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Supplementary Material

Reduced glutathione and procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze–thawing by stabilising disulfide bonds

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Annex A: Supplementary information for flow cytometry analyses

Unless otherwise stated, all flurochromes used for these analyses were purchased from Molecular Probes® (Invitrogen; Eugene, Oregon, USA) and were diluted with dimethyl sulfoxide (DMSO; Sigma).

Flow cytometry data were corrected following the procedure described by Petrunkina et al. (2010), as stated at the end of this section.

Sperm viability (SYBR-14/PI)

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/ PI), according to the protocol described by Garner and Johnson (1995). Briefly, sperm samples were incubated at 38ºC for 10 min with SYBR-14 at a final concentration of 100 nM, and then with PI at a final concentration of 10µM for 5 min at the same temperature. FL-1 was used for measuring the SYBR-14 fluorescence, while PI

fluorescence was detected through FL-3. After this assessment, three sperm populations were identified: i. viable green-stained spermatozoa (SYBR-14^{+}/PI^{-}); ii. non-viable red-stained spermatozoa (SYBR-14^{-}/PI^{+}), and iii. non-viable spermatozoa that were stained both green and red (SYBR-14^{+}/PI^{+}). Non-sperm particles (debris) were found in SYBR-14^{-}/PI^{-} quadrant.

Single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the PI channel (2.45%).

YO-PRO®-1/PI assay

Changes in the permeability of sperm plasma membrane were evaluated through co-staining with YO-PRO®-1 and PI from the Membrane Permeability/Dead Cell Apoptosis Kit with YO-PRO®-1/PI for flow cytometry (Martín *et al*. 2004). Samples were incubated for 5 min at 38ºC with YO-PRO®-1 (final concentration: 40 µM) and PI (final concentration: 10 µM). Green fluorescence (YO-PRO®-1) was collected with FL-1 sensor, while red fluorescence (PI) was collected with FL-3 sensor. In this probe, flow-cytometry dot plots yielded four differentiated sperm populations: i. Viable spermatozoa (YO-PRO-1^{-}/PI^{-}); ii. Viable spermatozoa with early changes in membrane permeability (YO-PRO-1^{+}/PI^{-}); iii. Non-viable spermatozoa with late changes in membrane permeability (YO-PRO-1^{+}/PI^{+}), and iiii. Non-viable spermatozoa (YO-PRO®-1^{-}/PI^{+}). Data from the two latter sperm populations are presented as grouped in a single category of non-viable spermatozoa (see Table 2).

Unstained and single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and for compensation of YO-PRO®-1 spill over into the PI channel (5.06%).

Acrosome integrity (PNA-FITC/PI)

Acrosome integrity was assessed by co-staining the spermatozoa with the lectin from *Arachis hypogaea* (peanut agglutinin) conjugated with fluorescein isothiocianate (FITC) and PI, according to the modified procedure described by Nagy *et al*. (2003). Briefly, spermatozoa were stained with PNA-FITC (final concentration: 2.5µg·mL^{-1}) and PI (final concentration: 10 µM) and incubated at 38ºC for 5 min. PNA-FITC fluorescence was collected through FL-1 and PI fluorescence was detected through FL-3. Spermatozoa were identified and placed in one of the four following populations: i. viable spermatozoa with intact acrosome (PI$^-$/PNA-FITC$^-$); ii. viable spermatozoa with damaged (exocytosed) acrosome (PI$^-$/PNA-FITC$^+$); iii. non-viable cells with intact acrosome (PI$^+$/PNA-FITC$^-$), and iiii. non-viable cells with damaged acrosome (PI$^+$/PNA-FITC$^+$).

Unstained and single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and for compensation of PNA-FITC-spill over into the PI channel (2.45%).

Membrane lipid disorder (M540/YO-PRO®-1)

Membrane lipid disorder was assessed using the co-staining protocol for Merocyanine 540 (M-540) and YO-PRO®-1 described by Harrison et al. (1996) and adjusted in our laboratory. Sperm samples were incubated for 10 min at 38ºC with M-540 and YO-PRO®-1 at a final concentration of 400 µM and 40 µM, respectively. The fluorescence of M-540 was detected through FL-2, while that of YO-PRO®-1 was detected through FL-1. Four sperm populations were observed in flow cytometry dot plots: i. viable spermatozoa with stable plasma membranes (M-540$^-$/YO-PRO®-1$^-$); ii. viable

spermatozoa showing an unstable plasma membrane M-540^{+}/YO-PRO$^{®}$-1^{-}); iii. non-viable spermatozoa with stable plasma membranes (M-540^{-}/YO-PRO$^{®}$-1^{+}), and iiii. non-viable spermatozoa with unstable plasma membranes (M-540^{+}/YO-PRO$^{®}$-1^{+}).

Data was not compensated.

Assessment of oxidative stress: peroxides (H_2DCFDA/PI) superoxides and (HE/YO-PRO$^{®}$-1)

Finally, ROS levels were determined through two different oxidation-sensitive fluorescent probes: 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and hydroethidine (HE), used to analyse the intracellular content of peroxides (H_2O_2) and superoxide anions ($O_2^{-\bullet}$), respectively (Murillo et al., 2007). Following a procedure modified from Guthrie and Welch (2006), a simultaneous differentiation of viable from non-viable spermatozoa was performed, by co-staining the spermatozoa either with PI or with YO-PRO$^{®}$-1.

In the first case, spermatozoa were stained with H_2DCFDA at a final concentration of 200 µM and PI at a final concentration of 10 µM and incubated at 25ºC for 60 min in the dark. H_2DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2',7'-dichlorofluorescein (DCF) upon oxidation (Guthrie and Welch, 2006). This DCF fluorescence was collected through FL-1, while PI fluorescence was detected through FL-3. Measurements were expressed as the geometric mean of green intensity fluorescence units (GMFI, geometric mean in FL-1) and this was used as the index of ROS generation. Unstained and single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and data was not compensated.

In the second probe, samples were stained with HE (final concentration: 4 µM) and with YO-PRO®-1 (final concentration: 40 µM) and incubated at 25ºC for 40 min in the dark (Guthrie and Welch, 2006). Hydroethidine is freely permeable to cells and it is oxidised by $O_2^{-\bullet}$ to ethidium (E) and other products (Zhao *et al*. 2003). Fluorescence of ethidium (E^+) was detected through FL-3 and that of YO-PRO®-1 was collected through FL-1. Data were expressed as the percentage of viable sperm with high $O_2^{-\bullet}$) (high ethidium fluorescence; E^+) and the geometric mean of red-intensity fluorescence (geometric mean channel in the FL-3). Data was not compensated.

Correction of Data: Identification of non-DNA containing particles

The percentage of non-DNA-containing particles (alien particles) was determined, since in some flow cytometry assessments, especially when working with cryopreserved spermatozoa, there may be an overestimation of sperm particles. According to Petrunkina and Harrison (2010), alien particles such as cytoplasmic droplets, cell debris, or diluent components (as egg yolk), will often show EV/FS and SS characteristics similar to those of spermatozoa and can not thus be excluded via light scatter. For this reason, 5 µL of each sperm sample coming from each of the ten treatments (extended, FT-C, G1, G2…), either at 30 or 240 min post-thawing, were diluted with 895 µL of milliQ®-distilled water. Samples were then stained with PI at a final concentration of 10µM and incubated at 38ºC for 3 min, according to the procedure described by Petrunkina *et al*. (2010). Percentages of alien particles (f) were used to correct the percentages of non-stained spermatozoa (q_1) in each sample and dual-staining analysis, except in SYBR-14/PI assay (i.e. YO-PRO®-1/PI, PNA-FITC/PI, M540/YO-PRO®-1, H_2DFCA/PI and HE/YO-PRO®-1), according to the following formula:

$$q_1' = \frac{q_1 - f}{100 - f} \times 100$$

Where q_1' is the percentage of non-stained spermatozoa after correction.

References

Garner, D. L., and Johnson, L. A. (1995). Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol. Reprod.* **53**, 276–284. doi:10.1095/biolreprod53.2.276

Harrison, R. A. P., Ashworth, P. J., and Miller, N. G. A. (1996). Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol. Reprod. Dev.* **45**, 378–391. doi:10.1002/(SICI)1098-2795(199611)45:3<378::AID-MRD16>3.0.CO;2-V

Martín, G., Sabido, O., Durand, P., and Levy, R. (2004). Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol. Reprod.* **71**, 28–37. doi:10.1095/biolreprod.103.024281

Murillo, M. M., Carmona-Cuenca, I., Del Castillo, G., Ortiz, C., Roncero, C., Sánchez, A., Fernández, M., and Fabregat, I. (2007). Activation of NADPH oxidase by transforming growth factor-beta in hepatocytes mediates up-regulation of epidermal growth factor receptor ligands through a nuclear factor-kappaB-dependent mechanism. *Biochem. J.* **405**, 251–259. doi:10.1042/BJ20061846

Nagy, S., Jansen, J., Topper, E. K., and Gadella, B. M. (2003). A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biol. Reprod.* **68**, 1828–1835. doi:10.1095/biolreprod.102.011445

Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vásquez-Vivar, J., and Kalyanaraman, B. (2003). Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular

fluorescence detection of superoxide. *Free Radic. Biol. Med.* **34**, 1359–1368. doi:10.1016/S0891-5849(03)00142-4

Annex B: Supplementary information for sperm motility assessment

Sperm-motility analysis was performed by utilising a commercial CASA system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system was based upon the analysis of 25 consecutive digitalised photographic images obtained from a single field at a magnification of 100× in a negative phase-contrast field. These 25 consecutive photographs were taken in a time lapse of 1 sec, which implied a velocity of image capturing of one photograph every 40 msec. Five to six separate fields were taken for each replicate, and three replicates were run per sample. Total and progressive motility together with other kinetic parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) were recorded.

Settings taken into account for all of the utilised motility parameters are stated in Table B1. Total motility was defined as the percentage of spermatozoa that showed a VAP >10 $\mu m \cdot s^{-1}$, whereas progressive motility was defined as the percentage of spermatozoa that showed a VAP >45 $\mu m \cdot s^{-1}$.

Table B1. Parameter ranges of sperm motility assessment

Parameter	Range
Range of particles area	10 μm^2–80 μm^2
Curvilinear velocity (VCL)	1–500 $\mu m \cdot s^{-1}$
Linear velocity (VSL)	1–500 $\mu m \cdot s^{-1}$
Average pathway velocity (VAP)	1–500 $\mu m \cdot s^{-1}$.
Straightness coefficient (STR)	10%–98%.
Linearity coefficient (LIN)	10%–98%.
Wobble coefficient (WOB)	10%–98%.
Amplitude of lateral head displacement (ALH)	0 μm –100 μm.
Beat cross frequency (BCF)	0 Hz–100 Hz.

Annex C: Supplementary tables for motility data

Table C1. Effects of the addition of GSH and/or PHCl in mean values of velocity parameters of boar spermatozoa subjected to freezing-thawing. Different superscripts (*a*–*d*) mean significant differences ($P < 0.05$) between a given treatment at a given time-point and the remaining of the treatments and time-points within the same category of spermatozoa. VSL: straight velocity. VCL: curvilinear velocity. VAP: average path velocity. Results are expressed as means ± S.E.M.

	VSL (μm·s^{-1})		***VCL (μm·s^{-1})***		***VAP (μm·s^{-1})***	
	30 min	***4 h***	***30 min***	***4 h***	***30 min***	***4 h***
Extended	26.2±2.4^{af}	21.8±1.2^{b}	53.3± 3.2^{a}	37.2±2.2^{bc}	35.9±2.3^{a}	29.0±1.5^{bc}
FT-C	20.3±1.7^{bc}	17.2±0.9^{d}	36.0 ± 2.2^{c}	30.9± 2.1^{d}	28.5±1.4^{ce}	23.5±1.3^{d}
G1	26.2±1.8^{af}	23.4±1.5^{be}	39.2± 2.4^{be}	38.2± 2.2^{b}	33.9±2.1^{ab}	32.4±1.9^{ab}
G2	28.5±1.5^{f}	24.9±1.8^{ae}	42.5± 2.6^{e}	39.2±1.9^{be}	36.2±2.1^{a}	32.8±2.0^{ab}
P1	22.6±1.2^{be}	18.1±1.0^{cd}	38.4± 2.2^{b}	33.6±1.9^{cd}	31.4±1.7^{b}	25.9±1.4^{de}
P2	24.5±1.3^{ae}	20.9±1.2^{bc}	42.1±2.6^{e}	37.1±2.0^{bc}	33.6±1.8^{ab}	29.4±1.5^{bc}
G1P1	25.4±1.4^{a}	22.2±1.1^{be}	39.8±2.3^{be}	36.4±1.8^{bc}	32.8±2.1^{ab}	29.6±1.8^{bc}
G1P2	27.0±1.4^{af}	23.5±1.5^{be}	42.6±2.3^{e}	39.3±1.8^{be}	35.0±1.9^{a}	31.7±1.7^{b}
G2P1	28.0±1.6^{f}	25.0±1.4^{ae}	42.2±2.6^{e}	40.3±2.4^{be}	35.7±2.2^{a}	33.9±2.0^{ab}
G2P2	28.9±1.6^{f}	25.6±1.3^{a}	43.0±2.6^{e}	39.6±2.1^{be}	36.6±2.3^{a}	32.8±1.9^{ab}

Table C2. Effects of the addition of GSH and/or PHCl in mean values of trajectory coefficients of boar spermatozoa subjected to freezing-thawing. Different superscripts (*a*–*d*) mean significant differences ($P < 0.05$) between a given treatment at a given time-point and the remaining of the treatments and time-points within the same category of spermatozoa. LIN: linearity coefficient. STR: straightness coefficient. WOB: wobble coefficient. Results are expressed as means ± S.E.M.

	LIN (%)		***STR (%)***		***WOB (%)***	
	30 min	***4 h***	***30 min***	***4 h***	***30 min***	***4 h***
Extended	48.6±3.0^{a}	60.4±3.7^{b}	71.5± 3.5^{a}	74.9±3.9^{ab}	67.2± 1.6^{a}	78.4± 4.4^{b}
FT-C	56.4±3.4^{c}	53.7±3.2^{ac}	71.1± 3.7^{a}	73.3±3.6^{ab}	80.8± 4.3^{b}	77.5± 4.2^{b}
G1	65.5±3.6^{bd}	61.2±3.5^{b}	76.2± 3.9^{ab}	72.1±3.8^{a}	83.0± 4.6^{b}	83.1± 4.3^{b}
G2	67.2±3.7^{d}	63.6±3.6^{bd}	77.5± 4.1^{ab}	75.1±3.9^{ab}	83.3± 4.3^{b}	82.5± 4.3^{b}
P1	58.6±3.4^{c}	53.9±3.1^{ac}	71.6± 3.7^{a}	70.0±3.6^{a}	82.9± 4.4^{b}	78.9± 4.2^{b}
P2	58.1±3.3^{c}	56.3±3.1^{c}	72.7± 3.7^{a}	71.2±3.8^{a}	79.7± 4.2^{b}	79.3 ± 4.2^{b}
G1P1	63.7±3.4^{bd}	60.9±3.3^{b}	77.4± 3.9^{ab}	74.8±3.7^{ab}	81.2± 4.2^{b}	80.1± 4.3^{b}
G1P2	63.1±3.8^{bd}	59.5±3.6^{bc}	76.5± 4.0^{ab}	72.9±3.8^{a}	81.3± 4.1^{b}	79.9 ± 4.2^{b}
G2P1	66.3±3.5^{bd}	61.8±3.6^{b}	78.3± 3.9^{b}	73.7±3.7^{ab}	82.8± 4.1^{b}	83.1± 4.2^{b}
G2P2	67.1±3.6^{d}	64.7±3.4^{bd}	78.5± 3.9^{b}	77.9±3.7^{b}	82.9± 4.2^{b}	81.5± 4.2^{b}