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Effect of dimethylsulfoxide on sphingomyelinase activity and cholesterol metabolism in Niemann-Pick type C fibroblasts

F.B. Scalco[2], R. Giugliani[1], P. Tobo[2] and J.C. Coelho[2]

Departamentos de [1]Genética e [2]Bioquímica, Universidade Federal do Rio Grande do Sul, e Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brasil

Correspondence
J.C. Coelho
Serviço de Genética Médica
Hospital de Clínicas de
Porto Alegre
Rua Ramiro Barcelos, 2350
90035-003 Porto Alegre, RS
Brasil
Fax: +55-51-316-8010

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Abstract

Niemann-Pick type C (NPC) fibroblasts present a large concentration of cholesterol in their cytoplasm due to a still unidentified deficiency in cholesterol metabolism. The influence of dimethylsulfoxide (DMSO) on the amount of intracellular cholesterol was measured in 8 cultures of normal fibroblasts and in 7 fibroblast cultures from NPC patients. DMSO was added to the fibroblast cultures at three different concentrations (1, 2 and 4%, v/v) and the cultures were incubated for 24 h. Sphingomyelinase activity was significantly increased in both groups of cells only when incubated with 2% DMSO (59.4 ± 9.1 and 77.0 ± 9.1 nmol h^{-1} mg $protein^{-1}$, controls without and with 2% DMSO, respectively; 47.7 ± 5.2 and 55.8 ± 4.1 nmol h^{-1} mg $protein^{-1}$, NPC without and with 2% DMSO, respectively). However, none of the DMSO concentrations used altered the amount of cholesterol in the cytoplasm of NPC cells (0.704 ± 0.049, 0.659 ± 0.041, 0.688 ± 0.063 and 0.733 ± 0.088 mg/mg protein, without DMSO, 1% DMSO, 2% DMSO and 4% DMSO, respectively). This finding suggests that sphingomyelinase deficiency is a secondary defect in NPC and shows that DMSO failed to remove the stored cholesterol. These data do not support the use of DMSO in the treatment of NPC patients.

Key words
- Cholesterol metabolism
- Sphingomyelinase
- Niemann-Pick disease
- Lysosomal storage disorders

Introduction

The Niemann-Pick group of diseases is characterized by a variable accumulation of lipids such as sphingomyelin and cholesterol. Niemann-Pick disease type C (NPC), an autosomal recessive disorder, is associated with a partial deficiency of sphingomyelinase activity and accumulation of unesterified cholesterol in the lysosomes of several tissues (1). Common clinical manifestations are neonatal jaundice, hepatomegaly, infiltration of foam macrophages in tissues, and slowly progressive neurological deterioration (2).

Despite the heterogeneity of the clinical manifestations, all NPC patients present deficient cholesterol processing in fibroblasts (2). Pentchev et al. (3) reported that excessive unesterified cholesterol is accumulated in NPC fibroblasts, suggesting a failure in the intracellular translocation of cholesterol

or in its processing within lysosomal cells (4,5).

Dimethylsulfoxide (DMSO) is a dipolar organic solvent which exists in liquid form at temperatures above 18°C. It can interact with water, salts, proteins, and lipids and can penetrate cell membranes and tissues without irreversible damage (6). DMSO has been used in clinical and biological studies and as a therapeutic agent in the treatment of some diseases (7-10).

Several investigations on the effect of DMSO on NPC fibroblast cultures have been reported. Most of these studies focused on the effect on sphingomyelinase activity (11-14). Diverging data have been reported about the effect of DMSO on the amount of intracellular cholesterol. According to Blanchette-Mackie et al. (13), incubation with 2% DMSO for 24 h does not alter the amount of intracellular cholesterol of NPC fibroblasts, whereas Yoshikawa (14) reported that 2% DMSO at the same incubation time reduces the fluorescence of unesterified cholesterol.

In the present study, NPC fibroblasts were incubated for 24 h with three different concentrations of DMSO. Our aim was to reproduce the results of other groups and to help establish whether the introduction of DMSO treatment for NPC patients is a viable option.

Material and Methods

Cell culture

Eight normal and 7 NPC fibroblast cultures were obtained from skin biopsies. NPC cells were obtained from the Paediatric Research Unit, Guy's Hospital, London, and from patients previously diagnosed at the Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Brazil. The control cell lines were obtained from volunteers. Cells were grown in HAM-F10 medium (Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum (FBS), obtained from Gibco BRL with mycoplasma, virus and endotoxin tested, at 37°C without 5% CO_2. When cells were confluent the flask was trypsinized and the content was transferred to cell culture dishes (NUNC) measuring 35 mm in diameter (about 20,000 cells/dish) in Eagle's minimum essential medium (MEM) supplemented with 5% (v/v) lipoprotein-deficient serum (LPDS), obtained from Gibco BRL and Sigma, respectively. These dishes were placed in a 5% CO_2 incubator at 37°C for 4 days.

This study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre and informed consent was obtained from all subjects that participated in this study.

DMSO addition

Following incubation, DMSO (Sigma) at one of three concentrations (1, 2 or 4%, v/v) and 50 µg/ml of culture medium (MEM) of low-density-lipoprotein (LDL, Sigma) from human plasma in 0.15 M NaCl with 0.01% (w/v) EDTA, pH 7.4, were added to each dish. Control cultures for normal and NPC fibroblasts did not contain DMSO. At this stage, cells were incubated with 5% CO_2 at 37°C for 24 h.

Fluorescent cytochemical staining

Cytochemical detection of unesterified cholesterol with Filipin was carried out according to Kruth et al. (15). Slides were analyzed by fluorescence microscopy and the fibroblasts were photographed with Kodak Ektachrome film.

Lipid analysis

Cells were harvested by trypsinization and washed with saline solution. After centrifugation, the cell pellets were disrupted by freezing and thawing in liquid nitrogen. Unesterified cholesterol was measured by the method of Gamble et al. (16) and protein

was measured by the method of Lowry et al. (17).

Sphingomyelinase activity

Sphingomyelinase activity was measured in order to detect the action of DMSO inside the cell. The assay was carried out with [choline-Me-^{14}C] sphingomyelin (0.37 µCi/mmol) obtained from Du Pont (Wilmington, DE), according to the method of Pentchev et al. (18).

Statistical analysis

Data are reported as mean ± SD. The comparison among treatments using three distinct DMSO concentrations and without DMSO was performed by one-way ANOVA followed by the Duncan test whenever necessary. The Student *t*-test for unpaired samples was used to compare two DMSO-free groups. The analysis was performed using the statistical software package SPSS/PC+, version 3.0, and the level of significance was set at $P<0.05$.

Results

NPC fibroblasts presented a large number of perinuclear granules which were not observed in normal cells (Figure 1). No effect was observed when 2% DMSO (v/v) was present in the culture medium. DMSO at 1 and 4% also did not reduce perinuclear fluorescence in NPC cells incubated for 24 h.

Sphingomyelinase activity was increased by DMSO (Table 1). Addition of 2% DMSO increased the enzyme activity in both groups, whereas 1% DMSO increased it only in normal cells. The 4% DMSO concentration reduced the cell enzyme activity significantly only in the NPC group.

The results of cholesterol measurement agreed with those obtained by the Filipin staining technique. Table 2 shows that NPC cells presented a larger amount of cholesterol than normal cells. DMSO did not affect this finding at any of the concentrations used.

Table 1 - Effect of DMSO on fibroblast sphingomyelinase activity of patients with Niemann-Pick type C disease.

Data are reported as means ± SD for the number of individuals given within parentheses. *P<0.05 compared to group without DMSO (Duncan test). **P<0.05 compared to control subjects (Student *t*-test).

DMSO	Control subjects (nmol h^{-1} mg $protein^{-1}$)	NPC patients (nmol h^{-1} mg $protein^{-1}$)
None	59.4 ± 9.1 (8)	47.7 ± 5.2** (7)
1%	82.7 ± 10.0* (8)	45.5 ± 4.1 (7)
2%	77.0 ± 9.1* (8)	55.8 ± 4.1* (7)
4%	61.3 ± 8.4 (8)	34.7 ± 5.3* (7)

Table 2 - Effect of DMSO on the amount of cholesterol in cultured fibroblasts from patients with Niemann-Pick type C disease.

Data are reported as means ± SD for the number of individuals given within parentheses. *P<0.05 compared to control subjects (Student *t*-test).

DMSO	Control subjects (mg/mg protein)	NPC patients (mg/mg protein)
None	0.347 ± 0.022 (8)	0.704 ± 0.049* (7)
1%	0.333 ± 0.043 (8)	0.659 ± 0.041* (7)
2%	0.305 ± 0.034 (8)	0.688 ± 0.063* (7)
4%	0.319 ± 0.057 (8)	0.733 ± 0.088* (7)

Discussion

Niemann-Pick disease type C is a lipidosis characterized by a block in the intracellular translocation of exogenously derived cholesterol that differentiates it from the other types of Niemann-Pick disease, where sphingomyelinase deficiency is the primary characteristic (19).

There is no specific treatment for the disease. Some palliative treatments directed

to reducing the cholesterol accumulated in the cell lysosome have been described. Sakuragawa et al. (9) and Hashimoto et al. (10) used 100-120 mg/day of DMSO for the treatment of NPC patients. Stabilization of neurologic disease was observed in an 8-year-old girl, but a 3-year-old boy showed no apparent benefit. Based on these data, no conclusion can be reached about the efficacy of treatment. Patterson et al. (20) used DMSO (100 mg/kg bid) alone or combined with cholesterol-reducing agents (lovastatin, cholestyramine and nicotinic acid) in NPC patients and concluded that DMSO alone does not reduce hepatic unesterified cholesterol in NPC patients but is effective when combined with other drugs.

Studies on the effects of DMSO on cell metabolism are very important because little is known about cholesterol homeostasis in NPC. It has been shown that DMSO corrects partial sphingomyelinase deficiency (11,12).

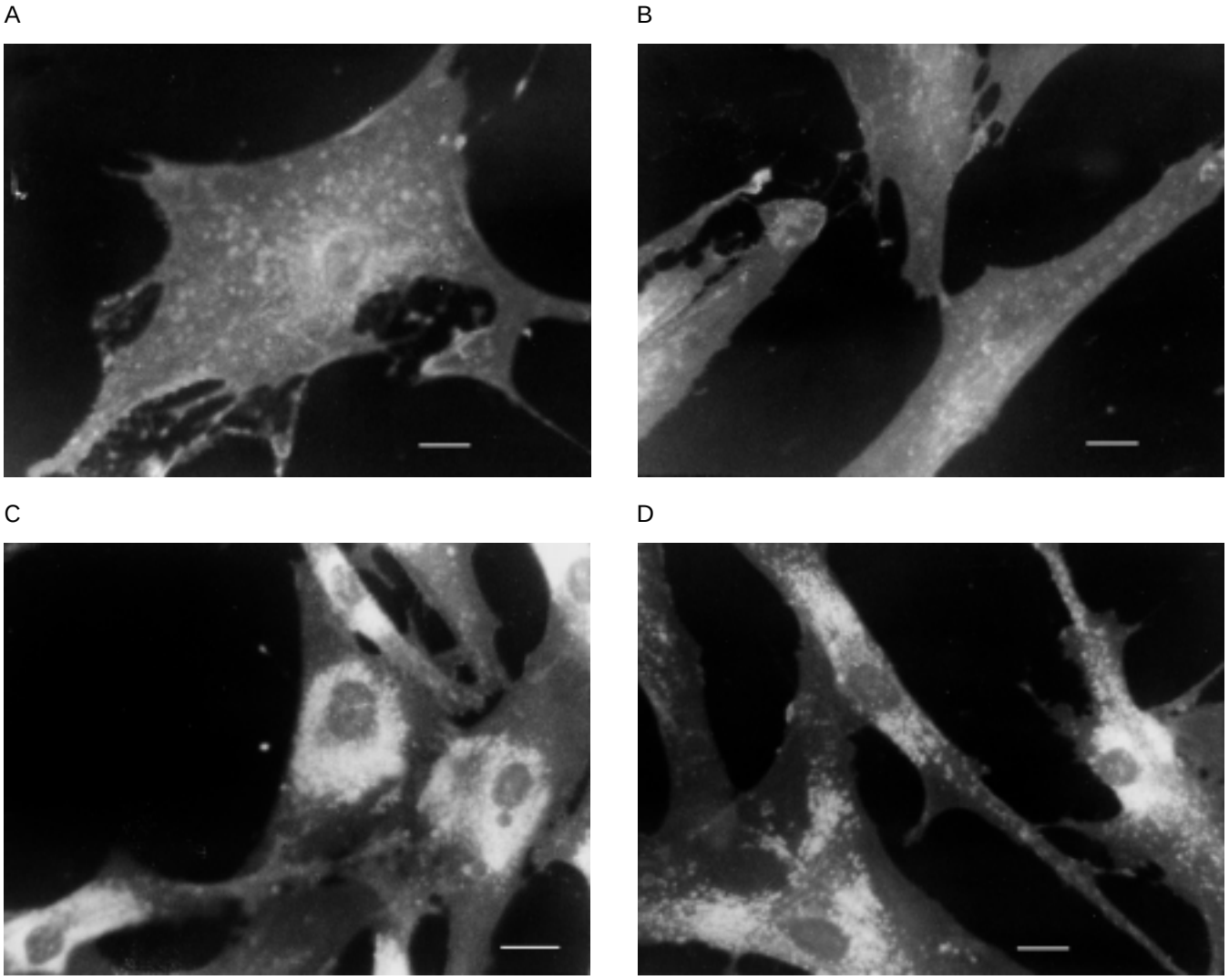

Figure 1 - Normal and Niemann-Pick type C fibroblast cultured with LDL for 24 h with or without DMSO. Cultures were stained with Filipin for fluorescence visualization of unesterified cholesterol. *A*, Normal fibroblasts incubated with 50 µg/ml LDL alone; *B*, normal fibroblasts incubated with 50 µg/ml LDL and 2% DMSO; *C*, Niemann-Pick type C fibroblasts incubated with 50 µg/ml LDL alone; *D*, Niemann-Pick type C fibroblasts incubated with 50 µg/ml LDL and 2% DMSO. Magnification, 40X. The bar corresponds to 25 µm.

However, regarding the reduction of cholesterol accumulated in the lysosomes of fibroblasts, results are controversial.

Blanchette-Mackie et al. (13) measured the cholesterol of NPC fibroblasts after cells were incubated with 2% DMSO and reported that the drug did not alter the amount of cholesterol when cultures were incubated for 12-24 h, but reduced the concentration of this compound after longer periods of incubation. Yoshikawa (14) observed that 2% DMSO added to the culture medium for 24 h of incubation partially reduced the amount of cholesterol in NPC cells. Up to that time, whenever the effect of DMSO on cholesterol amount was studied, 2% was the concentration utilized and the incubation time varied.

In the present study, cell cultures were incubated with DMSO for 24 h and different DMSO concentrations were used. No reduction in the amount of cholesterol was observed at any of the concentrations utilized (1, 2 and 4%). These results do not agree with those reported by Yoshikawa (14), but agree with those reported by Blanchette-Mackie et al. (13). DMSO at 2% concentration or at the other two concentrations used (1 and 4%) did not alter cholesterol inclusion in the lysosomes of NPC patients, in agreement with the unesterified cholesterol measured.

Our results confirm previous studies that have shown that 2% DMSO added to the culture medium increases sphingomyelinase activity after incubation from 6 h to 4 days (11,12). Using three different DMSO concentrations we observed that 1% DMSO is not sufficient to increase the enzyme activity in cells of NPC patients, but is sufficient to cause this effect in normal cells, and 2% DMSO increases sphyngomyelinase activity in both normal and NPC fibroblasts. We also reinforce the findings of the *in vivo* study of Hashimoto et al. (10) who found that DMSO, although it increases sphingomyelinase activity, fails to esterify exogenously administered cholesterol and to improve the clinical course of NPC disease.

After treatment of the cells with 4% DMSO, the enzyme activity showed a statistically significant drop in fibroblast cultures from NPC patients. In these experiments, no cell death was observed after this treatment, but 48 h of treatment with 4% DMSO caused death of more than 70% of cultured fibroblasts (data not shown), demonstrating the cytotoxic effect of 4% DMSO on cultured fibroblasts. Sakuragawa et al. (11) observed a similar effect, i.e., inhibition of cell growth, when normal cells were treated with 2% DMSO for 4 days.

If the deficiency of an enzyme involved in the degradation pathway of cholesterol is responsible for the storage of this substrate in cells of NPC patients, an increase in its activity would be expected after DMSO treatment in the same way as the effect previously shown on sphingomyelinase activity, which lowers cholesterol levels in fibroblasts. Recent studies by Carstea et al. (21) and Loftus et al. (22) showed that NPC-1 gene codes for a membrane structural protein which is involved in cholesterol transport through lysosomes and is defective in NPC patients. In this case, treatment of defective cells with DMSO would not be expected to modify intracellular cholesterol levels, as observed in our study. Even if DMSO could solubilize stored cholesterol its transport out of the lysosomes would not take place.

On the basis of present results, which are in agreement with those obtained by Blanchette-Mackie et al. (13), we conclude that DMSO probably has no effect on cholesterol levels, making no contribution to the treatment of these patients.

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