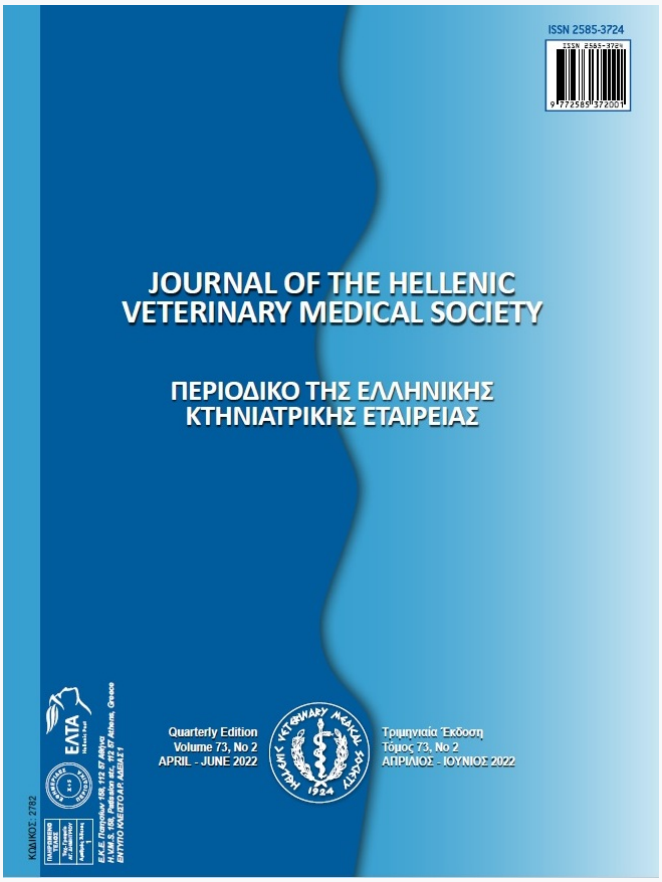
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Research article
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Oxidative stress in dogs during mid-gestation abortion: Fetal and maternal antioxidant alterations with clinical and hematological features

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ABSTRACT: The present study aimed to evaluate the GSH and MDA levels in dog's serum and vital organs of aborted fetuses, after application of two abortifacient. Animals of group I (n = 6) were treated twice with subcutaneous injection of 10 mg/kg aglepristone and group II (n = 3) received prostaglandin F2α at a dose of 125 μg/kg every 12 hours until abortion process completed. Hematologic examinations and sedimentation rates were performed of bitches. The duration between the first occurrence of vaginal discharge to first expulsion of fetuses ranged between 14-29 hours (mean: 22.6±5.5 h) and 24-33 hours in group I and II, respectively. Both treatments significantly increased the serum MDA levels (P<0.01); however aglepristone increased the MDA levels throughout the study, prostaglandin enhanced plasma MDA levels only on day 1. GSH levels were significantly lower in aglepristone-treated bitches than prostaglandin-treated animals (P<0.05). The treatments resulted in similar MDA levels in liver and heart tissues (P>0.05). GSH levels in kidney, muscle and heart tissues did not differ between groups (P>0.05), aglepristone resulted reduced GSH levels in liver tissue (P<0.05). High MDA levels could be evaluated as a useful marker for fetal suffering in pregnancy controls. The MDA and GSH levels, measured in vital organs of fetuses, could suggest possible toxic effects of abortifacients in newborns, as both medical agents are being used in parturition induction.

Keywords: Pregnancy termination, Malondialdehyde, Reduced Glutathione, Prostaglandin, Aglepristone, Dog

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INTRODUCTION

Unwanted pregnancy termination is a common procedure in canine reproduction. Safe and applicable methods consisting of inexpensive pharmacological agents for abortion are particularly desired. The administration of different abortifacients such as, prostaglandin F2α, dopamine agonists, dexamethasone and progesterone blockers were tested at different doses, alone or in combination for mid-gestation pregnancy termination in dogs (Kaya et al., 2014; Petterson and Tidholm, 2009; Galac et al., 2000; Cetin et al., 2000; Corrada et al., 2006; Hori et al., 2002; Gobello et al., 2002; Onclin and Verstegen, 1999; Feldman et al., 1993; Shille et al., 1984; Wanke et al., 1997; Zone et al., 1995; Onclin and Verstegen, 1996). Great oxygen consumption by feto-maternal unit and alterations in metabolic pathways are the reasons of increased oxidative stress during pregnancy period, leading imbalance between the formation of free radicals and the capacity for defense of the antioxidant mechanisms (Harma et al., 2004; Casanueva and Viteri, 2003; Vannucchi et al., 2007; Krieger and Loch-Caruso, 2001). Catalase is a major determinant in protecting ovary, embryo and sperm from oxidative damage. It prevents damages caused by oxidative stress by removing O2, generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Arslan et al., 2019). Decreased enzyme activity leads to the imbalance of elevated oxidative stress and reduced anti-oxidants which could be the reason of pathophysiology of pregnancy-related disorders, such as abortions, pre-eclampsia (Kolusarı et al., 2008; Liu et al., 2011; Khan et al., 2019; Issa et al., 2012).

The end product of polyunsaturated fatty acid is malondialdehyde (MDA) is a reliable biomarker of lipid peroxidation (LPO) (Acaroz et al., 2018). Increased free radicals generated during metabolic reactions, could have deleterious effects on cells, leading tissue damage and cell death (Turgut et al., 2006; Athar et al., 1993; Halliwell, 1997). As exogenous and endogenous factors such as stress, infectious, medical agents, UV rays, food additives could markedly increase lipid peroxidation in brain, lung, kidney and liver, MDA becomes an important marker due to enhanced oxidative stress (Acaroz et al., 2018). On the other hand, some antioxidant mechanisms exist, that are effective in cellular defense against oxidative injury (Turgut et al., 2006; Van Bladeren, 2000). A major non-enzymatic antioxidant, Glutathione (GSH), plays an important role in protection of cells against lipid peroxidation by free radicals and the toxicity of xenobiotic electrophiles, and maintaining redox homeostasis (Van Bladeren, 2000; Forman et al., 2009).

There are some reports about oxidative stress parameters during spontaneous miscarriage in women; however no information is available of these parameters during the abortion in dogs, not even investigated during canine pregnancy. The oxidative stress status during mid-gestation abortion in dogs has to the best of our knowledge not been studied before; therefore, the aim of this study was to investigate the GSH and MDA levels after application of two different abortifacients, used so often in small animal reproduction.

MATERIALS AND METHODS

Animals

Nine clinically healthy, mix-breed pregnant bitches between day 25 and 35 of gestation, aged 2-5 years, and with a body weight of 14-30 kg, were used in the study. All bitches, included to the study, were brought to Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Bursa/Turkey with a history of unwanted pregnancies. They were hospitalized during the process, fed a standard commercial dog food once daily, and given water *ad libitum*. Animals of group I (n = 6) were treated twice with subcutaneous injection of 10 mg/kg aglepristone (Alizin®, Virbac, Germany) with a 24 hours interval. Animals of group II (n = 3) received repeated intramuscular injections of prostaglandin F2α (Prostagin® Aksu Eczacılık ve İlaç San, Ankara, Turkey) at a dose of 125 μg/kg every 12 hours until abortion process completed.

Approval of the ethical committee of the Uludag University for using the animals was obtained (2014-03/02). Pregnancy confirmations were carried out by ultrasonographic examinations (5-7.5 MHz linear array transducer; Dynamic Imaging MCV-Concept, UK).

Sampling and examinations

The stage of pregnancy was defined according to reference equations by measuring fetal and extra-fetal structures (Luvoni and Grioni, 2000). The bitches were ultrasonographically examined every 12 hours. Not only the termination of pregnancies, but also the survivals of the fetuses were monitored by ultrasonography. The time period between first medical treatment-vaginal discharge and first abortion, number of aborted fetuses, body temperature of bitches and side effects were recorded. Aborted death fetuses

were collected; liver, kidney, heart, and skeletal muscle (*musculus gastrocnemius*) tissues of fetuses were immediately removed and stored at −20°C until analysis. In order to assess hematological consequences of treatments, collection of blood samples from saphenous vein of bitches were performed EDTA containing tubes. For MDA and GSH measurements, serum samples were stored at −20°C after centrifugation at 5000 rpm for 10 minutes.

Hematologic examinations

Numbers of erythrocyte and hematocrit values were estimated according to the methods reported by Jain (1986). A small amount of blood (about ¾ of tube) was placed in heparinized micro hematocrit capillary tube and centrifuged at 12000 rpm for 5 minutes. Also MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration), were estimated according to Jain (Jain, 1986).

Erythrocytes and leucocytes were counted by Thoma count slide. Blood samples were prepared with hayem and Turk solutions for erythrocyte and leucocyte examinations, respectively. Thoma slides were examined under a light microscope with magnification of 100x. The levels of hemoglobin were measured by Sahli method. A differential white cell count (leukocyte formula) consists of an examination of blood to determine the presence and the number of different types of white blood cells. Leukocyte formula was performed by peripheral blood smear stained by Pappenheimer panoptic staining method (Konuk, 1981). The erythrocyte sedimentation rate was measured by the Westergren method (Lewis, 1980) and reading was taken after 24 hours.

Determination of oxidative stress markers

Evaluation of malondialdehyde (MDA) levels in blood and tissue samples

Blood and tissue levels of MDA were determined after DNPH derivatization as described previously (Pilz et al., 2000). To prepare the blood samples, 360 µl serum and plasma was mixed 40 µl 10% sulfuric acid then centrifuged at 10000 rpm for 10 min. Two hundred µl supernatant was mixed with with 20 µl of 5 mM of DNPH prepared in 2 M hydrochloric acid and incubated for 24 h under dark. Tissues obtained from fetuses were homogenized in 2 ml of 1% sulfuric acid. Two hundred µl homogenate was mixed with 20 µl of 5 mM of DNPH prepared in 2 M hydrochloric acid and incubated for 24 h under dark. The mixture was then centrifuged at 10,000 × g for 10 min and 20 µl supernatant was injected onto HPLC system (model PU-980 liquid chromatography pump; Jasco, Japan). DNPH derivative of MDA was separated on a C18 reversed phase column (Macherey-Nagel GmbH, Duren, Germany) with a flow rate of 0.7 ml/min and UV detector (UV-1601 UV-Visible Spectrophotometer SHIMADZU, Japan)was set at 310 nm. The mobile phase was consisted of acetonitrile-distilled water (38: 62; v/v) containing 0.2% (v/v) acetic acid. MDA peaks were determined according to its retention time and confirmed by spiking with added exogenous standard. Concentrations of MDA were calculated from standard curve prepared from 1,1,3,3 tetramethoxypropane and expressed as pmol/mg protein for the tissues.

Evaluation of reduced glutathione (GSH) levels in blood and tissue samples

GSH levels of samples were determined according to the Ellman method (Ellman, 1959). For the collected blood samples from bitches, 360 µl serum and plasma was mixed 40 µl 10% sulfuric acid then centrifuged at 10000 rpm for 10 min. The tissues obtained from fetuses were homogenized in 2 ml of 1% sulfuric acid. Either 300 µl supernatant from blood samples or tissue homogenates were mixed 1 ml 0.3 M of Na2HPO4.2H2O (BioShop, Burlington, Ontario, Canada) and 0.2 mL of 0.4 mg/mL of 5,5- AQ18 dithiobis-2-nitrobenzoic acid (Sigma Chemical Co, St Louis, Mo) in 1% sodium citrate. After vortexing, the absorbance was read at 405 nm. The results were expressed as micromolar per milligram protein or ml serum and plasma.

Statistical analysis

The data obtained were analyzed using the GraphPad software, version 6.0 (GraphPad Software, Inc., La Jolla, CA) and expressed as the mean ± S.E.M. Statistical analysis was done using the 2-way analysis of variance test (ANOVA), followed by the post hoc Tukey's (aglepristone versus prostaglandin) or Dunnet's (versus baseline values) test. Student's unpaired t test was used for comparison between the two different treatment groups (e.g. MDA and GSH levels of fetus tissues). The P values < 0.05 were considered significant.

RESULTS

The effects of aglepristone and prostaglandin on

clinic parameters

The confirmation of pregnancy and abort process were carefully monitored by ultrasonography. Gestational sacs and fetuses with hearts beats were clearly detectable before treatments. Vaginal bleeding and discharge started 10-48 hours after first injection of aglepristone (mean: 32.8±13.8 hours), whereas beginning of bleeding was recorded between 25-30 hours after first injection of prostaglandin in bitches (mean: 27.6±2.5 hours). Pregnancy was terminated by aglepristone ranging from 49 hours to 99 hours after initiation of treatment in all bitches of group I (mean: 67.3±24.2). On the other hand abortion process completed 62 and 76 hours after first injection of prostaglandin in two bitches and after 4 days in one bitch. The duration between first occurrence of vaginal discharge to first expulsion of fetuses ranged between 14-29 hours (mean: 22.6±5.5 h) and 24-33 hours in group I and II, respectively. The mean timespan between initiation of aglepristone and prostaglandin treatments and observation of first aborted fetus were found 55.5±15.2 and 55±4.5 hours, respectively. The mean expulsion intervals between fetuses during abortion process was recorded 50±6.12 minutes (range: 45-60) for group I. However this interval was observed between 2.5-3.5 hours in two bitches in group II.

No gross abnormality was observed in the aborted fetuses in both groups during necropsy. The mean (± s.d.) crown rump length of the fetuses were 6.68±1.82 (range:3.5- 8.5cm) and 11.22±3.25 (range:16-8.5 cm) in groups I and II, respectively. The mean number of aborted fetuses per bitch was 3±2.12 (range:1-6) for group I and 4.5±2.12 (range: 3-6) for group II. Ultrasound examination showed fetuses with a decreasing amount of fetal fluid on consecutive examinations. Fetal heart beats were clearly detected although abortion had started. No dead fetuses were observed during the examinations until the abortions completed. No serious side effect was observed in bitches in group I, however uncompleted abortion process in one bitch and excessive salivation, prostration, vomiting, hyperpnoea, and anxiety were reported in all bitches in group II.

The effects of aglepristone and prostaglandin on hematologic parameters

Haematological parameters measured for both groups are shown in Table 1 and no significant difference was observed between groups. Although the parameters were within the normal ranges, the percentage of eosinophils has tendency an increase during the process in group I than group II (P>0.05).

The effects of aglepristone and prostaglandin on blood sedimentation time

Blood samples were collected from each animal and the erythrocyte sedimentation rate was determined from the blood samples, which collected before drug administrations (baseline) and on the day after abortus. The value of erythrocyte sedimentation rate was determined 24 hr after the blood collection. While prostaglandin induced a decrease in sedimentation rate after abortus (t=2.604, df=6, P< 0.05); conversely aglepristone failed to induce a decrease (t=0.4789, df=4, P> 0.05) (Fig 1). As shown in Fig 1, significant differences were noted for erythrocyte sedimentation rate 24 hr after abortus between aglepristone and prostaglandin treated animals (t=6.410, df=5, P< 0.01).

The effects of aglepristone and prostaglandin on MDA and GSH levels of plasma and serum in bitches

The blood samples were collected from the bitches which were in mid gestation period and the two treatment protocols were started to be applied just after first blood collection. The results of MDA and GSH levels of the first collected blood sample were record-

Table 1: Haematological parameters measured for groups Aglepristone and Prostaglandin

Observation	Treatment	WBC ($x10^9$/L)	RBC ($x10^{12}$/L)	Hb (g/l)	PCV (%)	MCV (fl)	MCH (pq)	Neut ($x10^9$/L)	Lymph ($x10^9$/L)	Mono ($x10^9$/L)	Eos ($x10^9$/L)
Day0	Aglepristone	9.77±1.49	6.35±0.45	13.48±0.77	44.83±2.29	71.69±4.19	21.43±0.76	70.50±3.66	20.00±2.98	2.83±0.87	6.67±2.32
	Prostaglandin	11.43±2.63	5.62±0.48	11.17±0.34	45,33±5,24	74.76±5.82	20.47±1.54	82.00±2.08	13.67±1.67	0.67±0.33	4.00±1.15
Day1	Aglepristone	11.65±0.64	6.54±0.27	12.42±0.40	43.58±2.08	66,84±2.87	19.07±0.63	75.00±4.03	17.83±2.77	0.83±0.65	6.17±2.41
	Prostaglandin	12.92±0.99	6.47±0.87	11.83±0.90	45.33±5.24	70.73±6.98	18.98±1.42	81.67±1.67	14.67±1.45	1.00±0.58	3.33±0.89
Day2	Aglepristone	11.95±0.95	6.72±0.41	14.00±0.76	43.50±1.99	65.29±2.88	20.94±0.84	68.33±4.19	21.50±5.05	1.67±0.21	8.50±1.86
	Prostaglandin	12.87±1.41	6.31±0.56	11.63±0.63	45.00±4.62	71.17±5.37	19.34±0.63	80.33±5.36	15.33±1.76	1.33±0.67	4.00±1.53
Day3	Aglepristone	9.57±0.91	6.68±0.28	13.25±0.96	41.50±2.05	62.07±1.53	19.74±0.84	69.33±2.09	21.17±1.44	1.67±0.49	7.67±0.95
	Prostaglandin	12.53±0.93	5.89±0.52	11.47±0.29	43.33±5.04	67.03±7.01	18.31±1.27	80.33±3.48	15.00±1.73	1.33±0.67	3.67±1.76
Day4	Aglepristone	9.34±1.12	6.61±0.46	12.07±0.63	40.75±1.79	62.58±3.08	18.43±0.64	68.50±3.98	21.83±3.53	2.00±1.03	7.33±2.12
	Prostaglandin	12.53±0.93	5.89±0.52	11.47±0.29	43.33±5.04	67.033±7.01	18.31±1.27	80.33±3.48	15.00±1.73	1.33±0.67	3.67±1.76

ed as baseline values. The evaluation of MDA and GSH levels in blood samples were performed daily. All animals showed induction of abortus in 5 days; hence the values were evaluated as before and after abortus for all animals.

As seen in Figure 2, no significant difference was found between baseline values for serum MDA (t=0.8325, df=5, $P<0.05$; student t test). However, aglepristone treatments increased significantly the MDA levels in serum and prostaglandin was able to increase only at 1st day [F (1,5) = 25.86, $P<0.01$; 2-way ANOVA]. Also, in a fore mentioned evaluations, an increase in MDA level by prostaglandin was observed, but this enhancement was limited only day 1.

We next determined the possible effects of aglepristone and prostaglandin on serum GSH levels at daily time points. Both aglepristone and prostaglandin did not alter the levels of GSH in serum specimens [over all 2-way ANOVA; F (1,5)=25.82, $P<0.01$; Fig 3]. There was only one significant time point which indicates that aglepristone and prostaglandin reduced the GSH levels in plasma (day 1; post hoc, $P<0.05$). On the other hand, GSH levels obtained from aglepristone-treated bitches were significantly lower than prostaglandin-treated animals ($P<0.05$).

The effects of aglepristone and prostaglandin on MDA and GSH levels of tissues obtained from fetuses

Figure 4 and 5 shows the effects of aglepristone and prostaglandin on MDA and GSH levels in kidney, liver, muscle and heart tissues which obtained from aborted fetuses. To determine possible different induction rate of MDA and GSH levels by aglepristone and prostaglandin, we simply evaluated the MDA or GSH levels of tissues and compared to each other using student t test. Aglepristone resulted higher MDA levels in kidney and muscle when compared to prostaglandin (t=2.405, df=15, $P< 0.05$ and t=2.155, df=15, $P< 0.05$; respectively, Fig 4). Aglepristone and prostaglandin treatments resulted similar MDA levels in liver and heart tissues (t=0.2422, df=14, $P> 0.05$ and t=0.2624, df=14, $P> 0.05$; respectively, Fig 4). As seen in Figure 5, while the GSH levels in kidney, muscle and heart tissues did not differ between aglepristone and prostaglandin treatment groups (t=1.508, df=12, $P>0.05$; t=0.4290, df=16, $P> 0.05$ and t=0.9818, df=16, $P> 0.05$; respectively, Fig 4), aglepristone resulted reduced GSH levels in liver tissue (t=2.234, df=14, $P< 0.05$, Fig 5).

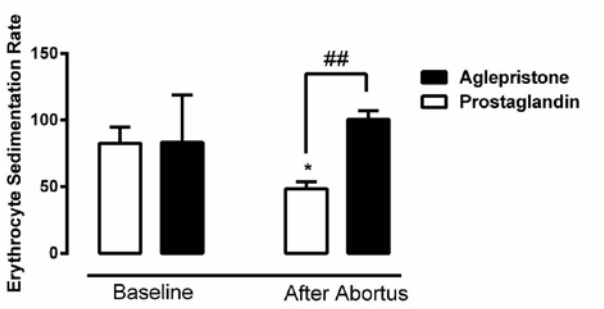


Figure 1: The effects of aglepristone and prostaglandin on blood sedimentation time

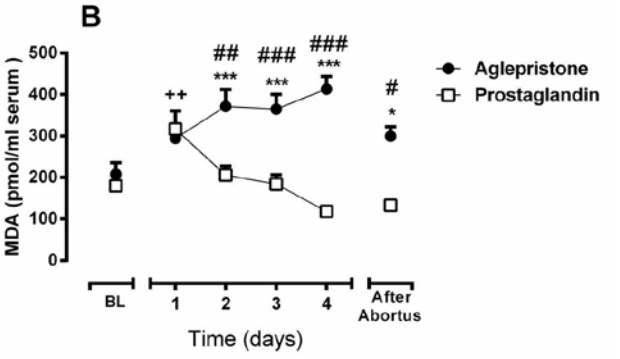


Figure 2: The effects of aglepristone and prostaglandin on MDA levels of serum in bitches

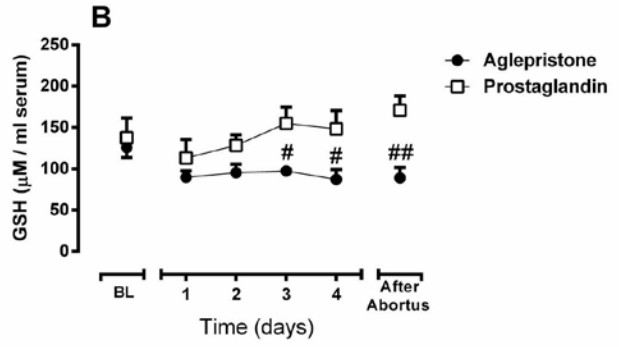


Figure 3: The effects of aglepristone and prostaglandin on GSH levels of serum in bitches

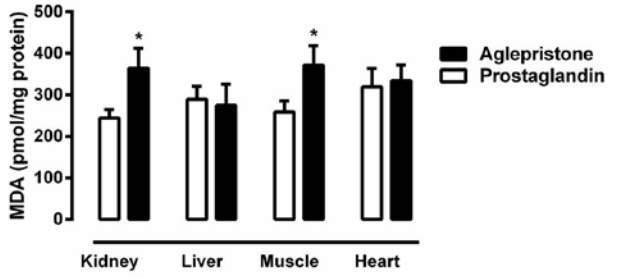


Figure 4: The effects of aglepristone and prostaglandin on MDA levels of tissues obtained from fetuses

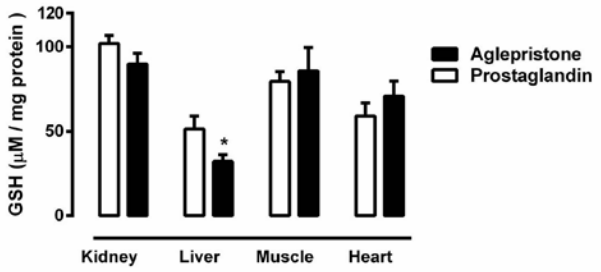


Figure 5: The effects of aglepristone and prostaglandin on GSH levels of tissues obtained from fetuses

DISCUSSION

Aglepristone and prostaglandin F2α are used as major abortifacients in dogs and cats (Galac et al., 2000; Georgiev and Wehrend, 2006; Fontbonne et al., 2009; Fieni et al., 2001; Baan et al., 2005; Concannon and Hansel, 1997; Romagnoli et al., 1991; Oettlé, 1982). The mean time table for completion of abortions and first vaginal bleeding and discharge in this study are similar with previous observations for both medical agents (Petterson and Tidholm, 2009; Corrada et al., 2005). Eosinophils have actually great role on allergic reactions and inflammation (Gleich, 2000). Eosinophil accumulation in uterus and mammary gland development had been reported during pregnancy (Gouon-Evans et al., 2000). Besides that, eosinophils contain lysosomal granules which support the tissue turnover, and thereby maintain the pregnancy process (Ross and Klebanoff, 1966).

In the present study, although there were no statistical differences in haemogram parameters, eosinophils were higher in group I than group II. This may be due to participating activation of eosinophils on the immunoglobulins, cytokines and platelet activating factors (Gleich et al., 1993). Erythrocyte sedimentation rate (ESR) is a marker of inflammation which also belonged to increased free radicals. Physiologically fibrinogen rises during pregnancy, and thereby sedimentation rate is high (Hameed and Waqas, 2006). Authors indicated that fibrinogen, which is an acute phase protein, and immunglobulins can promote the ESR during pregnancy (Lowlier et al., 1977). Interestingly ESR level was higher in aglepristone group than prostaglandin after abortus ($p>0.05$). It could be suggested that fibrinogen production could continue after abortus due to aglepristone, and organism could be protect aganist to inflammation. Pregnancy is a complex process for both animals and women, including physiological and biochemical changes for developing fetus and mother (Soma-Pillay et al. 2016). Lipid peroxidation during pregnancy is also expected to be increased, due to high oxygen demands for these changes (Vannucchi et al., 2007; Szczubiał et al., 2015). This study assessed lipid peroxidation during mid-gestation abortion in dogs, with clinical and hematological alterations by two treatment protocols. The blood and tissue sampling were performed only in mothers and their aborted fetuses, brought to our clinics with a history of unwanted pregnancies. No control group, without any medicine application, could be created because of ethical rules. On account of having no information about lipid peroxidation in normal pregnancy in this study, the results of MDA and GSH levels by the use of two important abortifacient had been reported. In order to eliminate this disadvantage, blood samples prior to the treatment protocol were collected as control samples or baseline values.

Aborted fetuses were also evaluated for the safety of drugs, as these two medical agents are being used for parturition induction. Severe DNA damage response, generated by uncontrolled reactive oxygen species (ROS) in cells, is mostly described as the alterations in the cellular redox state during hypoxia or oxidative stress. High lipid peroxidation rates determined by MDA, cause irreversible tissue damages in organs (Acaroz et al., 2019; Jackson et al., 1998). Reduced glutathione (GSH) and malondialdehyde (MDA) levels have been evaluated as markers for detecting severity of oxidative stress in this study. Both aglepristone and prostaglandin treatments increased MDA concentrations in dogs. However, high MDA and low GSH levels were found significantly different in aglepristone-treated dogs than prostaglandin-treated ones. Similar results had been also reported in mid-gestation termination in rabbits after aglepristone application (Vatan et al., 2015). High levels of MDA had also been detected during spontaneous abortion in women, which are in accordance with our results (Yusrawati et al., 2017). Not only lipid peroxidation marker levels but also comet assay of bone marrow cells had been indicated that aglepristone could increase DNA damage. As no publication about lipid peroxidation process after medicine dependent abortions exists, the measured values were compared with investigations conducted on spontaneous abortion in women. Placental or trophoblast oxidative stress has been suggested as an important factor in pregnancy loss. The suggested mechanism that is possibly in accordance with our results, high MDA and low GSH levels are associated with PGF2α stimulation. Corpus

luteum is the only source to produce progesterone and this steroid hormone is strong paracrine and/or autocrine regulator of CL function in the dog (Papa and Hoffmann, 2011).

Both abortifacients are altering progesterone levels during abortion and withdrawal of progesterone stimulates strongly PGF2α biosynthetic pathway by increasement of PGF metabolites (Kowalewski et al., 2009). In the light of this information, altered steroid hormone production by prostaglandin treatment could possibly trigger oxidative stress and the disruption of PGE2-PGF2α network could be the reason of abnormal MDA and GSH levels. On the other hand, aglepristone has been thought not only blocked progesterone mechanism via receptors, but also inducted oxidative stress in dogs. This important outcome could be based on our previous study, of which increase of oxidative stress and DNA damage on bone marrow in rabbits were reported (Vatan et al., 2015). Other important findings in this study were, MDA and GSH levels, measured in fetal heart, muscle, kidney and liver. Since the increase of lipid peroxidation due to prostaglandin release is determined, high production of MDA and protein carbonyls is a natural expectation during parturition both in mother and fetal circulation (Mongelli et al., 1997; Mehmetoglu et al., 2002; Rogers et al., 1999; Cindriva - Davies et al., 2007). Both abortifacients had been observed to be changed lipid peroxidation in fetal vital organs, however it was not known whether there was an increased oxidative stress exposure in fetuses.

Another important finding could be the increase of MDA levels in blood samples, indicating a marker for fetal suffering. This marker could be useful during control pregnancy in the dam and it could occur before the signs of decreasing heart rate of fetuses. Prostaglandin and antiprogestational agents are being widely used for not only termination of unwanted pregnancy, but also parturition induction in dogs (Fontbonne et al., 2009; Fieni et al., 2001; Baan et al., 2005). The risk of free-radical mediated diseases in babies has been reported, related with elevated levels of oxidative stress biomarkers. The detection of the high levels of oxidative parameters could provide the recognization of pathways of neonatal/perinatal diseases (Perrone et al., 2019).

CONCLUSION

If these abortifacients could cause increased oxidative stress in vital organs, parturition induction should be reevaluated because of possible toxic effects in newborns. However further studies are needed to confirm these findings and potential risk factors.

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CONFLICT OF INTEREST

None declared by the authors.

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