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An Improved Diluent for Long-Term Liquid Storage of Chicken Semen

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محلول مخفف لخزن السائل المنوي للدجاج لفترات أطول

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خلاصة: تم تطوير تركيبة محلول لتخفيف السائل المنوي للدجاج تأسيساً على نتائج دراسات مبدئية. أشارت هذه الدراسات إلى إمكانية إستخدام مادة القلوميت بدلاً عن مادتي الأسطتيت الفوسفين و التي تستخدم عادة في محاليل تخفيف السائل المنوي للدجاج. لقد حسن هذا الإستبدال مستويات خصوبة ما بعد التلقيح للسائل المنوي للدجاج بعد الخزن (in vitro) لفترة 24–96 ساعة. وأشارت تجربة أولية أخرى إلى إمكانية إحلال البوتاسيوم محل الصوديوم المستخدم عادة في هذه المحاليل بدون أي تأثير سلبي على مستوى الخصوبة. أظهرت هذه الدراسة أيضاً أن إحلال المحاليل الحيوية الثنائية BES و BIS-TRIS محل المحاليل الحيوية الأحادية في تخفيف السائل المنوي للدجاج له تأثير معاكس على حموضة المخفف و التي عندها يمكن معادلة الحموضة بدون تغير في الإسمولارية أو التركيب الكيميائي. تم تخفيف السائل المنوي الذي جمع من الذكور السائدة للسلالة المعلمة من مقاطعة مينوسوتا الأمريكية بنسبة 1:1 في مخففات ثم خزنت (in vitro) لفترة 96 ساعة تحت درجة حرارة قدرها 4 درجات مئوية، و بعدها تم تلقيح 24 دجاجة بهذه المخففات. أظهرت الدراسة بأن الإسمولارية بين 265–339 موسم لم يغير في مستويات الإخصاب. أشار التحليل الإحصائي إلى وجود زيادات معنوية في نسبة الإخصاب نتيجة لاستخدام المخففات المحفوظة بالطرق التقليدية عما هو محفوظ في BPSE-1. هناك حاجة لإجراء المزيد من الدراسات لتحديد أسس تفوق السائل المخفف الذي تم تطويره على BPSE-1.

ABSTRACT: A prototype chicken semen diluent formulation has been developed based on the results of preliminary experiments. Preliminary experiments indicated the substitution of glutamate for the acetate and phosphate generally present in chicken semen diluents improved post-insemination fertility levels obtained from chicken semen following 24-96 hours of in vitro storage. Additional preliminary experiments indicated potassium could be substituted for the sodium generally contained in chicken semen diluents without affecting post-insemination fertility levels. The dual biological buffers, BES and BIS-TRIS, that replace the single biological buffer sometimes contained in chicken semen diluents have opposing effects on the diluent's pH and permit adjustment of the prototype diluent's pH to a range of values without altering either its osmolarity or chemical composition. Semen collected from males of the Minnesota Dominant Marker Line was diluted 1:1 in the prototype diluent and stored in vitro for 96 hours at 4°C. Groups of twenty-four hens were inseminated with the stored semen samples. Osmolarities from 265-339 mOsm did not affect post-insemination fertility levels obtained from semen stored in the prototype diluent. The proportion of fertile eggs obtained post-insemination from semen stored in the prototype diluent was significantly greater than that obtained from chicken semen stored in BPSE-I in the Chi-square test. Further investigations are necessary to determine the basis for the superiority of the newly-developed poultry semen diluent formulation to BPSE-I.

Keywords: chicken semen, diluent, storage, fertility level, glutamate.

Whole chicken and turkey semen produces satisfactory post-insemination fertility levels for only short periods following semen collection (Lake and Ravie, 1982; Bakst, 1990; Bootwalla and Miles, 1992; Christensen, 1995; Etches, 1996). On the other hand, whole chicken (Lake, 1960; Van Wambeke, 1967) and turkey semen, diluted in an appropriate poultry semen diluent immediately after collection, can be stored *in vitro* for up to 24 hours with no decline in post-insemination fertility levels. The diluents that allow

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poultry semen to be stored for long periods commonly contain sodium, potassium, glutamate, phosphate, acetate and citrate. However, the levels of these components differ greatly among published formulations and the literature contains little empirical information to indicate the optimum concentrations of these components for maintaining fertility at maximal levels during *in vitro* storage.

The primary purpose of this work was to develop a high efficacy chicken semen diluent based on the results of preliminary experiments. The replacement of acetate and phosphate contained in BPSE-I (Beltsville Poultry Semen Extender), with glutamate, improved fertility levels following prolonged *in vitro* storage periods in preliminary experiments performed in this laboratory. Preparatory studies showed no adverse effect of replacing the sodium contained in BPSE-I with potassium and no beneficial effect of adding more than 2 g/l of fructose. Therefore, a prototype poultry semen diluent was formulated based on the results of the preliminary experiments. The effect of adjusting the diluent to various osmolarities on fertility following storage was determined and the performance of the prototype chicken semen diluent was compared with the performance of BPSE-I.

Materials and Methods

CHICKENS. The adult males that provided semen for all experiments were of the Minnesota Dominant Marker Line (Shoffner *et al.*, 1993). The line was kindly provided by Dr. Robert N. Shoffner formerly of the University of Minnesota, St. Paul, Minnesota. Hens were of the Lohman Brown strain (Lohman, Cuxhaven, Germany).

DILUENTS. The diluents prepared for preliminary experiments (results not shown), designed to determine the effect of chemical component substitutions (*e.g.*, glutamate for acetate) on post-insemination fertility levels obtained from stored semen samples were BPSE-I (Sexton, 1977), BPSE-II (Sexton, 1982) and modifications of both of these formulations. The diluents prepared for the experiment shown in Table 2 were the prototype chicken semen diluent formulation based on the results of the preliminary experiments and described in Table 1, and BPSE-I.

OSMOLARITY MEASUREMENTS. Diluent osmolarity was measured with a Model 3C2 Advanced Cryomatic Osmometer (Advanced Instruments Inc., Needham Heights, Massachusetts, USA).

SEMEN COLLECTION AND STORAGE. Minnesota Dominant Marker Line male semen was collected into 17 X 100 mm disposable plastic culture tubes (cat. no. #14-956-6D, Fisher Scientific, Pittsburgh, PA) with the abdominal massage method of Burrows and Quin (1935). Immediately after collection the pooled semen was dispensed in 1 ml volumes into disposable plastic culture tubes containing 1 ml of either the prototype diluent described in Table 1 or BPSE-I. The culture tubes were capped loosely to allow gas exchange. The length of tube containing the diluted semen sample was submerged in a 500 ml volume of 22°EC water and the submerged samples were placed in a 4°EC cold room for 96 hours prior to AI.

TABLE 1

Chemical composition of the prototype chickensemen diluent formulated on the basis of preliminary experiments.

Component	g/l	mM
Potassium glutamate	28.0 - 36.0	151.19 - 194.38
Fructose	2.00	11.10
Magnesium sulfate•7H_2O	0.20	1.67
BES free acid	1.07	5.00
BIS-TRIS free base	1.05	5.00
Phenol red	.005	-
pH		6.75

Addition of 28.0 and 36.0 g/l of potassium glutamate to the above diluent produces 265 and 339 mOsm solutions, respectively.

INSEMINATION. The diluted semen samples were administered intravaginally in 25 μl volumes per hen to groups of twenty-four hens with the method of Burrows and Quin (1935).

PREINCUBATION STORAGE AND INCUBATION. Eggs were stored at 55°EF prior to the onset of incubation. Incubation began on the final day of the egg collection period. The eggs were incubated at 99.7°EF in a Western Model TWO-8 Incubator with the wet bulb

TABLE 2

Effect of five osmolarities from 265-339 mOsm on Day 2-8, Day 9-15, and Day 2-15 post-insemination fertility levels obtained from chicken semen following 96 hours of in vitro storage.

Measured Osmolarity (mOsm)	No. Eggs		No. Fertile		% Fertile		2-15
	2-8	9-15	2-8	9-15	2-8	9-15	
265	124	127	62	18	50	14	32
288	137	127	66	17	48	13	31
303	130	136	69	22	53	16	34
321	132	130	66	15	50	12	31
339	131	124	52	9	40	7	24
265-339	654	644	315	81	48[a]	13	31[a]
BPSE-I	117	124	15	3	13	2	7

The 265, 288, 303, 321, and 339 mOsm solutions contained 28, 30, 32, 34, and 36 g/l of potassium glutamate, respectively.
[a]Significantly greater ($P < 0.01$) than the fertility levels produced with BPSE-I during the same post-insemination period.

reading maintained between 83-86°EF (~50-55 % relative humidity).

FERTILITY DETERMINATION AND DATA ANALYSIS. Eggs were broken and examined on Day 6 of incubation to determine whether they were fertile. Data was analyzed with the Chi-Square test (Snedecor and Cochran, 1989).

Results

FORMULATION OF A PROTOTYPE CHICKEN SEMEN DILUENT. Potassium glutamate was chosen as the major component to provide osmotic balance to the prototype chicken semen diluent because preliminary experiments indicated that replacement of the acetate and phosphate in BPSE-I with glutamate resulted in an improved diluent that produced greater post-insemination fertility levels than BPSE-I. Moreover, replacement of sodium in BPSE-I with potassium did not affect post-insemination fertility levels. The literature contains conflicting evidence showing the superiority of either fructose or glucose (Christensen, 1995) and fructose was chosen for addition to the prototype diluent until the sugar that maximizes post-insemination fertility levels obtained from stored semen is identified in future experiments. Fructose was added at twice the concentration of glucose in many cell and tissue culture media, 11.1 mM (2 g/l). Chicken semen contains only small quantities of sugars (Lake and Wishart, 1984) and the fructose concentration in 1:1 dilutions of chicken semen in the prototype diluent approximates the glucose concentration commonly contained in both mammalian and avian culture media. The addition of more than 2 g/l of fructose to the prototype diluent in a preliminary experiment did not improve post-insemination fertility levels obtained from stored semen.

The magnesium requirement of chicken spermatozoa during *in vitro* storage could not be determined from the literature (see Discussion) and 1.67 mM magnesium sulfate was added to the prototype diluent until the effect of magnesium on post-insemination fertility levels is determined in future studies. Citrate had detrimental effects on the efficacy of a chicken semen diluent (Lake and Ravie, 1981) and it was thus omitted from the prototype diluent. Equal concentrations of the biological buffers BES free acid and BIS-TRIS free base are contained in the prototype diluent and phenol red serves as the pH indicator. The complete chemical composition of the prototype chicken semen diluent is given in Table 1.

EFFECT OF OSMOLARITY ON FERTILITY AND COMPARISON WITH A PUBLISHED FORMULATION. Table 2 summarizes an experiment performed to identify the osmolarity that maximizes post-insemination fertility levels obtained from chicken semen stored in the prototype diluent and compares the efficacy of the prototype diluent with that of BPSE-I. Day 2-8, Day 9-15 and Day 2-15 post-insemination fertility levels produced with chicken semen samples, stored in aliquots of the prototype diluent, adjusted to five osmolarities in a 265-339 mOsm range, were not significantly different ($P < 0.05$) in the Chi-square test. On the other hand, post-insemination fertility results obtained from semen stored in the prototype diluent adjusted to all five osmolarities were pooled and Day 2-8 and Day 2-15 post-insemination fertility levels were significantly greater ($P < 0.01$) than Day 2-8 and Day 2-15 post-insemination fertility levels obtained from semen stored in BPSE-I in the Chi-square test.

Discussion

The prototype chicken semen diluent developed in this study is clearly superior to BPSE-I with respect to the post-insemination levels of fertility obtained from chicken semen following prolonged periods of *in vitro* storage and is likely to help fill a need in the poultry industry for improved poultry semen diluents (Christensen, 1995). The prototype diluent has other useful features in addition to its beneficial effects on the length of storage periods and post-insemination fertility levels. The pH of the prototype chicken semen diluent can be adjusted without altering its osmolarity or chemical composition and the diluent is relatively easy and inexpensive to prepare since it contains fewer and less expensive components than many other comm poultry semen diluents. Additional studies are neede to determine the biological basis for the superiority of the prototype chicken semen diluent.

Freezing point depressions of chicken semen diluents have been varied over a wide range with no affect on post-insemination fertility levels obtained from stored chicken semen (Wilcox and Shaffner, 1957; Harris and Hobbs, 1968) and osmolarities from 300-400 mOsm do not affect post-insemination fertility levels obtained from stored turkey semen (Graham and Brown, 1971). Likewise, five osmolarities in a 265-339 mOsm range did not affect post-insemination fertility levels obtained from chicken semen stored in the prototype diluent. Osmolarities that maximize percent hatch of fertile chicken and turkey eggs depend on the diluent's chemical composition (Harris *et al.*, 1963; Hobbs and Harris, 1963a and b; Harris, 1968) and the osmolarity that will maximize the percent hatch of fertile eggs obtained from chicken semen stored in the prototype diluent could not be obtained from literature values. Many of the semen diluents designed for long term storage of chicken semen are isotonic, ~340 (Van

Wambeke, 1967), or hypertonic to chicken seminal plasma (Lake and Ravie, 1982; Bakst, 1990; Bootwalla and Miles, 1992; Christensen, 1995; Etches, 1996). However, unpublished results from this laboratory indicate the prototype chicken semen diluent produces maximum percent hatches of fertile chicken eggs if its osmolarity is adjusted to 303 mOsm (the addition of 32 g/l of potassium glutamate to the formulation given in Table 1).

Magnesium is a co-factor for hundreds of enzymes including all that require ATP and it is required for anaerobic breakdown of glucose, protein synthesis and the motility of spermatozoa. However, the literature contains little information on the magnesium requirement of chicken spermatozoa during *in vitro* storage. The addition of 3.33 mM magnesium had no effect on post-insemination fertility levels and the addition of 10 mM magnesium slightly reduced post-insemination fertility levels in a previous study (Wilcox and Wilson, 1961). Yet, diluents designed for long term liquid storage of chicken semen often contain magnesium. Solutions that contain both calcium and high concentrations of phosphate have been shown to precipitate magnesium (Brink *et al.*, 1992) and the presence of seminal plasma calcium and high concentrations of diluent phosphate may have the potential to precipitate diluent magnesium from semen samples diluted and stored in some published chicken semen diluent formulations. For example, chicken semen diluted 1:1 in BPSE-I contains 0.84 mM diluent magnesium, 0.7 mM seminal plasma calcium (Lake and Wishart, 1984), and 60 mM diluent phosphate. The magnesium requirement of chicken spermatozoa during *in vitro* storage could not be established from the literature and 1.67 mM magnesium sulfate was added to the prototype chicken semen diluent until future studies determine the concentrations of magnesium that maximize post-insemination fertility and hatchability levels obtained from chicken semen samples following *in vitro* storage.

The pH of the prototype diluent can be raised or lowered without affecting its osmolarity since the diluent contains dual biological buffers with opposing effects on its pH. A 1 M solution of BIS-TRIS free base has a pH of approximately 9-11 and a 1 M solution of BES free acid has a pH of approximately 3.5-5.0. The prototype diluent contains equal amounts of BES and BIS-TRIS and has an intermediate pH of 6.75. The prototype diluent's pH can be raised to 6.75 with the addition of a solution prepared according to Table 1 with the exception that it contains 10 mM BIS-TRIS and no BES. The pH of the prototype diluent can be lowered to 6.75 with the addition of a solution prepared according to Table 1 with the exception that it contains 10 mM BES and no BIS-TRIS. The diluent's osmolarity is not affected by the pH adjustments since the osmolarities of the two solutions for adjusting its pH are identical to the osmolarity of the prototype diluent. Likewise, the pH of the prototype diluent can easily be adjusted to various values above or below 6.75 without affecting the diluent's osmolarity or chemical composition for experiments designed to determine the effect of diluent pH on post-insemination fertility and hatchability or for other experimentation requiring alteration of the pH.

These studies do not reveal the nature of the beneficial effect of potassium glutamate on chicken spermatozoa that enables greater post-insemination fertility levels to be obtained following *in vitro* storage. Glutamate chelates calcium ions (Martel, 1964; Miyazake *et al.*, 1974; Durham, 1983; Blaquiere and Berthon, 1987; Gu and Huang, 1991) which are toxic to chicken and turkey spermatozoa (Christensen, 1995), and heavy metal ions (Martel, 1964) that have toxic effects on animal cells, including blockage of ion channels and enzyme inactivation (Kiss and Osipenko, 1994). However, levels of glutamate that were beneficial for *in vitro* storage of chicken semen in the work reported here (151-194 mM) were considerably higher than the low levels that would be required to chelate the toxic ions present in stored chicken semen samples (the 2-4 mM citrate concentrations in several formulations for long term storage of chicken semen are apparently sufficient to chelate the calcium ions present in stored samples and eliminate their detrimental effects on post-insemination fertility levels). Finally, glutamate has been shown to both activate and up-regulate sodium-potassium ATPase in animal cells (Segal, 1981; Inoue and Matsui, 1990; Fukuda and Prince, 1992; Brines and Robbins, 1993; Marcaida *et al.*, 1996; Pellerin and Magistretti, 1997). However, both the function of the sodium potassium ATPase (Russell and Chambers, 1976) and protein synthesis are inhibited during storage at 4°EC.

Wilcox and Shaffner (1958) developed a simple semen diluent formulation containing only sodium phosphate and fructose that produced high levels of fertility following prolonged *in vitro* storage periods. The major difference between the prototype chicken semen diluent developed in this study and the Wilcox and Shaffner formulation is that potassium glutamate was the major component of the diluent developed for this study and sodium phosphate was the major component of the Wilcox and Shaffner formulation. Lake (1960) also developed a chicken semen diluent that produced high levels of fertility following prolonged *in vitro* storage periods. The glutamate concentration in Lake's Solution approached the glutamate concentration in the prototype chicken semen diluent developed in this study. Direct comparisons have not been made in this laboratory between the prototype chicken semen diluent

TABLE 3

Results obtained with the prototype chicken semen diluent and BPSE-I in this study alongside the results of three previous studies reporting the development of new chicken semen diluent formulations. The results show a gradual improvement in the efficacy of chicken semen diluents over the past 40 years. The results of Lake (1960), Van Wambeke (1967) and those of the present study were obtained with egg-type chickens.

Diluent (Year of Study)	Dose (μl)[e]	Post- A.I. Egg Collection Period (Days)	Fertility (%) Produced with Semen Stored for 0-96 Hours				
			Hours				
			0	24	48	72	96
Sodium Phosphate (1958)[a]	0.100	2-8	96.2	79.5[f]	-	-	-
Lake's Solution (1960)[b]	0.025	2-6	91.3	64.0	47	-	-
Diluent 2 (1967)[c]	.05 -.06	2-6	-	92-95	-	-	-
BPSE-I[d] (this study)	0.0125	2-8	-	-	-	69	13
Prototype Diluent (this study)	0.0125	2-8	-	-	-	85[g]	48

[a]Wilcox and Shaffner (1958).
[b]Lake (1960).
[c]Van Wambeke (1967).
[d]Sexton (1977).
[e]μl of whole semen (or μl of washed whole semen restored to its original volume) administered per hen. The total sample volumes administered per hen given in the original sources are in some cases larger than the figures given here since total sample volumes administered per hen are the sum of the volume of whole semen and the volume of diluent in the samples.
[f]Result obtained from a semen sample diluted in a sodium phosphate diluent supplemented with fructose.
[g]Unpublished result obtained prior to the completion of the empirical experiments that lead to improvements in the efficacy of potassium glutamate based diluents and formulation of the prototype chicken semen diluent.

formulation developed in the work reported here and either Wilcox and Shaffner's formulation or Lake's Solution. However, results obtained in this laboratory and results obtained in previous studies in other laboratories have been compiled in Table 3 so that indirect comparisons can be made between various chicken semen diluents that have been developed over the past 40 years.

In conclusion, a prototype chicken semen diluent formulation high in potassium glutamate has been developed and its superiority to BPSE-I for long term liquid storage of chicken semen has clearly been shown in the results reported here. The diluent fills a need in the poultry industry for improved chicken semen diluents. Dual biological buffers allow the prototype diluent's pH to be adjusted without altering its osmolarity or chemical composition and the prototype diluent is relatively easy and economical to prepare. Additional work is needed to determine the biological basis for the improved post-insemination fertility levels obtained from chicken semen stored in the prototype chicken semen diluent.

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