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Bile Acids and Premature Labor in Intrahepatic Cholestasis of Pregnancy

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BILE ACIDS AND PREMATURE LABOR IN INTRAHEPATIC CHOLESTASIS OF PREGNANCY

BY

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ABSTRACT

The association between serum bile acid levels and preterm birth has been reported. Clinical studies have shown that significant increase of serum bile acids during intrahepatic cholestasis of pregnancy (ICP) is associated with higher rate of preterm birth. However, the role of bile acids in preterm birth has not been established. In this study, we found that preterm birth was induced in mice by increasing plasma bile acids with cholic acid (CA) or carbon tetrachloride (CCl_4) treatment. CCl_4 treatment increased plasma bile acids by causing acute liver injury which down-regulated bile salt export pump (BSEP) in the liver. The data demonstrated that increased bile acid levels served as trigger for preterm birth. Consistent with the findings, reducing plasma bile acids by cholestyramine (CTM) was able to reverse the preterm birth induced by CA by decreasing plasma bile acid levels.fds

Mechanistic investigation revealed that preterm delivery induced by elevated bile acids was mediated by prostaglandins and prostaglandin receptor expressions. An increase in prostaglandin $F_{2\alpha}$ receptor (PTGFR) and a decrease in 15-hydroxyprostaglandin dehydrogenase (HPGD) and prostaglandin E2 receptor (PTGER2), all of which participate in normal parturition, was observed. More importantly, the expression change in PTGFR was reversed with CTM treatment, suggesting that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)/PTGFR signaling pathways are involved in mediating preterm birth induced by bile acids. Further studies showed an inverse correlation between plasma progesterone (P4) levels and PTGFR expression, which is consistent with significant P4 withdrawal and elevation of PTGFR before delivery in human.

In conclusion, we established the causative relationship of maternal bile acid levels and preterm delivery. The results from this study provide a molecular basis for new strategies to prevent preterm birth in pregnant women with ICP or other liver diseases by modulating bile acid homeostasis as well as the $PGF_{2\alpha}$/PTGFR signaling pathways.

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1. INTRODUCTION

Preterm birth is the greatest contributor to infant death. The shorter the term of pregnancy, the greater the risks of morbidity and mortality due to prematurity. Preterm birth accounts for 10% of neonatal mortality worldwide, 25% in the US, and surviving premature infants are at risk for neurological, cardiovascular, respiratory or metabolic complications (Matthew & MacDorman, 2006). Despite many efforts to understand the timing of parturition, the molecular mechanism is still not fully understood (Mesiano & Welsh, 2007; Chan, 2014).

Spontaneous preterm birth is one of the most common fetal complications of intrahepatic cholestasis of pregnancy (ICP), which occurs in about 19 to 60% of cases (Bacq et al., 1997; Fisk & Storey, 1988). One of the hallmark signs of ICP is elevation of serum bile acids due to impaired disposition of bile acids from the liver. Once maternal serum total bile acids (TBA) levels exceed 10 μmol/L, the patient is diagnosed as ICP. Severity of ICP is classified based on maternal serum TBA level. Serum TBA level of 10 to 39 μmol/L corresponds to mild, 40 to 99 μmol/L to moderate, and ≥ 100 μmol/L to severe ICP. Even with current treatments, spontaneous preterm birth occurs more than twice in ICP compared to normal pregnancies, and the incidences of fetal demise ranges from 2 to 11% (Geenes et al., 2014; Pata et al., 2011). The largest prospective cohort study in severe ICP to date concluded that risks for adverse perinatal outcomes including preterm delivery and stillbirth are significantly higher in severe ICP (Geenes et al., 2014).

Additional evidence connects the severity of the ICP conditions and timing of parturition. According to a study conducted in Sweden, risks of spontaneous preterm

birth and other fetal complications increase 1 to 2% for every additional μmol/L of maternal serum bile acids when it is ≥ 40 μmol/L (Glantz, Marschall & Mattsson, 2004). Another study conducted in San Francisco, CA concluded that severe ICP is associated with increased fetal complications (Rook et al., 2012).

The mechanism to trigger spontaneous preterm birth has not been fully understood. Evidence shows the association of elevated maternal serum TBA with preterm birth (Geenes et al., 2014; Glantz, Marschall & Mattsson, 2004; Rook et al., 2012). From these, it is reasonable to speculate that maternal serum TBA may play an important role in signaling preterm birth. However, currently there have been no studies to fully understand the role of bile acids in preterm birth. From this, we hypothesized that increase in serum bile acids play a direct role in inducing preterm labor.

In this study, we observed that direct treatment with bile acids in mice elevated maternal plasma bile acids and induced preterm labor. Treatments with hepatotoxin was also associated with preterm labor while increasing plasma bile acid levels from liver damage. In addition, preterm labor induced by elevated plasma bile acids was successfully reversed when plasma bile acid levels were decreased by bile acid sequestrant. The data demonstrated that elevation of plasma bile acids is a contributing factor in triggering preterm labor.

To study the mechanisms by which bile acids signal preterm labor, we investigated the changes in parturition-associated gene expressions in uteri following bile acid treatment. Changes in target gene expression revealed the involvement of prostaglandin signaling pathways in preterm labor. Understanding the pathological

mechanisms by which bile acids affect parturition will ultimately provide a molecular basis for development of new strategies to prevent preterm birth.

1.1 Review of Literature

Hormonal, Genetic, and Environmental Effect on ICP and Bile Acids

ICP patients have a defect in excretion of bile salts, which leads to increased serum bile acids. However, the pathophysiology of ICP is still unclear. Suggested mechanisms of ICP have three different factors – hormonal, genetic, and environmental effects. Increase in sex hormone levels during the later stages of pregnancy, genetic mutations and drug-induced liver injury have been studied as contributing factors for ICP (Reyes & Simon, 1993; Floreani & Gervasi, 2016; Paternosteer et al., 2002). Although these three factors pose different mechanistic pathway, all results in increased serum bile acids.

During late stages of pregnancy, sex hormones, mainly estrogens and progesterone, increase. This increase may slow normal flow of bile acids out of the liver by inhibiting bile salt export pump (BSEP, ABCB11) or multidrug resistance protein 3 (MDR3, ABCB4), which result in accumulation of bile acids in the liver and increased serum TBA levels (Stieger, Fattinger, Madon, Kullak-Ublick & Meier, 2000; Debry, Nash, Neklason & Metherall, 1997). The estradiol/estrogen receptor alpha transrepressive pathway directly interacts with farnesoid-X receptor (FXR) signaling pathway to reduce hepatic excretion of bile acid by inhibiting BSEP, which is positively regulated by bile acids/FXR signaling pathway (Chen et al., 2015; Song et al., 2014). Consistently, increase in estrogen and estrogen metabolites has been shown to cause cholestasis in animals (Stieger, Fattinger, Madon, Kullak-Ublick & Meier, 2000).

Progesterone level is also suggested to have a correlation with ICP. Progesterone treatment during third trimester resulted in development of ICP in some cases (Lammert, Marschall, Glantz & Matern, 2000). Progesterone metabolites, particularly sulfated form, are increased in ICP patients more than unaffected women (Reyes & Sjovall, 2000). High levels of 3α-sulfated progesterone metabolite epillopregnanolone sulfate (PM5S) inhibits bile acids/FXR mediated MDR3 and FXR itself. Decrease in FXR expression results in reduction of BSEP expression, which leads to excessive accumulation of hepatic as well as serum bile acids (Abu-Hayyeh et al., 2013).

Hormone levels are increased in twin or multiplet pregnancies, which contribute to 5 times higher incidence of ICP than singleton pregnancies (Gonzalez et al., 1989). This increase in sex hormones and their metabolites is resolved quickly after delivery when placental hormone production ceases, which is consistent with ICP resolving quickly after delivery. Based on this data, it can be concluded that sex hormone levels are closely related to increasing serum bile acid levels for development and resolution of ICP.

ICP patients may also present with elevated bile acids from genetic mutations. One of the risk factors for ICP is personal or family history (Abu-Hayyeh, Papacleovoulou & Williamson, 2013). Patients with a personal history of ICP present with 70% higher risk of developing ICP in subsequent pregnancies. In addition, ICP extends in families (Lammert, Marschall, Glantz & Matern, 2000; Reyes, 1992). It has been studied that many of ICP patients carry mutations in ABCB11 and/or ABCB4 gene. Those who possess p.V444A BSEP polymorphism were significantly at higher risk for developing in ICP (Meier et al, 2008; Dixon et al, 2009). Heterozygous p.G855R

mutation in BSEP can also increase sensitivity to estrogen to induce cholestasis or impair bile acid secretion from the liver, which result in increased serum bile acids (Reyes & Simon, 1993; Lang et al, 2007). Prevalence of ICP also depends greatly on geographic regions. ICP incidence rate ranges from 0.1% to 15.6% worldwide, showing higher prevalence in South America (especially Chile and Bolivia) or Scandinavia (Reyes et al., 1978). Even within the US, the rate of ICP in pregnancy ranges from 0.32% in Bridgeport Hospital, CT to 5.6% in Los Angeles where a majority are Latin population (Laifer, Stiller, Siddiqui, Dunston-Boone, & Whetham, 2001; Lee, Goodwin, Greenspoon, & Incerpi, 2006). These statistics suggest that genetic difference among races is also a contributing factor in development of ICP (Geenes & Williamson, 2009).

In addition to hormonal and genetic factors, some environmental factors may contribute to development of ICP (Hay, 2008; Lammert, Marschall, Glantz & Matern, 2000). ICP can be induced by drugs that cause liver damage, which is consistent with statistics that ICP occurs in higher rates in pregnant women with history of liver damage (Guntupalli & Steingrub, 2005; Rigby, Ehrenberg-Buchner, & VanBuren, 2014). Hormone replacement therapy or oral contraceptive usages have also been shown to cause cholestasis in some cases (Reyes & Simon, 1993; O'Donohue & Williams, 1997; Lindberg, 1992). Additionally, *in vitro* fertilization or advanced maternal age can increase the risk for developing ICP (Geenes & Williamson, 2009).

These three factors all contribute towards development of ICP, with common outcome of elevated bile acids.

Bile Acids and Parturition

A study has been published which showed higher rate of spontaneous preterm birth when serum bile acid levels were severely elevated in ICP (Geenes et al., 2014). Even during normal pregnancy, bile acid levels increase as the pregnancy progresses, reaching its maximum during third trimester and quickly returning back to normal upon delivery (Castaño et al., 2006; Heikkinen, Mäentausta, Ylöstalo & Jänne, 1981). During ICP, cholic acid (CA) levels are more significantly increased, which leads to marked elevation of CA/chenodeoxycholic acid (CDCA) ratio compared to normal pregnant women (Meng et al., 1997). Serum CA and CDCA levels are increased ten- and five-fold, respectively, in ICP patients (Heikkinen, Mäentausta, Ylöstalo, & Jänne, 1981). The average concentration of serum TBA is 7.44 μmol/L in normal pregnancy and 65.7 μmol/L in ICP. Table 1 lists the composition of each bile acids (CA, CDCA, deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA)) in TBA pool. Although CDCA, DCA, LCA, and UDCA present with slight or moderate increases during ICP, the extent is not as significant as CA, which is why CA is the choice of bile acids in inducing preterm birth in this study.

Hepatic Capacity and Preterm Birth

ICP incidences are more prevalent in patients with compromised liver function with acute or chronic liver diseases, and the outcomes to both mother and fetus are usually more severe (Guntupalli & Steingrub, 2005; Rigby, Ehrenberg-Buchner, & VanBuren, 2014). Either from genetic predisposition or from exposure to environmental factors, compromised functional capacity of liver results in increased serum bile acids (Bove et al., 2000). This further supports the relationship between elevated serum bile acids and preterm birth.

Parturition-Associated Genes

Parturition is commonly considered as an inflammatory process (Christiaens et al., 2008). Up-regulation of inflammatory genes such as interleukin-6 (IL-6), interleukin-1β (IL1β), tumor necrosis factor alpha (TNFα) or cyclooxygenase 2 (COX2) have been observed during parturition and also serve as biological markers for spontaneous preterm birth (Menon et al., 2011; Wei, Fraser, & Luo, 2011; Paternoster et al., 2002).

Some contraction-associated proteins (CAPs) such as oxytocin receptor (OXTR), prostaglandin E2 receptor (PTGER2), prostaglandin F receptor (PTGFR), and hydroxyprostaglandin dehydrogenase (HPGD) are also involved in spontaneous preterm birth (Soloff et al., 1983; Dong & Yallampalli, 2000). Progesterone is occasionally used to prevent contraction in normal pregnancies. When administered, progesterone inhibits OXTR and PTGFR expression to minimize myometrial contractility by desensitizing oxytocin and prostaglandin F2 receptors. Progesterone also increases HPGD expression to inactivate prostaglandins in myometrium to prevent contraction (Challis, Patel, & Pomini, 1999). This is consistent with normal pregnancy in which the onset of labor is preceded by a fall in progesterone levels.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Cholic acid (CA), carbon tetrachloride (CCl_4), cholestyramine (CTM), olive oil, chloroform, and isopropanol were purchased from Sigma-Aldrich. 1,2-propanediol was purchased from Acros Organics. Taqman Real-Time PCR Master Mix, RT-PCR probes, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) and fetal bovine serum (FBS) were purchased from Life Technologies. RNA-Bee for RNA isolation and dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific. Total bile acids (TBA) assay kit (Enzymatic Cycling, Cat #. BQ 042A-EALD) and aspartate aminotransferase (AST/SGOT) activity assay kit (Cat #. BQ K753-100) were purchased from BQ kit. Estradiol ELISA kit (Cat #. 582251) and Progesterone ELISA assay kit (Cat #. 582601) were purchased from Cayman Chemicals. Complementary DNA synthesis kits were purchased from Promega.

2.2 Timed Mating for CD-1 Mice

To ensure the time of treatment, mice were set up for timed mating. Mice at the age of two to six months old were used for mating. Two or three females and one male were placed in the same cage overnight. Females were checked for presence of vaginal plug the next morning as a confirmation of sexual activity before removing males from the cage. The first day of gestation was considered to be the day after the plug was found.

2.3 CA Treatment of Pregnant CD-1 Mice

Once the pregnancy was confirmed, pregnant mice were housed individually in each cage. The mice had free access to food and water and were on a 12-hour dark/light cycle. Twenty-four pregnant mice were randomly divided into three different groups of negative control, 86 mg/kg CA and 129 mg/kg CA. Nine mice were treated with 1,2-propanediol vehicle, 6 mice were treated with 86 mg/kg CA and 9 mice were treated with 129 mg/kg CA. The difference in number of mice in each group were due to limitations in timed pregnancy of mice. The two treatment groups of mice were injected in the intraperitoneal cavity with a dose of 129 mg/kg CA or 86 mg/kg CA, and the control group was injected with 1,2-propanediol, each administered every 12 hours for up to 4 injections. The end of the pregnancy term was determined when pregnant mice started the parturition process by visibly evaluating for signs of labor such as loss of blood, weight loss, presence of litters, or behavior changes for pushing. To evaluate for cannibalism, the weight of each mouse was monitored during the injection period.

When the parturition process was initiated, blood samples were collected in EDTA-treated anticoagulant tubes via cardiac puncture under isoflurane-induced anesthesia and spun at 3,500g for 9 min for plasma collection. Mice were euthanized by cervical dislocation and liver, uterus, placenta, and ovary were harvested for further studies. For mice that did not develop preterm labor, blood, liver, uterus, placenta and ovary samples were collected on day 18.7.

In addition, 6 additional pregnant mice were treated as control group with 1,2-propanediol every 12 hours for 4 injections. These mice were not sacrificed on day 18.7 and was observed for the full-term gestation period of control mice.

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Rhode Island.

2.4 CCl_4 Treatment of CD-1 Mice

Once pregnancy was confirmed, five, six and five pregnant females were injected in the peritoneal cavity with 1, 1.5 and 2 ml/kg CCl_4 in olive oil (40% v/v) once on day 16.3, respectively. The end of the pregnancy term was determined when pregnant mice started the parturition process by visibly evaluating for signs of labor. Mice were monitored for weight changes during injection period, and blood and tissue samples were harvested as previously described.

2.5 Plasma Total Bile Acids (TBA) Assay

After isolation of plasma from collected blood, four microliters of plasma were added to 270 µL of reagent R1. The mixture was then incubated at 37ºC for 3 minutes. After incubation, ninety microliters of reagent R2 were added to the mixture and the absorbance was immediately monitored at 405 nm for 2 minutes. ΔA405 nm/min was calculated for each sample and concentrations of TBA were calculated compared to the standard provided in the kit.

2.6 Plasma Aspartate Transaminase (AST/SGOT) Assay

AST reagent provided in the kit was reconstituted with 12 mL of distilled water. The reagent was then equilibrated to 37ºC. Ten microliters of plasma sample were mixed with 100 µL of reconstituted AST reagent. The absorbance was measured at 340 nm every minute for 3 minutes while the mixture was maintained at 37ºC.

ΔA340nm/min was calculated for each sample and concentrations of AST were calculated based on sample volume and instrument specification.

2.7 Plasma Progesterone (P4) ELISA Assay

Enzyme immunoassay (EIA) buffer, wash buffer, P4 tracer, P4 antiserum, Ellman's reagent and P4 standards were reconstituted per kit protocol. Each run included wells for blank, total activity, non-specific binding, maximum binding (B_0), 8 standards, and samples (B). For non-specific binding, a hundred microliters of EIA buffer and 50 µL of tracer were added to a well of ELISA plate coated with mouse monoclonal anti-rabbit IgG. For maximum binding, fifty microliters of EIA buffer, 50 µL of tracer, and 50 µL of antiserum were added. For each samples and standards, 50 µL of plasma or standard solution, 50 µL of tracer, and 50 µL of antiserum were added. Once all the reagents were added, the plate was incubated at room temperature for an hour on orbital shaker. After the incubation, the plate was washed with wash buffer for five times and 200 µL of Ellman's reagent was added to each well. For total activity, five microliters of tracer was added. Then the plate was incubated at room temperature for 90 minutes on orbital shaker. After the incubation, plate was read at 405 nm.

Standard curve was plotted for $\%B/B_0$ of standards vs. log of standard concentrations, and was fitted using 4-parameter logistic fit. Curve fitting was performed on SigmaPlot. Concentrations of P4 in each sample were calculated according to the logistic fit parameters.

2.8 Mouse Tissue mRNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

Mouse liver, uterus or placenta was homogenized in 1 mL of RNA-Bee per 50 mg of tissue for mRNA extraction. Three hundred microliters of chloroform was added per 1 mL of each homogenate, and the mixture was shaken vigorously for 30 seconds, followed by 5-minute incubation on ice. Samples were centrifuged at 12,000g for 15 minutes and the supernatant was mixed with 0.5 mL isopropanol in a clean tube. Samples were incubated at room temperature for 10 minutes and spun at 12,000g for five minutes. The supernatant was discarded. The remaining pellet was washed with 70% ethanol in diethylpyrocarbonate (DEPC)-treated water and was spun at 7,500g for five minutes. The washing step was repeated once to improve the quality of mRNA. The supernatant was then discarded and the pellet was dried under the air for 20-30 minutes until the pellet turned clear/opaque. The dried pellet was dissolved in 150 µL of DEPC-treated water at 4ºC overnight. RNA was quantified on Nano-Drop and 2.5 µg of total RNA was used for reverse transcription into complementary DNA (cDNA). ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) was used for Taqman RT-PCR. Using relative quantification (RQ) of cycle thresholds for each sample, transcript levels of each gene were normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.9 Cholestyramine (CTM) Treatment for CA or CCl_4-Induced Preterm Labor

CTM diet was prepared by mixing 2.5% (w/w) CTM resin in the regular diet. Twelve pregnant females were injected in the peritoneal cavity with 86 mg/kg CA in 1,2-propanediol every 12 hours for up to four injections. CTM diet was started 24 hours prior to the first injection. The end of the pregnancy term was determined when pregnant mice started the parturition process. Daily food consumption was monitored. Treated

mice were observed for weight and behavioral changes, and organs were harvested as previously described. To evaluate for cannibalism, the weight of each mouse was monitored during injection period.

2.10 E2 Treatment on Pregnant CD-1 Mice

Four pregnant female mice were injected in the peritoneal cavity with 5 mg/kg of E2 in 1,2-propanediol every 12 hours starting on day 15.7 for six consecutive injections. The end of the pregnancy term was determined when pregnant mice started the parturition process by visibly evaluating for signs of labor. Mice were monitored for weight or behavioral changes, and organs were collected as previously described for mRNA determination.

2.11 Treatment of Human Myometrial Cells (hTERT-HM)

The hTERT-HM human myometrial cell line was kindly provided by Dr. Jennifer Condon (University of Pittsburgh, Pittsburgh, PA) and was maintained in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin at 37ºC under humidifient 5% CO_2 and 95% air.

hTERT-HM cells were seeded in 10-cm petri dish in 10 mL phenol-red free DMEM/F12 supplemented with 10% (v/v) charcoal-stripped FBS and 1% (v/v) penicillin/streptomycin and cultured under 5% CO_2 until cells reached 75% confluency. Once the desired confluency was achieved, growth medium was replaced with 10 mL of phenol-red free DMEM/F12 supplemented with 1% (v/v) charcoal-stripped FBS and 1% (v/v) penicillin/streptomycin. Cells were then treated with 0.2% ethanol and 0.2% DMSO for controls or with 10, 50, 100, or 200 μM of CA in DMSO. Wells treated with

CA also received treatment with 100 nM of E2 in ethanol and 1 µM of P4 in ethanol to mimic pregnancy condition.

Progesterone-alone treatment was carried out in cell plates prepared as above. Cells were treated with 0.1% ethanol for control or with 200 nM, 1, 5, 10, and 50 µM of P4 in ethanol. All treatments were 0.1% of total volume medium.

2.12 Statistical Analysis

Welch's t-test with unequal variance was applied for comparison between two different groups. Grubbs' test was applied to detect any outliers within each groups. The values of outliers were repeated at least three times and confirmed. Standard deviation was used as the measure of variability. A p-value of less than 0.05 was considered statistically significant. MS Excel, Prism GraphPad or SigmaPlot were used for statistical analysis.

3. RESULTS

3.1 CA-Induced Preterm Labor

In order to test our hypothesis that bile acids cause preterm labor, pregnant mice were treated with CA and were observed for any preterm labor. CA was used to increase maternal TBA as it is most significantly increased form of bile acids in ICP (Table. 1). The first injection of CA was on day 16.3 of gestation based on ICP presentation, which correlates with elevation of bile acids in the last trimester of gestation (Brites, 2002).

To verify elevation of plasma TBA following CA injection, TBA concentrations in plasma were measured in CA-injected mice. The results showed significant increase in plasma TBA at the time of labor in pregnant mice treated with 86 mg/kg CA (20.7 ± 8.0 μM, $p = 0.0044$) and 129 mg/kg CA (29.7 ± 13.6 μM, $p = 0.0008$) compared to those treated with vehicle (6.73 ± 3.04 μM; Figure 1A). CA injection also resulted in an elevated plasma AST level; however, only high dose CA significantly increased AST at the time of labor (17.3±8.7 vs. 52.7±23.9 IU/L, $p = 0.002$; Figure 1B). This suggested that higher dose of CA treatment exerted mild liver injury due to overload of bile acids. In addition, higher dose of CA treatment resulted in higher rates of stillbirth than preterm labor. Therefore, lower dose CA treatment that did not show any toxicity to the liver with no risk for inducing stillbirth was used for further experiments.

Average full pregnancy term for normal CD-1 mice was 19.9 days, 95% CI [19.6, 20.1]. With CA treatment, gestation period was significantly decreased compared to vehicle treated mice (Figure 1C). Mice treated with 86 mg/kg CA had an

average term of 18.3 days (95% CI [18.2, 18.4]) and mice treated with 129 mg/kg CA had an average term of 17.8 days (95% CI [17.6, 18.0]; $p < 0.00005$ for both). Based on the results, it was established that direct CA treatment to intraperitoneal cavity of mice was able to induce preterm labor.

Changes in sex hormone levels were observed following CA treatment. Plasma P4 concentrations at the time of labor were significantly decreased from 20.1 ± 13.6 ng/ml in vehicle-treated mice to 0.34 ± 0.23 ng/ml and 2.39 ± 1.14 ng/ml in mice treated with 86 mg/kg and 129 mg/kg CA, respectively ($p = 0.002$ and 0.005; Fig. 1D). The result from this study is consistent with the changes in plasma P4 level in rodents during pregnancy, which maintains high progesterone level before delivery with a sudden decline once subjects initiate the labor. In addition, depending on the stage of labor, level of progesterone greatly differ. This may explain the wide variability in mice in control group.

In all plasma analysis, sample #4 in 86 mg/kg CA treatment group and sample #8 in 129 mg/kg CA treatment group showed abnormal values for both TBA and P4 assay, and were identified as outliers by Grubbs' test. These samples were excluded from the plasma analyses. Figures including these two samples are depicted in Fig 1.

The data collectively demonstrates that CA treatment to pregnant mice in the late stages of pregnancy elevated plasma bile acids without causing liver toxicity (low dose CA) and contributed to inducing preterm labor.

3.2 CCl_4-induced Preterm Labor

ICP is often accompanied by liver injuries, and risk of ICP increases in those who have previous or pre-existing liver damage (Guntupalli & Steingrub, 2005). As

liver injuries are accompanied by elevated bile acids, the effect of acute liver toxicity by CCl_4 in the length of pregnancy term was investigated. CCl_4 is one of the most potent hepatotoxins and is commonly used to induce acute liver injury in rodents (Mehendale, 1984; Mehendale et al., 1994). In treatments with varying concentrations of CCl_4, early parturition was achieved in 67%, 83.3%, and 100% of subjects treated with 1 ml/kg, 1.5 ml/kg, and 2ml/kg of 40% (v/v) CCl_4 in olive oil, respectively (Figure 2A). Compared to full term, mice treated with 1 ml/kg CCl_4 solution had an average pregnancy term of 18.9 days (95% CI [18.4, 19.4]; $p = 0.07$), mice treated with 1.5 ml/kg CCl_4 had an average term of 18.1 days (95% CI [17.9, 18.4]; $p = 0.0002$), and mice treated with 2 ml/kg CCl_4 had an average term of 17.6 days (95% CI [17.3, 17.9]; $p < 0.00005$). Although the highest dose showed 100% shorter term delivery, 90% of the litters were stillbirth. The lower two doses had 12% stillbirth with 1.5ml/kg CCl_4, and 4.8% stillbirth with 1 ml/kg CCl_4. Stillbirth or perinatal death in ICP occurs in about 2 to 11% of pregnancies, which is not the majority (Ehrenberg-Buchner & VanBuren, 2014). Therefore, the dose of 1.5 ml/kg of CCl_4 was used in future mouse studies.

To confirm CCl_4 induced acute hepatotoxicity, plasma AST levels at the time of labor were evaluated. As shown in Figure 3B, significant increases of serum AST levels were detected in pregnant mice treated with CCl_4 compared with those who received vehicle treatment (17.3 ± 8.7 vs. 111.2 ± 19.6 IU/L, $p = 0.00003$), verifying acute liver damage by CCl_4. Acute liver injury in turn was correlated with increase in plasma TBA. Figure 3C showed elevation of plasma TBA with CCl_4 treatment (6.73 ± 3.0 vs. 22.7 ± 6.2 μM, $p = 0.0003$). These results indicated that limiting liver

metabolic capacity induced preterm labor with increasing plasma TBA from acute hepatotoxicity.

Plasma P4 concentrations at the time of labor were decreased with CCl_4 treatment (6.37 ± 5.7 ng/mL, p = 0.02; Fig. 3D) as compared to those treated with vehicle (20.1 ± 13.6 ng/ml). This result was consistent with the P4 level change in CA-induced preterm labor, which the mice in labor showed significantly decreased plasma concentration of P4.

The data collectively demonstrated that CCl_4 treatment to pregnant mice in the late stages of pregnancy effectively increased plasma TBA by inducing acute liver toxicity, which likely contributed to triggering preterm labor, and showed progesterone withdrawal during preterm labor.

3.3 Changes in Gene Expressions in the Uterus for CA- or CCl_4-Induced Preterm Labor

With confirmation of preterm labor by elevated bile acids from treatments with CA or CCl_4, multiple parturition-associated genes in the uterus that have a role in delivery mechanism were analyzed for changes in expressions by real-time RT-PCR. Control samples were collected at the same time on day 18.7. However, samples in control group had not begun the parturition process, which adds to the limitation by comparing them with samples collected during labor for CA or CCl_4 treatment groups. Treatments of pregnant mice with CA or CCl_4 significantly increased OXTR mRNA expression by 2.7 and 2.0 fold, respectively (p = 0.004 and 0.04; Fig. 3A). Oxytocin is a hormone that causes contraction of the uterus, and OXTR, which encodes its receptor, is up-regulated during parturition (Blanks & Thornton, 2003). Our finding is

in agreement with reported evidence of increased OXTR expression during labor. It has been suggested that IL-6 plays a role in the regulation of onset of labor by increasing expression of genes that control prostaglandin synthesis (Robertson et al., 2010). Our data showed a significant increase of IL-6 expression by 17 fold in mice treated with CA ($p = 0.044$; Fig. 3B). In mice treated with CCl_4, IL-6 levels exhibited a trend of increase but did not reach statistical significance ($p = 0.14$; Fig. 3B). Sample #1 from CCl_4 treatment group was identified as an outlier for IL-6 expression, possibly due to pre-existing liver damage as spotty liver was observed during organ collection. The result including this data point is shown in figure 3B-1. Prostaglandin (PG) $F_{2\alpha}$ is known to play a major role in triggering parturition as a contractile PG, with increased expression of PTGFR as pregnancy term progresses (Brody-Eppley & Myatt, 1998). In this study, we found that PTGFR expression levels at parturition were significantly elevated by 1.5 and 2.1 fold in mice treated with CA and CCl_4, respectively ($p = 0.049$ and 0.040; Fig. 3C). On the other hand, the expression of HPGD, a major enzyme to metabolize prostaglandins, was significantly down-regulated by both CA and CCl_4 treatments compared to control group. The expression levels of HPGD were decreased by 49% and 77% in mice treated with CA and CCl_4 ($p = 0.0006$ and $p < 0.00005$; Fig. 3D). The existing data suggest elevated prostaglandin levels during parturition due to decreased prostaglandin metabolism (Challis, Patel, & Pomini, 1999). Our data is in agreement with the evidence which prostaglandin levels during parturition is elevated due to decreased prostaglandin metabolism by HPGD. We also found that PTGER2 expression was decreased with CA and CCl_4 treatment by 45% and 68%, respectively ($p = 0.043$ and 0.0065; Fig. 3E). It has been known that

PGE2 is a relaxatory prostaglandin during labor, and its receptor is decreased before and after the labor (Brodt-Eppley & Myatt, 1998). Taken together, decreased expression of PTGER2 and HPGD with concurrent increased expression of PTGFR in mice treated with CA and CCl_4 support the notion that labor process is mediated by prostaglandins and that CA-induced preterm labor undergoes the similar mechanism to labor in normal pregnancy. No consistent significant changes were observed for TNFα and COX2 with either CA or CCl_4 treatment (Fig. 3F, 3G). This result indicates that preterm labor induced by CA and CCl_4 may not involve other cytokines, as COX2 participates in synthesis of cytokines other than prostaglandins as well. Conclusively, all the data suggest that prostaglandin is closely related to preterm labor by CA or CCl_4 treatment. Further investigation of gene changes in full term labor is in need to claim whether the prostaglandins mediate the preterm labor or the parturition mediates the change in prostaglandin-associated genes.

3.4 Changes in Gene Expressions in the Liver of Mice with CA- or CCl_4-Induced Preterm Labor

In order to investigate the mechanisms of bile acid elevation and prostaglandin metabolism in mice with CA or CCl_4 treatment, BSEP, FXR and HPGD expressions in the liver were quantified by RT-PCR. As seen in figure 4A, no significant changes were observed for FXR after either treatment. However, with CCl_4 treatment, BSEP expression was significantly down-regulated by 81% ($p < 0.0003$; Fig. 4B). The results indicated that down-regulation of BSEP in the liver following CCl_4 treatment was a mechanism for increased plasma TBA by inhibiting hepatic bile acids excretion. Also, HPGD expression in the liver was down-regulated by 31% and 49% with

treatments with CA or CCl_4, respectively ($p = 0.04$ and 0.0022; Fig. 4C). This result suggest that systemic prostaglandin level is also decreased during labor due to decreased metabolism of prostaglandin in the liver, which is the major site for prostaglandin metabolism. In summary, the results suggest that CA and CCl_4 induced preterm labor is associated with inhibition of systemic prostaglandin metabolism and inhibition of BSEP in the liver by CCl_4 to increase plasma TBA.

3.5 Reversal of Preterm Labor in CA-treated Pregnant Mice by CTM

In order to confirm if preterm labor was truly triggered by elevation of plasma TBA, cholestyramine, a bile acid binding resin, was investigated to possibly reverse preterm labor. Cholestyramine is a bile acid sequestrant which forms insoluble complex with bile acids in the gastrointestinal tract to prevent reabsorption of bile acids. By administering cholestyramine, plasma TBA was expected to decrease and CA-induced preterm labor to be reversed if bile acid is truly the triggering factor.

CTM was added to diet simultaneously with CA injection, as previously described, to 12 pregnant females in the later stages of pregnancy. Preterm labor was reversed by 50% in mice treated with CA and CTM in comparison with mice treated with CA injection alone. More importantly, the reversibility of preterm labor by CTM was correlated with the amounts of CTM consumption of individual mouse. Of 12 mice given CTM diet and CA injection, the six that did not have preterm labor consumed significantly more food compared to the six that had preterm labor (6.76 ± 1.92 g vs. 2.65 ± 1.74 g over 72 hours, $p = 0.003$; Fig. 5A). Among six mice that had preterm labor with CA and CTM co-treatment, it was observed that the less CTM diet consumption, the gestation period was shorter ($R^2 = 0.97$; Fig. 5B). As expected, the

mice who consumed more CTM had decreased TBA levels (6.06 ± 3.03 μM) than the mice who consumed less CTM (17.9 ± 14.8 μM, p = 0.056; Fig. 5C). Taken together, the reversibility of preterm labor by CTM was directly correlated with the amount of food consumed and the resulting TBA levels. In all further analyses, tissue and plasma samples were performed only on the six mice that did not have preterm labor.

In the treatment with CTM, mice that did not have preterm labor, there was a decrease in plasma TBA to normal level (6.06 ± 3.03 μM) compared to TBA levels in mice that had CA-treated preterm labor (20.7 ±8.0 μM, p = 0.004; Fig. 6A). There were no significant changes in plasma AST (Fig. 6B), which indicates that CTM treatment did not exhibit liver toxicity. Plasma P4 level was also increased by 74 fold compared to CA-alone treatment (0.34 ± 0.23 vs. 25.12 ± 20.08 ng/mL, p = 0.03; Fig. 6C). Taken together, the plasma levels of TBA, AST and P4 of CTM treated full-term mice were compatible to that of regular full-term mice. The data indicated that by directly lowering plasma TBA, risk of preterm labor was reversed, which further strengthen the notion that elevated bile acids are the triggering factors in inducing preterm labor.

Consistently, RT-PCR revealed reversed trends in target genes compared to CA treatment. Samples were collected at the time of labor throughout the day. CTM treatment group samples that did not have preterm labor were collected uniformly at day 18.7 before initiation of parturition. The mRNA expression of OXTR was decreased by 51% in mice that had reversed preterm labor from CTM diet (p = 0.014; Fig. 7A). IL-6 expression showed a decreasing trend on average by 87% with CA and CTM treatment without statistical significance, which is consistent with the trend

when comparing with normal pregnancy (Fig. 7B). PTGFR was decreased by 56% compared to CA alone ($p = 0.0067$; Fig. 7C), and HPGD expression showed 1.7 fold increase ($p = 0.027$; Fig. 7D). The changes in expression of HPGD and PTGFR with CA treatment were reversed with CTM comparable to normal level, indicating that increase in PG level and PTGFR expression may play a more important role in initiation of labor process. COX2, which showed an increasing trend but did not have a statistically significant change with CA treated preterm-term mice compared to vehicle, was significantly decreased with CTM treatment (2.64 vs. 0.27, $p = 0.04$; Fig. 7E). Since COX2 participates in synthesizing prostaglandins, we expect that synthesis of prostaglandins would be decreased by decreasing plasma TBA. CTM treatment was able to reverse some changes in parturition-associated gene expressions by CA treatment. The results collectively indicated that CA-induced preterm labor was reversed by decreasing plasma TBA levels with CTM, while showing down-regulated PTGFR expression and possibly prostaglandin synthesis.

Collectively, the results demonstrate that CTM was able to effectively prevent preterm labor induced by elevation of bile acids. It can be expected that once constant delivery of CTM is guaranteed, complete prevention of preterm labor is possible.

3.6 Bile Acids for Appetite Suppression

Although this was not the purpose of our study, we had an interesting discovery while monitoring the food consumption of CA or CCl_4 treated mouse. During pregnancy, food intake is increased in humans. The same principle applies for mice. In the control group, pregnant female mice at later stage of pregnancy had average food consumption over 24 hours of 7.25 g. However, with CA or CCl_4

treatment, the diet pattern was altered. After CA injection, average food consumption over 24 hours was decreased to 2.44 g ($p < 0.00001$, Fig. 8A). With CCl_4 injection, average food consumption was decreased to 1.55 g over 24 hours ($p < 0.00001$, Fig. 8B). This was a rather surprising finding that diet intake was decreased with elevated TBA, to even lower than average intake for non-pregnant mice (4-5 g). This finding is consistent with previous publications which demonstrated appetite suppression effects of CDCA and DCA (George & Gallagher, 1968; Roberts et al., 2011). Decreased appetite in CCl_4-treated mice may be due to increased plasma TBA.

In addition to decreased diet intake, it was also noted that CA or CCl_4 treatment in pregnant mice were associated with no weight gain or slight weight loss. Typically during pregnancy, both mouse and human gain weight as the term progresses. We observed that in late stages of pregnancy, mice usually gain 1 – 3 g of weight every day. However, during the course of our study, we noticed that pregnant mice treated with CA or CCl_4 had either no change in weight or losing 1 – 2 g daily. This may be due to reduced food intake as described above.

3.7 Effect of E2 on TBA and AST

Previous studies have established that estrogen-induced cholestasis occurs by altering expression of BSEP (Song et al., 2014). To further investigate the effect of estrogen in increasing bile acids to trigger preterm labor, four pregnant females received 5 mg/kg E2 treatment every 12 hours starting day 15.7. Indeed, E2 treatment elevated plasma TBA from 6.73 μM to 14.8 μM ($p = 0.007$; Fig. 9A). However, E2 treatment did not cause preterm labor in any subjects. It should be noted that although bile acids were elevated in E2-treated mice, the levels were still lower than that in

mice treated with CA or CCl_4. Consistent with mild increase in TBA, no significant changes were observed for plasma AST level and P4 levels (Fig. 9B and 9C). Whether increasing E2 doses to further elevate bile acid levels will induce preterm labor remains to be determined.

Change in mRNA expression levels of target genes on day 18.7 was analyzed by RT-PCR. E2 treatment resulted in non-significant increase of OXTR expression by 2.2 fold ($p = 0.072$; Fig. 10A). Since these mice did not have preterm labor, OXTR expression was not expected to change much. IL-6 expression in uterus was significantly increased with E2 treatment by 9.7 fold, which indicates that E2 treatment contributes to increasing cytokines other than prostaglandins ($p = 0.015$; Fig. 10B). PTGFR expression, unlike mice treated with CA or CCl_4, was down-regulated by 52% ($p = 0.01$; Fig. 10C). Consistently with the change in CA or CCl_4 treated mice, HPGD expression level in E2 treated mice was decreased by 29% ($p = 0.044$; Fig. 10D). Similar changes in trends of OXTR, IL-6 and HPGD but not in PTGFR occurred with exposure to CA or CCl_4 treatment suggest that preterm labor may be more affected by expression level of PTGFR. COX2 mRNA expression level was decreased with E2 treatment by 90% ($p = 0.0008$; Fig. 10E), suggesting that E2 treatment contributed in decreasing synthesis of cytokines including prostaglandin.

The results collectively indicate that elevation of E2 alone may not be sufficient enough to trigger preterm labor. Although serum TBA is elevated, the magnitude of increase for E2 treated mice is 1.5-2 fold less than that for CA or CCl_4 treated mice. In addition, E2 treatment did not induce expected trend for PTGFR. Although prostaglandin metabolism was decreased by decreased HPGD expression,

PTGFR was significantly down-regulated, even compared to control. From the discrepancy in gene expression changes in PTGFR compared to mice that had preterm labor and all previous results of expression changes in prostaglandin-regulating genes, we suggest prostaglandin $F_{2\alpha}$/PTGFR signaling pathway is the determinant factor in triggering preterm delivery.

3.8 Bile Acids and Sex Hormone Treatments in Human Myometrial Cell Line (hTERT-HM)

To further confirm the correlation of current data to human, expression changes in target genes in hTERT-HM myometrial cells were investigated. First, cells were treated with varying concentrations of P4 to determine a dose-response relationship. As concentration of P4 treatment increased, OXTR was decreased (Fig. 11A) and IL-6 (Fig. 11B) and COX2 (Fig. 11C) were increased.

Cells were treated with varying concentrations of CA to study the effect of CA on target genes, while mimicking pregnancy condition with concurrent E2 and P4 treatment. E2 and P4 concentrations were chosen based on their effect on hTERT-HM cell individually. One millimolar of P4 was used in this treatment since this concentration did not cause any changes in expression of OXTR, IL-6 and COX2 by P4 itself. The result showed that as concentrations of CA were increased, all three targets – OXTR (Fig. 12A), IL-6 (Fig. 12B) and COX2 (Fig. 12C) – were increased. The data suggest that CA treatment in presence of E2 and P4 increase inflammatory genes. However, PTGFR, which was the target gene of our study, did not show any significant change with either P4 or CA with sex hormones combination treatment. HPGD was not even detectable in this specific cell line.

These results coincide with that from the in vivo experiment where P4 surge was associated with lower OXTR expression and increase in serum TBA caused elevation of OXTR, IL-6 and COX2. However, due to the discrepancy in PTGFR and HPGD expression, this cell might not be suitable for further investigation for our study. The effect of bile acids on endometrial cell line should be investigated in order to study more accurate mechanism of preterm labor induced by bile acids.

4. DISCUSSION

In this study, we successfully established the cause-effect relationship between bile acids and preterm labor. Elevation of bile acids was achieved by either direct introduction of CA or by inducing acute hepatotoxicity with CCl_4. The results of this study provided direct evidence to support the findings in several publications that severely elevated maternal serum bile acids were linked to higher rate of preterm birth and fetal complications (Geenes et al., 2014; Rigby, Ehrenbery-Buchner, & VanBuren, 2014; Glantz, Marschall, & Mattsson, 2004).

Treatments with CA or CCl_4 showed increase in several parturition-associated genes including OXTR, IL-6, and PTGFR, and decrease in HPGD and PTGER2, which confirms the involvement of these particular genes in preterm labor induced by bile acids.

Estrogen is known to increase bile acids. Our previous study demonstrated that estrogen induced cholestasis through transrepressive pathway with FXR inhibiting BSEP (Song et al., 2014). The results from this study showed no preterm labor with E2 treatment alone despite elevation of plasma TBA. However, it should be noted that the bile acid levels in E2-treated mice did not reach the levels observed in mice treated with CA or CCl4. In addition, there is a discrepancy in gene expressions between E2 treated mice and mice that had preterm labor. Although OXTR, IL-6 and HPGD showed the same trend as CA or CCl_4 treatment, PTGFR and COX2 were down-regulated by E2 treatment compared to control. Collectively, we suspect that the role of prostaglandin $F2_\alpha$/PTGFR signaling pathway in parturition is critical in inducing preterm labor.

Prostaglandins are important mediators for inducing labor by uterine contraction (Challis & Olson, 1988; Chan, 1983; Romero et al., 1996). It is often used to chemically induce labor. Especially, contractile PTGFR and relaxatory PTGER2 play the major role in uterine contractility (Coleman, Smith & Narumiya, 1994). Evidence supports that PTGFR expression was significantly increased until delivery and PTGER2 expression was significantly decreased from day 16 until postpartum in rats (Brody-Eppley & Myatt, 1998). Our results support this existing evidence in mice and suggests correlations of these receptors and bile acids in inducing labor.

$PGF_{2\alpha}$ also play an important role in progesterone level. Increases in $PGF_{2\alpha}$ metabolites is correlated with rapid decrease in progesterone during menstrual cycle (Koullapis & Collins, 1980). Also in mares, progesterone treatments reversed abortion induced by exogenous $PGF_{2\alpha}$ synthetic analog and inhibited endogenous $PGF_{2\alpha}$ secretion (Daels et al., 1996). PG-induced contraction in swine was reduced with progesterone administration (Künzel et al., 2014). Progesterone, specifically 17α-hydroxyprogesterone, is sometimes used to delay or prevent preterm labor in humans (Errol & Caughey, 2011). Our data showed a decrease in progesterone after initiation of parturition. For mice that did not have preterm labor maintained progesterone surge, whereas in mice that had preterm labor had a 10-15 fold decrease in plasma P4 compared to normal or CTM treated full term mice. This shows the same trend as previously published evidence of inverse correlation between progesterone and $PGF_{2\alpha}$, as bile acids induced preterm labor in our study was also associated with increased expression of PTGFR and decreased P4 concentration. This also explains why E2-treated mice did not have preterm labor despite elevation of bile acid level, since they

had significantly higher progesterone level even compared to control and down-regulated PTGFR expression. With our new data, we can conclude that a primary mechanism to trigger bile acids induced preterm labor, and possibly all other parturition, is mediated by the $PGF_{2\alpha}$/PTGFR signaling pathway. Elevation of bile acids alone may not be sufficient enough to induce preterm labor if there is still progesterone surge or up-regulation of PTGFR.

These changes in gene expression match the results from human myometrial cell treatment. Treatments with CA, E2 and P4 showed CA dose-response increase in OXTR, IL-6, and COX2 which further confirms the same effects of bile acids in humans. Further study to assess the translation of these gene changes in protein level is necessary.

Currently, treatment for ICP is to provide mostly symptomatic relief for mother. There are no conclusive results of the effects of treatment agents on improving fetal outcome. CTM is a bile acid sequestrant which is one of the first-line treatments for ICP. By directly removing bile acids in circulation, CTM relieves severe pruritus in ICP. Our study showed that CTM reversed 50% of CA-induced preterm labor by decreasing serum TBA with a strong positive relationship between amount of CTM intake and reversibility of preterm labor. Had 50% of mice that had preterm labor consumed more diet containing CTM, their preterm labor may have been reversed as well. Changes in expression for OXTR, IL-6, PTGFR and HPGD were also reversed compared to CA treatment alone. This further supports our hypothesis of bile acids directly related to causing preterm labor.

CCl_4 treatment in pregnant mice elevated plasma TBA by down-regulating BSEP expression in the liver. This information could be applied in preventing preterm labor in those who have liver injuries. Modifying BSEP expression may decrease the plasma TBA and could decrease risk of preterm labor.

During the course of our study, we noticed the unusual change in weight of pregnant mice treated with CA or CCl_4. Instead of gaining weight in the late stages of pregnancy, these treatments showed no weight gain or slight loss of weight in subjects. We suspected that this was due to excess bile acids. CDCA and DCA are known to suppress appetite (Bray & Gallagher, 1968; Roberts et al., 2011). Our results showed decreases in food intake with CA treatment compared to control in both during pregnancy and non-pregnancy. Although not conclusive, we suspect the relationship between increased plasma TBA and decreased appetite.

There are few limitations present in this study. When comparing the changes in parturition-associated gene expressions, samples were compared regardless of if the mice were actively in labor or before labor. Therefore, we cannot conclude that these changes in expression are due to preterm labor by CA treatment or due to the labor process itself. Further investigation with in vitro model is necessary to determine the mechanism of bile acids on these genes. Also the samples were collected throughout the day as they show signs of preterm labor. As bile acids are affected by circadian rhythm, this might have added to the variabilities of some analyses. In addition, toxicity profile of CCl_4 in other organs have not been fully established. Other chemicals such as acetaminophen should be investigated to further strengthen our findings that bile acids elevation due to limited liver metabolic capacity is associated

with preterm labor. Furthermore, as in ICP, bile acids other than CA is also elevated. However, this study only used CA to introduce exogenous bile acids in pregnant mice. For more accurate model, composite of bile acids pool should have been used.

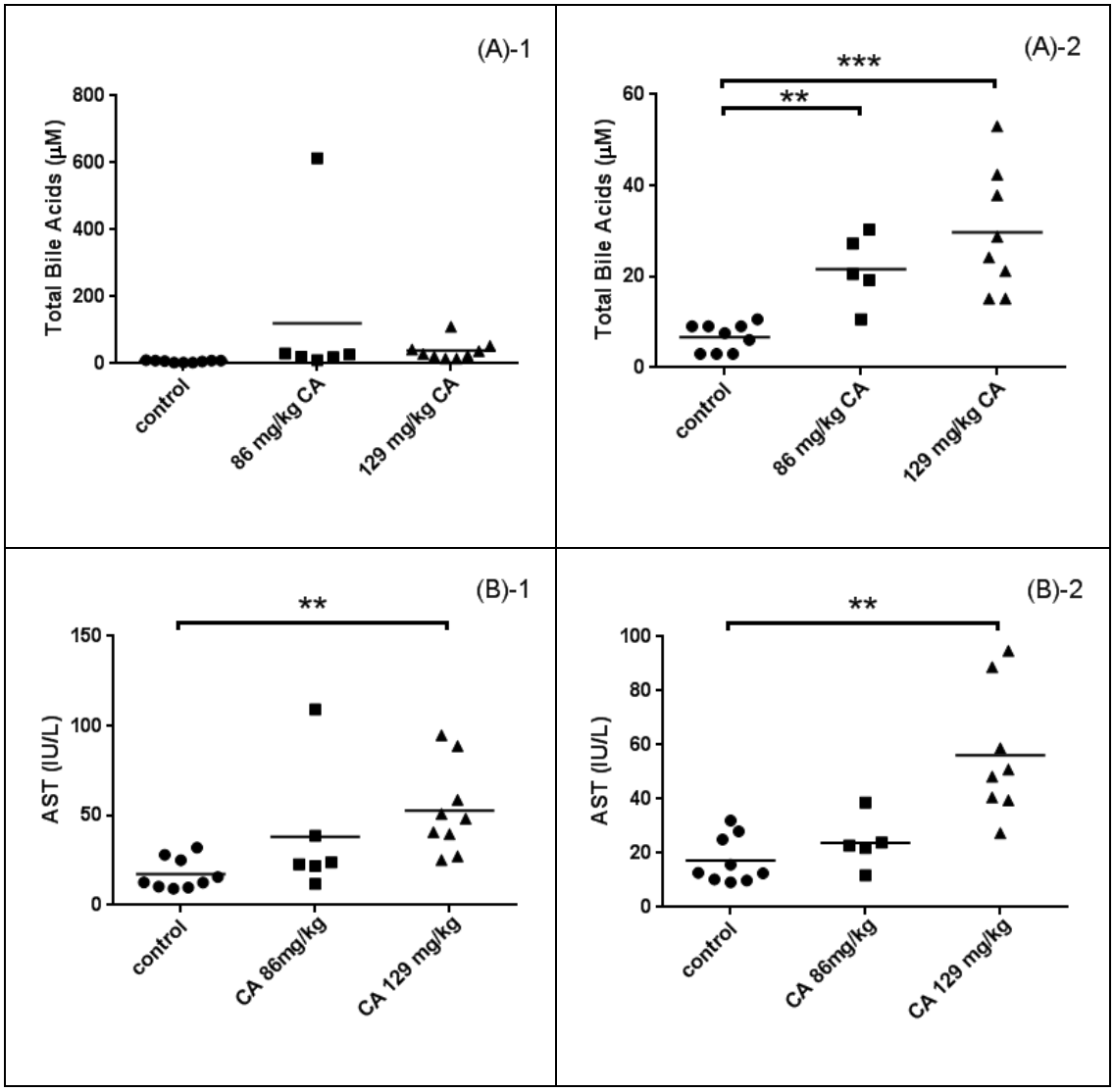
In conclusion, our study demonstrated that bile acids directly contribute in resulting in preterm labor. Although direct mechanism between bile acids and prostaglandin is still unclear, establishing this causative relationship may allow bile acids to serve as a predictor for preterm labor. Preventing spontaneous preterm birth is critical for fetal development in ICP. Bile acid levels serving as a biomarker for timing of parturition is a novel approach in studying molecular mechanism of spontaneous preterm labor. Understanding the pathological mechanisms by which bile acids affect parturition will ultimately provide a molecular basis for development of new strategies to prevent preterm labor. Upregulating BSEP, and decreasing bile acids, and antagonizing $PGF_{2\alpha}$/PTGFR signaling may represent new strategies for prevention of preterm labor in ICP or pregnant women with liver injuries such as cholestasis or hepatitis.

TABLES

	Normal Pregnancy		ICP	
Cholic Acid (CA)	30.4 %	2.26 µM	74.2 %	48.7 µM
Chenodeoxycholic Acid (CDCA)	40.1 %	2.98 µM	18.2 %	11.9 µM
Deoxycholic Acid (DCA)	20.5 %	1.52 µM	6.6 %	4.34 µM
Lithocholic Acid (LCA)	4.0 %	0.30 µM	0.5 %	0.33 µM
Ursodeoxycholic Acid (UDCA)	4.0 %	0.30 µM	0.55 %	0.36 µM

Table 1. Serum bile acids composition during normal pregnancy and intrahepatic cholestasis of pregnancy.

FIGURES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control
86 mg/kg CA
129 mg/kg CA
(A)-2
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control
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129 mg/kg CA
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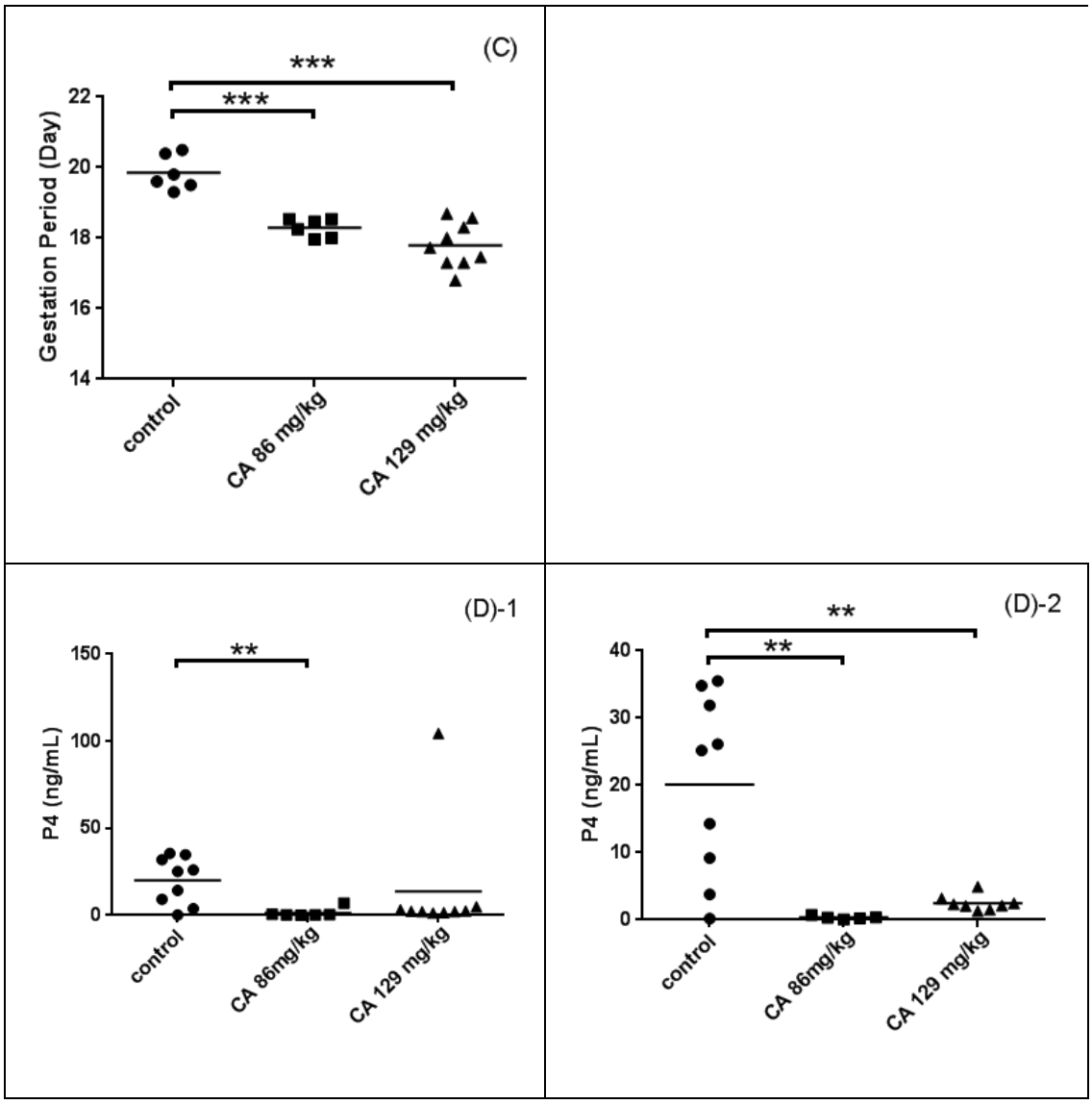


Figure 1. Effects of CA treatment on plasma concentration of TBA, AST, P4 and gestation period in CD1 mice.

Pregnant CD1 mice were treated with CA 86 mg/kg or 129 mg/kg in 1,2-propanediol or 1,2-propanediol vehicle IP. 6 mice received lower dose CA, 9 mice received higher dose CA or vehicle.Plasma samples were collected for TBA, AST and P4 assay. (A) Dose-dependent elevation of serum TBA with CA treatment, with outliers (1) and without outliers (2). (B) Dose-dependent elevation of serum AST with CA treatment, with outliers (1) and without outliers (2). (C) Shorter gestation period with CA treatment. (D) Decreased serum P4 with CA treatment, with outliers (1) and without outliers (2). (**$p < 0.005$, ***$p < 0.001$, Welch's t-test).

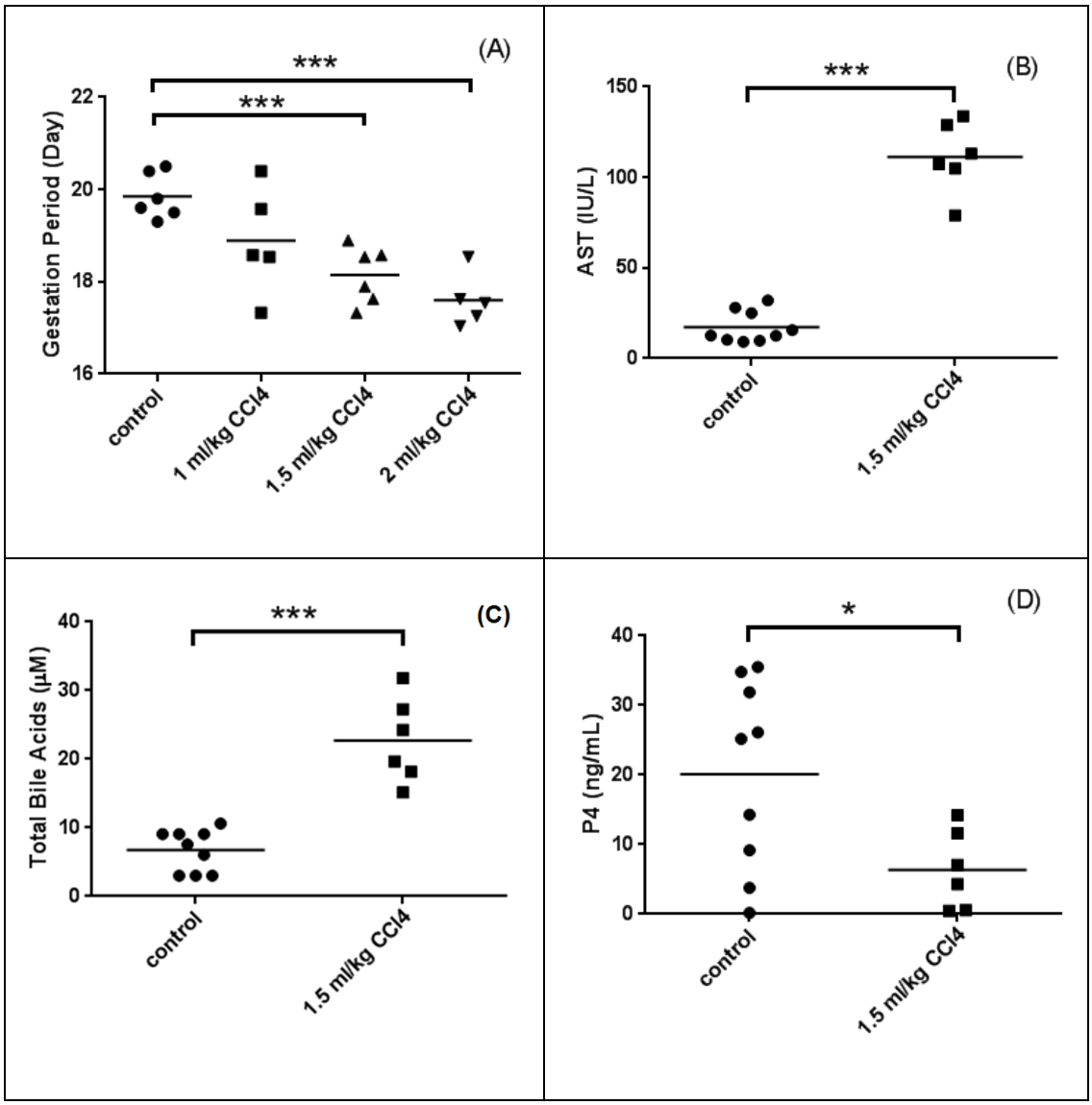
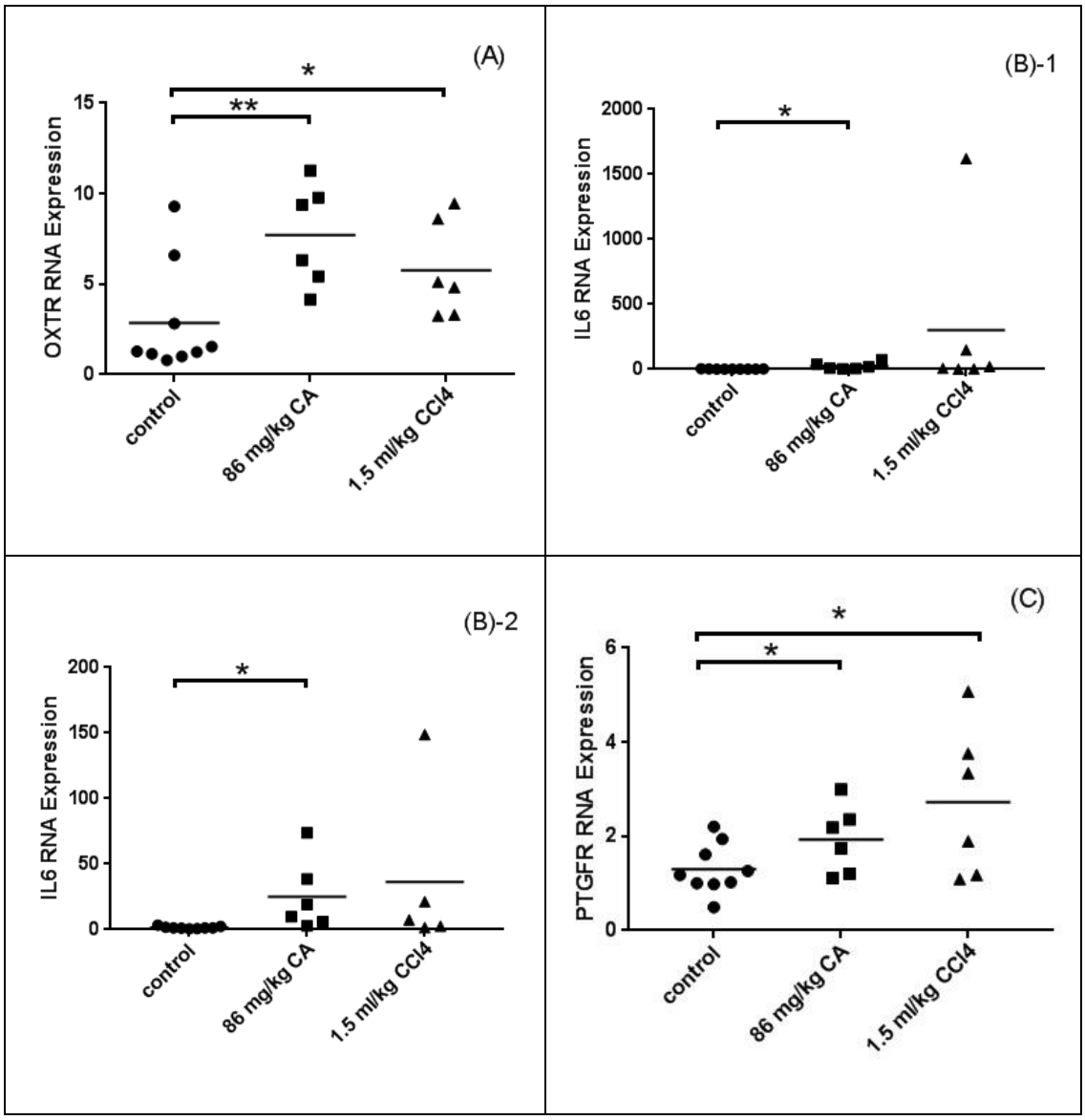


Figure 2. Effects of CCl_4 treatment on serum concentration of TBA, AST, P4 and gestation period in pregnant CD1 mice.

Pregnant CD1 mice were treated with 1, 1.5 or 2 ml/kg of 40% (v/v) CCl_4 in olive oil IP in 5, 6 and 5 mice per group, respectively. Plasma samples were collected for TBA, AST and P4 assay. (A) Dose-dependent decrease in gestation period with CCl_4 treatment. (B) Elevation of serum AST with CCl_4 treatment. (C) Elevation of TBA with CCl_4 treatment. (D) Decreased serum P4 with CCl_4 treatment. (*$p < 0.05$, ***$p < 0.001$, Welch's t-test).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5
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*
**
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(B)-1
IL6 RNA Expression
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1500
1000
500
0
*
control
86 mg/kg CA
1.5 ml/kg CCl4
(B)-2
IL6 RNA Expression
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150
100
50
0
*
control
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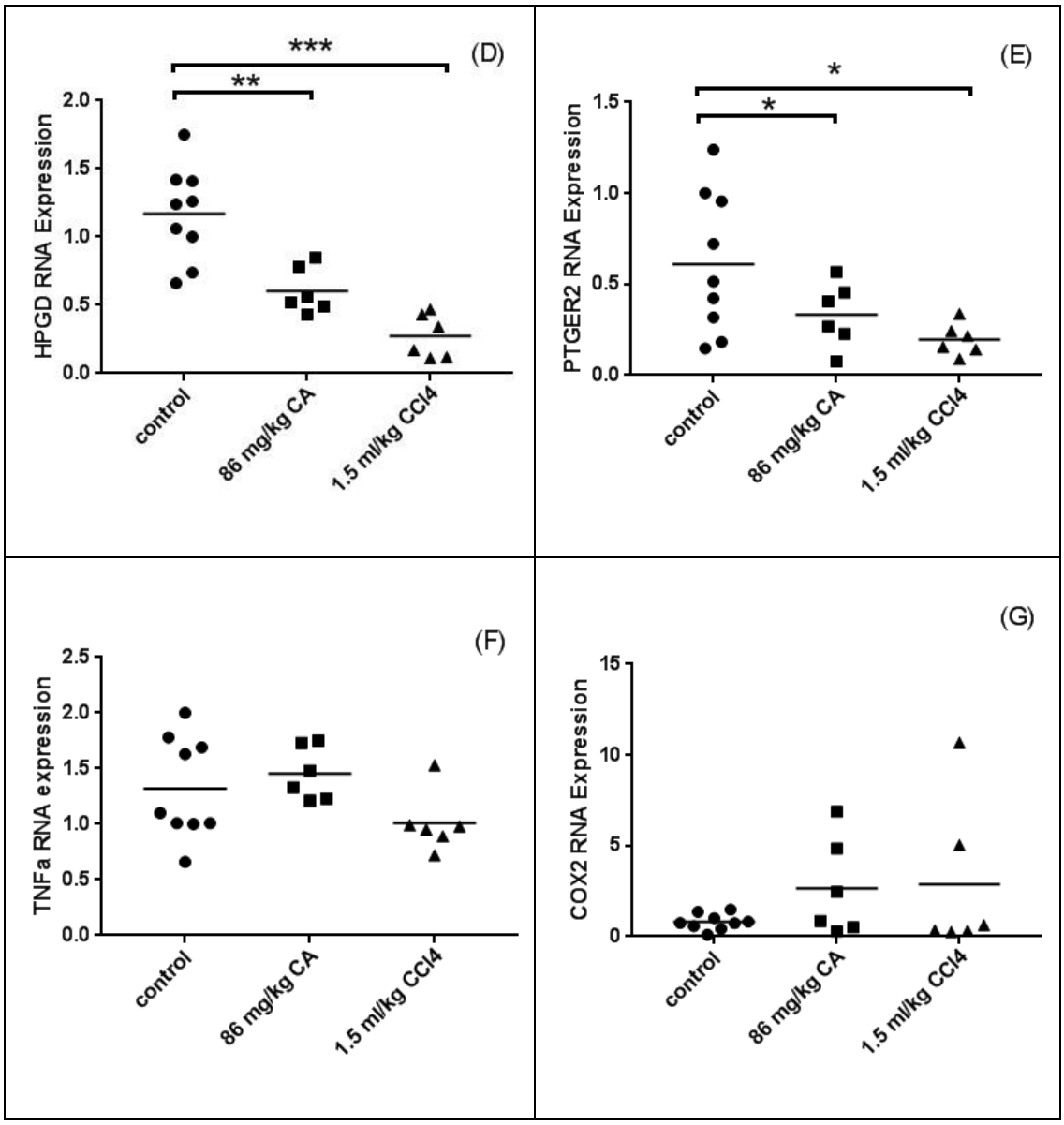

Figure 3. Effects of CA or CCl_4 treatment on parturition-associated genes in uterus of CD1 mice.

CD1 mice treated as above were used for mRNA analysis. Uterus were harvested during parturition or on day 18.7 and RNA was isolated using conditions stated in *Materials and Methods*. Endogenous OXTR (A), IL-6, with outliers (1) and without outliers (2) (B), PTGFR (C), HPGD (D), PTGER2 (E), TNFα (F) and COX2 (G) expressions were analyzed and normalized to GAPDH. The data are presented as a fold change in relative expression, each data points representing individual mice. (*$p < 0.05$,**$p < 0.005$, ***$p < 0.001$, Welch's t-test).

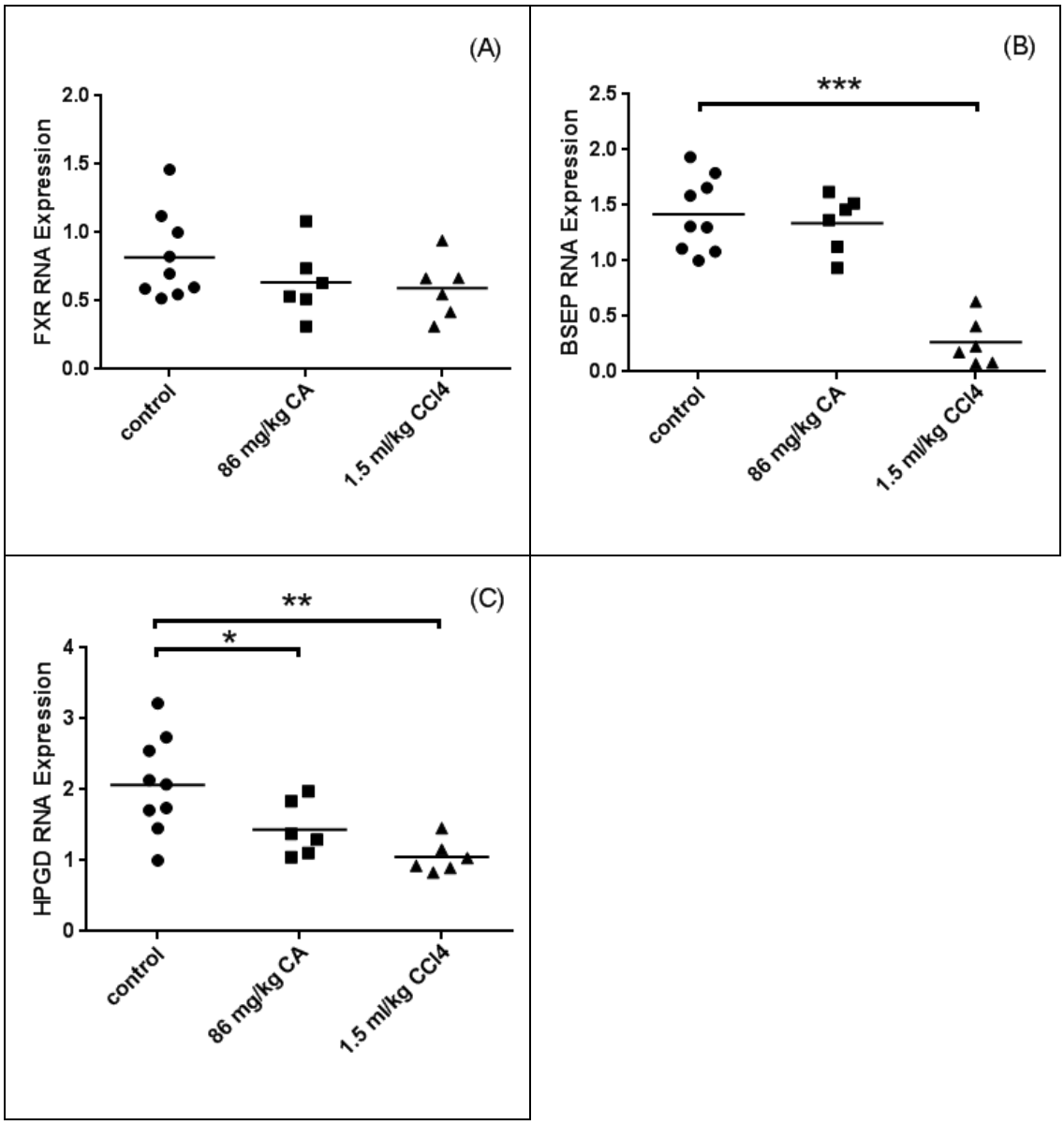

Figure 4. Effects of CA or CCl_4 treatment on endogenous FXR, BSEP and HPGD in liver of CD1 mice.

CD1 mice treated with CA or CCl_4 as above were used for mRNA analysis. Livers were harvested during parturition or on day 18.7 and RNA was isolated using conditions stated in *Materials and Methods*. Endogenous FXR (A), BSEP (B) and HPGD (C) expressions were analyzed and normalized to GAPDH. The data are presented as a fold change in relative expression, each data points representing individual mice. (***$p < 0.001$, Welch's t-test).

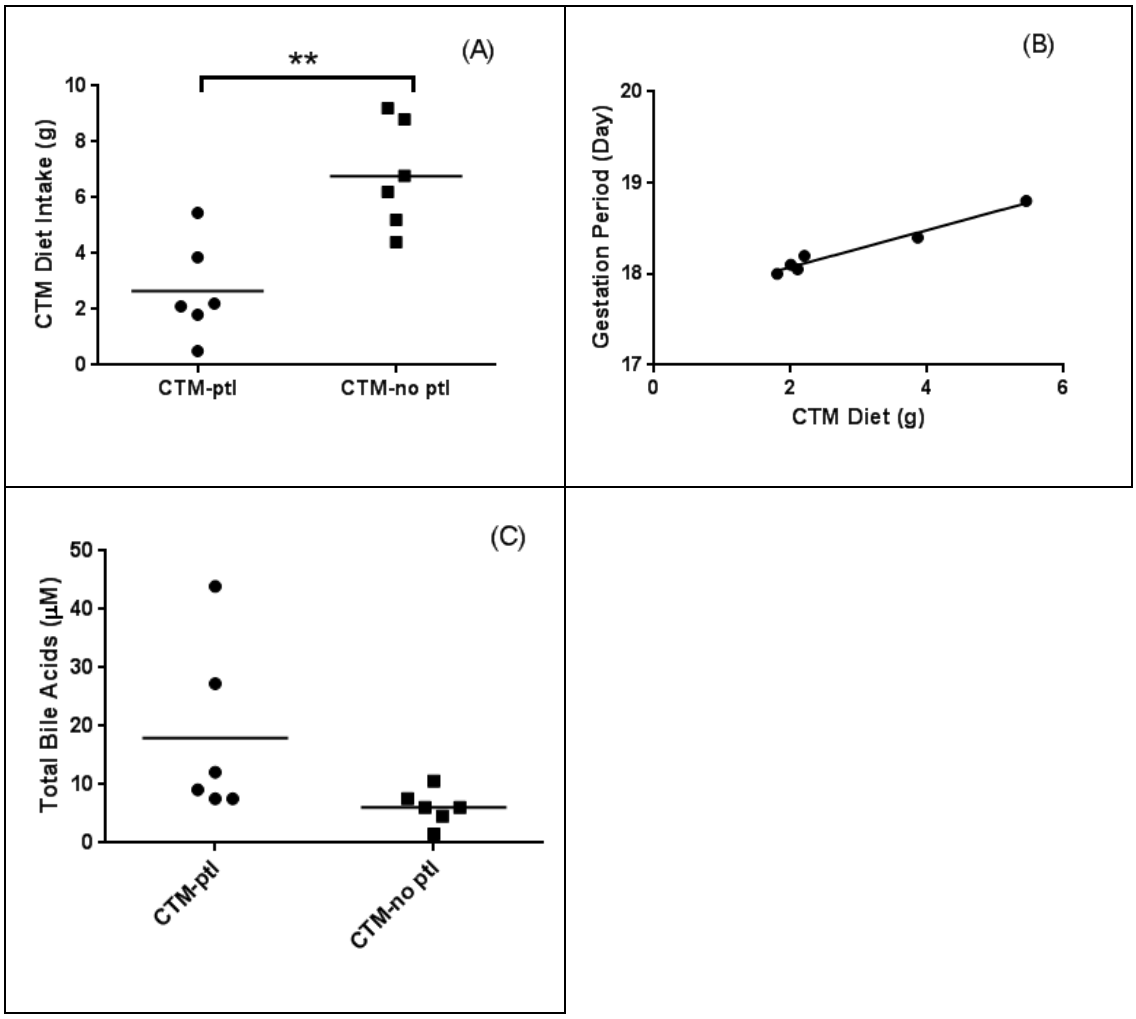

Figure 5. Effect of CTM intake amount on reversibility of preterm labor and plasma bile acids concentration.

CD1 mice treated with CTM and CA as above were used to assess correlation between amount of CTM intake and reversibility of preterm labor. (A) Amount of CTM intake difference between groups with preterm or full term labor. (B) Dose-response relationship of CTM treatment and gestation period ($R^2 = 0.95$). (C) Plasma serum samples were collected for determination of plasma TBA concentration. (**$p < 0.005$, Welch's t-test).

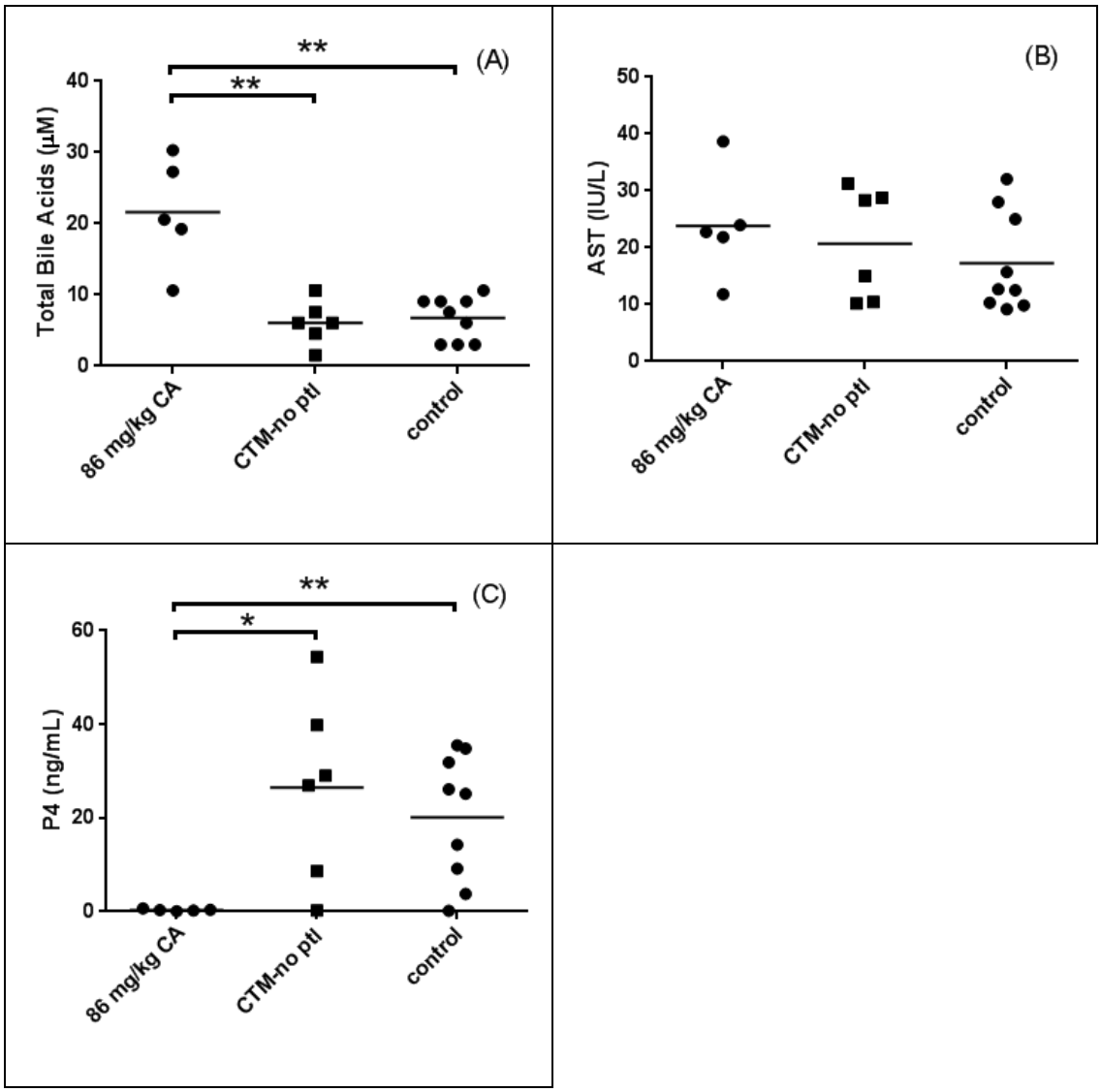

Figure 6. Effects of CTM treatment on serum concentration of TBA, AST, P4 and gestation period in CA-induced preterm labor in CD1 mice compared to control.

Pregnant CD1 mice were treated with 86 mg/kg CA IP and given regular or CTM diet (6 and 12 per group, respectively). For CTM treatment, only the mice that did not have preterm labor was used for analyses. Control group was included to assess the reversibility of CTM to normal level. Plasma samples were collected for determination of TBA (A), AST (B) and P4 (C) concentration. (*$p < 0.05$,**$p < 0.005$, Welch's t-test).

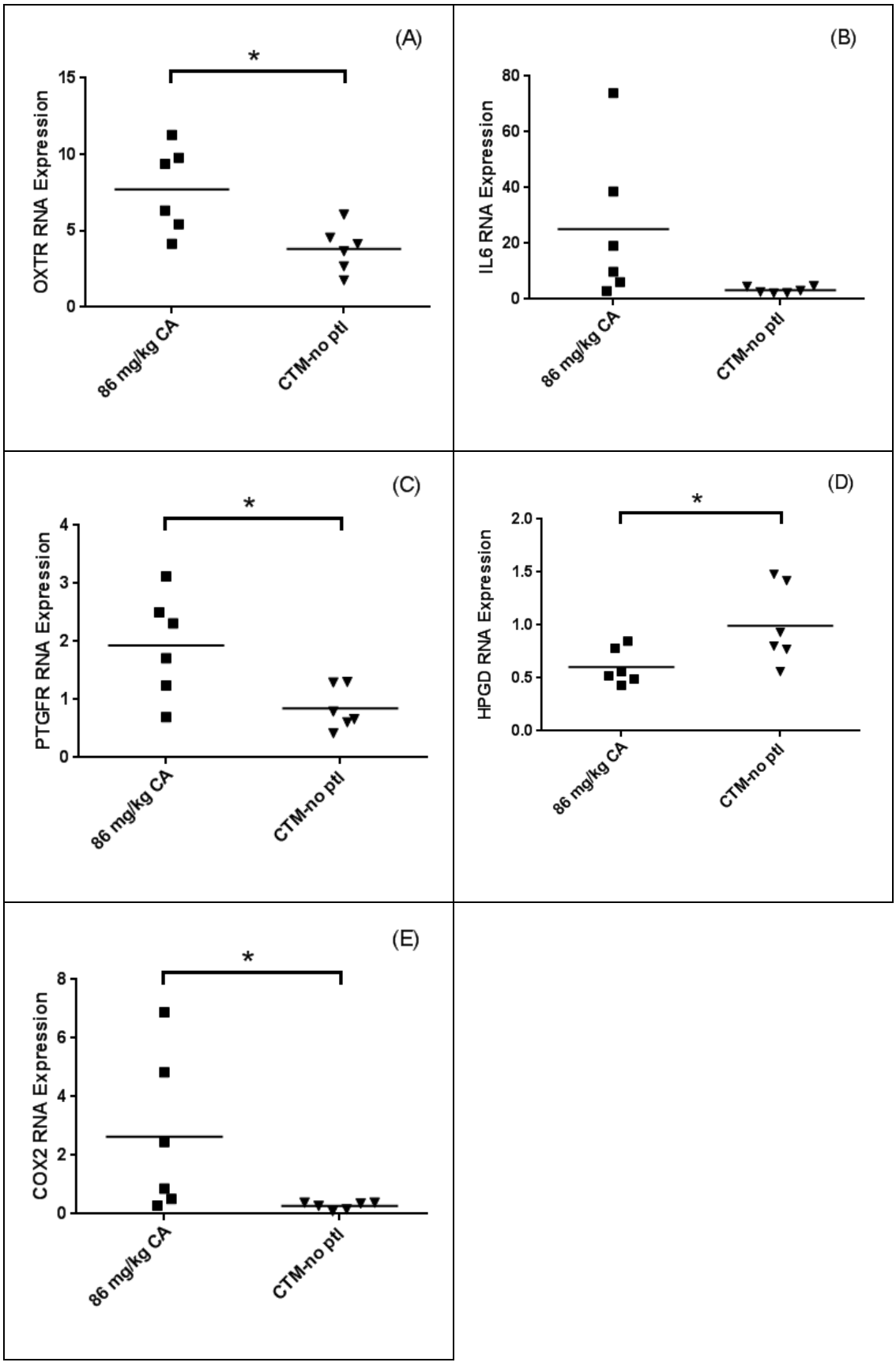
(A)
*
OXTR RNA Expression
15
10
5
0
86 mg/kg CA
CTM-no ptl
(B)
IL6 RNA Expression
80
60
40
20
0
86 mg/kg CA
CTM-no ptl
(C)
*
PTGFR RNA Expression
4
3
2
1
0
86 mg/kg CA
CTM-no ptl
(D)
*
HPGD RNA Expression
2.0
1.5
1.0
0.5
0.0
86 mg/kg CA
CTM-no ptl
(E)
*
COX2 RNA Expression
8
6
4
2
0
86 mg/kg CA
CTM-no ptl

Figure 7. Effect of CTM treatment on parturition-associated genes in uterus of CD1 mice.

CD1 mice treated with CTM and CA as above were used for mRNA analysis. Uterus were harvested during parturition or on day 18.7 and RNA was isolated using conditions stated in *Materials and Methods*. Endogenous OXTR (A), IL-6 (B), PTGFR (C), HPGD (D) and COX2 (E) expressions were analyzed and normalized to GAPDH. The data are presented as a fold change in relative expression, each data points representing individual mice. (*$p < 0.05$,**$p < 0.005$, ***$p < 0.001$, Welch's t-test).

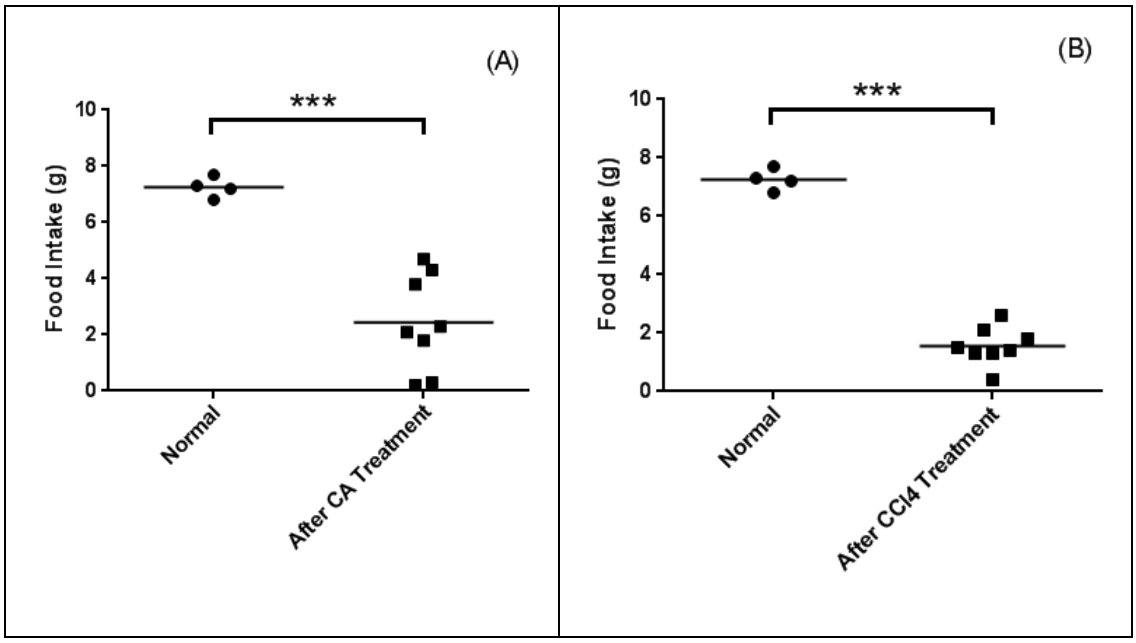

Figure 8. Effect of CA or CCl_4 treatment on food consumption.

Food consumption of CD1 mice treated with CA (A) or CCl_4 (B) were decreased. Each data points represent the diet intake of CD1 mice every 24 hours after CA or CCl_4treatment compared to normal consumption of pregnant mice. (***$p < 0.001$, Welch's t-test).

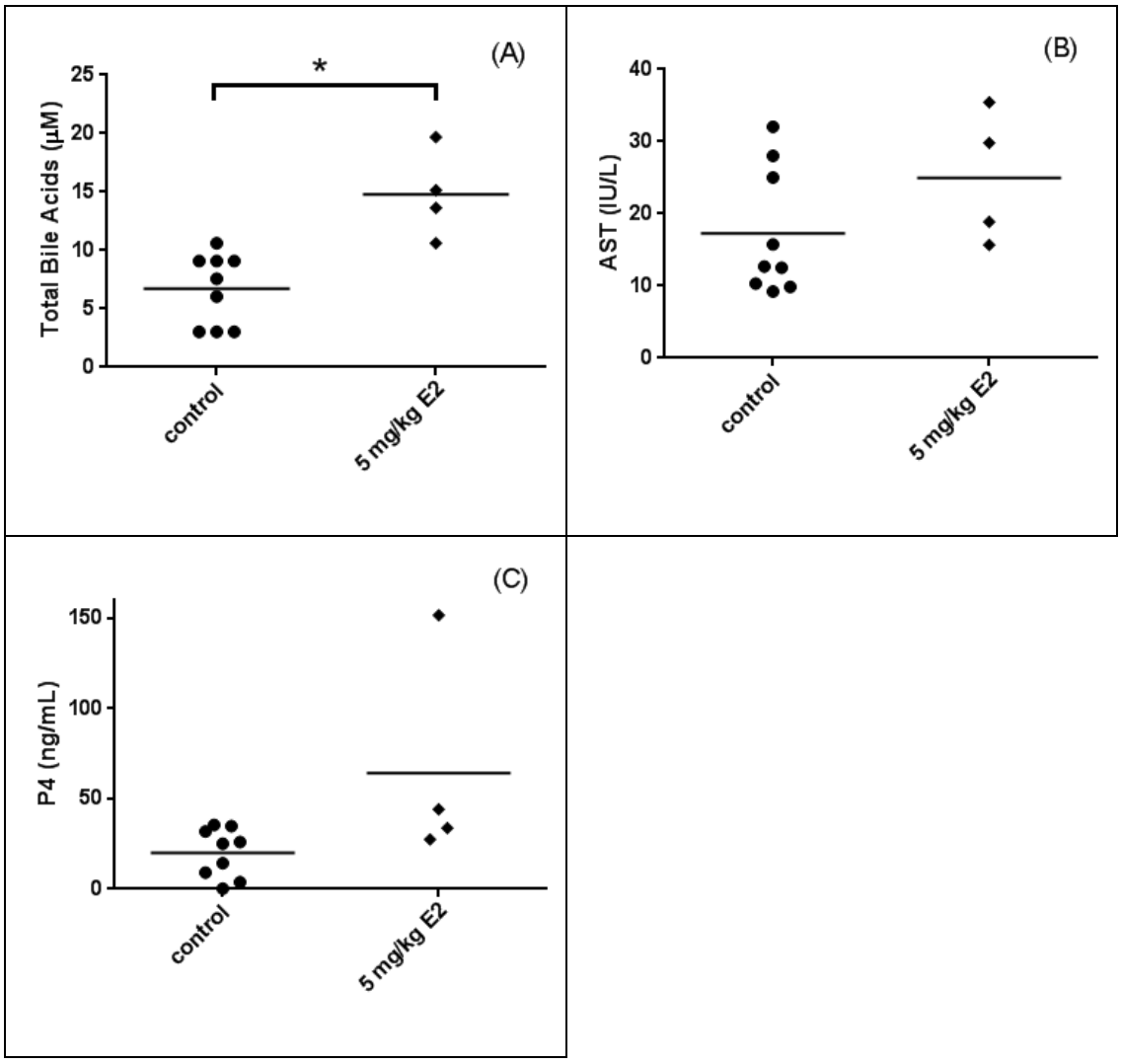

Figure 9. Effects of E2 treatment on plasma concentration of TBA, AST, and P4 in CD1 mice.

Pregnant CD1 mice were treated with 5 mg/kg E2 IP. Plasma samples were collected on day 18.7 for determination of TBA (A), AST (B) and P4 (C) concentration. (*$p < 0.05$, Welch's t-test).

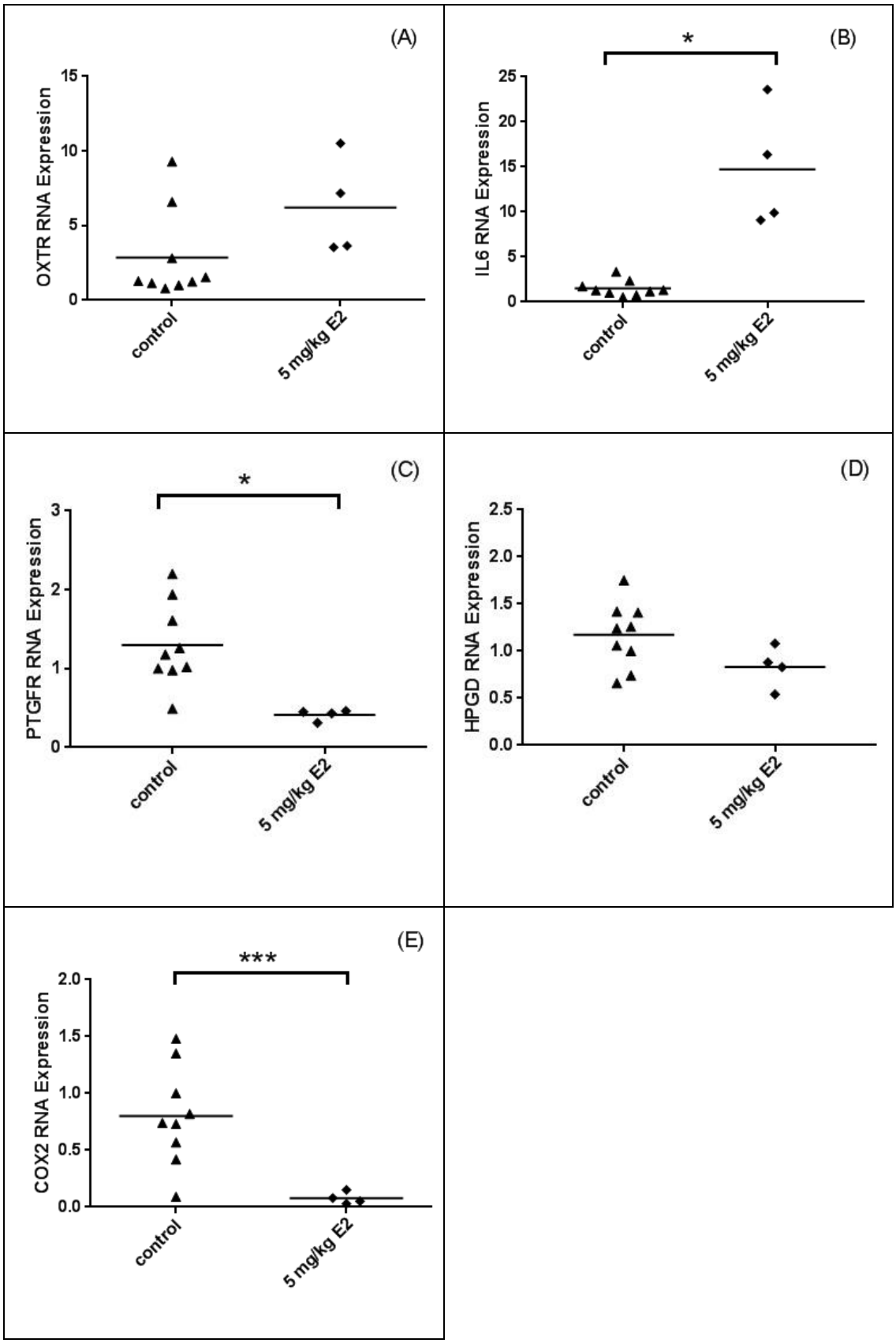
(A)
OXTR RNA Expression
15
10
5
0
control
5 mg/kg E2
(B)
*
IL6 RNA Expression
25
20
15
10
5
0
control
5 mg/kg E2
(C)
*
PTGFR RNA Expression
3
2
1
0
control
5 mg/kg E2
(D)
HPGD RNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
control
5 mg/kg E2
(E)
***
COX2 RNA Expression
2.0
1.5
1.0
0.5
0.0
control
5 mg/kg E2

Figure 10. Effect of E2 treatment on parturition-associated genes in uterus of CD1 mice.

CD1 mice treated with E2 were used for mRNA analysis. Uterus were harvested on day 18.7 and RNA was isolated using conditions stated in *Materials and Methods*. Endogenous OXTR (A), IL-6 (B), PTGFR (C), HPGD (D) and COX2 (E) expressions were analyzed and normalized to GAPDH. The data are presented as a fold change in relative expression, each data points representing individual mice. (*$p < 0.05$,**$p < 0.005$, ***$p < 0.001$, Welch's t-test).

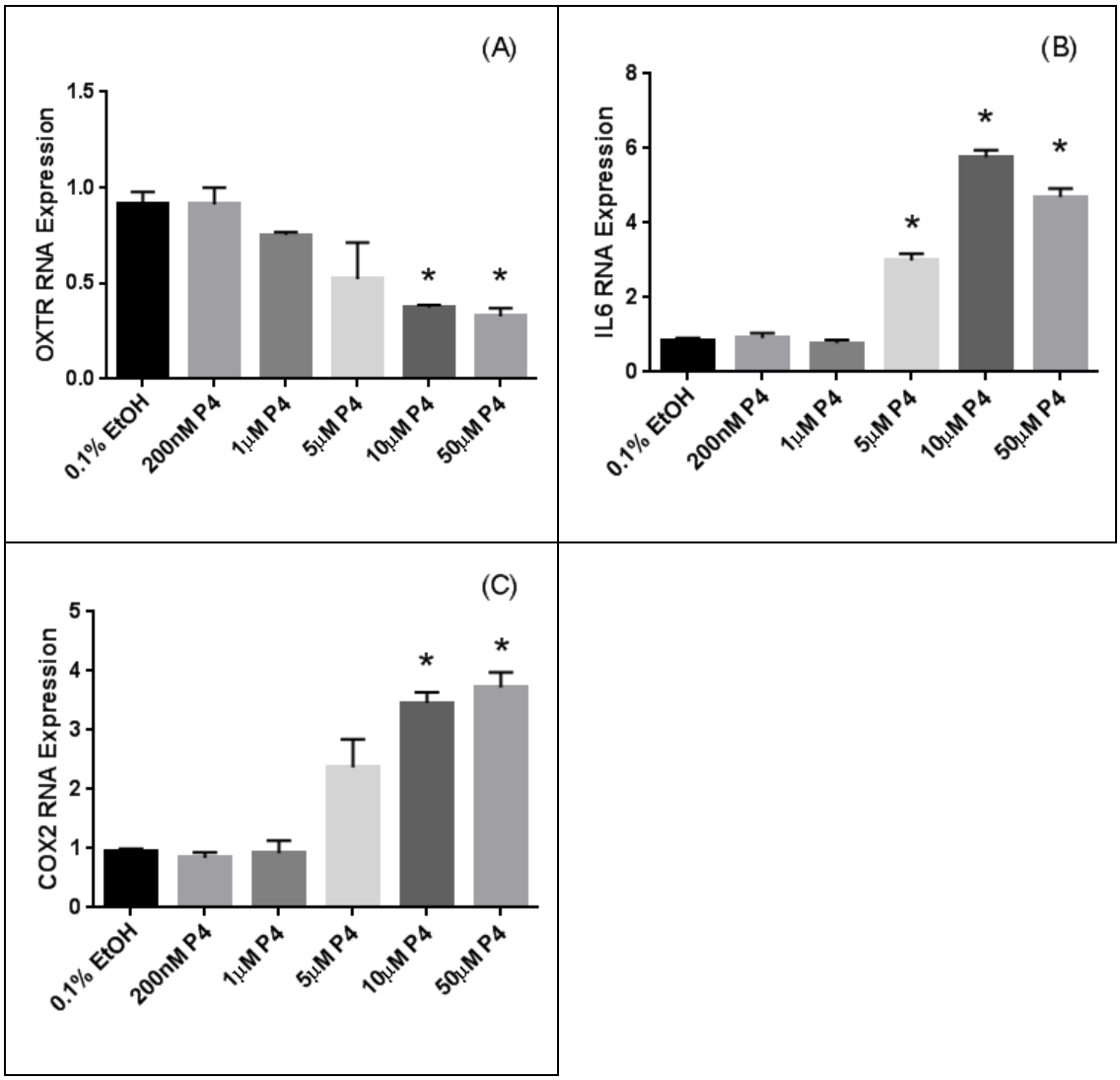

Figure 11. Effects of P4 treatment on RNA expression of OXT, IL-6 and COX2 in hTERT-HM cells.

hTERT-HM cells were treated with varying concentrations of P4 in ethanol or ethanol vehicle control. At 30 hours incubation, total RNA was isolated for RT-PCR quantification. The abundance of mRNA encoding OXTR (A), IL-6 (B) and COX2 (C) in hTERT-HM cells was measured and normalized against GAPDH. The data are presented as fold changes in relative expression of the mean of triplicates (*$p < 0.05$, Welch's t-test).

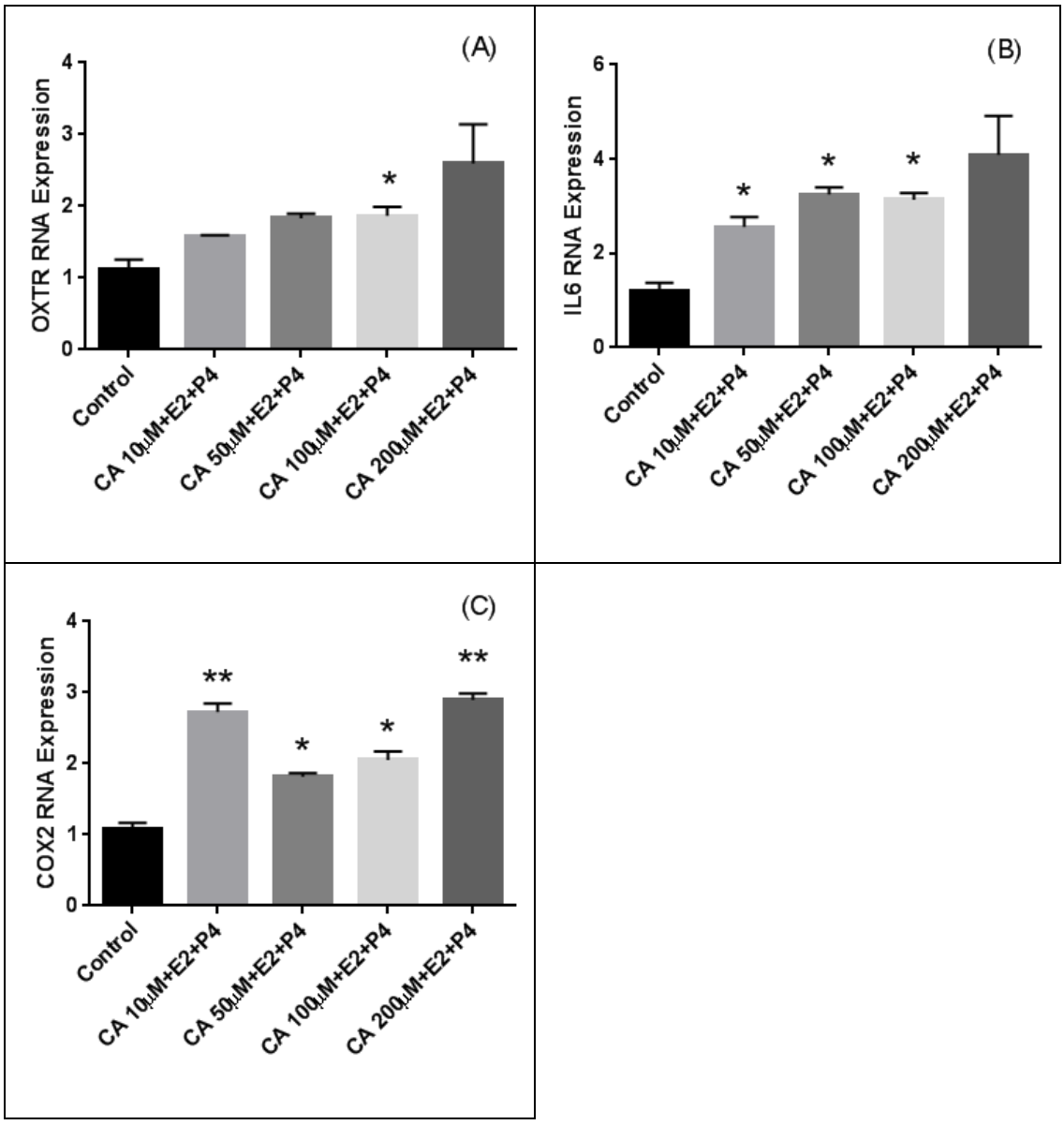

Figure 12. Effects of CA, E2 and P4 treatment on RNA expression of OXTR, IL-6 and COX2 in hTERT-HM cells.

hTERT-HM cells were treated with varying concentrations of CA in DMSO concurrently with consistent E2 and P4 dose in ethanol or DMSO and ethanol vehicle control. At 30 hours incubation, total RNA was isolated for RT-PCR quantification. The abundance of mRNA encoding OXTR (A), IL-6 (B) and COX2 (C) in hTERT-HM cells was measured and normalized against GAPDH. The data are presented as fold changes in relative expression of the mean of triplicates (*$p < 0.05$, Welch's t-test).

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