RESEARCH ARTICLE

BIOMARKERS OF OXIDATIVE STRESS IN DOGS

Author

W. Jean Dodds, DVM

Email: jeandodds@hemopet.org

Home Office: 310-828-4804

Hemopet, 938 Stanford Street, Santa Monica, CA 90403, USA

Abstract

Oxidative stress is the repair process that cells normally undergo after they metabolize oxygen and form free radicals called reactive oxygen species (ROS). When ROS production is excessive, however, cells undergo damage and release biomarker lipids and enzymes that lead to tissue inflammation, infections, obesity and cancers. This study describes a practical and rapid screening salivary test for cellular oxidative stress (OS) in dogs, which can be addressed to help enhance their health and longevity.

Of the 282 dogs tested for ROS, 79 had elevated levels of the biolipid isoprostane; and 38 of them also had saliva-based profiles for food sensitivity and intolerances to 24 primary foods. Only 3 dogs were found to be reactive to 20 or more foods. These results suggest that dogs with elevated ROS and clinical issues related to intense itching, scratching, chewing, and bowel irritability had relatively few identified foods as the culprits.

Key words: *oxidative stress, biomarkers, saliva*

Introduction:

In people and animals, cells are in homeostatic equilibrium when they are in oxidative balance, namely, when the cellular antioxidants (reducing agents) are balanced with the oxidants (oxidizing agents) [1-6]. However, when oxidant levels exceed antioxidants (termed cell redox), cells undergo oxidative stress (OS), which is the basic mechanism of all sickness and chronic disease [1-7].

Extensive investigations of OS and chronic tissue inflammatory responses have established the major underlying risk factors that play key roles in the etiology of a range of human (and animal) diseases that include rheumatoid arthritis, cancers, diabetes, obesity, neurodegenerative disorders, cardiovascular diseases, acute and chronic kidney injury and disease, and other chronic diseases [1,6,7, 9]. The expression of these diseases is influenced by a range of environmental, dietary, and lifestyle factors, and specific dietary components, along with exercise and a variety of nutraceuticals, can reduce these risk factors significantly [8-10].

Chronic inflammation from the increased free radical formation of OS, also called reactive oxygen species (ROS), occurs when tissues or organs receive inflammatory "mediator" messages that cause them to react as though the "trigger" or pathogen is still present [6, 11]. In healthy states, 25% of oxygen intake forms ROS, whereas this increases to 75% in unhealthy states and in aging.

Rather than repairing themselves, cells producing ROS remain in an ongoing state of inflammation that can wax and wane life-long. Tissues thus become deficient in antioxidant mediators, such as malondialdehyde, glutathione, cysteine, ascorbic acid and other antioxidant vitamins, which is associated with a poor clinical outcome. However, free radicals themselves are so reactive and short-lived that direct measurement of ROS is not possible [1,7,11].

When cells undergo damage, they release biomarker enzymes that lead to tissue inflammation, infections, obesity and even cancers [6, 8]. For example, biomarker levels in canine patients with multicentric lymphoma, oral fibrosarcoma, mast cell tumor, malignant melanoma, appendicular osteosarcoma, nasal tumors and peripheral ameloblastoma have been studied [12,13]. Each group consisted of 6 patients; antioxidant biomarkers were measured in serum and whole blood, and were compared to those of 31 healthy dogs. The increase of

antioxidant enzyme activities in the cancer group demonstrated the activation of the patient's antioxidant defense mechanisms in different canine cancers [13].

Importantly, the presence of high levels of these biomarkers has been successfully addressed with dietary and supplement changes that promote beneficial effects of added antioxidants [5,10]. These assays then are repeated periodically to assess the response and adjust nutritional and nutraceutical therapy, as needed [8].

Management of OS has recently focused on the development of functional foods, e.g. those containing natural Nrf-2 activators [8,10]. These include such antioxidant ingredients as: turmeric (Curcuma longa); and its relative, ginger (Zingiber officiale); chili peppers (Capsicum annuum); green tea (Camellia sinensis, which contains tannins and polyphenol catechins, and other teas); soybeans (Glycine max); tomatoes (Solanum lycopersicum, rich in lycopenes); grapes (not for pets); honey (not for infants or very young animals); cranberries (Vaccinium macrocarpon, contains pro-anthocyanidins); licorice (Glycyrrhiza glabra); celery, thyme, and parsley; garlic (Allium sativum, in moderation for pets); milk thistle (Silybum marianum); cabbages and broccoli.

Some Primary Oxidative Stress Biomarkers

- Isoprostanes are a series of prostaglandin-like compounds produced by peroxidation of arachidonic acid. This process is catalyzed by ROS, and they are considered the "gold standard" test for quantifying lipid peroxidation/OS in vivo in humans and animals. Isoprostane levels serve as a reliable surrogate biomarker for the presence of ROS [14-20].

- MicroRNAs are small non-coding RNA molecules found mostly in the cells of plants, animals and some viruses. They function in RNA silencing and post-transcriptional regulation of gene expression. The function of microRNAs is in gene regulation [21,22].

- Glutathione-5-Transferases (GST), previously called ligandins, belong to a superfamily of metabolic isozymes best known for their ability to catalyze conjugation of the reduced form of glutathione (GSH) to foreign substrates for the purpose of detoxification. They are found in plants, animals, fungi, and some bacteria. GSH prevents damage to cells by ROS, peroxides, lipid peroxides, and heavy metals, and plays an important role in cell signaling. Cytosolic GST is expressed primarily in heart, lung, and brain tissues;

whereas, GSTs in general function primarily in the liver. High levels of GST are associated with resistance to the apoptosis (cell death) induced by a range of substances, including chemotherapeutic agents. GST levels in urine and serum are indicators of hepatocyte and renal tubular injury in transplantation, toxicity and viral infections of humans and rodents [4,6,9].

Malondialdehyde, a reactive aldehyde, occurs naturally as a biomarker for lipid peroxidation and OS, and is formed when ROS degrade polyunsaturated lipids. Malondialdehyde is potentially mutagenic, and has been found in heated edible oils such as sunflower and palm oils. Corneas of human patients suffering from keratoconus and bullous keratopathy have increased levels of malondialdehyde, and this aldehyde also can be found in tissue sections of joints from human patients with osteoarthritis [23,24]. Levels in healthy canines are reported to be 1.00-1.77 µM in serum [12].

Tumor Necrosis Factor-Alpha (TNF-α), also called cachexin or cachectin, is a cell signaling protein involved in systemic inflammation and is one of the cytokines that make up the acute phase inflammatory response. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, natural killer (NK) cells, neutrophils, mast cells, eosinophils, and even neurons. TNF is an endogenous pyrogen that can induce fever, apoptotic cell death, cachexia, inflammation, inhibit tumorigenesis and viral replication, and respond to sepsis via interleukin IL-1and IL-6 producing cells. Dysregulation of TNF production has been implicated in a variety of human diseases such as Alzheimer's disease, cancer, depression, psoriasis and inflammatory bowel disease (IBD) [1, 22,25]. Levels of TNF-α in the serum of normal canines have been reported to range from 0–4 pg/mL [8].

Nuclear factor erythroid 2-related factor 2 (Nrf2), a protein that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation. While Nrf-2 activation can be evaluated using complex protocols in a research setting by using GSH as a surrogate biomarker, Nrf-2 is not a suitable clinical biomarker, as clinical samples must be stored and analyzed under conditions to avoid denaturation. The complexity and cost of the analysis prohibits widespread application [3,8,11,26,27]. The concentration of GSH in blood samples from

normal canines has been reported to be 0.128 ± 0.01 mM [8].

- Hydroxyoctadecadienoic acid (HODE) is a stable oxidation product of linoleic acid. Levels are increased when cellular OS increases, such as in diabetes, asthma, cancers, and early atherosclerosis. Gene expression is regulated via macrophages. Importantly, this biomarker can be elevated in the inflammation and OS of periodontal disease [28].

- Other Biomarker Enzymes include: Sorbitol Dehydrogenase, a cytosoli enzyme that converts sorbitol, the sugar alcohol form of glucose, into fructose; and 5' Nucleotidase, which catalyzes the phosphorolytic cleavage of 5' nucleotides, and is considered a maturation marker for T- and B-cells [22,25,29].

Measuring Biomarker Enzymes of Oxidative Stress

Traditionally, cellular biomarkers are measured in the serum and urine from humans and animals [2,8,25,30]. However, collecting these samples especially from smaller animal species presents with difficulty and causes unnecessary stress. Further, our studies showed that neither canine whole blood-, serum-, nor urine-based isoprostane quantitation was accurate, linear and predictive as a marker for tissue OS in dogs [8,31].

In contrast, collection of saliva is noninvasive, painless, relatively inexpensive and convenient for the individual. It is easily collected, stored and shipped, and provides a non-invasive means of multiple or serial sampling for use as a diagnostic tool for a variety of conditions in humans and animals [2,8,11,32]. Salivary biomarker testing can reveal the latent or pre-clinical form of developing OS.

Saliva also can be used as a diagnostic tool to assess the health or disease status of an individual.

The levels of salivary biolipids, like isoprostane, also are predicted to reflect the general mucosal immune response and can be induced in people and animals without parallel antibodies being detected in serum [2,33]. Only saliva-based isoprostane measurement results were found to be reliable, as shown below (see Results).

Methods:

1. Thirty healthy adult greyhounds, 15 of each sex and neutered/spayed served as the control group of dogs to establish the normal canine isoprostane reference range. These dogs were housed at the licensed animal blood bank facility maintained for a finite period

by the author and staff [34]. Other dogs with a variety of health conditions, of varying agers and both sexes, were recruited for this study from the local pet owners and by request of veterinarians in other areas of the country. The final patient group tested included 282 dogs.

Seventy-nine of the test group of 282 dogs had isoprostane levels above those of the control group, and were given the supplements, described below, intended to reduce their OS. After 5 - 6 months, the isoprostane levels of the saliva from these dogs were re-measured.

The saliva-based test assay developed for this study and for subsequent diagnostic purposes quantitates the isoprostane level in dog saliva as a lipid biomarker to determine the presence of harmful OS. The proprietary assay utilized an enzyme-linked immunosorbant assay (ELISA) testing system and affinity-purified rabbit anti-canine IgG antibody developed by the author and colleague (a), to detect the level of isoprostane in dog saliva.

This saliva-based test is a novel canine isoprostane assay, and is the first of a set of unique biomarker tests for pets that have been studied in dog saliva.

Results

After completing the initial clinical trial studies and analyzing 282 clinical patient samples; 79 of them (31.4%) were positive, having isoprostane biolipid levels above the canine normal reference range established by the author at 0.5-1.75 ng/mL of saliva.

Of the 79 positive testing dogs, there were: 33% spayed females, 31% neutered males, 21% intact males and 15% intact females. The ages ranged from 4 months to 15 years, although most were middle aged or older. The weight range was 4-143 pounds, with 79% being medium to large or giant in size; but no breed type predominated (Table 1).

The diets fed the 79 positive dogs included: 40% ate only a commercial raw diet; 24% only a commercial dry kibble; 23% a home cooked or home prepared raw diet, and 13% ate a combination of a commercial kibble and raw (Table 1).

Of the 79 positive dogs, 38 also had saliva-based profiles run for food sensitivity and intolerances to 24 primary foods [34]. Interestingly, only 3 of these 38 dogs had saliva-based test results that were reactive to 20 or more foods.

Table 1. Demographics of 79 Isoprostane Positive Dogs

Parameter	**(%)**	**(%)**	**(%)**	**Misc**
Pet Size (weight)	**Small**	**Medium**	**Large/Giant**	
	21	40	39	
Pet Age (years)	**< 2**	**2-4**	**4-10**	**>10**
	12	21	43	24
Pet Sex	**M**	**MN**	**F**	**FS**
	21	31	15	33
Pet Diet	**Only Raw**	**Only Dry Kibble**	**Kibble + Raw Combo**	**Homemade Cooked or Raw**
	40	24	13	23

Positive testing dogs were retested 5-6 months after being on foods and supplements designed to lower their cellular oxidative stress. Table 2 lists the outcomes in 15 dogs, when retested after use of these bioceutical supplements.

Table 2. Outcomes of 15 Isoprostane Positive Dogs After 5-6 Months on Supplements

Cases	***Isoprostane Before Supplements (ng/mL)***	***Isoprostane 5-6 Months After Supplements (ng/mL)***	***Clinical Comments***
1	*2.13*	*2.00*	*Cancer risk in family*
2	*2.90*	*2.11*	*Improved respiratory function*
3	*3.20*	*1.65*	*Reduced scratching/itching*
4	*1.76*	*1.10*	*Thyroid function improved*
5	*2.35*	*1.75*	*Hemangiosarcoma risk in family*
6	*1.90*	*0.70*	*No more urinary tract infections*
7	*3.04*	*2.26*	*Improved agility performance*
8	*2.45*	*2.75 **	*No change*
9	*2.30*	*1.60*	*Mammary carcinoma removed*
10	*1.95*	*1.40*	*Less foot itching /tear staining*
11	*2.26*	*1.73*	*Gut issues mostly resolved*
12	*2.60*	*1.48*	*Allergy issues reduced*
13	*1.89*	*0.80*	*Dermatitis issues improved*
14	*1.94*	*0.90*	*Itchy feet better; likes cool floor*
15	*2.09*	*2.20 **	*Still healthy*
	2.31 ± 0.04	***1.29 ± 0.48***	***Significant Reduction (t = <0.0001)***

**Used own supplements*

The list below includes supplements that can helped reduce the isoprostane level, as shown above after 5-6 months.

Supplements used in this study to bring down high levels of isoprostane included:

- Alpha-Lipoic acid
- Carnitine
- Co-Enzyme Q-10
- Ginger
- Green tea
- Licorice
- Milk thistle, garlic and honey.
- Resveratrol (as a natural supplement, or food like blueberries and cranberries)
- Soybeans
- Tomatoes
- Turmeric (curcumin) – without black pepper for pets
- Vitamin E

Discussion:

OS is the repair process that cells normally undergo after they metabolize oxygen and form free radicals, namely ROS. Healthy cells are in symbiosis (harmony) within the body and are maintained in this state primarily by the presence of a healthy, balanced microbiome. This microbiome is first present within the body during fetal development and at birth, and then continues to function throughout life [1,8,11,32].

The microbiome organisms (microbiota) that reside in the bowel are the most diverse and abundant, and so one should focus on the health benefits of the microbiome, such as by providing probiotics [1,5]. The benefits of this approach are easy for the consumer to observe and measure not only in themselves, but also in other people and pets. Observable benefits include lessening in the severity of eczema and other skin disorders, improved protein digestion (measurable by absent or reduced intestinal gas) in young human and animal athletes that consume protein-rich diets, and the alleviation of constipation in senior people and aging pets. These benefits improve the quality of life across all life stages [8,11,30].

However, when ROS production is excessive, cells undergo damage and release biomarker lipids and enzymes that lead to tissue inflammation, infections, obesity and even cancers [1,4,6-8]. This is known as dysbiosis [1,4,7,11]. Thus, when the healthy gut microbiome is disrupted in response to OS and exhibits dysbiosis, harmful microorganisms predominate. It creates an unhealthy gut barrier that can trigger cellular inflammation – which is directly related to

imbalances in the important neurotransmitters of the gut-brain axis. This in turn contributes to the cognitive and memory decline associated with aging and even to more serious issues such as depression [1,5,7,27,30]. This response is seen clinically and can be measured by the increased levels of cellular biomarkers like isoprostane [2,8].

This give-and-take response of microbiota can be enhanced by the addition of selected nutrients and supplements which balance the gut microbiome and can help counteract cellular OS and ROS production [8,10]. One proven way this can be accomplished is with the so-called functional foods that activate the body's critical Nrf-2 pathway, as listed above. The coined term for these foods is bioceuticals, a holistic approach to improving cognitive health and longevity [8].

Several dozen diagnostic methods have been reported for measuring biomarkers for OS, antioxidant capacity, and tissue inflammation and many have been used in biomedical research which supports that OS and inflammation are major risk factors for multiple diseases [22,25,26,30].

Urinary protein, when present in significant amounts is known to be a biomarker for kidney injury [30,31]. In healthy people, the normal urinary protein ranges from 0.03-0.26 µg/mg creatinine; that of healthy dogs can be up to a trace or +1 [9].

Except for C-reactive protein measured in blood and the albumin/creatinine ratio in urine, tests for these risk factors of ROS have generally been used in research settings on people and rodents with few clinical trials. These assays have not been routinely applied when monitoring human or animal health and wellness [8,22,25,30,33].

A few specialty laboratories have recently begun to offer tests for multiple biomarkers of OS and tissue inflammation [23,25,26.30]. But, these biomarkers for assessing OS and antioxidant capacity are unstable, because when air reacts with biofluids during transit, storage and processing, large artifacts can develop [8,25]. Thus, rapid sample analysis at the site of collection, or quick rapid freezing and transport of samples on dry ice, are important to obtain reliable results using serum or urine [22,30]. This testing for companion animals is impractical in a veterinary clinical diagnostic setting [8]. Thus, the development and use of a simple saliva-based test for OS in dogs, as shown here, offers a significant advancement in the field.

Regarding the sex breakdown and age demographics of the 79 isoprostane positive dogs shown in Table 1, this likely reflects the owner's increased decision for testing as older pets are more likely to be neutered or spayed and have some age-related health issues

Further, of the 79 positive dogs, only 3 of the 38 also tested for food sensitivity and intolerances to 24 primary foods had 20 or more identified food reactivities [34]. These results suggest that dogs with clinical issues related to intense itching, scratching, chewing, and bowel irritability had relatively few identified foods as the culprits. Environmental exposure to inhalants, fleas, ticks, mites and other insects as well as contact reactants also could be contributing to their clinical issues; and 3 dogs were taking an isooxazoline parasiticide.

End Note:

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