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The Effect of Semen Dilution on Morphology and Fertility of Japanese Quail (*Coturnix coturnix japonica*) Spermatozoa

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The effect of quail semen dilution with three extenders : Lake's, Ringer's and Tyrod's on morphology and fertility trials of unstored spermatozoa was evaluated.

Semen was collected twice a week by male stimulation by female method, from 60 quail males randomly divided into four groups (I-IV). Forty-eight females divided into four respective groups (A-D) were inseminated intravaginally with 10 μl of fresh or 20 μl of 2-folds diluted semen.

Semen dilution did not affect the number of live spermatozoa in total, but the number of live, morphologically normal spermatozoa depended on the diluent. The highest number of morphologically normal spermatozoa −65.3% vs. 67.5% in fresh semen was stated in semen diluted with Lake's extender. It was significantly higher ($P \leq 0.01$) than in semen diluted with Ringer's diluent (56.2%).

Egg fertility (99.2%), hatchability from set (91.2%) and fertile eggs (93.9%) in naturally mated group were significantly higher ($P \leq 0.01$) when comparing with females inseminated artificially both, with fresh and diluted semen. The highest fertility (60.1%) was obtained for spermatozoa diluted with Lake's extender, the lowest for spermatozoa treated with Ringer's solution (30.2%).

Key words : extenders, fertility, quails, spermatozoa morphology

Introduction

Although the experiments on quail artificial insemination were initiated long time ago (Wilcox and Clark, 1961) the effective method that allowing to obtain a high fertility is still missing. Published data vary significantly and fertility results are far away from that obtained in quail mated naturally. The application of the method of male stimulation by female (Chełmońska *et al.,* 2005 a) and intravaginal insemination with semen mixed with proctodeal gland foam (Chełmońska *et al.,* 2005 b) allows to obtain fertility equal to the results obtained by natural mating. Fertility depends also on semen quality, extenders and storage time *in vitro* (Baumgartner *et al.,* 1975 ; Kulenkamp *et al.,* 1966 ; Lepore and Marks, 1965 ; Marks and Lepore, 1965 ; McFarquhar and Lake, 1964). The authors mentioned above limited their experiments to short time storage and in the accessible literature, there is a lack of information on attempts on cryopreservation of quail spermatozoa.

Taking into account the particular advantages of quails as a model object in many fields of science, as well as their usefulness in different biotechnological methods of reproduction of other birds species, the experiments aiming at elaboration of a proper treatment within the subsequent steps of quails semen cryopreservation were undertaken.

In the first step of the experiment effect of the three semen extenders : Lake's (Lake, 1960), Ringer's and Tyrod's (Miętkiewski, 1978 ; Marks and Lepore, 1965) on morphology and fertility trials of quail spermatozoa was tested.

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Materials and Methods

Birds

Sixty quail males randomly divided into 4 experimental groups I-IV (15 each) and 48 females divided into 4 groups A-D (12 each) were used in the experiment. At the beginning of the experiment birds were 3-months-old. The experimental groups were as follows : group I (A- for females)- fresh, undiluted semen ; group II (B- for females)- semen diluted with Lake's extender ; group III (C- for females)- semen diluted with Ringer's solution and group IV (D- for females)- semen diluted with Tyrod's solution. In each group freshly collected semen was diluted 2-folds. Control group (E) consisted of 3 males and 12 females mated naturally. Males were kept individually in cages ($32\times44\times24$ cm), females in colony cages ($60\times45\times48$ cm, one for each group) at room temperature (20–22℃). During the reproductive period birds were treated with 14 hL/10 hD photoperiod. Water and feed, typical for reproductive quails were available *ad libitum* and supplemented with minerals and vitamins. Daily feed consumption was about 30 g/bird.

Semen Collection

Pooled semen was collected twice a week by male stimulation by female method (Chełmońska *et al.,* 2005 a), for 6 weeks, from every male group separately. For male stimulation, additional five sexually matured females are necessary. From groups II-IV semen was collected into small glass tubes filled with 0.2 m*l* of particular diluent. If the volume of collected ejaculates exceeded 0.2 m*l* the sample content was supplemented with diluent in order to obtain final dilution ratio 1 : 1. Twelve semen collections and inseminations procedures were performed.

Semen Evaluation

Spermatozoa integrity was assessed after collection, in vital-stained nigrosin-eosin semen smears (Jaśkowski, 1966) under a light microscope (Jenaval, Carl Zeiss ; ×1250 magnification, 300 sperm per slide). Within the live spermatozoa morphologically normal cells and cells with various deformations, like changes in head, acrosome, neck or midpiece were assessed (Gwara *et al.,* 2004). The results of the morphological examination were expressed as the percentage of particular classes of spermatozoa (300 cells＝100%). Additionally in every pooled ejaculates the total volume and spermatozoa concentration were counted.

Fertility Trial

Fertility trial of evaluated semen samples was scored on the basis of insemination results. Every female was inseminated intravaginally (Chełmońska *et al.,* 2005 b), immediately after semen collection (i.e. twice a week, in 3–4 days intervals) with a dose of 10 μl of fresh and 20 μl of diluted semen.

The number of set eggs, percent of fertility, percent of hatchability from set and fertile eggs were determined for clean, properly formed eggs collected daily starting at Day 2 after the first insemination to Day 4 after the last insemination. Eggs were stored at 12℃ and set weekly to the Type C 82 multistage incubators (Agraria, Gostyń, Poland). Seven egg sets were made weekly ; totally 2013 eggs were incubated. All fertile eggs were transferred to hatchery at Day 14 and incubated up to hatch, in order to determine the hatchability results.

Fertility and hatchability rates of the experimental groups were compared to naturally mated group (group E ; 3 males and 12 females).

Statistical Analysis

Semen quality, fertility and hatchability rates were analysed by ANOVA and Duncan multiple range test (SAS system, General Linear Models Procedure).

Results and Discussion

The volume of pooled semen collected from the group I was 220 μl on average (varying from 150 to 300 μl) ; in group II-230 μl (from 150 to 300 μl) ; in group III-240 μl (from 200 to 400 μl) and in group IV-280 μl on average (varying from 200 to 350 μl). Differences between groups were not significant.

Sperm concentration in diluted semen samples was similar in all groups and averaged at 1.29×10^9 ml^{-1} (varying from 0.84 to 1.70×10^9 ml^{-1})- in semen with Lake's extender ; 1.38×10^9 ml^{-1} (0.87 to 1.84×10^9 ml^{-1})- for Ringer's solution, and 1.33×10^9 ml^{-1} (0.95 to 1.78×10^9 ml^{-1})- for samples diluted with Tyrod's. In semen collected from group I (undiluted semen) spermatozoa concentration averaged at 1.72×10^9 ml^{-1} (varying from 0.90 to 2.46×10^9 ml^{-1}). Ejaculates volume and sper-

Table 1. Morphology of Japanese quail spermatozoa in the fresh and diluted semen (n=12 ; means ; ±SD)

Classes of spermatozoa (%)	Semen samples (Male groups)			
	I -Fresh undiluted	II I-Diluted with Lake's	III-Diluted with Ringer's	IV-Diluted with Tyrod's
Live in total	81.8±4.7	83.2±4.9	79.9±7.0	80.1±7.3
Live normal	67.5^{A*}±6.4	65.3^{a}±6.2	56.2^{Bb}±9.9	64.8±11.6
With different deformations	14.3^{A}±4.0	17.9^{a}±5.3	23.3^{Bb}±5.7	15.3^{A}±6.1

* Values within lines followed by different superscripts differ significantly (A, B-P≤0.01 ; a, b-P≤0.05).

matozoa concentration were similar to results obtained in previous experiments (Chełmońska *et al.,* 2005 a ; 2005 b) and to other results cited in literature (Baumgartner *et al.,* 1975 ; Bunaciu *et al.,* 1994 ; Buxton and Orcutt, 1975 ; Lepore and Marks, 1965 ; Tarasewicz *et al.,* 1997 ; Wentworth and Mellen, 1963).

The average data of morphology of semen collected from evaluated groups is given in Table 1. The percent of live spermatozoa in total was similar in evaluated groups, and existing differences were not significant among groups. It may suggest that tested diluents did not affect the number of live spermatozoa in total, however, the differences in number of live morphologically normal cells were observed. The highest percentage of the most desired form of spermatozoa was found in undiluted semen (it varied from 53.7 to 74.0), and this value was significantly higher (P≤0.01) when comparing with semen collected into Ringer's solution (from 43.0 to 74.0%) and Lake's extender (56.7–73.0%) (P≤0.05). Among live deformed cells, spermatozoa with broken or missing tail, bulb head and bent-neck were most frequently observed (Table 1).

It is difficult to discuss the results obtained in the present studies since the number of papers relating to quail spermatozoa morphology is limited. Brożek and Knote (1974) who evaluated sperm head deformations found from 0.14 to 0.34% such deformed cells, depending on quail age. Fujihara *et al.* (1989) and Bunaciu *et al.* (1994) described from 4.05 to 6.63% of deformed quail spermatozoa in total. Fujihara *et al.,* (1989) stated that after 24 hours storage of quail semen, over 70% of spermatozoa were deformed, and most of them had broken neck which is observed quite often in poultry semen stored in vitro (Saeki, 1960).

It is commonly known that the number of live morphologically normal spermatozoa in an insemination dose affects significantly the fertility, and in a consequence, the number of hatched chicks. From this point of view, our results indicate that from tested diluents, the less favourable for quail semen is Ringer's solution for which the lowest number of normal spermatozoa and the highest of deformed cells was stated (Table 1).

The highest fertility (from 87.5 to 100.0%) was obtained in naturally mated group (sex ratio was 1 : 4) and this value was significantly higher (P≤0.01) when comparing to each group inseminated artificially (Table 2). In relation to the fresh semen insemination, semen dilution decreased the fertilising ability of quail spermatozoa, but diluent effect was also observed. The best fertility traits were obtained for spermatozoa diluted with Lake's extender, and as it could have been expected, the spermatozoa of poorest fertilising potency were observed in Ringer's solution. Despite the semen treatment a great variability in fertility was observed within each inseminated group-it varied from 0.0 to 100.0% in particular egg sets.

The hatchability from set eggs and relations between the evaluated semen samples were similar as for fertility (Table 2). Semen dilution and kind of diluent however, did not affect the hatchability from the fertile eggs, but comparing to naturally mated group it was significantly lower (P≤0.01). Fertility results obtained in our experiment after fresh semen insemination are better than those

Table 2. Fertility and hatchability of Japanese quails inseminated with the fresh or diluted, non stored semen (means ; ±SD)

Semen sample and female group	Number of set eggs	Fertility (%)	Hatchability (%) from	
			Set eggs	Fertile eggs
A-Fresh undiluted	409	$72.82^{A}\pm21.09$	$64.05^{Aa}\pm20.99$	$85.64^{A}\pm18.41$
B-Diluted with Lake	407	$60.08^{A}\pm21.99$	$52.45^{Ab}\pm22.96$	$81.46^{A}\pm24.18$
C-Diluted with Ringer's	393	$30.19^{C}\pm20.24$	$25.38^{C}\pm20.05$	$78.21^{A}\pm35.81$
D-Diluted with Tyrod's	410	$37.21^{C}\pm25.08$	$31.61^{C}\pm25.56$	$76.74^{A}\pm32.33$
E-Natural mating	394	$99.18^{B}\pm2.97$	$91.18^{B}\pm8.29$	$93.92^{B}\pm8.03$

Values in columns followed by different superscripts differ significantly (A, B-P≤0.01 ; a, b-P≤0.05).

described by Marks and Lepore (1965) who obtained 56.1% of fertile eggs on average. However, after insemination with diluted semen this value was almost twice lower when comparing with 62.1% obtained by these authors for Ringer's and 72.3% for Tyrod's diluent. Much lower fertility — 24.35% after fresh semen insemination and 73.29% for natural mating, were achieved by Baumgartner *et al.,* (1975) and Wentworth and Mellen, (1963) who obtained only 23.1% of fertile quail eggs, after insemination with semen diluted in Krebs solution.

Although, fertility and hatchability traits for groups inseminated artificially with fresh or diluted semen were significantly lower in relation to naturally mated group, the results obtained with Lake's extender seem to be promising for further experiments on quail semen storage and cryopreservation.

Our results indicate the importance of proper choice of diluent, which was previously concluded by Łukaszewicz (1988) for rooster semen stored *in vitro* for 3 or 6 hours. This should encourage the authors to search for the more effective methods of quail semen short and long-term storage.

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