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Cold Storage Increases Albumin and Advanced Glycation-End Product-Albumin Levels in Kidney Transplants: A Possible Cause for Exacerbated Renal Damage

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Background. Prolonged cold storage (CS) of kidneys is associated with poor renal outcome after transplantation (Tx). We recently showed that in rats (Lewis), proteasome and renal function were severely compromised in kidney transplants subjected to CS (CS/Tx) as compared with those without CS exposure (autotransplanted [ATx]). **Methods.** Evaluation of whole-kidney extracts from our rat kidney transplant model showed a subset of proteins induced after CS/Tx when compared with ATx or sham groups; this study examined those proteins using mass spectrometry, western blotting, immunoprecipitation, and immunohistochemistry. **Results.** Mass spectrometry identified basal albumin levels in sham kidney extracts; western blots and immunohistochemistry confirmed this. Western blotting showed exceptionally higher albumin levels in both soluble and insoluble fractions of CS/Tx renal extracts when compared with ATx and sham groups. Surprisingly, levels of advanced glycation-end products (AGE) were higher in CS/Tx renal extracts. Furthermore, immunoprecipitation of albumin followed by western blotting for AGE revealed AGE-albumin in all 3 extracts; its levels were highest in CS/Tx extracts. Immunohistochemistry analysis of kidney sections revealed higher albumin or AGE levels in the CS/Tx group, and the protein was detected all over (within glomeruli, and intratubular and extratubular compartments) when compared with ATx and sham groups, which show confinement of these proteins to the extratubular compartment and within glomeruli. As expected, kidneys of the ATx group showed evidence of more macrophages, which was exacerbated in the CS/Tx group. **Conclusions.** These results suggested that CS/Tx increased AGE-albumin, which was correlated with increased inflammation and renal damage.

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Of necessity, kidneys to be transplanted which are procured from deceased donors must be placed in cold storage (CS) in the time between organ harvest and organ transplant. However, because prolonged CS of kidneys is detrimental to graft function,¹⁻⁹ >20% of kidneys must be discarded due to prolonged CS. Furthermore, our recent reports suggested that in rat models of transplantation (Tx), renal damage was more extensive in CS-exposed kidney transplants (CS/Tx) than transplants without CS (autotransplanted

[ATx]),^{10,11} highlighting the importance of preventing triggers of damage arising during CS. Our study also revealed compromised mitochondrial and proteasome function and disrupted mitochondrial protein homeostasis following CS/Tx.¹² Understanding the mechanisms that result in CS-mediated kidney damage would facilitate the development of new strategies and techniques to protect kidney tissues from damage and improve graft function and patient outcomes. These mechanisms currently are poorly understood. The goal of our

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laboratory is to elucidate the causes of CS-induced renal damage after transplant, so that new therapies can be designed to improve outcomes.

Albumin is a major plasma protein synthesized mainly by the liver and is responsible for oncotic pressure of the blood as well as a major source of carrier proteins. The presence of albumin in urine reflects the balance between glomerular filtration function and tubular reabsorption. Lowered serum albumin level is associated with decline in kidney function and has been described as an independent risk factor of mortality in different kidney diseases, including renal Tx.^{13,14} Similarly, posttranslational modification of serum albumin has been described in patients with chronic kidney disease, diabetes, and chronic liver disease,¹⁵ and some of these posttranslational modifications may cause unfavorable outcomes in these different diseases.^{16,17} No studies, to our knowledge, have considered albumin as a contributor to renal injury, including that which occurs as a result of CS and subsequent Tx (CS/Tx). Evaluation of whole-kidney extracts from our rat kidney transplant model showed a subset of proteins (~70 kDa) induced after CS/Tx when compared with ATx or sham groups; we hypothesized that albumin is one of those proteins. Here, we demonstrate, for the first time, that albumin levels (detected as advanced glycation-end products [AGE]-albumin) were higher in transplanted kidneys that first underwent CS than in those that did not, and this increased AGE-albumin correlates with poor renal outcome.

MATERIALS AND METHODS

Animals

Male Lewis rats (200–250 g) were used as transplant donors and recipients. All animal protocols were approved by the Institutional Animal Care and Use Committee, at the University of Arkansas for Medical Sciences (UAMS), and all animal experiments described below were performed in compliance with the Institutional Animal Care and Use Committee at UAMS using National Institute of Health guidelines. Rats were housed 3 per cage before surgery; after surgery, they were placed in individual cages in a climate-controlled room with a 12-hour artificial light/dark cycle. Rats were euthanized by exsanguination via withdrawal of blood from the posterior vena cava followed by ligation of the aorta while under isoflurane anesthesia (5% induction and 2% during procedure or surgery). Based on a power analysis of our preliminary CS/Tx data, a sample of 4 animals is needed for each group to detect a 20% change in albumin expression (from sham animals) with a power = 0.80, $\alpha = 0.05$, and $\beta = 0.20$. The research team used an unbiased approach of blinded analysis when possible. Rat surgeries were performed under isoflurane anesthesia and surviving rats were given subcutaneous buprenorphine (0.06 mg/kg) for pain.

Animal Surgeries

Orthotopic Renal Transplant Surgery

Surgeries were performed as previously described.^{10,11} For donor surgeries, the left and right kidneys were isolated and then flushed with and stored in University of Wisconsin solution at 4°C for 18 hours; the right kidneys of donor rats comprised the CS group (n = 9). For recipient surgeries, the native left kidney was removed, and the donor left kidney (exposed to CS) was flushed with cold saline and then transplanted by

end-to-end anastomosis as described;^{10,11} surgical ischemia time was <45 minutes. The native right kidney was immediately removed so that renal function was dependent upon the transplanted left kidney. The ureter was anastomosed end to end over a 5-mm PE-50 polyethylene stent. Postoperatively, animals were given 0.9% (w/v) NaCl in the abdominal cavity and placed under a heating lamp to recover from the anesthesia. After 24 hours of reperfusion, the transplanted left kidney and blood were collected under anesthesia and saved as the 18-hour CS/Tx group (n = 9). The rat survival rate was >95% for the CS/Tx groups 1 day posttransplant. For all groups, immediately after being harvested, kidneys were processed for high-resolution respirometry studies or were flash frozen for biochemistry assays and western blots.

Autotransplant Surgery

The ATx surgery was included in these studies so that the impact of CS could be isolated from the impact of transplant surgery alone. ATx was performed as described for orthotopic renal transplant surgeries, except that the left kidney was removed, flushed with saline, and immediately transplanted back into the same rat without CS exposure, followed by right nephrectomy.^{10,11} After 24 hours, the transplanted kidney was harvested under anesthesia; these kidneys comprised the ATx group (n = 9).

Sham Surgery

Rats underwent right nephrectomy with no renal Tx (sham operation); the removed right kidneys were saved as controls (n = 9). Twenty-four hours later, the left kidneys and blood from these rats were harvested and saved as the sham group (n = 9). Control kidneys were compared with CS kidneys, because both groups were harvested from healthy rats. Sham kidneys served as controls for both transplant models (ATx and CS/Tx) because all underwent a nephrectomy (removal of right kidney).

Organ/Sample Collection

One day following the surgery, kidneys, blood, and urine were collected under anesthesia. Kidneys were immediately snap frozen and saved at -80°C until further use. For serum collection, blood was allowed to clot on ice for 1 hour. The blood was then centrifuged (5000g) at 4°C for 10 minutes to remove the clot, and the serum (supernatant) was aliquoted and saved at -80°C until further use. Spot urine from bladder was collected and centrifuged (5000g) at 4°C for 10 minutes to remove debris, and the supernatant (urine) was saved at -80°C until further use.

Renal Extract Preparation

Renal extracts from whole kidney homogenates were prepared as described¹² for protein analyses with western blots, periodic acid-Schiff (PAS) reactions, and liquid chromatography mass spectrometry. Briefly, kidney homogenates were lysed with 1% Triton lysis buffer (50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5; 1% Triton X-100; 1 mM ethylenediaminetetraacetic acid; 2 mM EDTA; 12.5 mM glycerophosphate; 3.2 mM MgCl₂; 10% glycerol; 1 mM phenylmethylsulfonyl fluoride; 1 mM dithiothreitol; 1.2 mM Na₃VO₄; 2.5 mM NaF; all from Sigma, MO, USA) and protease inhibitor cocktail (Pierce, WI)¹⁸ for 30 minutes at 4°C on a rocking platform. Lysates

were centrifuged (16000g) at 4°C for 20 minutes and the supernatant was saved as the soluble fraction. The pellet was resuspended with urea-sodium dodecyl sulfate (SDS)-Triton lysis buffer (urea, 8 M; SDS, 3%; Triton, 1%) and was then put through 3 freeze-thaw cycles, followed by sonication on ice (3 cycles of 10-s sonication, 10-s pause). The sonicated extract was centrifuged at 4°C for 20 minutes, and the resulting supernatant was collected as the insoluble fraction. To obtain the total whole cell protein extract, the renal tissue homogenates were boiled with lysis buffer (8 M urea, 3% SDS, and 1% radioimmunoprecipitation assay) and centrifuged (16000g for 20 min at 4°C). Protein concentrations were determined with the bicinchoninic acid Protein Assay kit (Pierce). Renal tissue extracts were prepared for immunoprecipitation as described previously.¹⁹ Briefly, tissue extracts were prepared from whole kidney homogenates with 1% Triton lysis buffer (1 part tissue homogenate and 1 part of 2X lysis buffer) for 30 minutes at 4°C on a rocking platform. Lysates were centrifuged (16000g) at 4°C for 20 minutes to remove insoluble fractions and were precleared with Protein A/G agarose (Pierce) for 30 minutes.

Western Blot Analysis of Renal Tissue Extract, Serum, and Urine

Renal extract proteins (total protein, soluble, and insoluble fractions, 30 µg each) were resolved with SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to polyvinylidene fluoride (PVDF) membrane. Western blot analysis was performed with antibodies against albumin (1:1000; Abcam, MA, #ab207327, Rabbit monoclonal #EPR20195), AGE (1:1000; Abcam, Catalog# ab23722, Rabbit polyclonal), and β-actin (loading control, 1:1000; Sigma, #A5441, mouse monoclonal # AC-15). Serum was diluted 1:10 in phosphate buffered saline and protein concentration was measured using bicinchoninic acid reagent. For serum and urine samples evaluation, 50 µg of diluted serum sample or 10 µL of urine (spot collection from bladder) supernatant samples were resolved with SDS-PAGE and then transferred to PVDF membrane. Western blot analysis was performed with antibody against albumin (1:1000; Abcam, #ab207327, Rabbit monoclonal #EPR20195). Probed membranes were washed 3 times, and immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse-HRP, Cat# 074-1806; goat anti-rabbit-HRP, Cat# 5220-0337[074-1516]; Seracare KPL, MD) and enhanced chemiluminescence (Thermo Scientific Pierce, MA). Densitometry evaluation of scanned membrane was performed with AlphaEase FC software.

Immunoprecipitation Studies

Immunoprecipitation on renal tissue extracts was performed as described previously.¹⁹ Briefly, precleared supernatants (200 µg) were incubated overnight with albumin-specific antibody (1:200, Abcam). To bind immune complexes, Protein A/G agarose (Pierce) was added and incubated at 4°C for 4 hours. Agarose beads were collected by centrifugation. Pellets were washed 3 times with lysis buffer, resuspended in SDS sample loading buffer, and boiled before SDS-PAGE analysis. Proteins were separated by SDS-PAGE on 4%–12% gradient gels and transferred to PVDF membrane. Western blot analysis was performed with α-AGE (1:1000; Abcam, MA, Catalog#

ab23722, Rabbit polyclonal) and α-albumin antibodies. Probed membranes were washed 3 times, and immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Seracare KPL) and enhanced chemiluminescence (Thermo Scientific). Densitometry evaluation of scanned membranes was performed with AlphaEase FC software.

Periodic Acid-Schiff Reactions

Renal extract proteins (soluble and insoluble fractions, 30 µg each) were resolved with SDS-PAGE and then transferred to PVDF membrane. The membrane was processed for detection of protein glycation using the PAS reaction according to standard procedures. Briefly, the transferred membrane was incubated with periodic acid for 5 minutes at room temperature, followed by washing 3 times with deionized water. The membrane was then incubated with Schiff's reagent for 15 minutes at room temperature. Finally, the membrane was washed 3 times with deionized water and imaged using normal camera.

Liquid Chromatography Mass Spectrometry and Bioinformatics Identification of Proteins

The SDS-PAGE gel lane for each sample (n=3) was cut between ~55 and 90 kDa and excised slices were subjected to in-gel trypsin digestion as follows. Gel slices were destained in 50% methanol (Fisher Scientific, PA) and 100 mM ammonium bicarbonate (Sigma), followed by reduction in 10 mM Tris [2-carboxyethyl]phosphine (Pierce) and alkylation in 50 mM iodoacetamide (Sigma). Gel slices were then dehydrated in acetonitrile (Fisher Scientific), followed by addition of 100 ng porcine sequencing grade-modified trypsin (Promega, WI) in 100 mM ammonium bicarbonate (Sigma), and incubated at 37°C for 12–16 hours.

Peptide products were acidified in 0.1% formic acid (Pierce). Tryptic peptides were separated with reverse-phase Jupiter Proteo resin (Phenomenex, CA) on a 150 × 0.075 mm² column with a nanoAcquity UPLC system (Waters, MA). Peptides were eluted over a 30-minute gradient from 97:3 to 67:33 ratio of buffer A:B (buffer A: 0.1% formic acid, 0.5% acetonitrile; buffer B: 0.1% formic acid, 99.9% acetonitrile). Eluted peptides were ionized by electrospray (2.25 kV), followed by MS/MS analysis with higher-energy collisional dissociation on an Orbitrap Fusion Tribrid mass spectrometer (ThermoScientific) in top-seed data-dependent mode.

MS data were acquired with the Fourier Transform Mass Spectrometry analyzer in profile mode at a resolution of 240000 over a range of 375–1500 m/z. Following higher-energy collisional dissociation activation, MS/MS data were acquired with the ion trap analyzer in centroid mode and normal mass range with precursor mass-dependent normalized collision energy between 28.0 and 31.0. Proteins were identified through a database search with Mascot (Matrix Science, version 2.5.1) against the UniprotKB database restricted to *Rattus norvegicus* (36170 entries), a fixed modification of carbamidomethyl on C, variable modification of oxidation on M, a parent ion tolerance of 3 ppm, and a fragment ion tolerance of 0.5 Da. Scaffold (Proteome Software) was used to verify MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established with <1.0% false discovery (Scaffold Local FDR algorithm). Protein identifications were accepted if they could be

established with <1.0% false discovery and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.²⁰ Total spectral counts were exported from Scaffold and transformed to log₂ normalized spectral abundance factor values²¹ for analysis by ANOVA and Student's *t*-test.

Immunohistochemical Analysis of Tissue Sections

Two cross sections (4–5- μ m thickness) from each paraffin block were mounted on each glass slide (Fisherbrand Superfrost Plus; Fisher Scientific) and deparaffinized with xylene and a series of graded ethanol washes. The sections were further processed as described below.

For immunohistochemical analysis, antigens were retrieved by heating sections in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes. Endogenous peroxidase was quenched by incubating the sections with Peroxidase Suppressor (Thermo Scientific) for 15 minutes at room temperature. The slides were blocked with Non-Serum Protein Block (Dako) for 20 minutes at room temperature. Primary antibodies were prepared in antibody diluent solution (0.5% nonfat dry milk and 1% bovine serum albumin in tris buffered saline) and incubated overnight at 4°C. Antialbumin was diluted 1:500 (Abcam), anti-CD68 was diluted 1:200 (Abcam), and anti-AGE was

diluted 1:600. Immunoreactivity was detected with Envision+ System-HRP (Dako). Counterstaining was performed with Mayer's hematoxylin (Electron Microscopy Science, Hatfield, PA), and bluing was carried out by dipping in 0.125% ammonia blue solution. Finally, the slide-mounted sections were dehydrated, covered with Cytoseal-60 (Electron Microscopy Science), and mounted with a cover slip. All images were taken on Nikon Eclipse E800 microscope with Q Capture imaging and Nikon Elements software. Albumin staining was semiquantitatively evaluated based on the percentage of positive tubules in 10 high-power (400 \times) fields from cortex and medulla with the following scores: 0, null/negative; 1, <10% positivity; 2, 10%–50% positivity; 3, >50% positivity.

Transferase-mediated dUTP Nick-end Labeling Assay

For visualization of apoptotic and necrotic cells in situ terminal transferase-mediated dUTP nick-end labeling (TUNEL) method was utilized according to the protocol provided by the manufacturer (TACS TdT Kit, R&D Systems, MN). Counterstaining was performed using Gill's hematoxylin.

Statistical Analysis

Results are presented as mean \pm standard error of the mean (SEM) with GraphPad Prism software. Data were analyzed with

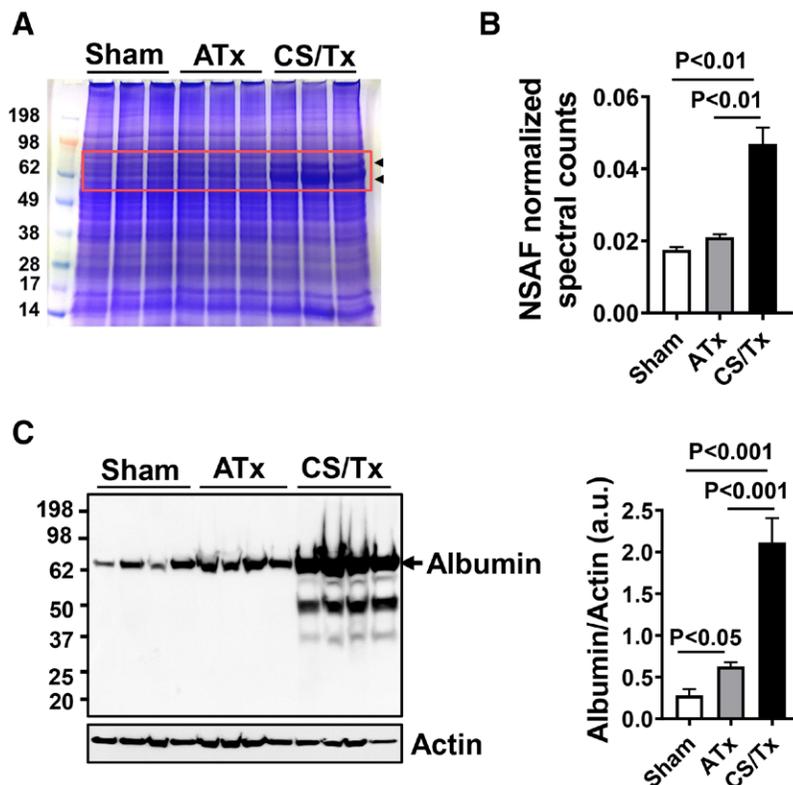


FIGURE 1. Total protein expression in kidneys is altered and the renal albumin level is increased after CS/Tx. Three experimental groups were considered: sham (right nephrectomy), 18-h CS followed by Tx (CS/Tx), and ATx (ie, Tx with no CS); in all groups, surgical procedures were followed by 1 day reperfusion; sham-operated rats were used as controls. A, Proteins of renal extracts (30 μ g) prepared from kidney homogenates were resolved with SDS-PAGE and visualized with Coomassie staining. B, Proteins in the indicated region (red box) of the gel in (A) were trypsin-digested and evaluated with LC-MS/MS, followed by normalized spectral count (NSAF). Values are expressed as mean \pm SEM (bars, $n=3$). Differences between the means of the groups were compared with one-way ANOVA followed by Tukey's post hoc test for multiple group comparisons; *P* value ($P<0.05$) indicates means are significantly different between sham and CS/Tx or between ATx and CS/Tx groups. C, Western blot analysis of total albumin in extracts (SDS/urea/Triton) of kidneys after sham, ATx or CS/Tx. β -actin was used as a loading control. A representative blot from 3 repeated experiments is shown, and the densitometry values are expressed as mean \pm SEM (bars, $n=4$). Differences between the means of the groups were compared with one-way ANOVA; *P* value indicates means are significantly different ($P<0.05$), when comparing CS/Tx to sham or ATx. ATx, autotransplantation; CS, cold storage; LC-MS/MS, liquid chromatography mass spectrometry; NSAF, normalized spectral abundance factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean; Tx, transplantation.

a one-way ANOVA followed by Tukey's post hoc test for multiple group comparisons, or an unpaired Student's *t*-test was used when comparing differences between the mean of 2 groups (eg, control vs CS) at a 95% level of confidence. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Albumin detected in kidneys was increased and altered after CS/Tx. Renal extract proteins were resolved with SDS-PAGE and stained with Coomassie. Remarkably, renal extracts from the CS/Tx group showed profound increases in higher molecular weight proteins (red box area, ~55–90 kDa range) when compared with the sham (control) and ATx groups (Figure 1A). Proteins within this region (red box area) were trypsin-digested and analyzed with mass spectrometry (see Methods). Albumin was detected in all 3 groups, and increased spectral counts were observed in the CS/Tx group (Figure 1B). Consistent with this, western blots of total protein extracted from renal homogenate showed significantly increased albumin levels in the CS/Tx group (Figure 1C).

CS/Tx shifts albumin to the insoluble fraction. We recently demonstrated that proteasome function is compromised and the key mitochondrial proteins involved in respiration are altered and shifted to the detergent-insoluble fraction after CS/Tx.¹² Here, we attempted to evaluate the effects of CS/Tx on the solubility of albumin as an indicator of its alteration. We solubilized kidney homogenates with 1% Triton X-100 to extract the detergent soluble and insoluble fractions. Western blot was used to evaluate the levels of albumin in the detergent-soluble and -insoluble fractions of the renal homogenates. ATx group showed increased albumin in the detergent-soluble fraction when compared with sham group (Figure 2A). Consistent with western blots of total albumin level, detergent-soluble (Figure 2A) and -insoluble (Figure 2B) fractions of renal homogenates indicated higher albumin levels in the CS/Tx group than in the sham or ATx groups.

CS/Tx exacerbates modification of albumin in kidney. PAS is a staining method used to detect polysaccharides or glycoproteins. We attempted to evaluate glycation of renal proteins after Tx. Proteins of renal extracts were resolved with SDS-PAGE, transferred to a membrane, and analyzed for protein glycation via PAS reaction (as described in Methods). Basal PAS-reactive proteins (~50–250 kDa) were detected in all 3 groups (arrow/arrow head); however, the CS/Tx group showed induction of most of these PAS reactive bands within this region (Figure 3A, arrows), suggesting that these renal proteins are modified with carbohydrate (possibly glycated) after CS/Tx. Interestingly, a glycoprotein band within ~50–75 kDa (arrow head) was prominently induced after CS/Tx. Western blotting of renal extracts detected AGE, and a prominent band was detected at ~70 kDa in all 3 groups (arrow head), with highest levels in the CS/Tx group (Figure 3B). Because the molecular weight of albumin is 69 kDa, we speculated that the prominent AGE band could be albumin. The membrane was stripped and probed for albumin. Interestingly, the prominent AGE-reactive band (arrow head) overlays with albumin (data not shown). To confirm this, albumin was immunoprecipitated from the renal extracts, and western blotting revealed that the immunoprecipitated albumin reacted with the AGE antibody, which was at the highest levels in the CS/Tx group (Figure 4A).

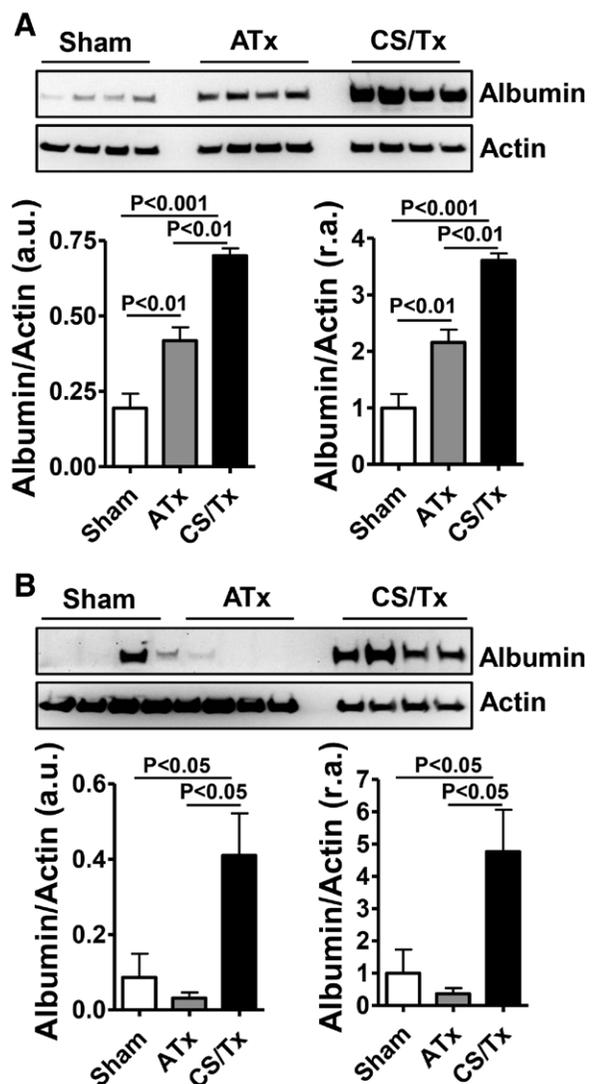


FIGURE 2. The albumin level is altered in kidneys after CS/Tx. Western blot analysis of albumin in 1% Triton soluble (A) and insoluble (B) fractions from kidney tissue extracts after sham (right nephrectomy), ATx (transplant with no CS) or CS/Tx (18-h CS/Tx); β -actin was used as a loading control. A representative blot from 3 repeated experiments is shown, and the densitometry values are expressed as mean \pm SEM (bars, $n=4$). Differences between the means of the groups were compared with one-way ANOVA; P value indicates means are significantly different ($P < 0.05$), when comparing CS/Tx to sham or ATx. ATx, autotransplantation; CS, cold storage; SEM, standard error of the mean; Tx, transplantation.

Increased Levels of Albumin in Urine

Because CS/Tx renal sections showed increased abundance of albumin, specifically within renal tubules and in the lumen, we attempted to examine the levels of albumin in urine samples (10 μ L) from rats. Indeed, western blot detected increased levels of albumin in the urine collected from the CS/Tx group when compared to sham or ATx groups (Figure 5). Interestingly, we did not detect any change in albumin levels in rat serum (Figure 6).

Exacerbated Increases in Albumin and AGE in Kidney Tubules After Prolonged CS/Tx

In kidney tissue sections from the sham group, immunohistochemistry detected basal levels of albumin (brown

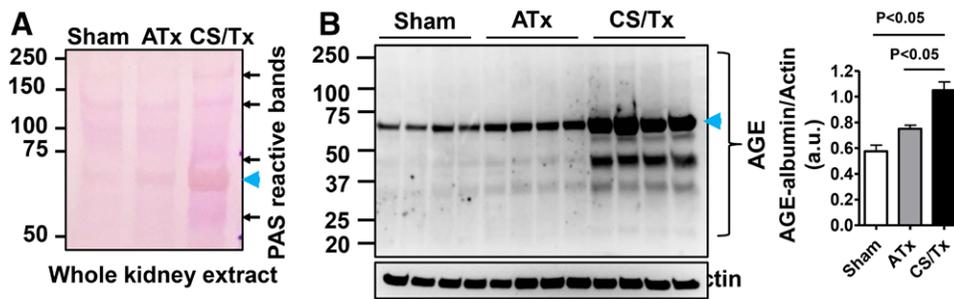


FIGURE 3. Kidney tissues from the CS/Tx group have altered albumin and AGE levels. Thirty micrograms of renal extracts from sham (right nephrectomy), ATx (transplant with no CS), or CS/Tx (18-h CS/Tx) groups were resolved with SDS-PAGE, and proteins were transferred to a PVDF membrane. A, The membrane was then processed for PAS reaction (see Methods). Arrows/arrow head indicate PAS reactive bands (glycoproteins) and arrowhead indicate possible albumin band. B, The membrane was used for western blotting with antibody against AGE (bands detected with chemiluminescence). β -actin was used as loading control. A representative blot from 3 repeated experiments is shown, and the densitometry values are expressed as mean \pm SEM (bars, $n=4$). Differences between the means of the groups were compared with one-way ANOVA; P value indicates means are significantly different ($P<0.05$), when comparing CS/Tx to sham or ATx. AGE, advanced glycation-end product; ATx, autotransplantation; CS, cold storage; PAS, periodic acid-Schiff; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean; Tx, transplantation.

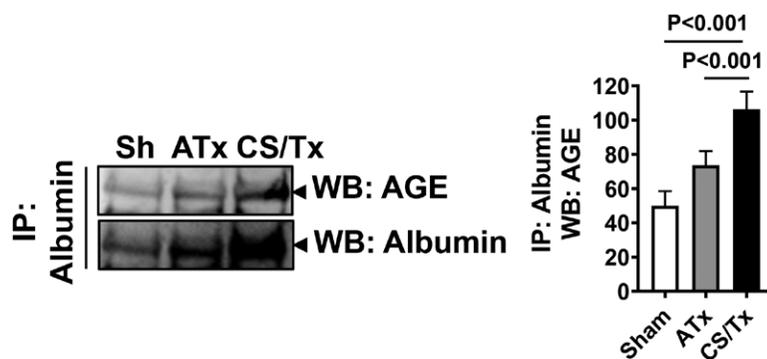


FIGURE 4. Increased AGE-albumin after CS/Tx. Albumin was immunoprecipitated from renal extracts of sham (right nephrectomy), ATx (transplant with no CS), and CS/Tx (18-h CS/Tx) groups ($n=3$ per group), and the product was analyzed by western blotting with AGE (upper panel) or albumin (lower panel) antibody. Densitometry values are expressed as mean \pm SEM ($n=3$). Differences between the means of the groups were compared with one-way ANOVA; P value indicates means are significantly different ($P<0.05$), when comparing CS/Tx to sham or ATx. AGE, advanced glycation-end product; ATx, autotransplantation; CS, cold storage; SEM, standard error of the mean; Tx, transplantation.

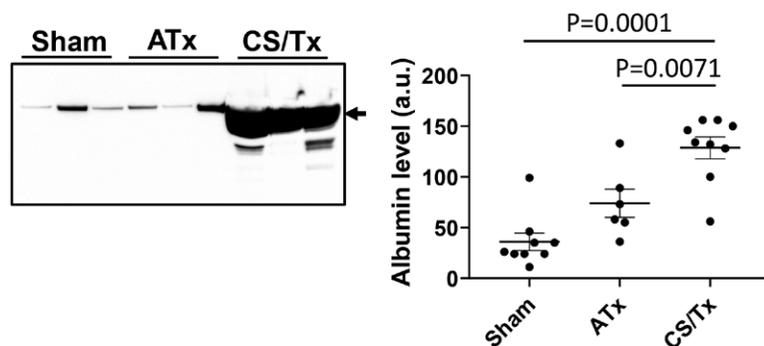


FIGURE 5. Urine levels of albumin are increased after CS/Tx. 10 microliters of urine (supernatant, see Methods) from Sham (right nephrectomy, $n=9$), ATx (Tx with no CS, $n=6$), and CS/Tx (CS/Tx, $n=9$) groups were resolved with SDS-PAGE, and proteins were transferred to a PVDF membrane. The membrane was then processed for western blotting with an antibody against albumin (bands detected with chemiluminescence). A representative blot from 3 repeated experiments is shown, and the densitometry values are expressed as mean \pm SEM (scatter plot). Differences between the means of the groups were compared with one-way ANOVA; P value indicates means are significantly different ($P<0.05$), when comparing CS/Tx to sham or ATx. ATx, autotransplantation; CS, cold storage; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean; Tx, transplantation.

color), which was localized in glomeruli or connective tissue (Figure 7A, upper row, left image). Consistent with western blot data (see Figures 1C and 2A), modest increases in albumin were detected in the ATx group, also confined to glomeruli and connective tissue (Figure 7A, upper row). Interestingly, further increased albumin levels were detected after 4-hour CS/Tx (4-h CS/Tx) when compared with sham and ATx, and

albumin localization was found in glomeruli, connective tissue, and the lumen of the tubules. Furthermore, albumin levels were dramatically higher in kidney tissue sections from the 18-hour CS/Tx group, and the protein was found within tubular cells, lumen of tubules, tubular casts, extratubular space, and glomeruli (Figure 7A, upper row). In kidney tissue sections from the sham group, immunohistochemistry

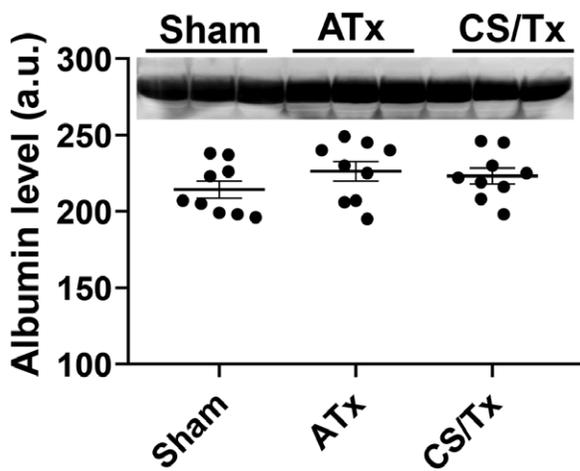


FIGURE 6. Serum levels of albumin are unaltered after CS/Tx. Fifty micrograms of diluted serum protein (1:10 in PBS) from sham (right nephrectomy), ATx (Tx with no CS), and CS/Tx groups were resolved with SDS-PAGE, and proteins were transferred to a PVDF membrane. The membrane was then processed for western blotting with antibody against albumin (bands detected with chemiluminescence). A representative blot from 3 repeated experiments is shown, and the densitometry values are expressed as mean \pm SEM (bars, $n=9$). Differences between the means of the groups were compared with one-way ANOVA. ATx, autotransplantation; CS, cold storage; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean; Tx, transplantation.

detected basal levels of AGE-albumin (brown color), and the levels were modestly higher in the ATx group (Figure 7A, lower row). Consistent with western blot data, AGE reactivity was the highest in the CS/Tx group and observed in glomeruli and intratubular and extratubular compartments, as well as within the tubular casts (Figure 7A, lower row).

Exacerbated Macrophage Infiltration After Prolonged CS/Tx

Because AGE products have the potential to induce inflammation, macrophage infiltration was evaluated by immunohistochemistry with CD68 antibody. In kidney tissue sections from the sham group, few macrophages were detected in the parenchyma (Figure 7B). As expected, sections from the ATx group had more CD68-positive cells (Figure 7B), and those from the CS/Tx group had dramatically more (Figure 7B). Consistent with albumin and AGE expression, the 18-hour CS/Tx kidneys showed more macrophages when compared with the 4-hour CS/Tx kidneys (Figure 7B).

Exacerbated Tubular Cell Death After Prolonged CS/Tx

Prolonged CS is detrimental to renal transplant outcome. We previously showed increased tubular necrosis after 18-hour CS/Tx.¹² Using the TUNEL staining kit, we evaluated TUNEL-positive nuclei/cells in paraffin embedded renal sections. As expected, a few sporadic TUNEL-positive nuclei were detected in sham kidneys and this number increased significantly with ATx, 4-hour CS/Tx, and 18-hour CS/Tx; however, the level of TUNEL-positive nuclei was the highest after 18-hour CS/Tx (Figure 8).

DISCUSSION

We have demonstrated, for the first time, that albumin and AGE-albumin levels are dramatically increased in the kidney after prolonged (18 h) CS combined with Tx. Our study revealed that basal albumin levels were observed primarily in the glomerulus and connective tissues in sham kidney; this renal albumin level was increased after ATx (no CS exposure) and 4-hour CS/Tx and detected at much higher levels after 18-hour CS/Tx. Our previous report demonstrated tubular necrosis and renal damage after ATx (transplants with no CS) and the necrosis/damage is exacerbated by CS/Tx.¹⁰⁻¹² Here, we demonstrated increased TUNEL-positive nuclei/cells after Tx; cell death was increased as the CS exposure was increased (18-h CS/Tx > 4-h CS/Tx). Together, our findings support the notion that increased CS time correlates with increased albumin level and severity of renal damage. Abundance of albumin in the casts of renal tubules after CS/Tx clearly suggests that the rats exhibited severe glomerular damage and albuminuria. It was intriguing to observe increased urinary albumin after CS/Tx, because it correlates with exacerbated renal dysfunction. This is notable because increased albumin excretion also correlates with increased incidence of patient death after Tx.^{22,23} Future studies should be designed to evaluate urinary albumin expression in patients after kidney transplant; such studies may help establish that increased renal/urinary albumin could serve as a potential biomarker of acute kidney rejection in these patients.

Albumin is considered to be a serum protein and liver is the main site of its production. Interestingly, the serum albumin levels did not change among the groups. Studies also provide evidence that other organs, including kidney, can actually synthesize albumin during fetal or neonatal stages.²⁴ In unprecedented findings, albumin was present in the tubular compartments of kidneys only after CS/Tx, suggesting that CS contributes for abnormal levels of albumin in rat kidney following CS/Tx. Jensen et al²⁵ have detected albumin mRNA in adult mouse renal cortex and have speculated that the cortical interstitium can synthesize albumin, whereas the tubular cells uptake the synthesized albumin from the basolateral site. If this is the case, we propose that CS treatment triggers a signal for posttransplant albumin synthesis in the renal interstitial compartment and that the albumin is then taken up by tubular cells. Future studies are warranted to elucidate the mechanisms of increased level of renal albumin in kidneys following CS/Tx.

Detection of albumin in detergent-insoluble fraction of kidney homogenate indicates modification of this protein. It was interesting to observe excess albumin level in the insoluble fraction only after CS/Tx. We previously reported that CS/Tx induces compromised proteasome function,¹² which lends support to the notion that localization of albumin to the insoluble fraction equates with loss of proteasome function. Taken together, these results suggest compromised clearance of the kidneys in both Tx models, ATx and CS/Tx, which both experienced warm ischemia/reperfusion injury; however, only the CS/Tx kidney was exposed to CS before Tx. Because renal ischemia/reperfusion injury leads to oxidative stress,^{26,27} we speculate that the threshold of oxidant generation is exceeded in the CS/Tx group, and therefore higher reactive oxygen species may have induced modification of albumin¹⁵ in this CS/Tx model. Of note, it has been reported in experimental settings that albumin abundance can act as a signal molecule and surprisingly this signaling cascade is involved

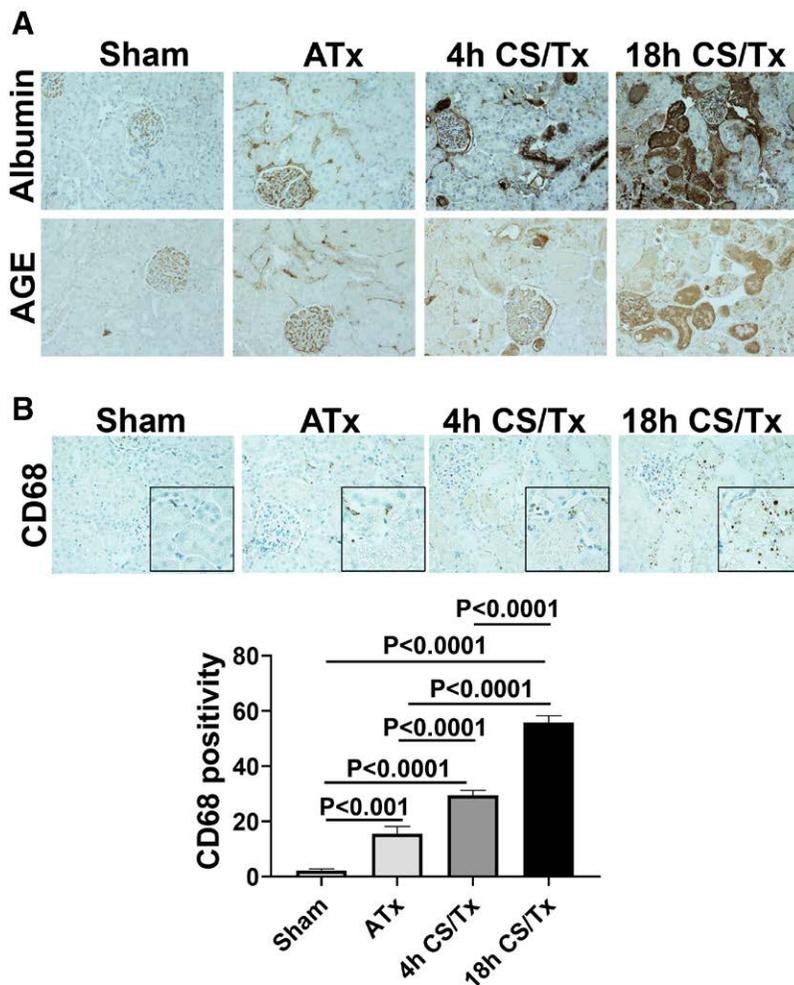


FIGURE 7. Increased albumin, AGE, and macrophage infiltration in the kidneys after CS/Tx. A, Kidney tissues (paraffin embedded section) from sham, ATx, 4-h CS/Tx, and 18-h CS/Tx groups were subjected to immunohistochemistry with antibody against albumin (upper panel) and AGE (lower panel). Brown staining indicates reactivity to albumin or AGE. B, Kidney sections from (A) were employed for CD68 immunostaining. Representative micrographs (n=6) shown with zoomed inset showing CD68 positive macrophages (brown staining, upper panel). Bar graph showing CD68 positive cells/fields, and the values are expressed as mean± SEM (bars). Differences between the means of the groups were compared with one-way ANOVA; *P* value indicates means are significantly different (*P*<0.05), when comparing 18-h CS/Tx to sham, ATx, or 4-h CS/Tx. AGE, advanced glycation-end product; ATx, autotransplantation; CS, cold storage; SEM, standard error of the mean; Tx, transplantation.

with reactive oxygen species generation in proximal tubular cells.²⁸⁻³⁰ These studies further show that in vitro treatment of renal epithelial cells with albumin activates the nuclear factor kappa-light-chain-enhancer of activated B cells or extracellular signal-regulated kinase pathway via epidermal growth factor receptor. Fewer reports further suggest that albumin modification alters the protein conformation, which likely triggers a pathogenic cascade. Interestingly, oxidized plasma albumin provides a prothrombotic effect and has been shown to induce endothelial tissue factor expression.³¹ Future studies are warranted to elucidate the type of albumin modifications during CS/Tx and whether this modification contributes to renal damage in our CS/Tx model.

ATx showed modest increases in AGE-albumin, but the detection was limited to glomeruli and extratubular compartment. Furthermore, dramatic increases in AGE-albumin were observed after CS/Tx, and they were detected in the tubular compartment and tubular casts. AGE-albumin has been reported to play a pathogenic role in disease models, such as limb and myocardial ischemia/reperfusion and diabetes. However, it was interesting to observe a basal level

of AGE-albumin in sham kidneys, and similar to albumin, the AGE-albumin was confined to glomeruli and extratubular compartment. However, prolonging the CS time led to increased AGE-albumin (18>4h) after Tx. Increased AGE-albumin in kidney correlated with augmented renal damage (Figure 8) and dysfunction^{10,11} after CS/Tx, suggesting that the excessive AGE-albumin possibly worsens kidney injury/dysfunction. Consistent with the increased AGE-albumin level, our CS/Tx model also displayed increased macrophage infiltration in kidneys. Son et al¹⁷ have reported in a limb ischemia/reperfusion model that M1 macrophages synthesize AGE-albumin and that its secretion leads to muscle cell death after postischemic reperfusion. In addition, AGE-albumin also augments vascular injury.^{32,33} Yeh et al³³ have reported that the long-term administration of AGE-albumin induces renal and vascular damage via activation of inflammasomes and endothelial dysfunction. Future studies are needed to elucidate mechanisms of AGE-albumin production in kidney, its specific role on renal function during CS/Tx, and whether renal macrophages contribute to excessive production of AGE-albumin in this CS/Tx model.

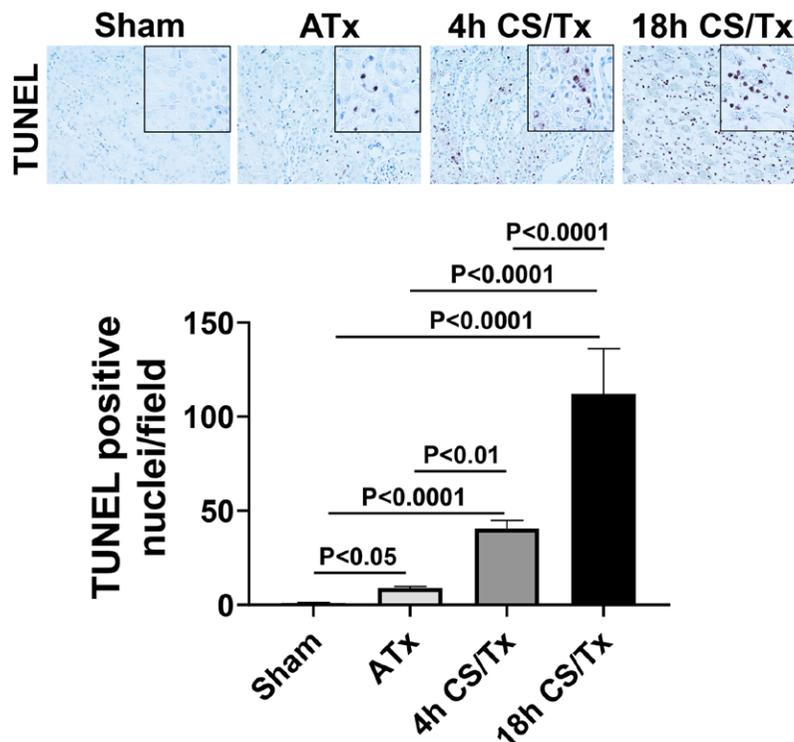


FIGURE 8. Increased CS time increases TUNEL positivity in the kidneys after CS/Tx. Kidney tissues (paraffin embedded section) from sham, ATx, 4-h CS/Tx, and 18-h CS/Tx groups were subjected to TUNEL reaction. Brown staining indicates reactivity to TUNEL. Representative micrographs ($n = 6$) showing TUNEL-positive nuclei/cells (brown staining). Bar graph showing TUNEL-positive nuclei/fields, and the values are expressed as mean \pm SEM (bars). Differences between the means of the groups were compared with one-way ANOVA; P value indicates means are significantly different ($P < 0.05$), when comparing 18-h CS/Tx to sham, ATx, or 4-h CS/Tx. ATx, autotransplantation; CS, cold storage; SEM, standard error of the mean; TUNEL, transferase-mediated dUTP nick-end labeling; Tx, transplantation.

In summary, our study suggests that prolonged CS triggers increased albumin level and its modification in kidneys after Tx, and this appears to be associated with acute kidney failure after CS/Tx.¹⁰ However, the mechanisms of the increased albumin and downstream consequences for renal pathology should be explored. Our study also suggested that CS/Tx increases the AGE-albumin level in kidneys, which could serve as a pathologic biomarker of acute kidney failure following CS/Tx. Published reports (in vitro nontransplant models) suggest that albumin and AGE-albumin have a pathologic role on renal tubular cells. Based on these reports and our recent findings, we hypothesize that the CS/Tx-mediated localization of albumin and AGE-albumin within kidneys promote inflammation and oxidative stress leading to renal damage. This then leads to a vicious cycle of albumin localization and its modification and finally renal damage.

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REFERENCES

- Acín-Pérez R, Fernández-Silva P, Peleato ML, et al. Respiratory active mitochondrial supercomplexes. *Mol Cell*. 2008;32:529–539.
- Bon D, Chatauret N, Giraud S, et al. New strategies to optimize kidney recovery and preservation in transplantation. *Nat Rev Nephrol*. 2012;8:339–347.
- Hanf W, Coda R, Meas-Yedid V, et al. Kidney graft outcome and quality (after transplantation) from uncontrolled deceased donors after cardiac arrest. *Am J Transplant*. 2012;12:1541–1550.
- Hoeger S, Petrov K, Reisenbuechler A, et al. The additional detrimental effects of cold preservation on transplantation-associated injury in kidneys from living and brain-dead donor rats. *Transplantation*. 2009;87:52–58.
- McAnulty JF. Hypothermic organ preservation by static storage methods: current status and a view to the future. *Cryobiology*. 2010;60(3 Suppl):S13–S19.
- Salahudeen AK. Cold ischemic injury of transplanted kidneys: new insights from experimental studies. *Am J Physiol Renal Physiol*. 2004;287:F181–F187.
- Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. *Kidney Int*. 2004;65:713–718.
- Stubenitsky BM, Booster MH, Brasile L, et al. I: negative effect of cold ischemia on initial renal function. *Asaio J*. 2000;46:60–61.
- Stubenitsky BM, Brasile L, Booster MH, et al. Deleterious effect of prolonged cold ischemia on renal function. *Transpl Int*. 2001;14:256–260.
- Parajuli N, Shrum S, Tobacyk J, et al. Renal cold storage followed by transplantation impairs expression of key mitochondrial fission and fusion proteins. *Plos One*. 2017;12:e0185542.
- Shrum S, Macmillan-Crow LA, Parajuli N. Cold storage exacerbates renal and mitochondrial dysfunction following transplantation. *J Kidney* 2016;2 pii:114.
- Lo S, Macmillan-Crow LA, Parajuli N. Renal cold storage followed by transplantation impairs proteasome function and mitochondrial protein homeostasis. *Am J Physiol Renal Physiol*. 2019;316:F42–F53.

13. Gujjarro C, Massy ZA, Wiederkehr MR, et al. Serum albumin and mortality after renal transplantation. *Am J Kidney Dis.* 1996;27:117–123.
14. Lang J, Katz R, Ix JH, et al. Association of serum albumin levels with kidney function decline and incident chronic kidney disease in elders. *Nephrol Dial Transplant.* 2018;33:986–992.
15. Watanabe H, Imafuku T, Otagiri M, et al. Clinical implications associated with the posttranslational modification-induced functional impairment of albumin in oxidative stress-related diseases. *J Pharm Sci.* 2017;106:2195–2203.
16. Cohen MP. Intervention strategies to prevent pathogenetic effects of glycated albumin. *Arch Biochem Biophys.* 2003;419:25–30.
17. Son M, Kang WC, Oh S, et al. Advanced glycation end-product (AGE)-albumin from activated macrophage is critical in human mesenchymal stem cells survival and post-ischemic reperfusion injury. *Sci Rep.* 2017;7:11593.
18. Parajuli N, Müller-Holzner E, Böck G, et al. Infiltrating CD11B+CD11C+ cells have the potential to mediate inducible nitric oxide synthase-dependent cell death in mammary carcinomas of HER-2/neu transgenic mice. *Int J Cancer.* 2010;126:896–908.
19. Haffner MC, Jurgeit A, Berlato C, et al. Interaction and functional interference of glucocorticoid receptor and SOCS1. *J Biol Chem.* 2008;283:22089–22096.
20. Nesvizhskii AI, Keller A, Kolker E, et al. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem.* 2003;75:4646–4658.
21. Zybailov B, Mosley AL, Sardiú ME, et al. Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. *J Proteome Res.* 2006;5:2339–2347.
22. Halimi JM, Buchler M, Al-Najjar A, et al. Urinary albumin excretion and the risk of graft loss and death in proteinuric and non-proteinuric renal transplant recipients. *Am J Transplant.* 2007;7:618–625.
23. Halimi JM, Matthias B, Al-Najjar A, et al. Respective predictive role of urinary albumin excretion and nonalbumin proteinuria on graft loss and death in renal transplant recipients. *Am J Transplant.* 2007;7:2775–2781.
24. Nahon JL, Tratner I, Poliard A, et al. Albumin and alpha-fetoprotein gene expression in various nonhepatic rat tissues. *J Biol Chem.* 1988;263:11436–11442.
25. Jensen TB, Cheema MU, Szymiczek A, et al. Renal type a intercalated cells contain albumin in organelles with aldosterone-regulated abundance. *Plos One.* 2015;10:e0124902.
26. Parajuli N, MacMillan-Crow LA. Role of reduced manganese superoxide dismutase in ischemia-reperfusion injury: a possible trigger for autophagy and mitochondrial biogenesis? *Am J Physiol Renal Physiol.* 2013;304:F257–F267.
27. Saba H, Batinic-Haberle I, Munusamy S, et al. Manganese porphyrin reduces renal injury and mitochondrial damage during ischemia/reperfusion. *Free Radic Biol Med.* 2007;42:1571–1578.
28. Reich H, Tritschler D, Herzenberg AM, et al. Albumin activates ERK via EGF receptor in human renal epithelial cells. *J Am Soc Nephrol.* 2005;16:1266–1278.
29. Morigi M, Macconi D, Zoja C, et al. Protein overload-induced NF-kappa activation in proximal tubular cells requires H(2)O(2) through a PKC-dependent pathway. *J Am Soc Nephrol.* 2002;13:1179–1189.
30. Tang S, Leung JC, Abe K, et al. Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells in vitro and in vivo. *J Clin Invest.* 2003;111:515–527.
31. Pasterk L, Lemesch S, Leber B, et al. Oxidized plasma albumin promotes platelet-endothelial crosstalk and endothelial tissue factor expression. *Sci Rep.* 2016;6:22104.
32. Goldin A, Beckman JA, Schmidt AM, et al. Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation.* 2006;114:597–605.
33. Yeh WJ, Yang HY, Pai MH, et al. Long-term administration of advanced glycation end-product stimulates the activation of NLRP3 inflammasome and sparking the development of renal injury. *J Nutr Biochem.* 2017;39:68–76.