to bicarbonate under capacitating conditions in liquid preserved boar spermatozoa in vitro

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Liquid-stored boar semen is commonly used for artificial insemination (AI) up to 72 h after dilution. Insemination with semen stored for longer periods generally results in reduced fertility. Standard semen parameters, i.e. motility and membrane integrity, usually give no indication of this reduction. Therefore, more sensitive methods are needed for detection of storage-induced changes in sperm quality. Capacitation has long been known to be an essential step in fertilization. In a number of studies bicarbonate has been shown to be the key capacitating agent in boar sperm in vitro (reviewed in Harrison & Gadella 2005). The ability of sperm to respond to bicarbonate in vitro by undergoing capacitatory changes can be measured as a sperm property crucial to fertilization (Petrunkina *et al.* 2005a; Silva & Gadella 2006). In this study we used calcium influx as a parameter to investigate the responsiveness of stored semen samples to bicarbonate. This parameter has been shown to be sensitive with respect to evaluating detrimental effect of cooling during liquid storage of boar sperm (Petrunkina *et al.* 2005b).

Three ejaculates from each of 14 boars of proven fertility were diluted in Beltsville Thawing Solution (BTS) extender to a concentration of 20 x 10^6 sperm / ml and stored at 17°C. After 12, 24, 72, 120 and 168 h of storage, motility was assessed in the diluted semen with a CASA-system, and membrane integrity was checked with propidium iodide (PI) and FITC-conjugated peanut agglutinin (FITC-PNA) using a flow cytometer. Samples were then washed through Percoll, loaded with the calcium probe Fluo-3-AM and PI, and incubated at 38°C in parallel in two variants of a Tyrode's medium. Medium A contained 15 mM bicarbonate as well as 2 mM Ca^{2+}, whereas bicarbonate was omitted from medium B; incubation in medium A was performed under 5% CO_2. Changes in Ca^{2+} influx were assessed on a flow cytometer at 3, 20, 40, 60, 90, 120, 150 and 180 min. The resulting kinetics of cell sub-populations were compared between media and storage time points, based on analyses of the non-agglutinated population.

During storage, motility declined only from 89.0 ± 3.2 to 74.4 ± 10.4% ($p = 0.001$) and membrane integrity from 83.1 ± 4.2 to 71.0 ± 20.9% ($p = 0.001$). However, bicarbonate induced marked changes in membrane permeability, as measured by increases in the population of Ca^{2+}-positive and PI-negative (live) cells as well as by increases in the population of PI-positive (dead) cells. After 12 and 24 h of storage, the population of Ca^{2+}-positive and PI-negative cells reached a maximum within 90 min of incubation in medium A, but as storage was prolonged the increase lessened although it reached its maximum more rapidly (after 40-60 min). A time point of 60 min was chosen for comparisons between storage periods and media. Values for the total % Ca^{2+}-positive / PI-negative cells in medium A declined significantly from 21.6 ± 6.4% at 12 h to 15.7 ± 2.78% at 72 h of storage ($p < 0.01$) after which they stayed at a constant level. At 3 min of incubation proportions of PI-positive (dead) cells in medium A varied between 9.8 ± 4.9% at 12 h of storage and 14.7 ± 3.3% at 168 h, whereas after 60 min of incubation their percentage had risen to around 46% regardless of storage period. In contrast, in medium B, 60 min values of both populations (i.e. Ca^{2+}-positive / PI-negative and PI-positive cells), though initially much lower than in Medium A, increased significantly through-

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out the whole storage period ($p < 0.01$) until they were comparable with the values in medium A. Response to capacitating conditions as measured by the change in % Ca^{2+}-negative / PI-negative cells between 3 min and 60 min of incubation (Δ_{60-3}) declined significantly in medium A during storage from 58.6 ± 8.2% after 12 h to 36.4 ± 8.6% after 168 h ($p < 0.001$), whereas in medium B it increased from 6.8 ± 4.4% to 24.0 ± 3.7% ($p = 0.001$). However, storage also resulted in a change in the sub-population distributions at the initial (3 min) incubation point. There were significantly higher ($p < 0.01$) percentages of Ca^{2+}-positive / PI-negative cells and PI-positive cells detected in both medium A and medium B: in medium A 10.1% Ca^{2+}-positive / PI-negative and 14.7% PI-positive after 168 h versus 3.3% and 9.8% respectively after 12 h; in medium B 9.1% Ca^{2+}-positive / PI-negative and 14.7% PI-positive after 168 h versus 2.1% and 7.2% respectively after 12 h. These data could be interpreted as indicating that storage has two effects. There was on the one hand a destabilization of the greater proportion of the population such that incubation even in the absence of bicarbonate caused membrane deterioration while incubation with bicarbonate caused such rapid membrane destabilization that the Ca^{2+}-positive / PI-negative state was increasingly short-lived before PI entered. These findings are in agreement with those of Petrunkina *et al.* (2005b), reporting that levels of Ca^{2+} uptake at the beginning of incubation under capacitating conditions are influenced by storage conditions. However, we also obtained evidence that the cohorts of cells that were less immediately responsive to bicarbonate became refractory, reducing the overall bicarbonate response in terms of appearance of Ca^{2+}-positive / PI-negative plus PI-positive cells.

As expected, motility and membrane integrity of the stored cells did not sufficiently reflect storage-dependent changes in sperm quality during prolonged storage. A recent review (Petrunkina *et al.* 2007) has proposed several requirements for the assessment of functional sperm parameters under capacitating conditions. In accordance with these proposals, we used a kinetic experimental approach with well-defined test and control media, considering equally both initial response and subsequent kinetics. Specific sperm response to bicarbonate as measured by intracellular increases in Ca^{2+} in live cells declined remarkably already after 72 h of liquid storage and dropped to almost zero in samples stored for 168 h. However, bicarbonate routinely caused large increases in dead cells within 20 min of incubation. In their original paper on fluo-3 detection of bicarbonate-mediated changes in boar sperm, Harrison *et al.* (1993) interpreted fluo-3-detectable Ca^{2+} entry as indicating the onset of a membrane destabilization eventually leading to cell death. Harrison (1996) proposed that if such destabilization were too rapid, fertilization would be compromised. Our findings suggest that boar semen contains a population of cells initially responsive to bicarbonate which becomes increasingly intrinsically unstable during storage and responds to bicarbonate too rapidly. In addition, more stable cohorts of cells exist that during storage become less responsive to bicarbonate. We suspect that the dual opposing effects of storage, namely destabilization and stabilization, may imply that after normal insemination life-time of most sperm in the female tract will be insufficient to ensure satisfactory fertilization levels, while the remainder may not respond to fertilizing conditions at all.

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