INVESTIGATION OF USEFULNESS OF SPERM ANALYSES IN DOGS FOR MALE FERTILITY STUDY

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ABSTRACT — The usefulness of sperm analyses in dogs, including sperm motion analysis, was investigated. Sperm motion analysis was performed with the CellSoft-4000 computer-assisted sperm analysis (CASA) system. First, we examined the conditions for preservation of optimal semen quality. We found that sperm retained more of their motility at 4°C than at 37°C. Secondly, we observed sperm motion, concentration and morphology in dog semen continuously for 11 weeks. We collected semen samples during the test period, and the samples retained sperm motion, concentration and morphology. Finally, we administered a-chlorohydrin, which decreases rodent sperm motion, at a single oral dose of 100 mg/kg or 150 mg/kg to dogs. Sperm motion was inhibited immediately after a-chlorohydrind treatment, and recovered after 2 weeks. None of the experimental animals were sacrificed in the above-mentioned examinations.

We thus confirmed that sperm analyses including motion analysis in dogs are useful in male fertility studies.

KEY WORDS: Sperm analysis, Beagle, CASA, CellSoft-4000

INTRODUCTION

In reproductive and developmental toxicity studies, that of fertility and early embryonic development to implantation (Segment I) is the only one available to investigate male fertility directly. Recently, sperm motion analyses have been performed to examine testicular function in Segment I. According to the ICH agreement (ICH guidelines, 29 November 1995), however, Segment I is not necessarily required to enter clinical trials. The adverse effects of under-development drugs on male fertility have been examined by histopathological examination of male reproductive organs in repeated-dose toxicity studies before clinical trials (Sakai et al., 2000). Sperm function and libido, however, cannot be analyzed by histopathological examination.

Dog semen can be collected from the same animal over a long period of time without sacrifice of the animal. Therefore, in evaluating toxic effects of agents on reproductive organs, manifestations of toxicity can be observed, and the period required for recovery can be observed as well. Moreover, it is possible to observe sexual behavior, i.e., erection and ejaculation, when semen samples are collected. Despite these advantages, however, there have been fewer studies concerning analysis of dog sperm than that of rodent sperm. In the present study, we attempted to confirm the usefulness of sperm analyses in dogs. For this purpose, we examined procedures of sperm analyses, characteristics of sperm motion, and changes in sperm motion induced by a-chlorohydrin over time in dogs.

MATERIALS AND METHODS

Experimental design

The present study included the following 3 experiments.

1. Validation of the procedure of sperm motion analysis in dogs (Experiment I)

The optimal conditions for preservation, from semen sampling to sperm motion analysis, were examined. Semen samples were collected from 6 beagles from 23 to 28 months of age and each sample was
aliquoted to 2 parts. One part was preserved in a 4°C-cooler box, and the other part was preserved in a 37°C-water bath. Every fraction was measured for percentage of motile sperm (% motile) and sperm curvilinear velocity (VCL) at 1, 3, and 6 hr after collection with a CellSoft-4000.

2. Confirmation of the characteristics of dog semen (Experiment II)
1) Experiment II-1
Sperm motion analysis was carried out using 20 dogs from 15 to 40 months of age. Semen samples were analyzed with a CellSoft-4000 having 9 motion parameters: percentage of motile sperm (% motile), curvilinear velocity (VCL), linearity, maximum and mean amplitude of lateral head displacement (ALH max and ALH mean), beat cross frequency (BCF), average radius, percentage of circular-swimming sperm out of motile sperm (circular/motile) and percentage of circular-swimming sperm out of all sperm (circular/all).

2) Experiment II-2
Three of the 20 dogs from 23 to 28 months of age were used to examine the effects of continuous semen sampling. Semen samples were collected twice a week during the first 4 weeks and once a week over the subsequent 7 weeks. Semen samples were analyzed for sperm motion, concentration and morphology.

3. Effects of α-chlorohydrin (Experiment III)
We examined the effects of α-chlorohydrin, which is known to reduce sperm motion in rodents, on dogs.
1) Experiment III-1
Seven dogs from 22 to 23 months of age were divided into a group of 3 as the control group and a group of 4 as the treatment group. The dogs in the treatment group were administered α-chlorohydrin at a single oral dose of 100 mg/kg. From each of the 7 dogs, semen was collected 3 times during the 2 weeks before administration and 6 times during the 3 weeks after administration. The sexual behavior of the dogs was observed when their semen samples were collected. The collected samples were analyzed for sperm motion, concentration and morphology.

2) Experiment III-2
The final semen samples collected before administration of α-chlorohydrin had lower sperm motion than usual in Experiment III-1. Because of this lower value, the change in sperm motion after α-chlorohydrin treatment was not dramatic. In order to demonstrate the effects of α-chlorohydrin on sperm motion more clearly, we administered α-chlorohydrin to the other 2 dogs of 22 and 33 months of age at a single oral dose of 150 mg/kg. The control group was not subjected to Experiment III-2.

Chemicals
α-chlorohydrin was purchased form Nacalai Tesque (Kyoto, Japan). Bovine serum albumin (BSA, cat # A2135) was purchased from Sigma-Aldrich (Tokyo, Japan). Dulbecco’s phosphate-buffered saline (D-PBS) containing glucose was obtained from Gibco BRL (Tokyo, Japan). All other reagents were purchased from commercial sources and were of at least analytical grade.

Animals
After quarantine and acclimatization, 33 healthy beagle dogs purchased from three breeding facilities were used for semen sampling; 15 animals from 15 to 24 months of age from Covance Research Products, Inc. (MI, U.S.A.), 10 animals from 11 to 29 months of age from Marshall Farms USA, Inc. (NY, U.S.A.) and 8 animals from 24 to 41 months of age from Yakken Farm, Inc. (Hyogo, JAPAN). Twenty of the 33 dogs from which semen samples could be collected easily with a few trials were used for the experiments. The dogs were individually housed in metal cages (W 700× H 800× D 650 mm) or concrete wall cages with a metal door without a ceiling (W 1000× D 800 mm). Solid food (DS, Oriental Yeast Inc., Tokyo) was fed at 0.25 kg/day and tap water was available ad libitum. The animal rooms were maintained at 23 ± 5°C, with 50 ± 20% relative humidity and a 12 hr light - dark cycle (lights on 7:15 to 19:15) and were cleaned and disinfected 6 days per week.

Semen sampling and observation of sexual behavior
Semen samples were collected in disposable tubes by digital manipulation; by holding the root of the bulbus glandis with two fingers and pressing the holding point until ejaculation or over about 5 min. Ejaculated semen was divided into 3 fractions based on color. The second fraction, which is milky white and originates in the epididymides, was used for sperm analyses. The sexual behavior of the dogs was classified into 4 grades at semen sampling: no erection, erection without ejaculation, ejaculation without the second fraction, and ejaculation with the second fraction.
Sperm motion in dogs.

Sperm motion analysis
The semen samples were preserved in a 4°C-cooler box immediately after collection (in Experiments II and III). The second fractions of semen were diluted over 100 times by 37°C-PBS containing 0.5% BSA immediately before motion analysis with the CellSoft-4000. The motion analysis was performed on a 37°C-hotplate within 1 min after the sample dilution. The parameter settings for the CellSoft-4000 are shown in Table I.

Sperm concentration and morphology
Sperm concentration in the second fraction of semen was counted with a hematocytometer using an optical microscope.

The morphology of the smeared specimens of semen was observed using an optical microscope. More than 200 sperm were observed per each specimen. The observed sperm were classified into 5 types: normal, isolated head, abnormal head, abnormal tail and others.

Table 1. CellSoft parameters for dog sperm analysis.

<table>
<thead>
<tr>
<th>Item</th>
<th>Establishment</th>
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<tbody>
<tr>
<td>General parameter</td>
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</tr>
<tr>
<td>Number of frames per second (Hz)</td>
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<tr>
<td>Video standard</td>
<td>NTSC/American-Canadian</td>
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<td>Minimum sampling - motile</td>
<td>2</td>
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<tr>
<td>Minimum sampling - velocity</td>
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</tr>
<tr>
<td>Maximum velocity (μm/sec)</td>
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</tr>
<tr>
<td>Threshold velocity (μm/sec)</td>
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</tr>
<tr>
<td>Threshold gray level</td>
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</tr>
<tr>
<td>Cell color</td>
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<tr>
<td>Pixel scale (μm/pixel)</td>
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</tr>
<tr>
<td>Cell size range (pixel)</td>
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<tr>
<td>End-point side</td>
<td>direction:0 velocity:0 mass:0</td>
</tr>
<tr>
<td>ALH parameter</td>
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<tr>
<td>Minimum number of points</td>
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<tr>
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<tr>
<td>Maximum radius (μm)</td>
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<tr>
<td>Camera lens</td>
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</tbody>
</table>

ALH: Amplitude of lateral head displacement.

Statistical analysis
In Experiment I, sperm motion data for each hour of observation were compared to those for time 0 by paired t-test analysis (Yoshimura, 1987). In Experiment III-1, numerical data for the treatment group were compared to the data for the control group of the same day with Student’s t-test (Yoshimura, 1987). The data of Experiment II, those of Experiment II-2, and the sexual behavior observed in all experiments were not analyzed statistically. All statistical tests were performed at the 0.05 or 0.01 levels of significance.

RESULTS

Validation of procedure of sperm motion analysis in dogs (Experiment I)
The effects of preservation temperature on sperm motion are shown in Fig.1. Sperm retained more motility at 4°C than at 37°C. The percentage of motile sperm (% motile) was significantly decreased at 1 hr after...
starting preservation at 37°C but retained at 3 hr after starting preservation at 4°C. Results for sperm curvilinear velocity (VCL) were the same at 4°C and 37°C. Thus, we decided to preserve all semen samples at 4°C immediately after collection and measured sperm motion within 2 hr after collection in Experiments II and III.

Confirmation of the characteristics of dog semen (Experiment II)

1. Experiment II-1
   Sperm motion analysis was carried out using the 20 dogs from which semen could be easily collected. Subsequent semen sampling trials increased the success rate of sampling, but there were also dogs from which it was impossible to collect semen.

   Sperm motion parameters of the 20 dogs are shown in Fig.2. There were differences of more than double between the lowest values and highest values of ALH max, ALH mean, radius, circular/motile and circular/linear.

2. Experiment II-2
   The collection of semen samples from the 3 acclimatized dogs was continuously possible over 11 weeks, although there were a few sporadic cases of lack of ejaculation or erection. Fig.2 shows changes in sperm motion parameters over 11 weeks. Continuous sampling did not influence the values of parameters, although some parameters were stable and others were not. Fig.3 shows changes in sperm concentration and morphology over 11 weeks. Sperm morphology was calculated following division into 2 types (normal or abnormal) in Fig.3, although it was classified into 5 types. As with sperm motion, sperm concentration and morphology were not influenced by continuous samplings over 11 weeks.

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**Fig. 1.** Comparison of changes of sperm motion parameters (% motile and curvilinear velocity) between different preservation temperatures. Each closed square (■) and bar show the mean and standard deviation of sperm motion parameters in the 4°C preservation during 6 hr and each open circle (○) and bar show these in the 37°C preservation. Motion data of each observation hour were statistically compared to the data of time 0. (* : p<0.05, ** : p<0.01).
Sperm motion in dogs.

Fig. 2. Nine sperm motion parameters analyzed for untreated dogs with the CellSoft-4000. Open circles (○) show the value of each dog in Experiment II-1. Closed squares (■) and bars show the mean and standard deviation of 3 dogs in Experiment II-2.
Effects of α-chlorohydrin (Experiment III)

1. Experiment III-1
A single oral dose of 100 mg/kg of α-chlorohydrin affected neither clinical signs nor sexual behavior of dogs. Sperm motion parameters, however, were affected by treatment with α-chlorohydrin (Fig.4). The curvilinear velocity (VCL) of the sperm of α-chlorohydrin-treated dogs was significantly decreased at 1 and 4 days after administration compared with that of control sperm. Linearity was significantly decreased at 1 day after administration as well. The decreased sperm motion parameters gradually recovered and achieved control values 2 weeks after administration. The other motion parameters, except for average radius, exhibited no significant changes following α-chlorohydrin treatment. Sperm concentration and morphology were not significantly changed (Data not shown).

2. Experiment III-2
Following a single oral dose of 150 mg/kg of α-chlorohydrin, vomitus was observed on the day of administration and the day after administration. As in Experiment III-1, sexual behavior was not affected by α-chlorohydrin treatment. VCL and linearity were decreased after administration and, as in Experiment III-1, recovered 2 weeks after administration (Fig.4).

DISCUSSION

Sperm motion analysis in rats with CASA has been common after issuance of the guidelines (ICH guidelines, 29 November 1995). Initially, we attempted to measure sperm motion in dogs with the CellSoft-4000 using the conditions for rats in our laboratory (Ban et al., 2001). Sperm of dogs, however, has its own characteristic shape, size and motion, so we could not measure sperm motion in dogs using the conditions appropriate for rats. We therefore had to change the objective lens and condition of the CellSoft-4000. Moreover, sperm motion is affected by dilution medium, temperature and period of preservation. The standardized method of sperm analysis is an important topic in clinical diagnosis. The WHO laboratory manual (4th ed.) provides a standardized protocol for human specimens: keep sperm at 20-40℃, measure within 1 hour, perform at least 2 times, etc. We attempted to standardize a realistic and easy method for sperm motion analysis in dogs. As a temperature until motion analysis, 37℃-preservation is more physiological than 4℃-preservation. However sperm motility decreased rapidly at 37℃ in ejaculated semen. It is difficult to analyze sperm motion immediately after ejaculation in toxicological studies of dogs. Thus, we selected a procedure in which semen samples were preserved at 4℃ immediately after ejaculation and diluted with 37℃-buffer.
Sperm motion in dogs.

Fig. 4. Change of sperm motion parameters in dogs administered $\alpha$-chlorohydrin.

Data were shown as the mean and standard deviation at a single dose of 100 mg/kg ($\circ$, n=4) or 150 mg/kg ($\triangle$, n=2) of $\alpha$-chlorohydrin and untreated (■, n=3). The semen samples of the day 0 in the 150 mg/kg were collected before the administration. The dotted lines show the mean ± 2SD of our background data. Statistical analysis was performed between the data of control and these of the 100 mg/kg group obtained at the same day (*: p<0.05, **: p<0.01).
just before motion analysis. There are some reports of analysis of dog sperm with CASA (Günzel-Apel et al., 1993; Dobrinski et al., 1993), in which specific media for semen dilution were used because motion had to measure for sperm frozen and thawed. Our purpose, however, was to return semen temperature to 37°C. As a result, we developed a procedure in which semen samples were preserved at 4°C within 2 hr, diluted with 37°C PBS containing 0.5% BSA immediately before analysis and analyzed on a 37°C-hotplate.

Semen sampling is a key technique in sperm analyses in dogs. Dogs must be trained to ejaculate with digital manipulation. We trained 33 dogs for semen sampling, analyzed the semen of 20 dogs, and continuously analyzed the semen of the 3 well-trained dogs in Experiment II. We were able to collect semen over 11 weeks from the same dogs and confirmed lack of effect of this sampling on sperm motion, concentration, and morphology. Dog sperm requires about 9 weeks for spermatogenesis in the testes (Takahashi et al., 1994). These facts mean that it is possible to monitor the effects of administered compounds on testis during all stages of spermatogenesis by continuous observation of semen quality using the same animal. Continuous sampling was possible, but there were a few sporadic cases of lack of erection or ejaculation over the 11 weeks. The reason for this is still unclear. Semen sampling was performed by the same pair of persons in the same rooms. The dogs might be influenced by small changes in environmental conditions, since dogs are generally nervous animals. If we estimate the toxic effects of under-development drugs on spermatogenesis or sexual behavior, we had better increase the number of animals in order to judge whether lack of erection or ejaculation is treatment-related or sporadic.

In the present examinations, we classified sperm morphology into 5 types. However, we observed a few abnormal sperm of each type per 200 sperm. A consensus report recommends that statistical analyses be limited to comparisons of 'normal' and 'abnormal' sperm in dogs (Seed et al., 1996). As with the consensus study, we compared morphological differences between normal and abnormal sperm in our examinations.

The antifertility effects of a-chlorohydrin, which reduces sperm motion, are well-known (Coppla, 1969; Samojlik and Chang, 1970; Vickery et al., 1974; Paz and Homonnai, 1982; Yamada et al., 1995). We analyzed sperm of rats administered a-chlorohydrin with the CellSoft-4000 in a collaborative study (Ban et al., 2001). The collaborative study found useful and sensitive sperm motion parameters for rats among the 9 parameters of the CellSoft-4000. The study suggested that % motile, VCL, ALH max and ALH mean were useful and sensitive parameters. In Experiment III, we intended to find useful and sensitive parameters of sperm motion in dogs. % motile, one of the most general motion parameters, was slightly but not significantly decreased within the mean of ±2SD of the background data by a-chlorohydrin treatment. % motile was not sensitive to a-chlorohydrin treatment as a parameter. This parameter is strongly influenced by impure particles contained in semen samples other than sperm themselves. Particles of size similar to sperm heads of dogs reduce apparent % motile. The characteristics of % motile, however, are different from those of the other parameters. This parameter objectively expresses how many sperm are motile among all sperm analyzed, although the 8 other parameters focus on motile sperm and characterize how such sperm move. Therefore, the low sensitivity of % motile does not mean that it is not useful for sperm motion analysis. VCL or linearity were significantly decreased by a-chlorohydrin treatment. In the above-mentioned collaborative study of rats, VCL was one of the most sensitive parameters, but linearity exhibited little or no change on treatment with 9 compounds including a-chlorohydrin (Ban et al., 2001). Therefore, linearity may be a uniquely sensitive indicator of dog sperm motion. ALH max and ALH mean were slightly increased, although not to a significant extent, even though these parameters were decreased in rats by a-chlorohydrin or other compounds (Ban et al., 2001). The cause of this different direction of change seems to originate from different shape of sperm between rats and dogs. This different direction of change is an interesting finding. But these parameters, while sensitive in rats, were not sensitive in dogs. BCF was not a sensitive indicator for a-chlorohydrin treatment. Average radius, circular/motile and circular/all were changed by a-chlorohydrin treatment. These parameters, however, were not considered useful and sensitive, since ranges of their background values were wider than that of treatment-related change. There is a limit to each parameter value in accuracy, because the CellSoft-4000 analyzes three-dimensional movement of sperm as two-dimensional images. It would be too hasty to conclude which parameters are generally useful for sperm motion analysis in dogs using only the results of this study.

The reason why Experiment III-2 was performed after Experiment III-1 is that the final semen samples collected before administration had lower sperm
motion than usual. Because of this lower value, the change in sperm motion after α-chlorohydrin treatment was not dramatic. A possible cause of the lower motion is an insufficient interval between the semen sampling points. We collected the semen samples that indicated lower sperm motion 2 days after from the last sampling point. At the other points in which at least a 3-day interval was allowed, sperm motion of untreated dogs was stable. One report insists that repeated semen ejaculations on alternate days do affect the density and volume of the semen, but not sperm motility in humans (Nnutu et al., 1991). In order to judge whether the 2-day interval is the cause of low sperm motion in dogs, it is necessary to carry out more detailed examination of intervals of semen sampling. In Experiment III-2, we collected semen samples twice a week at 3- or 4-day intervals. As a result, we were able to observe a clear result of α-chlorohydrin treatment.

Dose-related effects of α-chlorohydrin on sperm motion were not observed in Experiment III. We assumed that the vomiting which occurred after a dose of 150 mg/kg of α-chlorohydrin was the reason for this. Vomiting probably reduced the actual volume of exposure to α-chlorohydrin in Experiment II-2.

In the present study, we confirmed that it is possible to collect semen samples throughout the entire period of spermatogenesis from the same dogs with sperm motion, concentration and morphology maintained constant. It was also possible to detect sperm motion change induced by α-chlorohydrin. In studies of dogs, it is possible to observe sexual behavior, although it is difficult in fertility studies of rodents. Moreover, we performed all examinations without sacrificing any of the dogs. We conclude that sperm analyses, including motion analysis with CASA and observation of sexual behavior in dogs, are useful to assess adverse effects of under-development drugs on male fertility.

REFERENCES


