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Exploring the characters of sperm lipid peroxidation, enzymatic antioxidant activity, and spermatozoa zona binding capacity with aging in dogs

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This study aimed to assess differences in fresh semen quality between mature and senile dogs, focusing on oxidative stress markers, including spermatozoa lipid peroxidation and enzymatic antioxidant activity. Furthermore, the ability of frozen-thawed spermatozoa from these age groups to bind to the zona pellucida was investigated. Forty clinically healthy dogs were categorized into two groups: mature (3 ± 0.2 years; *n* = 20) and senile (10 ± 0.2 years; *n* = 20). A total of 107 semen samples (2–3 ejaculates per dog) were analyzed for motilities and kinetic parameters, sperm concentration, total sperm count (TSC), acrosome and membrane integrity, morphological defects, DNA fragmentation, lipid peroxidation, and the enzymatic activities of superoxide dismutase, catalase, and glutathione peroxidase in spermatozoa and seminal plasma. A zona-binding assay was performed using frozen-thawed semen from mature (*n* = 10) and senile (*n* = 10) dogs. Mature dogs exhibited significantly higher total motility, sperm concentration, TSC, membrane integrity, and zona binding capacity compared to senile dogs (*P* < 0.05). Conversely, senile dogs had significantly higher proportions of proximal cytoplasmic droplets and tail defects (*P* < 0.05). However, lipid peroxidation, enzymatic antioxidant activities, and acrosome integrity did not differ significantly between the groups (*P* > 0.05). In conclusion, the observed decline in fresh semen quality with age in dogs does not appear to be associated with increased susceptibility to oxidative stress. Furthermore, frozen-thawed spermatozoa from mature dogs exhibited superior zona binding capacity, likely due to a greater proportion of progressively motile sperm.

Keywords Oxidative stress, Canine semen, Zona binding assay, Aging

Several factors, including biochemical influences, have been identified as contributors to decreased sperm quality and infertility in various studies[1–5]. While extensive research in men and bulls has examined the impact of age on established oxidative stress markers[6–8]this aspect remains relatively underexplored in dogs. Studies on mouse models and bulls have demonstrated a correlation between aging, increased sperm DNA damage, and heightened susceptibility to oxidative stress[9–11]. This vulnerability arises from a decline in key antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which play a crucial role in protecting the sperm nucleus from oxidative damage. For example, antioxidant enzymes such as peroxiredoxin, which are essential for maintaining DNA integrity, are also affected by aging[6,12,13]. Recent studies in rats further indicate that germ cells in older males exhibit impaired DNA repair mechanisms and a diminished capacity to respond to oxidative stress compared to younger males[14,15].

Considerable evidence suggests that the lipid composition of the sperm membrane plays a crucial role in determining sperm sensitivity to cold, as well as its motility and overall viability[1,6,16,17]. Reactive oxygen species (ROS) are continuously generated in male reproductive tissues, primarily as byproducts of normal metabolic

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processes and during fertilization when sperm encounter elevated oxygen levels[2,18]. While low ROS levels can enhance sperm function by promoting hyperactivation, capacitation, and the acrosome reaction[19]excessive ROS levels are detrimental. They induce lipid peroxidation (LPO), particularly in the midpiece of the sperm cell, where mitochondria are concentrated[20]. This oxidative damage compromises sperm motility and viability by depleting intracellular ATP, damaging the axoneme, and causing morphological abnormalities. Consequently, robust antioxidant defense mechanisms within the sperm plasma membrane are essential for mitigating peroxidative damage and preventing sperm dysfunction[6,21].

The use of assisted reproductive techniques, such as artificial insemination (AI) with fresh and frozen-thawed semen and the freezing, storage, and shipment of canine semen, has increased in recent years[22,23]. This trend is primarily driven by the need to exchange genetic material and preserve semen from valuable stud dogs. Given the relatively long estrous cycles in dogs and the challenges of accurately determining the fertile period, assessing the impact of aging on semen quality is crucial. Using semen from aging dogs may compromise reproductive efficiency. Predicting in vivo fertility requires integrating multiple in vitro sperm functional tests to improve accuracy[24–26]. A key evaluation in canine reproduction is assessing the ability of spermatozoa to bind to the zona pellucida. Damage to molecular binding structures, undetectable through routine morphological sperm assessments, can significantly impair male fertility[26,27].

Therefore, this study investigates age-related differences in semen quality (viability, concentration, acrosome integrity, morphology) and oxidative stress markers in the semen of mature and senile dogs, as well as the ability of frozen-thawed spermatozoa from these groups to bind to the zona pellucida of oocytes. The analysis includes assessments of membrane LPO, sperm DNA fragmentation, and the activities of key antioxidant enzymes, such as SOD, CAT, and GPx, in both the cellular and plasma fractions of semen. Furthermore, a sperm-zona pellucida binding assay was performed to evaluate the impact of aging on fertilization potential in dogs.

Results

Experiment 1

Figure 1 summarizes the sperm characteristics for the mature and senile age groups. Spermatozoa from mature dogs exhibited a higher percentage of total motility than those from senile dogs ($P<0.001$). As reported in our previous study, mature dogs also showed superior progressive motility and kinematic parameters[28]. Sperm concentration and TSC were lower in senile dogs than in mature dogs ($P<0.001$) (Table 1). Mature dogs had a lower proportion of proximal cytoplasmic droplets and tail defects ($P<0.05$), while no differences were observed in head and midpiece defects.

Mature dogs exhibited a significantly higher percentage of sperm with intact acrosome and intact membrane (IAIM) compared to senile dogs ($P<0.05$; Table 2). Conversely, the proportion of sperm with intact acrosome and damaged membrane (IADM) and damaged plasma membrane (DM) was higher in senile dogs compared to mature dogs ($P<0.05$). However, no significant difference was observed in the percentages of sperm with damaged acrosome and intact membrane (DAIM), damaged acrosome and damaged membrane (DADM), and damaged acrosome (DA) between the two groups (Table 2).

As illustrated in Fig. 2, no significant differences were detected between the mature and senile groups in the following parameters: the proportion of live spermatozoa with lipid peroxidation ($P=0.838$), dead spermatozoa with lipid peroxidation ($P=0.238$), DNA fragmentation index (DFI) ($P=0.148$), and high DNA stainability (HDS) ($P=0.309$) (Table 1).

The activities of SOD, CAT, and GPx in seminal plasma and spermatozoa are summarized in Table 3. No significant differences were observed in the activities of these enzymes between the mature and senile age groups.

Among the covariates included in the statistical model, individual animal affected midpiece defects, TSO, and proximal droplets (Table 1).

Experiment 2

The results of the zona binding assay and post-thaw motilities and kinetic parameters are presented in Table 4. The mean number of spermatozoa bound to the zona pellucida was significantly lower in senile dogs than in mature dogs ($P<0.05$). Within both groups, variability was observed, with some oocytes not bound by any spermatozoa, while others were bound by multiple spermatozoa (Fig. 3a and b). The post-thaw motility and kinematic parameters of mature dogs were significantly higher than in senile dogs ($P<0.05$) (Table 4).

Discussion

To our knowledge, the present study is the first to examine the impact of aging on enzymatic antioxidant levels in dog spermatozoa and seminal plasma, as well as the ability of frozen-thawed spermatozoa to bind to the zona pellucida of oocytes. Additionally, parameters such as TSC, LPO, acrosome integrity, DNA fragmentation, and proximal cytoplasmic droplets were assessed in dogs of optimal reproductive age and those of advanced age, typically associated with a decline in semen quality. The increasing use of AI in dogs, along with the freezing, storage, and shipment of semen from valuable stud dogs, including those of advanced age, highlights the importance of this study. TSC has been well-documented to decline with age in humans, primarily due to alterations in testicular, epididymal, and reproductive accessory gland function[29–32]. In contrast, limited research has explored this parameter in dogs[33,34]. Previous studies combining data from fertile and subfertile dogs have indicated that age significantly influences TSC[33,34]. The findings of the present study align with those of Rijsselaere et al.[34]suggesting that aging in dogs may lead to reduced epididymal and accessory sex gland secretions (manifesting as decreased semen volume) and impaired spermatogenesis in the seminiferous tubules. These age-related changes, including diminished germ cell populations, likely contribute to the reduced TSC observed in senile dogs[3,29].

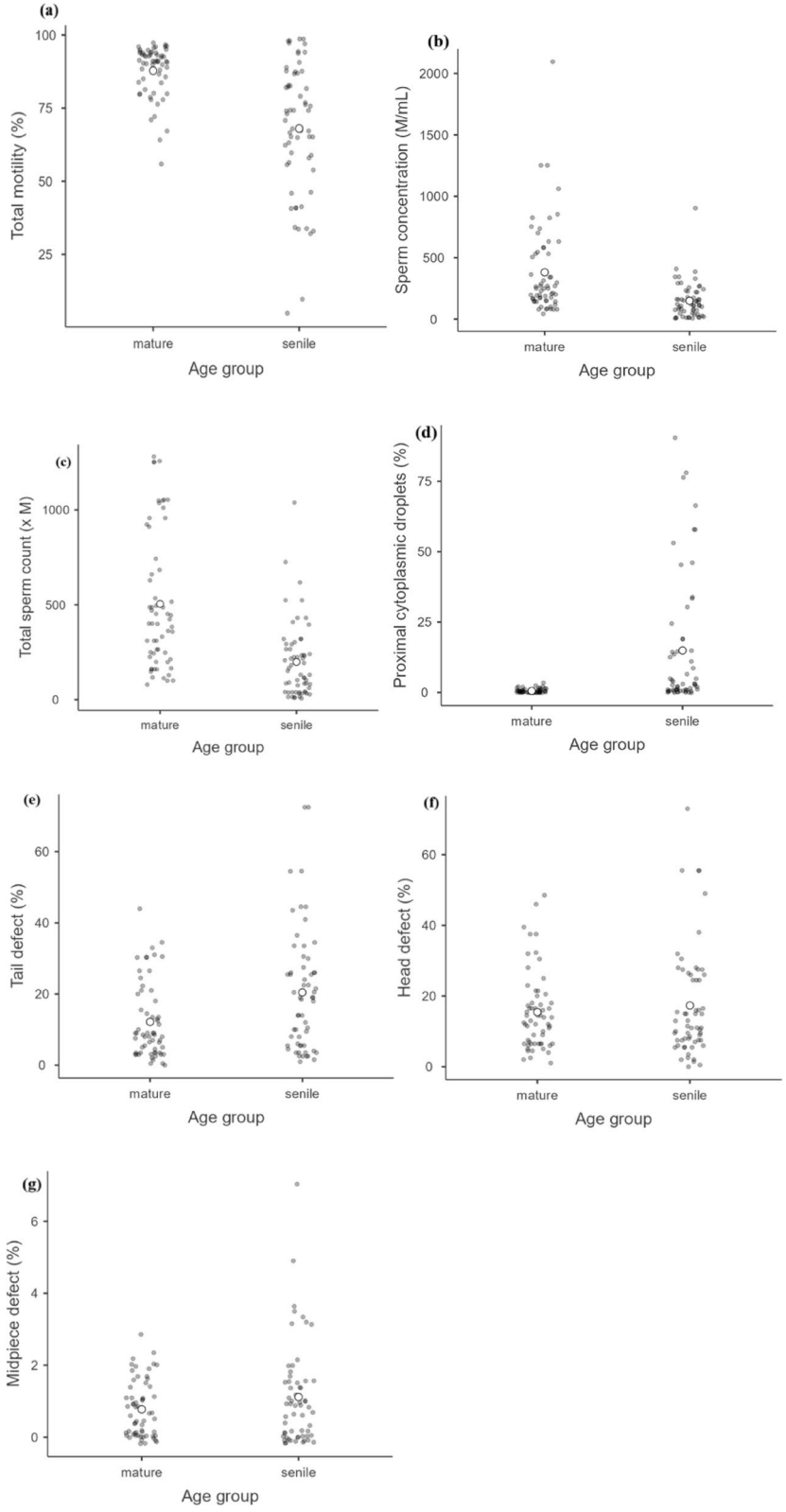

Fig. 1. (**a**) Total motility, (**b**) sperm concentration, (**c**) total sperm count, (**d**) proximal cytoplasmic droplets, (**e**) tail defects, (**f**) head defects, (**g**) midpiece defects in the fresh semen of mature ($n = 20$; 2–4 years) and senile ($n = 20$; ≥ 9 years) dogs. Dots represent individual ejaculates and the circle indicates mean values. $M = 10^6$.

Parameter	Age Group	Animal	Individual Age
Total motility	$P<0.001$	$P<0.001$	$P<0.001$
Sperm concentration	$P=0.003$	$P=0.003$	$P=0.004$
Total sperm count	$P<0.001$	$P<0.001$	$P<0.001$
Proximal cytoplasmic droplets	$P=0.006$	$P=0.009$	$P=0.009$
Tail defects	$P=0.048$	$P=0.067$	$P=0.035$
Head defects	$P=0.600$	$P=0.940$	$P=0.706$
Midpiece defects	$P=0.227$	$P=0.010$	$P=0.320$
Live lipid peroxidation	$P=0.838$	$P=0.829$	$P=0.858$
Dead lipid peroxidation	$P=0.238$	$P=0.234$	$P=0.418$
DNA fragmentation index	$P=0.148$	$P=0.106$	$P=0.148$
High DNA stainability	$P=0.309$	$P=0.275$	$P=0.446$

Table 1. Results of statistical comparisons of sperm characteristics in fresh semen of mature ($n=20$; 2–4 years) and senile ($n=20$; ≥ 9 years) dogs (repeated-measures ANOVA).

Parameters	Mature	Senile	*p*-value
IAIM (%)	77.7 ± 2.7	65.5 ± 2.7	0.003[a]
DAIM (%)	4.20 ± 0.9	4.31 ± 0.6	0.883[a]
IADM (%)	13.8 ± 2.5	25.3 ± 2.5	0.003[a]
DADM (%)	4.9 ± 0.9	6.3 ± 0.9	0.296[a]
Damaged acrosome (DA) * (%)	9.0 ± 1.2	10.4 ± 1.6	0.640[b]
Damaged membrane (DM) ** (%)	8.4 ± 1.9	30.7 ± 3.5	0.003[b]

Table 2. Mean and standard error (SEM) for the percentage of spermatozoa classified as intact acrosome and intact membrane (IAIM), damaged acrosome and intact membrane (DAIM), intact acrosome and damaged membrane (IADM), and damaged acrosome and damaged membrane (DADM), as well as overall acrosome damage (DA) and plasma membrane damage (DM) in mature ($n=20$; 2–4 years) and senile ($n=20$; ≥ 9 years) groups. [a]Repeated measures ANOVA. [b]t-test. * DA = DADM + DAIM. ** DM = DADM + IADM. Spermatozoa were stained with PNA-Alexa Fluor™ 488 and propidium iodide (PI) fluorescent probes and analyzed using flow cytometry.

The increased presence of proximal cytoplasmic droplets in sperm from senile dogs, along with the higher motility observed in mature dogs in this study, reinforces the hypothesis that impaired cytoplasmic droplet migration negatively affects sperm motility[35–37]. Cytoplasmic droplets are believed to harbor enzymes and substrates incorporated into sperm cells during droplet migration and sperm maturation, facilitating plasma membrane remodeling and the acquisition of motility[38–40]. Furthermore, cytoplasmic droplets are reported to modulate sperm motility by activating mitochondrial function during epididymal maturation[35,41]. Consistent with this, our previous report demonstrated that mature dogs exhibit significantly higher mitochondrial potential than senile dogs, further underscoring the critical role of cytoplasmic droplets in regulating sperm motility[28].

ROS production and LPO are widely believed to increase with age in mammals[42,43]. LPO can alter the physiological functions of sperm cell membranes and contribute to cellular damage, including DNA fragmentation[9,44,45]. Elevated LPO levels have been observed in the spermatozoa of aged Brown Norway rats and was associated with reduced sperm quality[15]. Similarly, increased LPO and oxidative DNA damage have been reported in the frozen-thawed semen of senior bulls[9]. In contrast, our study found no significant difference in sperm LPO levels between aged and mature dogs. These findings support the assertion by Rikans and Hornbrook[46] that increased LPO is not a universal feature of aging and varies depending on the species and tissue type. Our findings are consistent with two prior studies in dogs, which also reported no significant differences in LPO between age groups in fresh and cryopreserved semen[22,47]. Similarly, a study on Nili-Ravi buffalo bulls found no differences in LPO between aged and young individuals[48]. Furthermore, Neagu et al.[49] suggested that LPO appears to have a minimal role in sperm cryodamage in dogs and is unrelated to the antioxidant activity of seminal plasma. Based on our findings and these previous studies, we propose that LPO in dog spermatozoa is independent of age. The age-associated decline in semen quality in dogs may instead result from molecular changes and disruptions in apoptosis during spermatogenesis and sperm maturation, without significant involvement of the oxidative stress pathway.

The observations in this study regarding spermatozoa and seminal plasma enzymatic antioxidants differ from the findings of Kelso et al.[6] in bulls, where a significant reduction in seminal plasma and spermatozoa SOD and GPx levels was observed in older bulls (> 9 years of age). This discrepancy may be explained by the identical and relatively low levels of lipid peroxidation observed in both the senile and mature groups of dogs in the present study, suggesting comparable levels of antioxidant protection. In bulls, a reduction in antioxidant activity was linked to decreased concentrations of polyunsaturated and unsaturated fatty acids, such as arachidonic

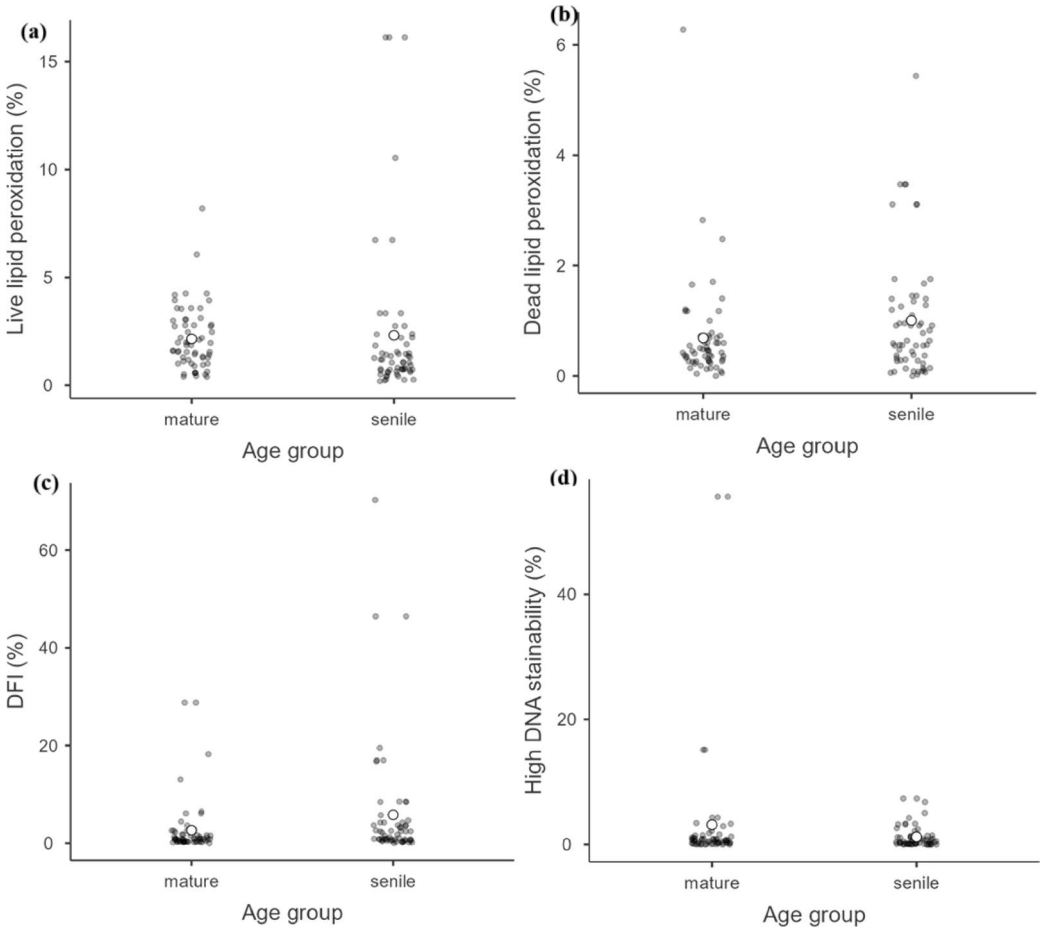

Fig. 2. (**a**) Live lipid peroxidation, (**b**) dead lipid peroxidation, (**c**) sperm DNA fragmentation (DFI), (**d**) high DNA stainability in the fresh semen of mature ($n = 20$; 2–4 years) and senile ($n = 20$; ≥9 years) dogs. Dots represent individual ejaculates and the circle indicates mean values.

Parameters	Mature	Senile	*p*-value[a]
Seminal plasma			
SOD (% inhibition rate)	63.7 ± 3.5	67.6 ± 3.7	0.454
CAT (U/mL)	15.0 ± 1.4	17.0 ± 1.4	0.250
GPx (U/L)	142 ± 3.8	141 ± 6.7	0.804
Semen			
SOD (% inhibition rate)	53.1 ± 5.2	61.6 ± 4.7	0.256
CAT (U/mL)	5.3 ± 0.1	5.2 ± 0.1	0.103
GPx (U/L)	289 ± 52.7	251 ± 62.0	0.351

Table 3. Mean and standard error (SEM) of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in seminal plasma and semen of mature ($n = 20$; 2–4 years) and senile ($n = 20$; ≥ 9 years) dogs. [a]Independent samples t-test. The first three parameters are of seminal plasma, and the others are of semen.

and docosahexaenoic acid, making spermatozoa more susceptible to peroxidative damage in older individuals[6]. Similarly, in senior mice, reduced antioxidant activity in sperm was associated with elevated ROS production and increased LPO[13]. Our findings highlight the species-specific differences in sperm metabolism and oxidative stress responses. Unlike bulls and mice, dogs may maintain consistent antioxidant levels and LPO across age groups, reflecting potential differences in sperm physiology and protection mechanisms.

The current study demonstrated that the semen of senile dogs is more susceptible to the freezing-thawing process than that of mature dogs. The immediate post-thaw sperm motility and kinematic parameters were significantly lower in senile dogs compared to mature dogs. This finding agrees with the report by Lechner et al.[50]who retrospectively analyzed data from frozen-thawed canine semen and found that the effects of

Parameters	Mature	Senile	*P*-value[a]
NBZ	14.7 ± 2.0	7.3 ± 2.0	0.016
Total motility PT (%)	53.5 ± 2.6	20.8 ± 2.6	< 0.001
Progressive motility PT (%)	37.1 ± 1.9	8.0 ± 1.9	< 0.001
VAP PT (µm/s)	88.5 ± 2.6	61.7 ± 2.6	< 0.001
VSL PT (µm/s)	82.7 ± 1.2	51.6 ± 1.6	< 0.001
VCL PT (µm/s)	129.0 ± 3.9	115.0 ± 3.9	0.030
SLOW PT (%)	5.8 ± 0.7	8.4 ± 0.7	0.022
STATIC PT (%)	55.2 ± 4.9	73.8 ± 4.9	0.016

Table 4. Mean and standard error (SEM) number of canine spermatozoa bound to the zona pellucida (NBZ); comparison between mature and senile dogs, and post-thaw kinematic parameters in mature (n = 10), and senile (n = 10) groups. [a]Repeated measures ANOVA. NBZ = mean number of spermatozoa bound to zona pellucida. PT = post-thaw. VAP = average path velocity. VSL = straight line velocity. VCL = curvilinear velocity. SLOW = slowly motile spermatozoa. STATIC = static sperm.

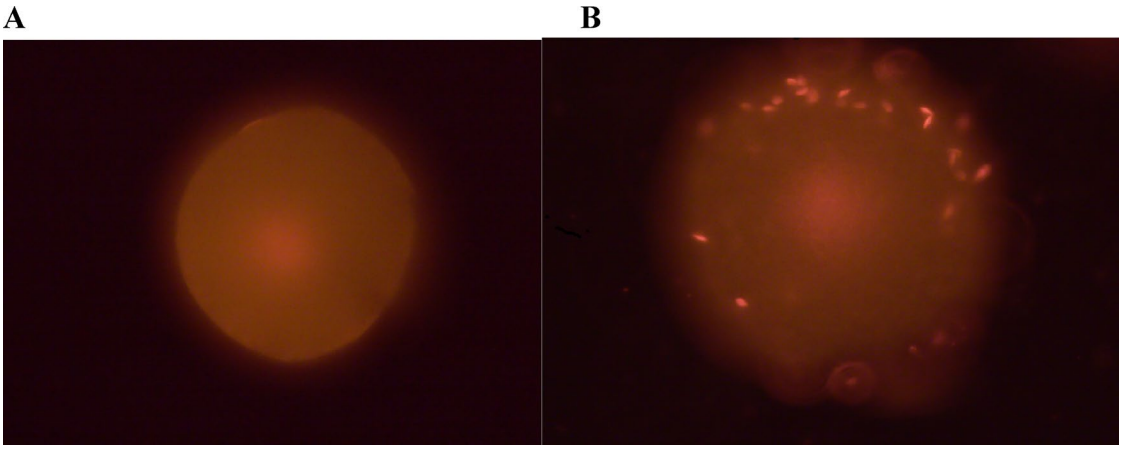

Fig. 3. (**A**) Representative oocyte without bound spermatozoa following a zona binding assay, visualized using PI staining. (**B**) Representative oocyte with multiple bound spermatozoa following a zona binding assay, visualized using PI staining.

cryopreservation on progressively motile sperm are more pronounced in dogs aged 10 and 11 years. Previous studies investigating the influence of aging on cryopreserved sperm motility have reported variable results, likely due to the inclusion of dogs ≥ 7 years in the senior group[22,47,51]. Consistent with Lechner et al.[50]our findings suggest that sperm quality and cryotolerance in dogs remain relatively stable throughout much of their lifespan but decline more noticeably in individuals aged nine years and older.

The ZBA provides insights into the fertilizing potential of spermatozoa[26]. Sperm–zona pellucida interaction is a critical step in fertilization, reflecting sperm competence in recognizing the oocyte[52,53]. This process begins with the interaction between receptors on the sperm membrane and glycoproteins of the zona pellucida[27]. Therefore, the ZBA can assess damage to molecular binding structures that routine sperm functional analyses may not detect. Additionally, the assay is useful in evaluating not only sperm damage caused by storage but also inherent dysfunctions in untreated spermatozoa that may contribute to reduced fertility[26,54].

In the present study, significantly more spermatozoa from mature dogs bound to the zona pellucida compared to those from senile dogs. While this finding may suggest reduced fertility in senile dogs in vivo, caution is warranted in its interpretation. The capacity of spermatozoa to bind to the oocyte varies between replicates due to multiple factors, including oocyte quality, the duration of ovarian cryostorage, sperm population characteristics, and individual male variation[27,55]. In domestic animals, attempts to establish a correlation between sperm–zona binding ability and field fertility have often yielded inconsistent results[26,56–59]. The discrepancy between in vivo and in vitro fertility assessments is unsurprising, as the ZBA does not account for sperm transport within the female reproductive tract or post–zona binding events[53,58].

Several factors influence sperm binding capacity to the zona pellucida, including in vitro capacitation, acrosomal status, and specific motile subpopulations[26,53,60,61]. Previous studies suggest that both acrosome-intact and acrosome-reacted dog spermatozoa can bind to the zona pellucida[62]and no correlation has been found between acrosomal status and sperm penetration of the oocyte[63]. Given the significant differences in the rapid and progressively motile sperm subpopulations between mature and senile dogs in this study, we propose

that this factor may explain the observed differences in zona pellucida binding, as this subpopulation has been shown to exhibit superior responses to in vitro capacitation[53].

Conclusions

In conclusion, no significant age-related changes in lipid peroxidation or enzymatic antioxidant activities were observed, suggesting that the observed decline in semen quality is not driven by increased susceptibility to oxidative stress. Furthermore, frozen-thawed spermatozoa from mature dogs exhibit a significantly higher zona pellucida-binding capacity than those from senile dogs, likely due to a greater proportion of progressively motile sperm subpopulations.

Materials and methods

Animals and experimental design

This study included 40 domestic dogs of various breeds presented for semen quality assessment at the Ambulatory of the Department of Reproduction and Clinic of Farm Animals, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences. Two groups of stud dogs were evaluated: the mature group (aged 2–4 years; 3 ± 0.2, $n = 20$) and the senile group (aged 9–11 years; 10 ± 0.2, $n = 20$), with a total of 107 ejaculates (2–3 per dog) collected for analysis. The dogs were similarly distributed among small, medium, and large breeds within each group: 8 small breeds (< 20 kg), 8 medium breeds (20–40 kg), and 4 large breeds (> 40 kg) in each group. All dogs were fed commercial diets without supplementation aimed at enhancing semen quality. Before semen collection, all dogs underwent a comprehensive examination, including ultrasonography to assess testicular and prostate health, confirming their reproductive health status. Semen was collected via preputial massage using a gloved hand in the absence of an estrous bitch. Ejaculates were obtained once per week from each dog. Semen collection during routine veterinary procedures was approved by the Animal Welfare Committee of Wroclaw University of Environmental and Life Sciences (Statement n. 15/2024). All experiments were conducted in compliance with the national Act on the Protection of Animals Used for Scientific or Educational Purposes (Dz. U. poz. 266) and are reported in accordance with the ARRIVE guidelines.

Additionally, semen from 10 mature (2–4 years; 3.3 ± 0.3 years) and 10 senile (9–11 years; 10.1 ± 0.2) dogs was cryopreserved and used in the zona pellucida binding assay (ZBA). Ovaries were collected from 20 bitches following routine ovariohysterectomy at the clinic and stored at -20 °C until use in the ZBA.

Experiment 1: assessment of sperm cell characteristics in fresh semen

Evaluation of spermatozoa

Immediately after collection, 10 µL of semen was diluted in Tris-citric acid-fructose extender, which consisted of Tris (hydroxymethyl)- aminomethane (0.2 M), citric acid monohydrate (0.06 M), fructose (0.05 M), and distilled water, and sperm concentration, TSC, and total motility were assessed using the Ceros II Computer-Assisted Sperm Analysis (CASA) system (Hamilton-Thorne Biosciences, Beverly, MA, USA). The software settings for the CASA system used in this study were presented in our previous study[28]. To evaluate sperm abnormalities, 200 spermatozoa were examined under a phase-contrast microscope (Nikon Eclipse E200). Morphological abnormalities were categorized as head defects, midpiece defects, tail defects, or the presence of cytoplasmic droplets[64].

Evaluation of acrosome integrity

For the simultaneous evaluation of acrosome and plasma membrane integrity, 10 µL of peanut agglutinin (PNA; 1 µg/mL) working solution (Life Technologies Ltd., Grand Island, NY, USA) was added to 500 µL of diluted semen containing 8×10^6 spermatozoa. The mixture was incubated for 5 min at room temperature in the dark. Subsequently, 3 µL of propidium iodide (PI) (1 mg/mL solution in water; P3566, Thermo Fisher Scientific Inc.) was added before analysis to differentiate between live and dead spermatozoa[65]. All samples were analyzed using a Guava EasyCyte 5® flow cytometer (Merck KGaA, Darmstadt, Germany), with 10,000 events recorded per sample. Fluorescence detection was performed using an argon-ion laser (488 nm). Based on a simultaneous assessment of acrosome and membrane integrity, spermatozoa were classified into four categories: (i) intact acrosome and intact membrane (IAIM), (ii) damaged acrosome and intact membrane (DAIM), (iii) intact acrosome and damaged membrane (IADM), and (iv) damaged acrosome and damaged membrane (DADM). Furthermore, parameters for overall acrosome damage (DA) and membrane damage (DM) were analyzed by grouping spermatozoa as follows: DA = (DAIM + DADM) and DM = (IADM + DADM).

Lipid peroxidation

LPO was assessed using the fluorescent lipid probe C11-BODIPY$^{581/591}$ (Life Technologies Ltd., Grand Island, NY, USA). This probe mimics fatty acids and integrates into the plasma membrane, emitting fluorescence upon lipid peroxidation. Under non-oxidized conditions, the probe emits red fluorescence; however, when subjected to oxidation, such as by peroxyl and alkoxyl radicals, the emission shifts to green fluorescence[66]. To conduct the assay, 1 µL of 2 mM C11-BODIPY$^{581/591}$ in ethanol was added to the diluted sperm samples, resulting in a final probe concentration of 4 µM. The samples were incubated for 30 min at 37 °C in the dark. Following incubation, the samples were centrifuged at 500×g for 3 min, and the pellet was resuspended in 500 µL of Tris buffer to remove unbound or excess dye. To assess cell viability, samples were stained with PI and further incubated for 5 min at room temperature before cytometric analysis. Dot plots of C11-BODIPY $^{581/591}$/PI -stained spermatozoa revealed four distinct cell populations: (1) live spermatozoa without LPO (PI-BODIPYred), (2) live spermatozoa with LPO (PI-BODIPYgreen), (3) dead spermatozoa without LPO (PI + BODIPYred), and (4) dead spermatozoa

with LPO (PI + BODIPYgreen). Green fluorescence was detected using an FL-1 detector, while red fluorescence was measured using an FL-3 detector[67].

Sperm chromatin status

Chromatin status was evaluated using the sperm chromatin structure assay (SCSA) as described by Evenson[68]which exploits the metachromatic properties of Acridine Orange (AO) dye (Life Technologies Ltd., Grand Island, NY, USA). Initially, 200 µL of denaturation fluid (0.1% (v/v) Triton X-100, 0.15 M NaCl, and 0.08 M HCl, pH 1.4) was added to 100 µL of diluted sperm. After a 30-second incubation, 600 µL of AO solution (6 µg AO/mL in buffer containing 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, and 0.15 M NaCl, pH 6) was added. Spermatozoa with intact DNA exhibited green fluorescence, whereas those with fragmented DNA displayed red fluorescence, classified as DFI[68]. A distinct subgroup of spermatozoa exhibiting high DNA stainability (HDS) was also identified. The high DNA-stainable sperm are believed to have abnormally high DNA staining due to defective protamination, resulting in an increased amount of retained histones. These cells appeared as a separate population positioned to the right of the normal sperm population (FL-3) and above the population characterized by fragmented DNA.

Enzyme quantification

Seminal plasma was obtained by centrifuging each semen ejaculate at 1,500 × g for 15 min. Enzymatic antioxidant activity was analyzed from a single ejaculate per dog. The activities of SOD, CAT, and GPx in semen samples (both seminal plasma and spermatozoa) of dogs were assessed spectrophotometrically using a BioTek ELx800 Absorbance Microplate Reader (Biotek, Winooski, VT, USA). SOD (19160-1KT-F) and GPx (MAK437) activities were measured using commercial Sigma-Aldrich kits (Darmstadt, Germany) at wavelengths of 450 nm and 340 nm, respectively. CAT activity was quantified using the catalase colorimetric activity kit (EIACATC, Invitrogen, USA) at a wavelength of 540 nm. CAT activity was assessed based on the enzyme's ability to catalyze the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. SOD activity was determined using the water-soluble tetrazolium salt WST-1[2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 H-tetrazolium, monosodium salt], which produces a water-soluble formazan dye upon reduction by superoxide anions. The rate of this reduction, which is proportional to xanthine oxidase (XO) activity, is inhibited by SOD. The IC_{50} (50% inhibition activity of SOD) was determined colorimetrically under the conditions specified by the manufacturer. GPx activity was measured by monitoring the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) by GPx. The resulting GSSG was subsequently reduced back to GSH through the action of glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH), with NADPH consumption measured spectrophotometrically. The enzyme activities were expressed as follows: SOD activity as % inhibition rate, CAT activity as U/mL, and GPx activity as U/L, in accordance with the manufacturer's guidelines.

Experiment 2: Zona pellucida binding assay (ZBA)

The ZBA was employed in this experiment to evaluate the ability of frozen-thawed spermatozoa from mature and senile dogs to bind to the zona pellucida. Frozen-thawed spermatozoa were utilized in this study because freshly collected semen underwent multiple quality assessments immediately after collection. Performing the ZBA several hours post-collection would have reduced semen viability. Additionally, the ZBA is a time-consuming procedure, making it impractical to conduct alongside other sperm functional tests on the same day. Furthermore, quality parameters of fresh semen could not be directly compared with frozen-thawed semen, as only approximately half of the senile dogs produced sufficient semen for both comprehensive quality assessments and cryopreservation.

Semen freezing

After semen collection and CASA evaluation, an aliquot containing approximately 200×10^6 spermatozoa was aspirated and placed in a tube, then centrifuged at 500 × g for 5 min to remove seminal plasma. The resulting pellet was diluted in a Tris-Fructose-Equex extender with egg yolk and frozen according to the method described by Nizanski[69], . Briefly, each extended semen sample was gradually cooled to 5 °C over 1 h, followed by the addition of 6% glycerol. After a 90-minute equilibration period at 5 °C, the samples were frozen in 0.5 mL straws at -140 °C using nitrogen vapor (5 cm above the liquid nitrogen surface) and subsequently stored in liquid nitrogen. Several months later, the samples were thawed at 37 °C in a water bath for 60 s for post-thaw motility and kinematic assessment, as well as for zona pellucida binding assay (ZBA).

Oocyte recovery

Ovaries from 20 bitches, aged 2 to 4 years and of various breeds, were collected following routine ovariohysterectomy procedures at the clinic. Among these, seven ovaries contained corpora lutea, three had follicles, and ten displayed no functional structures on the ovarian surface. The ovaries were rinsed with phosphate-buffered saline (PBS), immersed in 20 mL of NaCl solution, and stored at -20 °C. On the day of the experiment, ovaries were randomly selected and thawed at room temperature. For each trial, one pair of ovaries was designated for spermatozoa from mature and senile dogs. Cumulus-oocyte complexes (COCs) were retrieved by repeatedly slicing the ovaries in a Petri dish containing PBS with 0.5% (w/v) bovine serum albumin (BSA) under a stereomicroscope. Cumulus cells were removed by incubating the oocytes in 75 mmol/L sodium citrate for 15 min, followed by vortexing for an additional 15 min, as described by Strom Holst et al.[26]. The denuded oocytes were stored overnight at 4 °C in PBS containing 0.5% (w/v) BSA and used the following day for the ZBA.

Semen processing and ZBA

On the assay day, after thawing, semen was centrifuged at 300 × g for 5 min, and the supernatant was discarded. The sperm pellet was washed in 2 mL of Canine Capacitation Medium (CCM)[70] by centrifugation at 300 × g for 5 min, followed by the removal of the supernatant. The pellet was then resuspended in 2.5 mL of CCM to achieve a final concentration of 40×10^6 sperm/mL and incubated at 37 °C in 5% CO_2 for 2 h.

Forty-microliter droplets of CCM were prepared in four-well Petri dishes, with each well overlaid with 1.2 mL of mineral oil. Ten oocytes were added to each droplet and equilibrated at 37 °C in 5% CO_2 for at least 1 h, following the protocol described by Strom Holst et al.[26] Subsequently, a 25 µL aliquot of the sperm suspension was diluted with 75 µL of CCM, and a 10 µL aliquot of this dilution (containing approximately 50×10^3 spermatozoa) was added to each droplet[27]. The sperm-oocyte droplets were co-incubated at 37 °C in 5% CO_2 for 6 h.

Following incubation, sperm-oocyte complexes were transferred to 100 µL droplets of 1.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, fixed for 15 min, and stored in PBS containing 0.5% (w/v) BSA until evaluation. To remove loosely bound spermatozoa, sperm-oocyte complexes were washed by repeated pipetting in 100 µL droplets of PBS with 0.5% BSA. The oocytes were then stained with 10 µL of PI in 1 mL of PBS with 0.5% BSA for 10 min.

Stained sperm-oocyte complexes were placed on microscope slides and overlaid with a coverslip supported by four droplets of a Vaseline–paraffin mixture. Gentle pressure was applied to immobilize the oocytes in the medium. The number of spermatozoa strongly bound to the zona pellucida was determined using a fluorescence microscope equipped with a 488 nm excitation laser (100 mW output). Observations were performed at 400× magnification. Multiple optical sections were captured at 10 µm intervals for each oocyte. Images were compiled into a single visual layer using OLYMPUS cellSens Entry 1.18 software, and strongly bound spermatozoa were counted.

Statistical analysis

Data analysis was conducted using Jamovi version 2.3.21 (The Jamovi project, 2022). Statistical evaluation of all semen parameters was performed using repeated measures ANOVA, with age group specified as the between-subject factor, while individual dog and age were included as covariates. Ejaculate number was modeled as the within-subject factor. Data normality was assessed using the Shapiro-Wilk test, and non-normally distributed variables were log-transformed to meet parametric assumptions. Group comparisons of mean SOD, CAT, and GPx levels were performed using independent samples t-tests. Statistical significance was defined at $P \leq 0.05$, with exact P-values reported unless $P < 0.001$. Figures present scatter plots of individual ejaculates alongside corresponding mean values.

Data availability

The data that support the findings in this study are available upon reasonable request from the corresponding author [Kenneth Owoicho Abah].

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Author contributions

KOA: Writing—original draft, Resources, Methodology, Formal analysis, Funding acquisition, Data curation. ZLK: Resources, Investigation, Writing—review & editing. AP: Writing—review & editing, Resources. SP: Resources, Investigation, Writing—review & editing. AF: Supervision, review. WN: Writing—review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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