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EFFECT OF CRYOPROTECTANTS AND EQUILIBRATION PERIOD ON POST-THAWING CHARACTERISTICS OF NEW-ZEALAND WHITE RABBIT SPERMATOZOA

By

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ABSTRACT: The present study was conducted to investigate the effect of dimethylsulfoxide (DMSO) or glycerol as cryoprotectants on rabbit semen against the deleterious effect of freezing. Ten New- Zealand White mature male rabbits with an average body weight ranging from 2.500 to 2.750 kg were used. Semen was collected twice weekly for 20 weeks and were examined for advanced motility, percentages of alive spermatozoa and total abnormalities. Semen were diluted with sucrose extender containing 2 or 4% DMSO or glycerol. And exposed to equilibration period of one or two hours before freezing in liquid nitrogen (-196 °C).After freezing, semen samples were thawed at 37 °C for 10 seconds, advanced motility, percentage of alive spermatozoa and total abnormalities were assessed after thawing. Results obtained are summarized in the following:

1-Concerning cryoprotective agents.

a) The highest advanced motility (%) was recorded by using 4% DMSO (51.36± 0.97%) by using 1 hr equilibration followed in a significant ($p < 0.05$) decreasing order by 4% DMSO (43.18± 0.86%) by using 2 hr equilibration compared with all treatments.

b) The highest percentage of alive spermatozoa ($P < 0.05$) was recorded by using 4% DMSO (59.92±2.73) by using 1 hr equilibration followed by 4% DMSO (46.09± 0.35%) by using 2 hr equilibration, compared with all treatments.

c) The lowest ($P < 0.05$) percentage of abnormal spermatozoa was recorded by using 4% DMSO (15.82± 0.87%) and 2% DMSO (17.55± 0.84%) by using 1 hr equilibration compared with all treatments.

2-Concerning equilibration period. One hour gave the best results significantly ($p<0.05$) than two hours.

INTRODUCTION

The rabbit is considered to be a valuable laboratory animal in teratology, microbiology and medical and life sciences, many valuable mutants (Kurosawa *et al.*, 1995 and Watanabe *et al.*, 1997) including transgenic (Bosze *et al.*, 2003 and kitajima *et al.*, 2005) have been established in the rabbit. Therefore, a need has been recognized for reliable methods to bank rabbit genetic resources efficiently in the form of cryopreserved spermatozoa. Glycerol has been extensively used as a cryoprotectant for various cells, including mammalian spermatozoa and embryos, since Polge *et al.* (1950) who reported its cryoprotective properties. In early research on freezing rabbit spermatozoa, spermatozoa frozen with glycerol, resulted in extremely low fertility (Fox. 1961 and Stranzinger *et al.*, 1971). After 1980, studies employing acetamide (Handa and Nagasa, 1980 and Dalimata and Graham, 1997) or DMSO (Handa and Nagasa, 1980, Chen *et al.*, 1989 and Moce *et al.*, 2005) as a cryoprotectants have been encouraged. However, there is a little information on cryoprotectants used for freezing New Zealand White rabbit spermatozoa.

Equilibration of sperm with cryoprotectants is necessary for the sperm before freezing for giving adequate protection against crystallization and cold shock (Gilbert and Almquist, 1978). Several investigators have demonstrated that spermatozoa should be left in contact with cryoprotectants or a time interval before freezing is initiated for optimum post-thaw motility and fertility (Hafez, 1987; Salhab and Merilan, 1991).

The present study was conducted to compare 2% or 4% glycerol or DMSO as cryoprotectants in sucrose extender for ejaculated semen of the New zealand white rabbit. Advanced motility, alive percentage and total abnormalities of frozen/ thawed spermatozoa were examined to improve methods of sperm cyropreservation with the ultimate aim of banking genetic resources.

MATERIALS AND METHODS

Animals

Ten bucks of New Zealand White rabbits were used as semen

donars, and 4 female rabbits were used as teaser females. Rabbits were sexually mature, housed in individual cages and fed a commercial diet *ad libitum* (Table 1). Fresh water drinkers were also available continuously via automatic watering troughs.

Table (1):-Composition and chemical analysis of the experimental diet.

Ingredients	%
Berseem hay	34.50
Wheat bran	12.40
Soybean meal (44%)	17.50
Barely grains	30.00
Molasses	3.00
Daicalsium phosphate	1.00
Limestone	0.70
Sodium chloride salt	0.34
Premix[1]	0.30
Dl-Methionine	0.06
Antitoxins	0.10
Antifungi	0.10
Chemical analysis[2] (%)	
Crude protein(CP)	17.00
Crude fiber(CF)	12.91
Crude fat (EE)	2.13
Digestible energy(k.cal/kg fed)[3]	2415.00
Calcium phosphorous	1.07
Lysine	0.63
Methionine	0.85

1: Vitmain A 12000000 IU, V D3 220000 IU, V E 1000 mg, V k3 2000 mg, V B1 1000mg, V B2 4000mg, V B6 1000 mg, V B12 10 mg, pantototnenic acid 10000 mg, Niacin2000 mg, Biotin 50 mg, folic acid 1000 mg, Colin Chloride 500 mg, Selinum 100 mg, Copper 1000 mg, Iron 30000 mg, Manganese 55000 mg, Zinc 50000 mg, Iodine 1000 mg and carrier CaCo3 to 3000 gm.

2: Analyzed according to A.O.A.C (1990)

3: Digestable energy =TDN × 4.4 according to Church (1977)

Semen collection and evaluation.

Semen was collected using an artificial vagina two times per week for 20 weeks. Immediately after collection, gel plugs were removed using Pasteur pipettes and forceps. Fresh semen samples were examined for sperm advanced motility, survivability and total abnormalities. Sperm advanced motility (%) was immediately evaluated by placing a drop of fresh semen diluted with 1ml of a warm (37°C) sodium citrate dihydrate (2.9%) on a warm clean glass slide, covered with a cover slip and immediately examined under the high power (x 450) in different fields. Percentage of alive spermatozoa was counted in stained smears using the nigosin- eosin staining (Hacket and Macpherson, 1995). Total abnormal spermatozoa were counted from the slides prepared for alive spermatozoa test.

Semen extension.

Good semen samples (more than 70% motile spermatozoa were pooled and extended with sucrose extender (SYC) which contains 2.9% sodium citrate dihydrate, 0.04 gm citric acid, 1.25 gm sucrose and 20% egg yolk (vol/ vol). The cryoprotective agents were 2% or 4% DMSO or glycerol Also, 500 IU penicillin and 0.5 mg streptomycin/ml extender were added as antibiotics. The rate of extension was 1 semen: 4 extender and pH of the extender was ranged from 6.7- 6.9.

Equilibration period.

Extended semen with glycerol or DMSO was left at 5°C for 1 or 2 hours as equilibration period. The samples were then immediately frozen in liquid nitrogen (-196 °C) by using packaging method in straws (0.25 ml).

Cryopreservation of semen.

The straws were exposed to liquid nitrogen vapor at - 90 °C to – 100°C. The straws were kept at this level for 10 minutes, after which they were dipped directly into liquid nitrogen. At this time the freezing process was ended. Frozen semen straws were thawed by holding them at the closed end (not the plugged end) and dipped in a water bath at 37 °C for 10 seconds (Kashiwazaki *et al.*, 2006).

Examination of thawed-frozen semen was conducted in the same way as for fresh semen.

Statistical analysis

Duncan's multiple range test (Duncan, 1955)and analysis of variance were used for the statistical analysis of all the data, values were presented as means ± SE.

RESULTS

The present study was conducted to compare the effect of 2% or 4% DMSO or glycerol as cryopratactants for 1 or 2 hours as equilibration period on post- thawing characteristics of frozen spermatozoa of NZW rabbits. As shown in Table (2).

Concerning cryoprotective agents. Results of Table (2) show that the average of post- thawing motility, regardless the equilibration period was a) The highest advanced motility (%) was recorded by using 4% DMSO (51.36± 0.97%) by using 1 hr equilibration followed in a significant ($p < 0.05$) decreasing order by 4% DMSO (43.18± 0.86%) by using 2 hr equilibration. The highest percentage of alive spermatozoa ($P < 0.05$) was recorded by using 4% DMSO (59.92±2.73) by using 1 hr equilibration followed by 4% DMSO (46.09± 0.35%) by using 2 hr equilibration, compared with all treatments. The inverse trend was found in the percentages of total post- thawing abnormal spermatozoa. The lowest ($P < 0.05$) percentage of abnormal spermatozoa was recorded by using 4% DMSO (15.82± 0.87%) and 2% DMSO (17.55± 0.84%) by using 1 hr equilibration compared with all treatments.

Concerning equilibration period length, the results showed that one hour gave the best values than the two hours regardless of cryoprotective agents. Table (2) showed that the post- thawing motility of spermatozoa, post- thawing alive spermatozoa and total abnormalities spermatozoa frozen after one and two hours equilibration. Significant differences at ($p < 0.05$).The lowest values was recorded by using one hour and the highest values was recorded by using two hours equilibration.

DISCUSSION

In the present study, the advanced motility, and alive sperm percentages of the rabbit frozen semen with DMSO as a cryoprotective agent were the best in comparison with using glycerol as a cryoprotective agent. On other hand, percentage of abnormalities increased with using glycerol in comparison with DMSO.Increasing DMSO concentration (4%) gave the best results than the lowest one (2%).In contrast, increasing glycerol concentration gave bad results comparing with that obtained

Table (2): Effect of cryoprotectants and Equilibration period on Post-thawing characteristics of NZW Rabbit semen.

Semen characteristics	Fresh semen	Equilibration period	Post-thawing characteristics			
			DMSO		Glycerol	
			2%	4%	2%	4%
Advanced Motility (%)	72.09±1.3	1 hr	38.64±0.53 Ca	51.36±0.97 Aa	32.27±0.55 Da	21.36±0.77 Ea
		2 hrs	19.09±0.87 E F b	43.18±0.86 Bb	18.17±1.80 Fb	3.64±2.01 Gb
Alive sperms (%)	79.35±0.9	1 hr	46.00±0.52 Ba	59.73±2.15 Aa	37.36±0.98 Ca	25.55±0.86 Da
		2 hrs	23.28±1.2 D E b	46.09±0.35 Bb	21.45±1.44 Eb	5.00±1.07 Fb
Total abnormalities (%)	14.68±0.6	1 hr	17.55±0.84 Ca	15.82±0.87 Da	24.51±0.67 Aa	25.45±0.45 Aa
		2 hrs	19.36±0.87 Bb	19.18±0.63 Bb	24.91±0.21 Ab	27.18±1.02 Ab

a, b: within column any two overall means having different letters are significantly different ($p<0.05$).
A, B: within column any two overall means having different letters are significantly different ($p<0.05$).

using 2%glycerol.(Table.2).To date, the effects of some cryoprotectants on post- thawing sperm characteristics have been compared by Hanada and Nagase (1980) and Dalimata and Graham (1997).In the study of Hanada and Nagase (1980), the results indicated that DMSO was the highly cryoprotective agent for the New Zealand White rabbits semen, and one ml glycerol resulted in extremely low motility after thawing. Smith and Polge (1950) also, reported that less than 2% of the oocytes recovered from females that inseminated with rabbit semen frozen in the presence of glycerol were fertilized. Although glycerol has been successfully used to cryopreserve spermatozoa from several species, it is not an effective cryoprotectant for rabbit sperm freezing (Kashiwazaki *et al*., 2006).

Since, the discovery of cryoprotective actions of glycerol allowed freezing (Polge *et al*., 1950) and although hundreds of potential cryoproctants have been subsequently examined, glycerol has remained the cryoprotectant of choice for spermatozoa from almost all species, especially in domestic livestock (Curry, 2000). However, despite extensive experimentation on freezing of spermatozoa to optimize its use, the basis of the cryoprotective properties of glycerol and precisely why it would be more effective than other cryoprotectants for several species remains unclear. Glycerol is a permeative cryoprotectant that is able to cross the cell membrane in general. Recent identification of the water channel protein Aquaporin (AQP7), which also facilitates glycerol transport at the late spermtid stage in the rat (Ishibashi *et al*., 1997), may provide a specific route for glycerol to permeate sperm cells. Curry (2000) has also shown that spermatozoa from many domestic species have high permeability to water with low activation energy and insensitivity to inhibition by mercuric chloride (Watson *et al;* 1992 and Gilmore *et al*., 1996). These are membrane characteristics consistent with the presence of the AQP7 channel. It has been difficult to ascribe physiological role to the high water permeability of the sperm membrane, and if AQP7 is found to be widely expressed amongst domestic species, it may be that the transport of glycerol, not water, is its primary function However, rabbit spermatozoa have relatively lower water permeability and a higher activation energy than other domestic animals (Curry *et al*.,1995) The cryoprotective effects of glycerol are most evident at higher concentrations in domestic livestock species (Curry *et al*., 2000). On the other hand, glycerol was reported to be toxic to spermatozoa during cryopreservation in mice and rats, which are closely related species to the

rabbit (lagomorph). Mouse spermatozoa seem unable to withstand more than about 1.75% glycerol (Tada *et al.*, 1990 and Penfold *et al.*, 1993). Also, it has been reported that glycerol is toxic to spermatozoa in rats during sperm cryopreservation (Nakatsukasa *et al.*, 2001). In addition, glycerol has a marked antifertility effect on fowel spermatozoa (Lake *et al.*, 1980) without causing obvious morphological damage or impairing motility. The sensitivity of spermatozoa to these toxic effects varies with the species. For instance the susceptibility of boar spermatozoa to acrosomal damage at relatively low concentration of glycerol may explain the poor fertility of frozen/thawed boar spermatozoa compared with more resistant bull spermatozoa.However,it is believed from results of the present study that DMSO is able to penetrate the plasma membrane of the rabbit sperm , causing its cryopreservative action .Also it is observed that the cryoprotective effect of DMSO is most evident at higher concentration (4% than 2%). Consequently, it can be concluded that DMSO is not toxic to the rabbit sperm .

Concerning equilibration period, results of the present study revealed that averages of post-thaw motile, alive and abnormal rabbit sperm were found to be in preferable using equilibration period of one hour than that of two hours .This preferability was indicated in both samples of DMSO and glycerol agents (Table 2) .The differences between the two periods was significant ($p<0.05$).

Several studies were carried out to define the suitable equilibration period for different species .But few studies have been reported on the effect of equilibration period on post-thaw rabbit sperm characteristics.

Chen *et al.*(1989) found that cooling the extended semen required 4 hours as an equilibration period .Das and Rajkonwar (1994)reported that equilibration for one hour resulted in the least buck sperm damage before freezing but after freezing damage was the least among spermatozoa that had been equilibrated for 3 h, the percentage of spermatozoa with an a damaged acrosome increased with increasing period of equilibration (Duttal *et al.*,1996).Awad *et al.*(2000) reported that rabbit semen requires a short equilibration period 0-2 hours . Also, Abo El-Roose (2006)found that the one hour as an equilibration period gave higher results of post-thaw motility than 2 hours equilibration period.

In conclusion, 4%DMSO has higher cryoprotective effects than 2%DMSO, 2%glycerol and 4% glycerol when used as a cryoprotectant in

cryopreservation of New Zealand rabbit spermatozoa. Also, one hour as equilibration period gave the best results of post-thaw characteristics of NZW rabbit sperm.

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الملخص العربى

تأثير المواد الحامية وفترة الإتزان على صفات الحيوانات المنوية المجمدة بعد الإسالة للأرانب النيوزيلاندى البيضاء

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أجريت هذه التجربة بهدف دراسة تأثير المواد الحامية كالجلسرول والداى ميثيل سلفوكسيد وكذلك فترات الاتزان المختلفة على حيوية الحيوان المنوى المجمد وكذا النسبة المنوية للحى وايضاً النسبة المنوية للشواذ الكلية .

استخدم فى هذه التجربة عشرة ذكور من الارانب النيوزيلاندى البيضاء عند عمر ١٢-١٥ اسبوع ذات متوسط وزن ٢,٥٠٠ - ٢,٧٥٠ كجم .تم جمع عينات السائل المنوى لتقدير صفات الحيوانات المنوية الحديثة الجمع من حيث الحيوية والنسبة المنوية للحى وايضاً النسبة المنوية للشواذ الكلية.وتم تخفيف السائل المنوى بمخفف السكروز محتوياً على ٢%،٤% جلسرول او داى ميثيل سلفوكسيدثم بعد ذلك تركت العينات لمدة ساعة او ساعتين كفترات اتزان عى درجة حرارة ٥ م° وبعد التجميد باستخدام النيتروجين السائل(- ١٩٦ م°) تم عمل اسالة لهذه القصيبات فى حمام مائى على درجة حرار ٣٧ م° لمدة ١٠ ثوانى وقد اظهرت النتائج ما يلى:-

١- وجد ان اضافة ٤% داى ميثيل سلفوكسيد للمخفف كمادة حماية اعطى افضل النتائج للصفات المدروسة(% للحركة التقدمية-% للحيوانات المنوية الحية- % للشواذ الكلية) وهناك اختلاف معنوى عند ٥% بين كل المعاملات للمواد الحامية

٢- وجد أن التجميد بعد مرور ساعة واحدة فترة اتزان افضل معنوياً (٥%) من مرور ساعتين اتزان على الصفات % للحركة التقدمية-% للحيوانات المنوية الحية- % للشواذ الكلية.

نستخلص من هذه الدراسة انه لتطوير نظم التلقيح الاصطناعي والعمل على تطوير بنوك حفظ الأصول الوراثية، فلابد من الحصول على السائل المنوي العالي الجودة ومعالجته بأفضل الوسائل الحفظية فأن ٤% داى ميثيل سلفوكسيد أفضل المواد الحامية التي تم استخدامها وكذلك ساعة واحدة اتزان افضل من ساعتين.