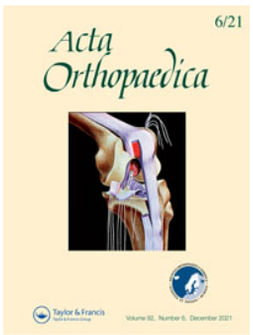

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Reduced NO accumulation in arthrotic cartilage by exposure to methylene blue

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ABSTRACT – Nitric oxide (NO) appears to be a final common inflammation mediator of cartilage degradation. Halting the pathological formation of excessive NO, by suppressing the inducible NO synthase (iNOS) activity, may help to preserve cartilage integrity. We used fresh ex-vivo human articular cartilage explants from normal and arthrotic joints for assessment of NO levels, as determined by its nitrite degradation products and nitric oxide synthase expression. We measured matrix proteoglycan content, assessed by image analysis of alcian blue staining, and proteoglycan synthesis, assessed by sulfate incorporation into proteoglycans. The effect of methylene blue, a nitric oxide synthase inhibitor, on matrix preservation was evaluated. Cartilage discs in vitro, derived from normal appearing joints, secreted about one tenth as much NO compared to discs derived from arthrotic cartilage. Cartilage explants showed a time-dependent reduction in the amount of aggrecan within the cartilaginous matrix. Addition of methylene blue to the growth medium lowered nitric oxide accumulation and prevented matrix degradation in the cultured cartilage discs. The cartilage matrix preservation effect was mediated through downregulation of all three isoforms of NOS, i.e., the neuronal NOS, endothelial NOS and inducible NOS and upregulation of TGF beta receptor in the chondrocytes. Our findings indicate that inhibition of NOS activity preserves cartilage matrix in vitro.

■

The pathogenesis of osteoarthrosis (OA) is not clear. However, cartilage breakdown is an unequivocal result. Arthrotic cartilage is characterized by its reduced content of extracellular matrix macromolecules, due to a reduction in synthesis and an increase in degradation. Recent data suggest that nitric oxide (NO) plays a key modulating role in the pathophysiology of arthrosis (Studer et al. 1999a).

NO is a soluble, short-lived free gaseous radical formed from L-arginine by a family of enzymes known as NO synthases (NOS) (Amin et al. 1999, Studer et al. 1999a). Constitutive NO synthase (cNOS) participates in intercellular signaling in neurons and endothelial cells, producing NO in picoMolar amounts (Bredt and Snyder 1990, Ignarro 1990). On the other hand, immunologic and inflammatory stimuli can induce the formation of an isoformic inducible NOS (iNOS), that can produce nanoMoles of NO (Radomski et al. 1990).

The fact that NO is produced in different amounts (variations in the order of 10^3) and over varying durations may explain the different roles and mode of action of the various NOS. In small amounts (pM) of NO, it can be beneficial as a modulator or messenger. On the other hand, in large amounts (nM), NO may be toxic and destructive, serving as a powerful mediator initiating inflammation (Pelletier et al. 1999b). A characteristic of iNOS is that it is not detectable in quiescent macrophage cell lines, but is induced by lipopolysaccharides (LPS) or certain cytokine agents, such as IL-1, TNF-α and IFN-γ (Shiraishi et al. 1997). The overexpression of iNOS in a variety of inflammatory tissues, including cartilage, has led to the assumption that the modulation of NO synthesis and activity may provide a new tool

to treat inflammation and autoimmune diseases. Methylene blue (MB) is a thiazine vital dye with antiseptic properties that is an inhibitor of nitric oxide synthase (Geng et al. 1998). NO leads to cartilage matrix degradation due to inhibition of transforming growth factor β and activation of matrix metalloproteinases (Studer et al. 1999b).

We evaluated the ability of methylene blue to inhibit the cartilage matrix degradation in human cartilaginous explants, by reducing inducible nitric oxide synthase and nitric oxide production.

Methods

To assess the role of nitric oxide (NO) in human cartilage metabolism and in osteoarthrosis, several assays are necessary. However, NO is a volatile gas. It is easier to determine its degradation products that are nitrates or nitrites. An easily performed colorimetric reaction exists for nitrite determination, but it requires reduction of all nitrates into nitrites as a first step. It is well known that incubation of cartilage in vitro results in leaching out of the matrix over time. Thus the effect of incubation on the explants should be assessed. Only then can the effect of nitric oxide inhibition be studied and compared to that found in incubated explants. NO is produced by living cells. To rule out cell death as the responsible factor for a reduction in NO, an assay to determine the viability of the discs over time is necessary. De-differentiation of chondrocytes might also be invoked as a reason for nitric oxide production. Thus we assessed sulfate incorporation into proteoglycans to ensure that the chondrocytes retained their phenotype. A reduction in NO level in tissue might be related to decreased production or increased breakdown. We therefore assessed inducible NO synthase by immunohistochemistry. We evaluated the effect of methylene blue, a known inhibitor of NO synthase, instead of more selective inhibitors because it can be readily used in clinical work.

Cartilage sampling

We collected cartilage specimens from 10 patients undergoing total hip replacement (5 patients) or total knee replacement (5 patients) due to primary osteoarthrosis. Patients using corticosteroids or non-steroidal anti-inflammatory drugs during the 2 weeks prior to surgery were excluded.

10 age-matched normal cartilage surfaces were collected during hip hemiarthroplasty due to subcapital fracture, performed within 24 hours after the fracture.

We used a specially produced hollow trephine with carbide coated cutting edge to remove shallow surface discs of 3 mm in diameter, from the articular surface, containing articular cartilage plus some subchondral bone. Each surface served as the source of several discs containing surfaces with macroscopically similar quality of cartilaginous tissue. No eburnated bone areas and osteophytes were used.

4 discs (75–150 mg wet weight) were incubated together in each well of the 24 wells/plate. The weight variation is explained by the varying thickness of the cylinder, i.e., in some cylinders more subchondral bone was removed. Each data-point was evaluated with 3 such wells, representing 12 separate discs. The total number of discs used was 12 at each point, times the number of check points (see below).

Nitric oxide levels in cartilage explants: nitrates assay

Cartilaginous discs were grown as explants in organ cultures in 2 mL of medium (F-12 supplemented with 0.1% bovine serum albumin (BSA), glutamine and antibiotics in 24 well-plates. The explants were incubated for 24, 48 and 72 hours. The incubator was kept at 37 °C and the atmosphere contained 5% CO_2 in air. Nitrites and nitrates were detected (as nitrites) in the cultures' supernatants. These supernatants were collected and stored at –20 °C, pending analysis.

A series of nitrite determinations in supernatants was carried out largely according to the previously described method of the Griess reaction before and after enzymatic reduction by E. coli nitrate reductase (in excess), with or without addition of exogenous nitrates. The results indicate that the total nitrites level, following nitrate reductase treatments, is the sum of exogenous nitrates and endogenous nitrates and nitrites. These findings enabled the direct utilization of the total nitrites values obtained after exposure of the super-

natant to nitrate reductase. In subsequent experiments, total nitrite levels were used to indicate the amount of NO in tissues.

Nitric oxide levels in cartilage explants: nitrate reduction and nitrite determination

The preparation of E. coli nitrate reductase (Bartholomew 1984) was done with an E. coli bacterial starter strain from one of the authors' laboratory (Y.W.), cultured under optimal conditions for producing high nitrate reductase activity. Bacteria were grown overnight in a 5-liter flask. Centrifugation at 1000 rpm for 15 minutes pelleted out the bacteria. The pellet was then resuspended in 5 cc of phosphate buffered saline. Aliquots of 0.5 mL/vial were frozen at –20 °C. Prior to use as a nitrate reducing agent, the bacterial suspension containing the nitrate reductase enzyme was further diluted in PBS (1:20 v/v). The diluted suspension was kept in an ice bucket, preserving its enzymatic activity for up to 10 hours.

To validate the methodology, nitrate standard curves were generated. Different amounts of $NaNO_3$ 1 mM were diluted in F-12 medium. The enzymatic nitrate reduction reactions contained 400 μL of the supernatant samples, 300 μL of the diluted bacterial suspension, and 20 μL of NADH (10 mg/mL). The mixture was allowed to interact for 1 hour at 37 °C, and then centrifuged at 10,000 rpm for 10 minutes at 4 °C. To 600 μL of supernatant samples, 300 μL of Griess reagent (Green et al. 1982) (1 volume of 0.1% naphthylethylenediamine dihydrochloride in distilled water plus 1 volume of 1% sulfanylamide in 5% H_3PO_4) were added, mixed and incubated in the dark at 23 °C for 10 minutes. The color intensity was measured spectrophotometrically at 550 nm by a plate reader device (BIO-TEK Instruments, Inc., Winoski, Vermont, Model ELX800). To control for possible bias due to the bluish hue of methylene blue itself, standard curves of exogenous sodium nitrate in F-12 were plotted, in the presence of various doses of methylene blue (0, 5, 15 and 30 μM).

Evaluation of nitric oxide synthase and matrix content of proteoglycans

We examined 20 samples (10 derived from patients with normal femoral head cartilage and 10 from patients with arthrotic cartilage from either hip or knee) per data-point. The inclusion of several donors was important to prevent bias of the conclusions due to a possible peculiarity of a specific patient. The discs had been incubated for 24, 48 or 72 hours, and exposed to methylene blue (0, 5, 15 and 30 mM). All specimens were fixed in 4% formalin, (pH 7.4) containing cetylpyridinium chloride (CPC) (0.5%) for 24 hours to prevent proteoglycans from leaching out. They were later processed for routine paraffin embedding (Butnariu-Ephrat et al. 1996). 5-μ thick sections were prepared for routine histologic and histochemical examinations. Sections glued to polylysine-coated glass slides were used for immunohistochemistry. Mayer's hematoxylin and eosin, Masson's trichrome, and alcian blue (pH 1.0 and pH 2.5) were used. Expression levels of 3 isoforms of nitric oxide synthase were assessed by immunohistochemistry: Brain IgG fraction anti-nitric oxide synthase (bNOS); endothelial IgG fraction anti-nitric oxide synthase (eNOS), and inducible IgG fraction anti-nitric oxide synthase (iNOS), rabbit polyclonal antibodies, 1:50 dilution (Sigma Israel Chemical Ltd., Park Rabin, Rechovot, Israel). Detection was performed with a peroxidase-antiperoxidase complex (1:150, Dakopatts with diaminobenzidine as a substrate (Dako, Glastrup, Denmark). Densitometeric evaluation of cell-membrane staining intensity was made by an image analyzer. Transforming growth factor beta (TGF β) is important in cartilage matrix synthesis and is inhibited by nitric oxide (Studer et al. 1999a). To assess the effect of NO inhibition on the TGF β pathway, TGF β receptor status was determined by immunohistochemistry. Intensity of staining was measured, using an image analyzer, after finding a region of interest around the chondrocytes. The average intensity was expressed in gray-scale values, with 0 indicating absolute black and 255 absolute white. The average intensity of 30 cells was determined and results are expressed as the mean staining intensity (SD).

Cell vitality assay

The XTT reagent kit for determination of cell vitality is based on the reduction ability of tetrazolium salts by mitochondrial enzymes (hydrogenases) of living cells, to form a soluble colored molecule—a formazan dye, measured at 450 nm by an

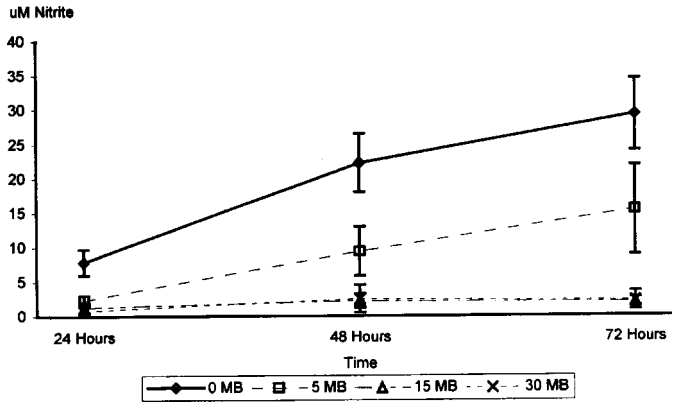


Figure 1. Nitrite accumulation in osteoarthrotic cartilage discs in vitro. Addition of methylene blue reduces nitrite accumulation in a dose-dependent fashion. At 15 and 30 micro molars, this is partly related to chondrocyte death (mean ± S.E., 0 = no methylene blue, 5 = 5 micromolars methylene blue, 15 = 15 micromolars methylene blue, 30 = 30 micromolars methylene blue). Statistical analysis using ANOVA with repeated measures: Alpha = 0.05, F = 16.9, $p < 0.001$.

ELISA plate reader. The optical density is proportional to the number of living cells and their metabolic status (Scudiero et al. 1988). Explants incubated for 24 hours and 48 hours were examined.

Explants were washed off their incubation medium, and medium containing the XTT reagent was added and incubated for 1 hour at 37 °C under 5% CO_2 in air. The supernatants were transferred to another 96 wells plate and read in the ELISA plate reader at 450 nm.

Proteoglycans synthesis by cartilage explants

The technique used to isolate and characterize ^{35}S-glycosaminoglycans from cultured cartilage disc was carried out, as previously described (Robinson et al. 1998).

Material

NADH, sodium nitrate, sodium nitrite, naphthylethylenediamine dihydrochloride, sulfanylamide, H_3PO_4 and BSA were purchased from Sigma Israel Chemical Ltd., Park Rabin, Rechovot, Israel. Phosphate buffered saline (PBS), F-12 medium, antibiotics, glutamine, and XTT cells proliferation assay kit were obtained from Biological Industries Co., Kibutz Beit Haemek 25115, Israel. Methylene blue reagent was bought from Fluka Chemicals, Buchs, Switzerland.

Statistics

The data were analyzed using ANOVA with repeated measures evaluating the effect of different concentrations of methylene blue (MB) over time in culture on nitric oxide accumulation (SPSS software).

Results

Nitric oxide levels in cartilage explants

Arthrotic cartilage in culture accumulated nitrates. Levels after a 72-hour incubation period were 4 times higher than after a 24-hour incubation, without methylene blue. Methylene blue 5 mM halved nitrite accumulation during the first 24 hours. In the presence of methylene blue, accumulation of nitrites was slower during the following 2 days of incubation (Figure 1).

Cartilaginous samples derived from normal subjects and incubated for 48 hours released small amounts of nitric oxide (0.3 (0.4) μM per milligram wet weight) and served as the baseline. Samples derived from osteoarthrotic patients showed up to a fiftyfold (7.5 (1.2) μM) increase in NO release, following the same incubation.

NO synthase in cartilage

Matrix staining intensity by alcian blue (pH 1) was directly related to the proteoglycan content of the tissue (Butnariu-Ephrat et al. 1996). When cartilage explants were cultured in vitro, leaching of proteoglycans occurred. Methylene blue partly prevented this degradation of the cartilage (Figure 2). Inducible NOS (iNOS) was expressed in explants in increasing amounts during 72 hours of incubation. Methylene blue reversed this accumu-

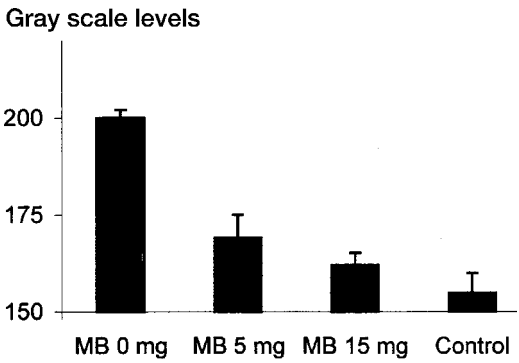
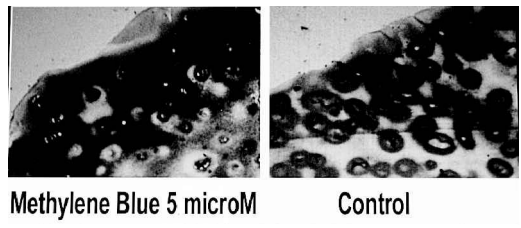


Figure 2.A. Methylene blue prevents cartilage matrix degradation after incubation of osteoarthrotic explants for 72 hours in a dose-dependent fashion. Quantitative evaluation of staining intensity by image analyzer using a 256 gray level scale (alcian blue pH 1 stain, 0 = absolute black, 255 = absolute white, mean ± S.D.)



B. Proteoglycans are leached out of cartilage discs in culture in vitro (on the left). Methylene blue 5 micromolars added to the medium partly prevents proteoglycan degradation (on the right) (alcian blue stain, methylene blue 5 microM= 5 micromolars added to the medium (× 40).

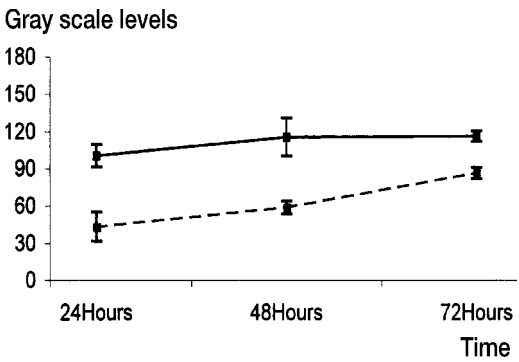
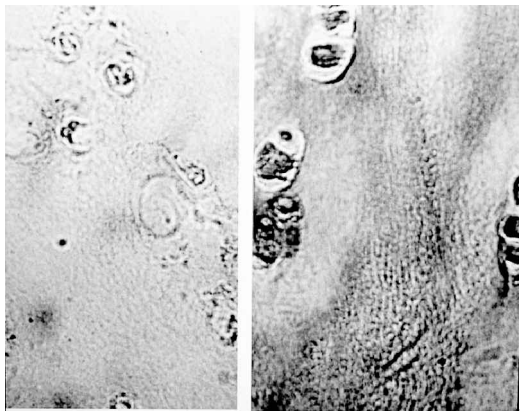


Figure 3. A. Inducible nitric oxide synthase expression in osteoarthrotic cartilage. Quantitative evaluation of staining intensity by image analyzer using a 256 gray level scale (polyclonal iNOS antibodies detected by DAB stain of PAP complex, 0 = absolute black, 255 = absolute white, mean ± S.D., - - - = no methylene blue added, —— = 5 micro-molars of methylene blue added).



B. Inducible nitric oxide synthase in chondrocytes' membranes of cultured cartilage discs (on the right). No expression of the enzyme when methylene blue is added to the culture medium (anti-iNOS antibodies 1:100, diaminobenzidine dye, methylene blue 5 microM = 5 micromolars methylene blue added to the medium (× 400).

lation of iNOS (Figure 3). Similar results were obtained in the two other constitutive isoforms of NOS evaluated (results not shown).

Effect of methylene blue on cellular viability and matrix synthesis

Explants incubated in the presence of carrier-free ^{35}sulfur (10 microCi/mL medium) during the last 24 hours of the incubation period were used to isolate ^{35}S-Glycosaminoglycan macromolecules as described in the Methods Section. The data summarized in the Table show a reduction in PG-GAG synthesis with increasing doses of MB.

The XTT reaction indicated that methylene blue adversely affects cell survival in concentrations of at least 10 μM (mean cell survival 75 (8)% of control), but not in concentrations of 5 μM or less (mean 92 (5) of control). Thus, the reduction in nitrate accumulation and NOS isoforms expression described above cannot be attributed to loss of cellular viability.

Glycosaminoglycan synthesis in cartilage is reduced by addition of methylene blue. Mean (SD)

Methylene blue (micro-molars)	Percent reduction in GAG synthesis	Average counts CPM S^{35} per mg wet weight of tissue
0		101
5	27 (8)	71
15	49 (10)	34
30	69 (4)	32

Correlation coefficient: r > –0.88

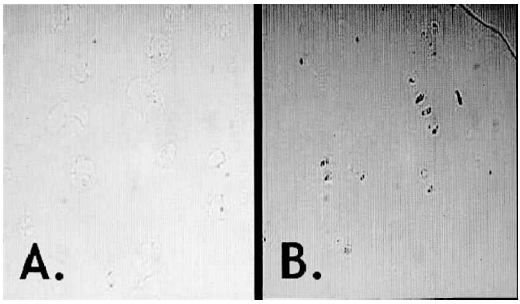


Figure 4. Methylene blue 5 micromolars restores chondrocytes' expression of TGF beta receptor (A. Incubation of cartilage explants for 24 hours without methylene blue, B. Similar explant cultures in the presence of methylene blue 5 micromolars (×400).

Effect of methylene blue on TGF beta receptor status of cartilage explants

Chondrocytes express TGF beta receptor before incubation in vitro. Expression in normal cartilage (130 (10)) is stronger than in osteoarthrotic specimens (194 (15)). Expression totally declines after 24 hours of incubation (222 (5)). Incubation with methylene blue 5 micromolars for 24 hours restores TFG β receptor expression (Figure 4) to values similar to those before incubation (158 (17)).

Discussion

The initiation of cartilage damage is multifactorial including abnormal joint mechanics, trauma and infectious arthritis. The existence of a final common denominator evoking a cascade of events leading to cartilage disintegration is hypothesized. The cartilage disintegration process can start with IL-1, followed by other inflammatory mediators (Horton et al. 1998). This causes the formation of an environment rich in free radicals and involving nitric oxide (NO) which is typical for an osteoarthrotic joint (Tanaka et al. 1998). The role of nitric oxide in osteoarthrosis is readily explained by its detrimental effect on chondrocyte metabolism—i.e.,inhibition of collagen and PG synthetic rates and enhancement of chondrocytes' apoptosis (Martel-Pelletier 1998). Controlling NO production is therefore a desirable therapeutic method.

Our findings confirm that arthrotic cartilage accumulates nitric oxide ex vivo (Martel-Pelletier 1998). Under conditions in which matrix loss of proteoglycans occurs (i.e., explant cultures), methylene blue partly reverses this trend. As methylene blue is a known inhibitor of NO synthase via a guanylyl cyclase-dependent pathway (Geng et al. 1998), it is reasonable to assume that nitric oxide reduction is the underlying mechanism of action. This assumption is borne out by the observation that nitric oxide synthase is reduced in cartilage discs exposed to methylene blue. Nitric oxide is synthesized from L-arginine by nitric oxide synthases (NOSs) (Jang and Murrell 1998). At least three isoforms of NOS are known (nNOS-neuronal, eNOS-epithelial and iNOS-inducible), differing in their rates of NO production, degree of cytochrome C reduction, NADPH utilization and calcium ion dependence. Most relevant to joint pathology is the inducible isoform (iNOS), which is capable of producing large amounts of NO (Jang and Murrell 1998). The iNOS is induced by the inflammatory mediators IL-1, IL-2, IL-6, TNFα and γ-interferon (Studer et al. 1999b). Nitric oxide secreted by both synovium and cartilage is important in the inflammatory process underlying cartilage destruction in inflammatory arthritis (Amin et al. 1999).

The effect of nitric oxide on joints is bimodal, depending on the concentration. In pico-molar amounts, the molecules serve as cytoprotective agents, interacting with oxygen free radicals and forming reactive nitrogen intermediates playing the role of endogenous anti-microbial substance. In excessive amounts and in combination with

TNFα, NO is cytotoxic to chondrocytes (Melchiorri et al. 1998)

Inducible NOS inhibitors should moderate the pathological process and serve as anti-inflammatory agents. Methylene blue has been used as a therapeutic drug for several decades, and has a good safety record. We have shown that it can also reduce iNOS expression and cartilage matrix degradation. In vitro, high doses of methylene blue can cause cellular death. This phenomenon is not known to occur in vivo, but is a cause of concern. Yet, it appears that overall, NO suppression by methylene blue is advantageous for the surviving cell population, which is probably more important than its cytotoxic effect. Our finding in this study, i.e., that inhibition of iNOS activity prevents matrix degradation in human cartilage, has previously been shown in animals (Pelletier et al. 1999a, b). It is suggested that, in situations of acute joint damage, septic or traumatic, joint lavage with a NOS inhibitor such as methylene blue can prevent later cartilage damage. Wheter a more selective iNOS inhibitor (Patel et al. 1999) is more appropriate for clinical use is unknown at this time and further investigations should be undertaken to clarify this point.

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