Effects of hyperbaric oxygen therapy on inflammatory factors and antioxidant status in dogs after ovariohysterectomy – a pilot study

by

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Abstract

Hyperbaric oxygen (HBO) therapy is proven to have anti-inflammatory, antioxidant and healing properties in human beings and laboratory animals. However, there are relatively few studies about the effects of HBO in companion animals. The goal of this study was to investigate the physiological effects of HBO in healthy dogs after ovariohysterectomy with two HBO treatments at 2.0 atmosphere of absolute pressure for 35 minutes. C-reactive protein, iron status, pro-inflammatory and anti-inflammatory cytokines, total oxidant status, total antioxidant status and oxidative stress index were measured before and after postoperative HBO. Hyperbaric oxygen therapy did not induce any detectable anti-inflammatory or antioxidant effects following ovariohysterectomy. No adverse effects were identified after HBO. Additional investigation using different inflammatory models is required to determine the role of HBO as an adjunctive therapy in dogs with more severe and complicated diseases, optimal therapeutic doses, and objective markers to quantify the response to HBO.

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List of abbreviations

ATA: atmosphere of absolute pressure

CRP: C-reactive protein

GM-CSF: granulocyte-macrophage colony-stimulating factor

h: hour

HBO: hyperbaric oxygen

IL: interleukin

IFN-γ: interferon γ

IP-10: interferon-γ-induced protein

KC-like: keratinocyte chemotactic-like

LMM: linear mixed models

LRS: Lactate Ringer's solution

MCP-1: monocyte chemoattractant protein 1

OHE: ovariohysterectomy

OSI: oxidative stress index

PDS: polydioxanone sutures

ROS: reactive oxygen species

SCF: stem cell factor

S. iron: serum iron

TAS: total antioxidant status

TIBC: total iron binding capacity

TOS: total oxidant status

TNF-α: tumor necrosis factor α

TSAT: transferrin saturation

UIBC: unsaturated iron-binding capacity

1. Introduction

Hyperbaric oxygen (HBO) therapy is a medical treatment modality gaining popularity in both human and veterinary medicine for its apparent acceleration of tissue healing (1, 2). During treatment, the patient breathes 100 % oxygen inside a chamber that is pressurized above normal sea level atmospheric pressure (1 atmosphere of absolute pressure [ATA]). Under these conditions the partial pressure of alveolar oxygen greatly increases from 102 mmHg on room air (21% oxygen) at 1 ATA to 1813 mmHg on 100% oxygen at 2.5 ATA leading to transient hyperoxia (3). The reported physiologic effects of HBO therapy include improved tissue oxygenation (3), short-term sublethal oxidative stress followed by a compensatory antioxidant response (4, 5, 6), modulation of inflammation and immune function (7, 8, 9), increased antimicrobial activity (10, 11), angiogenesis (12), enhanced fibroblast activation (13), upregulation of growth factors (14), vasoconstriction and reduction of vasogenic edema (15), and intravascular and tissue gas bubble reduction (16).

The cellular response to oxidative stress secondary to HBO has been principally investigated in animal models and human beings. Demonstrating the functions and clinical efficacy of adjunctive therapies such as HBO can be challenging. Limitations include, but are not limited to, the identification of specific and reliable biomarkers and validated measures of successful outcomes and the variability in individual immunologic response to treatment. Due to the lack of scientific evidence, limited indications for HBO therapy in people have been approved based on well-validated clinical experience by the Undersea and Hyperbaric Medical Society (Table 1) (17). In veterinary medicine the use of HBO is extrapolated from accepted indications in human medicine, experimental animal models, small retrospective evaluations and single case reports (18). Prospective randomized controlled clinical studies investigating the effects of HBO on inflammation, oxidation, and clinical outcome in companion animals are lacking.

The goal of this study is to investigate the physiological effects of HBO on surgically induced systemic inflammation and oxidation in dogs by comparing the expression of inflammatory biomarkers, pro-inflammatory and anti-inflammatory cytokines, total oxidant status, total antioxidant status and oxidative stress index before and after postoperative HBO. We hypothesized that HBO has anti-inflammatory and antioxidant properties in dogs undergoing ovariohysterectomy.

Table 1. List of accepted indications for hyperbaric oxygen therapy from the Undersea and Hyperbaric Medical Society (17)

Acute conditions	**Chronic conditions**
Air or gas embolism	Enhancement of healing in selected problem wounds
Carbon monoxide poisoning with or without cyanide poisoning	Osteomyelitis (refractory)
Clostridial myositis and myonecrosis (gas gangrene)	Skin grafts and flaps (compromised)
Crush injury, compartment syndrome, and other acute traumatic ischemias	
Decompression sickness	
Delayed radiation injury (soft tissue and bony necrosis)	
Exceptional blood loss (anemia)	
Intracranial abscess	
Necrotizing soft tissue infections	
Thermal burns	

2. Materials and Methods

2.1. Study population

The study was approved by the Auburn University Institutional Animal Care and Use Committee of the Department of Clinical Sciences.

Twelve 11- to 12-month-old intact female Beagle dogs were eligible for participation to the study. All dogs were up-to-date on vaccination as well as heartworm, flea and tick preventatives. They were fed the same diet [a], based on their daily energy requirement and body condition.

All dogs had been previously enrolled in a study comparing the efficacy of three different flea treatments. The dogs were appropriately treated, combed and moved in a different kennel at least three weeks prior to our study. No fleas were detected by the time of enrollment.

Each dog's health status was determined through review of medical history, physical examination, evaluation of hematological and biochemistry parameters, urine analysis with cytologic evaluation, ZnSO4/I2 fecal flotation and a SNAP®4Dx® assay [b]. A vaginal swab was also performed within the 24 hours preceding surgery to determine the stage of estrous cycle. These screening tests were performed to ensure the dogs were healthy and did not have any concomitant condition susceptible to influence the results of this study. Exclusion criteria are listed in Table 2.

Table 2. Exclusion criteria

Concurrent diseases that may influence inflammatory and antioxidant biomarkers including allergy and hypersensitivity, heartworm disease, severe intestinal parasite infections or ectoparasites associated with clinical signs
Medication other than heartworm and flea/tick preventative drugs
Recent vaccination
Pregnancy
Recent surgery < 1 month
Breaks in aseptic technique during surgery
Major post-operative complications: hemoabdomen, septic abdomen, wound dehiscence, abscess
Contraindications for HBOT: claustrophobia, pneumothorax, otitis, history of ear or thoracic surgery, dental cavities, nasal congestion

Few *Isospora (Cystoisospora) ohioensis* were detected by ZnSO4/I2 fecal flotation in one dog. While it is not unusual to see few oocysts in dogs less than 1-year-old, the clinical significance of this finding is unclear. All dogs were treated with oral ponazuril [c] (50mg/kg twice, 24 hours apart) prior to the study.

One dog was diagnosed with bacterial pododermatitis and was successfully treated with two subcutaneous injections of cefovecin [d] (8 mg/kg twice, 15 days apart) and topical therapy with 2% chlorhexidine twice daily for 21 days. Surgery was performed 15 days after clinical resolution of her pododermatitis.

Three dogs, including the dog treated for pododermatitis, entered estrus for the first time after inclusion in the study and prior to surgery. At least 30 days were allowed after the first day of estrus, prior to performing ovariohysterectomy (OHE) to ensure all dogs were in diestrus.

All dogs were healthy and not in estrus at the time of surgery.

2.2. Study design

Standard OHE was performed to induce a pro-inflammatory state and oxidative stress in healthy dogs. Using a random integer set generator (www.random.org), two groups of six dogs were generated. While the first group was the control group (Control group), the second group of dogs (HBO group) received 2 HBO treatments, 12 hours apart, starting six hours after surgery (Figure 1).

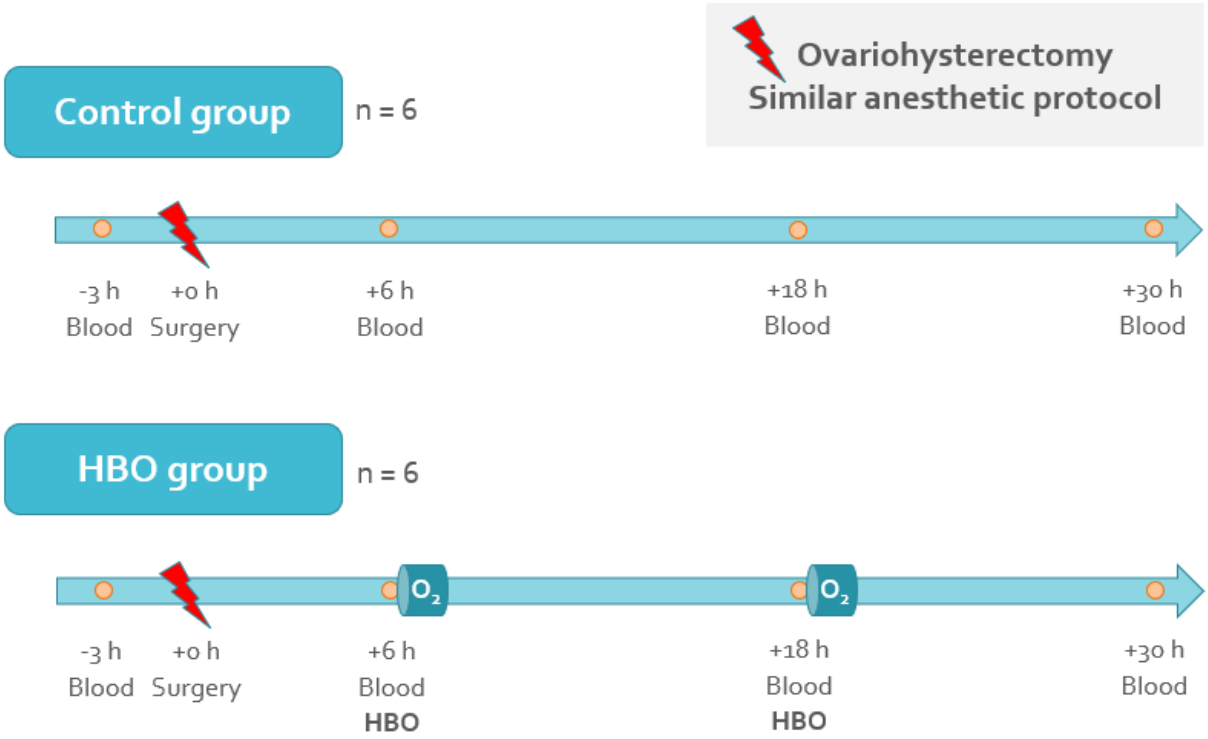


Figure 1. Protocol timeline

Dogs from the Control group (n=6) and hyperbaric oxygen (HBO) group (n=6) underwent an OHE at time 0 hours (red lightning bolt). Dogs from the HBO group received two HBO treatments (blue cylinder) six and 18 hours after surgery. Whole blood (yellow circle) was collected three hours prior to surgery (-3h), 6, 18 and 30 hours after surgery (+6h, +18h and +30h), and prior to HBO treatment when applicable.

The dogs were fasted 12 h prior to surgery with free access to water. Anesthesia was supervised by a single board-certified anesthetist. The dogs were pre-medicated with

hydromorphone [e] (0.1 mg/kg) and dexmedetomidine [f] (200 mcg/m^2) intra-muscularly, induced with midazolam [g] (0.2 mg/kg) and ketamine [h] (5 mg/kg) intravenously, and maintained on inhalant isoflurane. In the event of an adverse or refractory response to the drugs administered, action was taken at the discretion of the anesthetist and recorded. During anesthesia, the dogs received intravenous Lactate Ringer's Solution (LRS) [i] at a constant rate (5 mL/kg/h). Boluses of 10 mL/kg of LRS and an additional dose of hydromorphone were administered as needed for hypotension and rescue analgesia, respectively. Duration of surgery, adverse events, estimated blood loss, doses and time of drugs administered were recorded.

Ovariohysterectomies were performed by a single board-certified surgeon according to the standards of care. After ventral median laparotomy, the ovarian pedicles and uterine body were triple clamped, ligated and transfixed with 3-0 polydioxanone sutures (PDS) before transection. The uterus appeared more vascularized in the three dogs that had a previous estrous cycle and 2-0 PDS were used for uterine and pedicle ligations. The body wall was closed using 3-0 PDS in a simple interrupted pattern. The subcutaneous tissue and skin were closed using a 4-0 Monocryl in a simple continuous pattern. The length of the abdominal incision was standardized around 20 centimeters. Surgeries were completed without complication in 27 to 40 min, with a median time of 30.5 min and no difference between the groups (p-value = 0.59).

Dogs in the HBO group received two HBO treatments at 12-h intervals. The first treatment was performed six hours after recovery from surgery. Treatment consisted of administration of 100% oxygen at 2.0 ATA for 35 min with compression and decompression times of 10 min each using a purpose-built veterinary hyperbaric chamber [j]. The HBO treatments were supervised by a veterinarian who completed the Hyperbaric Medicine Team Training for Animal Applications [k]. The dogs were not sedated or treated with anxiolytics prior to entering the hyperbaric oxygen chamber. Treatments were performed at least four hours after the last administration of hydromorphone to decrease the risk for respiratory depression. The dogs were

observed through the windows of the chamber and via cameras during treatment, and monitored for respiratory distress, seizures, signs of barotrauma (head shaking, pawing at head, vocalization), and confinement anxiety. If these were observed, HBO treatment was discontinued. A full physical examination was performed immediately prior and after completion of the treatment. All adverse effects were recorded. To prevent the risk of spark formation and fire ignition in the chamber, the following precautions were taken: surgical incisions were closed with suture material and no staples, no electrical equipment nor blanket was allowed inside the chamber, metallic collars and leads were removed from the patient, intravenous catheters were wrapped in cotton bandaging material, and dogs were wearing plastic elizabethan collar.

Following surgery and recovery from anesthesia, the dogs were hospitalized in the Auburn University Veterinary Teaching Hospital Intermediate Care unit for 30 hours. Mentation was monitored hourly and physical examination was assessed and recorded every six hours. Incision sites were left uncovered, gently cleansed with gauze and saline if grossly contaminated, and photographed daily. Post-operative care and treatment were identical for all dogs and included administration of hydromorphone (0.05 mg/kg) intravenously every six hours for four doses and carprofen [l] (2.2 mg/kg) by mouth every 12 hours for four days starting 18 h after surgery (= after the last HBOT). Tramadol [m] (5 mg/kg) was given by mouth every eight to 12 h as needed for rescue analgesia.

After 30 h of hospitalization in the Intermediate Care unit, the dogs were relocated to the general boarding wards for continuous care and monitoring for 13 additional days. They were fed twice daily and allowed restricted exercise two to three times daily. Any abnormality was recorded and addressed according to the best standard of care. Sutures were removed 14 days after surgery.

2.3. Outcomes

2.3.1. Incision and pain scores

Postoperative wound healing and pain were evaluated four times daily for the first 30 h, then twice daily until suture removal 14 days after surgery using the pain scoring system developed by Davidson et al. in 2004, and the incision scoring system described by Sylvestre et al. in 2002 (Appendices 1 and 2). Both systems have been validated in post-OHE dogs (19, 20).

2.3.2. Inflammatory and oxidative biomarkers

Five milliliters of whole blood were collected from a jugular vein three hours prior to surgery, and six, 18 and 30 h after surgery (Figure 1). When applicable, blood was collected prior to HBO treatments. Blood was allowed 30 min to clot and centrifuged at 3,000 rpm for 9 min. Serum was collected, separated into aliquots of 300 to 600 µls, and stored at -80°C until sample analysis. Aliquots were thawed on ice for 30 to 60 min and vortexed prior to analysis. Serum progesterone was measured once prior to surgery, and all other biomarkers were measured at all time-points.

Cytokines, chemokines and growth factors

Serum cytokine, chemokine and growth factor concentrations were measured in-house using commercially available enzyme-linked immunosorbent assays and Luminex® multiplex technology. Milliplex® MAP Canine Cytokine/Chemokine Magnetic Bead Panels [n] were used to measure the following cytokines: interleukin (IL)-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, interferon γ (IFN-γ), interferon-γ-induced protein (IP-10), tumor necrosis factor alpha (TNF-

α), monocyte chemoattractant protein 1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and keratinocyte chemotactic-like (KC-like). The manufacturer's instructions were followed, and the results were read with a Luminex® MAP 96-well-microplate reader [o] at 511 nm. All samples were analyzed in duplicate. The data were analyzed using xPONENT® [p]. The observed concentration of each analyte for each sample was calculated using a standard curve generated from six standards provided by the manufacturer, and deionized water. After linear regression analysis, all standard curves had a coefficient of determination (R^2 value) above 0.997.

C-reactive protein (CRP)

Samples were sent to the University of Miami Acute Phase Protein Laboratory for serum CRP levels measurements using the Randox immunoturbidimetric Canine CRP assay [q] run on a Daytona Rx analyzer [r]. This assay, calibrated with canine specific control calibration material, is currently considered the reference method for canine CRP levels. Samples were run in duplicate.

Iron panel

Serum iron (S. iron) and unsaturated iron-binding capacity (UIBC) were measured using the colorimetric FerroZine® method with the Roche Cobas® 311 chemistry analyzer [s], at the on-site clinical pathology laboratory. Iron was dissociated from the transferrin-iron complex in weakly acid medium, and is reduced to the ferrous state by means of ascorbic acid. Ferrous ions then form a blue biochromatically quantifiable complex with the chromogen Ferrozine, or sodium 4-[3-pyridin-2-yl-5-(4-sulfophenyl)-1,2,4-triazin-6-yl]benzenesulfonate. After 5 min of

incubation at 37°C, the Fe2+-complex absorbance is measured at 560nm. The color intensity is proportional to the iron concentration in the sample.

Total iron binding capacity (TIBC) and transferrin saturation (TSAT) were calculated as follow:

$$TIBC = S.\ iron + UIBC$$

$$TSAT\% = [\ S.\ iron / TIBC\ ] \times 100$$

Oxidation status

Serum total antioxidant status (TAS) and total oxidant status (TOS) levels were determined by the colorimetric methods described by Erel et al. in 2004 and 2005 using commercially available kits [t, u]. Samples were run in duplicate. If the coefficient of variation was above 15%, samples were run in triplicate.

Antioxidants present in the serum sample react with dark blue-green colored 2,2'-azino-bis [3-ethylbenz- thiazoline-6-sulfonic acid (ABTS)] radical cation to form its colorless reduced form. The change of absorbance at 660 nm is measured spectrophotometrically at 37°C with an automatic analyzer SpectraMax® 384 [v], and is directly correlated to the total antioxidant level in the sample. The assay is calibrated with a stable antioxidant standard solution, a vitamin E analog called Trolox Equivalent, and the results are expressed in mmol Trolox equivalents/L.

Oxidants present in the serum sample oxidize the ferrous ion-o-dianisidine complex into ferric ion. These ferric ions form a colored complex with xylenol orange in an acidic medium. The change of absorbance at 530 nm is measured spectrophotometrically at 37°C with an automatic analyzer SpectraMax 384 [v], and directly correlated to the total oxidant level in the

sample. The assay is calibrated with hydrogen peroxide (H2O2), and the results are expressed in μmol H2O2 equivalents/L.

Oxidative stress index (OSI) is the percentage ratio of TOS to TAS (21, 22). The formula used is the following:

$$OSI = [\ (TOS, \mu mol/L) / (TAS, mmol\ Trolox\ equivalent/L)\ ] \times 100$$

Icteric index, hemolytic index, lipemic index, progesterone

These values were treated as potential covariates. Icteric, hemolytic and lipemic indexes were measured by American Society for Clinical Pathology certified medical technologists under the direction of an American Society for Clinical Pathology board-certified veterinary clinical pathologist at the on-site clinical pathology laboratory. These indexes are calculations of absorbance measurements that provide a semi-quantitative representation of levels of icterus, hemolysis, or lipemia (turbidity) in the sample. Serum is diluted with 0.9% NaCl, and the absorbance is measured by a Roche Cobas® 311 chemistry analyzer [s]. The bichromatic wavelength pairs used for serum icteric, hemolytic and lipemic index measurements are 480 nm and 505 nm, 570 nm and 600 nm, and 660 nm and 700 nm, respectively. Calculation formulas include corrections to compensate for the spectral overlap.

Serum progesterone levels were measured in-house, in duplicate, using an enzyme-labeled chemiluminescent technology and Immulite® 1000 [w].

2.4. Statistical analysis

A statistical power analysis was performed for sample size estimation [x]. Unfortunately, published data reporting the effects of OHE on inflammatory biomarkers and oxidation in dogs,

or the effects of HBO on these same biomarkers are sparse. One study assessing the changes of serum CRP concentration in bitches after OHE (23) and one study evaluating the effects of HBO on IFN-γ, IL-2 and IL-10 in rats with pancreatitis (7) were used for estimation of the sample size. A sample size of six dogs in each group seemed adequate to detect the effect of OHE and HBO on inflammatory biomarkers with a level of significance of 0.05 and power level of 0.75.

Cytokines concentrations below the limit of detection were substituted with the limit of detection divided by the square root of 2. This extrapolation produces estimates with smaller bias and error rates than other replacement techniques (ie. zero or absolute limit of detection) (24).

Unpaired Student t-tests were used to identify baseline differences in body weight and time of the surgical procedure between the two groups.

Outliers were identified prior to surgery using the median absolute deviation and a threshold of 3.5 (25) and excluded from analysis. This allowed the identification of two dogs in the Control group with pre-existing abnormal cytokine profile. These dogs had no identifiable disease at the time of inclusion, did not have pododermatitis, and were in anestrus. Both dogs had elevated levels of GM-CSF, IL-6, Il-15 and IL-18 prior to surgery. One dog also had elevated TNF-α, IL-2 and Il-7, and the other had elevated IL-10 prior to surgery.

Model residuals were examined to evaluate the assumption of normality. Linear mixed models (LMM) were used to analyze biomarker and covariate concentrations. The full LMM for each biomarker included fixed factors for group, time and a group by time interaction. Alternatively, baseline biomarker concentration was added as a covariate to adjust for baseline concentration. A random intercept for each dog was included to account for within dog correlations in repeated measurements over time. Lipemic index (at each time), hemolytic index

(at each time) and progesterone (at first time) were all initially included as fixed covariates LMM for each biomarker. Backwards elimination was used to remove the covariate with the highest non-significant p-value iteratively until only significant covariates were included in each LMM without and with baseline. These are listed in Table 3. Incorporation of covariates did not affect the results and the graphs below represent the values without covariate incorporation.

Table 3. Covariates and biomarkers

Biomarker	Covariate
S. iron	Lipemic index
	Hemolytic index
	Progesterone
UIBC	Lipemic index
TIBC	Hemolytic index
TSAT	Lipemic index
	Hemolytic index
	Progesterone
GM-CSF	Hemolytic index
IL-2	Hemolytic index
IL-8	Progesterone
TOS	Progesterone
OSI	Lipemic index

Satterthwaite degrees of freedom method was used for the comparison of means from two normal distributions with small samples and possibly different variances. Tukey's test was used for adjustment for multiple comparisons. Results are presented as mean ± standard deviation.

All analyses were performed using SAS V 9.4 [y]. A significance threshold of 0.05 was used. Graphs were created using GraphPad [z].

3. Results

3.1. Study population and clinical effects

The dogs' mean body condition score was 4.6/9 with no statistical difference between Control and HBO treated dogs. Control dogs weighed significantly more than HBOT treated dogs with a body weight of 10.3 (+/- 0.462) kgs and 8.98 (+/- 1.01) kgs, respectively (p-value = 0.017). All HBO treated dogs completed the HBO sessions without seizures, signs of discomfort or confinement anxiety detected. Physical examinations were normal for all dogs before and after HBO treatments. Pain and incision scores did not differ at any time between the Control and HBO treated groups. None of the dogs required rescue analgesia during hospitalization. All incisions were considered healed by the time of suture removal at 14 days with no signs of infection or dehiscence, and all dogs were clinically healthy two months after the study.

3.2. Ovariohysterectomy and HBO therapy effects on acute phase protein

Ovariohysterectomy induced a significant increase in CRP concentration 18 and 30 h following surgery ($p < 0.0001$) (Figure 2). Hyperbaric oxygen therapy did not significantly change serum CRP concentrations (p-value > 0.05 for each time).

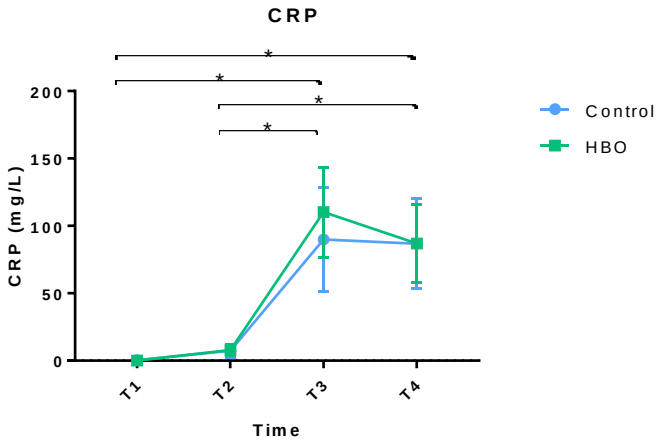


Figure 2. Mean and standard deviation of serum concentrations of C-reactive protein (CRP) prior to OHE (T1), 6, 18 and 30 hours after OHE (T2, T3 and T4, respectively) within the Control group and HBO group

* : statistically significant changes with time in both Control and HBO groups.

3.3. Ovariohysterectomy and HBO therapy effects on iron status

Ovariohysterectomy induced a significant decrease in S. iron concentrations at 6, 18, and 30 h following surgery (p-value < 0.0001), and a significant increase in UIBC at 6, 18, and 30 h following surgery (p-value < 0.0014). As a result, OHE induced a significant decrease in the calculated TSAT at 6, 18, and 30 h (p-value < 0.0015). TIBC was significantly decreased 18 and 30 h following surgery (p-value < 0.0372). There was no statistical difference between the Control group and the HBO group in iron status and at any time points. Mean values and standard deviation are shown in Figure 3.

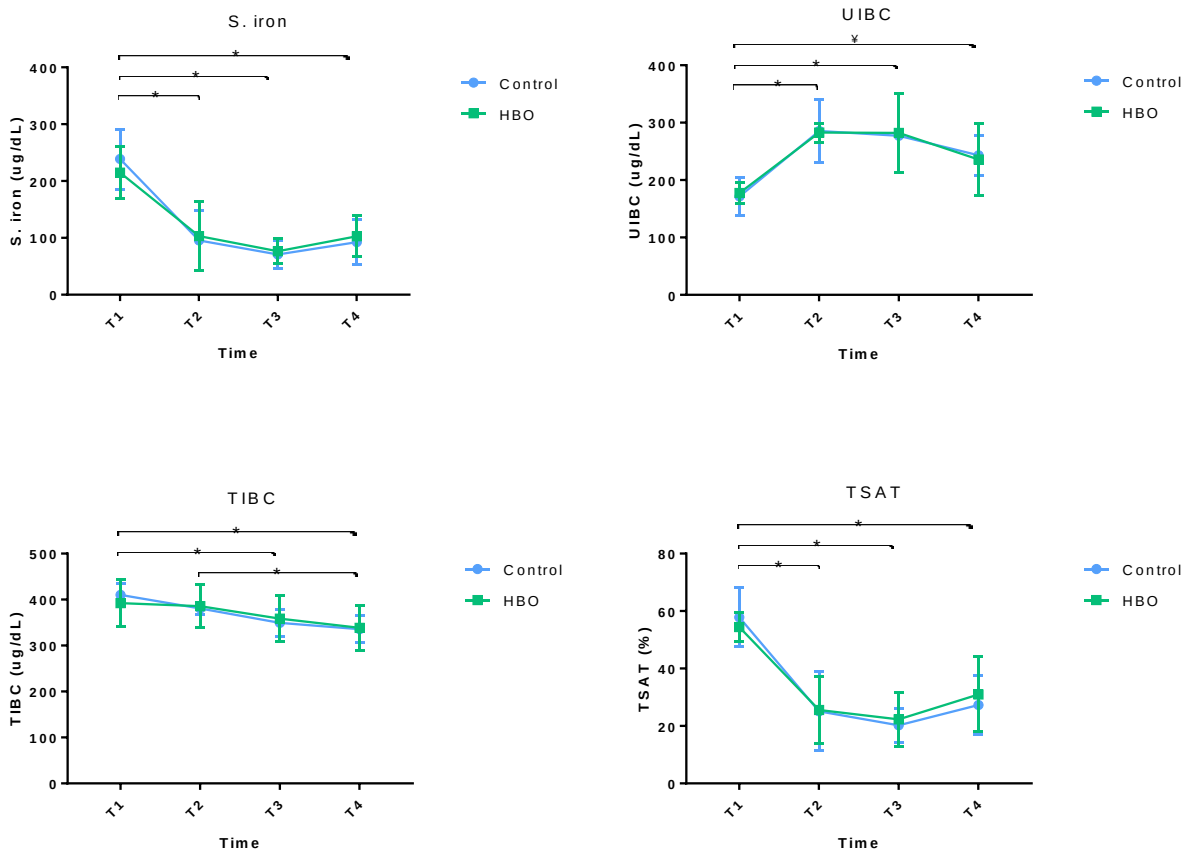


Figure 3. Mean serum iron (S. iron), unsaturated iron-binding capacity (UIBC), total iron-binding capacity (TIBC), and transferrin saturation (TSAT) prior to OHE (T1), 6, 18 and 30 hours after OHE (T2, T3 and T4, respectively) within the Control group and HBO group

* : statistically significant changes with time in both Control and HBO groups.

¥ : statistically significant changes with time in the HBO group.

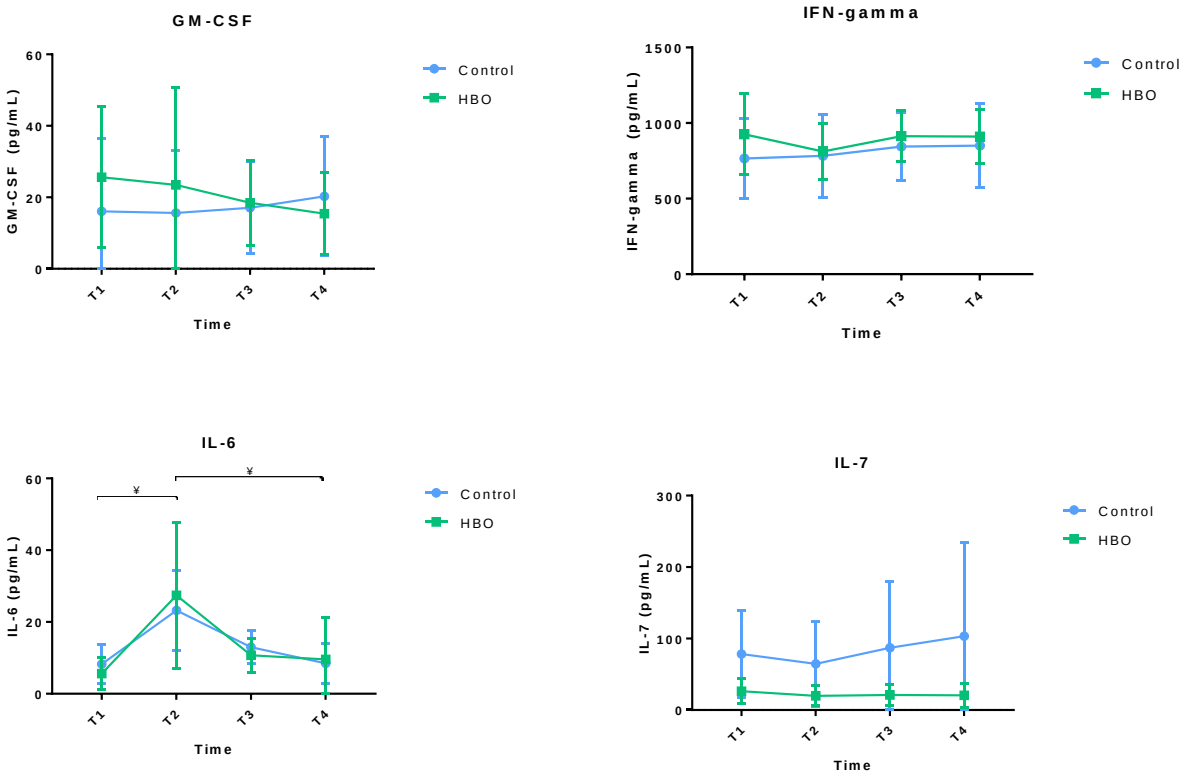
3.4. Ovariohysterectomy and HBO therapy effects on cytokines

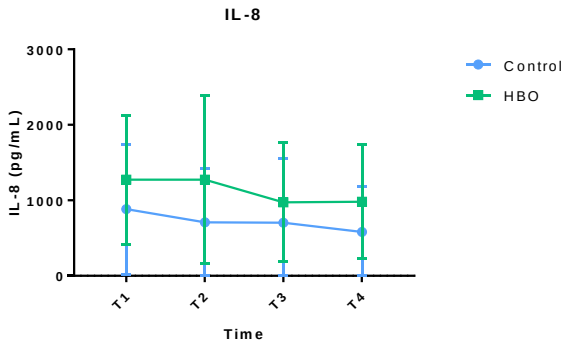
Of the 13 different cytokines evaluated, 11 cytokines were not affected by OHE.

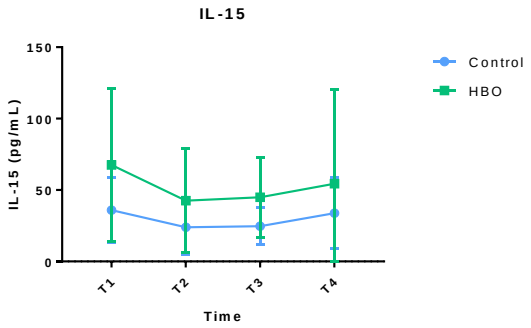
Ovariohysterectomy induced a significant increase in KC-like concentration six hours after surgery (p-value < 0.0076), with a subsequent decrease 30 h after surgery (p-value < 0.0103). In the HBO group, KC-like concentration was also significantly decreased 18 h after surgery compared to six hours after surgery (p-value = 0.0172). Similarly, OHE induced a significant increase in IL-6 concentration six hours after surgery (p-value = 0.0130), with a subsequent decrease 30 h after surgery (p-value = 0.0151).

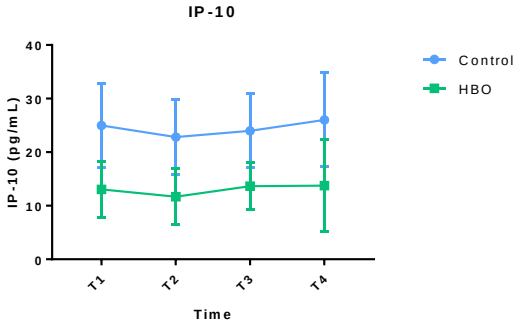
There was no statistical difference owing to HBO treatment in any of the cytokines and at any time points. Mean values and standard deviation of investigated cytokines concentrations are shown in Figure 4.

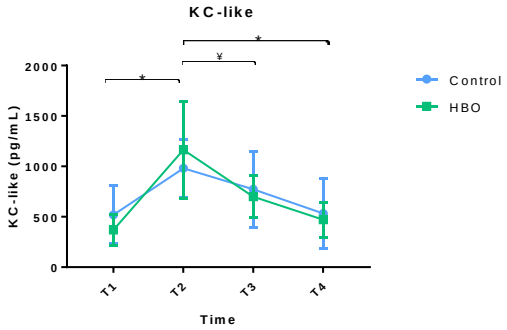
‘’ Pro-inflammatory ‘’ cytokines

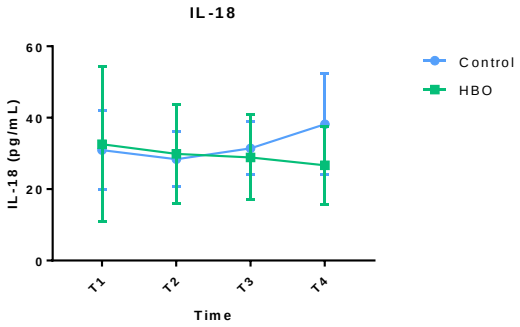


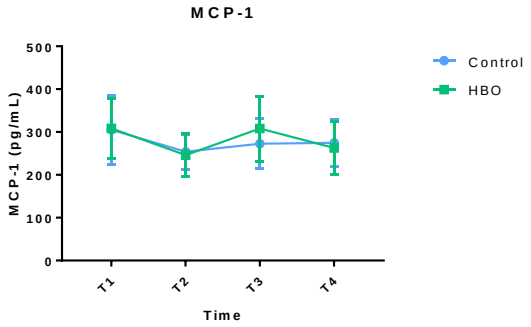

IL-8
IL-8 (pg/mL)
3000
2000
1000
0
T1
T2
T3
T4
Time
Control
HBO

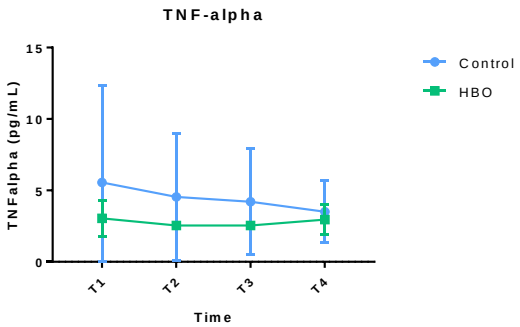

IL-15
IL-15 (pg/mL)
150
100
50
0
T1
T2
T3
T4
Time
Control
HBO


IP-10
IP-10 (pg/mL)
40
30
20
10
0
T1
T2
T3
T4
Time
Control
HBO


KC-like
KC-like (pg/mL)
2000
1500
1000
500
0
*
¥
*
T1
T2
T3
T4
Time
Control
HBO


IL-18
IL-18 (pg/mL)
60
40
20
0
T1
T2
T3
T4
Time
Control
HBO


MCP-1
MCP-1 (pg/mL)
500
400
300
200
100
0
T1
T2
T3
T4
Time
Control
HBO


TNF-alpha
TNFalpha (pg/mL)
15
10
5
0
T1
T2
T3
T4
Time
Control
HBO

‘’ Anti-inflammatory ‘’ cytokines

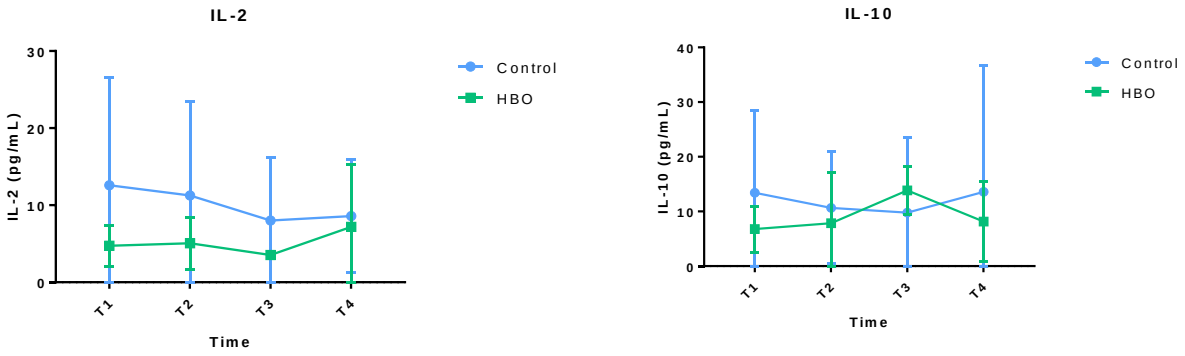


Figure 4. Mean and standard deviation of serum concentrations of cytokines: interleukin (IL)-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, interferon γ (IFN-γ), interferon-γ-induced protein (IP-10), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and keratinocyte chemotactic-like (KC-like) prior to OHE (T1), 6, 18 and 30 hours after OHE (T2, T3 and T4, respectively) within the Control group and HBO group

* : statistically significant changes with time in both Control and HBO groups.

¥ : statistically significant changes with time in the HBO group.

3.5. Ovariohysterectomy and HBO therapy effects on oxidation status

Ovariohysterectomy and HBO therapy did not induce any significant changes in TOS, TAS and OSI, with or without covariates incorporation ($p > 0.05$) (Figure 5).

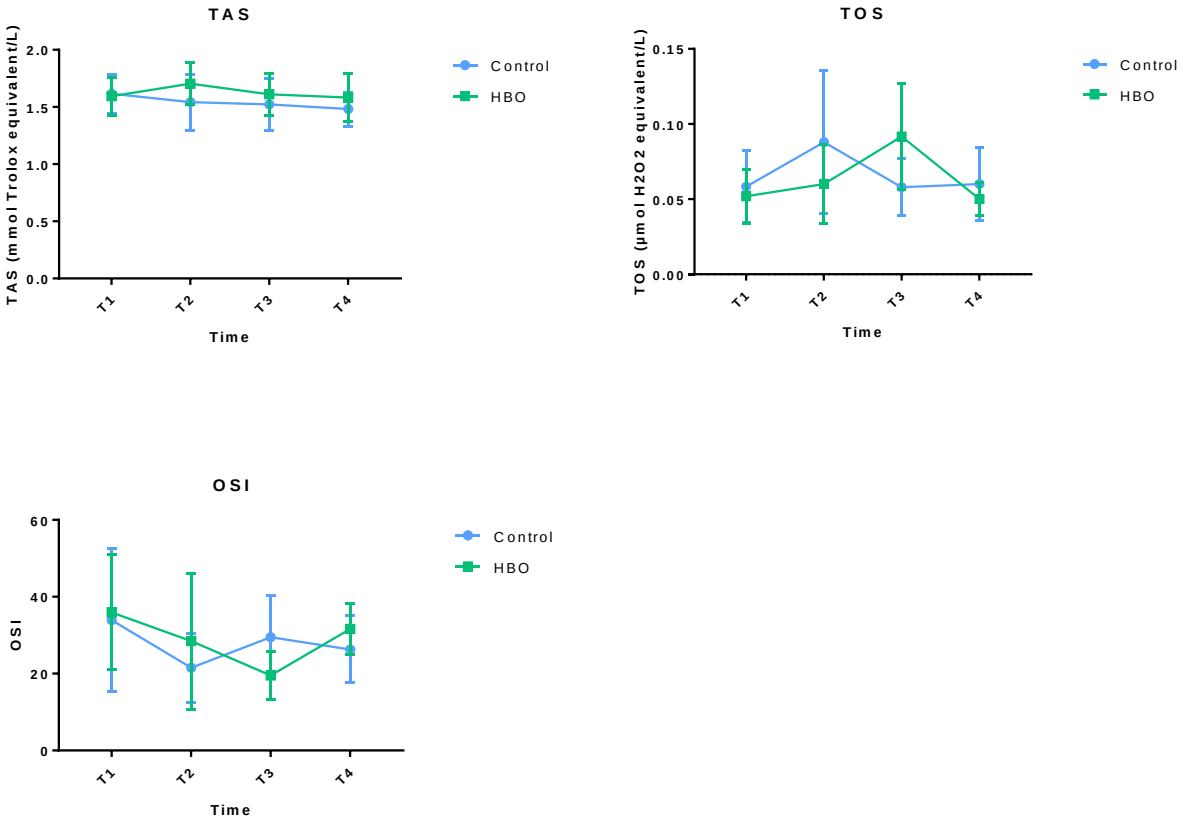


Figure 5. Mean and standard deviation of serum total oxidant status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI) prior to OHE (T1), 6, 18 and 30 hours after OHE (T2, T3 and T4, respectively) within the Control group and HBO group

4. Discussion

Ovariohysterectomy induced systemic inflammation, however, our study failed to demonstrate a change in TOS, TAS or OSI after surgery. Two treatments of HBO did not induce any detectable anti-inflammatory or antioxidant effects after OHE.

Hyperbaric oxygen is an adjunctive therapy, and designing a study to evaluate its therapeutic effects with adequate power and minimal bias is challenging. Ideally, HBO would be the only treatment administered to a large population affected by a disease comparable in severity and measurable with specific and sensitive biomarkers, and comparable to a placebo. These conditions are rarely met in a clinical situation. Most diseases reported to potentially benefit from HBO have various degrees of severity with no specific biomarkers identified (i.e. pancreatitis, brain injury, sepsis, lung injury, wounds, cardiopulmonary bypass surgery) (6, 7, 8, 9, 26, 27, 28, 29, 30). For this study, OHE was performed as a model of inflammation. Ovariohysterectomy is an elective surgical procedure performed in a controlled environment with relatively comparable amounts of stress and tissue injury between patients. There are also limited medical interventions involved outside of analgesics that could influence the results. In order to limit the variability of inflammation induced between dogs and between groups, the same board-certified surgeon operated on healthy dogs utilizing the same anesthetic and surgical protocol. There were no statistical differences in biomarker concentrations, incision score and pain score between the Control group and HBO group prior to surgery, and six hours after surgery prior to HBO therapy. Therefore, the clinical outcome, inflammation and oxidative stress induced by surgery were comparable immediately after OHE, and we speculated that differences between groups are attributable to HBO therapy.

In this study, HBO did not improve incision or pain scores. The absence of effects on incision score after OHE is consistent with one study in dogs in which HBO did not influence the healing of uncomplicated open and incisional wounds surgically created (31). There was no

difference at any time for contraction, epithelialization, subjective wound scores, histopathology scores, or bacterial loads between the control group and the group that received HBO therapy. Similar findings were found in rats with uncomplicated wounds (32). A Cochrane review included twelve randomized trials (577 participants) and concluded that HBO is more likely to benefit ischemic wounds, or chronic wounds characterized by hypoxic tissues and associated with venous diseases or diabetes in humans (33). Therefore, the absence of clinically detectable effects of HBO therapy on surgical laparotomy incision was expected in this study. Numerous studies have demonstrated the antinociceptive effect of HBO in relief for chronic pain (34, 35, 36) and acute inflammatory pain (37, 38). The mechanism of the reported analgesic effect of HBO is unknown, although some have attributed this effect to its anti-inflammatory properties and decreased expressions of inducible nitric oxide synthase and neuronal nitric oxide synthase (36, 37). There was no difference in pain scores between the Control and HBO dogs in this study. This is likely related to the adequate analgesia provided by opioid administration.. As it would be unethical to withhold analgesics post-surgery, opioids were administered to all dogs knowing the potential risk of masking the analgesic effects of HBO in this study.

All dogs had a significant increase in CRP 18 hours after surgery. These results are consistent with previous studies reporting an increase in CRP within the first 24 hours after OHE with a subsequent reduction in the absence of ongoing trauma (39, 40, 41, 42, 43). HBO therapy has been previously shown to reduce CRP serum concentration in people with traumatic brain injury treated with 4 HBO treatments at 3-day intervals (1 hour on 100% oxygen at 2.0 ATA) (9), and in children with autism after 40 HBO treatments at 1- to 2-day intervals (45 minutes on 100% oxygen at 1.3 and 1.5 ATA) (44). However, in our study, HBO did not affect the concentration of CRP. This finding may indicate a lack of effect of HBO on the acute phase protein at the dose used in this study after OHE, or result from a type II error. Further studies

involving greater inflammatory stimuli and/or chronic inflammation, different doses of HBO, and measurements of CRP over a longer period are needed before determining the effects of HBO on inflammatory acute phase proteins.

In both the Control and HBO groups, S. iron concentration and TSAT significantly decreased as early as six hours after surgery, while TIBC decreased 18 to 32 hours after surgery, and UIBC significantly increased six hours after surgery. Hypoferremia is a sensitive marker of systemic inflammation, including surgically induced inflammation (43, 45, 46, 47, 48). Changes in iron homeostasis occur within the first 24 hours after surgery and progressively normalize as inflammation subsides (43, 48), appears to correlate with the extent of surgery (45), and have been suggested as a valuable biomarker to monitor systemic inflammation and predict clinical outcome (46, 47). Inflammation-related hypoferremia is multi-factorial and is in part related to the upregulation of the acute phase proteins ferritin and hepcidin, leading to the sequestration of iron by the sarcoplasmic reticulum, and reduction of dietary iron absorption resulting in increased UIBC (49, 50, 51, 52), and in another part to the downregulation of the main iron binding transporter protein, transferrin, resulting in a decreased TIBC (53). As iron continues to decrease, the amount of transferrin saturated with iron decreases reflected by a decreased TSAT. To the author's knowledge, there is no published study reporting the effect of HBO on iron homeostasis. Iron and oxygen homeostasis are known to be strongly interconnected. Hypoxia is associated with decreased production of hepcidin and increased expression of transferrin. This results in an increase in dietary iron absorption and iron transport to erythroid tissues, ultimately leading to an increase in the capacity of red blood cells to transport oxygen (54). In the present study, the hyperoxemic state induced by HBO did not have a detectable effect on iron homeostasis 32 hours after surgery. Further studies are warranted to evaluate the effect of HBO on the expression of hepcidin, transferrin and ferroportin in patients with systemic inflammation for a longer period of study.

Ovariohysterectomy induced a significant increase in KC-like and IL-6 levels six hours after surgery, but not for the remaining 11 cytokines measured. Cytokines are key modulators of the inflammatory response to trauma. Via intracellular signaling pathways, cytokines regulate, augment (proinflammatory) or attenuate (anti-inflammatory) the inflammatory response, and promote proper healing (55). Studies in human medicine have yielded insights into potential applications of cytokines in the diagnosis, monitoring, prognostication, and therapy of a variety of diseases (56), but much remains to be elucidated regarding cytokines regulations, synergistic or antagonistic interactions, and roles in either development, progression, or control of pathologic conditions in veterinary medicine. Previous studies report an increase in serum concentrations of IL-6 and IL-10 in healthy dogs three days after ovariohysterectomy (57), and identified KC-like as being significantly higher in dogs with pyometra and sepsis compared to dogs without sepsis (58). Additionally, IL-6 and KC-like have been shown to correlate with the severity of disease and surgical trauma (59, 60). The temporary and mild increase limited to IL-6 and KC-like identified in our study suggests that the inflammation induced by OHE was minimal. No differences were detected at any time between the control and HBO groups, suggesting that HBO does not induce alterations in pro- and anti-inflammatory cytokines in dogs after OHE. These findings are opposed to previous studies reporting an anti-inflammatory effect of HBO detected in different species treated for pancreatitis, traumatic brain injury, sepsis and acute lung injury (7, 8, 9, 27, 62). The failure to detect more changes in cytokines levels in this study may be related to the nature of the surgery that did not induce enough inflammatory response, and/or the inclusion of cytokines that are not specifically stimulated by surgical trauma. Additionally, it is also possible that our sampling times did not correlate with some of the cytokines' delay of response and/or short half-life (61). Finally, there is a risk for a type II error, with the inclusion of a relatively low number of animals

along with a high inter-individual variability in the cytokine concentrations and response to stimulus.

One of the primary goals of this study was to determine the effects of HBO on oxidative stress. For this purpose, TAS and TOS were measured to estimate the global antioxidant and oxidant status, respectively. It is well accepted that HBO increases production of reactive oxygen species (ROS) at a sub-lethal concentration (5). At a low dose, via non-cytotoxic oxidative stimuli, increased ROS levels activate a negative feedback loop which leads to downregulation of oxidant enzymes and upregulation of antioxidant enzymes (32, 63, 64, 65). A large number of studies used individual antioxidant enzymes such as superoxide dismutase-1, catalase, and glutathione peroxidase, or oxidant enzymes such as malondialdehyde or F2-isoprostane to study and demonstrate the antioxidant effect of HBO (6, 8, 9, 66, 67). However, because the antioxidant and oxidant effects of each individual enzymes are additive, and because their measurement can be technically challenging, none of these are perfect surrogate biomarker of oxidative stress (68). More recently, TAS and TOS have been suggested to assess the antioxidant and oxidant status of biological samples (21, 22, 69). Lee et al. showed an increase in plasma TOS and OSI levels and decrease in TAS levels after ovariectomy in dogs, suggesting the oxidative stress induced by the surgery (70). However, our study failed to demonstrate a change in TOS, TAS or OSI after OHE. There was also no change after HBO therapy. These findings are in contrast with a previous study that showed significantly increased TAS levels and decreased TOS and OSI levels after five daily HBO treatments at 2.0 ATA for one hour in rats with induced acute lung injury (62). Our failure to detect changes in TAS and TOS may be related to the absence of oxidative stress induced by OHE and/or the lack of antioxidant effect of HBO after OHE. The technique used to measure the total antioxidant and oxidant capacity may also be a source of error. To this point, the ideal reference method to measure TAS and TOS in dogs has not been established and further studies are needed to

validate the technique described by Erel (69). Finally, TAS and TOS estimate the antioxidant and oxidant capacity of the serum but provide limited information regarding the intracellular oxidative stress, and do not evaluate the role of important enzymes such as superoxide dismutase, glutathione peroxidase, and catalases (71, 72). Ideally, individual antioxidants and oxidants would have been measured in complement to provide a wider picture of the antioxidant status.

A considerable number of studies suggests that the short exposure to hyperoxia during clinical HBO treatments can have therapeutic benefits. However, oxygen, particularly at supraphysiological pressures, has also the potential to increase systemic oxidative stress and induce hyperoxic cellular damage (73). Studies have shown that long-term exposure to repetitive HBO treatments (74, 75), and treatments at more than 3.0 ATA (27), result in cumulative oxidative stress and worsens clinical outcome. This study identified a safe HBO protocol for healthy dogs undergoing an elective surgical procedure.

This study has some important limitations. Because of the small population, a type II error could be present, resulting in failure to detect a difference in the different outcomes measured between the HBO and Control groups. In addition, OHE used as an inflammatory model may not induce enough inflammation and oxidative stress to effectively compare and study the effects of HBO as an adjunctive therapy. Collection of blood samples at more time points, closer to the HBO treatments and over a longer period of time could improve the ability to detect a difference in biomarkers concentrations. Finally, validated sensitive and specific biomarkers of inflammation, oxidative stress and HBO therapy effects are needed.

5. Conclusion

This is the first prospective randomized controlled study to investigate the effects of HBO on surgically-induced systemic inflammation and oxidation in dogs. Hyperbaric oxygen therapy did not induce any detectable anti-inflammatory or antioxidant effects after OHE and two treatments under the described protocol. These results cannot be extrapolated to more severe, complicated or chronic diseases. Additional investigation using different inflammatory models is required to identify objective markers to quantify the response to HBO, determine its role as an adjunctive therapy in dogs, and optimal dose (i.e., pressure; duration; number of treatments; frequency of treatment). A safe HBOT protocol that resulted in no adverse effects was identified.

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Footnotes

a. Marquis, ponazuril 150 mg/gm of paste (Merial, Inc., Duluth, GA, USA)

b. Convenia, cefovecin injection 80 mg/mL (Zoetis, Parsippany, NJ, USA)

c. Pro Plan Savor Shredded Blend Adult Chicken & Rice Formula (Nestlé Purina PetCare Company, St. Louis, MO 63164 USA)

d. SNAP®4Dx® assay (IDEXX Laboratories, Westbrook, ME, USA)

e. Hydromorphone HCl injection, USP, 40 mg/20 mL (West-Ward, Eatontown, NJ, USA)

f. Dexdomitor®, dexmedetomidine 0.5 mg/mL (Zoetis Inc., Kalamazoo, MI, USA)

g. Midazolam hydrochloride Injection, USP, 5 mg/mL (West-Ward, Eatontown, NJ, USA)

h. Ketaset®, ketamine HCl injection, USP, 1000 mg/10 mL (Zoetis Inc., Kalamazoo, MI, USA)

i. Vetivex®, Lactate Ringer's Solution (Dechra veterinary products, Overland Park, KS, USA)

j. Hvm model 3200 veterinary hyperbaric chamber (Hyperbaric veterinary medicine, Boca Raton, FL, USA)

k. Hyperbaric Medicine Team Training for Animal Applications (International ATMO, Inc. San Antonio, TX, USA)

l. OstiFen^{TM} caplets, 25 mg (Norbrook Laboratories, Newry, Co Down, Northern Ireland)

m. Tramadol hydrochloride tablets, USP, 50 mg (Sun Pharmaceutical Industries, Inc., Cranbury, NJ, USA)

n. Milliplex® MAP Canine Cytokine/Chemokine Magnetic Bead Panels (EMD Millipore Corporation, Burlington, MA, USA)

o. MAGPIX® Luminex MAP 96-well-microplate reader (Luminex Corporation, Austin, TX, USA)

p. xPONENT® (Luminex Corporation, Austin, TX, USA)

q. Canine C reactive protein immunoturbidimetric assay (Randox, Kearneysville, WV, USA)

r. Daytona Rx^{TM} analyzer (Randox, Kearneysville, WV, USA)

s. Roche Cobas® 311 chemistry analyzer (Roche/Hitachi Ltd., Indianapolis, IN, USA)

t. Total Oxidant Status Assay Kit (Rel Assay Diagnostics, Gaziantep, Turkey)

u. Total Antioxidant Status Assay Kit (Rel Assay Diagnostics, Gaziantep, Turkey)

v. SpectraMax Plus® 384 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA)

w. Immulite® 1000 (Siemens Healthcare Diagnostics Inc, Tarrytown, NY, USA)

x. http://clincalc.com/stats/samplesize.aspx

y. SAS V 9.4 (Cary, NC, USA)

z. GraphPad Software (San Diego, CA, USA)

Appendix 1
Pain scoring system (19)

PAIN SCORE

SUBJECTIVE PAIN SCORE

(0 or 1 for each criteria)

Behavior category	Changes in personality or attitude: Sudden aggressiveness in a docile animal or vice versa. Attempts to bite, especially if the painful area is palpated
Activity level	Restlessness, pacing, repeatedly getting up and down. Recumbent or reluctant to move. Guarding a painful area. Abnormal activity may not be noticed on brief observations
Inability to sleep	Would not lie down even when exhausted. Sits propped up in the corner of the cage
Facial expression	Dull eyes, dilated pupils, staring into space, pinning of ears or grimacing, sleepy, photophobic appearance
Abnormal vocalization	Vocalization, especially if the painful area is palpated or moved. This is an unreliable trait
Licking, biting, scratching, or shaking	Suspect discomfort, especially if directed at the painful area. Can lead to self-mutilation
Posture and ambulation	May limp or carry a painful appendage. May tense or tuck up the abdomen/back muscles
Physiologic parameters	Increased heart rate, blood pressure, respiration, and body temperature
Changes in hair coat	Ruffled or greasy fur indicates lack of grooming or piloerection
Appetite and thirst	Decreased food/water intake

OBJECTIVE PAIN SCORE

Physiologic Data

a) Pupils
Not dilated (0)
Dilated (2)

b) % increase in heart rate
< 20% (0)
> 20% (1)
> 50% (2)
> 100% (3)

c) % increase in respiratory rate
< 20% (0)
> 20% (1)
> 50% (2)
> 100% (3)

d) Rectal temperature
Within reference range (0)
Elevated rectal temperature (1)

e) Salivation
No salivation (0)
Salivation (2)

Response to Palpation
No change in behavior (0)
Guards/reacts when touched (2)
Guards/reacts before touched (3)

Activity
At rest – sleeping, semiconscious, or eating (0)
Awake (1)
Restless (paces, fidgets) (2)
Rolling, thrashing (3)

Mental Status
Submissive (0)
Overtly friendly (1)
Wary (2)
Aggressive (3)

Posture
Lateral recumbency (0)
Sternal recumbency (1)
Sitting or standing, head up (1)
Guarding or protecting affected area (includes fetal position) (2)

Vocalization
Not vocalizing (0)
Vocalizing when touched (2)
Intermittent vocalization (2)
Continuous vocalization (3)

Appendix 2
Incision scoring system (20)

INCISION SCORE
0 - Healed wound, primary healing with no granulation tissue of evidence of inflammation
1 - Mild redness, swelling, or pain on touch, intact fibrin seal and no discharge
2 - Mild-to-moderate redness, swelling, pain with slight dehiscence or serosanguinous discharge
3 - Moderate-to-severe redness, swelling, pain with discharge serosanguinous to mucopurulent discharge
4 - Dehiscence, infection