ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
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International Journal of Poultry Science 4 (4): 197-201, 2005
ISSN 1682-8356

Effects of Diluents, Cryoprotectants, Equilibration Time and Thawing Temperature on Cryopreservation of Duck Semen

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Abstract: A series of sequential experiments were carried out to determine optimum diluents, cryoprotectants, equilibration time, and thawing temperature for frozen duck semen in order to set up the commercial semen cryopreservating techniques which could be applied to the conservation of genetic resources, breeding, and commercial production in domestic ducks. In experiment 1, the seven semen extenders were studied to determine efficacy of the diluent on cryopreservation of duck Semen. The result showed that the diluent which contains 0.14% potassium citrate, 1.40% sodium glutamate, 0.98% disodium hydrogen phosphate, 0.21% sodium dihydrogen phosphate, 0.7% glucose, and 0.7% inositol was better than other six semen diluents. In experiment 2, the effects of various concentrations of cryoprotectants including glycerol, dimethyl sulphoxide (DMSO), dimethyl acetamide (DMA), and dimethyl formamide (DMF) on cryopreservation of bird semen were evaluated. The results showed that the cryoprotectant containing 10% DMSO was better than others. The experiment 3 was conducted to determine the effect of equilibration time and thawing temperature on cryopreservation of bird semen. The optimum equilibration time was 15 min and the optimum thawing temperature was 40°C

Key words: Duck, semen, diluent, cryoprotectant, equilibration time, thawing temperature

Introduction

Since Polge (1949) discovered that glycerol would preserve the motility of frozen chicken spermatozoa, much work has been conducted in the development of techniques for the preservation of poultry semen. To date, the most work has been performed on chicken spermatozoa, research on freezing storage of semen of duck and other avian species are relatively little. China has the largest water fowl production in the world and rich genetic resources of water fowl. However less work on breeding and resources conservation in China is conducted. At present conservation of poultry genetic resources is by living flock, which is costly. The cryopreservation of spermatozoa could play an important role in poultry breeding and genetic resources conservation as aids to economizing or introducing greater flexibility in special breeding programs. Therefore our experiment was conducted to study the cryopreservation of duck semen. Little research has been done on the effects of the prefreeze processing procedures such as extenders, cryoprotective agents, and thawing temperature. The purpose of this study was to determine the effects of a) extenders, b) various concentrations of glycerol, DMSO, DMA and DMF, c) equilibration time, d) thawing temperature on the motility of frozen-thawed spermatozoa.

Materials and Methods

Semen was obtained from Jinding Duck males by abdominal massage. Jinding Duck females were inseminated to determine fertility rate. All birds of the experiment were 43 wk of age at the start of the experiment and were housed in individual cages, whose dimensions were 52×52×44cm. Daily lighting was 16h light and 8h darkness. All birds received a layer diet (18% crude protein and 11.10MJ ME/kg) and had water available for *ad libitum* consumption. Care was taken during semen collection to minimize contamination by feces or urates. Clean and microscopically acceptable semen was collected into tubes maintained at 40°C and gently mixed. Motility was estimated before and after freezing by microscopical examination. 0.1 ml pooled semen was transferred into 1.5ml Eppendorf tube by micropipette and diluted with equal volume of extender. Diluted semen samples were held at 5°C for 2hr. Then 0.1ml DMSO was tipped into each Eppendorf tube and gently mixed except where cryoprotectants were studied. All samples were equilibrated at 5°C for 2hr except where equilibration time was studied. Following equilibration, the samples were frozen by immersion into an alcohol bath at -196°C and thawed at 40°C except where thawing temperature was studied. Freezing procedure referred to the procedure that was described by Sexton (1981), but adaptation was taken according to our experiments. All samples were processed as outlined in Table 1.

Four independent experiments were designed and conducted sequentially to test extender , cryoprotectants, equilibration time and thawing temperature.

Table 1: Freezing procedure

Step no	Treatment	Procedure
1	Diluent	Semen extender
2	Semen:diluent	1:1
3	Hold at 5°C	2h
4	Cryoprotectant	10% DMSO
5	Equilibrate at 5°C	2h
6	Semen package	Eppendorf tube
7	Freezing	Plunged, LN_2
8	Thaw temperature	40°C

Experiment 1: Seven extenders(Table 2) were prepared. Each extender was maintained at 40°C before dilution. Pooled semen was diluted 1:1 with each of the extenders. All samples were processed to step 8 as outlined in Table 1.
TES - [N - tris(hydroxy methyl)methyl - 2 - amino ethane sulfonic acid].
Tris - [Tris (hydroxy methyl) amino methane].
Dihydrostreptomycin sulfate 0.1g per 100ml
Benzylpenicillin Sodium 0.06g per 100ml

Experiment 2: On the basis of the results of the experiment on semen extenders, semen samples were diluted 1:1 with the extender and held at 5°C for 2hr, then 0.1ml of glycerol, DMSO, DMA or DMF were transported into the tubes and gave a final concentration of 4, 6, 8 or 10% (v/v).The semen-cryoprotectant was allowed to equilibrated for 2hr. The samples were processed to step 8 as outlined in Table 1.

Experiment 3: On the basis of the results of the previous two experiments, semen samples we redivided into five equal parts and processed (step 1 to 4), then DMSO was added to give a final concentration of 10%. The samples were equilibrated (Step 5) for 0, 15, 30, 60, 120min. After each equilibration period, the samples were frozen-thawed, then motility were assessed immediately.

Experiment 4: On the basis of the results of Experiment 1 through 3, semen samples were diluted and processed to(Step 1 to 7). The samples were thawed at 20, 30, 40, 50, 60, 70, 80, 90°C. After thawing, motilities were assessed immediately. After all experiments, using the above techniques, fertility was tested. Jinding female ducks were inseminated with 0.3ml of frozen semen or fresh semen diluted with saline. The fertility data represents eggs produced during the 2nd through 8th day after two inseminations. Fertility was determined by candling after 7 days incubation. Infertile and suspected fertile eggs were broken out into a pan of water to visually determine the signs of embryonic development(Gill *et al.*, 1996). In all experiments, significance ($P<0.05$) was determined by analysis of variance (ANOVA). Differences between treatment means were estimated by Duncan's multiple range test. Standard errors of the mean (SEM) were calculated from the variance derived from the ANOVA.

Results

Experiment 1: Seven semen extenders (Table 1) were prepared. In the seven extenders, No. 1 and 3 were extenders designed for freezing duck semen. No. 2 were Belstville Poultry Semen Extender (BPSE, Sexton, 1977), No. 4, 6 and 7 respectively were UNIIP-6, B-26, and IGGKP of the former USSR (Surai and Wishart, 1996), No. 5 was Lake's solution (Lake, 1960). Results (Table 3) indicated the extender containing glucose supported significantly higher ($P<0.05$) recovery of motility by frozen-thawed spermatozoa than other six extenders. Therefore modified IGGKP was used for the remaining trials.

Experiment 2: Motilities of semen frozen with various concentration of cryoprotectants are indicated in Table 4. Thawed duck spermatozoa after freezing with 10% DMSO or 8%glycerol showed no difference in postthaw motility, however DMSO at 10%concentration gave the highest motility of frozen-thawed spermatozoa. The motility of spermatozoa frozen in 8% glycerol or DMF was significantly higher than that obtained with glycerol or DMF levels of 4, 6 or 10% ($P<0.05$). DMSO or DMA at 10% was superior to lower concentrations of 4, 6, or 8%. Under the conditions employed, 10% DMSO was the best cryoprotectant tested for freeze-preservation of duck spermatozoa as judged by motility.

Experiment 3: Comparisons of the means of postthaw motility showed that 15min equilibration gave the highest average motility by frozen-thawed spermatozoa. Recovery of motility by frozen-thawed spermatozoa was poorest at 0 min equilibration. There was a trend seemed to be that as equilibration time increased beyond 15 min, there was a decline in postthaw motility.

Experiment 4: In experiment 4, the effects of thawing temperature on motility after freezing were studied. Data reported in table 6 demonstrated that there was a significant difference between 40 . and other thawing temperatures ($P<0.05$).

Experiment 5: The data in Table 7 showed that fertility of frozen-thawed semen was lower than that of fresh semen. When compared to the percent fertility obtained with fresh semen, after cryopreserving, including all the steps of previous treatments, only 39% of the fertilizing capacity for days 2 to 8 of frozen semen was recovered.

Discussion

Sexton(1975) reported that successful use of DMSO with chicken sperm was limited to its use with phosphate buffer as a suspension medium and he thought DMSO

Table 2: Chemical composition of extenders used for freezing duck semen

Constituent	1	2	3	4	5	6	7
Magnesium acetate	0.080		0.080			0.080	
Calcium chloride	0.040						
Magnesium chloride		0.034			0.068		
Sodium chloride				0.480			
Sodium bicarbonate				0.280			
Sodium acetate		0.430			0.851		
Potassium citrate	0.200	0.640	0.200		0.128	0.200	0.140
Sodium glutamate	0.200	0.867	0.200	0.820	1.920	0.200	1.400
Dipotassium hydrogen phosphate		2.270					
Potassium dihydrogen phosphate		0.065					
Disodium hydrogen phosphate							0.980
Sodium dihydrogen phosphate							0.210
Glucose							0.900
Fructose		0.500			1.000		
Lactose	3.000		3.000			3.000	
Inositol				0.280			0.900
Glycine	1.400		1.400	0.040		1.400	
Mannite	0.800		0.800				
TES	3.000	0.195					
Tris			3.000				
Ethylene diamine tetra-acetic acid. EDTA .				0.001			

Table 3: Effect of various extenders on motility of frozen duck semen

Extender No.	Motility (%)		Recovery rate of motility (%)
	Fresh	Postthaw	
1	80	35[b]	43.75[b]
2	80	23[c]	28.54[c]
3	80	7[d]	9.17[d]
4	80	9[d]	10.83[d]
5	80	13[d]	15.83[d]
6	80	32[b]	39.58[b]
7	80	59[a]	70.83[a]

a,d Means within columns with no common superscripts differ significantly (P<0.05)

chemically changed the suspension medium and these changes in extender affected the cell. In our experiment, the semen extender (No. 7) that gave the highest post-thawing motility included phosphate components and this could partly support the thought of Sexton. The n.2 extender also had phosphates, however it gave poorer motility, the contradiction was possibly explained by difference interaction of components of no. 2 and no. 7. Schramm (1976) reported that the degree of cytotoxicity of DMSO is influenced by the type of extender. Therefore it was possible that the appropriate combination of components of no.7 extender reduced the cytotoxicity of DMSO and yielded a high percentage of motile spermatozoa. Semen frozen with low DMA or DMSO levels did not exhibit vigorously swirling motility after freezing as did semen frozen with 10% level. Clark and Shaffner(1960) thought 7.5 to 8.5% glycerol was the optimum glycerol level. In our experiment 8% glycerol gave higher postthaw motility than other levels. The observation agree with the work of Clark and Shaffner, 1960. Polge (1949) found that insemination into hens of semen containing more than 5 percent glycerol, frozen or unfrozen, failed to result infertile eggs and 2 per cent glycerol in the semen was the maximum concentration compatible with the retention of full fertilization power. Glycerol is an effective cryoprotective for fowl spermatozoa, but glycerol is contraceptive for intra vaginally inseminated chicken or turkey spermatozoa (see reviews by Lake, 1986; Donoghue and Wishart, 2000). Tai *et al.* (2001) thought that DMA or DMSO was used as freezing cryoprotectant to replace glycerol and this was aimed at overcoming the contraceptive action of glycerol and simplifying the insemination protocol by avoiding the need to remove the cryoprotectant before insemination. In this experiment it was 10% DMSO that gave the highest motility and best protection for duck spermatozoa, therefore DMSO was selected as cryoprotectant for freezing drake semen. Optimum equilibration time before the freezing of semen has been reported. Graham *et al.* (1982)reported that there were no differences in recovery of motility by frozen-thawed spermatozoa over a wide range of times from 10 to 90 min, but motility was poorest at 0 and 90 min, the extremes of times tested. Sexton (1977) tested equilibration times of 10 and 60 min with 10 min being the optimum for his procedures. Sexton (1981) thought the cytotoxic effects of DMSO during equilibration was

Table 4: Effect of adding various concentrations of cryoprotectants on motility of frozen-thawed duck semen

Cryoprotectants	Concentration %	Motility (%)		Recovery rate of motility (%)
		(%) Fresh	Postthaw	
	4	80	11^{g}	14.06^{g}
	6	80	35^{ef}	43.75^{ef}
	8	80	54^{ba}	68.12^{ba}
Glycerol	10	80	50^{bc}	63.12^{bc}
	4	80	5^{gh}	6.56^{gh}
	6	80	8^{gh}	10.31^{gh}
	8	80	29^{f}	36.25^{f}
DMSO	10	80	58^{a}	73.12^{a}
	4	80	5^{gh}	6.87^{gh}
	6	80	13^{g}	15.94^{g}
	8	80	27^{f}	34.37^{f}
DMA	10	80	49^{bcd}	61.25^{bcd}
	4	80	3^{h}	3.44^{h}
	6	80	11^{g}	13.75^{g}
	8	80	46^{cd}	58.12^{cd}
DMF	10	80	42^{de}	52.19^{de}

a-h Means within columns with no common superscripts differ significantly (P<0.05).

Table 5: Effect of equilibration time on motility of frozen duck semen

Equilibration time prior to freeze min	Motility (%)		Recovery rate of motility (%)
	Fresh	Postthaw	
0	78	14d	18.59d
15	78	62a	80.13a
30	78	60ab	77.56ab
60	78	56b	72.12b
120	78	49c	63.46c

a-dMeans within columns with no common superscripts differ significantly (P<0.05)

Table 6: Effect of thawing temperature on motility of frozen duck semen motility (%)

Thawing temperature (°C)	motility (%)		Recovery rate of motility (%)
	Fresh	Postthaw	
20	78	29f	37.82f
30	78	39e	50.64e
40	78	62a	79.81a
50	78	57b	73.08b
60	78	28f	36.38f
70	78	45cd	58.01cd
80	78	41de	52.63de
90	78	50c	64.42c

a-eMeans within columns with no common superscripts differ significantly (P<0.05)

Table 7: Fertility of fresh and frozen duck semen

Treatment	No. hens	Fertility of days 2 to 8 (%)
Fresh	5	100
Frozen	5	39

related to both DMSO level and exposure time and short equilibration periods were necessary to minimize the cytotoxicity of DMSO on unfrozen chicken spermatozoa. The work of Mitchell and Buckland(1976) showed that as equilibration time beyond 45 min, there was a slight decline in reproductive performance. In our experiment the level of motility of for frozen-thawed semen were highest when samples were equilibrated for 15min(0.62) and equilibrating semen with 10% DMSO after 15min had a depressive effect on the post-thawing motility. These results indicated that 0min or longer equilibration time reduced the postthaw motility. The results support the work of above researchers, though they did not agree with those of Sexton (1981) who reported that motility was highest for semen diluted in BPSE and frozen by the Beltsville method with equilibration(2hr) periods at 5°C. Brown (1962) reported that the fertility of chicken spermatozoa thawed at 20°C was higher than 40°C. Tselutin *et al.* (1995) had duck semen thawed at 60°C. In our experiment thawing temperature at 40°C gave the highest motility of frozen duck semen. The difference of freezing system could result into variance of appropriate thawing temperature in different experiments. Tselutin *et al.* (1995) reported fertility level of 75 .83% after two artificial inseminations in 7 days of ducks with freezing-thawed semen. Sexton (1981) showed 61% fertility days 2 to 8 post artificial insemination with turkey frozen semen. Tai *et al.* (2001) reported that the percentage of fertility during days 3 to 9 after 2 consecutive insemination was 68 to 95% with frozen gander semen. Compared with above fertility, the fertility of 39% obtained in this study is low. Further work is needed to improve the overall recovery of fertility of duck semen subjected to freezing and thawing.

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