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Full Length Research Paper

Echinacea purpurea (L.) Moench attenuates lipopolysaccharide-induced inflammation in mice cervix

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Every minute approximately 1,400 babies are born prematurely world-wide and over 100 of these infants die. Causally, premature births are largely caused by infection-induced inflammation and current treatments are either unsafe or ineffective. Here, we test use of natural products [*Echinacea purpurea* (L.) Moench, root extract] (EP) with anti-bacterial and inflammatory activities and a long history of safe use to attenuate induction of inflammation in the cervix. Studies using three different complementary models, that is, non-pregnant in vivo, non-pregnant ex vivo and preterm labor models, were conducted. We also sought to decipher mechanisms likely to mediate EP's anti-inflammatory activities by blocking the activity of heme-oxygenase-1 (HO-1). Tissues were harvested and evaluated using real time-PCR, Western blot and/or histology. Here, we compare the suitability of the three models and show that EP attenuates the activity of the master inflammation transcription factor, nuclear factor kappa B (NFκB) (phosphorylated), and expression of select pro-inflammatory cytokines associated with inflammation-induced preterm labor. We also show that HO-1 may mediate EP anti-inflammatory activities in the cervix. These findings are significant as they provide important data that could potentially lead to the development of natural strategies for modulating infection-induced preterm labor.

Key words: Echinacea, cervical remodeling, preterm labor, Mice, lipopolysaccharide.

INTRODUCTION

Premature labor is a common and costly health care condition, the magnitude of which is staggering. Every minute more than 1,400 babies are born prematurely throughout the world and over 100 of these infants die (Beck et al., 2010). While the exact mechanisms that underlie cervical opening are not completely understood, it may be secondary to a microbial infection triggering the induction of inflammation (El-Bastawissi et al., 2000; Romero et al., 2006, 2009). Microorganisms may gain access to the amniotic cavity and fetus through many different routes, including ascension via the vagina, fetal membranes and amniotic cavity (Romero et al., 2006). The entry of lower genital tract bacteria into the decidua trigger the recruitment of leukocytes, notably via binding to toll-like receptors (TLRs), ultimately leading to an increase in cytokine production (Klein and Gibbs, 2004). TLRs recognize foreign invaders, including microorganisms, and this receptor system is believed to play a significant role in infection-mediated preterm birth (Challis et al., 2009). For example, TLR4 can be activated by lipopolysaccharide (LPS), a major cell wall component of gram-negative bacteria, as well as fragments of fetal fibronectin (Challis et al., 2009; Tsan and Gao, 2004). LPS can activate TLR4 and activate NFκB, a hallmark signal transduction pathway for innate immune responses (Chow et al., 1999; Da Silvera Cruz-Machado et al., 2010). NFκB is highly activated at sites of inflammation and can induce transcription of the pro-inflammatory cytokines involved in labor (Tak and Firestein, 2001). Current therapies for preterm labor are either unsafe to the fetus and/or mother, or are ineffective,

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so it is imperative that safe and effective therapies are developed to address this unmet medical need.

Plant extracts with a long history of safe use and well documented anti-microbial and anti-inflammatory activities, (EP) could potentially be used to attenuate inflammation-induced preterm labor in high-risk women (Barnes et al., 2005; Barret 2003; Goel et al., 2004; Shah et al., 2007). Interestingly, EP has been shown to decrease levels of some preterm labor-inducing or associated pro-inflammatory cytokines, such as TNFα, interleukin-1β (IL-1β), and IL-6, in non-reproductive tissues (Burger et al., 1997; Kim et al., 2002; Rininger et al., 2000), in part, by attenuating expression of a plethora of cytokines (Burger et al., 1997; Kim et al., 2002; Rininger et al., 2000; Zhai et al., 2007). It is, therefore, feasible to speculate that EP, which contains more than 19 anti-inflammatory bioactive compounds (Duke, 1992), could potentially be used to modulate infection-induced preterm labor. EP has been used for centuries by Native Americans for pain relief and wound treatment, as an antidote against various poisons, and for symptoms associated with the common cold (Barnes et al., 2005, Barret, 2003; Borchers et al., 2000; Goel et al., 2004; Shah et al., 2007). Recently, many of these claims have been confirmed experimentally using modern research technologies (Raso et al., 2012), including EP's, anti-cancer, anti-viral, antioxidant, and anti-inflammatory properties (Barrett, 2003; Raso et al., 2012). The current study focuses on EP's anti-inflammatory properties. For instance, EP has been shown to attenuate key inflammatory pathways, notably the NFκB signaling pathway, which results in the up-regulation of cyclo-oxygenase II (COX-II) and most of the other pro and occasionally anti-inflammatory cytokines (TNFα, IL-6, and IL-10) (Raso et al., 2012).

Because EP has been shown to decrease levels of preterm labor-inducing or associated pro-inflammatory cytokines in non-reproductive tissues (Burger et al., 1997; Kim et al., 2002; Rininger et al., 2000; Zhai et al., 2007), it is reasonable to speculate that EP could potentially be used to modulate infection-induced preterm labor.

Of the nine species of EP only three are used in herbal remedies, namely *Echinacea (E) angustifolia, E. pallida and E. purpurea,* referred to as EP from henceforth (Hobbs, 1989; McKeown, 1999). One possible factor that mediates EP's anti-inflammatory activities, among others, is heme-oxygenase 1 (HO-1) in the liver (Otterbein et al., 2003).

In the present study, we test the effectiveness of a whole hydro-ethanolic root extract of EP to attenuate lipopolysaccharide (LPS)-induced expression of some of the key pro-inflammatory factors associated with precocious cervical remodeling during preterm labor using three mice models, namely in vivo, ex vivo and preterm labor models. Our primary hypothesis is that EP can be used to modulate inflammation-induced precocious cervical remodeling. Here, we focus on investigating EP's ability to attenuate the expression of pro-inflammatory factors in the cervix and their likely underlying mechanisms.

MATERIALS AND METHODS

Animals Used in the Study

Mice from Charles River, strain C57BL6/129SvEv, were used in the present study (*n*=3-7) for all the experiments described below, including EP optimization (dose, route, frequency, duration) using the ex vivo and in vivo models and in mechanism studies, as described below. All animals used in *ex vivo* and mechanism (ex vivo + in vivo) studies were ovariectomized non-pregnant mice, whereas in vivo studies included both ovariectomized non-pregnant and preterm labor mice models (day 15 pregnant animals treated with LPS).

Animals were housed under constant room temperature (21°C), with a 12:12h light and dark cycle and had free access to water and feed. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the local institution (Appalachian State University) and the NIH guidelines (NIH publication number 86-23), and efforts were made to minimize both animal suffering and numbers of animals used**.** After their respective treatments and prior to tissue harvest, mice were administered a lethal dose of sodium pentobarbital (Sleep away®, Fort Dodge Laboratories Inc., Burlingame, CA), immediately followed by trans-cardio perfusion using 0.9% normal saline solution.

No trans-cardio perfusions were performed for ex vivo studies. All uterine cervical tissues were carefully harvested under a stereomicroscope, to avoid tissue contamination from vaginal or uterine tissues, and the tissues were then processed and analyzed appropriately using various techniques, including Western blot analysis and real time polymerase chain reaction (real time PCR), as described below. Immediately following tissue harvest, tissues for ex vivo studies were placed in 48 well plates containing fresh media (RPMI 1640 supplemented with 10% Fetal Bovine Serum) and incubated, as described below.

Surgery: Ovariectomies (ovary removal)

Before treatments or tissue harvest, sexually mature non-pregnant mice ~ 6 weeks old, weighing between 30-40g, were ovariectomized. Prior to surgery, animals were anaesthetized using a mixture of ketamine (43-129 mg/g body weight) and xylazine (8.6-26 mg/g body weight), followed immediately after surgery by administration of Baytril® antibiotic (Bayer, Leverkusen, Germany), to prevent post-surgery infections. Animals were then allowed

to rest for seven days post-surgery before performing experiments, to allow removal of residual ovarian sex steroid hormones, confirmed during tissue harvest by significant reduction in uterine size. Animals with normal uterine size, seven days after ovariectomy, were eliminated from the study.

Optimization Studies and Treatments

Based on previous studies, conditions were optimized for non-pregnant and pregnant in vivo models. The following conditions were found to be optimal and used in subsequent non-pregnant in vivo experiments: EP was given at a dose of 1 mg/mouse via intra-peritoneal (IP) injection route, followed by LPS administration at 100 µg/mouse four hours after EP dose, and one hour prior to cervical tissue harvest (see illustration in Figure 1). After treatment and prior to cervical tissue harvest, animals were euthanized with sodium pentobarbital (Sleepaway®, Fort Dodge Laboratories Inc., Burlingame, CA).

Pregnant animals from day 15 of pregnancy were used and induced with preterm labor, a slight modification of the previously reported animal model for preterm labor - 250 µg of intra-uterine LPS (Elovitz and Mrinalini, 2005). Preliminary studies in our lab also yielded optimal conditions for preterm model treatments, and were found to be as follows: initial injection of EP (1 mg/mouse) at time 0h, with boosters at times 4h and 8h, respectively, with animals euthanized at time 11h post initial EP injection (see Figure 2).

LPS was administered 1h after the last booster of EP, that is, time 9h. After treatment and prior to cervical tissue harvest, animals were euthanized with sodium pentobarbital (Sleepaway®, Fort Dodge Laboratories Inc., Burlingame, CA).

Development of Ex Vivo Model

The rationale for using the ex vivo model, which was an entire cervical tissue excised from a non-pregnant ovariectomized mouse, was to study EP's anti-inflammatory activities under a more controlled environment in a 48 well micro titer plate in which treatment conditions could be performed without interference from endogenous ovarian sex steroid hormones. With this being the first study of its kind, the development of the ex vivo model and the subsequent treatments had to be optimized, and were performed as follows:

Determination of optimal media for ex vivo studies

Two types of media were tested in order to determine the optimal medium for incubating ex vivo uterine cervical tissues. These included: HyClone RPMI [1640 1X with 2.05 ml L-glutamine (Thermo Scientific)], supplemented with 10% fetal bovine serum (LonzaBiowhittaker) and HyClone DMEM [High Glucose with 4.00 mM L-glutamine and 4500 mg/L glucose and sodium pyruvate (Thermo Scientific)], also supplemented with 10% fetal bovine serum (FBS) (LonzaBiowhittaker). The 0.1M PBS buffer only-treated group was used as a negative control. Harvested tissues were briefly rinsed in cold 0.1M PBS buffer and immediately placed into their respective wells in a 48-well plate, with each well either containing 250 µl of: **a**). 0.1M PBS, **b**). DMEM, or **c**). RPMI. The plates were incubated for 24h in HERA cell 150i CO_2 incubator (set at 5% CO_2) at 37°C (Thermo Scientific). At the end of the 24h period, all tissues were harvested and either stored at -80°C for molecular analysis (real time PCR and Western blot analysis) or fixed in 10% formalin for histological analysis. RPMI media was found to be the optimal medium, based on the morphological and molecular parameters, and thus was used in subsequent studies.

Determination of optimal duration for tissue integrity, viability and survival in ex vivo model

Animals were divided into six treatment groups (*n*=1) and uterine cervical tissues were harvested, briefly rinsed in 0.1M PBS buffer and incubated, based on the six treatment groups in the 48-well plates, as described earlier. Uterine tissues were incubated for: 1h, 4h, 8h, 12h, 16h or 24h, harvested and either fixed in 10% formalin for histological analysis or stored in -80°C for molecular analysis.

Determination of optimal dosage and time for EP and LPS

Uterine cervical tissues were harvested from mice, then the optimal time and dosage for EP and LPS treatments (*n*=3) for the *ex vivo* model were determined. To determine the optimal incubation time, tissues in the negative (only vehicle, i.e., 0.1M PBS buffer) and LPS alone (LPS, 1µg per well) control groups were incubated in the 48 well plates for 6h, 12h and 24h. Tissues were then harvested and stored at -80°C, processed for and examined using real-time PCR and Western blot analysis. The 6h incubation was determined to be the optimal duration, based on the molecular data and was, therefore, used in section 2 (optimal dosage).

To determine the optimal dosage for the incubations, tissues were incubated for 6h under varying treatment conditions. There was an EP only group (0.1 mg/well), and a dose response group, namely EP [0.01 (low), 0.1 (medium) or 1.0 (high) mg/well)] **+** LPS (1 µg per well), add-

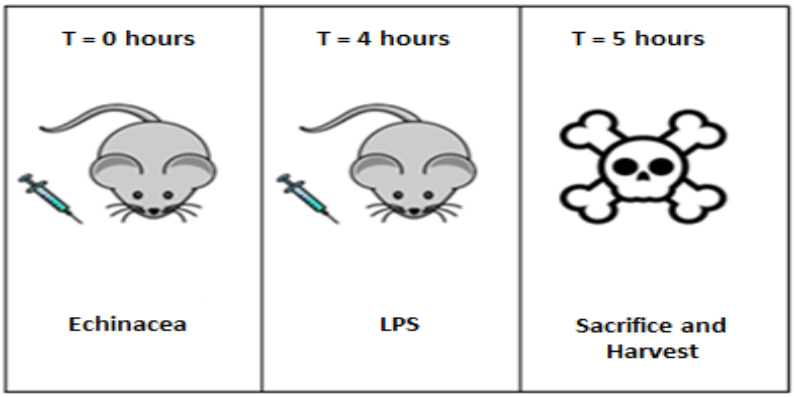

Figure 1. Illustration showing schedule of injections for non-pregnant, in vivo model.

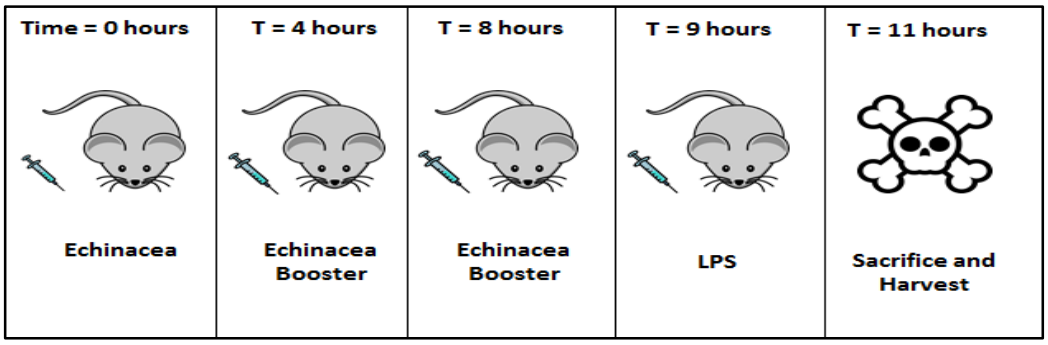

Figure 2. Illustration showing schedule of injections for preterm labor model.

ed an hour later, as well as a negative control (media only) and an LPS only group (1 µg per well). Tissues were then harvested 6h post LPS treatment, after which they were stored at -80°C, and examined later using real-time PCR and Western blot analysis.

For all subsequent *ex vivo* model experiments, the following optimal parameters were utilized: 6 h incubation, 1 µg of LPS per well and 1.0 mg of EP per well. This model was developed with assistance from Dr. Maryam Ahmed.

Mechanism of Action (In vivo non-pregnant mice)

In order to determine whether HO-1 mediates EP's anti-inflammatory activities in the cervix of mice treated with LPS, the HO-1 inhibitor zinc protoporphyrin, (ZnPP) was used to test if it can attenuate HO-1's anti-inflammatory mediatory actions in a dose-dependent manner. Mice were divided into six treatment groups, with all treatments administered via IP, in 50µl per mouse (*n*=3), as described here: negative control (ZnPP vehicle only, that is, only 1% DMSO); LPS only (100 µg LPS), ZnPP only control (0.125 mg/mouse**)**, low ZnPP + EP + LPS [ZnPP, 0.0125 mg/mouse; EP, 1 mg/mouse; LPS,100 µg of LPS per mouse], medium ZnPP + EP+ LPS [ZnPP, 0.125 mg/mouse; EP and LPS dosages, same as in sections above], high ZnPP + EP+ LPS [ZnPP, 1.25 mg/mouse; EP and LPS dosages, same as described above]. Treatment groups that received EP were administered first with EP extract (one injection), as well as ZnPP (one injection), followed two hours later by LPS (100 µg/mouse) and two hours post-LPS injection, the mice were euthanized. Cervical tissues were then harvested and stored at -80°C, and examined later using Western blot analysis.

Techniques Used in the Study

Harvested tissues were processed appropriately and analyzed using gene (real time PCR, qRT-PCR) and protein (Western blot) expression techniques, as well as basic histology (H&E staining), as described below:

Gene expression studies (real time PCR)

Gene expression analysis was performed using qRT-PCR to determine the extent to which EP influenced mRNA

Model	Advantages	Disadvantages
Non-pregnant (*in vivo*)	• Semi – controlled conditions, hormone free	• Not pregnant • Residual hormones (e.g. adrenal) • Non-hormonal factors (e.g. VEGF)
Preterm (*in vivo*)	• Simulates PTL	• Confounding factors (e.g. pregnancy hormones)
Non-pregnant (*ex vivo*)	• Ideal for deciphering mechanisms	• Does not reflect preterm

Figure 3. Comparison of three mice models.

expression of pro-inflammatory factors, such as IL-6 and COX-II, as well as HO-1 in the cervix of both pregnant and non-pregnant mice. Gene expression analysis was performed in three steps, as described below:

Tissue processing, messenger RNA isolation and quantification

Following treatments, animals were euthanized and trans-cardially perfused with normal saline (0.9% sodium chloride). The cervices were harvested immediately, snap-frozen and either processed or stored at -80°C until processing. Total RNA was isolated from individual cervices using the RNeasy Mini Kit (Qiagen, Valencia, CA) and then the quality and quantity of each sample was estimated using Nanodrop Spectrophotometer (NanoDrop 3000, Thermo Scientific). Aliquots of total RNA were diluted in RNase-free deionized (DI) water and either stored at -80°C or processed for reverse transcriptase PCR.

Reverse transcriptase PCR (RT-PCR)

Total RNA from the cervical tissue was reverse-transcribed and amplified in an Eppendorf Master Cycler (Hamburg, Germany) using reagents from Applied Bio-systems (Foster, CA). For generation of complementary DNA (cDNA), 1.0 µg of previously isolated total RNA was placed in a total volume of 9.5 µL per sample with RNase-free water, as determined by Nanodrop Spectrophotometer. The RNA was incubated for 5 minutes at 65°C and cooled to room temperature for 10 minutes. During the cooling period, 9.5 µL of a reverse transcriptase master mix was added to each tube, which was comprised of the following: reverse transcriptase buffer (2 µL per tube of RNA; Applied Biosystems, Foster, CA); $MgCl_2$ (2 µL per tube of RNA; Applied Biosystems, Foster, CA), dNTP (2 µL per tube of RNA; Applied Biosystems, Foster, CA); RNase inhibitor (0.5 µL per tube of RNA; Applied Biosystems, Foster, CA); RNAse-free water (2µL per tube of RNA; Applied Biosystems, Foster, CA); and random hexamers (1 µL per tube of RNA; Applied Biosystems, Foster, CA). Lastly, 1.0 µL of MuLV reverse transcriptase (Applied Biosystems, Foster, CA) was added to each tube. One tube did not receive reverse transcriptase enzyme, and therefore served as a non-template control for DNA contamination. The Thermocycler was programmed to run at 25°C for 10 minutes, 42°C for 2 hours, 95°C for 5 minutes, and stored at 4°C. The generated total cDNA was then used to evaluate mRNA levels of the genes of interest.

Real-time PCR (qRT-PCR)

Relative expressions of the genes of interest [IL-6, TNF, COX-II] were evaluated using qRT-PCR. TaqMan® Gene Expression Assays (Applied Biosystems, Foster, CA), which are pre-designed and pre-optimized gene-specific probe sets, were utilized and DNA amplification was performed using the Applied Biosystems qRT-PCR machine (ABI 7300 HT) with the GeneAmp 7300 HT sequence detection system software (Perkin-Elmer Corp.) The PCR reactions were set up in wells of 96-well plates in a volume of 25 µL per well. The reaction components included: 1000 ng (5.0 µL) of synthesized cDNA; 12.5 µL of 2X Taqman® Universal PCR Master Mix; 1.25 µL of 20X Assays-on-Demand™ Gene Mix (e.g. TNFα); and 6.25 µL of qRT-PCR-grade RNAse-free water. The program was set as follows: an initial step of 50°C for 2 min and 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 60 s. The relative amount was calculated from the threshold cycles with the instrument's software (SDS 2.0), according to the manufacturer's instructions. Relative expression levels of the target genes

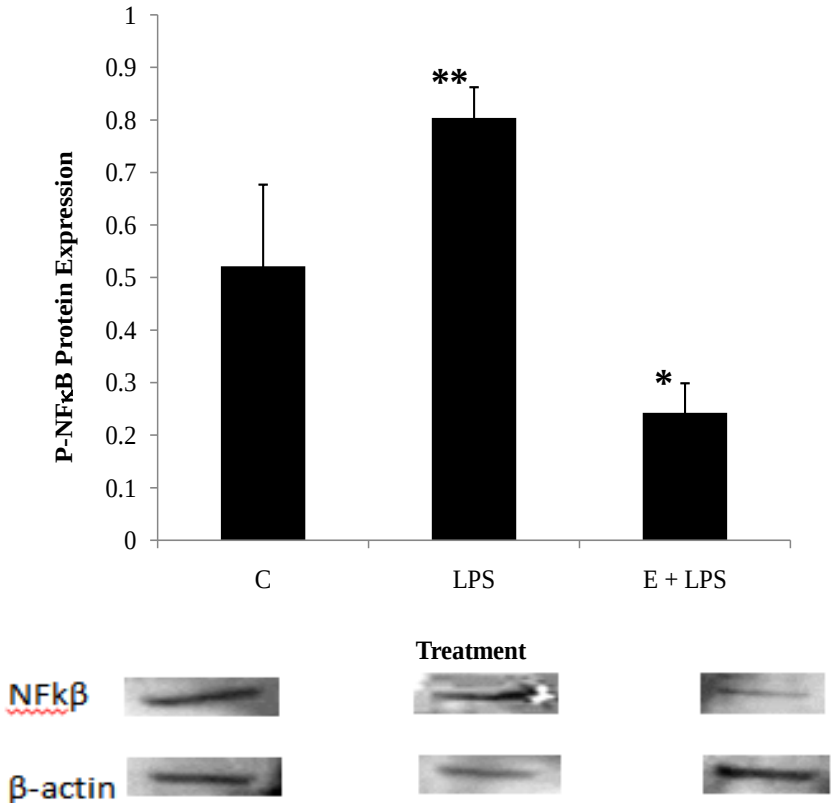

Figure 4. Effects of EPon phosphorylated NFκB in the cervix of*in vivo* non-pregnant ovariectomized mice, as revealed by protein expression studies (Western Blot). EPadministered IP down-regulated levels of p-NFκB protein. Negative control (C) =vehicle only, 0.9% NaCl, IP; LPS alone = 100 µg, IP and EP=1.0 mg/mouse, IP. *n*=3,** *p*< 0.05 LPS vs. C; **p*< 0.05 E + LPS vs. LPS. β-actin was used as a normalizer.

were normalized to the geometric mean of the endogenous control gene, Gusβ.

Protein expression studies (Western blot)

Protein expression studies were performed using Western blot for quantification of the proteins of interest in order to determine the extent to which EP influences the protein expression of various key pro-inflammatory factors (IL-6 and COX-II), as well as activity of phosphorylated NFκB and HO-1 in the uterine cervix of mice with or without LPS-induced inflammation, as described below:

Protein Extraction

Protein was extracted from cervical tissue using CellLytic™ M Cell Lysis Reagent (Sigma Aldrich) and protease inhibitor cocktail (Sigma Aldrich). Tissues were weighed individually, with lysis buffer calculated per tissue (1 g tissue: 20 ml buffer). Tissues were placed into pre-chilled conical tubes and mechanically homogenized in the buffer for 1-1.5 minutes, washing the homogenizer with distilled water in between uses. Once tissues were homogenized, tubes were centrifuged for 10 minutes at 20,000 x g at 4°C and the supernatant was transferred to pre-chilled eppendorf tubes and stored in -80°C freezer until ready for use.

Quantitative analysis of protein expression for the proteins of interest was performed as described here

Total protein was isolated from tissues and concentration determined, as described earlier. Ten (10) µg of each protein sample was run on gel electrophoresis at 125V for 90 min. Proteins were then transferred to PVDF membrane and incubated in blotto (5.0g non-fat dry milk and 100ml of 1xTBST) at 4°C. Membranes were stained with specific primary antibodies at 1: 500 dilution, according to the manufacturer and incubated overnight at RT. Membranes were then washed 3 times, 5 min each, with 1x TBST, prior to incubation with secondary antibody, that is, Donkey anti-rabbit IgG HRP conjugated and streptactin HPR

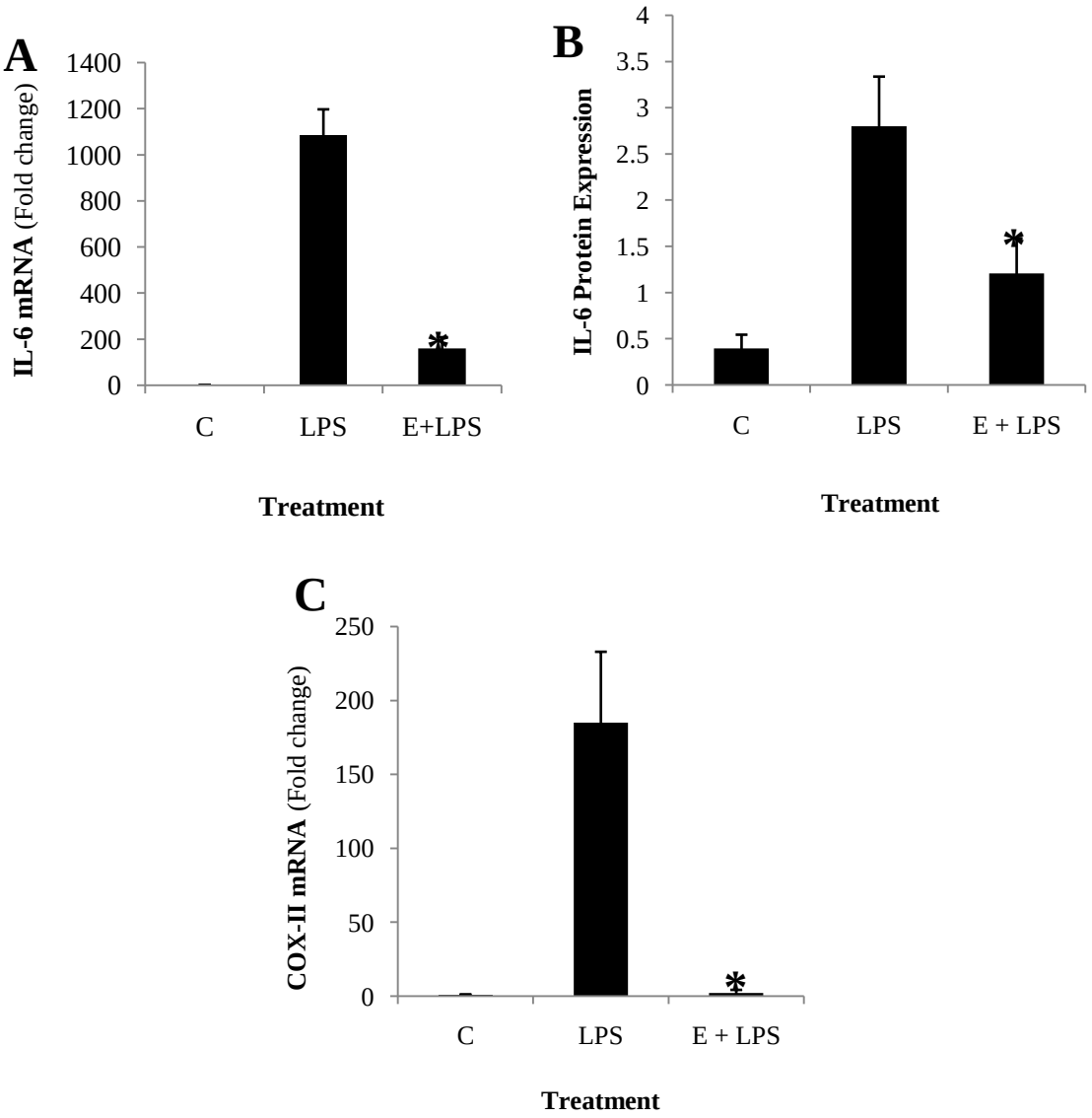

Figure 5. Effects of EPon expression of IL-6 mRNA and protein, as well as COX-II mRNA in the cervix of preterm labor mice. **A**) IL-6-mRNA and **B**) IL-6-protein, as revealed by real-time PCR (qRT-PCR) and Western blot analysis. EPadministered 2 times, IP, has a down-regulating effect on IL-6 mRNA levels and IL-6 protein expression compared to LPS only. *n*=3; * *p*< 0.05 LPS vs. C; ***p*< 0.05 E + LPS vs. LPS. β-actin was used as a normalizer in Western blot. **C**) COX-II mRNA expression, as revealed by real-time PCR (qRT-PCR). EP administered twice, IP, and has a down-regulating effect on COX-II mRNA levels compared to LPS only. *n*=3; **p*< 0.05 E + LPS vs. LPS.

antibodies for 1 hour at RT. Membranes were again washed 3 times, 5 min each, with 1xTBST, and the final wash was performed with 1xTBS. Finally, the membranes were then incubated for 5 min with luminescence-producing enzyme, namely limunol-enchancer/peroxidase solution and developed. Images were analyzed with ImageJ program (NIH). The specific bands of the proteins of interest were identified using a standard ladder with known molecular weight.

Basic Morphological Studies (Hematoxylin and Eosin staining, H & E)

Experiments were undertaken to examine the basic histology of uterine tissues following various treatments described earlier. Frozen sections were stained with the standard H & E staining procedure (VWR international LLC, USA) and imaged using Olympus DSU IX81 (Olympus, USA) to examine the overall tissue structure, their

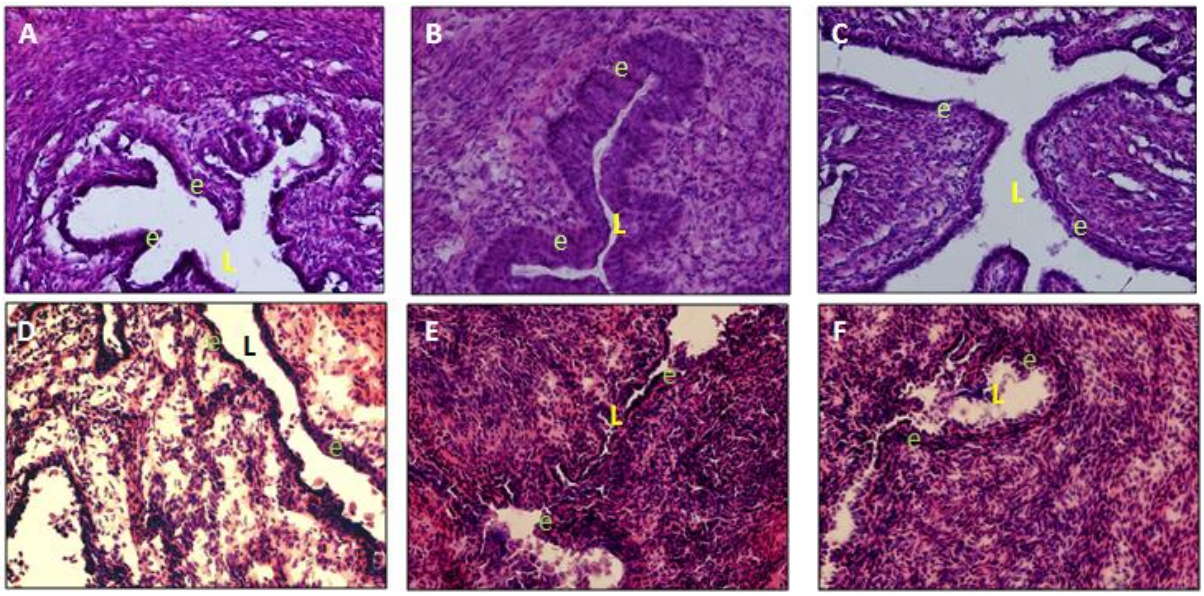

Figure 6. Histomorphogical profile of the cervix of non-pregnant ovariectomized mice treated *in vivo* (**A**-**C**) and *ex vivo* (**D**-**F**) mice treated with EP, as revealed by H & E stain. *In vivo* model: Tissues in EP+ LPS-treated animals (IP), closely resembles histology of negative but not LPS alone group. **A**) Negative Control (vehicle only, 0.9% NaCl, IP); **B**) LPS alone (100 µg, IP); **C**) EP (1 mg/mouse) + LPS (100 µg, IP). *Ex vivo* model: As in the *in vivo* model above, EP-treated cervices incubated in a 48-well plate resemble the histology of the negative control, but not the LPS alone treatment group. **D**) Negative Control (media only, 1640 RPMI); **E**) LPS alone (1 µg/well); **F**) EP (1 mg/well) + LPS (1 µg/well). All images were taken at 20x. "e" =epithelia, while "L"=cervical lumen.

characteristics and cellular subpopulations and blood vessels.

Statistical Analysis

Data were analyzed using Student's *t* test and ANOVA (single factor). *p*-values equal to or less than 0.05 were considered to be statistically significant.

RESULTS

Animal models

In order to determine whether EP inhibits infection-induced inflammation in the cervix, three animal model systems were utilized, including a non-pregnant ovariectomized in vivo model, a pre-term labor model and an ex vivo model. These animal models are complementary, with the advantages and disadvantages of each model highlighted in the Figure (see Figure 3).

EP suppresses the activity of NFκB in the cervix of non-pregnant in vivo model system

Here, we investigated the effects of EP on the levels of activated (phosphorylated) NFκB. The results from this experiment show that animals treated with 0.9% NaCl alone exhibited baseline levels of p-NFκB, whereas LPS-treated animals (LPS 100 µg, IP) showed a significant increase in p-NFκB levels compared to control. EP administered prior to LPS treatment, revealed a 4 fold (p= 0.0256) decrease in p-NFκB protein levels compared to LPS alone, showing that p-NFκB levels were significantly inhibited by EP compared to LPS alone (Figure 4).

EP down regulates expression of pro-inflammatory factors (COX-II and IL-6) in the cervix of preterm labor mouse model

Having shown EP's ability to suppress NFκB activity in the non-pregnant in vivo model, we wanted to test whether the preterm in vivo model would yield similar results when all confounding factors – sex steroid hormones, adrenal hormones, and adipose tissue – were present. Since IL-6 levels in preterm labor increase, we sought to investigate whether EP could suppress LPS-induced levels of IL-6 mRNA and protein, in our preterm labor in vivo model. We also looked at COX-II mRNA because COX-II is responsible for the conversion of arachidonic acid to prostaglandins, which stimulates myometrial contractions and ultimately ripening of the uterine cervix. Here, we show that EP, sharply inhibits IL-6 mRNA and protein levels by 11 (p= 0.00019) and 2.2 folds (p=0.0297), respectively (Figure 5a-b). We also show

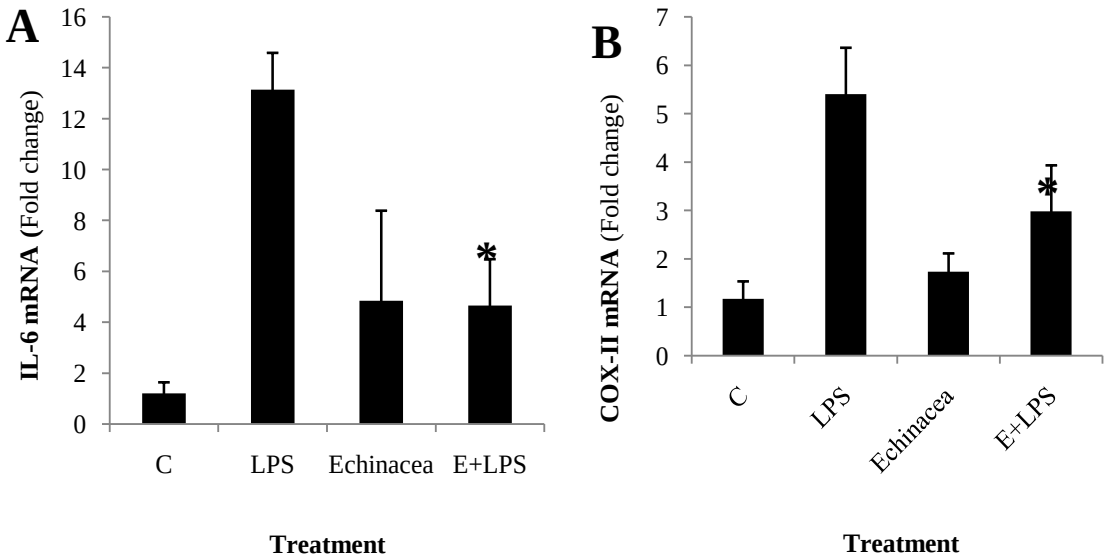

Figure 7. Effects of EP on **A**) IL-6 and **B**) COX-II mRNA expression in the mice cervix of the *ex vivo* model (non-pregnant ovariectomized), as revealed by real time-PCR. EP has a down-regulating effect on IL-6 and COX-II mRNA levels compared to the LPS alone group. Negative Control (vehicle only, RPMI 1640); LPS alone (LPS 1µg per well); EP only (0.1 mg/well); optimal dose of EP high (1.0mg/well) + LPS (1µg per well).*n*=3,* *p*< 0.05 E+LPS vs. LPS.

that EP robustly inhibits COX-II mRNA levels by almost 200 fold (*p*= 0.000258) (Figure 5).

Tissue integrity, viability and the anti-inflammatory activities profile of EP in the ex vivo model system

Having characterized some of EP's anti-inflammatory properties both in non-pregnant and pregnant in vivo models, we wanted to develop a model that could potentially eliminate the confounding factors seen in both in vivo models (sex steroids, adrenal hormones, adipose tissues). This led to the development of an ex vivo model, in which the entire cervical tissue was excised from the animal post-ovariectomy, with all treatments conducted in a microtiter plate. In this particular experiment, we first wanted to make sure the ex vivo model was a suitable model for evaluating EP's anti-inflammatory effects on cervical tissue, so we compared the cervical histomorphology of hematoxylin and eosin (H&E) stained tissues from non-pregnant in vivo and ex vivo models. Comparisons were based on tissue integrity and the cell types (epithelial cells, stromal cells, +/- immune cells) present. We show the presence of epithelial and stromal cells, indicative of tissue structure. We also show that the EP and LPS treated tissues look more like the negative control than the LPS only treated group, showing support for EP combating the effects of LPS on these tissues (Figure 6A-F).This is also demonstrated in Figure 6D-F, to a lesser degree, however, since EP + LPS-treated tissues appear similar to negative control than the LPS-only treated group. Basically, we show here that the integrity of structure remains intact in all samples, and the treatment with EP + LPS is more comparable to the negative control than the LPS-only group.

After confirming the proof of principle for the ex vivo model, showing that tissues remain viable after being removed from the body, we wanted to test EP's ability to decrease LPS-induced levels of IL-6 and COX-II in the ex vivo model, and compare the results to what we saw in the preterm in vivo model. The experimental treatments were as described before, except addition of a group treated with EP only, to study the effects of EP alone compared to control or baseline levels of IL-6 and COX-II mRNA. We show that EP significantly decreased LPS-induced IL-6 mRNA levels compared to LPS alone by 2.6 fold, with no statistical difference between the EP-alone treatment and the EP and LPS treated groups (Figure 7a). The same trend was noted with COX-II mRNA levels, with a fold change of 1.8 (Figure 7b). No significant difference was noted between EP alone group and the negative controls, for both pro-inflammatory factors.

Next, we sought to investigate whether EP could diminish p-NFκB levels in an ex vivo model. Here, we show that EP inhibits LPS-induced activation of NFκB, in a dose-dependent manner, with the medium and highest concentration of EP decreasing p-NFκB levels 2 and 3 fold, respectively, compared to LPS alone (*p*=0.0287) (Fig-

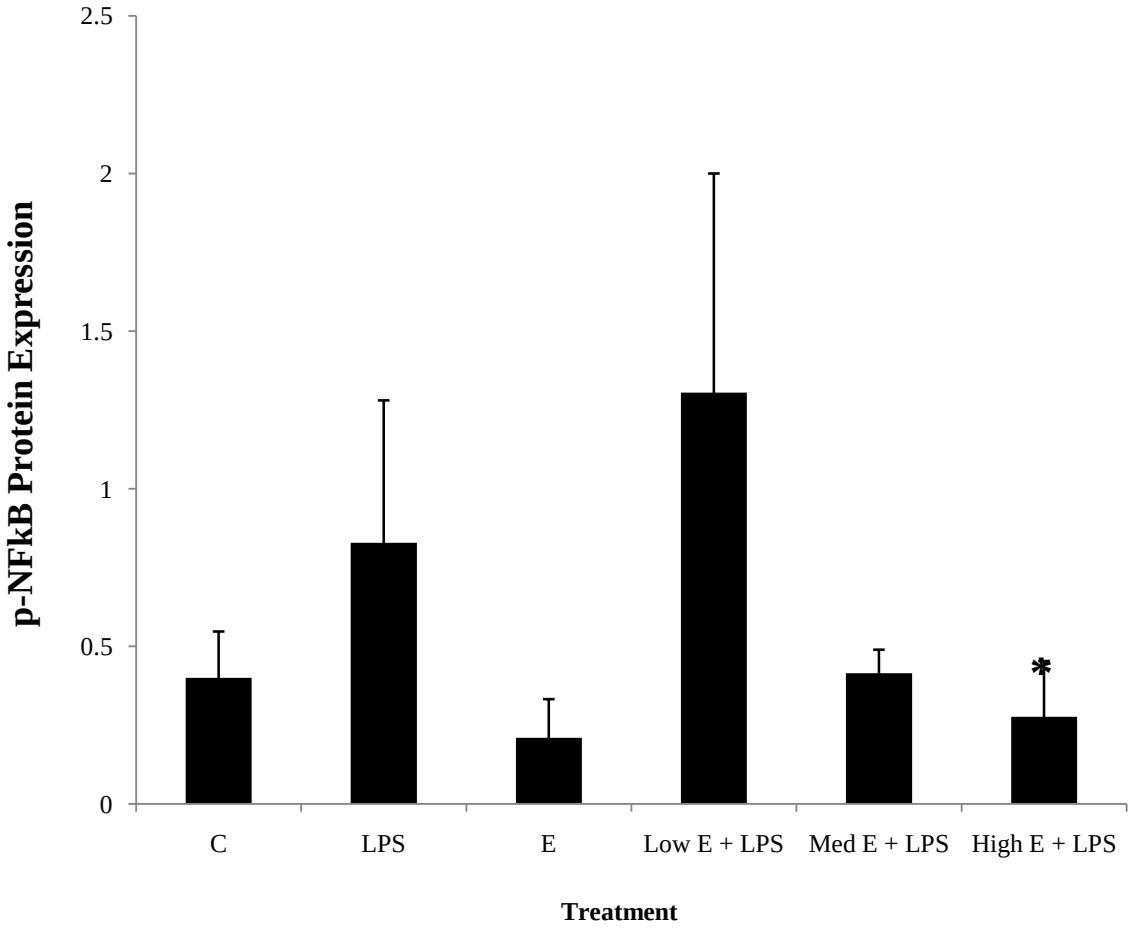

Figure 8. Effects of EP on phosphorylated NFκB in the cervix of *ex vivo* non-pregnant ovariectomized mice. EP down regulates the activity of NFκB, in the mice cervix of the *ex vivo* model, dose-dependently, as revealed by Western blot analysis. Negative control=vehicle only (RPMI 1640); LPS only = 1µg per well; EP only=0.1 mg/well; and EP [low=0.01mg/well; medium=0.1mg/well; high=1.0 mg/well] + LPS (1µg per well). *n*=3,* *p*< 0.05 High E+LPS vs. LPS; β-actin was used as a normalizer.

ure 8). We chose to use the highest dose; however, as these findings were consistent with our earlier observation in the non-pregnant in vivo model (Figure 8).

EP shows a down-regulatory effect on HO-1 protein (preterm *in vivo* model) and HO-1 mRNA (non-pregnant ex vivo model)

We investigated EP's effects on HO-1 protein and mRNA using both the preterm in vivo and non-pregnant ex vivo models, respectively. We show in the preterm in vivo model that EP: a) decreased HO-1 protein levels compared to LPS alone and b) combined with LPS significantly elevated levels of HO-1 protein compared to the negative control (Figure 9A). We observed similar results from our non-pregnant ex vivo model, showing EP: **a**) decreases HO-1 mRNA levels compared to LPS alone and **b**) combined with LPS significantly elevated levels of HO-1 mRNA compared to the negative control (Figure 9B).

HO-1 inhibitor blocks EP's down-regulatory effect on NFκB

Thus far, we have demonstrated EP's effects on HO-1 mRNA and protein expression in both the non-pregnant ex vivo and preterm in vivo models, respectively. Next, we sought to further confirm EP's ability to induce HO-1 activity by investigating the effects of blocking HO-1 on levels of p- NFκB, using our non-pregnant in vivo model. Animals treated with ZnPP, EP and LPS together showed an increase in levels of p- NFκB in a dose-dependent manner, with the highest dose of ZnPP exhibiting a 2 fold increase from the negative control. We also show a significant inhibition of activated levels of p- NFκB by ZnPP alone (Figure 10).

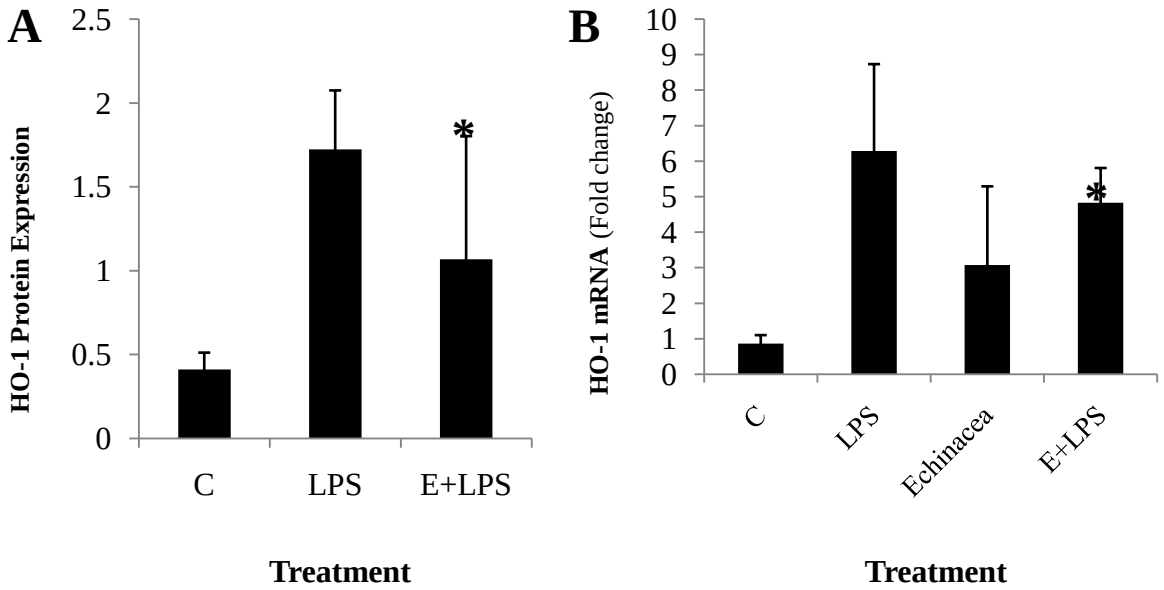

Figure 9. Effects of EP on HO-1 protein expression and mRNA levels in the mice cervix of the preterm *in vivo* and non-pregnant *ex vivo* models, respectively. EP: **A**) administered I.P down-regulates HO-1 protein levels compared to LPS only in the cervix of preterm labor mouse model, as revealed by protein analysis (Western blot). Negative control=vehicle only, 0.9 % NaCl, IP; LPS alone(250 µg/mouse, intrauterine); **B**) induces expression of HO-1 mRNA in an *ex vivo* non-pregnant ovariectomized mouse model compared to control (negative), as revealed by Western blot and real time PCR, respectively. n=3;* p< 0.05 E+LPS vs. LPS; β-actin was used as a normalizer.

DISCUSSION

Here, we examine the ability of EP to down-regulate the expression of LPS-induced pro-inflammatory cytokines in mice cervix and the underlying mechanisms. The key findings of the study are that EP: **1**) attenuates expression of key cytokines associated with precocious cervical remodeling (IL-6, COX-II) in vivo (non-pregnant and preterm labor model) and ex vivo; **2**) diminishes the activity of the master transcription factor of classical pro-inflammatory cytokines, NFκB (phosphorylated); **3**) promotes the expression of HO-1 in mice cervix and that **4**) HO-1 inhibitor blocks EP's attenuating effects by diminishing activity of p-NFκB in the cervix, in a dose dependent manner. These data suggest that inhibition of NFκB activity by EP is at least in part mediated by HO-1. The present study is the first to provide evidence for EP's anti-inflammatory activity and its likely underlying mechanism in the cervix. These data may prove useful in developing therapies that can be used to prevent and modulate inflammation-induced preterm labor.

Microbial infection and the subsequent precocious and abnormal presence of inflammatory factors, including TNFα, IL-1 and -6, are known to induce premature uterine contractions and birth canal opening (Menon and Fortunato, 2007; Raso et al., 2012; Rizzo et al., 1996) and ultimately, premature birth (Alderem and Ulevitch, 2000; Beutner, 2000; Menon and Fortunato, 2007; Rizzo et al., 1996; Zhang and Ghosh, 2001). For instance, in one study, of the 20% of subjects (women) that had microbial infection and abnormally elevated levels of IL-6 in the amniotic cavity and cervical secretions, 100% experienced preterm labor (Rizzo et al., 1996). For the most part, LPS binds to white blood cells (WBCs) expressing its receptor TLR4, activates NFκB and, subsequently, increases the expression of the downstream pro- and or anti-inflammatory cytokines (TNFα, IL-6 and IL-10) (Alderem and Ulevitch, 2000; Beutner, 2000; Zhang and Ghosh, 2001). Because the female reproductive tract (from fallopian tubes to vagina) richly expresses LPS receptor subtypes (TLR 1-6), it is endowed with an extensive immune surveillance, which plays a vital role in defense against infection (Pioli et al., 2004). Importantly, since preterm labor initiation can be triggered either in the cervix, upstream of the cervix, that is, uterus or both, this (presence of TLR) also implies that EP may act not only on the cervix, but also the rest of the female reproductive compartments. Taken together, the strong link between inflammation and preterm labor, and the ability of EP to modulate pro-inflammatory factors, makes the present findings of potential clinical importance.

Recently, there has been renewed interest in herbal remedies by the general public in North America, with EP topping the list (Cavaliere, 2009). Correspondingly, there has been an increase in the number of studies that have characterized various biological activities of EP, particularly its anti-inflammatory effects. For instance, studies have

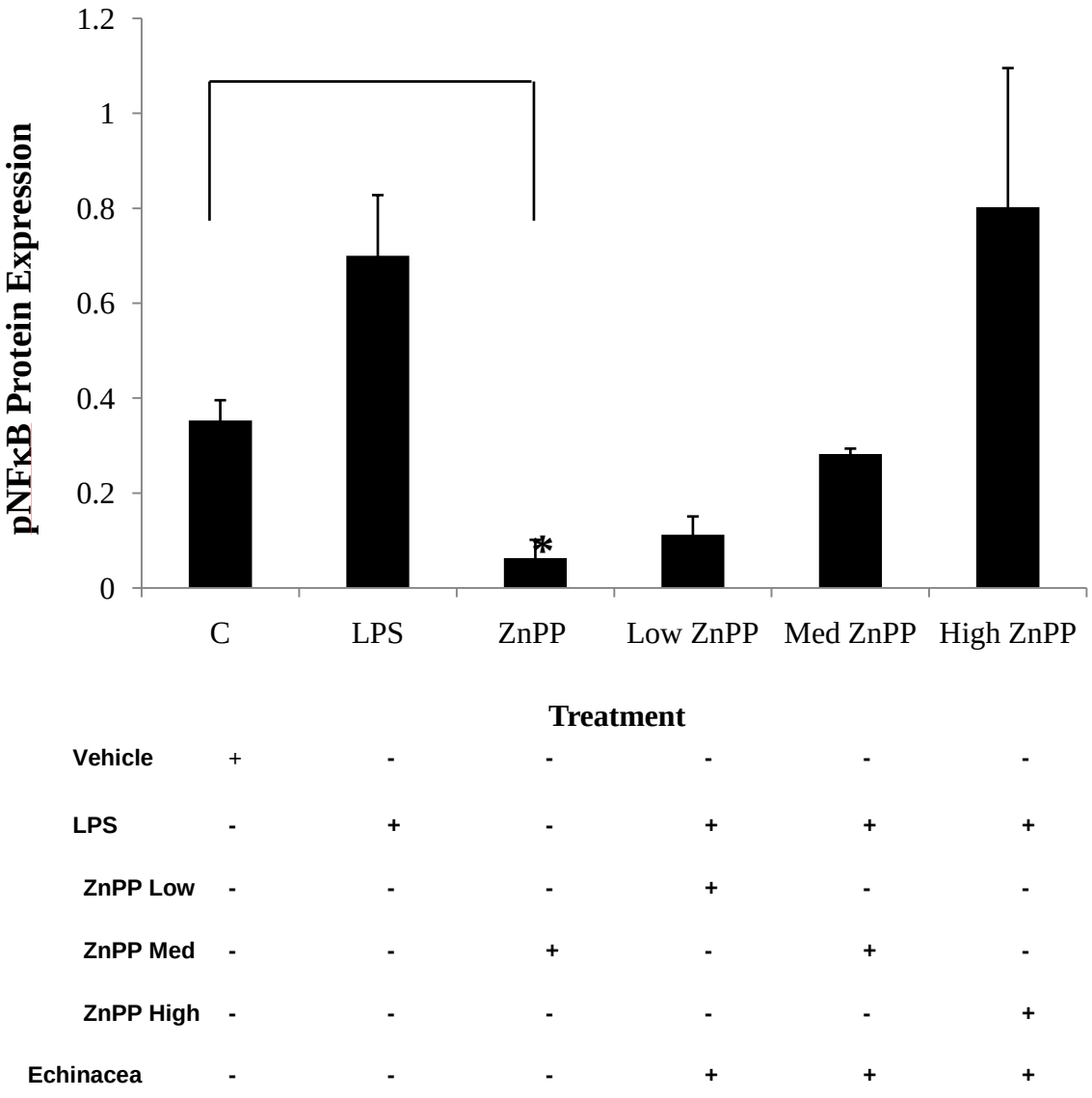

Figure 10. HO-1 inhibitor blocks EP's down regulatory effect on the activity of NFκB in cervix of mice treated with LPS as revealed by Western blot analysis. *n*=3;* *p*< 0.05 ZnPP vs. C. β-actin was used as a normalizer. The table below the figure indicates the solutions present in each treatment group, with a + indicating that a particular solution was used while – indicates it was not.

shown that EP attenuates expression of virus-induced pro-inflammatory cytokines by neutralizing rhinovirus 1a (RV1A)-induced cytokine secretion (Sharma et al., 2009), including an almost complete inhibition of IL-6 and TNFα activities at a dose of 40 µg/ml of EP extract (Sharma et al., 2009). Also, when murine RAW 264.7 macrophage cells, a cell line that is commonly used for studying lipid metabolism, inflammation and apoptosis, were activated to secrete high levels of pro-inflammatory factors using LPS, EP extracts inhibited the levels of the pro-inflammatory factors [TNFα, nitric oxide (NO), and inducible NO synthase (iNOS)] (Borchers et al., 2000), dose-dependently. Of note, EP does not only inhibit LPS-induced inflammation, but also other inflammation-inducing irritants, such as carrageenan and croton oil. Specifically, EP administered intravenously attenuated carrageenan and croton oil-induced rat paw edema and mouse ear tests (Barnes et al., 2005). The present data are consistent with these earlier data, and indicate that EP can modulate inflammation in different tissues of the body.

Alkylamides are major bioactive constituents in all EP ethanolic extracts, and significantly inhibit TNFα protein expression, as well as LPS-induced increase in NFκB

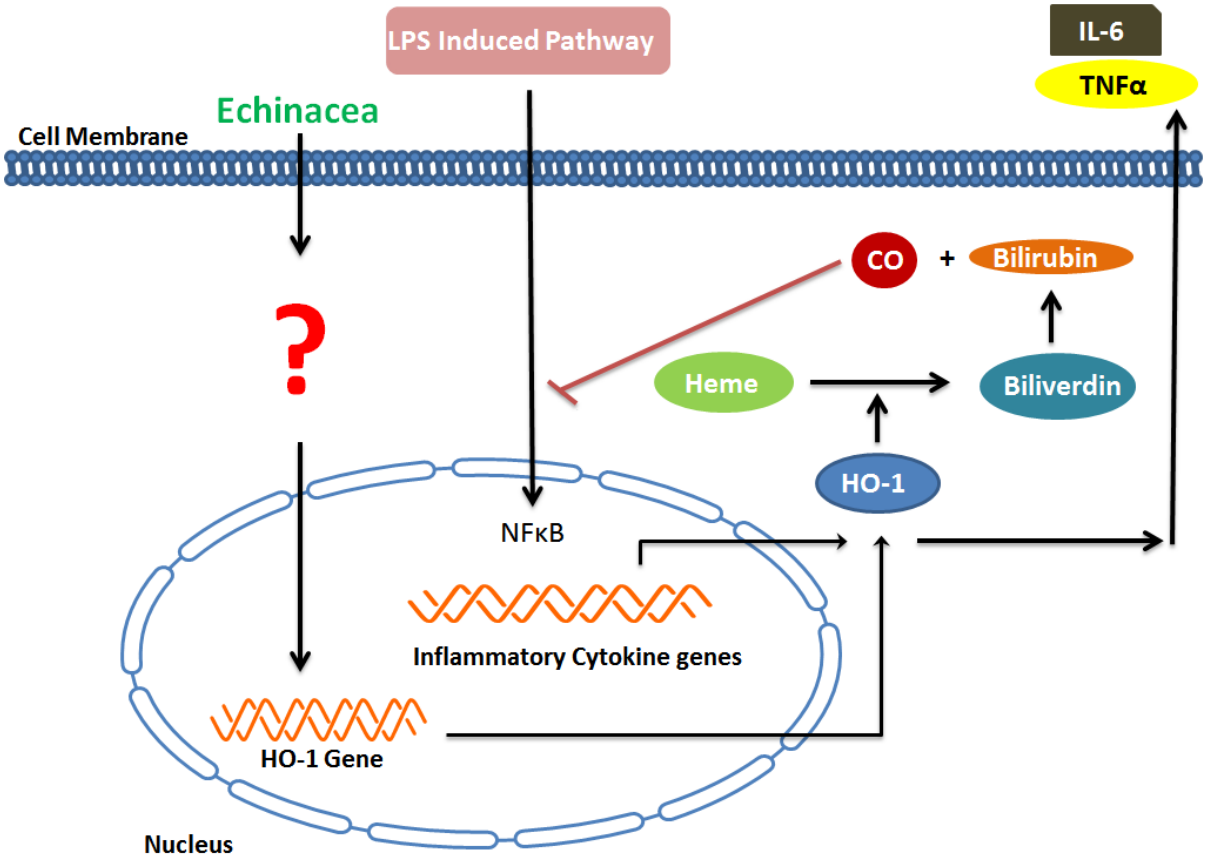

Figure 11. Proposed working model of EP in regulating expression of pro-inflammatory factors in the cervix. EP induces transcription of heme-oxygenase-1, which in turn blocks transcription of pro-inflammatory factors via its heme by product, carbon monoxide (CO).

activity (Gertsch et al., 2004). Earlier studies have suggested that the effects of EP are mediated by cannabinoid receptor CB2 and modulation of cyclic adenosine monophosphate (cAMP), activation of JNK and p38 MAP kinases, as well as downstream activation of NFκB (Gertsch et al., 2004). The study suggesting p-38 as a mediator of EP anti-inflammatory activities also observed a marked inhibition of LPS-induced NFκB activity and attributes this inhibitory activity to alkylamides, which were bioavailable in plasma following oral ingestion of EP (Toselli et al., 2009). Taken together, all of these studies show a strong support for EP (specifically alkylamides) as a modulator of inflammation.

Here, we also examined EP's effect on the expression of the enzyme HO-1, which has several anti-inflammatory links, including the reproductive tracts. However, its (HO-1) relationship with EP has not been reported previously in reproductive tissues, in general and cervix, in particular (Zenclussen et al., 2011). HO-1 is the rate limiting enzyme that breaks down heme from degraded red blood cells into carbon monoxide (CO), biliverdin, and free iron (Otterbein et al., 2003), and is believed to exert its anti-inflammatory properties through CO (Lee et al., 2004; Nath et al., 1992; Otterbein et al., 2003; Willis et al., 1996; Kapturczak et al., 2004). Indeed, mice lacking HO-1 develop chronic inflammatory diseases (Kapturczak et al, 2004); suboptimal placentation, as well as fetal lethality (Menon and Fortunato, 2007; Zenclussen et al., 2011), implying that EP could influence expression of a potentially important gene that may have broad impact on reproductive function and the tone of inflammation. In the liver, EP-derived alkylamides promote expression of HO-1, and is believed to contribute to the hepato-protective effects against LPS-induced inflammation (Hou et al., 2001) and infusion of the HO-1 inhibitor, ZnPP, in rats attenuates these beneficial hepato-protective actions of HO-1, showing that HO-1 plays a protective role against ischemia/reperfusion injury, which is also associated with inflammation (Menon and Fortunato, 2007). Further, Hou and others used murine macrophage cells to demonstrate that EP-derived alkylamides also induce expression of HO-1, which in turn inhibit expression of pro-inflammatory cytokines (Hou et al., 2001). These earlier observations are consistent with our present data, which show up-regulation of HO-1 mRNA (non-pregnant ex vivo) and protein (preterm in vivo) by EP. We also show

here that a known HO-1 inhibitor attenuates EP's anti-inflammatory effects dose-dependently via up-regulating the activity of NFκB (p-NFKB), implying that HO-1 mediates EP's anti-inflammatory activities. Data showing decrease of p-NFκB levels by ZnPP alone compared to negative control levels was not expected and is for now unclear. One possible explanation for this discrepancy could be post-surgery infection of the negative control animals, which could account for the observed increase in levels of p NFκB compared to the ZnPP-only treated animals. However, more studies are required since all animals were treated with antibiotics during surgery to control for infection.

We also report here development of an ex vivo model and demonstrate its suitability (tissue integrity and viability, and inflammatory response) as a bioassay for studying mechanisms and pathways underlying EP's anti-inflammatory activities. Although still in development, this model eliminates pregnancy-associated confounding variables by providing a tightly controlled environment, and will thus be especially useful as a target screening bioassay, as we attempt to follow, identify and isolate the exact extract candidate(s) in EP responsible for the anti-inflammatory activities. This will be accomplished through a series of sub-fractionation, chemical isolation steps and target screening. Data generated from this bioassay will then be used for the preterm labor mice model studies. We fully anticipate that isolated and concentrated sub-fractions and/or individual substances will induce higher and/or more prolonged anti-inflammatory activity on a per gram raw material basis. Whole hydro-ethanolic EP is known to have over 19 bioactive isolated compounds that demonstrate anti-inflammatory activities (Duke, 1992). These compounds have been shown to influence multiple inflammatory signaling pathways at various steps, including inhibiting the activity of IKKβ, interferon regulatory factor 3 (IRF3) and TLR3/TLR4-induced NFκB or IkB degradation (Lee and Chau, 2002). The inhibition of these upstream factors decreases the activity of a plethora of downstream pro-inflammatory factors (Alappat et al., 2010; Bouic and Lamprecht, 1999; Ha et al., 2008; Lee and Chau, 2002; Loizou et al., 2010; Moon et al., 2009; Shin et al., 2004; Yoon et al., 2010).

In conclusion, we report a novel finding that EP attenuates activity of NFκB and expression of its (NFκB) downstream pro-inflammatory cytokines, associated with infection-induced preterm labor in the mice cervix. We also developed an ex vivo cervical model and showed that the enzyme HO-1 could be one of the key mediators of EP's anti-inflammatory effects in the cervix (see proposed working model, Figure 11). Through our working model we propose that EP exerts its effects by increasing transcription of HO-1 (perhaps via the p38 MAP kinase pathway) to block inflammation via up-regulation of carbon monoxide, which in turn blocks transcription of pro-inflammatory factors via the NFκB signaling pathway. These findings are significant in that they provide insight that could potentially lead to the development of natural strategies for modulating infection-induced preterm labor.

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