Short Communication

Green tea extract attenuates MNU-induced photoreceptor cell apoptosis via suppression of heme oxygenase-1

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Abstract: The effects of green tea extract (GTE) on *N*-methyl-*N*-nitrosourea (MNU)-induced photoreceptor cell apoptosis were examined, and the possible mechanisms of action of GTE were assessed. Alterations in the retinal morphological architecture were determined by hematoxylin-eosin staining, vimentin immunoreactivity, and photoreceptor cell apoptosis (TUNEL labeling). Expression of oxidant marker, heme oxygenase (HO)-1, mRNA levels in outer nuclear cells was assessed by laser capture microdissection (LCM). Sprague-Dawley rats were given 40 mg/kg MNU at 7 weeks of age in the absence and presence of 250 mg/kg GTE treatment (once daily from 3 days prior to MNU for a maximum 10 days). Although photoreceptor cell degeneration began 24 hr after MNU, the morphological effects of GTE at the time point were not definitive. However, GTE lowered TUNEL labeling and HO-1 mRNA expression. At 7 days after MNU, photoreceptor damage was attenuated by GTE treatment. Therefore, the ability of GTE to reduce MNU-induced photoreceptor cell apoptosis may be due to its antioxidant properties. (DOI: 10.1293/tox.2015-0052; J Toxicol Pathol 2016; 29: 61–65)

Key words: apoptosis, green tea extract, heme oxygenase-1, laser capture microdissection, *N*-methyl-*N*-nitrosourea, photoreceptor cell

Retinitis pigmentosa (RP) is one of the most common forms of inherited blindness worldwide; the pathology begins with early night blindness followed by peripheral visual field alterations (tunnel vision) and eventually leads to blindness[1, 2]. Identification of an effective therapeutic agent against RP is desired because of the current absence of such treatments for RP. Therefore, animal models of RP are important for understanding human RP. Among the various RP animal models, the *N*-methyl-*N*-nitrosourea (MNU) model causes photoreceptor cell apoptosis similar to the mechanism of human RP[3, 4]. MNU-induced photoreceptor cell apoptosis is useful in the search for human RP treatments, as it is complete within 7 days following a single systemic administration of MNU to various animal species[5, 6]. Oxidative stress is thought to play a major role in patients with RP[7]. Oxidative stress contributes to the progression of MNU-induced photoreceptor cell apoptosis[8, 9], and chemicals such as edaravone, a free radical scavenger[8], and curcumin, a naturally occurring yellow pigment of turmeric and constituent of curry powder[10] that possesses an antioxidant property, suppress photoreceptor cell loss in the MNU model. Edaravone and curcumin inhibit MNU-induced photoreceptor cell apoptosis by suppressing oxidative stress by decreasing the levels of 8-hydroxy-2'-deoxyguanosine[8, 10]. Green tea extract (GTE) has been reported to suppress MNU-induced photoreceptor apoptosis in Sprague-Dawley rats[11]. GTE possess pleiotropic effects such as antioxidative, anti inflammatory, and anticancerous properties[12]. GTE contains epigallocatechin gallate (EGCG) as a major ingredient, and EGCG acts as an antioxidant to reduce photoreceptor cell damage[13–15]. However, the mechanisms of action of GTE on MNU-induced photoreceptor apoptosis have not been elucidated.

Heme oxygenase (HO), the rate-limiting enzyme in the heme degradative pathway, is induced in many cell types in response to a variety of stimuli. Three HO isoenzymes are expressed, including HO-1, HO-2, and HO-3. Among these subtypes, HO-1 is an inducible isoform that is involved in the response to oxidative stress[16] and may be a marker of cellular responses to oxidative damage. HO activation plays a role in photoreceptor cell loss and functions protectively by reducing oxidative injury[17]. However, it is not clear whether GTE-induced changes in HO-1 expression occur in photoreceptor cells after MNU damage. In the present study, the role of HO-1 in MNU-induced photoreceptor cell apoptosis was studied.

Six-week-old female Sprague-Dawley rats [Crl:CD(SD)] purchased from Charles River Laboratories Japan (Osaka, Japan) were housed in groups of 3 in plastic cages with paper bedding (Paper Clean, SLC, Hamamatsu, Japan) in a specific pathogen-free environment maintained at 22 ±

2°C and 60 ± 10% relative humidity, with a 12-hr light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.; illumination intensity less than 60 lux at the cage level). Animals were maintained on a commercial pellet diet (CMF 30 kGy, Oriental Yeast, Chiba, Japan) and had free access to water. The study protocol and animal procedures were approved by the animal care and use committee of Kansai Medical University (Permit Number: 15-021). Throughout the experiments, animals were cared for in accordance with the Guidelines for Animal Experimentation of Kansai Medical University.

MNU in powder form was obtained from Sigma-Aldrich (St. Louis, MO, USA) and stored at 4°C in the dark. A 5 mg/mL solution was prepared by dissolving MNU in physiologic saline containing 0.1% acetic acid immediately before use. The GTE used was THEA-FLAN 90S (ITO EN, Ltd., Tokyo, Japan), a decaffeinated product. Approximately 70% of the polyphenols in GTE are tea catechins with a galloyl moiety; these include EGCG (54%), epicatechin (12.4%), gallocatechin gallate (2.8%), catechin gallate (0.4%), epigallocatechin (0.3%), gallocatechin (0.1%), caffeine (0.0%), and others (0.3%)[11]. GTE was dissolved at 40 mg/mL in sterile distilled water just prior to use.

Female rats received a single intraperitoneal injection of 40 mg/kg MNU at 7 weeks of age. A daily dose of 250 mg/kg GTE was orally administered by gastric intubation starting 3 days prior to MNU administration, and administration was continued once daily for a maximum 10 days (Fig. 1). MNU and GTE doses were determined according to our previous report[11]. Control rats received an equivalent volume of saline instead of MNU and sterile distilled water instead of GTE at the same time points. Rats were divided into the following groups: control (CTR), MNU-exposed and GTE-untreated (MNU⁺/GTE⁻), and MNU-exposed and GTE-treated (MNU⁺/GTE⁺) groups. On the day of MNU exposure, GTE was administered 2 hr prior to MNU injection. All rats were observed daily for clinical signs of toxicity and were weighed on the day of MNU administration and on the day of sacrifice. Rats were sacrificed 24 hr and 7 days after MNU administration; GTE was not administered on the day of sacrifice. At the time of sacrifice, animals were euthanized under isoflurane (Wako, Osaka, Japan) anesthesia, and both eyes were quickly removed from each animal.

One eye was fixed overnight in 10% neutral buffered formalin, and the other eye was fixed overnight in methacarn (60% methanol, 30% chloroform, and 10% acetic acid). Formalin- and methacarn-fixed samples were embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin (HE), and used for vimentin immunohistochemistry or TUNEL staining.

Methacarn-fixed sections were utilized to detect dynamic activities of Müller cells using anti-vimentin monoclonal antibody (×500; Santa Cruz, Dallas, TX, USA). The antigen antibody complexes were identified using a streptavidin biotin (LSAB) staining kit (Dako, Carpinteria, CA, USA), and the reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB).

Formalin-fixed retinal tissues were evaluated by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP
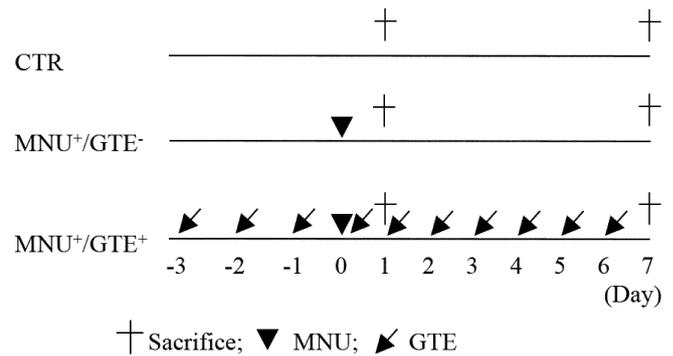

Fig. 1. Schematic representation of the experimental protocol. The experiment included control (CTR), MNU-exposed and GTE-untreated (MNU⁺/GTE⁻), and MNU-exposed and GTE-treated (MNU⁺/GTE⁺) groups. CTR rats received equivalent volumes of saline and sterile distilled water instead of MNU and GTE, respectively, at the same time points.

digoxigenin nick end-labeling (TUNEL) staining with an *in situ* apoptosis detection kit (TACS2 TdT; Trevigen, Gaithersburg, MD, USA), and the reaction products were visualized with DAB. Quantitative measurements were made from the central retina (approximately 400 μm from the optic nerve), and the number of TUNEL-positive cells per square millimeter was counted.

Three samples from 3 groups were used for laser capture microdissection (LCM). Formalin-fixed samples embedded in paraffin were sectioned at 10 μm × 3 slices. The sections were collected on Arcturus PEN Membrane Glass Slides (Applied Biosystems, Carlsbad, CA, USA), air dried overnight, and deparaffinized (2 minutes × 2 in xylol). Retinal layers were isolated using an ArcturusXT LCM System and Arcturus CapSure Macro LCM Caps (Applied Biosystems). LCM was performed by separately lifting the outer nuclear layer. RNA was isolated using a RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Carlsbad, CA, USA), according to the manufacturer's directions, and included a DNase treatment. Synthesis of cDNA was performed using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) and analyzed by real-time PCR.

Levels of mRNA were assessed by real-time quantitative PCR using a Rotor-Gene SYBR Green PCR Kit (QIAGEN, Hilden, Germany) and Rotor-Gene Q (QIAGEN) with two standard curve methods, according to the manufacturer's instructions. The following conditions were used: 95°C for 5 minutes (PCR initial activation step) followed by 40 cycles of 95°C for 5 sec (denaturation) and 60°C for 10 sec (combined annealing/extension). The mRNA level of the HO-1 gene was normalized to GAPDH. The GAPDH primers used were from the QuantiTect Primer Assay (QIAGEN). Master Mixes (22.5 μL) were added to 2.5 μL of cDNA. The HO-1 PCR primers produced a 110 bp product and were as follows: 5′-TCTATCGTGCTCGCATGAAC -3′ and 5′-CAGCTCCTCAAACAGCTCAA -3′[18, 19]. The intensity was quantified using image analysis software (Rotor-Gene Software ver 2.3.1.49, QIAGEN), and the results were standardized against the intensity of GAPDH.
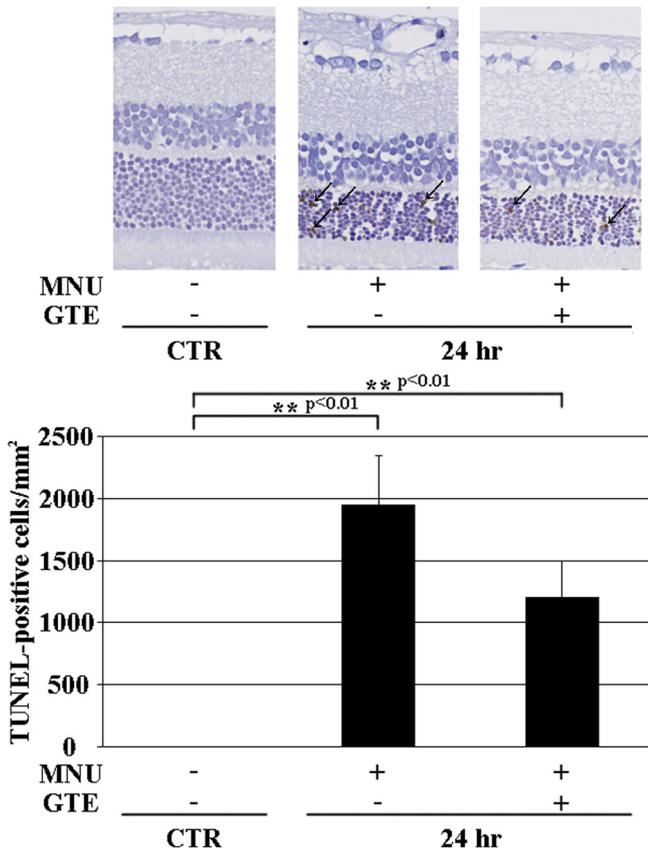
Fig. 2. Retinal morphology and vimentin immunoreactivity in normal animals and those treated with *N*-methyl-*N*-nitrosourea (MNU) (24 hr and 7 days), with or without green tea extract (GTE). Retinal regions depicted are as follows: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer; PEL, pigment epithelial layer.

Values were expressed as the mean ± SE and were analyzed using a one-tailed independent t-test for paired or unpaired samples after assuring the homogeneity of variances with the Excel Statistics Program File ystat 2008.xls. P values of <0.05 were accepted as statistically significant.

Twenty-four hours after MNU exposure, increased pyknosis and karyorrhexis of the photoreceptor nuclei and disarrangement of the photoreceptor segments were observed in HE sections (Fig. 2). However, although photoreceptor cell degeneration had begun by this time point, similar numbers of photoreceptor cells were found, and Müller cell proliferation was similar in both the GTE-treated and GTE-untreated rat retina according to HE staining and vimentin immunoreactivity, respectively. Therefore, the effect of GTE on disease suppression at this time point, from a structural perspective, was not conclusive. However, 7 days after MNU exposure, photoreceptor cells were virtually absent in the MNU$^+$/GTE$^-$ retina, while GTE treatment partially ameliorated MNU injury with partial retention of photoreceptor cells and suppression of Müller cell proliferation, as seen by vimentin staining, in the MNU$^+$/GTE$^+$ retina.

While the effect of GTE on disease suppression could be observed 7 days after MNU at the structural level, morphology alone could not be used to discern the effects of GTE 24 hr after MNU. Therefore, TUNEL staining and HO-1 expression were quantitatively evaluated 24 hr after MNU exposure. TUNEL-positive cells were not detected in the CTR retina, and MNU exposure produced more TUNEL-positive cells in the MNU$^+$/GTE$^-$ retina compared with the MNU$^+$/GTE$^+$ retina (Fig. 3). HO-1 mRNA expression was barely detectable in CTR photoreceptor cells, while significantly higher expression occurred in the MNU$^+$/GTE$^-$ retina; HO-1 expression tended to decrease in the MNU$^+$/GTE$^+$ retina compared with the MNU$^+$/GTE$^-$ retina (Fig. 4).

EGCG, a main component of GTE, has been shown to suppress photoreceptor cell damage caused by various agents[13–15]. In the present study, daily oral administration of 250 mg/kg GTE effectively attenuated MNU-induced photoreceptor cell apoptosis, and the compensatory proliferation of Müller cells was suppressed 7 days after MNU. At 24 hr after MNU, GTE suppressed photoreceptor cell apoptosis and downregulated HO-1 expression when retinal morphology was indistinguishable. LCM of the outer nuclear layer (photoreceptor cells) was performed, rather than isolation of
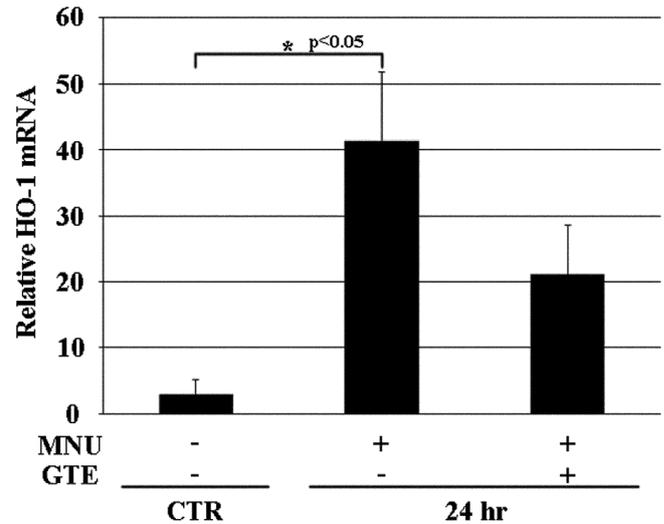
Fig. 4. mRNA expression of HO-1 in normal animals and those treated with *N*-methyl-*N*-nitrosourea (MNU) (after 24 hr) with or without green tea extract (GTE).

Fig. 3. TUNEL labeling in normal animals and those treated with *N*-methyl-*N*-nitrosourea (MNU) (after 24 hr) with or without green tea extract (GTE). Arrows indicate TUNEL-positive photoreceptor cells.

total retinal RNA[20], to obtain a more accurate comparison of mRNA HO-1 levels.

HO-1 mRNA and protein are barely detectable in the normal retina, whereas they are overexpressed in response to photooxidative (light) damage. The antioxidant dimethylthiourea suppresses light-induced photoreceptor damage by blocking the induction of HO-1; HO-1 induction is oxidatively driven[21] and produces an antioxidative effect. The iron chelators deferiprone[22] and α-lipoic acid[23], as well as curcumin[24], which has antioxidant properties, suppress light-induced photoreceptor damage accompanied by downregulation of HO-1 expression. The protease inhibitor bortezomib suppresses ischemia/reperfusion-induced inner retinal injury caused by oxidative stress; while expression of HO-1 mRNA is low in the normal retina, increased expression caused by ischemia/reperfusion is significantly lowered by bortezomib[9]. Antioxidants decrease oxidative stress and reduce induction of HO-1 in inner retinal cells and photoreceptor cells.

In general, antioxidant therapy has been reported to downregulate HO-1. This effect has been described extensively in oxidative stress-induced ocular disease[17]. However, upregulation of HO-1 is seen when curcumin treatment reduces H_2O_2-induced damage in cultured photoreceptor cells[24] and when resveratrol treatment reduces inner reti-

nal injury caused by retinal ischemic/reperfusion injury[25]. Adeno-associated virus moderates the levels of HO-1 expression in photoreceptors, resulting in cytoprotection from light damage by attenuating apoptosis[26]. However, while moderate levels of HO-1 expression protect against oxidative stress, higher levels of HO-1 expression result in susceptibility to oxidative stress, indicating the existence of paradoxical HO-1 actions[27]. As HO-1 expression increases after exposure to oxidative stress compared with the level in cells not exposed to stress, HO-1 acts as a defense mechanism. However, the present study and many other studies indicate that excessive stress can produce extremely high HO-1 expression, which may cause deleterious effects. Conversely, during moderate levels of stress, antioxidant treatment can produce increased HO-1 expression. Differences in the dose and degree of oxidative stress or in measurement timing may produce different results[27–29]. Using the present MNU dose regimen, GTE effectively attenuated photoreceptor cell apoptosis by lowering HO-1 mRNA expression.

Disclosure of potential conflicts of interest: In connection with this paper, there are no conflicts of interest with any companies to be disclosed.

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