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APPLICATION OF MULTIVARIATE ANALYSIS: TOWARD IMPROVEMENT OF ROOSTER SEMEN QUALITY ASSESSMENT

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ABSTRACT

Sperm cryopreservation is an important tool for storage of genetic material of animal species. Therefore, the aim of this study was to assess the association between extender and cryoprotective agents (CPAs) used in the term of sperm quality parameters. In our study, the comparison of two extenders (Kobidil$^+$ (K^+) and saline (S)) and their combinations with CPAs such as dimethyl sulfoxide (DMSO) or glycerol (GL) on the sperm motility and viability was analyzed. For detailed assessment of association between extenders with CPAs and sperm quality parameters of frozen/thawed semen, multivariate analysis was used. The obtained data showed that the both extenders with specific CPAs significantly affect the motility as well as the viability parameters. GL/K^+ (p-value=0.0168) had effect on the progressive motility, whereas GL/S (p-value=0.0174) influenced the total motility and velocity curved line parameters. Moreover, DMSO/K^+ (p-value=0.0346) had higher effect on the proportion of dead and apoptotic sperm compared to DMSO/S (p-value=0.036), which had higher impact on the proportion of acrosome damaged sperm. Future studies might benefit from these findings to establish the associations of these subpopulations with other rooster sperm quality parameters and thus improve the sperm quality. Association between the values can help us to better understand the effect of extenders and CPAs on the semen quality parameters.

Keywords: semen; extender; CPAs; motility; viability; multivariate analysis

INTRODUCTION

Previous studies have demonstrated that cryopreservation is the only effective method of storing avian sperm *ex situ* in an animal gene bank (Kowalczyk and Łukaszewicz, 2015; Iaffaldano et al., 2016 a). Following international agreements on animal biodiversity, there is a need to improve and standardize *in vitro* cryopreservation methods for genetic material. In the case of birds, *ex situ* sperm storage programs are currently being developed due to the failure of bird oocyte storage (Blesbois, 2007). Despite the wealth of research on sperm cryopreservation (Mphaphathi et al., 2012, Zaniboni et al., 2014; Mosca et al., 2016), the available methodologies are not sufficiently optimized due to induced sperm damage in the freezing process. As a result, the fertilization capacity of frozen sperm is highly variable and not sufficiently reliable for commercial purposes as well as for the use of sperm as a source of gene reserve (Long, 2006; Kowalczyk and Łukaszewicz, 2015; Mphaphathi et al., 2016). However, the efficiency of cryopreservation of the biological material of some animal species has not yet been fully achieved. The result is, for example, a reduced survival or reduced quality of thawed sperm.
The subphysiological temperatures can lead to arrest the cellular metabolic activities for a successful cryopreservation. However, this process can cause cell damage, such as intracellular ice formation, freezing injuries, and osmotic stress resulting in insufficient cell viability. Therefore, the extender and cryoprotective agents (CPAs) are essential components of cryo-medium for cell treatment before cryopreservation to keep high freezed/thawed (F/T) sperm recovery (Sexton, 1977; Bakst et al., 1979; Tselutin et al., 1999; Bailey et al., 2003; Abouelezz et al., 2015; Iaffaldano et al., 2016a, b).
For the assessment of the semen quality one- and two-way analysis of variance (ANOVA) is often used (Zaniboni et al., 2014; Abouelezz et al., 2015; Mphaphathi et al., 2016; Miranda et al., 2018), because the obtained data are independent. Due to the variation among populations, each extracted attribute is a different reflection of these few sources of variation. Therefore, the multivariate assay improves and highlights the main sources of variance in the collected data (Anderson, 1962). Interpretation of data variation is much easier and enables for better understanding of the interactions in biology, what is only possible by using the multivariate analysis.
Spermatozoa quality is essential for the maintenance of fertility. Extenders, concentration of CPAs, equilibrium time, freezing rate, and thawing temperature have been reported to influence the cryopreservation of rooster sperm. Due to the standardization and elimination of variability in the freezing process, the goal of our study was to evaluate the effect of two types of extenders on the motility parameters as well as on the proportion of apoptotic, dead and acrosome damaged sperm by multivariate analysis.

MATERIAL AND METHODS

Animals

Six mature clinically healthy males of Oravka line (12-18 months) reared in a breeding facility (NAFC – RIAP Nitra, Slovakia) were used in this experiment. All roosters were housed in separated cages, maintained under an artificial photoperiod (14 h of light at 10 lux and 10 h of dark) and were fed with a commercial standard diet (TEKRO Nitra, s.r.o., Slovakia) and watered *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 Slovak Animal Protection Regulation, RD 377/12 guidelines, which meet the requirements of the European Union Regulation 2010/63. Semen collection was performed by dorso-abdominal massage two times per week into prepared sterile tubes during 2 months (from March to May). Contaminated samples were excluded from the experiment and the minimum (60%) of sperm total motility was used for subsequent analysis.

Design of the experiment

The mixed suspension of sperm (heterospermia) from semen was prepared to eliminate the impact of individuals. Computer assisted sperm analysis (CASA) was used to analyze concentration and movement parameters of fresh (control) semen samples. The semen was then diluted (1:1; v/v) in commercial *Kobidil*$^+$ (group K^+) extender (Landata Cobiporc, France) and saline (group S, sodium chloride 0.9%,

B. Braun Medical Ltd, Bratislava, Slovak Republic) at room temperature (RT) and cooled down to 5 °C for 30 min and immediately again evaluate by CASA. The equilibrated semen suspensions, K^+ as well as S, were subdivided into 6 experimental groups according to the CPAs used as follows: dimethyl sulfoxide (DMSO/K^+), ethylene glycol (EG/K^+), glycerol (GL/K^+), and DMSO/S, EG/S, and GL/S, respectively. Afterwards, the freezing medium contained K^+/S and CPAs: DMSO (Sigma-Aldrich, Germany), EG (Sigma-Aldrich, Germany) or GL (Sigma-Aldrich, Germany) were added into the samples at the ratio of 1:1 (v/v) to give a final concentration of 8% and kept at 5°C for 45 min. Afterwards, the semen was loaded into 0.25 ml plastic straws during the equilibration time. The straws were exposed to liquid nitrogen vapours for 15 min (–125 to –130°C), then plunged into the liquid phase (–196°C) for storage. After 2–3 days, the straws were thawed at +5°C for 2 min and analyzed by CASA system and flow cytometry.

Computer assisted sperm analysis (CASA)

The fresh samples were diluted in K^+/S at the ratio of 1:100 (v/v). The sperm motility parameters were analyzed using the CASA system. An aliquot (2.5 µl) of this solution was placed on a Leja Standard Count Analysis Chamber (depth of 20 microns; MiniTüb, Tiefenbach, Germany) and evaluated using the CASA software under a Zeiss Axio Scope A1 microscope (Sperm Vision™; MiniTüb, Tiefenbach, Germany). For each sample, seven microscopic view fields were analyzed. Total motile spermatozoa (TM, motility > 5 µm/s), percentage of progressive moving spermatozoa (PM, motility > 20 µm/s), VCL (velocity curved line, µm/s), VSL (velocity straight line, µm/s), STR (straightness - VSL:VAP, velocity average path), LIN (linearity - VSL:VCL), BCF (beat cross frequency, Hz) were analyzed.

Flow cytometry analysis

Flow cytometric assessment represented the evaluation of apoptotic, dead and acrosome damaged sperm, as follows. Fluorochrome Yo-Pro-1 (Molecular Probes, Switzerland) for detection of apoptotic sperm and propidium iodide (PI) (Molecular Probes, Switzerland) for determination of dead sperm were used. The acrosome integrity using a fluorescein-labelled lectin from peanut agglutinin (PNA Alexa Fluor 488, Molecular Probes, Lucerne, Switzerland) was detected. Briefly, the sperm concentration was adjusted to $1x10^6$ cells/ml. One microliter of the Yo-Pro-1 solution (100 µmol/l) were added to 500 µl of the cell suspension to determine portion of apoptotic spermatozoa. Two microliters of PNA (0.5mg/ml) were added to 500 µl of the cell suspension to assess acrosome integrity. Samples were incubated in the dark at RT for 15 min. After incubation samples were washed in phosphate-buffered saline (PBS; Life Technologies, Slovak Republic), centrifuged at 600 x g for 5 min and the supernatant was removed. To determine number of dead sperm, 4 µl of the PI (50 µg/ml) were added to 500 µl of cell suspension. To analyze the overall sperm parameters FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) was used to measure at least 10,000 of sperm (events) per each sample.

Statistical analyses

Statistically significant differences between extenders were assessed using the paired t-test. The data were analyzed by the SigmaPlot software (Systat software Inc. Germany). Moreover, multivariate redundancy analysis (RDA) was used to determine the dependencies between objects (extender, CPA, motion and viability parameters) using the Monte Carlo permutation test in the Canoco program5 (ter Braak and Smilauer, 2012).

Results

Computer assisted sperm analysis (CASA)

In order to determine the effect of both extenders on the fresh sperm quality, CASA analysis was performed. Differences between the extenders were analyzed by the paired t-test. We observed the significant differences (P<0.05) between extenders in the parameters TM, PM, and VCL (Table 1).

Table 1 CASA analysis of fresh sperm diluted in different extenders equilibrated 0.5h at 5°C

Parameters	Control	Kobidil$^+$	Saline
TM (%)	72.63±2.14^a	72.24±1.64^a	47.80±5.76^b
PM (%)	56.20±2.17^a	50.24±3.62^a	24.67±5.93^b
VCL (µm/s)	77.02±5.30 ^{a}	81.17±3.10^a	63.45±6.75^b
VSL (µm/s)	26.37±0.85	25.01±2.54	21.14±1.42
STR (VSL:VAP)	0.57±0.02	0.60±0.03	0.55±0.01
LIN (VSL/VCL)	0.34±0.01	0.31±0.04	0.34±0.02
BCF (Hz)	28.05±0.63	32.36±2.29	25.88±1.12

Values are presented as least squares means ±SD. Different superscripts between experimental groups are significantly different (P≤0.05) ^{a}versus^b.

However, for comprehensive assessment, multivariate analysis of the F/T sperm parameters by redundancy analysis (RDA, SD=1.10 on the first ordination axis) was used. The values of the explained variability of data were 52% on the first ordination axis and 72.8% on the second ordination axis. Using the Monte Carlo permutation test, we identified a statistically significant effect between extenders K (p-value=0.0168), S (p-value=0.0174) and GL group. The selected variables were not mutually correlated with the maximum value of the inflation factor=1.8254. The ordination graph ordained into 3 clusters (triplot) (Figure 1).

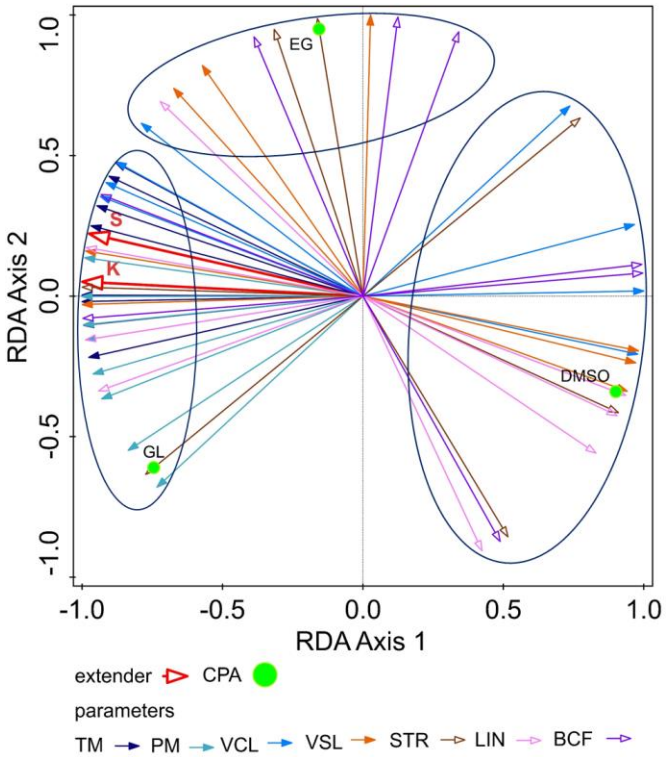

Figure 1 RDA plot of motility parameters in each cluster for each cryo-medium

According to the subpopulation dynamics, Figure 1 displays the distribution of individual extenders in combination with cryo-media in association to motility parameters. We detected high association between GL and both extenders. Moreover, Figure 1 demonstrates which parameter was influenced by which extender in combination with CPAs. GL/K^+ had effect on the PM, whereas GL/S influenced the TM and VCL parameters.

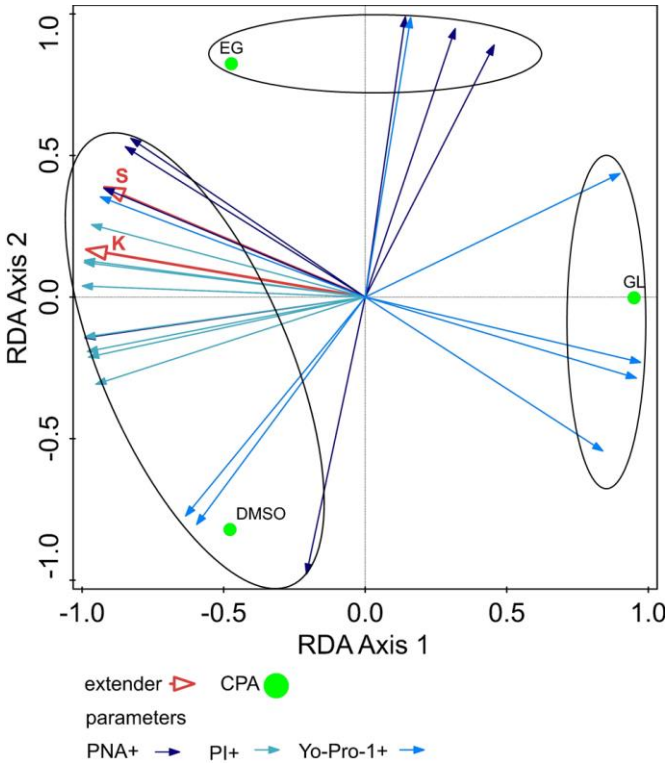

Figure 2 RDA plot of apoptotic, dead and acrosome damaged sperm proportion in each cluster for each cryo-medium

Flow cytometry analysis

Viability staining of sperm is one of the important tool for their quality assessment. Multivariate analysis of the proportion of dead, apoptotic and acrosome damaged sperm between the various extenders and CPAs used was determined by redundancy analysis (RDA, SD=1.25 on the first ordination axis). The values of the explained variability of data were 62.9% on the first ordination axis and 69.7% on the second ordination axis. The cumulative variability of the data set explained were represented in the first ordination axis 92.1% and in the 2nd axis 98.1%. Using the Monte Carlo permutation test, we identified a statistically significant effect between extenders K^+ (p-value=0.0346), S (p-value=0.036) and DMSO group. The selected variables were not mutually correlated with the maximum value of the inflation factor=1.9424.

In our study, we obtained three clusters, potentially representing sperm subpopulations. The subpopulation pattern was strongly preserved, even though, there were noticeable changes between the two extenders used. Figure 2 demonstrates a separation between individual extenders in combination with cryo-media in association to proportion of apoptotic, dead and acrosome damaged sperm. We detected the high association between DMSO and both extenders. DMSO/K^+ had the higher effect on the proportion of dead and apoptotic sperm compared to DMSO/S, which had higher impact to acrosome damaged sperm.

DISCUSSION

Cryopreservation significantly affects the number of viable sperm (Mocé et al., 2010). We tested different diluents in combination with permeable CPAs and their effect on motility parameters and sperm viability. There are differences between poultry species (broiler type, brood type) in viability and functional parameters of sperm after cryopreservation (Lake, 1960). Therefore, the analyses of sperm quality parameters using various extenders in association with CPAs as well as data evaluation are required.

Determination of sperm motility is one of the main indicators of the quality of the collected ejaculate, which, in our study, was analyzed by the CASA method. The method provides detailed evaluation of the basic sperm parameters to achieve objective results with sufficient repeatability (Amann and Waberski, 2013). The type of a suitable equilibration and freezing medium is an important part of freezing process to maintain the sperm functions. The storage of sperm during *in vitro* manipulations has a significant effect on motility as well as overall viability. Cooling the semen to + 5 ° C prolongs viability and maintains overall motility. In the case of rooster sperm, the lowest motility at 41°C was observed, however sperm suitable environment to maintain their viability is cooling to + 5°C. Moreover, extenders are substances for prolongation of sperm function thought the *in vitro* manipulations as well as artificial insemination (AI) (Foote, 1968; Graham 1978; Salisbury et al., 1978). For short-term (1 - 3 days) and long-term storage (4 <) extenders are used as a source of nutrients needed for metabolic activity of sperm (glucose), maintaining a constant pH (Tris, Hepes), osmotic pressure (NaCl, KCl), as well as inhibit the growth of microorganisms (antibiotics) (Gadea, 2003). Mphaphathi et al. (2016) used Kobidil $^+$ extender to dilute rooster sperm in the freezing process. However, Vasicek et al. (2015) compared two different diluents, saline and commercially available avian diluent with focus to the total motility in the given samples. They observed better effect for saline extender. However, other types of extenders, such as Nabi, Beltsville, Lake (Amini et al., 2015; Nabi et al., 2016; Fattah et al., 2017; Masoudi et al., 2019) for their energy supply, protection against thermal damage, reduction to physical stress are also applied for rooster semen freezing.

The present study aimed to contribute to sperm cryopreservation knowledge by the evaluation of rooster semen after cryopreservation using various combination of extenders and CPAs as well as the type of statistical method used. Based on our results, significant differences between used extenders were observed (Table 1). The lower values of TM and PM in saline could be the result of insufficient nutrients needed to maintain their viability. CPAs are one of the most important factors influencing sperm quality. CPAs such as DMA, DMSO, DMF, MA and EG, GL (Sasaki et al., 2010; Hanzawa et al., 2010; Mphaphathi et al., 2012; Mphaphathi et al., 2016; Masoudi et al., 2019) are permeable molecules used in the rooster semen cryopreservation (Purdy, 2009). However, due to the different conditions of the experiments, the results reported in the literature are very variable. We detected the highest association between GL and both extenders compared to DMSO and EG. Moreover, RDA analysis showed us which parameter was influenced by which extender in combination with CPAs. GL/K^+ had effect on the PM, however, GL/S influenced the TM and VCL parameters. This is important for the appropriate choice of extender to maintain high quality semen before and after semen cryopreservation. RDA analysis can help us to better understand the association between CPAs and extenders and can also be used for suitable choice for some supplements such as antioxidants or extender components to improved semen quality.

The high content of polyunsaturated fatty acids (PUFA) in the avian sperm membrane (Santiago-Moreno et al., 2012) is one of the main reasons for the dramatic reduction in the fertility of frozen sperm. This trend is attributed to the higher susceptibility to damage of rooster sperm compared to other species (Long, 2006; Hu et al., 2006). The integrity of sperm membranes can be affect by freezing and thawing resulted in the structural and substructural changes (Parks and Lynch, 1992). Fluorescence microscopy and flow cytometry are often used methods to assess sperm viability. According to Christensen et al. (2004, 2005) the flow cytometry seems to be more suitable than fluorescence microscopy due to the higher sensitivity. In previous studies, flow cytometry to evaluate specific parameters such as viability, apoptosis, mitochondrial membrane potential, lipid peroxidation, acrosomal status, ROS generation or DNA damage (Martinéz-Pastor et al., 2010; Shahverdi et al., 2015; Kulikova et al., 2015; Kuzelova et al., 2015) was used.

Several dyes are available to assess sperm viability, which can be used alone or in combination with other dyes for evaluation of different functional sperm properties. Live sperm can be distinguished by PI staining, an impermeable DNA-specific dye, alone or in combination with the membrane permeable dye SYBR 14 (Garner et al., 1995) as well as acrosome damage can be assess by plant lectins (Nagy et al., 2003). Apoptotic sperm can be detected by Yo-Pro-1 nuclear fluorochrome (Martin et al., 2004) because during this process sperm membrane becomes slightly permeable, while remaining impermeable to PI. Therefore, the Yo-Pro-1 and PI co-staining provides a sensitive detection for apoptosis (Idziorek et al., 1995). Moreover, PI offers one access to determine dead cells based on the membrane permeability (Vermes et al., 1995, 2000). Cells with intact plasma membranes exclude PI, however, during the late apoptosis membrane permeability increases and stains the sperm. Due to various fluorochromes, extenders and CPAs used, the suitable method for sperm quality dataset assessment is required.

It is known, that multivariate analysis for sperm quality assessment was already used (Abaigar et al., 2001; Beirão et al., 2009; Babamoradi et al., 2015). Permutations were used to obtain p-values for main effects (Zwanenburg et al., 2011). Our results obtained from multivariate analysis demonstrated a differences between individual extenders in combination with cryo-media in association to proportion of apoptotic, dead and acrosome damaged sperm. We detected the highest association between DMSO and both extenders in terms of sperm viability. DMSO/K^+ had the higher effect on the proportion of dead and apoptotic sperm compared to DMSO/S, which had the higher impact on the acrosome integrity. This information can help us in choosing a suitable extender and CPAs resulting in improvement of sperm quality. We can conclude that sperm subpopulations with distinct characteristics can be successfully identified by using a multivariate clustering analysis. The utility and biological importance of the identified subpopulations must be confirmed by further experimentation.

CONCLUSION

This study provides a detailed data analyzing method of sperm quality parameters. The analysis includes a clustering method for efficient identification of sperm populations. A multivariate data analysis to examine the effect of extender in association with CPAs type on the quality of rooster sperm was applied. The applicability of this methodology was demonstrated using CASA and flow cytometry datasets of sperm function. The results demonstrated that the proper statistic assay can effectively detect systematic variations based on extender and CPAs used. Our results provide the information how to measure sperm quality parameters in a more objective and interpretable manner, which can be used for sperm quality assessment for artificial insemination (AI) as well as genetic material storage in animal gene bank purposes.

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