The Role of Receptor Activation and the Inflammatory Response in the Maintenance of Pregnancy and Parturition

Katie Stutz
University of Redlands

Follow this and additional works at: https://inspire.redlands.edu/cas_honors
Part of the Biology Commons, and the Medicine and Health Sciences Commons

Recommended Citation

This work is licensed under a Creative Commons Attribution-Noncommercial 4.0 License
This material may be protected by copyright law (Title 17 U.S. Code).
This Open Access is brought to you for free and open access by the Theses, Dissertations, and Honors Projects at InSPIRe @ Redlands. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of InSPIRe @ Redlands. For more information, please contact inspire@redlands.edu.
The Role of Receptor Activation and the Inflammatory Response in the Maintenance of Pregnancy and Parturition

Katie Stutz

Research Advisors: Dr. Steven Yellon and Dr. Ben Aronson

Honors Thesis

4.15.2011
Abstract:

Progesterone is a steroid hormone that is responsible in the maintenance of pregnancy and prevention of preterm labor. The withdrawal of progesterone leads to the neuroinflammatory processes associated with cervical ripening and, ultimately, parturition[27]. Such actions of progesterone are thought to be exclusively mediated by binding to classical nuclear progesterone receptors (PR), even though progesterone also binds to glucocorticoid receptors (GR). The purpose of this study was to determine whether PR and/or GR regulate neutrophil immigration as part of the inflammatory mechanism that mediates preterm parturition and remodeling of the cervix. Time-dated pregnant CD-1 mice were ovariectomized (Ovx) on day 16 postbreeding and treated with either a pure GR agonist (dexamethasone, Dex; 0.5mg/kg/day i.m.) or a progestagen that binds to both PR and GR (medroxyprogesterone acetate, MPA; 10mg/kg/day i.m.). Sham-operated and Ovx mice given vehicle capsules served as controls. Female mice were killed before or after birth in order to obtain cervical tissue from both pre- and post-partum cervices. The cervix was sectioned, stained, and analyzed for cell nuclei density, collagen content and resident neutrophils which were counted by image analysis. Mice treated with Dex delivered 24h postovariectomy on day 17, as in Ovx controls. Similarly, mice given MPA delivered at normal term, as in Sham controls. The census of neutrophils in the cervix increased in Sham mice by day of birth (D19), decreased in Dex-treated mice before (D16.5) and after preterm birth, but was unaffected in mice after Ovx or MPA treatment. No differences were seen between groups when analyzed for either cell nuclei or extracellular matrix (ECM) suggesting these processes may not be regulated by PR or GR. These results do not support our hypothesis that neutrophil recruitment is associated with cervical remodeling in preterm. Additionally, although neutrophils may
affect the inflammatory response and degradation of the ECM in the cervix immediately leading up to birth, these results do not support our hypothesis that the PR and/or GR regulate neutrophil immigration in the process of preterm remodeling of the cervix or parturition. Thus, treatment with glucocorticoid receptor agonists are unlikely to be a useful treatment to block preterm cervical ripening or prevent preterm birth. This work supported in part by a NIH maternal and child health grant, HD054931.

**Introduction:**

Premature delivery affects 12.9% of all births and is the cause of at least 75% of neonatal deaths[24]. Preterm birth is defined as birth prior to 37 weeks of gestation and is a significant contributor to perinatal morbidity and mortality yet the mechanisms by which birth occurs remain unclear. The cascade of events that culminate in spontaneous preterm birth has several potential underlying pathways. Four of these pathways, which are supported by clinical and experimental evidence, include excessive myometrial and fetal membrane over-distention, decidual hemorrhage, precocious fetal endocrine activation and intrauterine infection or inflammation[23].

The most optimistic prospect for reducing the incidence of preterm birth is clearly the prevention of preterm labor with the ultimate goal of eliminating neonatal complications and death[16, 23]. The risk of neonatal death is extremely high, and those who survive commonly suffer life-long physical and mental disabilities which arise as complications of immaturity[16]. A major complication commonly seen in preterm infants is the underdevelopment of the lungs. The development of the respiratory system is not fully completed until the last weeks of fetal development, just before birth. Therefore, premature neonates experience difficulties associated with insufficient surfactant, a lipoprotein responsible for
reducing the alveolar surface tension, which is necessary for normal respiration to occur[10]. Infants born prematurely frequently require resuscitation and ventilation at birth. Although this is necessary for the survival of the premature infant, it can damage the immature lung and can prolong the infant’s dependence upon mechanical ventilation. Furthermore, the immature lungs are forced to grow and develop in an environment that is drastically different than that in utero and at a time when the lungs are required for gas exchange[10]. Lack of lung maturation at birth can lead to the inability for the premature infant to draw in its first breath which usually leads to death due to lack of oxygen delivery to the brain and other vital organs[8]. Hypoxia of vital organs can also put the infant at high risk for disabilities such as mental retardation, cerebral palsy, lung and gastrointestinal problems, vision and hearing loss[25].

Current management techniques are aimed at maintaining the pregnancy for a longer period of time so that the fetus can fully grow and develop in-utero before parturition.

Multiple efforts have failed to establish a single effective treatment for preterm labor, partly because the mechanisms regulating the uterus and cervix during pregnancy are not well understood[7]. Because the contracting uterus is the most frequently recognized antecedent of preterm birth, stopping contractions has been the focus of therapeutic approaches[23]. The inhibition of myometrial contractions is called tocolysis, and a drug administration to that end is referred to as a tocolytic agent. In the three decades tocolytics have been in use, none of the agents have lived up to the expectation that prematurity rates would be reduced by these drugs. Although more than 80% of women with preterm labor who are treated with tocolytic agents are able to maintain their pregnancies for an additional 24-48 hours after the drugs are administered, few data suggest that tocolysis maintains pregnancy for a longer period[23]. Additionally, tocolytics may have serious side effects including but not limited to arrhythmias.
in the mother and fetus, heart failure, chest pain, and shortness of breath. A further attempt to
decrease neonatal morbidity and mortality is to speed up the production of surfactant in the
lungs. To this effect, corticosteroids are administered to the mother between 24 and 34 weeks
of gestation. Currently, corticosteroid therapy is the only treatment shown to improve fetal
survival[24]. In a small trial study of pregnant women treated with corticosteroids, no
premature induction of labor or cervical ripening was seen[12]. However, there is no clear
evidence explaining the role of corticosteroids and glucocorticoid receptors in the maintenance
of pregnancy and an unripe cervix or cervical ripening and the induction of labor. The use of
progestational agents to prevent preterm birth among women with risk factors other than
previous preterm birth is currently an area of active research. Fonseca et al. reported that daily
administration of vaginal progesterone reduced the frequency of birth before 34 weeks of
gestation by more than 40% among asymptomatic women with a short cervix as seen on
ultrasonography[23]. In February 2011, the FDA approved a synthetic progesterone drug,
termed Makena, which has been shown to prevent preterm labor. Unfortunately, at this time the
drug is extremely expensive (ranging from $690 - $1500 per dosage) and is not being
administered acutely to women presenting with preterm birth. Currently, the drug is being
given to women who have experienced preterm labor in the past and are thus, at higher risk for
going into preterm labor once again.

Speculations regarding preterm birth are usually focused around asking questions about
the mechanism and determining the “how” and “why” premature birth occurs. Gonzales et al.
hypothesized that preterm cervical ripening may be due to an aberrant regulation in timing of
the same processes that occur at term, or may result from unique molecular mechanisms[9]. It
has been proposed that the earlier activation of the same key biochemical pathways accounting
for full term cervical ripening occur in preterm cervical ripening. At this time, it is unknown whether preterm cervical ripening is just an abnormal regulation in timing, or whether divergent mechanisms and pathways are involved in preterm versus full term birth[9].

In order to better identify the approximate time of the initiation of parturition, researchers have begun to separate parturition into various identifiable phases. The term parturition encompasses all aspects of the birthing process[16]. In the first phase, phase 0, the uterus is unresponsive to contractile stimuli and remains in a quiescent state.

The cervix begins to grow and swell (edema) while the collagen content is actually increasing in order to account for the hypertrophy of the tissue. In phase 1, the myometrium undergoes multiple changes which collectively bring about increased responsiveness, and the cervix begins to show a decrease in the collagen content, preparing for the cervix to become more elastic and dilated. Phase 1 appears to last for several days before the start of phase 2 and compromises the first 95% of pregnancy. Phase 2 is the time of active labor with an associated inflammatory response and the continuation of the degradation of the collagen matrix. Phase 3 follows delivery of the fetus; it is the time of uterine involution where the cervix is repairing itself and returning to its non-pregnant, unripe state. It is during this stage, the collagen content begins to rebuild itself and immune cells tend to disperse[16].

Cervical ripening is known to be characterized by three main changes in the cervix: growth of the cervical tissue (hypertrophy of the cervix), an increase in immune cells to the area (inflammatory response) as well as the degradation of the extracellular collagen matrix.
During gestation, the rigid, collagen-rich cervix acts as a gatekeeper between the fetus and the outside world[26]. However, towards the end of pregnancy, the collagen within the cervix begins to break down. This degradation of collagen matrix, known as collagenolysis, causes the cervix to become more soft and elastic, allowing it to relax and dilate in preparation for the fetus to exit the vaginal canal. This process, known as cervical ripening, provides a safe and relatively easy way for the fetus to travel through the cervix[14]. Failure of the cervix to ripen and remodel can result in dystocia, or difficult labor, and make vaginal births very painful or even impossible for the mother. Previous studies have indicated that cervical ripening precedes myometrial contractions by several weeks, suggesting that the process is of long duration, and the latter is a late event in the pathogenesis of preterm birth[9].

Cervical ripening resembles an inflammatory process in which immune cells, specifically macrophages and neutrophils, migrate into the cervix[26]. Increased activity of macrophages has previously been studied and is thought to be correlated with the breakdown of collagen in the cervix, as the cervix is dependent on collagen for its rigidity[13]. This may be attributed to macrophages increasing their production of both collagenase, an enzyme that breaks the peptide bonds within collagen, and cytokines, chemo-attractant proteins produced and secreted by immune cells[2]. The study of the distribution and alterations presented by the neutrophilic polymorphonuclear leukocytes in a study performed by Junqueira et al. suggest that neutrophils may have an important role in the process of cervical ripening[11]. Additionally, there is growing evidence that leukocyte influx is associated with cervical ripening at birth. In guinea pig experiments, monocytes and neutrophils are observed after normal ripening at term[13]. Because the neutrophil is a major source of collagenase, specifically MMP8, and the cervix is dependant on collagen for its rigidity, cervical ripening has been hypothesized to be
accompanied by an influx of neutrophils[13]. In many cases, a positive relationship between
the amount of infiltrated cells (neutrophils) and the degree of collagenolysis (cervical tissue
breakdown/cervical softening) could be observed[11]. This data suggests that the initiation of
birth could involve the neutrophil’s chemotactic agents[13]. Therefore, it is likely that these
enzymes play a pivotal role in tissue remodeling during cervical ripening.

Past research suggests that we need to study more about the mechanism of progesterone’s
actions in regulating cervical remodeling in order to understand these processes. It is well
documented that the steroid gonadal hormone progesterone plays a central role in the
reproductive events associated with establishment and maintenance of pregnancy[5].
Progesterone’s actions have been interpreted as blocking myometrium stimulants and inhibiting
cervical tissue breakdown[15]. Previous research has shown that an inflammatory process plays
an important role in the cervix during parturition. Because progesterone is found to inhibit the
release of inflammatory cytokines it may, therefore, act as an immunosuppresor in the cervix
during pregnancy[7].

Progesterone plays a dominant role during much of pregnancy, and the withdrawal of
progesterone’s functions appears to be essential for parturition to commence. The actions of
progesterone are mediated by multi-protein complexes that include progesterone-binding
components (receptors) that modify components (coregulators and adaptors) and effector
proteins[28]. The classically accepted mechanism of progesterone action involves a genomic
pathway involving nuclear progesterone receptors. It is hypothesized that progesterone binds
to nuclear receptors on the cell nuclei of the cervix and uterus which alters gene expression and
allows for maintenance of pregnancy[1]. There are currently two known isoforms of the nuclear
progesterone receptors that exist in humans: PRA and PRB. The PRB isoform contains an extra
164 amino acids over the PRA isoform; however functional differences remain uncertain. The significance in changing PR and coregulator activities in functional progesterone withdrawal can be assessed by the determination of whether similar changes occur in animals that deliver after the systemic withdrawal of the hormone[28]. In studies by Zakar et al., PRB does not seem to be essential for pregnancy maintenance in mice, and PRA may mediate the action of progesterone in the murine uterus during pregnancy. Further studies are currently being performed in order to better understand the specific functions that each nuclear isoform plays in maintenance of pregnancy[28].

In most non-primate placental mammals, the withdrawal of progesterone before the initiation of labor is manifest by a significant drop in circulating progesterone levels, which is due to either luteolysis or changes in placental steroidogenesis, which shunts precursors towards estrogen production. However, no such events can be demonstrated in human pregnancy[28]. Since systemic progesterone levels do not fall in women before birth, this does not account for the initiation of labor[13]. Instead, a theory of “functional progesterone withdrawal” has been proposed. The idea simply hypothesizes that the uterus and cervix gradually lose the capability to respond to progesterone’s effects, and as a result cervical ripening occurs despite systemic progesterone levels remaining high in the human body[28].

Because mice share such close physiological similarities to humans, mice were used as the experimental model in this study. We were able to use mice even though they show a drop in plasma progesterone levels because of the idea of the “functional progesterone withdrawal”
in humans. Additionally, mice share many physiological systemic similarities with humans including the reproductive, immune, endocrine and nervous systems. Rodents have long served as experimental models for studies of reproduction in mammalian species. Researchers are able to study these rodents because of the similarities that exist between the murine and human uteri including the multiple structural parallels in the cervix across species regarding collagen content and function. For these reasons, it was practical to assume that studies performed on mice would easily translate to outcomes that could be tested on humans in the future, allowing us to use the mouse as the practical model for this study. Studies have proven that rodents are useful for understanding the role of progestational agents in preterm parturition [26]. Furthermore, mice tend to have a highly predictable gestational period, around 19-21 days [9]. Their predictable gestational period allows researchers to easily predict outcomes and understand results concerning pregnancy, and parturition. The strain used in this study was the CD-1 mouse strain which has a normal gestation period of 18-19 days. It is important to note that because these mice were out-bred, their date of parturition was not as consistent as other strains might have been. However, CD-1 mice were used in this study despite this discrepancy because this strain has been used in previous studies and produced predictable results regarding pregnancy and parturition date using progestagens as well as more precise capsule doses which will be discussed in greater detail in the methods section[20].

The capability of progestational agent treatment to block ripening of the cervix and, as previously reported, arrest preterm birth, adds to accumulating evidence that progesterone may be effective early in the process to prevent preterm birth[26]. Previous studies by Shi et al. show that treatment with the pure progesterone agonist (only binds to the progesterone receptor), promegestone (R5020), was successful at maintaining an unripe cervix in pregnant
rats[22]. Previous research in this study by Jaclyn Cooperrider, lead to the conclusion that R5020 was also successful at maintaining an unripe cervix in ovariectomized mice as well. This data indicates that a compound capable exclusively of binding to progesterone receptors is able to maintain pregnancy, which suggests that the progesterone receptor explicitly plays a role in the maintenance of pregnancy. In a study by Yellon et al., progestational agonist treatment was found to forestall the endotoxin-induced immigration of neutrophils associated with cervical ripening[27]. It is well known that progesterone has a strong binding affinity for both progesterone receptors and glucocorticoid receptors. Additional research has illustrated that the progesterone hormone and other progestagens, such as medroxyprogesterone acetate, possess a strong mixed binding affinity for both the progesterone receptor and the glucocorticoid receptor [19]. Additionally, Dexamethasone, a pure corticosteroid capable of binding only to glucocorticoid receptors, has been studied by Challis et al. to treat pregnant rabbits in order to investigate its role in determining gestational length. Researchers found that treatment with dexamethasone in rabbits resulted in premature delivery[3]. However, because glucocorticoid receptor agonists have not yet shown an ability to mediate either maintenance of pregnancy or cervical ripening on their own, it is unlikely that the binding of progesterone to the glucocorticoid receptor alone will result in the receptor playing a role in these processes[12].

This particular study explored the activation of glucocorticoid receptors and their effect on neutrophil census in the cervix of pregnant mice. The purpose of this study was to determine if the glucocorticoid receptor agonist is able to maintain pregnancy through
sustaining an unripe cervix and whether or not the glucocorticoid receptor agonist would be an acceptable treatment to prevent preterm birth. We wanted to explore this idea because no other studies have been performed regarding the morphology and structure of the cervix after glucocorticoid agonist treatment. Based on previous research, it is unlikely that the glucocorticoid receptor agonist alone will maintain pregnancy and forestall preterm birth [27]. However, it may be possible that the combination of both progesterone and glucocorticoid receptor activation could in fact forestall preterm parturition and facilitate maintenance of an unripe cervix.

Despite the fact that progesterone treatment alone has produced positive results in the maintenance of pregnancy, supporting a strong argument for a solely progesterone receptor-mediated mechanism, it is likely that progesterone’s strong binding affinity for the glucocorticoid receptor plays a role in the mechanism as well. It is possible that the mechanism of action on the cervix by which the mixed progesterone/glucocorticoid receptor agonist, medroxyprogesterone acetate (MPA), acts may not be the same as the effects of the pure glucocorticoid receptor agonist, Dexamethasone (Dex). The glucocorticoid agonist may block the neutrophil recruitment but not inhibit the degradation of the collagen content and structure. This could lead to the novel discovery that neutrophil recruitment is not essential for cervical remodeling. The possible involvement of the glucocorticoid receptor must not be ignored and thus, explored to aid in the full explanation of the mechanism which mediates
preterm cervical ripening and parturition. Thus, the purpose tested in this study investigated the role of the glucocorticoid receptors and their effect on date of birth, remodeling of the extracellular matrix, and immune cell census in pregnant mice. Our hypothesis was then: the glucocorticoid receptor with the progesterone receptor regulates the neutrophil immigration in the mechanism for remodeling of the cervix and preterm parturition.

**Experimental Design and Methods**

**Animals, Surgeries, and Treatments**

Time-dated pregnant CD-1 mice were ordered from Harlan-Sprague Dawley and delivered to the lab on day 15 of gestation. On day 16, these mice were ovariectomized in order to eliminate their natural source of Estrogen and Progesterone. Preparation for surgery included anesthetizing the mice with isofluorine, shaving their back and cleaning the exposed skin with ethanol and betadine. Ovarectomies were performed by making a small, 5-10mm, incision in the mouse’s back and a second incision through the interperitoneal cavity directly below the first incision. Ovaries were identified by their small, spherical shape and white color. The uterine horn was then transected just below each ovary using surgical scissors. The interperitoneal cavity was closed with sutures. Silastic capsule implants were placed in between the cavity wall and the skin; the skin was then closed. There were no obvious effects from the surgeries on the mice or their offspring which was consistent with published observations [27].

Mice were distinguished by the treatment they received post-ovariectomy. The first group served as a control and was subjected to the same physical stress of the surgery including preparation, incisions, peanut oil-filled vehicle implants, sutures, and staples without removal of their ovaries; this group was termed the Sham Control Group. This group served as the control for normal cervical remodeling and term birth. Sham mice were expected to give birth
on day 19, the proposed day of normal term birth. The second group also served as a control. Mice in this group were ovariectomized unlike the sham group and had a silastic capsule filled with peanut oil was placed between the interperitoneal cavity and the skin. Because mice in this group underwent ovariectomies and no further treatment, this group was labeled Ovx. A third group underwent the bilateral ovariectomy and was then injected with medroxyprogesterone acetate (MPA), a mixed progesterone receptor/glucocorticoid receptor agonist. This group was referred to as MPA and received treatment at a concentration of 10 mg/kg/day[6]. The final group was injected with a pure glucocorticoid agonist, dexamethasone (Dex) after the bilateral ovariectomy. This group was termed Dex and was treated at a concentration of 4 mg/kg/day[4].

Table 1. Postbreeding day treatments, cervix harvest (X), and birth

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 16</th>
<th>Day 16.5</th>
<th>Day 17</th>
<th>Day 18</th>
<th>Day 19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham Control</td>
<td>Surgery</td>
<td>X (PreP)</td>
<td>X (PreP)</td>
<td>X (PreP)</td>
<td>X (PP)</td>
</tr>
<tr>
<td>Ovx Control</td>
<td>Surgery</td>
<td>X (PreP)</td>
<td>X (PP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPA</td>
<td>Surgery</td>
<td>X (PreP)</td>
<td></td>
<td>X (PreP)</td>
<td>X (PP)</td>
</tr>
<tr>
<td>Dex</td>
<td>Surgery</td>
<td>X (PreP)</td>
<td></td>
<td>X (PP)</td>
<td></td>
</tr>
</tbody>
</table>

Capsule Preparation

Capsules were prepared using Silastic tubing (1.57 mm inside diameter x 3.18 mm outside diameter – manufactured by Corning) and polyethylene tubing (1.57 mm inside diameter x 2.08 mm outside diameter – manufactured by Clay Adams). Research has previously determined that these capsules are capable of being used in studies regarding the maintenance of pregnancy[21]. Tubes
were cut in order to produce steroid-filled silastic capsules. The tip of a hot glue gun was used to heat-seal the tube. Vehicle capsules, used in Ovx mice, were filled with plain peanut oil using a 1 mL Tuberculin Syringe with an 18 gauge needle and were cut to 1 cm long[18]. The other end of the capsule was then heat-sealed at the end of injection and washed in 90% ethanol to sterilize. Capsules were immersed in phosphate buffered saline (PBS) for 24-hours incubation period prior to implantation in order to create a salt/pH balance that increased the chances for successful implantation of the capsule in the mouse.

**Injections and Implantations**

Ovx mice treated with progestational agonists were injected subcutaneously into the dorsum of the neck on gestational day 16. Mice given the progesterone/glucocorticoid receptor agonist treatment were injected with a 10 mg/kg concentration of MPA[6]. Those treated with the pure glucocorticoid agonist, were injected with a 4mg/kg concentration of Dex[4]. Capsules containing peanut oil vehicle were inserted into Ovx Control mice subcutaneously in the dorsal rump of the neck via a small 1-2 mm incision between the scapulas [27].

**Tissue Collection and Processing**

After ovariectomy and treatment, mice were euthanized with CO$_2$ on days 16.5, 17, 18, 18.5, or 19. The abdomen of each mouse was incised to access the abdominal and thoracic cavities. Upon visualization of the thoracic cavity, blood was drawn from the heart to determine serum progesterone and cortical levels. Following blood draw, the abdominal cavity was exposed to visualize the uterine horns and cervix. The cervix, attached to roughly 1 mm of the lower portions of the left and right uterine horns, was excised and post-fixed in 4% paraformaldehyde. Fixed tissues were embedded in paraffin, which allowed for easier
sectioning of the tissue, blocked, cut in 10 μm longitudinal sections, and placed onto glass slides for processing.

**Immunohistochemical Staining**

The prepared slides were processed by immunohistochemistry to identify neutrophils with the 7/4 antibody. Slides were heated at 60°C for 30-60 minutes to attach sections to the slides. Slides were then subjected to two 10-minute incubation periods with xylene, washed twice for 3 minutes at a time with 100% ethanol followed by 90-95% ethanol for two 3-minute periods. Next, the slides were incubated for two 3-minute washes in 70-75% ethanol; the process ended with one 2 minute rinse in deionized water in order to remove paraffin. In order to maintain a water resistant boundary around the tissue, a pep pen was used. The tissue then underwent a 2 minute incubation period using proteinase K for 2 minutes, 15 minute incubation with 3% hydrogen peroxide and finally, three 5-minute washes with PBS. Slides were then placed in a blocking serum (10% goat serum, 3% of 0.1 solution Triton-X) for 30 minutes and subsequently incubated in a 0.01 mg/ml concentration of the 7/4 antibody for 90 minutes before undergoing three more 5-minute rinse periods with PBS.

Next, the slides were incubated with biotyinylated anti-rat secondary antibody in a 1% concentration for 30 minutes and washed in three additional 5-minute periods of PBS. ABC and DAB (3,3-Diaminobenzidine) substrates were added with PBS washes in between. Slides were stained with hematoxylin (a nuclear stain which stains cell nuclei purple) for 2 seconds. The slides were then rinsed in running water for 15 minutes. Lastly, the tissue was dehydrated by two 5-minute rinses in 75% ethanol, two 5 minutes rinses in 95% ethanol, two 5 minutes rinses in 100% ethanol, and two 10-minute rinses in xylene.
Analysis of Data

Once the slides were stained and dried, images of the tissue sections were taken using a Zeiss microscope Axio Imager A1 and the Spot Advanced program. Eight non-overlapping images were taken on three different tissue sections per animal, allowing for a total of 24 images per animal. This allowed accurate and representative sampling of the cervical tissue and decreased bias in the counts.

Images were then stored and analyzed using Image Pro Plus 6.0. Neutrophil counts were then performed manually based on color, shape and size of 7/4 antibody (brown) stained areas. Cell nuclei counts were also performed manually based on color, shape and size of hematoxylin (purple) stained areas. Counts were then recorded in an Excel spread sheet and normalized to cell nuclei density. This data was analyzed using the “Graph Pad Prism” statistical computer program. Levene’s test, one-way Analysis of Variance (ANOVA) and appropriate t-tests were performed to identify statistical differences between the four groups.

Results:
Figure 1, Progesterone Withdrawal Induces Preterm Birth. Day of parturition post-breeding for mice that were Sham-operated, Ovx, or ovariectomized and treated with Medroxyprogesterone Acetate (MPA) or Dexamethasone (Dex). * p < 0.05 vs Sham group. Each bar represents the mean day of birth (=SE, number of mice/group specified) for that group. Where SE not shown, births occurred on the day indicated. A probability of *p<0.05 was used to determine significance between groups. Date of surgery indicated by red line, while normal term birth in blue line is based upon our previous reports [17].

Sham-operated mice delivered on gestational day 18 and 19. As expected, mice in the ovariectomy control group all gave birth on day 17, approximately 1-2 days prior to normal term delivery. Mice that underwent ovariectomies and later treated with MPA delivered on days 18 and 19 post-breeding, similar to the Sham control group. Dexamethasone treated mice experienced parturition on day 17, much like that of the ovariectomy control group (Figure 1). A one-way ANOVA of this data revealed that both the Ovx control group and Dexamethasone group were statistically different (p < 0.05) from the Sham-operated control.
Figure 2. Neither PR nor GR affect remodeling of the extracellular matrix (ECM). Optical density of the ECM in prepartum and postpartum mice subjected to Sham treatment, ovariectomy (Ovx), ovariectomy followed by Medroxyprogesterone Acetate (MPA) or Dexamethasone (Dex). Optical Density data was normalized to cell nuclei density in order to account for tissue hypertrophy of the cervix that occurs in the normal process of pregnancy. Each bar represents the mean optical density to cell nuclei density per μm³ of tissue (±SE, number of mice/group specified). * p < 0.05 vs. D16.5 Dex

Optical density data shows that neither activation of the PR or the GR is involved in ECM remodeling. This result can be seen through the lack of significant difference between groups treated with PR or GR and Sham. Data does demonstrate a slight significant different between D16.5 Dex and PP Dex groups however this may be due to the decreased of variability and smaller SE bars within the dexamethasone group as well as the decreased mean within the D16.5 Dex group. Dexamethasone has been noted to have an anti-inflammatory effect, which is why, D16.5 in this group has a lower optical density and greater collagen content. It is important to note that this data shows that dexamethasone is promoting more collagen and more structure within the cervix. However, based on previous research by Yellon et. al. the D16.5 cervix has ripened relative to a non-pregnant cervix and is sufficient for parturition Additionally, D16.5 is approximately 8 hrs after treatment with the GR agonist, whereas the postpartum group is 24 hrs
after treatment leading us to believe that this increase in OD may be related to dexamethasone wearing off. At this time, there is no clear evidence to support this hypothesis. It is important to note that the dexamethasone postpartum group is not significantly different from any of the other PP groups (Sham, Ovx, or MPA) suggesting that this statistical difference may not be biologically important for the process that remodels the cervix. No significant difference was found across postpartum groups using a one-way ANOVA (Figure 2).

Figure 3. GR agonist inhibits hypertrophy of cervix by day before birth. Cell nuclei counts in prepartum and postpartum mice subjected to Sham treatment, ovariectomy (Ovx), ovariectomy followed by Medroxyprogesterone Acetate (MPA) or Dexamethasone (Dex). Each bar represents the mean cell nuclei density per μm$^3$ of tissue (±SE, number of mice/group specified). $^b$ p < 0.05 vs. D17 Sham, $^b$ vs. D16.5 Dex.

Cell nuclei analysis provides data supporting that treatment with a GR agonist inhibits the hypertrophy of the cervix associated with normal term birth by the day before birth. Previous research in our laboratory has noted that D15 mice show a cell nuclei count of approximately 7 μm$^3$ x 10$^3$. Because of this, we have reason to believe that treatment with dexamethasone blocks the drop to 4 μm$^3$ x 10$^3$ that is seen in the other groups (Sham, Ovx, and MPA) by D16.5,
indicating an inhibition of the growth within the cervix. As previously stated, dexamethasone is known to have anti-inflammatory effects, therefore it would be expected that Dex D16.5 would be have a higher mean demonstrating decreased growth. Additionally, the mean of D16.5 cell nuclei is much higher than D16.5 in the other groups suggesting that the significant difference seen between D16.5 and the postpartum cervix in the dexamethasone group could be due to the decreased variability seen with treatment of Dexamethasone. The postpartum Dexamethasone group is not significantly different from Sham, Ovx or MPA suggesting that the cervix grows and stretches independent of the treatment received. No significant difference was found across postpartum groups using a one-way ANOVA (Figure 3).

![Graph showing Neutrophil/Cell Nuclei/μm² x 10²](image)

**Figure 4, Progesterone Withdrawal, Along with PR & GR Agonists, Prevents Neutrophil Immigration.** Neutrophil census in prepartum and postpartum mice subjected to Sham treatment, ovariectomy (Ovx), ovariectomy followed by Medroxyprogesterone Acetate (MPA) or Dexamethasone (Dex). Neutrophil counts were normalized to cell nuclei density in order to account for tissue hypertrophy of the cervix that occurs in the normal process of pregnancy. Each bar represents the mean neutrophil census normalized to cell nuclei density per μm² of tissue (±SE, number of mice/group specified). *p < 0.05 vs. D16.5 Sham, b vs. D17 Sham, c vs. PP Sham, a vs. D16.5 Dex.
Neutrophil counts displayed trends against what was previously speculated (see Figure 2). The census of neutrophils in the cervix increased by day of birth in Sham controls, which was expected. However, analysis between prepartum and postpartum groups for the Ovx and MPA groups using an unpaired t-test showed no statistical differences. This indicates that neutrophil numbers were unchanged in the cervix of mice after Ovx or MPA treatment, i.e. after progesterone withdrawal or progestational agonist treatment. Interestingly enough, an unpaired t-test between the day before birth (D17) in the Sham group and day before birth (D16.5) in the Dex group revealed a significant decrease in the number of neutrophils present after treatment with dexamethasone (p=0.0728). A significant decrease was discovered within the Dex group between prepartum and postpartum. Therefore, treatment with Dex, reduced neutrophils in the prepartum cervix. Again data demonstrates differences between D16.5 and PP Dex cervix. This is due to the decreased variability and smaller SE bars within the group. This again illustrates the consistent rebound effect across the data (seen in optical density, collagen data and neutrophil data) where dexamethasone wears off and the postpartum group is within the range of the normal controls. A one-way ANOVA within the prepartum group showed no significant difference across groups. Similarly, no significant difference was found across postpartum groups using a one-way ANOVA (Figure 4).

**Discussion:**

Findings in this study do not support the hypothesis that neutrophils are involved in the process of preterm remodeling of the cervix and/or parturition. In fact, data supports the alternative hypothesis. The lack of change in neutrophil numbers in the cervix before or after birth following progesterone withdrawal (Ovx) or receptor agonist treatment (MPA) after withdrawal does not support the hypothesis that the PR and GR, together, are involved in the
neutrophil recruitment that is associated with normal term remodeling of the cervix. Additionally, data supports that activation of the progesterone receptor, without activation of the glucocorticoid receptor, is capable of blocking the neutrophil recruitment and preventing preterm birth.

To determine the effects of progesterone withdrawal along with progestational agonist treatment, parturition days between groups were compared (Figure 1). Previous research on the Sham group leads us to expect normal term birth to occur on day 19 only[20]. However, CD1 mice delivered on days 18 and 19 post breeding. This did not prevent conclusions about the ability for these mice to maintain pregnancy because this outcome was a result of natural progesterone levels and natural processes in the body (because Sham mice did not have their ovaries removed). This information and statistical analyses performed versus the Sham control supported that the MPA group displayed the ability to maintain pregnancy through normal term and that progesterone withdrawal with a glucocorticoid agonist stimulated cervical ripening and preterm birth.

The ability of MPA to maintain pregnancy until normal term and inhibit the neutrophil recruitment associated with normal term cervical ripening indicates that a synthetic compound, which binds to both the progesterone receptor and the glucocorticoid receptor, would be able to maintain pregnancy (Figure 1, 2). Additionally, previous data in this study with R5020, a pure progesterone receptor agonist, demonstrated similar results suggesting that a progesterone receptor mechanism is responsible for maintaining pregnancy and blocking preterm cervical ripening. It was also noted that treatment with a pure glucocorticoid receptor agonist, Dexamethasone, induced preterm parturition and did not prevent the neutrophil immigration by the day of birth (Figure 1, 4).
In order to determine the mechanism by which pregnancy is maintained and preterm parturition occurs, optical density of the extracellular matrix, cell nuclei counts, and neutrophil counts were performed.

Optical density data through analysis of the extracellular collagen matrix (ECM) demonstrated that neither activation of the progesterone receptor (MPA) or the glucocorticoid receptor (Dex) affected the collagen density in the cervix, leading to the conclusion that it is possible the ECM is not affected by progesterone or glucocorticoid receptors and remodels in a mechanism independent of these receptors (Figure 2). Additionally, treatment with progestational agonist, MPA, did not show a decrease in cell nuclei when comparing prepartum and postpartum cervix, which suggests that the cervix grows in a similar mechanism to Sham after treatment with MPA (Figure 3). It is important to note that the major differences usually seen regarding the hypertrophy of the cervix are seen between D15 and D18. Our lab is currently in the process of obtaining data from these days. However, cell nuclei data demonstrated that treatment with Dex inhibits the hypertrophy of the cervix.

Analysis of neutrophils demonstrated that the mechanism of normal term birth is associated with an infiltration of neutrophils into the cervix by the day of birth. However, progesterone withdrawal (Ovx) and progestational agonist treatment (MPA) inhibit the neutrophil immigration associated with normal term birth as seen in the Sham group (Figure 4). Data from this study suggests that the mechanism of preterm birth may work through a mechanism, which does not involve neutrophils because neither progesterone withdrawal or progesterone receptor and glucocorticoid receptor agonist treatment prevents neutrophil recruitment by the day of birth. Since MPA sustains pregnancy and forestalls cervical...
remodeling through a neutrophil-independent mechanism, findings indicate that other inflammatory cells may regulate cervical remodeling.

Furthermore, treatment with Dexamethasone suggests that the glucocorticoid receptor is not involved in the mechanisms that regulate growth of the cervix, extracellular matrix remodeling or in progesterone withdrawal mediated preterm birth. Therefore, although glucocorticoids have anti-inflammatory actions, they are unlikely to be a useful treatment to block preterm ripening of the cervix or prevent preterm birth. Rather, data suggests that the progesterone receptor is critical to the processes that maintain pregnancy and prevent preterm parturition. From this, we can conclude that progestational agents may forestall specific aspects of inflammatory-mediated cervical remodeling and maintain pregnancy.

Although multiple animals were use for this study, not all of them could be properly imaged and analyzed for use in the final data set. Some cervices were more feasible for sectioning than others. In a minor number of cases, the cervix was not fully intact due to errors during surgery and excision of the cervix. As a result of this, some images had multiple holes in the tissue, which could not be avoided upon analysis. Because we felt this was not an accurate picture of the cervix, these animals were taken out of the study. In the majority of cases, we attempted to image areas of the cervix which were fully intact, minimizing the number of holes in the photos. In cases where holes were minutely present, but the majority of the cervix was intact, cervices were analyzed as accurately as possible.

Further analysis with other data in this study will need to be obtained and compared to determine if progesterone is acting through the progesterone receptor alone, or through the combined actions of both the progesterone receptor and the glucocorticoid receptor. Future work will include flow cytometry to determine if the known inflammatory response is due to activation
of neutrophils already present in the cervix rather than an immigration of neutrophils into the cervix. Once concrete conclusions can be drawn based on the results of this study, future studies can be aimed at determining if pure progesterone agonists, such as R5020, work through the same mechanisms to maintain pregnancy as progesterone. If and when future studies do establish the mechanism by which both normal term and pre-term pregnancy occurs, pharmacological studies can be used to determine possible ways to prevent pre-term birth from occurring.
Reference List

Ref Type: Generic


