Effect on fertility of storing fowl semen for 24 h at 5°C in fluids of different pH

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Summary. Improved storage of fowl semen above 0°C was achieved by adjusting the pH of the diluent. The fertility obtained with semen stored for 24 h at 5°C in diluents buffered at different pH values was compared with that of semen stored in a basic, unbuffered solution. The most satisfactory result was achieved with diluent buffered at pH 6·8 or 7·1. Worst fertility was obtained at pH 5·8 and pH 7·4 did not prove very satisfactory. There were indications that the effect of pH under the conditions of the experiment was to regulate metabolism and thereby influence the maintenance of the fertilizing ability of the spermatozoa.

Introduction

Early experiments on the storage of fowl semen reported the use of simple diluents, the compositions of which were based upon the content of certain inorganic ions and glutamic acid found in seminal plasma but with added fructose (Lake, 1958, 1960). Such diluents have been modified through the years and used for artificial insemination (AI), but they are not consciously buffered with conventional salt mixtures. However, the fertility achieved has been reasonably satisfactory after storing semen for up to 24 h. Van Wambeke (1967, 1972) reported better fertility results with fowl semen stored for a similar period using a more complex diluent containing milk and egg albumen. However, the hens he used were preselected for fertility and higher doses of diluted semen and thus greater numbers of spermatozoa were inseminated than in the work of Lake (1958, 1960).

Winchester & McKenzie (1941) demonstrated an influence of pH *per se* on the metabolic rate of ram and boar spermatozoa and similar interactions have been reported for fowl (Lardy & Phillips, 1943) and oyster (Humphrey, 1950) spermatozoa. Willett & Ohms (1958) and Salisbury & Kinney (1957) discussed the relationship between pH and lactic acid production *in vitro* as a factor influencing the motility and metabolism of bull spermatozoa. These authors suggested that spermatozoa might be inactivated by acid or a low pH during storage and reactivated by alkalinization before insemination. This is an interesting possibility for improving semen storage techniques. However, the composition of diluents, the types and strength of buffer salts, the semen dilution ratio, the storage temperature and duration of storage are all factors which could interact in particular circumstances to influence the activity of spermatozoa and the degree of acid production and pH changes in the medium during the period of storage. These interactions would have to be recognized and considered in attempting to use diluents with different initial pH values to manipulate the survival of spermatozoa and it is feasible that variations in fertility results in different laboratories where such methods have already been attempted are due to a lack of appreciation that such interactions may occur.

Recent work on the metabolism of fowl and turkey spermatozoa (McIndoe & Lake, 1973; Lake & McIndoe, 1976; unpublished observations) has prompted a re-examination of the importance of the buffering properties of a diluent and the significance of acid production during

24 h storage of semen with respect to retaining the fertilizing ability of the spermatozoa. It was considered that the use of pH as a means of regulating sperm activity and the retention of fertilizing ability during 24 h storage should be re-examined to see if better fertility could be achieved by modifying our unbuffered solutions (Lake, 1958, 1960). These had given better fertility than that obtained when diluents of different pHs were explored previously (Bogdonoff & Shaffner, 1954; Wilcox & Shaffner, 1957).

Materials and Methods

Animals. The 240 hens and the semen donors were of a commercial layer-type. All birds were maintained on a commercial breeder's ration and fed *ad libitum*. They were given 14 h light/24 h.

Semen treatment. Semen, uncontaminated with transparent fluid, was obtained by massage (Lake, 1957). The males were on a regular routine of semen collection and were not rested for more than 4 days before collections of semen were required for the experiments. This schedule was to minimize the number of degenerate spermatozoa in ejaculates (El Jack & Lake, 1966).

After collection the semen samples were pooled immediately and diluted (1:1 v/v) with diluent within 10 min. Both diluent and semen were at room temperature (18–22°C) when mixed. The diluents, at 4 different pHs and one unbuffered (Table 1), were tested simultaneously on split samples of semen. The biological buffers were chosen with a pK around the pH it was desired to maintain in order that the concentration of the buffer could be minimized and a more effective buffer medium obtained at each level. The pH of the fresh diluents and aliquots of the diluted semen after storage was measured with a pH meter using a combined reference and glass electrode suitable for handling small volumes of fluid (0.3 ml).

Table 1. Composition of the diluents used for storing fowl semen for 24 h at 5°C

Compound	Diluents				
	Basic (A)	7.4	7.1	6.8	5.8
Sodium glutamate · H ₂ O	0.96	0.76	0.76	0.66	0.98
Tri-potassium citrate · H ₂ O	0.064	0.064	0.064	0.064	0.064
Magnesium acetate · 4H ₂ O	0.04	0.04	0.04	0.04	0.04
Sodium acetate (anhydrous)	0.255	—	—	—	—
Glucose	0.3	0.3	0.3	0.3	0.3
1 N-NaOH	—	2.5 ml	2.9 ml	4.5 ml	1.22 ml
BES*	—	—	1.525	—	—
MES†	—	—	—	1.22	1.17
TES‡	—	1.425	—	—	—
Δ(mosmol/kg H ₂ O)	360	378	411	395	388
pH	7.23	7.4	7.05	6.8	5.8

Values are in g, unless otherwise stated, dissolved and made up to 50 ml with distilled water.

* NN-Bis (2-hydroxyethyl)-2-aminoethane sulphonic acid.

† 2-(N-Morpholino) ethane sulphonic acid.

‡ N-Tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid.

The freezing-point depression of fresh diluents was measured with an Advanced Osmometer, model 31 LAS. The osmotic pressures of the diluents were hypertonic to seminal plasma but within the range previously found not to affect adversely the retention of the fertilizing ability of fowl spermatozoa *in vitro* (Wilcox & Shaffner, 1957; Hobbs & Harris, 1963; Van Wambeke, 1977).

The diluted semen was placed in a refrigerator (5°C) after mixing, and allowed to cool gradually to 5°C, before being placed in a thermostatically controlled bath at 5°C for 24 h.

Each batch of diluted semen (about 1.7 ml) was stored in a round-bottomed, thin-walled tube measuring 7.5×1 cm which was plugged lightly with tissue paper.

After storage the motility of spermatozoa in each stored semen sample was observed in a hanging drop and the morphology of stored spermatozoa was examined in nigrosin–eosin smears (Lake & Stewart, 1978). The detailed results of these examinations are outside the scope of this paper.

Insemination procedure and design of experiment

The number of spermatozoa in a semen sample was determined using an electronic Coulter Counter (Model Fn). The optimum conditions for counting fowl spermatozoa were as follows: attenuation setting, 0.354; aperture setting, 16.0; threshold setting, 12.0; 50 μ m diameter orifice; dilution $\times 11\,000$ with 1% NaCl solution containing 0.1% sodium azide. The density of fowl spermatozoa varies between 4 and 6×10^6 /ml so that with an insemination dose of 0.06 ml of diluted semen each hen received between 120 and 180×10^6 spermatozoa.

Eggs were collected for 14 days after insemination. Fertility was estimated by candling between Days 4 and 7 of incubation; batches of eggs were held for 4 days before placing in the incubator. The fertility was expressed as the percentage of viable embryos; any cracked unincubated eggs were broken open and the blastoderms inspected for evidence of fertilization.

A population of caged hens received treated semen sequentially over a 15-month period; each treatment was repeated at least 5 times. At each insemination session the individuals were randomly assigned to treatment groups with respect also to their position in row and level of tier. Since the treatments were repeated several times simultaneously the possibility of seasonal or age effects contributing to the treatment results were eliminated.

At each AI session aliquots of the differently treated semen samples were inseminated consecutively and in rotation, rather than as one batch to eliminate any possible effect of holding time of diluted semen after storage.

The percentage of fertile eggs laid by each hen during Days 2–8 and 9–15 after insemination and the mean percentages for these periods for each treatment group were calculated. Percentage data were transformed to arc sine values for the analyses of variance. These were performed on the fertility data obtained for each session of semen administration and on the combined data for each treatment. In each case the pooled error mean square was used to test the level of significance of the differences between treatments by Student's *t* test.

Results

The most satisfactory fertility was achieved with the diluent buffered at pH 6.8 or 7.1 with an indication that the latter was best. The worst fertility was obtained after storage at constant pH 5.8 (Tables 2 and 3). The differences in fertility between split samples of semen stored at different pHs on separate occasions over the 15-month period showed relationships in general agreement with the analyses of the combined data.

The pH in the unbuffered diluted semen samples after 24 h was similar to that of the solution composed to remain at pH 5.8 throughout the entire period, and varied between pH 5.6 and 6.0, the variability presumably being due to different densities of spermatozoa in the replicated samples.

In a separate test the time course of the change of pH in semen diluted with the unbuffered solution was examined (Text-fig. 1); the spermatozoa were in a medium with a pH < 7 for only about half the 24-h period. The pH was also measured, after storage only, in 6 separate aliquots of semen diluted in each of the other media. On average, the pH in these samples after 24 h dropped by the following amounts; pH 7.4, 0.31 units; pH 6.8, 0.2 units; pH 7.1, 0.12 units.

Table 2. The numbers of fertilized eggs produced during Days 2–8 and 9–15 inclusive after single inseminations of semen stored for 24 h at 5°C in diluents of different pH

		Diluent					
		A*	5.8	6.8	7.1	7.4	B†
Total no. of hens		132	85	106	94	85	151
No. of hens producing ≥ 2 fertilized eggs		120	32	100	91	79	146
Days 2–8	Eggs of all hens	621/785 (81.9)	129/438 (29.5)	534/617 (86.6)	473/544 (87.0)	354/479 (73.9)	837/899 (93.1)
	Eggs of hens producing ≥ 2 fertilized eggs	608/676 (89.9)	107/189 (56.6)	531/581 (91.4)	462/505 (91.5)	337/414 (81.4)	831/865 (96.1)
Days 9–15	Eggs of all hens	187/769 (24.3)	24/470 (5.1)	182/581 (31.3)	170/562 (30.2)	86/481 (17.8)	514/892 (57.6)
	Eggs of hens producing ≥ 2 fertilized eggs	185/680 (27.2)	22/324 (6.8)	182/536 (34.0)	168/513 (32.7)	83/411 (20.2)	513/848 (60.5)

Values are total no. of eggs fertile/total no. of eggs laid, with the no. in parentheses representing the mean percentage fertility of the results from all AI sessions.

* Unbuffered basic solution.

† Unbuffered basic solution, freshly diluted.

Table 3. *P* values denoting levels of significance for the comparisons of fertility from the data in Table 2

		Diluents					
	Diluents	B	A	7.4	7.1	6.8	5.8
Days 2-8, all hens	B	—	0.001	0.001	N.S.	0.05	0.001
	A	0.001	—	N.S.	0.1	N.S.	0.001
	7.4	0.001	N.S.	—	0.1	N.S.	0.001
	7.1	N.S.	0.1	0.1	—	N.S.	0.001
	6.8	0.05	N.S.	N.S.	N.S.	—	0.001
Days 2-8, hens producing ≥ 2 fertilized eggs	B	—	0.001	0.001	0.01	0.02	0.001
	A	0.001	—	0.001	N.S.	N.S.	0.001
	7.4	0.001	0.001	—	0.001	0.001	0.001
	7.1	0.01	N.S.	0.001	—	N.S.	0.001
	6.8	0.02	N.S.	0.001	N.S.	—	0.001
Days 9-15, all hens	B	—	0.001	0.001	0.001	0.001	0.001
	A	0.001	—	0.02	0.1	0.05	0.001
	7.4	0.001	0.02	—	0.001	0.001	0.001
	7.1	0.001	0.1	0.001	—	N.S.	0.001
	6.8	0.001	0.05	0.001	N.S.	—	0.001

The pH rose by 0.06 units in the medium at pH 5.8 and this could be explained by the lack of motility of the spermatozoa in this medium and an associated depression of glycolysis and lactic acid production (unpublished observations). The, as yet, unknown factors leading to the tendency for a rise in pH in undiluted fowl semen (Wilcox, 1958) were presumably not sufficiently counteracted by the products of the very low metabolism of the spermatozoa in the acid pH 5.8 medium.

Discussion

Under the conditions of the present study diluents of pH 6.8 or 7.1 were the most satisfactory for maintaining the fertilizing ability of fowl spermatozoa during 24 h at 5°C. This is in general

Text-fig. 1. Time course of the change in pH in unbuffered diluent A-semen mixture over 24 h at 5°C. The initial pH of the diluent is indicated by the broken line.

agreement with earlier observations (Bogdonoff & Shaffner, 1954; Wilcox & Shaffner, 1957). Wilcox & Shaffner (1957) and Wilcox (1959) stored fowl semen in simple phosphate buffers and found small variations in fertility when semen was stored at between pH 6.47 and 7.95. However, the overall fertility was better in the present work than in the studies cited above, perhaps partly due to other factors in the diluents.

The best fertility obtained by us after 24 h was very satisfactory. The lower fertility obtained with those samples stored at pH 6.8 and 7.1 compared with the freshly diluted control is most probably accounted for by the death of a proportion of the spermatozoa in the former cases (unpublished observations) and thus it is highly probable that raising the insemination dosage would improve the fertility after storage, perhaps to the level of the control. This would make the method of storage a practical proposition for a breeder. The high fertility produced during Days 9–15 after insemination with the freshly diluted semen (Table 2) is presumably a reflection of the high number of viable spermatozoa in this inseminate.

The results of the present study suggest that the question of whether or not there is an optimal pH for maintaining the fertilizing ability of fowl spermatozoa *in vitro* is not a simple one. Obviously the often assumed 'physiological pH 7.4' is not ideal for the purpose. Studies reporting the optimal pH for sustaining maximum motility and/or some straightforward feature of metabolic activity, e.g. oxygen consumption, are not helpful in this respect. Maximum motility of spermatozoa was observed in those samples stored at pH 7.4. Only moderate motility was seen in those stored at 6.8 and 7.1 and even less in the unbuffered medium. These traits did not correlate with fertility. However, the samples at pH 5.8 were immotile and the fertility was correspondingly poor. In this case there was thus an apparent positive correlation between motility and fertilizing ability. However, the morphological condition of the majority of these spermatozoa, as seen in nigrosin-eosin smears, was good and comparable with those samples stored at pH 6.8 and 7.1. This suggests that, if the motility could be revived after storage at pH 5.8, good fertility might be obtained and this will be the subject of a future study. These observations re-emphasize the well known fact that there is not always a simple relationship between motility of spermatozoa and fertilizing ability of semen samples (Wilcox & Shaffner, 1957). However, the direct effect of pH may have been to regulate the activity of the spermatozoa; at pH values below 7.4 the motility of the cells in hanging drops was progressively reduced and was virtually nil in the pH 5.8 samples. In the unbuffered solution, with a changing pH during storage (Text-fig. 1), motility was low after 24 h and a proportion of the spermatozoa were morphologically damaged. Similar effects of low pH have been observed before (Bogdonoff

& Shaffner, 1954; Hobbs & Harris, 1963), albeit in sodium citrate solution which in itself has a tendency to reduce motility. In Van Wambeke's (1967) experiments the pH of diluted semen was about 6.25–6.65 after 24 h storage at 2–5°C and good levels of fertility were obtained.

It could be argued that the differences in osmotic pressure between the diluents of different pH (Table 1) may have partly influenced the fertilizing ability of the diluted semen after storage. However, this was not considered likely since there appeared to be no obvious correlation between the fertility trends and osmotic pressure of the diluents within the range covered. Also, previous work (Hobbs & Harris, 1963; Van Wambeke, 1977) showed that no serious effects on fertility were obtained using diluents with osmotic pressures ranging from 340 to 460 mosmol/kg H₂O. However, Hobbs & Harris (1963) used simple sodium citrate-citric acid solutions to vary pH and osmotic pressure simultaneously and showed that the highest fertility was obtained at 425 mosmol/kg H₂O with a pH 6 or 7. However, the overall fertility levels in the study of Hobbs & Harris (1963) were not as high as in the present work even with a higher insemination dosage. In past work, tonicity has not been varied without altering other chemical and physical characteristics of a fluid medium and this will have to be borne in mind in any future studies of this type to determine optimal tonicity *per se* for preserving the integrity of spermatozoa.

In conclusion, the pH for retaining fertilizing ability of fowl spermatozoa *in vitro* appears to be slightly lower than that reported to be optimal for maximizing features of metabolism (Lardy & Phillips, 1943; Bogdonoff & Shaffner, 1954). Amongst other things, there is presumably an optimum rate of metabolism to be encouraged, above and below which the retention of the functional status (ability to fertilize) of spermatozoa is affected. Control of this by regulation of the composition of the diluent would benefit the practice of poultry semen storage and artificial insemination.

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