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Impaired Forebrain Cellular Bioenergetics Following Acute Exposure to Ammonia

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Abstract

Introduction: The main purpose of this study was to report on the acute neurotoxicity of ammonia, using cellular respiration and ATP as surrogate metabolic biomarkers. Cellular respiration (mitochondrial O_2 consumption), ATP and glutathione (GSH) were measured in forebrain specimens after intraperitoneal administration of 3.8 to 28.8 µmol/g ammonia to Taylor Outbred mice; the lethal dose was ≥ 30 µmol/g.

Methods: Cortical fragments were collected in phosphate-buffered saline plus 5 mM glucose and immediately processed for measuring O_2 consumption, using a phosphorescence O_2 analyzer. Cellular ATP was determined with the luciferase/luciferin system. Cellular GSH was labeled with monobromobimane and the bimane derivatives were separated on HPLC and detected by fluorescence. In the presence of the tissue specimen in a vial sealed from air, O_2 concentration declined linearly with time, confirming zero-order kinetics of O_2 consumption. This process was inhibited by cyanide, confirming the oxidation occurred in the respiratory chain.

Results: For untreated mice, cellular respiration was 0.32 ± 0.12 µM O_2 min^{-1} mg^{-1} (n=36 mice), ATP was 238 ± 45 $pmol.mg^{-1}$ (n=9 mice) and GSH was 300 ± 50 $pmol.mg^{-1}$ (n=6 mice). For treated mice, cellular respiration was 0.26 ± 0.09 µM O_2 min^{-1} mg^{-1} (n=22 mice; 19% lower, p=0.034), ATP was 183 ± 34 pmol mg^{-1} (n=9 mice; 23% lower, p=0.008) and GSH was 320 ± 160 pmol mg^{-1} (n=6 mice, p=0.700).

Conclusion: Acute ammonia intoxication significantly lowered forebrain cellular bioenergetics. These changes would be are difficult to monitor at lethal doses, but the results are expected to be more pronounced.

Keywords: Ammonia; Neurotoxicity; Bioenergetics; Cellular respiration; Urea; Hepatic encephalopathy; Mitochondria.

Abbreviations: GSH: Glutathione; CNS: Central nervous system; NMDA: N-methyl-D-aspartate; ROS: Reactive oxygen species; mBBr: Monobromobimane; DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid); NEM: N-ethylmaleimide; MSA: Methanesulfonic acid; PBS: Phosphate-buffered saline; Pd phosphor: Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin; CN: cyanide; GO: Glucose oxidase

Introduction

Cellular bioenergetics describes the biochemical reactions involved in energy metabolism; and cellular respiration (mitochondrial O_2 consumption) describes the delivery of metabolites and O_2 to the mitochondria, oxidations of reduced metabolic fuels with passage of electrons to O_2, and the synthesis of ATP. Disturbances in these processes may alter tissue O_2 consumption and are investigated here in mouse forebrain specimens exposed to ammonia.

Hyperammonia invokes deleterious effects on CNS cells. This astrocyte-specific toxicity involves complex metabolic abnormalities (increased pyruvate and decreased citrate, suggesting impaired flux of the reducing equivalents to the mitochondria), mitochondrial dysfunction (permeability transition induction), cell swelling (intra-glial glutamine accumulation), activation of N-methyl-D-aspartate receptors, generation of reactive oxygen species and nitric oxide, and cerebral edema [1-7].

The encephalopathy in hyperammonia manifests as progressive lethargy, central tachypnea, hypothermia, convulsions, coma and death. The causes include fulminant hepatic failure, inborn errors of the urea cycle and organic aciduria. Ammonia also contributes to other encephalopathies, such as those induced by hypoxia, ischemia, hypoglycemia, hypercapnia and toxins [8,9]. Medical management of elevated ammonia levels involves providing agents that substitute for urea, such as benzoate and phenylacetate. These medications activate latent pathways, which divert nitrogen from urea to amino-acid acylation (benzoate, which conjugates with glycine to form hippuric acid) and acetylation (phenylacetate, which conjugates with glutamine to form phenylacetylglutamine) to form inert compounds [10].

The neurotoxicity of ammonia is incompletely understood. Five hepatic urea cycle enzymes (carbamyl phosphate synthetase, ornithine transcarbamylase, argininosuccinic acid synthetase, argininosuccinatelyase and arginase) detoxify ammonia to urea $[CO(NH_2)_2]$. Astrocytes, however, rely mainly on glutamine synthetase for removal of excess ammonia (glutamate + ATP + NH_3 → glutamine + ADP + phosphate).Several studies have shown glutamine contributes to ammonia neurotoxicity [4,5,7]. Hyperammonia causes mitochondrial accumulation of ammonia, which converts α-ketoglutarate to glutamate and consumes NADH [10]. This reaction is catalyzed by the mitochondrial enzyme glutamate dehydrogenase (α-ketoglutarate+NH_4^++NADH+H+ → glutamate+NAD^++H_2O). Due to its low affinity for ammonia, the catalysis occurs only with excess

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ammonia [10]. The net effect of converting glutamate to glutamine at the expense of mitochondrial α-ketoglutarate and NADH is inhibition of astrocyte respiration. These metabolic derangements (lesions) highlight the limited capacity of CNS cells to detoxify ammonia and the sensitivity of critical reactions to excess ammonia.

Cellular mitochondrial O_2 consumption is a highly sensitive surrogate biomarker of tissue damages [11,12]. This study examined the effect of acute ammonia exposure on mouse forebrain cellular respiration to explore the vulnerability of astrocyte bioenergetics to ammonia, using a highly-sensitive fluorescent O_2 analyzer [13]. The experiments included *in vitro* and *in vivo* ammonia treatments followed by determination of forebrain cellular respiration, ATP and GSH.

Materials and Methods

Reagents and Solutions

Ammonium acetate, glucose (anhydrous), 5,5'-dithio-bis(2-nitrobenzoic acid) [DTNB], GSH (m.w. 307.43; pKa 8.7), HPLC-grade methanol, dichloromethane, trifluoroacetic acid (TFA), methanesulfonic acid (MSA), and N-ethylmaleimide (NEM, forms thioether bonds with sulfhydryls) were purchased from Sigma-Aldrich (St. Louis, MO). Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin (Pd phosphor) was purchased from Porphyrin Products (Logan, UT). Monobromobimane (mBBr, 271.111) was purchased from Molecular Probes (Eugene, Oregon). Ammonia solution (NH3, about 35%, 18.1 M, specific gravity 0.88, m.w. 17.03) was purchased from Laboratory FSA Supplies (Loughborough, England). (The pKa of ammonia is 9.25; thus, at physiologic pH, the bulk of the compound exists as ammonium ion: $NH_3 + H^+ \rightarrow NH_4^+$).

Phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4) was prepared daily and kept on ice. Pd phosphor (2.5 mg/ml = 2 mM) was prepared in dH_2O and stored in aliquots at -20°C. The "Pd phosphor working solution" was PBS, 5 mM glucose, 3 μM Pd phosphor and 0.5% fat-free albumin (prepared daily and kept at 25°C). For O_2 measurement, a forebrain fragment was placed in 1.0 mL Pd phosphor working solution in a glass vial sealed from air.

Sodium cyanide (CN, 1.0 M) was prepared in dH_2O; the pH was adjusted to ~7.0 with 12 N HCl and stored at -20°C. Glucose oxidase (GO, 10 mg/mL) was dissolved in dH_2O and stored at -20°C. GSH was dissolved in dH_2O and stored at -80°C; GSH determination was by Ellman's reagent [14]. NEM (100 mM) was made in ethanol and stored at -20°C.The GS-bimane derivative (GSH standard), sodium methane sulfonate (NaMS), mBBr (0.1 M in acetonitrile) and DTNB (10 mM in 100 mM Tri-Cl, pH 8.0) were prepared and stored as previously described [15]. The GSH standard (2 μM) was linear from 10 to 200 picomoles.

Mice

The Taylor Outbred mice used in this study were maintained at the animal facility that was in compliance with NIH guidelines (http://grants.nih.gov/grants/olaw/references/phspol.htm). Animals were housed in rooms maintained at 22°C with ~60% relative humidity and a 12-hr light/dark cycle. They had ad libitum access to standard rodent chow and filtered water. The study was approved by the Animal Research Ethics Committee for care and use of laboratory animals at the College of Medicine and Health Sciences, UAE University.

Forebrain fragments

Specimens were collected at designated periods following intraperitoneal administration of ammonia. Mice were then anesthetized by intraperitoneal injection of 10 μL/g of 25% urethane. The scalp was removed and specimen was collected from the left top forebrain using a 4 mm Biopsy Punch (Miltex, Japan). The sample was weighed and immediately immersed in 1.0 mL PBS containing 5 mM glucose, 3 μM Pd phosphor and 0.5% fat-free bovine albumin for O_2 measurement at 37°C. Specimens were also immediately immersed in 2% trichloroacetic acid for ATP measurements and mBBr labeling reactions for GSH determination as described below.

Oxygen measurement

A phosphorescence oxygen analyzer was used to monitor O_2 consumption as previously described [11,12]. Oxygen was detected by the Pd phosphor (absorption maximum at 625 nm and phosphorescence maximum at 800 nm). Samples were exposed to 600 per min light flashes from a pulsed light-emitting diode array, peaking at 625 nm. Emitted phosphorescent light was amplified, digitized (at 1.0 MHz by a 20-MHz A/D converter) and analyzed as previously described [13].

The phosphorescence decay rate ($1/\tau$) was single exponential; $I=Ae^{-t/\tau}$, where I=Pd phosphor phosphorescence intensity. The values of $1/\tau$ were linear with dissolved O_2: $1/\tau = 1/\tau o + kq[O_2]$, where $1/\tau$=the phosphorescence decay rate in the presence of O_2, $1/\tau o$=the phosphorescence decay rate in the absence of O_2, and kq=the second-order O_2 quenching rate constant in $s^{-1} \bullet \mu M^{-1}$ [11]. Cellular respiration was measured at 37°C in 1 mL sealed glass vials. In the presence of a forebrain specimen and glucose, O_2 concentration decreased linearly with time. The rate of respiration (k, in μM O_2 min^{-1}) was, thus, the negative of the slope $d[O_2]/dt$. NaCN inhibited respiration, confirming O_2 was consumed in the mitochondrial respiratory chain.

GSH determination

For GSH labeling with mBBr, the reaction (final volume=250 μL) contained a tissue specimen in 10 mM Tris-MSA (pH 8.0) and 1.0 mM mBBr. The mixture was incubated at 25°C in the dark for 15 min. The reaction was stopped with 50 μL of 70% perchloric acid. The tissue was then disrupted by vigorous vortexing, diluted to a final volume of 1.0 mL with 10 mMTris-MSA and homogenized for 5 min. The acid-soluble supernatant was collected by centrifugation (13,000g at 4°C for 13 min) and stored at -20°C until HPLC analysis [15]. Specimens were also pre-incubated at 25°C in 10 mM or 0.1 mM NEM for 5 min prior to mBBr labeling.

The analysis was performed on HPLC (Waters, Milford, MA, USA) as previously described [15]. Ultrasphere IP column, 4.6x250 mm (Beckman, Fullerton, CA, USA) was operated at 25°C at 1.0 mL/min. The minimum quantifiable level was ~1 pmol. Solvent A was 0.1% (v/v) trifluoroacetic acid/water and solvent B was HPLC-grade methanol. The employed gradient was: 0 min, 10% B; 5 min 10% B; 13 min, 100% B; 15 min, 10% B; 20 min, re-inject. The excitation and emission wavelengths were 390 nm and 480 nm, respectively. The injection volume was 20 μL. The results were expressed as pmol/mg (pmol GSH corrected for the sample weight).

ATP determination

Forebrain fragments were homogenized in 2% trichloroacetic acid for 2 min and neutralized with 100 mM Tris-acetate, 2 mM EDTA, pH 7.7. The supernatants were collected by centrifugation (13,000xg at 4°C for 10 min) and stored at -20°C until analysis. The pH was adjusted to 7.75 immediately before ATP determination. ATP concentration was measured using the Enliten ATP Assay System

(Bioluminescence Detection Kit, Promega, Madison, WI). The acid-soluble supernatant (2.5 µl) was added to 25 µl of the luciferin/luciferase reagent. The luminescence intensity was measured at 25°C using GlomaxLuminometer (Promega, Madison, WI).The ATP standard was linear from 10 pM to 100 nM (R2 >0.999).

Statistical analysis

Data were analyzed using SPSS statistical package (version 19). The nonparametric test (2 independent variables) Mann-Whitney was used to compare samples.

Results

Forebrain cellular respiration

O_2 consumption by forebrain fragments was measured in phosphate-buffered saline (PBS) alone, in PBS + 5 mM glucose and in RMPI-1640 medium (11.1 mM glucose, 136 µM glutamate, and 2 mM glutamine). The specimen was placed in an O_2 vial immediately after tissue collection. The vials were then sealed from air and the O_2 concentration measured at 37°C as a function of time. In PBS alone (Figure 1A-1C), O_2 concentration declined exponentially with time; the values of R2 for exponential fits were >0.9457 (Panel A) and >0.9726 (Panel B). The corresponding values for linear fits were >0.9385 and >0.8506, respectively. Sodium cyanide (CN) inhibited respiration (86% inhibition), confirming O_2 was consumed mainly in the mitochondrial respiratory chain (Panel C). Glucose oxidase catalyzed the reaction D-glucose+O_2 → D-glucono-δ-lactone+H_2O_2, depleted the remaining O_2 in the vial at a rate of about 0.07 µM O_2 min^{-1}; the tissue was the source of this micromolar concentration of glucose. These results suggested that the O_2 consumption in Panels A-B was driven by endogenous brain metabolic fuels (including intracellular and extracellular residual glucose in the specimens), which were depleted with time.

In PBS+glucose (Figure 1D and 1E), O_2 concentration declined linearly with time; the R2 values for linear fits were >0.9597 (Panel D) and >0.9471 (Panel E).The corresponding values for exponential fits were >0.9185 and >0.9017, respectively. Thus, with glucose, the rate of O_2 consumption was constant (zero-order kinetics; k, in µM O_2 min^{-1}) and set as the negative of the slope d[O_2]/dt. The rate was corrected for sample weight (kc) and expressed in µM O_2 min^{-1} mg^{-1}. The values of kc in Panels D-E were 0.19 and 0.45 µM O_2 $min^{-1}mg^{-1}$, respectively. In Panel F, the measurements were performed in RPMI-1640 medium. The value of kc was 0.72 µM O_2min^{-1} mg^{-1}, which was inhibited by

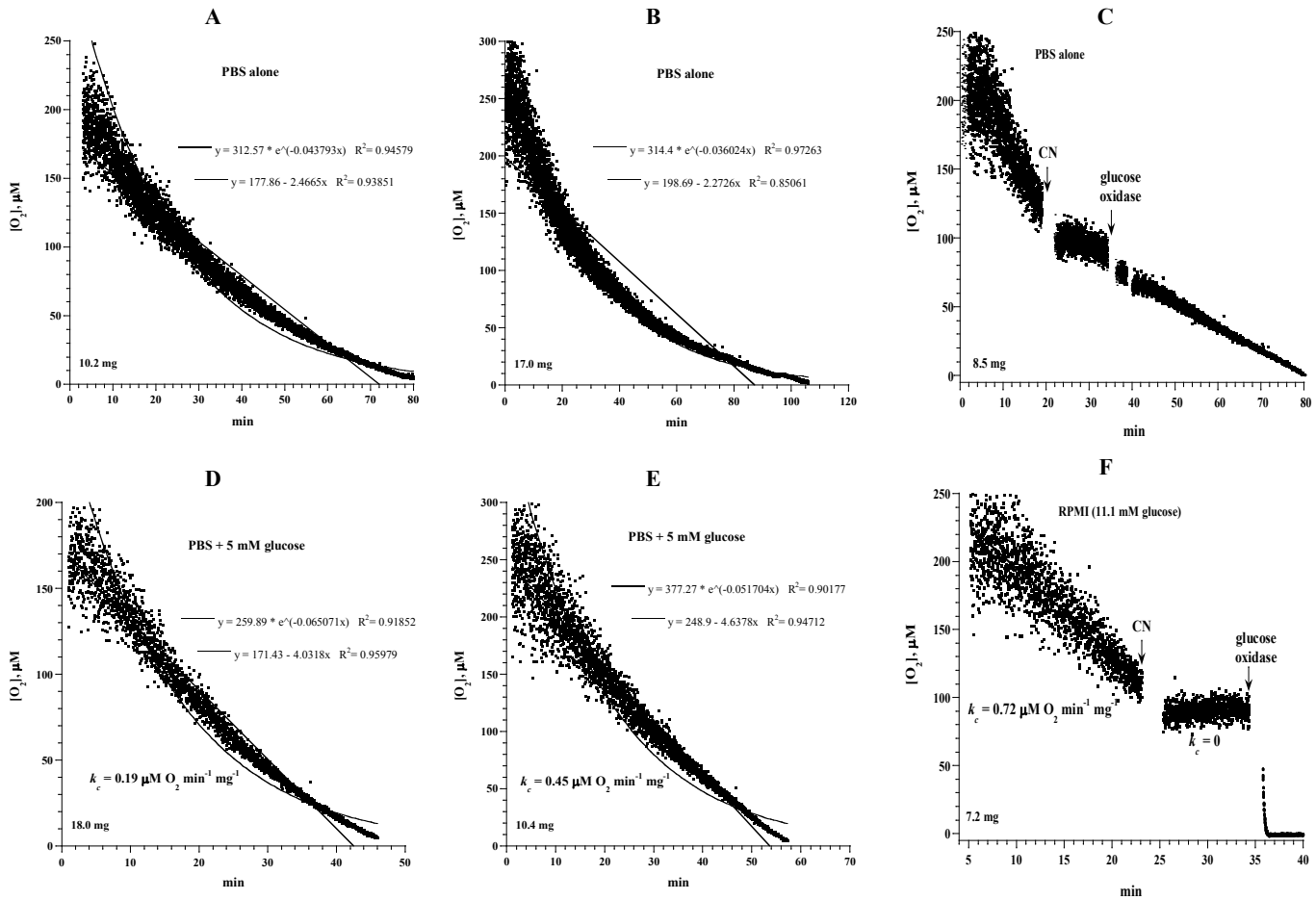

Figure 1: Cellular respiration without addition. In each Panel, a forebrain fragment was placed in the O_2 vial that contained 1.0 mL PBS alone, PBS + 5 mM glucose or RPMI medium (contained 11.1 mM glucose) supplemented with 0.5% fat-free bovine albumin and 3 µM Pd phosphor. The vials were sealed from air and the O_2 concentration was determined at 37°C as a function of time. The lines are exponential and linear fits. For Panels D-F, the rate of respiration (*k*, µM O_2 min^{-1}) was the negative of the slope of [O_2] *vs. t*. The values of k_c (µM O_2 min^{-1} mg^{-1}) and the additions of cyanide (CN) and glucose oxidase (catalyzed the reaction: D-glucose + O_2 → D-glucono-δ-lactone + H_2O_2) are shown. Glucose oxidase was added to confirm available O_2 in the solution. The "mg" values in the corner of the graph are the mg of tissue.

cyanide. The addition of glucose oxidase rapidly depleted remaining O_2 in the solution (Figure 1F). In 36 mice, the overall rate of cellular respiration was 0.32 ± 0.12 μM O_2 min^{-1} mg^{-1}.

In vitro treatment of forebrain specimens with ammonia

The *in vitro* effect of ammonia on cellular respiration was then investigated in PBS+5 mM glucose or MEM (Minimum Essential Medium Eagle Alpha Modification, which contained 5.6 mM glucose, 0.51 mM glutamate and 2 mM glutamine), Figure 2A-2F. Ammonia (3.6 to 18.0 mM) had insignificant immediate effect on cellular respiration (0.29 ± 0.08 vs. 0.27 ± 0.12 μM O_2 min^{-1} mg^{-1}, n =6 mice, p=0.589). Thus, the mitochondria (used here as a surrogate biomarker for cytotoxicity) were not immediately targeted by ammonia.

In vivo treatment with ammonia

The *in vivo* effect of ammonia on cellular respiration was investigated next. Ammonia was injected intraperitoneally (3.6 to 28.8 μmol/g) and forebrain specimens were collected 5 to 240 min after treatment. (Of note, injecting about 30 μmol/g resulted in convulsions and coma within 5 min.) O_2 consumption was measured in PBS+5 mM glucose; representative runs are shown in Figure 3A-3F. Respiration was inhibited by ammonia. The values of kc for treated mice were 0.26 ± 0.09 μM O_2 min^{-1} mg^{-1} (n=22 mice), ~19% lower than those for untreated mice (0.32 ± 0.12 μM O_2 min^{-1} mg^{-1}, n=36 mice), p=0.034.

Cellular ATP

Mice (3 separate experiments, n=3 mice per treatment group for each experiment) had intraperitoneal injections of 40 μL 0.9% NaCl or 40 μL of 9.0 M ammonium acetate (=360 μmol, or 16.5 to 24.8 μmol/g, 20.2±3.3 μmol/g). Forebrain specimens were collected 5 min after treatment and immediately placed in the trichloroacetic acid solution as described in Methods. Cellular ATP in untreated mice was 238 ± 45 pmol mg^{-1} and in treated mice 183 ± 34 pmol mg^{-1} (23% lower, p=0.008); n=9 mice for each group.

Cellular GSH

Mice (2 separate experiments, n=3 mice per treatment group for each experiment) had intraperitoneal injections of 40 μL 0.9% NaCl or 40 μL of 9.0 M ammonium acetate (=360 μmol, or 14 to 17 μmol/g). Forebrain specimens were then collected 5 min after treatment and placed immediately in the mBBr labeling reaction as described in Methods. Specimens were also incubated at 37°C with 0.1 mM NEM (forms thioether bonds with sulfhydryls) for 5 min prior to mBBr labeling. The GS-bimane derivatives were separated on HPLC and detected by fluorescence. Representative runs of the tissue acid-soluble supernatants for untreated and NEM-treated reactions are shown in Figure 4A and 4B. The GS-bimane peak had a retention time of 14.3 min. GSH contents were corrected for peak present in the NEM reactions. Cellular GSH in untreated mice was

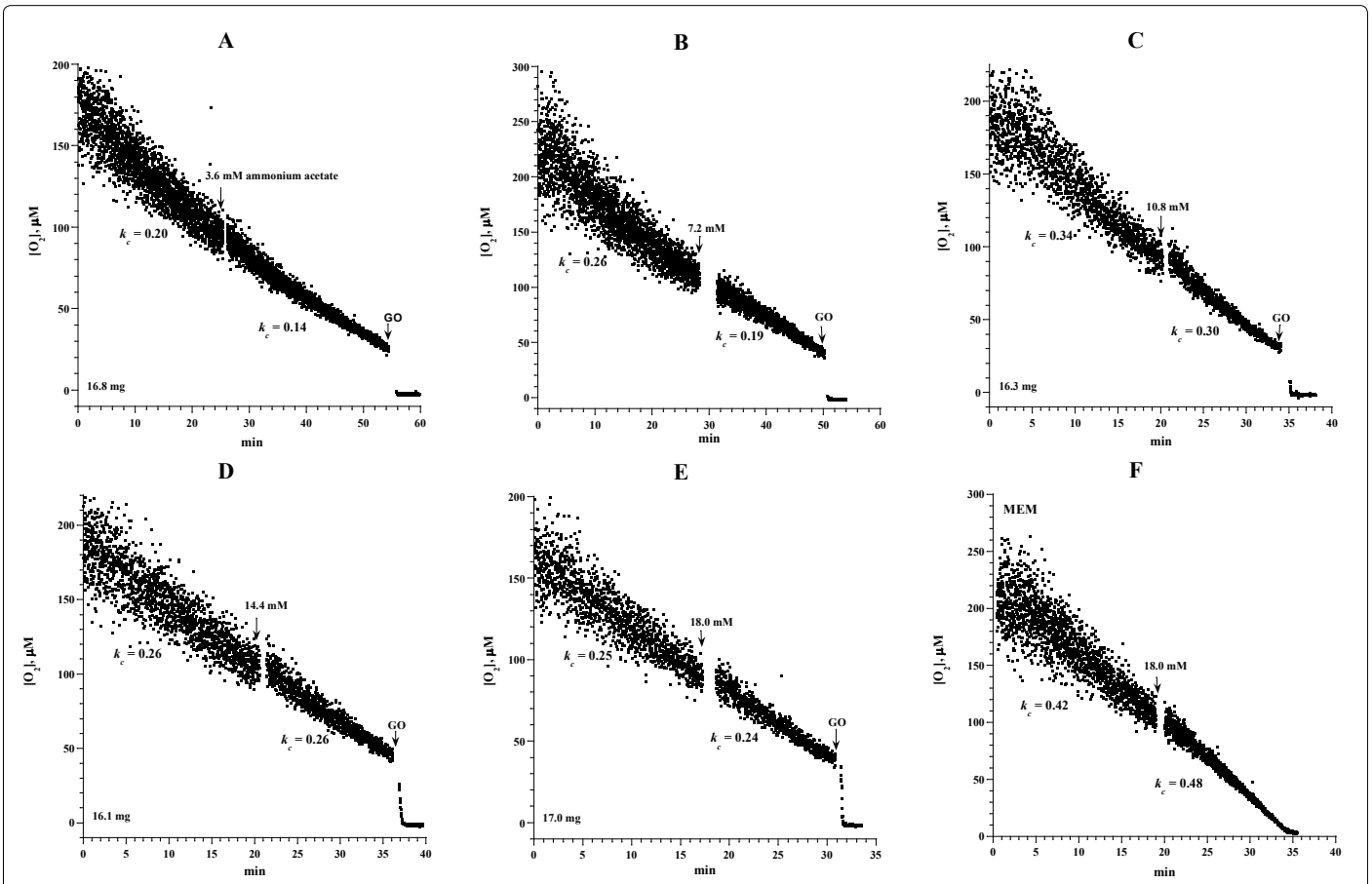

Figure 2: Cellular respiration after *in vitro* treatment with ammonia. Forebrain fragments were placed in the O_2 vials that contained 1.0 mL "PBS+5 mM glucose" or Minimum Essential Medium (MEM, contained 5.6 mM glucose) supplemented with 0.5% fat-free bovine albumin and 3 μM Pd phosphor. The vials were sealed from air and the O_2 concentration was determined at 37°C as a function of time. Various concentrations of ammonium acetate were injected directly into the vial at designated time periods. The values of k_c (μM O_2 min^{-1} mg^{-1}) and specimen weight are shown. GO, glucose oxidase.

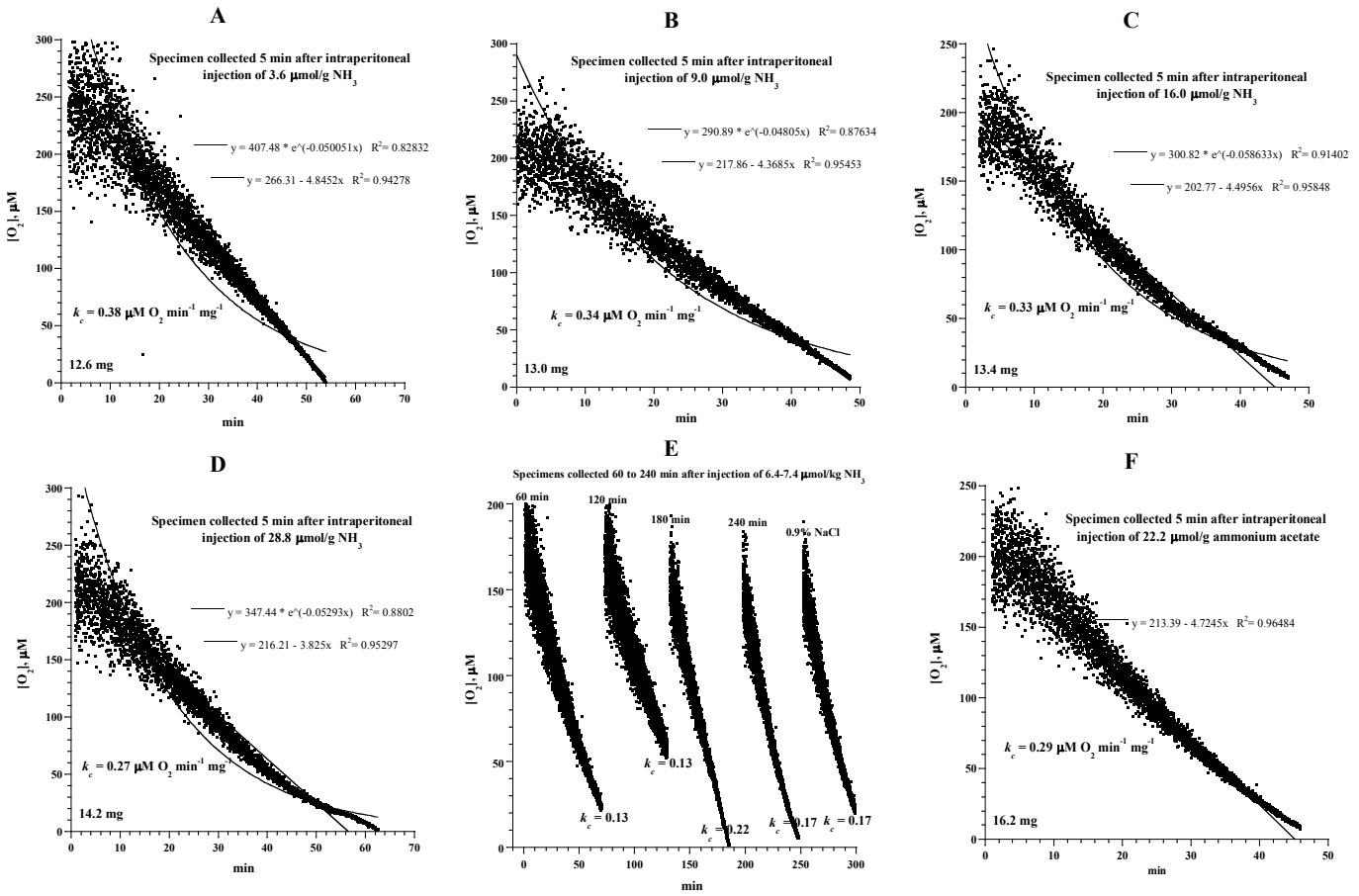

Figure 3: Cellular respiration after *in vivo* treatment with ammonia. Ammonia (3.6 to 28.8 µmol/g) was injected intraperitoneally and forebrain specimens were collected 5 to 240 min after treatment. Representative runs of O_2 consumption in PBS+5 mM glucose are shown. The lines are exponential and linear fits. The values of k_c (µM O_2 min^{-1} mg^{-1}), specimen weight and fitting equations are shown.

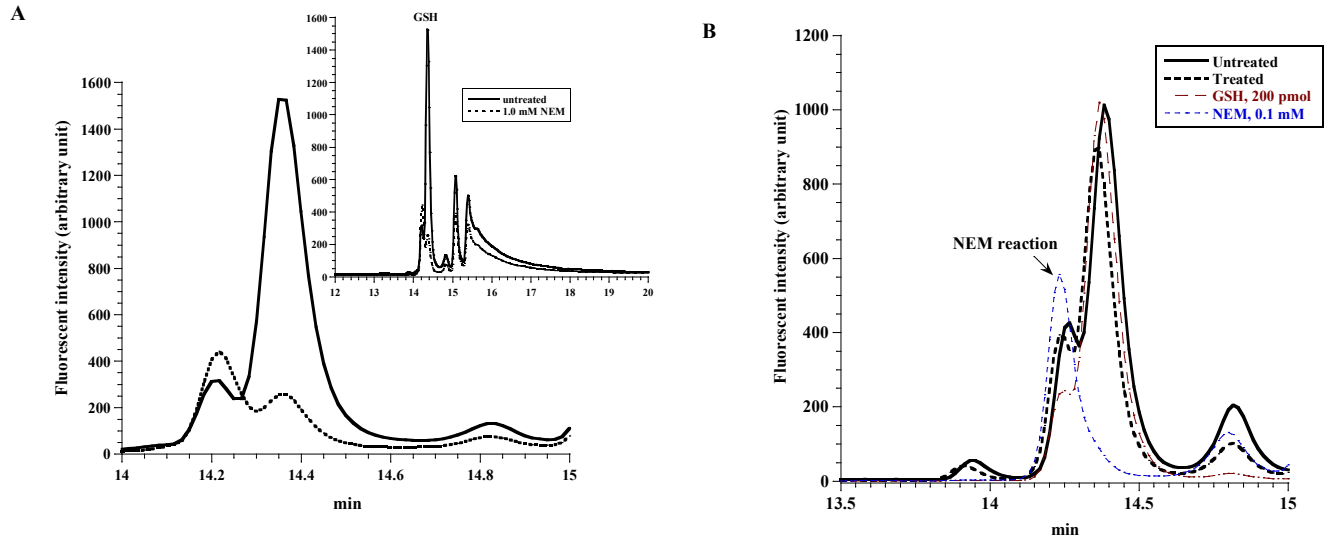

Figure 4: Cellular GSH. Representative HPLC runs of forebrain acid-soluble supernatants, showing the GS-bimane peak with a retention time of 14.3 min. Specimens were placed in the mBBr labeling reaction immediately after tissue collection. Panel A: Two specimens from untreated mouse; one sample was immediately incubated with mBBr and one was pre-incubated at 25°C in 10 mM NEM for 5 min before mBBr labeling. The calculation of cellular GSH (168 pmol mg^{-1}) was corrected for peak present in the NEM reaction. Panel B: Representative HPLC runs of treated (19.2 mg) and untreated (17.0 mg) specimens. NEM (0.1 mM) and GSH standard (200 pmol) runs are also shown.

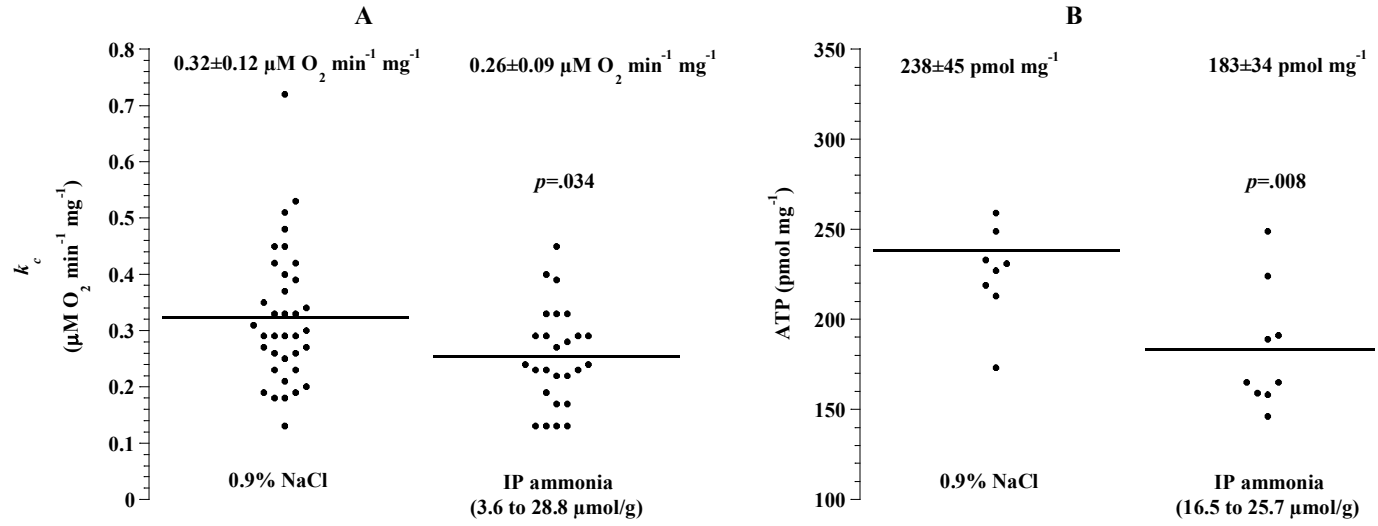

Figure 5: Summary of rates of cellular respiration (Panel A, k_c, in µM O_2 min^{-1} mg^{-1}) and contents of cellular ATP (Panel B, in pmol mg^{-1}).

300 ± 50 pmol mg^{-1} and in treated mice was 320 ± 160 pmol mg^{-1} (p=0.700); n=6 mice for each group.

Discussion

The main purpose of this study was to report on the acute neurotoxicity of ammonia, using cellular respiration and ATP as surrogate metabolic biomarkers. The success of this approach relied on rapidly collecting specimens free of ischemia and hypoxia (Figure 1).

The neurotoxicity of hyperammonia is a life-threatening condition. It is typically associated with altered astrocyte morphology and function due to limited capacity of CNS cells to handle ammonia. It also demonstrates the potency of excess ammonia in disturbing the equilibrium of critical reactions inside astrocytes.

The CNS metabolism of ammonia results in increased pyruvate (inhibition of pyruvate oxidation) and alanine (reductive amination of pyruvate), depletion of the neuroexcitatory amino acids aspartate and glutamate (transamination sequence reactions between pyruvate and aspartate, resulting in generation of alanine and oxaloacetate), accumulation of glutamine (condensation of glutamate and ammonia), and decreased flux of reduced equivalents (NADH) and other nutrients (malate, α-ketoglutarate, aspartate and glutamate) from the cytoplasm to the mitochondria [2]. Thus, molecules essential for transporting NADH to the mitochondria via the malate-aspartate cycle become limited. These metabolic derangements eventually lead to impaired O_2 consumption and ATP synthesis (oxidative phosphorylation) [2].

Several studies have confirmed that the complex biochemical lesions of ammonia lead to impaired cerebral cellular bioenergetics [1-3,16,17]. In one study, incubating cerebral cortex slices with 10 mM NH_3Cl resulted in decreased O_2 consumption, accumulation of pyruvate and increased utilization of glucose [3]. The authors concluded "that a primary toxic effect of ammonia on the brain may be direct interference with oxidative decarboxylation of pyruvic and α-ketoglutaric acids" [3]. Animal models have also revealed that ammonia alters the metabolism of putative neurotransmitters (glutamate and aspartate), activates N-methyl-D-aspartate receptors (NMDA) receptors and impairs cellular signaling through the excitatory neurotransmitter glutamate and its second messengers nitric oxide (NO) and cGMP [8,9].

The described methods permit comprehensive analyses of cellular respiration, ATP and GSH in viable brain specimens. These vital biomarkers can be used to study effects of drugs, toxins and pathogens. The results show ammonia inhibits brain cellular bioenergetics (cellular respiration and accompanying ATP synthesis) (Figure 5). The measured changes in respiration and ATP are expected to be more pronounced with lethal dosing.

Acknowledgements

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Authors' Contributions

MTA, SA-H, GBB, and AKS designed the study, carried out the analysis, interpreted the data and drafted the manuscript. SS programed the oxygen analyzer and performed data analysis. AA and TP performed the oxygen, GSH and ATP measurements. The authors read, edited and approved the final manuscript.

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