REPUBLIC OF TURKEY
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF VETERINARY OBSTETRICS AND GYNAECOLOGY

# MEASUREMENT OF PRINCIPAL PARAMETERS OF OXIDATIVE STRESS IN BLOOD SERUM, UTERINE AND PLACENTAL TISSUES OF PREGNANT CATS

**MASTER'S THESIS**

**Sanan RAZA**

**Samsun**

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The thesis prepared by **Sanan Raza** entitled as ***"Measurement of Principal Parameters of Oxidative Stress in Blood Serum, Uterine and Placental Tissues of Pregnant Cats"*** has been supervised by **Assoc. Prof. Nilgün GÜLTİKEN** and accepted by the panel as MASTER'S thesis following the examination on the date of ----/---- /2017.

Chairman: ----------

Member: ----------

Member: ----------

CONFIRMATION

This thesis has been approved by the members of the panel already stated above and determined by the Institute Executive Board.

…. / …. /.....

**Prof. Dr. Ahmet UZUN**
**Director of Institute of Health Sciences**

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## ÖZET

### GEBE KEDİLERDE KAN SERUMU, UTERUS VE PLASENTA DOKUSUNDA BAŞLICA OKSİDATİF STRES PARAMETRELERİNİN ÖLÇÜMÜ

**Amaç:** Bu çalışmanın amacı, sağlıklı gebe ve gebe olmayan kedilerde kan serumu, uterus ve plasentada oksidatif stres parametreleri olan malondialdehid (MDA), süperoksit dismutaz (SOD) ve glutatyon peroksidaz (GPx) ölçülmesi ve karşılaştırılmasıydı.

**Materyal ve Metod:** Toplam 20 kedi üç gruba ayrıldı: Grup 1 (n=7) 25-39 günlük gebe kedileri, grup 2 (n=6) 40-61 günlük gebe kedileri ve grup 3 (kontrol, n=7) gebe olmayan interöstrus dönemdeki kedileri içerdi. Serum örnekleri toplandıktan sonra bütün kedilere ovaryohisterektomi yapıldı. Plasentanın marjinal hematom ve zonar kısmından, interplasental bölgeden ve uterustan doku örnekleri toplandı. MDA konsantrasyonu ile SOD ve GPx aktiviteleri spektrofotometreyle ölçüldü.

**Bulgular:** Ortalama serum ve uterus MDA konsantrasyonu, Grup 2'de (5.78±0.67 nmol/ml, 5.86±0.83 nmol/mg) grup 1 (2.90±0.20 nmol/ml, 2.54±0.08 nmol/mg) ve grup 3'e (2.40±0.33 nmol/ml, 1.83±0.09 nmol/mg) göre belirgin olarak yüksekti ($P<0.05$). Interplasental ve zonar kısımda MDA konsantrasyonu, grup 2'de grup 1'den daha yüksekti ($P<0.001$, $P<0.01$). Serum, uterus, interplasental ve zonar kısımlardaki SOD ve GPx aktiviteleri grup 2'de grup 1 ve 3'e göre belirgin olarak yüksekti ($P<0.05$, $P<0.05$, $P<0.01$, $P<0.01$). Gebelik ilerledikçe, serum MDA konsantrasyonuyla SOD ve GPx aktivitelerinin hem gruplar arasında hem de grup içinde uterus ve plasental kısımlara kıyasla belirgin olarak yükseldiği tespit edildi. Bulgular, hem gebe ve gebe olmayan kediler arasında hem de orta dönem ve geç dönem gebe kediler arasında istatistiksel olarak fark olduğunu ortaya koydu.

**Sonuç:** Bulgular, kedilerde gebelik sırasında oksidatif stres oluştuğunu ve başlıca oksidatif stres parametrelerinin özellikle geç dönem gebeliklerde önemli oranda arttığını gösterdi.



**Anahtar kelimeler:** Oksidatif stres; Reaktif oksijen türleri; Gebelik; Plasenta ve kedi



**Sanan RAZA, Yüksek Lisans Tezi**
**Ondokuz Mayıs Üniversitesi - Samsun, Aralık- -2017**

## ABSTRACT

## MEASUREMENT OF PRINCIPAL PARAMETERS OF OXIDATIVE STRESS IN BLOOD SERUM, UTERINE AND PLACENTAL TISSUES OF PREGNANT CATS

**Aim:** The purpose of the study was to elucidate and compare the oxidative stress parameters malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the blood serum, uterine and placental tissues of healthy pregnant as well as of non-pregnant cats.

**Material and Method:** A total of 20 cats were divided into three groups: Group 1 (n=7) will consist of pregnant cats of 25-39 days, group 2 (n=6) pregnant cats of 40-61 days and group 3 (controls, n=7) non-pregnant interoestrous cats. After collection of serum samples, all cats underwent ovariohysterectomy. Placental samples from marginal hematoma, zonary placenta, inter-placental and uterine tissues were obtained. Analysis for MDA concentrations and SOD and GPx activities were made with spectrophotometer.

**Results:** The mean MDA concentrations in serum and uterine tissues of group 2 (5.78±0.67 nmol/ml, 5.86±0.83 nmol/mg) were significantly higher ($P<0.05$) than that found in group 1 (2.90±0.20 nmol/ml, 2.54±0.08 nmol/mg) and group 3 (2.40±0.33 nmol/ml, 1.83±0.09 nmol/mg). MDA concentrations in the interplacental region and zonary part were higher in group 2 than group 1 ($P<0.001$, $P<0.01$). SOD and GPx mean activities in serum, uterus, interplacental region and zonary tissues were significantly higher in group 2 than groups 1 and 3 ($P<0.05$, $P<0.05$, $P<0.01$, $P<0.01$) respectively. As pregnancy proceeded, serum MDA concentrations and SOD and GPx activities were found markedly higher between and in all groups than in uterine and placental tissues. The results revealed that there is statistically significant difference between both pregnant and non-pregnant cats and between midterm and late pregnancies. **Conclusion:** These results demonstrate the presence of oxidative stress during pregnancy in cats and the increase of principal parameters of oxidative stress during the late pregnancy is marked.



**Keywords:** Oxidative stress; Reactive oxygen species; Pregnancy; Placenta and cat

## ABBREVIATIONS

| | | |
|---|---|---|
| **AC** | **:** | Allantochorion |
| **BD** | **:** | Body diameter |
| **CAT** | **:** | Catalase |
| **CL** | **:** | Corpus luteum |
| **EC-SOD** | **:** | SOD: Extracellular enzyme |
| **GPx** | **:** | Glutathione dependent enzyme i.e. glutathione peroxidase |
| **GSH** | **:** | Reduced glutathione form |
| **GSR** | **:** | Glutathione reductase |
| **GST** | **:** | Glutathione-S-transferase |
| **$H_2O_2$** | **:** | Hydrogen peroxide |
| **HD** | **:** | Head diameter |
| **i.m.** | **:** | Intramuscular |
| **IUGR** | **:** | Intra-uterine growth restriction |
| **Kg** | **:** | Kilogram |
| **LH** | **:** | Luteinizing hormone |
| **mHz** | **:** | Megahertz |
| **MDA** | **:** | Malondialdehyde |
| **ml** | **:** | Milliliter |
| **mM** | **:** | Millimolar |
| **NADP+** | **:** | Nicotinamide adenine dinucleotide phosphate |
| **O2•−** | **:** | Superoxide ion |
| **OH•** | **:** | Hydroxyl ion |
| **OHE** | **:** | Ovariohysterectomy |
| **PZ** | **:** | Pigmented zone |
| **ROS** | **:** | Reactive oxygen species |
| **RBCs** | **:** | Red blood cells |
| **rpm** | **:** | Rotation per minute |
| **SOD** | **:** | Superoxide dismutase |
| **S.C.** | **:** | Subcutaneous |
| **TBARS** | **:** | Thiobarbituric acid-reactive substances |
| **TZ** | **:** | Transfer zone |

| | | |
|---|---|---|
| **U/l** | **:** | Units per liter |
| **w/v** | **:** | Weight by volume |
| **Yc** | **:** | Yolk sac |
| **µmol/l** | **:** | Micromoles per liter |
| **µL** | **:** | Microliter |
| **°C** | **:** | Centigrade |

**CONTENTS**

## 1. INTRODUCTION

In the cat, average gestation length is 65 days, however, the range is 56 to 69 days varying significantly amongst the breeds (Goericke-Pesch et al., 2010). Pregnancy duration is more uniform when days of gestation are counted from the first rise of progesterone concentration in blood (Feldman and Nelson, 2004). The optimum concentration of progesterone is required for the maintenance of pregnancy termed as "progesterone block" which is supported by corpus luteum (CL) in most of the domestic animals (Senger, 2003). The role of progesterone is cessation of myometrial contractions during gestation and it is mainly produced from ovaries but the source and concentrations may change in different species of animals like in cats (Jainudeen and Hafez, 2000). The conceptus in cat is enclosed by a precontact and primitive chorion. Then it becomes a part of a temporary choriovitelline placenta which is afterwards replaced by a chorioallantoic placenta acting as source of metabolic exchange (Leiser and Koob, 1993).

Concurrently a growing interest from breeders and researchers worldwide has prompted the promotion of feline fertility and the advancement of assisted reproductive techniques in this species. During physiological period of pregnancy, different modifications occur in metabolic pathways resulting in greater oxygen consumption and alteration in the consumption of energy substrates. Subsequently, pregnancy results in greater exposure to oxidative stress (Al-Gubory et al., 2010). Cats are more liable to be influenced by oxidative stress damage, possibly because of fastidious spleen structure in this peculiar species (Harvey and Kaneko, 1977; Christopher et al., 1995).

In aerobic cells, metabolic pathways are oxygen-dependent and physiologically synthetize a group of pro-oxidant molecules called reactive oxygen species (ROS) (Gate et al., 1999; Agarwal et al., 2006). Endometrial cells continuously produce ROS like in other aerobic systems as a sequel of their standard metabolism. A state of uneven balance between the concentrations of antioxidant defense mechanisms of the organism and ROS is characterized as oxidative stress (Agarwal et al., 2006; Agarwal et al., 2012; Al-Gubory, 2013). Oxidative stress can be provoked with higher concentrations of oxidants or/and reducing antioxidants and this imbalance critically impairs the normal

functions at cellular and molecular level (Sies, 1991; Dotan et al., 2004; Halliwell and Gutteridge, 2015).

Infertility and uterine diseases in domestic animals have urged scientists to evaluate the role of oxidative stress markers particularly during pregnancy and information concerning oxidative stress parameters in the feline uterus is almost inexistent (Santos et al., 2016). Recently, several research teams addressed this topic in other domestic species. It is imperative to determine the physiological limits or ranges of oxidative/antioxidative profiles in both pregnant and nonpregnant cats. This will help to elaborate and understand that how the oxidative stress might contribute to the likelihood of the complications in pregnancy.

## 2. BACKGROUND

Cats are induced ovulators and release of luteinizing hormone (LH) causes ovulation after 24-36 hours of copulation (Brown, 2006). However, there is a high degree of individual and breed variation and some cats might exhibit spontaneous ovulation. Gudermuth et al. (1996) and Kutzler (2007) reported that spontaneous ovulation might occur up to 60% of cats. After induced ovulation in oestrus, cats enter in stage of dioestrus, either pseudo-pregnant or pregnant depending upon fertility of breeding. Progesterone starts to be produced from CL following 1-2 days of ovulation and is a key in formation of pregnancy block until parturition (Feldman and Nelson, 2004).

In domestic animals, classification of the placenta is based on distribution of chorionic villi and in the cat and bitch villi encircle in form of broad zone or belt called zonary placenta. This consists of three different visible zones; a pigmented zone (PZ), a transfer zone (TZ) and nonvascular part of placenta called allantochorion (AC). PZ shows local regions of necrosis and maternal hemorrhage (Senger, 2003). Similarly, central part of cylindrical chorion is vascularized which grows from allantois functioning as placenta (Noakes et al., 2001). Endothelial type of placentation separates maternal and fetal blood by four tissue layers and marginal hematomas give brown color to borders of placenta (Wooding and Burton, 2008). Drawing of cat fetus along with placenta at 45 days of pregnancy has been shown in figure 1.

Oxidative stress is often defined as the imbalance between oxidants (which are formed as a normal product of aerobic metabolism) and antioxidants, either by overproduction of ROS, or by dysfunction of the antioxidant systems (Sies, 1991; Halliwell and Gutteridge, 2015). Anti-oxidizing defense systems are compensation mechanisms that antagonize ROS-induced cellular damage. However, the damage will take place if the production of deleterious oxygen species far exceeds the capacity of these defense mechanisms (Al-Gubory, 2013).

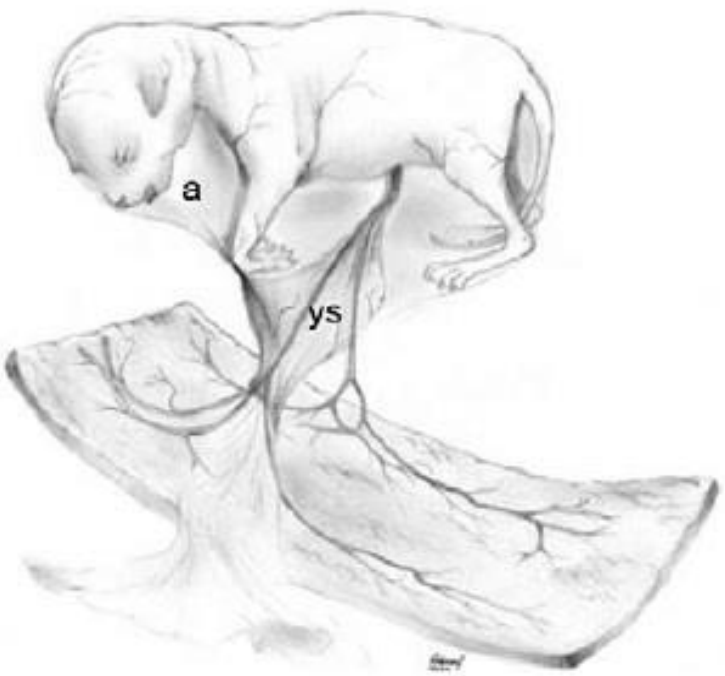


**Figure 1.** At 45 days of pregnancy drawing of cat fetus along with fetal membranes Amnion has covered the fetus (a) Placental girdle of allantois is connected to fetus with umbilical cord containing yolk sac (ys) and identifiable umbilical arteries and vein (Miglino et al., 2006)

Direct and indirect effects of ROS on cells can be summarized into three categories: RNA and DNA damage, lipid peroxidation, and protein damage (Riley and Behrman, 1991; Valko et al., 2007). Many different aldehydes, such as MDA, are originated as secondary products (Dotan et al., 2004; Ayala et al., 2014). Protein oxidation, caused by covalent modifications of a protein, can be induced with direct or indirect reaction of ROS with secondary by-products of oxidative stress (Shanlin et al., 1997; Shacter, 2000).

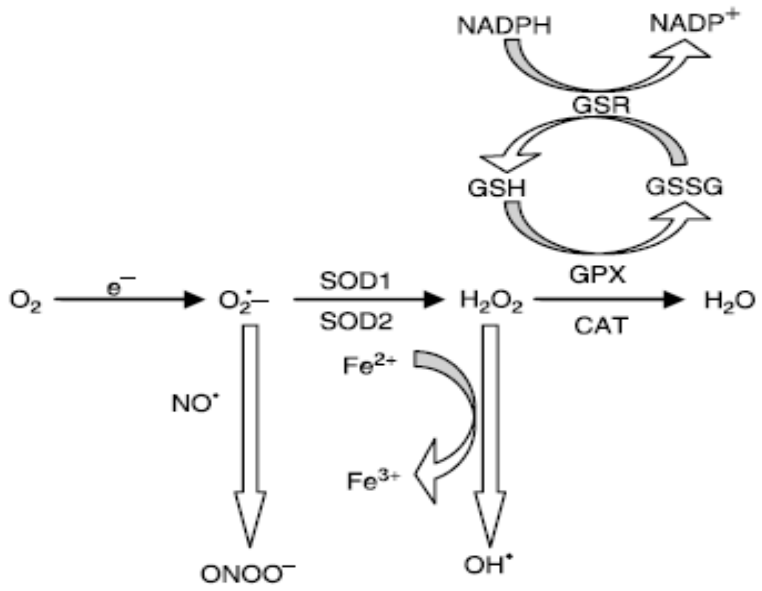


**Figure 2.** Diagrammatic illustration of reactive oxygen species (ROS) formation as well as propagation and vital cellular antioxidant enzymatic pathways (Garrel et al., 2010)

### 2.1. Reactive Oxygen Species (Free Radicals and Non-radicals)

Reactive oxygen species is an interchangeable term which is not only used to describe oxygen radicals' superoxide ($O_2$•−) and hydroxyl (OH•) but also some non-radical derivatives of oxygen i.e. hydrogen peroxide ($H_2O_2$). The former molecules contain one or more unpaired electrons in atomic or molecular orbitals giving a considerable degree of reactivity to the free radical oxidizing almost all classes of biologically important macromolecules, including proteins, lipids and nucleic acids (Halliwell, 1999). The production of (ROS) and important enzymatic pathways involved in cellular antioxidant activity are represented in figure 2.

*By-Products of Oxidative Stress*

Oxidative stress triggers cellular damage by acting on lipids and proteins, as well as other macromolecules (Halliwell, 2006). Lipid peroxidation is a non-enzymatic reaction which is manifested especially when unsaturated fatty acids of cell membranes undergo oxidation. Once initiated, lipid peroxidation remains as a chain reaction thus affecting many lipid molecules to produce lipid hydroperoxides and aldehydes. Briefly, a single event results in oxidation and damage of many macromolecules (Kruidenier and Verspaget, 2002). MDA is produced as a by-product of lipid peroxidation and its

analysis helps to determine the intensity of oxidative damage on the cellular membranes inside the body. MDA is measured as thiobarbituric acid-reactive substances (TBARS).

### 2.2. Reactive Oxygen Species Control

ROS control is an important approach which aims to preserve tissue homeostasis (Droge, 2002). The proportion of ROS produced and propagated in tissues is contained within physiological balanced limits; by a network of highly complex and integrated defense mechanisms. A particular set of detoxification pathways and scavenger reactions is used to maintain the balance which includes endogenous enzymatic and non-enzymatic antioxidant systems (Valko et al., 2007). The key endogenous enzymes which are directly involved in maintaining the balance and control of ROS production comprise of SOD, CAT, GPxs, glutathione reductase (GSR) and glutathione-S-transferase (GST), on the other hand, non-enzymatic endogenous antioxidant systems include, the nicotinamide adenine dinucleotide phosphate (NADP+) and the reduced form of NADP+ (NADPH) (Valko et al., 2007; Al-Gubory, 2013). Moreover, a number of different organic compounds such as vitamin C, vitamin E, carotenoids or the natural flavonoids are also part of the antioxidant system (Valko et al., 2007).

#### 2.2.1. Antioxidant Enzymatic Defenses

Mammalian cells have acquired a chain of antioxidant defense systems consisting of enzymatic and non-enzymatic antioxidants. Antioxidant defense system is used to prevent ROS production and control their dissemination and spread within and out of cellular organelles. Oxidative stress and cellular damage could be avoided by antioxidant enzymatic defenses (Agarwal et al., 2006; Browne et al., 2008; Al-Gubory, 2013). Superoxide anion and $H_2O_2$ are degraded by SOD, CAT and peroxidases such as GPx (Riley and Behrman, 1991). The composition of antioxidant defenses differs from tissue to tissue and one type of cell to another within the same tissue (Halliwell and Gutteridge, 2015).

*Superoxide Dismutase (SOD)*

SOD catalyzes the reaction of the superoxide anion to $H_2O_2$ which plays a key role in antioxidant reactions (Gate et al., 1999). In animal cells, most SOD is localized

in the cytosol and some is still present in lysosomes, nucleus, mitochondrial intermembrane space and peroxisomes (Halliwell and Gutteridge, 2015). Mammals produce three isoenzymes: SOD1 is mainly present inside the cytoplasm and contains metal cofactors, copper and zinc encoded as Cu, Zn-SOD; SOD2 encodes a Mn-SOD, a mitochondrial colonized Mn containing isoform; SOD3 encoding the extracellular enzyme (EC-SOD), also contains Cu and Zn as cofactors and it is structurally similar to Cu, Zn-SOD (Gate et al., 1999; Fujii et al., 2005; Halliwell and Gutteridge, 2015). The SOD catalyzed dismutation of superoxide anion gives rise to $H_2O_2$ also formed in vivo by several other enzymes, particularly oxidases (Halliwell and Gutteridge, 2015). Following equation describes the process of dismutation catalyzed by SOD.

$$2{\bullet}O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{(Dismutation Reaction)}$$

*Glutathione Peroxidase (GPx) and Catalase (CAT)*

GPx plays a key role in the detoxification of peroxides, $H_2O_2$ and lipid peroxides (Riley and Behrman, 1991; Halliwell and Gutteridge, 2015) using the reduced glutathione form (GSH) as electron donor (Fujii et al., 2005). This enzyme contains selenocysteine in its active center (Riley and Behrman, 1991; Fujii et al., 2005). GPx also exists in an insoluble form associated to the membrane (phospholipid hydroperoxide glutathione peroxidase) which acts on lipid hydroperoxides (Gate et al., 1999). The secondary antioxidative pathway include GPxs, which are located within the cytoplasm and mitochondrial matrix, and within peroxisomes CAT resides. Both catalyze the conversion of $H_2O_2$ to $H_2O$. Hence, GPxs and CAT depict the second main cellular defense pathways against oxidative damage (Michiels et al., 1994; Hayes and McLellan, 1999). These enzymatic antioxidants limit the production and propagation of toxic and highly reactive $H_2O_2$ derived ROS, mainly •OH radical. GR catalyses' the reduction of glutathione disulphide (GSSG) to GSH with NADPH as the reducing agent (Chance et al., 1979) and is considered to be an vital antioxidant enzyme for the GSH redox cycle that keeps adequate levels of GSH necessary for the maintenance of cells in a reduced state (Schafer and Buettner, 2001). Following equations describe the process of catalysis by antioxidant enzymes CAT and GPx respectively (Gate et al., 1999).

$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$ (CAT)

$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ (GPx)

In the female reproductive system, GSH is called to play a role in reducing oxidative stress, either by direct interaction with ROS, or by transfer of electrons to GPx, (Fujii et al., 2005).

### 2.2.2. Non-enzymatic Antioxidant Defenses

It is believed that antioxidant vitamins are among the significant dietary antioxidants that directly control ROS and provide an important source of protection against the damaging effects of ROS (Johnson et al., 2003). For the regulation of antioxidant enzymes, trace elements are important due to their function as cofactor and taking part in formation of active sites of enzymes. For normal physiological metabolism and growth, small quantities of trace elements are necessary for the developing organism (Mertz, 1981). A balanced diet with blend of fruits and vegetables incorporates polyphenols, which are important micronutrients as well as natural antioxidants because of their beneficial effects on health (Scalbert et al., 2005).

## 2.3. Association of Pregnancy with Oxidative Stress

Reactive oxygen species play a number of significant diverse roles in female reproductive biology including modulation of the uterine environment and embryo-maternal interaction at implantation (Riley and Behrman, 1991; Ufer and Wang, 2011). Oxidative stress has dual importance in the placenta. It plays both beneficial or regulatory role and adverse or destructive effects. Oxidative Stress is of high importance in the normal metabolism and cover of the placenta (Wu et al., 2016). The importance and regulatory roles of oxidative stress in the placenta are shown in figure 3.

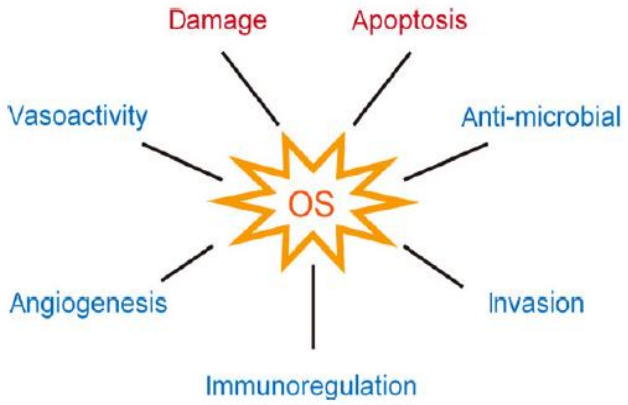


**Figure 3.** Importance and regulatory roles of oxidative stress in the placenta (Wu et al., 2016) Red color shows harmful and blue shows protective effects of oxidative stress

High metabolic demands are presumed to be responsible for triggering the production of ROS during pregnancy. During pregnancy various organs have been noticed to reveal higher basal consumption of oxygen and changes in substrate energy usage, ultimately resulting in the production of ROS (Toescu et al., 2002; Mutinati et al., 2013). Oxidative stress can occur as result of increased production of ROS and/or decreased antioxidant potential. The placenta is considered to be another local source of ROS in humans (Myatt and Cui, 2004). While pregnancy continues, fetal growth and demand for energy increase, which in turn cause excessive production of ROS in placental and fetal mitochondria (Kim et al., 2005).

Many studies have indicated that oxidative stress in pregnant women is mainly originated from placenta (Gitto et al., 2002; Myatt and Cui, 2004; Hung et al., 2010). Pregnancy-related oxidative stress has major contribution in total indicators of OS and these parameters can be derived from mitochondrial mass of placenta which increases with gestational age in humans (Wang and Walsh, 1998). Mitochondria are considered to be primary source of $\bullet O_2^-$, which is the main ROS radical (Cadenas et al., 2010). Oxidative stress has been found in placental tissues and along with pregnancy the intensity increases significantly (Qanungo and Mukherjea, 2000; Garrel et al., 2010). These studies indicate that lipid and protein peroxidation processes are presumed to be confined in placental tissues and in these specific tissue sites, the intensity of oxidative stress might be higher than that in blood plasma. Therefore, MDA can be a good indicator of oxidative stress in placental tissues.

The protection of CL as well as adaptation of embryonic, uterine and feto-placental antioxidant system to oxidative damage as pregnancy proceeds are presumed vital for successful pregnancy (Garrel et al., 2010). During pregnancy, antioxidant enzymes secure the sustenance of CL steroidogenesis along with rescuing it from luteolysis. For example, 1) SOD2, SOD3 and CAT levels increase during the early pregnancy in bovine (Rueda et al., 1995), 2) During pregnancy in sheep SOD1 can be identified and easily isolated (Al-Gubory et al., 2003), 3) SOD1 and SOD2 levels in CL follow the same pattern of progesterone concentration during pregnancy in rats (Sugino et al., 1993) and pseudopregnancy (Shimamura et al., 1995), 4) and in humans CL express higher levels of SOD1 during early pregnancy (Sugino et al., 2000).

### 2.4. Oxidative Stress Status of Different Species

#### 2.4.1. Oxidative Stress in Cats and Dogs

Changes in activity of antioxidant enzymes have been evidenced during dioestrus and pregnancy in bitches. Nevertheless, Kobayashi et al. (2014) demonstrated that SOD activity in the uterine fluid of bitches is higher in dioestrus than anoestrus or oestrus. In another study, serum profile of antioxidant biomarkers (vitamin A, vitamin E, magnesium and zinc) and markers of oxidative stress (carbonyl protein and TBARS) throughout the pregnancy displayed a markedly significant difference in bitches (Vannucchi et al., 2007).

Todorova et al. (2005) elucidated the presence of oxidative stress in clinically healthy dogs and cats with matched age and sex. Plasma concentrations of MDA in cats was found significantly higher than toms however, the opposite was seen in male dogs compared to bitches and the change was much higher for cats. However, in cats and dogs the activity of SOD did not show any change. Moreover, in female cats CAT values were higher than in male cats.

Serum concentrations of ROS during different phases of the oestrus cycle in the bitches have been determined by Rizzo et al. (2009). It was found that ROS were higher in oestrus than proestrual concentrations and anoestral values were statistically lowest ($P<0.001$). The highest concentrations of ROS were found during the time of peri-ovulatory period.

Tecles et al. (2015) determined that total antioxidant capacity in feline infectious peritonitis affected cats is lower than healthy ones. Similarly, changes in level of oxidative stress in pregnant bitches has been reported based on plasma markers for lipid and protein peroxidation. Interestingly, in pregnant dogs mean values of TBARS in plasma were found markedly higher ($P<0.05$) than in the non-pregnant ones (Szczubiał et al., 2015). The balance of oxidative stress within feline endometrial and placental tissues is poorly known.

In bitches, it has been demonstrated that both SOD and GPx are antioxidant enzymes of para-mount importance. Both are responsible for protection of cells/tissues and avoid accumulation of ROS hence, maintaining homeostasis (Santos et al., 2016). In another study, C-reactive protein, tumor necrosis factor-α, interleukin-6, SOD, CAT and TBARS in bitches with pyometra and control bitches were evaluated to explore the contribution of these parameters in the condition of multiple organ dysfunction syndrome However, oxidative stress was not detected in bitches with pyometra (Karabulut et al., 2016).

Tomsic et al. (2016) demonstrated the effect of sex and age on selected blood antioxidant parameters, i.e. total antioxidant capacity, GPx, and SOD in healthy dogs. It was found that activity of SOD significantly changed due to effect of growing age positively. Moreover, GPx showed relation with age (higher in older) as well as sex (higher in male dogs).

### 2.4.2. Oxidative Stress in Human and Rodents

Reactive oxygen species may modulate the growth of endometrial stroma. It is reported that increased oxidative stress in humans with pathologic conditions such as endometriosis results in exaggerated growth of endometrial stromal cells. Moreover, lack of protection due to depleted antioxidants favors the growth of endometrial stromal cells. Similarly, during late secretory phase of menstrual cycle before menses, rise of lipid peroxide and drop in SOD activity in the endometrium has been reported (Sugino et al., 1996). On the other hand, enhanced CAT, SOD and GPx activities in placental and fetal tissues have demonstrated protection against ROS toxicity in the fetoplacental system in humans (Qanungo and Mukherjea, 2000).

Humans have a rather different reproductive cycle than domestic animals but enhanced CAT and GPx activities, and GSH levels in placental tissue have been found to control higher concentrations of $H_2O_2$ and stimulation of placental differentiation (Jauniaux et al., 2000). Similarly, in CL, higher SOD1 activity during early pregnancy has been reported which favors the regulation of luteal function in humans (Sugino et al., 2000). Oxidative stress is considered to be a factor involved in number of human pregnancy related disorders, such as embryonic resorption, recurrent pregnancy loss, preeclampsia, intra-uterine growth restriction (IUGR) and fetal death (Gupta et al., 2007). It is also suggested that the depletion of placental antioxidant systems has been suggested as a key factor in early human pregnancy failure (Liu et al., 2006).

The importance of antioxidants can be seen from increased post-implantation embryonic death in SOD1 knock-out female mice, because of poor protection against $\bullet O_2^-$ during implantation (Ho et al., 1998).

Anadol et al. (2016) reported that surgical intervention of OHE in rats increased MDA concentration compared to sham group. While SOD and GPx activities were decreased in operated rats compared to sham group which clearly shows that OHE leads to oxidative stress indicating oxidative/antioxidative imbalance in rats.

### 2.4.3. Oxidative Stress in Farm Animals

Enhanced CAT and GPx activities and GSH levels in oviduct during mid (10-12 days) and end (18-20 days) of oestrus cycle in cows have been reported to control the amounts of $H_2O_2$ during fertilization (Lapointe and Bilodeau, 2003). A wide range of early embryonic loss occurs in dairy cattle and around 40% of cows remain pregnant after first insemination (Santos et al., 2004). The results of a study in buffaloes suggested that pregnancy is associated with oxidative stress and supplementation of vitamin E and selenium may be beneficial by alleviating oxidative stress (Dimri et al., 2010).

Higher concentrations of SOD1, GPx and GST activities in CL during early pregnancy of sheep were determined by (Al-Gubory et al., 2004), they suggested that these enzymes rescue the CL from apoptosis. Similarly, higher GPx and GSR activities and a concomitant drop in B-cell lymphoma 2 (BCL-2) associated X protein expression in early developing placentomes for the control of $H_2O_2$ and cell death during placental

development were determined in sheep. Indeed, the effectiveness of antioxidant enzymatic defenses against oxidative stress varies with the stage of placental development in sheep (Garrel et al., 2010).

We hypothesize that oxidative stress could be present in the pregnant cats, probably influenced by higher requirements of energy in pregnancy, and therefore serum, uterine and placental oxidative biomarkers may undergo changes. The aims of the present thesis research were;

- To evaluate oxidative stress through the activity of antioxidant enzymatic system, comprising superoxide dismutase (SOD) and glutathione dependent enzyme glutathione peroxidase (GPx),
- To analyze the oxidative damage caused to lipids i.e. malondialdehyde (MDA) at different time points of healthy feline pregnancy as well as in non-pregnant healthy cats.

## 3. MATERIALS AND METHODS

The study was supported by Ondokuz Mayis University, Project Management Office (Project no. PYO.VET.1904.17.027) and approved by Animal Experiments Local Ethics Committee of Ondokuz Mayis University (Approval no. 2017/23, Dated: 02.06.2017).

### 3.1. MATERIALS

#### 3.1.1. Animals

A total of 20 cats were divided into three groups: Group 1 (n=7) consisted of pregnant cats of 25-39 days, group 2 (n=6) pregnant cats of 40-61 days and group 3 (controls, n=7) non-pregnant interoestrus cats. Both pregnant and interoestrus cyclic cats were chosen from the ones that were brought to the clinic of the Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine with the request of spaying by their owners. All the cats were fed with dry food, clinically healthy and between the ages of 1-2. A limited range of age was preferred in order to reduce the effect on the parameters evaluated. The cats were not purebred, and breeds and coat patterns were determined according to their physical appearances (Table 1). Groups consisted of cats that had not been received any treatment or hormone administration in the past. The cats went through ovariohysterectomy with medial laparotomy under xylazine (2 mg/kg, im) and ketamine (10 mg/kg, im) anesthesia following ultrasonographical examination for detection of pregnancy age.

#### 3.1.2. Equipment and Materials

For clinical part of the study, ultrasonographic device, operation theatre equipment, postoperative care equipment and for the laboratory part of the study, ultrasonic homogenizer, spectrophotometer, centrifuge and vortex machine, weighing balance and incubator were used.

#### 3.1.3. Chemical Substances Used

Following chemical substances have been used to carry out the estimation of MDA and antioxidant enzymes activities.

### 3.1.4. Homogenization of Tissues Samples

Phosphate tampon 5% (w/v) and folin-fenol reactive were used.

### 3.1.5. MDA Estimation

In MDA concentration estimation, 1 ml of 0.67% thiobarbituric acid, n-butanol and 20% trichloroacetic acid were used.

### 3.1.6. SOD and GPx Estimation

Assay reagent mixture (0.15 mM nitroblue tetrazolium, 0.6 mM disodium ethylene diamine tetra-acetic acid, 0.3 mM xanthine, 0.4 M sodium carbonate and 1 g/l bovine serum albumin) and xanthine oxidase were used for SOD activity estimation. For GPx activity monitoring, phosphate buffer, GSH, sodium azide, glutathione reductase, ethylene diamine tetra-acetic acid, tertbutyl hydroperoxide and nicotinamide adenine dinucleotide were used.

## 3.2. METHODS

### 3.2.1. Ultrasound Examination of Pregnancy and Preparation of Animals

Pregnancy examinations were performed by real-time B-mode veterinary ultrasonography equipment and sector probe of 7.5 mHz (Falco Vet, Pie Medical, USA). Fetal parameters (gestational sac diameter, crown-rump length, body diameter and head diameter) were evaluated in order to determine pregnancy ages (Zambelli et al., 2002; Beccaglia and Luvoni, 2012). Afterwards, cats were starved for 12 hours before for ovariohysterectomy. The wound was covered with appropriate bandage and antibiotic was administered.

### 3.2.2. Dosage of Medicines Used

For induction of anesthesia xylazine (2 mg/kg, i.m.) was given and after 5 min interval ketamine (10 mg/kg, i.m.) was injected to perform ovariohysterectomy procedure. A broad spectrum antibiotic Synulox 1 ml/ 20kg or 8.75 mg/kg of bodyweight containing 7.0 mg amoxicillin and 1.75 mg clavulanic acid was administered S.C. for five consecutive days.

### 3.2.3. Trial Period

The trail period comprised of 2 months (early September to end of October 2017).

### 3.2.4. Principles of Methods Used

The principles used in the collection of samples and estimation of oxidative stress parameters are given as follows.

**Blood and Tissue Sampling**

Blood samples were taken from brachiocephalic vein before operation and to obtain serum, all samples were subjected to 10 min centrifugation at 1550 g. Samples of uterus and placenta were taken immediately after ovariohysterectomy i.e. uterine tissue and parts of placenta like zonary placenta, marginal hematoma and interplacental part. Each sample was in 1.0 x 1.0 cm size. Both serum and tissue samples were kept at -80°C until analyzed.

**Homogenization of Tissue Samples**

Samples were cut and homogenized in phosphate tampon 5% (w/v) ultrasonic homogenizer. Then supernatant was centrifuged at 18.000 rpm, at 4 °C for 10 min and stored at -80 °C until analyses. Protein concentrations of the tissues were determined according to the method described by Lowry et al. (1951). For that purpose, 1 ml of tissue extract, and standard solutions were transferred into sample and standard tubes and 1 ml of pure water into blank tubes, then 3 ml of reactive was added into tubes. 300 µl of folin-fenol reactive was added into tubes and mixed via vortex after the tubes waited in laboratory temperature for 30 minutes. Absorbances was read at 660 nm against blank after they were waited in laboratory temperature for 45 minutes and the concentrations were determined with the help of calibration curve.

**Determination of Antioxidant Enzymes Concentration**

*Determination of Malondialdehyde (MDA) Concentration*

Serum and tissue MDA concentrations were determined using the method of Yoshioka et al. (1979). 1 ml of 0.67% thiobarbituric acid and 2.5 ml of 20% trichloroacetic acid were mixed with tissue and serum samples, then heated at 95 °C for

30 min. The mixture was cooled then, and 4 ml of n-butanol was transferred in the content and subsequently, vortexed vigorously. Later, centrifugation was done to separate the butanol phase at 1550g for 10 mins. Spectrophotometric measurement of absorbance in the clear supernatant was done against n-butanol at 535 nm (Thermo Scientific, Genesys 10S UV-VIS, Madison, WI, USA). Afterwards, serum and tissue samples were evaluated for MDA concentrations from the standard curve of 1,1,3,3 tetraethoxypropane and results were demonstrated as µmol/l.

*Determination of Superoxide Dismutase (SOD) Activity*

SOD activity in serum and tissue was determined using the method suggested by Sun et al. (1989). In brief, 100 µl measured volumes of serum samples were poured in a mixture of 2.45 ml of assay reagent containing 0.3 mM xanthine, 0.6 mM disodium ethylene diamine tetra-acetic acid, 0.15 mM nitroblue tetrazolium, 0.4 M sodium carbonate and 1 g/l bovine serum albumin). Subsequently, 20 µl of xanthine oxidase was transferred to the new mixture and incubated for 20 min at 25 °C. Later, 1 ml of 0.8 mM copper (II) chloride was added after incubation, to stop the reaction. The activity SOD in serum and tissue supernatant was determined at 560 nm by detecting the reduction rate of the nitro blue tetrazolium inhibition. Lastly, SOD activity was expressed as U/l.

*Determination of Glutathione Peroxidase (GPx) Activity*

GPx activities in serum and tissue were determined using the method of Paglia and Valentine (1967). Briefly, 20 µl of serum samples was added 800 µl of reaction mixture (containing 0.1 M phosphate buffer, 10 mM GSH, 1 mM sodium azide, 1 mM ethylene diamine tetra-acetic acid, 1 unit of glutathione reductase and 1.5 mM nicotinamide adenine dinucleotide phosphate) with pH 7.0 and then incubated at 37 °C for 5 min. Then, another 10 µl of the 30 mM tertbutyl hydroperoxide was transferred to the mixture. Lastly, the absorbance was measured with spectrophotometry at 340 nm for 5 min at 37 °C and GPx activity was given as U/l.

### 3.2.5. Statistical Analysis

SPSS 22.0 package program was used for the statistical analysis of the data obtained in the study. Prior to the significance tests, for the parametric test hypotheses, all data were evaluated by Shapiro Wilk for normality and by Levene test for the homogeneity of variances. One-way analysis of variance (ANOVA) was used to check the significance of differences between groups for the parametric test assumptions. The Kruskal Wallis test was used for the variables that did not provide parametric test assumptions. Duncan test was used for variables that provided parametric test assumptions as a post-hoc test when differences between groups were significant. Mann-Whitney U test was used for each comparison in terms of variables that did not provide parametric test assumptions as a post-hoc test when differences in parameters were significant within the group. A minimum $p < 0.05$ criterion was used for all statistical evaluations. Correlation between the serum and tissue parameters was assessed using Pearson's correlation analysis.

## 4. RESULTS

### 4.1. Clinical Results

The individual clinical features of study and control group cats including the age of pregnancy and litter size are given in table 1.

**Table 1.** Individual clinical features of study and control group cats DSH (Domestic short hair), DMH (Domestic medium length hair)

| | Name | Age (years) | Breed and coat pattern | Weight (Kg) | Pregnancy Days | Litter size |
|---|---|---|---|---|---|---|
| **Group 1 (25-39 days, n= 7)** | Deniz | 2 | DSH, calico | 3 | 35 | 5 |
| | Bacaci | 1 | DMH, tabby | 3 | 38 | 3 |
| | Schila | 1 | DSH, calico | 4 | 28 | 3 |
| | Tatlis | 1 | DSH, calico | 3.4 | 32 | 3 |
| | Omtel | 1 | DSH, tabby | 3 | 33 | 5 |
| | Zeytin | 1 | DSH, tabby | 4 | 29 | 7 |
| | Tamed | 1 | DSH, calico | 3 | 35 | 5 |
| **Group 2 (40-61 days, n = 6)** | Pamuk | 1.5 | DSH, calico | 3.5 | 46 | 4 |
| | Akilli | 1 | DSH, tabby | 5 | 61 | 3 |
| | Queen | 1 | DSH, tabby | 3 | 53 | 4 |
| | Vahsi | 1 | DSH, tabby | 3 | 55 | 3 |
| | Rose | 1 | DSH, calico | 3 | 41 | 2 |
| | Sarikiz | 1 | DSH, calico | 3 | 54 | 7 |
| **Group 3 (Controls, n= 7)** | Kuyruksuz | 1 | DSH, tabby | 3 | Interoestrus | - |
| | Picasso | 1 | DSH, tortoiseshell | 3 | Interoestrus | - |
| | Tarcin | 1.5 | DSH, calico | 3 | Interoestrus | - |
| | Pixel | 1 | DSH, bicolor (Black-white) | 2.5 | Interoestrus | - |
| | Tekir | 1 | DSH, tabby | 2.3 | Interoestrus | - |
| | Poncik | 1 | DSH, calico | 2.8 | Interoestrus | - |
| | Ena | 0.5 | DSH, tabby | 2 | Interoestrus | - |

### 4.2. Results of Oxidative Stress Parameters

#### 4.2.1. Differences Between Groups

MDA concentrations in uterus were 2.54±0.08 nmol/mg, 5.86±0.83 nmol/mg, and 1.83±0.09 nmol/mg in group 1, 2 and 3 respectively and the difference was statistically significant (P<0.05). GPx activities in uterus were 5.07±0.52 U/mg, 5.56±0.55 U/mg and 1.24±0.11 U/mg in group 1, 2 and 3 respectively and the activities in group 1 and 2 were higher than group 3 (P<0.05). SOD activities in uterus were 7.91±0.86 U/mg, 8.17±0.44 U/mg and 6.43±0.62 U/mg in group 1, 2 and 3 respectively. The results of group 1 and 2 were significantly higher compared to group 3 (P<0.05), (Figure 4).

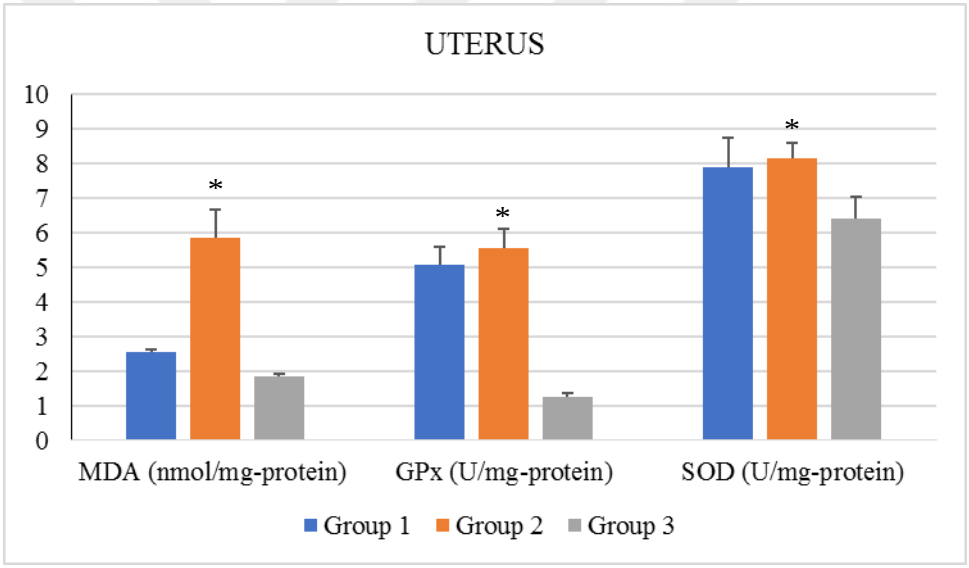


**Figure 4.** MDA concentrations (nmol/mg), GPx and SOD (U/mg) activities in uterine tissue of group 1, 2 and 3 (*P<0.05)

MDA concentrations in interplacental region were 1.79±0.08 nmol/mg and 4.32±0.29 nmol/mg in group 1 and 2 (P<0.001). GPx activities in interplacental region were 3.21±0.17 U/mg and 4.97±0.33 U/mg in group 1 and 2 (P<0.001). SOD activities in interplacental region were 5.43±0.39 U/mg and 6.31±0.33 U/mg in group 1 and 2 (P<0.01), (Figure 5).

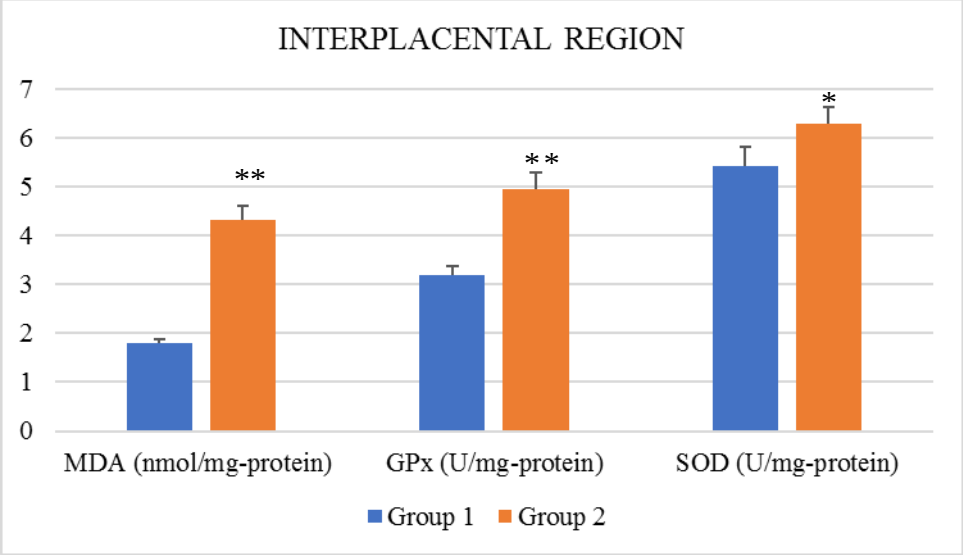


**Figure 5.** MDA concentrations (nmol/mg), GPx and SOD (U/mg) activities in interplacental region of group 1 and 2 (*P<0.01, **P<0.001)

MDA concentrations in zonary part were 1.68±0.08 nmol/mg and 3.87±0.33 nmol/mg and in group 1 and 2 (P<0.01). GPx concentrations in zonary part were 3.08±0.56 U/mg and 3.61±0.21 U/mg in group 1 and 2 (P<0.01). SOD concentrations in zonary part were 4.79±0.28 U/mg and 6.93±0.53 U/mg in group 1 and 2 (P<0.01), (Figure 6).

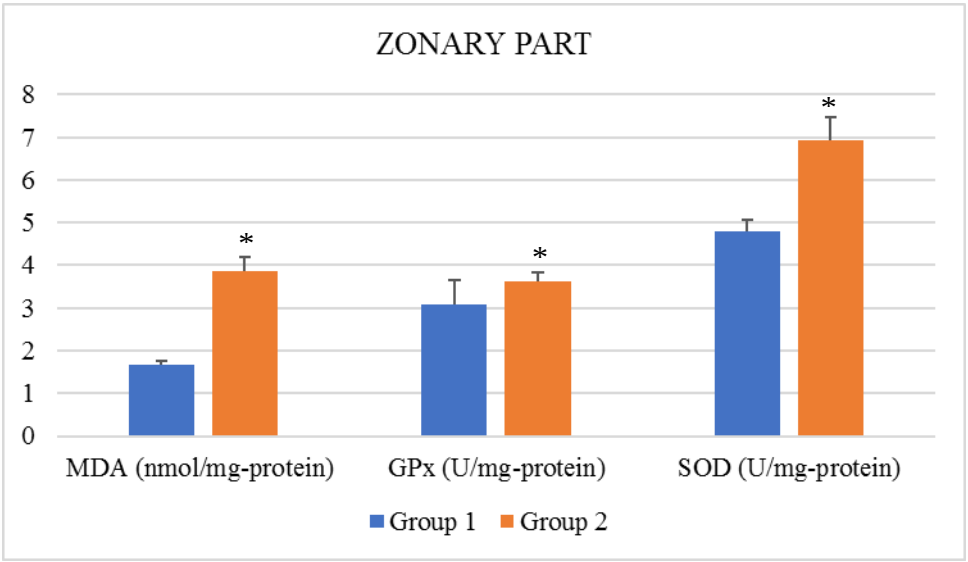


**Figure 6.** MDA concentrations (nmol/mg), GPx and SOD (U/mg) activities in zonary part of group 1 and 2 (*P<0.01)

**Oxidative Stress Parameters in Serum**

Serum MDA concentrations in group 1, 2 and 3 were 2.90±0.20 nmol/ml, 5.78±0.67 nmol/ml and 2.40±0.33 nmol/ml respectively. Concentrations in group 1 and 2 were found to be higher than group 3 (P<0.05). Serum GPx activities in group 1, 2 and 3 were 3.85±0.58 U/ml, 4.22±0.41 U/ml and 1.62±0.15 U/ml respectively. GPx activities in group 1 and 2 were determined to be higher than group 3 (P<0.05). Serum SOD activities in group 1, 2 and 3 were 7.56±0.92 U/ml, 8.25±0.68 U/ml and 5.45±0.39 U/ml respectively. SOD activities in group 1 and 2 were determined to be higher than group 3 (P<0.05), (Figure 7).

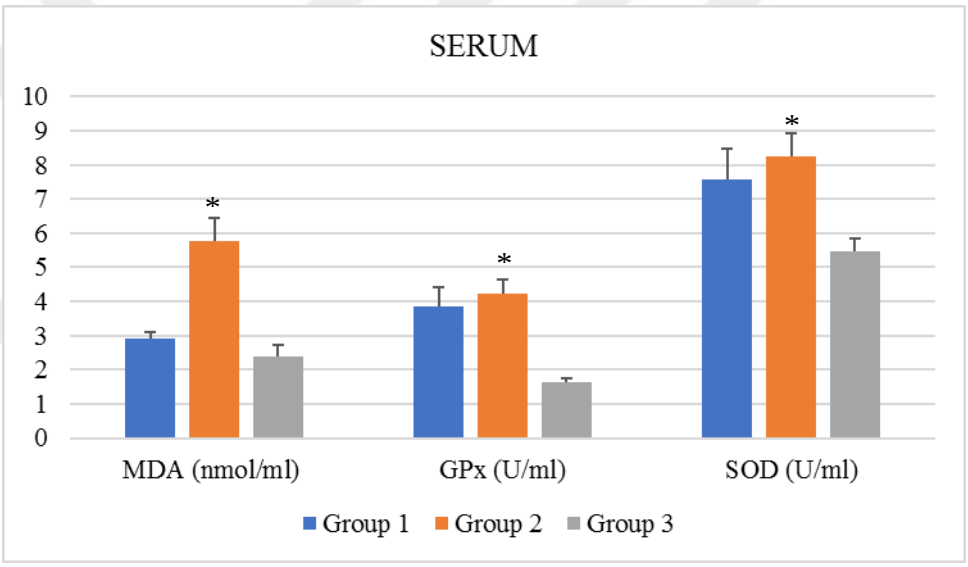


**Figure 7.** Serum MDA concentrations (nmol/ml), GPx and SOD (U/ml) activities in group 1, 2 and 3 (*P<0.05)

**4.2.2. Differences in Groups**

*Results in Group 1*

MDA concentrations in uterine tissue, interplacental region and zonary part of group 1 were 2.54±0.08 nmol/mg, 1.79±0.08 nmol/mg and 1.68±0.08 nmol/mg respectively and there was significant statistical difference among tissue samples (P<0.001). GPx activities in uterine tissue, interplacental region and zonary part of group 1 were 5.07±0.52 U/mg, 3.21±0.17 U/mg and 3.08±0.56 U/mg respectively and

there was significant statistical difference among tissue samples (P<0.001). SOD activities in uterine tissue, interplacental region and zonary part of group 1 were 7.91±0.86 U/mg, 5.43±0.33 U/mg and 4.79±0.28 U/mg respectively and there was significant statistical difference among tissue samples (P<0.001), (Figure 8).

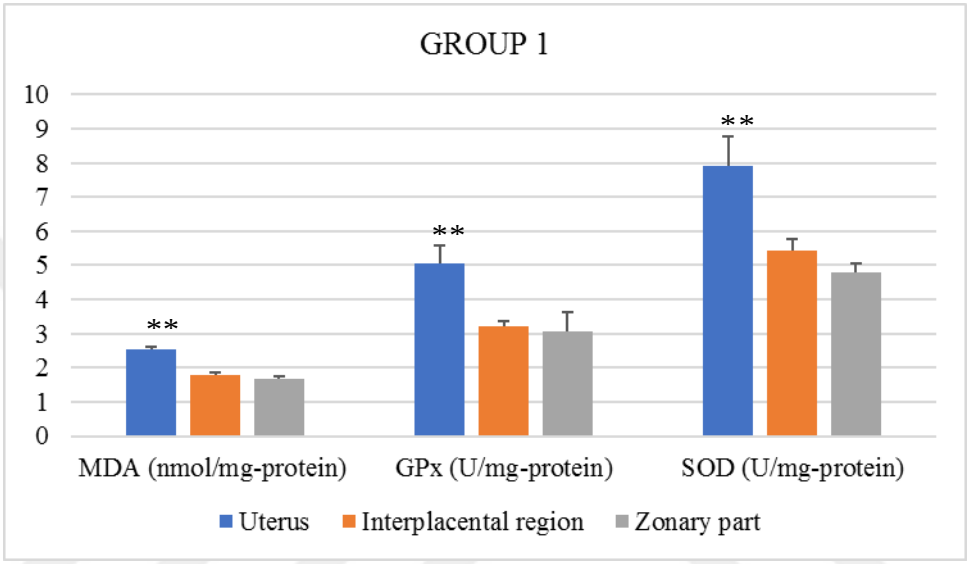


**Figure 8.** MDA concentrations (nmol/mg), GPx and SOD (U/mg) activities in uterus, interplacental region and zonary part of group 1 (**P<0.001)

*Results in Group 2*

MDA concentrations in uterine tissue, interplacental region and zonary part of group 2 were 5.86±0.83 nmol/mg, 4.32±0.29 nmol/mg and 3.87±0.33 nmol/mg respectively and there was significant statistical difference among tissue samples (P<0.01). GPx activities in uterine tissue, interplacental region and zonary part of group 2 were 5.56±0.55 U/mg, 4.97±0.39 U/mg and 3.61±0.21 U/mg respectively and there was significant statistical difference among tissue samples (P<0.01). SOD activities in uterine tissue, interplacental region and zonary part of group 2 were 8.17±0.44 U/mg, 6.31±0.33 U/mg and 6.93±0.53 U/mg respectively and there was significant statistical difference among tissue samples (P<0.01), (Figure 9).

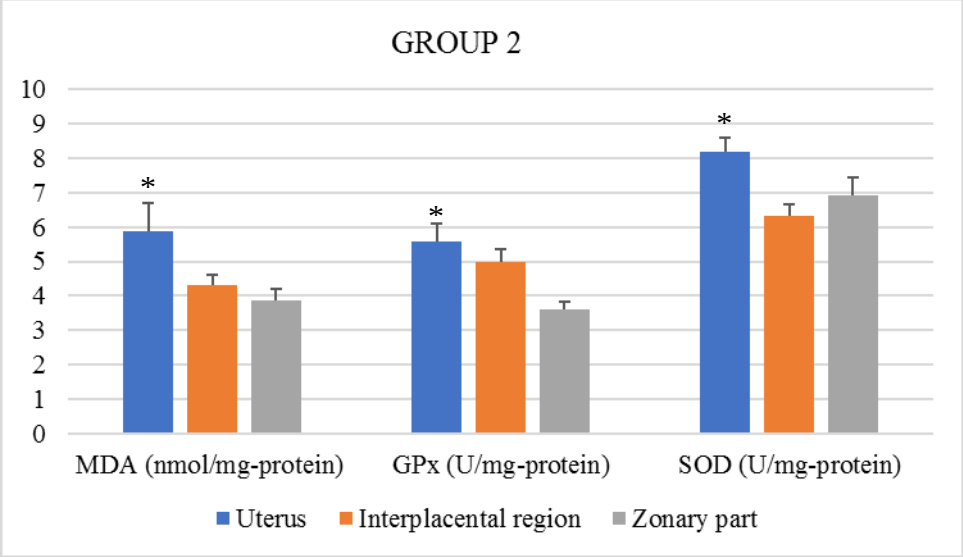


**Figure 9.** MDA concentrations (nmol/mg), GPx and SOD (U/mg) activities of uterus, interplacental region and zonary part of group 2 (*P<0.01)

The oxidative stress parameters (MDA, GPx and SOD) values were higher than the measurable upper limit in the marginal hematoma, therefore the results could not be evaluated in the study. We have thought that this result might be due to the fact that marginal hematoma is rich in erythrocytes and it is known that oxidative stress parameters like GPx is the most efficient antioxidant in the erythrocytes and prevents the harmful effects of $H_2O_2$ and organic peroxides (Ursini and Bindoli, 1987).

### 4.2.3. Correlation Between Serum and Tissue Results

The correlation between serum MDA concentrations and endometrium, interplacental region and zonary part of group 1 and group 3 was statistically non-significant. Similarly, the correlation between serum SOD, GPx activities and endometrium, interplacental region and zonary part in the group 3 was also non-significant. However, a positive correlation (r= 0.780, p= 0.038) between serum and zonary part GPx activities as well as highly positive correlation (r= 0.982, p= 0.000) between serum activity of SOD with endometrium activity of SOD in midterm pregnancy group 1 was found.

Correlations between serum MDA concentrations or serum SOD and GPx activity and endometrium, interplacental region, and zonally parts in the pregnancy

group 2 were not statistically significant. Although, the SOD and GPx activities have high increase in group 2 than group 1 during the course of pregnancy.

## 5. DISCUSSION

In this study, oxidative stress markers were measured during different time periods of normal pregnancy in cats. To assess the oxidative damage in the body, concentrations of MDA and activities of the SOD and GPx were measured using spectrophotometer. Lipid peroxidation intensity was determined by the measurement of MDA concentration, an end products of oxidative stress process. Llurba et al. (2004) stated that peroxidation of proteins and lipids can be evaluated by end products. As these products rapidly undergo metabolism and detoxification so determining their concentrations immediately after oxidative challenge is imminent.

None of the methods used for determining "oxidative stress" can be defined as the most appropriate standard in universal terms. In most of clinical trials selection of method depends on the research objective as well as its relevance. Assessment of oxidative stress parameters with only blood or only tissues cannot give a true picture of animal's health or disease status. Therefore, a combination of oxidative stress markers was evaluated in this study i.e. MDA along with antioxidant enzymes SOD and GPx. Lipid peroxidation processes may be restricted to specific tissues such as placenta, increasing oxidative stress markers concentrations in tissues compared to blood plasma. So MDA concentrations of uterus and placenta can be a good indicator of oxidative stress like in our study, along with antioxidant enzymes. Oztabak et al. (2005) stated that the concentrations of plasma lipid peroxidation marker (TBARS) were not different between pregnant and nonpregnant sheep. On the other hand, in cats it is unknown whether the concentration of oxidative stress parameters in blood serum, uterus and placenta are different or not between pregnant and nonpregnant cats. To our best knowledge, this is the first study stating antioxidant indices of cats during different time periods of pregnancy. There has been no report determining changes in the concentrations of MDA and activities of SOD and GPx enzymes in pregnant cats, throughout pregnancy.

Feline placenta grows rapidly in the third week of pregnancy and two brown hematoma are visible around 22-25 day of pregnancy (Miglino et al., 2006). In the cat, most of the smooth chorioallantoic consists of haemophagous area, performs phagocytosis and pinocytosis (Carter and Enders, 2016). Leiser and Enders (1980) stated that margins of zonary placenta or paraplacenta performs phagocytosis of

maternal RBCs and supply iron to fetus in cat and dog. In this study, MDA concentrations in feline placenta and uterus increased significantly during pregnancy and particularly the intensity of which increased towards the end of pregnancy in group 2. Group 2 MDA values in uterus, interplacental region and zonary part were found to be highest than group 1 and group 3. It is worth mentioning that specific sites like fetal or placental tissues can confine lipid and protein oxidation end products. As a matter of fact that, it may cause substantial change in blood levels of the major oxidative stress markers (Myatt and Cui, 2004). Therefore, MDA concentrations were found highest at these target sites of oxidative stress. Measuring oxidative stress parameters at sites like marginal hematoma, zonary and interplacental tissues or uterus provided an overview of oxidative stress in pregnant cats in this study. In general terms, the placenta can be considered as another local source of ROS. Garrel et al. (2010) reported that MDA concentration in sheep placentome significantly increased from days 35 to 80 and was very high from days 55 to 80 of pregnancy. Additionally, on day 60 and afterwards higher progesterone concentrations in placentomes are followed by an increase in MDA concentration. In our study serum MDA concentrations in group 2 were found highest which agrees with numerous studies conducted in pregnant humans. Studies show that the lipid peroxidation, TBARS concentration in hemolysate of erythrocytes increased significantly in healthy pregnant as well as diabetic women compared to non-pregnant women, (Djordjevic et al., 2004). In another study, Nakai et al. (2000) found MDA levels increased slightly from predelivery to 24 hours post-partum. Arikan et al. (2001) demonstrated that plasma and erythrocyte MDA levels were found to be significantly higher in the third trimester than in non-pregnant women ($P<0.02$). Many studies argue that several organs during pregnancy tend to reveal an increased basal oxygen consumption and changes in use of substrates for energy use, resulting in the production of ROS (Toescu et al., 2002; Mutinati et al., 2013). Szczubial et al. (2015) findings also proved that high metabolic demand during pregnancy leads to peroxidative damage of lipids and proteins in pregnant female dogs.

Christopher et al. (1995) stated that cats lack sinusoidal mechanism in spleen and efficient pitting function. Therefore, oxidative hemolytic anemia is more likely to occur in cats and up to 5% of red blood cells (RBSs) contain Heinz bodies in normal healthy cats. Therefore, higher concentrations of MDA in uterine and placental tissues

might be due to higher percentage of RBCs with Heinz bodies which is unique feature of cats.

Takehara et al. (1990) reported that concentration of lipoperoxides in maternal blood at 40 weeks gestation is 1.6 times higher than in nonpregnant women. Interestingly, the concentration in the cord blood was 70% lower than that in maternal blood. Similarly, our study MDA concentrations were significantly higher in serum compared to uterine tissue in group 1 but as the pregnancy continued the difference reduced for group 2. It means uterine and placental tissues suppress lipoperoxides formation in the late gestational age, thus lowering the concentration and protecting the fetus against oxygen toxicity. The present study revealed that the concentration of MDA was significantly higher in the serum than tissue samples of all groups, but the difference was significant only for pregnant cats.

Erisir et al. (2009), similar to Oztabak et al. (2005), reported no differences between plasma TBARS concentrations in pregnant and non-pregnant ewes. Similarly, Vannucchi et al. (2007) found that TBARS concentrations did not differ between different weeks of pregnancy in dogs. Furthermore, Castillo et al. (2005) reported that mean MDA concentrations before and after calving were not statistically different i.e. 10, 6, 2, 1 weeks before and 1, 2 after calving and even in dairy cattle in late lactation. In another study, Balal et al. (2004) has also demonstrated similar concentrations of MDA in last trimester of pregnant to the non-pregnant women. On contrary to above mentioned studies there was statistically significant difference in MDA concentrations between pregnant and nonpregnant cats in our study.

The SODs constitute the first and most important line of antioxidant enzyme defense systems against superoxide free radicals. In uterus, SOD activity was found higher in group 1 than group 3 and increased gradually reaching highest level at end of pregnancy in group 2. Additionally, SOD activity in group 2 showed higher differences in interplacental region with group 1 and 3 and much higher for zonary part of placenta. A marked, although not statistically significant increase in SOD activity was seen in group 1 and 2 compared to group 3 during the course of whole pregnancy. Many findings showed an increase in SOD activity in humans during pregnancy. In human placenta SOD activity increases 6 times by the end of gestation (Sekiba and Yoshioka,

1979; Takehara et al., 1990; Qanungo et al., 1999), whereas one study showed no change (Jauniaux et al., 2000).

According to Sekiba and Yoshioka (1979), SOD activity increases with growth of the placenta in humans and reaches a level of 3 times high by the end of gestation. Which is clearly in agreement with our study in which SOD activities in group 2 reached at higher level than group 1 during pregnancy. Moreover, it proves that oxygen requirement in the placenta at early stages of gestation is lower as compared to the end of gestation. Although, the differences of SOD activity between groups are not high especially in uterine and placental tissues as compared to serum levels.

Santos et al. (2016) suggested important role of SOD1 in endometrial physiology. Higher distribution pattern as well as immunoscores of SOD1 in the progesterone-associated stages of canine endometrium were reported. On the other hand, Sugino (2007) found that in the late secretory stage of menstrual cycle SOD activity decreases in humans, while lipid peroxidation and ROS levels increases. But in our study the SOD activities increased in pregnant cats and instead of controlling the oxidative stress, the byproduct MDA levels increased.

In our study the levels of antioxidants in serum and uterus samples were found relatively same. However, SOD and MDA values followed different trends in pregnant cats. As reported by Myatt et al. (1997) that no difference in concentrations or localization of SOD between normal and preeclampsia women but the ROS increased which shows loss of protection by antioxidant enzymes. Its might argued that persistent oxidative stress leads to weakening of antioxidant protection mechanism. Therefore, in this study the SOD activities remained similar in serum and tissues during the course of pregnancy, but MDA values increased rapidly. Which is quite like previous study reporting that oxidative stress results in depletion of antioxidants. Furthermore, high serum SOD levels show active and strong redox status in cats during pregnancy which can be used to evaluate the placental and uterine redox status.

Lekharu et al. (2014) stated a gradual increase in MDA concentration along the course of pregnancy and significantly higher concentrations from 1st to 3rd trimester in pregnant women while antioxidants SOD levels were found to be lowest in 3rd trimester of pregnancy. Contrary to humans, SOD levels in late pregnant cats increased during the course of pregnancy in our study.

In parallel to SOD, the GPx activities in uterus of group 1 and 2 were higher than group 3 moreover, interplacental region and zonary part of placenta showed higher activities in late pregnant cats. The increased GPx activities in group 2 may be explained by higher oxidative stress and even greater energy demands in pregnant cats with multiple litter size. Similarly, Djordjevic et al. (2004) reported gradual rise is seen in GPx activities, reaching the highest values in the 3rd trimester of pregnancy in humans. However, Arikan et al. (2001) demonstrated that plasma erythrocyte glutathione (GSH) levels were significantly lower in last trimester of pregnant women than in nonpregnant women ($P<0.03$).

Nemeth et al. (2001) found higher GSH concentrations found higher in pregnant women and suggested it as adaptation process to balance the redox status of red blood cells. During high metabolic demand of pregnancy, oxidative stress accelerates the antioxidants activity (Nakai et al., 2000). With respect to GPx activity conflicting results have been reported in whole homogenate of human placental tissue, varying from unchanged (Takehara et al., 1990; Qanungo et al., 1999) or increased (Jauniaux et al., 2000) activity, as pregnancy advances. Lastly, Santos et al. (2016) reported uniform distribution of GPx1 during the whole estrous cycle in the canine endometrium.

Antioxidants use microminerals for biological function, like $GP_X$ active site contains selenocysteine to perform function as antioxidant, so is selenium integral part of GPx antioxidant activity. The retention of the placenta in selenium-deficient cattle suggests a role for selenium–$GP_X$ in pregnancy outcomes (Eger et al., 1985). Therefore, study of microminerals along with oxidative stress can provide a better understanding of pregnancy related complications in cats.

Present study demonstrates that MDA concentrations, SOD and GPx activities in uterine and placental tissues increased from day 25 to 39 and very markedly from day 40 to 61. The serum concentration of three parameters also followed same pattern. Understanding the pattern of oxidative stress parameters in cat pregnancy will help to improve different nutrition regimes in pregnant cats.

A positive correlation between serum SOD and GPx activity was found with zonary part of placenta and uterine tissue in group 1. Moderate positive correlation was observed between GPx activity in serum and zonary part of placenta in group 1 which

clearly describes the maternal adaptation to oxidative stress and rise in GPx activity in zonary part of placenta and serum. Furthermore, SOD activities show high correlation between serum and interplacental part of placenta which could be controlled by other enzymatic and non-enzymatic antioxidants levels in the body. As stated by Erisir et al. (2009) rise in GPx causes reduction in activities of MnSOD during 35 and 55 days of pregnancy in ewes; like in our study GPx increased activities caused suppression of SOD activities in group 2 compared to group 1. This might be the reason of negative correlation of SOD and GPx activities with uterine and placental tissue SOD activities in group 2.

Endometrial GPx and SOD activities rise during pregnancy but at the same time serum and placental MDA concentrations increase (Biri et al., 2006). This indicates that antioxidants lose their potential towards the end of pregnancy. Briefly, it might be the reason of negative correlation between serum and placental activities of SOD and GPx in late pregnant group.

## 6. CONCLUSIONS AND RECOMMENDATIONS

A comparison of MDA, SOD and GPx levels in serum and tissues of pregnant and non-pregnant cats indicates that oxidative stress is present during pregnancy in healthy cats. This work tends to establish that feline placental tissues and blood serum contains antioxidant activity during different stages of pregnancy. The higher levels of antioxidant during the end of pregnancy can be linked to higher and gradual oxidative stress inflicted towards end of pregnancy. High stress consumes antioxidants so early that their protection to uterine and placental tissues becomes ineffective. Loss of balance in ROS and antioxidant protection poses risks of dystocia especially in cats with multiple kittens. As a whole these results revealed that oxidative stress biomarkers are subject to marked changes, a fact that definitely deserves further study including groups with different feeding regimes and litter size.

Infertility and uterine diseases in domestic animals has urged scientists to study the role of oxidative stress particularly during pregnancy. The results of our study might be a guidance for further research that will be performed for infertility in the cat.

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**APPENDIX**

Appendix 1. Certificate of ethical committee

**RESUME**

| | |
|---|---|
| Name and Surname: | SANAN RAZA |
| Place of Birth: | Kharian-PAKISTAN |
| Date of Birth: | 10-01-1990 |
| Marital status: | Married |
| Foreign Languages: | English/Urdu/Punjabi |
| Educational Status (Institution and Year): | Graduate in Veterinary Medicine from University of Veterinary and Animal Sciences Lahore Jhang-Campus). 2012. |
| Institution / Institution and Year of Work: | UVAS Lahore (2012-2016) |
| Email: | sanan.raza@uvas.edu.pk |