N-acetylcysteine prevents MAA induced male germ cell apoptosis: role of glutathione and cytochrome *c*

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Abstract Exposure to methoxyacetic acid (MAA), a major byproduct of the paint industry, causes testicular atrophy in multiple species. This study demonstrates DNA breakdown in rat germ cells after exposure to MAA in vivo within 12 h, leading to 40% germ cell death by 24 h. Within 4 h of treatment, cytochrome *c* is released from the mitochondria into the cytosol without the involvement of mitochondrial potential loss, reactive oxygen species generation or lipid peroxidation events. Peak activation of caspase-9 and caspase-3 is detectable post treatment at 4 and 8 h respectively. There is a decrease in germ cell glutathione levels within 2 h of MAA treatment. Replenishment of glutathione by pretreatment of the animals with the antioxidant *N*-acetylcysteine prior to MAA treatment could prevent the release of cytochrome *c*, DNA fragmentation and cell death.

Key words: Germ cells; Methoxyacetic acid; *N*-acetylcysteine; Apoptosis

1. Introduction

Glycol ethers are used in paints, lacquer, stains, inks, surface coatings, silk-screen printing, photographic processes, the semiconductor industry, textile and leather finishing and the production of food-contact plastics [1]. They are efficiently absorbed by inhalation as well as via dermal penetration and are rapidly converted in the body to metabolites that are equally toxic [1]. Exposure to ethylene glycol monomethyl ether and its major metabolite, methoxyacetic acid (MAA), cause testicular atrophy and infertility in multiple species including humans [2–7]. MAA primarily affects the spermatocytes and some population of round spermatids [2–7]. Even though MAA has been known for its effects on testicular germ cells, the mechanism by which it initiates germ cell death is not clear. Since the understanding of the pathway of toxin action in a cell is important in view of formulation of preventive measures for toxin exposure, this study was undertaken to investigate the mechanism of MAA induced germ cell death.

It is known that several biochemical parameters in the testis change in response to MAA treatment in animals. For example, upregulation of testicular protein kinase activity [8] and a thiol-specific antioxidant protein in germ cells after MAA treatment have been reported [9]. Expressions of a testicular germ cell-specific fatty acid-binding protein and Src (tyrosine kinase pp60) are also increased in response to MAA treatment [10,11]. Exposure to MAA causes depression of mitochondrial respiration [12] and complex formation between Bcl-w, Bax and Bak in the mitochondria [13]. Calcium channel blockers have been shown to be successful in preventing MAA induced germ cell death in rats [14]. In spite of all the above reports, the mechanism of germ cell death induced by MAA remains unknown. In this study, we demonstrate that in vivo, MAA induces an alteration in the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio in germ cells without inducing any change in reactive oxygen species (ROS) or lipid peroxidation levels. Release of cytochrome *c* from the mitochondria occurs resulting in the activation of caspases-9 and -3 leading to DNA fragmentation and apoptotic germ cell death. We also demonstrate the importance of GSH levels in the survival of the cells by pretreating rats with *N*-acetylcysteine (NAC) prior to MAA treatment, which increases cellular GSH levels and prevents germ cell death.

2. Materials and methods

2.1. Materials

Apoptosis detection kit was purchased from Promega (Madison, WI, USA). Vectastain ABC kit and diaminobenzidine-peroxidase substrate kit were procured from Vector Laboratories, Inc., (Burlingame, CA, USA). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide), H_2DCFDA (6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester)) and *cis*-parinaric acid (PnA) were obtained from Molecular Probes, Inc. (Eugene, OR, USA). FluorAce™ Apopain Assay kit was from Bio-Rad Laboratories (Hercules, CA, USA). Molecular weight markers and rabbit anti-cytochrome *c* IgG, rabbit anti-caspase-8 IgG, and rabbit anti-PARP IgG were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) respectively. All fine chemicals were from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Administration of MAA and NAC to adult rats

Rats were fed with MAA in PBS at 650 mg/kg body weight [50 mM phosphate buffer, pH 7.4; 0.9% NaCl] [5]. Control rats were fed with equal volumes of 50 mM PBS. NAC was injected intraperitoneally at a dose of 150 mg/kg of body weight for 3 days before challenging with a single dose of MAA.

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Abbreviations: MAA, methoxyacetic acid; NAC, *N*-acetylcysteine; TUNEL, TdT mediated dUTP nick-end labelling; ROS, reactive oxygen species; H_2DCFDA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester); JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GSH, glutathione; GSSG, oxidized glutathione

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2.3. Preparation of germ cells

Rat germ cells were prepared from the control and experimental animals as described previously [15].

2.4. TUNEL (TdT mediated dUTP Nick-End Labelling) assay and genomic DNA preparation

TUNEL staining and genomic DNA preparation were carried out as described previously [15]. For TUNEL, the incorporation of dUTP was assessed with avidin–horseradish peroxidase (HRP) at a dilution of 1:500. The sections were stained with DAB-peroxidase substrate kit and counter stained with hematoxylin.

2.5. Measurement of ROS, mitochondrial membrane potential, products of lipid peroxidation and GSH/GSSG levels

Intracellular levels of ROS were measured with H_2 DCFDA [16]. Briefly, 10^6 cells were incubated with 10 μg/ml dye at 33°C for 15 min. The cells were then washed once with PBS and the fluorescence emission was read at excitation of 488 nm and emission of 530 nm in a LS50B luminescent spectrofluorimeter (PE Biosystems, Norwalk, CT, USA). Mitochondrial membrane potential was measured by incubating the cells with 5 μg/ml solution of JC-I at 37°C for 10 min as described previously [17]. Lipid peroxidation was measured by MDA assay as described previously [15] and also by the oxidation of PnA. Briefly, 10^6 germ cells were incubated with 50 μg/ml PnA for 10 min at room temperature. The cells were washed with PBS and the exponential decline of fluorescence was measured at 530 nm at an excitation of 488 nm in a LS50B luminescent spectrofluorimeter. Intracellular levels of total GSH and GSSG were determined by the *o*-phthalaldehyde-derived fluorescence method of Senft et al. [18].

2.6. Preparation of germ cell cytosol, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot

Briefly, 10^8 germ cells were suspended in mitochondrial isolation buffer (150 mM sucrose; 10 mM succinate; 5 mM potassium phosphate; 10 mM HEPES–KOH, pH 7.4; 0.1% BSA) and lysed by nitrogen cavitation (450 psi for 30 min at 4°C) followed by centrifugation at $2500\times g$. The resulting supernatant was further centrifuged at $20\,000\times g$ and the pellet enriched in mitochondria was discarded. This supernatant was further centrifuged at $100\,000\times g$ for 1 h at 4°C in Optima™ XL-100K ultracentrifuge (Beckman Coulter Inc., Fullerton, CA, USA) and the supernatant obtained was stored at −70°C until further use. Protein assay was done using the bicinchoninic protein assay (Pierce Chemical Co., Rockford, IL, USA).

SDS–PAGE and Western blot were performed as described previously [19]. The anti-cytochrome *c* antibodies and the anti-rabbit IgG–HRP were used at a dilution of 1:2,500. The blots were developed using the ECL™ Western blotting detection reagents (Amersham Pharmacia Biotech., Uppsala, Sweden). Protein estimation was performed by the BCA method as described earlier [15].

2.7. Caspase assays

Activity of caspase-3 and caspase-9 were assayed according to the manufacturer's protocol provided with the Apopain assay kit. For the measurement of caspase-9 and -3 activity, substrates Ac-LEHD-AFC and Ac-DEVD-AFC were used respectively. The caspase activities were calculated from the standard curve of 7-amino-4-trifluoromethyl coumarin.

3. Results

3.1. Rat germ cells undergo apoptosis on exposure to MAA

To investigate the pathway of apoptosis initiated by MAA exposure, we first established the time kinetics of germ cell death. Around 40% of cell death occurred by 24 h post treatment (Fig. 1). DNA fragmentation was visible by 12 h as shown by the significant increase in the number of cells with TUNEL positive nuclei (Fig. 1, inset a) and laddering of the genomic DNA on agarose gels (Fig. 1, inset b). Therefore, it was clear that by 12 h changes leading to DNA fragmentation took place.

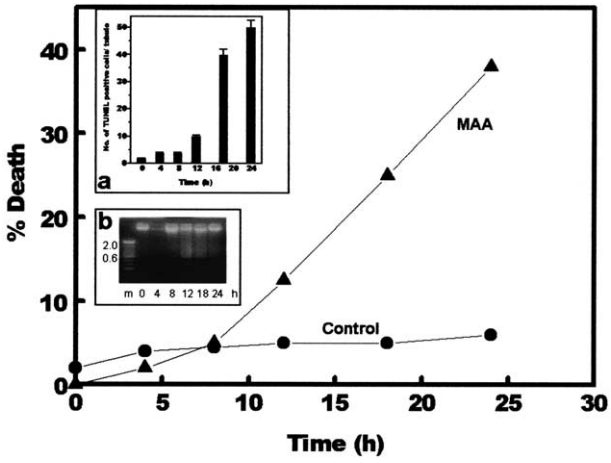

Fig. 1. Rat germ cells undergo apoptosis on exposure to MAA. Figure shows the viability of germ cells at different time points after MAA treatment. Results are mean ± S.D. of four experiments with each group containing six rats. Inset a: Quantitative estimation of TUNEL positive cells at different time points. The results are a mean ± S.D. of three experiments consisting of four rats each. At least 100 tubules from testicular section of each rat were counted. Inset b: DNA laddering pattern in germ cell genomic DNA on exposure to MAA in vivo. The time indicated represents the time of isolation of testis from MAA treated rats. Lane m, 100 bp marker. The results are that of a representative experiment of four repeats.

3.2. Cytochrome c release and caspase activation occurs in germ cells after exposure to MAA

To determine if germ cell apoptosis induced by MAA involved the release of mitochondrial proapoptotic factors, we checked for cytochrome *c* release from the mitochondria into the cytosol. Western blots showed the presence of cytochrome *c* in the cytosol from 4 h onwards in the treated group, whereas no cytochrome *c* was detectable in the cytosol of control cells (cells at 0 h)(Fig. 2A, inset). Once in the cytoplasm, cytochrome *c* catalyzes the oligomerization of apoptotic protease activating factor-1 (Apaf-1), thereby promoting the activation of procaspase-9 generating active caspase-9, which then activates procaspase-3 [20]. Our studies demonstrated that caspase-9 activity reached its peak by 4 h after exposure to MAA and the activity decreased from 8 h, falling to control levels by 12 h (Fig. 2A). Caspase-3 activity could be recorded at 4 h of exposure to MAA but reached its peak

Table 1
Levels of total, GSSG and reduced GSH in germ cells after exposure to a single dose of 650 mg/kg body weight MAA

Time (h)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	Total GSH content (nmol/mg protein)
0	0.371 ± 0.011	0.012 ± 0.002	0.395 ± 0.02
2	0.319 ± 0.02**	0.013 ± 0.003	0.345 ± 0.03
4	0.071 ± 0.003**	0.068 ± 0.005**	0.207 ± 0.04**

The total GSH content is expressed in GSH equivalents. At 4 h post exposure to MAA, GSH and GSSG levels are significantly different as compared to 0 h. Values represent mean ± S.D., $n = 4$. **$P < 0.001$.

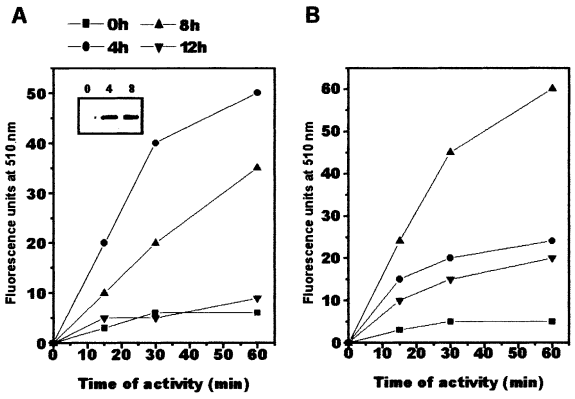

Fig. 2. Release of cytochrome *c* and activation of caspase-9 and -3 in MAA exposed rat germ cells. A, inset: Release of cytochrome *c* in the cytosol of germ cells exposed to MAA as recorded at 4 and 8 h. A,B: Activity of caspase-9 and -3 respectively in cytosolic extracts of germ cells exposed to MAA. Time represents the duration of the reaction.

at 8 h (Fig. 2B). The temporal relationship between cytochrome *c* release, activation of caspase-9 and -3 indicates that by 4 h the cells are committed to the apoptotic pathway.

3.3. Germ cell GSH levels change after exposure to MAA

One of the reasons why cytochrome *c* may have been released into the cytosol could be a change in the cellular redox state [21]. The cellular redox state is maintained by GSH that has multiple effects including radical scavenging, restoration of damaged molecules by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the reduced state [22]. Our studies show that the intracellular GSH levels decrease (Table 1) with a reduction in the ratio of GSH/GSSG by 20%. Therefore, from experiments described in this section it is obvious that after MAA exposure, the cells are in a state of apparent oxidative stress.

3.4. Exposure to MAA does not initiate a loss of mitochondrial membrane potential, production of ROS and lipid peroxidation

Accumulating evidence now suggests that ROS may act as signalling molecules for the initiation and execution of the apoptotic death program in many, if not all, current models of apoptotic cell death [21]. Since many toxins affect the mitochondria through the generation of ROS [23], we checked both mitochondrial activity and ROS generation. As a measure of mitochondrial activity, we used the dye JC-1 where intact mitochondria fluoresce red indicative of J-aggregates (at 590 nm emission) and mitochondria with low membrane potential fluoresce green showing increase in J-monomers (at 530 nm emission). Mitochondrial membrane potential was calculated as the ratio of aggregate to monomer [24]. MAA did not cause any loss of mitochondrial membrane potential as evidenced from the unchanged J-aggregate to J-monomer ratio (control, 5.5 ± 0.7, MAA treated, 5.2 ± 0.4). Since ROS increase could be induced by certain drugs, we checked the formation of intracellular ROS by using the dye H_2DCFDA. MAA in vivo did not induce the formation of intracellular ROS when checked at 1, 2 and 4 h (measurements at 2 h, fluorescence at 535 nm: controls, 40.25 ± 1.2; MAA treated, 48.24 ± 2.0). There were no lipid peroxidation events as tested by the estimation of malondialdehyde reactive products and

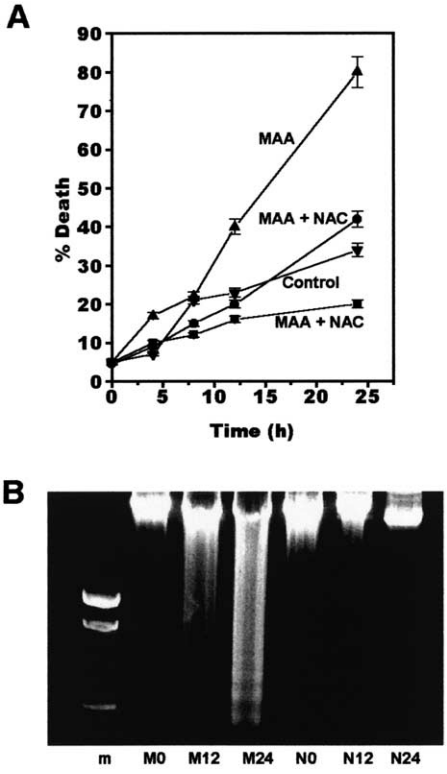

Fig. 3. Effect of NAC on MAA induced germ cell death and DNA fragmentation. A: Viability after treatment with MAA in NAC-pretreated rats. B: Inhibition of DNA fragmentation by treatment with NAC. m, marker; M0, DNA at 0 h; M12, DNA at 12 h post MAA administration; M24, DNA at 24 h post MAA administration; N0, DNA at 0 h post MAA administration; N12, DNA at 12 h post MAA administration in animals pretreated with NAC; N24, DNA at 24 h post MAA administration in animals pretreated with NAC.

oxidation of fluorescent *cis*-parinaric acid (data not shown). It has been shown by electron paramagnetic resonance studies that MAA has no effect on the lipid bilayer [25] and our studies indicate similar findings using other assays.

3.5. Treatment with NAC results in salvage of germ cell death

If a reduction in the intracellular levels of GSH was responsible for germ cell death, arguably, replenishing the levels of GSH by providing a substrate of the GSH biosynthetic pathway should result in the rescue of germ cells from undergoing apoptosis. NAC, a unique compound, which with its multiple activities can either work via its ROS scavenging ability, by increasing intracellular GSH levels or by serving as a reducing

Table 2
Quantitative estimation of TUNEL positive germ cells on exposure to MAA and NAC in vivo

Nature of treatment	No. of TUNEL positive cells per tubule
Placebo	5.0 ± 0.23
NAC	2.0 ± 0.12
MAA	56.0 ± 2.3
NAC pretreatment	19.0 ± 3.1**

At least 100 tubules per testicular section, prepared from a single testis from one rat, were counted for TUNEL positive cells. The results are mean ± S.D. of three repeats. *Significance: NAC+MAA compared with MAA, $^{**}P < 0.001$ as determined by unpaired two-tailed Student's *t*-test.

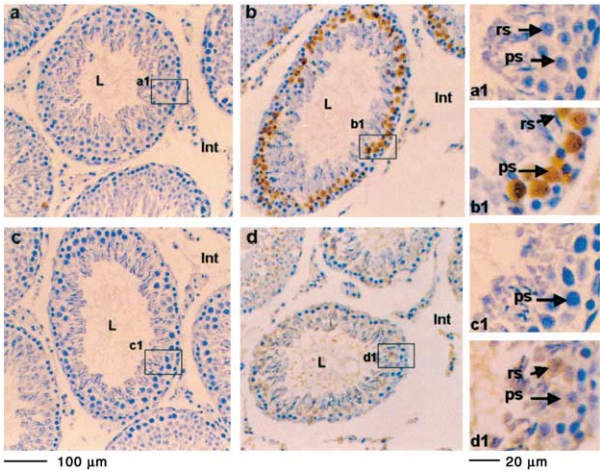

Fig. 4. TUNEL assay of testicular sections of animals exposed to MAA and NAC. a: Testis section from control animal; b: testis section from a MAA treated animal; c: testis section from a NAC treated animal; d: testis section from a MAA treated animal pretreated with NAC. a1–d1: Close-up of areas of seminiferous epithelium showing staining of different cell types in the seminiferous epithelium. ps, pachytene spermatocytes; rs, round spermatids; L, tubular lumen; Int, interstitium. Results are representative of six repeats.

agent [26], is widely used for treatment of various human disease conditions [27]. We used NAC to pretreat animals prior to exposure to MAA. A significant inhibition of cell death was found in the groups pretreated with NAC (Fig. 3A). DNA analysis of germ cells exposed to MAA after pretreatment with NAC showed a decrease in the formation of DNA ladders in agarose gels (Fig. 3B). The number of TUNEL positive nuclei/tubule was reduced by 77% as compared to cells from rats exposed to MAA only (Table 2). Sections from testis of treated and untreated rats with or without NAC clearly showed a decrease in the number of TUNEL positive cells (Fig. 4a–d). Clearly, a bulk of the spermatocyte and spermatid nuclei showing TUNEL positive response (Fig. 4b1) had significantly reduced as shown (Fig. 4d1). When GSH levels were measured after NAC preadministration in the treated rats, an 18% increase in the plasma GSH levels was observed. In germ cells of the NAC+MAA treated group as compared to only MAA treated rats, the level of total GSH was 0.250 ± 0.03 and 0.207 ± 0.04 nmol/mg protein ($n = 6$) respectively.

4. Discussion

Previous studies [2–7] including this study, show that MAA kills male germ cells by apoptosis. To investigate the pathway of apoptotic death, we first determined the time of DNA fragmentation as it is a late apoptotic event preceded by cellular changes like mitochondrial release of proapoptotic molecules and activation of caspases [28]. In the existing model where a single dose of MAA was given to rats, DNA fragmentation was visible around 12 h, therefore, it was obvious that proapoptotic changes occurred at an earlier time point. Detection of cytochrome *c* in the cytosol at 4 h after exposure to MAA showed that mitochondrial changes have occurred by 4 h, which correlates with earlier studies where dimerization of Bax and Bak, a phenomenon known to facilitate the release of cytochrome *c* has been demonstrated in response to MAA treatment [13]. However, the release of cytochrome *c* from the mitochondria was not preceded by any loss in the mitochondrial membrane potential in this model unlike in several mammalian cell types [28]. This is not surprising as, release of cytochrome *c* without mitochondrial membrane potential loss is also known [28]. Additional evidence for the involvement of the mitochondrial pathway comes from the activation of caspase-9 that reaches a peak at 4 h followed by peak caspase-3 activation at 8 h. Cytochrome *c* release initiates a chain of events which results in the activation of caspase-9 that in combination with Apaf-1 [20] can activate caspase-3 [20] leading to other apoptotic changes. In our studies, the temporal relationship between cytochrome *c* release, caspase-9 activation, caspase-3 activation and DNA degradation clearly establish that in response to MAA treatment, germ cells activate the mitochondrial pathway of apoptosis. There was no activation of the Fas–Fas L system as shown by our studies with Western blots of germ cell extracts (data not shown). Interestingly, we found that there was an alteration of cellular GSH/GSSG ratio within the first 2 h with a reduction in total intracellular GSH levels. Since such changes can bring about the release of cytochrome *c*, it is possible that in the event of MAA exposure, the release of cytochrome *c* was precipitated by the decline in the cellular GSH levels [22]. Arguably, if GSH depletion was the cause of the early apoptotic changes, supplementation of GSH precursors should be able to prevent such changes. NAC, which can serve as a precursor of GSH in the GSH biosynthetic pathway, increased germ cell and plasma GSH levels and was able to prevent several proapoptotic events including cytochrome *c* release (data not shown), DNA fragmentation and ultimately cell death. Other studies from our laboratory show that when germ cells are involved, GSH plays a very important role both during oxidative stress [15] and during exposure to toxins like 2,4-hexanedione [unpublished data].

Therefore, in summary, this study demonstrates the importance of the mitochondrial apoptotic pathway in MAA induced germ cell death that can be prevented by NAC, an antioxidant that is being widely used to treat a variety of human diseases.

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