Nimodipine rescues N-methyl-N-nitrosourea-induced retinal degeneration in rats

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ABSTRACT

Background: That nimodipine (NMD) is potentially useful for ophthalmic treatment. However, the effect of NMD is unknown on retinal degenerative diseases. **Objective:** The purpose of the present study was to investigate the effect of NMD on *N*-methyl-*N*-nitrosourea (MNU)-induced retinal degeneration (RD) and elucidate its possible mechanisms. **Materials and Methods:** Morphological observation of NMD on MNU-induced RD was evaluated by light microscopy and electron microscopy. Nonenzymatic antioxidant glutathione (GSH) was measured by a colorimetric method. Transforming growth factor-beta (TGF-β) was measured by enzyme-linked immunosorbent assay (ELISA). Telomerase was detected by reverse transcriptase polymerase chain reaction (RT-PCR). **Results:** The significantly protective effect of NMD on MNU-induced RD was demonstrated morphologically. NMD increased the content of GSH and decreased the level of TGF-β in rat retina. RT-PCR analysis demonstrated that NMD treatment significantly decreased mRNA level of telomerase. **Conclusion:** These data suggest that NMD inhibit MNU-induced RD in rats. The expressions of TGF-β, telomerase and GSH contents might partially contribute to its protective effects on MNU-induced RD.

Key words: Nimodipine, *N*-methyl-*N*-nitrosourea, retinal degeneration

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INTRODUCTION

Retinitis pigmentosa (RP) refers to a heterologous group of inherited human disorders which caused primary retinal degeneration and are characterized by loss of photoreceptor cells via apoptosis.[1] The effective medicines are in various stages of development, no drugs are actually available to treat people with RP.

In recent years, there has been an upsurge of interest in unraveling the roles of Ca^{2+} antagonists about the pathophysiology of different kinds of human diseases including neurodegenerative diseases, for example, retinal degeneration (RD).[2-4] Nimodipine (NMD) is a dihydropyridine calcium channel antagonist, which has been shown to dilate cerebral arterioles and increase cerebral blood flow in animals and humans.[5] Several studies *in vitro* and *in vivo* have demonstrated that NMD could protect retinal neurons from ischemic injury and was significantly effective vasodilators in human and pig retinal arterioles.[6,7] Several researchers reported that NMD had beneficial effect in visual field tests, color vision tests and contrast sensitivity,[8,9] and it has been shown to improve ocular hemodynamics and increase visual field in patients with normal-pressure glaucoma and primary open angle glaucoma.[10-12] *In vitro*, NMD has a direct neuroprotective effect against retinal ganglion cell damage related to hypoxia.[13] All these show that NMD is potentially useful for ophthalmic treatment. However, the effect of NMD is unknown on retinal degenerative diseases.

Herrold (1967) was the first to report RD in Syrian golden hamsters after systemic administration of the alkylating agent *N*-methyl-*N*-nitrosourea (MNU). A single systemic administration of MNU in rats leads to apoptosis and photoreceptor cell loss over approximately 7 days because it restricted deoxyribonucleic acid (DNA) methylation adduct formation in photoreceptor nuclei.[14] In previous studies, a dose of 60 mg/kg MNU was considered optimal to induce maximum damage in rat retinas.[15] MNU-induced

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photoreceptor cell death was attributed to the restriction of DNA adduct formation to photoreceptor cell nuclei leading to apoptosis.[15] Reactive oxygen species (ROS) play an important role in apoptosis induction under pathologic conditions,[16] which was related to cellular redox imbalance, as indicated by the depletion of glutathione (GSH), an essential regulator that maintains normal cellular redox status.[17-19] MNU also causes a decrease in reduced GSH, which effectively scavenges free radicals and other ROS.[20,21]

The transforming growth factor-b (TGF-β) family that participates in a number of biological events such as proliferation, differentiation, apoptosis, and cell death, plays physiologic roles during embryonic development and tissue homeostasis in the adult.[22,23] Previous studies reported that TGF-β decreased the intracellular GSH content in various cells, and GSH reduces TGF-β-stimulated ROS signal regulation.[24,25]

In addition, MNU has been implicated in various cancer models. A high expression level of telomerase is found within the germ line, embryonic stem cells, and cancer cells. According to these, we adopted the animal model of MNU-induced RD to assess the protective effect of NMD on morphology with electron microscopy, and the present work was to analyze whether resistance to MNU-induced damage might be due to the changes of GSH, TGF-β, and telomerase in retina.

MATERIALS AND METHODS

Chemical and dose formulation

MNU (Sigma,USA) was kept at −20°C in the dark, it was dissolved in phosphate-buffered saline (PBS) just before use. NMD was purchased from Shandong Fangming Pharmaceutical Group Co., Ltd, China. GSH and TGF-β test Kits were purchased from Nanjing Jian cheng Bioengineering Institute, China. All other chemicals were purchased from commercial sources.

Animals and procedures

Six-week-old female Sprague-Dawley rats were purchased from the animal facility at the China Medical University, P. R. China. Animal experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of animals in ophthalmic and vision research, and the study was approved by the local Medical Ethics Committee. Rats were reared under standard laboratory conditions (22 ± 2°C, 60% ± 10% relative humidity and a 12-h light–dark cycle) and had free access to food and water throughout the experiment.

Rats were randomly divided into three groups, including control group (NC group), MNU-treated group (MNU group), and NMD-treated group (NMD group). In the NMD group, rats received a single intraperitoneal (i.p.) injection of 60 mg/kg MNU, followed immediately by once daily i.p. of 2 mg/kg NMD for 5 days. In the MNU group, after a single i.p. of MNU injection, PBS (10 ml/kg, once a day) was administrated instead of NMD for 5 days. Rats in the NC group were injected with a corresponding bolus of PBS. The animals were sacrificed at 5 days after MNU treatment, rat eyes were enucleated to use for electron microscopy assay, reverse transcriptase polymerase chain reaction (RT-PCR) assay, determination of GSH and TGF-β.

Light microscopy assay

The eyes were fixed in 4% paraformaldehyde (PFA) for 24 h. Eyes were enucleated, a portion of cornea and lens were removed and the remaining eye cups containing the retinas were placed in 4% PFA overnight, the samples were incubated in 50% ethanol for 1 h, routinely dehydrated and embedded, and sagittal sections (4 μm thick) were cut near the optic nerve head. The slices were dewaxed, stained with hematoxylin and eosin (HE) for histological examination. To determine retinal damage, HE sections were examined under the optical microscopy (OLYMPUS, BX60 Japan) at 100-fold magnification.

Electron microscopy assay

A part of eyes reserved for electromicroscopy assay was kept in 2.5% glutaraldehyde overnight at 4°C, then the cornea and lens were removed and the remaining eye cups containing the retinas were placed in 2.5% glutaraldehyde overnight at 4°C. The samples were postfixed in 2% osmium solution for 20 min, then dehydrated and embedded in epon, and polymerased at 60°C. Thin sections (50 nm thick) stained with uranyl acetate and lead citrate were examined using a electron microscope (JEM-100CXII, Japan).

Determination of GSH content

A part of retinas were lysed directly on ice in 1 ml of PBS, after centrifugation, the supernatants were transferred to new tubes to examine TGF-β and GSH. The content of GSH was determined using the commercially available diagnostic kits and was measured by the method of Moron *et al*.[26]

ELISA analysis of TGF-β

The level of TGF-β was quantitated by a modification of a double ligand enzyme-linked immunosorbent assay (ELISA) method as previously described.[27]

RT-PCR analysis of telomerase

Total RNA from retinas was isolated using reagent Trizol

(Gibco, Inc., Grand Island, NY) according to manufacturer's instructions.[28] Ribonucleic acid (RNA) was quantitated by measuring the OD260 and reverse transcribed into a single-stranded complementary deoxyribonucleic acid (cDNA) with the RevertAid H Minus First Strand cDNA Synthesis Kit, K1632 (Fermentas AB, Vilnius, Lithuania). cDNA was amplified for 25 cycles so that the PCR product remained in the linear range. PCR (Golden DNA Polymerase, KT221, Tiangen, China) was performed with gene-specific primers for β-actin, telomerase gene [Table 1]. β-actin was used as an internal control to confirm mRNA integrity. The identity of all PCR products was confirmed by comparison with the correct size based on the known length of the DNA sequence on 1% agarose gel stained by ethidium bromide. The optical density of the bands was analyzed on the GeneSnap system (Syngene, Synoptics Ltd. Frederick, MD).

Statistical analyses

Values are expressed as mean ± standard error. One-way analysis of variance (ANOVA) was used followed by Fisher's least-significant difference (LSD) for the homogeneity testing of variance (Levene's test), and the data were analyzed by Dunnett's T3 for the heteroschedasticity of variance test. $P < 0.05$ was considered statistically significant. All statistical procedures were performed using SPSS 13.0 software for Windows (SPSS Inc., USA).

Table 1: Primer sequences for mRNAs amplified by RT-PCR

Gene	Primer sequences
hTERT	Forward: 5C| GCA GAA TTC ATGCCAGGGACTCCCCGCAGGTTG-3C|
	Reverse: 5C| CGGGTCGACTTACTC GTAGTTGAGGAC GCTGAAC-3C|
β-actin	Forward: 5C| -GGA AAT CGT GCG TGA C-3C|
	Reverse: 5C| -GGA AGG TGG ACA GTG AG-3C|

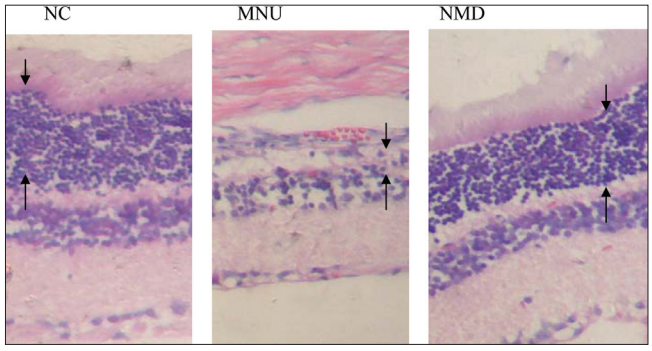

Figure 1: Effects of NMD on the retinal histological damage after MNU treatment in rats. Representative photographs of retinal sections stained with HE (H and E, each ×100): NC group, the normal outer nuclear layer; MNU group, the outer nuclear layer became thinned compared with NC group; NMD group, the outer nuclear layer became thicker compared with MNU group. Between arrowheads indicate the outer nuclear layer

RESULTS

Morphological observation of NMD on MNU-treated rats

Histological observations showed that i.p. administration of 60 mg/kg/day MNU caused the severe degeneration in the outer nuclear layer at 5 days after MNU injection [Figure 1]. At the same time, the number of surviving photoreceptor cells in the NMD group was significantly increased at 5 days after MNU. At the ultrastructural level [Figure 2], electron microscopy showed that morphology of photoreceptor cell was normal in the NC group, whereas vacuoles and fusion of mitochondrion were more numerously seen and heterochromatin are lost in the MNU group. Electron microscopic analysis disclosed that photoreceptor cells were less deteriorated in the NMD group than in the MNU group. At 5 days after MNU injection, 2 mg/kg NMD partially protected against MNU-induced retinal damage. These observations suggest that NMD has a beneficial effect on MNU-induced RD.

The effect of NMD on GSH content induced by MNU

The content of GSH was substantially decreased in MNU group compared with the NC group. Although the level of GSH in the NMD group was significantly different from that of the NC group, it was also significantly different from that of the MNU group (partial protection) [Figure 3].

The effect of NMD on TGF-β induced by MNU

As shown in Figure 4, the level of TGF-β was up-regulated in the MNU group compared with the NC group. Treatment with NMD decreased the expression of TGF-β in MNU-induced RD.

The effect of NMD on telomerase induced by MNU

RT-PCR result of telomerase mRNA expression is shown in Figure 5a, and quantitative data are summarized in Figure 5b. Telomerase upregulated significantly in MNU

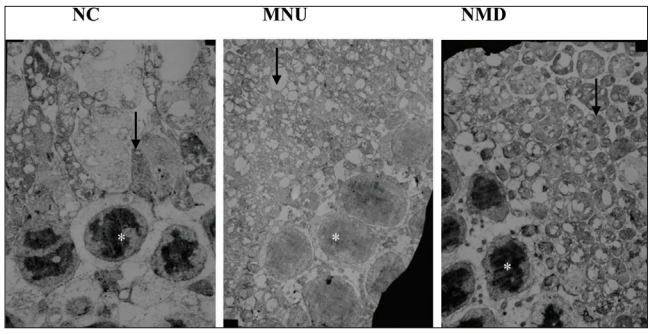

Figure 2: Effects of NMD on photoreceptor cells after MNU treatment at the ultrastructural level in rats. NC group, the normal photoreceptor cell; MNU group, the apparent vacuoles and fusion of photoreceptor cell mitochondrion, and heterochromatin was lost; NMD group, the less deterioration of photoreceptor cell. Asterisk (*), photoreceptor cell nucleus; arrow, mitochondrion (each ×4000)

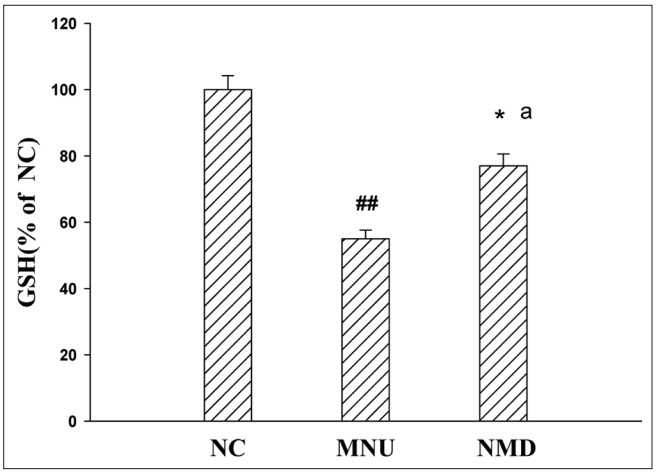

Figure 3: Effects of NMD on GSH level in MNU-induced RD. After MNU treatment, the GSH level showed reductions. In rats receiving NMD treatment, the GSH level was significantly higher than those rats in the MNU group and lower than in the NC group. a$P < 0.05$, ##$P < 0.01$, compared with the NC group, *$P < 0.05$, compared with the MNU group

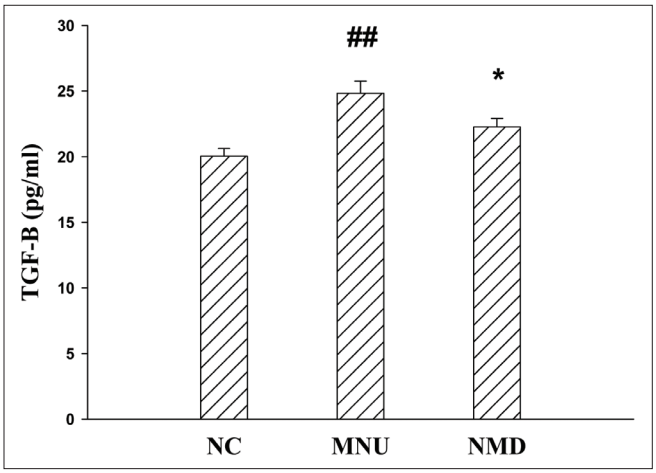

Figure 4: Liaoning effects of NMD on the level of TGF-β in MNU-induced RD. The retinal supernatants were assayed for TGF-β with ELISA. After MNU treatment, the level of TGF-β showed increase. In rats receiving NMD treatment, the level of TGF-β was significantly lower than those rats in the MNU group. ##$P < 0.01$ compared with the NC group, *$P < 0.05$ compared with the MNU group. There is no significant difference between NC and NMD group

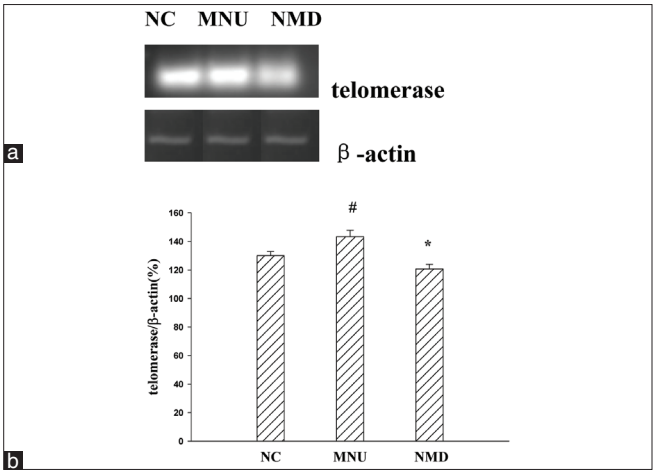

Figure 5: Effects of NMD on MNU-induced mRNA expression of telomerase in rat retina measured by RT-PCR, (a) DNA strips show expression levels of telomerase on agarose gel electrophoresis, (b) Effects of NMD on MNU-induced mRNA expression of telomerase in rat retina. #$P < 0.05$ versus NC group, *$P < 0.05$ versus MNU group. There is no significant difference between NC and NMD groups

group compared with the NC group. In the NMD group, telomerase was significantly lower than in the MNU group at 5 days.

DISCUSSION

NMD, an L-type calcium channel antagonist, has been mainly used in treatment of cardiovascular-related diseases, including angina pectoris, cardiac arrhythmia, and hypertension. NMD has been found to be beneficial in many central nervous system (CNS) disorders. The clinical studies demonstrated a favorable effect of NMD on the severity of neurological deficits caused by cerebral vasospasm following subarachnoid hemorrhage.[29,30] In the present study, systemic administration of NMD morphologically demonstrated a beneficial effect on RD caused by MNU. These results suggest that NMD has a protective effect against MNU-induced RD.

GSH, as a small molecule of radical scavenge, can act either as a nucleophile with the formation of conjugates or as a reducing agent to protect of cellular macromolecules, in which reaction, it is oxidized to its oxidized glutathione (GSSG). It has been reported that that GSH depletion causes increased secretion of vascular endothelial growth factor A (VEGF-A) in retinal pigment epithelium (RPE) and cellular redox status plays an important role in VEGF regulation in RPE cells.[31] In our study, we have found that neovascularization in retina was induced by a single i.p. injection of MNU in 3–4 weeks time period (data not shown). Viviane *et al*. confirm that NMD acts positively on lipid peroxidation.[32] In this present study, NMD treatment can increase the activity of GSH, thereby suggesting that this drug acts positively as a protective antioxidant.

TGF-β, a multifunctional cytokine, regulates cell proliferation, differentiation, and extracellular matrix synthesis. For ophthalmic study, the roles of TGF-β in retinal fibrosis, in proliferative retinal disorders and in macular degeneration are well documented.[33,34] Previous studies indicated that TGF-β and GSH mutual inhibit in hepatic stellate cells and murine embryonic fibroblasts.[24,25] In this study, MNU treatment can decrease the level of GSH, and TGF-β was expressed higher in the MNU group compared with the NC group. TGF-β was expressed lower in the NMD group compared with the MNU group.

For the above reasons, we speculate that GSH depletion may be induced by TGF-β mediate and TGF-β might be involved in the effect of NMD on MNU-induced RD. The relation between GSH and TGF-β in retina still need to be further studied.

Telomerase is an enzyme that adds a six-base DNA repeat sequence (TTAGGG) to chromosome ends and thereby prevents their shortening during successive rounds of mitosis.[35-37] The presence of telomerase activity in retina indicates that a fully functional form of telomerase can be found in the retina.[36] The expression of telomerase activity in retina implies other functions, such as regulation of cell cycle progression and maintenance of retinal cell phenotypes.[36,37] It has been reported that there are significant changes of gene expression in the early stage of MNU-induced RD.[38] In this present study, RT-PCR analyses revealed that telomerase mRNA levels decreased at 5 days after NMD injection. These results indicate that NMD treatment may modulate expression of telomerase to inhibit MNU-induced damage.

In conclusion, this study suggest that down-regulation of TGF-β and telomerase mRNA may play an important role in the protective effect of NMD against the MNU-induced damage, and it is also shown that the NMD protective effect can be responsible for influencing the content of GSH.

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