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Cadmium Exposure Activates Akt/ERK Signaling and Pro-Inflammatory COX-2 Expression in Human Gallbladder Epithelial Cells via a ROS Dependent Mechanism.

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Abstract

Gallbladder cancer (GBC) is the commonest biliary tract cancer with an ill-defined etiology. We examined the role of Cd^{+2} exposures in a primary human gallbladder (GB) cell line model in this study. Cd^{+2} exposures induced decreased cell viability, reactive oxygen species (ROS) generation, altered Akt/ERK signaling pathway activation, PGE_2 and COX-2 expression in a human primary gallbladder epithelial cell model. Pharmacological inhibitors were used to determine the key drivers of elevated COX-2 expression due to Cd^{+2} exposure. Our results show Cd^{+2} causes a dose-dependent reduction in GB cell viability (EC50 value – 18.6 μM). Dose-dependent activation of phospho-Akt and phospho-ERK signaling pathways via increased phosphoprotein expression was observed due to Cd^{+2}. Signaling activation of Akt and ERK was prevented by 5 mM N-Acetyl Cysteine (NAC) establishing the role of ROS as a key driver in the activation process. Importantly, we observed Cd^{+2} also caused a dose dependent change in the COX-2 and PGE_2 expression levels. PI3K-Akt and NF-kB signaling pathways play a key role in Cd^{+2} exposure induced COX-2 activation in the gallbladder epithelial cells. In conclusion, our study measures the toxicological effects of Cd^{+2} exposures on human GB epithelial cells for the first time and establishes the role of Cd^{+2} as a possible driver of the Akt/ERK pathway overactivity and chronic inflammation in gallbladder carcinogenesis.

Keywords

Gallbladder Cancer; Cadmium; Chronic Inflammation; Akt; COX-2; Reactive Oxygen Species

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Authors' contributions:

RRG conceived the study and provided the guidance for the design, oversight and funding of the study. PS, TSC and MR performed the necessary experimental work to generate the data in the manuscript. PS, TSC, MR and RRG performed the data analysis of the experimental data. RRG wrote the paper. All authors read and approved the manuscript.

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Conflict of Interests:

The authors declare that they have no competing interests.

I. Introduction:

Gallbladder cancer (GBC) is a relatively rare cancer [1]. Yet, it is the commonest malignancy in the biliary tract and the fifth commonest malignancy of the gastrointestinal (GI) tract [1-4]. Clinical outcomes in GBC patients are very poor with a 3-8% five-year survival and a mean patient survival of a mere six months [1]. This malignancy is often diagnosed at an advanced stage in patients and has a limited set of therapeutic options [1]. GBC incidence is elevated in distinct geographical locations across the globe with increased incidence observed in countries such as Chile, Bolivia, North India, Bangladesh, Peru, Japan and the state of New Mexico in the United States [1, 3-5]. The main underlying risk factor of GBC development is pre-existing gallstones [1, 3, 4]. While gallstones are an important risk factor, it does not fully explain the carcinogenic risk associated with the development of GBC [6]. It is highly likely secondary risk factors play an important role in GBC pathogenesis. Non-gallstone secondary GBC risk factors described include salmonella infections, female gender, obesity, poor socio-economic conditions, environmental risk factors such as diet, aflatoxins and potentially, heavy metal exposures [1-4, 7, 8].

In the state of New Mexico, the Native American populations have a high predisposition to develop gallbladder cancer compared to Caucasian populations (5-8 fold elevated incidence) [1, 9]. The Hispanic population within the state has an elevated predilection for GBC as well (2-4 fold higher) [1, 9]. Additionally, Native American women in New Mexico have one of the highest incidences of GBC in the continental United States for unknown reasons [1, 3, 9]. While many etiological factors are implicated in the GBC causation, very little attention has been paid thus far to the role of heavy metal exposures as a driver of this disease [1, 10]. Shukla et al in a case-control study showed significantly higher biliary concentrations of cadmium, copper and lead in the samples (serum, bile, tissue and gallstones) of GBC patients as compared to gallstones only group in India [11]. Other studies have explored the possible role of heavy metal toxicity in GBC pathogenesis [8, 10, 12]. Most of the studies examining the role of heavy metals in GBC causation thus far are of an epidemiological nature.

Cadmium (Cd) is a heavy metal toxin that is widely distributed in the environment [13-17]. Cd is a common by-product of many industrial processes including battery manufacture, leather-tanning industry, mining and smelting [13-17]. Cd contamination occurs in food products and water supplies. Cd is classified as a category I carcinogen by the International Agency for Research on Cancer (IARC) [13-17]. Cd is among the top ten dangerous substances of concern as classified by the Agency for Toxic Substances and Disease Registry (ATSDR). Chronic Cd exposure has been associated with cancers of the lung, prostate, kidney and pancreas [13, 15]. Cd exposures triggers stress protective and anti-apoptotic mechanisms in cells which over the long-term may lead to neoplastic transformation [15, 18, 19]. The primary sources of human exposure to Cd are via food consumption and inhalation of cigarette smoke [15, 18, 19]. Exposure to Cd has been shown to disrupt a variety of intra-cellular signaling processes including ROS generation, altered Ca^{+2} signaling, PI3K-Akt-mTOR pathways, MAP-kinase pathways and NF-kB pathways to name a few [17]. Cd can form complexes with proteins in the liver via sulfhydryl groups

(e.g., Glutathione – GSH) or high affinity metal binding metallothionein (MT) proteins [17]. The bound Cd is then excreted into the bile or the circulation as CdGSH or CdMT complexes [17, 20].

Previous studies on the molecular phenotyping of GBC showed dysregulated signaling of the Akt and ERK gene pathways in particular [21, 22]. Akt and MAPK pathways are part of a group of intracellular serine/threonine protein kinases that play a crucial role in the regulation of numerous cell signaling cascades essential for the cell functionality [17, 20, 23-25]. Activation of the cellular cascades of Akt and MAPK are responsible for diverse cellular homeostatic functions such as cell proliferation, differentiation and apoptosis [17, 20, 23-25]. The key members of the MAPK family include the extra-cellular regulated kinases (ERK), p38 kinases and the c-JUN (JNK) kinases. The ERK kinases are involved mainly in the cell proliferative effects due to growth factors while p38 and JNK modules are toxic cell stress responsive and pro-apoptotic [23, 24, 26]. Extensive cross talk is present between various cell signaling modules of the Akt and MAPK pathways. Cell signaling regulation of Akt and MAPK pathways is achieved via inhibitory feedback loops. However, in the presence of unopposed activation of these kinases, the cells tend to adopt a proliferative, pro-survival phenotype, which may lead to neoplastic transformation. Cd exposure has been previously shown to drive a dose and time dependent activation of the Akt and MAPK pathways (ERK is activated earlier than p38 and JNK) via a possible ROS mediated mechanism [27, 28]. Due to the established role of dysregulated signaling of Akt and ERK proteins in GBC and the epidemiological association of Cd and GBC, we sought to examine the effects of Cd exposures on the activation of these pathways in the current study. In particular, we focused mainly on the ERK portion of the MAPK signaling pathway due to its established relevance in the cell proliferative activity in GBC. We do not examine the p38 and JNK pathways in this manuscript.

A second well-established key feature of GB carcinogenesis is pre-existing chronic inflammation commonly seen in this form of cancer [7, 29]. The role of chronic inflammation as a key driver of carcinogenesis (e.g., pancreas, esophagus, liver, stomach and bladder and endometrium) is a well studied issue with extensive experimental evidence [30]. In GBC, the primary driver of chronic inflammation is likely the presence of gallstones [31]. Yet, the mere presence of gallstones alone is not sufficient to drive GB carcinogenesis [31]. There is also a clear need to study secondary drivers of chronic inflammatory processes in GBC, which is not well understood currently [7, 32]. Evidence from studies in India show a strong role for chronic salmonella infections of the GB as a driver of chronic inflammatory processes (Odds Ratio - 4.0) [4, 33]. However, chronic salmonella infections are infrequent in developed countries such as the United States. Thus, alternative drivers of GB chronic inflammation need to be examined in the western context. Cd exposure has been shown to cause activation of the NF-kB pathway via a ROS mediated mechanism [17, 20]. The NF-kB gene is a master regulator of chronic inflammatory gene expression. In GBC, a gene of particular interest is the PTGS2 gene (also known as COX-2) [7, 34]. COX-2 is an enzyme involved in tissue prostaglandin synthesis and is associated with many neoplastic conditions [35]. Normal gallbladder epithelium does not express COX-2 while aberrant GB dysplastic condition shows elevated COX-2 expression [34]. Thus, it is of much interest to determine

the existence of a mechanistic relationship between Cd exposure and COX-2 gene expression in gallbladder cells which is one of the objectives of this study.

While this study was underway, Lee et al conducted a large-scale epidemiological assessment which examined the levels of heavy metals in the serum of gallbladder cancer, gallstone only and matched normal population samples from the Shanghai Biliary Tract Cancer Study [8]. This study identified statistically significant elevated levels of cadmium, chromium, copper, molybdenum and vanadium heavy metals in GBC patients compared to matched gallstone controls [8]. This study concluded with the urgent need for mechanistic studies to examine these associations in detail. In the present study, we examine the effects of dose-dependent cadmium exposures in primary human gallbladder cells. In particular, we focus on the mechanistic role of Akt, ERK and COX-2 cell signaling pathway activation due to Cd exposures and the role of ROS in a GB primary epithelial cell line model for the first time. Our study demonstrates the effects of Cd exposure in the activation of Akt, ERK and COX-2 signaling pathways strengthening our hypothesis for a key heavy metal role in GB carcinogenesis supporting the recently published epidemiological findings [8].

II. Materials and Methods:

1) Chemicals and reagents:

The following chemical reagents used in the study were purchased from different vendors: Cadmium Chloride ($CdCl_2$) (cat # 202908) and N-acetyl cysteine (NAC) (cat # A9165) was purchased from Sigma Aldrich (St. Louis, MO, US). LY294002 (PI3K Inhibitor; cat #70920), PD98059 (MAPKK Inhibitor; cat # 10006726) and MG-132 (Proteosome inhibitor; cat # 13697) were purchased for Cayman Chemicals (Ann Arbor, Michigan, US). For the protein assays, the following primary antibodies were purchased from the following vendors: p-Akt (Ser473; cat # 4060S), Phospho-p44/42 ERK1/2 (cat # 4370S), and Anti-mouse IgG, HRP-linked Antibody (cat # 7076S) were obtained from Cell Signaling Technology (Beverley, MA, US). ERK1/2 (cat # sc-514302), Akt1 (cat # sc-5298), GAPDH (cat # sc-47724), COX-2 (cat # SC-376861) and anti-rabbit IgG, HRP linked antibody (cat # sc-2357) were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, US). The chemiluminescence (ECL) detection kit used for Western blot assays was from GE healthcare (cat # RPN2236, Chicago, Illinois, US). We used the 4–20% Mini-PROTEAN® TGX™ precast protein gels (cat # 4561094) from BioRad (Hercules, CA, US) for protein electrophoresis and separation.

2) Cell culture, cadmium and NAC treatments:

The human primary gallbladder cell line (GBP-9) used in this study was obtained from Celprogen (cat #36156-02, Torrance, CA, USA). The gallbladder cell line obtained from a 28-year-old human male donor. Gallbladder primary cells were grown in pre-coated cell culture chambers with specialized gallbladder primary cell culture medium (cat #M36156-02S, Celprogen, Torrance, CA, USA). Initial tests showed the gallbladder primary cells are viable for up to 12 passages. In this study, gallbladder primary cells beyond the 9^{th} passage were discarded and not used for experiments to avoid potentially confounding senescent effects. The primary gallbladder cells were grown in humidified atmosphere of

95% air ~ 5% CO_2 at 37°C. Cells in culture were collected by washing them in 1X PBS first followed by the use Cellstripper™ solution (cat #25056CI, Corning, NY, USA) as needed. The gallbladder primary cells were exposed to different concentrations (0, 0.5, 1, 2.5, 5 and 10 μM) of cadmium chloride solutions ($CdCl_2$) for variable duration (2, 4 and 24 hours) depending on the individual experimental assay. For the ROS blocking experiments, the GB primary cells were pre-incubated with N-acetyl cysteine (NAC) at a concentration of 5 mM for 1 hour prior to the initiation of the Cd exposure protocol.

3) Cell viability assay:

AlamarBlue© colorimetric assay (cat #786-922, G Biosciences, MO, US), was used to determine the cell viability of the GBP-9 GB cells used in the study in response to cadmium exposures. In brief, cells were seeded in 96 well plates at a seeding density of 1 x 10^4 cells/well and grown overnight to ensure attachment to the plate. The cells were then exposed to 0, 0.1, 0.5,1, 2.5, 5, 10, 25, 50 and 100 μM $CdCl_2$ and incubated for a further duration of 24 hours. After completion of the incubation period, 20 μl of AlamarBlue© reagent was added to the wells and incubated for a further duration of 6 hours. The assay works on the principle of the conversion of non-fluorescent AlamarBlue© reagent (blue) to a fluorescent red color by growing cells which is then measured by absorbance methods in a standard 96-well format plate reader. At the end of the incubation time, a plate reader (Biotek Synergy Neo2, VT, US) was used to measure the absorbance values at 570 and 600 nm wavelengths. The levels of dye reduction observed under different conditions of cadmium exposure was then used to calculate the cell viability in each well according to the manufacturer's instructions. Each treatment level was examined in triplicate. Negative, untreated and positive (100% reduction) controls were run in each plate for all of the cell viability assays as per the manufacturer's instructions.

4) Lactate dehydrogenase cytotoxicity assay:

The fluorometric LDH-cytotoxicity assay (cat # ab197004, Abcam, CA, USA) was performed to quantify the degree of cell death resulting from Cd exposures. In brief, cells were seeded in 96 well plates (seeding density ~ 1 x 10^4 cells/well) and grown overnight. The cells were then exposed to 0, 0.1, 0.5 1, 2.5, 5, 10, 25, 50 and 100μM $CdCl_2$ and incubated for a further 24 hours. The assay measures the extracellular LDH release in the media due to cell membrane disruption leading to cell death via the conversion of lactate to NADH and pyruvate NADH is then reduced by a proprietary probe to generate intense fluorescence. The excitation and emission wavelengths of fluorescence were 535 and 587 nm respectively. The plate reader (Biotek Synergy Neo2, VT, US) was used to obtain the fluorescence readings. Appropriate negative and positive readings were incorporated into the assay as per standard protocol recommendations from the manufacturer.

5) GB epithelial cell image morphology:

GBP-9 Gallbladder primary cells were cultured overnight in six-well plates until confluent. Cells were then exposed to Cd and Cd+NAC (5 mM) at 0, 5 and 10 μM concentrations) separately. To characterize differences in epithelial cell morphology, phase contrast imaging was performed at a time intervals of 2, 4, 8 and 24 hours after incubation on a Nikon

Diaphot microscope at 20× magnification. Qualitative differences in the epithelial cell morphology are described in the body of the text.

6) ROS measurement assay:

Production of reactive oxygen species was also measured quantitatively using the DCFH-DA assay method. The Cd^{+2} exposed cells were incubated with 0.5 μM DCFH-DA for a duration of 45 minutes at 37°C in dark. The fluorescence intensity from each well was measured on a microplate reader (Biotek Synergy Neo2, VT, US) at 485nm – 528nm excitation and emission wavelengths. THBP was used as positive control. Background fluorescence intensity measured from blank wells was corrected appropriately. The mean ± standard deviation (S.D) fluorescence intensity results are reported as a relative ratio between exposed and unexposed cells and one-way ANOVA statistical analysis was used to measure differences in the different exposure conditions. Reactive oxygen species (ROS) generation was also monitored semi-quantitatively by the dihydroethidium (DHE) assay. In brief, the primary gallbladder cells (1×10^4 cells/well) were cultured in a 96 well plate for 24 hours with $CdCl_2$ and $CdCl_2$+NAC at various concentrations. Then, the cells were further incubated with 0.5 μM DHE for a duration of 45 minutes at 37°C in dark. High content microscopy with automated image acquisition and quantification was performed using CellInsight CX5 High Content Screening (HCS) Platform (ThermoFisher Scientific, Waltham, MA, USA); >1000 cells were analyzed per well, and six replicates were analyzed in each exposure condition. Hydrogen Peroxide (H_2O_2) was used as positive control. Hoechst 33342 staining was used to stain the nucleus of the cells to define objects/cell based on background staining of the cytoplasm. ROS levels were assessed in a qualitative manner.

7) Glutathione Measurement Assay:

To study the effect of Cd^{+2} and NAC on total GSH levels, GBP-9 cells were plated at a cell density of 0.3×10^6 cells/mL into six-well plates. Cells were exposed to Cd^{+2} and NAC treatments for 4 and 24 hrs. The total intracellular GSH was quantified using a glutathione assay kit according to the manufacturer's instructions (Cayman chemicals, Ann Arbor, MI, USA). Cd^{+2} exposed cell pellets were sonicated in 2 mL of cold 50 mM 2-(N-morpholinoethanesulfonic acid buffer (MES buffer, pH 7.4) followed by centrifugation (10,000 × g, 15 min) at 4°C. The supernatant was then deproteinated using a buffer solution containing metaphosphoric acid (10% w/v) and triethanolamine (53.1% v/v) to remove proteins and avoid interference from protein-sulfhydryl groups. 50 μL of respective samples and 50 μL of standard were added to separate wells (n = 3 for each sample and standard) in a 96-well plate, followed by addition of 150 μL of freshly prepared GSH assay cocktail. The end point value at 30 min (Absorbance 405nm) was used to calculate the total intracellular GSH concentration in GBP-9 cell lysate.

8) Western blot analysis:

Cell lysates were prepared in RIPA lysis buffer (1M Tris-HCl, pH 7.6, 150mM NaCl, 5mM Ethylene Diamine Tetra-acetic Acid, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulphate) supplemented with a cocktail of protease inhibitors (cat #88665, Pierce™ Biotechnology Thermo Scientific, MA, US). The cell lysates were centrifuged at 10,000 × g for 5 min at 4^0C to remove cellular debris. The resulting supernatant was stored

at −20°C. Protein concentrations were determined using the bicinchoninic acid protein assay reagents (cat #23227, Pierce™ Biotechnology, ThermoScientific, MA, US) according to the manufacturer's instructions. For immunoblots, equal amount of protein (15~20 μg) was separated electrophoretically by SDS–polyacrylamide gel electrophoresis. The protein was transferred to a nitrocellulose membrane by the electroblotting procedure. Nonspecific binding was blocked by incubating the nitrocellulose blots with 5% milk in TBS- T (100 mM Tris, pH, 1 M NaCL, 1% Tween- 20) for 1 hour at room temperature. The membranes were incubated overnight with the following antibodies (1:500 dilution): p-Akt, p-ERK, total ERK ½, total Akt, COX-2 and GAPDH. GAPDH was used as a loading control for all of the assays in this study. After serial washes in TBS buffer containing 0.05% Tween-20 three times for 5 min each, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse antibody for a duration of one hour. The membranes were then washed for three times in TBS containing 0.05% Tween-20 for 5 min each, followed by signal development using an Amersham ECL detection kit (cat #RPN2232, GE Healthcare). Images were obtained using Bio-Rad ChemiDoc XRS+ imaging workstation (Bio-Rad, California, US). The intensity of the Western blot bands was quantitatively analyzed using the ImageJ software.

9) PGE2 Quantification using ELISA:

Levels of PGE2 released into the supernatants of Cd^{+2} treated cells were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Cells were seeded at a density of 0.05×10^6 cells/ml in a 24 well plate and treated with $CdCl_2$. The levels of soluble PGE_2 were measured after 4 and 24hrs in response to 0, 0.5, 1, 2.5, 5, and 10uM Cd^{+2} in the presence and absence of NAC supplementation. Supernatant was collected at the end of the incubation period and PGE_2 levels were measured according to the kit instructions. The ELISA plate was read by using a Synergy Neo2 plate reader (BioTek, VT, United States). Multiple replicates were analyzed and statistical analysis was performed.

10) Statistical analysis:

All data are represented as mean ± standard deviation (S.D) from the mean. Statistical analysis to determine differences due to dosing exposures were performed by a one-way ANOVA followed by strict Bonferroni pairwise t-test for multi-group comparisons. A value of $p < 0.05$ was considered significant for all of the tests. However, we show values of $p < 0.05$ (*), $p<0.01$(**) and $p<0.001$(***) as appropriate in the data. A minimum of three independent experimental replicates were performed for all assays with additional multiple technical replicates where appropriate. To calculate the EC50 values of the gallbladder epithelial cell viability, we fit a four-parameter log-logistic model to model to compute the cell viability of gallbladder primary cells in response to Cd^{+2}. The upper threshold (parameter *d* in equation below) was assigned a fixed value of 100% and all of the remaining parameters (*b, c and e*) were allowed to fit freely in an independent manner. The equation of 4-parameter log-logistic model is represented as follows. All statistical analysis was performed using R software and libraries (v.3.5.3)

$$f(x,(b,c,d,e)) = c + \frac{d-c}{(1+exp(b(log(x)-log(e))))}$$

Where,

f(x,(b,c,d,e) = Function of the model fit

b = Steepness of the curve fit

c = Lower limit of viability

d= upper threshold of viability and

e = ED50 characteristic of the curve fit

III. Results:

a. Cd^{+2} exposure causes gallbladder epithelial cytotoxicity in a dose-dependent manner which is alleviated by NAC co-incubation:

First, we established the cytotoxic effects of Cd^{+2} exposure on human primary gallbladder epithelial cells which is currently unknown. We examined the cytotoxic effects on GBP-9 cells across a wide range of $CdCl_2$ concentrations (0, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 μM). Gallbladder epithelial cells showed a consistent, dose-dependent reduction of cell viability due to Cd^{+2} exposure (see Fig 1). The 100 μM Cd^{+2} concentration showed a cell viability of only 0.2 ± 3.1 % (see Fig 1A) after 24-hour exposure as compared to the control cells. The results were consistently repeatable across multiple, independent experimental replicates (n = 5). One-way ANOVA analysis ($p < 0.05$) showed the higher concentrations of Cd^{+2} exposure lead to statistically significant differences in GBP-9 cell death above a Cd^{+2} concentration of 1 μM at the end of a 24-hour exposure period (see Figure 1A). To complement the cell viability results, we also examined the cellular death effects in Cd^{+2} exposed GBP-9 cells using the lactate dehydrogenase (LDH) release assay. Under conditions of cell death and lysis, the LDH enzyme is released which is then measured using a fluorescence assay. Concentrations similar to cell viability assay (0, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 μM) were used to monitor LDH levels at the end of a 24-hour Cd^{+2} exposure. As expected, a dose dependent increase in the LDH values was observed due to increasing Cd^{+2} dosing exposures (Fig 1A). Statistically significant levels of LDH ($p < 0.05$) was observed in Cd^{+2} doses of 1 μM and above similar to the cell viability results. A dose-response curve assessment of Cd^{+2} exposures was performed to quantify the EC50 cytotoxic doses of Cd^{+2} on primary GB epithelial cells (see Figure 1B). A 4-parameter log-logistic curve fit (see methods) was used to determine the EC10 and EC50 values of Cd^{+2} toxicity on GBP-9 cells. The EC10 and EC50 values for GBP-9 cells due to Cd^{+2} exposures were measured to be 2.27 μM and 18.9 μM respectively (see Figure 1B). This data establishes the cytotoxic potential of Cd^{+2} exposure on human gallbladder primary epithelial cells. Based on the calculated EC10 and EC50 values, we used 0, 0.5, 1, 2.5, 5 and 10 μM (sub-EC50 doses) in our subsequent experiments to understand the effects of sub-EC50 Cd^{+2} dose

toxicity on human gallbladder epithelial cells. Overall, these findings demonstrate a dose-dependent impact of Cd^{+2} ions on primary GB epithelial cell viability.

In addition to the effects of Cd^{+2} exposure on gallbladder primary cells alone, we tested the effects of simultaneous n-acetyl cysteine (NAC) co-incubation on GB epithelial cell viability at Cd doses tested earlier (0, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 μM) to examine the role of ROS species in gallbladder epithelial cell viability. NAC is a well-studied therapeutic molecule used to prevent oxidative stress in cells through the generation of anti-oxidant glutathione (GSH). The cells were pre-incubated with NAC for 2 hours followed by 24 hours of $CdCl_2$ exposure at different concentrations of $CdCl_2$. At the end of the 24-hour incubation period, we observed little to no reduction in the overall viability of the GBP-9 epithelial cells (see supplementary Fig 1). At low doses of Cd^{+2} (0.1 and 0.5 μM) exposure, we observe a slight elevation of cell viability (112 ± 2.2 % and 104 ± 3.9% respectively) probably corresponding to the low-dose hormetic effects of Cd similar to the effect described earlier [36]. However, we did not identify any statistically significant changes in the GB cell viability at all concentrations of Cd^{+2} exposures (0 – 100 μM). GBP-9 cells also showed a limited elevation in the LDH levels at 24 hours with 5 mM NAC pre-incubation (see supplementary Fig 1). The 100 μM concentration of Cd^{+2} exposure showed the highest increase of LDH level overall (19.8 ± 1.32 %). However, this is much lower compared to the 71 ± 5.9% seen with Cd^{+2} alone (see Fig 1). These results support the role of NAC co-incubation alleviates the Cd^{+2} exposure induced gallbladder epithelial cell cytotoxicity via a possible ROS generative mechanism which is explored in the next section.

b. Cd^{+2} exposure leads to elevated ROS and depletion of GSH levels in GB epithelial cells:

Toxic doses of Cd^{+2} are a well-known driver of ROS generation in different cell types [17]. However, since Cd^{+2} is not a Fenton type metal, the precise mechanisms of Cd^{+2} induced ROS generation are currently unclear [17]. To assess the role of ROS generation in Cd^{+2} induced gallbladder epithelial cytotoxicity specifically, we examined the Cd^{+2} dose dependent (0, 0.5, 1, 2.5, 5 and 10 μM $CdCl_2$) ROS levels in primary GB epithelial cells at 4- and 24-hour time points. We also used the well-established ROS scavenging agent, N-Acetyl Cysteine (NAC), to determine the impact of NAC pre-incubation on ROS suppressive activity due to Cd^{+2} ion exposure (see Fig 2). At the 4-hour time point, ROS elevation was seen only in the highest 10 μM Cd^{+2} dose (data not shown). However, at the 24-hour time point, we observed a steady, dose-dependent increase in the ROS levels due to Cd^{2+} exposures (see Fig 2A). Statistically significant increases ($p < 0.05$) in ROS levels were observed for the 2.5, 5 and 10 μM Cd^{2+} exposure doses relative to the unexposed GB primary cells (Fig 2A). In contrast, pre-incubation with anti-ROS NAC (5 mM) suppressed the overall ROS levels at 0.5, 1, 2.5, 5 and 10 μM Cd^{+2} exposure dose levels (see Figs 2A) as compared to Cd^{+2} exposure alone (Fig 2A). A slight elevation of ROS species was observed in 5 and 10 μM Cd^{+2} dose even with NAC incubation indicating the dose-dependent ROS generative effects of Cd^{+2} exposures in primary GB epithelial cells. In addition to the quantitative DCFH-DA assessment of the ROS levels, we also examined the ROS generation using an alternative, superoxide specific dihydroethidium (DHE) dye method which reports ROS species within the cell nucleus qualitatively (see supplementary data Fig 2). Similar to

the DCFH-DA assay, we also observed a dose-dependent elevation of the superoxide ROS in the cell nucleus due to Cd^{+2} exposure which was reduced by the addition of NAC supplementation (see supplementary Fig 2).

In addition to the quantitative and qualitative ROS levels, we also assessed the levels of the intracellular ROS protective molecule, glutathione (GSH), in GB epithelial cells due to Cd^{+2} exposures. We assessed the GSH levels at the 4- and 24-hour interval time points in Cd^{+2} exposed GBP-9 cells. Minimal changes in GSH level were seen at the 4-hour time point similar to ROS level (data not shown). However, we observed a dose dependent reduction in the GSH levels in at the 24-hour time point in response to ROS generation due to Cd^{+2} exposures (0.5, 1, 2.5, 5 and 10 μM Cd^{+2}; see Fig 2B). In contrast, supplementation with NAC prevented the reduction of GSH levels due to Cd^{+2} exposure relative to Cd^{+2} alone. This is in line with the established functional role of NAC in supplementation of intracellular GSH levels. In summary, our results confirm the existence of dose-dependent ROS generation and oxidative stress protective effects (via GSH generation) due to Cd^{+2} exposures and simultaneous NAC incubation respectively in human gallbladder epithelial cells.

c. Cd^{+2} exposures decrease GB epithelial cell monolayer integrity which is prevented by NAC supplementation:

Chronic inflammation is a key feature of gallbladder carcinogenesis [7, 32]. Thus, disruption in barrier integrity has the potential to drive chronic inflammation in the gallbladder. We examined the effects of Cd^{+2} exposure on altered GBP-9 cell monolayer characteristics at the 2, 4, 8 and 24-hour time points (0, 5 and 10 μM Cd^{+2} concentrations). GB epithelial cell monolayer features were assessed after Cd^{+2} exposure alone and Cd^{+2} co-incubated in the presence of NAC separately. The control cells (No Cd^{2+}; No Cd^{+2} + 5 mM NAC) showed a uniform monolayer of epithelial cells at the 24-hour time-point as expected (see Figure 3A and D). The classical cobblestone morphology commonly seen in cells of gut epithelial origin was preserved at 24 hours. In the cells treated with 5 μM Cd^{+2} alone, we observed loss in the cell-cell junctional contacts (see figure 3B) as early as 8 hours. Some scattered epithelial cells were observed to have a slightly rounded morphology after a 24-hour exposure. The 10 μM Cd^{+2} dose-exposure showed the biggest difference in the GB epithelial monolayer integrity. A majority of these cells demonstrated a rounded morphology with extensive loss of cell-cell junctional contacts (Fig 3C). In contrast, pre-treatment and co-incubation with 5 mM NAC preserved the GBP-9 cell-cell junctional contacts and the overall epithelial monolayer integrity at both 5 and 10 μM Cd^{+2} exposures (Fig 3E and F). Thus, we observed two key findings a) Cd^{+2} exposures have the potential to disrupt normal GB barrier homeostasis via disrupted epithelial monolayer integrity and b) pre-treatment with NAC protects the gallbladder epithelial cells from Cd^{+2} exposure induced loss of cell-cell junctional contacts indicating a potential role for ROS involvement in disrupted GB monolayer integrity. These effects may also be due to enhanced cell death and/or altered cell junction protein expression due to Cd^{+2} exposures and is the focus of our future studies in our lab.

d. Akt and ERK signaling pathway activation in GB epithelial cells due to Cd^{+2} exposures:

Akt and ERK pathways signaling overactivity is a consistent feature of gallbladder cancers [21, 22, 37]. Yet, the key molecular mechanisms responsible for the signaling dysfunction of Akt and ERK pathways in GBC are currently uncertain. Scanu et al showed the role of Salmonella infections as key driver of altered Akt and MAPK signaling in gallbladder organoids [22]. To evaluate the potential role of Cd^{+2} exposures as drivers of Akt/ERK signaling activation specifically, we exposed the GBP-9 epithelial cells to 0, 0.5, 1, 2.5, 5 and 10 μM $CdCl_2$ doses for 24 hours in the absence and presence of NAC co-incubation (5 mM) similar to previous experiments. Phosphorylated Akt activity of Ser473, a key phosphorylation activation site of the Akt protein, was measured using Western blot analysis along with the total Akt protein expression levels. We observed Cd^{+2} exposure causes a dose-dependent elevation in the p-Akt/total-Akt ratio at the 24-hour time point (see Fig 4A). Activated p-Akt expression was highest in the 10 μM Cd^{+2} exposure condition showing an approximately 3-fold increase compared to no Cd^{2+} exposure control (Fig 4A). In contrast, pre-treatment with NAC (5 mM) for 2 hours prior to Cd+2 exposure caused minimal activation of the phospho-Akt activity across all Cd+2 exposure levels with no statistical differences of phosphor-Akt expression. The Akt signaling protein is well known to contain a ROS-responsive signaling domain involved in causing a pro-survival cellular phenotype [17, 38]. Additionally, many of the negative regulatory protein phosphatases (e.g., PTEN) within the Akt pathway are also responsive to elevated ROS levels as well [17, 38]. Thus, these results indicate Cd^{+2} exposures may have a role as one of the mechanisms responsible for the signaling overactivity of Akt pathways commonly seen in the context of GB carcinogenesis [39]. Similarly, we also quantified the pro-proliferative p-ERK1/2 activation in response to varying doses of Cd^{+2} exposure. Similar to activation of p-Akt, we observe monotonic dose-dependent increase (0-10 μM Cd^{+2}) of the p-ERK1/2 activation after 24-hours of Cd^{+2} exposure (see Fig 4B). Pre-treatment with NAC (5 mM) suppresses the p-ERK1/2 phosphorylation activity similar to phospho-Akt indicating a likely ROS dependent mechanism of activation (Fig 4B). Thus, we conclude Cd^{+2} exposures may drive activation of Akt and ERK signaling pathways in gallbladder epithelia via a ROS driven mechanism. This data also supports the idea that heavy metal induced oxidative stress may be one of the mechanisms responsible for gallbladder carcinogenesis.

e. COX-2 expression is elevated in GB epithelial cells due to Cd^{+2} exposure:

As mentioned previously, long-term chronic inflammation has a well-established role in gallbladder carcinogenesis and is a characteristic feature of this form of cancer [1, 4, 32, 34, 40, 41]. However, the mechanisms (e.g., infections versus environment) underlying chronic inflammation associated with GBC are ill-understood. Gallstones, while important for GBC pathogenesis, do not fully explain the chronic inflammatory processes driving the process of GB carcinogenesis [1, 31]. Chronic GB inflammation is characterized by the elevated expression of PTGS2 (also commonly known as COX-2) which is overexpressed in the pre-neoplastic, dysplastic GB epithelial cells [34]. We evaluated the potential of Cd^{+2} exposures as a mechanism of elevated COX-2 expression in GBP-9 epithelial cells. GBP-9 cells were exposed to 0, 0.5, 1, 2.5, 5 and 10 μM $CdCl_2$ doses for 24 hours similar to other experiments. Since COX-2 expression is an early stress-response molecule, we examined the

COX-2 expression levels at two different time points after Cd^{+2} exposure (4- and 24-hours). We observed Cd^{+2} exposure causes a dose-dependent elevation of COX-2 expression at 4 hours (see Fig 5) across all of the tested concentrations (0 – 10 μM) and the 10 μM Cd^{+2} dose exposure showed the maximal COX-2 expression at 4 hours. However, at the 24-hour time point, we observe decreased COX-2 protein expression in the 5 and 10 μM Cd^{+2} exposure doses (see Fig 5). The reduced COX-2 protein expression seen with 5 and 10 μM exposures may be due to the cellular death and decreased viability seen in GBP-9 cells seen after 24 hours of Cd^{+2} exposure. Our findings are consistent with the possible role of heavy metals and Cd^{+2} exposure as a driver of chronic inflammation in gallbladder epithelial cells [35]. Thus, chronic Cd^{+2} exposures may be one of the important factors associated with chronic inflammation (and increased COX-2 expression) commonly seen in dysplastic and cancerous GB tissues. This may be of particular importance in regions with reduced overall Salmonella community burdens (e.g., Western hemisphere countries such as US) [1].

f. Identification of the key signaling pathways regulating elevated COX-2 expression due to Cd^{2+} exposures:

A previous study showed Cd^{+2} exposure may lead to elevated COX-2 expression via Akt phosphorylation and PTEN inactivation in a RAW264.7 murine macrophage model [42]. However, the major mechanism driving the regulation of COX-2 gene expression is through the well-established and studied NF-kB pathway signaling mechanism [17, 20, 35]. We used a pharmacological inhibitor-based approach to understand the signaling pathways regulating elevated COX-2 protein expression in gallbladder epithelial cells due to Cd^{+2} exposures. We sequentially tested LY294002 (a PI3K inhibitor), PD98059 (a p-ERK 42/44 inhibitor), MG132 (proteosome inhibitor and blocker of the NF-kB pathway signaling) and N-Acetyl Cysteine (NAC; an anti-oxidant factor) effects on COX-2 expression patterns in GB epithelial cells in conjunction with concomitant Cd^{+2} exposures. All of the inhibitors are specific to the individual signaling pathways of interest in this study (namely, the PI3K-Akt, ERK1/2 and NF-κB signaling pathways). GB epithelial cells were exposed to 10 μM Cd for a duration of 4 hours and the COX-2 protein expression was assessed thereafter in the presence and absence of the pharmacological inhibitors.

The PI3K-Akt pathway inhibitor (LY294002) alone reduced the baseline COX-2 expression level in a statistically significant manner ($p < 0.05$) indicating it plays a key role in COX-2 regulation (see Fig 6A). Co-exposure with Cd^{+2} did not elevate the COX-2 expression levels confirming a prominent role of the PI3K-Akt signaling pathway in regulation of COX-2 production in gallbladder epithelial cells. In contrast, the ERK selective inhibitor (PD98059) caused decreased COX-2 protein expression which was then rescued by the simultaneous exposure to Cd (see Fig 6B). This indicates the ERK signaling cascade likely has a limited role in regulating the COX-2 protein expression in the presence of Cd^{+2} exposures. Similar to the ERK inhibitor, we observed N-Acetyl Cysteine alone did not cause elevated COX-2 expression compared to the baseline (see Fig 6C). However, addition of Cd and NAC together caused statistically significant reduction in COX-2 protein expression (compared to Cd exposure alone; see Fig 6C) indicating a prominent role for Cd^{+2} exposure induced ROS generation as a driver of elevated COX-2 expression in GB epithelial cells. Finally, the NF-kB pathway inhibitor (MG132) in isolation had no impact on the baseline COX-2 protein

expression similar to the PI3K inhibitor and NAC (see Fig 6D). However, like Akt, simultaneous addition of Cd and MG132 caused a statistically significant reduction in the COX-2 expression indicating a functional role for the NF-kB pathway in regulation of the COX-2 protein expression in response to Cd exposure as well (see Fig 6D). In summary, the Akt and NF-kB pathways appear to be the key regulatory pathways involved in the COX-2 gene activation in GB epithelial cells in response to Cd^{+2} exposure. Since these two pathways are well-known to be free ROS radical responsive, our results prove Cd^{+2} exposure causes COX-2 gene activation via a likely ROS dependent mechanism.

g. Pro-inflammatory PGE2 levels are elevated in a dose-dependent manner due to Cd^{+2} exposure:

PGE2 is a pro-inflammatory lipid molecule which mediates chronic inflammation downstream of COX-2 expression [43, 44]. We examined PGE2 levels specifically since it is associated with a wide range of biological effects associated with inflammation and cancer [43, 44]. Similar to previous experiments, GBP-9 cells were exposed to multiple Cd^{+2} dose levels (0, 0.5, 1, 2.5, 5 and 10 μM) for a duration of 4 and 24 hours and the supernatants were collected. We observed a statistically significant elevation only in the 10 μM Cd^{+2} dose ($p < 0.05$) at the 4-hour time point (see Figure 7). However, at the 24-hour time point elevated levels of PGE2 expression were observed in the lower exposure doses (1 and 2.5 μM) and reduction in the higher doses (5 and 10 μM). However, these values failed to reach statistical significance using the stringent Bonferroni correction method. Reduced PGE2 levels at higher doses may be due to cellular death after 24 hours of Cd^{+2} exposure. Nevertheless, the trends of measured PGE2 levels in the GB epithelial cell culture supernatants are similar to those observed in COX-2 expression patterns described previously using Western blot analysis at the 4- and 24-hour time point (see fig 5). Co-incubation with NAC appears to alleviate the effects of Cd^{+2} exposures confirming again the key role of ROS in PGE2 production as well downstream elevation of COX-2 expression. These results show the potential of Cd^{+2} heavy metal exposures in the causation and maintenance of a chronically inflamed state within the gallbladder microenvironment which may be responsible for the eventual development of GBC.

IV. Discussion:

Gallbladder cancer (GBC) is a relatively rare cancer among the general population [1-4]. Yet, it shows distinct hotspots of geographical incidence globally in countries such as Chile, Bolivia, Japan, Poland, Northern part of India and the state of New Mexico in the United States [1-4]. Thus, it represents an interesting cancer model to understand the role of environmental factors in carcinogenesis. Multiple risk factors have been associated with GBC causation including salmonella infections, aflatoxins and other environmental factors [1-4]. However, no definitive associations have been established thus far. An additional challenge to improve our understanding of gallbladder carcinogenesis is the lack of GBC animal models [1]. An understudied risk factor is the role of heavy metal exposures in GBC pathogenesis [1]. Previous epidemiological data had showed elevated levels of heavy metals (cadmium, copper and lead) in a case-control study [11]. A recent large-scale epidemiological study by Lee et al has significantly strengthened the epidemiological

evidence for the role of heavy metal exposures in GBC causation [8]. However, the authors concluded this study with the urgent need for mechanistic studies to prove this association which is the main focus of this paper.

Expanding on the idea of a heavy metal mechanistic role in GBC carcinogenesis, we examined the effects of controlled exposures of Cd^{+2} in the human gallbladder epithelial cells in this study for the first time. The role of Cd^{+2} exposures in human carcinogenesis in multiple organs (e.g., lung, liver and testis) has been established in various clinical and epidemiological studies [45-47]. The cellular toxic potential of Cd^{+2} exposure is mediated through a variety of mechanisms including: free radical generation, reduced cellular antioxidant potential, cell signaling dysregulation, inhibition of DNA repair and prolonged cellular damage [13-15, 48]. Cd has a long biological half-life in the human body (9.0 – 28.2 years) and is associated with very low rates of removal from the human body [49]. Cd can systematically accumulate in various organs of the human body including the liver [50]. The human liver is a major site for storage and accumulation of environmental Cd over the long term [50, 51]. Since the gallbladder organ is anatomically adjacent to the liver, it may thus be exposed to higher levels of Cd via the hepatobiliary excretory route over long periods of time. The primary function of the gallbladder is to temporarily store and concentrate bile by reabsorbing water in the bile [52, 53]. We propose this physiological function of the gallbladder may also lead to unwanted secondary side-effects of heavy metal accumulation of water-soluble heavy metals such as Cd^{+2} ions in the gallbladder tissues over prolonged durations of exposure.

The data presented in this paper reinforces our primary hypothesis for a role of cadmium exposures in gallbladder carcinogenesis and is thus timely. We initially assessed the effects of Cd^{+2} exposure on the cell viability and cell death of GB epithelial cells. Similar to other studies in different primary human cells, we observe a dose dependent reduction of the GB epithelial cell viability due to Cd^{+2} exposure. Our studies establish an EC50 value of 18.9 μM in the GBP-9 epithelial cell line consistently. In the presence of 5 mM NAC, a clinically used anti-oxidant molecule, we observed the cellular cytotoxicity was greatly reduced across all the tested doses of Cd^{+2} exposure indicating a cytoprotective effect against Cd^{+2} induced ROS in GB epithelial cells. This was verified by measuring the ROS levels in GBP-9 cells using the DCFH-DA assay, which showed elevated ROS generation due to Cd^{+2} exposure at the 24-hour time point. Similar to the cell viability, pre-treatment with NAC reduced the total amount of ROS produced in GB epithelial cells confirming the role of ROS as a major driver of cytotoxicity. NAC's cytoprotective effects against Cd exposure are likely due to the free radical quenching effects as well as elevation of the natural antioxidant defenses as has been reported before [54].

The PI3K-Akt and ERK signaling cascades are critical for a variety of normal cellular functions[55]. Dysregulated PI3K-Akt and ERK signaling pathways are key players in carcinogenesis in a variety of cancer including GBC. The Akt and ERK pathways have been shown to be overexpressed, conferring a cancer cell survival advantage, as shown in previous studies of GBC [21, 22, 39]. Yet, the factors driving the overactivity of the Akt and ERK signaling pathways in the specific context of GBC remain ill understood. Scanu et al demonstrated Salmonella manipulates these pathways in a gallbladder organoid model

leading to cellular transformation [22]. In this study, we specifically examined the effects of Cd^{+2} exposure on the Akt and ERK phosphorylation status in gallbladder epithelial cells. Cd^{+2} exposure has been shown to drive increased production of reactive oxygen species (ROS) in multiple earlier studies [14, 15, 17]. Elevated ROS in turn has been implicated in the activation of the Akt and ERK signaling proteins via activation of oxidative status sensitive domains of these proteins [19, 38]. Regulatory feedback proteins in the Akt and ERK pathways (e.g., PTEN) also contain similar ROS sensitive structural protein domains and are negatively regulated due to ROS exposures [42]. We tested this hypothesis in our cells by examining the activation of the phosphorylated Akt and ERK1/2 activity in response to Cd^{+2} exposure. We observed a dose-dependent activation of the Akt and ERK1/2 molecules in gallbladder epithelial cells due to Cd^{+2} (0.1 – 10 μM) over the course of 24 hours. To establish the role of ROS in the activation of the Akt and ERK pathways, we pre-incubated GB epithelial cells with NAC prior to Cd^{+2} exposure. As expected, 5 mM NAC pre-treatment suppressed the ROS induced phosphorylation of Akt and ERK molecules confirming ROS generation as a key driving mechanism for the activation of these pathways. Our experiments thus establish a link to the potential role of long-term Cd^{+2} exposure in the Akt and ERK overactivation as one of the likely mechanisms underlying gallbladder carcinogenesis.

We also examined the effects of Cd exposures on a second key feature associated with gallbladder carcinogenesis, namely, chronic inflammation [1, 32, 34]. We specifically focused on the expression of COX-2, a pro-inflammatory enzyme involved in the conversion of arachidonic acid to prostaglandins both in acute and chronic inflammatory states [1, 32, 34]. COX-2 expression is upregulated by various pro-inflammatory cytokines, oxidative stress and mitogens. COX-2 protein overexpression plays a key role in various gastrointestinal (e.g., esophagus, liver and colorectal) carcinogenesis including gallbladder cancers [35, 43, 56]. In this study, we identified a dose-dependent increase in the COX-2 expression of the gallbladder epithelial cells due to Cd exposures (0 – 10 μM) as early as 2 hours. The maximal response across different concentrations of Cd exposure was observed after 4 hours of incubation. To understand the regulatory origins of Cd induced COX-2 gene expression, we used targeted pharmacological inhibitors to assess the effects on COX-2 expression. NF-kB pathway is a well-known, key regulator of COX-2 protein expression [35]. The promoter region of the COX-2 gene contains two NF-kB subunit binding sites, which indicates a direct binding transcriptional effect. Additionally, the PI3K/Akt signaling pathway also has been shown to play a key role in COX-2 gene expression in rat macrophages [42]. Cd^{+2} exposures of GB epithelial cells in the presence of NAC (ROS inhibitor), PI3K inhibitor (LY294002), ERK inhibitor (PD98059) and a specific NF-kB pathway inhibitor (MG132) in our studies identified that both the NF-kB and PI3K-Akt pathways are likely to play a key role in the COX-2 elevation seen in gallbladder epithelial cells due to Cd^{+2} exposure.

A key shortcoming of this study includes the use of primary gallbladder epithelial cells to study the effects of Cd^{+2} exposures. While not directly conclusive of a role in GB carcinogenesis, this study demonstrates for the first time the potential role of Cd^{+2} exposures as a driver of two well-established features of GB carcinogenesis reported in the literature. Namely, a) PI3K-Akt signaling pathway activation [22, 39] and b) COX-2 expression and

activation underlying chronic inflammation commonly seen in GBC [7, 32] in a human GB primary cell line model. Additional work is needed to demonstrate the role of Cd^{+2} in an animal model which is the focus of our future studies in our lab. A second shortcoming of this study is the use of acute Cd^{+2} dose exposures (0 – 10 μM) in this study which is unlikely to model the real-world case scenario. The effects of low-dose heavy metal exposures (~nanomolar exposures) over extended time periods (~4-6 weeks) are currently underway in the lab to model a much more realistic outcome of GB carcinogenesis.

In summary, the current study examines the effects of acute, sub-EC50 Cd^{+2} exposures on gallbladder epithelial cells for the first time. Very few research studies have thus far explored the role of heavy metal exposures in gallbladder carcinogenesis. We hypothesize heavy metal exposures may explain the unique geographical patterns of GBC incidence seen in the Western hemisphere (e.g., New Mexico, US) where salmonella infections tend to be rare. Our initial guiding hypothesis in the lab is now supported by the recent epidemiological publication showing statistically significant association of five heavy metals in the serum of GBC patients [8]. While we have chosen Cd^{+2} exposure as our “model” metal in this study to determine the role of heavy metal induced GB carcinogenesis, the real-world situation likely involves mixtures of metals (e.g., Vanadium and Uranium in New Mexico). Our future studies will seek to strengthen the role of these heavy metal mixtures in gallbladder carcinogenesis using low-dose exposure strategies as well as animal models of GBC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References:

1. Nemunaitis JM, et al., Gallbladder cancer: review of a rare orphan gastrointestinal cancer with a focus on populations of New Mexico. BMC Cancer, 2018 18(1): p. 665. [PubMed: 29914418]
2. Kanthan R, et al., Gallbladder Cancer in the 21st Century. J Oncol, 2015 2015: p. 967472. [PubMed: 26421012]
3. Roa I and de Aretxabala X, Gallbladder cancer in Chile: what have we learned? Curr Opin Gastroenterol, 2015 31(3): p. 269–75. [PubMed: 25693005]
4. Wistuba II and Gazdar AF, Gallbladder cancer: lessons from a rare tumour. Nat Rev Cancer, 2004 4(9): p. 695–706. [PubMed: 15343276]
5. Mehrotra R, et al., Genetic landscape of gallbladder cancer: Global overview. Mutat Res, 2018 778: p. 61–71. [PubMed: 30454684]
6. Shrikhande SV, et al., Cholelithiasis in gallbladder cancer: coincidence, cofactor, or cause! Eur J Surg Oncol, 2010 36(6): p. 514–9. [PubMed: 20537839]
7. Espinoza JA, et al., The inflammatory inception of gallbladder cancer. Biochim Biophys Acta, 2016 1865(2): p. 245–54. [PubMed: 26980625]

8. Lee MH, et al., A Metallomic Approach to Assess Associations of Serum Metal Levels With Gallstones and Gallbladder Cancer. Hepatology, 2019.
9. Nir I, et al., Diversification and trends in biliary tree cancer among the three major ethnic groups in the state of New Mexico. Am J Surg, 2012 203(3): p. 361–5; discussion 365. [PubMed: 22236535]
10. Pandey M, Environmental pollutants in gallbladder carcinogenesis. J Surg Oncol, 2006 93(8): p. 640–3. [PubMed: 16724354]
11. Basu S, et al., Heavy and trace metals in carcinoma of the gallbladder. World J Surg, 2013 37(11): p. 2641–6. [PubMed: 23942528]
12. Malhotra RK, et al., Gallbladder cancer incidence in Delhi urban: A 25-year trend analysis. Indian J Cancer, 2017 54(4): p. 673–677. [PubMed: 30082556]
13. Byrne C, et al., Cadmium--a metallohormone? Toxicol Appl Pharmacol, 2009 238(3): p. 266–71. [PubMed: 19362102]
14. Cuypers A, et al., Cadmium stress: an oxidative challenge. Biometals, 2010 23(5): p. 927–40. [PubMed: 20361350]
15. Hartwig A, Mechanisms in cadmium-induced carcinogenicity: recent insights. Biometals, 2010 23(5): p. 951–60. [PubMed: 20390439]
16. Ninkov M, et al., Toxicity of oral cadmium intake: Impact on gut immunity. Toxicol Lett, 2015 237(2): p. 89–99. [PubMed: 26051590]
17. Thevenod F, Cadmium and cellular signaling cascades: to be or not to be? Toxicol Appl Pharmacol, 2009 238(3): p. 221–39. [PubMed: 19371614]
18. Kiran Kumar KM, et al., Cadmium induces oxidative stress and apoptosis in lung epithelial cells. Toxicol Mech Methods, 2016 26(9): p. 658–666. [PubMed: 27687512]
19. Nemmiche S, Oxidative Signaling Response to Cadmium Exposure. Toxicol Sci, 2017 156(1): p. 4–10. [PubMed: 27803385]
20. Thevenod F and Lee WK, Cadmium and cellular signaling cascades: interactions between cell death and survival pathways. Arch Toxicol, 2013 87(10): p. 1743–86. [PubMed: 23982889]
21. Buchegger K, et al., The ERK/MAPK pathway is overexpressed and activated in gallbladder cancer. Pathol Res Pract, 2017 213(5): p. 476–482. [PubMed: 28285962]
22. Scanu T, et al., Salmonella Manipulation of Host Signaling Pathways Provokes Cellular Transformation Associated with Gallbladder Carcinoma. Cell Host Microbe, 2015 17(6): p. 763–74. [PubMed: 26028364]
23. Kim HG, et al., p38alpha MAPK is required for arsenic-induced cell transformation. Mol Carcinog, 2016 55(5): p. 910–7. [PubMed: 25969347]
24. Sui X, et al., p38 and JNK MAPK pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents. Cancer Lett, 2014 344(2): p. 174–9. [PubMed: 24333738]
25. Wagner EF and Nebreda AR, Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer, 2009 9(8): p. 537–49. [PubMed: 19629069]
26. Ki YW, et al., JNK and p38 MAPK regulate oxidative stress and the inflammatory response in chlorpyrifos-induced apoptosis. Toxicol Lett, 2013 218(3): p. 235–45. [PubMed: 23416140]
27. Ali I, et al., Cadmium at nanomolar concentrations activates Raf-MEK-ERK1/2 MAPKs signaling via EGFR in human cancer cell lines. Chem Biol Interact, 2015 231: p. 44–52. [PubMed: 25744307]
28. Huff MO, et al., Arsenite and Cadmium Activate MAPK/ERK via Membrane Estrogen Receptors and G-Protein Coupled Estrogen Receptor Signaling in Human Lung Adenocarcinoma Cells. Toxicol Sci, 2016 152(1): p. 62–71. [PubMed: 27071941]
29. Koshiol J, et al., Association of inflammatory and other immune markers with gallbladder cancer: Results from two independent case-control studies. Cytokine, 2016 83: p. 217–225. [PubMed: 27173614]
30. Marusawa H and Jenkins BJ, Inflammation and gastrointestinal cancer: an overview. Cancer Lett, 2014 345(2): p. 153–6. [PubMed: 23981579]
31. Venniyoor A, Cholesterol gallstones and cancer of gallbladder (CAGB): molecular links. Med Hypotheses, 2008 70(3): p. 646–53. [PubMed: 17855001]

32. Li Y, Zhang J, and Ma H, Chronic inflammation and gallbladder cancer. Cancer Lett, 2014 345(2): p. 242–8. [PubMed: 23981574]
33. Gonzalez-Escobedo G, Marshall JM, and Gunn JS, Chronic and acute infection of the gall bladder by Salmonella Typhi: understanding the carrier state. Nat Rev Microbiol, 2011 9(1): p. 9–14. [PubMed: 21113180]
34. Kim H, et al., Strong cytoplasmic expression of COX2 at the invasive fronts of gallbladder cancer is associated with a poor prognosis. J Clin Pathol, 2010 63(12): p. 1048–53. [PubMed: 20924037]
35. Hashemi Goradel N, et al., Cyclooxygenase-2 in cancer: A review. J Cell Physiol, 2019 234(5): p. 5683–5699. [PubMed: 30341914]
36. Mantha M and Jumarie C, Cadmium-induced hormetic effect in differentiated Caco-2 cells: ERK and p38 activation without cell proliferation stimulation. J Cell Physiol, 2010 224(1): p. 250–61. [PubMed: 20232314]
37. Bizama C, et al., Targeting specific molecular pathways holds promise for advanced gallbladder cancer therapy. Cancer Treat Rev, 2015 41(3): p. 222–34. [PubMed: 25639632]
38. Manning BD and Toker A, AKT/PKB Signaling: Navigating the Network. Cell, 2017 169(3): p. 381–405. [PubMed: 28431241]
39. Li M, et al., Whole-exome and targeted gene sequencing of gallbladder carcinoma identifies recurrent mutations in the ErbB pathway. Nat Genet, 2014 46(8): p. 872–6. [PubMed: 24997986]
40. Liu Z, et al., Circulating Levels of Inflammatory Proteins and Survival in Patients with Gallbladder Cancer. Sci Rep, 2018 8(1): p. 5671. [PubMed: 29618736]
41. Van Dyke AL, et al., Lipopolysaccharide-pathway proteins are associated with gallbladder cancer among adults in Shanghai, China with mediation by systemic inflammation. Ann Epidemiol, 2016 26(10): p. 704–709. [PubMed: 27793274]
42. Huang YY, et al., Cadmium selectively induces MIP-2 and COX-2 through PTEN-mediated Akt activation in RAW264.7 cells. Toxicol Sci, 2014 138(2): p. 310–21. [PubMed: 24449419]
43. Echizen K, et al., Inflammation in gastric cancer: Interplay of the COX-2/prostaglandin E2 and Toll-like receptor/MyD88 pathways. Cancer Sci, 2016 107(4): p. 391–7. [PubMed: 27079437]
44. Nakanishi M and Rosenberg DW, Multifaceted roles of PGE2 in inflammation and cancer. Semin Immunopathol, 2013 35(2): p. 123–37. [PubMed: 22996682]
45. Iavicoli I, Fontana L, and Bergamaschi A, The effects of metals as endocrine disruptors. J Toxicol Environ Health B Crit Rev, 2009 12(3): p. 206–23. [PubMed: 19466673]
46. Jaishankar M, et al., Toxicity, mechanism and health effects of some heavy metals. Interdiscip Toxicol, 2014 7(2): p. 60–72. [PubMed: 26109881]
47. Kim HS, Kim YJ, and Seo YR, An Overview of Carcinogenic Heavy Metal: Molecular Toxicity Mechanism and Prevention. J Cancer Prev, 2015 20(4): p. 232–40. [PubMed: 26734585]
48. Giaginis C, Gatzidou E, and Theocharis S, DNA repair systems as targets of cadmium toxicity. Toxicol Appl Pharmacol, 2006 213(3): p. 282–90. [PubMed: 16677676]
49. Suwazono Y, et al., Biological half-life of cadmium in the urine of inhabitants after cessation of cadmium exposure. Biomarkers, 2009 14(2): p. 77–81. [PubMed: 19330585]
50. Liver and Cadmium Toxicity. Journal of Drug Metabolism & Toxicology, 2013 03(06).
51. Hyder O, et al., Cadmium exposure and liver disease among US adults. J Gastrointest Surg, 2013 17(7): p. 1265–73. [PubMed: 23636881]
52. Grunhage F and Lammert F, Gallstone disease. Pathogenesis of gallstones: A genetic perspective. Best Pract Res Clin Gastroenterol, 2006 20(6): p. 997–1015. [PubMed: 17127184]
53. Jungst C, Kullak-Ublick GA, and Jungst D, Gallstone disease: Microlithiasis and sludge. Best Pract Res Clin Gastroenterol, 2006 20(6): p. 1053–62. [PubMed: 17127187]
54. <344.full.pdf>.
55. Engelman JA, Luo J, and Cantley LC, The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet, 2006 7(8): p. 606–19. [PubMed: 16847462]
56. Kiguchi K, et al., Therapeutic effect of CS-706, a specific cyclooxygenase-2 inhibitor, on gallbladder carcinoma in BK5.ErbB-2 mice. Mol Cancer Ther, 2007 6(6): p. 1709–17. [PubMed: 17575102]

Highlights

- Etiological factors driving gallbladder carcinogenesis in New Mexico are unknown
- Cd^{+2} exposure activates PI3K-Akt and MAPK signaling pathways in a dose-dependent manner
- Cd^{+2} drives COX-2 expression explaining the chronic inflammation seen in Gallbladder Cancer
- PI3K-Akt and NF-kB pathways regulate Cd^{+2} exposure induced COX-2 protein expression
- Thus, cadmium exposure may act as an environmental driver of gallbladder carcinogenesis

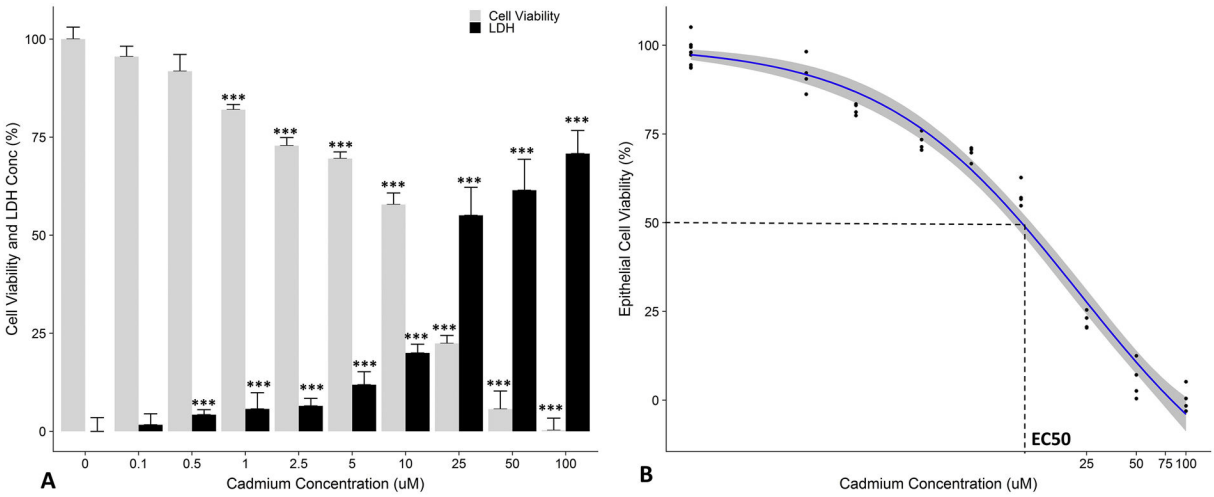

Figure 1.

A. Cell viability and LDH concentrations of GB epithelial cells in response to Cd^{+2} exposures (0-100 μM) over a 24-hour incubation period. GB epithelial cells show a dose dependent reduction in the cell viability and a corresponding increase the LDH concentrations in the media. Cell viability and LDH levels were tested in three independent replicates. Statistical significance of each Cd^{+2} exposure level was measured relative to the unexposed cells – (Significance levels: * p <0.05, ** p <0.01, *** p <0.001). B. A 4-parameter log-logistic fit of the cell viability in response to Cd exposures. GBP-9 epithelial cells show an EC50 value of 18.9 μM at the 24-hour time point.

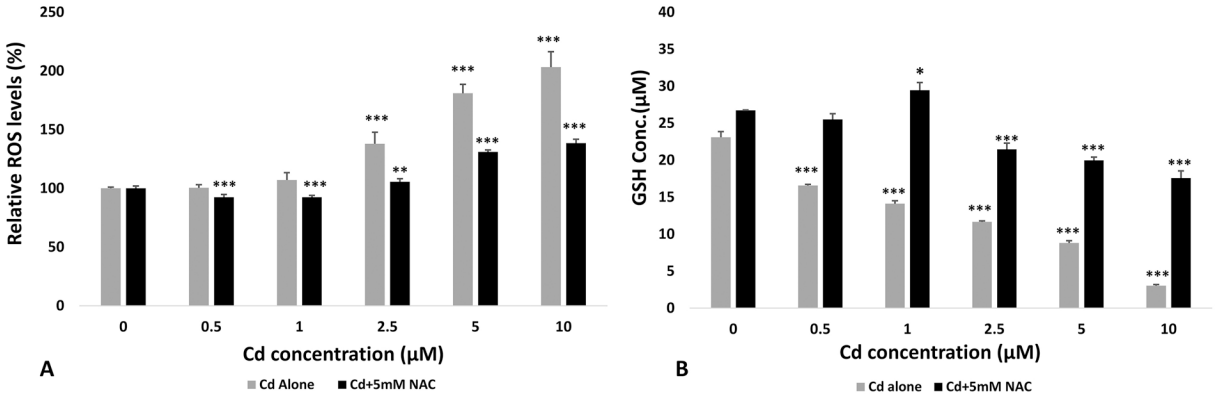

Figure 2:

A. Quantitative reactive oxygen species (ROS) levels due to Cd^{+2} exposures measured in the presence and absence of added NAC (5 mM) using the DCFH-DA assay. At low doses of Cd exposure with added NAC (0.5 and 1 μM), there is a reduction in the overall ROS levels. For all other Cd^{+2} concentrations, elevated ROS levels are observed both in the presence and absence of NAC. B. Effects of Cd^{+2} on glutathione (GSH) levels in the presence and absence of NAC supplementation after 24 hours. Cd^{+2} exposure alone causes sustained reduction of GSH levels while NAC supplementation leads to reduced overall decrease of GSH levels relative to Cd^{+2} unexposed cells (Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

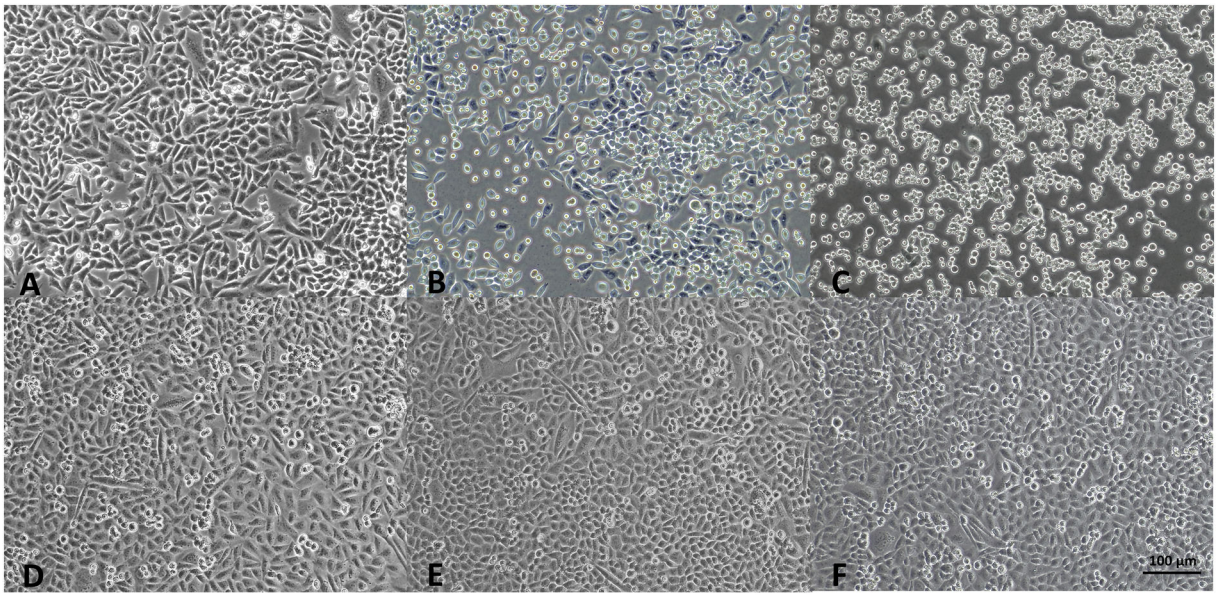

Figure 3:

Effect of Cd exposures on GB epithelial cell morphology. Cells were exposed to 0, 5 and 10 μM Cd in the absence (top row) and presence (bottom) of N-Acetyl Cysteine (5 mM) for a duration of 24 hours. Primary gallbladder epithelial cells exposed to 5 and 10 μM Cd^{+2} showed a rounded morphology and loss of cell-cell junctional contacts in a dose dependent manner (Panels B and C respectively). NAC prevented the loss of cell-cell contacts at the end of 24-hour exposure period (E and F). Images acquired at 10X magnification (scale bar – 100 μM shown in panel F).

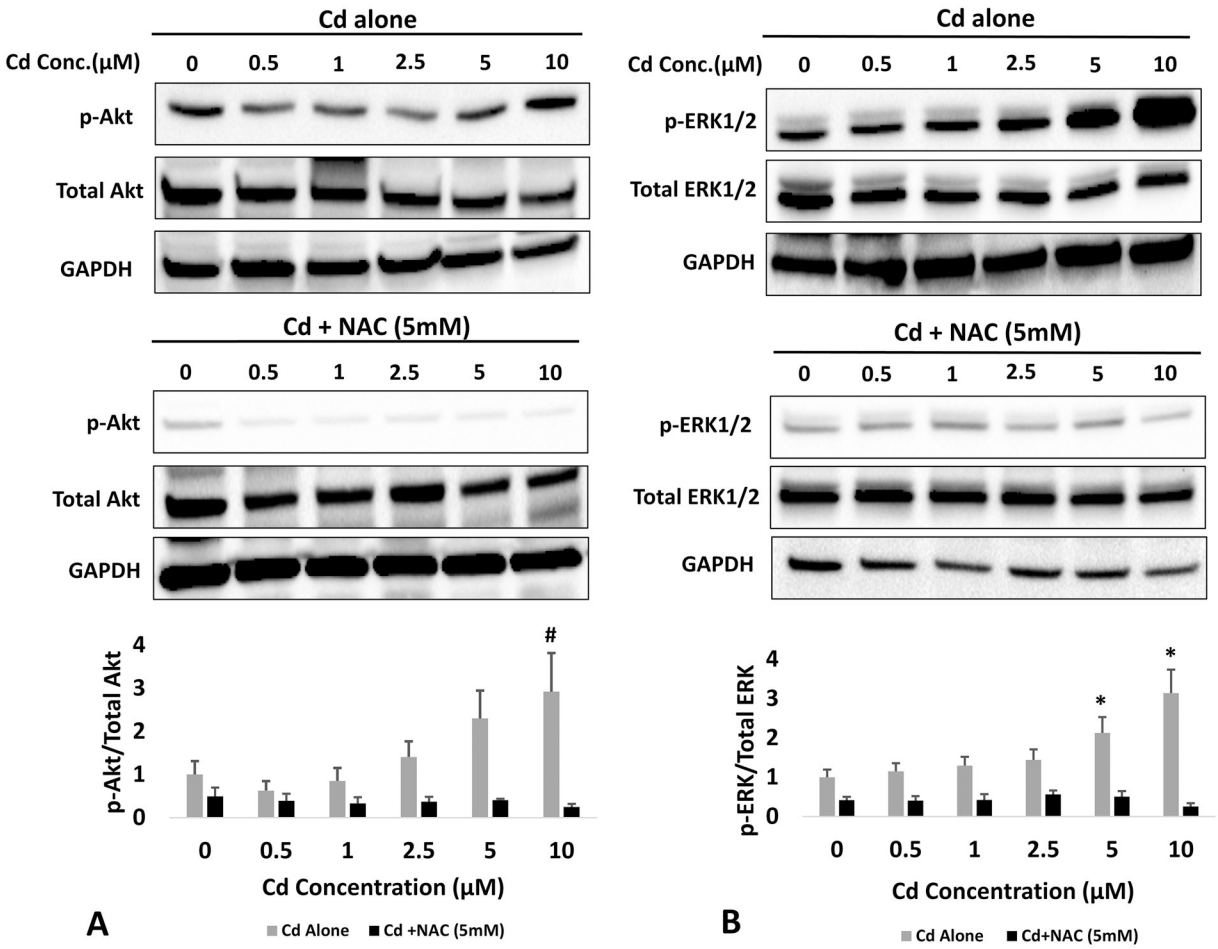

Figure 4:

A. Effect of Cd^{+2} exposures on expression of phosphorylated Akt in primary GB epithelial cells (top panel). GB epithelial cells showed a dose-dependent (0, 0.5, 1, 2.5, 5 and 10 μM) increase in the phosphorylation status of the Akt protein after 24-hour exposure. Co-incubation with N-Acetyl Cysteine (NAC) at 5 mM abrogated the phosphorylation of the Akt protein (middle panel). Reduction in phosphorylation was seen across all the concentrations of Cd^{+2} exposure (0-10 μM). Ratios of p-Akt/total Akt protein expression relative to unexposed cells (bottom panel). Data shows the ratios in the presence and absence of NAC co-incubation (# $p < 0.1$) **B.** Effect of Cd^{+2} exposures on phosphorylated ERK1/2 protein status in GB epithelial cells. (top panel) GB epithelial cells showed a dose-dependent (0, 0.5, 1, 2.5, 5 and 10 μM) increase in the phosphorylation of the ERK1/2 protein at the end of a 24-hour exposure duration. (middle panel) co-incubation with N-Acetyl Cysteine (NAC) at 5 mM abrogated the phosphorylation of the ERK1/2 protein similar to the Akt protein suggesting the role of Cd^{+2} induced ROS as a main mechanism driving the phosphorylation of Akt and ERK1/2. Reduction in phosphorylation was seen across all the concentrations of Cd^{+2} exposure (0-10 μM). (bottom panel) ratios of p-ERK1/2/total ERK protein expression relative to unexposed cells in the presence and absence of NAC co-incubation (* $p < 0.05$)

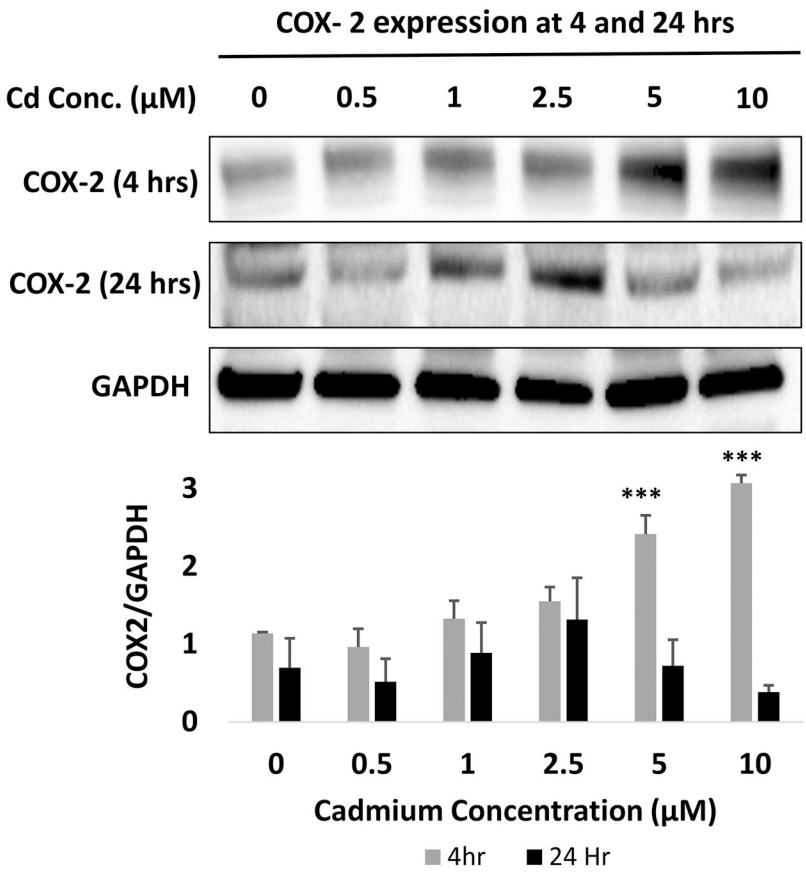

Figure 5:

COX-2 expression due to Cd^{+2} exposure. (top panel) GB epithelial cells showed a dose-dependent (0, 0.5, 1, 2.5, 5 and 10 μM) changes in COX-2 protein expression levels due to Cd^{+2} exposures. However, statistically significant differences were observed in the COX-2 protein expression levels only at the 4 hour time point in the 5 and 10 μM Cd^{+2} exposure. Decreased COX-2 expression was observed for the higher Cd dose levels (5 and 10 μM) at the 24-hour time point. This may be due to the progressive reduction of cell viability at higher dose levels of Cd exposures. (bottom panel) ratios of COX-2/GAPDH protein expression at the 4- and 24-hour exposure time point to Cd^{+2} (Statistical significance - *** $p < 0.001$).

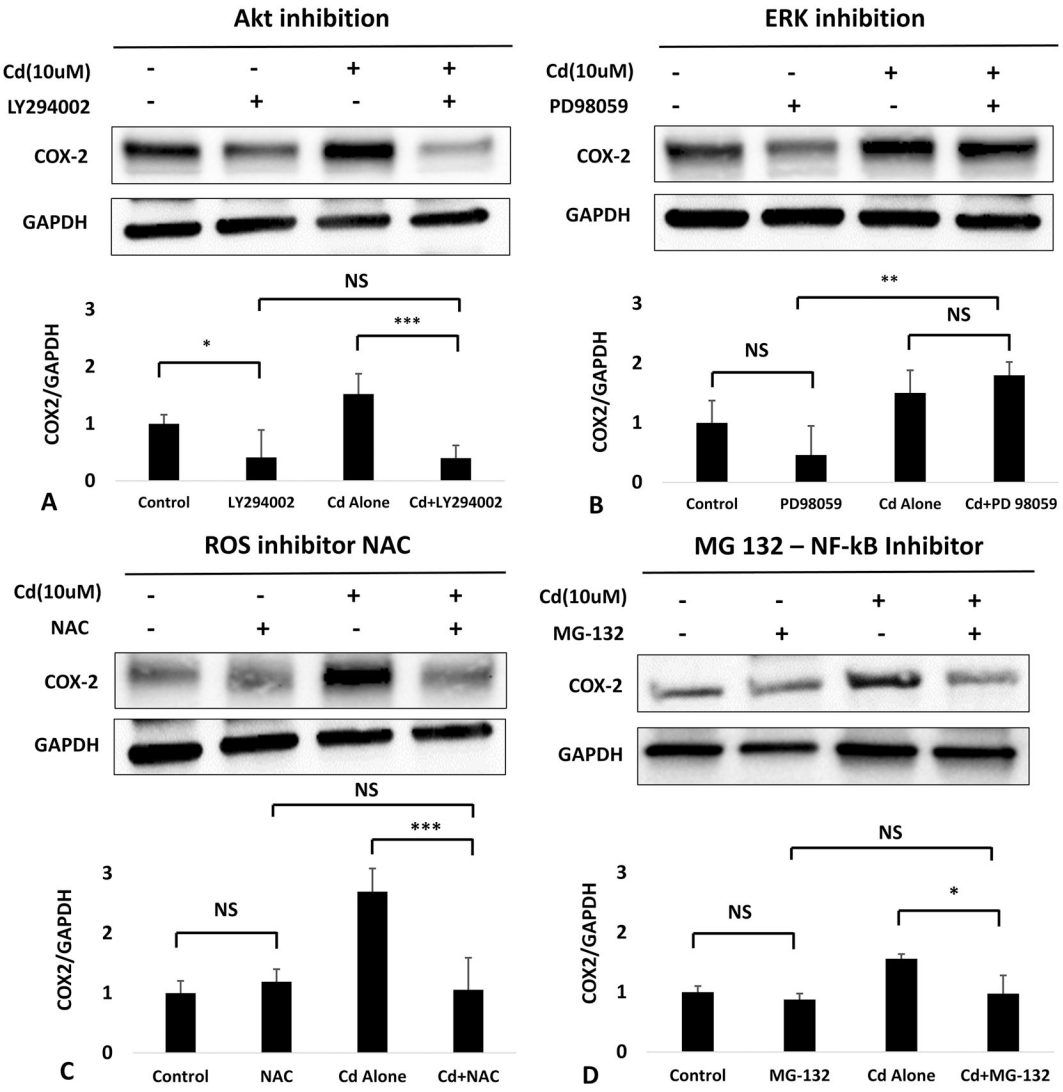

Figure 6:

A and B (top panels). Effects of pharmacological inhibitors of Akt and ERK signaling pathways on COX-2 protein expression in Cd^{+2} exposed GB epithelial cells. A. GB epithelial cells show lowered COX-2 protein expression when incubated with an Akt specific inhibitor (LY294002) indicating the Akt signaling pathway plays a key role in regulation of COX-2 expression due to Cd exposure. B. In contrast, GB epithelial cells incubated with an ERK pathway specific inhibitor (PD98059) did not show any reduction in the COX-2 expression due to Cd^{+2} exposure indicating the ERK pathway may not play a significant role in the regulation of COX-2 expression due to Cd^{+2} exposure in GB epithelial cells (Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). C and D (bottom panels). Effects of cellular ROS suppression using NAC and NF-kB pathway inhibition to determine effects on COX-2 protein expression in Cd^{+2} exposed GB epithelial cells. C. GB epithelial cells show lowered COX-2 protein expression when incubated with the global ROS scavenger, N-acetyl cysteine (NAC) indicating cellular ROS generation plays a key role in driving COX-2 expression in due to Cd^{+2} exposures. B. Similar to Akt inhibition, GB

epithelial cells incubated with a NF-kB pathway inhibitor (MG132) showed a significant reduction in the COX-2 expression with Cd^{+2} exposures. The NF-kB pathway, along with Akt pathway, is thus plays a significant role in elevated COX-2 protein expression due to Cd^{+2} exposure in GB epithelial cells most likely via a ROS mediated mechanism (Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

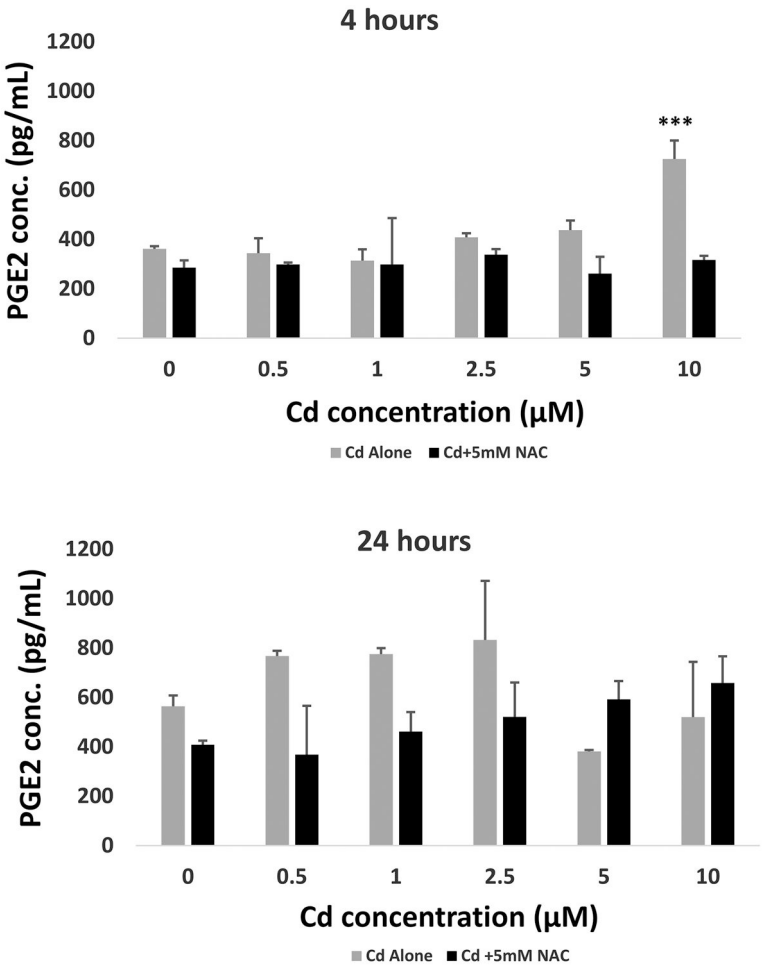

Figure 7:

Effects of Cd^{+2} exposure on PGE2 levels. PGE2 levels measured at 4 and 24 hours show elevated levels of PGE2 expression in the supernatants relative to unexposed cells. NAC supplementation causes a reduced expression of PGE2 relative to Cd^{+2} exposures alone (Significance levels: *** $p < 0.001$).