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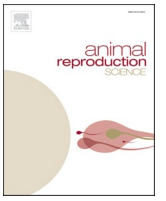

Antioxidant enzyme dynamics suggest the absence of oxidative stress in the canine endometrium across the estrous cycle

R. Payan-Carreira [a,*], C. Santos [b], D. Santos [c]

[a] CHRC - Comprehensive Health Research Centre, Department of Veterinary Medicine, University of Évora, Pole at Mitra, Évora 7002-774, Portugal
[b] CECAV, Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, P.O. Box 1013, Vila Real 5001-801, Portugal
[c] CITAB - DeBA, University of Trá~s-os-Montes and Alto Douro, P.O. Box 1013, Vila Real 5001-801, Portugal

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ABSTRACT

Oxidative stress plays a vital role in female fertility, yet the mechanisms regulating oxidative balance in the canine endometrium remain poorly understood. This study investigates the dynamics of the antioxidant enzyme system in the canine endometrium, focusing on superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GSR), and glutathione-S-transferase (GST), along with thiobarbituric acid reactive substances (TBARS) and total cellular thiols. Enzyme activities were revealed in five different phases of oestrus cycle in 25 dogs, using a spectrophotometric method: anestrus, proestrus, estrus, early diestrus and diestrus (n = 5 per group). Notably, a distinctive pattern in SOD and CAT activity was observed, with the former being characterised by a decrease from anestrus to estrus, and the later showed an opposite increase from anestrus to diestrus. In contrast, the activities of the glutathione-dependent enzymes GPX, GSR, and GST remained remarkably stable, although showing some fluctuations in different stages. TBARS analysis indicated an evident increase in oxidative stress-related lipid peroxidation in the canine endometrium only between anestrus and proestrus. Conversely, the thiol cell content remained consistent within the cycle stages. Our examination of enzyme ratios underscores a delicate balance in the normal canine uterus, effectively controlling oxidative stress without causing damage to lipids or proteins due to excessive reactive oxygen species. These findings contribute to our understanding of the unique physiological dynamics of the canine endometrium, offering valuable insights into the intricate regulation of oxidative stress in this context and its potential implications for female fertility.

1. Introduction

In physiological conditions, oxygen metabolism generates reactive oxygen species (ROS) through O_2 univalent reduction (Sies, 1991; Stanczyk et al., 2005), including radicals like $O_2^{\bullet-}$ and $OH^{\bullet}$, and non-radical derivatives like hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 2015). At moderate concentrations, ROS serve as crucial signaling molecules in cellular processes (Finkel, 1998; Droge, 2002; Poli et al., 2004)·H_2O_2 emerges as a primary messenger in redox signaling due to its unique properties (Lennicke et al., 2015). Unlike free radicals, H_2O_2 lacks unpaired electrons and can act as a paracrine signal, mediating growth factor signaling pathways through NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) and influencing cell migration,

* Corresponding author.
E-mail address: rtpayan@uevora.pt (R. Payan-Carreira).

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proliferation, and survival (Berridge, 2014; Di Marzo et al., 2018).

Living organisms maintain a dynamic balance between oxidative stress and antioxidant defense mechanisms. The antioxidant system comprises enzymatic and non-enzymatic components that regulate ROS flow and prevent cellular damage (Yu, 1994; Pamplona and Costantini, 2011). Key enzymatic components include:

- Superoxide dismutase (SOD), which detoxifies $O_2^{\bullet -}$
- Catalase (CAT), which reduces H_2O_2
- Glutathione-related enzymes [the glutathione peroxidase (GPX), glutathione reductase (GSR), and glutathione-S-transferase (GST)] that manage peroxide levels and regenerate glutathione (Halliwell and Gutteridge, 2015).

The conversion of H_2O_2 to water and oxygen occurs through the synergic activity of glutathione peroxidase and catalase (Zhu et al., 2012). Non-enzymatic antioxidants include glutathione, vitamins C and E, carotenoids, and flavonoids (Halliwell and Gutteridge, 2015). Oxidative stress emerges when ROS production significantly exceeds the antioxidant system's capacity, representing an imbalance between oxidants and antioxidants (Sies, 1991, 2020; Halliwell and Gutteridge, 2015).

Oxidative stress has been implicated in numerous pathological conditions, including tumor development, neurological diseases, diabetes mellitus, atherosclerosis, hypertension, and chronic kidney disease (Valko et al., 2007; Halliwell and Gutteridge, 2015). Understanding these mechanisms is crucial for developing therapeutic strategies targeting oxidative stress-related diseases (Valko et al., 2007; Halliwell and Gutteridge, 2015).

In females, cyclic changes in stromal-epithelial interactions, orchestrated by sex steroid hormones, are essential for endometrial function during implantation (Agarwal and Gupta, 2005; Agarwal et al., 2008). Antioxidant expression varies throughout the endometrial cycle (Al-Gubory et al., 2010; Cornelli et al., 2013), responding to changes in sex steroid dominance. Reactive oxygen species (ROS) play critical roles in female reproductive biology, modulating the uterine environment and embryo-maternal interactions during implantation (Riley and Behrman, 1991; Ufer and Wang, 2011; Rizzo et al., 2012).

Under pathological conditions like endometriosis, increased oxidative stress and antioxidant depletion may contribute to excessive endometrial stromal cell growth. While oxidative stress is associated with non-physiological endometrial modifications (Van Langendonckt et al., 2002; May et al., 2011) and various uterine diseases (Szczubial and Dabrowski, 2009), it also participates in physiological events such as menstruation and decidual implantation (Rizk et al., 2013).

In humans, excessive ROS can compromise early embryonic development by causing DNA fragmentation and apoptosis (Lin and Wang, 2020; Hardy et al., 2021), potentially thus compromising the embryo ability to implant successfully. Excessive ROS production in the uterine environment may affect endometrial receptivity by disrupting cytokine signaling and the balance of inflammatory mediators necessary for implantation (Artimovič et al., 2024). Although less studied, canine embryos may be similarly susceptible to oxidative stress during the implantation window.

Dogs exhibit a unique estrous cycle, with unique features compared to other domestic species. They are monoestrous, with a prolonged anestrus stage separating two estrous cycles and marked by basal levels of estrogen and progesterone (Concannon, 2011). The transition to a new follicular phase begins in the final third of anestrus with follicular recruitment (England et al., 2009). This anestrus period is critical for maintaining female dog fertility and facilitating endometrial regeneration (Okkens and Kooistra, 2006). The canine estrous cycle features a prolonged follicular stage, characterized by pre-ovulatory luteinization of dominant ovarian follicles, followed by an extended diestrus phase of consistent duration regardless of pregnancy status (Concannon, 2011). These distinctive characteristics, particularly the endometrial response to sex steroids, increase susceptibility to progesterone-associated degenerative conditions, including deciduoma formation (Nomura, 1997) and cystic endometrial hyperplasia (De Bosschere et al., 2001; Noakes et al., 2001).

Research interest has grown in studying oxidative stress in female reproduction, focusing on its relationship with infertility and uterine pathologies. However, understanding of oxidative stress in the endometrium of domestic species remains limited, both in healthy and diseased individuals (Al-Gubory et al., 2008; Ramos et al., 2015; Acar et al., 2020). Recent studies have advanced our knowledge through innovative approaches: immunohistochemistry revealing SOD and GPX distribution in the canine cyclic uterus (Santos et al., 2016), and ferrous-to-ferric ion oxidation assays examining total antioxidant changes in uterine tissue homogenates (Acar et al., 2020). While the latter provides a broader assessment of tissue oxidative stress homeostasis, it fails to elucidate the mechanisms of oxidative stress balance or the specific contributions of antioxidant enzymes in maintaining endometrial redox equilibrium.

Despite these advances, a comprehensive understanding of how oxygen scavenging enzymes coordinate throughout the canine estrous cycle to maintain oxidative balance and endometrial homeostasis remains elusive. This study aims to investigate the dynamics of antioxidant enzyme activities and measure protein and lipid oxidation levels across different stages of the canine estrous cycle. Moreover, it seeks to establish baseline data that could support future research on endometrial receptivity, uterine health, and the identification of markers for endometrial degenerative diseases.

2. Material and methods

2.1. Sample collection and preparation

Twenty-five healthy post-pubertal, nonpregnant nulliparous female dogs aged 10 months to 6 years underwent elective ovariohysterectomy (OVH) in this study (Supplementary Table 1). The animals were part of a municipal neutering program, and all surgical

procedures followed standard small animal surgical protocols, including appropriate aseptic techniques, anesthesia, pain management, and postoperative care. Surgical specimens were collected with owner's informed consent in accordance with European Ethical Standards (Directive 2010/63/EU).

Estrous cycle staging was determined through physical examination, vaginal cytology, ovarian inspection after OVH, and circulating progesterone levels (Table 1), with confirmation by ovarian and uterine histology (Payan-Carreira et al., 2011). Blood samples were collected from the jugular vein in serum-gel tubes (S-Monovette®, Sarstedt) and centrifuged at 2500 xg for 15 minutes. Serum was stored at −20°C, and progesterone levels were measured by immunoassay (Immulite® 1000, DPC) with < 6.0 % interassay variation. Diestrus was determined by progesterone levels between 16 and 2 ng.mL^{-1} and the presence of CL in the ovaries, with the LH surge day identified at 2 ng.mL^{-1}, this threshold used to establish the first day of estrus.

From the middle portion of each uterine horn, a 1.5 cm fragment was collected immediately after surgery and snap-frozen in liquid nitrogen for transport to the laboratory, where it was stored at −80°C until analysis. An adjacent uterine segment was collected for histological analysis, fixed in 10 % buffered formalin, embedded in paraffin wax, sectioned at 2 μm, and stained with hematoxylin and eosin for estrous cycle staging and to exclude uterine disease.

Uterine samples (n = 5 per group) were categorized according to five stages of the estrous cycle: anestrus, proestrus, estrus, early diestrus, and diestrus. Given that canine implantation occurs around post-ovulatory day 16 (Barrau et al., 1975; Concannon et al., 2001), diestrus was subdivided into two distinct phases as previously described (Payan-Carreira et al., 2011): early diestrus, corresponding to the embryo implantation period in pregnant cycles, and full diestrus, which corresponds to the mid-secretory stage.

2.2. Preparation of dog endometrial extracts and protein determination

Frozen tissue samples were dissected while still frozen to separate the endometrium from the myometrium and connective tissue. The isolated endometrium was finely minced with a surgical blade and homogenized in cold phosphate-buffered saline (PBS – 1.76 mM KH_2PO_4, 10 mM NaH_2PO_4, 2.7 mM KCl, 137 mM NaCl; pH 7.0). The homogenates underwent sonication for cell disruption (4 cycles of 5'' with 15'' intervals at 80 % amplitude under refrigeration), followed by centrifugation at 1500 xg for 15 minutes at 4°C. The resulting supernatant was used to determine protein concentration, measure enzymatic activities, and evaluate oxidative damage.

The protein concentration was determined according to Bradford (Bradford, 1976), with bovine serum albumin (BSA) as the calibration standard. 1000 μL of Bradford reagent [containing 0.01 % (w/v) Coomassie brilliant blue, 4.7 % (v/v) ethanol (95 %) and 8.5 % (w/v) phosphoric acid] was added to 10 μL diluted sample. Protein quantity was determined using absorbance at 590 nm.

2.3. Antioxidant enzyme activity assays

Enzymatic system activities (SOD, CAT, GST, GSR, and GPX) and molecular compounds affected by oxidative cell damage (TBARS and Thiols) serve as potential biomarkers of oxidative stress. Such stress occurs when the balance between oxidants and antioxidants is disrupted, either through excessive ROS accumulation or antioxidant defense depletion (Sies, 2020).

All enzyme activities were measured spectrophotometrically in duplicate, with the mean value representing each sample's result. Quality control measures included blank measurements and standard curve validation. For all enzyme analyses, a control assay was performed using PBS in place of tissue extract. Absorbance readings for enzyme activities, except for catalase, were obtained using a Varian Cary 10 spectrophotometer. Catalase activity was specifically measured using a YSI oxygen electrode system.

SOD (EC 1.15.1.1) activity was determined indirectly by measuring the decrease in nitro blue tetrazolium-formazan (NBT-diformazan) formation using the xanthine/xanthine oxidase system as a superoxide generator (Paya et al., 1992). Assays were conducted in a reaction mixture containing 100 nM potassium phosphate buffer (pH 7.8), 10 mM EDTA, 10 mM NBT, 10 mM hypoxanthine, and 0.023 U/mol xanthine oxidase. NBT reduction was monitored by measuring absorbance changes at 560 nm for 2 minutes at 25°C. The rate of NBT reduction in the absence of tissue served as a reference, with activity expressed as percentage inhibition of NBT-diformazan formation, following the equation.

Table 1
Criteria for staging the canine estrous cycle.

Stage of the cycle	Vaginal cytology	P_4	Ovary
Anestrus	> 90 % of the cells are parabasal or intermediate	Baseline [< 2 ng.mL^{-1}]	Smooth ovarian surface, devoid of visible structures
Proestrus	Presence of erythrocytes and raising percentage of superficial and intermediate cells	Below 2 ng.mL^{-1} until LH surge	Large antral follicles with 2–3 mm in diameter are clearly visible in the ovarian cortex
Estrus	> 90 % of superficial cells, most becoming cornified by ovulation, and sporadic erythrocytes	Above 2 ng.mL^{-1} (may reach 12–15 ng.mL^{-1})	Large follicles 5–8 mm in diameter with thick luteinized walls
Early diestrus	Sharp decrease in superficial cells, while intermediate and parabasal cells, along with neutrophils, are the major cell types visualized	> 16 ng.mL^{-1} and rising	Dark carmine, cavitary corpora lutea
Diestrus	Predominance of intermediate and parabasal cells; presence of variable number of neutrophils	above 16 ng.mL^{-1}	Carmine and compact corpora lutea

$$= \frac{\text{rate of NBT} - \text{diformazan formation}(control) - \text{rate of NBT} - \text{diformazan formation}(test)}{\text{rate of NBT} - \text{diformazan formation}(control)} x100$$

Test values were normalized to protein content (ΔAbs min^{-1}mg of protein^{-1}).

CAT (EC 1.11.1.6) activity was measured using a Clark-type oxygen electrode at 25°C (Delrio et al., 1977). One unit of CAT activity was defined as the amount of enzyme catalyzing the decomposition of 1 μmol H_2O_2 per minute.

GST (EC 2.5.1.18) activity was determined according to Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 1 mM CDNB, and 2 mM GSH. After 2 minutes of incubation, enzyme activity was measured for 2 minutes at 25°C. Activity was calculated using the molar absorptivity of CDNB (9.6×10^{-3} $M^{-1}.cm^{-1}$) at 340 nm and expressed as nM CDNB-conjugated glutathione.$min^{-1}.mg^{-1}$ of protein.

GSR (EC 1.6.4.2) activity was measured following Smith et al. (1988). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 2 mM EDTA, 0.1 mM NADPH, and 1 mM GSSG. Following 2 minutes of incubation, NADPH oxidation was monitored for 2 minutes at 25°C. Activity was calculated using NADPH molar absorptivity (6.22×10^3 $M^{-1}.cm^{-1}$) at 340 nm and expressed as nmol NADPH oxidized.$min^{-1}.mg^{-1}$ of protein.

GPX (EC 1.11.1.9) activity was determined using a modified method of Paglia and Valentin (1967) with *tert*-butyl hydroperoxide as substrate. The assay coupled GSH oxidation to NADPH oxidation catalyzed by GSR, monitoring NADPH decrease at 340 nm. The reaction mixture contained 1 mM *tert*-butyl hydroperoxide, 0.24 units yeast GSR, 0.5 mM NADPH, 2 mM EDTA, and 0.1 mM GSH in 100 mM potassium phosphate buffer (pH 7.5). After 2 minutes of incubation, NADPH oxidation was monitored for 2 minutes at 30°C. Activity was calculated using NADPH molar absorptivity (6.22×10^3 $M^{-1}.cm^{-1}$) at 340 nm and expressed as nmol NADPH oxidized. $min^{-1}.mg^{-1}$ of protein.

2.4. Lipid and thiol group oxidation

The lipid peroxidation index was assessed by measuring thiobarbituric acid reactive substances (TBARS) using an acid-heating reaction as described by Buege and Aust (1978) and modified by Doktorovova et al. (2014). Sample aliquots (0.5 mL) were combined with 2.5 mL of thiobarbituric acid (TBA) reagent containing 0.375 % w/v TBA, 0.25 M HCl, 15 % w/v trichloroacetic acid (TCA), and 6.8 mM butylated hydroxytoluene (BHT). The mixture was heated at 95°C for 15 minutes in a water bath. After cooling, the flocculent precipitate was removed by centrifugation (10,000 xg, 2 minutes), and the supernatant was collected. TBARS were quantified spectrophotometrically at 530 nm by measuring the absorbance of the MDA-TBA complex (absorbance coefficient = 1.56×10^5 $M^{-1}.cm^{-1}$). Results were expressed as nmol of TBARS.mg^{-1} protein.

Total cellular thiols (protein thiols + glutathione thiols) were measured as markers of redox status and oxidative stress (Baba and Bhatnagar, 2018). Total thiol group (-SH) oxidation was determined by measuring thiol content in canine endometrial extracts using 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB), following Ellman (1959) as modified by Sedlak and Lindsay (1968) and Suzuki et al., (1990). Endometrial suspension (0.5 mL) was mixed with 1 mL of 4 % w/v sulfosalicylic acid and centrifuged at 24,000 xg for 15 minutes. The pellet was resuspended and homogenized in 100 mM phosphate buffer (pH 8.0). The resulting protein suspension (0.5 mL) was incubated with agitation for 15 minutes with 4.5 mL phosphate buffer and 70 μL of 10 mM DTNB freshly prepared in absolute ethanol. After vortexing, samples were incubated for 15 minutes at room temperature. Absorbance was measured at 412 nm, and thiol concentration was calculated using an extinction coefficient of 1.36×10^5 $M^{-1}cm^{-1}$, with results expressed as nmol thiol.mg^{-1} protein.

2.5. Antioxidant enzymatic protection and oxidative stress ratios

Under physiological conditions, enzymatic antioxidants maintain equilibrium with locally generated oxidative stress. To evaluate

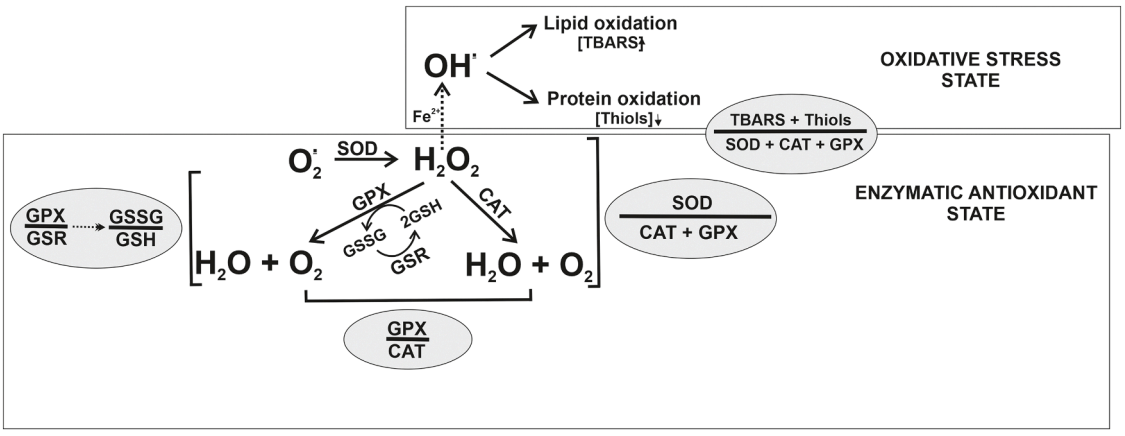

Fig. 1. The interplay of antioxidant enzymes (SOD, CAT, GPX, GSR), Thiols, and TBARS for oxygen scavenging. The schematic shows the relation between the enzyme ratios and the elimination of reactive species.

this balance across different stages of the canine estrous cycle, the following ratios were calculated using antioxidant enzyme activities, lipid oxidation, and thiol oxidation: $\frac{GPX}{CAT}$; $\frac{GPX}{GSR}$; $\frac{SOD}{(CAT+GPX)}$; $\frac{Thiols}{(SOD+CAT+GPX)}$; $\frac{TBARS}{(SOD+CAT+GPX)}$; $\frac{(TBARS+Thiols)}{(SOD+CAT+GPX)}$). These ratios provide information about the antioxidant enzyme system's scavenging ability and its protection against ROS damage in endometrial cells (Fig. 1).

The ***GPX/GSR*** ratio indicates tissue oxidative stress levels by describing the relationship between individual enzymatic activities and the glutathione redox cycle [GSH ↔ GSSG (Glutathione disulfide)]. The balance between GPX and GSR activities is essential for maintaining high cellular GSH levels, a critical non-enzymatic cellular antioxidant (Lushchak, 2012). An elevated ratio suggests increased oxidative stress, while lower ratios may indicate more effective antioxidant responses (Yang et al., 2006).

The ***GPX/CAT*** ratio assesses H_2O_2 degradation efficiency. GPX and CAT, the primary enzymes responsible for H_2O_2 detoxification, operate through distinct pathways and are particularly effective when cellular peroxide levels are elevated (Carter et al., 2004; Sies, 2017). CAT directly decomposes H_2O_2 into H_2O and O_2, while GPX removes H_2O_2 through GSH oxidation to GSSG, which GSR subsequently reduces back to GSH. GPX shows higher H_2O_2 affinity than CAT, and their cooperative mechanism enables GPX to maintain high catalase activity (Baud et al., 2004).

The ***SOD/(CAT + GPX)*** ratio reflects the coordinated activity of the three main antioxidant enzymes in managing ROS and oxidative stress. These enzymes form a protective shield against ROS, with CAT and GPX removing the hydrogen peroxide generated by SOD activity. This ratio expresses the equilibrium between SOD-catalyzed hydrogen peroxide formation and its decomposition by GPX and CAT (Muchová et al., 2001). An imbalance can lead to H_2O_2 accumulation, potentially forming harmful hydroxyl radicals through the Fenton reaction (Michiels et al., 1994; Muchová et al., 2001; Ighodaro and Akinloye, 2018).

The ***TBARS/(SOD + CAT + GPX)*** ratio indicates the balance between oxidative damage markers and key antioxidant enzyme activities. TBARS detect ROS action on cellular lipids (Dasgupta and Klein, 2014), with increased oxidative stress producing higher TBARS levels when enzymatic defenses (SOD+CAT+GPX) are compromised.

The ***Thiols/(SOD+CAT+GPX)*** ratio reflects the relationship between protein oxidative modifications and antioxidant enzyme protection. Physiologically, the cytosol's reducing environment maintains protein thiols in a reduced state. Elevated ROS can cause oxidative stress-induced protein modifications, reducing thiol levels and depleting glutathione (Baba and Bhatnagar, 2018). Active

Table 2
Basic descriptive statistics for endometrial oxidative stress enzymes, Thiols and TBARS in the endometrium of female dogs throughout the estrous cycle. Number of animals per group, n = 5.

	Stage	Mean	SEM	SD	Minimum	Maximum	p-value
SOD	AN	92.12	5.12	11.44	78.90	106.22	**0.002**
	PE	87.50	1.58	3.53	83.57	92.00	
	E	64.75	2.54	5.67	57.57	72.96	
	eDI	69.44	7.18	14.36	56.22	89.59	
	DI	69.79	6.38	14.26	51.96	87.91	
CAT	AN	3.37	0.60	1.33	1.94	4.93	**≤ 0.001**
	PE	3.29	0.47	1.05	2.32	4.97	
	E	9.77	3.31	7.40	4.94	22.64	
	eDI	16.71	4.51	9.03	4.32	24.54	
	DI	25.24	3.85	8.61	16.32	35.26	
GPX	AN	169.30	12.22	27.33	137.77	208.39	0.409
	PE	182.56	24.33	54.40	109.04	249.02	
	E	150.09	22.94	51.29	111.04	229.05	
	eDI	126.81	19.22	38.45	96.97	181.26	
	DI	150.27	18.83	42.11	116.00	215.20	
GSR	AN	10.00	3.73	8.34	3.19	23.60	0.110
	PE	6.60	1.29	2.89	4.47	11.58	
	E	11.35	1.25	2.80	8.16	15.18	
	eDI	17.87	4.59	9.17	12.63	31.57	
	DI	14.67	2.69	6.02	9.39	21.66	
GST	AN	17.76	5.41	12.10	6.52	37.29	**0.010**
	PE	18.99	3.94	8.82	9.20	32.48	
	E	60.89	13.08	29.24	21.77	91.74	
	eDI	59.73	12.82	25.63	43.98	98.00	
	DI	43.50	11.36	25.41	21.07	84.68	
TBARS	AN	4.24	0.14	0.31	3.84	4.62	0.298
	PE	9.69	2.16	4.82	4.65	15.79	
	E	6.00	1.48	3.31	2.47	10.72	
	eDI	6.46	1.02	2.04	4.49	8.78	
	DI	5.71	2.64	5.91	0.84	15.79	
Thiols	AN	445.30	102.34	228.84	142.66	745.56	0.491
	PE	585.59	53.44	119.49	466.07	720.33	
	E	473.66	43.97	98.32	374.10	614.93	
	eDI	458.17	83.75	167.49	307.83	645.56	
	DI	429.75	32.06	71.69	365.32	535.33	

Stages of the cycle: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). SOD – Superoxide dismutase; CAT – Catalase; GPX – Glutathione peroxidase; GSR – Glutathione reductase; GST – Glutathione-S-transferase; Thiols – Total sulfhydryls (-SH).

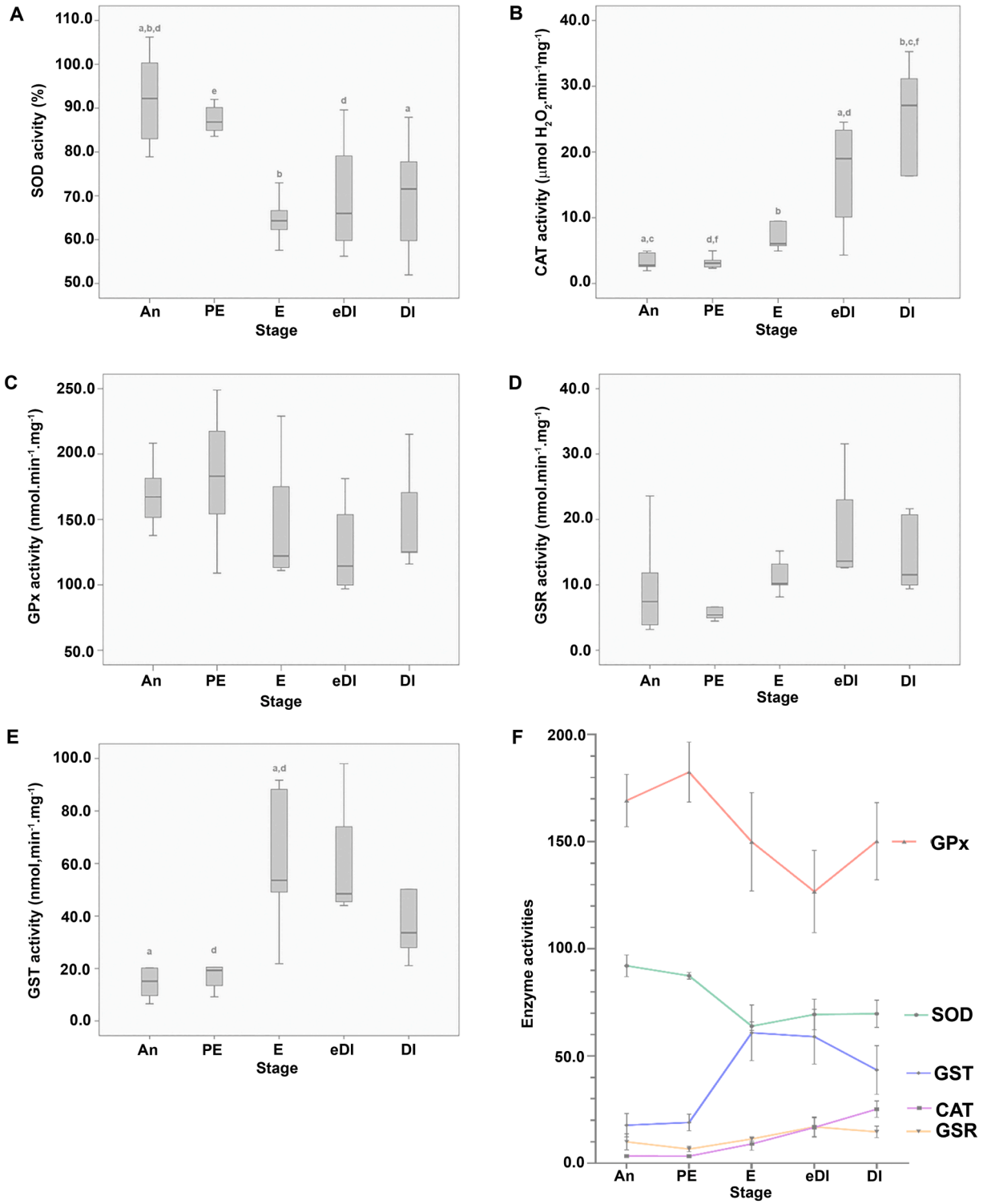

Fig. 2. Endometrial activity of oxidative stress enzymes at different stages of estrous cycle of bitch canine uterus [anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI)] Number of animals used per group, n = 5. A. Superoxide Dismutase (SOD) activity. B. Catalase (CAT) activity. C. Glutathione Peroxidase (GPX) activity. D. Glutathione Reductase (GSR) activity. E. Glutathione-S-Transferase (GST) activity. Significance levels are denoted by shared letters: $P < 0.05$ (a/d/e); $P < 0.01$ (b). F. Time series representation of enzymatic levels across the canine estrous cycle stages.

enzymatic defenses (SOD+CAT+GPX) help maintain stable cellular thiol levels.

The ratio ***(TBARS+Thiols)/(SOD+CAT+GPX)*** evaluates ROS effects on cellular lipids and proteins (Torres et al., 2011). The coordinated defense mechanism of SOD, CAT, and GPX removes superoxide radicals before their conversion to harmful hydroxyl radicals. This ratio compares ROS-induced oxidative effects to total antioxidant enzymatic defense, indicating tissue antioxidant status (Baba and Bhatnagar, 2018). An unchanged ratio suggests efficient neutralization of oxidative stress species without cellular damage.

2.6. Statistical analysis

Results from the biochemical analyses are expressed as mean ± standard error of the mean (SEM). For each sample, assays were run in duplicate, and the mean value was used for data pooling. Basic descriptive statistics (mean, standard error, and SEM) were used to characterize the samples and estrous stages.

Statistical analyses were performed using IBM SPSS Statistics version 22.0 for Mac OS X 10.8. Data distribution normality was assessed using the Shapiro-Wilk test, with p-values ranging from 0.061 for GPx to 0.702 for Thiols (df=25). Homogeneity of variances was confirmed using Levene's test based on medians, with results ranging from $F(4, 18) = 0.526$ (GR) to 2.145 (SOD), and

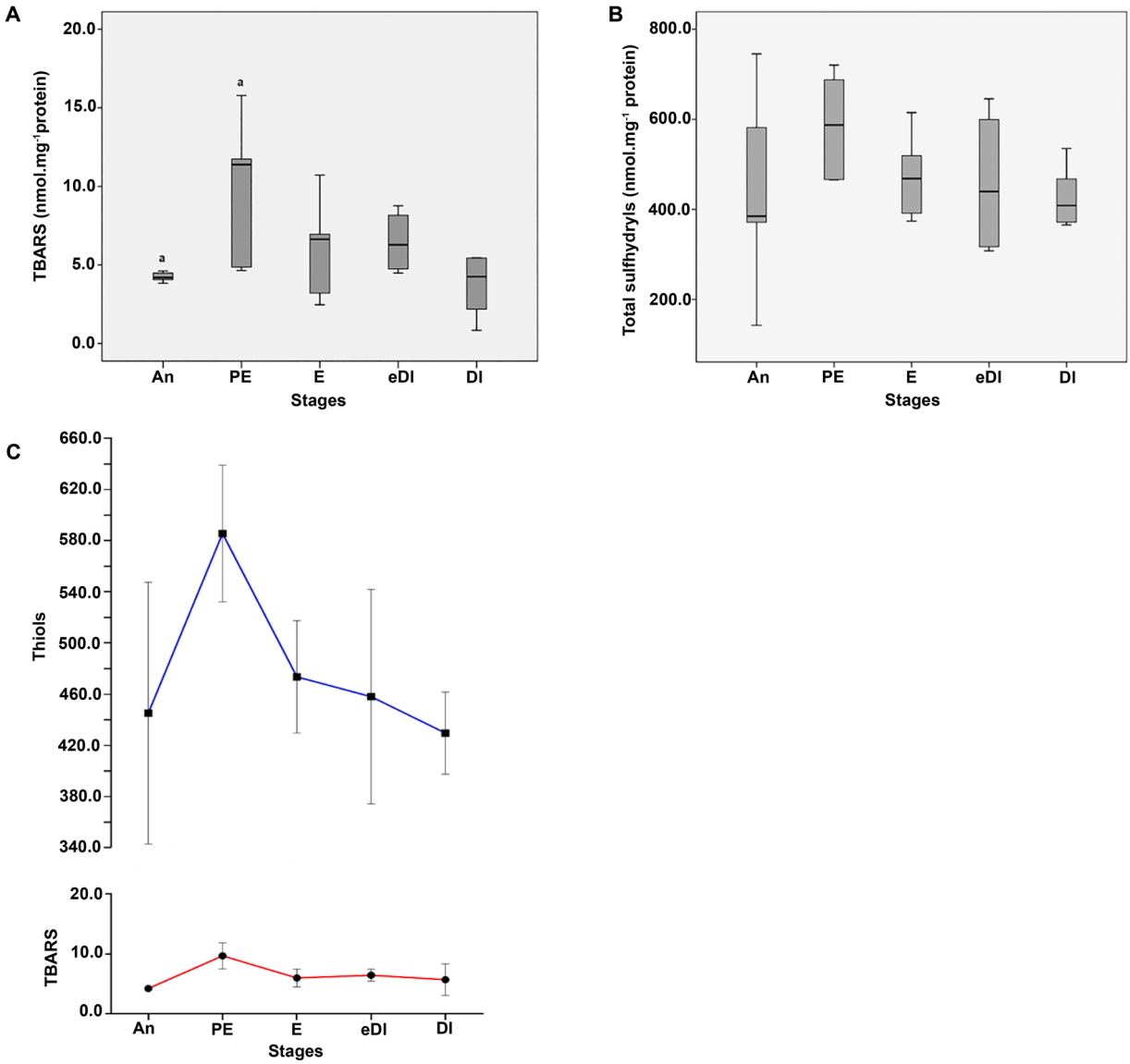

Fig. 3. Endometrial lipid and protein peroxidation levels measured from TBARS and Thiols at different stages of canine estrous cycle [anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI)]. Number of animals used per group, n = 5. **A**. TBARS levels; Significance levels are denoted by shared letters: $P < 0.05$ (a). **B.** Total sulfhydryls (-SH) (Thiols) levels. **C**. Time series representation of lipid (TBARS) and protein peroxidation (Thiols) across stages of the canine estrous cycle.

corresponding p-values from 0.718 (GR) to 0.117 (SOD). After confirming these assumptions, data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. Differences between groups were considered statistically significant at $p < 0.05$.

3. Results

3.1. Activity of oxidative stress enzymes

Antioxidant enzyme activities in canine endometrial homogenates were measured using spectrophotometric analysis of substrates or derived products (SOD, GPX, GSR, GST) and polarographic analysis of reaction products (CAT). Table 2 presents the activities of individual antioxidant enzymes, total cellular thiols and TBARS.

The estrous cycle stage significantly influenced the total superoxide radical-scavenging antioxidant enzyme activity ($p = 0.002$; Table 2). SOD activity was highest in anestrus and proestrus samples (Fig. 2A) compared to other cycle stages (estrus, early diestrus and diestrus). While no significant difference was found between anestrus and proestrus ($p = 0.956$), both stages showed higher activity than estrus ($p = 0.005$), early diestrus, and diestrus ($p < 0.01$). Among the remaining stages, only proestrus and estrus differed significantly ($p = 0.022$). No differences were detected among stages with elevated progesterone levels (estrus, early diestrus, and diestrus), reflecting generally decreased activity during these phases (Fig. 2A).

The activities of H_2O_2-scavenging antioxidant enzymes CAT and GPX are shown in Fig. 2B and C. Catalase activity in canine endometrium varied significantly throughout the estrous cycle ($p \leq 0.001$; Table 2). CAT activity was lowest during stages with basal progesterone levels (anestrus and proestrus), with no significant differences between these stages (Fig. 2B). During estrus, CAT activity showed a slight increase but remained statistically similar to anestrus, proestrus, and early diestrus levels. A significant increase in CAT activity occurred during early diestrus ($p < 0.05$ compared to anestrus or proestrus) and remained elevated through diestrus ($p < 0.001$ versus anestrus/proestrus; $p < 0.01$ versus estrus). In contrast, endometrial GPX activity remained stable across all estrous cycle stages ($p = 0.409$; Table 2), despite minor numerical variations between stages (Fig. 2C).

GSR activity remained relatively stable throughout the canine estrous cycle ($p = 0.110$; Table 2; Fig. 2D), though higher activity was observed during stages with elevated progesterone levels compared to anestrus and proestrus ($p = 0.038$).

GST activity showed significant variation across the estrous cycle ($p = 0.010$), with lowest levels during anestrus and proestrus compared to other cycle stages (Fig. 2E). The most pronounced increases in GST activity occurred during estrus and early diestrus, coinciding with rising progesterone levels (Fig. 2E). Significant differences were found between estrus and both anestrus ($p = 0.037$) and proestrus ($p = 0.044$). Early diestrus showed a trend toward higher GST activity compared to anestrus and proestrus ($p = 0.062$ and $p = 0.074$, respectively).

The time series graph in Fig. 2.F summarizes the trends in antioxidant enzyme activities across different stages of the canine estrous cycle, enabling a holistic comparison of the effects of sex steroid hormones (estrogen dominance vs. progesterone dominance vs. absence of hormonal influence).

The TBARS content in endometrial homogenates, which reflects ROS-induced lipid damage, showed no overall dependence on the canine estrous cycle stage ($P = 0.298$; Table 2), as illustrated in Fig. 3.A. However, TBARS levels were significantly higher during proestrus compared to anestrus ($P = 0.040$; Fig. 3.A), with no significant differences observed among other cycle stages.

The stage of the cycle did not significantly affect total sulfhydryl (-SH) levels in the canine endometrium ($P = 0.491$; Table 2), as shown in Fig. 3.B. Although proestrus showed slightly higher values compared to other cycle stages, these differences were not statistically significant.

Fig. 3C presents a time series graph showing trends in protein and lipid peroxidation across different stages of canine estrus. This visualization helps elucidate the effects of sex steroids (comparing periods of estrogen dominance, progesterone dominance, and

Table 3
Ratios of the activity of oxidative stress enzymes in the endometrium of female dogs throughout the estrous cycle. Data provided as Mean ± SEM. Number of animals per group, n = 5.

Ratios	AN	PE	E	eDI	DI	*P* value
$\frac{SOD}{(CAT + GPX)}$	0.56 ± 0.05	0.51 ± 0.08	0.44 ± 0.06	0.51 ± 0.09	0.41 ± 0.06	0.610
$\frac{GPX}{GSR}$	26.64 ± 7.15^{a,b}	33.24 ± 8.00^{b}	13.87 ± 2.47^{a,b}	8.41 ± 2.00^{a}	12.08 ± 2.94^{a,b}	**0.019**
$\frac{GPX}{CAT}$	55.02 ± 7.20^{b}	61.89 ± 14.17^{b}	19.30 ± 4.39^{a}	11.96 ± 5.82^{a}	6.78 ± 1.70^{a}	**≤ 0.001**
$\frac{Thiols}{(SOD + CAT + GPX)}$	0.02 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.625
$\frac{TBARS}{(SOD + CAT + GPX)}$	1.73 ± 0.41	2.19 ± 0.23	2.14 ± 0.14	2.15 ± 0.34	1.79 ± 0.17	0.249
$\frac{(TBARS + Thiols)}{(SOD + CAT + GPX)}$	1.74 ± 0.41	2.22 ± 0.23	2.17 ± 0.14	2.18 ± 0.34	1.81 ± 0.18	0.597

Stages of the cycle: anestrus (A), proestrus (PE), estrus (E), early diestrus (eDI) and diestrus (DI). SOD – Superoxide dismutase; CAT – Catalase; GPX – Glutathione Peroxidase; GSR – Glutathione reductase; GST – Glutathione-S-transferase; Thiols – Total sulfhydryls (-SH). Different subscripts within a row indicate statistical differences at $p < 0.05$.

minimal hormonal influence).

3.2. Oxidative stress ratios

Analysis of the total antioxidant enzymatic protection system in the canine uterus, measured through the SOD/(CAT+GPX) ratio, revealed minor, non-significant variations across estrous cycle stages (Table 3). However, peroxide-related enzyme ratios showed significant differences, with GPX/GSR ($p = 0.019$) and GPX/CAT ($p \leq 0.001$) varying notably across stages.

The GPX/CAT ratio was elevated in anestrus and proestrus compared to other stages (Table 3; Fig. 4.A), following a pattern similar to SOD activities (Fig. 2.A). This ratio reached its minimum in early diestrus and peaked in proestrus. The decreased values observed during early diestrus and diestrus likely resulted from increased CAT activity, as supported by individual CAT activity measurements (Fig. 2.B). The GPX/CAT ratio showed no significant differences among the remaining cycle stages.

The GPX/GSR ratio (Fig. 4.B) also exhibited a pattern similar to SOD activity, with significantly higher values in proestrus and anestrus compared to other cycle stages (Table 3). This ratio decreased during estrus and reached its lowest points in early diestrus and diestrus. These changes reflected stable GPX activity (Fig. 2.C), decreased GPX/CAT ratio (Table 3; Fig. 4.A), and a non-significant increase in GSR activity (Fig. 2.D).

The ratios used to assess oxidative stress state, namely $\frac{\text{Thiols}}{(\text{SOD}+\text{CAT}+\text{GPX})}$; $\frac{\text{TBARS}}{(\text{SOD}+\text{CAT}+\text{GPX})}$; $\frac{(\text{TBARS}+\text{Thiols})}{(\text{SOD}+\text{CAT}+\text{GPX})}$, showed minimal, non-significant variations across the canine estrous cycle stages (Table 3), indicating an effective enzymatic antioxidant protection and the existence of stable, non-oxidative stress conditions in the canine endometrium, in all the stages of the estrous cycle.

4. Discussion

ROS play an essential role in endometrial cell physiology and metabolic regulation when present at low concentrations (Sugino, 2007; Al-Gubory et al., 2010). Cellular systems maintain these levels within well-defined limits through antioxidant defense systems, which include intrinsic antioxidant compounds and enzymes that eliminate excess reactive species. These enzymatic antioxidants catalyze reactions converting ROS (such as superoxide radical and hydrogen peroxide) into water, preventing the formation of the more damaging hydroxyl radical that can harm chemical and cellular structures.

The results presented herein demonstrated that total glutathione-dependent enzyme activity in the endometrium remained relatively stable throughout the estrous cycle. However, anestrus and proestrus stages showed increased lipid peroxidation and SOD total activity, coupled with decreased CAT enzyme activity. Superoxide radical degradation occurs through two forms of superoxide dismutase: cytoplasmatic (SOD 1) and mitochondrial (SOD 2), with SOD activity frequently associated with mammalian reproduction (Fujii et al., 2005). The highest SOD activity was observed during anestrus, possibly because low sex steroid levels during this stage weaken the antioxidant protective role typically exerted by these hormones in the canine endometrium, leading to compensation through the SOD scavenging pathway (Pajović and Saicić, 2008).

Anestrus in dogs represents a period of intense endometrial remodeling, characterized by increased apoptosis in late diestrus and anestrus (Van Cruchten et al., 2003). The combination of lower CAT activity and increased SOD activity during anestrus and proestrus likely creates a local environment with higher oxidative potential, triggering compensatory survival mechanisms (Ramos et al., 2015). While low-level lipid peroxidation may favour adaptive stress responses in antioxidant enzymatic systems (Ayala et al., 2014), elevated SOD activity combined with reduced CAT activity might increase free hydroxyl radical levels, potentially enhancing lipid peroxidation (Ramos et al., 2015).

Unlike humans, where SOD activity increases from proliferative to mid-secretory stages (Sugino et al., 2002), the current study

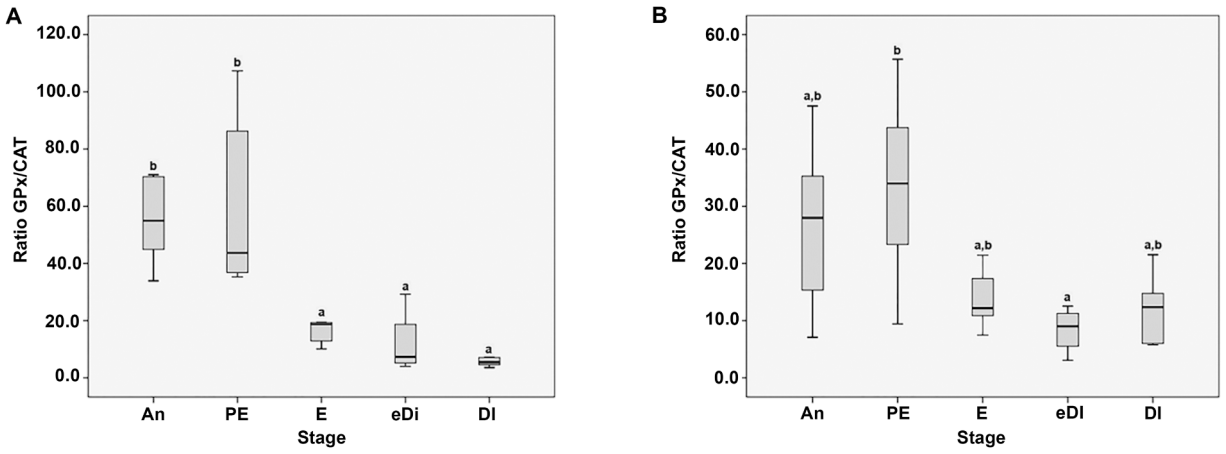

Fig. 4. Main oxidative stress ratios in the canine endometrium at different stages of canine estrous cycle. **A**. GPX/CAT ratio **B**. GPX/GSR ratio. Significance levels are denoted by shared letters: $P < 0.05$ (a); $P < 0.01$ (b).

revealed that canine SOD activity is inhibited by rising progesterone levels, which increase during estrus and remain elevated throughout early and full diestrus (Concannon, 2009). This species difference may reflect distinct physiological features, particularly regarding luteolysis. While human endometrial cells in culture show decreased SOD activity and increased ROS concentrations following estrogen and progesterone withdrawal in vitro (Sugino et al., 2004), canine luteolysis remains poorly understood, with PGF2α maintaining low values during late diestrus even as peripheral progesterone levels decline below 1–2 $ng.mL^{-1}$ (Luz et al., 2006). These differences in SOD activity pattern may reflect species-speciphic differences in reproductive physiology.

Knowledge about redox status and oxidative stress in the canine endometrium and their hormonal modulation remains limited. Studies in other species have shown various patterns: sheep exhibit upregulated endometrial SOD and GPX activity during implantation (Al-Gubory et al., 2010), a survival response deem crucial to prevent oxidative insult in early pregnancy (Al-Gubory et al., 2014) while also showing decreased SOD1 activity in the endometrium under physiological levels of estrogen and progesterone (Al-Gubory et al., 2008). Pigs demonstrate estrogen-mediated antioxidant activity through H_2O_2 synthesis inhibition in luteal and follicular cells (Murdoch, 1998), and mice show estrogen-decreased total SOD activity associated with increased endometrial cell membrane fluidity (Jain et al., 1999), which favours the fusion of trophectoderm and endometrial cells, with increased lipid peroxidation decreasing during SOD activity (Laloraya, 1990). Similarly, data from the present study showed a decrease in SOD activity from proestrus to estrus in dogs, where estrogen rises during proestrus, reaching a peak at the beginning of the estrus phase when progesterone levels start to increase in correspondence with the pre-ovulatory LH surge (Concannon, 2009; 2011).

An apparent discrepancy was observed between high catalase levels and decreased SOD activity, particularly during early diestrus and diestrus, suggesting an alternative pathway in H_2O_2 synthesis, as previously hypothesized (Gupta et al., 2011; Curieses Andrés et al., 2022). The increased SOD activity coupled with decreased CAT activity during anestrus likely promotes H_2O_2 production, potentially supporting endometrial cell regeneration through ROS-mediated mechanisms involving mitochondrial dysfunction, membrane damage, and cytochrome C release-induced apoptosis (Kruman et al., 1997).

Catalase activity in the endometrium may influence both proliferation and apoptosis pathways. In other tissues, CAT increases abort cell proliferation (Yano and Yano, 2002), and protect tumor cells from apoptosis in a hydrogen peroxide-mediated mechanism (Bechtel and Bauer, 2009). The increased CAT activity observed during diestrus may contribute to reduced endometrial proliferation, contrasting with peak proliferation during proestrus and early estrus (Van Cruchten et al., 2004). A similar role has been attributed to GST (Singh, 2015). The concurrent increase in GST activity during estrus, early diestrus, and diestrus suggests a supportive role alongside CAT, possibly linked to decreased glutathione-dependent activity on oxidative products-

The lower GPX/GSR ratio during progesterone-dominated stages suggests a more effective antioxidant response, with sufficient GSH regeneration capacity to support GPX's protective activities. Both GST and GPX regulate lipid and organic hydroperoxide concentrations, influencing cell proliferation, survival, and apoptosis (Brigelius-Flohe and Maiorino, 2013). Although best known for their GSH-dependent ability to conjugate or remove peroxides, GST can also bind nonsubstrate kinases in MAPK (*Mitogen-Activated Protein Kinase*) pathways, which regulate cell proliferation and apoptosis (Laborde, 2010). The increased activity during high-progesterone stages may reflect involvement in endometrial cell turnover regulation (Rider, 2002).

The lower GPX/CAT ratio in early diestrus and diestrus hints at a more prominent role for CAT in detoxifying hydrogen peroxide and counteracting ROS's damaging effects. The relatively unchanged ratios combining lipid and protein peroxidation markers with the collaborative enzyme shield of SOD, CAT, and GPX support this interpretation. Prolonged oxidative stress can damage cell proteins and lipids, potentially contributing to various health issues and diseases (Torres et al., 2011).

The SOD/(CAT+GPX) ratio evaluates the local scavenging enzymes' ability to prevent H_2O_2 accumulation—a highly reactive species originating from superoxide anion dismutation by SOD—through subsequent removal by CAT and GPX, which convert it into water and oxygen. Despite slight fluctuations across the canine estrous cycle, this ratio remains relatively stable, reflecting the enzymatic shield's efficiency in maintaining or restoring a healthy redox state in the canine endometrium (Dasgupta and Klein, 2014; Baba and Bhatnagar, 2018).

Individual antioxidant enzymes can have varied outcomes in maintaining tissue balance, with each enzyme playing a unique role across different cell populations. In the endometrium, stromal and epithelial cells mutually influence each other's functions. Data presented herein suggests that in the dog's endometrium, oxidative stress serves a regulatory role without causing tissue harm. This conclusion is supported by the absence of lipid or protein oxidation compounds, as indicated by thiol and TBARS activity patterns throughout the estrous cycle. The ratios used in this study indicate that under normal conditions, the canine endometrium maintains oxidative stress equilibrium. These findings align with existing knowledge about healthy uteri (Acar et al., 2020) and provide valuable insights into antioxidative stress enzyme activities, TBARS, and Thiols that sustain ROS equilibrium in non-diseased canine endometrium.

Recent research reveals a higher oxidative stress index in subclinical cystic endometrial hyperplasia (CEH) in dogs, characterized by increased total oxidant status and decreased total antioxidant status compared to mid-diestrus and 30-day pregnant samples (Kurt et al., 2021). CEH, particularly when associated with pyometra (CEH-P), depletes uterine antioxidants like superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH).

Chronic oxidative stress conditions may compromise uterine immune defenses, increasing the endometrium's susceptibility to bacterial colonization and infection. This disruption further exacerbates the antioxidant-prooxidant balance and weakens uterine defense mechanisms against pathogens and inflammation (Rautela and Katiyar, 2019). Studies have consistently demonstrated significant reductions in antioxidant enzymes (SOD, CAT, GPx, and GSH) in canine pyometra (Toydemir Karabulut, 2018; Kumar et al., 2024). These enzymatic changes impair ROS scavenging, resulting in free radical overproduction and increased lipid peroxidation (Kumar et al., 2024), which further damages uterine tissue.

Investigating the molecular mechanisms regulating redox balance in the canine endometrium during the estrous cycle can elucidate

peri-implantation processes, infertility mechanisms, and age-related endometrial degenerative changes. This research provides valuable information to support the development of new treatment and prevention strategies. Furthermore, these findings about canine endometrial redox balance may offer broader understanding of similar mechanisms across humans and other mammalian species.

The limited sample size (n = 5 per group) may represent a key limitation of this study, suggesting the need for future research with larger cohorts to further validate and generalize these oxidative stress parameter findings.

5. Conclusions

This study provides the first comprehensive data on antioxidant status and lipid and thiol group compound oxidation in healthy canine endometrium. The research demonstrates that the cyclic canine endometrium maintains an efficient oxidative stress control mechanism, preventing lipid and protein damage from reactive oxygen species. Moreover, cyclic fluctuations in antioxidant enzymes, particularly SOD and catalase, reflect the unique physiological regulatory mechanisms driven by sex steroid variations.

CRediT authorship contribution statement

R. Payan-Carreira: Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **C. Santos**: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **D. Santos**: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2025.107819.

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