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Free radical mediated oxidative stress and toxic side effects in brain induced by the anti cancer drug adriamycin: Insight into chemobrain

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Abstract
Adriamycin (ADR) is a chemotherapeutic agent useful in treating various cancers. ADR is a quinone-containing anthracycline chemotherapeutic and is known to produce reactive oxygen species (ROS) in heart. Application of this drug can have serious side effects in various tissues, including brain, apart from the known cardiotoxic side effects, which limit the successful use of this drug in treatment of cancer. Neurons treated with ADR demonstrate significant protein oxidation and lipid peroxidation. Patients under treatment with this drug often complain of forgetfulness, lack of concentration, dizziness (collectively called somnolence or sometimes called chemobrain). In this study, we tested the hypothesis that ADR induces oxidative stress in brain. Accordingly, we examined the *in vivo* levels of brain protein oxidation and lipid peroxidation induced by i.p. injection of ADR. We also measured levels of the multidrug resistance-associated protein (MRP1) in brain isolated from ADR- or saline-injected mice. MRP1 mediates ATP-dependent export of cytotoxic organic anions, glutathione S-conjugates and sulphates. The current results demonstrated a significant increase in levels of protein oxidation and lipid peroxidation and increased expression of MRP1 in brain isolated from mice, 72 h post i.p injection of ADR. These results are discussed with reference to potential use of this redox cycling chemotheraputic agent in the treatement of cancer and its chemobrain side effect in brain.

Keywords: *Adriamycin, cancer chemotherapeutics, oxidative stress, somnolence, chemobrain, multidrug resistance-associated protein 1*

Introduction

Free radical mediated oxidative stress has been implicated in many age-related neurodegenerative disorders [1]. Reactive oxygen species (ROS) leads to protein oxidation [2,3], lipid peroxidation [4,5], DNA and RNA oxidation in brain [6] with concomitant neuronal dysfunction and death. Oxidation of proteins leads to loss of activity of enzymes critical for cell functioning, and recently our lab identified some of the critical proteins that are oxidized in Alzheimer's disease (AD), using proteomics [7–9]. ROS generation hence becomes important in understanding oxidative stress and oxidative stress related disorders.

Adriamycin (ADR) is a cancer chemotherapeutic useful in treating various cancers, with especially good potency in the treatment of patients with solid tumors [10,11]. ADR is suggested to act as an antitumor agent by inhibiting DNA replication and DNA synthesis [12] by intercalating into grooves of DNA. Another important mechanism of action of ADR involves its interaction with topoisomerase II, which

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forms a DNA-cleavable complex [13,14]. One of the most accepted mechanisms of action of ADR on tumor cells is generation of free radicals by enzymatic electron reduction of ADR by variety of oxidases, reductases and dehydrogenases [15,16]. However, ADR can have serious side effects in various non-involved tissues, including brain, apart from its known cardiotoxic effects [17]. A free radical-mediated mechanism has been proposed to be responsible for ADR-induced toxicity in heart [18]. Cardiomyocytes treated with ADR show significant protein oxidation and lipid peroxidation [18]. Free radical scavengers are known to reduce this effect [19]. ADR also disrupts the mitochondrial electron transport system in heart tissues [20–22]. Increased expression of manganese superoxide dismutase (MnSOD) protects complex I against ADR-induced cardiomyopathy [20]. ADR contains a quinone in its structure, and as is well known, quinones undergo one-electron reduction to form semiquinones, which are free radicals [23,24]. Although there are reports of dizziness, confusion and dementia of cognitive function (somnolence) in patients treated with ADR [25], little has been reported on oxidative stress in brain due to this drug.

Therefore, in this study, oxidative stress parameters were studied in brain of ADR-treated animals. Protein carbonyl and 3-nitrotyrosine levels (indices of protein oxidation) and 4-hydroxynonenal levels (index of lipid peroxidation) were determined in brain from mice treated with ADR and from control mice.

Animals

Male mice (2–3 months of age), approximately 30 g in size, housed in the University of Kentucky Central Animal Facility in 12-h light/dark conditions and fed standard Purina rodent laboratory chow *ad libitum*, were used. The animal protocols were approved by the University of Kentucky Animal Care and Use Committee.

Materials and methods

Chemicals

Doxorubicin HCl (ADR) was purchased from Bedford Laboratories™. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. The protein oxidation detection kit was purchased from Intergen (Purchase, NY) and primary antibodies for HNE, 3-NT and the multidrug resistance-associated protein (MRP1) were purchased from Chemicon International (Temecula, CA).

Preparation of brain homogenates

Brains were isolated and dissected following sacrifice by decapitation from mice treated i.p. with ADR (20 mg/kg body weight), 72 h after injection, or from saline treated control mice, and placed in lysing buffer containing 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethylene glycol-bistetraacetic acid (EGTA) and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4. The dosage and time were chosen based on prior studies [20]. The brain was homogenized by 20 passes of a Wheaton tissue homogenizer, and the resulting homogenate was centrifuged at 1500*g* for 10 min. The pellet (nuclear fraction) was suspended in 1 ml PBS. The supernatant was retained and centrifuged at 20,000*g* for 10 min. The pellet (membrane fraction) was suspended in 1 ml phosphate buffered saline (PBS) containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBS) and the supernatant (cytosolic fraction) was retained for fluorescence studies. All the fractions suspended in PBS were washed twice with PBS at 32,000*g* for 10 min. The resulting fractions were assayed for protein concentration by the Pierce BCA method [26].

Protein carbonyls

Samples (5 μl) of brain homogenate (membrane fraction), 12% sodium dodecyl sulfate (SDS) (5 μl), and 10 μl of 10 times-diluted 2,4-dinitrophenyl-hydrazine (DNPH) from 200 mM stock were incubated at room temperature for 20 min, followed by neutralization with 7.5 μl neutralization solution (2 M Tris in 30% glycerol). This neutralized solution (250 ng) was loaded in each well on a nitrocellulose membrane under vacuum using a slot blot apparatus. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 1 h and incubated with a 1:100 dilution of anti-DNP polyclonal antibody in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 1 h. The membrane was washed three times in PBS following primary antibody incubation at intervals of 5 min each. The membrane was incubated following washing with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS in a 1:8000 ratio for 1 h. The membrane was washed for three times in PBS for 5 min and developed in Sigmafast tablets, [5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium substrate (BCIP/NBT substrate)]. Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (PC version of Macintosh compatible NIH image).

4-Hydroxynonenal

Samples (5 μl) of brain homogenate (membrane fraction), 12% SDS (5 μl), and 5 μl of modified Laemlli buffer containing 0.125 M Tris base pH 6.8,

4% (v/v) SDS, and 20% (v/v) glycerol were probed for protein-bound HNE in the same manner as discussed above.

3-Nitrotyrosine

Similarly, samples (5 μl) of brain homogenate (membrane fraction), 12% SDS (5 μl), and 5 μl of modified Laemmli buffer containing 0.125 M Tris base pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were probed for 3-NT in the same manner as described above.

The specificity for both 3-NT and HNE primary antibodies was performed and confirmed as described elsewhere [27]. For example, the brain samples were treated with either the HNE or 3-NT primary antibody that had been first reacted with free HNE or free 3-NT. The resulting HNE blot showed very faint, non-specific binding (data not shown), which was accounted in the background for each blot. The 3-NT blot showed no staining on the blot, suggesting that there was no non-specific binding of the primary 3-NT antibody (data not shown).

Western blots

Samples (100 μg) were incubated with sample loading buffer, and protein samples were denatured and electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane at 90 mA/gel for 2 h. The blots were blocked for 1 h in fresh wash buffer (10 mM Tris–HCl, pH 7.5), 150 mM NaCl, 0.05% Tween 20, pH 7.4, containing 3% bovine serum albumin) and incubated with a 1:1000 dilution of anti MRP1 monoclonal antibody in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBS) for 1 h. The membrane was washed three times in PBS and was incubated for 1 h with an anti-rabbit IgG alkaline phosphatase secondary antibody diluted in PBS in a 1:8000 ratio. The membrane was washed three times in PBS for 5 min and developed in Sigma fast tablets (BCIP/NBT substrate).

In all cases non-specific background labeling by the secondary antibody was negligible.

Statistical analysis

A two-tailed Student's *t*-test was used to assess statistical significance. P values <0.05 were considered significant for comparison between control and experimental data sets.

Results

Protein carbonyls

Protein carbonyls are the most often employed index of protein oxidation [2]. Treatment of mice with ADR (i.p.) revealed an increase in protein carbonyls in brain. Figure 1 shows the protein carbonyl levels in brain homogenate extracted from saline-injected mice (control) and brain homogenate extracted from mice injected with ADR. There was a significant increase in protein carbonyl levels in brain homogenates from ADR-injected mice when compared to control ($P < 0.001$, $n = 5$).

4-Hydroxynonenal

Arachidonic acid from phospholipids, on attack of free radicals, produces reactive alkenals such as HNE [28]. HNE binds to protein and inactivates them by

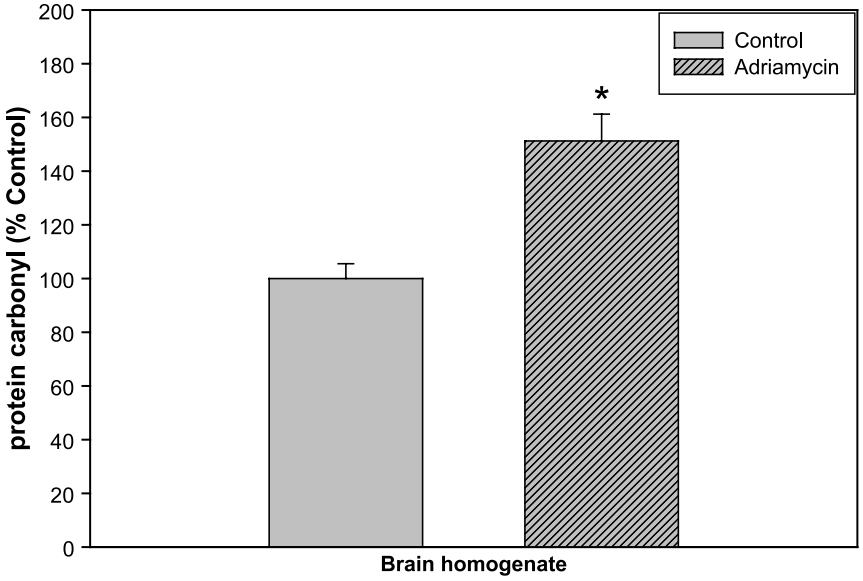


Figure 1. Increased *in vivo* protein oxidation in brain isolated from mice previously treated with adriamycin (20 mg/kg body weight) 72 h post i.p injection compared to brain isolated from saline injected mice. *$P < 0.001$, $n = 5$. (The mean value of 434 arbitrary units for control was taken as 100%).

changing their conformation [4,28,29]. Figure 2 shows the protein bound HNE levels in brain homogenate extracted from saline injected mice (control) and brain homogenate extracted from mice injected with ADR. There was a significant increase in protein bound HNE levels in brain homogenate from ADR-injected mice when compared to control ($P < 0.001$, $n = 5$).

3-Nitrotyrosine (3-NT)

Nitration of tyrosine residues is a marker of attack by RNS, such as peroxynitrite, that may have been formed by free radical mediated oxidative stress [7,8,30]. Figure 3 shows 3-NT levels in brain homogenate from saline-injected mice (control) and brain homogenate extracted from mice injected with ADR. There was a significant increase in 3-NT levels in brain homogenate from ADR-injected mice when compared to control ($P < 0.001$, $n = 5$).

MRP-1 expression

Glutathione conjugates of ADR are exported from cells via the multi drug resistance-associated protein-1 (MRP-1) [31]. Oxidative modification of MRP-1 would likely lead to accumulation of HNE [32], and in AD brain that is under oxidative stress [1,5,6], increased expression of MRP-1 was observed. Consistent with results in AD brain [32], Figure 4 shows that MRP-1 expression is significantly elevated in brain from ADR-injected mice compared to control ($P < 0.05$, $n = 3$).

Discussion

ADR is a commonly used cancer chemotherapeutic agent for various solid tumors. This agent is known to cause serious side effects in heart. However, ADR also shows side effects in other organs, including brain, which has not been studied extensively. ADR causes cardiomyopathy or congestive heart failure. Due to the presence of quinone in its structure, a free radical hypothesis for ADR-mediated oxidative stress and cytotoxicity is widely accepted. In the presence of Fe^{2+} free radical production increases [18]. Fe^{2+} acts as a catalyst for oxidative stress in neurodegenerative disorders [2,33]. An elevated level of iron is seen in several neurodegenerative disorders, including AD. Others showed that there is accumulation of iron in ADR-treated myocardial and neoplastic cells, which leads to increase in oxidative stress [34]. In the presence of an iron chelator the ADR induced oxidative stress is reduced in cardiomyocytes [35,36].

Nitric oxide synthase and cytochrome P450 reductase are some of the flavoprotein reductases that reportedly activate ADR-dependent redox cycling [37]. Since mitochondria are target organelles for ADR-induced cytotoxicity in cardiomyocytes [20,38,39], NADH dehydrogenase, a mitochondrial enzyme, stimulates ADR to form semiquinone radical and superoxide radical anion [40].

GSH is an endogenous antioxidant that is widely present in various tissues [41]. ADR was shown to induce depletion of glutathione (GSH) in heart tissue [42]. The level of glutathione peroxidases were also known to be reduced by ADR, and ebselen, a glutathione peroxidase mimetic, significantly inhibits ADR-induced oxidative stress and apoptosis [43].

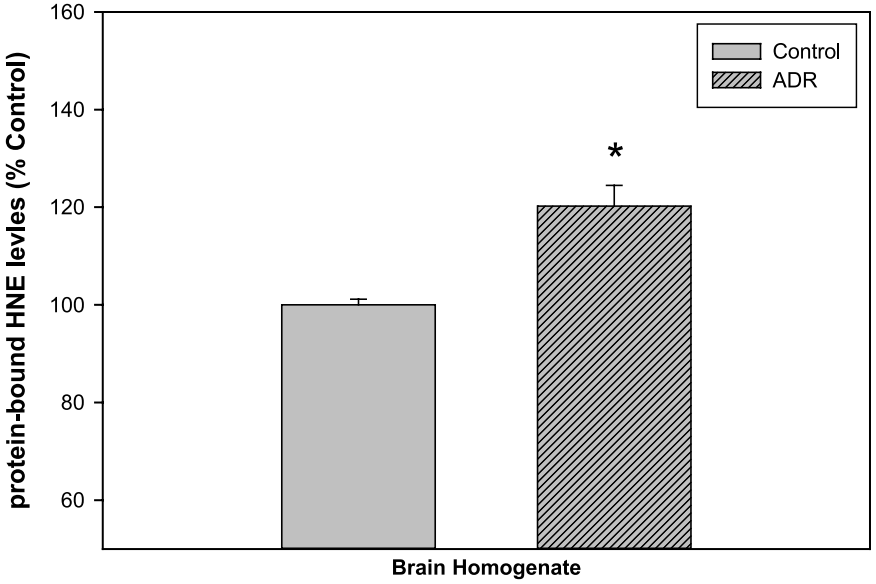


Figure 2. Increased *in vivo* protein bound HNE in brain isolated from mice previously treated with adriamycin (20 mg/kg body weight) 72 h post i.p. injection, compared to brain isolated from saline injected mice. * $P < 0.001$, $n = 5$. (The mean value of 526 arbitrary units for control was taken as 100%).

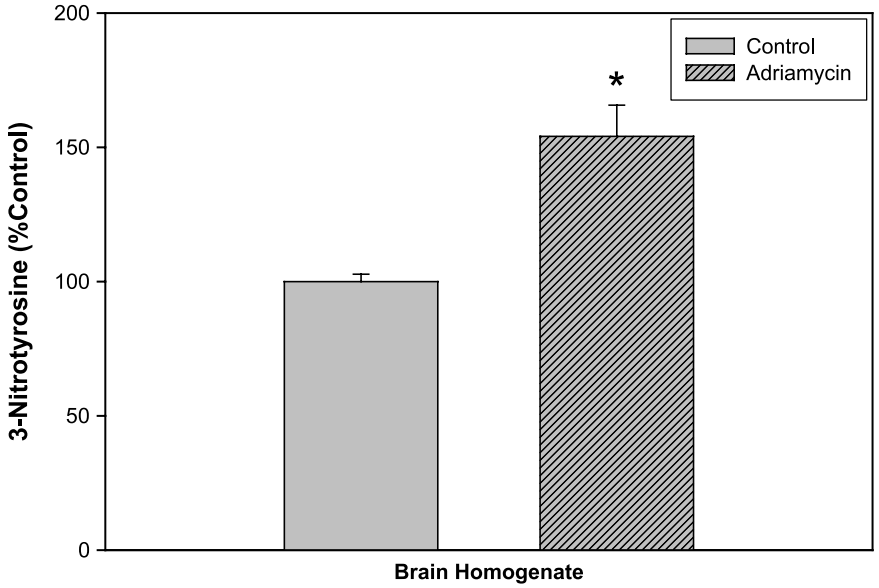
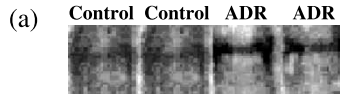


Figure 3. Increased *in vivo* 3-Nitrotyrosine levels in brain isolated from mice previously treated with adriamycin (20 mg/kg body weight) 72 h post i.p. injection, compared to brain isolated from saline injected mice. $^{*}P < 0.001$, $n = 5$. (The mean value of 152 arbitrary units for control was taken as 100%).

All these studies provide evidence for a free radical mechanism involved in ADR induced cytotoxicity.

Brain is particularly vulnerable to oxidative stress due to the presence of high levels of polyunsaturated fatty acids, relatively low antioxidant capacity, the presence of redox metal ions, and high oxygen utilization [2]. The free radicals generated in the brain cause damage to proteins, lipids and DNA and subsequently lead to cellular dysfunction or cell death [5,7,8,44]. Oxidative stress induced by ADR in brain could cause damage to proteins critical for cell functioning and may also lead to cell death.



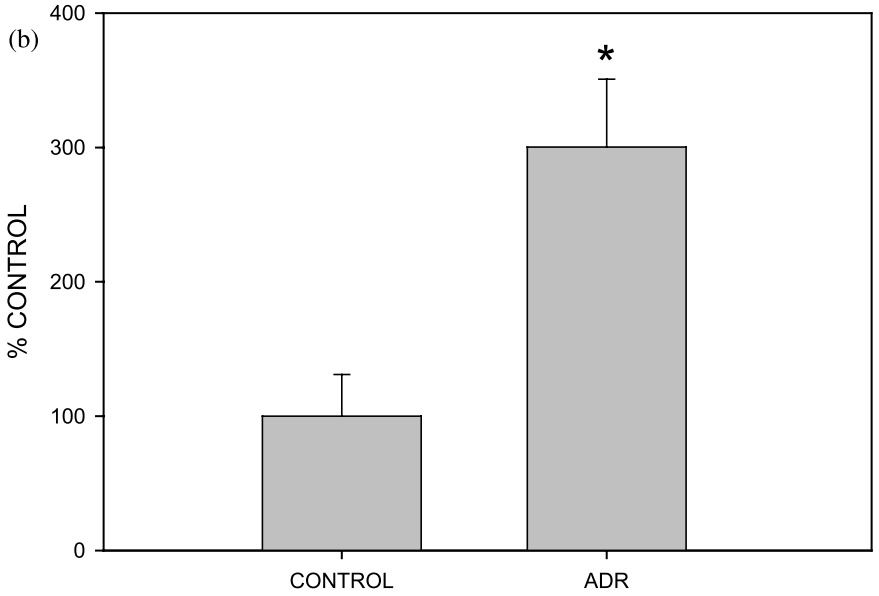


Figure 4. (a) Representative western blot showing increased MRP1 expression in brain isolated from mice injected with adriamycin. (b) Plot showing significant increase in MRP1 levels in brain isolated from mice injected i.p with adriamycin compared to saline injected control mice. $^{*}P < 0.05$, (The mean value of 272 arbitrary units for control was taken as 100%).

Involvement of the central nervous system in patients treated with ADR was previously shown [25]. These authors found that patients under treatment with ADR often complain of forgetfulness, lack of concentration and dizziness (collectively called somnolence). We demonstrated that ADR increases oxidative stress and antioxidants prevent cell death in ADR-treated neuronal cell culture. (*Vide infra*). The data presented in this paper shows that ADR *in vivo* causes oxidative damage to brain tissue.

Previously, we reported oxidized proteins in AD fit into critical pathways for cellular function and are dysfunctional [5–9]. The results shown in our current *in vivo* study are consistent with our *in vitro* results, showing that ADR acts as an oxidant. In the present study we demonstrated in brain homogenate isolated from rodents treated i.p with ADR a significant increase in protein carbonyl formation and 3-NT levels, markers of protein oxidation and RNS attack on tyrosine residues, respectively, and in HNE, a marker of lipid peroxidation. Protein carbonyls accrue via several mechanisms. In particular, some of the elevated protein-resident carbonyl groups may originate from HNE, which covalently binds to His, Cys, and Lys residues via Michael addition [2,28,32,44,47].

ADR increases superoxide production and enhances formation of nitric oxide [45]. As a consequence, the balance between superoxide and nitric oxide shifts and could lead to formation of peroxynitrite [30,45], from which 3-NT can arise. The significantly increased 3-NT levels in brain homogenate from ADR-treated mice compared to those from control mice could be due to reaction of peroxynitrite with tyrosine residues on polypeptide chains. The resulting nitration changes protein conformation [46] and, consequently, alters their function [5–9,30].

Lipid peroxidation products such as HNE and acrolein are known to be elevated in brain under oxidative stress in various neurodegenerative conditions, including AD [4–6,28,47]. These alkenals react with GSH [32,48] and are also known to be involved in apoptosis, which is correlated with GSH depletion [49]. In the current study, there was a significant increase in protein bound HNE levels in brain homogenate isolated from ADR-injected mice.

MRP1 is one of the 9-multidrug resistance proteins belonging to the ATP-binding cassette (ABC) transporter family [50]. MRP1 is an integral plasma membrane protein that mediates ATP-dependent export of cytotoxic organic anions, glutathione S-conjugates and sulphates [51,52]. MRP1 is expressed in various human tumors. Oxidized glutathione (GSSG) has been recognized as a co-substrate for MRP1 [52]. Translocation of therapeutic drugs into and out of the CNS mediated by MRP1 has been shown [53,54], and others have reported expression of MRP1 in cultured astroglial cells [55]. Involvement of MRP1 in the release of GSSG from brain astrocytes under oxidative stress has also been reported [56]. These studies suggest that MRP1 expression is altered during oxidative stress conditions. Consistent with this suggestion, we reported increased expression of MRP-1 in AD brain [32], which is under oxidative stress [1,2,4–8].

Studies have shown that MRP1 does not directly transport chemotherapeutic agents such as ADR [31]. However, it is not clear whether glutathione is either co-transported as a GS-conjugate with ADR or activates MRP1 for ADR transport [31,57].

In the current study, we observed a significant increase in the levels of MRP1 in brain isolated from ADR-injected mice compared to the level of MRP1 in brain isolated from saline injected control mice. The increased expression could be due to increase in GSSG because of oxidative stress caused by ADR. Alternatively, the increased expression could be the result of the increased GS-conjugate of ADR, which is transported out of brain via MRP1.

Patients under prolonged treatment with ADR show symptoms of forgetfulness, dizziness and loss of memory, a phenomenon commonly called chemo-brain [25]. ADR leads to post-chemotherapeutics problems in children. Often, the symptoms are noticed approximately 10 years after treatment. Our current *in vivo* studies show that ADR promotes oxidative stress in brain. Our data suggest that ADR, its metabolites or downstream sequelae is likely to enter the brain and cause oxidative stress, which may contribute to chemobrain. Further studies are necessary to find the oxidative stress mechanism induced by ADR, which will help in designing therapeutics to reduce the side effects of such anti-cancer drugs, including chemobrain.

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