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Short Paper

Nitrosative stress indices in dogs with neurological form of canine distemper

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Summary

This is a prospective, controlled, randomized clinical study to evidence the role of nitrosative stress in development of overt neurological sign of canine distemper (CD). The enrollment of cases wasa made on basis of overt sign of CD (n=139) and the findings were compared with apparently healthy dogs (n=15). The CD specific immunoglobulins resulted in 94 confirmed positive cases. The nitric oxide (NO) and neuronal nitric oxide synthase (nNOS) concentration in cerebrospinal fluid (CSF) (18.08 ± 2.76, 415.84 ± 46.24, respectively) and plasma (32.68 ± 4.31, 321.31 ± 102.30, respectively) were significantly ($P<0.05$) elevated as compared to healthy control group. The concentration of neuron specific enolase (NSE) in CSF and plasma were also significantly ($P<0.05$) higher in dogs suffering from CD. The significant differences in other biochemical parameters like total protein, albumin and globulin were found in the CSF of dogs compared with healthy control. The author concludes that NO plays a role in pathophysiology of neurological form of CD and nNOS and NSE estimation in CSF and plasma could help in making early diagnosis of clinical cases.

Key words: Canine distemper, Cerebrospinal fluid, Neurological, Nitric oxide, Nitrosative stress

Introduction

Nitric oxide (NO) is an unorthodox messenger molecule, which has numerous molecular targets, synthesized by a family of nitric oxide synthases (NOS) enzymes from substrate L-arginine in mammals (Forstermann and Seesa, 2012). Nitric oxide and NOS have been identified in many organ systems including liver, lungs, vascular tissue, skeletal muscle, and smooth muscle (Nelson *et al*., 2003) and participates in a wide range of signaling pathways, mediating physiologic processes such as vasodilation, memory and learning, neuronal development and regulation of immune response (Pacher *et al*., 2007). In brain NO is mainly synthesized in synaptic terminals by a neuronal NOS isoform neuronal nitric oxide synthase (nNOS), acting as a neuromodulator and transmitter (Forstermann and Seesa, 2012).

Additionally, NO could also lead to damage to cellular macrolomecule (proteins and DNA) which results in nitrosative stress. The presence of elevated NO and its derived compounds in the cerebrospinal fluid (CSF) of human patients with neurological disorders like Alzheimer's disease, amyotrophic lateral sclerosis, and multiplesclerosis is indicative of the possible role of NO in the pathophysiology of nervous disorders (Rejdak *et al*., 2008). NO being very labile rapidly oxidizes to more stable end product that is, nitrite and nitrate (Forstermann and Seesa, 2012). The measurement of plasma and CSF nitrite levels is reported to be a valid measure of NO generation in human studies (Shukla, 2007).

Canine distemper (CD), a common viral disease of canine (Latha *et al*., 2007) is known to cause heavy mortality in dogs suffering from its neurological form (Greene and Appel, 2006). Previous studies suggest the role of free radicals in the pathophysiology of CD (Vandevelde and Zurbriggen, 2005; Karadeniz *et al*., 2008). Neurological form of disease in dogs and other carnivores is characterized by multifocal demyelinating lesions of white and grey matters (Summer and Appel, 1994) which could be attributed to excessive production and accumulation of free radicals during process of viremia (Vandevelde and Zurbriggen, 2005).

Cerebrospinal fluid analysis has been found to have good sensitivity for the detection of neurological diseases (Terlizzi and Platt, 2006). Neuron specific enolase (NSE), a glycolytic isoenzyme located in central and peripheral neurons and neuroendocrine cells was found in CSF and systemic circulation in response to injury to neurons and can be used as a marker for detection of neuronal damage (Jauch *et al*., 2006; Zaheer *et al*., 2013). Further, significantly elevated serum levels of NSE have also been reported in human patients (González-García *et al*., 2012).

The present study aimed to establish the role of nitrosative stress in pathophysiology of neurologic CD and to examine the efficacy of NO, nNOS and NSE as biomarker in the screening of clinical cases of CD.

Materials and Methods

Place of study

The study was undertaken at Referral Veterinary Polyclinic (RVP), ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, Uttar Pradesh, India during 2012-2013. Dogs (n=139) of different age, sex and breed were enrolled for study including apparently healthy dogs (n=15) as control. The enrolment criterion adopted for the study was presence of nervous signs like tremors or seizures, myoclonus, ataxia, postural reaction defecit, tetraparesis or plegia. The exclusion criterion was death or failure of follow-up.

Blood collection

Blood was collected aseptically from saphenous vein and transferred to vial containing K_3 EDTA as an anticoagulant. Plasma was harvested by centrifugation at 3000 rpm for 15 min and stored at -20°C till analyzed.

CSF collection

Cerebrospinal fluid was collected from the cerebello medullary cistern as per the method of Terlizzi and Platt (2009) post sedation with Diazepam[@] 0.5 mg/kg b.wt. intravenously. Approximately 1 ml of CSF per 5 kg b.wt. was collected and stored at -20°C until further analysis.

Estimation of CD specific IgG and IgM

Canine distemper specific IgG and IgM in plasma and CSF was estimated by Indirect ELISA kits (Inmunologia Y Genetica Aplicada, S.A, Spain). The tests were performed as per manufacturer's instructions. The plate was read at 450 nm using automatic ELISA plate reader. Cut off values were calculated by multiplying mean optical density (OD) of positive control of respective ELISA kits with factor 0.2. The samples with OD less than cut off value were designated as negative and more than cut off value were designated as positive.

Estimation of NO, NSE and nNOS

Nitric oxide concentration in plasma and CSF was estimated by using Griess reagent as per Sastry *et al*. (2002). Whereas NSE and nNOS in plasma and CSF were estimated by using nNOS ELISA kit (Qayee-Bio Ltd., China) and USCN ELISA kit for NSE as per the recommendation of the manufacturer. The plate was read at 450 nm using automatic ELISA plate reader. Concentration of samples was calculated from standard curve by plotting the absorbance in semi logarithmic graph paper.

Estimation of total protein, albumin, globulin, albumin globulin ratio and albumin quotient (AQ)

Total protein, albumin and globulin in CSF and plasma were estimated by modified Biuret and Duma's method (Varley *et al*., 1980) using commercial kits procured from Span Diagnostics India. A/G ratio in plasma and AQ was calculated as per Thomas (1998).

Results

The screening of clinical cases of CD using canine specific immunoglobulin IgG and IgM resulted in 94 positive cases. Among 94 cases positive for both antibodies, 53 cases have shown low-medium titer (titer between 1/20 to 1/80) and the remaining 41 cases showed high titer (titer >1/160) for IgM. All healthy control dogs of present study were found negative for IgM antibodies and have medium titer of IgG (1/80 to 1/160).

The concentration of NO was significantly (P<0.05) higher in both CSF and plasma in dogs suffering from canine distemper virus (CDV) infection when compared with healthy control group (Tables 1 and 2). Whereas the NO concentration was found significantly higher in plasma than that of CSF in the CD cases as well healthy control dogs. Further, the study has also showed the significant increase in the activity of nNOS and NSE both in CSF and plasma of dogs suffering from CD as compared to healthy control dogs.

Table 1: Plasma biochemical parameters of healthy and CD affected dogs

Parameters	Healthy	CD affected group
Total protein (g/dl)	6.89±0.43[a]	8.34±1.84[b]
Albumin g/dl	3.85±0.22[a]	3.20±0.82[b]
Globulin g/dl	3.04±0.29[a]	5.14±0.89[a]
A:G ratio	1.27±0.25[a]	0.62±0.47[b]
NSE (ng/ml)	1.26±0.56[a]	5.08±1.04[a]
nNOS (ng/ml)	157.03±17.29[a]	321.31±102.30[a]
Nitric oxide (µ mol NO/ml)	16.54±2.17[a]	32.68±4.31[a]
AQ	0.50±0.58[a]	4.57±0.55[a]

Mean having same superscript in the same row differ significantly (P≤0.05). NSE: Neuron specific enolase, nNOS: Neuronal nitric oxide synthase, and AQ: Albumin quotient

Table 2: CSF biochemical parameters of healthy and CD affected dogs

Parameters	Healthy	CD affected group
Total protein (mg/dl)	25.32±0.42[a]	191.32±5.57[a]
Albumin mg/dl	19.26±1.28[a]	146.26±4.47[a]
Globulin mg/dl	6.06±0.38[a]	45.06±2.53[a]
NSE (ng/ml)	2.1 4±0.74[a]	14.34±2.48[a]
nNOS (ng/ml)	217.99±47.37[a]	415.84±46.24[a]
Nitric oxide (µ mol NO/ml)	9.46±2.32[a]	18.08±2.76[a]

Mean having the same superscript in the same row differ significantly (P≤0.05). NSE: Neuron specific enolase, and nNOS: Neuronal nitric oxide synthase

The alterations in biochemical parameters of dogs with CD are presented in Tables 1 and 2. Significant (P<0.05) increase in values of total protein, albumin, globulin, AQ was observed in CSF of dogs suffering from CD when compared with healthy control. Whereas the total protein (TP) and albumin did not vary significantly in plasma. Contrarily, the globulin concentration showed significant differences (P<0.05) as compared with healthy control. The non significant decrease in the A:G ratio was recorded in dogs suffering from CD than that of healthy control group.

Discussion

The presence of antibody to CDV in the CSF is considered as one of the most reliable indicators of nervous infection (Shell, 1990). Conventionally, serological diagnosis of distemper based on the detection of IgG antibody titre in paired serum samples or the detection of IgM in a single serum specimen has been practiced (Latha *et al*., 2007). Therefore, in the present study to rule out false positive IgG and IgM antibodies were estimated in both plasma and CSF. Present study revealed low to medium titer of IgM in CD affected dogs. One of the possible reasons for low to medium titer of IgM could be due to the immunosuppressive nature of the disease as well as the delayed presentation of clinical cases. Further, IgM antibodies persist in dogs with CD for five weeks to three months depending upon the strain of the virus (Greene and Appel, 2006) and may have appreciably lower titer thereafter.

Significant increase in the nitrates and nitrites level in blood of dogs infected with CDV has been documented (Karadeniz *et al*., 2008). In addition to many physiologic actions, free radical activity of NO can cause cellular damage through a phenomenon known as nitrosative stress (Shukla, 2007). There is substantial evidence that NO participates in neuronal damage by promoting the formation of peroxinitrite rather than acting directly. Peroxinitrite cause lipid peroxidation, alter membrane fluidity and permeability leading to improper neuronal function (Pacher *et al*., 2007).

Significantly higher concentration of NO in plasma than that of CSF in CD cases as well as in healthy control dogs could be attributed to the fact that in brain NO is synthesized only by nNOS whereas in other organs it may be formed by various isomers of NOS (Nelson *et al*., 2003).

The NSE level in CSF and plasma of present study was found significantly higher in dogs suffering from CDV than the healthy dog. An increase in activity of NSE in plasma has also been documented in diffused neuronal damage (Berger *et al*., 2002). Similarly, in our study the significant increase in the activity of nNOS and NSE both in CSF and plasma of dogs suffering from CDV when compared with healthy dogs suggests that there is a possible role of NO in neuronal damage. The involvement of NO in neurological damage has already been demonstrated in human patients suffering from Alzheimer and Parkinson diseases (Pacher *et al*., 2007).

The CSF has an extremely low protein concentration relative to serum and almost all of the proteins normally present in CSF are derived from plasma (Reiber, 2003).

The protein content of CSF have been found significantly elevated in human patents in compared to healthy which is suggestive of positive correlation of CSF protein level and nervous disorders (Pazzaglia *et al*., 1995). Our findings are in agreement with the earlier report (Thomas, 1998). The amount of albumin in the CSF may vary with the serum albumin, and the calculation of a ratio between CSF and plasma albumin, called an AQ, has been suggested as a more meaningful measure than CSF albumin alone. Alternations in AQ can be useful to interpret accurately the changes in CSF (Sorjonen, 1987; Terlizzi and Platt, 2009). As albumin is solely derived from plasma, increased level of CSF albumin is considered an indicator of blood-brain barrier (BBB) damage (Reiber, 2003). Similarly, in this study, the significant higher value of AQ was recorded in the dogs suffering from neurological form of CD as compared to healthy control group.

The author concluded that NO play a definite role in pathophysiology of nervous form of CD and NO, nNOS, NSE and AQ estimation in either of the biological samples (CSF and plasma) of dogs suffering from neurological form of distemper could be used as biomarker for screening of clinical cases. To the best of the authors' knowledge, this is the first documentation of its kind to investigate the role of nitrosative stress in pathophysiology of neurological form of distemper in India. The author believes this study could prove beneficial to ng further studies on the role of NO, nNOS and NSE in pathogenesis of distemper and role of NOS inhibitors in management of distemper.

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Conflict of interest

The authors disclosed that there are no conflicts of interest and all the authors have read and approved the manuscript before its submission.

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