Relationship between Canine Distemper and Oxidative Stress in dogs

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SUMMARY

The aim of the present study was to investigate the oxidative stress occurrence during canine distemper (CD) virus infection in dog. For that, plasma concentrations of markers for oxidative stress (malondialdehyde (MDA), nitrates and nitrites, and ceruloplasmin at a lesser extend) and of antioxidant compounds (glutathione (GSH), ascorbate, retinol and β-carotene) were measured and compared in 11 naturally CD infected dogs (4 with a predominant neurological form, 3 with a pulmonary form, 3 with an intestinal form and 1 with only general signs) and in 6 healthy controls. All infected dogs exhibited significantly elevated plasma concentrations of oxidant markers (MDA, nitrates and nitrites) and of ceruloplasmin compared to controls (p < 0.01) whereas antioxidant concentrations were significantly lowered in the diseased group (p < 0.05 for ascorbate and p < 0.01 for the others). Nevertheless, although nitrite concentrations were significantly decreased in dogs with neurological signs compared to the other infected dogs, the intensity of the variations of markers for oxidant and antioxidant status was independent of a specific clinical form of the disease. These results demonstrated the accumulation of reactive oxygen species leading to lipid peroxidation and to consumption of antioxidant system during acute CD in dogs.

Keywords: Canine distemper, dog, oxidative stress, MDA, NO, ceruloplasmin, antioxidant status, glutathione, ascorbate, vitamin A.

Introduction

Free radicals and their metabolites, also called reactive oxygen species (ROS) have been implicated in the pathogenesis of many diseases. The respiratory chain in mitochondria is mainly responsible for ROS production in organisms. Besides, ROS are generated throughout phagocytosis, redox reactions of xenobiotics and enzymatic reactions catalysed by lipooxygenases, cyclooxygenases, oxidases and dehydrogenases [38]. Although ROS exhibit several advantages in live organisms as signalling molecules, an excessive production may have harmful effects leading to oxidative damage of lipids, proteins, RNA and DNA [34]. The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by mechanisms called “redox regulation”, which protect living organisms from various oxidative stresses and maintain “redox homeostasis” by controlling the redox status in vivo [11].

ROS are known to cause cell damage by 3 main mechanisms: lipid peroxidation, protein oxidation and DNA oxidation. Therefore, cells have developed several defence and repair mechanisms to deal with oxidative stress: antioxidants represent the first line of defence and comprise enzymes such as superoxide dismutases, catalases, glutathione peroxidases and radical scavengers such as vitamins E and C, which are able to neutralise ROS and can be regenerated by the cellular antioxidant network. Oxidative stress can be evidenced by some markers (such as antioxidant enzyme activities and products of lipid and protein peroxidation) which can be measured in tissues, plasmas or haemolysates [15].

RÉSUMÉ

Les relations entre la maladie de Carré et le stress oxydatif chez le chien

Le but de cette étude est de rechercher l’implication du stress oxydatif au cours de la maladie de Carré chez le chien. Pour cela, les concentrations plasmatiques des marqueurs d’un stress oxydatif (malondialdéhyde (MDA), nitrates et nitrites, ainsi que la céruloplasmine dans une moindre mesure) et celles de composés anti-oxydants (glutathion (GSH), ascorbate, rétinol et β-carotène) ont été mesurées et comparées sur 11 chiens naturellement infectés par le virus de la maladie de Carré (4 présentaient essentiellement des signes neurologiques, 3 des signes pulmonaires, 3 des signes intestinaux et 1 seulement une atteinte générale) et sur 6 chiens en bonne santé. Les concentrations plasmatiques des marqueurs d’oxydation (MDA, nitrates et nitrites) et de la céruloplasmine ont été significativement augmentées chez tous les chiens infectés par rapport aux contrôles (p < 0.01) alors qu’une diminution significative des concentrations en anti-oxydants (p < 0.05 pour l’ascorbate, p < 0.01 pour les autres) a été observée en parallèle dans le groupe des chiens infectés. Néanmoins, bien que les concentrations en nitrites aient été significativement abaissées chez les chiens atteints d’une forme neurologique par rapport aux autres chiens malades, l’amplitude des variations des marqueurs d’oxydation et des anti-oxydants s’est avérée indépendante de la forme clinique de la maladie. Ces résultats démontrent l’accumulation d’espèces oxygénées hantant réactives conduisant à une peroxydation lipidique et à la consommation des systèmes anti-oxydants durant le stade aigu de la maladie de Carré chez le chien.

Mots-clés : Maladie de Carré, chien, stress oxydatif, MDA, NO, céruloplasmine, statut antioxydant, glutathion, ascorbate, vitamine A.
One of the diseases whose pathology is connected with ROS accumulation is canine distemper (CD) [39]. Canine distemper is a severe immunosuppressive and neurological disease in the dog and other carnivores that is characterized by multifocal demyelization lesions in grey and white matters of the central nervous system [35]. It is stated that these lesions were induced by the virus replication but also by an intense oxidative stress [39], a lot of evidence supporting that oxidative stress is involved in the pathogenesis of several neurodegenerative disorders [30]. The nervous system and especially the brain are vulnerable to the free radical damage for a number of reasons such as its high oxygen consumption rate, the rich abundance of polyunsaturated fatty acids and lipids, and the relatively limited antioxidant capacity compared to other organs [7]. WISNIEWSKI et al. [41] demonstrated that antiviral antibodies induced the release of ROS from CD-infected glial cells, leading to aggravation of the white matter damage seen during chronic distemper. The aims of the present study were to evidence the oxidative stress in dogs suffering from canine distemper disease and to relate it to the severity or to the clinical form of the disease.

Materials and Methods

ANIMALS AND PROTOCOL DESIGN

Eleven naturally CD infected dogs which are belongs to owners were presented to the clinical department of the Faculty of Veterinary Medicine, University of Ankara, Turkey between January and June 2005. The CD infected dogs (6 males and 5 females) are 8 Kangal dogs, 2 Siberian Husky and 1 German shepherd and they were 2-18 months old (the average age was: 6.23 ± 4.72 months) (Table I). The diagnosis was based on clinical signs (mainly neurological, pulmonary and intestinal signs) and was confirmed by virus detection in biological fluids with the Antigen Rapid Canine Distemper Ag test (Antigen Animal Genetics, Inc., Gyeonggi-do, Korea). The observing period lasted 30 days and mortality was 50%.

Six clinically healthy dogs (3 males and 3 females) were included in this study as a control group. Among them, there were 4 Kangal dogs, 1 Husky and 1 golden retriever and control dogs were 13.50 ± 6.44 months old in average (Table I).

Blood samples were collected by the saphena vein puncture in heparinized sterile tubes (100 IU heparin / mL blood) and immediately centrifuged (1 000 g for 10 min at 4°C). Thereafter, plasmas were carefully harvested and stored in polystyrene plastic tubes at -70°C until analysis.

CHEMICALS AND BIOCHEMICAL ANALYSES

Chemicals and reagents were purchased from Sigma and were of analytical grade.

Markers for oxidative stress

The MDA (Malondialdehyde) concentrations were measured according to the method of JAIN et al. [17]. The principle of this method was based on the spectrophotometric measurement
of the colour produced by the reaction of thiobarbituric acid with MDA. The concentration of thiobarbituric acid reactive substances (TBARS) was calculated by the absorbance coefficient of the malondialdehyde-thiobarbituric acid complex and expressed in μmol / L.

The nitric oxide (NO) and nitrate concentrations were detected by the method of MIRANDA et al. [25]. Nitrite and nitrate calibration standards were prepared by diluting sodium nitrate and sodium nitrite in pure water and the Griess solutions were premixed immediately prior to application to the plate. After loading the plate with samples (100 μL), 92.60 mM vanadium (III) chloride diluted in HCl 1M (100 μL) as well as Griess reagents [sulphanilamide diluted in 5% hydrochloric acid (2% w/v) (50 μL) and N-(1-Naphthyl) ethylenediamine dihydrochlorid diluted in water (0.1 % w/v) (50 μL)] were rapidly added to each well. For nitrite determination, the addition of vanadium chloride was omitted and samples and nitrite standards were only exposed to Griess reagents. The nitrites mixed with Griess reagents form a chromophore from the diazotization of sulphanilamide by acidic nitrite followed by coupling with bicyclical amines, such as N-1-(naphthyl) ethylenediamine. Blank sample values were obtained by substituting Griess reagents with the diluting medium. Nitrate and nitrite concentrations were proportional to the absorbance read at 540 nm.

Ceruloplasmin (CP) concentrations were assessed according to the spectrophotometric method of SCHOSINSKY et al. [33].

### Antioxidants

The estimation of reduced glutathione was measured by the spectrophotometric method of BEUTLER et al. [3]. The haemolysates were prepared from erythrocytes after three times of washing with 0.9% NaCl solution to remove leucocytes and platelets. Erythrocyte membranes were removed by centrifugation at 3000 g for 10 min at 4°C, then 0.3 M disodium hydrogen phosphate and DTNB (5,5’-Dithio-bis(2-nitrobenzoic acid)) solutions were added to samples and the obtained colour was read at 412 nm. The results were expressed in µmol / L.

The plasma ascorbic acid (vitamin C) concentrations were determined after derivatization with 2,4- dinitrophenylhydrazine [27]. After mixing plasma with ethanol and hexane (plasma: ethanol: hexane at the ratio of 1: 1: 3) absorbances were read at 425 nm and 325 nm for determining β-carotene and vitamin A (retinol) concentrations respectively [36].

### STATISTICAL ANALYSIS

All values were expressed as mean ± standard deviations. Statistical analysis of data was performed using a “U Mann Whitney” test. A value of p < 0.05 was considered statistically significant.

### Results

#### CLINICAL FINDINGS

The general status signs of all dogs with CD were exhaustion, hyperthermia and lack of appetite (n = 11). Moreover, 4 dogs (36.4%) exhibited neurological signs such myelitis, ataxia, nystagmus, tremor and paresis. In 3 dogs (27.3%), the main clinical signs were pulmonary (purulent flow in nose and eyes and coughing) and the intestinal form (lack of appetite and diarrhoea) was predominant in 3 other dogs (27.3%). One dog has only shown an alteration of the general status.

#### BIOCHEMICAL FINDINGS

The plasma concentrations of markers for the oxidative stress and antioxidants in control and infected dogs are presented in Tables I, II and III.

Plasma concentrations of MDA (the lipid peroxidation end product), of nitrates and of nitrites were markedly increased in the infected group compared to the control group (p < 0.01). All diseased dogs exhibited values above the superior limit of the 95% confidence interval calculated from controls (Tables II and III). Furthermore, a significant increase of plasma ceruloplasmin concentrations was observed in the infected group compared to the control group (p < 0.01) and all the values observed in this group were above 73.3 mg/L (superior limit of the 95% confidence interval in controls) (Tables II and III). Whereas plasma concentrations of MDA, of nitrates and of ceruloplasmin did not

### Table II : Biomarkers for oxidative stress and antioxidant status in healthy (n = 6) and canine distemper (CD) infected (n = 11) dogs.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CD infected dogs</th>
<th>Healthy dogs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (μmol/L)</td>
<td>22.47 ± 1.03</td>
<td>16.23 ± 1.44</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Nitrates (mg/L)</td>
<td>8.95 ± 0.38</td>
<td>6.15 ± 0.49</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Nitrites (mg/L)</td>
<td>3.58 ± 0.49</td>
<td>2.30 ± 0.12</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Ceruloplasmin (mg/L)</td>
<td>86.5 ± 7.8</td>
<td>64.3 ± 4.6</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GSH (mg/L)</td>
<td>148.3 ± 16.6</td>
<td>199.2 ± 18.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Ascorbate (mg/L)</td>
<td>3.9 ± 0.5</td>
<td>4.8 ± 0.6</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Retinol (μg/L)</td>
<td>195.6 ± 16.2</td>
<td>237.0 ± 12.0</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>β-carotene (μg/L)</td>
<td>129.1 ± 3.1</td>
<td>154.5 ± 7.4</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

m ± SD: mean ± standard deviation; N: number of values outside the limits of the 95% confidence interval calculated from healthy dogs; 95% IC: 95% confidence interval.
significantly differ in dogs with neurological signs compared to the other infected dogs, the concentrations of nitrites were significantly lowered in this subgroup (p < 0.05). By contrast, plasma concentrations of the antioxidants (GSH, ascorbic acid, retinol and β-carotene) were significantly decreased in infected dogs compared to healthy controls (p < 0.01 for GSH, retinol and β-carotene, p < 0.05 for ascorbic acid). The retinol and β-carotene concentrations were below the inferior limit of the 95% confidence interval calculated from controls in 90.9% and 100% of infected dogs respectively whereas 81.82% and 45.45% of diseased dogs presented decreased GSH and ascorbate concentrations respectively (Tables II and III). No significant difference for plasma antioxidant concentrations was evidenced between dogs with neurological damage and the other CD infected dogs.

### Table III: Comparison of the plasma concentrations of biomarkers for oxidative stress and antioxidant status according to the clinical form of the canine distemper.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Clinical forms of the canine distemper</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neurological form (n = 4)</td>
</tr>
<tr>
<td>MDA (µmol/L)</td>
<td>22.69 ± 0.39</td>
</tr>
<tr>
<td>Nitrates (mg/L)</td>
<td>8.66 ± 0.38</td>
</tr>
<tr>
<td>Nitrites (mg/L)</td>
<td>3.21 ± 0.22</td>
</tr>
<tr>
<td>Ceruloplasmin (mg/L)</td>
<td>87.2 ± 10.0</td>
</tr>
<tr>
<td>GSH (mg/L)</td>
<td>147.4 ± 15.1</td>
</tr>
<tr>
<td>Ascorbate (mg/L)</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>Retinol (µg/L)</td>
<td>189.9 ± 10.2</td>
</tr>
<tr>
<td>β-carotene (µg/L)</td>
<td>130.2 ± 0.9</td>
</tr>
</tbody>
</table>

Results are expressed as means ± standard deviations. Different superscripts a,b in the same line indicate significant difference (p < 0.05) between dogs with a particular clinical form with the other CD infected dogs.

### Discussion

In the present study, CD-infected dogs exhibited marked elevations of the markers for oxidative stress (plasma concentrations of MDA, nitrates and nitrites) and concentrations of ceruloplasmin (a positive acute phase protein) whereas antioxidant (GSH, vitamins A and C, β-carotene) concentrations were significantly lowered compared to healthy controls. But the investigated markers were not significantly more altered in dogs with neurological damage than in other CD infected dogs except for plasma nitrite concentrations which were significantly depressed in dogs with neurological signs compared to the other infected dogs. The polyunsaturated fatty acid enriched lipids are the most susceptible to the ROS toxic actions. The most commonly used assay for the evaluation of the intensity of lipid peroxidation

is the analysis of the content of TBARS represented by MDA [22]. The increased MDA concentrations observed in CD infected dogs in the present study clearly demonstrated the ROS accumulation and the oxidative stress occurrence during canine distemper. NO has a very important physiological function as a neuronal messenger molecule. The NO synthesis has been reported in astrocytes, microglia, a subset of oligodendrocytes, Schwann cells and cerebral endothelium [19]. However, when NO increases to unusually high concentrations within cell, it initiates a toxic cascade of events which can lead to the death of neurons [8]. There is substantial indirect evidence that NO participates to demyelization by promoting the formation of some ROS like peroxinitrite [40] rather than by directly acting on myelin or myelinating cells. Peroxinitrite can affect lipid peroxidation, membrane fluidity and permeability and it can alter the function of proteins embedded in the lipid bilayer [29]. In blood, the main metabolite of NO is NO3- and in the presence of a strong oxidative agent, NO3- is oxidized in NO2-. In our study, plasma concentrations of nitrates and nitrites were both dramatically enhanced in CD infected dogs (p < 0.01) suggesting that NO has contributed to systemic cellular lipid peroxidation, and also in nerve cells albeit the plasma nitrite concentrations were significantly depressed in dogs with neurological signs compared to the other infected dogs. The involvement of NO in neurological damage has already been demonstrated in patients suffering from Alzheimer [24], HIV [1] and Parkinson [4] diseases. In this way, GRIOT et al. [14] stated that the oligodendrocytes exhibited a selective vulnerability against ROS and that myelin was especially sensitive to the ROS induced lipid peroxidation. Although plasma concentrations of markers of oxidative stress (MDA and nitrates) were not significantly higher or were even lowered...
(nitrites) in dogs with a neurological form compared to the other CD infected dogs, it may be possible that ROS accumulation was sufficient enough in nervous system for inducing irreversible lesions considering its high susceptibility to oxidative conditions.

Ceruloplasmin plays an essential role in iron metabolism and possesses an oxidant activity by converting Fe$^{2+}$ into Fe$^{3+}$ [16]. This protein is also considered as a positive acute modulation was sufficient enough in nervous system for inducing (nitrites) in dogs with a neurological form compared to the central nervous system although KLOMP and GITLIN [20] stated that any disturbance in ceruloplasmin function might contribute to degenerative changes in the brain. However, the inflammation induced by viral replication would also be responsible for such variations of ceruloplasmin concentrations. In conclusion, the increased plasma concentrations of markers for oxidative damage (MDA, nitrites and nitrites, ceruloplasmin at a lesser extend) coupled to the decreased plasma concentrations of antioxidants (GSH, ascorbate, retinol and β-carotene) directly evidenced excessive ROS accumulation leading to impairment of antioxidant systems during canine distemper in dogs. As in the etiopathogenesis of well known neurodegenerative diseases (HIV, Parkinson and Alzheimer diseases), oxidative stress contributes to the tissue injury mainly in the central nervous system and may participate to demyelization. Nevertheless, further experiments are required on larger groups for correlating the intensity of the oxidative stress with the severity (aggravation) of the disease and for evaluating its implication during chronic forms of canine distemper.

References

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