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Quality and *in vitro* fertilising ability of cryostored Pinzgau bull insemination doses

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Abstract: Applicability of cryopreserved insemination doses is dependent on the quality of the semen after thawing. The aim of the study was to check the options of new markers for the assessment of sperm quality to predict their fertilisation ability. In five bulls of the Pinzgau breed, the sperm fertilising ability was determined using the *in vitro* fertilisation. Bulls were divided according to the achieved blastocyst rate: bulls with good fertilising ability (NOB – 38.6%, KAZ – 28.9%, GAL – 29.3%) and bulls with low fertilising ability (LOH – 19.4%, NUS – 22.1%). In addition to the sperm motility and morphology, we monitored common physiological sperm characteristics: viability, apoptosis, acrosomal status, capacitation, mitochondrial activity and generation of reactive oxygen species using the flow cytometry procedure. Novel fertility-related biomarkers, such as ubiquitination, overexpression of MKRN1, SPTRX-3 and PAWP proteins or histone modification (H3K4me2), were also analysed by flow cytometry. From all monitored parameters, more proper characteristics of impaired *in vitro* fertilising ability proved to be high incidence of apoptotic markers (YO-PRO, Caspase 3/7) and higher counts of morphologically abnormal spermatozoa. Although the *in vitro* fertilisation (IVF) test can be an advantageous method for evaluating the sperm fertilising ability, there are still differences between the *in vivo* and *in vitro* fertilisation processes, which must be considered for example, when evaluating the sperm capacitation status.

Keywords: biomarker; bovine; fertilisation; flow cytometry; sperm

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Storage of bull's insemination doses in liquid nitrogen and their use in insemination is a commonly used methodology and an integral part of reproduction management on beef farms. Thanks to long-term experience with cryopreservation of bovine semen, it is also one of the basic approaches for preserving genetic resources in the gene bank of animal genetic resources. It thereby contributes to the biodiversity conservation by preserving the insemination doses of bulls of endangered breeds, such as the Pinzgau breed in Slovakia, whose numbers are rapidly decreasing and has been registered as threatened with extinction since 1994 (Kasarda et al. 2008).

However, the applicability of such cryopreserved insemination doses is dependent on the quality of the preserved semen. A common approach to selecting the suitability of bull ejaculate for freezing is sensory assessment, determination of sperm concentration and motility. Motility and progressive motility are considered as most important characteristics associated with the fertilising ability of spermatozoa (Kathiravan et al. 2011). However, the overall quality of the semen and the related fertilising ability are much more complex features and include the ability of the sperm not only to fertilise and activate the egg but also to support embryo development (Butler et al. 2019).

There are large inter-individual differences in fertilisation ability between individual bulls, which can be observed in fresh ejaculates (Graham et al. 1990). The variability in the quality and fertilising ability of the sperm in the thawed insemination dose are then influenced by the individual characteristics of the male, the individual resistance of the sperm to freezing, but also the intensity of the damage occurring during the freezing/thawing process. With reduced cryoresistance, the quality can change fundamentally after thawing. Multiparametric flow cytometric analysis of bull spermatozoa provides quite rapid and complex screening of semen quality in comparison with time-consuming microscopic assessment (Bucher et al. 2019; Vasicek et al. 2022).

The aim of the study was to find potential markers for the assessment of sperm quality and-fertilization ability as a complex analysis of insemination doses of Pinzgau breed bulls based primarily on flow cytometry.

MATERIAL AND METHODS

Commercial insemination doses from five sexually mature bulls of the Pinzgau breed (Slovak Biological Services, Nitra, Slovakia) at the age of 2 to 3 years were used. All experiments were performed in three replicates.

Sperm motility assay by CASA

After removal from liquid nitrogen, the insemination doses were immediately thawed by immersion for 30 s in a water bath with a temperature of 37 °C. An aliquot taken from each semen sample was used for motility analysis immediately after thawing. Semen samples from individual bulls were diluted in a saline (0.9% NaCl; Braun, Melsungen, Germany) at a ratio of 1:40 (*v*/*v*), placed (at 10 µl) into a pre-warmed Makler counting chamber (depth of 10 µm; Microptic, Barcelona, Spain) and evaluated under a Zeiss AxioScope A1 microscope (Carl Zeiss, Oberkochen, Germany) using the computer-assisted sperm analysis (CASA) system (SpermVision™; Minitube, Tiefenbach, Germany) adjusted for bull sperm by the manufacturer. For each sample, six microscopic view fields were analysed for average concentration (10^9/ml), percentage of total motile spermatozoa (motility > 5 µm/s) and percentage of progressive motile spermatozoa (motility > 20 µm/s).

Flow cytometry assay

Sperm samples from each bull insemination dose were diluted to the concentration of 1×10^6 spermatozoa in a phosphate buffered saline (PBS; Ca- and Mg-free; Biosera, Nuaille, France) and incubated with chosen chemicals, which specifically identify common physiological sperm characteristics like the viability (SYBR-14), apoptosis (YO-PRO-1; Caspase3/7), acrosomal status (PNA), capacitation status (FLUO-4), mitochondrial activity (MitoTracker Green) and generation of reactive oxygen species (ROS; CellROX). The viability of spermatozoa was assessed using SYBR-14, a membrane-permeant nucleic acid green fluorescent dye (LIVE/DEAD® Sperm Viability Kit; Thermo Fisher Scientific, Waltham, MA, USA) and DRAQ7, a far-red fluorescent nucleic acid

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dye (BioStatus Limited, Shepshed, UK), which stains nuclei of dead or membrane-compromised cells. The proportion (%) of spermatozoa positive for SYBR-14 but negative for DRAQ7 was considered as the proportion of live spermatozoa (SYBR-14$^+$/DRAQ7$^-$), while SYBR-14$^+$/DRAQ7$^+$ and SYBR-14$^-$/DRAQ7$^+$ spermatozoa were considered as dead spermatozoa. YO-PRO-1 nuclear green dye (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect apoptotic-like changes in bull spermatozoa. The proportion (%) of spermatozoa positive for YO-PRO-1 (YO-PRO-1$^+$/DRAQ7$^-$ and YO-PRO-1$^+$/DRAQ7$^+$) was considered as the proportion of apoptotic-like spermatozoa. The caspase detection reagent, Caspase 3/7 (CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA), specifically recognizes active caspase-3 and caspase-7 proteins. The proportion (%) of spermatozoa positive for Caspase 3/7 (Caspase 3/7$^+$/DRAQ7$^-$ and Caspase 3/7$^+$/DRAQ7$^+$) was considered as the proportion of apoptotic-like spermatozoa. The acrosome integrity was inspected using fluorescent PNA probes (peanut agglutinin; Alexa Fluor 488 conjugate; Thermo Fisher Scientific, Waltham, MA, USA). After incubation, samples were washed and stained with ready-to-use DRAQ7 dye as mentioned above, and analysed by flow cytometry. The proportion (%) of spermatozoa positive for PNA (PNA$^+$/DRAQ7$^-$ and PNA$^+$/DRAQ7$^+$) was considered as the proportion of acrosome-damaged spermatozoa. Capacitation of bull spermatozoa was evaluated using FLUO-4 AM, specific Ca^{2+} green fluorescent probe (FLUO-4; Thermo Fisher Scientific, Waltham, MA, USA). The activity of mitochondria was assessed using MitoTracker® Green FM (MT Green; Thermo Fisher Scientific, Waltham, MA, USA). The proportion (%) of spermatozoa positive for MT Green (MT Green$^+$/DRAQ7$^-$) was considered as the proportion of spermatozoa with high mitochondrial activity. To measure the production of reactive oxygen species (ROS) in bull semen samples CellROX Green Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used.

In addition, the expression of novel fertility-related biomarkers, such as ubiquitination, overexpression of MKRN1, SPTRX-3 and PAWP proteins or histone modification (H3K4me2), were also analysed by flow cytometry as described in our previous study (Vasicek et al. 2022). To assess possible expression of fertility-related biomarkers, bull spermatozoa were stained with purified mouse monoclonal antibody against makorin ring finger protein 1 (MKRN1, clone OTI2C8, IgG2b; Thermo Fisher Scientific, Waltham, MA, USA) and rabbit polyclonal (IgG) antibodies against spermatid-specific thioredoxin-3 [SPTRX-3, known also as thioredoxin domain-containing protein 8 (TXNDC8); EMZ003; Kerafast, Boston, MA, USA] and against post-acrosomal WW domain-binding protein (PAWP, known also as WW domain-binding protein 2 N-Terminal Like (WBP2NL); 22587-1-AP; Proteintech Group, Rosemont, IL, USA), and rabbit monoclonal antibody against histone H3 dimethylated on lysine K4 (H3K4me2, clone Y47, IgG; Abcam, Cambridge, UK). To detect defective ubiquitinated spermatozoa in bull semen samples, purified mouse anti-ubiquitin monoclonal antibody (UBQ; clone P4G7-H11, IgG1) was used. The proportion (%) of spermatozoa positive for MKRN1 and SPTRX-3 was classified as defective spermatozoa. Using PAWP antibody, spermatozoa with low, moderate and high PAWP content can be distinguished, while spermatozoa with high PAWP content were classified as defective spermatozoa. Similarly, sperm samples with higher mean fluorescence intensity (MFI) values of H3K4me2 were classified as defective spermatozoa. The final value for MFI of H3K4me2 was obtained after subtracting the MFI of the control sample stained only with secondary antibody from the signal (MFI) of the experimental sample.

Samples were analysed immediately after staining and/or washing procedure using FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon ion laser and red-diode (635 nm) laser. Fluorescent signals were acquired by Cell Quest Pro™ software (BD Biosciences, San Jose, CA, USA) in green FL1 channel using 530/30 nm band pass filter, red FL3 channel using 670 nm long pass filter and/or far-red FL4 channel using 661/16 nm band pass filter. Calibration of the instrument was performed periodically using standard calibration beads (BD CaliBRITE™; BD Biosciences, San Jose, CA, USA). At least 10 000 events (spermatozoa) were acquired for each sample using log-scale and low flow rate (about 600–1 000 events/s) unless otherwise stated. Unstained samples or samples stained with secondary antibodies were used as a control to gate the positive cells according to the increased fluorescent intensity. Obtained flow cytometry data were evaluated using FlowJo™ software (v10.10.0) (BD Biosciences, San Jose, CA, USA).

https://doi.org/10.17221/142/2024-CJAS

In vitro fertilisation test

Immediately after slaughter, cow ovaries were brought to the laboratory at a temperature of 22–25 °C. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (2–8 mm in size) with a sterile 5 mL syringe and needle. The isolated COCs were washed several times in the holding medium (E-199 with HEPES and 10% foetal bovine serum – FBS). Oocytes with a complete and tightly connected cumulus cell layer and homogeneous medium-brown ooplasm were selected for *in vitro* maturation (IVM). The medium used for maturation was E-199 (Gibco) with L-alanyl glutamine added sodium pyruvate (0.25 mmol/l), gentamicin (50 mg/l), 10% FBS and FSH/LH (1/1 IU., Pluset). Morphologically good-looking oocytes surrounded by a compact layer of cumulus cells, with the medium-brown, finely granular homogeneous ooplasm, intended for IVF were washed in IVF–TALP medium (Tyrode-albumin-lactate-pyruvate solution, 10 mg/ml heparin, 50 mg per ml gentamicin) and put into 100 µl droplets of the IVF–TALP medium previously covered with mineral oil into Nunc 4-well plates (ThermoFisher Scientific, Bratislava, Slovak Republic). Afterwards, the sperm (at 2×10^6/ml) and PHE solution (20 mmol/l penicillamine, 10 mmol/l hypotaurine, 1 mmol/l epinephrine) were added and incubated at 38.5 °C with 5% of CO_2 in the atmosphere. In 18 h from the start of IVF, oocytes were cleaned of adhered spermatozoa and cumulus cell debris by vortexing in a microtube containing 75 µl of the holding medium for 30 seconds. The zygotes were subsequently placed in prepared 4-well culture dishes (NUNC) with a grown confluent monolayer of BRL feeder cells (BRL-1, EACCC). Menezo B2 medium, prepared according to their composition and supplemented with 10% FBS, was used for embryo culture. The development of *in vitro* fertilised oocytes was monitored up to the blastocyst stage. The zygote cleavage rate was recorded on day 2, while the blastocyst rate was evaluated on days 6, 7 and 8 after fertilisation.

Sperm morphology

A drop (5 µl) of the ejaculate diluted with distilled water at the ratio of 1 : 40 was placed onto the slide, covered with a coverslip and observed under a microscope with 100× magnification under immersion oil. We evaluated the morphology of the sperm malformations such as the separated tail, knob-twisted tail, torso tail, rounded tail, broken tail, retention of the cytoplasm drop, enlarged or reduced sperm head and other acrosomal sperm changes. For the determination of abnormal sperm incidence, a total of 400 sperm cells were examined.

Statistical analysis

In vitro fertilisation and embryo culture experiments were performed in three replicates. The distribution of embryos according to stages of development (cleavage and blastocyst rates) was analysed by Pearson's Chi-square (χ^2) test. Data obtained from flow cytometry analyses were evaluated using GraphPad Prism (v9.5.1) for Windows (GraphPad Software, San Diego, CA, USA) with two-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Results are expressed as the mean ± standard deviation (SD). *P*-values at $P < 0.05$ were considered as statistically significant.

RESULTS

In vitro fertilising ability

Based on the *in vitro* fertilisation experiments of matured bovine oocytes, we determined the average fertilisation ability of the individual tested bulls. Fertilising ability of Pinzgau bulls under *in vitro* conditions is presented in Table 1.

Bull sperm samples analysed in this study were divided into two groups according to the fertilising

Table 1. Fertilising ability of Pinzgau bull insemination doses tested using *in vitro* fertilisation test

Bull name	Fertilised oocytes (*n*)	Cleavage rate (*n*; %)	Total blastocyst rate (*n*; %)
NOB[a]	101	77 (76.2)	39 (38.6)
KAZ[b]	104	94 (90.4)	30 (28.9)
GAL[b]	99	70 (70.7)	29 (29.3)
NUS[c]	95	64 (67.4)	21 (22.1)
LOH[c]	134	89 (64.4)	26 (19.4)

[a–c]Groups with different superscripts are significantly different in distribution according to developmental stages ($P < 0.05$)

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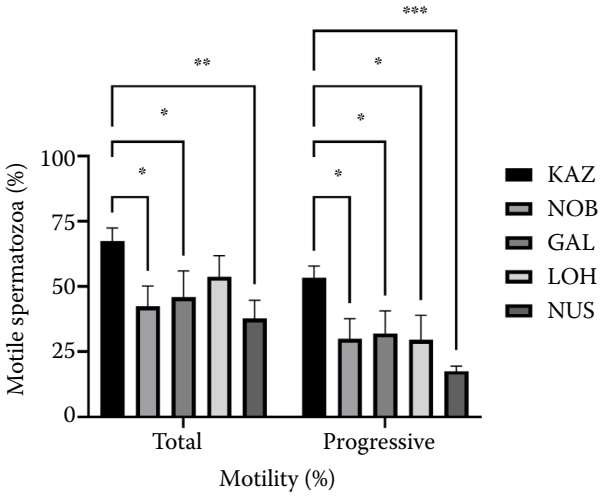

Figure 1. Results of motility analysis of Pinzgau bull semen samples

*, **, ***Differences between bulls marked with an asterisk are significant at $P < 0.05$, 0.01, 0.005, respectively

The bull KAZ showed an increased level of motility compared to other bulls, both in terms of total motility and progressive motility; the differences in motility between the other bulls were not significant

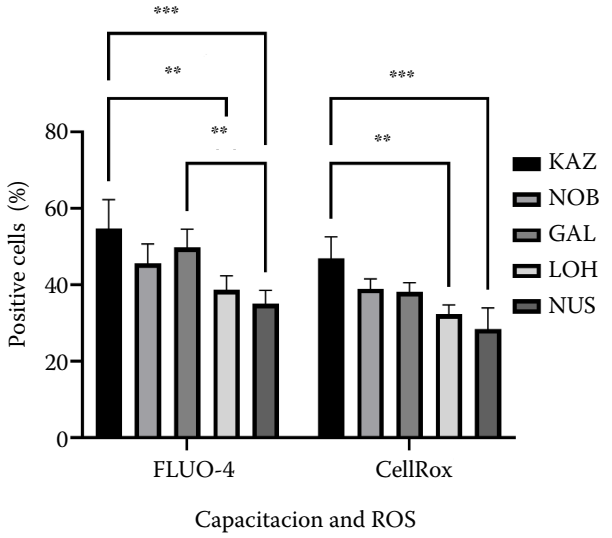

Figure 2. Results of flow cytometry analyses of capacitation status and generation of reactive oxygen species in Pinzgau bull semen samples

*, **, ***Differences between bulls marked with an asterisk are significant at $P < 0.05$, 0.01, 0.005, respectively

KAZ and GAl bulls showed significantly increased FLUO-4 marker positivity and KAZ bulls also had increased reactive oxygen species (ROS) production compared to NUS and LOH bulls from the group of bulls with lower *in vitro* fertilisation ability

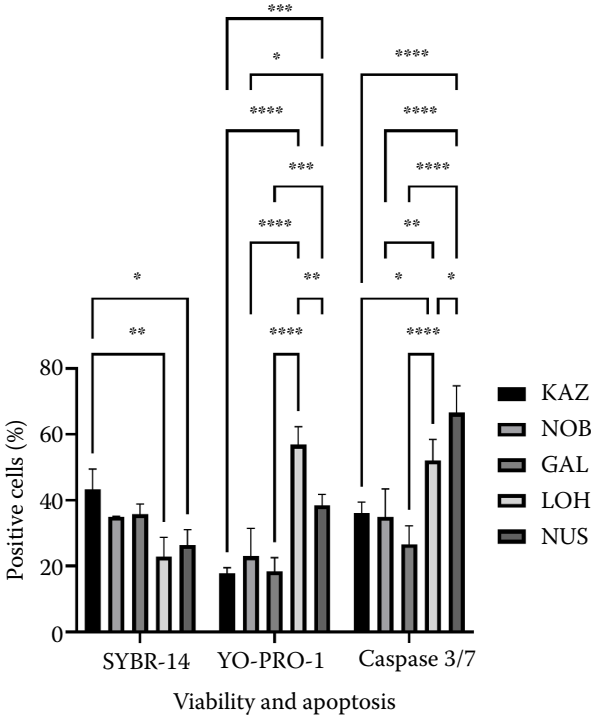

Figure 3. Results of flow cytometry viability and apoptosis analyses of Pinzgau bull semen samples

*, **, ***, **** Differences between bulls marked with an asterisk are significant at $P < 0.05$, 0.01, 0.005 and 0.001, respectively

NUS and LOH bulls with the lower *in vitro* fertilisation ability showed a lower viability rate compared to the KAZ bull and an increased incidence of apoptotic markers compared to the other bulls

ability assessed using *in vitro* fertilisation. Bulls achieving average fertilisation ability (around 30% blastocysts) were considered as bulls with good fertilisation ability NOB (38.61%), KAZ (28.85%) and GAL (29.29%). The NOB bull even achieved a significantly higher fertilisation capacity than KAZ and GAL bulls. Conversely, the LOH and NUS bulls were assessed as bulls with low *in vitro* fertilisation ability.

Motility and flow cytometry analyses

Sperm samples from each bull insemination dose were analysed for sperm motility and then stained with specific probes for identifying common physiological sperm characteristics like viability (SYBR-14), apoptosis (YO-PRO-1; Caspase3/7), acrosome status (PNA), capacitation status (FLUO-4), mitochondrial activity (MitoTracker Green), generation of reactive oxygen

https://doi.org/10.17221/142/2024-CJAS

species (ROS; CellROX), ubiquitination, expression of MKRN1, SPTRX-3 and PAWP proteins or histone modification (H3K4me2). These stained samples were analysed by flow cytometry. Results of complex analyses are presented in Figures 1–6. It is evident from the results that the KAZ bull clearly differed from all other bulls in the motility parameter, and especially in the progressive motility parameter, where it was demonstrably higher (Figure 1).

The same KAZ bull, from the group of bulls with better fertilisation ability, differed significantly in the CellROX parameter (Figure 2). The capacitation marker FLUO-4 was significantly lower in both bulls from the group with lower fertility (NUS and LOH) compared to bulls with higher fertility (KAZ and GAL) (Figure 2).

In the markers YO-PRO-1 and Caspase3/7, a very similar division into two groups of bulls can be discerned when we evaluated according to *in vitro* fertilisation ability (Figure 3).

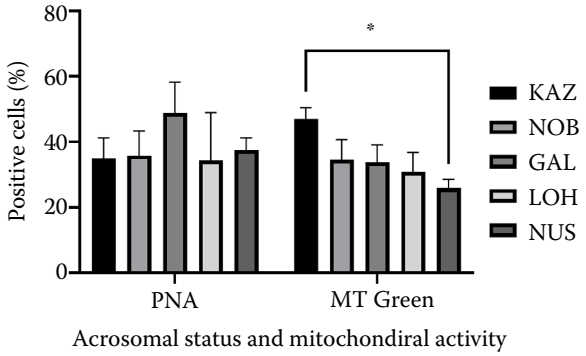

Figure 4. Results of flow cytometry analyses of the acrosomal status and mitochondrial activity in Pinzgau bull semen samples

*Differences between bulls marked with an asterisk are significant at $P < 0.05$

The KAZ bull showed increased mitochondrial activity compared with the other bulls; PNA = peanut agglutinin

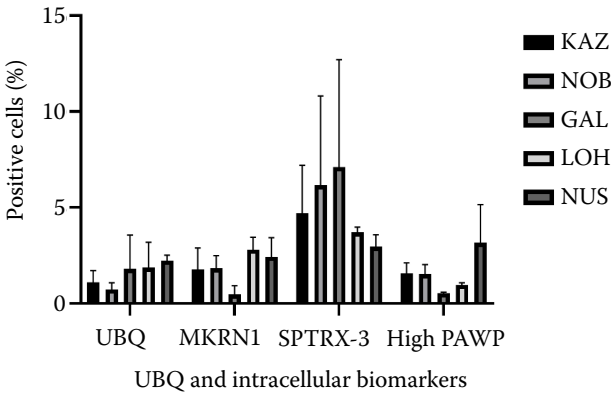

Figure 5. Results of flow cytometry analyses of fertility-related biomarkers in Pinzgau bull semen samples

Differences between bulls are insignificant; PAWP = post-acrosomal WW-domain binding protein; UBQ = ubiquitin

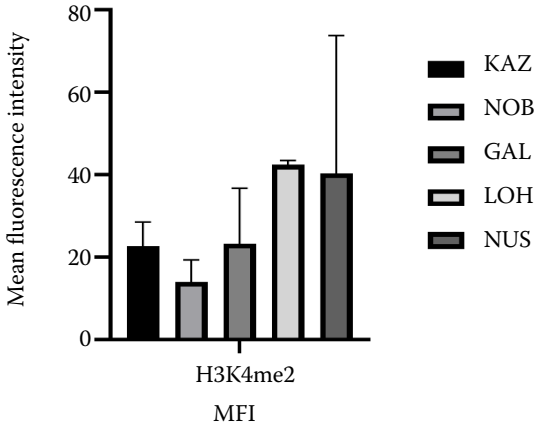

Figure 6. Results of flow cytometry analyses of the fertility-related biomarker H3K4me2 in Pinzgau bull semen samples

Differences between bulls are insignificant

In the other parameters of the flow cytometry analysis, no significant differences were found between the bulls (Figures 4, 5, 6).

The KAZ bull also showed increased mitochondrial activity (Figure 4).

Table 2. Occurrence of morphological abnormalities in the thawed insemination doses of Pinzgau-bulls (%)

Bull name	Separated tail	Knob twisted	Rounded tail	RC drop	Other	Total PS
NOB	2.00	0.25	1.75	0.13	0.75	4.87 ± 0.15^{a}
KAZ	2.25	2.75	0.88	0.38	0.63	6.87 ± 0.51^{b}
GAL	4.38	0.63	1.35	0.00	0.25	6.50 ± 1.01^{ab}
NUS	1.86	3.00	15.25	0.25	0.25	20.6 ± 2.30^{c}
LOH	3.50	1.00	4.75	0.25	0.50	10.0 ± 1.06^{d}

$^{a–d}$Groups with different superscripts are significantly different at $P < 0.05$

RC = retention cytoplasma; PS = pathological sperm

https://doi.org/10.17221/142/2024-CJAS

Sperm morphology

Results of sperm morphology abnormalities assessed in the insemination doses of five Pinzgau bulls are presented in Table 2.

DISCUSSION

Efforts to find a marker or a combination of markers in sperm to predict the fertilising ability of a bull have already been done in the past. In some cases, a correlation was found between viability parameters of bull frozen-thawed sperm and fertility after insemination (Januskauskas et al. 2003). However, testing the male fertility in every individual by artificial insemination is an expensive and demanding procedure. This method can be successfully replaced by *in vitro* fertilisation to assess sperm fertility since this procedure evaluates the spermatozoa-oocyte interactions occurring during the fertilisation process, allowing the determination of different endpoints in the early stages of the embryo development (Makarevich et al. 2011). The result of *in vitro* fertilisation of an undefined population of bovine oocytes is about 30% (usually 25–34%) of blastocysts, depending on the culture methodology used (Lopes et al. 2019; Oliveira et al. 2019; Yang et al. 2022).

Based on *in vitro* fertilisation experiments with insemination doses of Pinzgau bulls, we managed to distinguish bulls with good fertilisation ability (KAZ, GAL, NOB) from those with lower fertilisation ability (NUS and LOH). While the bull with the NOB designation achieved a well above-average level of blastocyst rate after *in vitro* fertilisation, NUS and LOH bulls showed lower fertilisation ability. In other analyses we compared the results between bulls from these two groups according to the rate of fertilising ability.

Based on the results of flow cytometry, the lower fertilising ability agreed with the increased incidence of sperm positive for apoptotic markers (YO-PRO, Caspases), where bulls with lower fertilising ability showed also a demonstrably higher incidence of cells labelled with pro-apoptotic markers. The occurrence of spermatozoa with apoptotic signs, therefore, showed to be suitable for assessing the fertilisation ability. Similar results were presented by Janskauskas et al. (2003), who found a correlation between fertility and the occurrence of annexin V-labelled (apoptotic) bull sperm after thawing.

A relatively extensive analysis of the interrelations between real fertility and markers detected by flow cytometry was offered by Bucher et al. (2019). In addition to traditional parameters, such as plasma membrane integrity, acrosome integrity, or mitochondrial membrane potential, like in our study, they also included the level of free Ca^{2+} by FLUO-4 staining into the multiple analysis. The level of Ca^{2+} indicates the capacitation status in sperm. Calcium release engages in signalling cascades that trigger a series of changes leading to hyperactivation and initiation of the acrosome reaction (Rahman et al. 2014). It is well known that before spermatozoa acquire the ability to fertilise, they must undergo this capacitation (Gervasi and Visconti 2016). The freezing process during sperm cryopreservation can affect these signalling pathways. In some spermatozoa after thawing, calcium is released and capacitation changes begin prematurely (Almadaly et al. 2015). As a result, their ability to survive in the reproductive tract of the female is reduced. This fact can lead to a significant reduction in fertility after insemination (Kadirvel et al. 2009). Therefore, the increased incidence of spermatozoa with a high Ca^{2+} level in the thawed insemination dose is considered rather a negative marker of bull fertility *in vivo* (Bucher et al. 2019). In our study, oppositely, the KAZ bull with higher fertilisation ability also had a demonstrably increased incidence of FLUO-4-positive sperm. We assume that the reason for this discrepancy is the difference in the methodology used for testing the fertilisation ability using *in vitro* fertilisation. During IVF the spermatozoa are immediately added to the co-culture with matured oocytes after being thawed and washed. In this case, their longevity is not essential and the capacitation changes that have taken place probably can even represent an advantage.

To assess the state of sperm acrosomes in the samples of the tested bulls, we used PNA staining (lectin *Arachis hypogaea* agglutinin), which specifically binds to the disaccharides with terminal galactoses, including disaccharides in the sperm acrosome. It has been known for a long time to bind to the acrosome membranes of the spermatozoa with damaged acrosome. PNA is routinely used for flow cytometry assays of acrosomal integrity (Graham et al. 1990). In our group of Pinzgau bulls, all individuals showed a relatively high percentage of PNA-positive sperm. This is probably related to the disruption of acrosome membranes

https://doi.org/10.17221/142/2024-CJAS

during freezing/thawing. The percentage of PNA-positive sperm is not usually as high as in fresh ejaculates, but this value usually increases after freezing/thawing (Nagy et al. 2003). However, we did not observe any differences between bulls with higher and lower fertilisation ability.

Mitochondrial activity in sperm is another important indicator of good quality. The activity of mitochondria was assessed through the use of a MitoTracker probe, which labels only active mitochondria. In our experiments MitoTracker-Green staining showed a higher incidence of active mitochondria in the KAZ bull compared to the other bulls, and this bull also had the highest motility. These parameters indicate good quality and fertility. At the same time, this bull showed an elevated ROS level (CellROX), which may be the result of its high mitochondrial activity. On the other hand, oxidative stress is considered as a negative marker of sperm fertilising ability because damage caused by ROS may have a negative effect on sperm fertilising ability (Koppers et al. 2008). These connections and interactions complicate the search for a universal marker of good fertility, and they show that the semen must be evaluated comprehensively, taking into account several factors.

Besides the traditional markers used to evaluate the quality of bull ejaculates/insemination doses in our groups of bulls we also tested several new potential intracellular markers proposed to predict the insemination ability of males (Sutovsky et al. 2015; Vasicek et al. 2022). Ubiquitin, MKRN1, SPTRX-3, PAWP and H3K4me2 are markers whose elevated expression in spermatozoa negatively correlated with fertility. Sutovsky et al. (2003) reported that ubiquitin can mark impaired sperm in the ejaculate. Ubiquitination is a mechanism by which the sperm plasma membrane is marked with a small protein ubiquitin. Ubiquitinated spermatozoa are then removed by proteasomes (Sutovsky et al. 2003). Therefore, the incidence of ubiquitinated spermatozoa may suggest poor semen quality. MKRN1 (Makorin1; C3HC4 RING finger protein) is highly conserved in mammals and expressed in the adult testis (Yoshida et al. 2003). MKRNs as RING finger E3 ligases mediate the substrate degradation that is related with conserved RING finger domains that control multiple cellular components through the ubiquitin-proteasome system (Wang et al. 2022). There are indications that it could also be a low fertility marker (Vasicek et al. 2022).

SPTRX-3, spermatid specific thioredoxin-3 (known also as TXNDC8), is a member of the thioredoxin family. It has been detected in the cytoplasm and nuclear vacuoles of defective spermatozoa, in round testicular spermatids in the testes of various mammals; increased content of SPTRX-3-positive spermatozoa was found in infertile males (Buckman et al. 2013).

Histone H3 dimethylated on lysine K4 (H3K4me2) is an epigenetic marker which may be associated with poor quality sperm. Histone dimethylation belongs to the post-translational modifications in the sperm head within spermatogenesis. Accidents in this process may also cohere with defects in chromatin integrity (Stiavnicka et al. 2020).

PAWP (post-acrosomal WW-domain binding protein) is noted in the bull sperm perinuclear theca, where it is transported in the phase of elongating spermatid during spermiogenesis. PAWP is known as an important protein in sperm for the oocyte-activating ability (Wu et al. 2007). While medium levels of PAWP are present in normal spermatozoa, low or high levels of PAWP were detected in semen with decreased fertility and with abnormality in sperm morphology (Sutovsky et al. 2015). Unfortunately, we did not find a clear match with the classification of bulls according to *in vitro* fertilisation ability in any of these new intracellular markers. One of the reasons for this observation can be the non-specificity of some antibodies against bull (mainly for anti- SPTRX-3, anti-MKRN1 and anti-H3K4me2). On the other hand, low numbers of ubiquitinated spermatozoa and spermatozoa with an abnormal level of PAWP expression as analysed by bull specific antibodies indicated that the decreased fertilising ability of some bulls in this study can be caused by their individual sensitivity to cryopreservation rather than by their genetically based infertility.

The interrelation between sperm morphology and their fertilising ability has been studied long ago. Soderquist et al. (1991) found a correlation between some abnormalities in the sperm morphology from samples of bulls used in artificial insemination and their real fertility. Even later, several authors confirmed that morphologically abnormal sperm can reduce rates of fertilisation and embryonic development (Thundathil et al. 2001; Walters et al. 2005). The quality of sperm morphology in bull ejaculate primarily reflects the health status of the testicles, seminiferous tubules and

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epididymis. Morphologically abnormal spermatozoa are present to some extent in every ejaculate, which does not exclude their use in reproduction. Usually, the ejaculate containing up to 30% of abnormal spermatozoa can be considered suitable for use (Menon et al. 2011). From our experimental results it is clear that the increased incidence of morphological abnormalities was correlated with reduced *in vitro* fertilisation ability. Bulls with reduced *in vitro* fertilising ability (NUS and LOH) showed a significantly higher percentage of morphologically abnormal sperm. Thus, when looking for a marker indicating the ability to fertilise, it would be appropriate to consider indicators of abnormal sperm morphology. Consistently with our findings, Tundathil et al. (2001) reported the reduced binding of morphologically abnormal sperm to the *zona pellucida*. Moreover, even morphologically normal sperm could be affected by the co-existence of a large number of abnormal sperm in the ejaculate, so that the resulting zygotes, after using such sperm in the IVF, did not develop to higher developmental stages. Similarly, Walters et al. (2005) reported impaired embryo development when using an insemination dose for fertilisation with an increased incidence of abnormal sperm mainly with defects in sperm heads.

CONCLUSION

By analyses of various important sperm traits, such as membrane integrity, apoptosis, mitochondrial activity, oxidative stress etc., in bull's cryostored semen samples, we found that the best marker of impaired *in vitro* fertilising ability is the elevated presence of apoptotic markers (YO-PRO, Caspase 3/7) and the occurrence of morphologically abnormal sperm. Unfortunately, we did not find a correlation between the proposed new intracellular markers and *in vitro* fertilising ability. It is necessary to point out that even though the *in vitro* fertilisation test can be an advantageous, more economical and less time-consuming option when evaluating the fertilisation ability, there are still substantial differences between the *in vivo* and *in vitro* fertilisation processes. Therefore, these differences and limitations of the *in vitro* fertilisation system, which we noted at evaluating the capacitation status of sperm, must be taken into account when interpreting obtained results to predict future fertilising ability.

Conflict of interest

The authors declare no conflict of interest.

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