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Nitric oxide in cerebrospinal fluid of central nervous system tuberculosis: correlations with culture, antibody response, and cell count

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Background/aim: The role of nitric oxide (NO) has been established in infection over the years. NO functions by inhibiting the growth of intracellular pathogens. The present study was undertaken to ascertain the role of NO in central nervous system (CNS) infection by *Mycobacterium tuberculosis*.

Materials and methods: A total of 781 chronic meningitis cerebrospinal fluid (CSF) samples suspected of CNS tuberculosis (TB) were categorized based on *M. tuberculosis* culture positivity, anti-TB antibody response, and CSF cell count and were analyzed for NO.

Results: We found that NO levels were positive in 10.88% of the CSF samples. Positivity for NO was 18%, 11.67%, 13.68%, 9.32%, and 9.66% in the cases with mycobacterial culture positivity, anti-TB antibody positivity, high cell count, low cell count, and zero cell count, respectively. Among the above cell count categories, NO levels were noticed to be elevated in high cell count samples with mononuclear cell predominance.

Conclusion: This study suggests that NO might play some role in the later stages of tuberculous meningitis. This is the first study to our knowledge in which NO was evaluated in CSF in relation to immune response and the presence of a pathogen with such a large number of subjects.

Key words: ELISA, mycobacteria, nitrite, tuberculous meningitis

1. Introduction

Pathogenesis of infections involves the host's innate defense against infection by host immune cells. Variations in immune response against various invading pathogens are seen in experimental animals and also in in vitro studies (1). In central nervous system (CNS) infections, brain cells such as astrocytes, microglial cells, and endothelial cells help in removal of pathogens by producing inflammatory cytokines and different gaseous molecules such as nitric oxide (NO) (2). NO is found to have diverse functions in the pathophysiology of diseases and is involved in many microbial infections. NO functions by inhibiting the growth of intracellular pathogens (3). It is produced as a gaseous molecule by the enzyme nitric oxide synthase (NOS) from L-arginine. There are three isoforms of NOS; one is of endothelial origin (eNOS) expressed constitutively in endothelium, one is of neuronal origin (nNOS) expressed in neurons, and the third is the inducible form (iNOS) expressed in macrophages and microglial cells (macrophages of the brain) (4). iNOS expression is induced by cytokines released in response to infections from macrophages (4). NO produced by iNOS has antibacterial activity, which causes breaks in the nucleic acid along with other physiological activities such as vasodilation and wound repair (2).

Knockout studies of murine models for the iNOS gene have shown its association with different infections such as tuberculosis, malaria, and leishmaniasis (5). NO acts indirectly through its reactive nitrogen intermediates formed after oxidation, such as peroxynitrite and nitrosothiols, which are responsible for damage to the CNS during meningitis (5). NO is very unstable and it gets converted to the NO end products, nitrite and nitrate, a few seconds after its release. Concentrations of NO end products are seen to be increased in meningitis (5).

The role of NO in tuberculous meningitis (TBM) is still not clearly understood. Macrophages play a major role in the host response against *Mycobacterium tuberculosis* (Mtb), the causative agent of TBM. Cytokines produced by macrophages and T-lymphocytes in response to Mtb infection activate macrophages to induce iNOS for the production of NO, which in turn acts along with other

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reactive nitrogen intermediates to inhibit/kill Mtb in murine models (2). However, in humans, the evidence is less clear; although iNOS expression is seen in astrocytes it is difficult to demonstrate in microglia (6).

The present study was undertaken to ascertain the role of NO in the cerebrospinal fluid (CSF) of TBM patients. The diagnosis of TBM is generally based on clinical features such as fever, headache, and altered sensorium and CSF findings such as pleocytosis, high protein, and low CSF/blood glucose and presence of basal exudates seen in imaging. Microbiological diagnosis is made by observing acid-fast bacilli (AFB) in the CSF by Ziehl–Neelsen staining, by culturing of CSF on suitable media such as Lowenstein–Jensen (LJ) or Middlebrook media, or immunologically by detection of antigens or antibodies specific for Mtb in the CSF or by using nucleic acid amplification methods (7). All the detection methods for Mtb have their own limitations with different sensitivity and specificity (8). The aim of the present study was to correlate the presence of NO in the disease process of CNS tuberculosis to a detailed extent using 781 CSF samples of various categories. The parameters considered for the present study in relation to NO were antibody response for Mtb, CSF culture positivity for AFB, and CSF cell count and cell type.

2. Materials and methods

2.1. Samples

Patients' CSF samples received in the Department of Neuromicrobiology, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India, were used for the study. The study group included prospectively collected chronic meningitis CSF samples of 781 consecutive patients of all age groups from June 2012 to June 2013. Ethical approval for the present study was obtained from the institutional ethics committee. Informed consent was obtained for lumbar puncture CSF samples from human subjects. Clinical diagnosis was made as per the disease guidelines. Patients were suspected of TBM based on clinical presentations such as fever and headache for more than 14 days, vomiting, neck stiffness, altered sensorium, and focal neurological deficits, along with preliminary laboratory evidence like decreased glucose and increased protein levels in the CSF samples. Closely mimicking diseases like neurocysticercosis and cryptococcal meningitis were ruled out. All the samples were processed for cell count, cell type, isolation of Mtb, presence of anti-Mtb antibody, and NO assay.

2.2. Cell count and cell type

The CSF was analyzed for cell count by using a Neubauer hemocytometer chamber using bright-field microscopy with a 40× objective as a routine diagnostic procedure in the lab. Samples with a total cell count of >20 cells/mm^3 were considered as having a high cell count and ≤20 cells/mm^3 as low cell count, and samples were also categorized as having predominantly polymorphonuclear or mononuclear cells or nil cells.

2.3. Isolation of Mtb by culture

CSF was cultured for Mtb by the conventional method on LJ medium and/or the automated mycobacterial growth indicator tube (MGIT) method. According to the standard protocol for the conventional method, 200–500 µL of CSF was inoculated aseptically on LJ medium, incubated at 37 °C, and observed weekly for 8 weeks. CSF was also cultured in the BACTEC MGIT 960 system for rapid growth of the bacteria as per the manufacturer's instructions (9). In all positive culture tubes, to confirm the presence or absence of mycobacteria, Ziehl–Neelsen staining was done.

2.4. Indirect ELISA for anti-Mtb antibody

The methodology used for ELISA was described earlier (10). Briefly, microtiter plates (TPP, Trassadingen, Switzerland) were coated with 50 µL (10 µg/mL) of mycobacterial (H37Rv) total sonicated antigen dissolved in phosphate-buffered saline by overnight incubation at 4 °C in a moist chamber. Unbound antigen was removed by washing with PBS-Tween 20 (PBST, 0.05%). Nonspecific free binding sites were blocked with freshly prepared PBST-milk (1%, w/v) at 37 °C for 2 h. The CSF samples to be tested were diluted (1:5) in PBST milk (1%, w/v) and the diluted samples were added to the plate in duplicates of 50 µL along with positive and negative controls. The plate was incubated for 90 min in a moist chamber at 37 °C and then washed 5 times with PBST. Secondary antibody (IgG) conjugated with horse radish peroxidase (1:3000 dilutions in PBST-milk; Dakopatts, Denmark) was added to each well (50 µL) and incubated in a moist chamber at 37 °C for 60 min. Substrate (O-phenylene di-amine; Sigma, St. Louis, MO, USA) dissolved in phosphate citrate buffer (0.1 M, pH 5) containing 0.01% hydrogen peroxide was added to each well (75 µL) and color development was allowed for 10–15 min. The reaction was arrested by adding 50 µL of 2 N sulfuric acid. The readings were taken by ELISA reader (Sunrise-Magellan, Tecan AG, Austria) at 492 nm. The positive cut-off value considered was ≥0.100 OD, calculated on the basis of the absorbance value (OD) of healthy controls by taking the mean of it plus three standard deviations (SDs).

2.5. Nitric oxide assay

NO is detected using a colorimetric assay based on a chemical diazotization reaction originally described by Griess in 1879 (11). NO was analyzed by the reaction of its end oxidation product (nitrite) for the formation of a purplish pink color with the Griess reagent. In this reagent, sulfanilic acid forms a diazonium salt with nitrite in the CSF sample, which then reacts with the azo dye

agent (N-alpha-naphthyl-ethylenediamine) to form a chromophore. This is detected colorimetrically at 540 nm (12).

The standard reference curve was prepared with each set of samples analyzed with 1:1 serial dilutions of sodium nitrite (10 mM) in distilled water. The first 2 columns in the 96-well plate were for the standards (50 µL/well). CSF samples (50 µL) were added to the remaining wells. Solution A containing 1% sulfanilamide in 5% phosphoric acid was mixed with an equal volume of Solution B containing 0.1% N-1-naphthylethylenediamine dihydrochloride in water prior to use. The prepared reagent (50 µL) was added to the plate containing the CSF sample. The final volume in each well was 100 µL. The reaction was allowed to proceed for 10 min, which forms a purplish pink color. Absorbance was measured at 540 nm in the ELISA plate reader (Sunrise-Magellan, Tecan AG).

2.6. Statistical analysis

Statistical analysis was done using GraphPad online free statistical software. Pearson's chi-square test was used to compare discrete variables (NO, anti-Mtb antibody, and Mtb culture report). Relations between mononuclear cells, polymorphocytes, and NO were analyzed by chi-square test. Categorical data were expressed as means with 95% confidence intervals. $P \leq 0.05$ was considered statistically significant.

3. Results

Among 781 CSF samples, 484 were from males with an age of 36.10 ± 16.66 years (mean ± SD) and 297 were from females of 35.06 ± 17.57 years of age. The NO standard curve was plotted with nitrite concentration on the x-axis and absorbance on the y-axis for the known concentrations of sodium nitrite. The concentration of NO in the CSF samples was determined using the linear regression curve. The correlation coefficient (R^2) of the values with that of the trend line obtained for the standard curve was 0.9974. The samples containing NO above 9.7 µM were considered positive for the assay based on the test samples and ruling out background values.

Out of 781 samples, 50 Mtb cultures were isolated by conventional or automated method. Out of 50 samples analyzed, 31 (62%) were positive for the anti-Mtb antibody and 9 (18%) were positive for NO. Among those, 5 (10%) samples were positive for both the anti-Mtb antibody and NO (Table 1). Among the culture-positive samples, 6 had polymorphs as the predominant cell type but none of them were positive for NO. Among 731 culture-negative samples, 167 (22.84%) samples were positive for anti-Mtb antibody and 76 (10.39%) were positive for NO. Among those, 18 (2.46%) samples were positive for both anti-Mtb antibody and NO.

The mean NO level obtained for all culture-positive samples (n = 50) was 1.5 µM, and the values ranged from 0 to 19 µM. Among the culture-positive samples, the mean value of NO in anti-Mtb antibody- and NO-positive samples was 11.2 µM. For anti-Mtb antibody-negative but nitrite-positive samples it was 9.8 µM. For anti-Mtb antibody- and nitrite-negative samples the value was around 2.9 µM. For anti-Mtb antibody-positive but nitrite-negative samples it was 0.34 µM. However, statistically, there was no significant correlation (P = 0.095) found between culture positivity and NO by the Pearson chi-square test.

The CSF samples were also grouped based on their cell count as having low and high cell counts as well as having mononuclear cells predominant or polymorphonuclear cells predominant, along with nil cells also considered as one of the categories. In 781 CSF samples analyzed,

Table 1. NO assay results among different categories of suspected tuberculous meningitis cases. The samples with NO above 9.7 µM were considered positive for the assay. Values in parentheses show percentages in comparison with the culture report.

Mtb culture	Cell count	Predominant cell type	Anti-Mtb antibody positive	NO assay positive
Positive n = 50	High n = 45	Mononuclear cells, n = 40	25 (50)	5 (10)
		Polymorphonuclear cells, n = 5	3 (6)	0
	Low n = 5	Mononuclear cells, n = 4	3 (6)	0
		Polymorphonuclear cells, n = 1	0	0
Negative n = 731	High n = 218	Mononuclear cells, n = 183	80 (10.94)	11 (1.5)
		Polymorphonuclear cells, n = 35	22 (3)	2 (0.27)
	Low n = 306	Mononuclear cells, n = 286	49 (6.7)	5 (0.68)
		Polymorphonuclear cells, n = 20	3 (0.41)	0
	Nil n = 207		13 (1.77)	0

311 samples had low cell counts, 263 samples had high cell counts, and 207 samples had nil cells (Table 1). NO positivity was higher among the samples with high cell count, followed by low cell count and then nil cell samples. However, statistically, no significant correlation (P = 0.355) was found between cell count and NO. Statistical values of P = 0.106 and P = 0.092 were obtained between mononuclear and polymorphonuclear predominant cells and NO, respectively, which were not statistically significant by Pearson chi-square test.

The graphical representation in the Figure was calculated by applying a 3-way Venn diagram for the CSF samples positive for any of the three tests among the 781 samples. The nonoverlapping region represents CSF samples positive for one of the tests. The areas that intersect represent the overlapping of two or three tests. Among them, 58 samples were positive only for nitric oxide, 148 samples were positive only for anti-Mtb antibody, and 15 samples were positive only for Mtb culture, while 18 samples were positive for both NO and anti-Mtb antibody (Figure). The remaining 506 samples were negative for all three of the tests.

Among the 781 CSF samples suspected of TBM, 198 (25.35%) samples were positive for the presence of anti-Mtb antibodies (Table 2). Among these samples, 23 (11.62%) were positive by NO assay. Out of 583 (74.65%) Mtb antibody-negative samples, 62 (10.63%) were positive for NO. However, no significant correlation was found (P = 0.702) between anti-Mtb antibody and NO assay by Pearson chi-square test. Overall, 85 (10.88%) samples showed positivity for the NO assay.

4. Discussion

Study of host response to pathogens like mycobacteria is essential due to high mortality of up to 50% and varying associated neurological sequelae among TBM survivors, especially in high-burden countries like India (13). The organism plays a vital role in modulating the host immune response. NO is found to play a role in TBM in mouse models, but there are few studies with human subjects. The role of NO has been established in disease processes over the years (14). It is shown that NO plays a role similar to cytokines in the disease process.

In the CSF, NO in the presence of oxygen gets converted to nitrite and then to nitrate, but the CSF nitrate levels may also be influenced by the damaged blood–brain barrier wherein the nitrate in the circulation can diffuse into the CSF (15). In the case of TBM, alteration in the blood–brain barrier permeability is known (16). CSF nitrate levels during bacterial meningitis are more varied than nitrite levels (17). Hence, CSF nitrite levels will be more specific and indicative of central nervous system NO production (18). Hence, in the present study we have considered nitrite measurements.

NO acts as an internal mediator in the CNS and in the case of excessive production it may be neurotoxic (19). The regulation of iNOS expression for the production of NO is a complex process, may depend on cell type and differentiation stage, and could be stimulus-dependent, making the extrapolation of animal studies to clinical settings difficult (5). It is also reported that NO levels in the CSF may vary depending on the levels of stimulatory and inhibitory cytokines. It is also reported that avirulent strains produce more NO in comparison to virulent strains (4). Furthermore, murine studies have shown that iNOS$^{-/-}$ specimens are good models of human TBM (20).

The metabolic pathways and the half-life of nitrite in the CSF are still unknown (21). In a study on TBM, increased levels of NO along with arginine were found to be of

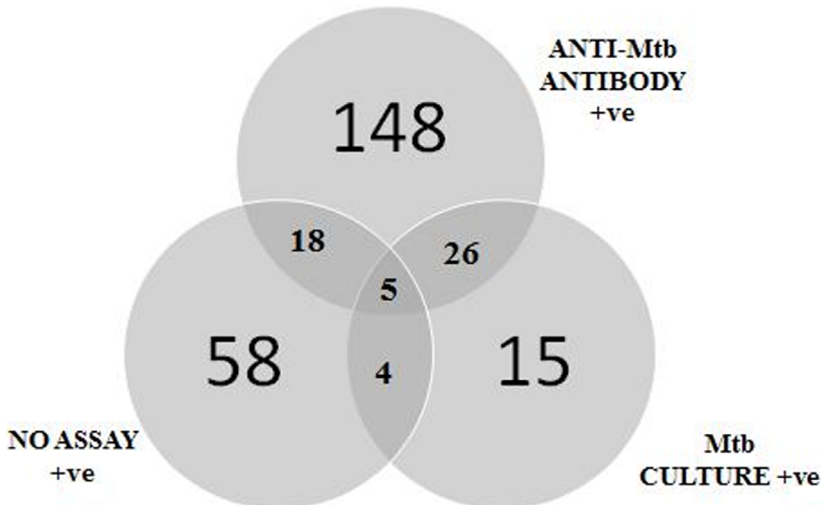

Figure. Venn diagram representing the number of CSF samples positive for anti-Mtb antibody, NO assay, and Mtb culture.

Table 2. Comparison of anti-Mtb antibody and NO among suspected tuberculous meningitis cases. Values in parentheses show percentages.

		NO assay		
		Positive	Negative	Total
Anti-Mtb antibody ELISA	Positive	23	175	198 (25.35)
	Negative	62	521	583 (74.65)
	Total	85 (10.88)	696 (89.11)	781

significance in neuronal death (22). According to previous studies, NO serves as a nonspecific immune modulator and it has an influence on prognosis. It is seen in other studies that the lack or reduction of NO production might play a role in latent infection (23). It was also observed that Mtb resistant to NO causes severe disease outcome (24).

The presence of AFB in the CSF, which is regarded as the gold standard for diagnosis of TBM, has revealed the presence of NO in about 18% of the cases. Even though Mtb is known to generate nitrite, the NO production by Mtb may be negligible as the bacterial load itself is very low in the CSF. In the present study, about 82% of the culture-positive samples were negative for the presence of NO. This could probably be regarded as a stage in which the immune response is not yet properly geared or it is damaged. It is interesting to note that mycobacterial culture positivity is also seen among a few CSF samples with low cell counts, but none of them were NO-positive.

Due to low culture positivity in the CSF of TBM cases, immunodiagnosis is regarded as an important marker for the disease diagnosis. The present study has revealed less difference between NO levels of both anti-Mtb antibody-positive and -negative cases. However, the anti-Mtb antibody in CSF samples did not show a significant correlation with NO. This is the first study to our knowledge in which antibody response against mycobacteria is correlated with NO.

It was reported that measuring NO in the CSF would not reliably distinguish bacterial meningitis from other causes of fever. It was also reported that there was no relationship between CSF NO concentration and CSF leukocyte count (25). Our study is in agreement with this finding. CSF cells, which are regarded as disease/immune markers in any CNS infection, presented a poor picture for NO with 90% of the 781 samples analyzed showing undetectable levels of NO. It is surprising to note that high cell count has no direct relation with the presence of NO. However, within the group marginally increased levels of NO were seen in high cell count CSF samples with mononuclear cell predominance and were least among CSF samples with high cell counts and polymorphonuclear cells predominant. This suggests that NO might play some role in the very later stages of TBM, where the disease sets in with high mononuclear cell response. However, in the initial stage of the disease, where an increased level of polymorphic cell response is seen, the role of NO is unclear.

In this study we focused on NO levels and its presence in the CSF of TBM cases. This is the first study to our knowledge in which NO was evaluated in the CSF in relation to antipathogenic antibody response, CSF cell count and cell type, and the presence of pathogen by culture with such a large number of subjects. It can be concluded from the present study that there is a marginal increase in NO among high CSF cell counts with mononuclear cells, though not statistically significant. Thus, it appears that NO does not directly influence the disease process. However, it appears that NO in association with cytokines might play an immunopathological role in the disease process.

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