Unique sex- and age-dependent effects in protective pathways in acute kidney injury

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Running Head: Age and Sex influence AKI response

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Abstract

Sex and age influence susceptibility to acute kidney injury (AKI), with young females exhibiting lowest incidence. In these studies, we investigated mechanisms which may underlie the sex/age based dissimilarities. Cisplatin (Cp)-induced AKI resulted in morphological evidence of injury in all groups. A minimal rise in plasma creatinine (PCr) was seen in Young Females, while in Aged Females, PCr rose precipitously. Relative to Young Males, Aged Males showed significantly, but temporally, comparably elevated PCr. Notably, Aged Females showed significantly greater mortality, while Young Females exhibited none. Tissue KIM-1 and plasma NGAL were significantly lower in Young Females than all others. IGFBP7 levels were modestly increased in both Young groups. IGFBP7 levels in Aged Females were significantly elevated at baseline relative to Aged Males, and increased linearly through day 3, when these levels were comparable in both Aged groups. Plasma cytokine levels similarly showed a pattern of protective effects preferentially in Young Females. Expression of the drug transporter MATE2 did not explain the sex/age distinctions. Heme oxygenase-1 (HO-1) levels (~28kD species) showed elevation at day 1 in all groups with highest levels seen in Young Males. Exclusively in Young Females, these levels returned to baseline on day 3, suggestive of a more efficient recovery. In aggregate, we demonstrate, for the first time, a distinctive pattern of response to AKI in Young Females relative to males which appears to be significantly altered in aging. These distinctions may offer novel targets to exploit therapeutically in both females and males in the treatment of AKI.

Keywords: Acute Kidney Injury, Heme Oxygenase-1, Cisplatin, Sex differences, Female, Autophagy, Aging, Cytokines, Kidney Injury Markers
Acute kidney injury (AKI) secondary to ischemia reperfusion and nephrotoxins is associated with high morbidity and mortality particularly when it occurs in critically ill patients (5, 6). Several chemical compounds, including drugs, have toxic effects on the kidneys due to its highly vascular nature and the presence of drug transporters on renal epithelia, which allow for accumulation of these drugs, particularly in the proximal tubule. These characteristics make drug-mediated AKI very common in patients. Cisplatin (Cp) is a cancer chemotherapeutic agent utilized in the treatment of solid organ tumors, and approximately one-third of patients who receive Cp develop toxic side-effects including Cp-induced AKI (61). There is growing evidence that differences in sex and age may influence the susceptibility, progression and response to AKI and/or to treatment (2, 15, 78). Because of well-known resistance of females to AKI, particularly in preclinical findings, a majority of the studies/models inducing AKI have largely been done in males (41, 71, 80, 81).

In preclinical models of ischemia-reperfusion injury (IRI) in C57BL/6 mice, under identical ischemia times, females exhibit a lesser decline in renal function as well as decreased evidence of tissue damage (3, 54). Other strains of mice similarly demonstrate a greater susceptibility in males than females to renal IRI and AKI (41, 81). The lesser susceptibility to ischemic insults to the kidney in females has been also seen in rat models of ischemic injury to the heart and brain (14, 32). Pre-clinical models of Cp-induced AKI have generated greater variability in the findings of sex-based protection. While several studies have suggested comparable or increased susceptibility in females, others demonstrate decreased susceptibility or increased protection (27, 28, 38, 51, 75, 77, 83, 84, 89, 100, 110). These studies vary by strain of rodents used, dosing regimen or absolute dose, type of renal injury or the age of the animals under study.

There are several reports that indicate that Cp-induced nephrotoxicity is related to sex [outlined in (76)]. Similarly in clinical studies, the medical history of patients, dosage and
methods of Cp administration also affect Cp-induced nephrotoxicity (94). Women show a higher incidence of nephrotoxicity in the presence of other co-morbid conditions such as types and stages of malignancies, cardiovascular diseases (like hypertension), and diabetes mellitus (30). Increased female susceptibility to Cp-induced nephrotoxicity has also been reported with other co-factors like dosage of Cp; prior treatment with Cp or other medications (co-treatment) (21, 67). Studies also report male sex to be a risk factor (70) or demonstrate increased susceptibility of male patients (69) to Cp-induced nephrotoxicity. In contrast, female protection in general is suggested by the reduced incidence of AKI in women relative to males as discussed by Anderson et al (2).

In preclinical models of AKI, aging confers an increased injury. In ischemic (16, 31, 68, 90) and nephrotoxic (29, 52, 74, 98, 103) preclinical models of AKI, aging animals exhibit greater mortality, prolonged renal dysfunction and increased severity of injury. In patients, aging also increases susceptibility to AKI and old age has been reported to be an independent predictor of AKI (42, 45). Further, recovery from AKI is decreased with aging, since among those who survive AKI, older individuals show a higher preponderance of a lack of full recovery than younger counterparts (42, 97). While aging is often accompanied by an increase in comorbid conditions and greater exposure to drugs with potential nephrotoxicity, changes in cellular and molecular pathways in the kidney may also influence injury and recovery (1, 98). Importantly, lower susceptibility to AKI in women is lost in aged women (78, 105). Based on these observations, open avenues of research (animal models or clinical studies) still remain to further explore factors influencing Cp-induced nephrotoxicity.

A common finding in animal models of AKI is the role of heme oxygenase-1 (HO-1) in protection against AKI (72). HO-1 is a microsomal enzyme that is responsible for the breakdown of heme and has been implicated to have cytoprotective, immunoregulatory, anti-oxidant, anti-apoptotic, and vasoactive properties [reviewed in (36, 62)]. Previous studies in young male mice demonstrated an integral role for HO-1 in protection from AKI (46, 62, 91, 111). In males, it is
well established that overexpression of HO-1 confers protection against kidney injury and lack of
HO-1 expression exacerbates AKI in animal models (62, 72). Interestingly, a recent study in
patients demonstrated that genetic polymorphisms in the HO-1 promoter that correlate with
decreased mRNA expression, associated with higher odds of AKI in patients who underwent
cardiac surgery. Further, this association was significantly stronger in women than in men (20,
60).

HO-1 has been shown to alter several important cellular pathways that are involved in
recovery of the proximal tubule in AKI, including, cell proliferation and apoptosis, pro- and anti-
inflammatory mechanisms, as well as autophagy, each of which may mediate the progression of
AKI (12, 13, 24, 56, 79, 82). These studies were conducted in males; whether sex-based
differences exist in these mechanisms is unknown. During and after AKI there is also a
concomitant autophagic response to replace or repair the damaged or dead cells which
promotes cellular proliferation in an attempt to restore and replenish the damaged tubular
epithelium (24). Autophagy is a lysosome-mediated destructive process by which the cell
maintains homeostasis by eliminating damaged proteins and organelles (35). The study of
autophagy frequently involves examination of critical proteins in the autophagy process (86). In
the sequence of steps necessary in autophagy, Beclin-1 (Becn1) 1 plays an early role in the
initiation of the autophagosome, Autophagy protein 5 (Atg5) is essential in the development of
the pre-autophagosome, while the cleavage of microtubule-associated protein 1 light chain 3
(LC3) to LC3-I and subsequently to LC-3-II is important in the transition from the mature
autophagosome until fusion with the lysosome. p62 is a protein that is degraded via an
autophagic process and its accumulation is thought to be indicative of defective autophagy. HO-
1 and autophagy, induced in response to Cp-mediated oxidative stress, have been shown to
confer protection against injury (12, 56, 82). Lack of HO-1 exposes the cells to increased heme
content and oxidative stress, leading to dysregulated or defective autophagy and cell death. In
contrast, HO-1 overexpression counteracts the heme over load and oxidative stress sustained
during Cp injury, promoting autophagy and cell survival (12). While no known sex-based
differences in autophagy have been identified, aging causes a decline in the autophagic activity
(19).

All these data provide indirect evidence of differences in susceptibility to AKI based on
age and sex. Given that the mechanistic studies of AKI in animals have predominantly been
done on males, and females show striking resistance to AKI, we sought to investigate and
perform a comparative analysis of the age- and sex-specific effects of Cp-induced AKI in young
and aged females and males on renal function measures and kidney injury parameters, on the
ensuing inflammatory response, on HO-1 induction, autophagy, cell proliferation and death.
Materials and Methods

General supplies were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA) unless otherwise identified.

Animal Model

Female and male C57BL/6J mice at 16-17 weeks old (Young) and 16-17 months old (Aged) were subjected to AKI with a single intra-peritoneal injection of Cp (20 mg/kg body weight; prepared as a 1 mg/ml solution in saline) or normal saline as vehicle between 9 and 11 am. These mice are housed under a 12hr:12hr light:dark schedule with lights on at 6 AM. Normal mouse chow and water was provided ad libitum. 1 and 3 days after Cp/vehicle injections, the mice were euthanized at the same time of day as the Cp injections. Kidneys and plasma were processed for further analyses as described below. Renal function was assessed by plasma creatinine (PCr) measured using LC-MS/MS performed by the Bioanalytical Core at UAB’s O’Brien Core Center for AKI Research (https://www.uab.edu/medicine/obriencenter/).

Mice were weighed daily and mortality was assessed using Kaplan Meier curves with adjustment for animal harvests. We chose the ages of the mice to be investigated based on survival data from Jackson Laboratories for the C57BL/6J strain as well as data from our own colony derived and refreshed from this stock. We chose the 16- to 17-month age for the “Aged” group, as this age range immediately precedes the decline in survival as a result of death due to age alone (10, 107): for female C57BL/6J mice, mortality begins at ~500 days or ~17 months; for males, mortality begins at ~700 days or ~23 months. Thus, the range of 16-17 months was chosen as a common age for the “Aged” group of both males and females. The 16- to 17-week age was chosen for the “Young” group to provide young adults that are beyond the stage of protection from AKI due to youth (29, 109), allowing identification of sex- and aging-specific susceptibility to AKI. All the mice used in this study were bred and maintained as an in-house colony at the University of Alabama at Birmingham from mice originating from Jackson...
Laboratories (stock# 000664). The colony was refreshed as appropriate with Jackson Laboratories stock. All protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Morphological Analysis

Kidney tissues were fixed in Neutral Buffered Formalin overnight at 4°C and processed for paraffin-embedding by the UAB Comparative Pathology Laboratory. 5 µm sections were stained using Periodic acid-Schiff (PAS) solution (Sigma-Aldrich; 395B-1KT; lot-SLBC9804) using the manufacturer’s instructions, dehydrated and mounted using VectaMount (Vector; H5000; lot-Y1016). Images were obtained using a LEICA DMI6000 B microscope with the LASV4.2 software. Using a semi quantitative scale of 0-4 (0, no damage evident; 1, dilated tubules and a few cells in lumen, one or two small casts; 2, dilated tubules and some vacuolated cells, few to several casts; 3, >50% dilated tubules with loss of brush border occasionally evident, many tubules with vacuolated cells, but cell layer intact, many casts; 4, extensive damage present, loss of brush border in some tubules, vacuolated cells, denuded basement membranes detected, casts in nearly all tubules), five representative images of each animal’s kidney were reviewed in a blinded fashion and assigned an injury score and a mean value was determined for each animal. A subset of samples was examined for histological scoring and were chosen based on having PCr values near the mean of the respective groups. Group mean±SEM (n=3-9/group) were then calculated from the animal mean scores.

Western Blot Studies

Western blotting was done as described previously (9, 46). Briefly, total kidney protein lysates were prepared by homogenizing tissues in RIPA buffer and 1x protease inhibitor cocktail (Thermo Scientific; 1861281; lot-QE217076). 30-40µg of protein or 2uL of plasma was resolved
on 4-15% Criterion TGX Precast Gels (Biorad) and transferred to nitrocellulose membranes (GE Healthcare Lifesciences). Blots were blocked in PBS Tween20 containing 5% non-fat dry milk. The blots were incubated overnight in the following primary antibodies with the respective dilutions: rabbit anti-HO-1 (Abcam; ab68477; lot-GR96795-19; 1:1000), rabbit anti-p62 (Sigma; P0067; lot-103M4785V; 1:500), rabbit anti-ATG-5/APG-5 (Santa Cruz; sc33210; lot-C2410; 1:1000), rabbit anti-Beclin-1 (Santa Cruz; sc11427; lot-l2614; 1:500), rabbit anti-LC3 (Sigma-Aldrich; L7543; lot-084M4798V; 1:500), rabbit anti-MATE2 (Sigma-Aldrich; PA5-24659; lot-SD2370533; 1:1000), goat anti-NGAL (R&D systems; AF1857; lot-JZP0316101; 1:1000), goat anti-KIM-1 (R&D systems; AF1817; 0.25ug/mL), rabbit anti-IGFBP7 (Abcam; ab74169; lot-GR268710-1; 1:500). For secondary antibody incubations, AlexaFluor 680 goat anti-rabbit IgG (Jackson ImmunoResearch; 111-625-144; lot-124312) or donkey anti-goat-HRP (Jackson ImmunoResearch; 705-035-003) or goat anti-mouse-HRP (Jackson ImmunoResearch; 115-035-062; lot-121006) was used. The blots were imaged using an Odyssey Infrared Imaging System Version1.1 and Image Studio Ver4.0 software or by exposing the blots in enhanced chemiluminescence substrate with radiographs. The blots were stained with Ponceau S solution (Sigma-Aldrich; P7170) for confirmation of equal loading. Densitometry on the bands of interest was done using ImageJ software (https://imagej.nih.gov/ij/) and normalized to the Ponceau S staining. Densitometry values on the graphs were represented as arbitrary units. Because the number of samples precluded inclusion of all samples on a single blot, in some cases, samples were run together first, by age/within sex, and then, by sex/within age. Results are discussed incorporating findings from both experimental designs.

Plasma cytokine levels measurements

Plasma cytokine levels were measured using the V-PLEX Proinflammatory Panel 1 Kit (mouse; Meso Scale Discovery; K15048D-1) as per manufacturer’s recommendations. Briefly, plasma samples were 2-fold diluted in diluent 41. 50uL of standards (calibrators) or samples were
loaded on the multi-spot plate and incubated at room temperature for 2 hours with shaking. After
washing with PBS containing 0.05% Tween-20 (PBS-T), 25uL of the detection antibody cocktail
was added to each well and incubated at room temperature for 2 hours with shaking. The wells
were washed as above and 150ul of the 2X read buffer T was added to each well to stop the
reaction. The plate was read using a MESO Sector S600 plate reader (Meso Scale Discovery).
Group mean±SEM (n=3-9/group) were calculated and cytokine levels are represented as
picograms/mL.

Neutrophil Gelatinase-Associated Lipocalin ELISA

Mouse plasma NGAL (Neutrophil Gelatinase-Associated Lipocalin) ELISA was
performed as per manufacturer’s instructions (Bioporto Diagnostics; KIT 042). Briefly, the
plasma samples were diluted in diluent provided in the kit. 100uL of calibrators and diluted
samples were incubated in microwells precoated with NGAL monoclonal capture antibody (1
hr). With intervening washes, biotinylated NGAL antibody was added to each well (1 hr),
followed by HRP-conjugated streptavidin (1 hr), and finally, a color-forming peroxidase substrate
with tetramethylbenzidine (TMB) (10 minutes), before stopping the reaction by adding dilute
sulfuric acid (Stop Solution). The plate was read at 450 nm (and reference wavelength of 650
nm) in a plate reader (Molecular Devices SpectraMax M2e). Group mean±SEM (n=4-6/group)
were calculated and plasma NGAL levels are represented as micrograms/µL.

Immunofluorescence staining

Kidney tissues were fixed in 4% paraformaldehyde in PBS overnight at 4°C and
cryopreserved in 20% sucrose solution at 4°C overnight before embedding in Tissue-Tek OCT
compound (Sakura Finetek). 5 µm thick sections were incubated in 0.1%TritonX 100 in
phosphate buffered saline (PBS) for 5 minutes and incubated in blocking solution, 2% normal
horse serum (Vector Laboratories; S-2000; lot-Y0323) in PBS, for 1 hour at room temperature.
Sections were incubated overnight at 4°C with rabbit anti-Ki67 antibody (Abcam; ab15580; lot-GR279879-1; 1:500) diluted in blocking solution and, subsequently, with fluorescently labeled secondary antibody AlexaFluor 488 Donkey anti-Rabbit (Jackson Immuno Research; 711-585-152; lot-109880; 1:200) for 15 minutes at room temperature. Slides were mounted with VECTASHIELD® Hard Set mounting medium with DAPI (Vector Laboratories; H-1500; lot-ZC0713) and slides were stored at -20°C until imaged. Images were acquired by using a LEICA DMI6000 B microscope with the LASV4.2 software. Ki67 positive nuclei were counted in a blinded fashion in 3-5 fields for each animal and group means±SEM were calculated from the animal mean scores.

**TUNEL staining**

Kidney tissues were fixed in Neutral Buffered Formalin overnight at 4°C and processed for paraffin-embedding by the UAB Comparative Pathology Laboratory (as above). Tissue sections were stained as per manufacturer's instructions (TACS®2 TdT-DAB in situ Apoptosis Detection Kit; Trevigen; 4810-30-K). The TUNEL reaction was visualized using HRP-labeled streptavidin and DAB solution and sections were counterstained with Methyl Green. After dehydration and clearing, slides were mounted with VectaMount permanent mounting medium (Vector Laboratories; H-5000) and images were acquired using a LEICA DMI6000 B microscope using the LASV4.2 software. TUNEL positive nuclei were quantified in a blinded fashion in the outer medullary region encompassing 3-5 fields per animal. Group means±SEM were calculated.

**HO enzyme activity**

HO enzyme activity was determined as described previously (57). Briefly, frozen kidney tissues were sonicated in buffer (200 mM KH₂PO₄, 135 mM KCl, 0.1 mM EDTA, pH 7.4), homogenized and subjected to ultracentrifugation to obtain microsomal pellets. The microsomal
pellet was incubated in a reaction mixture containing liver cytosol (source of biliverdin reductase), hemin, glucose-6-phosphate dehydrogenase, NADPH and glucose-6-phosphate. Following 1 hr of incubation in the dark, the resultant bilirubin formed was extracted with chloroform and optical densities at 464 and 530 nm were measured to determine HO activity. Group means±SEM were obtained and the HO enzyme activity levels are represented as picomoles (pmol) bilirubin formed/mg protein/hour.

Statistics

For statistical analyses, Graphpad Prism 6 (GraphPad Software, Inc.) was used. P values of ≤0.05 were considered significant. Data values indicate mean±SEM using 1-way or 2-way ANOVA with Tukey’s post-test for comparing more than 2 groups. Within group analyses utilized 1-way ANOVA to establish significant differences; 2-way ANOVA was utilized to identify significant differences across groups. Log-rank (Mantel-Cox) test was used to test the difference in the Kaplan Meier survival curves for the different groups.
Results

Renal function, mortality, histology and injury markers, and plasma cytokine levels differ by age and sex after Cp-induced AKI

Young and Aged mice of both sexes had comparable mean plasma creatinine (PCr) levels at baseline (Fig. 1A). In striking contrast to the other groups, significantly elevated PCr levels occurred earlier (day 1) in Aged Female mice whereas no significant elevation in mean PCr values was seen in other groups at this time point. Interestingly, PCr levels at day 1 in both Male groups demonstrated very little variance, while both Female groups had greater variance. The mean PCr observed in Young Females on day 1 did not reach significance for elevation above baseline. At day 3, PCr levels were significantly above baseline in all groups, with lowest levels seen in Young Females, moderate levels in Young Males, and high levels in both Aged groups. Young Female PCr was significantly lower than that of all other groups on day 3. Young Male PCr was significantly lower than that of both Aged groups. Importantly, death occurred earlier and at a higher rate in Aged Female mice compared with all other groups (Fig. 1B). Weight loss over 3 days post-Cp injections was comparable in these groups (Fig. 1C).

In addition to examining PCr levels to determine kidney function, we assessed other biomarkers, including kidney injury molecule 1 (KIM-1) and insulin-like growth factor-binding protein 7 (IGFBP7) protein levels in the kidney by Western blot. Plasma neutrophil gelatinase-associated lipocalin (NGAL) was evaluated by Western blotting and by ELISA. KIM-1 serves as a biomarker for proximal tubule injury (39, 92) and our data show that at day 3, kidney KIM-1 protein levels were increased above baseline in all groups (Fig. 2A and 2C). At day 3, Males (Young and Aged) showed significantly higher KIM-1 levels relative to the Females (Young and Aged, respectively) (Fig. 2A and 2C). These data indicate that proximal tubular injury in response to Cp was the highest at day 3 in all groups; however, the KIM-1 protein levels were
only modestly elevated in Young Female. Notably, the variance in the data of the Young Females at day 3 appeared greater than that of all other groups.

IGFBP7 is a marker of early tubule injury resulting from inflammation, ischemia or oxidative stress (26). Urinary levels of IGFBP7 and tissue inhibitor of metalloproteinase 2 (TIMP2) are currently being used in the clinical setting for assessing risk for AKI development (37). The mean protein expression values of IGFBP7 in kidneys from Young Male and Young Female were elevated at day 3 post Cp-AKI compared to the baseline levels (Fig. 2B and 2D). The mean protein expression values of IGFBP7 in Aged Female were significantly higher than Aged Male at baseline and day 1 post Cp-AKI, and increased linearly over the time course. In the Aged Male, the IGFBP7 protein expression showed a significant increase at day 3, as compared to baseline and day 1 post Cp-AKI, which achieved comparable levels to that of the Aged Female at this time point (Fig. 2B and 2D).

NGAL is upregulated in AKI due to proximal tubule injury and can be detected in urine and plasma (23). Evaluation of plasma NGAL by ELISA demonstrated that Aged Females on day 1 showed an elevation in these levels relative to baseline (although values did not attain statistical significance), which was not seen in any other group. At day 3 post Cp-induced AKI, there was a significant increase in NGAL levels, compared to baseline and day 1, in all groups except Young Female, which exhibited a large variance around the mean at day 3. Importantly, levels of plasma NGAL were significantly higher in all groups relative to Young Females at day 3 (Fig. 2E). Consistent with these findings, evaluation of plasma NGAL by Western blot indicated that Young Female and Aged Female had lower NGAL protein levels at day 3 compared to Young Male and Aged Male respectively (Fig. 2F).

To evaluate a role for inflammation in the susceptibility to Cp-induced AKI in our model, we analyzed the levels of plasma cytokines [TNF-α (Tumor Necrosis Factor-alpha), IL-2, IL-6,
IL-10, IL-12p70, IL-1β, IL-4, IL-5, IFN-γ (interferon-gamma) and chemokine (C-X-C motif) ligand 1 CXCL1/KC-GRO)] (Fig. 3A-F). Plasma TNFα was not significantly elevated above baseline in Young Female, while all other groups demonstrated elevated levels at day 1 in Aged Female, and at day 3 in Aged Female, Young Male, and Aged Male (Fig. 3A). IL-2 was not changed at day 1 post-Cp-induced AKI in any group, but was increased above baseline at day 3 in both Young Female and Young Male; no significant change was seen at day 3 in either Aged group (Fig. 3B). IL-6 appeared unchanged in the Young Female and Young Male groups throughout the time course (Fig. 3C). Interestingly, highest levels of IL-6 in the Aged Female group occurred on day 1 post-Cp-induced AKI and subsequently declined on day 3, but remained above baseline. Aged Male showed no change in plasma IL-6 levels at day 1, but these levels were significantly above baseline at day 3. Like IL-6, IL-10 levels in plasma appeared to have greater modulation in the Aged groups (Fig. 3D). In Young Female, IL-10 levels were not significantly changed although greater variance was observed at day 1. Young Male showed no change at day 1 post-Cp-induced AKI, but had a modest, but significant elevation at day 3. Likewise, Aged Male showed no change at day 1, but significant elevation in plasma IL-10 at day 3. Aged Female had a significant elevation in IL-10 at day 1 post-Cp-induced AKI, which declined at day 3, but remained above baseline. IL-12p70 was unaltered over the time course in both Young groups and in Aged Males, but was significantly elevated in Aged Females at day 1 post-Cp-induced AKI (Fig 3E). Significant changes in plasma levels of KC-GRO were temporally variable in each of the Young Female, Young Male, and Aged Male groups, with significant elevation at day 1 in Young Female and Aged Male, while day 3 levels were higher in Young Males (Fig. 3F). Aged Females demonstrated increased variance at day 1, but the changes didn’t reach significance. Levels of other cytokines tested in this study did not show any statistically significant differences (IL-1β, IL-4, IL-5, and IFN-γ; data not shown).
Periodic acid-Schiff (PAS) staining of kidneys revealed notable differences in structural morphology, including loss of brush border and cast formation in renal tubules, in all groups at Cp day 3. In Aged Female and Aged Male mice, kidney damage was evident at day 1 following Cp injections, but did not reach significance in the injury score quantitation (Fig. 4A and 4B). These data suggest that while both ages exhibit cellular damage and inflammatory response, after Cp injections, particularly with aging, retention of functional capacity and lesser modulation of cytokine levels occur only in Young Female mice.

**HO-1 protein expression in kidney differs with age and sex after Cp-induced AKI**

HO-1 protein expression was examined by Western blotting on total kidney lysates in all experimental groups and time points (Fig. 5A). HO-1 protein can be detected in Western blotting as a ~32kDa (full length) band and a ~28kDa (truncated) band in tissues and cells (64). The truncated form of HO-1 was elevated at day 1 of Cp injections in all groups, and remained above baseline levels in all but Young Female mice at day 3 (Fig. 5A, 5B and 5C), the typical point of a peak in PCr levels observed in this model (Fig. 1A). Baseline protein levels of full-length HO-1 appear to be higher in Young Male mice than in Aged Male mice, and decline below baseline in Young Male mice after day 3 of Cp administration (Fig. 5A, 5D and 5E). Expression of the full-length HO-1 did not change after Cp injections in females of either age or in Aged Male mice, but Young Male exhibited a significantly higher level than Young Female on day 1 following Cp injection (Fig. 5A, 5D and 5E). HO enzyme activity was examined on kidney lysates from all experimental groups. There were no significant differences in the HO enzyme activity in any of the groups, although increased variance in the data was apparent at day 3 in Young Females, and at day 1 and day 3 in Young Males (Fig. 5F). No data were obtained for the Aged groups at day 3 due to the high mortality rate; day 1 values, were elevated, but these changes did not reach significance.
Autophagy-related proteins differ with age and sex groups after Cp-induced AKI

Autophagy is upregulated during oxidative stress to promote cell survival and HO-1 can regulate autophagy (12, 50, 58, 82). To evaluate this mechanism in our model, Beclin-1 (BECN1), Atg5, LC3 ratio and p62 were examined by Western blot. Our data indicate that p62 levels are not elevated in Young Female or Young Male mice at day 1 after Cp injection, but are significantly elevated in both groups by day 3, with Young Male mice exhibiting significantly higher levels than Young Females (Fig. 6A and 6B). In Aged Male and Aged Female mice, a significant, steady rise in p62 expression occurred over the time course examined after Cp injection (Fig. 6A and 6B). Baseline levels of Becn1 in Aged Male and Aged Female were significantly higher than that in Young Male and Young Female mice, respectively (Fig. 6A, 6C and 6D). Within each age-sex experimental group, no significant change from baseline was observed on day 1 (Fig. 6A and 6C). On day 3, in both Aged Female and Aged Male, Becn1 levels declined modestly, but significantly from baseline, with Aged Female levels being similar to Young Female, while Aged Male levels remained above that of Young Male (Fig. 6A and 6D).

Comparable baseline levels of Atg5 were seen across all experimental groups (Fig. 6A and 6E). Atg5 was not elevated relative to baseline levels at day 1 within any group, but on day 3, all groups exhibited levels significantly above baseline levels (Fig. 6A and 6E). LC3 ratios (II/I) likewise showed no change at day 1 (data not shown). On day 3, there was no change in the LC3 ratios relative to baseline in Young Female and Young Male mice, but ratios in Aged Female and Aged Male mice were reduced significantly compared to baseline (Fig. 6A and 6F).

In aggregate, these data suggest that autophagy is deranged in this model of AKI with greater effect in aging mice. Importantly, the p62 findings appear to parallel the differences in functional changes seen in PCr, with lesser effect in Young Female.
Aging affects cell death and proliferation in Cp-induced AKI

Cell death by TUNEL assay was significantly increased at day 3 in Young Female, Aged Female and Aged Male; in Young Male, these data did not show a statistically significant increase. Increased variance in the data was observed at day 1 in both Aged groups, but no significant elevation from baseline was noted (Fig. 7A, 7B). Cell proliferation by Ki67 immunostaining was only found to be significantly elevated in Aged Female and Aged Male at day 3, relative to baseline and to day 1; no changes were seen in the Young groups (Fig. 7C and 7D). These data collectively indicate that aged mice, exhibit increased cell death and cell proliferation 3 days post-Cp injections, while young mice show only modest alterations in these parameters.

Expression of a Cp transporter in age and sex groups after Cp-induced AKI

Transport of Cp into renal proximal tubule from plasma occurs through basolateral transporters, principally Organic Cation Transporter 2 (OCT2), and clearance by excretion into urine by apical membrane transporters like Multidrug and Toxin Extrusion Protein-1 and 2 (MATE1 and 2) (63, 79). Up or down regulation of these transporters can effect accumulation of Cp in the tubules, thus potentially causing different degrees of toxicity and injury. We, therefore, evaluated the expression of kidney tissue MATE2 by Western blot in our Cp/saline mice cohorts. It has been previously reported that males have significantly higher MATE2 mRNA levels than in females in several organs (63). Kidney MATE2 protein expression was also significantly higher in Males compared to Females (Young or Aged) (Fig. 8A and 8B). Further, this difference in levels was maintained at all time points after injury with significantly higher levels in Males than in Females. MATE2 expression was unchanged in Young Female, although there was a steady (not significant) decline in the MATE2 mean expression values at day 1 and
day 3 compared to baseline levels in Aged Female. A decline from baseline in the level of this protein was significant in Young Males on day 3, but did not reach significance in Aged Male (Fig. 8A and 8B). These data collectively indicate that the dynamics of Cp excretion from the renal epithelium to the urine differs by sex, but this difference is maintained with renal injury and largely with age. Thus, the renal epithelium of females may have a greater time of exposure to Cp than that of males, but this time of exposure doesn’t play a role in the different status of protection in Young Females that appears to be absent in Aged Females.
The results of this study are the first to systematically characterize sex and age-related differences that relate to CP-induced AKI; specifically the (i) renal functional response; (ii) protein expression of functional kidney injury markers KIM-1, IGFBP7, NGAL; (iii) plasma cytokine levels reflecting the inflammatory component; (iv) protein and enzyme activity levels of HO-1; (v) autophagy markers; (vi) dynamics of apoptosis versus cell proliferation; and (vii) the role of transporters (specifically MATE2) in affecting accumulation of Cp in the tubules. The data presented show that while morphological damage is evident at both ages, Young Female mice show functional protection from AKI, and Aged Female mice as well as Young Male and Aged Male mice are more sensitive to functional decline. Patterns of HO-1 induction also differ between the age groups, and ineffective autophagy pathways appear to underlie this derangement. Young Female mice injected with Cp to induce AKI have preserved renal function; a lesser elevation of renal injury biomarkers (assessed by kidney protein expression of KIM-1, IGFBP7 and plasma NGAL levels), but definitive tissue damage; modest changes in markers of inflammation; no change in protein expression of a Cp transporter (MATE 2); and a pattern of HO-1 expression that differs from the pattern in aged females and both ages of males, which have significantly worse AKI. Furthermore, measures of autophagy, a process influenced by HO-1 and important in cell survival in AKI, show particularly deficient processing in aged female mice with Cp-induced AKI that parallels the strikingly accelerated decrease in renal function and the observed prolonged elevation of HO-1. In addition, these aged female mice exhibit early elevation of pro-inflammatory markers such as TNFα, IL-6 and IL-12p70, even while anti-inflammatory cytokines like IL-10 are elevated earlier in this group than in others. Our data also indicate that aged mice of both sexes exhibit increased cell death and cell proliferation 3 days after Cp administration.

Emerging evidence indicates that female physiology is different from that of males and may cause differences in the response to injury and/or to treatment. Clinical studies have largely
included a predominance of men (3, 34), perhaps due to the decreased incidence of AKI in women (2, 102). Because of previously observed evidence of sex differences in resistance and sensitivity to renal injury in animal models of AKI, preclinical investigations also largely have been conducted in males (49, 73, 93, 99, 101, 104, 106). In animal models of AKI, aging is also associated with greater susceptibility to AKI and decreased measures of repair. Importantly, our results demonstrate a model of protection from AKI that is lost with aging in which age and sex affects the severity of AKI. Consistent with previous studies (41, 46, 81), our data in Young males show that there is a significant worsening of kidney function and upregulation of renal injury markers (KIM-1, NGAL) at day 3 compared to baseline levels, which parallels the robust induction of HO-1 protein levels at day 1 and day 3 after Cp injections. In contrast, we demonstrate that there is an induction of HO-1 in Young Females at day 1 after Cp that declines rapidly returning to baseline levels at day 3, and these Young Female mice showed modest elevation in PCr even while tissue damage is evident at day 3. Where our findings diverge from that of others, we believe differences in the model characteristics may be important. Higher or different dosing regimen or younger aged females may exhibit increased renal injury parameters or death, but these changes may be due to the substantial injury to the intestine and concomitant dehydration rather than or in addition to a direct kidney injury. Further investigations are warranted to better establish the range of effects seen in preclinical Cp models of AKI in female mice. Likewise, a better elucidation of sex-based distinctions vs. age-based distinctions in the clinical literature remains an important goal.

All of the age/sex groups in this model demonstrated renal injury by PCr, tissue damage, elevated tissue KIM-1 and IFGBP7 and plasma NGAL. Yet in nearly all cases, elevation of these parameters was less in the Young Female group. Early elevations of PCr and plasma NGAL as well as significantly elevated baseline levels of IGFBP7 were uniquely observed in Aged Females. These distinctions suggest that the mechanisms of response to injury and/or the capacity of as yet undefined early protective compensatory mechanisms are more robust in
Young Females than in Aged Females. Our data also highlight the inflammatory component in Cp-induced AKI with the levels of plasma TNF-α following the same pattern of PCr levels. It is important to note that renal parenchymal cells produce TNF-α in Cp-induced nephrotoxicity (87, 112). Elevated levels of pro-inflammatory cytokines might enhance recruitment of immune cells to the site of injury and further amplify inflammation and injury. The elevation of plasma TNFα, IL-6 and IL-12p70 seen in Aged Female at day 1 are suggestive of an earlier, enhanced pro-inflammatory state in these mice, which may be insufficiently counterbalanced by the induction of IL-10 at this time point. The lack of response in IL-2 levels in Aged mice, while Young mice demonstrated a robust increase, may suggest an alteration in the role of T cells in this model with aging. Collectively, these data suggest a confluence of deleterious outcomes which may underlie the extreme mortality seen in Aged Female.

Sex- and age-based differences in the renal handling of drugs such as Cp have been shown to lead to alterations in pharmacokinetics (53), but whether these distinctions underlie the protection seen in Young Females that is lost in Aged Females is unknown. Our findings show that MATE2 protein expression is significantly higher in Males compared to Females (Young or Aged) at baseline, and with Cp-induced AKI. Our data on the expression of MATE2 do not support a role for this transporter in the dichotomy of responses between the Young and Aged Females, since low levels of MATE2 that are unchanged with injury, are evident in both groups of Females. The lower expression level in females of both ages may indicate that Cp is retained in the proximal tubule for longer duration than in males. However, these data may also be consistent with a role for another luminal transporter in the proximal tubule in the extrusion of Cp in females. It is important to note that we were not able to evaluate levels of OCT2 on the basolateral membrane, described as the primary transporter into proximal tubule cells, or MATE1 on the luminal membrane due to a lack of suitable antibodies being available for Western blotting or for immunostaining. Since the exposure of proximal tubule to Cp is largely derived by transport from peritubular capillaries rather than filtration through the glomerulus,
determining the level of the OCT2 or other similar transporter would be helpful to further understand these distinctions in the flux of Cp across the epithelium. Nonetheless, the decline in the mean expression of MATE2 in both Male groups may suggest that failure in this clearance mechanism may contribute to increasing exposure to Cp in these male mice and result in the occurrence of indicators of renal functional decline at later times in these mice.

HO-1 induction has been shown to be a protective response against AKI and has multifactorial and essential functions in animal models. As recently reviewed by Nath (72), HO-1 has effects within kidney cells as well as in other non-renal cell populations and functions in many pathways. A role for HO-1 in modulating the balance between pro- and anti-inflammatory mechanisms is also known to affect susceptibility to AKI (46, 88). HO-1 is expressed in tubular epithelia, endothelial cells, dendritic cells, and macrophages in the kidney, all of which are important in the manifestation and resolution of AKI (4, 12, 46, 91, 111). The effect of aging on HO-1 expression and effects in AKI is still unclear. The biology of HO-1 is complex due to its expression as different forms, which can be detected in tissues and cells by Western blot analysis as bands of ~32kDa and ~28kDa, as well as in serum as a recently described band at ~16kDa (108). The two immunoreactive bands of HO-1 detected in western blot analysis of kidney lysates using different primary antibodies as well as additional preliminary sequencing data in our laboratory has confirmed the identity of these two species as HO-1 (data not shown). It is important to note that the appearance of the truncated HO-1 band at ~28kD may be a doublet of two independent bands one day after Cp injection, but the resolution of these species was not possible under the conditions used in this study. Future studies are needed to explore this phenomenon and its role in AKI.

HO-1 has been shown to localize to endoplasmic reticulum, nuclei, and mitochondria, which may reflect the different intracellular functions of HO-1 (7, 8, 11, 18, 22, 25, 33, 47, 65). Importantly, age and/or sex may alter the localization of HO-1 intracellularly and extracellularly and thereby affect the biological activity of HO-1 (43, 44, 48, 66, 74, 85, 95). In this study, we
showed that the complex pattern of expression of HO-1 is altered differently in females than in males after Cp-induced AKI and may contribute to the unique protection seen in young females. The enzymatic activity of HO-1, thought to be found only or largely in the membrane-bound full-length (~32kDa) form, detoxifies heme moieties released from heme-containing proteins during cell death (96). In addition, the elaboration of the enzymatic products, CO and biliverdin, contributes to antioxidant properties within cells (40). Importantly, the greatest modulation in protein expression patterns occurs with the truncated, ~28kDa band. The lack of significant changes in females of either age and the more modest changes in males in the full-length ~32kDa band are consistent with no statistically relevant changes in HO enzyme activity in conferring protection in young females relative to others in this model of AKI.

In the Cp model of AKI, autophagy appears to be more markedly deficient in aged relative to young as has been reported previously and reviewed by Kroemer et al. (59). However, the accumulation of p62, as a marker of autophagy, seems to parallel the degree of renal injury in this model in that aged animals show early elevation of p62 and young males show higher levels than young females at day 3 when the greatest loss of renal function is evident in this model. These data suggest that young females may utilize autophagy more efficiently than other sex/age groups to repair the tubular damage leading to a more robust response to AKI and preservation of renal function despite this epithelial damage. Changes in the other proteins in autophagy did not parallel the unique functional preservation in young females. However, it is important to note that Beclin1 plays a role in apoptosis (55) and its modulation in aged animals may reflect both processes. Likewise, comparable changes seen in Atg5 across the studied groups indicate that autophagy is active in this model, but given the duality of roles of Atg5 in both autophagy and apoptosis (17), expression or levels of this protein may not reflect the distinctive functional protection seen in young females in this model of AKI. The differences in the ratio of LC3 species seen in aging again confirms the decreased flux in
autophagy seen in aging and does not provide insight into mechanisms that distinguish the unique young female biology in AKI.

In summary, our data demonstrate a pattern of injury and protection in response to AKI in females that differs from that in males, and which appears to be altered in aging. The dichotomy of response in young females vs. other sex-age groups in functional preservation while tissue injury is present, robust utilization of HO-1 induction and autophagy, as well as the cytokine profile that appears to be more anti- vs. pro-inflammatory, may all work in concert to lead to decreased susceptibility or enhanced recovery from AKI in young females. These findings will be beneficial for developing future studies to rigorously confirm the significance of age and sex and the role of HO-1 in mediating protection from AKI, and identify the underlying mechanisms of the unique protection observed in young females.
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Disclosure

No conflicts of interest, financial or otherwise, are declared by the authors.
References


Figure Legends

Figure 1. Age and sex influence renal function in Cp-induced Acute Kidney Injury (AKI).

(A) Plasma creatinine (PCr) in Cisplatin (Cp)-induced AKI (20mg/kg) is shown for Young and Aged, Males and Females. Data points are shown with horizontal lines representing the Group mean±SEM; n=5-17 mice/group. Significant differences (p≤0.05) between groups are shown as *: different from Day 0/ saline; #: different from other Cp time point; %: different across groups.

(B) Survival curve (% survival) of the mice in different age/sex groups 1, 2 and 3 days after Cp injection is represented in the Kaplan Meier curve. Log-rank (Mantel-Cox) test indicated that the survival curves are significantly different (p=0.0002); n=28-34 mice per group. (C) Comparison of weight loss over 3 days post-Cp/saline injections in different age- and sex- groups is shown; n= 5-9 mice for each sex/age saline group and n=2-16 mice for each sex/age Cp group.

Figure 2. Age and sex influence bio-markers of renal injury after Cp-induced Acute Kidney Injury (AKI).

Western blots on total kidney lysates for (A) kidney injury molecule 1 (KIM-1) and (B) insulin-like growth factor-binding protein 7 (IGFBP7) are shown with the Ponceau S stained blots. Densitometric values (arbitrary units) were normalized to Ponceau S staining of the membrane and represented in the graphs for (C) KIM-1 and (D) IGFBP7. Group mean values and standard errors are shown; n= 3-5 mice/group. (E) Mouse plasma NGAL (Neutrophil Gelatinase-Associated Lipocalin) levels (µg/mL) determined by ELISA are shown. Values indicate Group mean±SEM; n= 4-6 mice/group. (F) Result of Western blot on plasma samples for NGAL performed using equal volumes (2uL) of plasma for each mouse is shown. In all graphs, significant differences (p<0.05) between groups are shown as *: different from saline; #: different from other (cisplatin) Cp time point; %: different across groups. All the densitometry was performed using Image J.
Figure 3. Plasma levels of inflammatory cytokines in Cp-induced AKI. (A-F) Plasma cytokine levels, measured as picograms / ml (pg/mL), are depicted in the graph. Values indicate Group mean±SEM; n= 5-8 mice/group. Significant differences (p<0.05) between groups are shown as *: different from saline; #: different from other Cp time point; %: different across groups. TNF-α: tumor necrosis factor-alpha; IFN-ɣ: interferon-gamma; KC-GRO: Keratinocyte chemoattractant-growth-regulated oncogene or chemokine C-X-C motif ligand 1; IL: interleukin.

Figure 4. Structural morphology after Cp-induced AKI. (A) Representative images of Periodic acid–Schiff (PAS) stained kidney sections from Young (16-17 weeks) and Aged (16-17 months), male and female C57BL/6J mice injected with saline, or 1 day or 3 days after Cp (20mg/kg) injections are shown. Arrows=dilated tubules; Bold Arrow heads=tubular casts. Scale bar= 200µm. (B) Quantification using a semi quantitative scale (0-4) of indices of renal injury as seen as loss of brush border, expanded tubule lumen and cast formation in renal tubules is shown in the graph. n=3-9 mice/group. Significant differences (p<0.05) between groups are shown as *: different from saline; #: different from other Cp time point.

Figure 5. Heme oxygenase-1 (HO-1) levels in Cp-induced AKI. (A) Representative Western blot of HO-1 on kidney tissues with the Ponceau S stained blot used for normalization of the data is shown. (B, C) Densitometric values (arbitrary units) for ~28kDa, and (D, E) ~32kDa bands normalized to Ponceau S staining of the membrane are represented in the graphs. Group mean±SEM are shown; n= 3-6 mice/group. (F) Heme oxygenase (HO) activity levels, measured as picomoles (pmol) bilirubin formed/mg protein/hour, are depicted in the graph. Values indicate group mean±SEM; n= 2-6 mice/group. In all graphs, significant differences (p<0.05) between groups are shown as *: different from saline; #: different from other Cp time point; %: different across groups. Densitometry was performed using Image J.
Figure 6. **Western blot analyses of indicators of autophagy demonstrate sex and age distinctions.** (A) Representative Western blots of proteins involved in the autophagy pathway of p62, Beclin1 (BECN1), Atg-5 and LC3 (I, ~18kD, and II, ~16kD) with Ponceau S stained blot used for normalization of the data are shown. (B-F) Densitometric values (arbitrary units) normalized to Ponceau S are represented as graphs for the blots of p62, beclin1 (BECN1), Atg-5 and LC3 ratio (LC3II/LC3I). Data indicate Group mean±SEM; n=3-9 mice/group. Significant differences (p<0.05) between the groups are shown as *: different from saline; #: different from other Cp time point; %: different across groups. Densitometry was performed using Image J.

Figure 7. **Cell death and proliferation in Cp-induced AKI.** (A) Representative images of kidney sections in the TUNEL assay from Young (16-17 weeks) and Aged (16-17 months), male and female C57BL/6J mice injected with saline or 3 days after Cp (20mg/kg) injections are shown. Black arrow heads represent TUNEL positive nuclei. Scale bar represents 100 µm. (C) Representative images of kidney sections immunostained with Ki67 antibody from Young (16-17 weeks) and Aged (16-17 months), male and female C57BL/6J mice injected with saline or 3 days after Cp (20mg/kg) injections are shown. Red arrow heads represent Ki67 positive nuclei. Scale bar represents 200 µm. (B, D) Quantification of TUNEL- and Ki67-positive cells, respectively, are shown in the graphs. For TUNEL, counts were obtained for each animal within the outer medulla of one tissue section and Group mean±SEM were then calculated and analyzed (n=3 mice/Group). For Ki67 staining, representative images (3-5 fields/kidney) were obtained and stained nuclei were quantified to produce a mean value for each animal. Group mean±SEM were then calculated and analyzed (3 animals/group, Aged Female; 6 animals/group, all other groups). Significant differences (p<0.05) between groups are shown as *: different from saline; #: different from other Cp time point; %: different across groups.

Figure 8. **Expression of a drug transporter (MATE2) in Cp-induced AKI.** (A) Western blots on total kidney lysates for Multidrug and Toxin Extrusion Protein (MATE2) with the Ponceau S
stained blots are shown. (B) Densitometric values (arbitrary units) normalized to Ponceau S are represented as graphs for the blots. Group mean±SEM are shown; n=3-5 mice/group. Significant differences (p<0.05) between groups are shown as *: different from saline; #: different from other Cp time point; %: different across groups. Densitometry was performed using Image J.
Figure 3
Figure 6

(A) Western blot analysis showing the expression levels of p62, BECN1, ATG-5, and LC3 in young and aged females and males treated with Sal, Cpd1, and Cpd3. The molecular masses are indicated as 62 kDa, 60 kDa, 55 kDa, 18 kDa, and 16 kDa.

(B) Graphs showing the quantification of p62 levels in young and aged females and males treated with Sal, Cpd1, and Cpd3.

(C) Graphs showing the quantification of Beclin-1 levels in young and aged females treated with Sal, Cpd1, and Cpd3.

(D) Graphs showing the quantification of Beclin-1 levels in young and aged males treated with Sal, Cpd1, and Cpd3.

(E) Graphs showing the quantification of Atg-5 levels in young and aged females treated with Sal, Cpd1, and Cpd3.

(F) Graphs showing the quantification of LC3II/LC3I levels in young and aged females treated with Sal, Cpd1, and Cpd3.
Figure 7
Figure 8