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Cryopreservation of fluorescence activated cell sorted boar spermatozoa based on extracellular ubiquitination

A. Petelák, V. Krylov

Faculty of Science, Charles University in Prague, Prague, Czech Republic

ABSTRACT: The present study is focused on the methodology of fluorescence activated cell sorting (FACS) of spermatozoa stained by the antibody against extracellular surface marker ubiquitin (eUb) and subsequent protocol for their long term storage in liquid nitrogen (LN). High level of spermatozoa surface ubiquitination has been previously discussed as a negative quality marker. From a general point of view, any other outer membrane antigen would be compatible with our approach. Regarding our experimental design we found that only those insemination doses with at least 40% of motile spermatozoa after freezing and thawing (F/T) in the egg-yolk medium with lactose are suitable for the subsequent antibody staining and FACS. The sorting rate was sufficient for the preparation of up to 20 spermatozoa aliquots for intracytoplasmic sperm injections (ICSI). Two significantly different groups with good freezability were prepared and stored in LN (0.73% contamination of spermatozoa with high eUb level in non-ubiquitinated group and reversely 6.65% spermatozoa without eUb in highly ubiquitinated group). Sperm viability after FACS varied from 11 to 28% regardless of the used media ($P = 0.15$). Required viability of F/T sorted spermatozoa was obtained by using Solusem® extender as a load and collection medium. In this case 12% of viable spermatozoa with progressive motility in low eUb level group and 7% in high eUb level group ($P < 0.05$) were detected. Our approach allows obtaining sufficient number of viable spermatozoa for subsequent artificial fertilization by ICSI. This procedure could be used for a wide variety of spermatozoa sorting based on different surface markers.

Keywords: pig; antibody staining; quality marker; porcine sperm; FACS; ubiquitin; cryopreservation

INTRODUCTION

Domestic pig (*Sus scrofa domestica*) represents an important model organism in clinical applications and regenerative medicine (Nagashima et al. 2012). However, *in vitro* production of quality embryos is hampered by the low success in artificial reproduction techniques (ART) such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). With this regard the focus is given to the precise execution and monitoring of all key steps throughout the process.

A fundamental criterion for proper fertilization is the spermatozoa quality concerning the morphology, motility, and fertilization rate. Currently, spermatozoa evaluation is performed using a hemocytometer (mostly used), computer assisted semen analysis (CASA), photometric measurement or flow cytometry (FC) (Hansen et al. 2006). FC technique would be easily coupled with the analysis of spermatozoa extracellular ubiquitination (eUb) level since this indicator was found to be negatively correlated with their fertilization ability (Sutovsky et al. 2002; Hodjat et al. 2008). The eUb level determination would then improve the quality control in livestock reproduction and clinical medicine (Ozanon et al. 2005).

To confirm the direct effect of spermatozoa outer membrane ubiquitination on its quality and subsequent embryonic development, *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) could be utilized using spermatozoa labelled by anti

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Ub primary antibody (Ab). Secondary Ab coupled with fluorophore would enable their sorting, based on the presence or absence of fluorescence signal using fluorescence activated cell sorting (FACS). Currently, this technique is employed in spermatozoa sorting based on the sex chromosomes content, i.e. sperm sexing (Vazquez et al. 2009). Precisely separated populations of low eUb and high eUb spermatozoa are necessary for the subsequent comparison of fertilization tests using the techniques of *in vitro* assisted reproduction. In porcine model ICSI would be preferentially employed for its ability to overcome the lower spermatozoa motility after FACS procedure and to eliminate a polyspermic penetration, the common problem observed in standard porcine IVF protocols (Kren et al. 2003). ICSI provided similar fertilization and blastocyst forming rates when FACS sex sorted and unsorted spermatozoa were compared (Jo et al. 2014).

To overcome the problems with spermatozoa variability among particular insemination doses (Alkmin et al. 2014) it is necessary to have a well-established freezing and long-term storage protocol. In domestic animals a cryopreservation of cattle and horse semen is commonly used and improved (Barbas and Mascarenhas 2009), but the protocol for boar spermatozoa freezing is usually still based on egg-yolk extender (Pursel et al. 1972).

The low viability and conception rates of frozen and thawed (F/T) spermatozoa currently obtained after insemination are due to a combination of detrimental effects on sperm function and structural integrity during the freeze–thawing process (Watson 1995). In addition, boar spermatozoa membranes are highly sensitive to freezing stress (White 1993) and no more than 70% of spermatozoa are able to survive the cryopreservation (Gil et al. 2005; Martinez-Alborcia et al. 2012).

In this study we described a new combination of FACS procedure based on the surface marker and freezing protocol providing spermatozoa of sufficient quality for subsequent use in ART.

For the successful combination of cryopreservation and FACS it is important to optimize the extender and collection medium and make all processes with standard routine.

MATERIAL AND METHODS

Preparation of spermatozoa suspension. Insemination doses were purchased from local producer (Chovservis a.s., SOSK Brná, Hradec Králové, Czech Republic). Boar semen was collected by gloved-hand technique and all procedures were in accordance with Czech law. 20 ml of spermatozoa suspension was centrifuged for 5 min at 700 *g* and supernatant was aspired leaving approximately 1 ml of the original solution. Sediment was then resuspended and sorted on Percoll gradient (700 *g* for 15 min) consisting of 5 ml 40% Percoll (GE Healthcare Biosciences AB, Uppsala, Sweden) underlain with 2 ml of 80% Percoll. Dulbecco's phosphate buffered saline (DPBS) (Life Technologies, Carlsbad, USA) was used instead of standard PBS for the Percoll dilution. All media and solutions were tempered to 17°C. After centrifugation the supernatant was discarded and the spermatozoa pellet was resuspended with DPBS + 0.01% polyvinyl alcohol (PVA) (Sigma-Aldrich, St. Louis, USA) to a final concentration 1×10^8 spermatozoa/ml.

Antibody staining. Blocking of spermatozoa (30 min) was performed in 5% normal goat serum (5% NGS) (Sigma-Aldrich) in DPBS prior to staining. After a 5 min centrifugation of 5 ml spermatozoa suspension at 350 *g*, the supernatant was removed and pellet resuspended in 0.5 ml of 1% NGS with primary antibody (Anti Ubiquitin; Imgenex Corp., San Diego, USA) diluted 1:100 and incubated for 40 min at 17°C. Spermatozoa were then washed twice with 1% NGS and incubated for 60 min in 1% NGS with secondary antibody (Goat-anti-rabbit IgG FITC; Imgenex Corp.) diluted 1:200. After staining, cells were washed in appropriate load medium, DPBS + 0.01% PVA or Solusem® (Varkens KI Nederland, Deventer, the Netherlands), the standard extender routinely used by insemination doses producer.

FACS. To distinguish spermatozoa from other particles and debris, cells were stained for 15 min with Hoechst 33342 (Sigma-Aldrich) added to the appropriate load medium to a final concentration 1 µg/ml (Marchiani et al. 2007). To remove debris or clumped spermatozoa, samples were then filtered through a 40 µm mesh. A polychromatic high-speed cell sorter BD-Influx (BD Biosciences, San Jose, USA) equipped with lasers (355, 488 nm) was used. DPBS served as a sheath fluid. A standard nozzle was used for both groups during passage through the FACS. Sorted spermatozoa were deflected into 1.5 ml mini tubes containing 700 µl collection medium DPBS + 0.01% PVA or modified Tris-buffered medium (mTBM) (MilliQ water

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with 113.1mM $NaCl_2$, 3mM KCl, 20mM Trizma base, 5mM glucose, 5mM sodium pyruvate, bovine serum albumin in the concentration of 2 µg/ml, and 14.1mM phenol red – all Sigma-Aldrich) or extender Solusem®. The data were acquired using BD FACSDiva Software (BD Biosciences).

Spermatozoa were sorted into two groups to acquire the largest difference in the eUb level. In the first one (low eUb level) we selected spermatozoa with minimal fluorescein isothiocyanate (FITC) signal intensity and in the second group (high eUb) spermatozoa with strong signal were collected. Both groups included consistent population of spermatozoa with similar intensity of fluorescent signal to obtain enough intra-group similarity (Figure 2).

Freezing protocol. Sperm freezing medium was prepared according to study of Yi et al. (2002) with modification in surfactant component in fraction B where 8.3mM sodium dodecyl sulfate (SDS) (Sigma-Aldrich) was used instead of Orvus ES Paste.

We established our modified protocol for spermatozoa freezing and thawing which we used for freezability test. Twelve ml of spermatozoa suspension from insemination dose was centrifuged for 5 min at 700 *g*, supernatant was aspirated to a final volume of approximately 0.75 ml. Pellet was then resuspended and sorted on Percoll gradient tempered to 17°C, the supernatant was removed and pellet was resuspended in DPBS + 0.01% PVA tempered to 17°C to a final concentration of 5×10^7 spermatozoa/ml. Two ml of this suspension was centrifuged for 5 min and supernatant was discarded. The pellet was resuspended in 0.5 ml fraction A of freezing medium (20% (v/v) of egg yolk diluted in MilliQ water, 0.3M lactose monohydrate). Suspension was over a 2 h period slowly cooled in a refrigerator to 5°C and 0.5 ml of freezing medium fraction B (fraction A with 0.82M glycerol and 8.3mM SDS) was added. In a room tempered to 5°C, glass 50 µl capillaries (Drummond Scientific Co., Broomall, USA) were filled immediately with approximately 30 µl of suspension, the suspension was aligned to the centre, and Bunsen torch was used to seal the ends. Capillaries were horizontally placed on a plastic holder situated 5 cm above the liquid nitrogen (LN) surface in a tank for 10 min before they were immersed into LN and then stored.

For freezing FACS sorted spermatozoa some minor modification was performed. We did not make Percoll selection since this step was performed already before Ab staining. Collection medium with spermatozoa was centrifuged for 5 min at 700 *g*, supernatant was discarded, and pellet was gently resuspended in 200 µl fraction A of freezing medium. After refrigeration, 200 µl of fraction B was added. Subsequent procedures were the same as for the freezability test.

The spermatozoa suspension was thawed in 37°C water bath for 3 min and then immediately added to a test tube containing 250 µl mTBM-ICSI (mTBM with 2mM $CaCl_2$ $2H_2O$ and 1mM caffeine). Sperm motility was evaluated after a 20 min incubation.

Viability analysis. After the 20 min incubation in mTBM-ICSI at 38°C spermatozoa motility was evaluated using standard Bürker chamber. To determine spermatozoa concentration and motility, two independent measurements were performed, counting 200 spermatozoa in each replicate. Particular media combination for FACS was tried on 5 or 6 different insemination doses with good freezability.

Evaluation of spermatozoa viability based only on motility was sufficient for our purposes because this value is closely related to their viability and gives similar evaluation results (Gil et al. 2005; Martinez-Alborcia et al. 2012). Immotile spermatozoa were assessed as non-viable.

A comparison of viability between low- and highly-ubiquitinated spermatozoa was carried out in paired experiments.

Statistical analysis. Statistical analysis and graphs were carried out using the GraphPad Prism, Version 5.01 (GraphPad Software Inc., San Diego, USA). Data were analyzed by one-way ANOVA and *t*-test. Values were considered statistically significant at $P < 0.05$.

RESULTS

Selection of appropriate insemination dose. Insemination doses from different boars were prepared for cryopreservation, stored in LN for one day, and thawed. The motility of F/T spermatozoa varied from 0 to 55%. We arbitrary determined the presence of 40% motile spermatozoa after F/T as the lowest limit for using the original insemination dose for sorting procedure. Insemination doses with spermatozoa viability lower than 40% always revealed spermatozoa motility close to zero after FACS and F/T (data not shown).

FACS and the selection of collection medium. Representative patterns of spermatozoa immuno-

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fluorescence labelling with anti-ubiquitin antibody after FACS is shown in Figure 1. FACS analysis outcome (Figure 2) revealed distinct spermatozoa populations based on the FITC signal intensity derived from the staining by secondary antibody. Figure 2 shows gating of events determined as single cells and the intensity plot used for the sorting of high eUb level spermatozoa and cells with the minimal eUb level.

Sorting rate of high eUb level spermatozoa varied from 26 to 194 spermatozoa per second (sp/s) with the average value of 75 sp/s, low eUb level revealed values ranging from 63 to 318 sp/s with the average of 148 sp/s. These sorting rates kept spermatozoa in a relatively good condition and ensured a sufficient sorting resolution.

Sorted spermatozoa were reanalyzed by flow cytometry with identical configuration as for sorting. In the experimental groups with low ubiquitinated spermatozoa we found the average contamination 0.73% of spermatozoa with high level of eUb. Conversely, in the group of highly ubiquitinated spermatozoa low ubiquitinated counterparts were presented with 6.65% on average.

The viability of spermatozoa after sorting was the highest in the experimental group where load and collection medium was the Solusem® extender (Figure 3A).

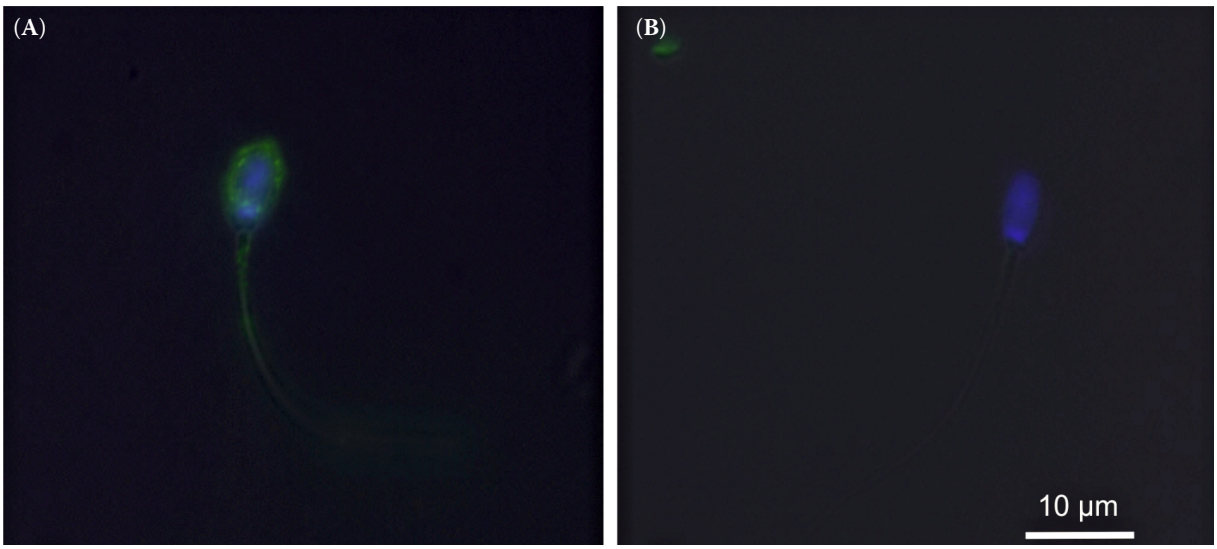

Figure 1. Commonly observed ubiquitination patterns (green) on sorted spermatozoa in the group with high ubiquitination level (**A**) and equally stained spermatozoa from low extracellular ubiquitination (eUb) level group (**B**). Chromatin was counterstained with DAPI (blue)

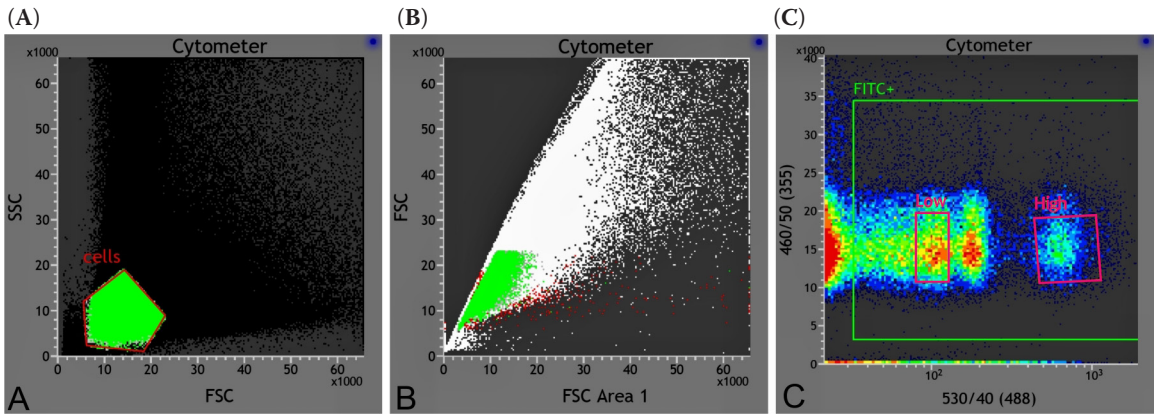

Figure 2. Flow cytometry analysis outcome of spermatozoa stained with Hoechst 33342 and FITC-conjugated secondary anti Ub primary antibody (Ab)

gating to distinguish debris and other bodies from spermatozoa (**A**), gating to select clumped spermatozoa from single cells (**B**), visible population of spermatozoa with low extracellular ubiquitination (eUb) level (Low) and high eUb level (High) (**C**)

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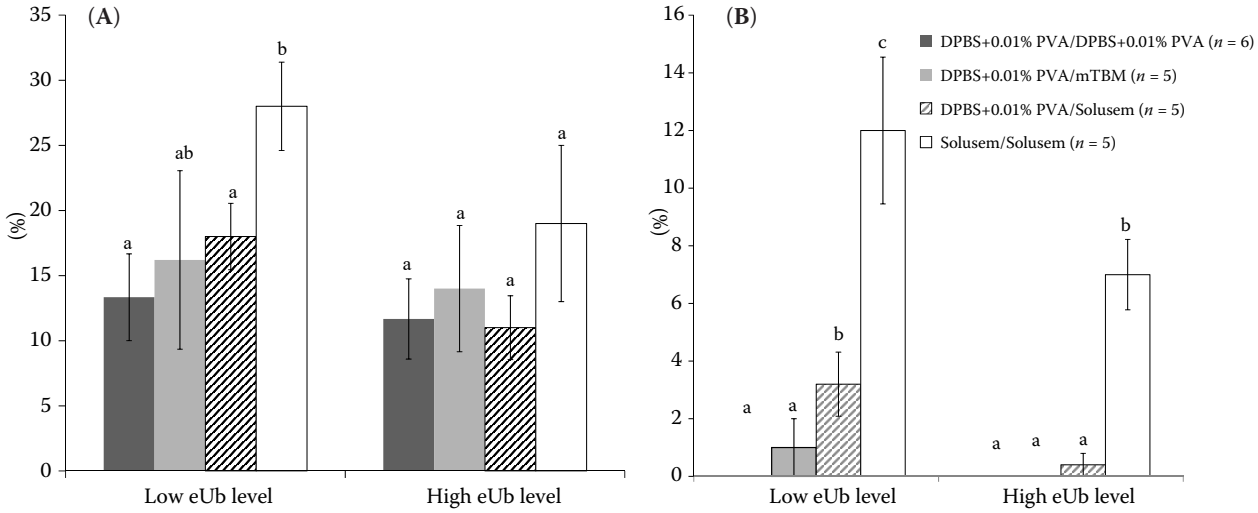

Figure 3. Percentage of viable low and highly ubiquitinated spermatozoa (**A**) and frozen/thawed spermatozoa (**B**) after fluorescence activated cell sorting (FACS) in different load/collection media (mean ± SEM). 200 spermatozoa were analyzed in each replicate (numbers of replicates are in brackets)

[a–c]significant difference ($P < 0.05$)

eUb = extracellular surface marker ubiquitin, DPBS = Dulbecco's phosphate buffered saline, PVA = polyvinyl alcohol, mTBM = modified Tris-buffered medium

The overall viability regardless of the used load and collection media was better for low ubiquitinated spermatozoa after FACS (18.6%) in comparison with the high eUb level experimental group (13.8%, $P < 0.005$) indicating a negative correlation between surface eUb level and spermatozoa quality.

Viability after thawing. The high viability rate of F/T spermatozoa after FACS is the key factor for the successful fertilization and early embryonic development. When DPBS + 0.01% PVA was utilized as a load and collection medium, no viable and motile spermatozoa were observed after FACS and F/T. Using DPBS + 0.01% PVA as a load and mTBM or standard extender as a collection medium, only few viable spermatozoa were detected but without progressive motility. The best viability rate with progressively moving spermatozoa was determined when Solusem® extender was employed as a load and collection medium (Figure 3B). In this case the viability of low ubiquitinated spermatozoa after FACS and F/T was 12.0% and in the high eUb level experimental group it was 7.0% ($P < 0.05$).

DISCUSSION

Plasma membrane of boar spermatozoa is sensitive to stress, which greatly limits the utilization of FACS and freezing and makes the IVF techniques more complicated (Vazquez et al. 2009; Balao da Silva et al. 2013). In addition, there is a wide individual variability among particular boars (Medrano et al. 2009) and even among individual semen collections from the same animal (Ciereszko et al. 2000). It is therefore important to evaluate the actual semen quality before subsequent fertilization or experimental trial. The first concern was to optimize spermatozoa survival rate during and after the freezing/thawing procedure.

Initial freezability test was important to overcome the above mentioned difficulties. Insemination doses without appropriate motility after F/T (below 40%) are not suitable for next procedures.

In contrast to cattle and horse, sorting of spermatozoa by FACS is used rarely in pig. Utilization of FACS to obtain sexed spermatozoa and piglets of desired sex was described by several authors (Vazquez et al. 2009), but only one approach was considered for commercial use (del Olmo et al. 2014). The necessity of large spermatozoa quantity for swine insemination (Vazquez et al. 2009) predetermines FACS especially in combination with cryopreservation to fertilization by ICSI. In our study we have shown that the average speed limit for the sorting rate regarding antibody staining of surface eUb is around 5×10^5 spermatozoa per hour which is incompatible with the preparation

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of standard insemination dose (minimum up to 5×10^8 F/T spermatozoa) (Knox and Yantis 2014) but it is sufficient for one laparoscopic insemination (del Olmo et al. 2014) or for many ICSI procedures.

A standard cytometer nozzle was used for all sorted groups because signal intensity of high eUb and low eUb populations was highly different and spermatozoon orientation during passage through the FACS detector had only minor contribution. In contrary to our experimental design, a standard nozzle is useless for the spermatozoa sexing where the signal intensity is very similar for XX and XY spermatozoa (Garner 2006). Staining of semen samples with Hoechst 33342 to distinguish spermatozoa from other particles and debris was also performed by Marchiani et al. (2007) or Martinez-Alborcia et al. (2012).

During FACS, spermatozoa are in the suboptimal condition and motility and viability decrease. On the other hand, high pressure during fast sorting has the same negative effect. We determined the optimal sorting duration up to 90 min. Above this time limit we observed serious decrease of spermatozoa motility and viability (personal observation).

To obtain two populations with maximal resolution regarding the ubiquitination level, only 1.51 and/or 0.91% of spermatozoa was selected into group with low and/or high eUb level. Interestingly, we observed significantly lower spermatozoa viability in the high eUb group compared to low eUb counterpart. This result corresponds to the hypothesis regarding the selection of epididymal spermatozoa based on eUb level (Sutovsky et al. 2002).

However, not all studies revealed this negative correlation between eUb level and spermatozoa quality. Muratori et al. (2005) and Varum et al. (2007) reported the opposite results. These discrepancies could be explained either by hypo-functionality of ubiquitin-based spermatozoa selection and degradation in epididymis (Eskandari-Shahraki et al. 2013) or by detection of ubiquitinated proteins, albeit not those on the sperm surface, that are intrinsic to normal spermatozoa (Sutovsky et al. 2015).

Our results are also in contradiction to study by Purdy (2008) concerning positive correlation between percentage of ubiquitinated spermatozoa in whole boar insemination dose and its freezability. The author highlighted the general beneficial effect of protein incorporation in the spermatozoa membrane (surface ubiquitin in this case) on its fluidity and cryopreservation success. With this regard it means that a separated group of highly ubiquitinated spermatozoa should reveal better survival rate after F/T in comparison with a non-ubiquitinated counterpart. However, our results indicated the opposite reliance, thus we disprove this hypothesis.

The viability after thawing greatly depends on initial semen parameters and precise manipulation during all processes. Unpublished data suggest temperature fluctuations during manipulation with spermatozoa as a crucial problem, especially after the initial cooling down to 5°C.

CONCLUSION

Spermatozoa undergo various kinds of stress during a relatively long procedure of Ab staining and FACS. Our approach allows obtaining a sufficient number of viable spermatozoa for subsequent artificial fertilization by ICSI. This procedure could be used for a wide variety of spermatozoa sorting based on different surface markers.

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Corresponding Author

Mgr. Aleš Petelák, Charles University in Prague, Faculty of Science, Viničná 7, 128 44 Prague 2, Czech Republic
Phone: +420 221 951 782, e-mail: petelak@natur.cuni.cz