Original Article

A Pilot Study on the Uptake of Propidium Iodide and YO-PRO-1 Iodide through the Pannexin Channels in Wallachian Frozen-Thawed Ram Spermatozoa

(flow cytometry / frozen-thawed spermatozoa / PANX1 / PANX2 / qRT-PCR)

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Abstract. Propidium iodide (PI) and YO-PRO-1 (YPI) dyes are routinely used to determine sperm viability in many livestock species. It is commonly accepted that these dyes penetrate only sperm cells with damaged plasma membranes. Recently, however, the mechanism of dye uptake unrelated to damaged plasma membranes, but instead related to pannexin channels in dog and stallion sperm cells was demonstrated. This pilot study aimed to evaluate the role of pannexins in the uptake of PI and YPI dyes on Wallachian frozen-thawed ram spermatozoa by flow cytometry using probenecid, a specific inhibitor of pannexin channels. Additionally, the expression of pannexins in Wallachian sperm was evaluated directly (by qRT-PCR). The results demonstrate the active role of pannexin channels in the uptake of PI and YPI dyes on frozen-thawed Wallachian ram sperm. In conclusion, when using the PI or YPI exclusion assay to determine Wallachian frozen-thawed ram sperm viability, the danger of overestimating the number of spermatozoa with the damaged plasma membrane must be considered. The observed breed-specific, and more importantly, individual differences in gene expression as well as in dye uptake indicate the need for further studies.

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Abbreviations: PBD – probenecid, PI – propium iodide, YPI – YO-PRO-1.

Introduction

PI and YPI dyes are routinely used to determine general sperm viability by flow cytometry or fluorescent microscopy in many species (Garner et al., 1986; Graham et al., 1990; Martin et al., 2004; Ortega-Ferrusola et al., 2008; Trzcińska et al., 2008; Martínez-Pastor et al., 2010; Hossain et al., 2011; García et al., 2012; Anel-López et al., 2017; Hossen et al., 2021; Vašíček et al., 2021; Buchelly Imbachí et al., 2022; Mańkowska et al., 2022; Petričáková et al., 2022; Pytlík et al., 2022) for their ability to penetrate only spermatozoa with destabilized plasma membranes (corrupted plasma membrane or early permeabilization of the plasma membrane). Live sperm cells are not stained with these dyes. Recently, however, the mechanism of PI uptake related to pannexin channels was demonstrated in frozen-thawed dog spermatozoa, and the danger of overestimating the

real number of non-viable spermatozoa assayed with the use of PI exclusion assay was claimed (Torres et al., 2017). Furthermore, Gallardo Bolaños et al. (2014) have demonstrated that pannexins allow the passage of YPI molecules inside the stallion sperm cells.

PI and YPI dyes are routinely used for the qualitative evaluation of frozen-thawed ram spermatozoa under the programme of conservation of native sheep breeds (Savvulidi et al., 2021; Vašíček et al., 2021; Vozaf et al., 2021, 2022). To the best of our knowledge, the dye uptake related to pannexin channels on Wallachian frozen-thawed ram spermatozoa has not been previously reported. Therefore, in the present study we aimed to evaluate the role of pannexins in fluorescent dye uptake by frozen-thawed Wallachian ram spermatozoa, to verify the reliability of PI- and YPI-based exclusion assays and to suggest the applicability of these assays for use within the programme of valuable genetic resource conservation.

Material and Methods

Ethics statements

All experimental protocols were approved by the review board of the Expert Commission Ensuring the Welfare of Experimental Animals at the Czech University of Life Sciences in Prague.

Rams

Two rams aged from 5 to 6 years were kept at the Demonstration and Experimental Centre of the Czech University of Life Sciences in Prague, under the conditions described previously (Savvulidi et al., 2021).

Semen

On each semen collection day, samples of the first ejaculate were obtained from each ram individually. A sheep/goat artificial vagina (Minitüb GmbH, Germany) was used. Semen was collected during the non-mating season (May–June 2020; March–June 2021) at 33 collection days (each collection day is considered a unique biological replicate, see Supplementary Table 1 on the article web page). A 24-hour pause was applied between any two adjacent semen collections. In total, 66 ejaculates (33 ejaculates from ram 1 and 33 ejaculates from ram 2) were used in the present study. Only the semen with good mass motility (scores 3 out of 5, or higher) was used for the following sperm freezing (David et al., 2015).

Sperm freezing-thawing

The initial semen processing was performed as previously described (Savvulidi et al., 2021), with some modifications. Briefly, immediately after collection (except the samples intended for mass motility evaluation), semen was initially diluted 1 : 4 (vol/vol) with an ANDROMED extender (Minitüb GmbH, Tiefenbach, Germany). Individual spermatozoa motility was not evaluated after the initial dilution. The sperm concentration was assessed once after the initial dilution using a pre-calibrated Genesys™ 10vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Based on the sperm concentration assessed after the initial dilution, samples were diluted by the ANDROMED extender using an appropriate dilution factor to a final concentration of 40×10^6/ml.

For calculating the dilution factor, the following formula was used:

$x = CONCaid \div CONCf$,

where: x – dilution factor; CONCaid – sperm concentration after initial dilution; CONCf – final concentration.

The diluted sperm were filled into 0.25 ml French straws (IMV Technologies, L'Aigle, France) under ambient temperature (25 °C). Straws were sealed with the sealing powder (IMV, Technologies, L'Aigle, France). The sealed straws were transferred to a refrigerator for cooling (from 25 °C to 6–8 °C; cooling rate 1.0 °C per min on average) and subsequent equilibration for 2 to 4 h (Lv et al., 2019). After equilibration, the straws were frozen in a special polystyrene freezing box (adapted from Animal Reproduction Systems, Inc., Ontario, CA, modified), as previously described (Ptáček et al., 2019; Savvulidi et al., 2021). The frozen straws were stored in liquid nitrogen for at least 24 h before thawing. Straws were thawed in a water bath at 60 °C for 5 s, and then were quickly transferred into a water bath at 38 °C for 25 s to normalize the temperature inside the straw.

Inhibition of pannexin channels by probenecid treatment in thawed spermatozoa

To investigate the impact of the pannexin channel inhibition on the process of PI and YPI uptake in frozen-thawed ram spermatozoa, probenecid (PBD, Sigma Aldrich, St. Louis, MO), a known specific inhibitor of pannexin channels (D'hondt et al., 2009; Torres et al., 2017), was used before the PI or YPI staining. PBD was prepared according to the manufacture's recommendations. One million thawed spermatozoa were co-incubated for 15 min (38 °C, in the dark) with PBD (Table 1).

PI and YPI uptake measurement by flow cytometry

The design of flow cytometry measurements follows our hypothesis: if the pannexin channels are expressed on ram spermatozoa, and if the status of pannexin channels on the ram spermatozoa is "active", then the uptake of the dye has to decrease after the PBD treatment. Immediately after PBD treatment and without washing, each sperm sample (2^6/ml) was stained separately with PI (Sigma Aldrich, St. Louis, MO) at a final concentration of 8 μg/ml, or YPI (Thermo Fisher Scientific, Waltham, MA) at a final concentration of 0.1 μM, for an additional 5 min (38 °C in the dark), so the sperm sample was treated with only one fluorescent dye before flow cytometry. The stained samples without washing

Table 1. Probenecid (PBD) concentrations used in the present study, and their corresponding repetitions. N = technical replicates, Σ = sum

PBD, final concentration (mM)	Ram 1, number of collection days	Ram 2, number of collection days	N, for both rams per particular concentration
0	33	33	250
0.4	5	5	22
1	11	11	70
2.5	12	11	74
3.75	8	7	60
5	11	11	88
6.25	8	8	64
6.87	7	7	56
7.5	6	6	48
10	5	5	40
–	Σ(N) = 392	Σ(N) = 380	Σ(N) = 772

were subsequently analysed using a NovoCyte digital flow cytometer, model 3000 (Agilent, Santa Clara, CA) with the NovoSampler Pro option accommodating 96-well plates. The flow cytometer was equipped with a blue (488 nm, 60 mW) solid-state laser and 615/20 nm and 530/30 nm BP optical filters for the detection of emitted PI or YPI fluorescence signals. The samples were run at low speed (14 µl/min, core diameter: 7.7 µm), and at least 10,000 gated events were recorded for each sample. Two to four technical replicates (wells) were analysed for each sample. NovoExpress software, v1.3.0 (Agilent) was used for automated cytometer setup and performance tracking as well as data acquisition. The same software was used to analyse the acquired flow cytometry data.

Statistical analysis

Statistical analyses were performed using the SAS 9.3. (SAS/STAT®; SAS Institute, Inc., Cary, NC) MIXED procedure. The statistical model with the randomized effect of the day of semen collection, fixed effects of the ram, PBD concentration and their interaction was used: Yijkl = DAYi + RAMj + CONCk + RAM*CONCjk + eijkl,

where: Yijkl = measured or assessed trait (PI or YPI uptake in frozen-thawed spermatozoa); DAYi = randomized effect of the day of semen collection; RAMj = fixed effect of the ram's ejaculate (j = ram No. 1, N = 392; j = ram No. 2, N = 380); CONCk = fixed effect of PBD concentration (k = samples with 0 mM PBD concentration, N = 250; k = samples with 0.4 mM PBD concentration, N = 22; k = samples with 1 mM PBD concentration, N = 70; k = samples with 2.5 mM PBD concentration, N = 74; k = samples with 3.75 mM PBD concentration, N = 60; k = samples with 5 mM PBD concentration, N = 88; k = samples with 6.25 mM PBD concentration, N = 64; k = samples with 6.87 mM PBD concentration, N = 56; k = samples with 7.5 mM PBD concentration, N = 48; k = samples with 10 mM PBD concentration, N = 40); RAM*CONCjk = interaction effect of the ram, eijkl = residual error.

The Tukey-Kramer method was used to evaluate the differences between the least squares means. The significance level of $P < 0.05$ was used to evaluate the differences among groups. For Figure 2, the Excel's unpaired TTEST option with 2-tails and similar dispersion was used to compare the means.

Results

Pannexin channels played an active role in the process of uptake of PI and YPI dyes in frozen-thawed spermatozoa. This phenomenon was sire-specific: PBD inhibition of pannexins in frozen-thawed sperm was numerically highest in ram 1, in comparison to ram 2 (Fig. 1, and especially Fig. 2).

In the sperm from ram 1, the PI and YPI uptake decreases were especially large when a 3.75 mM final concentration of PBD was used (1.06-fold decrease for PI; 1.07-fold decrease for YPI). Importantly, in the sperm from ram 2, the PI and YPI uptake fluctuated after probenecid treatment, without any clear dose-response curve. Interestingly, we observed a plasma membrane destabilizing effect of PBD in frozen-thawed sperm from ram 1 at as high a PBD concentration as 10 mM.

Additionally, the expression of *PANX1* and *PANX2* mRNA in freshly isolated Wallachian ram spermatozoa was confirmed by qRT-PCR (for details on the qRT-PCR methodology, see Supplementary Table 2 on the article web page). Importantly, expression was individually determined: *PANX2* mRNA was expressed at higher levels in ram 1 sperm cells than in those of ram 2 (Fig. 3).

Discussion

Our pilot study is amongst the first for the Wallachian native breed. To the best of our knowledge, there are no other publications concerning study of the mechanisms of dye uptake in the Wallachian ram sperm cells.

Due to the difficulties with the availability of endangered Wallachian local breed, a limited number of purebred Wallachian rams were used. In the present pilot study, only two mature original Wallachian rams were used. Rams with typical exterior signs of the breed and with an excellent breeding history were transported from the area of the Beskid Mountains. Animals were kept at the nearest distance to the laboratory of flow cytometry. This was done with special intention to reduce any variability due to the possible delay between the collection of semen and its subsequent evaluation. In addition, this enabled the use of rams in the same health condition under strict veterinary inspection, in the same breeding conditions, under the same feeding ration, with the semen collected and processed in the same manner

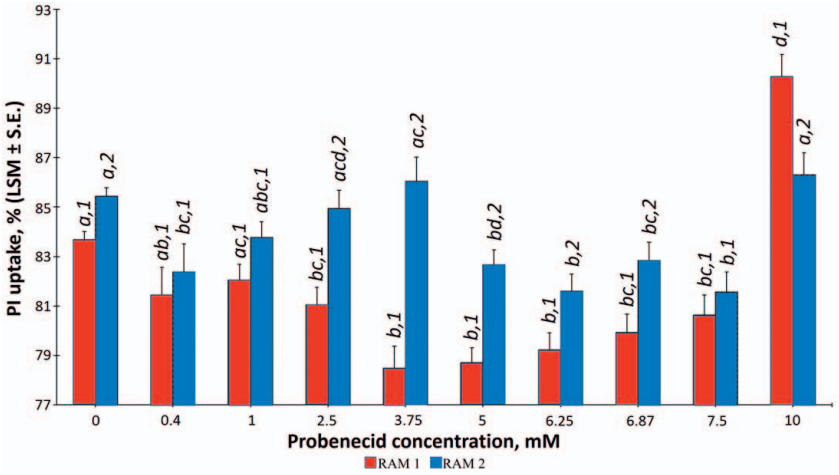

Fig. 1. PI uptake (LSM ± S.E.), as measured by flow cytometry. Values with the different notifications (*a–d* intra-ram differences; *1–2* ram-to-ram differences) indicate significant differences at the $P < 0.05$ level of significance.

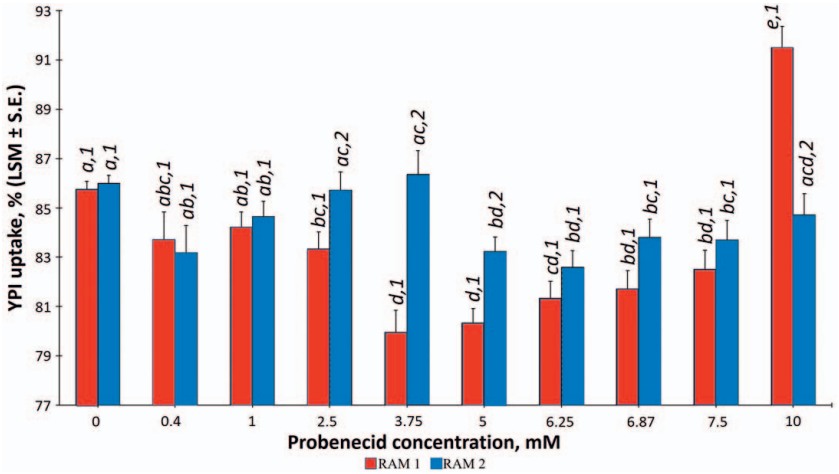

Fig. 2. YPI uptake (LSM ± S.E.), as measured by flow cytometry. Values with the different notifications (*a–d* intra-ram differences; *1–2* ram-to-ram differences) indicate significant differences at the $P < 0.05$ level of significance.

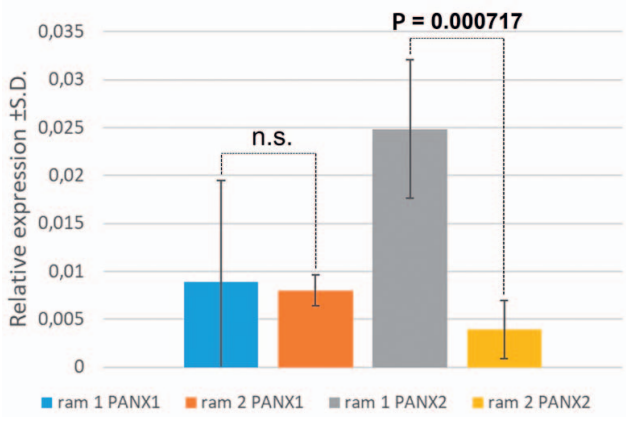

Fig. 3. Expression of *PANX1* and *PANX2* mRNA in freshly collected Wallachian ram spermatozoa. Expression of the genes is represented as relative expression ± standard deviation. *PANX1* and *PANX2* were normalized to the expression levels of the reference gene *ACTB*. n.s. – non significant ($P > 0.05$).

and in the same time duration. As we were aware of the use of a limited number of animals, the repetition of semen collection from both rams was highly increased in the present study (as described in section "Semen").

Our results indicate that pannexin channels play an active role in the process of PI and YPI dye uptake in frozen-thawed ram sperm cells and that this phenomenon is sire-specific: pannexin-mediated dye uptake was higher in sperm cells obtained from ram 1. We made this conclusion based on the sperm cell response to the PBD treatment: only inhibition of pannexins in ram 1 sperm cells (and not ram 2) led to the numerically largest decrease in the dye uptake with a clear U-shaped dose-response curve.

The decrease of the PI and YPI dye uptake after probenecid treatment was statistically significant ($P < 0.05$) in our study. The numerical value of the decrease observed in the present study was low (up to 6 % for PI, and up to 7 % for YPI; that is, the PI assay had a 6 %, and the YPI assay had a 7 % chance of detecting false

positives by flow cytometry). However, it is important to mention that the numerical value of the decrease of dye uptake after the pannexin inhibitor treatment might depend on many specific factors: breed, ram, inhibitor used, inhibitor concentration, time of incubation of cells with the pannexin inhibitor, and type of sperm samples (chilled or frozen). Ideally, a reliable assay must have a 0 % chance of detecting false positives. Here, we confirmed a non-0 % chance of detecting false positives by both assays, which are generally accepted as fully reliable. The decrease of PI and YPI dye uptake after probenecid treatment observed in our study was statistically significant, which suggests that the results of our pilot study could be a stimulus for other researchers to conduct additional, large-scale studies to evaluate the chance of detecting false positives by flow cytometry under their specific experimental settings.

Last but not least, it was shown previously that pannexins (namely, PANX1) play an important role in the process of adenosine triphosphate (ATP) molecule release in response to several stress types (of pathological, mechanical, or thermal nature), and that such a massive ATP release acts as a pro-inflammatory signal for recruiting immune cells to the damaged cells, as recently reviewed by Nadeali et al. (2022). We speculate that this might contribute to the formation of neutrophilic extracellular traps, which trap spermatozoa and initiate phagocytosis (Warr et al., 2023), thus decreasing the ability of cryopreserved spermatozoa to traverse the cervix after artificial insemination.

Conclusion

In conclusion, using the flow cytometry methodology, we were the first to show the active role of pannexins in the uptake of membrane-impermeable DNA dyes (PI and YPI) in Wallachian frozen-thawed ram spermatozoa. Therefore, when using PI or YPI exclusion assays to determine Wallachian ram sperm viability, the danger of overestimating the number of damaged spermatozoa must be considered. Moreover, the presence of pannexins *PANX1* and *PANX2* in Wallachian ram spermatozoa, again with individual sire differences, were confirmed with the use of qRT-PCR. However, the individual differences in gene expression as well as in dye uptake indicate the need for further studies. A larger sample size, including different native sheep breeds and possibly different domestic animal species, different breeding seasons, and different semen types (fresh, refrigerated, and frozen) can significantly contribute to a better understanding of the specific role of pannexin channels. The findings of the present pilot study support the scientific reasons for conducting large-scale experiments evaluating all these factors.

Author contributions

FGS and MP: flow cytometry experiments and flow cytometry data analysis; statistics; draft of the manuscript, figures and tables. AM, JP, MJ: sample collection. EG: molecular biology of samples, qRT-PCR. SN, JPB, LS: overall supervision. KSV: overall supervision, qRT-PCR-data analysis. All authors reviewed the manuscript.

Institutional review board statement

All methods were carried out following the relevant guidelines and regulations. All experimental protocols were approved by the review board of the Expert Commission Ensuring the Welfare of Experimental Animals at the Czech University of Life Sciences in Prague. Except for semen collection using an artificial vagina, the present study did not involve any animal *in vivo* experiments: no pharmacological procedures, surgical procedures, animal pathogen infection, or euthanasia were used in the present study. Therefore, the review board of the Expert Commission considers that this type of research does not fall under the legislation for the protection of animals used for scientific purposes (Act No. 246/1992 Sb.). It considers that this type of research has no impact on animal welfare because only non-experimental agricultural practices (following Act No. 166/1999 Sb. and Act No. 154/2000 Sb.) were used during the study.

Informed consent statement

Not applicable.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

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Conflict of interest

The authors declare no conflict of interest.

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