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EFFECTS OF CRYOPROTECTANTS AND FREEZING ON RABBIT SEMEN QUALITY (*).

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Abstract

The research was carried out to evaluate the influence of two cryoptotectants on rabbit semen quality before and after freezing and to assess the male effect on semen damages following dilution and freezing. For the trial 15 mature N.Z.W. bucks (aged 6-10 months), reared in an experimental rabbitry, were used. Semen was collected from each buck two times in May and June 1991. Three samples of individual semen were consecutively examined: immediately after collection - after dilution with cryoprotectant (at 37° C) - after freeze and thawing. Best ejaculates was then pooled togheter to verify the effect of the extender independently from the male effect. Acetamide (1 M, 2 M) or DMSO (1 M, 2 M), with or without glycerol (0, 2, 5% vol./vol.), were used as cryoprotectants. The qualitative traits examined were: live sperm, normal acrosomes and motility. The results pointed out a significance of the male effect on live sperm and motility in fresh semen and on normal acrosomes in frozen semen. The cryoprotectants influenced significatively live sperm and motility both in diluted and frozen semen, showing a low toxic effect (except DMSO 2 M and glycerol 5%) but an unsatisfactory protective action. The best results were obtained with DMSO 1 M without glycerol (live sperm 44.5%, normal acrosomes 70.4%, motility 53.8% in frozen semen).

Introduction

To date deep freezing of rabbit semen is the main impediment to the diffusion on large scale of artificial insemination, because rabbit semen responds differently than some other species. Rabbit spermatozoa are less sensitive to rapid cooling (from 30 to 0° C) than bull, dog or ram spermatozoa (6) but they are very susceptible to damages caused by hypertonic solutions. They also seem to be less permeable to glycerol (6) which lowers motility at high concentration. Despite of numerous attempts, an

(*) Research founded by MURST 40%.

effective and nontoxic cryoprotective agent has not yet been found; in fact all the tested substances explained negative effects on the spermatozoa survival and motility and on the acrosomal integrity. Therefore, the fertility levels with frozen semen were generally low (9, 12, 13) while some Researchers (1, 2, 3, 4, 6, 14, 15) obtained rather good results.
The most widely used cryoprotectants were glycerol (2, 4, 5, 14), ethylene glycol (5) and dimethylsulphoxide (4, 6, 7, 11). Nagase et al. (8) showed that sugars (xylose and raffinose) and polyols (erythritol, sorbitol, inositol), in diluent containing egg yolk, appear to give very little protection during freezing. Hanada and Nagase (6) investigated the effectiveness of eleven substances and reported that spermatozoa maintained a good motility at 20° C in hypertonic solutions of dimethylsulphoxide (DMSO), trimethylene glycol, acetamide and ethylene glycol suggesting that these compounds freely permeated the cell membrane and were relatively nontoxic. In contrast, sperm survival was low in hypertonic solution of formamide, propionamide and dimethyl formamide. The best recovery rates were obtained following freezing in media containing lactamide, DMSO and acetamide 1 M. The authors concluded that compounds containing hydroxyl groups were relatively less effective than those with amide or methyl groups. Chen (4), confirmed that acetamide was more protective than glycerol.
Hu et al. (7) when the semen was diluted with glucose, sucrose or lactose plus DMSO in several concentrations, did not observe significant differences in semen characteristics. The substitution of glucose with fructose increased conception rate; DMSO gave the best results at 3.3-3.9% levels.
The objectives of the present study were:

1. to assess the male effect on the semen resistance to damages caused by extender and freezing;
2. to evaluate the toxic and protective effects of some cryoprotectants.

Materials and Methods

For the investigations 15 mature N.Z.W. bucks (aged 6-10 months) were used; they ejaculated semen of good quality with a number of spermatozoa/ml ranged from 415 to 850 x 10^6. They were reared in an experimental rabbitry with natural ventilation. The photo-period was 16 hours/day and the animals were fed a commercial feed (17% crude protein, 16% crude fiber, 2550 DE kcal/kg) ad libitum. The semen was collected from each male two times on May and June 1991; in these months the environmental average temperature was 18.3 $\pm$ 2.9 and 24.1 $\pm$ 3.9 respectively.
Each individual sample was examined: immediately after the collection - after the dilution with the extender (at 37° C) - after freeze and thawing.
Best ejaculates were pooled to examine the effect of the extender independently from the buck effect.

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The examined traits were: live sperm, normal acrosomes and motility. The proportion of live sperm was estimated by staining them with tryphan blue, according to the method of Chen et al. (3). After centrifugation, resuspension and incubation, the sperm suspension was smeared on a glass slide, which was successively placed and stored overnight in a 10% solution of Giemsa stain in distilled water. One-hundred sperm from each ejaculate were examined to determine the percentage of live sperm and normal acrosomes.
The percentage of motile sperm was estimated subjectively placing a drop of semen on a microscope slide at 37° C and determining the progressive motile sperm with a video camera and a monitor connected to the microscope .
Semen was diluted at 1:6 with an extender consisting of tris buffer with 20% egg yolk and acetamide (1 M, 2 M) or DMSO (1 M, 2 M) with or without glycerol (0, 2, 5% vol./vol.). The pH was adjusted to 7.2. For deep freezing, semen was placed in tubes surrounded by water at 37° C and set in a refrigerator at 5° C. After about 4.5 hours semen was packaged in plastic straws containing 0.5 ml and immediately deep freezed. The freezing method was the same of that used for bull: straws were horizontally placed just above liquid nitrogen and were plunged into liquid nitrogen when the temperature was about -110° C (10-15 minutes).
Immediately before the analysis straws were thawed by transferring them from the liquid nitrogen to a water bath at 40° C for 20 s.

Statistical analysis was carried out according to the following linear model:

$$Y_{ijkl} = m + a_i + b_j + c_k + e_{ijkl}$$

Y_{ijkl} = experimental items;
m = overall mean;
a_i = fixed effect of buck (1..16);
b_j = fixed effect of acetamide and DMSO (1..4);
c_k = fixed effect of glycerol (1..3);
e_{ijkl} = residual effect.

Experimental data were processed by the PROC CORR and GLM (SAS, 10).

Results and Discussion

The significance of the effects reported in Table 1 shows that the buck effect strongly influenced live sperm and motility in fresh semen, and normal acrosomes in frozen semen, while cryoprotectant affected live sperm and motility in diluted and frozen semen.
The results of the semen evaluation at the three different stages are shown in Table 2. It is very clear that both cryoprotectants and low temperatures were

detrimental to the qualitative semen traits, and particularly the percentage of live spermatozoa and motility. The extender, in the pooled semen, lowered the survival value from 85 to 72%, normal acrosomes from 90 to 81% and motility from 84 to 71%.

Much more damage was caused by freezing; the sperm survival dropped down to 40%, normal acrosomes to 66% and motility to 36%. These results suggest that cryoprotectants were relatively nontoxic but not much effective.

Regarding the male effect on semen resistance to the negative action of chemical and physical factors, fresh semen showed differences between bucks, but the individual reaction to treatments differed little, except for a few ejaculates such as the semen of buck No. 12 which was strongly damaged by the extender and freezing. In fact, the trend of the values after dilution and freezing was quite similar to those of fresh semen: generally the best and the worst ejaculates were still very different after the two treatments.

This observation is confirmed from the correlation data reported in Table 3 which show that in fresh semen all the examined traits were positively correlated between them; in diluted semen only live sperm was significantly correlated with motility and in frozen semen the live sperm was correlated with both normal acrosome and motility.

Regarding treatment, it can be noted that percentages of live sperm and motility in frozen semen were significantly correlated to those of fresh semen; correlations also existed for motility vs live sperm and vs normal acrosomes.

The comparison between diluted and frozen semen shows significant correlations among motility and live sperm and motility and normal acrosomes.

From these results it can be deduced that ejaculate evaluation based on live and motile sperm permits to foresee the quality of semen (live and motile sperm) after freezing. Predicting acrosomal integrity is difficult because the modifications do not present a linear trend. However a definitive answer could be only obtained observing the fertility rate.

The influence of two cryoprotectants (acetamide and DMSO) at two concentrations (1M, 2M) and of the glycerol addition (2 and 5%) on the semen traits is shown in Table 4.

The data shows that both acetamide and DMSO gave rather unsatisfactory results with a moderately toxic effects and a rather low protective action. Acetamide had a more negative effect on sperm motility and its protective action for this trait was lower than DMSO.

DMSO 2 M was strongly toxic reducing very much live sperm (39%) and motility (28%). On the contrary DMSO 1 M was much less toxic and had a good protective action; the best performance after freezing was obtained with this substance at 1 M concentration.

The addition of glycerol did not produce any advantage and at the 5% level the quality of the frozen semen deteriorated greatly, particularly for live sperm.

The results obtained in this investigation agree fairly well with those of other Researchers but direct comparisons are often difficult.

Data on the motility of frozen semen by Hanada and Nagase (6) differed little from ours and showed the same trends (DMSO 1 M = 44 vs 54 %; Acetamide 1 M = 40 vs 30 %).
Weitze (14) reported that DMSO at 4.5 % vs 2.2 % improved motility (31.2 vs 16.1 %) but caused a decrease of normal acrosomes (41.2 vs 31.8%). In our trial very higher values of normal acrosomes (75.4%) and motility (53.8 %) were obtained with DMSO 1 M (about 16%).
Chen et al. (4) obtained 43% vs 32% motile spermatozoa with acetamide 1 M vs glycerol + DMSO; in a second experiment with acetamide only they obtained: motility 40% (fresh semen, 80%) and normal acrosomes 77% (fresh semen, 89%). In another research (3) motility was 40% (fresh semen 87%), live sperm 44% (fresh semen 84%) and normal acrosomes 54% (fresh semen 88%). Our values were similar: live sperm 38%, normal acrosomes 69% and motility 30% (fresh semen 81% - 88% and 74% respectively).
Based on the results presented it is possible to conclude that the analyzed traits are indicative of the semen quality and could be used to select the best ejaculates which have to be frozen. However evaluation methods require too much time and it is impossible to use them in large scale because numerous ejaculates must be evaluated before selecting a buck.
The use of computerized semen analyzers would probably provide more exact evaluations in a shorter time.
Another consideration is that, to date, sperm quality after freezing and thawing is still unsatisfactory.
Therefore it is necessary to test other diluents, to improve freezing techniques and to identify other semen traits that allow for the selection of the ejaculates which are more suitable for freezing.

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Table 1 - Significance of the effects.

Effects	Fresh semen			Diluted semen			Frozen semen		
	Live sperm %	Normal acrosomes %	Sperm motility %	Live sperm %	Normal acrosomes %	Sperm motility %	Live sperm %	Normal acrosomes %	Sperm motility %
Buck	**	n.s.	**	n.s.	n.s.	n.s.	n.s.	*	n.s.
Acetamide, DMSO	-	-	-	**	n.s.	*	n.s.	n.s.	*
Glycerol	-	-	-	n.s.	n.s.	**	**	n.s.	*

* P ≤ 0.05 ; ** P ≤ 0.01 ;
n.s. = not significant;

Table 2 - Effects of cryoprotectants and freezing on different males (LSMeans-DSE).

Buck No.	Fresh semen			Diluted semen			Frozen semen		
	Live sperm %	Normal acrosomes %	Sperm motility %	Live sperm %	Normal acrosomes %	Sperm motility %	Live sperm %	Normal acrosomes %	Sperm motility %
1	88.8	90.3	84.2	68.6	81.6	69.0	40.4	67.4	32.4
2	85.4	90.7	84.3	66.6	82.2	51.9	39.7	67.0	34.1
3	78.1	93.0	72.1	71.6	84.9	64.2	30.3	54.9	24.1
4	76.0	87.9	71.4	67.3	79.8	57.2	44.1	63.5	40.1
5	72.3	85.8	69.7	67.3	83.3	56.7	-	-	-
6	88.3	87.8	67.8	65.5	82.4	59.5	-	-	-
7	78.6	86.0	68.7	66.2	83.4	59.9	-	-	-
8	73.3	84.8	68.3	70.4	80.4	67.1	-	-	-
9	81.3	87.7	72.1	73.5	83.3	69.3	49.9	70.6	46.1
10	76.2	90.7	70.9	68.2	81.3	49.5	40.7	65.6	32.0
11	82.1	85.7	73.8	67.5	82.5	54.1	44.5	65.1	44.1
12	71.9	81.8	65.1	46.0	68.3	13.4	8.5	40.0	5.0
13	82.3	86.0	82.9	67.9	77.5	56.2	49.8	71.0	40.7
14	86.6	87.6	74.0	79.3	83.0	70.3	43.8	69.1	20.4
15	85.1	83.3	68.1	71.2	81.2	67.3	50.1	72.6	36.3
Pool	84.9	90.5	84.1	71.8	80.5	70.8	40.1	66.5	36.4
DSE	5.58	3.43	7.36	12.80	4.73	15.12	16.81	19.11	25.20
No.	40	40	40	183	143	180	127	127	135

Table 3 - Correlations between the examined traits (r).

	Fresh semen		Diluted semen			Frozen semen		
	Normal acrosomes	Motility	Live sperm	Normal acrosomes	Motility	Live sperm	Normal acrosomes	Motility
Fresh semen								
Live sperm	0.34*	0.50**	0.28*	0.05	0.15	0.40*	0.05	0.40*
Normal acrosomes	-	0.45**	0.26	0.18	0.27	-0.03	-0.10	0.30
Motility		-	0.38**	-0.01	0.39**	-0.12	-0.08	0.19
Diluted semen								
Live sperm			-	0.02	0.75**	0.31	-0.09	0.51**
Normal acrosomes				-	0.09	-0.09	0.10	0.59**
Motility					-	-0.09	-0.12	0.16
Frozen semen								
Live sperm						-	0.50**	0.54**
Normal acrosomes							-	-0.03

* $P \leq 0.05$; ** $P \leq 0.01$;

Table 4 - Effect of three different cryoprotectants at two concentrations on the semen quality before and after freezing (LSMeans).

Cryoprotectant	Fresh semen			Diluted semen			Frozen semen		
	Live sperm %	Normal acrosomes %	Sperm motility %	Live sperm %	Normal acrosomes %	Sperm motility %	Live sperm %	Normal acrosomes %	Sperm motility %
Acetamide 1 M	81.3	88.5	73.9	71.3B	82.6	58.8B	38.2	69.4	30.4b
Acetamide 2 M	80.0	87.9	72.7	72.5B	83.1	57.6B	37.6	68.6	26.1ab
DMSO 1 M	79.8	89.1	73.8	74.3B	81.0	63.4B	44.5	70.4	53.8c
DMSO 2 M	85.4	92.2	71.7	46.9A	78.2	43.5A	41.1	53.5	22.9a
Glycerol 0%	83.5	91.0	76.1	68.8	82.9	67.3B	48.9B	69.3	47.0b
Glycerol 2%	84.8	90.8	71.0	68.5	79.8	50.7A	50.1B	62.2	30.1a
Glycerol 5%	76.7	87.7	74.5	62.7	81.5	49.6A	25.0A	64.6	22.5a

A..B : $P \leq 0.01$;
a..c : $P \leq 0.05$.