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EVALUATION REDOX STATUS IN HEALTHY DOGS

VALUTAZIONE DELLO STRESS OSSIDATIVO IN CANI SANI

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SOMMARIO

INTRODUCTION

In recent years, there has been a growing interest in prevention in veterinary medicine. Preventive medicine consists of taking actions before the pathology occurs or detecting and treating it in early stages.

According to this, many researchers consider oxidative stress-related mechanisms to be important early events in disease development [1]. Several studies show that oxidative stress can also play an important role as a therapeutic target, as well as a prognostic index during serious pathologies [2].

There is growing evidence that oxidative stress (OS) significantly impairs organic function and plays a major role in the aetiology and pathogenesis of several metabolic diseases in veterinary medicine. In many of these cases, it is unclear if oxidants trigger the disease or if they are produced as a secondary consequence of the disease and from general tissue damage [3].

Therefore, studying redox status in healthy dogs can provide valuable instruments to understand mechanisms underlying the several diseases associated with oxidative stress (such as inflammatory, infectious and degenerative disorders) and may ultimately lead to the development of new treatments or prevention strategies.

The aim of this study is to determine if there are any differences between the redox status in healthy dogs in relation to age, sex and size, in order to set standard reference values. In particular, the two markers on which the study is based are d-ROMs (Reactive Oxygen Metabolites – derived compounds) and PAT (Total Antioxidant Power).

The possibility of measuring oxidative stress in vivo with simple, cheap and accurate tests, d-ROMs test and PAT test, provides veterinarians with a very suitable tool to monitor oxidative stress and to correctly choose eventual antioxidant supplementations in diseases proven related to oxidative stress in animals, particularly in dogs [4].

By studying redox status in healthy dogs, researchers may be able to identify new targets for these interventions and help improve the overall health and well-being of dogs.

OXIDATIVE STRESS

FROM OXIDATIVE DAMAGE TO REDOX REGULATION: HISTORIC BACKGROUND

The presence of free radicals in biological materials was discovered in the 50's. Soon therafter, Denham Harman [5] hypothesized that oxygen radicals may be formed as by-products of enzymic reactions in vivo. In 1956, he described free radicals as a Pandora's box of evils that may account for cellular damage, mutagenesis, cancer, and, finally, the degenerative process of biological aging.

The science of free radicals in living organisms entered a second era after McCord and Fridovich [6] discovered the enzyme superoxide dismutase (SOD). Numerous researchers were now inspired to investigate oxidative damage inflicted by radicals upon DNA, proteins, lipids, and other components of the cell.

A third era began with the first reports describing advantageous biological effects of free radicals. Mittal and Murad [7] provided suggestive evidence that the superoxide anion, through its derivative, the hydroxyl radical, stimulates the activation of guanylate cyclase and formation of the "second messenger" cGMP. Similar effects were reported for the superoxide derivative hydrogen peroxide.

Ignarro and colleagues [8] discovered independently the role of nitric oxide (NO) as a regulatory molecule in the control of smooth muscle relaxation and in the inhibition of platelet adhesion.

Roth and Dröge [9] found that in activated T cells the superoxide anion or low micromolar concentrations of hydrogen peroxide increase the production of the T-cell growth factor interleukin-2, an immunologically important T-cell protein.

At the beginning of the 21st century, there is now a large body of evidence showing that living organisms have not only adapted to an unfriendly coexistence with free radicals but have, in fact, developed mechanisms for the advantageous use of free radicals. Important physiological functions that involve free radicals or their derivatives include the following: regulation of vascular tone,

sensing of oxygen tension and regulation of functions that are controlled by oxygen concentration, enhancement of signal transduction from various membrane receptors including the antigen receptor of lymphocytes, and oxidative stress responses that ensure the maintenance of redox homeostasis. The field of redox regulation is also receiving growing attention from in view of the role that oxidative stress has been found to play in numerous disease conditions. These pathological conditions demonstrate the biological relevance of redox regulation [9].

REACTIVE OXYGEN SPECIES

ROS include superoxide, singlet O_2, H_2O_2, and the highly reactive hydroxyl radical [10].

The superoxide anion (SOD) is formed by the univalent reduction of triplet-state molecular oxygen ($^3O_2$). This process is mediated by enzymes such as NAD(P)H oxidases and xanthine oxidase or non-enzymically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. SODs convert superoxide enzymically into hydrogen peroxide. In biological tissues superoxide can also be converted non-enzymically into the non-radical species hydrogen peroxide and singlet oxygen ($^1O_2$). In the presence of reduced transition metals (e.g., ferrous or cuprous ions), hydrogen peroxide can be converted into the highly reactive hydroxyl radical (·OH). Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase. Because superoxide and NO are readily converted by enzymes or nonenzymic chemical reactions into reactive nonradical species such as singlet oxygen ($^1O_2$), hydrogen peroxide, or peroxynitrite ($ONOO^-$), i.e., species which can in turn give rise to new radicals. Most of the regulatory effects are indeed not directly mediated by superoxide but rather by its reactive oxygen species (ROS) derivatives. Frequently, different reactive species coexist in the reactive environment and make it difficult to identify unequivocally which agent is responsible for a given biological effect [9].

REACTIVE NITROGEN SPECIES

The NO radical (NO·) is produced in higher organisms by the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine. This process is catalyzed by the enzyme NOS. Depending on the microenvironment, NO can be converted to various other reactive nitrogen species (RNS) such as nitrosonium cation (NO^+), nitroxyl anion (NO^-) or peroxynitrite ($ONOO^-$).

The most relevant radicals in biological regulation are superoxide and NO. These radicals are formed by two groups of enzymes, i.e., the NAD(P)H oxidase and NOS isoforms, respectively. Many regulatory effects are mediated by hydrogen peroxide and other ROS that are chemically derived from superoxide.

REDOX STATE

The global concept of "Oxidative Stress" is defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage".

The basic idea is that, in the open metabolic system, a steady-state redox balance is maintained at a given setpoint, which provides a basal redox tone, and that a deviation from the steady-state redox balance is considered a stress, initiating a stress response. Implicit in the definition of oxidative stress is that a deviation to the opposite side of the balance is "reductive stress", and that there are physiological deviations, "oxidative eustress", and supraphysiological deviations, "oxidative distress". Oxidative eustress is an essential part of redox control and physiological redox signaling. Recent accounts were given not only for reactive oxygen species (ROS) and reactive nitrogen species (RNS), but also for reactive sulfur species (RSS), reactive electrophile species (RES), and reactive halogen species (RNS). Clearly, multiple interactions constitute checks and balances in redox regulation [11].

The "two-faced" character of ROS is clearly substantiated. For example, a growing body of evidence shows that ROS within cells act as secondary messengers in intracellular signaling cascades which

induce and maintain the oncogenic phenotype of cancer cells. However, ROS can also induce cellular senescence and apoptosis and can therefore function as anti-tumorigenic species [12].

There is also an appreciation that increased ROS may play an important role in the normal aging process and in the pathogenesis of numerous chronic diseases, including cancer, atherosclerosis, cardiovascular disease, diabetes mellitus, neurodegenerative diseases, liver injury, kidney disease and immune dysfunction [13].

ROS can severely alter the structure of molecules, such as proteins, lipids and deoxyribonucleic acid (DNA). These alterations can cause cell degeneration and ageing. Oxidative stress in dogs has been associated with osteoarthritis and carcinogenesis, one of the leading causes of death for these species [14].

In particular, ROS can break the lipid membrane and increase membrane fluidity and permeability. Protein damage involves site-specific aminoacidic modification, peptide chain fragmentation, cross-linked reaction products aggregation, electric charge alteration, enzymatic inactivation, and proteolysis susceptibility. Finally, ROS can damage DNA through oxidizing deoxyribose, breaking strand, removing nucleotides, modifying bases and crosslinking DNA-protein [15]; this will lead to phenotypical alterations and genetic diseases (by alteration of purine and pyrimidine in the structure of cellular DNA). If in this process, specific gene sequences are involved, the cell possibly turns into a neoplastic one.

Many cancers are thought to be the result of interactions between free radicals and DNA that lead to mutations that affect the cell cycle and which then leads to neoplasia.

Exposure to heavy metal ions, drugs, toxins, pesticides or insecticides may also contribute to the increase of ROS production in cells.

In cellular aging, two theories on the mechanisms of cellular aging are currently accepted: the mitochondrial theory and the free radical theory. They support the hypothesis that mitochondria are affected by an increased level of intracellular free radicals, which leads to the alteration of their function and a decreased cellular regenerative capacity. At the same time, the progressive accumulation of intracellular oxidizing factors that exceed the antioxidant capacity is also accepted. Under these conditions, the biological decline of the respective tissue and the reduction of the

adaptive capacity to stress appear. Subsequently, regardless of the mechanism involved, in mitochondrial DNA damage or in the direct involvement of prooxidant factors in cellular mechanisms, the cellular response to stress will produce an overexpression of proinflammatory genes with increasing levels of prooxidant factors [15].

At moderate concentrations, however, nitric oxide (NO), superoxide anion, and related reactive oxygen species (ROS) play an important role as regulatory mediators in signaling processes. Many of the ROS-mediated responses protect the cells against oxidative stress and reestablish "redox homeostasis." Higher organisms, however, have evolved the use of NO and ROS also as signaling molecules for other physiological functions. These include regulation of vascular tone, monitoring of oxygen tension in the control of ventilation and erythropoietin production, and signal transduction from membrane receptors in various physiological processes. NO and ROS are typically generated in these cases by tightly regulated enzymes such as NO synthase (NOS) and NAD(P)H oxidase isoforms, respectively. In a given signaling protein, oxidative attack induces either a loss of function, a gain of function, or a switch to a different function. Excessive amounts of ROS may arise either from excessive stimulation of NAD(P)H oxidases or from less well-regulated sources such as the mitochondrial electron-transport chain. In mitochondria, ROS are generated as undesirable side products of the oxidative energy metabolism [9].

THE ROS SOURCES

Free radicals are generally produced as a result of the influence of external factors, such as pollution, cigarette smoke, or internally, as a result of intracellular metabolism if the antioxidant mechanisms are overwhelmed [15].

EXOGENOUS ROS

Environmental triggers, such as exposure to cigarette smoke, UV radiation, heavy metal ions, ozone, allergens, drugs or toxins, pollutants, pesticides, or insecticides, may all contribute to the increase of ROS production in cells [16].

Ionizing radiation acts by converting hydroxyl radicals, superoxides and organic radicals into organic hydroperoxides and hydrogen peroxide [17].

Ultraviolet radiation (UVA) triggers oxidative reactions by stimulating riboflavin, porphyrins and NADPH-oxidase, with the production of 8-oxo-guanine as the main result and the decrease of intracellular glutathione (GSH) level with a return to normal after cessation of exposure [18]. Heavy metals play an essential role in the production of free radicals [19]. Iron, copper, cadmium, nickel, arsenic, and lead can induce free radicals by Fenton type reaction, but also by direct reactions between metal ions and cellular compounds with similar effects. Arsenic induces the production of peroxides, superoxides, nitric oxide and inhibits antioxidant enzymes such as glutathione-transferase, glutathione-peroxidase, and glutathione-reductase by binding to the sulfhydryl group. The free radicals generated from these reactions can affect DNA, with substitutions of some DNA bases such as guanine with cytosine, guanine with thymine and cytosine with thymine [20].

ENDOGENOUS ROS PRODUCTION

The main endogenous sites of cellular redox-reactive species generation-including ROS and reactive nitrogen species (RNS) comprise mitochondrial electron transport chain (ETC), endoplasmic reticulum (ER), peroxisomes, membrane-bound NADPH oxidase (NOX) isoforms 1–5, dual oxidases (Duox) 1 and 2 complexes, and nitric oxide synthases isoforms 1–5 (NOS1–3). The complexes I and III of mitochondrial ETC produces superoxide anion [21].

The mitochondrial ETC is considered to be the primary endogenous source of ROS but other internal sources are also present. Other sources of ROS, primarily H_2O_2, are microsomes and peroxisomes. Immune cells, such as macrophages and neutrophils, can also generate ROS due to their oxygen- dependent mechanisms to fight against invading microorganisms based on NOX2 isoform [22]. ROS are produced in mitochondria during aerobic metabolism [21]. Mitochondria serve as a major ROS generator and, at the same time, as a ROS receptor. Covalent and enzymatic changes in proteins during or after protein biosynthesis as well as during protein cleavage or degradation promote disease through oxidative damage and mitochondrial dysfunction. These post-translational changes participate in the regulation of mitochondrial function through free radical species and other messengers [23].

ROS AND CHRONIC DISEASES

CARDIOVASCULAR DISEASES

The main cardiovascular risk factors, such as hypertension and hypercholesterolemia contribute to enhancing ROS generation, leading to oxidative stress [24]. From all these cardiovascular risk factors, hypertension is an essential factor in the development of cardiovascular diseases (CVD) [25]. Small amounts of ROS in the cardiovascular system could provide remarkable benefits: anti-atherosclerotic, pro-angiogenesis and endogenous cardioprotective effects. Large numbers of ROS induce the loss of cell viability, since oxidative stress is involved in the development and/or progression of CVD, such as endothelial dysfunction, atherosclerosis, myocardial ischemia/reperfusion damage, heart failure, arrhythmias [15, 26].

ROS AND CANCER

It has been established that oxidative DNA damage is one of the key characteristics of carcinogenesis [27]. Cancer initiation and promotion are associated with chromosomal defects and activation of oncogenes by free radicals [28]. A common form of injury is the formation of hydroxylated DNA bases, considered an important event in chemical carcinogenesis. They interfere with healthy cell growth by causing genetic mutations and altering normal gene transcription. Oxidative lesions also produce many changes in the structure of DNA [29].

ROS contributes to cancer cell migration through various mechanisms: matrix degradation, cell-cell contact, cytoskeleton remodeling, regulation of gene expression [30].

In cancer cells, there is the condition of constant oxidative stress induced by mitochondrial dysfunction and metabolic changes. In fact, under normal circumstances, increased ROS levels stimulate cell death, but cancer cells overcome that by activating numerous oncogenes. NRF2 (nuclear factor erythroid 2-related factor) is the primary regulator of cell survival that raises cancer progression by protecting cancer cells from ROS and DNA damage [31].

DEGENERATIVE DISORDERS

In the brain, not all neuronal groups are equally sensitive to oxidative stress. For instance, neurons with longer axons and multiple synapses require more energy for axonal transport or long-term plasticity [32]. High ATP demand, in combination with dysfunctional mitochondria, make these neuron groups more sensitive to degeneration [33]. Correctly, dopaminergic neurons are exposed to additional oxidative stress produced by the dopamine metabolism, generating H_2O_2 and dopamine autoxidation, which generates superoxide [34]. During aging, mutations in DNA accumulate, cytosolic calcium dysregulates, and electron transport chain function decreases, making aging one of the major risk factors contributing to neurodegeneration [35].

Mechanisms of action of ROS: these affect proteins by modifying them in oxidative forms, which tend to form aggregates [36]. Protein aggregates then inhibit proteasomes, the main organelles in the cell for degradation of abnormal proteins. Accumulation of modified proteins with an inability to be destroyed in the proteasome stimulate more ROS formation and form a vicious cycle, a phenomenon included in neurodegenerative diseases related to oxidative stress [37].

DIABETES AND METABOLIC SYNDROME

Many metabolic contexts can lead to conditions of oxidative stress. A condition in which oxidation is an important pathogenetic link is type 2 diabetes. In this disease, insulin resistance is the basic component, to which a compensatory hypersecretion of insulin is linked. Reactive oxygen species can induce inactivation of signaling mechanisms between insulin receptors and the glucose transport system, leading to insulin resistance [38]. On the other hand, diabetes itself is a generator of oxidative stress. Hyperglycemia induces the generation of superoxide ions in endothelial cells at the mitochondrial level. Therefore, preventing the damage caused by oxidation is a therapeutic strategy in diabetes. Studies have shown that both glucose and free fatty acids can initiate the formation of free radicals through mitochondrial mechanisms and NADPH oxidase in muscles, adipocytes, beta cells and other cell types. Free fatty acids penetrate cellular organs, including mitochondria, where high levels of reactive oxygen species can cause peroxidation and damage. Recent studies show that type II diabetes and insulin resistance are associated with a decrease in mitochondrial oxidative function in skeletal muscle [39].

AGING

ROS is a cause of damage accumulation in cell constituents and connective tissues. This accumulation of losses in cells would be the reason for aging and aging-associated degenerative diseases. Aging can be caused by both genetic and external factors [40]. Today, while there are several theories of aging, the basic principle of most of them is still oxidative stress. In the aging process, it has been noticed that high-molecular protein aggregates accumulate in cells. Predominantly, these aggregates are made from proteins, with the remainder consisting of various lipids [41]. Most of the proteins aggregated are oxidized/modified by different reactive metabolites, and they could bind to cellular proteins. Thus, the crucial point for protein homeostasis maintenance is the degradation of these aggregates. The central place for cell damaged protein degradation is the proteasome, which recognizes only unfolded proteins as degradation targets [42]. Proteasome inhibition prevents further degradation of newly formed oxidized proteins and increases protein aggregation formation in cells [43]. Besides that, proteasome becomes dysfunctional during aging.

While proteasomal dysfunction is correlated with age progression and protein aggregation, proteasome activation slows the aging progress down and increases longevity [44].

ANTIOXIDANT DEFENSE

On the other side of redox balance, the defense against damaging levels of oxidants consists of several types of antioxidant enzymes in conjunction with their back-up systems, as well as of low-molecular-mass antioxidants, forming an antioxidant network [45].

Antioxidants break radical chain reactions. Their role requires acting both in hydrophilic and hydrophobic cellular environments, so their chemical structure is quite heterogeneous.

There are enzymatic and non-enzymatic antioxidants. From a nutritional perspective they are respectively endogenous and exogenous antioxidants [46]. The first class comprises all antioxidants that cells can synthesize from smaller building blocks. Accordingly, all enzymatic antioxidants are endogenous, as well as some non-enzymatic ones.

ENZYMATIC ANTIOXIDANTS

Several enzymes obstruct free radicals' formation. A further classification divides enzymatic antioxidants in in primary and secondary enzymes [46].

PRIMARY ENZYMES

Primary enzymes act directly in scavenging ROS arising from incomplete O_2 reduction, O_2^- and H_2O_2. SOD is a metalloenzyme, catalyzing superoxide anion dismutation to H_2O_2 and molecular oxygen.

SOD also competes for superoxide anion with NO. Therefore, SOD also indirectly reduces the formation of another deleterious ROS, peroxynitrite ($ONOO^-$), and increases the NO biological availability, an essential modulator for endothelial function.

GPX is a selenium-dependent oxidoreductase, which uses H_2O_2 or organic hydroperoxide as the oxidant, and the tripeptide GSH as the electron donor [47].

SECONDARY ENZYMES

Secondary enzymes play an indirect role by supporting other endogenous antioxidants. All the enzymatic activities described above rely on the continuous regeneration of the reduced form of reductants. This is usually performed by some reductases, NADPH-dependent (such as glutathione reductase).

So, enzymes responsible for the constant NADPH production can be considered secondary antioxidants, as their misfunction could affect the whole ROS balance. The main NADPH metabolic source is the pentose phosphate pathway, through the first two enzymatic activities: glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [48].

NON-ENZYMATIC ANTIOXIDANTS

Some chemical molecules of low-molecular-weight can also directly act as antioxidants. In this case, their action is not catalytic, always needing antioxidant regeneration or its supply from the diet. Non-enzymatic antioxidants can therefore be divided into endogenous (if the eukaryotic cell is able to synthesize it) and exogenous (if the antioxidant needs to be ingested mandatorily through the diet) [15].

ENDOGENOUS NON-ENZYMATIC ANTIOXIDANTS

GSH (γ-glutamyl-cysteinyl-glycine) is a tripeptide, mainly distributed in cytosol, but also in nuclei, peroxisomes and mitochondria. It has been described to be more effective in ROS scavenging than vitamin E, GSH, vitamin C and β-carotene.

Coenzime Q10 or ubiquinone is an antioxidant present in cell membranes, essential for ETC. In its active form (quinol), coenzyme Q10 can scavenge several ROS or regenerate other oxidized antioxidants (including vitamins C and E) [49].

Melatonin is a neurohormone derived from aminoacid tryptophan. It is involved in circadian rhythms but also acts as a potent antioxidant, protecting cell membranes against lipid peroxidation [50].

EXOGENOUS NON-ENZYMATIC ANTIOXIDANTS

Exogenous antioxidants need to be supplemented continuously through the diet since their synthetic pathways are usually present only in microbial or plant cells. Vitamins, two of which show prominent antioxidant effects, such as vitamins C and E, belong to essential class of molecules.

Vitamin C (ascorbic acid) exists in two redox forms: ascorbic acid (AA) is the reduced form.

Vitamin E is a fat-soluble vitamin, mostly found in several vegetable oils, nuts, broccoli and fish. Eight different forms have been reported but α-tocopherol has the highest antioxidant activity, especially in cell membranes [15].

Carotenoids are a broad class of tetraterpenes, widely distributed among plants. They have been suggested to be chemopreventive agents in cancer [51].

Flavonoids, in addition to its strong antioxidant properties, quench ROS formation inhibiting several enzymes and chelating metals involved in radical chain reactions [46].

WATER-SOLUBLE AND LIPOSOLUBLE ANTIOXIDANTS

Antioxidants can be divided into two categories depending on their solubility: water soluble and liposoluble. Water soluble antioxidants are best absorbed in the body because the vegetables and fruits that contain such antioxidants, also contain water. On the other hand, they are rapidly eliminated from the body through the urine. Water-soluble antioxidants include polyphenols, but also vitamin C. Liposoluble antioxidants, fat-soluble antioxidants are those that are absorbed in the presence of fats. Therefore, in the absence of fats, the body cannot absorb and use these antioxidants. It is important to note, however, that they are not easily removed from the body and can accumulate over time, exceeding the healthy level. Vitamin E is an example of a fat-soluble antioxidant [52].

ASSESSMENT OF OXIDATIVE STRESS

In recent years some assays panels have been developed to globally evaluate the oxidative balance by means of the concomitant assessment of ROS production and antioxidant system capability [53]. Traditional assessment of oxidative stress includes measurement of reaction products, measurement of ROS, or quantification of endogenous antioxidants. Newer testing, called oxidative stress profiling, encompasses assessment of several markers of oxidative stress as well as markers of inflammation and is believed to be a more thorough evaluation of oxidative stress [54]

Because ROS are short half-life molecules, the accurate detection of oxidative stress status (OSS) may prove technically challenging. Moreover, it must be kept in mind that a single test does not reflect the presence of OS [55]. It has been suggested that a much more extensive battery of tests is needed to evaluate oxidative stress and antioxidant status. Unfortunately, the cost of this extensive oxidative stress testing, which includes tests for 13 antioxidants, 22 trace elements, an iron profile, 9 markers of inflammation, and 11 markers of oxidative damage, is likely to be prohibitive to most researchers and clinicians [54].

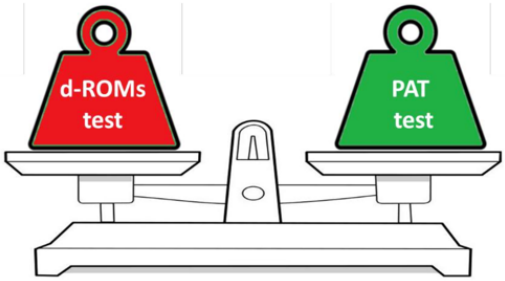
In human studies, the first step to assess OS requires the use of a large battery of assays including determination of enzymes and low molecular weight antioxidants, analysis of trace elements, evidence of oxidative damages to lipids, proteins or DNA and identification of the sources responsible for high ROS production. The second step includes careful pre-analytical treatment of samples since it may lead to artifactual values in the event of non-compliance with the protocol. This is of fundamental importance to determine values for blood or urine OS biomarkers and, therefore, to correctly interpret the results. In dogs, several studies have evidenced increased OS in systemic diseases. More precisely, sick dogs have increased oxidant marker levels while endogenous antioxidants and biomarkers of the oxidant response concomitantly decrease in whole blood, in serum, in plasma, in urine or in cerebrospinal fluid and spinal cord tissue [55].

The d-ROMs test provides a measure of the whole oxidant capacity of plasma against the N,N-diethylparaphenylendiamine (DMPD) in acidic buffer. Such oxidant capacity is mainly due to hydroperoxides, with the contribution of other minor oxidant factors [53].

The d-ROMs test is a valuable assay for the quantification of plasma or serum primary oxidative damaged molecules and, possibly, of other biological matrices, thus provides relevant information [56].

The PAT (Total Antioxidant Power) test is to evaluate the plasma antioxidant biological potential as the capacity of the plasma sample to reduce ferric ions to ferrous ions. Such a biological antioxidant potential is attributable to the major component of plasma barrier to oxidation (vitamin C, vitamin E, uric acid, bilirubin and so on). Successfully validated in humans, the d-ROMs test has been performed in several animal species, including mammalians [4].

MATERIALS AND METHODS



The assays were executed by H&D srl (Parma) with the aim of implementing a rapid test for the evaluation of d-ROMs and PAT in apparently healthy dogs.

The research was carried out by collecting blood samples (plasma) of 39 healthy dogs of different age, size, sex, fertility status and breed. Initially, 110 dogs were involved in the study, but a high number of samples could not be processed because hemolytic (due to irregularities of tubes lot).

The protocol of this study was submitted to the Committee for Animal Ethics of the University of Parma (approval number PROT. N. 17/CESA/2022 del 20 febbraio 2023).

BLOOD COLLECTION

For each dog a blood sample was taken from the cephalic or saphenous vein using a 2,5 ml syringe, a butterfly 21 G for medium/large size dogs and 22 G butterfly for the smaller ones. Ethyl alcohol was used as a disinfectant. The blood was collected in lithium heparine tubes and centrifugated at 2500 rpm for 10 minutes. Plasma was then separated from the cellular components and stored in a refrigerator at -20 °C.

Storage of plasma:

18-25°C	0-4°C	-20°C
12 hours	48 hours	2 months

The use of lithium heparine tubes is related to the incapability of this anticoagulant of chelating the iron and consequently, not to cause interference.

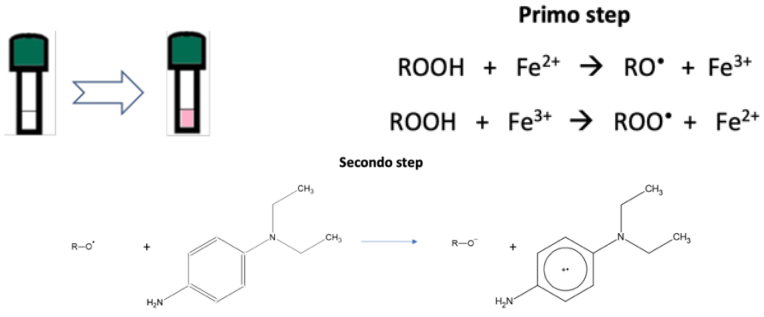
Anticoagulants such as K-EDTA do not allow peroxides detection, while citrate underestimate the values.

D-ROM TEST AND PAT TEST

The **d-ROMs test** carried out by H&D (Parma, Italy) quantifies the blood concentration of peroxides (the main ROS in plasma) by a colorimetric reaction.

The measure of peroxides gives a global insight of the underway oxidative processes, against lipids, proteins, peptides, nucleotides; actually, the main source of peroxides results from oxidative processes against membrane lipids because of their high susceptibility to oxidation. Besides, peroxides are the first detectable molecules during oxidative processes, so their quantification allows to intercept oxidative phenomena in advance of other oxidation markers, ensuring the possibility of early intervention. Finally, peroxides are themselves oxidant, so their measure is an indication of the potential damage [57].

d-ROM photometric test is based on two consecutive steps. In the first step, peroxides, in presence of iron released from plasma proteins by an acidic buffer, can generate alkoxyl and peroxyl radicals (ROOH), according to the Fenton's reaction. In the second step, such radicals, in turn, are able to oxidize an alkyl-substituted aromatic amine, the N,N-dietylparaphenylendiamine and develop a pink color. This amine is added to the test sample (previously diluted in acetic acid buffer - pH 4,8) and, in presence of ferric ions, changes color from colourless to more or less intense pink. This is due to N,N-dietylparaphenylendiamine oxidation, which involves the donation of one electron from the aromatic amine (colourless) to radical chemical species and finally to corresponding pink-colored radical cation. The ROM's concentration runs directly parallel with color intensity and is expressed as Carratelli Units (1 CARR U=0.08 mg hydrogen peroxide/dl) [4].



Analytical performance:

d-ROMS Test FAST:
Unit of measure: 1 U Carr = 0.08 mg / dl hydrogen peroxide
Linearity: the method is linear in the 50-600 U Carr range
Precision: CV% <5.0%
Interference: the addition of anticoagulants capable of chelating iron, such as EDTA or citrates, give rise to underestimates of the data; the use of disinfectants different by ethyl alcohol can lead to anomalous results.

The **PAT test** (Plasma Analytical Test) quantifies the global antioxidant potential. The quantified antioxidant potential is attributable to the major components of plasma barrier to oxidation (vitamin C, vitamin E, uric acid, bilirubin) [58]. PAT is not influenced by antioxidant enzymatic activity; it is just responsive to static antioxidant barrier. It means that this test measures the body's first response against oxidative phenomena. PAT Test measures the real plasma antioxidant power because excludes potential interfering reactions such as phosphates.

PAT test is also a photometric test based on iron's reactions: in particular, it measures the iron-reducing power by a colorimetric reaction: it is based on the ability to discolor of a colored solution of ferric ions (Fe^{3+}) complexed to thiocyanate derivative solution (a chromogen), when the ferric ions are reduced to ferrous ions (Fe^{2+}) in presence of reducing substances. The decolorization of the solution will be proportional to the concentrations of antioxidants [59].

Unit of measure: 1 U Cor = 1.4 μmol / liter of vitamin C.

Unico step

$$Fe(SCN)_n^{n+3} + AO_{red} \rightarrow Fe(SCN)_n^{n+2} + AO_{ox}$$

Analytical performance:

PAT TEST:
Unit of measure: 1 U Cor = 1.4 µmol / liter of vitamin C
Linearity: the method is linear in the 500-10,000 U Cor range
Precision: CV% <5.5%
Interferences: no interference was observed in the presence of a phosphate concentration of less than 40 mg/dl; the addition of anticoagulants capable of chelating iron, such as EDTA or citrates, give rise to underestimates of the data; the use of disinfectants different by ethyl alcohol can lead to anomalous results.

TEST PROCEDURE

In human medicine, the required amount of plasma to execute both tests is 10 µl. In this study, 10 µl was enough to process the PAT test, while in the d-ROM test the results were below the detection limit. In order to obtain acceptable values, the plasma amount for the d-ROM test has been raised to 30 µl.

Conservation: samples are still valid at -20 °C for 24 months according to d-ROM test and for 2 months for PAT test. At a temperature of 0-4 °C, both tests have validity of 48 hours. At 18-25 °C, the storage limit is 25 hours.

Both tests are executed on FRAS 5 or FRAS BRAVO, a specialized photometer for analysis of free radicals (using d-ROM test FAST) and antioxidant potential (using PAT test).

KIT COMPONENTS

Reagents:	**REDOXO BFP**	**REDOXO BFP20**	**REDOXO BFP10**
d-ROMs Test FAST:			
- Reagent R1 d-ROMs Test FAST Chromogenic mixture condensed in the cuvette, pre-dosed. Store at 15-25°C	50 pcs	20 pcs	10 pcs
- Reagent R2 d-ROMs Test FAST Buffer pH 4,8 , preservatives and stabilizers in ready prepared micro test tubes. Store at 15-25°C	50 pcs	20 pcs	10 pcs
-Reagent R3 d-ROMs FAST Catalyst solution Store at 15-25 °C	1x1,5ml	1x0,8ml	1x0,4ml
PAT TEST:			
- Reagent R1 PAT TEST Chromogenic mixture in the cuvette, pre-dosed. Store at 15-25°C	50 pcs	20 pcs	10 pcs
- Reagent R2 PAT TEST Ferric nitrate solution, stabilizers and preservatives. Store at 15-25°C	1x3ml	1x1,2 ml	1x0.6 ml
Materials:			
- tips, disposable	200 pcs	100 pcs	50 pcs
- sterile lancets, disposable	50 pcs	20 pcs	10 pcs
- heparinized microvettes, disposable	50 pcs	20 pcs	10 pcs

d-ROM test

The samples, previously collected in the freezer, were moved to the fridge (4 °C) for 24 hours. Before continuing, they were left out of the fridge for half an hour to reach room temperature. Then, in order to remove further interferences, plasma samples were centrifuged at 6000 rpm for 1 minute and a half.

A working solution was prepared by depositing 30 μl into a micro test tube containing reagent R2 10 μl of reagent R3 (catalyst solution) using a pipette and shaking for reverse for about 10 seconds.

After centrifuging and the preparation of the working solution, 30 μl of plasma was taken and deposited into micro test tubes containing the working solution and mixed by inversion for at least 10 seconds. Subsequently, the content was transferred into a cuvette containing the reagent R1, the condensed chromogen, and gently mixed by inversion for 10 seconds, so avoiding foaming.

Finally, the cuvette was inserted into the reading container making sure that the sides were oriented as indicated on the label. The reading is completed in 2 minutes and a half.

In order to guarantee the highest reproducibility and speed, the reading container thermostat is set at 37 °C.

PAT test

40 μl of reagent R2 (iron solution) must be added to the cuvette containing the reagent R1 (thiocyanate derivative pre-dosed solution). Once the cuvette is closed with the cap, it needs to be mixed by inversion for exactly 10 seconds. The cuvette is inserted into the reading cell of the instrument, which completes the first reading in about 2 seconds.

REFERENCE VALUES

d-ROMs Test FAST:

>500 U Carr	Very high oxidative stress
400-500 U Carr	High oxidative stress
340-400 U Carr	Oxidative stress
320-340 U Carr	Slight oxidative stress
300-320 U Carr	Borderline range
250-300 U Carr	**Normal values**

PAT TEST:

>2800 U Cor	Very high value
2800-2200 U Cor	**Normal value**
2200-2000 U Cor	Borderline range
2000-1800 U Cor	Slight deficiency status
<1800 U Cor	Deficiency status

For each dog included in the research, this table shows: breed, age, gender and fertility status, weight and d-ROMs and PAT results, that can be easily compared to the reference values in the previous table.

DOG N.	BREED	AGE	SEX	WEIGHT
1	Mongrel	8 y	SF	6 kg
2	Border Collie	7 y	SF	21 kg
3	Amstaff	7 y	SF	27 kg
4	Weimaraner	1 y	SF	22 kg
5	Golden Retriever	2 y	CM	36 kg
6	Labrador	10 y	SF	27 kg
7	Mongrel	4 m	F	7 kg
8	English Setter	3 y	M	25 kg
9	Mongrel	7 y	SF	35 kg
10	Pointer	4 y	SF	20 kg

11	French Hound	9 y	F	25 kg
12	Boxer	7 y	CM	35 kg
13	Boxer	9 m	M	23 kg
14	Border Collie	4 y	M	27 kg
15	Border Collie	7 y	M	27 kg
16	Cavalier King	8 y	SF	12 kg
17	Mongrel	5 y	M	30 kg
18	Labrador	11 y	M	40 kg
19	Mongrel	8 y	SF	40 kg
20	Border Collie	13 y	SF	26 kg
21	Labrador	8 y	M	37 kg
22	Mongrel	10 m	M	15 kg
23	German Shepherd	5 y	SF	37 kg
24	Dalmatian	9 y	M	32 kg
25	Mongrel	8 y	SF	25 kg
26	French Bulldog	8 y	SF	22 kg
27	Labrador	9 y	M	35 kg
28	American Staffordshire	5 y	F	30 kg
29	Labrador	11 y	SF	28 kg
30	Dalmatian	6 m	F	18 kg
31	Mongrel	9 y	CM	19 kg
32	Mongrel	1 y	M	15 kg

33	Schapendoes	6 y	SF	15 kg
34	Vizsla	6 y	M	30 kg
35	Maremma Sheepdog	9 y	SF	30 kg
36	Bernese Mountain dog	2 y	F	47 kg
37	Mongrel	5 y	SF	25 kg
38	French Bulldog	4 y	F	16 kg
39	Golden Retriever	13 y	SF	24 kg

M = Male / F = Female / CM = Castrated male / SF = Sterilized female

Puppies: until 1 year

Adult dogs: 1-10 years

Geriatric dogs: > 10 years

Small size dogs: 3-10 kg

Medium size dogs: > 10-25 kg

Large size dogs: > 25 kg

RESULTS

The results reported are part of an experimental project. The initial goal of this study was to analyze 100 plasma samples of healthy dogs, on which execute d-ROM test and PAT test, highlighting any possible gender differences, fertility status, age (puppies, adults, geriatrics), size (small, medium and large) and breed. Therefore, it would have been possible to set reference values in healthy dogs and consequently create a kit for veterinary clinics. Irregular results would warn about a possible pathological condition. Currently, we can rely on the following results. Ndr, we will carry on this project after graduation.

For each sample, we measured d-ROM and PAT values. The analyses were repeated three times for a more scientific validity. The following were calculated for all data: the average, the standard deviation (which shows how far the values differ from the average) and the percent coefficient of variation - CV - (which helps us to understand how the measure may vary in respect to the average).

Below is reported the Table 1 containing three averages for each analysis result, standard deviation, coefficient of variation, d-ROMs and PAT values.

DOG N.					dROMs	PAT		
1	Mongrel	8 y	SF	6 kg	145	2702	CV	
					151	2655	dROMs	PAT
					176	2786		
				AVERAGE	157,3333	2714,333	8,53264	1,996325
				ST. DEV.	13,42469	54,18692		
2	Border Collie	7 y	SF	21 kg	138	2616	CV	
					128	2489	dROMs	PAT
					139	2786		
				AVERAGE	135	2630,333	3,678929	4,625748
				ST. DEV.	4,966555	121,6726		
3	Amstaff	7 y	SF	27 kg	143	2736	CV	
					174	2991	dROMs	PAT
					170	3194		
				AVERAGE	162,3333	2973,667	8,481263	6,301278
				ST. DEV.	13,76792	187,379		
4	Weimaraner	1 y	SF	22 kg	215	2868	CV	
					215	2736	dROMs	PAT
					189	2412		
				AVERAGE	206,3333	2672	5,940154	7,170018
				ST. DEV.	12,25652	191,5829		
5	Golden Retriever	2 y	MC	36 kg	210	2285	CV	
					210	2670	dROMs	PAT
					180	2369		
				AVERAGE	200	2441,333	7,071068	6,770411
				ST. DEV.	14,14214	165,2883		
6	Labrador	10 y	SF	27 kg	258	2616	CV	
					196	2616	dROMs	PAT
					259	2736		
				AVERAGE	237,6667	2656	12,39787	2,12984
				ST. DEV.	29,46561	56,56854		
7	Mongrel	4 m	F	7 kg	239	3047	CV	
					205	3194	dROMs	PAT
					224	2894		
				AVERAGE	222,6667	3045	6,248095	4,022419
				ST. DEV.	13,91242	122,4827		
8	English Setter	3 y	M	25 kg	196	2752	CV	
					218	2840	dROMs	PAT
					214	2681		
				AVERAGE	209,3333	2757,667	4,570924	2,358336
				ST. DEV.	9,568467	65,03503		
9	Mongrel	7 y	SF	35 kg	215	2868	CV	
					236	2868	dROMs	PAT
					198	3330		
				AVERAGE	216,3333	3022	7,18431	7,20678
				ST. DEV.	15,54206	217,7889		
10	Pointer	4 y	SF	20 kg	283	2616	CV	
					260	2868	dROMs	PAT
					271	2573		
				AVERAGE	271,3333	2685,667	3,461671	4,844933
				ST. DEV.	9,392669	130,1187		
11	French Hound	9 y	F	25 kg	158	2529	CV	
					159	2971	dROMs	PAT
					172	2808		
				AVERAGE	163	2769,333	3,912296	6,590229
				ST. DEV.	6,377042	182,5054		
12	Boxer	7 y	CM	35 kg	260	2573	CV	
					231	2691	dROMs	PAT
					234	2894		
				AVERAGE	241,6667	2719,333	5,388145	4,875104
				ST. DEV.	13,02135	132,5703		
13	Boxer	9 m	M	23 kg	252	2816	CV	
					234	2868	dROMs	PAT
					263	2816		
				AVERAGE	249,6667	2833,333	4,787829	0,865166
				ST. DEV.	11,95361	24,51304		
14	Border Collie	4 y	M	27 kg	240	2403	CV	
					241	2489	dROMs	PAT
					243	2573		
				AVERAGE	241,3333	2488,333	0,516804	2,789169
				ST. DEV.	1,247219	69,40381		
15	Border Collie	7 y	M	27 kg	210	2316	CV	
					264	2227	dROMs	PAT
					248	2137		
				AVERAGE	240,6667	2226,667	9,410132	3,281893
				ST. DEV.	22,64705	73,07682		
16	Cavalier king	8 y	SF	12 kg	243	2736	CV	
					267	2768	dROMs	PAT
					254	2691		
				AVERAGE	254,6667	2731,667	3,851816	1,156221
				ST. DEV.	9,809293	31,5841		
17	Mongrel	5 y	M	30 kg	244	2489	CV	
					294	2452	dROMs	PAT
					259	2440		
				AVERAGE	265,6667	2460,333	7,8857	0,847608
				ST. DEV.	20,94968	20,85399		
18	Labrador	11 y	M	40 kg	249	2573	CV	
					267	2285	dROMs	PAT
					274	2627		
				AVERAGE	263,3333	2495	3,998878	6,016832
				ST. DEV.	10,53038	150,12		
19	Mongrel	8 y	SF	40 kg	235	2573	CV	
					227	2616	dROMs	PAT
					258	2655		
				AVERAGE	240	2614,667	5,475112	1,280838
				ST. DEV.	13,14027	33,48963		
20	Border collie	13 y	SF	26 kg	275	2418	CV	
					276	2529	dROMs	PAT
					282	2452		
				AVERAGE	277,6667	2466,333	1,11328	1,88276
				ST. DEV.	3,091206	46,43514		

DOG N.					dROMs	PAT		
21	Labrador	8 y	M	37 kg	132	2412	CV	
					150	2477	dROMs	PAT
					178	2440		
				AVERAGE	153,3333	2443	12,34354	1,089676
				ST. DEV.	18,92676	26,62079		
22	Mongrel	10 m	M	15 kg	149	2966	CV	
					206	2718	dROMs	PAT
					196	2868		
				AVERAGE	183,6667	2850,667	13,53031	3,577575
				ST. DEV.	24,85067	101,9847		
23	German Shepherd	5 y	SF	37 kg	184	2477	CV	
					181	2257	dROMs	PAT
					182	2212		
				AVERAGE	182,3333	2315,333	0,516804	0,516804
				ST. DEV.	1,247219	115,7824		
24	Dalmatian	9 y	M	32 kg	149	2346	CV	
					172	2280	dROMs	PAT
					204	2322		
				AVERAGE	175	2316	12,88779	1,177736
				ST. DEV.	22,55364	27,27636		
25	Mongrel	8 y	SF	25 kg	128	2191	CV	
					172	2191	dROMs	PAT
					163	2172		
				AVERAGE	154,3333	2184,667	12,29775	0,40998
				ST. DEV.	18,97952	8,956686		
26	French Bulldog	8 y	SF	22 kg	207	2322	CV	
					189	2634	dROMs	PAT
					237	2513		
				AVERAGE	211	2489,667	9,383408	5,158828
				ST. DEV.	19,79899	128,4376		
27	Labrador	9 y	M	35 kg	71	1552	CV	
					113	1907	dROMs	PAT
					110	1821		
				AVERAGE	98	1760	19,52156	8,591515
				ST. DEV.	19,13113	151,2107		
28	American Staffordshire	5 y	SF	30 kg	218	1930	CV	
					172	2511	dROMs	PAT
					246	2322		
				AVERAGE	212	2254,333	14,39001	10,73356
				ST. DEV.	30,50683	241,9702		
29	Labrador	11 y	SF	28 kg	79	1791	CV	
					88	1867	dROMs	PAT
					78	1702		
				AVERAGE	81,66667	1786,667	5,506423	3,774102
				ST. DEV.	4,496913	67,43062		
30	Dalmatian	6 m	F	18 kg	84	2602	CV	
					119	3080	dROMs	PAT
					117	2752		
				AVERAGE	106,6667	2811,333	15,0455	7,099899
				ST. DEV.	16,04854	199,6018		
31	Mongrel	9 y	CM	19 kg	225	2539	CV	
					250	2073	dROMs	PAT
					264	2372		
				AVERAGE	246,3333	2328	6,548608	8,280542
				ST. DEV.	16,1314	192,771		
32	Mongrel	1 y	M	15 kg	221	2477	CV	
					226	2257	dROMs	PAT
					215	2477		
				AVERAGE	220,6667	2403,667	2,037876	4,314616
				ST. DEV.	4,496913	103,709		
33	Schapendoes	6 y	FS	15 kg	309	2477	CV	
					322	2412	dROMs	PAT
					380	2299		
				AVERAGE	337	2396	9,158842	3,069434
				ST. DEV.	30,8653	73,54364		
34	Vizsla	6 y	M	30 kg	189	2480	CV	
					174	2621	dROMs	PAT
					196	2440		
				AVERAGE	186,3333	2513,667	4,925188	3,088438
				ST. DEV.	9,177267	77,63304		
35	Maremma Sheepdog	9 y	SF	30 kg	144	2440	CV	
					137	2350	dROMs	PAT
					161	2480		
				AVERAGE	147,3333	2423,333	6,839917	2,243398
				ST. DEV.	10,07748	54,36502		
36	Bernese Mountain dog	2 y	F	47 kg	287	2925	CV	
					259	2573	dROMs	PAT
					254	3009		
				AVERAGE	266,6667	2835,667	5,445755	6,660609
				ST. DEV.	14,52201	188,8727		
37	Mongrel	5 y	SF	25 kg	199	2723	CV	
					193	2616	dROMs	PAT
					195	2702		
				AVERAGE	195,6667	2680,333	1,274841	1,727074
				ST. DEV.	2,494438	46,29135		
38	French bulldog	4 y	F	16 kg	188	3029	CV	
					222	3047	dROMs	PAT
					204	3029		
				AVERAGE	204,6667	3035	6,785885	0,279581
				ST. DEV.	13,88844	8,485281		
39	Golden Retriever	13 y	SF	24 kg	191	2736	CV	
					197	2462	dROMs	PAT
					160	2816		
				AVERAGE	182,6667	2671,333	8,876195	5,674368
				ST. DEV.	16,21385	151,5813		

TABLE 1. DOGS USED IN THIS STUDY: BREED, AGE, SEX, SIZE, D-ROMS, PAT, AVERAGE, STANDARD DEVIATION, CV

These results have been used to create a Gaussian to underline the frequency with which these data are obtained. In fact, as shown by the curve, most of the values are collected in a range and to the sides of the curve they are increasingly less. d-ROMs and PAT average values follow normal distribution. This means that most of the observed data is clustered near the mean, while the data become less frequent when farther away from the mean. In fact, only few values deviate from the curve, and these are the ones not included in the reference range (Figure 1,2).

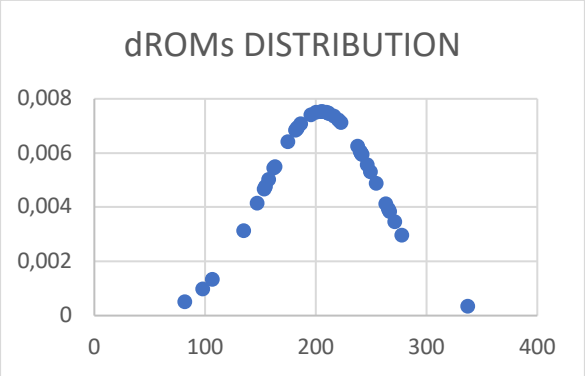


FIGURE 1. GAUSSIAN CURVE: D-ROMS DISTRIBUTION

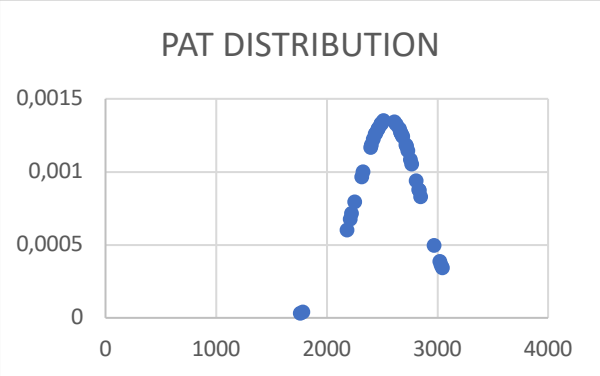


FIGURE 2. GAUSSIAN CURVE: PAT DISTRIBUTION

We have compared the d-ROMs and PAT of male and female dog's averages, specifying the average and standard deviation for both categories (Table 2). As we can notice, there are not remarkable gender differences.

	Average divided by sex				
	Male			Female	
Dog n.	dROMs	PAT	Dog n.	dROMs	PAT
5	200	2441,333	1	157,3333	2714,333
8	209,3333	2757,667	2	135	2630,333
12	241,6667	2719,333	3	162,3333	2973,667
13	249,6667	2833,333	4	206,3333	2672
14	241,3333	2488,333	6	237,6667	2656
15	240,6667	2226,667	7	222,6667	3045
17	265,6667	2460,333	9	216,3333	3022
18	263,3333	2495	10	271,3333	2685,667
21	153,3333	2443	11	163	2769,333
22	183,6667	2850,667	16	254,6667	2731,667
24	175	2316	19	240	2614,667
27	98	1760	20	277,6667	2466,333
31	246,3333	2328	23	182	2212
32	220,6667	2403,667	25	154,3333	2184,667
34	186,3333	2513,667	26	211	2489,667
			28	212	2254,333
			29	81,66667	1786,667
			30	106,6667	2811,333
			33	337	2396
			35	147,3333	2423,333
			36	266,6667	2835,667
			37	195,6667	2680,333
			38	204,6667	3035
			39	182,6667	2671,333
AVERAGE	211,6667	2469,133		201,0833	2615,056
ST. DEV.	46,42044	272,2826		58,41012	301,7376

TABLE 2. D-ROMS AND PAT IN MALE AND FEMALE DOGS

	Average divided by size				
	Small & Medium			Large	
Dog n.	dROMs	PAT	Dog n.	dROMs	PAT
1	157,3333	2714,333	3	162,3333	2973,667
2	135	2630,333	5	200	2441,333
4	206,3333	2672	6	237,6667	2656
7	222,6667	3045	9	216,3333	3022
8	209,3333	2757,667	12	241,6667	2719,333
10	271,3333	2685,667	14	241,3333	2488,333
11	163	2769,333	15	240,6667	2226,667
13	249,6667	2833,333	17	265,6667	2460,333
16	254,6667	2731,667	18	263,3333	2495
22	183,6667	2850,667	19	240	2614,667
25	154,3333	2184,667	20	277,6667	2466,333
26	211	2489,667	21	153,3333	2443
30	106,6667	2811,333	23	182,3333	2315,333
31	246,3333	2328	24	175	2316
32	220,6667	2403,667	27	98	1760
33	337	2396	28	212	2254,333
37	195,6667	2680,333	29	81,66667	1786,667
38	204,6667	3035	34	186,3333	2513,667
39	182,6667	2671,333	35	147,3333	2423,333
			36	266,6667	2835,667
AVERAGE	205,8947	2667,895		204,4667	2460,583
ST.DEV.	53,12832	224,7657		55,64438	319,4669

TABLE 3. D-ROMS AND PAT IN SMALL / MEDIUM SIZE AND LARGE SIZE DOGS

The next step was to compare the averages of small and medium sizes to the large ones, followed by average and standard deviation of both groups.

The results, even in this case, do not show any size differences (Tab. 3)

In the large dog group, we noticed a balance between male and female dogs, hence we calculated the average and standard deviation, which show very similar results (Tab.4)

	Large size males			Large size females	
	dROMs	PAT		dROMs	PAT
AVERAGE	206,5333	2386,367		202,4	2534,8
ST.DEV.	5,131601	64,9093		3,785939	85,33447

TABLE 4. D-ROMS AND PAT IN LARGE SIZE MALES AND LARGE SIZE FEMALES

Since the distribution of data was irregular (majority of females compared to males and preponderance of adults rather than puppies and geriatrics), the statistical analysis was performed on small/medium and large sizes, as a unique homogeneous group.

We calculated the average of this group and executed the t TEST, to see if the difference between the two averages is meaningful, i.e., if the difference is due to reasons related or to pure chance. The t TEST is a parametric statistical model, a value that has to be confronted to a standard range value. In turn, this depends on the degrees of freedom (observation numbers – groups numbers) and the P value (usually 5%). If the values obtained from the test are higher than the cut-off limit given by the degrees of freedom and P is 5%, the obtained difference is not due to chance.

In this statistical analysis, the t TEST value is below the threshold value. As we can see in Tab.5 and Tab.6, the P value in d-ROMs is 0,412 and in PAT is 0,131. In both cases, P is > 0,05, which means that there are not significant differences.

T TEST: two samples from the average (dROMs)		
	Variable 1	*Variable 2*
stat t	206,5333	202,4
Variance	2932,03	3595,106
Observations	10	10
Pearson Correlation	0,49811	
Hyperthetic difference for averages	0	
gdl	9	
Stat t	0,227784	
P(T<=t) one tail	0,412452	
Critical t one tail	1,833113	
P(T<=t) two tails	0,824905	
Critical t two tails	2,262157	

TABLE 5. T TEST (D-ROM)

T TEST: two samples from the average (PAT)		
	Variable 1	*Variable 2*
Average	2386,367	2534,8
Variance	64862,33	138355,5
Observations	10	10
Pearson Correlation	0,254961	
Hyperthetic difference for averages	0	
gdl	9	
Stat t	-1,19258	
P(T<=t) one tail	0,131763	
Critical t one tail	1,833113	
P(T<=t) two tails	0,263525	
Critical t two tails	2,262157	

TABLE 6. T TEST (PAT)

DISCUSSION

Although the key role of free-radical reaction in many biological processes is no longer in doubt, only few scientific studies support this topic in its practical application.

In veterinary medicine, data in presumably healthy dogs and standardized protocols are lacking [55]. Both photometric tests were validated previously in humans. Moreover, either d-ROMs test or PAT test showed reliable and suitable assays to evaluate globally and to monitor the oxidative stress either in health or in ill subjects [4].

Actually, in dogs, oxidative stress has been studied mainly in pathologic conditions. Thus, the primary objective of the work was to set baseline values in healthy dogs and verify their uniformity confronting them in a heterogeneous dog's population.

By the presented data, we can state that both d-ROMs and PAT test exhibit good analytical performances in the population involved in this study, with the possibility to obtain a reliable measure of the oxidative balance *in vivo*.

This is a preliminary stage. So far, the achieved results did not show significant differences among the groups.

This work is still in embryonic stage, and it is the first step to a wider project. The goal for the future is to enroll a higher number of dogs, in order to have a more balanced split of the categories, so as to provide an improved scientific value and accurate results to the research.

CONCLUSIONS

Oxidative stress plays an important role in the pathogenesis of potentially severe conditions. In the long term, increasing the level of prooxidant factors can cause structural defects in mitochondrial DNA and alterations in enzymatic functionality or cellular structures, with the appearance of functional, structural abnormalities or aberrations in gene expression.

It is crucial monitoring oxidative stress in dogs, and pets in general to improve the management. Oxidative stress is strictly correlated to animal wellness, so it can provide important information. The assessment of redox balance should be measured frequently in healthy dogs, in order to intercept inflammatory conditions and intervene with antioxidants supplementation and lifestyle changes, before serious pathologies occur. This mainly applies to older subjects, thus let them live a healthy old age.

Oxidative stress monitor can be useful also in diseases as additional data on which base the patient's management.

In conclusion, oxidative stress is an important pathogenetic link for dogs (and animals in general) and studies in this field may be important elements in the future, to better understand and manage various diseases.

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