“Comparison of the quality of frozen-thawed and cooled-rewarmed dog semen”

ELSEVIER

COMPARISON OF THE QUALITY OF FROZEN-THAWED AND COOLED-REWARMED DOG SEMEN

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ABSTRACT

Semen may be preserved for short-term storage either by cooling and rewarming or by freezing and thawing. The freeze-thaw process causes significant sperm damage, and it may be preferable to cool and rewarm samples, although cooled spermatozoa has a limited lifespan.

Ejaculates were collected from 6 adult male beagle dogs. The second fraction was divided into 2 aliquants, one of which was diluted with a Tris-egg yolk extender, placed into 0.5-ml straws and frozen in liquid nitrogen vapor, before being stored in liquid nitrogen. The second aliquant was diluted with a nonfat dried milk-glucose extender, cooled to and stored at 5°C. Portions of the cooled semen were removed on Days 0, 1, 2, 4, 6, 8 and 10, and the the semen quality was evaluated after rewarming. The semen quality of the frozen semen was assessed after thawing. A variety of assessments were made, including sperm motility, sperm morphology, acrosome status, hypo-osmotic swelling, and longevity at 39°C. Comparisons within ejaculates were made between the semen quality of the frozen samples and the samples that had been cooled and stored for different time periods.

Semen quality of the samples that had been cooled and stored deteriorated on a daily basis; however, in the first 2 d after collection, semen quality was always superior to that of the samples that had been frozen and thawed. The mean time taken for semen quality of samples that had been cooled and rewarmed to become equal to samples that had been frozen and thawed varied for each parameter of semen evaluation, but overall it was 118.7 $\pm$ 25.9 h.

Should short-term cryopreservation of dog semen be contemplated to allow sample transportation, it is clear that using conventional methodology dilution and cooling is the most suitable technique, provided that the sample is used within approximately 4.9 d of collection. Should the required length of storage exceed this time, it would be prudent for the sample to be frozen and then thawed.

Key words; dog, semen, cryopreservation, cooling

INTRODUCTION

The cryopreservation of dog semen is becoming increasingly popular since it allows for the transporting of genetic material both within and between countries. Semen transportation would

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reduce animal stress by eliminating travel and it may reduce disease risks as well as animal shipping costs.

There are generally 2 options for the transport of semen. It may be diluted, cooled and stored at 5°C before being rewarmed and used for insemination, or the semen may be dilnted and frozen at -196°C before being thawed and used.

The first success in freezing dog spermatozoa was reported by Rowson (24), while Seager (25) reported the first pregnancy resulting from artificial insemination (AI) with frozen-thawed dog semen. Since that time, there have been several studies that have investigated methods of preserving dog spermatozoa by freezing (see 8,9,26). Currently a limited number of extenders and freezing methods are being used, the most common of which was first described in 1972 (1-4). However, there has been little information published concerning the short-term storage of dog semen by dilution and cooling to 5°C (13-16,22). Once stored in this manner semen must be used within a short period of time. Nevertheless, this technique has become increasingly popular since it is possible to transport samples rapidly between countries.

There are several problems using frozen-thawed semen in the dog. These include poor post-thaw quality (21) and a significantly reduced life-span (longevity) of the spermatozoa after thawing (9). However, samples may be stored indefinitely and used when required. Cooled-rewarmed semen appears to be less damaged by the preservation process, and following the insemination of cooled-rewarmed semen the pregnancy rates appear to be higher than those of frozen-thawed semen (19). However, with increasing periods of storage, the quality of cooled semen deteriorates. It is likely that at a certain point in time the quality of cooled-rewarmed semen does not differ from that of frozen-thawed semen, and that after this particular time the quality of cooled-rewarmed semen is lower than that of frozen-thawed semen.

The aim of the present study thus was to establish the optimal storage time for cooled semen during which semen quality would be greater upon rewarming than had the sample been frozen and thawed.

MATERIALS AND METHODS

Six healthy 5-yr-old beagles of unknown fertility were used for the study. The dogs were housed in kennels with access to the outdoors. Semen was collected by digital manipulation twice weekly from each dog, and the 3 fractions of the ejaculate were collected into 3 separate plastic test tubes via glass funnels. Two ejaculates from each dog were used for the study. A drop of the second fraction was placed on a warmed glass slide, and the percentage of spermatozoa with normal, vigorous forward linear motion was subjectively assessed to the nearest 5% by the method of Taha (27). Spermatozoa showing circular, oscillatory and reverse movement were not considered to have normal motility. The sperm concentration of the second fraction was determined using an improved Neubauer haemocytometer counting chamber. The numbers of live and dead spermatozoa and spermatozoal morphology were examined on nigrosin/eosin stained smears using the classification of Christiansen (7). Using this classification, individual sperm cells were recorded as being either alive (unstained) or dead (stained), and their individual morphological abnormalities were tabulated according to their site (head, neck, midpiece, or tail). A further examination was made of the smear to evaluate the percentage of spermatozoa with normal intact acrosomes irrespective of the morphology of the cell using the classification of Bwanga (6). Spermatozoa with thickened acrosomes, vesiculated or disintegrating acrosomes, or detached and absent acrosomes were considered abnormal; acrosome status was recorded as either normal or abnormal. The percentage of spermatozoa with tails which were swollen following incubation with a hypo-osmotic

medium (hypo-osmotic swelling test) was assessed (12).

Sperm longevity was evaluated at dog body temperature by evaluation of sperm motility (as previously described) at times 0, 30, 60, 90, 120, 240 and 360 min during incubation at 39°C. From this, 2 summary measurements were calculated. These were the lag phase (the time taken for spermatozoal motility to fall to 75% of its initial value) and the period of decline (the time taken for spermatozoal motility to fall to 25% of the original value). This method has been previously described and shown to be useful for discriminating between samples (10).

The second fraction of each ejaculate was divided into 2 aliquants. The first was diluted 1:4 (volume:volume) with a Tris-egg yolk extender (1), placed into stoppered tubes, cooled slowly (half rate of cooling = 9 min) to 5°C, and 4 h after collection, placed into 0.5-ml straws before freezing 4 cm above liquid nitrogen in a wire basket. The straws were not seeded and were plunged into liquid nitrogen after 10 min. The second aliquant was diluted 1:4 (volume:volume) with a nonfat dried milk-glucose extender (5, 17), placed into stoppered tubes, cooled slowly (half rate of cooling = 9 minutes) to 5°C, and stored at that temperature.

Frozen semen was thawed by placing the straws into a water bath at 39°C and then emptying the contents into a test-tube within the same water bath. Assessments of sperm motility, morphology, hypo-osmotic swelling and longevity were made commencing 5 min after warming as previously described for pooled straws from each ejaculate. Portions of the cooled semen were rewarmed on Days 0, 1, 2, 4, 6, 8 and 10 by placing them into a test tube within a water bath at 39°C. Assessments of sperm motility, morphology, hypo-osmotic swelling and longevity were made commencing 5 min after warming as previously described.

For each dog, each parameter (percentage motility, percentage live normal spermatozoa, percentage total live spermatozoa, percentage normal acrosomes, percentage swollen spermatozoa, and 75 % lag and 25 % decline values), and each ejaculate, the time taken for the value of cooled-rewarmed semen to reach the value of frozen-thawed semen for the same ejaculate was calculated using an iterative method. The means were then calculated for each parameter for each dog, and the data were expressed as the mean (± SD) time in hours for cooled-rewarmed semen to reach the same value as the frozen-thawed semen.

RESULTS

During the study the initial characteristics of the ejaculates from all 6 dogs were within the following ranges: motility, 85 to 95%; concentration, 400 to 900 x10^6/ml; morphologically normal live spermatozoa, 80 to 94%; total live spermatozoa, 82 to 96%; normal acrosomes, 88 to 95%; osmotically swollen sperm 89 to 96%; 75 percent lag value, 140 to 183 min; 25 percent decline value, 410 to 484 min.

Semen quality following freezing-thawing was in the following ranges: motility 45 to 70%; morphologically normal live spermatozoa, 20 to 32%; total live spermatozoa, 26 to 52%; normal acrosomes, 52 to 84%; osmotically swollen sperm 46 to 73%; 75 percent lag value, 54 to 76 min; 25 percent decline value, 110 to 156 min.

Semen quality on Day 0 after cooling and rewarming was within the following ranges: motility, 85 to 90%; morphologically normal live spermatozoa, 78 to 90%; total live spermatozoa, 81 to 94%; normal acrosomes, 84 to 88%; osmotically swollen sperm 89 to 95%; 75 percent lag value, 132 to 193 min; 25 percent decline value, 431 to 468 min. On Day 10 semen quality after cooling and rewarming was within the following ranges: motility, 0 to 10%; morphologically

Table 1. The mean time in hours for various semen parameters of cooled-rewarmed samples to become equivalent to the values of frozen-thawed samples from split ejaculates. Two ejaculates were collected from each of six dogs. Values represent means of the six dogs, where values for each dog represent means from the two ejaculates.

Semen Parameter	n	Mean time in hours	SD
Percentage motility	6	124.5	19.2
Percentage swollen spermatozoa	6	123.1	54.3
Percentage live normal spermatozoa	6	106.8	61.1
Percentage total live spermatozoa	6	85.2	42.2
Percentage normal acrosomes	6	169.3	16.9
Longevity (75 % lag value)[a]	6	106.8	52.5
Longevity (25 % decline value)[b]	6	114.9	46.6

[a] 75 % lag value = the time taken for spermatozoal motility to fall to 75% of its initial value.

[b] 25 % decline value = the time taken for spermatozoal motility to fall to 25% of its initial value.

normal live spermatozoa, 0 to 8%; total live spermatozoa, 18 to 33%; normal acrosomes, 6 to 18%; osmotically swollen sperm 24 to 42%; 75 percent lag value, 0 to 12 min; 25 percent decline value, 0 to 30 min. For each parameter, the values were numerically higher for cooled-rewarmed semen than for frozen-thawed semen on Days 0, 1 and 2.

The mean times for semen quality of cooled-rewarmed samples to become equal to those of frozen-thawed samples are given in Table 1. For all 7 parameters, the mean time taken for semen quality of cooled-rewarmed samples to become equal to those of frozen-thawed samples was 118.7 $\pm$ 25.9 h.

DISCUSSION

In the present study, which utilized split ejaculates collected from 6 dogs on 2 occasions, we compared 2 methods of semen preservation. The aim of the study was to evaluate these methods using the effect of treatment upon standard semen characteristics as well as longevity of samples incubated at 39°C. This temperature (dog core temperature) was chosen since the increased spermatozoal metabolism which occurs at higher temperatures leads to a more rapid depletion of nutrients in the confined in vitro environment (20); therefore, treatment differences might be more marked. This method for the study of longevity has been suggested to mimic, to a certain extent, conditions encountered by the spermatozoa in the female genital tract (18). Poor survival of spermatozoa during incubation is associated with a lower fertilizing ability (23).

When the frozen samples were thawed, there was a marked deterioration in semen quality, as indicated by a reduction in spermatozoal motility, morphology, osmotic swelling and longevity compared with that of the original ejaculate. The values obtained, however, were similar to those previously reported following the freezing of dog spermatozoa (10), and are compatible with fertility (11). The changes in semen quality for samples that were cooled and then rewarmed were quantitatively similar to those previously reported (5).

For short periods of storage (up to 2 d) the quality of the cooled-rewarmed semen was superior to that of the frozen thawed semen. There was a reduction in each of the measures of semen quality with time. The rate of decline was different for each parameter, with spermatozoal morphology deteriorating most quickly. At approximately 110 h the longevity of the cooled-rewarmed semen was similar to that of the frozen-thawed semen; and, after approximately 4.9 d of storage at 5°C, the quality of the cooled-rewarmed semen was equivalent to the quality of frozen-thawed semen.

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