Magnesium Protects Against Cisplatin-Induced Acute Kidney Injury

By Regulating Platinum Accumulation

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Disclosures: The authors have no conflicts of interest.

Financial Support: The Feinstein Institute for Medical Research

Running title: Mg Status Regulates Cisplatin-Induced Nephrotoxicity

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Abstract

Despite its success as a potent antineoplastic agent approximately 25% of patients receiving cisplatin experience acute kidney injury (AKI) and must discontinue therapy. Impaired magnesium homeostasis has been linked to cisplatin-mediated AKI and because magnesium deficiency is widespread, we examined the effect of magnesium deficiency and replacement on cisplatin-induced AKI in physiologically relevant older female mice. Magnesium deficiency significantly increased cisplatin-associated weight loss and markers of renal damage (plasma blood urea nitrogen (BUN) and creatinine), histological changes, inflammation, as well as renal cell apoptosis, and modulated signaling pathways (e.g. ERK1/2, p53, and STAT3). Conversely, these damaging effects were reversed by magnesium. Magnesium deficiency alone significantly induced basal and cisplatin-mediated oxidative stress, while magnesium replacement attenuated these effects. Similar results were observed using cisplatin-treated LLC-PK1 renal epithelial cells exposed to various magnesium concentrations. Magnesium deficiency significantly amplified renal platinum accumulation, while magnesium replacement blocked the augmented platinum accumulation following magnesium deficiency. Increased renal platinum accumulation during magnesium deficiency was accompanied by reduced renal efflux transporter expression, which was reversed by magnesium replacement. These findings demonstrate the role of magnesium in regulating cisplatin-induced AKI by enhancing oxidative stress and thus, promoting cisplatin-mediated damage. Additional in vitro studies using ovarian, breast and lung cancer cell lines show that Mg supplementation does not compromise cisplatin’s chemotherapeutic efficacy. Finally, because no consistently successful therapy to prevent or treat cisplatin-mediated AKI is available for humans, these results support developing more conservative magnesium replacement guidelines for reducing cisplatin-induced AKI in cancer patients at risk for magnesium deficiency.

Keywords: apoptosis, hypomagnesemia, inflammation, nephrotoxicity, oxidative stress
INTRODUCTION

Cisplatin is a major chemotherapeutic drug used for treating cancers, including ovarian, breast, testicular, non-small cell lung, gastric, and others (12,50,57,69). In fact, testicular cancer has shown complete remission in approximately 70-80% of men following cisplatin treatment (23). Despite this remarkable success, cisplatin is associated with acute kidney injury (AKI) in approximately 25% of patients following repeated dosing (50,57,69). Many patients exhibit irreversible kidney injury due to cumulative damage to the proximal and distal tubules, requiring dose reduction or cisplatin discontinuation (50,69). While the pathogenesis of cisplatin-induced AKI includes localized inflammation, oxidative stress, DNA damage, and tubular epithelial cell apoptosis, along with impaired renal handling of magnesium (12,41,50,57,69,71), no therapies have been shown to consistently reduce or prevent cisplatin-induced AKI in humans.

Magnesium (Mg) is required (300-400 mg/day) for optimal metabolic function (e.g. synthesis/stability of DNA, RNA, and protein, mitochondrial function, and as a co-factor for ATP activity and over 300 enzymes) (4,26,33,53,67). Surprisingly, less than 50% of the US population consumes the reference daily intake (RDI) of Mg (68). Mg deficiency, characterized by increased inflammation and oxidative stress (47,49,56), can result from an imbalance between Mg intake, absorption, and renal losses, as well as increased metabolic demands (22,47,49,56,68). Magnesium deficiency is more common among the elderly (22,82) who are also more susceptible to AKI due to renal parenchymal loss, ATP depletion, and mitochondrial dysfunction (2,73).

The synergistic effects of cisplatin and magnesium deficiency are believed to contribute to renal dysfunction (42). Precisely how magnesium deficiency promotes cisplatin-induced AKI is not known and little has been done to prevent it. Because advanced age and female gender are risk factors for cisplatin-induced AKI (50,69), we investigated the effects of magnesium deficiency and Mg
replacement/supplementation following magnesium deficiency on cisplatin-induced AKI in older female mice.
METHODS

Animals and cell lines.

The Institutional Animal Care and Use Committee (IACUC) of The Feinstein Institute for Medical Research approved animal studies (IACUC #2012-009). C57BL/6 mice (females [retired breeders], 11 months old, Taconic Farms, Germantown, NY) were acclimatized under normal environmental conditions and allowed free access to standard chow and tap water for 1 wk before experimentation. The LLC-PK₁ renal epithelial and MCF-7 human breast cancer cell lines were purchased from ATCC (Manassas, VA, USA); the H460 human large cell lung cancer cell line was provided by Dr. H Simpkins (The Feinstein Institute for Medical Research, Manhasset, NY, USA). The A2780 human ovarian tumor cell line was obtained from Dr. TC Hamilton (Fox Chase Cancer Center, Philadelphia, PA, USA).

Experimental model of cisplatin-induced AKI.

Mice were randomized to receive either (A) control diet (normal chow containing 100% of the recommended Mg) (CTRL) or (B) Mg-deficient (MgD) diet (containing 10% of the recommended amount of Mg, prepared by Teklad/Harlan, Madison, WI, USA) for 2 wks prior to administration of either saline or cisplatin (12mg/kg, i.p.) (n=12-15 mice per group). All mice were weighed before cisplatin administration and just prior to euthanasia 48hrs post-cisplatin (thus, Mg-deficient mice were on the 10%Mg diet for a total of 16 days). In addition, one group of mice (n=10, Mg supplemented (MgS) or replacement group) received the Mg-deficient diet for 16 days followed by the control (100%Mg) diet along with 0.3%MgCl₂ (w/v) in their drinking water for 11 days prior to cisplatin (12mg/kg, i.p.). Following cisplatin treatment, this group continued 100%Mg-containing chow with 0.3%MgCl₂ (w/v) drinking water and received MgSO₄ (100mg/kg/day, s.c.) twice daily until euthanasia 48hrs later by CO₂ asphyxiation. Blood was collected by exsanguination via cardiac puncture into heparinized needles/syringes; after centrifugation, isolated
plasma was collected and frozen at -80°C until analysis. Kidneys were collected and either flash frozen in liquid N₂ (outer medulla and cortex only) or fixed in 10% formalin (1/2 kidney [sagittal sections]).

**Antibodies and reagents.**

ERK 1/2 (rabbit anti-mouse), p-ERK 1/2 (rabbit anti-mouse), STAT3 (rabbit anti-mouse), phospho-STAT3 Tyr705 (rabbit anti-mouse), p53 (mouse anti-mouse), p-p53 Ser15 (rabbit anti-mouse), MRP4 (ABCC4, rabbit anti-mouse) and GAPDH (rabbit anti-mouse) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). OCT1 (rabbit anti-mouse), CTR1 (rabbit anti-mouse), MRP2 (rabbit anti-mouse) and MRP6 (rabbit anti-mouse) antibodies were purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). OCT2 antibody (OCT21-A, rabbit anti-mouse) was purchased from Alpha Diagnostic International (San Antonio, TX, USA). Cisplatin (cis-Dicholorodiammineplatinum(II)) was purchased from Acros Organics (Pittsburgh, PA, USA). MgCl₂·6H₂O and MgSO₄ (anhydrous) were purchased from Fisher Scientific. 2’, 7’-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes/Invitrogen (Carlsbad, CA, USA). Neutral red was purchased from Sigma Aldrich (St. Louis, MO, USA).

**Determination of plasma Mg, blood urea nitrogen and creatinine levels.**

Plasma ionized Mg²⁺ (mg/dL) and blood urea nitrogen (BUN, mg/dL) levels were determined using the Quantichrom Magnesium and Quantichrom Urea Assay kits, respectively (BioAssay Systems, Hayward, CA, USA). Plasma creatinine (Cr, mg/dL) was measured using the enzymatic assay (Diazyme Laboratories, Poway, CA, USA).

**Real-time quantitative PCR (qPCR).**

High quality RNA was isolated from frozen kidneys using RNeasy Universal Plus Mini kit (Qiagen, Valencia, CA, USA). The purity/concentration of total RNA was assayed using the Nanodrop spectrophotometer.
qPCR reactions using specific primers (Table 1, Roche Universal Probe Library) were performed in duplicate/triplicate using the Eurogentec One Step RT qPCR mastermix, 100ng RNA and the Roche 480 Light Cycler using the following conditions: 48°C for 30min, 95°C for 10min followed by 45 cycles of 95°C for 15sec and 60°C for 1min. Relative changes in gene expression were calculated as fold-changes using the comparative Ct ($\Delta\Delta$Ct) method; mouse GAPDH was used as the housekeeping gene for normalizing transcript levels (16).

Cytokine and chemokine assays.

Frozen cortical renal tissue specimens (100mg) were homogenized in 250μl lysis buffer (Tris buffered saline pH 7.3 containing 0.25%Triton X-100 and protease and phosphatase inhibitor cocktail) on ice using a Dounce homogenizer; supernatants were collected after centrifugation. Kidney homogenates and plasma were assayed for cytokines and chemokines using the Meso Scale Discovery (MSD) multiplex platform and the MSD Sector Imager 2400 plate reader (Meso Scale Diagnostics, Rockville, MD, USA). The raw data were measured as electrochemiluminescence signals and analyzed using the Discovery Workbench 3.0 software (MSD). In addition, renal CXCL2 and CCL2 levels were determined by ELISA (R&D Systems, Minneapolis, MO, USA). Renal inflammatory mediator values were adjusted for protein concentrations (using the BioRad protein assay) (Bio-Rad, Hercules, CA, USA).

Measurement of tissue ATP levels and myeloperoxidase (MPO).

Renal cortical tissues were homogenized and ATP levels were measured using a colorimetric ATP assay kit (Biovision, San Francisco, CA, USA), according to manufacturer’s instructions. For assessment of MPO levels, renal cortex tissues were homogenized in lysis buffer (described above); protein concentrations were measured using the BioRad assay. MPO levels were measured by ELISA (Hycult Biotechnologies, PA, USA) and adjusted for protein concentrations according to manufacturer’s instructions.
**Western blotting.**

Renal cortex tissues were homogenized in lysis buffer (described above); protein concentrations were measured using the BioRad assay. Proteins (50μg/lane) were separated by SDS-PAGE electrophoresis (Invitrogen, Carlsbad, CA, USA) and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking for 1 h, the membranes were then incubated with each primary antibody (1:1000- Cell Signaling antibodies/ 1:300- Santa Cruz antibodies) overnight at 4°C. After washing, the blots were then incubated with the appropriate near-infrared-fluorescently labeled secondary antibody (1:15,000, LI-COR) for 1 h and washed before revealing the bands using the Odyssey infrared imaging system (LI-COR Biosciences). Band densities were determined using appropriate controls or housekeeping proteins; quantitation was determined using Image J Software (NIH).

**Histological assessment of renal cell apoptosis, neutrophil infiltration, and renal injury.**

Formalin-fixed kidneys were embedded in paraffin and sectioned (5µm). Renal apoptosis (in the cortex and outer medulla regions) was measured by TUNEL using the ApoTag kit (Millipore, Temacula, CA, USA). The slides were scored by counting the number of densely stained apoptotic cells per high power field (using >5 random fields/section, 4-5 mice per group). Neutrophilic infiltration was assessed by Napthol AS-D Chloroacetate esterase (Leder staining; Sigma Aldrich). The slides were scored by counting the number of neutrophils per high power field (HPF), as described for TUNEL. Similarly, sections were stained with H&E and scored using a semi-quantitative scale designed to assess AKI-associated tubular injury (tubular epithelial cell loss, necrosis, tubular epithelial simplification, intratubular debris and casts) by a pathologist unaware of the experimental groups (using > 5 random fields/section, 4-5 mice per group). Tubule injury
scores (ranging between 0 and 4) were based on the percentage of tubules affected (0: <10%; 1: 10-25%; 2: 25-50%; 3: 50-75%; 4: >75%).

Quantification of platinum accumulation in the kidneys.
Renal platinum (Pt) analysis was performed at the Biomarker Mass Spectrometry Facility of University of North Carolina at Chapel Hill using a modification of a previously described method (51). Briefly, frozen kidneys were digested with 100µL of concentrated (70%) nitric acid along with 150ng rhenium (Re) in a 7mL polypropylene screw cap vial and left at room temperature for 5 hrs. After venting, the vials were placed at 85°C overnight. Once cooled, 100µL of 30% H₂O₂ was added. The vials were vented 20 min after being returned to 85°C. Digestion continued for 4-6 hrs after which the samples were centrifuged at 3500 rpm for 5 min. Dilution to 3 mL with 18 MΩ water resulted in a final matrix of 2% nitric acid and 50 ng/g Re (which served as a yield monitor). Matrix matched calibration standards (Pt/Re, ranging from 0 to 1000ng/g) were prepared with 100µL of mouse plasma (Bioreclamation Liverpool, NY, USA). Samples and standards were processed in a single batch. Pt and Re were measured using an Agilent 7500cx (Tokyo, Japan) inductively coupled plasma mass spectrometer (ICP-MS) and quantified against an external calibration curve (r² = 0.9999). The validity of the data was assessed through use of the Re yield monitor, replicate sample analysis, and QC standards. The tissue concentrations of Pt were corrected individually for yield from the Re recovery. Re recovery for all samples was 98±1.5%. The difference in replicate sample analyses was ≤5%. QC standards varied <10% from known values.

In vitro LLC-PK₁ oxidative stress and cisplatin-induced cytotoxicity assays.
DCFH-DA (oxidative stress) assay: Briefly, LLC-PK₁ cells were grown in M199 containing 10%fetal bovine serum (FBS), penicillin-streptomycin (PS), and glutamine (Q). Four days prior to experimentation, the media was replaced with minimum essential media (MEM) containing 5%FBS, Ca (2.7g/dL), non-essential amino
acids, PSQ and either 100% (9.63mg/dL) or 10% of the recommended Mg (0.963mg/dL), as MgSO₄.

Approximately 16 hrs prior to experimentation, one set of cells maintained in 10%Mg was supplemented with MgSO₄ to 100%Mg (i.e. to a final concentration of 100%Mg, to mimic Mg replacement after Mg deficiency). On the day of the experiment, LLC-PK₁ cells were harvested, resuspended (1x10⁶ cells/ml) and then treated with either MEM (vehicle) or cisplatin (25µg/mL or 83.3µM) and incubated at 37°C/5%CO₂ for 3.5 hrs. The dose of cisplatin used for these experiments was based on the results of pilot studies testing the amount of cisplatin required to induce consistently measurable, yet modest increases in oxidative stress in these cells within 3.5hr, which allowed us to identify conditions that enhanced and reduced oxidative stress. After the incubation period, cells were washed with HBSS and labeled with DCFH-DA (20µM) for 20 min. Labeled cells were washed and analyzed for oxidative stress by the conversion of the non-fluorescent DCFH-DA into a fluorescent compound, dichlorofluorescein (DCF) by ROS intermediates, which was measured 0.5hr later in a quantitative manner using the Victor3 fluorescence plate reader (Perkin Elmer) at Exc485nm/Em535.

**LLC-PK₁ cytotoxicity assay:** LLC-PK₁ cells were grown as described above; media was replaced with 100%Mg-MEM media or 10%Mg-MEM media and the cells were incubated for 3 days. A portion of the cells maintained on 10% Mg media were supplemented with 190%Mg (final=200% Mg) one day prior to cisplatin treatment (prepared from a stock solution of 1mg/ml in pre-warmed saline). MEM or cisplatin (diluted in MEM 25µM final) was added and cells were assayed for cytotoxicity using the neutral red assay (65) 29 hrs later and read using a spectrometer (OD 570). The dose of cisplatin used for these experiments was determined through preliminary experiments; the dose that killed approximately 50% of the cells within approximately 30hr (which allowed us to identify conditions that improved cell viability or worsened cell viability).

A2780 cells were grown in RPMI 1640 media containing 10%FBS, PSQ in 96-well plates and allowed to reach 60% confluence. The media was replaced with MEM media containing either 100%Mg (4.9mg/dL) or 10%Mg (0.49mg/dL), supplied as MgSO₄, Ca (2.7g/dL), non-essential amino acids, and PSQ and the cells were incubated for 3 days. A portion of the cells maintained in 10%Mg media were supplemented with 90%Mg (final=100% Mg, as MgSO₄) approximately 36hrs prior to cisplatin treatment. MEM or cisplatin (diluted in MEM, 0-40µM final) was added and cells were assayed for cytotoxicity using the MTT assay (OD₅₇₀/₆₉₀) 24 hrs later. The IC₅₀ values for the tumor cell killing by cisplatin were calculated using non-linear regression to fit the data to the log (inhibitor-cisplatin dose) vs. response (variable slope-percent viable) curve using GraphPad Prism (GraphPad Prism Software, San Diego, CA, USA). The same procedures were followed for MCF-7 and H460 cell lines, except for MCF-7 breast cancer cells which were originally grown in DMEM media (where 100%Mg= 9.63mg/dL and 10%Mg= 0.963mg/dL).

Statistical analyses.

Experiments were performed at least twice and data are expressed as mean±SEM (or ±SD), as indicated. One-way ANOVAs were used for multiple comparisons followed by Bonferroni post-hoc testing using GraphPad Prism (GraphPad Software, San Diego, CA). P values <0.05 were considered significant.
**RESULTS**

*Mg status regulates cisplatin-induced markers of acute kidney injury.*

Consumption of the Mg-deficient (MgD) diet (10%Mg) by older female mice reduced plasma Mg\(^{2+}\) levels by 37% (1.39mg/dL±0.15 [MgD] vs. 2.21mg/dL±0.06 [controls], mean± SEM, p<0.001); Mg supplementation following Mg deficiency increased plasma Mg\(^{2+}\) levels by 23% (1.7±0.1 mg/dL, p=0.058) vs. MgD mice.

Cisplatin (CIS) induced significant weight loss (Fig. 1A, p<0.001); while Mg deficiency alone did not significantly affect weight, MgD+CIS significantly enhanced weight loss compared to cisplatin alone (Fig. 1A, p<0.05) which was attenuated by Mg supplementation (Fig. 1A, p<0.001). Mg deficiency alone had no effect on kidney function assessed by plasma BUN (Fig. 1B) and Cr levels (Fig. 1C). Cisplatin alone significantly elevated BUN levels (Fig. 1B, p<0.01) and only slightly (not significantly) elevated Cr levels (Fig. 1C) when compared to controls (CTRL). When combined with Mg deficiency, cisplatin significantly augmented BUN (Fig. 1B, p<0.05) and Cr levels (Fig. 1C, p<0.001) vs. cisplatin-treated mice, whereas Mg supplementation significantly attenuated cisplatin-induced BUN and Cr levels vs. MgD+CIS mice (Fig. 1, B and C).

*Renal tubular damage/necrosis following cisplatin is regulated by Mg status.*

Mg deficiency alone (Fig. 1, D and E) did not affect renal histology. However, cisplatin treatment induced significant renal injury vs. controls (Fig. 1, D and E). When combined with Mg deficiency, cisplatin significantly increased tubular injury when compared to cisplatin alone (Fig. 1, D and E). By contrast, MgDMgS+CIS kidneys showed significantly less tubular injury when compared to the MgD+CIS group (Fig. 1, D and E, p<0.001).
Cisplatin-mediated renal chemokine expression is up-regulated by Mg deficiency and down-regulated by Mg supplementation.

Renal expression of several chemokines is increased following cisplatin treatment and their expression is associated with increased renal injury (12,50,57,69). Cisplatin increased both renal Cxcl2 (Fig. 2A, p<0.05) and Cxcl10 mRNA expression (Fig. 2B, p<0.05), but only slightly up-regulated renal Ccl2 mRNA expression above controls (Fig. 2C). In combination with Mg deficiency, cisplatin significantly enhanced renal Cxcl2, Cxcl10, and Ccl2 mRNA expression above the levels observed following cisplatin alone; this was reversed by Mg supplementation (Fig. 2, A-C, p<0.001).

Mg deficiency alone significantly increased renal CXCL2 protein levels vs. controls (Fig. 2D). Cisplatin significantly enhanced renal CXCL2 levels compared to controls (Fig. 2D) and slightly increased CXCL1 (Fig. 2E, p=0.1), but did not increase CCL2 levels above controls (Fig. 2F). Mg-deficient mice treated with cisplatin showed enhanced renal CXCL2 (Fig. 2D, p<0.05), CXCL1 (Fig. 2E, p<0.001) and CCL2 (Fig. 2F, p<0.01) compared to cisplatin treated mice; chemokine expression induced by Mg deficiency was attenuated by Mg supplementation (Fig. 2, D-F, p<0.01).

Cisplatin-induced renal neutrophil infiltration is exacerbated by Mg deficiency and improved by Mg supplementation.

Because we observed a significant increase in CXCL1 and CCL2, neutrophil chemotactic factors, in the kidneys (Fig. 2, E and F) and because renal neutrophil infiltration is associated with cisplatin-induced AKI (25,44), we examined the effect of Mg status on cisplatin-induced renal neutrophil infiltration. Cisplatin slightly enhanced renal neutrophil infiltration compared to controls (p<0.05) without increasing renal MPO levels (Fig. 3, A-D). Both renal neutrophil accumulation and MPO levels were exacerbated when cisplatin
was given to Mg-deficient mice (Fig. 3, A-D, p<0.01) and these effects were improved by Mg supplementation (Fig. 3, A-D, p<0.001).

**Mg deficiency leads to dysregulated renal cytokine expression in cisplatin-treated mice: Reversal by Mg supplementation.**

Next, we investigated the effects of Mg deficiency (+ supplementation) on established cytokine markers of inflammation associated with cisplatin-induced AKI. Cisplatin up-regulated renal Tnfa mRNA expression vs. saline controls, but this was not significant (Fig. 4A). When cisplatin was combined with Mg deficiency, renal Tnfa mRNA was significantly enhanced (p<0.001) when compared to cisplatin alone (Fig. 4A) and this increase was reduced by Mg supplementation (Fig. 4A, p<0.001). Renal TNFα protein was not detectable under any conditions. Cisplatin alone did not alter renal IL-6 (Fig. 4B) or IL-1β (Fig. 4C) levels above controls. However, MgD+CIS mice showed significantly enhanced renal IL-6 (Fig. 4B) and IL-1β (Fig. 4C) levels compared to cisplatin-treated mice. Mg supplementation significantly attenuated cisplatin-mediated kidney cytokines following Mg deficiency (Fig. 4, A-C).

**Mg status regulates cisplatin-induced renal ERK1/2 and STAT3 activation.**

Based on the effect of Mg status on renal inflammation in the cisplatin model, we determined the effect of Mg on cisplatin-induced activation of ERK1/2 and STAT3, two pro-inflammatory signaling pathways implicated in cisplatin-induced AKI (38,59). Cisplatin alone slightly increased renal ERK1/2 phosphorylation (p-ERK1/2) over basal levels, but this was significantly enhanced by Mg deficiency (Fig. 5, A and B). By contrast, decreased renal p-ERK1/2 was observed following MgDMgS+CIS vs. MgD+CIS (Fig. 5, A and B). Total ERK1/2 (ERK1/2) was not affected by cisplatin or Mg deficiency; therefore, p-ERK1/2 expression was adjusted for ERK1/2 expression (Fig. 5, A and B). Phosphorylation of STAT3 (p-STAT3, Tyr705) was not altered
by Mg deficiency alone or cisplatin alone (Fig. 5, C, D and F), but was significantly enhanced by MgD+CIS (Fig. 5, C, D and F). Because cisplatin alone increased total STAT3 expression (Fig. 5, C and E), p-STAT3 levels were also corrected for GAPDH (Fig. 5F). Mg supplementation significantly decreased cisplatin-mediated renal p-STAT3 induced by MgD+CIS (Fig. 5, C, D and F).

Oxidative stress before and during cisplatin-mediated AKI is regulated by Mg status.

In conjunction with ongoing inflammation, oxidative stress is another characteristic feature of cisplatin-induced AKI (12). Mg deficiency alone and cisplatin alone significantly up-regulated renal Ncf1 mRNA expression (p<0.01 and p<0.05, respectively) when compared to controls (Fig. 6A). MgD+CIS further increased renal Ncf1 mRNA expression by ~2.5-fold vs. cisplatin alone (Fig. 6A) and this was reversed by Mg supplementation (Fig. 6A).

Using LLC-PK1 renal epithelial cells to further examine the effect of Mg status and cisplatin on oxidative stress in real time, we found that growth in 10%Mg media increased basal oxidative stress when compared to cells maintained in 100%Mg media (Fig. 6B, p<0.001) and 100%Mg following 10%Mg suppressed the elevated basal oxidative stress (Fig. 6B, p<0.001). Cisplatin treatment increased oxidative stress (p<0.001) in 100%Mg-LLC-PK1 cells (Fig. 6B) and even further enhanced oxidative stress in 10%Mg cells (Fig. 6B, p<0.001). By contrast, 100%Mg following 10%Mg reduced both basal and cisplatin-induced oxidative stress compared to untreated 10%Mg-exposed cells cisplatin-treated 10%Mg cells, respectively (p<0.001, Fig. 6B).
**Cisplatin-mediated renal ATP depletion and apoptosis is amplified by Mg deficiency and spared by Mg supplementation.**

Next, we investigated the effect of Mg status on renal ATP levels, p53 activation, and apoptosis in our model system. While Mg deficiency alone did not affect renal ATP levels when compared to controls, cisplatin reduced renal ATP levels by 30% vs. controls (Fig. 7A, p<0.05), which were further reduced in MgD+CIS mice (Fig. 7A, p<0.001); Mg supplementation significantly protected the MgD+CIS group against renal ATP loss (Fig. 7A, p<0.001).

Renal phospho-p53Ser15 (p-p53) was significantly increased by cisplatin (Fig. 7, B and C). Because cisplatin reduced total p53 expression (Fig. 7, B and D), p-p53Ser15 levels were corrected for GAPDH (Fig. 7E). MgD+CIS significantly enhanced the phosphorylation of p53 at Ser15 position, compared to cisplatin alone mice (Fig. 7E). Mg supplementation following Mg deficiency significantly decreased cisplatin-mediated phosphorylation of p53 when compared to MgD+CIS mice (Fig. 7E). Likewise, renal Bak mRNA expression was significantly increased by cisplatin, when compared to controls (Fig. 7F). While Mg deficiency alone had no effect on Bak mRNA expression, MgD+CIS significantly enhanced renal Bak mRNA expression compared to cisplatin alone (Fig. 7F) and this was reversed by Mg supplementation following Mg deficiency (Fig. 7F).

Using TUNEL staining, we confirmed the significant increase in cisplatin-induced renal cell apoptosis (Fig. 8, A, B and C), which was further and significantly increased by MgD+CIS (Fig. 8, A, B and C). Mg supplementation prior to and during cisplatin treatment significantly reduced the number of renal apoptotic cells when compared to MgD+CIS mice (Fig. 8, A, B and C).
Using the LLC-PK₁ cell line shown to exhibit increased cisplatin-induced oxidative stress following Mg deficiency (Fig. 6B); we examined the effect of Mg status on cisplatin-mediated renal epithelial cell cytotoxicity in vitro. LLC-PK₁ renal epithelial cells maintained in Mg-deficient media had significantly reduced cell viability (p<0.001) following cisplatin compared to 100%Mg+cisplatin-treated cells (Fig. 8D); Mg supplementation prior to cisplatin reversed cisplatin-induced LLC-PK₁ cytotoxicity (Fig. 8D, p<0.001).

**Mg status regulates renal platinum (Pt) accumulation.**

Because Mg status affected all aspects of cisplatin-induced AKI (e.g. inflammation, oxidative stress, and apoptosis), we investigated the effect of Mg status on platinum (Pt) accumulation in the kidneys. Cisplatin-treated mice showed renal Pt accumulation compared to controls (Fig. 9, p<0.05). Pt accumulation was amplified in MgD+CIS mice (Fig. 9, p<0.001) and this increase was completely blocked by Mg supplementation (Fig. 9, p<0.01).

**Magnesium status affects renal expression of cisplatin uptake and efflux transporters.**

Renal Pt accumulation is balanced by both cisplatin uptake and efflux. Mg deficiency alone and cisplatin alone did not affect Oct1 (Fig. 10A) or Oct2 (Fig. 10B) mRNA expression but significantly decreased Ctr1 (Fig. 10C, p<0.05) mRNA expression compared to controls. MgD+CIS mice showed significantly decreased Oct1 (Fig. 10A, p<0.0001) (vs. cisplatin alone and Mg deficiency alone), Oct2 (Fig. 10B, p<0.01) and Ctr1 (Fig. 10C, p<0.01) mRNA expression when compared to mice treated with cisplatin alone. OCT1 (Fig. 10, D and E, p<0.05), OCT2 (Fig. 10, D and F, p<0.0001) and CTR1 (Fig. 10, D and G, p<0.05) proteins were reduced by MgD+CIS vs. cisplatin alone and this was reversed by Mg supplementation (Fig. 10, D-G).
While cisplatin alone significantly increased renal $Abcc2$ (MRP2) (Fig. 11A, $p<0.0001$) and $Abcc4$ (MRP4) variants 1 and 3 (Fig. 11, $B$ and $C$, $p<0.01$) mRNA expression vs. saline-treated control mice, cisplatin alone only increased MRP4 and MRP6 protein levels but not MRP2 (Fig. 11, $E-H$). Mg deficiency alone did not affect efflux transporter expression at the mRNA or protein levels. When combined with cisplatin, Mg deficiency significantly decreased $Abcc2$, $Abcc4v3$, and $Abcc6$ (Fig. 11, $A-D$, $p<0.0001$) and slightly decreased $Abcc4v1$ (Fig. 11$B$, $p=0.1$) mRNA expression compared to cisplatin alone. Both MRP4 ($p<0.0001$) and MRP6 ($p<0.05$) protein levels were decreased by MgD+CIS vs. cisplatin alone (Fig. 11, $E$, $G$ and $H$). MgDMgS+CIS has significantly increased $Abcc2$ (Fig. 11A, $p<0.01$), $Abcc4v3$ (Fig. 11C, $p<0.0001$) and $Abcc6$ (Fig. 11$D$, $p<0.0001$) and slightly increased $Abcc4v1$ (Fig. 11$B$) mRNA expression vs. MgD+CIS. Mg supplementation significantly increased the protein expression of all the efflux transporters (MRP2, MRP4 and MRP6) (Fig. 11, $E-H$, $p<0.01$) compared to MgD+CIS mice.

**Mg supplementation does not compromise cisplatin-mediated tumor cell killing in vitro.**

Cisplatin is commonly used for treating ovarian cancer, breast cancer and lung cancer, among others. Using the human A2780 (ovarian), MCF-7 (breast) and H460 (lung) cancer cell lines, we investigated whether Mg status affects the anti-tumor efficacy of cisplatin in vitro. As expected the A2780, MCF-7, and H460 cancer cell lines grown in 100%Mg media were sensitive to killing by cisplatin (Table 2). Neither Mg deficiency (10%Mg) nor Mg replacement following Mg deficiency (10%Mg/100%Mg) significantly affected the IC$_{50}$ for cisplatin-mediated killing of A2780 ovarian and H460 lung cancer cells in vitro (Table 2). Mg deficiency significantly increased the IC$_{50}$ for cisplatin-mediated killing of MCF-7 breast cancer cells (Table 2, $p<0.05$), whereas Mg replacement slightly reduced the IC$_{50}$ for cisplatin-mediated killing of MCF-7 cells when compared to Mg-deficient cells (although not significantly) (Table 2). No significant difference in IC$_{50}$ values was observed between 100%Mg-treated MCF-7 cells and the Mg supplemented MCF-7 cells (Table 2).
**DISCUSSION**

In addition to increased oxidative stress, inflammation, and apoptosis in the kidneys (50,57,69), cisplatin leads to hypomagnesemia in up to 90% of patients (41). Although Mg deficiency has been proposed to amplify cisplatin-mediated renal damage, little is known regarding the mechanism(s) involved. Using a model of cisplatin-mediated AKI in older female mice, we report the deleterious effects of Mg deficiency on cisplatin-induced renal damage. Based on plasma Mg levels and previous reports, a Mg-deficient diet (10%Mg) given for 16 days induces mild-moderate Mg deficiency (9,76), which is not uncommon in the US, particularly among the elderly (22,82). A moderate dose of cisplatin (12mg/kg) was used to assess the effect of Mg deficiency (±Mg supplementation) because a higher dose of cisplatin (20mg/kg, commonly used) was lethal to Mg-deficient mice (data not shown), limiting our ability to study mechanism(s) and reversal by Mg supplementation. Conversely Mg supplementation, which increased serum Mg$^{2+}$ levels by almost 23% above that found in Mg-deficient animals, exerted significant renoprotective effects against cisplatin-induced AKI. Most cisplatin studies use male mice and to our knowledge, this is the first to use older mice to study cisplatin-mediated AKI. Older female C57BL/6 mice (11 months old) were used because (i) they better represent a cisplatin-treated cohort; (ii) older kidneys exhibit increased susceptibility to AKI (in humans and mice)(2,62,73,86); (iii) Mg deficiency is more common among the elderly (22,82); and (iv) women are more susceptible to cisplatin-induced AKI (50,69). Additional experiments were performed to examine the effect of Mg on cisplatin-induced oxidative stress and cytotoxicity using the LLC-PK$_1$ cell line. The LLC-PK$_1$ cell line is a porcine renal tubular epithelial cell line most commonly used to study nephrotoxic drugs and mechanisms to block drug-induced nephrotoxicity (34). The LLC-PK$_1$ cell line was chosen because it expresses both OCT2 (the major influx transporter expressed by the human kidney (32,77)), as well as numerous efflux transporters (75).
Numerous studies have connected Mg deficiency with enhanced inflammation in the intestines \((14,72)\), lungs \((54)\), and heart \((14)\) with elevated cytokine levels \((6,47,84)\). Although, cisplatin-induced AKI is typically accompanied by renal inflammation \((50,57,69)\), cisplatin, \((12\text{mg/kg})\) did not increase renal cytokines at 48hrs (Fig. 4). However, when cisplatin was combined with Mg deficiency, renal \(Tnfa\) mRNA, and renal IL-6 and IL-1\(\beta\) protein levels were significantly elevated (Fig. 4, A-C). In addition to renal cytokine dysregulation, MgD+CIS mice exhibited elevated renal chemokine expression (Fig. 2, A-F), with increased renal neutrophil infiltration and MPO levels (Fig. 3, A-D); these effects were reversed by Mg supplementation given with MgD+CIS. Consistent with our observations, several studies have reported the anti-inflammatory effects of MgSO\(_4\) treatment \((10,20,39,78)\).

In this study elevated cytokine/chemokine expression in the kidneys of MgD+CIS mice was accompanied by increased p-ERK and p-STAT3, while Mg supplementation suppressed both ERK1/2 and STAT3 activation (Fig. 5, A-F). ERK1/2 signaling has been linked to cisplatin-induced cytokine production \textit{in vivo} \((38)\) and the IL-6/STAT3 pathway has been implicated in renal inflammation \((55)\). In addition, both ERK1/2 and STAT3 activation have been implicated in oxidative stress \((21,70)\).

Mg deficiency alone was accompanied by enhanced renal oxidative stress, as shown by increased expression of \(Ncf1\) (which encodes for p47\(^{phox}\)) (Fig. 6A). p47\(^{phox}\), a cytosolic regulatory subunit of NADPH oxidase, is expressed by most cell types, including non-immune cells \((11)\), such as renal cells \((37)\) and auditory cells \((40)\) following cisplatin. Although Mg deficiency has been previously reported to increase oxidative stress in the liver, heart and skeletal muscles \((63,66)\), this is the first study to demonstrate increased renal oxidative stress during Mg deficiency, and exaggerated cisplatin-induced ROS production following Mg deficiency. Similar to previous studies showing that Mg deficiency enhances oxidative stress.
in several cell types (19,29,89), we observed increased basal ROS production by the LLC-PK₁ renal epithelial cells following Mg deficiency (Fig. 6B). Increased oxidative stress accompanying Mg deficiency in vitro and in vivo was further enhanced by cisplatin (Fig. 6, A-B), and this was reversed by Mg supplementation in vitro and in vivo. Consistent with our results, treatment of rats with the anti-oxidant, apocynin, which targets the NADPH oxidase system blocking p47phox translocation to the membrane, prevents cisplatin-induced AKI (13). Together, these observations suggest that an initial priming of the kidneys by Mg deficiency promotes ROS production which is exacerbated by cisplatin and this can be alleviated by Mg supplementation.

Both oxidative stress and inflammation have been shown to mediate cisplatin-induced renal injury (12,50,69). More specifically, ERK1/2 phosphorylates p53 at Ser¹⁵ (p-p53Ser¹⁵) (18,60) and the roles of both ERK1/2 and p53 in cisplatin-mediated renal apoptosis have been described (17,83,85). MgD+CIS mice showed enhanced renal ERK1/2 activation, total p53 and p-p53Ser¹⁵ compared to cisplatin-controls (Figs. 5 and 7, respectively). Cisplatin-induced apoptosis is associated with ATP depletion (8). In this study, Mg deficiency alone did not decrease renal ATP but when combined with cisplatin, Mg deficiency significantly decreased renal ATP (Fig. 7A). This decrease was attenuated by Mg supplementation, suggesting a role for Mg in reducing mitochondrial dysfunction during cisplatin-mediated AKI. In the mitochondrial pathway of apoptosis, BAK activation following cisplatin induces mitochondrial pore formation with concomitant cytochrome C release (50,57,69). We observed that renal Bak mRNA expression (Fig. 7F) and renal cell apoptosis (Fig. 8, A-C) were significantly increased in MgD+CIS mice compared to cisplatin treated mice and attenuated by Mg supplementation. Although previous studies showed that Mg deficiency induces apoptosis in the heart (79), thymus (48), and retina (30), to our knowledge this is the first study revealing the apoptotic role of Mg deficiency in cisplatin-mediated AKI. In rats, Mg supplementation given during
cisplatin-induced AKI had no nephroprotectant effect (5); however, prolonged Mg supplementation was provided prior to cisplatin in the absence of Mg deficiency, suggesting excess Mg may not be beneficial.

Mg is a required co-factor for over 300 enzymes in the body (26) and therefore, it is not surprising that Mg status regulates multiple pathways associated with cisplatin-induced AKI (e.g. oxidative stress, inflammation and apoptosis). It is equally plausible to hypothesize that Mg simultaneously regulates these pathways by controlling renal cisplatin/Pt accumulation (Fig. 9). Herein, we show that Mg deficiency increased and Mg supplementation decreased Mg deficiency-induced renal Pt accumulation despite decreased influx transporter expression in MgD+CIS mice. Although the precise mechanisms regulating cellular cisplatin uptake are not completely understood, it has been attributed, in part, to specific influx transporters including, OCT1, OCT2 and CTR1, as well as passive diffusion through the plasma membrane (Reviewed in (57,74)). Using a rat model, Yokoo et al (90) showed that Mg deficiency increased renal cisplatin accumulation and renal OCT2 expression following cisplatin. It is important to note that OCT2 knockout (OCT2\(^{-/-}\)) mice are incompletely protected from cisplatin-mediated AKI, with a 50% reduction in BUN and serum Cr levels compared to wild-type mice (27), indicating that OCT2 is only partially responsible. Likewise, CTR1 can mediate intracellular cisplatin accumulation in both human and rodent models (36,45,58); however CTR1 knockout cells can accumulate cisplatin (36,43). Caution should be taken when translating results obtained in mice to humans because the expression of cisplatin influx/efflux transporters is different (e.g. mouse kidneys express Oct1 and Oct2 (15,27,28), whereas human kidneys predominantly express Oct2 (3,31)). Based on our observations and the fact that diffusion into the cell accounts for up to 50% of cisplatin uptake (57), we propose that Mg deficiency enhances cisplatin diffusion into kidney epithelial cells through lipid changes in the cell membrane, as previously shown (46,64,81). Preliminary studies using LLC-PK\(_1\) renal epithelial cells showed that Mg deficiency significantly increased 16:1
(palmitoleic acid) by >200% and decreased 18:1ω-9 (oleic acid) by 20%. Although little is known about 16:1 with respect to the plasma membrane, 18:1ω-9 has been shown to alter the cell membrane’s lipid matrix (80). Thus, it is possible that Mg deficiency-mediated lipid changes influence cisplatin uptake despite decreased influx transporter expression. In addition, our data reveal a dramatic decrease in two important renal cisplatin efflux transporters MRP4 and MRP6 (1), by MgD+CIS (Fig. 11, E, G and H); and these effects were reversed by Mg supplementation (Fig. 11, E, G and H). Together, these results may explain the increased cisplatin accumulation in Mg deficient mice, and decreased cisplatin accumulation in Mg-supplemented mice. However, our results showing the protective role of Mg in cisplatin-mediated AKI do not rule out the possibility that other mechanisms, such as changes in osmolality, as previously described for mannitol (61), might also be involved.

It can be argued that the protective role of Mg could extend to tumors, whereby Mg supplementation may interfere with cisplatin-mediated killing of tumors. However, in highly proliferative tumor cells Mg concentrations are higher and more tightly regulated when compared to non-tumor cells (88). Therefore, they are more resistant to changes in Mg concentrations than non-tumor cells (88). Consistent with these observations, we did not observe any significant alterations in the IC50 of cisplatin for killing A2780 ovarian or H460 lung cancer cells related to Mg supplementation or Mg deficiency (Table 2). In the case of MCF-7 breast cancer cells, Mg deficiency increased the IC50 for cisplatin-mediated killing (Table 2, p<0.05), suggesting that cells were less sensitive to cisplatin killing when in Mg-deficient media. The IC50 for MCF-7 breast cancer cells was slightly reduced (although not significantly) by Mg replacement (Table 2) when compared to cells in Mg-deficient media. However, additional tumor cell lines and in vivo tumor models will be required to better understand the effects of Mg status on the chemotherapeutic efficacy of cisplatin.
Based on our findings we propose a mechanistic model describing the role of Mg in cisplatin-induced AKI (Fig. 12). These observations, together with the results of four small clinical trials reporting the renoprotective effects of Mg supplementation in cisplatin-treated patients with testicular (n= 16, Willox et al (87)), ovarian cancer (n=41, Bodnar et al (7)), head/neck cancer (n=23, Hirai et al (35)) and non-small cell lung cancer (n=50, Muraki et al (52)), strongly support maintaining Mg at homeostatic levels to reduce the number of patients who succumb to cisplatin-induced AKI. Studies are underway in our laboratory to examine the effect of Mg status on tumor cell growth and cisplatin-mediated cytotoxicity using a wide range of tumor cells, as well as the effect of Mg status on cisplatin-induced AKI in tumor-bearing animals. Finally, much larger randomized control trials with extended follow-up periods to study cancer progression and patient outcomes will be required to develop better guidelines for Mg supplementation on cisplatin-mediated AKI and to investigate the role of Mg on the antineoplastic efficacy of cisplatin in various cancer populations.
Financial support:

This work was supported by the Feinstein Institute for Medical Research

Disclosure:

The authors have no conflicts of interest.
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**Figure Legends**

**Fig. 1: Mg deficiency prior to cisplatin treatment enhances and Mg supplementation protects against cisplatin-induced weight loss and kidney damage**

Mice were maintained on either 100%Mg or 10%Mg-deficient [MgD] diets, as described in the methods and then treated with either saline [CTRL] or cisplatin (12 mg/kg [CIS]). Another group of mice maintained on 10%Mg was switched to the 100%Mg diet and given Mg supplementation [MgS], as described in the methods. (A) Change in weight from just prior to CIS to 48hrs post-CIS. All mice were euthanized 48h post-CIS (or saline) and (B) blood urea nitrogen (BUN) and (C) plasma creatinine levels were determined. Data are shown as mean (+SEM) mg/dL. Fixed kidney tissues were stained with H&E and evaluated for histology. Representative images for each group are shown in (D) (x200 magnification). Histological damage scores (ranging between 0 and 4) shown in (E) were based on the percentage of tubules affected (0: <10%; 1: 10-25%; 2: 25-50%; 3: 50-75%; 4: >75%). Data are shown as mean (+SEM). Scale bar (−) = 20 microns. ** p<0.01 vs. CTRL, *** p<0.001 vs. CTRL, † p<0.05 vs. CIS, †† p<0.01 vs. CIS, ††† p<0.001 vs. CIS, ‡‡‡ p<0.001 vs. MgD CIS

**Fig. 2: Cisplatin-induced renal chemokine expression is up-regulated by Mg deficiency and down-regulated by Mg replacement**

Mice were maintained on either 100%Mg or 10%Mg-deficient [MgD] diets, or were maintained on a 10%Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline) and (A) Cxcl2, (B) Cxcl10 and (C) Ccl2 mRNA expression in the renal cortical tissues was measured by qPCR. Data are shown as mean (+SEM) fold-change (vs. GAPDH housekeeping gene). (D) CXCL2 (E) CXCL1 and (F) CCL2 renal protein levels were measured by MSD/ELISA. Data are shown as mean (pg) per mg protein (+SEM). * p<0.05 vs. CTRL, † p<0.05 vs. CIS, †† p<0.01 vs. CIS, ††† p<0.001 vs. CIS, ††‡ p<0.001 vs. MgD CIS, ††† p<0.001 vs. MgD CIS, p=0.1 vs. CTRL
**Fig. 3: Cisplatin treatment following Mg deficiency is associated with enhanced neutrophil infiltration and renal myeloperoxidase (MPO)**

Mice were maintained on either 100% Mg or 10% Mg-deficient [MgD] diets, or were maintained on a 10% Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline); fixed kidney tissues were evaluated for neutrophils by Leder staining. Representative images for each group are shown in (A) at 200x magnification and (B) 400 x magnification and the mean number of neutrophils per high power field (HPF) (+SEM) is shown in (C). Frozen renal tissues were analyzed for myeloperoxidase (MPO) (D). Data are shown as mean MPO concentration (pg) per mg protein (+SEM).

Scale bar (——) = 20 microns. * p<0.05 vs. CTRL, †† p<0.01 vs. CIS, ††† p<0.001 vs. CIS, ‡‡‡ p<0.001 vs. MgD CIS

**Fig. 4: Mg deficiency prior to cisplatin treatment up-regulates the expression of renal cytokines: Reversal by Mg replacement**

Mice were maintained on either 100% Mg or 10% Mg-deficient [MgD] diets, or were maintained on a 10% Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline) and renal (A) Tnfa mRNA expression in the renal cortical tissues was measured using qPCR. Data are shown as mean (+SEM) fold-change (vs. GAPDH housekeeping gene); (B) IL-6 and (C) IL-1β renal protein levels were measured by MSD/ELISA. Data are shown as mean cytokine concentration (pg) per g protein (+SEM). †† p<0.01 vs. CIS, ††† p<0.001 vs. CIS, ‡‡ p<0.01 vs. MgD CIS, ‡‡‡ p<0.001 vs. MgD CIS

**Fig. 5: Mg status regulates cisplatin-induced activation of ERK1/2 and STAT3 inflammatory signaling pathways**

Mice were maintained on either 100% Mg or 10% Mg-deficient [MgD] diets, or were maintained on a 10% Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline) and ERK1/2 (ERK) and STAT3 protein expression
and phosphorylation in the renal cortical tissues were measured by Western blotting. GAPDH, total ERK and total STAT3 were used as loading controls. Representative blots for ERK are shown in (A) and quantitation of phospho-ERK/total ERK band densities (mean±SEM) is shown in (B). Representative blots for STAT3 (total STAT3), phospho-STAT3 \( ^{ Tyr^{305}} \) (p-STAT3), and GAPDH are shown in (C). Quantitation of (D) p-STAT3/total STAT3, (E) total STAT3/GAPDH; and (F) p-STAT3/GAPDH band densities. All data are expressed as mean band density (±SEM). † p<0.05 vs. CIS, ††† p<0.001 vs. CIS, ‡‡ p<0.01 vs. MgD CIS, ‡‡‡ p<0.001 vs. MgD CIS, p=0.08 vs. CTRL, p=0.09 vs. MgD CIS

**Fig. 6: Mg deficiency up-regulates and Mg supplementation down-regulates basal and cisplatin-induced oxidative stress in vivo and in renal epithelial cells**

Mice were maintained on either 100%Mg or 10%Mg-deficient [MgD] diets, or were maintained on a 10%Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with either saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline) and (A) *Ncf1* mRNA expression in the renal cortical tissues was measured using qPCR. Data are shown as mean (±SEM) fold-change (vs. GAPDH housekeeping gene). * p<0.05 vs. CTRL, ** p<0.01 vs. CTRL, † p<0.05 vs. CIS, ‡ p<0.01 vs. MgD CIS. (B) LLC-PK1 renal epithelial cells, grown in either 100%Mg media, 10%Mg (deficient) media or 10%Mg media followed by 100%Mg (10%Mg/100%Mg) for 4 days and then treated with MEM (vehicle, Veh) or cisplatin (CIS, 83.3 μM) and assayed for oxidative stress using the DCFH-DA assay. Data are shown as mean oxidative stress (or fluorescence) (±SD).

*** p<0.001 vs. Veh 100%Mg, ††† p<0.001 vs. Veh 10%Mg, ‡‡‡ p<0.001 vs. Veh 10%/100%Mg, §§§ p<0.001 vs. CIS 100%Mg, ¥¥¥ p<0.001 vs. CIS 10%Mg.

**Fig. 7: Mg deficiency prior to cisplatin treatment enhances renal ATP depletion, activation of the p53 pro-apoptotic signaling pathway and Bak mRNA expression in vivo: Reversed by Mg replacement**

Mice were maintained on either 100%Mg or 10%Mg-deficient [MgD] diets, or were maintained on a 10%Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline). (A) Renal ATP levels (shown as mean ATP
concentration (nmol/mg) (±SD), corrected for protein levels. (B) Kidney total p53 and phospho-53Ser15 (p-p53) were measured by Western blotting. GAPDH and total p53 were used as loading controls. Representative blots are shown.

The ratios of (C) phospho-p53/total p53, (D) total p53/GAPDH and (E) phospho-p53/GAPDH are shown as band densities (mean±SEM). (F) Renal Bak mRNA expression was measured using qPCR and expressed as mean (+SEM) fold-change (vs. GAPDH housekeeping gene). * p<0.05 vs. CTRL, † p<0.05 vs. CIS, ††† p<0.001 vs. CIS, ‡‡ p<0.01 vs. MgD CIS, ‡‡‡ p<0.001 vs. MgD CIS, p=0.1 vs. CTRL

**Fig. 8: Mg deficiency enhances cisplatin-induced renal cell apoptosis in vivo and increases cisplatin-killing of LLC-PK1 cells: Reversal by Mg replacement**

Mice were maintained on either 100%Mg or 10%Mg-deficient [MgD] diets, or were maintained on a 10%Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline). Renal apoptosis was measured by TUNEL staining and representative photomicrographs are shown (at 200x magnification) in (A) a complete representative section and (B) a selected area in the section. (C) Apoptosis was determined by counting the number of TUNEL positive cells per high power field using random sections and the mean apoptosis scores (±SEM) are shown. Scale bar (±) = 20 microns.

* p<0.05 vs. CTRL, † p<0.05 vs. CIS, ††† p<0.001 vs. MgD CIS. (D) LLC-PK1 cells were maintained in either 100%Mg or 10%Mg (Mg-deficient) media, or 10%Mg media followed by 100%Mg replacement (10%Mg/100%Mg). Cell viability was measured 24h post-CIS using the neutral red assay and data are shown as %viability (+SD). *** p<0.001 vs. 100% Mg CIS, ‡‡‡ p<0.001 vs. 10%Mg CIS

**Fig. 9: Cisplatin-induced renal platinum accumulation is enhanced by Mg deficiency and decreased by Mg replacement**

Mice were maintained on either 100%Mg or 10%Mg-deficient [MgD] diets, or were maintained on a 10%Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline) and renal platinum (195Pt) accumulation measured by ICP-MS is shown as mean±SEM (ng/g kidney tissue). * p<0.05 vs. CTRL, ††† p<0.001 vs. CIS, ‡‡ p<0.01 vs. MgD CIS
**Fig. 10: Mg status regulates cisplatin uptake transporter expression in the kidneys**

Mice were maintained on either 100%Mg or 10%Mg-deficient [MgD] diets, or were maintained on a 10%Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline) and renal cortical tissues were assessed for uptake transporter mRNA expression: (A) Oct1, (B) Oct2 and (C) Ctr1 by qPCR. Data are shown as mean (+SEM) fold-change (vs. GAPDH housekeeping gene). Representative Western blots showing renal OCT1, OCT2 and CTR1 protein expression are shown in (D). Quantitation of band ratios: (E) OCT1/GAPDH, (F) OCT2/GAPDH and (G) CTR1/GAPDH (mean band density (+SEM)) are shown. * p<0.05 vs. CTRL, † p<0.05 vs. CIS, ‡‡‡ p<0.0001 vs. CIS, ‡‡‡‡ p<0.0001 vs. MgD CIS, ‡‡‡‡ p<0.0001 vs. MgD CIS, p=0.1 vs. CTRL, p=0.07 vs. CTRL.

**Fig. 11: Mg status regulates cisplatin efflux transporter expression in the kidneys**

Mice were maintained on either 100%Mg or 10%Mg-deficient [MgD] diets, or were maintained on a 10%Mg diet followed by Mg supplementation (MgS), as described in the methods and then treated with saline [CTRL] or cisplatin (12 mg/kg [CIS]). All mice were euthanized 48h post-CIS (or saline) and renal efflux transporter (A) Abcc2, (B) Abcc4v1, (C) Abcc4v3 and (D) Abcc6 mRNA expression was measured by qPCR. Data are shown as mean (+SEM) fold-change (vs. GAPDH housekeeping gene). Representative Western blots for renal ABCC2 (MRP2), ABCC6 (MRP6) and ABCC4 (MRP4) expression are shown in (E). Quantitation of band ratios: (F) ABCC2 (MRP2)/GAPDH, (G) ABCC4 (MRP4)/GAPDH and (H) ABCC6 (MRP6)/GAPDH (mean band density (+SEM)) are shown. *p<0.05 vs. CTRL, **p<0.01 vs. CTRL, ****p<0.0001 vs. CTRL, †p<0.05 vs. CIS, †††p<0.0001 vs. CIS, †††p<0.0001 vs. MgD CIS, ††††p<0.0001 vs. MgD CIS, p=0.06 vs. CTRL, p=0.13 vs. CTRL, p=0.09 vs. CIS, p=0.1 vs. CIS.

**Fig. 12: Proposed mechanisms by which Mg regulates cisplatin-induced AKI**

Mg deficiency-MgD (↓) leads to enhanced renal cisplatin accumulation via decreased efflux transporter expression and increased cisplatin-induced inflammation and oxidative stress with reduced ATP levels in the kidneys. Activated
signaling pathways including, ERK1/2, STAT3 and p53 associated with inflammation and oxidative stress merge to
promote renal cell apoptosis/necrosis resulting in renal tissue damage and ultimately, AKI. Mg replacement
following Mg deficiency-MgS (MgS) protects against cisplatin-induced AKI by decreasing cisplatin accumulation,
increasing efflux transporter expression, inflammation, oxidative stress and the activation of pathways that lead to
kidney cell apoptosis/necrosis.
Fig. 1: Solanki et al

A

Weight Change (g)

CTRL MgD CIS MgD CIS MgD MgD

B

BUN (mg/dL)

CTRL MgD CIS MgD MgD

C

Creatinine (mg/dL)

CTRL MgD CIS MgD MgD

D

CTRL CIS MgD MgD CIS

MgD MgD CIS

E

Tubular Injury Score

CTRL MgD CIS MgD MgD

Tubular cell necrosis
Intratubular debris
Intratubular cast
Fig. 3: Solanki et al

A

CTRL   CIS    MgD MgS CIS
MgD    MgD CIS

↑ = Neutrophils

B

CTRL   CIS    MgD MgS CIS
MgD    MgD CIS

↑ = Neutrophils

C

# Neutrophils/HPF

CTRL    MgD    CIS    MgD CIS    MgD MgS CIS

D

IPO PW/HP

CTRL    MgD    CIS    MgD CIS    MgD MgS CIS

↑↑↑
Fig. 4: Solanki et al

A

![Bar graph showing Tnfa mRNA levels](image)

B

![Bar graph showing IL-6 levels](image)

C

![Bar graph showing IL-1β levels](image)
Fig. 5: Solanki et al

A

B

C

D

E

F

CIS   -   -   +   +   +   +   +
MgD  -   -   -   -   -   -   +
MgS  -   -   -   -   -   -   +

ERK
p-ERK
GAPDH

0   0.4   0.8   1.2
0   0.4   0.8   1.2
0   0.4   0.8   1.2

CTRL   MgD    CIS    MgD   MgD
CIS     MgS

CTRL  MgD    CIS    MgD    MgD
CIS      MgS

CTRL   MgD    CIS    MgD    MgD
CIS      MgS

p-STAT3/STAT3
p-STAT3/GAPDH
p-STAT3/GAPDH

p=0.08
p=0.09

CTRL   MgD    CIS    MgD   MgD
CIS     MgS

CTRL  MgD    CIS    MgD    MgD
CIS      MgS

CTRL   MgD    CIS    MgD    MgD
CIS      MgS

p-STAT3
STAT3
GAPDH

p-STAT3
STAT3
GAPDH

CTRL   MgD    CIS    MgD   MgD
CIS     MgS

CTRL  MgD    CIS    MgD    MgD
CIS      MgS

CTRL   MgD    CIS    MgD    MgD
CIS      MgS

p-STAT3
STAT3
GAPDH

p-STAT3
STAT3
GAPDH

CTRL   MgD    CIS    MgD   MgD
CIS     MgS

CTRL  MgD    CIS    MgD    MgD
CIS      MgS

CTRL   MgD    CIS    MgD    MgD
CIS      MgS

p-STAT3
STAT3
GAPDH

p-STAT3
STAT3
GAPDH

CTRL   MgD    CIS    MgD   MgD
CIS     MgS

CTRL  MgD    CIS    MgD    MgD
CIS      MgS

CTRL   MgD    CIS    MgD    MgD
CIS      MgS

p-STAT3
STAT3
GAPDH

p-STAT3
STAT3
GAPDH
**Fig. 6: Solanki et al**

**A**

![Bar chart showing Ncf1 (p47phox) mRNA expression](image)

- CTRL
- MgD
- CIS
- MgD CIS

Fold Change: 
- MgD: **
- CIS: ***
- MgD CIS: †††

**B**

![Bar chart showing Oxidative Stress](image)

- Veh
- CIS
- Veh CIS

MgD:
- 100% Mg: $$$
- 10% Mg: **
- 10% Mg/100% Mg: †††
Fig. 8: Solanki et al

A

CTRL  |  CIS  |  MgD  | MgS  | CIS
MgD  |  MgD  | CIS

B

CTRL  |  CIS  |  MgD  | MgS  | CIS
MgD  |  MgD  | CIS

C

Apoptosis Score

CTRL  |  MgD  |  CIS  |  MgD  |  MgD  |  MgD
CIS  |  MgD  |  MgS  |  CIS

D

Cell Viability (%)

CTRL  |  10% Mg  |  10% Mg  |  10% Mg  |  200% Mg
CIS  |  CIS  |  CIS  |  CIS  |  CIS

↑ = TUNEL +ve Cells
Fig. 9: Solanki et al
Fig. 10: Solanki et al

A. OCT1 mRNA (Fold Change)

B. OCT2 mRNA (Fold Change)

C. Ctr1 mRNA (Fold Change)

D. Summary of protein expression:

- OCT1
- OCT2
- GAPDH
- CTR1

E. OCT1/GAPDH

F. OCT2/GAPDH

G. CTR1/GAPDH

* p=0.1
† p=0.07
‡‡‡‡ p=0.1
‡‡‡‡‡ p=0.07
‡‡ ‡‡ ‡‡ ‡‡
‡‡‡‡ ‡‡‡‡

CTRL, MgD, CIS, MgD, MgS, CIS
**Fig. 11: Solanki et al**

**A**

![Graph showing Abcc2 mRNA expression](image)

**B**

![Graph showing Abcc4v1 mRNA expression](image)

**C**

![Graph showing Abcc4v3 mRNA expression](image)

**D**

![Graph showing Abcc6 mRNA expression](image)

**E**

![Western blot images with densitometry data](image)

**F**

![Graph showing MRP2/GAPDH ratio](image)

**G**

![Graph showing MRP4/GAPDH ratio](image)

**H**

![Graph showing MRP6/GAPDH ratio](image)
Fig. 12: Solanki et al

Cisplatin Treatment

Magnesium Deficiency Magnesium Replacement

Renal Cisplatin Accumulation

MgD: Efflux Transporter Expression
MgS: Efflux Transporter Expression

Inflammation
Chemokines/Cytokines
Inflammatory cells
ERK1/2
STAT3

Oxidative Stress
ERK1/2
STAT3
MPO
P53
Bak

Low ATP
Tubular Cell
Apoptosis/Necrosis

Renal Tissue Damage

AKI
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ncf1</strong> (p47phox)</td>
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<td>5-ctgccacatuccaggaacat-3</td>
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<td><strong>Gapdh</strong></td>
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<td>5-catatttctgtggtcacc-3</td>
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</tbody>
</table>
Table 2: Mg supplementation does not compromise cisplatin-mediated tumor cell killing \textit{in vitro}: Cisplatin IC\textsubscript{50} (μM, mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>100% Mg</th>
<th>10% Mg</th>
<th>10%/100% Mg</th>
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</thead>
<tbody>
<tr>
<td>A2780 Cells</td>
<td>12.71 ± 2.91</td>
<td>11.08 ± 4.39</td>
<td>12.4 ± 1.39</td>
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<td>MCF-7 Cells</td>
<td>32.78 ± 2.29</td>
<td>41.29 ± 4.08*</td>
<td>39.77 ± 1.33</td>
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<td>H460 Cells</td>
<td>20.24 ± 0.63</td>
<td>20.23 ± 8.45</td>
<td>19.23 ± 3.92</td>
</tr>
</tbody>
</table>

*p < 0.05 comparing 10%Mg-treated cells vs. 100%Mg-treated cells