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CRYOPRESERVATION OF GANDER SEMEN IN CRYOVIALS – COMPARATIVE STUDY

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The aim of the study was to find a practical and inexpensive method for freezing goose semen for use in routine inseminations under farm conditions. Two basic freezing protocols [(1) dynamic, programmable freezing and (2) static, nitrogen vapour method] were evaluated with varying concentrations of dimethylformamide (DMF) plus additional osmoprotectants such as betaine, trehalose, and sucrose, using cryovials as containers. Altogether eight different treatments were compared. Sperm viability before freezing and after thawing was examined by *in vitro* tests and, in the case of the simplest effective method, also by *in vivo* fertility test. There were no significant differences in sperm survival either in the dynamic (48–50%) or in the static protocol (43–46%), except for the treatment where the lowest DMF concentration was used without any osmoprotectant in the dynamic protocol (42.6%). The addition of osmoprotectants did not improve thawed sperm viability in any case. Fertility with frozen/thawed sperm using the simplest method was 58.5%, while that obtained with fresh, diluted semen was 66.9%. The study proved that the simple freezing of gander semen in nitrogen vapour with 9% DMF in cryovials could produce acceptable fertility. The newly elaborated method can be successfully used for routine inseminations by small- and large-scale goose breeders.

Key words: Gander, sperm, cryoprotectants, osmoprotectants, semen cryopreservation

Developing a cheaper and simple preservation protocol for gander semen is relevant and necessary for the following reasons: additional artificial insemination (AI) with frozen gander semen can be a solution for the species-specific reproduction problems such as monogamy and the poor semen quality in the first month after the onset and the last month before the end of egg production in both the spring (increasing day length) and the autumn (decreasing day length) active periods. Therefore, year-round fertile egg production in commercial geese is a challenge to breeders. Although AI is already routinely used in some goose

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breeding farms, the application of frozen-thawed semen could further improve the production parameters.

Of the different poultry species, the goose is known to have the poorest semen quality. The average ejaculate volume is only 0.2–0.3 mL with a relatively low sperm concentration (600–900 million/mL) and a live, intact sperm ratio around 60–70%. Despite these facts, some earlier cryopreservation studies showed better survival and more acceptable fertility rates with gander spermatozoa than with those of other poultry breeds (Tselutin et al., 1995; Sakhatsky et al., 1995; Tai et al., 2001; Łukaszewicz, 2002; Dubos et al., 2008; Barna et al., 2010).

There are only a few publications describing methods for the cryopreservation of gander semen and these generally use expensive programmable freezing devices that require technical expertise (Łukaszewicz, 2001*a*; Łukaszewicz, 2002; Łukaszewicz et al., 2004; Dubos et al., 2008).

This paper describes a further optimisation of the static nitrogen vapour method in polystyrene box for use by small-scale goose breeders and also a re-evaluation of the non-permeating osmoprotectants betaine, trehalose and sucrose (Blanco et al., 2011) when used in the freezing of gander semen.

According to our earlier investigations with domestic fowl (Végi et al., 2005) and guinea fowl (Váradi et al., 2013), insemination with frozen-thawed semen increased embryo mortality at the very early stage. In the present study this phenomenon was also investigated on goose embryos following an *in vivo* fertility test.

Materials and methods

Chemicals

Unless otherwise stated, chemicals used in this study were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary) and Reanal Laboratory Chemicals LLC (Budapest, Hungary).

Ethics statement

The geese were kept in conformity with the animal welfare prescriptions specified in the Hungarian Animal Protection Law (Act no. XXVIII of 1998). The research institution obtained permission for experimental animal researches from the National Food Chain Safety Office, Animal Health and Animal Welfare Directorate, Budapest, Hungary (permission number: 13/2015). All the experimental methods applied were approved by the Institutional Ethical Review Board (No. 2/2016).

Experimental birds and semen collection

Two-year-old Landes ganders (*Anser anser*) were used as semen donors. They were housed in individual pens and kept under a 9.5L:14.5D photoperiod.

The ganders were fed a commercial goose feed (300 g/gander/day) and had *ad libitum* access to water. Semen was collected twice a week for two months (n = 16) by a modified version of the abdominal massage technique (Burrows and Quinn, 1937). Individual gander semen quality was determined at the onset of semen collections and males producing poor-quality semen (< 40–50% motile and < 60% live, intact spermatozoa) were excluded from the experiment. Thereafter, pooled semen samples of 30 males were examined and used for the cryopreservation studies.

For artificial insemination (AI) females were penned separately in small groups (n = 20). The conditions were similar to those used for the ganders.

Semen qualification

The pooled samples were evaluated before freezing and after thawing. Sperm motility was examined by subjective scoring (1: 5–20%, 2: 20–40%, 3: 50–70%, 4: 75–85%, 5: 85–95% motile spermatozoa) and sperm concentration by spectrophotometer (Accucell IMV, France). Morphological abnormalities and live/dead sperm ratio were determined with aniline blue-eosin staining (Certistain, 115935 Eosin Y, Merck Ltd., Budapest, Hungary) (Váradi et al., 2013). Slides were evaluated microscopically (Zeiss, Axioscope; Carl Zeiss Microscopy GmbH, Germany) using an oil immersion objective and 1000× magnification. A total of 200 spermatozoa/slide were counted.

Freezing protocols

Two basic freezing methods were compared: programmable freezing as a dynamic protocol (D) and simple freezing in polystyrene box in nitrogen vapour as a static method (S). The pooled samples were diluted 1:1 with Łukaszewicz extender (Łukaszewicz, 2002) and cooled at 5 °C for 15 min. The permeating cryoprotectant dimethylformamide (DMF) was added using four different concentrations (7%, 9%, 10% and 12%) and equilibrated for 5 min at 5 °C based on our earlier study (Barna et al., 2010). Various combinations of non-permeating cryoprotectants, namely osmoprotectants (0.1 M betaine hydrochloride, 3% trehalose, 3% sucrose), were also evaluated based on the study of Blanco et al. (2011) as shown in Table 1.

Programmable freezing

From the diluted semen, 250-µL aliquots were transferred to cryovials and placed into a programmable freezing machine (Planer KRYO10, Planer Products Ltd., Middlesex, UK). Instead of the generally used straws, we used plastic cryovials as cryocontainer for easier handling. Cooling was started at 5 °C, with a –40 °C/min freezing rate until –140 °C, at which point the cryovials were quickly plunged into liquid nitrogen (LN).

Table 1

Experimental design of the semen freezing procedure

	Freezing protocols							
	Dynamic, programmable method D				Static, nitrogen vapour method S			
Diluents	Łukaszewicz diluent (see Łukaszewicz, 2002)							
Dilution rates	1:1							
Cooling period	for 15 min at 5 °C							
Treatments notation	D1	D2	D3	D4	S1	S2	S3	S4
Cryoprotectants	DMF	DMF	DMF	DMF	DMF	DMF	DMF	DMF
%	7%	10%	10%	10%	9%	12%	12%	12%
+			+	+			+	+
osmoprotectants			0.1 M betaine hydrochloride	3% trehalose + 3% sucrose			0.1 M betaine hydrochloride	3% trehalose + 3% sucrose
Equilibration period	5 min at 5 °C							
Freezing procedure	from 5 °C until – 140 °C, with – 40 °/min				3 min at 3 cm above the LN level			
Type of cryocontainer	1.5-mL plastic cryovials							
Thawing	2 min at 40 °C							

Freezing in a polystyrene box

Diluted semen samples (250 µL) in cryovials were prepared as above. The vials were placed 3 cm above the liquid nitrogen level in a polystyrene box for 3 min. The cooled cryovials were then plunged into liquid nitrogen.

Regardless of the freezing method, thawing was done at 40 °C in a water bath for 2 min.

Artificial insemination (AI)

Out of the eight treatments only the thawed semen frozen by the simplest static protocol (S1) was used for AI. To serve as controls, twenty females were inseminated with 40 million diluted, fresh spermatozoa. The diluent was the same as that used for the cryopreserved semen. Another 20 females were inseminated with 90 million frozen-thawed spermatozoa/female. The insemination doses were between 150 and 200 µl in all cases, depending on the sperm concentration. Artificial inseminations were performed twice weekly with a 72-h interval between inseminations for three consecutive weeks (n = 7).

Egg treatments and monitoring of embryo development

Egg collection was started two days after the first insemination and was performed three times a day during the insemination period. Eggs were stored at 18 °C before the start of incubation. Fertility was determined by candling on day 7 of incubation. After candling, clear eggs were opened and examined to differentiate unfertilised ova from early dead embryos that died before blood formation. Moreover, the ratios of fertilised eggs with normal embryonic development and embryos that died during the incubation were determined. Embryos dead in the oviduct were confirmed using a propidium iodide fluorescent staining method of the germinal discs according to Liptói et al. (2004).

Statistical analysis

The statistical analysis was performed using Statistica software version 10.0 (StatSoft Ltd., Hungary). The results of semen parameters were analysed by one-way ANOVA with Tukey's *post hoc* test. Percentage data were arcsine transformed and analysed (Harnos and Reiczigel, 2006). Data of fertility and embryonic mortality were analysed using the chi-squared test.

Results

In vitro *examinations*

By the examination of thawed sperm quality, 52.5 to 59% live cell ratios (live, intact + live, abnormal cells) were detected in the case of dynamic freezing,

while the static protocols produced 48.3 to 54.9% live cell ratios (Table 2). However, changes in sperm quality after freezing did not show significant differences between the dynamic and the static freezing protocols, with the exception of the treatment where the lowest DMF concentration was used without any osmoprotectants in the dynamic protocol (D1, $P \leq 0.01$).

Table 2

In vitro sperm assessment in fresh and frozen/thawed samples

		Live, intact	Live, abnormal	Dead
		%		
Fresh		82.7 ± 4.6	8.5 ± 4.9	8.8 ± 2.3
Frozen-thawed	D1	35.5 ± 3.5[a]	17 ± 4.6	47.5 ± 2.7
	D2	39.2 ± 1.9	16 ± 4.9	44.8 ± 3.1
	D3	39.7 ± 2.3	17.5 ± 2.9	42.8 ± 2.3
	D4	41.1 ± 0.9[b]	17.9 ± 5.9	41 ± 5
	S1	36.9 ± 1	18 ± 4.3	45.1 ± 3.5
	S2	37.6 ± 3.3	15.7 ± 3.3	46.7 ± 6.5
	S3	33.7 ± 3.3	14.6 ± 5.2	51.7 ± 8.4
	S4	37.8 ± 1	15.8 ± 3.3	46.4 ± 2.6

Different superscripts (a, b) denote statistically significant differences ($P \leq 0.01$)

In the programmable protocols sperm survival was not improved by osmoprotectants (42.6 ± 6.92, 47.9 ± 7.47, 48.5 ± 5.91, 50.3 ± 7.41%). Treatment D1 resulted in a lower ($P \leq 0.01$) survival rate of live, intact spermatozoa compared to treatment D4 (42.6 ± 6.92 vs. 50.3 ± 7.41%; Fig. 1A). Among the static protocols (S) no significant differences were found in sperm survival rates (43 to 46%; Fig. 1B).

In vivo *examination*

As there were no significant differences among the treatments in the live, intact sperm ratios the cheapest and simplest freezing protocol (S1) was selected and compared to the insemination results obtained with freshly used semen in fertility examinations. While the static protocol yielded 58.5% fertility, insemination with fresh, diluted semen resulted in 66.9% fertility.

In the control group the percentage of embryos with normal development was 46%, while the rates of very early and late embryonic deaths were 8% and 13%, respectively. In the case of frozen/thawed semen the proportion of embryos with normal development was 38%, while the rates of very early and late embryonic deaths were 13% and 8%, respectively. There were no significant differences between the two groups in any category (Table 3).

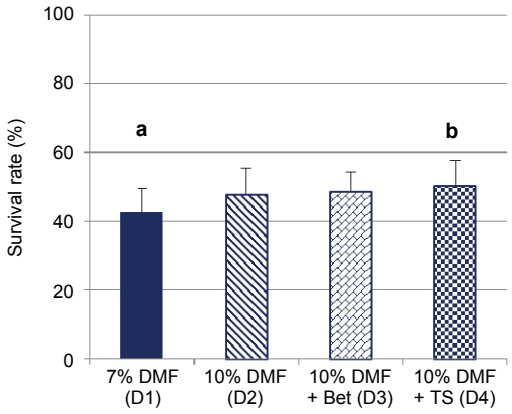

Fig. 1A. Survival rates of live, intact spermatozoa after freezing/thawing by dynamic methods

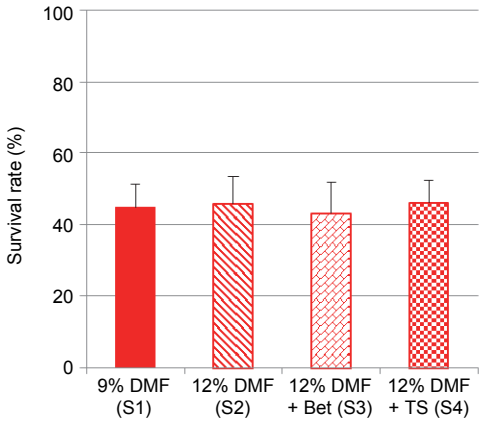

Fig. 1B. Survival rates of live, intact spermatozoa after freezing/thawing by static methods

Table 3

Fertility and embryo mortality following artificial insemination

	No. of eggs set	Normal development (%)	Eggs lost at candling (%)			Fertility (%)
			Embryos that died in the oviduct	Embryos that died during incubation	Infertile eggs	
Fresh	172	46.5	7.6	12.8	33.1	66.9 ± 9.19
Frozen/thawed	164	37.8	12.8	7.9	41.5	58.5 ± 12.73

Discussion

Studies comparing the static and the dynamic semen freezing methods have already been done in other poultry species. However, in those investigations the nitrogen vapour method seemed to be less effective in the case of *Galliformes* (Hanzawa et al., 2006; Barna et al., 2008; Sasaki et al., 2010; Santiago-Moreno et al., 2011; Váradi et al., 2013; Long et al., 2014), which indicates the need for the elaboration and/or improvement of species-specific semen freezing protocols.

In the present study the two basic freezing methods and the effects of various osmoprotectants were tested. Combinations of permeating and non-permeating cryoprotectants were used, since these components protect the spermatozoa in different ways. DMF was used as cryoprotectant, since it has no contraceptive effect in the oviduct and was proved to have an efficient protective function during the cryopreservation of gander spermatozoa (Łukaszewicz, 2001*a*; Santiago-Moreno et al., 2011). Of the osmoprotectants, betaine, trehalose and sucrose were proved to have a positive effect on turkey and crane spermatozoa (Blanco et al., 2011). However, to the best of our knowledge, the effect of the above-mentioned osmoprotectants has not been tested in gander semen freezing so far. Contrary to our expectations, neither betaine nor the trehalose plus sucrose combination improved the survival of frozen gander sperm significantly.

In earlier reports on gander semen freezing 8 to 25% live, morphologically normal sperm ratios were found in the case of programmable methods with significantly different fertility rates (Łukaszewicz, 2001*a*, *b*; Łukaszewicz et al., 2004). In the present study the live, intact sperm proportions were higher (35 to 41%) when the dynamic method was used. In the static freezing protocol the ratios of live, intact cells were nearly as high (34 to 38%) as with the programmable methods, with an acceptable fertility of 58.5%. In the few studies published on gander sperm cryopreservation the rates of sperm survival are usually not too high, while the fertility results are sometimes exceptionally good (Łukaszewicz, 2002). Using fast freezing on dry ice and 9% dimethylacetamide (DMA) as cryoprotectant, Tai et al. (2001) also achieved successful freezing. Acceptable fertility rates obtained by the use of a pellet method were also reported earlier (Tselutin et al., 1995; Sakhatsky et al., 1995). However, despite the simplicity of the method the use of pellets is not recommended for long-term storage due to the shrinkage of pellets over time and the consequential decrease in sperm survival (unpublished data). It is difficult to compare the above fertility data since it is known that the fertility rates depend on several factors including the type of breed, the age of the geese, the stage of the reproduction period, the insemination technique and the frequency of insemination.

Therefore, we think that the sperm survival data are more reliable for determining the efficiency of deep freezing than the fertility data, since the latter can be easily improved by modifying the technique of insemination (increased sperm vol-

ume and/or insemination frequencies). A good example is the study of Dubos et al. (2008) that compared varying numbers of frozen-thawed sperm and different insemination frequencies, and the best result was 60% fertility achieved with 40 million frozen-thawed spermatozoa/AI/female, using three inseminations per week.

In the present study fairly high (40 to 50%) sperm survivals were achieved by both freezing methods. The simplest static method was selected for the fertility trial because this can be more easily performed under farm conditions. On the basis of the sperm survival data the number of frozen-thawed spermatozoa used for insemination was more than twice that used in the case of fresh semen (90 vs. 40 million, respectively). Presumably, because of this there were no significant differences either in early and late embryonic mortality or in fertility data. Static freezing in a polystyrene box yielded nearly 60% fertility which approaches the average farm fertility of the same breed inseminated with fresh semen. The novelty of the method is the use of plastic cryovials as containers, which are much easier to apply under farm conditions compared to plastic straws. This simple and inexpensive method can be easily used even under farm conditions, thus it can be recommended for both the large- and the small-scale goose breeding industry as an additional option, reducing the maintenance and production costs.

This method may be suitable for the long-term storage of spermatozoa also in gene conservation works; however, this must be verified by further investigations.

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