Asian Journal of Andrology (2013) 15, 290–296

www.nature.com/aja

ORIGINAL ARTICLE

N-acetylcysteine protects against cadmium-induced germ cell apoptosis by inhibiting endoplasmic reticulum stress in testes

Yan-Li Ji*, Hua Wang*, Cheng Zhang, Ying Zhang, Mei Zhao, Yuan-Hua Chen and De-Xiang Xu

Cadmium (Cd) is a reproductive toxicant that induces germ cell apoptosis in the testes. Previous studies have demonstrated that endoplasmic reticulum (ER) stress is involved in Cd-induced germ cell apoptosis. The aim of the present study was to investigate the effects of *N*-acetylcysteine (NAC), an antioxidant, on Cd-induced ER stress and germ cell apoptosis in the testes. Male CD-1 mice were intraperitoneally injected with $CdCl_2$ (2.0 mg kg^{-1}). As expected, acute Cd exposure induced germ cell apoptosis in the testes, as determined by terminal dUTP nick-end labelling (TUNEL). However, the administration of NAC alleviated Cd-induced germ cell apoptosis in the testes. Further analysis showed that NAC attenuated the Cd-induced upregulation of testicular glucose-regulated protein 78 (GRP78), an important ER molecular chaperone. Moreover, NAC inhibited the Cd-induced phosphorylation of testicular eukaryotic translation initiation factor 2α (eIF2α), a downstream target of the double-stranded RNA-activated kinase-like ER kinase (PERK) pathway. In addition, NAC blocked the Cd-induced activation of testicular X binding protein (XBP)-1, indicating that NAC attenuates the Cd-induced ER stress and the unfolded protein response (UPR). Interestingly, NAC almost completely prevented the Cd-induced elevation of C/EBP homologous protein (CHOP) and phosphorylation of c-Jun N-terminal kinase (JNK), two components of the ER stress-mediated apoptotic pathway. In conclusion, NAC protects against Cd-induced germ cell apoptosis by inhibiting endoplasmic reticulum stress in the testes.
Asian Journal of Andrology (2013) **15,** 290–296; doi:10.1038/aja.2012.129; published online 28 January 2013

Keywords: antioxidant; apoptosis; cadmium; endoplasmic reticulum stress; *N*-acetylcysteine (NAC); testis; unfolded protein response

INTRODUCTION

In recent years, concerns about the adverse effects of various environmental contaminants on male reproduction have grown.[1] Cadmium (Cd) is a major environmental toxicant. The general population is exposed to Cd *via* drinking water, food and cigarette smoking. Cd is a reproductive toxicant in humans. Cd has been associated with male infertility and poor semen quality in humans.[2] According to several earlier epidemiological studies, there is a significant inverse correlation between blood Cd level and semen quality.[3,4] Even a low level of Cd accumulation in semen might contribute to male infertility by reducing sperm quality.[5] In rodent studies, Cd has been shown to be a testicular toxicant.[6] Animal experiments have revealed that Cd induces germ cell apoptosis in the testes.[7,8] However, the molecular mechanism of Cd-mediated testicular germ cell apoptosis remains unknown.

The endoplasmic reticulum (ER) is an important organelle required for many cellular processes. In the ER, nascent proteins are folded with the assistance of ER chaperones. In addition, the ER serves as a cellular Ca^{2+} store and plays an important role in homeostasis. The ER is sensitive to alterations in cellular homeostasis. When ER function is disturbed, ER stress occurs and unfolded protein response (UPR) signalling is activated to recover ER function.[9,10] UPR signalling is mediated by three transmembrane ER proteins: inositol requiring ER-to-nucleus signal kinase (IRE)-1, activating transcription factor-6 and double-stranded RNA-activated kinase-like ER kinase (PERK).[11,12] UPR signalling is involved in the process of apoptosis in somatic cells.[13,14] Recently, we found that crosstalk between ER stress and mitochondrial pathways mediated Cd-induced germ cell apoptosis in the testes.[15]

Excess reactive oxygen species (ROS) production and GSH depletion are associated with ER stress and UPR signalling activation.[16] Indeed, Cd induces the generation of ROS in multiple cell types.[17] In addition, Cd induces oxidative stress in the testes.[18,19] The aim of the present study was to investigate whether *N*-acetylcysteine (NAC), an antioxidant, can attenuate Cd-induced ER stress and germ cell apoptosis in testes. We demonstrate, for the first time, that NAC protects against Cd-induced germ cell apoptosis by inhibiting endoplasmic reticulum stress in testes.

MATERIALS AND METHODS

Chemicals and reagents

$CdCl_2$ and NAC were obtained from Sigma Chemical Co. (St Louis, MO, USA). Heme oxygenase (HO)-1, 3-nitrotyrosine (3-NT), X

Department of Toxicology, School of Public Health, Anhui Medical University, Hefei 230032, China
* These authors contributed equally to this work.
Correspondence: Professor DX Xu (xudex@126.com)
Received: 27 May 2012; Revised: 10 August 2012; Accepted: 31 August 2012; Published online: 28 January 2013

box-binding protein (XBP)-1 and phosphor-c-Jun N-terminal kinase (JNK) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Glucose-regulated protein 78 (GRP78) and phosphor-eukaryotic translation initiation factor 2α (p-eIF2α) antibodies were obtained from Cell Signaling Technology (Beverley, MA, USA). β-actin antibody was obtained from Boster Bio-Technology Co. Ltd (Wuhan, China). The chemiluminescence detection kit was obtained from Pierce Biotechnology (Rockford, IL, USA). TRIzol reagent was obtained from Molecular Research Center, Inc. (Cincinnati, OH, USA). RNase-free DNase was obtained from Promega Corporation (Madison, WI, USA). All other reagents were purchased from Sigma Chemical Co. unless otherwise stated.

Animals and treatments

Adult male CD-1 mice (8 weeks old, 28–32 g) were purchased from Beijing Vital River, whose foundation colonies were established by Charles River Laboratories, Inc (Wilmington, MA, USA). The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20 °C–25 °C) and humidity (50%±5%) environment. To investigate the protective effects of NAC on Cd-induced testicular germ cell apoptosis, 48 mice were randomly divided into four groups. In the Cd-alone group, all mice were intraperitoneally (i.p.) injected with $CdCl_2$ (2.0 mg kg^{-1}). In the NAC+Cd group, mice were i.p. injected with NAC (100 mg kg^{-1}) every 8 h beginning at 8 h before Cd injection (2.0 mg kg^{-1}, i.p.). In the NAC-alone group, mice were i.p. injected with NAC (100 mg kg^{-1}) every 8 h. The control mice were administered the same volume of normal saline (NS). Preliminary experiments showed that apoptotic cells were observed in the testes of mice treated with 2.0 mg kg^{-1} Cd. In addition, the number of apoptotic cells per tubule was highest at 24 h after Cd injection. Thus, testes were collected 24 h after Cd injection. The doses of NAC used in our experiments were determined in previous studies.[20,21] The testes were divided into two parts: half was kept at −80 °C for Western blotting and RT-PCR, and half was immersed in modified Davidson's fluid for 24 h for testicular histology and apoptosis analysis. All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Testicular histology and terminal dUTP nick-end labelling (TUNEL)

Two cross-sections from each testis were embedded in paraffin, and paraffin-embedded tissues were serially sectioned. At least two non-serial sections were stained with haematoxylin and eosin for morphological analyses. For the detection of apoptosis, paraffin-embedded sections were subjected to TUNEL using an *in situ* apoptosis detection kit from Promega Corporation according to the manufacturer's protocols. To assess apoptosis in testicular cells, 200 different seminiferous tubules were observed in each section. A histogram of the number of TUNEL-positive germ cells per seminiferous tubule and the percentages of the number of seminiferous tubules containing TUNEL-positive germ cells were analysed.

Immunoblotting

Immunoblotting was performed using testicular lysates. Briefly, protein extracts from each sample were added to a gel loading buffer (100 mmol l^{-1} Tris, pH 6.8, 20% glycerol, 200 mmol l^{-1} DTT, 4% SDS, 0.03% bromophenol blue) and boiled for 5 min. Proteins (50 μg per sample) in loading buffer were subjected to electrophoresis in 10%–15% SDS–polyacrylamide gel for 3 h. The gel was transferred by electrophoresis onto a polyvinylidene fluoride membrane from Millipore (Bedford, MA, USA) and blocked in 5% non-fat powdered milk in Dulbecco's PBS overnight at 4 °C. The membranes were incubated for 2 h with the following antibodies: HO-1, 3-NT, GRP78, p-eIF2α and p-JNK. β-actin was used as a loading control for total proteins. After washes in Dulbecco's PBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit or goat anti-mouse IgG antibody for 2 h. The membranes were then washed four times in Dulbecco's PBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an enhanced chemiluminescence detection kit from Pierce.

Isolation of total RNA and RT-PCR

Fifty milligrams of testis tissue was collected from each mouse. Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. RNase-free DNase was used to remove genomic DNA. The integrity and concentration of the RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. For the synthesis of cDNA, 1.0 μg of total RNA from each sample was resuspended in a 20 μl final volume of reaction buffer, which contained 25 mmol l^{-1} TrisHCl, pH 8.3, 37.5 mmol l^{-1} KCl, 10 mmol l^{-1} dithiothreitol, 1.5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} of each dNTP and 0.5 mg oligo$(dT)_{15}$ primer. After the reaction mixture reached 42 °C, 20 units of RT was added to each tube, and the sample was incubated for 60 min at 42 °C. Reverse transcription was stopped by denaturing the enzyme at 95 °C. The final PCR mixture contained 2.5 μl of cDNA, 1×PCR buffer, 1.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dNTP mixture, 1 U of Taq DNA polymerase, 1 μmol l^{-1} sense and antisense primers, and sterile water to 50 μl. The reaction mixture was covered with mineral oil. Primer pairs for PCR were designed based on known mRNA sequences (Genbank at PubMed) using the free online primer design software Primer 3 and BLAST. The following primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Company (Shanghai, China): *Gapdh*, 5′-GAG GGG CCA TCC ACA GTC TTC-3′ and 5′-CAT CAC CAT CTT CCA GGA GCG-3′; *Grp78*, 5′-GCG TGT GTG TGA GAC CAG AAC CG-3′ and 5′-TGC GTC CGA TGA GGC GCT TG-3′. For *Gapdh*, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. For *Grp78*, amplification was initiated by 3 min of denaturation at 94 °C for 1 cycle, followed by 45 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 1 min. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels for 45 min. pBR322 DNA digested with Alu I was used as a molecular marker (MBI Fermentas). Agarose gels were stained with 0.5 mg ml^{-1} ethidium bromide (Sigma Chemical Co.) TBE buffer. For analysis of *xbp-1* mRNA splicing, 2.5 μl of cDNA was amplified with specific primers described by others:[22] forward, 5′-CCA TGG GAA GAT GTT CTG GG-3′; reverse, 5′-ACA AGC TTG GGA ATG GAC AC -3′. PCR products were separated by electrophoresis on 2.5% agarose gels and visualized by ethidium bromide staining. The level of *sXBP-1* mRNA was normalized to *uXBP-1* mRNA level in the same samples. The level of *sXBP-1* mRNA was set to 1 as a control.

Determination of glutathione (GSH) content

GSH was determined by the Griffith method. Testicular homogenates (0.4 ml) were precipitated by the addition of 0.4 ml of a metaphosphoric acid solution. After 40 min, the protein precipitate was separated from the remaining solution by centrifugation at 4000*g* at 4 °C

for 5 min. Supernatant (400 μl) was combined with 0.4 ml of 300 mmol l^{-1} Na_2HPO_4, and the absorbance was read at 412 nm, a blank consisting of 0.4 ml supernatant plus 0.4 ml H_2O was used for background correction. Then, 100 μl DTNB (0.02%, w/v; 20 mg DTNB in 100 ml of 1% sodium citrate) was added to the blank and sample. Absorbance of the sample was read at 412 nm, and a blank sample was used for background correction. The GSH content was determined using a calibration curve prepared with a standard. GSH values were expressed as nmol mg^{-1} protein.

Statistical analysis

Each band from RT-PCR and immunoblotting was scanned, and band intensity was analysed by Image J software (http://rsb.info.nih.gov/ij/). For RT-PCR, the level of *Grp78* mRNA was normalized to *Gapdh* mRNA level in each sample. The level of *Grp78* mRNA in the control was set as 1. For immunoblotting, the levels of GRP78, p-eIF2α, C/EBP homologous protein (CHOP), HO-1, 3NT and p-JNK were normalized to β-actin in each sample. The level of the control was set as 1. All quantified data are expressed as the means±s.e.m. ANOVA and the Student–Newmann–Keuls *post hoc* test were used to determine the differences among groups.

RESULTS

NAC alleviates Cd-induced testicular histopathological damage

Testicular oedema was observed 24 h after $CdCl_2$ was administered to mice. Consistent with testicular oedema, the absolute and relative testis weights were higher in Cd-treated mice than in control mice. Interestingly, NAC pretreatment almost completely inhibited Cd-induced testicular oedema. In addition, NAC clearly alleviated the Cd-induced elevation of the absolute testis weight (**Figure 1a**) and the relative testis weight (**Figure 1b**). The effects of NAC on Cd-induced testicular histopathological damage are presented in **Figure 1c**. As expected, a slight necrosis of seminiferous tubules and a moderate haemorrhage in the interstitium were observed in the testes of mice treated with $CdCl_2$. Interestingly, NAC alleviated this Cd-induced histopathological damage in the testes.

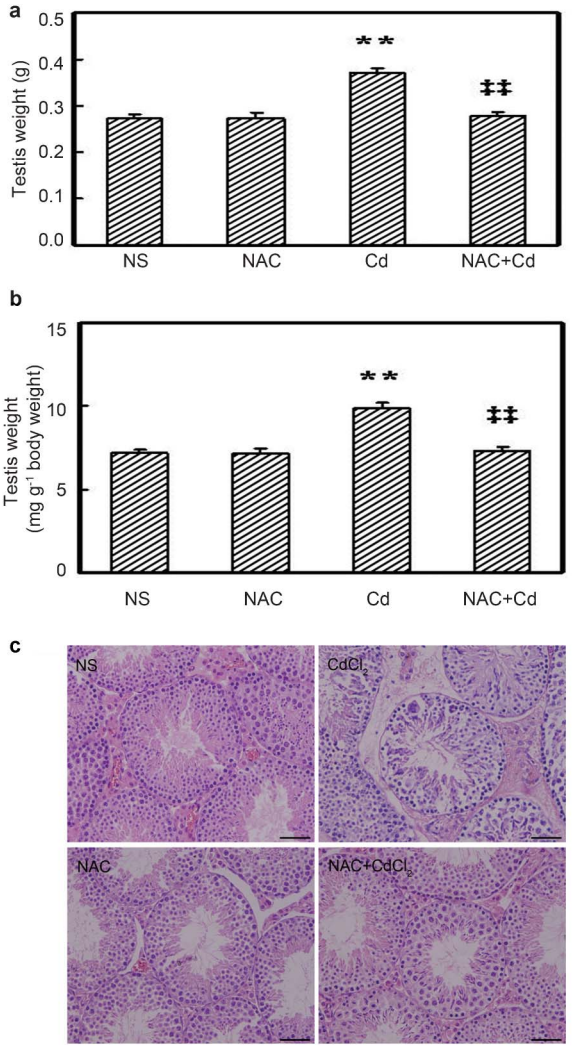

Figure 1 Effects of NAC on Cd-induced testicular histopathological damage. (**a**) Absolute weight of testis. (**b**) Relative weight of testis. $^{**}P<0.01$ *vs.* NS group. $^{‡‡}P<0.01$ *vs.* Cd group. (**c**) Testicular cross sections were stained with haematoxylin and eosin. Scale bar=50 μm. Cd, cadmium; NAC, *N*-acetylcysteine; NS, normal saline.

NAC protects against Cd-induced germ cell apoptosis in the testes

The effects of NAC on Cd-induced germ cell apoptosis in the testes were analysed. As shown in **Figure 2a**, a single dose of Cd increased the number of apoptotic cells in the testes. Most $TUNEL^+$ cells were germ cells in the seminiferous tubules. Further analysis showed that a single dose of Cd markedly increased the percentage of tubules with $TUNEL^+$ cells in the testes (**Figure 2b**). In addition, Cd increased the number of apoptotic cells per tubule (**Figure 2c**). Interestingly, pre-treatment with NAC clearly reduced both the percentage of tubules with $TUNEL^+$ cells (**Figure 2b**) and the number of $TUNEL^+$ cells per tubule (**Figure 2c**).

NAC alleviates Cd-induced ER stress in the testes

The effects of NAC on Cd-induced testicular ER stress were then analysed. As shown in **Figure 3a**, the level of GRP78 protein, an ER chaperone and activating transcription factor-6 target, was increased in the testes of mice treated with $CdCl_2$. Correspondingly, the level of *Grp78* mRNA was upregulated in the testes of mice treated with $CdCl_2$ (**Figure 3b**). Interestingly, NAC attenuated Cd-induced upregulation of GRP78 in the testes (**Figure 3a and 3b**). Next, the effects of NAC on XBP-1, a downstream target of the IRE1α pathway, were analysed in the testes of mice treated with $CdCl_2$. As expected, the level of spliced XBP-1 (sXBP-1) was markedly increased in the testes of mice treated with $CdCl_2$, indicating that IRE1α signalling was activated in the testes of mice treated with $CdCl_2$. NAC pre-treatment clearly alleviated Cd-induced elevation of sXBP-1 in the testes (**Figure 4a**). The effects of NAC on p-JNK, another downstream target of the IRE1 pathway, were then analysed in the testes of mice treated with $CdCl_2$. As shown in **Figure 4b**, the level of p-JNK was dramatically increased in the testes of mice treated with $CdCl_2$. NAC pre-treatment almost completely inhibited Cd-induced testicular JNK phosphorylation (**Figure 4b**). Finally, the effects of NAC on eIF2α, a downstream target of the PERK pathway, were analysed in the testes of mice treated with $CdCl_2$. As shown in **Figure 4c**, the level of phosphorylated eIF2α was increased in the testes of mice treated with $CdCl_2$. NAC pre-treatment attenuated Cd-induced testicular eIF2α phosphorylation (**Figure 4c**). The effects of NAC on CHOP, another downstream target of the PERK pathway, were then analysed in the testes of mice treated with $CdCl_2$. As expected, acute Cd exposure increased the level of CHOP in the testes. NAC pre-treatment almost completely inhibited Cd-induced upregulation of CHOP in the testes (**Figure 4d**).

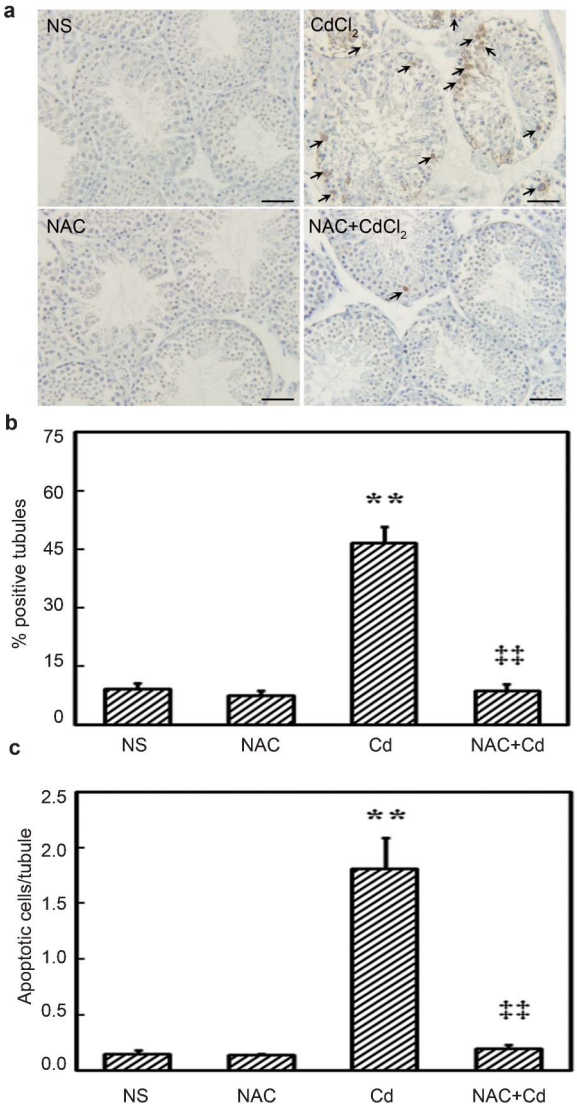

Figure 2 Effects of NAC on Cd-induced germ cell apoptosis in the testes. (**a**) Germ cell apoptosis in the testes was detected by TUNEL. Arrows indicate $TUNEL^+$ germ cells in seminiferous tubules. Scale bar=50 μm. (**b**) The percentage of seminiferous tubules containing $TUNEL^+$ germ cells. (**c**) The number of $TUNEL^+$ germ cells per seminiferous tubule. $^{**}P<0.01$ *vs.* NS group. $^{‡‡}P<0.01$ *vs.* Cd group. Cd, cadmium; NAC, *N*-acetylcysteine; NS, normal saline; TUNEL, terminal dUTP nick-end labelling.

Effects of acute Cd exposure on the level of caspase-12 in the testes

To investigate whether acute Cd exposure activates testicular caspase-12, the levels of procaspase-12 and cleaved caspase-12 were analysed in the testes of mice treated with $CdCl_2$. Unexpectedly, no cleaved caspase-12 was detected in the testes of mice treated with $CdCl_2$ (data not shown). Moreover, no significant difference in the level of testicular procaspase-12 was observed between Cd-treated mice and controls (**Figure 5**).

Effects of NAC on Cd-induced stress sensitive redox molecules in the testes

The effects of NAC on the level of HO-1, a marker of heat stress, were analysed in the testes of mice treated with $CdCl_2$. As shown in **Figure 6a**, the level of testicular HO-1 was increased in mice treated with $CdCl_2$. Interestingly, NAC pre-treatment alleviated Cd-induced upregulation of HO-1 in the testes (**Figure 6b**). 3-NT is a specific marker of protein nitration. The effects of NAC on Cd-induced testicular protein nitration are presented in **Figure 6a and 6c**. As expected, 3-NT intensity was enhanced in the testes of mice treated with $CdCl_2$. NAC pre-treatment attenuated Cd-induced protein nitration in the testes. The effects of NAC on the levels of GSH were then analysed in the testes of mice treated with $CdCl_2$. The level of GSH was dramatically decreased in the testes of mice treated with $CdCl_2$. NAC pre-treatment completely prevented Cd-induced loss of GSH in the testes (**Figure 6d**).

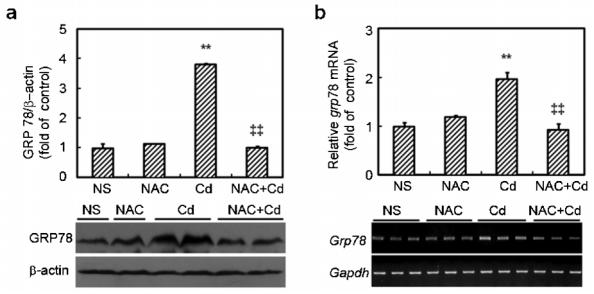

Figure 3 Effects of acute Cd exposure on the expression of GRP78 in the testes. (**a**) Testicular GRP78 protein was measured by immunoblotting. (**b**) Testicular *Grp78* mRNA levels were determined by RT-PCR. All experiments were repeated three times. $^{**}P<0.01$ *vs.* NS group. $^{‡‡}P<0.01$ *vs.* Cd group. Cd, cadmium; GRP78, glucose-regulated protein 78; NAC, *N*-acetylcysteine; NS, normal saline.

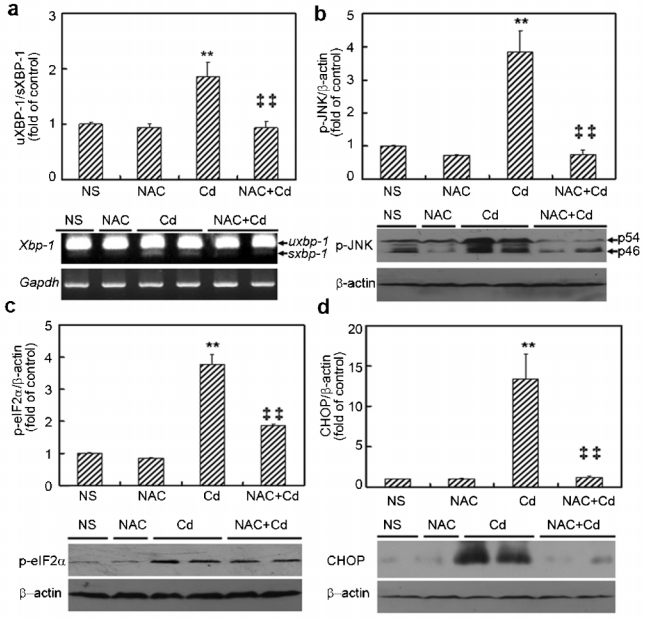

Figure 4 Effects of NAC on Cd-induced ER stress in the testes. (**a**) The levels of testicular *uXbp-1* and *sXbp-1* mRNA were measured using RT-PCR. (**b**) The level of testicular pJNK was measured by immunoblotting. (**c**) The level of testicular p-eIF2α was measured by immunoblotting. (**d**) The level of testicular CHOP was measured by immunoblotting. All experiments were repeated three times. $^{**}P<0.01$ *vs.* NS group. $^{‡‡}P<0.01$ *vs.* Cd group. Cd, cadmium; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; NAC, *N*-acetylcysteine; NS, normal saline; p-eIF2α, phosphor-eukaryotic translation initiation factor 2α.

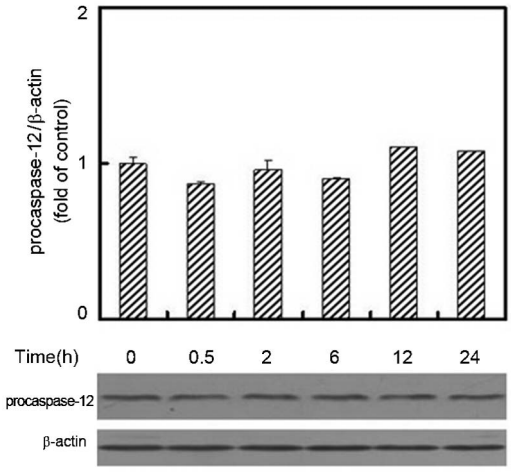

Figure 5 Effects of acute Cd exposure on the level of procaspase-12 in the testes. Mice were injected with $CdCl_2$ (2.0 mg kg^{-1}). Testes were collected at different times after Cd injection. The level of testicular procaspase-12 was detected by Western blotting. Cd, cadmium.

DISCUSSION

In the present study, we showed that exposure to a single dose of Cd elevated the level of GRP78, an important ER molecular chaperone, in the testes. Moreover, the level of phosphorylated eIF2α, a downstream target of the PERK pathway, was increased in the testes of Cd-treated mice, indicating that acute Cd exposure activates the PERK pathway in the testes. In addition, the level of spliced mRNA encoding XBP-1, a downstream target of the IRE1α pathway, was upregulated, suggesting that the IRE-1α pathway was activated in the testes of mice treated with $CdCl_2$. These results suggest that a single exposure to Cd induces ER stress and UPR signalling in the testes.

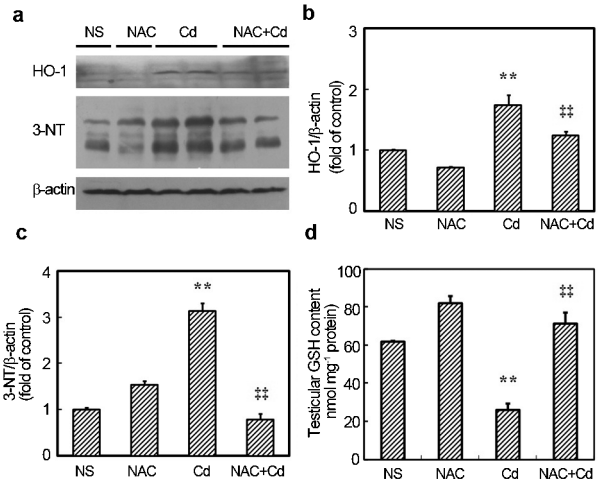

Figure 6 Effects of NAC on Cd-induced stress sensitive redox molecules in the testes. (**a**) Testicular HO-1 and 3-NT were measured by immunoblotting. A representative gel for HO-1, 3-NT and β-actin is shown. (**b**) HO-1 levels were normalized to β-actin in the same samples. (**c**) 3-NT was normalized to β-actin in the same samples. The densitometry unit of the control was set as 1. All experiments were repeated three times. (**d**) Testicular GSH content was measured. All data are expressed as the mean±s.e.m. (n=12). $^{**}P<0.01$ *vs.* NS group. $^{‡‡}P<0.01$ *vs.* Cd group. Cd, cadmium; GSH, glutathione; HO, heme oxygenase; NAC, *N*-acetylcysteine; NS, normal saline; 3-NT, 3-nitrotyrosine.

Numerous studies have demonstrated that the ER is especially sensitive to alterations in redox homeostasis.[16] When excess ROS are produced, the proteins formed in the ER may fail to fold properly.[23,24] The accumulation of unfolded and misfolded proteins in the ER lumen activates UPR signalling. Several studies have shown that antioxidants alleviate ER stress and UPR signalling.[25] Indeed, Cd induces oxidative stress in the testes. NAC is a potent antioxidant, and several studies have shown that pre-treatment with NAC scavenged the Cd-induced excess ROS and prevented apoptosis in somatic cells.[17,26–28] In the present study, we investigated the effects of NAC on Cd-induced ER stress in the testes. We showed that pre-treatment with NAC reduced the level of GRP78 in the testes of mice treated with Cd. Moreover, NAC alleviated the Cd-induced upregulation of *sXBP-1* mRNA in the testes. In addition, NAC attenuated the Cd-induced eIF2α phosphorylation in the testes. These results suggest that NAC inhibits Cd-induced ER stress and UPR signalling in the testes.

Several studies have demonstrated that UPR signalling is involved in β-cell apoptosis in the pancreas,[29,30] neuronal cell apoptosis in the brain,[31,32] preadipocyte apoptosis in adipose tissue,[33] renal epithelial cell apoptosis in the kidneys[34] and myocardial cell apoptosis in the heart.[35] Indeed, a recent report from our laboratory showed that Cd induced ER stress and activated UPR signalling in germ cells in the seminiferous tubules.[15] Furthermore, phenylbutyric acid, an ER chemical chaperone, protects mice against testis germ cell apoptosis.[15] In the present study, we found that NAC not only alleviated Cd-induced UPR signalling activation, but also protected against germ cell apoptosis in the testes. Therefore, it is reasonable to assume that NAC protects against Cd-induced germ cell apoptosis by inhibiting ER stress in the testes.

Caspase-12 is essential for ER stress-induced apoptosis in rodent animals. Several studies have demonstrated that caspase-12 is activated in ER stress-mediated apoptosis.[36,37] Interestingly, the present study showed that caspase-12 was not activated in the testes of mice treated with $CdCl_2$, suggesting that Cd-induced germ cell apoptosis is independent of caspase-12 activation. Several studies have demonstrated that IRE1α activates JNK and its downstream proapoptotic kinase apoptosis signal-regulating kinase.[38,39] The present study showed that the level of phosphorylated JNK, a downstream target of the IRE1 pathway, was markedly increased in the testes of mice treated with $CdCl_2$. Importantly, NAC almost completely inhibited Cd-induced JNK phosphorylation in the testes. These results suggest that JNK and its downstream proapoptotic kinase might be putative mediators of Cd-induced germ cell apoptosis in the testes.

CHOP, also known as growth arrest and DNA damage-inducible gene 153,[40] is one of the components in the ER stress-mediated apoptotic pathway and a downstream target of the PERK pathway.[14,41–44] The present study showed that the expression of CHOP was clearly upregulated in the testes of mice treated with $CdCl_2$. Moreover, NAC attenuated the Cd-induced upregulation of CHOP in the testes. Thus, CHOP contributes, at least partially, to Cd-induced germ cell apoptosis in the testes. How CHOP mediates Cd-induced testicular germ cell apoptosis remains unknown. One study demonstrated that overexpression of CHOP promoted translocation of Bax from the cytosol to the mitochondria. A recent study found that ER stress activated the BH3-only protein Bim and triggered apoptosis through CHOP-mediated direct transcriptional induction.[45] Thus, additional research is necessary to determine whether Cd induces the translocation of Bax from the cytosol to the mitochondria and upregulates the expression of Bim in the testes.

HO-1, a stress-inducible enzyme, is expressed at a low level in the testes, mainly in Sertoli and Leydig cells.[46] Testicular HO-1 expression

is upregulated in response to stress.[47] An earlier study demonstrated that Leydig cell-derived HO-1 regulates the apoptosis of premeiotic germ cells in response to stress.[8] Indeed, the present study showed that a single dose of Cd upregulated the expression of HO-1 in the testes. Moreover, NAC inhibited the Cd-induced upregulation of testicular HO-1. Thus, the present study does not exclude the role of HO-1 in Cd-mediated germ cell apoptosis in the testes.

The antioxidant activity of NAC primarily involves two mechanisms: (i) NAC acts as a free radical scavenger, directly scavenging hydrogen peroxide, hydroxyl free radicals and hypochloric acid;[48] and (ii) NAC acts as a precursor of GSH to facilitate intracellular GSH synthesis.[49] NAC decreases free radical levels by increasing GSH synthesis.[50] Indeed, the present study showed that NAC alleviated Cd-induced testicular GSH depletion. In addition, pre-treatment with NAC significantly attenuated Cd-induced upregulation of HO-1 and protein nitration in the testes. Thus, the protective effects of NAC against Cd-induced ER stress and germ cell apoptosis are not only attributable to its strong ROS-scavenging effect but also likely to the increased GSH synthesis in the testes. The protective effects of NAC against Cd-induced germ cell apoptosis may have therapeutic implications. According to a recent report from our laboratory, the antioxidant ascorbic acid protects mice from Cd-induced ER stress and germ cell apoptosis.[51] Thus, antioxidants may be useful as pharmacological agents to protect against Cd-induced reproductive toxicity in the testes.

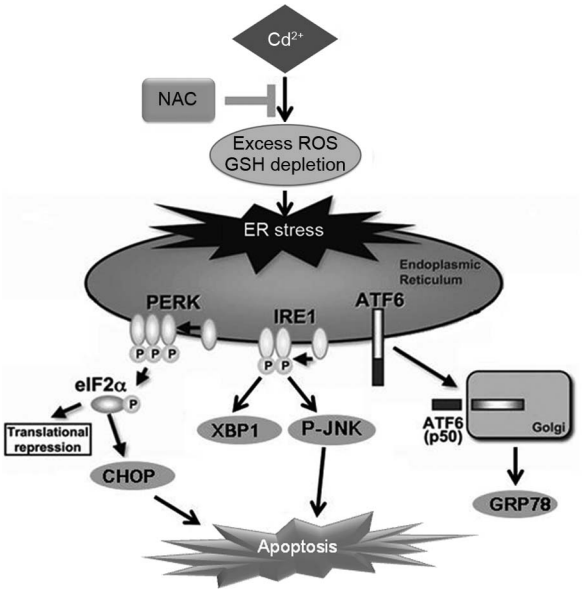

Figure 7 NAC protects against Cd-induced germ cell apoptosis by inhibiting UPR signalling in the testes. First, NAC attenuates the Cd-induced UPR signalling by inhibiting activation of PERK signalling and the IRE1α pathway in testis. Second, NAC protects against the Cd-induced germ cell apoptosis by inhibiting the Cd-induced upregulation of CHOP, a downstream target of PERK signalling, and phosphorylation of JNK, a downstream target of the IRE1α pathway. ATF, activating transcription factor; Cd, cadmium; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; GSH, glutathione; IRE, inositol requiring ER-to-nucleus signal kinase; JNK, c-Jun N-terminal kinase; NAC, *N*-acetylcysteine; p-eIF2α, phosphor-eukaryotic translation initiation factor 2α; PERK, double-stranded RNA-activated kinase-like ER kinase; ROS, reactive oxygen species; UPR, unfolded protein response; XBP, X box-binding protein.

In summary, the present study demonstrates that NAC protects against Cd-induced germ cell apoptosis by inhibiting the activation of UPR signalling in the testes (**Figure 7**). First, NAC attenuates the Cd-induced UPR signalling by inhibiting activation of PERK signalling and the IRE1α pathway in the testes. Second, NAC protects against the Cd-induced germ cell apoptosis by inhibiting the Cd-induced upregulation of CHOP, a downstream target of PERK signalling, and phosphorylation of JNK, a downstream target of the IRE1α pathway.

AUTHOR CONTRIBUTIONS

DXX and YLJ were responsible for the project application, the experimental design and the manuscript revision. YLJ carried out the study and analysed the data. HW and CZ solved all the technical issues and performed the statistical analysis. YZ was involved in the animal experiments, and MZ and YHC participated in the glutathione measurements. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

ACKNOWLEDGMENTS

This project was supported by the National Natural Science Foundation of China (No. 31000664) and Grants for Scientific Research of BSKY (No. XJ201114) and the Program of Young Top-Notch Talents from Anhui Medical University.

1 Mathur PP, D'Cruz SC. The effect of environmental contaminants on testicular function. *Asian J Androl* 2011; **13**: 585–91.
2 Pant N, Upadhyay G, Pandey S, Mathur N, Saxena DK *et al*. Lead and cadmium concentration in the seminal plasma of men in the general population: correlation with sperm quality. *Reprod Toxicol* 2003; **17**: 447–50.
3 Telisman S, Cvitković P, Jurasović J, Pizent A, Gavella M *et al*. Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men. *Environ Health Perspect* 2000; **108**: 45–53.
4 Xu B, Chia SE, Tsakok M, Ong CN. Trace elements in blood and seminal plasma and their relationship to sperm quality. *Reprod Toxicol* 1993; **7**: 613–8.
5 Wu HM, Lin-Tan DT, Wang ML, Huang HY, Wang HS *et al*. Cadmium level in seminal plasma may affect the pregnancy rate for patients undergoing infertility evaluation and treatment. *Reprod Toxicol* 2008; **25**: 481–4.
6 Siu ER, Mruk DD, Porto CS, Cheng CY. Cadmium-induced testicular injury. *Toxicol Appl Pharmacol* 2009; **238**: 240–9.
7 Kim J, Soh J. Cadmium-induced apoptosis is mediated by the translocation of AIF to the nucleus in rat testes. *Toxicol Lett* 2009; **188**: 45–51.
8 Ozawa N, Goda N, Makino N, Yamaguchi T, Yoshimura Y *et al*. Leydig cell-derived heme oxygenase-1 regulates apoptosis of premeiotic germ cells in response to stress. *J Clin Invest* 2002; **109**: 457–67.
9 Rasheva VI, Domingos PM. Cellular responses to endoplasmic reticulum stress and apoptosis. *Apoptosis* 2009; **14**: 996–1007.
10 Wek RC, Cavener DR. Translational control and the unfolded protein response. *Antioxid Redox Signal* 2007; **9**: 2357–71.
11 Kohno K. How transmembrane proteins sense endoplasmic reticulum stress. *Antioxid Redox Signal* 2007; **9**: 2295–303.
12 Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 2007; **8**: 519–29.
13 Kim I, Xu W, Reed JC. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov* 2008; **7**: 1013–30.
14 Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S *et al*. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest* 2002; **109**: 525–32.
15 Ji YL, Wang H, Zhao XF, Zhang C, Zhang Y *et al*. Crosstalk between endoplasmic reticulum stress and mitochondrial pathway mediates cadmium-induced germ cell apoptosis in testes. *Toxicol Sci* 2011; **124**: 446–59.
16 Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal* 2007; **9**: 2277–93.
17 Chen L, Liu L, Huang S. Cadmium activates the mitogen-activated protein kinase (MAPK) pathway *via* induction of reactive oxygen species and inhibition of protein phosphatases 2A and 5. *Free Radic Biol Med* 2008; **45**: 1035–44.
18 Ardais AP, Santos FW, Nogueira CW. Ebselen attenuates cadmium-induced testicular damage in mice. *J Appl Toxicol* 2008; **28**: 322–8.
19 Ognjanović BI, Marković SD, Ethordević NZ, Trbojević IS, Stajn AS *et al*. Cadmium-induced lipid peroxidation and changes in antioxidant defense system in the rat testes: protective role of coenzyme Q_{10} and vitamin E. *Reprod Toxicol*2010; **29**: 191–7.

20 Achat-Mendes C, Anderson KL, Itzhak Y. Impairment in consolidation of learned place preference following dopaminergic neurotoxicity in mice is ameliorated by *N*-acetylcysteine but not D_1 and D_2 dopamine receptor agonists. *Neuropsychopharmacology* 2007; **32**: 531–41.
21 Hacimuftuoglu A, Handy CR, Goettl VM, Lin CG, Dane S *et al*. Antioxidants attenuate multiple phases of formalin-induced nociceptive response in mice. *Behav Brain Res* 2006; **173**: 211–6.
22 Iwakoshi NN, Lee AH, Vallabhajosyula P, Otipoby KL, Rajewsky K *et al*. Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat Immunol* 2003; **4**: 321–9.
23 Dickhout JG, Hossain GS, Pozza LM, Zhou J, Lhoták S *et al*. Peroxynitrite causes endoplasmic reticulum stress and apoptosis in human vascular endothelium: implications in atherogenesis. *Arterioscler Thromb Vasc Biol* 2005; **25**: 2623–9.
24 Gorlach A, Klappa P, Kietzmann T. The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. *Antioxid Redox Signal* 2006; **8**: 1391–418.
25 Malhotra JD, Miao H, Zhang K, Wolfson A, Pennathur S *et al*. Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proc Natl Acad Sci USA* 2008; **105**: 18525–30.
26 Chen L, Xu B, Liu L, Luo Y, Zhou H *et al*. Cadmium induction of reactive oxygen species activates the mTOR pathway, leading to neuronal cell death. *Free Radic Biol Med* 2011; **50**: 624–32.
27 Kalariya NM, Nair B, Kalariya DK, Wills NK, van Kuijk FJ. Cadmium-induced induction of cell death in human lens epithelial cells: implications to smoking associated cataractogenesis. *Toxicol Lett* 2010; **198**: 56–62.
28 Oh SH, Lim SC. A rapid and transient ROS generation by cadmium triggers apoptosis *via* caspase-dependent pathway in HepG2 cells and this is inhibited through *N*-acetylcysteine-mediated catalase upregulation. *Toxicol Appl Pharmacol* 2006; **212**: 212–23.
29 Oyadomari S, Takeda K, Takiguchi M, Gotoh T, Matsumoto M. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc Natl Acad Sci USA* 2001; **98**: 10845–50.
30 Song B, Scheuner D, Ron D, Pennathur S, Kaufman RJ. Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *J Clin Invest* 2008; **118**: 3378–89.
31 Galehdar Z, Swan P, Fuerth B, Callaghan SM, Park DS. Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. *J Neurosci* 2010; **30**: 16938–48.
32 Higo T, Hamada K, Hisatsune C, Nukina N, Hashikawa T *et al*. Mechanism of ER stress-induced brain damage by IP_3 receptor. *Neuron* 2010; **68**: 865–78.
33 Guo W, Wong S, Xie W, Lei T, Luo Z. Palmitate modulates intracellular signaling, induces endoplasmic reticulum stress, and causes apoptosis in mouse 3T3-L1 and rat primary preadipocytes. *Am J Physiol Endocrinol Metab* 2007; **293**: 576–86.
34 Timmins JM, Ozcan L, Seimon TA, Li G, Malagelada C *et al*. Calcium/calmodulin-dependent protein kinase II links ER stress with Fas and mitochondrial apoptosis pathways. *J Clin Invest* 2009; **119**: 2925–41.
35 Avery J, Etzion S, DeBosch BJ, Jin X, Lupu TS *et al*. TRB3 function in cardiac endoplasmic reticulum stress. *Circ Res* 2010; **106**: 1516–23.
36 Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem* 2002; **277**: 34287–94.
37 Nakagawa T, Zhu H, Morishima N, Li E, Xu J *et al*. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000; **403**: 98–103.
38 Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K *et al*. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* 2002; **16**: 1345–55.
39 Urano F, Wang X, Bertolotti A, Zhang Y, Chung P *et al*. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 2000; **287**: 664–6.
40 Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ* 2004; **11**: 381–9.
41 Lin JH, Li H, Yasumura D, Cohen HR, Zhang C *et al*. IRE1 signaling affects cell fate during the unfolded protein response. *Science* 2007; **318**: 944–9.
42 Tajiri S, Oyadomari S, Yano S, Morioka M, Gotoh T *et al*. Ischemia-induced neuronal cell death is mediated by the endoplasmic reticulum stress pathway involving CHOP. *Cell Death Differ* 2004; **11**: 403–15.
43 Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T *et al*. Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. *Cell Death Differ* 2004; **11**: 1009–16.
44 Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT *et al*. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev* 1998; **12**: 982–95.
45 Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN *et al*. ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 2007; **129**: 1337–49.
46 Ewing JF, Maines MD. Distribution of constitutive (HO-2) and heat-inducible (HO-1) heme oxygenase isozymes in rat testes: HO-2 displays stage-specific expression in germ cells. *Endocrinology* 1995; **136**: 2294–302.
47 Maines MD, Ewing JF. Stress response of the rat testis: in situ hydridization and immunohistochemical analysis of heme oxygenase-1 (HSP32) induction by hyperthermia. *Biol Reprod* 1996; **54**: 1070–9.
48 Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of *N*-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic Biol Med* 1989; **6**: 593–7.
49 Song M, Kellum JA, Kaldas H, Fink MP. Evidence that glutathione depletion is a mechanism responsible for the anti-inflammatory effects of ethyl pyruvate in cultured lipopolysaccharide-stimulated RAW 264.7 cells. *J Pharmacol Exp Ther* 2004; **308**: 307–16.
50 Atkuri KR, Mantovani JJ, Herzenberg LA, Herzenberg LA. *N*-Acetylcysteine—a safe antidote for cysteine/glutathione deficiency. *Curr Opin Pharmacol* 2007; **7**: 355–9.
51 Ji YL, Wang Z, Wang H, Zhang C, Zhang Y *et al*. Ascorbic acid protects against cadmium-induced endoplasmic reticulum stress and germ cell apoptosis in testes. *Reprod Toxicol* 2012; **34**: 357–63.