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# The effect of preoperative antibiotic or antibiotic + vitamin C administrations on the inflammatory and oxidative state in the rabbits with experimentally induced pyometra

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**Summary:** In this study, rabbits were used as a model for canine pyometra to test the effects of preoperative therapy in the reduction of postoperative stress. Pyometra were induced in 18 healthy rabbits by the operative administration of *Pasteurella multocida* inoculum. Beginning on the third day after inoculation Group I (n=6) received enrofloxacin (2,5 mg/kg, s.c.), Group II (n=6) recieved enrofloxacin (2,5 mg/kg, s.c.) + vitamin C (100 mg, s.c.) injections for three days. Ovariohysterectomy were performed in groups I and II following this medical treatment. Group III (n=6) as the control group, did not recieve any medical therapy and were operated on the third day after inoculation. Blood samples were collected before inoculation, on the day of operation, 24$^{th}$ h, 72$^{th}$ h and at 7$^{th}$ day following the operations. Ceruloplasmin (CERU), malondialdehyde (MDA), vitamin C, haptoglobin (Hp) and serum amyloid-A (SAA) levels were analyzed. In the postoperative 72$^{th}$ h period mean CERU level of Group III were found significantly higher ($p<0,05$) than Group I and II. Mean MDA level were significantly higher in Group III on the day of operation compared to the treatment groups ($p<0,05$). SAA levels rised in all of the groups following inoculation, on the day of operation ($p<0,05$) which is followed by a gradual decline until the 7$^{th}$ day. Overall CERU and MDA levels tend to be higher in the control group compared to the treatment groups whereas Hp and SAA levels were not significantly different. These findings may indicate that preoperative therapy may reduce the oxidative stress mildly however it did not significantly alter the acute phase protein response definitively and a significant positive effect of both preoperative treatments compared to the control group could not be pronounced.

Keywords: Acute phase proteins, antioxidants, ovariohysterectomy, pyometra, rabbit.

## Tavşanlarda deneysel oluşturulan pyometra olgularında, preoperatif antibiyotik veya antibiyotik + C vitamini enjeksiyonlarının yangısal ve oksidatif duruma olan etkileri

**Özet:** Bu çalışmada pyometra operasyonu sonrası stresin azaltılmasında preoperatif sağaltımın etkisini araştırmak amacıyla tavşanlar model olarak kullanıldı. Bu amaçla onsekiz sağlıklı tavşanda *Pasteurella multocida*' nın operatif yöntemle uterusa inokulasyonu gerçekleştirildi. İnokulasyondan sonra 3. günden başlanarak Grup I' e (n=6) enrofloksasin (2,5 mg/kg, s.c.), Grup II' ye (n=6) aynı dozda enrofloksasin ve vitamin C (100 mg, s.c.) enjeksiyonları 3 gün süreyle devam ettirilmiş ve bundan sonra ovaryohisterektomi uygulanmıştır. Kontrol grubu olan Grup III' e (n=6) medikal sağaltım uygulanmamış ve inokulasyondan sonra üçüncü günde ovaryohisterektomi uygulanmıştır. Kan örnekleri inokulasyondan önce, ovaryohisterektomi gününde, postoperatif 24. saat, 72. saat ve 7. günde alınmıştır. Bu örneklerde serüloplazmin (CERU), malondialdehit (MDA), vitamin C, haptoglobin (Hp) ve Serum Amiloid A (SAA) düzeyleri belirlenmiştir. Serüloplazmin bütün gruplarda inokulasyonu takiben yükselirken Grup I ve II' de postoperatif 72s' de düşmüş ancak Grup III' de yüksek bulunmuştur ($p<0,05$). Yine Grup II' de MDA düzeyleri operasyon gününde sağaltım gruplarına göre yüksektir ($p<0,05$). SAA düzeyleri zamana bağlı olarak bütün gruplarda operasyon gününde yükselmiş daha sonra postoperatif 7. güne kadar düşmüştür. Genel olarak CERU ve MDA düzeylerinin kontrol grubunda diğer gruplara göre daha yüksek olduğu, Hp ve SAA düzeylerinde ise gruplar arasında önemli bir farklılık görülmediği saptanmıştır. Bu bulgular preoperatif sağaltımın oksidatif stresi önlemede sınırlı düzeyde etkili olurken akut faz yanıtı üzerinde önemli düzeyde etkili olmadığını göstermektedir.

Anahtar sözcükler: Antioksidanlar, akut faz proteinleri, ovaryohisterektomi, pyometra, tavşan.

## Introduction

In the final stages of canine pyometra loss of appetite, depression and fever are encountered acutely and ovariohysterectomy is indicated in most of the cases (4). These symptoms are similar to those of acute pyometra cases observed in rabbits mostly caused by *Pasteurella multocida* (10).

Some aminoacids and omega 3 acids, antioxidants, vitamins and minerals may alter inflammatory reactions and oxidative stress by enhancing the the immunity (25). Besides, possible alleviating effects of antioxidant vitamins were evident in the recovery of experimentally induced tissue damage or intestinal anastomozes in animal models (21, 24). Monitoring acute phase proteins

such as Serum Amyloid A (SAA), C - reactive protein (CRP) and Haptoglobin (Hp) may be of importance in predicting postoperative complications following ovariohysterectomy in bitches with pyometra (4).

In practice, where ovariohysterectomy as a tretment for pyometra becomes mandatory, it is often a serious decision for the clinician whether to operate immediately or to pre-treat the patient with antibiotics before the surgery. In addition, the minimalisation of early postoperative stress and complications may be possible by the supplementation of antioxidants reducing the tissue damage such as vitamin C. In this study rabbits were used as a model for canine pyometra and it was aimed to test these hypotheses by demonstrating the effects of preoperative vitamin C + antibiotic or antibiotic administrations, on the postoperative antioxidant and inflammatory status following ovariohysterectomy in rabbits with experimentally induced pyometra. Ceruloplasmin, MDA, vitamin C and acute phase proteins Hp and SAA were used to assess the oxidative and inflammatory status.

## Material and Method

*Animals and feeding:* In this study 18 New Zeland White Rabbits weighed at least 2.5 kg and aged 8 months were used. The animals were obtained from a conventional rabbit farm and caged individually, fed with water and commercial calf growing pellet feed, both given *ad-libitum*.

*Inoculations and induction of pyometra: P. multocida* strain were isolated and identified from a rabbit showing severe respiratory disease symptoms which were brought to the Small Animal Clinic of Adnan Menderes University Faculty of Veterinary Medicine. The inoculum were prepared from live passages of the strain on every morning prior to the surgical administration, finally adjusted to the concentration of McFarland = 2. The rabbits were sedated and anesthetised by the administration of xylazine (2 mg/kg, i.m.) and ketamine (44 mg/kg i.m.). Both uterine horns were exteriorised from the midline incision in dorsal recumbency. Initially a 20 G intravenous canula was directed cranially into the lumen of the right uterine horn and inoculum administered at a volume of 0.5 to 1 ml. Exactly the same administration were performed to the left uterine horn and both horns were ligated cranially to the punctuaton holes. Only crystal penicilline were administered inside the abdominal cavity before the closure of peritoneum.

Following inoculation rectal temperatures were taken on the days following inoculations: Day 0 (D0), Day 1 (D1), Day 2 (D1); first and second days of antibiotic administrations (AD 1), (AD 2); The day of ovariohysterectomy (OHE) and at 24$^{th}$ h (PO24h), 48$^{th}$ h (PO48h), 72$^{th}$ h (PO72h) and at 7$^{th}$ day (POD7) following ovariohysterectomy in Group I and II. In Group III AD1 and AD2 were omitted as no antibiotic or Vitamin C administrations were given.

The diameter of both uterine horns were measured and the thicker values were recorded for each examination (Figure 1). The measurements were performed on following days: D1, D2, AD1 and AD2 for Group I and Group II, while in Group III AD1 and AD2 were omitted as aforementioned.

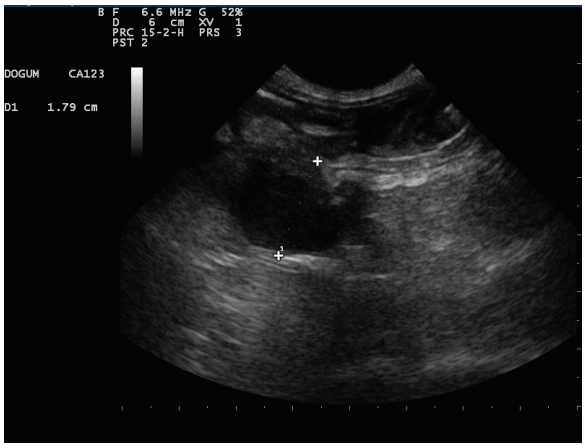


Figure 1. Ultrasonogram of the infected uterine horn.
Şekil 1. Enfekte edilmiş olan cornu uterinin ultrasonografisi.

*Treatments:* Starting on Day 3 after inoculations, below treatments and administrations were given:

Group I (n=6): Ovariohysterectomy on Day 6 (OHE) following daily enrofloxacin (2.5 mg/kg, s.c.) injections for three consecutive days.

Group II (n=6): Ovariohysterectomy on Day 6 (OHE) following daily enrofloxacin (2.5 mg/kg, s.c.) and Vitamin C (100 mg, s.c.) injections for three consecutive days.

Group III (n=6): Ovariohysterectomy on Day 3 (OHE) (Control group: No preoperative treatments).

*Ovariohysterectomy:* The same midline incision were re-incised and both uterine horns and ovaries extripated. Postoperative enrofloxacin (2.5 mg/kg, s.c.) were administered for three consecutive days to all rabbits following surgery.

*The collection of blood samples:* Blood samples were collected from the ear artery of rabbits into EDTA containing tubes and plasma obtained. First samples were taken prior to the inoculation (Day 0) while 2$^{nd}$., 3$^{rd}$., 4$^{th}$ and 5$^{th}$ samples were collected just before the OHE and PO24h, PO72h and POD7 following OHE respectively.

*Biochemical analyses:* Plasma MDA, Vitamin C and CERU analyses were performed immediately after collecting blood samples while separate plasma samples for Hp and SAA were stored at -20° C until analyzed. Plasma concentrations of Hp were determined by the hemoglobin-binding method based on the phase range Hp kit (Haptoglobin kit, Tridelta Development Ltd., Greystones, Ireland) using microtiter plates and an ELISA reader (Optic Ivyman System, Spain). Plasma concentrations of SAA were measured by the solid phase sandwich ELISA kit (SAA kit, Tridelta Development Ltd., Greystones, Ireland). Ceruloplasmin concentrations

were estimated in plasma *ad modum* (22). MDA, formed from the breakdown of polyunsaturated fatty acids, was considered as an index for the peroxidation reaction. The absorbance of the reaction product of MDA with TBA was measured at 532 nm. Quantification was based upon a molar extinction coefficient of 1.56 x 105 M-1 cm-1. The plasma VIT C concentrations were measured by the phosphotungstic acid method of Kway (13).

*Statistical Analyses:* Intergroup relationships between biochemical parameters (VIT C, MDA, Hp, SAA and CERU) at five different samplings were calculated by variance analysis using One-Way ANOVA. In group differences for every group were analysed separately by means of variance tests for reperated measures, which is followed by Bonferroni pairwise comparisions. The changes according to time were also analysed by variance tests for repeated measures and Bonferroni pairwise comparisions concerning rectal temperatures and ultrasonographic measurement of uterine diameters in all groups througout the study.

## Results

In all animals by Day 2 uterine lumen reached a diameter of 1 cm, detectable in USG and/or body temperature reached over 40°C, as clinical manifestations of pyometra. Significant differences in Groups II and III were observed between D0 (39.96 ± 0.27 ºC) and PO72 (39.06 ± 0.58 ºC) ($p<0,05$); Day 0 (39.6 ± 0.29ºC) and PO48h (38.53 ± 0.36ºC) respectively ($p<0.05$). Mean diameter of uterine horns rised up to 1.59 ± 0.48 cm on AD2 which is signficantly different from D1 (0.96 ± 0.27 cm) ($p<0.05$) in Group I. In Group III also there was a statistically significant change of mean uterine diameter in between D1 (0.78 ± 0.14 cm) and D2 (1.33 ± 0.11 cm) ($p<0.05$) (Figure 2).

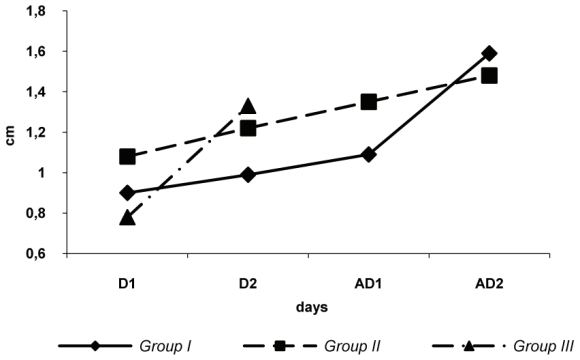


Figure 2. Uterine diameters in all groups following inoculation.
Şekil 2. Bütün gruplarda inokulasyon sonrasında uterus çapları.

Table 1. Plasma concentrations of CERU (mg/dL), MDA (µmol/L), VIT C (mg/dL), HP (g/L) and SAA (µG/mL) of rabbits throughout the experiment.
Tablo 1. Deney süresince tavşanlarda CERU (mg/dL), MDA (µmol/L), VIT C (mg/dL), HP (g/L) and SAA (µG/mL)' nın plazma konsantrasyonları.

| | | *Day 0 (pre-inoculation)* | *OHE Day* | *Post-op 24 h* | *Post-op 72 h* | *Post-op Day 7* | p (time effect)** |
|---|---|---|---|---|---|---|---|
| CERU (mg/dL) | *G I* | 44.37 ± 16.82$^{a}$ | 197.25 ± 33.24$^{b}$ | 201.92 ± 39.18$^{b}$ | 102.81 ± 23.39$^{Bc}$ | 110.35 ± 13.16$^{c}$ | P<0.001 |
| | *G II* | 66.91 ± 29.79 | 140.33 ± 99.59 | 156.09 ± 87.95 | 90.84 ± 41.1$^{B}$ | 85.83 ± 39.61 | P<0.05 |
| | *G III* | 81.24 ± 30.89$^{a}$ | 132.16 ± 41.21 | 175.96 ± 30.99$^{b}$ | 164.63 ± 39.43$^{A}$ | 103.44 ± 12.87$^{a}$ | P<0.005 |
| p (group effect)* | | NS | NS | NS | P < 0,05 | NS | |
| MDA (µmol/L) | *G I* | 5.08 ± 1.26 | 2.74 ± 0.86$^{Ba}$ | 4.03 ± 2.36 | 7.97 ± 2.33$^{b}$ | 3.57 ± 4.7 | P<0.05 |
| | *G II* | 3.84 ± 1,59 | 2.25 ± 1,81$^{B}$ | 7.84 ± 4.62 | 11.52 ± 6.24 | 9.29 ± 5.15 | P<0.05 |
| | *G III* | 4.22 ± 0.63$^{a}$ | 4.93 ± 1.84$^{A}$ | 7.15 ± 2.89 | 7.72 ± 1.68$^{b}$ | 6.39 ± 3.97 | NS |
| p (group effect)* | | NS | P < 0,05 | NS | NS | NS | |
| VIT C (mg/dL) | *G I* | 0.1 ± 0.04 | 0.14 ± 0.02 | 0.25 ± 0.14 | 0.29 ± 0.19 | 0.21 ± 0.1 | NS |
| | *G II* | 0.21 ± 0.16 | 0.38 ± 0.27 | 0.53 ± 0.24 | 0.24 ± 0.19 | 0.25 ± 0.07 | NS |
| | *G III* | 0.12 ± 0.06 | 0.21 ± 0.09 | 0.21 ± 0.3 | 0.11 ± 0.05 | 0.13 ± 0.05 | NS |
| p (group effect)* | | NS | NS | NS | NS | NS | |
| Hp (g/L) | *G I* | 0.91 ± 0.48$^{a}$ | 3.65 ± 0.96$^{b}$ | 4.2 ± 2.96 | 2.06 ± 1.43 | 1.03 ± 0.061$^{c}$ | P<0.05 |
| | *G II* | 1.28 ± 0.96$^{a}$ | 2.72 ± 2.51 | 5.96 ± 2.19$^{b}$ | 3.11 ± 2.19 | 1.82 ± 2.23 | P<0.05 |
| | *G III* | 1.49 ± 1.08 | 2.87 ± 2.12 | 2.56 ± 1.47 | 1.85 ± 1.1 | 1.2 ± 0.45 | NS |
| p (group effect)* | | NS | NS | NS | NS | NS | |
| SAA (µg/mL) | *G I* | 4.04 ± 1.97$^{a}$ | 56.95 ± 21.14$^{b}$ | 55.46 ± 17.89$^{b}$ | 17.88 ± 7.57$^{c}$ | 4.3 ± 3.74$^{a}$ | P<0.001 |
| | *G II* | 13.03 ± 14.38 | 38.38 ± 28.8 | 61.78 ± 31.68 | 30.66 ± 21.58 | 6.55 ± 2.89 | P<0.05 |
| | *G III* | 23.09 ± 25.67$^{a}$ | 40.81 ± 29.56 | 80.59 ± 19.84$^{b}$ | 24.53 ± 25.82 | 5.23 ± 2.84$^{c}$ | P<0.05 |
| p (group effect)* | | NS | NS | NS | NS | NS | |

* Different superscripts A,B in the same column indicate significant differences (p: group effect).
** "p" level indicates significant differences calculated by ANOVA repeated measures;
Different superscripts a,b,c in the same row indicate significant differences between measuraments at different times within a group (Multiple comparisions calculated by Bonferroni pairwise comparisions' adjusted to mean siginifance level of $p<0.05$)

Significant changes in biochemical parameters throughout the study were observed in CERU, MDA, Hp and SAA concentrations. Additionally, significant differences were observed at PO72h in CERU, and on the the OHE Day in MDA concentrations in between groups which are all demonstrated in Table 1.

## Discussion

Various methods have been described to induce CEH/pyometra in the bitch. These include the inoculation of bacterial strains especially *E. coli*, insertion of irritant substances into the uterus and hormonal applications or combinations of the above methods (2, 19). Following a series of experiments De Bosschere et al. (6) have administered *E. coli* inoculum into one uterine horn succeeding with the ligation of the horn in metestural bitches. By this procedure the researchers have achieved to induce a histologically and immunohistochemically similar CEH/pyometra to the natural occuring entity in the bitch (6).

In the present study it was primarily aimed to inoculate the rabbit uterus via an intrauterine catather guided through cervix with real time USG, which would avoid the possible influence of surgical stress on the antioxidant and acute phase proteins. However no fluid accumulation in the uterus and no increase in the body temperature were observed following inoculation and on the $4^{th}$ day explorative laparatomy have revealed no macroscopical difference from the uninoculated uterine horn. The reason for the failure to achieve an infection were postulated as the high motility of the rabbit uterus under normal cyclical hormonal activity. Consequently, it did not seem possible to inoculate cyclical rabbits via cervical catatherisation thus the researchers have used a similar procedure which De Bosschere et al. (6) have described. This resulted in a distinct development of pyometra manifested by increase in the uterine diameter detected by ultrasonography together with increase in the body temperature. Macroscopically the fluid filled in the uterus had a creamy or foamy appereance. These symptoms seem to mimic the conditon of carnivores that are brought to the clinics with decreased appetite and mild depression. The condition was easy and fast to reproduce in about 3 days time and thought to be a model for the CEH/pyometra studies concerning general condition of the individual, like the present study.

VIT C is required for the biosynthesis of collagen during tissue development and at sites of tissue damage besides it prevents and reduces the oxidation of biomolecules (15). Under physiological conditions rabbits produce sufficient VIT C for their daily maintanence however it is always considered as a marker of oxidative stress and the reduction of its content may indicate an increase in oxidative stress (7). Research has been focused on nutritional supplementation of rabbits with VIT C in various disease or traumatic conditions (12, 18). In the present study it was aimed to show the changes in blood VIT C during pre and postoperative course and to test if the preoperative parenteral administration of VIT C have altered postoperative stress. VIT C levels were higher in the VIT C + antibiotic administered group compared to the other groups, as would be expected however these differences were insignificant. Also no statistically significant changes in VIT C levels were observed in any of the groups throughout the study. It is notable and interesting that VIT C levels rised in all groups following inoculation until the OHE Day and PO24h, and decreased again at POD7, insignificantly. This increase contrasts with the expectations that more consumption of antioxidants during infections should end up in decrease of plasma VIT C levels. However as this condition could not be verified or rejected by previous data, further investigations concerning biochemical interactions of ascorbic acid biosynthesis are required.

Plasma CERU have been shown to rise in acute and chronic infections and also in experimentally induced carcinoma (8, 23). Endotoxin-induced ocular inflamation caused a decrease in the concentration of ascorbic acid in the aqueous humor and an increase in the vitreous humour of rabbits as well as CERU increased together with the copper concentration in the aqueous humor as a result of influx from the blood. (17). In the present study plasma CERU levels have rised in all three groups following inoculation and then decreased near pre-inoculation concentrations on POD7, a result in accordance with the above studies. In Group I the differences throughout time were statistically significant and peak concentration was reached on PO24h whereas in the control group (Group III) a similar profile was observed and the peak concentration was reached also on PO24h. The differences throughout time were insignificant in Group II which also have shown a similar CERU profile. It was also remarkable that a significant difference in mean plasma CERU on PO72h between the control and the other groups was observed which indicates that preoperative VIT C and antibiotic or solely antibiotic administrations have caused to alleviate the severity of acute phase protein response.

MDA is a degradation product of lipid peroxidation and has been used as a marker of oxidative stress. In studies where rabbits were used as experimental models concerning, amelioration by various compounds in ischemic reperfusion of various tissues MDA is used to demonstrate the degree of oxidative stress (9, 14). Similarly toxiciological effects of some cancer drugs have also been studied (11). However there are a limited number of studies on the action of MDA during inflammatory processes. Yaralioglu-Gurgoze et al. (26) have reported that in mares with endomteritis MDA

levels were significantly higher compared to that of healthy mares. Yoruk et al. (27), experimentally induced staphylococcal maxillary sinusitis in rabbits and observed that treatment with antibiotics revealed lower tissue concentrations of MDA compared to methylprednisolone treated or untreated animals. In the present study mean plasma MDA levels in the control group were significantly higher than the two other treatment groups on the OHE Day ($p<0,05$), a data seems to be in accordance with the above findings. However mean plasma MDA concentrations of all groups significantly rises through time making its peak level on PO72h. Interestingly, highest MDA levels at this time were observed in Group II compared to the other groups that were moderate. The decrease in MDA concentrations of Groups I and II following the inoculation until the OHE Day can be attributed to the administration of medications while cessation of exogen VIT C after this point may have lead to the increase in MDA levels in Group II.

Dabrowski et al. (5) have monitorized the acute phase protein concentrations in the healthy or pyometra suffering bitches that have been undergone ovariohysterectomy. The Hp concentrations of healthy bitches did not differ from the pyometra affected ones prior to the surgery. However a significant rise in both groups' Hp levels were observed beginning from the 3$^{rd}$ day postoperatively and reaching peak levels on the 5$^{th}$ day and consequently declining to preoperative concentrations. This condition were attributed to the fact that, as Hp is a type II acute phase protein which slowly reaches peak levels in 4-6 days and at this time periods can be used to observe postsurgical stress. In this study, Hp levels in the control group did not increase significantly following inoculation or ovariohysterectomy instead a significant rise compared to the initial values in Group I at the OHE Day and in Group II at the Post op 24$^{th}$ hour were observed. Hp levels declined to initial values before inoculation on the post-op 7$^{th}$ Day. This finding may be explained as Group III animals were exposed to pyometra 2 days less than the other groups while not recieving any antibiotics or VIT C and until the ovariohysterectomy day Hp levels did not have the time to rise significantly.

The acute rise in SAA concentrations have been reported in most mammals during various acute inflammatory conditions (1, 3, 20). It is believed that SAA is one of two major APPs in the rabbit while the other one is C-reactive protein (16). Dabrowski et al (5) have reported that SAA concentrations together with CRP, were significantly higher in bitches with pyometra prior to ovariohysterectomy while it similarly rised in healthy bitches following 24 h and declined to normal levels in both groups after 10 days following ovariohysterectomy. Same researchers also have revealed the potential importance of SAA and CRP as early predictors of postoperative complications, based on the higher levels observed in complicated ovariohysterectomy cases compared to non complicated patients (4). In the present study only SAA concentrations were measured. In none of the time periods no significant difference have been observed inbetween groups. However A significant rise of SAA levels in Group I was observed on PO24h and gradually declined to similar levels prior to the inoculation, by POD7. A similar trend were also observed in the two other groups and significant differences throughout time were also oberved in Group III, at PO72h. Interestingly all groups were at similar levels by POD7. These findings re-indicate the importance of SAA levels in acute phase reactions while a benefical effect of VIT C administration in Group II with respect to SAA levels could not be pronounced. It can be emphasised that the operative inoculation process may have caused an abundant rise of inflammatory proteins particularly SAA which may mask the possible effect of preoperative therapy.

In conclusion significant changes were observed in all parameters throughout the experiment beginning by the initiation of experimental infection and continuing in and after the ovariohysterectomy treatment process in all groups. CERU and MDA levels tend to be higher in the control group compared to the treatment groups whereas Hp and SAA levels were not significantly different. These findings may indicate that preoperative therapy have achieved to reduce the oxidative stress to a limited extent however it did not significantly alter the acute phase protein response definitively. Regarding the lower profile of acute phase proteins in the control group it may be benefical to immediately operate animals suffering from pyometra rather than an extended preoperative therapy either with antibiotics or VIT C, however before this can be stated it must be noted that the possible masking effect of operative inoculation should be elucidated by the progression of a non surgical procedure.

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