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QUALITY EVALUATION OF FRESH GANDER SEMEN OF SLOVAK WHITE GOOSE BY CASA AND FLOW CYTOMETRY: SHORT COMMUNICATION

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ABSTRACT

In this preliminary study, the quality of fresh gander semen from the original Slovak White goose (SW) breed was analysed. Semen was collected from three (SW, n = 3) ganders into prepared sterile tubes by dorso-abdominal massage. Firstly, volume of individual gander semen was determined. Afterward, the concentration, motility parameters and sperm viability were evaluated using computer assisted semen analyser (CASA) and flow cytometry, respectively. Our results showed that the volume of individual semen samples varied from 0.05 to 0.38 ml. No significant differences in concentration and total motility of fresh semen was found among the ganders tested. However, significant differences ($P \leq 0.05$) in progressive movement of fresh semen between two males (SW2 and SW3) were observed. Moreover, differences ($P \leq 0.05$) in the percentage of apoptotic and necrotic sperm between two males (SW1 and SW3) were revealed by a flow cytometry. These preliminary data suggest that the objective assessment of fresh gander sperm motility may be an effective indicator of frozen-thawed semen quality. Therefore, regular semen assessment is required in order to preserve good-quality insemination doses from native breeds.

Key words: gander; semen; motility; CASA; flow cytometry

INTRODUCTION

Semen quality is an important factor affecting cryopreservation and fertilizing ability. Compared to mammalian or chicken semen, the data of gander semen characterisation are limited. Previous studies have demonstrated significant differences in semen characterisation among ganders (Łukaszewicz, 2002; Łukaszewicz and Kruszynski, 2003). Moreover, ganders produce a small volume of semen (0.05–1.0 ml) with a low sperm concentration (0.03– 0.8 x 10^9.ml^{-1}) and a low number of live normal cells (10–60 %). Sustained fertility in the avian female depends on its ability to store adequate viable sperm in straws and to supply the infundibulum with sufficient numbers of sperm to fertilize an ova. Only morphologically normal and vital sperm are capable of ascending through the vagina of a goose and fertilize it (Bakst *et al.*, 1994). Sperm vitality and viability are the primary determinants of fertility in domestic species (Froman and Feltmann, 1998, 2000; Froman *et al.*, 2003).

The semen analyses, essential for the study of gander quality, generally includes the evaluation of sperm motility and viability. There are many methods of assessing semen quality and estimating the fertilising potential of sperm. Common methods of semen evaluation have involved determination of the percentage of motile sperm (on a pre-warmed

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glass slide), the sperm morphology (with various staining techniques) and the concentration in using counting chamber. However, conventional semen assessment is being increasingly replaced by fluorescent staining techniques, computer assisted semen analysis (CASA) or flow cytometry.

Moreover, standard techniques of sperm viability analysis are fluorescence microscopy or flow cytometry using fluorescent staining probes. The flow cytometry technique is an automatic system able to provides precise assessment of sperm quality (Petrunkina and Harrison, 2007) because of its high sensitivity, repeatability (Christensen *et al.*, 2004; 2005) and determination of a large number (10,000) of sperm in a short period of time (Rijsselaere *et al.*, 2005). Several stains are available for evaluating cell viability and can be used alone or in combination with other dyes for the assessment of sperm quality.

In the present study, quality evaluation of Slovak White goose semen was done using CASA and flow cytometry methods. (DRAQ5, Yo-Pro-1, Sytox Green).

MATERIAL AND METHODS

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines on the care and use of laboratory animals. The treatment of the animals was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic, no. SK P 28004 and Ro 1488/06-221/3a. The experiments were carried out in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments.

Animals

Three clinically healthy males of breeds Slovak White goose breed (individuals marked as SW1, SW2, SW3) from 1 to 11 years, reared in a private breeding facility, were used in this experiments. All ganders were maintained in the flock and fed with wheat and oats and water given *ad libitum*.

Experimental design

Semen was collected from sexually mature gander males by dorso-abdominal massage into prepared sterile tubes twice a week in a regular manner. Samples contained urine and cell debris were removed. An aliquot taken from an individual gander semen was used for motility analysis immediately after collection. The fresh semen was diluted at the ratio of 1:4 (v:v) in a saline (sodium chloride 0.9%, B. Braun Medical Ltd. Bratislava, Slovak Republic). A part of this solution (3.4 µl) was placed on a pre-heat Standard Count Analysis Chamber Leja (depth of 20 µm) (MiniTüb, Tiefenbach, Germany) and evaluated using the CASA software under a Zeiss Axio Scope A1 microscope (Sperm Vision™; MiniTüb). Seven microscopic view fields were analysed for each sample and concentration (CON), percentage of total motile sperm (TM; motility > 5 $\mu m.s^{-1}$) and percentage of progressive motile sperm (PM; motility > 20 $\mu m.s^{-1}$) were assessed in each sample.

To assess a viability of the frozen–thawed sperm, each sample was stained fluorescently with DRAQ5 (nucleated cells) in co-stained with Yo-Pro-1 (apoptotic sperm) and Sytox Green (necrotic sperm) dyes. Samples were washed and centrifuged in a $PBS^{(-)}$ (Sigma-Aldrich, Germany) at 600 x g for 5 min and subdivided into three tubes for subsequent flow cytometric assessment of DRAQ 5-positive, DRAQ 5-positive apoptotic and DRAQ 5- positive necrotic sperm, as described below. The detection of apoptotic and necrotic sperm was performed using the specific nuclear fluorochrome Yo-Pro-1 (Molecular Probes, Switzerland) and specific nuclear fluorochome Sytox Green (Molecular Probes, Switzerland) in combination with the DRAQ5 nuclear dye (Biolegend, Germany) in order to detect only the sperm from seminal plasma.

One microliter of the Yo-Pro-1 solution (100 $\mu mol.l^{-1}$) and 0.1µl of DRAQ5 (5mM) were added to 500µl of the cell suspension to determine a proportion of apoptotic sperm. Samples were mixed and incubated in the dark at room temperature for 15 min. After incubation samples were washed in $PBS^{(-)}$ and centrifuged at 600 x g for 5 min; the supernatant was discarded. To detect portion of the necrotic sperm, firstly 0.1 µl of DRAQ5 were added to the cell suspension and incubated 15 min in the dark at room temperature. Afterwards, samples were

washed in PBS$^{(-)}$ and centrifuged at 600 x g for 5 min; the supernatant was discarded and 2.5 µl of SYTOX Green (30µM) were added to 500µl to the cell suspension and incubated for 15 min in the dark at room temperature. After the second incubation a flow cytometry assay was performed.

At least, 10,000 events were analysed for each sample. The emitted green fluorescence of YO-PRO-1, Sytox green-positive cells and the red fluorescence from DRAQ5-positive cells were recorded in the FL-1 and FL-3 channels, respectively. The different labelling patterns in bivariate analysis (e.g. Yo-Pro-1/Sytox Green) identified different sperm populations: single-positive nucleated cells (DRAQ5$^+$); double-positive apoptotic sperm (DRAQ5$^+$/Yo-Pro-1$^+$) and double-positive necrotic (DRAQ5$^+$/Sytox Green$^+$) cells.

Statistical analysis

Sperm quality among the individuals was statistically evaluated by a Tukey test using a Sigma Plot Software (Systat Software Inc., Germany). Differences at $P \leq 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

The CASA analysis and flow cytometry using fluorescence probe are commonly used for assessment of more detailed sperm characteristics in avian semen.

The present study describes the use of this technique for gander semen assessment. The quantity and quality of fresh semen depends on individual gander features as was reported in other species (Łukaszewicz *et al.*, 2002; 2004; Waberski *et al.*, 2011).

In our study, the quality parameters of fresh Slovak white gander semen were analysed. The volume of individual semen samples varied from 0.05 to 0.38 ml. No significant differences in CON and TM of fresh semen were found among the ganders tested. However, significant differences in PM of fresh semen between two males (SW2 and SW3) were observed (Table 1).

Compared with males from other poultry species such as chickens, ducks or turkeys, ganders have a relatively limited testicular development in adulthood, which results in fewer sperm produced per unit time than in other species (Łukaszewicz *et al.*, 2002). Moreover, this phenomenon may affect the reduction in motility parameters of individual ganders.

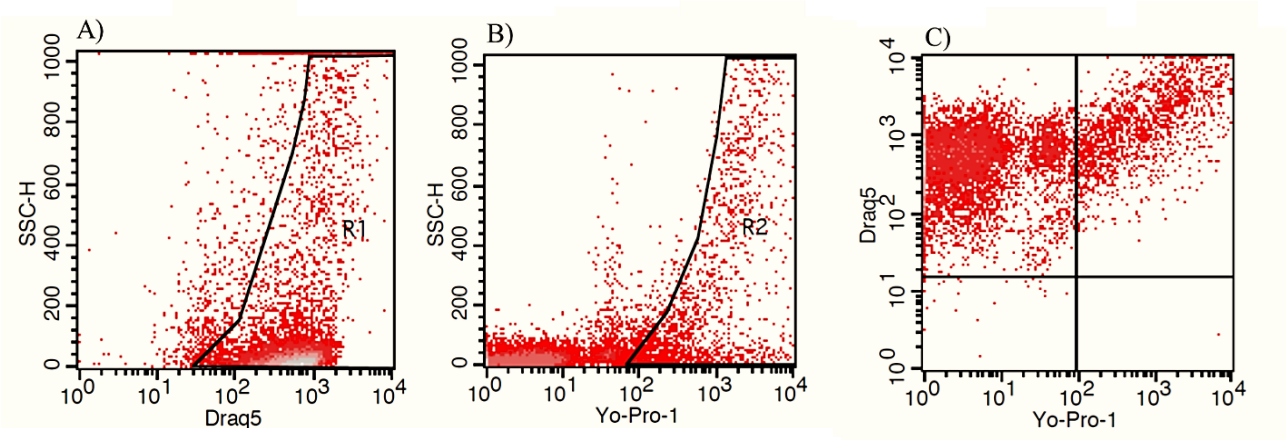

Figure 1. Representative dot plots of a flow cytometric examination of gander sperm A) Region R1 represents nuclear cells (DRAQ5$^+$) B) Region R2 represents apoptotic cells (Yo-Pro-1$^+$) C) double-positive nucleated apoptotic cells (DRAQ5+ and Yo-Pro-1$^+$). Markers of necrosis were evaluated by the same method

Table 1. The fresh gander semen characteristic

Gander	SW1	SW2	SW3
Vol (ml)	0.18 ± 0.71	0.16 ± 0.42	0.31 ± 0.23
CON (x10^9)	1.762 ± 0.68	0.96 ± 0.26	1.7 ± 0.43
TM (%)	55.37 ± 2.57	70.01 ± 3.82	55.86 ± 6.04
PM (%)	33.59 ± 3.06	49.38 ± 4.92^a	20.08 ± 0.93^b

Different superscripts indicate significant differences; ^{a} vs ^{b}, $P < 0.05$
VOL – volume; CON – concentration; TM – total motility; PM – progressive movement

We applied flow cytometry to examine proportion of apoptotic and necrotic sperm. Many authors used flow cytometry protocols to analyse specific parameters of sperm quality such as viability, apoptosis, acrosomal status, capacitation, mitochondrial membrane potential, lipid peroxidation, reactive oxygen species generation (ROS) or DNA damage (Martínez-Pastor *et al.*, 2010).

In our study the quality of the fresh Slovak white gander semen was not excellent (Table 2). We found 68.16 % of live sperm, which was lower than that obtained by Gee and Sexton (1990), who reported 92.9 % of live sperm cells in Aleutian Canada goose (*Branta canadensis leucopareia*) on eosin-nigrosine-stained slides. Other authors also showed a higher percentage of live sperm: in White Italian (*Anser anser*) gander semen – 92.2 % (Łukaszewicz, 2002), in Chinese Brown Geese – 83 % (Tai *et al.*, 2001) and in Greylag ganders – from 90.3 to 93.3 % (Łukaszewicz *et al.*, 2004).

It can be concluded that this study provides the first characteristics of Slovak white goose semen quality. Basing on the results from CASA and flow cytometry our findings revealed differences between individuals. Therefore, we suggest that the objective assessment of fresh gander sperm quality may be an effective indicator of the fertility of fresh and frozen–thawed semen from individual males. Consequently, regular semen assessment is required in order to preserve good-quality insemination doses collected from native breeds.

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Table 2. The fresh gander sperm viability

Gander	SW1	SW2	SW3
DRAQ5$^+$ (nucleated)	92.96 ± 0.41	91.25 ± 1.46	79.33 ± 3.72
DRAQ$^+$/Yo-Pro-1$^-$/ Sytox$^-$ (live)	68.16 ± 2.98	58.73 ± 3.19	25.34 ± 7.73
DRAQ$^+$/Yo-Pro-1$^+$ (apoptotic)	17.5 ± 1.35^a	21.77 ± 1.85	37.19 ± 1.43^b
DRAQ$^+$/Sytox$^+$ (necrotic)	14.34 ± 1.63^a	19.50 ± 1.34	37.46 ± 6.30^b

Different superscripts indicate significant differences; ^{a} vs ^{b}, $P < 0.05$

REFERENCES

Bakst, M. R., Wishart, G. & Brillard, J. P. 1994. Oviductal spermatozoa selection, transport, and storage in poultry. *Poultry Science Review*, 5, 17–143.

Christensen, P., Hansen, C., Liboriussen, T. & Lehn-Jensen, H. 2005. Implementation of flow cytometry for quality control in four danish bull studs. *Animal Reproduction Science*, 85, 201–208.

Christensen, P., Stenvang, J. P. & Godfrey, W. 2004. A flow cytometric method for rapid determination of sperm concentration and viability in mammalian and avian semen. *Journal of Andrology*, 25(2), 255–264.

European Commission. *Legislation for the protection of animals used for scientific purposes. Horizontal legislation on the protection of animals used for scientific purpose*. 2016. Available at: http://http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm

Froman, D. P. & Feltmann, A. J. 2000. Sperm mobility: Phenotype in roosters (*Gallus domesticus*) determined by concentration of motile sperm and straight line velocity. *Biology of Reproduction*, 62, 303–309.

Froman, D. P. & Feltmann, A. J. 1998. Sperm mobility: A quantitative trait of the domestic fowl (*Gallus domesticus*). *Biology of Reproduction*, 58, 379–384.

Froman, D. P., Bowling, E. R. & Wilson, J. L. 2003. Sperm mobility phenotype not determined by sperm quality index. *Poultry Science*, 82, 496–502.

Gee, G. F. & Sexton, T. J. 1990. Cryogenic preservation of semen from the Aleutian Canada goose. *Zoo Biology*, 9, 361–71.

Idziorek, T., Estaquier, J., De Bels, F. & Ameisen, J. C. 1995. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *Journal of Immunological Methods*, 2, 249–258.

Łukaszewicz, E. 2002. An effective method for freezing White Italian gander semen. *Theriogenology,* 58, 19–27.

Łukaszewicz, E., Chrzanowska, M., Jerysz, A. & Chełmonska, B. 2004. Attempts on freezing the Greylag (*Anser anser* L.) gander semen. *Animal Reproduction Science*, 80, 163–173.

Łukaszewicz, E., Kruszyński, W. & Fujihara, N. 2003. Effect of age on quality of fresh and frozen-thawed semen in White Italian ganders. *Asian Journal of Andrology,* 5, 89–93.

Martin, G., Sabido, O., Durand, P. & Levy, R. 2004. Cryopreservation induces an apoptosis like mechanism in bull sperm. *Biology of Reproduction*, 71, 28–37.

Martínez-Pastor, F., Mata-Campuzano, M., Alvarez-Rodriguez, M., Alvarez, M., Anel, L. & De Paz, P. 2010. Probes and techniques for sperm evaluation by flow cytometry. *Reproduction in Domestic Animals*, 45, 67–78.

Roth, B. L., Poot, M., Yue, S. T. & Millard, P. J. 1997. Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. *Applied and Environmental Microbiology*, 63, 2421–2431.

Tai, J. J. L., Chen, J. C., Wu, K. C., Wang, S. D. & Tai, C. 2001. Cryopreservation of gander semen. *British Poultry Science*, 42, 384–388.

Waberski, D., Schapmann, E., Henning, H. & Riesenbeck, A., Brandt, H. 2011. Sperm chromatin structural integrity in normospermic boars is not related to semen storage and fertility after routine AI. *Theriogenology*, 75, 337–345.

Rijsselaere, T., Van Soom, A., Tanghe, S., Coryn, M., Maes, D. & De Kruif, A. 2005. New techniques for the assessment of canine semen quality: a review. *Theriogenology*, 64(3), 706–719.