The Effect of D-Methionine on Cochlear Oxidative State with and without Cisplatin Administration: Mechanisms of Otoprotection

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Abstract

D-methionine (D-met) protects against cisplatin (CDDP) ototoxicity, but the mechanisms are not well understood. This study investigated D-met protection of cochlear oxidative state as measured by superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and malondiadehyde (MDA) levels. The design comprised four groups of five rats each: (1) a saline control group, (2) a CDDP-only-treated group, (3) a CDDP group pretreated with D-met, and (4) a group receiving only D-met. Auditory brainstem response testing (ABR) was performed before and 3 days after injection. CDDP alone caused marked hearing loss; significantly reduced SOD, CAT, and GR levels; and increased MDA levels, but D-met pretreatment protected against these changes. These studies suggest that D-met protects cochlear antioxidant enzyme levels from CDDP-induced decrements. The excellent correlation of enzyme levels with hearing loss and weight loss suggests that antioxidant enzyme level protection may underlie, at least in part, D-met's otoprotective action.

Key Words: Antioxidants, auditory brainstem response, catalase, cisplatin, enzymes, glutathione peroxidase, glutathione reductase, hearing, malondiadehyde, ototoxicity, superoxide dismutase

Abbreviations: ABR = auditory brainstem response; CAT = catalase; CDDP = cisplatin; DDTC = diethylthiocarbamate; D-met = D-methionine; EDTA = ethylenediaminetetraacetic acid; GR = glutathione reductase; GSH = glutathione; GSH-Px = glutathione peroxidase; GSSG = oxidized form of glutathione; MDA = malondiadehyde; NADPH = reduced nicotinamide adenine dinucleotide phosphate; SOD = superoxide dismutase

Sumario:

La D-metionina (D-met) protege contra la ototoxicidad por cisplatino (CDDP) pero los mecanismos involucrados no son bien comprendidos. Este estudio investiga la protección con D-metionina del estado oxidativo de la cóclea, mediado de acuerdo a los niveles de dismutasa de superóxido (SOD), catalasa (CAT), peroxidasa de glutatión (GSH-Px), reductasa de glutatión (GR) y dialdehído malónico (MDA). El diseño incluyó cuatro grupos de cinco ratas: (1) un grupo de control con solución salina, (2) un grupo tratado solamente con CDDP, (3) un grupo de CDDP pre-tratado con D-metionina, y (4) un grupo que recibió sólo D-metionina. Se realizó una evaluación con respuestas auditivas del tallo cerebral (ABR) antes y 3 días después de la inyección. El CDDP causó una marcada hipoacusia, redujo significativamente los niveles de SOD, CAT y GR, y elevó los niveles de MDA, pero el pre-tratamiento con D-metionina protegió contra todos estos cambios. Estos estudios sugieren que la D-metionina protege a los niveles de enzimas antioxidantes cocleares del
Among therapeutic agents in common clinical use, cisplatin (CDDP) is the most ototoxic (See reviews by Moroso and Blair, 1983; Koegel, 1985; Griffin, 1988). Initially nephrotoxicity was the primary dose-limiting factor for CDDP administration. However, improved nephrotoxicity control through hypertonic saline, hydration, and mannitol administration has allowed higher clinical CDDP dosing. Unfortunately, these protective regimens do not prevent or ameliorate ototoxicity, and higher CDDP dosing protocols frequently result in permanent, irreversible, hearing loss (Berry et al, 1990; Rybak et al, 1992; Ravi et al, 1992). The incidence and degree of hearing loss correlate highly with CDDP dose, particularly cumulative dose (Vermorken et al, 1983; Schroder et al, 1986; Hoeve et al, 1988). Incidence figures range from 9 percent to over 90 percent depending on ototoxicity criteria, as well as dosing and patient factors (Rybak, 1981). An approximate 50 percent incidence of cisplatin ototoxicity is reported in head and neck cancer and gynecologic oncology patients (Blakley and Meyers, 1993; Blakley et al, 1994). Because CDDP-induced ototoxicity is almost invariably irreversible (Vermorken et al, 1983), prevention is critical. Because ototoxicity is now the most common dose-limiting factor, CDDP otoprotection could not only protect hearing but also could allow more patients to safely complete a full treatment course for their cancer.

In 1996, it was first established that D-methionine (D-met) protects against CDDP-induced hearing loss (Campbell et al, 1996). D-met effectively prevented virtually all CDDP-induced auditory brainstem response (ABR) threshold shift and cochlear hair cell loss and markedly reduced CDDP-induced damage to the stria vascularis (Campbell et al, 1996, 1999). Since that time, numerous articles have been published on D-met’s highly effective otoprotective action not only for CDDP-induced but also for aminoglycoside-induced and noise-induced hearing loss (Kopke et al, 1997, 2002; Gabaizadeh et al, 1997; Campbell et al, 1999; Reser et al, 1999; Sha and Schacht, 2000; Coleman et al, 2002). However, the mechanisms of D-met’s otoprotective action are still being investigated.

An oxidative mechanism could explain why D-met protects against CDDP-induced, aminoglycoside-induced, and noise-induced hearing loss because free radical formation appears to be a common mechanism underlying all three of these toxicities (Yamane et al, 1995; Kopke et al, 1997; Ohlemiller and Dugan, 1998; Sha and Schacht, 2000). Methionine is reversibly oxidized and serves as a free radical scavenger (Vogt, 1995). Methionine’s free radical scavenging ability could explain why it is protective against multiple types of cochlear toxins. For CDDP, methionine may play an additional antioxidant role. D-met may react directly with the hydrated CDDP; D-met’s sulfur groups may bind to the CDDP, thus protecting the sulfur-containing enzymes and proteins (Melvik and Pettersen, 1987; Jones and Basinger, 1989; Miller and House, 1990; Jones et al, 1991).

In general, there are several factors to consider in the role of oxidative damage in toxicity (see reviews by Oberley and Oberley, 1986; Ross, 1988). All active oxygen species — O\textsuperscript{2-}, H\textsubscript{2}O\textsubscript{2}, and OH\textsuperscript{-} — can be damaging or lethal to cells. The superoxide radical O\textsuperscript{2-}, is produced during normal metabolism, but levels may be increased by certain disorders.
Three basic enzymes are responsible for converting superoxide radical into water: superoxide dismutase (SOD), which converts the superoxide radical into peroide; catalase (CAT), which converts peroide into water; and glutathione peroxidase (GSH-Px) in the GSH pathway, which also converts peroide to water by oxidizing the reduced form of GSH to glutathione disulfide (GSSG), which is the oxidized form. Glutathione reductase (GR) reduces the GSSG back to GSH, which is then again available for oxidation with concomitant conversion of peroide to water. If any of these systems fail, the hydroxy radical, which is the most damaging, can form. Lipid peroxidation also results and can be measured by increased malondialdehyde (MDA) levels. Lipid peroxidation, which can be reduced by free radical scavengers, is caused by active oxygen species reacting with polyunsaturated fatty acids in biological membranes.

CDDP alters the antioxidant system of the auditory pathway. CDDP reduces GSH levels in both the cochlea and the inferior colliculus (Ravi et al, 1991). Ravi and colleagues (1995) investigated changes specifically in the cochlear antioxidant system. Systemic CDDP administration decreased GSH levels and reduced activity of the enzymes GSH-Px and GR. GSSG was not found, suggesting that the overall GSH levels decreased rather than being oxidized. Ravi and colleagues (1995) also reported increased MDA levels, reflecting increased lipid peroxidation. The increased lipid peroxidation could indicate that CDDP reduced levels of GSH and GSH enzymes, largely inactivated the GSH antioxidant system, thus allowing the increased levels of free radicals to cause lipid peroxidation. Thus, CDDP may inactivate one of the cochlea’s primary detoxification systems.

CDDP does increase the level of free radicals in general (see introduction by Hannemann and Baumann, 1988). Kopke and colleagues (1997) demonstrated that CDDP altered the antioxidant enzyme activity in unprotected cochlear explants and demonstrated that D-met provided superior protection against hair cell loss as compared to other agents. However, they did not test the effect of D-met on the antioxidant enzyme activity. In vitro studies may also yield somewhat different findings than in vivo studies, because of the latter’s potential for metabolic interactions.

The role of free radicals and antioxidants in CDDP ototoxicity is not fully resolved, but SOD, CAT, and GSH-Px are prominent in both the organ of Corti and the stria vascularis (Pierson and Gray, 1982). Ikeda and colleagues (1993) suggested that increased levels of superoxide free radicals might alter outer hair cell motility by increasing the Ca2+ influx. This influx was blocked by SOD administration. CDDP inhibition of outer hair cell calcium transduction channels may play a role in ototoxicity (McAlpine and Johnstone, 1990; Saito et al, 1991; Yamamoto et al 1994).

Clearly, the oxidative process plays a role in CDDP ototoxicity. It is also established that D-met provides excellent otoprotection that is equal or superior to any other otoprotective agent yet tested and with no known side effects (Campbell et al, 1996) or interference with anti-tumor activity in vivo (Jones and Basinger, 1989; Gillette-Cloven et al, 2000) when appropriate tumor models and dosing strategies are employed. However, the role of D-met in the cochlear oxidative process in the presence and absence of CDDP has not been explored in an in vivo model.

The purposes of this study were to determine if D-met alone alters the oxidative state/antioxidant status of the cochlea; to determine if D-met preadministration protects the cochlea against CDDP-induced oxidative changes, specifically SOD, CAT, GSH-Px, GR, and MDA; and to determine if the degree of CDDP-induced hearing loss correlates with any of the measures of cochlear oxidative state. The long-term objective is to prevent CDDP-induced ototoxic hearing loss in patients receiving chemotherapy.

METHOD

Subject Groups

Male Wistar rats (250 to 350 g) were divided into four groups of five rats each. Group 1 comprised a normal control group receiving saline injection only. Group 2, a treated control group, received a 30-minute intraperitoneal infusion of 16 mg/kg CDDP. Group 3 received a 300 mg/kg intraperitoneal D-met injection only. Group 4 received 300 mg/kg D-met intraperitoneally 30 minutes prior to CDDP infusion. This D-met dose is known to fully protect against CDDP-induced ABR threshold shift and outer hair cell loss.
D-met and Cochlear Oxidative State/Campbell, Meech, Rybak and Hughes

(Campbell et al, 1996) and to protect the stria vascularis (Campbell et al, 1999). No mortality occurred in any animal group, so replacement animals were not needed.

Rectal temperatures were monitored throughout recordings. A warming pad maintained animal core temperature.

All animals were fully anesthetized throughout all ABR procedures, drug administration, and sacrifice with a 1 ml/kg intramuscular injection of Rompun cocktail (a solution containing 86.21 mg/ml ketamine and 2.76 mg/ml xylazine), which was supplemented as needed with half doses.

Each animal’s weight was measured just prior to the initial and the final ABR testing.

ABR Testing

ABR thresholds were measured before and 3 days after drug administration in response to 100 msec clicks, and for tone bursts centered at 1, 4, 8, and 14 kHz, with 1 msec rise-fall times and a 0 msec plateau gated by a Blackman envelope. Stimuli were presented through a Koss Pro/4X plus earphone, coupled to each rat’s external auditory meatus with a speculum and recorded with a Biologic Brain Atlas evoked potential unit. The 14 kHz stimulus was generated by a custom-built high frequency stimulator (courtesy of Dr. Stephen Fausti). An intensity series was obtained for each animal from 100 to 0 dB peak equivalent sound pressure level for click stimuli and sound pressure level for tone bursts in 10 dB decrements at 10/sec. Threshold was defined as the lowest intensity capable of eliciting a replicable, visually detectable response.

A total of approximately 500 sweeps constituted each average. The recording epochs were 15 msec following stimulus onset. Responses were analogue filtered with a 30 to 3000 Hz bandpass.

Enzyme Analysis

After the final ABR testing, the animals were euthanized by decapitation while still under general anesthesia, as described above. After sacrifice of the rat, the cochleae were surgically harvested and frozen in liquid nitrogen. Then the cochlear soft tissues were removed, placed in phosphate buffer (0.05 M, 0.1 mM ethylenediaminetetraacetic acid (EDTA) sodium phosphate buffer (pH 7.0)), and homogenized. These cochlear tissue extracts were examined for a Hitachi 2000 Spectrophotometer for SOD, CAT, GSH-Px, and GR antioxidant enzyme activity and MDA levels.

SOD activity was determined according to the method of Misra and Fridovich (1972). Epinephrine (20 µls of 30 mM in 0.05% acetic acid) was added to 10 ml of tissue extract with 970 µl carbonate buffer and the mixture measured at 480 nm for 4 minutes. The rate of reaction was calculated between 90-100 seconds where linearity usually occurs. SOD activity is expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50 percent, which is equal to 1 U/mg protein.

CAT activity was determined at room temperature according to the method of Aebi (1984) using 10 µl of 100 percent ethyl alcohol, added to 100 µl of tissue extract and then placed in an ice bath for 30 minutes. After warming to room temperature, 10 µl of Triton X-100 RS was added. Ten µl of this mixture was added to a solution of 240 µl sodium phosphate buffer and 250 µl of 0.066 M H2O2 (in phosphate buffer), and measured at 240 nm for 60 seconds. Activity was determined using the molar extinction coefficient of 43.6 M⁻¹ cm⁻¹. One unit of CAT activity degrades 1 mmol of H2O2/min./mg protein.

Protein estimation was estimated according to the method of Read and Northcole (1981) using bovine serum albumin as a standard.

GSH-Px activity was determined according to the method of Flohe and Gunzler (1984). GSH-Px activity was determined by adding 50 µl of tissue extract to a reaction mixture of 550 µl sodium phosphate buffer, 100 µl 0.01 M GSH (in phosphate buffer), 100 µl 1.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 100 µl GR (0.24 units in phosphate buffer). After addition of the tissue extract, the mixture was incubated at 37º C for 10 minutes. Fifty µl of 12 mM t-butyl hydroperoxide was added to 450µl of the mixture and then measured at 340 nm for 180 seconds. Activity was determined using the millimolar extinction coefficient 6.22 M⁻¹ cm⁻¹. One unit of activity is the mmol of NADPH oxidized/min/mg protein.

Glutathione reductase (GR) activity was determined by the method of Carlberg and Mannervik (1985) using 50 µl of NADPH (2mM) in 10 mM Tris-HCL buffer (pH 7.0), added to 50 µl of GSSG (20mM dissolved in potassium phosphate buffer) and 850 µl of potassium phosphate buffer (0.5M, pH 7.0,
0.2 mM EDTA). Fifty microliters of tissue extract were added to the NADPH-GSSG buffered solution and measured at 340 nm for three minutes. The millimolar extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$ was used to determine activity. One unit of GR activity was equal to the mmol of NADPH oxidized/min/mg protein.

**Lipid Peroxidation**

The extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive products (malondialdehyde) using the method of Ohkawa and colleagues (1979). One hundred microliters of tissue homogenate was added to 100 µl of double distilled water and 50 ml of 8.1 percent sodium dodecyl sulfate and incubated at room temperature for 10 minutes. Three hundred and seventy-five microliters of 20 percent acetic acid and 375 µl of thiobarbituric acid (0.6%) were added to the tissue solution and placed in a boiling water bath for 60 minutes. Following incubation, 250 µl of double distilled water and 1.25 ml of 15:1 butanol-pyridine solution were added to the mixture and centrifuged for 5 minutes at 1000 rpm. The resulting supernatant was measured at 532 nm using the U2000 spectrophotometer. MDA concentrations were determined using 1,1,3,3-tetraethoxypropane as standard. Results were expressed as nmoles of MDA/mg protein.

**Results**

**ABR Threshold Shift**

As expected, CDDP induced marked ABR threshold shift in response to all stimuli, including clicks, 1, 4, 8, and 14 kHz tone bursts, as measured from preadministration baseline to post-testing 3 days later. D-met alone did not alter ABR thresholds. Administering D-met 30 minutes prior to the CDDP completely prevented ABR threshold shift for all stimuli. Both the D-met alone group and the group pretreated with D-met 30 minutes prior to the CDDP were not significantly different from the saline control group (Fig. 1). ABR threshold shift for the CDDP group was significantly greater than for all other groups (p < .001).

**Weight Loss**

D-met administered 30 minutes prior to the CDDP markedly ameliorated weight loss (Fig. 2). Both the D-met alone group and the D-met pretreated group were not significantly different from the saline control group. Weight loss for the CDDP group was significantly greater than all other groups (p < .001).

**Enzyme Analysis**

CDDP significantly reduced SOD (p < .05) and CAT (p < .01) enzyme activity levels (Fig. 3). In rats pretreated with D-met and those receiving D-met alone, enzyme activity levels of both SOD and CAT tended to be higher but were not significantly different than those of the saline control group. D-met preadministration fully protected both SOD and CAT enzyme activity levels. CDDP also significantly reduced the GR.

**Approvals**

All procedures were approved by the Southern Illinois University School of Medicine Animal Care and Use Committee, and are in compliance with “The Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (1996) prepared by the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press Washington, D.C.

**Statistical Analysis**

Electrophysiologic (ABR thresholds for the various stimuli) and oxidative measures (SOD, CAT, GSH-Px, GR, and MDA levels) were analyzed using a repeated measures factorial analysis of variance (ANOVA) design controlling overall significance level at the .05 level. Correlational analyses were performed using two-tailed Pearson correlation coefficients and included all ABR measures and measures of oxidative state. Statistical analysis controlled for the number of correlations.
Figure 1. Average auditory brainstem response (ABR) threshold shift (± 1 SD) from pre- to post-testing in response to click, 1, 4, 8, and 14 kHz stimuli for the animal groups. A black circle above the bar indicates that results were significantly different from the control group (p < .05). CDDP = cisplatin; D-met = D-methionine.

Figure 2. Average animal weight loss (± 1 SD) from pre- to post-test is plotted for each animal group. A black circle above the bar indicates that results were significantly different from the control group (p < .05). CDDP = cisplatin; D-met = D-methionine.
**Figure 3.** A graph of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) enzyme activity levels (average ± 1 SD) for each animal group. A black circle located above the bar indicates that results were significantly different from the control group (p < .05). SOD activity is expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50 percent, which is equal to 1 U/mg protein. One unit of CAT activity degrades 1 mmol of H₂O₂/min/mg protein. For GSH-Px, one unit of activity is the mmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized/min/mg protein. One unit of GR activity was equal to the mmol of NADPH oxidized/min/mg protein. CDDP = cisplatin; D-met = D-methionine.

**Figure 4.** A graph of average malondialdehyde levels (± 1 SD) for the different experimental groups. A black circle above the bar indicates that results were significantly different from the control group (p < .05).
enzyme activity level (p < .01) compared with control activity levels. GSH-Px enzyme levels tended to be lower in the CDDP group than in the saline control group but did not reach statistical significance (see Fig. 3). In rats pre-treated with D-met before CDDP administration, GR enzyme levels were significantly protected (p < .01). GSH-Px and GR enzyme activity levels in the D-met alone group tended to be higher but were not significantly different from those of saline controls.

**Lipid Peroxidation**

CDDP significantly elevated MDA levels (p < .01). MDA levels in the D-met alone group were not significantly different than those in the saline control group (Fig. 4). The pretreated D-met group's MDA levels were between those of the control and CDDP groups and were not significantly different from either group.

**Correlation of ABR Threshold Shifts to Cochlear Oxidative Status**

In general, the degree of ABR threshold shift across groups significantly correlated with enzyme levels. ABR threshold shift for all stimulus conditions correlated with CAT and GR levels. ABR threshold shift for all stimulus conditions, except 1 kHz, correlated with SOD levels and with MDA levels. Animals with greater hearing loss had lower CAT, GR, and SOD levels and higher MDA levels. ABR threshold shifts did not correlate with GSH-Px levels for any stimulus frequency. Additionally, weight loss calculated in both absolute and percentage change from initial weight inversely correlated with initial weight inversely correlated with SOD, CAT, GSH-Px, GR, and MDA levels (Table 1). The animals with greater weight loss had lower cochlear antioxidant enzyme levels and higher MDA levels.

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<td>GR</td>
<td>Pearson Correlation</td>
<td>.682**</td>
<td>.514*</td>
<td>.682**</td>
<td>.611**</td>
<td>.792**</td>
<td>-.624**</td>
<td>-.633**</td>
<td>.707**</td>
<td>.539*</td>
<td>.445*</td>
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<td>Significance (two-tailed)</td>
<td>.001</td>
<td>.020</td>
<td>.001</td>
<td>.004</td>
<td>.000</td>
<td>.003</td>
<td>.000</td>
<td>.014</td>
<td>.049</td>
<td>.007</td>
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<tr>
<td>MDA</td>
<td>Pearson Correlation</td>
<td>.515*</td>
<td>-.305</td>
<td>-.540*</td>
<td>-.562*</td>
<td>-.677**</td>
<td>.759**</td>
<td>.756**</td>
<td>-.553*</td>
<td>-.520*</td>
<td>-.544*</td>
<td>-.580**</td>
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<tr>
<td></td>
<td>Significance (two-tailed)</td>
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<td>.191</td>
<td>.014</td>
<td>.010</td>
<td>.001</td>
<td>.000</td>
<td>.011</td>
<td>.019</td>
<td>.013</td>
<td>.007</td>
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*Indicates results significant at the .05 level; ** Indicates results significant at the .01 level. Statistical analysis controlled for the number of correlations. ABR = auditory brainstem response; CAT = catalase; GR = glutathione reductase; GSH-Px = glutathione peroxidase; MDA = malondiadehyde; SOD = superoxide dismutase.
DISCUSSION

The results of this study demonstrate that D-met preadministration significantly protects cochlear SOD, CAT, and GR anti-oxidant enzyme levels from CDDP-induced decrements. The correlation of the antioxidant enzyme levels to the degree of ABR threshold shift and to cochlear MDA levels strongly suggests that D-met’s protection of the cochlear antioxidant system may underlie its otoprotection. Although not reaching significance, there was a trend for D-met alone to increase all four of the antioxidant enzyme levels over the normal baseline levels, as reflected in the saline group. Interestingly, GSH-Px activity was not protected from CDDP-induced decrease. This differential effect on the antioxidant enzymes merits further investigation. However, because the effect is specific to most but not all of the anti-oxidant enzymes, a simple deactivation of CDDP by binding could not fully explain these results.

Methionine can bind directly to platinum, but this mechanism could not explain the protections against aminoglycoside-induced (Sha and Schacht, 2000) and noise-induced hearing loss (Kopke et al, 2002) or the differential effect on the various antioxidant enzymes, nor could it explain the tendency of D-met alone to seemingly increase antioxidant enzyme activity levels. However, these effects could be explained if D-met protected or up-regulated the anti-oxidant pathways.

Nonetheless, the issue of methionine-CDDP binding needs to be considered, particularly in retaining CDDP antitumor activity. Melvik and Pettersen (1987), using an in vitro model, demonstrated that methionine preadministration sensitized NHIK 3025 human uterine cervix carcinoma cells to CDDP treatment, but simultaneous administration of methionine and CDDP in solution inhibited CDDP cytotoxicity. Deegan and colleagues (1994) studied a methionine-CDDP complex using both in vivo and in vitro models. The methionine-CDDP complex eliminated CDDP-induced nephrotoxicity, whereas most, but not all, of CDDP’s antitumor activity was retained. They concluded, “These results indicate that cisplatin-methionine is significantly cytotoxic yet lacks cisplatin-associated renal toxicity and may, therefore, have a role in the treatment of human malignancies.”

Ekborn and colleagues (2002) measured the area under the curve for CDDP with and without D-met administration 60, 10 and 0 minutes prior to CDDP in an in vivo model using pigmented guinea pigs. Data were subsequently collapsed across all three time intervals. They dose-adjusted CDDP administration to compare toxicity in groups at the same unbound methionine levels. In doing so, they compared toxicities in the D-met groups with much higher CDDP dosing to lower-dose CDDP-alone groups. Unfortunately, they assumed that antitumor activity was solely dependent on unbound platinum levels, although Deegan and colleagues (1994) clearly demonstrated that a methionine-CDDP complex retained most of its antitumor activity. Ekborn and colleagues (2002) did not actually test antitumor efficacy in their study. Further they used an extremely high D-met to CDDP ratio of 37.5:1, twice what we reported as necessary for complete otoprotection with a 30-minute preadministration interval between D-met and CDDP (Campbell et al, 1996, 1999). Therefore, given the half-life of D-met as 58 minutes, they effectively had a simultaneous administration ratio of D-met to CDDP of 18.75:1 even for the 60-minute preadministration interval, which they then collapsed with the 10- and 0-minute preadministration intervals with even higher effective simultaneous administration. Complete otoprotection can be obtained with a dosing ratio of 18.75:1 with a 30 minute preadministration of D-met, thus resulting in an effective ratio of 14:1 at the time of CDDP delivery.

D-met preadministration does not significantly interfere with CDDP’s antitumor action when tested in appropriate tumor models and with appropriate dosing ratios (Jones and Basinger, 1989; Gillette-Cloven et al, 2000). Some concern has been expressed that D-met may act simply by inactivating CDDP. Reser and colleagues (1999) reported that D-met provided excellent protection from CDDP-induced hearing loss but partially inhibited CDDP’s anti-tumor action. However, they used only three animals per cell in the tumor model, showed a small insignificant effect, and did not have adequate statistical power. More importantly, they used a breast cancer tumor model, which is clinically irrelevant. Breast cancer, even metastatic breast cancer, is not treated with CDDP because it is ineffective. Occasionally, CDDP is tried for breast cancer when all other therapies have
failed, but generally with poor result. In cancer research, an array of different tumor models have been developed because it is critical that therapies be tested on the same type of cancer for which they will be used. Gillette-Cloven and colleagues (2000), of the Division of Gynecologic Oncology at University of California Irvine Cancer Research Institute, used an ovarian tumor model with adequate sample size and statistical power and showed no significant antitumor interference of D-met preadministration. Ovarian cancer is one of the primary clinical applications of CDDP, particularly high-dose CDDP. Jones and Basinger (1989) also found no interference with D-met preadministration using appropriate dosing ratios in another clinically relevant tumor model of the Walker 256 carcinoma. Only randomized clinical trials will be able to fully address D-met’s role in protection from CDDP side effects and retention of therapeutic efficacy.

Systemic administration of D-met would be preferable because systemic D-met could provide not only otoprotection, but also nephroprotection, weight loss protection, and possibly improved survival (Jones and Basinger, 1989; Campbell et al, 1996; Gillette-Cloven et al, 2000). However, if otoprotection is the only goal, D-met applied directly to the round window also provides excellent CDDP ototoxicity (Korver et al 1998, 2002; Reser et al 1999).

One of the concerns for many protective agents is whether the proposed agent itself will cause side effects. For example, systemic diethyldithiocarbamate (DDTC) initially showed promise as a CDDP otoprotective agent. However, DDTC side effects were severe enough to preclude clinical use (Qazi et al, 1988; Rothenberg et al, 1988). D-met, even at the high levels utilized thus far, has not demonstrated side effects. Further, D-met, both parenteral and oral, has been safely used in humans for other applications, including radiographic imaging studies (Meyer et al, 1985), and nutritional studies (Kies et al, 1975; Stegink et al, 1986; Kaji et al, 1987). D-met is also safer than the essential amino acid L-met. Many studies have indicated that D-met is not toxic unless converted to the L-isomer (Stekol and Szaran, 1962; Walser et al, 1973; Benevenga, 1974; Blom et al, 1989; Friedman, 1999).

D-met as a micronutrient is already present in a wide variety of foods and is thus not alien to the human system. For example, dietary-high quality protein contains 26mg/g methionine (National Research Council, 1980). The D-isomer of methionine is present in particularly high amounts in fermented foods such as yogurts and cheeses. Thus, D-met is part of our daily nutritional intake and has already been safely used in humans for other medical applications, but its roles in normal oxidative processes and protection from oxidative damage have not been fully explored.

In this study, we have documented that D-met does protect the activity levels of the anti-oxidant enzymes SOD, CAT, and GR from CDDP-induced decrements. However, further investigation is warranted regarding the effects of D-met on GSH and GSSG levels. CDDP does reduce both cochlear GSH and GSSG levels (Ravi et al, 1991; 1995). Methionine does play a critical role in synthesizing GSH. Methionine can be converted to cysteine, and GSH synthesis is dependent on cysteine availability (Lu, 1998). Consequently, increased methionine levels may increase or protect GSH levels. Further, methionine can specifically increase GSH levels within the mitochondria, and mitochondrial GSH levels can reduce the risk of oxidative injury, resulting in apoptotic cell death (Fernandez-Checa et al, 1998). Methionine also reduces injury-induced GSH transport out of the cell (Ghibelli et al, 1998). Perhaps the reason that methionine is an effective antidote for acetaminophen toxicity (WHO Expert Committee on the Use of Essential Drugs, 1998) is its role in GSH synthesis and protection. Acetaminophen overdose depletes intracellular hepatic GSH, which underlies its toxicity (Kroger et al, 1997). We are currently conducting further research to determine the role of D-met in cochlear GSH levels.

SUMMARY

Cisplatin alone caused significant hearing loss at all stimulus frequencies, and D-met provided complete otoprotection. Administered alone, D-met did not alter ABR thresholds. Cisplatin significantly reduced SOD, CAT, and GR levels and increased MDA levels. D-met pretreated animals or those receiving D-met alone, had SOD, CAT, GR, and MDA levels that were not significantly different from the saline-injected control group. D-met preadministration did not pro-
tect GSH-Px levels. Significant correlations existed between ABR threshold shifts for all stimuli and SOD, CAT, GR and MDA levels across all groups but not GSH-Px levels. Significant correlations also existed between weight loss and all ABR threshold shifts as well as SOD, CAT, GR, GSH-Px, and MDA levels.

These studies suggest that D-met, at least partially, protects most cochlear antioxidant enzyme levels from CDDP-induced decreases. The excellent correlation of enzyme levels with hearing loss suggests that antioxidant enzyme level protection may underlie, at least in part, D-met’s otoprotective action.

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REFERENCES


