EVALUATION OF EFFECTS OF CRYOPRESERVATION ON RABBIT SPERMATOZOA MEMBRANES WITH TRYPAN BLUE-GIEMSA STAINING

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ABSTRACT

The effects of cryopreservation on membrane of rabbit spermatozoa were studied by Trypan blue-Giemsa staining. Semen samples of 11 White New Zealand bucks (N=31) were evaluated before freezing and after freezing-thawing. The applied cryopreservation method based on two cryoprotectants (DMSO, glycerol). The integrity of the head and tail membrane and the acrosome status were evaluated on smears stained by trypan blue and Giemsa stains according to KOVACS, FOOTE (1992). The freezing-thawing process increased the number of dead cells from 18% to 46%. At the same time live cell heads were also observed and significant damage of acrosomal and tail membrane integrity ($p<0.001$) was detected. The most destroying effect was shown on sperm tail, which was found as the doubled number of stained tail spermatozoa. The freezing-thawing effects were not influenced by collection date but some differences were detected between semen of different bucks in term of tolerance to freezing.

Key words: rabbit semen, cryopreservation, membrane integrity, staining.

INTRODUCTION

In the last decade the artificial insemination became a general reproduction technique in commercial rabbit breeding. The insemination can be performed at any date and place; therefore the propagation can be timed, what is more simple and cheaper. At the same time the semen production and quality of different bucks are controlled, and the fertility of male rabbits can be estimated by *in vitro* methods. Importance of evaluation in advance is underlined by the fact that one single male affects the fertility and prolificacy of about one hundred females. Several studies were published about freezing of rabbit semen, although in the practice it is not applied (CHEN and FOOTE, 1994; VICENTE and VIUDES-CASTRO, 1996; MOCÉ and VICENTE, 2002). The reasons can be that very different data were published about evaluation of fresh or frozen semen (ALVARIÑO, 2000). Cryopreservation of rabbit semen has more interest: this procedure could help preservation of valuable breeds presenting unique genetic characteristics (JOLY *et al.*, 1996) or for commercial rabbit production to balance the often different needs of volume

required for artificial insemination, when working with batch system. Freezing and thawing process on the other hand can damage membranes which are particularly sensitive to temperature changes. The possibility for cryopreservation with good result can be different in case of semen collected from different bucks (CHEN *et al*., 1989). The evaluation of rabbit bucks from this point of view preceding their involvement in artificial insemination or in semen freezing program is very important. This can be done faster and cheaper by a suitable *in vitro* method. The effect of cryopreservation on spermatozoa quality was studied by different *in vitro* methods, estimating mainly the percent of motile cells. Motility/progressive motility of wet preps is estimated under light microscope, subjectively or assisted by computer aided image analysis, the later being quite expensive in case of rabbit semen. Percentage of live cells with intact acrosome could be evaluated under light microscope on stained smears; however, this does not give information about the motility of the cells in most cases (except apparent morfological tail defects).

KOVACS-FOOTE (1992) worked out a method for estimation of sperm cell viability and acrosome status, and which was found to be applicable also for detection of nonmotile cells bearing tails without apparent morphologycal anomaly, based upon the tail's staining (NAGY *et al*., 1999). The two-step staining with trypan-blue and Giemsa is simple, although it's evaluation quite laborious and time consuming.

In the present experiment, the staining pattern was evaluated in fresh and frozen-thawed rabbit semen samples, which were collected from 11 trained bucks at five different occasions.

MATERIAL AND METHODS

Bucks and semen collection

The semen samples were collected from 11 mature and trained New Zealand White breeding bucks selected for weight gain on the experimental farm of ISAR. IMV type artificial vagina was used. The collection was repeated on every second Fridays for five times. Within this period, the bucks were used once for artificial insemination of the Institute's breeding stock. Number of samples collected on each day was 5-7, and number of samples per each buck was 1-5, depending on the mounting willingness of the bucks. The animals were housed individually in wire cages equipped with self-eater and nipple drinker and were offered *ad libitum* with commercial fattening feed. The room had artificial ventilation system. The lighting program was 16 hours light and 8 hours dark.

Staining of semen samples

The collected semen samples were put in the water bath adjusted to 37 C. A part of the samples were diluted (1:20) with 0.9% NaCl, and were stained by Kovács-Foote method (KOVÁCS and FOOTE, 1992).

First, one drop diluted samples were mixed on a microscope slide with one drop of isoosmotic 0.2% trypan blue (preparated from 0.4% trypan blue SIGMA T-8154 diluted

with 0.9% NaCl 1:1) and were smeared. After vertical air-drying the slides were fixed for two minutes with fixative composed of 86 ml of 1 N HCL plus 14 ml of 37% formaldehyde solution and 0.2 g neutral red (SIGMA, N-2880) and then were rinsed with tap and distilled water. The slides were stained in 7.5% Giemsa (SIGMA, GS-500) for 3.5 hours at 37 C or overnight at room temperature. The slides were rinsed with tap water and distilled water and then were held for two minutes in a jar of distilled water for the best differentiation. Finally, the slides were dried in air, and covered with DePeX (SERVA, 18243) and coverslip.

Semen samples evaluation

Stained smears were evaluated by light microscopy at 1000x magnification. The status of the head and tail of 200 spermatozoa was classified in each smear. The samples were evaluated before freezing as well as after freezing-thawing. By this method the spermatozoa were classified as: live cell with intact acrosome (ALINT), live cell with intact acrosome and stained tail (ALST), live cell with damaged or missing acrosome (ALDAM), dead cells (D) have been categorized in the same way. The Cinderella sperm counter software (elaborated by BODÓ, SZ. http://sperm.abc.hu) was used for assistance of counting.

Semen cryopreservation

Only semen samples showing more than 70% subjective motility were frozen. This percentage was estimated subjectively by examining spermatozoa on a microscope slide warmed to 37°C.

A two step cryopreservation was used essentially based on the method published by ANDRIEU and COUROT (1976), with some modifications (adopted from Besenfelder's personal communication). Initially, one part of semen was mixed with two parts of a 9 v/v% DMSO and 15 v/v% egg yolk extender in a small conical tube at room temperature, and was cooled to 5 °C over a period of 90 minutes by placing in a regular refrigerator, and the same time, the required devices (tubes, pipette, straws) and solutions were also cooled. After cooling, the semen was diluted carefully with a 5 °C, 4 v/v% glycerol and 15 v/v% egg yolk extender of original volume of semen. Sample was distributed into 0.25 ml straws and was left at 5 C for 30 minutes. The straws were placed horizontally in a rack approximately 4 cm above a reservoir of liquid nitrogen. After 20 minutes, the straws were plunged into liquid nitrogen. Both of the extenders were based on Tris-citrate.

The semen was thawed by direct transfer of straws from liquid nitrogen to water bath at 39 C. After 10-12 sec, the straws were removed from the bath and dried.

Statistical analysis

The influence of freezing-thawing process on frequency of spermatozoa damage in total, or by date and male was evaluated by Chi-square test tool of GeneStat software.

RESULTS AND DISCUSSION

Categories of sperm cells by different staining result

The rear part of head of live cells is not stained, and the dead ones are stained dark. The intact acrosomes are purple, the damaged ones are lavender, and the posterior part of the heads without acrosomes is light grey. The damaged tail membrane is stained black, and these cells are not motile (NAGY *et al*., 1999). The categories of sperm cells are showed in Figure 1.

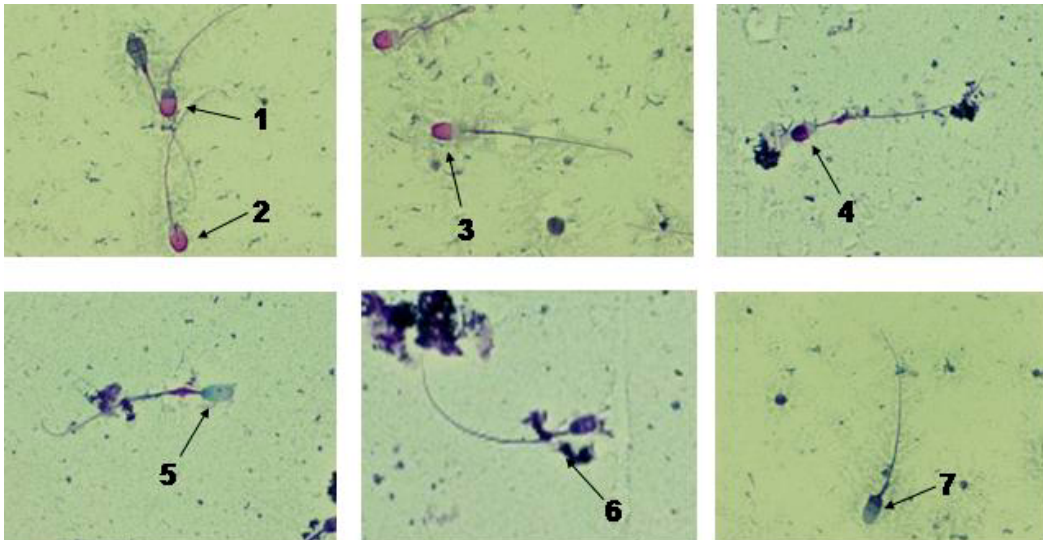

Figure 1. Microphotographs of semen smears stained according to Kovács and Foote

1. dead cell with intact acrosome (DINT), **2**. live cell with intact acrosome (ALINT), **3.** live cell with intact acrosome and stained tail (ALST), **4**. live cell with damaged acrosome (ALDAM) **5.** live cell with missing acrosome (ALDEN) **6**. dead cell with damaged acrosome (DDAM), **7.** dead cell with missing acrosome (DDEN),

Influence of freezing-thawing on the number live and dead spermatozoa

The frequency of live and dead spermatozoa can be a rough judgment of a freezing method's effectivity. The number of live and dead cells was counted in the fresh and the frozen-thawed samples, and evaluated by Chi-square statistics. The freezing-thawing process increased the number of dead cells, consequently reducing the number of live sperm cells(Table 1).

Table 1. Number of cells stained as live or dead before and after freezing-thawing procedure within the total scored sperm-cell population.

Cell status	Fresh	Frozen-thawed
Live	5235	3363
Dead	1136	2851

(Pearson Chi-square value is 1143.50 with df 1, probability level under null hypothesis is $p < 0.001$)

The proportion of dead cells was 18% in the fresh semen and increased to 46% after freezing-thawing, what is in good agreement with values found by CHEN *et al*. (1989).

Quality of live cells after freezing-thawing procedure
Information about the damage of sperm cells caused by freezing-thawing is not complete if only the death of these cells is considered. When the stained smears were evaluated, presence of live cells having membrane injuries was apparent. Acrosome and tail membrane integrity had more often failed in the semen samples after freeze thawing ($p<0.001$), Figure 2.

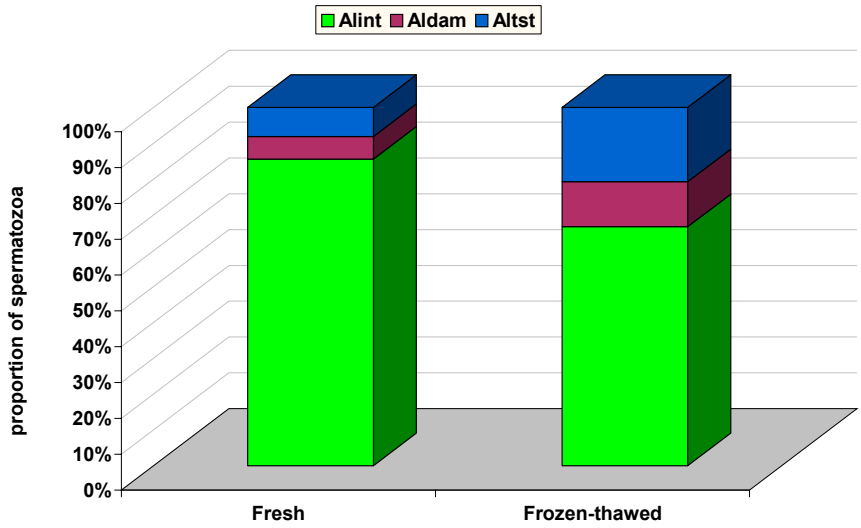

Figure 2. Proportion of live intact spermatozoa and sperm cells with membrane injury within the live cell's compartment before and after freezing-thawing. (ALINT: live cell with intact acrosome, ALDAM: live cell with damaged acrosome, ALST: live cell with stained tail)

However, number of sperm cells with missing or damaged acrosome has changed at lower level than what was published by CHEN *et al.* (1994), who found a significant 34% increase, although with different freezing method. The most destroying effect of freezing-thawing was detected on sperm tails, resulting in significantly increased frequency (contribution to Pearson Chi-square value is 151.5) of live but immotile spermatozoa, without fertilizing ability. The number of sperm cells with straight and stained tail has almost doubled by this procedure. In summary, the proportion of intact spermatozoa capable to fertilize, has decreased by 18%. Analysis of rabbit semen evaluating different classes within live cells has not been published, yet.

Influence of collection date and sampled buck on the freezing-thawing effect
The semen collection was repeated five times, each sampling following the previous one of 2 weeks (Fig. 3). Considering the fresh samples at the first date (the first three stars on the Fig. 3), the frequency of the dead cells was higher ($p< 0.001$), and this difference was entirely attributed to one buck with exceptionally wrong semen sampled only at that time as proved by the comparison of different bucks (Fig. 4). At the evaluation of frozen-

thawed samples, the last occasion showed a significantly higher number of dead cells (the last three starts on the Fig. 3). At that time, however, any differences between semen samples could not be detected. Instead, the very complex nature of freezing-thawing procedure and any mistake done with this particular batch of samples was supposed to cause even significant differences in the result.

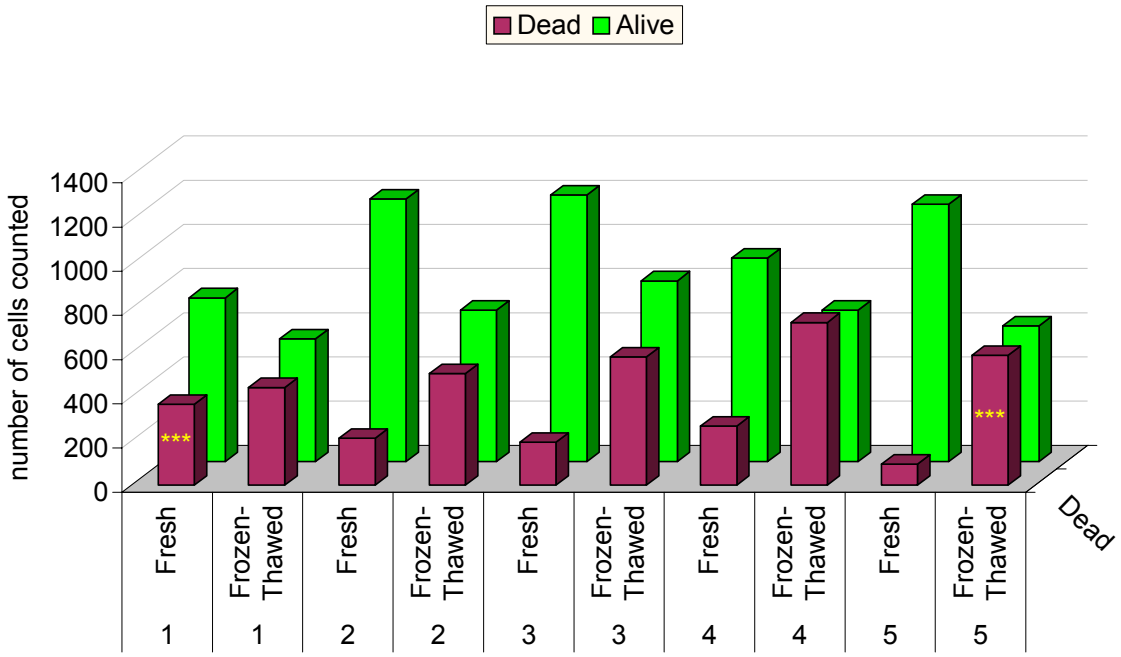

Figure 3. Number of live and dead spermatozoa counted before and after freezing-thawing of samples collected at different times

The results of subjective motility estimation were well reflected in the relative frequency of live cells counted on stained smears made of fresh semen, except one sample (buck C on Fig. 4). The decrease of relative frequency of live spermatozoa after freezing-thawing was different only in two males (buck A and B on Fig. 4). Semen samples collected from buck A were more resistant to the effects of freezing-thawing, contrary to this, samples of buck B were very sensitive.

The staining method here used unequivocally detected the membrane damaging effect of the freezing-thawing process of semen, what was earlier shown only by electromicrospic studies (COURTENS and THEAU-CLEMENT, 1996). Moreover, differences in the degree of damage according to the date of semen sampling and freezing or to the mounting buck have also been found. This could be important in the light that protocols available for rabbit semen cryopreservation show contradictory results (BESENFELDER *et al*., 2000). Furthermore, evaluation by artificial fertilization seems inadequate, because the results are influenced by a lot of confounding factors (sexual receptivity, parity, insemination dose, etc) (THEAU-CLEMENT *et al.* 1996).

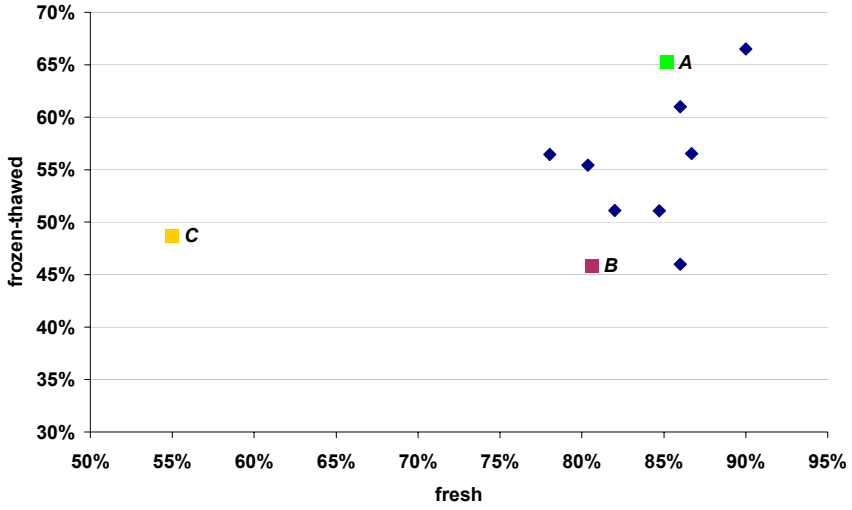

Figure 4. Correlation of live spermatozoa in fresh and frozen- thawed semen samples collected from different bucks

CONCLUSION

The Trypan blue-Giemsa staining was proved to be highly sensitive to detect the harmful effects of freezing method. Frequency of the dead cells was increased by the effects of freezing-thawing procedure, and the number of damaged cells, unable to fertilize, were increased also within the decreasing population of live cells.
The tail membranes were especially damaged by the applied cryopreservation procedure, what is shown by the doubled number of stained tail spermatozoa.
Practically, the effects of freezing-thawing procedure were not influenced by the date of collection. However, differences appeared among tolerance of freezing of semen collected from different bucks.

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