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PRINCIPAL INVESTIGATOR: Richard J Johnson MD

CONTRACTING ORGANIZATION: University of Colorado, Denver
Aurora, CO 80045

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14. ABSTRACT Our studies evaluate how recurrent dehydration can cause chronic kidney disease, an important question for the military and public. We hypothesize, based on preliminary data, that dehydration associated renal injury results from hyperosmolarity induced activation of renal aldose reductase-fructokinase. We made excellent progress this last year. First, we now have the floxed KHK KO mouse and have generated successful litters so we can proceed with selective renal knockout of fructokinase in dehydration induced kidney disease. Aim 2 investigates the role of vasopressin receptors and uric acid, and we have completed studies with the Vasopressin 2 receptor (submitted) and have completed the experiments with V1a and V1b knockout. We have also performed some hypothalamic explant studies and documented a functional fructokinase system in the hypothalamus (manuscript submitted). Aim 3 tests the role of rehydration with fructose solutions with or without blocking of vasopressin receptors. The administrative delay with our collaborators has been resolved and experiments are ongoing. In summary, we are proceeding with how recurrent dehydration causes chronic kidney disease via vasopressin and fructokinase and remain on target for finishing in time.

15. SUBJECT TERMS
Dehydration, Chronic kidney disease, vasopressin, fructose

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1. INTRODUCTION:

Our studies are aimed at identifying how recurrent dehydration may lead to chronic kidney disease. This is important for the military as well as for the general population. In preliminary studies we developed the first model of chronic kidney disease from recurrent dehydration and found evidence that the renal injury is mediated by hyperosmolarity that activates an enzyme pathway in the kidney (aldose reductase-fructokinase) that leads to tubular injury. Here we proceeded with three aims to identify the role of this pathway. Aim 1 evaluated the role of fructokinase in the renal tubule on dehydration induced kidney disease. Aim 2 investigated the role of downstream mediators, including the role of vasopressin receptors and uric acid in driving dehydration induced kidney injury. Aim 3 evaluated the role of rehydration with fructose solutions with or without blocking of vasopressin receptors. These studies provided important insights into how recurrent dehydration cause chronic kidney disease

2. **KEYWORDS:** Dehydration, Heat Stress, Chronic Kidney Disease, Fructose, Uric acid, Vasopressin, Rehydration, Mesoamerican Nephropathy

3. ACCOMPLISHMENTS:

a. **What were the major goals of the project?**

Our primary goal was to better understand the cause of a major epidemic occurring in Central America among rural workers working in hot conditions. Our hypothesis was that recurrent severe heat stress may be a cause of chronic kidney disease. The importance of this to the Defense Department is that soldiers are frequently in hot,hostile conditions where they are at risk for heat stress and dehydration. A better understanding of the role of heat stress in chronic kidney disease, how it causes injury, and potential ways to prevent it could be of great benefit to man.

To study this, we proposed three aims.

The purpose of Aim 1 was to test the role of fructose metabolism in heat stress associated kidney disease. While the common source of fructose is from added sugars in the diet, fructose can also be produced in the body in response to high glycemic foods, high salt diet, and heat stress. We had previously show that mice that cannot metabolize fructose are protected from kidney disease induced by heat stress. In Aim 1 we planned to make mice that lack fructose metabolism in their kidney to see if they might be protected from heat stress nephropathy.

The purpose of Aim 2 was to test the role of vasopressin and uric acid in driving the kidney disease of heat stress. Here the plan was to perform 4 sets of studies. To study vasopressin, we would use mice lacking vasopressin receptors (V1a and V1b) and to study the vasopressin 2 receptor we would administer a V2 agonist (desmopressin) in our murine model of chronic kidney disease. To study uric acid, we would determine if lowering uric acid with a xanthine oxidase inhibitor (allopurinol) would be protective. These were some of the more important experiments of the grant.

The goal of aim 3 was to test the power of rehydration of water compared to fructose as a rescue solution for heat stress nephropathy. Here the hypothesis was that rehydration with water containing fructose might worsen the kidney disease associated with heat stress. We also wanted to test whether rehydration with fructose might affect vasopressin and whether additional vasopressin blockade would provide protection from fructose induced rehydration. Some of these latter studies were performed in collaboration with our collaborator, Dr Laura Gabriela Sanchez-Lozada., at the Laboratory of Renal Physiology at the Instituto Nacional de Cardiología Ignacio Chavez in Mexico City.

b. **What was accomplished under these goals?**

1) Major activities.

Most of the major activities were based on performing studies in mice and/or rats exposed repeatedly to heat stress. Our classical model in mice involves exposing mice to extreme hot conditions (38-39 degree C) for periods of 30 minutes at a time, and for as many as 7 or 8 times in a given day, usually for a period of 5 weeks. Mice are allowed water only at night, and although the conditions are severe, there is no mortality and the study was approved by our IACUC and the DOD Accuro system. Our studies were focused on identifying what might increase the risk for kidney disease, and what might make the kidney disease better. Our studies included studying the role of diet and rehydration (focusing on fructose) as well as hormones involved in dehydration and their receptors (especially vasopressin receptors 1a, 1b, and 2) and uric acid, a biologically active substance that increases in the setting of heat stress and dehydration. While we were working on these studies for the DOD, we also were performing separate studies funded by Solidaridad on human samples of subjects exposed to recurrent heat stress who were developing kidney disease. The net effect was the discovery of a new mechanism for chronic kidney disease, potential new treatments, and insights into how heat stress causes kidney damage and how it can be prevented.

2) Specific objectives;

a). The goal of Aim 1 was to further explore the role of fructokinase (also known as KHK) in driving heat stress associated kidney disease. This was based on our discovery that mice lacking fructokinase (KHK-KO) were protected from chronic kidney disease associated with heat stress.[1] What was exciting about that discovery was that the animals had never received fructose in their diet, so the fructose was endogenously produced. Indeed, we realized that heat stress could raise serum osmolarity and that activates a pathway known as the polyol pathway in which glucose is converted to sorbitol and then to fructose. We have subsequently found that heat stress induces fructose generation in the kidney where it is metabolized, resulting in local oxidant and uric acid generation that can damage local kidney tubules.[2] In aim 1 our goal was to make a mouse that lacked fructokinase only in the kidney (proximal tubule) to prove that it is the local fructose metabolism in the kidney that is important for the development of kidney disease.

b). The goal of Aim 2 was to evaluate the role of vasopressin receptor 1A, vasopressin receptor 1B, vasopressin 2 receptor, and uric acid in the model of heat-dehydration induced kidney disease. As mentioned, heat stress is known to raise serum osmolality and a rise in serum vasopressin. Vasopressin, while having an important role in urinary concentration, has also been postulated to cause kidney disease if levels are high and prolonged. Therefore the objective was to test this, and to tease out the specific receptors. Heat stress is also known to result in some generation of uric acid from tissues and lead to an increase in uric acid excretion—and so we also wanted to test whether uric acid might have a role in heat stress. This was particularly true because the rural workers in the sugarcane fields were often developing hyperuricemia.

c) The primary goal of Aim 3 was to investigate the safest way to hydrate animals that were undergoing recurrent heat stress. The concept was that if fructose was indeed injurious to the kidney, that rehydration with sugar- or fructose-containing liquids might actually worsen the kidney disease. The specific objectives of aim 3 was to perform studies comparing fructose to water in rehydrating mice and rats undergoing recurrent heat stress and determining if one type of hydration is superior and also how it interrelated with vasopressin.

3) Significant results/key outcomes

We have had a number of very significant results and key outcomes.

a. **Generation of a Fructokinase (KHK)-LoxP mouse for tissue specific knockout of fructose metabolism.** Fructokinase (KHK) knockout mice were initially developed and validated by Dr

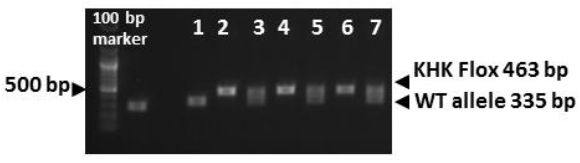


Figure 1: Representative gel showing offspring for heterozygous KHK loxP containing alleles (463 bp) and WT alleles (335 bp). Lanes 2,4, and 6 are homozygouse (both alleles) loxP containing sequences, lanes 3,5 and 7 hemizygous and lane 1 wild type alleles.

Bonthron at Leeds[3, 4]. We have published several manuscripts employing these mice[1, 5-10] including their phenotypic response to fructose. However, one of the aims of the DOD grant was to be able to knockout the fructokinase in the kidney by developing a tissue-specific fructokinase knockout mouse. This turned out to be challenging, but after a few attempts, we succeeded. Specifically, we generated a set of mice with loxP sequences flanking exons 3 and 4 of the fructokinase gene. As seen in the figure below, we have successfully obtained homozygous mice with both pairs of chromosomes containing the lox P sequences for crossing with specific kidney specific cre-recombinase expressing mice. Specifically the Ksp1.3 Cre transgenic mouse is available at the Jackson laboratory in the B6 strain (012237-B6.Cg-Tg(Cdh16Cre)91lgr/J) and we are in the process of knocking out KHK from the kidney to evaluate its role in the kidney disease of heat stress. Figure 1 shows a representative gel showing the loxP allele as compared to the wild type allele, thereby documenting that we have both heterozygous and homozygous LoxP KHK mice.

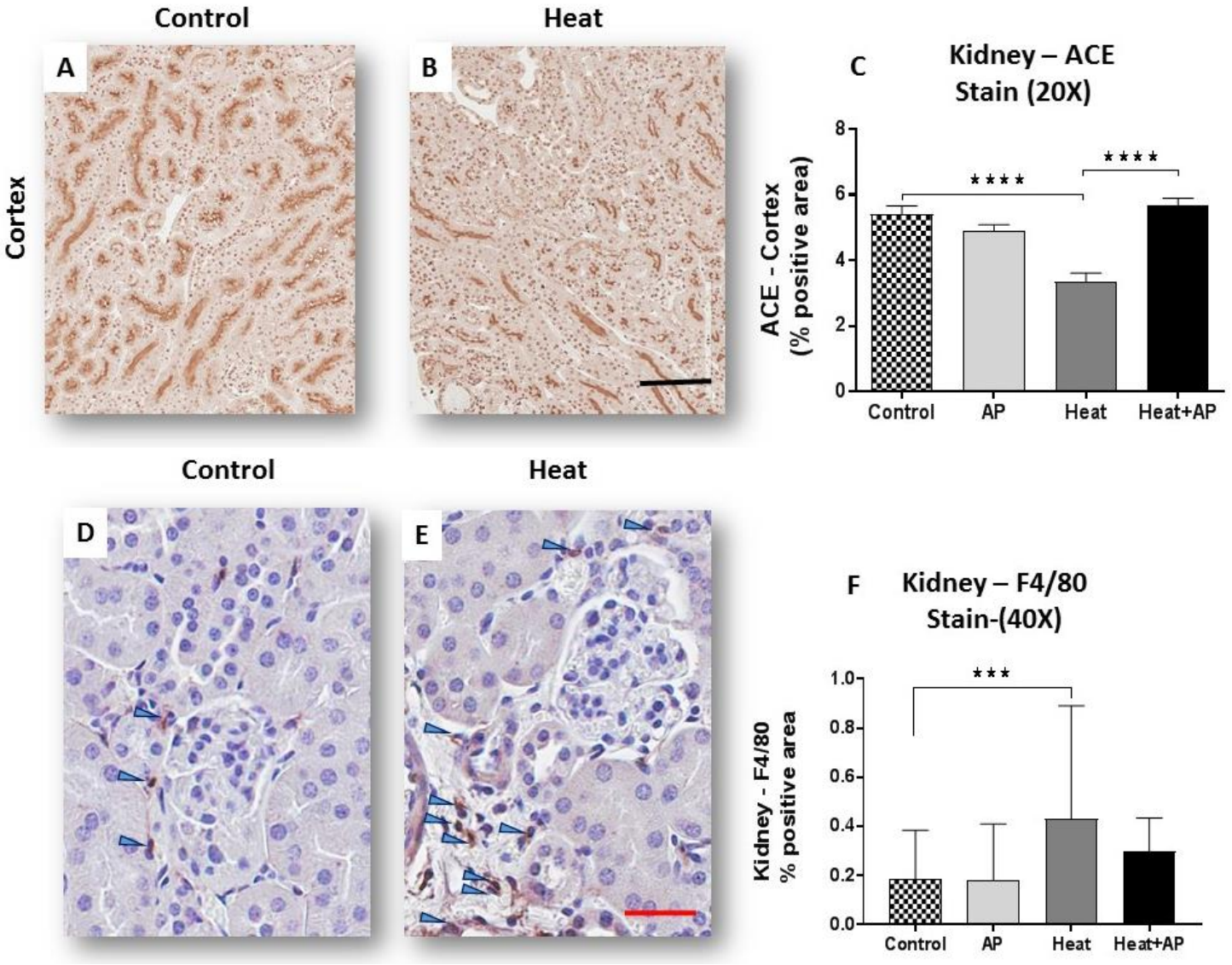
b. **Role of Uric acid in Heat Stress Nephropathy**. There has been a lot of excitement that uric acid might be a toxin for kidney disease, both acute and chronic, and there was a major symposium on this topic held by the National Kidney Foundation in 2016. One of the main interests has been in the role of uric acid in heat stress induced kidney disease, and especially among sugarcane workers in Mesoamerica. One of our aims for the DOD proposal was to investigate the role of uric acid in a murine model of heat stress and dehydration induced kidney disease. We therefore performed a study in which wild type mice were subjected to recurrent heat stress (39.5°C for 30 min, 7 times daily, for 5 weeks) with or without allopurinol treatment and were compared to control animals with or without allopurinol treatment. Kidney histology, liver histology, and renal function were examined.

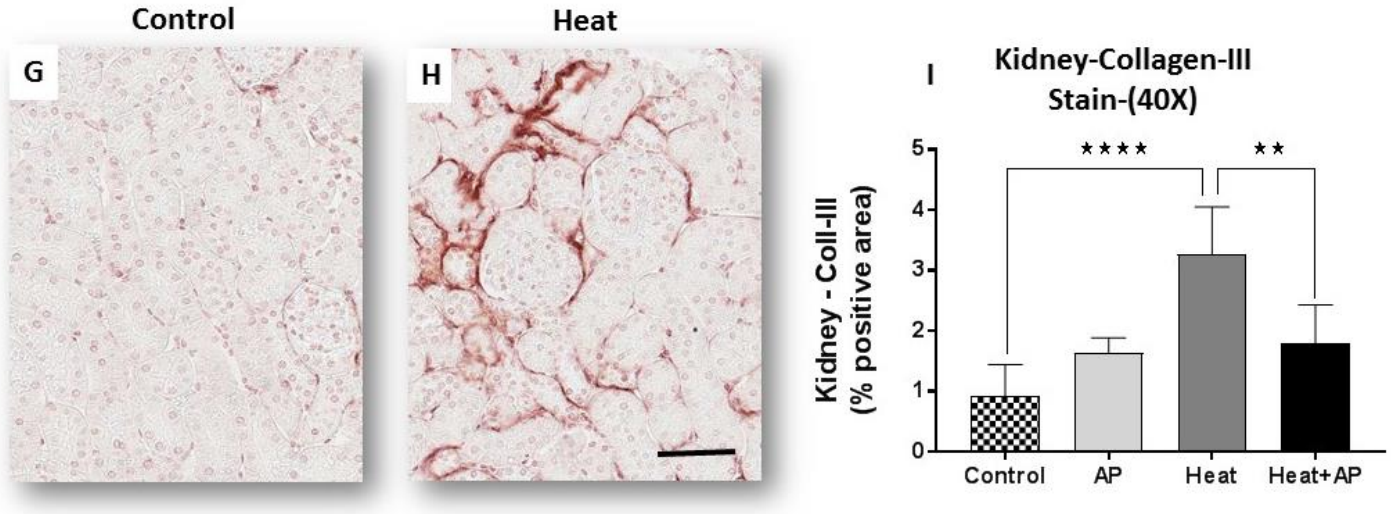
The procedure was well tolerated, and over the 5 weeks of the study, there was no mortality. The heat stress group developed evidence for chronic renal injury, with worsening renal function, and the development of modest interstitial fibrosis in their kidneys. Remarkable, the allopurinol treated group showed better renal function and less fibrosis. Shown is a table of the renal functional changes.

Parameters	Control	AP	Heat	Heat+AP	Anova P values
Serum Creatinine (ug/ml)	0.86 ± 0.08 **a**	0.81 ± 0.09	0.97 ± 0.18 **b**	0.77 ± 0.12	<0.0268
Urine NGAL (ng/ml)	34 ± 3.8 **a**	28.7 ± 10.2	60.1 ± 15.9	60.8 ± 15.8	<0.0001
Urine Albumin (µg/ml)	46.6 ± 12.9 **a**	39.9 ± 13.7	59.5 ± 6.8	55.8 ± 5.4	< 0.001

a, p< 0.05 comparing **control** vs **heat**; b, p<0.05 comparing **heat** to **heat plus allopurinol**

As shown below, there was an improvement in much of the histology in the allopurinol treated group. For example, there was preservation of the proximal tubule in heat stressed mice given allopurinol, as shown by evidence of preserved brush border (ACE staining). This was accompanied by less inflammation (F4/80 staining) and less fibrosis (collagen III staining) (see figures). This was also associated with less uric acid in the kidney. One of the other surprise findings was that there was liver fibrosis also developing in these mice, and allopurinol corrected this as well.





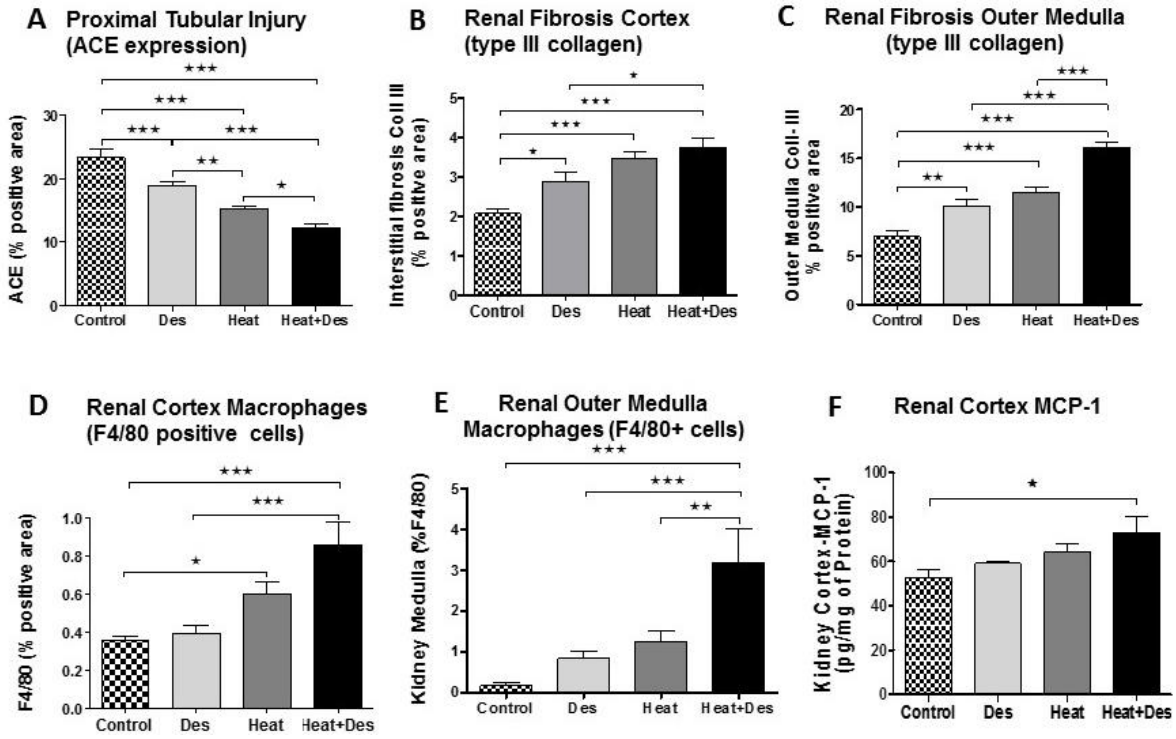
Key: AP, allopurinol alone in absence of heat stress. Heat, heat stress and recurrent dehydration for 5 weeks. Heat + AP, heat stress with allopurinol. Statistics: *p<0.05, **p<0.01; ***p<0.001

The discovery that allopurinol can prevent chronic kidney disease from heat stress was met with great interest both by the journal (American Journal of Physiology) where it is presently in revision, and also by the research community, and the International Society of Nephrology is currently developing protocols for administering allopurinol to sugarcane workers at risk for developing heat stress nephropathy. One of the most important findings in this grant, this discovery is also likely to have a significant impact on military who deployed to hot areas of the world, such as the Middle East.

c. **Vasopressin Drives Kidney Disease in Heat Stress Nephropathy via the Vasopressin 2 Receptor.** There has been some data that chronic elevations of vasopressin may actually not be good for kidney function, and the paradigm that vasopressin may be similar to the renin angiotensin system, in that acute stimulation is usually a protective mechanism, but that excessive stimulation over time may be injurious. One of our hypotheses was to investigate if chronic heat stress and dehydration may lead to chronically elevated levels of vasopressin that might cause kidney disease. This hypothesis had not been tested and was consider one of the key objectives of the DOD proposal. In addition, an unknown aspect was to determine if the effect of vasopressin to cause kidney disease was through the receptor involved in urinary concentration (V2 receptor) or whether it involved other receptors (specifically V1a and V1b), and this was also part of our DOD proposal.

To investigate the role of V2, we administered a V2 agonist (desmopressin) to mice undergoing heat stress according to the DOD protocol. Treatment was extended for 5 weeks. The striking finding was that desmopressin treatment was associated with worsening tubulointerstitial fibrosis, with more proximal tubular injury, fibrosis in both the cortex and medulla, and a greater inflammatory response. There was also an interesting glomerular finding, with worse albuminuria and more mesangiolytic changes. The importance of the finding is that it shows

that vasopressin is like the renin angiotensin system, with acute increases aiding urinary concentration, but persistent stimulation of the V2 receptor causing chronic kidney disease. This emphasizes the importance of this pathway in the pathogenesis of kidney disease and the key importance of adequate hydration for those working in hot climates. The desmopressin paper was published in American Journal of Physiology last year.[11]

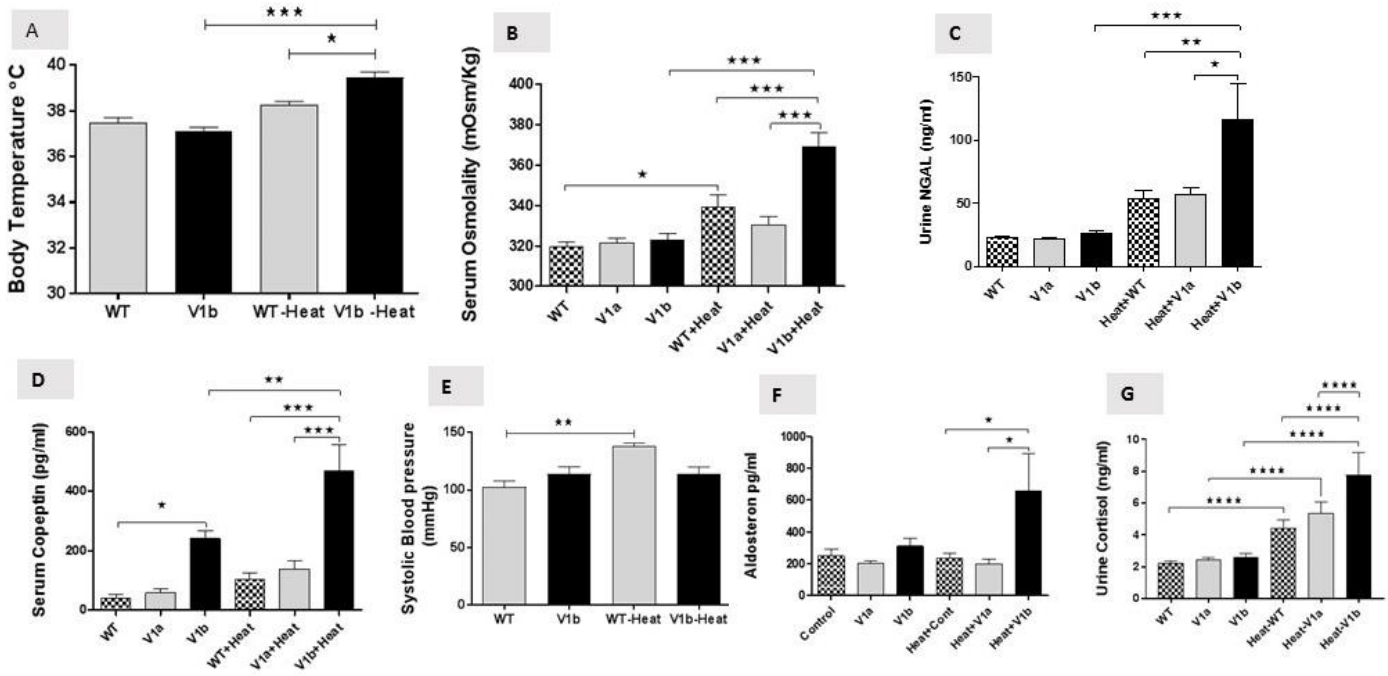


Key: DES, desmopressin alone in absence of heat stress. Heat, heat stress and recurrent dehydration for 5 weeks. Heat + Des, heat stress with desmopressin. Statistics: *p<0.05, **p<0.01; ***p<0.001

d. Vasopressin Receptor 1b has protective effects in heat stress induced kidney damage. There are two other important vasopressin receptors, V1a, which is on blood vessels and may regulate blood pressure, and V1b, which is involved in acute responses (release of ACTH, glucagon) but also helps regulate temperature. The latter might be considered important in subjects in heat stress, and so we had also included studies in our DOD proposal to evaluate the role of these two receptors.

The V1a and V1b receptor knockouts are commercially available from Jackson labs (0255101 and 006160, respectively) and we obtained them and bred them for our studies. We also have completed our studies using the V1a and V1b knockout mice. Here we have had another exciting finding. Specifically, the V1b KO mouse was found to be very sensitive to heat, and showed a greater rise in body temperature compared to the wild type mouse on heat exposure (Fig A). This was associated with greater water loss (from sweat) during heat exposure, resulting in greater serum osmolarity (Fig B), which was associated with greater urinary NGAL (which can mark prerenal

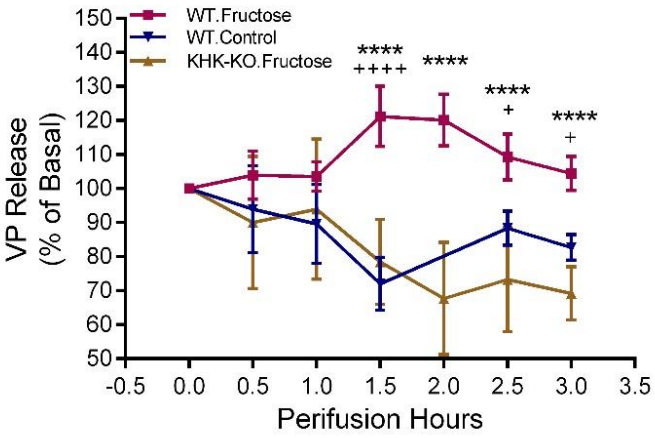
dehydration) (Fig C) , greater albuminuria and greater copeptin (vasopressin) response (Fig D). Systolic BP was consequently lower (Fig E) and resulted in compensatory increases in aldosterone and urinary cortisol (F,G).



The study analysis is being completed and will be submitted soon. However, the exciting finding is the discovery that the temperature regulatory function of vasopressin 1b receptor is a clinically significant function and that it protects animals from heat stress. This shows that simple blocking of vasopressin will likely be injurious for people with recurrent heat stress.

e. A Novel Mechanism for Stimulating Vasopressin Secretion.

Another aspect of Aim 2 was to dissect out the fructose-vasopressin axis during acute dehydration and to include hypothalamic explant studies to look at direct control of hypothalamic function as it related to the role of fructose in stimulating vasopressin. We completed this study and discovered that vasopressin is regulated by fructose at the level of the hypothalamus. Specifically, we first discovered that the aldose reductase and fructokinase are both expressed in the supraoptic nucleus (SON) of the hypothalamus where vasopressin is synthesized. We then showed that acute dehydration and chronic heat stress/dehydration activate the aldose



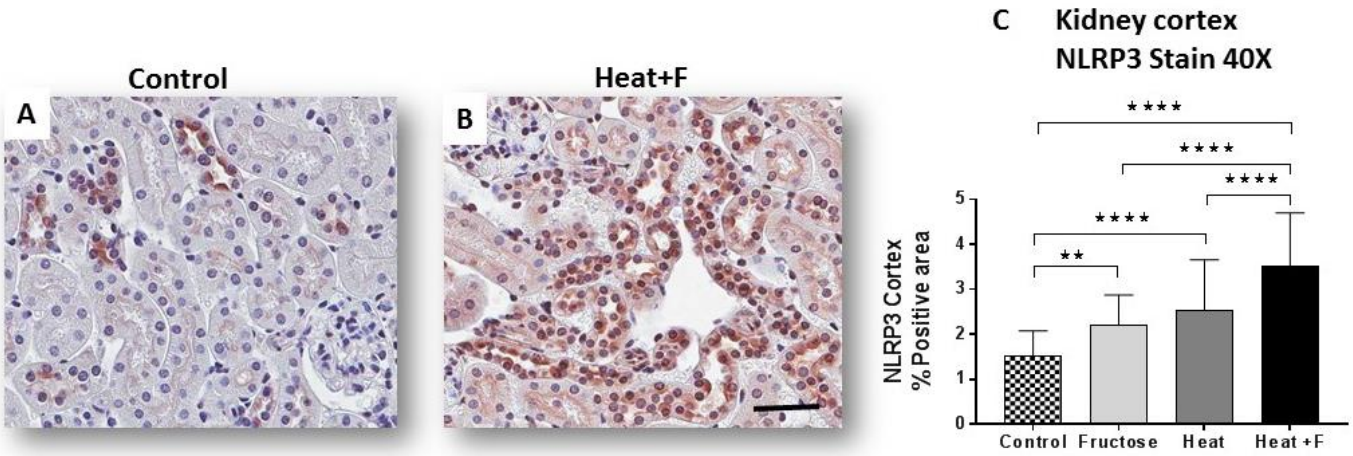
reductase and fructokinase pathway in the hypothalamus in association with vasopressin production. Then, in accordance with our DOD proposal, we performed hypothalamic explants and showed that fructose could stimulate vasopressin directly, but not in hypothalamic from animals that could not metabolized fructose (fructokinase knockout mice, or KHK-KO fructose). In these mice no vasopressin was released from the fructose, showing the mechanism for vasopressin release requires the ability to metabolize fructose and is not simply an osmotic effect. This discovery was viewed as major in the world of water regulation and vasopressin, and the paper was published in the Journal of Neurophysiology.[8]

f. Role of fructose rehydration in heat stress nephropathy

As mentioned, aim 3 of our DOD proposal was aimed at comparing rehydration solutions, and specifically to determine if rehydration with water was superior to fructose-containing solutions. To evaluate this, we compared rehydration with equivalent amounts of water or fructose (15%) containing water to mice for 5 weeks. Our primary finding was that rehydration with fructose made kidney disease worse, with more proximal tubular brush border loss, more inflammation, and greater fibrosis.

Parameters	**Control**	**Fructose**	**Heat**	**Heat+F**	**Anova p values**
Serum Creatinine (µg/ml)	0.38 ± 0.1	0.36±0.1	0.69 ± 0.2	0.72 ± 0.2	P<0.001
Urine Albumin (µg/mg of Cr)	26.1±5.1	27.5±7.5	54.3±28.1	65.5±32.3	P<0.05
Proximal tubule Brush border % (ACE)	10.3±2.3	6.6±1.8	6.9±1.6	4.5±3.1	P<0.0001
Inflammation (F4/80) %	0.13±0.3	0.13±0.2	0.25±0.3	0.55±0.6	P<0.05
Interstitial Fibrosis (Coll-III) %	0.23±0.1	0.28±0.3	0.43±0.3	0.53±0.4	P<0.0001

One of the more interesting findings was the induction of inflammasomes by the fructose rehydration, including greater expression of NLRP3, caspase 3 and interleukin-18. The observation that inflammasomes might be



induced with heat stress had not previously been known, and the observation it was worsened with fructose rehydration was consistent with the greater inflammatory response.

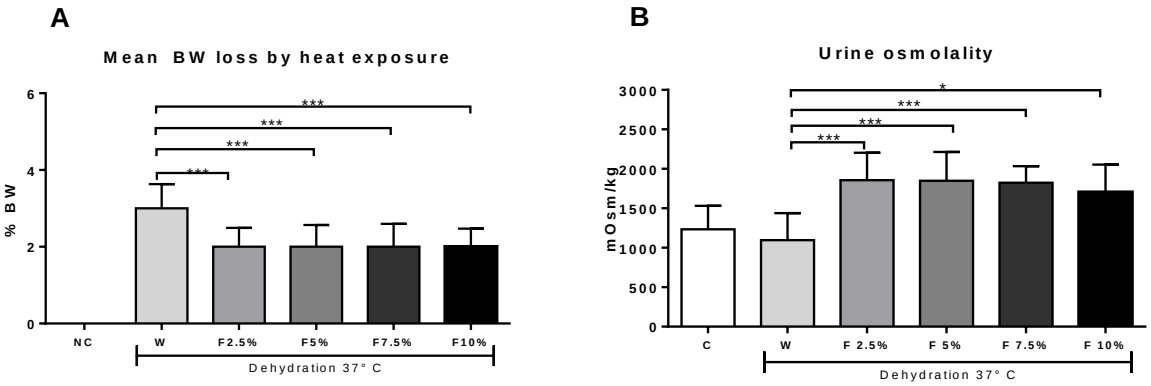
The net effect of this study was to document that the type of rehydration fluid may make a key difference in the outcome of the kidney in subjects experiencing recurrent heat stress. Since the World Health Organization rehydration packets contain fructose, these raises concerns that current hydration practices may need to be reassessed from the standpoint of their potential effects on the kidney.

The fructose study is currently under review at BMC Nephrology.

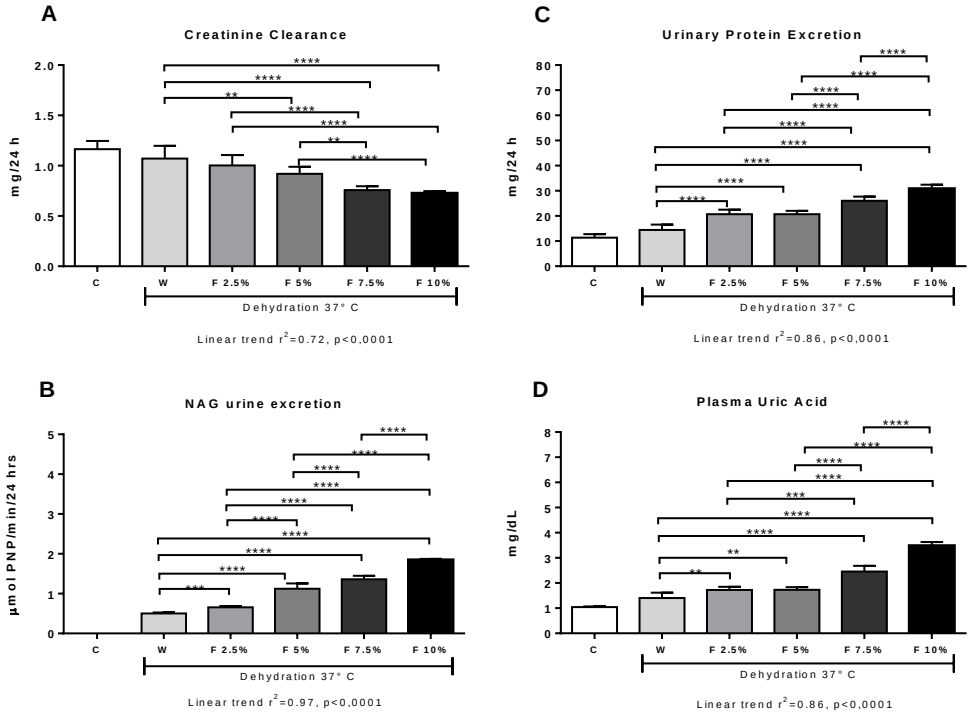
We also had a subcontract with Dr Laura (Gaby) Sanchez-Lozada in the Department of Renal Pathophysiology at the Institute Nacional Cardiologia in Mexico City. Her participation was delayed because the funds were received in her lab until July 2016. She and her group previously showed that after mild heat stress rehydration with a fructose-containing beverage further activated vasopressin and aldose reductase-fructokinase pathways that resulted in greater oxidative stress and injury to the kidney. Therefore, the purpose of these experiments was to explore the effect of different concentrations of fructose in the rehydration fluid and correlate them with parameters of kidney damage. We used the model of heat stress in rats previously developed in our lab that consists in exposing rats to 37°C during one hour in a closed chamber. This manipulation induces a loss of 2-3% of body weight after heat exposure, which is approximately the volume of fluids that extenuating work or exercise under heat is induced in humans. As rats have preference for sweet-flavored beverages, the studies were conducted offering the same volume that rats intake of water only at night (from 17:00 pm to 9:00 am) using the following

fructose concentrations 2.5, 5, 7.5 and 10%. By this approach we could tightly control the fructose intake in rats. A group of the normal non-dehydrated rats and water offered as hydration fluid was included as a reference.

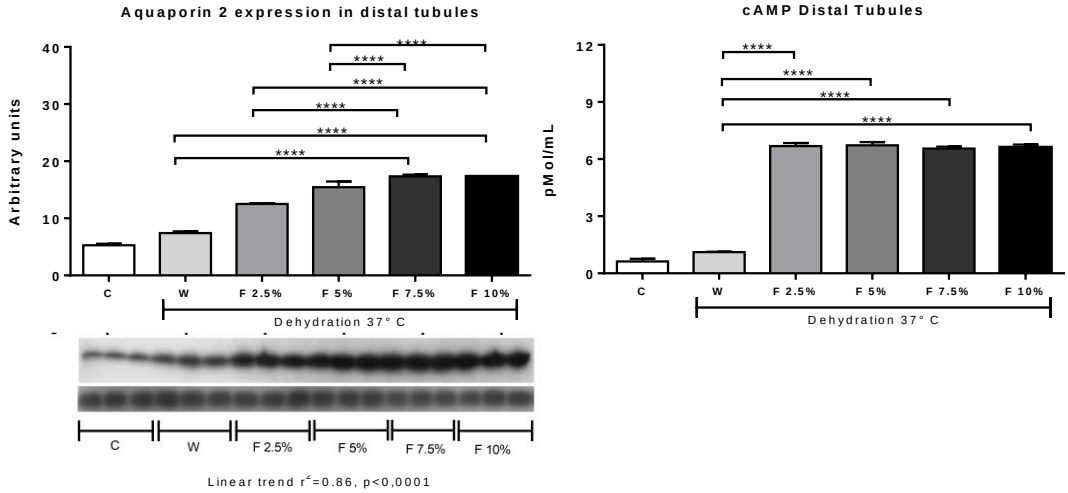
As expected, fluid intake was similar among all groups (A). Interestingly, heat stress induced a greater mean loss of body weight in water rehydrated rats in comparison with all groups receiving different concentrations of fructose. Despite this fact, urine osmolality was increased in fructose rehydrated groups in comparison to water rehydrated group (B), suggesting that body water might be sequestered in a body compartment in fructose rehydrated rats.



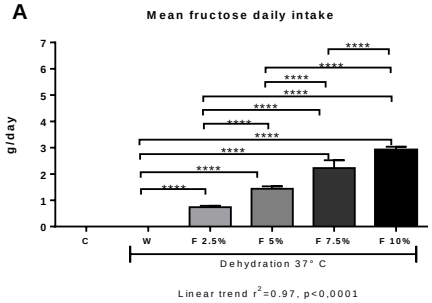
Fructose content in rehydration fluid inversely correlated with renal creatinine clearance (A), and positively correlated with the urinary excretion of the marker of tubular damage NAG (B) and proteins (C). Uric acid is a by-product of fructose metabolism such as also depicted a positive correlation with fructose concentrations in rehydration fluid.



Plasma vasopressin (copeptin) also depicted a dose-response relationship with fructose dose. In addition, we evaluated the expression, by western-blot, of aquaporin 2 as a proxy of renal V2 receptor activation in homogenates of distal tubules. A dose-response in aquaporin 2 expression was also observed, suggesting that increased concentrations of renal vasopressin had a dose-response effect in the activation of V2 receptor. cAMP is one of the second messengers after V2 receptor activation by vasopressin, interestingly, in all doses of fructose, and putatively of vasopressin, we obtained a similar concentration in cAMP in distal tubule homogenates, which was significantly increased in comparison to rats that were rehydrated with water. That observation suggests that other pathways activating adenylyl cyclase might be also overactivated in heat stressed rats rehydrated with fructose. In this regard, it is known that an assortment of adenylyl cyclase isoforms activated by diverse stimulus are expressed along distal nephron.



As animals drunk a similar fluid volume in all groups, fructose intake correlated with its content in the different beverages (A) correlated with fructose intake. These data suggest that fructose content in rehydration fluids should be lower than 2.5% when there is an additional stimulus for the activation of vasopressin pathway, such as heat stress, as fructose has an amplificatory effect on vasopressin secretion. The synergistic effect of fructose content in rehydration fluids had detrimental consequences in renal function. These data were presented at the American Society of Nephrology Kidney Week 2018. Currently we are completing the studies to prepare the manuscript.



4) Other achievements.

The development of the Floxed fructokinase mouse will have major implications for future studies that extend well beyond the DOD grant. First, it will allow us to explore the role of CNS fructokinase in sugar craving, and of islet cell fructokinase in the development of diabetes. The liver fructokinase can also be targeted and may be responsible for the fatty liver and insulin resistance. Of course, targeting the kidney might provide the insight of how dehydration causes kidney injury via the fructokinase pathway.

In addition, during the last two years we have determined that the epidemic of kidney disease due to dehydration and heat stress is present in multiple countries and, working with climatologists, we linked it to climate change. The paper was published in CJASN and led to numerous interviews on NPR and elsewhere. We also reviewed the evidence vasopressin and fructokinase are involved in the mechanisms of dehydration, and this paper was published in JASN. The identification of these ongoing epidemics, coupled with the research in this grant identifying potential mechanisms by which kidney damage may be occurring, should be of great benefit in the development of clinical trials to prevent chronic kidney disease that is occurring from recurrent dehydration. Indeed, as mentioned, there are now several groups planning studies to investigate the protective role of allopurinol in subjects repeatedly exposed to heat who are developing kidney disease.

5) Challenges/ stated goals not met.

Research funded by the grant was initially delayed with some administrative challenges during the first year that related to getting approval from the IACUC and ACCURO for the dehydration studies in our animals, but all was eventually approved. There were also some challenges with getting our subcontract with Mexico approved by the two Universities—however, this was also resolved. Finally, the generation of the KHK-LoxP mouse took longer than expected. Nevertheless, almost all of the projects were finished, analyzed, and have been submitted and/or are published. The exception if the fructokinase-tissue specific knockout in the kidney—while we have the mouse, we have not been able to perform the heat stress study with it yet, but this is planned this coming year.

c. **What opportunities for training and professional development has the project provided?**

The grant was not meant to provide training or professional development, and so at one level there is nothing to report. However, the studies are opening up information on the role of dehydration in kidney disease. This last year our work was selected to be the primary topic for a meeting in Aspen organized by the Aspen Global Change Institute, and our work has also led to invited presentations at the American Society of Nephrology in Chicago in November 2016 and at the European Dialysis and Transplant meeting in Madrid in June 2017. We were also invited to speak at the American Public Health Association Conference in Denver in September 2016. I have also been asked to speak in several graduate school courses, including a T32 funded course at the University of Colorado on our work to help in the education of physicians and graduate students interested in the effects of heat

stress on health. Thus, our work led to presentations to medical students, graduate students and fellows, and this in turn has initiated interest from a number of individuals who now want to pursue studies on the mechanisms driving kidney disease from heat stress.

d. **How were the results disseminated to communities of interest?**

There have been multiple news reports on our work, including in NPR and various journals. The discovery of multiple epidemics of chronic kidney disease among workers in Central America, Mexico, India and Sri Lanka has been international news, and the strongest evidence to date is that it is mediated by heat stress and dehydration. Our work linking it with vasopressin and fructose metabolism has often been quoted. The work on allopurinol, as well as on fructose rehydration, was presented at the American Society of Nephrology meeting in New Orleans, resulted in much excitement in the field and is stimulating similar studies in humans. The data on vasopressin has not yet been published or presented, but the finding that V1b receptor is protective will almost certainly stimulate interest from renal physiologists, neurophysiologists, and physicians. .

e. **What do you plan to do during the next reporting period to accomplish the goals?**

Not applicable as this is the final report. However, we will still conduct the study in which mice that have been manipulated so that they cannot metabolize fructose specifically in their kidney are evaluated for their response to heat stress.

4. IMPACT:

a. **What was the impact on the development of the principal discipline(s) of the project?**

Our studies document for the first time the importance of chronic recurrent dehydration as a mechanism for causing chronic kidney disease. This has led to huge interest from the academic nephrology societies and public media around the world, especially as it is becoming apparent that there are epidemics of chronic kidney disease emerging in Central America, India and Bangladesh, Sri Lanka, and elsewhere. As such, there is great interest in our research, especially with the realization that this disease may increase with global warming and worldwide water shortages. The discoveries made from the DOD proposal have had significant impact. First, our research has identified uric acid as a key mediator of heat stress associated renal disease, and this has led to several groups planning clinical trials. Our work showing a yin-yang effect of vasopressin is very interesting, for the V2 receptor pathway is deleterious whereas the V1b pathway is protective. This could lead to new trials involving manipulating the vasopressin receptors as opposed as to blocking vasopressin per se. The scientific discovery that vasopressin is regulated by fructose at the level of the hypothalamus will lead to changes in the textbooks on the importance of fructose in vasopressin synthesis and release. The discovery that fructose in rehydration fluid

can worsen heat stress associated renal disease is likely to impact the sports drink industry and rehydration solutions, and our work has already led certain sugarcane industry leaders, like Pantaleon, to change their recommended hydration practices for their workers. Indeed, our discovery of a cutoff of 2.5 percent fructose content may well lead to changes in recommendations for fructose content in sports drinks. Thus, it is evident that the impact of the DOD studies is quite significant, as it is elucidating mechanisms of kidney damage from recurrent dehydration. With the recent reports that there have been over 40,000 deaths from these epidemics worldwide, the importance of our work is considered high.

b. **What was the impact on other disciplines?**

Our work is identifying global warming as a factor driving kidney disease, and as such is causing some concern as the first epidemic disease induced by climate change. This has generated interest in many other disciplines (general medicine) as well as by the lay public (NPR, BBC). The Aspen Global Change Institute invited our group as well as climatologists, anthropologists, epidemiologists, and interested physicians to a week-long conference on climate change and health in 2016 in which our work received much attention. The DOD grant is exploring the mechanisms, and as the results are generated, will likely have an impact on our understanding of the cause of disease and the importance and risks associated with dehydration. We believe our reports will identify chronic kidney disease as one of the first major human diseases due to global warming.

c. **What was the impact on technology transfer?**

Our work is heightening interest into the correct ways for rehydration when in the dehydrated state. Indeed, our work is generating concern that the current use of WHO sugar rehydration packages for dehydration as they contain fructose which might be injurious to the kidney. While this data is not generating new intellectual property, it is generating interest in current approaches to the treatment of dehydration.

d. **What was the impact on society beyond science and technology?**

Our studies could lead to a reevaluation of sports drinks and rehydration packages for the hydration of individuals who are exposed to heat and dehydration.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

No significant changes were made other than a minor revision of the Statement of Work plan as it related to Aim 3. The proposed change to the SOW was submitted and approved by the DOD in the summer of 2015.

Actual or anticipated problems or delays and actions or plans to resolve them

There were two delays in the first year of the study—the first was obtaining animal care protocol by both our local IACUC and ACCURO. This however, was approved in mid-2015. The second delay related to obtaining an agreement between my institution (University of Colorado) and our collaborator's institution (Cardiologia University in Mexico City) as it related to the subcontract. This also was resolved in early 2016.

Changes that had a significant impact on expenditures

There were no changes that had impact on expenditures during the three years the grant was active.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

6. PRODUCTS

1. PRODUCTS:

Publications, conference papers, and presentations

1. Milagres T, Lanaspa MA, Garcia G, Ishimoto T, Andres Hernando A, Kuwabara M, Jensen T, Sato Y, Sanchez-Lozada LG, Johnson RJ, Roncal C. Rehydration with Fructose worsens Dehydration-induced Renal Damage. BMC Nephrol (submitted)

2. Roncal-Jimenez CA, Milagres T, Andres-Hernando A, Kuwabara M, Jensen T,Song Z, Bjornstad P, Garcia GE, Sato Y, Sanchez-Lozada LG, Lanaspa MA and Johnson RJ. The effects of exogenous desmopressin on a model of heat stress nephropathy in mice Am J Physiol Renal Physiol 2017; 1312:F418-F426.

3. Song Z, Roncal-Jimenez CA, Lanaspa-Garcia M, Oppelt SA, Kuwabara M, Jensen T, Milagres T, Andres-Hernando A, Ishimoto T, Garcia GE, Johnson G, MacLean PS, Sanchez-Lozada LG, Tolan DR, Johnson RJ. Role of Fructose and Fructokinase in Acute Dehydration Induced Vasopressin Gene Expression and Secretion in Mice. J Neurophysiol. 2017 Feb 1;117(2):646-654

4. Roncal-Jimenez CA, Sato Y, Milagres T, Andres Hernando A, Garcia G, Bjornstad P, Butler Dawson J, Sorensen C, Newman L, Krisher L, Madero M, Glaser J, Garcia-Trabanino, Jarquin Romers E, Song Z, Jensen T, Kuwabara M, Rodriguez-Iturbe B, Sanchez-Lozada LG, Lanaspa MA, Johnson RJ. Experimental Heat Stress Nephropathy and Liver Injury are Improved by Allopurinol. Am J Physiol Renal Physiol (in revision)

Books and book chapters

Nothing to report

Other publications, conference papers, and presentations. These are papers related to our research but not specifically funded by the DOD.

1. Glaser J, Lemery J Rajagapolan B, Diaz HF, Garcia-Trabanino R, Taduri G, Madero M, Amarasinghe M, Abraham G, Anutrakulchai S, Jha V, Stenvinkel P, Roncal-Jimenez C, Lanaspa MA, Correa-Rotter R, Sheikh-Hamad D, Burdmann EA, Andres Hernando A, Milagres T, Weiss I, Kanbay M, Wesseling C, Sanchez-Lozada LG, Johnson RJ. Climate Change and the Emergent Epidemic of Chronic Kidney Disease from Heat Stress in Rural Communities: The Case for Heat Stress Nephropathy. CJASN 2016 Aug 8;11(8):1472-83
2. Johnson RJ, Stenvinkel P, Jensen T, Lanaspa MA, Roncal C, Song Z, Bankir L, Sanchez-Lozada LG. Metabolic and Kidney Diseases in the Setting of Climate Change, Water Shortage, and Survival Factors. JASN 2016 Aug;27(8):2247-56
3. Johnson RJ. Pro: Heat stress as a potential etiology of Mesoamerican and Sri Lankan nephropathy: a late night consult with Sherlock Holmes Nephrol Dial Transplant. 2017 Apr 1;32(4):598-602
4. Wesseling C, Aragon A, Gonzalez M, Weiss I, Glaser J, Rivard CJ, Roncal-Jimenez C, Correa-Rotter R, Johnson RJ. Heat stress, hydration and uric acid: a cross-sectional study in workers of three occupations in a hotspot of Mesoamerican nephropathy in Nicaragua. BMJ Open 2016 Dec 8;6(12):e011034
5. Roncal-Jimenez C, Garcia-Trabanino R, Barregard L, Lanaspa MA, Wesseling C, Harra T, Aragon A, Grases F, Jarquin E, Gonzalez MA, Weiss I, Glaser J, Sanchez-Lozada LG, Johnson RJ. Heat Stress Nephropathy from Exercised-Induced Uric Acid Crystalluria: a Perspective on Mesoamerican Nephropathy? Am J Kid Dis 2016 Jan;67(1):20-30
6. García-Arroyo FE, Cristóbal M, Arellano-Buendía AS, Osorio H, Tapia E, Soto V, Madero M, Lanaspa MA, Roncal-Jiménez C, Bankir L, Johnson RJ, Sánchez-Lozada LG. Rehydration with Soft Drink-like Beverages Exacerbates Dehydration and Worsens Dehydration-associated Renal Injury. Am J Physiol Regulatory, Integrative and Comparative Physiology 2016 Jul 1;311(1):R57-65
7. Roncal-Jimenez CA, Garcia-Trabanino R, Wesseling C, Johnson RJ. Mesoamerican Nephropathy or Global Warming Nephropathy? Blood purification 2016; Jan 15; 41(1-3):135-138.

Website(s) or other Internet site(s)

We do not have a website that details our results of the DOD study

Technologies or techniques

We have not introduced any new technologies or techniques.

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Our research is generating great interest in the role of dehydration and global warming in chronic kidney disease. Specifically, by identifying vasopressin and uric acid as potential targets, it may stimulate interest in the use of vasopressin antagonists or uric acid lowering therapy (e.g. allopurinol) to prevent renal injury from heat stress.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Richard J Johnson*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	0000-0003-3312-8193
Nearest person month worked:	2 months
Contribution to Project:	*Dr. Johnson has overseen the design, performance and analysis of the studies*
Funding Support:	*DOD funding*

Name:	*Zhilin Song*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6 months
Contribution to Project:	*Dr. Song is performing studies to identify the effects of dehydration on the vasopressin axis in the hypothalamus.*
Funding Support:	*DOD funding*

Name:	*Carlos Roncal*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6 months
Contribution to Project:	*Mr. Roncal is performing all of the experiments in Aim 2 and overseeing their analyses.*
Funding Support:	*DOD funding*

Name:	*Laura G Sanchez-Lozada*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 month

Contribution to Project:	*Dr. Sanchez Lozada oversaw the administrative aspects of executing the subcontract.*
Funding Support:	*DOD funding*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

For Dr. Richard Johnson, M.D., the following new projects have been initiated:

NIDDK 1RO1DK109408-01A1 (Johnson) 04/01/2016-3/30/2021 2.4 calendar

Dietary Salt has an Unrecognized Role in Modulating $300,000

Energy Intake and Metabolic Syndrome

Goal: To investigate the role of salt in inducing obesity and metabolic syndrome

NIDDK RO1 DK108859-01 (Lanaspa) 04/01/2016-3/30/2021 0.6 calendar

$279,325 Targeting fructokinase, endogenous fructose production and purine degradation for the prevention and treatment of hereditary fructose intolerance

Goal: To investigate the role of fructokinase in mice with aldolase B deficiency using aldolase B KO, fructokinase KO and lowering uric acid. This grant has no overlap and does not involve generation of inhibitors.

NIH R42 DK104432-01 (Johnson) 09/20/2017-08/31/2019

Year 1 289,363 Year 2 769,627 1.2 Calendar months

Scientific Merit and Feasibility of Fructokinase Inhibition for Obesity (STTR phase II application)

Goal: To develop fructokinase inhibitors for the development of obesity.

There have been no changes in active support for Carlos Roncal, Zhilin Song or LG Sanchez Lozada.

What other organizations were involved as partners?

We have a collaboration supported by the DOD with Gaby Sanchez-Lozada at the Cardiologia University as part of our DOD proposal.

Organization Name: Instituto Nacional de Cardiología Ignacio Chavez

Location of Organization: Mexico City, Mexico

Partner's contribution to the project: Will be responsible for completion of aim 3.

Financial Support; Was supported by DOD grant (subcontract)

In-kind support: None

Facilities :None

Collaboration None (other than our DOD collaboration with Dr Sanchez-Lozada)

Personnel exchanges None

Other. None

8. SPECIAL REPORTING REQUIREMENTS

1. **COLLABORATIVE AWARDS:** Not applicable (the DOD grant was a single PI awarded grant)
2. **QUAD CHARTS:** Not applicable

9. APPENDICES:

References

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9. 2. Cirillo, P, Gersch, MS, Mu, W, Scherer, PM, Kim, KM, Gesualdo, L, Henderson, GN, Johnson, RJ, Sautin, YY: Ketohexokinase-dependent metabolism of fructose induces proinflammatory mediators in proximal tubular cells. *J Am Soc Nephrol,* 20**:** 545-553, 2009.

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Papers currently under review

1. Milagres T, Lanaspa MA, Garcia G, Ishimoto T, Andres Hernando A, Kuwabara M, Jensen T, Sato Y, Sanchez-Lozada LG, Johnson RJ, Roncal C. Rehydration with Fructose worsens Dehydration-induced Renal Damage. BMC Nephrol (submitted)

2. Roncal-Jimenez CA, Sato Y, Milagres T, Andres Hernando A, Garcia G, Bjornstad P, Butler Dawson J, Sorensen C, Newman L, Krisher L, Madero M, Glaser J, Garcia-Trabanino, Jarquin Romers E, Song Z, Jensen T, Kuwabara M, Rodriguez-Iturbe B, Sanchez-Lozada LG, Lanaspa MA, Johnson RJ. Experimental Heat Stress Nephropathy and Liver Injury are Improved by Allopurinol. Am J Physiol Renal Physiol (in revision)

Rehydration with Fructose worsens Dehydration-induced Renal Damage

Tamara Milagres[1], Miguel A. Lanaspa[1], Gabriela Garcia[1], Takuji Ishimoto[1], Ana Andres Hernando[1], Masanari Kuwabara[1], Thomas Jensen[1], Yuka Sato[1], Jason Glaser[2], Laura G. Sánchez-Lozada[3], Richard J. Johnson[1], Carlos Roncal Jimenez[1]

[1]Division of Renal Diseases and Hypertension, University of Colorado, Aurora, CO; [2]La Isla Network, Léon, Nicaragua;[3] Laboratory of Renal Physiopathology, Instituto Nacional de Cardiologia, Ignacio Chavez, Mexico City

Contact: Carlos Roncal Jimenez, Division of Renal Diseases and Hypertension, 12700 East 19th Ave 7th Floor Offices of the Nephrology Division, Mail Stop C281, University of Colorado Anschutz Medical Campus, Aurora CO USA 80045 Carlos.Roncal@ucdenver.edu

Running Title: Fructose and Inflammasomes

Abstract; Word Count 2400; Figures 4, Tables 2

Abstract

Background: We reported that recurrent heat stress and dehydration induces chronic kidney disease (CKD) due to polyol-induced fructose generation with metabolism by fructokinase. Here we test if rehydration with fructose worsens renal injury compared to water alone.

Methods: Mice were recurrently exposed to heat (39.5 C^0 for 30 min/h, 5 times daily for 5 wks) with rehydration consisting of 6 ml each night of water (n=7) or fructose (10%, n=7), and were compared to control mice on water (n=7) or fructose (n=7). Various markers of renal injury were assessed.

Results: Compared to control animals, there was a progressive worsening of renal injury (inflammation and fibrosis) with fructose alone, heat stress alone, and heat stress with fructose rehydration ($P<0.01$ by ANOVA). The combination of heat stress with rehydration with fructose was associated with increased intrarenal expression of the inflammasome markers, NLRP3 and IL-18, compared to heat stress alone. In addition, heat stress with or without fructose was associated with increased expression of caspase -3 and monocyte chemoattractant protein-1 levels. Fructose administration was also associated with an increase in serum copeptin levels (a biomarker of vasopressin) and elevated copeptin was also observed in mice undergoing heat stress alone.

Conclusions: These studies suggest that heat stress may activate intrarenal inflammasomes leading to inflammation and renal injury, and provide evidence that rehydration with fructose may accelerate the renal injury and inflammatory response.

Key words: Fructose, Mesoamerican Nephropathy, Heat stress, Vasopressin

Introduction

Heat stroke is a concern throughout the world, not only as a complication of exercise (such as marathon running) and of military campaigns, but also for individuals working outside in both rural settings and in hot urban environments [1-6]. Epidemics of heat stroke associated with extreme heat events (heat waves) were reported in Chicago in 1995 and Europe in 2003 [1, 7-10]. Indeed, heat stroke is likely to become more common as temperatures rise, for now nearly 75% of heat extremes can be attributed to climate change [11, 12].

Most emphasis on heat stroke relates to the acute risk for multi-organ failure and death. However, heat stroke has also been associated with residual liver and kidney damage [13, 14]. More recently, we have raised the hypothesis that chronic recurrent heat stress might be able to induce chronic kidney disease (CKD), and we have hypothesized this may be of relevance to the epidemics of CKD being observed in among agricultural workers in various hot communities in Central America, India, Sri Lanka and elsewhere [15-17].

To investigate this hypothesis, we developed a model of CKD induced in mice by repetitive heat stress and dehydration over a 5 week period. Using this model, we found that if mice were hydrated during the heat stress period, they did not develop renal injury, whereas if they hydrated after the heat/dehydration period, that they developed mild tubulointerstitial kidney disease [18]. In this same study we found evidence that heat stress/dehydration caused activation of aldose reductase in the kidney, an osmotically-sensitive enzyme that converts glucose to sorbitol, which is then further metabolized in the renal cortex to fructose. Fructose is known to be metabolized in the proximal tubule by the enzyme fructokinase, and this results in a drop in ATP levels, the generation of uric acid, and a burst of oxidative stress that can cause tubular injury [19]. Indeed, mice lacking fructokinase were protected from the renal injury of recurrent heat stress [18].

Given that many individuals working in the rural communities hydrate themselves with sugary beverages, including soft drinks and fruit juices, one might hypothesize that rehydration with fructose-containing solutions might exacerbate our model of renal injury of heat stress and dehydration. In this regard, recently Garcia-Arroyo et al developed a model of mild heat stress and dehydration in rats in which rats were exposed for only one hour a day to heat (36º C) followed by 2 hours rehydration with water or a soft drink mixture containing 11 percent fructose-glucose mixture [20]. This mild model of dehydration does not result in chronic interstitial fibrosis but does manifest with tubular injury and intrarenal oxidative stress [20]. Importantly, this study found that rehydration with a sugary beverage resulted in worse tubular injury and oxidative stress.

We therefore tested the hypothesis that fructose might accelerate heat stress induced CKD in our model. Furthermore, recently it has been recognized that subjects developing CKD in Central America often have evidence of intermittent acute renal injury with a predominance of inflammatory cells on their biopsy [17, 21]. This was viewed as counter to the generally held idea that the acute renal injury might be more similar to acute

tubular necrosis. However, heat stroke is also associated with a pronounced renal inflammatory infiltrate [17]. We thus investigated if heat stress might be associated with an increase in inflammasome-associated proteins, and tested the hypothesis that this might be worsened by fructose ingestion.

Materials and Methods

Animals. Male 8-week old C57BL/6J wild type mice (WT, Jackson Lab, Bar Harbor, ME) were maintained in temperature- and humidity-controlled specific pathogen-free conditions on a 14-hour dark/10-hour light cycle and fed regular diet *ad libitum* (Harlan Teklad; no. 2918, containing 58 percent carbohydrate, 24 percent protein, and 18 percent fat), with free access to tap water.

Experimental Design. The experimental study consisted of four groups (n=7 each): WT control (**Control**); WT fructose (**F**); WT mice exposed to heat and water restriction with water rehydration (**Heat**); and WT mice exposed to heat and water restriction with fructose-containing drinking water (10% fructose) as the rehydration fluid (**Heat+F**). The heat stress-dehydration protocol has been previously reported and consisted of placement of mice in a heat chamber set at 39.5º Celsius for 30 minutes per hour over 8 hours (7 episodes/day), 5 days/week, for 5 weeks [18]. In between heat periods mice were allowed to rest at room temperature. Fluids were restricted during the heat/dehydration period, and then 6 ml of rehydration fluid per day were provided per mouse afterwards, consisting of water in the **Heat** group and fructose water in the **Heat + F** group. This degree of rehydration fluid was calculated based on the overall 24 hour fluid intake required by mice of this age and weight in our past study [18]. Mice were sacrificed after the 5^{th} cycle of heat stress at the end of the 5th week by anesthesia (isoflurane) with cardiac exsanguination and collection of serum, puncture of the bladder for collection of urine, and removal of kidney tissues for analyses.

All experiment were conducted with adherence to the NIH Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado.

Biochemical analyses. Urine was collected at the end of the study from the bladder and were analyzed for urine osmolality using the Freezing-Point Osmometry method (Advance Instruments Micro Osmometer-Norwood Massachusetts USA). Serum and urine creatinine concentrations were analyzed with the high-performance liquid chromatography–tandem mass spectrometry method [22]. Urinary albumin was measured using a Colorimetric Albuwell Assay Kit (Exocell Co., PA). Serum copeptin levels were measured using Elisa enzyme- linked immunosorbent Assay kit for copeptin (Cloud-Clone Corp., Houston TX) Serum fructose was

measured using the EnzyChrom Fructose Assay Kit (Bioassay Systems, Hayward, CA) and serum uric acid was measured using QuantiChrom Uric Acid assay kit (BioAssay Systems).

Kidney tissue samples were homogenized in a buffer containing 2 mM MgCl2, 1 mM EGTA, 1 mM DTT, and 0.5% (vol/vol) Triton X-100. Homogenates were centrifuged at 13,000 rpm for 10 min (4°C) and protein in the collected supernatant quantified (BCA protein assay Kit - Pierce, Rockford, IL). Renal fructose and uric acid levels were assessed by utilizing the Bioassay Systems kits (see above) and values normalized to protein lysate concentration. Renal MCP-1 was measured on cortical lysates by Mouse MCP-1 ELISA Kit (Invitrogen ThermoFisher – Grand Island NY-USA) and corrected for total protein (Pierce, BCA protein assay Rockford, IL-USA).

Histology. Tissues were fixed in 10% formalin or methyl Carnoy's and embedded in paraffin. Three μm sections were stained with periodic acid-Schiff reagent (PAS). On Axial sections of the kidney, (Aperio Technologies, Vista, CA). Proximal tubular brush border loss was assessed by immunostaining for angiotensin-converting enzyme (ACE) using antimurine ACE antibody; (R&D, Minneapolis, MN). Renal fibrosis was determined by immunohistochemical staining for type III collagen with a goat anti-type III collagen antibody (Southern Biotech, Birmingham-AL-USA). Macrophage infiltration were detected using Rat Anti-Mouse F4/80 antibody (Serotec, Oxford, UK). The number of positive cells for F4/80 was counted using an Aperio scanner (Aperio Technologies, Vista, CA). The software allows color recognition and positive cells were identified as % positive color saturation at 20 magnification in a blinded manner using at least 15 fields for each biopsy sample. For the fibrosis and ACE staining, digital images at 20X magnification of approximately 10 cortical fields were analyzed using Image scope of Aperio Scanner software. The percent positive area was determined as the 3,3-diaminobenzidine-positive pixel values per negative pixel values in each section.

Statistical analysis. Statistical analyses were performed using the GraphPad Prism version 6 (GraphPad Software, Inc. La Jolla, CA). All data are presented as the mean ± s.e.m. Independent replicates for each data point (n) are identified in figure legends and one-way analysis of variance (ANOVA) with the Bonferroni post hoc test used for individual comparisons. We also used the Student's t-test to specifically compare heat alone (**Heat**) to heat plus fructose (**Heat+F**) groups since the experiment was designed specifically for this comparison (see comments in limitation section). $P < 0.05$ was regarded as statistically significant.

Results

General Findings. Recurrent heat stress and dehydration were induced in two groups of mice over a 5 week period in which the only difference was that one group received water rehydration (Heat) and the other

group received the same amount of drinking water containing fructose (10%) (Heat+F). We also studied two groups of mice not exposed to heat stress/dehydration administered the same dose of water or fructose-containing water. While each day the mice undergoing heat stress and dehydration would lose significant weight (about 10% body weight), they were able to fully rehydrate at night and no mortality was observed. At the end of the study there were significant differences in weight by one way ANOVA, with the fructose control showing higher weight compared with the heat stress/dehydration group ($p=0.003$) and heat stress/dehydration groups plus fructose group ($p<0.001$), but with no differences between the other two groups (**Table 1**).

Effect on Dehydration Markers. Both groups of mice exposed to heat stress and dehydration showed increases in serum osmolarity, and serum vasopressin (determined by measuring serum copeptin) levels (**Table 1**). Serum copeptin levels increased more in the fructose rehydrated control group compared to the water rehydrated group (**Table 1**), consistent with studies suggesting fructose can stimulate vasopressin release [23, 24]. The effect of fructose to increase serum copeptin was also greater in the Heat+F compared to the Heat alone group when analyzed by Student's t-test ($p=0.049$).

Effect on Renal Function. Serum creatinine was elevated in both heat stress groups compared to the control and fructose alone groups (**Table 2**). Also, the groups exposed to heat stress and dehydration plus fructose had a tendency for elevated albuminuria compared to the control group ($P=0.055$) or fructose group ($P=0.051$) by Bonferroni post hoc test. However, no significant differences were noted between the heat stress and heat stress plus fructose group.

Effect on Renal Histology. Compared to controls animals, mice exposed to heat stress and dehydration (heat) and (Heat+F) showed evidence for focal proximal tubular injury (**Fig.1)** with loss of proximal tubular brush border as noted by immunostaining for angiotensin converting enzyme (ACE) (**Fig 2A-C, Table 2**). The mice receiving fructose as rehydration (Heat+F) showed more severe loss of proximal tubule brush border compared to heat alone (HEAT) ($P<0.001$) (**Table 2**). This was associated with interstitial macrophage infiltration (F4/80 positive cells) (**Fig 2D-F, Table 2**). Interstitial fibrosis (noted by collagen III staining) was also increased in the renal cortex of heat stressed mice, but there was no difference between HEAT and HEAT+F groups (**Table 2**, **Fig. 2 G-I**).

Effect on Sorbitol, Fructose and Uric acid Levels in the Renal Cortex. The polyol (aldose reductase) pathway has been reported to be induced in the renal cortex of this heat-stressed model in mice [18]. Sorbitol, the product of aldose reductase, was elevated in both groups of mice exposed to heat stress and tended to be higher in the fructose treated group (HEAT+F) compared to heat stress alone (HEAT), although this was not significant (**Fig. 3A**). We also measured fructose levels in the renal cortex. Surprisingly, fructose levels in renal cortex were not higher in the heat stress group compared to the control group and other groups (**Fig. 3B**). Fructose is

metabolized in the proximal tubule by fructokinase with the generation of uric acid [19]. Uric acid levels were higher in the renal cortex of heat stress and fructose (HEAT+F) group compared to control group (**Fig. 3C**).

We have previously reported that fructose generated uric acid can stimulate MCP-1 production in proximal tubular cells [19, 25]. Soluble uric acid has also been reported to stimulate inflammasome responses in tubular cells [26]. We therefore measured the renal cortical content of the chemokine, MCP-1, (**Fig. 3D**) as well as markers of the inflammasome, including NRLP3 (**Fig. 4A-C**); Caspase 3 (**Fig. 4D-F**), and the macrophage product, IL-18 (**Fig 4G-I)**. Specifically, NLPR3, caspase 3 and IL-18, and all were higher in the mice with heat stress that were rehydrated with fructose as opposed to water, with NLRP3 and IL-18 levels were significantly higher in the HEAT+F group compared to HEAT alone.

Discussion

Heat Stress and dehydration are well known to cause a 'prerenal' type of kidney injury, but it has historically been considered to not be associated with tubular injury and to be fully reversible with hydration. Recently some have proposed that recurrent heat stress and dehydration may be a cause of CKD, based primarily on epidemiological studies performed on sugarcane workers who are developing CKD in Central America [27-30]. To explore this possibility, our group developed a model of CKD in mice induced by repetitive exposure to heat stress over a 5 week period [18]. These mice developed chronic tubulointerstitial inflammation and interstitial fibrosis, both which are characteristic of the CKD in Central America (Mesoamerican Nephropathy)[31].

The mechanisms by which repetitive heat stress may cause kidney damage has been a 'hot' topic. Some studies suggest a role for vasopressin [32, 33], which has long been known to induce some tubular and glomerular injury [34]. Indeed, a recent study found that conivaptan, which blocks V1a and V2 receptors, could reduce renal injury associated with recurrent heat stress [33]. The role of fructose has also been considered, especially since heat stress/dehydration can activate the polyol (aldose reductase-sorbitol dehydrogenase) pathway in the kidney, resulting in endogenous fructose production. Indeed, mice that cannot metabolize fructose easily (fructokinase knockout) appear to be protected from heat stress induced renal disease [18]. Furthermore, in a model of mild heat stress, rehydration with fructose resulted in intrarenal oxidative stress and mild tubular injury [35].

Here we evaluate the effect of equal hydration with fructose containing water or regular water in our chronic model of heat stress. The primary finding was that fructose worsened the renal tubular injury (as noted by ACE staining) and resulted in marked tubulointerstitial inflammation and a tendency for worse renal fibrosis (although the latter two findings were not significant). In addition, we found evidence for an increase in

inflammatory pathways in the renal cortex, including the chemokine MCP-1 and inflammasome markers (NLRP3, caspase 3 and IL-18). Thus, dietary fructose should be viewed as a potential amplifier of the renal injury process, and the mechanism is tightly linked with stimulation of inflammatory processes.

We recognize that the renal fibrosis observed in our fructose-rehydrated mice was not significantly worse than heat stress alone, and as such we cannot conclude that fructose definitely worsens heat stress nephropathy. It is possible that a difference would have been shown if the study had been conducted over a longer period. Nevertheless, the overall trend is suggestive that fructose, and perhaps other sugary beverages, are not likely to benefit and tend to worsen renal injury associated with heat stress and dehydration.

We should state a limitation about statistics. We conducted Bonferroni correction for multiple comparisons. However, since our primary interest was whether there was worse renal injury with heat stress with rehydration with fructose compared to heat stress with water rehydration, we also performed a statistical assessment of these two groups using the t-test. We understand this method is not fair if we accounted for the effect of both heat and fructose. However, we believe that this t-test is acceptable in this case because our aim is to know only the effects of fructose intake under the heat stress condition.

In conclusion, rehydration with fructose in a model of heat stress and dehydration was associated with significant increased renal inflammation and a tendency for worse renal outcomes. We recommend additional studies to investigate what concentrations of fructose and glucose may provide benefit for maintaining glucose stores while at the same time minimizing the risk for renal injury in the dehydrated patient.

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Disclosure: Dr Johnson, Lanaspa and Sanchez-Lozada are members of Colorado Research Partners LLC that is making inhibitors of fructose metabolism. Dr Johnson is also on the Scientific Board of Kibow, Inc

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Tables

Table 1 General Characteristics of Experimental Groups.

Parameters	Control	Fructose	Heat	Heat+F	Anova p values
Body weight Basal (g)	29.5 ± 2.1	29.3 ± 2.0	30.0 ± 2.3	30.2 ± 2.1	NS
Body weight After 5 W (g)	31.3 ± 3.6	33.3±2.1	28.8 ± 1.9	28.3 ± 1.2	<0.001
Serum Osmolality (mOsm/Kg)	307 ± 6.1	312.2 ± 6	343.6±16.8	340±29.8	<0.0001
Serum Copeptin (pg/ml)	10 ± 3.9	25.3 ± 1.4	18.4 ±4.5	25±4.2	<0.0001

Body weight after 5 weeks: The fructose group has a higher weight at the end of the study compared to the heat group ($p<0.01$) and heat plus fructose group ($p<0.001$) by Bonferroni post hoc test.

Serum Osmolality: The control group has significantly lower serum osmolality than the heat group ($p<0.001$) and heat plus fructose group ($p<0.01$) by Bonferroni post hoc test. The fructose group is significantly lower than heat group ($p<0.05$).

Serum Copeptin: The control group has significantly lower serum copeptin than the fructose group ($p<0.001$), heat group ($p<0.01$) and heat plus fructose group ($p<0.001$) by Bonferroni post hoc test ($p<0.05$). The fructose group is significantly higher than heat group ($p<0.05$). The heat group tends to be lower than heat plus fructose group ($p=0.08$) by Bonferroni post hoc test, but it shows significant difference by t-test ($p=0.049$).

Table 2 Effect of Fructose with or without Heat on Renal Disease

Parameters	Control	Fructose	Heat	Heat+F	Anova p values
Serum Creatinine (μg/ml)	0.38 ± 0.1	0.36±0.1	0.69 ± 0.2	0.72 ± 0.2	P<0.001
Urine Albumin (μg/mg of Cr)	26.1±5.1	27.5±7.5	54.3±28.1	65.5±32.3	P<0.05
Proximal tubule Brush border % (ACE)	10.3±2.3	6.6±1.8	6.9±1.6	4.5±3.1	P<0.0001
Inflammation (F4/80) %	0.13±0.3	0.13±0.2	0.25±0.3	0.55±0.6	P<0.05
Interstitial Fibrosis (Coll-III) %	0.23±0.1	0.28±0.3	0.43±0.3	0.53±0.4	P<0.0001

Serum Creatinine: The serum creatinine of the control group is significantly lower than heat group (P<0.05) and heat plus fructose group (P<0.05) by Bonferroni post hoc test. Fructose group is significantly lower than heat group (P<0.05) and heat plus fructose group (P<0.05).

Urine Albumin: The control group tends to have lower urinary albumin than the heat plus fructose group (P=0.055) and the fructose group tends to be lower than heat plus fructose group (P=0.051) by Bonferroni post hoc test.

Proximal tubule Brush border: The control group has significantly higher proximal tubular brush border (ACE positivity) than the fructose group (P<0.001), heat group (P<0.001) and heat plus fructose group (P<0.001) by Bonferroni post hoc test. The fructose group is significantly higher than heat plus fructose group (P<0.01). Heat group is significantly higher than heat plus fructose group by t test as well (P<0.001).

Inflammation: The control group has significantly lower interstitial inflammation than the heat plus fructose group (P<0.05) by Bonferroni post hoc test.

Interstitial Fibrosis: The control group has significantly lower interstitial fibrosis than the heat group ($P<0.01$) and heat plus fructose group ($P<0.001$) by Bonferroni post hoc test.

Figure Legends

Figure 1. Tubular Damage. Proximal tubular injury, consisting of loss of brush border with tubular dilation (black arrows in PAS stain, 200) is observed in fructose-treated controls (Figure B), and is worse in mice exposed to heat stress/dehydration with water rehydration (Figure C) or fructose (D) (PAS, 200).

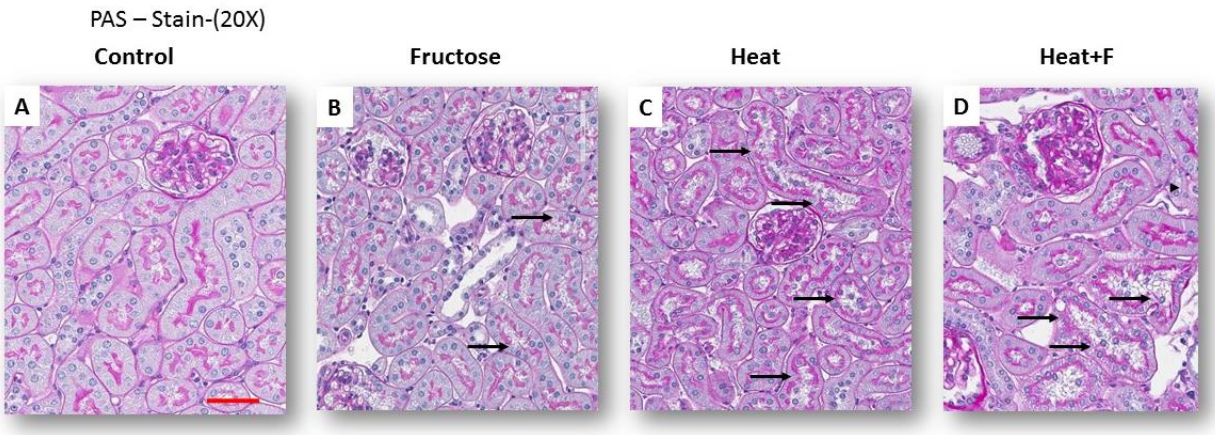


Figure 2. Renal injury. Proximal tubular injury was assessed by staining for proximal tubular brush border for ACE (Fig.2A-C, 200), interstitial inflammation was determined by staining for F4/80 positive macrophages (Figure 2D-F, 400x), and interstitial fibrosis by staining for type III collagen (Fig 2G-H, 200). Heat stress was associated with loss of proximal tubular brush border, interstitial inflammation and interstitial fibrosis which tended to be worse with fructose rehydration. Scale bar 50µm. Statistical analysis was performed using ANOVA with Bonferroni correction for individual comparisons. Key: *, p<0.05; **, p<0.01; ***p<0.001, ****, p<0.0001

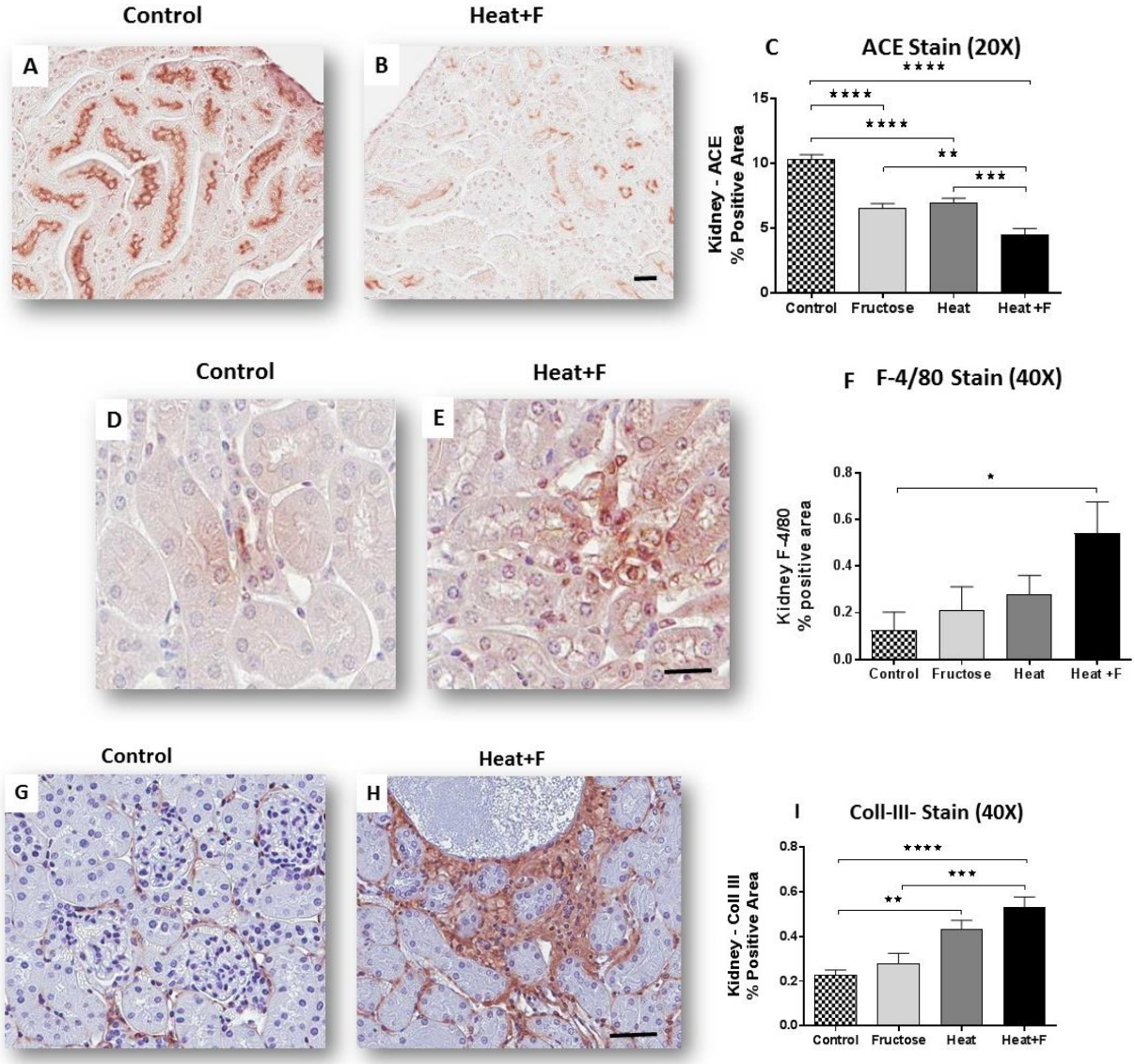


Fig. 3 Renal Cortex Sorbitol, Fructose and Uric Acid Levels. (A) Sorbitol levels were increased in heat plus fructose (Heat+F) exposed mice compared to wild type controls by Bonferroni post hoc test (p=0.013). The Fructose control group also had lower levels of sorbitol than the Heat+F group (p=0.015). **(B)** There was no significant differences of fructose in renal cortex for individual group comparisons by Bonferroni post hoc test.

(C) Heat+F group had higher uric acid in renal cortex compared to the control by Bonferroni post hoc test (p=0.042). **(D)** Heat+F group had higher MCP-1 in renal cortex than control group (P=0.013) and fructose group (P=0.016) by Bonferroni post hoc test. Key: *p<0.05

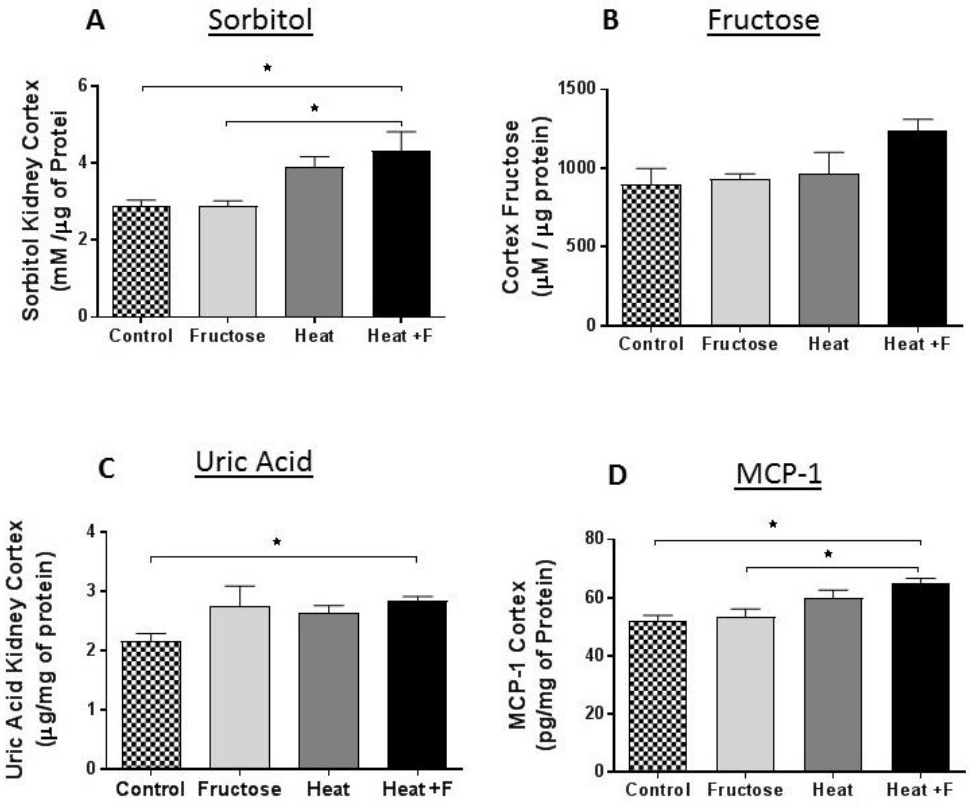
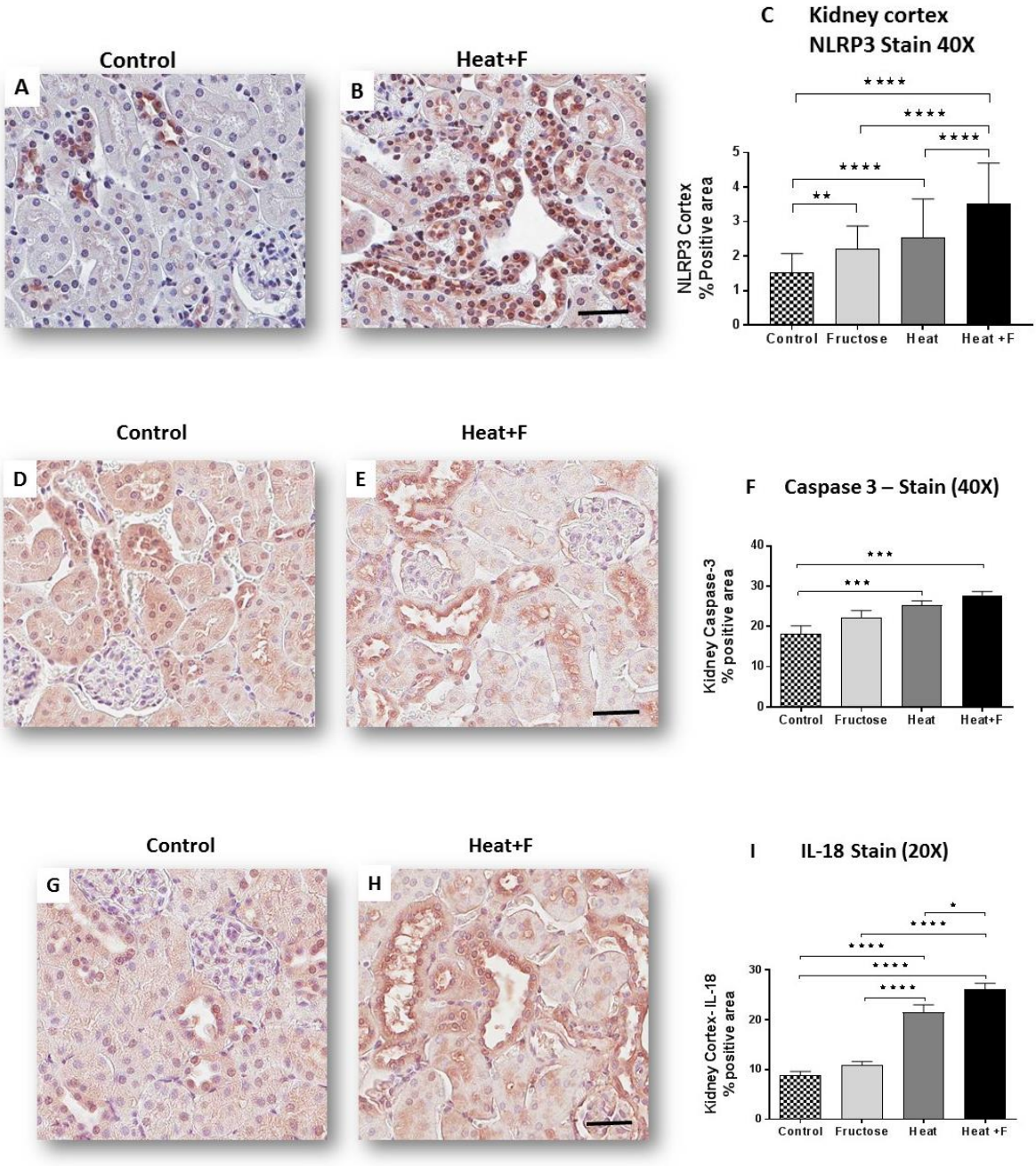


Figure 4. Inflammatory response and renal injury: Renal cortical NLRP3 expression showed stepwise increase with the highest level in the HEAT+F group by quantitation of % NLRP3 positive stain area (Fig.4A-C, 400x, Scale bar 50µm). Caspase 3 expression was higher in heat stress + Fructose (Heat+F) group compared to the other groups (Fig.4D-F, 400x Scale bar 50µm). Similar findings were shown for Interleukin 18 (Fig.4G-I, 400x, Scale bar 50µm). Key: *, p<0.05; **, p<0.01; ***p<0.001, ****, p<0.0001



Experimental Heat Stress Nephropathy and Liver Injury are Improved by Allopurinol

Carlos A. Roncal-Jimenez[1], Yuka Sato[1], Tamara Milagres[1], Ana Andres Hernando[1], Gabriela García[1], Petter Bjornstad[2], Jaime Butler Dawson[3], Cecilia Sorensen[3], Lee Newman[3],Lyndsey Krisher[3], Magdalena Madero[4], Jason Glaser[5], Ramón Gárcía-Trabanino[6], Emmanuel Jarquin Romero[7], Zhilin Song[1], Thomas Jensen[1], Masanari Kuwabara[1], Bernardo Rodriguez-Iturbe[8], Laura Gabriela Sanchez-Lozada[9], Miguel A Lanaspa[1*], Richard J Johnson[1*]

[1]Division of Renal Diseases and Hypertension, University of Colorado Anschutz Medical Campus, Aurora CO; [2]Department of Pediatric Endocrinology, University of Colorado School of Medicine, Aurora, Colorado, [3]Center for Health, Work and Environment, Colorado School of Public Health, [4]Dept of Nephrology, Institution Nacional de Cardiologia, Ignacio Chavez, Mexico City; [5]La Isla Network, Léon, Nicaragua; [6]Centro de Hemodiálisis, San Salvador, El Salvador, San Salvador, El Salvador; [7]Agency for Agricultural Health and Development (Agdysa), San Salvador, El Salvador; [8] Nephrology Service Hospital Universitario and IVIC-Zulia , Maracaibo, Venezuela; [9]Laboratory of Renal Physiopathology, Instituto Nacional de Cardiologia, Ignacio Chavez, Mexico City.

*Co-last authors

Correspondence to: Carlos Roncal, Division of Renal Diseases and Hypertension, University of Colorado Anschutz Medical Campus, Aurora CO 80045 carlos.roncal@ucdenver.edu

Running Title: Allopurinol Protects Heat Stress Nephropathy

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Disclosure: Dr Johnson, Lanaspa and Sanchez-Lozada are members of Colorado Research Partners LLC that is making inhibitors of fructose metabolism. Dr Johnson is also on the Scientific Board of Kibow, Inc

Abstract

Background: An epidemic of chronic kidney disease (CKD), termed Mesoamerican nephropathy, has been observed in Central America among workers in the sugarcane fields. One hypothesis is that the CKD may be caused by recurrent heat stress and dehydration, and potentially by hyperuricemia. Accordingly, we developed a murine model of kidney injury associated with recurrent heat stress. In the current experiment, we tested whether allopurinol treatment provides renal protection against recurrent heat stress and dehydration by blocking the production of uric acid.

Methods: Wild type mice were subjected to recurrent heat stress (39.5°C for 30 min, 7 times daily, for 5 weeks) with or without allopurinol treatment were compared to control animals with or without allopurinol treatment. Kidney histology, liver histology, and renal function were examined.

Results: Heat stress conferred both kidney and liver injury. Kidneys showed loss of proximal tubules, infiltration of monocyte/macrophages, and interstitial collagen deposition, while livers of heat stressed mice displayed an increase in macrophages, collagen deposition and myofibroblasts. Allopurinol provided significant protection, and improved renal function in the heat stressed mice. The renal protection was associated with reduction in intrarenal uric acid concentration and heat shock protein 70 expression.

Conclusions: Heat stress-induced renal and liver injury can be protected with allopurinol treatment. We recommend a clinical trial of allopurinol for individuals developing renal injury in rural areas of Central America where the epidemic of chronic kidney disease is occurring.

Key Words: Mesoamerican nephropathy, uric acid, chronic kidney disease of unknown etiology, heat stress, heat shock

Introduction

Epidemics of chronic kidney disease (CKD) of unknown etiology have emerged in rural areas in Central America, Sri Lanka, India and other sites (2, 18). The primary histologic finding is chronic tubulointerstitial disease, often associated with some degree of global glomerulosclerosis (41). The etiology remains unknown, although some believe it may relate to exposure of toxins, heavy metals, or subacute infections (18, 42). Our group has postulated that it may be the consequence of recurrent heat stress, resulting in renal injury driven by effects of hyperosmolarity (via activation of the polyol pathway), heat, vasopressin, and/or hyperuricemia (9, 15, 30), and we have established experimental models in which some of these pathways can be shown to induce chronic tubulointerstitial damage (11, 12, 31, 33).

One potential mechanism for renal injury could relate to chronic hyperuricemia and/or uricosuria. Hyperuricemia is common among subjects working in the sugarcane fields in Central America and is associated with increased risk for the development of Mesoamerican nephropathy (13, 23, 40). Hyperuricemia has also been shown experimentally to have important intrarenal hemodynamic effects, including reducing effective renal blood flow, increasing glomerular pressure, in addition to to inducing intrarenal oxidative stress, local inflammation and tubular cell injury and activation (20, 34-37). Therefore, allopurinol is commonly prescribed to subjects with Mesoamerican nephropathy by the local nephrologists.

Accordingly, we tested the hypothesis that allopurinol treatment might attenuate heat stress induced renal injury in a murine model. Whereas liver injury is not commonly reported in subjects with Mesoamerican nephropathy, heat stroke and heat stress are known to induce liver damage (6, 14, 21). Thus, we also evaluated the effect of heat stress on the liver and the potential protective effect of allopurinol therapy.

Materials and Methods

Experimental Design: Eight week old male wild type (WT) mice (C57BL/6, Jackson Laboratory, Bar Harbor, ME) were used. The mice were kept under temperature and humidity-controlled specific pathogen-free conditions and maintained on a 14-h dark/10-h light cycle. Mice were allowed *ad libitum* access to normal laboratory chow (Harlan Teklad; 2920X, Madison, WI) and water *at libitum* until the start of the experiment. Single mice were housed per cage and all were treated simultaneously under the same conditions. The experimental protocol was approved by the University of Colorado Animal Care and Use Committee.

The heat stress and dehydration protocol consist of placing mice in a heated environment (Isotemp- Fisher Scientific Incubator, Dubuque, IA) at 39.5 °C for 30 minutes each hour for a total of 7 hours, 5 days a week for a total duration of 5 weeks (31, 33). This procedure resulted in the animal sweating (on its paws) with an acute loss

of 10 to 13% of body weight by the end of the heating period each day, in absence of hydration. However, following the heat period the animals were allowed to drink, and they restored their weight loss by hydrating through the night (31, 33).

Mice were divided into four groups (n=7 each). Group 1 consisted of control mice that were never dehydrated, and water was provided *ad libitum* (Control). Group 2 consisted of control mice that were provided allopurinol solution (32 mg/ Kg) in their drinking water in the absence of heat treatment or dehydration (AP group). Group 3 consisted of mice that were dehydrated but provided water for 12 hours each night (Heat); and Group 4 were mice that were dehydrated but provided allopurinol solution 12 hours at night (Heat+AP). Standard mouse chow (Harlan Teklad, 2920X) was provided day and night (except during the periods of heat exposure).

Blood Testing: Mice were sacrificed at the end of the dehydration period (that is after the 7 hours dehydration protocol at 5 weeks) by anesthesia and cardiac exsanguination. Serum uric acid was measured by EnzyChrom Uric Acid Assay kit (BioAssay System, Hayward, CA); this method utilizes 2,4,6-tripyridyl-s-triazine that forms a blue colored complex specifically with iron in the presence of uric acid, and the intensity of the color, measured at 590 nm, and is directly proportional to the uric acid concentration. Serum creatinine concentration was measured by high-performance liquid chromatography–tandem mass spectrometry (24, 38). Serum osmolality was measured using the Advance Micro Osmometer (Model 3300, Advanced Instruments, Norwood, MA). Serum copeptin was measured using (Mouse Enzyme Immunosorbent Assay Kit; Cloud – Clone Corp; Houston TX.77084-USA).

Urine Testing: Urine was collected at the end of dehydration period directly by bladder puncture and was analyzed for urine osmolality using the Advanced Micro Osmometer (Model 3300, Advanced Instruments, Norwood, MA); urinary neutrophil gelatinase-associated Lipocalin (NGAL) was measured using the mouse neutrophil gelatinase-associated Lipocalin Elisa (Quantikine ELISA Kit, R&D Systems, Inc., Minneapolis, MN) Urine creatinine concentration was analyzed by high-performance liquid chromatography–tandem mass spectrometry (38).

Histology: The kidneys were removed, and tissue sections were fixed in 10% formalin or methyl Carnoy's fixative solutions and embedded in paraffin with cutting sections of 3 µm thickness. Immunostaining was performed on 3 micron paraffin-embedded sections of methylcarnoy's fixed tissue using classical immunoperoxidase techniques with 3, 3-diaminobenzidine (DAB) for color enhancement. Proximal tubular brush border loss was assessed by immunostaining for angiotensin-converting enzyme (ACE) using antimurine ACE antibody; (R&D, Minneapolis, MN). Renal fibrosis was determined by immunohistochemical staining for type III collagen with a goat anti-type III collagen antibody (Southern Biotech, Birmingham-AL-USA). Macrophage infiltrates were detected using Rat Anti-Mouse F4/80 antibody (Serotec, Oxford, UK). Myofibroblasts and

mesangial cells expressing alpha smooth muscle actin were detected using the SMC-Alpha Actin Rabbit Polyclonal Antibody RB-9010-P0 (Thermo Fisher-Waltham, MA-02451 USA). Heat shock protein 70 (HSP-70) expression was determined using Anti-HSP-70 antibody (2A4, antibody 5442, Abcam, Cambridge, MA). Similarly, liver tissue sections were stained by immunohistochemistry for F4/80+ macrophages, alpha smooth muscle actin myofibroblasts (stellate cells), and type III collagen. Digital images were obtained at 20X and 40X magnification (200-400) and were analyzed using Aperio scanner Image scope software (Aperio Technologies, Vista, CA). The software allows color recognition and positive tubule stain were identified as % positive color (DAB positive) saturation in a blinded manner using at least 10 fields for each biopsy sample.

Tissue Studies: Renal MCP-1 was measured on cortical lysates by ELISA (BD Biosciences Pharmingen, San Diego, CA) and corrected for total protein (Pierce, Rockford, IL). Renal fructose content was measured with the EnzyChrom fructose assay kit (BioAssay Systems, Houston, TX) and renal sorbitol was measured using the Biovision (Mountain View, CA) enzymatic assay based on the conversion of sorbitol to fructose with the generation of NADH (color) by recombinant sorbitol dehydrogenase. Renal cortical uric acid was measured by uric acid assay kit from BioAssay Systems (QuantiChrom- Hayward, CA-USA).

Statistical analysis: All data are presented as means ±SE. Data graphics and statistical analysis were performed using Prism 7 (GraphPad). One-way ANOVA with the Mann Whitney test was used to compare ranks.

Results

General Findings: Mice were subjected to intermittent heat episodes (39.5 °C for 30 minutes, 7 times during the day, 5 days per week for 5 weeks). This treatment resulted in a 10-15% weight loss during the dehydration period which recovered fully following rehydration overnight (33). Mice exposed to heat were lethargic immediately after the heat stress but showed normal behavior and activity the following morning and no mortality occurred over the course of the study. Water intake after 5 weeks was not significantly different between groups although intake tended to be higher in the groups exposed to heat (**Table 1**). Mice exposed to heat stress weighed less at the end of the 5-week period (23.4 ± 0.7 and 22.5 ± 0.3 g/mouse in the heat and heat +AP groups, respectively) compared to controls (26.6 ± 1.6 and 25.9 ± 1.7 g/mouse in the control and AP groups, respectively, $p < 0.0001$ comparing the two heat-exposed groups compared to controls) (**Table 1**).

Osmolarity and uric acid: The daily dehydration/heat stress resulted in an increase in serum and urine osmolarity at the end of the dehydration period at 5 weeks (**Table 1**). Urine pH was also lower in the heat stress group compared to controls (**Table 1**). Serum osmolarity was lower in the heat plus AP group versus the heat alone group, despite similar urine osmolarity. Serum copeptin, which is a stable precursor for vasopressin, was

also increased with heat stress (**Table 1**). Serum copeptin was no different between the heat alone and heat plus AP groups, although the serum copeptin was slightly higher in the AP group compared to the wild type controls.

Serum uric acid concentrations collected at the end of the heat stress period were slightly but significantly greater in the heat stress group compared to the controls (Mann Whitney test p=0.016). Serum uric acid concentrations were reduced by allopurinol regardless of whether they were exposed to heat (**Table 1**). Furthermore, urinary uric acid concentrations were similar in all groups except for the AP alone which tended to be lower although this did not reach statistical significance (**Table 1**).

Effect on Renal Injury: Mice exposed to recurrent heat stress develop low grade renal injury and inflammation (33). Indeed, we found that heat stress was associated with a loss of proximal tubular brush border (noted by staining for ACE) (**Figure 1A-C**), an influx of F4/80 positive macrophages **(Figure 1D-F**) and an increase in interstitial fibrosis (type 3 collagen deposition) (**Figure 1G-I)**. Allopurinol treatment was associated with significant preservation of the proximal tubule and less fibrosis compared to heat stress alone, and the macrophage infiltration was largely prevented. Heat stressed mice also resulted in higher serum creatinine levels that was significantly lower with allopurinol treatment (**Table 2**). However, urinary NGAL was elevated and urine albumin excretion tended to be higher in the heat stress exposed mice compared to control mice, but there was no significant difference in heat exposed mice who also received allopurinol (**Table 2**). This may be due to the fact that urinary NGAL increases with dehydration, i.e. a 'prerenal' injury (26).

Potential Mechanisms of Renal Injury: We had previously reported that heat stress and dehydration can stimulate the endogenous production of fructose in the kidney (33). Consistent with this finding, fructose levels were elevated in the renal cortex of heat stressed mice compared to control mice (**Figure 2A**) in association with accumulation of uric acid, which is generated during fructose metabolism (**Figure 2B**). Renal cortical uric acid levels in heat stressed mice were reduced by allopurinol (**Figure 2B**). Uric acid has been reported to activate the polyol pathway (16) and while there was a tendency for lower fructose levels in the heat plus AP group, it did not reach statistical significance.

We previously reported that uric acid generated during fructose metabolism by the proximal tubule can stimulate the expression of the chemokine, monocyte chemoattractant protein-1 (MCP-1) (8). However, while we found a tendency for higher MCP-1 protein in the renal cortex by ELISA, it did not reach statistical significance (**Figure 2C**).

Heat stress is also known to induce heat shock proteins (HSPs), which can also induce a renal inflammatory response (27). Indeed, we found HSP70 upregulated in the tubules of heat stressed mice and this

was significantly reduced with allopurinol. However, an interesting finding was that allopurinol itself could also induce mild HSP70 upregulation (**Figure 2 D-F**).

Effect on Liver Damage: We also evaluated the effect of recurrent heat stress on the liver. Heat stress was associated with a significant increase in F4/80 positive monocyte/macrophage infiltration (**Figure 3A-C**), with alpha smooth muscle actin positive myofibroblasts (**Figure 3D-F**), and with type III interstitial collagen deposition (**Figure 3G-I**). Heat stressed mice that were administered allopurinol showed significantly less inflammation and fibrosis.

Discussion

Heat stroke is a common medical problem associated with severe heat exposure and can result in hypotension and multiorgan failure, with the liver and kidney being particularly affected (3-7, 10). This type of disease can affect military recruits, marathon runners, rural workers, and sedentary individuals during periods of heat waves (3-7, 10), and is likely to become a more serious problem with climate change and increasing heat extremes (1, 15, 17, 25, 28). While the effects of acute heat stroke are well known, there is some evidence that repetitive exposure to heat stress may lead to progressive CKD (15, 19).

Our group has developed a murine model of CKD induced by repeated heat stress (33). The model places mice under stress but there is no mortality and it replicates working in hot environments with limited hydration. We have previously shown that renal injury in this model is associated with intrarenal production of fructose and uric acid from activation of the aldose reductase-sorbitol dehydrogenase-fructokinase (polyol) pathway in the kidney (33). Indeed, mice lacking fructokinase were largely protected from heat stress-induced injury. We have also found evidence that elevated vasopressin activity, which is stimulated by both heat and hyperosmolarity, may also be implicated in the pathogenesis, as desmopressin (a vasopressin analogue) can accelerate renal injury (31), while blocking vasopressin 1 and 2 receptors with conivaptan can protect renal injury in a similar model (12).

Heat stress is also known to raise serum uric acid, which may result from muscle or tissue breakdown (22). Hyperuricemia is also common in subjects with Mesoamerican nephropathy (13, 23, 40) and some subjects also develop hyperuricosuria (30), which has prompted local nephrologists to use allopurinol as part of the treatment for these patients. We therefore performed a study to determine if allopurinol, a xanthine oxidase inhibitor, could protect against heat stress-induced renal injury. Since heat strokes can also induce chronic liver damage we also evaluated the effect of allopurinol on liver injury (6, 14, 21).

Mice exposed to recurrent heat stress developed CKD associated with low grade renal and liver injury. The kidneys showed proximal tubular injury with brush border loss (noted by ACE immunostaining), low grade

inflammation and renal fibrosis, and similarly an increase in inflammatory macrophages and fibrosis was observed in the liver. There was also an increase in alpha smooth muscle actin positive cells that likely represent a myofibroblast-type activation of the liver Ito cells (29). Interestingly, allopurinol was able to protect against both the renal and hepatic damage.

The potential mechanism for renal and hepatic protection by allopurinol could be via several mechanisms. Of interest, while serum uric acid was higher in the heat stress group versus healthy controls, the differences were modest. The inability to show differences in serum uric acid could be due to the fact that, unlike humans, mice expresse uricase, which is an enzyme that degrades uric acid as it passes through the liver. However, there was an increase in renal uric acid that was lowered by allopurinol. The uric acid could be produced from the polyol pathway, and indeed renal fructose levels were elevated in the heat stress treated group compared to controls. In turn, renal uric acid has been proposed to cause local injury and inflammation (8). We did find less HSP70 expression in the kidneys of heat stressed mice treated with allopurinol. We have reported that uric acid can induce MCP-1 in tubular cells (8, 32), and there was a tendency for less MCP-1 expression and monocyte-macrophages in the heat stress group treated with allopurinol compared to the untreated heat stress group. Of note, while allopurinol was overall protective for both renal and liver injury from heat stress, there appeared to be subtle renal injury induced by allopurinol alone, although it did not appear significant. Allopurinol in high doses can induce renal injury in rats (39). It is possible we were observing very mild nephrotoxicity from allopurinol.

In summary, allopurinol treatment protects mice from renal and hepatic injury associated with repeat heat stress. Based on these data, we believe a clinical trial to determine if lowering uric acid in subjects with Mesoamerican Nephropathy, or in people at risk for this disease, is warranted.

Table 1. Water Intake, Osmolality and Serum and Urinary Uric Acid. ***Key***: AP, allopurinol alone; Heat, Heat alone; Heat +AP, heat stress/dehydration with allopurinol.

Parameters	Control	AP	Heat	Heat+AP	Anova P values
24 hours Water intake (ml)	3.7 ± 0.4	3.6 ± 0.9	4.4 ± 0.3	4.6 ± 0.6	NS
Body Weight after 5 Weeks (g)	26.6 ± 1.6**a**	25.9 ± 1.7	23.4 ± 0.7	22.5 ± 0.3	< 0.0001
Serum Osmolality (mOsm/kg)	324.3 ± 5.3 **a**	324.2 ± 3.5	344.5 ± 6.0 **c**	336.3 ± 4.1	< 0.0001
Urine Osmolality (mOsm/kg)	2228.6 ± 440.6**a**	1554.3 ± 669.6**b**	3006.7 ± 470	2893.3 ± 314.6	< 0.0001
Serum Uric Acid (mg/dl)	2.0 ± 0.07 **a**	1.6 ± 0.15 **b**	2.2 ± 0.05 **c**	1.7 ± 0.15	< 0.0001
Urine Uric Acid (mg/dl)	11.2 ± 1.0	9.6 ± 3.0	11.7 ± 1.4	11.9 ± 1.9	NS
Serum Copeptin (pg/ml)	56.5 ± 15 **a**	85.2 ± 14.6 **b**	126.1 ± 22.7 **c**	141.3 ± 19.1	< 0.0001

a, $p< 0.05$ comparing **control** vs **heat** by Mann Whitney; **b**, $p<0.05$ comparing **allopurinol** alone with **control**, and **c**, $p<0.05$ comparing **heat** to **heat plus allopurinol**

Table.2 Effect on Renal Injury.

Parameters	Control	AP	Heat	Heat+AP	Anova P values
Serum Creatinine (ug/ml)	0.86 ± 0.08 **a**	0.81 ± 0.09	0.97 ± 0.18 **b**	0.77 ± 0.12	<0.0268
Urine NGAL (ng/ml)	34 ± 3.8 **a**	28.7 ± 10.2	60.1 ± 15.9	60.8 ± 15.8	<0.0001
Urine Albumin (µg/ml)	46.6 ± 12.9 **a**	39.9 ± 13.7	59.5 ± 6.8	55.8 ± 5.4	< 0.001

a, p< 0.05 comparing **control** vs **heat** by Mann Whitney; b, p<0.05 comparing **heat** to **heat plus allopurinol.**

Fig. 1 Renal Histologic Changes from Heat Stress. The proximal tubular brush border, denoted by staining for ACE, was decreased in the wild type mice exposed to heat stress (B); compared to controls (A). Similarly, the expression of F4/80, which marks monocyte/macrophages, was increased in the interstitium of the heat stress group (see the blue arrows) compared to control wild type mice (Fig. D, E); Type III collagen deposition was also increased in mice exposed to heat and was reduced by allopurinol (G, H). Quantitation of the histologic changes are shown in Figs C, F and I. Scale bar, 50 μm. ***Key***: AP, allopurinol alone; Heat, Heat alone; Heat +AP, heat stress/dehydration with allopurinol. Comparisons only shown for control vs heat, heat vs heat + AP, and control vs AP.

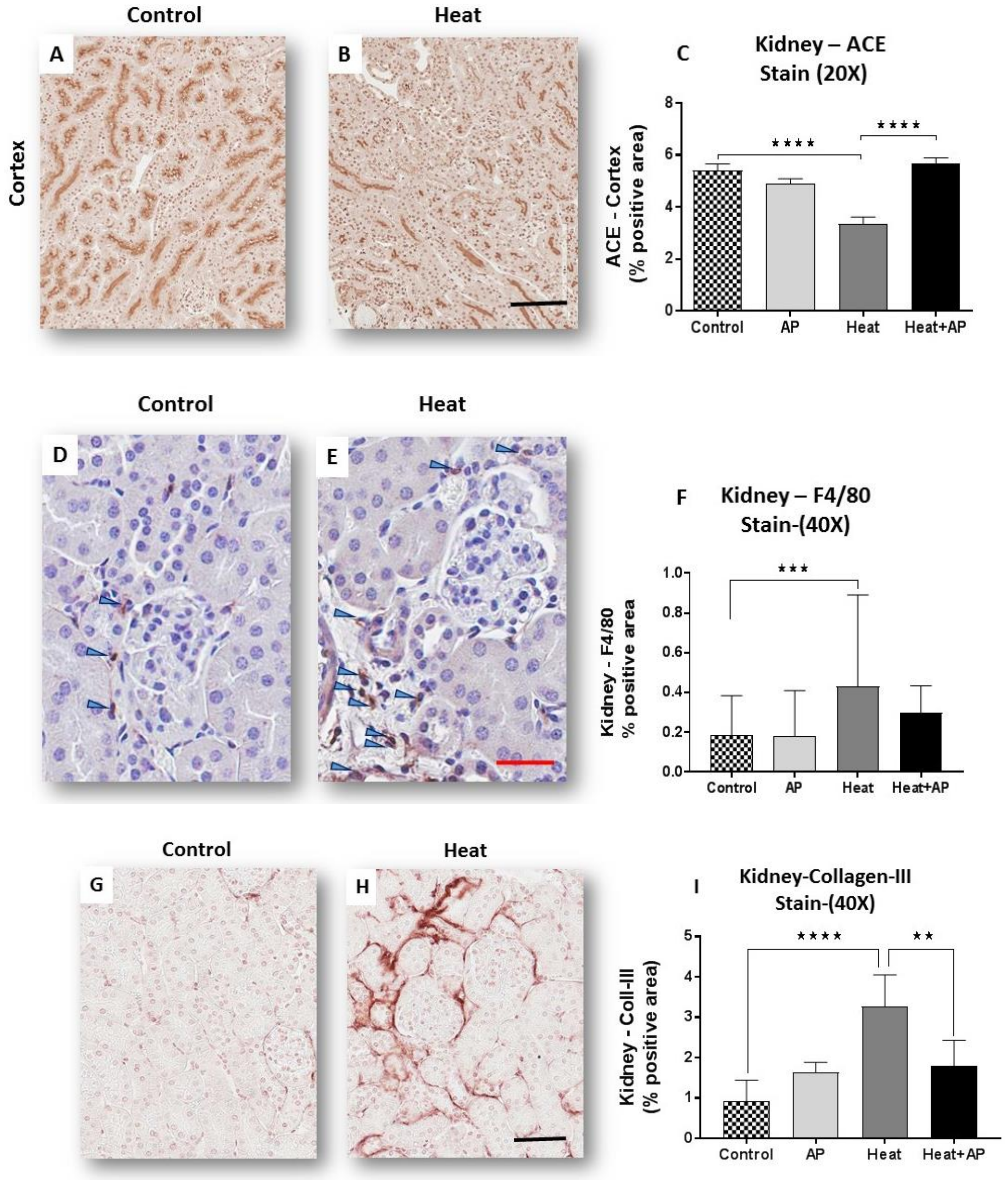


Fig. 2 Inflammatory Mechanisms in Heat Stress Exposed Mice. Renal Cortex Fructose and Uric Acid Levels. (A) Fructose levels were increased in heat exposed rats compared to wild type controls. (B) Uric acid was also high in the renal cortex of the heat stress group compared with controls, Allopurinol and Heat + Allopurinol respectively. (C) Renal cortical MCP-1 tended to be higher in the heat alone group but this did not reach significance compared to control and Heat plus allopurinol groups. (Mann Whitney p values =0.088 and 0.066 respectively). HSP-70 was induced in heat stress animals (D, E) and was reduced in the heat plus allopurinol group. Interestingly, there was some induction of HSP70 in the allopurinol alone. Quantitation of % HSP-70 positive stain area is also shown (F). Scale bar, 50 μm. ***Key***: AP, allopurinol alone; Heat, Heat alone; Heat +AP, heat stress/dehydration with allopurinol.

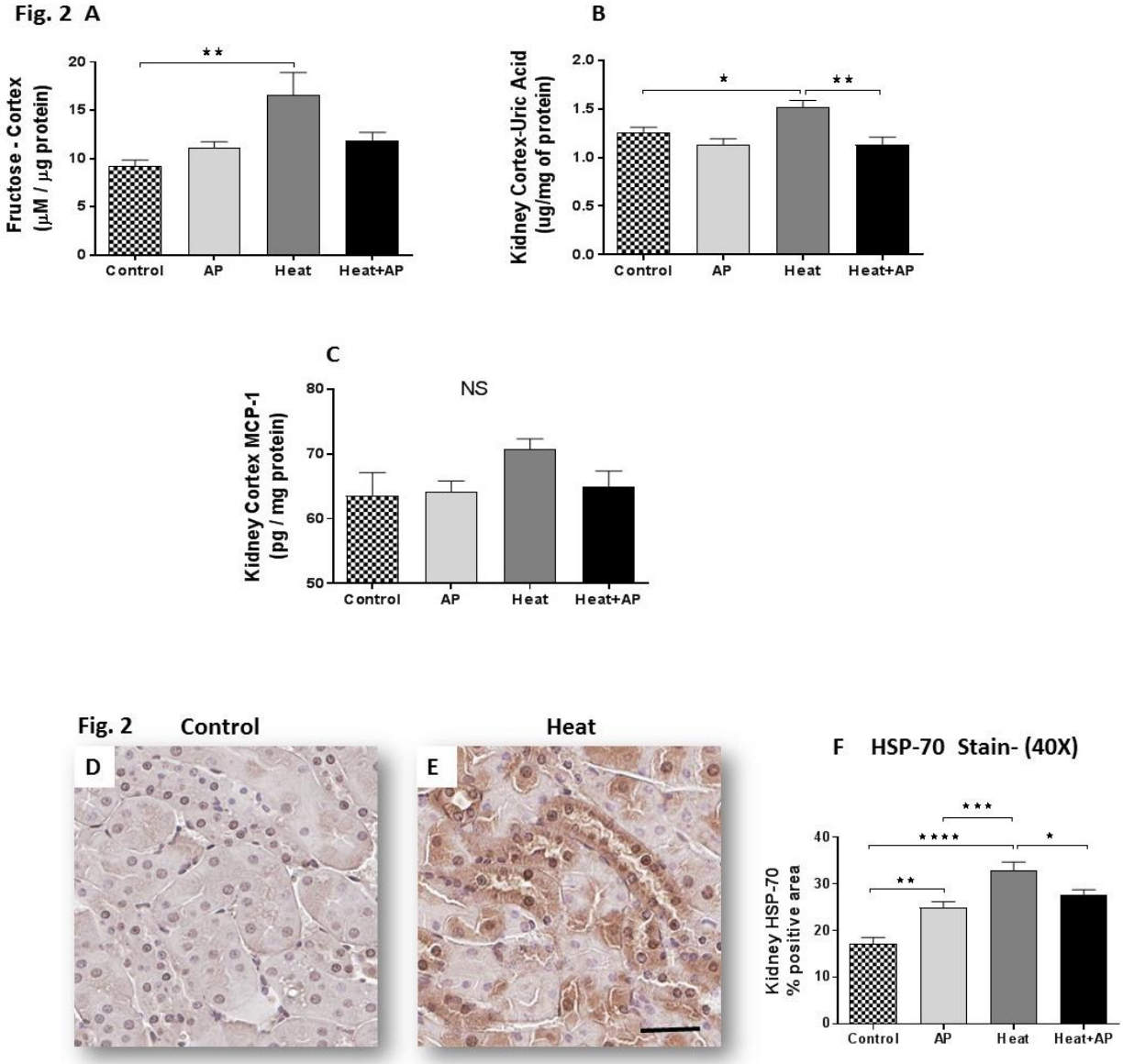
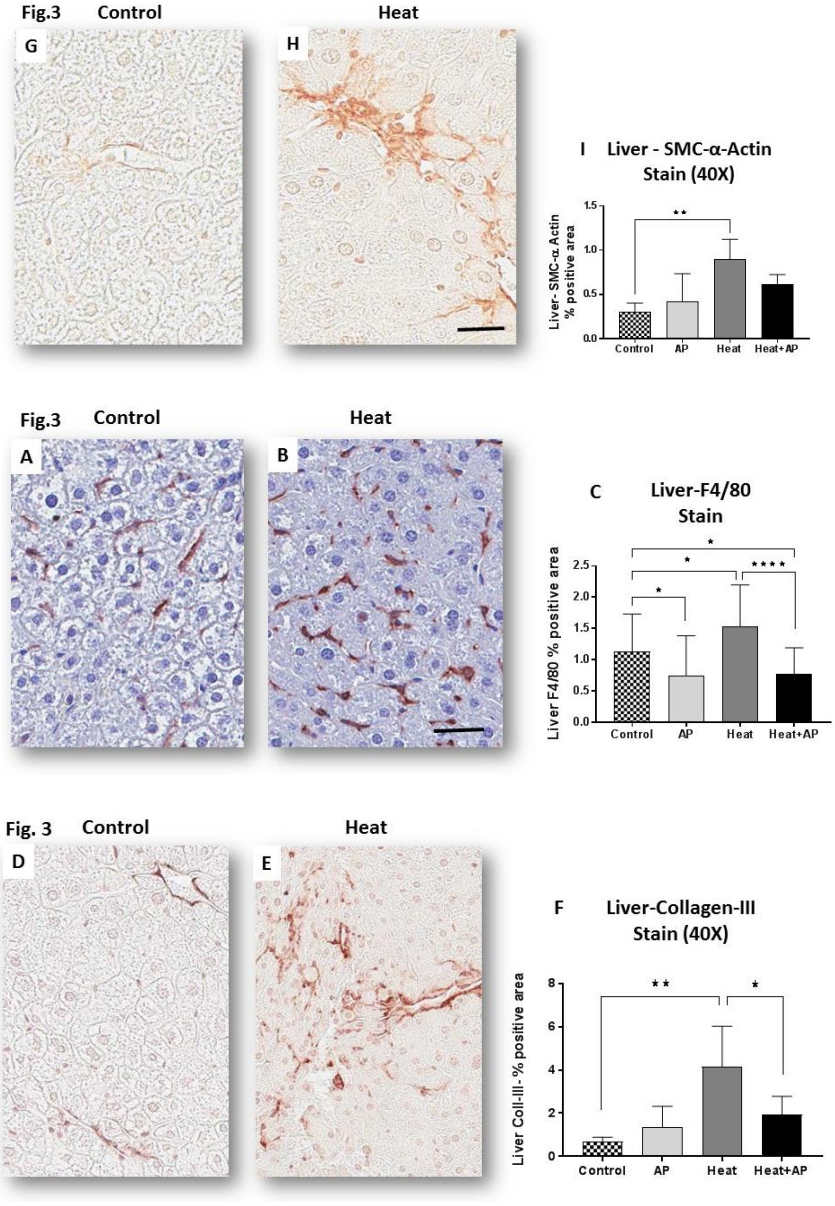


Fig.3 Liver Histology. The liver showing marked increase in F4/80positive cells with heat stress compared to the other groups (Fig. 3A, B). Quantitation of histologic changes are shown in Fig. 3 C. Type III collagen deposition was increased in heat stressed animals compared to wild type controls and was reduced by allopurinol treatment (Fig. D; E). Quantitation of histologic changes are shown in Fig. 3 F. Alpha smooth muscle cell actin expression was increased in heat stress exposed mice, consistent with activation of the Ito cells into a myofibroblast phenotype. Allopurinol tended to reduce the number of myofibroblasts compared to heat stress alone, although this did not reach statistical significance (Figures 3 G, H). Quantitation of histologic changes are shown in Fig. 3 I. Scale bar, 50 μm. ***Key***: AP, allopurinol alone; Heat, Heat alone; Heat +AP, heat stress/dehydration with allopurinol.



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