Effect of different fractions of seminal plasma on the fertilizing ability of fowl spermatozoa stored *in vitro*

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Summary. This paper describes the effects of whole seminal plasma and of dialysed seminal plasma on the fertilizing ability of fowl spermatozoa stored for 24 h at 4°C. The fertilizing ability of fowl semen diluted 1:1 with Beltsville Poultry Semen Extender and stored for 24 h at 4°C was enhanced after replacement of the homologous seminal plasma by the diluent (89 versus 77% fertilization rate). Better results were obtained with seminal plasma dialysed against water before sperm storage to discard the <1 kDa or the <50 kDa fractions. It was concluded that low molecular weight seminal plasma fractions could damage the fertilizing ability of spermatozoa during storage at 4°C, whereas high molecular weight fractions appeared to enhance fertilizing ability.

Keywords: seminal plasma; fertilizing ability; fowl

Introduction

During short-term *in vitro* preservation, fowl semen is generally stored at 2–5°C after dilution with an artificial diluent. Using whole semen uncontaminated with the transparent fluid exuded from the lymph folds of the cloaca during ejaculation, Lake & Ravie (1979) and Van Wambeke (1984) obtained high fertilization rates (87–97%) after artificial insemination of hens with semen stored for 24 h at 4°C. In contrast, Blesbois (1986) and Sexton (1988) found that the fertilizing ability of fowl spermatozoa stored for 24 h at 4°C is impaired by the presence of seminal plasma. This occurs even if the transparent fluid is absent from the medium (Blesbois, 1986).

Preliminary results from our laboratory indicated that fresh semen diluted 1:1 in seminal plasma uncontaminated by the transparent fluid and inseminated within 30 min after the semen was collected, gave fertilization rates as high as those from semen diluted 1:1 in Beltsville Poultry Semen Extender (BPSE, Sexton, 1977). After semen was stored for 4 h at 4°C, the fertilization rates obtained with semen diluted in seminal plasma were 50% lower, whereas those obtained with semen diluted in BPSE were similar to those obtained with unstored semen.

The deleterious effects of seminal plasma may result from the accumulation of metabolic wastes (Riddle, 1968; Fujihara & Howarth, 1978). To prevent the accumulation of toxic metabolic products, Graham & Shangren (1986) dialysed whole semen during storage but could not retain the fertilizing ability of fowl spermatozoa. The purpose of the present paper was to determine whether the dialysis of seminal plasma only before storage could improve the fertilizing ability of spermatozoa stored for 24 h at 4°C with dialysed seminal plasma and a diluent.

Materials and Methods

Animals

Each experiment was conducted with 48 meat-type cockerels from ISA I99 which was originally derived from a Cornish strain for the males and 300 ISA Vedette for the females. The birds used were between 30 and 80 weeks old. They were housed in individual battery cages. The lighting regimen was 14 h light:10 h dark. All birds were fed the

same standard diet (11.7 MJ of metabolizable energy and 150 g crude protein per kg) in restricted amounts, i.e. 110 g per male and 120 g per female once a day to prevent excessive fattening of the birds.

Experimental design

Experiment 1. Breeders were force-moulted at 45 weeks of age and used for inseminations at the age of 75–80 weeks, i.e. at the peak of their second production period (laying rate 75%). The semen was used either fresh (undiluted or diluted) or stored for 24 h at 4°C after dilution.

The following three dilution treatments were applied. In the first treatment, semen was diluted 1:1 in Beltsville Poultry Semen Extender (BPSE, Sexton, 1977). In the second treatment, seminal plasma was replaced by BPSE (centrifuged for 10 min at 500 g) before dilution 1:1 in BPSE. For the third treatment, homologous seminal plasma was replaced by dialysed seminal plasma (see below) and diluted 1:1 in BPSE. During storage the pressure of oxygen of semen samples was 400 nmol O₂ ml⁻¹ as measured with a Clarke's electrode (Rank Brothers, Cambridge, UK).

Experiment 2. Breeders were 30–40 weeks old and their mean laying rate was 75%. They were inseminated either with fresh undiluted semen or with semen stored for 24 h at 4°C after dilution as follows: (i) dilution 1:1 with BPSE; (ii) centrifugation, resuspension of spermatozoa in BPSE up to the initial volume of semen followed by dilution 1:1 of this reconstituted semen in BPSE; (iii) as above, but the resuspension of spermatozoa was done in homologous dialysed seminal plasma (see below) with low molecular weight fractions removed (≤ 1 kDa); (iv) as above, low and mean molecular weight fractions (≤ 50 kDa) were removed from the homologous dialysed seminal plasma.

Semen collection. Semen was collected by massage (Burrows & Quinn, 1937). Care was taken to avoid any contamination of semen with the cloacal products and particularly with the transparent fluid. The presence of glucose in the ejaculate indicates contamination of semen by the transparent fluid (Lake, 1966). Glucose determination was therefore systematically performed on all samples (glucose analyser 2, Beckman, Palo Alto, CA, USA) so that any sample containing transparent fluid could be discarded. Ejaculates from 10–12 birds were mixed in the same collection tube, and constituted a 'pool'. Collection and mixing of these 10–12 ejaculates took about 5 min. The spermatozoa concentration of each 'pool' was evaluated using a calibrated photometer ($\lambda = 535$ nm) and was found to be $5\text{--}7 \times 10^9$ spermatozoa per ml.

Preparation of seminal plasma

Centrifugation. Seminal plasma was separated from spermatozoa by three successive centrifugations. The first centrifugation was performed within 10 min after semen collection, at 500 g and 20°C for 10 min. The supernatant was then centrifuged again under the same conditions, giving a second supernatant which was centrifuged at 3000 g and 4°C for 30 min. The final supernatant was defined as 'centrifuged seminal plasma'.

Dialysis. The centrifuged seminal plasma was then dialysed for 24 h at 4°C against double distilled water. Three types of membrane were used, type 1 for Expt 1 and types 2 and 3 for Expt 2 ('Poly-Labo'-BP36, 67023 Strasbourg Cedex, France). Type 1 was a 'Visking tube' with an approximate mean pore diameter of 240 nm allowing salts and the smallest molecules to be eliminated. Types 2 and 3 were more precisely calibrated membranes (Spectrapor 6) that can eliminate molecules of less than 1 kDa (type 2) or 50 kDa (type 3). After dialysis, the volume of seminal plasma was 20% above its original volume following the absorption of water through the membrane. The osmolality of the dialysed seminal plasma was therefore readjusted to 370 mosmol l⁻¹ by adding 30 to 35 g l⁻¹ N-tris[hydroxymethyl-2-aminoethane]sulphonic acid (TES). pH was adjusted to 7.2–7.3 by adding NaOH. The seminal plasma was then mixed with the centrifuged sperm until the initial semen volume was obtained. This reconstituted semen was then diluted 1:1 with BPSE before being stored at 4°C.

Artificial inseminations

The fertilizing ability of semen was evaluated after intravaginal artificial inseminations of 24 hens per treatment. Every hen was inseminated weekly for 5 to 7 weeks using 200×10^6 spermatozoa per dose. Eggs were collected on days 2 to 8 inclusive after each insemination (500 to 1200 eggs per treatment in each experiment). The fertility (fertilized eggs/incubated eggs $\times 100$) was determined by candling the eggs 7 days after the incubation started. The hatchability (hatched eggs/fertilized eggs $\times 100$) was determined after 21 days of incubation. Fertility and hatchability were compared using χ^2 tests (for review see Dagnelie, 1975).

Results

Experiment 1. Storage of spermatozoa without seminal plasma or with dialysed seminal plasma

After artificial insemination (Table 1) with fresh undiluted semen, the fertility of eggs was very high (95.5%). This rate was reduced when semen was diluted with BPSE (90%, $P < 0.05$). But this

reduction did not occur when the seminal plasma was eliminated or replaced by dialysed seminal plasma before dilution (95 or 94%, respectively, of fertilized eggs). The adverse effect of BPSE upon recently ejaculated sperm therefore probably results from an interaction between this diluent and some dialysable components of the seminal plasma.

Table 1. Effect of the presence or absence of whole or dialysed seminal plasma in the storage medium on the fertilizing rates of eggs laid 2 to 9 days after artificial insemination with fowl spermatozoa stored for 24 h at 4°C in BPSE* diluent

Treatment of semen	Fresh semen		Spermatozoa stored for 24 h at 4°C	
	Fertility	Fertilized eggs: incubated eggs	Fertility	Fertilized eggs: incubated eggs
Undiluted	95.5 ^a	600:628		
Diluted 1:1 with BPSE	90 ^b	508:565	72 ^d	411:569
Seminal plasma replaced by BPSE before dilution	95 ^a	545:572	79 ^c	498:631
Seminal plasma replaced by dialysed seminal plasma before dilution in hypertonic BPSE	94 ^{ab}	543:579	81 ^c	541:667

Values with the same superscript did not differ significantly ($P < 0.05$). *BPSE: Beltsville Poultry Semen Extender.

However, after 24 h of *in vitro* storage at 4°C, the fertility of diluted semen was reduced in all treatments. This fall was less marked when the seminal plasma was eliminated and replaced by BPSE alone or by dialysate + BPSE (79 or 81% of fertilized eggs, respectively) than when seminal plasma was still present (72% fertilized eggs). This results from a detrimental effect of seminal plasma on stored sperm.

Experiment 2. Seminal plasma dialysed at 1 or 50 kDa

As in Expt 1, the fertility (Table 2) was high when hens were inseminated with fresh undiluted semen (93.5%) and lower when whole semen was diluted with BPSE and stored for 24 h (77.5%). The replacement of seminal plasma by BPSE alone before storage still improved the fertilizing ability of sperm stored for 24 h (fertility: 89%). However, the highest fertilizing ability of stored spermatozoa was obtained when the original seminal plasma had been replaced by dialysed seminal plasma plus BPSE. The elimination of the seminal plasma fraction below 1 or 50 kDa before sperm storage resulted in 92 or 93%, respectively, of fertilized eggs, i.e. very near the percentage of fertilized eggs obtained with fresh undiluted semen in this experiment. The dialysis of seminal plasma before storage can therefore prevent the spermatozoa from losing their fertilizing ability.

The results obtained after storage were higher than in Expt 1 (77.7–89 versus 72–79%). This may result from the use of younger birds (30–40 weeks old) than in Expt 1 (75–80 weeks old), although the hens were used just after the laying peak in each case.

Discussion

The results presented here show that the seminal plasma and the interaction between the seminal plasma and the diluent influence the fertilizing ability of fresh and stored semen. According to Sexton (1977) and Blesbois (1986), the dilution of whole semen (spermatozoa plus seminal plasma) with an artificial diluent impairs the fertilizing ability of fresh semen. In addition, if spermatozoa are resuspended in the artificial diluent after the elimination of seminal plasma, the fertility is as high as the fertility obtained with fresh undiluted semen. These last two observations show that the

Table 2. Effects of seminal plasma dialysed at 1 kDa or 50 kDa on fertilization rates of eggs obtained 2–9 days after artificial insemination with fowl spermatozoa stored for 24 h at 4°C diluted 1:1 in BPSE* diluent

	Fertility	Fertilized eggs: incubated eggs
Fresh undiluted semen	93.5 ^a	1075:1147
Semen stored for 24 h at 4°C diluted 1:1 in BPSE:		
whole semen diluted with BPSE	77.5 ^c	873:1128
seminal plasma replaced by BPSE before dilution	88 ^b	970:1104
seminal plasma replaced by seminal plasma dialysed at 1 kDa	92 ^a	982:1070
before dilution in hypertonic BPSE		
seminal plasma replaced by seminal plasma dialysed at 50 kDa	93 ^a	992:1067
before dilution in hypertonic BPSE		

Values with the same superscript did not differ significantly ($P < 0.05$). *BPSE: Beltsville Poultry Semen Extender.

artificial diluent alone is not responsible for the decrease in fertility. The interaction between seminal plasma and the artificial diluent is responsible for the deleterious action of dilution on fresh semen.

The results obtained with stored semen clearly demonstrate that seminal plasma has a deleterious effect on spermatozoa stored *in vitro*.

Dialysis of seminal plasma before storage indicates that the deleterious effect results from the dialysable fraction of seminal plasma (molecular weight < 1000). This may be related to the presence in seminal plasma of toxic factors such as zinc (Blesbois & Mauger, 1989) and peroxides (authors' unpublished observations). The use of dialysed seminal plasma improves the fertilizing ability of stored sperm, which seems to indicate that larger molecules in seminal plasma (> 50 kDa) may improve the storage of spermatozoa.

Effect of seminal plasma on fresh semen

The composition of seminal plasma has been used as a basis for the earliest cock semen diluents (Lake, 1960). Seminal plasma has been reported to maintain the fertilizing ability of fresh diluted semen (Munro, 1938; Weakly & Shaffner, 1952) and especially highly diluted semen (Lake & Ravie, 1987). Moreover, seminal plasma stimulates the motility and the respiration of fowl spermatozoa (Fewless *et al.*, 1975; Terada & Watanabe, 1978; Ashizawa & Wishart, 1987; Wishart & Ashizawa, 1987). The results of our preliminary experiments and those of Sexton (1988) also indicate that the dilution of fresh semen with seminal plasma at a ratio of 1:1 does not change the fertilizing ability of fresh semen.

Effects of seminal plasma on *in vitro* storage of spermatozoa

The situation is more confusing for stored semen. Lake & Ravie (1979) and Van Wambeke (1967, 1984) reported high fertility (89–97%) for cock semen stored for 24 h at 2–5°C in semen containing homologous seminal plasma and artificial diluents.

The authors attributed their success partly to the lack of transparent fluid in the semen used. Sexton (1988), using seminal plasma containing some transparent fluid, reported a deleterious effect of stored seminal plasma on fresh and stored semen. This effect disappeared after filtration.

Our results were obtained with seminal plasma without any transparent fluid (attested by careful manipulations and glucose concentration). In these conditions, as in previous experiments (Blesbois & Mauger, 1987, 1989), seminal plasma is toxic to fowl spermatozoa stored for 4 h and 24 h at 4°C.

Most of the other reports on seminal plasma toxicity concern the resistance of spermatozoa to cold shock. Toxic effects of seminal plasma on the motility and morphology of thawed spermatozoa have been reported in many species including the ram (Schmehl *et al.*, 1988) and the bull

(Garcia & Graham, 1987a, b, c). In each case, the use of dialysis revealed that the deleterious effect of seminal plasma was located in the low molecular weight seminal plasma fraction (12–14 kDa for cocks and rams, 1 kDa for bulls).

Results of dialysis were obtained in original conditions since the storage of semen was at 4°C and fertility was tested. Results indicate that, in cocks, the deleterious effect of seminal plasma is located in the low molecular weight fraction (< 1 kDa) of seminal plasma. Moreover, the fraction of molecular weight higher than 1 or 50 kDa appears to be beneficial to the *in vitro* storage of fowl spermatozoa. The same tendency has been previously reported by Garcia and Graham (1987c) concerning the resistance of bull semen to freezing. Storage of cock semen with dialysed seminal plasma (1 or 50 kDa) therefore led to fertilization rates as high as those obtained with fresh semen.

In conclusion, the present study emphasizes the complexity of the role of seminal plasma and its duality of effects on the storage of fowl semen. Seminal plasma appears to contain fractions of molecular weight higher than 50 kDa that are beneficial to the *in vitro* storage of spermatozoa and, in contrast, fractions of molecular weight lower than 1 kDa which are toxic to *in vitro* storage of spermatozoa.

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