ECHINACEA AND PRETERM LABOR: A NATURAL REMEDY

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Abstract

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Causally, premature births largely result from inflammation and current treatments are either unsafe or ineffective. Here, our goal was to test whether the use of natural products [*Echinacea purpurea* (L.) Moench, root extract] with anti-bacterial and -inflammatory activities and a long history of safe use could attenuate induction of inflammation in the cervix (birth canal). Studies using three different complementary models, specifically non-pregnant *in vivo*, non-pregnant *ex vivo* and preterm labor models, were conducted. We also sought to decipher mechanisms likely to mediate *Echinacea'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 *Echinacea* attenuates levels of the activated (phosphorylated) master inflammation transcription factor, nuclear factor kappa B (NFκB), and expression of select pro-inflammatory cytokines associated with inflammation-induced preterm labor. We also show that HO-1 may mediate *Echinacea's* 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.

Dedication

To: Mom, Dad, and Julie, for all of the support, love, kindness and understanding you've shown me as I've grown. I couldn't have made it nearly as far in life as I have if it weren't for all of you, and I love you so much. Thank you for guiding me and supporting me as I've pursued my dreams.

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Introduction

Overview

Premature labor is a common and costly health care condition, the magnitude of which is staggering. Every minute approximately 1,400 babies are born prematurely throughout the world and over 100 of these infants die (Beck *et al.* 2010). Causally, half of these premature births are known to result from precocious and abnormal presence of infection-induced inflammatory factors, such as tumor necrosis factor alpha (TNFα), during contractions and cervical (birth canal) opening. 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 (Romero *et al*. 2006). Current therapies for preterm labor are either unsafe to the fetus and/or mother, or are ineffective, 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, such as *Echinacea*, could potentially be used to attenuate inflammation-induced preterm labor in high-risk women (Barrett 2003, Goel *et al.* 2004, Barnes *et al.* 2005, Shah *et al.* 2007). Interestingly, *Echinacea* 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, Rininger *et al.* 2000, Kim *et al.* 2002), in part, by attenuating expression of a plethora of cytokines (Burger *et al*. 1997, Rininger *et al.* 2000, Kim *et al.* 2002, Zhai *et al*. 2007). It is,

therefore, feasible to speculate that *Echinacea* could potentially be used to modulate infection-induced preterm labor.

Basics of Cervical Remodeling and the Birth Process

During pregnancy, the quiescent uterus provides a favorable environment for the growing fetus and the non-pliable cervix ensures retention of the fetus *in utero*. However, at term the cervix undergoes tissue remodeling or cervical ripening with a progressive dissociation and disorganization of collagen fibers and bundles that leads to softening and dilation of the cervix. At this time the uterus begins to contract and the cervix relaxes, and, collectively, these alterations facilitate a timely passage of the fetus at parturition (Kelly 2002). Failure or disruption of these processes causes complications at parturition, such as preterm or protracted labor, that account for 75% of all infant deaths (Challis 2000).

Physiological Changes Associated with Labor and Delivery in the Cervix

The cervix is a fibrous, connective tissue mainly composed of collagen and proteoglycans (Sennstrom *et al*. 2000). Cervical remodeling occurs in two general steps, the first being a slow stage, which extends over much of pregnancy, followed by a final rapid process, immediately preceding labor (Sennstrom *et al*. 2000). The slow process shows a change in the turnover of matrix components, which results in reorganization of the collagenic fibrillar network, while the final ripening state shows an influx of neutrophils capable of secreting collagenase and elastase (Sennstrom *et al.* 2000). During cervical remodeling, there is a 50-70% decrease in collagen and proteoglycan concentration, with an increase in collagen synthesis and this increased proteolytic activity coincides with an increase in solubility of collagen (Sennstrom *et al.* 2000). Cervical remodeling is also

characterized by an increased accumulation of leukocytes in the cervical stroma before the onset of labor (Thomson *et al.* 1999, Young *et al.* 2002). These leukocytes are responsible for the breakdown and subsequent remodeling of cervical tissue via the release of matrix metalloproteinases, prostaglandins, cells adhesion molecules, and nitric oxide (NO) (Thomson *et al.* 1999, Osman *et al.* 2003).

Pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNFα, have been identified in reproductive tissues during labor (Young *et al* 2002). In the cervix, each of these cytokines has been found in different locations within the tissue (Young *et al.* 2002). For example, IL-1β, IL-6, IL-8 and TNFα have all been shown to be localized to a subpopulation of leukocytes (possibly neutrophils), as well as glandular epithelial cells, luminal epithelia and stromal cells within the cervix (Young *et al.* 2002). It has been suggested that the invading leukocytes are principally responsible for the noted increase in these pro-inflammatory cytokines (Young *et al.* 2002, Osman *et al.* 2003). However, for the most part, their specific underlying mechanisms and function under physiological conditions remain obscure; it is unclear whether they are simply the byproducts of another pathophysiological event (Young *et al.* 2002). Either way, it is likely that they do function to stimulate uterine activity, either directly or indirectly, via perhaps an increase in prostaglandin production, attraction of leukocytes, membrane rupture and/or tissue remodeling under pathological conditions, notably during infection (Young *et al.* 2002). Specifically, IL-1β and TNFα stimulate arachidonic acid release and the subsequent prostaglandin production in human myometrial cells, which then stimulate myometrial contractions and ultimately ripening of the uterine cervix (Young *et al.* 2002). IL-1β and TNFα are also known to increase production of matrix metalloproteinase-9 in human

myometrial smooth muscle cells and may therefore play a role in precocious tissue remodeling associated with obstetrical complications, such as preterm labor (Young *et al.* 2002).

Preterm Labor

In humans, preterm labor is defined as labor occurring prior to 37 weeks gestation (Simhan & Caritis 2007) and is the leading cause of death in children under 5 years of age, second only to pneumonia (Blencowe *et al.* 2012).

Epidemiology: According to the World Health Organization, 15 million babies are born prematurely each year worldwide, with the majority of these births coming from Nigeria, China, India, the United States and Brazil (Blencowe *et al.* 2012). Currently, the global average percentage of preterm birth rate is 11.1% (Wen *et al.* 2004) and according to the March of Dimes report card, in 2012 the United States (US) earned a grade of "C" at 11.7% (March of Dimes, 2012).The US intends to reduce its rate down to 9.6% by 2020 (March of Dimes, 2012). Nationally, Mississippi has the highest rate at 16%, while Vermont has the lowest rate, at 8%. North Carolina's rate is at 12.6%, earning it a grade of "C" and is slightly higher than the national average (March of Dimes, 2012). Premature births cost the United States healthcare system $26 billion a year (Beck *et al.* 2010).

Preterm Labor Biology: *Pathogenesis*: While nearly 40% of premature births have an unknown cause, studies suggest that there are four main causes of spontaneous preterm labor, namely: a) maternal and/or fetal stress, b) bleeding, c) stretching and d)

infections/inflammation (Goldenberg *et al.* 2002, Simhan & Caritis 2007, Beck *et al.* 2010). Chronic psychosocial stress of the mother or physical stress in the fetus induce production of corticotropin-releasing hormone (CRH), which in turn may trigger other hormones, such as prostaglandins, which trigger uterine contractions and eventually preterm birth (Goldenberg *et al.* 2002). Uterine bleeding as a result of complications such as placental abruption (placenta peels away from the uterine wall before delivery), which may trigger release of proteins involved in clotting, such as thrombin, which in turn stimulates uterine contractions (Goldenberg *et al.* 2002). Uterine distension by multi-fetal pregnancies may lead to increased gravitational weight exerted on the cervix and a positive feed-forward release of the hormone oxytocin, which stimulates uterine contractions (Goldenberg *et al.* 2002). The bulk of preterm labor is induced by bacterial infections that lead to inflammation and preterm labor and account for the preterm premature rupture of membranes (PROM) (25-40%), and obstetrically indicated preterm delivery (20-25%) (Goldenberg *et al.* 2002, Wen *et al*. 2004).

Risk factors: Several lifestyles and factors have been identified to put a woman at risk for preterm birth, including: a) a history of preterm birth; b) size or multi-fetal pregnancies; c) certain uterine or cervical abnormalities (such as shortened cervix); d) ethnicity, with the highest rate in black women; e) age, with teenage or older mothers at the greatest risk; f) education and socio-economic status highest in women with low education and socio-economic status; g) habits, such as cigarette smoking increase the risk; h) marital status: unmarried women or those not living with a partner are at a higher risk; i) occupation: women with stressful occupations are at a higher risk; j) body weight: low maternal pre-pregnancy body mass index, and poor or excessive weight gain increase the likelihood of a preterm birth (Goldenberg *et al.* 2002, Wen *et al*. 2004). Of all these risk factors cited above,

the top three are: a) history of preterm birth, b) size or multi-fetal pregnancies, and c) certain uterine or cervical abnormalities (such as shortened cervix) (Goldenberg *et al.* 2002, Wen *et al*. 2004).

Diagnostics: Risk scoring systems, uterine contraction monitoring, cervical sonography and fetal fibronectin testing are several tools frequently utilized in the prediction and early detection of preterm birth, with the most promise seen from cervical length measurement and fibronectin tests (Wen *et al.* 2004). Cervical length is determined using trans-vaginal ultrasound (Shennan & Jones 2004). According to cervical assessments in women without contractions, studies of both lower- and higher-risk women have shown that the shorter the cervix (less than 25 mm), the more likely a preterm birth will occur (Shennan & Jones 2004). Fetal fibronectin is produced by fetal and placental tissue and acts as the "glue" that adhere the placental membranes to the endometrium and is normally detected in vaginal secretions up to week 22 of gestation, and again 1 to 3 weeks before delivery (Iams 2003, Shennan & Jones 2004). Fibronectin found in cervicovaginal secretions after 22 weeks is an indicator of disruption of the decidual-chorionic interface and has been shown to be associated with a six-fold increased risk of preterm birth before 35 weeks, and a 14-fold increased risk of preterm birth before 28 weeks (Iams 2003). In addition to cervical measurements and fetal fibronectin tests, there are various but less reliable markers that can be used to predict spontaneous preterm birth (Kim *et al* 2011). These include leukocyte differential counts and neutrophil to lymphocyte ratio (Kim *et al* 2011), levels of serum IL-6 and TNFα, which are elevated during preterm labor, IL-1β, IL-6, IL-8, and IL-18 in cervicovaginal secretions, where they are elevated during preterm labor (Kim *et al* 2011).

Current therapies: Stopping uterine contractions has been the focus of current therapeutic approaches, based on the assumption that clinically apparent contractions correspond with the initiation of labor, and therefore the prevention of contractions should prevent labor (Simhan & Caritis 2007). Inhibition of myometrial contractions is called tocolysis, and drugs administered for such a purpose are called tocolytics (Simhan & Caritis 2007). The aim of this treatment regimen is to delay delivery so that interventions, such as corticosteroid shots can be implemented (Wen 2004). Corticosteroids help enhance development of lungs and particularly secretion of pulmonary surfactant, which reduces the risk of neonatal respiratory distress syndrome, intra-ventricular hemorrhage, necrotizing entercolitis, and overall perinatal death (Wen 2004). There are many classes of tocolytics, each with their own advantages and short-comings, including: a) beta adrenergic receptor agonists (terbutaline), b) nitric oxide (NO) donors, c) magnesium sulfate, d) calcium channel blockers (nifedipine), e) cyclooxygenase (COX) inhibitors (indomethacin), and f) oxytocin-receptor antagonists (Simhan & Caritis 2007). While tocolytics are good for temporal inhibition of contractions, most do not help decrease preterm birth and pose serious negative side effects both to the mother and fetus, notably low birth weight, neonatal complications, and increase in risk of death (Wen *et al.* 2004, Simhan & Caritis 2007). Currently, the only therapy that targets the cervix to prevent spontaneous preterm birth is elective cervical cerclage (Wen *et al.* 2004). In cerclage, a stitch is inserted in the cervix to keep it closed, and removed around 37 weeks of pregnancy (Wen *et al.* 2004). Studies have shown that this procedure may have some significant effect in reducing preterm births before 34 weeks of gestation. However, more studies are needed to identify women who would most benefit from cerclage (Simhan & Caritis 2007). Lastly, for infection-induced preterm labor, which

accounts for the bulk of spontaneous preterm birth and the focus of the current study, antibiotics are currently the treatment of choice. However, for the most part, they also have side effects; especially to the fetus (Wen *et al.* 2004).

Infection/inflammation-induced preterm labor: As stated earlier, intrauterine infection/inflammation is frequently associated with preterm labor (Romero *et al.* 2002, Romero *et al.* 2006). Studies have suggested that preterm labor is often triggered by the body's response to certain bacterial infections, such as those involving the genital, urinary tracts and fetal membranes (Goldenberg *et al.* 2002). 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 & Gibbs 2004).

Toll-like receptors 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 well component of gram-negative bacteria, as well as fragments of fetal fibronectin (Tsan & Gao 2004, Challis *et al.* 2009). LPS can activate TLR4 and trigger NFκB, a hallmark signal transduction pathway for innate immune responses (Chow *et al.* 1999, Da Silveira Cruz-Machado *et al.* 2010). NFκB is highly activated at sites of inflammation and can induce transcription of the pro-inflammatory cytokines previously described to be involved in labor (Tak & Firestein 2001).

Upon LPS recognition, TLR4 recruits its downstream adaptors through interactions with toll-interleukin-1 receptor (TIR) domains (Poltorak *et al.*1998). One of the five TIR domain-containing adaptor proteins is myeloid differentiation primary response gene 88 (MyD88) (O'Neill & Bowie 2007). MyD88 also contains a death domain (DD), which can recruit and activate a death domain-containing kinase, IL-1 receptor associated kinase-4 (IRAK-4) (Suzuki *et al.* 2002). Another adaptor protein, TNF receptor-associated factor 6 (TRAF-6) is critical for the MyD88-dependent pathway downstream of IRAK-4 (Lu *et al.* 2008). TRAF6 activates transforming growth factor-β-activated kinase 1 (TAK1), which then activates downstream IKK (IκB kinase) and mitogen-activated protein kinase (MAPK) pathways (Lu *et al.* 2008). NFκB exists in the cytoplasm in an inactivated form associated with inhibitors of κB (IκB) (Tak & Firestein 2001). Phosphorylation of IκB leads to the degradation of IκB proteins and translocation of the transcription factor NFκB into the nucleus, which controls the expression of pro-inflammatory cytokines via binding of NFκB to κB enhancer elements of target genes, inducing transcription of pro-inflammatory cytokines (Tak & Firestein 2001). Activation of the downstream MAPK pathways leads to induction of another transcription factor, activator protein-1 (AP-1), which also has a role in the expression of pro-inflammatory cytokines (Chang & Karin 2001).

There are three major subfamilies of MAP Kinases: extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (Kyriakis & Avruch 2001). These pathways can be activated by a wide variety of stimuli acting through different receptor families, such as hormones and growth factors acting through receptor tyrosine kinases, or cytokine receptors (Kyriakis & Avruch 2001). It is generally accepted that there are many MAPK pathways existing in parallel and in conjunction with the NFκB pathway, and that

these pathways are important to stress and inflammatory responses, including up regulation of cytokines, commonly seen in the inflammation-induced preterm labor (Kyriakis & Avruch 2001).

Some of the relevant pro-inflammatory cytokines associated with preterm labor include TNFα, IL-1β, IL-6 and COX-II (Romero *et al.* 2006). IL-10 is one of the many anti-inflammatory cytokines which acts to counter-balance an overreaction from pro-inflammatory cytokines during infection or pregnancy (Thaxton & Sharma 2010). For instance, IL-10 levels are higher during preterm labor, perhaps as a response to high circulating levels of pro-inflammatory cytokines, such as IL-1β (a pro-inflammatory cytokine) (Dubicke *et al.* 2010). Cytokines have been found to trigger prostaglandin synthesis in the amnion, chorion, decidua, myometrium, and cervix, among other things (Young *et al.* 2002, Klein & Gibbs 2004). This then leads to uterine contractions, cervical dilation, membrane exposure, and greater entry of microbes into the uterine cavity. Cytokines, such as IL-6, have also been found to stimulate production of matrix metalloproteinases by the chorion and amnion (Young *et al.* 2002, Klein & Gibbs 2004). Matrix metalloproteinases are implicated in cervical ripening as well as degradation of the fetal membranes (Young *et al.* 2002, Klein & Gibbs 2004). There has also been evidence supporting a relationship between periodontal disease and preterm delivery (Romero *et al.* 2006). However, intrauterine infections caused by bacteria are currently considered to be the leading cause of infection-induced preterm birth (Romero *et al.* 2006). The most common microbial isolates from the amniotic cavity and intact membranes of women with preterm labor are *Ureaplasma urealyticum (U. urealyticum), Fusobacterium* species and *Mycoplasma hominis* (Romero *et al.* 2002, Romero *et al.* 2006). In contrast, vaginal colonization by

organisms such as *U. urealyticum* and *Candida* species has not been shown to cause preterm birth. However, *U. urealyticum* is found in the amniotic fluid and has a prevalence of 6% of patients with preterm birth (Romero *et al.* 2006).

Antibiotic trials to prevent preterm birth have been conducted using three different designs: a) antenatal treatment of lower genital tract infection caused by specific organisms; b) treatment of preterm labor with intact membranes; c) treatment after preterm premature rupture of membranes (PPROM) (Klein & Gibbs 2004). Based on data from these trials, compelling evidence supports intervention in clinical practice.

Antenatal treatment of lower genital tract infection caused by specific organisms: Certain organisms and/or infections have been associated with preterm birth and make up the list of candidate infections for antenatal treatment, such as: *U urealyticum,* group B streptococci, *Neisseria (N) gonorrhoeae*, *Chlamydia (C)trachomatis, Trichomonas (T) vaginalis,* bacteriuria, and bacterial vaginosis (BV) (Klein & Gibbs 2004). Although *U urealyticum* does not appear to cause preterm birth, it can become pathogenic if it gains access to the upper genital tract (Klein & Gibbs 2004). For this reason, following clinical trials, it was found that screening and/or treatment of lower genital *U urealyticum* to prevent preterm birth, is not helpful and should, therefore, be avoided, in the absence of upper genital tract infection (Klein & Gibbs 2004). Rectovaginal group B streptococci should not be treated antenatally either to prevent preterm birth; however, group B streptococcal bacteriuria should be screened for and treated to prevent preterm birth (Klein & Gibbs 2004). Also, the sexually transmitted organisms *N gonorrhoeae* and *C trachomatis* should be screened for and treated to prevent spread and vertical transmission (Klein & Gibbs 2004). Screening for and treatment of bacteriuria in pregnancy has been found to prevent symptomatic urinary tract

infection as well as preterm birth (Klein & Gibbs 2004). Women with symptomatic *T vaginalis* infections in pregnancy have successfully been treated with oral metronidazole, which has been shown to be safe in the first trimester of pregnancy (Klein & Gibbs 2004). Asymptomatic *T vaginalis*, however, should not be screened for or treated based on a large clinical trial that shows the absence of benefit as well as increased risk of preterm birth (Klein & Gibbs 2004). The association between BV and preterm birth is an interesting one, with BV being found in 10-25% of women – BV can be diagnosed via gram stain, and approximately 50% of women with BV are asymptomatic. Unfortunately, results of treatment trials for BV have been conflicting, with some trials showing a reduction in preterm birth when BV was screened for and treated and some trials showing no benefit of screening and treating overall (Klein & Gibbs 2004). On the basis of a number of clinical studies, however, it is suggested that screening and treatment of high-risk women are warranted with a recommended oral regimen lasting seven days (Klein & Gibbs 2004).

Antibiotic treatment in preterm labor with intact membranes: Several studies have reported the use of antibiotics to prolong pregnancy in women in preterm labor with intact membranes (Kenyon *et al.* 2001, King & Flenady 2003). The overall use of antibiotics, however, did not decrease preterm birth, delivery within 48 hours, or perinatal mortality rates compared to women not treated with antibiotics (Kenyon *et al.* 2001). In a meta-analysis from the Cochrane Library, many trials were assessed, with the overall conclusion that the routine administration of antibiotics to women with preterm labor and intact membranes could not be recommended, as there were no clear improvements in neonatal outcomes and potentially a trend toward increased neonatal deaths (King & Flenady 2003).

Antibiotic treatment after preterm premature rupture of membranes (PPROM): Several large trials have been conducted to assess this area (Klein & Gibbs 2004). One such trial assessed patients taking ampicillin and erythromycin for seven days compared to placebo in women with PROM from 24-32 weeks gestation, finding women given antibiotics were far more likely to remain undelivered at 2, 7, 14 and 21 days compared to placebo (Mercer *et al.* 1997). Another trial showed small but significant benefits in oral antibiotic therapy for 10 days with erythromycin or amoxicillin or both compared to placebo (Kenyon *et al.* 2001). As a result of comparing these studies, it has been suggested that the routing prescription of antibiotics (specifically erythromycin) in women with PPROM can be associated with better maternal and fetal outcome (Klein & Gibbs 2004).

With mounting evidence that antibiotic treatment can be beneficial only in certain cases of preterm labor, the question remains as to why antibiotics do not regularly decrease premature birth. There are several explanations that have been suggested, such as: a) bacterial lysis by antibiotics, which can hasten preterm labor; b) prescription of the wrong antibiotic, since the organism responsible for infection-induced preterm labor is not known in many cases; and most convincingly c) the fact that once clinical signs have manifested, the inflammatory cascade has progressed too far to be affected by antibiotics (Klein & Gibbs 2004). This, in conjunction with the magnitude of redundancy in the cytokine network, demonstrates the limitations of blocking one single factor in preventing infection/inflammation-induced preterm labor (Klein & Gibbs 2004, Romero *et al.* 2006). This reality calls for alternative approaches to managing infection-induced preterm labor, such as the use of natural remedies with anti-bacterial and –inflammatory activities, e.g.,

Echinacea. One possible factor that mediates Echinacea's anti-inflammatory activities, among others, is heme-oxygenase 1 (HO-1) in the liver (Otterbein *et al.* 2003).

Heme oxygenase (HO)-1

HO-1 is an enzyme that catalyzes the breakdown of heme into three products: carbon monoxide (CO), biliverdin, and free iron (Otterbein *et al.* 2003). HO-1 plays tissue-protective roles under normal and physiological conditions, including anti-inflammatory, anti-apoptotic, and anti-proliferative actions in endothelial, epithelial and smooth muscle tissues, to name a few (Otterbein *et al.* 2003).The anti-inflammatory properties of HO-1 have been further supported by the fact that HO-1 deficient mice develop a chronic inflammatory disease state that progresses with age (Otterbein *et al.* 2003).

There is strong evidence suggesting that the by-products of heme catabolism mediate the protective function of HO-1 (Otterbein *et al.* 2000). One study suggests that carbon monoxide (CO) can selectively modulate the cascade of pro-inflammatory and anti-inflammatory cytokines. Of interest to the current study, CO has been shown to inhibit LPS-induced production of TNFα, IL-1β, while increasing IL-10 production in these same cells (Otterbein *et al.* 2000). In this particular study, the anti-inflammatory effects of CO involve the MAP kinase signaling pathway, specifically the MKK3/p38 MAP kinase pathway (Otterbein *et al.* 2000). This is based on several observations: CO had no effect on ERK1/ERK2 or JNK MAP kinases. It is also known that HO-1 is mediated by the p38 MAP kinase pathway in response to oxidative stress (Otterbein *et al.* 2000). Unfortunately, the precise mechanism used by CO to modulate the MAP kinases remains unclear (Otterbein *et al.* 2000) and *Echinacea*'s effects and the role of HO-1 in mediating its (*Echinacea*) anti-

inflammatory activities in infection-induced preterm labor in the cervix have not been studied.

Echinacea

Overview*: Echinacea angustifolia*, a member of the *Compositae* family and known as the purple coneflower, has been used for centuries by Native Americans for pain relief and wound treatment, an antidote against various poisons, and for symptoms associated with the common cold (administrative routes used unclear) (Borchers *et al.* 2000, Barrett 2003, Barnes 2005, Goel *et al.* 2004, Shah *et al.* 2007). Of the nine species of *Echinacea* only three are used in herbal remedies, namely *Echinacea (E) angustifolia, E. pallida and E. purpurea*, referred to as *Echinacea* from henceforth (Hobbs 1989, McKeown 1999). What is commonly called *Echinacea* in the United States is likely one of the three mentioned species, or a combination of two or even all three of them (Borchers *et al.* 2000).

Biological activities: There are substantial differences in the chemical compositions and biological activities between different species, as well as between their roots and aerial parts (Borchers *et al.* 2000). Recently, many of these claims have been confirmed experimentally using modern research technologies (Raso *et al.* 2002), including *Echinacea's* anti-fungal, anti-cancer, anti-viral, antioxidant, and anti-inflammatory properties (Raso *et al.* 2002, Barrett 2003). The current study focuses on *Echinacea's* anti-inflammatory properties. For instance, *Echinacea* has been shown to attenuate key inflammatory pathways, notably the NFκB signaling pathway, which results in up-regulation of COX-II and other pro- and anti-inflammatory cytokines (TNFα, IL-6, and IL-10) (Raso *et al.* 2002). Because *Echinacea* has been shown to decrease levels of preterm labor-inducing or associated pro-inflammatory

cytokines, such as TNFα, IL-6, IL-10 and IL-1, in non-reproductive tissues (Burger *et al.*1997, Rininger *et al.* 2000, Kim *et al.* 2002, Zhai *et al.* 2007), it is reasonable to speculate that *Echinacea* could potentially be used to modulate infection-induced preterm labor.

Subfractions: Within a hydro-ethanolic extract of *Echinacea,* there are many sub-fractions which may be responsible for the well-documented anti-inflammatory properties. For example, hydro-ethanolic extract of *Echinacea* (which is the most commonly used vehicle) contains two major groups of compounds, namely caffeic acid conjugates and alkylamides, the two main bioactive alkylamides being dodeca-2E, 4E, 8Z, 10Z-tetraenoic acid isobutylamide and dodeca-2E, 4E- dienoic acid isobutylamide (Stevenson *et al.* 2005, Matthias *et al.* 2007). Although these isolates have been shown to have immunomodulatory effects, their exact underlying pathway or their specific bioactive ingredients are yet to be identified (Stevenson *et al.* 2005, Matthias *et al.* 2007). Currently, no industry standardization is required for the amount of individual chemical constituents in commercial preparations of *Echinacea* (Toselli *et al.* 2009) but many *Echinacea* extracts are standardized to cichoric acid (or total phenols), implying that cichoric acid may account for its biological activities (Stevenson *et al.* 2005, Matthias *et al.* 2007).

Metabolism: Among the phytochemicals found in *Echinacea*, the bioavailable alkylamides are thought to be the compounds responsible for the immunomodulatory effects in the human (Toselli *et al.* 2009). Human liver microsomes have been shown previously to degrade certain alkylamides in a time-dependent manner, suggesting that these components are metabolized by cytochrome p450 enzymes (Toselli *et al.* 2009, Toselli *et al.* 2010). There are two main groups of active compounds in the hydro-ethanolic extracts of *Echinacea*: i.e., a) lipophilic compounds, such as alkylamides, and b) hydrophilic compounds

mainly consisting of caffeic acid derivatives (Toselli *et al.* 2009). These two main groups work together and are found mainly in commercial ethanolic extracts. Alkylamide concentrations are highest in the roots of the plant and at least eight alkylamides present in hydro-ethanolic *Echinacea* preparations were bioavailable after oral ingestion, while, in contrast, caffeic acid derivatives were not detected in circulation. Absorption of alkylamides was found to be rapid after ingestion, and serum alkylamide concentrations typically reached their maximum levels 20-45 minutes after ingestion. Another study showed plasma levels of people who ingested *Echinacea* tablets showed no caffeic acid conjugates after ingestion; alkylamides were rapidly absorbed and were measureable in plasma 20 minutes after ingestion and detectable for 12 hours afterward (Matthias *et al.* 2005). These studies collectively suggest that caffeic acid conjugates and cichoric acid have poor bioavailability, leaving alkylamides as the likely mediator of *Echinacea*'s therapeutic effects (Matthias *et al.* 2007).

Purpose of Study

In the present study, we test the effectiveness of a whole ethanolic root extract of *Echinacea* 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 *Echinacea* can be used to modulate inflammation-induced precocious cervical remodeling. Here, we focus on investigating *Echinacea's* ability to attenuate the expression of pro-inflammatory factors in the cervix and their likely underlying mechanisms.

Methods and Materials

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 *Echinacea* 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 (Sleepaway®, 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: *Echinacea* was given at a dose of 1 mg/mouse via inter-peritoneal (IP) injection route, followed by LPS administration at 100 µg/mouse four hours after *Echinacea* dose, and one hour prior to cervical tissue harvest, as illustrated in Figure 1. After treatment and prior to cervical tissue harvest, animals were euthanized with sodium pentobarbital (Sleepaway®, Fort Dodge Laboratories Inc., Burlingame, CA).

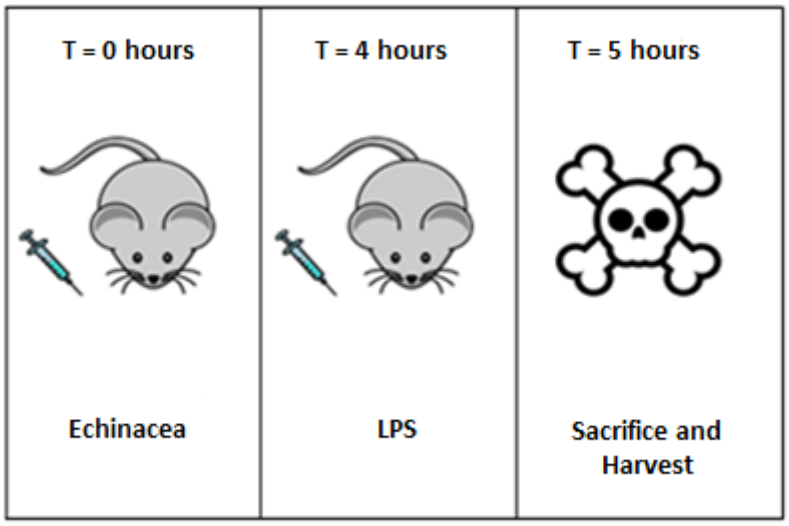

Figure 1 Illustration showing schedule of injections for non-pregnant, *in vivo* model

Pregnant animals from day 15 of pregnancy were used and induced with preterm labor, based on the previously reported animal model for preterm labor - 250 μg of intra-uterine LPS; (Elovitz & 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 *Echinacea* (1 mg/mouse) at time 0 h, with boosters at times 4 h and 8 h, respectively, with animals euthanized at time11 h post initial *Echinacea* injection, as shown in Figure 2 below. LPS was administered one hour after the last booster of *Echinacea*, i.e., time 9 h. After treatment and prior to cervical tissue harvest, animals were euthanized with sodium pentobarbital (Sleepaway®, Fort Dodge Laboratories Inc., Burlingame, CA).

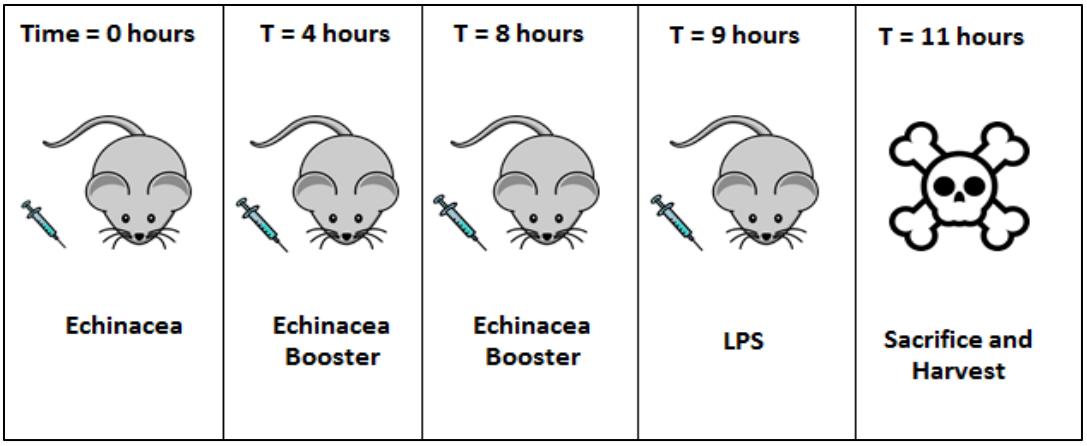

Figure 2 Illustration showing schedule of injections for pre-term labor model

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 *Echinacea's* anti-inflammatory activities under a more controlled environment (a 48 well microtiter 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 (Lonza Biowhittaker) 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) (Lonza Biowhittaker). 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 24 h in HERAcell 150i CO_2 incubator (set at 5% CO_2) at 37°C (Thermo Scientific). At the end of the 24 h 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: 1 h, 4 h, 8 h, 12 h, 16 h or 24 h, 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 *Echinacea* and LPS: Uterine cervical tissues were harvested from mice, then the optimal time and dosage for *Echinacea* and LPS treatments (*n*=3) for the *ex vivo* model were determined. To determine the optimal time for incubating, 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 6 h, 12 h and 24 h. Tissues were then harvested and stored at -80°C, processed for and examined using real-time PCR and Western blot analysis. The 6 h 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 6 h under varying treatment conditions. There was an *Echinacea* only group (0.1 mg/well), and a dose

response group, namely *Echinacea* **[**0.01 (low), 0.1 (medium) or 1.0 (high) mg/well)**]** + LPS (1 μg per well), added an hour later, as well as a negative control (media only) and an LPS only group (1 μg per well). Tissues were then harvested 6 h 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 *Echinacea* per well.

Mechanism of Action (*In vivo*, non-pregnant mice)

In order to determine whether HO-1 mediates *Echinacea'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 50 μl per mouse (*n*=3), as described here: negative control (ZnPP vehicle only, i.e., only 1% DMSO); LPS only (100 μg LPS), ZnPP only control (0.125 mg/mouse**)**, low ZnPP + *Echinacea* + LPS [ZnPP, 0.0125 mg/mouse; *Echinacea*, 1 mg/mouse; LPS,100 μg of LPS per mouse], medium ZnPP + *Echinacea* + LPS [ZnPP, 0.125 mg/mouse; *Echinacea* and LPS dosages, same as in sections above], high ZnPP + *Echinacea* + LPS [ZnPP, 1.25 mg/mouse; *Echinacea* and LPS dosages, same as described above]. Treatment groups that received *Echinacea* were administered first with *Echinacea* 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) 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 *Echinacea* influenced mRNA 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^{o}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^{o}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 Biosystems (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 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-DemandTM 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 were normalized to the geometric mean of the endogenous control gene, GusB.

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 *Echinacea* 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 xg at 4°C. Once centrifuged, the supernatant was transferred to a pre-chilled eppendorf tubes and stored in -80°C freezer until ready for use.

BCA Protein Assay: Once protein was extracted from the individual cervical tissues, a BCA assay was run to quantify protein concentration. Using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA) protein standards made up of varying concentrations of albumin and distilled water were made in eppendorf tubes, as was a working reagent (25 ml Reagent A, 500 µl Reagent B). Protein sample concentrations in either 1:10 or 1:5dilutions were made in eppendorf tubes. A 96 well micro plate was loaded with each standard in

triplicate (25 µl per well) and protein samples were loaded in triplicate (25 µl per well). After loading the plate, 200 µl of working reagent was added to each well. The plate was then incubated at 37°C for 30 minutes. At the end of the 30 minutes incubation period, the plate was read in a spectrophotometer (Thermo Scientific, USA) at 562 nm, giving protein concentrations in ng/µl.

Gel Electrophoresis: Once protein sample concentrations were quantified via BCA assay, sample proteins were prepared for gel electrophoresis. The volume of 10 µg of sample protein was calculated using values generated from the BCA assay. Once protein volume was calculated, volume of distilled water was also calculated [10 µl Total in well – desired protein volume (µl) – 2.5 µl (Sample buffer) – 1.0 µl (reducing agent)]. NuPage LDS Sample Buffer (4x) was added to each tube, followed by the correct amount of distilled water. Protein samples were then vortexed prior to the addition of the calculated volume of protein. Once tubes had all their respective solutions added, they were vortexed for 10-15 seconds before being centrifuged for 30 seconds (or until all liquid had settled at the bottom of the tube). Tubes then sat at room temperature while the electrophoresis chamber was set up. While setting up the chamber, we first removed the pre-made gel from its plastic before taking off the comb and white tape; then the wells were flushed via pipette with running buffer. The gels were then loaded into the electrophoresis apparatus, with the clamp down. We then carefully poured a small amount of 1x NuPage running buffer in between the two gels before making sure there were no leaks. If there were no leaks, running buffer was added halfway up the chamber before adding 500 µl of NuPage Antioxidant; once antioxidant was added, the remainder of the chamber was filled with running buffer just above the wells. At this time, the electrophoresis chamber set up was complete, so we

incubated protein samples in the water bath at 65°C for 10 minutes. While the protein was incubating, 1.3 µl of ladder was loaded into the gels into their designated wells (dependent on the gel map designated for each particular gel). After incubation but before loading of protein samples, we added 1.0 µl of NuPage Reducing Agent to sample tubes. Then we loaded the 10ul samples consisting of protein, distilled water, sample buffer and reducing agent to each well. After the gels were loaded, the outer chambers were filled with running buffer. Gels were then run at 125 volts for approximately 1 hour (we stopped once the blue dye was about to the bottom of the gels). The final step in the gel electrophoresis process involves the use of an iBlot machine. The first step was to set up the machine by putting the bottom cathode on the bottom and soaking a piece of filter paper in distilled water until the gel was ready. Gels were removed from the electrophoresis chamber one at a time, and using the spatula provided, the cassette was slowly peeled off being careful not to tear the gel; then the tops of the wells were cut off to form a nice square shape. The gel was then placed above the bottom cathode (cassette up, gel facing down) and slowly transferred from the cassette to the bottom cathode. Once the gels were placed onto the bottom cathode, making sure no loose pieces of gel were on top or around the gel, filter paper was placed on top of the gel, with the air bubbles rolled out using the rolling pin. The top cathode was then removed from its packaging and placed on top of the filter paper (shiny side up), with all the bubbles rolled out with the rolling pin. At this point we placed the sponge on top with the metal part of the sponge touching the metal part of the iBlot. The machine was then latched shut and a red light shined indicating proper assembly of the apparatus. The machine was set to P3, 7:00, and then ran for 7 min. At the end of the 7 min run time, the sponge and top cathode were removed, exposing the filter paper, which was removed carefully. At this point, before

removing the gels, a razor blade was used to cut the membrane apart separating both membranes, and then slowly and carefully the gels were removed. Next the membrane was submerged in blotto and incubated overnight in 4^{o}C.

Primary Antibody Incubation: During this stage of western blot analysis, primary antibodies were diluted to the appropriate dilutions previously optimized in our lab as suggested by the manufacturer [Santa Cruz Biotechnology, USA (NFKB 1:500, IL-6 1:1000. COX-II 1:500, Beta-actin 1:1000)] and added 2 ml per membrane; 1 ml front, 1 ml back. Using plastic sandwich bags, small bags were prepared for the membrane and primary antibody. The membrane was then removed from blotto and put into the middle of the bag with one end towards the spine of the bag. As antibody dilution accuracy is key, it was important to remove as much blotto as possible without drying out the membrane. Next, both sides of the bag were sealed before primary antibody was pipetted into the bag (1 ml in the front, 1 ml in the back). Before sealing the top of the bag, all air bubbles were removed. Once the top was sealed, the membrane was placed on a shaker with a heavy flat surface on top (such as a huge text book) to ensure even distribution of the antibody. The membrane was then left overnight on the shaker.

Secondary Antibody Incubation and Imaging: Once membranes were allowed to incubate overnight with primary antibody, we removed them from the shaker and removed the membrane from its bag into a small container containing 1x TBST for a 10 minute wash. The membrane was then washed twice more for 10 minutes each after the initial wash. During the wash period, the secondary antibodies were diluted to the appropriate dilutions previously optimized in our lab as suggested by the manufacturer [Santa Cruz Biotechnology, USA (NFKB 1:10,000, IL-6 1:10,000. COX-II 1:10,000, Beta-actin

1:10,000)]). Sandwich bags were once again used to make bags for the membrane and secondary antibody to be incubated in. After the third wash in 1x TBST, the membrane was placed in the middle of the open bag, with both sides sealed (as described for primary antibody incubation). Secondary antibody was pipetted in (1 ml in front, 1 ml in the back). Before sealing the top of the bag, all air bubbles were removed. These membranes were then placed on a shaker underneath a heavy textbook for one hour. After the hour long incubation period, the membrane was removed from the bag and washed in 1x TBST for 20 minutes. After the initial wash, the membrane was washed twice more in 1x TBST for 10 minutes each wash. After the three washes in 1x TBST, the membrane was washed in 1x TBS for 10 minutes. During this last wash, the developer was booted up according to manufacturer's instructions. While membranes were washing we prepared a peroxidase/luminol enhancer solution in the dark, as this solution is light sensitive (2 ml peroxidase solution, 2 ml of luminol/enhancer solution). The solution was vortexed and kept in the dark. Once all washing was complete, membranes were placed on saran wrap and 1 ml of peroxidase/luminol enhancer solution was added to each membrane, soaking for 5 minutes. At the end of the 5 minute period, membranes were picked up to drip off as much solution as possible without drying the membrane out. The membrane was then placed on a new piece of saran wrap which was folded over to cover the membrane, being careful not to have any creases or bubbles. The membranes were then places in a developing cassette and taken to the developer machine.

Developing Western Blot Membranes: The developer is housed in a dark room. Water container was checked and verified to be empty prior to starting the development process. The machine was further prepared for use following manufacturer's instructions.

Once the machine was ready to use, we exposed the membranes onto photo paper for varying increments of time. Once this designated time period had passed, the photo paper was removed from the developing cassette and placed into the developer. Once the paper completed its run through the developer, we were able to take the film which now showed bands of protein indicative of each target proteins; we were able to quantify the changes in protein expression as compared to beta-actin using ImageJ (National Institute for Health, USA) for analysis.

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 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

In order to determine whether *Echinacea* 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 outlined in Figure 3.

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 model system

***Echinacea* suppresses the activity of NFκB in the cervix of non-pregnant in vivo model system.**

We used the non-pregnant *in vivo* model, as this was the most commonly used model in our lab at the time. Here, we investigated the effects of *Echinacea* on the levels of

activated (phosphorylated) NFκB. In the experiments where we examined NFκB, we used protein expression as an indicator of activity, as phosphorylation is indicative of activity. Our hypothesis is that *Echinacea* regulates LPS-induced transcription of pro-inflammatory factors by reducing the levels of activated NFκB, such as IL-6 and TNFα. For this experiment, the control (C) was only treated with 0.9% NaCl for baseline levels. LPS was used as a positive control, as it robustly simulates infection-induced inflammation. Finally, the treatment group was initially administered *Echinacea* followed four hours later by LPS, in order to determine whether *Echinacea* can effectively block LPS-induced NFκB activities. 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. *Echinacea* administered prior to LPS treatment, revealed a 4 fold (p= 0.0256) decrease in p-NFκB protein levels compared to LPS alone, showing p-NFκB levels were significantly inhibited by *Echinacea* compared to LPS alone (Appendix, Figure 4).

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

Having shown *Echinacea'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 *Echinacea* could suppress LPS-induced levels of IL-6 mRNA and protein, in our preterm labor *in vivo* model, which is normally associated with increases in matrix metalloproteinases that are responsible for degradation of collagen in the cervix during cervical remodeling. 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. Successful suppression of these two pro-inflammatory factors (IL-6 and COX-II) by *Echinacea* could imply its potential for modulating inflammation-induced cervical remodeling. The negative control (C) in this experiment was treated as described above, however for the positive control (LPS only) and the treatment group (*Echinacea* + LPS) a higher dose of LPS (250 μg/mouse) was administered. Here, we show that *Echinacea*, sharply inhibits IL-6 mRNA and protein levels by 11 (p= 0.00019) and 2.2 fold (p=0.0297), respectively (Appendix, Figure 5a-b). We also show that *Echinacea* robustly inhibits COX-II mRNA levels by almost 200 fold (p= 0.000258).

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

Having characterized some of *Echinacea'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 *Echinacea'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. The negative and positive controls, as well as *Echinacea* treatment in the non-pregnant *in vivo* model were performed as described earlier (see above), whereas in the *ex vivo* model, the negative control was vehicle-treated only (RPMI 1640), while the LPS treatment was ten-fold lower than the *in vivo* model (1 μg/well), and *Echinacea* treatment was 1 mg/well. We show the presence of epithelial and stromal cells, indicative of tissue structure. We also show that the *Echinacea* and LPS treated tissues look more like the negative control than the LPS only treated group, showing support for *Echinacea* combating the effects of LPS on these tissues (Appendix, Figure 6A-F).

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 *Echinacea'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 *Echinacea* only, to study the effects of *Echinacea* alone compared to control or baseline levels of IL-6 and COX-II mRNA. We show that *Echinacea* significantly decreased LPS-induced IL-6 mRNA levels compared to LPS alone by 2.6 fold, with no statistical difference between the *Echinacea* alone treatment and the *Echinacea* and LPS treatment groups (Appendix, Figure 7a). The same trend was noted with COX-II mRNA levels, with a fold change of 1.8 (Appendix, Figure 7b). No significant difference was noted between *Echinacea* alone group and the negative controls, for both pro-inflammatory factors.

Thus far, we have demonstrated *Echinacea's* ability to inhibit expression of LPS-induced IL-6 and COX-II mRNA in both the *in vivo* preterm and *ex vivo* non-pregnant model, as well as the levels of p-NFκB in the non-pregnant *in vivo* model. Next, we sought

to investigate whether *Echinacea* could diminish p-NFκB levels in an *ex vivo* model. Cervices were essentially treated as described earlier, except for the addition of a dose-dependent treatment of *Echinacea* (doses ranging from 0.01 mg/well – 1.0 mg/well), with LPS dosage unchanged. Here, we show that *Echinacea* inhibits LPS-induced activation of NFκB, in a dose-dependent manner, with the medium and highest concentration of *Echinacea* decreasing p- NFκB levels 2 and 3 fold, respectively, compared to LPS alone (p=0.0287) (Appendix, Figure 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 (Appendix, Figure 8).

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

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

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

Thus far, we have demonstrated *Echinacea'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 *Echinacea'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 were essentially treated as previously described, except for the addition of a ZnPP only treatment, as well as a dose-dependent treatment of ZnPP (doses ranging from 0.0125 mg/mouse to 1.25 mg/mouse) with *Echinacea* and LPS doses unchanged. Animals treated with ZnPP, *Echinacea,* 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 (Appendix, Figure 10).

Discussion

The purpose of the present study was to examine the ability of *Echinacea* to down-regulate the expression of LPS-induced pro-inflammatory cytokines in mice cervix and its likely underlying mechanisms. The key findings of the study are that *Echinacea*: 1) attenuates expression of certain cytokines associated with precocious cervical remodeling (IL-6, COX-II), in three animal models, including non-pregnant *in vivo, ex vivo* and preterm labor models, 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 *Echinacea's* attenuating effects on the levels of p-NFκB in the cervix, in a dose dependent manner. These data suggest that the NFκB inhibitory activity of *Echinacea* is at least in part, mediated by up-regulation of the inflammatory mediator HO-1 by *Echinacea*. The present study is the first to provide evidence for *Echinacea'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 (Rizzo *et al.* 1996, Raso *et al.* 2002, Menon & Fortunato 2007) and, ultimately, premature birth (Rizzo *et al.* 1996, Alderem & Ulevitch 2000, Beutner 2000, Zhang & Ghosh 2001, Menon & Fortunato 2007). When LPS, a cell wall component of gram negative bacteria, binds to white blood cells (WBCs) expressing its receptor [toll-

like receptor 4 (TLR4)], it activates NFκB and, subsequently, increases the expression of the downstream pro- and or anti-inflammatory cytokines (TNFα, IL-6 and IL-10) (Alderem & Ulevitch 2000, Beutner 2000, Zhang & 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 or upstream of it, i.e., uterus, this (presence of TLR) also implies that *Echinacea* may act not only on the cervix, but also the rest of the female reproductive compartments. Notable pro-inflammatory cytokines associated with preterm labor in humans include IL-6 and TNFα, among others (Rizzo *et al.* 1996). 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). IL-6 activates the immune system through its receptor complex, IL-6Rα and gp130 (Kamimura *et al.* 2004), which in turn activate downstream transcription factors, such as STAT3 and MAPK (Kishimoto *et al.* 1995). Another key factor induced by infection is COX-II, the enzyme responsible for converting arachidonic acid to prostaglandins, which play an important role during labor (Gross *et al.* 2000). These pro-inflammatory factors are expressed in a variety of female reproductive tissues, including myometrium, fetal membranes and the cervix before and after labor (Young *et al.* 2002). In the same study, Young *et al* (2002) showed that invading leukocytes secrete pro-inflammatory cytokines in multiple reproductive tissues, notably the myometrium, cervix, placenta, and fetal membranes, including IL-1β, IL-6, IL-8, and TNFα in biopsies taken after labor than in those obtained before labor (Young *et al* 2002). Most importantly, an increase in IL-6

concentrations in cervicovaginal fluid (most likely from leukocytes) has been observed during labor at term and preterm (Young *et al.* 2002). There is also support for the cytokines themselves acting as mediators of leukocyte recruitment at term, in a positive feed-forward manner – i.e., cytokines mediate leukocyte infiltration and the leukocytes in turn produce more cytokines, ultimately stimulating uterine contractions by inducing prostaglandin production (Young *et al.* 2002). Taken together, the strong link between inflammation and preterm labor, and the ability of *Echinacea* to modulate pro-inflammatory factors, makes the present findings of potential clinical importance.

Medicinal plants have been used in various regions of the world for several centuries, including North America (Foster 1991, Hutchens 1992, Flannery 1998). However, the development of modern synthetic chemistry and target screening assays led to a sharp decline in the pursuit of herbal remedies (Binns *et al.* 2002, Woelkart *et al.* 2008, Altamirano-Dimas *et al.* 2009, Hudson 2012). Recently, there has been renewed interest in herbal remedies by the general public in North America, with *Echinacea* topping the list (Cavaliere 2009). Correspondingly, there has been an increase in the number of studies that have characterized various biological activities of *Echinacea*, particularly its anti-inflammatory effects. For instance, studies have shown that *Echinacea* attenuates expression of virus-induced pro-inflammatory cytokines by neutralizing rhinovirus 1a (RV1A)-induced cytokine secretion (Sharma *et al* 2009b), including an almost complete inhibition of IL-6 and TNFα activities at a dose of 40 μg/ml of *Echinacea* extract (Sharma *et al* 2009b). 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, *Echinacea* extracts inhibited the levels of the pro-inflammatory factors [TNFα,

nitric oxide (NO), and inducible NO synthase (iNOS)] (Zhai *et al.* 2009), dose-dependently. Of note, *Echinacea* does not only inhibit LPS-induced inflammation, but also other inflammation-inducing irritants, such as carrageenan and croton oil. Specifically, *Echinacea* administered intravenously attenuated carrageenan- and croton oil-induced rat paw edema and mouse ear tests (Barnes *et al.* 2005). Collectively, the present data are consistent with these previous data, and indicate that *Echinacea* can modulate inflammation in different tissues of the body.

Echinacea's ability to reduce inflammatory cytokines has been demonstrated in a variety of *in vivo* and *in vitro* models. For instance, the effects of the three commonly used *Echinacea* species, *E. angustifolia, E. pallida, and E. purpurea*, both *in vivo* and *in vitro*, to inhibit the production of inflammatory mediators – such as nitric oxide (NO), TNFα and IL-1β in activated macrophages, has been demonstrated (Zhai *et al.* 2007). Another study using an *in vitro* model sought to test the effects of *Echinacea* treatment and rhinovirus infection on the activation of transcription factors in the BEAS-2B human bronchial epithelial cell line (Sharma *et al.* 2006). The findings from this study showed an increase in 12 different transcription factors by both *Echinacea* extracts (uninfected) from basal levels and a decrease in transcription factors by both *Echinacea* extracts when infected with rhino virus – most dramatically with STAT4 and NFκB (Sharma *et al.* 2006). This implies that *Echinacea* can act to down-regulate specific transcription factors associated with inflammation (Sharma *et al.* 2006). Another study using BEAS-2B human bronchial epithelial cells looked at the effects of *Echinacea* treatment and rhino virus infection on pro-inflammatory cytokines IL-6 and IL-8 (Sharma *et al.* 2009a). This study found rhino virus induced IL-6 and IL-8 increases can be reversed by *Echinacea* (Sharma *et al.* 2009a). Other models have also been

studied. For example, male BALB/c mice were used in a study conducted by Raso *et al*, where the anti-inflammatory effect of *Echinacea* was evaluated against carrageenan-induced paw edema in mice (Raso *et al.* 2002). Mice were treated by gavage with *Echinacea* three days before injection with carrageenan and 72 hours post-injection (Raso *et al.* 2002). This study found *Echinacea* to significantly inhibit edema and decrease COX-II and inducible nitric oxide synthase (iNOS), as well as inhibition of LPS-induced COX-II expression in peritoneal macrophages (Raso *et al.* 2002). Another study using peripheral blood mononuclear cells (PBMC) attempted to track down the molecular method of action of *Echinacea* (Gertsch *et al.* 2004). In this particular study, cells treated with *Echinacea* alone showed an increase in TNFα, β-actin and IL-8, with a marked decrease in IL-2 levels; however, *Echinacea* did inhibit LPS-mediated TNFα protein levels (Gertsch *et al.* 2004). Alkylamides, one of the bioactive constituents in all *Echinacea* ethanolic extracts, were found to significantly inhibit TNFα protein expression, as well as LPS-induced increase in NFκB activity (Gertsch *et al.* 2004). This study suggested that the effects of *Echinacea* 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). It is this final study, perhaps, that is most important to the present study. Not only does it show marked inhibition of LPS-induced NFκB activity, but also attributes the activity to alkylamides, which are bioavailable in plasma following oral ingestion of *Echinacea* (Toselli *et al.* 2009). Taken together, all of these studies show strong support for *Echinacea* (specifically alkylamides) as a modulator of inflammation.

Here, we also examined *Echinacea's* effect on the enzyme HO-1, which has several anti-inflammatory links, but has not been reported previously in reproductive tissues

(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 these catabolic by-products (Nath *et al.* 1992,Willis *et al.* 1996, Lee & Chau 2002, Otterbein *et al.* 2003). Indeed, mice lacking HO-1 develop chronic inflammatory diseases (Kapturczak *et al.* 2004). In the liver, *Echinacea*-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 (Amersi *et al.* 1999). Further, Hou and others used murine macrophage cells to demonstrate that *Echinacea*-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. In Figure 9, we show up-regulation of HO-1 mRNA (non-pregnant *ex vivo*) and protein (preterm *in vivo*) by *Echinacea*, which is consistent with previous studies; however, no significant difference in levels of both HO-1 mRNA and protein in cervices treated with *Echinacea* plus LPS compared to LPS alone were observed. This implies that LPS has no significant effect on *Echinacea's* ability to up-regulate HO-1 mRNA and/or protein. We also show that a known HO-1 inhibitor (Figure 10) attenuates *Echinacea's* anti-inflammatory effects dose-dependently via up-regulating levels of activated or p-NFκB, implying that HO-1 mediates Echinacea'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.

Currently, our knowledge and understanding of HO-1's action in reproductive biology is very limited. Notably, and of relevance to the current study, are reports that suggest that miscarriages in both human and mice, which are commonly triggered by microbial infection and inflammation (Menon & Fortunato 2007, Zenclussen *et al.* 2011), are associated with diminished local levels of HO-1. This implies that HO-1 may be an important player in modulating inflammation and the resultant loss of pregnancy. Further, this may perhaps explain why deletion of HO-1 leads to suboptimal placentation, as well as fetal lethality in mice. The present study is the first to demonstrate HO-1's mediatory anti-inflammatory activities for *Echinacea* in reproductive tissues in general, and cervix, in particular. These findings are of interest in that they show, for the first time, that *Echinacea* influences expression of a potentially important gene that may have broad reproductive functions, i.e., HO-1 does not only modulate inflammation, but also other key reproductive events associated with pregnancy, such as placental and fetal growth and viability (Zenclussen *et al.* 2011).

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 *Echinacea'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 *Echinacea* 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. To date, whole hydro-ethanolic *Echinacea* has over 19 bioactive isolated compounds that demonstrate anti-inflammatory activities, of note, luteolin, apigenin, caffeic acid, beta-sitosterol and limonene (Duke 1992). Generally, according to recent reports, the most probable sub-fractions of *Echinacea* likely to mediate its anti-inflammatory properties are alkylamides and polysaccharides (Clifford *et al.* 2002, Raso *et al.* 2002, Kraus *et al.* 2006, Hinz *et al.* 2007). These compounds have been shown to influence multiple inflammatory signaling pathways and at various steps, including inhibiting the activity of IKKβ, interferon regulatory factor 3 (IRF3), and TLR3/TLR4-induced NFκB or IkB degradation (Lee *et al.* 2009). The inhibition of these upstream factors decreases the activity of a plethora of downstream pro-inflammatory factors, such as TNFα, IL-6, IL-12, NOS, COX-II (PGE_2) (Bouic & Lamprecht 1999, Shin *et al.* 2004, Ha *et al.* 2008, Lee *et al.* 2009, Moon *et al.* 2009, Alappat *et al.* 2010, Loizou *et al.* 2010; Yoon *et al.* 2010). Looking at Figure 5 and *Echinacea's* effect on p-NFκB levels we see a strange increase in NFκB levels in cervices treated with low *Echinacea* prior to LPS compared to cervices treated with LPS alone. Here, we expected levels to still be high compared to measurement of NFκB activity in the presence of a higher dose of *Echinacea.* One possible explanation for this could be post-surgery infection in the mouse after ovariectomy that had not been resolved prior to excision of the cervix from the animal. While the increase is strange, it does not

appear to be statistically significant compared to LPS alone, however conducting further studies may be necessary.

In the present study, we use three different animal models that complement each other. For example, in our preterm model, the response to LPS was seen in the 100 fold range, and *Echinacea* drastically reduced expression of pro-inflammatory cytokines, whereas in both non-pregnant *in vivo* and *ex vivo* models, the response to LPS treatment was mild, with only a 2-3 fold difference compared to Echinacea plus LPS. On average, the *Echinacea* response was similar to the negative control (vehicle only, 0.9% NaCl). This difference in the magnitude of response to infection between preterm and non-pregnant *in vivo* may reflect the differential influence of two major sex steroid hormones on inflammation: i.e., estrogen and progesterone. While estrogen has anti-inflammatory activities, progesterone on the other hand is pro-inflammatory. Therefore, one possible explanation for the remarkably high IL-6 and COX-II mRNA levels in the preterm *in vivo* model (Figure 2) could be due to the influence of estrogen and progesterone on the expression of pro-inflammatory factors in the cervix (Pettipher *et al.* 1996, Deshpande *et al.* 1997, Kershaw & Flier 2004, Gillgrass *et al.* 2005, Nilsson 2007).

In conclusion, we report a novel finding that *Echinacea* attenuates activity of NFκB and expression of its (NFκB) downstream pro-inflammatory cytokines, associated with infection-induced preterm labor in the cervix of mice. We also developed an *ex vivo* cervical model and showed that the enzyme HO-1 could be one of the key mediators of *Echinacea's* anti-inflammatory effects in the cervix (see proposed working model, Figure 11). Through our working model (Figure 11) we propose that *Echinacea* acts through 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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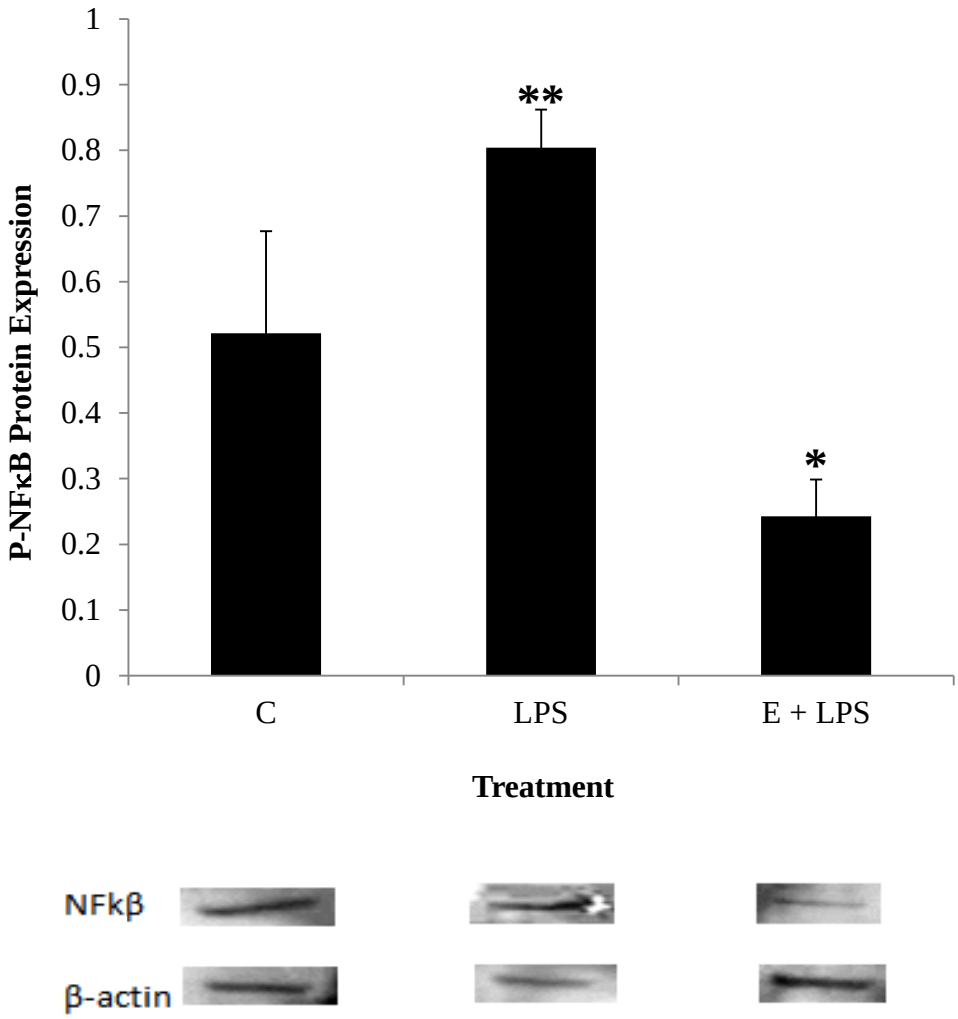


Figure 4 Effects of *Echinacea* on phosphorylated NFκB in the cervix of *in vivo* non-pregnant ovariectomized mice, as revealed by protein expression studies (Western Blot). *Echinacea* administered IP down-regulated levels of p-NFκB protein. Negative control (C) = vehicle only, 0.9% NaCl, IP; LPS alone = 100 µg, IP and *Echinacea* =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.

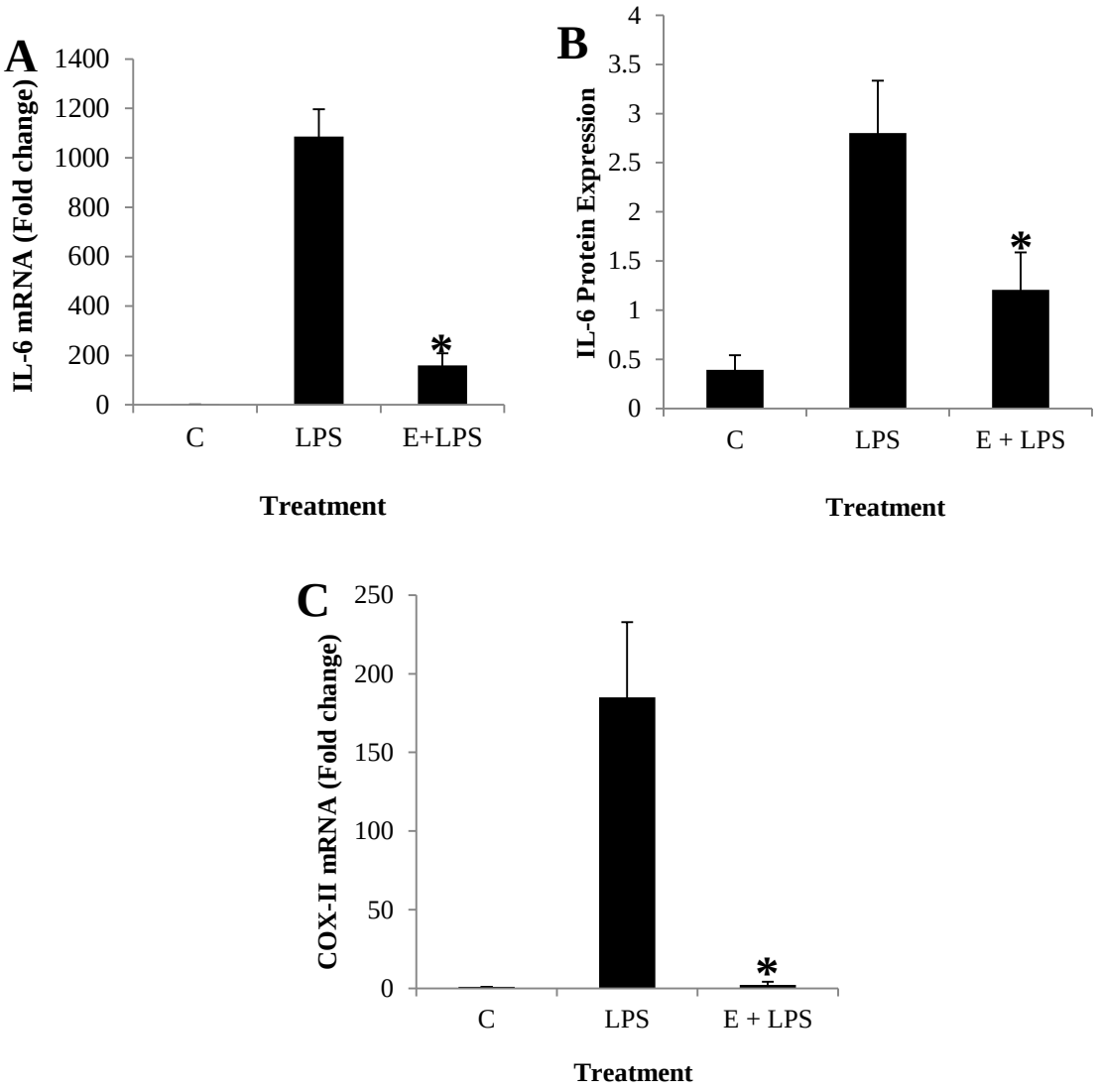


Figure 5 Effects of *Echinacea* on expression of IL-6 mRNA and protein, as well as COX-II mRNA in the cervix of preterm labor mice. **A**) IL-6-mRNA and **B**) IL-6-protein, as revealed by real-time PCR (qRT-PCR) and Western blot analysis. *Echinacea* administered 2 times, IP, has a down-regulating effect 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). *Echinacea* 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.

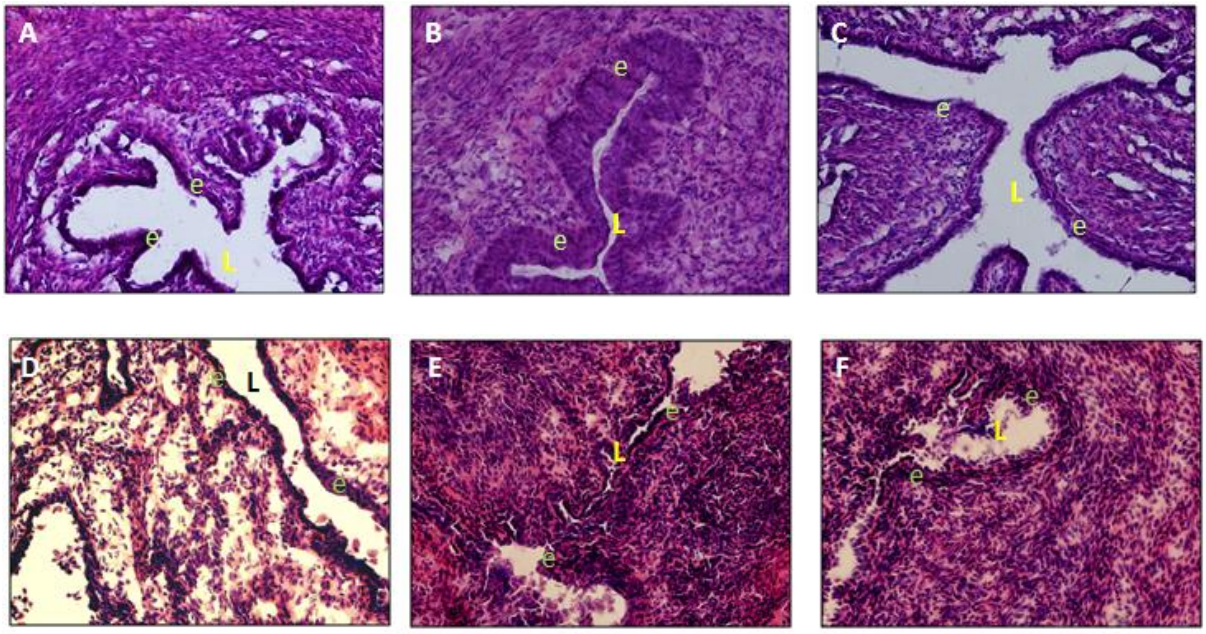


Figure 6 Histomorphogical profile of the cervix of non-pregnant ovariectomized mice treated *in vivo* (**A**-**C**) and *ex vivo* (**D**-**F**) mice treated with *Echinacea*, as revealed by H & E stain. *In vivo* model: Tissues in *Echinacea* + LPS-treated animals (IP), closely resembles histology of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

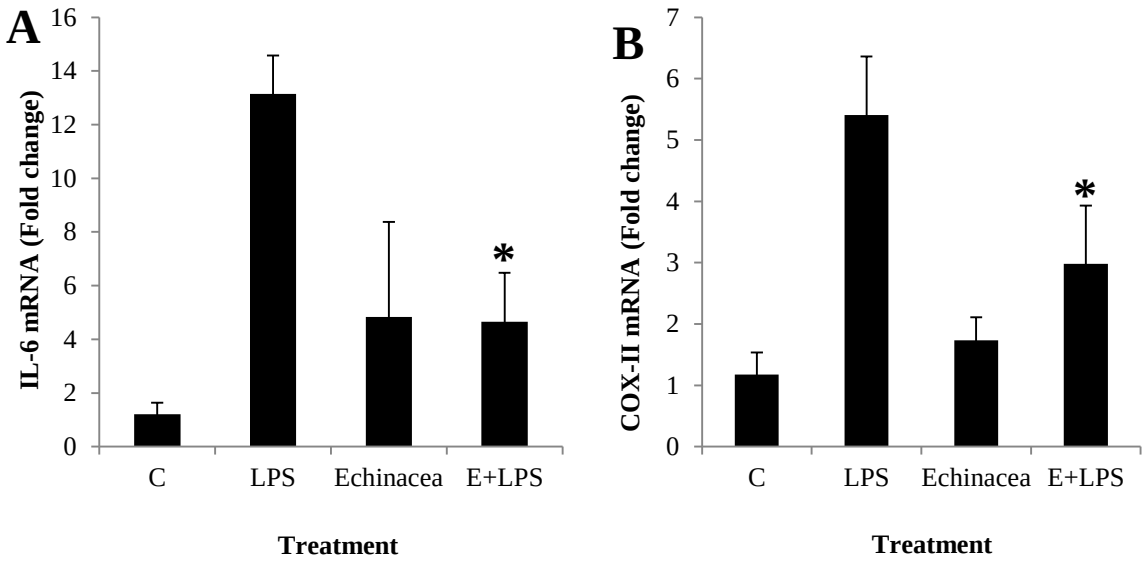


Figure 7 Effects of *Echinacea* on **A**) IL-6 and **B**) COX-II mRNA expression in the mice cervix of the *ex vivo* model (non-pregnant ovariectomized), as revealed by real time-PCR. *Echinacea* has a down-regulating effect on IL-6 and COX-II mRNA levels compared to the LPS alone group. Negative Control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

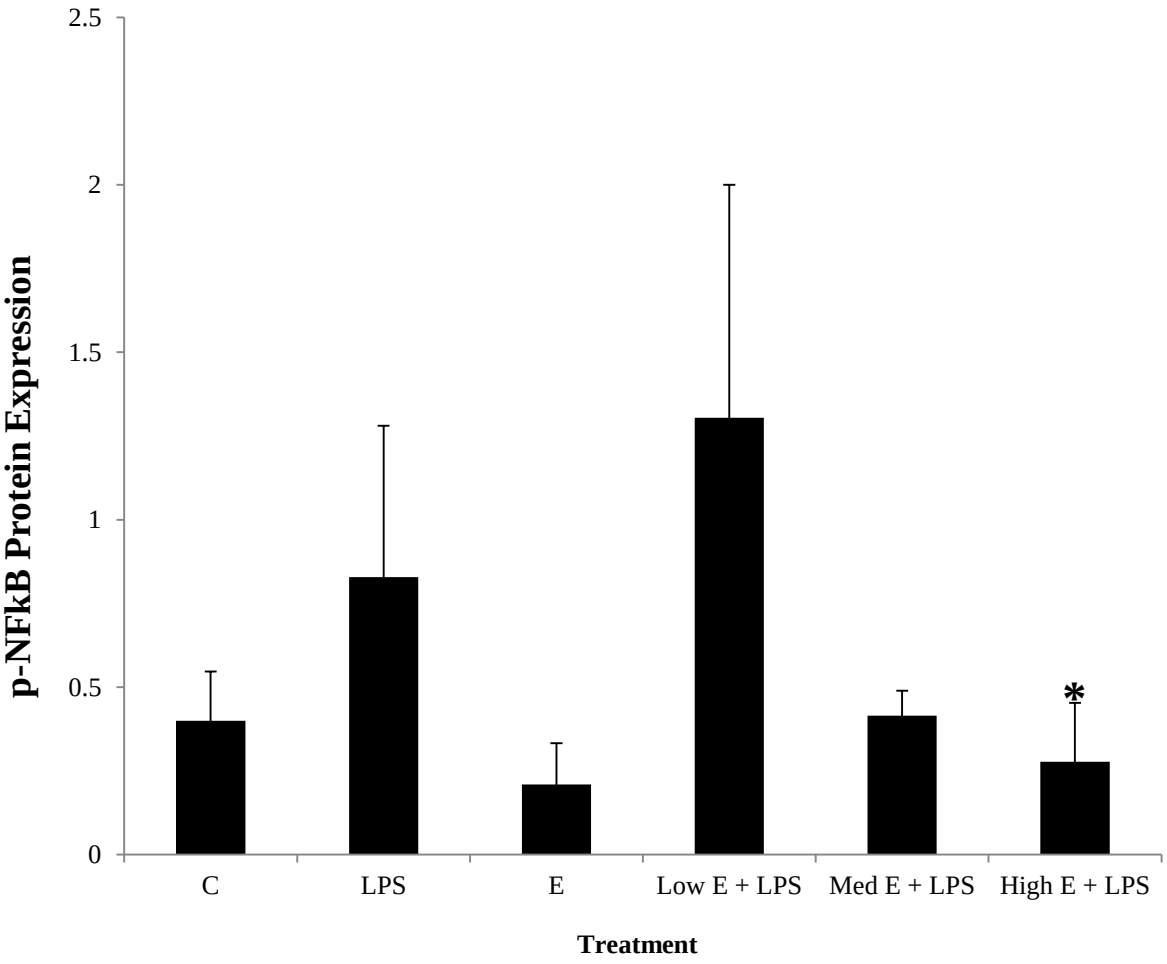


Figure 8 Effects of *Echinacea* on phosphorylated NFκB in the cervix of *ex vivo* non-pregnant ovariectomized mice. *Echinacea* down regulates the activity of NFκB, in the mice cervix of the *ex vivo* model, dose-dependently, as revealed by Western blot analysis. Negative control=vehicle only (RPMI 1640); LPS only = 1μg per well; *Echinacea* only=0.1 mg/well; and *Echinacea* [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.

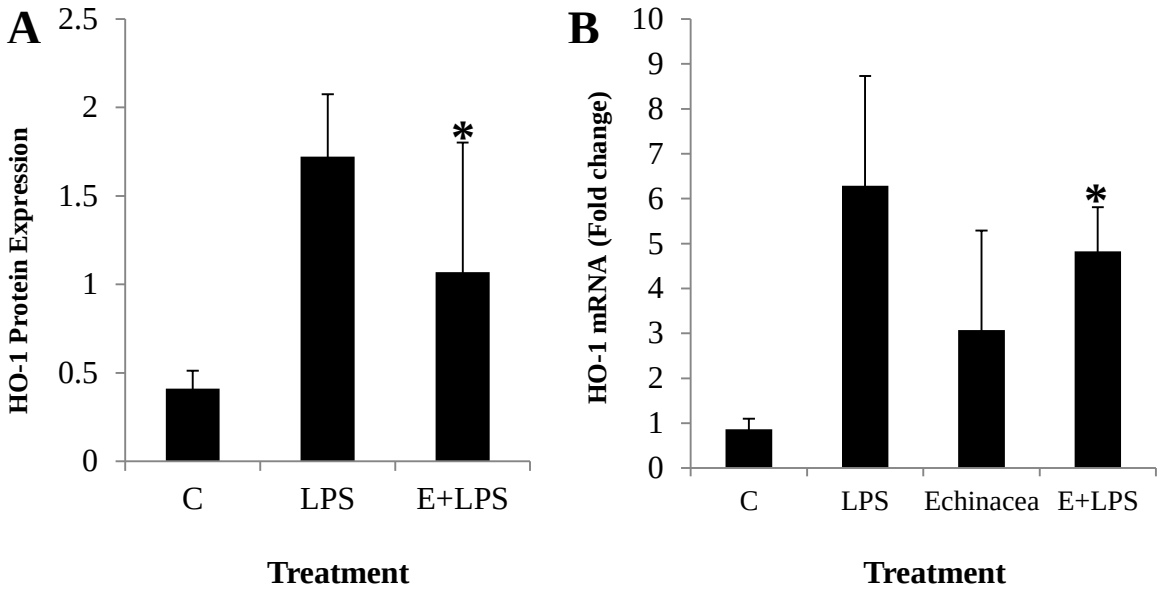
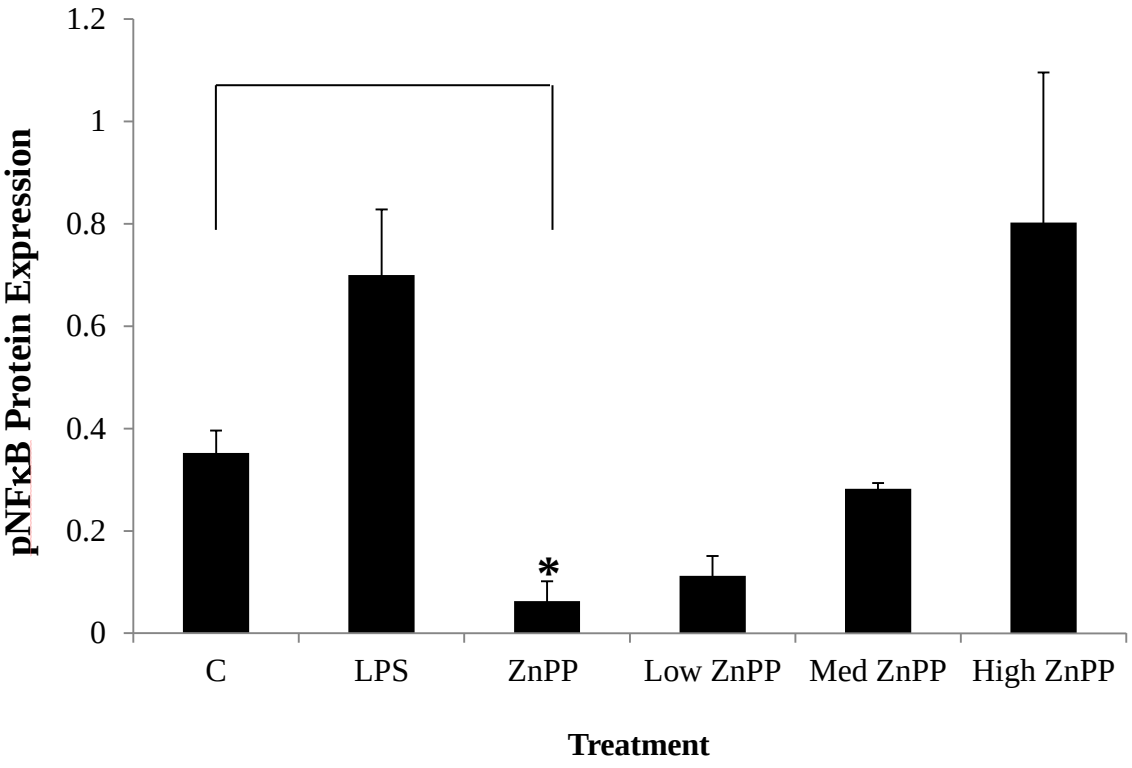


Figure 9 Effects of *Echinacea* on HO-1 protein expression and mRNA levels in the mice cervix of the preterm *in vivo* and non-pregnant *ex vivo* models, respectively. *Echinacea*: **A**) administered I.P down-regulates HO-1 protein levels compared to LPS only in the cervix of preterm labor mouse model, 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.



Vehicle	+	-	-	-	-	-
LPS	-	+	-	+	+	+
ZnPP Low	-	-	-	+	-	-
ZnPP Med	-	-	+	-	+	-
ZnPP High	-	-	-	-	-	+
Echinacea	-	-	-	+	+	+

Figure 10 HO-1 inhibitor blocks *Echinacea'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.

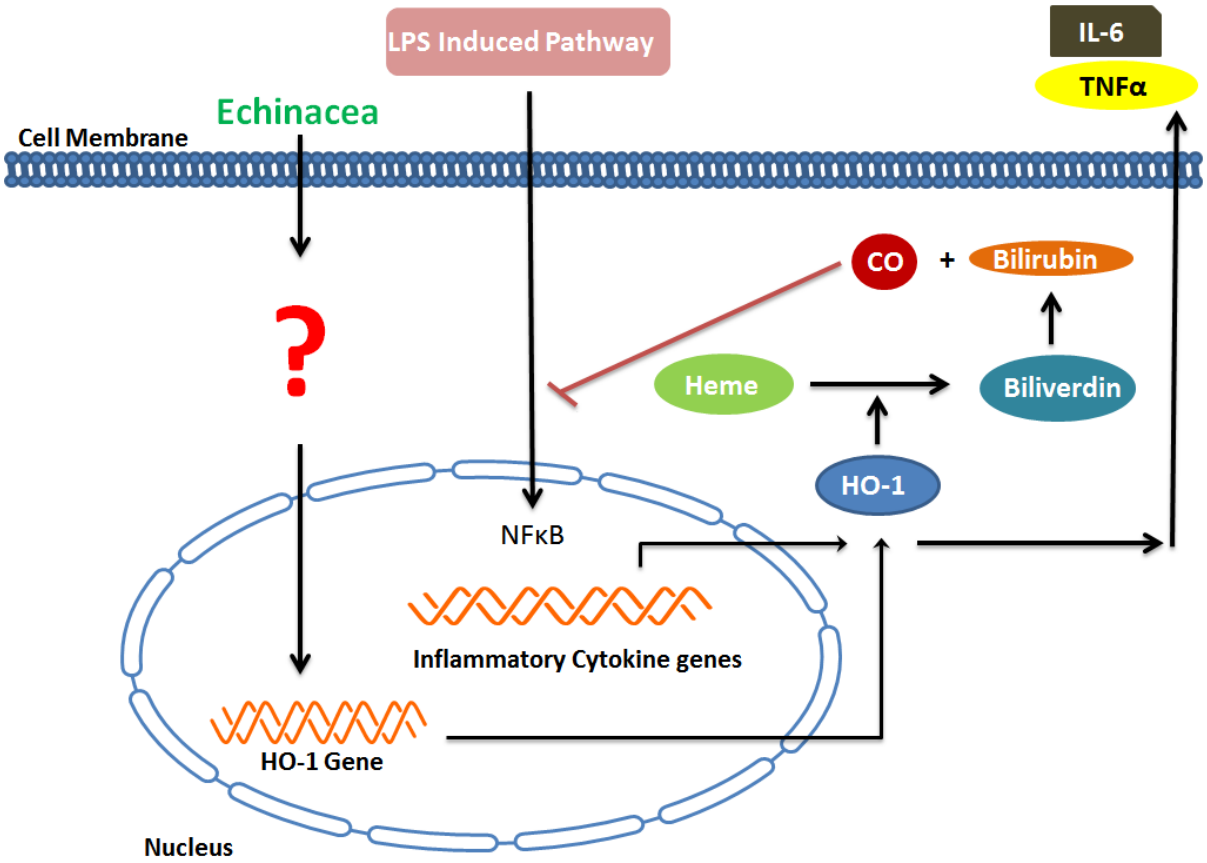


Figure 11 Proposed working model of *Echinacea* in regulating expression of pro-inflammatory factors in the cervix. *Echinacea* induces transcription of heme-oxygenase-1, which in turn blocks transcription of pro-inflammatory factors via its heme byproduct, carbon monoxide (CO).

Vita

Jordan Anne Estes was born in Pittsburgh, Pennsylvania to parents Julia Fries and James Estes on April 20. Jordan is the older sister of Julie Estes, who is pursuing a career in party/event planning, and is five years younger than Jordan. Jordan and Julie started their lives in Pennsylvania and moved around frequently. From Pittsburgh, PA to Greensboro, North Carolina in 1996; from there, they moved to Matthews, North Carolina in 1998, Walnut Cove, NC in 1999 and Kernersville, NC in 2005 where Jordan completed her high school education, graduating from East Forsyth High School in 2007.

During her high school years, Jordan participated in dance team, even making captain her senior year. In the fall of 2007 after graduating high school, Jordan became a freshman at Lenoir-Rhyne University in Hickory, NC where she spent three years working very hard to graduate early. Jordan graduated from Lenoir-Rhyne University in 2010 with her Bachelors of Science degree in biology with minors in both chemistry and classics.

After graduating from Lenoir-Rhyne, Jordan attended Appalachian State University where she pursued a Master's of Science degree in cell and molecular biology. Specifically focusing on reproductive biology combined with herbal remedies, Jordan worked under the guidance of her advisor Dr. Chishimba Nathan Mowa for the last three years and received her Master of Science degree in May 2013. After graduation, Jordan will pursue a career in the cell and molecular field of research.