ROS

RESEARCH ARTICLES

Acetaminophen, the Active Ingredient of Tylenol, Protects against Peroxynitrite-Induced DNA Damage: A Chemiluminometric and Electron Paramagnetic Resonance Spectrometric Study

Xueqing Dou[1,1a], Jason Z. Li[1,1b], Igor Danelisen[2], Michael A. Trush[3], Hara P. Misra[1], Hong Zhu[2], Zhenquan Jia[2,4], and Y. Robert Li[2,4–6]

[1]Virginia Tech CRC Research Building II, Blacksburg, VA 24060, USA; [1a]Presently at Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300020, China; [1b]Presently at University of Virginia, Charlottesville, VA 22903, USA; [2]Campbell University School of Osteopathic Medicine, Buies Creek, NC 27506, USA; [3]Department of Environmental Health Sciences, The Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205, USA; [4]Department of Biology, University of North Carolina, Greensboro, NC 27412, USA; [5]Virginia Tech–Wake Forest University School of Biomedical Engineering and Sciences, Blacksburg, VA 24061, USA; [6]Department of Biomedical Sciences and Pathobiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

Correspondence: zhu@campbell.edu (H.Z.), z_jia@uncg.edu (Z.J.), yli@campbell.edu (Y.R.L.)

Dou X et al. Reactive Oxygen Species 3(8):127–134, 2017; ©2017 Cell Med Press
http://dx.doi.org/10.20455/ros.2017.819
(Received: December 21, 2016; Revised: January 20, 2017; Accepted: January 21, 2017)

ABSTRACT | This study investigated the hypothesis that acetaminophen at pharmacologically relevant concentrations may act as a protector against peroxynitrite toxicity. Our results showed that acetaminophen inhibited SIN-1 (a peroxynitrite generator)-induced DNA cleavage in a concentration-dependent manner in an in vitro model. With bicarbonate-enhanced luminol-dependent chemiluminometry, we further showed a nearly complete blockage of peroxynitrite-derived chemiluminescence by acetaminophen at 25–100 μM with minimal effects on SIN-1-mediated oxygen consumption, suggesting acetaminophen as a potent scavenger of peroxynitrite. Electron spin resonance spectrometry in combination with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO)-spin-trapping supported the ability of acetaminophen at high concentrations to diminish the production of a free radical species (likely hydroxyl radical) from SIN-1. Fenton chemistry-based DMPO-spin trapping further demonstrated the hydroxyl radical-scavenging capacity of acetaminophen. Collectively, the results of this study for the first time revealed the potential of acetaminophen to protect against peroxyitrite toxicity, which may have important implications in neuroprotection associated with the use of this popular analgesic and antipyretic drug.

KEYWORDS | Acetaminophen; Chemiluminometry; DNA damage; Electron paramagnetic resonance; Peroxynitrite; SIN-1; Spin-trapping

ABBREVIATIONS | CL, chemiluminescence; DMPO, 5,5-dimethylpyrroline *N*-oxide; EPR, electron paramagnetic resonance; PBS, phosphate-buffered saline; SIN-1, 3-morpholinosydnonimine

ROS

RESEARCH ARTICLES

CONTENTS

1. INTRODUCTION

Acetaminophen, the active ingredient of Tylenol, is among the most widely used over-the-counter analgesic drugs. On the other hand, the drug is also well known for its ability to cause liver injury upon over dosage. Recent studies suggest that acetaminophen at therapeutic doses may protect against dopaminergic neuron degeneration and neuroinflammation in animal models [1–3]. In this context, peroxynitrite, a reactive species formed from the bi-radical reaction between superoxide and nitric oxide, plays an important role in neurocytotoxicity and inflammatory injury via, at least partially, inducing DNA strand breakage and the subsequent activation of poly ADP ribose polymerase [4–6]. Accordingly, this study investigated the potential effects of acetaminophen at pharmacologically relevant concentrations on peroxynitrite-induced DNA damage and the underlying mechanisms. Our results for the first time demonstrated that acetaminophen inhibited peroxyitrite-induced DNA damage which appeared to result from its scavenging of peroxynitrite and/or peroxynitrite-derived secondary radical species.

2. MATERIALS AND METHODS

2.1. Materials

ϕX-174 RF I plasmid DNA was from New England Biolabs (Beverley, MA, USA). Authentic peroxynitrite was from Calbiochem (San Diego, CA, USA). 3-Morpholinosydnonimine (SIN-1), acetaminophen, and other chemicals were from Sigma–Aldrich (St. Louis, MO, USA). SIN-1 was dissolved in cold phosphate-buffer saline, pH 5.5, and stored at –80°C. The concentration of authentic peroxynitrite was determined spectrophotometrically at 302 nm (extinction coefficient = 1670 $M^{-1}cm^{-1}$). The peroxynitrite stock solution was aliquot and stored at –80°C under nitrogen and used within 3 months.

2.2. DNA Strand Cleavage Assay

DNA strand breaks were measured by the determining the conversion of supercoiled ϕX-174 RF I double-stranded DNA to open circular and linear forms, according to the method described before [7]. In brief, 0.2 µg DNA was incubated with SIN-1 in the presence or absence of acetaminophen in phosphate-buffered saline (PBS, pH 7.4) in a final volume of 24 µl at 37°C for 60 min. Following the incubation, the samples were immediately loaded in a 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate and 2 mM ethylenediaminetetraacetic acid (EDTA), and electrophoresed in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer. After electrophoresis, the gels were stained with a 0.5 µg/ml solution of ethidium bromide for 30 min, followed by another 30 min destaining in water. The gels were then photographed under ultraviolet illumination and quantified using an Alpha Innotech Imaging system (San Leandro, CA, USA).

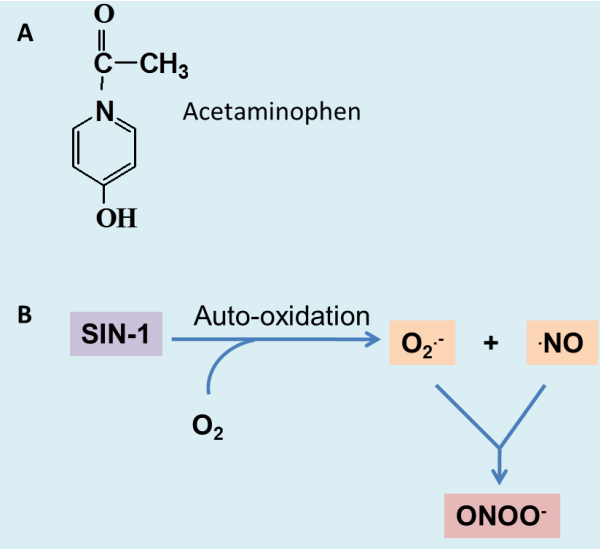

FIGURE 1. Structure of acetaminophen (A) and formation of peroxynitrite ($ONOO^-$) from SIN-1 autoxidation (B). As depicted, autoxidation of SIN-1 results in the formation of both superoxide ($O_2^{\bullet -}$) and nitric oxide ($^{\bullet}NO$). Reaction between superoxide and NO forms peroxynitrite.

2.3. Chemiluminometry

SIN-1 autoxidizes at a physiologically relevant pH to give rise to both superoxide and nitric oxide which react with each other at a diffusion-limited rate to form peroxynitrite (**Figure 1**). The bicarbonate-enhanced luminol-dependent chemiluminescence (CL) assay [8] was employed to detect the formation of peroxynitrite from SIN-1 autoxidation in PBS containing 15 mM sodium bicarbonate. The real-time CL response was continuously monitored at 37°C for 60 min with a Berthold Biolumat LB9505C multi-channel luminometer (Wildbad, Germany).

2.4. Oxygen Polarography

Oxygen consumption caused by SIN-1 autoxidation was monitored with a Clark oxygen electrode (YSI 5300, Yellow Springs, OH, USA) upon mixing SIN-1 in 2.5 ml air-saturated PBS at 37°C in the presence or absence of acetaminophen. The oxygen consumption caused by SIN-1 was expressed as percentage of saturation oxygen [9].

2.5. EPR SPIN-Trapping

The spin trap 5,5-dimethylpyrroline *N*-oxide (DMPO) was used to measure hydroxyl radicals derived from the decomposition of peroxynitrite or the Fenton reaction (H_2O_2/Fe^{2+}) in the presence or absence of acetaminophen. The electron paramagnetic resonance (EPR) spectra were recorded at room temperature with a spectrometer (Bruker D-200 ER, IBM–Bruker), operating at X-band with a TM cavity and capillary cell, as described by us before [9]. The EPR spectrometer settings were as following: modulation frequency, 86 kHz; microwave frequency, 9.845 GHz; microwave power, 29.8 mW; modulation amplitude, 2.17 G; and scan time, 400 s. The reactants were mixed in test tubes to a final volume of 0.2 ml and then transferred to a capillary cell for EPR spectral acquisition at room temperature under conditions described above.

2.6. Statistical Analysis

All data are expressed as mean ± SD from at least three separate experiments unless otherwise indicated. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. Statistical significance between the values was considered at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Induction of DNA Strand Cleavage by SIN-1

Induction of single-strand and double-strand breaks of supercoiled φX-174 RF I plasmid DNA led to the formation of open circular and linear forms, respectively. Previous studies showed that autoxidation of SIN-1 at a physiological pH led to the formation of peroxynitrite, which caused DNA cleavage [9]. To set the stage for determining the effects of acetaminophen on peroxynitrite-induced DNA damage, we first characterized the induction of DNA strand cleavage by SIN-1 at various concentrations. As shown in **Figure 2**, incubation of ϕX-174 RF I plasmid DNA with SIN-1 at 25–500 µM for 60 min resulted in the increased conversion of supercoiled DNA (SC) to both open circular (OC) and liner (L) forms in a concentration-dependent manner. Thus,

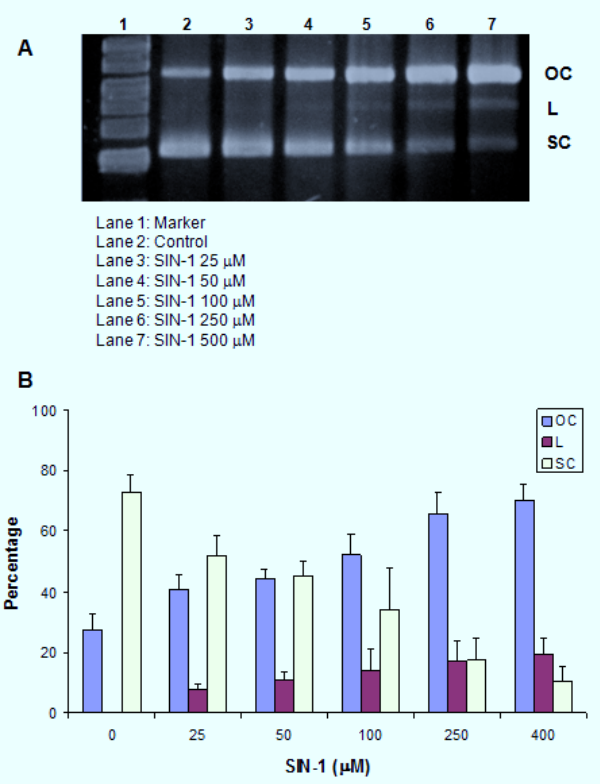

FIGURE 2. SIN-1-mediated DNA strand cleavage in ϕX-174 RF I plasmid DNA. The DNA was incubated with the indicated concentrations of SIN-1 for 60 min followed by detection of the conversion of the supercoiled (SC) DNA to open circular (OC) and linear (L) forms. (A) shows a representative gel picture and (B) shows quantitative data.

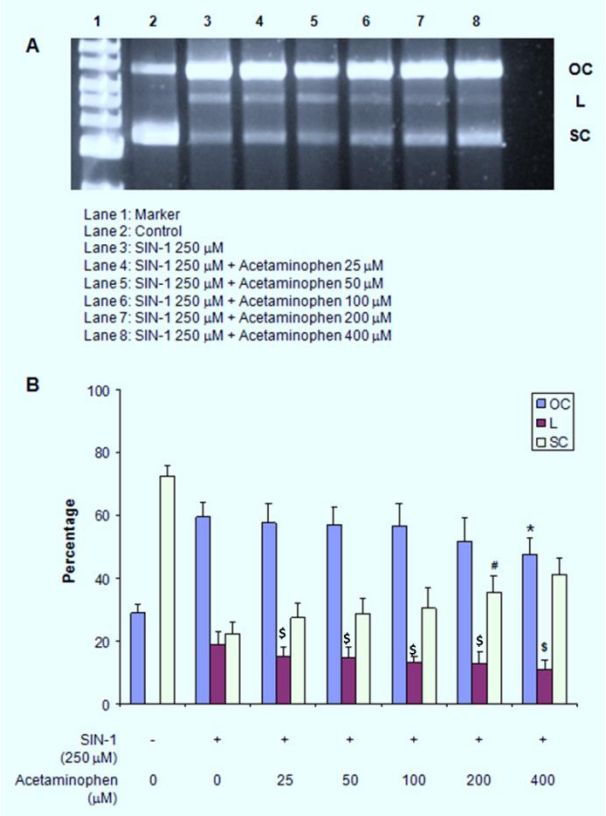

FIGURE 3. Effects of acetaminophen on 250 μM SIN-1-induced DNA strand cleavage in ϕX-174 RF I plasmid DNA. The plasmid DNA was incubated with 250 μM SIN-1 for 60 min in the presence or absence of the indicated concentrations of acetaminophen followed by detection of the conversion of the supercoiled (SC) DNA to open circular (OC) and linear (L) forms. (A) shows a representative gel picture and (B) shows quantitative data. * or #, $p < 0.05$ versus SIN-1 along; $ (ratio of L/SC), $p < 0.05$ versus SIN-1 alone.

under the present experimental conditions, SIN-1 autoxidized to form the DNA-cleaving peroxynitrite.

3.2. Inhibition of SIN-1-Mediated DNA Cleavage by Acetaminophen

A high concentration of SIN-1 (250 μM) was chosen to determine the effects of acetaminophen on SIN-1-mediated DNA cleavage. As shown in **Figure 3**, the presence of acetaminophen significantly inhibited SIN-1-mediated DNA strand cleavage in a concentration-dependent manner. Notably, the ratio of linear form to supercoiled form (L/SC) was significantly reduced by acetaminophen even at 25 μM. As 250 μM SIN-1 caused drastic DNA cleavage, which might be too severe to be pathophysiologically relevant. As such, a lower concentration of SIN-1 (25 μM) was used to cause mild DNA damage. As shown in **Figure 4**, the presence of acetaminophen at 400 μM nearly completely prevented SIN-1-induced DNA cleavage. The above results thus suggested that acetaminophen could markedly inhibit peroxynitrite-induced DNA damage. Plasma concentrations of acetaminophen can reach up to 100–200 μM after therapeutic dosages [10–12]. Hence, the concentrations

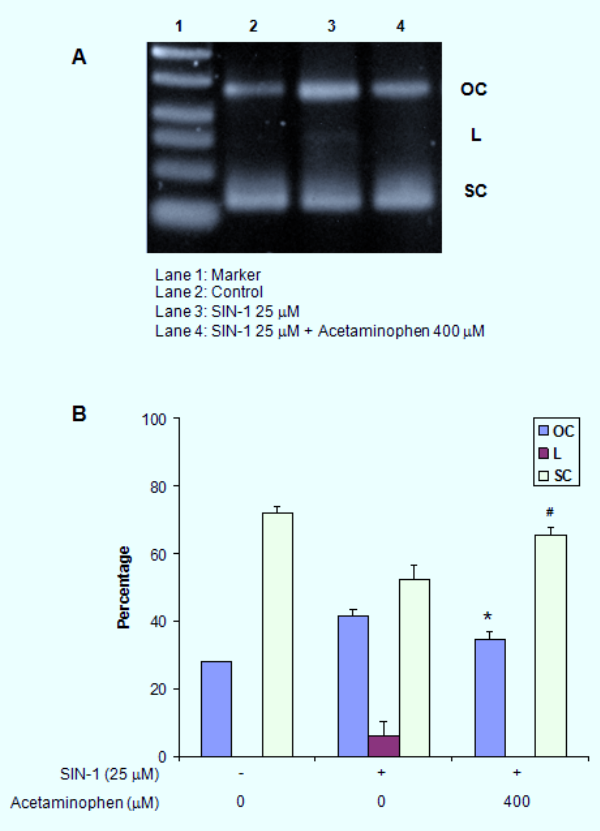

FIGURE 4. Effects of acetaminophen on 25 μM SIN-1-induced DNA strand cleavage in ϕX-174 RF I plasmid DNA. The plasmid DNA was incubated with 25 μM SIN-1 for 60 min in the presence or absence of the indicated concentrations of acetaminophen followed by detection of the conversion of the supercoiled (SC) DNA to open circular (OC) and linear (L) forms. (A) shows a representative gel picture and (B) shows quantitative data. * or #, $p < 0.05$ versus SIN-1 alone.

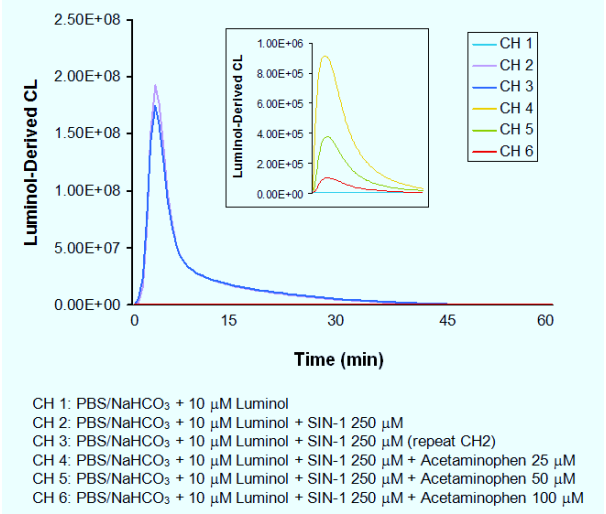

FIGURE 5. Effects of acetaminophen on bicarbonate-enhanced, peroxynitrite-dependent, luminol-derived CL from SIN-1. The real-time CL responses from SIN-1 (250 μM) in PBS containing 15 mM $NaCO_3$ and 10 μM luminol in the presence or absence of the indicated concentrations of acetaminophen were recorded continuously for 60 min.

of acetaminophen used in the present study were pharmacologically relevant.

3.3. Inhibition of Peroxynitrite-Dependent CL by Acetaminophen

To investigate the mechanisms underlying acetaminophen-mediated inhibition of SIN-1-induced DNA damage, we next determined the effects of acetaminophen on peroxynitrite-dependent CL. Bicarbonate-enhanced luminol-derived CL is indicative of peroxynitrite formation [8]. As shown in **Figure 5**, acetaminophen at 25–400 μM nearly completed inhibited the CL response. The nearly complete abolishment of bicarbonate-enhanced CL response from SIN-1 autoxidation by acetaminophen at a concentration as low as 25 μM suggested that this drug might act as a potent scavenger of peroxynitrite. The minimal effects of acetaminophen at 100 and 400 μM on SIN-1-mediated oxygen consumption (**Figure 6**) indicated that the inhibitory effect of acetaminophen on peroxynitrite-dependent CL was unlikely due to its inhibition of SIN-1 autoxidation. As depicted in **Figure 1**, consumption of oxygen is a critical step leading to the formation of peroxynitrite by SIN-1.

3.4. Effects of Acetaminophen on Peroxynitrite-Derived Free Radical Production

It has been suggested that decomposition of peroxynitrite may lead to the formation of hydroxyl radicals [13], and this radical formation may be responsible, at least partly, for peroxynitrite-induced DNA cleav-

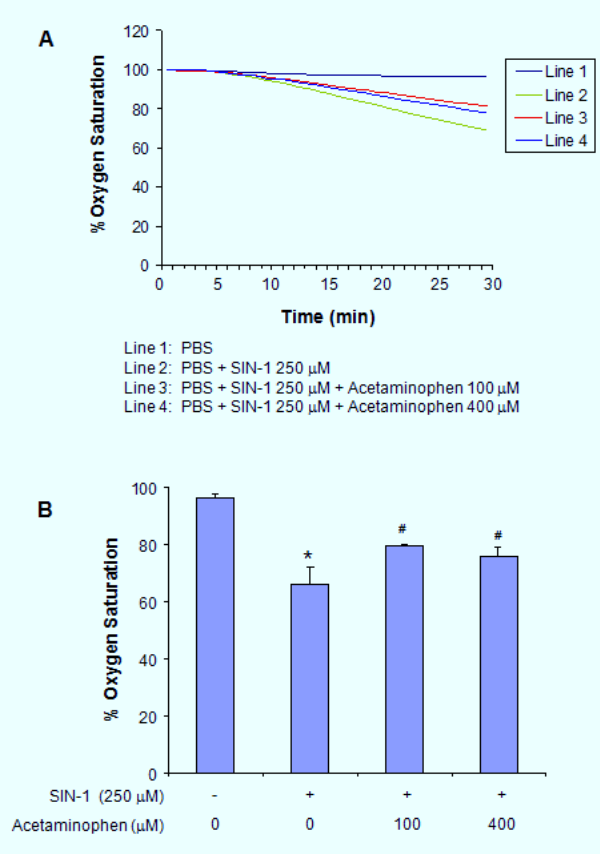

FIGURE 6. Effects of acetaminophen on SIN-1-mediated oxygen consumption. The SIN-1 (250 µM)-induced oxygen consumption was monitored continuously for 30 min in PBS in the presence or absence of the indicated concentrations of acetaminophen. *, $p < 0.05$ versus control; #, $p < 0.05$ versus SIN-1 alone.

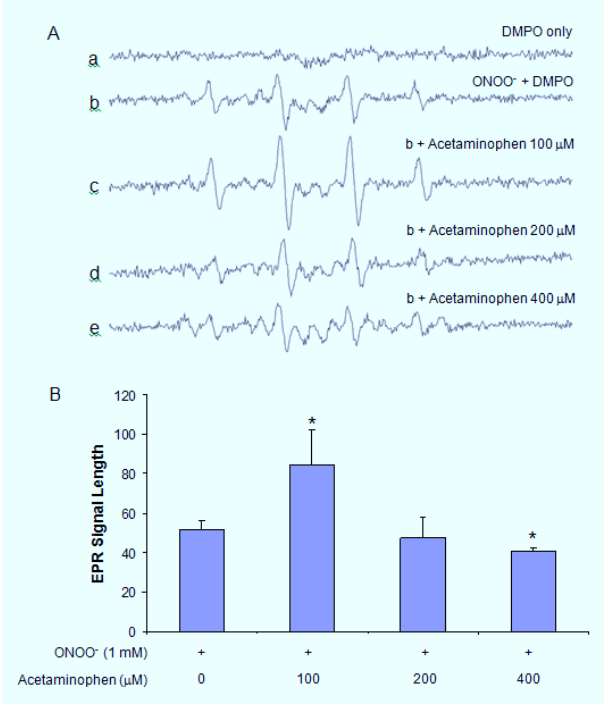

FIGURE 7. Effects of acetaminophen on peroxynitrite-derived radical production. DMPO-spin trapping detection of radical species was carried out following incubation of authentic peroxynitrite ($ONOO^-$, 1 mM) with DMPO (40 mM) in the presence or absence of the indicated concentrations of acetaminophen. *, $p < 0.05$ versus $ONOO^-$ alone.

age. Hence, we used the DMPO-spin trapping technique to determine the effect of acetaminophen on the above radical formation from peroxynitrite. As shown in **Figure 7**, incubation of DMPO with authentic peroxynitrite led to the formation of a DMPO-spin adduct characteristic of a hydroxyl radical adduct. Interestingly, the presence of acetaminophen as 100 µM significantly increased the formation of this spin adduct, whereas 400 µM acetaminophen diminished this radical production. It remains unknown how acetaminophen caused this biphasic response. To further investigate the effect of acetaminophen on hydroxyl radicals, we employed the Fenton chemistry to generate hydroxyl radicals. As shown in **Figure 8**, incubation of DMPO with hydrogen peroxide and Fe^{2+} led to the detection of a strong EPR spectrum characteristic of DMPO-hydroxyl radical adduct. It is well-established that the Fenton reaction between hydrogen peroxide and Fe^{2+} generates hydroxyl radicals. Addition of acetaminophen at 100 and 400 µM significantly inhibited the formation of the hydroxyl radicals, suggesting that acetaminophen might be a potential hydroxyl radical scavenger.

3.5. Conclusion

Taken together, this study demonstrated that acetaminophen at pharmacologically relevant concentra-

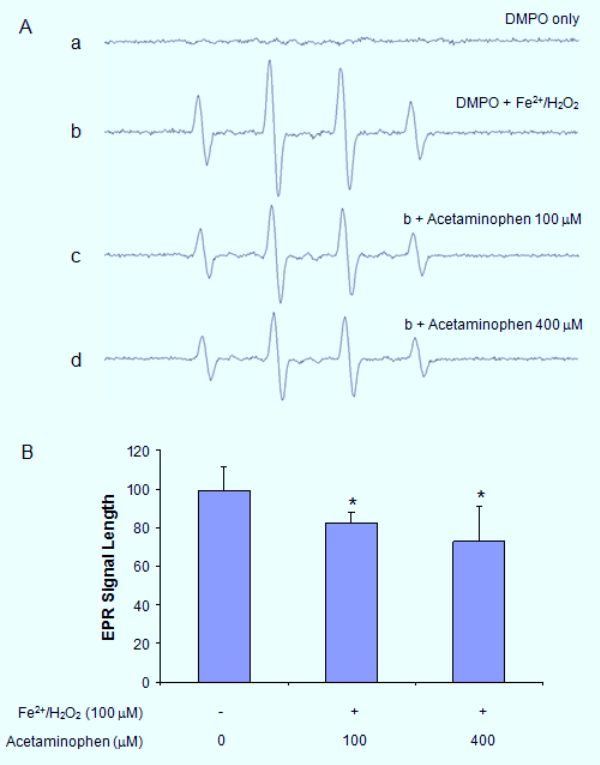

FIGURE 8. Effects of acetaminophen on H_2O_2/Fe^{2+}-derived hydroxyl radical formation. DMPO-spin trapping detection of radical species was carried out following incubation of H_2O_2 (100 µM)/FeSO4 (10 µM) with DMPO (40 mM) in the presence or absence of the indicated concentrations of acetaminophen. *, $p < 0.05$ versus control.

tions is able to inhibit peroxynitrite-induced DNA damage in vitro. The results of both chemiluminometric and EPR spectrometric experiments also indicated that acetaminophen might directly scavenge peroxynitrite or the secondary hydroxyl radical generation, especially at high concentrations. While the detailed chemical mechanisms of acetaminophen as an inhibitor of peroxynitrite toxicity remain to be elucidated, the findings of the present study might have important implications in neuroprotection as well as other beneficial effects associated with the use of acetaminophen at therapeutic dosage. Further studies are also warranted to determine if acetaminophen protects against peroxynitrite toxicity in cultured cells as well as in vivo animal models.

REFERENCES

1. Locke CJ, Fox SA, Caldwell GA, Caldwell KA. Acetaminophen attenuates dopamine neuron degeneration in animal models of Parkinson's disease. *Neurosci Lett* 2008; 439(2):129–33. doi: 10.1016/j.neulet.2008.05.003.
2. Tripathy D, Grammas P. Acetaminophen inhibits neuronal inflammation and protects neurons from oxidative stress. *J Neuroinflammation* 2009; 6:10. doi: 10.1186/1742-2094-6-10.
3. Tripathy D, Sanchez A, Yin X, Martinez J, Grammas P. Age-related decrease in cerebrovascular-derived neuroprotective proteins: effect of acetaminophen. *Microvasc Res* 2012; 84(3):278–85. doi: 10.1016/j.mvr.2012.08.004.
4. Szabo C, Zingarelli B, O'Connor M, Salzman AL. DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. *Proc Natl Acad Sci USA* 1996; 93(5):1753–8.
5. Mandir AS, Przedborski S, Jackson-Lewis V, Wang ZQ, Simbulan-Rosenthal CM, Smulson ME, et al. Poly(ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism. *Proc Natl Acad Sci USA* 1999; 96(10):5774–9.
6. Li J, Baud O, Vartanian T, Volpe JJ, Rosenberg PA. Peroxynitrite generated by inducible nitric oxide synthase and NADPH oxidase mediates microglial toxicity to oligodendrocytes. *Proc Natl Acad Sci USA* 2005; 102(28):9936–41. doi: 10.1073/pnas.0502552102.
7. Li Y, Trush MA. DNA damage resulting from the oxidation of hydroquinone by copper: role for a Cu(II)/Cu(I) redox cycle and reactive oxygen generation. *Carcinogenesis* 1993; 14(7):1303–11.
8. Li Y, Zhu H, Kuppusamy P, Zweier JL, Trush MA. Mitochondrial electron transport chain-derived superoxide exits macrophages: implications for mononuclear cell-mediated pathophysiological processes. *Reactive Oxygen Species* 2016; 1(1):81–98. 10.20455/ros.2016.815
9. Jia Z, Zhu H, Li Y, Misra HP. Potent inhibition

of peroxynitrite-induced DNA strand breakage and hydroxyl radical formation by dimethyl sulfoxide at very low concentrations. *Exp Biol Med (Maywood)* 2010; 235(5):614–22. doi: 10.1258/ebm.2010.009368.

10. Holmer Pettersson P, Jakobsson J, Owall A. Plasma concentrations following repeated rectal or intravenous administration of paracetamol after heart surgery. *Acta Anaesthesiol Scand* 2006; 50(6):673–7. doi: 10.1111/j.1399-6576.2006.01043.x.
11. Montgomery CJ, McCormack JP, Reichert CC, Marsland CP. Plasma concentrations after high-dose (45 mg.kg-1) rectal acetaminophen in children. *Can J Anaesth* 1995; 42(11):982–6. doi: 10.1007/BF03011069.
12. Stocker ME, Montgomery JE. Serum paracetamol concentrations in adult volunteers following rectal administration. *Br J Anaesth* 2001; 87(4):638–40.
13. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87(4):1620–4.