Original Article

Optimizing system for sperm quality evaluation using crystalloid diluent for pigeons (*Columba livia domestica*)

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Abstract

Semen quality is a crucial factor for predicting fertility and hatchability in several avian species including pigeons. To evaluate pigeon semen quality, prior dilution of semen was required due to its small volume with highly concentrated spermatozoa. Conventional crystalloid solutions—Normal saline (NSS) and Ringer's solution (LRS) are common diluents for such a task; however, the unoptimized dilution rate of such diluents was a major cause contributing to inaccurate semen quality evaluation. Due to the lack of such knowledge in pigeons this study aimed to optimize the dilution rate of Normal saline (NSS) and Ringer's solution (LRS) for pigeon semen quality evaluation. The semen of a total of 20 male racing pigeons was collected and diluted in NSS or LRS at 12.5-, 25-, 50-, 100- and 200-fold dilutions. Immediate percentages of sperm motility, progressive motility and viability were determined for each fold dilution. The 12.5- and 25-fold-diluted semen were further evaluated at 0, 0.5, 1 and 3 h of storage. The results showed that NSS and LRS rendered indifferent effects on immediate semen quality. However fold dilutions higher than 25 resulted in a great decline in semen quality both in NSS and LRS. Storage of 12.5-fold and 25-fold diluted semen resulted in a marked decrease of semen quality as soon as 0.5 h; however, LRS was better than NSS in terms of maintaining semen quality. This study thus supports immediate quality evaluation of pigeon semen—by which 12.5-25 fold-dilutions were suggested.

Keywords: Crystalloid diluent, Semen dilution, Storage time, Semen quality, Pigeon

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Introduction

Assisted reproductive techniques (ARTs), especially semen storage and artificial insemination have been implemented as beneficial tools both for poultry production and endangered bird conservation (Chowdhury *et al*., 2014). Climate change and global warming have impacted most animal species (Bolger *et al*., 2005). More than 2,528 animal species were on the list of threatened species (IUCN, 2017). According to the report, at least 9% of bird species worldwide are now in crisis and showing significant long-term decline. Artificial fertilization has become important to the wildlife conservation program (Herrick, 2019). Regarded as the ideal model for avian ARTs development, the pigeon is generally selected due to its constant semen production and easy handling size. Moreover, the pigeon itself is well-recognized for its employment in fancy contests, racing and even consumption (Cheng *et al*., 2002; Klimowicz *et al*., 2012; Klimowicz *et al*., 2008; Klimowicz *et al*., 2005; Sontakke *et al*., 2004).

Semen quality can determine avian fertility and hatchability (Parker et al., 2002; Parker and McDaniel, 2002) and semen quality evaluation is one of the most crucial processes for breeder selection and improvement. Due to the viscous small volume with highly concentrated spermatozoa of avian semen, semen dilution is usually required prior to quality evaluation steps to prevent semen dehydration and sperm movement alleviation (Blanco *et al*., 2009; Gee *et al*., 2004). Conventional crystalloid solutions like Normal saline and Ringer's solution are routinely applied as semen diluent for such purposes among various bird species (Paranzini *et al*., 2018; Neuman *et al*., 2002; Parker and McDaniel, 2004; Klimowicz *et al*., 2005). Interestingly, the solution type and dilution rate are profound factors influencing semen quality evaluation. For example, 10-fold dilution of broiler's semen in Normal saline (0.85% saline) results in optimal broiler semen quality evaluation (Parker and McDaniel, 2002, 2004, 2006; Parker *et al*., 2002), while higher fold dilution results in deteriorated and thus inaccurate semen quality evaluation (Parker and McDaniel, 2003). Despite its wide recognition, Normal saline is still limitedly studied in pigeon semen quality evaluation. On the other hand, Ringer's solution is the primary crystalloid diluent for semen handling in pigeons—for which immediate evaluation is generally suggested (Klimowicz *et al*., 2005). As far as we knew, little is known about dilution rate effects such crystalloid diluent on pigeon semen quality. Due to the lack of such crucial comprehension, this study aimed to determine the dilution effect of Normal saline and Ringer's solution on pigeon semen quality. With the knowledge acquired from this study, we hope to improve the conventional system used to correctly evaluate pigeon semen quality.

Materials and Methods

Experimental procedures were approved by Rajamangala University of Technology Tawan-OK Animal Ethics Committee (RMUTTO-ACUC-2-2019-003), and care was taken to minimize the number of animals used.

Experimental design: This study aimed to determine the optimal dilution rate of Normal saline (NSS) and Ringer's solution (LRS) along with their storage time for pigeon semen quality analysis. The fresh ejaculates obtained from 20 racing pigeons were diluted with NSS or LRS at 12.5-, 25-, 50-, 100- and 200-fold dilutions. For storage time determination, the 12.5- and 25-fold diluted semen were maintained at 25 °C and evaluated for sperm motility and viability at 0, 0.5, 1 and 3 h. The semen samples were individually evaluated and the entire experiment was repeated three times.

Experimental birds: A total of 20 adult male racing pigeons weighing 400 to 442 g were used. The birds were housed individually in metal cages, exposed to natural environmental conditions and provided with 10 g of complete feed twice daily with water available ad libitum.

Semen collection and evaluation: To reduce technical variations, the same operator performed semen collection throughout the study. Semen was collected from the birds twice a week by lumbosacral massaging and gentle squeezing at the base of the cloaca (Cheng *et al*., 2002; Sontakke *et al*., 2004). The ejaculates were analyzed for their semen quality–volume, color, consistency, pH, percentage of total motile sperm, progressive motility, percentage of sperm viability, sperm concentration, total sperm and percentage of normal and abnormal sperm. The ejaculate volume was determined by aspirating the semen into a calibrated displacement pipette and the pH using pH indicator strips (Riedel-De-Haën AG, Germany). Each semen's characteristics was individually evaluated by the same evaluator in order to standardize the performance.

The percentage of total motile sperm and progressive motility was determined by putting 10 µl of sperm suspension on a slide and then covering it with cover glass (18 mm x 18 mm). Total motile sperm and progressive motility were evaluated under light microscope at 100 x magnification for 8-10 fields. Total motile sperm were expressed as percentages ranging from 0 to 100%. Progressive motility of the sperm was graded on a 5-point score (where 0 indicates no motility, 1 indicates non progressive motility, 2 indicates slow progressive motility, 3 indicates side to side movement accompanied by slow progressive motility, 4 indicates faster progressive motility, and 5 indicates very fast progressive motility according to (Sontakke *et al*., 2004). Diluted semen at each dilution was promptly evaluated within 5 minutes.

The viability and morphology of the pigeon sperm were assessed on the same microscope slide, stained with eosin-nigrosin dye. The proportions of live (eosin-impermeable) and dead (eosin-permeable) sperm in a sample were assessed on the basis of 200 sperm and individually categorized as: normal, amorphous head, bent head, macrocephalic head, acrosomal defect, loosed head, abnormal midpiece, proximal droplet, coiled tail, bent tail, distal droplet and loosed tail in a sample assessed on 300 sperm under light microscope at 1,000 x magnification (Cheng *et al*., 2002; Sontakke *et al*., 2004).

Sperm concentration was assessed by dilute 10 µl of sperm suspension in 90 µl of formal saline (100-fold dilution). Subsequently, 10 µl of the diluted sperm suspension was transferred to a counting chamber (Boeco, Germany) and the sperm concentration was then evaluated under light microscope at 400 x magnification.

Statistical analysis: Data was presented as mean ± standard deviation (S.D.) and mean ± standard error of mean (S.E.). Normal distribution of the data ($P < 0.05$) was confirmed using the D'Agostino & Pearson omnibus normality test. Differences of sperm motility and viability between two sample groups of interest were analyzed using student's *t*-test, while differences among sample groups were analyzed using one-way analysis of variance. Significant differences among experimental groups were analyzed using Tukey's test. $P < 0.05$ was considered statistically significant.

Results

Semen characteristics: The stock solutions were measured for the pH before use. The pH of LRS was approximately 6.4-6.5, while the pH of NSS was approximately 5.5-5.6. The majority of ejaculates had white to cream color with high viscosity. The mean semen volume and pH were 11.75 ± 4.51 µl and 7.66 ± 0.16, respectively. Mean percentages of total motile sperm and sperm viability were 86.25 ± 9.40 and 82.75 ± 6.74. The mean score of progressive motility was 4.91 ± 0.27, while mean sperm concentration was 1.65 ± 0.9 x 10^9 sperm per milliliter. For sperm morphology, the mean percentage of normal sperm was 88.50 ± 3.39, while the mean percentage of abnormal sperm was 11.50 ± 3.39 (Fig. 1; Table 1).

Dilution effect on semen quality: Immediate comparisons (0 h) of total sperm motility, progressive motility and viability acquired from NSS and LRS diluted semen revealed no differences in each of the fold-dilutions (Fig. 2, supplementary Table 1-3). On the other hand, comparison of total sperm motility acquired among the fold dilutions revealed a significant decline of total sperm motility as early as 50-fold dilution (Fig. 2, supplementary Table 1). Progressive motility started to decrease earlier (1:25-fold dilution), while sperm viability was maintained until 100-fold dilution (supplementary Table 2-3)

Effect of storage time on semen quality: Since the series of fold-dilutions implied 12.5- and 25-fold dilutions as favorable dilutions for immediate semen quality evaluation, we further determined their efficiency in maintaining semen quality over time. However, a decline of total motile sperm was noticeable in both types of diluent as early as 0.5 h (Fig. 3-4, supplementary Table 4). Similarly, percentages of progressive motility and viability also significantly reduced within 0.5-1 h of storage (supplementary Table 5-6).

Effect of diluent types on semen quality maintenance over storage time: A comparison between NSS and LRS on their ability to maintain semen quality at 25-fold dilution manifested LRS as a better diluent than NSS in maintaining total sperm motility over storage time — by which a rapid decline of sperm motility was noticeable in NSS-diluted semen when compared to that of LRS at 1-3 h ($P < 0.05$) (Fig. 5, supplementary Table 7).

Table 1 Semen characteristics of racing pigeons.

Parameter	Mean ± S.D.	Range
Ejaculate volume (µl)	11.75 ± 4.51	5 – 20
Semen pH	7.66 ± 0.16	7.4 – 7.8
Total motile sperm (%)	86.25 ± 9.40	70 – 100
Progressive motility (0-5)	4.91 ± 0.27	4 – 5
Sperm viability (%)	82.75 ± 6.74	72 – 93.5
Sperm concentration (x 10^9 / ml)	1.65 ± 0.90	0.62 – 4.03
Total sperm (x 10^9 sperm)	22.31 ± 20.58	3.10 ± 80.63
Morphologically normal sperm (%)	88.50 ± 3.39	83.33 – 95
Morphologically abnormal sperm (%)	11.50 ± 3.39	5 – 16.67
Amorphous head (%)	1.56 ± 1.36	0 – 4.33
Bent head (%)	1.31 ± 0.69	0 – 2.33
Macrocephalic head (%)	1.39 ± 1.02	0 – 4
Acrosomal defect (%)	1.23 ± 1.16	0 – 3.67
Loosed head (%)	2.35 ± 1.46	0.33 – 5
Abnormal midpiece (%)	0.48 ± 0.55	0 – 1.67
Proximal droplet (%)	0.02 ± 0.08	0 – 0.33
Coiled tail (%)	0.64 ± 0.73	0 – 2.67
Bent tail (%)	1.56 ± 2.15	0 – 6.33
Distal droplet (%)	0.02 ± 0.08	0 – 0.33
Loosed tail (%)	0.37 ± 0.39	0 –1

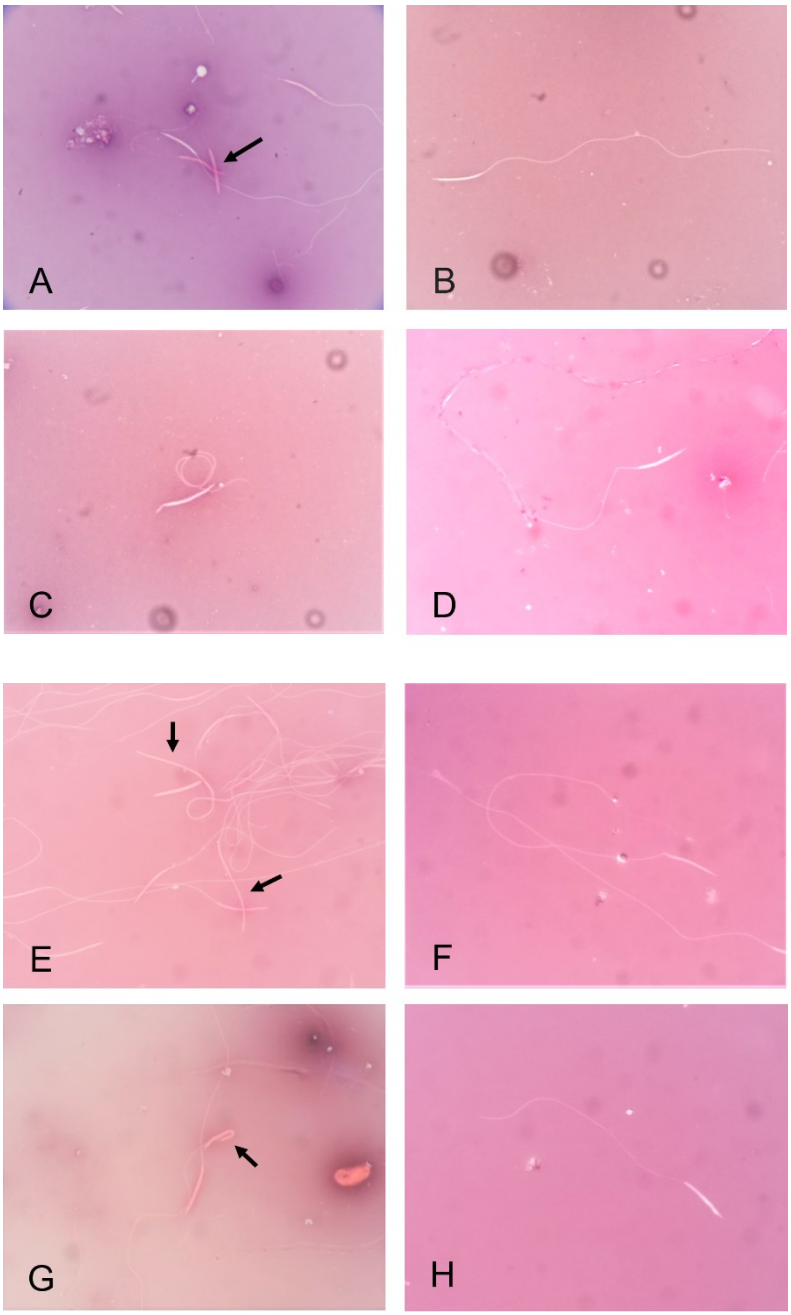

Figure 1 Morphological appearance of pigeon sperm under light microscope at 1,000 x magnification. (A) Dead sperm stained with red dye of eosin (arrowed); (B) normal sperm; (C) coiled tail; (D) bent tail; (E) macrocephalic head (arrowed); (F) abnormal midpiece; (G) bent head (arrowed); and (H) acrosomal defect.

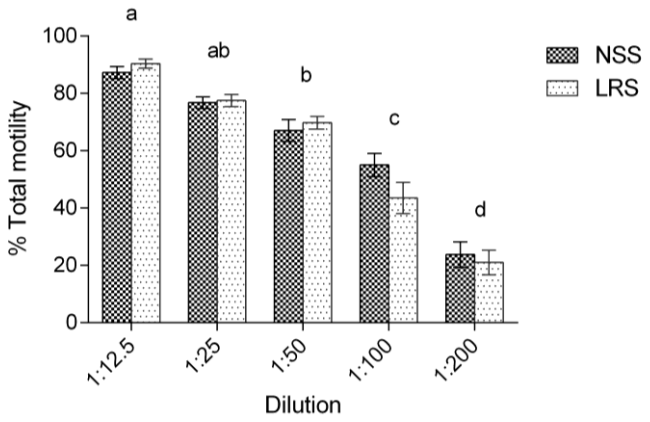

Figure 2 Total motility (%) of pigeon spermatozoa diluted in NSS and LRS at 12.5-, 25-, 50-, 100- and 200-fold dilution. Data was expressed as mean ± SE. Small letters above the bar indicate significant difference among dilutions ($P < 0.05$).

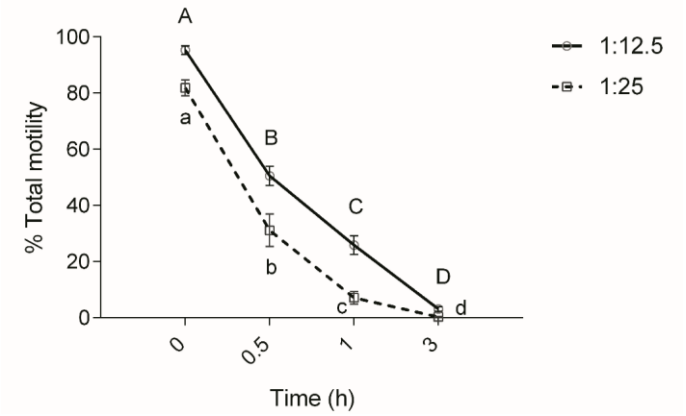

Figure 3 Total motility (%) of pigeon spermatozoa diluted in NSS (12.5- and 25-fold dilution) stored at 0, 0.5, 1 and 3 h. Data was expressed as mean ± SE. Capital letters indicate significant difference among storage times of 12.5-fold dilution, while small letters indicate significant difference among storage times of 25-fold dilution ($P < 0.05$).

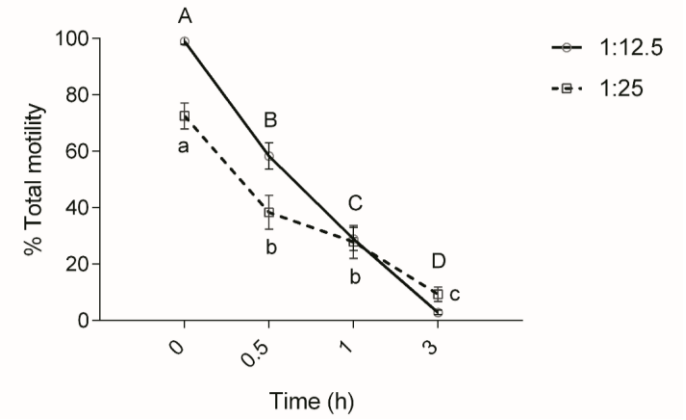

Figure 4 Total motility (%) of pigeon spermatozoa diluted in LRS (12.5- and 25-fold dilution) stored at 0, 0.5, 1 and 3 h. Data was expressed as mean ± SE. Capital letters indicate significant difference among storage times of 12.5-fold dilution, while small letters indicate significant difference among storage times of 25-fold dilution ($P < 0.05$).

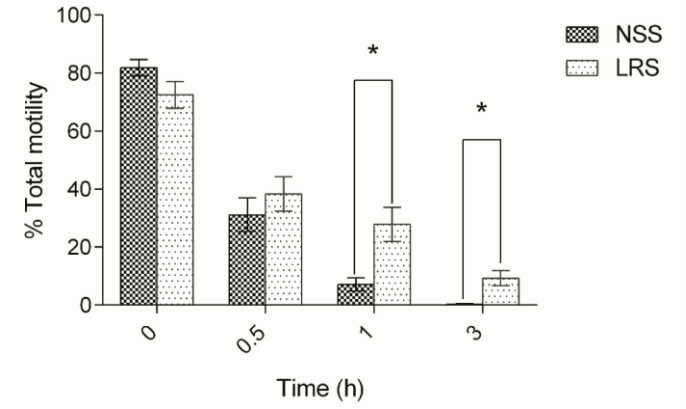

Figure 5 Total motility (%) of pigeon spermatozoa diluted in NSS and LRS at 25-fold dilution stored at 0, 0.5, 1 and 3 h. Data was expressed as mean ± SE. Asterisks above the bar indicate significant difference between groups ($P < 0.05$).

Discussion

Semen quality is regarded as an important parameter to evaluate fertility and hatchability in several avian species, including pigeons (Neuman *et al.*, 2002; Parker and McDaniel, 2002, 2003, 2004). Previous studies in poultry have indicated that semen quality was more predictive of fertility at lower dilutions, since a high dilution rate could negatively affect sperm motility rendering the false quality of the semen (Parker and McDaniel, 2003; Parker and McDaniel, 2006). Agreeing with evidence in poultry, the detrimental effect of high dilutions on pigeon semen quality was also manifested in the current study.

Semen quality of racing pigeons acquired from this study had similar characteristics to several previous reports including mean ejaculated volume (Cheng *et al.*, 2002; Sontakke *et al.*, 2004), mean sperm concentration (Klimowicz *et al.*, 2005) and semen pH (Sontakke *et al.*, 2004). The mean percentage of morphologically normal sperm (88.50 ± 3.39 %) was, however, higher than that reported by Sontakke et al.,

(2004) (75.2 ± 0.2 %) with predominant sperm abnormalities including the presence of amorphous head, bent tail and loosed head (Table 1 and Fig. 1.) These results implied adequate semen quality to other pigeon semen quality studies.

Similar immediate semen quality acquired from NSS and LRS diluted semen in each fold dilution implied their substitutability and comparability between each other for immediate semen quality evaluation. The result also indicated the contribution of dilution rate effect on semen quality and suggested 12.5- and 25-fold dilutions proper for semen quality evaluation in this study, of which the acquired percentages of sperm motility both in NSS and LRS were comparable to those previously reported (Sontakke *et al*., 2004). Interestingly, the acquired result noted a rapid decline in pigeon semen quality as early as 50-fold dilution—which was still a much lower dilution rate than that in other pigeon semen quality studies (Klimowicz *et al*., 2008; Klimowicz *et al*., 2005; Sontakke *et al*., 2004).

Adverse dilution effect is well-recognized in other avian species (Parker and McDaniel, 2002, 2004, 2006; Parker *et al*., 2002; Sexton, 1976). The harmful effects are believed to contribute to disturbance of seminal plasma's characteristics and the sperm's environment by the diluent, itself (Blesbois and de Reviers, 1992; Garner *et al*., 2001). Previous studies indicated that avian sperm motility is dependent on oxygen and ion concentration (Blesbois and de Reviers, 1992; Thomson and Wishart, 1991). Immediate change in gas exchange, ionic balance and ATP metabolism due to the altered of dilution rate can thus greatly impair sperm motility (Parker and McDaniel, 2006).

Alkalinity was also reported to enhance avian sperm velocity (Holm and Wishart, 1998). By means of this, the high acidity of NSS and LRS compared to that of pigeon semen was likely to reduce sperm motility, which was even more aggravated as fold dilutions increased. Moreover sperm motility could be even further inhibited by excessive ATP generation stimulated by the crystalloid diluent (Parker and McDaniel, 2006; Wishart, 1982, 1984). In addition to the higher alkalinity, LRS also had Ca^{2+} which was not present in NSS. The Ca^{2+} in LRS might have contributed to better sperm motility maintenance via enhancement of mitochondrial calcium cycling (Froman, 2013) and active sliding of microtubules (Ashizawa *et al*., 2013).

Despite utilizing 12.5- and 25-fold dilution, a decline of sperm motility was noticeable as early as half an hour of storage (Fig. 3-4). This supports the cruciality of immediate semen evaluation after collection similar to other previous studies (Klimowicz *et al*., 2008; Klimowicz *et al*., 2005). Interestingly, LRS was likely to maintain better semen quality over time compared to that provided by NSS. This was likely to contribute to higher acidity of NSS when compared with LRS—which resulted in a more rapid decline of sperm movement over time as previously described (Holm and Wishart, 1998; Singh and Davis, 2020).

In conclusion, semen diluted in NSS and LRS had the same immediate semen quality, however increasing fold-dilution beyond 50-fold or storing sperm rendered significant adverse effects on semen quality. This study thus suggests 12.5- to 25-fold dilution as suitable dilution rates for immediate pigeon semen quality evaluation.

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