Universidade de Aveiro Departamento de Biologia
2011

Verónica Isabel Correia Bastos

Levels and effects of indoor contaminants in European pets

Níveis e efeitos de contaminantes ambientais domésticos em animais de companhia

Universidade de Aveiro Departamento de Biologia
2011

Verónica Isabel Correia Bastos

Levels and effects of indoor contaminants in European pets

Níveis e efeitos de contaminantes ambientais domésticos em animais de companhia

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Ana Catarina Almeida Sousa, bolseira de Pós Doutoramento do CESAM e Departamento de Biologia da Universidade de Aveiro, da Doutora Helena Cristina Correia de Oliveira, bolseira de Pós Doutoramento do CESAM e Departamento de Biologia da Universidade de Aveiro e do Prof. Doutor António José Arsénia Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho aos meus pais, José Bastos e Lurdes Correia, e, ainda, ao meu namorado, Fernando Torres, pelo incentivo e apoio incondicionais na sua realização.

o júri

João António de Almeida Serôdio, Prof. Auxiliar (Presidente), Departamento de Biologia da Universidade de Aveiro

Vera Lúcia de Almeida Maria (Arguente Principal), Investigador Pós-Doutoramento, CIMA - Centro de Investigação Marinha e Ambiental, Faculdade de Ciências e Tecnologia, Universidade do Algarve

Ana Catarina Almeida Sousa (Orientador), Investigador Pós-Doutoramento, CESAM - Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro

Helena Cristina Correia de Oliveira (Co-Orientador), Investigador Pós-Doutoramento, CESAM - Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro

António José Arsénia Nogueira (Co-Orientador), Prof. Associado c/ Agregação, Departamento de Biologia da Universidade de Aveiro

agradecimentos

A realização deste trabalho deve agradecimentos a:

Doutora Ana Sousa, pelo apoio constante, orientação e, acima de tudo, pela amizade e compreensão.

Doutora Helena Oliveira, Doutor João Paulo Teixeira, Prof. Doutor António Nogueira e Prof. Conceição Santos, por toda a ajuda e contribuição neste trabalho.

À Doutora Vera Maria e à Doutora Bebianno pela quantificação de metalotioninas.

Aos Médicos Veterinários Lisete Vieira, Joana Tuna, Inês Santos, Inês Freitas, Joana Alegrete, Hugo Vilhena e Sónia Miranda que permitiram e realizaram as colheitas de amostras de sangue aos animais domésticos.

Ao Doutor Ramiro Pastorinho e Doutor James Ranville pela deteminação de metais.

À Doutora Inês Domingues pelo apoio prestado no laboratório LETAL na quantificação de proteínas e à Sónia Coelho por todo o auxílio laboratorial.

À Susana Pinho pela incansável ajuda no laboratório do INSA.

À Nádia, que de certa forma iniciou todo este processo, pois se não a tivesse conhecido não estaria a escrever esta tese. A ela agradeço todo o apoio e confiança que depositou em mim.

À minha amiga, Rita Figueiredo, que esteve sempre disponível para me apoiar e ajudar e a todos os meus amigos que de certa forma me acompanharam.

À Maria, a gata que inspirou o tema do estudo.

Ao Chiwi, pela companhia e pelos momentos de descontracção que me proporcionou na escrita da tese.

agradecimentos

Ao meu irmão, Ricardo Bastos e à minha cunhada Paula Bastos por me apoiarem, incentivarem e por me acompanharem sempre.

Ao meu maravilhoso sobrinho por me fazer mais feliz sempre que o vejo sorrir.

À minha prima, Lia Temudo, por todos os momentos de compreensão e de incentivo.

Ao meu querido namorado por me aturar nos piores momentos, me chamar à razão nos meus devaneios e partilhar comigo as diferentes fases da minha vida.

E, finalmente, aos melhores pais do mundo, por me terem incentivado desde o início a matricular-me no mestrado e por me ajudarem e me amarem incondicionalmente durante toda a minha vida.

palavras-chave

Gatos, Cães, Contaminação Interior, Exposição a Metais, dano no DNA, Ensaio de Cometas

resumo

Os humanos e os animais estão frequentemente expostos a contaminantes potencialmente perigosos, incluindo metais que podem induzir efeitos nocivos tal como danos no DNA. Gatos e cães têm sido considerados como bons sentinelas para a espécie humana na identificação de substâncias potencialmente perigosas dentro de casa, uma vez que eles partilham o mesmo ambiente que os seus donos. No presente estudo foram avaliados os níveis e os potenciais efeitos dos metais em animais de companhia. As concentrações de metais na ração de cães e no seu sangue foram determinados através de inductively coupled plasma optical emission spectrometry (ICP-OES) e os níveis de metalotioninas (MTs) quantificados através de differential pulse polarography (DPP).

Os níveis de crómio (Cr), cobre (Cu), lítio (Li), níquel (Ni), chumbo (Pb) e zinco (Zn) estavam mais aumentados na ração seca do que na ração húmida. Contrariamente, o cádmio (Cd) apresentou valores mais elevados nas amostras de ração húmida. Para todas as amostras analisadas a concentração de metais foi sempre inferior ao nível máximo recomendado pelo National Research Council, o que sugere que a ração não é responsável por uma excessiva ingestão de metais.

As concentrações de Cd, Cr, Cu, Li, Ni, Pb e Zn em amostras de sangue de cães variou entre: $<0.2x10^{-3}$ - $17.9x10^{-3}$; $430x10^{-3}$ - $771x10^{-3}$; $<0.9x10^{-3}$ – $10.1x10^{-3}$; $<0.6x10^{-3}$ - $480\ x10^{-3}$; $16.9x10^{-3}$ – $72.4x10^{-3}$ and 3.06 – 4.46 $\mu g.g^{-1}$. Estes valores estão dentro do intervalo de referência e não estão correlacionados estatisticamente com os níveis de MTs.

No presente estudo os danos de DNA em gatos e cães também foram determinados pelo ensaio de cometas. Os danos no DNA foram relativamente baixos (<22.9% em gatos e <32.3% em cães) e não foram detectadas diferenças significativas para ambas as espécies. Do mesmo modo, não se detectaram diferenças significativas relativamente aos gatos e cães e os seguintes factores: género, idade, estado de saúde, residência, localização geográfica e dieta.

De modo geral, esta tese demonstrou que a contaminação por metais não parece ser um problema para os animais estudados e, ainda, que estes animais, actualmente, não estão expostos a químicos que induzem danos severos no DNA.

keywords

Cats, Dogs, Indoor Environment, Metal Exposure, Metallothioneins, DNA damage, Comet assay

abstract

Humans and animals are frequently exposed to harmful contaminants; including metals that may induce deleterious effects as for example DNA damage. Pet cats and dogs have been reported as good sentinels for human beings in the identification of potential health hazardous substances, since they share the same indoor environment as their owners. In the present work the levels and potential effects of metals in companion animals were evaluated. The concentrations of metals in dog food and blood were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) and the levels of metallothioneins quantified by differential pulse polarography (DPP). The levels of chromium (Cr), cooper (Cu), lithium (Li), nickel (Ni), lead (Pb) and zinc (Zn) in dry food samples were higher than the ones found in wet ones. An opposite trend was established for cadmium (Cd) that exhibited higher levels in wet food samples. For all samples analyzed, the concentrations of metals were always below the maximum recommended levels set by the National Research Council suggesting that food is not being responsible for an excessive intake of metals.

The concentrations of Cd, Cr, Cu, Li, Ni, Pb and Zn in blood samples varied between $<0.2x10^{-3}$ - $17.9x10^{-3}$; $430x10^{-3}$ - $771x10^{-3}$; $<0.9x10^{-3}$ – $10.1x10^{-3}$; $<0.6x10^{-3}$ - $480\ x10^{-3}$; $16.9x10^{-3}$ – $72.4x10^{-3}$ and 3.06 – 4.46 $\mu g.g^{-1}$. Those values are within the reference range and were not significantly correlated with MTs levels.

In this study the DNA damage in pet cats and dogs was also assessed, using comet assay. The DNA damage is relative low (<22.9% in cats and <32.3% in dogs) and no significant differences were detected between the two species. Similarly, no significant differences were detected for both cats and dogs and the following factors: gender, age, health status, residence, location and diet. Overall this thesis demonstrates that for the studied animals' metal contamination doesn't seem to be a major problem and that they are not currently exposed to chemicals that induce severe DNA damage.

Index

List of Figures

List of Tables

Acronym list

AAFCO: Association of Feed Control Officials

AI: Adequate intake

AP: Apurinic/apyrimidinic

DHEA: Dehydroepiandrosterone

DNA: Deoxyribonucleic acid

DPP: Differential Pulse Polarography

EPA: Environmental Protection Agency

FDA: Food and Drug Administration

ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry

MT: Metalothionein

MTSA Commitee: Committee on Minerals and Toxic Substances in Diets and Water for Animals

Na_2EDTA: Ethylene Diamine Tetraacetic Acid disodium salt

PBMC: Peripheral Blood Mononuclear Cells

PBLs: Peripheral Blood Lymphocytes

p-PD: para-Phenylenediamine

RDA: Recommended Dietary Allowances

ROS: Reactive Oxidative Species

SCGE: Single cell gel electrophoresis

SSB: Single-strand breaks

Chapter I

General Introduction

Chapter I. General Introduction

1. Pets as Human Sentinels

Animals have been reported as good sentinels for human beings, according to the United States National Academy of Sciences "like humans, domestic animals, fish and other wildlife are exposed to contaminants in air, soil, water, and food, and they can suffer acute and chronic health effects from such exposures" (Glickman et al., 1991). A classic example is the "canary in the coal mine" case: in the 19th century, coal miners brought canaries into mines as an early-warning signal for toxic gases including methane and carbon monoxide. The birds would become sick before the miners, who would then have a chance to escape or put on protective respirators. Another classical example of sentinels that had significance for human exposure is the one from Minamata Bay in Japan during the 1950s. The cats that had consumed methylmercury-contaminated fish displayed neurobehavioral symptoms: they start "dancing" in the streets and sometimes collapsed and died. Besides these two classical examples, the presence of reproductive abnormalities in fish, birds, and alligators has been attributed to the presence of endocrine-disrupting chemicals in the environment (van der Schalie et al., 1999).

Sentinel animals can be used to identify potential health hazards to other animals or humans in any type of environment, including homes, since they provide early warnings of chemical exposure due to their quickly response (more quickly than humans who are similarly exposed), i.e. decreased latency, and to their response at lower doses (increased susceptibility) (Glickman et al., 1991).

Cats and dogs, in particular, are good sentinels to assess adverse human health outcomes related to low-levels exposure to many indoor contaminants as they share the same indoor environment as their owners.

The arguments for the use of cats as sentinels can be summarized as follows (Barthold et al., 2009):

- Cats are similar to humans with the same neurological, cardiovascular, respiratory and immune system;
- Large database exists based on studies using cats as models of human disease;

- The genetic diversity of the general cat population has provided several valuable genetically based models of human disease.

The arguments for the use of dogs as sentinels can be summarized as follows (Barthold et al., 2009):

- Random source animals represent potentially important models for research on naturally occurring diseases such as cancer, infectious diseases, and age related diseases;
- Dogs are a genetically diverse study group;
- Dogs are physically and physiologically similar to humans;
- The dog's cardiovascular system is similar to that of humans in both size and function;
- Random source dogs may have age-related chronic or persistent disease conditions such as congestive heart failure, arthritis, allergy, dementia, and neoplastic conditions that may make them desirable for investigations into similar human conditions;
- Random source dogs exposed to outdoor environments and various vectors that may carry diseases can be effective models of naturally occurring infectious diseases.

To provide an integrated measure of exposure and extrapolation to humans, the animal's blood or animal's tissues can be collected for analysis. Exposure pathways to such contaminants include not only the indoor environment (that pets share with their owners) but also food, which in recent years is not shared by pets and owners. In the next section a brief description of both exposure pathways is provided.

2. Exposure pathways:

2.1. Indoor environment:

The indoor environment is an important source of contaminants that can be generated by indoor sources (for example from the leaching of many consumer products) or can be a result of the migration from the outdoor air (Le Cann et al., 2011). There are three types of agents that cause indoor contamination: chemical agents, biological agents and physical agents. A chemical agent refers to metals, volatile organic compounds (VOCs), semi volatile organic compounds (SVOCs), pesticides, brominated flame retardants (BFRs), etc. Molds, bacterial endotoxins and allergens are considered biological agents. Considered as physical agents are the exposure to noise, extremely low frequency (ELF) fields and to radiofrequency (RF) fields.

Indoor exposure to chemicals occurs through inhalation, ingestion and dermal pathways. Dust is considered an important exposure route for some chemicals especially for children due to the hand to mouth behavior. Pets have the identical exploratory behavior as children (frequent mouthing of hands and other objects), consequently they are more vulnerable to environmental exposure (Le Cann et al., 2011).

House dust is a repository and a concentrator of many chemicals amongst which metals are included. Dust can be originated from external sources (through the wind and feet) or be generated within the house. Many metals are higher concentrated in indoor than exterior dust or soil in urban environments (Ibanez et al., 2010). In general, metals (at least in their inorganic forms) are in particulate, irrespirable form and may be ingested especially by children and pets during their hand to mouth activity (Le Cann et al., 2011)

Recent studies evaluated the levels of selected chemicals in household pets, including metals (Park et al., 2005; López-Alonso et al., 2007; Bischoff et al., 2010); organochlorines (Kunisue et al., 2005; Storelli et al., 2009), brominated flame retardants (Dye et al., 2007; Venier and Hites, 2011) and perfluorochemicals (Guruge et al., 2008).

2.2. Pet Food

Nowadays most pets are feed with a different diet than the one of their owners and therefore in order to use pets as sentinels for human exposure it is important to check if the contamination profile is not due to the food they are consuming.

In recent years, the pet food industry has become a multimillion dollar business all around the world (Duran et al., 2010). There are several pet foods available in the market and it is hoped that food has the nutritional and metals levels which ensure the pets health, appropriate development and the comfort they need (Duran et al., 2010).

Different types of food are available such as dry, wet, canned, moist, semi-moist, frozen-chilled pet and treats [Figure 1].

Figure 4: An example of the variety of pet foods available in supermarkets.

Pet food must have proteins, fat, carbohydrates, vitamins, and minerals at the right proportion, such as some metals (especially iron, copper, and manganese) which are known to be essential for pet's health (Duran et al., 2010). The functions of minerals in the body can be summarized in:

- Formation of bone and cartilage;
- Maintenance of fluid and acid/base balance;
- Transportation of oxygen in the blood;
- Normal functioning of muscles and nerves;
- Production of hormones.

It is extremely important that minerals are in a proper balance in pet's body, since excess or deficiency of one mineral can affect the action of others. The amount of minerals in a pet's body depends on the intake from diet, the ability of the animal to absorb minerals form the intestine and any conditions (diseases, for example) that can cause excess, loss or retention of minerals.

Minerals can be classified in three major categories: macrominerals (sodium, chloride, potassium, calcium, phosphorous, magnesium) which are required in gram (g) amounts/day; trace minerals (iron, zinc, copper, iodine, fluorine, selenium, chromium) required in milligrams (mg) or micrograms (μg) amounts/day; and other important trace minerals that have a unclear role in companion animal nutrition (cobalt, molybdenum, cadmium, arsenic, silicon, vanadium, nickel, lead, tin). One mineral intake influences others minerals action, so the intake of any mineral should be in a balanced amount, otherwise mineral imbalance is likely to occur (Merk, 2011). The main effects of deficiency and toxicity of each mineral for pet cats and dogs are described as follows.

Sodium and Chloride → Dietary deficiency of sodium and chloride is extremely rare. A sodium or chloride deficiency can occur during an excess loss of these two minerals from the body, resulted from prolonged (or chronic) severe diarrhea and/or vomiting. Sodium and chloride toxicity generally does not occur in normal animals with access to good quality drinking water, because any excess intake of sodium or chloride is filtered through the kidneys and excreted into the urine (National Research Council, 1980).

Calcium and Phosphorous → Deficiency of these minerals is uncommon, except in animals that consume high-meat diets (high in phosphorous and low in calcium) and diets high in phytates. The decrease of calcium absorption (when the supplies of calcium are insufficient or when phosphorous are in excess) can result in hyperesthesia, and loss of muscle tone with temporary or permanent paralysis associated with nutritional secondary hyperparathyroidism, skeletal demineralization, particularly of the pelvis and vertebral bodies, develops with calcium deficiency. Excessive intakes of calcium can cause signs more severe of osteochondrosis and decreased skeletal remodeling in young animals (Merk, 2011).

Potassium → according to the National Research Council (1980), potassium toxicity may interfere with the magnesium absorption and utilization. At the excessive intake of potassium, the heart and adrenal glands are adversely affected.

Magnesium → Is a cofactor of many intercellular metabolic enzyme pathways. In a balanced diet, magnesium is rarely deficient. Mineral complexes can be formed within the intestine and may decrease magnesium absorption when calcium or phosphorus supplementation is excessive, insoluble and indigestible. Clinical signs of magnesium deficiency in puppies are depression, lethargy, and muscle weakness. Excessive magnesium is excreted in the urine (Merk, 2011).

Iron and Copper → Deficiency of these minerals are rare, except in animals which diet is composed almost entirely of milk and vegetables. When iron and copper are in deficiency, they can cause microcytic, hypochromic anemia and a reddish tinge to the hair in a white-haired animal (Merk, 2011). Acute copper poisoning causes severe gastroenteritis characterized by abdominal pain, diarrhea, anorexia, dehydration and shock. If animal survives to the gastrointestinal disturbances may develop hemolysis and hemogobinuria. Animals that survive the acute episode may die of subsequent renal failure. Normal blood copper levels, in many species, range between 50 - 150 $\mu g.dl^{-1}$ (Merk, 2011). Copper levels of 26 to 38 ppm in dietary for sheep can elevated the hepatic copper levels, otherwise levels of 500 ppm copper in rats diet are well tolerated (Merk, 2011).

Zinc → Deficiency results in emesis, keratitis, achromotrichia, retarded growth, and emaciation. Decreased zinc availability has been noted in canine diets containing excessive levels of phytate (Merk, 2011). Signs of toxicosis with zinc include gastrointestinal distress, emesis, decrease food consumption, decrease growth, anemia, poor bone mineralization, damage to the pancreas, arthritis, white muscle disease, internal hemorrhaging and nonviable newborn. For most species, signs of toxicosis of zinc first appear when levels around 1000 ppm are incorporated in a normal diet with many nutrients above required levels. A maximum tolerable level of zinc in the diet that appears to be safe, depending on species, is between 300 to 1000 ppm (National Research Council, 1980).

Iodine → Deficiency is rare when pets are feed with a balanced diet, but when they are feed with a diet composed of high meat or diets containing saltwater fish, iodine deficiency can appear (Merk, 2011).

Fluorine → Fluorine comes from the environment and originates naturally from rocks, soil or from industrial processes. The normal levels of fluorine are <0.2 mgL^{-1} in plasma (Merk, 2011).

Selenium → "Selenium deficiencies are extremely rare in dogs and basically unknown in cats. If a selenium deficiency occurs, the signals are poor reproduction, puppy death, muscle weakness, and abnormalities of the heart muscle. Selenium toxicity in dogs or cats is rare, but can occur if the dietary intake exceeds 1.99 mg kg^{-1} of food eaten (on a dry matter basis) for prolonged periods of time. Signs of selenium toxicity include hair loss, lameness, anemia, liver cirrhosis" (Association of Feed Control Officials, 2011).

Lead → Lead is widespread in the urban environment. Previously the main lead source was associated with leaded gas but nowadays the renovation of old houses, painted with lead-based paint, is probably the most important one. Lead based paints have been associated with lead poisoning in small animals and children. The lead concentration in the blood of 30 ppm is consistent with a diagnosis of lead poisoning for most species of domestic animals (National Research Council, 1980; Merk, 2011).

Cadmium → At high levels, cadmium can cause anemia, neutrophilia, lymphocytopenia, enteropathy, renal tubular damage, bone marrow hypoplasia, decrease granulation of the adrenal medulla, hypertrophy of the heart ventricles, hypertension and splenomegaly; cadmium can also reduce growth rate in young animals. At very high levels cadmium can cause death. Cadmium, at concentrations as low as 1 ppm in the diet or drinking water produce adverse effects in monogastric animals. At 5 ppm more severe effects can be observed (National Research Council, 1980).

Chromium → Maximum tolerable dietary levels are set at 3000 ppm chromium as the oxide and 1000 ppm as the chloride for domestic animals. The signs of acute toxicosis included inflammation and congestion of the stomach, ulceration of the rumen and

abomasum and increased concentration of blood and liver chromium (National Research Council, 1980).

Cobalt → The National Research Council (1980) considers that a deficiency of cobalt is more likely to occur than toxicosis. Toxic levels appear to be at least 300 times the requirement. The signs of toxicosis are polycythemia in simple-stomached animals, reduce intake and body weight, emaciation, anemia, debility, increased hemoglobin and packed cell volume and elevated liver cobalt in ruminants.

Nickel → This element is essential in iron absorption and required in growth. Relatively to maximum tolerable levels in most experiments levels of 1000 ppm induce adverse effects, including decreased growth rate or even weight loss, changes in red blood cell numbers and hemoglobin, accumulation of nickel and alterations in tissue concentrations of several essential elements. 2500 ppm of nickel can produce emesis in dogs (National Research Council, 1980).

Table 6: Daily recommended allowances of minerals for pet cats (A) and dogs (B) according to Beitz, Baeur et al (2006a, 2006b).

A

Minerals	Daily Recommended Allowance
Calcium	0.18 g
Phosphorous	0.16 g
Potassium	0.33 g
Sodium	42 mg
Chloride	60 mg
Magnesium	25 mg
Iron	5 mg
Copper	0.3 mg
Iodine	88 µg
Zinc	4.6 mg
Manganese	0.3 mg
Selenium	19 µg

**Daily needs for an adult cat weighing 4 Kg at maturity, consuming 250 Calories per day*

B

Minerals	Daily Recommended Allowance
Calcium	0.75 g
Phosphorous	0.75 g
Potassium	1 g
Sodium	100 mg
Chloride	150 mg
Magnesium	150 mg
Iron	7.5 mg
Copper	1.5 mg
Iodine	220 µg
Zinc	15 mg
Manganese	1.2 mg
Selenium	90 µg

**Daily needs for an adult dog weighing 15 Kg at maturity, consuming 1,000 Calories per day*

Diverse studies determined metals concentrations in a variety of food, such as:

- Human food;
- Baby food;
- Water;
- Milk;
- Wine.

However, only a limited number of scientific papers are available for pet foods. Atkins et al. (2011a, 2011b) have two publications on this subject. In the first study (Atkins et al. 2011a): the raw materials used at the manufacturing process of pet food and all the potential source of contamination were analyzed. Part II (Atkins et al. 2001b) discussed how the data related to the dietary exposure of toxic metals in those pet foods when calculated for the daily intake of an average-sized dog and cat. In that study, the presence of a smaller group of toxic elements including Arsenic (As), Beryllium (Be), Cadmium (Cd), Mercury (Hg), Lead (Pb), Antimony (Sb), Thallium (Tl), and Uranium (U) was studied in pet foods because of their severe toxicity on humans. Nickel was found in the various types of pet food, which, according to those authors, was the cause of inclusion as a toxic element. The Atkins et al (2011b) investigation, shown that "the trace-metal content of many of the different pet foods is significantly higher than EPA (Environmental Protection Agency) and WHO (World Health Organization) values, when adjusted for the average weight of a dog and cat" and "this is of particular relevance when the data are compared with the results obtained with the cans of human tuna fish, sardines and chicken (which show heavy metal concentrations at or below EPA and WHO values) and are adjusted for the average weight of a human adult" (Atkins et al., 2011b). This study originated a controversial memorandum from FDA Committee (Food and Drug Administration Committee): the Target Animal Safety Review Memorandum. FDA Committee reported critical mistakes in the Atkins et al (2011b) study part II, such as: (1) the authors used EPA and WHO values for people to compare animals diet and physiology rather than the values proposed by the Committee on Minerals and Toxic Substances in Diets and Water for Animals (MTSA Committee); (2) the lack of scientific basis for the amount of consumed food ("each of the products that were tested should have contained recommended amounts to feed for a scientific weight of dog or cat in a feeding directions

section of the label"); (3) the uncorrected disparity of differences in moisture in the expressed concentrations of elements in wet and dry products; among others. FDA Committee also concluded that when they compare the information reported by Atkins et al (2011b) with the information available in the scientific literature regarding mineral toxicities in domestic animals, there is no reason to concern for long term safety of any of the products based on any of the measured quantities for antimony, arsenic, beryllium, cadmium, cesium, cobalt, lead, molybdenum, nickel, thallium, thorium, and uranium, or for mercury in non-reproducing dogs and cats. Only one cat food exceeds the maximum tolerance established by MTSA Committee for mercury in reproducing cats. The FDA Committee added "Any potential safety concern may arise only from insufficient data in the target species to demonstrate a species-specific tolerance and results from a theoretical tolerance being extrapolated from other species".

Despite the controversy associated with those particular papers the fact is that the FDA didn't established the maximum levels of metals in pet food, furthermore the maximum metals daily requirements are highly variable depending on the breed, size, age and physical status, amongst other factors.

Another study about metal levels in pet foods was published by Duran et al (2010). Those authors determined the trace metal concentrations in pet foods commercially available in the Turkish market. The researchers concluded that "trace metal concentrations of the investigated commercial pet food samples available in Turkey appeared to meet the regulated nutritional values by AAFCO" and added that "more pet specific research is needed to better address the potential problems that dogs and cats food may have".

3. Organisms' responses to contaminants

When exposed to contaminants, organisms respond in different ways: the responses to high contaminant concentrations include gross morphological or physiological changes or even death; on the other hand, low level exposures leads to subtle responses (sometimes with hard quantification), such as, induction of catabolic enzymes and/or protective proteins (Forbes and Calow, 1996).

Regarding metals, high doses can be acutely lethal, whereas at lower doses metals have a wide range of health effects in humans and animals, such as, mutagenicity, carcinogenicity, teratogenicity, immunosuppression, poor body condition and impaired reproduction (López-Alonso et al., 2007).

After exposure, the organism initiates a series of responses that include in the first stage the development of protection mechanisms against the toxic agent. One protection mechanism to metal exposure is the induction of metallothioneins, since these non-enzymatic proteins are responsible for metal detoxification. There are situations, however, that the animals' detoxification ability is not enough to prevent chemicals to induce damage. In such case it is important to access the damage extension. Metals, like other contaminants, are genotoxic agents. Their presence in indoor environment may cause oxidative stress and consequently, DNA damage.

4. Metallothioneins

Metalothioneins (MTs) were firstly isolated from horse kidney by Margoshes and Vallee (1957). MT is a cysteine-rich cytosolic and nuclear metal-binding protein in which the thiol groups (-SH) of cysteine residues enable MTs to bind particular metals (Amiard et al., 2006). MTs have typically, a low molecular weight (<7000 Da) and high metal content comprising predominantly Zn, Cu or Cd (Coyle et al., 2002). MTs appear to be present in all eukaryotic organisms (Cummings and Kovacic, 2009) which have multiple MT genes coding for different isoforms (Atif et al., 2006). There are four known isoforms of MT which are MT-1, MT-2, MT-3, MT-4. MT-1 and -2 are particularly abundant in liver, pancreas, intestine and kidney, instead MT-3 and -4 are present in brain and skin (Davis and Cousins, 2000).

The main role of metallothioneins is the sequestration and detoxification of excess of environmental toxic metals – non-essential metals - (e.g. Cd, Hg) or of physiologically important metals - essential metals - that are chemically disruptive in ionized form (Cu, Zn) (Coyle et al., 2002; Amiard et al., 2006). MTs have different affinities for metal cations: $Hg^{2+} > Cu^{+} > Cd^{2+} > Zn^{2+}$. MT is also an acute phase protein, since it is highly

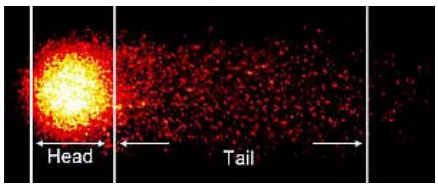
inducible with tissue injury, sepsis, inflammation and neoplastic disease (Cummings and Kovacic, 2009).

The functional mechanism of MT leads to considerate that different isoforms of this protein may be involved in the binding to different metals (Serafim and Bebianno, 2009). Consequently, metallothioneins have been considered a relevant biomarker of environmental metal exposure (Ivankovic et al., 2003).

5. Comet assay

The single cell gel electrophoresis (SCG), is a rapid, simple, sensitive and inexpensive method that visualizes and measures DNA strand-breaks at single cells, through gel electrophoresis and using fluorescence microscopy (Singh, 1998).

This technique is commonly known as comet assay since the appearance of the damaged cells resembles a comet where DNA fragments migrate out of the cell nucleus (head) to the tail (McKelveymartin et al., 1993)



©http://www.toxikologie.uniwuerzburg.de/en/general_information/

Figure 5: DNA damage evaluated through comet assay. DNA fragments, when exposed to electrophoresis, migrate out of the nucleus into a tail, resembling a comet.

5.1. Historical overview

DNA damage in individual cells was firstly quantified by Rydberg and Johanson (1978) that calculated the ratio of fluorescence using a photometer. Latter, Ostling and Johanson (1984) used a microgel electrophoresis technique to improve the evaluation of DNA damage in single cells, detecting only DNA double-strand breaks. The technique has then been modified to improve the sensitivity and reproducibility of the method and therefore to measure DNA damage more objectively (Olive and Didenko, 2002). The most important

improvements were introduced by Singh et al (1988). These authors introduced the main version of single cell gel (SCG) using alkaline electrophoresis in the detection of DNA damage, caused by chemicals and physical agents. This technique detects single-strand DNA breaks, alkali labile sites (apurinic/apyrimidic (AP) sites), crosslinks, and incomplete DNA repair sites in individual cells.

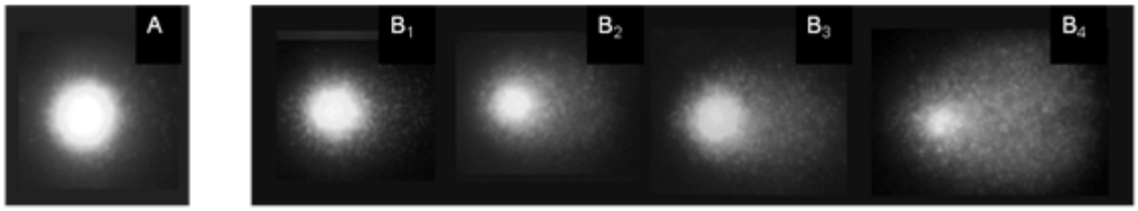


Figure 6: Examples of cells obtained by the comet assay technique. (A) Intact cell (B: B1 – B4) Cells with increasing degree of DNA damage.

5.2. Technical description

The single cell gel electrophoresis (SCGE) assay or comet assay measures and analyses DNA damage in the individual cells, and the alkaline version – pH>13 (introduced by Singh et al (1988)), "is capable of detecting DNA single-strand breaks (SSB), alkali labile sites, DNA-DNA/DNA protein cross-linking, and SSB associated with incomplete excision repair sites" and was considered as the "optimal version for identifying agents with genotoxic activity" (Tice et al., 2000).

Any nucleated cells can be studied through comet assay, such as neurons, hepatocytes, pulmonary cells, and spermatozoa (Marlin et al., 2004). Peripheral blood mononuclear cell (PBMC), such as other leukocytes are also frequently used (Marlin et al., 2004; Dusinska and Collins, 2008) due to their cellular and nuclear state (DNA integrity) that reflects body exposure, since these cells circulate in whole body (Kassie et al., 2000; Dusinska and Collins, 2008).

Fresh cells are usually used to perform the comet assay, however and due to practical reasons it is preferable to use frozen isolated cells (see for example isolation protocols described by Dusinska and Collins (2008) and Duthie et al (2002)).

Leukocytes are embedded in a thin layer of agarose on a glass slide, lysed with a solution containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, 10M NaOH, pH 10 and 2% Triton X-100. Thus membranes and soluble cell constituents, as well as histones,

are removed, leaving the DNA still supercoiled and attached to the nuclear matrix. Subsequent alkaline incubation and electrophoresis causes DNA loops containing breaks to move towards the anode. After electrophoresis, DNA is stained using ethidium bromide, and observed under a fluorescence microscope trough a software such as *CometAssay®, CASP, Comet IV* among others. The images resemble comets, and the relative content of DNA in the tail indicates the frequency of breaks. Strictly speaking, "breaks" in the context of the alkaline comet assay include apurinic/apyrimidinic (AP) sites, which are alkali labile (Olive and Didenko, 2002; Dusinska and Collins, 2008).

5.3. Data analysis

The comet assay results are most usually expressed in computerized image analysis (see Figures 3 and 4) as % DNA in tail, tail length and tail moment - product of the tail length and the fraction of total DNA in the tail (Collins et al., 1997; Lee et al., 2000; Hartmann et al., 2003; Dusinska and Collins, 2008), although the ratio of the length to width migrating DNA and tail inertia can quantify the migration of DNA (Valverde and Rojas, 2009). Table 2 describes the main parameters used and their advantages and limitations.

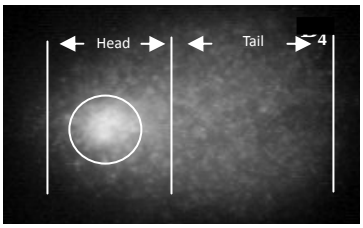


Figure 4: Image analysis with *Comet Assay IV* software.

Table 7: Description of the comet assay main parameters with their advantages and limitations. Cited from: [(1)] Dusinska and Collins (2008); [(2)] Salagovic et al. (1997); [(3)] Collins et al. (1995); Collins et al. (1997); Valverde and Rojas (2009); [(4)] Collins et al. (1997); Hartmann et al (2003); [(5)] Valverde and Rojas (2009); [(6)] García et al. (2004).

Parameters	Description	Advantages	Limitations
Tail Length	Length of the comet tail measured from the right border of head area to the end of tail (in pixels)	Allows the association with fragmented DNA	Depends on electrophoresis conditions, which poses some difficulties when comparing different works[(1)]
Tail	The product of the fraction of total DNA in tail and	i) Good correlations with the dose of	i) There are several methods to calculate tail moment and

Parameters	Description	Advantages	Limitations
Moment	the tail length: TailDNA% x TailLength ([percent of DNA in the tail] x [tail length])	genotoxic agents[1] ii) Gives the possibility both of a short tail containing a high fraction of DNA, or of a long tail containing a low fraction of highly fragmented DNA[2]	different image analysis systems that may give different values[1] ii)Eliminates useful information on the relationship between the extent of migration and the percentage of migrated DNA[5]
% DNA in Tail	Percent of DNA in the comet tail	i) Good correlations with the dose of genotoxic agents[1] ii) More meaningful and easier to compare comet assay results[1,6] iii) Correlation with visual score evaluation[3] iiii) Correlated with DNA break frequency over a wide range of damage levels[4,6]	Not possible to establish a relation with the tail length

Recently, an alternative method to evaluate DNA damage was proposed and it is based on a visual scoring. This method, less laborious and much faster, relies on the categorization of comets into five different classes, depending on tail intensity: from 0 (zero detectable damage – no visible tail) to 4 (head of comet very small which most of DNA is in tail) (Collins et al., 1995; Collins et al., 1997; Heaton et al., 2002; Hartmann et al., 2003; García et al., 2004; Dusinska and Collins, 2008). Although some authors recommend image analysis (Hartmann et al., 2003), other authors compared visual scoring and computer image analysis in the same cells and concluded that there is a close correspondence to both scores (Collins et al., 1997; Heaton et al., 2002) [Table 3].

Table 8: Comparison between visual scoring and image analysis of comet assay technique. Cited from: (1) Collins et al. (1995); Collins et al. (1997); Heaton et al. (2002); Hartmann et al. (2003); García et al. (2004); Dusinska and Collins (2008); (2) Hartmann et al. (2003); (3) Collins et al. (1997); Heaton et al. (2002).

Visual Scoring[1]	Image Analysis
i) Less laborious and much faster **ii) Close correspondence at the visual scoring and computer image analysis[3]**	Recommended by some authors as a rigorous method[2]
More susceptible to human error	Requires more work than the visual scoring and a specific software for the analysis

5.4. Advantages and limitations of the comet assay technique

This technique has proven to be sensitive for genotoxicity screening on the effect of chemicals and complex mixtures (Cotelle and Ferard, 1999) and as many advantages, including the following (Heaton et al., 2002):

1) It requires a low number of cells per sample and therefore a small blood volume;
2) It is sensitive enough to detect low levels of DNA damage;
3) It is an highly productive assay;
4) It is easy to apply;
5) It enables the use of different cells types;
6) It has a low cost.

Besides those advantages the comet assay has some limitations:

1) It is susceptible to the type and timing of sampling. The optimal timing to collect samples is few hours after the acute or chronic exposure to the genotoxic agent terminates (when an equilibrium state between the induction and repair of DNA damage is presumed to be maintained). The optimal moment to analyze the samples decreases between exposure termination and sample processing due to repair enzyme, which the DNA damage in a population of cells reduces (Valverde and Rojas, 2009).

2) The exposure agent can influence the loss of damaged cells through DNA repair processes, apoptosis, necrosis, or cell turnover (Valverde and Rojas, 2009).

3) Standardized methods for lyses, electrophoresis and other procedures are required for the application of comet assay in genetic toxicology and for easier comparison of data between laboratories (Albertini et al., 2000; Tice et al., 2000; Olive and Didenko, 2002).

5.5. Applications

The applications of comet assay include different areas of research such as molecular epidemiology, biomedical and environmental health (radiation biology; genetic toxicology; and genetic ecotoxicology). It allows the establishment of correlations between DNA damage, oxidative stress, diseases and antioxidant protection (clinical applications such as aging, exercise, training, measurement of cell-growth, DNA-repair mechanisms, effects of diet and antioxidant supplementation on oxidative DNA damage; human monitoring) (Cotelle and Ferard, 1999; Olive and Didenko, 2002; Marlin et al., 2004; Dusinska and Collins, 2008; Valverde and Rojas, 2009). The comet assay is used as a biomarker of effect in several studies ranging from environmental to human toxicology ones (Salagovic et al., 1996).

Some studies, for example, used the comet assay to evaluate primary DNA damage associated with air pollution parameters (Binkova et al., 1996; Valverde et al., 1997; Vineis and Husgafvel-Pursiainen, 2005; Brauner et al., 2007; Valverde and Rojas, 2009). A brief description of those studies can be found in Table 4.

Table 9: Cases reported associating DNA damage and air pollutants.

Reference	Contribution
Koehler et al (2010)	The comet assay is a good screening method which gives the indication of a NO_2 potential genotoxic effect.
Mondal et al (2010)	DNA damage evaluated through the comet assay is positively associated with indoor air pollution from biomass fuel use.
Coronas et al (2009)	The comet assay is a sensitive tool to detect DNA damage in subjects exposed to an oil refinery, with high genotoxic activity.

Reference	Contribution
Valverde and Rojas (2009)	The comet assay is a method of choice for population-based studies of environmental and occupational exposure to air pollutants, metals, pesticides, radiation and other xenobiotics.
Brauner et al (2007)	The levels of oxidative DNA damage (measured with comet assay) are associated to PAH (Polycyclic aromatic hydrocarbons) exposure.
Vineis and Husgafvel-Pursiainen (2005)	Particulate air pollution induces oxidative damage to DNA.
Pandey et al (2005)	DNA damage in lymphocytes is at least partially related to exposure to BMF (biomass fuels) smoke.
Anderson et al (1998)	The Comet assay has high sensitivity for carcinogens, but its specificity is uncertain because few non-carcinogens have been tested.
Awara et al (1998)	Plastics workers who were exposed to vinyl chloride monomer (VCM) for different periods of time showed significantly increased levels of DNA damage.
Valverde et al (1997)	Comet assay (assessing DNA damage in leukocytes, nasal and buccal epithelial cells) has a great potential at monitoring humans exposed to genotoxic pollutants.
Binkova et al (1996)	Establishment of a correlation between respirable particles and tail intensity in lymphocytes.

The comet assay is used not only in human studies but also in a huge variety of organisms from different taxa. Several review papers have been published, and the applications of this technique fully described for different organisms, ranging from bacteria to man (Figure 5) (Cotelle and Ferard, 1999; Wada et al., 2005; Waters et al., 2005; Paetau-Robinson et al., 2008; Dhawan et al., 2009; Chen et al., 2010; Kimura et al., 2010; Pate et al., 2010).

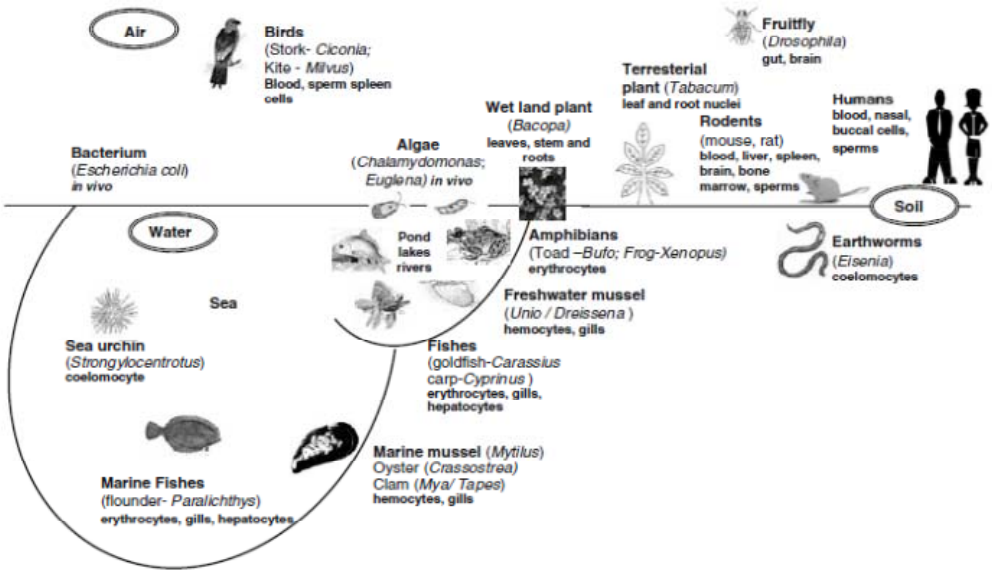


Figure 5: Schematic diagram of the use of Comet assay in assessing DNA damage in different models from bacteria to humans after Dhawan, Bajpayee et al. (2009).

Concerning pets, some studies using the comet assay technique are available and summarized in Table 5.

Table 10: Studies in pet cats and dogs using the comet assay technique.

Reference	Study description/goals	Major Results
Kreja et al (1996)	Evaluation of DNA damage through the comet assay (CA) technique in canine peripheral blood and bone marrow (BM) cells exposed to total body x-ray irradiation.	A continuous reduction of DNA damage was observed in the course of progressive leucopenia and granulocytopenia.
Kreja et al (1996b)	Evaluation of DNA damage through CA in canine buffy coat cells from BM aspirates and cells harvested from CFU-Fs (fibroblastoid colony-forming unit) colonies or from mixed populations of adherent BM stromal cell layers after exposure to increasing doses of X-rays.	Immediate DNA damage was much lower in cultured stromal cells than in hematopoietic cells in BM aspirates; and the differences in clonogenic survival could be due to differences in the type of their initial DNA damage.
Shen et al	Evaluation of DNA damage trough CA in order to understand	DHEA can significantly reduce

Reference	Study description/goals	Major Results
(2001)	if the treatment with neuroactive adrenal steroid (DHEA), reduces DNA damage in the brain and Peripheral Blood Lymphocytes PBLs of early dogs.	steady state levels of DNA damage in the mammalian brain.
Heaton et al (2002)	Evaluation of DNA damage through CA in canine and feline leukocytes to report the application of the technique.	CA is a useful tool for measure end products on a reliable biomarker of oxidative stress such as cellular DNA status in cats and dogs.
Li et al (2003)	Evaluation of DNA damage through CA in canine CVSMCs (cerebral vascular smooth muscle cells) in order to determine whether or not these cells undergo apoptosis when exposed to peroxynitrite ($ONOO^-$).	$ONOO^-$ formation in the brain may play important roles in neurodegenerative processes and strokes.
Waters et al (2003)	Evaluation of DNA damage through CA in aging canine prostate cells and PBLs, in order to study if dietary selenium (Se) supplementation decreased DNA damage and increased epithelial cell apoptosis.	Se may benefit the aging prostate by decreasing the accumulation of DNA damage in epithelial cells.
Alves et al (2004)	Evaluation of DNA damage through CA in dogs with confirmed cases of acute bacterial cystitis (BC) to determine the relationship among inflammation, genotoxicity, and carcinogenesis.	DNA damage is not associated with the infection by specific bacteria.
Waters et al (2005)	Evaluation of DNA damage through CA in a canine model in order to mimic Se intake of men. The authors "tested the hypothesis that the relationship between Se intake and DNA damage within the prostate and brain is non-linear, i.e. more Se is not necessary better."	Not all men will necessary benefit from increasing their Se supplementation.
Wada et al (2005)	Evaluation of DNA damage through CA in three canine tumor cell lines to study the DNA double strand breaks after □-ray and carbon ion radiation. Since tumor tissues have different radiosensitivities the researchers' intent to determine the intrinsic radiosensitivity of tumors in individual patients in advance of radiotheraphy.	The initial and residual (4hr) DNA damage due to □-ray and carbon ion radiation were higher in a radiosensitive cell line than in radioresistant cell line.
Yu and Paetau-Robinson	Evaluation of DNA damage through CA in fed cats with a dry cat food supplemented with antioxidants, vitamin E, vitamin C and β-carotene, in order to test their influence on DNA	The supplements may be beneficial to cats with renal disease.

Reference	Study description/goals	Major Results
(2006)	damage reduction in the progression of chronic renal failure.	
Waters et al (2007)	Evaluation of DNA damage through CA with *in vivo* canine model to evaluate whether the susceptibility of PBLs to oxidative stress-induced DNA damage could identify those individuals with the highest prostatic DNA damage and get closer to the goal of individualized risk prediction for prostate cancer.	The sensitivity of PBLs to oxidative stress challenge provides a noninvasive surrogate marker for prostatic DNA damage.
Sekis et al (2009)	Evaluation of DNA damage through CA in feline Peripheral Blood Mononuclear Cells PBMC and feline lymphoma cell line. The study intended to determine the pharmacokinetics of metronidazole (antibiotic commonly used in cats) administered intravenously, and after oral administration of metronidazole benzoate.	The PBMC collected from cats after 7 days of oral metronidazole induces, apparently, reversible DNA damage in lymphocytes, but is unlikely to produce clinical signs of acute toxicity.
Chen et al (2010)	Evaluation of DNA damage through CA in Mardin-Darby canine kidney cells (MDCK)-treated with para-phenylenediamine (p-PD), a suspect carcinogen that is present in many permanent hair dyes.	p-PD induced oxidative DNA damage and apoptosis in MDCK cells and the pre-treatment of these cells with antioxidants vitamin C or E, significantly inhibited the generation of citotoxicity and ROS when added p-PD.
Kimura et al (2010)	Evaluation of DNA damage through CA on the olfactory and respiratory epithelia of dogs from different regions of São Paulo, Brazil.	Air pollution, measured by PM10 (particulate matter index), could be responsible for the lesions on DNA.

6. Organization and the objectives of the thesis

The goal of the present thesis is to access the levels and effects of some environmental pollutants in pet cats and dogs. In this study, the blood of dogs, collected by a certified health professional (a veterinary medical doctor, V.M.D.), was used in order to access the levels of some contaminants, namely metals, and to evaluate possible adverse effects on

pets' health. Metallothioneins were used as a biomarker of metal exposure and DNA damage was also evaluated in a different group of animals – pet cats and dogs - using the comet assay technique.

The present thesis is organized in four chapters. The first chapter provides a general introduction of the use of pets as sentinels for human exposure, and to the different topics addressed in the thesis. Chapter II and III are structured as scientific papers. Chapter II describes the results of metals levels in blood and food of pet dogs and the concentration of metallothioneins. Chapter III provides information on DNA damage of pet cats and dogs.

Chapter I → introduces, briefly, the use of pets as sentinels for human exposure; the indoor exposure to contaminants of pet cats and dogs and the organisms' response to contaminants.

Chapter II → Describes the levels of selected metals in dog's blood and analyses the significance of pet food as a potential metal source. The response of dogs to metal exposure is also studied trough the assessment of metallothioneins levels.

Chapter III → Evaluates the extension of DNA damage in pet cats and dogs, comparing the levels in both species and their relation with individual factors such as gender, age, health status, food type regularly consumed, residence and geographic location.

Chapter IV → Provides a global conclusion of Chapter II and III.

References

Albertini, R.J., Anderson, D., Douglas, G.R., Hagmar, L., Hemminki, K., Merlo, F., Natarajan, A.T., Norppa, H., Shuker, D.E.G., Tice, R., Waters, M.D., Aitio, A., 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. Mutation Research-Reviews in Mutation Research 463, 111-172.

Alves, A., Gontijo, A., Salvadori, D.M.F., Rocha, N.S., 2004. Acute bacterial cystitis does not cause deoxyribonucleic acid damage detectable by the alkaline comet assay in urothelial cells of dogs. Veterinary Pathology 41, 299-301.

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160-202.

Anderson, D., Yu, T.W., McGregor, D.B., 1998. Comet assay responses as indicators of carcinogen exposure. Mutagenesis 13, 539-555.

Association of Feed Control Officials, 2011. Essencial Minerals. Wisconsin.

Atif, F., Kaur, M., Yousuf, S., Raisuddin, S., 2006. In vitro free radical scavenging activity of hepatic metallothionein induced in an Indian freshwater fish, Channa punctata Bloch. Chem.-Biol. Interact. 162, 172-180.

Atkins, P., Ernyei, L., Driscoll, W., Obenauf, R., Thomas, R., 2011a. Analysis of Toxic Trace Metals in Pet Foods Using Cryogenic Grinding and Quantitation by ICP-MS, Part I. Spectroscopy 26, 46-56.

Atkins, P., Ernyei, L., Driscoll, W., Obenauf, R., Thomas, R., 2011b. Analysis of Toxic Trace Metals in Pet Foods Using Cryogenic Grinding and Quantitation by ICP-MS, Part II. Spectroscopy 26, 42-+.

Awara, W.M., El-Nabi, S.H., El-Gohary, M., 1998. Assessment of vinyl chloride-induced DNA damage in lymphocytes of plastic industry workers using a single-cell gel electrophoresis technique. Toxicology 128, 9-16.

Barthold, S.W., Bolser, D.C., Garcia, K.D., Haywood, J.R., Leland, S.E., Miller, L., Nelson, R.J., Serpell, J., Talcott, M.R., 2009. Scientific and Humane Issues in the Use of Random Source Dogs and Cats in Research, Washington, D.C.

Beitz, D.C., Baeur, J.E., Behnke, K.C., Dzanis, D.A., Fahey, G.C., Hill, R.C., Kallfelz, F.A., Kienzle, E., Morris, J.G., Rogers, Q.R., 2006a. Your cat's nutricional needes. Nacional Research Council.

Beitz, D.C., Baeur, J.E., Behnke, K.C., Dzanis, D.A., Fahey, G.C., Hill, R.C., Kallfelz, F.A., Kienzle, E., Morris, J.G., Rogers, Q.R., 2006b. Your dog's nutricional needes. Nacional Research Council.

Binkova, B., Lewtas, J., Miskova, I., Rossner, P., Cerna, M., Mrackova, G., Peterkova, K., Mumford, J., Meyer, S., Sram, R., 1996. Biomarker studies in northern Bohemia. Environmental Health Perspectives 104, 591-597.

Bischoff, K., Priest, H., Mount-Long, A., 2010. Animals as sentinels for human lead exposure: a case report. Journal of Medical Toxicology : Official Journal of the American College of Medical Toxicology 6, 185-189.

Brauner, E.V., Forchhammer, L., Moller, P., Simonsen, J., Glasius, M., Wahlin, P., Raaschou-Nielsen, O., Loft, S., 2007. Exposure to ultrafine particles from ambient air and oxidative stress-induced DNA damage. Environmental Health Perspectives 115, 1177-1182.

Chen, S.C., Chen, C.H., Tioh, Y.L., Zhong, P.Y., Lin, Y.S., Chye, S.M., 2010. Para-phenylenediamine induced DNA damage and apoptosis through oxidative stress and enhanced caspase-8 and-9 activities in Mardin-Darby canine kidney cells. Toxicology In Vitro 24, 1197-1202.

Collins, A., Dusinska, M., Franklin, M., Somorovska, M., Petrovska, H., Duthie, S., Fillion, L., Panayiotidis, M., Raslova, K., Vaughan, N., 1997. Comet assay in human biomonitoring studies: Reliability, validation, and applications. Environmental and Molecular Mutagenesis 30, 139-146.

Collins, A.R., Ma, A.G., Duthie, S.J., 1995. The kinetics of repair of oxidative DNA-damage (strand breaks and oxidized pyrimidines) in human-cells. Mutation Research-DNA Repair 336, 69-77.

Coronas, M.V., Pereira, T.S., Rocha, J.A.V., Lemos, A.T., Fachel, J.M.G., Salvadori, D.M.F., Vargas, V.M.F., 2009. Genetic biomonitoring of an urban population exposed to mutagenic airborne pollutants. Environment International 35, 1023-1029.

Cotelle, S., Ferard, J.F., 1999. Comet assay in genetic ecotoxicology: A review. Environmental and Molecular Mutagenesis 34, 246-255.

Coyle, P., Philcox, J.C., Carey, L.C., Rofe, A.M., 2002. Metallothionein: The multipurpose protein. Cell. Mol. Life Sci. 59, 627-647.

Cummings, J.E., Kovacic, J.P., 2009. The ubiquitous role of zinc in health and disease. J. Vet. Emerg. Crit. Care 19, 215-240.

Davis, S.R., Cousins, R.J., 2000. Metallothionein expression in animals: A physiological perspective on function. J. Nutr. 130, 1085-1088.

Dhawan, A., Bajpayee, M., Parmar, D., 2009. Comet assay: a reliable tool for the assessment of DNA damage in different models. Cell Biol Toxicol 25, 5-32.

Duran, A., Tuzen, M., Soylak, M., 2010. Trace element concentrations of some pet foods commercially available in Turkey. Food and Chemical Toxicology 48, 2833-2837.

Dusinska, M., Collins, A.R., 2008. The comet assay in human biomonitoring: gene-environment interactions. Mutagenesis 23, 191-205.

Duthie, S., Pirie, L., Jenkinson, A., Narayanan, S., 2002. Cryopreserved versus freshly isolated lymphocytes in human biomonitoring: endogenous and induced DNA damage, antioxidant status and repair capability. Mutagenesis 17, 211.

Dye, J.A., Venier, M., Zhu, L., Ward, C.R., Hites, R.A., Birnbaum, L.S., 2007. Elevated PBDE levels in pet cats: Sentinels for humans? Environ. Sci. Technol. 41, 6350-6356.

Forbes, V.E., Calow, P., 1996. Costs of living with contaminants: implications for assessing low-level exposures. Belle Newsletter 4, 221–227.

García, O., Mandina, T., Lamadrid, A., Diaz, A., Remigio, A., Gonzalez, Y., Piloto, J., Gonzalez, J., Alvarez, A., 2004. Sensitivity and variability of visual scoring in the comet assay:: Results of an inter-laboratory scoring exercise with the use of silver staining. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 556, 25-34.

Glickman, L.T., Fairbrother, A., Guarino, A.M., Bergman, H.L., Buck, W.B., Cork, L.C., Hayes, H.M., Legator, M.S., Mcconnell, E.E., Mcnelis, D.N., Temple, S.A., 1991. Animals as sentinels of environmental health hazards. National Academies Press.

Guruge, K.S., Manage, P.M., Yamanaka, N., Miyazaki, S., Taniyasu, S., Yamashita, N., 2008. Species-specific concentrations of perfluoroalkyl contaminants in farm and pet animals in Japan. Chemosphere 73, S210-S215.

Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, A., Speit, G., Thybaud, V., Tice, R.R., 2003. Recommendations for conducting the in vivo alkaline Comet assay. Mutagenesis 18, 45-51.

Heaton, P.R., Ransley, R., Charlton, C.J., Mann, S.J., Stevenson, J., Smith, B.H.E., Rawlings, J.M., Harper, E.J., 2002. Application of single-cell gel electrophoresis (comet) assay for assessing levels of DNA damage in canine and feline leukocytes. J. Nutr. 132, 1598S-1603S.

Ibanez, Y., Le Bot, B., Glorennec, P., 2010. House-dust metal content and bioaccessibility: a review. Eur. J. Mineral. 22, 629-637.

Ivankovic, D., Pavicic, J., Raspor, B., Falnoga, I., Tusek-Znidaric, T., 2003. Comparison of two SH-based methods for estimation of metallothionein level in the digestive gland of naturally occurring mussels, Mytilus galloprovincialis. Int. J. Environ. Anal. Chem. 83, 219-231.

Kassie, F., Parzefall, W., Knasmüller, S., 2000. Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. Mutation Research/Reviews in Mutation Research 463, 13-31.

Kimura, K.C., Fukumasu, H., Chaible, L.M., Lima, C.E., Horst, M.A., Matsuzaki, P., Sanches, D.S., Pires, C.G., Silva, T.C., Pereira, T.C., Mello, M.L., Matera, J.M., Dias, R.A., Monnereau, A., Sasco, A.J., Saldiva, P.H.N., Dagli, M.L.Z., 2010. Evaluation of DNA damage by the alkaline comet assay of the olfactory and respiratory epithelia of dogs from the city of Sao Paulo, Brazil. Experimental and Toxicologic Pathology 62, 209-219.

Koehler, C., Ginzkey, C., Friehs, G., Hackenberg, S., Froelich, K., Scherzed, A., Burghartz, M., Kessler, M., Kleinsasser, N., 2010. Aspects of nitrogen dioxide toxicity in environmental urban concentrations in human nasal epithelium. Toxicology and Applied Pharmacology 245, 219-225.

Kreja, L., Selig, C., Nothdurft, W., 1996. Assessment of DNA damage in canine peripheral blood and bone marrow after total body irradiation using the single-cell gel electrophoresis technique. Mutation Research-Environmental Mutagenesis and Related Subjects 359, 63-70.

Kreja, L., Selig, C., Plappert, U., Nothdurft, W., 1996b. Radiation-induced DNA damage in canine hemopoietic cells and stromal cells as measured by the comet assay. Environmental and Molecular Mutagenesis 27, 39-45.

Kunisue, T., Nakanishi, S., Watanabe, M., Abe, T., Nakatsu, S., Kawauchi, S., Sano, A., Horii, A., Kano, Y., Tanabe, S., 2005. Contamination status and accumulation features of persistent organochlorines in pet dogs and cats from Japan. Environ. Pollut. 136, 465-476.

Le Cann, P., Bonvallot, N., Glorennec, P., Deguen, S., Goeury, C., Le Bot, B., 2011. Indoor environment and children's health: Recent developments in chemical, biological, physical and social aspects. International Journal of Hygiene and Environmental Health.

Lee, R., Kim, G.B., Maruya, K.A., Steinert, S.A., Oshima, Y., 2000. DNA strand breaks (comet assay) and embryo development effects in grass shrimp (Palaemonetes pugio) embryos after exposure to genotoxicants. Mar. Environ. Res. 50, 553-557.

Li, J.F., Su, J.L., Li, W.Y., Liu, W.M., Altura, B.T., Altura, B.A., 2003. Peroxynitrite induces apoptosis in canine cerebral vascular muscle cells: possible relation to neurodegenerative diseases and strokes. Neuroscience Letters 350, 173-177.

López-Alonso, M., Miranda, M., García-Partida, P., Cantero, F., Hernández, J., Benedito, J.L., 2007. Use of dogs as indicators of metal exposure in rural and urban habitats in NW Spain. Sci. Total Environ. 372, 668-675.

Margoshes, M., Vallee, B.L., 1957. A cadmium protein from equine kidney cortex. J. Am. Chem. Soc. 79, 4813-4814.

Marlin, D.J., Johnson, L., Kingston, D.A., Smith, N.C., Deaton, C.M., Mann, S., Heaton, P., Van Vugt, F., Saunders, K., Kydd, J., Harris, P.A., 2004. Application of the comet assay for investigation of oxidative DNA damage in equine peripheral blood mononuclear cells. J. Nutr. 134, 2133S-2140S.

McKelveymartin, V.J., Green, M.H.L., Schmezer, P., Poolzobel, B.L., Demeo, M.P., Collins, A., 1993. The single-cell gel-electrophoresis assay (comet assay) - a European review. Mutation Research 288, 47-63.

Merk, 2011. The Merk veterinary manual. National Academy Press, Washington, D.C. retrieved 21-11-2011. available online: http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/182902.htm

Mondal, N.K., Mukherjee, B., Das, D., Ray, M.R., 2010. Micronucleus formation, DNA damage and repair in premenopausal women chronically exposed to high level of indoor air pollution from biomass fuel use in rural India. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 697, 47-54.

National Research Council, 1980. Mineral Tolerance of Domestic Animals. National Academy of Sciences, Washington, D.C. retrieved 21-11-2011. available online: http://www.nap.edu/openbook.php?record_id=25&page=R1

Olive, P.L., Didenko, V.V., 2002. The comet assay: an overview of tecniques. In: Didenko, V.V. (Ed.). In situ detection of DNA damage: methods and protocols. Humana PressINC., Totowa,N.J., pp. 179-194.

Ostling, O., Johanson, K.J., 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian-cells. Biochemical and Biophysical Research Communications 123, 291-298.

Paetau-Robinson, I., Yamka, R.M., Friesen, K.G., 2008. Foods with lipoic acid and elevated levels of vitamin E and vitamin C correlate with whole blood antioxidant concentrations and may protect geriatric dogs from oxidative stress. International Journal of Applied Research in Veterinary Medicine 6, 93-100.

Pandey, A.K., Bajpayee, M., Parmar, D., Rastogi, S.K., Mathur, N., Seth, P.K., Dhawan, A., 2005. DNA damage in lymphocytes of rural Indian women exposed to biomass fuel smoke as assessed by the Comet assay. Environmental and Molecular Mutagenesis 45, 435-441.

Park, S.H., Lee, M.H., Kim, S.K., 2005. Studies on the concentrations of Cd, Pb, Hg and Cr in dog serum in Korea. Asian-Austral. J. Anim. 18, 1623-1627.

Pate, J., Rademacher, N., Pariaut, R., Saelinger, C., Strickland, K., Gaschen, L., 2010. Comet-tail artifacts in normal dogs and dogs with cardiogenic pulmonary edema. Journal of Veterinary Internal Medicine 24, 190.

Rydberg, B., Johanson, K., 1978. Estimation of DNA strand breaks in single mammalian cells. DNA repair mechanisms, 465–468.

Salagovic, J., Gilles, J., Verschaeve, L., Kalina, I., 1996. The comet assay for the detection of genotoxic damage in the earthworms: a promising tool for assessing the biological hazards of polluted sites. Folia Biologica (Praha) 42 17-21.

Salagovic, J., Maes, A., van Gorp, U., Verschaeve, L., Kalina, I., 1997. The cell cycle positions influence DNA migration as measured with the alkaline comet assay in stimulated human lymphocytes. Folia Biologica (Praha) 43, 79-82.

Sekis, I., Ramstead, K., Rishniw, M., Schwark, W.S., McDonough, S.P., Goldstein, R.E., Papich, M., Simpson, K.W., 2009. Single-dose pharmacokinetics and genotoxicity of metronidazole in cats. Journal of Feline Medicine and Surgery 11, 60-68.

Serafim, A., Bebianno, M.J., 2009. Metallothionein role in the kinetic model of copper accumulation and elimination in the clam Ruditapes decussatus. Environ. Res. 109, 390-399.

Shen, S., Cooley, D.M., Glickman, L.T., Glickman, N., Waters, D.J., 2001. Reduction in DNA damage in brain and peripheral blood lymphocytes of elderly dogs after treatment with dehydroepiandrosterone (DHEA). Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 480, 153-162.

Singh, N.P., 1998. A rapid method for the preparation of single-cell suspensions from solid tissues. Cytometry 31, 229-232.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantification of low-levels of DNA damage in individual cells. Experimental Cell Research 175, 184-191.

Storelli, M.M., Storelli, A., Barone, G., Franchini, D., 2009. Accumulation of polychlorinated biphenyls and organochlorine pesticide in pet cats and dogs: Assessment of toxicological status. Sci. Total Environ. 408, 64-68.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis 35 206-221.

Valverde, M., Lopez, M.D.C., Lopez, I., Sanchez, I., Fortoul, T.I., OstroskyWegman, P., Rojas, E., 1997. DNA damage in leukocytes and buccal and nasal epithelial cells of individuals exposed to air pollution in Mexico City. Environmental and Molecular Mutagenesis 30, 147-152.

Valverde, M., Rojas, E., 2009. Environmental and occupational biomonitoring using the Comet assay. Mutation Research-Reviews in Mutation Research 681, 93-109.

van der Schalie, W.H., Gardner, H.S., Bantle, J.A., De Rosa, C.T., Finch, R.A., Reif, J.S., Reuter, R.H., Backer, L.C., Burger, J., Folmar, L.C., Stokes, W.S., 1999. Animals as sentinels of human health hazards of environmental chemicals. Environmental Health Perspectives 107, 309-315.

Venier, M., Hites, R.A., 2011. Flame Retardants in the Serum of Pet Dogs and in Their Food. Environ. Sci. Technol. 45, 4602-4608.

Vineis, P., Husgafvel-Pursiainen, K., 2005. Air pollution and cancer: biomarker studies in human populations. Carcinogenesis 26, 1846-1855.

Wada, S., Van Khoa, T., Kobayashi, Y., Funayama, T., Ogihara, K., Ueno, S., Ito, N., 2005. Prediction of cellular radiosensitivity from DNA damage induced by gamma-rays and carbon ion irradiation in canine tumor cells. Journal of Veterinary Medical Science 67, 1089-1095.

Waters, D.J., Shen, S., Glickman, L.T., Cooley, D.M., Bostwick, D.G., Qian, J.Q., Combs, G.F., Morris, J.S., 2005. Prostate cancer risk and DNA damage: translational significance of selenium supplementation in a canine model. Carcinogenesis 26, 1256-1262.

Waters, D.J., Shen, S., Xu, H., Kengeri, S.S., Cooley, D.M., Chiang, E.C., Chen, Y., Schlittler, D., Oteham, C., Combs, G.F., Glickman, L.T., Morris, J.S., Bostwick, D.G., 2007. Noninvasive prediction of prostatic DNA damage by oxidative stress challenge of peripheral blood lymphocytes. Cancer Epidemiology Biomarkers & Prevention 16, 1906-1910.

Waters, D.J., Shen, S.R., Cooley, D.M., Bostwick, D.G., Qian, J.Q., Combs, G.F., Glickman, L.T., Oteham, C., Schlittler, D., Morris, J.S., 2003. Effects of dietary selenium supplementation on DNA damage and apoptosis in canine prostate. Journal of the National Cancer Institute 95, 237-241.

Yu, S., Paetau-Robinson, I., 2006. Dietary supplements of vitamins E and C and beta-carotene reduce oxidative stress in cats with renal insufficiency. Veterinary Research Communications 30, 403-413.

Chapter II

Environmental contamination by metals and metallothioneins induction in European dogs

Chapter II. Environmental contamination by metals and metallothioneins induction in European dogs

Abstract

Metals are widespread in the environnent and they may affect both humans and pets that frequently share the same indoor environment. In the present work, the levels of metals in food and blood of dogs were measured by ICP-OES (inductively coupled plasma optical emission spectrometry). Metallothioneins (MTs) concentrations were also determined. The levels of cadmium (Cd), chromium (Cr), cooper (Cu), lithium (Li), nickel (Ni), lead (Pb) and zinc (Zn) in food samples varied between: $2.42x10^{-3}$ – $183x10^{-3}$; 0.104– 3.06; 3.57 – 30.9; 25.4 $x10^{-3}$ – $579x10^{-3}$; $81x10^{-3}$ – $768x10^{-3}$; $134x10^{-3}$ – $603x10^{-3}$; 34.3 – 248 $\mu g.g^{-1}$, respectively. In blood samples the levels of Cd, Cu, Li, Ni, Pb and Zn varied between: $<0.2x10^{-3}$ - $17.9x10^{-3}$; $430x10^{-3}$ - $771x10^{-3}$; $<0.9x10^{-3}$ – $10.1x10^{-3}$; $<0.6x10^{-3}$ - 480 $x10^{-3}$; $16.9x10^{-3}$ – $72.4x10^{-3}$ and 3.06 – 4.46 $\mu g.g^{-1}$, respectively. Daily intake of the studied metals present in dog food samples are below the maximum recommended levels. Concerning dog blood samples, metals concentrations are within the reference values and were not significantly correlated with MTs levels

1. INTRODUCTION

Environmental contamination by metals may be originated from human activities and/or natural geological sources (López-Alonso et al., 2007). Metals at high doses can be acutely lethal, however at lower doses they have a wide range of health effects in animals and human beings (López-Alonso et al., 2007). The continuous exposure to metals can result on bioaccumulation, which leads to health concerns in humans and other animals directly or indirectly (through food chain for example) (Park et al., 2005). For each metal there are several exposure pathways that depend on the particular contaminated media as for example air, soil, water, dust or food (Caussy et al., 2003). Metals in their inorganic forms

are irrespirable particles but may be ingested (from house dust) by children and pets, since they have the same "hand-mouth" behavior (Le Cann et al., 2011).

Pets are thus useful indicators of potential metal health hazards to other animals or humans in any type of environment, including homes (Glickman et al., 1991). Dogs, in particular, are good sentinels to assess adverse human health outcomes related to low-levels exposure to many indoor contaminants as they share the same indoor environment as their owners.

However in order to use dogs as sentinels it is necessary to study the exposure pathways that are not common to pets and humans. Nowadays, pets are feed with a different diet than the one of their owners; hence the characterization of metal levels in pet food should be performed. The quantification of blood metal levels can thus be used to detect possible metal contamination in the pet's environment and consequently their owner's environment (after ruling out the role of food as a source). However, the quantification of metal blood concentrations does not take into account the organism's biological response to metals and their metabolic availability (Oliveira et al., 2010). Therefore, the quantification of metallothioneins (MTs) is of great importance as their induction is one of the first detectable signs of the presence of metals (Vijver et al., 2004). The aim of this study is determine metals and metallothioneins levels in pet dogs through blood analysis and investigate the importance of pet food in these levels.

2. MATERIAL AND METHODS

2.1. Sample's collection

2.1.1. Blood

Animals presented for medical procedures at Hospital Veterinário do Eucalipto (Aveiro, Portugal) and Clínica Veterinária Os Bichos (Chaves, Portugal) were recruited for this study. Twenty-one blood samples were collected from the jugular or cephalic veins into EDTA tubes (BD *Vacutainer®* plastic EDTA tube) by certified health professionals and immediately frozen. The samples were kept at -20^{o}C until analysis.

2.1.2. Food

Fifteen food samples (purchased in supermarkets and veterinary clinics) were freeze dried, grinded into fine power and preserved in falcon tubes at -20ºC until chemical analysis.

2.2. Metal analysis

2.2.1. Sample's preparation and digestion

About 1 to 2 g of whole blood was digested at atmospheric pressure with 2 ml of optima grade nitric acid (HNO_3) in sealed Teflon containers for 12-24h at 100°C. The digestion mixture was then transferred to falcon tubes and diluted up to 10ml with nanopure water. Food digestion followed the protocol described by Duran et al (2010) with some modifications. About 1g of food was digested with 6ml of HNO_3 and 2ml of hydrogen peroxide in Teflon containers in two stages: in the first one, the vials were kept open at room temperature for about 4hours whereas in the second one the vials were sealed and placed in the oven at 100°C for 24h. After cooling, the digested solutions were water-diluted up to 10ml in falcon tubes.

2.2.2. Quantification

Food and blood samples were analyzed for seven metals, cadmium (Cd), chromium (Cr), copper (Cu), zinc (Zn), nickel (Ni), lead (Pb) and lithium (Li), by inductively coupled plasma optical emission spectrometry (ICP-OES) using Indium (In) and Scandium (Sc) as internal standards. The analyses were validated using European Union, Institute for Reference Materials and Measurements - Certified Reference Materials (CRMs) BCR 634, BCR 635, and BCR 636. Recoveries varied between 81 and 83% and the precision of the method was better than 10%. All reagents used were trace analysis grade or better quality and all aqueous solutions were prepared using ultra-pure water (>18Ω cm^{-1}).

The method detection limit was: $0.2x10^{-3}$ $\mu g.g^{-1}$ for Cd, $0.4x10^{-3}$ $\mu g.g^{-1}$ for Cr, $0.4x10^{-3}$ $\mu g.g^{-1}$ for Cu, $0.9x10^{-3}$ $\mu g.g^{-1}$ for Li, $0.6x10^{-3}$ $\mu g.g^{-1}$ for Ni, $1.7x10^{-3}$ $\mu g.g^{-1}$ for Pb and $0.3x10^{-3}$ $\mu g.g^{-1}$ for Zn.

2.3. Metallothioneins

MT analysis was performed by differential pulse polarography (DPP) according to the method described by Bebiano and Langston (1999). MT levels were determined after homogenization in 20 mM Tris buffer (pH 8.6) containing 150 mM of NaCl. The homogenates were centrifuged for 45 min at 30,000 g at 4 ºC. Aliquots of the supernatant were heat-treated at 80 ºC for 10 min and re-centrifuged at 30,000 g for 45 min at 4 ºC. MT quantification was made using a 646VA Processor autolab type II and an ECO Chemie IME663 mercury drop electrode. Comparisons of peak heights with those of standard additions of purified rabbit MT enabled the quantification. Accuracy of the DPP analytical method was obtained by internal calibration using commercial rabbit liver MT-I. Recovery studies of our cytosols spiked with the MT-I rabbit liver standards revealed good recovery results ranging from 97 to 103%. Results are expressed as μg MT/mg protein.

2.4. Statistical analysis

Statistical analyses were performed using *SigmaPlot® 11* software for a significance level of 0.05. The correlation analysis refers to the non-parametric Spearman rank order correlation.

3. RESULTS AND DISCUSSION

The levels of cadmium, chromium, cooper, lithium, nickel, lead and zinc in food samples varied between: $<0.2x10^{-3}$ – $183x10^{-3}$; $<0.4x10^{-3}$ – 3.06; 3.57 – 30.9; $<0.9x10^{-3}$ – $579x10^{-3}$; $81x10^{-3}$ – $768x10^{-3}$; $<1.7x10^{-3}$ – $603x10^{-3}$; 34.3 – 248 $\mu g.g^{-1}$, respectively (Table 6). Cd and Zn were negatively correlated (Spearman rank order, r=-0.537, p=0.012) and Cu and Zn were positively correlated (Spearman rank order, r=-0.436, p=0.0474). No significant correlations were found between the other metals.

Table 6: Characterization of food type and metals concentrations (μg.g^{-1} dry weight (dw)) in the fifteen analyzed dog food samples.

Sample	Food type	Cd	Cr	Cu	Li	Ni	Pb	Zn
Fd1	Dry	$2.42x10^{-3}$	$615x10^{-3}$	22	$94.2x10^{-3}$	$643x10^{-3}$	$604x10^{-3}$	160
Fd2	Dry	$26.1x10^{-3}$	$261x10^{-3}$	15	$112x10^{-3}$	$398x10^{-3}$	$229x10^{-3}$	147
Fd3	Dry	$16.3x10^{-3}$	$383x10^{-3}$	22.1	$579x10^{-3}$	$559x10^{-3}$	$472x10^{-3}$	193
Fd4	Dry	$2.64x10^{-3}$	$288x10^{-3}$	22	$122x10^{-3}$	$431x10^{-3}$	$221x10^{-3}$	165
Fd5	Dry	$45.5x10^{-3}$	$174x10^{-3}$	24.4	$505x10^{-3}$	$308x10^{-3}$	$585x10^{-3}$	197
Fd6	Dry	$22.1x10^{-3}$	$340x10^{-3}$	30.9	$208x10^{-3}$	$576x10^{-3}$	$134x10^{-3}$	220
Fd7	Dry	$53.8x10^{-3}$	$153x10^{-3}$	19.5	$503x10^{-3}$	$378x10^{-3}$	$<1.7 x10^{-3}$	201
Fd8	Dry	$62.6x10^{-3}$	$<0.4x10^{-3}$	21	$527x10^{-3}$	$358x10^{-3}$	$<1.7 x10^{-3}$	219
Fd9	Dry	$117x10^{-3}$	$143x10^{-3}$	21.8	$410x10^{-3}$	$548x10^{-3}$	$<1.7 x10^{-3}$	248
Fd10	Wet	$58.7x10^{-3}$	$<0.4x10^{-3}$	13.3	$118x10^{-3}$	$81x10^{-3}$	$<1.7 x10^{-3}$	183
Fd11	Wet	$132x10^{-3}$	$<0.4x10^{-3}$	19	$74.5x10^{-3}$	$232x10^{-3}$	$<1.7 x10^{-3}$	244
Fd12	Wet	$183x10^{-3}$	$636x10^{-3}$	20.7	$156x10^{-3}$	$702x10^{-3}$	$<1.7 x10^{-3}$	76.2
Fd13	Wet	$45.3x10^{-3}$	$104x10^{-3}$	12.6	$<0.9 x10^{-3}$	$531x10^{-3}$	$<1.7 x10^{-3}$	165
Fd14	Wet	$<0.2x10^{-3}$	$<0.4x10^{-3}$	19.1	$25.4x10^{-3}$	$309x10^{-3}$	$<1.7 x10^{-3}$	186
Fd15	Wet	$105x10^{-3}$	3.06	3.57	$88.9x10^{-3}$	$768x10^{-3}$	$<1.7 x10^{-3}$	34.3

Based on the recommendations of the food manufacturer it is possible to calculate the daily intake of each metal by multiplying the metal levels (per gram on a dry weight basis) with the corresponding amount of each food daily intake (Table 7).

Table 7: Recommended amount (g) of each dog food for a medium sized dog (about 15kg) and the correspondent amount of metals (μg) ingested for each food type per day. For those food samples in which the metal concentrations were below the detection limit (DL), a value of half the DL was assigned (e.g. sample Fd 14: the concentration of Cd used for the calculation of the amount of ingested metal per day was 0.0001 (μg).

Sample	Recommended daily amount (g)	Cd	Cr	Cu	Li	Ni	Pb	Zn
Fd1	247	0.6	152	5430	247	159	149	39500
Fd2	280	7.31	73.1	4200	31.4	111	64	41200
Fd3	270	4.40	103	5970	156	151	127	52100
Fd4	240	0.634	69.1	5280	29.3	103	53.0	39600
Fd5	285	13	49.6	6950	144	87.8	167	56100
Fd6	240	5.30	81.6	7420	49.9	138	32.2	52800
Fd7	250	13.5	38.3	4880	126	94.5	0.213	50300
Fd8	230	14.4	0.046	4830	121	82.3	0.196	50400
Fd9	230	26.9	32.9	5010	94.3	126	0.196	57000
Fd10	190	11.2	0.038	2530	22.4	15.4	0.162	34800
Fd11	150	19.8	0.03	2850	11.2	34.8	0.128	36600
Fd12	135	24.7	85.9	2790	21.1	94.8	0.115	10300
Fd13	200	9.06	20.8	2520	0.09	106	0.17	33000
Fd14	225	0.0225	0.045	4300	5.72	69.5	0.191	41900
Fd15	23	2.42	70.4	82.1	2.04	17.7	0.02	789

In order to evaluate if the food ingested by each dog being or not responsible for the intake of an excessive dose of metals we used the average grams of dry food (samples Fd1

to Fd9) consumed per day for a 15 Kg dog (252 g) and multiplied that amount by the metal's concentrations (Table 7).

Table 8: Minimum, maximum and median amount of metals (µg) consumed per day according to the specifications of daily intake provided by each food manufacturer (see Table 7). The maximum recommended daily intake according to National Research Council. (1980)[(1)] and Association of American Feed Control Officials (2008a)[(2)] is also shown (µg.g^{-1}).

	Cd	Cr	Cu	Li	Ni	Pb	Zn
Minimum	0.598	20.8	82.1	2.04	15.4	32.2	789
Maximum	26.9	152	7420	247	159	167	57 x10^3
Median	8.175	49.6	4830	31.3	94.8	0.196	41.1 x10^3
Maximum recommended in the literature	0.5[(1)]	1000 chloride; 3000 oxide[(1)]	250[(2)]	-	50 for cattle[(1)]	30[(1)]	1000[(2)]
Maximum based on average food intake (252g) (µg)	126	>252x10^3	>63 x10^3	-	12600	7560	>252 x10^3

As represented in Table 8, Cd, Cr, Cu, Ni, Pb and Zn daily intake for all food samples are below the maximum recommended level. This fact allows us to exclude food as major exposure pathway to toxic levels of metals. Therefore, and considering that besides food, humans and their dogs are exposed to metals in a similar way; dogs are thus very good sentinels for human exposure in the domestic environment.

Table 9 summarizes the metal levels detected in pet food samples from different areas around the word. In general, the metals detected in this study are lower than the ones previously reported with only one exception for copper. Our Cu maximum level –(30.9 µg.g^{-1}, in Fd 6) is higher than the one reported for Turkish food samples (10.2 µg.g^{-1}). However, the Cu value obtained for sample Fd6 is lower than the maximum level recommended by Association of Feed Control Officials (250 µg.g^{-1}) (Association of American Feed Control Officials, 2008a). For the other metals our values are also lower than the maximum levels recommended by the National Research Council (1980).

Table 9: Metal levels found in different pet foods (µg.g^{-1}).

Reference	Country	Cd	Cr	Cu	Li	Ni	Pb	Zn
Duran et al (2010)	Turkey	0.60 – 2.47	0.58 – 3.73	3.33 – 10.2		7.78 – 19.7	1.66 – 15.5	
Alomar et al (2006)	Chile			54 (max level)				354.5 (maxlevel)
This study	Portugal	2.42x10^{-3} – 183x10^{-3}	104x10^{-3} – 3.06	3.57 – 30.9	25.4 x10^{-3} – 579x10^{-3}	81x10^{-3} – 768x10^{-3}	134x10^{-3} – 603x10^{-3}	34.3 – 248

The metals levels were also determined in dogs' blood. Table 10 shows the concentrations of Cd, Cr, Cu, Li, Ni, Pb and Zn detected in dog blood samples.

Table 10: Metals and metallothioneins (MTs) concentration in dog blood samples.

Sample	Metals (µg.g^{-1})							MTs (µg MT/mg protein)
	Cd	Cr	Cu	Li	Ni	Pb	Zn	
AVE-DOG-01	<0.2x10^{-3}	<0.4x10^{-3}	552x10^{-3}	<0.9x10^{-3}	43.2x10^{-3}	32.7x10^{-3}	3.39	10.47
AVE-DOG-02	<0.2x10^{-3}	<0.4x10^{-3}	609x10^{-3}	<0.9x10^{-3}	13.8x10^{-3}	37.2x10^{-3}	3.79	9.04
AVE-DOG-03	<0.2x10^{-3}	<0.4x10^{-3}	665x10^{-3}	<0.9x10^{-3}	44.1x10^{-3}	30.6x10^{-3}	3.42	6.57
AVE-DOG-04	<0.2x10^{-3}	<0.4x10^{-3}	518x10^{-3}	<0.9x10^{-3}	82.4x10^{-3}	31.7x10^{-3}	3.31	7.12
AVE-DOG-05	1.91x10^{-3}	<0.4x10^{-3}	430x10^{-3}	<0.9x10^{-3}	<0.6x10^{-3}	16.9x10^{-3}	3.06	5.68
AVE-DOG-06	1.98x10^{-3}	<0.4x10^{-3}	507x10^{-3}	<0.9x10^{-3}	<0.6x10^{-3}	65.3x10^{-3}	3.7	6.35
AVE-DOG-07	17.9x10^{-3}	<0.4x10^{-3}	757x10^{-3}	<0.9x10^{-3}	232x10^{-3}	35.4x10^{-3}	4.44	6.70
AVE-DOG-08	13.6x10^{-3}	<0.4x10^{-3}	744x10^{-3}	<0.9x10^{-3}	323x10^{-3}	23.8x10^{-3}	4.22	10.01
AVE-DOG-09	1.99x10^{-3}	<0.4x10^{-3}	474x10^{-3}	<0.9x10^{-3}	17.5x10^{-3}	18.6x10^{-3}	3.62	5.79
AVE-DOG-11	2.99x10^{-3}	<0.4x10^{-3}	475x10^{-3}	<0.9x10^{-3}	17.5x10^{-3}	18.6x10^{-3}	3.62	10.23
CHA-DOG-01	<0.2x10^{-3}	<0.4x10^{-3}	698x10^{-3}	<0.9x10^{-3}	480x10^{-3}	72.3x10^{-3}	3.68	3.91
CHA-DOG-02	<0.2x10^{-3}	<0.4x10^{-3}	771x10^{-3}	10.1x10^{-3}	<0.6x10^{-3}	52.1x10^{-3}	3.36	5.85
CHA-DOG-03	<0.2x10^{-3}	<0.4x10^{-3}	600x10^{-3}	8.37x10^{-3}	128x10^{-3}	72.4x10^{-3}	4.01	3.07
CHA-DOG-04	<0.2x10^{-3}	<0.4x10^{-3}	760x10^{-3}	5.93x10^{-3}	37.3x10^{-3}	49.5x10^{-3}	4.46	9.04
CHA-DOG-05	<0.2x10^{-3}	<0.4x10^{-3}	674x10^{-3}	10.1x10^{-3}	355x10^{-3}	32.1x10^{-3}	3.33	8.86
CHA-DOG-06	<0.2x10^{-3}	<0.4x10^{-3}	617x10^{-3}	<0.9x10^{-3}	<0.6x10^{-3}	50.3x10^{-3}	3.82	5.51
CHA-DOG-07	<0.2x10^{-3}	<0.4x10^{-3}	557x10^{-3}	<0.9x10^{-3}	88.8x10^{-3}	44.7x10^{-3}	4.1	4.14
CHA-DOG-08	<0.2x10^{-3}	<0.4x10^{-3}	578x10^{-3}	<0.9x10^{-3}	<0.6x10^{-3}	45.4x10^{-3}	3.88	5.81
CHA-DOG-10	<0.2x10^{-3}	<0.4x10^{-3}	698x10^{-3}	6.58x10^{-3}	19.7x10^{-3}	58.4x10^{-3}	3.83	2.84
CHA-DOG-11	<0.2x10^{-3}	<0.4x10^{-3}	524x10^{-3}	9.89x10^{-3}	<0.6x10^{-3}	36.8x10^{-3}	3.73	3.43
CHA-DOG-13	7.95x10^{-3}	<0.4x10^{-3}	566x10^{-3}	<0.9x10^{-3}	11.4x10^{-3}	29.4x10^{-3}	4.04	5.02

Chromium was below the technique detection limit (<0.4x10^{-3}) in all blood samples analyzed and therefore will not be further discussed. The levels of Cd, Cu, Li, Ni, Pb and Zn in blood samples varied between: <0.2x10^{-3} - 17.9x10^{-3}; 430x10^{-3} - 771x10^{-3}; <0.9x10^{-3} – 10.1x10^{-3}; <0.6x10^{-3} - 480 x10^{-3}; 16.9x10^{-3} – 72.4x10^{-3} and 3.06 – 4.46 µg.g^{-1}, respectively (Table 10).

Table 11: Minimum, maximum and median amount of metals (µg.g^{-1}) and the reference values for dogs blood samples according to [(1)] (Matsuno et al., 1991); [(2)] (Farver, 2008).

	Cd	Cr	Cu	Li	Ni	Pb	Zn
Minimum	<0.2x10^{-3}	<0.4x10^{-3}	430x10^{-3}	<0.9x10^{-3}	<0.6x10^{-3}	16.9x10^{-3}	3.06
Maximum	17.9x10^{-3}	-	771x10^{-3}	10.1x10^{-3}	480 x10^{-3}	72.4x10^{-3}	4.46
Median	0.0001	0.0002	0.6	0.00045	0.0197	36.8 x10^{-3}	3.73
Reference values	5.1 x10^{-3} [(1)] in control group	-	1 – 2 [(2)]	-	-	0-0.5 [(2)]	Without values for dogs[(2)]

Matsuno et al (1991) exposed dogs to several cadmium concentrations, their results for the control group were $5.1x10^{-3}$ $\mu g.g^{-1}$, although when they exposed dogs (through diet supplement) to 1 mg per day, the Cd blood concentration increased to levels of 25.8 $x10^{-3}$ $\mu g.g^{-1}$ in females and 30.1 $x10^{-3}$ $\mu g.g^{-1}$ in males. These results lead us to conclude that the two samples of the present study that have Cd blood concentrations superior to 5.1 $x10^{-3}$ and inferior to 25 $x10^{-3}$ (AVE-DOG-07 and AVE-DOG-08 with $17.9x10^{-3}$ and $13.6x10^{-3}$ respectively) are exposed to low doses of Cd (inferior to 1 mg per day), being apparently safe (Table 11).

The copper blood concentrations obtained in the present study ($430x10^{-3}$ - $771x10^{-3}$ $\mu g.g^{-1}$), are lower than the normal range for blood copper levels.

Concerning to Pb, apparently, there was no contamination with this metal between the dogs studied, since their Pb blood concentration are within the reference levels.

Table 12 reports the concentration of metals in pets blood samples found in the literature.

Table 12: Metals concentration found in pet blood samples (µg/ml = ppm).

Reference	Cd	Cr	Cu	Li	Ni	Pb	Zn
Park, Lee et al (2005)	0.22± 0.01	0.50±0.06				0.61+-0.08	
Berny et al (1995)						33% of dogs ≥0.1	
Matsuno et al (1991)	$5.1x10^{-3}$						
This study	0.0001	0.0002	0.6	0.00045	0.0197	$36.8x10^{-3}$	3.73

Metallothioneins concentration were not significantly correlated with the blood metals levels detected in the studied dogs with the exception of lead that was negatively correlated, although the significance was close to 0.05 (Spearman rank order, r=-0.438, p=0.0466).

Metallothioneins levels varied between 2.84 and 10.47 (µg $MT.mg^{-1}$ protein).

The sample that has the highest value of MTs was AVE-DOG-01 (10.47 µg MT/mg protein). This animal didn't had any quantifiable level of Cd, Cr and Li (<$0.2x10^{-3}$,

<$0.4x10^{-3}$ and <$0.9\ x10^{-3}$, respectively) in its blood. The values of Cu, Ni, Pb and Zn were, respectively: $552x10^{-3}$; $43.2x10^{-3}$; $32.7x10^{-3}$; 3.39 $\mu g.g^{-1}$. Considering that MTs have different affinities for metal cations ($Hg^{2+} > Cu^{+} > Cd^{2+} > Zn^{2+}$) (Cummings and Kovacic, 2009), we can hypothesize that the MTs increased levels are mostly due to copper and finally to zinc. However, the highest Cu concentration found in the present study wasn't followed by the highest MTs level (CHA-DOG-02). This might be due to the fact that the metal levels detected in blood are not high enough to trigger detoxification mechanisms.

Table 13: Cases reported associating metallothioneins concentration in animal's blood samples.

Reference	Study	MT
Blain et al (1997)	Determined metallothionein concentration in mouse embryos after exposure to Zn.	93.9 – 688 $\mu g.g^{-1}$
Croteau et al (2002)	Determined metallothionein concentration in larvae of *Chaoborus* in order to monitor Cd in lakes.	100 – 270 nmol/g
Yoshida et al (1998)	Determine metallothionein concentration in liver of 55 forensic autopsies.	250±313 $\mu g.g^{-1}$
Smith et al (1993)	Determine metallothioneins in rats plasma after exposure to Cd.	0.55 µmol MT/L

The comparisons between MTs levels found in the present study (2.84 and 10.47 µg MT/ mg protein) and the levels reported in the literature (Table 13) are sometimes difficult as different animal's models were used. Bebianno and Machado (1997) for example, stated that "metallothionein concentration from 2-3 mg/g have been proposed as background levels against which metal contamination should be assessed". Considering that range in our study seventeen samples provide early warnings of metal exposure. Otherwise the MT role is unclear at the lower doses of metals, since this protein can be induced by hormone-like compounds and environmental stress factors beyond metals. Furthermore, that value was based on studies performed in invertebrates and therefore may not be comparable to ours. As far as we are aware few studies addressed the levels on MTs in healthy dogs, and the great amount of work performed with MTs in companion animals refers to cancer studies (see Dincer et al. (2001) and references therein).

Overall, our study suggests that there is no warning metal contamination between the studied samples. However, the measurement of metals in blood reflects only the recent absorption of such compounds by the animals (Park et al., 2005). In cause of this, metal levels in blood samples can be inferior to the maximum tolerable level but these values don't provide us information about previous exposure, since metals in blood stream can be accumulated in high affinity organs (such as liver, kidney, bone and brain) and metabolized, excreted via sweat, feces, urine and can be deposited in hair (Park et al., 2005). Therefore a more integrated approach should be used in order to access not only the recent exposure scenario but also previous exposures that may have negative impacts on the animal's health. Further studies should include for example the quantification of trace metal levels in hair samples.

ACKNOWLEDGMENTS

The authors are grateful to the veterinarians Lisete Vieira VMD, Joana Tuna VMD, Inês Santos VMD, Inês Freitas VMD and Joana Alegrete VMD for the samples collection. Authors thanks to Dr. James Ranville and Dr. Ramiro Pastorinho for the metals determination by ICP-OES analysis. Thanks are also to Dr. Vera Maria and Dr. Bebianno for the metallothioneins quantification.

References

Alomar, D., Hodgkinson, S., Abarzua, D., Fuchslocher, R., Alvarado, C., Rosales, E., 2006. Nutritional evaluation of commercial dry dog foods by near infrared reflectance spectroscopy. J. Anim. Physiol. Anim. Nutr. 90, 223-229.

Association of American Feed Control Officials, 2008a. AAFCO Dog Food Nutrient Profiles. Retieved 5-12-2011. Available online: http://www.peteducation.com/article.cfm?c=2&aid=662

Bebianno, M.J., Langston, W.J., 1999. Metallothionein induction in mussels exposed to a metal mixture. Birkhauser Verlag Ag, Basel.

Bebianno, M.J., Machado, L.M., 1997. Concentrations of metals and metallothioneins in *Mytilus galloprovincialis* along the south coast of Portugal. Marine Pollution Bulletin 34, 666-671.

Berny, P.J., Cote, L.M., Buck, W.B., 1995. Can household pets be used as reliable monitors of lead-exposure to humans. Sci. Total Environ. 172, 163-173.

Blain, D., 1997. Metallothionein Pre-induction by Zinc and Isotretinoin Teratogenicity in CD-1 Mice.

Caussy, D., Gochfeld, M., Gurzau, E., Neagu, C., Ruedel, H., 2003. Lessons from case studies of metals: investigating exposure, bioavailability, and risk. Ecotox. Environ. Safe. 56, 45-51.

Croteau, M.N., Hare, L., Campbell, P.G.C., Couillard, Y., 2002. Metallothionein-like metal-binding protein in the biomonitor Chaoborus: Occurrence and relationship to ambient metal concentrations in lakes. Environ. Toxicol. Chem. 21, 737-741.

Cummings, J.E., Kovacic, J.P., 2009. The ubiquitous role of zinc in health and disease. J. Vet. Emerg. Crit. Care 19, 215-240.

Dincer, Z., Jasani, B., Haywood, S., Mullins, J.E., Fuentealba, I.C., 2001. Metallothionein expression in canine and feline mammary and melanotic tumours. J. Comp. Pathol. 125, 130-136.

Duran, A., Tuzen, M., Soylak, M., 2010. Trace element concentrations of some pet foods commercially available in Turkey. Food Chem. Toxicol. 48, 2833-2837.

Farver, T.B., 2008. Concepts of normality in clinical biochemistry. Retrieved 5-12-2011. Available online: http://www.google.pt/books?hl=pt-PT&lr=&id=spsD4WQbL0QC&oi=fnd&pg=PA1&dq=Concepts+of+normality+in+clinical+biochemistry&ots=T_84aJli7i&sig=suAeSomcEaZfWVrDp1pAlSVsNOk&redir_esc=y#v=onepage&q=Concepts%20of%20normality%20in%20clinical%20biochemistry&f=false

Glickman, L.T., Fairbrother, A., Guarino, A.M., Bergman, H.L., Buck, W.B., Cork, L.C., Hayes, H.M., Legator, M.S., Mcconnell, E.E., Mcnelis, D.N., Temple, S.A., 1991. Animals as sentinels of environmental health hazards. National Academies Press.

Le Cann, P., Bonvallot, N., Glorennec, P., Deguen, S., Goeury, C., Le Bot, B., 2011. Indoor environment and children's health: Recent developments in chemical, biological, physical and social aspects. International Journal of Hygiene and Environmental Health.

López-Alonso, M., Miranda, M., García-Partida, P., Cantero, F., Hernández, J., Benedito, J.L., 2007. Use of dogs as indicators of metal exposure in rural and urban habitats in NW Spain. Sci. Total Environ. 372, 668-675.

Matsuno, K., Kodama, Y., Kawamoto, T., Kayama, F., Tsuchiya, K., 1991. Absorption of cadmium after long-term oral administration of cadmium to dogs. Biol. Trace Elem. Res. 28, 99-108.

National Research Council, 1980. Mineral Tolerance of Domestic Animals. National Academy of Sciences, Washington, D.C.

Oliveira, M., Ahmad, I., Maria, V.L., Serafim, A., Bebianno, M.J., Pacheco, M., Santos, M.A., 2010. Hepatic metallothionein concentrations in the golden grey mullet (Liza aurata) - Relationship with environmental metal concentrations in a metal-contaminated coastal system in Portugal. Mar. Environ. Res. 69, 227-233.

Park, S.H., Lee, M.H., Kim, S.K., 2005. Studies on the concentrations of Cd, Pb, Hg and Cr in dog serum in Korea. Asian-Austral. J. Anim. 18, 1623-1627.

Smith, L.M.J., 1993. The Dynamics of Cadium and Metallothionein Accumulation in Peripheral Blood Cells and Selected Organs of Rats.

Vijver, M.G., Van Gestel, C.A.M., Lanno, R.P., Van Straalen, N.M., Peijnenburg, W., 2004. Internal metal sequestration and its ecotoxicological relevance: A review. Environ. Sci. Technol. 38, 4705-4712.

Yoshida, M., Ohta, H., Yamauchi, Y., Seki, Y., Sagi, M., Yamazaki, K., Sumi, Y., 1998. Age-dependent changes in metallothionein levels in liver and kidney of the Japanese. Biol. Trace Elem. Res. 63, 167-175.

Chapter III

DNA damage in European pets assessed by comet assay

Chapter III. DNA damage in European pets assessed by comet assay

Abstract

The indoor environment is an important source of contaminants not only for humans but also for their pets. Animals and humans respond similarly to toxic agents; however, animals, due to their shorter lifespan, develop more rapidly pathological conditions caused by environmental agents. Consequently, household cats and dogs are good sentinels to assess adverse human health outcomes related to low-levels of many indoor contaminants, some of which are known genotoxic agents. In the present work, blood samples of forty one pets - sixteen cats and twenty five dogs – were collected and DNA damage was evaluated trough the comet assay technique.

This is the first study that compares DNA damage in pet cats and dogs. The DNA damage is relative low (<22.9% in cats and <32.3% in dogs) and no significant differences were detected between the two species. Similarly, no significant differences were detected for both cats and dogs and the following factors: gender, age, health status, residence, location and diet.

1. INTRODUCTION

Animals have been reported as good sentinels for human life (Glickman et al., 1991). Pet cats and dogs can be used to identify potential health hazardous, such as chemical exposure, in any type of environment, including homes. They can provide early warnings of contaminants exposure, since they develop, more rapidly, pathological conditions caused by environmental contaminants, due to their shorter lifespan and to their response at lower doses (increased susceptibility) (Glickman et al., 1991).

Pet cats and dogs share the same environment as their owners; therefore they are exposed to the same contaminants. It is known that some of these contaminants are genotoxic agents, causing DNA damage. DNA damage may be related to oxidative stress, which with few antioxidant defenses may be one of the factors for disease (Cooke et al.,

2003). The evaluation of DNA damage can be performed through several different techniques such as: sister chromatid exchange (SCE), micronuclei (MN), and single cell gel electrophoresis (SCGE). The single cell gel electrophoresis (SCGE) is a method that visualizes and measures DNA strand-breaks at single cells, through gel electrophoresis and using fluorescence microscopy (Singh, 1998). This technique is usually named as comet assay since the appearance of the damaged cells resembles a comet where DNA fragments migrate out of the cell nucleus (head) to the tail (McKelveymartin et al., 1993). This technique has many advantages: it requires a low number of cells per sample and therefore a small blood volume; it is sensitive enough to detect low levels of DNA damage; it is a highly reproductive assay; it is easy to apply; it enables the use of different cells types; and it has a low cost (Heaton et al., 2002).

In this work, the single cell gel electrophoresis (SCG) or comet assay work was used in order to assess the DNA damage in leukocytes of indoor pet cats and dogs. The main objectives of the present work are: (i) to compare the DNA damage in both species and (ii) to understand which factors are responsible for the observed damage.

2. MATERIAL AND METHODS

2.1. Animals

All the animals used in the present work were registered at the "Hospital Veterinário do Baixo Vouga", Aveiro, Portugal and the animals' owners signed an informed consent and answered a questionnaire about the general health status, residence and dietary habits of the respective animal. A total number of forty one samples were collected between January and August 2011, among which sixteen were cats and twenty five were dogs. Table 14 and 15 summarizes the data relative to each animal including gender, age, breed, health status, food type regularly consumed, residence and geographic location.

Table 14: Data relative to each cat including age, sex, breed, health status and food type regularly consumed. CAT-A corresponds to cats samples in which the comet assay technique was performed according to Heaton et al (2002) and CAT-P corresponds those in which Costa et al (2008) protocol was used (see text for details). F=Female; M=Male; H=Healthy; U=Unhealthy.

Sample	Gender	Age (years)	Breed	Health Status	Food type
CAT-A-01	F	2	European Shorthair	H	Dry
CAT-A-03	M	4	mixed breed (Persian/Siamese)	H	Dry
CAT-A-04	M	6	Sphynx	H	Dry
CAT-A-05	F	14	European Shorthair	U	Dry/wet/homemade
CAT-A-08	F	12	European Shorthair	U	Dry
CAT-A-09	F	13	European Shorthair	U	Dry
CAT-A-10	M	2	European Shorthair	U	Dry/wet
CAT-A-11	F	1	European Shorthair	H	Dry
CAT-A-12	M	6	European Shorthair	U	Dry/wet
CAT-P-01	F	13	European Shorthair	U	Dry
CAT-P-02	F	14	European Shorthair	U	Dry/wet/homemade
CAT-P-04	F	8	European Shorthair	H	Dry/homemade
CAT-P-05	M	0.4	Sphynx	U	Homemade
CAT-P-06	M	8	European Shorthair	U	Dry/homemade
CAT-P-07	M	1.5	British Shorthair	U	Dry
CAT-P-08	F	7	European Shorthair	U	Dry/wet

Table 15: Data relative to each dog including age, sex, breed, health status and food type regularly consumed. DOG-A corresponds to dog samples in which the comet assay technique was performed according to Heaton et al (2002) and DOG-P corresponds those in which Costa et al (2008) protocol was used (see text for details). F=Female; M=Male; H=Healthy; U=Unhealthy.

Sample	Gender	Age (years)	Breed	Health Status	Food type
DOG-A-02	F	11	Weimaraner	U	Dry
DOG-A-03	M	5	Labrador Retriever	U	Dry
DOG-A-08	F	4	Labrador Retriever	U	Dry
DOG-A-09	F	3	Great dane	U	Dry
DOG-A-10	F	8	Rottweiler	U	Dry/Homemade
DOG-A-11	M	2	Boxer	U	Dry
DOG-A-12	M	14	Poodle	U	Dry/Homemade
DOG-P-01	F	9	Boxer	U	Dry
DOG-P-02	F	0.3	Labrador Retriever	U	Dry
DOG-P-03	M	2	Labrador Retriever	U	Dry
DOG-P-04	M	1	Dalmatian	U	Dry
DOG-P-05	M	4	Great dane	U	Dry
DOG-P-06	M	3	Unknown	U	Dry/Homemade
DOG-P-07	F	13	Unknown	U	Dry
DOG-P-08	F	7	Unknown	H	Dry/Homemade
DOG-P-09	M	2	French Bulldog	H	Dry
DOG-P-10	M	5	Poodle	U	Homemade
DOG-P-12	M	4	Estrela mountain dog	U	Dry
DOG-P-13	M	10	Boxer	H	Dry
DOG-P-14	F	3	Pinscher	U	Dry/Homemade
DOG-P-15	M	7	Shar-pei	U	Dry
DOG-P-16	M	3	Unknown	H	Dry/Wet
DOG-P-17	F	10	Golden Retriever	U	Dry
DOG-P-18	M	5	Unknown	H	Dry
DOG-P-19	F	8	Husky	U	Dry

2.2. Blood collection

About 2 mL of cats' and dogs' blood were collected by a certified health professional (V.M.D.) with needle and syringe from the jugular vein into EDTA tubes (BD *Vacutainer®* plastic EDTA tube). All samples were kept at 4ºC until cell isolation procedure (processed within 4 hours).

2.3. Leukocytes isolation

Leukocytes were isolated over Histopaque 1083 gradients (*Sigma®*) and stored at -80ºC after the addition of a freezing solution (50% FBS, 40% RPMI and 10% DMSO). The detailed isolation protocol is provided in Annex I.

2.4. Comet assay

Two different approaches were used: for the samples collected between January and June 2011 the comet assay was performed according to Heaton et al (2002) with slight modifications (see Annex II for the detailed protocol). Briefly, leukocytes were embedded in a thin layer of agarose on a glass slide and lysed with a solution containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, 10M NaOH, pH 10 and 1% Triton X-100. Following lysis slides were incubated 40 min in alkaline electrophoresis buffer, before being electrophoresed at 11 V during 30 min. DNA was stained using ethidium bromide, and observed under fluorescence microscope trough *Comet IV* software. For the remaining 25 samples, collected during July and August 2011, the comet assay was performed according to protocol described by Costa et al (2008) (see Annex IV for a detailed description). Briefly, leukocytes were embedded in a thin layer of agarose on a glass slide and lysed with a solution containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, 10M NaOH, pH 10 and 2% Triton X-100. Following lysis, slides were washed twice and then incubated 20 min in alkaline electrophoresis buffer, before being electrophoresed at 30 V during 20 min. DNA was stained using ethidium bromide, and observed under fluorescence microscope trough *Comet IV* software.

2.5. Statistical analysis

Statistical analyses were performed using *Minitab® 14* software. The non-parametric Mann-Wittney U test was used to compare DNA damage between cats and dogs; and the Kruskall Wallis test was used to test differences in observed DNA damage between genders, ages, residences, locations, diets and health status. Statistical analyses were performed for a significance level of 0.05.

3. RESULTS AND DISCUSSION

This is the first study that compares DNA damage in pet cats and dogs. The DNA damage (evaluated through %DNA in tail) is relative low, varying between 7.3 and 22.9% in cats and between 7.4 and 32.3% in dogs.

No significant differences (U-Statistic=189.000; p=0.779) in DNA damage between cats and dogs were detected (Figure 6). For both species, the median and the interquartile values are almost the same indicating that the DNA damage is situated in the same range at least for three quarters of the animals. However, the highest DNA damage levels were detected in dogs, with a quarter of the dogs with values above 18.5% of DNA in tail, furthermore, the highest damage was also observed in a dog sample (DOG-A-11) with 32% of DNA in tail represented as outlier (*) in Figure 6. As previously mentioned, there are few studies describing the DNA damage levels in pets and only two analyzed the influence of stressors using *in vivo* models (Blount et al., 2004; Kimura et al., 2010). However, from those two studies only the one from Blount et al (2004) can be compared with ours as they use similar endpoints to access the DNA damage. Generally, the results they obtained for DNA damage in dogs are comparable to ours with values lower than 50%.

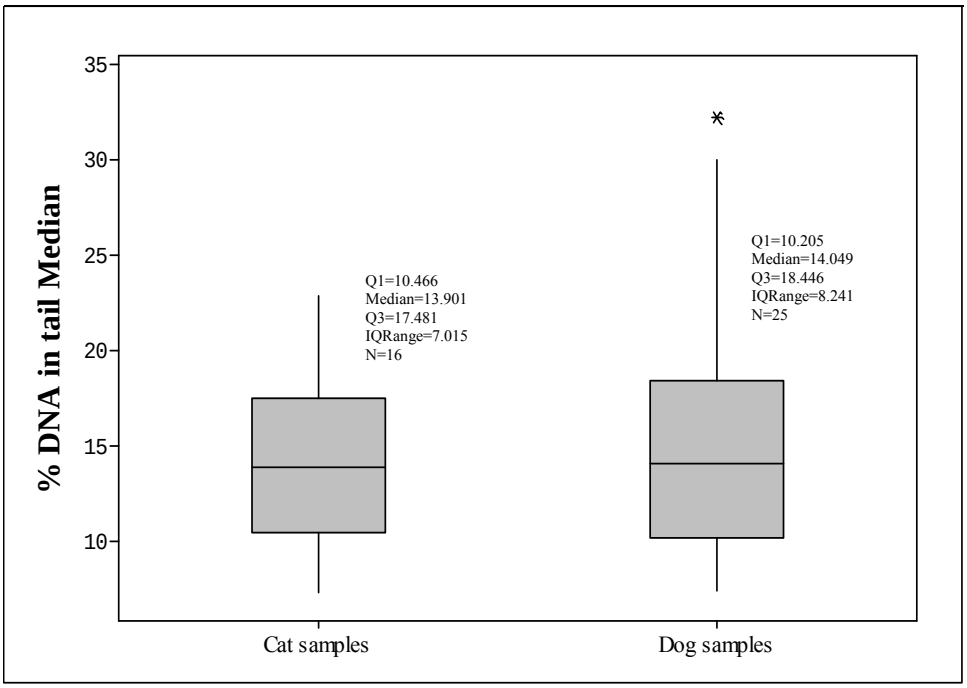


Figure 6: Boxplot summarizing the % DNA in tail of pet cats and dogs. Maximum, minimum, median, outliers (*) and the 1st quartile (Q1) and 3rd quartile (Q3) are presented. (Q1= the median of the lower half of the data set; Q3= the median of the higher half of the data set; Median = the value separating the higher half of a sample from the lower half; IQRange= inter quartile - the difference between the upper and the lower quartiles; N= number of samples.).

Owners questionnaire responses allowed to categorize pets according to their diet (if they eat dry, wet, homemade or mixed food); residence (if they live mostly inside the house (indoor), outside (outdoor) or a mix between indoor and outdoor); and geographic location (if pets leave in a urban, sub-urban or rural environment).

Concerning food, and since all cats eat dry food, the categorization was based on the consumption of other food types, hence, we considered four categories: "no supplement" when cats eat only dry food, "wet food" when cats eat dry and wet, "homemade" when they eat dry and homemade food and "mixed" when cats eat the three types of food. As for dogs the majority of dogs eat only dry food (n=19) and six other dogs eat dry and homemade food.

Regarding cats' habitat the majority lives in sub-urban environments: nine cats in sub-urban, five in urban and two in rural areas. Only two cats live exclusively outdoors, six are allowed to stay both indoors and outdoors and eight live permanently indoors. Concerning dogs, three live in urban, eighteen in sub-urban and four in rural environments. The

residence of dogs is mostly mixed between indoors and outdoors (n=15), five dogs live exclusively indoors and five exclusively outdoors.

In order to understand which factors (gender, age, residence, geographic location, diet and health status) are responsible for the observed damage, the non-parametric Kruskall Wallis test was performed. The obtained results are show in Table 16 and Table 17. There are no significant statistical differences for both cats' and dogs' samples concerning the following factors: gender, age, health status, residence, location and diet.

Table 16: Values of H, DF and P for the different factors in cat samples evaluated through non-parametric Kruskall wallis test (using chi-square approximation).

Factor	Kruskall Wallis test (H)	DF (degrees of freedom)	P
Gender	0.07	1	0.791
Age	1.99	4	0.738
Residence	0.28	2	0.870
Location	0.04	2	0.979
Diet	1.56	3	0.669
Health Status	0.26	1	0.610

Table 17: Values of H, DF and P for the different factors in dog samples evaluated through non-parametric Kruskall wallis test (using chi-square approximation).

Factor	Kruskall Wallis test (H)	DF (degrees of freedom)	P
Gender	3.07	1	0.08
Age	6.50	4	0.164
Residence	1.39	2	0.500
Location	0.69	2	0.709
Diet	1.31	1	0.252
Health Status	1.85	1	0.174

Despite the fact that no significant differences were obtained for any of the factors studied empirical differences were detected and will be further described. Figure 7, 8, 9 and 10 summarizes the levels of DNA damage in both species in relation to the different factors.

A B

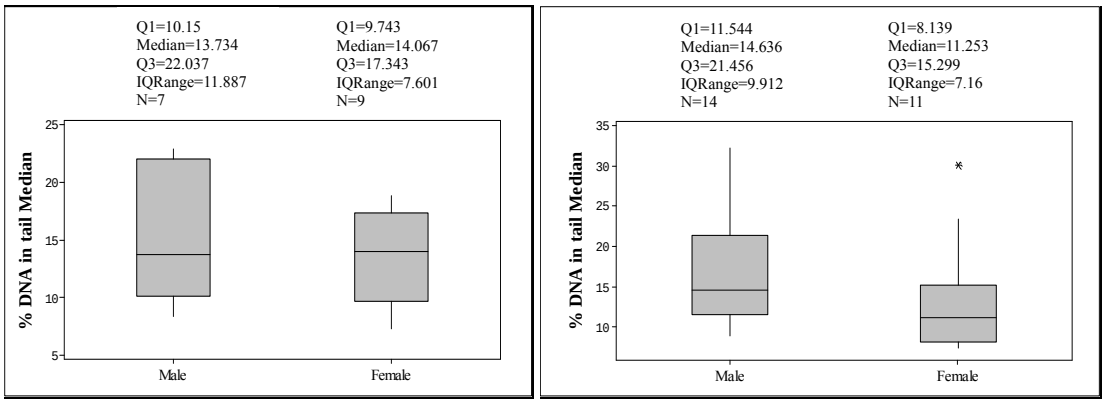


Figure 7: Boxplot summarizing % of DNA in tail in relating to the gender of pet cats (A) and dogs (B). Maximum, minimum, median, outliers (*) and the 1st quartile (Q1) and 3rd quartile (Q3) are presented.

Of the sixteen studied cats, nine are females and seven are males. DNA damage range in male cats is wider than for females, registering also the highest values. Concerning dogs, the same trend is noticeable with males (n=14) exhibiting higher DNA damage than females (n=11). The effect of gender in DNA damage levels is highly variable as some studies suggest and that there are higher levels in males than in females, as for example Scheirs et al (2006); Bajpayee et al (2002), although they couldn't establish the causal nature of these differences. Scheirs et al (2006) determined genotoxic damage in mice along polluted areas of Belgium and concluded that genetic damage (determined through comet assay) was higher in males than in females at the most polluted site. Bajpayee et al (2002) also detected higher DNA damage levels in males than females in an Indian human population. However, this trend is not universal amongst mammals as many studies didn't found any difference between gender (Diem et al (2002); Pastor et al (2001) and Festa et al (2003), just to cite a few) and others observed higher levels of DNA damage in rodents females (Heuser et al., 2002).

The age of pet cats ranged between 0.4 to 14 years and between 0.3 and 14 years for dogs. No relationship was found between the age factor and DNA in tail for both cats and dogs. Such results disagree with the ones obtained by Blount et al (2004) when investigating the relationship between DNA damage and age. Those authors determined DNA damage in thirty-four Labrador Retriever dogs related with age from an *in vivo* perspective. In their study, although the goal is different from ours, they investigated DNA

damage through comet assay related to age in presence or absence of oxidative challenge and detected higher levels of DNA damage in older dogs, concluding that senior animals are more susceptible to DNA damage than young ones, although the DNA damage levels were inferior to 50% of DNA in tail. Similar results (increased damage with age) were obtained by Kimura et al (2010) when they evaluated the extended of DNA damage of the olfactory and respiratory epithelia of thirty-three dogs from different regions of São Paulo, Brazil.

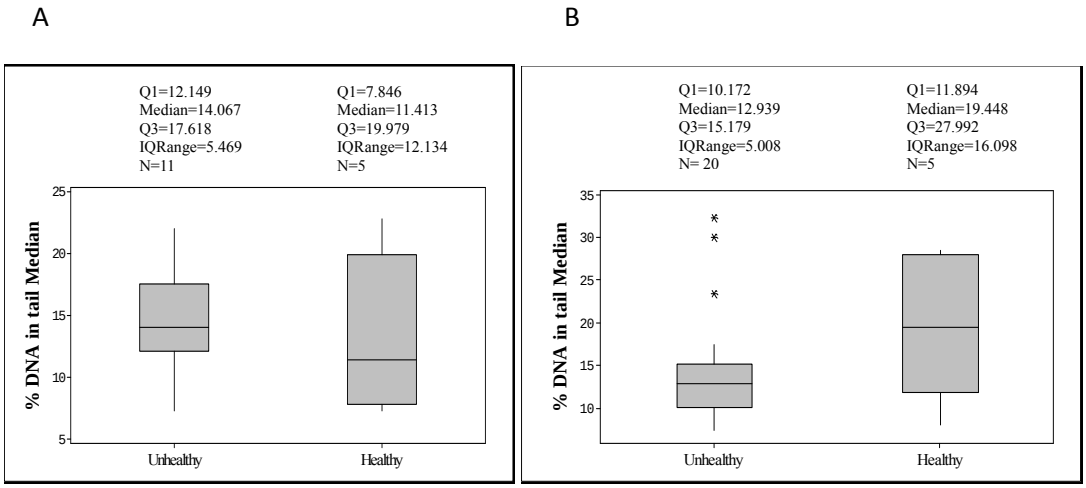


Figure 8: Boxplot summarizing % of DNA in the tail in relation to the health status for pet cats (A) and dogs (B). Maximum, minimum, median, outliers (*) and the 1st quartile (Q1) and 3rd quartile (Q3) are presented.

At the moment of blood collection only five cats were healthy against eleven unhealthy cats. The healthy ones have a higher DNA damage range and, also, the highest value of DNA in tail (22.9%). Despite this, the median of healthy cats is inferior to unhealthy median. As for dogs five were healthy and twenty unhealthy. Healthy dogs have higher DNA damage range, in which the median is superior to the 75th percentile of unhealthy dogs, although unhealthy dogs presented outliers with levels as high as 30% of DNA in tail. The apparently contradictory results in the DNA damage and health status can be explained by the fact that the categorization of healthy/unhealthy is not informative enough. An animal can be considered healthy but it can be affected by some pathology not diagnosed that can induce DNA damage. Furthermore, unhealthy animals can suffer from a condition that doesn't affect DNA integrity. More detailed information on the health status

is necessary; ongoing work will provide us the necessary insights to better understand the overall health condition of the pets (hormonal analysis and biochemical data).

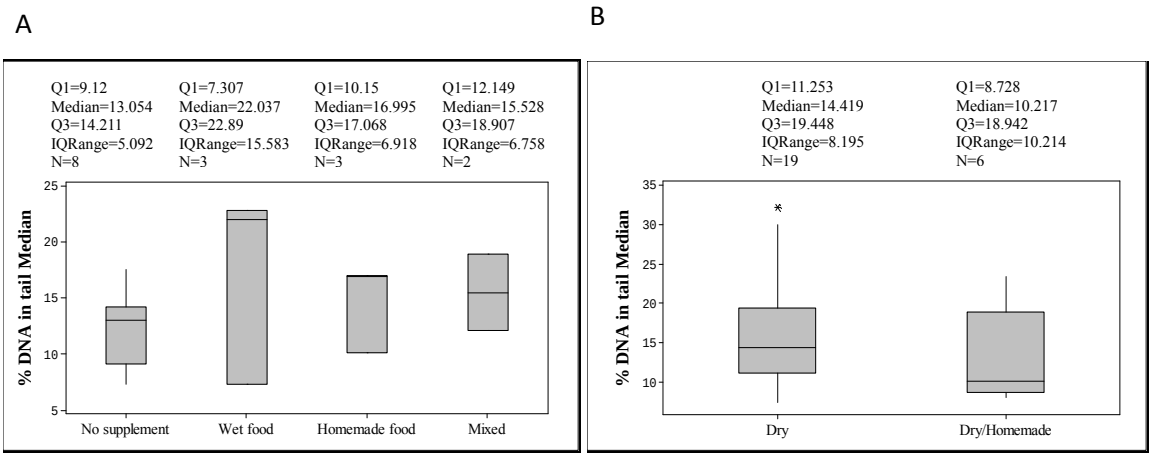


Figure 9: Boxplot summarizing the % of DNA in tail in relation to diet for pet cats (A) and dogs (B). Maximum, minimum, median, outliers (*) and the 1st quartile (Q1) and 3rd quartile (Q3) are presented.

The evaluation of diet's influence on cat's DNA damage is impaired by the low number of samples (only three cats eat wet food). Therefore, and even though it seems that cats that eat wet food have the highest DNA damage values, more animals are necessary to drawn such conclusions. As for dogs, the median that eat dry and homemade food is lower than the 25th percentile of dogs that eat only dry food. Such observation might suggest that a mixed diet (dry + homemade) is somewhat better for dogs. However, due to the low sample size (six dogs eat dry + homemade against the nineteen that eat only dry) it is impossible to draw such conclusion.

A B

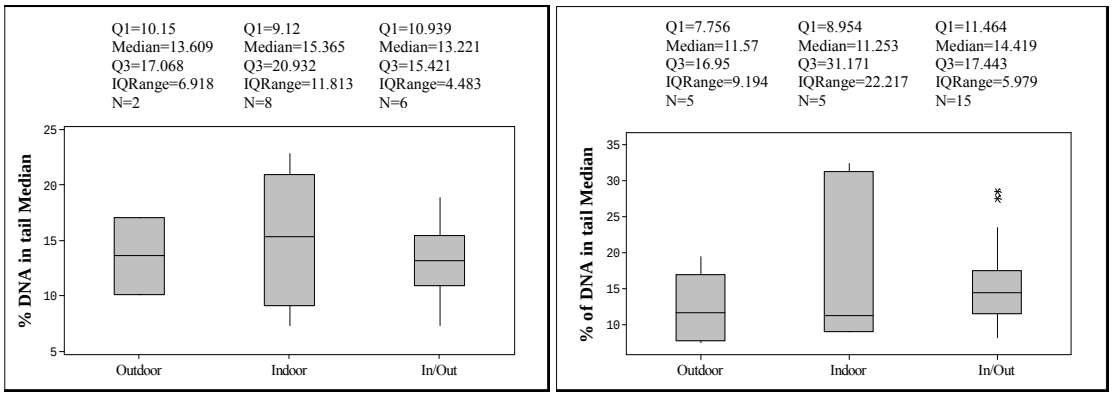


Figure 10: Boxplot summarizing the % of DNA in tail in relation to the residence for pet cats (A) and dogs (B). Maximum, minimum, median, outliers (*) and the 1st quartile (Q1) and 3rd quartile (Q3) are presented.

The eight cats that live indoors have the lowest and the highest value of DNA damage, although the median of outdoor, indoor and in/out is similar. The same trend is observed in dogs boxplot. An increased number of samples is necessary in order to better understand if the residence factor is or not determinant for DNA damage. There were no differences between pets living in urban, sub-urban or rural environmental, mostly because the majority of pets on this study live in a sub-urban environment.

In conclusion, this study demonstrates that the levels of DNA damage in pets cats and dogs from central Portugal are relatively low (< 35% of DNA in tail) and that there are no significant differences between the two species. Furthermore, no determinant factors for DNA damage could be established, probably due to the limited number of samples. Future work should use an increased number of animals and also a parallel study exposing *ex vivo* pet cats and dogs leukocytes to environmental contaminants in order to verify their exposure effects, since that with *in vivo* studies it is very difficult to establish a cause-effect relationship.

ACKNOWLEDGMENTS

The authors are grateful to the veterinarians at Hospital Veterinário do Baixo Vouga, Hugo Vilhena, VMD and Sónia Miranda, VMD, for the samples collection, and also to the pets owners and the pets themselves for their participation. Thanks are also due to Dr. João Paulo Teixeira and Susana Pinho for the assistance with the comet assay technique performed at Instituto Nacional de Saúde Dr. Ricardo Jorge, Porto.

References

Bajpayee, M., Dhawan, A., Parmar, D., Pandey, A.K., Mathur, N., Seth, P.K., 2002. Gender-related differences in basal DNA damage in lymphocytes of a healthy Indian population using the alkaline Comet assay. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 520, 83-91.

Blount, D.G., Heaton, P.R., Pritchard, D.I., 2004. Changes to levels of DNA damage and apoptotic resistance in peripheral blood mononuclear cells and plasma antioxidant potential with age in labrador retriever dogs. J. Nutr. 134, 2120S-2123S.

Cooke, M.S., Evans, M.D., Dizdaroglu, M., LUNEC, J., 2003. Oxidative DNA damage: mechanisms, mutation, and disease. The FASEB journal 17, 1195.

Costa, S., Coelho, P., Costa, C., Silva, S., Mayan, O., Santos, L.S., Gaspar, J., Teixeira, J.P., 2008. Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde. Toxicology 252, 40-48.

Diem, E., Ivancsits, S., Rudiger, H.W., 2002. Basal levels of DNA strand breaks in human leukocytes determined by comet assay. J. Toxicol. Env. Health Pt A 65, 641-648.

Festa, F., Cristaldi, M., Leradi, L.A., Moreno, S., Cozzi, R., 2003. The Comet assay for the detection of DNA damage in Mus spretus from Donana National Park. Environ. Res. 91, 54-61.

Glickman, L.T., Fairbrother, A., Guarino, A.M., Bergman, H.L., Buck, W.B., Cork, L.C., Hayes, H.M., Legator, M.S., Mcconnell, E.E., Mcnelis, D.N., Temple, S.A., 1991. Animals as sentinels of environmental health hazards. National Academies Press.

Heaton, P.R., Ransley, R., Charlton, C.J., Mann, S.J., Stevenson, J., Smith, B.H.E., Rawlings, J.M., Harper, E.J., 2002. Application of single-cell gel electrophoresis (comet) assay for assessing levels of DNA damage in canine and feline leukocytes. J. Nutr. 132, 1598S-1603S.

Heuser, V.D., da Silva, J., Moriske, H.J., Dias, J.F., Yoneama, M.C., de Freitas, T.R.O., 2002. Genotoxicity biomonitoring in regions exposed to vehicle emissions using the comet assay and the micronucleus test in native rodent Ctenomys minutus. Environmental and Molecular Mutagenesis 40, 227-235.

Kimura, K.C., Fukumasu, H., Chaible, L.M., Lima, C.E., Horst, M.A., Matsuzaki, P., Sanches, D.S., Pires, C.G., Silva, T.C., Pereira, T.C., Mello, M.L., Matera, J.M., Dias, R.A., Monnereau, A., Sasco, A.J., Saldiva, P.H.N., Dagli, M.L.Z., 2010. Evaluation of DNA damage by the alkaline comet assay

of the olfactory and respiratory epithelia of dogs from the city of Sao Paulo, Brazil. Experimental and Toxicologic Pathology 62, 209-219.

McKelveymartin, V.J., Green, M.H.L., Schmezer, P., Poolzobel, B.L., Demeo, M.P., Collins, A., 1993. The single-cell gel-electrophoresis assay (comet assay) - a European review. Mutation Research 288, 47-63.

Pastor, N., Lopez-Lazaro, M., Tella, J.L., Baos, R., Hiraldo, F., Cortes, F., 2001. Assessment of genotoxic damage by the comet assay in white storks (Ciconia ciconia) after the Donana Ecological Disaster. Mutagenesis 16, 219-223.

Scheirs, J., De Coen, A., Covaci, A., Beernaert, J., Kayawe, V.M., Caturla, M., De Wolf, H., Baert, P., Van Oostveldt, P., Verhagen, R., Blust, R., De Coen, W., 2006. Genotoxicity in wood mice (Apodemus sylvaticus) along a pollution gradient: Exposure-, age-, and gender-related effects. Environ. Toxicol. Chem. 25, 2154-2162.

Singh, N.P., 1998. A rapid method for the preparation of single-cell suspensions from solid tissues. Cytometry 31, 229-232.

Chapter IV

General Conclusion

Chapter IV. General Conclusion

Harmful compounds present in indoor environment, such as metals, can be acutely lethal when in high doses and even at low doses they have a wide range of health effects in humans and pets (who share the same environment of their owners and have the "hand-mouth" behavior like children) (López-Alonso et al., 2007).

In the present work pet cats and dogs were used as sentinels for human contamination by studding levels and effects of metals, through the determination of metals concentrations, the respective induction of MTs and trough the assessment of DNA damage. The importance of pet food as a potential source of metals was also studied.

Cadmium (Cd), Chromium (Cr), Copper (Cu), Lithium (Li), Nickel (Ni), Lead (Pb) and Zinc (Zn) levels detected in dog food samples (Chapter II) are within the range proposed by the National Research Council (1980). Thus, food can be excluded as responsible for the metal contamination. Concentrations of metals in dogs' blood were bellow the maximum limit recommended in literature, leading us to conclude that, apparently, there is no warning metal contamination in the dogs studied.

Results from the analysis of DNA damage (Chapter III) also disclose the same tendency, with damage levels below 35% in both cats and dogs. However, DNA damage levels were highly variable and it was not possible with the limited number of animals that could be selected for this study to assess which parameters – gender, age, health status, diet, residence or geographic location - were significantly affecting DNA integrity.

Since no significant differences between pets living indoor and outdoor were detected, the relation between DNA damage and a possible indoor contamination cannot be established. Therefore, pets and their owners, apparently, are not exposed to indoor contamination leading to DNA damage (since both share the same indoor environment and pets are sentinels for human exposure). The studies using pets and their owners become of relevant interest once they will allow early warnings of contaminants exposure.

References

López-Alonso, M., Miranda, M., García-Partida, P., Cantero, F., Hernández, J., Benedito, J.L., 2007. Use of dogs as indicators of metal exposure in rural and urban habitats in NW Spain. Sci. Total Environ. 372, 668-675.

National Research Council, 1980. Mineral Tolerance of Domestic Animals. National Academy of Sciences, Washington, D.C.

Annex

Annex

Annex I

Table 1: Macrominerals: Functions, Body Content, Deficiency Symptoms, and Recommended Dietary Allowances (RDA) (Sareen S. Gropper et al., 2009)

Mineral	Selected Physiological Functions	Approximate Body content	Selected Enzyme Cofactors	Deficiency Symptoms	Selected Food Sources	RDA/AI
Calcium	Structural component of bonés and teeth; role in cellular processes, muscle contraction, blood clotting, enzyme activation	1,400 g	Adenylate, cyclase, kinases, protein kinase, Ca^{2+}/Mg^{2+} -ATPase	Rickets, osteomalacia, osteoporosis, tetany	Milk, milk products, sardines, clams, oysters, turnip and mustard greens, broccoli, legumes, driedmfruits	1,000 mg, * 19-50 years
Chloride	Primary anion; maintains pH balance, enzyme activation, component of gastric hydrochloric acid	105 g		In infants: loss of apetite, failure to thrive, weakness, lethargy, severe hypokalemia, metabolic acidosis	Table salt, seafood, milk, meat, eggs	
Magnesium	Component of bones; role in nerve impulse transmission, protein synthesis; enzyme cofactor	35 g	Hydrolysis and transfer of phosphate groups by phosphokinase; important in numerous ATP-dependent enzyme reactions	Neuromuscular Hyperexcitability, muscle weakness, tetany	Nuts, legumes, whole-grain cereals, leafy green vegetables	400 mg males; 310 mg females; 19-30 years
Phosphorus	Structural component of bone, teeth, cell membranes, phospholipids, nucleic acids, nucleotide coenzymes, ATP-ADP phosphate transferring system in cells, pH regulation	850 g	Activates many enzymes in phosphorylation and dephosphorylation	Neuromuscular, skeletal, hematologic, and cardiac manifestations; rickets, osteomalacia	Meat, poultry, fish, eggs, milk, milk products, nuts, legumes, grains, cereals	700 mg, 19^{+} years
Potassium	Water, electrolyte, and pH balances; cell membrane transfer	245 g	Pyruvate kinase, Na^{+}/K^{+} -ATPase	Muscular weakness, mental apathy, cardiac arrhythmias, paralysis, bone fragility	Avocado, banana, dried fruits, orange, peach, potatoes, dried beans, tomato, wheat bran, dairy products, eggs	4,700 mg,* 19^{+} years
Sodium	Water pH and electrolyte regulation; nerve transmission, muscle contraction	105 g	Na^{+}/K^{+} -ATPase	Anorexia, nausea, muscle atrophy, poor growth, weight loss	Table salt, meat, seafood, cheese, milk, bread, vegetables (abundant in most foods except fruits)	1,500 mg,* 19-50 years
Sulfur	Component of sulfur-containing amino acids, lipoic acid, and 2 vitamins (thiamin, biotin)	175 g		Unknown	Protein foods-meat poultry, fish, eggs, milk, cheese, legumes, nuts	Not established

* indicates adequate intake

Table 2: The Microminerals: Aproximate Body Content, Selected Function, Deficiency Symptoms, Food Sources, and Recommended Intake Dietary Allowance (RDA) or Adequate Intake (AI) (Sareen S. Gropper et al., 2009)

Mineral	Approximate Body content	Selected Physiological Roles	Selected Enzyme Cofactor Roles	Selected Deficiency Symptoms	Food Sources	RDA/AI (Adults)
Chromium	4-6 mg	Normal use of blood glucose and function of insulin		Glucose intolerance, glucose and lipid metabolism abnormalities	Mushrooms, pures, asparagus, organmeats, whole-grains breads and cereals	35 µg* male; 25 µg* female
Copper	50-150 mg	Utilization of iron stores, lipids, collagen, pigment, neurotransmitter synthesis	Oxidases, monooxygenases, superoxide dismutase	Anemia, neutropenia, bone abnormalities	Liver, shellfish, whole grains, legumes, eggs, meat, fish	900 µg
Fluoride	Unknown	Maintenance of teeth and bone structure		Dental caries, bone problems	Fish, meat, legumes, grains, drinking water (variable)	4 mg* male; 3 mg* female
Iodine	15-20 mg	Thyroid hormones synthesis		Enlarged thyroid gland, mydexema, cretinism, increase in blood lipids, gluconeogenesis, and extracellular retention of NaCl and H_2O	Iodized salt, salt-water seafood, sunflower seeds, mushrooms, liver, eggs	150 µg
Iron	2.4 g	Component of hemoglobin and myoglobin for O_2 transport and cellular use	Heme enzymes, catalase, cytochromes, myeloperoxidase, nonheme enzymes camitine and collagen synthesis	Listlessness, fatigue, anemia, palpitations, sore tongue, angular stometitis, dysphagia, decreased resistance to infection	Organ meats (liver, meat, molasses, clams, osters, nuts, legumes, seeds, green leafy vegetables, dried fruits, enriched/whole-grain breads/cereals	8 mg male; 18 mg female
Manganese	10-20 mg	Brain function, collagen, bone, growth, urea, synthesis, glucose and lipid metabolism, CNS function	Arginase, pyruvate carboxylase, PEP, carboxykinase, superoxide dismutase	In animals, possibly humans: impaired growth, skeletal abnormalities, impaired CNS function	Wheat barn, legumes, nuts, lettuce, beet tops, blueberries, pineapple, seafood, poultry, meat	2.3 mg* male; 1.8 mg* female
Molybdenum	Unknown	Metabolism of purines, pyrimidines, pteridines, adehydes, and oxidation	Xanthine dehydrogenase/oxidase, adehyde oxidase, sulfide oxidase	Hypermethioninemia, ↑ urinary xanthine, sulfite excretion, ↓ urinary sulfate and urate excretion	Soybeans, lentils, buckwheat, oats, rice, bread	45 µg
Selenium	15 mg	Protects cells against destruction by hydrogen peroxide and free radicals	Glutathione peroxidase, 5'-deiodinase, thioredoxin reductase	Myalgia, cardiac myopathy, ↑ cell fragility, pancreatic degeneration	Grains, meat, pouly, fish, dairy products	55 µg
Zinc	1.5-2.5g	Energy metabolism, metabolism, protein synthesis, collagen formation, alcohol detoxification, carbon dioxide elimination, sexual maturation, taste and smell functions	DNA-RNA polymerase, carbonic anhydrase, carboxypeptidase, alkaline phosphatase, deoxythymidine kinase	Poor wound healing, subnormal growth, anorexia, abnormal taste/smell; changes in hair, skin, nails; retarded reprodutive system development	Oysters, wheat germ, beef, liver, poultry, whole grains	11 mg/day male; 8 mg/day female

* indicates adequate intake

Table 3: Ultratrace elements: Selected Functions, Deficiency Symptoms, and Food Sources (Sareen S. Gropper et al., 2009)

Mineral	Selected Possible Physiological Roles	Selected Deficiency Symptoms in Animals	Food Sources
Arsenic	Methyl groug use, normal growth	Curtailed growth	Seafood
Boron	Bone development, cell membrane, embrogenesis, metabolic regulator, inflammation	Altered bone mineral metabolism, depressed growth	Fruits, vegetables, legumes, nuts
Nickel	Possibly involved in hormonal membrane or enzyme activity	Depressed growth, impaired hematopoiesis	Nuts, legumes, grains, cacoa products
Silicon	Connective tissue and bone formation, prolylhydroxylase activity	Decreased collagen, long bone and skull abnormalities	Beer, unrefined grains, root vegetables
Vanadium	Mimics insuli action, inhibition of Na^{+}/K^{*} - ATPase	Reduced growth, hematologic changes, metabolism changes	Shellfish, spinach, parsley, mushrooms, whole grains

Annex II

Cadmium (Cd), lead (Pb) and lithium (Li) are regularly found in food and consequently in blood, although they are not essential. They don't have a physiological role, enzyme cofactor role and animals don't have deficiency symptoms since these metals are not necessary.

Cadmium (Cd)

This element is present in almost all food, although the concentrations vary depending on the food type (Jarup, Berglund et al. 1998). The cadmium average daily intake is 70 μg day^{-1} [corresponding to the present PTWI (provisional tolerable weekly intake)], but according to Jarup et al (1998) this value is unacceptable, since with 70 μg day^{-1}, 7% of the general human adult population would be expected to develop cadmium-induced kidney lesions.

Lead (Pb)

The main lead exposure is from food and water, although the intake estimate is difficult, since food is not contaminated in a controlled or predictable agricultural or manufacturing process (WHO, 2000)

Lithium (Li)

Drinking water, grains and vegetables are the main source for lithium intake. The endogenous serum levels in humans as well as daily intake of this alkaline metal were found highly variable: from 1.1 to 59.7 μg L^{-1}, or 104.1 μg day^{-1} to 1596–2568 μg day^{-1} respectively, greatly depending on the environmental occurrence of lithium and on dietary habits (Norra, Feilhauer et al. 2010). Recommended dietary allowance is 1 $mgday^{-1}$ for a 70-kg adult. Lithium may be a biological trace element for mammals, since it is useful for normal health and life expectancy, growth, weight and reproduction (Norra et al., 2010).

The toxicity of each mineral can be summarized as follows:

- Calcium (Ca): Large intake can result in hypercalcemia and deposition of calcium in soft tissues, along with systemic alkalosis. Constipation can also occur (Sareen S. Gropper et al., 2009).
- Phosphorous (P): Characterized by hypocalcemia and tetany (Sareen S. Gropper et al., 2009).
- Magnesium (Mg): Magnesium salts (3-5 g), such as from $MgSO_4$, may have a cathartic effect, leading to diarrhea and possible dehydration. Other signs including nausea, flushing, double vision, slurred speech, and weakness, usually appear at plasma magnesium concentrations of 9 to 12 mg dL^{-1}. Acute magnesium toxicity results in nausea, depression and paralysis (Sareen S. Gropper et al., 2009).
- Sodium (Na): Hypertonic solutions produce violent inflammation of the gastrointestinal track and vomiting. Effects on the animals include convulsions, muscular twitching and rigidity, cerebral and pulmonary edema, stupor and death (WHO, 1979).
- Potassium (K): Hyperkalemia (high serum potassium concentration), results in severe cardiac arrhythmias and even cardiac arrest (Sareen S. Gropper et al., 2009).
- Chloride (Cl): Hypertonic salt solutions cause convulsions, diarrhea, muscular twitching and rigidity, prostration and death; dehydration and congestion occur in most internal organs, particularly in the meninges and brain; death may occur from respiratory failure secondary to acute encephalopathy (WHO, 1979).
- Iron (Fe): Hemochromatosis (genetic disorder which increased iron absorption). The absorbed iron is progressively deposited within joints and tissues, especially the liver, heart, and pancreas, causing extensive organ damage and ultimately organ failure (Sareen S. Gropper et al., 2009).
- Zinc (Zn): Acute: metallic taste, nausea, vomiting, epigastric pain, abdominal cramps, and bloody diarrhea. A chronical ingestion leads to copper deficiency (Sareen S. Gropper et al., 2009).

- Copper (Cu): Intake of 64 $mgday^{-1}$: epigastric pain, nausea, vomiting and diarrhea. Hematuria, liver damage resulting in jaundice and kidney damage resulting in oliguria or anuria. In amounts about 900 mg day^{-1} is lethal. Chronic ingestion of 30 mg for more than 2.5 years can result in liver failure (Sareen S. Gropper et al., 2009).
- Selenium (Se): Nausea, vomiting, fatigue, diarrhea, hair and nail brittleness and loss, paresthesia, interference in sulfur metabolism and inhibition of protein synthesis. Acute poisoning is lethal, with damage occurring to most organ systems (Sareen S. Gropper et al., 2009).
- Chromium (Cr): Cr^{3+} picolinate: plasma concentrations between 600 to 2,400 μg have been associated with renal failure and hepatic dysfunction. Cr^{6+}: respiratory disease or dermatitis and skin ulcerations. Liver damage may also occur. CrO_3: severe acidosis, gastrointestinal hemorrhage, hepatic injury, renal failure, and death (Sareen S. Gropper et al., 2009).
- Iodine (I): Acute: burning of the mouth, throat and stomach; nausea, vomiting, diarrhea and fever. Problems with thyroid gland, including both hyper- and hypothyroidism and inflammation of the thyroid (Sareen S. Gropper et al., 2009).
- Manganese (Mn): accumulation within the liver and other organs such as the brain (resulting in neurologic abnormalities) (Sareen S. Gropper et al., 2009).
- Molybdenum (Mo): Gout (inflammation of the joints caused by accumulation of uric acid) (Sareen S. Gropper et al., 2009).
- Fluoride (F): changes in bone, kidney and possibly nerve and muscle function. Acute: nausea, vomiting, diarrhea, acidosis and cardiac arrhythmias. Ingestion of 5 and 10 g sodium fluoride or 32 to 64 mg fluoride Kg^{-1} body weight (bw) can lead to death, although it may occur with an intake as low as 5 mg fluoride Kg^{-1} bw (Sareen S. Gropper et al., 2009).
- Arsenic (As): Inorganic forms are more toxic and appear to be carcinogenic; acute: gastrointestinal distress, encephalopathy, anemia and hepatotoxicity. Is fatal at intakes of 70 – 300 mg day^{-1}. Chronic: skin hyperpigmentation, hyperkeratosis, muscle weakness, peripheral neuropathy, excessive sweating,

liver damage, delirium, encephalopathy, vascular changes and cancers of the oral cavity, skin, lungs, colon, bladder and kidney (Sareen S. Gropper et al., 2009).

- Boron (B): acute: nausea, vomiting, diarrhea, dermatitis and lethargy. Increased urinary excretion of riboflavin. Chronic: nausea, poor appetite and subsequent weight loss, anemia, patchy dry edema and seizures (Sareen S. Gropper et al., 2009).
- Nickel (Ni): In humans: nausea, vomiting and shortness of breath. In animals: lethargy, ataxia, irregular breathing, and hypothermia, among others, possibly including death. It is a carcinogen with effects on DNA: hypermethylation of DNA, inhibition of acetylation of histones, condensation of chromatin and gene silencing (Sareen S. Gropper et al., 2009).
- Silicon (Si): kidney stones. Associated with diminished activities of several enzymes that prevent radical damage, including glutathione peroxidase, superoxide dismutase and catalase. Inhaling dust high in silica cause silicosis characterized by a progressive fibrosis of the lungs that leads to respiratory problems (Sareen S. Gropper et al., 2009).
- Vanadium (V): green tongue (from deposition of green-colored vanadium in the tongue), diarrhea, gastrointestinal cramps, disturbances in mental function, hypertension and renal toxicity (Sareen S. Gropper et al., 2009).
- Cobalt (Co): Rats exposed to cobalt sulfate in the diet at higher doses experienced adverse cardiac effects; cobalt has been shown to be mutagenic in somatic and germ cells in *in vivo* and *in vitro* experiments; clastogenic effects in bone marrow cells were observed in mice orally exposed to cobalt; causes genotoxic effects in mammals; mice and rats exposed to high oral doses of cobalt chloride for 2–3 months experienced testicular degeneration and atrophy; rabbits exposed at high doses were found to have increased mortality, fetal resorption, and number of fetuses with decreased body weight; soluble cobalt has also been shown to block inorganic calcium channels, which can affect neuromuscular transmissions (Kim et al., 2006).
- Cadmium (Cd): renal tubular damage; skeletal damage (osteoporosis) may be a critical effect; classified in European Union as a carcinogen. Studies in experimental animals indicated that a single high dose can give rise to necrosis

of the testicles, and a long-term low-dose exposure can cause changes in male sex hormone levels in animals. Animal studies have shown that cadmium may give rise to several adverse effects in ovaries and placentas and also to teratogenic and developmental effects (Jarup et al., 1998)

- Lead (Pb): The lowest observed lethal doses in animals after short-term oral exposure to lead acetate, lead chlorate, lead nitrate, lead oleate, lead oxide, and lead sulfate range from 300 to 4000 $mgkg^{-1}$ bw. In several studies, increased frequencies of renal tumors were noticed in both male and female rats, with a greater increase in males. It has effects on haem synthesis, neurological and behavioral effects, renal effects, cardiovascular effects, and effects on the reproductive system. In addition, lead has been shown to have effects on bone and on the immune system in laboratory animals (Sareen S. Gropper et al., 2009).
- Lithium (Li): Lithium depletion in rats led to neurobehavioral alterations, including motor activity, response to handling, avoidance behavior and social aggression (Norra, Feilhauer et al. 2010); can cause renal failure; decrease free triiodothyronine (FT3) and free tetraiodothyronine, thyroxine (FT4) levels in rats; reduces iodide uptake, iodine organification and *de novo* hormone synthesis; Li^+ can substitute Na^+ in the Na^+/I^- cotransporter, which can decrease the affinity of the transporter for iodide and/or decrease the iodide kinetic uptake in thyroid cells. It also causes an arrest of spermatogenesis and increase in female's blood estradiol levels (after 1 month exposure to Li^+) (Allagui et al., 2005)

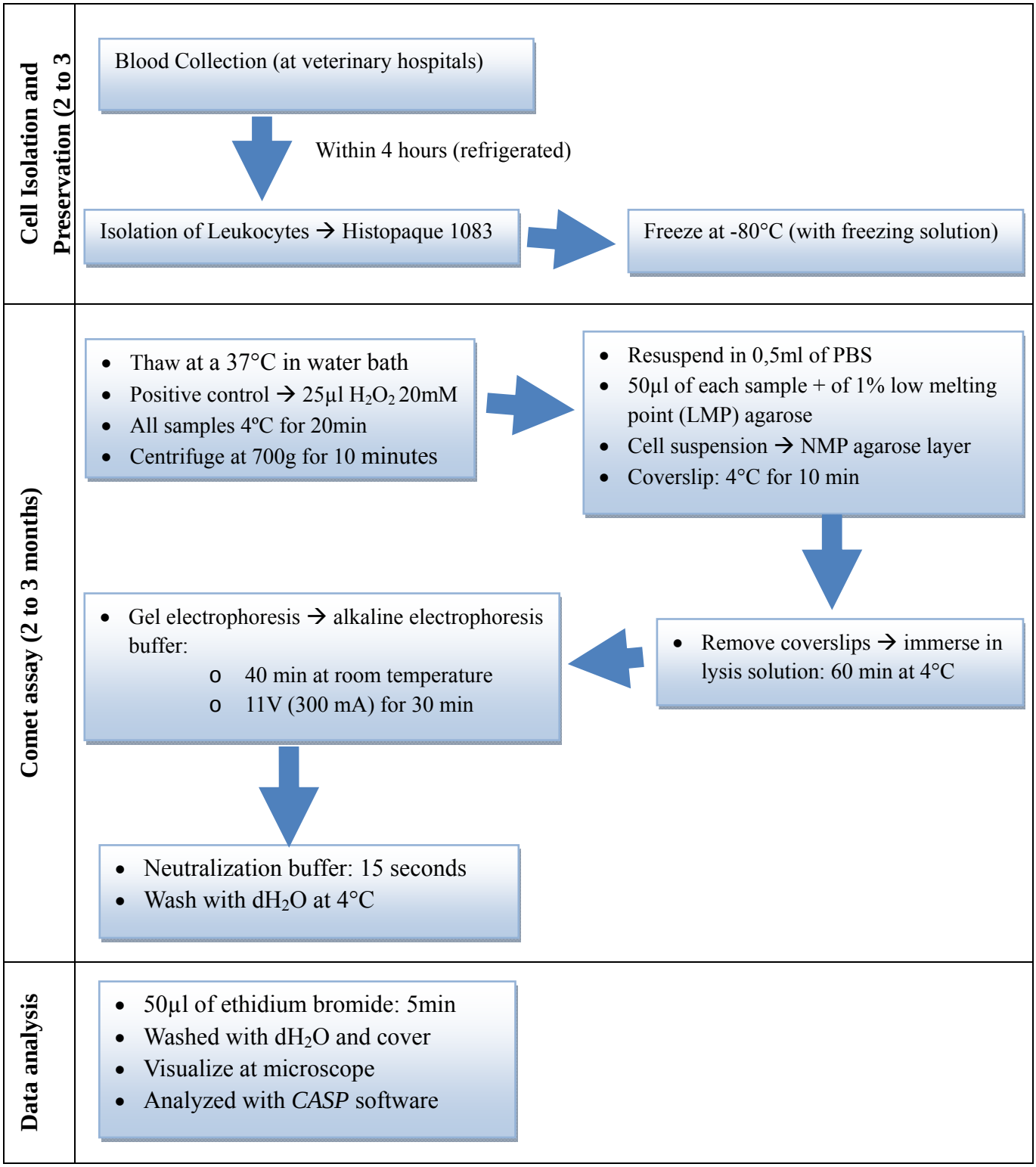
Annex III

Comet assay protocol (Heaton et al., 2002):

Solutions:

- **Freezing solution** → 50% FBS, 40% RPMI, 10% DMSO.
    - E.g.: 8 vials **(**4ml) → 2ml of FBS; 1,6ml of RPMI; 0,4ml of DMSO;
- **Layer of Agarose Normal Melting Point (NMP)** →1% (1g agarose in H_2O) normal-melting point (NMP) agarose (Sigma) prepared at 50ºC in PBSa
- **Layer of Agarose Lower Melting Point (LMP)** → 1% low melting point (LMP) agarose;
- **Lysis Solution** → 2.5 M NaCl, 100 mM sodium EDTA, 10 mM Tris, pH adjusted to 10 using NaOH pellets, 1% Triton X-100 (v/v) (added immediately before use);
- **Alkaline Electrophoresis Buffer** → 30ml 10N NaOH, 5ml 200mM EDTA:
    - 10N NaOH → 200g NaOH in 500ml dH_2O. Store at room temperature.
    - 200mM EDTA (disodium salt 2 . H_2O) → 14.89g in 175ml dH_2O, slightly warm, set to pH10 with 10N NaOH, add dH_2O up to 200ml. Store at room temperature.
- **Neutralization Buffer** → 0.4 M Tris–HCl, pH 7.5.

Method:


Cell Isolation and Preservation (2 to 3
Blood Collection (at veterinary hospitals)
Within 4 hours (refrigerated)
Isolation of Leukocytes → Histopaque 1083
Freeze at -80°C (with freezing solution)
Comet assay (2 to 3 months)
Thaw at a 37°C in water bath
Positive control → 25µl H_2O_2 20mM
All samples 4ºC for 20min
Centrifuge at 700g for 10 minutes
Resuspend in 0,5ml of PBS
50µl of each sample + of 1% low melting point (LMP) agarose
Cell suspension → NMP agarose layer
Coverslip: 4°C for 10 min
Remove coverslips → immerse in lysis solution: 60 min at 4°C
Gel electrophoresis → alkaline electrophoresis buffer:
40 min at room temperature
11V (300 mA) for 30 min
Neutralization buffer: 15 seconds
Wash with dH_2O at 4°C
Data analysis
50µl of ethidium bromide: 5min
Washed with dH_2O and cover
Visualize at microscope
Analyzed with CASP software

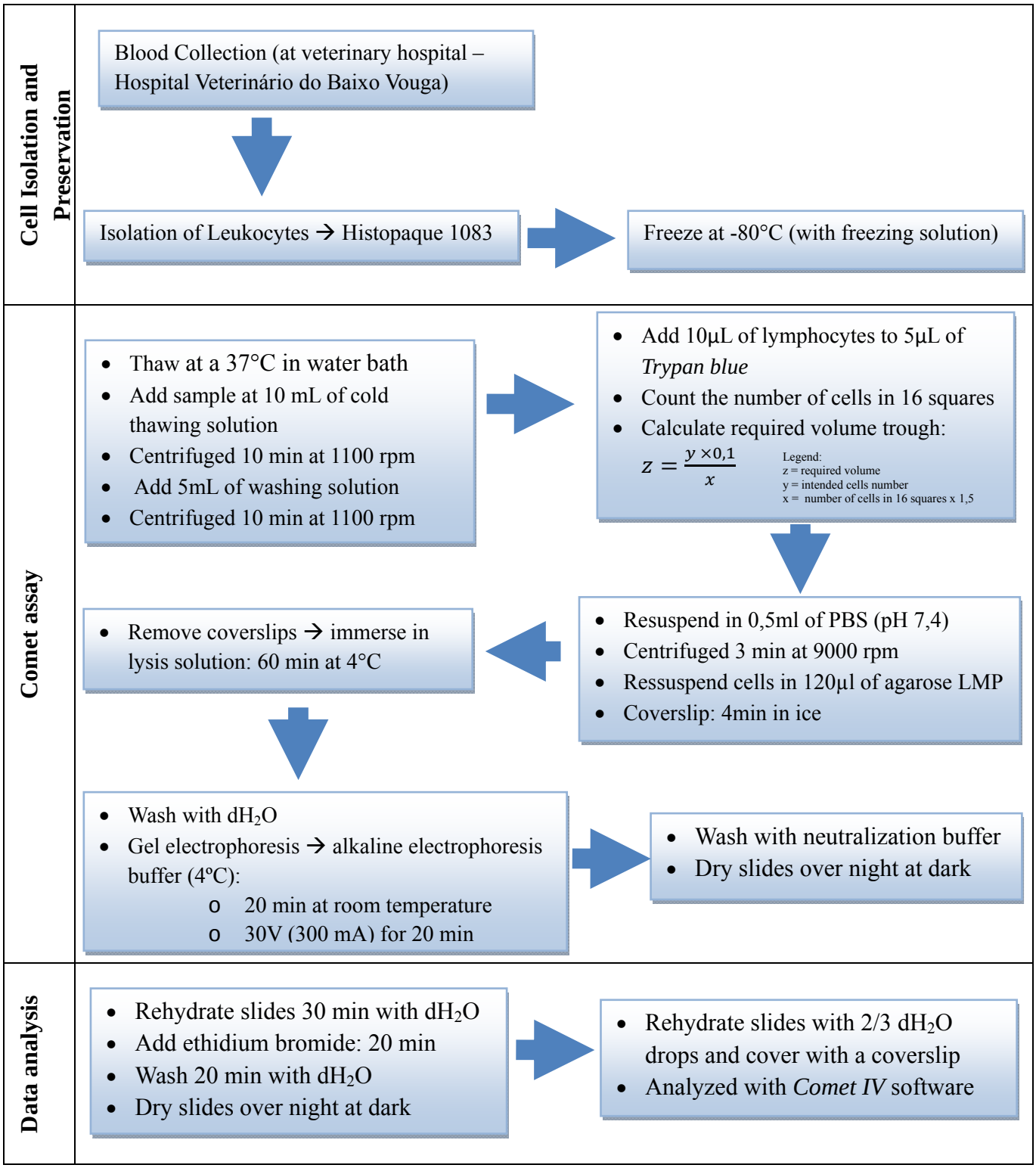
Annex IV

Comet assay protocol (Costa et al., 2008):

Solutions:

- **Freezing solution** → 50% FBS, 40% RPMI, 10% DMSO.
  - E.g.: 8 vials **(**4ml) → 2ml of FBS; 1,6ml of RPMI; 0,4ml of DMSO.
- **Thawing solution** → 50% FBS, 40% RPMI, 10% dextrose.
  - E.g.: 20 samples (200mL) → 120mL of FBS; 96mL of RPMI; 24g of dextrose.
- **Washing solution** → 90% of RPMI; 10% of FBS.
  - E.g.: 20 samples (110mL) → 99mL of RPMI; 11mL of FBS
- **Layer of Agarose Normal Melting Point (NMP)** → 2g of agarose in 200mL deionized water (dH_2O)
- **Layer of Agarose Lower Melting Point (LMP)** → 0.06g of agarose low melting point in 10mL of PBS (PBS pH 7.4);
- **Lysis Solution** → 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, 10M NaOH, pH 10
  - E.g.: A 500mL solution: 73,05g NaCl; 18,6g Na_2EDTA; 0,6g Tris-base; 5g NaOH. Sum to the volume with dH_2O and adjust pH to 10 with HCl 32%. Save at 4ºC and add 2% Triton X-100 before use
- **Alkaline Electrophoresis Buffer** → 30ml of 10mM NaOH, 5ml of 200mM Na_2EDTA:
  - 250mL of 10mM NaOH → 100g NaOH in dH_2O.
  - 100mL of 200mM Na_2EDTA → 7,4g of Na_2EDTA in dH_2O
- **Neutralization Buffer** → 0.4 M Tris–base, pH 7.5
  - 1Lof solution: 48,452g of Tris-base in dH_2O. Adjust pH to 7,5 with HCl 32%.

Method:


Cell Isolation and Preservation
Blood Collection (at veterinary hospital – Hospital Veterinário do Baixo Vouga)
Isolation of Leukocytes → Histopaque 1083
Freeze at -80°C (with freezing solution)
Comet assay
Thaw at a 37°C in water bath
Add sample at 10 mL of cold thawing solution
Centrifuged 10 min at 1100 rpm
Add 5mL of washing solution
Centrifuged 10 min at 1100 rpm
Add 10µL of lymphocytes to 5µL of Trypan blue
Count the number of cells in 16 squares
Calculate required volume trough:
$z = \frac{y \times 0{,}1}{x}$
Legend:
z = required volume
y = intended cells number
x = number of cells in 16 squares x 1,5
Resuspend in 0,5ml of PBS (pH 7,4)
Centrifuged 3 min at 9000 rpm
Ressuspend cells in 120µl of agarose LMP
Coverslip: 4min in ice
Remove coverslips → immerse in lysis solution: 60 min at 4°C
Wash with dH_2O
Gel electrophoresis → alkaline electrophoresis buffer (4ºC):
20 min at room temperature
30V (300 mA) for 20 min
Wash with neutralization buffer
Dry slides over night at dark
Data analysis
Rehydrate slides 30 min with dH_2O
Add ethidium bromide: 20 min
Wash 20 min with dH_2O
Dry slides over night at dark
Rehydrate slides with 2/3 dH_2O drops and cover with a coverslip
Analyzed with Comet IV software

References

Allagui, M.S., Hfaiedh, N., Croute, F., Guermazi, F., Vincent, C., Soleilhavoup, J.P., El Feki, A., 2005. Side effects of low serum lithium concentrations on renal, thyroid, and sexual functions in male and female rats. Comptes Rendus Biologies 328, 900-911.

Costa, S., Coelho, P., Costa, C., Silva, S., Mayan, O., Santos, L.S., Gaspar, J., Teixeira, J.P., 2008. Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde. Toxicology 252, 40-48.

Heaton, P.R., Ransley, R., Charlton, C.J., Mann, S.J., Stevenson, J., Smith, B.H.E., Rawlings, J.M., Harper, E.J., 2002. Application of single-cell gel electrophoresis (comet) assay for assessing levels of DNA damage in canine and feline leukocytes. J. Nutr. 132, 1598S-1603S.

Jarup, L., Berglund, M., Elinder, C.G., Nordberg, G., Vahter, M., 1998. Health effects of cadmium exposure - a review of the literature and a risk estimate - Preface. Scandinavian Journal of Work Environment & Health 24.

Kim, J.H., Gibb, H.J., Howe, P.D., 2006. Cobalt and inorganic cobalt compounds. WHO.

Norra, C., Feilhauer, J., Wiesmuller, G.A., Kunert, H.J., 2010. Differential effects of endogenous lithium on neurobehavioural functioning: A study on auditory evoked potentials. Psychiatry Research 178, 176-181.

Sareen S. Gropper, Jack L. Smith, Groff, J.L., 2009. Advanced nutrition and human metabolism Wadsworth, Cengage Learning, Australia.

WHO, 1979. Sodium, chlorides, and conductivity in drinking-water. 63.

WHO, 2000. Safety evaluation of certain food additives and contaminants. International Programme on Chemical Safety.