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Original article

Evaluation of uterine antioxidants in bitches suffering from cystic endometrial hyperplasia-pyometra complex

A. Kumar[1], J.K. Prasad[2], S. Verma[3], A. Kumar[4], A. Gattani[5], G.D. Singh[6], V.K. Singh[6]

[1] Department of Veterinary Gynaecology and Obstetrics, Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar 800014, India
[2] Dean, Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar 800014, India
[3] Department of Veterinary Medicine, College of Veterinary Science and Animal Husbandry, Deen-dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-anusandhan Sansthan (DUVASU), Mathura, U.P. 281001 India
[4] Department of Veterinary Biochemistry, Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar 800014, India
[5] Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur, M.P., 483220 India
[6] Veterinary Clinical Complex, Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar 800014, India

Correspondence to: S. Verma. e-mail: vetsonika@gmail.com

Abstract

Cystic endometrial hyperplasia-pyometra complex (CEH-P) is a common disease in sexually mature bitches. Disease progression leads to oxidative stress, resulting in the depletion of uterine antioxidants and lipid peroxidation of associated cells, which further aggravates the condition. The concentration of antioxidant enzymes, the level of lipid peroxidation within the uterine tissue, and its reflection in the serum and urine need to be elucidated. The aim of this study was to analyze the concentration of antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx), and the lipid peroxidation marker malonaldehyde (MDA) in three types of samples, i.e., serum, urine, and uterine tissue. For this purpose, 58 pyometra-affected and 44 healthy bitches were included in the present study. All animals underwent ovariohysterectomy (OVH). Our data indicated highly significant difference ($p<0.01$) in the antioxidant concentrations of uterine, serum and urine samples. Furthermore, there was a highly significant ($p<0.01$) difference in the serum levels of ferric reducing antioxidant power (FRAP) and free radical scavenging activity (FRSA) indicated poor capacity to overcome oxidative stress in the CEH-Pyometra condition. We showed that CEH-P induces oxidative stress, which further depletes the antioxidant enzyme reserves in the uterus. Thus, the weak antioxidant defence predisposes to uterine damage and disease progression. The simultaneous depletion of antioxidants and an increase in lipid peroxidation in the serum and urine may also act as early indicators of uterine pathology.

Keywords: antioxidants, oxidative stress, ovariohysterectomy, pyometra

Introduction

The cystic endometrial hyperplasia-pyometra complex (CEH-P) is one of the most frequently occurring uterine disorders in sexually mature bitches, leading to life-threatening conditions (Egenvall et al. 2001, Sasidharan et al. 2023). CEH-P is a hormonally mediated uterine pathology that starts with uterine wall thickening, followed by further inflammatory conditions associated with bacterial invasion and subsequent deposition of a semisolid pus-like substance within the uterine lumen (Kumar and Saxena 2018). The damaging effect at the initial stage of the disease is localized in the uterus; however, as the condition becomes chronic, it includes systemic illness along with damaging effects on several vital organs (Prasad et al. 2017). The most common treatment of choice for canine pyometra with systemic illness is ovariohysterectomy (Jitpean et al. 2014). CEH-P is a multifactorial disease; however, oxidative stress is the most important predisposing factor to its occurrence (Al-Gubory et al. 2010). Oxidative stress is a consequence of excessive free-radical production. Free radicals are classified into two groups: reactive oxygen species (ROS) and reactive nitrogen species (RNS), both of which are often referred to as RONS (Agarwal et al. 2005). Excessive ROS production has been associated with various reproductive disorders in female dogs. The overproduction of free radicals leads to oxidative stress, which causes a surge in ROS production (Agarwal et al. 2005, Todorova et al. 2005). A basic level of ROS is required for various physiological processes occurring in cells and tissues, such as proliferation, maturation, and programmed cell death (Todorova et al. 2005). The balance between free radicals and antioxidants is essential for cellular homeostasis (Al-Gubory et al. 2010). The produced ROS in tissue is kept to the normal physiological level by a highly complicated system that includes the neutralization of free radicals using non-enzymatic (vitamin C, E, and microminerals, i.e., Zn, Cu) and enzymatic antioxidants (SOD, CAT, GPx, etc.) (Ercan and Fidanci 2012). Excessive ROS produced during oxidative stress rapidly depletes serum and tissue antioxidants, further aggravating pathological conditions. Excess ROS binds with the unsaturated fatty acid moiety of the lipid bilayer of the cell membrane, leading to lipid peroxidation (Al-Gubory et al. 2010). In the case of CEH-P, uterine tissue damage becomes a favorable site for colonization by pathogens, causing further damage to the endometrial tissue (Ďuračková 2010). It is now well established that oxidants and antioxidant equilibrium are key to the healthy environment within the uterus; thus, antioxidant potential is directly associated with the reproductive well-being of an animal (Halliwell 2006).

The present study evaluated and compared the antioxidative capacity of the uterus along with its reflection in serum and other body secretions, i.e., serum and urine, among the bitches suffering from pyometra and healthy bitches at the diestrus stage. The study hypothesizes that oxidative stress and depletion of the antioxidant defense system in uterine tissue succumb the animal to uterine pathological conditions, i.e., CEH-P.

Materials and Methods

Animals and sample collection

This study was conducted with the approval and guidelines of the Institutional Animal Ethics Committee of the Bihar Animal Sciences University (IAEC/BVC/2023/01). The present study was conducted on 58 CEH-P-affected and 44 healthy diestrus bitches of different breeds like Labrador Retriever, German Shepherd, Golden Retriever, and Rottweiler, weighting 30-45 kg in the 4-7-year age group (Table 1). Each animal in the study underwent a standard clinical evaluation protocol and was diagnosed on the basis of an ultrasonographic evaluation followed by vaginal cytology. Healthy bitches brought to clinics for spaying showed the presence of parabasal cells on vaginal cytology. A preliminary diagnosis of CEH-P was made based on history, clinical signs, physical examination, and abdominal palpation, along with haematological (Nihon Kohden Hematology Analyzer) and blood biochemical (Biochem Analyzer SS-168 Vet) evaluations. A confirmatory diagnosis of CEH-P was made based on the presence of hypoechogenic content in the uterus upon transabdominal ultrasound examination (SonoScape S12 Vet). During the clinical examination of bitches, the presence of a small volume of pus-like semisolid substance was also observed in some cases of open-type pyometra. Healthy bitches brought for spaying were categorized as group C, and those diagnosed with CEH-P were categorized as group P. The diestrus phase of the estrus cycle in both groups were confirmed based on vaginal cytology, indicating an abundance of parabasal cells, and a serum progesterone assay.

Three types of samples were collected: blood/serum, urine, and uterine tissue. All samples were obtained using standard veterinary procedures, with prior permission from pet owners. Blood and urine samples from both groups were collected on the day of diagnosis. Approximately 3 ml of whole blood was collected by puncturing the cephalic vein. One ml of whole blood was then placed in an ethylene diamine tetra-acetic acid (EDTA) coated vial for haematological

Table 1. Detailed information of all the dogs (bitches) underwent ovariohysterectomy (OVH) having physiological and cystic endometrial hyperplasia-pyometra complex (CEH-P) uterine condition.

SI. No.	Breed	Age	Body weight
1	Labrador Retriever	6 yrs	41 kg
2	Rottweiler	4 yr 3 months	36 kg
3	Golden Retriever	5 yr.7 months	37.5 kg
4	Rottweiler	6 yr 9 months	44 kg
5	German Shepherd	6 yr 2 months	42 kg
6	Labrador Retriever	5 yr 8 months	39 kg
7	German Shepherd	6 yr2 months	38 kg
8	Labrador Retriever	4 yr 6 months	32.5 kg
9	Labrador Retriever	5 yr 3 months	36 kg
10	Golden Retriever	6 yr 6 months	42 kg
11	German Shepherd	5 yrs	39 kg
12	Golden Retriever	5 yr 3 month	40 kg
13	Labrador Retriever	5 yr 5 month	38.5 kg
14	Labrador Retriever	6 yr 5 month	42 kg
15	Labrador Retriever	6 yrs	38 kg
16	German Shepherd	4 yr 6 month	36 kg
17	German Shepherd	5 yr 2 month	34 kg
18	Rottweiler	4 yr 8 month	37 kg
19	Labrador Retriever	5 yrs	32 kg
20	Rottweiler	4 yr 6 month	38 kg
21	Golden Retriever	6 yr 9 month	42 kg
22	German Shepherd	7 yrs	44.5 kg
23	Golden Retriever	6 yr 2 month	38 kg
24	Labrador Retriever	5 yr 9 month	37 kg
25	German Shepherd	6 yr 8 month	41.5 kg
26	Labrador Retriever	4 yr 3 month	33 kg
27	Labrador Retriever	5 yr 2 month	38 kg
28	Labrador Retriever	6 yr 7 month	42 kg
29	Rottweiler	5 yr 1 month	37 kg
30	Labrador Retriever	6 yr 8 month	44 kg
31	Labrador Retriever	4 yr 5 month	34 kg
32	German Shepherd	5 yrs	36 kg
33	Rottweiler	6 yr 2 month	40 kg
34	German Shepherd	6 yrs	38 kg
35	Labrador Retriever	5 yr 4 month	34 kg
36	German Shepherd	6 yr 11 month	43 kg
37	Rottweiler	4 yr 7 month	34 kg
38	Rottweiler	5 yr 2 month	36 kg
39	Labrador Retriever	4 yr 9 month	33 kg
40	Golden Retriever	6 yr 4 month	38 kg
41	Labrador Retriever	4 yr 8 month	34 kg
42	Rottweiler	5 yr 9 month	39 kg
43	Golden Retriever	5 yr 8 month	35 kg
44	Rottweiler	4 yr 6 month	34 kg
45	German Shepherd	4 yr 3 month	35.5 kg
46	German Shepherd	6 yr 8 month	41 kg
47	Golden Retriever	6 yr 2 month	39 kg
48	Golden Retriever	5 yr 8 month	35 kg
49	Labrador Retriever	5 yr 3 month	37 kg
50	German Shepherd	4 yr 6 month	34 kg
51	German Shepherd	6 yr 4 month	39 kg

cont. Table 1.

SI. No.	Breed	Age	Body weight
52	Labrador Retriever	6 yr 1 month	37 kg
53	German Shepherd	6 yr 3 month	39 kg
54	Golden Retriever	6 yr 8 month	42 kg
55	German Shepherd	5 yr 7 month	36 kg
56	Labrador Retriever	4 yr 3 month	31 kg
57	German Shepherd	4 yr 4 month	35 kg
58	Rottweiler	5 yr 6 month	39 kg
59	Golden Retriever	5 yr 6 month	36 kg
60	German Shepherd	5 yr 2 month	35 kg
61	German Shepherd	6 yr 8 month	42 kg
62	Labrador Retriever	6 yr 3 month	36 kg
63	Labrador Retriever	7 yr	44 kg
64	Rottweiler	6 yr 4 month	37 kg
65	Golden Retriever	6 yr 8 month	39 kg
66	Golden Retriever	6 yr 9 month	38 kg
67	German Shepherd	4 yr 6 month	34 kg
68	Labrador Retriever	5 yr 2 month	38 kg
69	Rottweiler	4 yr 4 month	35 kg
70	German Shepherd	5 yr 10 month	39.5 kg
71	German Shepherd	6 yr 7 month	43 kg
72	German Shepherd	6 yr 2 month	38 kg
73	German Shepherd	4 yr 7 month	34 kg
74	Golden Retriever	4 yr 2 month	32 kg
75	Labrador Retriever	4 yr 3 month	36 kg
76	Golden Retriever	6 yr 11 month	42 kg
77	Labrador Retriever	6 yr 9 month	39 kg
78	German Shepherd	6 yr 7 month	41 kg
79	Rottweiler	6 yr 2 month	36 kg
80	German Shepherd	4 yrs	32 kg
81	Labrador Retriever	6 yr 8 month	40 kg
82	Labrador Retriever	6 yr 6 month	37 kg
83	Golden Retriever	6 yr 10 month	44.5 kg
84	Rottweiler	6 yr 8 month	42 kg
85	Labrador Retriever	6 yr 2 month	35.5 kg
86	Golden Retriever	6 yr 9 month	42 kg
87	German Shepherd	6 yr 1 month	37 kg
88	Golden Retriever	5 yr 8 month	36 kg
89	Labrador Retriever	6 yr 11 month	43 kg
90	Golden Retriever	6 yr 4 month	41 kg
91	German Shepherd	4 yr 2 month	36 kg
92	Labrador Retriever	4 yr 2 month	33.5 kg
93	German Shepherd	6 yr 1 month	42 kg
94	German Shepherd	5 yr 6 month	38 kg
95	German Shepherd	6 yr 8 month	42 kg
96	Labrador Retriever	6 yr 2 month	39 kg
97	Golden Retriever	4 yr 3 month	32.5 kg
98	German Shepherd	5 yr 4 month	36 kg
99	Golden Retriever	6 yr 3 month	41 kg
100	Rottweiler	4 yr 9 month	33.5 kg
101	German Shepherd	5 yr 7 month	37.5 kg
102	Labrador Retriever	5 yr 9 month	38 kg

analysis. The remaining sample was stored in a plain vial and left for 15 minutes at room temperature, followed by centrifugation for 10 minutes at 2000 g. The supernatant was used for biochemical evaluation, and the remaining samples were stored at -20°C for hormonal and antioxidant evaluation. Urine was collected by per-vaginal catheterization using a Ryle's tube using proper aseptic precautions and stored at -20°C for antioxidant analysis. Ovariohysterectomy was carried out as per the standard surgical procedure followed at our clinic. Briefly, a combination of atropine sulphate (0.4 mg/kg) and butorphanol (0.35 mg/kg) was administered as pre-anaesthetic medication. After 15 minutes, induction of anaesthesia was performed with a combination of tiletamine and zolazepam (1:1, w/w ratio) at the rate of 4 mg/kg body weight, followed by maintenance with the above combination at the rate of 7.5 mg/kg. Uterine samples were collected in a normal saline solution and placed in the laboratory in a styrofoam box filled with ice. Longitudinal incisions were made to evaluate macroscopic findings such as endometrial thickening and the presence of pus or pus-like substances. Similarly, uterine samples intended for assessing antioxidant levels were also collected by excising a 1 cm^2 area at four different parts of the uterus (two from the body of the uterus and one each from the uterine horns), followed by thorough rinsing with normal saline to remove traces of blood or pus in order to avoid any effect on antioxidant levels. These parts were stored in an Eppendorf tube at -80°C until further processing.

Homogenization and sample processing

Uterine samples intended for antioxidant analysis were rinsed thoroughly with phosphate-buffered saline (pH 7.4), followed by homogenization. Homogenates were then centrifuged at 4°C for 30 min at 3000 g, and the supernatants were analysed for TBARS, SOD, CAT, and GPx. Similarly, serum and urine samples were analysed for MDA, SOD, GSH, CAT, GPx, LDH, FRAP, ABTS radical scavenging activity, and FRSA (in serum), as well as urea, GGT, and creatinine (in urine) according to the standard protocol followed in our lab along with additional methodologies described by Karabulut (2018), with slight modifications.

Histo-pathological evaluation of uterus

Histopathological evaluation was conducted by tissue sectioning, followed by haematoxylin and eosin staining, as described by Woźna-Wysocka et al (2021).

Statistical analysis

The results obtained from both groups (C and P) were subjected to statistical analysis. An independent sample t-test was calculated using SPSS version 20 (IBM Corporation). Results were presented with the mean, standard deviation of the mean (mean ± SD), p value, as well as lower and upper values of the 95% confidence interval (CI). The confidence interval (CI) set at 95% was calculated based on the difference of means between the C and P groups. Results with a value of $p<0.05$ were used to indicate statistically significant, and $p<0.01$ values were treated as highly significant, and $p>0.05$ was calculated as non-significant (NS).

Results

Histopathological evaluation of the samples from group C revealed no degenerative changes in the endometrium, and the findings were suggestive of a typical diestrus stage of the estrous cycle (Fig. 1). The P group showed cystic endometrial hyperplasia along with flattened epithelium of the endometrial gland at several locations in the endometrium. Almost all the samples in this group showed proliferative endometrium with signs of acute to chronic inflammation. Furthermore, multiple samples also showed signs of endometrial hemorrhage (Fig. 2)

The serum biochemical evaluation conducted on both groups i.e., C and P, shown in Table 2, indicated that creatinine, blood urea nitrogen, and serum glutamic pyruvic transaminase (SGPT), blood glucose, albumin and phosphorus values differed highly significantly ($p<0.01$). Similarly, hematological evaluations shown in Table 3 indicated highly significant difference ($p<0.01$) in TEC, TLC, hemoglobin, PCV, neutrophils and lymphocytes. Highly significant differences ($p<0.01$) between two groups were observed in the serum antioxidant capacity parameters, as indicated in Table 4. The antioxidant concentration in uterine tissue, as indicated in Table 5, showed highly significant difference ($p<0.01$) in SOD, CAT, GSH, GPx, and TBARS (MDA estimation) concentrations between both groups. Urine samples were also evaluated for the presence of urea, creatinine, and GGT, and all values differed highly significantly ($p<0.01$), as indicated in Table 6. However, non-significant (>0.05) differences were observed in serum SGOT, total protein, calcium and siodium levels.

Discussion

The present work evaluated the antioxidant status in the blood, uterus, and urine of healthy and CEH-Pyo-

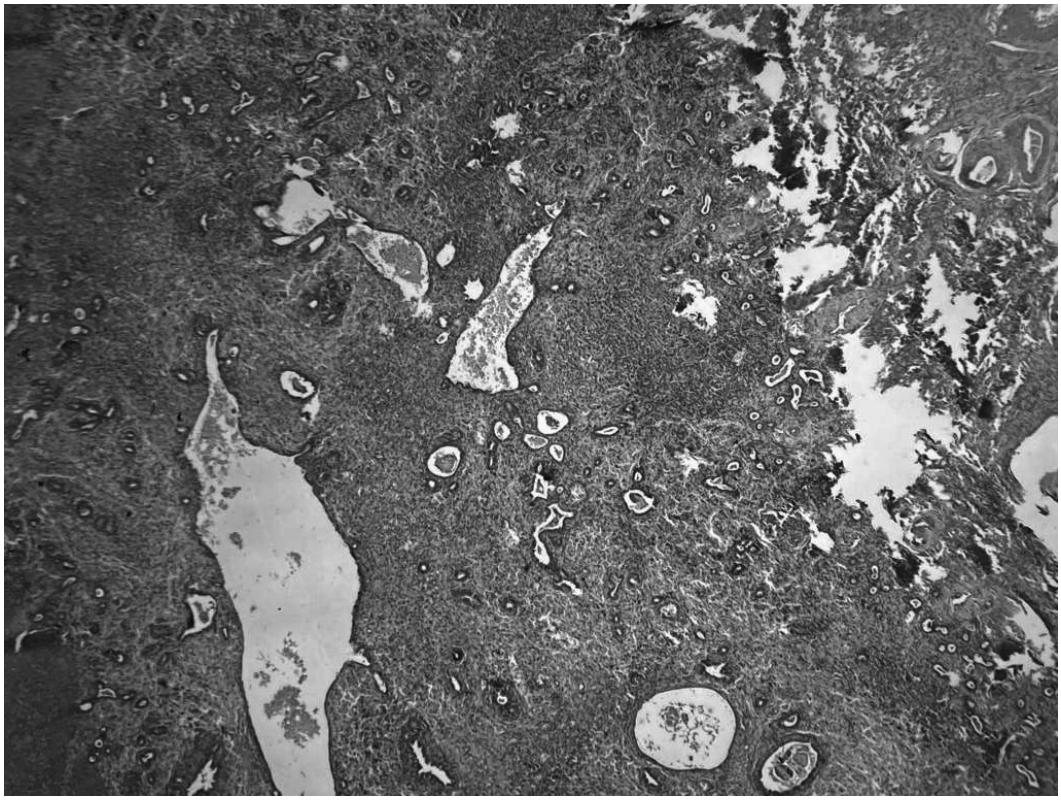

Fig. 1. Representative photograph showing the physiological bitch endometrium during the diestrus stage of the estrous cycle. Arrow indicating clear lumen surrounded by stroma with few small cystic structures. The samples of the uterus were sectioned for examination under higher magnification (×100).

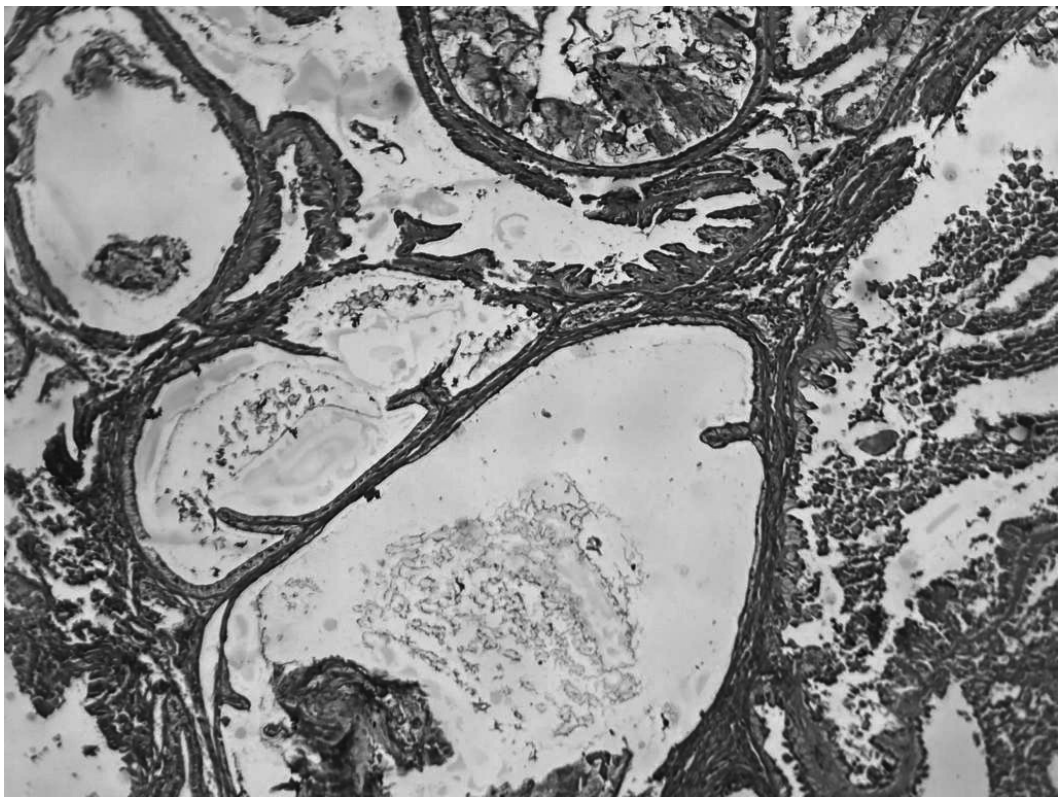

Fig 2. Representative photograph showing cystic hyperplasia (arrow) along with flattened epithelium (white arrowhead) of the endometrial gland at several locations in the bitch endometrium. The samples of the uterus were sectioned for examination under higher magnification (×100).

Table 2. Serum biochemistry (mean±SD) in bitches.

Parameters	C group (n=44)	P group (n=58)	*P* value	95% CI Lower & upper	Significance
Creatinine (mg/dl)	0.72 ± 0.10	1.61 ± 0.21	0.00	[0.956, 0.85]	**
BUN (mg/dl)	15.69 ± 2.71	74.65 ±10.95	0.00	[61.29, 56.63]	**
SGOT (IU/L)	40.99 ± 5.81	40.66 ± 5.69	0.38	[1.11, 2.01]	NS
SGPT (IU/L)	33.34 ± 7.54	21.31 ± 4.18	0.00	[10.53, 13.6]	**
ALP (U/L)	108.54 ± 8.23	100.86 ± 9.39	0.01	[1.89, 13.46]	*
Total Protein (g/dL)	8.34 ± 0.15	8.65 ± 0.23	0.54	[0.45, 0.2]	NS
Albumin (mg/dl)	2.76 ± 0.57	2.23 ± 0.44	<0.001	[0.44, 0.6]	**
Glucose (mg/dl)	79.79 ± 10.17	57.98 ± 11.64	<0.001	[17.43, 26.18]	**
Calcium (mmol/L)	9.91 ± 2.62	9.49 ± 1.53	0.158	[0.02, 0.83]	NS
Phosphorus (mg/dl)	2.46 ± 0.39	3.54 ± 0.68	<0.001	[1.3, 0.85]	**
Sodium (mEq/L)	127.59±11.43	126.79 ± 8.11	0.351	[3.3, 4.94]	NS
Potassium (mEq/L)	4.51 ± 0.56	4.87 ± 0.84	0.002	[0.7, 0.13]	**
Chloride (mEq/L)	129.35 ± 13.44	117.15 ± 10.40	<0.001	[8.02, 17.11]	**

* p<0.05; ** p<0.01, NS-p>0.05. 95% CI was calculated based on the difference of means between C and P groups.

Table 3. Hematological evaluation (mean±SD) in bitches.

Parameters	C group (n=44)	P group (n=58)	*P* value	95% CI Lower & upper	Significance
TEC (10^6/µL)	6.11 ± 0.50	4.74 ± 0.55	<0.001	[0.91, 1.82]	**
TLC (10^3/µL)	10.26± 0.71	20.94 ± 2.29	<0.001	[11.39, 9.97]	**
Haemoglobin (gm/dL)	14.25± 0.59	11.54 ± 1.07	<0.001	[2.29, 3.03]	**
PCV (%)	43.5 ± 2.50	40.8 ± 2.43	<0.001	[1.71, 4.64]	**
Neutrophils (%)	81.25± 2.29	78.8 ± 1.46	0.006	[1.63, 3.11]	**
Lymphocytes (%)	15.75± 2.02	16.8 ± 1.07	<0.001	[1.67, 0.44]	**
Monocytes (%)	1.5 ± 0.82	3.2 ± 0.74	<0.001	[1.99, 1.37]	**

* p<0.05; ** p<0.01, NS-p>0.05. 95% CI was calculated based on the difference of means between C and P groups.

Table 4. Serum concentrations of antioxidants and antioxidant capacity (mean±SD) in bitches.

Antioxidants	Parameters				
	C group (n=44)	P group (n=58)	*P* value	95% CI Lower & upper	Signifi-cance
Lipid peroxidation Malondialdehyde (MDA) (nmol/mL)	4.34±0.61	9.23±0.77	<0.001	[5.16, 4.6]	**
SOD (ng/mL)	90.16±7.21	69.66±7.01	<0.001	[17.68, 23.39]	**
GSH (mM)	0.19±0.21	0.28±0.06	0.001	[0.14, 0.03]	**
Catalase (ng/mL)	177.37±9.82	156.39±10.80	<0.001	[16.85, 25.09]	**
Glutathione peroxidase (U/L)	4.66 ±0.57	2.29±0.32	<0.001	[2.19, 2.54]	**
Lactate dehydrogenase (LDH) (U/L)	505.17±68.86	1236.92±136.33	<0.001	[775.52, 686.36]	**
Ferric reducing antioxidant power (FRAP) (µmol/L)	527.14±54.27	487.45±54.28	<0.001	[18.54, 61.6]	**
ABTS radical scavenging activity (TE)	1.76±0.46	1.46±0.52	0.002	[0.10, 0.49]	**
Free radical Scavenging activities (FRSA) (TE)	104.72±9.70	82.53±7.27	<0.001	[18.85, 25.52]	**
Progesterone (ng/ml)	11.06±1.44	11.52±1.02	0.034	[0.93, 0.03]	NS

* p<0.05; ** p<0.01, NS-p>0.05. 95% CI was calculated based on the difference of means between C and P groups.

Table 5. Antioxidant capacity of uterine tissue (mean±SD) in bitches.

Parameters	C group (n=44)	P group (n=58)	P value	95% CI Lower & upper	Significance
Lipid peroxidation (Malondialdehyde) (nmol/mL)	22.43±2.80	30.80±6.19	<0.001	[9.42, 5.3]	**
SOD (ng/mL)	3.9±0.51	1.37±0.43	<0.001	[2.35, 2.71]	**
GSH (mM)	13.0±2.20	8.81±1.11	<0.001	[1.04 , 0.92]	**
Catalase (ng/mL)	3.79±0.48	1.21±0.29	<0.001	[2.42 , 2.73]	**
Glutathione peroxidase (U/L)	5.42±0.46	3.85±0.67	<0.001	[1.33, 1.8]	**

* p<0.05; ** p<0.01, NS-p>0.05. 95% CI was calculated based on the difference of means between C and P groups.

Table 6. Evaluation of biochemical parameters in bitch urine (mean±SD).

Parameters	C group (n=44)	P group (n=58)	P value	95% CI Lower & Upper	Significance
Urea (mg/dl)	209.63± 33.27	353.93± 94.25	<0.001	[173.81, 114.77]	**
Creatinine (µmol/L)	17.38 ± 1.96	48.5 ± 4.43	<0.001	[32.57, 29.72]	**
GGT (U/L)	22.47 ± 5.99	83.82 ± 7.01	<0.001	[57.62, 52.43]	**

* p<0.05; ** p<0.01, NS-p>0.05. 95% CI was calculated based on the difference of means between C and P groups.

metra-affected dogs and it was observed that the later condition significantly compromised the antioxidant capacity. CEH-Pyometra affected dogs revealed oxidative stress-related changes in uterine tissue.

Production of ROS is a continuous process in response to basic physiological activities in animals and humans; overproduced oxidants are controlled by antioxidants present within tissues, including reproductive organs (Halliwell and Whiteman 2004, Santos et al. 2016). Excessive ROS production indicates concurrent inflammation, resulting in oxidative stress, leading to a massive release of ROS (El-Bahr and El-Deeb 2016). Oxidant scavenging enzymes in the uterus regulate the level of oxidants to a limited extent; however, higher levels of ROS quickly deplete these scavengers, which is indicated by the release of several biochemical substances which act as markers (Halliwell 2006). The present study aimed to evaluate the antioxidant status of the uterus affected by CEH-pyometra. This study evaluated the status of antioxidants and inflammatory markers in three important tissues: blood/ /serum, uterine tissue, and urine. ROS-induced damage to polyunsaturated fatty acids present within the cell membrane causes the release of MDA, which is evaluated by measuring thio-barbituric acid reactive substance levels in sample tissues. Our findings indicated that the uterine MDA value was significantly higher in group P, revealing the occurrence of lipid peroxidation in the pyometra-affected uterus, which is in agreement with the findings of Nielsen et al (1997). In contrast, Karabulut (2018), observed a non-significant increase in MDA levels in the uterine tissues of pyometra--affected bitches. Oxidant scavenging enzymes play a vital role in the regulation of ROS levels by neutralizing them. Superoxide dismutase (SOD) and catalase are two important cellular antioxidant enzymes present in cells. SOD is an antioxidant enzyme with high protective ability; therefore, it is considered as the first line of defense against the damaging effects of ROS. These enzymes, i.e., SOD and catalase, are primarily involved in controlling the production of hydrogen peroxide and oxygen radicals through the digestion of cellular and extracellular superoxide radicals, thereby inhibiting the formation of H_2O_2. Catalase rapidly catalyzes the conversion of preformed H_2O_2 into water and oxygen molecules, thus preventing damage to surrounding cells. Our biochemical findings of serum and uterine cells showed that antioxidant enzymes such as SOD and catalase were significantly ($p<0.01$) decreased in CEH-Pyometra affected dogs compared to the control, which was in agreement with the findings of Karabulut (2018). Contrary to these findings, Szczubial and Dabrowski (2009), reported non-significant differences in SOD and CAT enzyme concentrations between pyometra and control groups. The trend in the latter study may have been observed due to the estimation of these enzymes at an early stage of pathology, as the duration of inflammation considerably affects the levels of several antioxidant enzymes (Szczubiał et al. 2019). Glutathione peroxidase (GPx) is another antioxidant enzyme that removes hydrogen peroxide production and, therefore, acts as a defensive agent against lipid peroxida-

tion. The lower level of GPx in our study indicated oxidative stress in the pyometra affected group. GSH is an important antioxidant enzyme that is essential for the prevention of cellular oxidative damage. GSH rapidly neutralizes ROS, prevents DNA damage, restores cellular activity, and controls apoptotic changes caused by oxidants (Dringen 2000). Levels of GSH and GPx are related to each other, as earlier is involved in the synthesis of later enzymes. Ghezzi (2011), observed that immunocompromised animals with higher ROS production were deprived of tissue GSH. Similarly, previous studies have reported the progression of CEH-Pyometra in bitches with lower levels of GSH and GPx enzymes (Smith 2006, Jitpean et al. 2012). These studies potentiate our findings, as we observed a highly significant difference ($p<0.01$) in serum and uterine tissue concentrations of GSH and GPx between the P and C groups. The reduced level of antioxidant enzymes, namely, CAT, SOD, GPx and GSH, in the P group of our study reflects overproduction of free radicals in bitches suffering from CEH-Pyometra as compared to the C group. Valko et al. (2007), opined that overproduced free radicals rapidly deplete antioxidant enzymes, which is in agreement with our results for these enzyme levels. (Emanuelli et al. 2012, Gogoi et al. 2018). Once ROS production overpowers uterine antioxidant capacity, it initiates cellular damage through lipid peroxidation, resulting in sudden surge of leukocytes i.e., polymorpho-nuclear cells (PMN), lymphocytes, eosinophils, monocytes and macrophages (Biswas et al. 2016). PMN cells and macrophages, during their phagocytic activity, release ROS which adds to oxidative stress-induced cellular damage (Hussain et al. 2016). These findings are in accordance with our hematological findings, which reported a highly significant ($p<0.01$) increase in the proportion of PMN cells, lymphocytes and monocytes in the P group as compared to the healthy C group. Similarly, a highly significant ($p<0.01$) increase in MDA levels in the P group indicates considerable cellular damage in uterine tissues in CEH-Pyometra affected dogs. Our hematological findings as well as MDA levels in serum and uterine tissue are in relation to earlier findings by Hussain et al. (2016). A higher level of ROS leads to lipid peroxidation, causing the decomposition of the lipid moiety of the cellular architecture and exerting a significant impact on the properties of the cell membrane and the connections among cell organelles (Ghezzi 2011). Damage to the lipid moiety, specifically polyunsaturated fatty acids, results in the production of malondialdehyde (MDA), a low-molecular-weight final product from damaged cells. MDA was measured by estimating thiobarbituric acid reactive substances (TBARS). We observed a significantly high level of TBARS in the serum, indicative of lipid peroxidation. Our findings were consistent with those reported by Nielsen et al. (1997). TBARS estimation of uterine tissue revealed that lipid peroxidation of uterine tissue is associated with pyometra conditions.

Serum BUN and creatinine concentrations indicate the filtration rate of the glomerulus; however, these parameters should not be treated as markers for assessing the cellular and functional impairment of kidney tubules because of their poor specificity. The presence of enzymes like gamma-glutamyl transferase (GGT) and alkaline phosphatase in urine is considered a marker of renal tubular impairment. Proximal convoluted tubules in the kidney possess GGT, but this enzyme fails to cross the glomerular surface due to its higher molecular weight, thus concentration of urinary GGT in physiologically normal kidney remains negligible. However, elevated levels of GGT in urine indicates damage to convoluted tubules of kidney, reflecting renal impairment (Rivers et al. 1996). The present study evaluated Urea, GGT and creatinine levels in urine and all these parameters were highly significant ($p<0.01$) between the two group. Our findings were in agreement with previous works which observed enhanced level of urinary creatinine and GGT levels in dogs suffering with kidney damage (Clemo 1998). Our result of urinary GGT, creatinine and urine indicate possible impairment of renal tubular function of dogs affected with CEH-Pyometra. Although findings of our study are prominent yet inclusion of cytokines study as well as expression study of mRNA regulating antioxidant capacity of uterus, would have potentiated our work with special reference to early disease diagnosis.

Depletion of the antioxidant reserve was reflected as weakened antioxidant capacity of the uterus, which renders it more suitable for CEH-P pathogenesis.

Conclusions

In conclusion, CEH-P progression depends on several factors including ROS production. Our findings suggest that excessive ROS induces oxidative stress, which further depletes the antioxidant enzyme reserves in the uterus and serum. Weak antioxidant defences may be the major factor responsible for uterine damage through lipid peroxidation and disease progression with further damage to other vital organs including Kidney.

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