# Mechanism of cold storage-mediated proteasome dysfunction: a plausible role of p38MAPK signaling

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## ABSTRACT

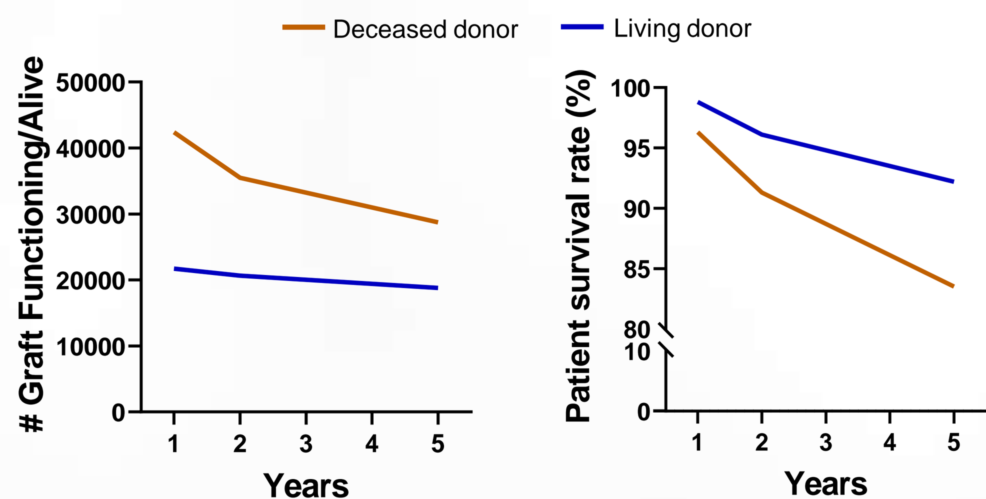
Kidneys derived from deceased donors must undergo cold storage (CS) process before being transplanted. However, clinical and experimental reports suggest that prolonged CS of kidneys often leads to poor graft outcomes after transplantation. In a previous study, we showed that rat kidney CS followed by transplantation reduces renal proteasome function, which correlates with graft damage. The objective of this study was to understand the role of proteasome and molecular mechanisms of its dysfunction during renal CS+Tx. We followed the established in vivo and in vitro models of rat kidney CS followed by transplantation (CS+Tx) or rewarming (CS+RW). To understand the role of proteasome function during CS+Tx, rat donor kidneys were flushed with bortezomib (a proteasome inhibitor) followed by immediate transplantation in a new recipient rat. Ex vivo treatment of the donor rat kidneys with bortezomib prior to transplantation (without CS) increased renal dysfunction after transplantation, indicating that normal proteasome function is essential to prevent renal injury in transplants. To assess molecular mechanisms of CS+Tx-mediated proteasome dysfunction, normal rat proximal tubular (NRK) cells were exposed to various CS times followed by rewarming (CS+RW). Results showed a time-dependent increase of p38MAPK phosphorylation (activation) during CS. The p38MAPK phosphorylation in NRK cells also correlated with CS+RW-mediated proteasome dysfunction and cell injury. Treatment of NRK cells with VX-745, a specific inhibitor of p38MAPK during CS followed by rewarming (CS+VX-745+ RW) blunted p38MAPK activation, increased proteasome function, and improved cell viability compared to CS+RW alone. Finally, our in vivo data revealed that CS activates p38MAPK in rat kidneys, and its downstream target molecules, Hsp27 and ATF2, are activated (phosphorylated) during CS+Tx. These results clearly indicated that CS activates p38MAPK, which contributes to the decline of proteasome function leading to graft injury following transplantation. Future studies should test the therapeutic potential of VX-745 during CS to improve allograft survival, benefiting the ESKD patients.

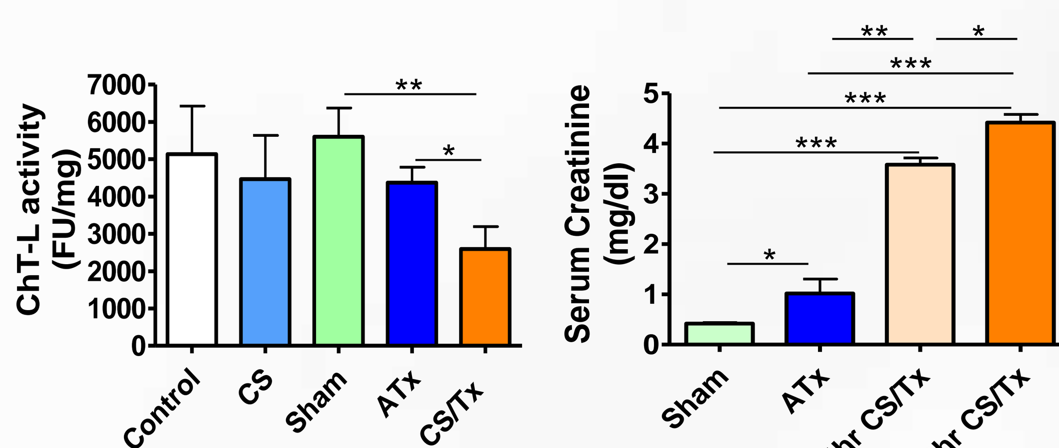
## BACKGROUND

- **End-Stage Kidney Diseases (ESKD)** is the 9th leading cause of death with over 700,000 Americans affected and predicted to be 5th leading cause of death by 2040.
- As many as 9 in 10 adults with Chronic Kidney Disease (**CKD**) are unaware of the disease and progress to ESKD each year (CDC, 2021).
- Renal replacement therapy (Dialysis/Transplantation) is the treatment modality for patients with ESKD.
- Kidney transplantation is the preferred because it increases longevity, quality of life, and reduces medical cost for patients with ESKD.
- However, 7 out of 10 patients remain on dialysis due to shortage of transplantable kidneys and ~40,000 patients/year die while on the waiting list for kidney transplant.

**Why Renal Cold preservation is required?**

- Large number of kidneys come from deceased donors, which are routinely flushed and stored in a cold preservation solution to prolong the viability while being matched for recipient.
- Prolonged cold preservation can cause extensive renal tissue damage following transplantation.
- ~25% of procured donor kidneys (deceased) are annually discarded.
- Molecular mechanisms for cold preservation-induced damage are largely unknown!



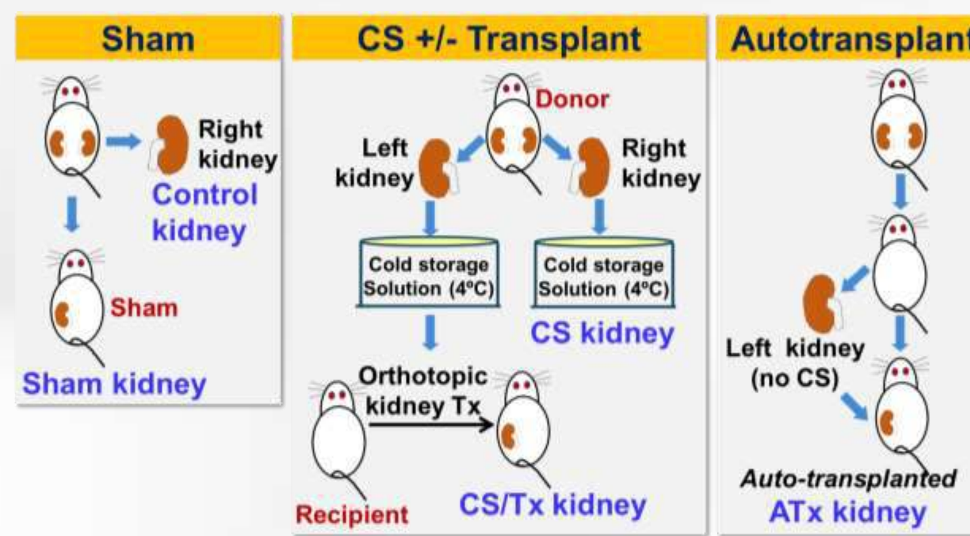

(*Shrum S et al, Journal of Kidney, 2016, Parajuli N et al., PLoS One, 2017*), and Lo S et al., AJP-RP, 2018.

**Overall goal:** Improve the outcomes of kidney transplants by identifying mechanisms related to cold-storage-induced renal damage and adopting targeted therapies, thereby decreasing the incidence of cold-storage/transplant associated graft failure.

## METHODS

Transplant Models: The kidneys of male Lewis rats were isolated, flushed with and stored in cold storage (CS) solution followed by transplantation in male Lewis recipient rats (in vivo model of CS+Tx). Rat renal proximal tubular (NRK) cells were exposed to CS solution followed by rewarming (CS+RW; in vitro model of transplant).

**In vivo rat kideny transplant model**




Protein Detection: For denatured western blot, renal extract extracts from whole-kidney homogenates or NRK cells were prepared with RIPA-based lysis buffer and proteins were resolved with SDS-PAGE and then transferred to polyvinyl fluoride membrane. For native gel western blot, renal extracts from whole-kidney homogenates were prepared with 0.9% digitonin lysis buffer.

Proteasome Function Assay: A fluorescent based peptide substrate was used to measure Chymotrypsin-like peptidase activity of the proteasome.

Cell Viability Assay: ATP Glo Assay was performed with NRK cells with/without drug treatment in CS or CS+RW conditions.

## RESULTS

### Proteasome subunit levels after CS plus transplantation

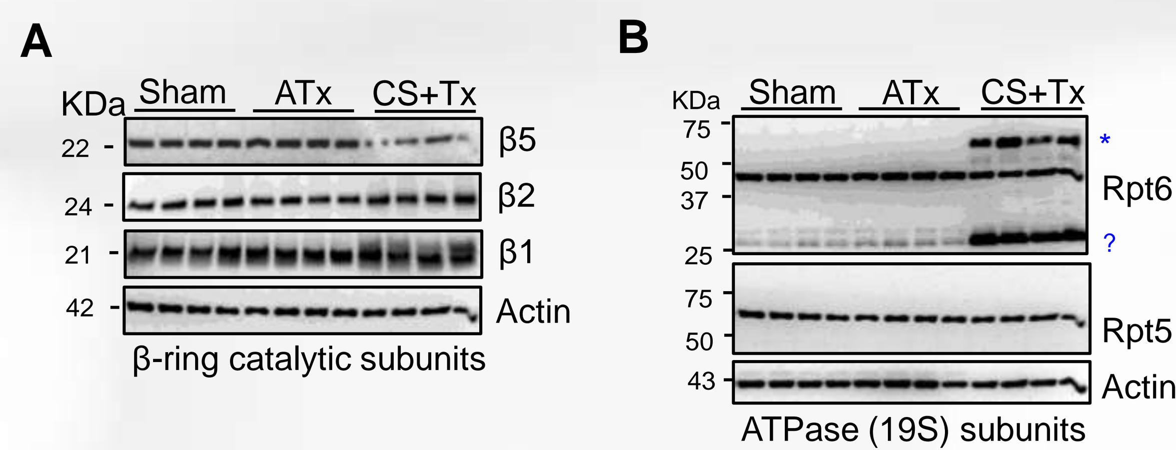



**Figure 1**. Cold storage effects in aberrant levels of proteasome subunits in renal grafts. Denatured renal homogenates from Sham (right nephrectomy), 18-h cold storage (CS) combined with transplantation (CS+Tx), and auto-transplant (ATx; transplant with no CS) groups 1-day post surgery were evaluated for protein levels of **(A)** catalytic subunits of β-ring (20S) and **(B)** ATPase subunits (19S) using SDS-PAGE western blot (n=3).

### Abnormal Rpt6 subunit after CS plus transplantation

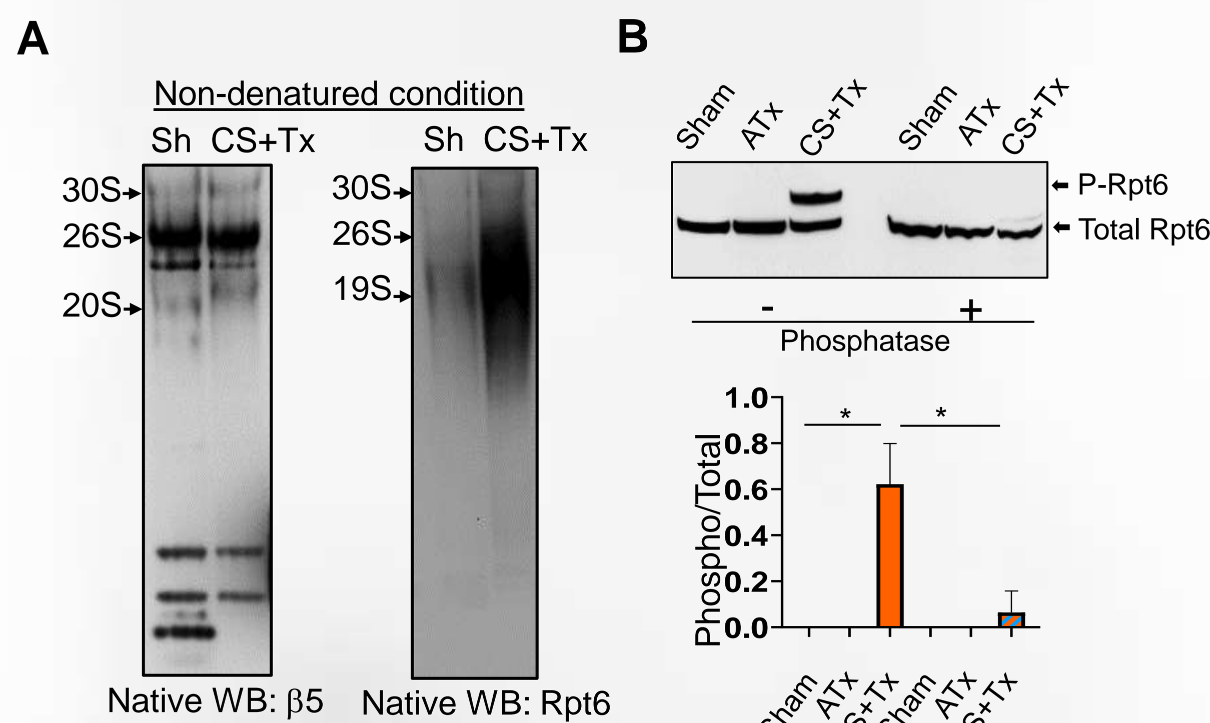



**Figure 2** Cold storage plus transplantation effects in post-translational modification of Rpt6 subunit of the renal proteasome. **(A)** Non-denatured renal homogenates from Sham (right nephrectomy) and 18-h cold storage (CS) plus transplantation (CS+Tx) groups 1-day post surgery were evaluated for native levels of renal proteasome using western blot and Rpt 6 (left) or β5 (right) antibody (n=3/group). **(B)** Representative western blot (denatured) for Rpt6 subunit using renal extracts treated with or without phosphatase from Sham, ATx (auto transplant, transplant with no CS), and CS+Tx groups 1-day post-surgery (n=3/group).

### The p38MAPK pathway during CS plus transplantation

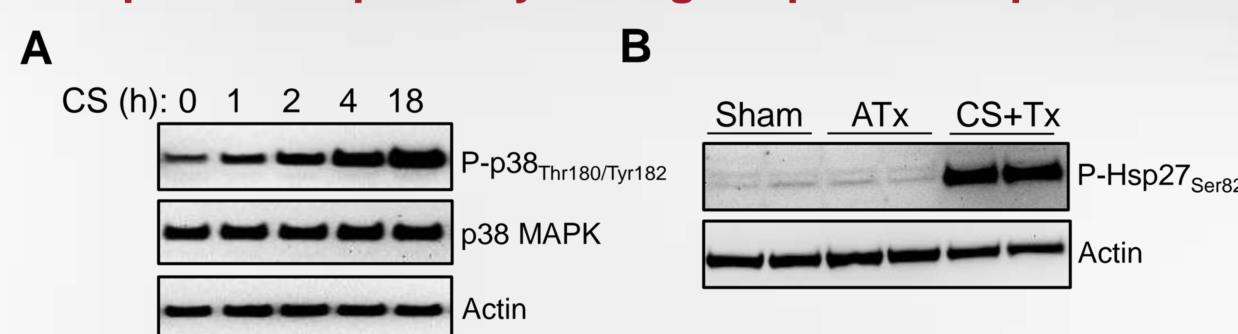



**Figure 3.** Activation of p38MAPK during cs/CS+Tx **(A)** NRK-52E cells were exposed to 0-18 h cold storage (CS). NRK cell extracts were employed for SDS-PAGE western blot using p38MAPK antibodies (phosphorylated or total). Actin was used as loading control. **(B)** Denatured western blots for phosphorylated Hsp27 or ATF2 (downstream target proteins of p38 MAPK pathway) using renal extracts from Sham (right nephrectomy), 18-h cold storage (CS) combined with transplantation (CS/Tx), and auto-transplant (ATx; transplant with no CS) groups 1-day post surgery (n=2/group).

### Proteasome activity and cell viability after p38MAPK inhibition

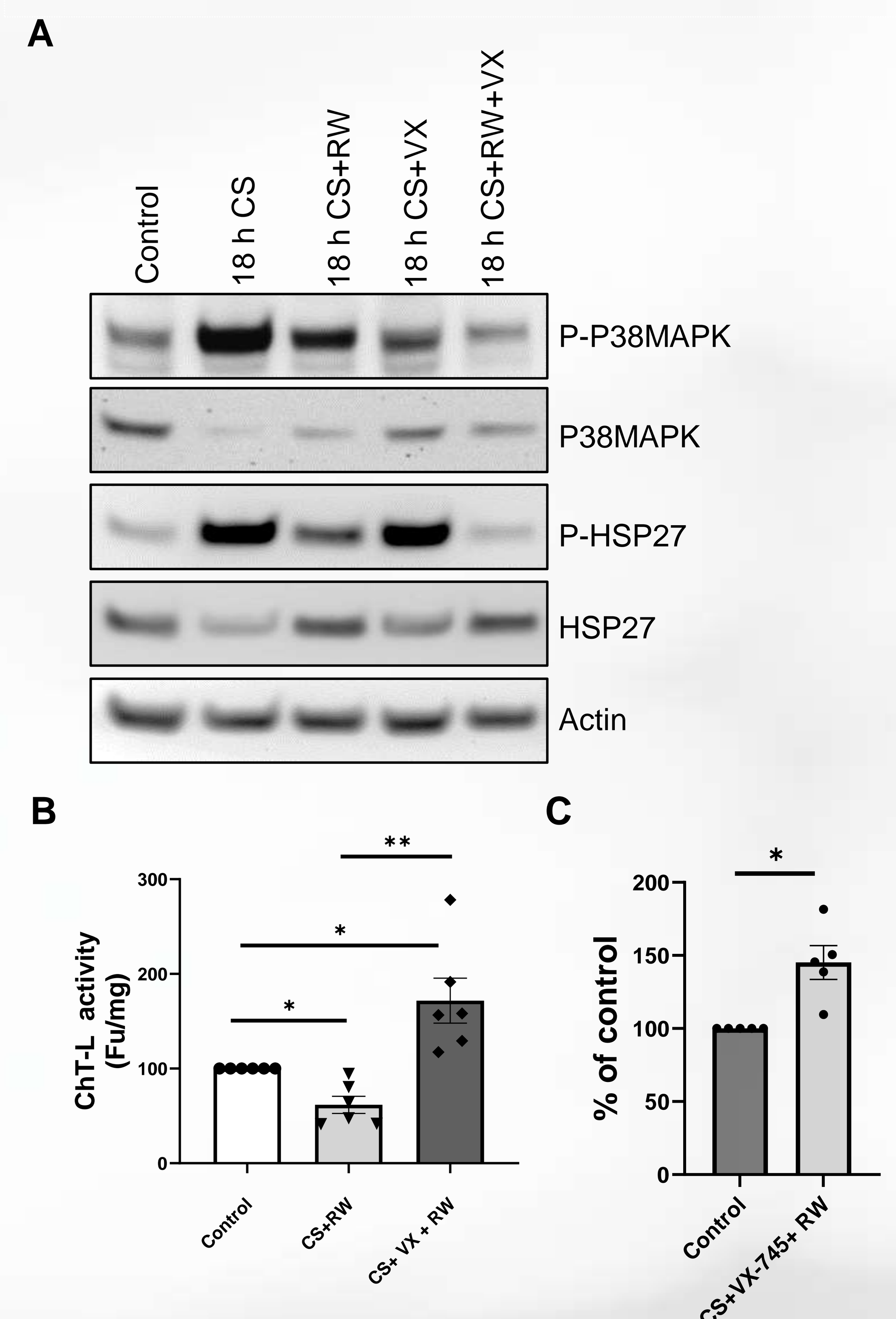



**Figure 4.** Inhibition of p38MAPK during CS improves proteasome function and cell viability after rewarming. NRK-52E cells were exposed to 18 hr cold storage (CS, 4˚C) with or without VX-745, a p38-MAPK inhibitor, [5 μM] or DMSO (vehicle control). After 18 hr, the cells were washed with cold PBS followed by rewarming with cell growth medium for 6 hr at 37˚C (CS+RW). **(A)** NRK cell extracts were employed for SDS-PAGE western blot using p38MAPK antibodies (phosphorylated or total). Actin was used as a loading control. **(B)** Chymotrypsin-like peptidase activity (proteasome function) in NRK cells after CS+/-VX-745+RW (n=6/group). **(C)** Cell viability is increased with inhibition of p38MAPK in NRK cells (n=5). Renal cell survival study using the CellTiter-Glo® Luminescent Cell viability Assay kit.

### Normal proteasome function is essential for kidney function

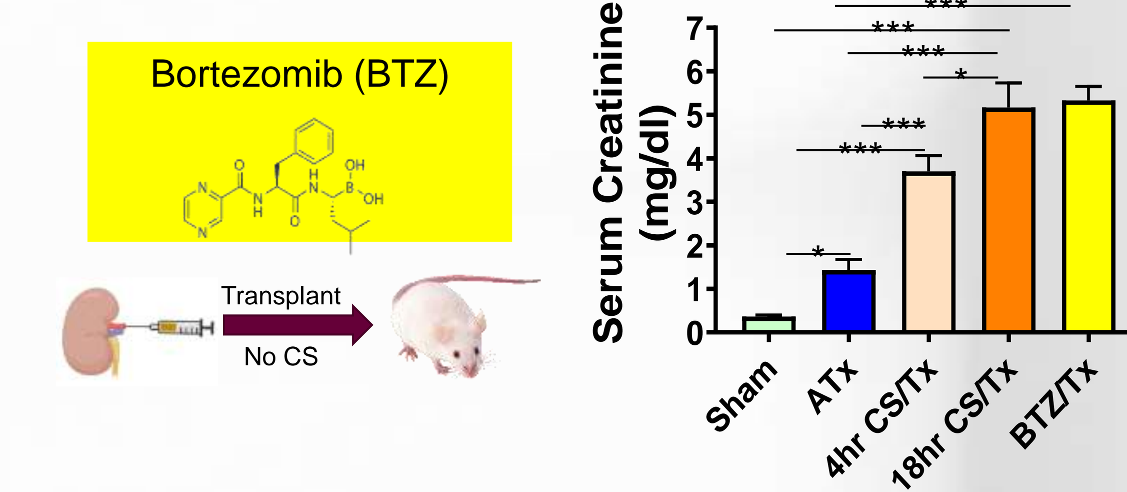


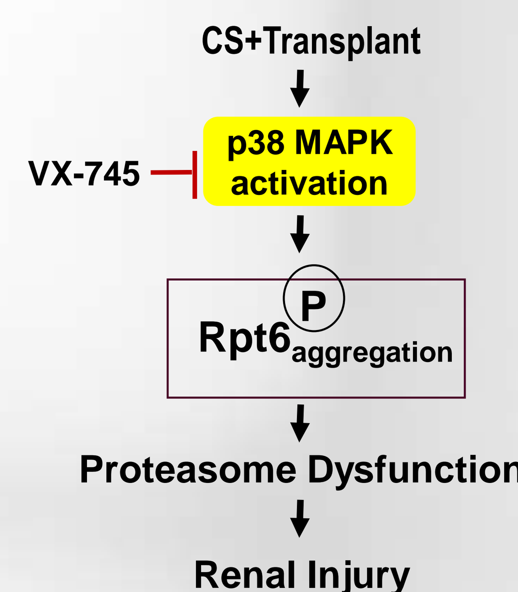

**Figure 5.** Inhibition of proteasome function using Bortezomib in donor kidneys before transplant increases kidney dysfunction. **(A)** Schematic showing perfusion of donor rat kidneys with Bortezomib (reconstituted in warm saline, without CS) followed by transplantation in a recipient rat. **(B)** The serum creatinine level in rats using iSTAT serum chemistry analyzer (n=3).

## CONCLUSIONS

- CS-mediated activation of the p38MAPK may contribute to Rpt6 phosphorylation/aggregation leading to compromised proteasome function.
- p38MAPK could be a novel therapeutic target during CS to reduce CS+Tx-mediated graft failure.

## FUTURE DIRECTION

- Delineate the mechanisms of Rpt6 phosphorylation and aggregation during renal CS and transplantation.
- Impact of Rpt6 modification on proteasome/renal dysfunction during transplantation.




## ACKNOWLEDGEMENTS

- NIH DK123246 (Parajuli)
- UAMS – Barton Pilot Award (Parajuli)
- TPA19TPA34850057(Parajuli)

NIH National Institute of Diabetes and Digestive and Kidney Diseases

UAMS College of Medicine Office of Research

American Heart Association.