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Original article

Semen characteristics and selected biochemical markers of canine seminal plasma in various seasons of the year

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Abstract

The aim of this study was to evaluate the influence of season on selected qualitative semen characteristics and biochemical markers of canine seminal plasma. Whole ejaculates were collected from 5 crossbred dogs aged 2-8 years. The study covered a period of one year divided into four seasons: spring (March, April, May), summer (June, July, August), autumn (September, October, November) and winter (December, January, February). Semen samples were subjected to macroscopic and microscopic analyses to determine semen volume, total sperm counts and sperm morphology parameters. The study also involved the determination of sperm motility parameters (CASA system), sperm plasma membrane integrity (SPMI, fluorescent staining SYBR-14/PI), sperm mitochondrial membrane potential (MMP, fluorescent staining JC-1/PI) and the ATP content of sperm cells. Total protein content (TPC) and the activity of alkaline phosphatase (AP) and acid phosphatase (AcP) were determined in biochemical analyses of seminal plasma. No significant differences in ejaculate volume, SMPI or ATP content of sperm cells were observed between seasons. The highest total sperm counts were reported in ejaculates acquired in summer and autumn. The lowest MMP values were determined in summer ejaculates. No significant differences in sperm motility (MOT) were observed throughout the experiment, but ejaculates collected in autumn and winter were characterized by the highest progressive motility (PMOT). AP activity and TPC were not significantly affected by season. However, AcP activity levels were significantly lower in autumn than in the remaining seasons. Seasonal variations in the analyzed macroscopic and microscopic parameters of ejaculates and biochemical markers of seminal plasma did not exert a clear negative effect on the quality of canine semen.

Key words: dog, season, semen quality, seminal plasma

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Introduction

In most mammalian species, reproductive capacity peaks in selected seasons. The sexual activity of males is triggered mainly by changes in day length (Malpaux et al. 1999). Clear seasonal changes in semen quality are observed in undomesticated canids. Reproductive seasonality was noted in red fox, silver fox, coyote and gray wolf males (Mitsuzuka 1987, Smith et al. 1987, Lefebvre et al. 1999, Minter and DeLiberto 2008). In the above species, spermatogenesis and testosterone production peak during the breeding season (Lincoln 1989). In coyotes, the reproductive season lasts from December to April (Bekoff et al. 1986, Minter and DeLiberto 2008), and ejaculates collected during that time are characterized by the highest sperm counts, highest sperm motility and the highest percentage of morphologically normal spermatozoa (Minter and DeLiberto 2008).

In the domestic dog (*Canis familiaris*), the reproductive cycle ceased to be controlled by photoperiod in the domestication process (Linde-Forsberg and Reynaud 2012). Male dogs are sexually active throughout the year, but several authors have reported seasonal variability in selected semen quality parameters (Kuroda and Hiroe 1972, Takeishi et al. 1975, Taha et al. 1981). The above observation could play an important role in the process of selecting ejaculates for insemination and long-term sperm preservation. In dogs, the success of artificial insemination depends on the choice of the optimal insemination period and the use of high quality semen, including well-preserved semen. Low quality semen can significantly decrease the effectiveness of insemination (Niżański et al. 2004).

This study was undertaken to expand our knowledge of qualitative changes in the canine semen in the annual cycle. The aim of this study was to determine selected qualitative semen parameters and biochemical markers of canine seminal plasma in different seasons of the year.

Materials and Methods

Animals and semen sampling

The experiment was performed over a period of 12 months on 5 crossbred dogs aged 2 to 8 years. The animals were housed individually in indoor-outdoor runs with natural lighting. They were fed twice a day with commercial dry dog feed and had *ad libitum* access to water. The experiment was conducted in accordance with the guidelines of the Local Ethics Committee.

Whole ejaculates (including pre-sperm, sperm-rich and post-sperm fractions) were obtained by manual stimulation twice per month, at two-week intervals, over a period of 12 months. The collected semen samples were subjected to macroscopic and microscopic analyses.

Semen characteristics

The volume of ejaculates was determined with a measuring cylinder. Spermatozoa concentrations were determined cytometrically in a Burker counting chamber after dilution with 0.85% sodium chloride (NaCl). The total ejaculate sperm count was calculated by multiplying sperm concentrations by ejaculate volume.

Sperm morphology was evaluated by staining sperm smears with the Giemsa staining method described by Watson (1975). Morphological abnormalities were expressed as a percentage of total sperm counts.

Sperm plasma membrane integrity (SPMI) was assessed using the dual fluorescent staining technique described by Garner and Johnson (1995) with the use of SYBR-14 and propidium iodide, PI (Live/Dead Sperm Viability Kit; Molecular Probes, OR, USA). Aliquots (10 μL) of sperm samples stained with SYBR-14/PI were examined under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). For each aliquot, approxi-mately 200 sperm cells were classified as spermatozoa with an intact or damaged plasma membrane.

Sperm mitochondrial membrane potential (MMP) was assessed in semen samples using a dual fluorescent staining technique with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbo-cyanineiodide (JC-1) (1 mg/ml DMSO) (Molecular Probes, Eugene, OR, USA) and PI (10 μl of PI solution in 0.5 mg/ml PBS), according to a previously described method (Thomas et al. 1998). Aliquots (10 μL) of stained sperm samples were examined under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). Sperm cells displaying only orange-red fluorescence at the mid-piece region were considered viable spermatozoa with high MMP, while those exhibiting green fluorescence were considered as nonviable spermatozoa with low MMP.

The ATP content in the spermatozoa was assessed using a bioluminescence kit (ATP Bioluminescence Assay Kit CLSII; Roche Molecular Biochemical). Measurements of ATP content were performed using a Junior Bioluminometer (Berthold Technologies, Germany), according to the assay kit protocol. The ATP content in the spermatozoa was calculated from

the ATP standard curve and expressed as nmol ATP/10^8 spermatozoa.

Sperm motility characteristics were evaluated using the Hamilton-Thorne Sperm Analyzer IVOS version 12.3 (Hamilton-Thorne Biosciences, Beverly, MA, USA). Software settings for the semen analyzer were chosen based on the manufacturers' recommendations for dog sperm analyses: frame acquired – 30, frame rate – 60 Hz, minimum cell contrast – 75, minimum cell size – 6 pixels, straightness threshold – 75%, path velocity threshold – 100 μm/s, low VAP cut-off – 9.9 μm/s, low VSL cut-off – 20 μm/s, static size gates – 0.80-4.93, static intensity gates – 0.49-1.68, static elongation gates – 22-84. The motility parameters determined by the IVOS analyzer were: total motility (MOT, %), progressive motility (PMOT, %), average path velocity (VAP, ,m/s), straight line velocity (VSL, μm/s), curvilinear line velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %). A droplet of approximately 5fl was placed in a Makler counting chamber (Sefi-Medical Instruments Ltd., Haifa, Israel) at 37°C, and six fields were examined per sample.

Biochemical analysis of seminal plasma

Seminal plasma was separated from ejaculate by centrifugation at 1000 x g for 15 min at room temperature. The recovered seminal plasma was centrifuged at 10 000 x g for 10 min at room temperature and stored at -80°C, until required for further analysis.

Total protein content was measured according to the method proposed by Weichselbaum (1946), using serum bovine albumin (BSA, Serum and Vaccine Production, Cracow, Poland) as the standard.

The activity of alkaline (AP) and acid (AcP) phosphatases was determined by the method described by Bessey et al. (1946). Disodium salt p-nitrophenyl phosphate (Sigma, St Louis, MO, USA) was used as substrate. Absorbance was measured at a wavelength of 410 nm against a control sample. Phosphatase activity was derived from a standard curve obtained using p-nitrophenol solution (Sigma, St Louis, MO, USA) as the standard.

Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM). To determine seasonal changes in semen parameters, 4 periods of 3 months each were identified to represent spring (March, April, May), summer (June, July, August), autumn (September, October, November) and winter (December, January, February). Seasonal means of semen characteristics were analyzed by ANOVA and Duncan's multiple comparison test using Statistica software (StatSoft Incorporation, Tulsa OK., USA). Differences between means were considered significant at $P \leq 0.05$.

Results

No significant ($P>0.05$) differences in sperm volume, counts of morphologically normal sperm and SPMI were observed between seasons (Table 1). The highest total sperm counts (ejaculate volume x sperm counts) were noted in ejaculates collected in summer and autumn. Ejaculates obtained in summer were characterized by the lowest percentage of spermatozoa with high MMP (fluorescent staining with JC-1/PI). No significant ($P>0.05$) differences in the ATP content of sperm cells were reported between seasons, but the highest ATP concentrations were determined in spermatozoa sampled in winter.

No significant ($P>0.05$) differences in sperm motility (MOT) were reported between seasons (Table 2). The highest PMOT values were noted in ejaculates collected in autumn and winter, whereas the lowest PMOT values were observed in summer ejaculates. Spermatozoa from summer ejaculates were also characterized by the lowest STR and LIN values and the lowest velocity parameters (VAP and VSL).

The total protein content of seminal plasma did not fluctuate on a seasonal basis (Table 3), but the highest TPC values were noted in winter and spring, and the lowest in autumn. Significant ($P>0.05$) variations in alkaline phosphatase (AP) activity were not reported between seasons, whereas the activity levels of acid phosphatase (AcP) were season-dependent. Significantly ($P<0.05$) lowest AcP levels were noted in autumn relative to the remaining seasons of the year.

Discussion

No significant changes in the volume of whole ejaculates were observed between seasons. Similar results were noted by Taha et al. (1981), where the sperm-rich fraction remained constant throughout the year, but significant variations in the volume of the prostatic fraction were reported. In a study by Takeishi et al. (1975), canine ejaculates sampled in summer were characterized as having the highest volume.

Table 1. Characteristics of canine semen in different seasons.

Season	Semen volume (ml)	Total sperm count (x10^6 spz)	Sperm with normal morphology (%)	SPMI (%)	MMP (%)	ATP content (nmol/10^8 spz)
Spring	14.57 ± 2.69	605.5^{a} ± 51.6	92.44 ± 1.19	88.72 ± 0.83	83.12 ± 1.07^{ab}	3.09 ± 0.42
Summer	13.41 ± 2.16	897.7^{b} ± 88.7	91.69 ± 1.21	88.23 ± 0.95	79.75 ± 2.41^{a}	3.37 ± 0.35
Autumn	13.19 ± 2.16	802.9^{ab} ± 63.9	89.20 ± 1.50	87.04 ± 1.14	84.83 ± 1.45^{b}	3.33 ± 0.35
Winter	15.75 ± 2.39	637.8^{a} ± 67.7	89.54 ± 1.25	89.35 ± 1.78	85.05 ± 1.43^{b}	3.70 ± 0.31

Values in different seasons represent the mean (± SEM) of 30 ejaculates from 5 crossbred dogs.
Values with different superscripts (a-b) within columns differ significantly at P<0.05.
SPMI – sperm plasma membrane integrity, MMP – sperm mitochondrial membrane potential

Table 2. Sperm motility parameters of canine semen samples in different seasons.

Season	MOT (%)	PMOT (%)	Kinematic parameters						
			VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
Spring	91.4 ± 0.69	65.03 ± 2.11^{ab}	135.5 ± 2.53^{ab}	117.0 ± 2.89^{ab}	194.6 ± 4.14^{a}	7.1 ± 0.32^{a}	17.9 ± 0.77^{a}	85.1 ± 0.90^{a}	62.2 ± 2.07^{a}
Summer	92.0 ± 1.37	59.76 ± 2.93^{a}	130.8 ± 2.67^{a}	110.9 ± 2.45^{a}	186.8 ± 4.45^{ab}	6.7 ± 0.20^{ab}	12.5 ± 0.50^{b}	84.3 ± 0.90^{a}	62.6 ± 1.53^{a}
Autumn	93.4 ± 0.49	71.17 ± 1.47^{bc}	138.3 ± 2.07^{ab}	120.6 ± 2.18^{bc}	188.8 ± 2.94^{ab}	6.5 ± 0.19^{ab}	13.8 ± 0.52^{b}	86.6 ± 0.71^{ab}	66.4 ± 1.46^{ab}
Winter	93.5 ± 0.57	74.75 ± 1.59^{c}	140.0 ± 3.03^{b}	126.6 ± 2.62^{c}	179.5 ± 3.29^{b}	6.1 ± 0.19^{b}	17.6 ± 0.72^{a}	88.8 ± 0.83^{b}	70.7 ± 1.62^{b}

Values in different seasons represent the mean (± SEM) of 30 ejaculates from 5 crossbred dogs.
Values with different superscripts (a-c) within columns differ significantly at P<0.05.
MOT – total motility, PMOT – progressive motility, VAP – average path velocity, VSL – straight line velocity, VCL – curvilinear line velocity, ALH – amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness, LIN – linearity.

Table 3. Biochemical parameters of canine seminal plasma in different seasons.

Season	TPC (mg/ml)	Enzyme activity	
		AP (U)	AcP (U)
Spring	32.06 ± 1.45	6770.03 ± 1135.95	1491.45 ± 139.13^{a}
Summer	30.75 ± 1.72	6065.25 ± 999.65	1774.05 ± 171.8^{a}
Autumn	28.15 ± 1.93	6550.71 ± 1263.63	811.06 ± 141.64^{b}
Winter	32.10 ± 1.76	6145.16 ± 1215.27	1668.76 ± 188.44^{a}

Values in different seasons represent the mean (± SEM) of 30 ejaculates from 5 crossbred dogs.
Values with different superscripts (a-b) within columns differ significantly at P<0.05.
TPC – total protein content, AP – alkaline phosphatase, AcP – acid phosphatase

The highest total sperm counts were noted in ejaculates collected in summer and autumn. Taha et al. (1981) noted an increase in total sperm counts in spring and early summer. In a study by Albrizio et al. (2013), ejaculates obtained from 12 crossbred dogs were characterized by similar volume (1-6 ml) and sperm concentrations (100-500 x 10^6/ml) throughout the year. In our study, high levels of alkaline phosphatase (AP) activity in seminal plasma throughout the year are indicative of normal epididymal function. The epididymides are the main source of AP in the canine reproductive system (Frenette et al. 1986). Relatively high values of standard deviation point to considerable differences in AP activity levels in ejaculates collected from the same animal and from different individuals. Similar variations in phosphatase activity levels have been reported in the seminal plasma of boars and wild boars (Glogowski et al. 1997, Kozdrowski and Dubiel 2004).

A microscopic analysis revealed that the percentage of morphologically normal spermatozoa did not decrease below 80% in any of the evaluated seasons.

The highest counts of morphologically normal sperm cells were reported in ejaculates collected in spring and summer. Similar results were reported by Taha et al. (1981), in whose study ejaculates collected in May and June were characterized by the highest counts of morphologically normal spermatozoa. By contrast, Takeishi et al. (1975) observed the lowest number of morphologically normal sperm cells in summer, and they attributed their findings to high ambient temperatures. In a year-long study conducted in a tropical climate, Ortega-Pacheco et al. (2006) did not report significant differences in the percentages of morphologically normal spermatozoa in canine ejaculates. In mammals, testicular function is influenced by intratesticular temperature which should be 2.5-5.3°C lower on average than body temperature (Brito et al. 2004, Henning et al. 2014). Despite these observations, the influence of elevated temperature on spermatogenesis in male gonads and the maturation of spermatozoa in canine epididymides has not yet been fully elucidated. However, aberrations in spermatogenesis resulting from short-term heat stress are observed less frequently in dogs than in other mammals (Henning et al. 2014).

Evaluations of SPMI, similarly to evaluations of sperm morphology, revealed that environmental factors, including ambient temperature, did not influence the above parameter. No significant changes in the analyzed parameter were reported between seasons.

The number of sperm cells with high MMP (fluorescent staining with JC-1/PI) varied significantly between seasons. This parameter decreased in summer despite an absence of changes in oxidative phosphorylation and the glycolytic pathway, as manifested by the ATP content of spermatozoa. These results could indicate that energy metabolism in canine sperm cells is maintained at an optimal level in all seasons of the year. According to Kuroda and Hiroe (1972), the highest levels of metabolic activity in canine spermatozoa are observed between autumn and spring, whereas the lowest levels are noted in summer. In the cited study, the highest rates of oxygen consumption and glucose transformation were observed in sperm cells from spring ejaculates, whereas fructolysis was intensified in ejaculates collected in autumn.

In this study, no significant changes in sperm motility (MOT) were observed between seasons. Taha et al. (1981) demonstrated that season had no effect on sperm motility in ejaculates collected from 5 Beagle dogs. Our findings are also consistent with the results reported by Albrizio et al. (2013), in whose study ejaculates collected in autumn and winter were characterized by the highest counts of progressively motile sperm (PMOT). The remaining motility parameters evaluated in the CASA system are difficult to interpret. According to the literature, motility parameters, excluding BCF, LIN and STR, are significantly correlated with fertility in dogs (Rijsselaere et al. 2007). The threshold values of VAP, VSL and VCL, which are indicative of low and high levels of fertility in dogs, have not yet been clearly defined. In our study, the lowest motility values were noted in ejaculates collected in summer. According to several authors, sperm motility could deteriorate in response to environmental factors, such as high ambient temperature in summer (Taha et al. 1981, Ortega-Pacheco et al. 2006). In this study, the remaining quality parameters (sperm morphology, plasma membrane integrity) in summer ejaculates were within the norm.

No significant changes in TPC were observed in seminal plasma. Average TPC values ranged from 28.1 mg/ml to 32.7 mg/ml and were lower than those noted in the seminal plasma of arctic foxes during the breeding season (33.2 to 48.9 mg/ml) (Stasiak et al. 2010). Minor variations in TPC values between seasons could point to moderate changes in epididymal or prostatic secretions. Higher TPC values in winter and spring could indicate that protein stabilizes the integrity of plasma membranes, thus contributing to healthy sperm function. Similar observations were made by Koziorowska-Gilun et al. (2011) in boar semen.

The radical drop in AcP activity in canine seminal plasma in autumn is difficult to interpret. Annual changes in AcP activity in canine seminal plasma have never been discussed in the literature. The prostate gland is the main source of acid phosphatase in the canine reproductive system (Strzeżek and Janowski 2003). Prostatic secretions are regulated by androgens (Takeishi 1980, Johnston et al. 2000). A decrease in AcP activity in autumn could point to limited secretory activity of the prostate, probably due to a drop in androgen concentrations. The decrease in the secretory capacity of the prostate gland in autumn could also lower the total protein content of seminal plasma. Taha et al. (1981) and Albrizio et al. (2013) did not observe significant differences in testosterone levels in canine blood serum between seasons, but a significant decrease in serum testosterone concentrations was noted in autumn in coyotes, a seasonally breeding species (Minter and DeLiberto 2008).

It can be concluded that seasonal variations in the analyzed macroscopic and microscopic parameters of ejaculates and biochemical markers of seminal plasma did not exert a clear negative effect on the quality of canine semen. Unlike wild canids, domestic dogs produce ejaculates that are characterized by normal sperm parameters throughout the year. The results of this study were difficult to interpret, and further research on a larger population, including pedigree

dogs, is required. Seasonal changes in the synthesis and secretion of biochemical components of canine semen, which play an important role in canine reproduction, remain unexplained. These processes need to be examined in greater detail to select ejaculates of the highest quality for insemination and long-term semen preservation.

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