Glutathione Ester But Not Glutathione Protects Against Cisplatin-Induced Ototoxicity in a Rat Model

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Abstract
Glutathione (GSH) provides an important antioxidant and detoxification pathway. We tested to determine if direct administration of GSH or GSH ester could reduce cisplatin- (CDDP) induced ototoxicity. We tested eight groups of five rats each: a control group, a group receiving 16 mg/kg ip CDDP infused over 30 minutes, and six groups receiving either GSH or GSH ester at 500, 1000, or 1500 mg/kg intraperitoneally 30 minutes prior to 16 mg/kg CDDP. Auditory brainstem response thresholds were measured for click and tone-burst stimuli at baseline and 3 days later. Outer hair cell (OHC) loss was measured for the apical, middle and basal turns. The 500 mg/kg GSH ester reduced hearing loss and OHC loss, but protection decreased as dosage increased, suggesting possible toxicity. GSH was not significantly protective. The best GSH ester protection was less than we have previously reported with D-methionine.

Key Words: Cisplatin, glutathione, glutathione ester, hearing loss, ototoxicity, outer hair cells

Abbreviations: ABR = auditory brainstem response; CAT = catalase; CDDP = cisplatin; GSH = glutathione; GR = glutathione reductase; GSH-Px = glutathione peroxidase; GSSG = oxidized form of glutathione; HSD = honestly significant difference; peSPL = peak equivalent sound pressure level; Pt = platinum; -SH groups = thiols (sulfur-hydrogen); SOD = superoxide dismutase; SPL = sound pressure level

Sumario:
El glutatión (GSH) brinda una importante vía antioxidante y de cetoxificación. Realizamos una prueba para determinar si la administración directa de GSH o del éster de GSH podía reducir la ototoxicidad inducida por cisplatino (CDDP). Hicimos una evaluación en ocho grupos de cinco ratas cada uno: un grupo control, un grupo que recibió CDDP intraperitoneal a 16 mg/kg en una infusión durante 30 minutos y seis grupos que recibieron intraperitonealmente GSH o el éster de GSH a 500, 1000 o 1500 mg/kg, 30 minutos antes del CDDP a 16 mg/kg. Se midieron umbrales de respuestas auditivas del tallo cerebral tanto para clicks como para bursts tonales, al inicio y 3 días después. La pérdida de células ciliadas externas (OHC) fue establecida a nivel de las vueltas apical, media y basal. La dosis de 500 mg/kg de éster de GSH disminuyó la hipoacusia y la pérdida de OHC, pero la protección disminuyó conforme la dosis se incrementó, sugiriendo una posible toxicidad. EL GSH no resultó significativamente protector. El mejor efecto protector del éster de GSH fue menor que el previamente reportado con D-metionina.

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cisplatin (CDDP) is the most ototoxic agent in common clinical use. The incidence of ototoxicity in various studies has been reported to occur in approximately 62 percent of cases ranging from 11-97 percent (Schweitzer, 1993). However there are no effective otoprotective agents currently in clinical use.

As documented in both human and animal studies, cochlear anatomical changes occur in both the organ of Corti and stria vascularis (Schuknecht, 1993; Campbell et al, 1996; Meech et al, 1998). The primary histologic findings include hair cell degeneration and damage to the supporting cells that are dose related (Anniko and Sobin, 1986). Both inner, and to a greater degree, outer cochlear hair cell loss can occur, predominantly in the basal turn (Fleischman et al, 1975; Estrem et al, 1981; Komune, 1981; Schweitzer, 1993). The cuticular plate may soften and more lysosomal bodies may be present in the outer hair cells’ apical portion (Estrem et al,1981). CDDP may also alter cochlear supporting cells and Reissner’s membrane (Estrem et al, 1981; Komune, 1981; Schweitzer, 1993). High dosage CDDP can even cause the membranous labyrinth to collapse (Anniko and Sobin, 1986).

Clinically, the high incidence of CDDP-induced hearing loss among patients receiving high-dose CDDP chemotherapy (e.g., gynecologic oncology patients) is well known (Rubin et al, 1995; Kaern et al, 1996; Shapiro et al, 1998). Ototoxicity in humans can occur even in the absence of other toxicities (Piel et al, 1974). Because CDDP-induced loss is almost invariably permanent (Vermorken et al, 1983), prevention of the hearing loss is of prime importance. Consequently, a number of studies have been exploring the possible use of CDDP otoprotective agents (Schweitzer et al, 1986; Rybak et al, 1995; Church et al, 1995; Campbell et al, 1996, 1999).

One proposed protective agent is glutathione (GSH). The tripeptide GSH, L-gamma-L-cysteinyl-glycine, an endogenous nonprotein free thiol, has multiple cellular roles. These roles include protection of –SH (sulfur-hydrogen) groups of proteins, catalysis, metabolism, transport, detoxification of foreign substances, repair and protection against reactive oxygen species by inactivation of peroxides, and free radicals (Meister 1983, 1988; Ross, 1988; Reed, 1990; Tedeschi et al, 1990; see review by Meister and Anderson, 1983). GSH is highly reactive with many endogenous and exogenous compounds (see review by Meister, 1983). GSH does not alter the pharmacokinetics of platinum (Pt) distribution in humans but may increase both the rate of Pt elimination and the extent of Pt distribution. The increased Pt distribution may underlie prolong the residence of Pt in the body (Leone et al, 1992).

Theoretically, sulfur-containing compounds, like GSH, may be protective because of the high affinity of their sulfur groups for platinum complexes. But reduced GSH is also an important part of the antioxidant pathways. Glutathione peroxidase (GSH-Px) in the GSH pathway also converts peroxide to water by oxidizing the reduced form of GSH to the oxidized form (GSSG). Glutathione reductase (GR) reduces the GSSG back to GSH, which is then again available for oxidation with concomitant conversion of peroxides to water. If any of these systems fail, the hydroxy radical, the most damaging free radical, can form via the Haber-Weiss reaction. Lipid peroxidation also results and can be indirectly measured by increased malondialdehyde (MDA) levels. Reactive oxygen species reacting with polyunsaturated fatty acids in biological membranes can cause lipid peroxidation. Free radical scavengers can decrease lipid peroxidation.
GSH clearly plays a role in CDDP-induced nephrotoxicity. Depletion of GSH, an endogenous free radical scavenger, and increased free radical production with resultant increased lipid peroxidation appear to underlie nephrotoxicity (Hannemann and Baumann, 1988; Nakano and Gemba, 1989). Yet Litterst and colleagues (1982) reported increased renal GSH at 8 and 12 days after CDDP administration but reduced GR levels at 5, 8 and 12 days post-treatment. Mistry and colleagues (1988) reported that CDDP increases protein thiol in the proximal tubule 1-3 hours after administration, but the concentration drops by 8 hours and at 120 hours reaches a nadir 29% below controls. Schweitzer (1993) suggested that platinum binding to protein sulfhydryl groups may cause CDDP nephrotoxicity, accounting for the nephroprotective action of thiols (Gandara et al, 1989). Babu and colleagues (1995) found that biweekly CDDP administration for 15 days decreased all the antioxidant enzymes they measured, including superoxide dismutase (SOD), GSH-Px, and catalase (CAT) as well as GSH. Administration of GSH ester significantly ameliorated all of these effects. Dietary depletion of selenium, a cofactor in GSH-Px activity, increases both nephrotoxicity and lethal toxicity in the rat (Satoh et al, 1987). According to Jones and colleagues (1992), intracellular GSH furnishes the “first line of protection” against initial nephrotoxicity.

CDDP decreases renal GSH thus increasing lipid peroxidation (Sugihara et al, 1987a, 1987b; Hannemann and Baumann, 1988). Boogard and colleagues (1991) reported that CDDP rapidly diminished GSH after 15 minutes in rat renal proximal tubule cells. However, if GSH precursors, cysteine, glutamine, and glycine, were added to the incubation medium (Hank’s N-2-hydroxyethylpiperazine-N(165)-2-ethanesulfonic acid bovine serum albumin buffer), GSH levels recovered completely within 30 minutes rather than only partially in an hour.

Diethylmaleate, a GSH depleter, increases nephrotoxicity and lethality secondary to CDDP (Litterst et al, 1986) and increases CDDP-induced lipid peroxidation in rat renal slices (Nakano and Gemba, 1989). Decreased GSH also exacerbates CDDP-induced mitochondrial calcium uptake impairment (Kameyama and Gemba, 1991) in rat renal slices.

GSH administration can provide CDDP nephroprotection in animals (Zunino et al, 1983, 1989; Tedeschi et al, 1990; Jones et al, 1991b) and in humans (Zunino et al, 1989; Jones and Basinger, 1989; Di Re et al, 1990; Tedeschi et al, 1990). GSH, when administered at a 20:1 molar ratio to CDDP, provided only moderate nephroprotection relative to other sulfur-containing nucleophiles (Jones and Basinger, 1989). However, dose-response curves were not obtained, and maximal efficacy may not have been achieved. Other studies have shown excellent GSH nephroprotection (Zunino et al, 1983, 1989; Di Re et al, 1990; Tedeschi et al, 1990).

The GSH antioxidant pathway may also play a role in CDDP ototoxicity. Ravi and colleagues (1995) investigated changes specifically in the cochlear antioxidant system. They found that systemic CDDP administration decreased reduced GSH levels and reduced activity of the enzymes GSH-Px and GR when measured at 72 hours post-administration. GSSG was not found, suggesting that the overall GSH levels decreased rather than merely being oxidized. In a separate study, they found that CDDP also reduced GSH levels in the inferior colliculus (Ravi et al, 1991). However, Ford and colleagues (1997) did not find changes in SOD, CAT, or GSH-Px at either 24- or 72-hour epochs. Lauterman and colleagues (1995), in an animal dietary study, demonstrated that CDDP ototoxicity correlated with low GSH and albumin levels.

GSH ester is more effective than GSH as a nephroprotectant (Anderson et al, 1990) but has not been tested for protection against CDDP-induced hearing loss. Theoretically, GSH ester may be more effective because it is more readily transported into cells and then split into GSH intracellularly. Intracellular GSH is required for protection against heavy metals (as reviewed by Anderson et al, 1990). Babu and colleagues (1995) reported that GSH ester restored kidney GSH, SOD, CAT, and GSH-Px to near normal levels in CDDP-treated animals.

GSH has been reported to reduce weight loss (Zunino et al, 1983) and does not inhibit CDDP’s antitumor activity as measured by survival time (Zunino et al, 1983, 1989; Jones et al, 1991a) by tumor survival in animals (Jones and Basinger, 1989) and in human clinical trials (Di Re, 1990). In fact, Tedeschi and colleagues (1990) reported a high response rate in ovarian cancer because the
nephroprotection enabled the use of higher CDDP levels yet reported ototoxicity and nephrotoxicity in 13 of 51 and 29 of 51 patients, respectively. The specifics of the auditory testing were not provided.

Another reason for investigating GSH and GSH ester otoprotection is that D-methionine is a highly effective otoprotective agent. One of the ways D-methionine may provide CDDP otoprotection is through the GSH pathways. Methionine plays a critical role in synthesizing GSH (Lu, 1998) and can specifically increase mitochondrial GSH. Mitochondrial GSH may protect against oxidatively induced apoptotic cell death (Fernandez-Checa et al, 1998). Further, methionine decreases GSH transport out of the cell following injury (Ghibelli et al, 1998). If the primary mechanism of D-methionine protection is secondary to its role in synthesis and retention within the cell, then perhaps direct administration of GSH or GSH ester could provide equal or better protection.

Direct administration of GSH or GSH ester may be otoprotective, but further research is needed. Therefore, the purpose of this study was to compare the relative efficacy of GSH and GSH ester in ameliorating CDDP-induced hearing loss and outer hair cell loss.

**RESEARCH DESIGN AND METHODS**

**Subjects**

Forty male Wistar rats (275-420 g) served as subjects. Group 1 (CDDP-treated control group) comprised 5 animals given 16 mg/kg cisplatin dissolved in 1 mg/ml of normal sterile saline administered intraperitoneally over a 30-minute period. Because of the 50 percent mortality in this group, 10 animals were initially injected to obtain 5 animals that survived the study period. Group 2 (saline-injected control group) comprised 5 control animals injected with an equivalent volume of saline. Group 3 (GSH-pretreated groups) comprised three groups of 5 animals each injected intraperitoneally with either 500, 1000, or 1500 mg/kg GSH 30 minutes prior to CDDP administration. The timing of administration was chosen because Zunino and colleagues (1989) found maximal nephroprotection with no direct CDDP interaction with this timing and because this timing has proved to be effective for D-methionine otoprotection (Campbell et al, 1996, 1999). These GSH administration levels were selected based on nephrotoxicity studies by Zunino and colleagues (1983, 1989), Tedeschi and colleagues (1990), and Di Re and colleagues (1990). Group 4 (GSH ester-protected group) comprised 3 groups of 5 animals each injected intraperitoneally with either 500, 1000, or 1500 mg/kg GSH ester 30 minutes prior to CDDP administration. The GSH ester timing and levels were selected to match those for GSH based on the studies cited above but also on the GSH ester dosing that was effective for CDDP nephroprotection in studies by Anderson and colleagues (1990) and Babu and colleagues (1995).

All drug administrations and auditory brainstem response (ABR) measures were performed while the animal was under general anesthesia, following injection of Rompun cocktail delivering 86.2 mg/kg ketamine and 2.76 mg/kg xylazine supplemented as needed with half doses.

**Ototoxicity measures:**

**Electrophysiologic Measures**

Auditory Brainstem Testing (ABR) was used to assess auditory threshold while the animal was under general anesthesia, as described above. Testing occurred just prior to drug administration and again 3 days later. After the final ABR data collection, the animal was sacrificed by decapitation (guillotine) while still under general anesthesia.

ABR data collection was obtained with a customized Biologic Traveler system with an additional custom-made high frequency stimulus for 14 kHz. Stimuli were transmitted through a Koss PRO/4XPLUS earphone. All testing was performed with the animal in a double walled booth. ABR thresholds were measured in response to 100-msec clicks and to tone-bursts centered at the frequencies of 1, 4, 8, and 14 kHz presented at 10/sec. Tone-burst stimuli were gated by a Blackman envelope with 2-msec rise-fall for 1 kHz and 1-msec rise-fall for 4, 8, and 14 kHz and 0-msec plateau. An intensity series was obtained for each animal from 100 dB peak equivalent SPL (peSPL) for click stimuli and sound pressure level (SPL) for tone-bursts, proceeding in 10 dB decrements.
to below threshold. The term peSPL means that the amplitude of the click stimulus from the prestimulus baseline to the first peak is equivalent to the SPL of a pure-tone stimulus having the same prestimulus baseline to peak amplitude. Threshold was defined as the lowest intensity capable of eliciting a replicable, visually detectable response.

A total of 512 sweeps constituted each average. The recording epochs were 15 msec following stimulus onset. Responses were analog filtered with a 30 to 3000 Hz bandpass. Platinum/iridium needle electrodes were placed at the vertex (noninverting) to a point directly below the ipsilateral pinna (inverting) with a ground electrode placed in the hind leg.

Rectal temperatures were monitored throughout recordings with animal temperature maintained by a warming pad.

Perfusion Technique and Scanning Electron Microscopy

After sacrifice, animals were decapitated and cochleae perfused with fixative through the perilymphatic spaces. The primary fixative was 2.5% glutaraldehyde at 4º C in 0.1M cacodylate (Cac) buffer (pH 7.4). We hand-drilled a small hole into the first turn of the otic capsule with a sharpened pick. In vitro perfusion was performed intermittently within 5 minutes of sacrifice through the small hole in scala tympani, allowing the fluid to exit through the opened oval window. After perfusion fixation, the round window membrane was removed and the cochleae were immersed and then placed in the refrigerator overnight.

After overnight fixation in glutaraldehyde, the cochleae were rinsed in 0.1 M cacodylate buffer and gently perfused with the buffer through the perilymphatic spaces by loosely fitting the tube end of the perfusion syringe over the opening drilled in the scala tympani. Cochleae were then rinsed in buffer three times. After rinsing, the cochleae were post-fixed by a perfusion of 1.5% OsO4 (at 4º C) in cacodylate buffer under a fume hood. Fixation was continued by immersion and rotation in the same fixative for 15 minutes. The cochleae were rinsed in the same fashion as after glutaraldehyde fixation.

Under the dissecting microscope, the bony capsule of each cochlea was carefully removed. The tissue was then serially dehydrated in 30, 50, 75, 85, 2 x 95 and 3 x 100 percent ethyl alcohol. Each specimen was then dried using Peldri and placed on a stub for sputter coating with 13 nm platinum. The tissue was then viewed through a Hitachi S-500 scanning electron microscope and photographs taken on Polaroid-type 55 film.

Semiquantitative analysis per turn for the outer hair cells was performed in the following manner: For each turn of the cochlea, apical (20% from apex), middle (50% down from apex), and base (70% down from apex), a representative sample was examined. Based on frequency maps in the rat, these cochlear areas correspond approximately to 4, 8, and 14 kHz (Muller, 1991), which corresponds to the frequencies chosen for ABR analysis. For each sample, 11 inner hair cells served as a guide to count a section of 33 outer hair cells or 11 per row. The number of damaged or missing outer hair cells, within each sample, were then counted. This sampling technique was selected based on a power analysis of our initial data.

Weight

Each animal’s weight was measured in an Ohaus triple beam balance scale before administration of the anesthetic for the pretest and again before the post-test 3 days later. The weight was used as an indicator of general health.

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) National Academy Press, Washington, D.C., prepared by the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council.

Analysis of Data

ABR data were analyzed using a three-factor analysis of variance (ANOVA) with one between-subject factor (groups) and two within-subject factors (frequency and pre-vs. post-test). Each dependent variable was analyzed independently. Tests subsequent to the ANOVA were carried out in accordance with the Tukey honestly significant difference (HSD) procedure. Weight loss was measured
using the same type of statistical analysis as the ABR measures. Scanning electron microscopic data were analyzed for each turn using a one-way ANOVA with post hoc Tukey HSD analysis. The criterion for statistical significance for all measures was $p < .05$.

RESULTS

ABR

For both GSH and GSH ester, the 500 mg/kg level provided the best protection from threshold shift, with GSH ester providing better protection than GSH. GSH ester, at 500 mg/kg, provided significant protection from ABR threshold shift for all stimulus conditions (Fig. 1). With our five animals per group, this protection reached significance ($p \leq .05$) for 1, 8, and 14 kHz stimulus conditions. However, the trend in data was the same for all other stimuli. GSH alone, at any level, did not provide significant protection for any ABR stimulus condition.
Hair Cell Counts

Low level (500 mg/kg) GSH ester, but not GSH, provided significant protection from outer hair cell loss in the basal and middle turns (Fig. 2). As expected, significant outer hair cell loss did not occur in the apical turn for any group, including the CDDP-treated control group. Consequently, no difference between groups was found for the apical section. Hair cell counts were performed only on the most effective levels of GSH and GSH ester, which was 500 mg/kg for each of these agents.

Weight

Neither GSH nor GSH ester provided significant protection from weight loss (Fig. 3).

Survival

All of the rats in the saline-injected control group survived until sacrifice. Half of the animals in the CDDP-treated control group survived until sacrifice; thus, 10 animals were required to obtain the 5 survivors. In the GSH-pretreated groups, one animal in the 500 mg/kg group and two animals in the 1000 mg/kg group died before the 72 hour final assessment. In the GSH ester-pretreated groups, one animal in the 1500 mg/kg group died prior to the 72-hour final assessment, but all others survived. Therefore, it appears that all levels of GSH ester and, to a lesser degree, GSH reduced mortality.

**DISCUSSION**

The results of this study demonstrate that GSH ester is an effective otoprotective agent for CDDP-induced ototoxicity and is more effective than GSH. However, these results also suggest that both GSH ester and GSH may be toxic for increasing dosing levels. For some stimulus conditions, the GSH 1000 and 1500 mg/kg pretreated groups actually showed greater hearing loss than the CDDP-treated control group. Although this exacerbation did not occur for any of the GSH-ester pretreated groups, the degree of otoprotection for GSH ester decreased as dosing increased.

However, all of the GSH- and GSH ester-pretreated groups showed better survival than the CDDP-treated control group, suggesting...
at least some systemic protection. Significant protection from weight loss was not obtained.

These results suggest that although GSH ester is more effective than GSH, hearing protection is only partial. Increasing dose does not improve protection, but rather decreases it. Consequently, it appears unlikely that GSH ester or GSH can be as effective as other agents such as D-methionine that provide complete protection against CDDP-induced hearing loss, although part of D-methionine’s otoprotective action may be related to its role in GSH synthesis and cellular retention (Fernandez-Checa et al, 1998; Ghibelli et al, 1998; Lu, 1998).

Although previous studies have not examined the otoprotective effects of GSH ester in an in vivo model, our data are consistent with other in vitro work.

Kopke and colleagues (1997), using organotypic explants from the rat organ of Corti, reported that GSH and GSH ester protected outer hair cells from CDDP ototoxicity. However, they also found D-methionine to be more effective. Their work is consistent with our earlier in vivo work demonstrating that D-methionine provides complete otoprotection from ABR threshold shift and outer hair cell loss (Campbell et al, 1996). Kopke and colleagues (1997) also demonstrated that administration of L-buthionine sulfoximine, an inhibitor of GSH formation, exacerbated CDDP ototoxicity in their model. This finding may support the present study’s results in that a requisite amount of GSH may be required in the system to control CDDP ototoxicity, but excessive amounts may not be advantageous and, in fact, may be damaging.

Kopke and colleagues (1997) also demonstrated that GSH ester blocked the generation of reactive oxygen species in the outer hair cells of the cochlear explants. Yet, in our study, otoprotection using GSH ester was incomplete. Several factors may account for this discrepancy. In their in vitro model, drug pharmacokinetics may be a lesser issue than in the in vivo model we used. In an in vivo model, drug delivery can be controlled, but the respective uptake of the various agents into the cochlea may vary. Furthermore, the metabolism of the various drugs in a whole-animal model may be an important factor. In our study, many interactions may have occurred prior to cochlear uptake or metabolites may have played a role. CDDP can directly bind to sulfur-
containing compounds. Further, other systemic toxicities, including nephro- and gastrointestinal toxicities, may affect the outcome of an in vivo study of platinum compounds. The differences between in vivo and in vitro models could affect the apparent relative efficacy of various protective agents.

Another factor that may play a major role in distinguishing between otoprotective agents is the stria vascularis. CDDP damages the stria vascularis by reducing density of the cytoplasmic organelles in marginal cells, damaging nuclei and mitochondria, inducing edema, and causing bulging and compression along the luminal edge (Meech et al, 1998). D-methionine can protect against this type of CDDP ototoxic damage (Campbell et al, 1999), but the effects of other protective agents on the stria have been largely ignored. We are currently conducting further study of GSH and GSH ester in protecting this structure.

In summary, GSH ester provides superior CDDP otoprotection to GSH; however, GSH ester otoprotection decreases as GSH ester dosing increases. Even at the most efficacious dose, GSH ester is not as effective as the D-methionine otoprotection we have demonstrated in our earlier studies.

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