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Sperm quality of Zemplin Rabbit and Liptov Bold-Spotted Rabbit breeds

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Abstract

Zemplin Rabbit and Liptov Bold-Spotted Rabbit are Slovak national breeds. Our aim was to characterize the sperm quality of these two breeds, as these characteristics are important for artificial insemination and cryopreservation as biodiversity conservation tools. For this evaluation, sperm samples of sexually mature Zemplin Rabbit (ZR) males (n = 6) and Liptov Bold-Spotted Rabbits (LR) (n = 4) were used. According to progressive motility (PM) data (CASA), samples were divided into two groups: A (>30% PM) and B (<30% PM). In addition to PM (ZR-A: 48.6±3.8%, ZR-B: 16±3.2%, LR-A: 35.38±2.6%, LR-B: 4±2.2%), total motility (TM) (ZR-A: 69.1±4.1%, ZR-B: 35.5±4.1%, LR-A: 59.1±3%, LR-B: 26.9±4.1%) and morphological abnormalities (ZR-A: 29.7±1.2%, ZR-B: 40±4%, LR-A: 34.3±4.3%, LR-B: 48.3±4.7%) were also assessed using the CASA system. The proportion of dead/live, apoptotic and oxidative damaged spermatozoa (Spz) was assessed by flow cytometry using fluorescent dyes: SYBR-14, Sytox Green, Yo-Pro-1 and CellROX Green, respectively. The results of flow cytometry correspond to the values of motility and morphometry. Sperm with PM less than 30% does not show proper quality values, while the sperm with PM higher than 30% is suitable for further analysis and use.

Keywords: rabbit, spermatozoa, CASA, flow cytometry

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Introduction

Biodiversity is one of the most important factors of sustainable agriculture. Farm animals, which in the past were mainly used for meat production, were developed on a relatively narrow genetic basis and the genetic management of genetic resources has been receiving attention recently. There are several major concerns regarding genetic resources. This is one of the most important reasons why the breeding of local breeds is so important for maintaining biodiversity.

Rabbit breeding has a long tradition in our region. Originally bred for meat, rabbits are currently also bred for sport and exhibitions (ALVES et al., 2015) as well as for specialized genotypes for biological research (TŮMOVÁ et al., 2011). Zemplin Rabbit (ZR) is a medium-sized breed, so that the Viennese Blue Rabbit, the New Zealand Red Rabbit and the Slovakian Grey-Blue Rex were particularly involved in its breeding. ZR has been an officially recognized breed since 1987. It is included in the European Breed Standards and is currently bred in the Czech Republic and Hungary in addition to Slovakia (CHRENEK et al., 2019). Liptov Bold-Spotted Rabbits (LR) is one of the youngest Slovak national rabbit breeds. The breed was recognized in 2005. The following combinations proved successful in its breeding: Vienna‘s wild-coloured and Dutch wild-coloured, or Vienna Wild-Blue and Dutch Pearl. It is a small or medium breed with good productivity and fertility. Several colour variations of LR are known: wild-coloured, black-and-blue and grey-blue (CHRENEK et al., 2019).

The CASA system (computer assisted sperm analysis) was used as an input analysis for the assessment of total and progressive motility and, in addition, for the evaluation of morphological changes. Standard sperm flow-cytometric analyses involved assessment of different spermatozoa characteristics, which affect the overall semen quality. The viability of spermatozoa basing on their plasma membrane integrity is commonly analysed either directly, using SYBR-14 dye, staining of live metabolically active cells, or in combination with dead cell dyes, entering cells via disrupted membrane (GARNER and JOHNSON, 1995). Sytox Green nuclear fluorochrome was used to evaluate dead cells. In addition to viability analysis, apoptosis-like changes in spermatozoa should be analysed, because such spermatozoa can be hidden within the live cell population. One of the most frequently used mechanisms for determining apoptotic cells is increased membrane permeability revealed by the nuclear dye - YO-PRO-1 iodide (PENA et al., 2005). Oxidative stress or damage, triggered by reactive oxygen species (ROS), may have unfavourable impact on spermatozoa fertilizing ability. There are several probes, which accumulate in cells and become fluorescent after oxidation and, thus, may be used for the detection of ROS in spermatozoa. In our case, CellROX Green was used as a probe that should bind DNA after oxidation (RILEY et al., 2021). Since rabbit

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ejaculate can contain a large number of granular bodies, we used a DRAQ5 far-red dye to distinguish nucleated cells from other events.
This work should serve as a basic evaluation of the microscopic sperm properties of the above-mentioned breeds. Following these results, it is possible to continue with more detailed analyses and then try to cryopreserve the samples for the purpose of biodiversity protection.

Material and methods

Clinically healthy and sexually mature males of ZR and LR were used in this experiment. The age of the individuals ranged from 10 to 18 months. Semen was collected two times per week through artificial vagina with warm water (50°C). Semen was immediately evaluated for volume, concentration, motility (initial checking) and divided into two groups: A (>30% PM) and B (<30% PM). A total of 32 ejaculates (16 from each breed) were used in our experiments.

Sperm motility
The motility and sperm movement were analysed by CASA (SpermVision™ software, Minitube, Tiefenbach, Germany) with light microscope (at the 200× magnification; AxioScope A1, Carl Zeiss Slovakia, Bratislava, Slovakia) and Makler counting chamber (Microptic, Barcelona, Spain). Samples were diluted by saline (0.9% NaCl; Braun, Nuaille, Germany) at ratio 1:20 (v/v). A drop of diluted semen (10 μL) was transferred to a counting chamber and analysed with manufacturer's pre-set parameters for rams. We mainly focused on total (TM) and progressive motility (PM).

Morphological changes
After motility analysis, an aliquot of samples was placed in the refrigerator and stored until the next day (approx. 24 h) to immobilize sperm for morphological analysis. Morphological abnormalities were measured by the same microscope as CASA was performed. In this analysis morphological abnormalities of sperm cells, e.g. detached flagellum from head, twisted flagellum, shortened flagellum, broken flagellum, cytoplasmic droplet flagellum, reduced or enlarged sperm head, or other pathological sperm were evaluated.

Flow cytometry
Individual ejaculate samples were washed in PBS($^-$) (phosphate buffered saline, Ca^- and Mg^- free; Biosera, Nuaille, France) (300x g, 5 min.). Each sample was divided into 5 tubes with DRAQ5 staining solution (100 μmol.ml^{-1}; BioLegend, San Diego, USA) to identify nucleated cells. The proportion of apoptotic cells was evaluated using YO-PRO-1 (100 μM; Molecular Probes, Lucerne, Switzerland), the proportion

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of dead cells was determined by Sytox Green staining solution (30 µmol.ml^{-1}; Thermo Fisher Scientific, Waltham, MA, USA) and oxidatively damaged sperm - using CellROX Green staining (Thermo Fisher Scientific, Waltham, MA, USA). We used staining with SYBR-14 (50 µg.ml^{-1}; Thermo Fisher Scientific, Waltham, MA, USA) to evaluate viability. The control sample (without staining solutions) was resuspended in 250 µl of PBS($^-$). The cells were incubated for 15 min at RT (room temperature) and in the dark (SYBR-14 - 15 min. in the dark at 37 °C). After the incubation period, the samples were washed in 1 ml of PBS($^-$) and analysed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). At least 10,000 spermatozoa were analysed in each sample.

Results and discussion

Sperm motility of both breeds is shown in Figure 1. Progressive motility of B groups is below 20%, therefore it is considered unsuitable for fertilization, if we assume that the minimum PM value for successful fertilization is 30%. According to Theau-Clément et al. (2016), rabbit fertility is significantly higher, when total motility is above 80% and progressive motility is above 70%. Lavara et al. (2005) claim that insemination centres use a high percentage of total motile cells (more than 70%) as a selection parameter of ejaculates before their inclusion in the final pools and the use of heterospermic doses to perform inseminations.

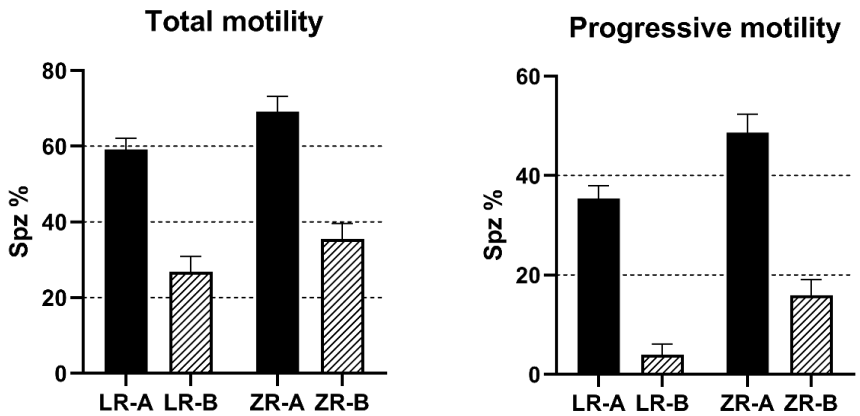

Figure 1. Motility parameters of analysed rabbit semen samples. The data are expressed as the means ± SEM.

The total value of morphologically abnormal sperm (Figure 2) is over 20% in both breeds and groups, which is not within the range of commercial insemination dose standards (malformation rate ≤ 20%). The most common malformation in both

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breeds and groups was a cytoplasmic droplet. An increase in the incidence of abnormalities in spermatozoa and abnormal trajectories has been observed when semen samples presented a high incidence of cytoplasmic droplets in rabbits (FAUSTO et al., 2001). The percentage of sperm cells with normal morphology is an important indicator of semen fertility (BARTH and OKO, 1989) and the sperm characteristic is most highly correlated with fertility in humans (MORTIMER and MENKVELD, 2001).

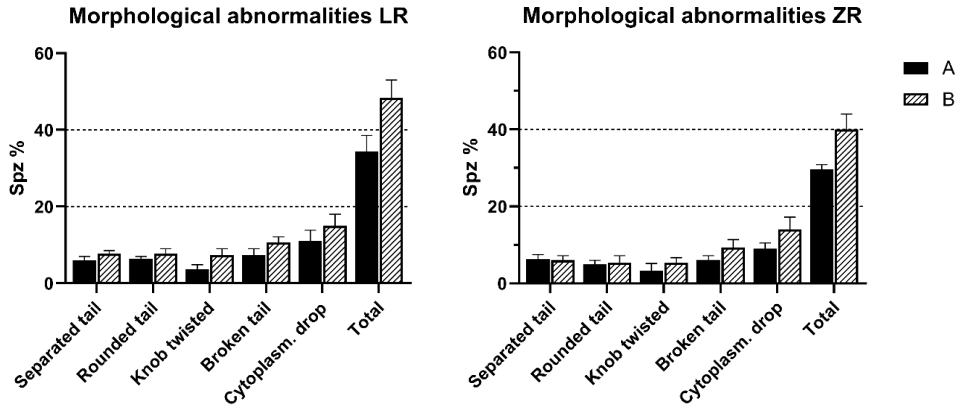

Figure 2. Percentage of individual abnormalities and all abnormal spermatozoa. The data are expressed as the means ± SEM.

Beside the motility and morphological abnormalities, viability was also evaluated. The proportion of live rabbit sperm was assessed by the SYBR-14 probe, which is most widely used marker of live cells, mainly in combination with PI (Propidium iodide),7-AAD (MARTÍNEZ-PASTOR et al., 2010) or DRAQ7 (VAŠÍČEK et al., 2022). Only sperm that maintain an intact acrosome can take part in fertilizing an oocyte. Therefore, the percentage of sperm with damaged acrosome should be low in order to maintain high fertility levels. Sytox Green, a green dead cell dye, was used in combination with the DRAQ5 red fluorescent dye to analyse dead sperm. This dye has been previously reported to be useful for sperm analysis either alone, or in combination with other specific probes, e.g. Annexin V or DHE (VARUM et al., 2007; DE IULIIS et al., 2006). In addition, a green apoptotic-like changes dye, Yo-Pro-1, was used for apoptosis assessment (KUŽELOVÁ et al., 2017). All the above-mentioned parameters were evaluated by flow cytometry. Although in oxidatively damaged cells we detected negligible values in both groups A and B, other markers corresponded to motility parameters because the proportion of apoptotic and dead cells was higher in group A (Figure 3).

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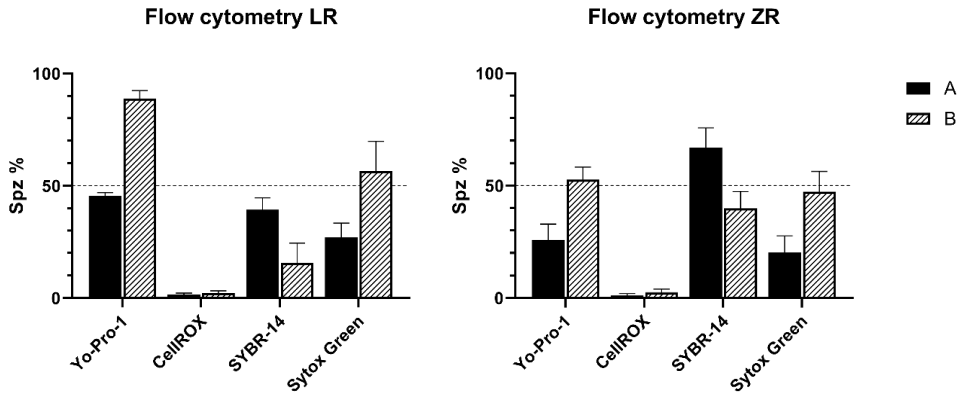

Figure 3. Evaluation of rabbit sperm quality parameters by flow cytometry. The data are expressed as the means ± SEM.

Conclusion and recommendation

Our results show inappropriate quality of group B in both breeds. Individuals from this group should not be used for artificial insemination. Group A could be used for insemination, but it is likely that these sperm samples would not be suitable for cryopreservation. However, we cannot confirm this claim until further analysis is carried out using a wider panel of markers or cryopreservation itself.

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