

Evaluating Biomarkers and Treatments for Acute Kidney Injury in a Zebrafish Model

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SUMMARY

Coronary Artery Disease (CAD) is the leading cause of death in the United States. Despite the prevalence of CAD, there have been few clinical advancements to treat this disease, and mortality due to CAD is rising. Cohort studies have shown that 81% of Acute Kidney Injury (AKI) patients in the renal fibrosis stage later develop CAD. In this investigation, we aimed to create a cost-effective strategy to treat AKI and thus prevent CAD in this patient population. To achieve this aim, we conducted a three-pronged study in a model of *Danio rerio*. In the first phase, we tested whether AKI is induced in *Danio rerio* upon exposure to the environmental toxins arsenic, aristolochic acid, and cadmium. In the second phase, we evaluated nitrotyrosine as an early biomarker for toxin-induced AKI in *Danio rerio*. Nitrotyrosine levels increased 3.5 times its baseline after induction of AKI using arsenic, aristolochic acid, and cadmium. In the third phase of this study, we evaluated 4 treatments of renal fibrosis, the last stage of AKI, because it is a gateway connecting AKI and CAD. Upon conducting a Picro Mallory Trichrome Stain to assess the effects of these treatments on renal fibrosis, we found that the compound SB431542 was the most effective treatment (reduced fibrosis by 99.97%). The approach to treat AKI patients, and potentially prevent CAD, described in this investigation is economically feasible for translation into the clinic in both developing and developed countries.

INTRODUCTION

Coronary Artery Disease (CAD) is the leading cause of mortality in many developed countries and a major burden in developing countries. Despite the prevalence of CAD, there have been few clinical advancements to treat this disease, and mortality rates due to CAD are expected to rise (1). Recent cohort studies suggest that there is a correlation between kidney injury and cardiovascular diseases (2). Notably, 81% of Acute Kidney Injury (AKI) patients go on to develop CAD, indicating a strong correlation between renal and cardiovascular diseases. Thus, treating AKI effectively may prevent progression to CAD, as well as lower cardiovascular mortality rates. There are two main issues related to current treatments for AKI. The first issue is the most commonly used AKI biomarker, serum creatinine, may be inadequate. Serum creatinine is considered the gold

standard for evaluating kidney function, yet existing literature suggests that it is a late biomarker of kidney injury and that it is highly unreliable (3). Serum creatinine increases only after about half of kidney function is lost (4). This biomarker also fluctuates significantly with meat consumption and anabolic agents, resulting in inaccurate diagnostic test results (5). The second issue related to treating AKI is a lack of therapeutics. Most patients are treated with hemodialysis but this is not an optimal option as it exacerbates reactive oxygen species presence and debilitates daily activities.

As a result, I conducted a three-pronged study in a model of zebrafish (*Danio rerio*) to induce, detect, and treat AKI. Zebrafish were utilized only during their invertebrate stage, until 168 hours post-fertilization, at which time they are deemed vertebrates. The simplicity of the zebrafish kidneys, structural similarities to that of humans, and similar glomerular filtration processes of the zebrafish make them a good model organism to conduct renal studies (6).

In the first phase of my project, I used the three most universal pollutants in the world – arsenic, aristolochic acid, and cadmium – to induce AKI in *D. rerio* (7-9). These pollutants are infamous for their carcinogenic effects and induction of respiratory issues, so I decided to investigate their effects on renal function (10-14). The induction of AKI by pollutants has not been tested, therefore we hypothesized that if AKI was induced through the pollutants, then these pollutants would also induce post-injury fibrosis.

In the second phase of my project, we evaluated a novel biomarker for pollutant-induced AKI. In researching potential biomarkers, we wanted to identify an early intervention biomarker to detect the presence of renal injury before significant damage occurs. We investigated the potential of nitrotyrosine, a compound that marks oxidative stress, as a marker of the pathogenesis and progression of AKI. We hypothesized that if AKI is induced through the environmental pollutants arsenic, aristolochic acid, and cadmium, then nitrotyrosine levels will significantly increase.

In the third phase of my project, we evaluated treatments that target the last stage of AKI, known as renal fibrosis. Fibrosis involves the damage-induced scarring of healthy tissue and the inappropriate accumulation of connective tissue that occurs after injury (15). It impacts most, if not all, vital functions of the kidney. No FDA-approved drugs have been developed to target renal fibrosis, and there are no clinical trials evaluating treatments to target renal fibrosis. It is imperative to therapeutically target this stage of injury because it is the

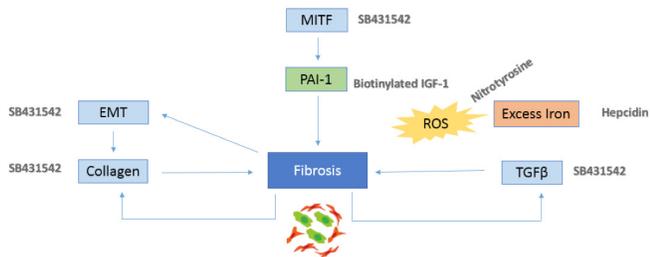


Figure 1. Four treatments were devised to inhibit different pathways of fibrogenesis such as excess iron production, transforming growth factor Beta, and collagen buildup.

progression stage from Acute Kidney Injury to higher mortality linked diseases, such as CAD as shown previously (16). I evaluated the effects of four therapies – Hepcidin, SB431542, Biotinylated IGF-1, and IV-fluid – on fibrosis (Figure 1).

For treating AKI, I used Hepcidin as an iron chelator, a compound that modulates excess iron levels in the human body. Excess iron can lead to reactive oxygen species generation, cellular death, apoptosis, or fibrosis (17). All four are harmful to the kidneys, so quantifying Hepcidin found naturally in the body could have mitogenic effects.

Additionally, I investigated biotinylated insulin-like growth factor (IGF-1). IGF-1 has proven to have several mitogenic properties such as stimulating cell proliferation and cellular regeneration (18). However, IGF-1 readily diffuses through tissues, an undesirable quality as localized targeting of the kidneys is more effective (19). Streptavidin is a homotetramer that has binding zones for four biotin molecules. There are multiple benefits of using such a delivery system. Streptavidin’s versatility allows it to survive in many conditions within the human body such as extreme pH levels. Its counterpart, biotin, can easily be conjugated to bind with other molecules (20). The two compounds have high affinity binding due to Van der Waals interactions and morphological complementarity between the binding pocket and biotin conjugate (21). Davis et al. applied this delivery system to treat myocardial infarctions and reported 28 days of sustained delivery of IGF-1 in cardiomyocytes (22). This novel approach has not been tested in current renal research and thus holds promising data in renal treatments.

Another treatment I tested was SB431542, a novel

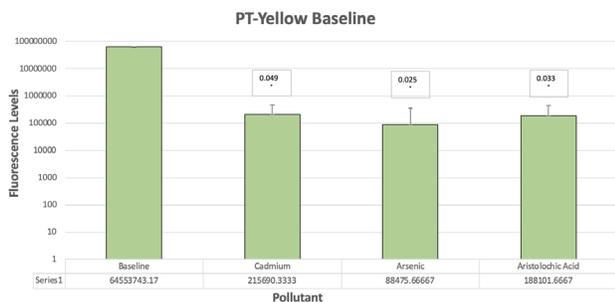


Figure 2. According to the PT-Yellow assay, the pollutants lowered fluorescence levels extensively.

molecule designed for inhibiting transforming growth factor beta 1-induced collagen 1α1 (collagen 1 alpha 1), production of fibronectin, and epithelial to mesenchymal transition (EMT), which are all interconnected with fibrogenesis (23). EMT is prominent during pathological conditions; when renal fibrosis sets in, about 36% of new fibroblasts come from local EMT (24). SB431542 can prevent such excess aggregation of collagen and extracellular matrix buildup and alleviate fibrogenesis.

The positive control I administered was IV fluid as it is currently used in treatments to replenish fluids and balance electrolytes (25). From these four treatments, I hypothesized that if post-injury fibrosis is present and treated with biotinylated Insulin Growth Factor-1 complexed with streptavidin, SB431542, Hepcidin, or IV fluid, then SB431542 will ameliorate the presence of fibrosis with the greatest efficacy.

RESULTS

In order to confirm that arsenic, cadmium, and aristolochic acid induce AKI, we performed fluorescence analysis with PT-Yellow. PT-Yellow is a substance absorbed by the proximal renal tubules in the zebrafish, and it labels kidney function in relation to fluorescence emissions (26). Labeling of the proximal tubule correlates with levels of blood supply to the kidneys, such that lack of labeling (low absorption of the dye) correlates with reduced glomerular filtration rate and decreased kidney function. We used fluorescence analysis on ImageJ to measure PT-Yellow labeling. Decreases in kidney function were quantified by marking decreases in labeling in pollutant zebrafish embryos, in comparison to control embryos. Kidney filtration was impaired after exposure to pollutants, which supported the first hypothesis that the pollutants induce AKI (Figure 2). Of the three pollutants tested, arsenic caused the most extensive damage, lowering fluorescence levels by 99.68%.

Next, I verified nitrotyrosine’s potential as a biomarker by using a reactive oxygen species (ROS) detection solution. Single electron reduction between the ROS detection solution and nitrotyrosine produces chemiluminescence (27). A

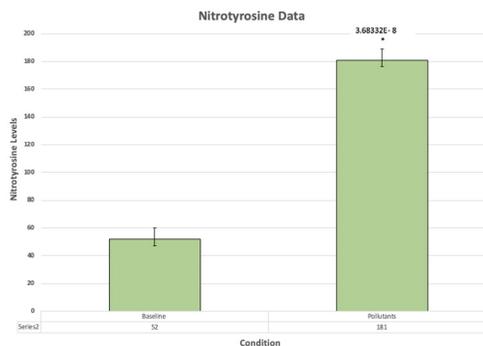


Figure 3. The Reactive Oxygen Species Detection Solution shows a direct correlation between the disease and nitrotyrosine levels (248.08% increase). A two tailed t-test proved a significant p-value of 3.68332E-8.

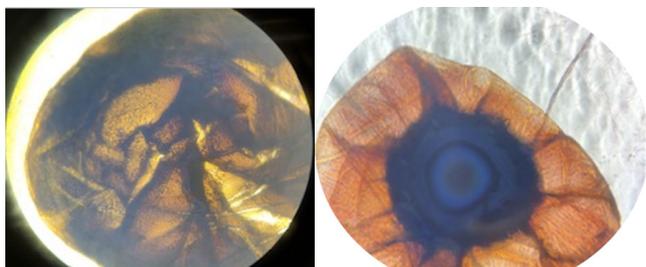


Figure 4. The picture on the left is a Picro Mallory Trichrome Stain conducted on a negative control embryo whereas on the right, the embryo is induced with fibrosis. Here, there are concentric rings of blue that is characteristic of collagen 1alpha1 presence. This is also the blastoderm region of an embryo where Epithelial to Mesenchymal Transition (EMT) occurs. However, the same degree of blue regions cannot be found in an embryo without fibrogenesis induced.

luminometer then measured levels of luminescence as a result of nitrotyrosine presence. “Nitrotyrosine levels were significantly increased by 248.08% following pollutant-induced (two-tailed student’s t-test, p -value = 3.68332E-8; **Figure 3**.)” Thus, we determined that there is a strong correlation between the proposed biomarker and AKI.

A Picro Mallory Trichrome stain was utilized to colorimetrically detect collagen 1alpha1 (COL1A1), an accepted marker of fibrosis. When stained samples were analyzed, we observed blue areas in the samples (**Figure 4**). The picture on the left of **Figure 4**, the control, did not have the concentric blue rings whereas the sample on the right did, indicating localized patterns of COL1A1 and therefore higher degrees of fibrosis. We used the Picro Mallory Trichrome stain to conclude that Cadmium, Arsenic, and Aristolochic Acid induced fibrosis by measuring COL1A1 (**Figure 5**).

In order to test the effect of four potential treatments (hepcidin, SB435142, biotinylated-IGF1, and IV fluid) on fibrosis, we measured COL1A1 levels after treating *D. rerio* at various concentrations of each. 0.004M of SB435142 reduced fibrosis with the greatest efficacy, specifically by 99.96 5%. 0.003M and 0.0019M of SB435142 lowered levels by 99.927% and 99.923%, respectively (**Figure 6**). Thus, the third hypothesis was supported that SB431542 was most effective in treating fibrosis (two-tailed student’s t-test, p -value = 0.014).

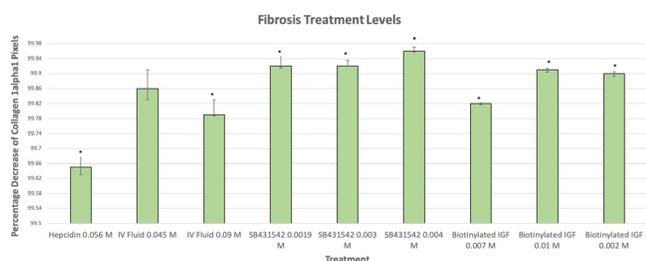


Figure 6. The Picro Mallory Trichrome stain showed an average decline in fibrosis levels for SB431542 at 99.97%. A two tailed t-test proved a significant p -value of 0.014.

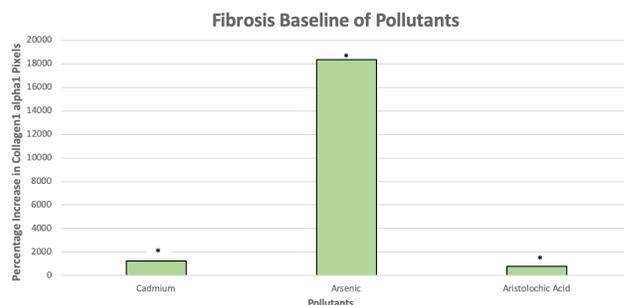


Figure 5. The Picro Mallory Trichrome Stain supported that the pollutants significantly compounded fibrosis levels. A two tailed t-test proved a significant p -value of 0.0126.

The three other treatments also hold potential as biotinylated IGF-1, Hecpidin, and IV Fluid lowered fibrosis by 99.913%, 98.5%, and 98.53% respectively. Interestingly, the zebrafish treated with SB431542 also survived for a longer amount of time. Compared to samples that were not treated, addition of SB431542, biotinylated IGF-1, Hecpidin, or IV fluid extended average life span by 58%, 42.5%, 33%, and 10%, respectively.

Unexpectedly, when renal fibrosis was induced, the zebrafish also exhibited ‘fibrous’ eye shapes (**Figure 7**, right). These ‘fibrous’ eye shapes were recurrent throughout the data and are markers of microphthalmia-associated transcription factor (MITF) upregulation, which is consistent with existing literature (16). These peculiar eye shapes are only present in AKI-induced zebrafish, not in wildtypes such as the zebrafish (**Figure 7**, left), indicating that the upregulation in melanocytes is a result of renal damage. This observation can reshape research with diseases characterized by drastic increases in MITF, such as melanomas and chronic eye diseases, to refocus on renal injuries as a possible root cause.

DISCUSSION

Through statistical analysis, fluorescence averages, and COL1A1 calculations, we concluded that the results supported each of the three hypotheses. Arsenic, cadmium, and aristolochic acid were shown to induce AKI, nitrotyrosine was shown to be a biomarker for AKI, and SB431542 was

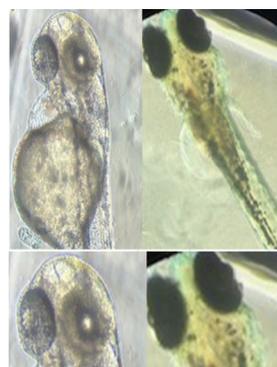


Figure 7. The fish on the left is a wildtype zebrafish with normal eye shape and MITF expression. On the right is an AKI induced zebrafish that exhibits a peculiar phenotypic eye shape known as fibrous eyes due to an increase in MITF expression.

most effective in alleviating fibrosis.

The PT-Yellow assay served as a proof-of-concept that the environmental pollutants arsenic, cadmium, and aristolochic acid function as nephrotoxins and induce AKI, because they significantly lowered fluorescence. For example, arsenic levels dropped by 99.68%. Constant exposure to this water contaminant can cause substantial nephrological damage urging social and legal reform to ensure that the issue of pollution will be mediated for their nephrological impacts.

The PT-Yellow test also determined each treatment's ability to restore kidney function, measured by increases in fluorescence levels. Hepcidin, IV fluid, SB431542, and biotinylated IGF-1 were able to increase fluorescence rates of pollutant-induced samples by 384.7%, 209.4%, 654.5%, and 494.5%, which restored fluorescence levels to 89%, 99%, 97%, and 88% of the negative control's levels, respectively.

The ROS detection solution identified nitrotyrosine's robust presence with a 248.08% increase after AKI had been induced. This supports the first hypothesis because of a *direct* correlation between the proposed biomarker and AKI. Further research should establish that the nitrotyrosine increase is mediated by kidney damage.

The substance's easy detection, quantification, and lack of fluctuation compared to serum creatine affirm its potential in clinical treatments. Additionally, serum creatinine only elevates by 50% on average between 4 hours (baseline measurement) to 27 hours (28). Thus, the tested biomarker in this experiment is more strongly correlated with AKI and increases in a shorter time span.

Furthermore, the Picro Mallory Trichrome Stain supported the third hypothesis which predicted that SB431542 would be most effective in treating fibrosis. The higher concentrations of this solution were more effective- 0.004M decreased the collagen levels by 99.965%. Further studying of the pathways SB431542 inhibits, summarized in **Figure 1**, can open up new paths for developing AKI treatments.

Biotinylated IGF-1 also holds promising value in fibrosis amelioration. IGF-1 diffuses through tissues easily, which is not optimal considering the size of the zebrafish pronephros. In order to combat this characteristic, a biotin-streptavidin complex was used as a delivery mechanism for IGF-1 due to their high-affinity binding.

Additionally, we demonstrated that the pollutants not only induced AKI, but they also induced post-injury fibrosis which was, until now, not directly supported (**Figure 3**). Furthermore, the data collected reconceptualized why there are such high mortality rates associated with CAD. Further testing may prove that pollutants could be a factor in the etiology of AKI and potentially CAD. Thus, AKI prominence correlates to an increase in CAD mortality, furthering the need for comprehensive treatment for AKI.

By observing the recurrent 'fibrous' eye shapes, two conclusions were deduced: first, that the kidneys regulate a multitude of processes throughout the body, reinforcing the importance of integrative strategies while treating

diseases such as CAD, and second, that studies on diseases characterized by drastic increases in MITF (such as melanomas and chronic eye diseases) could refocus research on possible underlying renal complications and bring about advancements to both fields.

A shortcoming was due to the large sample size, combinations of the treatments were not feasible. Each of the four treatments were able to target a specific aspect of AKI effectively, however, combining them may produce better results. Synergistic reaction combining these treatments may be the path to effectively treat the pathways, so future research can include a matrix of different combinations.

Additionally, the ability to administer these treatments may be limited on a patient to patient basis. For example, Hepcidin cannot be administered in large quantities to patients who have iron deficiency, nor can IGF-1 be prescribed to diabetics due to the risk of hypoglycemia.

However, the current treatment, dialysis, is associated with nosocomial infections, ROS generation, and many other secondary issues. Nevertheless, dialysis is not effective in targeting fibrotic pathways because it only targets blood filtration. Additionally, it costs up to \$72,000 annually to patients (28), and if CAD is subsequently developed, coronary artery bypass grafts costs between 70,000 to 120,00 dollars (29). All treatments utilized in this experiment, however, are readily accessible and cheap for translation into clinical applications for patients in both developing and developed countries.

These discoveries can function as catalysts, catapulting CAD research into renal fields. The results obtained may be transferred from bench to bedside insofar as the detection and utility of the therapy plans can fit into existent treatment structures yet produce more desirable results.

METHODS

All procedures utilizing the three pollutants required a respirator and appropriate personal protection (proper shoes, safety goggles, gloves, and a lab coat). The pollutants were handled under a chemical fume hood to limit contact and inhalation. All trash generated was discarded in a chemical waste container in the Biohazard trash. All zebrafish embryos were used within the first 168 hpf while they are considered invertebrates. A total of 940 embryos were used; 6 per condition induced.

The pollutants were dissolved in sterile, distilled water. For dose titrations, embryos were collected, randomly divided into groups, and exposed to water containing pollutants at appropriate concentrations- 50 pbb of arsenic, 60 ppb of cadmium, and 35 pbb of aristolochic acid.

PT-Yellow was used to determine whether or not the pollutants induced AKI. 0.5 mL of PT-Yellow was added to 1.5 ml of embryo medium. After taking pictures with a fluorescent compound microscope equipped with a digital camera, images were input into ImageJ to quantify integrated density and area. Corrected Total Cell Fluorescence (CTCF) was determined by the following equation: $CTCF = \text{Integrated Density} - (\text{Area of}$

selected cell X Mean fluorescence of background readings).

Nitrotyrosine detection started at 48 hpf. For the ROS assay, 5 ml of ROS-detection solution was prepared by dissolving a generic ROS-sensitive probe stock solution in Hank's Balanced Salt Solution. Embryos were incubated in the dark for 15 minutes at 28°C. At the end of the incubation time, the solution was removed and washed twice with 2 ml of Hank's Balanced Salt Solution. Embryos were transferred to a black well plate and put into a luminometer.

To prepare a working solution of biotinylated IGF-1, streptavidin needed to be bound to this biotinylated growth factor. The compound was mixed with streptavidin in a 1:1 molar ratio and incubated for one hour in order for complete binding at each site. For SB431542, 0.0019M, 0.003 M, and 0.004M was isolated and added to 1.5 ml of zebrafish medium. For the next treatment, Hecpudin, 0.056M was added to 1.5 ml of zebrafish medium. The positive control, IV fluid had 0.045M and 0.09 M were added to 1.5 ml of zebrafish medium. The treatment stage started at 48 hpf as the kidneys start to form at 40 hpf.

For treated embryos, fixation started at 50 hpf, whereas for control samples, this process started at 48 hpf to account for treatment administration time periods for the fixation process, every 3 embryos were fixed with 4% paraformaldehyde and PBS. After leaving them overnight at 4°C, they were washed twice in PBS, 5 min each, at room temperature. Embryos were transferred to vials with 100% methanol and then cooled to -20°C for 30 minutes. After bringing them back to room temperature, they were immersed for 5 minutes in 50% methanol in phosphate buffered saline with Tween (PBST), and then 5 min in 30% methanol in PBST. The embryos were rinsed twice in PBST for another 5 min, and fixed for 20 min in 4% paraformaldehyde in phosphate buffered saline. A final rinse of PBST was administered twice for 5 minutes each.

After primary fixation protocols, a Picro Mallory Trichrome Stain was used to detect fibrosis by marking COL1A1 presence in samples. Embryos were placed on glass slides and submerged in saturated picric acid solution overnight. A wash with tap water (3 changes of running water for 2 minutes), then distilled water for 30 - 60 seconds was conducted. Then, Picro Mallory Solution A stained the embryos for 2 minutes. Excess dye solution was removed and slides were transferred directly into Picro Mallory Solution B for 30 minutes. Slides were washed with 0.5% glacial acetic acid, and were subsequently dehydrated with 100% alcohol. To quantify the fibrosis levels, ImageJ was used with the RGB plugin (30).

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