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Investigating the Effects of Paraquat on Kidney Disease Biomarkers in HEK293 Cells

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INVESTIGATING THE EFFECTS OF PARAQUAT ON KIDNEY DISEASE BIOMARKERS IN HEK293 CELLS

by

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ABSTRACT

Farmworkers in Apopka, FL, have been subjected to overhead pesticide exposure since the 1940s. Pesticides including Paraquat (PQ), Metribuzin and Aldicarb were sprayed onto the field while farmworkers worked. In "Fed Up: The High Cost of Cheap Food," farmworkers recalled the physical toll these conditions took on their bodies, blaming pesticides for their diseases, such as chronic kidney disease (CKD). While established that pesticides, specifically PQ, may be involved in some forms of Parkinson's disease, no explicit connection has been identified for SLE, CKD, and other diseases experienced by farm workers. This study evaluated whether pesticides could contribute to kidney disease. We quantified the fluorescence of reactive oxygen species (ROS) following varying PQ exposure in human embryonic kidney 293 (HEK293) cells using a microplate reader. Dosages of 75 and 150 μM were chosen based on previous literature. We also measured expression of KD biomarkers *KIM-1* and *NGAL* upon PQ exposure with RT-qPCR. Glutathione-S-transferase pi 1 (*GSTP1*) served as an indicator of ROS. We predicted that ROS would increase with increasing PQ concentration, as would the fold change in the expression of the mRNA biomarker levels. The results showed a trend of increased expression of *NGAL*, *KIM-1* and *GSTP1* as PQ concentration increased. This study suggests metabolic panels may be an option when assessing patient health, especially patients susceptible to kidney disease. Future *in vitro* and *in vivo* examinations of these biomarkers are needed to clinically correlate physiological concentrations of these pesticides, and progression of kidney disease.

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LIST OF ABBREVIATIONS

HEK293—Human Embryonic Kidney 293

PQ—Paraquat

BACTIN—Beta Actin

KIM—Kidney Injury Molecule

NGAL—Neutrophil Gelatinase Associated Lipocalin

GSTP1—Glutathione-s-Transferase pi 1

DMEM—Dulbecco's modified eagle serum

FBS—Fetal Bovine Serum

PBS—Phosphate-Buffered Saline

CKD— Chronic Kidney Disease

PC— Positive Control

INTRODUCTION

Kidney disease (KD) is characterized by the progressive deterioration of kidney function. While initially a patient may feel and present asymptomatic, this can gradually worsen to edema, appetite loss, and kidney failure, requiring dialysis or even transplantation[1]. Factors and preexisting conditions that may increase the chance of chronic kidney disease (CKD) development include diabetes, hypertension, cardiac disease, and a family history of disease[2]. Moreover, individuals have been identified as at increased risk of CKD based on ethnicity, gender and age[2]. In 2021, it was reported that nearly 40 million people in the US have CKD[3]. In farmworkers, the CKD relevant to this group known as is referred to as CKD of the unknown (uCKD), as the classical symptoms CKD such as hypertension, and diabetes are absent[4]. However, patients with uCKD may share symptoms of hyperuricemia, reduced kidney size, and electrolyte deficiencies[4]. Heat stress seems to be a common denominator amongst farmworker communities in India, Sri Lanka, and Central America[4]. Research is needed to further investigate and establish symptoms of this population that share similar labor conditions.

Most studies use eGFR (estimated glomerular filtration rate) to establish a diagnosis of CKD, where the value for this measure tends to span the following range in units of ml per minute per 1.73 m^2 of body-surface area. The normal range is 90 to 120 mL/min/1.73 m^2 whereas 60-90 mL/min/1.73 m^2 indicates early stage of KD, 15-60 mL/min/1.73 m^2 points to KD, and 0-15 mL/min/1.73 m^2 is the range of kidney failure[5-6]. eGFR is calculated using a patient's age, sex, race, and creatinine levels of blood serum[7]. However, values may differ between laboratories due to different methods, instruments, and formulas for calculation[7]. In patients with poorer kidney function, the reported eGFR is lower. Some clinical paradigms may also opt for an albumin analysis to calculate an albumin to creatinine ratio, normally 30 - 300 mg/g[8].

Clinical management of uCKD consists of eGFR monitoring, kidney biopsies, and urinalysis[4]. A history of the patient may be analyzed to identify prior labor in heat-intensive environments, NSAIDs and tobacco use, and pesticide exposure through urine and/or serum levels[9]. Due to the variety of etiologies, there is no current consensus on treatment[10]. Management of CKD is derived from treatment of consequential symptoms, such as anemia (treated by erythropoietin stimulating agents or ESAs) and high blood pressure (treated by ACE inhibitors). Advanced stages may require dialysis or kidney transplants, both of which are expensive in the American healthcare system and out-of-reach options for many farmworkers[10].

Pesticides

Paraquat

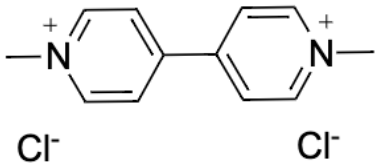

Figure 1. Chemical structure of Paraquat.

Of the proposed causes, toxin exposure is an etiological suspect of CKD[4]. Pesticides used in Florida include Paraquat (PQ) and Metribuzin[11-12]. These pesticides were sprayed onto the fields using planes[11]. One of the most common methods of exposure is the dermal route[13]. In the book "Fed Up: The High Cost of Cheap Food," amongst many farmworkers in Apopka, one farmworker recalls being drenched in a liquid mixture of pesticides without proper PPE when employed on a farm in the 1990s[11]. About 20,000 farmworkers are estimated to be exposed to pesticides each year[14]. A variety of pesticides are used such as Paraquat for soybeans and cotton and Metribuzin for potatoes and soybeans[11-12,15].

Paraquat (1,1′-Dimethyl-4,4′-bipyridinium dichloride hydrate; Gramoxone; Methyl viologen dichloride; Paraquat dichloride) is an herbicide available in the United States only to licensed commercial entities[16]. Due to its toxic nature, it is dyed blue, has a pungent odor, and contains an emetic agent in case of ingestion[16]. It is banned in many countries including the United Kingdom, China, and the European Union[17]. Protection guidelines after contact mention cutting away clothing rather than removing it from over the head[16]. Paraquat causes damage to the gastrointestinal (GI) tract via ingestion and accumulates in pneumocytes via an active transport mechanism[16]. Long-term side effects include scarring of the GI tract and lungs as well

as failure of the kidneys and heart. PQ can also cross the placental barrier and reach the fetus indicating its teratogenic capabilities[18]. PQ's half-life in the soil is about 20 years and varies from about 2 to ~800 years in water, depending on depth and UV exposure[18]. It has been suspected to cause Parkinson's disease[19]. Jang et. al (2015) showed *in vitro* that the dosage of 75 and 150 μM of PQ in RAW264.7 cells induced cellular damage through a ROS-mediated pathway involving caspase-3 and mitochondria.

Metribuzin

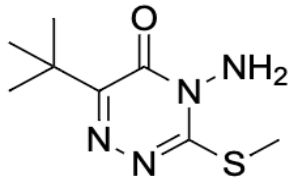

Figure 2. Chemical structure of Metribuzin.

The effects of Metribuzin (4-Amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one) are controversial. Delancey et. al (2009) concluded there may be a link between Metribuzin and lymphohematopoietic carcinogenicity, consistent with Sookhtanlou et al.'s (2022) findings of increased risk of health hazards to farmworkers who used Metribuzin at higher than recommended concentrations. Another study demonstrated negative genotoxic results associated with Metribuzin[20]. In Environmental Protection Agencies (EPA) mammalian studies, tumorigenic effects, along with body mass decrease, organ dysfunction and mortality were observed[21].

Pesticides chosen for this study reflect their usage in field conditions and have been explored in the literature. Paraquat is more well-established in mammalian studies and has been

shown to correlate to Parkinson's disease[22-23]. Metribuzin, although widely explored in agricultural studies, needs to be further explored in human studies as there is a significant gap in literature. This study aims to provide more information about this pesticide in *in vitro* conditions as well.

Biomarkers

The National Institutes of Health (NIH) Biomarkers Definitions Working Group defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[24]. These biological entities can be used to detect, diagnose, evaluate, and track diseased states within organisms. In addition to evaluation of glomerular filtration rate (GFR) and urine output, many biomarkers exist for kidney function, most of which are detectable in urine and/or serum. GFR is the most accepted and employed measure of kidney function, yet is limited due to racial discrepancies[24]. Serum creatinine also has its drawbacks, including age, sex, and diet. Moreover, serum creatinine may be in concordance with GFR, however, the strength of the correlation may decrease with deteriorating kidney function[25]. Novel and validated biomarkers are needed to assist with diagnoses of CKD, especially considering the possibility of pesticide use in the farmworker population contributing to uCKD.

Glutathione-S-transferase pi 1

Endogenous proteins exacerbated in response to oxidative stress triggers include glutathione-S-transferase (GSTP), glutathione (GSH), and superoxide dismutase (SOD). *GSTP1* delivers and conjugates GSH to other endogenous or exogenous electrophilic entities to detoxify them[26]. GSH is ubiquitous in most cells and organelles. It serves to maintain cellular oxidation and reduction reactions and plays a role in various stages of the cell cycle, such as S and M

phase[27]. SOD enzymes are present in kidney cells and participate in the first line of defense against ROS. Elevations of ROS levels are a pivotal factor during the development of acute kidney injury, putting patients at higher risk for CKD[27]. Thus, exploring the effect of ROS in relation to pesticides and CKD *in vitro* can elucidate potential relationships between the pathophysiology and the oxidative cellular stress induced. The pi 1 variant was chosen as it is the most highly expressed GST variant in the HEK293 cell line[28].

Kidney Injury Molecule-1

Kidney Injury Molecule-1 (*KIM-1*) is a transmembrane glycoprotein expressed after acute injury to the kidneys. Although absent in normal conditions, *KIM-1* is upregulated in instances of ischemia, where it is hypothesized to ameliorate the immunity and morphological framework of the kidneys[26]. *KIM-1* is an established biomarker for acute kidney injury (AKI) and is still being evaluated for CKD: Sabbisetti et al. (2014) alludes to *KIM-1* being elevated in cases of uCKD. *KIM-1* elevation was also able to predict CKD risk and imminent eGFR decline in healthy middle-aged patients[29]. *KIM-1's* potential as a biomarker of CKD is also strengthened by its increase in expression during interstitial fibrosis and inflammation in CKD murine models and patients[30].

Neutrophil Gelatinase-Associated Lipocalin (NGAL)

NGAL, also known as lipocalin-2, is a proposed biomarker existing in either its monomeric (25 kDa) or dimeric form: the former is associated with an origin from renal tubules, whereas the latter is expressed by neutrophils[31]. Like *KIM-1*, *NGAL* is also associated with AKI, yet has shown promise of CKD, as it mimics the former's trend in increasing over time as AKI progresses into CKD, and consequently wanes[31]. Murine models of kidney lesions from nephron reduction exposed *NGAL* as an indicator of kidney disease advancement[31].

Farmworkers in Apopka, FL have been subjected to overhead pesticide exposure since the 1840s[32]. Pesticides such as Paraquat (PQ), Metribuzin, Aldicarb, and more were sprayed onto the field whilst farmworkers were working[11-12]. Due to poor labor conditions, chemical exposure was not limited to the farm: it spread to farmworkers' families through washing contaminated clothes at home. Some farmworkers suspect it spread generationally through learning defects in children of mothers who worked on the fields while pregnant[11].

In "Fed Up: The High Cost of Cheap Food," farmworkers recalled the arduous physical toll the conditions took on their bodies and blamed pesticides for diseases such as systemic lupus erythematosus (SLE), chronic kidney disease (CKD), arthritis, and other chronic ailments, but no explicit link has been made[11]. There is considerable research associated with pesticides, especially PQ and DDT, being associated with Parkinson's disease (PD)[33]. In murine models, PQ caused apoptosis of dopaminergic neurons in the substantia nigra and deterioration of motor skills via the intrinsic mitochondrial pathway[19]. However, most murine studies were correlations rather than exploration of causation mechanisms, did not test a variety of pesticides, and links to other illnesses remained unexplored in depth. This study aims to clarify the aforementioned gaps in literature and test whether pesticides, such as PQ and Metribuzin, could cause kidney disease.

METHODS

Cell Culture

HEK293 are cells derived from human embryonic kidneys. Cells were gifted by Dr. Chai's lab at UCF. They were maintained in DMEM (Fisher Scientific) with 10% FBS (Fisher Scientific), and 1% penicillin, streptomycin, and Gibco Amphotericin B (Fisher Scientific) cultured in a humidified incubator at 37˚C and 5% CO_2. Experiments were conducted once a confluence of 80% was achieved. Experiments were concluded before passage 15.

CellROX ROS Assay

Black, flat-bottom, 96-well plates were coated with 100 µL of 100 µg/mL poly-L-lysine (PLL; Sigma) for 2 hours in a humidified incubator at 37 °C and 5% CO_2. After rinsing 3 times with sterile water, HEK293 cells were seeded at 15,625 cells per cm^2. After 24 hours, treatments of 100 µM Menadione (Sigma) used as a positive control (PC), Paraquat (PQ; Sigma), and Metribuzin (Fisher) dissolved in complete media were applied at a final *in vitro* concentration of 75 and 150 µM. After 24 hours, CellROX Deep Red (Invitrogen) dissolved in complete media was applied to wells at a final concentration of 5 µM to detect ROS. After 30 minutes of dark incubation at 37˚C and 5% CO_2, the microplate was washed with prewarmed phosphate-buffered saline (PBS) twice, and then analyzed by a Tecan Infinite Pro m200 plate reader at 644/665 nm excitation and emission wavelength, with the most confluent positive control well set as the Gain (reference well). Figure 3 depicts a visual workflow of this method.

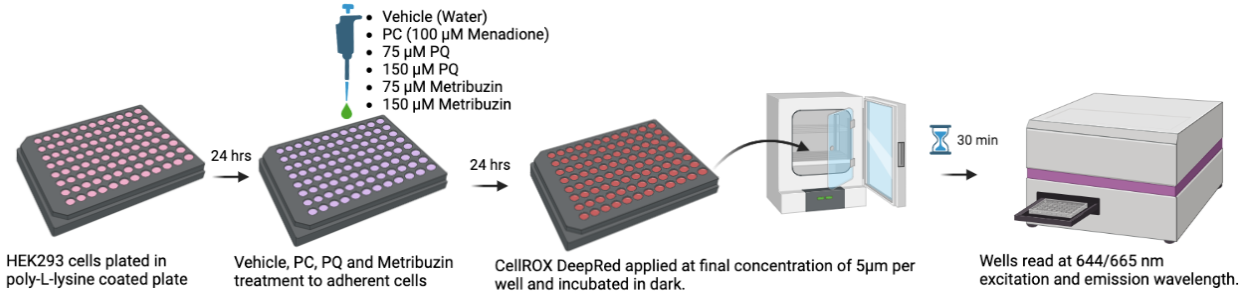

Figure 3. Visual workflow of ROS Assay method. Image created using BioRender.

Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

Clear 6-well plates were coated with 3 mL of 100 µg/mL poly-L-lysine (Sigma) for 2 hours in a humidified incubator at 37 °C and 5% CO_2. After rinsing 3 times with sterile water, HEK293 cells were plated at 36,458 per cm^2. After 24 hours, bright field pictures of cells were obtained at 20x magnification for qualitative, visual analyses. Cells were then treated with or without 100 µM Menadione as a positive control, as well as 75 or 150 µM of PQ, all respectively dissolved in complete media.

After 24 hours, RNA was extracted using the RNeasy Extraction kit (Qiagen). For RNA to cDNA conversion, 1 mg of RNA was converted using the iScript cDNA synthesis kit (Bio-Rad). A final concentration of 50 µg of cDNA was used for qPCR experiments using the Sso Fast EvaGreen Supermix (BioRad). Figure 4 illustrates the visual guide for the RT-qPCR method utilized in this experiment. The primers and genes examined in this experiment are in Table 1. Analysis was done using the $2^{-\Delta\Delta Ct}$ method by normalizing to the untreated *BACTIN* conditions. In the case of examining *KIM-1*, where the untreated *BACTIN* condition average Ct values were too low to be detected, the *BACTIN* in the positive control condition (Menadione at 100 µM) was used to normalize Ct values of samples.

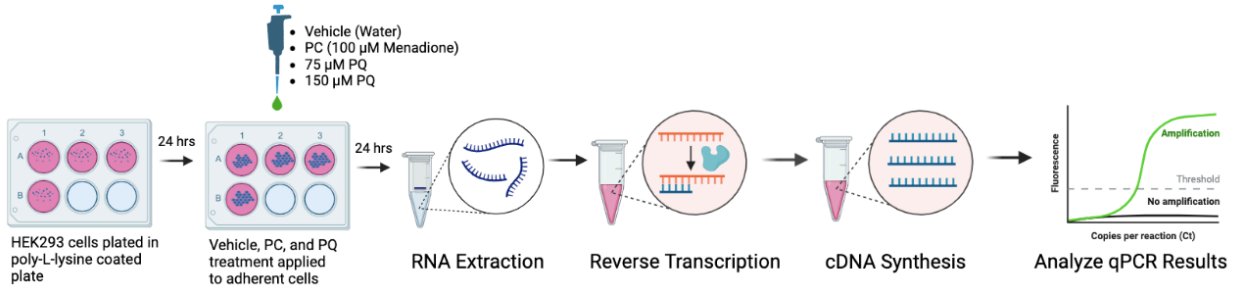

Figure 4. Visual workflow of RT-qPCR method. Image created using BioRender.

Human Gene	**Primers**
Beta Actin (*BACTIN*)	Forward: 3'- CAC AGA GCC TCG CCT TTG CC - 5' Reverse: 3'- GAC GAG CGC GGC GAT ATC AT- 5'
Neutrophil Gelatinase-Associated Lipocalin (*NGAL)*	Forward: 3'- GTG GGC AGA GAC CCC AAG AA- 5' Reverse: 3' - TGC TGA GGA GCC AAG GTG TC- 5'
Kidney Injury Molecule-1 (*KIM-1*)	Forward: 3'- GCC CAG GCA GAA CCA TGA AC- 5' Reverse: 3' - ATT GCT CCC TGC AGT GTC GT- 5'
Glutathione-S-transferase (*GSTP1*)	Forward: 3'- CGG AGA CCT CAC CCT GTA CC- 5' Reverse: 3'- CAC GCC GTC ATT CAC CAT GT- 5'

Table 1: Primers for qPCR.

RESULTS

Brightfield pictures were obtained of HEK293 cells in 6-well plates before RNA extraction on 20x magnification (Figure 5). Qualitatively, the cells appear healthy in the Vehicle condition, as expected, due to their lack of vacuoles, and presence of projections, and adherence to the vessel's surface. However, cells were unhealthy in the positive control condition, appearing mostly suspended, morphologically round, and of irregular density across the vessel's surface. In the 75 µM PQ condition, cells did not appear as damaged as in the PC condition. Cells were slightly dispersed, and irregular in their morphology but were overall more than 80% confluent. There was a slight instance of vacuolization as evident in the bottom-right corner of this condition. Lastly, cells also appeared less damaged than the PC condition; however, they appeared to be more spaced out and seemed to have a higher incidence of vacuoles.

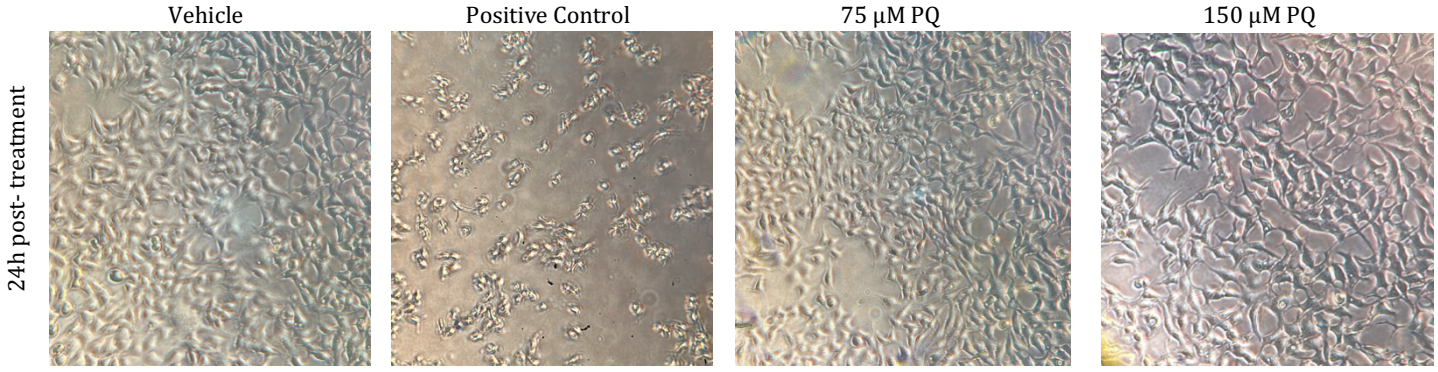

Figure 5. HEK 293 cells in the Vehicle, PC, and PQ conditions.

To quantitatively assess the levels of intracellular, cytoplasmic ROS, we conducted a ROS assay. HEK293 cells were treated with pesticide after confluence was achieved. The CellROX Deep Red reagent was used to detect intracellular ROS. The fluorescence emitted was detected at an excitation wavelength of 644, and emission wavelength of 665. Statistical analyses were carried out by a one-way ANOVA, and a Tukey post-hoc test. No significant differences were detected between the different conditions. Thus, in this experiment no increase in ROS was detected as a result of increasing pesticide exposure (Figure 6).

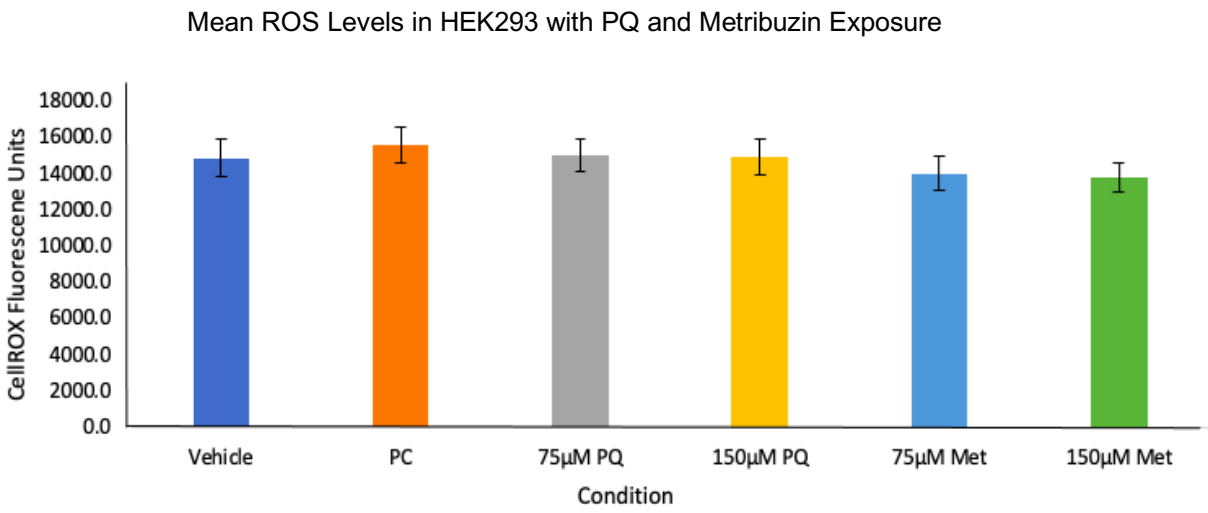

Figure 6. Graph of CellROX fluorescence units against experiment conditions. Values were not significantly different (one-way ANOVA, and Tukey post-hoc test, $p > 0.05$).

Next, we checked for increased expression of chronic kidney disease markers. As evident in Figure 7, there was a trend for *GSTP1* expression levels to increase as the concentration of PQ increased, with the highest expression fold change at 150 µM of PQ. It was expected that transcription of *GSTP1* would increase, acting as a positive control, as the concentration of PQ increased; since high ROS levels have been evident mammalian PQ studies[34-35]. The *GSTP1* expression in all pesticide conditions was lower than the vehicle condition as a control, as evident by the fold change being under 1 in all conditions. The PC (Menadione at 100 µM) condition had the lowest *GSTP1* expression, which was about a third of the expression levels of vehicle. *GSTP1*'s relative fold change was about half as much as vehicle in the 75 µM PQ condition. Lastly, *GSTP1* expression fold change was the nearest to vehicle in the 150 µM PQ condition at 0.81, which was the highest fold change obtained for *GSTP1* relative to previous treatment conditions.

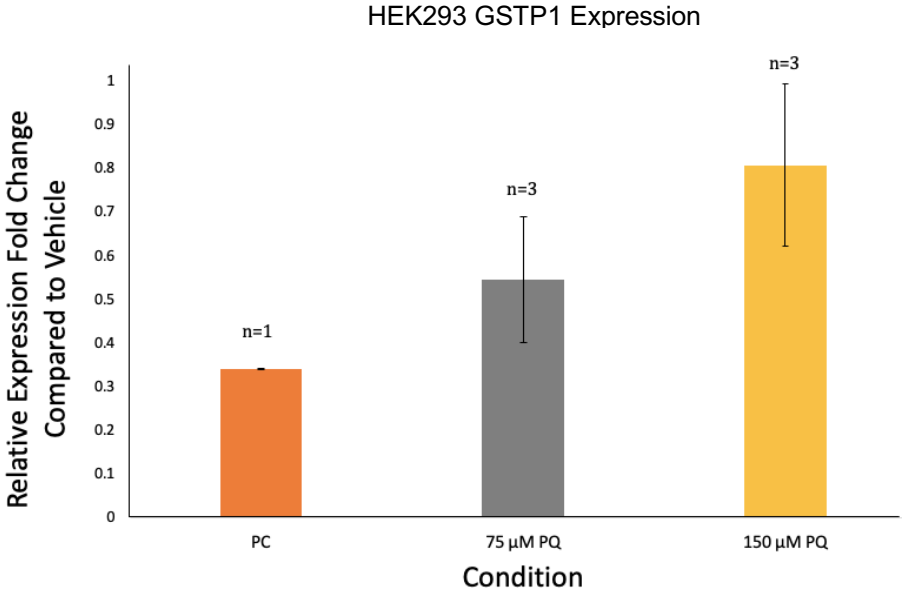

Figure 7. Relative fold expression graph comparing average $2^{-\Delta\Delta Ct}$ values of *GSTP1* in HEK293 cells. Pesticide exposure was compared to vehicle control, with *BACTIN* as the housekeeping gene. There is a trend for increasing amounts of *GSTP1* as the amount of PQ increases.

The average Ct values of the vehicle *KIM-1* condition were too high to be used in analysis, which meant the transcriptional activity was too low to be detected without pesticide exposure. Therefore, in both PQ concentration conditions, the relative expression fold change was compared to the PC (Menadione at 100 µM) condition. There was a slight trend for increasing *KIM-1* mRNA with more PQ exposure (Figure 8). *KIM-1* expression was lower in the PQ conditions than in the PC Menadione. However, the fold change in the 75 µM PQ condition was only 0.09 times the PC condition. Increasing PQ concentration to 150 µM produced more expression of *KIM-1*, 0.15 times the PC condition. Thus, expression of *KIM-1* was induced a 66.7% fold increase from 75 to 150 µM PQ.

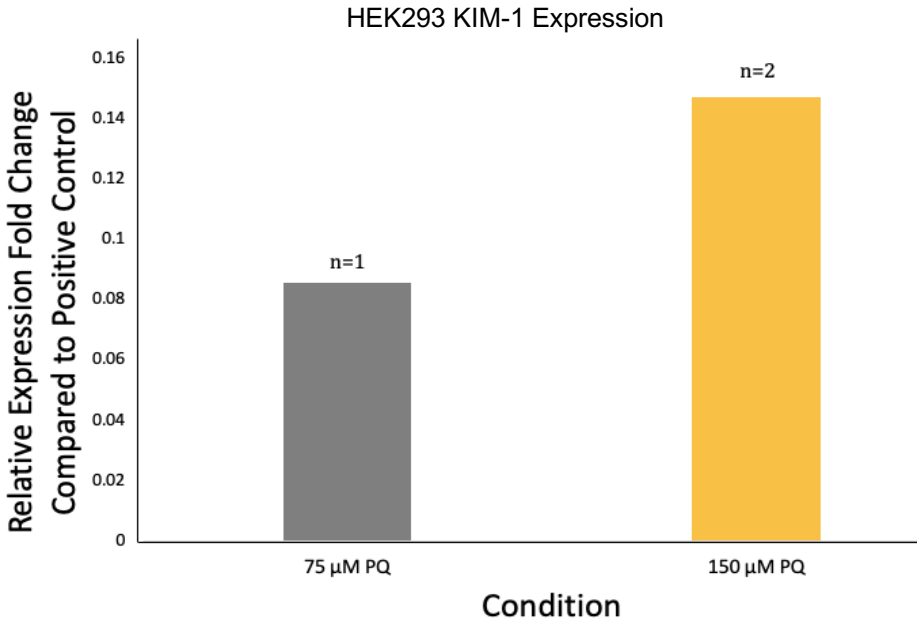

Figure 8. Relative fold expression graph comparing average $2^{-\Delta\Delta Ct}$ values of *KIM-1* in HEK293 cells. The control mRNA was *BACTIN*. There is a trend for increasing *KIM-1* expression with increasing amounts of PQ.

The relative fold change in *NGAL* expression compared to vehicle was observed to increase when the concentration of PQ increased, as evident by the much higher relative fold change expression of *NGAL* in the 150 µM PQ condition compared to the 75 µM PQ condition (Figure 9). *NGAL* expression in the 150 µM PQ condition was the greatest, as expected. In the 150 µM PQ condition, *NGAL*'s fold change in expression was 12.12 times higher than vehicle, whereas the 75 µM PQ condition was only 0.33 times as high as vehicle. *NGAL* was upregulated as evident by the 3572% fold increase from 75 µM to 150 µM PQ.

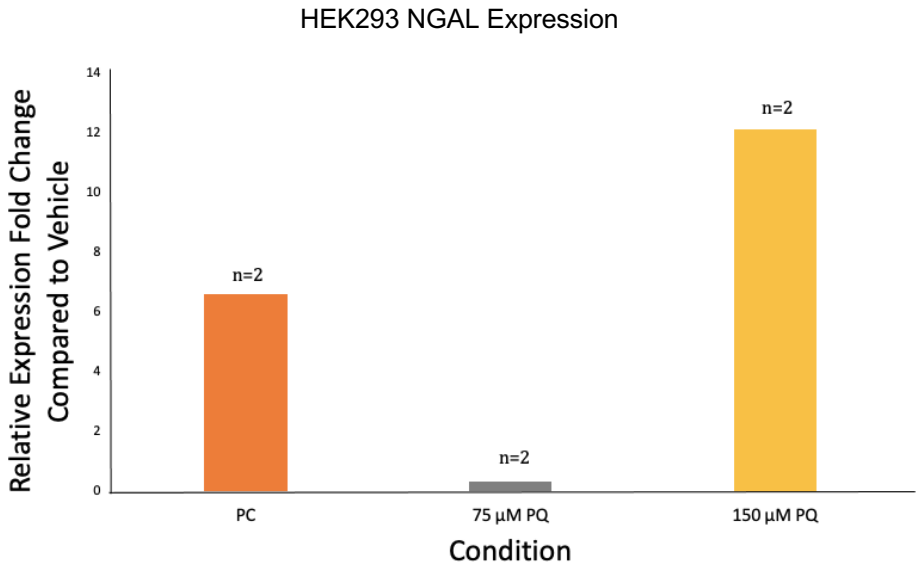

Figure 9. Relative fold expression graph comparing average $2^{-\Delta\Delta Ct}$ values of *NGAL* in HEK293 cells. The control mRNA was *BACTIN*. There is a trend for both PC and 150 µM PQ to increase expression of *NGAL*.

DISCUSSION AND FUTURE DIRECTIONS

The study aimed to investigate a potential link between pesticides like Paraquat and kidney disease biomarkers *KIM-1*, *NGAL*, *GSTP1*, and intracellular ROS. *KIM-1* and *NGAL* were hypothesized to increase as the concentration of PQ increased. This change was expected to be manifested through an increased fold change in mRNA ($2^{-\Delta\Delta Ct}$) compared to vehicle control with *BACTIN* as the control mRNA. These biomarkers are also candidates to explore when clinically assessing kidney disease. ROS and *GSTP1*, measured by microplate reader and RT-qPCR experiments, respectively, were also suspected to increase as indicators of oxidative and cellular stress when pesticide treatments became more concentrated. A higher level of intracellular ROS is also associated with acute kidney disease, which is a significant contributor to CKD[27].

In this study, a significant relationship could not be detected between ROS and increasing levels of PQ and Metribuzin. This could be due to an absence of an acute response: perhaps PQ and Metribuzin evoke a stronger, chronic response in HEK293 cells. This study chose a 24h dosage time due to it being explored in previous pesticide studies with mammalian cells[34-35]. It is recommended that if this study were to be replicated, a variety of dosage times are explored and physiologically correlated to body retention times in farmworkers.

Another reason may be that the CellROX Deep Red reagent was not sensitive enough to discriminate ROS levels between different conditions in the microplate environment is that the reagent is suited for microplate experiments as well as flow cytometry, thus amplifying any shortcomings due to plate reader sensitivity. Previous issues that were troubleshot included substantial loss of HEK293 cells during washing steps, because of which PLL was used to precoat cell culture dishes before experiments. To minimize any background fluorescence, black-

walled microplates were used. Finally, to minimize variability in seeding densities across the cell culture vessel, a 96-well plate was utilized with a plate reader setting of 10 reads per well to curtail discrepancies introduced by any uneven cell densities across the well. Because the CellROX Deep Red reagent can be used for plate reader experiments as well as fluorescence-activated cell sorting (FACS) and cell cytometry, another future direction is repeating this ROS experiment using FACS technology. Results may also be improved by using an alternate or less harsh chemical as a positive control condition, such as TBHP (*tert*-Butyl hydroperoxide), or using a lower concentration of Menadione. The current positive control of Menadione at 100 μM was harsh for cells, as evident in Figure 5. After 24h, about 40% of confluent cells were detached from the well. This may have led to a lower relative fluorescence intensity (RFU) reported by the PC condition due to a lower cellular count, as well as discrepancies introduced by the loss of uniformity in cell densities.

Lastly, RT-qPCR experiments indicated a trend between the conditions, where the highest concentration of pesticides resulted in a trend of an increased fold change in *GSTP1*, *KIM-1* and *NGAL* compared to lower, 75 μM PQ condition. For the *GSTP1* RT-qPCR assay, the PC condition had a low fold change increase; this could be because the cellular morphology was heterogenous across the wells, thus introduced a differing *BACTIN* (cytoskeletal structural protein) expression. Thus, the baseline, *BACTIN* housekeeping control expression may have differed in repeated trials of Menadione at 100μM. Another observation is that the expression levels of *GSTP1* are all lower than *BACTIN* in the untreated condition. It is unclear as to exactly why this is, however, a prior study by Yang and Tiffany-Castiglioni (2007) also found that *GSTP1* enzyme activity decreased after the 24h timepoint upon PQ exposure, as does GSH (substrate for *GSTP1*). Since *GSTP1* utilizes GSH to scavenge ROS, the decrease in GSH may

have led to decreased activity (enzyme, and perhaps transcriptional) of *GSTP1*[36]. Moreover, the expression levels for *GSTP1* may have upregulated quicker than 24 hours and thus were unable to be detected as expected. It could also be proposed that Paraquat may upregulate the *GSTP1* expression levels to a greater extent than Menadione (PC) or participate in a more potent negative feedback mechanism involving *GSTP1*. Statistically, more trials are needed to confidently establish the fold change of *GSTP1* in the PC condition as currently, n=1.

In the *KIM-1* experiments, the untreated trials had low expression and had average Ct values above 34, and thus could not be utilized. This could have been due to irregular, rounder morphologies of cells and thus decreasing *BACTIN* content, which could have led to higher average Ct values than expected. Thus, the fold change of *KIM-1* in 75 μM and 150 μM conditions was normalized against the positive control condition (Menadione). A similar trend was observed, where expression levels of *KIM-1* were exacerbated at a higher PQ concentration condition. Given *KIM-1* was transcriptionally undetected in vehicle conditions, in these experiments, the basal expression levels of *KIM-1* were very low, yet upon PQ treatment KIM-1 mRNA was upregulated in response to pesticide exposure. In this study, *KIM-1* may be the strongest biomarker of uCKD in cases of pesticide exposure, as expression of *KIM-1* is activated upon PQ exposure.

In the *NGAL* experiments, as the fold change increase compared to vehicle conditions, a much higher $2^{-\Delta\Delta}$Ct for the 150 PQ μM condition was detected, compared to the 75 μM treatment. The PC in this assay dramatically increased the levels of *NGAL* as well, and the results imply that upon cellular damage, *NGAL* expression levels may increase in HEK293 cells. There was a 36.7-fold increase in the 150 μM PQ condition compared to the 75 μM, and it may be hypothesized that there exists a pesticide threshold at which *NGAL* is upregulated. Future studies

are needed to track transcriptional activity across various pesticide concentrations and time points to detect the triggers for *NGAL* upregulation.

A shortcoming of the RT-qPCR experiments was sample size as more trials need to be conducted to attain statistical analysis. In addition to repeating these experiments, a future direction would employ a more sensitive fluorescent RT-qPCR probe like TaqMan to detect lower mRNA expression levels.

In conclusion, there was no detectable significant relationship between intracellular ROS levels and increasing PQ and Metribuzin concentrations. These results do not corroborate the trend of increasing ROS seen in previous PQ studies, possibly due to experimental limitations[34-35]. However, there was a trend for increased mRNA expression of *KIM-1*, *NGAL* and *GSTP1* between the 75 μM and 150 μM of PQ conditions, which may indicate that perhaps an increase in these KD biomarkers is correlated to increased PQ exposure in HEK293 cells. Moreover, this study establishes that although at minimal levels due to the low expression fold changes, *NGAL* and *KIM-1* can be detected in HEK293 after pesticide exposure cells via RT-qPCR. *NGAL* did show an increase in expression with increased PQ exposure, which was indicated in a prior study as a precondition to uCKD[31]. *KIM-1* also had a trend to slightly increase, yet more trials are needed to establish any statistical relationships. More studies are recommended to clinically correlate these findings in the *in vivo* environment, especially in farm workers. These preliminary findings do raise concerns about the safety of pesticide exposure and the potential risk for developing kidney disease.

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