

# N-acetyl-L-cysteine amide protects retinal pigment epithelium against methamphetamine-induced oxidative stress

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## ABSTRACT

**Methamphetamine (METH), a highly addictive drug used worldwide, induces oxidative stress in various animal organs. Recent animal studies indicate that methamphetamine also induces oxidative stress in the retina, which is an embryonic extension of the forebrain. The aim of this study, therefore, was to evaluate the protective effects of N-acetylcysteine amide (NACA) against oxidative stress induced by METH in retinal pigment epithelium (RPE) cells. Our studies showed that NACA protected against METH-induced oxidative stress in retinal pigment epithelial cells. Although METH significantly decreased glutathione (GSH) levels and increased reactive oxygen species (ROS) and malondialdehyde (MDA) levels, these returned to control levels with NACA treatment. Overall observations indicated that NACA protected RPE cells against oxidative cell damage and death by inhibiting lipid peroxidation, scavenging ROS, increasing levels of intracellular GSH, and maintaining the antioxidant enzyme activity and the integrity of the bloodretinal barrier (BRB). The effectiveness of NACA should be further evaluated to determine its potential for the treatment of numerous retinal diseases caused by oxidative stress.**

**Keywords:** Methamphetamine; Oxidative Stress; Antioxidant; Reactive Oxygen Species; N-Acetylcysteine Amide; Blood-Retina Barrier

## 1. INTRODUCTION

Methamphetamine (METH), a highly addictive psychostimulant, is abused by approximately 35 million people

worldwide [1]. A dramatic increase in METH-related emergency department visits is alarming, with >50% involving young adults between the ages of 18 - 34 years [2]. The relative ease of METH's availability, coupled with its toxicity, has resulted in increased numbers of associated medical complications and fatalities [2,3].

Oxidative stress is believed to play a crucial role in METH-induced toxicity and is supported by several studies which reported decreased glutathione (GSH) levels, reduced levels and activities of antioxidant enzymes, and increased lipid peroxidation and protein carbonylation, all hallmarks of oxidative stress [4-8]. In the case of the brain, it is believed that, initially, METH causes a massive release of dopamine by inhibiting monoamine oxidase activity and dopamine uptake [9]. With higher doses, however, it causes dopamine depletion by degenerating dopaminergic terminals, damaging dopaminergic neurons, and decreasing dopamine transporter numbers [10,11]. Dopamine then reacts with molecular oxygen to form reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, and hydroxyl free radicals, resulting in a condition known as oxidative stress [11] and causes neuronal death by apoptosis [12].

Oxidative stress acts as a propagating force in the pathogenesis of many ocular disorders, including cataracts, glaucoma, diabetic retinopathy, HIV-related retinopathy, and age-related macular degeneration (AMD) [13-15]. Oxidative stress possibly contributes to the death of retinal cells and degeneration of the macula [16-20]. Free radical formation in a developing retina has been reported to be induced by xenobiotics, e.g., environmental pollutants, pharmacological substances, and alcohol [21].

METH abuse is implicated in a number of serious ocular pathologies, including corneal ulceration, retinal vasculitis, episcleritis, scleritis, panophthalmitis, endophthalmitis, and retinopathy [22-24]. It has been reported that METH induces oxidative stress in the retina and

adversely affects the dopaminergic system of the rat retina [25], particularly during central nervous system (CNS) development. Prudencio *et al.* [26] demonstrated that METH altered retinal plasma membrane integrity, esterase activity, and/or pH in rat retina homogenates. Another study reported exacerbation of damaging effects of kainic acid on the retina by METH [27]. Under physiological conditions, high levels of antioxidant enzymes and small antioxidant molecules, particularly glutathione (GSH), in the Muller (glial) cells protects retinal pigment epithelium (RPE) cells against oxidative stress. GSH in the Muller cells is depleted under oxidative stress and, since GSH cannot be transported directly into the cells, the need arises for permeable compounds that can increase intracellular GSH levels. One such compound, known to increase intracellular GSH levels in cells, is the low molecular weight thiol antioxidant, N-acetylcysteine amide (NACA). Previous work by our group has demonstrated that NACA restored the levels of GSH, and scavenged the ROS produced in human brain endothelial cells, upon treatment with METH [4].

Even though the adverse effects of METH on the brain are linked to oxidative stress, little is known about its effect on the retina. Considering the ability of NACA to protect cells from oxidative stress [28-30], the effectiveness of this antioxidant was evaluated as a treatment option for METH-induced oxidative damage to RPE cells. Understanding NACA's protective role against METH-induced oxidative damage to RPE cells would help develop NACA as a potential therapeutic agent for treating RPE cells against numerous oxidative stress-related ocular diseases.

## 2. MATERIALS AND METHODS

### 2.1. Materials

The retinal pigment epithelial cell line, ARPE-19, was purchased from the American Type Culture Collection (ATCC # CRL-2302) (Manassas, VA), and the N-acetylcysteine amide (NACA) from Dr. Glenn Goldstein of David Pharmaceuticals in New York, NY. HPLC chemicals were obtained from Fisher Scientific (Pittsburgh, PA). Cell culture reagents were purchased from ATCC in Manassas, VA, and Calcein AM was bought from Biotium, Inc. (Hayward, CA). The National Institute on Drug Abuse (NIDA) provided methamphetamine, while other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

### 2.2. ARPE-19 Cell Culture and Treatment

The ARPE-19 cells were cultured in a one-to-one ratio of DMEM: F-12 culture medium, and supplemented with 10% (v/v) FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Cells were maintained in a 37°C incubator

and supplied with 95% air and 5% CO<sub>2</sub>. Cells between passage numbers 26 and 32 were used for experiments.

### 2.3. Determination of Cell Viability

The ARPE-19 cells were seeded in a 96-well tissue culture plate, at a density of approximately  $1.25 \times 10^4$  cells/well, for a day. The media was then discarded and the cells were treated with various concentrations of METH and NACA in serum-free media for 24 hours and 2 hours, respectively. Protective effects of NACA were studied by pre-incubating cells with NACA for 2 hours, followed by treatment with 500 µM of METH for 24 hours. After 24 hours of METH treatment, the medium was discarded and a Calcein AM assay KIT (Biotium, Inc. CA) was used to determine cell viability relative to the control group [31]. The cells were then washed three times with PBS, and 100 µL of 2.0 M Calcein AM in PBS were added to each well for 30 minutes at 37°C. The fluorescence was measured with an excitation wavelength at 485 nm and an emission wavelength of 530 nm, using a microplate reader (FLUOstar, BMG Labtechnologies, Durham, NC, USA).

### 2.4. Intracellular ROS Measurement

Intracellular ROS generation was measured using a well-characterized probe, 2', 7'-dichlorofluorescein diacetate (DCFH-DA) [32]. ARPE-19 cells were seeded at a density of  $1.25 \times 10^4$  cells/well in a 96-well plate. DCFH-DA is hydrolyzed by esterases to dichlorofluorescein (DCFH), which was trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by the action of cellular oxidants. In groups with NACA pretreatment, media containing 1 mM NACA was added and incubated for 2 hours. Once pretreated, the cells were washed twice with PBS and incubated with a solution of 50 µM DCFH-DA in phenol red free media for 30 minutes. This was followed by washing the cells twice with PBS, and the respective groups were then dosed either with 500 µM METH or plain media for 4 hours and then fluorescence was determined at 485 nm excitation and 520 nm emission, using a microplate reader (FLUOstar, BMG Labtechnologies, Durham, NC, USA).

### 2.5. Experimental Design for Oxidative Stress Parameters

Parameters, including GSH, MDA, and activities of glutathione peroxidase and catalase, were measured after the cells were treated, as described below. After seeding the cells, the flasks were divided into the following four groups: 1) control; 2) NACA-only; 3) METH-only; and 4) METH + NACA. In groups with NACA pretreatment, media containing 1 mM NACA was added and incubated

for 2 hours. After pretreatment, the media in the control and NACA-only groups were replaced with plain media, while both of the remaining two groups received media containing METH for 24 hours. The cell pellets obtained were then further processed for appropriate assays.

### 2.5.1. Determination of Glutathione (GSH)

The levels of GSH in the tissues were determined by RP-HPLC, according to the method developed in our laboratory [33]. The HPLC system (Thermo Electron Corporation) consisted of a Finnigan Spectra System vacuum membrane degasser (model SCM1000), a gradient pump (model P2000), autosampler (model AS3000), and a fluorescence detector (model FL3000) with  $\lambda_{\text{ex}} = 330$  nm and  $\lambda_{\text{em}} = 376$  nm. The HPLC column used was a Reliasil ODS-1 C18 column (5  $\mu\text{m}$  packing material) with 250 mm  $\times$  4.6 mm i.d. (Column Engineering, Ontario, CA). The mobile phase (70% acetonitrile and 30% water) was adjusted to a pH of 2 with acetic acid and o-phosphoric acid. The N-(1-pyrenyl)-maleimide (NPM) derivatives of GSH were eluted from the column isocratically at a flow rate of 1 mL/min. The cell samples were homogenized in a serine borate buffer (100 mM Tris HCl, 10 mM borate, 5 mM serine, 1 mM diethylenetriaminepentaacetic acid), centrifuged, and 50  $\mu\text{L}$  of the supernatant were added to 230  $\mu\text{L}$  of HPLC grade water and 750  $\mu\text{L}$  of NPM (1 mM in acetonitrile). The resulting solution was incubated at room temperature for 5 minutes, and the reaction was stopped by adding 10  $\mu\text{L}$  of 2 N HCl. The samples were then filtered through a 0.45  $\mu\text{m}$  filter and injected into the HPLC system.

### 2.5.2. Determination of Malondialdehyde (MDA)

The MDA levels were determined according to the method described by Draper *et al.* [34]. Briefly, 550  $\mu\text{L}$  of 5% trichloroacetic acid (TCA) and 100  $\mu\text{L}$  of 500 ppm butylated hydroxytoluene (BHT) in methanol were added to 350  $\mu\text{L}$  of the cell homogenates, and boiled for 30 minutes in a water bath. After cooling on ice, the mixtures were centrifuged, and the supernatant collected was mixed 1:1 with saturated thiobarbituric acid (TBA). The mixture was again heated in a water bath for 30 minutes, followed by cooling on ice. 500  $\mu\text{L}$  of the mixture was extracted with 1 mL of nbutanol and centrifuged to facilitate the separation of phases. The resulting organic layers were first filtered through 0.45  $\mu\text{m}$  filters and then injected into the HPLC system (Shimadzu, US), which consisted of a pump (model LC-6A), a Rheodyne injection valve and a fluorescence detector (model RF 535). The column was a 100 mm  $\times$  4.6 mm i.d. C18 column (3  $\mu\text{m}$  packing material, Astec, Bellefonte, PA). The mobile phase used contained 69.4% sodium phosphate buffer, 30% acetonitrile, and 0.6% tetrahydrofuran. The fluorescent product was monitored at  $\lambda_{\text{ex}} = 515$  nm and  $\lambda_{\text{em}} = 550$

nm. The concentrations of the TBA-MDA complex in the mixture was determined by using the calibration curve obtained from a 1,1,3,3,-tetraethoxypropane standard solution. This method differs from commonly used TBARS assay in that it specifically determines TBA-MDA adducts by HPLC.

### 2.5.3. Measurement of Catalase (CAT) Activity

Catalase activity was measured according to the method described by Aebi [35]. Briefly, it was measured spectrophotometrically at a wavelength of 240 nm, in the supernatant of the cell homogenate, following the exponential disappearance of hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 10 mM). The catalase activity was calculated from the equation  $A_{60} = A_{\text{initial}}e^{-kt}$ , where  $k$  represents the rate constant,  $A_{\text{initial}}$  is the initial absorbance and  $A_{60}$  is the absorbance after 60 seconds have passed.

### 2.5.4. Measurement of Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity was determined using a glutathione peroxidase colorimetric assay kit purchased from Oxis Research (Foster City, CA). The cell samples were homogenized in 50 mM of phosphate buffer (pH 7.4), containing 1.0 mM EDTA, and then centrifuged at  $7500 \times g$  for 10 minutes. The resulting supernatant was collected to be used for the assay while the remaining debris was discarded. In brief, the assay buffer, supernatant, and NADPH reagent (containing glutathione reductase, GSH, and NADPH) were placed in a cuvette and the reaction initiated by the addition of t-butylhydroperoxide (tBHP). The decrease in absorbance at 340 nm was recorded for 3 minutes and the change in  $A_{340}/\text{min}$  from the initial linear portion of the curve was used to calculate the GPx enzyme activity. The GPx activity was calculated using the extinction coefficient of NADPH ( $6220 \text{ M}^{-1}\cdot\text{cm}^{-1}$ ) and expressed as U/mg of protein.

### 2.5.5. Dextran Permeability Study and Trans-Endothelial Electrical Resistance (TEER) Measurement

ARPE-19 cells were seeded at a density of  $1.5 \times 10^5$  cells/well onto collagen-coated inserts with a pore size of 0.4  $\mu\text{m}$  and allowed to form a monolayer. Trans-endothelial electric resistance (TEER) measurement by an EVOM voltohmmeter (World Precision Instrument, Sarasota, FL, USA) assessed the tightness of the ARPE monolayer [4]. The cell monolayer was then treated as described in Experimental Design. After this, the media was replaced with 150  $\mu\text{L}$  of fresh medium. The insert containing the cell monolayer was then transferred to a fresh plate containing 500  $\mu\text{L}$  of serum-free medium. The TEER reading was recorded immediately and TEER

values were calculated as: Resistance  $\times$  0.32 cm<sup>2</sup> (insert surface area). Thus, resistance is proportional to the effective membrane.

## 2.6. Determination of Protein

Protein levels of the cell samples were measured by the Bradford method [36] using bovine serum albumin as standard.

## 2.7. Statistical Analysis

Statistical significance was calculated using an unpaired two-tailed student's *t* test in which "\*" represents a *p*-value  $\leq$  0.05 when compared to the control group, and "\*\*\*" represents a *p*-value  $\leq$  0.05 when compared to the METH group. All reported values were represented as mean  $\pm$  S.D. of triplets.

## 3. RESULTS

### 3.1. Protection from METH Toxicity

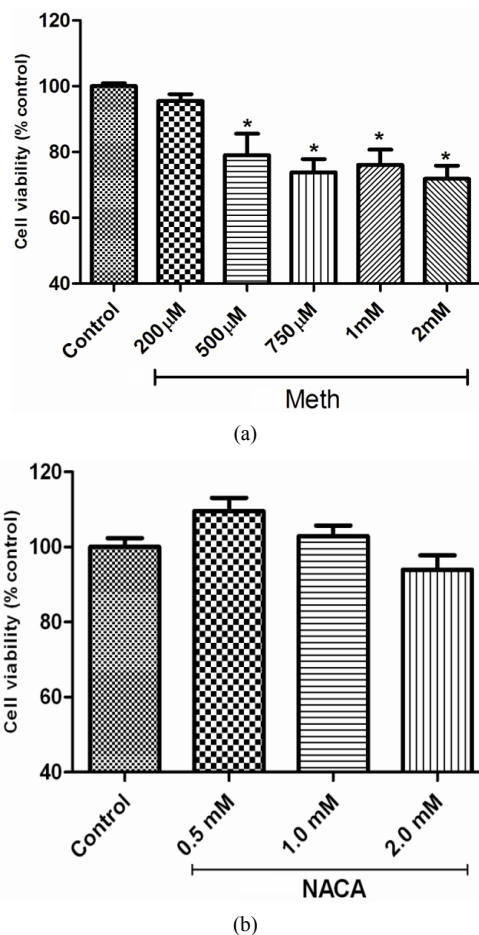
Cell viability was measured as a function of METH and NACA dose to determine the optimum concentrations for both METH and NACA, respectively. **Figures 1(a)** and **(b)** revealed that a concentration of 500  $\mu$ M METH and 1 mM NACA are the minimum toxic doses of METH and NACA, for ARPE-19 cells and, therefore, these doses were selected for further experiments. **Figure 2** shows that pretreatment with 1.0 mM of NACA successfully protected the ARPE-19 cells from METH-induced death, thereby increasing viability to  $\sim$ 92% of that of control levels.

### 3.2. Intracellular ROS Measurements

After treatment with 500  $\mu$ M of METH, ROS production was found to have increased. METH (500  $\mu$ M) induced an increase in the DCF fluorescence by approximately 30%, as compared to that of the control (**Figure 3**), while pretreatment with 1 mM NACA (2 hours prior to exposure) completely erased this increase. NACA alone did not significantly alter DCF fluorescence compared to that of the control.

### 3.3. Intracellular Glutathione

**Figure 4** shows the effect of METH on cellular GSH levels in ARPE-19 cells in the presence and absence of NACA. A 24-hour exposure to METH resulted in an increase in cellular oxidative stress. Treatment with 500  $\mu$ M of METH altered the GSH level to 80% of that of the control. A 500  $\mu$ M treatment of METH, with a 1 mM pretreatment of NACA, was significantly different from that of the METH group alone, with *p*  $\leq$  0.05 and a GSH concentration of 98% of that of the control group. The

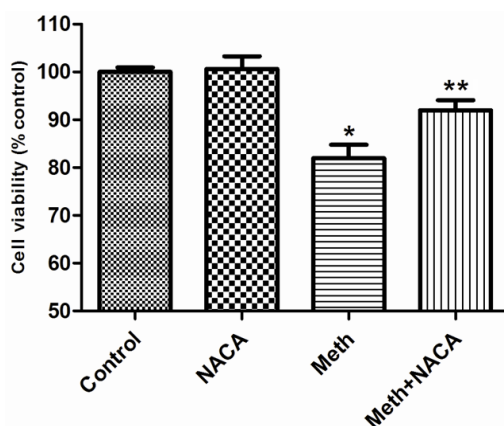


**Figure 1.** Cell viability of ARPE-19 cells. (a) Cell viability after varying treatments with METH for 24 hours. A concentration of 500  $\mu$ M was found to be the minimum toxic dose of METH for ARPE-19 cells and was used as the optimum dose for all experiments. Values are represented as mean  $\pm$  S.D. (\*) refers to significant differences from the control with *p*  $\leq$  0.05. The graph is representative of at least three independent experiments; (b) Cell viability of ARPE-19 cells after treatment with NACA for 2 hours. ARPE-19 cells were treated with various concentrations of NACA (0.5 mM, 1 mM, and 2 mM). After 2 hours of treatment, cell viability was quantified by a Calcein AM assay and a concentration of 1 mM NACA was used for the rest of the experiments in this study. Values are represented as mean  $\pm$  SD.

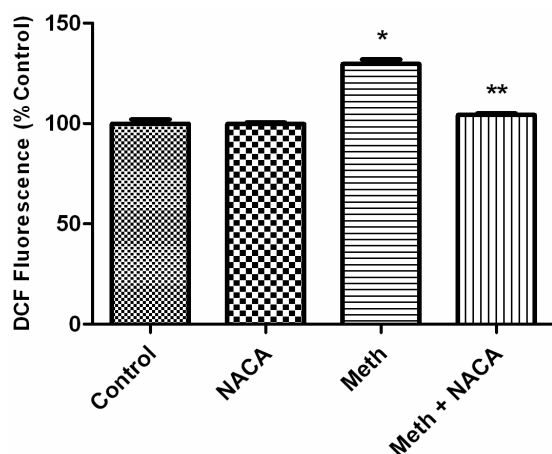
NACA alone treated group was found to have no significant changes in the GSH levels, as compared to that of the control group.

### 3.4. Lipid Peroxidation Byproduct: MDA

MDA was used as an index of lipid peroxidation. MDA levels were determined in ARPE-19 cells pretreated with NACA for 2 hours, followed by exposure to METH for

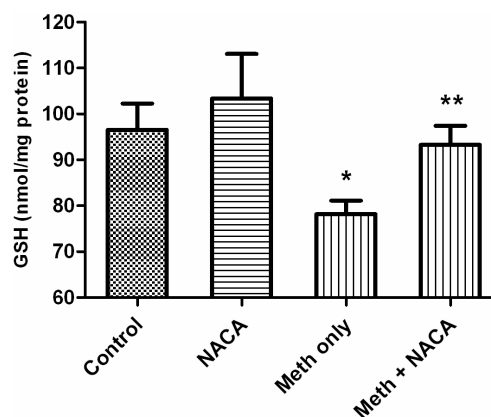


**Figure 2.** Cell viability of ARPE-19 cells after treatment with METH and NACA. Cell viability was quantified by a Calcein AM assay 24 hours after exposure to METH, following a 2-hour pretreatment with NACA. Treatment with METH (500  $\mu$ M) alone was seen to significantly decrease cell viability. NACA (1.0 mM) was seen to protect against some METH-induced cell toxicity. \* $p \leq 0.05$  compared to the control group, and \*\* $p \leq 0.05$  compared to the corresponding value of the METH group.

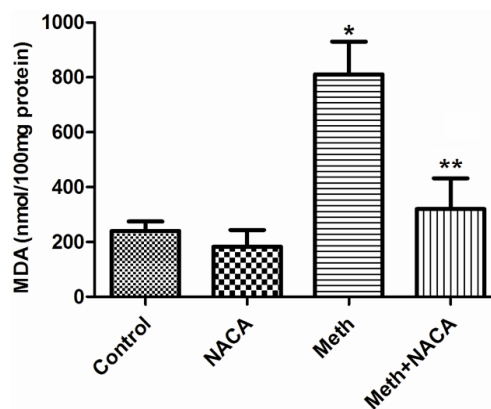


**Figure 3.** ROS levels in ARPE-19 cells after treatment with METH and NACA. ROS levels were measured after 4 hours of treatment for control, NACA, METH, and METH + NACA groups. The ROS level in the NACA-only group was very similar to that of the control group. However, a concentration of 500  $\mu$ M of METH significantly decreased the ROS level (\* $p \leq 0.05$ ). Pretreatment with 1 mM of NACA returned the ROS level to near that of the control level (\*\* $p \leq 0.05$ ). The results are representative of at least three independent experiments.

24 hours. METH-treated cells had significantly higher levels (about four fold) of MDA, as compared to those of the control (Figure 5). Pretreatment with 1 mM of NACA completely reduced this increase, with MDA levels becoming nearly the same as those of the control. The NACA-only treated group showed no significant difference when compared to the control.



**Figure 4.** Intracellular GSH levels in ARPE-19 cells after treatment with METH and NACA. GSH levels were measured after 24 hours of treatment for control, NACA, METH, and METH + NACA groups. The GSH level in the NACA-only group was similar to that of the control. Exposure to METH (500  $\mu$ M) significantly decreased intracellular GSH (\* $p \leq 0.05$ ). Pretreatment with NACA (1 mM), 2 hours before the addition of METH, prevented such a dramatic decrease (\*\* $p \leq 0.05$ ). At least three independent experiments were performed.



**Figure 5.** MDA levels in ARPE-19 cells after treatment with METH and NACA. It was found that after 24 hours the MDA levels significantly increased. MDA levels in the NACA-only group were very similar to those of the control group; therefore, this was not included in the figure. METH (500  $\mu$ M) induced a significant increase in the MDA level. Addition of 1 mM of NACA pretreatment decreased lipid peroxidation significantly below that of METH alone. Values represent the mean  $\pm$  SD. “\*” refers to significant differences from the control with  $p \leq 0.05$  and “\*\*” refers to the significant differences from the METH-only group. The graph is representative of three independent experiments.

### 3.5. Catalase and Glutathione Peroxidase Activity

Effects of METH on the enzyme activities of catalase

(CAT) and glutathione peroxidase (GPx) were measured (Table 1). METH administration markedly decreased the levels of GPx and CAT enzyme activities by about 30%, as compared to the control group. Pretreatment with NACA significantly attenuated this decrease to 10% of the control GPx activity level but didn't alter the CAT activity level. In both of these experiments, NACA alone did not significantly alter the results from those of the control group; all experiments were conducted in triplicate.

### 3.6. Dextran Permeability and TEER Assays

The cell permeability assay and TEER are especially important in this study because they simulated the integrity of the BRB. Permeability studies showed that treatment with 500  $\mu$ M of METH increased permeability by approximately 15%, as compared to that of the control (Figure 6(a)), while pretreatment with NACA significantly reduced this permeability to levels similar to that of the control group. As in the permeability study, TEER results (as shown in Figure 6(b)) also indicate the protective effect of NACA. Exposure to 500  $\mu$ M resulted in a decrease in TEER by about 10%, as compared to that of the control, while pretreatment with NACA gave resistance values only slightly less than control levels. Treatment with NACA alone did not significantly alter TEER, as compared to that of the control. All experiments were conducted in triplicate.

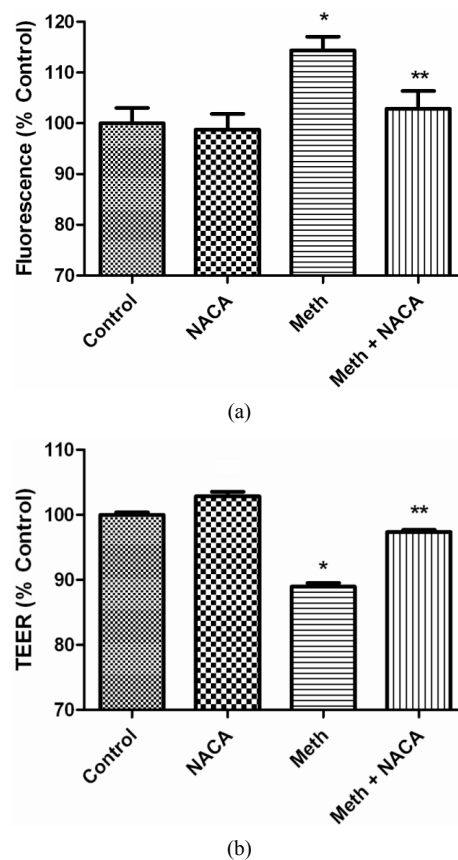
## 4. DISCUSSION

Retinal pigment epithelial (RPE) cells form a nonrenewable monolayer of specialized cells derived from the neuroectoderm and are present at the interface between choroidal capillaries and the neurosensory retina. They play numerous roles, including regulation of ion and metabolite transport, phagocytosis of shed photoreceptor outer segments, metabolism of retinol, formation of the outer blood-retinal barrier, maintenance of the extracellular matrix, and metabolism of numerous potential

**Table 1.** Catalase and GPx activity in ARPE-19 cells after treatment with Meth and NACA.

Group	CAT (mU/mg protein)	GPx (mU/mg protein)
Control	19.5 $\pm$ 3.1	56.6 $\pm$ 9.8
NACA only	20.1 $\pm$ 5.2	59.5 $\pm$ 8.4
Meth only	14.1 $\pm$ 0.6*	39.2 $\pm$ 1.7*
Meth + NACA	19.1 $\pm$ 7.8	51.0 $\pm$ 7.4**

Values represent the mean  $\pm$  SD. "\*" refers to significant differences from the control with  $p \leq 0.05$  and "\*\*" refers to the significant differences from the METH-only group. The values are representative of three independent experiments.



**Figure 6.** Protective effects of NACA on dextran permeability or TEER for ARPE-19 cells treated with METH. Cells were seeded onto a collagen-coated insert with a pore size of 0.4  $\mu$ m at a density of  $1.5 \times 10^5$ /well, and allowed to culture until a monolayer formed. The cell monolayer was then treated with 500  $\mu$ M of METH with or without the 2-hour pretreatment using 1 mM of NACA for 24 hours. (a) Significantly higher permeability of dextran across the cell monolayer was observed after treatment with METH, as compared to the control (\* $p \leq 0.05$ ). Pretreatment with NACA, however, was shown to reduce this permeability (\*\* $p \leq 0.05$ ) to levels similar to control values; (b) Cells treated with only METH resulted in significantly decreased TEER values, as compared with both control (\* $p \leq 0.05$ ) and METH + NACA (\*\* $p \leq 0.05$ ) groups. At least three independent experiments were performed for dextran permeability or TEER assays.

toxins, such as the free radicals and peroxides [37]. Dysfunction of the retinal pigment epithelium (RPE) is implicated in many ocular pathologies, including vision loss.

Oxidative effects of METH on RPE cells have not been extensively studied, despite the fact that METH abuse is quite prevalent worldwide, due to the euphoria produced, its wide availability, and relatively low cost. Herein, we report the *in vitro* effects of METH on human

retinal cells (ARPE-19 cell line) and the role of a novel thiol antioxidant, N-acetylcysteine amide (NACA), in preventing RPE cell death and maintaining RPE redox status.

METH-treated ARPE cells showed a significant increase in ROS accumulation, indicating oxidative stress. This resulted in depletion of GSH, the key thiol antioxidant, leading to disruption of the thiol redox state and, ultimately, cell death. A possible explanation for a decrease in GSH levels is the reduced activity of the enzymes involved in GSH synthesis and/or the oxidation of GSH to GSSG under oxidative stress. Pretreatment with NACA increased the GSH levels and cell viability in the METH-treated group, indicating that NACA had replenished the GSH levels in these cells and prevented oxidative stress-induced cell death. These results are also in accordance with our previous study on METH-induced toxicity [4]. The protective effect of NACA is probably mediated by NACA's ability to increase GSH biosynthesis by reducing extracellular cystine to cysteine, and/or by supplying the sulfhydryl groups that can stimulate GSH biosynthesis, or by conversion of GSSG to GSH [38].

Lipid peroxidation is thought to play an important role in the pathogenesis of age-related macular degeneration, and is a consequence of increased levels of ROS in the macula [39]. The membranes of outer segments of photoreceptors are rich in polyunsaturated fatty acids (PUFAs), which are highly susceptible to radical damage and peroxidation [40]. Increased levels of lipid peroxidation products can also lead to lysosomal dysfunction in the RPE [39]. Therefore, MDA, an important lipid peroxidation by-product, showed a significant increase in the levels when measured in ARPE-19 cells treated with METH. Lipid peroxidation is a key mechanism by which METH and its radicals induce cell death. It has been postulated that radicals attack membrane lipids and initiate a chain of events leading to lipid peroxidation [41,42]. Pretreatment with NACA successfully reversed the increase in MDA levels, indicating that NACA can protect RPE cells from lipid peroxidation. Preventing this is important for improving RPE lysosomal function, as highlighted in a study of the effects of N-acetylcysteine on lysosomal volume and metabolism in RPE cells loaded with regular or oxidized human and porcine rod outer segments [43]. These results are also in line with a previously reported increase in MDA levels [25]. Concomitant reduction of GSH levels (a substrate for glutathione peroxidase) might have hampered the decomposition of lipid peroxides in METH-treated animals. NACA was able to break lipid peroxidation chain reaction by supplying an adequate amount of GSH as a substrate for glutathione peroxidase to effectively decompose lipid peroxides, thus reducing MDA levels.

Antioxidant enzymes are involved in the detoxification

of lethal peroxides inside the cells. A significant reduction in the activity of GPx and catalase was observed after METH treatment. A decrease in GPx activity may have been partially due to diminished GSH levels that GPx needs as a substrate. We previously reported decreased GPx activity, due to METH, in human brain microvascular endothelial cell culture [36]. Our results are also in accordance with the previously reported decrease in GPx activity after METH administration [44-47]. In contrast, the increased GPx activity in rat brains, observed by Flora *et al.* [48] after METH administration, could have been due to the enhanced levels of cellular glutathione observed in their study. On the other hand, no change in GPx activity in rat brains was reported by Moszczynska *et al.* [6] in response to METH.

A decrease in catalase activity has been reported for various tissues undergoing oxidative stress [49]. However, an increase in catalase activity in RPE cells, challenged with H<sub>2</sub>O<sub>2</sub>, has also been reported [50]. On the other hand, Melo *et al.*, [51] were unable to find differences in the catalase activity in the retinas of METH-treated rats. The response of this antioxidant enzyme to oxidative agents could be tissue/organ specific and may be adaptive. So far, it is poorly understood through which mechanisms METH affects the function of catalase. However it is thought that the tyrosyl radical in the catalase enzyme is involved in the catalytic activity of the enzyme [52,53]. Nitration or a reaction of free radicals with this tyrosine residue could lead to inactivation of the enzyme. Phosphorylation of the tyrosine residue on catalase has also been reported and may lead to decreased activity of the catalase enzyme [54]. NACA pretreatment maintained GPx and catalase activity in the METH + NACA group, possibly due to the scavenging of free radicals by NACA and by providing GSH, which is a substrate for GPx.

In the retina, the RPE forms the blood-retinal barrier (BRB) by forming tight junctions that control the exchange of nutrients and metabolites between the retina and the underlying choriocapillaries. It is thought that oxidative stress may induce changes in the BRB, which may contribute to the pathogenesis of retinal degeneration. It was, therefore, important to determine if NACA could protect the junctional integrity of ARPE-19 cells exposed to oxidative stress. NACA prevented a METH-induced decrease in TEER which led to protection of cellular homeostasis and outer BRB integrity. A similar decrease in TEER has been reported in another study, where ARPE-19 cells were exposed to hydrogen peroxide [55].

## 5. CONCLUSIONS

This study demonstrates that METH induces oxidative stress in ARPE cells by depleting GSH and that NACA, a

GSH prodrug, protected ARPE cells against METH-induced oxidative damage, possibly by a variety of mechanisms, including scavenging of ROS; increasing the activity of the detoxification enzyme, glutathione peroxidase (GPx), by providing GSH which is a limiting substrate for GPx activity and halting the production of further ROS and lipid peroxidation. GSH might also provide protection by recycling other key antioxidants like vitamin E and vitamin C.

A logical approach, therefore, to enhancing antioxidant protection, would be the use of pharmacologic doses of highly-active antioxidants that can be delivered to the sites of damage caused by ROS. Epidemiological data supports the beneficial effects of antioxidant supplementation to decrease these oxidative processes in the retina and retinal pigment epithelium [56]. Studies have shown protective effects of NACA against radiation-induced toxicity, HIV-protein-induced oxidative stress, allergic airway disease, pollutant-induced lung disease, and lead-induced cell death [29-31,49].

The development of a useful antioxidant agent in the form of eye drops would significantly improve the severity of eye disorders in METH abusers. Our current and future work would provide additional momentum for research on antioxidant-based approaches to treating degenerative eye conditions. Further investigation could help determine NACA's efficacy in METH-abuse treatment and an evaluation of the histology in METH-treated animals would provide additional information on its therapeutic potential. Remarkable protection shown by NACA in previous studies indicates that NACA may be developed into a potential treatment option in the prevention or delay of numerous retinal diseases caused by oxidative stress, including METH abuse.

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