

Doxycycline in autoimmune central nervous system disorders in children: an *in vitro* study

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SUMMARY: Anlar B, Şenbil N, Güven A. Doxycycline in autoimmune central nervous system disorders in children: an *in vitro* study. Turk J Pediatr 2007; 49: 274-277.

Tetracyclines have antiinflammatory properties. To test the *in vitro* effect of doxycycline in autoimmune neurological disorders of childhood, peripheral blood lymphocytes from multiple sclerosis (n=11), acute disseminated encephalomyelitis (n=12), and control patients (epilepsy and headache, n=12), all aged 5-17, were examined for proliferation, migration, and apoptosis after culture with doxycycline, concanavalin A and myelin basic protein for 48 hours. Doxycycline increased proliferation in the control group, and less in the multiple sclerosis group but not in the acute disseminated encephalomyelitis group (p<0.03). It increased apoptosis in multiple sclerosis patients (p<0.02). According to this preliminary study, doxycycline might have immunomodulatory effects in children, justifying future studies with larger and more homogeneous patient groups.

Key words: tetracyclines, doxycycline, lymphocyte, multiple sclerosis, acute disseminated encephalomyelitis, autoimmune.

Treatment options are limited in autoimmune neurological disorders of childhood, as data on disease-modifying drugs commonly used in adults are insufficient in children. Tetracyclines have anti-inflammatory and immunomodulatory activities independent of their antibiotic properties¹. Various actions on monocytes and T lymphocytes have been reported, as well as beneficial effects in certain experimental and clinical conditions such as pemphigus, rheumatoid arthritis, and acute lung injury². Minocycline is the most well studied member of the tetracycline family; however, it may cause autoimmune disorders³. Doxycycline, a semi-synthetic structural isomer, has well-known absorption and excretion patterns and wide clinical use in chlamydial, mycoplasmal, or bacterial infections. We investigated the effect of doxycycline by *in vitro* experiments as a potential drug in childhood autoimmune neurological disorders.

Material and Methods

Three groups of patients aged 5-17 years: relapsing-remitting multiple sclerosis (MS, n=11), acute disseminated encephalomyelitis in remission (ADEM, n=12), and neurological

controls (epilepsy and headache, n=12) were studied. The diagnoses were based on previously established criteria^{4,5}. Their characteristics are summarized in Table I. Patients were similar in age and gender distribution. None of the epilepsy cases was on phenytoin.

Peripheral blood lymphocytes were isolated from 10 ml heparinized blood on Ficoll-Hypaque gradient, resuspended at 10⁶ cells/ml, and cultured in RPMI with 5% fetal calf serum (RPMI-FCS) with or without concanavalin A (Con A, 2 µg/ml), myelin basic protein (MBP, 20 µg/ml), or doxycycline hydrochloride (2.5 µg/ml) for 48 hours at 37°C and 5% CO₂. These concentrations of Con A and MBP were determined in preliminary experiments to induce submaximal proliferation in order not to mask the effects of the other compounds. The concentration of doxycycline was chosen according to the serum level obtained by clinically used doses⁶. At the end of 48 hours, cells were washed, resuspended in RPMI-FCS and used in duplicates in the assays described below.

Proliferative response was assessed by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay⁷. Briefly, cells were incubated with 1 mg/ml MTT in 96-

Table I. Descriptive Features of Patient Groups

Diagnosis (n)	Median age (range)	M/F	Treatment (n)	Latest attack (n)
MS (10)	16 (9–17)	5/5	Beta interferon (4) Prednisone 5 mg on alternate days (2) No treatment (4)	1–6 months ago (5) 6 months–5 years ago (5)
ADEM (12)	11 (5–15)	5/7	No treatment	1–6 months ago (5) 6 months–5 years ago (7)
Control (12)	12 (9–17)	7/5	Valproate (3), carbamazepine (3), ethosuximide (1), none (5)	–

M/F: Male/female. MS: Multiple sclerosis. ADEM: Acute disseminated encephalomyelitis.

well plates (37°C, 5% CO₂, 4 hours), the dye solubilized with 50 µg dimethylsulfoxide per well, and optic density was measured at 560 nm by an automated reader. Proliferation indexes were calculated as: average count_{experimental}: average count_{medium}. The change in proliferation index (% change) induced by the addition of doxycycline to each experimental condition was calculated, and changes over 10% were interpreted as significant.

Migration of lymphocytes in gelatin-coated polycarbonate filters was assessed as described before⁸. Briefly, filters (Sartorius, Gottingen, Germany, 5 µm-pore size) were pre-coated overnight in gelatin and placed in Boyden chambers. The upper chamber was filled with 1 ml of cell suspension and the bottom chamber, with RPMI + 10% FCS. Chambers were incubated in 5%CO₂ at 37°C for 4 hours. The filter papers were collected, stained with hematoxylin, and the distance covered by cells was measured under light microscope. The average of five high power fields (400x) was taken for each filter.

Apoptosis was assessed by morphology and DNA laddering on agarose gels. For morphology, lymphocyte suspensions were mixed 1:1 with acridine orange (100 µg/ml) in phosphate buffered saline (PBS), and one drop of the dyed suspension was placed on a microscope slide and examined under fluorescent microscope (Nikon 800). The average percentage of apoptotic cells in a count of minimum 200 cells was calculated. DNA laddering was examined by electrophoresis: peripheral blood leukocytes were treated with lysis buffer (50 mmol/L Tris, 20 mmol/L EDTA, 1% NP-40, pH 7.5), centrifuged at 1600 g for 5 minutes, and the supernatant was treated with proteinase K. After ethanol precipitation, 5 µg of DNA

was separated on a 1.5% TBE/agarose gel, stained with ethidium bromide, visualized on UV transilluminator and photographed. The presence of laddering was evaluated by a blinded examiner. The results of the two methods were in agreement in 80% of the experiments. Samples where discrepancies persisted in repeated experiments were excluded from the analysis. All chemicals were obtained from SIGMA, St. Louis, MO.

Differences between group mean values were analyzed with the t test and differences between the frequency of particular responses with the chi square test using Microsoft Excel 2000 and SPSS 10.0 for Windows.

Results

Doxycycline compared to medium alone increased proliferation by at least 10% in certain control (3/12 patients) and MS (2/10) patients, but not in ADEM (0/12); the difference between ADEM and control groups was significant ($p < 0.03$, chi square test). The combination of doxycycline and Con A increased proliferation in more MS patients (4/11), while doxycycline+MBP had no effect (increased proliferation in 0/10 and 2/12, respectively) (ADEM and MS vs. control, $p < 0.000$).

Migration of lymphocytes increased with the addition of doxycycline to MBP in all patient groups (Table II). The small sizes of the groups precluded statistical analysis. Apoptosis tended to increase with the addition of doxycycline in all groups, more often in MS patients (7/10 patients, $p < 0.02$, chi square test).

When patients under no immunomodulatory treatment were evaluated separately, similar trends persisted although statistical analysis was not possible (data not shown).

Table II. Distance Covered by Mononuclear Cells in Gelatin-Coated Membranes and Percentage of Apoptotic Cells Under Various Culture Conditions

Distance (μm , mean \pm SEM)	Con A		MBP	MBP + Doxycycline	
MS	12.2 \pm 2.6		14.8 \pm 4.4	17.3 \pm 6.6	
ADEM	6.8 \pm 1.8		6.7 \pm 2.1	11.4 \pm 2.3	
Control	11.5 \pm 3.1		9.5 \pm 3.3	15.7 \pm 4.1	

Apoptotic cells (% of total) (average \pm SEM)	Con A	Doxycycline	Con A + Doxycycline	MBP	MBP + Doxycycline
MS	18.2 \pm 5.4	30.4 \pm 9.6	28.9 \pm 7.8	22.9 \pm 10.6	28.4 \pm 9.3
ADEM	19.6 \pm 7.6	29.3 \pm 9.1	24.3 \pm 8.2	32.5 \pm 11.1	36.3 \pm 9.7
Control	21.2 \pm 6.4	32.1 \pm 11.9	24.2 \pm 7.4	26.0 \pm 9.0	47.1 \pm 9.0

MS: Multiple sclerosis. ADEM: Acute disseminated encephalomyelitis. SEM: Standard error of the mean.
Con A: Concanavalin A. MBP: Myelin basic protein.

Discussion

Despite certain limitations, this study provides some preliminary data on the *in vitro* effects of doxycycline. The rarity of childhood MS and ADEM and ethical considerations in obtaining healthy control cases precluded the investigation of larger treated and untreated groups, and the amount of blood available from children limited the number of our laboratory tests. Different *in vitro* concentrations of doxycycline could not be tried, and lymphocyte migration, tested across gelatin membranes to mimic *in vivo* function, could not be combined with matrix metalloproteinase activity by zymography or enzyme linked immunosorbent assay (ELISA). We arbitrarily took 10% change as difference, but this may be discussed. Beta interferon or steroid therapy in MS cases might have influenced lymphocyte functions; we addressed this issue to some extent by comparison of treated and untreated groups, but larger numbers are needed. The effect of treatment in epileptic cases is probably negligible because anticonvulsant drugs other than phenytoin do not significantly affect lymphocyte functions⁹.

Tetracyclines can affect pathways targeted in the treatment of autoimmune disorders and MS: lymphocyte proliferation, migration, apoptosis, or cytokine production. Among tetracycline analogues, doxycycline has the most potent inhibitor effect on lymphocyte proliferation at therapeutic concentrations. In contrast, minocycline shows a slight increase in antigen-specific proliferation of T cells

and synthesis of Th2 cytokines, and slightly reduced synthesis of inflammatory cytokines¹⁰. Doxycycline is a nonspecific inhibitor of matrix metalloproteinases implicated in MS, infections, and cancer metastasis. This drug has also been shown to eliminate activated T lymphocytes through apoptosis. However, the effects of tetracyclines are pleiotropic, depending on the timing of the application, the nature of the stimulus, and the activation status of lymphocytes. For instance, phytohemagglutinin-activated Jurkat cells are more susceptible to doxycycline-induced apoptosis¹¹, and lymphocyte tumor necrosis factor (TNF) secretion can diminish or remain unaffected, depending on the type of stimulus¹². Our results with doxycycline in MS patients indicated a moderate increase in proliferation and migration. This might be due to an already activated immune status in MS, while our ADEM patients in remission had generally lower responses to doxycycline. Increased apoptosis might be beneficial since remissions in MS and ADEM are associated with apoptotic elimination of autoreactive lymphocytes. In order to estimate the net clinical effect of doxycycline in these disorders, proliferation, migration and apoptosis should be assessed separately in helper-effector or regulator lymphocyte subsets, and the production of Th1 and Th2 type cytokines should be measured.

This is a preliminary study of a commonly available drug with a well-known safety profile. Future, preferably multicentric, studies, in order to include larger and homogeneous patient groups, and more

detailed immunological assays appear worthwhile given the safety and the neuroprotective potential of this family of drugs.

Acknowledgement

We are indebted to Emin Kansu, MD, and Lale Dogan, MSc, for their establishment of the MTT assay, and to Şerife Dinler and Semra Ülger for their technical assistance.

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