Lactoferrin is an iron-binding protein (molecular mass of 80 kDa) that is found in various exocrine fluids, blood plasma and specific granules of neutrophils [15]. Lactoferrin has been identified in the seminal plasma of humans [10,19], swine [21] and horses [14]. Lactoferrin seems to be synthesized mainly in the epididymis of mice [27], swine [16] and horses [7], and it is present in the prostate and seminal vesicles, but not in the epididymis, of humans [26]. The physiological role of lactoferrin in the male reproductive tract remains unclear, although lactoferrin has been identified as the sperm-coating antigen in humans [10] and swine [16, 21].

Transferin is homologous to lactoferrin and is a major iron transport protein in the circulation [1], and is also found to be present in the seminal plasma of humans [12] and cattle [9]. Transferin is secreted by the Sertoli cells [13, 22] and plays an important role in spermatogenesis by providing iron to developing germ cells, which have specific transferrin receptors [11, 24]. Seminal plasma transferin is known to be a good index of gonadal function in humans [2, 6] and cattle [9], although 20% and 40% of transferin in semen is contributed by the accessory sex glands of humans [12] and cattle [9], respectively.

No information is available about either lactoferrin or transferin in canine semen. In the present study, an enzyme immunoassay for measuring the concentrations of lactoferrin and transferin in canine seminal plasma was developed and the relationships between seminal plasma lactoferrin and transferin concentrations, the number of sperm and sperm motility were examined.
body as a probe, were concentrated to about 3 ml by ultrafiltration (Centrisil YM-30, Millipore Corporation, Bedford, MA, U.S.A.). The concentrated lactoferrin solution was applied to a Sephacryl S-200 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) column (2.0 × 100 cm) equilibrated with 20 mM sodium phosphate, 500 mM NaCl, pH 7.2, at a flow rate of 13.2 ml/hr. The pooled eluates containing lactoferrin were dialyzed twice against 1/10 of 10 mM sodium phosphate, pH 6.5, and then applied to a CM Sepharose CL-6B (Sigma, Chemical Co.) column (1.0 × 1.3 cm) equilibrated with 10 mM sodium phosphate, pH 6.5, at a flow rate of 28.8 ml/hr. After washing the column with 30 ml of the same buffer, lactoferrin was eluted with a linear gradient of NaCl prepared by mixing equal volumes (150 ml) of 10 mM sodium phosphate, pH 6.5 and 500 mM NaCl in 10 mM sodium phosphate, pH 6.5. The lactoferrin-containing eluates were concentrated to about 3 ml by ultrafiltration as described above and used in subsequent experiments.

Protein and iron measurement: Protein was measured according to the method of Lowry et al. [18] with bovine serum albumin as the protein standard. Nonheme iron in purified lactoferrin preparations was measured according to the method of Bomford et al. [3].

Enzyme-linked immunosorbent assay (ELISA): To detect the immunological cross-reaction of canine lactoferrin with antiserum to equine seminal plasma lactoferrin, a double-antibody ELISA was performed. First, the Immuno Plate Maxisorp F96 microtiter plates (Nunc, Roskilde, Denmark) were coated by adding 100 μl of 100 ng/ml canine or equine seminal plasma lactoferrin in phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) to each well and storing overnight at 4°C. The lactoferrin-coated plates were then washed with PBS containing 0.05% Tween 20 (PBST), and masked by incubation with ELISA buffer containing 0.5 M ammonium sulfate, preincubated overnight at 4°C with an equal volume of canine seminal plasma or a canine lactoferrin standard diluted with the same buffer, and then added to the plates.

A sandwich ELISA was used to measure the concentration of transferrin in canine seminal plasma according to the procedures for equine serum ferritin determination [20], with the following exceptions. The plates were coated with affinity-purified antibody to canine serum transferrin at 1,000 ng/ml, and the concentration of the alkaline phosphatase-conjugated anti-canine serum transferrin antibody was 100 ng/ml. ELISA buffer was used to mask the antibody-coated plates and to dilute the enzyme-labeled antibodies. ELISA buffer containing 0.5 M ammonium sulfate was used to dilute canine seminal plasma and to prepare the purified canine transferrin standards.

Statistical analysis: Correlation coefficients were determined by simple linear regression.

RESULTS

Characterization of purified canine seminal plasma lactoferrin: Purified canine seminal plasma lactoferrin produced a single band with a molecular mass of 75.2 kDa on an SDS-PAGE gel. The migration rate was almost identical to that of canine serum transferrin (Fig. 1A). The iron saturation of purified canine lactoferrin was 70.4%.

![Fig. 1. SDS-PAGE (A) and immunoblotting (B) of canine lactoferrin and transferrin. (A) Purified samples of lactoferrin (2 μg, lane 1) and transferrin (2 μg, lane 2) were visualized along with molecular mass standards (1 μg each, lane 3). (B) A blotting membrane containing purified equine lactoferrin (200 ng, lane 1), canine lactoferrin (200 ng, lane 2), and canine transferrin (200 ng, lane 3) was immunostained with rabbit antiserum to equine lactoferrin as a probe. Anode at bottom.](image-url)
Canine lactoferrin was found to cross-react significantly with the antibody to equine seminal plasma lactoferrin in immunoblotting (Fig. 1B) and in a double-antibody ELISA (Fig. 2).

Quantification of canine seminal plasma lactoferrin: A competitive ELISA was developed for measuring the concentration of lactoferrin in seminal plasma. A representative standard curve is shown in Fig. 3, indicating that the detection limit was about 10 ng/ml. This figure also shows that anti-lactoferrin antibodies did not react at all with transferrin under the conditions of this assay. Recoveries of 45 ng/ml, 90 ng/ml, and 180 ng/ml lactoferrin added to seminal plasma diluted 800-fold with ELISA buffer containing 0.5 M ammonium sulfate were 111.1 ± 8.5% (mean ± S.D., n=4), 93.1 ± 7.4% (n=4), and 100.0 ± 6.5% (n=4), respectively. Intra-assay coefficients of variation from twelve measures of lactoferrin in two canine seminal plasma samples were 8.8% (34 ± 3 µg/ml) and 4.9% (41 ± 2 µg/ml). Inter-assay coefficients of variation from ten measures of lactoferrin in two canine seminal plasma samples were 5.4% (37 ± 2 µg/ml) and 11.9% (42 ± 5 µg/ml).

Seminal plasma lactoferrin from 14 apparently healthy dogs varied from 12 to 197 µg/ml, with a mean value of 77 ± 59 µg/ml. Figures 4A and 4B show a positive correlation between the seminal plasma lactoferrin concentration and sperm concentration (r=0.7025, P<0.01) and between total seminal plasma lactoferrin and the total sperm number (r=0.8049, P<0.001), respectively. Nevertheless, there was no significant correlation between the seminal plasma lactoferrin concentration or total seminal plasma lactoferrin content and sperm motility (data not shown).

Quantification of canine seminal plasma transferrin: A sandwich ELISA was developed for measuring the concentration of transferrin in seminal plasma. A representative standard curve shown in Fig. 5 indicates that the detection limit was about 1 ng/ml. This figure also shows that antibodies raised to canine serum transferrin did not cross-react at all with canine seminal plasma lactoferrin under the conditions of this assay. Recoveries of 5.3 ng/ml, 10.7 ng/ml, and 21.3 ng/ml transferrin added to seminal plasma diluted 51-fold with ELISA buffer containing 0.5 M ammonium sulfate were 102.4 ± 7.3% (n=4), 102.3 ± 9.4% (n=4), and 102.7 ± 9.5% (n=4), respectively. Intra-assay coefficients of variation from twelve measures of transferrin in two canine seminal plasma samples were 1.63 ± 0.03 µg/ml and 1.3% (0.75 ± 0.01 µg/ml). Inter-assay coefficients of variation from eight measures of transferrin in two canine seminal plasma samples were 4.3% (1.62 ± 0.07 µg/ml) and 2.7% (0.73 ± 0.02 µg/ml).

The concentration of transferrin in seminal plasma samples from 14 dogs varied from 0.32 to 12.60 µg/ml, with a mean value of 2.44 ± 3.25 µg/ml. There was no significant correlation between the seminal plasma transferrin concentration and the sperm concentration (r=0.2982, P>0.05, Fig. 6A) or between total seminal plasma transferrin and total sperm number (r=0.3709, P>0.05, Fig. 6B). There was also no significant correlation between the seminal plasma transferrin concentration or total seminal plasma transferrin content and sperm motility (data not shown).

Furthermore, there was no significant correlation between seminal plasma lactoferrin and transferrin concentrations (data not shown).
DISCUSSION

The molecular mass (75.2 kDa) of lactoferrin purified from canine seminal plasma in the present study is equivalent to that of lactoferrin purified from human (76 kDa) [23] and equine (80 kDa) [14] seminal plasma. It was recently found in swine that lactoferrin is first secreted as a 75-kDa glycoprotein but carbohydrate moieties are gradually digested resulting in a 70-kDa protein in the cauda epididymis [16].

Lactoferrin and transferrin were found to coexist in seminal plasma of dogs in the present study as well as in human seminal plasma [10, 12, 19]. In this study, specific immunoassays were developed to measure these two homologous iron-binding proteins with no immunological cross-reaction (Figs. 3 and 5), although these proteins have common antigenic determinant(s) in their denatured forms [25].

The present study is the first to measure the concentration of lactoferrin in canine seminal plasma and to clarify the positive correlation between the concentration of lactoferrin and the sperm count in canine semen. The concentration range of lactoferrin in canine seminal plasma appears to be lower than that in swine [21] or human [19] seminal plasma. Buckett et al. [4] found high seminal plasma lactoferrin concentrations in human semen samples with oligospermia and oligoasthenospermia compared to normospermic samples. Nevertheless they did not explain whether there was any relationship between the seminal lactoferrin concentration and sperm density in the normal or abnormal samples.

High levels of seminal lactoferrin have been shown to be a diagnostic sign of prostatitis in humans [8]. It has been shown that administration of short-term high-dose testosterone propionate to normal men reduces not only the sex accessory-gland secretory proteins, including lactoferrin, prostatic-acid phosphatase, and prostate-specific antigen, but also zinc and fructose, which are prostatic and vesicular secretion markers, respectively [5]. Lactoferrin mRNA levels in mouse epididymis are increased by estrogen, at least in the prepubertal stage, and are not influenced by androgen [27]. Determination of the lactoferrin concentration in...
canine semen under various disease conditions and examination of the effects of androgen or estrogen administration on canine seminal lactoferrin are necessary to clarify the physiological significance of this protein in the male reproductive tract.

In the present study, canine seminal plasma transferrin was quantified for the first time. The seminal transferrin concentrations in normal dogs seem to be much lower than those in normal humans [2, 6, 12] or cattle [9]. Although Kawakami et al. [17] reported that the mean concentration of transferrin in normal canine testis was $321 \pm 32 \text{ ng/g}$, testicular transferrin in animals other than dogs has not been measured. A positive correlation exists between seminal plasma transferrin concentrations and sperm density and between total seminal transferrin and total numbers of sperm in ejaculate in humans [2, 6] and cattle [9]. But in the present study no significant correlation was found between seminal transferrin and the sperm count in dogs, indicating that canine seminal transferrin is not an index of gonadal function unlike human and bovine seminal transferrins. In contrast, the levels of seminal lactoferrin positively correlated with sperm density in dogs, suggesting that canine seminal lactoferrin reflects spermatogenesis. The origin of seminal lactoferrin in dogs has not yet been determined. If canine lactoferrin is secreted mainly in the epididymis as in mice [27], swine [16] and horses [7] or in the prostate and seminal vesicles as in humans [26], it is reasonable to consider that there is a factor that relates lactoferrin secretion to spermatogenesis.

The individual difference in seminal plasma lactoferrin and transferrin concentrations and sperm density was found to be large in dogs used here. Further studies are required to determine whether there are age-related or breed-related variations in these seminal parameters in dogs.

REFERENCES


