Changes in Semen Quality and *In Vitro* Sperm Capacitation during Various Frequencies of Semen Collection in Dogs with Both Asthenozoospermia and Teratozoospermia

Eiichi KAWAKAMI, Tatsuya HORI and Toshihiko TSUTSUI

*Department of Reproduction, Nippon Veterinary and Animal Science University, 1–7–1, Kyonan-cho, Musashino-shi, Tokyo 180, Japan*

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**ABSTRACT.** Eight male dogs with both asthenozoospermia and teratozoospermia were used in this study. In experiment 1, semen was collected 10 times at intervals of 48 hr, 24 hr and 12 hr in 4 of the 8 dogs, and the semen quality was evaluated. In experiment 2, semen was collected 5 times at 24-hr intervals in the other 4 dogs. The spermatozoa collected on day 1 and day 5 were incubated for 4–6 hr in Canine Capacitation Medium, and the percentages of hyperactivated sperm (%HA), acrosome-reacted sperm (%AR), and the zona pellucida-binding sperm count (ZP sperm count) were assessed. The results of experiment 1 showed that the percentage of motile sperm increased and the percentage of abnormal sperm decreased markedly as the intervals between semen collections became shorter. When semen was collected at 12-hr intervals, the percentage of motile sperm increased from about 65%, the value before frequent collection was started, to about 80%, and the percentage of sperm with abnormal tails decreased from 30% to 15%. In experiment 2, the percentages of HA and AR, and the ZP sperm count in specimens collected on day 5 were higher than those in specimens collected on day 1, and the differences in % HA and in ZP sperm count were significant (P<0.05). The results demonstrated that sperm motility, abnormality, and potential fertility in dogs with asthenozoospermia and teratozoospermia can be temporarily improved by frequent semen collection. — KEY WORDS: asthenozoospermia, canine, frequent semen collection, teratozoospermia.

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Spermatozoa with abnormal tails are known to occur as a result of epididymal dysfunction in the bull [13, 14, 30] and the boar [7]. It has been reported that epididymal function in the dog is maintained by testosterone secreted by the testis [35]. The authors [21, 22] have reported that peripheral plasma testosterone levels were lower in dogs that had a high percentage of sperm with abnormal tails in the cauda epididymis than in normal controls. Frequent semen collection in bulls with poor semen quality has been reported to temporarily increase sperm motility and decrease sperm tail abnormalities [13, 14, 30]. Although there have also been reports on frequent semen collection in dogs [3, 10, 31, 37], the semen quality of the dogs in these studies was normal. The semen quality of infertile dogs has also been investigated [1, 16, 21, 22, 29, 34]. However, there have been no reports on the effect of frequent semen collection on spermatogenic dysfunction in the dogs.

The present study was conducted on 8 dogs whose semen quality was judged to be poor, mainly due to high percentages of low motility sperm (asthenozoospermia) and sperm with abnormal tails in their ejaculates (teratozoospermia). Sperm from 4 of 8 dogs with both asthenozoospermia and teratozoospermia was collected at intervals of 48 hr, 24 hr, and 12 hr, and changes in semen quality were examined. The other 4 dogs were used to investigate *in vitro* sperm capacitation.

**MATERIALS AND METHODS**

**Animals:** Eight dogs (5 Beagle dogs, 2 mongrel dogs and a Shetland sheep dog) with poor semen quality, aged 3–6 years, were used. The body weight of the Beagle dogs and the mongrel dogs ranged from 12 kg to 15 kg, and the Shetland sheep dog weighed 20 kg. All of the dogs were diagnosed as having both asthenozoospermia and teratozoospermia, based on a comparison of their semen quality (Table 1) and with other reports on normal semen quality in dogs [17, 20, 38]. The dogs were housed in pens with runs under natural lighting, and water was available *ad libitum*.

**Semen evaluation:** The male dogs were trained so that their semen could be collected by digital manipulation without an estrous bitch. Semen samples were examined for total semen volume, total number of sperm, and percentages of actively motile sperm, viable sperm, and morphologically abnormal and immature sperm using methods previously described [23]. Briefly, the concentration of sperm in the semen was determined by hemocytometer counts. The percentages of motile sperm and viable sperm were estimated by counting actively motile sperm on a warmed slide glass and by the eosin-nigrosin staining method, respectively. Sperm morphology was examined by the aniline blue and eosin staining method [5]. Spermatozoa with a cytoplasmic droplet attached to the mid-piece were counted as immature sperm [39]. It has been known that the ejaculate in dogs can be divided into 3 fractions [15]. In the present study, the pH and osmolarity (Vapor Pressure Osmometer; Model 5100, Wescor, Inc., U.S.A.) of the second semen fraction, which contains the sperm, were measured.

**Experiment 1**

**Frequency of semen collection:** Semen was collected at
various frequencies in 2 Beagle dogs (Nos. 1 and 2) and 2 mongrel dogs (Nos. 3 and 4). Semen was collected 5 times at 5-day intervals in these four dogs before beginning the frequent collection trials. The concentrations of Na⁺ and K⁺ in the seminal plasma (obtained after removing the sperm from the second semen fraction by centrifugation) was measured using an Ektachem DT analyzer (Kodak, Inc., U.S.A.). Since peripheral plasma testosterone levels fluctuate daily in dogs [42], peripheral blood was collected 4 times at 3-hr intervals, on the semen collection days to assay plasma testosterone.

Semen was collected 10 times in each dog at 48-hr, 24-hr or 12-hr intervals. There was a 10-day sexual rest period between each of the three series of collection.

Plasma testosterone assay: Plasma testosterone levels were determined by radioimmunoassay using the method described by Makino et al. [27]. Rabbit anti-serum against T-11-succinate-BSA was used. The intra-assay and inter-assay coefficients of variation were 3.0% and 9.6%, respectively.

Percentages of abnormal sperm and immature sperm in the genital tract: The testes, epididymides and vasa deferentia of Nos. 3 and 4 dogs were excised under halothane inhalation anesthesia. Smear preparations of the testes, the caput, corpus and cauda epididymis and the proximal portion of the vas deferens were examined immediately. Morphologically abnormal sperm and sperm with cytoplasmic droplets were identified by the aniline blue and eosin staining method [5]. One thousand sperm per slide were scored with each staining procedure.

Experiment 1

Semen quality: The mean (±SEM) values for semen quality and peripheral plasma testosterone in Nos. 1–4 dogs before the collections at different frequencies were carried out are shown in Tables 1 and 2. Over 90% of morphological defects in the sperm of all 8 dogs consisted of bent or coiled tails. No erythrocytes or leukocytes associated with infectious diseases of the testis, epididymis or the prostate were found in the ejaculates.

Changes in semen quality during collections at the different frequencies are shown in Figs. 1–4. Although the volume of semen and the number of sperm decreased gradually with frequency of semen collection, the semen was characterized by increases in the pH and in the sperm motility in the second semen fraction. There were marked decreases in the percentages of sperm with abnormal tails as the intervals between collections became shorter.

Percentages of abnormal sperm and immature sperm in smear preparations: The percentages of abnormal sperm and immature sperm in smear preparations of the testis, epididymis and the proximal portion of the vas deferens are shown in Fig. 5. The percentages of sperm with abnormal tails increased markedly in the caput epididymis and in the proximal portion of the vas deferens.

Experiment 2

In vitro sperm capacitation: The mean (±SEM) values for semen quality and plasma testosterone on day 1 and day...
The normal semen quality values in adult Beagle dogs are reported to be: total semen volume, 7–12 ml; total number of sperm, 300–500 × 10⁶; sperm motility, 80–90%; and percentages of abnormal sperm and immature sperm, 5–12% and 1–2%, respectively [3, 17, 20, 22, 38, 40]. Normal values of pH, osmotic pressure, and Na⁺-K⁺ concentrations in seminal plasma have been reported to be associated with the production of sperm with abnormal motility and morphology [7, 13, 14, 30, 32]. There is a report [11] on dogs stating that the epididymis and the proximal portion of the vas deferens are not only passage way for spermatozoa but accessory reproductive organs of spermatozoal maturation and storage.

In this study, many sperm with abnormal tails were observed in the epididymis and the proximal portion of the vas deferens, suggesting that the decrease in sperm motility and defects in sperm tails were related to the abnormal values of pH, osmotic pressure and Na⁺-K⁺ concentrations in the epididymal fluid and the seminal plasma stored in the proximal portion of the vas deferens. Some of morphologically abnormal sperm in the dog [16, 29] have been reported to be caused by orchitis, epididymitis or scrotitis. No inflammation of the gonads or accessory reproductive glands was observed in the 8 dogs in this study. However, they were found to have lower peripheral plasma testosterone levels than those of normal dogs (2–4 ng/ml) [19, 36, 42]. Epididymal function in dogs is maintained by testosterone secreted by the testis [35], as is the case in other animals, and thus the poor semen quality in the 8 dogs in this study may be related to their low plasma testosterone levels. Hormone therapy [21, 37] may be effective for such dogs.

It has been reported that frequent semen collection temporarily induces an increase in sperm motility and a decrease in sperm tail abnormalities in bulls with poor semen quality [13, 14, 30]. In addition, frequent semen collection is thought to induce normal secretory function of epithelial cells lining the epididymal duct, thereby normalizing the concentrations of Na⁺-K⁺ and the values of pH and osmotic pressure in the epididymal fluid. This normalization may be a secondary factor in the improvement of semen quality. The improvement in semen quality is thought to be unrelated to androgen secretion by the testis, because there was no change in plasma testosterone levels during frequent semen collection.

The zona pellucida-binding assay has recently been reported to be more useful as a diagnostic method for testing the in vitro fertilizing ability of spermatozoa or semen samples with low fertilizing potential than for examination of HA and AR in the spermatozoa of several species [8, 9, 12, 28, 44]. The present study showed that the zona
pellucida-binding assay is a useful method for assessing the fertilizing ability of dog sperm and that the potential fertility of sperm in dogs with both asthenozoospermia and teratozoospermia can be temporarily improved by frequent semen collection.

The results of this study have confirmed that renewal of seminal plasma by semen collection at regular intervals is important for maintaining satisfactory quality of semen.

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FREQUENT COLLECTION OF SEMEN IN INFERTILE DOGS


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Fig. 3. Changes in percentages of abnormal sperm and immature sperm at three different frequencies of ejaculation, i.e., at 48-hr, 24-hr, and 12-hr intervals, in dogs (Nos. 1–4) with both asthenozoospermia and teratozoospermia. The values are mean ± SEM.

**P<0.05, ***P<0.01, in comparison with the first collection in frequencies of ejaculation.

Fig. 6. Mean (±SEM) percentages of hyperactivated sperm (HA), acrosome-reacted sperm (AR) and total number of sperm bound to zona pellucida (ZP) after 4–6 hr incubation on day 1 and day 5 during ejaculation at 24 hr intervals in dogs (Nos. 5–8) with both asthenozoospermia and teratozoospermia. *P<0.05, in comparison with day 1.

Fig. 5. Mean percentages of abnormal sperm and immature sperm in the testis, epididymis and ductus deferens of two dogs with both asthenozoospermia and teratozoospermia.

Table 3. Semen quality and plasma testosterone levels (means ± SEM) on day 1 and day 5 during frequent ejaculation at 24-hr intervals in dogs (Nos. 5–8) with both asthenozoospermia and teratozoospermia

<table>
<thead>
<tr>
<th>Semen collection</th>
<th>Total semen volume (mL) (2nd fraction)</th>
<th>Total sperm count (×10⁶)</th>
<th>Motile sperm (%)</th>
<th>Viable sperm (%)</th>
<th>Abnormal sperm (%)</th>
<th>Testosterone (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>11.3 ± 0.4</td>
<td>6.4 ± 0.1</td>
<td>356 ± 149</td>
<td>53 ± 4</td>
<td>22 ± 2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Day 5</td>
<td>9.7 ± 0.3*</td>
<td>6.7 ± 0.1**</td>
<td>211 ± 92</td>
<td>75 ± 3**</td>
<td>13 ± 2*</td>
<td>1.9 ± 0.2</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, in comparison with day 1.