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High percentage of morphological defects in sperm of the Zemplin rabbit breed: A major obstacle to fertility?

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Abstract: This research investigates the high prevalence of morphological defects in the sperm of a particular rabbit breed, namely the Zemplin rabbit (ZR). We tried to uncover the possible causes and consequences of the observed abnormalities. Ejaculate samples from ten adult males were divided into two qualitative groups according to the motility parameters revealed by CASA (computer-assisted sperm analysis). Group A contained samples with total motility (TM) above 50% and progressive motility (PM) above 30%. Group B contained samples with TM and PM below these values. Viability (SYBR-14/DRAQ5™ and SYTOX™ Green/DRAQ5™ staining) and apoptosis (YO-PRO ™-1/DRAQ5 ™ stainig) analyses through flow cytometry served as an additional insight into the characteristics of the qualitative groups. In both cases, we noted significant differences ($P < 0.05$) between groups which corresponded to motility values. Both groups showed an increased incidence of abnormal spermatozoa, and we did not observe any significant differences between them. Simultaneously, we assessed the practical implications of these sperm defects on artificial insemination (AI), and also, we did not notice any significant differences in these parameters. The use of transmission electron microscopy enhanced our understanding of the morphological defects at the ultrastructural level. In summary, the study provides an understanding of the qualitative differences in rabbit semen, highlighting variations in quality parameters. While these differences exist, they may not necessarily translate into significant variations in AI outcomes. Our study shows higher values of cytoplasmic droplets in ZR semen, but it also shows that these conditions do not necessarily mean a fundamental obstacle to fertility in the case of AI.

Keywords: biodiversity; conservation; cytoplasmic droplet; morphology; spermatozoa

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While the majority of male infertility cases result from quantitative issues leading to either the absence of sperm or a decrease in sperm count, a significant number of cases are attributed to morphological or qualitative defects in sperm that impede its ability to achieve successful fertilisation (Ray et al. 2017). The evaluation of sperm morphology proves to be the most distinguishing sperm parameter when comparing two groups of males, one fertile and the other infertile (Guzick et al. 2001). Aberrant spermatozoa may indicate disruptions in spermatogenesis caused by several factors such as heat stress, malnutrition or genetic background (Dewaele et al. 2022). It affects not only those spermatozoa exhibiting abnormalities but also others within the same ejaculate that display a normal or near-normal appearance (Saacke 2008). The association between poor sperm morphology and a decline in fertility rates has been established (Nagy et al. 2013; Attia et al. 2016). Examining sperm morphology abnormalities and their frequency may serve as a predictive factor for semen fertilizing capacity (Hough et al. 2002). In general, the maximum acceptable volume of abnormal sperm per insemination dose is 30% (Chenoweth et al. 1992).

During the process of spermatogenesis, Sertoli cells phagocytose the cytoplasm of germinative cells, leaving a residual remnant known as the sperm cytoplasmic droplet (CD) (Cooper 2005). This abnormality constituted the majority of abnormalities that we recorded. This structure was firstly described by Retzius in 1909 and given a name (Retzius 1909). In the initial stage of mauration within the caput epididymis, a significant proportion of spermatozoa exhibit a proximal droplet. However, as the maturation progresses along the epididymis, the CD undergoes migration from a proximal to a distal position on the midpiece (Cooper 2011). Simultaneously to CD migration, in the corpus epididymis, spermatozoa gain motility (Angrimani et al. 2014). The precise mechanism underlying CD migration and the acquisition of motility remains uncertain, as noted by Xu et al. (2013). In mice, Yuan et al. (2013) proposed a function of the CD as an energy source during sperm maturation in the epididymis, suggesting that the droplet contains enzymes and substrates essential for the proper functioning of sperm mitochondria. Additionally, various studies point towards a correlation between failure in CD translocation and reduced sperm motility (Cooper 2005; Xu et al. 2013), highlighting the significant interplay between the CD, mitochondrial function and sperm motility.

Zemplin rabbit (ZR) is a breed of domesticated European rabbit (*Oryctolagus cuniculus*), so it is the Slovak national breed. Our team is engaged in the biodiversity conservation through *in vitro* cell preservation of national livestock breeds using cryopreservation. Our goal was to analyse the sperm characteristics of these animals in as much detail as possible for the purposes of later successful sperm cryopreservation.

MATERIAL AND METHODS

Study site and animal management

Ten mature and clinically healthy males of Zemplin rabbit (ZR) breed aged (12–18 months) and kept in a breeding facility (NPPC – RIAP Nitra, Slovak Republic) were used in this study. All males were housed in separate cages, under an artificial photoperiod (14 h of light at 10 lux and 10 h of dark) and were fed a commercial standard diet, water was available *ad libitum*. The animal treatment was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic (No. SK U 18016) in accordance with the guidelines of Slovak Animal Protection Regulation RD 377/12, which meet the requirements of the European Union Regulation 2010/63.

Semen collection and treatment

Sample collection was performed twice a week using an artificial vagina (IMV Technologies, L'Aigle, France) filled with water (approx. 50 °C) into prepared sterile tubes. Obviously contaminated samples were excluded from the experiments. Following the collection, the sperm samples were transported to the laboratory and promptly assessed for volume, concentration, and motility parameters (initial checking). The samples were divided according to motility into two qualitative groups in order to avoid bias caused by the uneven representation of pathological spermatozoa in the samples. Group A contained samples with total motility (TM) above 50% and progressive motility

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(PM) above 30%. Group B contained samples with motility parameters below these values. A total of 32 ejaculates were used in our experiments.

CASA (computer-assisted sperm analysis)

Sperm motility and movement were assessed using CASA (SpermVision™ software, Minitube, Tiefenbach, Germany), along with a light microscope (at 200 × magnification; AxioScope A1; Zeiss, Oberkochen, Germany) equipped with phase contrast and the Makler counting chamber (Microptic, Barcelona, Spain).

The samples were diluted with saline (0.9% NaCl; Braun, Nuaille, Germany) at a ratio of 1 : 20 (v/v). A 10 µl drop of the diluted semen was placed onto a counting chamber and analysed according to the manufacturer's preset parameters for rabbits. Six microscopic fields were examined for each sample, with the analysis conducted automatically, focusing primarily on total motility, progressive motility, and concentration.

Flow cytometry

Portions of semen were diluted to a concentration of 1 × 10^6 spermatozoa in phosphate-buffered saline (PBS, devoid of calcium and magnesium; Biosera, Nuaille, France) in prepared tubes for flow cytometric assessment of sperm viability and apoptosis. These aliquots were then incubated with selected fluorescent markers. The viability of spermatozoa was determined using SYBR-14 (Garner and Johnson 1995), a green fluorescent dye that permeates cell membranes (LIVE/DEAD® Sperm Viability Kit; Thermo Fisher Scientific, Waltham, MA, USA). Specifically, 1 × 10^6 spermatozoa were incubated with 2.5 µl of SYBR-14 (resulting in a final concentration of 100 nM) for 10 min in darkness at 37 °C. Apoptotic cells were identified using the green nuclear fluorochrome YO-PRO-1 (100 µM; Thermo Fisher Scientific, Waltham, MA, USA).

Samples were incubated at room temperature for 15 min in darkness. To detect dead sperm, SYTOX Green dead cell stain (at a final concentration of 30 nM; Thermo Fisher Scientific, Waltham, MA, USA) was incubated with 1 × 10^6 spermatozoa diluted in 500 µl of PBS for 15 min in darkness at room temperature. Given the high abundance of granules in rabbit seminal plasma, DRAQ5 (BioStatus Limited, Shepshed, UK), a far-red fluorescent DNA stain for live or fixed cells, was used in all samples to identify nucleated cells and exclude seminal debris from the analysis.

Following the incubation, the samples underwent washing (600 × *g*, 20 °C, 5 min), after which the supernatant was carefully removed, and the cells were resuspended in PBS (devoid of calcium and magnesium) for analysis. The prepared samples were analysed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon ion laser and a red diode (635 nm) laser. The data obtained were evaluated using Cell Quest Pro™ software (BD Biosciences,

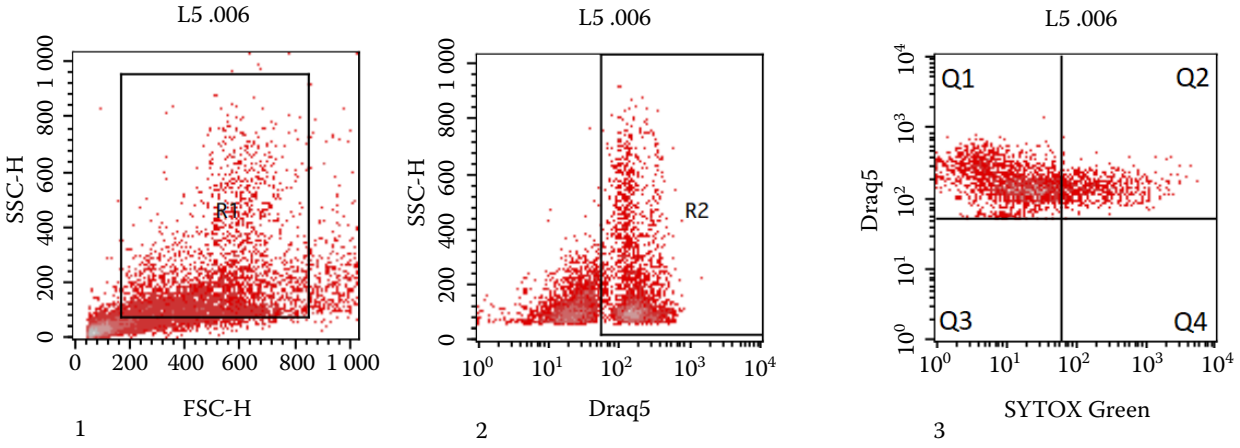

Figure 1. Strategy for evaluating results obtained from flow cytometry

Dot plot 1: Gating the sperm population based on their size and granularity; Dot plot 2: Gating the population of nucleated cells (DRAQ5$^+$); Dot plot 3: Nucleated (Q1 + Q2; DRAQ5$^+$) and dead (Q2; DRAQ5$^+$/SYTOX Green$^+$) cells

FSC-H = forward scatter – height; SSC-H = side scatter – height

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San Jose, CA, USA). Each sample was analysed for 10 000 events at least (sperm cells). Figure 1 depicts flow cytometric dot plots illustrating the strategy for evaluating the analysed samples.

Morphological changes

Following the motility assessment, an aliquot of sperm was placed in the refrigerator and kept until the next day (approximately 24 h) to immobilize the spermatozoa for the purpose of examining their morphological structure. The examination of morphological abnormalities was conducted using the same microscope that was used for CASA. This assessment involved the evaluation of various abnormalities in sperm morphology, such as flagellum detached from the head, flagellum with twists, shortened or broken flagellum, flagellum with cytoplasmic droplets, variations in sperm head size (reduced or enlarged), and other pathological conditions. For our purposes, we mainly focused on the flagellum abnormalities, namely detached tail, coiled tail, twisted end piece, broken tail and cytoplasmic droplet. The number of sperm with morphological changes was compared to the total count of sperm (200), and subsequently, the percentage of specific sperm abnormalities was determined.

Transmission electron microscopy

Transmission electron microscopy was employed to assess the ultrastructural morphology of rabbit sperm. The method was previously outlined by Olexikova et al. (2019). In brief, fresh sperm samples obtained from male rabbits were fixed in a fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 mol/l sodium cacodylate buffer, pH 7.1–7.3) for 1 h at 4 °C. Following the fixation, the sperm underwent three washes in cacodylate buffer for 15 min each. Sperm pellets were then post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h and embedded in 2% agar. The samples were dehydrated through an acetone series, embedded in PolyBed resin (Polysciences), and sectioned into ultrathin sections (70 nm) placed on nickel grids. After contrast staining with uranyl acetate and lead citrate, the samples were examined using a transmission electron microscope (JEM-2100; JEOL, Tokyo, Japan). Electronograms were captured at a magnification of × 10 000 for each group.

Artificial insemination

Samples of ejaculate were used to create a heterospermic batch. These batches were diluted in a commercial insemination medium (Minitube, Tiefenbach, Germany) at a ratio of 1 : 10. The sperm concentration was approx. 50×10^6 spermatozoa per ml.

Hormonal preparation of the females began 48 h before artificial insemination (AI), with each female receiving an injection of 25 IU PMSG (Pregnant mare's serum gonadotropin, Sergon; Bioveta, Ivanovice na Hané, Czech Republic). Each female was inseminated with 0.5 ml of ejaculate using an insemination plastic pipette, simultaneously with an intramuscular injection of 2.5 µg (0.1 ml) of synthetic GnRH (Gonadotropin-releasing hormone, Supergestran; Ferring-Léčiva, Jesenice, Czech Republic).

The number of live births, stillbirths, and weaned offspring was recorded. Conception is defined as the ratio of pregnant females to the total number of inseminated females × 100. Pregnancy checks were performed on day 17 after AI using abdominal palpation. The number of stillborn and live-born offspring was recorded immediately after birth. On day 40 after birth, the number of weaned offspring was recorded.

Statistical analysis

Experiments were performed in four different replicates. The Shapiro-Wilk test was used to assess the normality distribution of the data. Subsequently, the obtained data were analysed by comparing qualitative groups using an unpaired *t*-test with GraphPad Prism v9.0.0 for Windows (GraphPad Software, San Diego, CA, USA). Differences between individual abnormalities were assessed using two-way ANOVA with Tukey's multiple comparisons test. A total of 32 ejaculates from ten males were used in our experiments, with eight females used for artificial insemination.

The data are presented as means ± SEM, and statistical significance was considered at $P < 0.05$.

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RESULTS

Sperm motility evaluation

The rabbit semen samples examined in this study were categorized into two groups based on sperm motility. We observed a significant difference ($P < 0.05$) in motility parameters between the qualitative groups (Figure 2). In terms of concentration, we did not notice any significant differences (approx. 0.5×10^9 spermatozoa per ml in both groups).

Flow cytometry

Sperm viability and apoptosis were evaluated using specific fluorescent markers. Flow cytometric assessment revealed significant differences between qualitative groups in the populations of apoptotic (YO-PRO-1$^+$ cells), live (SYBR-14$^+$ cells) and also dead (SYTOX Green$^+$ cells) sperm cells (Figure 3).

Evaluation of morphological changes

Analysis of morphological changes revealed only slight but not statistically significant differences between qualitative groups (Figure 4). In this analysis, we compared the total number of sperm with flagellar morphological abnormalities. In Figure 5, we can see that cytoplasmic droplets account for the largest proportion of abnormalities. CD represent a significantly higher ($P < 0.05$) proportion of morphological abnormalities in both groups than the other ones.

Transmission electron microscopy

At the ultrastructural level (Figure 6), we observed the occurrence of spermatozoa with a pathological morphology, which fully corresponded to the morphological evaluation using light microscopy. The occurrence of a retained cytoplasmic drop was extremely frequent, especially in the proximal part, the mitochondrial compartment of the flagellum. Similarly, flagellum formation disorders, especially

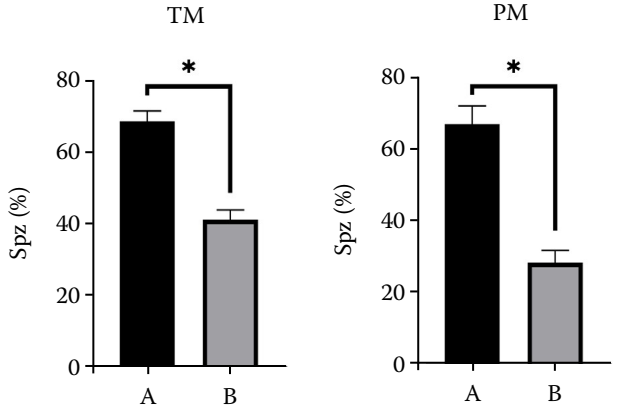

Figure 2. Comparison of total and progressive motility between the qualitative groups

*Significant difference ($P < 0.05$) between the A and B groups. The data are expressed as means ± SEM

PM = progressive motility; Spz = spermatozoa; TM = total motility

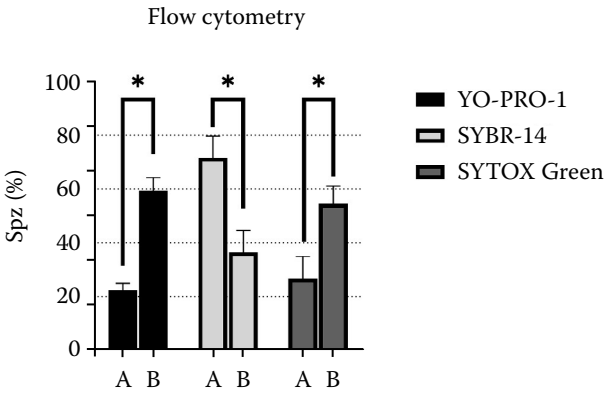

Figure 3. Comparison of biomarkers revealed by flow cytometry between the qualitative groups

*Significant difference ($P < 0.05$) between the A and B groups in the incidence of YO-PRO-1, SYBR-14 and SYTOX Green positive cells. The data are expressed as means ± SEM

Spz = spermatozoa

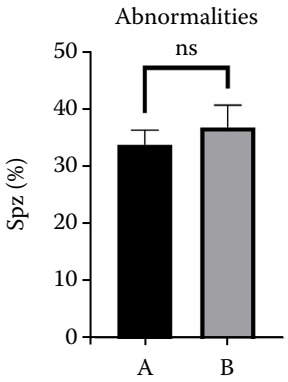

Figure 4. Comparison of morphological abnormalities between the qualitative groups

No significant differences between groups were found. The data are expressed as means ± SEM

Spz = spermatozoa

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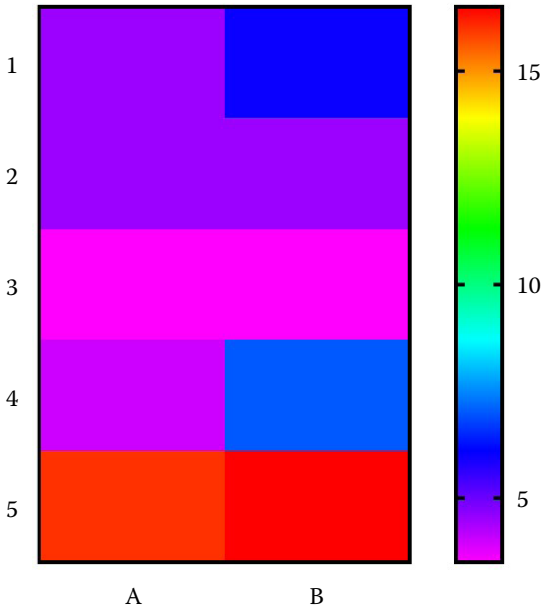

Figure 5. Heatmap of the incidence of individual abnormalities

1 – detached tail, 2 – coiled tail, 3 – knob-twisted tail, 4 – broken tail, 5 – cytoplasmic droplet; The data are expressed as means of individual abnormalities

twisted flagellum or twisted coil in the distal part, occurred frequently.

We also detected the occurrence of other morphological abnormalities, for example a double flagellum (Figure 6C), but also spermatozoa with disrupted plasma membrane, acrosome membranes and loose acrosome mass. The overall microscopic picture indicates a high representation of pathological spermatozoa.

Artificial insemination

The results of artificial insemination (Table 1) did not reflect any differences between groups. It also seems that the increased proportion of morphological abnormalities, namely CD, did not represent a serious problem for the fertility (at least not in the case of artificial insemination) of rabbits, as the number of live births did not differ significantly from the results of artificial insemination using doses without an increased proportion of abnormal sperm (data not shown).

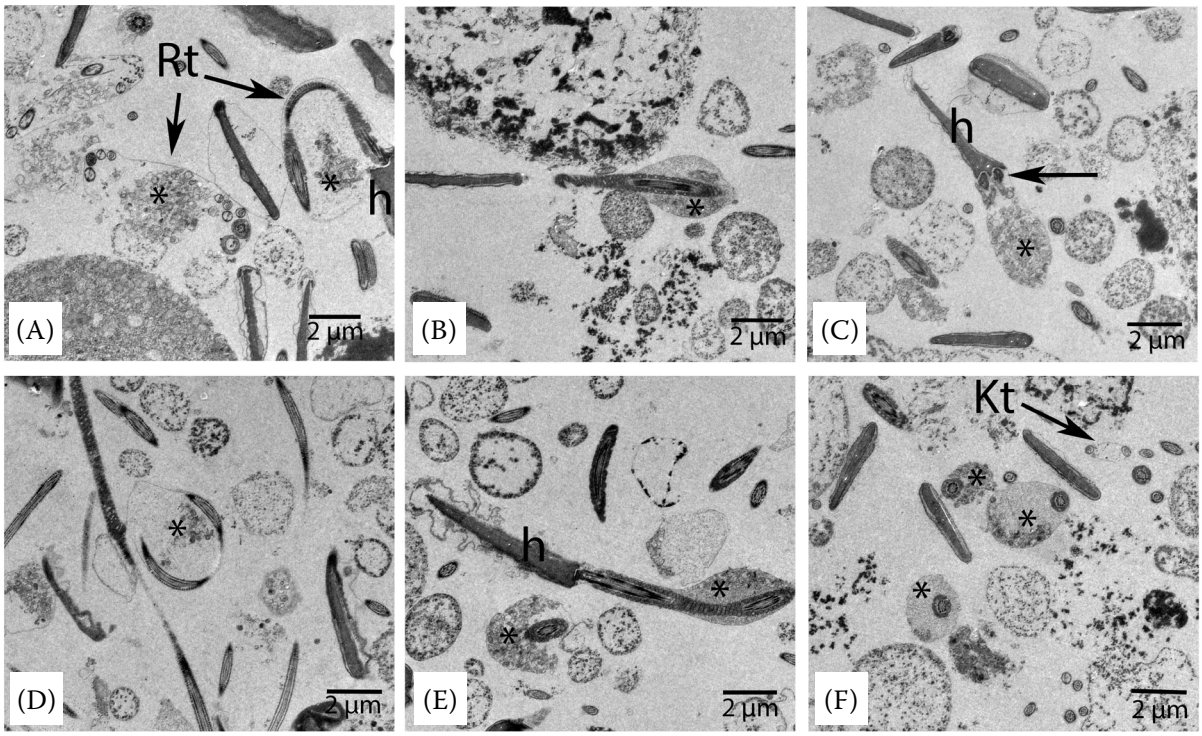

Figure 6. Ultrastructure of rabbit spermatozoa

(A) Coiled tail (Ct) marked with an arrow. Sperm head (h). Remains of cytoplasm from a retained CD (*). (B) Retained CD in the mitochondrial compartment of the flagellum (*). (C) Sperm head (h) with an undulating membrane. Incorrectly formed flagellum – double flagellum (arrow). Retention of cytoplasm (*). (D) coiled flagellum with cytoplasm retention (*). (E) Sperm head (h) with loosened acrosome. Retained CD in the mitochondrial compartment of the flagellum (*). (F) Three transversally cut sperm flagella with a retained cytoplasmic drop (*)

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Table 1. Comparative analysis of live births, stillbirths and weaned offsprings between the qualitative groups

Group	LB	SB	W
A	10.3 ± 1.3	0.3 ± 0.3	10 ± 1.2
B	8.8 ± 1.1	0.5 ± 0.3	8.3 ± 1.3

No significant differences between groups were found. The data are represented as means ± SEM

LB = live births; SB = stillbirths; W = weaned

DISCUSSION

Our research team is actively involved in preserving the biodiversity of livestock through the cryopreservation of sperm cells, oocytes, embryos, and stem cells. It is crucial that the cell samples intended for these purposes are maintained in optimal conditions, considering that cryopreservation is a potentially harmful process. Hence, it is imperative to gather comprehensive information about these samples to assess their ability to fulfil their biological function both in the fresh state and potentially after the process of freezing and thawing. Currently, one of our goals is to create a collection of Zemplin rabbit sperm samples. After sperm collection, we noted a higher incidence of abnormal sperm. As far as we know, there is no such research with this breed from this area, so we could not clearly determine the reason for this phenomenon and its consequences.

Lavara et al. (2005) claimed that motility parameters, determined by the CASA system, in combination with sperm morphology analyses can provide information about the fertilizing potential of rabbit sperm. Significant correlations were observed between kindling rate and the percentage of total motile cells (r = 0.31; P < 0.05) and the percentage of abnormal sperm in the sample (r = −0.32; P < 0.05), but correlations between sperm morphology and fertility can also vary widely (Rodriguez-Martinez 2003). A correlation was noted between the percentage of sperm containing CDs and the linearity index of sperm (r = −0.28; P < 0.05) and there was also a significant negative correlation between kindling rate and the linearity index of sperm (r = −0.32) (Lavara et al. 2005). Elevated occurrences of abnormalities in spermatozoa and abnormal trajectories were observed in semen samples with a high prevalence of CDs in both rabbits (Fausto et al. 2001) and bulls (Amann et al. 2000). A high percentage of abnormal sperm could suggest inadequate epididymal maturation.

CDs were initially identified as bulges of cytoplasm that consistently remain attached to the neck area of spermatozoa subsequently to spermiation (Retzius 1909). Further studies conducted at both the ultrastructural and biochemical levels have suggested that CDs are derived from the Golgi apparatus and endoplasmic reticulum, exhibiting enzymatic properties (Oko et al. 1993). In the last twenty years, research using gene knockout mice has revealed that CDs are involved in the volume regulation, allowing spermatozoa to rapidly adjust to osmotic changes as they travel through the epididymis and later through the female reproductive tract (Cooper 2011). There is an ongoing discussion in the scientific literature (Rengan et al. 2012) regarding the correlation between the presence of CDs and sperm function, whether CDs serve a functional purpose or are merely residual cytoplasmic remnants, and their prevalence across mammalian species. This debate partly arises from the predominant use of epididymal spermatozoa in animal studies to examine CDs, whereas ejaculated spermatozoa are typically used in human studies due to practical limitations in obtaining epididymal samples. Xu et al. (2013) suggested that CD is a normal organelle found in the majority of spermatozoa in the initial segment of the epididymis, with a prevalence of 90% in mice and 85% in monkeys. As spermatozoa progress through the epididymis, CDs appear to migrate from an upper position (neck or mid-piece) to a more distal position (mid-principal junction, principal, and end pieces) in both mice and monkeys. Notably, the presence of CDs is linked to the potential for motility development. Hence, CDs appear to indicate sperm motility and may contribute to the development of sperm motility during epididymal maturation. The absence of CDs in ejaculated spermatozoa, compared to their prevalence in most caudal epididymal spermatozoa, suggests that CDs are shed or released during ejaculation (Cooper 2011).

Discovering a test with a strong correlation to fertility poses a challenge. The perfect sperm assay should assess multiple sperm characteristics concurrently in a substantial number of cells within a sample, gauging the proportion possessing all the necessary attributes for fertilizing an oocyte (Graham 1996). Flow cytometry offers the capability to assess various sperm attributes simultaneously in tens of thousands of sperm cells

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(Vasicek et al. 2022). The viability of spermatozoa in this study was evaluated by the SYBR-14 probe, which is most widely used in combination with PI or 7-AAD (Martinez-Pastor et al. 2010). However, in our sample analysis, a new far-red nucleated cell dye, DRAQ5, was incorporated in the flow cytometry process to effectively eliminate the common issue of seminal plasma debris found in rabbit semen. On the other hand, SYTOX Green, a green dead cell dye, was used in combination with DRAQ5 to identify dead cells. This dye has also been previously reported to be useful for sperm analysis either alone, or in combination with other specific probes like DRAQ5, Annexin V or DHE (De Iuliis et al. 2006). To measure apoptotic alterations in the samples, YO-PRO-1 has been utilized. Recent studies have emphasized its capacity to identify changes in apoptotic cells in frozen-thawed spermatozoa under various *in vitro* capacitation conditions (Peris-Frau et al. 2021) or in rams with differing levels of fertility (Ledesma et al. 2016).

On the other hand, to identify differences between semen samples, a relatively low number of sperm per insemination is required. However, to optimize fertility rates, the quantity of sperm in each insemination significantly surpasses the number needed to achieve the highest attainable fertility for most males. Therefore, the fertilizing potential of individual sperm in most samples is masked by the excess sperm inseminated in different species like in rabbits (Farrell et al. 1993) and in cattle (Shannon and Vishwanath 1995). This may be one of the reasons why we did not see any significant differences in AI outputs between qualitative groups.

CONCLUSION

In an effort to create a collection of the Zemplin rabbit breed sperm samples, our initial analysis revealed a significant percentage of flagellar morphological defects. This prompted a multifaceted investigation using the computer-assisted sperm analysis, flow cytometry, evaluation of morphological abnormalities by light and transmission electron microscopy, and artificial insemination. We tried to uncover the possible causes and consequences of the observed abnormalities. The occurrence of CDs in ejaculated semen may have genetic reasons. The small population size of Zemplin rabbits could cause inbreeding depression, or it is a natural phenomenon of the breed due to its genetic background. Further research is needed to confirm this claim. Despite the differences in the quality of the individual groups and the high number of abnormal spermatozoa, we achieved satisfactory results of artificial insemination, probably due to a significant excess of the number of spermatozoa required for successful fertilisation.

Conflict of interest

The authors declare no conflict of interest.

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