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OPTIMIZATION OF FACS SORTING FOR THE IMPROVEMENT OF LIVESTOCK SEMEN QUALITY

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ABSTRACT

Fluorescence-activated cell sorting (FACS) of spermatozoa was mainly used for sex sorting. Recently, FACS has been used to eliminate DNA-damaged human spermatozoa using YO-PRO-1 staining. Fragmentation of sperm DNA is considered as one of the reasons of male infertility. Moreover, YO-PRO-1 can effectively mark apoptotic as well as dead spermatozoa. Till now, only two FACS instruments were used for the spermatozoa sorting. However, both of them are not more commercially available from different reasons. In this study, we used novel FACSMelody Cell Sorter for the elimination of apoptotic and dead cells from the rabbit and ram semen samples in order to improve their overall quality. Briefly, semen samples were stained using YO-PRO-1 dye (apoptotic and dead cells) and/or propidium iodide (PI; only dead cells). Three different sorting experiments were performed: E1 – YO-PRO-1 and PI stained rabbit sperm cells were sorted into the tubes containing 1 ml of PBS; E2 – PI stained rabbit sperm cells were sorted into tubes that were washed with FBS prior adding PBS; and E3 – YO-PRO-1 and PI stained ram sperm cells were sorted into tubes washed with FBS prior adding PBS. As a sheath fluid sterile PBS was used. All samples, control (before sorting), negatively and positively sorted fractions, were analysed using CASA for motility assessment. Moreover, all sorted samples were re-stained with PI for viability assessment. In conclusion, elimination of dead (PI^+) sperm from rabbit samples might improve their quality, since their progressive motility increased significantly ($P<0.001$) after sorting from 40 to 65%. However, ram spermatozoa seem to be sensitive to sorting procedure thus further optimisation of this procedure is required.

Keywords: FACS, sperm quality, motility, viability, YO-PRO-1, PI

INTRODUCTION

Fluorescence-activated cell sorting (FACS) of sperm cells is used mainly for sperm sexing since turn off the 80s and 90s (**Johnson *et al.*, 1989; Johnson *et al.*, 1994; Johnson *et al.*, 1999; Johnson, 2000; Garner, 2006)** using Beltsville sorting technology. This method is based on the differences in DNA content of X- and Y-bearing sperm cells that are stained with Hoeschst 33342 dye (**Seidel Jr, 2007; Sharpe and Evans, 2009**). Beginning with the first report in rabbits (**Johnson *et al.*, 1989**) to date, this method has become a commercially used in the bovine (**Frijters *et al.*, 2009**), swine (**Garcia *et al.*, 2007; Vazquez *et al.*, 2009**) and ovine (**de Graaf *et al.*, 2006; Leahy *et al.*, 2010**) industry. In recent years, the Magnetic-activated cell sorting (MACS) has been used to improve the quality of human or livestock semen based on the elimination of apoptotic spermatozoa via binding of Annexin V-conjugated nanoparticles to externalized phosphatidylserine (**Grunewald *et al.*, 2001**). This method can enhance the reproductive outcomes in human clinical practice (**Said *et al.*, 2006; Oseguera-López *et al.*, 2019**), but not in livestock production as reported in rabbits (**Vasicek *et al.*, 2014**) or boar (**Mrkun *et al.*, 2014**). On the other hand, it was observed that bull sperm cells with damaged membrane can be effectively removed using propidium iodide-conjugated magnetic nanoparticles (**Fox *et al.*, 2012**). However, the reproductive outcomes regarding this method are unknown.
As an alternative to MACS, FACS sorting of YO-PRO-1 positive cells were recently used to remove apoptotic cells and thus reduced the number of DNA-fragmented spermatozoa in order to improve the outcomes in assisted reproduction (**Ribeiro *et al.*, 2013; De Geyter *et al.*, 2019**). Thus, beside the sperm sexing FACS sorting might be used also to improve semen quality. However, for both sorting procedures only two instruments were used as mentioned in published studies (**de Graaf *et al.*, 2007; da Silva *et al.*, 2013; Ribeiro *et al.*, 2013; Quan *et al.*, 2015; de Geyter *et al.*, 2019**). First of them, a MoFLo SX DP sorter (Beckman Coulter, Miami, FI, USA) with a patented nozzle specially improved for sperm sorting is not available for research use. The second one, a BD Influx flow cytometer (BD Biosciences, San Jose, CA, USA) intended for research applications is not more available at the market. For this reason, a novel flow-cytometric sorter FACSMelody (BD, New Jersey, USA) with constant pressure and nozzle size was tested in the present study.
The main objective of this preliminary study was to optimize the sorting of rabbit and ram spermatozoa using FACSMelody sorter in order to enhance the output semen quality by removal of apoptotic and dead cells while maintaining and/or improving their motility parameters.

MATERIAL AND METHODS

Animals

Clinically healthy and sexually mature rabbits (n = 6) and rams (n = 6) were used in this study as follow: six bucks of New Zealand White rabbit breed, one ram of Native Wallachian sheep breed, two rams of Improved Wallachian sheep breed and two rams of Slovak Dairy sheep breed. All animals were reared at the breeding facility (NPPC, RIAP Nitra, Lužianky, Slovak Republic) and semen samples were collected as described previously (**Kuželová *et al.*, 2017; Baláži *et al.*, 2020**). The experiments were carried out in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments (https://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm).

Experimental design

Rabbit and ram semen samples were sorted using YO-PRO-1 dye staining apoptotic cells and dead cells and/or propidium iodide (PI) that stains only dead cells. Three different sorting experiments were performed. At first, both YO-PRO-1 and PI stained rabbit sperm cells were sorted into the 5 ml round-bottom

BD Falcon polystyrene tubes (BD, New Jersey, USA) containing 1 ml of phosphate-buffered saline (PBS without Ca and Mg; Biosera, France) (Experiment 1 – E1). Since tube walls could be sticky for sorted cells, PI stained rabbit sperm cells were sorted into Falcon tubes that were washed with fetal bovine serum (FBS; Sigma-Aldrich, Germany) prior the addition of PBS (Experiment 2 – E2). At last, both YO-PRO-1 and PI stained ram sperm cells were sorted into Falcon tubes washed with FBS as in E2 (Experiment 3 – E3).

Computer assisted sperm analysis (CASA)

Fresh semen samples (control samples) were diluted and analysed using CASA system (Sperm Vision^{TM}, MiniTübe, Germany). For each sample, concentration of spermatozoa (10^9 per mL), percentage of total motility (motility > 5 µm/s) and progressive motility (motility > 20 µm/s) of spermatozoa were measured as described previously (**Kuželová *et al.*, 2017; Baláži *et al.*, 2020**). The motility parameters were observed also in stained (YO-PRO-1 and PI) samples before sorting and subsequently in negative fractions of sorted samples in order to assess the effect of staining and sorting on semen quality.

YO-PRO-1 and PI staining

Semen samples were diluted in PBS to a final concentration of 10^7 sperm/mL and incubated with YO-PRO-1 (Molecular Probes, Eugene, Oregon, USA) at final concentration of 100 µmol/L for 15 min. in the dark at room temperature. Samples were washed after incubation in PBS at 600x g for 5 min. and resuspended in 1 ml of PBS for sorting procedure. Other diluted semen samples were mixed with staining solution of PI (Molecular Probes, Eugene, Oregon, USA) at final concentration of 50 µg/mL and sorted immediately without subsequent incubation and washing steps.

Fluorescent activated cell sorting (FACS)

The stained samples were sorted using BD FACSMelody Cell Sorter (BD, New Jersey, USA) equipped with 488-nm blue direct diode laser (20 mW), 640-nm red direct diode laser (40 mW) and 100 µm nozzle operating at 23 psi. The lasers were aligned using calibration beads provided by BD prior to each sorting. Sterile PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM $Na_2HPO_4 \cdot 12H_2O$; pH = 7.4) was used as sheath fluid for sorting and additional analyses. To eliminate debris and noise, the sperm cells were gated using a forward and side scatter and only singlets (single cells) were chosen in order to exclude the agglutinated sperm from the analysis (Figure 1A and 2A). The average sorting rate was 500–3500 events/s. Sorting was continued until 200,000 to 500,000 sperm cells negative for YO-PRO-1 or PI were collected not exceeding the total sorting volume of 0.5 – 1 ml. Aliquot of negative fraction from each sort was analysed using CASA as mentioned before. Both sorted fractions (positive and negative) of each sort were stained again with PI at final concentration of 50 µg/mL and analysed again (10,000 cells) in order to control the viability and/or efficiency of sorting (Figure 1B and 2B). All sorting experiments were acquired and evaluate using BD FACSChorus Software (BD, New Jersey, USA).

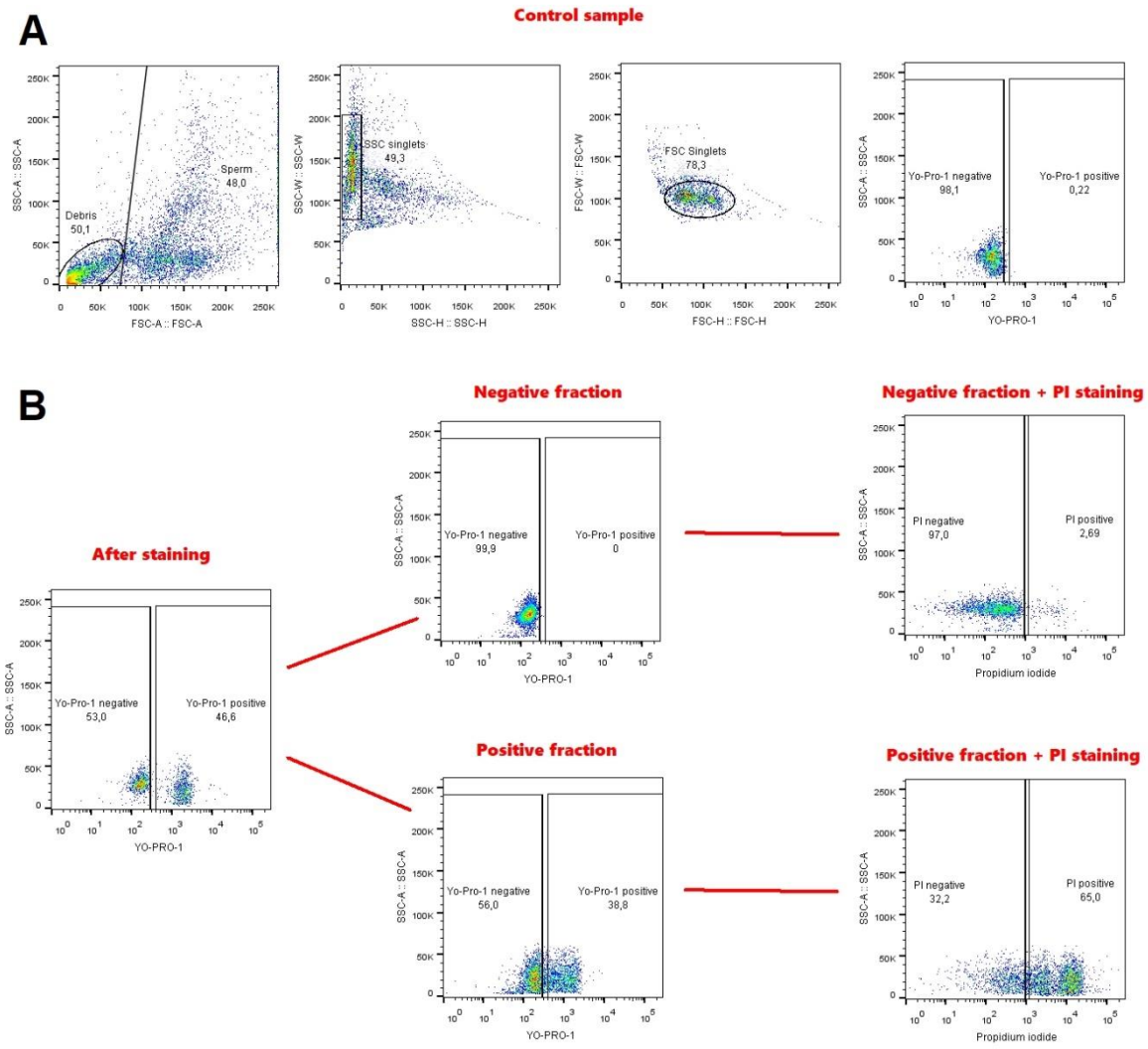

Figure 1 Sorting strategy of YO-PRO-1 stained rabbit spermatozoa: A) Sperm cells were gated in order to eliminate debris and only singlets were chosen for analysis and sorting. B) YO-PRO-1 stained cells were sorted according to the positivity into negative and positive fraction, analysed for YO-PRO-1 positivity and after-stain with PI for viability assessment.

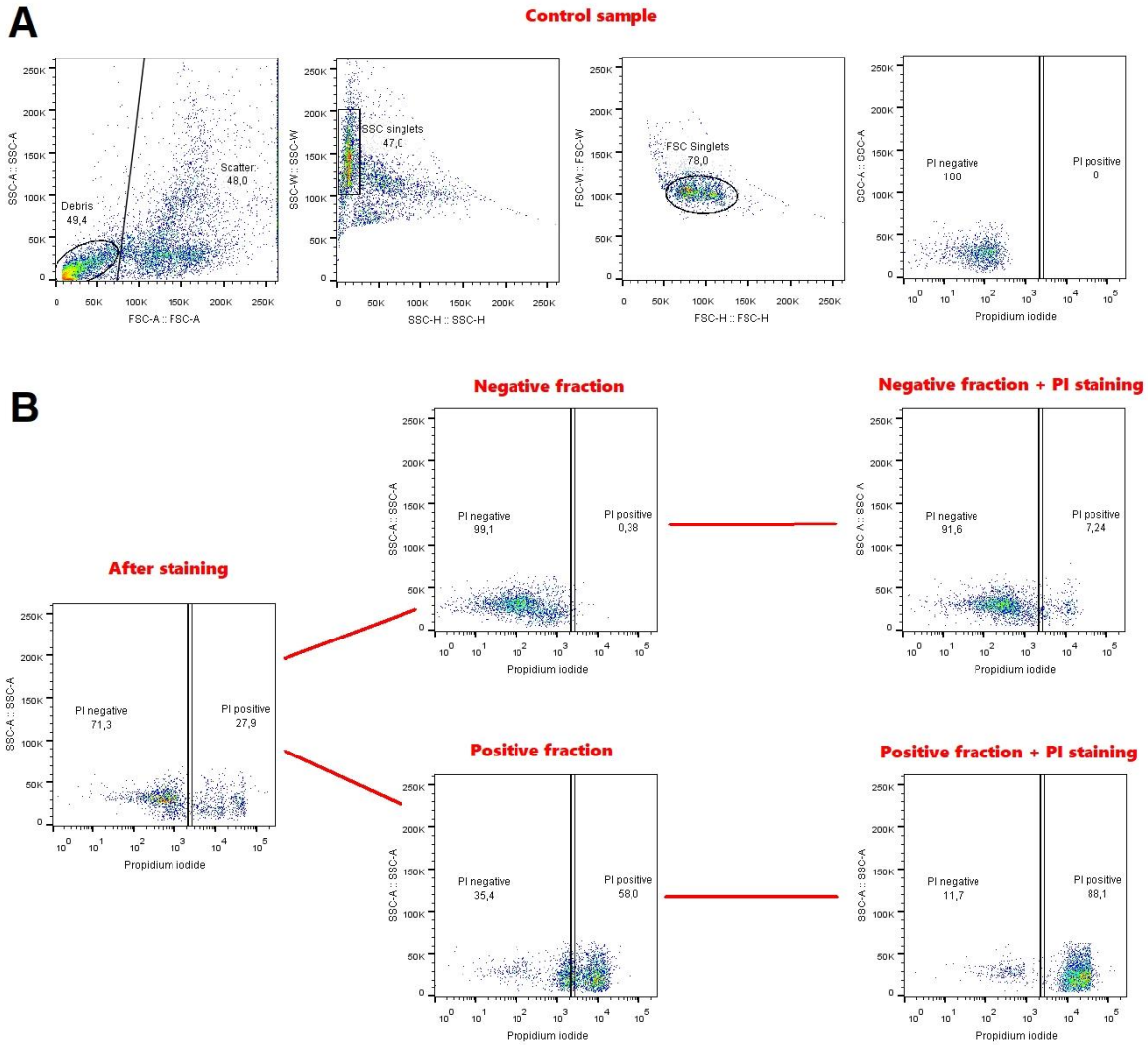

Figure 2 Sorting strategy of PI stained rabbit spermatozoa: A) Sperm cells were gated in order to eliminate debris and only singlets were chosen for analysis and sorting. B) PI stained cells were sorted according to the positivity into negative and positive fraction, analysed for PI positivity to check the sorting efficiency and after-stain again with PI for viability assessment.

Statistical analysis

Obtained results, sorting efficiency and viability of sorted sperm cells were as well as motility parameters (CASA), were evaluated using the SigmaPlot software (Systat Software Inc., Germany) with one-way ANOVA (Holm-Sidak method) and expressed as the means ± SEM. *P*-values at $P<0.05$ were considered as statistically significant.

RESULTS

Experiment 1

In this experiment, rabbit sperm cells were sorted into the prepared tubes with PBS after staining with YO-PRO-1 or PI in order to remove apoptotic or dead cells, respectively (Fig. 3). A high sorting efficiency was achieved for both of YO-PRO-1 and PI as negative fractions showed zero positivity (Fig. 3A, C). However, the percentage of YO-PRO-1$^+$ cells in positive fraction did not change when compared to the control sample (Fig. 3A). On the other hand, PI$^+$ cells increased from 11% to about 50% in positive fraction in comparison to control (Fig. 3C). Concerning the viability of sorted samples, the proportion of dead cells (PI$^+$) reached about 90 % in negative and 70 % in positive fractions of YO-PRO-1 sorted spermatozoa (Fig. 3B). The viability of negative fraction after PI sorting was significantly better in comparison to the positive fraction, although the percentage of dead cells achieved almost 45% (Fig. 3D). The motility analyses of sorted spermatozoa corresponded with the decrease in cell viability (Fig. 3E). In both sorting experiments, a slight decrease in total and progressive movement of spermatozoa was observed immediately after staining. Moreover, all reported motility parameters dropped to zero values in negative fractions after both sorting procedures. Thus, the sorting technique presented in E1 could not be useful to improve the quality of spermatozoa in terms of their viability and motility.

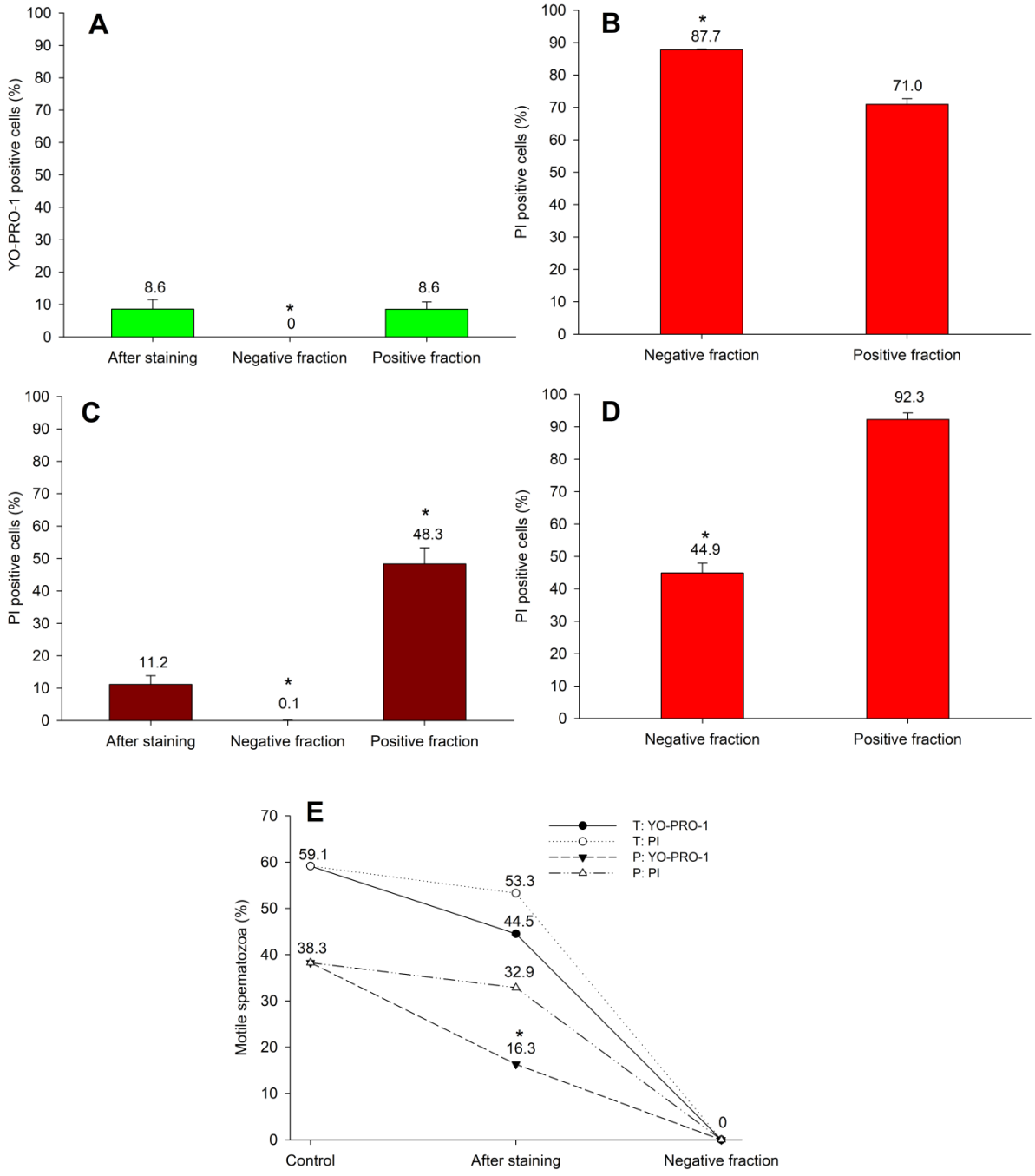

Figure 3 Sorting of rabbit spermatozoa (Experiment 1)
A) Sorting efficiency of YO-PRO-1 sorted spermatozoa (*different at P<0.05); B) viability of YO-PRO-1 sorted spermatozoa (*different at P<0.001); C) Sorting efficiency of PI sorted spermatozoa (*negative fraction different to stained sample at P<0.05 and positive fraction different to stained sample at P<0.001); D) viability of PI sorted spermatozoa (*different at P<0.001); E) Sperm motility before and after staining and sorting. T – total and P – progressive motility of YO-PRO-1 and PI stained and sorted spermatozoa (*different to control sample at P<0.05).

Experiment 2

In E2 experiment, tubes used for retaining the sorted spermatozoa were washed with FBS prior to the sorting of PI stained rabbit spermatozoa (Fig. 4) in order to increase the viability and yield of sorted sperm cells. Again, the sorting efficiency was high as negative fraction showed zero PI positivity. However, PI positivity in positive fraction increased only twofold from 9 to 17% (Fig. 4A). On the other hand, the cell viability retained significantly high (about 90%) in negative fraction compared to the positive fraction (Fig. 4B). This tendency was confirmed by motility analysis, since both total and progressive (P<0.05) motility of sorted spermatozoa in negative fraction increased in comparison to the control sample (Fig. 4C).

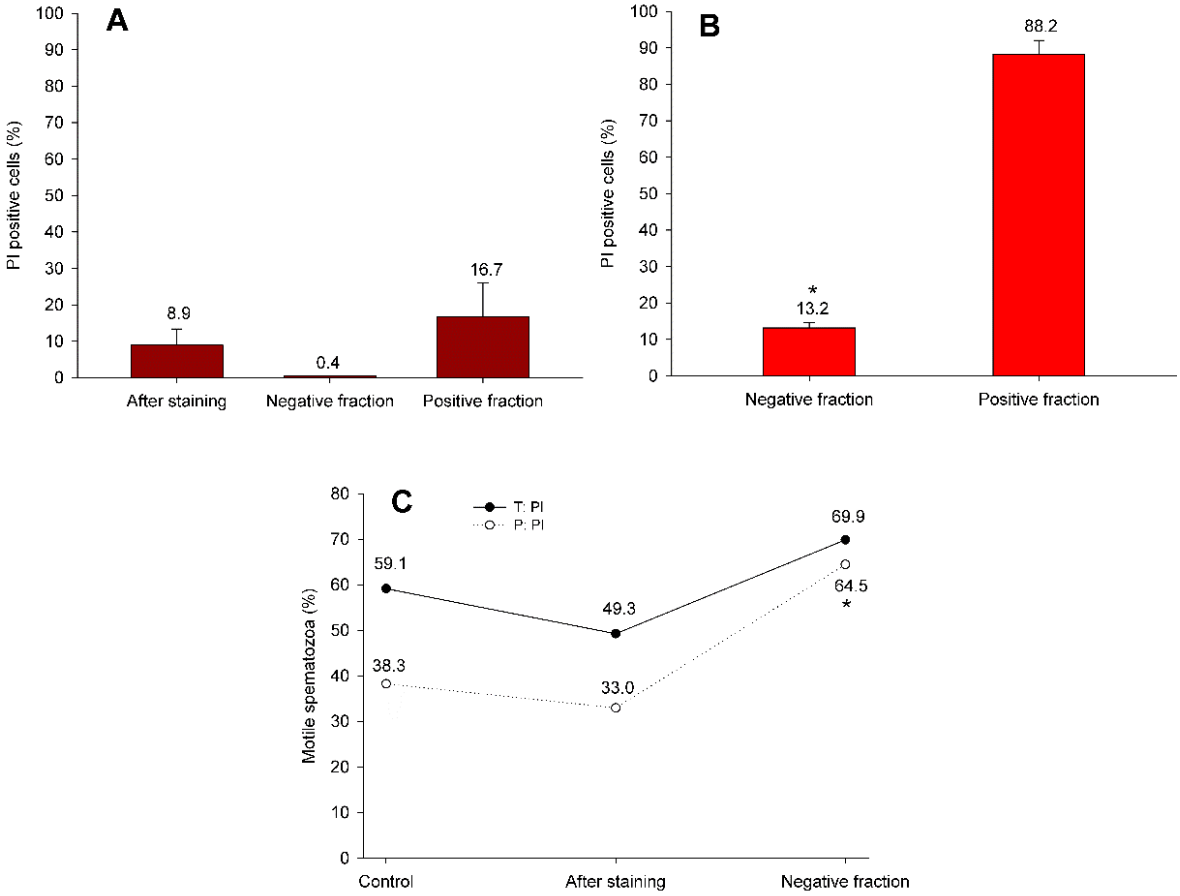

Figure 4 Sorting of rabbit spermatozoa (Experiment 2)
A) Sorting efficiency of PI sorted spermatozoa; B) viability of PI sorted spermatozoa (*different at P<0.001); C) Sperm motility before and after staining and sorting. T – total and P – progressive motility of PI stained and sorted spermatozoa (*different to control sample at P<0.05).

Experiment 3

In the last experiment, ram spermatozoa were sorted into FBS washed tubes (Fig. 5) as in E2. In this experiment both YO-PRO-1 and PI stained ram spermatozoa were sorted in order to confirm the sorting procedure that seems to be optimized for rabbit spermatozoa as shown in E2. Although, repeatedly high sorting efficiencies were noticed in negative fractions of YO-PRO-1 and PI sorted sperm (Fig. 5A, C), significantly decreased positivity was observed in YO-PRO-1 positive fraction or no change in the proportion of positive cells was noticed in PI positive fraction when compared to the control samples. The viability of both YO-PRO-1 and PI sorted ram sperm cells was significantly better in negative fractions in comparison to the positive fractions (Fig. 5B, D), but did not significantly improve in comparison to the control sample (35% and 63%, respectively in comparison to the control sample viability of 49%; Fig. 5C). On the other hand, although the motility parameters decreased after staining, the motility values of both YO-PRO-1 and PI sorted spermatozoa were restored to the basal values before sorting without any improvement (Fig. 5E).

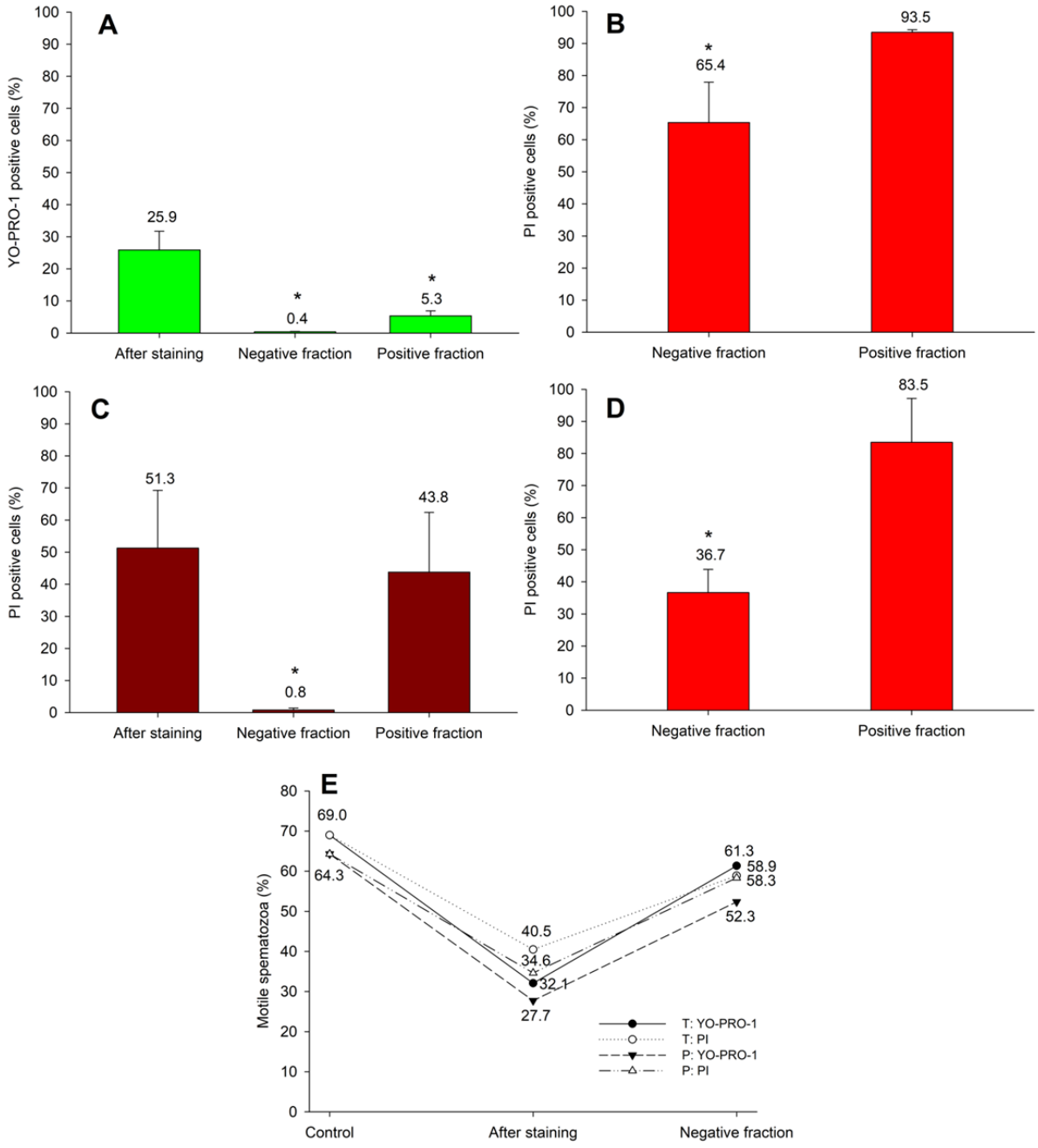

Figure 5 Sorting of ram spermatozoa (Experiment 3)
A) Sorting efficiency of YO-PRO-1 sorted spermatozoa (*different to stained sample at P<0.05); B) viability of YO-PRO-1 sorted spermatozoa (*different at P<0.05); C) Sorting efficiency of PI sorted spermatozoa (*different at P<0.05); D) viability of PI sorted spermatozoa (*different at P<0.05); E) Sperm motility before and after staining and sorting. T – total and P – progressive motility of YO-PRO-1 and PI stained and sorted spermatozoa.

DISCUSSION

For the first time, FACSMelody sorter was used to sort apoptotic and dead spermatozoa of different animal species for the purpose of potential improvement of the semen quality. Until recently, FACS sorting procedure of spermatozoa was mainly used for sex sorting. However, a few recent studies (**Ribeiro *et al.*, 2013; De Geyter *et al.*, 2019**) showed that using a YO-PRO-1 staining and FACS sorting the proportion of DNA-fragmented spermatozoa in human semen samples can be reduced to 5% as a result of apoptotic cell removal. In general, fragmentation of nuclear DNA is one of the main features of apoptosis (**Ribeiro *et al.*, 2013**). Increased amount of apoptotic sperm cells within the semen that is associated with higher number of spermatozoa with DNA fragmentation can resulted in male infertility (**Aziz *et al.*, 2007; Schulte *et al.*, 2010; Aitken *et al.*, 2011; Varshini *et al.*, 2012; Zorn *et al.*, 2012**) as well as in early pregnancy loss (**Zini *et al.*, 2008; Robinson *et al.*, 2012**). Thus, DNA integrity is crucial not only for successful fertilization, but also for further development of embryos (**Morris *et al.*, 2002; Virro *et al.*, 2004; Enciso *et al.*, 2012**). YO-PRO-1 staining has been shown to be an effective, simple, fast and trustworthy method for the quantification of spermatozoa apoptosis (**Martin *et al.*, 2004; Martin *et al.*, 2007**) and seems to be nontoxic when used for short-term incubation (less than 3 days) (**Idziorek *et al.*, 1995; Gawlitta *et al.*, 2004**). In addition, this dye co-stains apoptotic as well as dead spermatozoa (**Ribeiro *et al.*, 2013**).

In the presented study, we tried to optimize the sorting of YO-PRO-1 (apoptotic and dead cells) and PI (only dead cells) stained rabbit and ram spermatozoa. We used nontoxic YO-PRO-1 dye that requires incubation and washing steps. Oppositely, the PI staining omits incubation, as this toxic agent can be harmful to cells and long-term culture could stain also live cells (**Zhao *et al.*, 2010**). In all experiments (E1-3), positively stained sperm cells were effectively sorted as negative fractions did not exceed 1% of positive cell. High purity of YO-PRO-1 sorted spermatozoa (about 98.3%) was similarly observed by **Ribeiro *et al.* (2013)**. On the other hand, the positive fractions either YO-PRO-1 or PI sorted cells (E1-E3) achieved only 5-50% purity (understand as % of positive cells). However, reanalysis with PI staining of positively sorted fractions in all E1-E3 revealed noticeably higher (70-94%) percentage of dead cells when compared to those that were just sorted (5-50%). Thus, it seems that dyes YO-PRO-1 (data not shown) and/or PI were somehow washed out from the positively stained cells during the sorting procedure. This phenomenon might be possibly caused by the pressure of sorting instrument that impacts the sorted cells. Unfortunately, the

published studies did not observe the purity of positive fractions after sorting and so we could not confirm this hypothesis by other findings.

In general, highly diluted spermatozoa are during sorting procedure exposed to the potential dye toxicity, electrical charge, different media and/or mechanical forces (**Leahy *et al.*, 2011**). Additional damaging factors for the sorted spermatozoa can be their acceleration through the microfluidic system, laser beam exposure, their distribution into the droplets and at last their falling down to collecting tubes (**Gosalvez *et al.*, 2011**). Previous studies aimed on sex sorted spermatozoa revealed that high pressures (about 50 psi) have detrimental effect on their viability and motility (**Suh *et al.*, 2005**), as well as on embryo development (**Campos-Chillon and de la Torre, 2003**) and fertility (**Seidel *et al.*, 2003**). However, those effects were eliminated by reducing the sorting pressures below 30 psi (**Suh *et al.*, 2005**). The reanalysis of sorted positive and negative fractions in all our experiments with PI enabled to assess the viability of sorted spermatozoa and thus to evaluate the possible harmful impact of sorting procedure. First of all, it has to be mentioned that the original sheath fluid designed for FACS sorters contains some type of preservatives that may have adverse cytotoxic effect on certain type of cells. To eliminate this effect, a sterile preservative-free PBS was used as sheath fluid as it can be found in several published studies focused on sperm sorting. Secondly, we observed significant drop in viability of negatively sorted rabbit spermatozoa in E1 when compared to sample before sorting (from 90% to 13% for YO-PRO-1 or 55% for PI). On the contrary, the viability of PI sorted rabbit sperm (E2) in negative fraction was comparable to sample prior sorting (about 90%). Since, we used the same staining procedure in both experiments (E1 and E2), washing the collection tubes with FBS prior the sorting most probably facilitates the dropping of spermatozoa into the tubes and thus retained their initial viability. Similarly, the viability of sorted ram spermatozoa in E3 varied in negative fractions between 40-60% (YO-PRO-1 and PI, respectively) in comparison to control sample with 50% of viability. However, it means that either FBS washing of the collecting tubes did not improve significantly the sperm viability after sorting.

In this study, FACSMelody sorter with constant pressure at 23 psi was used. According to the above-mentioned studies, this pressure size might not negatively influence the viability or motility of sorted spermatozoa. Similarly, **Ribeiro *et al.* (2013)** reported only about 6-7% of damaged spermatozoa in human YO-PRO-1 negatively sorted spermatozoa after re-staining with YO-PRO-1 when a sorting pressure at 20 psi was used. Moreover, they observed about 25% of dead sperm in negatively sorted fraction; however, they did not state the viability of spermatozoa before sorting. In addition, they noticed that motility of those spermatozoa decreased after sorting. Another study of **da Silva *et al.* (2013)** aimed on sex sorting revealed that this procedure increased the membrane permeability of stallion spermatozoa. The authors demonstrated that the viability of sex sorted spermatozoa was reduced from 70 to 60%. At the same time, a significant decrease in the spermatozoa motility was observed after sorting, but even more also already after the staining of samples before the sorting itself. Other authors (**Quan *et al.*, 2015**) observed that independently of the dye concentration (Hoechst 33342 in this study) or the staining duration, the staining procedure itself negatively affects the sperm motility and viability, as well as the presence of apoptotic spermatozoa in the stained ram semen sample. In all our experiments (E1-E3), we also found a decrease in sperm motility immediately after staining of rabbit or ram semen samples. Since the YO-PRO-1 dye is relatively nontoxic for short-term use as it was demonstrated above, probably the washing steps and the dilution of spermatozoa itself might negatively affect their motility. In case of PI, a slight toxic effect of this dye could be taken into consideration as a reason for the decline in the sperm motility. However, more important fact is that the progressive motility of rabbit sperm cells increased significantly after the FACS elimination of PI^+ cells (E2). Moreover, the sorted ram spermatozoa got back the motility parameters that were similar to their initial motility prior sorting and staining (E3). On the other hand, the majority of sorted spermatozoa in E1 were non-motile, that correlates with their pure viability after sorting probably caused by omitting the FBS washing step of collecting tubes. In general, we did not observe major improvement (except for E2) either reduction (except for E1) in the sperm motility after sorting procedure. However, some immobilizing effect of FACS procedure that is correlated with the initial sperm quality was previously observed by **Ribeiro *et al.* (2013)**. Much earlier, **Auger *et al.* (1993)** suggested according to their findings that only sperm with disturbed function could be immobilized or even killed by FACS.

In summary, from the presented experiments (E2 and E3) we can conclude that rabbit sperm quality might be improved by the elimination of dead spermatozoa via FACS. On the other hand, although the viability of PI sorted ram spermatozoa slightly increased, the viability of ram spermatozoa that were sorted using YO-PRO-1 decreased and their motility in both cases was not improved. Thus, ram spermatozoa seems to be much more sensitive to the sorting procedure as rabbit sperm cells. In general, it has been already proved that ram spermatozoa are very sensitive to any oxidative damage (**Hamilton *et al.*, 2016**) or cold shock (**Mendoza *et al.*, 2013**). Anyway, it seems that PI sorting of ram spermatozoa seems to be less sensitive to sperm cells, maybe due to the omitting of washing steps. Moreover, **De Geyter *et al.* (2019)** stated that removal YO-PRO-1^+ human spermatozoa with damaged DNA using FACS did not significantly enhance the fertility results of assisted reproduction.

Broadly speaking, YO-PRO-1 could be a convenient alternative for removal of apoptotic cells in contrary to Annexin V staining, since this dye labels both apoptotic cells with intact membrane as well as cells with comprised membrane (**Idziorek *et al.*, 1995; Ribeiro *et al.*, 2013)** thus omitting the staining of dead cells with additional necrotic marker such as propidium iodide. On the other hand, although the PI staining technique is easy and relatively quick, the potential carcinogenic effect of PI on the stained living cells should be taken into consideration. However, at the present sorting of apoptotic cells using YO-PRO-1 staining is possible only *via* FACS as there is no available similar commercial kit conjugated with nanoparticles as for example the Annexin V MicroBead kit (Miltenyi Biotec, Germany) for the MACS removal of apoptotic cells. Although, the latter mentioned kit is successfully used in human assisted reproduction (**Said *et al.*, 2006)**; the largescale application of this method for the improvement of livestock semen quality has been proven as unsatisfying for rabbit (**Vasicek *et al.*, 2014**) or boar (**Mrkun *et al.*, 2014**). On the other hand, the MACS technique offers a much bigger yield of sorted cells if automatic instrument is used for a comparable time as FACS that usually resulted with a lower concentrated cell fraction. Moreover, the MACS technique for the removal of PI positive dead cells has been already patented (**Fox *et al.*, 2012**), although their practical outcomes are unknown. Anyway, the alternative to the FACS or MACS sorting of PI^+ cells could be another commercially available product named Dead Cell Removal Kit (Miltenyi Biotec, Germany) for removal of dead cells *via* MACS. However, the potential use of this kit for the improvement of the farm animal semen quality has to be explored yet. In any case, it is obvious that several factors such as the yield, purity, toxic effect or even species etc. should be taken into consideration before one's decided for the sorting strategy.

CONCLUSION

In conclusion, the improvement of sperm quality by FACS sorting of rabbit spermatozoa using FACSMelody instrument seems to be possible. However, several aspects of the sorting have to be taken into consideration. At first, sterile PBS should be used as a sheath fluid and collection tubes should be washed with FBS prior the sorting. An interesting fact is that the dyes are somehow washed out from the stained cells during the sorting probably due to the higher pressure necessary for sorting. On the other hand, the sorting pressure at 23 psi did not significantly affect the viability of negatively sorted spermatozoa. However, the ram sperm sorting is still a big challenge in order to improve the semen quality of individual breeding male and thus for example increase the cryopreservation efficiency of the insemination doses due to the high physiological sensitivity of ram spermatozoa. For further optimisation of sorting experiments we therefore suggest to try on the use of some specific ram sperm diluent for staining procedure as well as a liquid for sorted sperm in the collection tubes to minimise any additional negative effect on the sperm viability and motility. Moreover, a fertilizing ability of the sorted sperm of any animal species should be assessed after each kind of sorting.

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